Non-viral delivery methods for the manufacture of reprogrammed chimeric antigen receptor (CAR) T-cells

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Declaration

I, Rosie Woodruff confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
Project Abstract

CAR T-cell therapy involves the genetic manipulation of T-cells, to redirect specificity to tumour-associated antigens, and subsequent expansion in an *ex vivo* process. Most CAR T-cell therapies rely on the use of viral transduction or transposition to insert heterologous DNA sequences into the genome; however, there are inherent risks with these approaches, including insertional mutagenesis and lack of copy number control. This thesis explores the use of non-viral delivery methods to manufacture CAR T-cells and approaches to prevent the premature differentiation of T-cells to terminal effector cells, which is known to reduce the efficacy of CAR T-cell therapy.

Proprietary transfection technologies, including nucleofection and soluporation, were evaluated for gene delivery to T-cells. Nucleofection (electroporation) is a highly efficient process to facilitate the introduction of DNA (~50%), RNA (~50%) or ribonucleic protein complexes (RNPs) (~90%). The transfection of DNA significantly impacted cell viability (~75%) which was vastly improved by the transfection of RNA (~90%). Soluporation was investigated as a method to improve cell viability, however, the viability was comparable to electroporation (~90%), transfection efficiencies of RNA/RNPs were typically lower (~25% and ~50%, respectively), and the introduction of dsDNA was extremely inefficient (<10%). Novel viral approaches to deliver the CAR or accessory genes were investigated as a comparison to the explored non-viral systems. The soluporation of lentivirus did not enhance the efficiency of integration (~30%), and transduction by non-integrating vectors was inefficient (~20%) and resulted in low transgene expression in primary T-cells.

The nucleofection of RNA was found to be well tolerated by T-cells and can be exploited for their reprogramming. We explored the use of circular RNA, which has greater stability than linear mRNA, in two different applications. The first involved the reprogramming of T-cells to a less differentiated state by the expression of transcription factors which were known to be involved in controlling lineage commitment. We demonstrate a maintenance of the naïve and stem cell-like subsets, thereby slowing T-cell commitment to the effector cell lineage during *ex vivo* modification and expansion. The second approach delivered the cytosine base editor, BE4max, to disrupt splicing sites or to
introduce premature stop codons into the genes of inhibitory receptors, including PD-1 and TIM3, to disrupt expression and prevent T-cell exhaustion.

Building on the established RNA delivery protocols, we combined base editing with the introduction of an anti-CD30 CAR, for the treatment of Hodgkin lymphoma. Our optimised protocol aims to address challenges associated with the limited efficacy of CAR T-cells, including T-cell inhibition and exhaustion, to improve product persistence and therapeutic outcomes.
Impact Statement

CAR T-cell manufacturing processes remain heavily dependent on retroviral and lentiviral vectors for the genetic modification of T-cells. The drawbacks of viral transduction methods include: the inherent risk of insertional mutagenesis due to random integration, lack of copy number control and costly manufacturing processes. Non-viral approaches are under development, enabling the targeted integration of transgenes, that circumvent many of these issues. While advances are being made, considerable work is required to improve the delivery of nucleic acid and proteins to T-cells, as well as the stability of these components. This thesis aims to address to two elements required for the manufacture of CAR T-cell products using non-viral methods: the delivery of molecules to T-cells and the stability of RNA expressing gene editing components.

Novel delivery approaches were explored for the purpose of CAR T-cell manufacturing. Soluporation was investigated for the transfection of RNA/DNA and RNPs to T-cells and a comparison was made to the widely adopted method, nucleofection. Unfortunately, the transfection of RNA/RNPs was less efficient than nucleofection (~25% and ~50% lower) and the cell viability was comparable (~90%). We concluded that soluporation, without further improvements in efficiency, is not a viable method for the manufacturing of CAR T-cells. In comparison to the non-viral approaches, we explored alternative viral methods, including the soluporation of lentivirus and the use of non-integrating vectors for transient gene expression. Both approaches resulted in low transduction efficiencies and transgene expression, therefore, favouring a non-viral strategy.

Upon comparison of delivery methods, we found T-cells were more tolerant of the transfection of RNA than DNA. We explored the use of circular RNA (circRNA) to prolong protein expression and different applications of this approach. Initial experiments investigated the use of circRNA-encoding transcription factors, to reprogram T-cells to a less differentiated state during ex vivo modification. The delivery of transcription factors can counteract the effects of T-cell expansion and can be employed to “re-boot” differentiated leukapheresis at the start of the manufacturing process or prior to cryopreservation. This method allows for T-cell differentiation and expansion in vivo to prevent premature exhaustion and to improve CAR T-cell persistence.
In a second application we investigate the delivery of a cytosine base editor, BE4max. Cytosine base editors can be employed to disrupt the genes encoding T-cell inhibitory receptors, and when combined with the introduction of CAR genes, can generate an improved drug product with tolerance to tumour-associated immunosuppressive signals. The manufacture of base-edited CAR T-cell therapies is limited by the large-scale production of base editor-encoding mRNA, which is a costly process and requires complex mRNA characterization. Our protocol provides an efficient, scalable, and financially viable method to produce large quantities of the base editor RNA. Upon nucleofection to T-cells, we demonstrate enhanced base conversion using circRNA compared to linear mRNA, which allows for a reduction in the amount of RNA required for editing, therefore, reducing the cost to manufacturing.

In conclusion, we developed a non-viral gene delivery method and demonstrate two potential applications in the context of CAR T-cell manufacturing. Our developed approach can be combined with insertion of an anti-CD30 CAR to revolutionize the treatment of Hodgkin lymphoma.
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<td>AAV</td>
<td>Adeno-Associated Virus</td>
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<td>Adenine Base Editor</td>
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<td>APOBEC</td>
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<td>ARCA</td>
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<td>CRISPR</td>
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<td>CRS</td>
<td>Cytokine Release Syndrome</td>
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<td>CtIP</td>
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CTL Cytotoxic T Lymphocyte
CTLA Cytotoxic T Lymphocyte Associated Protein
CTS Cas9 Target Sequences
CVB3 Coxsackievirus B3
CXCR3 C-X-C Motif Chemokine Receptor 3
dCas9 Deficient Cas9
DHX37 DEAH-Box Helicase 37
DMEM Dulbecco’s Modified Eagle’s Medium - high glucose
DMSO Dimethyl Sulfoxide
DNA Deoxyribonucleic Acid
DNA-PKcs DNA-Dependent Protein Kinase Catalytic Subunit
DNMT3A DNA-Methyltransferase 3A
dNTP Deoxynucleoside triphosphate
ds Double-Stranded
DSB Double-Stranded Break
DTT Dithiothreitol
DVD Dishevelled
E: T Effector: Target
EBV Epstein Barr Virus
EF1α Eukaryotic Translation Elongation Factor 1 alpha 1
eGFP Enhanced Green Fluorescent Protein
ELISA Enzyme-Linked Immunosorbent Assay
EOMES Eomesodermin
ERCC1 Excision Repair Cross Complementation (ERCC) Group 1
EXO1 Exonuclease 1
Fab Fragment Antigen Binding
FAS Fas Cell Surface Death Receptor
FBS Foetal Bovine Serum
FDA Food and Drug Administration
FGL1 Fibrinogen-Like Protein 1
Fox Forehead Box
FOXO1 Forkhead Box O1
FOXP1 Forkhead Box P1
FZD Frizzled
Gal Galectin
GC Germinal Centre
GM-CSF Granulocyte Macrophage Colony-Stimulating Factor
GMP Good Manufacturing Practice
GP Gag-Pol
gRNA Guide RNA
GSK Glycogen Synthase Kinase
GTP Guanosine Triphosphate
GvHD Graft-versus-Host Disease
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<td>h</td>
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<td>MRE11-RAD50-NBS1 Complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MYB</td>
<td>MYB proto-oncogene</td>
</tr>
<tr>
<td>NBS1</td>
<td>Nibrin</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear Factor of Activated T-cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa B</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-Homologous End-Joining</td>
</tr>
<tr>
<td>NILV</td>
<td>Non-Integrating Lentiviral Vectors</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NLPHEL</td>
<td>Nodular Lymphocyte-Predominant HL</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localisation Sequence</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>NR4A</td>
<td>Nuclear Receptor Subfamily 4A</td>
</tr>
<tr>
<td>NRD</td>
<td>Negative Regulatory Domain</td>
</tr>
<tr>
<td>NT</td>
<td>Non-Transduced</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotides</td>
</tr>
<tr>
<td>NT sgRNA</td>
<td>Non-Targeting sgRNA</td>
</tr>
<tr>
<td>NuRD</td>
<td>Nucleosome Remodelling Complex</td>
</tr>
<tr>
<td>OCT4</td>
<td>Octamer-Binding Transcription Factor 4</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Opti-MEM</td>
<td>Opti-MEM™ I Reduced Serum Medium</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
</tr>
<tr>
<td>ORR</td>
<td>Overall Response Rate</td>
</tr>
<tr>
<td>PAM</td>
<td>Protopspacer Adjacent Motif</td>
</tr>
<tr>
<td>PARP1</td>
<td>Poly(ADP-Ribose) Polymerase 1</td>
</tr>
<tr>
<td>PB</td>
<td>PiggyBac</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
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<tr>
<td>pCCL</td>
<td>Lentiviral 3rd Generation Vector Backbone</td>
</tr>
<tr>
<td>pCMV</td>
<td>Mammalian Expression Vector Backbone</td>
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<td>Polymerase Chain Reaction</td>
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<td>PCSK9</td>
<td>Proprotein Convertase Subtilisin/Kexin Type 9</td>
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<td>PD-1</td>
<td>Programmed Cell Death Protein 1 (protein)</td>
</tr>
<tr>
<td>PDCD1</td>
<td>Programmed Cell Death Protein 1 (gene)</td>
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<tr>
<td>PD-L</td>
<td>Programmed Death-Ligand</td>
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<td>Prime Editor gRNA</td>
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<td>pEQ</td>
<td>Retroviral Packaging Vector Backbone</td>
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<tr>
<td>PGK</td>
<td>Phosphoglycerate Kinase</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-Kinase</td>
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<tr>
<td>PIC</td>
<td>Pre-Integration Complex</td>
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<td>PIE</td>
<td>Permuted Intron-Exon</td>
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<td>pJet</td>
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<td>PKR</td>
<td>Protein Kinase R</td>
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<tr>
<td>PMA</td>
<td>Phorbol Myristate Acetate</td>
</tr>
<tr>
<td>pMA</td>
<td>Cloning Vector Backbone Lacking Gene Expression Elements</td>
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<tr>
<td>pMAX</td>
<td>Maximal Mammalian Expression Vector Backbone</td>
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<td>pMD2</td>
<td>Lentiviral 3rd Generation Envelope Vector Backbone</td>
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<td>pMDL</td>
<td>Lentiviral 3rd Generation Packaging Vector Backbone</td>
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<td>Polyadenylation</td>
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<td>PPT</td>
<td>Polypurine Tract Region</td>
</tr>
<tr>
<td>PRDM1</td>
<td>PR Domain Zinc Finger Protein 1</td>
</tr>
<tr>
<td>pRSV</td>
<td>Lentiviral 3rd Generation Accessory Element Vector Backbone</td>
</tr>
<tr>
<td>PSMA</td>
<td>Prostate-Specific Membrane Antigen</td>
</tr>
<tr>
<td>PtdSer</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PTP</td>
<td>Phosphatase Domain</td>
</tr>
<tr>
<td>pUCminusMCS</td>
<td>Plasmid Cloning Vector Backbone with Multiple Cloning Site</td>
</tr>
<tr>
<td>pVac</td>
<td>Compact Plasmid Cloning Vector Backbone</td>
</tr>
<tr>
<td>rAAV</td>
<td>Recombinant AAV</td>
</tr>
<tr>
<td>RAD</td>
<td>DSB Repair Protein</td>
</tr>
<tr>
<td>RBP s</td>
<td>RNA-Binding Proteins</td>
</tr>
<tr>
<td>RCRs/RCLs</td>
<td>Replication-Competent Retroviral or Lentiviral Particles</td>
</tr>
<tr>
<td>RD114</td>
<td>Feline Endogenous Virus Envelope Glycoprotein</td>
</tr>
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RFP  Red Fluorescent Protein
RIG-1  Retinoic Acid-Inducible Gene I
R-loop  DNA-RNA Triplex Structure
RNA  Ribonucleic Acid
RNAi  RNA Interference
RNAP  RNA Polymerase
RNP  Ribonucleic Protein
ROS  Reactive Oxygen Species
RPA  Replication Protein A
RPM  Revolutions Per Minute
RPMI  Roswell Park Memorial Institute
RRE  Rev Responsive element
RT  Room Temperature
RUNX  Runt-Related Transcription Factor
RV  Retroviral Vector
S520A  Serine-520-Alanine
SA  Splice Acceptor
SAR  Scaffold/Matrix Attachment Protein
SB  Sleeping Beauty
SCD  Sickle Cell Disease
scFv  Single Chain Variable Fragment
SCID  Severe Combined Immunodeficiency
SD  Splice Donor
± SD  ± Standard Deviation
SDS-PAGE  Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SFG  Retroviral Vector Backbone
SFGmR  Retroviral Vector Backbone with SAR
gRNA  Single Guide RNA
SH2  Src Homology 2
SHP  Small Heterodimer Partner
shRNA  Short Hairpin RNA
SIN  Self-Inactivating
SLE  Systemic Lupus Erythematosus
SMAD  Suppressor of Mothers Against Decapentaplegic
snRNA  Small Nuclear RNA
SOCS  Suppressors of Cytokine Signalling
SOX2  SRY-Box 2
SPACE  Synchronous Programmable Adenine and Cytosine Editor
SPRI  Solid Phase Reversible Immobilization
ss  Single-Stranded
SSTR  Single-Stranded Templated Repair
STAT  Signal Transducer and Activator of Transcription
STK  Stalk Spacer Domain
SV40  Simian Virus 40
TALEN  Transcription Activator-like Effector Nuclease
TB  Terrific Broth
TBE  Tris/Borate/EDTA
TBX21  T-Box 21
TCF  T-cell Factor
TCM  Central Memory T-cells
TCR  T-cell Receptor
TD  Transduction
TE  Transfection Efficiency
TEFF  Effector T-cells
TEM  Effector Memory T-cells
TENT  Terminal Nucleotidyl-Transferase
TET2  Tet Methyl cytosine Dioxygenase 2
TGFβ  Transforming Growth Factor Beta
TGFβR  TGFβ Receptor
Th17  T Helper Type 17
TILs  Tumour-Infiltrating Lymphocytes
TIM3  T-cell Immunoglobulin and Mucin Domain-Containing Protein 3
TLRas  Toll-Like Receptors
TME  Tumour Microenvironment
TN  Naive T-cells
TNF  Tumour Necrosis Factor
TNFRSF  TNF Receptor Superfamily
TORC1  Target-of-Rapamycin Complex 1
TOX2  TOX High Mobility Group Box Family Member 2
TRAC  TCR Alpha Chain
tracrRNA  Trans-activating crRNA
TRβC  TCR Beta Chain
Treg  Regulatory T-cell
Tris-HCL  Tris Hydrochloride
tRNA  Pre-Transfer RNA
TRUCKs  T-cells Redirected for Antigen-Unrestricted Cytokine-Initiated Killing
TSCM  Stem Cell-Like Memory T-cells
UGI  Uracil DNA Glycosylase Inhibitor
UTR  Un-Translated Region
VH  Variable Heavy Chain
VL  Variable Light Chain
VSV-G  Vesicular Stomatitis Indiana Virus Envelope Glycoprotein
WNT  Wingless/Integrated Protein
WPRE  Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element
WT  Wild Type
| **XLF-XRCC4** | X-Ray Repair Cross Complementing 4-like factor |
| **XPF** | ERCC Group 4, DNA Repair Endonuclease XPF |
| **ZAP70** | Zeta-Chain Associated Protein Kinase 70 |
| **ZEB2** | Zinc-Finger E-Box Binding-Homeobox 2 |
| **ZFN** | Zinc Finger Nuclease |
| **β2M** | Beta 2 Microglobulin |
| **γC** | Common Gamma Chain |
| **Ψ** | Psi Sequence |
| **ΨU** | Pseudouridine |
Thesis Structure

This thesis explores non-viral genome engineering approaches to enhance the performance of adoptive T-cell therapies. The first section investigates gene delivery methods and identifies circular RNA as a desirable approach for transient transgene expression. Two applications of circular RNA are investigated, to modulate the T-cell phenotype by the expression of transcription factors, and to disrupt T-cell inhibitory receptors using cytosine base editors. In the final chapter, the developed RNA protocol is employed to generate a genome-edited anti-CD30 CAR T-cell product that will be investigated in a clinical study for classical Hodgkin lymphoma. The thesis has the following structure:

Chapter 1 provides a background to the CAR T-cell manufacturing process, a comparison of viral and non-viral gene integration methods, a literature review of the latest genome editing technologies and an introduction to RNA transfection.

Chapter 2 defines the materials and experimental methods used in this thesis.

Chapter 3 explores methods for the genetic manipulation of T-cells, including the transfection of RNA/DNA and ribonucleic protein complexes using two proprietary delivery technologies, nucleofection and soluporation. Alternative viral approaches are explored for CAR T-cell manufacturing, including the soluporation of lentivirus and the use of non-integrating viral vectors.

Chapter 4 investigates the use of circular RNA to transcriptionally reprogram T-cells to a less differentiated cell state, with the aim of improving CAR T-cell persistence.

Chapter 5 presents the use of circular RNA to enhance base editing outcomes and establishes a protocol for multiplexed gene disruption using the cytosine base editor, BE4max.

Chapter 6 describes the development of a genome-edited anti-CD30 CAR T-cell product, in preparation for the clinical study.
Chapter 1. Introduction

1.1 The genetic manipulation of T-cells for adoptive cell therapy

The promise of chimeric antigen receptor (CAR) T-cell therapy depends on the ability to deliver novel binding domains to T-cells, which enhances their ability to recognise and attack antigen-specific tumour cells. Heterologous DNA sequences are integrated into the genome of donor T-cells through viral or non-viral methods. There are now six FDA-approved CAR T-cell products for the treatment of haematological malignancies including Abecma® (idecabtagene vicleucel), Breyanzi® (lisocabtagene maraleucel), Carvykti™ (ciltacabtagene autoleucel), Kymriah™ (tisagenlecleucel), Tecartus™ (brexucabtagene autoleucel) and Yescarta™ (axicabtagene ciloleucel), all of which rely upon retroviral or lentiviral transduction to deliver the CAR genes. There are risks associated with viral integration including insertional mutagenesis and lack of copy number control. With the advent of genome engineering developments, non-viral methods including clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated nuclease 9 (Cas9)-mediated homology-directed repair (HDR) and transposition have received increasing attention for the application of CAR integration and provide advantages including copy number control and regulation of transgene expression.

Following genetic manipulation to introduce the CAR, the donor T-cells are expanded ex vivo, which can lead to T-cell differentiation into the memory and effector subsets. The T-cell effector subsets exhibit the highest cytotoxicity, but their proliferative capacity is limited. Therefore, this can lead to poor expansion and persistence upon adoptive transfer and limits the efficacy of CAR T-cell therapy. Non-viral gene delivery technologies can be applied to improve the therapeutic efficacy of CAR T-cell products by facilitating reprogramming during the manufacturing process, through the delivery of RNA/DNA and proteins.

This thesis will investigate non-viral delivery methods for the manufacture of CAR T-cell therapies and will investigate approaches to improve the function of donor T-cells.
In the final section of this thesis, the developed non-viral gene delivery approach will be incorporated into a manufacturing process for the development of a next-generation CAR T-cell therapy for the treatment of Hodgkin lymphoma.

This section presents a brief introduction into the genetic manipulation of T-cells by (i) describing the structure of CARs and the general process of CAR T-cell manufacture, (ii) comparing viral and non-viral methodologies for the introduction of heterologous DNA sequences, (iii) exploring novel genome engineering technologies for improving CAR T-cell function and (iv) providing a background to RNA transfection for the reprogramming of T-cells.

1.1.1 Chimeric Antigen Receptors (CARs)

This section will provide a background to the structure of CARs and their evolution in design since the discovery of the first single chain variable fragment (scFv) CAR in 1993 (Eshhar et al., 1993). Generally, a CAR consists of four distinct components, an extracellular antigen-binding domain, a spacer region, a transmembrane domain, and the intracellular signalling domain (illustrated in Figure 1.1). The antibody or ligand specificity is harnessed to direct T-cells to specific tumour antigens and to generate a robust and potent response through the cytotoxic effector mechanisms. Unlike αβ T-cell receptors (TCRs), CARs are capable of recognising antigen in a major histocompatibility complex (MHC)-independent manner, and therefore can be directed to most surface molecules.
Chapter 1. Introduction

Figure 1.1 Structural evolution of CARs.

Chimeric antigen receptors (CARs) are introduced to T-cells to reprogram their specificity to tumour-associated antigens. CARs are formed of four main components, the extracellular antigen-binding domain, the spacer and transmembrane regions, and the intracellular signalling domain. The first-generation CARs contain the CD3ζ endodomain derived from the endogenous T-cell receptor. Structural evolution of CARs involves the incorporation of co-stimulatory modules to improve T-cell signalling and are characterised as second-generation (one co-stimulatory domain) and third-generation CARs (two costimulatory domains). Further addition of inducible modules has formed fourth-generation CARs, which provide functional benefits such as the secretion of cytokines including interleukin-12 (IL-12).

1.1.1.1 Antigen-binding domain

The extracellular domain consists of the targeting moiety, which is commonly generated by fusing the light and heavy chain variable regions of an immunoglobulin (VL and VH) into a scFv through a flexible polypeptide linker. Alternatively, the domain can be generated using an IgG Fab fragment, VHH single-domain antibody or using natural
ligands. The engineered domain retains its specificity, as determined by the hypervariable complementarity determining regions of the antibody. The antigen recognition domain can be derived from murine or humanised antibodies, and the synthesised scFvs are screened for their binding affinity using phage display libraries. The scFv is carefully selected based on their binding affinity and kinetics, to tune CAR sensitivity according to target antigen density present on the tumour cells. Low affinity antigen binding domains will only allow for CAR T-cell signalling in the presence of high antigen density, whilst high affinity scFvs will signal in environments featuring high or low antigen density (Chmielewski et al., 2004; Caruso et al., 2015). In addition to antigen affinity, the kinetics of binding can influence the efficiency and safety of CAR T-cell therapies. Fast-off rates are desirable to improve cytolytic capacity by preventing T-cell exhaustion, and to reduce CAR-mediated toxicities (Ghorashian et al., 2019).

1.1.1.2 Spacer and transmembrane domains

A spacer region separates the binding domain from the transmembrane and intracellular domains to determine the distance and flexibility of the CAR from the T-cell plasma membrane. The most common spacer regions include the hinge or constant regions of human IgG and the immunoglobulin-like extracellular regions of CD8α, CD4 or CD28 (Alabanza et al., 2017). The optimal spacer length can vary depending on the proximity of the target antigen and aims to maintain the dimensions of the native TCR and peptide-bound MHC interaction. For membrane-proximal epitopes, a longer spacer would be desirable to enable formation of the CAR: epitope synapse (Guest et al., 2005). The transmembrane domain connects the extracellular and intracellular signalling domains and is often derived from CD3ζ, CD8, CD4 or CD28 molecules. Although primarily functioning as a structural element, some studies have shown that the transmembrane domain can influence CAR surface expression and function (Pulè et al., 2005; Fujiwara et al., 2020).

1.1.1.3 Intracellular signalling domains

The intracellular signalling domain has been extensively studied and has led to the evolution of CARs and the improvement in their signalling output. The first-generation
CARs contained a single CD3ζ-activating domain from the TCR complex and lack co-stimulatory domains. This was sufficient to lyse tumour cells in vitro, however, cytokine secretion was suboptimal and CAR T-cells lacked activation and proliferation, leading to cell anergy (Harding et al., 1992; Eshhar et al., 1993; Brocker and Karjalainen, 1995). The clinical performance of first-generation CARs was limited by poor expansion and persistence (Kershaw et al., 2006; Kowolik et al., 2006; Savoldo et al., 2011).

Second-generation CAR T-cells were engineered to include the signalling domain of co-stimulatory molecules in cis, including CD28 and 4-1BB (CD137). This significantly improved CAR T-cell proliferation, production of IFN-γ and IL-2, in vivo persistence and cytolytic capacity (Maher et al., 2002; Porter et al., 2011). Other co-stimulatory domains have been tested including OX-40 (CD134) and inducible costimulatory (ICOS) (Guedan et al., 2014).

An additional intracellular signalling domain has been added to generate third-generation CARs, the most studied combinations are CD28-CD3ζ with either 4-1BB or OX-40. Reports have indicated that the function of second and third generation CARs are comparable (Pulè et al., 2005; Carpenito et al., 2009).

To further augment T-cell function, second or third-generation CARs have been engineered to include either a fourth co-stimulatory domain or an anti-tumour payload. These CARs have been termed fourth-generation CARs or T-cells redirected for universal cytokine-mediated killing (TRUCKs) (Chmielewski and Abken, 2015). Activation through these CARs will initiate production and secretion of cytokines, including IL-12, to promote anti-tumour activity (Chmielewski and Abken, 2012; Pegram et al., 2012).

1.1.2 An overview of CAR T-cell manufacturing

The success of CD19-targeted CAR T-cells had led to a significant increase in the number of CAR trials. There are now 554 registered trials (clinicaltrials.gov) that are: not yet active, active not recruiting, or recruiting/enrolling by invitation. CARs have been generated to target cell surface receptors, cell adhesion molecules and gangliosides whose expression is upregulated on the surface of tumour cells (Ahmed et al., 2015; Junghans et al., 2016; Heczey et al., 2017; Ramos et al., 2017, 2020; Raje et al., 2019). Regardless
of the CAR binding domain and structure, the general principles of the manufacturing process remain consistent. The CAR T-cell manufacturing process involves the genetic modification of peripheral blood mononuclear cells (PBMCs) through an *ex vivo* procedure. In brief, PBMCs are collected, and T-cells are processed by selection and/or activation through stimulation of the CD3 and CD28 receptors. Stimulated T-cells are then engineered to express a CAR to redirect specificity to tumour associated antigens, followed by expansion to meet the requirements of the clinical dose (Figure 1.2).

**Figure 1.2** CAR T-cell manufacturing process.

The manufacture of CAR T-cell therapies begins with the extraction of T-cells from the patient by apheresis. The T-cells are then isolated and activated through stimulation of the CD3 and CD28 receptors and the addition of interleukins. The activated T-cells are genetically modified to introduce the CAR transfer cassette, and this can be performed through viral or non-viral methods. The CAR T-cells are then expanded to a number sufficient to meet the clinical dose and cryopreserved before thawing and re-infusion to the patient. At Autolus these processes are performed predominately on the CliniMACs Prodigy (centre).
The production of high-quality clinical products relies on a reproducible and robust manufacturing process. Process development efforts have focused on optimising each manufacturing step to generate a scalable and standardised process that limits the risk of product variability. Commercial-scale manufacturing has previously been limited by labour-intensive procedures which have relied on numerous manual and open processing steps that require highly skilled operators and extensive training. Recent developments have incorporated closed-system technologies, such as the Miltenyi CliniMACS Prodigy, which allows for integrated cell washing, selection, activation, and expansion on a single device, to automate CAR T-cell processing and to minimise the risk of contamination.

The next section describes the individual steps for CAR T-cell processing in greater detail:

1.1.2.1 Cell source and selection

Autologous CAR T-cell products are personalised medicines that require the collection of PBMCs from the patient in a process termed leukapheresis. The extracted cells can be used fresh or cryopreserved prior to washing and processing. Closed systems including the Biosafe Sepax and Miltenyi Prodigy are often employed to automate the washing and cell separation steps. These systems can incorporate either size-based fractionation or bead enrichment for the depletion of monocytes and isolation of lymphocytes. Generally, CAR T-cells are produced from the CD3+ population, however other groups are selecting for specific subsets of T-cells including CD8+ cells. One clinical study has further selected for CD8+ cytotoxic lymphocytes and CD4+ helper lymphocytes using magnetic bead enrichment, and are infusing B-cell acute lymphoblastic leukaemia (ALL) patients with a defined CD4+: CD8+ composition of one CD4+ CAR T-cell to every CD8+ CAR T-cell (Turtle et al., 2016). An alternative strategy is to isolate T-cells with a defined phenotype including naïve (T\(_N\)) (Hinrichs et al., 2011), stem cell memory-like (T\(_{SCM}\)) (Berger et al., 2008), or central memory T-cells (T\(_{CM}\)) (Gattinoni et al., 2011). This can be achieved by selection at the start of the process or through enrichment during the manufacturing process. One published report has described a sorting strategy for the enrichment of T\(_N\) and T\(_{CM}\) subsets through bead selection for CD4- CD62L+ CD45RA+ and CD4- CD62L+ CD45RA- populations, respectively (Casati et al., 2013).
Immunomagnetic selection was also employed by Wang et al., to select for the T<sub>CM</sub> subset by first depleting the CD14+, CD45+ and CD4+ cells, then positively selecting for CD62L+. This technique has been incorporated into FDA-approved clinical trials for CD19-specific CARs and highlights the importance of the T-cell source (Wang et al., 2012).

1.1.2.2 T-cell activation

T-cell activation is a necessary step to enable efficient CAR integration and cell expansion. The activation signal must be of adequate strength and requires a primary signal through the TCR combined with costimulatory signals including CD28 or 4-1BB. Soluble anti-CD3 and anti-CD28 monoclonal antibodies (mAbs) can provide stimulation in the presence of cytokines. The strength of the activation signal can be improved by immobilisation to plates or beads such as the commercially available reagents Invitrogen’s CTS Dynabeads® CD3/CD28 and Miltenyi MACS GMP TransAct™ CD3/28. Optimisation of the cytokine conditions can provide functional advantages to CAR T-cells. Low concentrations of interleukin-2 (IL-2) can favour the formation of early T-cell memory subsets and prevent differentiation by over-stimulation (Kaartinen et al., 2017). Other studies support the use of IL-7 and IL-15 to enrich for early lineage cells (Singh et al., 2011), or the combination of IL-15 and IL-21 for improved in vivo cytolytic capacity (Xu et al., 2016).

1.1.2.3 Genetic modification

The genetic introduction of a CAR can be performed using viral vectors or non-viral systems; the methods are discussed in detail in section 1.2. Currently, all of the approved CAR T-cell therapies rely upon retroviral and lentiviral vectors, which enables highly efficient and permanent gene transfer. Although the transduction process is simple, the cost of GMP-grade vector is extremely high and exacerbates the cost of CAR T-cell manufacturing. Pre-clinical development has seen a rise in the use of non-viral systems, including CRISPR-mediated homology-directed repair and transposition; these systems provide specific advantages as detailed in section 1.2.2.
Non-viral methods rely on the use of transfection technologies to deliver genome engineering components. Components for transient expression may include DNA-encoding the CAR and RNA/RNPs encoding an endonuclease/transposase, for stable gene integration by CRISPR-mediated HDR or transposition. Alternatively, mRNA-expressing novel genes, such as transcription factors, dominant negative proteins, or anti-apoptotic proteins, may be supplied to the cell to provide additional functions. Electroporation remains the most widely used method for the genetic manipulation of T-cells, due to its high efficiency of gene delivery for a range of substrates (DNA/RNA and protein). Many commercial delivery systems have been developed based on this approach, including Lonza’s Nucleofector™, MaxCyte’s electroporator and Miltenyi’s electroporation platform. Alternative methods of cell permeabilization are under development and include chemical cell permeabilization (soluporation), carrier-based gene transfer (e.g., lipid nanoparticles) and mechanoporation (e.g., microfluidic squeezing). A detailed introduction to transfection techniques is provided in Chapter 3, and an evaluation of two proprietary technologies is provided in section 3.9.2.

### 1.1.2.4 Expansion and cryopreservation

Following introduction of the CAR, an ex vivo expansion period is required to ensure sufficient CAR T-cell numbers are reached to meet multiple dose requirements. Most protocols require 7 to 10 days of expansion which can be performed using bioreactors such as the GE WAVE or G-Rex systems, or integrated technologies including the CliniMACS Prodigy. The potential of CAR T-cell therapies is limited by poor in vivo expansion and persistence, which is correlated with the state of T-cell differentiation. Efforts have been made to reduce the duration of ex vivo expansion to sustain the early T-cell memory phenotype and prevent in vivo exhaustion. A published study by Ghassemi et al., has investigated the effect of reducing the manufacturing time on the efficiency of anti-CD19 CAR therapies. Results demonstrated that CAR T-cells can be harvested at 48 hours post-transduction to prevent differentiation and improve tumour control (Ghassemi et al., 2018). Other engineered platforms include Gracell’s FaST CAR-T and Novartis’ T-Charge™, which have reduced manufacturing times to 1 day and 2 days, respectively.
These processes support T-cell stemness and rely upon expansion in vivo (Novartis, 2021; Gracell Biotechnologies, 2022).

The continuous improvement to manufacturing processes supports the evolution of CAR T-cell therapies with improved functional capacity. This section has briefly summarised the individual steps to CAR T-cell processing and the next section will compare viral and non-viral methods for CAR insertion.

1.2 Genetic introduction of heterologous DNA sequences

The genetic engineering technologies used to deliver CARs can be divided into two classes: viral vectors and non-viral systems. The following section compares current methods for the insertion of heterologous DNA sequences and describes the latest developments of non-viral mediated gene knock-in.

1.2.1 Viral vectors

1.2.1.1 Retroviral vectors

Retroviral vectors are commonly derived from the Moloney murine leukaemia virus (MoMLV) and aim to achieve stable integration of a replication deficient provirus into the chromosomal DNA. Based upon the structure of an infectious virus, the life cycle of the vector begins upon attachment to cellular surface receptors, to trigger endocytosis and release of the single-stranded RNA genome (ssRNA). Following entry, the RNA genome is reverse transcribed into complementary DNA (cDNA), which associates with viral and host cell proteins to form the pre-integration complex (PIC). The retroviral PIC is relatively unstable and requires breakdown of the nuclear membrane to allow for nuclear import, and therefore requires the cycling of T-cells. The CAR is then integrated into the host genome and is transcribed and translated using the host cell machinery for presentation on the surface of T-cells.

Retroviral vectors have been engineered to ensure they are replication incompetent by providing the packaging and genome components in trans on separate plasmids for transfection and viral assembly in HEK293T cells. However, safety concerns remain,
relating to the integration profile and preference for insertion at cellular promoters. MoMLV vectors have previously been used for T-cell immunotherapy of severe combined immunodeficiency- (SCID-) X1 disease, by genetic correction of the IL-2 receptor gamma (IL2RG) in CD34+ cells. Although the immunodeficiency was successfully treated, several patients developed T-cell related leukaemia due to insertional mutagenesis into the LIM domain-only 2 (LMO2) proto-oncogene (Cavazzana-Calvo et al., 2000; Hacein-Bey-Abina et al., 2008). The genotoxicity of clinical retroviral vectors has been reduced by the development of self-inactivating (SIN) vectors, where the promoter and enhancer regions of the 3-prime (3’) long terminal repeat (LTR) has been removed to prevent their activity (Xu and Eiden, 2012).

Retroviral vectors provide high transfer efficiency and transgene expression, which has been achieved through optimisation of the retroviral cassette. Modifications include promoter optimisation and the mutagenesis of viral elements including the central polypurine tract region (PPT), which is important for the priming of reverse transcription (Robson and Telesnitsky, 2000).

1.2.1.2 Lentiviral vectors

The CAR T-cell field has seen a rise in the application of lentiviral vectors for CAR integration. The most widely used lentiviral vectors are derived from the ssRNA genome of human immunodeficiency virus-1 (HIV-1), which is considerably more complex than gammaretroviral genomes and has received extensive engineering to improve both biosafety and transgene expression. Like retroviral vectors, SIN particles have allowed for integration of the desired sequence without continued viral replication. In contrast to gamma retroviruses, the favoured integration sites are distant from transcriptional start sites or cellular promoters, reducing the risk of insertional mutagenesis and thus improving the safety of gene integration (Beard et al., 2007).

HIV-1-derived vectors are commonly pseudotyped with the vesicular stomatitis virus G protein (VSV-G) envelope and confer broad tropism across various types of non-proliferating and proliferating cells. Their PIC is relatively stable compared to gamma retroviruses and can pass through nuclear pores of quiescent cells although, studies have
shown that transduction efficiencies benefit from minimal T-cell activation (Beard et al., 2007). The main limitation of lentiviral vectors is the difficulty of generating stable producer cell lines for GMP-grade vector manufacture.

The production of both retroviral and lentiviral vectors requires a complex manufacturing process and release testing. This includes an assessment of vector sterility, mycoplasma testing, quantification of replication competent retroviral or lentiviral particles (RCRs/RCLs) and quality testing for contaminants including residual host and plasmid DNA.

Viral vectors are an attractive tool for CAR integration; however, several key features discourage their use in favour of novel non-viral approaches. The main limitation of viral vectors is the packaging limit, which allows for the expression of two or rarely three transgenes. The safety profile and risk of insertional mutagenesis remains a concern, although genotoxicity is limited in T-cells. Virus production scale and cost is also a key limiting factor for a completely scalable CAR T-cell manufacturing process.

1.2.2 Non-viral systems

Non-viral methods for CAR insertion are currently also under research and early clinical development. CRISPR/Cas9 technology can be used to successfully target a CAR to the T-cell receptor (TCR) α chain (TRAC) locus whilst simultaneously knocking out the TCR in a process termed homology-directed repair (HDR) (Eyquem et al., 2017). Alternatively, transposon-based systems such as Sleeping Beauty and piggyBac involve the electroporation of transposon (containing transgene) and transposase plasmids and have shown proof-of-concept as a financially-viable CAR manufacturing approach (Nakazawa et al., 2011; Magnani et al., 2020).

Genome editing components can be commercially sourced or produced in-house using simple and convenient methods compared to the complex and expensive production of GMP-grade viral vectors. Non-viral systems also provide the benefits of reduced insertional mutagenesis and control of copy number through site-specific integration. The main limitation to these approaches is reaching a high efficiency of modification without compromising cellular viability, as well as off-target modification. Recent studies have
also highlighted the risk to chromosomal abnormalities, particularly during multiplexed editing mediated by DNA double-stranded break (DSB) formation. A background to the current non-viral genome engineering methods is provided in the following section, including a discussion of the advantages and disadvantages of each approach.

1.2.2.1 Homology-directed repair

The insertion of a CAR-encoding sequence into the genome can be achieved by HDR, which first involves the generation of a targeted DNA DSB using an endonuclease such as Cas9 (Eyquem et al., 2017; Roth et al., 2018; Stadtmauer et al., 2020), Cas12a/Cpf1 (Dai et al., 2019), a transcription activator-like effector nuclease (TALEN) (Poirot et al., 2015; Berdien et al., 2014), zinc finger nuclease (ZFN) (Yamamoto et al., 2019), or a homing endonuclease (MacLeod et al., 2017). DSBs are repaired predominately via the non-homologous end joining (NHEJ) pathway, an error prone process that can introduce indels or deletions to the genomic target at high frequency resulting in gene disruption (Figure 1.3). Alternatively, the host cell can be provided with a DNA donor template, which can be copied into the genome by HDR during DNA replication. This process can be used to correct gene function or to insert new genetic elements into the genome of T-cells. The programmable simplicity of Cas9 in comparison to zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), makes it a desirable gene perturbation tool. The following section will describe the mechanism for CRISPR/Cas9-mediated gene insertion by the formation of DSBs and templated repair.
Figure 1.3 Alternative repair pathways of Cas9-mediated DNA double-stranded breaks.

The single guide RNA (sgRNA) directs Cas9 endonuclease to the complementary DNA target site upstream of the 5'-NGG protospacer adjacent motif (PAM sequence) and unwinds the DNA. Cas9 cleaves the target and non-target strands to generate a DNA double-stranded break (DSB). DSBs are repaired via non-homologous end joining resulting in gene disruption by nucleotide deletion or addition (indel formation) or by homology-directed repair when supplied with a DNA repair template.

1.2.2.1.1 Cas9-mediated DNA DSBs

The type II CRISPR endonuclease Cas9 derived from *Streptococcus pyogenes* (Spy) associates with an engineered single guide RNA (sgRNA) to form a ribonucleic protein (RNP) complex. The sgRNA contains a 20-nucleotide targeting sequence in the CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA) scaffold. This directs Cas9 to the desired genomic target, located directly adjacent to an NGG protospacer adjacent motif (PAM). Upon binding to the target sequence, the HNH and RuvC nuclease domains cleave the target and non-target strands respectively to form a blunt-ended DNA DSB.
The introduction of mutations in the nuclease domains has yielded nickase and catalytically inactive variants (dCas9) of the enzyme. The RuvC domain is formed of three discontinuous segments and the D10 mutation disrupts its catalytic activity, generating a nickase that cleaves only the target strand. On the other hand, the HNH domain occurs at the C-terminus and is inactivated by the H840A mutation, generating a non-target strand-cleaving nickase (Jinek et al., 2012). Cas9 nickases can be paired to form staggered single-stranded breaks (SSBs) on opposite strands of the DNA (forming a DSB) to initiate HDR. Nickase pairing is an approach used to increase the specificity of targeting, as it is unlikely that two off-target sites will be close enough to each other to result in the formation of a double-stranded DNA break.

1.2.2.1.2 Repair mechanisms of DSBs

DNA repair machinery is recruited to reverse DSBs by re-ligation of the ends through pathways such as NHEJ or microhomology-mediated end joining (MMEJ), or by templated repair from a donor template through HDR (illustrated in Figure 1.4). The initial recognition and processing of DSBs is thought to determine the repair pathway. Unprocessed blunt-ended DSBs undergo NHEJ, whilst 5’ to 3’ resection can generate overhangs to initiate repair via MMEJ or HDR.

NHEJ is the default repair mechanism in human cells and is activate at all stages of the cell cycle. Repair begins within 30 minutes of the DSB and is initiated by the binding of the Ku70/Ku80 complex (Mao et al., 2008). The Ku70/Ku80 complex protects the blunt ends from DNA resection to prevent MMEJ and HDR and recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and XLF-XRCC4. XLF-XRCC4 subsequently binds to DNA ligase IV to repair the DSB. If DNA ends are incompatible, Artemis can be recruited to generate blunt ends for direct ligation.

MMEJ is an alternative end-joining pathway that shares aspects of both NHEJ and HDR. DSB repair by MMEJ does not require a donor template however, similarly to HDR relies upon DNA end resection to initiate the process. Resection involves the processing of 5’ ends to generate ssDNA overhangs to reveal homologies and involves the MRE11-RAD50-NBS1 (MRN) complex. MRN binds adjacent to the DSB and becomes activated.
by the co-factor, CtIP. The MRN/CtIP complex initiates strand nicking upstream of the break on the 5’ end and simultaneously prevents Ku70/Ku80 binding, therefore inhibiting NHEJ. The resection process begins from the nick by the 3’-to-5’ exonuclease, MRE11, and generates short 3’ overhangs of less than 20 base pairs that are homologous on opposite strands. The DNA flaps are then removed by the endonuclease, ERCC1-XPF, and DNA polymerase copies the missing bases from the template for ligation by Ligase I or Ligase III. Other cofactors such as PARP-1 have been described to prevent Ku70/Ku80 binding and to aid recruitment of MRE11 (Ranjha, Howard and Cejka, 2018).

HDR is restricted to S and G2 phases of the cell cycle and is a templated process that can incorporate heterologous DNA sequences into the host cell genome using donor DNA templates that contain flanking regions of homology. The source of DNA repair templates includes the sister chromatid that becomes available during replication, exogenous plasmid or linear dsDNA, or ssDNA. The mechanism of repair differs between the structure of the donor template; chromosomal or dsDNA is copied by homologous recombination whilst the latter involves single-stranded templated repair (SSTR). HDR is a relatively slow process that requires a minimum of 7 hours and is initiated by MRN/CtIP-mediated end resection (Mao et al., 2008). Further resection occurs by exonuclease 1 (EXO1) to generate long ssDNA overhangs on each side of the DSB. The exposed ends are then coated and stabilised by the replication protein A (RPA) complex, followed by the recruitment of RAD52, which can be replaced with RAD51. RAD51 is required for homologous recombination whereas RAD52 is required for SSTR. RAD51 filaments search for a donor template and subsequent strand invasion occurs to form a D loop. Strand capture occurs at the second DSB end and forms a Holliday junction to mediate repair by the DNA polymerase and Ligase I. The mechanisms of SSTR are still under investigation and depend upon association with RAD52 and multiple rounds of annealing and extension from the ssDNA donor template (Yeh, Richardson and Corn, 2019). The discovery of these repair mechanisms had led to the improvement to HDR efficiencies.
Figure 1.4 Alternative pathways for repair of DNA DSBs.

An overview of DNA repair machinery involved in repair of DNA double-stranded breaks (DSBs) via non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), or homology-directed repair (HDR).

1.2.2.1.3 Improving HDR efficiencies

The design of sgRNAs often involves the ranking of sequences based on the measure of total indels produced by the nuclease. The prediction of sgRNA editing efficiencies therefore is based upon the repair by NHEJ. One study has improved the prediction of sgRNA efficiency for HDR outcomes based on MMEJ repair, which shares greater similarity to HDR than NHEJ (Tatiossian et al., 2021).

Furthermore, commercial sgRNAs can be chemically modified to contain 2’-O-methyl 3’phosphorothioate bonds between three terminal nucleotides of the 5’ and 3’ ends to induce high levels of genome editing. Modified sgRNAs have demonstrated enhanced
intracellular stability to achieve over 98% knockout frequency in primary cells (Hendel et al., 2015; Yin et al., 2017).

The main challenge of non-viral gene targeting is the delivery of gene-editing components, which is commonly performed by electroporation of plasmid donor or linear DNA templates. The presence of DNA in the cytoplasm can elicit immune responses and severe toxicity. Adeno-associated viral (AAV) vectors have low immunogenicity thus, have become the preferred method for donor template delivery and require a simple transduction step following the transfection of Cas9 RNPs (Eyquem et al., 2017).

As an alternative strategy to AAV, work has been performed to improve the knock-in efficiencies and the absolute yield of edited cells through the optimisation of DNA repair templates. Shy et al., have reported the use of Cas9 target sequences (CTS) at the 5’ and 3’ ends of the HDR template for recruitment and shuttling of the complex into the nucleus (Shy et al., 2022). However, double-stranded DNA (dsDNA) repair templates caused high toxicity and limited the knock-in efficiencies in T-cells. Toxicity could be partially attenuated by coating HDR templates in anionic polymers such as polyglutamic acid. As an alternative approach, CTS-containing single-stranded DNA (ssDNA) templates could improve knock-in efficiencies by over 5-fold, reaching over 80% HDR-edited cells, and improved live cell yields by 7-fold (Shy et al., 2022). Other template developments include the incorporation of RNP-binding sequences to tightly folded DNA nanostructures, which can be delivered using virus-like particles to reduce toxicity and increase insertion efficiencies (Lin Shiao et al., 2021).

Differences have been observed in the repair pathways of dsDNA and ssDNA templates. In contrast to dsDNA templates, repair from ssDNA templates is highly efficient and independent from the classical RAD51 pathway, and instead requires the Fanconi Anaemia DNA repair pathway (Richardson et al., 2018). Richardson et al., have studied the interaction of Cas9 and dsDNA and revealed slow dissociation that initiates from the 3’ end of the non-target DNA strand. Following this observation, optimisation of the ssDNA templates was performed. ssDNA templates complementary to the non-target strand provided a 2.6-fold increase in HDR compared to those that were complementary
to the target strand. Previous studies have focused on using donor templates that were symmetric around the break. By engineering asymmetric ssDNA templates with 36 bp on the PAM-distal site and 91-bp on the PAM-proximal side of the break, HDR efficiencies of up to 60% were reached (Richardson et al., 2016).

Achieving efficient HDR can be challenging as due to its competition with NHEJ and its cell-cycle restriction. Strategies to favour HDR outcomes include the use of pharmacological inhibitors targeting components of NHEJ including inhibitors of the Ku70/Ku80 complex and DNA-PKcs such as NU7441 (Robert et al., 2015; Fu et al., 2021). Ligase IV can be inhibited using the small molecule, SCR7, and has increased the HDR efficiency in human cells (Li et al., 2017; Riesenberg and Maricic, 2018). Other studies have used HDAC class I/II inhibitor, Trichostatin A, or CDC7 inhibitor, XL413, to improve knock-in outcomes by up to 3.5-fold (Wienert et al., 2020). Additionally, IDT have developed a proprietary HDR enhancer, which has been reported as a NHEJ inhibitor, to enhance knock-in studies (Kath et al., 2022).

1.2.2.1.4 Targeted insertion

Popular target sites for CAR insertion include the TRAC and TCR β chain (TRβC) loci (Berden et al., 2014; Eyquem et al., 2017; MacLeod et al., 2017; Roth et al., 2018). Placing the CAR under transcriptional control of the endogenous TRAC promotor has the benefit of mimicking the TCR expression level for optimal signalling. Eyquem et al., have monitored the dynamics of TRAC-targeted CAR expression upon encounter with antigen. Within 12 hours of exposure, CAR expression was downregulated to provide a physiological rest to T-cells which was subsequently returned to the TCR baseline within 36 hours. In comparison, retroviral CAR integration resulted in significantly higher expression (Eyquem et al., 2017).

Unlike retroviral gene insertion where multiple copies of the transgene are integrated in a non-specific manner, the number of insertions is relatively controlled, therefore improving the safety profile (Eyquem et al., 2017). The transgene can be inserted at one or two alleles, generating a heterozygous or homozygous edit, respectively. The TCRα chain is subject to allelic exclusion, where only a single TCRα chain is paired with a
TCRβ chain and expressed on the cell surface. However, the mechanism of TCRα chain allelic exclusion is fallible and in mice, expression of two TCRα chains can occur in up 10% of T-cells (Brady, Steinel and Bassing, 2010). In early experiments of this project, we co-transfected T-cells with a pool of two CAR-encoding donor templates and TRAC-targeted RNPs and observed a small portion of T-cells (<4%) that were double positive for the CAR templates. This demonstrates the failure to exclude one copy of the allele, resulting in a homozygous edit.

The TRAC locus has facilitated the screening of novel synthetic circuits to reprogram T-cell function. Roth et al., have developed a pooled knock-in strategy that uses CRISPR/Cas9 to introduce barcoded libraries of knock-in templates encoding switch receptors (reprogramming inhibitory receptors for TCR stimulation), cytokines and transcription factors, to enhance T-cell fitness (Roth et al., 2020). A selective pressure can be applied to the knock-in population in vitro including various resting, stimulation and immunosuppressive conditions, alternatively the engineered T-cells can be transferred to immunodeficient mice-bearing tumours for in vivo competition. Single-cell sequencing allows for the identification of beneficial knock-in templates and reveals cellular phenotypes induced by each construct. The developed method, PoKI-seq, identified of a novel chimeric TGFβRII-41BB receptor to enhance activity against solid tumours (Roth et al., 2020). A following study has combined this approach with the introduction of CAR-encoding sequences to identify advantageous transcription factor insertions in a context-dependent manner (Blaeschke et al., 2022).

Safe harbour sites have also been investigated for transgene insertion as a strategy to overcome clonal expansion due to insertional mutagenesis of retroviral and lentiviral vectors (Lombardo et al., 2011; Eyquem et al., 2013). Odak et al., have screened 373 genomic safe harbour sites for Cas9-mediated CAR insertion and found one site that maintained long-term expression and provided a potent anti-tumour response. CAR expression diminished from most of the identified sites, demonstrating the importance of the integration site on transgene expression (Odak et al., 2020).
Off-target effects remain a concern, however, new iterations of the endonuclease protein have been developed to improve specificity, such as Cas9 nickase (Ran et al., 2013), split Cas9 (Zetsche, Volz and Zhang, 2015), and dimeric CRISPR RNA-guided Fok1 (Guilinger, Thompson and Liu, 2014; Tsai et al., 2014). By splitting the activity of the endonuclease into two independent domains, two sgRNAs are required to target the insertion site. Another recent technology is Cas-CLOVER, which consists of two deficient Cas9 proteins that are fused to the Clo51 nuclease domains and facilitate DSB generation upon dimerization. Cas-CLOVER has been shown to reduce off-target effects and yields edited T-cells of a stem-like phenotype when combined with a piggyBac transposon encoding a CAR (Madison et al., 2022).

Repair of DSBs can lead to on-target mutagenesis including deletions, duplications, inversions, and chromosomal translocations. The first-in-human phase I clinical trial tested the safety of lentiviral-transduced NY-ESO-1 CAR T-cells with multiplexed editing of TRAC, TRβC and PDCD1 loci (Stadtmauer et al., 2020). Although durable engraftment was observed, chromosomal translocations were reported, with the most abundant resulting in a 9.3 kB deletion caused by rearrangements between TRβC1: TRβC2. A further study, using the same sgRNA sequences as Stadtmauer et al., to target the TCR and PDCD1 have observed frequent aneuploidy including a loss of chromosome 14 in 9% of the cells and gain of chromosome 14 in 1.4% of cells (Nahmad et al., 2022). This highlights the requirement to monitor chromosomal aberrations in clinical protocols and drives the development of alternative gene modification tools for multiplexed editing. An alternative gene delivery method is transposition, which is discussed in detail below.

1.2.2.2 Transposition

Transposon-based systems are the most common alternative to viral vectors due to their safe and reliable transfer of DNA and have been reported for manufacturing of CAR T-cell therapies (Singh et al., 2015). The general mechanism of transposition is exemplified in Figure 1.5, which involves the binding of transposase proteins to inverted terminal repeats (ITRs) that flank the transgene sequence, to initiate DNA excision and insertion into the target DNA. CAR T-cells generated using this approach have shown anti-tumour activity in reported in vitro and in vivo studies (Chicaybam et al., 2019).
1.2.2.1 Sleeping Beauty

The Sleeping Beauty (SB)/piggyBac (PB) vector contains the transgene flanked by inverted terminal repeats (ITRs) (orange triangles) that are recognised by the transposase proteins (green spheres) to form a circular pre-excision complex. The transposon is excised, generating double-stranded DNA breaks, which are subsequently repaired. The transposition complex recognises the target DNA, and the transposase proteins mediate integration.

Figure 1.5 General mechanism of transposition.

The Sleeping Beauty (SB) transposon system relies on the electroporation of two plasmids, the first encoding the CAR sequence between ITRs and the second that expresses the transposase. The delivery of clinical-grade plasmids is a relatively cost-effective method and requires less release testing than viral vectors, however, the electroporation of DNA plasmid can be toxic to T-cells and can result in substantial cell loss. The native SB systems were limited by low integration efficiencies, however, transposition rates have been improved with the transposase iteration, SB11 (Geurts et al., 2003) and a further 10- to 100-fold with SB100X (Jin et al., 2011). To overcome uncontrolled transposase gene activity by plasmid DNA delivery, published studies have successfully expressed the SB100X transposase recombinant protein for direct delivery to T-cells with the CAR-encoding transposon (Querques et al., 2019). The main advantage of this approach is the improved safety compared to viral vectors, the
integration profile is biased towards AT-rich palindromes and the integrated transposon has reduced promoter activity (Yant et al., 2005).

The SB technology entered the clinic in 2011 for the generation of patient- or donor-derived anti-CD19 CAR T-cells, which were administered as an adjuvant immunotherapy after autologous (NCT00968760) or allogeneic (NCT01497184) HSC transplantation (Kebriaei et al., 2016). The 30-month progression-free and overall survivals were 83% and 100%, following the autologous HSCT, and 53% and 63%, following the allogeneic HSCT. No acute or late toxicities were observed, demonstrating the safety and potential of SB to manufacture autologous and allogeneic CAR T-cells (Kebriaei et al., 2016). These pilot studies relied on plasmid vectors to deliver the SB components, which limited cell recovery and the number of stable CAR T-cells, therefore requiring a prolonged expansion period. Recent studies, including the CARAMBA trial, have addressed this manufacturing concern by engineering small transposon DNA vectors called minicircles. Minicircles are derived from their parental plasmids but are depleted in their vector backbone to reduce their size and associated toxicity in primary T-cells (Monjezi et al., 2017). The CARAMBA trial engineered SLAMF7-targeted CAR T-cells for the treatment of multiple myeloma by co-delivery of the transposon minicircle DNA with mRNA encoding the SB100X hyperactive transposase to CD8+ T-cells by nucleofection. The CD8+ CAR T-cells were then expanded for a period up to 12 days before formulation of the drug product (Prommersberger et al., 2021).

1.2.2.2.2 PiggyBac

The piggyBac (PB) transposon system has been developed as an alternative transposition method and uses a simple cut-and-paste mechanism for the insertion of DNA sequences into TTAA sequences and CpG islands (Galvan et al., 2009). Comparative studies have demonstrated similarities in the integration profile of PB transposons to MoMLV gamma retroviral vectors due to their preference for insertion into transcriptional start sites (Huang et al., 2010; Gogol-Döring et al., 2016). In mammalian cells, the PB system allows for the integration of large payloads (>13 kB) at superior transposition activity compared to SB transposases (Wu et al., 2006; Nakazawa et al., 2009). For example, the 7PB transposase variant outperforms SB100X by 2- to 3-fold (Doherty et al., 2012).
Due to low efficiencies in PBMCs, CAR T-cell manufacturing protocols have been optimised to enrich for the CAR+ population following transposition. This has been achieved by stimulation with artificial antigen-presenting cells (APCs) or optimised cytokine cocktails (Manuri et al., 2010; Morita et al., 2018). One study presents a significantly improved protocol for the selective expansion of edited T-cells using a combination of IL-4, IL-7 and IL-21 and achieves >90% CD19-CAR+ cells of an early memory phenotype with the low expression of exhaustion markers (Ptáčková et al., 2018). PB transposons have been used to express multiple integrated transgenes in T-cells, including CAR genes and iCasp9 safety switches. Here, the edited T-cells were enriched by 2-fold through expansion in IL-15 (Nakazawa et al., 2009). This approach enables the elimination of edited T-cells in the event of severe cytokine release syndrome (CRS) and demonstrates the application of PB transposons to improve the success of CAR T-cell therapies.

This section has provided a background to the methodologies for the insertion of heterologous DNA sequences, either by viral transduction or non-viral systems including CRISPR/Cas9 HDR and transposition. The clinic currently relies on retroviral and lentiviral mediated delivery, although, non-viral systems are of increasing interest, albeit low editing efficiencies and off-target concerns. The following section will discuss novel genome engineering technologies for the application of gene disruption.

1.3 Novel genome engineering technologies

The genome engineering field is rapidly advancing and has recently led to the discovery and application of novel CRISPR/Cas9-derived genome editing agents including nucleases, transposases, prime editors, and base editors. These technologies can be applied to insert CAR genes, as discussed in the previous section, and/or to reprogram T-cell functions through engineering gene knockouts. This section will focus on the genetic manipulation of cells with the focus of disrupting genes that are associated with poor CAR T-cell function including inhibitory receptors, such as programmed cell death protein-1 (PD-1).
1.3.1 Gene perturbation by CRISPR/Cas9

CRISPR/Cas9 offers unprecedented opportunities to reprogram T-cell functions for the benefit of cellular therapies, including the disruption of genes to modulate T-cell signalling. Lessons from the clinic have led to the identification of targets for gene knockout. In a study of chronic lymphocytic leukaemia, one patient treated with an anti-CD19 CAR T-cell therapy achieved rapid expansion originating from a single clone. It was found that the lentiviral vector inserted the CAR into the tet methylcytosine dioxygenase 2 (*TET2*) gene, which provided a proliferative advantage. Upon disruption by CRISPR/Cas9 *in vitro*, the functional advantages were recapitulated thus, highlighting *TET2* as a potential gene target (Fraietta, Nobles, *et al.*, 2018). Other gene targets have been identified through high-throughput gene knockout screens, which have discovered important regulators of T-cell proliferation, differentiation, and exhaustion. Dong *et al.*, found an RNA helicase, *DHX37*, that suppresses CD8+ T-cell activation, cytokine secretion and cytotoxicity by modulating NF-κB signalling, which was restored upon gene disruption (Dong *et al.*, 2019). Other studies have taken a targeted approach to knock out the expression of known drivers of exhaustion. Prinzing *et al.*, show that disruption of the epigenetic modifier, *DNMT3A*, can decrease methylation at the promoter regions of *TCF7* and *LEF1*, to promote T-cell stemness and prevent exhaustion (Prinzing *et al.*, 2021). In addition, Khan *et al.*, have shown that ablation of the thymocyte selection-associated high mobility group box protein (TOX) transcription factors can suppress exhaustion without inhibiting the formation of effector T-cells (Khan *et al.*, 2019). An alternative approach is to engineer T-cells to resist the inhibitory signals of the immunosuppressive tumour microenvironment (TME) and this approach is discussed in greater detail below.

1.3.1.1 Targeting inhibitory receptors

CAR T-cell therapies have shown remarkable responses for liquid tumour indications; however, the lack of persistence remains a challenge for the treatment of some haematological and solid tumour malignancies. Upon successful homing and infiltration, repetitive antigen stimulation can lead to exhaustion and loss of effector functions characterised by reduced proliferation, cytokine secretion and cytolytic capacity,
differentiation into terminal effectors, as well as the upregulation of multiple inhibitory receptors including PD-1, T-cell immunoglobulin and mucin domain 3 (TIM3) and lymphocyte-activated gene 3 (LAG3). These factors contribute to the lack of robust and durable responses in vivo.

The targeting of solid tumours is further limited by the physical barriers and immunosuppressive signals of the TME, which is formed of numerous cell types including extracellular matrix components and inflammatory mediators. The TME expresses ligands for binding to T-cell inhibitory receptors, to downregulate cytotoxic functions and to induce an exhaustive phenotype. Monoclonal antibodies targeting PD-1, TIM3 and LAG3 have demonstrated clinical efficacy to augment CAR T-cell therapies to prevent exhaustion, although, their effect can exacerbate adverse reactions and toxicities in patients. The genetic knockout of inhibitory receptors presents a new method to alleviate this risk and to provide a means to modulate the T-cell response to immunosuppressive signals.

The upregulation of inhibitory receptor ligands, including PD-L1 and PD-L2, has been correlated with a poor prognosis in patients, particularly in response to PD-1 blockade therapy. PD-1 is a member of the immunoglobulin superfamily and upon interaction with its ligands, strongly interferes with TCR signal transduction. PD-1 expression is transiently upregulated after T-cell activation and is a known marker of T-cell exhaustion. Rupp et al., have demonstrated that PD-L1 expression of tumour cells can impair CAR T-cell function, which can be restored via CRISPR/Cas9-mediated PD-1 disruption, and has highlighted the role of the PD-1/PD-L1 axis in tumour control (Rupp et al., 2017).

Cytokines secreted by the TME resident and tumour cells can suppress the function of T-cells. The transforming growth factor beta (TGFβ) family includes TGFβ1, TGFβ2 and TGFβ3, which are secreted by stromal cells including fibroblasts, mesenchymal stem cells, blood and lymphatic epithelial cells and pericytes. TGFβ signals through the type 1 and type 2 receptors. Signalling begins with the binding of TGFβ to TGFβ receptor 2 (TGFβR2), a serine/threonine receptor kinase, which subsequently recruits TGFβR1 for phosphorylation and activation of SMAD signalling. TGFβ can suppress T-cell function
by downregulating genes encoding cytokines, including perforin and granzymes in CD8+ cells, and by preventing differentiation and inducing Treg conversion in CD4+ cells. Treg conversion is an evolutionary conserved mechanism to prevent the exhaustion of CAR T-cells.

Alishah et al., have demonstrated that CAR T-cells retain their cytolytic capacity following CRISPR/Cas9-mediated knockout of TGFβR2, and can resist the anti-proliferative effects of exogenous TGFβ administration, thus, outcompeting the wild-type CAR T-cells. As identified by repeated stimulation assays, TGFβR2 knockout cells displayed less activation-induced exhaustion (Alishah et al., 2021). Furthermore, the attenuation of TGFβ signalling can enhance the efficacy of CAR T-cell therapy in solid tumour indications. In a patient-derived xenograft model of pancreatic cancer, Tang et al., observed an improvement in antitumour activity by TGFβR2-edited CAR T-cells compared to the non-edited control, and this effect was correlated with an increase in the central memory and effector memory subsets (Tang et al., 2020). CRISPR/Cas9-mediated disruption has also been applied to reduce the toxicities of CAR T-cells by knockout of the gene encoding granulocyte macrophage colony-stimulating factor (GM-CSF) and has abrogated the effects of cytokine release syndrome and neurotoxicity in vivo (Sterner et al., 2019).

1.3.1.2 Allogeneic approaches

Largely, the application of CRISPR/Cas9 technologies has been applied to the generation of allogeneic CAR T-cell therapies, which requires knockout of the endogenous TCR (through TRAC-targeting) and elimination of the major histocompatibility complex (MHC) genes to prevent graft-versus-host-disease (GvHD) and T-cell-mediated rejection, respectively. The MHC class I genes, also referred to as human leukocyte antigen (HLA) class I (HLA-A, HLA-B, HLA-C), have largely been disrupted by targeting Cas9 to the beta 2 microglobulin (β2M) locus. Studies demonstrate that double (TRAC and β2M) and triple knockout (TRAC, B2M, PDCD1) CAR T-cells have reduced alloreactivity and enhanced antitumour activity in vitro and improved engraftment and proliferation in vivo (X. Liu et al., 2017; Ren, Liu, et al., 2017).
Ren et al., have further developed a one-shot protocol for the generation of Fas-resistant universal CAR T-cells by encoding multiple sgRNAs within the CAR lentiviral cassette and transfecting the cells with Cas9 mRNA. Activation of the T-cell receptor, CD95, by Fas ligand can induce terminal differentiation and apoptosis thereby limiting the activity of CAR T-cells. Multiplexed gene disruption of TRAC, PDCD1 and CD95 has attenuated activation-induced cell death and has improved Fas-resistance (Ren, Zhang, et al., 2017). This study also attempted the multiplexed editing of four gene loci (TRAC, β2M, PDCD1 and CTLA4), however, this resulted in very low efficiencies and highlights the importance of efficient delivery methods.

Complete ablation of HLA-class I molecules by β2M knockout can render allogeneic therapies to NK-mediated rejection through “missing self” recognition. Intellia Therapeutics, a CRISPR-based biotechnology company, have developed an allogeneic platform that simultaneously disrupts the TRAC locus and integrates a CD30-targeted CAR by HDR, combined with the knockout of HLA class II and an undisclosed receptor “X”, designed to reduce but not abrogate HLA class I expression. This approach eliminates GvHD concerns, avoids recognition by host CD4+ and CD8+ T-cells and provides protection from NK-mediated rejection in comparison to the β2M knockout control (Schultes, Birgit, 2022). An alternative approach shown by Jo et al., uses TALENs and AAV to insert the NK inhibitor, HLA-E, into the genome of TRAC- and β2M-edited T-cells, to compensate for “missing self” (Jo et al., 2022).

It is widely recognised that multiplexed genome editing with Cas9 can lead to chromosomal translocations occurring from multiple DSBs. Hence, Intellia Therapeutics’ process involves sequential editing steps to circumvent this issue. The delivery of genome editing components is commonly performed by electroporation and can have detrimental effects on cellular viability and recovery. To support their complex engineering requirements (TRAC, β2M, “X”), Intellia Therapeutics report the use of lipid nanoparticles to enhance cellular functionality and produce allogeneic products with efficient gene knockouts (>90%) and high insertion rates (>80%) whilst maintaining a high TSCM phenotype (>70%) (Schultes, Birgit, 2022).
1.3.2 The safety of CRISPR/Cas9 multiplexed genome editing

Genome-edited T-cells have been safely infused into patients and clinical trials are ongoing to investigate their improvement to therapeutic outcomes (Y. Lu et al., 2020; Stadtmauer et al., 2020). Genome engineering efficiency, specificity and safety are key requirements for their application to ex vivo cellular therapies. Off-target effects can lead to genome instability, the activation of oncogenes, inactivation of tumour suppressor genes, as well as the disruption of essential genes. The analysis of sgRNA off-target effects has been achieved by sequencing methods including GUIDE-seq (Tsai et al., 2015), Digenome-seq (Kim et al., 2015), or CIRCLE-seq (Tsai et al., 2017). In addition to gRNA-dependent off-target effects, the imprecise repair of DSBs can lead to indels, duplications, inversions, or chromosomal translocations (Kosicki, Tomberg and Bradley, 2018). Chromosomal rearrangements are a particular challenge for multiplexed editing where concurrent DSBs can yield multiple ssDNA ends for replication and repair (Leibowitz et al., 2021; Nahmad et al., 2022). These rearrangements have been observed in CRISPR-engineered CAR T-cell products targeting the TRAC, TRβC1, TRβC2 and PDCD1 genes for disruption (Stadtmauer et al., 2020), and have been observed in preclinical studies of CAR T-cells engineered by TALENs (Poirot et al., 2015; Qasim et al., 2017). Genome engineering technologies that do not require DSBs, such as cytosine and adenine base editors, offer a potentially safer alternative for the multiplexed genome modification of CAR T-cells.

1.3.3 Base editing

Base editing involves the precise and irreversible conversion of one base to another without introducing any DSBs (Komor et al., 2016; Gaudelli et al., 2017). Base editors (BEs) are formed of three main components, a catalytically impaired form of the Cas nuclease, a programmable sgRNA to direct specificity, and a deaminase protein that can act upon ssDNA within a narrow editing window, termed the BE window. Cytosine and adenine base editors (CBEs and ABEs) were the first to be developed, although recent work has expanded the scope of BEs.
1.3.3.1 Mechanism of action

BEs employ deaminases to convert either cytosine-to-thymine, or adenine-to-guanine. Cytosine deaminases currently used for base editing purposes include apolipoprotein B editing complex 1 (APOBEC1), activation induced deaminase (AID) or apolipoprotein B editing complex 3 (APOBEC3G) (Komor et al., 2016). Adenine base editors typically rely on the TadA tRNA adenosine deaminase from *Escherichia coli* (*E. coli*) (Gaudelli et al., 2017, 2020). The activity of the deaminases is restricted to certain sites in the genome by fusion to a catalytically inactive Cas9 (deficient Cas9) or a Cas9 nickase. This prevents cleavage of the modified strand and removal of the converted base. The sgRNA directs the BE to the target site by binding to the complementary DNA of the protospacer, upstream of the PAM. An R-loop is formed, and the non-target strand is displaced, allowing base conversion on the ssDNA by the deaminase (cytosine-to-uridine or adenine-to-inosine). In BEs that utilize a Cas9 nickase, the non-edited strand will be nicked, and this will initiate DNA repair mechanisms. The base substitution is then carried over into the target strand when the modified base is read by the DNA polymerase. Uridine will be read as thymidine, resulting in the insertion of an adenine on the target strand, and inosine will be read as guanine, resulting in the insertion of a cytosine (Komor et al., 2016; Gaudelli et al., 2017). The mechanism of base editing with a cytosine base editor, BE4max, is exemplified in Figure 1.6.
Figure 1.6 Mechanism of base editing with a cytosine base editor BE4max

Cytosine base editors (CBEs) convert cytosine-to-thymine, via a uracil intermediate, using a Cas9 nickase or deficient Cas9 (dCas9) (blue) fused to a cytidine deaminase (red), which is guided to the protospacer through a complementary single guide RNA (sgRNA) (purple). The BE4max CBE uses an APOBEC1 cytidine deaminase and has two copies of the uracil glycosylase inhibitor domain (UGI) (lilac) to prevent excision of the converted uracil, therefore increasing the enzyme’s efficiency. The binding of the Cas9 sgRNA to the genomic DNA facilitates R-loop formation and dissociates the DNA strands. The single-stranded DNA becomes available for deamination of the target cytosine, converting the cytosine (C) to a uracil (U). The DNA is nicked on the opposite strand and DNA replication and repair will convert the G: U heteroduplex to a A: T base pair. Figure adapted from (Komor et al., 2016).

1.3.3.2 Applications of base conversion

1.3.3.2.1 Gene correction

Base editing was originally harnessed as a method to correct pathogenic mutations that lead to human disease. BEs have been used to correct genetic diseases by \textit{ex vivo} genetic
modification or by in vivo editing. ABEs can successfully convert the pathogenic sickle variant of the β-globin gene \(HBB^s\) to the benign Makassar variant \((HBB^G)\) in haematopoietic stem and progenitor cells (HSPCs) of sickle cell disease (SCD) patients. An editing efficiency of 80% was achieved through the electroporation of ABE mRNA with a targeted gRNA. Sixteen weeks after transfer to immunodeficient mice, 68% of cells contained \(HBB^G\) and the sickling of reticulocytes was decreased by 5-fold (Newby et al., 2021). CBEs have also been employed to treat other blood disorders including β-thalassaemia (Zeng et al., 2020).

In vivo base editing has shown promise for the treatment of Hutchinson-Gilford progeria syndrome which is caused by a dominant-negative C > T (c.1824; p.G608G) mutation in the gene that encodes nuclear lamin A (\(LMNA\)). This mutation results in the mis-splicing of RNA and leads to the production of the toxic progerin protein that results in premature ageing. In fibroblasts from children, lentiviral delivery of the directed ABE resulted in 87-91% correction of the allele. To investigate the potential of in vivo delivery, mice that were homozygous for the pathogenic allele were treated with a single retro-orbital injection of ABE-containing AAV9. A reduction of progerin protein levels was observed combined with an extended lifespan from 215 to 520 days (Koblan et al., 2021).

The treatment of genetic diseases relies on the ability to deliver BEs in vivo to the relevant cell types. Despite promising studies, the delivery of ABE vectors remains a challenge and can limit editing efficiencies. A dual AAV system has been developed to improve ABE packaging, where the CBE protein was split into the N and C terminal for reassembly using intein sequences for reconstitution by trans-splicing. This approach has enabled therapeutically relevant editing efficiencies in the liver (38%), retina (38%), heart (20%) and skeletal muscle (9%) (Levy et al., 2020).

Recently, base editing has been performed in non-human primates to edit the \(PCSK9\) gene to lower cholesterol levels. ABE mRNA and a gRNA targeting \(PCSK9\) was delivered to macaques by lipid nanoparticle-based delivery. PCSK9 and low-density lipoprotein levels were reduced by 32% and 14% respectively with no detection of off-target
mutations. This work supports the application of ABEs for in vivo correction of monogenic liver diseases (Rothgangl et al., 2021).

David Liu, the pioneer of base editing technology, has founded a clinical-based base editing company, Beam Therapeutics, whose colleagues are developing several base-editing treatments including the ex vivo cellular therapy, BEAM-101, which has received IND clearance for the treatment of SCD or β-thalassaemia (Beam Therapeutics Inc., 2022). Other applications of base editors are under preclinical study and include the correction of pathological mutations for Glycogen Storage Disease Type 1a (Aratyn-Schaus, 2022) and α-1 antitrypsin deficiency (Beam Therapeutics Inc., 2022; Packer et al., 2022). BEs have also been employed to disrupt gene expression, as discussed in the following section.

1.3.3.2.2 Gene knockout

CBEs and ABEs can be employed to facilitate precise and controlled gene disruption by targeted deamination. The sgRNA sequence can be designed to position point mutations in precise locations of the coding sequence to either disrupt mRNA splicing or to introduce premature stop codons (UAA, UGA, UAG), resulting in the attenuation of translation (Billon et al., 2017; Kuscu et al., 2017; Kluesner et al., 2018).

BEs provide the possibility of multiplexed editing without the associated risk of DNA rearrangements. Preece et al., have employed CBEs to install stop codons in the TRβC1/2 chains of T-cells, to simultaneously disrupt the endogenous TCR upon replacement with a hepatitis B-virus specific recombinant TCR by lentiviral delivery (Preece et al., 2020). Alternatively, BEs have been employed to combine disruption of TRAC, β2M and PDCD1 with transduction of a CD19-targeted CAR. In contrast to CRISPR/Cas9-edited cells which resulted in ~2% translocation frequencies, the base-edited CAR T-cells showed no chromosomal rearrangements (Webber et al., 2019).

Beam Therapeutics are developing a base-edited allogeneic CAR T-cell product, BEAM-201, for the treatment of T-cell acute lymphoblastic leukaemia (T-ALL). A recent press release has reported high ex vivo editing efficiencies of 96-99% across four loci and 85% transduction of the CD7-targeted CAR, whilst 77% of the polyclonal product was
estimated to contain all five genetic modifications. *In vitro* studies demonstrated robust cytokine secretion and rapid cytotoxicity, with the absence of chromosomal translocations and aberrant p53 activation (Beam Therapeutics Inc., 2020).

Similarly, Georgiadis *et al.*, report the preclinical development of base-edited CD3- and CD7-targeted CARs for combinatorial therapy of T-ALL. Base editing of the endogenous TCR/CD3 and CD7 prior to lentiviral transduction to introduce the anti-CD3 and anti-CD7 CARs, provides a fratricide-resistant universal CAR-T approach. The edited population was self-enriched to yield populations of 99.6% TCR-CD3-CD7- and exhibited high cytotoxicity in a human xenograft murine model. Upon molecular interrogation, no translocations were observed, supporting the application of BEs compared to previous CRISPR/Cas9 and TALENs approaches (Georgiadis *et al.*, 2021).

### 1.3.3.3 Improving base editors

Since their development in 2016, BEs have received extensive optimisation to advance their technical capabilities. Various iterations of base editors have been engineered to improve efficiency, targeting scope and specificity. Research efforts continue to develop new variants of base editors, such as those that support C-to-G transversion editing (Kurt *et al.*, 2021) or dual editing at a single protospacer, for example, the CRISPR-Cas9-based synchronous programmable adenine and cytosine editor (SPACE) (Grünewald *et al.*, 2020).

#### 1.3.3.3.1 Improving efficiency

A strategy to improve the editing efficiencies of CBEs involves inhibiting endogenous enzymes of the base-excision repair pathway. Base-excision repair corrects small lesions in the DNA sequence and is initiated by a DNA glycosylase to remove the damaged base. To prevent excision of the modified base by uracil DNA N glycosylase, BE enzymes have been fused with copies of the uracil DNA glycosylase inhibitor (UGI). The most widely used CBEs include BE3, a spCas9 nickase with rat APOBEC1 and a single copy of UGI, and BE4, which contains two copies of the UGI (Komor *et al.*, 2017). Optimisation of codon usage can further improve base conversion efficiencies, BE4max was produced by
Genscript’s codon optimisation method and demonstrated 1.8-fold higher editing compared an engineered BE4 with IDT codons (Koblan et al., 2018).

Combined with codon-optimisation efforts, improvements to early generation ABEs have involved the mutagenesis of the TadA deaminase (Gaudelli et al., 2017; Koblan et al., 2018). ABE7.10 was engineered to include two copies of the deaminase, an N-terminal wild-type TadA that is fused to an evolved TadA (TadA-7.10) at the C-terminal. ABE7.10 facilitated a base conversion of approximately 50% in human cells with high product purity (Gaudelli et al., 2017). Through screening a library of TadA deaminases, ABE7.10 was further evolved into the ABE8 variant, which contains a single engineered TadA domain (Gaudelli et al., 2020). Other strategies have involved phage-assisted evolution to improve deamination efficiencies by up to 590-fold (Richter et al., 2020).

### 1.3.3.3.2 Expanding targeting scope

When selecting a BE, it is important to consider the position of the target base and its surrounding sequence. The base editing window is defined as the region of bases upstream of the PAM where the target base may occur. The position of the window will vary depending on the deaminase, but typically this lies between bases 4 and 8 (where the PAM is 21-23). Deaminases also vary in their sequence preference, and this will influence the base conversion efficiency. Some deaminases such as APOBEC1 favour a thymidine before the target cytosine and others prefer a guanosine (Anzalone, Koblan and Liu, 2020). An appropriate base editor should be selected considering the PAM requirement of the endonuclease and the sequence preference of the deaminase.

Although the *Streptococcus pyogenes* Cas9 offers the least restrictive PAM sequence, some genes remain challenging to target. Kim *et al.*, have generated novel BE3 BEs with natural or engineered Cas9 variants to expand the number of target sites by 2.5-fold (Kim *et al.*, 2017). The directed evolution of Cas9 has led to the generation of new PAM variants to widen the targeting scope of CBEs. Hu *et al.*, have used phage-evolution to develop the xCas9 variant which relaxes the PAM requirement to NG, GA and GAT sequences whilst retaining high DNA specificity (J. H. Hu *et al.*, 2018). Other studies
have developed a Cas9 variant with a near-PAMless sequence requirement, named SpRY, using structural-guided engineering methodologies (Walton et al., 2020).

1.3.3.3 Reducing off-targeting

gRNA-independent off-target effects have been observed with APOBEC deaminases and can occur in both DNA and RNA sequences, thus driving the development of CBE and ABE mutants (Grünewald, Zhou, Iyer, et al., 2019; Zhou et al., 2019; Gaudelli et al., 2020). Grünewald et al., have engineered a CBE variant, SECURE-BE3, that contains R33A and K34A mutations in the rat APOBEC1 enzyme to decrease the frequency of off-target RNA edits by 3800-fold (Grünewald, Zhou, García, et al., 2019). Structural-guided evolution of SECURE-BE3 has yielded further improved variants (Grünewald, Zhou, Iyer, et al., 2019). Whereas Yu et al., describe eight next-generation BE4 CBEs with either RrA3F, AmAPOBEC1, SsAPOBEC3B or PpAPOBEC1 deaminase iterations that provide a 45-fold reduction in off-target DNA effects and a 69-fold reduction in C-to-U edits in the transcriptome (Yu et al., 2020).

1.3.3.4 Base editor delivery

The delivery of the BE enzyme has historically been achieved by plasmid DNA transfection, which presents the highest off-target effects on both DNA and RNA sequences. Alternative methods including mRNA and protein delivery have significantly improved the safety profile of BEs (Rees et al., 2017; Villiger et al., 2021). Rees et al., have reduced off-target base editing of BE3 by installing four mutations (N497A, R661A, Q695A and Q926A) to reduce Cas9 DNA binding affinity. The engineered high-fidelity-BE3 (HF-BE3) was complexed with the gRNA and delivered into mammalian cells using the cationic lipid transfection reagent, Lipofectamine 2000. Lipid-mediated delivery of the RNP was sufficient to reduce off-target editing compared to plasmid DNA delivery (Rees et al., 2017). It was hypothesised that APOBEC overexpression is the main cause of off-target editing. The production of the BE protein has been reported in two studies, in HEK293Ts and E. coli, and remains a key challenge due to its instability (Zeng et al., 2020; Jang et al., 2021). Delivery efforts have moved towards the co-transfection of gRNA and BE mRNA, which is typically achieved by electroporation. In human cells
Webber et al., and Gaudelli et al., found no detectable off-target mutations at the DNA or RNA level following the transfection of BE4max or ABE8 mRNA (Webber et al., 2019; Gaudelli et al., 2020).

In summary, the function of CAR T-cells can be reprogrammed through the transient expression of genome editing components including Cas9 endonuclease or CBEs. This study uses the BE4max editor to knock out inhibitory receptors in T-cells and aims to incorporate base editing into a clinical manufacturing process for the generation of CAR T-cells for Hodgkin Lymphoma therapy. The following section introduces the transfection of RNA for the delivery of such components.

1.4 RNA for T-cell reprogramming

Non-viral gene delivery approaches have typically relied on the electroporation of DNA plasmids. This is a simple and cost-effective technique, however, the presence of DNA in the cytoplasm can cause toxicity, resulting in dramatically reduced cell viability and survival. Recently, the transfection of DNA has been surpassed by the delivery of messenger RNA (mRNA), which is well tolerated in the cytoplasm and improves the survival of transfected cells. The transfected RNA is translated using the host-cell machinery; however, the RNA is relatively unstable and is rapidly degraded by exonucleases, leading to transient expression. The stability of RNA can be modestly improved through modifications to the RNA sequence, as described below.

1.4.1 Improving RNA stability

Previous efforts to improve the stability of mRNA include the incorporation of a 5’ cap, 5’ and 3’ untranslated regions (UTRs), nucleoside modifications, and a 3’ polyadenylation (poly(A)) tail. In vitro transcription typically involves the addition of a 5’ N7-methylguanosine (m7G) cap, also known as a Cap 0 structure, which is added to the first nucleotide via a 5’ to 5’ triphosphate bridge. The 5’ cap is important for the recruitment of translational initiation factors and for preventing RNA degradation. The Cap 0 structure and can be incorporated into transcripts in two orientations, of which only one is functional. Some studies use the anti-reverse cap analog (ARCA) which results in
a modified Cap 0 mRNA, ensuring its incorporation in the correct orientation, to yield only translatable transcripts (Hadas et al., 2019). Phosphorothioate ARCA cap analogs have also been described to further promote transcript stability (Kuhn et al., 2010). Alternatively, the Cap 0 structure can be converted to a Cap 1 structure by the addition of a methyl group on the 2’O position of the initiating nucleotide. Generally, transcripts of the T7 promoter initiate with a guanine at the +1 transcript position (cytosine in the template DNA), hence, the cap analog is supplied in the transcription reaction at an excess over GTP to promote its incorporation. A recent study reports the CleanCap® Cap 1 AG trimer technology to improve the capping efficiency compared to ARCA protocols (~95% efficiency compared to ~70% using ARCA capping) (Vaidyanathan et al., 2018). Vaidyanathan et al., further demonstrate the importance of the Cap 1 structure to evade innate sensing of synthetic mRNA encoding the Cas9 sequence (Vaidyanathan et al., 2018).

Other studies have turned towards the incorporation of UTRs to tune the sequence for optimal protein expression. One study has developed a model for screening of over 35,000 truncated 5’ UTRs and has predicted the ribosome loading on these sequences (Sample et al., 2019). Screening studies have facilitated the discovery of novel synthetic UTRs for optimising protein expression in non-viral cassettes (Cao et al., 2021). Other reports have incorporated 3’ UTRs including the sequence derived from human beta globin mRNA (Holtkamp et al., 2006).

Nucleoside modifications such as pseudouridine (ΨU) can reduce the immunogenicity of the RNA molecule by preventing signals through Toll-like receptors (TLRs) which recognise double-stranded RNA (recognised by TLR3) or single-stranded RNA (recognised by TLR7 and TLR8) (Karikó et al., 2005). Upon administration to mice, only unmodified mRNA resulted in an interferon response (Karikó et al., 2008). Additional studies have combined modifications including N1-methylpseudouridine (m1ΨU) and 5-methylcytidine (m5C) to further augment RNA stability by evading innate immunity. The expression of luciferase mRNA was improved by 118-fold compared to ΨU-modified mRNA or 23-fold compared to m1ΨU-modified mRNA (Andries et al., 2015). Codon
optimisation of the transcript sequence has also significantly improved expression in yeast cells (Presnyak et al., 2015).

In eukaryotic cells a poly(A) tail of 100-250 bases is added post-transcriptionally to enhance mRNA stability and translation. The poly(A) tail interacts with polyadenosyl-binding proteins that associate with translational initiation factors at the 5’ cap, therefore generating a closed mRNA structure (Gallie, 1998). Whilst the *in vitro* production of proteins can occur from mRNAs with much shorter tails, studies have reported a positive correlation between poly(A) tail length and translational efficiency (Peng, Murray and Schoenberg, 2008). Recent studies report the benefit of adding non-adenosine residues into poly(A) tails, also known as mixed tailing, which is can be facilitated by terminal nucleotidyl-transferase (TENT) 4A and TENT4B (Lim et al., 2018). Further work has involved engineering transcripts to contain multiple modifications including cap analogs, nucleoside modifications and poly(A) tails, for maximal benefit (Andries et al., 2015; Vaidyanathan et al., 2018; Omer-Javed et al., 2022).

In recent years, focus has honed upon the development of exon containing circular RNAs (circRNAs) as a novel method to improve RNA stability by protecting the 5’ and 3’ ends from exonuclease degradation. The following section will provide a literature review of circRNAs including their endogenous functions and synthetic applications, alongside exploring the current methods for producing translatable circRNAs *in vitro*.

### 1.4.2 Introduction to circular RNA

mRNA is widely used in biological systems to transiently express desired proteins and can be transfected with high efficiency and low toxicity; however, one fundamental limitation is its instability and rapid turnover. CircRNA is structurally different to mRNA and is formed by back-splicing of pre-mRNA (Wang and Wang, 2015). The 5 prime (5’) and 3 prime (3’) ends are ligated together with a phosphodiester bond to form a covalently closed single-stranded structure. CircRNA lacks the exposed ends of linear RNA and is therefore resistant to exonuclease degradation, improving the half-life and transgene expression. CircRNAs have been documented throughout nature (Salzman *et al*., 2012;
Jeck et al., 2013) and have received increasing interest in the biotechnology field due to their diverse functions.

1.4.2.1 Functions of endogenous circRNA

Endogenous circRNAs typically contain 1 to 5 exons and are on average 500 nucleotides (nt) long. The biogenesis of circRNA is a slow process that occurs post-transcriptionally by back-splicing (Zhang et al., 2016). The back-splicing reaction is mediated by spliceosome machinery and is facilitated by the presence of intronic complementary sequences (ICSs), such as Alu repeats, which enables base pairing to bring the downstream splice donor and upstream splice acceptor sites into close proximity (Jeck et al., 2013; Liang and Wilusz, 2014). Back-splicing is a relatively inefficient process and competes with canonical splicing, leading to a relatively low abundance of endogenous circRNAs (Ashwal-Fluss et al., 2014). RNA-binding proteins (RBPs) can bind to ICSs to increase or dampen circRNA expression and can act as a bridge between distal splice sites to enhance the back-splicing reaction (Conn et al., 2015).

Although some intron-containing and intron-lariat circRNAs have been reported in the nucleus, the majority of exon-containing circRNAs are localised in the cytoplasm (Salzman et al., 2012). RNA interference (RNAi) screens have identified factors involved in the nuclear export of circRNAs. The factors UAP56 and URH49 have been identified as key modulators of circRNA localisation and are responsible for controlling the export of long and short circRNAs, respectively (Huang et al., 2018). CircRNAs are resistant to exonuclease degradation, and this has led to their relatively stable half-life and accumulation in non-dividing cells. One study compared the expression of 60 circRNAs compared to their cognate linear RNAs and found that half-lives ranged from 18.8 to 23.7 hours compared to 4.0 to 7.0 hours with linear RNA (Enuka et al., 2016).

1.4.2.1.1 Non-coding functions

RNAi screens using short hairpin RNA (shRNA) and small interfering RNAs (siRNAs) to target the back-splicing junction (BSJ) have facilitated knockout studies to study the function of circRNAs. Using an shRNA library, Chen et al., discovered 171 circRNAs out of 1500 circRNAs to be involved in cellular proliferation of prostate cancer cell lines
(S. Chen et al., 2019). Further studies have utilised the RNA-targeting type VI CRISPR/Cas13 system for the specific knockout of circRNAs whilst retaining the linear counterparts, and has identified a group of circRNAs that are important for proliferation in a cell-type-specific manner (Li et al., 2021). Base editors can be directed to install point mutations in back-splice sites to facilitate the knockout of circRNA isoforms. A recent study has performed a small-scale base editor screen and identified an exon of the ZNF292 gene locus that represses cellular proliferation (Gao et al., 2022).

Although their functional mechanisms are yet to be fully elucidated, circRNAs are thought to influence the activity of RNA polymerase (RNAP) II and the rate of gene transcription, and to participate in the sequestering of micro RNAs (miRNAs) and proteins. CircRNAs have been shown to interact with nucleic acid to activate or repress gene expression, or to interfere with the splicing of linear RNA isoforms. A class of non-coding intron-containing circRNAs have been shown to promote transcription of their paternal genes in cis by binding with small nuclear RNAs (snRNA) and forming ribonucleic complexes with RNAP II to enhance gene expression (Li et al., 2015). On the other hand, circRNAs can anneal with genomic DNA to form a DNA: RNA triplex structure (R-loop) which results in transcriptional pausing and exon skipping (Conn et al., 2017). Alternatively, transcription can be modulated by the activation of transcription factors. One published study identifies circKcnt2, a circRNA derived from exons 4 to 8 of Kcnt2 pre-mRNA, that recruits the nucleosome remodelling deacetylase (NuRD) complex to the Baf1 promoter to repress gene expression (B. Liu et al., 2020).

CircRNAs can also act as miRNA or protein sponges by binding and sequestering their activity. Some circRNAs contain miRNA binding sites such as circBIRC6, which is expressed in human embryonic stem cells and directly interacts with miR-34a and miR-145 to modulate differentiation and pluripotency (Yu et al., 2017). Other circRNAs can bind directly to mRNAs to reduce their translation or to outcompete mRNAs for binding with target proteins. cia-cGAS was identified in murine bone marrow and binds to the cGAS protein via a dsRNA region to prevent cGAS-mediated exhaustion in quiescent haematopoietic stem cells (HSCs) (Xia et al., 2018). Additional studies have demonstrated that circRNAs can dampen innate immune responses by binding to
pathogen recognition receptors including the dsRNA receptor protein kinase R (PKR) to suppress signalling. Upon viral infection and RNase L-mediated circRNA degradation, this leads to PKR activation and initiation of the innate immune response (Liu et al., 2019). CircRNA-protein binding can interfere with signalling pathways including the regulation of cell-cycle progression (Du et al., 2016), cellular proliferation (Tsitsipatis et al., 2021) and mitochondrial metabolism (Zhao et al., 2020).

1.4.2.1.2 Translatable circular RNAs

Translatable circRNAs are localised to the cytoplasm and have been discovered by mass spectrometry studies through the identification of BSJ junctions, internal ribosome entry sites (IRES), and N6-methyladenosine (m6A) modifications. CircRNAs do not contain the 5’ cap structure of linear RNAs, and therefore require an IRES sequence to initiate translation in a cap-independent manner. The addition of IRES sequences to synthetic circRNAs enables sufficient protein expression in vitro and demonstrates the translatable potential of endogenous IRES-containing circRNAs (Wesselhoeft, Kowalski and Anderson, 2018). Examples in nature include circZNF609 and circFGFR1, which are respectively involved in myogenesis and the control of proliferation during cellular stress (Legnini et al., 2017; C.-K. Chen et al., 2021). Alternatively, m6A modifications are abundant in human cells and have been shown to recruit initiation factors including eLFG2 and an m6A reader, YTHDF3, to promote translation (Yang et al., 2017).

Endogenous circRNAs have diverse functions and can interact with nucleic acids and proteins to modulate gene transcription, RNA splicing, to sponge miRNAs or proteins, whilst some have translational capacity. Studies have demonstrated their interference with signalling pathways including cellular proliferation, innate sensing, pluripotency and differentiation, and cell-cycle regulation.

1.4.2.2 Applications of synthetic circRNAs

The functions of endogenous circRNAs can be harnessed and enhanced by synthetic engineering of the RNA sequence. CircRNAs can be generated using chemical, enzymatic or ribozymatic methods to ligate the 5’ and 3’ ends of the RNA sequence into a covalently closed circular RNA. This thesis uses a self-splicing permuted intron-exon
sequence derived from the *Anabaena* group I intron to generate circRNA *in vitro*, as illustrated in Figure 1.7. The generation of circRNA is described in detail in section 1.4.2.3 and the following section summarises the current applications of synthetic circRNAs in the biotechnology field. In brief, the exogenous delivery of circRNAs can be applied to manipulate cellular functions using aptamer technologies, to suppress signalling pathways and pathological miRNAs as therapeutic agents, to modulate immune responses, or to enable continued protein expression *in vivo*.

**Figure 1.7 The structure of circular RNAs and their production**

Circular RNA is produced using an optimised permuted intron-exon (PIE) splicing method, described by Wesselhoeft et al., 2018. The construct contains the self-splicing intron from *Anabaena* pre-transfer RNA (tRNA) flanking an internal ribosome entry site (IRES) from coxsackievirus B. Splicing of the *Anabaena* PIE sequence produces a circular RNA where the 5-prime end of the CVB3 IRES is linked to the 3-prime end of the open reading frame (ORF).
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The spacer sequences separate the PIE intron sequences from the IRES and the ORF to reduce interference. Internal homology (IH) sequences and homology arms (HA) promote the folding of the RNA into a splicing bubble. When pre-mRNA is provided with magnesium ions (Mg\(^{2+}\)) and guanosine triphosphate (GTP), two transesterification reactions occur and because the exons are already fused together and orientation of the splicing sequences is reversed, this facilitates back-splicing and the release of circular RNA from the introns.

1.4.2.2.1 Imaging cellular signalling and manipulating function

Synthetic biologists have engineered circRNA aptamers to overcome current limitations including expression and stability in mammalian cells. Litke and Jaffrey have developed the Twister-optimised RNA for durable overexpression (Tornado) system to probe cell function. The Tornado system contains the RNA of interest flanked by Twister ribozymes to mediate autocatalytic cleavage and ligation into RNA circles by endogenous RNA ligase, RtcB. The aptamers are potent inhibitors of protein expression as exemplified by the NF-κB circRNA aptamer, which binds to the p65 subunit and supresses NF-κB signalling (Litke and Jaffrey, 2019). This approach can be used to image cellular processes by co-expression of a fluorescent Broccoli aptamer and can improve aptamer expression by 200-fold compared to linear RNA in HEK293T cells (Filonov et al., 2014; Litke and Jaffrey, 2019).

1.4.2.2.2 Aptamer therapeutics

Non-coding circRNA aptamers have also demonstrated potential for therapeutic purposes to sponge disease-related miRNAs or to modulate innate immune responses. A proof-of-concept study has shown that circRNAs can sequester viral miRNAs to inhibit viral protein production, including miRNA-122 from the Hepatitis C virus (Jost et al., 2018), whilst others have focused on the inhibition of cancer-associated miRNAs, such as miRNA-21, to reduce proliferation of tumour cell lines (Liu et al., 2018). Some autoimmune diseases such as systemic lupus erythematosus (SLE) suffer from a downregulation of endogenous circRNAs, including those that bind to the dsRNA receptor PKR to prevent aberrant innate responses in the absence of viral infection. Endogenous circRNAs can be overexpressed in SLE-derived T-cells to dampen PKR activation and correct cytokine signatures (Liu et al., 2019). To support clinical
manufacturing of therapeutic aptamers, a streptavidin sequence can be inserted into the sequence to facilitate \textit{in vitro} or \textit{in vivo} purification (Umekage and Kikuchi, 2009).

\textbf{1.4.2.2.3 Gene therapy}

Circular aptamers can be engineered to contain anti-sense gRNA sequences to direct endogenous adenosine deaminases acting on RNA (ADAR) enzymes and improve adenosine-to-inosine editing efficiencies by 3.5-fold compared to linear gRNA (Katrekar \textit{et al.}, 2022). This study used AAV vectors to deliver circular gRNAs \textit{in vivo} for the correction of the amber nonsense mutation in a mouse model of mucopolysaccharidosis type-1-Huler syndrome. Alternatively, RNA circularisation can enhance prime editing efficiencies by improving the stability of the prime editor guide RNA (pegRNA), as well as providing flexibility to their design. The pegRNA can be split into two components, a gRNA sequence and a circular reverse transcription template for copy into the genome (B. Liu \textit{et al.}, 2022).

\textbf{1.4.2.2.4 Translatable circRNAs}

CircRNAs can serve as templates for continuous protein expression by removal of the stop codon sequence to generate an infinite open reading frame (ORF) (Nakamoto \textit{et al.}, 2020). The most common application for translatable circRNAs is the production of cell factories, predominately in bacterial cell-free expression systems. Abe \textit{et al.}, have demonstrated the continuous translation of circRNAs in \textit{E. coli} lysate by rolling circle amplification to increase protein production by over 100-fold compared to a linear control (Abe \textit{et al.}, 2013). Further work has evaluated the protein expression in mammalian lysate and in live human cells (Abe \textit{et al.}, 2015). Through incorporation of 2A self-cleaving peptide sequences, this approach can improve the production of secreted multimeric recombinant proteins, as exemplified by the expression of the human glycoprotein Erythropoietin in CHO cells (Costello \textit{et al.}, 2019).

Alternatively, circRNAs can be used to express viral proteins for vaccine therapy. The COVID-19 pandemic has exacerbated the development of modified RNA therapeutics, including the development of circRNA vaccines. A recent study has engineered RNA
circles encoding the receptor binding domain of the SARS-CoV2 Spike protein for exogenous vaccination to induce a robust and durable antibody response (Qu et al., 2022).

These studies highlight some of the potential applications of synthetic circRNAs, either as non-coding aptamers or as templates for translation. The next section will describe the formation of circRNAs *in vitro*.

### 1.4.2.3 Generation of circRNAs *in vitro*

Exogenous RNA circles can be generated by chemical methods (Nakamoto et al., 2020), by the ligation of linear mRNA ends using T4 DNA or RNA ligases, or by ribozymatic methods using group I self-splicing introns (Petkovic and Müller, 2015; Müller and Appel, 2017). Self-splicing introns can produce RNA circles more efficiently than enzymatic methods, particularly when circularising sequences longer than 1 kB (C.-X. Liu et al., 2022). The following section describes the mechanism of group I intron splicing and the permutation of these sequences to enable the circularisation of RNA *in vitro*.

#### 1.4.2.3.1 Group I introns and their splicing mechanism

Group I introns are self-splicing ribozymes that excise themselves from RNA precursors, such as ribosomal RNA (rRNA), pre- transfer RNA (tRNA) or mRNA. The splicing mechanism involves two successive transesterification reactions and is initiated with nucleophilic attack by the 3’ hydroxyl group of the guanosine on the phosphorous of the 5’ splice site. This cleaves the precursor RNA and ligates guanosine to the 5’ end of the intron, leaving a free 3’ hydroxyl group on the 5’ exon. A second transesterification occurs on the 5’ exon’s hydroxyl group by the phosphorous on the 3’ splice site. This results in exon ligation and release of the intron (Puttaraju and Been, 1992). This mechanism can be exploited to generate exogenous coding circRNAs.

#### 1.4.2.3.2 Engineering circRNAs from group I introns

CircRNA was first synthetically engineered by Puttaraju et al., using the tRNA group I intron sequence derived from *Anabaena* cyanobacterium (Puttaraju and Been, 1992). The self-splicing intron was re-engineered to generate RNA circles through end-to-end fusion of exons which interrupt half-intron sequences (also known as permuted intron-exon
(PIE) sequences). The splice donor and acceptor sites were positioned in the reverse orientation to allow back-splicing to occur when heated to 55°C with guanosine triphosphate (GTP) and magnesium ions (Mg$^{2+}$). The back-splicing reaction releases covalently-closed RNA from the cleaved intron sequences (Puttaraju and Been, 1992). Ford and Ares have extended this work by incorporating foreign sequences into a permuted intron self-splicing system based on the catalytic intron of the T4 phage thymidylate synthase gene (Ford and Ares, 1994). This work demonstrated the ability to produce RNA circles in vitro and in vivo in bacteria and yeast.

In recent work, Wesselhoeft et al., has shown that higher splicing efficiencies can be achieved from the permuted *Anabaena* group I intron sequence compared to the system derived from T4 phage (Wesselhoeft, Kowalski and Anderson, 2018). Further work has involved optimising the *Anabaena* PIE sequence to support the stable translation of circRNAs in eukaryotic cells. Homology and spacer sequences were added to the PIE sequence to improve the circularisation of exons up to 5 kB in length by promoting the formation of a splicing bubble. The incorporation of strong homology sequences resulted in splicing efficiencies of up to 95%, however, efficiencies were negatively correlated with RNA length. Spacer sequences were also included to prevent steric hindrance between the highly structured 3’ PIE splice site and the IRES. Furthermore, by replacing the encephalomyocarditis virus (EMCV) IRES sequence with the IRES from Coxsackievirus B3 (CVB3), luciferase translation efficiencies were increased by 1.5-fold in HEK293T cells (Wesselhoeft, Kowalski and Anderson, 2018).

This thesis will investigate the application of circRNAs to enhance the manufacturing process of CAR T-cells. The circRNA was designed based on Wesselhoeft’s optimised *Anabaena* PIE sequence, as exemplified in Figure 1.7 (Wesselhoeft, Kowalski and Anderson, 2018).

1.4.2.3.3 Purification and validation of RNA circularity

In addition to optimising the circRNA sequence, Wesselhoeft et al., have highlighted the importance of producing high quality RNA. Although the literature reports conflicting evidence on the immunogenicity of exogenous circRNAs (Y. G. Chen et al., 2017;
Wesselhoeft et al., 2019), it is widely accepted that circRNA should be of high purity prior to transfection. Previous studies have shown reduced immunogenicity of RNA circles produced by enzymatic methods compared to those that originate from PIE sequences (C.-X. Liu et al., 2022). The extraneous introns remaining from circRNA biosynthesis can be detected by PRRs such as Retinoic acid-inducible gene I (RIG-1) and TLRs to initiate an anti-viral response. However, the removal of intron sequences by RNase degradation can purify circRNA, thus preventing innate immune activation (Wesselhoeft et al., 2019). For in vivo applications, high-performance liquid chromatography (HPLC) purification may be required to further reduce interferon responses (Karikó et al., 2011).

Further studies have described methods for analysing the quality of exogenous RNAs and to verify their circularity (Quabius and Krupp, 2015; Zhang, Yang and Chen, 2021; Abe et al., 2022). Capillary electrophoresis is commonly performed to confirm the correct size of circRNA and the release of intron sequences, however, this technique relies upon a difference in size from the contaminants. RNase R degrades all RNA with free ends of 7 nucleotides or greater and can be employed to confirm RNA circularisation and to enrich circRNA prior to use (Suzuki, 2006; Wesselhoeft et al., 2019). RNase R has however, been reported to degrade circRNAs, which can result in a reduction in yield (Zhang, Yang and Chen, 2021). Alternatively, oligonucleotide-directed RNase H can be used to distinguish between linear and circRNA. RNase H will digest RNA in a DNA hybrid and will yield two products from digestion of linear RNA compared one product from circRNA due to strand nicking. Digestion products can be detected and quantified by Northern blotting or by gel electrophoresis. Another method to validating RNA circularisation is the use of divergent PCR, where primers are designed to amplify the BSJ region. In linear RNA, the primers are divergent and do not produce an amplicon (Quabius and Krupp, 2015).

This section has summarised efforts to improve linear RNA stability through the incorporation of 5’ and 3’ transcript modifications combined with nucleoside substitution to evade innate sensing and degradation. CircRNAs have been discovered in nature as an endogenous mechanism to promote RNA stability and have diverse functions including
the regulation of gene transcription and splicing, sequestration of miRNAs and proteins, whilst a small number have demonstrated translational capacity. CircRNAs can be synthetically engineered to produce non-coding aptamers to investigate cell functions, therapeutic tools to modulate signalling pathways and immunity, or as a method to express proteins *in vitro* and *in vivo*. The manipulation of the group I self-splicing sequence has produced circRNAs with high efficiency and optimal transgene expression.
1.5 Project objectives

This thesis aims to address two challenges associated with the manufacture of cellular therapies: the delivery of RNA/DNA and RNP to T-cells and the stability of gene editing components. The first chapter explores novel gene delivery methods, including nucleofection and soluporation, and their application to CAR T-cell manufacturing. Viral approaches are also explored for comparison to the non-viral method. Following determination of the suitable delivery protocol, two applications of circular RNA are explored to improve the function of CAR T-cells. CircRNAs were found to be highly stable in T-cells and were used to express transcription factors or genome editing components. When combined with insertion of the CAR, these approaches will facilitate the manufacture of reprogrammed CAR T-cells with an early memory phenotype, or with resistance to inhibitory signals by engineering gene knockouts. In the final chapter, the circRNA transfection protocol is applied to manufacture PD-1 base-edited anti-CD30 CAR T-cells, to enhance the therapeutic efficacy of treatment for Hodgkin lymphoma, where the tumour microenvironment is known to downregulate T-cell responses. In summary, this study aims to:

1.5.1 Evaluate gene delivery methods, including nucleofection and soluporation, circular RNA, and alternative viral approaches for CAR T-cell manufacturing (summarised in Table 1) (Chapter 3).

1.5.2 Reprogram the T-cell phenotype using transcription factor-encoding circRNA (Chapter 4).

1.5.3 Develop a multiplexed base editing protocol using circRNA-encoding BE4max, to disrupt the expression of four T-cell inhibitory receptors (Chapter 5).

1.5.4 Generate a PD-1-edited anti-CD30 CAR product using the circRNA base editing protocol and an optimised CAR cassette (Chapter 6).
Table 1. Summary of the explored transient delivery methods.

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<td>Nucleofection</td>
<td>DNA, RNA, Protein/RNPs</td>
<td>- High efficiency (&lt;95%)&lt;br&gt;- Requires a 4-day expansion period for recovery&lt;br&gt;(~50% cell loss)</td>
<td>- Large-scale device available&lt;br&gt;- Closed system&lt;br&gt;- Compatible with Miltenyi Prodigy</td>
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<td>Soluporation</td>
<td>RNA, Protein/RNPs</td>
<td>- Lower efficiency (&lt;70%)&lt;br&gt;- Variable cell recovery (~25-75%)&lt;br&gt;- Maintains T-cell phenotype</td>
<td>- High cost associated with minimal cargo requirements&lt;br&gt;- Large-scale device available&lt;br&gt;(closed system)</td>
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<td>Circular RNA</td>
<td>RNA</td>
<td>- Higher transfection efficiencies compared to linear mRNA&lt;br&gt;- Prolonged duration of expression&lt;br&gt;- Donor-to-donor variability</td>
<td>- Circumvents the requirement for RNA capping/polyadenylation&lt;br&gt;- Circularisation efficiency decreases with transgene size</td>
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<td>NILV</td>
<td>RNA</td>
<td>- Poor transduction efficiencies in T-cells&lt;br&gt;- Low level of expression&lt;br&gt;- Minimal cell loss</td>
<td>- Difficult to scale for GMP manufacturing&lt;br&gt;- High cost &amp; safety concerns&lt;br&gt;- Ease of transduction process</td>
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Chapter 2. Materials & Methods

The following section outlines the materials and experimental methods used in this thesis.

2.1 Materials

2.1.1 Materials and reagents

Table 2 Molecular biology materials, reagents, and kits

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<td>STARLAB (Milton Keynes, UK)</td>
<td>S1182-1830-C</td>
</tr>
<tr>
<td>5 mL Stripette™ Paper/Plastic-Wrapped Sterile</td>
<td>Corning (distributed by Fisher Scientific UK Ltd., Loughborough, UK)</td>
<td>10084450</td>
</tr>
</tbody>
</table>
### Chapter 2. Materials and Methods

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
<th>Code</th>
</tr>
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<tbody>
<tr>
<td>10 mL Stripette™ Paper/Plastic-Wrapped Sterile</td>
<td>Corning (distributed by Fisher Scientific UK Ltd., Loughborough, UK)</td>
<td>10084450</td>
</tr>
<tr>
<td>25 mL Stripette™ Paper/Plastic-Wrapped Sterile</td>
<td>Corning (distributed by Fisher Scientific UK Ltd., Loughborough, UK)</td>
<td>10606151</td>
</tr>
<tr>
<td>50 mL Stripette™ Paper/Plastic-Wrapped Sterile</td>
<td>Corning (distributed by Fisher Scientific UK Ltd., Loughborough, UK)</td>
<td>10636391</td>
</tr>
<tr>
<td>Sterile TC Grade T-25</td>
<td>Thermo-Scientific (Loughborough, UK)</td>
<td>156367</td>
</tr>
<tr>
<td>Sterile TC Grade T-75</td>
<td>Thermo-Scientific (Loughborough, UK)</td>
<td>156499</td>
</tr>
<tr>
<td>Sterile TC Grade T-175</td>
<td>Thermo-Scientific (Loughborough, UK)</td>
<td>159910</td>
</tr>
<tr>
<td>1.5 mL Microcentrifuge Tubes</td>
<td>STARLAB (Milton Keynes, UK)</td>
<td>S1615-5599</td>
</tr>
<tr>
<td>2 mL Microcentrifuge Tubes</td>
<td>STARLAB (Milton Keynes, UK)</td>
<td>E1420-2700</td>
</tr>
<tr>
<td>15 mL Falcon Tubes</td>
<td>Corning (distributed by Fisher Scientific UK Ltd., Loughborough, UK)</td>
<td>430791</td>
</tr>
<tr>
<td>50 mL Falcon Tubes</td>
<td>Corning (distributed by Fisher Scientific UK Ltd., Loughborough, UK)</td>
<td>430829</td>
</tr>
<tr>
<td>150 mL Storage Bottle</td>
<td>Corning (distributed by Fisher Scientific UK Ltd., Loughborough, UK)</td>
<td>431175</td>
</tr>
<tr>
<td>250 mL Storage Bottle</td>
<td>Corning (distributed by Fisher Scientific UK Ltd., Loughborough, UK)</td>
<td>430281</td>
</tr>
<tr>
<td>3 mL Graduated Pasteur Pipette</td>
<td>STARLAB (Milton Keynes, UK)</td>
<td>E1414-0311</td>
</tr>
<tr>
<td>Sealing Film Parafilm-M 100 mm Wide 38 Metre Roll</td>
<td>Thermo-Scientific (Loughborough, UK)</td>
<td>11772644</td>
</tr>
<tr>
<td>Nunc MaxiSorp™ 96-Well, Flat-Bottomed</td>
<td>Thermo-Scientific (Loughborough, UK)</td>
<td>44-2404-21</td>
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</table>
## Materials and Methods

<table>
<thead>
<tr>
<th>Description</th>
<th>Supplier</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile Tissue Culture Treated 96-Well Culture Plate, Flat Bottomed with Lid</td>
<td>Thermo-Scientific (Loughborough, UK)</td>
<td>10687551</td>
</tr>
<tr>
<td>Sterile Tissue Culture Treated 96-Well Culture Plate, Round Bottomed with Lid</td>
<td>Thermo-Scientific (Loughborough, UK)</td>
<td>10360691</td>
</tr>
<tr>
<td>Sterile Tissue Culture Grade 24-Well Culture Plate</td>
<td>Thermo-Scientific (Loughborough, UK)</td>
<td>10377841</td>
</tr>
<tr>
<td>Sterile Tissue Culture Grade 6-Well Culture Plate</td>
<td>Thermo-Scientific (Loughborough, UK)</td>
<td>10284901</td>
</tr>
<tr>
<td>Sterile Non-Tissue Culture Treated 24-Well Culture Plate</td>
<td>VWR (Leicestershire, UK)</td>
<td>734-2779</td>
</tr>
<tr>
<td>Sterile Non-Tissue Culture Treated 6-Well Culture Plate</td>
<td>VWR (Leicestershire, UK)</td>
<td>734-0948</td>
</tr>
<tr>
<td>Flow-Cytometry Plates, 96-Well V-Bottom</td>
<td>Merck Life Science (Watford, UK)</td>
<td>M8185-100EA</td>
</tr>
<tr>
<td>Sterile Tissue Culture Treated 100 mm Petri Dish</td>
<td>Corning (distributed by Fisher Scientific UK Ltd., Loughborough, UK)</td>
<td>430167</td>
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<tr>
<td>50 mL SepMate™ PBMC Isolation Tubes</td>
<td>STEMCELL Technologies (Cambridge, UK)</td>
<td>85450</td>
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<tr>
<td>Sterile 50 mL Reservoir</td>
<td>STARLAB (Milton Keynes, UK)</td>
<td>E2310-1010</td>
</tr>
<tr>
<td>1.8 mL Cryovial with External Thread, Self-Sealing Cap, Skirted</td>
<td>STARLAB (Milton Keynes, UK)</td>
<td>E3090-6222</td>
</tr>
<tr>
<td>10 mL Cryovial</td>
<td>Simport (distributed by Fisher Scientific UK Ltd., Loughborough, UK)</td>
<td>T310-10A</td>
</tr>
<tr>
<td>NC-Slide A8</td>
<td>Chemometec (Allerod, Denmark)</td>
<td>942-0003</td>
</tr>
<tr>
<td>50 mL Luer-Lock Syringes</td>
<td>Thermo-Scientific (Loughborough, UK)</td>
<td>15899152</td>
</tr>
<tr>
<td>Combi-Stopper Closing Cones</td>
<td>B Braun™ (distributed by Fisher Scientific UK Ltd., Loughborough, UK)</td>
<td>4495152</td>
</tr>
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</table>
### Materials and Methods

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOLUPORE® Transfection Pods</td>
<td>Avectas (Kildare, Ireland)</td>
<td>N/A</td>
</tr>
<tr>
<td>SOLUPORE® Recovery Base</td>
<td>Avectas (Kildare, Ireland)</td>
<td>N/A</td>
</tr>
<tr>
<td>SOLUPORE® Recovery Tubing Set</td>
<td>Avectas (Kildare, Ireland)</td>
<td>N/A</td>
</tr>
<tr>
<td>Fisherbrand™ Sterile PES Syringe Filter, 0.2 µM</td>
<td>Thermo-Scientific (Loughborough, UK)</td>
<td>15206869</td>
</tr>
<tr>
<td>Fisherbrand™ Sterile PVDF Syringe Filter, 0.45 µM</td>
<td>Thermo-Scientific (Loughborough, UK)</td>
<td>15191499</td>
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### Table 4 Tissue culture reagents and kits

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco's Phosphate Buffered Saline (PBS)</td>
<td>Sigma-Aldrich (distributed by Merck Life Science UK Ltd., Watford, UK)</td>
<td>D8537-500ML</td>
</tr>
<tr>
<td>Trypsin-EDTA (0.25%), phenol red</td>
<td>Sigma-Aldrich (distributed by Merck Life Science UK Ltd., Watford, UK)</td>
<td>T4049-100ML</td>
</tr>
<tr>
<td>Iscove's Modified Dulbecco's Medium (IMDM)</td>
<td>Lonza Bioscience (Basel, Switzerland)</td>
<td>BE12-726F</td>
</tr>
<tr>
<td>Dimethyl sulfoxide anhydrous, ≥99.9%</td>
<td>Sigma-Aldrich (distributed by Merck Life Science UK Ltd., Watford, UK)</td>
<td>276855</td>
</tr>
<tr>
<td>100X GlutaMAX Supplement</td>
<td>Gibco (distributed by Thermo-Scientific, Loughborough, UK)</td>
<td>35050-061</td>
</tr>
<tr>
<td>Fetal Bovine Serum (FBS)</td>
<td>Sigma-Aldrich (distributed by Merck Life Science UK Ltd., Watford, UK)</td>
<td>F7524</td>
</tr>
<tr>
<td>Roswell Park Memorial Institute (RPMI) 1640 Medium</td>
<td>Gibco (Loughborough, UK)</td>
<td>21870-084</td>
</tr>
<tr>
<td>100X Penicillin-Streptomycin-Glutamine</td>
<td>Sigma-Aldrich (distributed by Merck Life Science UK Ltd., Watford, UK)</td>
<td>12090216</td>
</tr>
<tr>
<td>50X Hybridoma Fusion and Cloning Supplement</td>
<td>Sigma-Aldrich (distributed by Merck Life Science UK Ltd., Watford, UK)</td>
<td>11363735001</td>
</tr>
<tr>
<td>Material/Kit Name</td>
<td>Supplier/Details</td>
<td>Catalog Number</td>
</tr>
<tr>
<td>----------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagle’s Medium - high glucose (DMEM)</td>
<td>Thermo-Scientific (Loughborough, UK)</td>
<td>11574486</td>
</tr>
<tr>
<td>Leukocyte Cones</td>
<td>NHS Blood and Transplant (London, UK)</td>
<td>N/A</td>
</tr>
<tr>
<td>Ficoll-Paque PLUS</td>
<td>Sigma-Aldrich (distributed by Merck Life Science UK Ltd., Watford, UK)</td>
<td>17-1440-02</td>
</tr>
<tr>
<td>TexMACSTM GMP Medium 2000 mL</td>
<td>Miltenyi Biotec (Woking, UK)</td>
<td>170-076-306</td>
</tr>
<tr>
<td>Human Serum AB Off the Clot 100 mL</td>
<td>Sera Laboratories (Sussex, UK)</td>
<td>SM-512-HS</td>
</tr>
<tr>
<td>T Cell TransAct™, Human</td>
<td>Miltenyi Biotec (Woking, UK)</td>
<td>130-111-160</td>
</tr>
<tr>
<td>Anti CD3 Pure (OKT3) (100 μg/mL)</td>
<td>Miltenyi Biotec (Woking, UK)</td>
<td>130-093-387</td>
</tr>
<tr>
<td>Anti CD28 Pure (15E8) (100 μg/mL)</td>
<td>Miltenyi Biotec (Woking, UK)</td>
<td>130-093-375</td>
</tr>
<tr>
<td>Human IL-2, 50 μg</td>
<td>Miltenyi Biotec (Woking, UK)</td>
<td>130-097-743</td>
</tr>
<tr>
<td>Human IL-7, 25 μg</td>
<td>Miltenyi Biotec (Woking, UK)</td>
<td>130-095-367</td>
</tr>
<tr>
<td>Human IL-15, 25 μg</td>
<td>Miltenyi Biotec (Woking, UK)</td>
<td>130-095-760</td>
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<tr>
<td>GeneJuice® Transfection Reagent</td>
<td>Merck Millipore (distributed by Merck Life Science UK Ltd., Watford, UK)</td>
<td>70967</td>
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<tr>
<td>Polybrene (10 mg/mL)</td>
<td>Merck Millipore (distributed by Merck Life Science UK Ltd., Watford, UK)</td>
<td>TR-1003-G</td>
</tr>
<tr>
<td>Opti-MEM™ I Reduced Serum Medium</td>
<td>Gibco (Loughborough, UK)</td>
<td>31985070</td>
</tr>
<tr>
<td>RetroNectin</td>
<td>Takara (London, UK)</td>
<td>T100</td>
</tr>
<tr>
<td>SF Cell Line 96-Well Nucleofector™ Kit</td>
<td>Lonza Bioscience (Basel, Switzerland)</td>
<td>V4SC-2096</td>
</tr>
<tr>
<td>SF Cell Line 4D-Nucleofector™ X Kit S</td>
<td>Lonza Bioscience (Basel, Switzerland)</td>
<td>V4XC-2032</td>
</tr>
<tr>
<td>Material Description</td>
<td>Supplier</td>
<td>Catalog Number</td>
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<tr>
<td>--------------------------------------</td>
<td>-----------------------------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>P3 Primary Cell 96-Well Nucleofector™ Kit</td>
<td>Lonza Bioscience (Basel, Switzerland)</td>
<td>V4SP-3096</td>
</tr>
<tr>
<td>P3 Primary Cell 96-Well Nucleofector™ Kit (960 RCT)</td>
<td>Lonza Bioscience (Basel, Switzerland)</td>
<td>V4SP-3960</td>
</tr>
<tr>
<td>P3 Primary Cell 4D-Nucleofector™ X Kit S</td>
<td>Lonza Bioscience (Basel, Switzerland)</td>
<td>V4XP-3032</td>
</tr>
<tr>
<td>P3 Primary Cell 4D-Nucleofector™ X Kit L</td>
<td>Lonza Bioscience (Basel, Switzerland)</td>
<td>V4XP-3024</td>
</tr>
<tr>
<td>P3 Primary Cell 4D-Nucleofector™ LV Kit L</td>
<td>Lonza Bioscience (Basel, Switzerland)</td>
<td>V4LP-3002</td>
</tr>
<tr>
<td>Alt-R® Cas9 Electroporation Enhancer</td>
<td>Integrated DNA Technologies (Leuven, Belgium)</td>
<td>1075916</td>
</tr>
<tr>
<td>20X S-Buffer</td>
<td>Avectas (Kildare, Ireland)</td>
<td>N/A</td>
</tr>
<tr>
<td>Nuclease Free Water</td>
<td>Integrated DNA Technologies (Leuven, Belgium)</td>
<td>11-05-01-04</td>
</tr>
<tr>
<td>Absolute Ethanol</td>
<td>Thermo-Scientific (Loughborough, UK)</td>
<td>10644795</td>
</tr>
<tr>
<td>DasherGFP mRNA (2.08 μg/mL)</td>
<td>Aldevron (ND, US)</td>
<td>3870-1000</td>
</tr>
<tr>
<td>RBS™ 25 Solution</td>
<td>Sigma-Aldrich (distributed by Merck Life Science UK Ltd., Watford, UK)</td>
<td>83460</td>
</tr>
<tr>
<td>CTS OpTmizer Media + Supplement</td>
<td>Thermo-Scientific (Loughborough, UK)</td>
<td>A1048501</td>
</tr>
<tr>
<td>Physiologix XF hGFC</td>
<td>Nucleus Biologics (CA, US)</td>
<td>10159</td>
</tr>
<tr>
<td>L-glutamine 200 mM</td>
<td>Thermo-Scientific (Loughborough, UK)</td>
<td>25030-024</td>
</tr>
<tr>
<td>Antibiotic-Antimycotic (100X)</td>
<td>Thermo-Scientific (Loughborough, UK)</td>
<td>15240062</td>
</tr>
<tr>
<td>Light Duty Tissue</td>
<td>Thermo-Scientific (Loughborough, UK)</td>
<td>17445653</td>
</tr>
<tr>
<td>Single Guide RNA, modified</td>
<td>Synthego (CA, US)</td>
<td>N/A</td>
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<tr>
<td>TE Buffer</td>
<td>Synthego (CA, US)</td>
<td>638471</td>
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### 2.1.2 Solutions and buffers

**Table 5 Molecular biology buffers**

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<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
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<tbody>
<tr>
<td>Lysogeny Broth (LB)</td>
<td>LB tablets were purchased from Melford (L24065-5000.0) and contain 10 grams of Tryptone, 5 grams of Yeast Extract, 5 grams of NaCl and 1.5 grams of TRIS/TRIS HCL. LB tablets were dissolved in deionised water with 5 grams of NaCl and the pH was adjusted to 7.2.</td>
</tr>
<tr>
<td>Tris/Borate/EDTA (TBE) buffer</td>
<td>TBE buffer contained 9.3 grams of EDTA, 55 grams of Boric Acid and 108 grams of Tris-base in 1 litre of deionised water.</td>
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Table 6 Tissue culture medium composition

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition</th>
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</thead>
<tbody>
<tr>
<td>Complete RPMI 10%</td>
<td>10% filtered heat inactivated fetal bovine serum, 1% GlutaMAX</td>
</tr>
<tr>
<td>Complete RPMI 20%</td>
<td>20% filtered heat inactivated fetal bovine serum, 1% GlutaMAX</td>
</tr>
<tr>
<td>Complete IMDM</td>
<td>10% filtered heat inactivated fetal bovine serum, 1% GlutaMAX</td>
</tr>
<tr>
<td>Complete TexMACSTM</td>
<td>3% Human AB Serum</td>
</tr>
<tr>
<td>Complete DMEM</td>
<td>10% filtered heat inactivated fetal bovine serum, 1% Penicillin-Streptomycin-Glutamine, 1X Hybridoma Fusion and Cloning Supplement</td>
</tr>
<tr>
<td>Complete CTS OpTmizer</td>
<td>5% Physiologix XF hGFC, 1% L-glutamine, 1% Antibiotic-Antimycotic</td>
</tr>
<tr>
<td>Freezing medium</td>
<td>FCS, 10% DMSO</td>
</tr>
</tbody>
</table>

2.1.3 Antibodies

Table 7 Flow-cytometry antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorochrome</th>
<th>Supplier</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-AAD Viability Staining Solution</td>
<td>B3 channel</td>
<td>BioLegend (London, UK)</td>
<td>420404</td>
</tr>
<tr>
<td>CAT-19 (Rat Fc)</td>
<td>Unconjugated</td>
<td>Autolus (in-house) (London, UK)</td>
<td>N/A</td>
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<tr>
<td>CCR7</td>
<td>PE</td>
<td>Miltenyi Biotec (Woking, UK)</td>
<td>130-119-583</td>
</tr>
<tr>
<td>CD2</td>
<td>PE</td>
<td>BioLegend (London, UK)</td>
<td>300208</td>
</tr>
<tr>
<td>CD223 (LAG3)</td>
<td>Brilliant Violet 421</td>
<td>BioLegend (London, UK)</td>
<td>369314</td>
</tr>
<tr>
<td>CD25 (IL-2RA)</td>
<td>Brilliant Violet 421</td>
<td>BioLegend (London, UK)</td>
<td>302630</td>
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</table>
### Chapter 2. Materials and Methods

<table>
<thead>
<tr>
<th>CD27</th>
<th>VioBright 515</th>
<th>Miltenyi Biotec (Woking, UK)</th>
<th>130-120-028</th>
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<tbody>
<tr>
<td>CD279 (PD-1)</td>
<td>PE</td>
<td>BioLegend (London, UK)</td>
<td>329906</td>
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<tr>
<td>CD3</td>
<td>PE/Cy7</td>
<td>BioLegend (London, UK)</td>
<td>344816</td>
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<tr>
<td>CD3</td>
<td>VioGreen</td>
<td>Miltenyi Biotec (Woking, UK)</td>
<td>130-113-142</td>
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<tr>
<td>CD30 (Ki-1)</td>
<td>PE</td>
<td>BioLegend (London, UK)</td>
<td>333906</td>
</tr>
<tr>
<td>CD366 (TIM3)</td>
<td>Brilliant Violet 421</td>
<td>BioLegend (London, UK)</td>
<td>345008</td>
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<tr>
<td>CD45RA</td>
<td>APC Vio770</td>
<td>Miltenyi Biotec (Woking, UK)</td>
<td>130-117-747</td>
</tr>
<tr>
<td>CD8</td>
<td>VioBlue</td>
<td>Miltenyi Biotec (Woking, UK)</td>
<td>130-110-683</td>
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<tr>
<td>CD8a</td>
<td>APC/Cy7</td>
<td>BioLegend (London, UK)</td>
<td>301016</td>
</tr>
<tr>
<td>CD95</td>
<td>PE Vio770</td>
<td>Miltenyi Biotec (Woking, UK)</td>
<td>130-113-006</td>
</tr>
<tr>
<td>FMC63</td>
<td>AF647</td>
<td>Autolus (in-house) (London, UK)</td>
<td>N/A</td>
</tr>
<tr>
<td>IgG1, Isotype Control</td>
<td>PE</td>
<td>BioLegend (London, UK)</td>
<td>400112</td>
</tr>
<tr>
<td>IgG1, Isotype Control</td>
<td>Brilliant Violet 421</td>
<td>BioLegend (London, UK)</td>
<td>400158</td>
</tr>
<tr>
<td>IgG1, Isotype Control</td>
<td>PE/Cy7</td>
<td>BioLegend (London, UK)</td>
<td>400126</td>
</tr>
<tr>
<td>Rat Fc</td>
<td>APC</td>
<td>BioLegend (London, UK)</td>
<td>405407</td>
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<tr>
<td>Rat Fc</td>
<td>PE</td>
<td>BioLegend (London, UK)</td>
<td>405406</td>
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<tr>
<td>REA Control</td>
<td>PE</td>
<td>Miltenyi Biotec (Woking, UK)</td>
<td>130-113-438</td>
</tr>
<tr>
<td>REA Control</td>
<td>VioBright 515</td>
<td>Miltenyi Biotec (Woking, UK)</td>
<td>130-113-445</td>
</tr>
<tr>
<td>REA Control</td>
<td>PE Vio770</td>
<td>Miltenyi Biotec (Woking, UK)</td>
<td>130-113-440</td>
</tr>
<tr>
<td>REA Control</td>
<td>APC Vio770</td>
<td>Miltenyi Biotec (Woking, UK)</td>
<td>130-113-435</td>
</tr>
</tbody>
</table>
Chapter 2. Materials and Methods

| Recombinant CD30/TNFRSF8 Protein, Human | Biotinylated | Sino Biological (Eschborn, Germany) | 10777-H08H-B |
| Streptavidin | PE | BioLegend (London, UK) | 405203 |
| SYTOX™-AADvanced™ Viability | B3 channel | Thermo-Scientific (Loughborough, UK) | S10274 |
| SYTOX™-Blue Viability Dye | V1 channel | Thermo-Scientific (Loughborough, UK) | S34857 |
| V5 Tag | APC | Abcam (Cambridge, England) | ab72560 |
| Zombie™ NIR Fixable Viability Dye | R3 | BioLegend (London, UK) | 423106 |

### 2.1.4 Cell lines

**Table 8 Details of purchased cell lines**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Supplier</th>
<th>Catalogue Number</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK293T</td>
<td>ATCC® (VA, US)</td>
<td>CRL-3216™</td>
<td>The 293T cell line is a highly transfectable derivative of human embryonic kidney 293 cells and contains the SV40 T-antigen. 293T cells can replicate vectors containing the SV40 region of replication, and are used for gene expression, production of retroviral vectors and protein production.</td>
</tr>
<tr>
<td>SUP-T1</td>
<td>ATCC® (VA, US)</td>
<td>CRL-1942™</td>
<td>Human T lymphoblast derived from the malignant cells of an eight-year-old boy with T-cell Lymphoblastic Lymphoma.</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>DSMZ (Braunschweig, Germany)</td>
<td>ACC-326</td>
<td>Human T-cell leukaemia cell line derived from the peripheral blood of a 19-year-old man with acute lymphoblastic leukaemia (ALL) in relapse in 1971.</td>
</tr>
<tr>
<td>HDLM-2</td>
<td>DSMZ (Braunschweig, Germany)</td>
<td>ACC-17</td>
<td>Human Hodgkin lymphoma cell line established from the pleural effusion of a 74-year-old man (nodular sclerosing, stage IV) in 1982.</td>
</tr>
</tbody>
</table>
Chapter 2. Materials and Methods

<table>
<thead>
<tr>
<th>RPMI-8226</th>
<th>PHE Culture Collections (Salisbury, UK)</th>
<th>87012702</th>
<th>Human myeloma cell line derived from the peripheral blood of a 61-year-old male with the disease. The cells produce and secrete Ig lambda light chain.</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-428</td>
<td>DSMZ (Braunschweig, Germany)</td>
<td>ACC-197</td>
<td>Human Hodgkin lymphoma cell line established from the pleural effusion of a 37-year-old woman (nodular sclerosing, stage IVB) in 1978.</td>
</tr>
<tr>
<td>L-540</td>
<td>DSMZ (Braunschweig, Germany)</td>
<td>ACC-72</td>
<td>Human Hodgkin lymphoma cell line established from the bone marrow of a 20-year-old woman (nodular sclerosing, stage IVB).</td>
</tr>
</tbody>
</table>

### 2.1.5 Equipment

#### Table 9 Specialist equipment

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>NanoDrop™ 8000 Spectrophotometer</td>
<td>Thermo-Scientific (Loughborough, UK)</td>
</tr>
<tr>
<td>Azure c200</td>
<td>Azure Biosystems (CA, US)</td>
</tr>
<tr>
<td>Azure c600</td>
<td>Azure Biosystems (CA, US)</td>
</tr>
<tr>
<td>C1000 Touch Thermal Cycler</td>
<td>Bio-Rad (Watford, UK)</td>
</tr>
<tr>
<td>C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module</td>
<td>Bio-Rad (Watford, UK)</td>
</tr>
<tr>
<td>Qubit™ Fluorometer</td>
<td>Invitrogen (distributed by Thermo-Scientific, Loughborough, UK)</td>
</tr>
<tr>
<td>2200 TapeStation System</td>
<td>Agilent (Cheshire, UK)</td>
</tr>
<tr>
<td>96-Well Vortexer</td>
<td>Sigma-Aldrich (distributed by Merck Life Science UK Ltd., Watford, UK)</td>
</tr>
<tr>
<td>EasyEights™ EasySep™ Magnet</td>
<td>STEMCELL Technologies (Cambridge, UK)</td>
</tr>
<tr>
<td>Automated ELISA Plate Reader</td>
<td>Thermo-Scientific (Loughborough, UK)</td>
</tr>
</tbody>
</table>
### Chapter 2. Materials and Methods

<table>
<thead>
<tr>
<th>Equipment Description</th>
<th>Supplier Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mini-PROTEAN System Vertical Electrophoresis Cell</td>
<td>Bio-Rad (Watford, UK)</td>
</tr>
<tr>
<td>Trans-Blot Transfer System</td>
<td>Bio-Rad (Watford, UK)</td>
</tr>
<tr>
<td>CoolCell™ FTS30</td>
<td>Corning (distributed by Fisher Scientific UK Ltd., Loughborough, UK)</td>
</tr>
<tr>
<td>MACSQuant®10 Flow-Cytometer</td>
<td>Miltenyi Biotec (Woking, UK)</td>
</tr>
<tr>
<td>MACSQuant®X Flow-Cytometer</td>
<td>Miltenyi Biotec (Woking, UK)</td>
</tr>
<tr>
<td>LSRFortessa™ X-20 Flow-Cytometer</td>
<td>BD Biosciences (Wokingham, UK)</td>
</tr>
<tr>
<td>Amaxa™ 4D Nucleofector™ Core Unit</td>
<td>Lonza Bioscience (Basel, Switzerland)</td>
</tr>
<tr>
<td>Amaxa™ 4D Nucleofector™ X Unit</td>
<td>Lonza Bioscience (Basel, Switzerland)</td>
</tr>
<tr>
<td>Amaxa™ Nucleofector™ 96-Well Shuttle System</td>
<td>Lonza Bioscience (Basel, Switzerland)</td>
</tr>
<tr>
<td>4D Nucleofector™ LV Unit</td>
<td>Lonza Bioscience (Basel, Switzerland)</td>
</tr>
<tr>
<td>SOLUPORE® Technology</td>
<td>Avectas (Kildare, Ireland)</td>
</tr>
<tr>
<td>Analytical Balance</td>
<td>VWR (Leicestershire, UK)</td>
</tr>
<tr>
<td>NucleoCounter NC-250</td>
<td>Chemometec (Allerod, Denmark)</td>
</tr>
<tr>
<td>High-Speed Centrifuge</td>
<td>Thermo-Scientific (Loughborough, UK)</td>
</tr>
</tbody>
</table>
### 2.1.6 Plasmids

Table 10 Plasmid details

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUCminusMCS.T7.AnabaenaPIE_pAC.CVB3_IRES.NLS-mClover3-NLS</td>
<td>Construct containing the self-splicing intron from <em>Anabaena</em> tRNA (PIE stands for permuted intron-exon) flanking an IRES from coxsackievirus B3 and mClover3 (mClover) with nuclear localisation sequences. Splicing of the <em>Anabaena</em> PIE produces a circRNA where the 5-prime end of the CVB3 IRES is linked to the 3-prime end of the mClover sequence.</td>
</tr>
<tr>
<td>pMA.T7promoter.NLS-mClover3-NLS</td>
<td>T7 primer site followed by an mClover sequence for mRNA synthesis using the T7 RNA polymerase.</td>
</tr>
<tr>
<td>pVac.mClover3_Hu_alpha_globin_3UTR</td>
<td>mClover sequence with a human alpha globin 3 prime UTR for mRNA synthesis.</td>
</tr>
<tr>
<td>pVac.mClover3_Hu_beta_globin_3UTR</td>
<td>mClover sequence with a human beta globin 3 prime UTR for mRNA synthesis.</td>
</tr>
<tr>
<td>pMAXFP-eGFP</td>
<td>Lonza pMAX control plasmid expressing <em>Aequorea victoria</em> jellyfish eGFP.</td>
</tr>
<tr>
<td>pCCL.PGK.aCD19cat-CD8STK-41BBZ</td>
<td>Simple CAT-19 scFv in VH-VL orientation in pCCL.PGK. Used as a linear dsDNA template for transfection and to produce anti-CD19 encoding lentivirus.</td>
</tr>
<tr>
<td>pMA.2A-aCD19_CAT19_CD8STK-41BBz_pA_TRAC_HDR_repair_1000bp</td>
<td>T-cell receptor alpha chain HDR template with 1000 bp left and right homology arms designed to integrate the anti-CD19 2nd generation CAR (CAT-19) at the 5-prime end of the constant region. Used as a template for mRNA synthesis.</td>
</tr>
<tr>
<td>pUCminusMCS.T7.AnabaenaPIE_pAC.CVB3_IRES.aCD19_CAT-CD8STK-41BBz</td>
<td>CAT-19 CAR cloned into a construct containing a self-splicing intron from <em>Anabaena</em> tRNA. Used as a template for circRNA synthesis.</td>
</tr>
<tr>
<td>pJet1.2.P2A-aCD19_FMC63_CD8STK-41BBz_HBBpa_TRAC_HDR_repair_1000bp</td>
<td>T-cell receptor alpha chain HDR template with 1000 bp left and right homology arms designed to integrate anti-CD19 2nd generation CAR (FMC63) at the 5-prime end of the constant region. Used as a template for mRNA synthesis.</td>
</tr>
<tr>
<td>pUCminusMCS.T7.AnabaenaPIE_pAC.CVB3_IRES.aCD19_FMC63-CD8STK-41BBz</td>
<td>FMC63 CAR cloned into a construct containing a self-splicing intron from <em>Anabaena</em> tRNA. Used as a template for circRNA synthesis.</td>
</tr>
<tr>
<td>Plasmid Name</td>
<td>Description</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>RDF</td>
<td>RD114 envelope expression plasmid for retroviral pseudotyping.</td>
</tr>
<tr>
<td>pEQ-Pam3-E</td>
<td>Moloney Murine Leukemia Virus Gag-Pol expression plasmid.</td>
</tr>
<tr>
<td>pMD2.G</td>
<td>VSV-G envelope expression plasmid for lentiviral pseudotyping.</td>
</tr>
<tr>
<td>pMDLgp.RRE</td>
<td>Gag-Pol plasmid containing the REV response element for 3rd generation lentivirus production.</td>
</tr>
<tr>
<td>pRSV.Rev</td>
<td>REV plasmid for 3rd generation lentivirus production.</td>
</tr>
<tr>
<td>pMDLgp_Integrase_D64V.RRE</td>
<td>Wild-type HIV Gag-Pol with D64V mutation in the DDE domain of integrase to render it catalytically inactive and enable the production of non-integrating lentivirus.</td>
</tr>
<tr>
<td>pCCL.PGK.iRFP670</td>
<td>Lentiviral construct containing the iRFP670 fluorescent protein.</td>
</tr>
<tr>
<td>pCCL.EF1a.iRFP670</td>
<td>Lentiviral construct containing the iRFP670 fluorescent protein.</td>
</tr>
<tr>
<td>pCCL.MND.iRFP670</td>
<td>Lentiviral construct containing the iRFP670 fluorescent protein.</td>
</tr>
<tr>
<td>pCCL.MND.mClover3</td>
<td>Lentiviral construct containing the mClover fluorescent protein.</td>
</tr>
<tr>
<td>pCCL.MND.RFP670-T2A-BACH2</td>
<td>Lentiviral construct containing iRFP670 and the BACH2 transcription factor.</td>
</tr>
<tr>
<td>pCCL.MND.RFP670-T2A-FOXO1</td>
<td>Lentiviral construct containing iRFP670 and the FOXO1 transcription factor.</td>
</tr>
<tr>
<td>pCCL.MND.RFP670-T2A-TCF1</td>
<td>Lentiviral construct containing iRFP670 and the TCF1 transcription factor.</td>
</tr>
<tr>
<td>pCCL.MND.RFP670-T2A-BCL-XL</td>
<td>Lentiviral construct containing iRFP670 and the BCL-XL transcription factor.</td>
</tr>
<tr>
<td>pCCL.MND.RFP670-T2A-EOMES</td>
<td>Lentiviral construct containing iRFP670 and the EOMES transcription factor.</td>
</tr>
<tr>
<td>pCCL.MND.RFP670-T2A-ID3</td>
<td>Lentiviral construct containing iRFP670 and the ID3 transcription factor.</td>
</tr>
<tr>
<td>Construct Name</td>
<td>Description</td>
</tr>
<tr>
<td>----------------</td>
<td>-------------</td>
</tr>
<tr>
<td>pCCL.MND.RFP670-T2A-LEF1</td>
<td>Lentiviral construct containing iRFP670 and the LEF1 transcription factor.</td>
</tr>
<tr>
<td>pCCL.MND.RFP670-T2A-c-MYB</td>
<td>Lentiviral construct containing iRFP670 and the c-MYB transcription factor.</td>
</tr>
<tr>
<td>pCCL.MND.RFP670-T2A-c-MYB_1-330</td>
<td>Lentiviral construct containing iRFP670 and a truncated c-MYB transcription factor (constitutively active).</td>
</tr>
<tr>
<td>pCCL.MND.RFP670-T2A-c-MYB_1-330_GP</td>
<td>Lentiviral construct containing iRFP670 and a mutated and truncated c-MYB transcription factor (GP insertion in the transactivation domain).</td>
</tr>
<tr>
<td>pCCL.MND.RFP670-T2A-WNT3A</td>
<td>Lentiviral construct containing iRFP670 and the WNT3A transcription factor.</td>
</tr>
<tr>
<td>pCCL.MND.RFP670-T2A-BACH2_S520A</td>
<td>Lentiviral construct containing iRFP670 and a mutated BACH2 transcription factor (constitutively active).</td>
</tr>
<tr>
<td>pCCL.MND.RFP670-T2A-RUNX3</td>
<td>Lentiviral construct containing iRFP670 and the RUNX3 transcription factor.</td>
</tr>
<tr>
<td>pCCL.MND.RFP670-T2A-STAT3</td>
<td>Lentiviral construct containing iRFP670 and the STAT3 transcription factor.</td>
</tr>
<tr>
<td>pCCL.MND.RFP670-T2A-BCL6</td>
<td>Lentiviral construct containing iRFP670 and the BCL6 transcription factor.</td>
</tr>
<tr>
<td>pUCminusMCS.T7.AnabaenaPIE_pAC.CVB3_IRES.BACH2</td>
<td>The BACH2 transcription factor cloned into a construct containing a self-splicing intron from Anabaena tRNA. Used as a template for circRNA synthesis.</td>
</tr>
<tr>
<td>pUCminusMCS.T7.AnabaenaPIE_pAC.CVB3_IRES.FOXO1</td>
<td>The FOXO1 transcription factor cloned into a construct containing a self-splicing intron from Anabaena tRNA. Used as a template for circRNA synthesis.</td>
</tr>
<tr>
<td>pCCL.MND.mClover3-T2A-BACH2</td>
<td>Lentiviral construct containing mClover and the BACH2 transcription factor.</td>
</tr>
<tr>
<td>pCCL.MND.mClover3-T2A-FOXO1</td>
<td>Lentiviral construct containing mClover and the FOXO1 transcription factor.</td>
</tr>
<tr>
<td>pCMV_BE4max</td>
<td>Construct containing the cytosine base editor (CBE) for C: G-to-T: A editing. Addgene plasmid #112093.</td>
</tr>
<tr>
<td>SFGmR.V5-CD8STK-T2A-BE4max</td>
<td>CBE cloned into SFGmR. Used to generate stable cell lines.</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Construct Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUCminusMCS.T7.AnabaenaPIE_pAC.CVB3_IRES.BE4max</td>
<td>CBE cloned into a construct containing a self-splicing intron from <em>Anabaena</em> tRNA. Used as a template for circRNA synthesis.</td>
</tr>
<tr>
<td>pCCL.MND.aCD30-CD8STK-41BBz</td>
<td>MP codon optimised anti-CD30 CAR.</td>
</tr>
<tr>
<td>pCCL.EF1a.aCD30-CD8STK-41BBz</td>
<td>MP codon optimised anti-CD30 CAR.</td>
</tr>
<tr>
<td>pCCL.MND.huIL15-T2A-aCD30-CD8STK-41BBZ</td>
<td>MP codon optimised anti-CD30 CAR with 5-prime secreted IL-15.</td>
</tr>
<tr>
<td>pCCL.EF1a.huIL15-T2A-aCD30-CD8STK-41BBZ</td>
<td>MP codon optimised anti-CD30 CAR with 5-prime secreted IL-15.</td>
</tr>
<tr>
<td>pCCL.MND.aCD30-CD8STK-41BBz-T2A-huIL15</td>
<td>MP codon optimised anti-CD30 CAR with 3-prime secreted IL-15.</td>
</tr>
<tr>
<td>pCCL.EF1a.aCD30-CD8STK-41BBz-T2A-huIL15</td>
<td>MP codon optimised anti-CD30 CAR with 3-prime secreted IL-15.</td>
</tr>
<tr>
<td>pCCL.MND.aCD30-CD8STK-41BBz_Genscript_co</td>
<td>Genscript codon optimised anti-CD30 CAR.</td>
</tr>
<tr>
<td>pCCL.EF1a.aCD30-CD8STK-41BBz_Genscript_co</td>
<td>Genscript codon optimised anti-CD30 CAR.</td>
</tr>
<tr>
<td>pCCL.EF1a.aCD30-CD8STK-41BBz-T2A-huIL15_Genscript_co</td>
<td>Genscript codon optimised anti-CD30 CAR with secreted human IL-15.</td>
</tr>
<tr>
<td>pCCL.MND.aCD30-CD8STK-41BBz_GeneArt_co</td>
<td>GenArt codon optimised anti-CD30 CAR.</td>
</tr>
<tr>
<td>pCCL.EF1a.aCD30-CD8STK-41BBz_GeneArt_co</td>
<td>GeneArt codon optimised anti-CD30 CAR.</td>
</tr>
<tr>
<td>pCCL.EF1a.aCD30-CD8STK-41BBz-T2A-huIL15_GeneArt_co</td>
<td>GeneArt codon optimised anti-CD30 CAR with secreted human IL-15.</td>
</tr>
<tr>
<td>SFG.CD30</td>
<td>Human CD30 cloned into SFG. Used to generate stable cell lines.</td>
</tr>
</tbody>
</table>
2.2 Methods

2.2.1 Molecular Biology

2.2.1.1 Molecular cloning

2.2.1.1.1 Polymerase chain reaction

PCR fragments were required for molecular cloning of new plasmids and the generation of templates for transfection or \textit{in vitro} transcription. Oligonucleotides were supplied by Integrated DNA Technologies (IDT) and were stored at -20°C at a concentration of 100 µM in TE buffer. Oligonucleotides were diluted to 10 µM prior to PCR set-up. PCR amplification was performed using Q5® DNA High-Fidelity Polymerase (NEB, M0491L) in 50 µL reactions. PCR reactions were prepared on ice using the volumes and corresponding buffer concentrations outlined in Table 11. Amplification was performed in a thermocycler using the thermocycling conditions described in Table 12. PCR reactions were then purified using Qiagen’s QIAquick PCR Purification Kit and were checked for sizing by gel electrophoresis.

\textbf{Table 11 PCR protocol using Q5® DNA polymerase}

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume used</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water</td>
<td>to 50 µL</td>
<td>N/A</td>
</tr>
<tr>
<td>5X Q5® Reaction Buffer</td>
<td>10 µL</td>
<td>1X</td>
</tr>
<tr>
<td>5X Q5® High GC Enhancer</td>
<td>10 µL</td>
<td>1X</td>
</tr>
<tr>
<td>10 µM Forward Primer</td>
<td>2.5 µL</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>10 µM Reverse Primer</td>
<td>2.5 µL</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>1 µL</td>
<td>200 µM (each)</td>
</tr>
<tr>
<td>Template DNA (1-100 ng)</td>
<td>1 µL</td>
<td>0.1 – 2 ng/µL</td>
</tr>
<tr>
<td>Q5 High-Fidelity DNA Polymerase</td>
<td>0.5 µL</td>
<td>0.02 units</td>
</tr>
</tbody>
</table>
Table 12 Thermocycling conditions for PCR with Q5® DNA Polymerase

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>98°C</td>
<td>30 – 90 seconds</td>
</tr>
<tr>
<td>Denaturation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>25 – 35</td>
<td>15 seconds</td>
</tr>
<tr>
<td>Extension</td>
<td>cycles 98°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td></td>
<td>50-72°C</td>
<td>30 seconds / kilobase</td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>2 minutes</td>
</tr>
<tr>
<td>Hold</td>
<td>12°C</td>
<td>∞</td>
</tr>
</tbody>
</table>

2.2.1.1.2 Restriction endonuclease digestion

Restriction digestion was performed to generate cloning fragments with compatible “sticky ends” and to generate linearised templates for in vitro transcription. 3-5 μg plasmid DNA was digested with NEB’s high-fidelity restriction enzymes, where the final concentration of each enzyme was 3% of the final reaction volume (50 μL for plasmid digestion) (Table 13). For PCR products, the entire sample was digested, and the final reaction volume was scaled up to 100 μL with molecular grade nuclease-free water. The buffer selection and incubation parameters were defined by the manufacturer. If double digests were required and the buffers were incompatible, digests were performed serially. Digested DNA was separated by gel electrophoresis and purified using Qiagen’s Gel Extraction Kit. Where only one restriction enzyme was used to linearise a plasmid, the digested product was purified using Qiagen’s PCR Purification Kit.

Table 13 Restriction endonuclease digestion protocol

<table>
<thead>
<tr>
<th>Component</th>
<th>50 μL Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water</td>
<td>to 50 μL</td>
</tr>
<tr>
<td>DNA</td>
<td>3-5 μg</td>
</tr>
</tbody>
</table>
Chapter 2. Materials and Methods

### 10X Buffer
<table>
<thead>
<tr>
<th>Component</th>
<th>5 µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Restriction Enzyme 1</td>
<td>1.5 µL (15 units)</td>
</tr>
<tr>
<td>Restriction Enzyme 2</td>
<td>1.5 µL (15 units)</td>
</tr>
</tbody>
</table>

#### 2.2.1.1.3 DNA ligation

Following gel extraction, the digested vector DNA was dephosphorylated using Antarctic Phosphatase (NEB, M0289S) at a final enzyme concentration of 5 units per 1-5 µg DNA in a 50 µL reaction (Table 14). The reaction was incubated at 37°C for 1 hour (h), followed by inactivation of the enzyme at 80°C for 120 seconds. The vector and insert fragments were then ligated using T4 DNA Ligase (Roche, 10716359001) in a final volume of 20 µL (Table 15). Typically, vector backbone DNA was diluted to <100 ng/µL and reactions were set up using an insert to vector molar ratio of 3:1 to 10:1. Ligation reactions were incubated at 16°C for a minimum of 1h prior to transformation.

**Table 14 Protocol for DNA dephosphorylation using Antarctic Phosphatase**

<table>
<thead>
<tr>
<th>Component</th>
<th>50 µL Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water</td>
<td>to 50 µL</td>
</tr>
<tr>
<td>DNA</td>
<td>30-44 µL (1-5 µg)</td>
</tr>
<tr>
<td>10X Buffer</td>
<td>5 µL</td>
</tr>
<tr>
<td>Antarctic Phosphatase</td>
<td>1 µL (5 units)</td>
</tr>
</tbody>
</table>

**Table 15 Protocol for DNA ligation using T4 DNA Ligase**

<table>
<thead>
<tr>
<th>Component</th>
<th>20 µL Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water</td>
<td>to 20 µL</td>
</tr>
</tbody>
</table>
2.2.1.1.4 Small scale DNA preparation

For small scale DNA preparations, 3-5 mL Lysogeny Broth (LB) containing the appropriate selective antibiotic was inoculated with a single plasmid colony and was incubated overnight at 37°C and 250 rpm. The NucleoSpin® Plasmid EasyPure Mini Kit (Machery-Nagel) was used to extract DNA from the bacterial culture following the manufacturer’s protocol. The DNA was eluted from the column using 50 µL nuclease-free water or TE buffer.

2.2.1.1.5 Large scale DNA preparation

For large scale DNA preparations, a single colony was picked from a bacterial agar plate and was added to 100 mL LB containing the appropriate selective antibiotic. The inoculated broth was then placed in a shaking incubator at 37°C and 250 rpm for 14-18h. The DNA was isolated with a low endotoxin concentration using the NucleoBond® Xtra Midi Kit (Machery-Nagel) using the manufacturer’s protocol. The DNA was resuspended in TE buffer at 1000 ng/µL.

2.2.1.1.6 DNA quantitation

The DNA concentration can be determined using the absorbance of light at wavelength 260 nanometre (nm), whilst the purity can be determined by the ratio of absorbance at 260 nm and 280 nm. A ratio of 1.8 indicates a high degree of DNA purity with low contamination of RNA or protein. The Thermo-Scientific Nanodrop 8000 spectrophotometer was used to determine both the DNA concentration and purity.
2.2.1.7 Gel electrophoresis

To analyse and separate DNA fragments, a 1% agarose gel was prepared by adding 1.5 g agarose to 150 mL 1 X TAE and microwaving until dissolved. 7 µL SYBR™ Safe DNA Gel Stain (Invitrogen) was added once the mixture had cooled and was left to set. Bioline DNA Loading Buffer was diluted 1:5 with DNA and samples were run at 140 volts for 40 minutes alongside an appropriate ladder (1 kB Hyperladder, Bioline). At the end of the run, a blue light transilluminator (Azure c200 Gel Imaging System) was used to capture an image of the gel and to excise fragments of the correct size. The excised DNA was then purified using the QIAquick Gel Extraction Kit (Qiagen).

2.2.1.2 Bacterial manipulation

2.2.1.2.1 Growth and maintenance of E. coli

*E. coli* bacteria were grown in liquid LB media or in LB-agar plates supplemented with the appropriate antibiotic and were cultured at 37°C with agitation at 250 rpm, where appropriate.

2.2.1.2.2 Bacterial transformation

High efficiency chemically competent bacteria (NEB 5-alpha Competent *E. coli*, C2987) were thawed on ice and transferred to PCR tubes. 2 µL of the ligated DNA products were added to 25 µL of bacteria and were left to incubate on ice for 30 minutes. The *E. coli* and ligation mixture was heat shocked at 42°C for precisely 35 seconds before placing on ice for an additional 5 minutes. 0.5-1 mL SOC outgrowth media was added, and bacteria was recovered for 1h in a shaking incubator at 37°C and 250 rpm. Following the outgrowth step, the transformed bacteria were plated on ampicillin (100 µg/mL) or kanamycin-laden (50 µg/mL) agarose plates.

2.2.1.3 RNA Synthesis

2.2.1.3.1 In vitro transcription of linear RNA

Linear mRNA requires a 7-methylguanylate cap structure at the 5-prime (5’) end and a polyadenylation (poly(A)) tail at the 3-prime (3’) end to promote its stability. 5’ capped mRNA was synthesised by *in vitro* transcription from 0.2 µg purified PCR product or 1.0
µg linearised plasmid DNA. A T7 promoter and consensus Kozak sequence (T7 promoter sequence with initiating guanine in bold and underlined, TAATACGACTCACTATAGGGAGA; Kozak, GCCACC) was incorporated into the construct or added into the forward PCR primer to facilitate synthesis using the mMESSAGE mMACHINE™ T7 Transcription Kit (Invitrogen). The reactions were prepared at RT using the volumes outlined in Table 16, which could be scaled as necessary. In vitro transcription was performed by incubating the reactions at 37°C for 2h in a thermocycler. Template DNA was degraded by treatment with TURBO DNase (supplied with the kit), by adding 1 µL of the enzyme to each 20 µL reaction and incubating for a further 15 minutes. mRNA was then polyadenylated using the Poly(A) Tailing Kit (Invitrogen) which could be directly added to the un-purified mMESSAGE mMACHINE™ reaction (Table 17). Polyadenylation was performed by incubating the reactions at 37°C for 1h.

**Table 16 mMESSAGE mMACHINE™ protocol for in vitro transcription**

<table>
<thead>
<tr>
<th>Component</th>
<th>20 µL Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water</td>
<td>to 20 µL</td>
</tr>
<tr>
<td>2X NTP/CAP</td>
<td>10 µL</td>
</tr>
<tr>
<td>10X Buffer</td>
<td>2 µL</td>
</tr>
<tr>
<td>DNA</td>
<td>Up to 6 µL (0.2-1 µg)</td>
</tr>
<tr>
<td>T7 RNAP Enzyme</td>
<td>2 µL</td>
</tr>
</tbody>
</table>

**Table 17 Protocol for polyadenylation of RNA**

<table>
<thead>
<tr>
<th>Component</th>
<th>100 µL Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>mMESSAGE mMACHINE reaction</td>
<td>20 µL</td>
</tr>
</tbody>
</table>
Chapter 2. Materials and Methods

Alternatively, NEB’s HiScribe™ T7 Quick High Yield RNA Synthesis Kit was used to produce RNA from 1.0 µg of PCR product. Reactions were set up according to the volumes in Table 18, and were incubated at 37°C for 2h. To remove template DNA, 30 µL of nuclease-free water and 2 µL DNase I (NEB, M0303S) was added to each 20 µL reaction, followed by an incubation at 37°C for 15 minutes. RNA was purified and subsequently capped using the Vaccina Capping System (NEB, M2080S). This method generated RNA with a Cap 0 structure which was then treated with Cap 2'-O-Methyltransferase (NEB, M0366S) to produce a Cap 1 structure. 10 µg RNA was diluted to a final volume of 14 µL in nuclease-free water and was denatured by heating to 65°C for 5 minutes. Denatured RNA was placed on ice for 5 minutes prior to adding the capping components (Table 19) and incubating for 1h at 37°C. In this instance, the poly(A) tail was incorporated into the 5’ end of the reverse primer through the addition of 120 thymine bases.

**Table 18 Protocol for *in vitro* transcription using the HiScribe™ T7 Kit**

<table>
<thead>
<tr>
<th>Component</th>
<th>20 µL Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water</td>
<td>to 20 µL</td>
</tr>
<tr>
<td>2X NTP Buffer Mix</td>
<td>10 µL</td>
</tr>
<tr>
<td>DNA</td>
<td>Up to 8 µL (1 µg)</td>
</tr>
</tbody>
</table>
2.2.1.3.2 *In vitro transcription of circRNA*

The HiScribe™ T7 Quick High Yield RNA Synthesis Kit was used to transcribe RNA from 0.2 µg purified PCR product or 1.0 µg linearised plasmid DNA containing the modified permuted intron exon sequence from *Anabaena* cyanobacterium (Wesselhoeft, Kowalski and Anderson, 2018). Since circRNA does not need to be capped or polyadenylated, DNase I treated RNA was immediately purified prior to circularisation.

2.2.1.3.3 *RNA purification*

Purification is necessary to remove unincorporated nucleotides, proteins, and salts from the transcription reactions and to concentrate the RNA for downstream applications. Linear mRNA that was produced using the mMESSAGE mMACHINE™ T7 Transcription Kit was purified by lithium chloride (LiCl) precipitation or using the MEGAclear™ Transcription Clean-Up Kit (Invitrogen). For the latter, RNA was diluted
to 100 µL with elution solution before adding 350 µL of binding solution and 250 µL 100% ethanol. This facilitated RNA binding to the membrane of a column filter cartridge. The sample was passed through the filter by centrifugation at 10,000 x g and was washed twice by adding 500 µL wash solution. RNA was then eluted by adding 50 µL elution solution and the column was heated to 70°C for 5 minutes before centrifugation for 1 minute.

RNA that was transcribed using the HiScribe™ T7 Quick High Yield RNA Synthesis Kit was purified before and after capping/circularisation by LiCl precipitation. 25 µL LiCl (supplied with the kit) was added per 50 µL reaction (DNase I treated RNA) and was incubated at -20°C for a minimum of 30 minutes. Reactions were pooled into a 1.5 mL microcentrifuge tube before centrifugation at maximum speed for 15 minutes at 4°C. The RNA pellet was then washed with 500 µL ice-cold 70% ethanol and was resuspended in 50 µL elution solution (supplied with Invitrogen’s MEGAclear™ Transcription Clean-Up Kit) per 50 µL reaction.

2.2.1.3.4 RNA circularisation

CircRNAs have greater stability in eukaryotic cells compared to linear RNA because their closed ends protect the RNA from exonuclease degradation. RNA can be circularised by chemical methods using condensing agents, by enzymatic methods using ligases, or by ribozymatic methods using self-splicing introns (Müller and Appel, 2017). Here, circularisation was performed using the self-splicing permuted group I catalytic intron sequence and the protocol published by Wesselhoeft et al., (Wesselhoeft, Kowalski and Anderson, 2018). Purified RNA was circularised at a final concentration of 0.3-1.0 µg/µL in a buffer containing 50 mM Tris-HCl pH7.5, 10 mM MgCl₂, 1 mM DTT and 2 mM GTP. The sample was heated to 55°C for 15 minutes to allow back-splicing and release of the intronic sequences.

2.2.1.3.5 RNA quantitation

The Qubit™ Fluorometer and Qubit™ RNA Broad Range Assay Kit (Invitrogen) were used to determine the RNA concentration. Qubit™ Fluorometers detect fluorescent dyes that only fluoresce when they are bound selectively to RNA. This technique is more
specific than UV absorbance and can distinguish between RNA and contaminating template DNA. The sample concentration is calculated based on the fluorescence of negative and positive standards that are used to calculate a standard curve on the Qubit™ Fluorometer.

2.2.1.3.6 RNA gel electrophoresis

The Agilent 2200 TapeStation system and RNA ScreenTape reagents were used to check the integrity and size of the RNA. 5 μL RNA ScreenTape Sample Buffer was added to 1 μL RNA (100-500 ng) or RNA ScreenTape Ladder in PCR tubes. Samples were mixed thoroughly, and RNA was denatured by heating to 72°C for 3 minutes. RNA was chilled on ice for 2 minutes prior to electrophoresis.

2.2.1.4 Preparation of DNA templates

DNA templates for transfection studies were generated by PCR using Q5® DNA High-Fidelity Polymerase (NEB, M0491L) as previously described (Table 11, Table 12). Here, the reaction volumes were scaled to 100 μL for amplification using a 96 deep-well thermocycler. PCR products were checked for sizing by gel electrophoresis before purification using Agencourt AMPureXP solid-phase reversible immobilisation (SPRI) paramagnetic beads (Beckman Coulter) at a final bead concentration of 0.5X the PCR reaction volume. To elute the DNA, 3 μL elution solution per 100 μL PCR reaction was added to the magnetic beads and was incubated for a minimum of 1h before magnetic bead removal. Residual beads were removed from the eluted DNA by centrifugation at 10,000 x g for 60 seconds and transferring the DNA supernatant to a fresh 1.5 mL microcentrifuge tube.

2.2.2 Tissue Culture

2.2.2.1 Propagation of cell lines

2.2.2.1.1 Maintenance of adherent cell lines

HEK293T cells were cultured in complete IMDM in a 175cm² tissue culture treated flask in a humidified incubator at 37°C and 5% CO₂ atmosphere. Twice a week at 80% confluency, cells were passaged 1:10. Cells were washed with 1X PBS then incubated at
37°C for 5 minutes with 5 mL trypsin-EDTA. 10 mL of complete IMDM was added to harvest the cells. Cells were pelleted at 400 x g for 5 minutes and resuspended into fresh medium.

2.2.2.1.2 Maintenance of suspension cell lines

Suspension cell lines were maintained in complete RPMI 10% (SUP-T1, MOLT-4, RPMI-8226, L-428) or in complete RPMI 20% (HDLM-2, L-540) in 75cm² tissue culture treated flasks at 37°C and 5% CO₂ atmosphere. Culture densities were maintained between approximately 0.2-1.0x10⁶ cells/mL by 1:5-1:10 dilution with fresh medium, as determined by the discolouration of the phenol red indicator dye.

2.2.2.1.3 Maintenance of hybridoma cell lines

Rat hybridomas were generated by Genovac and were maintained in complete DMEM in 75cm² tissue culture treated flasks at 37°C and 5% CO₂ atmosphere. When the phenol red indicator dye became discoloured, cells were passaged 1:5-1:10 by media replacement.

2.2.2.1.4 Single cell cloning

Single cell cloning was required to generate highly expressing stable cell lines. Single cell isolation was achieved by limiting dilution to ensure that on average every well of a tissue culture-treated flat bottom 96-well plate would contain 1 cell in 100 μL medium. Cell clones were cultured for approximately 10 days until a visible population had been established. Clones were screened by flow-cytometry to identify wells displaying positive clones and were further expanded into 48- or 24-well plates. Subsequent rounds of expansion and screening were performed until cells were ready for cryopreservation.

2.2.2.1.5 Cryopreservation and recovery of cell lines

Cells were cryopreserved in cryovials at 5x10⁶ cells/mL in cell freezing medium. Cryovials were placed in a CoolCell™ in the -80°C freezer to allow for a controlled drop in temperature of 1°C/minute before transfer to liquid nitrogen. Upon recovery, cells were thawed quickly in a water bath at 37°C to prevent toxicity caused by DMSO. Once defrosted, cells were transferred to a 15 mL Falcon tube and washed with warm medium before transferring to a flask.
2.2.2.2 **Primary cell culture**

2.2.2.2.1 **Isolation of mononuclear cells**

Peripheral mononuclear cells (PBMCs) are isolated from NHSBT leukocyte cones by density gradient centrifugation using Ficoll-Paque PLUS (GE Health Care) and SepMate-50 Tubes (STEMCELL Technologies). PBMCs were aspirated from the monolayer and resuspended for downstream processing. PBMCs were cultured in either complete TexMACS™ or complete RPMI 10% at approximately 1x10^6 CD3+ cells/mL.

2.2.2.2.2 **Stimulation of T-cells**

T-cells are activated through engagement of the CD3 and CD28 receptors, combined with a cytokine signal. Thawed cells were resuspended at 2-3x10^6 total viable cells/mL and recovered overnight or used fresh and activated on the day of isolation. T-cell activation was performed at 1-2x10^6 total viable cells/mL using TransAct™ (research grade, 1:100) and 10 ng/mL interleukin-7 (IL-7) and interleukin-15 (IL-15) (Miltenyi Biotec). Alternatively, PBMCs were activated with soluble anti-CD3 (aCD3) and anti-CD28 (aCD28) antibodies at a final concentration of 50 ng/mL and 50 iU/mL interleukin-2 (IL-2) (Miltenyi Biotec). T-cells were activated for 24h prior to lentiviral transduction or 48h prior to retroviral transduction or non-viral gene delivery.

2.2.2.3 **Retroviral work**

2.2.2.3.1 **Generation of viral supernatant by transient transfection**

The generation of retroviral and lentiviral supernatants can be achieved by the transient transfection of HEK293T cells with DNA encoding the packaging and genome components. Lentiviral vectors are produced using a 3rd generation system, where three packaging plasmids; Gag-Pol, REV and VSV-G are co-transfected with the plasmid expressing the transgene of interest. The Gag-Pol plasmid encodes the structural polyprotein “gag” and the replication components; reverse transcriptase, RNase H, and integrase, from the “pol” gene. REV encodes an accessory protein required for the packaging of viral particles and VSV-G forms the envelope to mediate cellular receptor binding and membrane fusion. For retroviral vectors, the REV plasmid is not required, and retroviral particles are pseudo-typed with the RD114 envelope to enable transduction.
of human cells. HEK293T cells were plated into 10 cm tissue culture treated plates in aim to achieve >70% confluency or >50% confluency on the day of transfection for lentiviral or retroviral packaging, respectively. Transient transfection was conducted using GeneJuice® (Merck Millipore), a non-lipid-based transfection reagent that complexes with DNA. Packaging and transgene plasmids were mixed in a 15 mL Falcon tube using the amounts detailed in Table 20 and Table 21, and the GeneJuice® master mix was prepared by diluting 30 µL in 470 µL of plain IMDM media, per 10 cm plate. Following an incubation at RT for 5 minutes, the GeneJuice® mix was added to the DNA mix and was incubated for 15 minutes before adding to the cells in a dropwise manner.

Table 20 DNA ratios for packaging of lentiviral vectors

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>µg/plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>REV</td>
<td>1.560</td>
</tr>
<tr>
<td>Gag-Pol</td>
<td>3.125</td>
</tr>
<tr>
<td>VSV-G</td>
<td>1.560</td>
</tr>
<tr>
<td>Transgene</td>
<td>6.250</td>
</tr>
</tbody>
</table>

Table 21 DNA ratios for packaging of retroviral vectors

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>µg/plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gag-Pol</td>
<td>4.7</td>
</tr>
<tr>
<td>RD114</td>
<td>3.1</td>
</tr>
<tr>
<td>Transgene</td>
<td>4.7</td>
</tr>
</tbody>
</table>

2.2.2.3.2 Purification of viral supernatants

Lentiviral supernatant was harvested at 48h post-transfection, and retroviral supernatant was harvested at 72h. Supernatants were pelleted at 400 x g for 5 minutes to remove
cellular debris and were filtered through a 0.45 µM filter before freezing at -80°C. To increase viral titres, lentiviral supernatants were concentrated by high-speed centrifugation at 10,000 x g for 20 hours, where noted. Viral pellets were resuspended by gentle agitation in Opti-MEM medium using a 50X or 100X reduction in volume for integrating and non-integrating supernatants, respectively.

2.2.2.3.3 Titration of viral supernatants

HEK293T cells were plated at 50,000 cells per well in a tissue culture treated 24-well plate in complete IMDM containing 8 µg/mL polybrene (Merck Millipore). Supernatants were thawed and kept on ice and serial dilutions were prepared in 96-well V-bottom plates. Supernatants were typically diluted 1:4 to 1:8, serially for 6 points. This ensured transduction efficiencies were below 20%, to aim for a vector copy number of one integration per cell. 100 µL of diluted supernatants were added to cells, followed by centrifugation at 1000 x g for 10 minutes and transfer to a 37°C incubator with 5% CO₂. To determine functional titre, flow-cytometry was performed at 72h post-transduction to determine the percentage of transduced cells in each well. The average functional titre was calculated using the following equation:

\[
\text{Viral titer (Transducing units (TU)/mL) = } \frac{(\text{number of cells plated } \times \text{ (% transduction efficiency/100)}]}{(\text{vector volume in mL})}
\]

2.2.2.3.4 Preparation of RetroNectin plates

RetroNectin has been reported to improve retroviral transduction efficiencies by aiding the co-localisation of viral particles with the cells (Hanenberg et al., 1996). RetroNectin plates were prepared by coating non-tissue culture treated 24-well plates with 500 µL PBS containing 4 µL RetroNectin (1 mg/mL stock, Takara). Alternatively, non-tissue culture treated 6-well plates were coated with 1.5 mL of the RetroNectin mix. RetroNectin-coated plates were incubated at 4°C overnight, left at RT for 2h or placed in a humidified 37°C incubator for 1h prior to the transduction of suspension cells.
2.2.2.3.5 Retroviral transduction of suspension cell lines

Retroviral supernatant was thawed in a water bath at 37°C and then placed on ice. RetroNectin was aspirated and 1.5 mL or 3 mL supernatant was added to each well of a 24-well or 6-well plate, respectively. For 24-well plates, cells were harvested and resuspended at 0.6x10⁶/mL, before adding 0.5 mL to each well. For 6-well plates, cells were resuspended at 1x10⁶/mL and 1 mL was plated. Transduction was performed by spinoculation at 1000 x g for 40 minutes at RT, before returning to the incubator for 72h before analysis.

2.2.2.3.6 Lentiviral transduction of PBMCs

PBMCs were transduced at a multiplicity of infection (MOI) ranging from 0.2-10 virions per cell depending on the desired transduction efficiency and vector copy number. The MOI was calculated using the following equation:

$$\text{Vector Volume (mL)} = \frac{(\text{desired MOI} \times \text{number of cells per well})}{(\text{viral titer (TU/mL)})}$$

RetroNectin was aspirated from non-tissue culture treated 24-well or 6-well plates and the calculated vector volume was added to the wells in a final volume of 1.5 mL or 3 mL complete RPMI 10% or complete TexMACS™. PBMCs were stimulated as previously described and were harvested and resuspended at 0.6x10⁶/mL or 1x10⁶/mL in complete medium. For 24-well plates, 0.3x10⁶ cells were plated per well, compared to 1x10⁶ cells per well in a 6-well plate. The cell culture medium was supplemented with cytokines to a final concentration of 50 iU/mL IL-2 (in complete RPMI 10%) or 10 ng/mL IL-7 and IL-15 (in complete TexMACS™). The plated PBMCs were transduced by spinoculation at 1000 x g for 40 minutes at RT, prior to an incubation at 37°C and 5% CO₂ for 72hr. Flow-cytometry was then performed to detect the integrated transgene of interest.

2.2.2.4 Transient transfection

2.2.2.4.1 Nucleofection using Lonza’s Nucleofector™ device

Nucleofection is an efficient method for the delivery of DNA, RNA, or protein into primary cells. The protocol involves the preparation of delivery substrates alongside
washing the cells to remove serum-containing medium that may contain nucleases. The substrates are combined with the cells in a nucleofection buffer prior to delivering an electrical pulse. The samples can be transfected using either 20 µL or 100 µL nucleofection cuvettes, for the electroporation of 1x10^6 cells or 5x10^6 cells, respectively. Substrates were prepared in PCR tubes or 96-well V-bottom plates in a maximum volume of 7 µL PBS per 20 µL cuvette (volumes were scaled accordingly). Immediately prior to nucleofection, cells were harvested and washed twice in 15 mL PBS by centrifugation for 5 minutes at 400 x g. Meanwhile, the nucleofection buffer was prepared by combining the appropriate volume of nucleofection solution and supplement (Table 22). Alt-R® Cas9 Electroporation Enhancer (IDT) was added to the nucleofection buffer at a final concentration of 5 µM, to enhance the transfection efficiency of proteins. Cells were then resuspended at 1x10^6 cells per 20 µL nucleofection buffer and were added to the substrates. Samples were transferred to nucleofection cuvettes and were nucleofected using Lonza’s Amaxa 4D-Nucleofector™ or 96-well shuttle device using optimised programs and pulse codes (Table 23). Immediately after nucleofection, 80 µL or 400 µL complete RPMI 10% or complete TexMACS™ (without cytokines) was added to 20 µL or 100 µL cuvettes. Nucleofection cuvettes were placed in a humidified 37°C incubator and cells were left to recover for 10-15 minutes. Transfected cells were then transferred to 24-well tissue culture plates or T-25 tissue culture treated flasks at a concentration of 1-2x10^6 cells/mL.

Table 22 Preparation of nucleofection buffers

<table>
<thead>
<tr>
<th>Reagent</th>
<th>20 µL cuvettes</th>
<th>100 µL cuvettes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleofector Solution</td>
<td>16.4 µL</td>
<td>82.0 µL</td>
</tr>
<tr>
<td>Supplement</td>
<td>3.6 µL</td>
<td>18.0 µL</td>
</tr>
<tr>
<td>Alt-R® Cas9 Electroporation Enhancer (10 nmol)</td>
<td>1.0 µL</td>
<td>5.0 µL</td>
</tr>
</tbody>
</table>
Table 23 Optimised programs and pulse codes

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Program/Buffer</th>
<th>Pulse Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUP-T1</td>
<td>SF</td>
<td>CM-150</td>
</tr>
<tr>
<td>PBMC</td>
<td>P3</td>
<td>EH-115</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>SF</td>
<td>CA-137</td>
</tr>
<tr>
<td>RPMI-8226</td>
<td>SF</td>
<td>DN-100</td>
</tr>
</tbody>
</table>

2.2.2.4.2 Soluporation using Avectas’ proprietary technology

The SOLUPORE® technology has been developed by Avectas, as a novel non-viral delivery method using a low concentration of ethanol to temporarily permeabilise the cellular membrane.

2.2.2.4.2.1 Cell preparation

For technology transfer experiments, PBMCs were provided by Avectas as a frozen culture and T-cell activation was prepared according to the Avectas protocol. PBMCs were thawed and immediately activated at 1x10^6 cells/mL with 100 ng/µL soluble aCD3 and aCD28 antibodies in complete CTS OpTmizer containing 250 iU/mL IL-2. Cells were transferred to a T-175 tissue culture treated flask and were placed in an incubator at 37°C and 5% CO₂ for 72h-96h. Following initial technology transfer experiments, PBMCs were stimulated at 1x10^6 cells/mL for 48h using complete TexMACS™ supplemented with TransAct™ (research grade, 1:100) and 10 ng/mL IL-7 and IL-15 (Miltenyi Biotec).

On the day of soluporation, 1x10^6 cells were sampled for flow-cytometry to determine the CD3 content and the level of activation through the expression of CD25. The remaining tissue culture was pelleted by centrifugation at 350 x g for 7 minutes and washed twice with an equal volume of PBS, prior to resuspension in 20 mL pre-warmed CTS OpTmizer medium (no supplementation). Cells were counted and resuspended at a
concentration of $0.6 \times 10^6$ cells/mL before aliquoting 10 mL for each transfected sample into a 50 mL Falcon tube. 10 mL cell culture was pelleted, resuspended in 10 mL PBMC CTS OpTmizer maintenance medium, and was transferred to a T-25 tissue culture treated flask as the un-transfected control.

2.2.2.4.2.2 Research tool calibration

The SOLUPORE® research tool is a pressurised system that delivers the substrate-containing cargo by atomisation. The research tool must be calibrated prior to use, and the air pressure adjusted, to ensure the correct volume of cargo is delivered. As a starting point, the air pressure was set to 1730 mbar and the sample pressure to 60 mbar. To calibrate the machine, 1 mL calibration solution (preparation described in Table 24) was added to a microcentrifuge tube and was weighed using an analytical balance. The tube was placed in the tube holder on the top of the machine and atomisation was initiated by pressing the “spray” button on the display screen. A container was placed below the atomiser to collect the waste. The tube was then re-weighed, and the volume dispensed was calculated by subtracting the post-calibration volume from the pre-calibration volume, assuming 1 mg = 1 µL. The calibration process was repeated for a minimum of three times, and the sample pressure adjusted until 50 µL was reliably dispensed.

Table 24 Preparation of SOLUPORE® calibration solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water</td>
<td>4150</td>
</tr>
<tr>
<td>20X S-buffer</td>
<td>250</td>
</tr>
<tr>
<td>Absolute ethanol (final concentration 12%)</td>
<td>600</td>
</tr>
</tbody>
</table>
2.2.2.4.2.3 Transfection process

The SOLUPORE® delivery solution was prepared immediately before transfection and involves resuspending the cargo for delivery in soluporation buffer (S-buffer) and ethanol. The volume of delivery solution prepared should account for the number of 50 µL transfection sprays, an excess volume of 20% per sample, a dead volume of 150 µL and an additional two 50 µL sprays to prime the tubing. The delivery solution was prepared in the same way as the calibration solution, by diluting 20X S-buffer in nuclease-free water and adding ethanol to a final concentration of 12%. The substrate was added at a concentration that allows the desired amount to be delivered in a 50 µL spray volume. The microcentrifuge tube containing the calibration solution was removed from the machine and replaced with a tube containing the cargo delivery solution. The tubing line that connects the delivery solution to the atomiser was primed by actuating the “spray” button twice before transfection. A single-use transfection pod was placed on a holder on the research tool and the pod membrane was primed by adding 2 mL CTS OpTmizer medium. The pod base was blotted with sterile tissue to start the draining process and was visually inspected to ensure the membrane was dry before adding the cells. Transfections were performed sequentially, the prepared cells in a single 50 mL Falcon tube were mixed by pipetting and were gently poured onto the primed pod membrane. The draining process was timed for a period of 2 minutes, after which the medium should have passed through into the waste container below, leaving the cells on the membrane as an exposed monolayer. The pod was temporarily removed from the research tool for a period of 30 seconds and the residual medium was absorbed from beneath using a sterile tissue. The pod was immediately returned to the machine and the spray button was actuated to deliver the cargo in the form of droplets. The transfection was then incubated for exactly 30 seconds before adding 2 mL complete CTS OpTmizer to stop the transfection.

2.2.2.4.2.4 Cell recovery

Prior to soluporation, 50 mL syringes were filled with 12 mL complete CTS OpTmizer, preparing one syringe for each transfection. Each syringe was attached to a recovery base through a tubing line with a closed clamp. During the 30 second transfection incubation,
the recovery base was loosely screwed onto the pod, and following the addition of complete medium, was screwed on tightly to ensure a sufficient seal. The tubing line clamp was opened, and the medium was flushed into the pod using the syringe plunger. The cells were recovered from the filter membrane by pipetting and transferring cells to a tissue culture treated T-25 flask. The pod membrane was washed with a further 2 mL complete medium and was added to the flask. Cells were returned to a humidified 37°C incubator and were left for 24h prior to analysis.

2.2.2.5 Non-viral genome editing

2.2.2.5.1 Guide RNAs

Guide RNAs (gRNAs) are required for CRISPR-mediated genome editing or base editing using cytosine or adenine base editors. gRNA sequences for CRISPR-mediated genome engineering were designed using Synthego’s CRISPR knockout guide design tool (https://design.synthego.com/#/). The top four rating sequences were subsequently tested for their editing efficiencies. Base editing gRNAs were designed to introduce premature stop codons or to disrupt splice donor or acceptor sites using the BE-Designer web tool (http://www.rgenome.net/be-designer/) and the splice-site disruption single gRNA design program SpliceR (https://z.umn.edu/splicer) (Hwang et al., 2018). The first step in the generation of gRNAs is to anneal the CRISPR RNA (crRNA) with the trans-activating RNA (tracrRNA). Pre-annealed single guide RNAs (sgRNAs) were supplied by Synthego and were modified to contain three phosphorothioate modifications at the 5-prime and 3-prime ends to increase their stability (Hendel et al., 2015). sgRNAs were reconstituted to 80 µM in TE and were stored at -80°C.

2.2.2.5.2 CRISPR/Cas9-mediated genome editing

The Cas9 enzyme is an RNA-guided endonuclease that can be re-directed to a chosen site where it will generate a double-stranded break (DSB). The DSB is predominately repaired by non-homologous end joining (NHEJ), an imprecise process that will result in the formation of indels and disruption of the gene. To genome edit 1x10^6 cells, ribonucleic proteins (RNPs) were produced by annealing 100 pmol of non-targeting or targeting sgRNA with 50 pmol Alt-R S.p HiFi V3 Cas9 nuclease (61 µM, IDT) at a molar ratio of
2:1. RNPs were formed by incubation at RT for 20 minutes, prior to delivery by nucleofection or soluporation (2.2.2.4.1 and 2.2.2.4.2). Gene knockout efficiencies were determined by flow-cytometry at 96h post-transfection.

2.2.2.5.3 Base editing

Base editing requires the co-transfection of sgRNA and RNA encoding the base editor protein. This can be achieved by nucleofection or soluporation as outlined in sections 2.2.2.4.1 and 2.2.2.4.2. To base-edit 1x10^6 stimulated T-cells, 30 pmol sgRNA and 4 µg base editor RNA was delivered, unless otherwise noted. T-cells were expanded for 96h post-transfection, at which point cells were re-stimulated with TransAct™ (research grade, 1:100) and 10 ng/mL IL-7 and IL-15 (Miltenyi Biotec), to upregulate the expression of the target genes. Flow-cytometry was performed 24h and 72h later to determine the gene knockout efficiencies. Due to the heterogeneous expression of T-cell inhibitory receptors, the base editing efficiencies were calculated by normalisation to the relevant non-targeting (NT) sgRNA control:

\[
\text{Editing efficiency (\%)} = \left( \frac{(\% \text{ protein expression in NT sample} - \% \text{ protein expression in edited sample})}{\% \text{ protein expression in NT sample}} \right) \times 100
\]

2.2.2.5.4 Genotyping

Genotyping was performed to confirm CRISPR-mediated genome editing and to calculate base conversion efficiencies. Genomic DNA was isolated from T-cells at 7 days post-nucleofection using Invitrogen’s PureLink™ Genomic DNA Mini Kit. Oligonucleotides (Integrated DNA Technologies) were designed to amplify a region of <800 bp across the target site by PCR (PCR-1). If non-specific product bands were observed, a nested PCR was performed by amplifying PCR-1 using primers internal to the sequence. The PCR product was sent for Sanger sequencing at Source BioScience and was subsequently analysed for gene editing. For CRISPR-mediated genome editing, sequencing traces were uploaded to Synthego’s Inference of CRISPR Edits (ICR) analysis tool to calculate the overall editing efficiencies (https://ice.synthego.com/#/) (Conant et al., 2022). To analyse base conversion efficiencies, Sanger sequencing traces
Chapter 2. Materials and Methods

were uploaded to the web app EditR (http://baseeditr.com/) (Kluesner et al., 2018). EditR can determine the outcomes of base editing by comparing the frequency of each base in a single position relative to the target sequence.

2.2.3 Antibody generation using hybridomas

An anti-idiotype antibody was developed to identify expression of the anti-CD30 CAR. Hybridomas were generated by the service provider, Genovac, through the immunisation of rats with CAR-encoding plasmid DNA. Following immunisation, rats displaying sera conversion were taken forward for the generation of hybridomas. Hybridomas are formed by the fusion of mortal B-cells with immortal myeloma cells to facilitate continued antibody secretion into the cellular supernatant. We screened 880 neat hybridoma supernatants on SUP-T1 cells transduced with the CAR, co-expressed with eGFP, and an anti-Rat PE-conjugated secondary antibody for detection by flow-cytometry. 54 hybridomas were identified and taken forward for sub-cloning at Genovac. Upon re-screening of sub-clones, 5 positive candidates were identified and shipped as frozen cultures. Upon cellular revival, the supernatant from the optimal clone, 2A11, was collected. Cellular supernatant was pelleted by centrifugation and passed through a 0.22 µM filter prior to use.

2.2.4 Flow-cytometry

Flow-cytometry was performed using the Miltenyi Biotec MACSQuant®10 or MACSQuant®X instruments or using the Becton Dickinson (BD) Fortessa flow-cytometer.

2.2.4.1 General staining method

Typically, 0.2-1.0x10^6 cells per sample were harvested and pelleted in 96-well V-bottom plates. Cells were washed in 150 µL PBS before resuspension in 100 µL antibody mix. All antibodies were prepared at dilutions recommended by the manufacturer. All staining steps were performed at RT in the dark for 15-30 minutes prior to washing with 150 µL PBS. Samples were resuspended in 50-100 µL PBS or viability stain and 80% of the volume was analysed using the MACSQuant® instruments. Alternatively, 30,000 live
cell events were captured using the BD Fortessa flow-cytometer. Isotype and/or non-transduced samples were included as negative controls for flow-cytometry gating.

### 2.2.4.2 Preparation of counting beads

Counting beads typically fluoresce in all flow-cytometry channels and were used to normalise the median fluorescence intensity (MedFI) of each channel, allowing for comparison between samples that were run on separate days. CountBright Absolute Counting Beads (Thermo-Scientific) were diluted 1:10 and 50 µL was added to each well of a 96-well V-bottom plate, prior to the addition of cells for staining. Normalisation was calculated by dividing the Sample MedFI by the bead MedFI.

### 2.2.4.3 Compensation

Using multiple fluorophores in a flow-cytometry panel can lead to spill over of fluorescence into adjacent channels. Compensation can be calculated and applied to the samples to control for the interference. Unlabelled and single stain controls were prepared for every flow-cytometry plate where more than three fluorophores were used. Single stains were performed by staining cells or one drop of UltraComp eBeads™ Compensation Beads (Invitrogen) with 1 µL of antibody. Spill over can then be calculated automatically by identifying the positive and negative populations in FlowJo software.

### 2.2.4.4 Gating strategy

Flow-cytometry data was analysed using FlowJo Software, version 10.8.1. The gating strategy first involved the identification of the cell population based on size and granularity (FSC-A versus SSC-A), before isolating the singlets (FSC-A versus FSC-H) and live cells (negative for viability dye). Where appropriate, T-cells were identified as CD3+ prior to subsequent gating on the markers of interest.

### 2.2.5 In vitro functional assays

#### 2.2.5.1 Cytotoxicity assay

Measuring the cytolytic capacity of CAR T-cells is a key part of the characterisation process. Co-cultures were set up with CAR T-cells and cell lines that express the target
antigen. A flow-cytometry based assay was performed to determine cytotoxicity by measuring the percentage of viable target cells remaining in co-cultures after a given time. This can be expressed as a percentage of remaining target cells compared to the non-specific killing observed with the non-transduced T-cells.

Co-cultures were set up using a defined ratio of effector to target cells, where the number of target cells remains at 50,000 cells per well. Typically, ratios of 1:1-1:8 effector to target cells were used. Where multiple CAR constructs were included for comparison, the percentage of transduced cells was normalised to the lowest transduction efficiency through the addition of non-transduced cells prior to plating. 50,000 target cells were first plated into 96-well tissue culture treated flat bottom plates in 100 µL complete RPMI 10%. Effector dilutions were prepared in complete RPMI 10%, in the absence of cytokines, and 100 µL cell suspension was added on top of the targets. A single 96-well V-bottom plate was prepared for each flow-cytometry time point, and cultures were returned to the 37°C incubator at 5% CO₂ until analysis.

2.2.5.2 T-cell proliferation

In small-scale experiments, the relative T-cell proliferation was assessed by counting the number of live T-cells (gated on CD3+) in a set uptake volume (80% of the sample volume) by flow-cytometry using the MACSQuant® instrument. In large-scale experiments, the cell counts were recorded using the NucleoCounter® NC-250™ and the fold-expansion was normalised to the cell count on the day of nucleofection.

2.2.5.3 Cytokine secretion

Cytokine secretion was assessed by removing 150 µL supernatants from co-cultures on the day of harvest and storing at -20°C. Enzyme-linked immunosorbent assays (ELISAs) were performed to measure the concentration of secreted cytokines, including IL-15, IL-2 and interferon-γ (IFN-γ). The human ELISA MAX Deluxe Kits were ordered from BioLegend and were performed according to the manufacturer’s instructions. Briefly, Nunc MaxiSorp™ flat-bottom plates were coated with 100 µL diluted capture antibody and were coated overnight at 4°C. The plate was washed and blocked, and diluted samples were added and incubated for 2h at RT on a plate shaker. Samples were typically diluted
1:5-1:100 to ensure concentrations fell within range of the standard curve. The plate was washed, and the detection antibody was added for a further 1h. Excess detection antibody was removed by washing and the Avidin-HRP solution was added. After a 30-minute incubation and a further wash, the detection substrate was added. 15 minutes later, sulfuric acid stop solution was added and the colorimetric changes were detected using a plate reader at 450 nm absorbance. A standard curve was prepared by assaying serial dilutions of the standard cytokine (supplied with the kit) at a known concentration and was used to determine the concentration of unknown samples.

2.2.6 Statistical tests

GraphPad Prism software (version 9.3.0) was used to calculate statistical analyses, using a p value of less than 0.05 to indicate statistical significance. Un-paired two-tailed Student’s t-tests were used to compare two datasets or two-way analysis of variance (ANOVA) was used for the comparison of multiple datasets. Paired t-tests were performed on circRNA-encoding transcription factor datasets to account for donor variability in RNA expression.
Chapter 3. Novel gene delivery methods for the genetic manipulation of T-cells

3.1 Overview

Proprietary delivery methods can be employed to deliver RNA/DNA and RNPs into donor T-cells to facilitate CAR integration, or alternatively to deliver genes that can reprogram T-cells and improve their function. This chapter compares the delivery of RNA and dsDNA substrates and presents the advantages of using circRNA for the genetic manipulation of primary cells. Two non-viral delivery technologies are also evaluated, nucleofection and soluporation, with a particular focus on the efficiency of transfection, as well as cellular viability and recovery. The final section of this chapter explores alternative viral methods including the soluporation of lentivirus and the use of non-integrating lentiviral vectors as a transient approach.

3.2 Introduction

3.2.1 Summary of viral and non-viral methods for CAR integration

Most CAR T-cell manufacturing processes involve the permanent modification of primary human T-cells ex vivo, as transient approaches have proven to result in poor efficacy. T-cells are engineered to express a CAR to redirect specificity to tumour associated antigens, for the purpose of adoptive cell therapy. The genetic engineering technologies used to deliver CARs can be divided into two classes: viral and non-viral. Retroviral and lentiviral vectors remain the most widely used system in the clinic, owing to their high efficiency. Genetic modifications are permanent, and the transduction process is simple and relatively non-toxic to the cell. The main limitation with viral vectors is their packaging limit, with lentiviral vectors for instance this is approximately 10 kilobases (kB). Although these vectors are replication incompetent, safety concerns have been highlighted, albeit primarily in the stem cell field (Wang and Rivière, 2016). A detailed summary of viral vectors is provided in section 1.2.1.
Non-viral approaches have also been developed, including CRISPR/Cas9-mediated HDR (described in section 1.2.2.1) (Roth et al., 2018), and transposition using the Sleeping Beauty (SB) and piggyBac (PB) systems (described in section 1.2.2.2) (Nakazawa et al., 2011; Deniger et al., 2015). These approaches often rely on the electroporation of dsDNA encoding the transfer cassette, as well as the transient expression of an endonuclease or transposase. Despite efforts to optimise the electroporation conditions, the transfection of dsDNA remains suboptimal and can cause cellular toxicity (Zhang et al., 2018). Other considerations include the risk of off-target gene modification and chromosomal rearrangements (occurring during the repair of DNA DSBs). On the other hand, non-viral approaches offer the advantages of copy number control and increased payload size, and do not require the costly generation of viral vector.

### 3.2.2 Transient gene delivery methods for T-cell reprogramming

The function of CAR T-cells can be reprogrammed through transient gene expression. The encoded protein can include transcription factors to promote an early effector T-cell phenotype, pro-survival factors (X. L. Li et al., 2018), dominant negative proteins to block exhaustion, or genome editing components to facilitate gene disruption and/or integration (Komor et al., 2016; Eyquem et al., 2017). This study uses the developed non-viral protocols to transiently express the desired components, to ensure the encoded proteins are not carried over to the patient. There are several transient delivery methods that have been previously reported and are discussed below.

#### 3.2.2.1 Viral methods

##### 3.2.2.1.1 Adeno-associated virus

The viral method that has attracted most significant attention for transient delivery is the Adeno-Associated Virus (AAV). AAV has a small single-stranded DNA (ssDNA) genome of approximately 4.8 kB. The recombinant version of the virus (rAAV) lacks all viral genes (Rep, Cap and Aap) and is used as a protein nanoparticle engineered to traverse the cell membrane, where it can deliver its DNA cargo into the nucleus. This method is particularly useful for the delivery of HDR templates for CRISPR/Cas9-mediated insertion to alleviate the toxicity associated with dsDNA transfection (Eyquem
et al., 2017; MacLeod et al., 2017). The AAV genomic DNA exists in episomes in the nucleus which are diluted through cell division. AAV vectors are generally considered safer than adenovirus, however, they have been shown to integrate into the genome. This occurs specifically at the q arm of chromosome 19 where they usually remain latent. rAAV vectors are an attractive tool for transient DNA delivery due to their low immunogenicity and their ability to transfect both dividing and non-dividing cells across a broad host and of cell type tropism range. The main disadvantages are the limited packaging size and the slow onset of transgene expression, which is approximately 7 days at peak (Reimsnider et al., 2007; Lee et al., 2017).

3.2.2.1.2 Non-integrating lentivirus

Lentiviral vectors such as those derived from the single-stranded RNA (ssRNA) retrovirus human immunodeficiency virus-1 (HIV-1) have been extensively used for stable gene transfer. HIV-1 integrates its proviral DNA into the host cell genome in a non-specific manner. Lentiviral vectors have the advantage of being able to infect dividing and non-dividing cells with high efficiency. This feature can be harnessed by non-integrating lentivirus (NILV) for highly efficient and transient gene transfer by preventing integration activity (Figure 3.1).
Figure 3.1 Illustration of the lifecycle of integrating versus non-integrating lentiviral vectors.

Integrating and non-integrating lentiviral vectors share the initial stages of the lentiviral life cycle (blue arrows) which involve virion binding to the host and entry through direct fusion with the membrane. The viral core is then released to reveal the ssRNA genome which is reverse transcribed to viral DNA (pre-integration complex) and translocated to the nucleus. With integrating lentivirus (red arrows) the pre-integration complex is non-specifically inserted into the genome by the integrase protein. The integrated proviral DNA will be transcribed and translated to produce the desired protein. In non-integrating vectors (green arrows), the proviral DNA can undergo recombination to form viral episomes. Episomes containing 1 LTR are generated through homologous recombination (HR) whilst those with two LTRs are formed by non-homologous end joining (NHEJ). Episomes are then transcribed and translated to express the transgene of interest (Rittiner et al., 2020).
3.2.2.1 The lifecycle of integrating and non-integrating vectors

The viral lifecycle, as depicted in Figure 3.1, involves virion fusion with the cell membrane to deliver the viral core and the RNA genome. The RNA genome is reverse transcribed to dsDNA and forms the pre-integration complex (PIC), which is shuttled to the nucleus for integration. Integration is catalysed by the viral enzyme, integrase, which is a 32 kDa protein encoded by the Pol gene. Integrase binds to the viral attachment (att) sites at the U3 and U5 regions within the PIC’s long terminal repeats (LTRs) and nicks the linear viral DNA to recess the 3’ ends, producing overhangs that are 2 bases long. Integrase then mediates transfer of the viral DNA to the host’s genome by facilitating attack of the phosphodiester backbone by the exposed hydroxyl groups of the recessed 3’ ends. Ligation of the viral DNA, including removal of the 5’ ends, is carried out by host proteins.

Non-integrating vectors are integration deficient and rely on the generation of viral episomes that reside in the nucleus. Episomes were discovered as by-products of integration and are transcribed to allow transgene expression. They are generated either by homologous recombination of the LTRs, leading to the formation of 1 LTR circles, or by NHEJ to generate 2 LTR circles (Farnet and Haseltine, 1991; Li, 2001). Protein expression is dependent of the rate of turnover of the episomes and their dilution during cell division. Depending on the rate of cell division the transgene may be expressed for shorter or prolonged periods.

3.2.2.1.2 Impairing integration

Cis and trans acting regions of the HIV-1 genome are important for mediating integration. These regions include the viral DNA att sites (Brown, Chen and Engelman, 1999) and the conserved catalytic domain of the integrase protein (Leavitt et al., 1996). Key nucleotides have been pin-pointed by mutational studies and highly efficient mutants have been characterised to render integration incompetence (Masuda, Kuroda and Harada, 1998; Nightingale et al., 2006). A 12-base pair U3 attachment site deletion (ACTGGAAAGGGCT) was identified by Shaw et al., and decreased integration by 2-fold, whilst providing higher transgene expression compared to a previously reported D116N
integrase catalytic core mutant (Shaw et al., 2017). Apolonia et al., showed that
dinucleotide mutation of the attachment sites (from CA to TG in U3 and U5) reduced the
level of integration approximately 200-fold compared to wild-type HIV lentiviral genome
vector. This vector can be combined with integration deficient integrase (D64V) to reduce
the level of integration even further so that it is approximately 1000-fold lower than that
of wild-type (Apolonia et al., 2007). An additional benefit of combining mutations is the
improved safety profile by preventing reversion to the integrating phenotype.

3.2.2.1.2.3 Applications of NILV

Non-integrating vectors are suitable for transient gene expression or for the transduction
of non-proliferating cells. Studies have demonstrated the application of NILV to
effectively deliver a Cas9 endonuclease to a murine renal cell carcinoma cell line (J. Hu
et al., 2018), and to improve efficiencies of gene integration in human embryonic stem
cells, through efficient delivery of the HDR donor template (Y. Wang et al., 2017). Most
applications have been shown for post-mitotic cells, and further evaluation is required to
assess applications in proliferating human T-cells.

3.2.2.2 Non-viral systems

3.2.2.2.1 Transfection and electroporation

Non-viral methods for the transient introduction of nucleic acid and proteins to eukaryotic
cells include transfection or electroporation. Transfection involves the encapsulation of
molecules in charged lipid polymers which are taken up by the cell through endocytosis.
Popular lipid-based transfection reagents include Lipofectamine™ and PEIpro®,
although, non-lipid chemical transfection reagents are also available, for example, the
GeneJuice® Transfection Reagent. Transfection is a simple and cost-effective technique
that works well for many cell lines, such as HEK293T packaging cells, but does not work
well for the modification of primary cells. On the other hand, electroporation is a physical
transfection process which involves suspending host cells with DNA, RNA or protein in
a conductive solution and applying an optimised electrical pulse. This generates
temporary pores in the plasma membrane and moves charged molecules into the
cytoplasm via an electrical field.
Electroporation has been used to deliver various types of effector molecules, including SB and PB DNA transposons, for the generation of CAR T-cells (Nakazawa et al., 2009; Manuri et al., 2010). The transfection of mRNA encoding TALENs has mediated disruption of the endogenous TCR and CD52, for the production of allogeneic CAR T-cells that can evade lymphodepleting regimens (Poiriot et al., 2015; Qasim et al., 2017). Electroporation works well for the delivery of large nucleic acid fragments, however, has limited application in primary cells due to high toxicity.

Proprietary technologies have been developed to improve the survival of pulsed cells and have demonstrated success with human T-cells. For example, the Neon™ transfection system enables electroporation using a pipette tip as the electroporation chamber which generates a more uniform electric field (Kim et al., 2008). Similar technologies include MaxCyte’s electroporation platform which provides a scalable solution for the transfection of $7.5 \times 10^4$ to $2 \times 10^{10}$ cells. Beatty et al., use the MaxCyte STX system for the delivery of mesothelin-specific CAR mRNA and achieve TEs of >99% whilst maintaining 91% cell viability (Beatty et al., 2014). The MaxCyte system was also used by Stadtmauer et al., to deliver Cas9 RNPs targeting the TRAC, TRBC and PDCD1 loci in the first-in-human clinical trial of CRISPR-edited CAR T-cells. Editing efficiencies of 45%, 15% and 20% were achieved, respectively, with a cell viability of >70%. A popular transfection technique for high efficiency editing is the Nucleofector™ technology developed by Lonza which is described in the following section.

3.2.2.2 Nucleofection

Nucleofection is an alternative method that combines both approaches of transfection and electroporation. Lonza’s Nucleofector™ technology uses combinations of cell type-specific buffers and optimised electrical parameters for the introduction of molecules directly into the cells’ nucleus. Cells are suspended in a transfection buffer designed to provide a protective environment for electroporation whilst maintaining physiologically relevant cellular conditions.

Nucleofection has been used to introduce plasmids encoding CAR transposons in quiescent, unstimulated primary human T-cells, achieving 43% CAR integration after
enrichment on CD19+ artificial antigen presenting cells (Singh et al., 2008). Following further process optimisation, Singh et al., led a first-in-human SB clinical trial of CD19-targeted CAR T-cells with 84% CAR expression after 28 days of expansion (Kebriaei et al., 2016; Srour et al., 2020). Nucleofection is a popular technique for the delivery of genome editing endonucleases, such as Cas9 RNPs, to disrupt the expression of inhibitory receptors, such as PD-1 (Hendel et al., 2015; Rupp et al., 2017). Studies have combined RNP delivery with the transduction of AAV6 vectors encoding donor DNA templates to simultaneously disrupt the endogenous TCR and knock-in an anti-CD19 CAR (Eyquem et al., 2017). Additional studies have developed a completely non-viral approach by electroporation of Cas9 RNPs and double-stranded or single-stranded DNA donor templates (Roth et al., 2018; Shy et al., 2022).

Electroporation and nucleofection are the most common non-viral techniques for the transfection of primary cells. Nucleofection is highly efficient for the delivery of both nucleic acid and protein; however, electroporation conditions must first be optimised, otherwise the technique will cause significant cell death (Seki and Rutz, 2018; Zhang et al., 2018). Seki and Rutz have investigated the impact of different combinations of nucleofection buffers and pulse codes on the TE, median fluorescence intensity (MedFI) and cellular viability of murine and human T-cells, transfected with labelled RNP complexes, and identified the optimal nucleofection conditions for both species.

While nucleofection/electroporation provides high transfection efficiencies, there is a cost. The high voltage pulses and serum-free electroporation buffers used during the process can cause cellular deterioration. Zhang et al., investigate the impact of nucleofection on CD4+ T-cells and observed morphological changes (such as wrinkled plasma membranes) and increased intracellular calcium levels, leading to an altered activation state within 24 hours of receiving the electrical pulse (Zhang et al., 2014). The limitations must be carefully considered before selecting a technology for the manufacture of cellular therapies and recent efforts have led to the exploration of novel transfection technologies that do not rely on electrical pulses.
3.2.2.2.3 Soluporation

SOLUPORE® is a non-viral, cell engineering platform that has been developed by Avectas, to enable the *ex vivo* production of T-cell and natural killer (NK) cellular therapies. The technology is a reversible permeabilization method to allow efficient entry of DNA, RNA or protein without compromising cell viability (O’Dea *et al.*, 2017; Kavanagh *et al.*, 2020). The soluporation method is of particular interest because it supports genome editing applications and allows multiple transfections to be performed without perturbing product fitness and functionality (Kavanagh *et al.*, 2021). An image of the SOLUPORE® research tool and schematic of the transfection process is shown in Figure 3.2.

![Image of SOLUPORE® research tool and schematic of the transfection process](image)

**Figure 3.2 SOLUPORE® technology for T-cell transfection.**

Reversible permeabilization by soluporation. (a) Photo of the SOLUPORE® research tool. The research tool is connected to an air compressor (not shown) to provide a pressurised air flow to
the atomiser and to deliver the hypotonic permeabilization buffer containing cargo in the form of droplets. (b) Schematic representation of the reversible permeabilization procedure. Cells are added to the transfection pod in cell culture medium (light purple), medium is removed by blotting underneath the pod membrane with sterile tissue. The spray is actuated on the screen of the research tool to deliver the hypotonic permeabilization buffer containing a low concentration of ethanol (red) and cargo (dark purple). The membrane is temporarily permeabilised, causing osmotic swelling and intracellular delivery of the cargo. After a short incubation, cell culture medium is added to stop the reaction and osmotic gradients are restored. Adapted from (O’Dea et al., 2017).

Soluporation is an electroporation-independent method that uses a low concentration of ethanol to generate temporary membrane pores. The cargo of interest is suspended in 10-12% ethanol and is placed in a microcentrifuge tube at the top of the SOLUPORE® research tool, which delivers a high-pressure pulse of the transfection mixture to the cells. In preparation for transfection, stimulated T-cells ($6 \times 10^6$) are pipetted on to the membrane of a proprietary transfection pod. The pod consists of polyester track etched membrane that is permeable to culture medium but is impermeable to cells. After removal of the medium, the cargo is then delivered to the exposed cell monolayer through atomisation into droplets. Small cargos will enter by diffusion through membrane pores, whilst larger molecules will enter by osmotically driven water influx. Cell culture medium is then added to the transfection pod to stop the process and to reseal the cell membrane. Transfected cells are then manually collected from the transfection pod. Excess water will be removed from the cells using host regulatory processes and electrolyte osmotic gradients will be restored.

3.2.2.2.4 Selection of transfection technologies for evaluation

Electroporation is generally considered the “gold-standard” process for the genetic manipulation of primary T-cells, due to its high efficiency of transfection. Several commercial technologies have become available for research purposes and cell manufacturing, such as Lonza’s Nucleofector™ (Nakazawa et al., 2011; Chicaybam et al., 2013), MaxCyte’s electroporator (Stadtmauer et al., 2020), BioRad’s Gene Pulser (Krug et al., 2014) and Thermo-Scientific’s Neon™ pipette electroporation system.
Lonza’s Nucleofector™ remains the most popular technique for T-cell reprogramming and has been applied for the targeted integration of large DNA sequences (Roth et al., 2018), to deliver transposon/transposase components (Singh et al., 2008; Manuri et al., 2010; Madison et al., 2022), and to engineer base-edited CAR T-cells (Georgiadis et al., 2020; Diorio et al., 2022). As nucleofection has wide applications in T-cells, we selected the technology for evaluation. In comparison to nucleofection, we investigate soluporation, a novel method that does not rely on an electrical current, to improve the viability and recovery of transfected cells. Soluporation uses a chemical permeabilization method for the intercellular delivery of RNA/RNPs and has been reported to maintain the T-cell phenotype (Kavanagh et al., 2021).

The first section of this thesis will investigate gene delivery methods and their application for the manufacturing of CAR T-cells. This chapter will compare the transfection of RNA (linear or circular) and DNA substrates (section 3.6) and will evaluate the Nucleofector™ and SOLUPORE® technologies (section 3.7). Nucleofection was chosen as the state-of-the-art transfection technology for primary T-cells, for comparison to the novel soluporation platform. Section 3.8 will summarise the chapter by investigating alternative viral approaches for comparison to the explored non-viral methods.
3.3 Chapter Aims

This study aims to compare non-viral gene delivery approaches and explores their potential applications to enhance the function of CAR T-cells. The first results chapter will evaluate the transfection of RNA/DNA and RNPs to primary T-cells using two proprietary delivery systems, nucleofection and soluporation. The final section of this chapter investigates alternative viral approaches, such as the soluporation of lentiviral vectors and the use of non-integrating lentivirus for transient gene expression. In summary, this chapter aims to:

3.3.1 Optimise the electroporation conditions for primary T-cells.

3.3.2 Produce and evaluate the use of circRNA.

3.3.3 Compare the delivery of RNA (linear and circular) and DNA substrates.

3.3.4 Assess soluporation as a method to introduce RNA/DNA and RNP complexes.

3.3.5 Investigate alternative viral methods for gene delivery.
3.4 Nucleofection using Lonza’s 4D-Nucleofector™ Device

3.4.1 Pulse code optimisation using the P3 Primary Cell Nucleofector™ Kit

Work was carried out to optimize the delivery of nucleic acid to stimulated T-cells. Here, the focus was to identify suitable pulse codes for use with Lonza’s P3 Primary Cell Nucleofector™ Kit, to achieve high TEs without compromising cell viability. Lonza recommends pulse code optimisation using the supplied enhanced green fluorescent protein (eGFP) plasmid. Here, pulse codes were first tested with the eGFP plasmid and then an mClover3-encoding mRNA that was in vitro transcribed.

PBMCs were activated for 48h with TransAct™ (1:100, research grade) in complete TexMACSTM supplemented with IL-7/15. On the day of nucleofection, cells were harvested and washed twice in phosphate buffered saline (PBS) to remove traces of nucleases in serum-containing medium, prior to resuspension in the P3 nucleofection buffer. PBMCs were nucleofected with eGFP plasmid (0.4 µg) or mClover3 mRNA (2 µg), according to the method outlined in section 2.2.2.4.1.

At 24h post-transfection, flow-cytometry was performed to identify the TE and cellular viability. The pulse codes EH-100, EH-115 and EO-115 facilitated the delivery of eGFP plasmid with efficiencies of up to 75% (Figure 3.3). The viability was inversely correlated with the TE and was variable amongst donors. The pulse codes, CM-150 and FI-115, did not result in a high efficiency of transfection and were deemed unsuitable for the nucleofection of PBMCs. Hence, two additional pulse codes, DN-115 and EO-100, were investigated for RNA delivery. All pulse codes facilitated the delivery of mClover3 mRNA with up to 85.5% TE and minimal differences were observed in the cellular viability. The expression of mRNA varied between the two donors; one donor maintained mClover3 fluorescence at 96h post-nucleofection (Figure 3.4). In agreement with published data (Roth et al., 2018), we found that the pulse code EH-115 provided a high transfection efficiency (~75%), with reasonable viability (~50%). Hence, we concluded that the pulse code EH-115 was suitable for the delivery of nucleic acids to stimulated T-cells.
Figure 3.3 Pulse code optimisation using DNA substrates.

Assessment of pulse codes for the delivery of 0.4 µg pmaxGFP plasmid to 1x10^6 PBMCs. GFP fluorescence (left) and cellular viability (7AAD-) (right) were measured by flow cytometry at 24h post-nucleofection, represented as mean (n=3 independent biological donors, represented as different symbols).
Figure 3.4 Pulse code optimisation using RNA substrates.

Assessment of pulse codes for the delivery of 2 µg mClover3 mRNA to 1x10⁶ PBMCs. (a) Structure of the mRNA template encoding the mClover3 sequence flanked by a 5’ simian virus 40 nuclear localisation sequence (NLS) and a 3’ NLS derived from c-Myc. mClover3 fluorescence (left) and cellular viability (Live, 7AAD-) (right) were measured by flow cytometry at (b) 24h and (c) 96h post-nucleofection, represented as mean (n=2 independent biological donors, represented as different symbols).
3.5 CircRNA for the prolonged duration of transgene expression

After ascertaining the optimal PBMC nucleofection conditions for plasmid DNA and RNA, we began investigating approaches to increase the stability of mRNA and prolong expression in PBMCs. To achieve this, we produced linear and circular RNA encoding two different cargos, either the green fluorescent protein mClover3 or an anti-CD19 CAR and compared their expression kinetics in nucleofected SUP-T1 cells and PBMCs. An introduction to circular RNA is detailed in section 1.4.2.

3.5.1 Circularisation of RNA increases stability in SUP-T1 cells

3.5.1.1 Structure of RNA templates

The circRNA template was generated by inserting the coding sequence of SV40 NLS-mClover3-c-Myc NLS (mClover3) into the Anabaena PIE sequence, downstream of the T7 transcription start site and Kozak consensus sequence (a background to self-splicing introns is provided in section 1.4.2.3.2) (Wesselhoeft, Kowalski and Anderson, 2018). The split intron-exon sequences flank the coxsackievirus B IRES and mClover3 sequence. The presence of internal homology sequences and homology arms aid RNA folding and the formation of a splicing bubble. The back-splicing reaction ligates the 5’ end of the IRES to the 3’ end of the mClover3 sequence through two transesterification reactions, and the intronic sequences are released. The addition of spacer regions reduces the interference of the IRES-mClover3 sequence with the PIE sequences. For comparison, linear mClover3 mRNAs were generated, and these did not contain the PIE or IRES sequences. Linear RNAs contained a 10 bp 5’UTR followed by the mClover3 sequence which leads into a 3’UTR and poly(A) tail. Two different 3’UTRs, derived from globin genes, were tested to promote linear RNA stability (Mayr, 2016, 2019). The structure of RNA templates is exemplified in Figure 3.5.
The circRNA template was designed based on the *Anabaena* PIE sequence and was transcribed from a T7 transcription start site adjacent to the Kozak consensus sequence (Wesselhoeft, Kowalski and Anderson, 2018). The construct contains the split intron and exon (E2/E1) sequences flanking a coxsackie virus B3 (CVB3) IRES and the mClover3 sequence. A polyAC spacer (S) separates the *Anabaena*-derived sequences from the open reading frame. Internal homology (IH) sequences and homology arms (HA) promote the formation of a splicing bubble and circularisation of the RNA. The linear constructs contain a 5’ Cap 0 structure and a poly(A) tail consisting of 150 adenine bases. Constructs either lack the 3’ UTR (No 3’ UTR) or contain a human α-1 or β globin 3’ UTR.

### 3.5.1.2 Production of RNA

The commercially available mMESSAGE mMACHINE™ T7 Transcription Kit was used to generate 5’ 7-methyl guanosine capped mRNA from linearised plasmid (method 2.2.1.3). Templates for the synthesis of linear RNA were produced by PCR using a forward primer to introduce the T7 and Kozak sequences and a reverse primer to incorporate a 3’ poly(A) tail. To facilitate circularisation, RNA was resuspended in a buffer containing magnesium ions and GTP, prior to heating the RNA to 55°C for 15
minutes (Wesselhoeft, Kowalski and Anderson, 2018). The linear controls (pre-circular and linear RNA) were resuspended in the circularisation buffer without the GTP and were kept on ice. To confirm circularisation, RNA was analysed by gel electrophoresis using Agilent’s TapeStation (Figure 3.6). Although in the absence of GTP, spontaneous splicing was observed in the pre-circular control, as shown by the release of the intronic sequences. When preparing the samples for gel electrophoresis, the RNA is heated to 72°C to relax the secondary structures which may catalyse the back-splicing reaction. In addition, contaminants were observed at approximately 3.5 kB and may occur due to incomplete plasmid linearisation. Although, these fragments are observed at a low abundance compared to the full-length RNA. Prior to nucleofection, the linear and circular RNA templates were purified using the MEGAclear™ Transcription Clean-Up Kit.

Figure 3.6 Production of mClover3 RNA.

Linear (left) and circular (right) RNA templates were analysed by gel electrophoresis using the Agilent TapeStation. The circRNA template was analysed pre- and post-circularisation to
confirm circularity by the release of the introns. Linear No 3’ UTR (None), human α-1 (Hu α-1) or human β globin (Hu β) 3’UTR RNA template.

3.5.1.3 Nucleofection of mClover3-encoding linear and circular RNA

To investigate the stability of linear and circular templates, SUP-T1 cells were nucleofected with 0.25 µg-4.00 µg of RNA (method 2.2.2.4.1). In brief, cells were resuspended in the SF Cell Line Nucleofector™ Kit and mixed with the RNA prior to electroporation using Lonza’s recommended pulse code, CM-150. The expression of the fluorescent protein, mClover3, was then monitored by flow-cytometry over the course of 7 days. As shown in Figure 3.7, SUP-T1 cells were highly permissible to RNA electroporation and TEs of >75% were achieved with 0.25 µg RNA. The MedFI of the protein indicated greater expression from circRNA compared to the pre-circular control. Protein expression is positively correlated with the amount of RNA and reaches a peak at 2 days from transfection. A greater RNA input also results in the increased duration of expression. CircRNA was more stable than the pre-circular RNA control, at the experiment endpoint 90% of SUP-T1 cells transfected with 4.00 µg circRNA were mClover3+, compared to 60% mClover3+ using pre-circular RNA.

Previous efforts to improve RNA stability include nucleoside modification, codon optimisation and the addition of stabilising 3’ UTRs (see section 1.4.1). As a further comparison, linear mRNA templates were engineered to contain different 3’ UTRs taken from human α or β globin sequences (Mayr, 2016, 2019). SUP-T1 cells were nucleofected with 4 µg RNA, and circRNA outperformed all linear controls by at least 10-fold (One-Way ANOVA; versus pre-circular, p = 0.0220, versus linear No 3’UTR, p = 0.0218, versus linear human α-1 globin 3’UTR, p = 0.0202, and versus linear human β globin 3’UTR, p = 0.0204) (Figure 3.7).
Figure 3.7 Comparison of linear and circular mClover3 RNA in SUP-T1 cells.

Comparison of mClover3 expression from linear and circular RNA. (a) Transfection efficiency (left) and MedFI of mClover3 expression (normalised to CountBright beads) (right) following nucleofection with 0.25 µg-4.00 µg of RNA, represented as mean ± SD, n=3 technical replicates. (b) Comparison of mClover3 expression (normalised MedFI) using circular or linear RNAs containing different 3’ UTR sequences to enhance the stability, represented as mean ± SD (n=2 technical replicates).
3.5.2 Prolonged translation from RNA circles in stimulated T-cells

To compare the stability in stimulated T-cells, a comparison was performed to determine the duration of expression from circular and linear RNAs encoding a second-generation anti-CD19 CAR (FMC63 binder). CAR-encoding templates were chosen for the proof-of-concept study as their expression can be easily detected on the cell surface by flow-cytometry staining using an anti-idiotype antibody.

3.5.2.1 Structure and production of RNA templates

The CAR sequence consisted of the anti-CD19 FMC63 scFv, a human CD8 stalk and transmembrane domain, and 4-1BB and CD3ζ costimulatory and activation endodomains. In circular templates, this sequence was cloned into the *Anabaena* PIE sequence, as previously described. A linear RNA control contained the human β globin 3’ UTR for increased stability and a non-circular template contained the PIE sequence at the 5’ end (3’ intron) but lacked the PIE sequence at the 3’ end (5’ intron) (Figure 3.8). RNA was synthesised using the mMESSAGE mMACHINE™ Kit as previously described (method 2.2.1.3). The linear template was resuspended in circularisation buffer minus the GTP and was kept on ice, and the non-circular template was resuspended in complete circularisation buffer and was heated to 55°C.

<table>
<thead>
<tr>
<th>Circular RNA template</th>
<th>Pre-circularisation: 2904 bases, Post-circularisation: 2420 bases</th>
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<tr>
<td>HA 3’intron E2 S CVB3 IRES Anti-CD19 CAR S E1 5’intron HA</td>
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<table>
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<th>Non-Circular RNA template</th>
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</table>

Figure 3.8 The structure and length of RNA templates encoding the FMC63 CAR for nucleofection into primary T-cells.
Chapter 3. Results

The CAR-encoding sequence was cloned into the *Anabaena* PIE sequence and was transcribed from a T7 transcription start site adjacent to the Kozak consensus sequence (Wesselhoeft, Kowalski and Anderson, 2018). Constructs were engineered to lack the 3’ PIE sequence (5’ intron) (non-circular) to prevent circularisation, or to contain a human β globin 3’ UTR (Hu β globin 3’ UTR) to promote the stability (linear). The non-circular and linear RNAs contained a 5’ Cap 0 structure and a poly(A) tail consisting of 150 adenine bases.

3.5.2.2 Nucleofection of CAR-encoding linear and circular RNA

PBMCs were activated with TransAct™ and nucleofected 48 hours later with 4 µg of CAR-encoding mRNA (method 2.2.2.4.1). CAR expression was followed over the course of 7 days by staining with an anti-anti-CD19 FMC63 AF647-conjugated idiotype antibody. At 24h post-nucleofection, a high TE was observed with circRNA, on average 96% of T-cells were CAR+ compared to 53% with non-circular RNA and 25% with linear RNA (Figure 3.9). CAR expression was maintained at 97% for 4 days with circRNA but was undetectable by day 7. In contrast, CAR expression from the non-circular and linear RNA controls declined rapidly after 24h and cell surface expression was undetectable by day 4. At 24h, circRNA provides the highest TE (One-Way ANOVA; versus linear, p = 0.0016, versus non-circular, p = 0.0087) and MedFI of CAR expression (One-Way ANOVA; versus linear, p = 0.0006, versus non-circular, p = 0.0005). Expression of the transgene was approximately 10-fold higher from circRNA compared to linear RNA. The following section (Section 3.6) describes a further comparison using an alternative anti-CD19 binder, CAT-19 (developed in-house), and evaluates the use of linear or circular RNA compared to DNA templates.
Figure 3.9 Dynamics of CAR expression from linear or circular RNA in stimulated T-cells.
Chapter 3. Results

Time course of anti-CD19 CAR (FMC63 binder) expression in T-cells nucleofected with 4 µg of RNA. (a) Flow-cytometry plots (FSC-A versus FMC63-AF647) are shown for the CD3+ compartment at 1 day and 2, 4, and 7 days post-nucleofection. (b) Percentage of CAR+ T-cells (left) and MedFI of CAR expression (normalised to CountBright beads) (right), represented as mean (n=3 independent biological donors, represented as different symbols).

This work demonstrates that circRNA is more stable in primary T-cells compared to linear RNA. Using the Anabaena PIE-based system, expression was detected for a minimum of 4 days. The next section will directly compare the nucleofection of RNA and DNA, with a focus on the TE and cellular viability.
3.6 DNA versus RNA for transient protein expression

A direct comparison was performed to compare the nucleofection of RNA (linear and circular) and DNA substrates in stimulated T-cells. Templates were generated in vitro and encode the second generation anti-CD19 CAR (CAT-19 binder) that is in clinical trial for the treatment of adult acute lymphoblastic leukaemia (ALL) and non-Hodgkin lymphoma (Roddie et al., 2019).

3.6.1 Structure of RNA templates

Expression of the CAR was driven by a phosphoglycerate kinase 1 (PGK) promoter in the DNA template or a T7 transcription start site in RNA templates, which enables in vitro transcription. The CAR sequence consisted of a human T-cell receptor β chain variable region signal peptide, the anti-CD19 CAT-19 variable heavy and variable light sequences separated by a 16 amino acid serine glycine linker, a human CD8 stalk and transmembrane domain, and 4-1BB and CD3ζ endodomains. Two types of RNA were investigated, and they differed in their structure. Linear RNA contained a 5’ 7-methyl guanosine cap and a 3’ human β globin poly(A) tail to increase its stability. An engineered circRNA was designed to enable the formation of closed RNA circles to increase the RNA half-life (Puttaraju and Been, 1992; Wesselhoeft, Kowalski and Anderson, 2018). The structure of templates for nucleofection are exemplified in Figure 3.10.

<table>
<thead>
<tr>
<th>Circular RNA template</th>
<th>Pre-circularisation: 2783 bases, Post-circularisation: 2402 bases</th>
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</tr>
</thead>
<tbody>
<tr>
<td>PGK Anti-CD19 CAR WPRE 3’ LTR SV40 poly(A)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.10 The structure and length of RNA and DNA templates for nucleofection into primary T-cells.

The CAR-encoding sequence (CAT-19 binder) was cloned into the *Anabaena* PIE sequence to facilitate the production of circRNA (Wesselhoeft, Kowalski and Anderson, 2018). The linear RNA contained a 5’ Cap 0 structure, a human β globin 3’UTR (Hu β globin 3’UTR), and a poly(A) tail to promote its stability. Expression of the CAR was driven by a T7 transcription start site and Kozak consensus sequence or a PGK promoter. The DNA template contains the Woodchuck pre-processing element (WPRE) which leads into the self-inactivating 3’ long-terminal repeat (3’LTR) and a SV40 poly(A) signal sequence.

### 3.6.2 Production of DNA and RNA templates

DNA templates were produced through PCR amplification and were purified using 0.5x SPRI paramagnetic beads and an elution volume of 3 µL P3 nucleofection buffer per 100 µL PCR reaction (method 2.2.1.4). 5’ capped mRNA was synthesised using the mMESSAGE mMACHINE™ T7 Transcription Kit and the linear format was polyadenylated using Invitrogen’s Poly(A) Tailing Kit (method 2.2.1.3). RNA was circularised, as previously described (Wesselhoeft, Kowalski and Anderson, 2018). Prior to transfection, RNAs were purified using the MEGAClear™ Transcription Clean-Up Kit and their sizing and integrity was confirmed by gel electrophoresis using Agilent’s TapeStation (Figure 3.11).
Figure 3.11 Production of DNA or RNA templates.

Analysis of DNA and RNA templates prior to nucleofection. The DNA template was analysed on 1% agarose (left). The linear RNA template was analysed pre- and post-polyadenylation (middle), and the circRNA template was analysed pre- and post-circularisation on the Agilent TapeStation (right). The release of intronic sequences (introns) indicates successful RNA circularisation.

3.6.3 Comparison of substrates for transient transfection

In line with Autolus’ manufacturing process, fresh or thawed PBMCs were stimulated with TransAct™ and IL-7/15 for 48h, prior to nucleofection with DNA/RNA (method 2.2.2.4.1). At 24h, flow-cytometry was performed to identify the CAR expression using an anti-idiotypic antibody (targeting CAT-19, developed in-house) and an anti-Rat PE-conjugated secondary antibody. The percentage of CAR+ T-cells was positively correlated with the amount of nucleic acid, and this was also reflected in the MedFI of the protein. A plateau in the expression was reached at 2 µg with circular and linear RNA, whilst DNA expression continued to increase in three donors. Generally, donors that were activated immediately after isolation (fresh) showed higher TEs than those that were previously cryopreserved (thawed).
Some donors were recalcitrant to RNA transfection; however, circRNA provided an improvement to the efficiency of transfection compared to linear RNA (4 µg template; p = 0.0299) (Figure 3.12). The highest TE (80% CAR+ T-cells) was achieved in a fresh donor transfected with 4 µg circRNA. At the maximum amount of nucleic acid, CAR expression from circRNA was comparable to DNA, however, DNA transfection resulted in severe toxicity and the expression from linear RNA was significantly lower than DNA (percentage CAR+ T-cells; p = 0.0131, MedFI of CAR expression; p = 0.0262) (Figure 3.12, Figure 3.13).

Figure 3.12 Comparison of CAR-encoding DNA and RNA substrates at 24h post-nucleofection.
Expression of the anti-CD19 CAR (CAT-19 binder) in T-cells. Flow cytometry plots (FSC-A versus CAT-19-PE) are shown for one fresh donor in the CD3+ compartment at 24h post-nucleofection. No nucleic acid (no cargo) and no pulse samples were included as nucleofection controls.
Figure 3.13 DNA versus RNA: CAR expression at 24h.
Comparison of anti-CD19 CAR (CAT-19 binder) expression from DNA and RNA templates in fresh or thawed donors. (a) Percentage of CAR+ T-cells (left) and MedFI of CAR expression (normalised to CountBright beads) (right) at 24h post-nucleofection, where each connected line represents an individual donor, n=6 independent biological donors (thawed), n=4 (fresh). (b) Comparison of DNA and RNA templates using 4 μg of template, represented as mean (n=10 independent biological donors, represented as different symbols). (One-Way ANOVA; *p ≤ 0.05).

Decay of the CAR expression was investigated at 96h post-nucleofection, and a 5- to 10-fold reduction in the MedFI was observed for all samples. Expression from linear RNA was undetectable and was significantly reduced in the DNA group. Expression from circRNA was significantly higher than linear RNA (percentage CAR+ T-cells; p = 0.0299), demonstrating the advantage of using closed structures to improve RNA stability (Figure 3.14, Figure 3.15).
Figure 3.14 Comparison of CAR-encoding DNA and RNA substrates at 96h post-nucleofection.

Expression of the anti-CD19 CAR (CAT-19 binder) in T-cells. Flow cytometry plots (FSC-A versus CAT-19-PE) are shown for one fresh donor in the CD3+ compartment at 96h post-nucleofection. No nucleic acid (no cargo) and no pulse samples were included as nucleofection controls.
Figure 3.15 DNA versus RNA: CAR expression at 96h.
Comparison of anti-CD19 CAR (CAT-19 binder) expression from DNA and RNA templates in fresh or thawed donors. (a) Percentage of CAR+ T-cells (left) and MedFI of CAR expression (normalised to CountBright beads) (right) at 96h post-nucleofection, where each connected line represents an individual donor, \(n=6\) independent biological donors (thawed), \(n=4\) (fresh). Samples that no longer expressed the template were omitted from the MedFI graphs. (b) Comparison of DNA and RNA templates using 4 \(\mu\)g of template, represented as mean \((n=10\) independent biological donors, represented as different symbols). (One-Way ANOVA; *\(p \leq 0.05\)).

To assess the toxicity of nucleic acid delivery, the percentage of dead cells was determined by flow-cytometry staining using the viability dye, SYTOX™-Blue, which permeates compromised membranes in apoptotic cells. DNA delivery was found to cause substantial cell death compared to linear and circular RNA (24h and 96h post-nucleofection; \(p < 0.0001\)). At 24h, DNA transfection (4 \(\mu\)g) increased the number of dead cells by 2.51-fold compared to circRNA delivery (and 2.86-fold at 96h). Toxicity was positively correlated with the amount of DNA and was accompanied by a drop in the absolute live cell count (adjusted to the final culture volume). Samples transfected with RNA had an improved cellular recovery at 96h (circRNA versus DNA, \(p < 0.0001\), \(p = 0.0016\), circRNA vs DNA and linear RNA vs DNA at 96h, respectively) (Figure 3.16, Figure 3.17). Compared to DNA transfection (4 \(\mu\)g), circRNA delivery facilitated a 9.9-fold increase in the number of live cells at 24h and a 16-fold increase at 96h. Hence, we concluded that RNA delivery can improve the cellular viability and recovery compared to the transfection of DNA.
Figure 3.16 DNA versus RNA: cell viability and recovery at 24h.
Comparison of toxicity from DNA and RNA templates in fresh or thawed donors. (a) Percentage of Dead T-cells (SYTOX™-Blue+) (left) and live cell count (adjusted to the entire culture volume using CountBright beads) (right) at 24h post-nucleofection, where each connected line represents an individual donor, $n=6$ independent biological donors (thawed), $n=4$ (fresh). (b) Comparison of DNA and RNA templates using 4 µg of template, represented as mean ($n=10$ independent biological donors, represented as different symbols). (One-Way ANOVA; ****$p \leq 0.0001$).
Figure 3.17 DNA versus RNA: cell viability and recovery at 96h.
Comparison of toxicity from DNA and RNA templates in fresh or thawed donors. (a) Percentage of Dead T-cells (SYTOX™-Blue+) (left) and live cell count (adjusted to the entire culture volume using CountBright beads) (right) at 96h post-nucleofection, where each connected line represents an individual donor, $n=6$ independent biological donors (thawed), $n=4$ (fresh). (b) Comparison of DNA and RNA templates using 4 $\mu$g of template, represented as mean ($n=10$ independent biological donors, represented as different symbols). (One-Way ANOVA; **$p \leq 0.01$, ****$p \leq 0.0001$).

In summary, nucleofection is a highly efficient method for the transfection of nucleic acid into primary human T-cells. However, the process can be relatively toxic and results in substantial cell loss. This work demonstrates the advantage of using circRNA compared to DNA. CircRNA was probably more stable than linear RNA, as higher transfections efficiencies were observed, and the MedFI of CAR expression was higher. Together, these data suggest that expression from circRNA could improve cellular viability and recovery, compared to DNA. The major drawback of RNA transfection is the variability between donors, however, studies suggest the immunogenicity of circRNA could be reduced by the inclusion of modified nucleosides and by ensuring a high purity of circRNA (Karikó et al., 2008, 2011).

Although a direct comparison was not performed to evaluate the T-cell source, it appeared that fresh PBMC donors were more amenable to nucleic acid delivery than cryopreserved PBMCs. As demonstrated by Panch et al., cryopreservation may directly impact the viability of PBMCs, which may reduce their amenability to RNA expression and delay their recovery post-nucleofection. Upon comparison of fresh and cryopreserved CAR T-cells, the authors found an elevated expression of apoptosis signalling, mitochondrial dysfunction and cell cycle damage pathways in cryopreserved products, despite comparable in vivo persistence (Panch et al., 2019). Likewise, Xu et al., have compared the function of fresh and cryopreserved anti-BCMA CAR T-cells and found those that were used fresh to have superior cytotoxicity in vitro, but these differences were not observed in vivo (Xu et al., 2018). These studies indicate that the thawing process can be detrimental to the cell function.
The following section will investigate an alternative transfection technology termed soluporation. RNA/DNA and RNPs will be delivered in a comparative study to investigate the efficiency of transfection, the cellular viability and recovery, and to evaluate the practical applications of each technology. Considerations include the reliability of each method and the potential for process scale-up.
3.7 Evaluation of SOLUPORE® technology

3.7.1 Technology transfer using linear eGFP mRNA

To validate the SOLUPORE® research tool, we investigated the efficiency of mRNA transfection, firstly in one PBMC donor supplied by Avectas, followed by two Autolus donors. The soluporation protocol was previously optimised and provided by Avectas with modifications, where indicated, to adapt to the Autolus process (see section 2.2.2.4.2 for a detailed protocol). In brief, PBMCs were stimulated for 72h using aCD3/aCD28 soluble antibodies (100 ng/µL) in complete CTS OpTmizer supplemented with 250 iU/mL IL-2. In preparation for transfection, the stimulated T-cells were washed with PBS, prior to loading (6x10^6 cells) onto a proprietary transfection pod. The culture medium was removed and the cargo suspension, containing eGFP mRNA in S buffer and 12% ethanol, was delivered as a high-pressure pulse to the cell monolayer. At 24h post-transfection, the TE was analysed by flow-cytometry to calculate the percentage of eGFP+ cells. The cellular recovery was calculated as a percentage of viable cells using NucleoCounter® NC-250™ cell counts pre- and post-transfection (24h).

Three independent runs were performed using the Avectas donor and 13 µg mRNA. The efficiency of transfection was approximately 65% and aligned with Avectas’ internal results. Cell recovery was also variable between runs and ranged between 30% and 75% of cellular input. To validate the process, two Autolus donors were transfected with 20 µg mRNA. Here, the amount of RNA was increased to match the RNA requirements for the nucleofection protocol, which ranged between 3-4 µg per 1x10^6 cells. At 24h, the achieved TEs were somewhat variable (45%-75%) and the cell recovery ranged between 30% and 75% (Figure 3.18). Despite differences in the amount of transfected RNA, the Avectas and Autolus donors were comparable in terms of TE (un-paired t-test; p = 0.1653) and cell recovery (un-paired t-test; p = 0.6894).
Figure 3.18 Transfection of eGFP RNA using the SOLUPORE® delivery system.

Validation of the SOLUPORE® research tool using (a) Avectas and (b) Autolus donors. Flow-cytometry plots (FSC-A versus GFP) are shown for one representative sample in the CD3+ compartment at 24h post-transfection with eGFP (GFP) mRNA. GFP fluorescence (left) and cellular recovery (percentage viable cells recovered, calculated using NucleoCounter® NC-250™) (right), represented as mean ± SD (Avectas; n=1 biological donor prepared as two or
three technical replicates, Autolus; \( n=2 \) independent donors prepared as three or four technical replicates, replicates are represented as different symbols).

### 3.7.2 Comparison of delivery methods using CAR-encoding templates

Following validation of the SOLUPORE® research tool, a direct comparison was performed to compare soluporation to nucleofection. As described in an earlier section, we investigated the delivery of circRNA or linear dsDNA templates encoding the anti-CD19 CAR (CAT-19 binder) (shown in Figure 3.10). The optimized protocols differed in their stimulation methods and the number of T-cells for transfection (methods 2.2.2.4.1, 2.2.2.4.2). For soluporation, PBMCs were stimulated for 72h with aCD3/aCD28 in complete CTS OpTmizer containing IL-2, whereas those for nucleofection were stimulated for 48h using TransAct™ in complete TexMACSTM containing IL-7/IL-15. The RNA/DNA substrates were prepared using 4 µg nucleic acid per 1x10⁶ cells (nucleofection) and the amounts were scaled accordingly for soluporation (6x10⁶ cells).

To determine the efficiency of each method, flow-cytometry was performed at 24h to detect the CAR+ population. Soluporation facilitated the introduction of circRNA (26%) with comparable efficiency to nucleofection (36%) (\( p = 0.6412 \)), however, did not support the delivery of large dsDNA templates. The transfection of circRNA presented minimal toxicity as shown by the percentage of dead cells (10%), although, soluporation did not further improve the viability compared to nucleofection (\( p = 0.9411 \)) (Figure 3.19).
Figure 3.19 Transfection of CAR-encoding templates using proprietary technologies.

Comparison of CAR-encoding (CAT-19 binder) circRNA and DNA delivery by nucleofection or soluporation. (a) Flow-cytometry plots (FSC-A versus CAT-19-PE) for representative samples in the CD3+ compartment at 24h post-soluporation. (b) Percentage of CAR expression (left) and percentage of cell death (SYTOX™-Blue+) (right), represented as mean ± SD (nucleofection; n=6 independent biological donors from three experiments; soluporation; DNA; n=2, RNA; n=4 independent biological donors from one experiment. Individual donors are represented as different symbols). Un-transfected (UT), no cargo (NC). (Two-Way ANOVA; **p ≤ 0.01).
3.7.3 Improving DNA delivery

This section of the work aimed to assess the SOLUPORE® research tool for various non-viral delivery applications including targeted gene integration. As CRISPR/Cas9-mediated HDR requires the delivery of DNA donor template and Cas9 RNP complexes, we set out to improve the transfection of dsDNA. Solutoporation did not facilitate the delivery of large linear dsDNA templates, hence, we investigated the transfection of a small, supercoiled plasmid to improve the entry through temporary membrane pores. The standard soluporation process requires the resuspension of cargo in S buffer and 12% ethanol (method 2.2.2.4.2). To prevent precipitation of the Cas9 protein, we lowered the ethanol concentration to 10% and investigated the impact on DNA transfection in the absence of RNP complexes. The amount of DNA was reduced from 4 µg DNA to 2-3 µg DNA per 1x10^6 cells, to reduce toxicity and to improve the survival of transfected cells.

Identification of eGFP fluorescence at 24h revealed limited levels of transfection. Although increasing the amount of DNA improved the efficiency of transfection, the percentage of GFP+ T-cells did not surpass 7% and reducing the concentration of ethanol did not appear to affect the cargo delivery (Figure 3.20). We concluded that soluporation requires further development to achieve efficient DNA transfection and suggest the investigation of ssDNA templates to mimic the structure of mRNA. However, the production of ssDNA is expensive, and an alternative and cost-effective gene integration method would be to transfecf Cas9 RNPs, followed by transduction with AAV vectors or non-integrating lentivirus carrying the DNA donor templates. In the next section we evaluate the SOLUPORE® research tool for the delivery of Cas9 RNP complexes, in comparison to our existing method.
Figure 3.20 Transfection of an eGFP plasmid DNA using the SOLUPORE® research tool.

Efficiency of transfection using an eGFP-encoding supercoiled dsDNA plasmid. (a) Flow-cytometry plots (FSC-A versus GFP) for representative samples in the CD3+ compartment at 24h post-soluporation. (b) Percentage of eGFP (GFP) expression (n=1 biological donor). Un-transfected (UT), no cargo (NC).

3.7.4 Genome editing using Cas9 RNPs

We investigated the SOLUPORE® research tool for the delivery of TRAC-targeted Cas9 RNPs, in a side-by-side comparison to nucleofection. The TRAC target sequence (TCTCTCAGCTGGTACACGGC) was designed using Synthego’s CRISPR design tool and the sgRNA contained phosphorothioate modifications to improve its stability (Hendel et al., 2015). Following the previously optimised CRISPR/Cas9 gene knockout protocol (method 2.2.2.5.2), T-cells were stimulated for 48h using TransAct™ in complete
TexMACS™ and IL-7/15. The RNP complexes were formulated by combining non-targeting (NT) or TRAC-targeting sgRNAs with Cas9 endonuclease at a ratio of 2:1 and were resuspended in S buffer containing 10% ethanol to prevent precipitation. Soluporation and nucleofection were performed concurrently (methods 2.2.2.4.1, 2.2.2.4.2), and transfected T-cells were expanded for a period of 4 days, at which point knockout efficiencies were determined by flow-cytometry staining for the CD3 protein. Soluporation of RNPs facilitated disruption of the TCR with 50% efficiency, however, this was significantly less efficient than nucleofection, which resulted in approximately 90% gene knockout (p < 0.0001) (Figure 3.21).

![Figure 3.21 Comparison of proprietary technologies for TRAC-Cas9 RNP delivery.](image)
Disruption of the TCR through delivery of TRAC-targeted RNPs by nucleofection or soluporation. (a) Flow-cytometry plots (FSC-A versus CD3-PE-Cy7) for one representative donor in the CD3+ compartment at 96h post-transfection. (b) Percentage of CD3 expression, represented as mean ± SD (n=3 independent biological donors, represented as different symbols). Un-transfected (UT), no cargo (NC), non-targeting (NT). (Two-Way ANOVA; ****p ≤ 0.0001).

To summarise, soluporation can facilitate the delivery of RNA templates with efficiencies ranging from 45-75% for a small single-stranded eGFP mRNA and 14-36% for a CAR-encoding circRNA. We validated this approach for gene knockout as demonstrated by the delivery of TRAC-targeted RNPs and disruption of the endogenous TCR, however, efficiencies were approximately half of that achieved by nucleofection. Unfortunately, dsDNA could not be introduced by soluporation, therefore, limiting the application for non-viral gene integration. The next section turns towards an alternative application to mediate CAR delivery by enhancing lentiviral gene transfer.
3.8 Alternative viral approaches

3.8.1 SOLUPORE®-mediated delivery of viral vectors

The generation of GMP-grade lentiviral vectors is expensive, time-consuming, and prone to failure resulting in low titres. Techniques to reduce the multiplicity of infection (MOI) required for successful transduction of primary T-cells are therefore desirable. Current processes typically rely on an MOI ranging between 2 and 9. We sought out to investigate whether soluporation could enhance lentiviral transduction, by improving the contact between the virus and cell membrane, to enable a reduction in the MOI. The first experiment tested the requirement of the S buffer and ethanol (soluporation buffer) for the delivery of lentiviral particles by atomisation.

To achieve an MOI of 3 in a volume of 50 µL, the anti-CD19 CAR (CAT-19 binder) lentiviral supernatant was purified by high-speed centrifugation to reduce the volume 50 times (methods 2.2.2.3.1-2.2.2.3.4). The titre improved from $7.18 \times 10^6$ TU/mL to $5.66 \times 10^8$ TU/mL, aided by the removal of transduction inhibitors in the viral supernatant. The vector was prepared in the presence or absence of soluporation buffer and was delivered to T-cells after 24h of stimulation with TransAct™ in complete TexMACS™ containing IL-7/15 (method 2.2.2.4.2). As a control process, $1 \times 10^6$ cells were transduced using an equivalent MOI by spinoculation in RetroNectin-coated 6-well plates (method 2.2.2.3.6).

At 96h post-transduction, the efficiency of gene integration was determined by flow-cytometry staining using the anti-idiotype antibody. Soluporation resulted in 6% and 17% CAR+ T-cells in the presence or absence of soluporation buffer, respectively. However, spinoculation resulted in higher transduction efficiencies compared to soluporation ($p < 0.0001$), averaging at 44% CAR+ T-cells (Figure 3.22). We confirmed that soluporation buffer was not required for SOLUPORE® delivery of lentiviral particles ($p = 0.2247$).
Figure 3.22 Assessing the requirement of soluporation buffer for lentivirus delivery.

Transduction efficiencies achieved by soluporation or spinoculation in the presence or absence of soluporation buffer. (a) Flow-cytometry plots (FSC-A versus CAT19-PE) are shown for one representative sample in the CD3+ compartment at 96h post-transduction. (b) Transduction efficiency, represented as mean ± SD (n=3 independent biological donors prepared as three technical replicates, represented as individual points with donors indicated by different symbols). (Two-Way ANOVA; ****p ≤ 0.0001). With/without soluporation buffer (w/ S Buffer, w/o S Buffer).

Next, we hypothesised that lentiviral transduction could be enhanced by increasing the incubation time between the droplets of viral vector and the exposed cell monolayer. The
standard process requires an incubation of exactly 30 seconds (s) after atomisation of the cargo, to allow the generation of membrane pores and diffusion of the cargo into the cell. For lentiviral delivery, viral particles should not enter the cell by diffusion due to their size, and instead an incubation will allow time for virion fusion and release of the viral core. Here, the incubation time was increased from 30s to 60s and 90s, in aim to improve viral unloading. Following the previous experiment, purified lentiviral supernatant was prepared in plain TexMACSTM in the absence of soluporation buffer.

Soluporation using the standard 30s incubation resulted in an average transduction of 35.5% and increasing the incubation time to 60s and 90s did not significantly improve lentiviral transduction (32.1% for 60s and 46.9% for 90s) (p = 0.9833 30s versus 60s, p = 0.1082 30s versus 90s) and instead had a detrimental effect on cellular viability (Figure 3.23). A 90s incubation resulted in a high percentage of dead cells (74.3% SYTOX™-Blue+), compared to 34.12% using the standard 30s process. A transduction efficiency of 68.6% was achieved by spinoculation, which outperformed soluporation for all incubation conditions (p < 0.0001 for 30s and 60s, p = 0.0013 spinoculation versus 90s).
Figure 3.23 Investigating the incubation time for SOLUPORE® delivery of lentivirus.

Transduction efficiencies achieved by increasing the lentivirus-to-cell incubation time. (a) Flow-cytometry plots (FSC-A versus CAT19-PE) are shown for one representative donor in the CD3+ compartment at 96h post-transduction. (b) Transduction efficiency (left), represented as mean ± SD and percentage of dead cells (SYTOX™-Blue+) (right), represented as mean +SD (n=3 independent biological donors, represented as different symbols). (Two-Way ANOVA; **p ≤ 0.01, ****p ≤ 0.0001). Spinoculation (Spin).

RetroNectin has been previously reported to improve viral transduction efficiencies by improving the localisation of the virion with the cell (Hanenberg et al., 1996). A final attempt to optimise lentiviral delivery by soluporation involved coating the PETE
membrane of the transfection pod with RetroNectin. The surface area of the pod membrane was equivalent to the area of a single well of a 6-well plate, thus 8 µL RetroNectin (1 mg/mL stock, Takara) per 1.5 mL PBS was used for coating. Transfection pods were left to incubate for 2h at room temperature, prior to transduction as described above.

RetroNectin did not improve transduction by soluporation and instead slightly reduced the efficiency (p = 0.0399), 22.9% transduction was observed compared to 32.45% without RetroNectin. As predicted, RetroNectin significantly enhanced viral transduction by spinoculation, which outperformed soluporation by approximately 2-fold (p < 0.0001). However, in the absence of RetroNectin, transduction efficiencies were comparable between the two processes (p = 0.6329) (Figure 3.24).
Figure 3.24 Assessing the efficiency of transduction using RetroNectin.

Transduction efficiencies achieved using RetroNectin-coated membranes. (a) Flow-cytometry plots (FSC-A versus CAT19-PE) are shown for one representative donor in the CD3+ compartment at 96h post-transduction. (b) Transduction efficiency, represented as mean ± SD (n=2 independent biological donors prepared in duplicate for RetroNectin conditions. Replicates are represented as individual points with donors indicated by different symbols). (Two-Way ANOVA; *p ≤ 0.05, ****p ≤ 0.0001). With/without RetroNectin (w/ R, w/o RN).

In summary, soluporation was able to transduce T-cells by atomisation of lentiviral particles encoding CAR genes, however, transduction efficiencies were 2-fold lower than those obtained by using our standard process (spinoculation with RetroNectin). The
following section continues to explore alternative viral delivery approaches and investigates NILV for transient gene expression.

### 3.8.2 Non-integrating lentiviral vectors for transient RNA delivery

#### 3.8.2.1 Duration of expression from NILV

Next, we investigated an alternative method for transient transgene expression using a viral approach. To generate NILV vectors, the *Gag-Pol* (GP) integrase D64V mutant was cloned into the HIV-1 pMDL.GP plasmid. A 3rd generation lentiviral packaging system was used to produce anti-CD19 CAR (CAT-19 binder) supernatants, by substituting the wild-type GP plasmid for one containing the D64V mutation (method 2.2.2.3.1). Upon assessment of the viral titre, a 2-fold reduction was observed for the non-integrating vector (1.23x10^7 TU/mL) compared to the integrating counterpart (2.26x10^7 TU/mL). To investigate transgene expression over time, SUP-T1 cells were transduced by spinoculation with RetroNectin using a range in MOI between 2 to 9 virions per cell (method 2.2.2.3.5). These MOIs reflect the differences in the amount of integrating vector supernatant used in the CAR T-cell manufacturing process.

Flow-cytometry at 24h revealed greater than 90% transduction for all MOIs of 3 or greater. With an MOI of 1, non-integrating vectors already showed a reduction in the transduced population compared to the integrating control, this is also evident to some extent with an MOI of 3. The percentage of transduced cells peaks at 48h and is stably expressed by the integrating vector, whilst the non-integrating CAR expression declines rapidly. At an MOI of 9 this reduces from 97% (day 2) to 45% (day 3) and with an MOI of 3, from 90% (day 2) to 10% (day 3). This demonstrates that expression can be tuned to some extent by optimisation of the MOI. Through normalisation to CountBright beads, the MedFI measurements revealed a lower level of CAR expression from non-integrating vectors compared to the integrating phenotype; this is clearly shown at 24h. The expression from an integration deficient vector is reduced by approximately 10-fold compared to its competent counterpart (Figure 3.25). This work demonstrates that the expression window from non-integrating HIV-1 vectors is approximately 3-4 days in dividing cells. Transduction is a simple and effective method and does not necessarily
require a spinoculation step, hence, repeat transductions could be easily performed to enhance the expression window using an optimised dosing regimen.
Figure 3.25 Expression dynamics from non-integrating lentiviral vectors in SUP-T1 cells.

Transduction efficiencies achieved using integrating and non-integrating vectors. (a) Flow-
cytometry plots (FSC-A versus CAT19-PE) are shown for an MOI of 3, gated on the live cells
(SYTOX™-Blue-). (b) Transduction efficiency (left) and MedFI of CAR expression (normalised
to CountBright beads) (right), where one line represents one indicated MOI (n=1 sample).
Integrating lentivirus (ILV), non-integrating lentivirus (NILV).

3.8.2.2 Feasibility of NILV in stimulated T-cells

Through proof-of-concept work in SUP-T1 cells, we have identified that NILV can
provide expression for a duration of approximately 3 days. This section aims to optimise
the transgene expression by incorporating different promoters, which vary in their
strength, into the genome plasmid. Here we test the human phosphoglycerate kinase 1
(PGK) and elongation factor 1 alpha (EF1α) promoters and the Murine leukaemia virus
derived MND promoter. These sequences were tested in integrating vectors to determine
the strongest promoter for expression in primary T-cells, prior to NILV investigation.

PBMCs were stimulated using soluble aCD3/aCD28 in complete RPMI 10% containing
IL-2 for 24h prior to spinoculation using integrase-proficient lentiviral supernatants
encoding a near infrared fluorescent protein, iRFP670, at an MOI of 3 (method 2.2.2.3.6).
Transduction efficiencies were determined at 72h and an efficiency of 50% was observed
for both integrating and non-integrating vectors. The MND promoter provided the highest
expression, as determined by the MedFI of the iRFP670+ population. Expression from
the MND promoter was approximately 5-fold higher compared to the PGK promoter,
which provided the lowest expression (p = 0.0124). No significant difference was
observed between the EF1α and PGK or MND promoters (Figure 3.26).
Figure 3.26 Optimisation of the iRFP670 construct for optimal transgene expression.

Transduction of T-cells using integrase-proficient vectors containing different promoters. (a) Flow-cytometry plots (FSC-A versus iRFP670) are shown for one representative donor in the CD3+ compartment at 72h post-transduction. (b) Transduction efficiency (left) and MedFI of iRFP670 (RFP) expression (right), represented as mean ± SD (n=3 independent biological donors, represented by different symbols). (Two-Way ANOVA; *p ≤ 0.05).

Next, we generated NILV supernatants as previously described, using the iRFP670 construct containing the MND promoter. Previous studies report the purification of NILV by ultracentrifugation prior to transduction of human cell lines (Vink et al., 2009; Ortinski et al., 2017). Here, we concentrate supernatants by high-speed centrifugation for 20h at 10,000 x g and resuspend supernatants in a 100X volume reduction using plain OptiMEM medium (methods 2.2.2.3.1-2.2.2.3.3).

PBMCs were stimulated for 24h using aCD3/aCD28 antibodies in complete RPMI 10% containing IL-2, and 250 μL of supernatant was used to transduce 0.3x10^6 cells by spinoculation in RetroNectin-coated 24-well plates. The supernatant was split by volume between three PBMC donors and SUP-T1 cells to maximise the level of transduction. As
Chapter 3. Results

A positive control, T-cells were transduced with an integrase-proficient vector at an MOI of 3 (methods 2.2.2.3.5-2.2.2.3.6).

Transduction efficiencies were determined at the peak of transgene expression at 48h post-spinoculation (Figure 3.25). In SUP-T1 cells, the transduction efficiencies of integrating and non-integrating vectors were near 100%, however, a reduction in the MedFI was observed with NILV. In PBMCs, both the transduction efficiency and MedFI were significantly lower from the non-integrating vector compared to the integrase proficient counterpart (transduction efficiency, \( p = 0.0143 \); MedFI, \( p < 0.0001 \)). On average, the NILV resulted in 19% transduction compared to 45% with the integrating vector, and MedFI was reduced by 16-fold (Figure 3.27).
Figure 3.27 Comparison of protein expression from integrating and non-integrating lentiviral vectors.

Comparison of iRFP670 (RFP) expression from non-integrating vectors in SUP-T1 cells and PBMCs. (a) Flow-cytometry plots (FSC-A versus iRFP670) are shown for one representative donor in the live (SYTOX-Blue-, SUP-T1) or the CD3+ compartment (PBMC) at 48h post-transduction. (b) Transduction efficiency (left) and MedFI of iRFP670 (RFP) expression (right), represented as mean ± SD (SUP-T1, n=1 sample; PBMC, n=3 independent biological donors represented by different symbols). (Two-Way ANOVA; *p ≤ 0.05, ****p ≤ 0.0001). Integrating lentivirus (INT LV), non-integrating lentivirus (NINT LV).

This work concluded that NILV is an unsuitable method for transient gene delivery in primary T-cells, due to the challenge of achieving sufficient transduction and expression, combined with the difficulty of producing NILV at GMP-scale.
3.9 Discussion

3.9.1 Optimising the substrate for gene delivery

3.9.1.1 CircRNA enhances transgene expression

The work in this chapter has explored the suitability of different substrates for gene delivery including the transient expression of RNA/DNA and protein. These substrates can be delivered to primary T-cells by non-viral proprietary technologies or by viral methods including non-integrating lentiviral vectors. We investigated the application of a PIE self-splicing RNA derived from the Anabaena pre-tRNA group I intron to deliver circRNA to primary T-cells and enable prolonged transgene expression (Puttaraju and Been, 1992; Wesselhoeft, Kowalski and Anderson, 2018). When compared to 5’ capped and 3’ polyadenylated mRNA, we found that circRNA provided higher TEs and expression (as determined by an increase in MedFI) which increased the duration of protein expression (section 3.5.1). In PBMCs, delivery of the anti-CD19 CAR (FMC63 binder) (approximately 2 kB in size) resulted in expression for a period of 4-7 days, compared to 3-4 days with the linear format (3.5.2).

3.9.1.2 RNA delivery preserves cell viability

To address the hypothesis that RNA is less toxic than DNA, circRNA encoding an anti-CD19 CAR (CAT-19 binder) was compared to linear mRNA and DNA using Lonza’s Nucleofector™, which is a popular technology for the transfection of primary cells. The process of nucleofection can result in at least 50% cell loss, and this is exacerbated by DNA delivery. Using 4 µg template, TEs were comparable between circRNA and DNA, delivery of circRNA significantly reduced cell death and generally improved cell recovery (section 3.6).

3.9.1.3 Minimising donor-to-donor variability of circRNA expression

The amenability to circRNA transfection was somewhat variable between donors. The highest TEs were observed in PBMC donors that were used fresh without prior cryopreservation. Studies have shown that cryopreserved PBMCs have reduced cell viability within two days of thawing compared to those used fresh (Panch et al., 2019).
Fresh donors may be more resilient to the nucleofection process, indicating the importance of the T-cell source and cell fitness. Although studies report conflicting evidence for the immunogenicity of circRNAs (Y. G. Chen et al., 2017; Wesselhoeft et al., 2019), variability could be attributed to differences in innate sensing of the intronic ssRNA sequences, which are derived from the Anabaena cyanobacterium group I intron. The quality of RNA is a critical requirement for clinical applications and requires purification by removing the introns and un-circularised RNA. This can be achieved by RNase R treatment (Wesselhoeft, Kowalski and Anderson, 2018); however, some reports have shown this can degrade circRNA and can result in a reduction in yield (Zhang, Yang and Chen, 2021). Alternatively, introns could be removed by magnetic bead purification using complementary biotinylated oligonucleotides, or high-performance liquid chromatography (HPLC) could separate and purify the RNA circles (Karikó et al., 2011).

Previous studies have improved the expression of linear mRNAs by \textit{in vitro} transcription using modified bases such as pseudouridine and 5-methylcytidine, which prevents innate sensing and enhances RNA stability (Andries et al., 2015; Omer-Javed et al., 2022). We tested the incorporation of modified nucleotides into circRNA, however this prevented circularisation, which was in agreement with published reports (Wesselhoeft et al., 2019). This may be due to alterations in the chemical structure which prevent transesterification and back-splicing. Further work could investigate the use of alternative circularisation methods, such as RNA ligation using single-stranded oligo splints, to generate modified circRNAs (Petkovic and Müller, 2015), however, some studies suggest that this may not be necessary in circRNAs (Wesselhoeft et al., 2019). This work concluded that circRNA is a desirable substrate for delivery to T-cells, for extended protein expression whilst maintaining cell viability.

3.9.2 Selection of a non-viral gene delivery system

Next, we evaluated two proprietary gene delivery systems, nucleofection and soluporation, before exploring the potential applications of circRNAs in T-cells (sections 3.6, 3.7). Important considerations for the assessment of transfection technologies include the therapeutic cell yield (TE and cell viability), cargo flexibility, quality of the
T-cell product (phenotype, proliferation, and cytotoxicity), and scalability (Raes et al., 2021).

3.9.2.1 **TE and cargo flexibility**

A high yield of transfected cells is important due to the limited cell numbers that are collected from lymphogenic patients. Whilst nucleofection is a highly efficient method for RNA (~50%), DNA (~50%) and protein delivery (~90% TCR knockout), soluporation introduced ssRNA (~25%) and RNPs (~50% knockout) with reduced efficiency and dsDNA was extremely inefficient (<10%).

3.9.2.2 **Cell viability and recovery**

To assess the therapeutic yield, cell viability and recovery are key factors, however assays are currently lacking. The NucleoCounter® NC-250™ relies on acridine orange (AO) and 4',6-diamidino-2-phenylindole (DAPI) staining to measure the viability and concentration of nucleated eukaryotic cells. Assessing viability at 24h post-transfection can be problematic, due to the incomplete recovery of the cell membrane and its permeability to DAPI staining, resulting in an overestimation of cell death. This was particularly challenging for the assessment of cells following nucleofection, where the cell and nuclear membranes had been disrupted. Hence, we use flow-cytometry to detect the percentage of dead cells by positive staining for the SYTOX™-Blue live/dead dye. Following the transfection of circular CAR-encoding RNA, our results indicate comparable levels of cell death between technologies at approximately 10-15%, although cell loss is substantial at ~50%. Drawbacks of this method include an overestimation of cell viability, due to the exclusion of lysed and fragmented cells during population gating. This highlights the importance of functional assays such as cytotoxicity and proliferation, to complement estimation of the cell viability.

3.9.2.3 **T-cell phenotype**

T-cell expansion can aid the recovery of cells post-transfection, a 4-day culture period was sufficient to expand the transfected cells for analysis post-nucleofection. Prolonged cell culturing combined with the physical process of electroporating the cells can lead to progressive differentiation, loss of proliferative capacity and exhaustion. Zhang et al.,
show the impact of nucleofection on the cell state and function of un-stimulated CD4+ cells. Observations included wrinkled plasma membranes, an upregulation in the activation markers, CD69 and CD40L (CD154), increased transcriptional activity and intracellular calcium levels (Zhang et al., 2014).

Soluporation was developed as a gentle transfection method to prevent transcriptional alterations and to preserve the T-cell phenotype. Kavanagh et al., compared the gene expression profiles of transfected cells and found mis-regulation of 263 out of 574 immune related genes at 6h post-nucleofection, including those involved in T-cell activation, metabolism, and exhaustion (Kavanagh et al., 2020). Only 8 genes were mis-regulated by soluporation indicating preservation of T-cell function, however, this approach is significantly less efficient than nucleofection and is suited to applications which allow for the subsequent selection of edited cells. On the other hand, the nucleofection of resting T-cells combined with shorter culturing periods may help maintain the product phenotype. Alternatively, we describe a method to reprogram the phenotype of T-cells during nucleofection, which is described in the next chapter (Chapter 4).

Other approaches that do not rely on electrical or chemical membrane disruption, including carrier-based transfection (e.g., lipid nanoparticles (LNPs)) and mechanoporation (e.g., microfluidic squeezing), have been shown to preserve the functionality of T-cells compared to nucleofection. Intellia Therapeutics use LNPs to delivery genome engineering components to T-cells, and report gene knockout and knock-in efficiencies of >90% and >80%, respectively. The efficiencies were comparable to nucleofection, and the engineered T-cells displayed reduced DNA damage, an improved T-cell phenotype (>70% T_{SCM} cells), and increased cell expansion (Schultes, Birgit, 2022). As LNPs can be applied for ex vivo and in vivo gene modification, their application for CAR T-cell manufacturing is of particular interest.

On the other hand, mechanoporation facilitates the delivery of molecules by squeezing the cells through microfluidic devices in a solution containing the cargo of interest (Sharei et al., 2013, 2014). DiTommaso et al., demonstrate comparable editing efficiencies of
cell squeezing and nucleofection (~45% PD-1 knockout). Compared to the non-edited T-cells (PD-1+), the squeeze-edited T-cells (PD-1-) displayed a tumour-killing advantage in vivo, which was not observed with PD-1-edited T-cells generated by nucleofection (DiTommaso et al., 2018). Cell squeezing technologies have now become commercially available; SQZBiotech have developed an automated, fully closed, and integrated GMP system, as well as a point-of-care system that bypasses the requirement for engineering in a clean room (Gilbert et al., 2022). Alternative technologies include Indee Labs’ Hydropore™ system and CellFE’s microfluidic platform. The Hydropore™ system uses microfluidic vortex shedding to promote cargo uptake, including mRNA and Cas9 RNPs, in primary T-cells (Jarrell et al., 2019, 2021). Whilst the CellFE platform uses rapid cell compressions to reduce the duration of membrane permeabilization to less than 1 second, in order to improve the cell viability compared to other devices (Loo et al., 2021).

3.9.2.4 Manufacturing capabilities

To develop a GMP-compatible manufacturing process, throughput and scalability are necessary considerations of a transfection system. Typically, CAR T-cell doses require $1 \times 10^8$-$1 \times 10^9$ cells per patient for autologous therapies, and this is significantly higher for allogenic approaches which requires scale-up for dosing multiple patients. A robust and reliable process is required to manufacture high-quality T-cell products. To minimise variability, closed cell processing and automation are desirable. Lonza’s 4D-Nucleofector™ LV Unit can support the manufacture of up to $1 \times 10^9$ cells using GMP-grade proprietary transfection buffers and Avectas are currently developing a clinical SOLUPORE® system for the engineering of $1 \times 10^8$ cells, both of which support closed cell processing. The main limitation of soluporation is the high cost of reagents to account for dead volumes in the system. The research tool requires an additional three transfection sprays which can significantly increase the cost of manufacturing.

An alternative system is Miltenyi’s electroporation device; the system has been described for the generation of off-the-shelf anti-CD19 CAR T-cells. In the published study, T-cells were transfected with TRAC-targeting TALEN mRNA, which resulted in >60% CD3 disruption and >80% viability, prior to lentiviral transduction to deliver the CAR genes (Alzubi et al., 2021). This system has the advantages of allowing users to modify the
electrical parameters to optimise the protocol, as well as providing the option for integrated manufacturing with the Prodigy system.

The engineered cell product must comply with GMP regulations and pass safety release testing, which comprises bacterial and mycoplasma sterility, and quantitation of residual DNA, proteins, and endotoxins. Combined with greater potential for scale-up, we confirmed that nucleofection was more efficient than soluporation, had greater cargo flexibility, and the impact to cell viability could be minimised by delivery of RNA.

### 3.9.3 Alternative viral approaches

As a comparison to non-viral delivery, we explored two novel viral approaches. Firstly, we investigated the delivery of anti-CD19 CAR (CAT-19 binder) encoding lentivirus by soluporation, in aim to improve the efficiency of transduction (section 3.8.1). Although transduction efficiencies were comparable to those achieved by spinoculation in the absence of RetroNectin, RetroNectin-coating of cell culture plates can enhance transduction efficiencies by up to 2-fold, hence, soluporation was deemed unsuitable.

Secondly, we investigated the application of NILV for transient expression in proliferating T-cells (section 3.8.2). We engineered non-integrating lentiviral vectors encoding fluorescent proteins and monitored the duration of expression in SUP-T1 cells and PBMCs. We hypothesised that NILV could offer the advantages of a simple transduction process and reduced cell loss compared to nucleofection. However, the level of protein expression was significantly impaired in PBMCs compared to integrase-proficient vectors encoding iRFP670 or mClover3 (Appendix, Figure 8.1).

This study aims to investigate gene delivery approaches to improve CAR T-cell manufacturing. One application includes the expression of transcription factors to reprogram T-cells to a less differentiated cell state. Gene delivery by NILV was inefficient and resulted in low expression, hence, this approach was deemed unsuitable for the delivery of transcription factors. Introduction of a scaffold attachment region may improve the expression profile of these vectors by providing the episome with an origin of replication (Jenke et al., 2004; F. Chen et al., 2017).
With further optimisation to improve the efficiency, this approach could be used to transiently deliver Cas9 or base editors, since genome editing does not necessarily require high expression of the editing enzyme. A recent study has demonstrated the application of NILV for delivery of base editors to primary T-cells, however using an alternative LentiFlash® system that uses the MS2 aptamer sequence to package the transgene sequence into viral particles (Prel et al., 2015; Whisenant et al., 2022). With additional optimisation of our system, NILV vectors encoding base editors could be combined with integrating lentiviral transduction for delivery of the CAR. In this case, the non-integrating vector will require additional mutations in the LTR att sites to prevent the reversion of an integrase deficient vector through borrowing of the IN protein from the competent vector. Alternatively, this issue could be resolved by performing double transductions of non-integrating lentiviral vector with a CAR-encoding retrovirus.

The cost of GMP-compliant viral vector manufacture is extremely expensive and the safety profile of NILV requires extensive testing. We conclude that non-viral techniques provide advantages over viral methods, including a high efficiency of transfection, an improved safety profile, and reduced economic costs.

3.9.4 Conclusions

In summary, the work in this chapter has identified circRNA as the most optimal substrate for gene delivery by nucleofection. Nucleofection supports both T-cell reprogramming and genome editing protocols through the efficient delivery of different effector molecules including RNA/DNA and RNPs. The following results chapters detail two applications of circRNA delivery, to facilitate transcriptional reprogramming and the knockout of inhibitory receptors by base editing. These approaches can be combined with the stable expression of the CAR, either by CRISPR/Cas9-mediated HDR or lentiviral transduction, to generate a T-cell product with an optimal phenotype, fitness, and function.
Chapter 4. Transcription factor expression from circRNA maintains T-cell naivety/memory phenotype

4.1 Overview

The previous chapter evaluated the applications of two proprietary delivery methods for the delivery of RNA/DNA and proteins into primary T-cells. The nucleofection of circRNA was found to prolong the duration of transgene expression, whilst maintaining cellular viability. The second section of this study will explore two applications of circRNA for T-cell reprogramming. The first application uses circRNA to transiently express transcription factors known to control T-cell differentiation. By promoting an early memory T-cell phenotype, we can improve the in vivo persistence and efficacy of CAR T-cells therapies, to generate robust and durable remissions in patients.

4.2 Introduction

4.2.1 T-cell differentiation

Anti-tumour activity and long-term memory are achieved by the generation of phenotypically and functionally distinct CD8+ T-cell populations. Naïve T-cells (T_N) are the most immature of subsets and are defined by the expression of the RA isoform of transmembrane tyrosine phosphatase, CD45, the costimulatory receptors, CD27 and CD28, and the lymph node homing molecules, L-selectin (CD62L), C-C motif chemokine receptor 7 (CCR7) and C-X-C motif chemokine receptor 3 (CXCR3) (Gattinoni, Klebanoff and Restifo, 2012). This enables circulating T_N cells to enter the lymphoid organs where they become activated by antigen-presenting cells, thus, initiating a progressive differentiation process to form the memory and effector subsets (illustrated in Figure 4.1). As T-cells become more differentiated they lose their proliferative capacity, coincident with an increase in cytotoxicity. The pre-mRNA encoding the CD45RA isoform is alternatively spliced to form CD45RO, which is characteristic of the memory compartment. The memory compartment is classically divided into two subsets known as central memory (T_CCM) and effector memory (T_EM) T-cells. T_CCM cells maintain
expression of CD62L, CCR7 and CXCR3 and circulate in the body to provide rapid cytokine secretion (predominantly IL-2) upon activation. TEM cells lose their lymphoid homing ability through loss of CD62L and CCR7 but have an increased capacity for interferon-γ (IFN-γ) release and cytotoxicity (Gattinoni, Klebanoff and Restifo, 2012). An early subset of TCM have been described as stem cell memory T-cells (TSCM) and these can both self-renew and replenish more differentiated subsets of memory T-cells, by retaining long telomeres and telomerase activity (Ahmed et al., 2016). The TSCM compartment expresses the CD45RA isoform, costimulatory receptors and homing receptors, and differ from naïve cells based on the expression of CD95 (also known as FAS) and the IL-2 receptor subunit beta (CD122), both of which are markers of memory T-cells (Gattinoni et al., 2011; Lugli et al., 2013). The memory T-cells differentiate to form the subset known as terminal effectors (TEFF), which exhibit the highest cytotoxicity and become rapidly exhausted.

Figure 4.1 Schematic of T-cell differentiation and flow-cytometry markers for identification of T-cell subsets.

Naïve T-cells are stimulated by antigen presenting cells (APCs) and undergo differentiation into the memory cell subsets (stem cell memory, central and effector memory), before the transition into terminal effector cells. As T-cells differentiate they lose their proliferative and self-renewal
capacity, and in the case of CD8+ T-cells their cytotoxicity increases. The phenotypic attributes to identify T-cell populations can be detected by flow-cytometry and include cluster of differentiation 95 (CD95), protein tyrosine phosphatase receptor type C (CD45) RO and RA isoforms, CD27, C-C motif chemokine receptor 7 (CCR7), L-selectin (CD62L), CD28 and C-X-C motif chemokine receptor 3 (CXCR3). Expression is indicated as (+) and no expression is indicated as (-).

4.2.2 The molecular regulation of differentiation

The differentiation process is controlled by the complex interaction of transcription factors to co-ordinate a balance between self-renewal and effector functions. Transcriptional signatures of memory compartments have identified key regulators that are involved in regulating T-cell stemness (Bonnal et al., 2015; Ranzani et al., 2015). Transcription factors that are upregulated in naïve cells include T-cell factor 1 (TCF1) (encoded by TCF7), inhibitor of DNA-binding 3 (ID3), Kruppel-like factor 7 (KLF7), lymphoid enhancer-binding factor 1 (LEF1) and forkhead box P1 (FOXP1). Whereas, inhibitor of DNA-binding 2 (ID2), eomesodermin (EOMES), zinc finger E-box binding homeobox 2 (ZEB2), PR domain-containing 1 with ZNF domain (PRDM1, also known as BLIMP-1), T-box 21 (TBX21, also known as T-bet) and killer cell lectin-like receptor subfamily G member 1 (KLRG1) are highly expressed in effector memory cells (Gattinoni et al., 2011). In early subsets, signalling pathways operate to promote self-renewal whilst repressing differentiation. The self-renewal pathways include WNT-β-catenin (Gautam et al., 2019), signal transducer and activator of transcription factor 3 (STAT3) (Siegel et al., 2011) and forkhead box O (FOXO) signalling (Kerdiles et al., 2009). Pathways promoting differentiation are driven by pro-mitotic cytokines such as IL-2 and include PI3K-AKT-mTOR and RAS-RAF-MAPK signalling. Pro-differentiation signalling can be counteracted by TGFβ and downstream SMAD signalling to promote the expression of ID3 (Gattinoni, Klebanoff and Restifo, 2012). A less differentiated phenotype is desirable for CAR T-cell therapies and can be achieved by the induction of self-renewal signalling or the inhibition of pro-differentiation signalling. A summary of the molecular control of differentiation is illustrated in Figure 4.2.
Figure 4.2 Transcription factor signalling regulates the expression of self-renewal genes.

WNT/β-catenin signalling activates the downstream transcription factors, TCF and LEF, to promote the expression of genes involved in regulation of T-cell stemness, including the transcription of STAT3. Cytokine signalling through the STAT3 and SMAD4 pathways play an important role in promoting the expression of self-renewal genes. On the other hand, activation of the PI3K-AKT-mTOR pathway leads to T-cell differentiation and causes the phosphorylation and sequestration of BACH2 and FOXO1 in the cytoplasm. BACH2 is an AP-1 repressor and prevents the expression of effector genes associated with terminal differentiation through competing with AP-1 for the enhancer sites. The transcription factor, FOXO1, plays an important role in the regulation of gluconeogenesis and glycogenolysis, as well as the expression of self-renewal genes.

4.2.3 Naïve and early memory T-cells for adoptive cell therapy

The CAR T-cell manufacturing process involves the activation and expansion of donor T-cells (outlined in section 3.8.2), and extended manufacturing times can lead to naïve cells differentiating to memory and effector subsets. Although the effector subsets provide a potent cytotoxic response, mediated by the release of IL-2 and IFN-γ, T\textsubscript{EFF} cells...
become rapidly exhausted upon in vivo administration, leading to poor engraftment and persistence (Klebanoff et al., 2005). In a study of acute lymphoblastic leukaemia (ALL), 40-60% of patients that received anti-CD19 CAR therapy relapsed due to poor product persistence or the appearance of CD19 negative clones (Ghorashian et al., 2019). The study of persistence has been achieved in ALL patients using cellular phenotyping combined with retroviral integration site analysis to track the fate of infused CAR T-cells. T_{SCM} cell clones were found to play a critical role in mediating the early anti-leukemic response and long-term CAR T-cell persistence in responding patients (Biasco et al., 2021).

The persistence and efficacy of immunotherapies can be improved by generating CAR T-cells with a less differentiated phenotype (containing high numbers of T_N and T_{SCM} cells). The advantage of T_N and T_{SCM} cell subsets is conferred by their long-life span, capacity for self-renewal, strong proliferative potential, and ability to form memory subsets in vivo. Gattinoni et al., generated CD8+ T-cells at progressive stages of differentiation through repeated antigen stimulation and transferred individual subsets to melanoma-bearing mice. Whilst in vitro assays demonstrated highest cytotoxicity from the effector subset, the greatest tumour control was observed with naïve and early effectors in vivo (Gattinoni, 2005). This work was further support by Klebanoff et al., who found CD8+ T-cell differentiation status to be inversely correlated with in vivo anti-tumour activity (Klebanoff et al., 2011). Graef et al., have investigated the self-renewal capacity and multipotency of T_N and T_{CM} cells by in vivo fate mapping across three serial single-cell adoptive transfers in mice. Their results indicated that CD62L+ T_{SCM} cells reside in the central memory compartment, which remain multipotent and able to restore immunocompetence throughout serial adoptive transfers (Graef, 2014). Other studies have engineered T_N cells in vitro by culturing recombinant TCR-transduced cells with IL-7 and IL-15, and have shown their ability to differentiate into T_{EM} and T_{EFF} in vivo (Cieri et al., 2013). These studies demonstrate that engineered T_N and T_{SCM} cells can recapitulate the full memory repertoire upon adoptive transfer.
4.2.4 Enforcing an early T-cell memory phenotype

Strategies to induce early memory formation include a variety of methods to alter cell signalling and metabolic pathways (McLellan and Ali Hosseini Rad, 2019). Cell culture medium can be supplemented with cytokines or small molecule inhibitors during CAR T-cell expansion. The cytokines, IL-7 and IL-15 (Gomez-Eerland et al., 2014; Xu et al., 2014; Alizadeh et al., 2019) and/or IL-21 (Hinrichs et al., 2008; Alvarez-Fernandez et al., 2015) are commonly used to generate T<sub>SCM</sub> cells. Small molecules used to modulate the T-cell phenotype were first reported in 2009 byGattinoni et al. The glycogen synthase kinase 3β (GSK-3β) inhibitor, TWS119, was employed to increase the T<sub>SCM</sub> compartment through activation of WNT-β-catenin signalling (Gattinoni et al., 2009). The use of TWS119 has since been incorporated into a clinical manufacturing process for the generation of anti-CD19 CAR T-cells (Sabatino et al., 2016). Other studies report the use of PI3K/AKT inhibitors to promote T-cell naivety through metabolic reprogramming by FOXO1 (Klebanoff et al., 2017; Mousset et al., 2018; Funk et al., 2022). Alternative strategies include optimisation of ex vivo T-cell activation (Alvarez-Fernandez et al., 2015; Kagoya et al., 2017), and modulation of the TCR signal strength. Freucht et al., describe mutation of the tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAM) to reduce the number of functional ITAMs from three to one in a CAR bearing a CD28 endodomain to generate 1XX CAR T-cells. The fine tuning of TCR signalling in these CAR T-cells resulted in more balanced effector and memory programs (Feucht et al., 2019). In a further study, 1XX CAR T-cells have shown superior efficacy in pancreatic and melanoma mouse models, demonstrating their application for the treatment of solid tumours (Duan et al., 2022).

A recent study has shown that terminally exhausted CD8+ TILs can be reinvigorated by metabolic reprogramming using a half-life-extended interleukin-10-Fc fusion protein, to restore proliferative expansion by promoting oxidative phosphorylation (Guo et al., 2021). Furthermore, Weber et al., demonstrate phenotype modulation and restoration of anti-tumour function by transiently inhibiting CAR expression using a small molecule, dasatinib, to provide a physiological “rest” to exhausted CAR T-cells (Weber et al.,
2021). These studies suggest that differentiated and exhausted T-cells can be “re-booted” to a less differentiated state to restore functionality.

Whilst these methods have demonstrated modest improvements to the T-cell phenotype, an alternative strategy that provides greater specificity and control of T-cell function is desirable. This study aims to promote an early memory phenotype and to restore the function of exhausted T-cells through transcriptional reprogramming.

### 4.2.5 Transcriptional reprogramming

The reprogramming of cells has typically referred to the induction of pluripotency by the enforced expression of the four Yamanaka transcription factors, OCT4, KLF4, c-MYC and SOX2, which was first achieved by retroviral integration (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Following the initial reprogramming studies, the field has moved towards the use of synthetic mRNAs to repeatedly deliver pluripotency factors (Warren et al., 2010; Mandal and Rossi, 2013). This has been achieved by incorporating modified nucleotides, such as m$^5$C and pseudouridine (ΨU), to suppress innate sensing and immunogenicity of exogenous RNAs (Karikó et al., 2005). Warren et al., demonstrate that modified mRNA can induce pluripotency from human somatic cells, as well as directing the fate of induced pluripotent stem cells to terminally differentiated myogenic cells (Warren et al., 2010).

Using a similar approach, we can reprogram late memory T-cells (TEM and TEff) into earlier cell fate lineages and/or enrich for T$_N$ and T$_{SCM}$ subsets through enforced expression of transcription factors. One study has delivered a mix of unmodified mRNAs encoding eight transcription factors (LEF1, KLF7, ID3, EOMES, BCL6, TCF1, FOXP1 and FOXO1) to stimulated T-cells and observed an increase in CCR7 expression from day 5 to day 7 post-transfection (H. Lu et al., 2020). Here, we characterise the individual effects of transcription factors on the T-cell phenotype, selected to promote self-renewal signalling or to counteract pro-differentiation signalling. The key signalling pathways are described in greater detail below and include WNT-β-catenin, STAT3, SMAD and PI3K-AKT-mTOR signalling (Figure 4.2).
4.2.5.1 Promoting self-renewal pathways

4.2.5.1.1 WNT-β-catenin signalling

WNT-β-catenin signalling has been associated with self-renewal in embryonic stem cells and in T-cell subsets. In the absence of WNT signalling, β-catenin is phosphorylated by the destruction complex, which contains the scaffold protein, Axin, APC and the kinases, casein kinase (CK1α) and GSK-3β. Activation of WNT signalling by WNT ligation to the Frizzled receptor and low-density lipoprotein co-receptors initiates a signalling cascade that disrupts the destruction complex and results in the accumulation of β-catenin in the nucleus. Nuclear β-catenin tethers TCF1 and LEF transcription factors to DNA for the transcription of self-renewal genes (Gattinoni, Klebanoff and Restifo, 2012). Other WNT-β-catenin pathway components such as c-MYB, have been found to increase the early memory compartment, through the transcriptional activation of TCF7 and repression of ZEB2 (Gautam et al., 2019). A recent study has demonstrated the role of c-MYB to maintain the self-renewal capacity of a population of CD62L+ PD-1+ cells, which are responsible for the proliferative burst in response to PD-1 checkpoint inhibition and for long-term antiviral immunity (Tsui et al., 2022). WNT3A, TCF1, LEF1 and c-MYB (including constitutively active and inactive mutants) are investigated in this study.

4.2.5.1.2 STAT3 signalling

STAT3-dependent cytokines (such as IL-21, IL-10 and IL-6) have been shown to enhance the formation of the early memory compartment in CD8+ T-cells (Hinrichs et al., 2008; Cui et al., 2011; Siegel et al., 2011). Cytokines bind to their corresponding receptors causing receptor dimerization and the transphosphorylation and activation of JAK proteins. The activated JAKs subsequently phosphorylate the receptor and generate binding sites for STAT proteins via their SH2 domains. In contrast to other γc cytokines, IL-21 preferentially activates STAT3 proteins, whereas IL-2, IL-7, IL-9 and IL-15 activate STAT5A and STAT5B, indicating their differing roles (Lin and Leonard, 2000; Zeng et al., 2007). Bound STATs then become phosphorylated by JAKs, causing dissociation from the receptor and the formation of homo- or heterodimers. STAT3 homodimers translocate to the nucleus to activate genes that withhold differentiation such
as the KLF family proteins and BCL6 (Ichii et al., 2004; Cui et al., 2011). Here, STAT3 and BCL6 are investigated as candidates for overexpression in T-cells.

### 4.2.5.2 Counteracting pro-differentiation pathways

#### 4.2.5.2.1 SMAD signalling

Activation of the SMAD pathway can counteract the pro-differentiation signals of the RAS-RAF-MAPK pathway, to inhibit T-cell effector functions. SMAD2 and SMAD3 are activated upon binding of TGFβ to the TGFβ receptor and translocate to the nucleus with SMAD4. The SMAD complex induces the expression of early memory markers, such as ID3 and CD62L, and suppresses the expression of pro-differentiation genes, such as EOMES and KLRG1 (Chandiran et al., 2021). ID3 has been shown to block differentiation by repressing the activity of E-protein transcription factors such as E2A (Yang et al., 2011). The transcriptional regulation of ID3 in effector subsets occurs through BLIMP-1 and studies have shown that enforced ID3 expression can rescue the survival of terminally differentiated KLRG1+ T-cells (Ji et al., 2011).

SMAD4 signalling can also occur independently of TGFβ and has been shown to regulate the same genes in the opposite direction (upregulation of EOMES and KLRG1), to promote the formation of the T\textsubscript{CM} and effector subsets (Chandiran et al., 2021). The T-box transcription factor, EOMES, induces IFN-γ, perforin and granzyme expression, and is transcriptionally regulated by RUNX3, which functions to control CD8+ effector functions (Pearce et al., 2003; Cruz-Guilloty et al., 2009). We investigate the overexpression of ID3 and RUNX3, to mediate early memory formation, and EOMES, to promote formation of the T\textsubscript{CM} compartment.

#### 4.2.5.2.2 PI3K-AKT-mTOR signalling

The PI3K-AKT-mTOR pathway regulates the cell cycle to promote metabolism, proliferation, differentiation, and cellular survival (Kim and Suresh, 2013). PI3K phosphorylates and activates AKT, resulting in phosphorylation and nuclear exclusion of BACH2 and FOXO1 (causing inactivation), and activation of the nutrient-sensitive kinase, mTOR. Downregulation of mTOR activity (for example, rapamycin mTOR
inhibition) and nuclear localisation of BACH2 and FOXO1 have been associated with the generation of T\textsubscript{SCM} cells (Michelini et al., 2013; Roychoudhuri et al., 2016; Alizadeh et al., 2019). BACH2 is a multifunctional transcription factor and has been shown to compete with the AP-1 transcription factor for binding to the AP-1 enhancer, to prevent the expression of genes associated with terminal differentiation and exhaustion (Roychoudhuri et al., 2016; Sidwell and Kallies, 2016). Overexpression of BACH2 has enhanced the stem-like transcriptional profile in CD8\textsuperscript{+} T-cells (Yao et al., 2021). Whereas, FOXO1 has been shown to control homeostasis and survival of T\textsubscript{N} cells by sensing oxidative stress and nutrient availability, and regulating the homing and survival signals, CD62L, CCR7 and TCF1 (Kerdiles et al., 2009; Kim et al., 2013). This study investigates BACH2, a constitutively active BACH2 mutant (S520A), and FOXO1.

This section of the study will investigate circRNA delivery to transiently express transcription factors and reprogram T-cells to a less differentiated cell state. To identify suitable transcription factors, we first screened a pool of transcription factors by stable expression in T-cells, prior to investigation of the transient approach.
4.3 Chapter Aims

This chapter aims to deliver circRNA-encoding transcription factors to reprogram T-cells and slow the commitment to the effector memory T-cell lineage during \textit{ex vivo} modification. A transient approach aims to support the formation of the highly cytotoxic effector cells \textit{in vivo}, for a durable anti-tumour response. The transcription factor candidates will be screened initially by lentiviral transduction before the selection of optimal candidates for the transient approach. In summary, this chapter aims to:

4.3.1 Screen fifteen transcription factors using a stable expression system.

4.3.2 Produce circRNA-encoding the selected transcription factors.

4.3.3 Evaluate the use of circRNA-encoding transcription factors for T-cell reprogramming.
4.4 Screening candidate transcription factors by stable expression

Fifteen transcription factors were initially screened by lentiviral integration to enforce stable expression in T-cells, and their effects were characterised by monitoring phenotypic changes after a culturing period of 7 days. Thirteen candidates were investigated to promote self-renewal signalling and formation of the T_N and T_SCM subsets, EOMES was investigated to increase the T_CM subset, and the anti-apoptotic protein, BCL-XL, was investigated to improve the viability of nucleofected cells (X.-L. Li et al., 2018). The transcription factor candidates and their known functions are listed in Table 25.

**Table 25. List of candidates for expression in primary T-cells.**

<table>
<thead>
<tr>
<th>Candidate</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>WNT3A</td>
<td>WNT-β-catenin signalling</td>
<td>(Gattinoni et al., 2009)</td>
</tr>
<tr>
<td>LEF1</td>
<td>WNT-β-catenin signalling</td>
<td>(Gattinoni et al., 2009)</td>
</tr>
<tr>
<td>TCF1</td>
<td>WNT-β-catenin signalling</td>
<td>(Gattinoni et al., 2009)</td>
</tr>
<tr>
<td>c-MYB</td>
<td>WNT-β-catenin signalling</td>
<td>(Tsui et al., 2022; Gattinoni et al., 2009; Gautam et al., 2019)</td>
</tr>
<tr>
<td>c-MYB (1-330)</td>
<td>WNT-β-catenin signalling, constitutively active c-MYB lacking the negative regulatory domain</td>
<td>(Gautam et al., 2019)</td>
</tr>
<tr>
<td>c-MYB (1-330 GP)</td>
<td>WNT-β-catenin signalling, constitutively inactive c-MYB mutant that lacks the negative regulatory domain and contains a GP insertion at amino acid position 304 in the transactivation domain.</td>
<td>(Gautam et al., 2019)</td>
</tr>
<tr>
<td>STAT3</td>
<td>Cytokine signalling</td>
<td>(Hinrichs et al., 2008; Cui et al., 2011; Siegel et al., 2011)</td>
</tr>
</tbody>
</table>
BCL6 | Transcriptional repressor of pro-differentiation genes (Ichii et al., 2004)
---|---
ID3 | DNA binding protein inhibitor (Ji et al., 2011; Yang et al., 2011)
RUNX3 | Forms heterodimeric complex core-binding factor with CBFβ (Pearce et al., 2003; Cruz-Guilloty et al., 2009)
EOMES | T-box transcription factor (Cruz-Guilloty et al., 2009)
BACH2 | AP-1 repression (Roychoudhuri et al., 2016; Yao et al., 2021)
BACH2 (S520A) | AP-1 repression, constitutively active BACH2 containing a S520A mutation to prevent phosphorylation by AKT and subsequent nuclear exclusion (Roychoudhuri et al., 2016)
FOXO1 | Metabolic homeostasis and expression of homing markers (Kerdiles et al., 2009; Michelini et al., 2013)
BCL-XL | Anti-apoptotic protein (X.-L. Li et al., 2018)

The transcription factor sequences were cloned into a pCCL lentiviral genome plasmid containing the MND promoter, which drives expression of an iRFP670 (RFP) transduction marker and T2A self-cleaving sequence, upstream of the transgene flanked by nuclear localisation sequences. Lentivirus was produced and titrated, and PBMCs were transduced at a fixed MOI of 3 virions per cell (methods 2.2.2.3.1, 2.2.2.3.3, 2.2.2.3.6). Flow-cytometry was performed at 7 days post-transduction using the panel described in Figure 4.1 and the memory phenotype was compared between the non-transduced (RFP-) and transduced (RFP+) populations of each sample. T_N cells were detected as CD45RA+, CCR7+, T_CM as CD45RA-, CCR7+, T_EM as CD45RA-, CCR7- and T_EFF as CD45RA+, CCR7-.
As shown in the CD3+ compartment (Figure 4.3), an increase in the T\textsubscript{N} subset was observed in T-cells transduced with BACH2, BACH2 (S520A), FOXO1, or RUNX3, which was not observed in T-cells transduced with RFP alone. T-cells transduced with BACH2 or BACH2 (S520A) demonstrated a 2-fold increase in the naïve subset, and transduction with FOXO1 resulted in a 2.7-fold increase. There was also a concomitant increase in the T\textsubscript{CM} compartment (1.5-fold for BACH2 and BACH2 (S520A), 1.8-fold for FOXO1), which was observed in T-cells transduced with TCF1, LEF1, c-MYB, c-MYB (1-330) or c-MYB (1-330 GP) as well (~ 1.8-fold). Conversely, a 2-fold reduction in the T\textsubscript{CM} subset was observed in T-cells transduced with EOMES or BCL6.
Figure 4.3 Stable introduction of transcription factors promotes transcriptional reprogramming.

Phenotypic changes in T-cells transduced with lentiviral genomes encoding transcription factors. (a) General structure of the transcription factor-encoding lentiviral genomes. Expression of the transcription factor is driven by an MND promoter and is separated from the
iRFP670 (RFP) transduction marker by a 2A self-cleaving peptide sequence (T2A). The transcription factor sequence is flanked by a 5’ SV40 NLS and a 3’ NLS derived from c-MYC.

(b) The memory phenotype was compared between the non-transduced (RFP-) and transduced (RFP+) populations of each sample in the CD3+ compartment at day 7 post-transduction. Flow-cytometry plots (CD45RA-APC-Vio770 versus CCR7-PE) are shown for four transcription factors from one representative donor. One construct lacked the transcription factor sequence and was included as a control (RFP only). (c) Graphical representation of the T-cell memory phenotype in the non-transduced and transduced populations at day 7 post-transduction, represented as mean ± SD (n=3 independent biological donors).

Analysis of the CD8+ and CD4+ compartments revealed differences in the effects of overexpressed transcription factors (Figure 4.4, Figure 4.5). In the CD8+ compartment, an increase in the T_N population was observed for T-cells transduced with TCF1 (2-fold, p = 0.0079), LEF1 (2.4-fold, p = 0.0004), WNT3A (1.9-fold, p = 0.0146), RUNX3 (2.2-fold, p = 0.0002), STAT3 (1.9-fold, p = 0.0349), BCL-XL (1.7-fold, p = 0.0256), and was particularly evident in cells transduced with BACH2 (2.3-fold, p ≤ 0.0001), BACH2 (S520A) (2.6-fold, p ≤ 0.0001) or FOXO1 (3-fold, p ≤ 0.0001). On the other hand, an increase in the T_CM population was observed upon over-expression of FOXO1 (2.5-fold, p = 0.0235), TCF1 (2.5-fold, p = 0.0210), c-MYB (2.3-fold, p = 0.0019), c-MYB (1-330) (2.6-fold, p = 0.0005) or c-MYB (1-330 GP) (2-fold, p = 0.0394) (Figure 4.4). In the CD4+ compartment, a 2.8-fold increase in the T_N population was observed for FOXO1-transduced T-cells (p ≤ 0.0001) and a reduction in the T_CM compartment was observed for EOMES- (2.3-fold, p = 0.0104) and BCL6-transduced T-cells (2.7-fold, p = 0.0007) (Figure 4.5).
Figure 4.4 Effects of stable transcription factor overexpression in CD8+ T-cells.

Changes in the proportion of naïve and central memory T-cells following transduction with transcription factor-encoding lentivirus. The transduced population was identified by expression of iRFP670 (RFP) which was co-expressed with the transcription factor. One construct lacked the transcription factor sequence and was included as a control (RFP only). The percentage of T_N (CD45RA+, CCR7+) or T_CM (CD45RA-, CCR7+) cells was compared between the non-transduced (RFP-) and transduced (RFP+) populations of each sample in the CD8+ compartment at day 7 post-transduction, represented as mean ± SD (n=3 independent biological donors, represented as different symbols). (Two-Way ANOVA; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).
Surprisingly, we also saw an improvement in T-cell naivety with c-MYB (1-330 GP), which lacks the negative control region and contains an insertion in the transactivation domain that renders it non-functional. This mutant differs from the negative control described by Gautam et al., where the insertion is engineered into the full-length transcription factor (Gautam et al., 2019). The negative regulatory domain may
participate in downregulating the activity of the transcription factor, which may explain the failure to render the transcription factor non-functional.

Initial screening of transcription factors by stable expression identified BACH2, BACH2 (S520A), FOXO1, TCF1, LEF1 and RUNX3 as promising candidates. BACH2 and FOXO1 generated the most notable phenotypic changes and were chosen for further study.

4.5 Transient expression of BACH2 and FOXO1 facilitates transcriptional reprogramming

Following screening of transcription factors in a stable expression system, BACH2 and FOXO1 were evaluated in a transient system by the nucleofection of \textit{in vitro} transcribed circRNA.

4.5.1 Production of transcription factor-encoding circRNA

The BACH2 and FOXO1 sequences were cloned into the \textit{Anabaena} PIE sequence (Wesselhoeft, Kowalski and Anderson, 2018) and circRNA generated by \textit{in vitro} transcription (method 2.2.1.3). As shown in Figure 4.6, RNA circularisation was confirmed by release of the intronic sequences (observed at 181 and 624 bases) and a shift in the size of RNA. Full length RNA was observed at 3511 bases for BACH2 and 2953 bases for FOXO1.
4.5.2 Nucleofection of BACH2- and FOXO1-encoding circRNA

To investigate the application of circRNA for phenotype modulation, BACH2- and FOXO1-encoding circRNAs were delivered by nucleofection to PBMCs activated with TransAct™ and IL-7/15 for 48h (method 2.2.2.4.1). This provided a strong stimulation signal, promoting differentiation towards an effector T-cell phenotype during expansion.

Figure 4.6 Transcription factor-encoding circRNAs.

(a) Diagram of circRNA templates containing the BACH2 and FOXO1 coding sequences. HA, homology arm; E2, exon 2, CVB3 IRES, coxsackievirus B3 internal ribosome entry site; NLS, nuclear localisation sequence; E1, exon 1. (b) TapeStation bioanalyser image of in vitro transcribed RNA pre- and post-circularisation, showing the increased mobility of the circular RNA, compared with the linear RNA, and the released introns after circularisation.
After nucleofection, the PBMCs were rested for 24h before resuspending in fresh medium containing IL-2, to drive pro-differentiation signalling.

4.5.2.1 **Grouping donors according to the level of RNA expression**

As we previously observed donor-to-donor variability in the amenability to circRNA transfection (Figure 3.13), we included a control to determine the transfection efficiency and level of circRNA expression in six independent donors. The same donors that were transfected with transcription factor-encoding circRNA were separately nucleofected with an mClover3 circRNA and were analysed by flow cytometry 24h later. Although all donors were amenable to RNA transfection (TEs >85%), variability was observed in the MedFI of mClover3 expression (16.8-fold difference between Donor 1 (highest) and Donor 6 (lowest)). To account for the variability, we classified donors into high, medium, and low RNA expression groups according to the mClover3 MedFI, relative to one another (Figure 4.7). This would allow us to determine the phenotypic changes in donors that are highly amenable to RNA transfection versus those that expressed RNA at low levels.
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Figure 4.7 Classification of donors according to the level of RNA expression.
Donors were grouped based on their amenability to RNA transfection, as determined by the relative MedFI of mClover3 expression at 24h post-nucleofection with 3 µg mClover3 (mClover) circRNA. Flow-cytometry plots (FSC-A versus mClover3) are shown for donors in the (a) high, (b) medium or (c) low RNA expression groups. (d) Transfection efficiency and MedFI of mClover3 expression (normalised to CountBright beads), represented as mean ± SD (n=6 independent biological donors, represented as different symbols).

Following 7 days of expansion, the phenotype of transfected T-cells was determined using the memory phenotyping panel (as described in Figure 4.1). The CD45RA and CCR7 markers were used to identify the T_N, T_CM, T_EM, and T_EFF subsets, as previously described. In the CD8+ compartment, staining of the no pulse and no RNA controls show that there was considerable phenotypic heterogeneity between donors, with donors 3, 5 and 6 having >50% T_EM, while donors 1, 2 and 4 had >68% T_CM and T_N cells (Figure 4.8). Few T_N cells were observed in the CD4+ compartment and there was an increased proportion of T_CM cells, except for donors 5 and 6 where >72% of cells were T_EM (Figure 4.9).

Transient of expression of either FOXO1 or BACH2 led to transcriptional reprogramming, particularly in the medium and high RNA-expressing donors. In donors 1, 2 and 4, the enforced expression of BACH2 resulted in a reduction of T_N cells and an increase in T_CM cells within the CD8+ compartment. FOXO1-encoding circRNA also increased the T_CM compartment, except in donor 2, which showed an increase in the T_N subset (Figure 4.8). In the CD4+ compartment, a reduction in the CCR7 expression of T_CM cells was observed for BACH2-transfected cells, whereas an increase was observed for FOXO1-transfected cells (Figure 4.9).
Figure 4.8 Transcriptional reprogramming in CD8+ T-cells.
Chapter 4. Results

Phenotypic changes in stimulated T-cells nucleofected with 3 µg transcription factor-encoding circRNA. The memory phenotype was compared between the no RNA and the transfected samples in donors that express RNA to a (a) high, (b) medium, or (c) low level. Flow-cytometry plots (CD45RA-APC-Vio770 versus CCR7-PE) are shown for the CD8+ compartment at day 7 from nucleofection.
Figure 4.9 Transcriptional reprogramming in CD4+ T-cells.
Phenotypic changes in stimulated T-cells nucleofected with 3 µg transcription factor-encoding circRNA. The memory phenotype was compared between the no RNA and the transfected samples in donors that express RNA to a (a) high, (b) medium, or (c) low level. Flow-cytometry plots (CD45RA-APC-Vio770 versus CCR7-PE) are shown for the CD4+ compartment (gated on CD8-) at day 7 from nucleofection.

Generally, differences in the phenotype were only observed in the medium and high RNA-expressing donors. BACH2 overexpression resulted in a significant reduction in the percentage of T<sub>N</sub> cells in the CD8+ (p = 0.04043) and CD4+ (p = 0.02804) compartments, which was coincident with a general increase in the T<sub>CM</sub> subsets. A slight reduction in the CCR7 expression was also observed in T<sub>CM</sub> CD4+ T-cells (Figure 4.10). Upon expression of FOXO1, a significant increase in the CCR7 expression was found in the CD8+ T<sub>N</sub> (p = 0.04645) and T<sub>CM</sub> (p = 0.04145) subsets. Donors 1 and 3 showed a reduction in the percentage of T<sub>N</sub> cells and an increase in the T<sub>CM</sub> subset. This agrees with published data, implicating FOXO1 in controlling transcription of the CCR7 gene. In the CD4+ compartment, FOXO1 appeared to reduce naivety and promote formation of the T<sub>CM</sub> cells (Figure 4.11).
Figure 4.10 Transcriptional reprogramming using BACH2-encoding circRNA.
Phenotype modulation in T-cells transfected with 3 µg BACH2-encoding circRNA. Changes in the percentage of cell subsets and their corresponding level of CCR7 expression (MedFI, normalised to CountBright beads) were determined in the (a) CD8+ or (b) CD4+ (gated on CD8-) compartment at day 7 from nucleofection. Donors were grouped into high, medium, or low RNA expression groups as determined based on their TE at 24h. Each line represents one donor (n=6 independent biological donors, represented as different symbols). Paired t-test; *p ≤ 0.05. Naïve (T_N, CD45RA+, CCR7+) and central memory subsets (T_CM, CD45RA-, CCR7+).
Figure 4.11 Transcriptional reprogramming using FOXO1-encoding circRNA.
Phenotype modulation in T-cells transfected with 3 μg FOXO1-encoding circRNA. Changes in the percentage of cell subsets and their corresponding level of CCR7 expression (MedFI, normalised to CountBright beads) were determined in the (a) CD8+ or (b) CD4+ (gated on CD8-) compartment at day 7 from nucleofection. Donors were grouped into high, medium, or low RNA expression groups as determined based on their TE at 24h. Each line represents one donor (n=6 independent biological donors, represented as different symbols). (Paired t-test; *p ≤ 0.05). Naïve (T_N, CD45RA+, CCR7+) and central memory subsets (T_CM, CD45RA-, CCR7+).

4.5.3 Summary of transcriptional effects on T-cells

The results of this section demonstrate that circRNA can enforce expression of transcription factors and promote phenotype modulation in amenable donors. Following TransAct™ stimulation, BACH2 significantly reduced naivety and promoted the formation of T_CM cells, whereas FOXO1 increased CCR7 expression of CD8+ T_N and T_CM cells and improved the frequency of the T_CM subsets. Interestingly, the T-cells of donors that expressed low levels of the transgenes encoded by the circRNAs predominantly exhibited a T_EM phenotype, potentially suggesting reduced translational activity in these cells or increased RNA endonuclease activity, leading to destruction of the circular RNA.
4.6 Discussion

4.6.1 Identification of transcription factors to promote T-cell naivety

This chapter has identified transcription factors for overexpression in T-cells to promote a less differentiated phenotype. Fifteen candidates were selected for screening by stable expression, and BACH2, BACH2 (S520A), FOXO1, TCF1, LEF1 and RUNX3 were identified as promising candidates (section 4.4). Overexpression of BACH2 and FOXO1 led to the greatest improvement in CD8+ T-cell naivety, hence, these candidates were chosen for further study. The observed phenotypic differences can be explained by their inherent functions; BACH2 has been shown to compete with AP-1 binding sites to prevent the expression of pro-differentiation genes, whereas FOXO1 promotes the expression of genes associated with self-renewal (TCF7), homing (CCR7), and metabolic reprogramming (Kerdiles et al., 2009; Michelini et al., 2013; Roychoudhuri et al., 2016).

We investigated the co-delivery of BACH2 and FOXO1, however, co-transduction of lentiviral supernatants was unsuccessful (Appendix, Figure 8.2), and co-transfection of circRNAs did not further augment reprogramming compared to FOXO1 transfection alone. As FOXO1 has been shown to inhibit the activity of AP-1 and to upregulate the expression of BACH2, enforced expression of BACH2 may not be necessary (Delpoux et al., 2021). A previous study describes the co-delivery of eight transcription factor-encoding RNAs to T-cells (LEF1, KLF7, ID3, EOMES, BCL6, TCF1, FOXP1 and FOXO1) (H. Lu et al., 2020), however, this approach will result in a high translational burden and several transcription factors, including EOMES and BCL6, may be redundant or disadvantageous. Screening the effects of individual transcription factors provides a refined approach and we identify the lead candidate as FOXO1, to promote the formation of naive and early memory T-cells.

4.6.2 Expression of BACH2- and FOXO1-encoding circRNA facilitates T-cell reprogramming

CircRNAs were generated to encode BACH2 and FOXO1 flanked by nuclear localisation sequences to promote their intranuclear activity and override their inhibition by AKT-
mediated phosphorylation. CircRNAs could be efficiently produced (section 4.5.1), and we investigated the phenotypic effects of transient FOXO1 or BACH2 over-expression in TransAct™ and IL-2 stimulated T-cells (section 4.5.2). BACH2 was shown to promote the T<sub>CM</sub> subset (and reduced the T<sub>N</sub> subset) whilst FOXO1 increased the T<sub>CM</sub> and T<sub>N</sub> subsets. It may be challenging to drive T-cells to a truly naïve state since the CAR T-cell manufacturing process requires stimulation and expansion. Regardless, a T<sub>CM</sub> phenotype will provide advantages over a T<sub>EM</sub> and T<sub>EFF</sub> phenotype <em>in vivo</em>. Wang <em>et al.</em>, have compared the adoptive transfer of T<sub>CM</sub> cells compared to T<sub>N</sub> and T<sub>SCM</sub> cells and found that early memory cells had improved engraftment, likely due to their proliferative capacity (Wang <em>et al.</em>, 2016). As T<sub>SCM</sub> cells are an early subset of T<sub>CM</sub> cells, we attempted identification of the T<sub>SCM</sub> subset using CD27 and CD95 expression (gated on the CD45RA+, CCR7+ population), however, T<sub>SCM</sub> identification was unclear and will require additional markers such as CD62L.

### 4.6.3 Transcription factor-encoding circRNA provides transient and specific molecular control

The delivery of circRNA will prolong the duration of transcription factor expression (compared to linear mRNA) but will also ensure that the proteins will not be carried over into the patient. This will allow T-cell differentiation into the effector subsets <em>in vivo</em> to maintain anti-tumour activity. Advantages of this approach include greater specificity and molecular control compared to existing methods, such as small molecules inhibitors targeting GSK-3β (Sabatino <em>et al.</em>, 2016) and PI3K-AKT pathways (Klebanoff <em>et al.</em>, 2017; Mousset <em>et al.</em>, 2018; Funk <em>et al.</em>, 2022). Other approaches include the site-specific integration of transcription factors into the genome. Using their pooled CRISPR/Cas9 knock-in screen, Roth <em>et al.</em>, have identified TCF7 to provide a selective advantage under excessive stimulation conditions (Roth <em>et al.</em>, 2020). TCF7 has been incorporated into the TRAC locus of HIV-specific CD8+ T-cells, and has improved T-cell expansion and persistence during adoptive cell therapy (Rutishauser <em>et al.</em>, 2021). However, stable expression may result in undesirable effects in the patient and will require safety switches to eliminate T-cells in case of adverse events. An alternative strategy would be to place
the transcription factor under the control of an inducible promoter (such as a granzyme B inducible promoter) for conditional expression in effector subsets.

4.6.4 Confirming functional advantages

The results of this chapter conclude that circRNAs can drive phenotype modulation through the expression of BACH2 and FOXO1. Further work is required to assess the function of reprogrammed T-cells by combining transcription factor delivery with the introduction of the CAR. *In vitro* functional experiments may include RNA sequencing to assess the effects on gene expression, metabolic measurements such as mitochondrial oxygen flux and extracellular acidification rate, cytotoxicity, cytokine release, and re-stimulation assays. However, as highlighted by Gattinoni *et al.*, *in vivo* assays will likely be required to confirm the functional advantages (Gattinoni, 2005).

4.6.5 Applications of transcriptional reprogramming for cellular therapies

Transcriptional reprogramming could be applied to “re-boot” exhausted leukapheresis at the start of the manufacturing process or to reprogram CAR T-cells prior to cryopreservation. Although published studies report the ability to reverse exhaustion (Guo *et al.*, 2021; Weber *et al.*, 2021), further work is required to assess the function of BACH2- and FOXO1-encoding circRNA in terminally differentiated T-cells. As shown in the first results chapter, nucleofection can be detrimental to T-cell viability and survival, especially during CRISPR/Cas9 integration protocols which involve the delivery of large dsDNA templates (section 3.6). Omer-Javed *et al.*, describe the use of modified mRNA encoding CXCR4 to improve HSC migration, and also found expression to rescue the effects of nucleofection (Omer-Javed *et al.*, 2022). Transcription factors could be co-delivered with RNPs and/or HDR templates to improve the phenotype and viability. As an alternative to nucleofection, lipid nanoparticles (LNPs) could be used to deliver transcription factors during *ex vivo* manufacturing to preserve the cell viability. A biotechnology company, ORNA, has developed an *in vivo* approach using LNPs packaged with circRNA encoding the CAR (Mabry *et al.*, 2022). The function of *in vivo*-generated CAR T-cells could be improved by the co-delivery of circRNAs encoding the CAR and desired accessory molecules (e.g., cytokines, transcription factors). CircRNAs
can be directed specifically to T-cells by engineering the lipid nanoparticles with antibodies directed to CD5 (Mabry et al., 2022; Rurik et al., 2022).

### 4.7 Conclusions

This chapter has successfully screened fifteen transcription factors to modify the T-cell phenotype by lentiviral integration. CircRNAs encoding BACH2 and FOXO1 were generated and tested in T-cells stimulated using TransAct™. BACH2 promoted generation of the T\textsubscript{CM} subset, whereas FOXO1 improved the formation of the T\textsubscript{CM} and T\textsubscript{N} subsets.
Chapter 5. Development of a multiplexed base editing process using circRNA-encoding BE4max

5.1 Overview

Recent improvements to the manufacturing process aim to generate high quality T-cell products with an early memory phenotype to improve \textit{in vivo} persistence. This can be achieved by preventing T-cell differentiation and exhaustion. The previous chapter has demonstrated the potential of circRNA to transiently express transcription factors to reprogram cells into a less differentiated cell state. This chapter explores a second application of circRNA, to express cytosine base editors for the disruption of exhaustion markers. Tumour cells can exploit the expression of T-cell inhibitory receptors to manipulate cytotoxic T-cells and to suppress the immune response. Base editors can be employed to knockout the expression of exhaustion markers/inhibitory receptors to provide resistance to the immunosuppressive tumour microenvironment, enhancing the efficacy of CAR T-cell therapies. This chapter aims to screen sgRNAs for multiplexing the gene knockout of four inhibitory receptors using circRNA-encoding BE4max.

5.2 Introduction

5.2.1 T-cell exhaustion

T-cell exhaustion is a progressive dysfunctional cell state triggered by persistent antigen stimulation during chronic viral infection or in response to tumours (Blank \textit{et al.}, 2019). Firstly, T-cells lose their cytotoxicity and ability to proliferate. This is followed by the loss of IL-2, tumour necrosis factor (TNF) and IFN-\(\gamma\) secretion, and a switch to the production of the immunosuppressive cytokine, IL-10. \textit{In vitro} models using repeated antigen stimulation have revealed distinct epigenetic and transcriptional profiles of exhausted T-cells, and have found the transcription factors, ID3, SOX4 and TOX, to be key regulators of the exhaustive phenotype (Khan \textit{et al.}, 2019; Good \textit{et al.}, 2021). Exhausted T-cells are further characterized by the sustained expression of inhibitory receptors, including programmed cell death 1 (PD-1), T-cell immunoglobulin and mucin domain-containing protein 3 (TIM3) and lymphocyte-activation gene 3 (LAG3), which
function to downregulate effector activities. In vivo models have demonstrated that CAR T-cells acquire an exhausted phenotype in the tumour microenvironment (TME) (Moon et al., 2014; J. Chen et al., 2019) and has highlighted exhaustion as a major limitation concerning therapeutic efficacy (Long et al., 2015; Fraietta, Lacey, et al., 2018; Lynn et al., 2019).

5.2.1 Inhibitory receptors

5.2.1.1 Modulation of TCR signalling by inhibitory receptors

T-cell activation is tightly regulated by the expression of stimulatory and inhibitory receptors that interact to modulate TCR signalling. T-cell activation involves the presentation of antigens on the major histocompatibility complex (MHC) to the TCR by antigen-presenting cells (APCs). The CD3ζ becomes phosphorylated which recruits and activates zeta-chain associated protein kinase 70 (ZAP70). ZAP70 promotes the assembly of downstream adaptor and scaffold proteins to activate signalling through the PI3K-AKT-mTORC1, MAPK and NFκB pathways. To generate a sufficient signal for T-cell activation, a secondary co-stimulatory signal is required. Co-stimulation is achieved by engagement of CD28 on the surface of T-cells with the CD80 (B7-1) or CD86 (B7-2) receptors expressed on APCs. Inhibitory receptors interfere with TCR signalling to modulate effector functions and will be described in more detail below (Error! Reference source not found.).
Inhibitory receptors become upregulated following T-cell activation and are frequently associated with T-cell exhaustion. Some T-cell inhibitory receptors include programmed cell death protein 1 (PD-1), T-cell immunoglobulin and mucin domain-containing protein 3 (TIM3), lymphocyte activating 3 (LAG3) and transforming growth factor beta receptor 1/2 (TGFβR1/2). Upon interaction with their ligands, which are secreted or expressed on tumour cells or antigen presenting cells (APCs), these signal to suppress T-cell activation and downstream functions. Programmed cell death ligand 1/2 (PD-L1/2), high-mobility group box 1 protein (HMGB1), galectin-9 (Gal-9), phosphatidylserine (PtdSer), carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1), peptide-bound major histocompatibility complex class II (pMHCII) (expressed on APC only), T-cell receptor (TCR), C-type lectin receptor (LSECtin), galectin-3 (Gal-3), fibrinogen-like protein 1 (FGL1).

### 5.2.1.2 PD-1

PD-1, also known as cluster of differentiation 279 (CD279), is encoded by the programmed cell death 1 (PDCD1) gene, which consists of five exons. PD-1 is a type I transmembrane protein (288 amino acids) and comprises an extracellular
immunoglobulin variable (IgV) domain, a transmembrane domain, and an intracellular tail. The protein is expressed on the surface of activated T-cells and B-cells and has also been identified on natural killer (NK) cells, macrophages, monocytes, and dendritic cells. PD-1 is expressed when T-cells become activated and functions as an immune checkpoint to downregulate the inflammatory immune response and to limit autoimmunity by promoting self-tolerance. Activated T-cells release IFN-γ which upregulates the expression of PD-1 ligands on tumour infiltrating lymphocytes (TILs) and tumour cells. The ligands include PD-L1 (CD274) and PD-L2 (CD273), which are members of the B7 family (B7-H1 and B7-DC, respectively), and cause PD-1 to associate with the TCR in the immunological synapse to directly inhibit T-cell activation (Riley, 2009).

The cytoplasmic tail of PD-1 contains two tyrosine phosphorylation sites; the membrane proximal tyrosine located in the immunoreceptor tyrosine-based inhibitory motif (ITIM), and the distal tyrosine located in the immunoreceptor tyrosine-based switch motif (ITSM). T-cell stimulation results in phosphorylation of the ITIM and ITSM motifs, which recruits src homology 2-domain-containing tyrosine phosphatase (SHP)-1 and SHP-2. SHP-1 is expressed primarily in hematopoietic cells, and mutational studies have found that the inhibitory function of PD-1 relies on the binding of SHP-2 to ITSM (Okazaki et al., 2001; Chemnitz et al., 2004). SHP-2 contains two tandem SH2 domains and one phosphatase domain (PTP) and functions to dephosphorylate key signal transducers of TCR signalling (Patsoukis et al., 2020).

5.2.1.3 TIM3

TIM3 is encoded by the hepatitis A virus cellular receptor 2 (HAVCR2) gene, which consists of seven exons. The type I transmembrane glycoprotein (301 amino acids) consists of an extracellular Ig-like V-type domain, a serine/threonine-rich mucin stalk, a transmembrane domain, and a C-terminal cytoplasmic tail. Although originally identified as a receptor of CD4+ and CD8+ T-cells, B-cells, NK cells, dendritic cells, macrophages, and mast cells have also been shown to express TIM3. TIM3 is known to induce immunological tolerance to prevent autoimmunity, and to inhibit T-cell responses by mediating T-cell exhaustion. Ligands of TIM3 include soluble proteins and/or cell surface receptors including galectin-9 (Gal-9), high-mobility group box 1 protein (HMGB1),
phosphatidylserine (PtdSer), and carcinoembryonic antigen cell adhesion molecule 1 (CEACAM-1).

The cytoplasmic tail of TIM3 contains five tyrosine phosphorylation sites; Tyr256 and Tyr263 allow interactions with HLA-B-associated transcript 3 (BAT3) and tyrosine kinase FYN (Rangachari et al., 2012). During T-cell activation, TIM3 is recruited to the immunological synapse and BAT3 binds to the cytoplasmic tail to recruit the active form of LCK, maintaining TCR signalling. Gal-9 can be secreted or expressed on tumour cells or antigen-presenting cells and can bind to TIM3 glycan-binding sites via its carbohydrate recognition domains (Yasinska et al., 2019). Bound Gal-9 can promote TIM3 oligomerization, thus supporting binding with other ligands such as CEACAM-1. Upon binding, Try256 and Try263 become phosphorylated and BAT3 is released. This enables the recruitment of tyrosine kinase FYN, which disrupts the immune synapse by activating phosphoprotein associated with glycosphingolipid-enriched microdomains I (PAG). The tyrosine kinase, CSK, is then recruited, which phosphorylates LCK on an inhibitory residue to disrupt TCR signalling and to promote T-cell anergy (Wolf, Anderson and Kuchroo, 2019).

5.2.1.4 LAG3

LAG3 is a homolog of CD4 and is encoded by the LAG3 gene, which consists of eight exons. The type I transmembrane protein (525 amino acids) comprises an extracellular region that contains four Ig-like domains termed domain 1 (D1) to D4, a transmembrane domain and a cytoplasmic domain. LAG3 is predominantly expressed on CD4+ and CD8+ cells and is upregulated following TCR activation and cytokine stimulation (IL-2 and IL-12). The protein associates with the TCR to negatively regulate activation, proliferation, effector functions, and homeostasis in CD4+ and CD8+ T-cells (Maruhashi et al., 2020).

LAG3 has been proposed to interact with peptide-bound MHCII via the D1 domain and binds with higher affinity than CD4 to inhibit T-cell activation. Additional ligands include FGL1, LSECtin, galectin-3 and α-synuclein fibrils, which are secreted or expressed to high levels on tumour cells and interact with LAG3 on CD8+ and NK cells (Anderson,
Joller and Kuchroo, 2016; Burnell et al., 2022). The cytoplasmic tail of LAG3 mediates negative signal transduction and contains three conserved motifs, the FxxL motif containing a serine phosphorylation site (Ser484), a KIEELE motif and a glutamate-proline (EP) dipeptide multiple repeats motif (Workman, Dugger and Vignali, 2002; Chocarro et al., 2021). The functions of these motifs are yet to be fully elucidated, however mutational studies have demonstrated their importance in mediating the inhibitory signal (Maeda et al., 2019).

5.2.1.5 TGFβR2

The type II TGFβ receptor is encoded by the TGFβR2 gene, which consists of eight exons. TGFβR2 is a transmembrane protein containing a serine/threonine protein kinase domain (567 amino acids) and forms a heterodimeric complex with the type I receptor, TGFβR1, to transduce the signal of the TGFβ cytokines. TGFβ cytokines (TGFβ1, TGFβ2 and TGFβ3) are secreted in the TME from stromal cells such as CAFs, mesenchymal stem cells, pericytes and blood endothelial cells (Turley, Cremasco and Astarita, 2015). TGFβ binds to TGFβR2, which subsequently recruits two units of TGFβR1 and phosphorylates the serine residues in the serine-rich motif. Phosphorylation of the ligand-receptor complex leads to phosphorylation of receptor regulated SMADs (R-SMADs), which then form complexes with co-mediator SMADs (co-SMADs) (Tzavlaki and Moustakas, 2020). The homo-oligomeric or hetero-oligomeric SMAD complexes are translocated to the nucleus where they interact with DNA-binding co-factors and transcriptional coactivators or corepressors to regulate the expression of genes associated with differentiation, apoptosis and cell proliferation (Akhurst, 2017). TGFβ signalling can be attenuated by disruption of the TGFβ receptor, through targeting either the TGFβR1 or TGFβR2 for knockout.

5.2.2 The tumour microenvironment

The TME is a heterogenous population of infiltrating and resident host cells (immune, stromal and tumour cells), extracellular matrix, and secreted factors. The tumour niche presents multiple challenges to CAR T-cells, including hypoxic conditions, induction of
metabolic reprogramming and immunosuppressive signalling through checkpoint inhibitors.

The formation of the immune privileged tumour niche relies on the function of multiple cell types. Cancer-associated fibroblasts (CAFs) have been shown to secrete C-X-C motif chemokine ligand 12 and C-X-C receptor type 4, whilst tumour-associated macrophages secrete C-C motif chemokine ligands 17, 18 and 22, to recruit regulatory T-cells (Tregs). In addition to the activation of Tregs, myeloid-derived suppressor cells (MDSCs) can produce reactive oxygen species (ROS) and secrete anti-inflammatory cytokines such as IL-10 and TGFβ. TGFβ secretion induces angiogenesis, remodelling of the extracellular matrix (inducing fibrosis), and downregulates T-cell effector functions by signalling through the TGFβ receptor (Morgan and Schambach, 2018).

Tumours can exploit inhibitory T-cell signalling to induce exhaustion and to protect tumour cells from elimination. The key inhibitory receptor-ligand interactions include PD-1-programmed cell death ligand 1/2 (PD-L1/L2), cytotoxic T-lymphocyte antigen 4 (CTLA-4)-CD80/CD86, TIM3-Galectin-9/phosphatidylserine and LAG3-LECtin. The induction of exhaustion further upregulates PD-1, LAG3 and TIM3, and high expression of these receptors correlates with a poor prognosis in patients (Li et al., 2012; Wang et al., 2018). A promising strategy to improve cellular therapies is the blockade of the inhibitory receptors, which has restored the cytotoxic function and cytokine secretion of exhausted CD8+ T-cells.

5.2.3 Disruption of inhibitory receptors

5.2.3.1 Checkpoint blockade

Inhibitory receptors downregulate TCR signalling, and their blockade can restore T-cell effector functions. CTLA-4 is a homologue of CD28 and functions to inhibit T-cell activation by binding to CD80/CD86 with higher affinity. CTLA-4 and PD-1 have been proven as effective targets for checkpoint blockade by monoclonal antibodies (mAbs), and the most widely used PD-1 inhibitors include pembrolizumab and nivolumab (Prasad and Kaestner, 2017; Rotte, Jin and Lemaire, 2018; Patel et al., 2019). Studies have investigated the effect of combining inhibitors targeting multiple inhibitory pathways.
The combination of nivolumab and ipilimumab (inhibiting CTLA-4) resulted in an overall response rate (ORR) of 74% in Hodgkin lymphoma patients, although toxicity was significantly higher than nivolumab treatment alone (Armand et al., 2021). Patients refractory to PD-1 blockade have been found to upregulate TIM3 expression (Koyama et al., 2016), and the combination of anti-PD-1 and anti-TIM3 immunotherapies has improved therapeutic outcomes (Sakuishi et al., 2010; Fourcade et al., 2014). A recent study has identified binding of PD-1 to galectin-9, a known ligand of TIM3, which highlights galectin-9 as a promising target (Yang et al., 2021). Furthermore, clinical trials are evaluating mAbs that block the interaction of LAG3 with MHCII, whilst those targeting non-canonical ligands, such as FGL1 (binding partner of TIM3), are under investigation in research studies (Wang et al., 2019). Although checkpoint blockade provides modest tumour control, when administered in combination with CAR-T therapies, this can exacerbate toxicities.

5.2.3.1 Dominant negative approaches

Inhibition of TGFβ signalling can be achieved through the overexpression of a dominantnegative form of TGFβR2 (TGFβR2DN), which lacks the kinase signalling domain and competes with endogenous TGFβR2 for the cytokine (Wieser et al., 1993; Bollard et al., 2002; Kloss et al., 2018). A recent paper describing the development of an anti-prostate-specific membrane antigen (PSMA) CAR for the treatment of prostate cancer, showed that overexpression of TGFβR2DN was superior to disruption of the TGFβR2 gene, as the latter approach reduced the proliferative potential of the CAR T-cells (Narayan et al., 2022). While disruption of the TGFβR2 gene may negatively impact proliferation, there are ramifications to the overexpression of TGFβR2DN, which can sequester TGFβ, reduce levels of the cytokine in the serum, and potentially exacerbate the effects of cytokine release syndrome should it occur during CAR T-cell therapy (Narayan et al., 2022).

5.2.3.2 Gene knockout

An attractive alternative to checkpoint blockade is to disrupt the expression of inhibitory receptors in CAR T-cells using CRISPR/Cas9 or base editors (described in detail in
sections 1.3.1 and 1.3.3). Knockout of PD-1 by CRISPR/Cas9 has enhanced the function of CD19-targeted CAR T-cells (Rupp et al., 2017), whilst those directed to multiple myeloma have shown a 2-fold increase in the secretion of TNF and IFN-γ, compared to the non-edited control (Zhao et al., 2018). In a recent study, an anti-CD19 CAR was targeted to the PDCD1 gene by CRISPR/Cas9-mediated HDR and resulted in tumour elimination in B-cell non-Hodgkin lymphoma patients in the absence of significant toxicities (Zhang et al., 2022). Disruption of the PDCD1 gene by insertion of the anti-CD19 CAR expression cassette, consisting of the EF1α promoter, CAR coding sequence and SV40 poly(A) signal sequence, resulted in the production of CAR T-cells that were resistant to PD-L1 and maintained the ability to lyse CD19+ target cells bearing the ligand. Furthermore, these gene edited cells were found to be predominantly CD8+ memory T-cells, which were able to rapidly proliferate in response to antigen, and the advantage of this was borne out in the results of the clinical trial. The initial manufacturing process used for generating the engineered CAR T-cells was inefficient and patients could only be infused with low doses (0.56x10⁶ CAR T-cells/kg); despite this, all patients receiving limited doses of CAR T-cells exhibited complete responses, suggesting the memory CAR T-cells were able to expand in vivo and effectively eliminate the tumour cells (Zhang et al., 2022).

PD-1 disruption by CRISPR/Cas9 has been largely evaluated in the allogenic setting (section 1.3.1.2), where multiplexed editing is required to knockout the endogenous TCR and MHC molecules alongside PD-1 (X. Liu et al., 2017; Ren, Liu, et al., 2017). Other studies have disrupted the TGFβ receptor, which has improved the function of anti-mesothelin CAR T-cells by reducing Treg conversion and preventing exhaustion (Tang et al., 2020).

Due to the safety concerns with generating multiple DSBs, an increasing number of studies are investigating base editing to attenuate protein expression by introducing point mutations into the gene. Allogeneic CAR-T approaches have benefited from the base editing of inhibitory receptors. Webber et al., report the disruption of PDCD1 in CD19 CAR T-cells (Webber et al., 2019), and Diorio et al., describe the development of the first CAR T-cell therapy (BEAM-201) to include four simultaneous genetic edits by base
editing (Diorio et al., 2022). CD7 expression was disrupted to prevent fratricide of the T-cell product following lentiviral transduction with a CD7-specific CAR. The TRAC locus was silenced to prevent GvHD, knockout of PDCD1 was included to improve in vivo persistence, and the fourth gene target, CD52, was silenced to enable preconditioning with an anti-CD52 monoclonal antibody, alemtuzumab. This study did not disrupt the expression of HLA class molecules, as BEAM-201 aims to bridge treatment to curative allogeneic stem cell transplantation. Approximately 93% of T-cells contained all four genomic edits and multiplexed genome editing did not induce chromosomal translocations. Compared to CRISPR/Cas9 editing, the base editing protocol maintained high cell viabilities, improved proliferation, as well as optimised CAR T-cell yields (Diorio et al., 2022). This is likely due to the reduction in the DNA damage response and associated genotoxicity, therefore improving cellular fitness. These studies demonstrate the potential application of base conversion to enhance the performance of autologous therapies, where base editing is in its infancy.

5.2.4 Targets for gene disruption by base editing

This study investigates base editing to generate an autologous CD30-targeted CAR-T product, that can resist the inhibitory signals of the TME. The targets chosen for disruption include PD-1 (PDCD1), TIM3 (HAVCR2), LAG3 (LAG3) and TGFβR2 (TGFβR2).
5.3 Chapter Aims

This chapter will describe the application of circRNA for the transient expression of cytosine base editors in primary T-cells. Our previous work demonstrated that circRNA is more stable than linear mRNA, and we hypothesise that circRNA could be beneficial for base editing purposes, by either increasing the expression of base editors or reducing the amount of RNA required to produce gene edited CAR T-cells. The work in this thesis will contribute towards the development of a GMP-compliant manufacturing process for the generation of a genome-edited anti-CD30 CAR T-cell product, for the treatment of classical Hodgkin lymphoma (cHL). This chapter aims to:

5.3.1 Identify optimal sgRNAs for efficient gene disruption of *PDCD1*, *HAVCR2*, *LAG3* and *TGFβR2*.

5.3.2 Develop a multiplexed genome engineering protocol using circRNA-encoding BE4max.

5.3.3 Determine the amount of circRNA required for the manufacture of base-edited CAR T-cells.
5.4 Guide RNAs for efficient gene disruption

5.4.1 Design strategy

It has previously been shown that gene knockout can be facilitated using RNA-guided endonucleases and targeting single guide RNAs (sgRNAs). In the case of base editing, the sgRNAs direct the base editor to a target site, containing the appropriate nucleotide, to install a point mutation to disrupt gene expression (see sections 1.3.3.1 and 1.3.3.2.2). There are two widely adopted ways of disrupting gene expression: introducing mutations into mRNA splice donor (SD) or splice acceptor (SA) sites (Gapinske et al., 2018; Webber et al., 2019) or converting a codon to a premature stop codon (pmSTOP) (Billon et al., 2017; Kuscu et al., 2017). Webber et al., demonstrate that sgRNAs targeting SD sites have superior knockout efficiencies compared to those that target SA sites (which can result in exon skipping) or those that introduce pmSTOP codons (Webber et al., 2019). It is not always possible to target a SD in a particular gene due to the dependencies of the BE enzyme, including the restriction of the NGG PAM and the BE activity window (BE4max has a BE window of C4-C8). An alternative way of perturbing gene expression is to mutate key functional amino acids. Such an approach was taken to disrupt PD-1 by targeting N-linked glycosylation sites, which resulted in decreased cell surface expression of the inhibitory receptor (Shi et al., 2019). It is important to screen multiple sgRNAs to optimise gene disruption.

The sgRNAs shown in Figure 5.2 (PDCD1), Figure 5.3 (HAVCR2), Figure 5.4 (LAG3) and Figure 5.5 (TGFβR2) show the sgRNA target sites on the respective genes and the desired editing outcomes. The BE-Designer web tool (http://www.rgenome.net/be-designer/) was used to design sgRNAs to introduce stop codons into the sense strand of the gene by identifying point mutations that result in TAA, TGA or TAG outcomes (encoding UAA, UGA, UAG) (Webber et al., 2019). While SpliceR (https://z.umn.edu/splicer) was used to identify sgRNAs that disrupt mRNA splicing sites (Hwang et al., 2018; Webber et al., 2019). The SD site comprises the GT sequence at the 5’ end of the intron whilst the SA site is defined by the AG sequence at the 3’ end. The CBE must be directed to the cytosine occurring on the antisense strand of the gene to
generate a G > A conversion on the sense strand, converting the SD from GT to AT and the SA from AG to AA.

![Diagram of PDCD1 gene knock out](image)

**Figure 5.2 sgRNAs designed for gene knockout of PDCD1 using BE4max.**

sgRNAs were designed using SpliceR or the CRISPR RGEN BE-Designer Tool are compatible with cytosine base editors that use the SpCas9 NGG PAM variant. The target base within the target sequence is shown in red and potential by-stander cytosines are shown in orange. Two sgRNAs were previously described by (Webber et al., 2019). The optimal sgRNAs are highlighted in green. Premature stop codon (pmSTOP), splice donor (SD), splice acceptor (SA).
Figure 5.3 sgRNAs designed for gene knockout of HAVCR2 using BE4max.

sgRNAs were designed using SpliceR or the CRISPR RGEN BE-Designer Tool are compatible with cytosine base editors that use the SpCas9 NGG PAM variant. The target base within the target sequence is shown in red and potential by-stander cytosines are shown in orange. The optimal sgRNA is highlighted in green. Premature stop codon (pmSTOP), splice donor (SD), splice acceptor (SA).
Figure 5.4 sgRNAs designed for gene knockout of LAG3 using BE4max.

sgRNAs were designed using SpliceR or the CRISPR RGEN BE-Designer Tool are compatible with cytosine base editors that use the SpCas9 NGG PAM variant. The target base within the target sequence is shown in red and potential by-stander cytosines are shown in orange. The optimal sgRNAs are highlighted in green. Premature stop codon (pmSTOP), splice acceptor (SA).
Figure 5.5 sgRNAs designed for gene knockout of TGFβR2 using BE4max.

sgRNAs were designed using SpliceR or the CRISPR RGEN BE-Designer Tool are compatible with cytosine base editors that use the SpCas9 NGG PAM variant. The target base within the guide RNA sequence is shown in red and potential by-stander cytosines are shown in orange. The optimal sgRNAs are highlighted in green. Premature stop codon (pmSTOP), splice donor (SD).

5.4.2 Initial screening of PD-1 and TIM3 sgRNAs in cell lines

To establish a base editing protocol, sgRNAs targeting PDCD1 and HAVCR2 were first screened in commercially available cell lines that upregulate the expression of target genes, prior to validating the selected sgRNAs in stimulated primary T-cells. We found MOLT-4 cells to upregulate PD-1 expression following phorbol myristate acetate (PMA) and ionomycin treatment, whilst RPMI-8226 cells were found to constitutively express TIM3 (Appendix, Figure 8.3). Cell lines were engineered to stably express a fourth-generation CBE, BE4max, by retroviral transduction (method 2.2.2.3.5). As shown in Figure 5.6, a V5 transduction marker was incorporated upstream of the BE4max sequence.
to identify the transduced population (transduction efficiencies are shown in Appendix, Figure 8.4).

**Figure 5.6 Structure of the BE4max retroviral construct.**

Diagram of the BE4max construct used to produce retroviral supernatant. The construct consists of a 5-prime long terminal repeat (LTR) followed by the packaging signal of Moloney murine leukaemia virus (Ψ), a T-cell receptor beta variable signal peptide (SP), a V5 tag positioned on a CD8 stalk (8STK), a 2A self-cleaving peptide sequence (T2A), a SV40 nuclear localisation signal (NLS), and the BE4max base editor sequence. The BE4max sequence consists of the APOBEC1 deaminase, Cas9 (D10A) and two copies of the uracil glycosylase inhibitor domains (UGI). Downstream of the sequence is a second NLS and a scaffold attachment region (SAR), upstream of the 3’ LTR.

The eight PD-1 and four TIM3-targeting sgRNAs were screened by nucleofection, where 30 pmol sgRNA was delivered to 1x10⁶ cells using the optimised pulse codes (method 2.2.2.4.1) (Appendix, Figure 8.5 and Figure 8.6). Following appropriate re-stimulation, the loss of PD-1 and TIM3 expression was observed in the transduced population. The optimal PD-1 sgRNA targeted the SD site of E1 and was as previously described (Webber et al., 2019), whilst the optimal TIM3 sgRNA targeted the SA site of E2 (indicated in green on the sgRNA maps, Figure 5.2, Figure 5.3). As target gene expression was heterogenous, the knockout efficiency was normalised to the percentage of cells expressing the target gene in the non-targeting (NT) sgRNA sample. The optimal sgRNAs resulted in up to 80% and 60% gene knockout of *PDCD1* and *HAVCR2*, respectively (Appendix, Figure 8.5, Figure 8.6). This work validated the function of BE4max CBE and highlighted sgRNAs for investigation in primary T-cells.
5.5 *PDCD1* and *HAVCR2* disruption using circRNA-encoding BE4max

Two key readouts were used to determine the efficiency of base editing: loss of protein expression by flow-cytometry and the percentage of base conversion in the genome. To measure protein loss, we first identified a method to upregulate gene expression in primary T-cells using various stimulation cocktails.

5.5.1 Upregulation of target gene expression following stimulation

To validate the target gene expression, PBMCs were stimulated for 72h in complete TexMACS™ supplemented with 10 ng/mL of IL-7 and IL-15 with the addition of either TransAct™ (1:100, research grade) with or without 20 ng/mL interferon-γ (IFN-γ), 20 ng/mL IFN-γ alone, 10 ng/mL phytohemagglutinin (PHA) or PMA and ionomycin (40.5 µM PMA and 669.3 µM ionomycin). A non-activated control was included where PBMCs were cultured in complete TexMACS™ containing IL-7/IL-15. Compared to the non-activated control, PD-1 and TIM3 expression was upregulated under all conditions except when cytokines were administered alone (Figure 5.7). We concluded that TransAct™ stimulation was the most effective at driving gene expression and proceeded to develop a base editing protocol using circRNA-encoding BE4max.
Figure 5.7 Upregulation of target gene expression in stimulated T-cells.

(a) Upregulation of PD-1 or (b) TIM3 expression in T-cells following 72h of stimulation. PBMCs were cultured in complete TexMACS™ supplemented with 10 ng/mL of IL-7 and IL-15 and were either left non-activated (NA) or were stimulated using TransAct™ (TA) with/without IFN-γ, PHA or PMA and ionomycin (P+I). Supplementation with dimethyl sulfoxide (DMSO) was included as a negative control. Flow cytometry plots are shown for one representative donor and the percentage of expression is shown below, represented as mean ± SD (n=6 independent biological donors, represented as different symbols). (Two-Way ANOVA; **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).

### 5.5.2 Production of BE4max circRNA

The coding sequence of the cytosine base editor BE4max was cloned into *Anabaena* PIE sequence to facilitate the production of circRNA (Wesselhoeft, Kowalski and Anderson, 2018). RNA was prepared by *in vitro* transcription using either Invitrogen’s mMESSAGE mMACHINE™ T7 Transcription Kit or NEB’s HiScribe™ T7 Quick High Yield RNA Synthesis Kit (method 2.2.1.3). Synthesised RNA was purified by either using a column-
based method, MEGAclear™ Transcription Clean-Up Kit, or by lithium chloride (LiCl) precipitation. The chosen method for RNA synthesis was the HiScribe™ T7 Quick High Yield kit and subsequent LiCl precipitation, as the yield and quality of the RNA was improved compared to the mMESSAGE mMACHINE™ T7 Transcription Kit and MEGAclear™ Transcription Clean-Up Kit (Appendix, Figure 8.7). Circularisation was performed by resuspending RNA in a buffer containing magnesium ions and GTP, and heating the RNA to 55ºC for 15 minutes (Wesselhoeft, Kowalski and Anderson, 2018). After purification, RNA was analysed by gel electrophoresis using the Agilent TapeStation. Full-length CBE RNA was observed at 6.5 kB in length and circularisation was confirmed by release of the intron sequences shown as 181 and 624 bases. After circularisation, some linear RNA remains, but it is estimated that approximately 75% of the RNA is circular (Figure 5.8).

Figure 5.8 Production of BE4max cytosine base editor circular RNA.

(a) Diagram of the BE4max construct for in vitro transcription. The circRNA template was designed based on the Anabaena permuted-intron exon (PIE) sequence. The BE4max sequence
consists of the APOBEC1 deaminase, Cas9 nickase (D10A) and two copies of the uracil glycosylase inhibitor domains (UGI). (b) Production of circular base editor RNA using the HiScribe™ RNA Synthesis Kit and lithium chloride precipitation. Following the back-splicing reaction, circularisation was confirmed by the identification of the intronic sequences on the TapeStation gel. (c) Efficiency of circularisation calculated as a percentage of full-length RNA.

5.5.3 PD-1 and TIM3 base conversion efficiencies in stimulated T-cells

A base editing protocol was developed using PD-1 sgRNA-2 (Webber E1 SD) and TIM3 sgRNA-1 (SPICER E2 SA) sequences, as selected from the sgRNA screen in MOLT-4 and RPMI-8226 cell lines. The protocol aimed to closely follow the CAR T-cell manufacturing process to enable efficient technology transfer. PBMCs were thawed and allowed to rest overnight in complete TexMACS™, prior to stimulation with TransAct™ and IL-7/15 for 48h. Stimulated T-cells were nucleofected with 30 pmol sgRNA and 4 μg of the CBE circRNA per 1x10^6 cells (method 2.2.2.5.3). As a positive control to aid flow-cytometry gating, non-targeting and targeting Cas9 RNPs were formulated by combining 150 pmol sgRNA with 50 pmol Alt-R® HiFi Cas9 endonuclease in PBS prior to nucleofection (method 2.2.2.5.2). *PDCD1* sgRNA-1 (RGEN E1 pmSTOP) and *HAVCR2* sgRNA-2 (RGEN E2 pmSTOP) were chosen to direct Cas9-mediated DSBs to exons, to disrupt protein expression by the generation of indels. Following nucleofection, cells were expanded for a period of 4 days before re-stimulation with TransAct™ to upregulate target gene expression, for detection of gene knockouts by flow-cytometry 24h later (Figure 5.9).
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Figure 5.9 Process workflow for base editing of stimulated T-cells.

The generation of base edited T-cell products begins with *in vitro* transcription of base editor-encoding RNA and commercial sgRNA synthesis, which can be performed concurrently with the isolation and activation of PBMCs. Base editor RNA is delivered to T-cells along with the sgRNA by nucleofection using Lonza’s 4D-Nucleofector™ device. Conventional studies use linear BE4max mRNA, here we investigate the use of circular RNA. Following recovery post-nucleofection, the edited cells are expanded for a period of 4 days prior to re-stimulation of half of the cells. This will enable the detection of gene knockouts by flow-cytometry 24h later. The non-activated cells show the constitutive gene expression and serve as a negative control for flow-cytometry gating.

At day 5 post-nucleofection, PD-1 was expressed in 9.0-14.8% of non-activated samples and in 56.5-68.3% of activated samples of one representative donor (Figure 5.10). Base editing using circRNA and sgRNA-2 or CRISPR/Cas9 gene disruption reduced PD-1 expression in activated samples to 21.4% and 16.6%, respectively (Figure 5.10a). On average, PD-1 expression was reduced from 63.5% in the NT sgRNA sample to 17% (p = 0.0002), resulting in an editing efficiency of 73.2%, whilst Cas9 RNPs resulted in a
protein loss from 61.6% to 23.7% (p = 0.0011) and an average editing efficiency of 61.6% (Figure 5.10b).

Figure 5.10 Phenotyping T-cells for base editing of PDCD1.

Base editing of stimulated T-cells using the sgRNA that targets the SD site of E1 in PDCD1. Four days after co-transfection of the sgRNA and circRNA-encoding BE4max, half of the cells were re-stimulated with TransAct™ and IL-7/IL-15 to identify gene disruption. (a) Flow-cytometry plots (FSC-A versus PD-1-PE) at 24h after re-stimulation, one representative donor
is shown. Non-activated cells served as a negative control and show the basal level of gene expression. (b) PD-1 expression in re-stimulated cells at day 5 post-nucleofection, represented as mean ± SD (n=5 biological donors from two independent experiments, donors are represented as different symbols). (Two-Way ANOVA; **p ≤ 0.01, ***p ≤ 0.001).

In contrast to the heterogenous expression of PD-1, TIM3 was highly expressed in non-activated T-cells (64.5-77.1%) and was upregulated to 81.3-89.0% expression following 24h of TransAct™ stimulation. The HAVCR2 sgRNA sequence targets the SA site of E2 and co-transfection with CBE-encoding circRNA reduced TIM3 expression from 86.4% to 22.8% in one representative donor, compared to 81.3% to 14.0% with the Cas9 RNPs (Figure 5.11a). TIM3 knockout efficiency averaged at 64% and the expression was reduced from 89% to 32% (p < 0.0001). Using Cas9 RNPs, protein expression was reduced from 88% to 45.8% (48% editing efficiency (p < 0.0001) (Figure 5.11b).
Figure 5.11 Phenotyping T-cells for base editing of HAVCR2.

Base editing of stimulated T-cells using the sgRNA that targets the SA site of E2 in HAVCR2. Four days after co-transfection of the sgRNA and circRNA-encoding BE4max, half of the cells were re-stimulated with TransAct™ and IL-7/IL-15 to identify gene disruption. (a) Flow-cytometry plots (FSC-A versus TIM3-BV421) at 24h after re-stimulation, one representative donor is shown. Non-activated cells served as a negative control and show the basal level of gene expression. (b) TIM3 expression in re-stimulated cells at day 5 post-nucleofection, represented
as mean ± SD (n=8 biological donors from four independent experiments, donors are represented as different symbols). (Two-Way ANOVA; ****p < 0.0001).

To confirm base conversion at the genomic level, genomic DNA was extracted from the non-targeting, PD-1 or TIM3 base-edited samples at day 7 post-nucleofection. An 800-bp region spanning the target site was amplified by PCR, Sanger sequenced and analysed by EditR to determine editing efficiencies (method 2.2.2.5.4) (Hwang et al., 2018; Kluesner et al., 2018). The BE4max base editing window lies between positions 4 and 8 inclusive, thus, any cytosines that occur within this region can be subject to deamination by APOPBEC-1. Base conversions in addition to on-target indels are termed by-stander edits.

The PDCD1 sgRNA-2 sequence is complementary to the antisense strand and targets the cytosine at position 7 within the protospacer, thereby resulting in a G•C > A•T conversion on the sense strand and disruption of the SD site of E1 (GT > AT). Figure 5.12a shows the percentage base conversion for the PD-1 edited sample of one representative donor. An on-target G > A conversion was observed at 57% frequency and 13% of the population contained a by-stander edit at position 8. When analysed against the non-edited sequencing trace, indels were present only in the target edited sample, suggesting there was no gRNA-independent deamination (Figure 5.12b). As shown in Figure 5.12c, the average on-target editing efficiency was 74.5% and ranged between 35-100%. The most frequent by-stander edit occurred at position 8 and minimal transversions were detected in the protospacer sequence.
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Figure 5.12 Genotyping T-cells for base editing of PDCD1.

Genotyping base-edited T-cells at 7 days post-engineering. A region spanning E1 of the PDCD1 gene was amplified from the genomic DNA, Sanger sequenced and analysed using EditR. (a) EditR analysis showing 57% G > A base conversion at the target guanine and 13% G > A by-stander editing, in one representative donor. (b) The PDCD1 sgRNA is complementary to the sense strand (targeting the SD site of E1). The sequencing traces of the non-edited and edited samples were aligned to the target gene in SnapGene. The appearance of the adenine peaks
(green) on the sense strand demonstrates successful base editing in the polyclonal population.  
(c) Antisense base conversion efficiencies for all cytosines in the protospacer, represented as mean ± SD (n=5 biological donors from two independent experiments), with the target cytosine highlighted in red.

To disrupt the SA site of E2, the HAVCR2 sgRNA-1 sequence targets the cytosine at position 6 in the antisense orientation, to mutate the sequence from AG to AA (G•C > A•T editing) on the sense strand. In one representative donor, the EditR analysis revealed an on-target G > A conversion frequency of 63%, whilst by-stander edits at positions 4 and 5 occurred at 37% and 58% frequency, respectively. This donor was found to contain a G•C > T•A mutation at position 5, occurring at 4% frequency (Figure 5.13). Visual analysis of the sequencing traces in SnapGene was performed by aligning the non-edited and edited sequencing traces to the sequence of the HAVCR2 gene and confirmed editing by the appearance of the adenine peaks and a reduction in the guanine frequency (Figure 5.13b). The TIM3 sgRNA-1 sequence resulted in an on-target editing efficiency of 76%, which ranged between 45-100%. We found >99% on-target base conversion in half of the donors. By-stander edits occurred at positions 4 and 5 with 50-100% frequency (Figure 5.13c).
Figure 5.13 Genotyping T-cells for base editing of HAVCR2.

Genotyping base-edited T-cells at 7 days post-engineering. A region spanning E2 of the HAVCR2 gene was amplified from the genomic DNA, Sanger sequenced and analysed using EditR. (a) EditR analysis showed 63% G > A base conversion at the target guanine and 58% and 37% G > A by-stander editing, in one representative donor. (b) The HAVCR2 sgRNA is complementary to the sense strand (targeting the SA site of E2). The sequencing traces were aligned to the target gene in SnapGene. The appearance of the adenine peaks (green) on the sense
strand demonstrates successful base editing in the polyclonal population. (c) Base conversion efficiencies for all cytosines in the protospacer, represented as mean ± SD \((n=8\) biological donors from four independent experiments), with the target cytosine highlighted in red.

Following screening of the sgRNA sequences in cell lines, we validated the chosen PD-1 sgRNA and TIM3 sgRNAs, targeting the SD site of E1 in \(PDCD1\) and SA site of E2 in \(HAVCR2\), in stimulated primary T-cells using circular CBE RNA. To summarise, the PD-1 sgRNA resulted in an average protein loss of 73.2% efficiency, and on-target base conversion of 74.5%. The TIM3 sgRNA provided an editing efficiency of 64% (protein loss) and 76.5% base conversion at the genomic level.

### 5.6 LAG3 disruption using circRNA-encoding BE4max

Next, we set out to screen sgRNAs targeting LAG3 using our validated circRNA approach. Here, seven sgRNAs were screened in stimulated T-cells following the same method as above (method 2.2.2.5.3). As shown in Figure 5.14a, LAG3 expression was upregulated after re-stimulation to 73.8-84.1% in the control samples of one representative donor (no pulse, no RNA, NT sgRNA, NT RNP). Protein loss varied between sgRNAs and LAG3 expression was reduced to 6.51% (sgRNA-3), 6.79% (sgRNA-5) and 6.83% (sgRNA-7) using the most optimal sequences, the sgRNAs -2 and -6 did not facilitate protein knockout. The Cas9 RNP control reduced protein expression to 8.62% using sgRNA-1. An average of five independent donors demonstrated a reduction in LAG3 expression from 67% in the NT gRNA control down to 20% and 14% using sgRNA-3 \((p = 0.0205)\) and sgRNA-5 \((p = 0.0078)\) that introduce pmSTOP codons into E3 and E4 respectively, and 19% using sgRNA-7 \((p = 0.0173)\) that disrupts the SA site of E7 (Figure 5.14c).
Figure 5.14 Screening guide RNAs for base editing of LAG3 using circular base editor RNA.

Base editing of stimulated T-cells using sgRNAs that disrupt SA sites or to introduce pmSTOP codons into the LAG3 gene. Four days after co-transfection of the sgRNA and circRNA-encoding BE4max, half of the cells were re-stimulated with TransAct™ and IL-7/IL-15 to identify gene disruption. (a) Flow-cytometry plots (FSC-A versus LAG3-BV421) at 24h after re-stimulation, one representative donor is shown. (b) Table displaying the single guide RNA sequence names. (c) LAG3 expression in re-stimulated cells at day 5 post-nucleofection,
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represented as mean ± SD (n=5 biological donors from two independent experiments, donors are represented as different symbols). (Two-Way ANOVA; *p ≤ 0.05, **p ≤ 0.01).

Genomic DNA was extracted on day 7 post-nucleofection and an 800 bp region was amplified spanning each target site for analysis by Sanger sequencing and EditR (method 2.2.2.5.4). On-target editing efficiencies are shown in Figure 5.15 (top left), sgRNAs -3 and -5 mediated 79.4% and 83.2% C > T conversion on the sense strand and sgRNA-7 resulted in 79.6% conversion on the antisense strand. Whilst sgRNA-2 did not disrupt the expression of LAG3, sgRNA-6 facilitated an on-target edit with 74.6% efficiency, however, while editing was observed at the genomic level, protein loss did not occur and LAG3 was still detectable at the cell surface. By-stander editing was observed for all sgRNA sequences at high frequencies. Where the target base is adjacent to a cytosine, both cytosines are efficiently converted. The sgRNA-5 sequence contains four sequential cytosines in the BE window and base conversion occurs at 53%, 86%, 85.6% and 83.2% efficiency for cytosines at positions 5 to 8. The cytosines at positions 6, 7 and 8 were converted with 98-100% efficiency in four out of five donors.

Our screening highlighted sgRNAs -3, -5, and -7 as the most efficient sequences for targeting LAG3. Protein loss was achieved at 70.2%, 79.2% and 71.1% efficiency, respectively, and all three sgRNAs resulted in at least 79% C > T conversion.
Figure 5.15 Genotyping T-cells for base editing of LAG3 using circular BE4max-encoding RNA.
Genotyping base-edited T-cells at 7 days post-engineering. A region spanning each target site was amplified from the genomic DNA, Sanger sequenced and analysed using EditR software. Base conversion efficiencies are shown for all cytosines in the protospacer, represented as mean ± SD (n=5 biological donors from two independent experiments), with the target cytosine highlighted in red. The most optimal guide RNAs were sgRNA-3, sgRNA-5, and sgRNA-7.

### 5.7 TGFβR2 disruption using circRNA-encoding BE4max

Following the identification of optimal LAG3 sgRNAs, we screened seven TGFβR2 sgRNAs in stimulated PBMCs. As TGFβ receptor expression can be challenging to identify by flow-cytometry, gene knockouts were verified at the genomic level (using method 2.2.2.5.4). As shown in Figure 5.16, four sgRNAs successfully installed point mutations at the target site within the TGFβR2 sequence, two of which target the SD site of E2. The sgRNA-2 sequence facilitated an average editing efficiency of 66.8% at C5 with 100% C > T conversion observed in three out of five donors; however, by-stander editing was found outside of the BE window at C11 (50.4%) and C13 (3.6%). The sgRNA-3 sequence facilitated on-target cytosine conversion at 79.3% efficiency (ranging from 22% in one donor to 74-100% in four donors) and no by-stander effects were identified. The sgRNAs -4 and -6 introduced TAG stop codons into E3 and E5 with 69% and 71.6% efficiency, respectively. Some sgRNA sequences failed to induce mutations in TGFβR2, including sgRNAs -1, -5, and -7 where the target cytosine occurs on the edge of the BE window. This screen highlighted four potential sgRNAs (sgRNA-2, -3, -4 and -6) for disruption of TGFβR2.
Figure 5.16 Genotyping T-cells for base editing of TGFβR2 using circular BE4max-encoding RNA.
Genotyping base-edited T-cells at 7 days post-engineering. A region spanning each target site was amplified from genomic DNA, Sanger sequenced and analysed using EditR software. Base conversion efficiencies are shown for all cytosines in the protospacer, represented as mean ± SD (n=5 biological donors from two independent experiments), with the target cytosine highlighted in red. The most optimal guide RNAs were sgRNA-2, sgRNA-3, sgRNA-4, and sgRNA-6.

We have designed and screened sgRNAs to knock out the expression of four inhibitory receptors by splice site disruption or the introduction of pmSTOP codons. The validated sgRNAs are PD-1 sgRNA-2 (Webber E1 SD) (*PDCD1*), TIM3 sgRNA-1 (SPLICER E2 SA) (*HAVCR2*), LAG3 sgRNAs -3 (RGEN E3 pmSTOP 02), -5 (RGEN E4 pmSTOP 02), and -7 (SPLICER E7 SA) (*LAG3*) and TGFβR2 sgRNAs -2 (SPLICER E2 SD 01), -3 (SPLICER E2 SD 02), -4 (RGEN E3 pmSTOP), and -6 (RGEN E5 pmSTOP 02) (*TGFβR2*).

### 5.8 Multiplexed gene disruption of T-cell inhibitory receptors

To develop a multiplexed base editing process using circRNA-encoding BE4max, we used four sgRNAs that were previously validated: PD-1 sgRNA-2 (Webber E1 SD) (*PDCD1*), TIM3 sgRNA-1 (SPLICER E2 SA) (*HAVCR2*), LAG3 sgRNA-5 (RGEN E4 pmSTOP 02) and TGFβR2 sgRNA-2 (SPLICER E2 SD 01). Although multiple sgRNAs facilitated highly efficient LAG3 and TGFβR2 editing, we chose LAG3 sgRNA-5 as it resulted in the greatest reduction in protein expression. SD site targeting can result in more efficient gene disruption compared to the introduction of pmSTOP codons, which can lead to translational readthrough (Webber *et al.*, 2019), hence, we selected the TGFβR2 sgRNA-2 that targets the SD of E2 at the 5’ end of the gene.

Following the optimised process, stimulated T-cells were nucleofected with 30 pmol of each sgRNA and 4 µg of circular BE4max (method 2.2.2.5.3). Figure 5.17 shows the expression of PD-1, TIM3 and LAG3 following 24h of re-stimulation to upregulate target gene expression (day 5 post-nucleofection). TGFβR2 expression was not investigated due to its high turnover on the cell surface. Flow-cytometry plots from one representative donor demonstrate efficient multiplexed editing of all genomic targets. The expression of PD-1 was heterogenous (36.4-40.9% in non-edited samples) and was reduced to 6.7% in
the multiplexed edited sample, compared to 13.4% with the Cas9 RNP control (Figure 5.17b). TIM3 expression was reduced from 81.3-89.3% in control samples to 21% in the multiplexed sample and was approximately 2-fold more efficient than editing with the RNP (Figure 5.17c). LAG3 expression varied between 64.2-69.6% in control samples and multiplexed editing reduced expression to 18% with circRNA compared to 10.2% with the Cas9 control (Figure 5.17d).

Figure 5.18 shows a graphical representation of the multiplexed editing results. On average, PD-1 expression was reduced from 63.5% to 20.5% ($p = 0.0026$), TIM3 expression was reduced from 86% to 32% ($p < 0.0001$) and LAG3 expression was reduced from 71% to 27% ($p < 0.0001$) (Figure 5.18a). To account for the heterogenous expression of target genes, percentage protein loss was calculated and resulted in 67.8%, 62.8% and 62%, PD-1, TIM3 and LAG3 protein loss, respectively. To assess the editing efficiencies at the genomic level, EditR analysis carried out on day 7 samples (method 2.2.2.5.4). In the multiplexed edited sample, average on target C > T point mutation frequencies were 82% for $PDCD1$, 73% for $HAVCR2$, 60% for $LAG3$ and 74% for $TGFβR2$. By-stander editing was observed at the $PDCD1$, $HAVCR2$ and $LAG3$ target sites with varying efficiency (Figure 5.18b). This work validates the use of circRNA to multiplex the disruption of four inhibitory receptors in T-cells.
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a. Diagrams showing the design of sgRNAs targeting different genes.

b. Flow cytometry analysis comparing effects of No Pulse and No RNA treatments with NT sgRNA and Multi sgRNAs.

c. Scatter plots illustrating the expression levels of TIM3 under different conditions.

d. Similar analysis as in (b) for LAG3.
Figure 5.17 Phenotyping T-cells for multiplexed base editing of PDCD1, HAVCR2, LAG3 and TGFβR2 using circular BE4max RNA.

Base editing of stimulated T-cells using multiplexed sgRNAs that target the inhibitory receptors PD-1, TIM3, LAG3 and TGFβR2. 4 days after co-transfection of the sgRNA and circRNA-encoding BE4max, half of the cells were re-stimulated with TransAct™ and IL-7/IL-15 to identify gene disruption. (a) Diagram of sgRNAs for co-transfection with BE4max. (b)-(d) Flow-cytometry plots (FSC-A versus PD-1-PE, TIM3-BV421 or LAG3-BV421) to demonstrate inhibitory receptor expression at 24h after re-stimulation, one representative donor is shown. Flow-cytometry was not performed for TGFβR2 due the low sensitivity of the detection method.
Figure 5.18 Efficient multiplexed base editing of *PDCD1*, *HAVCR2*, *LAG3* and *TGFβR2* using circular BE4max-encoding RNA.
Base editing of stimulated T-cells using multiplexed sgRNAs that target the inhibitory receptors PD-1, TIM3, LAG3 and TGFβFR2. (a) Table of sgRNAs for co-transfection with BE4max (top left). Percentage PD-1 (top right), TIM3 (bottom left) and LAG3 (bottom right) expression in re-stimulated samples at day 5 post-nucleofection, represented as mean ± SD (n=5 biological donors from two independent experiments, donors are represented as different symbols). (b) Genotyping base-edited T-cells at 7 days post-engineering. Base conversion efficiencies are shown for all cytosines in the protospacer, represented as mean ± SD (n=5 biological donors), with the target cytosine highlighted in red.

5.9 CircRNA outperforms linear CBE-encoding mRNA

The multiplexed genome editing protocol was validated using 4 µg circRNA, consistent with a published base-edited CAR T-cell study (Webber et al., 2019). The previous chapter demonstrated the improved half-life of circRNA and increased duration of transgene expression. To address the hypothesis that circRNA delivery would result in greater BE4max protein expression, we titrated the amount of circRNA in comparison to linear mRNA. HAVCR2 was selected for genome knockout, as TIM3 expression is highly upregulated in stimulated T-cells.

Firstly, linear and circRNA encoding BE4max was produced by in vitro transcription (method 2.2.1.3). To generate linear mRNA, the BE4max sequence was amplified by PCR (from pCMV_BE4max, Addgene #112093) using a forward primer that annealed to the T7 promoter and a reverse primer that introduced a polyadenylation tail of 120 bases. RNA was synthesised from 1 µg PCR product using the HiScribe™ T7 High Yield RNA Synthesis Kit and was subsequently capped and 2’-O-methylated using NEB’s Vaccinia Capping Enzyme and mRNA Cap 2’-O-Methyltransferase. Capped and polyadenylated RNA was purified by LiCl precipitation and was analysed by gel electrophoresis using the Agilent TapeStation. As shown in Figure 5.19, full-length base editor RNA was observed at 6.3 kB in length. CircRNA was produced as described in section 5.5.2.
Figure 5.19 Production of BE4max cytosine base editor messenger RNA.

(a) Diagram of BE4max capped and polyadenylated messenger RNA. The BE4max sequence consists of the APOBEC1 deaminase, Cas9 (D10A) and two copies of the uracil glycosylase inhibitor domains (UGI) and is flanked by simian virus 40 (SV40) nuclear localisation sequences (NLS). The 5’ and 3’ untranslated regions (UTRs) were included to improve RNA stability and the His tag at the 3’ end enables detection (derived from pCMV_BE4max Addgene #112093).

(b) Production of capped and polyadenylated linear base editor RNA using the HiScribe™ RNA Synthesis Kit and lithium chloride precipitation.

To compare circRNA to linear mRNA, 1x10^6 stimulated PBMCs were transfected with 30 pmol NT or HAVCR2 sgRNA and 0.25 µg-4.00 µg BE4max RNA (method 2.2.2.5.3). Figure 5.20a shows TIM3 expression at day 5 post-nucleofection in one representative donor. In the control samples, TIM3 was upregulated to 77.9%-84.4%, and in edited samples 4.00 µg linear RNA facilitated a reduction in protein expression to 30.7% compared to 12.6% with circRNA (64% and 84.5% knockout efficiencies, respectively). Using 0.25 µg, an amount 16-fold lower than that of published studies, editing using linear and circular RNAs reduced expression to 52.3% and 19.9%, equivalent to 35% and 75% editing. As shown in Figure 5.20b (left), circRNA significantly outperformed linear RNA at 0.25 µg (p = 0.0067) and 0.50 µg (p = 0.0047). On average, 0.50 µg circRNA
reduced TIM3 expression to 19% (75% knockout efficiency) and increasing the RNA amount to 1.00 µg and 2.00 µg reduced expression to 12.9% and 11%, at which point the addition of RNA did not further improve knockout efficiencies (4.00 µg circRNA reduced TIM3 expression to 10%). Editing with 0.50 µg circRNA was comparable to the level of editing using 4.00 µg of linear mRNA, facilitating an 8-fold reduction in the RNA amount.

Genomic DNA was extracted and sequenced across the SA site of E2 to identify the efficiency of C > T conversion at the on-target site (method 2.2.2.5.4) (Figure 5.20). A positive correlation is observed between the amount of RNA and the editing efficiency; with linear mRNA, editing efficiencies increase from 25% using 0.25 µg to 85% using 4.00 µg. On the other hand, circRNA editing efficiencies increase from 58% using 0.25 µg to 100% using 2.00 µg. CircRNA facilitated greater editing than linear RNA, most notably at the lower amounts of CBE-encoding RNA (0.25 µg, p = 0.0356; 0.50 µg, p = 0.0405; 1.00 µg, p = 0.0422). As shown in one representative donor, the electropherograms demonstrate improved base conversion at every amount of RNA (Figure 5.21). This data suggests that 0.50 µg-1.00 µg BE4max RNA is sufficient to knockout protein expression with high efficiencies (75% protein loss and 76% C > T conversion using 0.50 µg, 84% protein loss and 91% C > T conversion using 1.00 µg).
Figure 5.20 Linear versus circular RNA-encoding BE4max for base editing of HAVCR2.
Base editing of stimulated T-cells using the sgRNA that targets the SA site of E2 in HAVCR2. Four days after co-transfection of the sgRNA and 0.25 µg-4.00 µg circRNA-encoding BE4max, half of the cells were re-stimulated with TransAct™ and IL-7/IL-15 to identify gene disruption. (a) Flow-cytometry plots (FSC-A versus TIM3-BV421) at 24h after re-stimulation, one representative donor is shown. (b) TIM3 expression in re-stimulated cells at day 5 post-nucleofection and corresponding C > T base conversion (day 7), represented as mean ± SD (n=5 biological donors, represented as different symbols, from two independent experiments). (Two-Way ANOVA; *p < 0.05, **p < 0.01).
Figure 5.21 Base conversion of *HAVCR2* using linear or circular BE4max-encoding RNA.

Genotyping of base edited T-cells using increasing amounts of BE4max CBE-encoding linear or circular RNA. Electropherograms of the converted sequence are shown for one representative donor. At every amount of RNA, improved editing is observed with circular BE4max. The base editing window is indicated in a red box and the target base is highlighted using a red triangle. Adenine (green), thymine (red), cytosine (blue), guanine (black).
To investigate the effect on the level of by-stander editing, all C > T mutations in the protospacer were analysed for their efficiency. As shown in Figure 5.22, on-target editing efficiencies at C6 were positively correlated with the amount of RNA (linear and circular), however, by-stander efficiencies (at C4 and C5) also increased. At each amount of RNA, circRNA had improved editing at all three cytosines within the BE window (C4-C8). Highest editing efficiencies occur at cytosines in the middle of the BE window at positions C5 and C6. Using 0.25 µg RNA, minimal C > A editing was observed and transversion edits have been previously reported in the literature (Komor et al., 2017). Using 2.00 µg and 4.00 µg circRNA, 100% editing at C5 and C6 was observed for all donors. The editing profile of 0.50 µg circRNA was comparable to 2.00 µg of the linear format, approximately 75% editing was observed. This work demonstrates that higher editing efficiencies are achieved using circRNA compared to linear mRNA, hence, facilitating a reduction in the amount of RNA required for efficient editing.
Figure 5.22 Genotyping T-cells for base editing of *HAVCR2* using linear or circular BE4max-encoding RNA.

Genotyping base-edited T-cells using an increasing amount of BE4max-CBE encoding linear or circular RNA at 7 days post-engineering. A region spanning E2 of *HAVCR2* was amplified from
the genomic DNA, Sanger sequenced and analysed using EditR software. Base conversion efficiencies are shown for all cytosines in the protospacer, represented as mean ± SD (n=5 biological donors from two independent experiments), with the target cytosine highlighted in red.

To further validate the use of circRNA, we investigated a second target gene for disruption, *PDCD1*, using 0.25 µg-4.00 µg CBE-encoding RNA (method 2.2.2.5.3). In line with previous data, circRNA provided an enhancement to the disruption of PD-1 compared to linear RNA at the protein level (0.25 µg, p = 0.0151; 0.50 µg, p = 0.0439; 1.00 µg, p = 0.0457) and at the genomic level (1.00 µg, p = 0.0410) (Appendices, Figure 8.8-Figure 8.10). The greatest editing efficiencies were achieved with 1.00 µg circRNA, PD-1 expression was reduced from 56% (NT sgRNA) to 4% using circRNA compared to 63% (NT sgRNA) to 32% with linear RNA. The on-target base conversion efficiencies averaged at 97% for 1.00 µg circRNA compared to 44% with linear RNA (method 2.2.2.5.4) (Appendix, Figure 8.8).

This chapter has successfully screened eight PD-1 sgRNAs, four TIM3 sgRNAs, and seven LAG3 and TGFβR2 sgRNAs using the circRNA-encoding BE4max protocol. Multiplexed editing of all four inhibitory receptors resulted in a polyclonal edited population with knockout efficiencies ranging from 62%-68%, and base conversion efficiencies of >60% for all gene targets. As circular RNA is more stable than linear RNA we titrated the amount of CBE-encoding circular RNA and compared editing efficiencies to corresponding amounts of linear RNA. CircRNA outperformed the linear counterpart for the disruption of two target genes, *HAVCR2* and *PDCD1*. Circularisation enabled a reduction in the amount of BE4max RNA required to achieve equivalent levels of editing.
5.10 Discussion

5.10.1 Protocol development and selection of sgRNAs

This chapter describes the development of a novel base editing protocol using circRNA-encoding BE4max, for the disruption of four inhibitory receptors in stimulated primary T-cells. We designed sgRNAs to target splice sites or to introduce pmSTOP codons into the exons of PDCD1, HAVCR2, LAG3 and TGFβR2, to attenuate protein expression. Firstly, we screened PDCD1 and HAVCR2 sgRNAs in cell lines that were engineered to stably express BE4max (section 5.4.2). The optimal PDCD1 sgRNA disrupted the SD site in E1 and was previously described by Webber et al., (Webber et al., 2019) and the most efficient HAVCR2 sgRNA disrupted the SA site of E2. In T-cells using BE4max circRNA, we observed a 73% reduction in PD-1 expression and 75% on-target base conversion at the genomic level, which was comparable to published data (Webber et al., reported 78.6% protein loss and 63.7% on-target C > T conversion). Minimal by-stander editing was observed at C3 and C4 (<20% editing) and the by-stander edit at C8 (62% C > T conversion) was consistent with previous reports (Webber et al., 2019). Whereas the HAVCR2 sgRNA (SPLICER E2 SA) resulted in 64% TIM3 knockout and 75% on-target C > T conversion in the antisense strand (50-75% by-stander editing at C4 and C5). No transversion edits were observed at any site in the protospacer for both PD-1 and TIM3 sgRNAs (section 5.5).

Guide RNAs targeting SD sites are more efficient to knockout protein expression than those disrupting SA sites or introducing pmSTOP codons (Webber et al., 2019). If a SA site is non-functional, exon skipping can occur leading to the splicing out of an exon, which may still result in the expression of functional protein (Gapinske et al., 2018). The insertion of pmSTOP codons may also fail to disrupt gene expression, as codon suppression can occur that promotes translational readthrough and protein expression (Loughran et al., 2014).

It should be noted that sequence dependencies of the selected CBE limit its targeting scope. BE4max and its requirement for an NGG PAM and an editing window of C4-C8 (as determined by the rat APOBEC1 deaminase), restricted the design of LAG3 sgRNAs
to the introduction of pmSTOP codons and the disruption of SA sites. Many groups are working towards new iterations of base editors with relaxed PAM variants or modified BE windows to improve the targeting scope and editing efficiencies (Walton et al., 2020; Z. Liu et al., 2020). Furthermore, the rat APOBEC1 deaminase favours the conversion of a cytosine adjacent to a thymine (TC), and a cytosine adjacent to a guanine (GC) is unfavoured. The sgRNAs can be designed to improve efficiencies by targeting sequences containing the TC motif. Nevertheless, our screening efforts identified three suitable sgRNAs that edit LAG3 with efficiencies ranging from 70%-80% protein loss and >75% on-target base conversion. One sgRNA disrupted the SA in E7 and the two optimal sgRNAs introduced pmSTOP codons; sgRNA-5 (RGEN E4 pmSTOP 02) facilitated the greatest loss of LAG3 expression and was selected for multiplexed editing. The target site contains four adjacent cytosine bases within the BE window, and we observed significant by-stander editing at C5, C6, C7 (section 5.6).

To disrupt TGFβR2, seven sgRNAs were screened by genotyping. We identified four sgRNAs to facilitate base editing with approximately 75% efficiency (section 5.7). Two sgRNAs targeted the SD site of E2 and two sgRNAs introduced pmSTOP codons into E3 and E5. We hypothesised that higher knockout efficiencies could be achieved by targeting exons nearest the 5’ end of the gene (before the transmembrane domain), to prevent shuttling of truncated proteins to the cellular membrane, hence, we selected sgRNA-2 (SPLICER E2 SD 01) for multiplexed editing. Our sgRNA screen identified by-stander mutations occurring outside of the BE window, at varying frequency from C3 to C13. This highlights the requirement to predict the outcomes of C > T mutations for all positions within the protospacer. To reduce by-stander editing, CBE variants have been engineered through mutation of the deaminase to narrow the BE window. Triple mutations in BE4max have yielded YFE-BE4max, which has a BE window of three nucleotides (C4-C6) (Z. Liu et al., 2020). By-stander mutations are a significant challenge for gene correction; however, this is less of a concern for gene disruption, as additional mutations may enhance the efficiency of protein knockout. We next investigated multiplexed editing with the four most efficient sgRNAs, using the circRNA process.
5.10.2 Efficient multiplexed editing of four inhibitory receptors

To multiplex the disruption of T-cell inhibitory receptors, stimulated T-cells were co-transfected with four sgRNAs and circRNA-encoding BE4max (section 5.8). We observed 62%-68% protein loss for PD-1, TIM3 and LAG3 and 83%, 73%, 60% and 75% base conversion in the PDCD1, HAVCR2, LAG3 and TGFβR2 genes, respectively. Efficiencies were comparable to single target disruption, and multiplexing did not visibly impact cell viability or proliferation. Published studies have evaluated the off-target effects in multiplexed base-edited cells compared to those generated by CRISPR/Cas9 (simultaneous disruption of TRAC, B2M and PDCD1), and found BE4max to significantly reduce the frequency of gRNA-independent off-target mutations and translocations (Webber et al., 2019). Further work is required to validate sgRNAs by whole-genome next-generation sequencing (NGS) and chromosomal aberrations can be evaluated by optical genome mapping to ensure multiplexing does not increase genotoxicity (Nahmad et al., 2022).

5.10.3 Circular RNA increases editing efficiencies by BE4max

To optimise our process for CAR T-cell manufacturing, we investigated the amount of RNA required for efficient genome engineering (section 5.9). In line with published studies, our research process involved transfecting 1x10⁶ T-cells with 30 pmol of sgRNA and 4 µg BE4max circRNA (Webber et al., 2019). Based on previous results of this thesis, which show that circRNA has greater stability than linear mRNA, we hypothesised that RNA circularisation would increase BE4max expression. However, increased expression may lead to greater off-target deamination, which has been previously identified at the DNA and RNA level (Grünewald, Zhou, Iyer, et al., 2019; Zhou et al., 2019; Gaudelli et al., 2020). By reducing the amount of circRNA for transfection, we can fine-tune the expression for the desired editing efficiency and minimise off-target deamination. Our results demonstrate that circularisation facilitates at least an 8-fold reduction in the amount of RNA required to achieve levels of editing comparable to the current linear mRNA process. When targeting HAVCR2 for disruption, 0.50 µg of circRNA resulted in 80% knockout of the TIM3 protein (76% on-target base conversion), which was comparable to the level of editing using 4.00 µg of linear mRNA. Furthermore, 1.00 µg
of circRNA was sufficient to induce 91% base conversion at the genomic level, and 1.00 µg circRNA resulted in 100% on-target editing in three out of five donors. Assessment at an additional target site, *PDCD1*, also demonstrated increased editing with circRNA compared to linear mRNA. This work demonstrates the application of circRNA to improve base conversion efficiencies, which will significantly reduce the cost of clinical manufacturing.

We also tested the SOLUPORE® system for delivery of TIM3 sgRNA and linear BE4max RNA, co-transfected with an mClover3 RNA to identify the transfected population (Appendix, Figure 8.11). Base editing can be performed by soluporation, which may provide manufacturing benefits such as maintenance of the early memory phenotype. The following chapter in this thesis will combine base editing using circRNA-encoding BE4max, with the introduction of an anti-CD30 CAR, for the development of a Hodgkin lymphoma CAR T-cell therapy.

### 5.11 Conclusions

This chapter has described a second application of circRNA, to enable the efficient disruption of four T-cell inhibitory receptors, PD-1, TIM3, LAG3 and TGFβR2. Guide RNAs were designed and screened in primary T-cells and editing efficiencies were validated by the loss of protein expression and the frequency of C > T conversion at the target base. Our circRNA process enabled the multiplexing of four gene targets with high on-target editing efficiencies of greater than 70% for *PDCD1*, *HAVCR2* and *TGFβR2*, and 60% editing of *LAG3*. When compared to linear mRNA, editing with circRNA was more efficient, therefore enabling a reduction in the required amount of base editor RNA.

6.1 Overview

Despite its high efficiency, multiplexed editing using Cas9 endonuclease can result in chromosomal translocations and significant genotoxicity. The recent discovery of CBEs has facilitated gene knockout by the precise and direct conversion of a cytosine to a thymine, leading to the disruption of splicing sites or the introduction of premature stop codons. CBEs do not rely on the formation of DSBs, and chromosomal translocations are infrequent, therefore, base editing is an attractive tool for multiplexed editing (an overview is provided in section 1.3.2). The previous chapter demonstrated the application of circRNA to improve base conversion efficiencies by enhancing the expression of BE4max. This chapter employs the circRNA base editing protocol to generate an autologous CAR T-cell product, with improved persistence and cytotoxicity.

6.2 Introduction

This chapter describes the development of a base-edited anti-CD30 CAR T-cell therapy for the treatment of Hodgkin lymphoma. CD30-directed CAR T-cells have been tested in clinical studies but currently lack durable responses (discussed in section 6.2.3) (C.-M. Wang et al., 2017; Ramos et al., 2017, 2020; Hombach, Rappl and Abken, 2019). The tumour microenvironment is a particular challenge to cHL treatment; immunosuppressive signalling can downregulate T-cell effector functions and can induce premature exhaustion, resulting in poor product persistence. We hypothesised that CD30-directed CAR T-cells would benefit from multiplexed base editing to prevent T-cell inhibition and to restore cytotoxicity. This chapter optimises the anti-CD30 CAR sequence and addresses the challenges of T-cell fratricide (caused by CD30 expression on T-cells) and inhibition by the TME, to improve the therapeutic efficacy and outcome in HL patients.
6.2.1 Hodgkin lymphoma

Hodgkin lymphoma (HL) is a B-cell cancer occurring in the lymphatic system. In 2020, 83,087 new cases were diagnosed worldwide, with the highest incidence occurring in Southern Europe (Huang et al., 2022). HL and can be classified into classical HL (cHL) and nodular lymphocyte-predominant HL (NLPHL). cHL has a bimodal age distribution, most patients are diagnosed during adolescence (15-35 years of age), followed by a peak in adults >55 years of age, who often present with more advanced disease and a poor prognosis (Punnett, Tsang and Hodgson, 2010). The tumour cells of cHL, termed Hodgkin and Reed-Sternberg (HRS) cells, are characterised by CD30+CD15-CD20+ expression and derive from germinal centre (GC) B-cells which have lost their B-cell phenotype (Weniger et al., 2018). Hodgkin cells are mononucleate, whereas Reed-Sternberg cells are binucleate or multinucleate, and are typically derived from Hodgkin cells. In approximately 40% of cases, HRS are infected with Epstein Barr Virus (EBV), which contributes to pathogenesis by rescuing GC B-cells from apoptosis (Küppers, Engert and Hansmann, 2012; Weniger and Küppers, 2021). The TME comprises infrequent HRS cells (~1%) which are surrounded by a milieu of non-malignant cells, including T-cell subsets, eosinophils, mast cells, fibroblasts, extracellular matrix, and benign B-cells, that are distinct from normal lymphoid tissue (Scott and Gascoyne, 2014) (illustrated in Figure 6.1). Section 5.2.2 describes the challenges of the immunosuppressive TME to CAR T-cell therapy.
Figure 6.1 Schematic of the classical Hodgkin lymphoma tumour cell niche.

The tumour microenvironment (TME) comprises a heterogeneous population of tumour cells and non-malignant immune and stromal cells, extracellular matrix, blood vessels and secreted factors (shown as pink spheres). In classical Hodgkin lymphoma the Hodgkin and Reed-Sternberg cells represent ~1% of the population. The TME poses many challenges to CAR T-cells, including the physical barrier to T-cell infiltration, hypoxic conditions, and immunosuppressive signalling which downregulates T-cell responses.

Although most patients are cured with first line therapies, which involves combinatorial chemotherapy and localised radiotherapy, 15-20% of patients either have refractory disease or relapse after treatment (Glimelius et al., 2015). The second line of treatment for relapsed patients typically includes intensified chemotherapy followed by an autologous haematopoietic stem cell transplant (HSCT) (Schmitz et al., 2002). Approximately 50% of patients relapse after transplant and the prognosis of these patients is poor. Relapsed or refractory patients can receive treatment with brentuximab vedotin, an anti-CD30 antibody-drug conjugate that delivers the antimitotic agent, monomethyl auristatin E. In a pivotal phase II study, brentuximab vedotin was used as monotherapy in patients with advanced disease, and led to an overall response rate of 75% with
complete remission in 34% of patients (Younes et al., 2012). Following immunotherapy, relapsed patients can receive an allogeneic HSCT (Sarina et al., 2010), although allogeneic transplantation improves the survival of cHL patients, treatment relies on the availability of a HLA-matched donor. Thus, a small group of patients will require alternative therapy options such as CAR T-cell therapy. CAR T-cell therapy targeting CD30 on the surface of HRS has shown promise for refractory and relapsed cHL patients (C.-M. Wang et al., 2017; Ramos et al., 2017, 2020; Hombach, Rappl and Abken, 2019).

6.2.2 CD30 as a clinical target

CD30 (encoded by tumour necrosis factor (TNF) receptor superfamily, member 8 (TNFRSF8)) is a type I transmembrane glycoprotein (595 amino acids) belonging to the TNF receptor family (Muta and Podack, 2013). In 1982, CD30 was discovered as a target for cHL during the production of the monoclonal antibody, BerH2, that was directed to the Hodgkin cell line, L-428 (Su et al., 2004). The structure of CD30 comprises an elongated extracellular domain consisting of six cysteine-rich repeats, a transmembrane region, and a cytoplasmic tail. The extracellular region can be cleaved to produce a soluble version of the protein (sCD30), which is present in the serum of cHL patients (Pizzolo et al., 1990; Hombach et al., 1998). The ligand of CD30, CD30L (CD153), is a type II transmembrane glycoprotein and occurs in both membrane-bound and soluble forms. Binding of CD30L to CD30 induces receptor trimerization, which results in the recruitment of TNF receptor-associated factors (TRAF1/2/5) and downstream signalling through the NFκB and MAPK/ERK pathways (Gedrich et al., 1996; Pierce and Mehta, 2017).

CD30 is highly expressed on HRS cells and various other lymphomas including anaplastic large cell lymphoma. In normal tissues, limited expression has been observed on specific subsets of activated T-cells and B-cells, and haematopoietic stem cells (HSCs) (Hombach et al., 2016; Weyden et al., 2017). CD30 has pleiotropic effects depending on the cellular context and can provide both pro-survival and anti-apoptotic signals. In T-cells, CD30 expression peaks at four to five days post-stimulation and is thought to play a role in co-stimulation as well as the regulation of proliferation (Gilfillan et al., 1998).
Other functions include the induction of cytokine secretion (IL-2, TNF and IFN-γ), as well as a role in T helper 1 and 2 (Th1/Th2) responses (Kumar and Younes, 2014).

6.2.3 Anti-CD30 CAR T-cell therapy

The study of CD30-targeted CAR T-cells began in 1990 by Hombach *et al.*, who demonstrated the cytolytic capacity of first generation CARs in HL cells lines (Hombach *et al.*, 1998). Due to the lack of co-stimulatory endodomains, the function of CAR T-cells was limited. Hence, Savoldo *et al.*, engineered CD30-directed EBV-specific cytotoxic T-cells to promote co-stimulation and effector functions against EBV+ and CD30+ tumours (Savoldo *et al.*, 2007). Further studies have incorporated co-stimulatory endodomains within the CAR and have been assessed in clinical trials (CAR evolution is described in section 1.1.1) (C.-M. Wang *et al.*, 2017; Ramos *et al.*, 2017). Wang *et al.*, generated anti-CD30 CAR T-cells by lentiviral transduction using a CAR containing the 4-1BB endodomain and treated 17 HL patients (and 1 anaplastic large cell lymphoma (ALCL) patient) which had received various pre-conditioning treatments. Patients received a dose of 1.1x10⁷ to 2.1x10⁷ CAR T-cells/kg and achieved an overall response rate (ORR) of 39% (7 patients had a partial response and 6 patients had stable disease) (C.-M. Wang *et al.*, 2017). Using an alternative scFv, endodomain and transduction process, Ramos *et al.*, treated 9 patients (7 HL patients, 1 ALCL patient, 1 DLBCL patient which evolved to HL) with up to 2x10⁸ CAR T-cells/m², with no lymphodepleting regimens administered before infusion. A second-generation CAR containing the CD28 endodomain and was delivered using a gammaretroviral vector. Of the 9 treated patients, an ORR of 33% was achieved (2 patients had a complete response and 3 patients had stable disease) (Ramos *et al.*, 2017). Both studies demonstrate the tolerability of anti-CD30 CARs with some anti-tumour activity.

Studies of CD30-directed CAR T-cells are ongoing (Grover and Savoldo, 2019). Wang *et al.*, are investigating the clinical benefit of third generation CARs using the CD28-4-11Bζ endodomains. In their pilot study, of 9 treated patients (6 HL patients and 3 ALCL patients), 7 patients achieved a complete response, however, 4 patients relapsed after a minimum period of 10 weeks (Wang *et al.*, 2020). Following their phase I study that demonstrated clinical safety, Ramos *et al.*, have conducted two parallel phase I/II studies.
(NCT02690545 and NCT02917083) where 41 patients received $0.2 \times 10^8$ to $2 \times 10^8$ CAR T-cells/m$^2$ following various lymphodepleting regimens. Patients who received fludarabine-based lymphodepletion had an ORR of 72%, including 59% of patients with complete response, however, the 1-year progression-free and overall survival was 36% and 94%, respectively (Ramos et al., 2020). Although initially effective, durable responses of CD30-targeted CAR T-cells are lacking and the treatment of relapsed and refractory HL remains an unmet clinical need (Grover and Savoldo, 2019).

### 6.2.4 Manufacturing a next-generation anti-CD30 CAR T-cell therapy

This study aims to develop a next-generation anti-CD30 CAR T-cell therapy with enhanced efficacy. We address the challenges of limited T-cell persistence and early exhaustion by enforcing the expression of a secreted IL-15, combined with base editing of inhibitory receptors to provide resistance to immunosuppressive signals.

#### 6.2.4.1 Inclusion of secreted IL-15 to enhance therapeutic efficacy

IL-15 is a 14-15 kDa glycoprotein belonging to the common gamma chain ($\gamma$C) family of cytokines, consisting of IL-2, IL-4, IL-7, IL-9, and IL-21. IL-15 and IL-2 share the receptor complex subunits, IL-2/IL-15 receptor $\beta$ (CD122) and the $\gamma$C (CD132), and only differ in their $\alpha$ subunits. IL-15R$\alpha$ is a transmembrane protein that aids trafficking of the IL-15 from the endoplasmic reticulum to the cell surface and plays an important role in trans-presentation. The IL-15/IL-15R$\alpha$ complex can also be cleaved to form a soluble protein with a short half-life, which can provide a burst in cytokine activity (Robinson and Schluns, 2017). Whilst the IL-2/IL-15R$\beta$ and $\gamma$C receptors are highly expressed on CD8+ T-cells and NK cells, they can also be found on the surface of haemopoietic cells. Receptor dimerization leads to activation of JAK1 and JAK3 and downstream signalling through STAT5, as well as stimulation of the PI3K-AKT and RAS-MAPK pathways (Dwyer et al., 2019). IL-15 functions to promote the activation, proliferation, and migration of CD8+ T-cells and has been shown to inhibit activation-induced cell death and reverse T-cell anergy. Exogenous delivery of IL-15 can selectively induce proliferation in CD8+ T-cells and NK cells, making the cytokine an attractive molecule for immunotherapy. Klebanoff et al., demonstrate the transgenic expression of IL-15 can
improve the *in vivo* function of adoptively transferred CD8+ T-cells in IL-15 knockout hosts (Klebanoff et al., 2004). Compared to IL-2 which drives the proliferation of effector T-cells, IL-15 supports the maintenance of the memory compartment. Hurton *et al.*, have engineered a chimeric IL-15, consisting of the full-length native IL-15 peptide sequence fused to the IL-15Rα sequence via a flexible linker, and have co-expressed an anti-CD19 CAR using *Sleeping Beauty*. Their engineered T-cells showed improved persistence *in vivo* and displayed a T<sub>SCM</sub> phenotype (Hurton *et al.*, 2016). In addition to CD19-targeted therapies, transgenic expression of IL-15 has also been included in the context of glioma-targeted CAR T-cells (Krenciute *et al.*, 2017), as well as CAR-NK cells (E. Liu *et al.*, 2017).

**6.2.4.2 Genome engineering solutions**

The therapeutic efficacy of CAR T-cell therapies remains limited for cHL and will require complex genome engineering solutions to develop next-generation cellular therapies. This study aims to apply base editing to disrupt gene expression and improve *in vivo* persistence of anti-CD30 CAR T-cells. A potential reason for the limited efficacy of current CAR-T therapies is the interference of CD30 expression on activated T-cells, which may lead to fratricide in the T-cell product. Following identification of an optimised anti-CD30 CAR, this chapter aims to assess the requirement of CD30 knockout prior to CAR delivery. To further improve the function of CD30-targeted CAR T-cells *in vivo*, T-cell inhibitory receptors can be disrupted using CBEs to prevent inhibition from the immunosuppressive TME.
6.3 Chapter Aims

Previous work of this thesis describes a protocol using circRNA-encoding BE4max, to enhance the efficiency of gene disruption. This chapter aims to combine the introduction of a novel anti-CD30 CAR with base editing, to improve the *in vivo* performance and clearance of HRS cells. The first section of this chapter describes the optimisation of the CAR cassette, followed by testing the selected CARs in functional assays. To identify gene targets for disruption, the requirement of CD30 knockout is first evaluated to prevent T-cell fratricide. The thesis is summarised by the application of circRNA to knockout T-cell inhibitory receptors in a CAR T-cell manufacturing process, to generate CAR T-cells with improved effector functions. Potential gene targets include *PDCD1*, *HAVCR2*, *LAG3* and *TGFβR2*. In summary, this chapter aims to:

6.3.1 Identify a suitable CAR cassette for expression in T-cells.

6.3.2 Investigate the function of the selected anti-CD30 CARs.

6.3.3 Assess the requirement for CD30 disruption before transduction of the anti-CD30 CAR.

6.3.4 Combine base editing of inhibitory receptors with the introduction of the anti-CD30 CAR.
6.4 Screening anti-CD30 CARs for optimal expression

To identify a lentiviral cassette for maximal anti-CD30 CAR expression, we screened fourteen CAR constructs using the anti-CD30 CAR that was previously developed at UCL (Figure 6.2a). Lentiviral constructs were engineered to drive CAR expression from either from the MND hybrid viral promoter or the human elongation factor 1 alpha (EF1α) promoter.

The MND promoter consists of the Moloney murine leukaemia virus long terminal repeat with its U3 replaced with that of the myeloproliferative sarcoma virus (MPSV) (Challita et al., 1995; Robbins et al., 1998). This substitution increases the level of expression in haematopoietic stem cells due to the introduction of binding sites for the Sp1 transcription factor, and the negative control region present in the MPSV U3 was deleted to further enhance the activity of the promoter (Haas et al., 2003).

The human EF1α promoter was selected for comparison with the MND promoter as it does not impact viral titre, can increase transduction efficiency, and has a high level of transcriptional activity in primary T-cells (Rad S. M. et al., 2020). A comparative study showed that the human EF1α promoter outperformed the strong cytomegalovirus (CMV) promoter in human T-cells, providing higher levels of expression of a tri-cistronic cassette comprising an eGFP transduction marker, aHer2 CAR, and the anti-apoptotic protein Mcl-1, which translated to more efficient cytolysis of target cells and increased cytokine secretion (Rad S. M. et al., 2020).

To improve in vivo performance, the construct was engineered to incorporate a secreted human IL-15 module at either the 5’ or 3’ end of the CAR scFv, separated by a T2A self-cleaving peptide. The CAR sequence was optimised for mammalian expression using either a proprietary tool developed by Martin Pulè (MP), or the GenScript or GeneArt codon optimisation tools. GenScript codon optimisation was selected as it was previously shown to be superior to other optimisation algorithms for codon optimisation of the BE4 cytosine base editor, and ultimately resulted in the creation of BE4max (Koblan et al., 2018). Figure 6.2b shows the CAR sequence alignments, from the CD30 signal peptide to the end of the 3’ IL-15 and reveals 79.35% similarity between the MP and GenScript
sequences and 78.35% similarity between the GenScript and GeneArt sequences. The IL-15 coding sequence that was cloned into the MP-codon optimised construct was derived from the GeneArt sequence, therefore, the MP and GeneArt sequences show the greatest homology (86.12%). The sequences have similar GC content, ranging from 57% (GeneArt) to 58% (Genscript and MP).

Figure 6.2 Anti-CD30 CAR lentiviral cassettes for screening in primary T-cells.
(a) Structure of lentiviral cassettes encoding a second-generation anti-CD30 CAR. Sizes correspond to the length of cassette from the 5’ LTR to the 3’ LTR. Expression of the CAR is driven by either a murine leukaemia virus derived MND promoter or an elongation factor 1 alpha (EF1α) promoter. The CAR sequence (anti-CD30 CAR) includes a CD30 signal peptide, anti-CD30 variable heavy and variable light sequences separated by a 16 amino acid serine glycine linker, a human CD8 stalk and transmembrane domain, and 4-1BB and CD3ζ costimulatory and activation domains, respectively. The CAR sequence has been codon optimised (COpt) for mammalian expression using either the Martin Pulè proprietary (MP), GenScript or GeneArt codon optimisation tools. Where noted, a secreted human IL-15 module was included at either the 5’ or 3’ end of the CAR, separated by a T2A self-cleaving peptide. Lentiviral components following the 5’ LTR include the packaging signal of human immunodeficiency virus type 1 (ψ), a Rev response element (RRE), a central polypurine tract and central termination sequence (cPPT), and the Woodchuck pre-processing element (WPRE) which leads into the self-inactivating 3’ LTR. (b) Sequence homology between the MP-codon optimised (MP), Genscript and GeneArt codon-optimised CAR sequences containing the IL-15 at the 3’ end.

Activated PBMCs were transduced with lentiviral supernatants at an MOI of 2.5 (method 2.2.2.3.6), to enable comparison of the codon optimised sequences. Transduction efficiencies were determined 72h after transduction by flow cytometric analysis of cells stained with soluble CD30 protein (sCD30). The transduction efficiencies were generally comparable between the promoters and codon-optimisation strategies, ranging between 55% and 68% (Figure 6.3), except when IL-15 was incorporated at the 5’ end. Those that contained the 5’ IL-15 had significantly lower transduction efficiencies averaging at 30% (MND) and 43% (EF1α) (5’ MND versus MND, p < 0.0001; 5’ EF1α versus EF1α, p = 0.0142). This positional effect was also reflected in the level of CAR expression, as determined by MedFI measurements of the transduced populations (5’ MND versus MND, p = 0.0263; 5’ EF1α versus EF1α, p = 0.0123) (Figure 6.3b). The incorporation of IL-15 generally dampened the expression of the CAR, although this was better tolerated at the 3’ end.

The results of these experiments failed to reveal any improvement in transduction efficiency or CAR expression through codon optimisation of the coding sequences. This may have been due to the choice of the promoters used in the lentiviral constructs, as both
the MND and EF1α promoters provide a high level of transcriptional activity, potentially masking any increase in mRNA stability or rate of translation. As no significant difference was observed between the promoters or codon-optimisation strategies, the constructs chosen for further investigation were the MP-codon optimised CAR, whose CD8α spacer and transmembrane, 4-1BB and CD3ζ sequences were used in the clinical trial of an anti-CD19 CAR (Ghorashian et al., 2016), with EF1α promoter driving expression. The anti-CD30 CAR with or without IL-15 (3’ end) were taken forward for further analysis.
Figure 6.3 Anti-CD30 CAR expression in stimulated T-cells.

T-cells transduced with anti-CD30 CARs at an MOI of 2.5. (a) Flow cytometry plots (FSC-A versus anti-CD30 CAR, detection by sCD30-Bio and streptavidin-PE) at 72h post-transduction, one representative donor is shown. (b) Transduction efficiencies (percentage anti-CD30 (aCD30) CAR positive T-cells) and CAR expression (MedFI, normalised to CountBright beads)
at 72h post-transduction, represented as mean ± SD \( (n=10 \) biological donors, represented as different symbols, from three independent experiments). (Two-Way ANOVA; \(*p \leq 0.05, \text{****}p \leq 0.0001\). Martin Pulè (MP), GenScript (GS), GeneArt (GA) codon optimisation.

6.5 Validating the functional capacity of selected anti-CD30 CARs

To test the function of anti-CD30 CAR T-cells, we engineered and characterised cell lines expressing CD30, and conducted several standard CAR characterisation assays using transduced primary T-cells.

6.5.1 Generation and characterisation of CD30-expressing cell lines

To assess their functional capacity, CAR T-cells were co-cultured with target cells either constitutively expressing CD30 or engineered to express the antigen. Firstly, the SUP-T1 cells were transduced with a retroviral construct encoding human CD30 (method 2.2.2.3.5). Before proceeding to functional assays, the percentage of transduced cells was analysed by flow-cytometry and the constitutive expression of CD30 was determined in three Hodgkin lymphoma cell lines (HDLM-2, L-428, L-540) (Figure 6.4). Varying levels of CD30 expression were observed, transduced SUP-T1 cells expressed CD30 in 52.7% of the population, compared to >98% of HDLM-2, L-428 and L-540 cells. The highest CAR expression was observed in the L-540 cell line, closely followed by HDLM-2, and L-428 demonstrated the lowest antigen expression, as determined by the MedFI of the CD30 protein.
Figure 6.4 Cell lines expressing the CD30 target antigen.

(a) Diagram of the CD30 construct for transduction of the SUP-T1 cell line. The construct consists of a 5-prime LTR followed by the packaging signal of Moloney murine leukaemia virus (MoMLV) (Ψ), the MoMLV pol region containing the splice acceptor site (Pol), the human CD30 sequence and the central polypurine tract sequence (cPPT) which leads into the 3’ LTR. 

(b) Flow cytometry plots (FSC-A versus CD30-PE) to demonstrate the level of CD30 expression in the transduced SUP-T1 cell line and three Hodgkin lymphoma (HL) cell lines (HDLM-2, L-428 and L-540).

6.5.2 Cytotoxicity assessment of EF1α-driven anti-CD30 CARs

Measurement of the cytotoxic capacity of CAR T-cells is a key component of CAR characterisation and is performed by co-culturing a defined ratio of transduced effector cells with antigen-expressing target cells. The MP-codon optimised CAR, selected from the screening assay, contains the EF1α promoter with or without the addition of the secreted IL-15 module (aCD30 and aCD30-IL-15, respectively). PBMCs were transduced with lentiviral supernatant at an MOI of 2.5 (method 2.2.2.3.6), T-cells were harvested 72h later and transduction efficiencies were determined by flow-cytometry (64-76%
transduction was observed) (Figure 6.5). After resting the cells overnight, the transduction efficiencies were normalised, and co-cultures were prepared at effector to target cell ratios of 1:1 and 1:4 (method 2.2.5.1). Five target cell lines were investigated, including non-transduced SUP-T1 cells, which do not constitutively express CD30, SUP-T1 CD30+ cells, and three Hodgkin lymphoma cell lines, HDLM-2, L-428 and L-540.

Cytolytic capacity was measured at 24h, 48h and 72h, at which point flow-cytometry was performed to identify the percentage of remaining target cells. Using a 1:1 ratio, cytotoxicity was observed at 24h using both the aCD30 and aCD30-IL-15 CARs against CD30-expressing cell lines, and the non-transduced SUP-T1 cells were resistant to cytotoxicity. The greatest cytotoxicity was observed against the L-540 cell line, demonstrating 23-24% target survival, compared to 30% survival of SUP-T1 CD30+ cells and 41% survival of HDLM-2 cells. Although comparable between CARs, cytotoxicity was less evident against the L-428 cell line, which expressed a lower target antigen density (77% target survival with aCD30 and 78% with aCD30-IL-15) (Figure 6.6). At 48h, non-specific killing is observed against the SUP-T1 cells and the cell survival across

Figure 6.5 CAR transduction efficiencies in stimulated T-cells.

(a) Flow cytometry plots (FSC-A versus anti-CD30 CAR, detection using sCD30-Bio and streptavidin-PE) to demonstrate the percentage of CAR expression, one representative donor is shown. (b) Graphical representation of CAR transduction efficiencies, represented as mean ± SD (n=5 biological donors, represented as different symbols, from two independent experiments).
all target cell lines is greatly reduced (Figure 6.7). By 72h, effective clearance of the target cell lines was observed, with less than 25% target survival across all CD30-expressing cell lines (Figure 6.8). To analyse the relative cell proliferation, the absolute number of effector cells was determined in a set uptake volume by flow-cytometry (method 2.2.5.2). At 24h and 48h, comparable expansion was observed between CARs, whilst the non-transduced T-cells expanded at a much slower rate. At 72h, the T-cells transduced with aCD30-IL-15 demonstrated slightly greater proliferation compared to the aCD30 CAR alone when co-cultured with L-540 cells (p = 0.0131).
Figure 6.6 Cytolytic capacity of anti-CD30 CARs using an effector to target ratio of 1:1 at 24h.

Cytotoxicity of T-cells expressing aCD30 CARs. (a) Flow cytometry plots (CD2-APC versus CD3-PE/Cy7) at 24h of co-culture, one representative donor is shown. Target cell lines are identified as CD3 negative and express the CD2 marker to varying levels. (b) Percentage of remaining target cells (normalised to non-transduced T-cells) and absolute number of effectors (in a set flow-cytometry uptake volume), represented as mean ± SD (n=5 biological donors, represented as different symbols, from two independent experiments). Non-transduced SUP-T1 (SUP-T1), SUP-T1 CD30+ (SUP-T1-CD30).
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(a) A series of scatter plots showing cytotoxicity and effector responses for different cell lines and treatments, including NT, aCD30, and aCD30-IL-15.

(b) Bar graphs depicting target survival and absolute cell count for the same treatments and cell lines.
Figure 6.7 Cytolytic capacity of anti-CD30 CARs using an effector to target ratio of 1:1 at 48h.

Cytotoxicity of T-cells expressing aCD30 CARs. (a) Flow cytometry plots (CD2-APC versus CD3-PE/Cy7) at 48h of co-culture, one representative donor is shown. Target cell lines are identified as CD3 negative and express the CD2 marker to varying levels. (b) Percentage of remaining target cells (normalised to non-transduced T-cells) and absolute number of effectors (in a set flow-cytometry uptake volume), represented as mean ± SD (n=5 biological donors, represented as different symbols, from two independent experiments). Non-transduced SUP-T1 (SUP-T1), SUP-T1 CD30+ (SUP-T1-CD30).
Figure 6.8 Cytolytic capacity of anti-CD30 CARs using an effector to target ratio of 1:1 at 72h.

Cytotoxicity of T-cells expressing aCD30 CARs. (a) Flow cytometry plots (CD2-APC versus CD3-PE/Cy7) at 72h of co-culture, one representative donor is shown. Target cell lines are identified as CD3 negative and express the CD2 marker to varying levels. (b) Percentage of remaining target cells (normalised to non-transduced T-cells) and absolute number of effectors (in a set flow-cytometry uptake volume), represented as mean ± SD (n=5 biological donors, represented as different symbols, from two independent experiments). (Two-Way ANOVA; *p ≤ 0.05). Non-transduced SUP-T1 (SUP-T1), SUP-T1 CD30+ (SUP-T1-CD30).

Compared to the 1:1 effector to target cell ratio, the 1:4 ratio resulted in slower killing kinetics across all cell lines. Minimal cytotoxicity was observed against the L-428 and HDLM-2 cell lines at 24h; 51% and 57% SUP-T1 CD30+ survival was observed with aCD30 and aCD30-IL-15 CARs; and 54% and 89% survival was observed with the L-540 cell line, respectively (Figure 6.9). From 48h onwards, effective clearance of the target cell lines was demonstrated with both CARs and greater cytotoxicity was observed against the L-540 cell line with aCD30 compared to aCD30-IL15 at 24h and 48h (24h, p = 0.0016, 48h, p = 0.0224). Effector cells expanded at a comparable rate, at 72h using the 1:4 ratio aCD30 CAR T-cells expanded within the range of 0.97- to 5.10-fold relative to non-transduced T-cells and aCD30-IL-15 CAR T-cells expanded 0.67- to 5.39-fold (Figure 6.10, Figure 6.11).
Figure 6.9 Cytolytic capacity of anti-CD30 CARs using an effector to target ratio of 1:4 at 24h.

Cytotoxicity of T-cells expressing aCD30 CARs. (a) Flow cytometry plots (CD2-APC versus CD3-PE/Cy7) at 24h of co-culture, one representative donor is shown. Target cell lines are identified as CD3 negative and express the CD2 marker to varying levels. (b) Percentage of remaining target cells (normalised to non-transduced T-cells) and absolute number of effectors (in a set flow-cytometry uptake volume), represented as mean ± SD (n=5 biological donors, represented as different symbols, from two independent experiments). (Two-Way ANOVA; **p ≤ 0.01). Non-transduced SUP-T1 (SUP-T1), SUP-T1 CD30+ (SUP-T1-CD30).
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a

NT  aCD30  aCD30-IL-15

SUP-T1  Effectors 15.9  Effectors 26.0  Effectors 27.4

Targets 81.0  Targets 72.0  Targets 58.2

SUP-TL-CD30  Effectors 17.4  Effectors 78.2  Effectors 75.1

Targets 52.4  Targets 9.49  Targets 9.09

HDLM-2  Effectors 26.8  Effectors 89.1  Effectors 79.1

Targets 72.4  Targets 9.49  Targets 9.09

L.428  Effectors 46.7  Effectors 46.8  Effectors 45.2

Targets 58.4  Targets 28.7  Targets 23.3

L-S40  Effectors 42.4  Effectors 56.6  Effectors 75.9

Targets 75.0  Targets 23.3  Targets 23.3

b

Cytotoxicity

Target Survival (%)  0  25  50  75  100  125  150

SUP-T1  SUP-TL-CD30  HDLM-2  L.428  L-S40

NT  aCD30  aCD30-IL-15

Effectors

Absolute cell count  0  10000  20000  30000  40000  50000

SUP-T1  SUP-TL-CD30  HDLM-2  L.428  L-S40

NT  aCD30  aCD30-IL-15
Figure 6.10 Cytolytic capacity of anti-CD30 CARs using an effector to target ratio of 1:4 at 48h.

Cytotoxicity of T-cells expressing aCD30 CARs. (a) Flow cytometry plots (CD2-APC versus CD3-PE/Cy7) at 48h of co-culture, one representative donor is shown. Target cell lines are identified as CD3 negative and express the CD2 marker to varying levels. (b) Percentage of remaining target cells (normalised to non-transduced T-cells) and absolute number of effectors (in a set flow-cytometry uptake volume), represented as mean ± SD (n=5 biological donors, represented as different symbols, from two independent experiments). (Two-Way ANOVA; *p ≤ 0.05). Non-transduced SUP-T1 (SUP-T1), SUP-T1 CD30+ (SUP-T1-CD30).
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(a) 

(b)
Figure 6.11 Cytolytic capacity of anti-CD30 CARs using an effector to target ratio of 1:4 at 72h.

Cytotoxicity of T-cells expressing aCD30 CARs. (a) Flow cytometry plots (CD2-APC versus CD3-PE/Cy7) at 72h of co-culture, one representative donor is shown. Target cell lines are identified as CD3 negative and express the CD2 marker to varying levels. (b) Percentage of remaining target cells (normalised to non-transduced T-cells) and absolute number of effectors (in a set flow-cytometry uptake volume), represented as mean ± SD ($n=5$ biological donors, represented as different symbols, from two independent experiments). Non-transduced SUP-T1 (SUP-T1), SUP-T1 CD30+ (SUP-T1-CD30).

6.5.3 Inflammatory cytokine secretion

Secretion of the inflammatory cytokines, IFNγ and IL-2, released by CD4+ and CD8+ T-cells following specific activation, was measured by ELISA of co-culture supernatant harvested at 72h (method 2.2.5.3). Whilst non-transduced T-cells did not produce cytokines when co-cultured with antigen-expressing target cell lines, aCD30 and aCD30-IL-15 CAR T-cells specifically released IFN-γ and IL-2 when incubated with the HL cell lines only. Surprisingly, no cytokines were secreted when CAR T-cells were co-cultured with the SUP-T1 CD30+ cell line. This could be explained by the expression of CD80 and CD86 on HL cell lines, which will provide a co-stimulatory signal to CAR T-cells expressing CD28, unlike SUP-T1 cells which do not express CD80/CD86. Despite minimal differences in cytotoxicity, greater IL-2 secretion was observed with the aCD30-IL-15 CAR during co-culture with L-428 cells at a 1:1 ratio and with L-540 cells at a 1:4 ratio (1:1 L-428, p = 0.0151; 1:4 L-540, p = 0.0097) (Figure 6.12). In summary, no significant functional differences were observed between the anti-CD30 CARs.
Figure 6.12 Effector cytokine secretion at 72h of co-culture with target cells.

Cytokine release in co-cultures of aCD30 CAR T-cells and CD30-expressing cell lines at 72h. (a) Interferon-γ and (b) interleukin-2 secretion by ELISA, represented as mean ± SD (IFN-γ, n=5 biological donors, represented as different symbols, from two independent experiments; IL-2, n=3 biological donors). (Two-Way ANOVA; *p ≤ 0.05, **p ≤ 0.01).

6.5.4 Investigating the advantage of secreted interleukin-15

IL-15 is an inflammatory cytokine of the IL-2 superfamily which preferentially promotes activation, proliferation, and cytotoxicity of CD8+ T-cells and NK cells. The addition of an IL-15 module may therefore improve the in vivo function of our anti-CD30 CAR T-cells. To investigate the secretion of IL-15, ELISA was performed on cell culture supernatant collected from CAR T-cells at 72h post-transduction (method 2.2.5.3) (PBMCs stimulated with aCD3/aCD28 and IL-2). The aCD30-IL-15 CAR secreted an average of 767 pg/mL of IL-15 compared to 64 pg/mL with the aCD30 CAR (Figure 6.13a).
6.5.4.1 Antigen-independent proliferation

To determine if the anti-CD30 CAR T-cells expressing IL-15 were capable of antigen-independent proliferation, a starvation assay was set up where the cells were cultured in the absence of cytokines. Control samples, where the cells were cultured in the presence of exogenous IL-15 (10 ng/mL), were also set up. CAR T-cells were removed from IL-2 supplementation 72h after transduction, and the transduction efficiency normalised to 20%. Cells were re-seeded once every week at a cell density of 0.5x10^6 cells/mL in complete RPMI 10%. Cellular proliferation was analysed by NucleoCounter® NC-250™ cell count measurements over the course of three weeks.

In the absence of IL-15 supplementation, non-transduced T-cells did not proliferate and those transduced with the aCD30-IL-15 CAR exhibited a minor improvement in expansion. At 168h, the aCD30-IL-15-transduced cells had expanded 5-fold, compared to a 3-fold expansion in aCD30-transduced cells. In the presence of IL-15 supplementation, the aCD30 CAR rapidly expanded and outperformed the aCD30-IL-15 CAR, most notably at 336h and 504h (336h, p = 0.0010; 504h, p = 0.0070) (Figure 6.13b).

The results of this experiment are largely in agreement with published data showing that IL-15 expressing anti-CD19 CAR T-cells undergo limited expansion in the absence of antigen stimulation, while they can expand up to 10-fold when challenged with antigen (Hoyos et al., 2010).

Further assays will be required to evaluate the aCD30 and aCD30-IL-15 CARs in vivo, where the by-stander T-cells and NK cells will benefit from IL-15 trans-presentation. Hence, we decided to continue the comparison of aCD30 CARs in our in vitro functional experiments.
Figure 6.13 *In vitro* expansion of CAR T-cells constitutively expressing IL-15.

Proliferative advantage of T-cells transduced with anti-CD30 CARs. (a) IL-15 secretion in cell culture supernatants collected from CAR T-cells at 72hr post-transduction, represented as mean ± SD (n=7 biological donors, represented as different symbols). (Un-paired t-test; ****p ≤ 0.0001). (b) 3-week expansion of transduced T-cells in the absence (-) or presence (+) of IL-15 supplementation, represented as mean ± SD (n=3 biological donors). (Two-Way ANOVA, aCD30 versus aCD30-IL-15; *p ≤ 0.05).

6.6 Assessing the requirement for CD30 knockout prior to CAR delivery

6.6.1 CD30 expression dynamics in stimulated T-cells

CD30 is known to be expressed on subsets of T-cells and B-cells. Although current clinical studies do not disrupt CD30 expression, transduction with an anti-CD30 CAR may lead to fratricide, reduced expansion during the manufacturing process, and poor *in vivo* persistence. We conducted a series of experiments to address the requirement of CD30 disruption, beginning with the analysis of CD30 expression in stimulated T-cells.
PBMCs were stimulated for 48h using two methods; soluble aCD3/aCD28 antibodies and IL-2 or TransAct™ and IL-7/15, prior to resuspension in cytokine-supplemented medium. Both stimulation methods resulted in the upregulation of CD30, compared to the cytokine-alone controls. CD30 expression was greatest following TransAct™ stimulation and peaked at day 3, averaging at 37%, compared to 21% expression after 2 days of treatment with soluble antibodies (TransAct™ versus aCD3/aCD28 at day 3 and day 4, p < 0.0001) (Figure 6.14). Since both stimulation methods result in the upregulation of CD30, the disruption of CD30 may be desirable before introduction of the CAR.
Figure 6.14 Expression of CD30 on stimulated T-cells.
Upregulation of CD30 expression in stimulated T-cells (a) Flow cytometry plots (FSC-A versus CD30-PE) 1-4 days post-stimulation, one representative donor is shown. (b) Percentage of CD30-expressing T-cells over the course of 4 days, represented as mean ± SD (n=3 biological donors). (Two-Way ANOVA, aCD30 versus aCD30-IL-15; ****p ≤ 0.0001). Soluble aCD3/aCD28 and IL-2 (sAb + IL-2), TransAct™ and IL-7/15 (TA + IL-7/15).

6.6.2 Functional comparison of non-edited and CD30-edited CAR T-cells

6.6.2.1 Cytotoxicity assessment of edited anti-CD30 CAR T-cell products

To assess the requirement of CD30 knockout, a cytotoxicity assay was performed to compare the function of non-edited and CD30-edited anti-CD30 CAR T-cells. Previous work screened nine CD30-targeted sgRNAs by base editing and resulted in low editing efficiencies (Appendices, Figure 8.12-Figure 8.14). Hence, this assay employed CRISPR/Cas9 technology to disrupt CD30 expression (method 2.2.2.5.2). The experimental schematic is shown in Figure 6.15.

![Figure 6.15 Process workflow for the functional comparison of CD30-edited and non-edited anti-CD30 CAR T-cells.](image)

The nucleofection of non-targeting or CD30-targeted Cas9 RNP complexes was performed on resting T-cells. T-cells were stimulated using soluble aCD3/aCD28 antibodies after a 4-5h incubation. At 48h post-nucleofection, CD30-edited or non-edited T-cells were transduced with lentiviral supernatant by spinoculation with RetroNectin. CAR T-cells were harvested at 72h post-transduction and were analysed for their transduction efficiencies. Cytotoxicity assays were prepared the following day using CD30-expressing target cells. Samples for genotyping were taken on day 8 to confirm CD30 ablation by sequencing.
Here, the nucleofection of non-targeting or CD30-targeted RNPs (using sgRNA-1 targeting E1) was performed on resting T-cells to allow sufficient time for recovery and expansion, prior to lentiviral transduction. Cells were rested for a period of 4-5h post-nucleofection, prior to T-cell activation using soluble aCD3/aCD28 antibodies. After 24h, IL-2 was added to the culture medium and T-cells were rested overnight prior to lentiviral transductions of aCD30, aCD30-IL-15 or aCD19 CARs at an MOI of 2.5 (methods 2.2.2.5.3, 2.2.2.3.6). The aCD19 CAR was included as a negative control for the cytotoxicity assay. Transduction efficiencies ranged between 40-72% and were normalised to 45% (+/-5%) to analyse the cytotoxicity against SUP-T1 cells and the three cHL target cell lines at an effector to target cell ratio of 1:4 (method 2.2.5.1).

![Figure 6.16](image_url)  
**Figure 6.16** CAR transduction efficiencies in CD30-edited or non-edited T-cells.
(a) Flow cytometry plots (FSC-A versus CAR-PE, detection using sCD30-Bio and streptavidin-PE or aCAT-19 + aRat-PE) to demonstrate the percentage of CAR expression, one representative donor is shown. (b) Graphical representation of CAR transduction efficiencies, represented as mean ± SD (n=6 biological donors, represented as different symbols).

On the day of cytotoxicity set up, 1x10^6 T-cells were harvested for genotyping to determine the efficiency of CD30 disruption (method 2.2.2.5.4). After extracting genomic DNA, an 800 base pair region of exon 1 was amplified by PCR, Sanger sequenced and analysed using the Inference of CRISPR Edits (ICE; Synthego). The frequencies of indel formation and editing were greater than 75% for five donors, with only one donor exhibiting a lower editing efficiency of 58% (Figure 6.17).

Figure 6.17 Efficiency of CD30 disruption in stimulated T-cells.

CRISPR/Cas9 editing efficiencies in anti-CD30 CAR T-cells at day 8 post-nucleofection (n=6 biological donors). A region spanning E1 of the TNFRSF8 gene was amplified from the genomic DNA of non-edited or edited T-cells and was sent for Sanger sequencing. Sequencing traces were analysed using the Synthego ICE knockout tool to calculate the percentage of indels and the associated knockout (KO) score.
Consistent with the CAR validation assays, slower killing kinetics were observed for the L-428 cell line and their clearance was evident at 48h. Whilst the aCD19 CAR did not reduce the survival of CD30-expressing target cells, the aCD30 CAR reduced the survival to 22% (HDLM-2), 34% (L-428) and 24% (L-540) using non-edited T-cells, compared to 15%, 41% and 22%, using CD30-edited T-cells. The aCD30-IL-15 CAR resulted in 37% (HDLM-2), 59% (L-428) and 40% (L-540) target cell survival using non-edited T-cells, compared to 27%, 62%, and 35% survival using CD30-edited T-cells (Figure 6.18, Figure 6.19). The cytolytic capacity of non-edited or CD30-edited CAR cells was comparable. The number of effector cells (CD2+CD3+) in a defined flow-cytometry uptake volume was used to assess the relative proliferation (method 2.2.5.2). Although not statistically significant, lower cell numbers were observed for CD30-edited T-cells compared to the non-edited samples, indicating that CD30 knockout may be detrimental to cell proliferation (Figure 6.19).
Figure 6.18 Cytolytic capacity of non-edited and CD30-edited anti-CD30 CAR T-cells using an effector to target ratio of 1:4 at 48h.
Cytotoxicity of non-edited (NT sgRNA) or CD30-edited (CD30 sgRNA) T-cells expressing aCD30 CARs. Flow cytometry plots (CD2-APC versus CD3-PE/Cy7) at 48h of co-culture with (a) HDLM-2, (b) L-428 or (c) L-540, CD30-expressing target cells, one representative donor is shown. An aCD19 CAR was included as a negative control.

Figure 6.19 Functional comparison of non-edited and CD30-edited anti-CD30 CAR T-cells using an effector to target cell ratio of 1:4 at 48h.

Cytotoxicity of T-cells expressing aCD30 CARs. (a) Percentage of remaining target cells (normalised to non-transduced T-cells) and (b) absolute number of effectors (in a set flow-cytometry uptake volume), represented as mean ± SD (n=6 biological donors, represented as different symbols). An anti-CD19 CAR (aCD19) was included as a negative control.

6.6.2.2 Inflammatory cytokine secretion

Cell culture supernatants were harvested at 48h of co-culture with CD30-expressing target cells and were analysed for the release of two inflammatory cytokines, IFN-γ and IL-2 (method 2.2.5.3). Whilst the non-transduced and aCD19 CAR T-cells did not release
effector cytokines, comparable profiles were observed between the non-edited (NT sgRNA) and CD30-edited samples (CD30 sgRNA), demonstrating that CD30 disruption does not affect the cytotoxicity of CAR T-cells in vitro. The aCD30 CAR T-cells released greater levels of IFN-γ than the aCD30-IL-15 CAR (NT sgRNA L-540, p = 0.0114; CD30 sgRNA L-428, p = 0.0158), and this correlates with small differences observed in the cytotoxicity assay. The release of IL-2 was comparable between CARs (Figure 6.20).

Figure 6.20 Cytokine secretion at 48h of co-culture of non-edited or CD30-edited anti-CD30 CAR T-cells with target cells.

Cytokine release in co-cultures of aCD30 CAR T-cells and CD30-expressing cell lines at 48h. (a) Interferon-γ and (b) interleukin-2 secretion by ELISA, represented as mean ± SD (n=6 biological donors, represented as different symbols). (Two-Way ANOVA; *p ≤ 0.05).

CD30 expression is upregulated in stimulated primary T-cells and is positively correlated with strength of the activation signal (Figure 6.14). However, our assays demonstrate that CD30 disruption does not improve the cytotoxicity of anti-CD30 CAR T-cells, as
determined by the clearance of CD30-positive target cells and associated cytokine release profiles. In fact, the knockout of CD30 appeared to impair the recovery of cells after nucleofection, resulting in reduced proliferation. Since the knockout of CD30 is inefficient using BE4max and did not appear to provide a functional advantage, we concluded that CD30 disruption was not required for manufacturing.

6.7 Generation of PD-1-edited aCD30-IL-15 CAR T-cells

6.7.1 Small-scale manufacture of PD-1-edited aCD30-IL-15 CAR T-cells

The final section describes the development of a base-edited CAR T-cell protocol. PD-L1 expression is frequently observed in HRS cells and anti-PD-1 monoclonal antibodies, such as pembrolizumab, can reverse T-cell inhibition by disrupting the PD-1-PD-L1 axis. Hence, PD-1 was selected for our proof-of-concept study to demonstrate that base editing can be combined with insertion of a CAR. Selected T-cells were base-edited following our small-scale research protocol (method 2.2.2.5.3) and were subsequently transduced at 24h post-nucleofection with anti-CD30-IL-15 encoding lentiviral supernatant (method 2.2.2.3.6), prior to flow-cytometry phenotyping on day 7 and day 9 (Figure 6.21).

**Figure 6.21** Schematic of the base-edited CAR T-cell manufacturing process.

The manufacture of genome-edited CAR T-cell products begins with T-cell selection using the Miltenyi Prodigy and CD4+/CD8+ microbeads. Immediately after isolation, the T-cells are activated in a 100M G-Rex using TransAct™ and IL-7/15 for 48h prior to editing. A non-targeting or PD-1-targeting sgRNA is then co-delivered with 1.00 µg BE4max-encoding circular RNA per 1x10⁶ T-cells. Edited T-cells are recovered overnight before spinoculation with
aCD30-IL-15-encoding lentiviral supernatant in RetroNectin-coated 6-well plates. Edited T-cells were expanded until day 9, at which point they were cryopreserved.

To ensure efficient editing, we co-transfected 2x10^6 T-cells with 60 pmol PD-1 sgRNA (targeting SA of E2) and 2.00 µg of BE4max-encoding circRNA (1.00 µg per 1x10^6 T-cells). Although non-edited CAR T-cells expressed low levels of PD-1 (<10% at day 7), we found disruption of the gene to be extremely efficient, where expression was reduced to <1.5% (Two-Way ANOVA, day 7, p = 0.0692, day 9, p = 0.1005). By the end of the process (day 9), PD-1 expression was downregulated to <6% in non-edited CAR T-cells (Figure 6.22, Figure 6.23a). At this point, genomic DNA was extracted and analysed by genotyping as previously described (method 2.2.2.5.4). We found 100% C > T conversion at the target position, C8, and at the adjacent by-stander edit (C7), in all three donors (Figure 6.23b).

Furthermore, we show that base-edited T-cells were amenable to lentiviral transduction. To identify the anti-CD30 CAR, we developed an anti-idiotype antibody by hybridoma generation and sub-cloning (Genovac), and screened 880 clones by staining an aCD30 scFv-eGFP expressing SUP-T1 cell line with neat cellular supernatant and an anti-Rat-PE secondary antibody (method 2.2.3) (described in Appendices, Figure 8.15, Figure 8.16). The cellular supernatant from the optimal clone, 2A11, was used to determine the transduction efficiencies; >50% of T-cells expressed the aCD30 CAR, using an MOI of 2.5 (Two-Way ANOVA, p = 0.6025) (Figure 6.23c).

As a measure of T-cell proliferation, viable cell counts were recorded, and fold-expansion was calculated by normalisation to the number of nucleofected T-cells (method 2.2.5.2). As shown in Figure 6.23d, an initial drop in cell number was observed at 24h post-nucleofection (~50% cell loss), which was consistent with previous data. Expansion was limited for 4 days post-nucleofection, followed by rapid T-cell proliferation. Non-edited CAR T-cells expanded by 15.0-fold compared to 14.6-fold in PD-1-edited CAR T-cells (One-Way ANOVA; p = 0.9890).
Figure 6.22 Phenotyping PD-1 base-edited anti-CD19 CAR T-cells.

Phenotyping was performed at day 7 and day 9 of the manufacturing process (5- and 7-days post-editing) to identify PD-1 disruption and CAR transduction. Flow-cytometry plots show (a) PD-1 (FSC-A versus PD-1-PE) or (b) aCD30-IL-15 expression (FSC-A versus aCD30 CAR-APC, detection using aCD30 CAR anti-idiotype and aRat-APC) in T-cells, one representative donor is shown.
Chapter 6. Results

Figure 6.23 Characterisation of PD-1-edited CAR T-cells.

Product characterisation was performed at day 7 and/or day 9 of the manufacturing process (5- and 7-days post-editing). (a) PD-1 and (b) anti-CD30-IL-15 CAR expression, represented as mean with donors represented as different symbols, (c) base conversion efficiencies and (d) T-cell proliferation calculated as fold-expansion using the number of viable cells (NucleoCounter® NC-250™) normalised to the number of nucleofected cells, represented as mean ± SD (n=3 biological donors from two independent experiments).
6.7.2 PD-1 disruption does not prevent T-cell activation

As a demonstration that base-edited T-cells retain their ability to signal through the TCR, we re-stimulated CAR T-cells upon thawing with TransAct™ and IL-7/15 and measured the upregulation of activation markers, CD25 and CD69, in addition to PD-1. CD25 functions as the alpha chain of the high-affinity IL-2 receptor, whilst CD69 functions as a C-type lectin protein, both are known as early markers of T-cell activation (expression 4-6h post-activation) (Motamedi, Xu and Elahi, 2016). A small population of un-stimulated T-cells expressed the markers PD-1 or CD69 (<5%), whilst >95% of T-cells expressed CD25. Following stimulation, PD-1 was upregulated to >30% in non-edited CAR T-cells, and those with the base-edit could not express the protein. TransAct™ stimulation resulted in a 10-fold increase in the MedFI of CD25 expression, and a 2-fold increase in CD69 (Figure 6.24). We concluded that base editing of the PD-1 exon 1 splice donor site effectively disrupted gene expression, as limited upregulation of the inhibitory receptor was observed in the PD-1 edited sample (~1.7% PD-1+) compared with the non-targeting control (~41% PD-1+) after stimulation of the CAR T-cells with TransAct™. In contrast, upregulation of the activation markers CD69 and CD25 was unperturbed in the PD-1 base-edited CAR T-cells, compared with the non-targeting control, indicating that signalling through to TCR and CD28 co-receptor was still intact and functional.
Figure 6.24 Expression of activation markers in stimulated PD-1-edited CAR T-cells.
Base-edited CAR T-cells were re-stimulated for 24h using TransAct™ and IL-7/15 and their ability to induce T-cell activation was determined by the expression of (a) PD-1, (b) CD25 and (c) CD69. Flow-cytometry plots (FSC-A versus PD-1-PE, CD25-BV421 or CD69-FITC) are shown for one representative donor. Isotype controls were used for the gating strategy (data not shown).
Figure 6.25 PD-1 disruption does not prevent the activation of base-edited CAR T-cells.

To induce TCR activation, base-edited CAR T-cells were re-stimulated for 24h using TransAct™ and IL-7/15. PD-1, CD25 and CD69 expression is shown in (a) un-stimulated or (b) stimulated T-cells, represented as mean (n=3 biological donors in triplicate, represented as individual points with donors in different symbols). (Two-Way ANOVA; **p ≤ 0.01, ****p ≤ 0.0001). The intensity of expression (MedFI) was calculated by normalisation to CountBright beads.
6.8 Discussion

6.8.1 Optimisation of the CAR cassette

This chapter has screened fourteen lentiviral constructs containing the anti-CD30 scFv binder that was previously developed at UCL (section 6.4). A secreted module encoding the inflammatory cytokine, IL-15, was incorporated at the 5’ or 3’ end of the scFv to enhance the in vivo performance. Three codon optimisation strategies, alongside two commonly used promoters, MND and EF1α, were compared to optimise CAR expression and the number of molecules on the T-cell surface. The optimisation of codon usage did not appear to improve CAR expression, and minimal differences were observed between promoters. Although an MOI of 2.5 was used to ensure comparable transduction efficiencies across samples, the percentage of transduced T-cells was relatively high and greater differences may be observed by reducing the MOI and level of transduction. The EF1α promoter was selected to drive expression of the CAR, because its transcriptional activity was comparable to that of the MND promoter and it has already been validated by its use in a clinical setting with the anti-CD19 CAR, tisagenlecleucel (Milone et al., 2009; Maude et al., 2016). The MP-codon optimised anti-CD30 CAR constructs were taken forward for further investigation and were validated by confirming their cytotoxicity against three cHL cell lines, HDLM-2, L-428 and L-540 (section 6.5).

In vitro starvation assays indicated an initial improvement to the expansion of CAR T-cells containing the secreted IL-15 module; however, at the experiment endpoint there was no difference in the cell number (section 6.5.4). These results are in agreement with published data showing that CAR T-cells expressing IL-15 do not proliferate in the absence of antigen stimulation but rapidly expand up to 10-fold more than control CAR T-cells upon challenge with antigen (Hoyos et al., 2010). As IL-15 is involved in the maintenance of T-cell memory, with IL-15 knockout mice exhibiting reduced numbers of CD8+ memory T-cells and NK cells (Lodolce et al., 1998), expression of IL-15 in anti-CD30 CAR T-cells is probably skewing them to a memory phenotype. While we did not conduct phenotyping of the T-cells with the memory panel described in chapter 4, this would be important to validate the inclusion of secreted IL-15 with the anti-CD30 CAR to expand the memory subset. It has been shown that overexpression of IL-15 in T-cells

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helps maintain the expression of the lymphoid homing markers, CD62L and CCR7, which may be beneficial in the context of Hodgkin lymphoma (Hoyos et al., 2010).

Follow up work, validating the inclusion of the IL-15 gene with the anti-CD30 CAR, would include an in vitro restimulation assay to demonstrate that the cytokine elicits increased proliferation compared to CAR alone controls, and an in vivo comparison of the elimination of engrafted Hodgkin lymphoma cells in NSG mice. As IL-15 would be presented in trans by the anti-CD30 CAR T-cells it would stimulate by-stander T-cells and NK cells, potentially leading to a more profound anti-tumour response, as these cell types are engaged. This may be particularly important in the immunosuppressive tumour microenvironment of Hodgkin lymphoma. We have begun work in immunodeficient NSG mice engrafted with L-428 or L-540 cell lines, where additional stimulatory and inhibitory signals are present in the TME.

### 6.8.2 Assessing the requirement for CD30 knockout

As described in the literature, we found CD30 to become upregulated on stimulated T-cells (section 6.6.1. The expression of CD30 on T-cells can cause multiple challenges to the function of anti-CD30 CAR T-cells, including T-cell fratricide, blocking of the anti-CD30 scFv and pro-apoptotic signalling (upon engagement with CD30L). It is known that membrane bound CD30 can be cleaved to form a soluble version of the protein. Hombach et al., have conducted experiments to test the function of CAR T-cells in the presence of high concentrations of sCD30 (up to 6000 units/mL) and found cytolytic activity to be retained (Hombach et al., 1998). Hombach et al., have further shown that removal of the CD30 positive cells by anti-CD30 immunotoxins or CAR T-cells can improve the function of carcinoembryonic antigen-targeted CARs against CD30 negative solid tumours, by disrupting the regulatory CD30-CD30L axis and removing a population of IL-10-secreting T-cells (Hombach, Rappl and Abken, 2019). Despite the reported challenges, clinical studies do not disrupt CD30 expression.

This chapter aimed to address the reported concerns and compared the function of non-edited and CD30-edited anti-CD30 CAR T-cells in functional assays (cytotoxicity and cytokine release). We did not observe a functional advantage to CD30 ablation, and
instead found a reduction in the number of CAR T-cells, indicating that CD30 is required for proliferation (section 6.6.2). As CD30 is known to be involved in T-cell co-stimulation, this result was not surprising. Ongoing work will compare the transcriptomes of edited and non-edited CAR T-cells to determine the impact on effector mechanisms. Although previous results demonstrate CD30 upregulation post-activation, expression was not detected in the donors of this assay, suggesting that genetic knockout may not be necessary.

We did not observe T-cell fratricide in the non-edited culture. Regardless of inconsistent CD30 expression, T-cells may be protected from fratricide by several mechanisms. CD30L is also expressed on activated T-cells as well as cHL tumour cells, and engagement with CD30 on T-cells may prevent fratricide by steric hindrance (Gruss et al., 1996). Hematopoietic stem and progenitor cells (HSPCs) also express CD30 and targeting the antigen would result in long lasting blood aplasia, however, activated HSPCs are protected by sub-threshold levels of CD30 and by high levels of the granzyme B inhibitor, SP-6/P19, to prevent CAR T-cell degranulation (Hombach et al., 2016). The level of CD30 expression on T-cells is relatively low compared to tumour cells, and like HSCs, may be below the cytotoxic threshold (Savoldo et al., 2007).

### 6.8.3 Improving the durability of anti-CD30 CAR T-cells

In the final section of this chapter, base editing of PD-1 was combined with the introduction of the anti-CD30-IL-15 CAR using our circRNA protocol (section 6.7). We observed 100% on-target base conversion using 1.00 µg BE4max-encoding circRNA. In a parallel study, we co-transfected 50x10⁶ T-cells of one donor with 12.5 µg circRNA and 1500 pmol PD-1 sgRNA using Lonza’s Large Volume Nucleofector™. We also observed 100% on-target base conversion and these results suggest we can lower the amount of RNA for large-scale studies, further reducing the RNA-associated cost to the manufacture of base-edited immunotherapies.

We found PD-1 expression to be relatively heterogenous among donors (1.7% to 27% T-cells), which was consistent with previous reports. In patients, Ramos et al., have shown that PD-1 is expressed on 33% of infused anti-CD30 CAR T-cells (Ramos et al., 2017).
These findings highlight the susceptibility of CAR T-cells to inhibition through the PD-1/PD-L1 pathway. Results of immune checkpoint blockade studies support the disruption of PD-1 (Armand et al., 2016; Lesokhin et al., 2016), as well as other T-cell inhibitory receptors, including CTLA-4 (Bashey et al., 2009) and LAG3 (NCT02061761). The combination of PD-1, LAG3 and TIM3 inhibitors are also under evaluation (NCT03311412) and allude to a potential benefit of multiplex gene disruption in anti-CD30 CAR T-cells.

To determine the functional advantage of PD-1 and/or multiplexed gene disruption, we will require the generation of in vivo models to compare the persistence and efficacy of non-edited and base-edited CAR T-cells. Current murine models of cHL are limited to NSG mice engrafted with cHL cell lines, such as HDLM-2, L-540 and L-428 (Stasi et al., 2009; Alvarez-Fernandez et al., 2015; Guo et al., 2022). However, cHL cell lines are typically slowly dividing (doubling times of 75h (HDLM-2), 70h (L-540) and 38h (L-428) and engraftment can take up to 3 weeks. We express the requirement of humanised murine models, such as patient-derived xenograft (PDX) models, to recapitulate the tumour microenvironment and the associated stimulatory and inhibitory signals. However, PDX models of cHL are currently challenging, largely due to tumour heterogeneity (Diorio et al., 2022).

Modification of the manufacturing conditions can further improve the persistence of anti-CD30 CAR therapies. Alvarez-Fernández et al., generated an anti-CD30 CAR product with a T_{SCM} phenotype through culturing in low doses of IL-7, IL-15 and IL-21, and providing the T-cells with a short CD3/CD28 co-stimulation (Alvarez-Fernández et al., 2021). In future work, we could co-deliver BE4max-encoding circRNA with transcription factor-encoding circRNA to generate an anti-CD30 product with an early memory phenotype and tolerance to tumour-associated immunosuppressive signals.
Chapter 7. Discussion and Final Conclusions

This thesis has explored non-viral delivery methods to manufacture CAR T-cells, with a focus on non-integrative approaches to reprogram and improve the T-cell function. Alternative viral approaches were also investigated, including the generation of NILV for transient expression, and the soluporation of lentivirus for stable CAR integration. Non-viral approaches will circumvent the issues associated with viral vectors, including insertional mutagenesis, lack of copy number control and the costly generation of GMP-grade vector. To generate a non-viral manufacturing process, we recommend combining the transient expression of circular RNA with CRISPR-associated integration to insert the CAR by homology-directed repair, transposition, or recombination. Here, we provide a summary of the results and discuss the potential of novel genome engineering tools for targeted CAR insertion. The proposed viral and non-viral methods for the manufacture of reprogrammed cell therapies are summarised below (Figure 7.1).
### Figure 7.1 Proposed viral and non-viral approaches for manufacturing CAR T-cells.

CAR T-cell manufacturing involves the permanent integration of the CAR genes and the transient expression of accessory molecules to reprogram CAR T-cells and improve their function. Delivery by viral vectors involves a simple transduction process; integrase-proficient vectors can deliver the CAR, whereas the low level and transient nature of non-integrative vectors is suited to the delivery of cytosine base editors. Alternatively, proprietary delivery technologies can be utilised to facilitate targeted integration of the CAR using novel genome editing tools (CRISPR-mediated HDR or CRISPR-associated transposases/recombinases) for co-delivery with circRNAs encoding base editors or transcription factors.
Chapter 7. Discussion

7.1 Summary of results

This thesis provides manufacturing insights for T-cell reprogramming by non-viral engineering. We have identified suitable substrates for transient expression alongside the evaluation of proprietary transfection technologies for optimal delivery. Applications of our developed approach include the expression of transcription factors to modulate the T-cell phenotype, and base editors to disrupt the genes of inhibitory receptors. When combined with the introduction of a CAR, these approaches can prevent premature T-cell differentiation and exhaustion, which are limiting factors to therapeutic efficacy.

7.1.1 Evaluating delivery approaches

7.1.1.1 Investigating proprietary delivery tools

The first results chapter investigates the expression of DNA, RNA, and protein in primary T-cells and evaluates two proprietary technologies for their delivery. We found nucleofection to facilitate the introduction of DNA (~50%), RNA (~50%) and RNPs (~90% TCR knockout) with high efficiency. Soluporation was investigated as a method to improve cell viability, however, the viability was comparable to electroporation (~90%), and dsDNA (<10%), RNA (~25% CAR-encoding circRNA) and RNPs (~50% TCR knockout) were introduced with reduced efficiency. Soluporation has been previously reported to introduce GFP-encoding mRNA (~75%), CAR-encoding RNA (~60%) and TRAC-targeted RNPs (~75% TCR knockout) to T-cells (Kavanagh et al., 2021). Whilst the efficiency of GFP-encoding mRNA transfection was comparable to our results (~50-75%), we observed significantly lower transfection efficiencies with CAR-encoding circRNA (~25%). The results indicate that the transcript length may negatively impact the transfection efficiency, as well as the structure of the nucleic acid.

Soluporation was ineffective at delivering dsDNA to T-cells. To extend the applications of soluporation to CRISPR/Cas9-mediated HDR, ssDNA could be investigated to mimic the structure of mRNA for transport through the membrane pores. However, strategies to recruit the HDR template into the nucleus will likely be required, as soluporation does not permeabilise the nuclear envelope. Alternatively, HDR templates could be delivered after soluporation of Cas9 RNPs, using AAV vectors. Nevertheless, soluporation requires
highly concentrated cargo in a small volume (50 µL) and an excess of three transfection sprays (150 µL) to prime the SOLUPORE® research tool. The associated cost of additional Cas9 nuclease is extremely high, therefore favouring alternative delivery tools, such as nucleofection, where the excess volume is kept to a minimum (cargo is premixed with the cells).

The impact of transfection on T-cells is an important factor to consider when selecting a delivery tool. Although nucleofection is a widely adopted technology in cell manufacturing, the process of electroporation affects the morphology of CD4+ T-cells, and can induce a calcium influx and activation-like state (upregulation of CD69 and CD154) (Zhang et al., 2014). Kavanagh et al., have compared the transcriptional profiles of T-cells at 6 hours post-soluporation or nucleofection; only 10 of 582 genes were differentially expressed in soluporated T-cells, compared to 265 of 582 genes in nucleofected T-cells. The mis-regulated genes included those associated with T-cell activation, exhaustion, metabolism, and apoptosis. The transcriptional profile of nucleofected cells was somewhat restored by 24 hours (Kavanagh et al., 2021). Despite temporary transcriptional changes, nucleofection was recommended as an efficient delivery approach that offered cargo flexibility for genome engineering applications. Alternatively, we could investigate the use of lipid nanoparticles (LNPs) to deliver circRNA to T-cells, which does not require nuclear entry for expression, to avoid the impact of the electrical pulse.

LNP delivery has become of increasing interest in recent years as a method to deliver mRNA ex vivo and in vivo. The main advantage of LNP-based delivery is the high efficiency of transfection (~90%) whilst preserving cellular viability (~80% at 24h) (Geczy, Reka, 2022). Billingsley et al., screened a library of LNPs and identified an optimal formulation for the delivery of CAR mRNA to primary T-cells. When compared to electroporation, CAR expression was comparable and cell viability was significant improved (~75% compared to 35%) (Billingsley et al., 2020). LNP-based engineering has shown promise to maintain the health and function of engineered T-cells by retaining a high proportion of T_{SCM} cells, reducing DNA damage, and increasing the capacity for expansion compared to electroporation (Schultes, Birgit, 2022). LNPs are a promising
delivery method for mRNA, however gene editing components, including DNA transposons and HDR templates, are required in the nucleus, therefore favouring the nucleofection technology which delivers two electric pulses to facilitate entry through the nuclear envelope.

### 7.1.1.2 Development of circRNA

We have explored the use of circRNA for applications to T-cell engineering. CircRNAs can be generated in vitro using a self-splicing PIE sequence and are resistant to exonuclease degradation (Wesselhoeft, Kowalski and Anderson, 2018). We discover that T-cells can tolerate the nucleofection of circRNA, which is modified in its structure, to enforce the expression of transgenes. Compared to the transfection of DNA, which resulted in substantial toxicity and cell loss, circRNA improved the transgene expression and cell viability, albeit with variability in expression between donors.

When compared to the linear counterpart in primary T-cells, the duration of CAR expression was extended from 3-4 days with linear mRNA to 4-7 days using circRNA. A previous study compared the translation efficiency of Gaussia luciferase-encoding linear or circular RNAs in HEK293T cells. In agreement with our data, comparison of the luminescence over time revealed prolonged luciferase activity from circRNA, which continued for 5-6 days, compared to 3-4 days with linear mRNA (Wesselhoeft, Kowalski and Anderson, 2018). Furthermore, Wesselhoeft et al., demonstrate that the circularisation efficiency decreases with the length of the coding sequence; the study reports ~70% circularisation of the Cas9 nuclease (5 kB). Here, we demonstrate that long transcripts, such as the BE4max sequence (6.4 kB), can be circularised with >75% efficiency, demonstrating the versatility of our approach for a wide range of transcripts.

Published studies describe applications of circRNAs as non-coding aptamers to manipulate cellular functions, as translatable templates for continued protein synthesis, and as immunogenic agents for vaccination (Costello et al., 2019; Litke and Jaffrey, 2019; Qu et al., 2022). The work in this thesis describes novel applications of translatable circRNAs in primary T-cells to enhance ex vivo cellular therapies. Circular RNAs may
encode transcription factors to promote specific cell phenotypes, anti-apoptotic proteins to improve T-cell survival, and gene editing components to facilitate gene knockouts.

With further research, the circRNA approach could facilitate targeted CAR integration, to circumvent the use of toxic dsDNA repair templates. CircRNA-encoding HDR templates could be co-delivered with a reverse transcriptase protein and Cas9 RNP, to facilitate reverse transcription and site-specific integration inside the cell. A primer binding site and RNA aptamer sequence can be included in the circRNA templates to recruit a reverse transcriptase fused to an RNA binding protein (RBP). Upon DSB formation, the reverse-transcribed cDNA can be inserted by HDR via flanking homology arms. Tethering components in this way has been demonstrated by the Pin-point base editing system, where the deficient Cas9 complexes with a guide RNA containing a recruiting RNA aptamer for the RBP-tethered cytidine deaminase (Collantes et al., 2021).

The circRNA approach has potential to improve the manufacture and efficiency of transient CAR T-cells, which can be generated ex vivo or in vivo using RNA-encapsulated LNPs (Mabry et al., 2022; Rurik et al., 2022). Production of circRNA circumvents the requirement for capping and polyadenylation to simplify the manufacturing process, as well as reducing the cost. CircRNA is more stable than linear mRNA and can enhance CAR expression to promote in vivo cytotoxicity, using an appropriate re-dosing regimen. This approach is particularly valuable where target antigens are also expressed at low levels on healthy cells, to reduce on-target off-tumour toxicity by allowing CAR activity to decay to extinction.

7.1.1.3 Alternative viral approaches

Non-integrating lentiviral vectors were evaluated in this study as an alternative transient approach. We used the D64V mutation to render the catalytic domain of the integrase protein inactive. Unfortunately, we observed a 2-fold reduction in viral titre of our CAR-encoding D64V mutant vector (1.23x10⁷ TU/mL), compared to the wild-type integrating vector (2.26x10⁷ TU/mL); however, this was in agreement with previous reports (Apolonia et al., 2007). Alternative mutants, such as the 12 bp U3 deletion described by Shaw et al., do not appear to impact the viral titre; however, the reduction in integrative
activity was less effective compared to D64V (2-fold compared to 1000-fold) (Apolonia et al., 2007; Shaw et al., 2017).

In rapidly dividing SUP-T1 cells, we observed transgene expression for up to 4 days using a CAR-encoding NILV (>90% CAR+ at day 2 decayed to <15% by day 4), whilst expression remained stable from the integrating counterpart. The duration of expression from the D64V mutant was shorter than that of published studies; Apolonia et al., show that GFP expression in HEK293T cells decayed from ~35% at day 2 to extinction by day 9 (Apolonia et al., 2007). These results demonstrate the transient nature of NILV in dividing cells. On the other hand, NILV vectors are suited to gene therapy applications in non-dividing cells, where the viral episomes are retained and expression is stable. Apolonia et al., show that GFP fluorescence steadily declines in dividing muscle cells (~40% GFP+ at peak to ~5% at day 12), but once muscle cells are differentiated to non-dividing myotubes, the GFP expression is retained (~30% GFP+ at day 12). The NILV expression profile differs based on the stability of the encoded protein and the rate of cellular proliferation.

Transduction by NILV was inefficient in T-cells and is only suited to applications which require a low level of transgene expression; we observed a 16-fold reduction in iRFP670 fluorescence from the D64V mutant compared to the integrating counterpart. To improve the expression from our non-integrating vectors, a scaffold attachment region could be incorporated into the transgene DNA to promote episomal replication (Jenke et al., 2004; F. Chen et al., 2017). Due to the low level and transient expression in primary T-cells, we recommend the use of NILV for the expression of genome engineering components, such as Cas9 and CBE proteins. This approach will minimise the risk of off-target editing. Recently, Whisenant et al., describe the use of NILV, generated using the proprietary LentiFlash® system, to transiently express base editors for the treatment of Hutchinson-Gilford progeria syndrome (Whisenant et al., 2022). The proprietary LentiFlash® system offers an alternative method for generating non-integrative vectors; the lentiviral particle is engineered to contain the bacteriophage MS2-coat protein to allow interaction with an RNA genome containing multiple copies of the MS2 RNA aptamer (Prel et al., 2015). Non-integrative retroviral particles (virus-like particles) have also been engineered to
package adenine base editor RNPs; the base editor protein is fused to the C-terminus of MLV gag polyprotein via a linker, which is cleaved by the MLV protease during particle maturation. The authors’ optimised VLP architecture has facilitated 45% (B2M) and 60% (CIITA) A > G base conversion in T-cells (Banskota et al., 2022).

NILV is costly to produce at clinical-grade and presents safety risks. Mutation of the attachment sites may improve the safety profile, to alleviate the risk of integrase mutants reverting to the wild-type phenotype. Different mutations are more effective than others; Shaw et al., describe a 12-base pair deletion in the U3 attachment site which reduces integration by 2-fold (Shaw et al., 2017), whilst dinucleotide mutations in the U3 and U5 regions (CA to TG) have been shown to reduce integration by 200-fold (Apolonia et al., 2007). Attachment site mutations have been combined with those disrupting the catalytic domain of the integrase (D64V), to reduce integration by 1000-fold, albeit a reduction in the transgene expression (Apolonia et al., 2007).

Nevertheless, we conclude that circRNA provides an inexpensive and safer alternative to NILV vectors for improved expression in primary T-cells. A summary of the explored approaches is provided in Table 26.
Table 26. Summary of transient delivery methods and their recommended applications.

<table>
<thead>
<tr>
<th>Method</th>
<th>Cargo</th>
<th>Therapeutic Yield</th>
<th>Throughput &amp; Scalability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleofection</td>
<td>DNA/RNA Protein/RNPs</td>
<td>- High efficiency (&lt;95%)</td>
<td>- Large-scale device available</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Requires a 4-day expansion period for recovery (~50% cell loss)</td>
<td>- Closed system</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Compatible with Miltenyi Prodigy</td>
</tr>
<tr>
<td>Soluporation</td>
<td>RNA Protein/RNPs</td>
<td>- Lower efficiency (&lt;70%)</td>
<td>- High cost associated with minimal cargo requirements</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Variable cell recovery (~25-75%)</td>
<td>- Large-scale device available (closed system)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Maintains T-cell phenotype</td>
<td></td>
</tr>
<tr>
<td>Circular RNA</td>
<td>RNA</td>
<td>- Higher transfection efficiencies compared to linear mRNA</td>
<td>- Circumvents the requirement for RNA capping/polyadenylation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Prolonged duration of expression</td>
<td>- Circularisation efficiency decreases with transgene size</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Donor-to-donor variability</td>
<td></td>
</tr>
<tr>
<td>NILV</td>
<td>RNA</td>
<td>- Poor transduction efficiencies in T-cells</td>
<td>- Difficult to scale for GMP manufacturing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Low level of expression</td>
<td>- High cost &amp; safety concerns</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Minimal cell loss</td>
<td>- Ease of transduction process</td>
</tr>
</tbody>
</table>

7.1.2 Applications of circRNA for T-cell reprogramming

7.1.2.1 Phenotype modulation by transcription factor expression

Two applications of circRNAs were investigated for T-cell reprogramming. The first approach involved the enforced expression of transcription factors, BACH2 and FOXO1, which were initially identified from a pool of fifteen candidates by lentiviral integration. Delivery of circRNA encoding either FOXO1 or BACH2 was sufficient to drive changes in the T-cell phenotype. BACH2 reduced naivety and enriched the TCM subset, whereas FOXO1 promoted the TCM subset and CCR7 expression in TN and TCM cells. A previous study describes the electroporation of eight transcription factor-encoding mRNAs to T-cells; LEF1, KLF7, ID3, EOMES, BCL6, TCF1, FOXP1 and FOXO1, and reports an increase in CCR7 expression by day 5 (H. Lu et al., 2020). This approach will result in a high translational burden and certain transcription factors may be disadvantageous; we found overexpression of EOMES and BCL6 in T-cells to promote the effector memory subset. Our approach has characterised the individual effects of transcription factors and has identified FOXO1 as the lead driver of T-cell naivety. FOXO1 has been shown to directly upregulate the expression of self-renewal genes (TCF7) and lymphoid homing markers, such as CCR7; which was confirmed in our results by an increase in the
receptor’s expression. Furthermore, FOXO1 promotes the expression of BACH2 and competitively binds to the genomic loci of AP-1 subunits, to prevent the expression of pro-differentiation genes (Delpoux et al., 2021). Combining transcription factors may not be necessary to enhance reprogramming, as FOXO1 controls multiple transcriptional networks.

Another interesting transcription factor that was identified in our lentiviral screen was TCF1 (encoded by *TCF7*). *TCF7* has also been highlighted in pooled knock-in screens; insertion of the transcription factor into the TRAC locus provided T-cells with a minor proliferative advantage under excessive stimulation conditions, which mirrors antigenic abundance in the tumour microenvironment (Roth et al., 2020). The *TCF7* knock-in also increased the number of CCR7+ T-cells in solid tumours, suggesting the transcription factor promotes a naïve T-cell state. Roth *et al.*, have taken forward this transcription factor for overexpression in HIV-specific CD8+ T-cells and have demonstrated an improvement in their proliferation upon adoptive transfer (Rutishauser *et al.*, 2021). Transcription factor insertions have also been screened in combination with different antigen specific TCRs or CARs. Using their modular screening platform, Blaeschke *et al.*, found the transcription factor AP4 to enhance long term T-cell fitness, and when combined in a single polycistronic construct with BATF, further enhanced the anti-tumour activity *in vivo* (Blaeschke *et al.*, 2022). The sustained expression of transcription factors may be detrimental to long-term T-cell function, either by preventing their differentiation to the cytotoxic effector subsets, or by inducing T-cell exhaustion due to sustained proliferation. Our circRNA approach will ensure the effects of transcription factors are transient and are not carried over into the patient.

Transcriptional reprogramming using circular RNAs was only observed in donors that were highly amenable to RNA transfection. The COVID-19 pandemic has accelerated the development of RNA therapeutics and study of the responses to vaccination have provided insights to the donor variability observed in this study. It was found that some individuals cannot elicit an immune response to mRNA vaccines without the inclusion of modified bases such as pseudouridine (Dolgin, 2021). Furthermore, published studies provide conflicting results regarding the immunogenicity of circRNAs, and highlight the
importance of the circRNA purity (Y. G. Chen et al., 2017; Wesselhoeft, Kowalski and Anderson, 2018). The observed donor variability could be attributed to differences in the innate sensing of exogenous circRNAs. Unfortunately, the addition of nucleoside modifications prevents the back-splicing of RNA, and has a negative impact on the translational capacity (Wesselhoeft et al., 2019). Alternatively, circRNAs can be purified from the immunogenic intron sequences by HPLC fractionation, and this may address the variability in expression between donors.

Nevertheless, our results indicate that transcription factor-encoding circRNA can modulate the expression of naïve and early memory markers, CD45RA and CCR7. Analysis of additional markers such as CD95 and CD62L will support the identification of the TSCM subset. Alternative methods to promote naivety during CAR T-cell manufacture include medium supplementation with cytokines (IL-7, IL-15 and IL-21) (Hinrichs et al., 2008; Gomez-Eerland et al., 2014; Xu et al., 2014; Alizadeh et al., 2019) and small molecules that inhibit GSK-3β and PI3K-AKT signalling (Gattinoni et al., 2009; Klebanoff et al., 2017; Mousset et al., 2018; Funk et al., 2022). In comparison, the circRNA approach provides greater specificity and can fine-tune T-cell signalling. Further work will be required to confirm the functional advantages of transcriptional reprogramming. RNA sequencing could be performed to identify changes in the expression of self-renewal genes and pro-differentiation factors, as well as the assessment of functional capacity. As noted by Gattinoni et al., in vitro assays alone may not identify functional advantages, and in vivo assays of persistence will likely be required (Gattinoni, 2005).

7.1.2.2 Disruption of T-cell inhibitory receptors by cytosine base editors

The second application of circRNA involved the expression of a cytosine base editor, BE4max. When paired with a sgRNA to direct point mutations to splicing sites or to introduce premature stop codons, base editing could be employed to disrupt the expression of inhibitory receptors that become upregulated on exhausted T-cells. We screened multiple sgRNAs targeting PDCD1, HAVCR2, LAG3 and TGFβR2, in primary T-cells using the circRNA approach. The optimal sgRNAs resulted in efficient loss of PD-1 (~73%), TIM3 (~64%) and LAG3 (~80%) protein expression and demonstrated
base conversion efficiencies of >75% (TGFβR2 by genotyping only). Multiplexing of the four gene targets resulted in comparable editing efficiencies and did not appear to impact the viability or proliferation of edited cells. Further investigation is required to assess the functional benefits of individual and multiplexed gene knockouts, alongside the assessment of genotoxicity.

Base editors can induce sgRNA-independent off-target deamination in both DNA and RNA, as well as unpredicted nucleotide conversions in the target sequence (Grünewald, Zhou, Iyer, et al., 2019; Zhou et al., 2019; Gaudelli et al., 2020). RNA and RNP delivery of the base editor has been shown to reduce genome-wide off-target deamination compared to plasmid or viral delivery, as RNA and protein is rapidly degraded in the cytoplasm (Rees et al., 2017; Villiger et al., 2021). As circRNA is more stable than linear mRNA, we hypothesised that circularisation would increase BE4max expression and improve base conversion efficiencies. We titrated the amount of circRNA, in aim to achieve efficient editing whilst preventing off-target deamination. We found that circularisation could facilitate at least an 8-fold reduction in the amount of RNA to achieve comparable HAVCR2 editing efficiencies. Although ~75% base conversion could be achieved using 0.50 µg circRNA, increasing the amount to 1.00 µg resulted in 100% conversion in three out of five donors. RNA circularisation shows promise to significantly lower the cost of base-edited CAR T-cell therapies by reducing the amount of GMP-grade RNA required for manufacturing. In addition to the reduction in RNA amount, circular RNA does not require capping or polyadenylation, hence, the cost of RNA synthesis is further reduced by approximately 2-fold.

A recent pre-clinical study has described the clinical-scale manufacture of quadruple-edited allogeneic anti-CD7 CAR T-cells (TRAC, CD52, PDCD1 and CD7) for the treatment of T-ALL (BEAM-201) (Diorio et al., 2022). Diorio et al., incorporate 5’ and 3’ UTRs into the sequence and in vitro transcribe BE4max-encoding mRNA using NEB’s HiScribe™ High-Yield Kit and TriLink’s proprietary CleanCap® technology, prior to purification by lithium chloride precipitation (Holtkamp et al., 2006; Vaidyanathan et al., 2018; Sample et al., 2019). The described process closely follows our protocol and therefore validates our RNA production strategy. Their study reports high editing
efficiencies of >90% using 2 µg of RNA per 1x10^6 T-cells in their initial experiments, which was reduced to 1 µg per 1x10^6 cells for their clinical product. In comparison, we generated PD-1-edited T-cells at scale (50x10^6 cells) using 0.25 µg of circRNA per 1x10^6 cells and observed 100% base conversion, suggesting we can lower the amount of RNA by at least 4-fold compared to the published study. Although only tested in one donor, these results highlight the high efficiency of editing in CD4+ and CD8+ selected T-cells.

7.1.3 Manufacturing genome-edited aCD30 CAR T-cells

Genome-editing can enhance the function of CAR T-cells through the ablation of inhibitory receptors such as PD-1 and TGFβR2, which has historically been achieved using CRISPR/Cas9 technology (Rupp et al., 2017; Tang et al., 2020; Alishah et al., 2021). However, multiplexed editing using Cas9 endonuclease results in the formation of multiple DSBs which can cause aneuploidy, chromosomal translocations, and significant genotoxicity (Kosicki, Tomberg and Bradley, 2018; Leibowitz et al., 2021; Nahmad et al., 2022). Base editing does not rely on the formation of DSBs, therefore offers a safer alternative for multiplexed genome engineering. This study combines base editing with the introduction of an anti-CD30 CAR for the treatment of Hodgkin lymphoma. To our knowledge, this thesis describes the first application of base editing in the context of an autologous therapy. An optimised anti-CD30 CAR construct was first identified, followed by the investigation into T-cell fratricide caused by CD30 expression, prior to the development of a scalable base editing manufacturing process.

7.1.3.1 Optimisation of the anti-CD30 CAR coding sequence

Firstly, we screened fourteen lentiviral CAR constructs which shared the anti-CD30 scFv binder but varied in their codon optimisation strategy, promoter usage (MND or human EF1α) and inclusion of the secreted IL-15. No differences were observed between the codon optimisation strategies or promoters, except when IL-15 was incorporated at the 5’ end, which dampened CAR expression. IL-15 could be incorporated to the 3’ end of the scFv without impacting CAR expression. The MP-codon optimised CAR was validated by assessing the cytotoxicity and release of effector cytokines during co-culture with three Hodgkin lymphoma cell lines (HDLM-2, L-540, L-428).
The benefit of incorporating IL-15 was investigated in a cytokine starvation assay, although minimal differences were observed between T-cells expressing the anti-CD30 CAR and those that were engineered to secrete IL-15. Hoyos et al., describe the inclusion of an IL-15 accessory module into the anti-CD19 CAR cassette, and report a 10-fold improvement in expansion upon co-culture with antigen-expressing target cells (Hoyos et al., 2010). In agreement with our study, we do not observe a significant proliferative advantage of CAR-IL-15 T-cells in the absence of antigen stimulation. To validate inclusion of the module, an in vitro restimulation assay is required; Hoyos et al., show that differences in expansion are only observed after five weeks of re-stimulation with antigen-expressing targets, at which point the proliferation of anti-CD19 CAR T-cells diminishes. It would also be interesting to monitor how the secretion of IL-15 changes with stimulation of the CAR; in the absence of antigen stimulation, we observe IL-15 secretion in the cell culture supernatant at ~770 pg/mL. In accordance with the published study, we would expect the secretion of IL-15 to increase after antigen stimulation, hence, improving the expansion of the T-cell culture. Secreted IL-15 can be presented to neighbouring T-cells and NK cells in trans, and in vivo assays will be required to assess the benefit of IL-15 secretion in the tumour microenvironment.

7.1.3.2 Addressing T-cell fratricide

We found that T-cells upregulate CD30 (up to ~37%) following stimulation through the TCR and CD28 co-receptor, which appeared to be positively correlated with the strength of the stimulation signal. Stimulation with soluble antibodies (aCD3 and aCD28) provided a weaker stimulation signal than TransAct™ and correspondingly resulted in a reduced frequency of CD30 upregulation. Other genome-edited cell products, such as the anti-CD3 and anti-CD7 dual-CAR approach described by Georgiadis et al., report base editing of endogenous CD3 and CD7 receptors to avoid fratricide upon introduction of the CARs. However, sufficient CAR+ T-cells could still be obtained in the in the CD7+ cohort albeit using an extended culturing period (Georgiadis et al., 2021). To prevent fratricide of the anti-CD30 CAR product, we investigated the knockout of CD30. Although multiple sgRNAs were screened, base editing of CD30 using BE4max (~35%) was inefficient compared to Cas9-mediated disruption (~80%). The inefficient editing
could be attributed to the scarcity of suitable target sites using the BE4max base editor and point towards the use of an alternative base editor with less stringent editing requirements, such as a wider editing window or different PAM usage (Anzalone, Koblan and Liu, 2020).

To assess the functional requirement of CD30 disruption, CD30-edited CAR T-cells (using Cas9 endonuclease) were compared to non-edited CAR T-cells. No differences in the cytolytic capacity were observed, however CD30 gene disruption reduced the proliferative capacity of the CAR T-cells, suggesting it is positively involved in this process. Interestingly, we did not observe CD30 upregulation in these donors, suggesting CD30 expression is dependent on multiple factors. Further work will involve transcriptional profiling to identify the functional role of CD30 and the downstream effects of CD30 disruption. Furthermore, ongoing studies involve the comparison of CD30-edited and non-edited CAR T-cells in an immunodeficient murine model engrafted with L-540 or L-428 cell lines.

7.1.3.3 Generation of base-edited aCD30 CAR T-cells

To address the challenges of T-cell inhibition and exhaustion and to improve the therapeutic efficacy of CAR T-cells, we aimed to combine base editing of inhibitory receptors with the introduction of the anti-CD30 CAR. We successfully manufactured \( \sim 40 \times 10^6 \) PD-1 base-edited CAR T-cells and achieved an editing efficiency of 100% and transduction efficiency of >55%. Further work will involve multiplexing the editing of \( PDCD1, HAVCR2, LAG3 \) and \( TGF\beta R2 \) and will compare the functional capacity of edited CAR T-cells \textit{in vitro}, by assessing their ability to eliminate tumours in the presence of the inhibitory ligands.

Prior to clinical translation, the individual and combinatorial effects of gene knockouts will be determined using \textit{in vivo} models, to assess gene targets relevant to Hodgkin lymphoma. Published studies using PDX models have shown limited benefits of PD-1 disruption, although, the clinical success of checkpoint inhibition suggests functional advantages may be observed in patients (Diorio \textit{et al.}, 2022). The response to checkpoint inhibition has provided further insights into the potential gene targets for multiplexed
editing. Patel et al., have investigated the classical Hodgkin lymphoma cell niche and have identified a population of CTLA-4+ cells in patients refractory to anti-PD-1 blockade (Patel et al., 2019). Hodgkin lymphoma patients may benefit from CAR T-cells containing the multiplexed knockout of PD-1 and CTLA-4.

Recently, the FDA has provided guidance on the release criteria of gene therapy products (Food and Drug Administration, 2022). The ex vivo-modified cell products must be evaluated for the on-target editing efficiency, off-target editing frequency, chromosomal rearrangements, quantitation of residual gene editing components and the number of genome-edited cells. Off-target editing must be assessed by multiple orthogonal methods, including an unbiased genome-wide assessment to identify the type, frequency and location of off-target events (Food and Drug Administration, 2022). The guide RNAs selected for our clinical study will be validated by bioinformatic prediction of the off-target sites, followed by the identification of off-target events by GUIDE-seq or CIRCLE-seq, alongside whole-genome sequencing. It may also be beneficial to sequence the RNA transcripts to detect off-target deamination by APOBEC1 (Zhou et al., 2019).

In summary, this thesis has explored the use of proprietary technologies to develop a circRNA delivery protocol for the application of T-cell reprogramming. Using the developed approach, we combined base editing with the introduction of an anti-CD30 CAR. Our T-cell product addresses key challenges limiting therapeutic success, including T-cell inhibition and exhaustion, in aim to improve CAR T-cell persistence and generate robust and durable responses in patients.
7.2 Future perspectives

The following section describes proposed areas for future work, including development of the delivery method, circRNA for \textit{in vivo} CAR-T, improvements to circRNA, strategies for combinatorial T-cell reprogramming and a discussion of the potential non-viral approaches for targeted CAR insertion.

7.2.1 Transient delivery methods

Our favoured approach to developing a base-edited CAR T-cell manufacturing process is to transfect cells with a sgRNA and circRNA encoding the BE4max CBE, to disrupt the expression of inhibitory genes, and subsequently transduce the cells with lentivirus carrying the CAR and accessory modules. As the approach involves the transfection of T-cells, it is possible to co-deliver additional RNAs encoding transcription factors or anti-apoptotic proteins to either modulate the phenotype of the cells (reprogram to a memory subset) or to prevent apoptosis. The current transfection method is nucleofection (electroporation), although, this method has its drawbacks as it causes transient activation of the T-cells, resulting in the release of intracellular Ca$^{2+}$ stores, and leads to substantial cell death. An alternative transfection method, soluporation, was investigated although it was found to have its limitations, notably lower transfection efficiencies compared with nucleofection, and incompatibility with the delivery of DNA. As both nucleofection and soluporation have their drawbacks, further work is required to optimise the delivery method. Alternative approaches could be LNPs or non-integrating virus, such as non-integrating lentivirus. We explored the use of non-integrating lentivirus to deliver genomes encoding fluorescent proteins, iRFP670 and mClover3, to T-cells and found the method to be inefficient. LNPs would be a preferable approach, as they can be functionalised to target specific cell types, making them compatible with \textit{in vivo} delivery, and they have a lower toxicity than other transfection methods.

7.2.2 \textit{In vivo}-generated CAR T-cells

LNPs can deliver mRNA to the cytoplasm of T-cells whilst maintaining the cell viability and phenotype. As they can be functionalised to target specific cell types, we recommend their application for the \textit{in vivo} delivery of circRNAs. A circRNA biotechnology
company, ORNA, are developing an in-situ CAR approach using LNPs encapsulating circRNA encoding the CAR sequence (Mabry et al., 2022). The circRNA approach will prolong the duration of CAR expression compared to linear mRNA, to enhance the cytotoxicity of in vivo-generated CAR T-cells. The transient CAR approach may improve the safety of treatment as the CAR will decay to extinction. As the CAR expression does not persist, the LNPs can be repeatedly dosed to improve the efficacy. LNPs have been specifically targeted to T-cells in vivo, for the treatment of chronic heart disease caused by fibrosis (Rurik et al., 2022). Rurik et al., target T-cells using anti-CD5-coated LNPs which release mRNA encoding an anti-fibroblast activation protein CAR. We suggest packaging antibody-directed LNPs with CAR- and/or accessory molecule-encoding circRNA to provide additional functions to CAR T-cells. Accessory molecules may include transcription factors, anti-apoptotic proteins, genome editing components or cytokines to recruit and activate resident immune cells. This approach will bypass the requirement of ex vivo cell manufacturing, significantly reducing the cost, and instead will require the characterisation of mRNA encapsulated LNPs prior to in vivo delivery.

### 7.2.3 Improvements to circRNA

This thesis investigates the applications of circRNA, with initial work comparing its stability to unmodified linear mRNA. Work by Kariko and Weissman et al., has revolutionised the field of mRNA therapeutics by the inclusion of nucleoside modifications to enhance RNA stability (Karikó et al., 2005, 2008). The benefit of nucleoside modifications has come to light through the study of responses to COVID-19 vaccination, where modified mRNA has led to improved immune responses (Dolgin, 2021). To expand our knowledge of circRNA, a comparison to modified linear mRNA would be desirable. Published studies have addressed this question; Wesselhoeft et al., demonstrate that circRNA provides superior protein expression to unmodified and nucleoside-modified linear mRNAs (pseudouridine, 5-methycytosine). In HEK293T cells, the production of luciferase from circRNA was improved by 811.2% compared to unmodified mRNA, or by 54.5% compared to nucleoside-modified mRNA at 24 hours post-transfection (Wesselhoeft, Kowalski and Anderson, 2018).
An alternative method to enhance the translation efficiency of circRNAs is to engineer the sequence. Chen et al., have improved the translation efficiency of circRNAs by several 100-fold through optimisation of the IRES, the inclusion of 5’ and 3’ UTRs and synthetic aptamers to recruit translation initiation machinery. The optimized human erythropoietin-encoding circRNA (based on the group I intron of T4 phage) contained the 5’ UTR poly(A)-binding protein motif and the human α-globin 1 3’ UTR, as well as a human rhinovirus B3 IRES engineered with an eIF4G-recruiting aptamer to aid localisation of the ribosome. Upon in vivo delivery, circRNA led to an initially lower level of protein compared to N1-pseudouridine-modified linear mRNA, however, the linear mRNA expression declined within 48 hours and circRNA expression remained stable for 96 hours (Chen et al., 2022).

Unmodified circRNA has been shown to elicit an immune response in various cell lines; circRNA resulted in a significantly stronger IFN-α and IL-6 cytokine response in A549 cells compared to an un-transfected control, as well as the induction of IFN-β1 (30-fold) and RIG-1 transcripts (3-fold) in HeLa cells (compared to nucleoside-modified linear mRNA) (Wesselhoeft, Kowalski and Anderson, 2018). Although circRNA does not contain the triphosphate motif required for RIG-1 activation, RIG-1 may distinguish foreign circRNA from self-circRNA by the lack of host nuclear proteins (Y. G. Chen et al., 2017; Wesselhoeft et al., 2019). To reduce the immunogenicity of circRNAs, modified nucleosides could be incorporated during in vitro transcription. Pseudouridine and 5-methylcytidine modifications can reduce the immunogenicity of exogenous RNAs by preventing RIG-1 and Toll-like receptor signalling and may address the donor-to-donor variability observed in this study (Karikó et al., 2005, 2008).

Previous reports have attempted the incorporation of N1-methylpseudouridine modifications to circRNA generated by the PIE method. A 100% replacement of uridine abolished ribozyme activity and prevented circularisation, whilst partial replacement reduced the back-splicing efficiency (Wesselhoeft et al., 2019). The published study investigated an alternative method of circularisation using T4 RNA ligase I and oligo splints to bring the 5’ and 3’ ends of the transcript into close proximity (Petkovic and Müller, 2015). The 1.5 kB transcript was successfully circularised with 40% efficiency,
however, modified circRNA failed to express the luciferase protein in HEK293T or A549 cells (Wesselhoeft et al., 2019). In comparison to nucleoside-modified linear mRNA, unmodified circRNA provided prolonged expression and a reduced cytokine response, indicating that circRNA does not require modification. The immunogenicity was restored upon linearisation of circRNA by heat and RNase H treatment, as determined by the activation of TLR8 reporter cells, indicating that the circular conformation is involved in TLR evasion (Wesselhoeft et al., 2019).

Wesselhoeft et al., highlight the importance of RNA purity to further reduce immunogenicity, which may arise by the presence of intron sequences, derived from the Anabaena cyanobacterium, and dsRNA. Upon the delivery of intron- or circRNA-containing HPLC fractions, Wesselhoeft et al., have demonstrated that the non-circular components of the splicing reaction significantly contribute to immunogenicity in A549 cells (Wesselhoeft et al., 2019). Proposed methods to remove non-circular RNA include treatment with RNase R, however, reports have shown that RNase R can also degrade circRNA and severely impact RNA yield (Zhang, Yang and Chen, 2021). Whilst RNase R treatment alone is insufficient to prevent immunogenicity, HPLC purification of the circRNA fraction offers a more practical solution and can confirm RNA identity by transcript length. However, as HPLC separates impurities based on size, it may be challenging to distinguish nicked circRNA. To further improve the purity, HPLC-purified RNA can be treated with phosphatases to remove contaminating triphosphates (present on precursor RNA), which can stimulate RIG-1 responses. As shown by Wesselhoeft et al., phosphatase treatment was highly effective in reducing the immunogenicity of circRNA; the secretion of IL-6, IFN-α, TNF-α and IFN-γ inducible protein 10 was reduced to baseline levels in A549 cells transfected with HPLC-, phosphatase- and RNase R-treated circRNA compared to HPLC- and RNase R-treated circRNA (Wesselhoeft et al., 2019).

The process outlined in this thesis can support GMP-grade RNA synthesis through use of NEB’s GMP-compliant nucleosides and HiScribe™ T7 Transcription Kit (our proposed method is described in Figure 7.2). We require a high yield of circularised product and note that longer transcripts are more challenging to circularise due to the distance between
the 3’ and 5’ introns and the reduced ability to form a splicing bubble. In our experience, we can successfully circularise a transcript of >6 kB with ~75% efficiency (encoding the BE4max CBE). To improve the circularisation efficiency, we suggest separating the base editor protein into the N- and C-terminal regions and incorporating intein sequences to enable self-assembly inside the cell (Villiger et al., 2021). Furthermore, Wesselhoeft et al., demonstrate the reduction in splicing efficiency from ~95% to ~70% with transcripts ranging from 1.1 kB (GLuc) up to almost 5 kB (Cas9). After RNase R treatment, the Cas9-encoding circRNA yield was reduced to ~20%, indicating a high proportion of nicked transcripts (Wesselhoeft, Kowalski and Anderson, 2018). Whilst we do not determine the portion of nicked circRNA, nicking would likely occur within the base editor sequence and would abolish the protein activity. The highly efficient editing in our study indicates a low proportion of nicked circRNA. To translate the circRNA approach to the clinic, several release criteria must be met, including, validation of the RNA identity by RT-PCR and sequencing, evaluation of RNA integrity by capillary gel electrophoresis, the quantification of endotoxins, residual DNA template, dsRNA and host proteins, and the assessment of RNA sterility.

![Figure 7.2 Schematic of circRNA manufacturing.](image)

The stability of RNA can be enhanced by the circularisation of transcripts containing the PIE sequence derived from the cyanobacterium, Anabaena. Firstly, RNA is in vitro transcribed from a linearised DNA template (generated by enzymatic digestion or PCR), prior to degradation of the DNA template by DNase treatment. RNA is purified by lithium chloride precipitation and circularised by heating to 55˚C for 15 minutes in a buffer containing magnesium ions and GTP. After purification, RNA is analysed on the TapeStation to confirm the RNA transcript length
and integrity. If removal of the intron sequences is required, RNA can be purified by high performance liquid chromatography.

### 7.2.4 Combinatorial T-cell reprogramming

The work in this thesis highlights two potential applications of circRNAs to enhance the function of CAR T-cell therapies. The cHL tumour microenvironment poses multiple challenges to treatment including physical barriers to T-cell homing, an infrequent population of target cells (~1%) and the induction of immunosuppressive signalling. These factors lead to premature differentiation, T-cell inhibition, and poor persistence. Alvarez-Fernandez et al., demonstrate the functional advantage of a high proportion of T<sub>SCM</sub> cells in the anti-CD30 CAR product (Alvarez-Fernández et al., 2021). Transcriptional reprogramming could be combined with the disruption of inhibitory receptors by base editing, to generate an anti-CD30 CAR product with optimal phenotype, fitness, and function.

T-cell differentiation is controlled by the graded expression of opposing transcription factors. Hence, we could employ base editing to disrupt pro-differentiation genes and/or transcription factors, combined with the simultaneous delivery of circRNAs encoding those to promote self-renewal. Additional gene targets could include PRDM1, which encodes for BLIMP-1, for combination with circRNA-encoding BCL-6. Yoshikawa et al., show that genetic ablation of PRDM1 supported the expansion of early memory CAR T-cells in vivo, which improved cytolytic capacity in multiple adoptive immunotherapy models (Yoshikawa et al., 2022). Other combinations could include the disruption of ID2 and delivery of ID3 to promote naivety (Yang et al., 2011), or the disruption of T-bet combined with the overexpression of EOMES to enhance the T<sub>CM</sub> subset (Kaech and Cui, 2012).

Other targets to control T-cell differentiation include TET2 and the suppressors of cytokine signalling (SOCS) family of proteins. Insights from an anti-CD19 CAR T-cell study revealed that the complete remission of one patient was accompanied by the expansion of CAR T-cells originating from a single clone. Lentiviral vector-mediated insertion of the CAR transgene had disrupted the methyl cytosine dioxygenase TET2
gene, and led to the formation of a central memory T-cell phenotype (Fraietta, Nobles, et al., 2018). On the other hand, SOCS can attenuate the activity of cytokines to regulate the formation of Tregs and the maturation of CD8+ T-cells. Cytokines belonging to the γc family (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21) transduce signals through STAT phosphorylation, which are directly and indirectly regulated by SOCS proteins (Palmer and Restifo, 2009). Generally, STAT3 phosphorylation promotes naivety whilst STAT4 activity enhances differentiation. SOCS family members could be disrupted and combined with STAT3 overexpression to dictate the differentiation of CAR T-cells and to modulate cytokine secretion.

To combine our circRNA protocols, timing is a key factor. To minimise the negative impact of nucleofection, circRNA-encoding transcription factors could be co-delivered with base editor proteins. Alternatively, as our transcription factor-encoding circRNAs contain a nuclear localisation sequence, we could use a less invasive transfection method such as lipid nanoparticles, and “re-boot” exhausted T-cells at the beginning of the manufacturing process or prior to CAR T-cell cryopreservation.

7.2.5 Targeted CAR insertion

The proposed plan for developing base-edited CAR T-cells is to introduce CBE mRNA by nucleofection and then transduce the T-cells to introduce the CAR sequence. A targeted approach would be more appropriate and leverage the transfection method used to introduce the mRNA/RNP complex and components required for the insertion of the CAR coding sequence at the same time. The CAR could be inserted by CRISPR/Cas9-mediated HDR, or alternatively using novel genome editing technologies which combine Cas effector machinery with transposases or serine recombinases. The section below describes the latest advances in targeted CAR insertion.

7.2.5.1 CRISPR-directed transposition

The bacterial CRISPR/Cas system provides sequence-specific adaptive immunity for the defence against invasive genetic elements by nucleolytic degradation. However, recent studies have discovered nuclease-deficient Cas effectors which have been co-opted by transposases to enhance the mobility of transposons into specific target sites. Two
subtypes of CRISPR-associated transposon (CAST) systems have been described and facilitate RNA-guided targeting of Tn7-like transposons. The type I systems contain components of the Cascade effector, whilst type V-K systems utilise a catalytically inactive Cas12k protein (Figure 7.3). In these systems, the inactive Cas protein binds to target DNA through the complementary gRNA to form an R-loop, thus enabling transposases to act upon the displaced ssDNA.

The Tn7 transposon from *E. coli* Tn7 mediates non-replicative cut-and-paste transposition using the transposase proteins TnsA, TnsB and TnsC. TnsA and TnsB are enzymes which excise the transposon via DSBs, TnsB further mediates DNA integration and TnsC is an ATPase that co-ordinates transposase/transposon complex formation (Peters, 2014). Together, these proteins associate with TnsD, a DNA-binding protein that directs the transposon to the chromosomal homing site (attTn7), or TnsE, which facilitates transposition into mobile genetic elements and plasmids (Saito *et al.*, 2021).

The Tn7-like CAST systems lack TnsE and instead use TniQ (a homolog of *tnsD*) which acts as an adaptor between the Cas effector and the transposase/transposon complex to target transposition using acquired spacers in the CRISPR array (Halpin-Healy *et al.*, 2020; Petassi, Hsieh and Peters, 2020). Through the study of *Vibrio cholerae* Tn6677 transposon in *E. coli*, Klopme *et al.*, have identified the type I-F RNA-guided complex known as Cascade and have demonstrated its ability to integrate genetic payloads of varying length at targeted sites in the bacterial genome. The maximal expression was observed with a 775 bp transposon and resulted in 25% integration (Klompe *et al.*, 2019).

Strecker *et al.*, have characterised a novel type V-K CRISPR-associated transposase derived from *Scytonema hofmanni* cyanobacterium. The ShCAST complex consists of the Cas12k effector bound to Tn7-like transposase subunits (TnsB/TnsC/TniQ) (Strecker *et al.*, 2019). ShCAST does not contain TnsA and cannot facilitate DNA cleavage to release the transposon from the genome, thus indicating transposition using an alternative pathway. Instead, ShCAST utilises a direct replicative copy-and-paste mechanism enabling duplication of the transposon. Insertion sites were defined as segments of DNA 60-66 bp downstream of the PAM and efficiencies of up to 80% were achieved in the *E.*
coli genome (Strecker et al., 2019). Type V-K systems do not encode a recombinase; hence, duplications are thought to arise by homologous recombination, requiring host DNA repair machinery. Work has been carried out to study the structural basis of target site selection and has revealed the interacting roles of TnsB/TnsC/TniQ proteins (Park et al., 2021; Querques et al., 2021).

The Cas12K systems provides the advantage of a reduced gene requirement compared to Cascade systems and therefore requires a smaller coding size. However, studies have
reported an additional type I-F system that contains a single TnsAB polypeptide with endonuclease- and integrase-family domains to mediate non-replicative cut-and-paste transposition (Vo et al., 2021). Typically, these systems rely on the co-transfection of multiple transposon/transposase plasmids. Alternatively, the DNA-editing all-in-one RNA-guided CRISPR-Cas transposase (DART) system has been engineered as a single plasmid for delivery to microbial communities to probe the role of bacterial species by species- and site-specific genome editing (Rubin et al., 2022). This work highlights a promising avenue for streamlining the components required for high-fidelity RNA-guided DNA integration.

Similar systems have been adopted for mammalian expression by combining the Cas9 effector with an engineered PB transposase. This approach harnesses the transposon payload cassette size to facilitate the precise integration of long DNA sequences. The Find and Cut-And-Transfer (FiCAT) technology consists of a nuclease-proficient Cas9 protein fused to a C-terminal PB transposase. The PB transposase contains mutations (R372A, K375A, D450N) to reduce DNA binding and increase excision, hence, promoting targeted insertion and preventing random integration. Editing is achieved by co-delivery of FiCAT-encoding mRNA or plasmid DNA with the transposon DNA containing the transgene flanked by ITRs. Although deficient Cas9 and nickase variants were tested, efficient integration relied upon nuclease activity and suggested a role of DSBs for on-target activity. The developed technology was compared to Cas9-based HDR using truncated GFP (tGFP)-encoding transposons varying in size from 2.5 to 9.5 kB, for insertion at the AAVS1 safe harbour site. FiCAT was able to correct GFP expression in an engineered tGFP HEK293T reporter cell line and showed higher editing efficiencies of up to 2-fold whilst maintaining accuracy. Editing was further demonstrated in human fibroblast and mouse myoblast cell lines and in vivo in mouse liver (Pallarès-Masmitjà et al., 2021).

Further studies report transposase-CRISPR mediated targeted integration (TransCRISTI) in the human genome using a fusion of Cas9 endonuclease and a double mutant PB transposase. Two Cas9-PB effector molecules regulate the excision of the transposon from the donor plasmid through binding to the ITRs. The transposon is then shuttled to
the genomic gRNA binding site where a DSB is formed to initiate transposition. Although TransCRISTI knock-in efficiencies could be enriched to 72% following antibiotic selection, editing efficiencies in HEK293Ts remain low at approximately 4% GFP knock-in to the AAVS1 safe harbour site (Rezazade Bazaz, Ghahramani Seno and Dehghani, 2022).

7.2.5.2 CRISPR-directed serine recombinases

The CRISPR/Cas9 system has been widely adopted for genome engineering applications by exploiting the repair mechanisms of DNA DSBs, however, the ability to target large DNA sequences into the genome is limited by the efficiency of homologous recombination. On the other hand, the SB and PB transposase systems offer a large cargo capacity, however, their integration profile is relatively uncontrolled. CRISPR-associated transposases and serine recombinases offer alternative mechanisms that exploit the precision of Cas9, combined with the ability to scale the cargo size. The development of CRISPR-associated serine recombinases was driven by the recent discovery of prime editor technology.

Prime editors (PEs) have been developed to replace short DNA sequences at specified locations in the genome by fusion of a catalytically inactive Cas9 endonuclease to an engineered reverse transcriptase. The enzyme is programmed to the target site through design of the prime editing guide RNA (pegRNA) that also acts as a template for copy into the genome. The reverse transcription template encodes the desired edit and can include point mutations, deletions of up to 80 bp and insertions of up to 44 bp. Upon binding of the prime editor-pegRNA complex to the target DNA, the PAM-containing strand is nicked by the Cas9 endonuclease, allowing for hybridisation of the primer binding site (PBS) in the pegRNA. The bound PBS therefore primes reverse transcription of the pegRNA template for copy into the genome. The first-generation PE (PE1) has evolved into new and improved PE iterations by installing mutations in the reverse transcriptase to improve editing efficiencies (PE2), or by incorporating additional nicking sites to initiate DNA repair and installation of the edit (PE3). In contrast to HDR, this system does not require the formation of DSBs or the delivery of a DNA repair template. PE3 can efficiently correct for pathogenic human variants with efficiencies of
approximately 30-50% in HEK293Ts, with significantly reduced off-target indels compared to Cas9 HDR (Anzalone et al., 2019). PE4 and PE5 iterations have further improved editing outcomes of PE2 and PE3 by the co-expression of dominant negative MLH1 to evade DNA mismatch repair which can inhibit prime editing and generate off-target indel by-products (P. J. Chen et al., 2021).

Building upon PE technology, Programmable Addition via Site-specific Targeting Elements (PASTE) was developed as a method to expand the payload size. The DNA PASTE system consists of a Cas9 nickase fused to a reverse transcriptase and serine recombinase and can facilitate the introduction of DNA sequences up to 36 kB in size, albeit low editing efficiencies in the range of 10-50% in human cell lines and 5% in primary cells (Yarnall et al., 2022). Serine recombinases typically insert sequences containing attP attachment sites into regions of target DNA containing attB landing sites. Through design of the pegRNA, PEs can install the 46 bp attB landing sites at targeted locations for recognition by the Bxb1 integrase. In comparison to HDR, PASTE mediated GFP insertion into the N-terminus of ACTB and LMNB1 with comparable or lower efficiencies respectively, however, the generation of off-target indels was significantly lower. Integration by PASTE is independent from DSB repair pathways and can facilitate gene integration in dividing and post-mitotic cells, providing an advantage over Cas9-mediated HDR which is dependent on cellular division. To evaluate the payload limitations, cargos of up to 13.3 kB in size were tested in dividing and non-dividing cells and resulted in at least 10% integration. Efficiencies can be improved by delivering larger template DNA amounts, however at the expense to cellular viability (Yarnall et al., 2022).

Further developments include the generation of twin prime editors (twinPE) which have expanded the capability of replacing large DNA sequences. TwinPES require two pegRNAs to template the synthesis of DNA on the opposing strands, resulting in the replacement of the DNA sequence between the two PE strand-nick sites (~100 bp). The inserted sequences can encode one or multiple recombinase recognition sites which can be used as substrates for Bxb1. This approach has facilitated a 5.6 kB sequence replacement at the AAVS1, CCR5 and ALB loci in HEK293T cells with efficiencies of approximately 6% (Anzalone et al., 2022). Although CRISPR-associated integrases can
be used to deliver large cargos, editing in human cell lines and primary cells occurs at approximately 5-6% efficiency.

Whilst further development is required to improve the efficiency of CRISPR-associated transposases or recombinases, these approaches are a promising future avenue for targeted CAR insertion in primary T-cells. When combined with the transient expression of circRNAs encoding transcription factors or cytosine base editors, the non-viral manufacture of next-generation T-cell products can be achieved.

7.3 Final conclusions

This thesis has explored non-viral delivery approaches for the improvement of cellular therapies. CircRNA was identified as a suitable substrate to prolong transgene expression whilst maintaining cellular viability, unlike the transfection of DNA or linear mRNA that resulted in substantial toxicity and a low level of transgene expression, respectively. Novel SOLUPORE® technology (developed by Avectas) was evaluated in comparison to Lonza’s 4D Nucleofector™. Although the technology could facilitate the delivery of linear and circular RNA, transfection efficiencies were lower than those of nucleofection, and cell viability and recovery were comparable. Two applications of the developed circRNA approach were investigated to address the challenges of premature T-cell differentiation and exhaustion. CircRNA facilitated the introduction of transcription factors, BACH2 and FOXO1, to drive early memory formation, and the expression of the BE4max cytosine base editor. Using the established RNA protocols, we combined base editing with the introduction of an optimal anti-CD30 CAR, for the treatment of Hodgkin lymphoma. Our circRNA approach facilitated at least an 8-fold reduction in the amount of CBE-encoding RNA required for sufficient base editing compared to linear mRNA and will significantly reduce the cost of manufacturing. Transcriptional engineering and base editing can be combined to develop next-generation CAR T-cell products with improved persistence to revolutionise the treatment of haematological and solid malignancies.
Chapter 8. Appendix

Figure 8.1 Comparison of protein expression from integrating and non-integrating lentiviral vectors.

Comparison of mClover3 (mClover) expression from non-integrating vectors in SUP-T1 cells and PBMCs. (a) Flow-cytometry plots (FSC-A versus mClover3) are shown for one representative donor in the live (SYTOX-Blue-, SUP-T1) or the CD3+ compartment (PBMC) at 48h post-transduction. (b) Transduction efficiency (left) and MedFI of mClover3 expression (right), represented as mean ± SD (SUP-T1, n=1 sample; PBMC, n=3 independent biological donors, represented as different symbols). (**p ≤ 0.01, ***p ≤ 0.001). Integrating lentivirus (INT LV), non-integrating lentivirus (NINT LV).
Figure 8.2 Co-transduction of BACH2 and FOXO1 lentiviral supernatants.
Efficiency of co-transduction in T-cells using an MOI of (a) 3 or (b) 6 virions per cell per lentiviral supernatant. Constructs encoded either an mClover3 (mClover) or iRFP670 (RFP) transduction marker alone or in combination with BACH2 or FOXO1. Flow-cytometry plots (iRFP670 versus mClover3) are shown at 72h post-transduction for one representative donor (n=3 independent biological donors).
Figure 8.3 Expression of gene targets in cell lines after treatment with PMA and ionomycin

(a) Expression of PD-1 in MOLT-4 cells or (b) TIM3 in RPMI-8226 cells following stimulation. 0.5x10^6 cells were treated with phorbol myristate acetate (PMA) and ionomycin (40.5 µM PMA and 669.3 µM ionomycin) or dimethyl sulfoxide (DMSO) only as a negative control. Flow-cytometry was performed at 24h post-stimulation. MOLT-4 cells can be stimulated with PMA
and ionomycin to upregulate the expression of PD-1 whereas RPMI-8226 cells do not further upregulate TIM3 expression upon activation. Non-activated (NA), PMA and ionomycin (P+I).
Figure 8.4 Generation of MOLT-4 and RPMI-8226 cell lines that stably express BE4max.

Transduction efficiencies of MOLT-4 and RPMI-8226 BE4max cell lines. 1x10^6 MOLT-4 or RPMI-8226 cells were transduced with 2 mL of supernatant in 6-well plates by spinoculation with RetroNectin. Transduction efficiencies were determined through detection of the V5 tag by flow-cytometry at 72h.
Figure 8.5 Phenotyping stimulated MOLT-4 cells for base editing of PDCD1.

MOLT-4 cells constitutively expressing V5-BE4max were nucleofected with sgRNAs targeting PDCD1. Four days after nucleofection, half of the cells were stimulated with PMA and ionomycin and 24h later flow-cytometry was performed to identify gene knockouts. (a) Flow-cytometry plots (FSC-A versus PD-1-PE) for the stimulated samples at day 5 are shown. PD-1 knock-out was detected in the transduced population by gating on the V5+ population (V5-APC). Non-activated samples were used as gating controls (data not shown). (b) Table displaying the sgRNA sequence names. (c) Percentage of transduced cells expressing PD-1 at day 5 post-nucleofection (n=1). Webber E1 SD (sgRNA-2) and Webber E2 pmSTOP sgRNA sequences
(sgRNA-4) (Webber et al., 2019) enabled a reduction in PD-1 expression from approximately 65% to 36%.
Figure 8.6 Phenotyping unstimulated RPMI-8226 cells for base editing of HAVCR2.

RPMI-8226 cells constitutively expressing V5-BE4max were nucleofected with sgRNAs targeting HAVCR2. Five days post-nucleofection, TIM3 knockout was detected by flow-cytometry. (a) Flow-cytometry plots (FSC-A versus TIM3-BV421) at day 5 are shown. TIM3 expression was analysed on the transduced population by gating on the V5+ population (V5-APC). (b) Table showing the sgRNA sequence names. (c) Percentage of transduced cells expressing TIM3 at day 5 post-nucleofection (n=1). The SPLICER E2 SA sgRNA sequence (sgRNA-1) enabled a reduction in TIM3 expression from approximately 55% to 23%.
Figure 8.7 Optimising the purification of BE4max cytosine base editor RNA.

Base editor linear and circular RNA was *in vitro* transcribed from 1 µg linearized plasmid. Capped linear mRNA was produced using the mMESSAGE mMACHINE T7 Transcription Kit (mM) and was polyadenylated using the Invitrogen Poly(A) Tailing Kit. Uncapped linear RNA was transcribed using the HiScribe RNA Synthesis Kit (HS) and was subsequently capped using vaccinia capping enzyme and 2′-O-methyltransferase to generate cap 1 mRNA. To produce circular RNA, the HiScribe RNA Synthesis Kit was used to produce uncapped RNA which was circularised by resuspending RNA in a buffer containing GTP and magnesium ions and incubating the RNA at 55°C for 15 minutes. Two different methods for RNA purification were tested. RNA was either purified using the MEGAclear Transcription Clean-Up Kit (MC) which utilises RNA-binding columns, alternatively, RNA was resuspended in lithium chloride (LiCl) for precipitation. RNA that was purified using the MC method appeared to be sheared as demonstrated by smearing on the Agilent TapeStation electrophoresis image. The preferred method for RNA purification was LiCl precipitation.
Figure 8.8 Linear versus circular RNA-encoding BE4max for base editing of *PDCD1*.

Base editing of stimulated T-cells using the sgRNA that targets the SD site of E1 in *PDCD1*. 4 days after co-transfection of the sgRNA and 0.25 µg-4.00 µg circRNA-encoding BE4max, half of the cells were re-stimulated with TransAct™ and IL-7/IL-15 to identify gene disruption. (a) Flow-cytometry plots (FSC-A versus PD-1-PE) at 24h after re-stimulation, one representative donor is shown. (b) PD-1 expression in re-stimulated cells at day 5 post-nucleofection and corresponding C > T base conversion (day 7), represented as mean ± SD (*n=3* biological donors, represented as different symbols, from two independent experiments). (Two-Way ANOVA; *p* < 0.05).
Figure 8.9 Base conversion of *PDCD1* using linear or circular BE4max-encoding RNA.

Genotyping of base edited T-cells using increasing amounts of BE4max CBE-encoding linear or circular RNA. Electropherograms of the converted sequence are shown for one representative donor. Greater editing was observed with circular BE4max compared to the linear counterpart. The base editing window is indicated in a red box and the target base is highlighted using a red triangle. Adenine (green), thymine (red), cytosine (blue), guanine (black).
Figure 8.10 Genotyping T-cells for base editing of \(PDCD1\) using linear or circular BE4max-encoding RNA.

Genotyping base-edited T-cells using an increasing amount of BE4max-CBE encoding linear or circular RNA at 7 days post-engineering. A region spanning E1 of \(PDCD1\) was amplified from the genomic DNA, Sanger sequenced and analysed using EditR software. Base conversion
efficiencies are shown for all cytosines in the protospacer, represented as mean ± SD (n=2 biological donors from two independent experiments), with the target cytosine highlighted in red.
Appendix

Figure 8.11 Soluporation of base editor RNA for gene knockout of HAVCR2.

Base editing by soluporation of linear CBE-encoding RNA. 6x10^6 stimulated T-cells were co-transfected with 180 pmol of NT sgRNA or TIM3 sgRNA (targeting SA site of E2), 24 µg of BE4max CBE-encoding linear mRNA and 6 µg mClover3 (mClover) linear mRNA. The RNA mix was suspended in 12% ethanol for delivery by soluporation in a final volume of 50 µL. Four days after engineering, half of the cells were re-stimulated with TransAct™ and IL-7/IL-15. Flow-cytometry was performed 24h later to identify gene knockouts. Co-transfection of mClover3 RNA enabled identification of the transfected population for subsequent gating. (a) Flow-cytometry plots (FSC-A versus TIM3-BV421) at 24h post-stimulation to show TIM3 expression in the un-transfected (mClover-) and transfected populations (mClover+). (b) TIM3
expression in re-stimulated cells at day 5 post-nucleofection, gated on the transfected population where appropriate \((n=1\) biological donor). A 30% reduction in TIM3 expression was observed. Un-transfected (UT), no cargo (NC).
Figure 8.12 sgRNAs designed for gene knockout of TNFRSF8 using BE4max.

sgRNAs were designed using SpliceR or the CRISPR RGEN BE-Designer Tool and are compatible with cytosine base editors that use the SpCas9 NGG PAM variant. The target base within the guide RNA sequence is shown in red and potential by-stander cytosines are shown in orange. The optimal sgRNA is highlighted in green. Premature stop codon (pmSTOP), splice donor (SD), splice acceptor (SA).
Figure 8.13 Screening guide RNAs for base editing of TNFRSF8 using circular base editor RNA.
Base editing of stimulated T-cells using sgRNAs that disrupt splicing sites or introduce pmSTOP codons into the \textit{TNFRSF8} gene. Four days after co-transfection of the sgRNA and circRNA-encoding BE4max, half of the cells were re-stimulated with TransAct\textsuperscript{TM} and IL-7/IL-15 to identify gene disruption. (a) Flow-cytometry plots (FSC-A versus CD30-PE) at 24h after re-stimulation, one representative donor is shown. (b) Table displaying the single guide RNA sequence names. (c) CD30 expression in re-stimulated cells at day 5 post-nucleofection and corresponding base conversion at the target cytosine (day 7), represented as mean ± SD (\textit{n}=3 biological donors, represented as different symbols (left)). CD30 expression was minimally upregulated in no pulse samples. T-cells that received the NT sgRNA expressed CD30 in 34% of the population, and this was reduced to 19% using sgRNA-7 that targets the SD in exon 10.
Figure 8.14 Genotyping T-cells for base editing of TNFRSF8 using circular BE4max-encoding RNA.

Genotyping base-edited T-cells at 7 days post-engineering. A region spanning each target site was amplified from the genomic DNA of non-edited or edited T-cells and was sent for Sanger
sequencing and analysis using EditR software. Base conversion efficiencies are shown for all cytosines in the protospacer, represented as mean ± SD (n=3 biological donors), with the target cytosine highlighted in red. The most optimal guide RNA was sgRNA-7.
Figure 8.15 Monoclonal antibody generation using hybridoma technology.

The generation of hybridomas begins with the vaccination of rats with DNA plasmid encoding the antigen of interest (aCD30 CAR). The antibody-secreting B-cells are isolated from the rat spleens and fused with myeloma cells to generate an immortal antibody-secreting cell line. Single cell dilutions are prepared to isolate hybridoma clones, which secrete antibody into the cellular supernatant. Cellular supernatants are screened by flow-cytometry using a cell line engineered to express the antigen. The positive clones are expanded, and the cellular supernatant containing the anti-idiotype antibody is harvested and frozen for downstream applications.
Figure 8.16 Identification of hybridoma clones secreting an anti-idiotype aCD30 CAR antibody.

A SUP-T1 cell line was engineered to co-express eGFP and the anti-CD30 scFv on the cell surface to allow screening of the hybridoma cellular supernatants. (a) Diagram of the construct for transduction of the SUP-T1 cell line. The construct consists of a 5-prime LTR followed by the packaging signal of Moloney murine leukaemia virus (MMLV) (Ψ), the MMLV pol region containing the splice acceptor site (Pol), the anti-CD30 scFv sequence, CD19 transmembrane (TM) domain and a scaffold/matrix attachment region (SAR) which leads into the 3’ LTR. Flow cytometry plots (anti-CD30 CAR versus eGFP) to demonstrate aCD30 CAR staining using (b) sCD30-Bio and streptavidin-PE or (c) neat hybridoma cellular supernatant and an anti-Rat-PE secondary antibody. In a total of 880 clones, 2 clones were identified as antibody-secreting (2A11 and 10C2), an example is shown for one clone that failed to produce an aCD30 CAR antibody (1C10).


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