Editing T Cell Specificity

Roua Abdullah Alsubki

Institute of Child Health
University College London

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Declaration:

I, Roua Abdullah Alsubki, confirm that the work presented in this thesis is my own. Where information has been extracted from other sources, I confirm that this has been directed in the thesis.

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Abstract:

Allogeneic hematopoietic stem cell transplantation is considered to be the main means of treatment for hematological malignancies. However, disease relapse and graft versus host disease remain the major cause of death post transplantation. To reduce the risk of graft versus host disease and in order to improve the graft versus leukemia effect, genetically engineered T-cells are used to express tumor specific antigens. This is either through the transfer of a recombinant antigen-specific T cell receptor (TCR) or through the introduction of antibody-like recognition in chimeric antigen receptors (CARs) toward tumor-associated antigens. These methods have made substantial advances. Nevertheless, the complexity in modifying and producing autologous specific T-cell products for each patient is a major barrier to the broader application of this approach. In this context, the ability to generate an "off-the-shelf" mismatched donor-derived therapeutic T cell product was investigated. To overcome HLA barriers and to eliminate the risk of graft versus host disease, tailored transcription activator-like effector nucleases (TALENs) knocking out the expression of endogenous T cell receptor was utilised. Also, the potential of engineered meganucleases and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) to disrupt the genomic sequence of the T cell receptor was evaluated.

Large numbers of recent clinical trials have suggested that *in vivo* persistence and expansion having a potent anti-tumor activity of the genetically engineered T cells is crucial to have a robust clinical response. In order to generate a T cell product possessing these previous features, we have investigated the ability to engineer naïve cord blood T cells toward specific tumor antigens. Due to their naivety, the higher telomeres activity, low graft-versus-host-disease, amongst several other features, have the potential of making cord blood an optimal source for the production of a universal allogeneic engineered T cell therapy. Moreover, preclinical models have demonstrated that culturing naïve T cells in the presence of interleukin-7 and interleukin-15 might retain the modified cells in their naïve like phenotype.

In conclusion, delivery of CD19CAR genes using lentiviral vectors into naïve cord blood T cells could form the basis of generating a universal cell bank of therapeutic T cells against B cell lymphoma. With further optimisation to improve efficiency, this could be combined with TALENs for site-specific disruption of the endogenous T cell receptor to eradicate the risk of graft-versus-host disease.

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Abbreviations:

Acute myeloid leukaemia (AML)

Adult peripheral blood mononuclear cells (PBMC)

Alpha single constant gene (Cα)

American Society of Hematology (ASH)

Antigen-processing (TAP)-deficiency

Capsid (CA)

Carboxy-anhydrase-IX (CAIX)

Chimeric Antigen receptor (CAR)

Complementarity-determining regions (CDRs)

Cord blood T cells (CB-T cells).

Cord blood transplantation (CBT)

Cytotoxic T lymphocytes (CTL)

Deoxyribonucleic acid (DNA)

Diversity (Dβ) J gene segments (Jβ)

Double strand break (DSB)

Epstein Barr virus (EBV)

Epstein-Barr virus specific cytotoxic T cells (EBV-CTLs)

Envelope protein (env).

Glycoproteins 120 and 40 (Gp120 and gp41)

Graft-versus-host disease (GVHD)

Hematopoietic stem cell transplantation (HSCT)

Hematopoietic stem cells (HSCs)

Homologous recombination (HR)

Human immunodeficency (HIV-1) virus

Human leukocyte antigen (HLA)

Immunoreceptor tyrosine-based activation motifs (ITAMs).

In-vitro transcription (IVT)

J gene segments (Jα)

Major histocompatible (MHC)

Matrix (MA),

Megnaucleases (MGN),

Moloney murine leukemia virus (MMLV)

Myelodysplastic syndrome (MDS)

Negative regulatory factors (nef)

Neucleocapsid (NC) proteins. Integrase (IN).

Non-homologous recombination end joining (NHEJ)

Protospacer adjacent motif (PAM)

Polyethylenimine (PEI) optimum

Pre-integration complex (PIC) Long-terminal repeat sequences (LTRs). Double strands (ds)

Protease (pro)

Recombination signal sequence (RSS)

Recombination-activating gene 2 (RAG1 and RAG2)

Regulator of expression of virion proteins (rev)

Reverse transcriptase (RT) enzyme

Ribonucleic acid (RNA)

Severe combined immunodeficiency (SCID-X1)

Single chain antibody (scFv)

Single beta constant gene 1 and 2(Cβ1 and Cβ2)

Small interfering RNA (siRNA)

Spleen focus-forming virus LTR (SFFV)

Surface protein (SU)

Group-specific antigen (gag)

Polymerase (pol)

T cell receptor (TCR)

T helper 1 (Th1)

Trans membrane (TM)

Transactivator of transcription (tat)

Transcription activator-like type III effector (TALE) nucleases (TALENs).

Transduced (TD)

Un-transduced (UT)

Repeat-variable di-residue (RVD)

Transfer RNA (tRNA)

Tumor reactive infiltrating T lymphocytes (TIL)

Tumor-associated antigens (TAA)

Tumour necrosis factor α (TNF α)

Type 1 cytotoxic T (Tc1)

Variable (V), diversity (D) and joining (J)

Vesicular stomatitis virus (VSV-G Self-inactivating (SIN)

Viral protein R (vpr)

Viral protein U (vpu)

Virion infectivity factor (vif)

Wilms tumour antigen-1 (WT-1)

Wiskott-Aldrich syndrome (WAS)

Woodchuck hepatitis virus post transcriptional regulatory element (WPRE)

Zinc finger nucleases (ZFNs)

Chapter 1: Introduction

Over the past two decades, the transplant of allogeneic hematopoietic stem cells has become a successful therapeutic option for relapsed hematological malignancies in high-risk leukemic patients (Weber et al., 2009). However, the difficulty in finding matched HLA-donors, coupled with the high risk of graft-versus-host disease (GVHD), remains a significant therapeutic limitation (Khouri et al., 2001). Thus, novel, more efficient and less toxic remedies are required to eliminate cancerous cells without any relapse or side effects.

Evidence suggests that the anti-tumour immune surveillance system in the human body recognises tumour-associated antigens (TAA) - proteins expressed by malignant cells - and discriminates them from normal and healthy cells. This provides a solid foundation to develop new, robust and active therapies for cancer such as Adoptive-T cell therapy (Morris et al., 2006). Adoptive cellular therapy uses autologous or allogeneic T cells, with highly specific tumour recognition, and expands them *in vitro* then they are administrated back to the patients, with the aim of targeting cancerous cells and killing them specifically (Grupp and June, 2011). It has been successful in achieving tumour regression in transplant-related malignancies, leukaemia, and melanoma. For example, Rosenberg successfully performed Adoptive-T cell immunotherapy in patients with advanced metastatic melanomas. Tumour reactive infiltrating T lymphocytes (TIL) were isolated from surgically removed tumours, expanded *in vitro* and re-infused back into the patients conditioned by treatment with immunosuppressant drugs (Figure 1). The results of this trial demonstrated the clinical benefit of adoptively transferring T cells in approximately 50% of the patients (Rosenberg and Dudley, 2004). Although Adoptive-T cell immunotherapy has led to remarkably successful outcomes in treating certain categories of malignancies, the inability to isolate and expand a sufficient number of high-avidity specific T lymphocytes for therapeutic purposes is the major obstacle to the success of the therapy (Aqui and June, 2008).

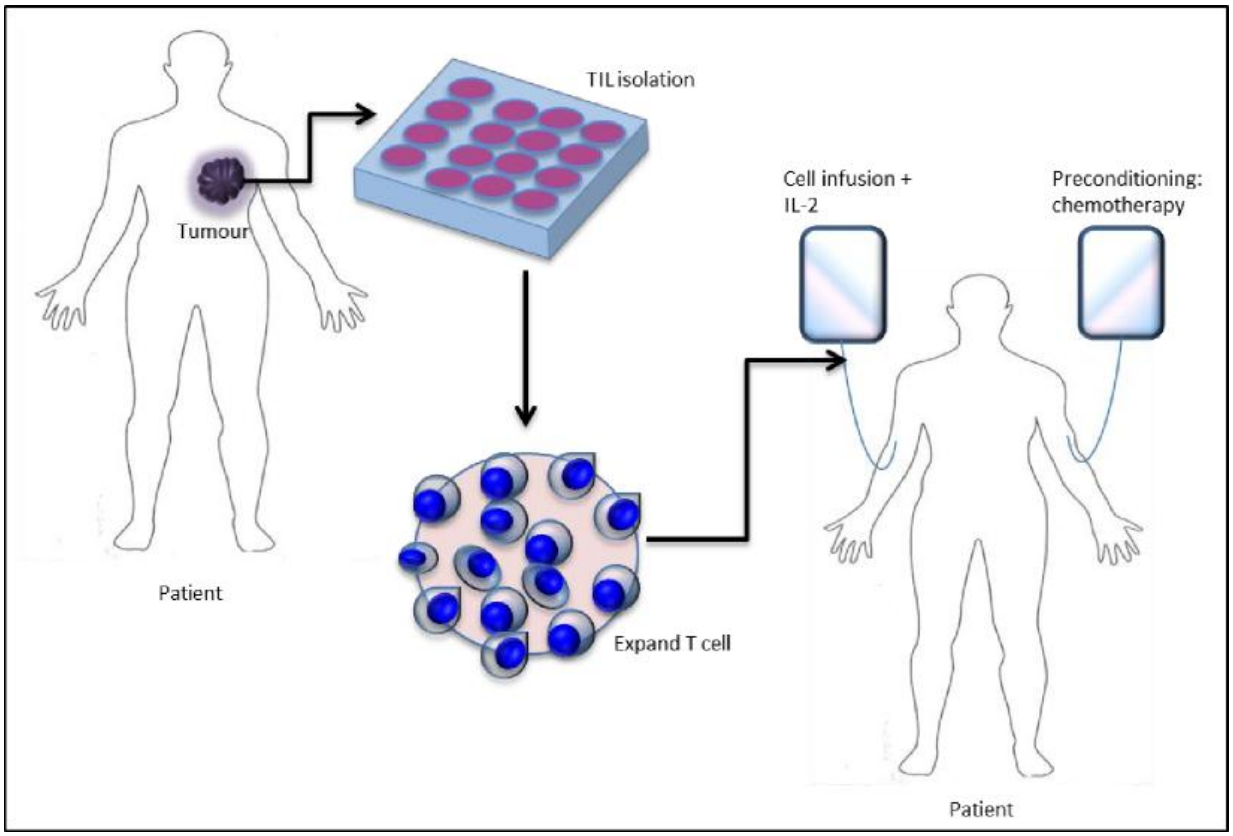


Figure 1: Clinical application of gene modified T-cells. Diagram illustrates the usage of natural T cells for treatment of cancer.TIL=tumour infiltrating lymphocytes.

An alternative approach to generate large numbers of potent and specific-redirected T cells is to genetically engineer T lymphocytes to express an antigen-specific T cell receptor (TCR), which is called TCR therapies, or protein-infused chimeric antigen receptors (CARs) (Figure 2). Consequently, the immune reactivity will be redirected towards a defined TAA. Genetically engineered T lymphocyte not only has the potential to redirect T cells' specificity toward tumors, it also has the potential to improve natural immune response and correct impaired immunity. These approaches could overcome the fundamental limitations associated with central and peripheral tolerance, and produce more efficient redirected-T cells that will specifically target tumours without the requirement for *de novo* T-cell activation in the patient (Sharpe and Mount, 2015).

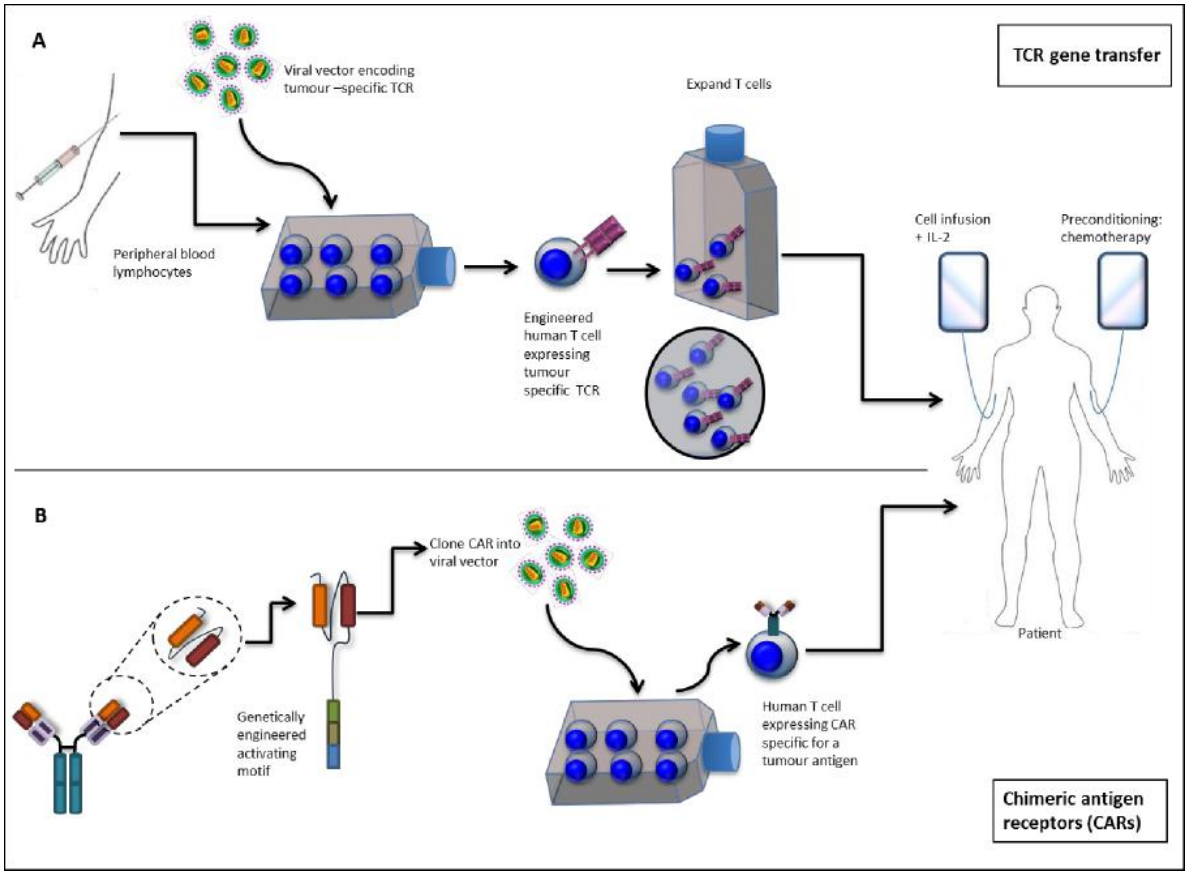


Figure 2: Different ways to genetically engineer T cells to confer specificity on tumour-associated antigens. Current trials have shown several ways in which T cells can be genetically engineered to recognise tumour-associated antigens: i) allogeneic T cells can be transduced with viral vector-encoding, tumour-specific TCR, resulting in the production of T cell populations expressing tumour-specific TCR; ii) sequences encoding the variable regions of antibodies are engineered to encode a single chain, which is then genetically grafted onto the TCR intracellular domains that are capable of activating T cells. These CARs have antibody-like specificities, which enable them to recognise MHC-unrestricted structures in the surfaces of target cells.

In 1986, Dembic and colleagues were the first group to demonstrate the feasibility of redirected T cell antigen specificity in favour of tumour-specific antigens (Dembic et al., 1986). Based on this concept, several scientific groups conducted trials attempting to prove the feasibility and safety of antigen-specific T cell therapy.

In addition to discussing the structure and gene rearrangement of TCRs and CARs, the next section will review several successful clinical trials that have utilised antigen-specific T cell therapy.

1.1 T-lymphocytes:

1.1.1 Structure of T cell receptors (TCRs):

T lymphocytes recognise a major histocompatible (MHC) epitope complex via a multi-chain glycoprotein expressed on their surface. This glycoprotein is known as T cell receptor. TCR plays a crucial role in T cell activation, antigen specificity and T cell survival. In 1996, monoclonal antibodies targeting a cloned T cell line were used in order to identify the structural basis of TCR. This study of clonotypic antibodies concluded that TCR is a member of the immunoglobulin gene super family, and it is a heterodimer molecule composed of two covalently linked, transmembrane polypeptide chains, denoted α and β (Abbas et al., 2000). An analysis of the amino acid sequences of different αβ TCRs demonstrated that each chain was composed of: i) an amino-terminal, variable domain (V); ii) a constant region (C), and; iii) a short hinge region of cysteine residue, which forms a disulphide bond. Moreover, this sequence analysis revealed that TCR is anchored to the cell membrane by hydrophobic, short trans-membrane domain ends in a cytoplasmic tail that contributes to the signal transduction process (Abbas et al., 2000). TCR's combining-antigen binding site, the buried surface of the variable region, encompasses hyper-variable loops known as complementarity-determining regions (CDRs) 1, 2, and 3 within both the Vα and Vβ regions, and an additional loop termed HV4 on the β chain. CDRs are responsible for direct recognition of different antigens and binding to the MHC peptide complex. Sequence examination of CDRs revealed that CDR1 and CDR2 regions are less variable than CDR3. This result suggested that CDR1 and 2 preferentially bind to the MHC helices and provide a constant interaction point between MHC and TCR, while CDR3 binds to the peptide presented in the MHC groove (Garcia et al., 1999). TCR is a multi-component assembly of α and β chains and four other membrane proteins: CD3ε, CD3δ, CD3γ and an invariant ζ chain. The CD3εδγ-ζ complex promotes the stability and cell surface expression of TCR, and also contributes to carrying out the signaling transduction mechanism on antigen recognition by TCR.

TCR crystallization studies revealed another intra-cellular component of CD3 complex attached to TCR, known as immunoreceptor tyrosine-based activation motifs (ITAMs). ITAMs play a significant role in the intra-cellular activation of the T lymphocyte and involve the phospholyrating of two tyrosine residues on the binding of TCR with peptide MHC complex, leading to the generation of docking sites for transduction molecules. These initiate downstream signaling and eventually the activation of T lymphocytes (Cantrell, 1996) (Figure 3).

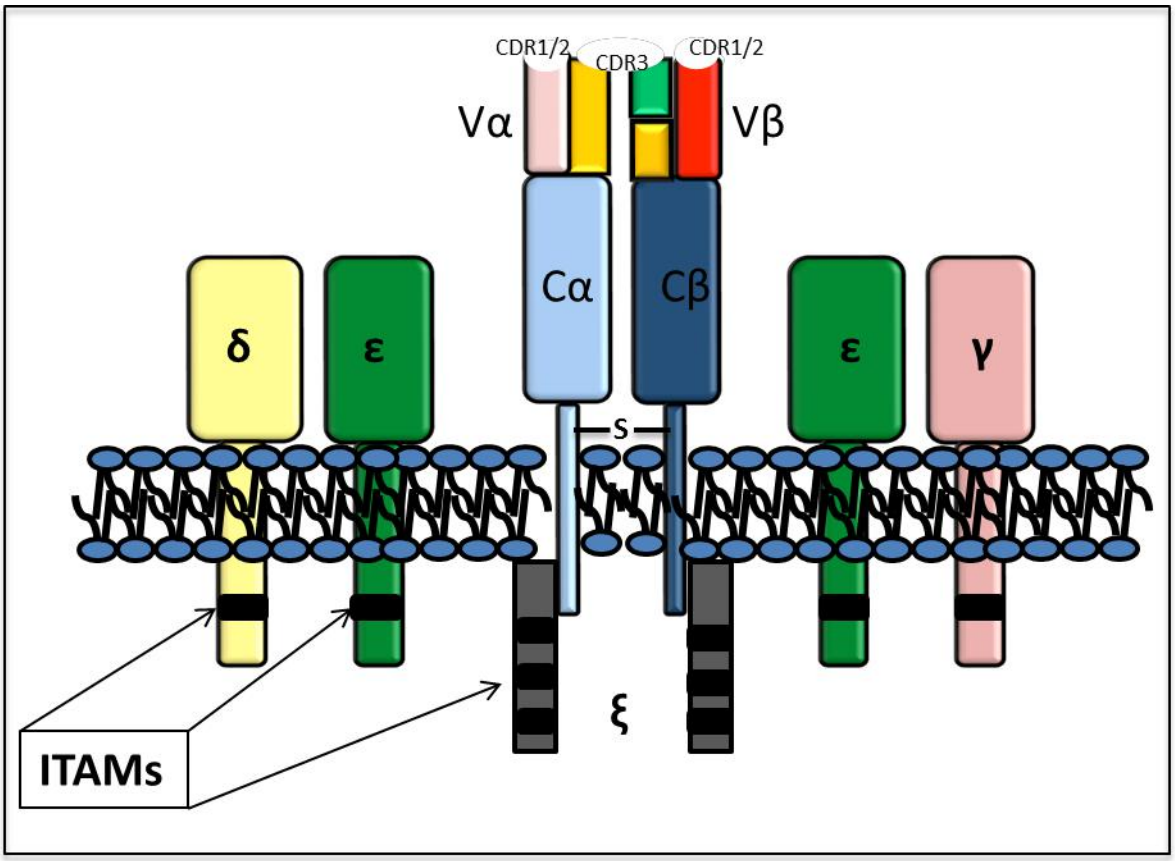


Figure 3: Schematic representation of TCR-CD3/ ξ complex on the T cell's surface.TCR is a disulphide-linked heterodimer αβ chain. The α chain contains a constant domain (light blue) and a variable region (pink and yellow), and the β chain is composed of a constant region (dark blue) and a variable domain (green, yellow and red). The aβ TCR is not covalently linked to CD3 proteins (CD3δ (yellow), CD3ε (green), CD3 γ (pink), and the ξ chain (grey)). The ITAM regions (black horizontal blocks) are phospholyrated at specific residues creating a docking site for downstream effectors leading to T cell activation. The CDRs consist of complementarity-determining regions 1, 2, and 3 within both the Va and Vβ regions, and are responsible for the direct recognition of different antigens and binding to the MHC peptide complex. ITAM= immunoreceptor tyrosine-based activation motifs

1.1.2 TCR Gene Rearrangement:

Given the importance of highly diverse repertoires of lymphocyte receptors in the protection against infection, a complex genetic mechanism has evolved for producing highly variable proteins. The TCR antigen recognition variable region is encoded by a combination of discontinuous, multiple gene segments known as variable (V), diversity (D) and joining (J) for the TCR β chain, or V and J segments only for TCRα. These genetic segments are assembled together via a somatic DNA rearrangement mechanism during T lymphocyte maturation to form a complete V region sequence.

Gene segments encoding a TCR α locus are located on chromosome 14. The variable domain is encoded by 70–80 gene segments named as variable or V gene segment Vα and a cluster of 61 joining or J gene segments (Jα) followed by a single, constant gene (Cα) expressing the constant region. In contrast, the TCR β locus, located on chromosome 7, has a different organization, with a cluster of 52 Vβ segments positioned far away from another two separated gene clusters, each composed of a single diversity (Dβ) gene segment and 6–7 J gene segments (Jβ), followed by single constant genes (Cβ1 and Cβ2) (Jung and Alt, 2004, Yoshikai et al., 1985).

TCR gene rearrangements take place in the thymus during T cell development, and the beta chain locus undergoes genetic recombination before the alpha locus. At some point during T cell differentiation, Dβ juxtaposes with one of the 6–7 Jβ segments forming DβJβ recombination, and this is followed by rearrangement of one of the 52 Vβ segments with the DβJβ to assemble a complete, variable gene exon abbreviated as VβDβJβ. Subsequently, the DNA sequence will undergo a transcription process leading to the formation of a primary nuclear transcript that contains a non-coding sequence between VβDβJβ and Cβ1. In the mature form of mRNA, the non-coding sequence will be spliced out to give rise to VβDβJβCβ mRNA, which is then ready for translation. Once a functional β chain has been rearranged, genetic recombination of the α chain begins. Alpha locus genes will rearrange via a homologous mechanism to the gene recombination of the beta locus. This is initiated by the recombination of one segment of 70–80 Vα and one of the 61 Jα genes forming VαJα, which is then transcribed into RNA including Cα. After splicing the introns, Cα will become proximal to VαJα and will form a complete mRNA which is ready for translation (Blom et al., 1999) (Figure 4). The V(D)J recombination process is regulated by two important components: a specific DNA sequence that flanks gene segments ready to recombine, known as the recombination signal sequence (RSS), and V(D)J recombination enzymes, encoded by two genes, recombination-activating gene 1 and recombination-activating gene 2 (RAG1 and RAG2) (Leiden et al., 1988).

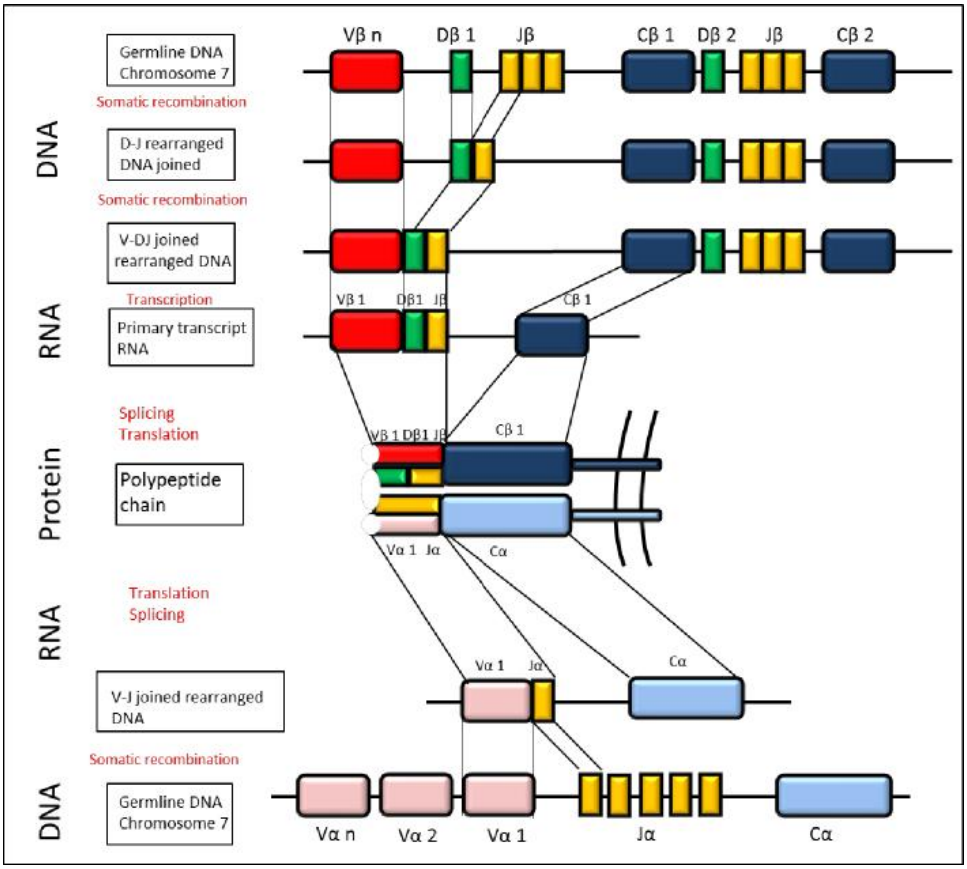


Figure 4: T cell receptor chain rearrangement and expression. The TCR α and β genes are the result of somatic recombination of various gene segments: variable segment (V: red and pink blocks), diversity segment (D: green block), joining segment (J: yellow blocks). For the β chain (upper part of the figure) a Dβ gene segment rearranges to form a Jβ segment, creating a DβJβ segment that will combine with one of the Vβ to create a functional V region exon. Transcription and splicing of the VβDβJβ exon to Cβ generates mRNA that is translated to yield a TCR β chain protein. For the α chain (lower part of figure), a Vα gene segment rearranges to form a Jα gene segment to create a functional V region exon. Transcription and splicing of the VαJα to Cα generates mRNA that is translated to yield the TCR α-chain receptor. The α and β chains pair soon after this synthesis to yield an αβ TCR heterodimer.

1.2 T-cell immunotherapy:

The elimination of non-self molecules that are expressed not only by the virally infected cells, but also by transformed (tumor) cells is one of the immune system's functions. Through an immune surveillance system, immune cells have the ability to recognise the non-self and neoantigens and to destroy them. However, cancerous cells have developed strategies to escape and suppress the immune system, such as downregulating the expression of neoantigen; hence, they can evade immune cell detection (Pinzon-Charry et al., 2005, Blankenstein et al., 2012). As the field of immunology continues to developed, a large number of innovative therapies are being discovered that utilise the immune cells and optimise their specificity toward specific diseases through genetic modifications (Katsnelson, 2013). Over the past two decades, proof-of-concept studies have demonstrated the potential of immune-cell therapies against malignancies, based on editing the specificity of immune

cells toward specific tumor associated antigens (TAAs). Immune cells that have been widely used as therapeutic tools are included in natural killer cell therapies (Cheng et al., 2013), dendritic cell therapies (Vacchelli et al., 2013) and T cell immunotherapies (Maus et al., 2013). Due to the remarkable clinical success of genetically modified T cell immunotherapies in treating certain forms of cancer, the main focus of this project is to improve their treatment potential and to evaluate the ability to generate large numbers of T cell therapeutic products to be used in the human clinical field.

1.2.1 Immune Surveillance Hypothesis:

"It is no means inconceivable that a small accumulation of tumour cells may develop and because of their possession of new antigenic potentialities provoke an effective immunological reaction with regression of the tumour and no clinical hint of its existence "

By sir Frank Mac Farlane Burnet

Several decades ago, the idea that the immune system could potentially identify and eradicate cancerous self-cells from the host, was first proposed by Paul Ehrlich. In 1957, the formal hypothesis of cancer immune surveillance was proposed by Sir Macfarlane and Lewis Thomas. Their concept demonstrates that adaptive immunity is a primary line of defense against cancer and it is responsible for preventing cancer development in the host (Schreiber et al., 2011). In mice, immune surveillance was directly demonstrated especially with the advent of the knock out technologies; however, this is not the case in humans. Although natural immune surveillance is difficult to examine in human cancers, there are now significant studies to strongly support this hypothesis in humans (Reiman et al., 2007). One group argued that the direct correlation between non-viral tumour incidence and immune-competence status of transplanted patients might be a proof supporting the immune surveillance notion (Birkeland et al., 1995). Others postulate that the correlation between patient survival and the quantity, quality and spatial distribution of tumour-infiltrating T lymphocytes (TILs) might be the strongest evidence of cancer immunoediting in humans. The presence of cytokines that promote cancer control such as interferon gamma (INF-γ) and tumour necrosis factor alpha (TNF-α) has been linked with having a better outcome for patients with different types of malignancies. The presence of elevated levels of antibodies and T cell reactions against tumour associated antigens in cancer patients might be considered another indication of immunoediting in humans (Schreiber et al., 2011). Dunn and his colleagues have suggested that transplant recipients maintained on

immunosuppressants, and individuals with primary immunodeficiency disease are at higher risk of developing malignancy (Dunn et al., 2002).

In 2001, the concept that the immune system not only protects the human body against tumour formation, but also shapes tumour immunogenicity has been evaluated. Cancer immunoediting proceeds sequentially through three phases; elimination, equilibrium and escape: "the three Es of cancer immunoediting". The elimination phase is the process in which both innate and adaptive immunity are working together to detect and destroy the presence of a developing tumour before it becomes clinically apparent. However, if rare cancer cell variants survive the elimination phase, they enter the equilibrium phase. In the equilibrium phase, the outgrowth of tumour cells is prevented by adaptive immunity and maintained in a functional state of dormancy. Finally, tumour cells that have the ability to circumvent immune recognition and elimination and still emerge as a growing and visible tumour, proceed to the escape phase (Dunn et al., 2004). Now with the understanding of the cancer immunoediting concept, and the clarification of the molecular and cellular mechanism of the three Es, it should be feasible to explore new cancer immunotherapies with high efficiency and safety standards (Hanahan and Weinberg, 2011).

1.2.2 Tumor associated antigens (TAA):

The identification of a variety of tumour antigens in human and animal cancers has provided a solid foundation for cancer immunotherapy. Tumour antigens are peptides derived from proteins that are processed in the cytosol and expressed by neoplastic cells due to some genetic alternating events such as, mutations or chromosomal rearrangements (Morris et al., 2006).

Based on their patterns of expression, tumour antigens can be divided into two categories:

One category is called tumour specific antigens (TSAs); which are novel non-self-antigens that are expressed only by malignant cells, but not by normal cells. On the other hand, the tumour antigens that are expressed by both self and malignant cells are called tumour associated antigens (TAAs); these antigens are normal cellular components whose expression is abnormal or deregulated in tumours (Van den Eynde and van der Bruggen, 1997).

The modern classification of tumour antigens is based on the molecular structure and source of antigens. Some tumour antigens originate due to mutation in oncogenes or tumour suppressor genes such as mutation in Ras gene or p53 tumor suppressor gene. Other malignant peptide-derived proteins may be the product of alteration in cellular genes not involved in tumourgenesis such as, different mutant proteins in melanomas. Some tumour

antigens may be normal cellular constitutes that are overexpressed or aberrantly expressed in neoplastic cells; for instance, Wilm's Tumour antigen-1 (WT-1) that is overexpressed in a number of malignancies such as leukaemia (Abbas et al., 2014).

For tumour associated antigens to be ideal candidates for immunotherapeutic approaches, they should be selectively expressed in tumor tissue and detectable in most patients. For example, NY-ESO-1 cancer-testis antigens, are neoantigens that are only expressed in neoplastic cells and testis; hence, they are among the most ideal candidates for immunotherapeutic applications (Chen et al., 1998). Some studies suggest that most TAAs have broader expression in healthy tissue; as a result, using these antigens as targets of an immune response might lead to the induction of autoimmune phenomena. Although this risk must be taken very seriously, several animal studies have shown that there might be a difference in the susceptibility of normal and tumour tissue to the effector arms of immune response (Krackhardt et al., 2002).

1.2.3 Adoptive T-cell Therapy:

Traditionally, there have been three means to treat malignancies: surgery, chemotherapy and radiotherapy. Although to date these are the most beneficial treatments, in the medical field as remedies for cancer, they suffer from limitations; such as killing the dividing, functional normal cells; blocking the cell cycle and having severe implications in the proliferation of normal cells (Perica et al., 2015). In hematological settings, allogeneic hematopoietic stem cell transplant (HSCT) is considered the standard treatment to mediate the graft-versus-leukaemia effect (GVL) and hence improve a patient's conditions (Kennedy-Nasser and Brenner, 2007). However, the difficulty in finding a HLA-matched donor, the lethal consequences of graft versus host disease (GVHD), and the risk of relapse after disease treatment are some critical unresolved limitations of HSCT. As a result of these serious limitations, new novel and robust therapies are required (Forman et al., 2016).

The concept of targeting cancer by harnessing the immune system to recognize and destroy neoplastic cells (cancer immunotherapy) has been established over many years and could overcome the obstacles of the current therapies. Adoptive T cell immunotherapies have proven their success in the clinical field and therefore, are considered the most powerful kind of treatment in the field of immunotherapy (June, Yee, 2005).

Approaches to mitigating the effect of GVHD involve expansion of T cell lines following anti-genetic stimulation in order to produce antigen-specific populations, while eliminating

alloreactive clones. Although this treatment has been used successfully to treat viral reactivation (Bollard and Heslop, 2016) after transplant, and more recently to treat leukemic relapse (McLaughlin et al., 2015), the only problem with this approach is the long amount of time needed to prepare antigen specific T cells; which can take several weeks of culture (Jorritsma et al., 2011).

Redirecting T cell specificity toward TAAs through the transfer of antigen-specific receptor genes is considered as an alternative approach to circumvent the need to culture, grow and enrich antigen-specific T cell clones. These strategies may involve the transfer of recombinant variants of conventional heterodimeric αβ T cell receptor chains (TCR gene transfer therapy) (Stauss et al., 2015), or hybrid composites of anti-body-like receptor chains linked to trans-membrane and activation domains (CARs) (Maus and June, 2016). One of the major differences between αβTCRs therapy and CARs is that αβ TCRs have lower affinities and recognise processed peptide antigens expressed in the context of the major histocompatibility complex (MHC); whereas CARs are high affinity, MHC-unrestricted and capable of engaging target cell surface proteins independently of presentation pathways (Sadelain et al., 2013).

1.2.4 TCR Gene Transfer:

Genetically modified TCR therapies are based on conferring T-cells' specificity toward TAAs through introducing exogenous genes expressing antigen-specific TCR α and β chains (Gross et al., 1989). The tumor specific α and β TCR chains are designed, isolated and cloned into a viral vector backbone; replication defective gamma retrovirus or lentivirus vectors, to transduce T cells and generate a tumour specific-antigen T cell clone. To produce effective, engineered T cells with tumor-specific TCR, an appropriate and specific target sequence should be selected and identified. This target sequence might be found integrated within rare tumor-reactive T cells; which would be a difficult approach to isolate the sequence from (Thomas et al., 2010).

Alternative technologies have been developed to generate highly active anti-tumor T cell clones. For instance, one tactic is to immunise transgenic mice that express human leukocyte antigen (HLA) with human tumor peptides to produce antigen-specific T cell clones that could be harvested and later isolating the TCR αβ chains' specific sequence (Tey, 2014). Retroviral gene transfer of exogenous α and β TCR encoding sequences targeting a tumor associated antigen named MART1 in melanoma was the first trial to prove TCR gene transfer efficacy in humans. Tumor regression was detected in 2/15 subjects treated with engineered

autologous T cells targeting melanoma (Morgan et al., 2006). A higher affinity modified TCR targeting MART1 mediated the anti-tumor effect in clinical trials with melanoma and with tumor regression of 6/20 patients; however, on target anti-melanin effect on hair, skin and eyes was detected (Johnson et al., 2009a). Another side effect was also reported after infusing carcinoembryonic antigen-specific TCR T cells targeting colorectal carcinoma, where bowel inflammation was noticed after therapy (Parkhurst et al., 2011). Another study spotted a minor toxicity following treatment of cancer-testis patients with genetically modified T cells targeting Ny-ESO-1 TAA (Robbins et al., 2011). Similar approaches have been used by other scientific groups to treat viral complications such as Epstein-Barr virus (EBV) (Hart et al., 2008). Engineered autologous T cells with introducing exogenous αβ TCR to target Hepatitis B antigen in patients with hepatocellular carcinoma has also proved the success of TCR gene transfer therapy (Qasim et al., 2015b).

In hematological settings, low numbers of protein antigens have proven suitable as candidates for T cell immunotherapies. One of the most common TAAs used as a target in treating leukemia is Wilm's Tumor-1 antigen (WT-1); a normal protein that is overexpressed on tumor leukemic cells (Casalegno-Garduno et al., 2010). Due to its importance as a potential treatment for leukemia; especially acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS), and its emerging clinical success, WT-1 TCR therapy is a focus of this project and will be discussed in detail in the next section.

1.2.4.1 Clinical studies using αβ TCR gene-modified T cells:

The following table summaries some of the recent clinical trials that have utilised TCR gene-modified T cells:

Data from: Clinicaltrial.gov 10/05/2016

Malignancy	Target antigen	Vector	Comments	References/ClinicalTtrial.govidentifier
Melanoma	MART-1	TCR-RTV	17 subjects: two partial. 30% response rate without toxicity	(Morgan et al., 2006)/NCT00509288
Melanoma, oesophageal and synovial sarcoma	MAGEA3	TCR-RTV	9 patients. One complete response. Our partial response	(Morgan et al., 2013)
Metastatic Melanoma	GP100	TCR-RTV	16 subjects: one complete response and two partial responses.	(Johnson et al., 2009b)/ NCT00509496
Colorectal cancer	CEA	TCR-RTV	Significant response associated with no off-tumor toxicity	(Parkhurst et al., 2011)/NCT00923806
Synovial sarcoma and melanoma	NY-ESO-1	TCR-RTV	17 subjects: two complete response and 7 partial responses. 50% response without toxicity	(Robbins et al., 2011)/NCT00670748

Table 1: Examples of published clinical studies of positive responses to TCR gene transfer therapy.

1.2.4.2 WT1-TCR:

In 2005, the elimination of human leukemic cells using autologous T cells, which were genetically modified to express a novel αβ TCR encoding sequence targeting a Wilms tumour antigen-1 (WT-1), was reported in a humanised mouse model. WT-1 is a zinc finger transcription factor that plays a crucial role in urogenital development. It has an oncogenetic function and it is abundantly expressed in different types of solid tumours such as lung, breast cancers, and in the vast majority of leukaemia cells, with restricted expression in normal, hematopoietic progenitor and stem cells. Thus, WT1 antigen is considered an attractive target antigen for immunotherapy against these malignancies. Clinical trials of TCRs gene transfer therapy to treat relapse of AML/MDS by targeting HLA-A2/WT1 peptides are being planned in centers in Japan (Ochi et al., 2011), the USA (Schmitt et al., 2013) and the UK (Xue et al., 2005).The notable anti-leukemic effects that were detected without toxicity following the infusion of non-manipulated donor-derived WT1-specific CD8

T cells in post-transplant patients provides a solid foundation for this approach (Tawara et al., 2015).

In 2004, the first phase I clinical trial of cancer immunotherapy using peptide vaccination targeting the WT1 protein in patients with leukaemia, myelodysplastic syndrome (MDS), lung or breast cancer was reported. Peptides in combination with an appropriate adjuvant were injected to stimulate CD8+ CTLs specific to the MHC-class-I-restricted peptides. Amongst eight patients with evaluable disease, five achieved reduction in acute myeloid leukaemia (AML). Notably, in two MDS patients, the numbers of leukocytes in their peripheral blood, the majority of which was likely derived from MDS clones, significantly decreased after the first administration of the WT1 peptide. A significant correlation between immunological and clinical responses was observed in a cohort of all the patients examined (n=26). The correlation between the increase in WT1-specific cytotoxic T lymphocytes (CTL) frequencies and clinical responses provided evidence indicating that WT1-specific CTLs induced by WT1 vaccination played an important role in the clinical responses. In conclusion, this study demonstrated that WT1 vaccination can induce WT1-specific CTLs and result in cancer regression without damage to normal tissues in the clinical setting (Oka et al., 2004).

Two groups reported mouse models of adoptive transfer of T cells with WT1-specific TCR genes: one group developed a novel strategy to induce both tumour-specific T helper 1 (Th1) and type 1 cytotoxic T (Tc1) cells by lentiviral transduction of HLA-A24–restricted TCR α and β chain genes isolated from a WT1-specific Tc1 clone. WT1-TCR gene-transduced Th1 and Tc1 cells, exhibited both cytotoxicity and cytokine production in response to WT1 tumour peptide–pulsed HLA-A24+ cells and freshly isolated HLA-A24+ WT1+ leukemic cells (Morris et al., 2006). Another group confirmed the feasibility of producing WT1-specific cytotoxic T lymphocytes (CTL) by transferring the WT1-TCR into T cells isolated from CML and AML patients. WT1-transduced T cells displayed HLA-A2–restricted cytotoxicity against leukaemia cell lines and against CD34+ cells isolated from leukaemia patients. This strongly suggests that patient's T cells are functionally active and that WT1-TCR gene transfer is likely to enable them to recognize autologous leukaemia cells. The survival and efficacy of TCR-transduced human T cells is likely to be greater in patients than in the murine model experiments used in this study (Xue et al., 2005). Recently, a translational study using mouse xenograft models; which transfused with WT1-TCR transduced T cells, for AML therapy was reported. This group developed a safe and effective WT1-TCR retroviral vector for adoptive immunotherapy trials with tailored T cells. They

generated various types of retroviral constructs encompassing unmodified or codon optimised WT1-TCR α/β genes that had been functionally analysed *in vitro*. The best active construct then tested in an autologous primary leukaemia model *in vivo*. This study provides a solid foundation for the planned WT1-TCR gene therapy trial in leukaemia patients (Xue et al., 2010).

One of the most significant recent studies using WT1 in adoptive T cell therapy was to study the correlation between self-antigen expression in normal tissues and phenotype and function of WT1-specific T cells in thymus and peripheral blood. A recent study designed a murine model experiment to analyse the thymus development of WT1-specific TCR and to define the fate of these cells in periphery. Murine hematopoietic stem cells (HSCs) were purified and transduced with WT1-TCR genes; these modified cells were then injected into HLA- A0201 transgenic mice. The outcome of this experiment showed that WT1-TCR specific T cell differentiate into memory T cells and are able to exhibit antigen-specific effector activity; moreover, they were not diminished by central or peripheral tolerance. In brief, this study demonstrated that WT1 specific T cells did not show any signs of self-antigen tolerance and differentiated into fully functional memory phenotype T cells (Pospori et al., 2011).

1.2.5 Chimeric Antigen Receptors (CARs):

TCR-based anticancer therapies are constrained by HLA restriction, which limits the applicability of TCR therapy to patients who express a particular HLA type. Additionally, antigen expression of tumours can be lost by HLA down-regulation (Garrido et al., 1997). Alternatively, chimeric antigen receptors (CARs) can overcome these considerable barriers because they can redirect T cell specificity towards non-HLA-restricted antigens. Moreover, the affinity of antigen binding mediated via antibody-derived recognition is much stronger than that afforded by conventional αβ TCR (Kalos et al., 2011).

Eshhar and colleagues were the first scientific group to explore CARs, conducting their research in 1989. CARs design merges both antibody-like detection with a T cell activation role (Maher, 2012). CARs are hybrid molecules composed of a tumour-antigen binding domain of a single chain variable fragment (scFv) antibody, originating out of a murine monoclonal antibody (ectodomain), and fused to one or more T cell intracellular-signaling domains. They are responsible for activation, persistence, trafficking and effector functions (Finney et al., 1998), (Krause et al., 1998) in transduced T cells (endodomain) after stimulation through a transmembrane domain, which anchors CARs to T cells, and which is linked to a spacer protein (Figure 5) (Eshhar et al., 1993, Bridgeman et al., 2010).

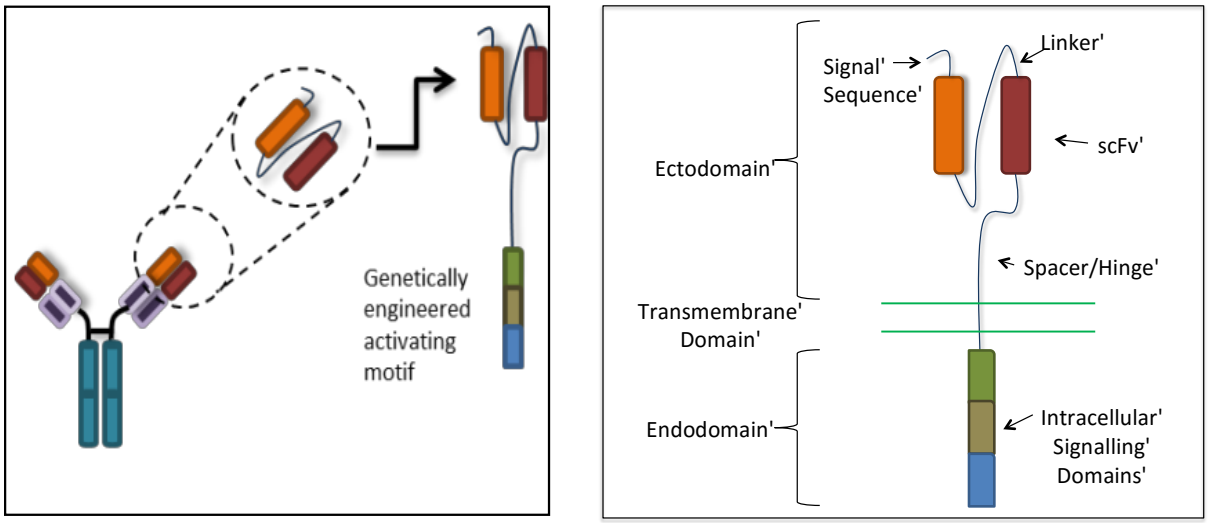


Figure 5: Schematic diagram exhibits the different components of chimeric antigen receptors (CARs): Left: the sequence of the antigen target motif typically is derived monoclonal antibody which determines CAR specificity. Right: variable regions (H+L) from antibody used to provide specificity to an antigen and are joined by peptide linkers. Spacer protrudes construct from cell surface and intracellular domains allow activation of signaling pathways.

1.2.5.1 Three Generations of CARs:

CAR specificity is determined by the antigen-targeting domain (scFv), which consists of cloned variable regions of heavy and light chain antigen-specific monoclonal antibodies. The scFv is linked to the transmembrane and intracellular signaling domain via linker/spacer hinge proteins. The transmembrane domain is derived from the CD4 or CD8 and CD3-ζ activation region, in addition to the co-stimulatory molecules which involve CD27, CD28, 4-1-BB and/or OX40, depending on the CAR generation configuration (Sadelain et al., 2013, Qasim and Thrasher, 2014).

CAR's design has evolved over many decades. It was first described as a murine-derived receptor, and is now known as a complicated third generation configuration with humanised sequence. The main aim of developing more complex configurations of CAR design is to enhance the T cell signaling mechanism which leads to sustained *in vivo* persistence of engineered T cells and robust anti-tumour activity. In first-generation CARs, signals occurring when activated are transmitted through ITAM domains on CD3-ζ only. Early clinical trial data relating to the function of CARs demonstrated that first-generation CARs produce a number of signals insufficient to sustain the *in vivo* persistence of T cells. Therefore, another intracellular T cell co-stimulatory molecule, such as CD28 or CD137, has been incorporated into CARs to produce second-generation CARs. Animal studies have suggested that T cells bearing second-generation, antigen-specific CARs exhibited improved anti-tumour activity compared with T cell-expressing, first-generation CARs, as well as, longer *in-vivo* persistence of CAR engineered T cells. Third generation CARs incorporate

additional signaling domains from receptors such as the tumour necrosis factor receptor family members like 4-1BB or OX40 (Figure 6), to ensure their effective function and survival. Pre-clinical data has suggested that the inclusion of 4-1BB signaling domains might reduce the susceptibility of CARs to trigger the secretions of IL-2 and tumour necrosis factorα (TNF α) and, as a result, precipitate cytokine release and enhance antitumor efficacy (Milone et al., 2009).

CARs have shown to be extremely robust anti-tumour reagents in the field of T cell immunotherapy and it is likely that they will be a major platform to engineer anti-cancer T cells (Jena et al., 2010).

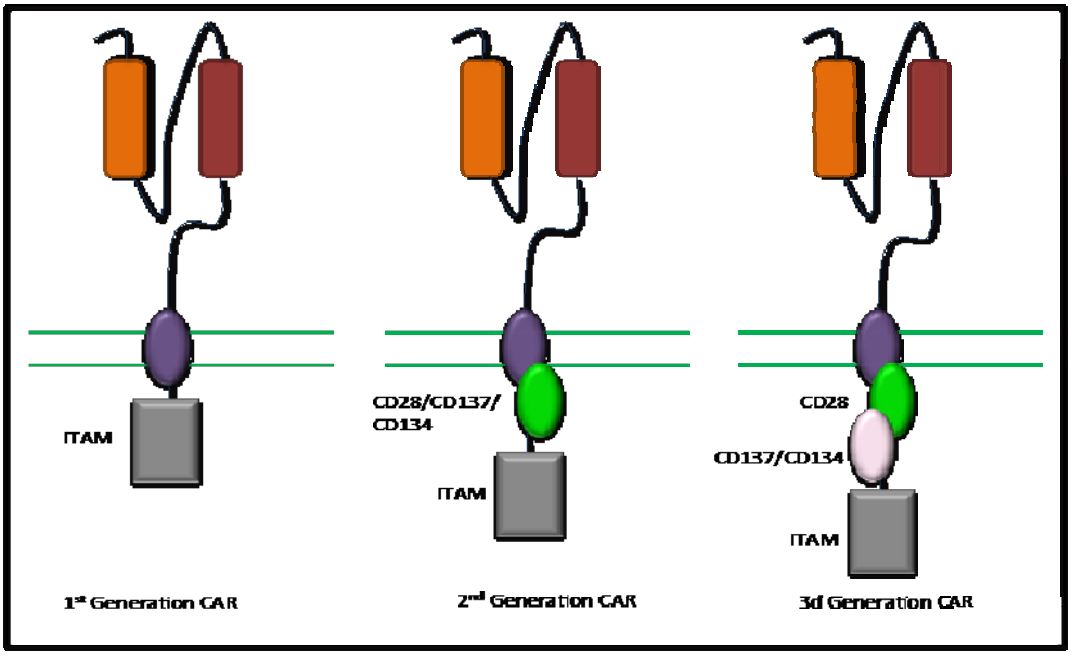


Figure 6: Description of 1st, 2nd and 3rd generations CARs. The extracellular domain (scFv) segments are colored orange and the various TCR-signaling components are colored in violet, green and pink. Second-generation CARs contain one co-stimulatory endodomain (illustrated with CD28 or CD137 or CD134), cloned with the scFv and the CD3-ζ endodomain. Third-generation CARs contain at least two co-stimulatory endodomains, such as the endodomains of CD28 and CD 134 or CD137.

1.2.5.2 Clinical studies using CARs:

1.2.5.2.a CD4-CD3ζ CARs against HIV-1:

In the early 1990s, several studies examined the potential of engineering CD4 and CD8 positive T cells by targeting glycoprotein 120 on the surface of HIV. They did so by inserting a gene containing the extracellular domains of human CD4 linked to the zeta chain of the T cell receptor (2^{nd} generation CAR configuration) targeting HIV-1 envelop glycoprotiens in to T lymphocytes. Pre-clinical studies demonstrated that CD4-zeta T cells addressing the

elimination of gp120 are powerfully able to kill HIV-1 *in vitro* and inhibit viral replication in HIV infected cells. The CD4-CD3ζ approach has since been transferred to the clinic (Walker et al., 2000). In 2002, a randomised phase II study of CD4-ζ T cells in 40 patients confirmed that CD4-ζ T cell infusions resulted in elevated CD4+ T cell counts. In addition, this trial observed a prolonged and stable persistence of CD4ζ T cells in the blood and in the movement of matter towards the gut mucosa (Deeks et al., 2002). Together with other CD4+ T cell adoptive-transfer studies (not discussed here), these trials provided substantial data illustrating the safety and feasibility of adoptive therapy with CD4ζ gene-modified cells for HIV-infected subjects.

1.2.5.2.b GD2 CARs:

In 2008, Pule laboratory (Cancer Institute, UCL, London, UK) conducted a clinical trial in which a chimeric antigen receptor was designed against disialoganglioside GD2 (a non-viral, tumour-associated antigen) for the treatment of neuroblastoma. In this trial, cytotoxic T cells (CTLs) against Epstin Barr virus (EBV) (EBV-CTLs) were engineered to express GD2CAR. Two sets of engineered cytotoxic T lymphocytes (CTLs) were applied to eleven patients with neuroblastoma: human virus-specific CTLs expressing CAR directed GD2 and non-viral-activated T cells expressing the same GD2-CAR. The outcome data of this study showed that four subjects had evidence of tumour necrosis or regression, and none of them developed any detectable antibodies to CAR-CTLs; additionally, the study showed that EBV-CTLs expressing GD2CAR are able to survive longer than the non-viral activated GD2CAR T cells (Pule et al., 2008). Another ongoing study using CAR T cells targeting GD2 antigen in neuroblastoma patients has proved the efficiency of CAR T cell therapy; among the 19 subjects enrolled; three of them with active disease achieved complete remission, and persistence of GD2CAR beyond six weeks was associated with superior clinical outcomes (Louis et al., 2011). This study is registered at http://www.clinialtrials.gov/as NCT00085930.

Cancer	Target Antigen	Gene construct/generation	Gene transfer	Comments	References
RCC	CAIX	scFv-CD3 1st generation CAR	Gamma RTV	No objective responses, limited persistence.	(Lamers et al., 2013)
Indolent B- NHL and mantle cell lymphoma	CD20	scFv-CD3ξ 1st generation	EP	7 patients: 2 maintained CR, 1 PR, 4 SD. Successful demonstration of non- viral gene transfer. Car T cell persisted for 1–3 weeks alone/5–9 weeks with IL-2.	(Till et al., 2008)
Indolent B- NHL and mantle cell lymphoma	CD20	scFv-CD28- 41BB-CD3ξ 3rd generation	EP	4 patients: 2 SD, 2PR. Car T cell persisted for 1 year.	(Till et al., 2012)
Ovarian cancer	FR	scFv-CD3ξ 1st generation	Gama RTV	14 patients were assigned to 1 of 2 cohorts in the study. 8 patients in cohort 1 received a dose escalation of T cells in combination with high-dose interleukin-2. 6 patients in cohort 2 received dual-specific T cells (reactive with both FR and allogeneic cells). Toxicity mostly due to IL-2 injections. Limited persistence of CAR T cells.	(Kershaw et al., 2006)
Colorectal cancer	ErbB2	scFv-CD28- 41BB-cd3ξ 3rd generation	Gamma RTV	Fatal , early respiratory distress and lung inflammation	(Morgan et al., 2010)

Table 2: Summary of some selected clinical trials that have utilised CARs gene-modified T cells.
Abbreviations: RCC: renal cell carcinoma; CLL: chronic lymphoblastic leukaemia; ALL: acute lymphoblastic leukaemia; EP: electroporation

1.2.5.c This CD19CAR:

At the present time, human CD19 antigen is considered the most investigated TAA in T-cell CAR therapy and this is due to several factors: CD19 antigen expression is restricted to B-cells and B-cell progenitors and it is not expressed by other hematopoietic stem cells, which allows scientists to target only B-cell lineage without affecting other hematopoietic lineage (Stamenkovic and Seed, 1988, Muschen et al., 2002). Also, CD19 is abundantly expressed by neoplastic cells that originate from B-cell lineage, such as lymphoma and lymphocytic leukaemia (Campo et al., 2011). B cell malignancies were one of the first types of cancers eradicated by CAR T cell therapy in murine models. Nowadays, impressive reports have emerged highlighting the remarkable response rate in 60-90% of B-cell malignancies in patients with relapsed or refectory lymphoblastic leukaemia. Another CD19CAR trial is explained in the following table.

Cancer	CAR construct	Gene transfer	Comments	References
CLL or ALL	scFv-CD28- CD3ξ 2nd generation	Gamma-RV	Ten patients: 1 death, 3 SD. CAR T cells persist for 40 days.	(Brentjens et al., 2011)
NHL	scFv-CD3ξ 1st generation vs. scFv-CD28- CD3ξ 2nd generation	Gamma-RV	6 patients enrolled. CD28 co- stimulation improved *in vivo* expansion and persistence of CAR+T cells for more than 6 months.	(Savoldo et al., 2011)
NHL	scFv – CD3ξ 1st generation	Electroporation	4 patients: 2 maintained CR. 1–7 days cellular immune response against CAR T cells	(Till et al., 2008)
CLL	scFv-41BB- CD3ξ 2nd generation	LV	3 patients: 2 CR, 1 PR. CAR T cells persisted for more than 6 months.	(Kalos et al., 2011)
ALL	scFv-41BB- CD3ξ 2nd generation	LV	4 patients: 2 CR, 1 durable >18 months. 1 relapse, with blast cells that no longer expressed CD19	(Grupp et al., 2013)
FL,CLL, B-cell lymphoma	scFv-CD28- CD3ξ 2nd generation	Gamma-RV	1 patient shows complete regression of FL. 8 patients: 6 obtained remission, ` patient developed long-term depletion of normal polyclonal CD19+ B cells and 1 has SD. CAR T cells persisted for 14 weeks.	(Kochenderfer et al., 2010).clinicaltrials.gov as #NCT00924326. (Kochenderfer et al., 2011) ClinicalTrials.gov as NCT00924326.

Table 3: Summary of some selected clinical trials that have utilised CD19CARs gene-modified T-cells. Abbreviations: CR: complete response; PR: partial response; CLL: chronic lymphoblastic leukaemia; ALL: acute lymphoblastic leukaemia; FR FL: follicular lymphoma.

1.3 Limitations of antigen-specific gene transfer therapy:

Despite the growing number and length of remissions using antigen-specific T cell therapy to treat lymphomas and leukemia, considerable barriers might affect the efficacy of the genetically modified T cells. One factor that might have an impact on treatment is the tumor microenvironment itself. Tumor microenvironment is an immune suppressive media which is composed of neoplastic cells, vasculature and immune cells and also contains some molecules that affect the antigen presenting process to T-cells. As a result, T cells will not be able to recognise sick cells; leading to the propagation of the cancerous cells (Zou, 2005). Additionally, tumor microenvironment is characterised by the presence of large numbers of T regulatory cells (T_{regs}); which have a huge impact in reducing anti-tumor activity of genetically modified T cells (Curiel, 2007). Before applying the treatment, lymphodepletion regimes might decrease the number of T_{regs} and improve the efficacy of anti-tumor activity of the genetically modified T cells (Schmitt et al., 2009). Moreover, clinical studies have demonstrated that reducing the numbers of circulating T cells (lymphodepletion) as part of preparative regimes prior to the treatment could promote *in vivo* persistence, survival and expansion of the transferred genetically modified T cells by decreasing the competition for IL-7 and IL-15 cytokines that are responsible for T cell proliferation and existence (Klebanoff et al., 2005b, Gattinoni et al., 2005a, Paulos et al., 2007).

Another factor that can influence the long-term outcome of efficacy following genetically modified T cell therapy is that cancerous cells might down regulate the expression of the targeted TAA (Grupp et al., 2013). In one case study following anti-CD19 CAR T cell therapy, scientists noticed that after a complete remission of the lymphoplastic cells, blast cells emerged again without the expression of CD19 (Maude et al., 2014).

The risk of losing TAA expression remains to be elucidated. Also, cell dose of the therapeutic product might have an impact on the efficacy of T cell based immunotherapies. Pre-clinical experiments have shown that transferring more numbers of genetically engineered T cells results in a progressive improvement in tumour regression (Klebanoff et al., 2011).

Although this technique holds promise as a strategy to eradicate malignancies, there are potential safety risks associated with using these genetically engineered T-cells as a therapy. Safety hazards include the following: on-target off-tumor reactivity, off-target reactivity and cytokines release syndrome (Bendle et al., 2010). Antigen-specific gene transfer therapy involves a genetic alteration of donor-derived T cells to express an antigen-specific receptor that is directed against a particular TAA. As a result, immunoreactivity of the therapeutic,

genetically modified T cells would be induced to eliminate cancerous cells only without affecting other normal cells. Choosing an optimal antigen target that is only expressed by tumor cells is a demanding and challenging task. Most of the TAAs are expressed by both cancer and normal cells; as a result, genetically modified T cells recognise TAAs on normal body tissue; even if they express at a very low level, resulting and triggering an immune response against normal healthy body tissue (Johnson et al., 2009b); this phenomenon is called "on-target off tumor toxicity" (Casucci et al., 2015). Engineering T cells with higher selectivity towards certain tumours might be one solution to improve the treatment and avoid on-target off-tumor toxicity. One study has suggested a dual-CAR targeting strategy (Lanitis et al., 2013, Pegram et al., 2012); where T cells are engineered to express two CARs targeting two different target-antigens on tumour cells to ensure that modified T cells will only activate once engaged to both antigens on tumour cells. The proof-of-concept of this strategy has shown a promise that modified T-cell therapies might be safer using this approach (Kloss et al., 2013).

The risk of off-target reactivity is mainly related with TCR gene transfer T cell therapies where the newly introduced TCR might react against a peptide in proteins other than the targeted ones. Some authors have demonstrated that the other cause of off-target reactivity is the formation of mixed dimers between the introduced and the endogenous TCR alpha and beta chains. The assembly and surface expression of an exogenous TCR is a complex and intricate process, requiring the pairing of the introduced alpha and beta chains to form a heterodimer that is then joined with the four invariant CD3 chains. In transduced T cells, the introduced alpha or beta chains have the potential to form heterodimers with the complementary alpha or beta chain. This results in the production of novel TCR with unknown specificity, which might provoke off-target autoimmunity (van Loenen et al., 2010, Bendle et al., 2010). Additionally, levels of CD3 are finite within T cells, and consequently, wrongly mis-paired TCRs might compete with the exogenous TCR for CD3 leading to the usage of CD3 stock by endogenous TCR; as a result, the expression level of introduced TCR will be reduced (Morris et al., 2003). Several strategies have been explored with the aim of overcoming these hurdles: one aims to reduce the mis-pairing phenomenon between α and β chains of therapeutic-exogenous TCR and endogenous TCR; others aim to eliminate endogenous TCR expression and improve the graft-versus-leukaemia/-lymphoma effect. Furthermore, the endogenous αβ TCR may recognise major and minor histocompatibility antigens in the recipient, leading to GVHD in the allogeneic setting. As a consequence, most clinical trials conducted at present are infusing autologous, re-engineered, antigen-specific T

lymphocytes to diminish the possibility that endogenous αβ TCR will recognise normal tissues after adoptive transfer (Torikai et al., 2012). Brentjens and his group suggested that using autologous, genetically modified; antigen-specific T cells have achieved initial success in clinical trials targeting hematological malignancies (Brentjens et al., 2011). However, it could be argued that this approach is limited by the time and expense required to manufacture an antigen-specific T cell product for each patient.

Finally, cytokines release syndrome is considered the main drawback of genetically modified CAR T cell therapy (Maus et al., 2014). Clinical studies have shown that T-cell therapies can induce a fast and potent eradication of tumor cells resulting in the production of high levels of cytokines release and macrophage activation syndrome. Following CAR T cell therapy, clinical trials have demonstrated that patients might suffer from high fever, nausea, diarrhea and rigors which might be due to high levels of INF-γ and IL-6 production (Maude et al., 2014). Some recent studies have exhibited that administration of anti-IL6 receptor (Teachey et al., 2013) might prevent these reactions, but the timing of applying this reagent is not yet known, neither is it known whether it has any impact on the efficacy of the tumor regression and elimination by the genetically engineered T cells.

T cell therapy	Disease	Antigen-target	Adverse event	Reason	Reference
Genetically modified T cells	Melanoma	MAGE-A3	3 patients developed neurological toxicity: 2 died, 1 had full neurological recovery	On-target off-tumor reactivity. MAGE-A3 antigen is expressed by neurological cells.	(Morgan et al., 2013)
Genetically modified T cells	Myeloma and melanoma	Affinity enhanced TCR against MAG-A3	2 subjects died of cardiac toxicity after 5 days of infusion	Off-target reactivity; peptide from heart muscle protein was identified as an alternative target of MAGE-A3 TCR T cells	(Linette et al., 2013, Cameron et al., 2013)
Genetically modified T cells	Metastatic colorectal cancer	CEA	Diarrhea, fever	On-target off tumor reactivity;	(Parkhurst et al., 2011)
CAR-T cell	ALL	CD19	Continuous depletion of normal B cells	On-target of target toxicity. CD19 is expressed by normal B cells	(Grupp et al., 2013)
CAR- T cell	CLL	CD19	Persistence fever associated with hypotension and respiratory distress and subsequently died	Cytokines release storm	(Brentjens et al., 2010)
CAR-T cell	Metastatic renal cell carcinoma	CAIX	Liver enzyme disruption	Cytokines release storm	(Lamers et al., 2013)

Table 4: Summary of some adverse events from T-cell therapy clinical trials. CLL: chronic lymphoblastic leukaemia; ALL: acute lymphoblastic leukaemia; CAIX: carboxy-anhydrase-IX

In addition to the previously mentioned challenges facing antigen-specific T cell therapy, efficient gene transfer methodologies to T cells and their acceptable safety profile is still a matter of concern. Several means of gene transfer in human T lymphocyte will be discussed later in the text.

1.4 Improving the efficacy of T-cell therapies:

Multiple research groups have attempted, by different means, to promote the preferred assembly and surface expression of the exogenous alpha and beta chains in TCR (Govers et al., 2010). The first strategy to lessen mixed dimer formation was to use hybrid TCRs with human variable domains and a murine constant region. Compared with human TCR, a murine-human TCR hybrid enhanced the homologous pairing of introduced TCR and it expressed more efficiently in human T-cells: however, proven immunogenicity against the murine compartment might be a major concern (Cohen et al., 2006, Sommermeyer and Uckert, 2010, Thomas et al., 2007). Another approach is to replace the threonine-48 on the alpha chain and serine-57 on the beta chain with cysteines, forming an additional intramolecular disulphide bond within the constant compartment of the exogenous TCR alpha beta chains. The modified peptides pair more efficiently, resulting in boosted TCR expression and improving its functional activity (Kuball et al., 2009). An alternative approach focused on improving the translation of the transgenic TCR mRNA. Codon optimisation of the exogenous TCR genes favours the pairing of the introduced chains and increased TCR reactivity by introducing a point mutation into constant domain of α and β chain (Gustafsson et al., 2004). Recently, an alternative platform has been explored; and has proven its efficiency in reducing TCR mis-pairing by knocking down the expression of the endogenous TCR by targeted gene therapy using specific DNA nucleases (Provasi et al., 2012, Bunse et al., 2014). Several means of disrupting endogenous α and β TCR expression on T lymphocyte will be discussed later in the text. Although these strategies led to a significant increase in the expression of the introduced TCR, they fall short of ensuring the high expression levels that are essential for high-avidity tumour-specific T cell effectors. Most crucially, the mis-pairing phenomenon with the endogenously expressed TCR is not fully eradicated in these strategies.

Another important factor in order to improve T-cell therapy is to manipulate or retain T-cell differentiation in favor of antigen-specific T cell. Generally, it has been shown that adoptively transferred genetically modified T cells are highly differentiated into effector memory T cells which display abbreviated survival, impotent anti-tumor activity and immune

exhaustion post-infusion. Culturing engineered T cells in an environment containing γ cytokines just as IL-7 and IL-15 (Kaneko et al., 2009); or IL-15 and IL-21 (Pouw et al., 2010) pre-infusion to drive T-cell differentiation might retain the central memory T-lymphocytes phenotype, prolonged peripheral persistence and potent anti-tumor activity (Zoon et al., 2015, Montes et al., 2005). Another different strategy is focused on engineering naïve cord blood T cells to express CAR or antigen-specific TCR instead of adult antigen-experienced T cells (Thompson et al., 2015, Tammana et al., 2010). CB-T cells have a naïve phenotype and they might retain their less differentiated phenotype following *in vitro* stimulation and genetic manipulation prior to the adoptive T-cell transfer; which results in generating potent anti-tumor engineered T cells with naïve or stem cell memory phenotype and great capacity of *in vivo* survival and proliferation following infusion (Frumento et al., 2013). Moreover, CB-T cells mediate potent antiviral and anti-leukemia effects following allogeneic transplantation, and further reduce the possibility of graft-versus-host disease occurring, as compared to the risk posed by peripheral blood grafts (de Haar et al., 2015). Since single-cord blood graft has a limited hematopoietic stem cell dose, it is not unusual to pool matched or partially HLA-matched donations, and this may provide an opportunity to supplement grafts using engineered T cells with additional HLA- matched donations. To mitigate the GVHD effect in the allo-setting and to generate "off-the-shelf" universal T cells expressing antigen-specific receptors, we proposed a strategy based on using genetically engineered cord blood T cells (CB-T cells)(Eapen et al., 2007, Hiwarkar et al., 2015).

1.5 Eliminating endogenous TCR expression:

As it has been mentioned before in the text; one of the strategies to reduce the mis-pairing adverse event that is related to TCR gene transfer therapy is to knock down the expression of endogenous αβ TCR. Gene editing technology has emerged recently; where specific molecular reagents are capable of driving a highly specific DNA cleavage using targeted nucleases.

Gene-specific targeting is a precise gene spotting strategy in which the mutant version of the gene might be replaced with the normal, functional copy at the exact site of the endogenous gene, the consequence being that the gene restores its normal function. Moreover, by targeting the specific locus of the desired gene, unpredictable genetic effects, such as the random integration of the transgene, and the production of mutagenesis, might be controlled. These tailored endonucleases induce a double strand break (DSB) at a specific locus within the genome: consequently, DNA-repairing mechanisms will take place, resulting in either

switching off of the gene's function via the second DNA-repairing pathway, a process called non-homologous recombination end joining (NHEJ), or replace the mutant form of the gene via homologous recombination (HR) (Figure 7) (Takata et al., 1998).

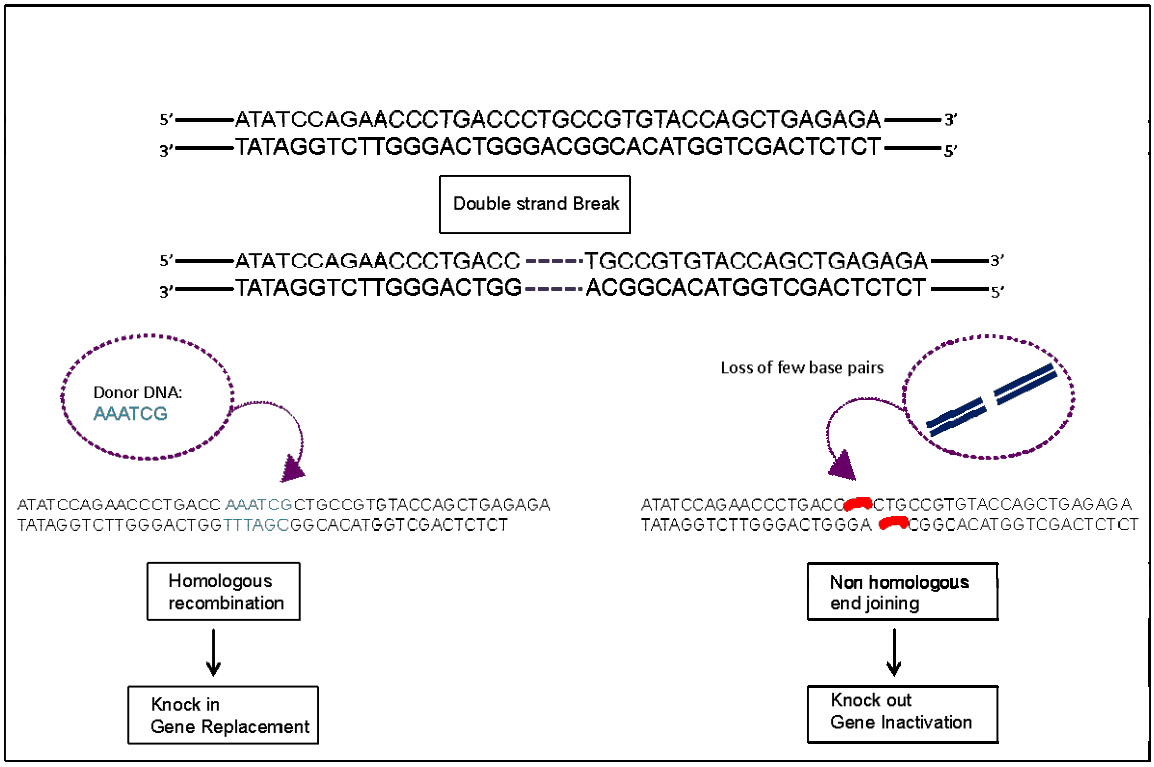


Figure 7: The mechanism of DNA double strand break (DSB) repair. DSB is repaired by two major pathways: homologous recombination (left) or non-homologous end-joining (NHEJ) (right). HR can result in gene replacement, while NHEJ leads to gene inactivation.

Several types of tailored endonucleases have been engineered and used as DNA-targeting tools in different studies and include meganucleases (MGNs) (Silva et al., 2011), zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) (Joung and Sander, 2013), and clustered regularly interspaced short palindromic repeats (CRISPR) (Sander and Joung, 2014, Jinek et al., 2012, Mali et al., 2013) reagents.

Another advantage of eliminating the internal αβ TCR expression is that it might provide a new strategy to produce "universal" off-the shelf therapeutic antigen-specific T cell products from unrelated donors to be administrated to multiple subjects without causing GVHD (Torikai et al., 2012).

In this project, the feasibility of using other endonucleases such as MGN, TALENs and CRISPR/Cas9 targeting constant regions of α and/or β chain of TCR was investigated. In the next section, a brief description of each gene-editing tool will be discussed.

1.5.1 siRNA knocking down endogenous αβ TCR expression:

As an alternative to protein engineering, a Japanese group reported that small interfering RNA (siRNA) has the ability to down-regulate endogenous TCR expression, which leads to a decrease in the potential for mis-pairing, thus improving the expression and reactivity of introduced TCR. They designed a system that can express therapeutic TCR while silencing the expression of endogenous TCRs by using a siTCR retroviral vector. This vector encodes the alpha and beta chains for TCR, recognizing the tumor specific antigen named MAGE-A4, and siRNA simultaneously targets conserved regions in the wild-type TCR constant domain. The results of this study demonstrated that a primary human T cell transduced with a MAGE-A4-TCR-siRNA vector led to an elevated expression of introduced TCR and reduced, but did not eradicate, the expression of endogenous TCR (Ochi et al., 2011). Recent studies incorporated siRNA to a Wilms Tumor antigen 1 (WT-1)-specific TCR vector. The promising data produced by this study illustrated an increase in anti-leukaemia activity *in vivo*: a reduction in the potential loss of antigen specificity after expansion was also noticed, possibly reflecting the diminished generation of mis-paired TCRs(Okamoto et al., 2012). These data from RNA interference lend theoretical support to a novel strategy that uses genome-targeting technology.

1.5.2 ZFNs knocking down endogenous αβ TCR expression:

ZFNs are hybrid artificial DNA endonucleases that are comprised of adjusted-design (customized) zinc finger DNA binding domains that fuse to non-specific cleavage endonuclease domains of a restriction enzyme called Fok1.

In 2012, Provasi et al. designed zinc-finger nucleases to edit T cell specificity at the DNA level by knocking out the endogenous TCR. Although this study yielded promising data, T cells were exposed to two rounds of stimulation with CD3/CD28 beads and two viral vectors were used to deliver ZFNs and antigen-specific alpha beta TCR genes: adenovirus and lentivirus, respectively (Provasi et al., 2012). Consequently, the yield population of therapeutic T cells will be highly differentiated and exhausted; unfavorable conditions for adoptive immunotherapy. In addition, ZFNs have been criticized in past studies for their propensity to cleave off-target sequences, resulting in toxicity (Beumer et al., 2008). Moreover, designing highly specific ZFNs targeting a novel DNA sequence can be time and resource intensive, limiting the development of additional targeting tools (Isalan, 2012).

1.5.3 Meganucleases:

MGN are sequence-specific endonucleases recognizing large and unique DNA sequences (12–45 base pairs)(Paques and Duchateau, 2007). Their recognition sites are extremely rare within the genome, and consequently the chance of an off-target toxicity effect occurring is seldom or almost non-existent. This feature makes meganucleases superior to ZFNs as a gene-targeting tool (Meissner et al., 2014). In nature, MGNs promote the propagation of mobile genetic elements via a process known as homing. Homing endonucleases can be redesigned to target a specific sequence within the genome. The MGNs that are used in this study are called I-CreI and belong to the LAGLIDADG family of homing endonucleases. I-CreI has been exposed to different mutations in its DNA-interacting region to allow the cleavage of a precise sequence within the T cell receptor beta chain gene, resulting in gene inactivation (Barzel et al., 2011).

The process of engineering and designing a novel MGN with high specificity requires empirical and selection-based approaches that can be time and resource consuming (Epinat et al., 2003). TALENs (see directly below for definition) thus far appear not to be subject to the previous limitations suffered by both MGN and ZFNs.

1.5.4 TALENs:

Another chimeric molecule known as TALENs, composed of a non-specific cleavage domain of endonuclease FokI, and fused to a specifically designed DNA-recognition domain, is

becoming an attractive tool for gene targeting and manipulating. Transcription activator like effectors (TALEs) are a unique class of DNA-binding proteins that are naturally produced by plant-pathogenic bacteria in the genus *Xanthomonas*. Their main function is to directly modulate host gene expression to enhance virulence (ability to cause disease). TALE's DNA-binding domain is comprised of a central region of 15.5–19.5 highly conserved units in a unique tandem array. Each repeat consists of 33–35 amino acids, which confers DNA-binding specificity, flanked by an N-terminal domain and a nuclear localisation domain prior to being flanked by C-terminal, transcriptional activation domain (Sun and Zhao, 2013, Holkers et al., 2013a). The repetitive units' sequence of TALE is identical except for two extremely variable amino acids at positions 12 and 13 known as 'repeat- variable di-residue' (RVD) that establishes the base-recognition specificity of each unit (Figure 8). In other words, each RVD preferentially recognises one of the four bases in the target site. This one-repeat-to-one-base relationship allows one to predict how the TALE site will bind to a specific nucleotide within the genome and easily customises the TALE domain targeting a sequence of interest (Miller et al., 2011, Li et al., 2012). Because of this, as well as the minimal, off-target cleavage activity of TALENs compared to ZFNs, TALENs have attracted great interest as a potential DNA-targeting tool. In December 2015, at the meeting of the American Society of Hematology (ASH) in Orlando, a conference paper was presented on the first clinical application of "universal" CD19-targeted CAR T cells modified by TALENs targeting alpha constant regions to knock out the expression of endogenous T-cell receptors, which effectively eliminates the risk of GVHD (Qasim et al., 2015a). The therapy was used to treat an infant with refractory, relapsed B-cell acute lymphocytic leukemia under the basis of UK special therapy regulations. Although the follow-up period is still quite short, the intervention, comprising lymphodepletion and infusion of the universal CD19 CAR T cells, has induced molecular remission where all other treatments have failed.

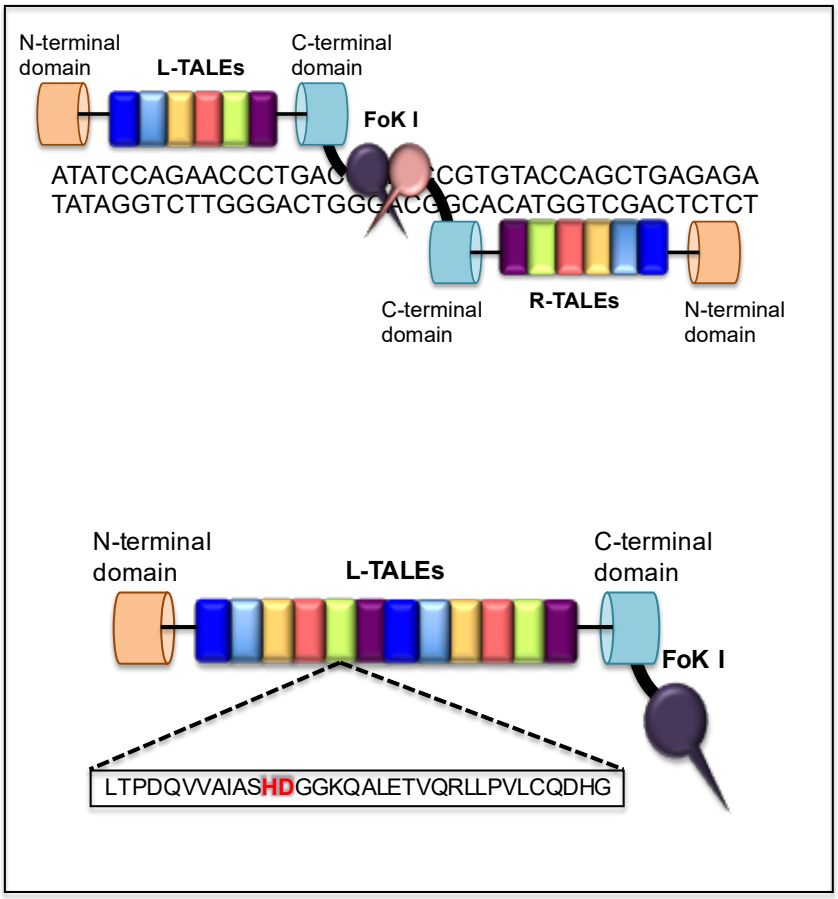


Figure 8: Schematic diagram of a transcription activator-like effector nucleases (TALENs). TALE repeats (top) are represented as coloured rectangles flanked with N- and C-terminal motifs. Bottom diagram exhibits the TALE-derived, DNA-binding domain. The amino acid sequence of a single TALE repeat is expanded with the two hypervariable residues, highlighted in red text.

1.5.5 CRISPR/Cas9

The CRISPR/Cas9 genetic engineering tool is comprised of two main compartments: Cas9 nucleases; the part that is responsible for DNA cleavage; and guide RNAs: small molecules that direct Cas9 toward a specific sequence within the genome (Jinek et al., 2012). Clustered regularly interspaced short palindromic repeats (CRISPR) are an adaptive defense mechanism that evolved by many species of bacteria and archaea to protect them against exogenic mobile genetic elements derived from invading foreign DNA. Once foreign nucleic acid is invading these microbes, few fragments of the invader DNA sequence are captured and inserted as a "spacer" between identical repeats that are specific to a particular CRISPR system. Once the DNA locus is transcripted, a precursor RNA is produced then processed to small fragments of one spacer linked to a portion of the repeats sequence. When the same virus or parasite infect the host again, the corresponding RNA guides the destruction process of the invading DNA or RNA. The production of spacer RNAs and the cleavage of the

corresponding invading sequence are mediated by Cas proteins (CRISPR-associated) (Sander and Joung, 2014, Carroll, 2012). Researchers have discovered that this complex DNA cleavage process can be recapped *in vitro* by using a combination of purified Cas9 and a single guide RNA that has minimal features of both spacer and trans-CRISPR activator RNA (that is full complementary to the repeat sequence). The cleavage site should be a DNA sequence that matches the spacer and a minimum 16 target base pairs are required to ensure recognition of the specific sequence within the complex genome. Additionally, a crucial 2 to 3 base pairs (or more) located just right to the DNA-RNA hybrid region called proto-spacer adjacent motif (PAM) is required for Cas9 to recognize target DNA sequence (Jinek et al., 2012, Wiedenheft et al., 2012). In addition to the simplicity in designing guide RNA targeting specific locus and the high efficiency of the system (Yu et al., 2013, Jiang et al., 2013), CRISPR/Cas9 system is able to target and hit multiple loci within the genome at the same time by injecting multiple guide RNAs (Wang et al.). Given all the previous features of CRISPR/Cas9, scientists have predicted that this system can potentially be used to replace MGNs, ZFNs or TALENs for targeted genomic cleavage in higher organisms (Deltcheva et al., 2011).

1.6 Gene delivery to T-cells:

The further success of gene therapy is partially dependent on the understanding of vector biology and of the interaction mechanism between host and delivery agent. Multiple means have been explored to deliver a particular gene into human T lymphocytes:

1. 6.1 Non-viral gene delivery system:

1.6.1.1 DNA Plasmid:

Naked DNA plasmid can be transferred in T cells by a method known as electroporation or nucleoprotein transfection. Transfected T cells can be selected based on the accompanying insertion of drug resistant genes. Although the inexpensive cost of this method is a significant advantage, it simultaneously suffers from several disadvantages. As naked DNA integrates at very low frequency in T cells, this approach is considered inefficient and extended culture for several weeks is required to generate sufficient numbers of cells. Consequently, subsequent capacity to survive long-term *in vivo* might be significantly compromised. Moreover, the inclusion of antibiotic-resistant genes in T cells might generate immunogenicity leading to the premature elimination of these cells *in vivo* (Jensen et al., 2010).

1.6.1.2 Transposons/Transposase systems (Sleeping Beauty and PiggyBac)

This is a non-viral integration system of gene delivery that relies on two non-viral components: i) donor transposon plasmid and ii) helper transposase. This system provides a higher integration rate than naked DNA with efficient long-term transgene expression. In comparison to some viral vectors, sleeping beauty additionally allows for the insertion of larger DNA fragments (Field et al., 2013).

1.6.2 Viral gene delivery system:

An optimal viral vector should harness its viral infection and integration pathways without pathogenicity or subsequent expression of viral genes, or the formation of infectious, replication competent viral particles. Producing a virus vector with the previous features requires the deletion of some coding regions, while leaving the sequences that are required for in cis functions, such as the movement of the packaging vector genome into the virus capsid, or the integrated viral genome into the host DNA (not all vectors genome is able to integrate, for example, recombinante associated adenovirus genome don't integrate to the host genome). Replacement of these sequences with expression cassette allows the expression of therapeutic transgenes. Besides having the ability to carry foreign genes and express them efficiently and steadily, the production of stable, propagated vectors, and the ability to be purified to high titers are some of the additional characteristics required to generate an ideal gene delivery vehicle. There are five main popular categories for vector engineering: oncoretroviruses, lentiviruses (LV), adenoviruses, adeno-associated viruses and herpes simplex viruses (Walther and Stein, 2000). For T cells, gamma RV and LV have been most useful.

1.6.2.1 Gamma-Retroviruses:

Because of their ability to stably integrate into the host cell genome, retroviruses are considered the most useful vehicle to deliver genes into mammalian cells. Retroviruses are RNA viruses with the ability to reverse transcribe their genome into double-strands (ds) DNA (proviral). In addition to the stable, long-term expression of the transgene, retroviruses possess other features that make them a useful tool for therapeutic applications: for example, they have the ability to infect a wide variety of cell types with a low cytotoxic profile compared to other virus vectors. Also, they are capable of carrying foreign genes of reasonable sizes (8 kilobases (kb)) and of generating viral vectors with significant titres for efficient gene transfer. However, a gammaretrovirus like Moloney murine leukemia virus (MMLV) has the tendency to integrate near-regulatory sites of the active gene: therefore, if

the genes adjacent to integration site are porto-oncogenes, this might lead to insertional mutagenesis, which is defined as the chance integration of a provirus adjacent to a cellular proto-oncogene, perturbing its expression and thus potentially resulting in oncogenesis. This phenomenon has been observed in four patients with severe combined immunodeficiency (SCID-X1) and one Wiskott-Aldrich syndrome (WAS) patient who was treated with gammaretrovirus and developed T cell leukaemias. The study revealed that the retrovirus integrated near the promoter pg LMO2 gene, which is a proto-oncogene related to T cell leukemias (Hacein-Bey-Abina et al., 2003). Another drawback feature associated with gammaretrovirus vectors is that they can only gain access to the cell nucleus during the cell division cycle at the point where the nuclear membrane breaks down: consequently, they don't have the ability to transduce non-dividing cells (Park et al., 2000). On the other hand, vectors derived from HIV-1 (lentiviral vectors) have the ability to efficiently transduce both quiescent and dividing cells: hence, the possible target cell populations will rise remarkably to include haematopoietic cells, muscle cells and neurones. Moreover, lentiviral vectors possess other advantages that make them superior to retroviral vectors, including their ability to carrying genes up to 10kb (which is accompanied by a decreasing of the titre), and the possibility of developing insertional mutagenesis with lentiviral vectors is decreased because they do not tend to target promoter regions within the genome.

1.6.2.2 HIV-1 lentiviral vectors:

1.6.2.2.1) Human Immunodeficiency (HIV-1) Virus structure:

HIV-1 virus is a lentivirus, which is a member of the retrovirus family. Lentiviruses are characterised by a period of latency between the infection and the onset of the clinical disease. In a manner common to all viruses, HIV-1 utilises cellular machinery in order to reproduce and can only replicate within the host cell. Retroviruses are unique in virology, in that they allow the genetic material composed of ribonucleic acid (RNA) to be integrated into the host deoxyribonucleic acid (DNA) through the employment of the reverse transcriptase (RT) enzyme. However, HIV-1 has a unique structure compared with other lentiviruses: it is approximately 120 nanometres in diameter and typically described as spherical. The genetic material is in the form of 9749 nucleotides, long, single-stranded RNA molecules, of which there are two copies in the centre of the virus, which is in turn surrounded by the conical viral capsid (protein shell) composed of the protein p24 (Ratner et al., 1985a). The proteins contained in the capsid help to ensure that the virion (the extracellular, viral infective agent)

is protected against degradation by nucleases. When the virus fuses with the host cell membrane, it utilises the plasma membrane to form a protective layer around itself, containing the proteins gp120 and gp41 (glycoproteins), which protrude from the cell surface in a characteristic spike pattern. The combination of gp120 and gp41 are known as the env protein (envelope protein) consisting of a stem of gp41 (3 molecules) and a cap of gp120, although the precise configuration of this arrangement is open to interpretation depending on the imaging techniques that are utilised (Zhu et al., 2006). However, by using the host membrane as a means of protecting the viral genome, it is possible to evade immune detection to a certain degree.

The two distinct components of the HIV-1 virus are thus: the viral envelope, formed by the host cell plasma membrane; and the viral core containing the genetic material. The following section will consider the structure of the genomic material in HIV-1, along with the characteristic enzymes that ensure successful host cell integration of genetic material and evasion of degrading enzymes.

1.6.2.2.2) HIV-1 genome:

Although HIV belongs to the retrovireade family, there are some distinct features that distinguish HIV-1 from other members in this family. HIV genome has 9 open reading frames that give rise to 15 different proteins, with the env gene encoding gp120 and gp 40 (Watts et al. 2009). Common to all retroviruses are the four essential genes: group-specific antigen (gag), polymerase (pol), protease (pro) and envelope (env). In addition to these, HIV-1 carries six other auxiliary genes: negative regulatory factors (nef), regulator of expression of virion proteins (rev), transactivator of transcription (tat), virion infectivity factor (vif), viral protein R (vpr), and viral protein U (vpu) (Turner and Summers, 1999).

Four fundamental genes are responsible for encoding the virus structure: gag encodes internal structure proteins such as matrix (MA), capsid (CA), and neucleocapsid (NC) proteins. Pol gene is translated to generate the viral enzymes: for instance, reverse transcriptase (RT), integrase (IN) and the RNase H. Gag, pol, pro coding sequence is expressed as a polyprotein known as gag-pol-pro, which is self-cleaved by a protease enzyme that encodes via the Pro gene. Proteins that are associated with the viral envelop, the trans membrane (TM) and surface protein (SU), are expressed by the env gene. The remaining six auxiliary genes are accountable for encoding proteins that are involved in a broad spectrum of viral mechanisms. The first supplementary gene is nef, which plays a role in modifying the host immune response. It encodes nef proteins which reduce the expression of CD4 and Major histocompatibility complex I and II (MHCI and II): as a result, the virus can escape from the

host immune system. The second auxiliary gene is rev, and this gene is in charge of encoding proteins that play a major function in regulating the gene expression of other virion proteins. The third non-essential gene is tat: proteins encoded by this gene are at the helm of regulating the viral transcription mechanism while proteins encoded by vpr are responsible for guiding the viral compartments to their directions inside the cell via a signal called “the nuclear localization signals”. Therefore, vpr assists in transporting the pre-integration complex (PIC) from the cytoplasm toward the nuclear pore. Moreover, it induces the arrest of the cells in the G2 phase of the cell-cycle: thus, the virus can increase its transcription and protein production. Vpr also has more than one function, down-regulating CD4, and helping in the budding process of the virus. Finally, vif proteins inhibit a defensive cell factor called APOBEC3G, resulting in the production of an infectious progeny of the virus (Watts et al., 2009).

Once the RNA viral genome is reverse transcripted and integrated in the host’s genome, the provirus gene is flanked by two equivalent, non-coding, long-terminal repeat sequences (LTRs). LTR is comprised of three regions: U3, R and U5. In the proviral DNA, the 5’-U3 region incorporates the promoter and enhancer sequences that are essential for the transcription of viral genes. On the other hand, the 3’-U5 serves as a transcription termination site. The R region is arranged in repeats at both ends and contains the essential sequence for strand transfer during the reverse transcription process (Figure 9).

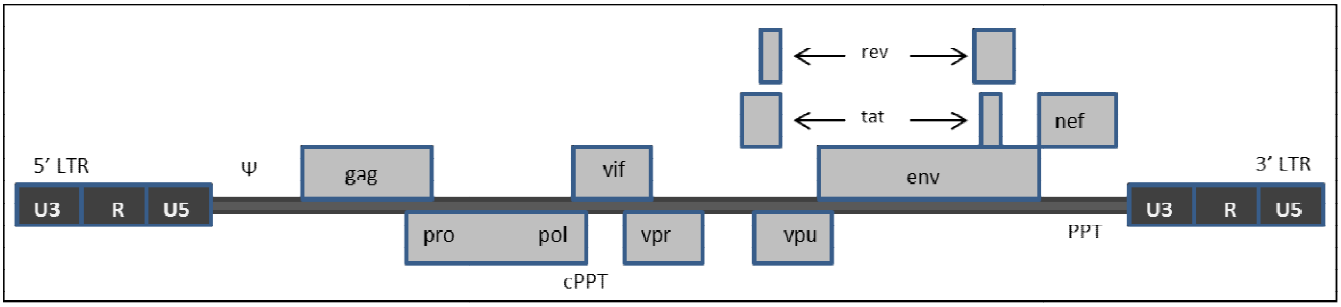


Figure 9 Schematic of HIV-1 genome encodes the structural genes gag, pol, env. In addition to the regulatory genes, tat and rev, there are accessory genes, vif, vpr, vpu and nef. The provirus is flanked by two identical, long-terminal repeats (LTRs); which are subdivided into U3, R and U5 regions. Moreover, the genome also contains the packaging signal ψ, polypurine tract (PPT) and central PPT (cPPT).

1.6.2.2.3 Virus life cycle:

The replication of retroviruses is a multi-stage process. Retroviruses commence their life journey by attaching their surface glycoprotein to specific receptors on target cells. Once the virus is attached to its target, the viral envelop membrane fuses to the host cell membrane and allows virus entry (Dragic et al., 1996). Following entry of the virion into the host cell, un-

coating occurs where the viral particle is disassembled. As a result, the viral RNA genome is freed in the host cytoplasm and ready for reverse transcription. Reverse transcription starts at the 5' end of RNA (3' end of tRNA), where the primer-binding site (PBS) is located. Transfer RNA (tRNA) will bind to PBS and use reverse transcriptase (RT) to perform its function, which converts RNA to (ds) DNA capable of integration into the host genome. Once reverse transcription has taken place, a viral complex known as pre-integration complex (PIC) will be generated. In order for retroviral PICs to enter the nuclear membrane, mitosis (cell division) should occur; on the other hand, lentiviral PICs have the ability to penetrate the host nuclear membrane without requiring cell division; this is one of the reasons behind the preference for lentiviral vectors over retroviral vectors. PIC has a larger diameter than the nuclear pore, and thus, nuclear entry does not occur via simple penetration of PIC through these pores. Although the exact mechanism utilised by lentivirus to enter the nucleus is not yet fully understood, it has been suggested that many viral and host molecules contribute to driving PIC to the nucleus. Once the provirus is inside the nucleus boundaries, viral integrase will play its role by chopping part of the host genome and sealing the provirus genome instead. Consequently, viral gene expression patterning will commence using host cellular machinery (Haseltine, 1991).

1.2.2.4 The development of third generation lentiviral vector:

In the first generation of HIV-derived vectors, the replication-defective, recombinant, chimeric lentiviral vector is built up from three different components: the first is the packaging plasmid expressing the HIV-1 core proteins, enzymes and accessory factors; the second plasmid is the envelop plasmid which carries the G envelop glycoprotein gene from another virus called the vesicular stomatitis virus (VSV-G) (Figure 10.D); and the last expression plasmid is the transfer vector plasmid that encodes the transgene of interest and cis-acting elements for HIV-1 that are essential for packaging, reverse transcription and integration.

Generating a vector derived from the pathogenic human virus carrying all its genes, except for env, raises biosafety concerns because it would only require a minimal number of recombination events to build a functional virulent viral particle. Therefore, re-designing of the packaging system has occurred, resulting in the production of a second generation of lentiviral vectors. In the second generation, the four accessory non-essential genes are eliminated from the packaging system, leaving the four essential HIV-1 genes, gag and pol, for expressing all structural and enzymatic components of the virion, and tat and rev, which

are crucial for transcriptional and post-transcriptional functions (Figure 10.B). The deletion of vif, vpr, vpu and nef from the packaging system has no effect on the production of high titres of lentiviral vectors, but it improves the safety profile. Although this second version of the viral vector is widespread and there is no evidence for detecting any recombination events, additional alterations have been applied to the lentiviral vector for further reduction of the recombination effect and the formation of the replication competent lentivirus (RCL).

To improve the safety profile of viral vectors, 3rd-generation lentiviral vectors have emerged (Figure 10.C). One of the differences in the 3rd-generation design is that it has four separate plasmids: two packaging plasmids, an envelope plasmid and a vector transfer plasmid. Another improvement in the 3rd-generation system is the replacement of the U3 in 5' LTR in the transfer vector plasmid with an active promoter, removing the need of tat to carry on an efficient transcription. Additionally, it has been suggested that expressing rev in trans would lead to the production of high-titer vector stock. This gene delivery system conserves only three of the nine HIV-1 genes, and has four separate transcriptional units for the production of vector particles: thus, it offers a remarkable advantage from the point of view of biosafety (Dull et al., 1998).

To address the unwanted outcome of inserted mutagenesis, self-inactivating (SIN) vectors have been engineered (Figure 10.A). SIN is viral vectors that incorporate a deletion in U3 region at 5 ' LTR, where viral promoter and enhancer are located. Upon reverse transcription and integration, this modification will lead to the inactivation of the viral promoter/enhancer elements. At the 5' region of SIN, U3 can be replaced with strong promoters from other viruses such as the spleen focus-forming virus LTR (SFFV), which is widely used due to its ability to produce high titers and to provide a constant expression. However, SFFV is not preferably used in clinical settings due to two reasons: first, SFFV is derived from the gamma retrovirus, thus leading to safety concerns; second, it tends to methylate, silencing the gene expression process. As a result, a new, human-derived promoter is required. Human phosphoglycerate kinase (PGK) promoter can be used to provide a high level of gene expression and has been approved for use in clinics (Cooray et al., 2012).

In addition to the reduction in the number of HIV-1 genes to improve safety, some elements are added for different purposes. A mutant version of the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) has been used to increase vector titre and boost gene expression (Higashimoto et al., 2007).

Lentiviral vectors have been widely used in a large number of clinical trials to treat a range of diseases. In 2006, the first clinical trial to treat HIV-1 by lentiviral vector was published. This

study has revealed crucial information about the efficiency and safety of using these vectors. Moreover, it builds a solid foundation to assist the following gene therapy studies with lentiviral vectors (Levine et al., 2006).

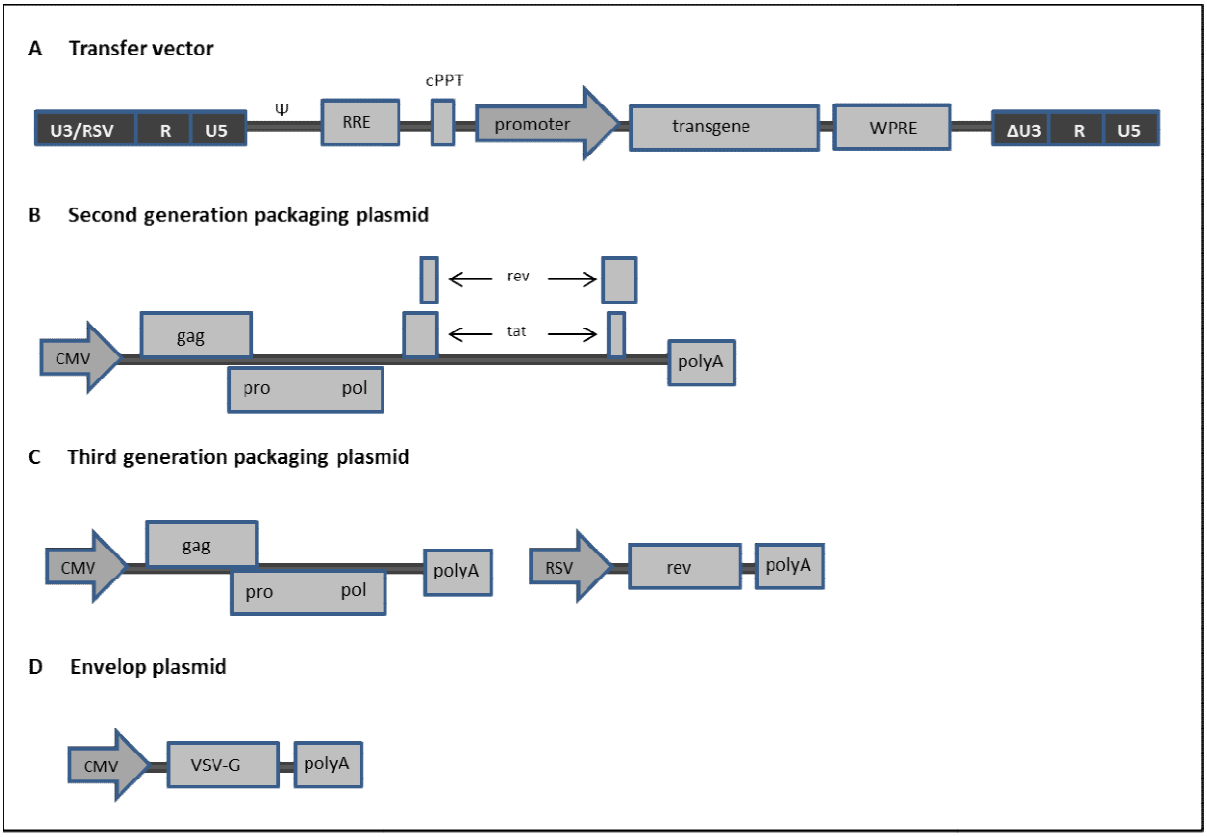


Figure 10: Second and third generation lentiviral vectors. A. Self-inactivating lentiviral transfer vector. The second generation system uses the HIV-1 original U3, while the third-generation packaging system substitutes U3 in the 5' LTR with Tat independent Rous Sarcoma virus (RSV) promoter. RRE: rev responsive element, cPPT=central polypurine tract, WPRE= Woodchuck hepatitis virus post transcriptional regulatory element. B. Second generation lentiviral packaging plasmid.CMV= Cytomegalovirus promoter.C. Third generation lentiviral packaging plasmid. Rev gene is provided in packaging cell in trans using the RSV-rev plasmid. D. Envelop plasmid with vesicular stomatitis virus glycoprotein (VSV-G)

1.7 Aims of the project

To investigate the feasibility of generating off-the-shelf, universal, antigen-specific T cells from healthy donors that can be administered to multiple patients without causing GVHD.
The main questions addressed in this project are:

i. Is it feasible to prevent endogenous TCR expression using nuclease technologies?
ii. Can TCR knockout cells be redirected against target antigens via lentiviral gene transfer of antigen specific receptors?
iii. Do engineered cord blood T cells retain their naïve phenotype after subjected to multiple manipulations?
iv. Do naïve cord blood T cells tolerate multiple manipulations that require knocking out TCR and introducing new receptor better than adult T cells?

Chapter 2: Materials and Methods

2.1 Materials:

All cell culture reagents were supplied by Gibco BRL (Invitrogen) and all general chemicals by Sigma-Aldrich. Enzymes for molecular cloning and other restriction digestion reactions were supplied by Promega, New England Biolabs and ThermoFisher Scientific. Primers were provided by Invitrogen. DNA sequencing reactions were performed by Eurofins MWG Opero, while next generation sequencing were completed by MiSeq illumena

2.1.1 General reagents:

1-Kb plus DAN Ladder	Invitrogen
Agar	Invitrogen
Agarose	Melford
Ampicillin	Sigma-Aldrich
Bovin Serum Albumin (BSA)	Sigma-Aldrich
Carbenicillin	Sigma-Aldrich
dNTPs	Applied Biosciences
Ethylenediaminetetraacetic acid (EDTA)	
Ficoll	GE Healthcare
Interleukin-2 (IL-2)	Proleukin,Chiron
Interleukin-7 (IL-7)	Proleukin,Chiron
Interleukin-15 (IL-15)	Proleukin,Chiron
Kanamycin	Sigma-Aldrich
LIVE/DEAD fixable blue dead cell stain (Tryban blue)	Invitrogen
MES SDS Running buffer (20x)	Invitrogen
NuPAGE 4-12% Bis-Tris gels	Invitrogen
NuPAGE Transfer buffer (20x)	Invitrogen
SeeBlue® Plus2 Protein standard	Invitrogen

2.1.2 Buffers:

10x TAE: 400mM Tris-acetate, 10mM EDTA in H2O

2x B & W: 10 mM Tris-HCL (pH7.5), 1 mM EDTA, 2 M NaCl

1x B&W with 0.005% Tween 20:5 mM Tris-HCL,0.5mM EDTA, 1 M NACL,0.005%Tween20

Laemmli buffer: 60mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue

MACS buffer: phosphate-buffered saline (PBS), 0.5% bovin serum albumin (BSA), 2 mM EDTA. Mix and filter them through 0.45µm Filter.

2.1.3 Antibodies:

sc-9100	T cell receptor alpha rabbit IgG	Santa Cruz Biotechnology
sc-9101	T cell receptor beta rabbit IgG	Santa Cruz Biotechnology
130-098-219	Anti-TCR-biotin mouse IgG2b	Miltenyi Biotec
130-090-485	Anti-biotin microbeads	Miltenyi Biotec
	Cre-I (Meganuclease) rabbit IgG	Cellectis
NXA931	Sheep HRP-linked Anti-Mouse	GE Healthcare
NA934	Donkey HRP-linked Anti-Rabbit	GE Healthcare
115-066-072	Biotin-SP-conjucated AffiniPure F(ab')$_2$ fragment goat anti-mouse IgG, F(ab')$_2$ fragment specific	Jackson Immunoresearch laboratorie
555547	FITC Mouse Anti-Human TCR α/β	Becton Dickinson UK LTD
555335	APC Mouse Anti-Human CD3	Becton Dickinson UK LTD
LS-C107386	PE-Cy7 Mouse Anti-Human CD45RA	LifeSpan Bioscience
305624	Brilliant violet 421 Mouse Anti-Human CD95	Biolegend
MHCD0431	PerCP Mouse Anti-Human CD4	Thermofisher scientific
557734	Alexa Fluor® 647 Rat Anti-Human CD197 (CCR7)	BD Pharmingen™
561914	FITC Mouse Anti-Human CD62L	BD Pharmingen™
555635	PE Mouse Anti-Human CD8	BD Pharmingen™

2.1.4 Kits:

Amaxa cell line nucleofector kit V	Lonza
Amaxa nucleofector kit for human T cells	Lonza
CD34 microbead kit and MD columns	Miltenybiotec
DNeasy Blood & Tissue kit	Qiagen
Endofree plasmid preparation kit	Qiagen
Real Assembly TALEN kit	Joung lab
Mini-elute PCR purification kit	Qiagen
Mini / maxi Plasmid preparation kit	Qiagen
Mini-elute PCR purification	Qiagen
Pierce BCA protein assay kit	Thermo Fisher Scientific
QiaQuick PCR purification kit	Qiagen
QiaQuick Gel purification kit	Qiagen
RNeasy Mini Kit	Qiagen
Surveyor mutation detection kit	Thermo Fisher scientific
T7 mScript™ Standard mRNA Production System	Cambio
The GenElute™ Mammalian Genomic DNA Purification kit	Sigam-aldrich
TCR depletion kit and LD columns	Miltenybiotec
TOPO TA cloning	Invitrogen

2.1.5 PCR Primers:

Unless otherwise stated, all primers were supplied by Invitrogen and most of PCR reactions were processed using phusion high-fidelity DNA polymerase (Promega/New Biolab) or Go Taq DNA Polymerase (Promega)

Primer name	Primer sequence	
CAPNS1 fwd	5'-ACCGCGGCCGCAATTGCAGT-'3	Meganuclease target site
CAPNS1 rev	5'- AAAGGGGACCAGTGGGTGCTTCG-3'	Meganuclease target site
oJS2581 fwd	5'-TCTAGAGAAGACAAGAACCTGACC-'3	TALEN α unit 1- biotin adds
oJS2582 rev	5'-GGATCCGGTCTCTTAAGGCCGTGG-'3	TALEN α unit 2
T7 CRISPR TRAC fwd	5'-TTAATACGACTCACTATAATCCGTCAGGGTTCTGGATATC-'3	Adds T7 promoter

T7 CRISPR TRAC rev	5'-AAAAGCACCGACTCGGTGCC-'3	Adds T7 promoter
TRAC fwd	5'-AACGTTCACTGAAATCATGGCC-'3	Meganuclease / TALENs target site
TRAC rev	5'-GCTGGGGAAGAAGGTGTCTT-'3	Meganuclease / TALENs target site
TRBC1 fwd	5'-CTGTCAGAGGAAGCTGGTCTGG-'3	Meganuclease target site
TRBC1 rev	5'- CTTACCATGGCCATCAACACAAG-3'	Meganuclease target site
TRBC2 fwd	5'-GTGGGAGAGACCAGAGCTACCTG-3'	Meganuclease target site
TRBC2 rev	5'- CCATTGACCACACAAATAGTAAGG-3'	Meganuclease target site

2.1.6 Cells:

2.1.6.1 Bacterial cells:

Most of the transformation process was done using One Shot® Stbl3™ Chemically Competent *E. coli* (Invitrogen, UK). These cells are excellent host strain to use for cloning unstable inserts, such as lenitviral DNA containing direct repeats. This strain is derived from the HB101 *E.coli* group and its genotype is F- mcrB mrr hsdS20 (rB-, mB-) recA13 supE44 ara-14 galK2 lacY1 proA2 rpsL20 (Str) xyl-5 λ- leu mtl-1r .

Second bacterial cells called XL1-Blue Competent Cells (Agilent Technologies, USA) were used in the transformation process on cloning homemade TRBC TALENs DNA fragment into Fok-nuclease expression vector. These cells are designed for routine cloning application. The XL1-Blue genotype is recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lacF′ proAB lacIq ZΔM15 Tn10

2.1.6.2 Mammalian cell lines and culture conditions:

Multiple cell lines derived from hematopoietic origin were used: **Jurkat cells** are an immortalized human, T lymphoblastoid cell line derived from an acute T cell leukaemia, and they are suspension lymphoblasts. This cell line was established from the peripheral blood of a 14 year-old boy by Schneider et al (Schneider et al., 1977).

An antigen-processing (Tap) deficient human cell line expresses HLA-A2 named **T2** was also used. T2 cells are a mutant human cell line that have a defect in antigen presentation in the context of class I major histocompatibility complex (MHC) molecules. As a consequence,

the HLA-A2 molecule of these cells can be efficiently loaded with exogenous peptides (Amrolia et al., 2003).

A human, TCR-negative, **Jurkat-76** cell line was also utilised in addition to the previous cell lines: this is a cloned, human, T cell leukaemia line that is deficient in endogenous TCR expression.

All these cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen life technologies, UK) and were supplemented with 10% of heat-inactivating, foetal-calf serum (FCS) (Sigma Life Sciences, USA) and 1% of penicillin/Sterptomycin (Invitrogen Life Technologies, UK).

Cell line named **Sup-T1** was also applied in some experiments. It consists of human, T cell, lymphoblastic lymphoma cells that are derived from a malignant pleural effusion taken from an eight year-old Caucasian male with T cell lymphoblastic lymphoma. These cells were stably transfected with CD19 antigen and they were obtained from Cancer Institute, UCL, UK. The SupT1-CD19 cell line was used to evaluate the functionality of a T cell transduced with CD19-CAR lentivirus. A non-transfected SupT1 cell line; CD19 negative SupT1, was also used as a negative control for these experiments. SupT1 cells were treated in similar conditions to those of other cell lines derived from the human T cell population.

For lentiviral virus production, **HEK293T cells** were carefully treated and cultured. These cells are human embryonic-kidney cells which are grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Life Technologies, UK), supplemented with 10% FCS (Sigma Life Sciences, USA) and 1% Penicillin/Streptomycin. They are adherent cells and their suitable environment for incubation is at 37^{o}C with 5% CO_2. Their proliferative capacity and permissibility to transfection make them a good choice as a packaging cell line for virus production.

2.1.7 Cord blood and primary human T cells and culture conditions:

Cord blood (CB) is a well-established origin for hematopoietic stem cells transplantation. One of its immunological advantages is its ability to tolerate antigen mismatch that leads to a decrease in GVHD incidence, while maintaining to pursue an anti-tumour effect (Barrett and Bollard, 2015). The widespread use of CB-Transplantation is obstructed by the delay in hematologic engraftment and by high, treatment-related mortality (Okas et al., 2010).

CB contains a comparatively higher number of mononuclear cells including T cells, B cells, and natural killing cells than peripheral blood (PB) or bone marrow (Harris and Rogers,

2007). CB-T lymphocytes are the main concern of this project, so the next paragraph will discuss its properties.

CD3+ percentage is similar in both CB and PB; however, the absolute number of T cells is higher in CB than PB. Markedly, the CD4 to CD8 ratio is the same in both CB and PB. CB-T cells are naïve CD45RA+CD62L+. Surprisingly, it has been reported that even after exposing CB-T cells to stimuli, they maintain their CD45RA compartments and therefore, maintain their naïve phenotype (Cairo et al., 2016); a fundamental requirement for a successful adoptive T cell therapy.

The Anthony Nolan Cord Blood Bank, based in the UK, provided us with umbilical cord blood units (CB). Adult peripheral blood mononuclear cells (PBMC) were obtained from healthy, adult volunteer donors who signed a consent, and they are based at the Institute of Child Health, UCL. Both CB-T cells and PBMCs were treated in similar ways: the units were diluted in phosphate buffer saline (PBS) (Invitrogen Life Technologies, UK) at a 1:1 ratio and incubated for 20–30 min at room temperature. Diluted blood was then gently layered onto 15 ml ficoll-paque (GE Healthcare, UK) in 50 ml falcon tubes. After being subjected to 35 min of centrifugation at 400 gravity, mononuclear cells were collected from the layer between the plasma and the ficoll, and washed with PBS three times. After the final wash, the harvested monocular cells were resuspended in X-VIVO 10 or 15 medium (Invitrogen, UK) and supplemented with 5% AB human serum (Invitrogen, UK), together with 100 units per ml of human, recombinant interleukin 2 (IL-2) (Proleukin, Novartis, USA). Afterwards, cells were activated with different stimulators (A detailed discussion regarding T cell activation will be presented further in the text) for 48 hours before transduction or transfection.

2.1.8 Media:

All starter culture for bacterial growth is made of LB broth: 1% tryptone, 0.5% yeast extract; 1% NaCl. A 1.5% LB agar is added to the previous recipe to form an agar plate.

For Mammalian cells, all cell culture media were obtained from Gibco BRL and Invitrogen, unless otherwise stated. The medium used for culturing cell lines was supplied with foetal calf serum (FCS) (Sigma-Aldrich). For cord blood and primary cells, X-VIVO 10 or 15 medium (Lonza) was supplied with human AB serum (Lonza). Dynabeads ® Anti-CD3/anti-CD28 beads (Invitrogen) were used as a one mean for T cell activation in addition to IL-2 (100U/ml).

2.1.9 Parental plasmids:

pLNT/SFFV-MCS-WPRE: self-inactivating (SIN), second generation lentiviral backbone derived from pHR plasmid. It includes a SFFV promoter followed by a multiple cloning site and Woodchuck hepatitis virus post transcriptional regulatory element (WPRE).

pLNT/SIEW: (SFFV-IRES-eGFP-WPRE) lentiviral vector with the same backbone as pLNT/SFFV-MCS-WPRE, with an IRES-eGFP inserted at the multiple cloning site.

pLNT/SFFV-TRAC-TALENs: expression plasmid encoding the left arm TRAC TALENs, under the control of SFFV promoter; cloned into self-inactivating next generation lentiviral vector backbone. Provided by Cancer Institute, UCL.

pLNT/SFFV-TRAC-TALENs: expression plasmid encoding the right arm of TRAC TALENs, under control of SFFV promoter; cloned into self-inactivating next generation lentiviral vector backbone. Obtained from the Cancer Institute, UCL.

pCRISPR-TRAC: All-in-one sgRNA expression plasmid encoding TRAC sgRNA under the control of U6 promoter and encoding Cas9 under the control of CMV promoter Provided by GeneCopoeia.

pCRISPR-TRBC1: expression plasmid encoding TRBC1 sgRNA under the control of U6 promoter and encoding Cas9 under the control of CMV promoter. Provided by GeneCopoeia.

2.1.10 Generated plasmids:

Sub-cloning is a method in which any DNA fragment, promoters or selectable markers are re-cloned from one plasmid to another expression plasmid. This technique has been applied to move MGN open reading frames (CAPNS, TRBC01 and TRBC02) from their original expression plasmid and clone them into lentiviral vector backbone named pSEIW. SIEW is self-inactivating (SIN) second generation lentiviral backbone derived from pHR plasmid. It includes a SFFV promoter followed by IRES-eGFP and Woodchuck hepatitis virus post transcriptional regulatory element (WPRE).

BamHI and AscI (Promega, UK) restriction enzymes were used to linearize the vector and prepare it for ligation. MGN open reading frames were chopped out from their original expression plasmid with EcoRI and SacII (Promega, UK) restriction enzymes. Afterwards, the digested vector and appropriate inserts were mixed at 5:1 ratio and incubated overnight with Ligase (Promega, UK) at 16^{o}C. On the following day, ligation reactions were transformed into competent *E.coli* (Invitrogen) and cultured on ampenicillin culture agar. The picked-up colonies were exposed to restriction reaction with EcoRI (Promega, UK) and run on 1% gel to define the correct sizes of vector and inserts.

2.2 Methods:

2.2.1 Escherichia coli (*E.coli*) growth and maintenance:

A single colony of *E.coli* was picked up and placed in 5 mls LB broth. This starter culture was incubated at 37°C with agitation at 250 rpm for 16-18 hours. Another way that bacterial culture was obtained; was by streaking it out on an agar plate (1.5% becto agar) and incubating it in incubator at 37°C overnight. For selection of transformed *E.coli*, the same media was supplemented with appropriate antibiotics: 50µg/ml Ampicillin or kanamycin, or 100 µg/ml Carbenicillin was used. A 15% glycerol was added to the starter cultures in order to store bacterial cultures in -80°C.

2.2.2 Transformation of chemically competent *E.coli*:

One vial of component *E.coli* bacteria was thawed on ice and 1-5 µl of DNA was added. The bacteria and DNA mixture was incubated on ice for 30 min. After 30 min of incubation, the bacterial cells were heat-shocked at 42°C for 45 seconds, then cooled on ice for two minutes. Afterwards, a 250 µl SOC medium was mixed with the cells and incubated on horizontal manner with agitation at 37°C for 1 hour. A 100 µl bacterial culture was plated out in LB agar supplemented with appropriate selection antibiotics and incubated overnight at 37°C.

2.2.3 Plasmid DNA preparation:

A single colony of *E.coli* containing plasmid DNA was grown overnight at 37°C in LB broth supplied with an appropriately selected antibiotic. Next, the plasmid was purified and extracted by alkaline lysis using a Qiagen Miniprep or Maxiprep kit according to the manufacturer's instructions. Then, a Nanodrop ND-1000 spectrophotometer at a wavelength of 260nm was used to measure DNA concentration.

2.2.4 Restriction endonuclease digest:

In order to prepare DNA for traditional cloning procedure or for any other DNA analysis, restriction enzymes have been used to cut DNA at a specific site. DNA was digested in a reaction containing one or two restriction enzymes (<10% final volume), 0.1mg/ml BSA, 1x buffer (supplied by manufacturer) and water to reach a final volume of 20 µl or 50 µl reaction. The reaction was then incubated for 1 to 4 hours at 37°C.

2.2.5 Filling in of 5' DNA overhangs to form blunt ends:

The 5' DNA protruding ends generated by restriction enzyme digests were filled-in using DNA polymerase I Large (Klenow) fragment. 1 unit (U) of enzyme per microgram of DNA

was mixed with DNA, 1x buffer (supplied by Promega) and 40µM of each dNTP. After 10 min of incubation at room temperature, the reaction was stopped by heat inactivation of the enzyme for 10 min at 75^{o}C.

2.2.6 Dephosphorylation of 5' phosphate groups from DNA:

Prior to ligation and to avoid re-ligation of linearised vector DNA with compatible ends, 5' phosphate groups from DNA were removed. Thermosensitive alkaline phosphatase was added directly to restriction reactions at 1U per microgram of DNA. Then, after 15 min of incubation in at 37°C, the enzyme was inactivated at 75°C for 10 minutes.

2.2.7 DNA Ligation:

1U of T4 DNA Ligase and 1x T4 DNA ligase buffer (supplied by manufacturer) were used to ligate insert DNA into plasmid backbone in approximately a 3:1 molar ratio in a final reaction volume of 20 µl. For sticky ends, the reaction was incubated overnight at 4^{o}C, while for blunt ends, an overnight incubation at 16^{o}C was considered. After overnight incubation, the reaction was transformed into chemically component *E.coli* bacteria.

2.2.8 Agarose gel electrophoresis and purification of DNA fragments:

One of the standard lab procedures is to separate DNA fragments by size for visualization and purification using gel electrophoresis. Agarose is dissolved in 1xTAE buffer by heating, and ethidium bromide or syber green was added to the final concentration of 0.5µg/ml before it was allowed to solidify. Alongside 1kb Plus DNA ladder, samples were loaded onto gel after they were mixed with DNA loading buffer. Negatively charged DNA fragments move toward positive electrodes by electrophoresis at 100-150V in 1x TAE buffer and then visualised under UV light using a UviDoc gel documentation system. In some cases, DNA fragments were cut out from the gel using a scalpel under UV light. Qiaquick gel extraction kits were used to purify them.

2.2.9 Genomic DNA extraction:

Before incubating the cell's lysate at 70^{o}C for 10 min, a cell pellet was resuspended in 200µl resuspension solution and mixed with 20 µl proteinase kinase and 200 µl lysis buffer. Afterwards, 200 µl ethanol was added and the sample then transferred into a binding column and spun in a bench top centrifuge at centrifugal force of 6500 x gravity for 1 minute. The column was washed twice by adding 500µl washing solution and finally, DNA was eluted in 180 µl H2O and stored at -20^{o}C.

2.2.10 Topo TA cloning:

DNA fragment was extracted from 1% agarose gel, then amplified by PCR using Taq-polymerase enzyme and purified according to the manufacturer's instructions. The PCR amplified DNA fragment was then ligated into pCR™4-TOPO® vector by incubating the PCR insert with salt solution, water and vector for 5 minutes at room temperature. The Topo cloning reaction was then placed on ice and used to transform One shot Topo10 *E.coli* by heat shock, which then were plated on ampicillin LB agar and incubated overnight. The insert was sent for sequencing after extracting DNA from some selected colonies.

2.2.11 Surveyor Nuclease assay:

The CAPNS1, TRAC and TRBC MGN target sites as well as the TRAC TALENs target sites were amplified by PCR using corresponding primers (mentioned in the materials section) and PCR product was resolved by electrophoresis through a 1% or 2 % agarose gel. Then, 15 µl (approximately 200 ng) of PCR product (DNA) was denatured in a thermocycle PCR machine for 10 minutes at 95^{o}C and cooled down to 85°C with a decrement of 2°C/s then 85°C to 25°C with a decrement of 0.1°C/s and 4°C to allow re-annealing. Surveyor nuclease recognizes and cleaves a mismatched double strand DNA created when NHEJ DNA containing mutations re-anneals with the native sequence. Therefore, amplified DNA was exposed to 2 µl Enhancer S and nuclease S at 1:1 ratio and incubated at 42^{o}C for 1 hour to digest mismatched DNA and producing two bands. Cleaved DNA was visualized on 2% agarose gel with 0.5µg/ml ethidium bromide under UV light.

2.2.12 mRNA production and In-vitro transcription:

Reports and studies have proven that delivering gene of interest as mRNA is more effective and less toxic to the cells than using a naked DNA plasmid nuclofection system (Mock et al., 2015). Therefore, it has been suggested that mRNA electroporation might be a reliable alternative method as a gene-transferring technique into T cells. T7 mScript™ Standard mRNA Production System and RNeasy kit were used to produce mRNA from expression plasmid encoding TRAC TALENs. Plasmids; which contain T7 promoter sequence, first were linearised by restriction digest using AvrII and then purified using QIAquick PCR Purification Kit. Then, the DNA template was subjected to In-vitro transcription (IVT) by adding the following reagents: 10x mScript T7 transcription buffer, NTP solution, 100mM DTT, RNase inhibitor and finally the mScript T7 enzyme solution and incubated at 37^{o}C for 30 min. DNase I was added the previous IVT reaction to remove any DNA template residues. Before capping the In-vitro transcript RNA, it was heat-denatured at 65^{o}C for 10

minutes then capped by adding 10U/µl ScriptCap capping enzyme and incubated at 37°C for 30 minutes. After capping, mScript™ poly (A) polymerase enzyme was added to the poly(A) tails sequence with the desired length. At the end of each reaction, approximately 60 µg of fully capped and polyadenylated mRNA was produced.

2.2.13 Quantification of protein by using Pierce BCA protein assay:

Standard reagents were prepared by diluting the content of albumin standard (BSA) into clean vials from 2000µg/ml to25 µg/ml in milliQ water. The volume required for working reagents was first determined by the following equation:

(# standards + # unknowns) × (# replicates) × (volume of WR per sample) = total volume WR required, then, in order to prepare the working solution, 50 parts of BCA reagent A (supplied with the kit) with 1 part of BCA reagent B (supplied with the kit).

In a microplate well, 25 µl standard or sample were added in replicates and were mixed with 200 µl working solution on a plate shaker for 30 seconds. Covered Plate was allowed to cool down at room temperature after an incubation period of 30 minutes at 37°C. Finally, the absorbance was read on plate reader at or near 562 nm.

2.2.14 Western blot analysis:

$1x10^{6}$ cells were harvested 24 hours after transfection and lysed using RIPA buffer (Santa Cruz Biotechnology, CA). 20µg of protein and SeeBlue Plus2 Pre-stained Standard were separated by 4-12% Bis-Tris NuPAGE gel electrophoresis and 1x MES buffer at 200V for 45 min. Proteins then transferred from the gel onto immobiolon-P PVDF membrane by using X-Cell II Blot module and 2x NuPAGE transfer buffer plus 10% methanol at 18V for 45 min (Merck Millipore, UK). In order to avoid a non-specific antibody binding, membrane was blocked in 4% milk powder in PBST (PBS containing 0.05% Tween-20) for an hour at room temperature. Afterwards, membranes were incubated with primary, rabbit, polyclonal antibody against I-CreI at 1: 20,000 dilution for meganuclase detection or with 1:5000 for anti-TCR alpha and beta rabbit IgG and incubated overnight at room temperature. On the following morning and before adding the horse-radish peroxidase conjugated goat anti-rabbit secondary antibody (diluted 1:2000 in 4% milk in PBST), the membrane was washed three times by adding 20 ml of PBST. After the one-hour incubation of the membrane with secondary antibody, it was washed in PBST and the specific protein bands were visualized with an enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific, USA). A rabbit antibody against β-tubulin was used for the loading control.

2.2.15 Propagation and storage of mammalian cell lines:

Adherent cell lines were passaged when they were 80-90% confluent by washing with PBS then detaching them by incubating with trypsin/EDTA. Cells were diluted and maintained in fresh warm DMEM, then cultured at suitable concentration into a tissue culture flask. Non-adherent cells were passaged when the media color changed by transferring a proportion of the cells into a fresh and warm medium. All cells were cultured at 37^{o}C in 5% CO2 incubators. As mentioned above, all media were supplemented with 10% FCS and 1% penicillin/streptomycin. In order to create a cell bank, a 1-10x10^{6} cells' pellet was re-suspended in freezing medium; which was made up of 90% FCS and 10% DMSO, then frozen slowly in an isopropanol freezing container at -80^{o}C overnight. The cells were then transferred into liquid nitrogen. If cells were required, they were moved from liquid nitrogen and rapidly thawed in a water bath, then added in drop by drop manner to warm fresh complete medium. Cells then were centrifuged to remove DMSO and resuspended in fresh warm compete appropriate medium and cultured in a T25 tissue culture flask.

2.2.16 Cell counting:

A haemocytometer (0.0025 mm2, Marienfeld, Germany) was used as the counting chamber to count the viable cells' density. A 10 µl cell suspension was mixed with 90 µl Trypan blue dye (Sigma Life Sciences, USA) and loaded into the counting chamber. Dead cells ingest the Trypan blue dye and are discriminated from intact living cells by using direct-light microscopy. The total number of viable cells was counted in the four large squares within the haemocytometer and the resulting figures input into the following equation:

Average of cell number in the 4 squares x 10 (dilution factor) x10^{4}= total number of cells/ml in the original suspension.

2.2.17 Isolation of cord blood and primary human T cells and culture conditions:

The Anthony Nolan Bank, based in the UK, provided us with umbilical cord blood units (CB). Adult peripheral blood mononuclear cells (PBMC) were obtained from healthy, adult-volunteer donors based at the Institute of Child Health, UCL after signing consent. Both CB-T cells and PBMCs were treated in a similar way: units were diluted in phosphate buffer saline (PBS) (Invitrogen Life Technologies, UK) at a 1:1 ratio and incubated for 20 min at room temperature. Diluted blood was then gently layered onto 15 ml ficoll-paque (GE Healthcare, UK) in 50 ml falcon tubes. After being subjected to 40 min of centrifugation at 400 x gravity, mononuclear cells (buffy coat) were collected from the layer between the plasma and the ficoll, and washed with PBS 3 times. After the final wash, the harvested

monocular cells were resuspended in X-vivo 10 or x-vivo 15 medium (Invitrogen, UK) and supplemented with 5% AB human serum (Invitrogen, UK), together with 100 units per ml of human recombinant interleukin 2 (IL-2) (Proleukin, Novartis, USA). 10^6 cells per well were placed in a 24-well plate or for scale up experiments, $50x10^6$-$100x10^6$ cells were cultured in a 100 ml cell differentiation bag (Miltenyi Biotec, Germany) and activated with different stimulators (this section will later discuss the means by which T-lymphocytes are stimulated) for 48 or 72 hours before transduction or transfection.

2.2.18 CB and PBMCs stimulation conditions:

CB-T cells and PBMCs were activated under three different conditions. The standard way of activating human lymphocytes is to use Dynalbeads® ClinExVivoTM CD3/CD28 (Invitrogen, UK) at a ratio of 1:1. For small scale experiments, 10^6 CD3/CD28 beads per ml were added to 10^6 CB-T cells or PBMCs; while for large scale experiments, $50x10^6$ or $100x10^6$ beads per ml were added to 50x 10^6 or $100x10^6$ CB-T cells, in addition to 100 U/ml of IL-2 supplementation every 48 hrs. Another means of stimulation involved the use of a combination of cytokines cocktail: 5 ng/ml of human recombinant interleukin-7 (IL-7) (Peprotech, USA), 5 ng/ml of human, recombinant interleukin 15 (IL-15) (Peprotech, USA), and 100 u/ml of IL-2 were added to the previously isolated CB-T cells and PBMCs. Finally, a combination of CD3/CD28 beads and IL-7 and IL-15 cytokines were added to the third batch of isolated CB-T cells and PBMCs. After 48 hours of activation, cells were ready for either virus exposure or transfection.

2.2.19 Nucleo-transfer of DNA into cord blood, PBMCs and Jurkat cells:

Three different means were used to introduce various types of DNA-nucleases to Jurkat cells, CB-T cells and PBMCs. After stimulating CB-T cells and PBMCs with anti-CD3/anti-CD28 beads or cytokines, cell density ranged from 2-5 x 10^6 cells were centrifuged and resuspended in 100 µl human-T cell nucleofector solution for amaxa transfection system, and in E2 buffer for neon transfection system. Afterwards, cells were exposed to different types of gene-specific nucleases as a DNA: 10 µg of each pair of TRAC TALENs (Cellectis, France) (TriLink, USA) (Hamburg, Germany), and TRAC and TRBC CRISPR/Cas9 (Genecopoiea, USA). DNA was delivered directly to the nucleus of the cells using a Nucleofector II device (amaxa) (Lonza, Belgium) with program T-20 or by using the following electroporation parameters with a neon-transfection system:

Pulse voltages (v)	Pulse width (ms)	Pulse number
2150	20	1

Following transfection, the cells were immediately transferred to 1 ml of pre-warmed, complete X-VIVO 10 or 15 media with IL-2 and cultured at 37^{o}C using 5% CO2. In the same experiments with Jurkat cells, cells were nucleofected directly without any stimulation by using the x-001 program with amaxa, while with neon transfection, the following electroporation parameters were used:

Pulse voltages (v)	Pulse width (ms)	Pulse number
1350	10	3

After nucleofection, the cells were placed in complete RPMI 1640. The cells were kept in culture for 24 and 48 hours prior to analysis for marker genes such as eGFP or red-Cherry and for seven days before evaluation of T cell receptor knock out (TCR KO) efficiency. In the case of the beads' stimulation, cells were rested overnight following semi-automated, magnetic bead removal using a Dynal ClinExVivo MPC (Invitrogen, UK) prior to analysis.

2.2.20 mRNA electroporation with BTX electroporation system:

For delivering mRNA into the cells, the BTX electroporation system was used. Two days after activating the cells or five days after stimulating and transducing, 10x10^{6} cells were aliquoted, centrifuged and resuspended in 400 µl electroporation buffer. Then, 20 µg TRAC TALENS (10µg/pair) or 500 ng TRAC guide RNA and Cas9 were added to the corner of the electroporation cuvettes. Cells were mixed with the RNA constructs in the cuvettes then moved quickly into the electroporation chamber that is connected to BTX machine. After electroporation, cells were transferred quickly into a pre-warmed, completely fresh X-VIVO 10 or 15 in T12.5 tissue culture flask. Two hours later, 100 U/ml of IL-2 was added. Cells were kept in this culture condition until moved to G-rex10 the next morning for expansion of the cells for scale up purposes.

2.2.21 Production of lentiviral vector:

Unless otherwise stated, all plasmids were supplied by Plasmid Factory, Germany

12-15 x 10^6 packaging cells; HEK 293T cells, were cultured into 175T tissue culture flasks in 25 ml of supplemented DMEM and incubated overnight at 37°C in 5% CO_2 atmosphere to reach ~80% confluency. For second generation lentivirus production, three different DNA constructs: 25 µg vector plasmid, 32.5 µg gag-pol packaging plasmid pCMV-dR8.74 (for integrating virus), or pCMV-dR8.74 D64V (for non-integrating virus) and VSV-G2 envelop plasmid were mixed together in 5 ml OptiMem and sterilized thorough a 0.22µm filter. For the production of third generation lentivirus particles, a 25 µg pRSV-Rev fourth plasmid was added and filtered in addition to the previous packaging plasmids. The DNA mixture was incubated with a mixture of 5 ml OptiMEM and 1 µl 10 mM polyethylenimine (PEI) for 20 min at room temperature. After washing the packaging cells with warm OptiMEM, 10 ml of DNA-PEI mixture were added to each T175 flask and the cells were incubated for 4 hours at 37°C using 5 % CO2. The medium was then replaced with 30 ml of supplemented DMEM. Following overnight incubation of the transfected cells, the culture medium was replaced with supplemented DMEM and kept for another period of overnight incubation at 37°C using 5 % CO2. To harvest the lentiviral vector, 48 and 72 hours after transfection the supernatant was collected and filtered through 0.45 µM filter (Merck Millipore,UK) and placed into an ultracentrifuge (Sorvall, Thermo Fisher Scientific, USA) for spinning at 23,000 RPM for 2 hrs. The final stage of the production involved re-suspension of the virus pellet in 100 µl OptiMEM, which was incubated on ice for 20 min and eventually stored as 20 µl aliquots at-80°C.

For scale-up lentivirus production, 20x10^6 HEK293ts cells were cultured in a 5-layer culture flask (Millicell HY Flasks, USA) for three days before transfection. 55 µg pMD.G envelop, 90 µg gag-pol packaging plasmid, 45 µg Rev and 180 µg vector plasmid were mixed together in 25 ml OptiMUM and filtered through 0.22 µm filter then incubated with 25 ml of OptiMUM containing 5 µl PEI at room temperature for 20 min. The 50 ml DNA-PEI mixture was added to the packaging cells. After 4 hours incubating cells with DNA-PEI mixture, the transfection reagent was exchanged with 150 mls completely warm DMEM. Unlike the small scale production, there was no medium change on the next day of transfection. For virus harvesting, the 48 and 72 hours after transfection virus was subjected to overnight spin at speed of 10.000 g. Finally, the virus pellet was re-suspended in 900 µl OptiMEM, which was incubated on ice for 30 min and eventually stored as 80 µl aliquots at -80°C.

2.2.22 Virus titration:

$1x10^5$ HEK293ts were plated in 24 wells plate the day before transduction, while for $5x10^4$ Jurkat or TCR –ve cells, the cells were plated on the same day for virus titration. To define the number of functional viral particles per ml, an aliquot of concentrated virus particles was used in a serial dilution process using amounts of virus particles ranging from 0,0032 µl to 10 µl in volume. Viral particles were serially diluted to add 10, 2, 0.4, 0.08, 0.016 and 0.0032 µl of the virus stock in 100 µl of OptiMEM per well. The cells were incubated with the dilutions prepared at 37°C/5% CO2 for 6 hours in 300 µl volume. Following 6 hours of incubating cells with the lentivirus, 700 µl supplemented DMEM was added to each well and the cells were kept in the incubator for 3 days. Seventy-two hours later, transduced cells were collected and stained with mAb against targeted antigens (Vβ 2.1 PE FC gamma chain of CAR-CD19 PE-Cy5 or CD34 QBen-10-APC) for FACS analysis by using an LSR II cytometer (Becton Dickinson). Analyses of the results were carried out using FlowJo software and to calculate the original, infectious virus units the following equation was applied:

Number of infectious units per ml = ((Percentage of infected cells/100) x number of cells) X 1000))/amount of virus

2.2.23 INF-gamma capture assay:

In a 96-well plate, 1.5 x 10^6 cells of WT1-TCR transduced CB-T cells (n=3) were seeded overnight at 37°C using 5% CO2. The next morning, effector CB-T cells were incubated with 1.5 x 10^6 T2 cells at a ratio of 1:1. These cells were loaded for 3–6 hours with 100 µM of WT1 peptide, and the remaining $1.5x10^6$ T2 cells were loaded with ADV, a non-specific peptide. Cells were then harvested, washed and stained with INF-γ catch reagent (Miltenyi Biotech, Germany) and incubated on ice for 5 minutes. 10 ml of pre-warmed RPMI 1640 supplemented with 1% AB serum was added to the cells and incubated at 37°C on a MACS (Miltenyi Biotech, Germany) rotor for 45 min. The cells were washed and stained with anti-INF-γ phycoerythrin (PE) (Miltenyi Biotech, Germany) and anti-CD8 fluorescein isothiocyanate (FITC) (BD Biosciences, UK) for 10 minutes on ice. Flow cytometric analysis was performed using the Becton Dickinson LSR II cytometer and FlowJo software.

2.2.24 ^{51}Chromium release assay:

$1.6x10^6$ T2 cells-target cells- were peptide-loaded with 100µM of either relevant peptide, WT126p, or an irrelevant ADV peptide and incubated at 37°C using 5% CO_2 for 1 hour. While for SupT1-CD19 positive and Sup-T1 negative target cells were counted and $1.6x10^6$

aliquots were prepared. During the incubation period, $6x10^5$ cells/ ml of effector T cells (WT1-TCR- or CD19-CAR transduced CB-T cells), were placed in 100 µl of culture medium RPMI 1640 in a 96-well, U bottom plate at different ratios, ranging from 20:1 to 1.25:1. Target cells were labelled with 100µCi of ^{51}Chromium (PerkinElmer Las, UK) and incubated for one hour at 37^{o}C. Labelled target cells were then washed twice with PBS (Invitrogen, UK) and diluted to reach $5x10^4$ cells/ml. 100 µl of diluted target cells were applied to all the wells of the previously seeded 96-well plate and spun to facilitate cell-to-cell contact. Six wells were plated with triton; toxic reagent, to measure the spontaneous ^{51}Cr release. After 4 hours of incubating labelled target and effector cells at 37^{o}C and using 5% CO_2, 50 µl supernatant were added to 150µl scintillation fluid in 96-well isoplates and incubated at room temperature overnight. The next morning ^{51}Cr release effect was measured by beta-counter.

The specific killing activity of the cells is calculated as follows:

Experimental ^{51}Chromium release – spontaneous ^{51}Chromium release

% specific killing=Maximum ^{51}Chromium release – spontaneous ^{51}Chromium release

2.2.25 Flow cytometry:

Fluor-chrome, labelled-monoclonal antibodies were used for staining the cells with indicated specificity and for controlling the appropriate isotypes. Samples were acquired through using a FACS BD LSRII cytometer (BD Biosciences) and using FACSDiva software. All data were analysed using FlowJo software version 7.6.5 or 10.

Briefly, cells were pelleted by centrifugation then stained with the appropriate monoclonal antibody diluted in PBS and incubated for 20 min at 4^{o}C in the dark. Stained cells were washed with PBS before resuspending for FACS analysis.

Viable cells were gated on forward/side scatter; therefore, measuring cell death was based on the shift on the forward and side scatter axis as cells became smaller and more complex.

2.2.26 Transduction of lentiviral T-cell receptor or chimeric antigen receptor constructs into cord blood T-cells and peripheral blood:

In small scale experiments, T-cells derived from cord blood unit and PBMCS were obtained by centrifugation through ficoll and plated $1x10^6$ cells per well in 24 well plate on Day 1, then activated with different activation methods (explained in detail in chapter 4). Forty-eight hours post-activation, donor-derived cord blood T-cells or PBMCs were exposed to a single round of lentiviral vectors transduction carrying WT1-TCR or CD19-CAR genes, with multiplicity of infection (MOI) 5 or 10. Three days later, transduced T-cells were stained with mAbs conjugated to PE targeting the Vβ2.1 region and mCβ-APC to monitor WT1-TCR

expression. For evaluating CD19CAR transduction efficiency, different mAbs were used: mAB conjugated to a Cy-5-targeted spacer portion (Fc gamma) of CD19-CAR; mAB conjugated to APC targeting CD34 as RQR8 (short suicide gene) was linked to CD19CAR; and/or indirect staining of Fab fragment of the CAR using streptavidin-PE as the secondary antibody. The expression of WT1-TCR or CD19-CAR transgenes was analysed using flow cytometry on an LSR II cytometer, and FACS data were analysed using FlowJo or FlowJo software. Throughout the culture period, half of the culture medium was exchanged with fresh complete x-vivo 10 or 15 medium, and 100 U/ml IL-2 or other cytokines cocktail depending on the way of activation. Cells were then exposed to different functional assays.

The scalability of CB-T engineering experiment was performed either within a closed bag system or in 10m G-rex as part of feasibility studies for 'off-the-shelf therapies' for the treatment of haematological malignancies. Forty-eight hours after activation, 50 or $200x10^6$ activated-CB-T cells were transduced with SIN LNT-SFFV-WT1TCR with MOI 4 in a closed bag system or with Lnt-EFLα-RQR8-2A-CD19CAR with MOI of 5. Three days following transduction, the cells were stained for transduction efficiency and moved into the G-rex10 system in order to expand them. Flow cytometric analysis using a LSR II cytometer (BD Bioscience) was conducted and FACS data were analysed using FlowJo software. Transduced cells were then sorted and frozen down for functionality and cytoxcity assays.

2.2.27 TALENs production:

Engineering of TALENs-recognising sequences from the constant region of TCR (TRAC, TRBC):

A pair of TALENs targeting the constant region of alpha chain of TCR was provided by Cellictes, a French company. Another pair of TALENs targeting the constant region of both α and β chains of TCR has designed and constructed in-house with the help of information contained at www.TALengineering.org (Figure11). The sequence within exon 1 on chromosome 14 was chosen to be the target place for TALENs targeting TRAC, while a conserved sequence within TRBC1 and TRBC2 genes on exon 1 at chromosome 7 was used as a platform to design TALENs targeting TRBC (Figure 12).

Figure 11: Process of designing TALENs pairs targeting sites within genomic loci of TCR-alpha (left) and TCR-beta constant region (right).

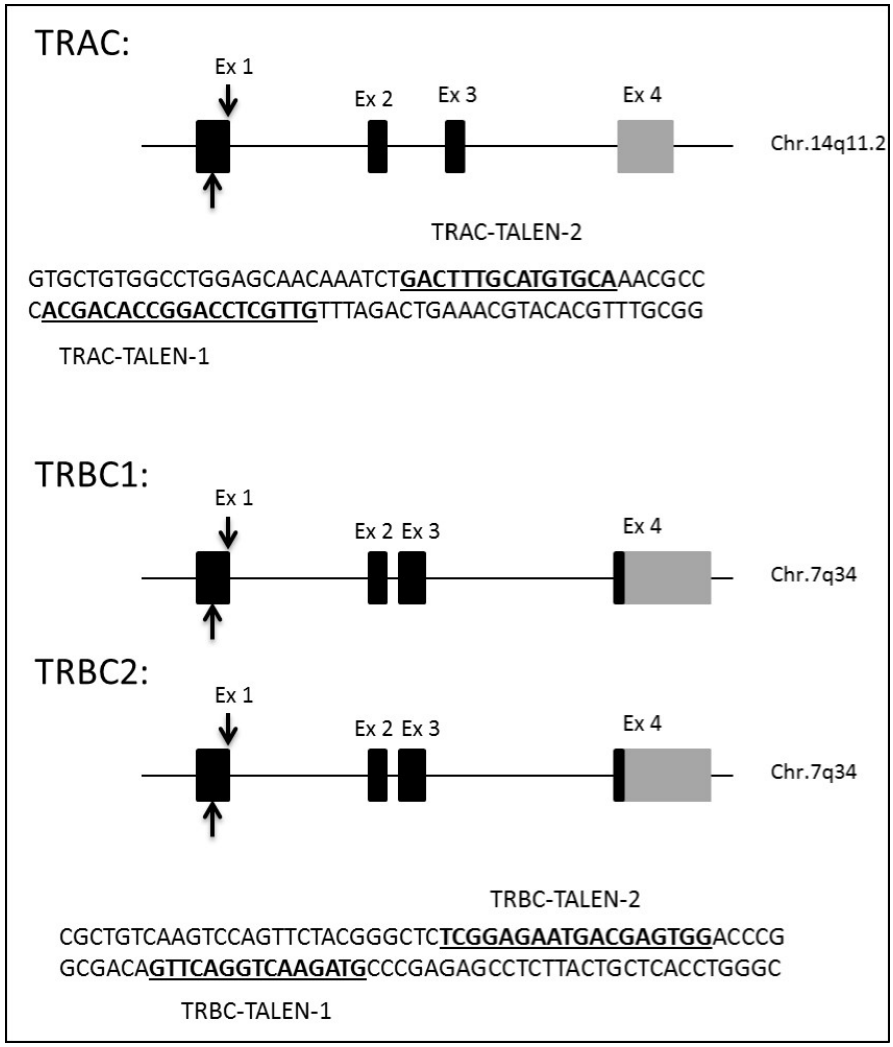


Figure 12: TALENs pairs targeting sites within genomic loci of the TCR-alpha and TCR beta constant region. Each exon is represented by a block. Black boxes represent coding regions, whereas grey blocks show non-coding regions. One TALENs pair was designed to bind to exon 1 of the TCR a constant region (TRAC) and another TALENs pair binds a conserved sequence on exon 1 of the TCR β constant regions 1 (TRBC1) and 2 (TRBC2). Underlined nucleotide sequences represent the intended binding sequence of each TALEN.

One of the suggested, customised graphical plans (Figure 13) has been used as a platform to assemble these particular nucleases.

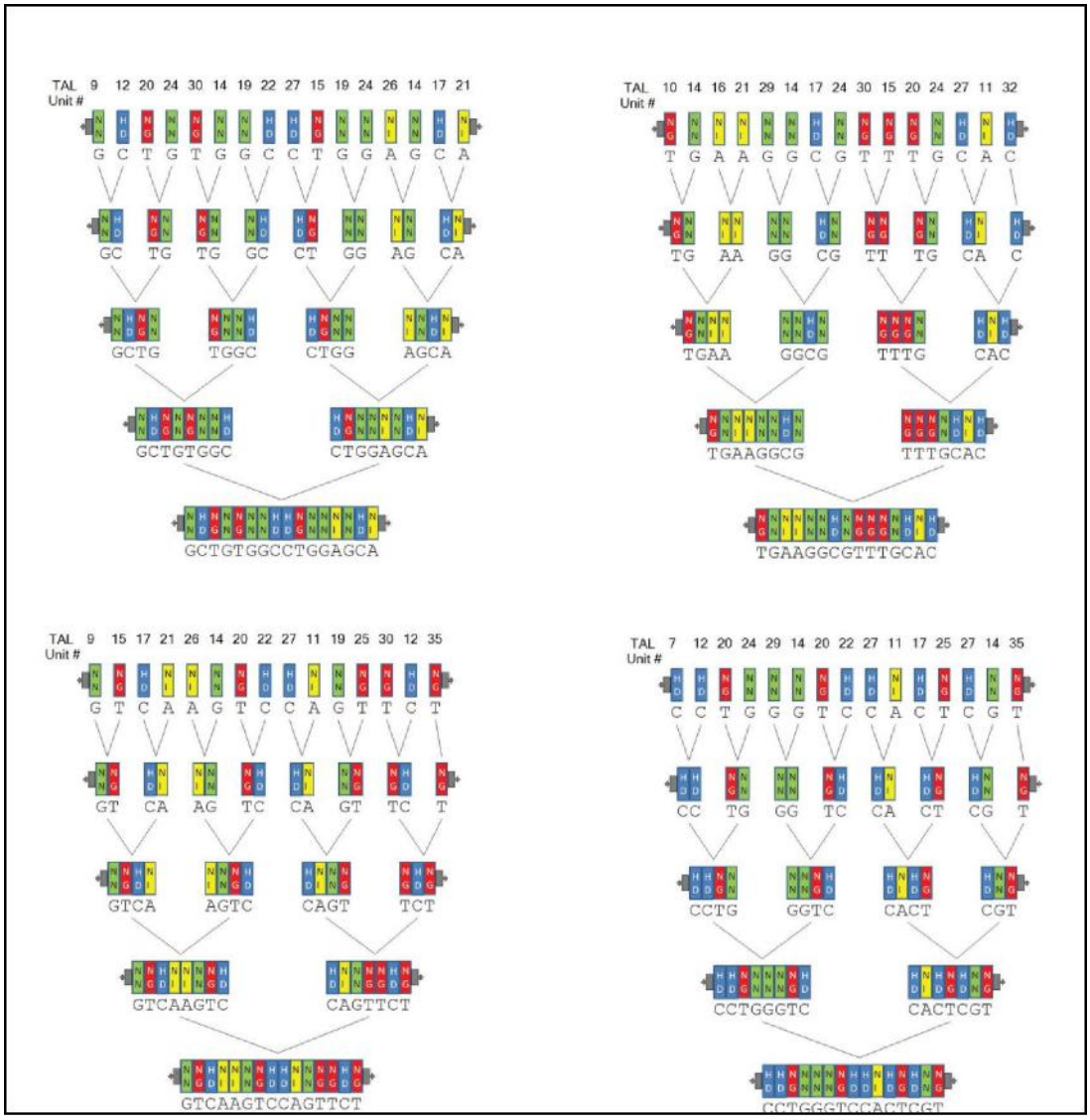


Figure 13: The customised graphical output provided by TALengineering resource.TAL effector repeats are shown as coloured rectangles. Diagram A represents the arrangement of TAL effector repeats to assemble pair of TALENs targeting sites within genomic loci of TCR-a while diagram B represents the arrangement of TAL effector repeats to assemble pairs of TALENs targeting conserved sequences within the genomic loci of TCR-β.

Using the graphical outputs provided by www.talengineering.org as a guide (Figure 13), TALENs were assembled following the next steps:

To prepare DNA fragment encoding the first TAL effector repeat, 20 rounds of PCR using the following primers: oJS2521 (5'-biotin-TCTAGAGAAGACAAGAACCTGACC-3') and oJS2582 (5'-GGATCCGGTCTCTTAAGGCCGTGG-3') were conducted. The resulting PCR products, which are biotinylated on the 5' end, are then exposed to 40 units of the restriction enzyme BsaI-HF (New England Biolab, USA) for digesting and producing 4-base pair

overhangs. Afterwards, the digested DNA fragment was purified using a QIAquick PCR purification kit (Qiagen, UK) according to the manufacturer's instructions.

To prepare DNA fragments encoding the rest of TAL effectors repeats (2nd, 3rd, 4th,... etc), 10 µg of each plasmid was digested with 50 units of BbsI restriction enzyme (New England Biolab, USA) for 2 hours at 37°C, followed by serial restriction digests performed in NEBuffer 4 (New England Biolab, USA) at 37°C using 100 units each of XbaI, BamHI-HF and SAlI-HF enzymes (New England Biolab, USA), added at 5-minute intervals. These restriction digest reactions were then purified using a QIAquick PCR purification kit (Qiagen, UK) according to the manufacturer's instructions. At this stage, the DNA fragments encoding DNA TAL effector repeats were ready to be assembled in an iterative fashion on solid-phase magneticbeads; the strategy requires restriction digestion, purification and ligation to take place, in that order (Figure 14).

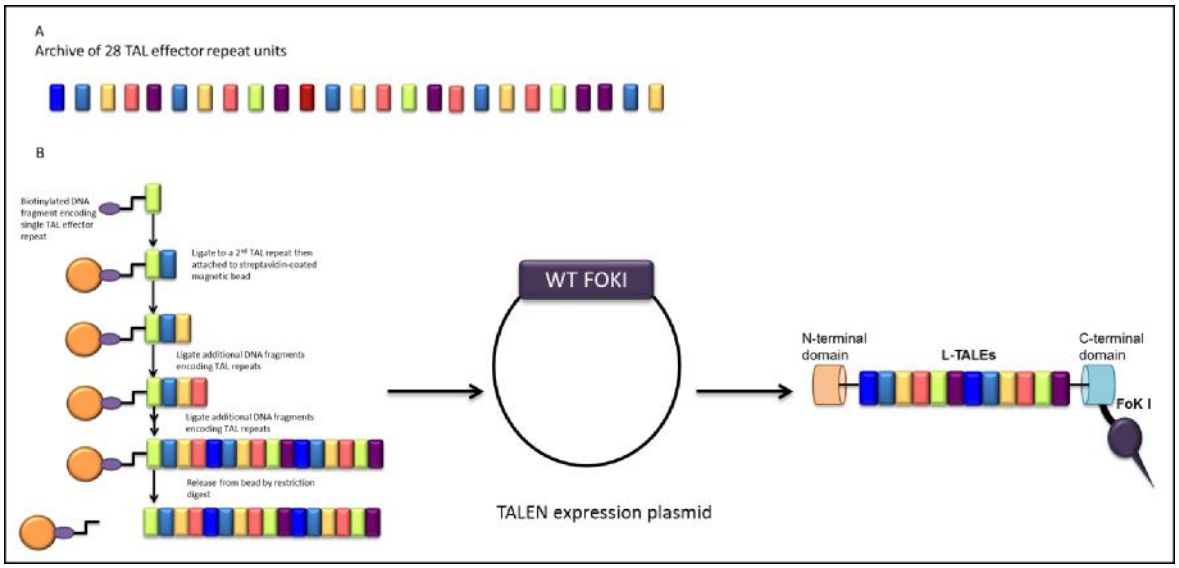


Figure 14: Graphical overview of the TALENs assembly method. A) Archive of 28 TAL effector repeat-encoding plasmids required to assemble TALE**.** (B) Schematic overview of the TALE assembly process. A DNA fragment encoding a single TAL effector repeat and labelled on its 5′ end with biotin (purple oval) is initially ligated to a second DNA fragment encoding one specific TAL effector repeat and then attached to a streptavidin-coated magnetic bead (orange sphere). Additional DNA fragments encoding the rest of TAL effector repeats are ligated until an array of the desired length is assembled. The DNA fragment encoding the full-length TAL effector repeat array is then cleaved from the bead by restriction digestion and ligated to TALEN expression plasmid, which encodes wild-type nuclease FOKI, to produce fully functional TAL effector nuclease.

In a PCR tube set-up, the initial ligation was done by mixing the first biotinylated DNA fragment, which encodes the first TAL effector repeat with T4 DNA ligase (Invitrogen, UK), QLB (quick ligase buffer) (Invitrogen, UK), and the 2nd TAL effector repeat. The mixture was incubated for 15 minutes at room temperature. During the incubation period, anti-

CD3/nti-CD28 beads (Life Technologies, UK) were washed and placed on a SPRI plate®, a 96-ring magnet plate (Beckman Coulter, UK) for 3 minutes, then re-suspended in buffer. After 15 minutes of incubation, the initial ligation reaction was added to the washed-beads at a ratio of 1:1 and incubated for 15 minutes at room temperature. Afterwards, the beads were washed again using buffer. For the next ligation, the digestion of bead-bound DNA fragment using BsaI-HF (New England Biolab, USA) was performed, followed by a beads-washing step, then the ligation of the extension DNA fragment by T4 DNA ligase (Invitrogen, UK). The previous steps were repeated to complete the assembly of the entire TAL effector array. Finally, the magnetic beads were released from the DNA fragments by using BbsI (New England Biolab, USA). Fully assembled TAL effector repeats were then ligated to the TALEN expression plasmid vector using T4 DNA ligase (New England Biolabs, USA) to produce fully functional TALE effector nucleases.

2.2.28 Production of CAR CD19 cord blood T-cells with TCR negative phenotype (Cellectis protocol):

T-cells derived from cord blood were obtained through centrifugation using Ficoll as was described earlier. Due do the presence of a high percentage of nucleated red blood cells, CD3 staining was carried out to determine the exact number of lymphocytes. Fifty to 100 $x10^6$ cells were activated with anti CD3/CD28 Dynal beads at 1:1 ratio and cultured in 100 ml differentiate culture bag (Milteny biotec, UK) at a concentration of $1x10^6$ cells/ml in 100 ml completely fresh X-VIVO 15 and 100 U/ml IL-2. Two days after activation, the bags were coated with retronectin (Takara, Japan) and incubated for 2 hours at room temperature. Later the cells were subjected to Lnt-EFLα-RQR8-CD19CAR 3rd generation lentivirus with titer of $2x10^8$ IU/ml and added to the retronectin-coated bags. Three days after transduction, 500 µl of cells were analysed for transduction efficiency by staining the cells for CD19CAR expression and flow cytometry. On Day 4 and 24 hours prior to exposing cells to TALENs, the cells were counted and re-cultured at a density of $1.7x10^6$/ml to be prepared for electroporation. On electroporation day, $10x10^6$ cells were aliquoted in a 15 mls falcon tube, centrifuged and resuspended in a 400 µl electroporation buffer. 10 µg TRAC TALENS per pair were added to the corner of the electroporation cuvette, then the cells were added and mixed by pipetting. Afterwards, cuvettes were transferred to cuvette chamber of BTX machine and electroporated with the following program:

	Group 1	Group 2	Group 3	
Amplitude	1200	1200	130	V
Duration	0,1	0,1	0,2	ms
Interval	0,2	100	2	ms
Number	1	1	4	

300 µl of pre-warmed X-VIVO 15 was added to the electroporated cells and they were immediately transferred to pre-warmed x-vivo 15 plus 5% human serum medium in T12.5 flasks and incubated for 2 hours at 37°C before adding 100 U/ml of IL-2. The next day, cells were moved to G-rex10 (WilsonWolf, USA); an expansion system that provides cells with unlimited neutrinos and unlimited O2, compared to the T-flasks. The cells were allowed to expand until Day 17 from activation. During this period and exactly at Day 12, the cells were stained with mAB targeting TCR α/β-FITC and CD3-APC to monitor the reduction in TCR expression. In the final step, TCR positive (+ve) cells were separated from TCR negative (–ve) population by running the cells through an LD column and using a TCR depletion kit.

2.2.29 TCR depletion:

In the final step of CD19CAR+ve TCR-ve cell production, cells were collected from G-rex10 and pelleted by centrifugation at 300xg for 10 minutes. Cell pellet was then resuspended in 100 µl MACS buffer. The cell suspension was then incubated with 10 µl Anti-TCRα/β biotin for 10 minutes in the dark in the refrigerator. The cells were washed in 1-2 mls of MACS buffer, and centrifuged at 300xg for 10 minutes, then resuspended in 80 µl buffer. 20 µl anti-biotin microbeads were added and incubated with biotin labelled cells for 15 minutes at 4°C. The cells were washed as previously explained and resuspended in 500 µl MACS buffer and proceeded to depletion with LD column. LD columns were inserted into a midi MACS separator, then rinsed with 2 mls of MACS buffer and let it run through. After applying the cells onto the prepared LD column, the column was washed twice with 1 mls of MACS buffer. Afterwards, unlabeled cells which passed through the column were collected and labelled as depleted TCR negative fraction. In order to collect TCR positive population that trapped in the column, cells were forced out by applying a plunger to the column after

removing it from the MACS separator. Finally, the cells were cultured in complete x-vivo15 in 24 well plate for overnight. The cells then were frozen down and stored in liquid nitrogen.

2.2.30 CD34 selection with MS column:

After transducing T cells - either derived from CB or PBMCs- with CD19CAR lentivirus construct that has CD34 RQR8 as a selectable marker - cells were exposed to positive selection by using a CD34 microbead kit and MD column. After adding 100 µL of FcR blocking reagent to the 300 µl cell suspension, CD34 positive cells were labelled with 100 µl CD34 microbeads and incubated in the dark at 4^{o}C for 30 minutes. Cells were washed with MACS buffer then resuspended in 500 µl MACS buffer before proceeding to magnetic separation. MS columns were first rinsed with 500 µl buffer then labelled cells were applied onto the column and the through flow was collected to use it as a control of CD34 negative population. The column was washed three times with 500 µl buffer. And later separated from the separator and placed into collection tube. 1 ml of buffer was added to the column and positive fraction cells were immediately flushed out from column by pushing the plunger into the column. Both positively and negatively labelled fraction cells were cultured in X-VIVO 10 before they were analysed.

2.2.31 Engineering Cord Blood T cells with CD19CAR:

T cells derived from different cord blood donors were engineered to express CD19CAR construct. The T cells were separated from whole blood through ficolling, as previously described. Two days after T cell activation with 1:1 ratio anti-CD3/anti-CD28 beads, they were transduced with third generation Lnt-EFLα-RQR8-CD19CAR. Cells were moved to expand into G-rex10 and throughout the 12 days of culture, the cells were fed with IL-2 every 48 hours and phenotyped at Day 5 and Day 12. On Day 12, the cells were frozen in freezing medium and stored at -80^{o}C then moved to liquid nitrogen.

2.2.32 High throughput next generation sequencing (NGS):

Cord blood T cells were activated with CD3/CD28 beads or the cytokines cocktail (IL-7, IL-15 and IL-2), transduced with CD19CAR lentivirus, electroporated with TRAC TALENs as mRNA, and ultimately a TCR negative population was selected. DNA genomic material was extracted by using a DNeasy blood and tissue kit as previously stated from cell pellet of genetically altered cells by TALENs. A high generation sequencing process was carried out using MiSeq illumina (USA) and DNA samples were prepared with the help of Dr. Claire Deakins and Dr. Anastasia Petrova. An Illumina Miseq Machine was used to perform the

NGS procedure. Raw sequencing data was analysed using software named CRISPR-GA; where the raw sequencing reads were uploaded and then underwent quality control by trimming to discard the results that contained any sequencing errors. The software then mapped the reads to the reference sequence (TRAC). The mapping was done by a specialised BLAT platform, which is suitable to support indels (deletions and insertions). The software processes the mapped BLAT results to detect the insertions and deletions. It then uses pattern matching to compute the number of reads matching the expected sequence or generating variants. This information is then integrated to create plots and reports. Pattern matching is used to calculate the number of reads matching the expected sequence and other variants generated. NHEJ and HR are estimated by computing specific equations. The final result consists of three parts. First, the results report the analysis of indel sizes. Second, they illustrate the analysis of indel locations within the edited genomic locus. Lastly, they show an integrated plot representing indels, NHEJ and HR. Furthermore, a FASTA file is created with all reads containing indels, as well as an XML text output with HR, NHEJ values and the indel relative location in genomic locus.

2.2.33 Phenotyping Assay to determine T cell subset:

As the project proceeded, new phenotyping panels were improved and used:

After activating and transducing T cells either derived from cord blood or adult peripheral blood with different vectors, the cells were subjected to phenotyping staining to determine their characteristics. In the first panel, cells were stained with the following markers: CD4, CD8, CD27, CD25 and CD45RA. Later on, another panel was used to characterise T-cell subsets: CD4, CD8, CD45RA, CD62L, CD95 and CCR7. In addition, Fab fragment of CD19CAR stain was also added to characterise cells in a more specific manner.

Chapter 3: Engineering Cord Blood T cells for Tumor Immunotherapy

3.1) Introduction:

Although adoptive-T cell immunotherapy has led to remarkably successful outcomes in treating cancer and viral infections, the inability to isolate and expand a sufficient number of high-avidity, specific T lymphocytes for therapeutic purposes poses a fundamental limitation for this therapy (Rooney and Leen, 2012). An alternative approach is to generate genetically modified T lymphocytes expressing an exogenous specific TCRα and β, or an artificial protein-fusion derived chimeric receptor known as CARs, aimed at targeting specific tumor associated antigens (Morris et al., 2003, Ghorashian et al., 2015). The limitations associated with this fast-growing field are mainly time and the high expense of manufacturing the patient-specific therapeutic product. In addition, endogenous αβTCR chains in the allogeneic infused T-cells might lead to GVHD (Torikai et al., 2012). One of the possible solutions to overcome this hindrance is to use a third party donor that would be easily accessible and have suitable application for more than one recipient. We hypothesised that this could possibly be achieved by engineering unrelated cord blood T-cells towards specific antigens (Serrano et al., 2006).

Unrelated cord blood transplantation (CBT) has in recent years become an attractive therapy for hematological disorders. This is due to the fact that CB-T cell permits HLA disparity between the recipient and the donor, and a noticeably lower risk of graft-versus-host disease (GVHD) for a given degree of mismatch, compared to hematopoietic cell transplantation (HCT) from adult donors (Rubinstein et al., 1998, Rocha et al., 2001, Hiwarkar et al., 2015). In fact, the absence of memory T cell fraction may be the reason for the low incidence and severity of GVHD that is noticed in cord blood transplantations (Kloosterboer et al., 2004). Additionally, the majority of cord blood T cells possess a naïve phenotype, which is characterized by expressing CD45RA, CCR7 and CD62L. Consequently, the rate of persistence and survival of the genetically engineered CB-T cells after engraftment might be higher than the engineered adult T cell (Eisenthal et al., 2003, Frumento et al., 2013). Also, CB-T cells preserve their long telomeres. Consequently, they have higher telomeres activity, which is a good indicator of higher proliferative activity (Weng, 2012). These factors, in addition to the ethnic diversity and relative ease of collection, have made CB-T cells amongst the best candidates for adoptive T cell therapy to generate an allogeneic universal therapeutic product (Shpall et al., 2011).

Here we hypothesised that CB-T cells possess attributes that are fundamental for the generation of a universally genetically modified therapeutic T cell product. We investigated the advantages of engendering genetically engineered CB-T cells over adult donor engineered T-cells. This chapter describes the outcomes of redirecting the specificity of cord blood T cells through expression of: 1) Recombinant TCRs using exogenous antigen specific T-cell receptors against Wilm's tumour antigen (Wilm's tumour antigen is the tumour associated antigen over-expressed by leukemic cells); and 2) Chimeric antigen receptor (CAR) targeting CD19 antigen molecule expressed by normal and neoplastic B cells. WT-1 TCR and second generation CD19CAR were introduced to the activated CB-T cells via lentiviral vector transduction ensuing second and third generations lentiviral vectors were produced. Interferon gamma and 51Chromium release assays were used as a means to evaluate cytotoxic activity and specific-target specificity of the genetically engineered cells in order to assess the functionality of the genetically engineered CB-T cells. Engineered CB-T cell properties were explored via investigating the expression profile of naïve chemokines by phenotyping assay.

3.2) Engineering CB-T cell with WT1-TCR:

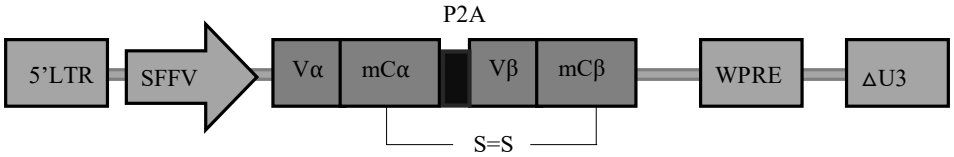
3.2.1) Vector Production and Characterisation:

Lentiviral vector stocks were produced by transient transfection of HEK293T cells with lentiviral vector plasmid, a second generation lentiviral packaging plasmid p8.74 and VSV-G envelope plasmid, pMDG. Lnt-PGK-eGFP construct was used to produce GFP virus for use as a control. Virus particles were harvested at forty-eight and seventy-two hours after transfection and concentrated by ultracentrifugation. The titre of each virus batch was quantified by transducing the appropriate type of cells with serial dilutions of virus stock and the expression of the desired antigen-receptor was analysed by flow cytometry seventy-two hours after transduction. After that, the number of infectious units per ml was calculated.

Plasmid encoding WT1-TCR alpha and beta genes used in this project were originally cloned from an allo-restricted, cytotoxic T lymphocyte line specific for the WT1 antigen-derived, HLA2-2 presented peptide pWT126. WT1-TCR is comprised of a Vα1.5/Vβ2.1 heterodimer (Xue et al., 2005). In addition to fusing murine constant-region genes (mCα and mCβ) to the cloned, variable-region genes of WT1-TCR, cysteine residues were introduced to the WT1-TCR construct. The WT1TCR construct was previously ligated to a lentiviral vector with an SFFV in an internal promoter (Figure 15.A). FACS analysis was performed on a TCR-

negative Jurkat cell line and was used for virus titration (Figure 15.B). A typical titre of 7.1 x 10^8 infectious units (IU) per ml was achieved.

A) Lnt-SFFV-WT1-TCR modified construct



B) Virus titration of Lnt-SFFV-WT1-TCR

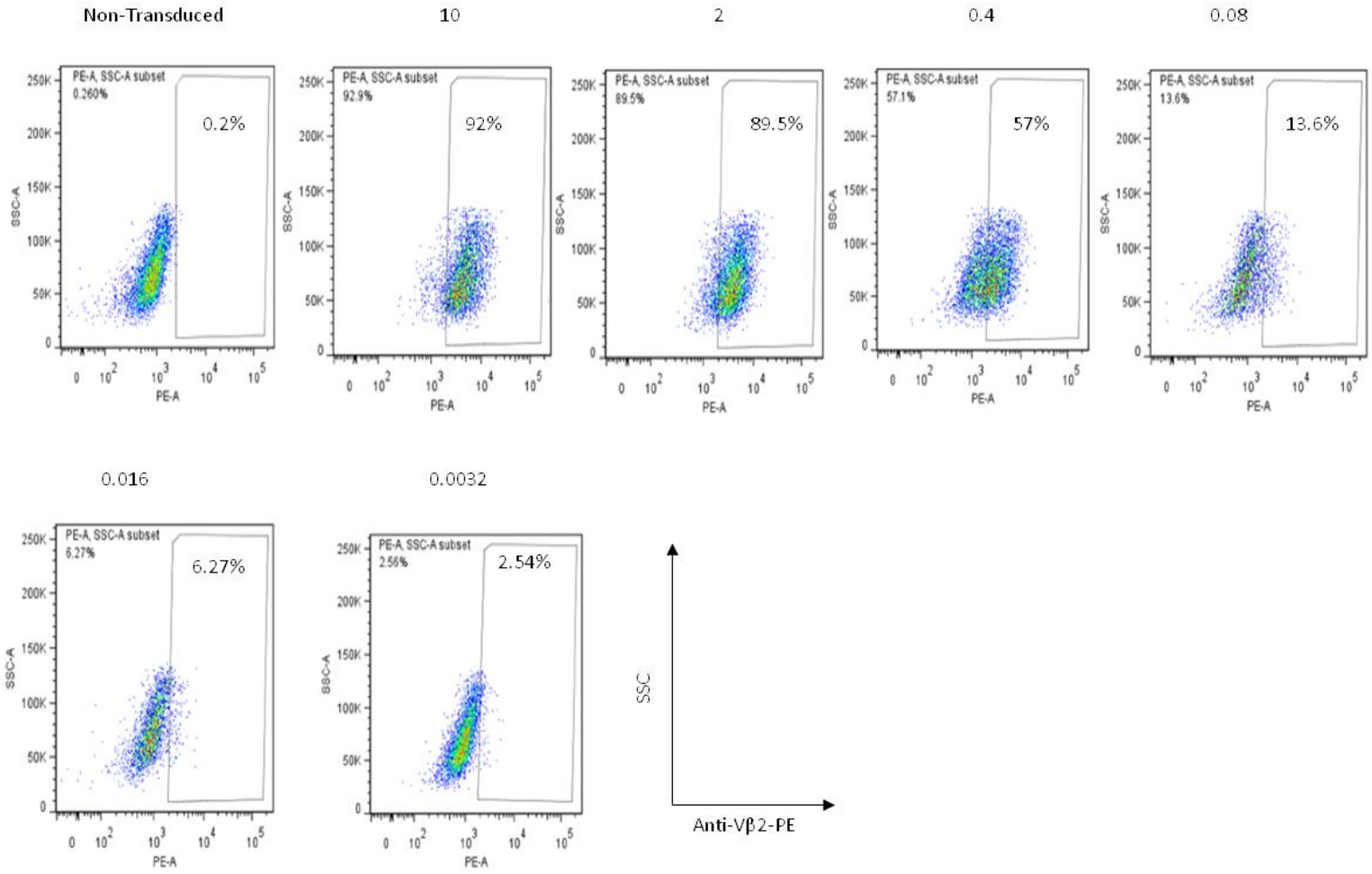


Figure 15: Lnt-SFFV-WT1-TCR virus titration. A) Schematic map of Lnt-WT1-TCR modified construct. B) FACS plot data demonstrated WT1-TCR transduction efficiency using serial dilutions of concentrated LNT-SFFV-WT1-TCR virus supernatant on the Jurkat negative cell line.

Forty-eight hours after activating CB-T cells with anti CD3/CD28 Dynal beads at a 1:1 ratio, the activated cells were transduced with lentiviral vectors containing human, Vα1.5/Vβ2 TCR genes and murine, constant α/β genes at MOI of 10. Three days post transduction; the transduced cells were harvested for further analysis including transduction efficiency, evaluation and some functional assessment. This experiment was conducted on five cord blood units and the following section will discuss the outcome of editing the specificity of these cells toward WT1 antigen.

3.2.2) WT1-TCR Transduction Efficiency of all Five Cord Blood Donors after Activation of CD3/CD28 Beads and Transduction with LNT-WT1-TCR (MOI 10):

Three days after transduction, cells were stained with anti-Vβ2.1 and anti-mCβ (murine constantβ) antibodies to determine the transduction efficiency. Gating on the viable cells on the LSR II cytometer, a significant increase in the percentage of the T cells-expressed Vβ2.1/mCβ led to between 49–95% gene transfer (mean=60%, n=5) compared to the non-transduced T cell population in the CD3/C28 beads stimulation condition was detected (Figure 16). This result indicated that WT1-TCR was successfully transduced into CB-T cells.

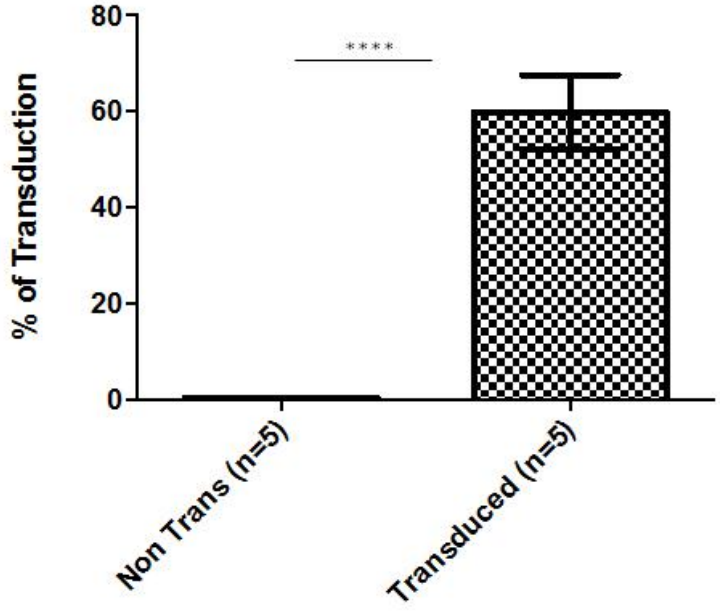


Figure 16: Expression level of WT1-TCR after transducing activated cord blood T cells (n=5) with Lnt-SFFV-WT1TCR. Ggrouped graph show a summary of the expression levels of WT1-TCR on the surface of activated cord blood T cells (n=5) post transduction with MOI 10 of WT1-TCR lentiviral vector. A considerable proportion (49-95%) of WT1-TCR cells in WT1-TCR transduced T-cells were positive for staining with mAb to Vβ1 and mCβ in comparison to non-transduced CB-T cells. Student T test was calculated to be $p<0.0001$

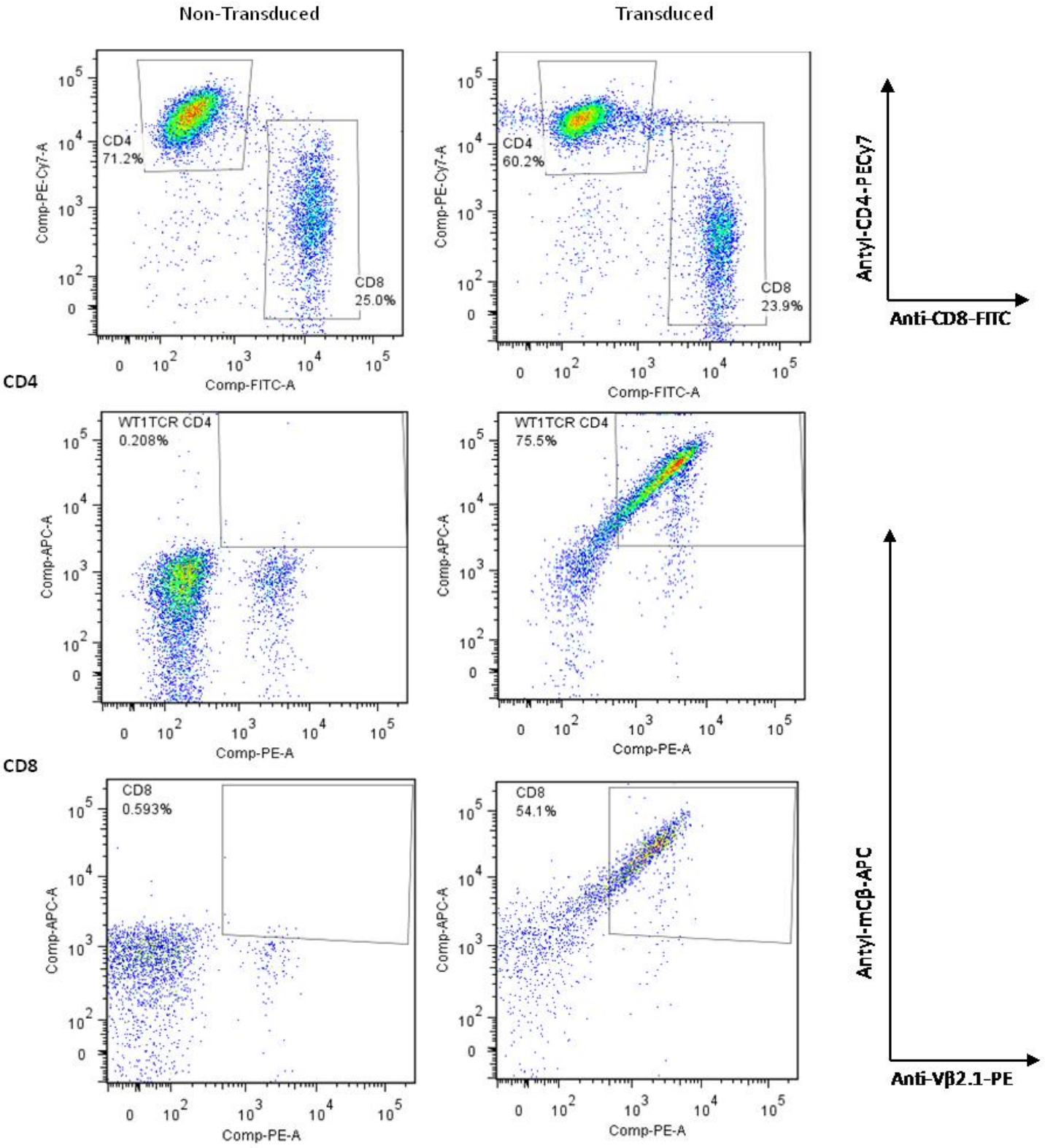


Figure 17: Expression level of WT1-TCR on CD3/CD28 beads + IL-2 activated CB-T cells. Representative FACS blot of one CB donor displaying the percentage of activated T-cells expressing both Vβ2 and mCβ and the level of antigen expression is indicated by mean fluorescent intensity (CD3/CD28 beads +IL2 stimulation condition). Plots in the first row exhibit the percentage of viable CD4+ and CD8+ cells. The percentage of CD4+WT1-TCR+ T cells are shown in the FACS plots in the second row, while the last row illustrates the FACS plots of CD8+WT1TCR+ T-cells.

To assess the function of WT1-TCR CB-T cells, antigen-specific cytokine production from the WT1-TCR transduced T-cells and the antigen- specific cytotoxicity were examined.

INF-γ capture assay:

WT1-TCR transduced CB-T cells of three donors were stimulated by T2 cells loaded with the modified WT126 peptide or irrelevant ADV peptide for six hours and examined for production of IFN-γ. The cells were then harvested and stained with INF-γ catch reagent then with anti-INF-γ phycoerythrin (PE) and anti-CD8 fluorescein isothiocyanate (FITC) before flow cytomeric analysis of INF-γ release. The FACS plots suggested that WT1-TCR transduced cells stimulated with the WT126 peptide produced IFNγ, whereas cells stimulated with the irrelevant peptide (ADV) did not do as well as non-transduced cells. INF-γ release percentages from WT1-TCR transduced cells of 6.3%, 12.5% and 2.3% for donors 1, 2 and 3 respectively, were detected by the LSRII flow cytometric machine. Conversely, a percentage of less than 1% of INF-γ was produced by the non-specific peptide (ADV). Both negative and positive controls were used in parallel to the genetically engineered cells and their INF-γrelease results were reasonable and supportive of the assay (Figure 19). Un-transduced cells were mixed with T2 cells to act as the negative control group. To simulate a positive control, one drop of superantigen named staphelylococcal enterotoxin B (SEB) was mixed with the un-transduced cells and T2 cells.

A representative FACS plot of CB-T cells obtained from the 3rd donor showed that the functional cytokines secretion of the WT1- TCR transduced cells activated by CD3/CD28 beads was 2.30 % of the Vβ2.1 positive cell population (Figure 18).

A summary of the functional INF-γ secretion of the WT1-TCR-transduced cells activated by CD3/CD28 beads is explained in figure 19. The bar chart shows that WT1-TCR-transduced T cells were able to recognise and kill peptide-loaded T2 cells in an antigen-specific manner without affecting or killing the non-specific peptide-loaded cells.

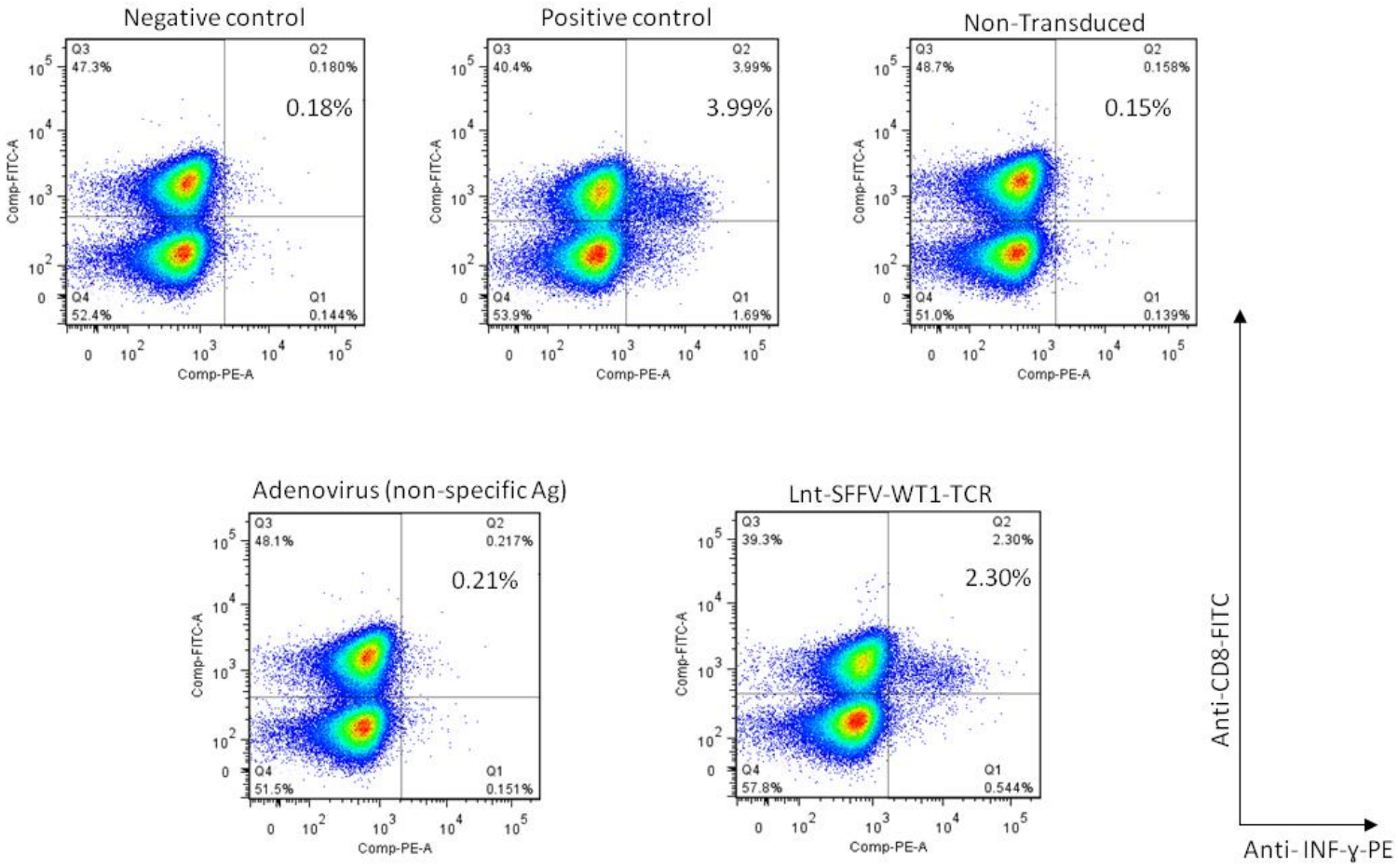


Figure 18: antigen-specific responses of WT1-TCR transduced CB-T cells. The WT1-TCR construct (indicated at the top) was transduced into CB-T cells and its functional activity was determined. An antigen-specific interferon γ (INF-γ) production determined after 6 hrs of stimulation with specific PWT126 peptide or non-specific ADV peptide. Representative FACS plot data of intracellular INFγ staining are shown.

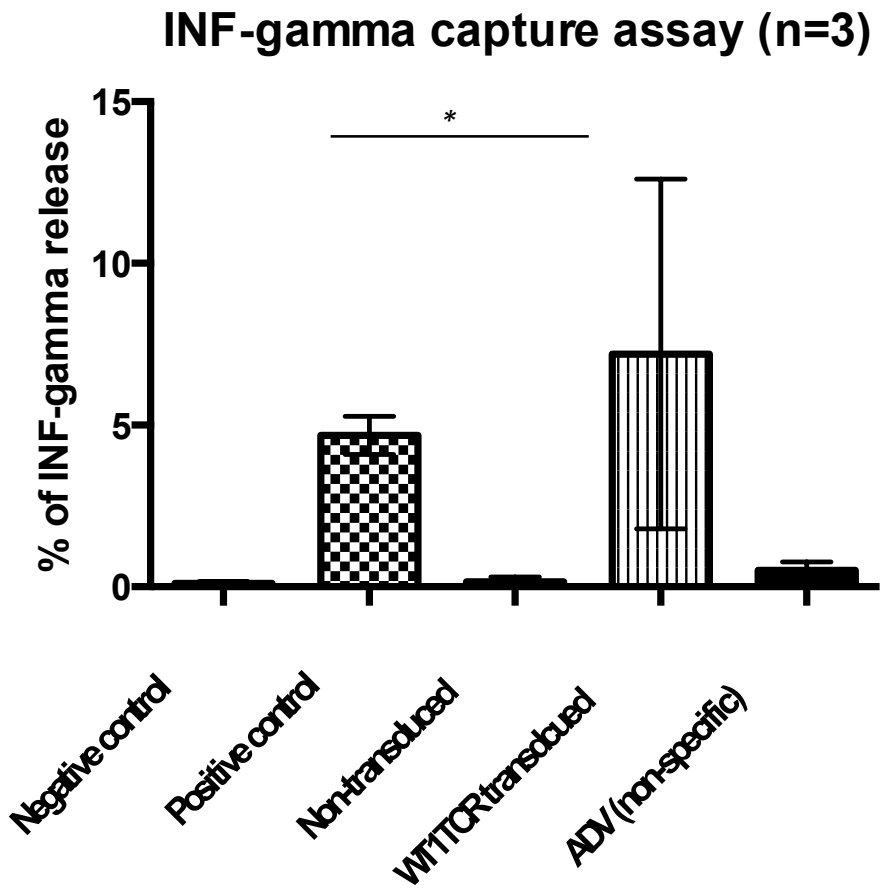


Figure 19: Summary of INF-gamma secretion produced by three CB-T cells transduced with Lnt-SFFV-WT1-TCR after stimulation with specific peptide (pWT126) or irrelevant peptide (ADV). Bar graph demonstrates the level of INF- γ secretion of WT1-TCR transduced CB-T cells (n=3) in response to specific and non-specific antigen. Student t test was calculated to be equal to 0.0147 ($p<0.05$).

The previous data might suggest that WT1TCR-transduced CB-T cells are highly capable of recognising their target antigens and discriminating them from non-target cells. To further confirm this observation, an *in vivo* trial is required.

^{51}Chromium Release Assay:

The *in vitro*, antigen-specific functionality of WT1-TCR-transduced CB-T cells was also demonstrated in antigen-specific killing activity in a ^{51}chromium release cytotoxicity assay using a relevant and non-relevant peptide loaded onto target cells (T2 cells). As demonstrated in figure 20, the human, TAP-deficient T2 cells were killed after being coated with pWT126 (specific peptide), but not after being coated with ADV peptide (control, non-specific peptide). The increase in the percentage of the killing activity; or of ^{51}Cr release, of CB-T cells that expressed WT1-TCR was found to be 85% compared with the killing activity of non-transduced T cells, and the killing behaviour of WT1-TCR CB-T cells against target cells loaded with non-specific peptides (Figure 20).

The previous data might suggest that WT1- TCR transduced CB-T cells are highly capable of recognising their target antigens and discriminating them from non-target cells. To further

confirm this observation, an *in vivo* trial is required. Moreover, it has been noticed that levels of killing reflect the levels of gene transfer.

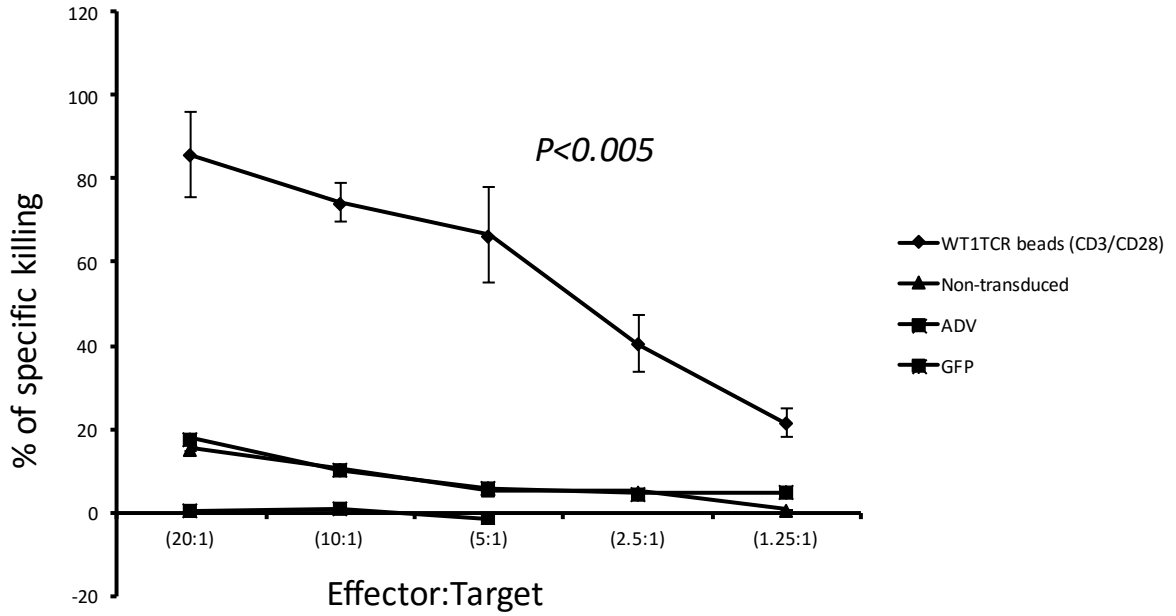


Figure 20: Percentage of specific killing of Lnt-SFFV-WT1TCR CB-T cells. WT1-TCR engineered CB-T cells (n=5) recognised their specific tumour antigen and selectively kill their target, cancerous cells but not non-specific control targets in vitro.

3.2.3) WT1 TCR transduced CB-T cells show naïve phenotype post activation and of lentiviral transduction:

Phenotype classification of transduced CB-T cells, such as naïve, effector and memory T cells is useful in determining the fate and the exhaustion status of the cells prior to transferring them into a patient's body. The first panel of phenotyping used in this project was staining the cells to determine the expression of CD4, CD8, CD27 and CD45RA. The cells were categorised as "naïve" if they possessed a CD4+CD8+CD45RA+CD27+ phenotype; as a "memory" if they lost the expression of the CD45RA marker; or as an "effector" if they were negative phenotypic for both CD45RA and CD27. The expression of these surface markers was detected by flow cytometry after staining the cells with mAbs and targeting them. The following table demonstrates the percentages of each T cell subsets in detail for the three donors:

	Naive			Effector			Memory		
	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3
Un-activated	70%	77.2%	61.8%	12.6%	1.3%	5.33%	1.18%	10.44%	1.69%
Un-Td	68%	13.7%	69.7%	23.6%	5.7%	6.13%	1.56%	62.2%	0.73%
WT1TCR-Td	71%	0.16%	47%	0.7%	0.23%	19.4%	18.1%	79.3%	3.6%

Table 5: The percentages of CB-T cells different subsets prior and post activation and transduction.

As Table 5 shows, CB-T cells from donor 1 and donor 3 behaved in almost the same manner and exhibited similar percentages of CD27 and CD45RA expression. CB-T cells in any of the three conditions (un-activated, un-transduced or WT1TCR transduced) from both donors appear to express a higher percentage within the naïve category compared to the memory and the effector subsets. By contrast, CB-T cells from the 2nd donor behaved in a different manner. The activated cells either transduced or un-transduced, expressed CD27 in higher percentages (62.2%-79.3%) than CD45RA.Thus, they were classified as memory T cells, but the un-activated T-cells were categorised as naïve T cells based on their expression of the surface markers, CD27 and CD45RA (Figure 21).

The results from this assay might suggest that CB-T cells could conserve their naïve phenotype even after activation and lentiviral transduction. The naïve phenotype is a crucial requirement for T cell immunotherapy in which infused engineered T cells live longer within the patient's body, and it might also show better results in the homing process.

As it appears from the previous results, cells from different donors behave in various manners and produce different results. For this reason, and because the phenotyping panel was so basic, results from this assay might not be precise. A more advanced phenotyping panel was used for the future experiments and it will be discussed in the next chapters.

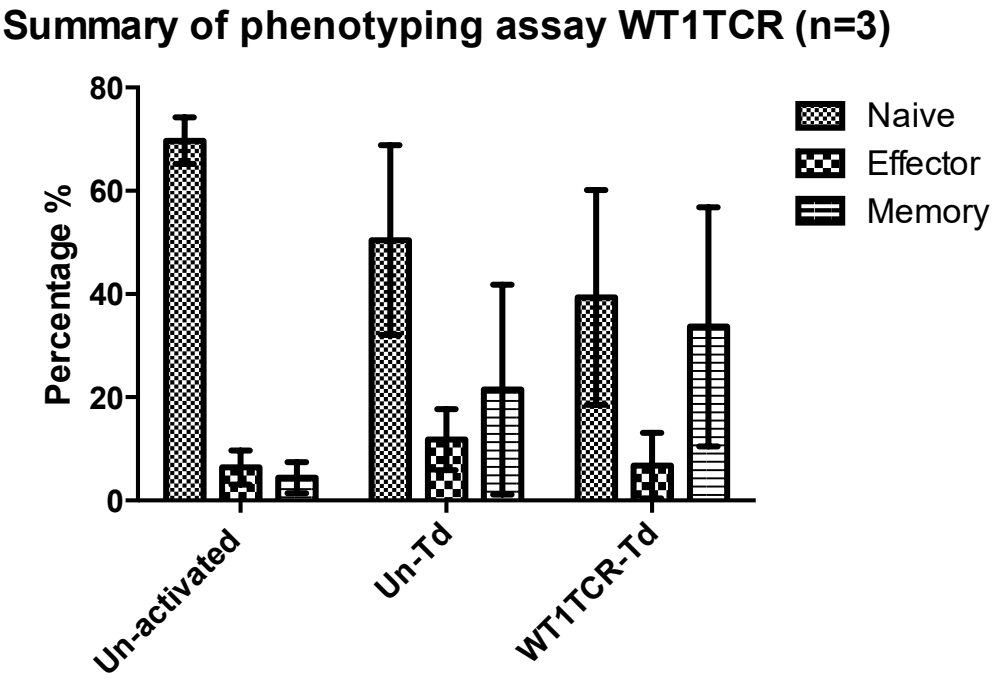


Figure 21: Classification of WT1TCR transduced CB-T cells prior and post activation (n=3). The frequency of CD27 and CD45RA cells in CD4+ T cells and CD8+ T cell populations from three donors. CB-T cells were isolated from three CB units and then stained with anti-CD4, anti-CD8, anti-CD27, and anti-CD45RA mAb. The mean percentage and SD of each condition in each subset are shown.

After these promising results, the production of substantial numbers of engineered cord blood T cells was discussed.

3.2.4) Scale-up Experiment to Engineer CB-T Cells with WT1TCR in a Closed Bag System:

In the scalability experiment relating to CB-T cell engineering, performed with 200 x10^6 CB-T cells in a closed bag system, the ficolled CB-T cells were activated with anti-CD3 and anti CD28 in a 100 ml differentiation bag. Two days later, activated T cells were transduced with Lnt-SFFV-WT1TCR (MOI 4) and kept in culture for ten days with IL-2 supplemented every forty-eight hours. On Day five of the experiment, cells were collected and stained with anti-Vβ2.1 and anti-murine constant β chain to evaluate the transduction efficiency by flowcytometry.WT1-TCR-transduced CB- T cells demonstrated a remarkable increase in the percentage of the positive WT1-TCR cell population, compared with non-transduced cells. The FACS plot in figure 22 illustrates that up to 58.5 % of CB-T cells express TCR composed of Vβ2.1 and mCβ chains targeting WT1 antigens.

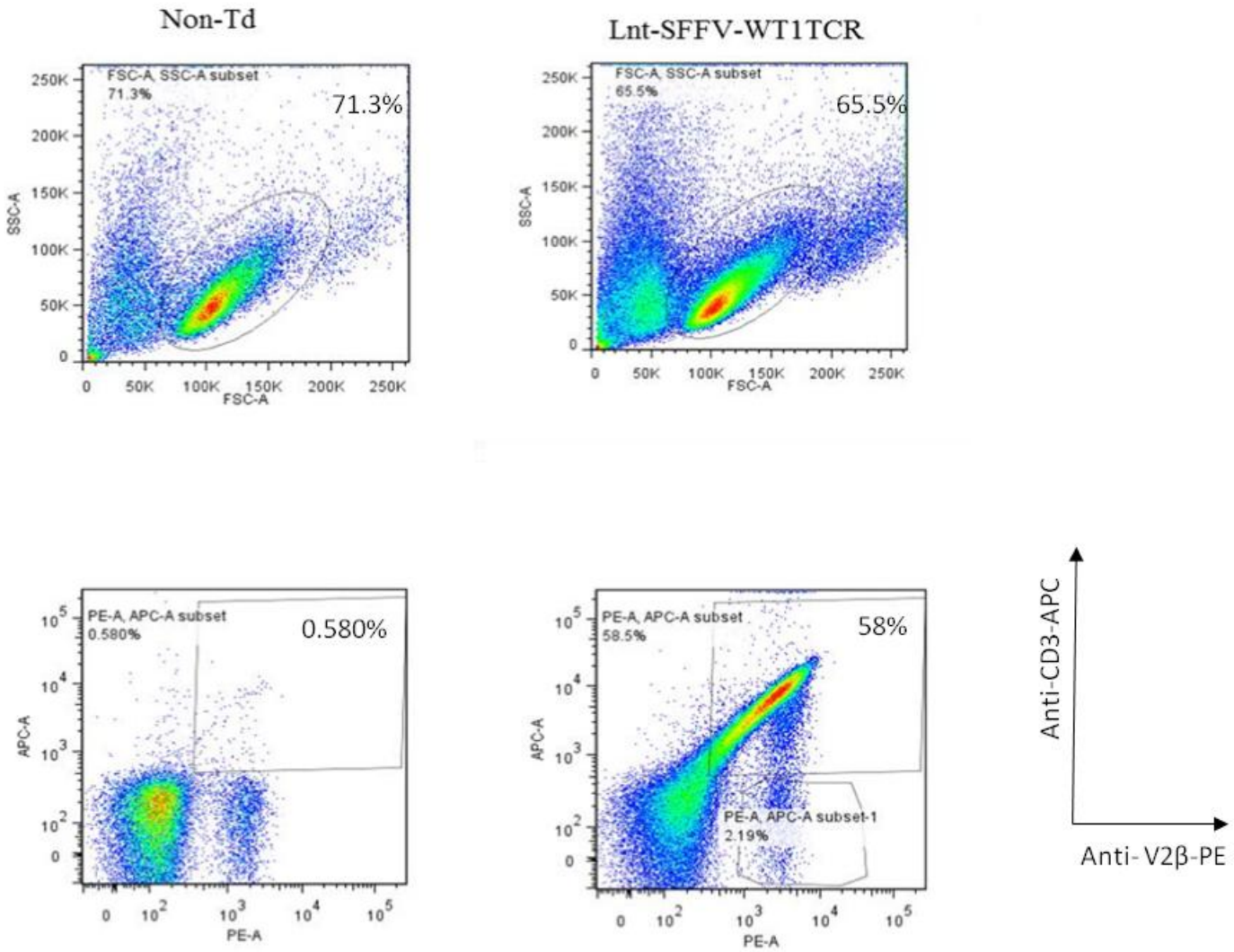


Figure 22: Expression of WT1-TCR in closed bag system for scale up purposes. 200x10^6 CB-T cells were activated with CD3/CD28 beads and transduced with LNT-SFFV-WT1TCR (MOI 4). A) Gating on the viable cell population, B) FACS plots data demonstrated the remarkable efficiency of WT1-TCR on CB-T cells after transduction.

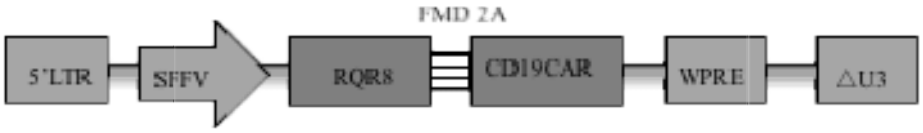
WT1-TCR transduced cells were later selected based on the existence of murine constant region within the introduced TCR that were expressed by the cells. WT1-TCR transduced cells were stained with anti-mCβ-APC mAbs then labeled with anti-APC microbeads. The cells that expressed WT1-TCR were then positively selected or enriched by using MS MACS column. 70x10^6 positive selected cells were frozen down in 90% FCS and 10% DMSO for further analysis.

3.3) Engineering CB-T cell with CD19CAR:

3.3.1) Vector production and characterization:

Two lentiviral vectors were utilised in this project to engineer CB-T cells with CD19CAR. The first lentiviral vector expressing CAR-CD19 with under the control of SFFV promoter was produced by PULE LAB (UCL Cancer Institute, UK). The parental plasmid is a self-inactivating (SIN), second generation lentiviral backbone derived from a pHR plasmid. It includes a SFFV promoter, followed by a RQR8 sort suicide gene (CD34 marker) connected to CD19CAR via foot and mouth disease 2 like peptide (FMD-2A) (Figure23.A). Vector titration of Lnt-CAR-CD19 with an SFFV promoter (Figure 23.B) was performed on a CD19CAR-negative-SupT1 cell line. The FACS analysis provided a titre of 1.8 x 10^9 IU per ml.

A) Lnt-SFFV-CD19-CAR construct



B) Virus titration of Lnt-SFFV-CD19-CAR

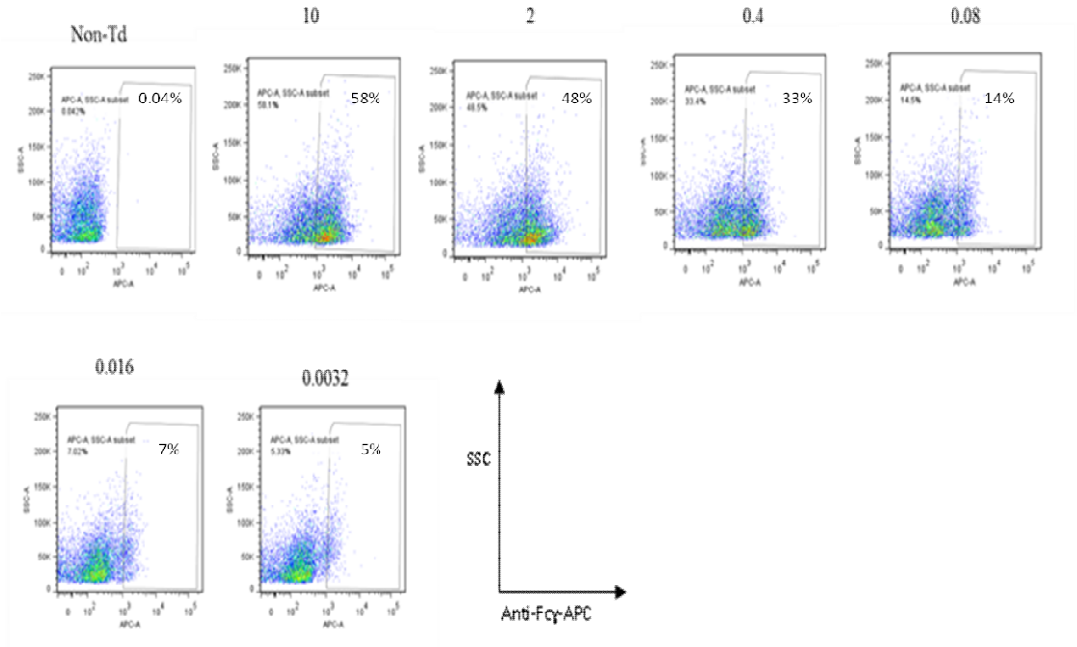
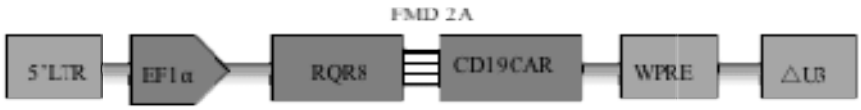


Figure 23: Lnt-SFFV-CD19-CAR virus titration. A) Schematic map of Lnt-SFFV-CD19CAR construct. B) FACS plot data demonstrated CD19CAR transduction efficiency using different amounts of concentrated LNT-SFFV-CD19CAR virus on a CD19CAR-negative SupT-1 cell line.

The second CD19CAR lentiviral vector utilised in this project was a pCCL third generation lentiviral vector configuration expressing CAR-CD19 transgene under the control of an EFLα promoter. The CD19CAR transgene is linked to the RQR8 suicide gene via a viral P2A sequence for dual gene expression. In addition to an EFlα promoter, the vector also includes a Rev responsive element (RRE), Central Polypurine Tract (CPPT) and a mutated Woodchuck Post Regulatory Element (mWPRE) (Figure 24.A). After the first and second viral harvests, the number of infectious viral particles per ml was determined by transduced HEK 293ts, or Jurkat cells with a serial dilution of the prepared concentrated vector. The transduction percentage was determined by flow cytometry after staining the transduced cells with anti-CD34 to detect the RQR8 portion of the receptor. A typical titre of $2x10^8$ infectious units (IU) per ml was achieved (Figure 24.B).

A) Lnt-EFLα-CD19-CAR construct



B) Virus titration of Lnt-EFLα-CD19-CAR

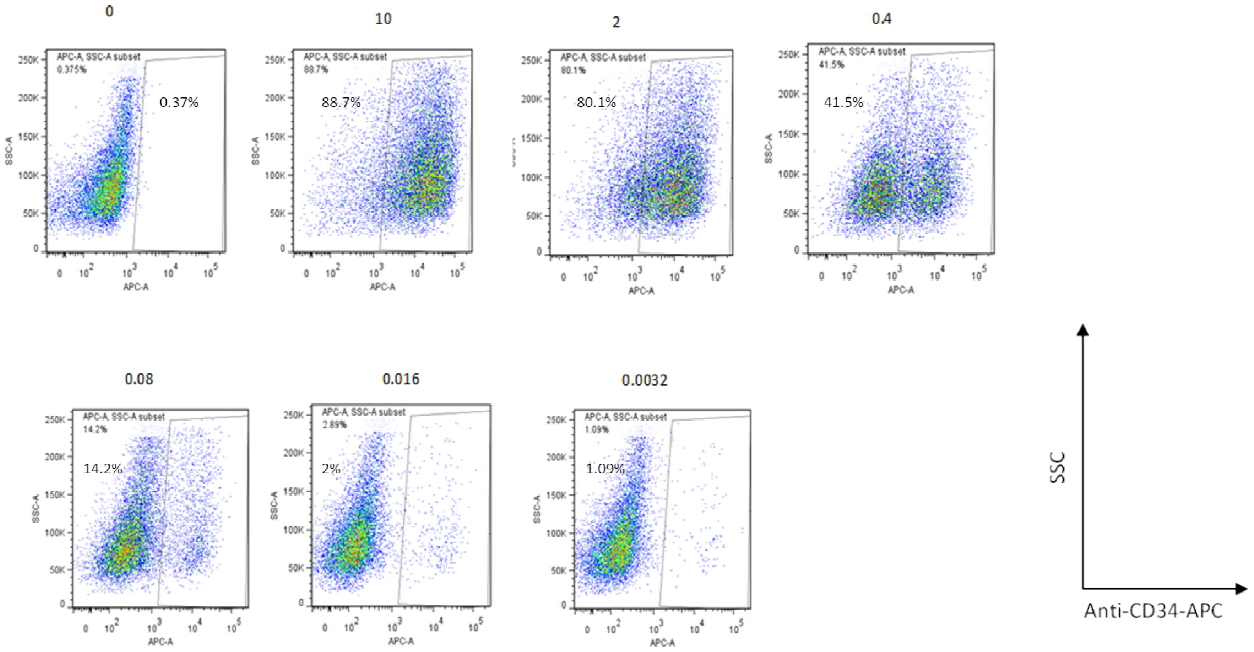


Figure 24: Lnt-EFLα-CD19-CAR virus titration. A) Schematic map of Lnt-EFLα-CD19-CAR construct. B) FACS plot data illustrate CD19-CAR transduction efficiency using different amounts of concentrated Lnt-EFLα-CD19-CAR virus on a Jurkat cell line.

3.3.2) CD19-CAR transduction efficiency after activation of CB-T cells with CD3/CD28 beads and transduction with LNT-SFFV-CD19CAR (MOI 10):

One unit of CB was ficolled and $2x10^6$ cells were cultured in X-VIVO 15 + 5% HS, 100 units of IL-2 and CD3/CD28 Dynal beads at a 1:1 ratio per well were placed in 24 well plate. Two days later, activated cells were exposed to a Lnt-SFFV-CD19CAR vector at MOI of 10. Three days post transduction; transduced cells were harvested for further analysis, including transduction efficiency evaluation and some other functional assessments, such as ^{51}Cr release assay. To investigate the transduction efficiency, both transduced and non-transduced cells were stained with antibodies that are designed to target the spacer/linker region of CD19-CAR, named scFv-APC mAb, for flow cytometry analysis. The flow cytometric characterization of CD19CAR-CB-T-cells revealed a remarkable increase in the percentage of T cells-expressed as CD19CAR – up to 70%, compared with non-transduced cells in the CD3/CD28 beads stimulation condition (Figure 25).

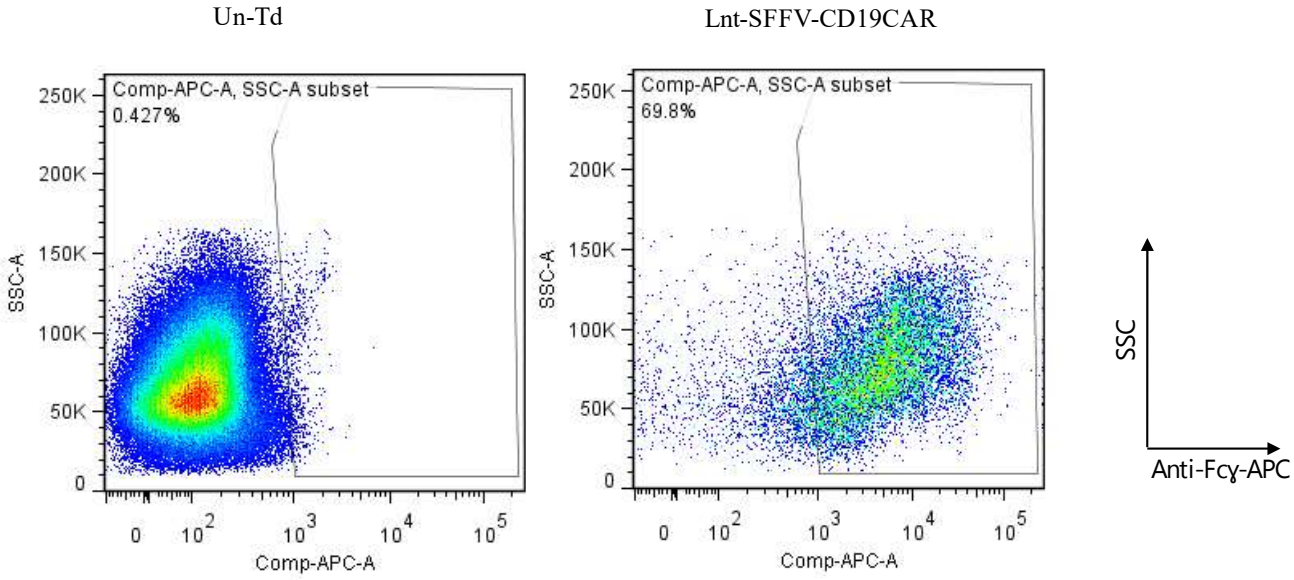


Figure 25: Expression level of CD19CAR transgene after lentiviral transduction of CB-T cells. FACS blot on the left demonstrate the level of CD19CAR expression in the un-transduced population (0.427%); which is considered as the background. FACS blot on the left illustrate the clear population of the transduced cells that expressing CD19CAR (69.8%).

3.3.3) CD19-CAR T cells generated from cord blood have the potential for significant and specific *in vitro* lysis of their target:

The functionality of the CD19CAR transduced CB-T-cells was also investigated by determining the specific killing behavior of the transduced CB-T cell population against

target cells expressing CD19 on their surfaces (SupT1-CD19 cell line). CD19-CAR CB-T cells generated after lentiviral transduction were incubated for four hours with a ^{51}Cr labeled target. The SupT1-CD19 cells stably transfected with the CD19 antigen, or SupT1 cell line that served as positive and negative controls, respectively. Manufactured anti-CD19-CAR CB-T cells were able to kill their targets in a CD19-specific manner with a remarkably high percentage of killing activity (70%). More impressively, very high levels of cytotoxicity were appreciated even at very low effector-to-target (E:T) ratios. In contrast, the corresponding un-transduced controls demonstrated little cytotoxicity (Figure 26). The previous data might suggest that CD19CAR transduced CB-T cells are highly capable of recognising their target antigens and discriminating them from non-target cells. To further confirm this observation, an *in vivo* trial is required. Moreover, it has been noticed that levels of killing reflect the levels of gene transfer.

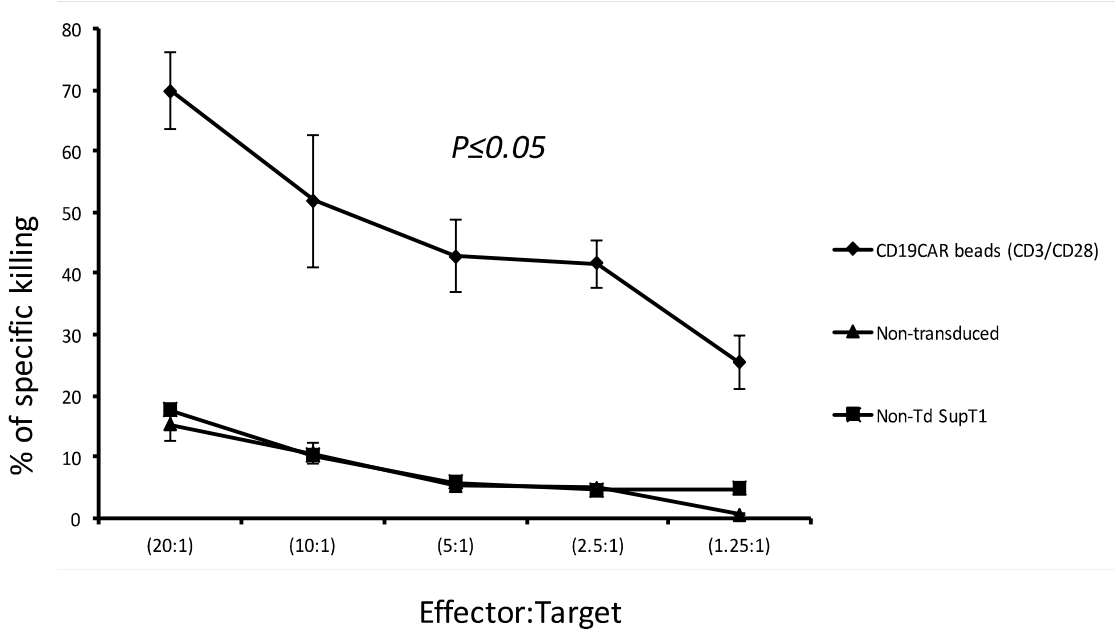


Figure 26: Percentage of specific killing of Lnt-SFFV-CD19-CAR CB-T cells. LNT-SFFV-CD19CAR-transduced normal donor CB-T cells killed CD19-SupT1 cells expressing CD19 on their surface, but not CD19 negative-SupT1 cells (control target).

Experiments using the lentiviral vector with a SFFV promoter were conducted only on one cord blood unit, while the other vector that utilizes EFlα as a promoter was used to edit the specificity of more than one CB-T cell donor. In the following section, we will discuss the outcome of editing the specificity of these cells towards a CD19 antigen.

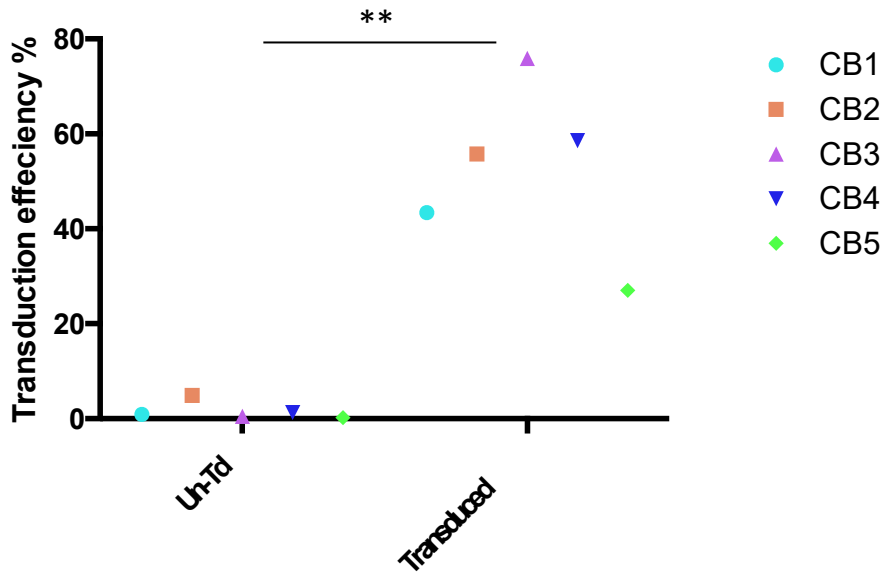
3.3.4) CD19-CAR Transduction efficiency after activation CB-T cells with CD3/CD28 beads and transduction with LNT-EFLα-CD19CAR (MOI 5):

After the promising outcome of the preliminary data, a scale-up plan to manufacture large quantities of engineered CB-T cells was discussed. On the first day, up to 100 $x10^6$ CB-T cells (n=5) were activated in 100 ml differentiation bags with anti-CD3/anti-CD28 beads. Two days later, the activated cells were transduced with Lnt-EFlα-CD19CAR lentivirus vector at MOI 5. After transduction, the cells were moved to be cultured in G-rex 10m flasks for ten more days. The G-rex expansion system is considered to be one of the most robust systems to multiply cell growth rate by providing unlimited gas exchange and nutrition via its gas permeable membrane. Finally, the cells were harvested to analyse their killing activity and to investigate their naïve phenotype, and some cells were frozen for *in vivo* assay.

Before proceeding to the final step, two donors were subjected to a positive selection of CD34 in order to have a pure population of cells expressing CD19CAR alone using the MACS separation system for positive selection. However, the number of positive selected cells was not reflective of the high percentages of transduction efficiency. In fact, the numbers of the positive cells were very low. It had been later discovered that CD34 was expressed by the cells for only a short period of time, and then it disappeared in an unexplainable manner. One of the notions that had been suggested regarding the reason of its disappearance was that CD34, or to be more specific RQR8, was recycled by the cells, so it may have been subjected to a period of disappearance. Nevertheless, to date the exact reason behind the disappearance of the RQR8 region after a short time of transduction remains unclear.

To determine the levels of CD19-CAR expression on the cell surface, cells were stained with CD34-APC monoclonal antibodies (mAb) and/or anti-Fab antibody for flow cytometric analysis two days after transduction. Flow cytometry detected RQR8 and/or Fab on up to 75.9% CD19CAR transduced CB-T cells, and less than 1% background detected on un-transduced cells (27%-75.9%, mean=52.14) (Figure 27)

A) Percentage of CD19CAR expression



B) FACS blot represent the expression of CD19CAR

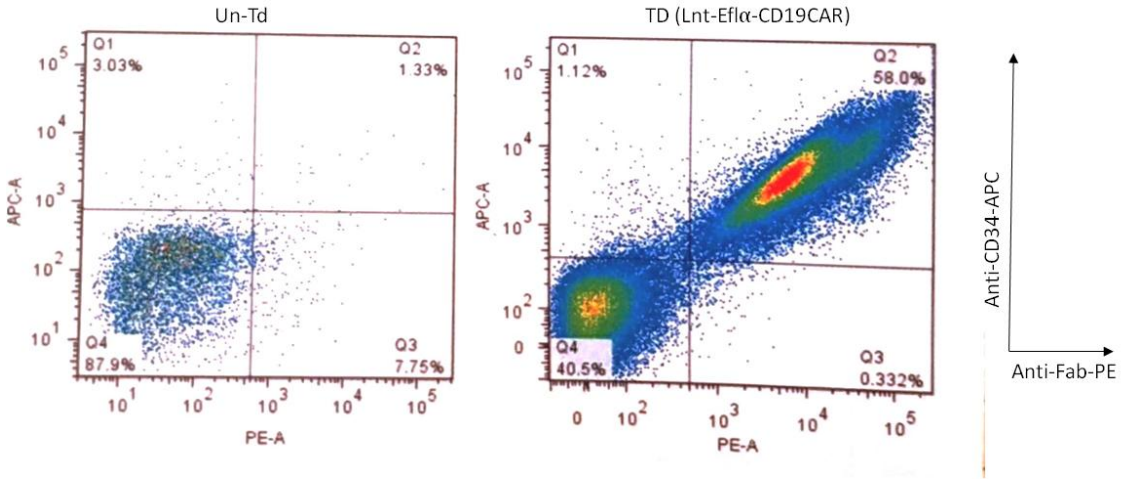


Figure 27:CD19CAR expression levels on five different donors of CB-T cells. A) Grouped dot graph illustrates the percentages of CD19CAR expression in 5 different CB units transduced with Lnt-EFlα-CD19CAR. Un-transduced cells from the same donors were used as a control group. A significant increase in CD19CAR expression levels were observed within the transduced group, compared to the percentages within the un-transduced batch (P value ≤ 0.01). B) Representative FACS plots that explain the flow cytometric characterisation of CD19CAR expression of CB-T-cells from donor number 4. FACS plots show 58% of the transduced cells are expressing CAR19 with 1% background detected within the control group by staining with anti-Fab-PE and anti-CD34-APC antibodies.

3.3.5) CD19CAR Engineered T cells Phenotype Post Activation and Transduction (n=3):

Twelve days post activation and after engineering the cells with CD19CAR, the cells were stained with CD4, CD8, CD45RA, CD62L and CCR7 to investigate the effect of CD19CAR on them, as well as to determine their fate after being engineered. As shown in the bar chart display in Figure 28, there is a slight difference in the percentage of the naïve population between the engineered cells and the un-transduced cells. Un-activated cells were used as a control and the results resembled the un-engineered cells. It may be concluded from this assay that CB-T cells still retained their naïve phenotype and it is feasible to expand the naïve CB-T cells *ex vivo*.

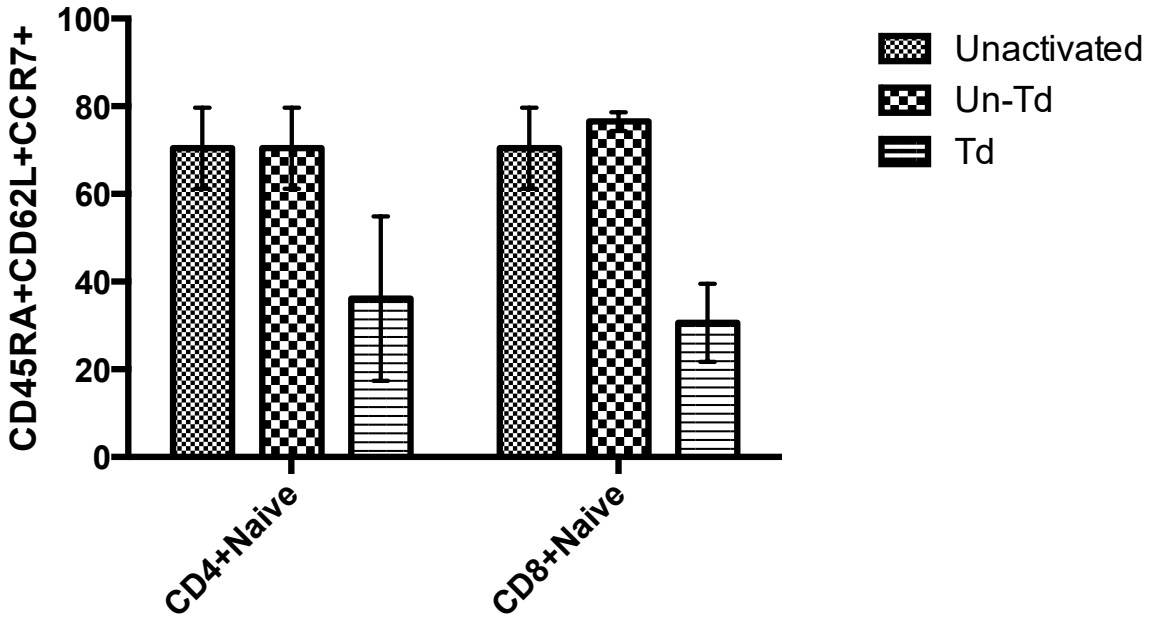


Figure 28: Phenotypic analysis of CAR19 engineered CB-T cells. Bar chart demonstrates the expression of naïve markers: CD45RA, CD62L and CCR7 on CD4 and CD8 subsets of CB-T cells transduced with CD19CAR lentiviral vector.

3.3.6) Ability of CAR19 Engineered CB-T Cells to Specifically Kill their Target (n=3):

A Sup-T1-CD19 cell line was used as the target cells to investigate the functionality of the CD19CAR engineered T cells. The specific killing behavior of the transduced CB-T cells against their target was determined after incubating them with a ^{51}Cr labeled CD19-SupT1 cell for four hours. The SupT1 cells that negatively express CD19 were used as a negative control. The engineered CAR19 CB-T cells were able to detect and kill over 40 % of their target cells and rarely showed killing behavior against the negative control cell line. Similar to the killing activity of the un-transduced cells, the previous data might suggest that CD-19CAR transduced CB-T cells are highly capable of recognising their target antigens and discriminating them from non-target cells. To further confirm this observation, an *in vivo* trial is required. Moreover, it has been noticed that levels of killing reflects the levels of transduction efficiency (Figure 29).

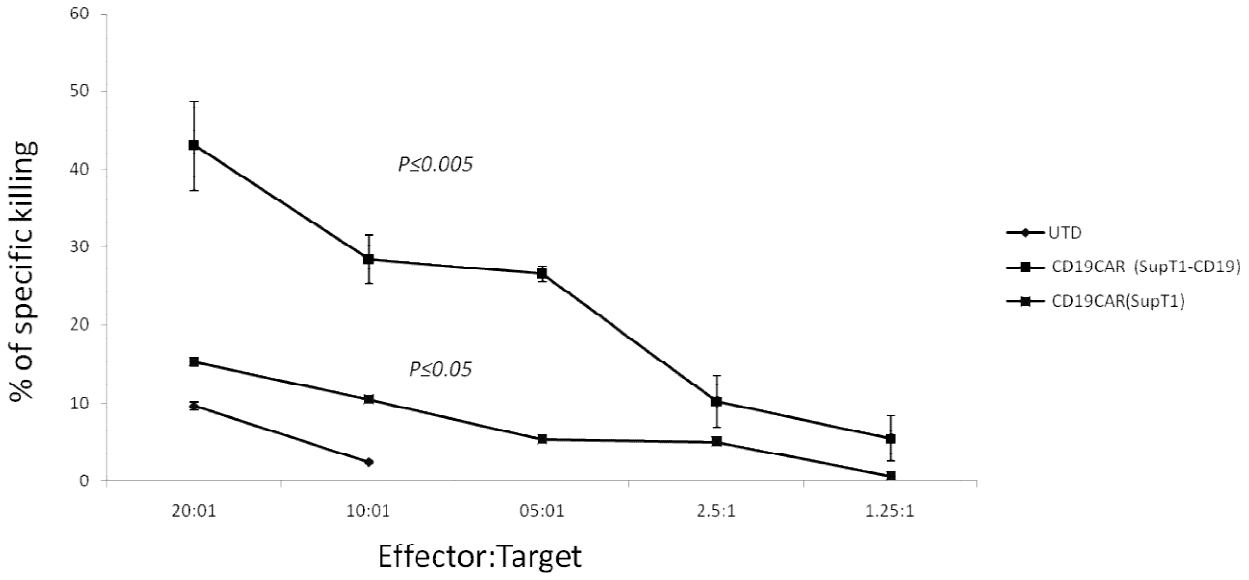


Figure 29 : Percentage of specific killing of Lnt-EFαL-CD19CAR CB-T cells. Lnt -EFlα- CD19CAR-transduced normal donor CB-T cells killed CD19-SupT1 cells expressing CD19 on their surface, but not CD19 negative-SupT1 cells (control target). P value≤ 0.005 reflects the difference of killing activity between untransduced CB-T cells and CD19CAR CB-T cells. P value ≤0.05 reflects the difference of killing behavior between CAR19 cells against SupT1-CD19 and CAR19 calls against SupT1 not expressing CD19.

3.4) Discussion:

Graft-versus-host-disease (GVHD) is the end of a complex interplay of donor and host factors that leads to massive tissue damage and impaired immunological recovery. Despite decades of research studies examining GVHD's role in delaying immune reconstitution, it remains a fundamental cause of morbidity and mortality after allogeneic transplantation and limits the success of this therapeutic approach (Parmar et al., 2014). Umbilical cord blood cells have become a major source of hematopoietic stem cells that are utilised for the treatment of patients with high-risk hematologic diseases lacking a human leukocyte antigen (HLA)-matched bone marrow donor (Eapen et al., 2007). Cord blood lacking memory T cell subsets is responsible for the low incidence and severity of GVHD after allogeneic hematopoietic stem cell transplantations (HSCT) from CB. Additionally, the naïve phenotype of CB-T cells, ease of accessibility and the prolonged survival rate after engraftment, amongst several other characteristics, have made CB-T cells an optimal source to generate allogeneic universal "off-the-shelf" pharmaceutical T cell products.

Although unrelated cord blood transplantation has proven to be a great source of hematopoietic stem cells for patients with B-lineage acute lymphoblastic leukaemia (B-ALL), a T cell-subset in circulating CB-T cells prevents tumour identification that might lead to disease-relapse. In 2006, a scientific group hypothesised that using a single-chain chimeric immunoreceptor targeting specific antigen (i.e. CD19), will overcome this limitation. Therefore, CD19CAR derived from CB-T cells has already been generated and tested successfully in a murine model (Serrano et al., 2006), providing a solid foundation to generate universal off- the-shelf genetically engineered CB-T cells, which is the main goal of this chapter.

In order to achieve our aims, we have investigated the feasibility of genetically engineered CB-T cells for direct anti-tumour therapy using lentiviral transfer of both WT1-αβ TCR and CD19-CAR. The self-inactivating lentiviral vectors expressing an optimised TCR targeting Wilms' tumour antigen 1 (WT1-TCR) or the CD19-specific chimeric antigen receptor (CAR), were produced and tested on TCR-negative Jurkat T cells. CB-T cells were activated with anti-CD3/-CD28 beads in the presence of interleukin-2 and exposed to a single round of lentiviral transduction. Three days after transduction, a flow cytometric analysis found between a 49–95% gene transfer (mean=60%, n=5) with high levels of viability and

functional integrity. Engineered CB-T cells exhibited antigen-specific interferon-γ release and mediated appropriate, target-specific cytotoxicity in chromium release assays. The scalability of CB-T engineering was confirmed in a closed-bag system appropriate for therapeutic use as part of feasibility studies for 'off-the-shelf therapies' to treat haematological malignancies. Findings related to engineering CB-T cells with third generation CAR CD19 reveal high gene transfer percentages found between 27–75% (mean=52.14%, n=5) with high viability. In the primary *in vitro* ^{51}Chromium release experiment, anti-tumour activity of genetically altered CB-T cells was observed to be highly specific in targeting the CD19 antigen expressed by the SupT1 cell line. Interestingly, the levels of chromium released by the antigen-specific engineered cells reflect the percentage of gene transfer. These findings suggest that naïve CB-T cells have a high potential to be engineered and manipulated towards a specific antigen with a great anti-tumour effect and high specificity.

In adoptive T cell therapy, it has been shown that the circulating, infused engineered adult T cells undergo memory-effector differentiation in a rapid manner, which is linked to a reduction in the survival rate and anti-tumour efficacy after engraftment. Furthermore, immune exhaustion of engrafted T cells is associated with a late differentiated phenotype (Klebanoff et al., 2005a, Gattinoni et al., 2005b).

Here our findings suggest that engineered CB-T cells retain their less differentiated phenotype, naive and stem cell memory T cell subsets after *in vitro* activation with genetic manipulation, which is favourable for immunotherapy. In summary, the outcomes of this chapter may suggest that CB-T cells are more amenable to alteration and more easily lend themselves to transduction. This would lead to an accessible and fixable production of off-the-shelf therapeutic, antigen-specific T lymphocytes that are ready for infusion into multiple recipients without the risk of GVHD occurring. However, the potency of the anti-tumour effect of these engineered cells should be evaluated in an *in vivo* mouse model before being applied in clinical trials. For further work, CB-T cells should be evaluated to reject B-cell lymphoma (Raji cells line) in a xenograft tumour model in the immunodeficient mouse strain NOD/SCID/IL2rγ^{null} (NSG). Another issue is that the endogenous αβ TCR will still be expressed by the engineered T cells, resulting in a possibility that this endogenous TCR might lead to some problems, such as GVHD or a pairing with the exogenous newly introduced TCR leading to the production of unspecific TCRs (Heemskerk, 2010). One of our

aims in this project is to disrupt the expression of the endogenous TCR-CD3 complex by using engineered nucleases as part of genome editing technology. The outcome of these experiments will be discussed later in the text.

Chapter 4: Comparison of CB-T Cell Activated with CD3/CD28 Beads and IL-2 versus CB-T Cell Activated with Cytokines (IL-7 and IL-15) and IL-2

4.1) Introduction:

The age-related differences in engineered T-cell differentiation, activation, adhesion, and exhaustion status have had a major influence on their ability to achieve fully competent anti-tumor activity in adoptive immunotherapy (Harris et al., 1992). As it has been mentioned earlier, one of the fundamental keys for successful adoptive T cell therapy against cancer is the expansion, persistence and long survival of the tumour-directed therapeutic T cells after infusion. Ordinarily, T-cells from ficolled blood are activated via cross-linking of anti-CD3 and anti-CD28 conjugated beads prior to transduction or transfection. Once the gene transfer process is performed, cells are usually expanded by adding γc chain cytokine IL-2 every forty-eight hours (Savoldo et al., 2011). The T cells' activation signal is triggered via the TCR-CD3 complex. The T cell expansion and proliferation, co-stimulatory CD28 is responsible for this function (Frauwirth and Thompson, 2002). Anti-CD3/anti-CD28 conjugated beads are considered to be a strong stimulant; therefore, cells will divide and differentiate in a rapid manner towards effector-memory T cells (T_{EM}) and expressing CD45RO chemokine with less than 10% of CCR7 and CD62L co-expression. As a consequence, these cells may have short persistence *in vivo* after engraftment. Although effector-memory T cells have a robust anti-tumour activity, only central-memory T cells and other less differentiated T-cell subsets, such as naïve and the recently defined "T-memory stem cells," are critical for *in vivo* expansion, survival, and long-term persistence (Gattinoni et al., 2011, Xu et al., 2014).

Interesting reports have suggested that activation of adult T cells via common-γ cytokines such as IL-7 and IL-15 will preserve the stem cell memory T cells subsets, a subpopulation that is favorable for adoptive T cell therapy due to its high survival rate and efficient anti-tumour activity (Xu et al., 2014, Gattinoni et al., 2009). These chemokines implant due to their activation mechanism that has been reviewed in the literature. For example, IL-15 promotes proliferation of naive CD8+ subsets, endorses T cell survival via up-regulation of Bcl-2 and also blocks the inhibitory effect of regulatory T cells and effector-memory T cells; while the induction of homeostatic expansion of naïve T cells is one of the roles of the IL-7.

Most interestingly, CB-T cells don't lose their CCR7+ expression after being activated with IL-7 and IL-15; suggesting that using a cytokines cocktail as a stimulant might be a better alternative to activate CB-T cells prior to viral transduction for adoptive T cell therapy.

In this project, the gene transduction efficiency of CB-T cells activated with CD3/CD28 beads was compared to cytokine cocktail-activated CB-T cells. Prior to transduction, ficolled

cord blood was subjected to either anti-CD3/CD28 beads at a 1:1 bead ratio or to a cytokines cocktail composed of IL-7, IL-15 at a concentration of 5ng/ml and IL-2 (1 00U/ml). The functionality and the anti-tumour activity of these transduced cells were also examined.

4.2) Cytokines (IL-7 and IL-15) Titration:

In order to determine the ideal combination of the cytokines cocktail that produced a robust activation of CB-T cells with a reasonable survival rate, a cytokines titration experiment was performed. Different concentrations of IL-7 (0, 0.5, 5, 50 ng/ml) were mixed with 100 U/ml IL-2 and with different concentrations of IL-15 (0, 0.5, 5, 50ng/ml) to activate $2x10^6$ CB-T cells in a 24-well plate. Three days post-activation, a third generation CD19CAR lentiviral vector was used to transduce these activated cells. Forty-eight hours after transduction (Day 5 after activation), the cells' viability and CD19CAR expression level were investigated by flow cytometry after staining the cells with mAb targeting the RQR8 portion (anti-CD34) of the CD19CAR construct. The interleukins were topped up every 48 hours for each condition. IL-2 was added to all conditions at a concentration of 100U/ml and topped up every 48 hours. The first column of table 6 demonstrates the effect of different concentrations of IL-7 & IL2 (100U/ml) as a stimulator on CD19CAR transduced CB-T cells by measuring the expression levels of CD19CAR. The second column illustrates the consequence of different concentrations of IL-15 & IL2 (100U/ml) alone as a stimulator on CD19CAR transduced CB-T cells by measuring the expression levels of CD19CAR. The third column shows the percentage of CD19CAR expression levels on CB-T cells that were activated with various dosages of IL-15, 5 ng/ml of IL-7 and 100 U/ml of IL-2.

The final results suggest that 5 ng/ml of IL-7 + 5 ng/ml of IL-15 + 100 U/ ml of IL-2 is the ideal condition to stimulate activation of cord blood T-cells (Table 6). Surprisingly, the data suggested that the highest percentage of CD19CAR expression is reached when the cells are activated with IL-15, in combination with IL-2, but not IL-7 (Figure 30). We chose to use IL-7, IL-15 and IL-2 as the most suitable conditions for the survival and function of T cells.

	IL-7+IL-2 (100 U/ml)	IL-15+IL-2 (100U/ml)	IL-7 (5ng/ml)+IL-15 IL-2 (100U/ml)
0 ng/ml	3.90 %	1.68 %	5%
0.5 ng/ml	5.9%	15.9 %	7.3%
5 ng/ml	6.14 %	20%	**17%**
50ng/ml	10.60 %	12%	8.40%

Table 6: Transduction efficiency of CB-T cells transduced with 2nd generation Lnt-SFFV-CD19CAR and cultured under various concentrations of IL-7, IL-15 or both.

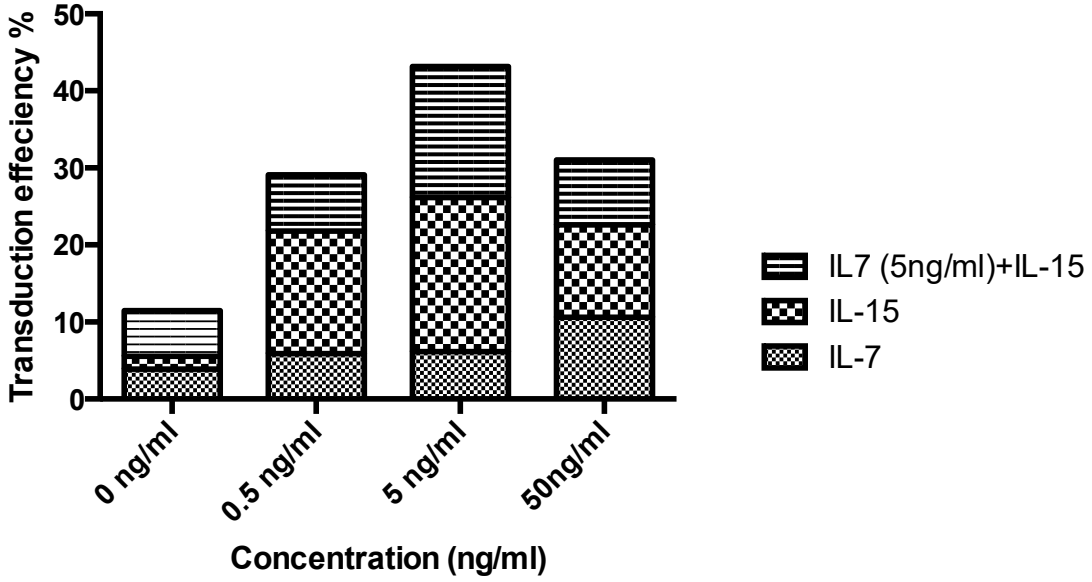


Figure 30: The percentage of CD19CAR expression level of CB-T cells after activation with different combination of various dosages of IL-7 or/and IL-15 stimulators in addition to 100 U/ml of IL-2 with all conditions. Plot demonstrates CD19CAR transduction efficiency percentage at Day 5 after activation of CB-T cells with different concentrations (0, 0.5,5,50 ng/ml) of different cytokines.

4.3) Comparison of WT1-TCR and CD19CAR transduction efficiency of CB-T cell stimulated with different conditions before transduction:

Cord blood T cells were activated using different stimulators, either CD3/CD28 antibodies (n=5) and IL-2 (100 U/ml), or a combination of IL-7 (5 ng/ml), IL-15 (5 ng/ml) (n=3) and IL-2 (100 U/ml). Forty-eight hours post activation, the activated T cells (with either condition) were transduced with lentiviral vectors containing human, Vα1.5/Vβ2 TCR genes and murine, constant α/β genes (MOI 10), or lentiviral vectors carrying scFv CD-19CAR genes (MOI 10). Three days after transduction, the cells were stained with anti-Vβ2.1 and mCβ antibodies or an antibody targeting the spacer portion of the CD19-CAR molecule (Fcγ). Gating on the viable cells on the LSR II cytometer, a significant increase in the percentage of T cells-expressed Vβ2.1/mCβ led to between a 49–95% gene transfer (mean=60%, n=5)

compared with the non-transduced T cell populations in the anti-CD/anti-C28 bead stimulation condition (Figure 31). Comparing this result with the transduction efficiency of the T cell population stimulated with cytokines alone, CB-T cells showed T cells-expressed in 26–47% gene transfer (mean=38.5%, n=3) on the basis of Vβ2.1/mCβ expression (Figure 31). Representative FACS plots of one cord blood donor displaying the percentage of engineered CB-T cells expressing both V2β and mCβ is illustrated in Figure 32.

Previous studies on peripheral blood found less than around 30% gene transfer levels in cytokine-stimulated cells. WT1-TCR transduced cells were utilised in some functional assays such as INF-gamma and ^{51}Cr release or cytotoxic assay as well as CD19CAR transduced CB-T cells.

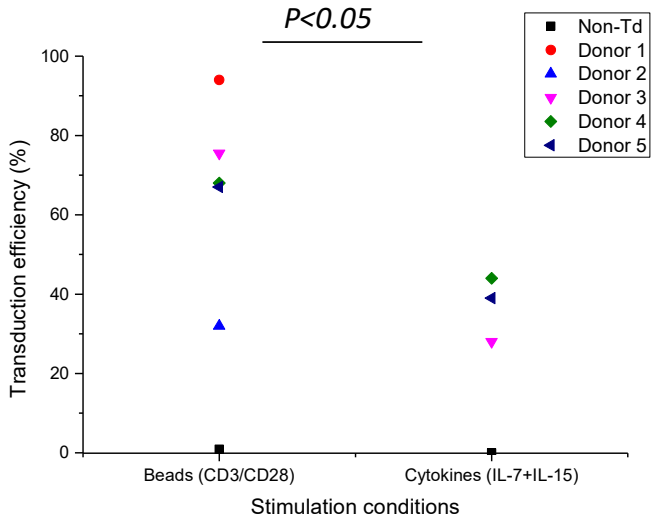


Figure 31: Expression of WT1-TCR in five human cord blood donors. Lnt-SFFV-WT1TCR modified construct was transduced into CB-T cells and its expression was analysed by anti Vβ2, and mCβ antibody staining. Graph shows comparison of WT1-TCR transduction efficiency of CB-T cell stimulated with different conditions before transduction; five CB-T cells stimulated with CD3/CD28 beads +IL- 2, while three other CB-T cells were stimulated with a combination of IL-7 and IL-15.

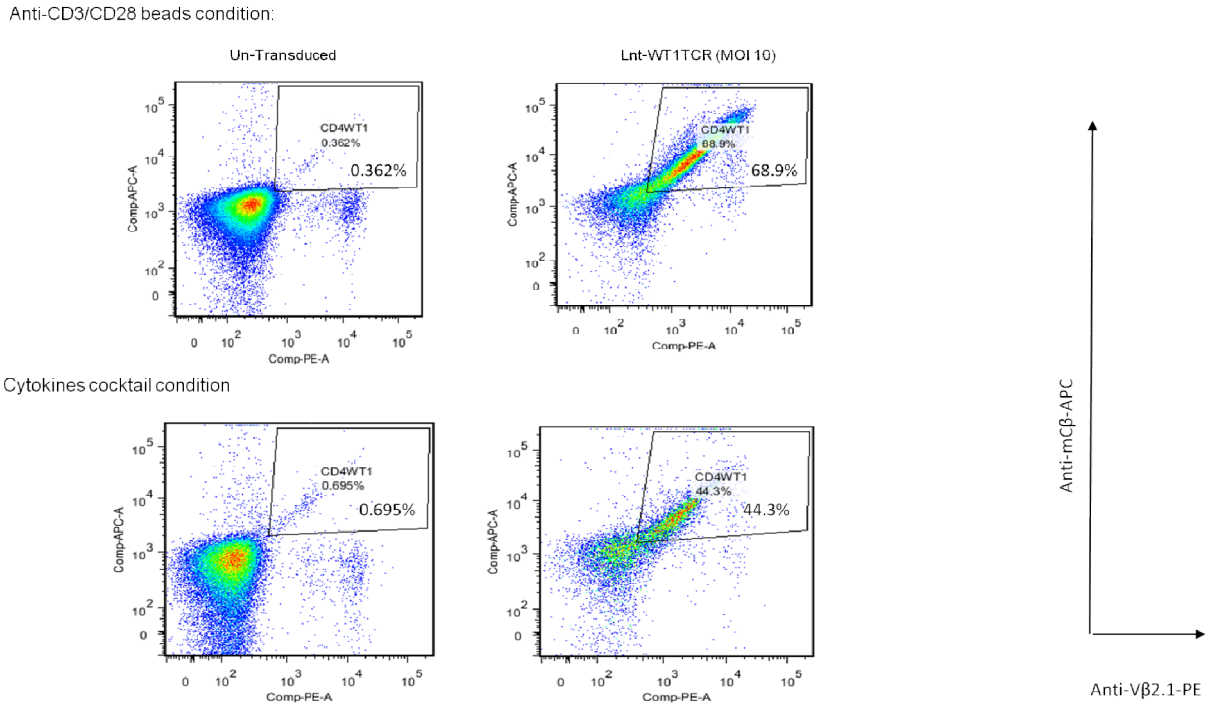


Figure 32: Representative FACS plots of one cord blood donor displaying the percentage of engineered CB-T cells expressing both V2β and mCβ. Upper plots display the transduction efficiency of CB-T cells after lentiviral transduction and using anti-CD3/CD28 as a stimulus. Same donor CB-T cells were stimulated with a cocktail of cytokines (IL-7+IL-15+IL-2) and transduced with Lnt-sffv-WT1TCR; WT1TCR expression level is represented in the lower FACS plots for both transduced population and control

Figure 33 illustrates the expression level of CD19CAR on the surface of five different donors of CB-T cells transduced with Lnt-SFFV-CD19CAR or Lnt-EFlα-CAD19CAR vectors and activated under two different conditions: with CD3/CD28 beads+IL-2 (100U/ml) and with a cytokines cocktail (IL-7 (5ng/ml) +IL-15 (5ng/ml)+IL-2 (100U/ml). Gating on the viable cells on the LSR II cytometer, a significant increase in the percentage of T cells-expressed CAR19 led to between a 32.7-75.9% gene transfer (mean=52.9%, n=5) compared with the non-transduced T cell populations in the CD3/C28 bead stimulation condition. Comparing this result with the transduction efficiency of the T cell population stimulated with cytokines alone, CB-T cells showed T cells-expressed in 15.8–48.6% gene transfer (mean=29.1%, n=3) on the basis of CD19CAR expression. A representative FACS analysis of the expression levels of CD19CAR on CB-T cells stimulated with two different conditions of one donor is explained in figure 34.

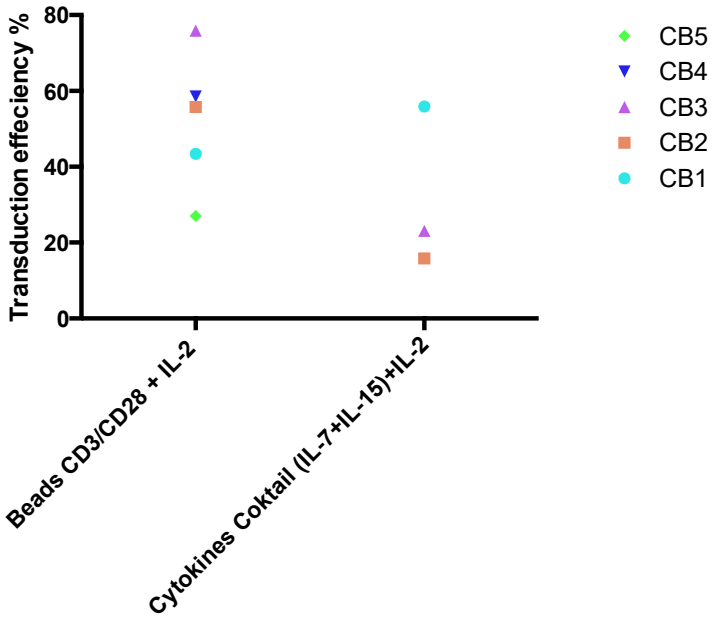


Figure 33: Expression level of CAR19 in five human cord blood donors. Lnt-SFFV-CD19CAR or Lnt-EFlα-CAD19CAR vectors were transduced into CB-T cells and CAR19 expression was analysed by anti-scFv or anti-CD34 and/or anti-Fab antibody staining. Graph shows comparison of CD19CAR transduction efficiency of CB-T cells stimulated with different conditions before transduction; five CB-T cells stimulated with CD3/CD28 beads +IL- 2 (100U/ml), while three other CB-T cells were stimulated with combination of IL-7 (5ng/ml) and IL-15 (5ng/ml) in addition to IL-2 (100U/ml).

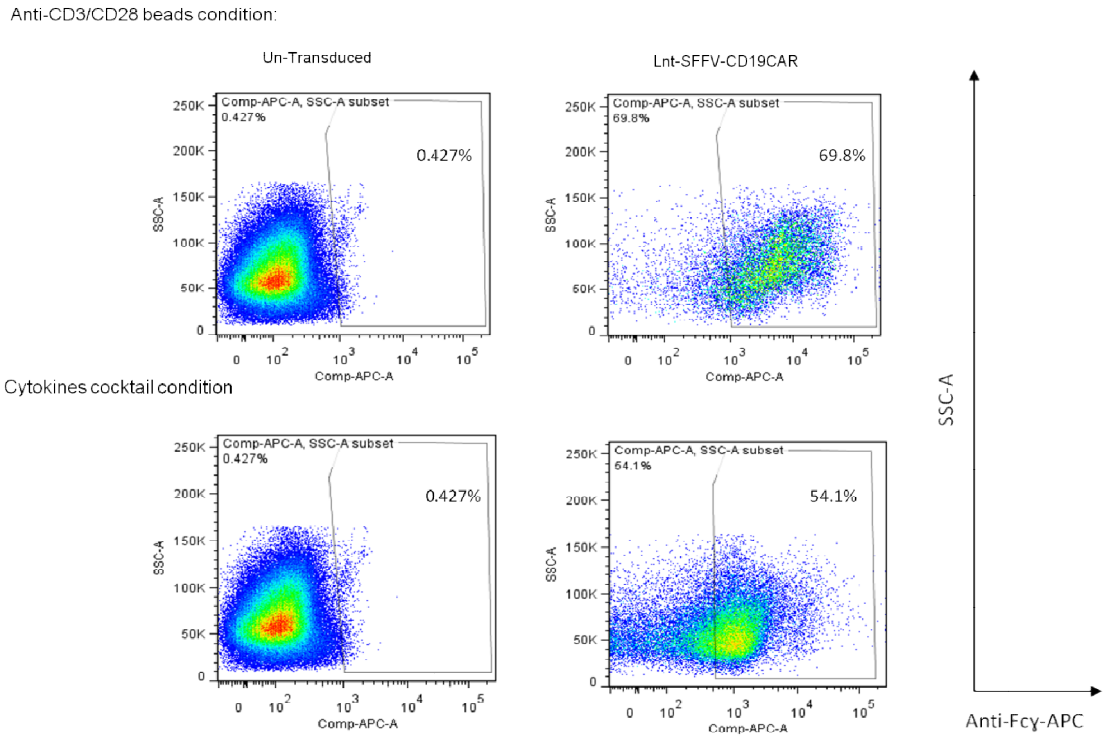


Figure 34: FACS analysis of CD19CAR CB-T cells stained with scFv-APC mAb. FACS plots revealed a remarkable increase in the percentage of T cells-expressed CD19CAR – up to 70% compared with non-transduced cells in the CD3/CD28 beads stimulation condition (upper plots). In comparison, the T cells population activated with IL-7, IL-15 and IL-2 showed an increase in the percentage of T cells-expressed to 50 % vs. with non-transduced cells (lower plots).

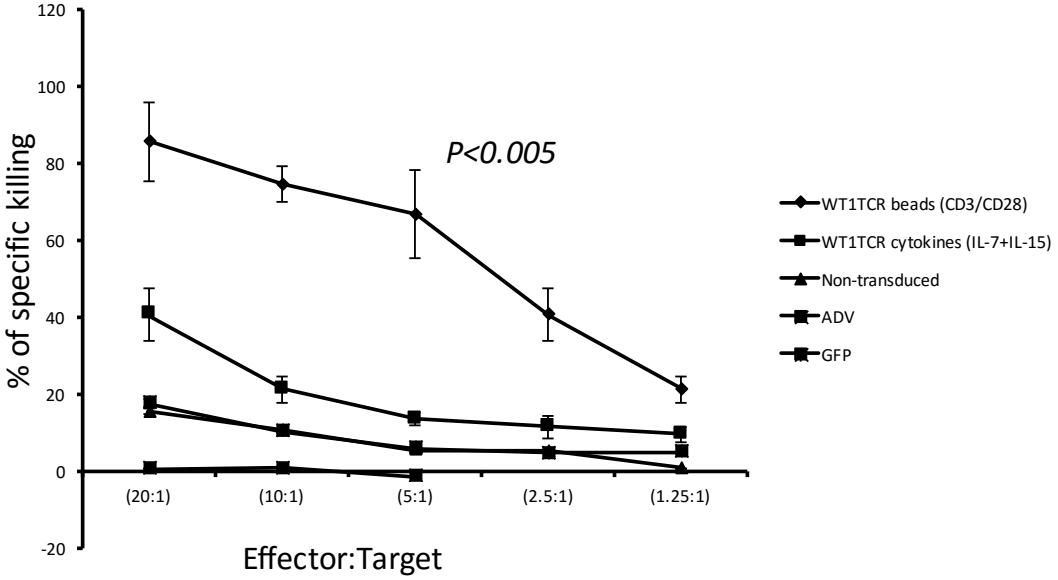
4.4) CB-T Cells engineered with WT1-TCR and CD19CAR have the ability to recognise their targets:

The antigen-specific killing activity of the cord blood that was transduced with WT1-TCR was evaluated in cytotoxicity assays using a relevant and non-relevant peptide loaded onto target cells (T2 cells). As demonstrated in Figure 35.A, the human, TAP-deficient T2 cells were killed after being coated with pWT126 (specific peptide), but not after being coated with ADV peptide (control, non-specific peptide). For the WT1-TCR CB-T cells that were activated with beads, a substantial increase in the percentage of the killing activity of T cells-expressed WT1-TCR was found to be 85% compared with the killing activity of non-transduced T cells and the killing behavior of WT1-TCR T cells against target cells loaded with a non-specific peptide. In comparison to the specific killing activity of beads-stimulated WT1-TCR CB-T cells, the specific lysis behavior of cytokines-stimulated WT1- TCR CB-T cells showed a percentage of 40% specific killing activity, in keeping with the lower transduction efficiency in these cells ($p<0.0005$)

Figure 35.B illustrates the specific killing behavior of two different donors of CB-T cell that transduced with two different lentiviral vectors expressing CAR-CD19 and activated under two different conditions: with CD3/CD28 beads+IL-2 (100U/ml) and with a cytokines cocktail (IL-7 (5ng/ml) +IL-15 (5ng/ml)+IL-2 (200U/ml)). The specificity and anti-cancer activity of CD19-CAR T cells was analysed by using target cells expressing CD19 on their surfaces (SupT1CD19 cell line) and as a negative control, SupT1 cells that do not express that CD19 was used (SupT1 cell line). An Lnt-SFFV-CD19CAR vector was used to transduce the first donor and a high percentage of killing activity (70%) was observed in CD19-CAR T cells stimulated with anti-CD3/CD28 beads. T cells stimulated with cytokines showed a significant killing activity, compared with the killing activity of the non-transduced T cell population (48%) (Figure 35 B.i). Again, both groups had levels of killing reflective of the levels of gene transfer. The second donor CB-T cells were transduced with Lnt-EFlα-CD19CAR and a significantly high percentage of anti-tumor activity was detected in both bead stimulated T cells (43%) and cytokines stimulated T cells (49%). In both experiments, CAR19 engineered T cells did not recognize the SupT1 cells and, as a result, no cytotoxic activity was noticed from these cells (Figure 35 B.ii).

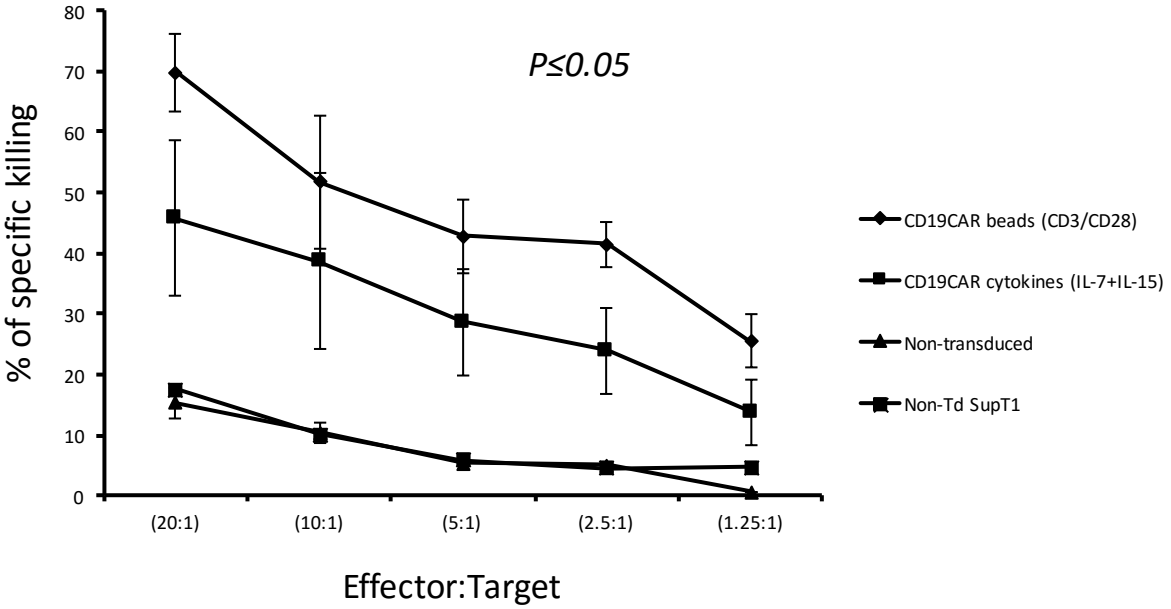
The previous data may imply that either bead stimulated engineered CB-T cells or cytokines activated CB-T cells, are able to recognize their target cancerous cells and kill them in a perfect manner.

A) Specificity of WT1-TCR transduced CB-T cells toward their target (PWT126):



B) Specificity of CD19CAR transduced CB-T cells toward their target (CD19) in two donors:

i)



ii)

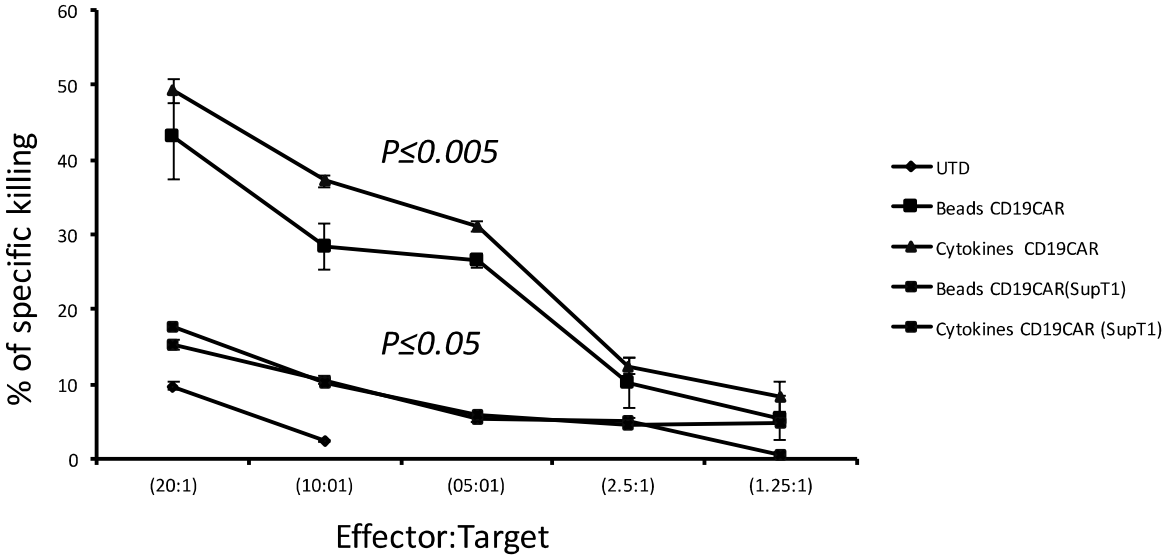


Figure 35: WT1-TCR or CD19CAR can recognise their specific tumour antigen and selectivity kill their target, cancerous cells but not non-specific control targets *in vitro*. A) LNT-SFFV-WT1TCR transduced normal donor CB-T cells killed T2 cells loaded with WT1 peptide but not non-specific control targets (ADV). B) i.LNT-SFFV-CD19CAR-transduced normal donor CB-T cells killed CD19-SupT1 cells expressing CD19 on their surface, but not CD19 negative-SupT1 cells (control target). * p value refers to the significant differences between the antiCD3/antiCD28 stimulation condition and the cytokines stimulation condition. ii. Engineered-T cells stimulated with cytokines cocktail show a higher percentage of anti-cancer activity toward specific target SupT1-CD19 than CB-T cells anti-CD3/anti-CD28 beads. Both groups demonstrate little cytotoxic activity toward negative control SupT1 cells, which is almost similar to the anti-tumour activity of the un-transduced cells toward SupT1CD19 cells. P ≤ 0.005 reflects the significant levels of antitumor activity of engineered CB-T-cells stimulated with cytokines cocktail toward SupT1CD19 cells, versus the negative control. P value ≤0.05 reflects the difference of killing behavior between CAR19 cells stimulated with anti-CD3/anti-CD28 beads against SupT1-CD19 and CAR19 cells against SupT1 not expressing CD19.

4.5) Compression of expression levels of INF-γ secretions of WT-TCR transduced CB-T cells stimulated with CD3/CD28 beads vs. cytokines cocktail:

The *in vitro*, antigen-specific functionality of WT1-TCR-transduced T cells was also demonstrated using intracellular cytokines staining applied to three CB-T cells donors (n=3). The first two CB-T cells were exposed to a CD3/CD28 activated condition, while the third donor's CB-T cells were exposed to two differently activated conditions: using CD3/CD28 beads and IL-2 (100U/ml) or with IL-7, IL-15 and IL-2 cytokines. After six hours of stimulation with relevant, (PWT126) or irrelevant (ADV), peptide-loaded T2 cells, INF-γ secretion was observed in response to PWT126 peptide only. A representative FACS plot of CB-T cells obtained from the 3rd donor showed that the functional cytokines secretion of the WT1- TCR transduced cells activated by anti-CD3/anti-CD28 beads was observed to be

2.30% of the Vβ2.1 positive cell population. The same percentage of activity has been detected in the WT1TCR CB-T cells stimulated with cytokines (2.10% INF-γ secretion) (Figure 36). The outcome of both negative and positive controls supported the results of the experiment. The previous data might suggest that WT1TCR-transduced CB-T cells are highly capable of recognising their target antigens and discriminating them from non-target cells either stimulated with anti-CD3/anti-CD28 beads, or with the cytokines cocktail. To further confirm this observation, an *in vivo* trial is required.

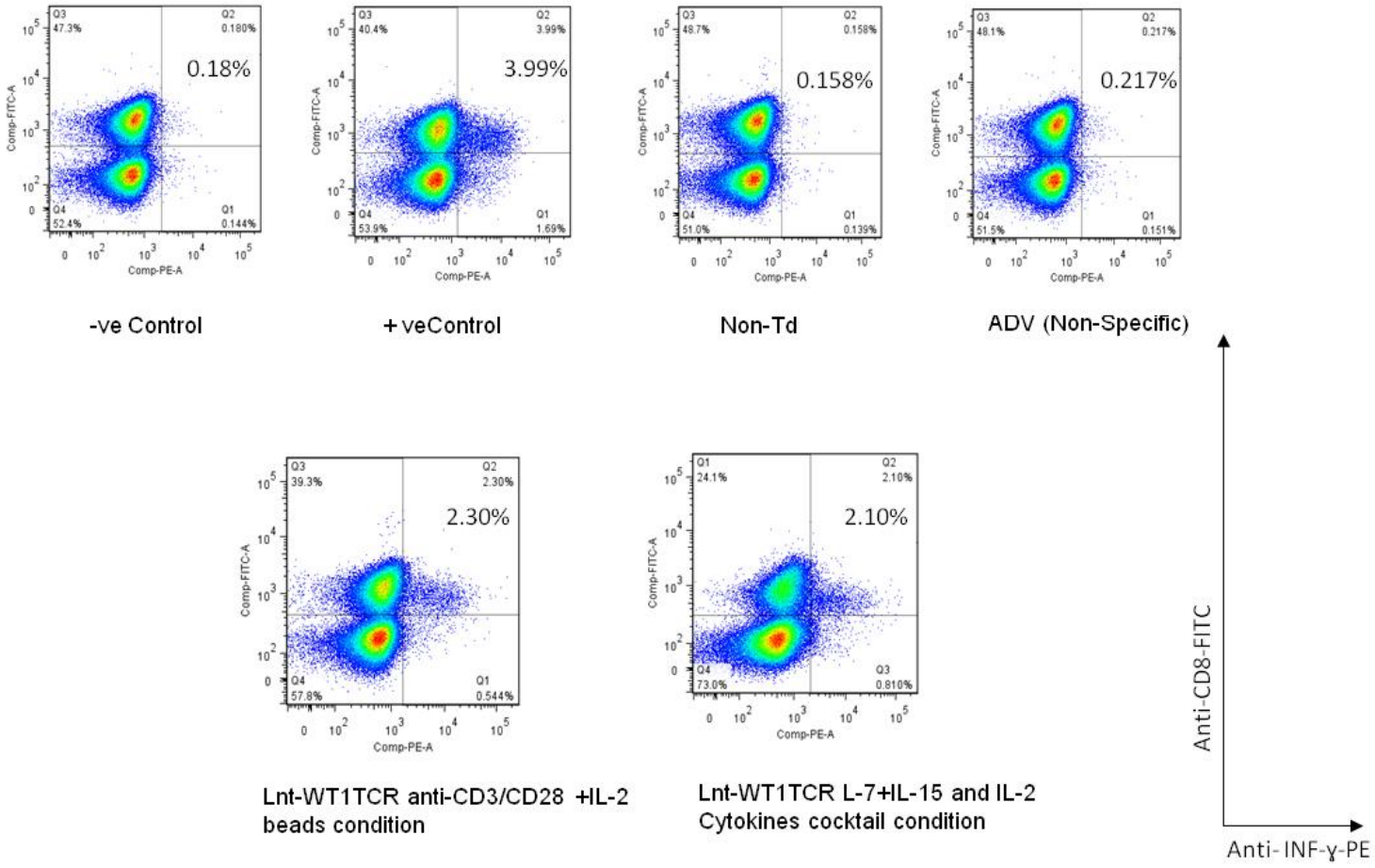
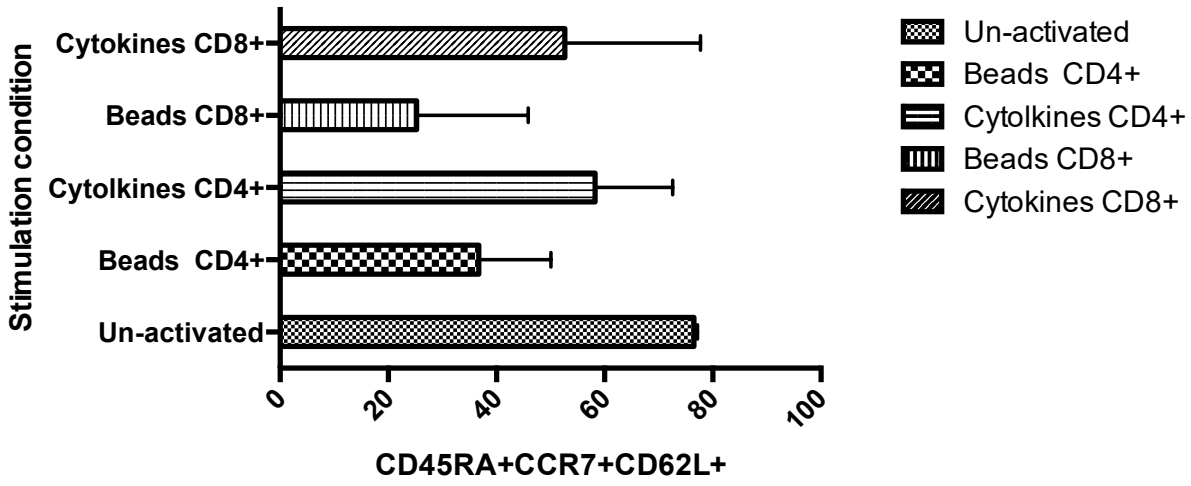


Figure 36: Antigen-specific responses of WT1-TCR transduced CB-T cells. The WT1-TCR construct (indicated in chapter 3) was transduced into CB-T cells and its functional activity was determined. A) antigen-specific interferon γ (INF-γ) production determined after 6 hrs of stimulation with specific PWT126 peptide or non-specific ADV peptide. Representative FACS plot data of intracellular INFγ staining are shown. FACS plots in the top row illustrate the percentage of INF-γ secretion of the controls that used in the experiment. FACS plots on the bottom row demonstrates the percentage of INF-γ secretions of WT1-TCR CB-T cells stimulated with anti-CD3/antiCD28 beads + IL-2 (100U/ml) (right) versus WT1-TCR CB-T cells stimulated with cytokines cocktail+IL-2 (100U/ml) (left).

4.6) CB-T cells stimulated with IL-7(5ng/ml) IL-15 (5ng/ml) IL-2 (100U/ml) show more naïve phenotype characteristic than cells stimulated with anti-CD3/anti-CD28 beads:

The presence of naïve-like phenotypes engineered CB-T cells were detected by staining the cells (n=3) with the following markers: CD8, CD4, CD45RA, CD62L and CCR7 antibodies. Flow cytometric analysis demonstrated that cytokine stimulated cells acquire a higher percentage of naïve phenotype than engineered cells that were activated with anti-CD3/anti-CD28 beads. Un-activated cells were stained after being exposed to liquid nitrogen for freezing purposes. These cells were not stained with CD4 and CD8 because they lack the expression of these two markers. The expression of the naïve like markers was determined by flow cytometry. Cytokines stimulated CB-T cells appeared to express the naïve markers (CD4+ subset: 44%-73%, mean=58.2, n=3 /CD8+subset: 25.5%-75%, mean= 52.6%,n=3), in a high percentage similar to the naïve markers on the surface of un-stimulated cells (76%-77%, mean= 76.5%, n=3). On the other hand, a lower percentage of CD45RA,CD62L and CCR7 was detected on the surface of the bead stimulated CB-T cells (CD4+subset: 0.32%-49.5%, mean= 24%, n=3/ CD8+subset: 60.1%-47%, mean= 34%, n=3) (Figure 37 A,B). It could be concluded from the previous data that using cytokines to stimulate the cells before transduction might be better than anti-CD3/anti-CD28 beads in terms of conserving the naïve phenotype of the cells and retaining the cells with their naïve-like markers. However, the level of cell survival and the number of cells at the end of the experiment is not very promising in the case of cytokine stimulating conditions.

A)



B)

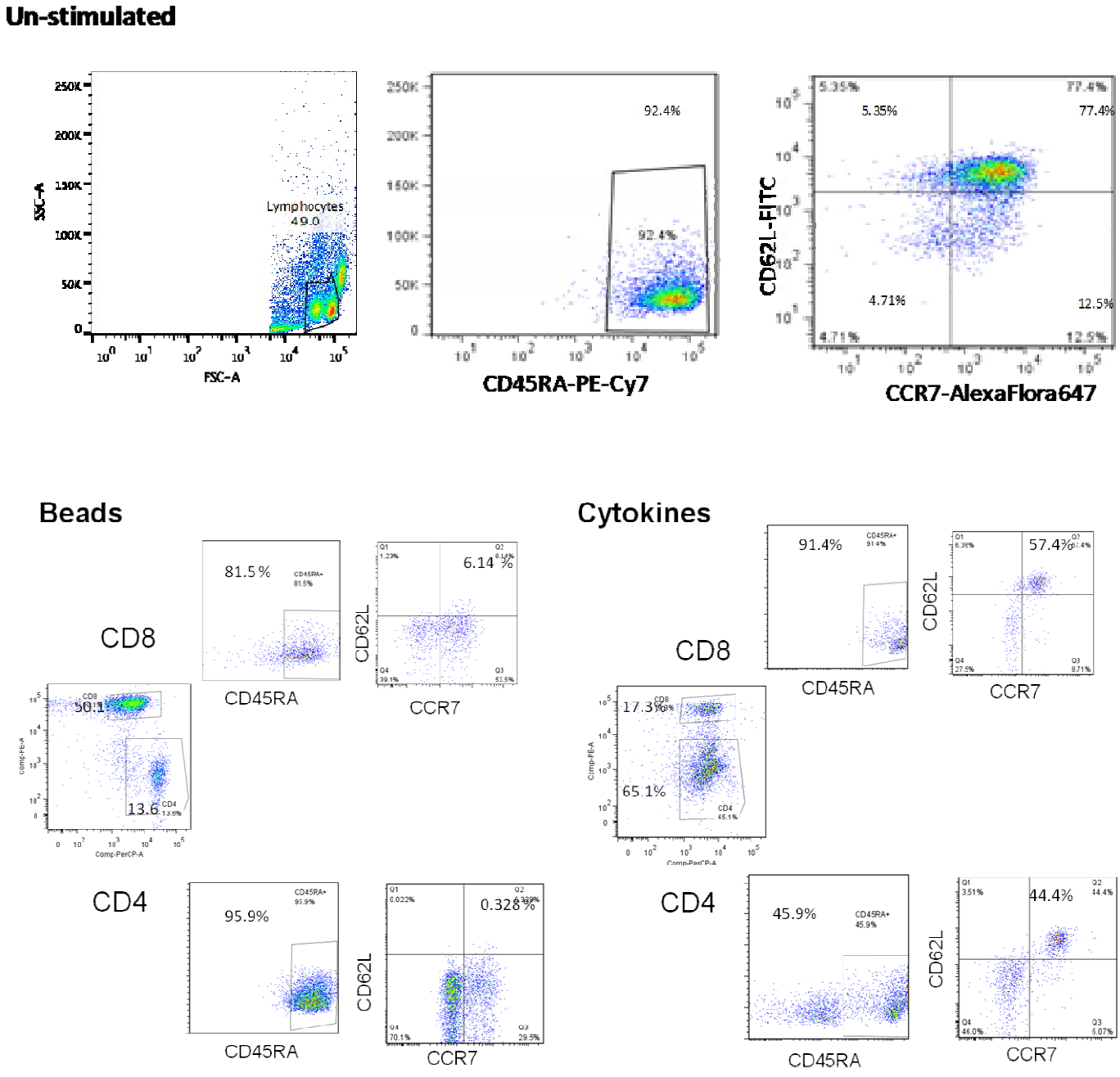


Figure 37: Comparison of expression levels of CD45RA, CD62L and CCR7 on the surface of CD19CAR transduced CB-T cells that were stimulated with anti-CD3/anti-CD28 beads vs. cytokines cocktail. A) Bar chart characterizes the percentage of naïve like phenotype cells in two different groups: CAR19 engineered CB-T cells activated with anti-CD3/anti- CD28 beads vs. CAR19 engineered CB-T cells activated with cytokines cocktail including IL-7, IL-15 and IL-2. The latter group show a higher percentage of naïve like cells than the beads activated group. Un-stimulated cells were used as a control. B) Representative FACS plots of CAR19 transduced CB-T cells. Top plots illustrate the gating process and the percentage of the CD45RA+CD62L+CCR7+ cells in the un-manipulated cells. Gating on the live cells then on CD45RA positive population, FACS plots suggest that more than 70% of the cells are naïve. The lower plots demonstrate the expression of CD45RA, CD62L and CCR7 on the cell surface of engineered CB-T cells that were stimulated with beads versus cytokines stimulated CD-T cells

4.7) Discussion:

One of the main cornerstones of an effective adoptive T cell therapy is the ability to generate a high number of antigen-directed therapeutic T cells. Hence, it is crucial to provide the optimal activation condition that will ensure the best proliferation and the maximum transgene expression while T cells are being cultured *ex vivo* (Ritchie et al., 2013). The outcome of this chapter might indicate that using anti-CD3/anti-CD28 beads as an activation condition surplus IL-2 generates a strong signal that may lead to fully differentiated T cells. However, as a result, T cells will be exhausted and they will reach their apoptotic fate fairly soon after *in vivo* infusion. On the other hand, stimulating naïve CB-T cell precursors with IL-7, IL-15 and IL-2 and continuous feeding of the cells with the cytokines cocktail every forty-eight hours, retains the cells in the naïve like phenotype for a long period of time, promising an *in vivo* expansion and persistence. In addition, cells activated with the interleukins show high percentages in transduction efficiency and have proved their ability to recognise their targets and kill them in specific manner.

In light of these findings, the only advantage of using cytokines over anti-CD3/anti-CD28 beads as stimuli would be the ability to preserve the interleukins stimulated naïve T-cells within their naïve phenotype and not shift them toward the terminal phenotype. Consequently, the naïve-like engineered T cells might provide better anti-tumour responses. Although stimulating CB-T cells with the interleukins cocktail provided naive-like engineered cells with high transgene expression and high anti-tumour activity, expanding these cells *ex vivo* was the biggest obstacle.

It has been noticed that the numbers of cells and their survival rate dropped as the experiment continued over twelve days. This might be due to the absence of a strong signal, which is the key to allowing T cells to survive and proliferate; however, CAR19 should provide the transduced T cells with a strong survival signal. Some recent reports have suggested that in order to preserve CAR19 T cells in culture and expand them *ex vivo,* the naïve T cell precursors should be activated with anti-CD3/anti-CD28 Dynalbeads, and engineered cells should be kept in culture in the presence of IL-7 and IL-15 (Cieri et al., 2013).

Another group has proposed that exposing CAR19 T cells to serial antigen stimulation and culturing the cells in the presence of IL-7 and IL-15 will reduce the CD8+ cell death rate and increase the survival rate of CD19-CAR T cells. Moreover, this group highlighted the importance of CD8 positive subsets after demonstrating that CD8+CD45RA+CCR7+ is the only cellular group that expanded after repetitive stimulation with the CAR-antigen,

advocating that it's the only subset that should be preserved to encounter multiple tumour cells *in vivo* (Xu et al., 2014).

Optimising culture conditions of naïve T cell precursors to generate an immense number of effective engineered T cells with a high transduction efficiency and high anti-tumour activity, while preserving their naïve or stem cell memory phenotype is an important goal for the future. Two other different stimulation conditions could be suggested: The first method is to activate the cells with anti-CD3/anti-CD28 beads in the presence of IL-7 and IL-15 and with IL-2 (using the same concentrations that were used in this project). The second process is to follow Xu and colleagues' method by challenging the cells with plate bound-OKT3, then re-stimulating them with irradiated Raji cells and keeping them in a culture in the presence of IL-7 and IL-15. It would also be useful to investigate the importance of the CD8+ population by positive selection or by isolating CD8+ cells prior to activation and transduction in order to demonstrate if these cells in any way benefit the optimisation and expansion of naïve or stem memory engineered T cells.

In conclusion, the transduction efficiency and cellular anti-tumour activity of cultured CB-T cells from five different cord blood units were compared after activating them using either anti-CD3/anti-CD28 beads plus 100 U/ml of IL-2 or cytokines cocktail (including: 5ng/ml IL-7+ 5 ng/ml IL-15 and 100 U/ml IL-2). The data from this chapter might prove that CB-T cells activated with an interleukins cocktail provide favourable data regarding generation of effective and robust, naïve engineered T cells that have high potential to kill their target with high levels of transgene expression. However, these cells could not be cultured and expanded *in vitro* like the beads activated cells, which show a very high survival and expansion rate. Future studies should explore how to overcome this main obstacle and to obtain a robust expansion of the engineered CB-T cells, while preserving their naïve or stem cell memory phenotype.

Chapter 5: Disruption of Endogenous T-Cell Receptor with Site Specific DNA Nucleases.

5.1) Introduction:

An innovative type of gene therapy technique that might overcome some of the obstacles of current gene therapy protocols is genome editing or DNA targeting nuclease technology (Alwin et al., 2005) . Genome editing includes repair technology, a precise gene correction strategy in which the mutant version of the gene is replaced with the normal functional copy at the exact site of the endogenous gene; the consequence being that the gene restores its normal function. By targeting the specific locus of the desired gene, unpredictable genetic effects, such as the random integration of the transgene, and the production of mutagenesis, might be controlled. Recently, nucleases including: meganuclases (MGN), zinc-finger nuclease (ZFN), transcription activator–like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeat (CRISPR)-associated nuclease Cas9 (CRISPRCas9), have become attractive genetic tools for gene targeting and manipulating (Cox et al., 2015).

Nucleases-based genome editing technology enables precise genetic changes by delivering a double strand break (DSB) at specific genomic loci. DSBs subsequently generate lesions within the specific site which may be repaired either by non-homologues end joining (NHEJ), our mechanism of interest, or by homology-direct repair (HDR) depending on the aim of the genomic editing, cell status, and the presence of the repair template.

The NHEJ mechanism to repair the lesion involves the direct joining of the two DSB ends in a process that does not require a repair template. NHEJ-mediated DSB eventually results in the creation of a small insertion or deletion mutations (indels) connecting the break locus. The indels introduced into the coding sequence of a gene can lead to frame-shift mutations that consequently lead to mRNA degradation by non-sense-mediated decay, or result in the production of non-functional truncated proteins. Thus, NHEJ may be used to disrupt gene function, as it may also lead to permanent inactivation by introducing loss-of- function mutations into the gene in targeted cells (Byers, Rouet et al., 1994).

To date, there are two broad categories of nuclease systems that have been developed to enable site-specific genome editing. The first group includes MGNs and their derivatives, ZFNs and TALENs where their mode of DNA recognition is based on protein-DNA interaction. The second group includes CRISPR-Cas9. In this group, specific DNA binding is achieved via guiding Cas9 to the targeted DNA sequence by short RNA guide molecules that base-pair directly to the target DNA and by protein-DNA interactions (Miller et al., 2007, Cade et al., 2012, Boissel et al., 2014, Cong et al., 2013).

Meganucleases (MGNs) are endonucleases with large (>14-bp) recognition sites. The DNA binding domains which are also responsible for cleavage of target sequences (Smith et al., 2006). ZFNs and TALENs are chimeric enzymes consisting of a DNA binding domain fused to the sequence-agnostic FokI nuclease domain (Wolfe et al., 2000, Moscou and Bogdanove, 2009). CRISPR Cas9 technology is based on an enzyme called Cas9 that relies on a guide RNA molecule to home it on its target DNA, then edit the DNA to disrupt genes or insert desired sequences (Mali et al., 2013). Re-engineering meganusleases and ZFNs requires protein engineering, while a complex molecular cloning is essential to re-targeting TALENs (Isalan, 2012, Sun and Zhao, 2013). In contrast, the Cas9 protein is invariant and can be easily re-targeted to new DNA sequences by changing a small portion of the sequence of an accompanying RNA guide in those base-pairs directly with the target sequence. Another potential advantage of Cas9, in addition to its ease of accessibility and its low cost, is its ability to introduce multiple DSBs in the same cell via expression of distinct guide RNAs (Yin et al., 2014). All four types of engineering nucleases have been shown to accomplish efficient genome editing in a wide range of model organisms and mammalian cells, which make them a valuable reagent in the field of targeted gene therapy. Efforts are underway in both industry and academia to develop these tools as therapeutics reagents (Cox et al., 2015).

The main aim of this chapter is to assess the efficiency of different types of the gene-specific nucleases, meganucleases, TALENs and CRISPR/Cas9, at disrupting the TCR locus within the genome. Editing the genetic sequence of the endogenous T cell receptors may lead to the production of T cells devoid of allo-reactive TCRs by targeting the constant region of the TCR α and/or β chain (Provasi et al., 2012). Introduction of antigen-specific receptors or chimeric antigen receptors into such TCR-disrupted cells could yield populations of "universal T cells" that are suitable for infusion into multiple HLA disparate recipients without the risk of GVHD (Torikai et al., 2012).

To test the feasibility of generating such a disrupted T cell, we started by testing the effectiveness of meganucleases in disrupting the genetic sequence of the constant region of a T cell receptor; followed by evaluating TALENs as an alternative platform for knocking down TCR expression with less toxicity. Then CRISPR/Cas9 nuclease, which is designed to target the constant region of the TCR alpha chain, was also involved in the experimental process to investigate its ability to disrupt its specific site within the genome. Finally, a comparison between TALENs and CRISPR/Cas9 from the prospective of genome disruption and cell toxicity was also evaluated.

5.2) Meganucleases:

5.2.1) Expression of Meganucleases by Amaxa Nucleofection of coding DNA into Jurkat cells:

Three different specific meganucleases expression plasmids that target specific sites within the genome were generated by Cellectis (France).

Two are mutated meganucleases that are targeting a conserved sequence within the genes of the beta chains constant region of T cell receptor; β1 and β2 (called TRBC01 and TRBC02). The third construct is targeting a housekeeping gene called calpain, small subunit 1 (CAPNS1) and is used as a control. Two µg of DNA of meganuclease expression plasmids, TRBC01, TRBC02 or CAPNS1, were introduced to the nucleus of Jurkat cells by using the amaxa nucleofection system according to the manufacturer's instructions. Transfected cells were kept in a culture for seven days before being harvested. Western blotting was used to confirm the expression of meganuclease protein from the plasmid using anti-CreI antibodies. As Figure 38 shows, there was a highly specific band containing the correct size present in the transfected population, but not in the non-transfected cells. As a control, loading expression of beta-tubulin proteins was also verified. The molecular weight of all three constructs is 39 kilodalton (kDa), but the beta-tubulin molecular weight is 50 kDa.

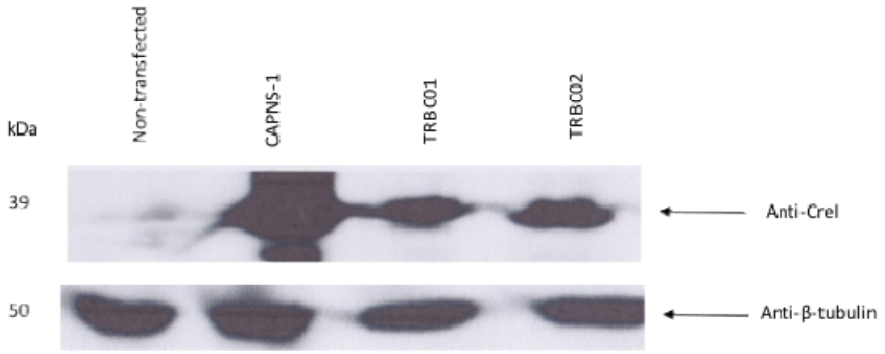


Figure 38: Western blotting using Anti-CreI on meganuclease transfected Jurkat cells. Non-nucleofected cells were used as a control. Beta-tubulin was used as a loading control.

5.2.2) Detection of Meganuclease's molecular signature within transfected Jurkat cells:

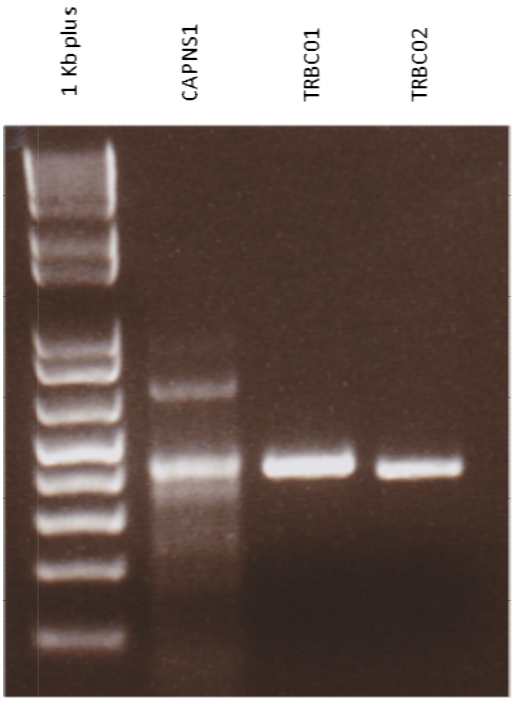
To endorse the molecular signature of meganuclease within the genome of the transfected cells, a surveyor mutation detection assay was used. This enzyme mismatch cleavage system uses plant single-strand specific endonucleases of the S1 nuclease family named CELII- or Survoyer nuclease - that cleave heteroduplex DNA at mismatches and extrahelical loops formed by single or multiple nucleotides. Surveyor nuclease technology involved the following steps: DNA samples were extracted from the transfected cells and the

meganuclease specific-site (target DNA) was amplified by PCR. The PCR product was then used to form heteroduplex by being exposed to a denaturation and hybridization process. Afterwards, the annealed DNA was treated with Surveyor Nuclease S to detect the presence of the miss-matched DNA binding feature of any NHEJ. At the end, digested DNA product was analyzed using conventional gel electrophoresis platform to detect any TCR knockout effect (Figure 39 A) (Vouillot et al., 2015).

Another mechanism to evaluate mutation in the genome after the delivery of the engineered nuclease is by traditional Sanger DNA sequencing after cloning PCR product into a topo-cloning vector. The DNA sequences were analysed by vector NTI software where they were aligned with the 20 base pair target sequence (the specific site of meganuclease) and the original sequence (obtained from NCBI).

Although the western blot has shown a well expressed meganuclease as a protein, the molecular signature of genome disruption could not be confirmed as both DNA sequencing and nuclease assay failed to detect genomic breaks (Figure 39 B).

A) Surveyor mutation detection assay:



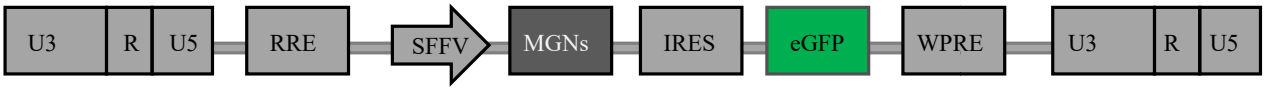
B) DNA Sequencing:

Figure 39: The molecular signature of meganuclease transfected jurkat cells show a failure of meganuclease to cleave their target sequence. A) Amount of targeted gene disruption measured by a mismatch-selective endonucleases assay on DNA, amplified from transfected Jurkat cells. Gel's figure demonstrated the expected size of the intact amplicon, which is 400 bp in all transfected samples, instead of showing the nuclease cleavage product of heteroduplexes carrying the mismatch, which should be two bands of 254 and 146 in size. PCR un-specific bands were detected in the control lane. B) DNA sequencing of the same MGN-transduced Jurkat cells has confirmed nuclease assay results. Alignment of MGN targeted sequence, original sequence (downloaded from NCBI) and DNA sequence of transfected cells (test sequence). No disruption can be detected within the targeted sequence.

5.2.3) Cloning Meganuclease (MGNs) constructs into a 2nd generation Lentivirus vector:

These three meganucleases were cloned into a 2nd generation self-inactivating lentivirus backbone that expresses the transgene under the control of an SFFV promoter and includes an IRES-eGFP at a multiple cloning site (pLNT/SIEW).They were also cloned in the same plasmid, but without the eGFP (Figure 40 A,B). The generated plasmids were used to produce a lentivirus vector by transient transfection of HEK293Tcells in combination of envelope VSV-G plasmids (pMDG) and gag-pol packaging plasmids. The vector expressing MGN-CAPNS1 with an SFFV promoter was tittered on a Jurkat cell line and FACS analysis illustrated a titre of 0.31 x 10^7 IU per ml. For the two other vectors that express MGN-TRBC01 and MGN-TRBC02, a TCR-negative Jurkat cell line was used for virus titration. A typical titer of 0.1x10^7 IU per ml was produced for LNT-SFFV-TRBC01/TRBC02 and 1.1x10^8 IU per ml was produced for LNT-SFFV-TRBC01-IRES-eGFP as well as for Lnt-SFFV-TRBC02-IRES-eGFP.

A)



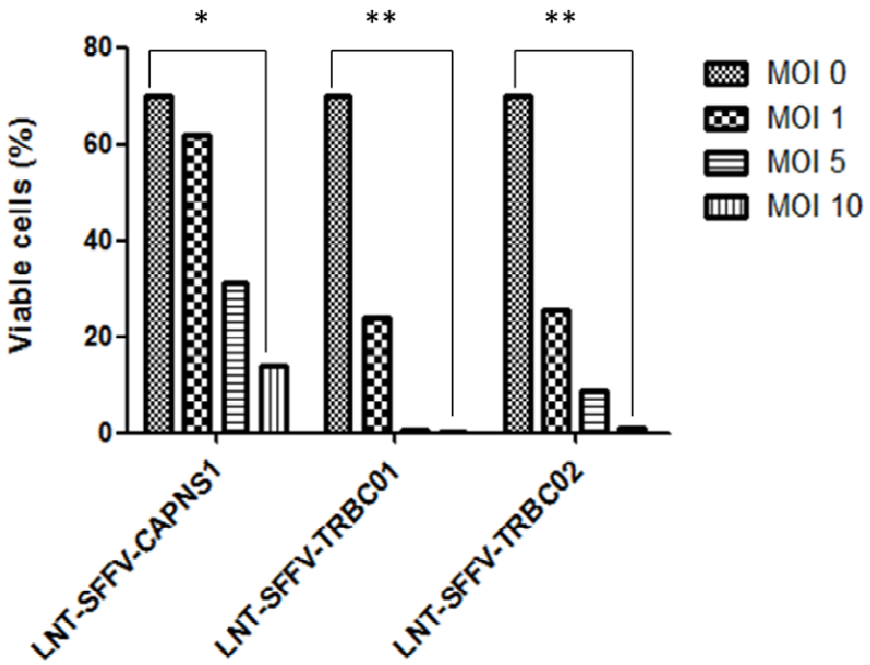
B)

Figure 40: Schematic maps of the cloned meganuclease into different lentivirus vector backbones. A) Schematic map represents successful cloning of different MGNs into lentivirus vector backbone that includes IRES-eGFP. B) Representative map of meganucleases cloned into lentivirus vector backbone without IRES-eGFP.

5.2.4) CD3 Expression levels and cell viability after transduction of Jurkat cells with Lnt-SFFV-MGN:

Jurkat cells were transduced with three different rates of infection (MOI's) (0, 1, 5, 10) of the Lnt-SFFV-MGN virus, and cultured in complete RPMI. Seven days following transduction, the MGNs knock-out effect of endogenous TCR within the transduced cells was evaluated by measuring the reduction in the percentage of CD3 expression by FACS analysis. Flow cytometric analysis demonstrated that the viability of MGN-transduced cells was reduced dramatically compared to the non-transduced cells (Figure 41 A), and there was no sign of CD3 expression reduction that could be detected within MGN-transduced cells (Figure 41 B). The previous results may suggest that MGN is toxic to the cells and this might explain the lack of activity observed. The results suggest that an alternative platform may be required.

A) Viability of Jurkat cells transduced with several constructs of MGNs



B) CD3 level expression of Jurkat cells transduced with various constructs of MGNs

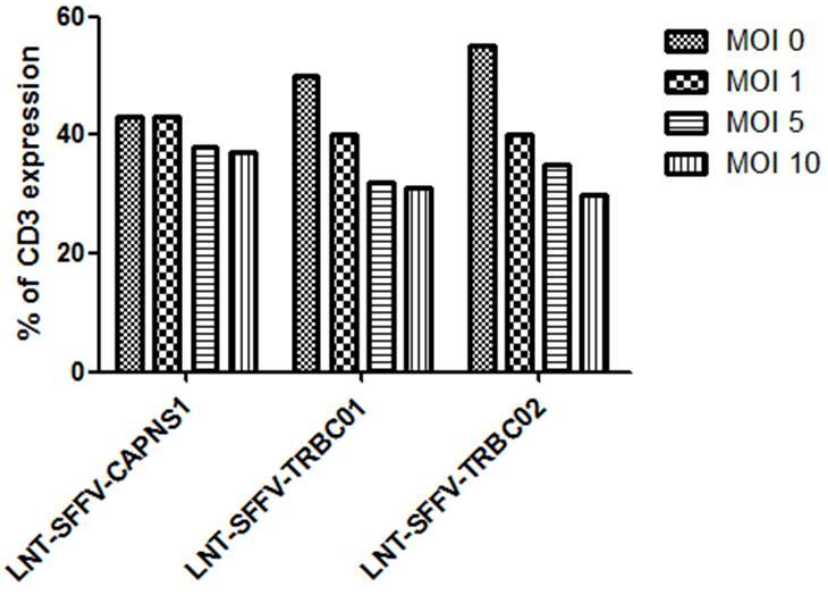


Figure 41: Unsuccessful attempts using MGNs to reduce CD3 expression. A) Bar Chart demonstrating the percentage of CD3 expression within transduced and non-transduced Jurkat cells. There is no significant decrease in CD3 expression between the transduced and non-transduced cell population. B) Bar charts demonstrating the percentage of viable Jurkat cells following transduction with the LNT-MCS-MGN virus at different MOIs. A significant drop in the viability of the cells after transduction can be concluded from the graph. (n=1)

5.3) TRAC-TALENS

To disrupt the expression of the endogenous TCR, a pair of engineered TALENs targeting a sequence found within the constant-region genes of the alpha chain of TCR was designed and obtained from Cellectis (Paris, France) and Fehse labs in Hamburg (Germany).

Cellectis provided TRAC TALENs as expression plasmids and they were cloned into a second generation, SIN, lentiviral vector backbone by Gordon Weng-Kitn (Pule Laboratory, Cancer Institute, UCL). Each pair of TALENs was connected to a tag molecule, which helps identify the expression level of TALENs within the transfected cells. V5 is attached to the right pair of TRAC TALENs, whereas, MYC is ligated to the left pair of TRAC TALENs (Figure 42).

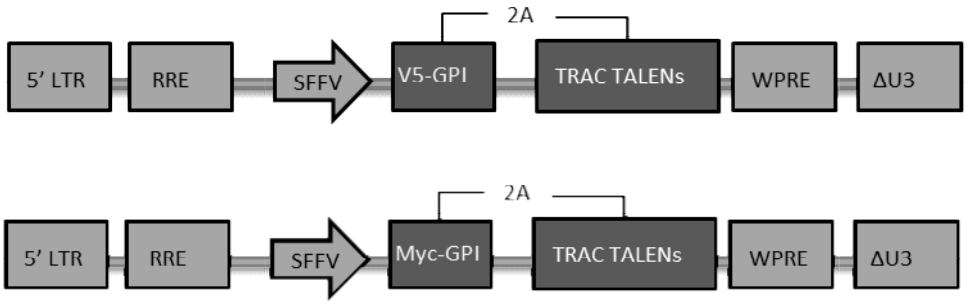


Figure 42: Schematic figure of TALENs-TRAC constructs ligated to 2nd generation, SIN, lentiviral vector backbone. Each pair of TALENs is connected to tag molecule; which helps identify the expression level of TALENs within the transfected cells.V5 is attached to the right pair of TALENs-TRAC (top), whereas, MYC is ligated to the left pair of TALENs-TRAC (bottom). V5= is synthetic epitope tag derived from a small epitope (Pk) present on the P and V proteins of the paramyxovirus of simian virus (SV5). MYC= is synthetic peptide (EQKLISEEDL), derived from the C-terminus of the human c-mycprotein . Amaxanucleofection system was used to deliver the Tagged TARC TALENs on to three various cell types: Jurkat cells, primary cells and cord blood T cells. 2A= self-cleaving peptide - 18-22 amino acid sequence- that is readily cleaved as translation occurs, separating the two proteins. WPRE= Woodchuck hepatitis virus post transcriptional regulatory element added at the 3' non-coding region of a vector to increase vector titre and gene expression.

5.3.1) The Effect of TRAC TALENs plasmids on Jurkat cells:

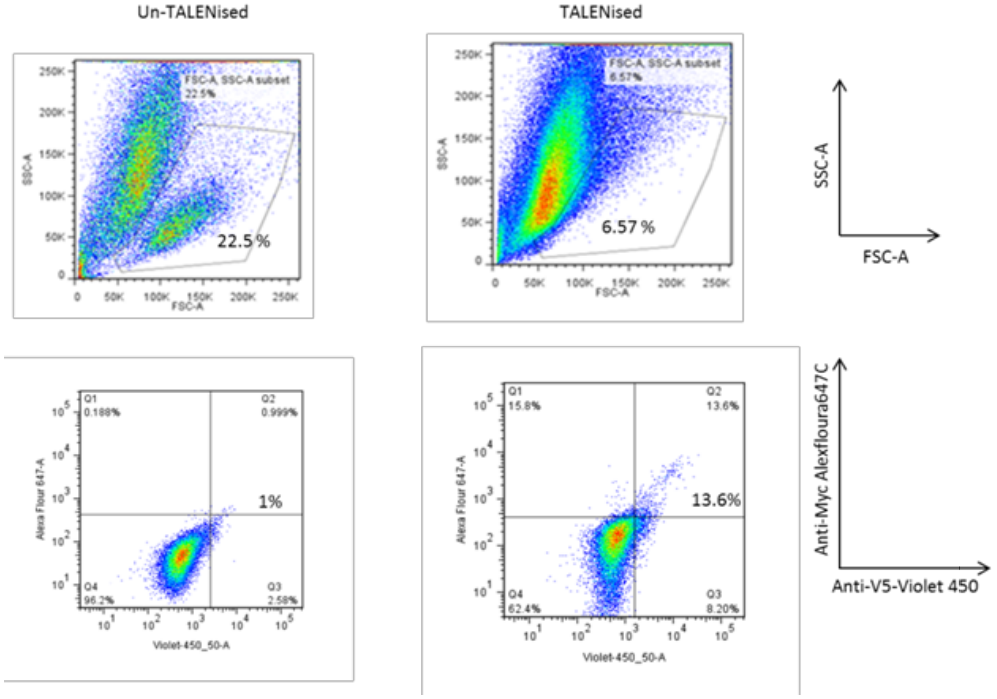
Although viral vectors, particularly, lentiviral vectors, are very attractive tools for the transfer of nucleic acids to a variety of mammalian cells, the delivery of TALENs is prevented by repetitive elements of the individual TAL effector nuclease. To avoid this problem, TRAC TALENs were tested as plasmid rather than viral vector (Mock et al., 2014). $2x10^6$Jurkat cells were nucleofected or TALENised with a 5µg of TRAC TALENs (each pair) as DNA construct using x-001 program on an amaxa nucleofection instrument and nucleofection solution of Lonza kit V according to the manufacturer's instructions. As a control, cells were nucleofected with each pair alone. Forty-eight hours after nucleofection, the TALENs-tag expression was analysed by staining the cells with 0.5µl of Anti-V5-Daylight 405 and 0.5µl of Anti-Myc-AlexaFloura 647 mAb. The control cells were stained with Anti-V5-Daylight 405 or Anti-Myc-AlexaFloura 647 mAb alone. Seven days after TALENisaition, the cells were stained with anti-TCR α/β-FITC and/or anti-CD3-APC to gain knowledge about the percentage of the TCR negative population after nucloefection.

This experiment was conducted three times to estimate the efficiency of TRAC TALENs in editing TCR gene expression and the monitor cells' viability before applying the same conditions to adult or naïve T cells.

The flow cytometric analysis suggested that the tag expression percentage is aligned with the percentage of the TCR negative population percentage. The first experiment showed that 25% of the cells had expressed TALENs and there was a 27% reduction in the TCR positive population. The FACS plots from the second experiment showed that 11.2% of the nucleofeceted cells had expressed tag-TRAC TALENs, and 19% of the TCR negative population was detected. The third experiment showed that the TCR negative population had increased by 16 % in Jurkat TALENised, compared to the non-TALENised population; and 13 % of the cells exposed to TRAC TALENs had expressed tag V5 and tag Myc on their surfaces (Figure 43 A,B) (Figure 44). TALENised cells' viability was reduced by 4.5 fold on day two (Figure 45).

This promising data led to the next step of testing the effect of TRAC TALENs on the adult and cord blood T cells. The next section will discuss the results of knocking-out the expression of TCR from primary and cord blood T cells, as well as some optimisation to the protocol to provide better outcomes.

A) Myc and V5 tag Expression Levels in both TALENised and Un-TALENised JurkatCells:



B) Percentage of TCR negative population levels in both TALENised and Un-TALENised Jurkat cells

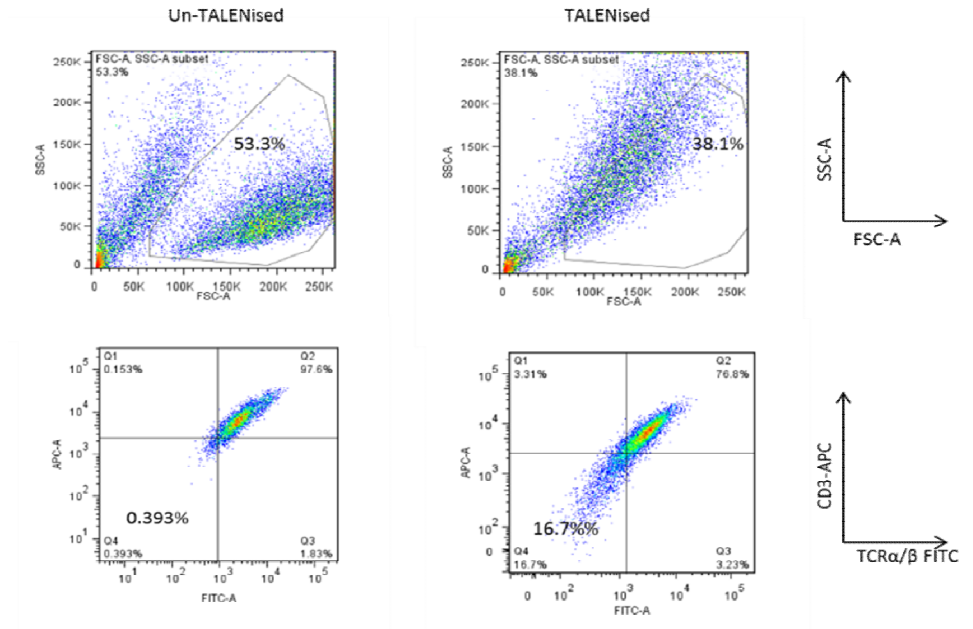


Figure 43: Representative FACS plots of Jurkat cells TALENised with TRAC-TALENs as a DNA construct. A) Gating on viable cells; FACS plots represent the levels of TALENs expression by showing the percentage of tag expression in both TALENised and control group at Day 2 of the experiment. B) At Day 7 after nucleofection and gating on the viable cells, FACS plots represent the percentage of TCR negative population that produced after TALENisation of Jurkat cells in comparison with non-TALENised population.

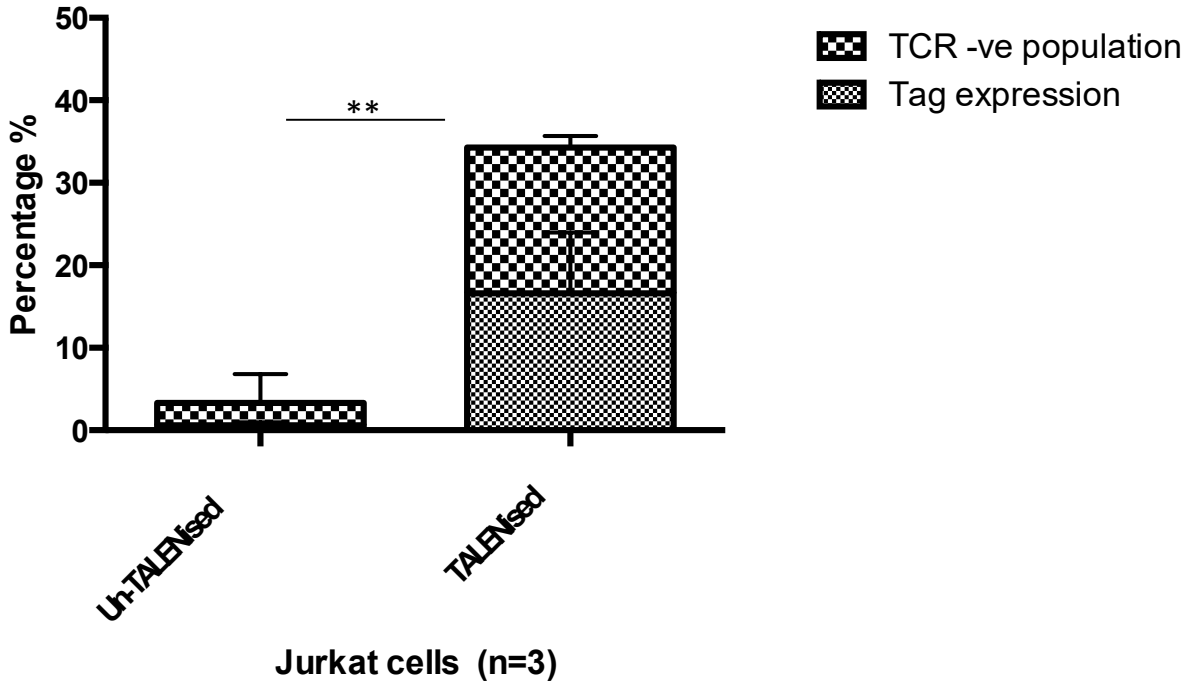


Figure 44: Bar chart exemplifies the correlation between TRAC TALENs tag expression levels and the increase in the percentage of TCR negative population after TALENising Jurkat cells with TRAC TALENs as a DNA via amaxa nucleofection system. The bar chart shows, an approximately similar percentage between tag expression levels and the percentage of TCR negative group in TALENised population and in the control group as well. P value was calculated by GraphPad software ($P<0.01$).

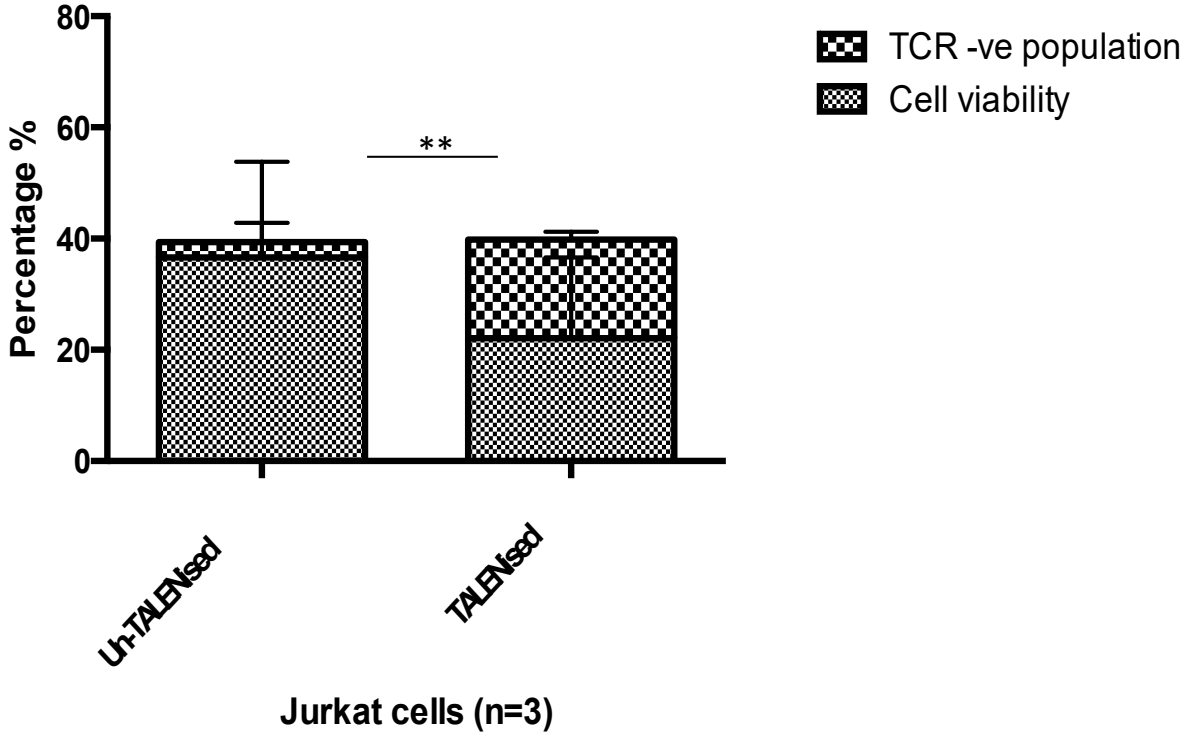


Figure 45: The effect of TRAC TALENs as a DNA construct on the viability of Jurkat cells and on the percentage of TCR expression. Bar charts represent the consequence of TRAC TALENs (as DNA) on the cell viability and the expression of TCR on Jurkat cells in both treated and non-treated populations. There is a significant increase in the percentage of TCR negative population within the TALENised

population in comparison to the un-TALENised cells ($P<0.01$). There is no significant difference between TALENised and un-TALENised cells in cell viability.

The previous preliminary data had suggested that TRAC TALENs are effective albeit with reduced cell viability and the TCR knock out effect. Thus, the same experiment was conducted on both adult and cord T cells before considering a scale-up process.

5.3.2) Comparison of the Effect of TRAC TALEN on PBMCs and CB-T Cells:

Mononuclear cells were collected from peripheral blood or cord blood units after separation of blood layers using Ficoll. For small scale experiments, $2x10^6$ cells were activated with anti-CD3/CD28 Dynal beads at a 1:1 ratio and incubated for 48 hours before TALENisation in a 24 well culture plate. 5µg of TRAC TALENs DNA (each pair) was introduced to naïve or adult T cells via an amaxa nucleofection system using the Lonza human T cell kit according to manufacturer's instructions. Two days later, the tag expression of TRAC TALENs was analysed by flow cytometry after staining the cells with anti-Myc and anti-V5 antibodies. For cord blood T cells, FACS blot results suggested that 22.3% of the nucleofected cells had expressed TALENs on their surface with 2.53% of the background detected on the non-nucleofected population. Down regulation of TCR expression was detected seven days after TALENisation; the cells were stained for TCR using anti-TCR α/β-FITC and anti-CD3 APC and analysed by flow cytometry. Interestingly, the percentage of the TCR negative population was correlated with the percentage of the cells that were expressed TRAC TALENs. These results showed that the TCR-negative population had increased approximately by three-folds within nuclofected cells, compared to non-treated cells. Whereas, FACS plots revealed that 33% of TCR-negative cells were detected by flow cytometry within treated cells with an 11.8% background detected within the non-treated cells (net of TCR negative population=21.20) (Figure 46). Regarding the cell survival percentage, the FACS plots showed that cell viability of nuclofected naïve cells was reduced by only 10% compared to the non-nuclofected cells at Day 7, since beads activation and Day 5 from nucleofection.

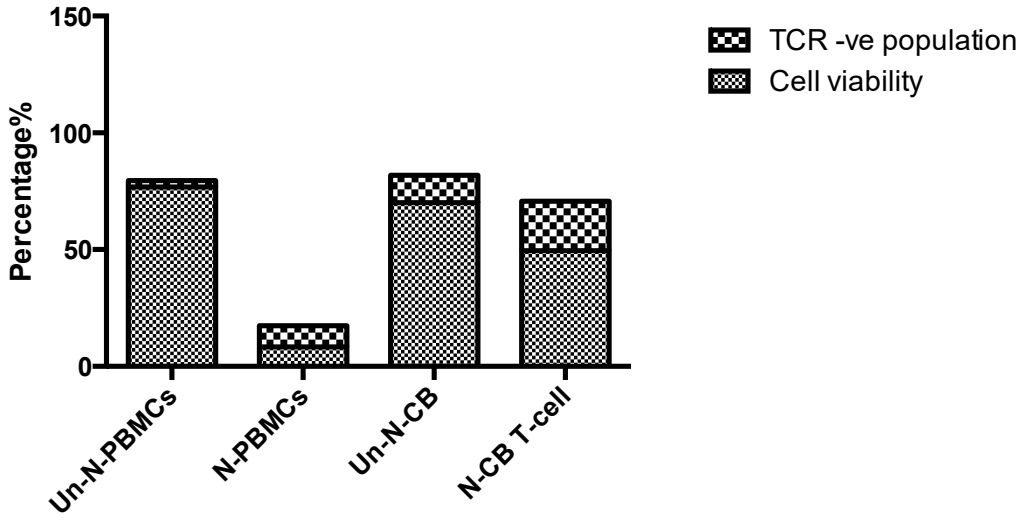


Figure 46 : The differences of cell viability and TCR TALENs KO efficiency within nucleofected -CB-T cells and nucleofected PBMCs. Grouped graph demonstrates the differences of two factors: cell viability and TCR TALENs KO efficiency within nuclofected CB-T cells (N-CB-T cell) and nucleofected PBMCs (N-PBMCs).Un-nucleofected cells were used as a control (Un-N-cells). Graph shows a remarkable difference in cell viability between the two groups. It appears from the blot that TRAC TALENs has approximately the same effect on both N-CB-T cell and N-PBMCs since both of them showed a similar TCR negative population percentage. The statistics applied to this data include unpaired t-test and Mann-Whitney test. T test result ($p > 0.99$) suggested there are no significant differences between the two groups.

This data might have suggested that naïve cells are more prone to transfection with a better result of cell viability compared to adult T cells. Also, it might prove that TRAC TALENs has the ability to cause a disruption in TCR alpha constant region genes leading to the down regulation of its expression. This result might be promising for showing the effect of TALENS on TCR expression, but not for cell viability and DNA toxicity to the cells. For that reason, most of the experiment has shifted to use mRNA instead of DNA in transfection process.

In the next section, the feasibility of delivering TRAC as mRNA and its effect on cell viability and TCR expression will be discussed.

5.3.3) Delivering TALENs as mRNA:

Tag-TRAC-TALENs mRNA was tested on Jurkat cells and then on PBMCs using nucleofection. 10 µg of TRAC TALENs per pair as mRNA were delivered to $5x10^6$ Jurkat cells using the BTX electroporation system (voltages as in the methods chapter). The cells were incubated in warm, complete RPMI for seven days before FACSing for observation of TCR expression. The TALENised cells were stained with anti-TCR α/β and anti-CD3 to evaluate the efficiency of TRAC TALENs as mRNA in down-regulating the TCR expression and in maintaining the viability of the cells within a reasonable percentage. The results proposed a remarkable increase in the TCR-negative population after electropotration with

mRNA compared to the non-electroporated population with reasonable viability percentages. The FACS plots of mRNA electroporated Jurkat cells showed a 50.8% shift from TCR+CD3+ quadrant toward TCR-ve CD3-ve quadrant compared to the non-electroporated cells with a 13.4% background. This makes the pure net of the TCR-negative population around 37.4%. Regarding cell viability and based on gating the viable cells, the plots demonstrated that TRAC TALENs mRNA are not toxic to the cells since there was only a 1-fold reduction in viable cell percentages in electroporated cells compared to the non-electroporated group (Figure 47).

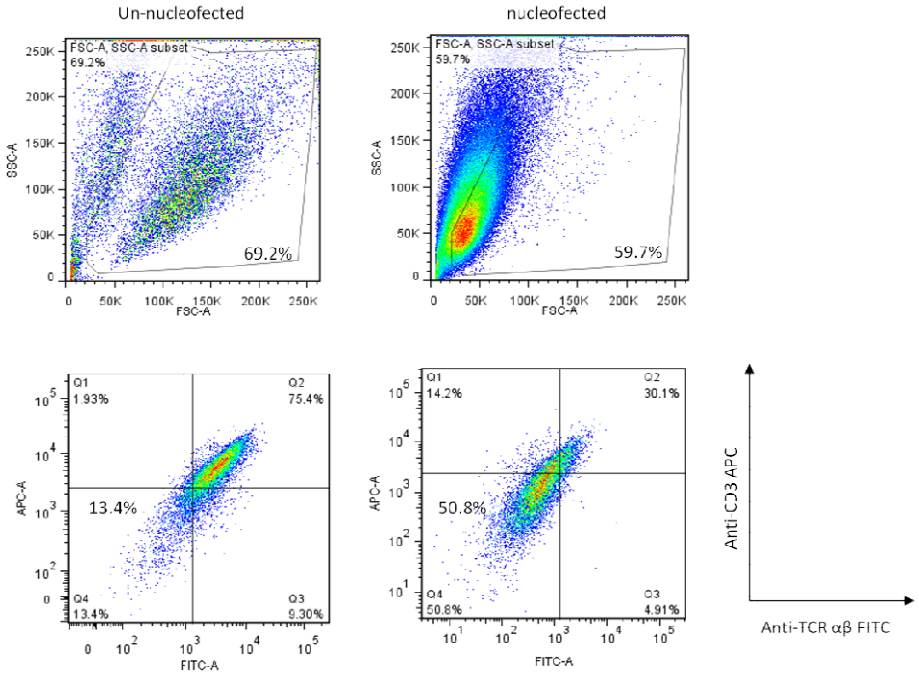


Figure 47: FACS plots of mRNA-TRAC-TALENs electroporated Jurkat cells. The representative FACS plots are illustrating the status of Jurkat cells with and without delivering TRAC TALENs as an mRNA. The upper plots represent the viability of the treated and the non-treated cells, while the lower plots show the TCR knock out efficiency in electroporated cells vs un-electroporated cells. Data from upper plots suggest that there is no significant difference in cell viability between the treated and the control group. Gating on the viable cells, lower plots propose that there is an increasing TCR negative population by 37.4% in electroporated cells compared to un-electroporated cells.

The adult T cells were first activated with anti-CD3/CD28 beads then exposed to TRAC TALENs mRNA at a volume of 10 µg per pair and incubated for seven days. The cell viability and down-regulation of cell surface expression of TCR α/β and CD3 was determined by FACS analysis after staining them with anti-TCR α/β and anti-CD3 mAbs. While gating on viable cells, the outcomes of this test proposed that within the 49% of TALENised viable

cells, the TCR/CD3 negative population of TALENised PBMCs had increased three-fold in comparison to the non-TALENised PBMCs. The data from the adult T cells showed that there was no significant difference in cell viability between TALENised and non-TALENised cells (Figure 48).

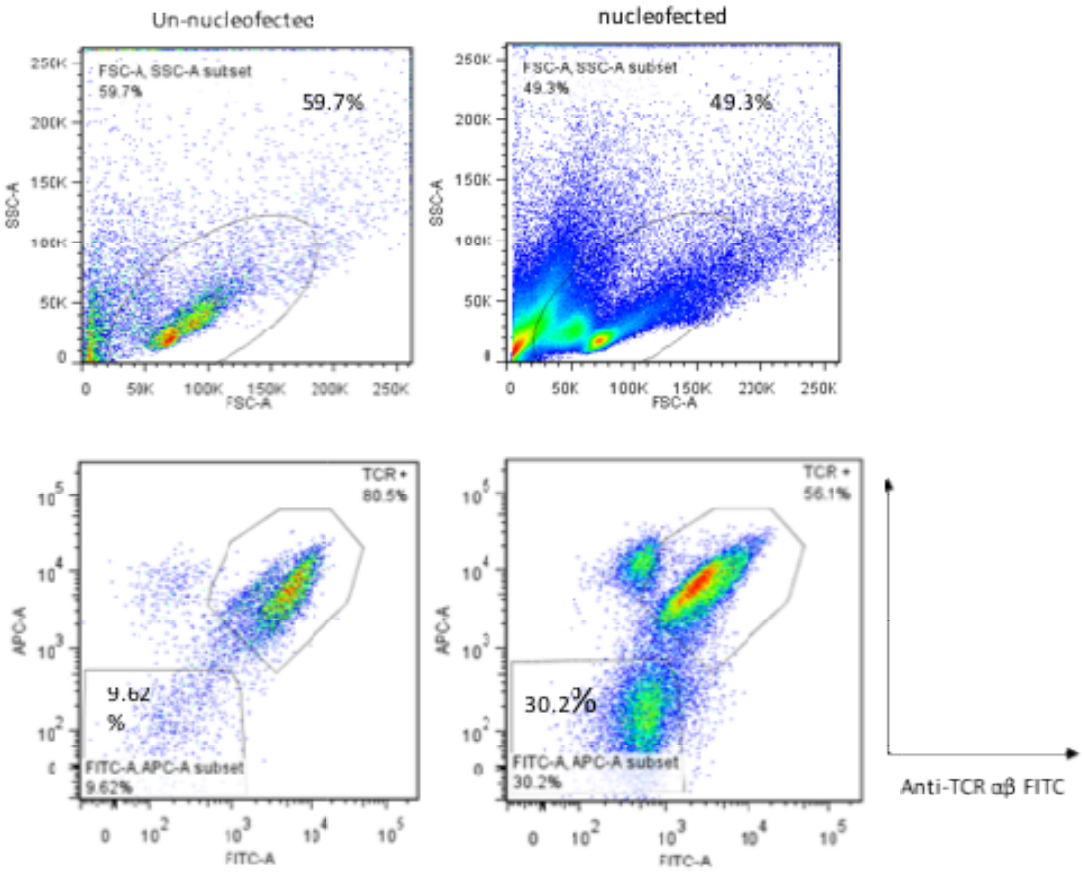


Figure 48: FACS plots of mRNA-TRAC-TALENs electroporated PBMCs cells. The representative plots are illustrating the status of activated PBMCs cells before and after delivering TRAC TALENs as an mRNA. The upper plots represent the viability of treated and non-treated cells, while the lower plots show the TCR KO efficiency in nucleofected cells vs. un-nucleofected cells. Data from upper plots suggest that there is no significant difference in cell viability between the treated and the control group. Gating on the viable cells, lower plots propose that there is an increasing TCR negative population by 21.2% in nucleofected cells compared to un-nucleofected cells because of the shift of the cell populations from the double positive quadrant to the double negative quadrant.

5.3.4) Comparison of the TRAC TALENs Effect as mRNA Vs DNA on PBMCs:

In order to compare the effect of TRAC TALENs as DNA or mRNA on TCR knock down and cell viability, the same experiment using the same donor's cells was conducted using DNA TRAC TALENs parallel to mRNA TRAC TALENs (mentioned above)(Figure 48). The data showed a three-fold decline in cell viability and an 18% escalation in the percentage of TCR/CD3neg nucloefected PBMCs compared to the control population (Figure 49.B). Furthermore, nucleofected cells were stained with Anti-MYC and anti-V5 to measure the expression level of TALENs within the cells; the FACS plots suggested that 17% of the cells

were able to express TALENs (Figure 49.A), which was equivalent to the percent of the TCR-neg population.

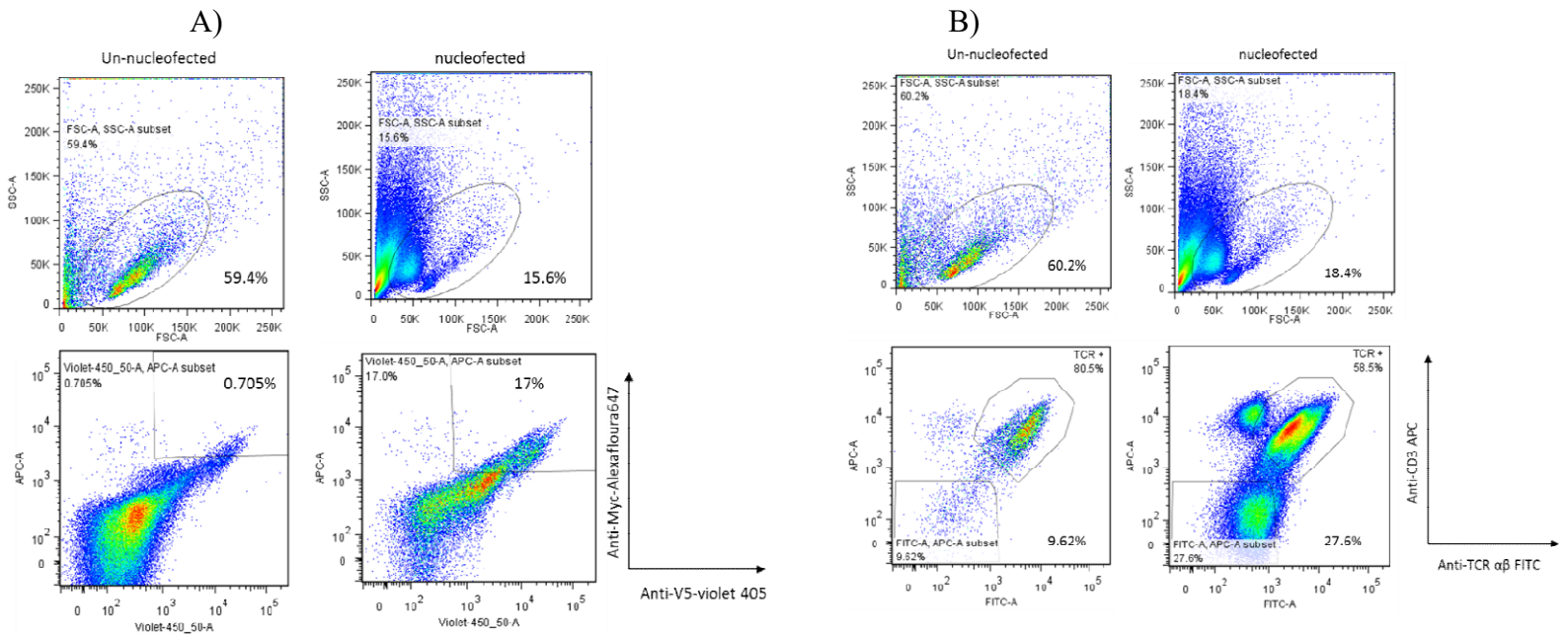


Figure 49: Delivering TRAC TALENs as DNA in 2nd donor PBMCs. A) TWO days after nucleofection with TRAC TALENs, cells were stained with 0.5 µl anti-Myc and 0.5 µl anti-V5. Upper plots demonstrate the gating on the viable cells, while lower plots show the percentage of TRAC TALENs expression by TALENised cells vs. non-TALENised cells. Results show that 17% of the treated cells are expressing both V5 and Myc; which are the markers for TRAC TALENs expression, on their surface with 0.7% of background in the non-treated cells. B) At Day 7 after nucleofection, nucleofected and un-nucleofected cells were collected and stained for TCR and CD3 expression. Upper plots show the gating strategy of the viable cells; lower plots show the percentage of the viable cells that are expressing TCR α/βand CD3. Cell viability has decreased from 60% of un-TALENised cells to reach 18.4% of the TALENised population. For the evaluation of the efficiency of TRAC KO, TCRneg population was monitored. As the gates on the FACS plots in the lower part illustrate, there was an increase in the TCRnegative population by 27.6 % with 9.62% background in the non-TALENised group.

In conclusion, this data might suggest that delivering TALENs as a DNA construct could be toxic to the cells leading to enormous cell loss, but it has no effect on the TALENs and their ability to disrupt the specific site within the genome. In addition, it might demonstrate that nucleases in the form of mRNA could be a better alternative for a more stable expression and higher cell viability (Figure 50).

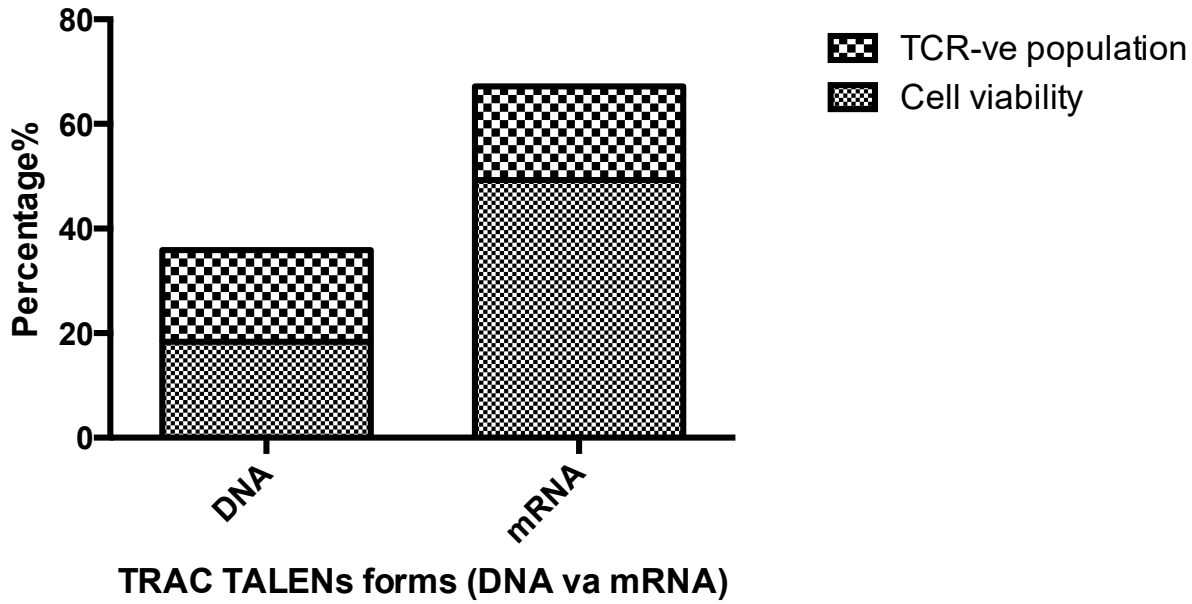


Figure 50: Comparing the effect of delivering TALENs as mRNA or DNA on cells' viability and the disruption of alpha constant region within the cells' genome. This graph reveals that cells TALENised with mRNA show better viability than cells TALENised with DNA. No significant difference could be spotted in the actual effect of TRAC TALENs on disruption of the gene either TALENs deliver as an mRNA or DNA. U npaired t test was conducted on this data and showed non-significance differences within the data ($p>0.99$)

Nowadays the focus of most researchers and scientists has shifted towards a new designer nuclease named CRISPR Cas9 in relation to targeted genome editing platforms. Due to its ease of use and its ability to target multiple specific sequences at the same time, it has been seen as an attractive tool in the field of genome engineering (Cong et al., 2013). In the next section, the potential of CRISPR Cas9 in targeting a specific sequence within the alpha and the beta constant region will be investigated.

5.4) TRBC and TRAC CRISPR/Cas9:

CRISPR/Cas9 nuclease constructs targeting the constant regions of TCR either α or β were generated for TRAC, TCAGGGTTCTGGATATCTGT and TRBC GCAAACACAGCGACCTCGGGT (GeneCopoeia US)(Figure 51).

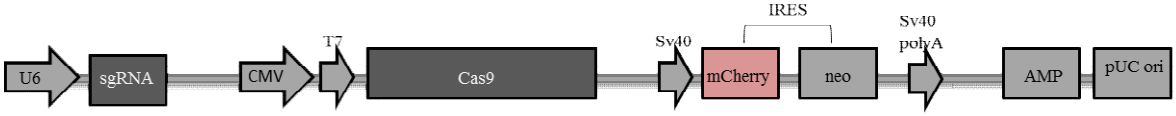


Figure 51: Illustration figure of CRISPR/Cas9 construct targeting sequence within the alpha constant region of TCR.

5.4.1) Delivering CRISPR/Cas9 into Jurkat cells as a DNA construct:

During a seven day experiment, the efficiency of CRISPR/Cas9 in disrupting their target sites within the Jurkat cells genome was determined. On Day one, $2x10^6$ cells were exposed to 5 µg of TRAC and/or 5 µg of TRBC CRISPR/Cas9 as DNA construct using programme x-001 on the amaxa nucleofector machine. Two days later, the CRISPR/Cas9 expression was analysed by detecting the expression levels of mCherry on flow cytometry. The FACS plots manifested that the levels of the mCherry expression within the nucleofected cells demonstrated that up to 21% of the nucleofected cell population was able to express CRISPR/Cas9, in a comparison with the non-nucleofected cells (Figure 52).The cells were stained with anti-CD3 conjugated to APC flourochrome after seven days of exposing them to CRISPR/Cas9 in order to examine the ability of CRISPRs in recognising and breaking up their specific site within the genome. Notably, the cells transfected with TRAC CRISPR/Cas9 or TRBC CRISPR/Cas9 alone showed a higher percentage of the CD3neg population (21.5% or 20.5%), in comparison to the cells that were transfected with both CRISPRs/Cas9 TRBC and TRAC (CD3neg=13.2%). As a control, some cells were exposed to the same nucloefection conditions, but without adding any DNA constructs. A background of 5% was detected within this control group and also among the non-treated group. The cells' viability was not as promising as the knock out efficiency data. The cell viability dropped dramatically within the cells exposed to transfection (either with or without DNA) compared to the control group (Figure 53).

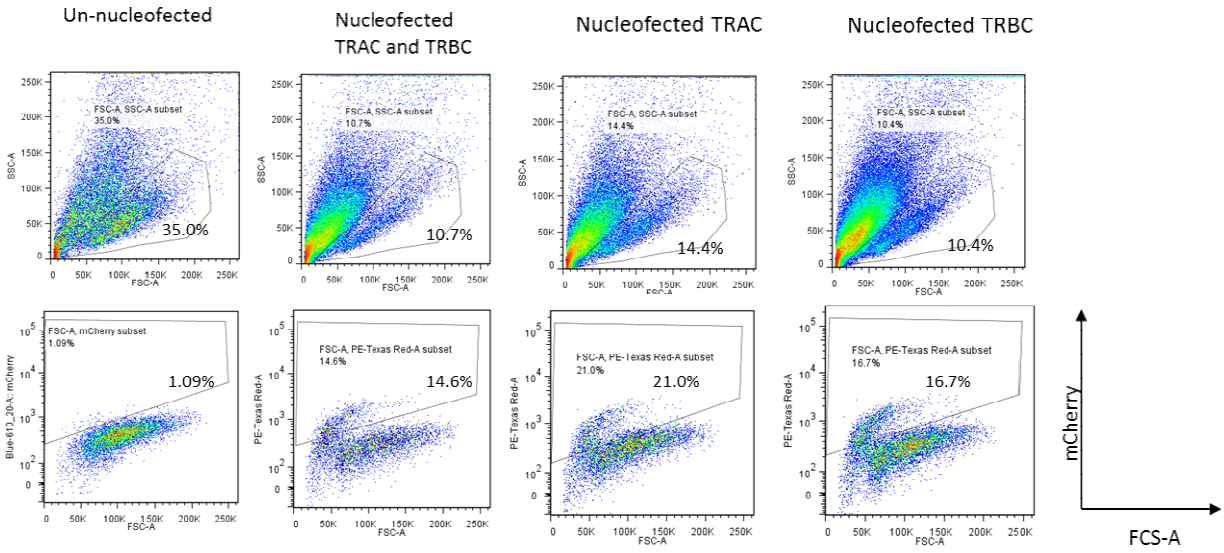


Figure 52: Expression of TRAC and TRBC CRISPR /Cas 9 construct within Jurkat cells. Gating of the live cells population (upper plots); lower plots illustrate the percentage of Jurkat cells in expressing mCherry marker within treated and non-treated cells. Background was detected to be around 1.09% in control group, while for nucleofected cells, mcherry level was elevated up to 14.6% within cells exposed to both TRAC and TRBC CRISPR/Cas9, up to 21% in TRAC CRISPR/Cas9 nucleofected cells and within cells nucleofected with TRBC CRISPR/Cas9 alone, mCherry level was 16.7%. As upper plots exemplify, cell viability was ranged between 10.7%-14.4% for nucleofected group, whereas for control group, cell viability was 35%.

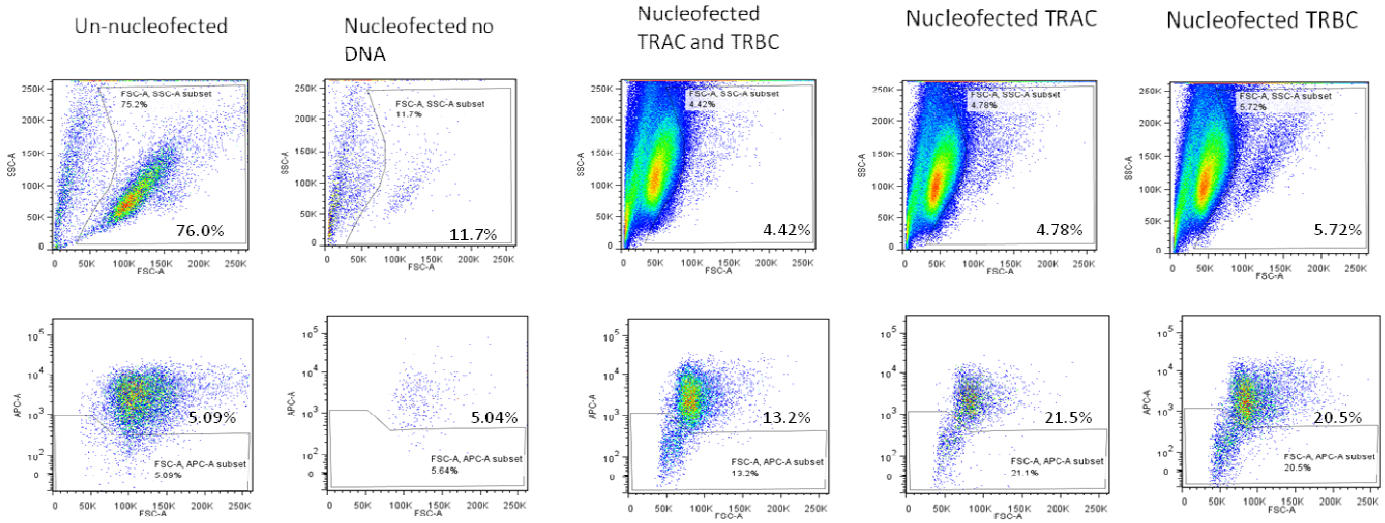


Figure 53: The effect of CRISPR/Cas9 TRAC and/or TRBC (as a DNA construct) on the genome of Jurkat cells Upper FACS plots demonstrate the gating on the viable cells and they show the dramatic reduction in cell viability percentage. Lower plots show the shift in the CD3 expression level from positive to negative phenotype within the treated group compared to the non-treated group.

Due to the low percentages of cell viability, it was not feasible to keep the cell pellet for any further functional experiments. It has been suggested to produce mRNA from CRISPR/Cas9 DNA constructs, and then test their effect on the cell's viability in addition to TCR knock out efficiency.

5.4.2) Delivering CRISPR/Cas9 as mRNA:

A T7 promoter sequence was included within the CRISPR plasmid. According to the CRISPR/Cas9 map, the T7 promoter sequence was found just behind Cas9, but not the guide RNA (sgRNA). For this reason, the T7 promoter sequence was added to the previous construct ahead of the sgRNA sequence by PCR cloning. In the end, 1.5 µg of mRNA of the sgRNA was produced after following the MEGAscript kit protocol for mRNA production. Following Zhang's laboratory protocol (See reference) in delivering CRISPR/Cas9 as mRNA for the purpose of gene disruption by NHEJ, 50 ng/µl of sgRNA of CRISPR/Cas9 TRAC mRNA was mixed with 100 ng/µl of Cas9 mRNA (TriLink, USA) in the cuvette of electroporation. Afterwards, $2x10^6$Jurkat cells were added to the nucleases and exposed to an electroporation condition using the BTX system. Seven days later, the cells were stained with anti CD3-APC mAb to detect the percentages of the CD3neg population. The flow cytometry data was analysed by FlowJo and there was no noticeable difference in cell viability between the control and the TRAC CRISPR/Cas9 electroporated cells. For evaluating TCR surface expression, lower plots of electroporated Jurkat cells show a shift toward the CD3neg gate with 26.7% and a 2.77% background detected within the CD3neg gate of the non-nucleofected group (Figure 54).

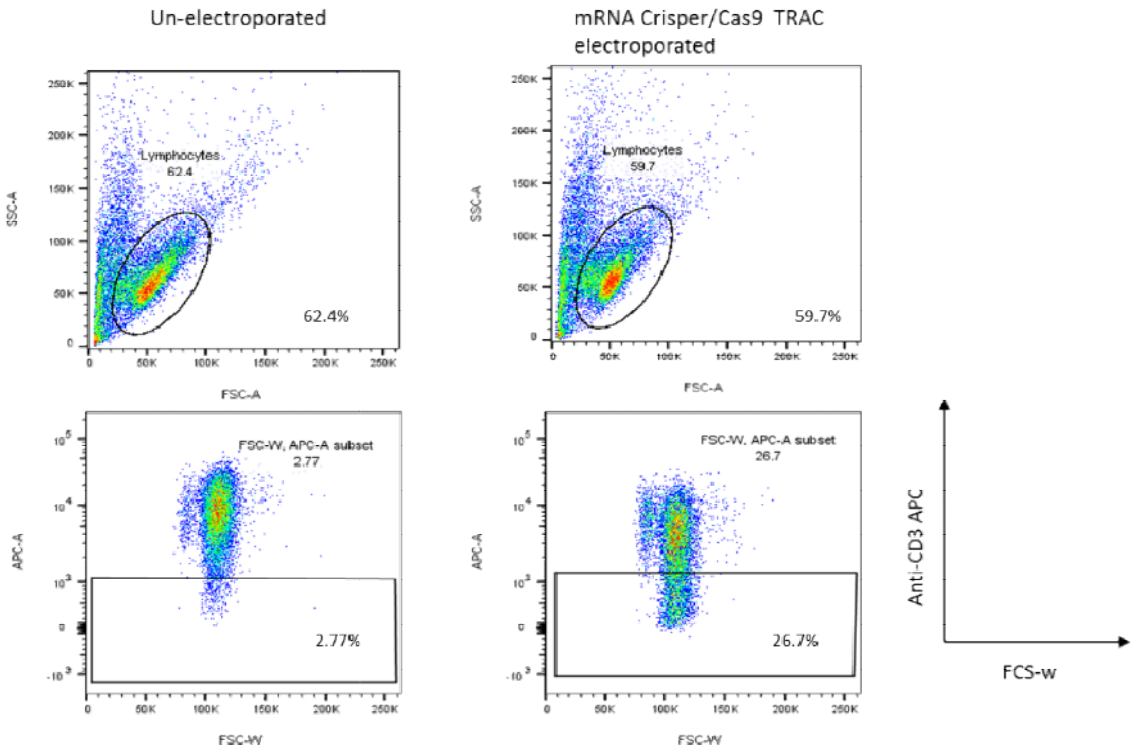


Figure 54: the effect of TRAC CRISPR/Cas9 as mRNA construct on TCR expression on the surface of Jurkat cells. Upper plots display the percentage of viable cells by gating on live Jurkat; control group show a cell viability percentage of 62.4% and for CRISPR/Cas9 electroporated cells the percentage of viable cells is 59.7%. Lower plots show that 26.7% of the electroporated cells have shifted toward CD3neg gate; while for non-electroporated cells, there was only 2.77% background of CD3neg population.

In summary, data illustrates in figure 55 shows that delivering CRISPR/Cas9 TRAC as DNA might be toxic to the cells and lead to immediate cell death. On the other hand, mRNA constructs seem to cause no harm to the cells and maintain cell viability within a reasonable percentage after electroporation, with a decent percentage of a TCR-disrupted population.

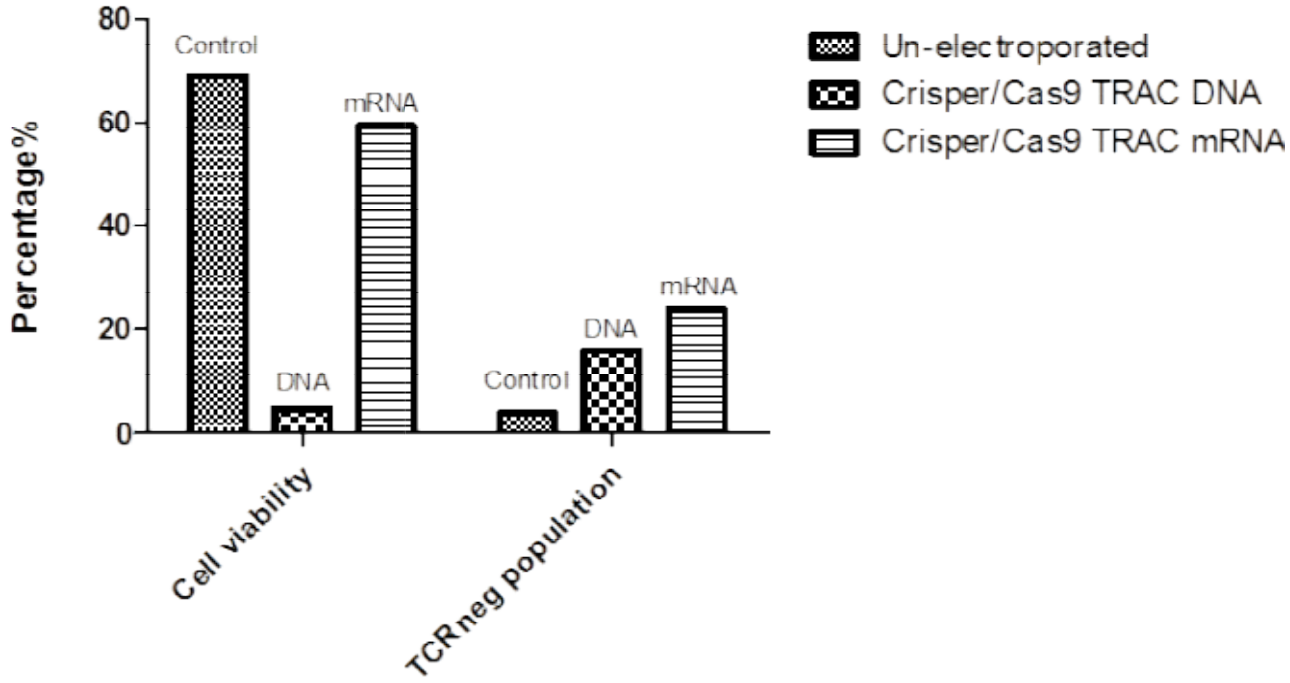


Figure 55: Comparison between the effects of TRAC CRISPR/Cas9 as a DNA construct vs as mRNA on TCR surface expression and cell viability of Jurkat cells. In comparison with the control group, the chart shows a notable reduction in cell viability within the DNA treated group but not in mRNA electroporated cells; which show a percentage approximately similar to the control labeled bar. Regarding TCR KO efficiency, the bar labeled with mRNA shows the highest percentage of TCR-neg population among the other groups (un-electroporated cells and cells treated with TRAC CRISPR/Cas9 as DNA).

5.5) TRAC TALENs vs TRAC CRISPR/Cas9:

In addition to the pair of TRAC TALENs that were obtained from the UCL Cancer Institute (London-TALENs or Cellectis-TALENs), another pair was provided by Dr. Boris Fehse's laboratory in Hamburg (Germany) and was named Hamburg-TALENs. The effect of Hamburg TALENs in knocking out the expression of TCR was compared to the previous pair of TRAC TALENs and to TRAC CRISPR/Cas9. As previously stated, $2x10^6$Jurkat cells were nucleofected with 5µg DNA of TRAC London-TALENs, TRAC Hamburg-TALENs and TRAC CRISPR/Cas9 and incubated for seven days before analysis. Surprisingly, FACS plots showed that both TALENs have the same knock out activity on the TCR expression, while TRAC CRISPR/Cas9 showed less knocking out activity. Both TRAC TALENs; London and Hamburg, appear to increase the percentage of the TCRneg-CD3neg population by 13.4% comparing to un-TALENised cells, whereas FACS plots of cells nucleofected with TRAC CRISPR/Cas9 show 9.03% of the cells in the TCRneg-CD3neg quadrant. As previously stated, DNA might be toxic to the cells which could be the reason for low cell viability within the nucleofected cells (Figure 56).

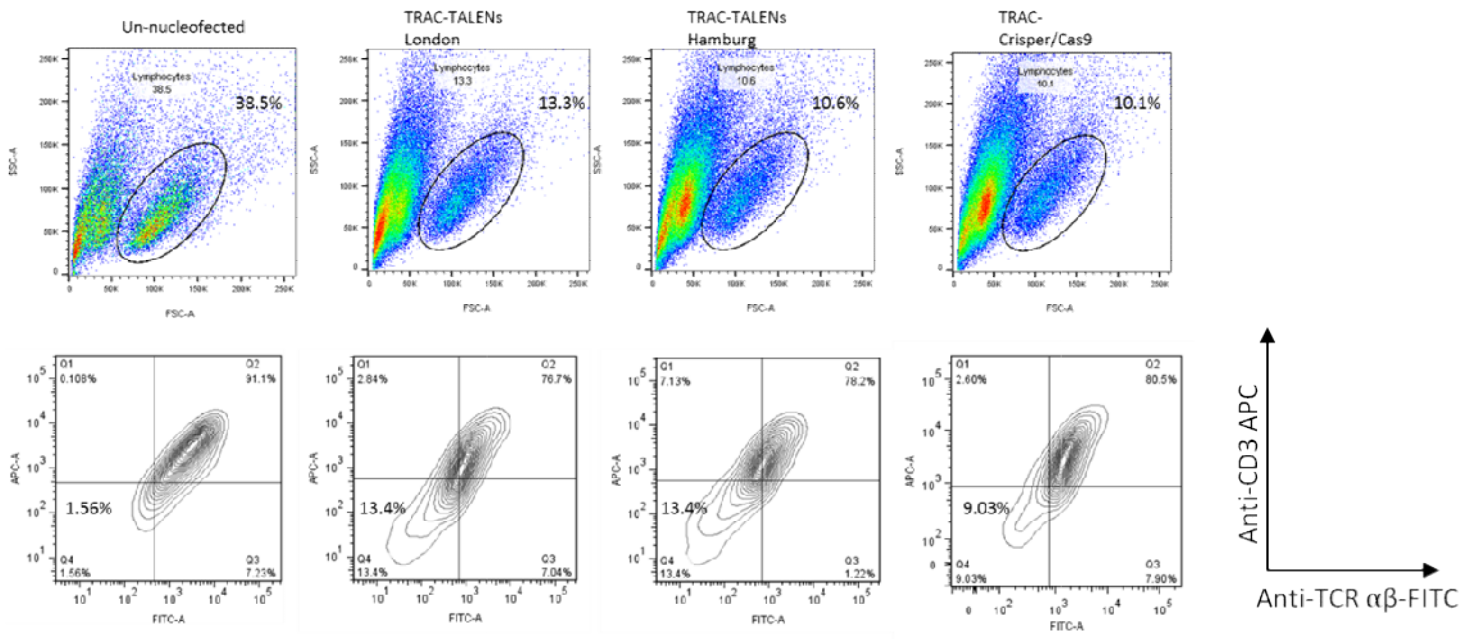


Figure 56: Comparison between the effects of various kinds of nucleases in knocking out TCR expression on the surface of Jurkat cells. Representative FACS plots of Jurkat cells that were exposed to TRAC TALENs London, TRAC TALENs Hamburg and TRAC CRISPR/Cas9 as a DNA. Upper plots represent the gating on the viable cells, while lower plots demonstrate nucleases activity by showing the percentage of TRCneg-CD3neg population.

The same experiment was conducted again, but instead of using DNA, mRNA constructs were used. TRAC-Hamburg mRNA was produced in order to conduct this experiment. The DNA was first linearised by using an AvrII enzyme, later it was used as a template to produce 60 µg of mRNA. 10µg mRNA of TRAC TALENs, either London or Hamburg, was used to electroporate $5x10^6$ Jurkat cells, while for TRAC CRISPR/Cas9, the Jurkat cells were electroporated with 50ng/µl of mRNA sgRNA plus 100ng/µl Cas9 mRNA. The electroporation process was performed using the BTX electroporation system and the cells were assessed by flow cytometry seven days later. The FACS plots showed a remarkable cell viability percentage within treated cells in comparison to the un-electroporated cells. The mRNA TRAC TALENs obtained from Cellectis were shown to produce the highest knock out activity by showing a sharp shift of the cells toward the TCR-negative quadrant in the FACS plots after gating of the viable cells. 35.5% TCR-negative cells were detected within the viable cells' gate of TRAC TALENs-Hamburg electroporated cells and within cells exposed to TRAC CRISPR/Cas9; the percent of the TCR-negative population was 21.9% (Figure 57).

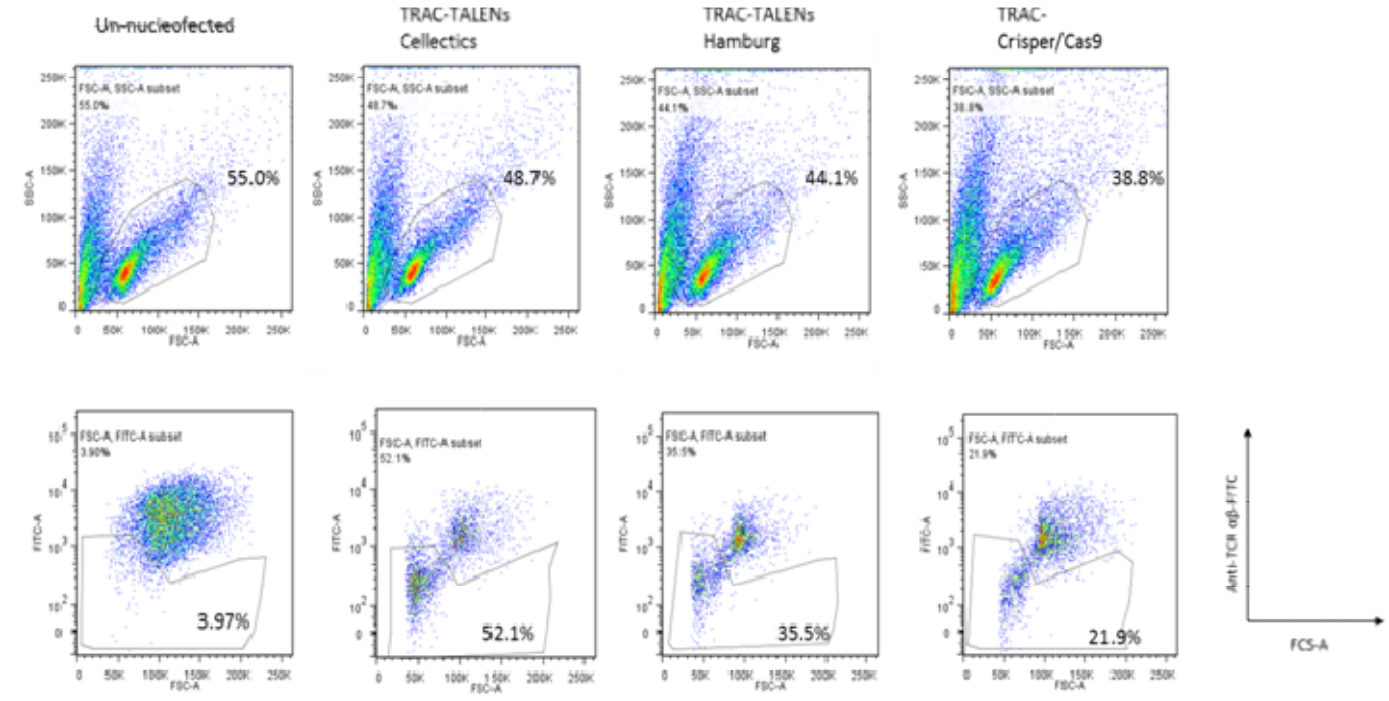


Figure 57: Comparison between different nucleases activity for knocking out TCR expression on the surface of Jurkat cells. Representative FACS plots of Jurkat cells that were exposed to TRAC TALENs London, TRAC TALENs Hamburg and TRAC CRISPR/Cas9 as an mRNA. Upper plots represent the gating on the viable cells while lower plots demonstrate nucleases activity by showing the percentage of TRCneg-CD3neg population.

To conclude, this data might suggest that TALENs show a slightly better TCR knock out activity than CRISPR/Cas9. Moreover, it appears that delivering nucleases as an mRNA form produces a higher cell viability percentage and superior knock out activity than delivering them as DNA.

5.6) Discussion:

A large number of strategies have emerged and investigated the efficacy of genome-editing technology rather than the conventional gene therapy approach. Molecular reagents including: meganuclase, zinc-finger nuclease, TALENs and CRISPR/Cas9 are becoming attractive genetic tools for gene targeting and manipulation (Kay, 2011). All four types are under evaluation for becoming a broad spectrum in gene therapy application. In the field of adoptive T cell immunotherapy, ZFNs and TALENs were applied to knockout the expression of endogenous T cell receptors either α or β or both. Consequently, the population yield of therapeutic T cells can be applied to multiple non-related recipients without causing GVHD (Qasim and Thrasher, 2014).

This section of the thesis discusses the feasibility of targeting the constant region of the endogenous TCR locus by several different gene-editing tools including: meganucleases, TALENs and CRISPR/Csa9. Meganucleases are engineered to target TCR constant beta locus (C1 and C2) in the Jurkat cell line. TALENs and CRISPR/Cas9 were used to disrupt

the sequence within the TCR alpha constant region in Jurkat, PBMCs and CB-T cells and the results are described. The outcome of the experiments conducted in this chapter suggested that meganuclease might be toxic to the cells and that this is due to the acceleration in cellular death rate after they had been subjected to the meganuceleases plasmid. In general, introducing nucleases as an extrachromosomal DNA to the cells will lead to reduction in cell survival and this is because the DNA will be introduced directly to the cells at the nuclear level. This could prove to be toxic to the cells. For this reason, most of the therapeutic genome editing tools have been transformed to an mRNA species where the electroporation is acquired at the cytoplasmic level. Nevertheless, the same results were achieved by using a lentiviral vector as the transferring vehicle of megnaucleses to the respective cells.

TALENs and CRISPR/Cas9 were selected to be the new platforms to suppress the expression and inactivate the function of TCR. Several attempts were performed to assemble in-house TALENs targeting both constant chains of TCR (TRAC and TRBC). A positive result was accomplished in building the right arm of the TALENs designed to target the beta constant chain; whereas for the left arm, no success was achieved despite all attempted endeavors. Building and assembling TALENs fragments through complex digestion and cloning procedures was not as easy as designing them. The process of digesting one TAL effector fragment to be ligated to the following TALE effector fragment was a labour-intensive and tedious procedure with a very dismal percentage of success. Commercially applied TALENs were used to conduct the experiments.

TRAC TALENs from different sources were utilised to disrupt alpha constant regions of TCR on Jurkat cells, CB-T cells and adult T cells. The emerging data was outstandingly promising given that the percentage of disrupted-TCR reached 70% after being TALENised with TRAC TALENs as mRNA. CRISPR/Cas9 was also designed to target TRAC, but its efficacy was not as remarkable as the efficacy of TALENs in knocking down the expression of TCR. However, experiments aiming to compare the efficacy of CRISPR/Cas9 to TALENs were only conducted once. Consequently, more experiments are required to achieve a solid conclusion. The CRISPR/Cas9 plasmid required an optimization due to the presence of a large number of accessory elements such as mCherry. Additionally, the alteration of the DNA plasmid into mRNA demands optimization in order to achieve high quality and purity.

Despite the numerous successes and remarkable excitement surrounding genome editing based therapy, it poses several challenges that require further work. Off-target cleavage of genome editing tools is one of the main safety concerns for clinical application. Most of the studies that evaluate the target specificities of ZFNs, TALENs, and CRISPR/Cas9 have

concluded that TALENs have the lowest incidence of off-target cleavage compared to ZFNs and CRISPR/Cas9 (Pattanayak et al., 2011, Guilinger et al., 2014, Fu et al., 2013, Veres et al., 2014). Therefore, the understanding of the specificity of engineered genome editing nucleases needs to be improved. Another major obstacle facing genome targeted technology is the means to introduce these designer nucleases to the respective target cells. Currently, the electroporation method to deliver extrachromosomal DNA-based nuclease expression vectors, mRNA or proteins might be consider the most popular method to transiently express nucleases within the cells (Gaj et al., 2012). Although using integration-competent and integration-deficient lentiviral vectors as a delivering system was successful and a more potent method of delivery than electroporation, they are still under consideration because they may lead to constitutive expressions resulting in producing more off-target activity (Weber et al., 2008, Mock et al., 2014).

Recently, an Adeno-associated virus (AAV) vector proved its efficiency in delivering designer nucleases into different tissue types such as the liver and the brain (Holkers et al., 2013b). Although this class of life-changing medicine is still in its infancy, genome editing presents alluring opportunities for tackling a number of diseases that are beyond the reach of previous therapies. However, their efficacy, safety and specificity require them to be optimized until they can be considered for use in human clinical applications.

Chapter 6: Generation of “off-the-shelf” universal engineered CAR19 Cord Blood T cells

6.1) Introduction:

In recent years, promising data has emerged from many studies investigating the ability of editing T cell specificity towards a variety of malignancies such as B-cell malignancies. Engineering autologous T cells to express chimeric antigen receptor (CAR) against CD19, a leukemic antigen on B-cells, has recently been shown to be highly effective as a cell-based therapeutic tool for relapsed leukaemia. Harvesting an adequate number of autologous T-cells from heavily treated cancer patients or from small infants may be not feasible to manufacture a sufficient number of therapeutic engineered CAT19 T-cells. Utilising donor T cells from allogeneic HLA matched haematopoietic stem cell donors as part of transplant strategy might be feasible. However, in some circumstances it is not possible to find HLA matched allogeneic hematopoietic stem cell donors. It has been proposed that generating "off-the-shelf" engineered CAR19 T cells from non-matched donors might be an attractive alternative approach, but it must overcome HLA-barriers. It is important to engineer T cells with the ability to evade host-mediated immunity, and to provide anti-leukemic activity without graft-versus host disease. The endogenous TCR αβ on the infused allogeneic T-cells might cause graft-versus-host disease by recognising minor and major histocompatibility antigens in the recipient (Torikai et al., 2012). In order to preclude TCR-mediated deleterious recognition of normal tissues after adoptive transfer, and to reduce the time and expense to manufacture patient-specific T-cell as a therapeutic product, our goal is to generate universal "off-the shelf" T-cells expressing CD19 CAR from allogeneic healthy cord blood donors. Consequently, it can be administrated to HLA non-matched patients without causing GVHD.

CD19 is a tumour specific antigen that is constitutively expressed on most acute and chronic B-cell malignancies. Therefore, to target malignant B cells, we have produced third generation lentiviral vectors to express a second generation CD19 CAR (designed with CD28 as a costimulatory molecule) and also the vector incorporated an abbreviated sort/suicide gene RQR8, comprising a 136 amino acid cell surface protein inclusive of a CD34 epitope for clinical applications. This same approach has been used in clinical trials to treat acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL) by Cellectis, a biopharmaceutical company focused on developing immunotherapies using universal allogeneic adult T-cells engineered to target CD19 marker on malignant cells. In June 2016, Cellectis announced that the first patient had been treated in the Phase I study of UCART19 in paediatric acute B-lymphoblastic leukemia (B-ALL) at the University College of London (UCL).

To test the feasibility of generating universal allogeneic CD19-CAR+CB-T cells, the culturing process for generating CD19 CAR+ T cells was modified to include the editing of the genome of CD19-CAR+ T cells to irreversibly eliminate the expression of the endogenous αβ TCR. After activating CB-T with either anti-CD3/anti-CD28 beads or with a cytokines cocktail; which includes IL-7 + IL-15 and IL-2, CB-T cells were transduced with a lentiviral vector expressing CD19CAR with a remarkably high transduction efficiency. Later, to knockout the alpha TCR loci, the transcription activator-like effector nucleases (TALEN)s was used targeting genomic sequences in the constant regions of the endogenous α subunits of the TCR. The TALENs mediated genome editing by catalysing the formation of a DNA double strand break (DSB) in the genome. Targeting a DSB to a predetermined site within the coding sequence of a gene was previously shown to lead to permanent loss of the functional target gene expression via repair by non-homologous end joining (NHEJ), an error-prone cellular repair pathway that results in the insertion or deletion of nucleotides at the cleaved site (Cade et al., 2012).

Here we demonstrate that cord blood T cells are highly prone to multiple manipulations by delivering significantly high transduction efficiency and show a high TCR knock out efficiency. Moreover, we demonstrate that TALENs targeting the alpha chain of endogenous TCRs in CB-T cells resulted in the desired loss of TCR expression. As expected, these modified CD19CAR TCR negative CB-T cells maintained their CAR-mediated redirected specificity for CD19.

6.2) Manufacture of Universal CD19CAR T ($TCR^{neg}CAR19^{pos}$) Cells using the G-rex Expansion System: (Figure 58)

CB-T cells were activated with antiCD3/anti-CD28 beads (n=5) or with a cytokines cocktail composed of 5ng/ml IL-7, 5 ng/ml IL-15 and IL-2 (n=3) and cultured at a concentration of $1x10^6$ cells/ml in a 100 ml differentiation bag (Miltenyi Biotec) for two or three days in a cytokines activation condition. On the day of viral transduction, the 100 ml differentiation bags were coated with 10 mls of retronectin and incubated at room temperature for a couple of hours. Later, activated cells were exposed to self-inactivating third generation lentiviral vectors carrying CD19CAR as a transgene under the control of an internal EFL alpha promoter and linked to the sort/suicide gene RQR8 at MOI of 5 (Figure 24.A). Transduced cells were stained with anti-CD34 and/or anti-Fab antibodies for transduction efficiency and assessment. Flow cytometric characterization of transduced cells showed that cells expressing CD19CAR between 32.7%-75.9% (n=5, mean=52.92) for anti-CD3/anti-CD28 beads activated cells, while for cytokines activated cells, the CD19CAR expression level ranged between 15.8%-48.6% (n=3, mean= 29.1) (Figure 59).

After transduction and prior to electroporation, transduced cells were prepared for receiving TRAC TALENs by culturing them at a concentration of $1\text{-}1.7x10^6$ cells/ml in complete X-VIVO 15.

After analysing the transduction efficiency, cells were exposed to 20 µg of mRNA encoding TRAC TALENS (10µg/arm) and electroporated using the BTX system, then cultured in G-rex m10 for expansion for seven days. Twelve days after activation, the down expression of endogenous TCR was assessed by evaluating the percentage of CD3negative and/or TCR-negative population by flow cytometry. Flow cytometry analysis revealed that up to 60% of the bead activated cells lost their TCR/CD3 surface expression (22%-60%, n=5, mean=40.28), although for cells activated with the cytokines cocktail, TCR/CD3negative percentages ranged from 12%-56% (n=3 mean=36.2)(Figure 60). To enrich the TCR/CD3negative population, a depletion column and negative selection using anti-TCR paramagnetic beads was used and more than 99% enrichment was achieved.

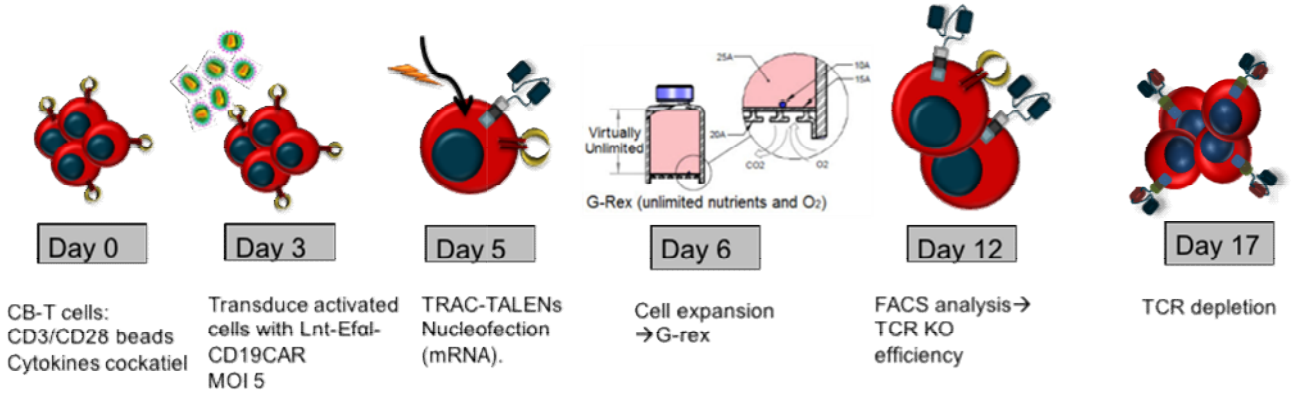


Figure 58: Schematic Presentation of the generation of "off-the-shelf" CD19CAR CB-T cells. A pair of TALENs-encoding mRNA was electro transferred 5 days after stimulation and transduction of CB-T cells. T cells were then cultured with 200 IU/ml of IL-2 and/or 5ng/ml of IL-7 and 5 ng/ml of IL-15, and incubated at 37°C 5%CO2 in G-rex m10 expansion flask, as indicated. CD3 and/or TCR expression was analysed by flow cytometry on Day 7 after electroporation. On Day 17, TCR negative population was negatively selected to be separated out from TCR positive cells.

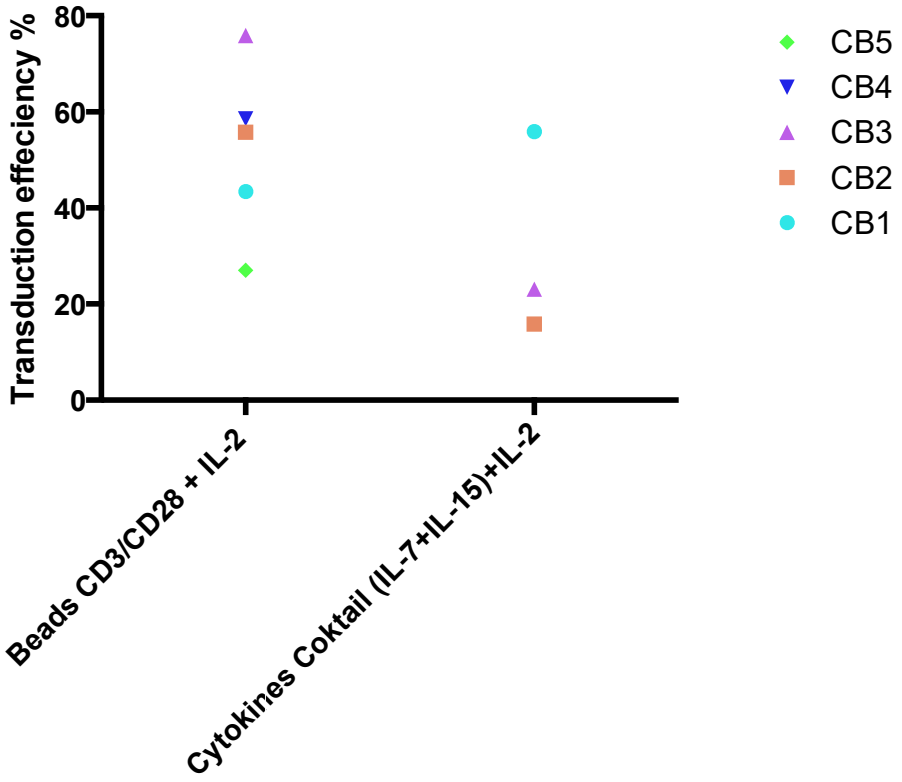
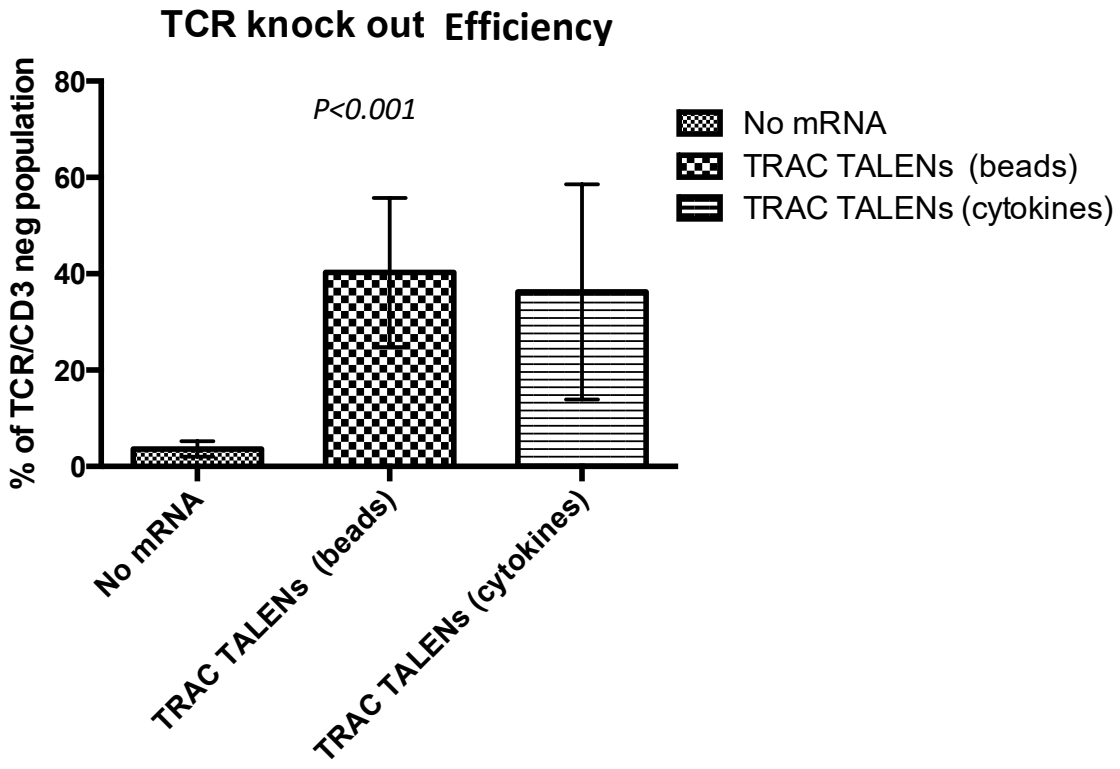


Figure 59: Expression of CAR19 in five human cord blood donors. Lnt-SFFV-CD19CAR or Lnt-EFlα-CAD19CAR vectors were transduced into CB-T cells (n=5) and CAR19expression was analysed by anti-scFv or anti-CD34 and/or anti-Fab antibody staining. Graph shows comparison of CD19CAR transduction efficiency of CB-T cell stimulated with different conditions before transduction; five CB-T cells stimulated with CD3/CD28 beads +IL- 2 (100U/ml), while three other CB-T cells were stimulated with combination of IL-7 (5ng/ml) and IL-15 (5ng/ml) in addition to IL-2 (100U/ml).

A)



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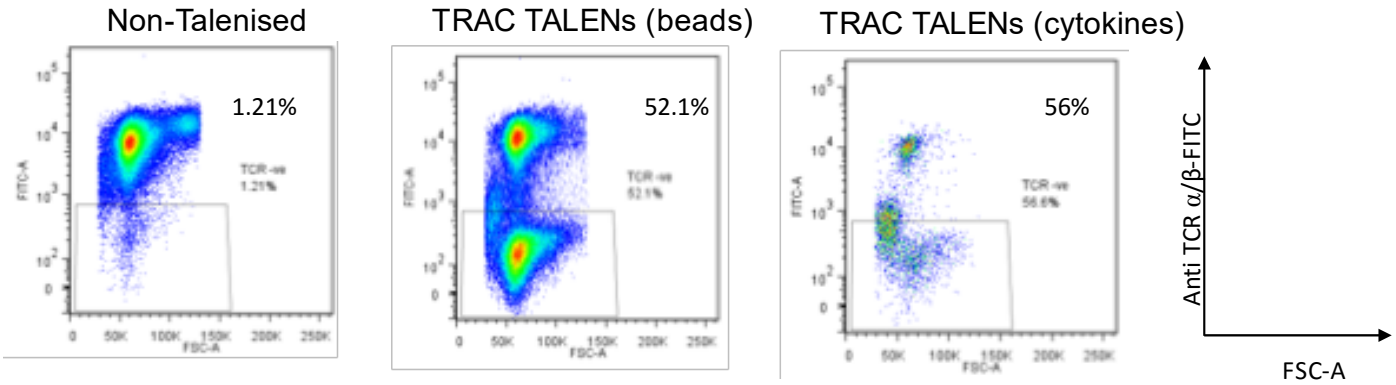

Figure 60: Down-regulation of CD3/TCR expression after electrotransfer of mRNA encoding the TCR alpha/beta targeted TALENs. Day 7 after electrotransfer of the indicated doses of mRNA coding for TRAC targeted TALENs pairs, TCR αβ-CD3 expression was analysed by either TCR αβ or by co-staining TCRαβ and CD3. A) Bar chart shows TCR/CD3 expression in T cells cultured with anti-CD3/anti-CD28 beads (n=5) vs. T cells cultured in interleukins cocktail after TRAC TALENs transfer (n=3). B) Representative flow data at Day 7 after TALENs electrotransfer is shown. Flow cytometry data are gated on cells viable cells. Percentages in the lower half represent the percentage of TCR negative cells in T-cell populations.

6.3) Enrichment of TCR negative CB-T cells:

Isolating the TCR-disrupted population is an essential requirement for future clinical application to prevent GVHD in human leukocyte disparate recipients. To address this issue, TCR/CD3 negative cells were negatively selected using anti-TCR αβ paramagnetic beads and a LD depletion column. Flow cytometry analysis showed >80% of TCR-depleted cells validating the elimination of the TCR positive population (Figure 61).

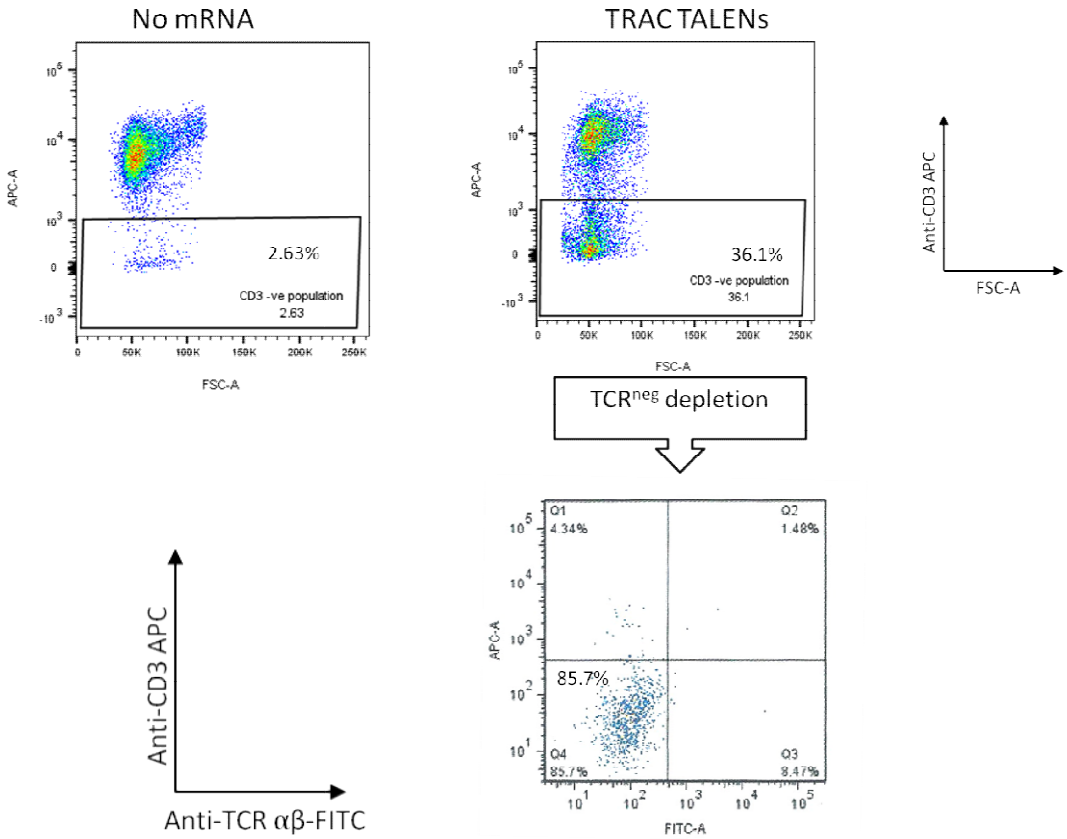

Figure 61: TCR negative CB-T cells can be enriched by depleting TCR positive T cells population. TCRαβ/CD3 expression after depletion using TCRαβ-specific paramagnetic beads. Flow cytometry reveals expression of TCRαβ and CD3 in CB-T cells 17 days after activation by anti-CD3/anti-CD28 beads (10 days after TRAC TALENs transfection). Percentage in the bottom-left quadrant represents the percentage of TCRαβ/CD3neg T cells.

6.4) Disruption of the αβ TCR-CD3 Complex on T cells using TALENs Targeting TRAC:

To confirm that electroporated CB-T cells had been genetically modified at the intended TRAC TALENs target sites, a surveyor nuclease assay was performed using specific oligonucleotide primers flanking target sites within TRAC. $TCR^{neg}CD19CAR^{pos}$ cells $TCR^{pos}CD19CAR^{pos}$ cells and bulk, non-selected TCR $CD19CAR^{pos}$ cells (that stimulated with cytokines) were exposed to PCR to amplify the TRAC TALENs-target site, then they were exposed to Nuclease N mutation detection enzyme. The Nuclease N digestion products, representative of genetic changes induced by the TRAC TALENs, were present only after electrotransfer of the TCR-specific TALENs pairs on a 1% agarose gel. To evaluate the efficiency of TRAC TALENs by this enzyme, two fragments at size of 300 and 200 base pairs should be detected on the gel. As the gel demonstrates, two fragments of the right sizes (very faint band at the size of 200 bp) were observed in the $TCR^{neg}CD19CAR^{pos}$cells, but not in the $TCR^{pos}CD19CAR^{pos}$ population, as only one band could be seen (Figure 62). It could not be confirmed if the very faint two bands that appeared at the cytokines activated cell lane were products of Nuclease N enzyme, as they appeared as a smear of a DNA.

This experiment on naive T cells confirmed that TALENs designed to target TRAC lead to permanent disruption of αβ TCR expression, as assessed by the Nuclease mediated surveyor assay and confirmed by flow cytometry analysis of TCR/CD3.

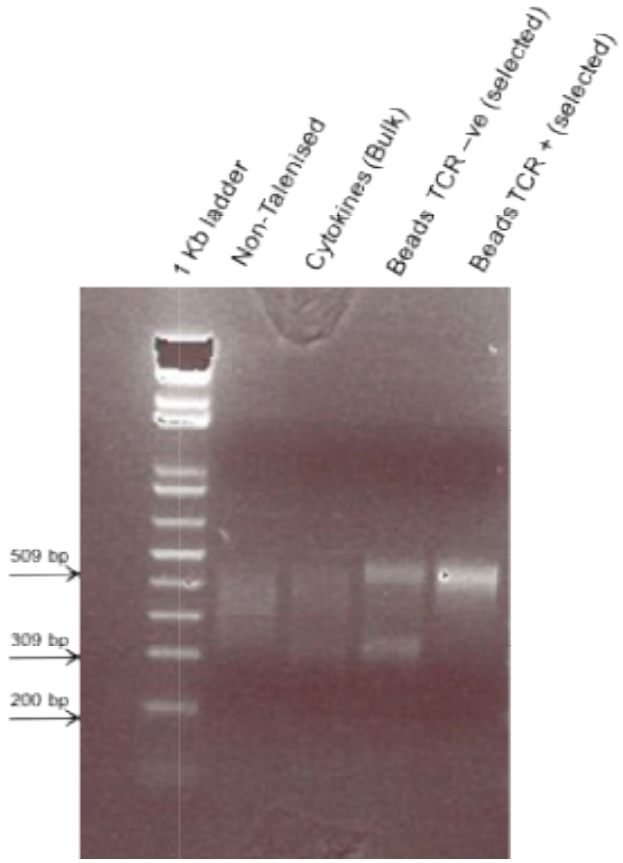

Figure 62: Surveyor nuclease assay to detect TALENs-mediated modification of TCR target sites in CB-T cells. CB-T cells activated with anti-CD3/anti-CD28 beads (electroporated cells were selected for TCR) or with cytokines cocktail (the bulk of the cells have not been selected for TCR negative population). Arrows indicate the fragments produced by a surveyor nuclease digest of amplicons bearing a mismatch at the intended site of TALENs cleavage in the TRAC locus.

6.5) Next Generation Sequencing:

As a confirmation of the efficacy of TRAC TALENs in editing its specific target, genomic DNA of the manipulated CD19CAR T cells were subjected to next generation sequencing. The first run was conducted by Dr. Claire Deakins. The genomic DNA of a purified TCR negative population (after αβTCR depletion), pure TCR positive fraction and of cytokines-stimulated CD19CAR CB-T cells were amplified by PCR then exposed to deep sequencing using an Illumina Miseq Machine. NHEJ was detected at a frequency of >70% of TRAC locus for pure negative TCR population. In the cells with non-disrupted TCR fraction, NGS detected NHEJ events at a frequency of < 1% of TRAC locus, which was similar to the control non-modified cells. NGS interrogation followed by nested PCR amplification of TRAC target sites of cytokines stimulated CD19CAR CB-T cells, confirmed high levels of NHEJ signatures for TRAC and the high efficiency of TRAC TALENs in gene editing (>50%) (Figure 63) (Table 7).

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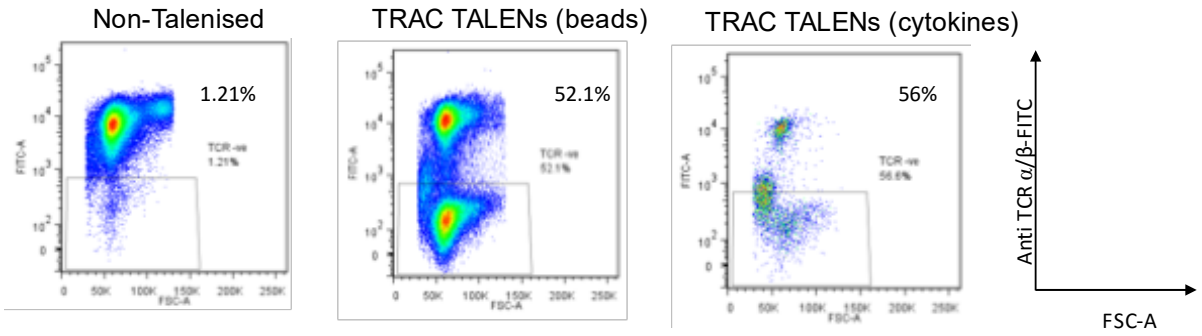


	Neg ctrl		TCR neg		TCR pos		Cytokines	
Total analysed reads	1595473		975598		1458492		1004869	
Proportion of reads accounted for by expected sequence	**99.74%**		**22.50%**		**99.71%**		**22.04%**	
Number of non-matching sequences detected	130		2273		137		2013	
Number of raw reads	2002521		1577976		1812864		1631300	
Number of reads after first anchoring step (first 6nt)	1917659	95.8%	1500034	95.1%	1746596	96.3%	1561718	95.7%
Number of reads after Q30 filtering	1616164	80.7%	1093441	69.3%	1478741	81.6%	1128061	69.2%
Number of reads after second anchoring step (7nt before cleavage site)	1600894	79.9%	980523	62.1%	1463845	80.7%	1009227	61.9%
Number of reads after matching read 1 and read 2	1595473	79.7%	975598	61.8%	1458492	80.5%	1004869	61.6%

Table 7: Summary of next generation sequencing data of the genome of TRAC TALENised CB-T cells. Table summarises how many reads were accounted for by the un-mutated sequence and how many mutated sequences were detected within the genome of TALEN-manipulated CB-T cells, as well as un manipulated population. It also describes how many reads were passed at the various stages of filtering and trimming of sequence reads. FACS plots represent percentages of TCR-disrupted T cells in both stimulation conditions and the result is almost consistence with the NGS outcome.

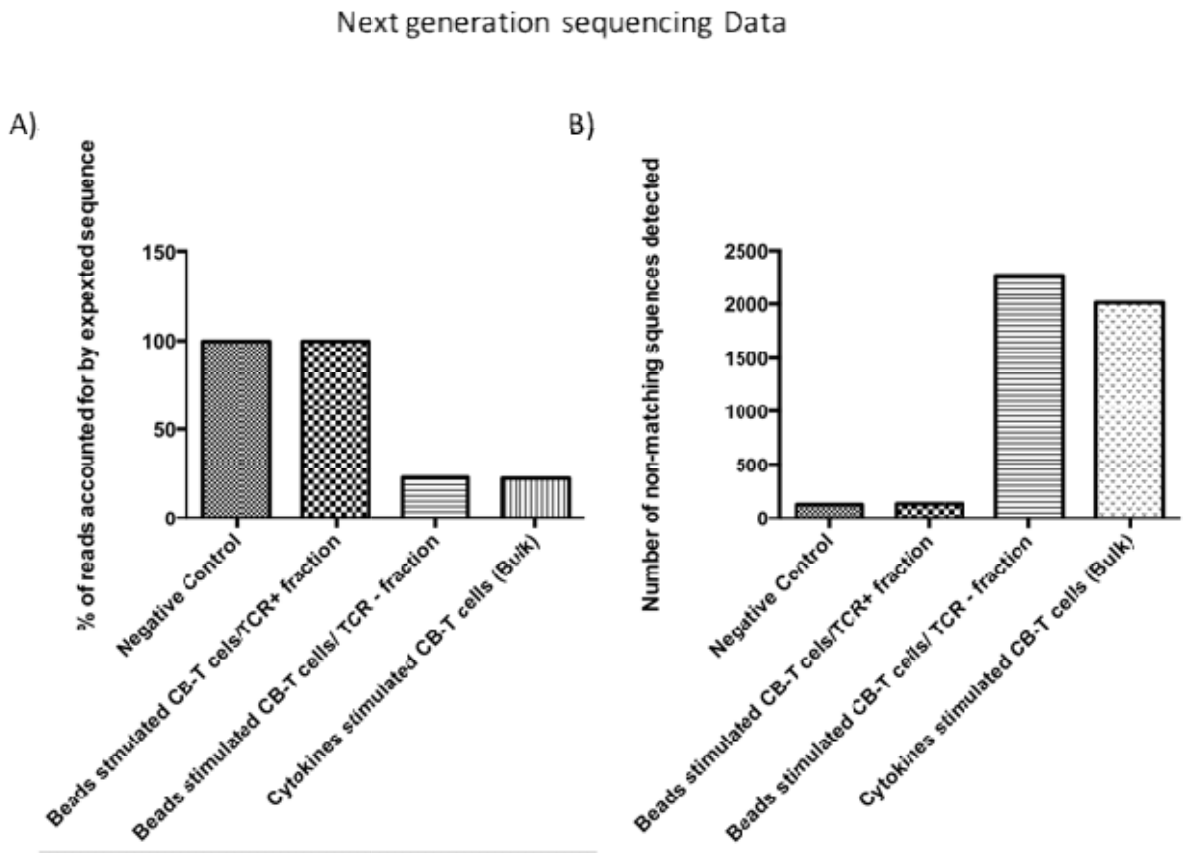
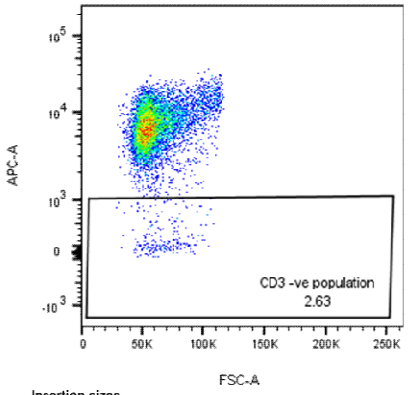


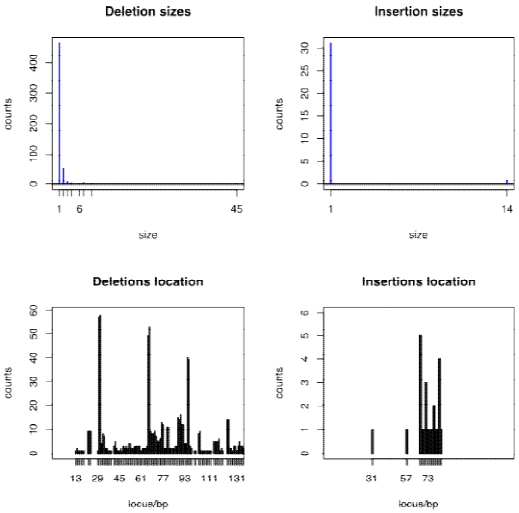
Figure 63: Next generation sequencing data of DNA extracted from TALENised CB-T cells. Histograms illustrate summary of Next generation sequencing data of DNA extracted from TALENised cord blood T-cells (n=1) that has been stimulated with anti-CD3/anti-CD28 beads or cytokines cocktail (IL-7+IL-15 and IL-2). A) Histogram demonstrates the percentage of the similarities between the original sequence of TRAC and the DNA sequence of the manipulated CB-T cells. There is a significantly low percentage of similarity between the original TRAC sequence and CB-T cells DNA that have been exposed to TRAC-TALENs. B) Histogram demonstrates the number of non-matching sequences detected within the genome. As the graph shows; there is a high percentage of non-matching sequence spotted within the genome of the manipulated cells (TALENised cells; TCR negative population) in comparison with the genome of the un-manipulated cells negative control and TCR positive fraction.

Two other donors' genomic DNA extracted from TALENised-genetically edited CD19CAR CB-T cells were also subjected to next generation sequencing using a CRISPR-GA pipeline plaform in an experiment performed by Dr. Anastasia Petrova using an Illumina Miseq Machine. Raw sequencing data was analysed using software named CRISPR-GA; where the raw sequencing reads were uploaded and then underwent quality control by trimming to discard the results that contained any sequencing errors. The software then mapped the reads to the reference sequence (TRAC). The mapping was done by a specialised BLAT platform, which is suitable to support indels (deletions and insertions). The software processes the mapped BLAT results to detect the insertions and deletions. It then uses pattern matching to compute the number of reads matching the expected sequence or generating variants. This information is then integrated to create plots and reports. Pattern matching is used to calculate the number of reads matching the expected sequence and other variants generated. NHEJ and HR are estimated by computing specific equations. The final result consists of three parts. First, the results report the analysis of indel sizes. Second, they illustrate the analysis of indel locations within the edited genomic locus. Lastly, they show an integrated plot representing indels, NHEJ and HR. Furthermore, a FASTA file is created with all reads containing indels, as well as an XML text output with HR, NHEJ values and the indel relative location in genomic locus (Güell et al., 2014).

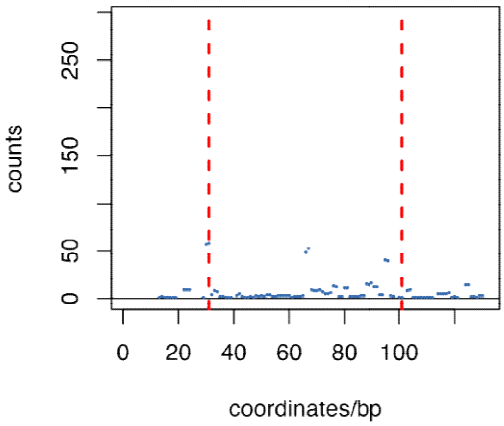
The batch was subjected to detailed release characterization to quantify and exemplify deletions and insertions at the intended target site. Next Generation Sequencing (NGS) detected on-target events consistent with NHEJ (Insertions, Deletions and Indels) at a frequency of >60% for (36-61%) TRAC locus and for similar events at a frequency of just 0.096-0.11% for control non-modified cells (Figure 64).

A) Non-modified CB-T-cells (control)

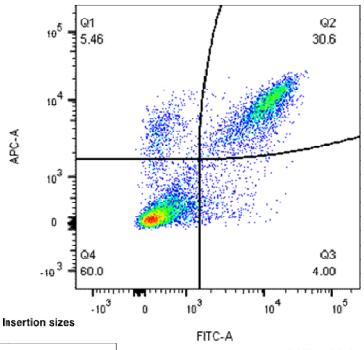


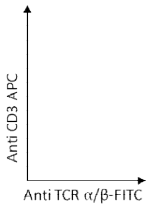


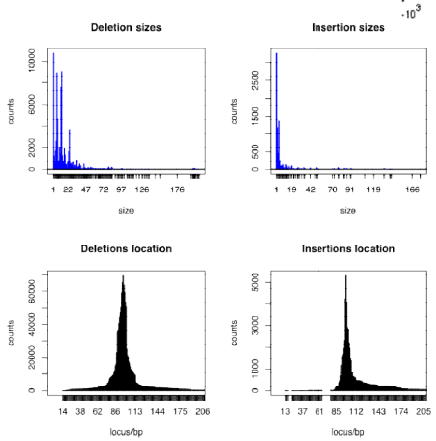
HR: NA%(in red) NHEJ: 0.1%(in blue)



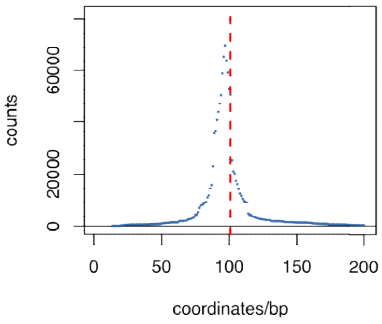
B) Genetically modified CD19CAR CB-T cells by TRAC TALENs (mRNA) (Donor 1)







HR: NA%(in red) NHEJ: 61%(in blue)



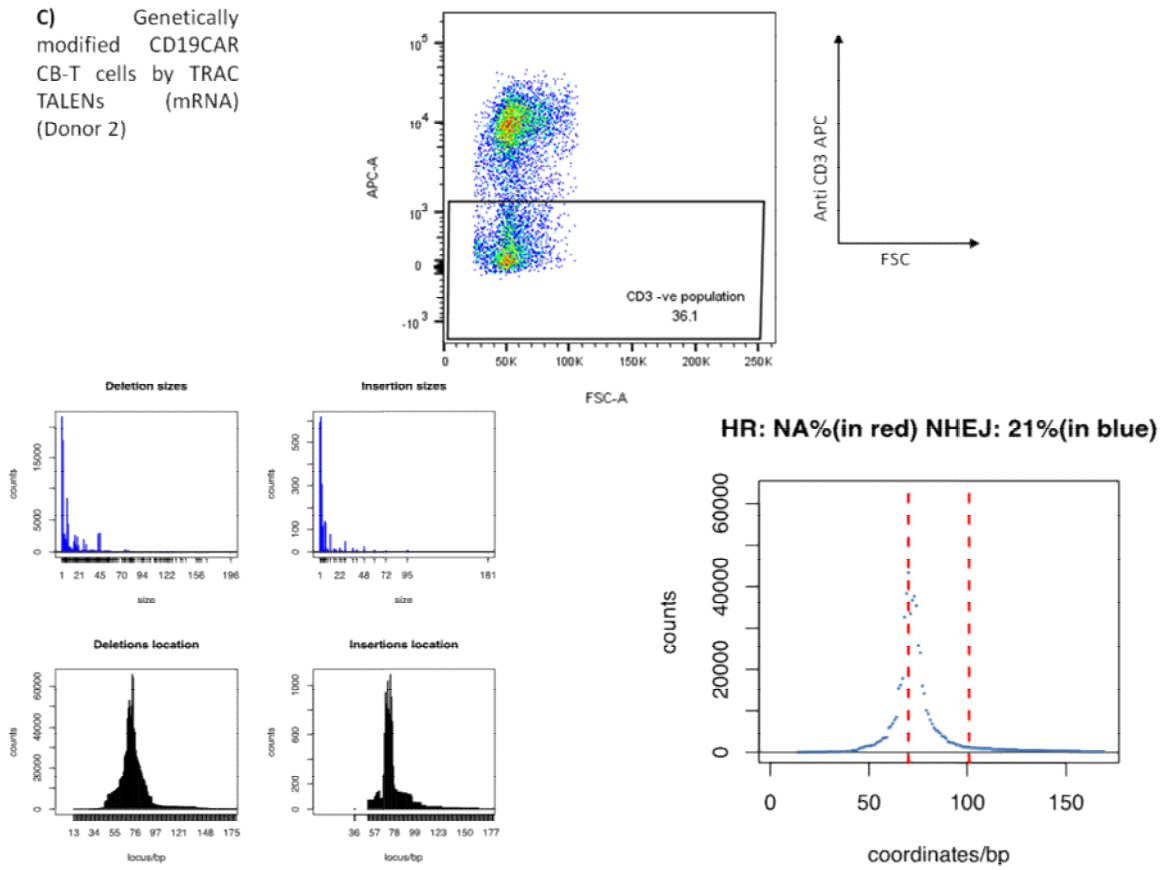


Figure 64: NGS TRAC target site sequences. NGS TRAC target site sequences found a high frequency (>60%) of events consistent with NHEJ repair of double stranded DNA scission. The results were consistent with the flow cytometric quantification of gene-editing considering that expression at the TRAC locus is subject to allelic exclusion. A) Control group represented by un-manipulated CB-T cells. FACS blot (top) shows the percentage of CD3 negative population before genome editing using TRAC TALENs. NGS data (bottom) estimated the frequency of NHEJ (right) in the normal condition of the cells as well as the analysing indels sizes and their respective frequencies and their location within your reference locus (left). B) Flow cytometric analyses demonstrated that 60% of T cell population had a TCR negative characteristic (top). NGS data was consistence with flow results where there are around 11000 deletions of 1base pair also have around 3250 insertions of 1base pair; most of the indels are concentrated around 100base pair of the reference sequence (bottom-left).we have around 50000 indels concentrating around 100bp of our target sequence and NHEJ percentage is 61% (bottom right). C) FACS blot demonstrate percentage of TCR-disrupted engineered T cells (top) which is consistence with the NHEJ frequency that determined by NGS (bottom). Right graph demonstrates the NHEJ frequency 21% while graphs on the left illustrate indels sizes and their respective frequencies and their location within your reference locus; there are > 60,000 deletion of 1base pair and around 1000 insertions of 1 base pair; most of the indels are concentrated around 60-100 base pair of the reference sequence (bottom-left).

6.6) TCR-disrupted CD19CAR CB-T Cells Appear to Retain Naïve Phenotype Characteristics

In order to assess differentiations of disrupted CB-T cell populations by flow cytometry, a panel of the following markers was used: CD45RA, CD62L, CCR7 as well as T cell subtype CD4 and CD8. After isolating TCR negative cells from the TCR positive population, the cells were stained with the CD4, CD8, CD45RA, CD62L and CCR7 to determine the percentage of naïve cells. Our data suggests that the TCR negative population was co-expressing naïve markers in a more abundant manner (n=3, CD4+:43%-60%, mean= 53%/ CD8+:23%-45%, mean=35.6%) than the pure TCR positive population (n=3, CD4+:25%-31%, mean= 27.6% / CD8+:25%-37%, mean=29%). The un-activated cells were thawed and analysed right away at the same time and included with the other samples (n=3, 26.6%-31.7%, mean=28.4%) (Figure 65). Although the data outcome from the un-activated cells was not as expected, it helps in gating the cells in an accurate manner once analysed by the FlowJo software.

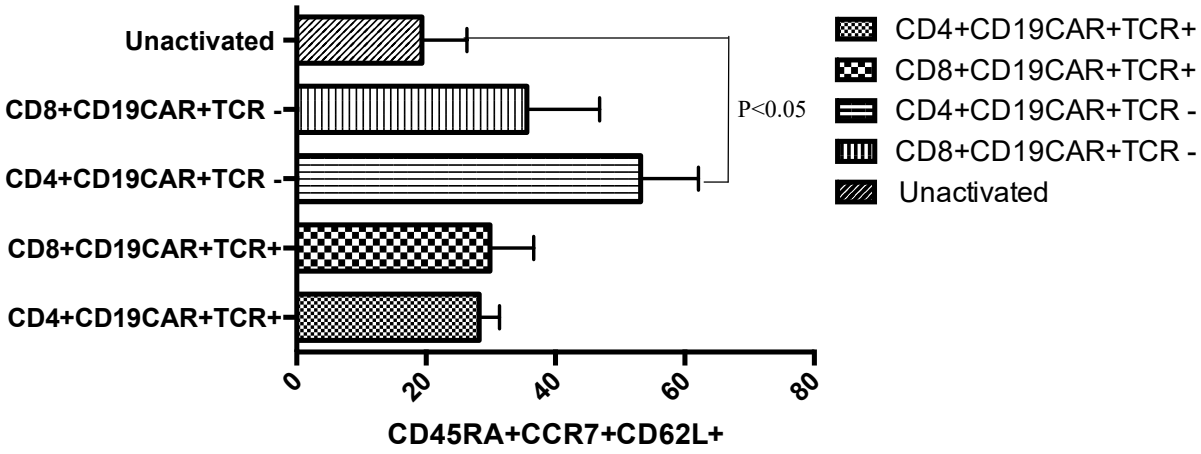


Figure 65: Co-expression of naive phenotype markers on CAR19 CB-T cells. Bar chart illustrates phenotypic analysis of CB-T cells product after being transduced with Lnt-EFlα-CD19CAR, TALENised with TRAC TALENs mRNA and TCR depleted (n=3). Un-manipulated cells (unactivated) were used as a control to gate the cells correctly on FlowJo software. Disrupted TCR cells (TCR negative population) show higher percentages of naïve like phenotypic features than TCR positive fraction.

Another phenotypic panel to determine the immunological naivety is displayed by a stem cell memory T cell (T_{SCM}) subset. The expression of additional protein surface markers, including the death receptor CD95 (also known as FAS), in the context of naive-appearing T cells was recently found to delineate a new memory T cell subset in humans and mice (Gattinoni et al., 2011). T_{SCM} has superior survival potential and efficiently engrafts in allogeneic transplant models resembling naive T cells in that they are CD45RA+CD45RO-, and they express high levels of the co-stimulatory receptors CD62L and CCR7. Stem cell memory T cells have a high proliferative capacity and are both self-renewing and 'multipotent'. They are able to differentiate into other T cell subsets, including central memory T cells and effector memory T cells. These properties define their 'stem cell-like' abilities (Gattinoni et al., 2011). Antibodies targeting CD95 were used in addition to anti-CD45RA and anti-CD62L to define the phenotype properties of disrupted-CAR19 engineered T cells after being negatively selected for TCR expression. FACS plots indicated that the negatively expressed TCR group, especially the CD4 subset, is significantly superior in expressing stem cell memory T cells markers than the pure TCR positive population (Figure 66).

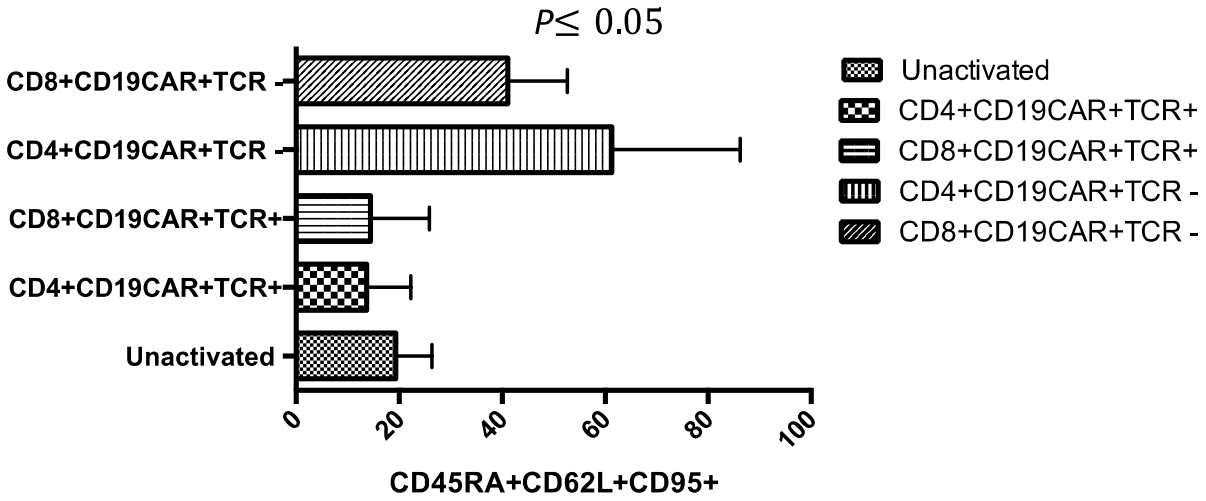


Figure 66: Co-expression of stem cell memory T cell phenotype markers on CAR19 CB-T cells. Bar chart illustrates phenotypic analysis of CB-T cells product after being transduced with Lnt-EFlα-CD19CAR, TALENised with TRAC TALENs mRNA and TCR depleted. Un-manipulated cells (unactivated) were used as a control to gate the cells correctly on FlowJo software. Disrupted TCR cells (TCR negative population) show higher percentages of naïve like phenotypic features than TCR positive fraction.

It was later suggested that phenotyping data might be more useful and more precise if the cells were stained with anti-Fab marker in addition to the other T cells differentiation markers. Thus, the exact phenotype of only CAR19+ cells will be determined. Bulk amounts of CD19CAR+ CB-T cells (n=2) that had been exposed to TRAC TALENs (Td-TRAC

Talenised) were stained with another phenotypic panel: Fab, CD45RA, CD62L and CCR7 antibodies. Flow cytometric results showed that TALENised CD19CAR+ T cells were superior to non-TALENised non-engineered CB-T cells in preserving the CD45RA+CCR7+ CD62L+ population in *ex vivo* expanded CAR-T cells (Figure 67).

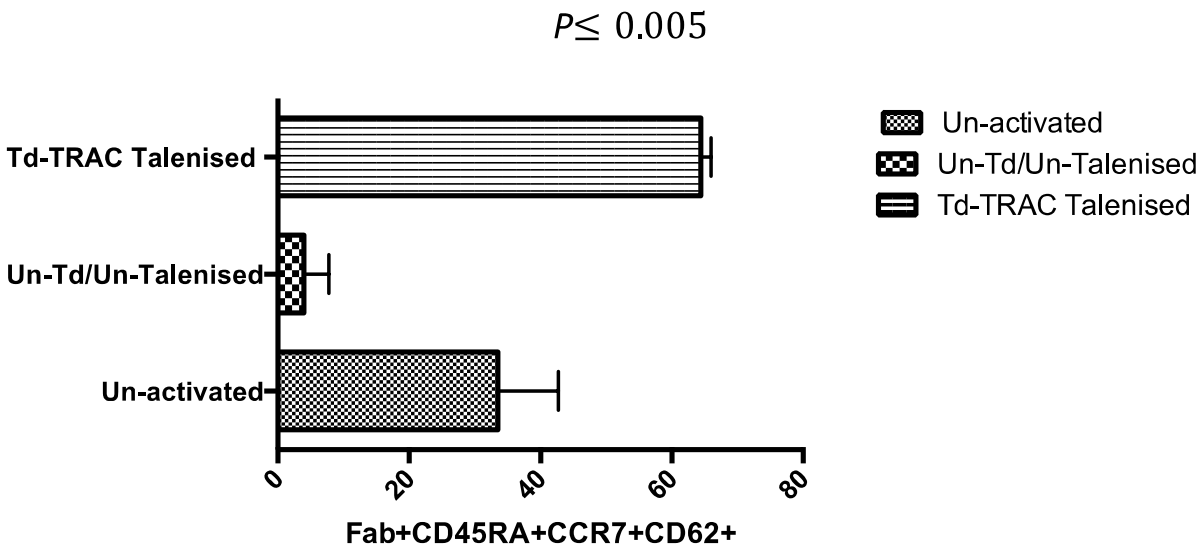


Figure 67: Co-expression of naive phenotype markers on Fab positive CAR19-enginereed CB-T cells. Bar chart illustrates phenotypic analysis of CB-T cells product after being transduced with Lnt-EFlα-CD19CAR and TALENised with TRAC TALENs mRNA. Un-manipulated cells (unactivated) were used as a control to gate the cells correctly on FlowJo software. Disrupted TCR cells (Td-TRAC Talenised) show remarkably high percentages of naïve-like phenotypic features, in comparison to the un-transduced and un-talenised cells.

6.7) Discussion:

Clinical trials involving the usage of chimeric antigen receptors targeting CD19 as a targeted immunotherapy for B-cell malignancies show a high therapeutic potency with a complete remission rate of >90 % (Nguyen et al., 2008, Maude et al., 2015). However, expertise and additional support from clinicians, researchers, pharmaceutical companies and investors are required for this therapy to have a solid infrastructure. Consequently, it emerges as a powerful tool to treat B-cell malignancies. Moreover, the low number of T cells from some individuals might limit its potency as a therapy (Qasim and Thrasher, 2014, Humphries, 2013). It has been proposed that generating pre-manufactured or engineered T cells might be a robust alternative approach, but it should overcome the HLA barrier and preclude the undesired αβ TCR on the infused T cells from leading to GVHD. In this chapter, the specificity of naïve T cells (cord blood T cells) was redirected towards the CD19 antigen by delivering chimeric antigen receptors targeting CD19 to the cells. In addition, it has been proven that they can be genetically edited by TRAC TALENs to eliminate the expression of the endogenous αβTCR. This has a therapeutic implication in regards to donor-derived T cells that are infused to achieve an antitumor effect without causing GVHD.

The TCR αβ assembly requires it to be a heterodimer in order to form a functional receptor on the cell surface. In this manner, disrupting either TRAC or TRBC will be sufficient to eliminate the expression of TCR αβ. Recent studies have shown that a mutation in the TRAC genome sequence has led to the loss of TCR αβ expression (Morgan et al.).

TALENs have been demonstrated to interrupt target gene expressions as an outcome of error-prone DNA DSB repair by a non-homologous end joining (NHEJ).This in most cases leads to a frame shift mutation resulting in a premature stop of translation. TALENs target a specific sequence within the genome and thus disrupt gene expression at the genomic level, which is an advantage over techniques that involve transcriptional repression and require sustained expression of the inhibiting factor (i.e. the enforced expression of shRNA to mediate TCR down-regulation). Due to the ability of TRAC TALENs to create a permanent disruption in the gene expression after transient delivery and expression, it enabled us to use *ex vivo* transcribed mRNA in a “hit-and-run” manner for electrotransfer of TRAC TALENs into CB-T cells.

As an alternative to allogeneic bone marrow transplant for hematopoietic reconstitution, an un-related human umbilical cord blood (CB) sample has been used successfully in various haematological disorders. This clinical application is approached because CB is readily

available and has a rich source of hematopoietic stem cells with exceedingly proliferative capacities (Hiwarkar et al., 2015). There are some reports that suggest that CB is comprised of phenotypically and functionally immature cytotoxic T lymphocytes (CTLs) with decreased alloantigen-specific cytotoxicity. In contrast to the CTLs in peripheral blood, the CTLs derived from cord blood show a high expression of CD45RA (a naïve T cell marker) and a low expression of CD45RO (an activated T cell marker).It has been proven by several studies that cord blood may be used for immunotherapy to treat a variety of hematopoietic disorders (Lee et al., 2011).

In this project, the strength of products from both CB-T cells and TALENs, were combined together to produce universal "off-the-shelf" engineered CB-T cells targeting CD19 antigen that are expressed by B-cell malignancies. The human application of "universally" engineered CD19-CAR+ CB-T cells that have been genetically modified with TRAC TALENs will rely on efficacy as well as safety. The genetically edited T cells have a specific cytotoxic behaviour toward primary targets and cell lines. Their therapeutic potential is also dependent on persistence after adoptive transfer. The second generation CAR designed for this study activates CB-T cells through chimeric CD28 and CD3-ζ. It remains to be determined in side-by-side clinical trials whether other CAR designs, such as signalling through CD137 and CD3-ζ, are superior. Safety depends on selective elimination of endogenous TCR and minimizing TALENs-mediated enzymatic activity at off-target sites. We did observe that the efficiency of enzymatic activity at TRAC TALEN genomic loci was approximately 22-60% (mean=40.28%) for CB cells activated with anti-CD3/anti-CD28 beads, while for cytokines stimulated CB cells, the percentages of TCR-disrupted T cells was 12-56% (with mean= 36.3%) after a single electrotransfer of an mRNA species coding for TRAC TALENs pairs. However, continued cell-surface expression of TCRs from HLA-disparate unrelated CB-T-cell donors may cause GVHD after adoptive immunotherapy. Therefore, to prevent GVHD after infusion we used αβTCR-specific paramagnetic beads to deplete and re-deplete T cells with residual expression of TCRs.

Most importantly, we also identified that the genetically edited CD19CAR-CB-T cell were retained in the expression of the naïve CD45RA isoform CD62L and CCR7, which might be the subset that correlates with *in vivo* expansion of TCR negative CD19CAR-T cells in lymphoma patients (Xu et al., 2014).

CD95 is another marker that was under investigation in this study. It has been suggested to be associated with a subset named “T-memory stem cells”. Moreover, our data from genetically edited CD19CAR-T CB-cells subsequently generated from healthy CB donors, show that the subset of CAR-redirected CD45RA+CCR7+ also co-express CD95, further indicating that this antigen-experienced population is indeed closely related to “T-memory stem cells” as it retains both phenotypic and functional elements of “naïvity” or “stem-ness.”

In conclusion, it has been demonstrated that TCRnegative-CD19 CAR+ CB-T cells can be generated using a genetic approach to diminish the expression of the undesired endogenous TCR with TALENs using a robust electrotransfer platform and to introduce CD19 CAR using a lentiviral vector gene delivery system. Our approach abolishes the danger of GVHD caused by adoptive transfer of large numbers of allogeneic T cells, while maintaining the desired effector functions mediated by CD19RCD28 CAR to target malignant B cells. This strategy provides an important step to developing a “universal” CD19CAR+ T cell, which can be manufactured from one unrelated CB donor and administered on demand to multiple patients. Subsequent studies are focusing on preventing rejection of the infused allogeneic TCR-negative CD19CAR+ CB-T cells by the recipient's immune system recognizing disparate HLA. For example, this may be accomplished by using genetic modifications including a nucleases-mediated knockout of HLA. Other issues require focus on the future studies for finding a robust means to extract T cells out from a CB-unit. Consequently, the activation process will be done accurately without adding an excessive number of beads. This can be achieved by using various, but specific kits, such as anti-CD3 or anti-CD62L paramagnetic beads before activating the cells with anti-CD3/anti-CD28 beads.

Chapter 7: Discussion

7) Discussion:

Therapeutic success following allogeneic HSCT is defined as achieving a GVL effect without risking the potentially lethal effects of GVHD. Several strategies have been tried with the aim of addressing the separation of GVL and GVHD after engraftment of allogeneic hematopoietic stem cells, such as adoptive transfer T-cell therapy (Walter et al., 1995). Although adoptive-T cell immunotherapy has led to remarkably successful outcomes in treating cancer, the inability to isolate and expand a sufficient number of high-avidity, cytotoxic-specific T lymphocytes (infiltrating T lymphocytes) for therapeutic purposes is the biggest obstacle to the success of the therapy (Pardoll, 2003). An alternative approach to generating large numbers of potent and specific T cells is to genetically engineer T lymphocytes to express an exogenous tumour antigen specific TCR or an artificial receptor known as CARs targeting precise TAA (Kapp et al., 2009, Willemsen et al., 2003)

The high risk involved with generating GVHD by the endogenous TCR in allogeneic settings after HSCT, the potential generation of harmful, unpredicted TCR specificities and the suboptimal activity toward their specific target in both autologous and allogeneic settings are the major drawbacks of antigen-specific TCR gene transfer therapy. Furthermore, the cytokines release storm is a serious adverse event that's associated with CAR T cell therapy (Gross and Eshhar, 2016, Klebanoff et al., 2016).

Here, we used TALENs mediated cord blood T cell engineering coupling with lentiviral transduction to produce universally applicable CAR19 T cells that can be administered to multiple patients without causing GVHD.These strategies could form the basis of a new gene therapy treatment against haematological malignancies and this discussion illustrates some of the aspects that must be further considered before application of this technology.

7.1) Delivery and processing of cord blood units:

Cord blood units should be as fresh as possible to ensure the presence of high numbers of efficient viable T-cells. Cord blood units used in this study were supplied by the Anthony Nolan Cord Blood Bank and delivered to our facility from Nottingham. As a result, in some events the time between collection and processing of the blood might have been over forty-eight hours, which might led to a huge loss of the desired cells. Likewise, it is a challenging task to extract the buffy coat out from the ficolled old blood. Another issue associated with cord blood is the existence of enormous immature nucleated red blood cells, or "red blood cell contamination". Cord nucleated red blood cells might be counted false positively as lymphocytes leading to miscalculation of the amount of anti-CD3/anti-CD28 beads that are

required to activate T-cells at 1 bead: 1 cell ratio. The addition of excessive beads may result in producing highly differentiated and exhausted engineered cells that may not be able to survive and enter the apoptosis phase in a quick manner. For these reasons, several strategies are required to eliminate the superfluous red blood cells that might lead to undesirable effects. Staining the cord blood mononuclear cells with anti-CD3 prior to activation was used as one tactic to determine the definite percentage of lymphocyte. Automated calculation of lymphocytes using the C-max machine was also considered as one technique to mitigate red blood cell amounts. Another strategy to eliminate red blood cell contamination might be to positively select out red blood cells from the mononuclear cells by using MACS system and immunological beads against red blood cells. All these techniques have not yet proven their efficiency and need more optimization. A more proficient approach might be a positive extraction of the naïve lymphocytes by using microbeads to select CD62L or CD45RA positive cells.

7.2) Optimisation of culturing and expansion system for engineered universal CAR19 CB-T cells:

Culturing the genetically altered cells in an optimal environment to have a large number of cells is a crucial requirement to generate off- the- shelf T cells as a therapeutic product (Hodi et al., 2010). Recently, the G-rex expanding system has emerged as one of the most effective culturing techniques in adoptive immunotherapy (Bajgain et al., 2014). This culture container provides the cells with unlimited gas exchange via a gas transmembrane. Although the effectiveness of this device has been proven in many experiments; here, following electroporation, TALENised and CD19CAR modified T cells were not be able to expand as much as expected. Several factors might account for this: treated cells that have been activated with anti-CD3/anti-CD28 beads may have been exhausted and entered the apoptosis phase rapidly and therefore, could not tolerate 17 days in culture (Wherry, 2011). In the other case; where cells that were stimulated with the cytokines cocktail alone, the stimuli may not have been considered to be not strong enough to generate survival signals in order to keep the naïve cells viable. As well as modified CD19CAR cells expressing CD19CAR, they carry RQR8, a compact/marker suicide gene moiety transcriptionally linked to CAR19 and it is an epitope from the CD34 molecule (Philip et al., 2014). Following lentiviral transduction, uncoupling of RQR8 from CAR19 of detectable cell surface expression of CD34 presumably resulted in reduced sensitivity to select CD34 CAR19 T cells after transduction. Consequently, a number of cells that would be selected based on the expression of CD34 will

be affected. New strategies need to be considered. Interestingly, one study suggested that naïve lymphocytes are required to be activated at Day one with anti-CD3/anti-CD28 beads, later kept in culture with the presence of IL-7 and IL-15; gamma cytokines responsible for proliferation and expansion of T cells (Cieri et al., 2013). Another advantage of culturing modified cells in the presence of IL-7 and IL-15 is the significant lower levels of Tregs; a favorable condition for adoptive T cell therapy, when compared to culturing the cells in IL-2 culture condition (Cha et al., 2010).

7.3) Viral vectors:

The fact that self-inactivating second generation WT1 TCR coding LV vector and a third generation CD19- CAR coding LV vectors have been used to transduce the CB- T cells appears not to imitate the transduction efficiency. The only main differences between these two viruses concern safety issues. In addition, both viruses were coated with the VSV-G envelope glycoprotein, therefore should be able to infect CB-T lymphocytes with the same efficiency (Ratner et al., 1985b). The only concern regarding viral vectors is the possible variation that could be introduced into data as a result of using different preparations of lentiviral vector. However, preparing enough vector stock to perform all the experiments could help to eliminate this.

7.4) Engraft of antigen-specific CB-T cells in NOD/SCID mice:

In anticipation of further testing of our results relating to the use of CB-T cells as an off-the-shelf therapy, an *in vivo* assessment is essential. In future research, a SCID/NOD mouse will be injected with tumour cells (BV173 leukaemia cells or SupT1 cells), leading to the production of specific cancerous cells expressing TAA. A large number of expanded CD19-CARs or WT1-TCRs CB-T lymphocytes will be injected intravenously into the mouse followed by the monitoring of the elimination of cancerous cells without causing axenoreactivity.

An *in vivo* experiment to investigate the anti-tumour activity of engineered cord and adult CD19CAR T cells was conducted by Dr.Christos Georgiadis and Mr.Roland Preece using NOD SCID gamma male (NSG) mice. PBMCs or CB (effector cells) were processed by Ficoll for the isolation and culture of T cells. Cells were activated using Dynal beads (1:1 based on lymphocyte count; therefore, 3:1 based on CD3 count) in X-VIVO 15 media supplemented with 5% HS and 100 units/ml of r-IL2. Cells were transferred to retronectin coated bags the following day and transduced at an MOI of 5 with pCCL-EF1a-CAR19 vector. Cells were transferred and expanded in G-Rex 10 flasks for twelve days and

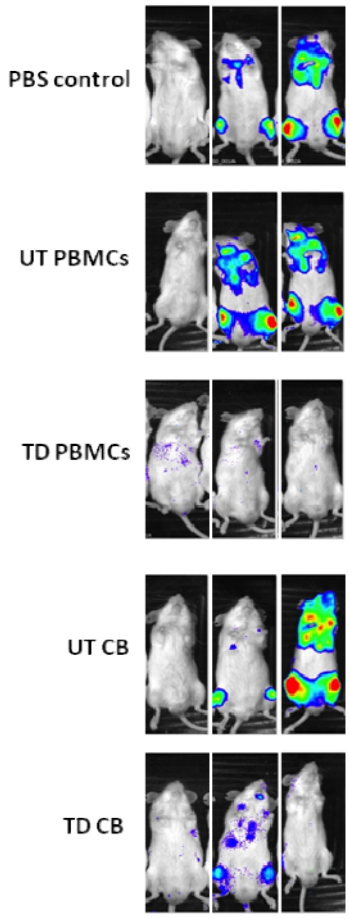
phenotyped for transduction efficiency evaluation using anti-Fab; Fab expression was 50% for both naïve and adult cells. Cells were cyropreserved in $5x10^6$/ml or $10x10^6$/ml aliquots in 90% FCS/10% DMSO. Both UT and TD populations were cultured and expanded in the same manner.

For *in vivo* studies, male NSG mice were ordered at 4 weeks for use at 6 weeks. $5x10^5$ Luciferace transduced Daudis were inoculated intravenously (IV) via tail vein on Day 0. Animals were imaged by *in vivo* imaging instrument (IVIS) on Day 1. On Day 2, effector T cells were thawed and counted for IV infusion. Cell numbers required were $4x10^6$ transduced (TD) cells (therefore $8x10^6$ cells assuming the 50% TD efficiency). An equal number ($8x10^6$) cells/animal were counted for the control untransduced (UT) animals. Due to cells numbers, only one mouse was injected with no Daudi + PBS, three with Daudi + PBS, five with Daudi + UT PBMCs, three with Daudi + TD PBMCs, five with Daudi + UT CB, and seven with Daudi + TD CB.

Mice were imaged by IVIS on Day 6, Day 9 and Day 14 by IP injection of luciferin at an optimal time-point and concentration.

Marrow and spleen were harvested on Day 14. Marrow and spleen were lysed and processed for staining according to Dr.Brian's phillip (UCL Cancer Institute, UK) protocol and full panel FACS staining was carried out. Tumour cells and T cells were identified in both marrow and spleen in animals receiving UT effectors. Tumour cells were cleared in both cohorts of mice receiving TD PBMCs, or CB cells (Figure 68).

A)



B)

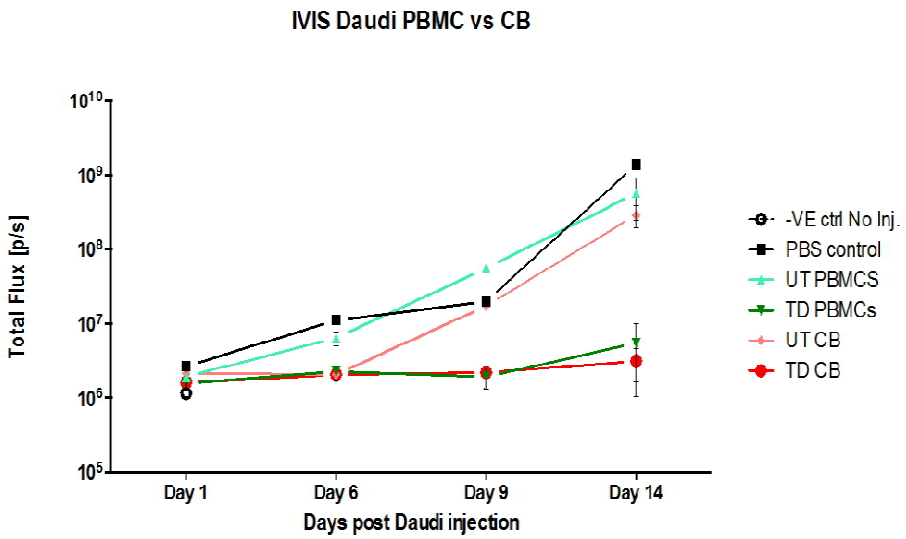


Figure 68: ***In vivo*** **activity of CD19CAR engineered CB and adult T cells on Daudi cells**. A) Representative experiment illustrates slower tumor growth followed by rapid rejection of B-cell lymphoma in mice receiving allogeneic CAR19 engineered CB-T cells and allogeneic PB T cells compared with mice receiving and control mice. B) Graph shows a drop in the percentages of Daudi cells post the injection of CAR19 engineered cells (CB and PBMCs); while for control cohort and UT group, the percentages of Daudi were still high even after receiving the modified CAR19 cells.

7.5) Genome editing technology to knock out TCR expression:

Mis-pairing endogenous and exogenous TCR chains has been clearly linked to sever toxicity in a pre-clinical model using mice (Aggen et al., 2012).Therefore, the absence of the endogenous TCR expression on TCR-edited cells is essential for the more robust and homogenous expression of the introduced antigen-specific receptor and for completely overcoming the safety issue raised by the risk of inducing autoimmune diseases. Although a large number of genetic modifications of exogenous antigen-specific receptors have been identified as a means of reducing the extent of this impairing effect, strategies that are able to completely abrogate this risk have not been previously reported. Several reports and studies have evaluated the potential use of a siRNA and ZFNs expression to specifically down regulate endogenous TCR expression. Despite their encouraging outcomes, both approaches reduced, but did not eliminate, the expression of endogenous TCR (Gaj et al., 2013).

In this project, we tested the feasibility of genome-editing tools such as meganucleases TALENs and CRISPR/Cas9 to knock out the expression of endogenous TCR allowing for rapid generation of a large numbers of tumour-specific T lymphocytes expressing high functional levels of only the desired TCR, which eliminates the risk of TCR miss-pairing. Concluded results associated with the usage of meganucleases demonstrated that this tool is not effective to knock out TCR expression and it might have a toxic effect on the cells. So, an alternative platform was selected.

TALENs have been designed and assembled to target sequences within the TCR α and β chains genes. A pair of TALENs targeting sequences within the constant region of the alpha chain was nucleofected as DNA or mRNA into various types of T cells: Jurkat, PBMCs and cord blood T cells. FACS analysis for the expression of endogenous TCR of the cells used in this study suggested that TALENs has the ability to recognise its target and reduce the expression of endogenous TCR in a remarkable manner. However, further investigation is required to ensure the safety of using this genetic-modified tool in clinics.

Distinct from site-specific nucleases that have been described earlier, CRISPR/Cas9 system has widely emerged as an effective alternative approach to MGNs, ZFNs, and TALENs. Here, the forcefulness of CRISPR/Cas9 to knock out the expression of TCR was tested. Promising results were produced with a satisfying percentage of knock out; however, further experiments are required to elucidate the effect of CRISPR/Cas9 on TCR at the molecular level. To further investigate CRISPR/Cas9 system, some unnecessary elements, such as the

marker genes red cherry and its promoter sv40 should be removed. Another issue that should be taken in consideration is the way to deliver sgRNA and Cas9 in to T cells. A further experiment should test the feasibility of CRISPRs (gRNA) and Cas9 into third generation lentiviral vector backbones and produce a robust batch of lentiviral vectors that carry CRISPR/Cas9 system.

Further investigation should be taken to decide either to shift to CRISPR/Cas9 or proceed with TRAC TALENs.

The ethical use and safety of genome editing technology remains a fundamental issue that pulls this technology away. These problems were shoved into the spotlight in April, when news broke that scientists had used the CRISPR/Cas9 system to engineer the endogenous beta globin gene; the cause of beta thalassemia once it is mutated, in human zygots (Liang et al., 2015). The embryos whose genomes were edited were unable to result in a live birth; in addition, a surprisingly large number of off-targets were detected, and the paper has generated heated debate over whether the CRISPR/Cas9 system should be used to make heritable changes to the human genome.

There are other concerns about genome editing technology, such as its potential risks to genome edits and the concern that edited organisms could disrupt entire ecosystems (Baltimore et al., 2015). Scientists are eager to conduct more studies to prove whether these concerns are warranted and to develop strategies for better targeted genome technology.

Major safety concerns have been obviated in a recent clinical trial conducted by Professor Waseem Qasim and his team (Great Ormond Street Hospital, UCL, UK) to treat an infant with refectory relapsed B-ALL. It is the first scientific team to use TALENs edited cells in a human with remarkable success (Qasim et al., 2015a).

Additional improvements to generate universal, genetically engineered T cells may include denuding T cells of HLA molecules; consequently, these cells might evade immunological recognition and reduce the risk of rejection (Torikai et al., 2013).

7.6) Conclusion:

To sum up briefly, this project indicates that, in the field of T cell immunotherapy, engineered, antigen-specific cord blood T cells should be the first choice in terms of cells that should be used in “on-the-shelf” therapy. This holds the added benefit of eradicating the time and expense limitations of antigen-specific gene transfer therapy. Supporting this conclusion is the fact that CB-T cells have shown a significant expression level of WT1-TCR and CD19-CAR genes following experiments with lentiviral gene delivery. Moreover, remarkably

positive results from functional trials support the previous hypothesis. It has been proposed that genome-editing technology can be used as a solution to the limitations of antigen-specific gene transfer therapy. The feasibility of MGN and TALE nucleases' ability to disrupt the sequence within the genome of TCR alpha or beta chains has been tested *in vitro*. The use of MGN was shown to be unsuccessful in causing genome disruption and this might be due to the toxic effect of MGN on the cells. TALENs studies demonstrated that the knocking-out expression of endogenous TCR might be feasible. Although this study appears to lead to the conclusion that CB-T cells are a valuable tool in T cell immunotherapy, and that genome-editing technology might be the solution to most of the drawbacks involved in antigen-specific gene transfer therapy, further research and monitoring is essential for the development of a more comprehensive understanding of this field.

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