KU LEUVEN

FACULTEIT BIO-INGENIEURSWETENSCHAPPEN

Generation of T cell receptor-redirected human T cells and the characterisation of their anti-tumour activity

Ontwikkeling van humane T cellen met gemodificeerde T-celreceptor en karakterisering van hun anti-tumoractiviteit

Promotoren:
Prof. Jan Paeshuyse
Departement Biosystemen
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Prof. Viggo Van Tendeloo

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Masterproef voorgedragen
tot het behalen van het diploma van
Master of science in de bio-ingenieurswetenschappen:
cel- en gentechnologie

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Preface

Improvise. Adapt. Overcome. Citizens of the internet know these three words all too well. In some way, research is not that different from survival in the wild. Unexpected things happen all the time and as a researcher you need to deal with them. Nonetheless, some of these surprises may mean that a lot of work has to be redone and this can be demotivating. Therefore, I'd like to devote this page to the people that have supported me throughout this year to keep up the motivation.

Diana, it is hard to explain in words how grateful I am that I had you as my supervisor. *"Hakuna matata*", it means "no worries", was your motto and it became mine as well. You've taught me so much by answering all my questions and by brainstorming to solve my stupid mistakes in the last step of an experiment. Your repeated advices, such as to "pipet up and down" to "resuspend well" still haunt me in my dreams, but it greatly improved my lab work. Lastly, I admire your patience with me and your self-sacrifice during this year. It inspired me and made me realise even more how much I want to stay in the field of research for the upcoming years. Thank you.

The whole Laboratory of Experimental Haematology deserves a spot in this list as well for giving me a warm welcome at the beginning of my internship and the guidance and friendship throughout the year. In particular, I would like to thank prof. dr. Viggo Van Tendeloo for taking a leap of faith by accepting the first bioscience engineer in his lab; Hans for his very appreciated help in the lab and the interesting or comical discussions during lunch; Heleen for her tips and tricks and all the silly moments inside and outside of the office; and the rest of the TIGr group for the continuous help and support.

I want to send some love to my girlfriend, Rani, for biking with me through sun and rain to the hospital and spending lunchtime together whenever our schedules allowed us to. I adore you for your perseverance and attempts to make the impossible possible. Thank you for being you!

My friends and family actually deserve an apology for the hours of complaining about how nothing is going as planned. Having a well-cooked meal, either with my parents or together with my fellow thesis students, followed by a relaxing evening always cheered me up and was well appreciated.

Finally, I would like to thank all the people that I could not or forgot to mention.

English Abstract

Background: Immuno-gene therapy using T cell receptor (TCR)-redirected T cells is currently being tested as a cancer therapy in early phase clinical trials. Most studies are exploiting integrating retro- or lentiviral vectors to permanently introduce therapeutic TCR in the T cells. However, permanent expression of the transgene TCR may pose serious safety issues when treatment-related toxicities would occur. This thesis mainly investigated whether 1) human CD4 T cells can be efficiently transfected with a Wilms' Tumour 1 (WT1)-specific HLA-A2-restricted TCR using a nonviral RNA-based transfection method, and 2) if these TCR-transfected CD4 T cells can be activated upon recognition of their cognate antigens.

Method: The Jurkat E6.1 cell line, resting or pre-activated CD4 T cells were transfected according to our in-house developed double sequential electroporation (DSE) method, first with (S) or without (M) wild type TCR DsiRNA to suppress endogenous TCR expression and then 24h later with $WT1_{37\text{-}45}$ *TCR* mRNA (T37) in conjunction or not with *CD8* mRNA (CD8). Subsequently, TCR-redirected CD4 T cells were co-cultured with $WT1_{37\text{-}45}$ presenting tumour cells. Flow cytometric analysis determined surface expression of T37 TCR, CD8 and activation markers CD69, CD137 and CD154. Lastly, IFN-γ and granzyme B secretion was quantified via ELISA for the pre-activated CD4 T cells.

Results: TCR transfection efficiency in Jurkat E6.1 cells, serving as a model for CD4 T cells, reached almost maximal values (>98% tetramer(+) cells) after DSE with ST37+CD8. Substantially lower levels of TCR expression were observed when transfection of DsiRNA and/or co-transfection with CD8 was omitted. Importantly, we were able to successfully engineer difficult-to-transfect primary CD4 T cells with a WT1-specific TCR using our DSE protocol, albeit with a lower efficacy than seen in Jurkat cells. Transfection rate in primary resting CD4 T cells was variable and ranged from 3 to 46% (average 24%), while preliminary data show a more robust and reproducible efficiency of >60% when CD4 T cells undergo a pre-activation step prior to DSE. When co-cultured with WT1-positive target cells, only pre-activated DSE CD4 T cells co-transfected with CD8 displayed an upregulation of CD69, CD137, CD154. Granzyme B appeared to be released upon antigen recognition in conditions independently of CD8 co-transfection, unlike IFN-γ secretion. Nonetheless, DsiRNA transfection improved secretion levels for both molecules.

Conclusion: Our DSE protocol involving transfection of DsiRNA, T37 *TCR* mRNA and *CD8* mRNA represents a valid method to efficiently engineer CD4 T cells with a transgene

TCR. CD8 co-expression and suppression of endogenous TCR expression by siRNA positively influences transgene TCR expression and function. Notably, high TCR expression levels and effector functions upon TCR triggering by cognate antigen seems to require pre-activation of the CD4 T cells prior to TCR engineering. The latter data on pre-activation warrant confirmation in a larger cohort of donors.

Dutch Abstract

Achtergrond: Immuno-gen therapie met T cel receptor (TCR) gemodificeerde T cellen wordt momenteel in vroege fase klinische studies getest als kanker therapie. De meeste studies exploiteren integrerende retro- en lentivirale vectoren om permanent therapeutische TCR in de T cellen te introduceren. Permanente expressie van de transgene TCR kan echter ernstige veiligheidskwesties opleveren wanneer behandelingsgerelateerde toxiciteit zich voor doet. Deze thesis onderzocht of 1) humane CD4 T cellen efficiënt getransfecteerd kunnen worden met een Wilms' Tumor 1 (WT1)-specifieke HLA-A2-gelimiteerde TCR door middel van een nonviraal RNA-gebaseerde transfectiemethode, en 2) of deze geëngineerde CD4 T cellen geactiveerd kunnen worden bij de herkenning van hun antigen.

Methode: De Jurkat E6.1 cellijn, rustende of gepreactiveerde CD4 T cellen werden getransfecteerd volgens onze zelf ontwikkelde dubbele sequentiële elektroporatie (DSE) methode, eerst met (S) of zonder (M) wild type TCR DsiRNA om de endogene TCR expressie te onderdrukken en 24h later met $WT1_{37\text{-}45}$ *TCR* mRNA (T37) in combinatie met of zonder *CD8* mRNA (CD8). Vervolgens werden TCR gemodificeerde CD4 T cellen in cultuur gebracht met $WT1_{37\text{-}45}$ presenterende tumor cellen. Via flowcytometrische analyse werd de oppervlakte-expressie van de WT1 TCR, CD8 en de activatiemerkers CD69, CD137 en CD154 op de CD4 T cellen bepaald. Ten slotte werd IFN-γ en granzyme B secretie voor de pre-geactiveerde T cellen via ELISA gekwantificeerd.

Resultaten: TCR transfectie efficiëntie in Jurkat E6.1 cellen, die dienen als model voor CD4 T cellen, bereikte bijna maximale waarden (>98% tetrameer(+) cellen) na DSE met ST37+CD8. Er werd substantieel minder TCR expressie waargenomen wanneer de transfectie van DsiRNA en/of cotransfectie van CD8 werd weggelaten. Belangrijk is dat we erin slaagden om moeilijk te transfecteren primaire CD4 T cellen te engineeren met een WT1-specifieke TCR met ons DSE protocol, zij het met een lagere efficiëntie dan in Jurkat cellen. De efficiëntie in primaire rustende CD4 T cellen was variabel en varieerde tussen 3 en 46% (gemiddeld 24%), terwijl preliminaire data een robuustere en efficiëntere transfectie van >60% laat zien wanneer de CD4 T cellen worden gepreactiveerd voor DSE. Na co-cultuur met WT1-positieve target cellen vertoonden enkel gepreactiveerde DSE CD4 T cellen met CD8 een opregulatie van CD69, CD137 en CD154. In tegenstelling tot IFN-γ secretie, leek granzyme B onafhankelijk van CD8 cotransfectie te worden vrijgezet bij herkenning van het antigen. Desalniettemin verbeterde DsiRNA transfectie de secretie van beide moleculen.

Conclusie: Ons DSE protocol met transfectie van DsiRNA, T37 *TCR* mRNA en *CD8* mRNA is een bruikbare methode om CD4 T cellen efficiënt te engineeren met een transgene TCR. CD8 co-expressie en suppressie van de endogene TCR door siRNA verbeterd de transgene TCR expressie en functie. Merk op dat een hoog TCR expressie niveau en de effector functies bij herkenning van het verwante antigen een preactivatie van de CD4 T cellen vereisen. Deze laatste observaties rond preactivatie dienen bevestigd te worden in een grotere groep donoren.

Abbreviations and symbols

Abbreviation	Full
ABL	Abelson murine leukaemia viral oncogene homolog
ALL	Acute lymphoid leukaemia
Allo	Allogeneic
AML	Acute myeloid leukaemia
AP	Accelerated phase
AP-1	Activator protein 1
APC	Antigen presenting cell
Auto	Autologous
BCR	Breakpoint cluster
BCR	B cell receptor
BM	Bone marrow
BP	Blast phase
CAR	Chimeric antigen receptor
Cas-9	CRISPR associated protein 9
CDK4	Cyclin dependent kinase
CLL	Chronic lymphoid leukaemia
CML	Chronic myeloid leukaemia
CP	Chronic phase
CR	Complete response
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
CTL	Cytotoxic lymphocyte
CyR	Cytogenetic response
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DSB	Double stranded break
DSE	Double sequential electroporation
DsiRNA	Dicer-substrate siRNA
E2A	Equine rhinitis A virus 2A sequence
FBS	Foetal bovine serum
FDA	U.S. Food and Drug Administration
FISH	Fluorescence *in-situ* hybridisation
FLT3	FMS like tyrosine kinase 3
gp100	Glycoprotein 100
GVHD	Graft-versus-host disease
GVT	Graft-versus-tumour
hAB	Human AB serum

Abbreviation	Full
HLA	Human leukocyte antigen
HSC	Haematopoietic stem cell
HSCT	Haematopoietic stem cell transplantation
IFN	Interferon
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
Ion	Ionomycin
IPO8	Importin-8
IRES	Internal ribosomal entry site
ITAM	Immunoreceptor tyrosine-based activation motifs
LTR	Long terminal repeats
MAGE	Melanoma associated antigens
MAPK	Mitogen-activated protein kinase
MART-1	Melanoma-associated antigen recognised by T cells 1
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
MHC-I	MHC class I
MHC-II	MHC class II
miRNA	Micro RNA
MR	Molecular response
MRD	Minimal residual disease
mRNA	Messenger RNA
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor κB
NKG2D	Natural Killer Group 2D
ORR	Objective response rate
OS	Overall survival
P2A	Porcine teschovirus 2A sequence
PAM	Protospacer adjacent motive
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
Ph	Philadelphia
PMA	Phorbol myristate acetate
pMHC	Peptide:MHC
PR	Partial response
RB	Retinoblastoma
RBC	Red blood cells
RISC	RNA-induced silencing complex

Abbreviation	Full
RPL13A	Ribosomal protein L13A
RPMI	Roswell Park Memorial Institute 1640
RSR	Relative survival rate
RT-qPCR	Real-time quantitative PCR
sgRNA	Single-guide RNA
siRNA	Silencing RNA
SNP	Single nucleotide polymorphisms
T37	$WT1_{37\text{-}45}$-specific TCR
TAA	Tumour associated antigen
TALEN	Transcription activator like effector nucleases
TAP	Transporter associated with antigen presentation
T_{CM}	Central memory T cell
TCR	T cell receptor
T_{EFF}	Effector T cell
T_{EM}	Effector memory T cell
TETAR	T cells expressing two additional T cell receptors
TF	Transcription factor
TIL	Tumour infiltrating lymphocyte
TKI	Tyrosine kinase inhibitor
TM	Transmembrane
TP53	Tumour protein p53
TRAC	T cell receptor alfa constant region mRNA
tracrRNA	Transactivating crRNA
TRBC	T cell receptor beta constant region mRNA
TRP-1/2	Tyrosine related protein 1/2
TSP-1	Thrombospondin-1
VEGF-A	Vascular endothelial growth factor-A
WBC	White blood cells
WHO	World Health Organisation
wt	Wild type
WT1	Wilms' tumour 1
ZFN	Zinc finger nuclease

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Literature review

This part has the purpose of providing the reader of some of the basic information required for this thesis. First, the general aspects of cancer development are briefly covered based on the eight hallmarks and two enabling characteristics defined by Hanahan and Weinberg (1). Subsequently, more information about the development, incidence and treatments of the most prevalent types of leukaemia will be discussed. Lastly, several approaches in adoptive cell transfer will be covered, including tumour-infiltrating lymphocytes, T cells with chimeric antigen receptors and T cell receptor-modified T cells. As the latter approach is a main component of this thesis, it will receive considerably more attention. This part provides a brief introduction to T cells and the isolation and delivery of TCRs, as well as some problems encountered during the development of this approach and their solutions. To conclude this overview, several studies about TCR-redirected CD8 and/or CD4 T cells are mentioned to illustrate the potential of this approach.

1 Cancer

1.1 Cell cycle

Multicellular organisms rely on dividing cells for their growth and development. By communicating with each other, cells determine the size of their population, when they have to divide or when they have to die (2). Once a tissue or organ reaches its normal size, cell proliferation slows down to a level that maintains the population as it is. This tightly regulated process is known as the cell cycle. The cycle consists of a G1-, S-, G2- and M-phase and is, with a few exceptions, always followed in this order. A major part of the cycle is the G1-phase, which consists of many (conditional) processes. Cells in the G1-phase perform tasks that are specific to the organ or tissue. A cell can stay at the beginning of this phase, often called G0, as long as it does not receive any proliferative signals. Neurons and muscle cells, for example, are permanently in the G0-phase and cannot be malignantly transformed (2). These (anti-)proliferative signals influence the growing and differentiation behaviour of the cells and cause them to proceed to the DNA synthesis phase, or S-phase. In between the G1- and S-phase, however, there is a restriction point (R-point), or checkpoint, where the cell determines whether the preceding steps in the cell cycle were performed correctly. Noticeably, some of these mechanisms are crucial, and any abnormality may result in malignantly transformed cell types. In the S-phase, all the genetic

material is carefully doubled before entering the G2-phase, where the cell will divide. Errors in the DNA of the daughter cells can result in either cell death or disease. The G2-phase is fairly similar to the G1-phase, where proteins are produced to prepare for mitosis. The cell cycle ends with the mother cell dividing into two daughter cells, each with a copy of the DNA and half of the proteins and organelles.

1.2 Hallmarks of cancer

Tumour cells are not limited in their growth like normal cells. They are, among other things, insensitive to restriction of growth due to population density (3). These neoplasms can be benign or malignant. Benign tumours are generally harmless as they grow in a confined area and do not show invasive behaviour (3, 4). Conversely, malignant neoplasms do invade surrounding tissues and can spread, or metastasise, to other parts of the body through the bloodstream or lymphatic system.

Already in the year 2000, Hanahan and Weinberg defined six hallmark capabilities of cancer that normal cells have to acquire to eventually progress to malignant cells (Figure 1): sustaining proliferative signalling, evading growth suppressors, resisting cell death,

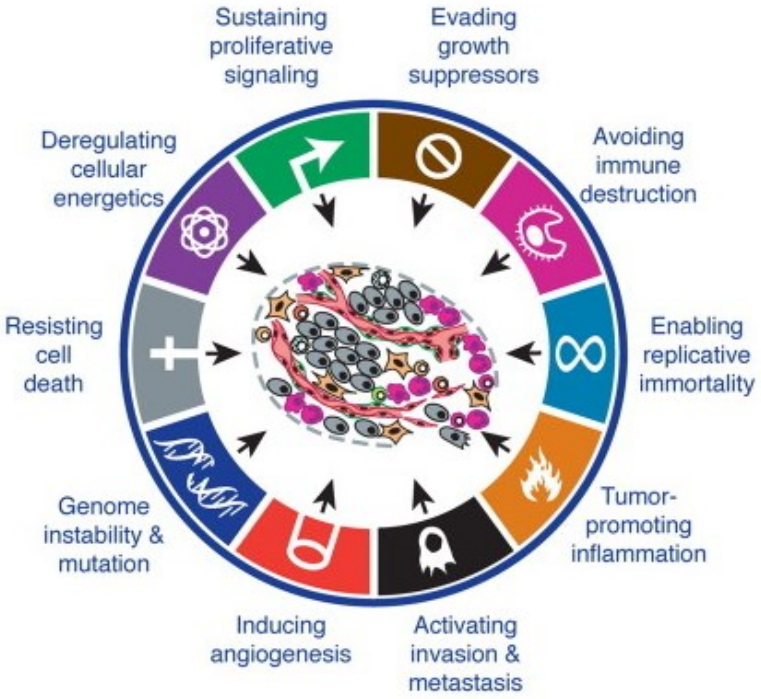

Figure 1 | Hallmarks of cancer and their enabling characteristics.

Malignant cells are the result of the acquisition of eight hallmark capabilities by normal cells. This process is facilitated by two enabling characteristics: Genome instability and inflammation. Adapted from Hanahan & Weinberg (2011) (1).

enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (5). They revisited this topic in 2011 and added reprogramming of the energy metabolism and the evasion of destruction by the immune system as two emerging hallmarks to the list (1). They also described two important processes, or "enabling characteristics", to obtain these hallmarks: genome instability and inflammation (Figure 1). What follows is a brief overview of these enabling characteristics and hallmarks.

1.2.1 Enabling characteristics

1.2.1.1 Genomic instability

Genetic instability is described by Hanahan and Weinberg (1) as possibly the most important enabling characteristic of multiple hallmarks of cancer. Subsequent mutations in the genome that are not repaired or problems with the stability of the chromosomes can lead to cancer (3). The cells will either die or, because of their mutant genotypes, gain a selective advantage over their surrounding cells and become a dominant subpopulation in a local tissue environment (1). Some mutation and genetic alterations in leukaemia will be briefly covered later in this review.

1.2.1.2 Inflammation

For more than three decades, it has been known that cells of the innate and adaptive immune system infiltrate the tumours in a way that are similar to inflammatory conditions (6). Later discoveries surprisingly indicated that the tumour-associated inflammatory response could enhance tumorigenesis and progression, and promote the hallmark acquisition with the supply of bioactive molecules in the tumour environment (1). For example, reactive oxygen species and reactive nitrogen intermediates are able to induce damage to the DNA and accelerate the growing mutational load in nearby cancer cells (7). In their book, Krawczyk *et al.* cover several cytokines, chemokines and transcription factors that play a role in the effect of inflammation in the development of leukaemia (8).

1.2.2 Hallmarks

1.2.2.1 Sustaining proliferative signalling

Probably one of the best-known characteristics of cancer cells is their capacity of sustaining their proliferation. As mentioned above, normal cells follow a tightly controlled sequence of events and require growth-promoting signals to progress through the cell cycle. Cancer cells, however, develop several ways to deregulate these signals (1). Among them are the autocrine proliferative stimulation by producing growth factors that can bind the receptors on the cell surface or the stimulation of normal cells by cancer cells to produce these ligands

for them. Another possibility is the hyperresponsiveness to the available concentration of ligand by upregulating the number of receptors displayed on the cell surface or the independency of the ligand by altering the structure of the receptor molecules resulting in activation of the proliferative signalling pathways without the presence of the ligand (1). Alternatively, this ligand independence can be acquired by constitutively activating parts of the signalling pathway downstream of the receptor.

1.2.2.2 Evading growth suppression

Next to promoting stimulating signals for growth, cancer cells have to evade pathways that are engineered to limit cell proliferation. These pathways often depend on tumour suppressor genes, including retinoblastoma (*RB*) and tumour protein p53 (*TP53*) (1). The cell uses RB to decide whether to progress through the cell cycle in a response to the integration of internal and external signals (9, 10). On the other hand, the cell measures the damage to its DNA, levels of nucleotides, glucose and oxygenation. By combining these inputs, TP53 can stop the cell cycle progression or, in cases of irreparable damage, induce apoptosis, a type of programmed cell death (1).

1.2.2.3 Resisting cell death

As mentioned above, TP53 is one of many proteins that can induce apoptosis (11). A balance between the pro- and antiapoptotic proteins of the Bcl-2 family control the apoptotic trigger (12). Bcl-2, for example, is an inhibitor by suppressively interacting with proapoptotic proteins, like Bax and Bak, in the outer membrane of the mitochondria. When this suppressive state is lifted, the proteins can disrupt the outer membrane resulting in a release of signalling molecules of which cytochrome c is the most important one. Subsequently, these molecules induce a cascade of caspases that ultimately results in the death of the cells.

Tumour cells develop several strategies to avoid apoptosis with a loss-of-function mutation in the TP53 protein among the most frequently occurring ones (1), eliminating an important sensor of damage from the apoptosis-inducing network. As an alternative, by downregulating proapoptotic factors, the cell can upregulate the expression of antiapoptotic regulators or survival signals, or it can completely block the ligand-induced apoptotic pathway.

1.2.2.4 Enabling replicative immortality

Most of the normal cells in the human body have the capacity to divide only a limited number of times. The telomeres, which consist of multiple hexanucleotide tandem repeats,

protect the chromosomal ends from erosion and end-to-end fusions. After every cell division, the telomeres get shorter until the ability to protect the chromosome is lost. This mechanism plays a crucial role in the unlimited proliferation capacity of cancer cells (13). Unlike most normal cells, a large portion of spontaneous immortalised cells, including human cancer cells, succeed in significantly expressing telomerase, an enzyme that is able to extend the telomeric DNA. Therefore, the presence of the enzyme is associated with a resistance to both senescence (a non-proliferative but viable state) and apoptosis, which are two important defence mechanisms against cancer. Alternatively, cancer cells can maintain their telomere length by means of a recombination-based mechanism by exchanging inter- or intrachromosomal sequence information (5, 14).

1.2.2.5 Inducing angiogenesis

A constant flow of nutrients and oxygen, and a way to dispose of carbon dioxide and metabolic waste are required by both normal and cancer cells. The process of angiogenesis (the formation of new blood vessels from existing ones) does only occur during embryogenesis, wound healing and the female reproductive cycle (1). During cancer development, an "angiogenic switch" is turned on resulting in the continuous formation of new blood vessels that support the neoplastic growth (15). Factors that induce or suppress angiogenesis are thought to control this switch. The best-known inducer and suppressor are vascular endothelial growth factor-A (VEGF-A) and thrombospondin-1 (TSP-1), respectively (16, 17).

1.2.2.6 Activating invasion and metastasis

Already in 1889, Paget noticed that the frequency of metastasis in certain organs is higher than in others (18). The discrepancy between the blood supply to the organs and the prevalence of metastases in these organs was remarkable. Therefore, he proposed his "seed and soil" hypothesis in which he describes that, similar to plants, in order to allow survival and proliferation of the cancer cell (the seed), the new environment (the soil) has to be compatible. Even more than 100 years later, this hypothesis is still widely accepted (19).

Successful metastasis requires several, sequential steps that have to be completed without any problems, some of which were already discussed above (20). A primary tumour – that was formed by a cellular transformation of a cell to a neoplastic cell – uses nutrients that are supplied by diffusion into the tissue (19). Then, by forming new vascular tissue in the process of angiogenesis, sufficient nutrients are transported to the tumour to maintain its growth. Eventually, in case of malignant cells, local invasion of the host tissue will occur

followed by detachment of the cells into the bloodstream. Most of the cancer cells, however, are killed relatively quickly. Surviving cells will become trapped in the capillary beds of distant organs by adhering to the walls of the blood vessel (21). Subsequently, they will extravasate into the new host organ. Finally, the metastatic process is complete when proliferation in this new tissue is successful, a process called colonisation (1).

1.3 Cancer and the immune system

Development of cancer can be prevented by the immune system in three ways (22): 1) Eliminate tumour-inducing viral particles from the host; 2) Prevent the formation of an inflammatory environment by removing pathogens; 3) Identification and elimination of tumour cells based on the expression of tumour-associated antigens (TAAs). Paul Ehrlich already hypothesised in the early 1900's that tumour growth was limited by the immune system (22, 23). The now called immunosurveillance hypothesis was only recognised more than 50 years after the concept was published, although it was still heavily debated in the following decades. Not surprisingly, the concept was validated multiple times with the technological advances in the 21st century (23). The presence of a tumour and tumour progression despite having immunosurveillance indicates that malignant cells develop an escape mechanism or some form of resistance against the immune system (22). These two properties were incorporated into the cancer immune editing hypothesis as three consecutive phases: elimination (immunosurveillance), equilibrium and escape (Figure 2).

All normal cells, if exposed to oncogenic conditions, can be transformed into malignant cells (23). The tumour cells can alarm the immune system by expressing tumour antigens or by producing certain molecules. Subsequently, both the innate and adaptive immune system can react to these signals by destroying the malignant cells to prevent tumour formation. However, it may occur that the elimination is incomplete and remaining tumour cells can survive in a maintained equilibrium with the immune system. In this scenario, these cells will accumulate mutations over time that generate TAAs (22). Finally, the selective pressure of the immune system will give rise to a tumour population that is resistant to the anti-tumour response or that is able to avoid or suppress it. The immune system is no longer able to control the growth of the tumour.

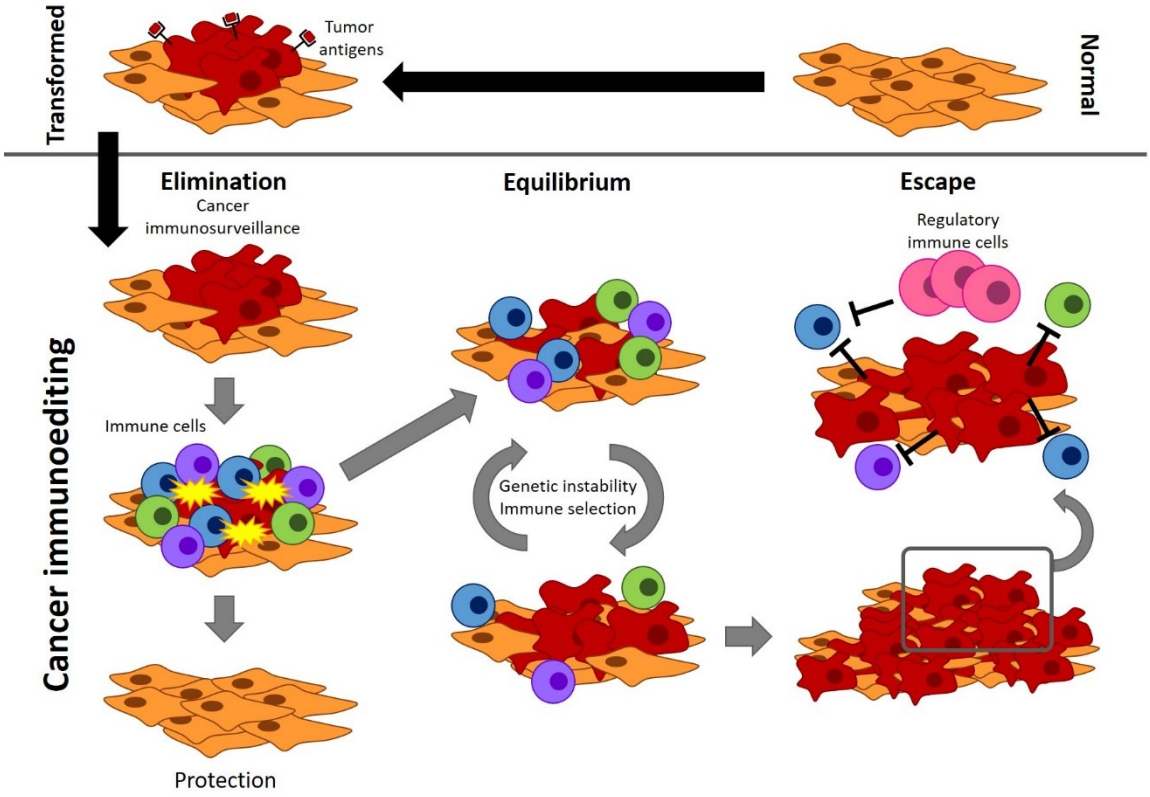

Figure 2 | Visualisation of the immune editing hypothesis.

Exposing cells to oncogenic conditions may result in malignant transformation (above). Normally, the immune system recognises the tumour cells and is able to remove them without harming the surrounding cells in the process of cancer immunosurveillance (below). However, if some malignant cells remain, they can either survive in equilibrium or develop characteristics that enable them to avoid or suppress the immune system. Adapted from Dunn *et al.* (23) by Diana Campillo-Davo.

1.3.1 Tumour-associated antigens

Five groups of TAAs that are recognised by the immune system are currently defined based on the expression patterns of the antigen (22, 24).

1.3.1.1 Cancer-testis antigens

Some genes are silent in most tissues in the body but can be activated in several tumours (24). For example, testicular germ cells and placental trophoblasts express genes that are not present in other healthy cells. However, some tumour cells reactivate expression of these genes. Since the testes do not express major histocompatibility complex (MHC) molecules (in humans: human leukocyte antigen, HLA) on their surface, the antigens are not presented to the immune system. Conversely, tumour cells do present the peptide:MHC (pMHC) complex on their surface, which makes these peptides tumour-specific antigens

(e.g. melanoma associated antigens (MAGE)). It is important to note that these antigens are expressed on the medullary epithelium of the thymus as well, in function of the negative selection of T cells (22).

1.3.1.2 Differentiation antigens

Differentiation antigens are specific for melanomas and healthy melanocytes, and are highly immunogenic despite being self-antigens (22). Well-characterised examples are tyrosinase, glycoprotein 100 (gp100) and tyrosine related protein 1 and 2 (TRP-1/2).

1.3.1.3 Antigens from mutations

Antigenic peptides are often a result of point mutations in a gene. In tumours, these mutations are common in oncogenes. A single point mutation in cyclin dependent kinase 4 (CDK4) can prevent it from binding to its inhibitor (25). Chromosomal rearrangements are also a source of tumour-specific antigens (22, 24).

1.3.1.4 Overexpressed tumour antigens

The expression level of some genes in tumour cells exceeds that of normal cells, also generating considerably more antigens (24). The Wilms' tumour 1 (WT1) protein is highly expressed in solid and haematopoietic tumours but has little expression in normal tissues (22). T cells that survive the strong negative selection in the thymus will probably have a low affinity for the antigen and occur in lower numbers to reduce the risk of autoimmunity.

1.3.1.5 Viral antigens

The final group are antigens that are generated by the infection of viruses that have oncogenic properties (22). Common viral associated cancers are caused by the Epstein-Barr virus, hepatitis B and the human papillomavirus.

2 Leukaemia

The blood in our body mainly consists of three types of cells, each with their specific function: erythrocytes, or red blood cells (RBC), for oxygen transport; thrombocytes (platelets) to slow down or stop bleeding by coagulation; and lymphocytes, white blood cells (WBC), that fight off infections (26). These cells arise from the haematopoietic stem cells (HSC) – that are primarily located in the bone marrow (BM) – by following either the myeloid or lymphoid differentiation pathway. On one hand, the RBC, thrombocytes and myeloblasts are derived from a common myeloid progenitor, and follow the myeloid pathway. The myeloblasts can further differentiate into granulocytes, including monocytes, eosinophils, basophils and neutrophils (27). On the other hand, HSC can follow the

lymphoid pathway to form a common lymphoid progenitor and then differentiate into lymphoblasts and, subsequently T, B or NK cells (3).

2.1.1 Characteristics and classification

Leukaemia is caused by the quick proliferation of precursor cells in the BM. Due to the rapid increase in numbers, the leukaemic cells will crowd out the normal cells (26). Eventually, the cancerous cells will leak out to the blood stream where they are responsible for some of the symptoms due to their lack of or reduced functionality.

The disease can be subdivided into two main groups, depending on the speed of disease development and the developmental stage of the cells: acute (quick, immature) or chronic (slow, more mature) leukaemia (26, 27). Although a rise in the number of WBC can be detected in both types of leukaemia, the acute form shows the release of poorly differentiated blasts from the BM into the bloodstream that are unable to function properly whereas chronic leukaemia often results in partially functional WBC (26). Eventually, the increased proportion of (partly) dysfunctional cells can lead to anaemia, thrombocytopenia and/or granulocytopenia (27). Further classification is based on whether myeloid or lymphoid precursor cells are affected. The most common types of leukaemia are acute/chronic myeloid leukaemia (AML/CML) and acute/chronic lymphoid leukaemia (ALL/CLL) (26). Further classification and details of these haematological malignancies are thoroughly covered by Berger *et al.* (27).

2.1.1.1 Acute myeloid leukaemia

AML is defined as a disease in which the differentiation of haematological cells in the BM is halted in an early developmental stage (28). The mechanism behind this arrest has still to be elucidated, although chromosomal translocations resulting in the (in)activation of genes and other (epi)genetic aberrations are frequently observed (29). A characteristic of the disease is a blast count of more than 20% in the BM (28, 29). Lower values are now classified by the World Health Organisation (WHO) as myelodysplastic syndrome (MDS). Notably, patients with high risk MDS, showing an excess in blasts in the BM, are more likely to develop AML. Other risk factors include familial syndromes (e.g. Down syndrome (28)), environmental exposures (e.g. smoking (30)) and drug exposures (e.g. chemotherapy) (31, 32).

2.1.1.2 Chronic myeloid leukaemia

CML is one of few cancers that is known to be caused by a single chromosomal translocation (33). It is an acquired cytogenetic aberration in the HSC that leads to

proliferating, poorly differentiated precursor cells of the myeloid lineage. A translocation of the long arm of chromosome (chr) 9 with the long arm of chr 22 is the cause of more than 90% of CML cases. The remainder of the cases are often due to variant translocations (27). The combination of chr 22 with the long arm of chr 9 is commonly known as the Philadelphia (Ph) chromosome. The fusion of the Abelson murine leukaemia viral oncogene homolog (*ABL*) oncogene – a tyrosine kinase – on chr 9 with a specific breakpoint cluster region (*BCR*) on chr 22 forms a new gene called *BCR-ABL* encoding the BCR-ABL protein with constitutive tyrosine kinase activity (29, 34), resulting in the CML phenotype in a process that is yet to be fully elucidated (33).

CML progression is divided into three phases: the chronic phase (CP), accelerated phase (AP) and blast phase (BP) (35). In developed countries, most patients are diagnosed with CP-CML during a regular check-up showing an abnormal complete blood count, which is a test to determine the number of WBC, RBC and platelets and the concentration of certain proteins (35, 36). With current standards of care (see 2.1.3 Treatment methods), CP-CML patients can have a rather normal lifespan (35). However, mutations – mostly single amino acid changes in the BCR-ABL kinase domain (37) – can turn these regimens ineffective, leading to progression of the disease from CP-CML, through AP-CML, to BP-CML. BP-CML is an acute form of leukaemia in which blasts are myeloid in two thirds of patients and lymphoid in one third (34). Furthermore, the BP is defined by the WHO as a presence of at least 20% of blasts in the peripheral blood or BM (29, 36), yet there is still some debate about the exact cut-off value.

2.1.1.3 Acute lymphoid leukaemia

It is known that chromosomal translocations, structural variations and/or sequence mutations are responsible for the arrest of maturation of lymphoid precursor cells in ALL (38, 39). Notably, the Ph chromosome is also present in some cases of ALL (40). However, unlike in the BP of Ph^+-CML, only lymphoid blasts are formed in this type of leukaemia. Other chromosomal rearrangements include genomic alterations that result in activation of cytokine receptors or tyrosine kinases (41). These cases show similar gene expression profiles to Ph^+-ALL and are consequently termed "Ph^+-like ALL" (38). These rearrangements may not be sufficient to induce leukaemia and, as indicated by the work of Wiemels *et al*. (42), other mutations may be required.

2.1.1.4 Chronic lymphoid leukaemia

CLL is a disease of $CD5^+$ B cells that can be subdivided based on the expression of a "mutated" or "unmutated" immunoglobulin heavy-chain variable region gene (43). These mutations occur in the germinal centres in the lymph nodes through deamination of cytosine residues in the variable region and error-prone repair in a process called somatic hypermutation (44). However, as this is a natural process, the classification of CLL is solely based on the stage of maturation of the B cell of origin. Notably, B cells in CLL patients show a limited repertoire of B cell receptors (BCR), indicating that there is a selection of B cells based on their expressed BCR (45). These BCRs possibly recognise autoantigens, resulting in the constitutive signalling of the BCR (46).

Although the exact origin of CLL remains unknown (47), several risk factors and aspects of the pathophysiology have been clarified over the years (43). Chromosomal rearrangements, somatic mutations and micro RNA (miRNA) alterations are some of the genetic alterations in CLL. Only four chromosomal rearrangements are identified in more than 80% percent of CLL patients (43). The most common, a deletion in chromosome 13q14.3 (del(13q)), can be found in >50% of patients (48). This region encodes miRNAs that regulate the expression of anti-apoptotic proteins or proteins important in cell cycle progression (47, 49). A smaller fraction of patients have a del(11p), 12q trisomy or del(17p) (43, 48). Furthermore, several studies have shown high genetic variability in CLL patients (43). This includes, among others, mutations in genes with a role in DNA damage, messenger RNA (mRNA) processing, chromatin modification and B cell-related signalling and transcription.

2.1.2 Incidence and relative survival rates

In 2015, the Belgian Cancer Registry released a detailed report dedicated to haematological malignancies covering the incidence, trends and relative survival rates of several of these diseases between 2004 and 2012 in Belgium (50). With a little over 6 500 new diagnoses each year, haematological malignancies account for 10% of all malignant tumours. About 69% of these are lymphoid malignancies, 30% have a myeloid origin and less than 1% are histiocytic or dendritic cell (DC) neoplasms. From the leukaemias discussed above, CLL and AML are the most common with 12% and 8%, respectively, and are followed by ALL and CML (both 3%).

Although the risk of illness generally increases with age, children and adolescents are the major group in ALL with 54% of cases, which explains the low average age at diagnosis

of 27 years and 31 years for males and females (M/F), respectively. In contrast, CLL has a relatively high mean age of 68/70 years (M/F) with higher age-specific incidence rates from 40 years. On the other hand, the myeloid malignancies, AML and CML show correspondingly an average age for M/F of 64/63 years and 59/61 years. While the incidence of CML intensifies with age, AML shows a rapid increase from the age of 50, especially for males. Notably, the ratio of M/F incidence rates of all four malignancies is considerably tilted towards males: 1.3 for AML, ALL and CML, and 1.7 for CLL.

Advancements in treatment methods have improved the 5-year relative survival rate (RSR) across all haematological malignancies from 57% in 2000-2003 to 66% in 2008-2012. Chronic leukaemias have rather high 5-year RSR, although they may vary between age groups or subtypes. For instance, the 5-year RSR in CLL is 84%/85% (M/F) but the prognosis decreases from the age of 65 to around 60%. Moreover, BCR-ABL negative CML shows overall poorer outcomes than CML in general (80%/82% M/F 5-year RSR). Notwithstanding the high incidence in children of ALL, with almost 90%, they show great 5-year relative survival rates compared to adults (~40%) . Interestingly, ALL from a B-cell precursor is more common in children, while T cell ALL is more frequently diagnosed in adults. Lastly, AML has the worst 5-year RSR with only 24%/29% M/F. However, these numbers increase up to 55% for patients younger than 50 and differ between AML subgroups.

2.1.3 Treatment methods

The diagnosis of any type of leukaemia is quickly followed by some form of treatment, called induction therapy. The aim of induction therapy is to eliminate all signs of cancer. Typical first-line therapies include chemotherapy, irradiation and/or surgery (51). However, since leukaemia is a cancer of the blood and rarely forms solid tumours, surgery is usually not a viable treatment. Chemotherapy, on the other hand, is generally seen as the standard of care for all types of leukaemia (52-55). Consolidation therapy is the next step in the process, used in an attempt to kill any cancer cells that might remain after induction therapy. This includes chemotherapy, irradiation or a stem cell transplant (51). Subsequently, a long-term and less aggressive maintenance therapy, such as antibodies targeting cancer cells, is started to reduce the risk of relapse (51).

2.1.3.1 Consolidation therapy: HSC transplantation

In addition to chemotherapy and radiation regimens, HSC transplantation (HSCT) is often used as a consolidation therapy for haematological malignancies. Stem cells can either be

obtained from donors (allogeneic) or from the patient (autologous) (56). Allogeneic HSCT (allo-HSCT) requires that the patient undergoes cytoreductive conditioning prior to the transplant to minimise the chance of rejection of the donor graft (56, 57). Although the preconditioning often comprises high doses of chemotherapeutics, reduced intensity conditioning may be advisable for patients of age or with a lower health status (56-58). Furthermore, considering the risks of allo-HSCT, the prognostic markers of the disease are also taken into account (e.g. allo-HSCT is standard of care for high-risk AML patients in first complete remission (CR1), but not advised for CR1 low-risk AML), which has recently been discussed in detail by Majhail and colleagues (58).

In order to avoid the recognition of host histocompatibility antigens by immunocompetent donor T cells, known as graft-versus-host disease (GVHD), allo-HSCT requires an HLA-matched donor (a close family member or matched unrelated donor) (57). Although HLA-mismatching is the main cause of GVHD, minor histocompatibility antigens (e.g. single nucleotide polymorphisms (SNP) in antigens) can also contribute to this effect (57). These same donor T cells target remaining cancer cells (graft-versus-tumour (GVT) effect), making it difficult to treat or prevent the GVHD without abolishing the beneficial effects of the GVT effect (56).

Notwithstanding that allo-HSCT is the best post-remission therapy, autologous HSCT (auto-HSCT) offers a viable alternative for patients who lack a suitable donor or who are not eligible for allo-HSCT (59). Stem cells are extracted from the patient prior to preconditioning procedures and are administered again afterwards (59). This type of transplantation lacks the benefits of GVT effects, but also offers lower morbidity and mortality rates due to the absence of GVHD. Nonetheless, Cioch *et al*. reported that auto-HSCT shows similar results to allo-HSCT in the aforementioned target groups (59).

2.1.3.2 Therapy development

Over the years, several new therapies have been developed to either improve the results obtained with chemotherapy or as an alternative to it. Every promising compound has to meet strict criteria before it can be marketed. The toxicity and dosage of the substances are tested *in vitro* and *in vivo* in preclinical trials (60). How the drug will behave in the human body cannot be derived from these studies, hence the need for further clinical trials, which generally consist of three phases (phase I-III). In phase I, the potential new drug is administered to a small group of less than 100 volunteers (60). This part of the trial is required to verify the safety, dosage and possible side effects of the drug, and can take some

months to complete. Next, the first results of efficacy are collected from the administration to several hundreds of patients in a phase II clinical trial that can take up to two years (60). Furthermore, the researchers more precisely determine the therapeutic dose and continue to monitor safety. Finally, before moving to the market, the benefit of the therapy has to be proven in a phase III trial, involving up to 3 000 patients for 1 to 4 years (60). This large number of volunteers also ensures that most (long-term) side effects are identified. Post marketing, the drug is still followed up and can be seen as phase IV in the process. In 2006, a new phase 0 was introduced in an attempt to accelerate the translation of biomedical advancements into a product. These trials are carried out in a small population (<15 people) to identify promising therapeutics quicker, based on pharmacokinetics, pharmacodynamics and target localisation (61).

The endpoint that is used in these trials depends on several factors such as the type of tumour, the speed of disease progression and the expected survival of the patients (62). Overall survival (OS) is an example of a patient-centred endpoint and is defined as the time from randomisation until death from any cause (62). OS is seen as the golden standard, but measurements might be impeded by longer survival of the patients, crossovers from the control group and ethical issues. As an alternative, and although initially intended for the use in accelerated approval processes for the FDA, tumour-centred or surrogate endpoints are frequently used as an alternative (62). Two examples are objective response rate (ORR) and minimal residual disease (MRD). ORR is defined as the percentage of patients in which the therapy induced a reduction in tumour size by a predefined amount and for a minimum duration. With regard to MRD, on the other hand, new techniques are used to detect the remaining traces of cancer cells after initial treatment, normally undetectable with standard laboratory methods. Evidently, each endpoint has its benefits and shortcomings, which are covered elsewhere (62).

The results of a clinical trial are also expressed in terms of the extent and the level of the response. When the tumour has decreased in size or the tumour burden in the body was lowered, a partial response (PR) is obtained, while a CR means all signs of cancer are eliminated (51). For leukaemia patients, the response to a particular treatment can be further categorised (63): [1] a molecular response (MR) is based on a polymerase chain reaction (PCR) to detect the amount of remaining genes or transcripts of a cancer gene in the blood or BM; [2] a cytogenetic response (CyR) is based on the detection of chromosomal translocations using fluorescence *in situ* hybridisation (FISH) or cytogenetics; [3] a haematological response is determined by the complete blood count.

2.1.3.3 Recent treatment approaches

Recent treatment methods tend to focus on combinatorial therapies that often achieve improved ORR, CR and/or OS. These approaches include the use of tyrosine kinase inhibitors (TKI) and unconjugated monoclonal antibodies (mAb), immunotoxins, peptides or ligands bound to toxins of plant, bacterial or human origin and bispecific antibodies (64). Due to the rapid development of these novel approaches, some recommend that patients should be enrolled in well-designed clinical trials when possible (53, 65).

TKIs, a type of small molecule inhibitor, interfere with malfunctioning tyrosine kinase receptors. Mutations activating the FMS like tyrosine kinase 3 (FLT3) are relatively common in AML patients and mostly occur in the juxtamembrane domain or occasionally in the activation loop of the tyrosine kinase domain (8, 52). An example of a TKI is the U.S. Food and Drug Administration (FDA)-approved first generation FLT3 inhibitor midostaurine. In a phase III clinical trial, midostaurine was added in all phases of standard treatment and showed an improved 4-year OS and ORR (52, 66). In CML, interferon (IFN)-α was the standard treatment between 1995 and 2001 in addition to chemotherapy until the approval of the TKI imatinib mesylate, targeting the BCR-ABL fusion protein (67). Treatment with imatinib is significantly better compared to IFN-α and chemotherapy, shown by complete CyR in a phase III clinical trial (68). With continued treatment of responding patients, part of them can achieve very low levels of MRD or even undetectable MRD (69). Moreover, approximately 40% of patients in deep MR stay in treatment free remission after ceasing their TKI treatment (53). Since long term therapy comes with a high financial cost, unforeseen side-effects and a lowered quality of life, some argue that it might be beneficial for some patients to consider stopping their treatment and aim for a durable treatment free remission (53, 65). Intolerant or non-responsive patients to imatinib may be helped with second generation TKIs like nilotinib and dasatinib, which are more potent but often induce more side effects (65, 67). Meanwhile, the combinatorial therapy of pegylated IFN-α and TKIs is under investigation, showing mixed results with faster rates of deep response but often discouraging side effects (67).

Although treatment with fludarabine (a purine analogue) combined with cyclophosphamide (an alkylating agent) is still the standard of care in CLL, they may be combined with monoclonal antibodies. The addition of rituximab, an antibody targeting CD20, to the treatment results in significantly improved ORRs and CRs (70, 71). However, toxicity in these and other chemoimmunotherapy trials was higher in older patients, which suggest that it is a more viable option for younger, fit patients (72). Nivolumab and

pembrolizumab are antibodies targeting the immune checkpoint molecule called programmed cell death protein 1 (PD-1) (73). PD-1 is expressed on activated T cells, while its ligands, PD-L1 and PD-L2, are mainly expressed on macrophages and dendritic cells but also on other tissue types and tumour cells. The antibodies inhibit the PD-1/PD-L1/2 interaction, effectively inhibiting negative regulation of T cell activity by the tumour cells (74).

3 Adoptive T cell immunotherapy

Without any doubt, there are many possible treatment methods for cancer, partly illustrated above. However, research for new, more effective therapies is still required since a one-fits-all approach is not available. The use of monoclonal antibodies was already briefly touched upon and is part of a range of therapies involving or exploiting the immune system, called immunotherapies. Furthermore, there are adoptive cell based immunotherapies that make use of DCs or T cells to treat cancer. These autologous or allogeneic derived immune cells can be used to combat several types of malignant diseases. In brief, DC vaccination strategies use DCs that are matured and loaded *ex vivo* with tumour-specific antigens followed by reinjection, or that are stimulated *in vivo* for the uptake of the tumour-specific antigen (75). They can subsequently present these antigens to T cells that will contribute to tumour rejection (75). In what follows, the use of T cells in immunotherapy will be discussed more in depth, including tumour-infiltrating lymphocytes (TILs), chimeric antigen receptor (CAR) T cells and T cells with a modified T cell receptor (TCR). In light of the focus of this thesis, the latter approach will be covered more thoroughly than the others.

3.1 Introduction to T cells

The T cell receptor is a heterodimer of the immunoglobulin gene superfamily (76), expressed on the surface of T lymphocytes that determines the specificity and target cell of the T cell (77). Which combination of receptor chains is used, is the result of a complex maturation process in the thymus (44). Depending on the expression of several surface molecules, the T cells can either carry the strongly conserved γδ TCR or the heavily variable αβ TCR. The latter group is subdivided based on the expression of CD4 or CD8 as their co-receptor. In both cases, the association with the CD3 complex (composed of four CD3 proteins forming two heterodimers and one homodimer) is necessary for the presentation of the αβ TCR on the cell surface (Figure 3) (44, 78).

The TCR acquires its specificity during the maturation process by somatic rearrangements on the α and β chain between the variable (V), joining (J) and – only in the beta chain – diversity (D) segments (44). Due to the large TCR variety from these rearrangements, it is suspected that T cells should be able to recognise most antigens presented to them (77). In a process called central tolerance, potentially useful thymocytes are first positively selected for the recognition of autologous pMHC complexes. However, the organism needs to ensure that self-antigens do not elicit an immune response, therefore positive-selected cells are then negatively selected on strong recognition of self-antigens and undergo apoptosis (44). After leaving the thymus, the now mature T lymphocytes can travel around the body in search for its antigen, including the cancer antigens discussed in 1.3. It is possible that some T cells recognising self-antigens leading to T cell activation were not eliminated in the central tolerance process. This can be resolved by peripheral tolerance mechanisms (79): for naive T cell activation, both the primary signal from the recognition of the pMHC complex by the TCR and a secondary signal from co-stimulatory molecules on the cell surface are required (44). When naive T cells encounter their antigen in the absence of the secondary signal, the T cell will enter an anergic state in which it is hyporesponsive to new stimuli from their antigen (79). Using this mechanism, the tumour can circumvent recognition by limiting the co-stimulation of the T cells in the tumour environment (80, 81).

Importantly, the TCR can only recognise peptides bound to one of two classes of MHC molecules (44). The TCR is aided during peptide recognition by co-receptors CD8 and CD4, that they acquire in the course of clonal selection mentioned above (44). CD8 T cells can recognise MHC class I (MHC-I) molecules, which are present on almost all cells in the body, while CD4 T cells can interact with cells that carry MHC class II (MHC-II) molecules on their surface, which are mostly specialised antigen presenting cells (APC), such as DCs (44). Another important difference is the origin of the presented peptides. MHC-I is presented on the cell surface after being loaded with a peptide coming from the cytosol, originating from viruses, intracellular bacteria or cancer proteins (44). On the other hand, specialised APC present MHC-II carrying peptides derived from phagocytosed or endocytosed particles.

Correctly identifying the corresponding pMHC by the TCR, together with matching MHC molecule and co-receptor, will trigger the first activation signal of naive T cells through the CD3 complex (44). The signalling cascade is initiated by phosphorylation of tyrosine residues on the immunoreceptor tyrosine-based activation motifs (ITAMs) on the

cytoplasmic part of the CD3 complex (44). This is followed by a complex phosphorylation cascade (fully covered in Janeway's Immunobiology (44)), that requires a second activation signal through a co-stimulatory receptor (e.g. CD28) on the T cell surface to activate three transcription factors (TF): [1] nuclear factor of activated T cells (NFAT), a cytoplasmic TF, is translocated into the nucleus after dephosphorylation induced by a calcium influx into the cell; [2] the TF activator protein 1 (AP-1) is activated through a mitogen-activated protein kinase (MAPK) cascade; [3] protein kinase C activates nuclear factor κB (NF-κB). Simultaneous activation of all three TF will stimulate the expression of interleukin (IL)-2, which promotes T cell proliferation and differentiation to effector T cells (44). Following activation, CD8 effector cells can recognise pMHC on target cells and induce apoptosis by targeted release of various molecules. CD4 T cells on the other hand can have many functionalities, including helping APC to stimulate naive CD8 T cells, upregulating the activity of other cells to fight off pathogens or regulatory functions.

3.2 Tumour-infiltrating lymphocytes

The therapeutic use of TILs, which are explained in more detail further in the text, was pioneered by Dr. Steve Rosenberg's team by demonstrating the curative properties of *ex vivo* expanded lymphoid cells in mice (82). With the earliest results in 1988, they showed that this approaches also had potential in humans (83). Using optimised culturing conditions, preconditioning of the patient by lymphodepleting chemotherapy and systemic administration of IL-2 after TIL reinfusion, objective regressions of 50-70%, with some patients obtaining a durable CR, were achieved in metastatic melanoma (84-86). So far, there has been little success trying to expand these findings into other malignant diseases due to the limited ability to extract sufficient TILs, poor antigenicity or the location of the tumour (74, 87).

3.3 Chimeric antigen receptors

CAR T cell therapy is, so far, one of the most promising immunotherapies from the past couple of decades, especially in haematological disorders. Together with the advances in genetic engineering, CAR T cells allow for an effective cancer therapy by patient-derived cells (77). The receptor consists of three parts (Figure 3): [1] an extracellular target binding domain, which is made from a modified antigen binding portion of an antibody from a library or a ligand that can bind to its receptor; [2] a hinge region consisting of a spacer and transmembrane domain required to achieve the optimal distance between the binding and signalling domain; [3] an intracellular signalling/activation domain (88-90). So far, there

have been three generations of CAR T cells with the main difference in the intracellular domains. The first generation was based on the finding that the CD3-ζ chain or Fc receptor γ chain are sufficient to activate T cell signal transduction pathways (90-92). Although the signalling was sufficient to elicit a cytotoxic activity, the threshold for other T cell functions was not reached (90). This issue was solved with the second generation CARs that included the cytoplasmic domain of a co-stimulatory receptor (e.g. CD28 or 4-1BB) giving an increased signalling strength and persistence of the T cells, improving their potency as well. Third generation receptors make use of two co-stimulatory domains combined with the activation domain. However, different combinations of signalling domains are being tested to obtain the maximal anti-tumour response (74, 90).

In 2014, a clinical trial by Maude *et al.* with CAR T cells recognising CD19 in patients with ALL reported a CR in 90% of the patients and a 67% event-free survival (93).

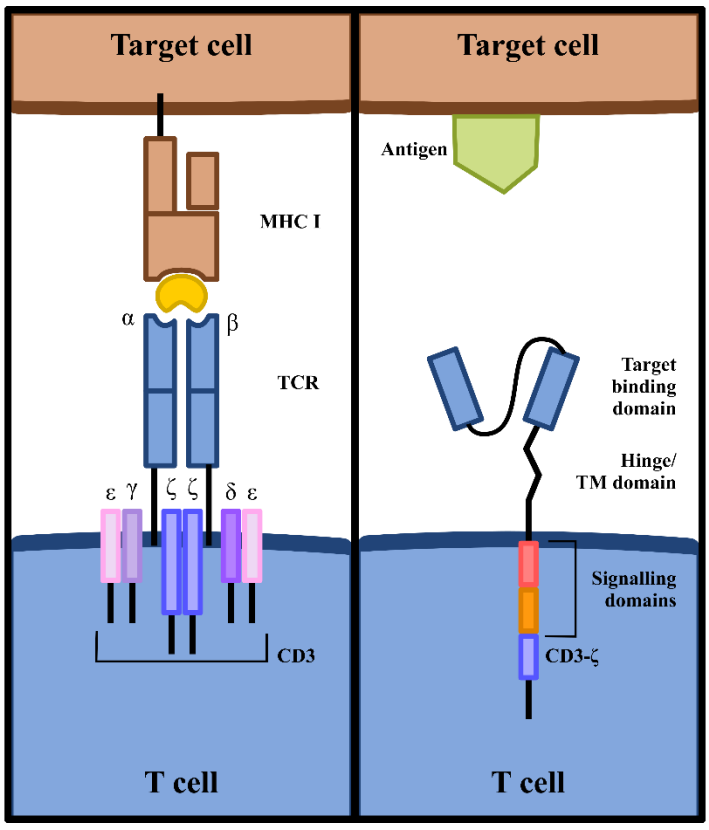

Figure 3 | T cell receptor (TCR) and chimeric antigen receptor (CAR).

The TCR is made up of two receptor chains (α and β) and is associated with the CD3 signalling complex used to elicit an immune response on encountering its specific peptide:MHC molecule. CAR consist of an extracellular target binding domain coupled to the CD3-ζ activation domain only (first generation), one (second generation) or two (third generation) co-signalling domains through a spacer/transmembrane (TM) domain. These receptors can recognise antigens on the cell surface in an MHC-independent manner.

These impressive results led to the development of CARs for many other haematological malignancies (88). Furthermore, in September (94) and October (95) of 2017, the first (by Novartis) and second (by Kite Pharma) CD19-CAR T cell therapy were approved by the FDA, both targeted at B cell malignancies. In contrast, the advancements in CAR development for solid tumours are rather unsuccessful, encountering toxicity and a lack of therapeutic response (74, 88). Among the challenges that are faced are heterogeneous expression of the antigen, inability of the T cells to reach the tumour and the immune inhibiting tumour microenvironment, which are all further discussed in the review by Xia *et al.* (88).

CARs have several advantages as well as disadvantages over other therapies (74). The first advantage is that the binding portion of the CAR can interact with its binding partner on the surface of the cell, independent of the presentation of the epitope on an MHC molecule. Moreover, unlike small molecule inhibitors, the target of CAR is not required to have a critical function in the tumour cell (77). CARs are also not restricted to protein antigens but can be modified to recognise carbohydrates and lipids (74, 96). Expression on normal tissues, however, should be limited in all cases. Secondly, appropriate CAR design can counteract the downregulation of co-stimulatory molecules by the cancer cell, which is one cancer mechanism to evade immune detection (88). On the other hand, with regards to the disadvantages, CARs are only limited to the recognition of antigens that are on the cell surface, which allows the cancer cell to escape CAR detection if the antigen is lost, while TCR modified T cells can respond against the presentation of intracellular derived peptides as well (74). Furthermore, normal TCR-mediated signalling utilises a wider range of signalling molecules than used in CAR-mediated responses, which might imply that the full potential of the CAR-modified T cells has not been reached yet.

3.4 TCR-modified T cells

T cell receptor (TCR)-modified T cells are another novel approach in immunotherapy treatments. In general, autologous lymphocytes are extracted from the patient and a new, tumour-reactive TCR is introduced. The T cells are subsequently expanded *ex vivo* and infused back into the patient (74).

3.4.1 Isolation of tumour-specific TCRs

As evident from the existence of TILs, our body already generates tumour-specific T cells that recognise tumour-associated antigens with their TCR. There are, however, some other

methods to obtain new or high-affinity TCRs that we can subsequently introduce in the bulk of T cells to redirect their specificity.

The isolation of the TCR from TILs is fairly straightforward. In general, a piece of the tumour is resected and cultured with high-dose IL-2 (97). Subsequently, the TILs are divided in an array by limiting dilution and co-cultured with cells displaying different tumour antigens. Then, the monoclonal T cell clones displaying reactivity to the desired antigen are expanded and the TCR α and β chains are sequenced.

To overcome the limitation of TILs to melanoma (74, 87), HLA transgenic mice can be used to generate tumour-specific T cells by injecting tumour-associated proteins (98). After injection, spleen cells of the mice are harvested and co-cultured with cells peptide-pulsed with the priming peptide. Next, as it happens with TILs, the protein-specific effector cells are expanded and single specific cytotoxic lymphocytes (CTL) are obtained by limiting dilution. Finally, the anti-tumour TCRs are isolated and sequenced.

TCRs generated from TIL isolation often only demonstrate low affinity for self-peptides due to the tolerance mechanisms in the body (99, 100). However, altering the regions that interact with the peptide or the MHC molecule through phage display might result in higher affinities (100). In general, changes in several amino acid residues of the variable regions of the TCR can be introduced via PCR using mutagenic primers in site-directed mutagenesis (100). Subsequently, these mutated sequences are linked to a gene coding for a coat protein and cloned into phagemid vectors that are transfected into bacteria (100, 101). The bacteriophages are then recovered from the bacteria and pooled together. Selection occurs by adding the pool to the desired target pMHC allowing the TCR, attached to the phage, to bind to the pMHC complex (100). Interacting phages are then harvested and allowed to infect their host, which is cultivated in a selective medium. Finally, the selection procedure is repeated several times to determine the affinity and specificity of the TCR (100, 101).

3.4.2 Delivering genes into T cells

Throughout the years, several gene delivery methods have been developed. Some relevant methods will be briefly discussed, covering the general concept and highlighting advantages and disadvantages. These methods can be used to transfer the obtained TCR genes into T cells to be used for T cell therapy.

3.4.2.1 Viral vectors

The γ-retroviral and lentiviral vectors are made by removing the *gag, pol* and *env* genes from the viral genome, allowing the insertion of exogenous genes and effectively making them replication-defective (102). The production of infectious viral particles is accomplished using packaging cells that are either transiently transfected with two other helper plasmids containing the *gag*/*pol* and *env* genes respectively or modified to express these genes by stable integration in their genomes (102). Through recognition of a packaging sequence, the viral vector is loaded into the budding virions (102, 103). The viral vector also contains long terminal repeats (LTR) required for the integration in the host genome that have promotor and enhancer functionality (102).

Although retroviral and lentiviral vectors are useful for stable integration in the genome, the reverse transcriptase is relatively error-prone, which makes these vectors less suitable for corrective gene therapy (104). Additionally, nearly random integration carries the risk of insertional mutagenesis by upregulating oncogenes or deactivating tumour suppressor genes (102, 103).

3.4.2.2 Transposons

One upcoming non-viral DNA delivery platform is the use of transposons, which are DNA elements that are able to move from one spot in the genome to another (105). Similar to viral vectors, they are often prepared in a system using two plasmids, one containing a transcriptional element with the gene of interest flanked by terminal inverted repeats, another containing the transposase. The transposase is an enzyme responsible for the excision and reintegration of the transposable element into the host genome. Due to the use of plasmids, they are easily manufactured in large quantities and thus considerably cheaper compared to viral vectors (106). They are, in addition, less immunogenic and can generally carry larger cargo genes. The ability to efficiently insert new genes in vertebrates came with the reconstruction of the *Sleeping Beauty* transposon system, derived from fish (107). Early results in animal models show little risk using this system due to a lower chance of genotoxicity associated with the random integration in one of many integration sites available in the genome (105, 106). However, gene encoded transposase has a chance to incorporate in the genome that can result in hopping of the transposon, which is undesirable for therapeutically purposes and thus the development of additional safety measures is required (105, 108).

3.4.2.3 Transient transfection

Despite the increasing success of adoptive T cell therapies, there are still some problems that have to be tackled. For example, various toxicities have been observed following adoptive transfer of engineered T cells with both CARs and TCRs (109), among which cytokine release syndrome, on-target/off-tumour and off-target toxicities (110). It can be assumed that transient expression of TCRs through transfection of DNA or mRNA could only lead to transient toxicities and is therefore more manageable (109). Moreover, mRNA has some advantages over DNA, mostly because it does not need to be translocated to the nucleus (111). Moreover, it poses no risk of integration into the genome, expression levels are easily adjusted with the amount of supplied mRNA, expression is almost instant and it does not rely on promotor strength.

One delivery method is the chemical encapsulation of the coding sequence with positively charged chemicals (e.g. lipofection with cationic lipids) that are attracted by the negatively charged cell membrane and results in the uptake by the cell (111). There are also physical delivery methods including micro-injection (injecting the nucleic acids in the cell) and electroporation (applying a current over the cells, inducing transient pores through which the DNA/mRNA can pass the cell membrane) can be employed (111). The latter approach is very appealing since an optimised protocol results in easy and rapid modification of a large amount of cells. Moreover, compared to the use of viral vectors, electroporation of mRNA is more convenient in production (112) and requires a less specialised infrastructure in contrast to viral delivery methods.

3.4.3 Hurdles to overcome in TCR engineering

By introducing an exogenous TCR, there are in total four different TCR chains that are expressed in the cell. Without taking appropriate measures, this has some implications for the expression of the transgene TCR because of competition for the CD3 complex (required for proper signalling) and the mismatching of the endogenous and introduced TCR chains (113). Erroneous combinations lead to a reduction in antigen-specific TCR levels that will consequently reduce the overall strength of interaction of the T cell with its antigen, called avidity (113). Furthermore, autoimmunity is a serious concern as mispairing may result in a TCR with unknown specificities. In order to solve this problem, some techniques have been developed and will be further discussed below.

3.4.3.1 Disruption of the endogenous TCR

With the emergence of zinc finger nucleases (ZFN), transcription activator like effector nucleases (TALEN) and the clustered regularly interspaced short palindromic repeats combined with the CRISPR associated protein 9 (CRISPR-Cas9), it has become relatively easy to effectively eliminate expression of the endogenous TCR (114-116). These platforms induce double strand breaks (DSB) in the genome that are repaired based on homology – based on a DNA template – or by non-homologous end joining, which in essence pastes both sides of the DNA chain together, often inducing some form of mutations (117). Two catalytic domains of the FokI restriction enzyme are responsible for this DSB in ZFN and TALEN, guided by the recognition of triplet nucleotides by single zinc fingers or a single nucleotide by two variable amino acids in 33-35 amino acid repeats, respectively (118). The CRISPR system recognises a ±20 base pair target region via the single-guide RNA (sgRNA) which is composed of the CRISPR RNA (crRNA) and the transactivating crRNA (tracrRNA) (118). When complexed to the Cas9 protein and adjacent to a protospacer adjacent motive (PAM) sequence (typically 5'-NGG-3'), it will generate a DSB. In contrast, the ZFN and TALEN approach require complex engineering of two separate proteins, which can result in off-target cleavage (119). Another benefit of the CRISPR-Cas9 system is that it is active as a monomer and thus can edit multiple targets – using multiple sgRNAs – with little chance of off-target effects (118). Transient expression of these proteins is sufficient to obtain a modified cell population, which can be achieved by means of integration-deficient viruses, DNA or RNA transfection – of which the latter is the least toxic for the cells (118). However, in these cases, additional cell manipulation is needed to introduce the new gene, which might result in higher cell toxicity.

Although abovementioned techniques offer the possibility to completely knock out the TCR genes, they might not be suited for therapeutic use due to their complexity and/or the relatively long time required to obtain the knock-out. Alternatively, as demonstrated by Ochi *et al.*, knocking down the endogenous TCR can be achieved by adding silencing RNA (siRNA) sequences to their viral vector encoding the isolated TCR genes (120). SiRNAs are one of two RNA interference mechanisms present in nature (121). Endogenous siRNAs are formed by splicing of larger RNA precursors to 21 nt RNA duplexes by the large endoribonuclease Dicer (121). Subsequently, an Argonaut family protein associates with the complex and forms the RNA-induced silencing complex (RISC) (121). Finally, single stranded RNA (ssRNA) is formed by degradation or ejection of the passenger strand, leaving only the guide strand that is able to interact with the target RNA, tagging it for

degradation. First generation artificial siRNAs are very similar to these naturally occurring ones, being 21 nt RNA duplexes with two 3'-overhangs (122). After the hypothesis emerged that Dicer substrates would have different properties than the artificial Dicer products, a series of experiments were conducted by Integrated DNA Technologies and the laboratory of John Rossi (121). These resulted in the finding that the optimised Dicer-substrate siRNA (DsiRNA) consists of a 27 nt guide strand and a 25 nt passenger strand with a 3'-overhang on the guide strand and two DNA bases at the 3'-end of the passenger strand (123). In a study by Hefner *et al.* they demonstrated that DsiRNA, generated with 21 nt from the 3'-overhang, was more potent than cognate siRNA for most targets, in particular at lower cellular concentrations (124). Furthermore, it was shown that the silencing duration after transfection was linked to the potency as well. These results indicate that siRNA, and especially DsiRNA, offer an appropriate alternative to previously described methods. The DsiRNA can be delivered to the cell through a viral vector or electroporation (125, 126). Although the latter only transiently silences the target gene, it could be combined with other strategies, such as the delivery methods discussed in 3.4.2.

3.4.3.2 Improving TCR expression

One way of improving the overall expression of the exogenous TCR is the use of an internal ribosomal entry site (IRES) or a 2A peptide. Including an IRES between both chains of the TCR allows the binding of a ribosome and the translation from the second gene (127). It was a popular method because it provided a reliable way for the co-expression of two or more genes and the possibility to add different subcellular localisation sequences to the genes (127, 128). An important downside is the size of more than 500 bp, which is disadvantageous in viral vectors that only have a limited cargo capacity (128). In addition, it was found that the translation of the gene after the IRES was significantly lower, which is undesirable in the case of TCR chains that are preferably present in equimolar quantities (113, 128). The 2A peptide is a self-cleaving small peptide of only 18-22 amino acids from picornavirus that resolves these issues (127). In contrast to IRES, no ribosome is recruited for the second gene. Instead, the ribosome continues from the first gene, but skips the synthesis of the glycyl-prolyl peptide bond at the C-terminal end of the 2A peptide, resulting in a proline residue on the N-terminal side of the second protein (129). The effect of the residual tags on both peptides has to be evaluated for every protein separately. Nonetheless, this mechanism results in an improved equimolar expression of both genes, which reduces the risk of mispairing (113, 127).

Replacing the TCR gene codons with synonymous codons that are more frequently encountered in the human genome – a process called codon optimisation – can considerably improve TCR expression as well (130). Moreover, codon-optimised introduced TCRs are not affected by DsiRNA designed to target the wild type *TCR* sequences and can therefore be combined (120). Removing cryptic splice sites, changing mRNA secondary structures and instability motifs that can reduce expression of a protein can also be achieved using codon optimisation (131). It is not impossible, however, that this process generates new open reading frames that might generate an immune response to the new TCR (113).

As mentioned before, the association between the TCR and CD3 complex is necessary for the cell surface expression of the TCR (44, 78). The introduced TCR has to compete with the endogenous TCR for the limited amount of available CD3 complexes (113). To overcome this problem, the cells can be transduced with an additional vector encoding the CD3 genes to increase the expression in the cells, resulting in an increased presentation of the introduced TCR (113). Alternatively, the endogenous TCR can be inactivated or silenced using various techniques that were discussed above.

3.4.3.3 Improving correct TCR chain mispairing

Mispairing of the TCR chains can be largely overcome with a combination of the following approaches. First, murinisation of parts of the constant region of the TCR can improve specific pairing as they prefer interacting with another murinised chain, relative to the human counterpart (132). It was shown that the decreased mismatching combined with a better interaction with the endogenous CD3 complex improves the surface expression of the murinised TCR as well (113, 132, 133). However, the murine parts can elicit an immune response and thus have to be limited as much as possible (133). Another option is the introduction of new cystine residues in the TCR constant region that can form disulphide bonds, improving pairing and the avidity of the TCR (113, 133).

3.4.4 Optimal T cell phenotype

While there are already therapies being developed using modified T cells, it is still rather unclear which subtype of T cells should be engineered. Ideally, engineered T cells would migrate towards tumour sites and peripheral lymphoid organs upon injection (84). Subsequently, they would start to proliferate and differentiate into effector T cells (T_{EFF}), effector memory T cells (T_{EM}) or central memory T cells (T_{CM}). Then, the cells have to show cytotoxic activity and persist for immunosurveillance. T_{EM} can proliferate and have limited self-renewal capacity (134). However, as they are perforin deficient, they show

little cytotoxic activity. On the other hand, T_{EFF} and terminally differentiated T cells show the best killing, but lack proliferative capacity and it is thought their persistence is not sufficient for a long term tumour response (134). In mouse models it has been shown that T_{CM} are superior T cells if they encounter their antigen in the lymph nodes on APCs (134). Recently, another little differentiated subset, the stem cell memory T cells, was discovered that showed self-renewal and the capacity to differentiate into effector cells on antigen encounter (84, 135). Furthermore, retrospective analysis of clinical trials indicates that less differentiated subsets show better anti-tumour responses and long term persistence (84, 136). However, it is still unclear if separation of these subtypes is beneficial to the therapy, or if it would only increase the cost and time needed.

3.4.5 Examples of approaches with TCR-engineered T cells

TCR modified T cells were first used in a clinical trial targeting melanoma-associated antigen recognised by T cells 1 (MART-1), a melanoma differentiation antigen, in patients with metastatic melanoma by Morgan *et al*. (76, 137). By adoptive transfer of transduced peripheral blood lymphocytes, they achieved 13% of the patients with an objective regression, which served as a proof of principle (76). Later, the same group tried to improve these results with the use of TCRs also specific for the MART-1 antigen, but with a higher avidity (76). However, with the increased number of patients having an objective regression, they observed several on-target off-tumour toxicities as well. Some of the side-effects could be overcome by local administration of steroids. These results indicate that targets have to be carefully selected to prevent or minimise these toxic events. More recently, in 2011, Robbins *et al.* presented a response in 66% and 45% of synovial cell sarcoma patients and melanoma patients, respectively, using T cells redirected against the cancer-testis antigen NY-ESO-1 (138). In contrast to the melanoma differentiation antigens, there were no signs of toxicity related to the therapy. To their knowledge, they were also the first to find an effective therapy against a non-melanoma tumour. Despite the efforts of trying to target these differentiation or cancer-testis antigens, potentially the best approach would be targeting neoantigens and thus provide personalised treatment. Höfflin and colleagues studied the effects of CD8 T cells transfected with two different TCRs, one targeting a common melanoma antigen and one against a patient specific antigen, called T cells expressing two additional T cell receptors (TETAR) (139). The T cells are not restricted by tolerance mechanisms since the neoantigen can contribute to the cancer phenotype (139). The study showed that the TETAR were functionally secreting cytokines

and showed antigen specific cytotoxic activity *in vitro*. In addition, this setup proved to be better compared to a 1:1 mixtures of T cells expressing each TCR separately. Another alternative approach was recently reported by transfecting γδ T cells with mRNA encoding an αβ TCR specific for gp100 (109). These cells were shown to kill tumour cells by stimulating either their introduced αβ TCR or endogenous Natural Killer Group 2D (NKG2D) receptor. If the αβ TCR fails to recognise its antigen due to downregulation or loss of expression by the cancer cell, then the endogenous receptor can still mediate anti-tumour reactivity since its ligand is often upregulated by some tumours (109). Additionally, the probability of therapy induced toxicity is expected to be reduced by the transient expression of the αβ TCR, absence of mispairing of TCR chains and lack of autoimmunity by activation of γδ T cells (109). However, these observations have to be confirmed *in vivo* as well.

While the focus of immunotherapy was mainly on CD8 CTL, it has become clear that CD4 T cells play an important role in tumour eradication as well (140, 141). They improve CD8 CTL cytotoxic activity, enhance anti-tumour immunity in the tumour micro-environment and induce tumour cell lysis through granzyme B and perforin secretion (141, 142). Tumours often do not express MHC-II molecules, preventing recognition by CD4 T cells (81). Using TCR engineering, they can be redirected towards MHC-I recognition, allowing them to target the same antigen as CD8 T cells and thus activate their effector functions in the same conditions (141). Additionally, it has been shown in mice that adoptive transfer of CD4 T cells can reverse CD8 T cell tolerance for TAAs, expressing the same TCR (143). These and future findings might result in the development of new combinatorial therapies with both engineered CD4 and CD8 T cells.

Context and goals

Every year around 500 people are diagnosed with AML. A one-fits-all treatment still remains to be found for many, if not all, forms of cancer, including AML. There are many ongoing studies that investigate novel treatment methods that are aimed towards a combinatorial treatment with chemotherapy, or a complete replacement thereof. One promising field of study is adoptive T cell therapy in which T cells of the patient are equipped with a TCR that specifically recognises a TAA, such as WT1, or neoantigen resulting in the activation of the T cell and subsequent killing of the tumour cell. In general, research has focussed on CD8 CTL since they are characterised by their direct capacity to kill tumour cells that express MHC-I-restricted epitopes on their surface. However, in the past decade the importance of the helper function of CD4 T cells for, among other things, the persistence of co-transferred CD8 T cells became clear (144). Furthermore, it was found that CD4 T cells can have a cytolytic capacity as well (145). TCR-redirected T cell therapies for AML mostly target WT1 as it has been crowned as one of the most promising AML antigens based on factors such as specificity, expression patterns and levels, oncogenicity, immunogenicity and clinical relevance (146).

The majority of studies investigating TCR-redirected T cells make use of viral transduction in order to permanently express the TCR on the surface of the cell. This technique, however, raises many safety concerns and requires specialised infrastructure. Moreover, in some cases off- or on-target toxicities or excessive release of cytokines are observed during clinical trials. These adverse effects are disadvantageous for the patient and alleviation by, for example, administration of corticosteroids jeopardises the treatment. Transient expression of the TCR would minimise the risks for the patient as the possible side-effects would disappear over time in accordance with the loss of TCR expression.

In this master thesis we redirected CD4 T cells through double sequential electroporation (DSE) of DsiRNA, targeting the wild type (wt) TCR, and mRNA encoding the codon-optimised HLA-A2-restricted $WT1_{37\text{-}45}$ TCR (T37) in combination with or without *CD8* mRNA. The goal of this research mainly consists of two parts: 1) to assess the mRNA transfection efficiency in CD4 T cells through the expression level of the T37 TCR, and the effect of CD8 co-transfection; and 2) to investigate if these CD4 T cells can be activated via antigen-specific triggering of the transgene TCR and to determine the functionality of the activated T cells.

Materials and methods

A large portion of the materials and methods are adapted from an, at the time of submission, unpublished paper by Campillo-Davo *et al.* (147). Each section that contains information from this paper is foreseen of the appropriate reference.

1 Study Design

In this study, it was hypothesised that CD4 T cells, double sequential electroporated with DsiRNA and codon-optimised WT1-specific *TCR* mRNA with or without *CD8* mRNA, would be activated and show a cytolytic effector function similar to CD8 counterparts upon recognition of their target antigen. We tested transfection of DsiRNA and *TCR* mRNA using a cell line and primary samples from anonymous donors provided by the Blood Service of the Flemish Red Cross (Mechelen, Belgium), following the approval of the Ethics Committee of the Antwerp University Hospital and the University of Antwerp (Antwerp, Belgium) under reference number 16/35/357 (147). Information regarding number of independent replicates can be found in the figure legends. Epitope-specific T cell activation was analysed by co-culture of cells with a tumour cell line in the presence of a relevant peptide.

2 T cell isolation and cell lines

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood using Ficoll density gradient centrifugation (Ficoll-Paque PLUS; GE Healthcare). CD4 T cells were positively selected using human CD4 magnetic microbeads (Miltenyi Biotec), following manufacturer's instructions. Purity of isolated CD4 T cells was analysed by staining with anti-human CD3-PerCP, CD4-PE and CD8-FITC monoclonal antibodies (BD Biosciences). Samples were measured on a CytoFLEX flow cytometer (Beckman Coulter) and subsequently stored at -80°C in cryomedium, consisting of foetal bovine serum (FBS; Gibco Invitrogen) supplemented with 10% dimethyl sulfoxide (DMSO), for at least four days. The human acute T cell leukaemia cell line Jurkat Clone E6.1 (ATCC, TIB-152) was maintained in Roswell Park Memorial Institute 1640 (RPMI) culture medium (Gibco Invitrogen) supplemented with 10% FBS. HLA-A*02:01-positive T2 cells, a human lymphoblastoid cell line with transporter associated with antigen presentation (TAP) deficiency that can be loaded with exogenous MHC class I-restricted peptides, were kindly provided by Dr. Pierre Van der Bruggen (Ludwig Institute for Cancer Research, Brussels,

Belgium) and were maintained in Iscove's Modified Dulbecco's Medium (IMDM; Gibco Invitrogen) supplemented with 10% FBS. The human lymphoblastoid cell line U266 which was kindly provided by Dr. Wilfred T.V. Germeraad (GROW School for Oncology & Developmental Biology, Maastricht University, Maastricht, The Netherlands), was maintained in IMDM with 10% FBS. All cell lines were maintained in logarithmic growth phase at 37°C in a humidified atmosphere supplemented with 5% CO2 (147).

3 CD4 T cell pre-activation

12-well flat bottom plates (Greiner Bio-one) were coated with purified no azide, low endotoxin anti-human anti-CD3 antibody (BD Biosciences) by incubating 800 µL of a 1 µg/mL of the antibody in phosphate buffered saline (PBS; Gibco Invitrogen) solution for 2 hours at 37°C. Subsequently, 2 mL of CD4 T cells resuspended in AIM-V + 10% human AB serum (hAB; Gibco Invitrogen) with 2 µg/mL purified no azide, low endotoxin anti-human anti-CD28 antibody (BD Biosciences) at a concentration of 2-18 x 10^6 cells/mL was added to the coated well. Every two to three days, the cells were harvested and the wells were washed with PBS. Then, a sufficiently diluted cell suspension in AIM-V + 10% hAB with 2 µg/mL anti-CD28 was added to freshly coated wells.

4 *In vitro* mRNA transcription

SoloPack Golden supercompetent *E. coli* cells were transformed with pST1 DNA plasmids, kindly provided by Dr. F. Fujiki (Division of Health Sciences, Osaka University, School of Medicine, Osaka, Japan), according to manufacturer's instructions. The plasmids contain the codon-optimised (cop) $WT1_{37\text{-}45}$- (T37)-specific *TCRα* and *TCRβ* genes separated with a 2A sequence from porcine teschovirus-1 (P2A) or *CD8α* and *CD8β* genes separated by a 2A sequence from equine rhinitis A virus (E2A). Transformed *E. coli* cells were cultured in LB-kanamycin agar plates and incubated overnight at 37ºC and amplified in LB-kanamycin cultures at 37ºC under constant motion. Plasmid DNA isolation and purification from bacterial cells were performed using the Nucleobond Xtra Midi EF and Nucleobond finalizer kits (Macherey-Nagel). Next, plasmid DNAs were digested with Sap-I restriction enzyme (Thermo Fisher Scientific) for 16 h at 37ºC. Capped mRNA transcripts were synthesized from linearized plasmids and purified by DNase digestion and LiCl precipitation using a mMessage mMachine T7 *in vitro* transcription kit (Life Technologies) following manufacturer's recommendations (147).

5 Electroporation

Cryopreserved cells were thawed in pre-warmed AIM-V medium (Gibco Invitrogen) with 10% hAB and left to recover for at least one hour at 37°C and 5% CO_2. Before DSE, 10 x 10^6 viable Jurkat E6.1 or 10-20 x 10^6 viable CD4 T cells were washed in cold serum-free Opti-MEM I medium (Gibco Invitrogen), resuspended in 200 µL of the same medium and transferred to a 4.0 mm electroporation cuvette (Cell Projects). Next, the cells were first electroporated with 100 µM of DsiRNA (1:1 pool of 100µM TCRα:TCRβ DsiRNA; Integrated DNA Technologies) and twenty-four hours later with 1 µg of *in vitro* transcribed *TCR* mRNA, with or without *CD8* mRNA, per 10^6 cells. Electroporations were performed in a Gene Pulser Xcell™ device (Bio-Rad Laboratories) using Square Wave protocol (500 V, 5 ms, 0 gap, 1 pulse). Immediately after each electroporation, cells were transferred to 5 mL of RPMI medium supplemented with 10% FBS (Jurkat E6.1 cells) or AIM-V medium with 10% human AB serum (CD4 T cells) and incubated for a minimum of 20 min at 37ºC and 5% CO_2 prior to analyses. Between the first and second electroporations or when cells had to be used on the next day after electroporation, cells were transferred to 6-well plates (Greiner Bio-one) and incubated at 37ºC and 5% CO_2. Cell yield 24 hours after the second electroporation ranged from approximately 40 to 60% and 20 to 30% cells from the total input resting or activated CD4 T cells before electroporation, respectively, with an average viability of 88 and 98% after second electroporation (147). Electroporations with water (mock) are indicated with M and transfection of DsiRNA, *T37 TCR* and *CD8* mRNA with S, T37 and CD8 respectively.

6 Analysis of transgene TCR surface expression

The Jurkat E6.1 or CD4 T cells were harvested and stained with the following mAbs: anti-human TCR Vβ 21.3-FITC (Miltenyi Biotec), CD8-PE, CD3-PerCP, CD4-PECy7 for 15 mins at room temperature. After washing, samples were resuspended in 200 µL of FACS buffer (FACSFlow sheath fluid, BD; 0.1% bovine serum albumin (BSA), Sigma-Aldrich; 0.05% sodium azide, Merck) and measured on a CytoFLEX flow cytometer. Alternatively, Jurkat E6.1 cells were incubated with $WT1_{37\text{-}45}$/HLA-A*02:01 tetramer-PE (monomers kindly provided by Prof. D. A. Price, Division of Infection and Immunity, Cardiff University School of Medicine, Cardiff, UK) for 30 min at 37ºC, washed and stained with anti-human CD3-PerCP, CD8-FITC, CD4-PECy7 mAbs (BD Biosciences) for 15 min at room temperature. After washing, cells were resuspended in 200 µL of FACS buffer for

flow cytometric analysis using a CytoFLEX flow cytometer. CD4 T cells were harvested after electroporation at different time points and stained with $WT1_{37-45}$/HLA-A*02:01 tetramer-PE for 30 min at 37ºC. Next, cells were washed in FACS buffer and stained with anti-human CD3-PerCP, CD8-FITC and CD4-PECy7 mAbs (BD Biosciences) for 15 min at room temperature. After washing, cells were resuspended in 200 µL of FACS buffer for flow cytometric analysis using a CytoFLEX flow cytometer. First, an electronic gate was set on the lymphocytes, followed by selection of the CD3(+)CD4(+) cells. TCR(+) and CD8(+) fractions were then analysed from this final population (147).

7 RT-qPCR analysis

Twenty-four hours after electroporation, total RNA was extracted from human primary resting or pre-activated CD4 T cells using RNeasy Micro kit (QIAGEN), according to the manufacturer's instructions. Complementary DNA (cDNA) was synthetized by reverse transcription from total RNA samples using iScript cDNA synthesis kits (Bio-Rad) and diluted in water to equal concentrations. Real-time quantitative PCR (RT-qPCR) reactions were performed in duplicate on a CFX96™ real-time PCR detection system (Bio-Rad) using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) and PrimePCR™ primers (Bio-Rad) to detect and quantify the relative abundance of T cell receptor alpha constant region mRNA (*TRAC*; forward primer: 5'-CTGTCTGCCTATTCACCGATT-3', reverse primer: 5'-GTCAGATTTGTTGCTCCAGG-3'), T cell receptor beta constant region mRNA (*TRBC*; forward primer: 5'-GGTGAATGGGAAGGAGGTG-3', reverse primer: 5'-GTATCTGGAGTCATTGAGGGC-3') and T37 *TCR* mRNA (forward primer: 5'-AAGAGAACCCTGGCCCTATG-3', reverse primer: 5'-CCGCTGTTCTGTCTGTACCA-3') transcripts. Importin-8 (IPO8, Hs.505136; Biorad, qHsaCED0056515) and ribosomal protein L13A (RPL13A, Hs.523185; Biorad, qHsaCED0020417) were chosen as reference genes (148). Results were analysed using CFX Manager (v3.1, Bio-Rad) (147).

8 Flow cytometric analysis of activation markers

For the analysis of TCR specificity, T2 or U266 cells were peptide-pulsed with 10 µg/mL of $WT1_{37-45}$ (JPT Peptide Technologies) per 10^6 cells in 1 mL of serum-free IMDM medium (Gibco Invitrogen) for 60 min at room temperature under constant motion. Next, the cells were washed and resuspended in AIM-V medium with 10% hAB and added to electroporated human primary CD4 T cells at an effector-target ratio of 4:1 and incubated

for 6 or 24 hours at 37ºC and 5% CO_2. After incubation, supernatants were collected for analysis of cytokine secretion and cells were stained with either anti-human CD8-Pacific Blue (Life Technologies), CD3-PerCP-Cy5.5, CD4-FITC, CD69-APC-Cy7, CD154-PE mAbs (BD Biosciences), and LIVE/DEAD fixable aqua dead cell stain kit (Thermo Fisher Scientific) for 15 min at room temperature (6 hour co-cultures) or CD8-Pacific Blue, CD127-FITC (Miltenyi Biotec), CD137-PerCP-Cy5.5 (Biolegend), CD154-PE, CD4-PECy7, CD25-APC (Biolegend), CD69-APC-Cy7 mAbs and LIVE/DEAD fixable aqua dead cell stain kit for 15 min at room temperature (24 hour co-cultures). Cells were washed and analysed using a FACSAria II flow cytometer (BD Biosciences). The gating strategy consisted of gating on singlet viable lymphocytes. Then, analysis of the activation markers was performed on the CD4 T cells through a gate on CD3(+)CD4(+) for 6 hour co-cultures or CD4(+) cells for 24 hour co-cultures (147).

9 Cytokine secretion assays

Secretion of IFN-γ and granzyme B by electroporated human primary pre-activated CD4 T cells was determined by enzyme-linked immunosorbent assay kits (ELISA; respectively, Peprotech, Affimetrix and R&D Systems) following manufacturer's instructions in supernatants of co-cultures used for the analysis of activation markers. All ELISA plates were measured using a Victor 3 multilabel plate reader (Perkin Elmer) (147).

10 Statistical analysis

Flow cytometry data were analysed using FlowJo software (v10.2, TreeStar Inc). Prism software (v7, GraphPad) was used for graphing and statistical calculations. Graphs represent mean values ± SEM or individual measurements per donor. Data were analysed using Welsh's unpaired parametric t test or repeated measures one-way or two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc comparisons between different electroporation conditions. Results were considered statistically significant when p-value was less than 0.05 (147).

Results

1 The Jurkat E6.1 cell line as a model for primary CD4 T cells

The Jurkat E6.1 cell line, which is TCRαβ(+), CD4(+) and CD8(-), was used as a model for primary human CD4 T cells to study the expression of the transgene TCR. As described in the methods, they were first electroporated with either water (mock, M) or DsiRNA (silenced, S) and subsequently with the T37 *TCR* mRNA with or without *CD8* mRNA. Transgene TCR surface expression was observed through staining with pMHC tetramers and mAb targeting the Vβ chain of the TCR (TCR Vβ21.3). DsiRNA transfection significantly increased the %tetramer(+) Jurkat E6.1 cells, but only in the absence of CD8 (MT37 vs ST37: $p < 0.0001$) (Figure 4A). Meanwhile, Jurkat E6.1 cells electroporated with ST37+CD8 showed significantly higher tetramer staining than conditions where CD8 was not transfected (MT37 vs MT37+CD8: $p < 0.0001$; ST37 vs ST37+CD8: $p < 0.01$). It was further confirmed that CD8 introduction caused this increase as all tetramer(+) cells were also co-expressing CD8 (data not shown). On the other hand, the TCR Vβ21.3 chain was present on nearly all cells for all conditions (Figure 4B). No considerable differences were found between all treated groups.

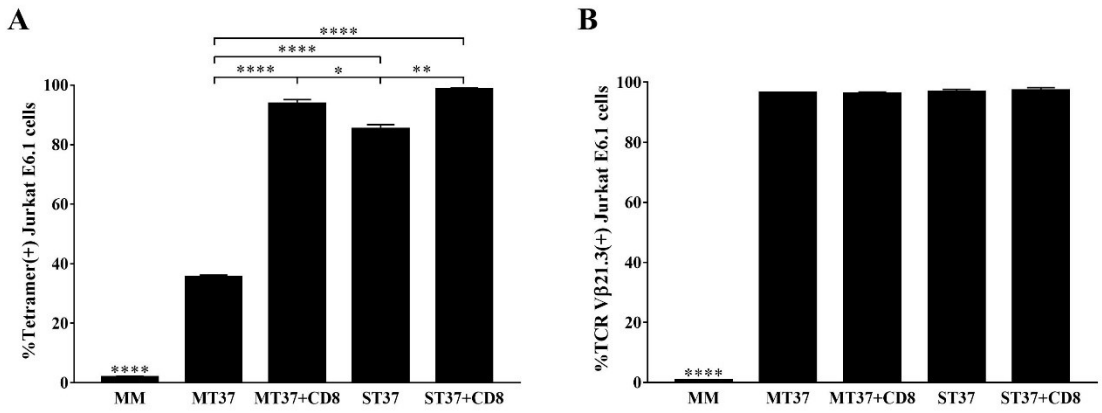

Figure 4 | TCR expression in DSE Jurkat E6.1 cells.

DSE Jurkat E6.1 cells (N = 2) were stained with either pMHC tetramers or TCR Vβ21.3 mAb. **(A)** Tetramer staining of the ST37+CD8 condition showed significantly higher transgene TCR expression levels compared to all other conditions, except MT37+CD8. Furthermore, DsiRNA electroporation or co-introduction of *CD8* mRNA, but not both, also significantly improved tetramer binding capacity. **(B)** Nearly all Jurkat E6.1 cells were TCR Vβ21.3(+) in all treated groups and no considerable differences were found between them. Electroporation conditions: M = Mock, S = DsiRNA, T37 = T37 *TCR* mRNA, CD8 = *CD8* mRNA.

2 The effect of cryopreservation and DSE on the viability and yield of CD4 T cells

After confirmation of expression of the T37 TCR in the model, cells from healthy volunteers were used for further experiments. Freshly isolated primary CD4 T cells from PBMC were immediately pre-activated, electroporated or cryopreserved at -80°C. At least one hour after thawing, the cryopreserved cells showed a significant decrease in cell viability ($p < 0.01$) (Figure 5A). However, during the thawing of the first three replicates, the DMSO in the cryopreservation medium was only diluted between 1:2 and 1:4 instead of 1:10. This did not have a major effect on the viability, as the difference remains significant after excluding these samples from the analysis (data not shown). As an initial attempt to improve the yield after freezing overall, hAB + 10% DMSO cryopreservation medium was compared to the standard, FBS + 10% DMSO, for one donor, including two replicates (Supplementary table 1). The viability and yield for the experimental medium were on average nine and 14 percentage points lower, respectively, than the FBS + 10% DMSO.

Despite the discrepancy in viability between fresh and frozen cells, there was no significant difference between fresh DSE (85%) and frozen DSE cells (86%) (Figure 5A). Note that the cryopreserved cells improved in viability after second electroporation. Similarly, when comparing before and after DSE, there was little difference in the amount of cells lost between fresh and frozen material (46% and 43%, respectively) (Figure 5B).

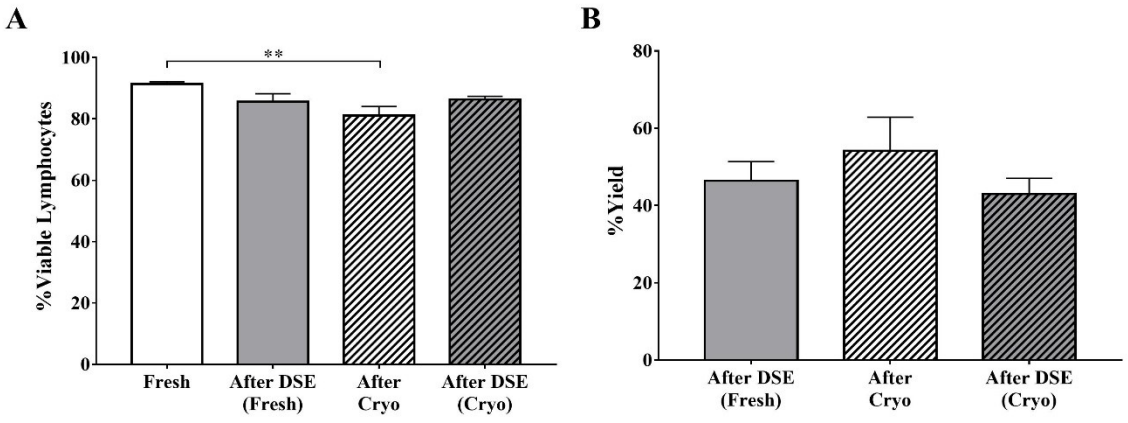

Figure 5 | Viability and yield of CD4 T cells before and after cryopreservation or DSE.
(A) The viability of CD4 T cells after cryopreservation dropped significantly, but recovered to the same level after DSE as fresh material. **(B)** Freezing of the cells causes a considerable loss of CD4 T cells. The yield after DSE is similar between fresh and cryopreserved cells compared to the amount of viable cells before electroporation. DSE = Double sequential electroporation. N = 4-8 independent replicates.

3 Transgene TCR expression levels in primary CD4 T cells following DSE

Similar to the Jurkat E6.1, primary CD4 T cells were stained with T37 TCR-specific tetramers or an anti-TCR Vβ21.3 mAb. Surface expression kinetics of the TCR indicated a maximal expression level around 24 hours after the second electroporation (Supplementary figure 1). Therefore, samples were stained between 14h and 30h after the second electroporation in all remaining experiments. Variation in measurement timepoints was due to technical difficulties or unavailability of the CytoFLEX flow cytometer. Freshly isolated (circles) and cryopreserved cells (squares) were pooled as a resting group (Figure 6, left), while CD4 T cells of two donors were subjected to expansion using anti-CD3 and anti-CD28 antibodies, forming the pre-activated group (Figure 6, right). The small size of this group did, however, not allow for reliable statistical analysis.

Staining with a panel consisting of the tetramers and anti-CD3, CD4 and CD8 mAbs showed a significant increase in stained cells for the ST37+CD8 electroporation condition compared to the control (MM) and ST37 condition (both $p < 0.001$) (Figure 6A). Additionally, the MT37+CD8 condition (13.0%±1.0%; N = 2; Data not shown) showed a smaller proportion of positive cell compared to its silenced counterpart (23.7%±5.8%; N = 8; Figure 6A). Furthermore, a relatively large variation was seen between donors within the ST37+CD8 condition, ranging from 2.7% (DR1) to 46.4% (DR15). Conversely, with an average of 63.5%±3.1% (N = 2), the two pre-activated donors showed considerably higher levels of tetramer staining (Figure 6B). While no TCR(+) cells were observed for the resting ST37 condition, there was moderate staining for the pre-activated group. One donor (DR14) was excluded from the analysis since RT-qPCR analysis showed no or limited levels of introduced T37 *TCR* mRNA and as a consequence the absence of protein expression.

On the other hand, multiparametric analysis with a mAb cocktail containing anti-TCR Vβ21.3, CD3, CD4 and CD8 mAbs did not show a significant difference between the electroporation conditions for non-activated cells. Although it could be expected to detect higher amounts of TCR Vβ21.3(+) CD4 T cells compared to the tetramer staining due to the fact that mispaired chains are stained as well, this was only valid for two out of three donors (DR8b and DR9) (Figure 6C). The pre-activated cells were first stained for the transgene variable TCRβ chain and subsequently for CD3, CD4 and CD8. Similar to the non-activated cells, a lower expression level was detected.

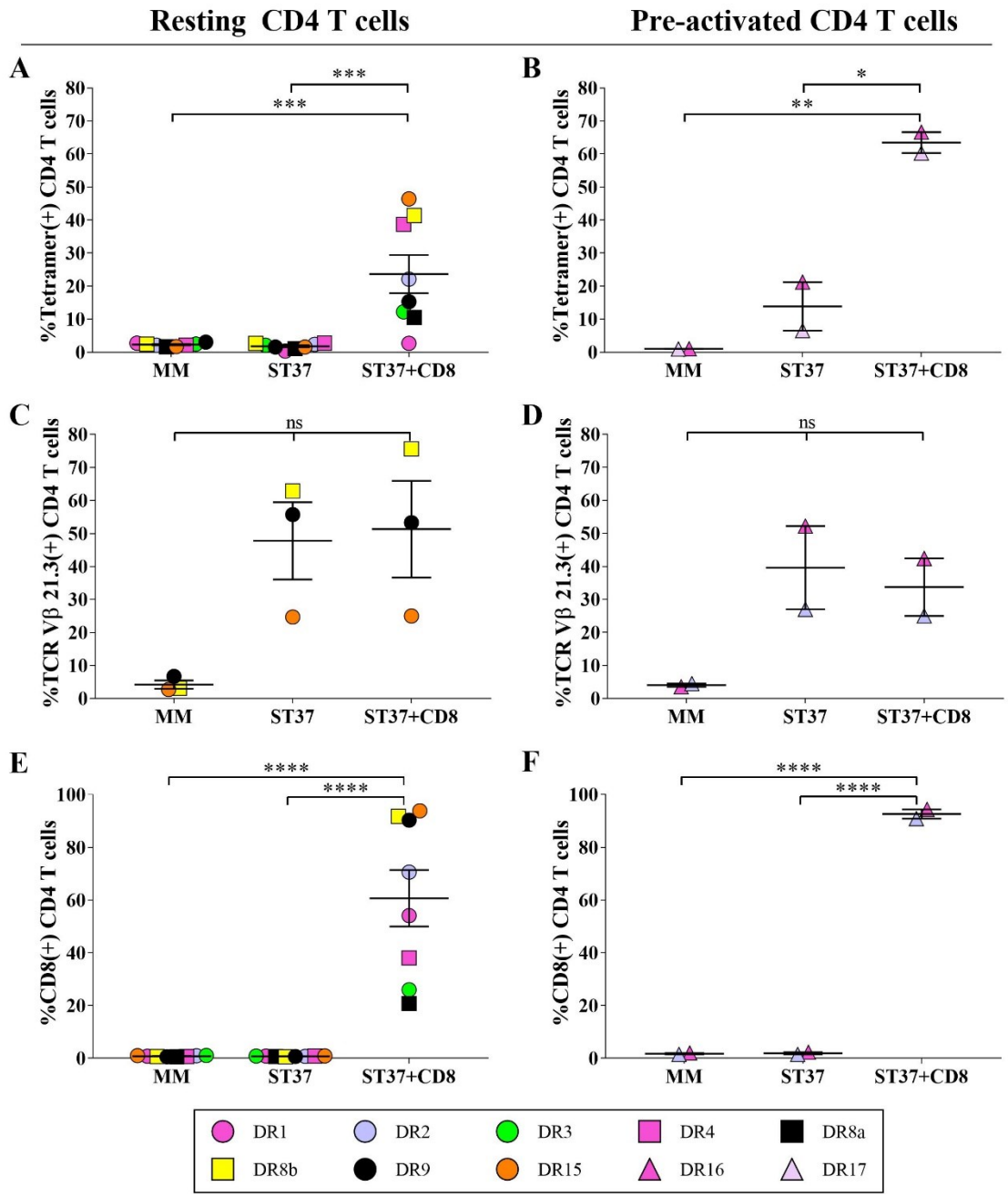

Figure 6 | T37 TCR and CD8 surface expression in DSE primary CD4 T cells.

Flow cytometric analysis of the $WT1_{37\text{-}45cop}$-specific TCR surface expression with tetramer or anti-TCR Vβ21.3 antibody showed a considerable amount of cells expressing the TCR, in the presence or absence of CD8, after DSE **(A-D)**. CD8 was significantly more present on the cells after electroporation of the mRNA **(E,F)**. Both resting (N = 8) and pre-activated (N = 2) CD4 T cells were assessed (left and right column, respectively). Resting T cells consisted of pooled data from fresh (circles) and cryopreserved (squares) DSE cells. Data from pre-activated CD4 T cells are depicted as triangles. Electroporation conditions: M = Mock, S = DsiRNA, T37 = T37 *TCR* mRNA, CD8 = *CD8* mRNA. DR = Donor; ns = not significant.

CD8 expression levels were measured simultaneously with tetramer staining (Figure 6E,F), as well as in the TCR Vβ21.3-panel (data not shown). Similar to tetramer staining levels in non-activated cells, variation in CD8 expression is relatively large, ranging from 20.8% (DR3) to 93.4% (DR15). Thus far, pre-activated cells show consistently high levels of CD8 (92.6%). Furthermore, as demonstrated by DR1 and DR4, low CD8 expression is not always linked to low TCR expression or *vice versa* (Figure 6A,E).

4 RT-qPCR analysis of RNA transfection efficiency

The effect of DsiRNA and introduction of T37 *TCR* mRNA were verified using RT-qPCR. Samples from human primary CD4 T cells were collected 24 hours after first electroporation to confirm the downregulation of the endogenous TCR mediated by the DsiRNA or 24 hours after the second electroporation to assess the transfection efficiency of the T37 *TCR* mRNA. Since the DsiRNA are designed to specifically downregulate transcripts encoding the TCRα and TCRβ chain constant region (*TRAC* and *TRBC*, respectively), we analysed the levels of these transcripts compared to untreated (mock) cells. Significant downregulation ($p < 0.0001$) of both *TRAC* and *TRBC* transcripts in DSE CD4 T cells was detected, as shown in Figure 7A,B, which confirmed the silencing effect

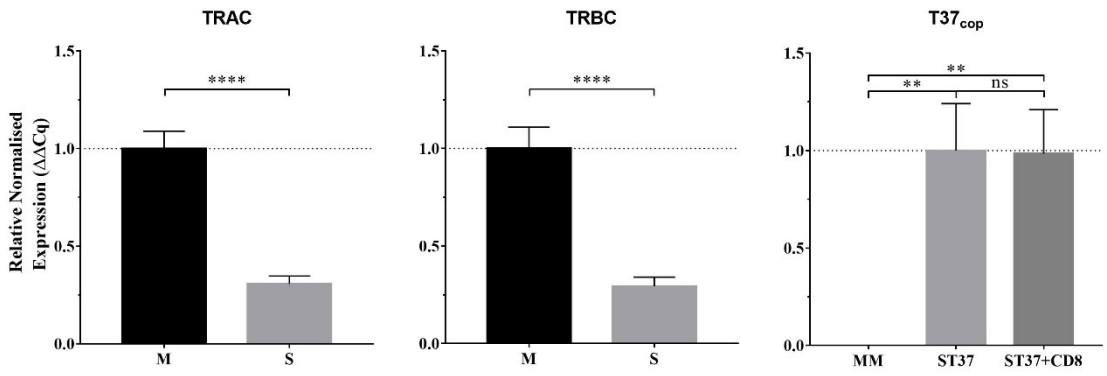

Figure 7 | RT-qPCR analysis of DsiRNA silencing capacity and T37 *TCR* mRNA transfection efficiency in resting DSE CD4 T cells.

Introduction of DsiRNA targeting the alfa and beta constant regions of the wt *TCR* mRNA (*TRAC* and *TRBC*, respectively) proved to significantly downregulate *TRAC* (left) and *TRBC* (middle) expression (N = 7). Furthermore, T37 *TCR* mRNA was efficiently transfected as shown by the significantly increased expression levels of the transcript in both treatment conditions (right) (N = 6). Electroporation conditions: M = Mock, S = DsiRNA, T37 = T37 *TCR* mRNA, CD8 = *CD8* mRNA.

of the DsiRNA on the endogenous *TCR* chains and the *de novo* expression thereof. Similar results were observed in pre-activated DSE CD4 T cells (Supplementary figure 2).

With regard to the transfection efficiency of the introduced T37 *TCR* mRNA, custom primers targeting a sequence comprising the P2A and part of the *TCRα* chain were used to specifically quantify the relative amount of this mRNA and to exclude the quantification of the endogenous *TCR* transcripts. The significant ($p < 0.01$) increase in *T37* expression present showed that T37 *TCR* mRNA was efficiently introduced in the appropriate conditions of all donors (Figure 7C), except for the excluded DR14, as mentioned earlier. The variability in the levels of introduced T37 *TCR* mRNA was consistent with the variable expression levels of the TCR on the cell surface.

5 Antigen-specific upregulation of activation markers on CD4 T cells

Upon recognition of their target antigen, antigen-specific CD4 T cells will upregulate defined activation markers independent of the functional specialisation of the cell (149). To correctly analyse the antigen-specific activation of CD4 T cells, Bacher & Scheffold (149) recommended targeting CD69, CD137 (4-1BB) and CD154.

For this experiment, the donors were divided into three groups: resting CD4 T cells co-cultured for 6 hours or 24 hours and pre-activated CD4 T cells co-cultured for 24 hours. As a late activation marker, the upregulation of CD137 was only assessed in the 24 hour co-cultures (149).

CD69 is one of the earliest activation markers to observe after antigen specific activation (149). There was only a noticeable increase in CD69 expression for the ST37+CD8 condition of the pre-activated CD4 T cells in all co-culture conditions, but none of them were significantly different from the control (double mock electroporated CD4 T cells) (Figure 8A). Meanwhile, only a subtle increase is apparent for both ST37 and ST37+CD8 electroporated resting CD4 T cells when co-cultured with peptide-pulsed T2 cells (Supplementary figure 3A,B).

CD154, also known as CD40L, is a recommended marker to assess CD4 T cell activation with a peak expression between four and twelve hours (149). Therefore, it can be used in combination with other markers, such as CD69 and CD137. Due to the rapid internalisation when binding to its receptor, CD40, blocking of this interaction with an mAb is required (149). In the co-cultures with resting CD4 T cells, none of the conditions showed

an increase in CD154 after 6 or 24 hours (Supplementary figure 3C,D). In contrast, as depicted in Figure 8B, CD154 was significantly upregulated in the pre-activated CD4 T cells upon TCR triggering with peptide-pulsed T2 cells. Furthermore, MT37+CD8 electroporated CD4 cells co-cultured with peptide-pulsed T2 cells showed only slightly lower CD154 levels than the ST37+CD8 condition (30% vs 38%; data not shown). Remarkably, the peptide-pulsed U266 co-culture did not result in an increased level of CD154(+) cells (data not shown).

Lastly, also CD137 was significantly upregulated in the ST37+CD8 pre-activated T cells (2.7% vs 17.6%) upon specific recognition of the WT1-antigen on T2 cells compared to the control (Figure 8C), while the difference in expression for co-cultures with U266 was smaller (4.0% vs 8.7%; data not shown). Importantly, despite the fact statistical relevance was obtained for two activation markers, it needs to be emphasised that only two replicates were studied and caution should be taken when interpreting the results.

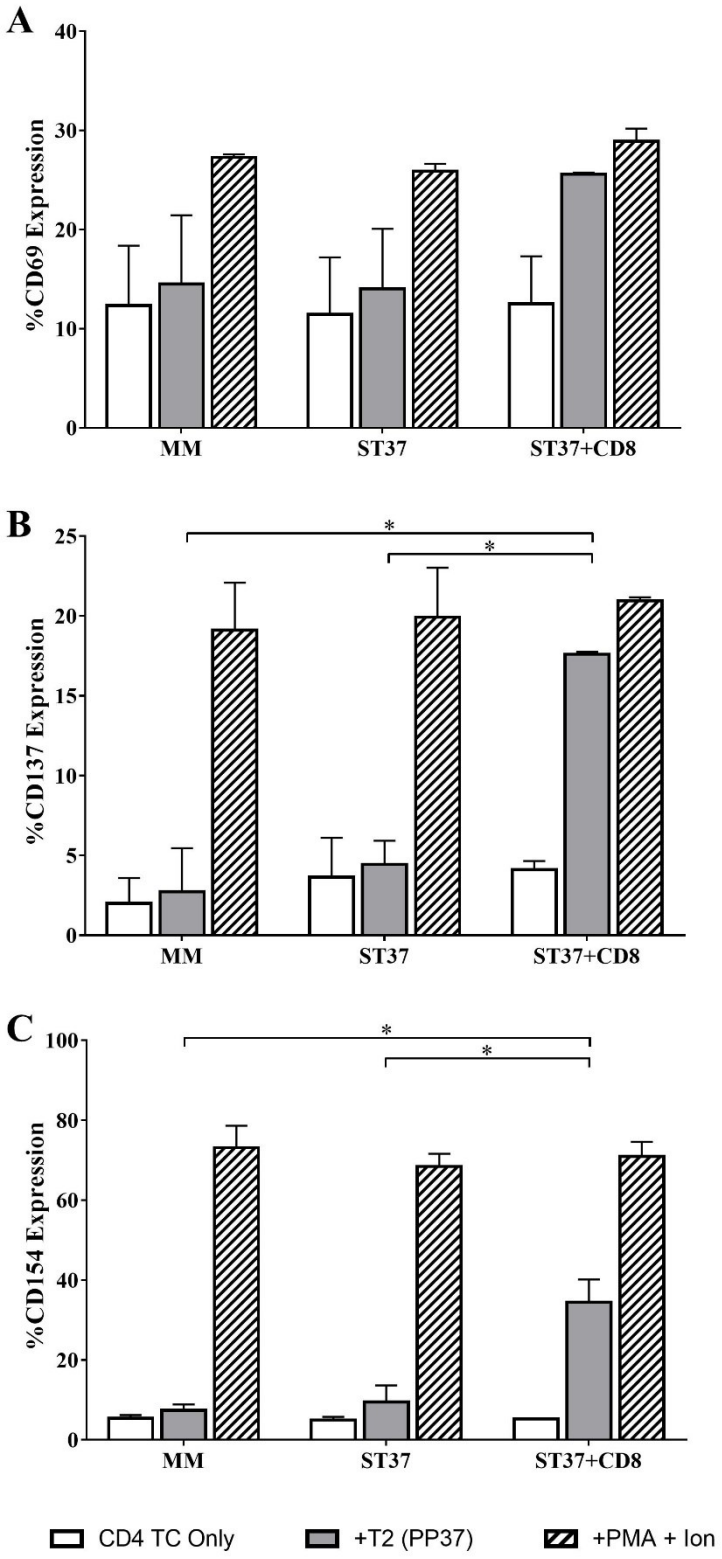

Figure 8 | Antigen-specific upregulation of activation markers in DSE CD4 T cells.

In the presence of peptide-pulsed T2 cells, only pre-activated ST37+CD8 electroporated CD4 T cells showed a significant increase in CD137 and CD154, and a considerable increase in CD69. All conditions containt 2 independent replicates. Electroporation conditions: M = Mock, S = DsiRNA, T37 = T37 *TCR* mRNA, CD8 = *CD8* mRNA. TC = T cells; PP37 = peptide-pulsed with $WT1_{37\text{-}45}$; PMA = phorbol myristate acetate; Ion = ionomycin.

6 Secretion of granzyme B and IFN-γ upon antigen recognition

Following co-culturing of pre-activated DSE CD4 T cells with $WT1_{37-45}$ peptide-pulsed T2 or U266 cells, the supernatant was harvested from duplicate wells. Subsequently, granzyme B (Table 1) and IFN-γ ELISA (Table 2) were performed to further assess the cytotoxic functionality and activation of the CD4 T cells, respectively. Only the results of the T2 co-cultures compared to the negative (T cells only) and positive (PMA + ionomycin) control are depicted.

With regard to granzyme B secretion, both donors showed considerably increased levels compared to the control for the ST37+CD8 condition when CD4 T cells were co-cultured with peptide-pulsed T2 cells, exceeding the concentration of the highest standard (2.5 ng/mL) as shown in Table 1. Furthermore, MT37+CD8 electroporated CD4 T cells showed an improved release, similar to their silenced counterparts (data not shown).

By contrast, IFN-γ was only synthesised by CD4 T cells with both T37 TCR and CD8 in the presence of antigen-bearing T2 cells (Table 2). Downregulation of the endogenous TCR largely improved the amount of IFN-γ detected in the supernatant (data not shown).

Table 1 | Granzyme B release of DSE pre-activated CD4 T cells.

Compared to the negative control, i.e. T cells cultured without tumour cells, the ST37+CD8 condition of both donors showed considerably elevated levels of granzyme B secretion after co-culture with peptide-pulsed T2 cells. Unexpectedly, the MM and ST37 condition of donor 16 displayed secretion of granzyme B as well. Data was obtained from hard duplicates for each donor. Electroporation conditions: M = Mock, S = DsiRNA, T37 = T37 *TCR* mRNA, CD8 = *CD8* mRNA. TC = T cells; PMA = phorbol myristate acetate; Ion = ionomycin; DR = donor.

Granzyme B (ng/mL)				
Co-culture	**Donor**	**MM**	**ST37**	**ST37+CD8**
TC Only	DR16	0.229	0.137	0.130
	DR17	0.144	0.086	0.099
+T2	DR16	1.015	1.506	>2.500
	DR17	0.240	0.107	>2.500
+PMA+Ion	DR16	>2.500	>2.500	>2.500
	DR17	>2.500	2.455	>2.500

Table 2 | IFN-γ release of DSE pre-activated CD4 T cells.

Only cells that express the T37 TCR (ST37 with or without CD8) release IFN-γ in the presence of peptide-presenting T2 cells. Data was obtained from hard duplicates for each donor. Electroporation conditions: M = Mock, S = DsiRNA, T37 = T37 *TCR* mRNA, CD8 = *CD8* mRNA. TC = T cells; PMA = phorbol myristate acetate; Ion = ionomycin; DR = donor.

IFN-γ (ng/mL)				
Co-culture	**Donor**	**MM**	**ST37**	**ST37+CD8**
TC Only	DR16	0.000	0.000	0.000
	DR17	0.074	0.051	0.050
+T2	DR16	0.000	0.000	0.911
	DR17	0.000	0.060	>3.000
+PMA +Ion	DR16	0.526	0.590	0.702
	DR17	0.158	0.481	0.470

Discussion

During this master thesis, we investigated whether primary CD4 T cells, that were equipped with an HLA-A2-restricted T37 TCR through electroporation, could be activated upon recognition of their antigen presented by tumour cells and examined the role of the CD8 co-receptor in this process. Furthermore, overall transfection efficiency and the effect of cryopreservation on CD4 T cells was studied.

In view of future applications of this research, cryopreservation of CD4 T cells might be important. For example, when moving to the clinical setting, it may be desirable to store material of multiple patients for later reinfusion or simultaneous immunological assays to minimise variation (150, 151). However, the freeze-thawing process is often accompanied by a substantial loss of cell material. It is also of importance that the electroporation protocol is optimised to minimise cell death during the process.

Although the methods used in this study resulted in high cell viability, the yield after thawing remained considerably lower than the expected 70-80%, as observed in earlier experiments using CD8 T cells within our lab and reported in PBMC cryopreservation literature (151). An alternative cryopreservation medium was tested, containing hAB instead of FBS, in light of future clinical applications and in an attempt to improve cell recovery. However, this new medium did not improve the yield and had a negative effect on the viability as well. Therefore, it is advised to further optimise the freezing medium for the cryopreservation of CD4 T cells. However, other factors could have also influenced the obtained results. For instance, improper handling of the cells during the entire cryopreservation process could have exerted negative effects on both the viability and yield (151). Moreover, as biological time is not completely stopped below -130°C (152), additional cell losses could be caused by the prolonged storage (up to 50 days) at -80°C. In contrast, Weinberg *et al.* showed that storing PBMC for up to three weeks at -70°C compared to liquid nitrogen did not result in any significant difference in either viability or yield across multiple laboratories (151). However, as cryopreservation is complex and optimal conditions differ between cell types (152), it might be required to assess the effect of storage temperature on CD4 T cells. Lastly, part of the variation in yield might be attributed to biological differences between donors. Regardless of these issues, the combined loss of cells by the cryopreservation process and DSE should be accounted for during future experiments.

Efficient transfer of the DsiRNA, T37 *TCR* and *CD8* mRNA is crucial for further assays. Electroporation of these RNAs in the Jurkat E6.1 model, using the protocol described in the *Materials and methods* section, showed excellent results. Staining with MHC-I tetramers and TCR Vβ21.3 mAb showed that nearly all cells were positive for the transgene TCR when combined with the DsiRNA and CD8 co-receptor. In the absence of DsiRNA, mispairing of the transgene TCR chains with those of the endogenous TCR was more prevalent, which consequently results in a decreased tetramer staining. Furthermore, binding between the TCR and tetramer not only relies on the affinity of the TCR, but also on the clustering of the TCR and co-receptor stabilisation (153). Therefore the staining has a direct relationship with the avidity of the TCR of interest and thus the stability of the interaction between the TCR and pMHC. The CD8 co-receptor stabilises this interaction and explains the improved results in conditions with *CD8* mRNA.

In stark contrast to Jurkat E6.1 cells, primary resting CD4 T cells were less efficiently electroporated. Nonetheless, similar to CD8 T cells, TCR expression was maximal around 24 hours after DSE (154). Previous reports confirm that transfection efficiencies are generally lower for resting cells as compared to stimulated cells (155, 156). Therefore it was not surprising that the transfection efficiency of pre-activated CD4 T cells was considerably higher, resulting in TCR:pMHC complex formation even in the absence of CD8. Earlier studies describe that high affinity TCRs can bind tetramers without any co-receptor, but the presence of CD8 further enhances the formation of the TCR:pMHC complex (145). In our observations, the CD8 co-receptor further stabilises the complex, as shown by the larger fraction of tetramer(+) cells, and indicates that the T37 TCR is CD8-dependent (153). The introduction of DsiRNA effectively silences the endogenous TCR chains, confirmed by RT-qPCR analysis and the consistent improved tetramer(+) fractions for the ST37+CD8 condition compared to the MT37+CD8 condition. Furthermore, relative quantification of T37 *TCR* mRNA expression levels did not show a difference between ST37 and ST37+CD8 conditions, showing that CD8 co-introduction does not impede the transfection of the T37 *TCR* mRNA.

Surprisingly, the number of TCR Vβ21.3(+) cells was much lower than expected as the antibody should also stain mispaired chains of the transgene TCR. This might be caused by steric hindrance between the fluorochromes of the antibodies that target nearby epitopes (157), in this case of the TCR complex. However, this hypothesis seems unlikely for several reasons. For instance, FITC is a small fluorochrome compared to PE (including its conjugates) and PerCP (158), which should not be seriously affected by other

fluorochromes. Moreover, staining with the TCR Vβ21.3 mAb prior to staining with other antibodies did not improve the results. A comparison between single and double stains should rule out this possibility completely.

Although there was a marked difference in tetramer staining between MT37+CD8 and ST37+CD8 conditions of pre-activated CD4 T cells, there was a much smaller discrepancy in upregulation of activation markers. These results suggest that the introduction of DsiRNA is not required for the functional activation of the CD4 T cell through the transgenic TCR. This finding is further confirmed by the levels IFN-γ secretion in both conditions. Nonetheless, DsiRNAs are essential to maximise the number of available CD3 molecules for the transgenic TCR, limit mispairing with endogenous TCR chains and prevent the potential consequences of off-target specificities. The upregulation of the activation markers and the production of IFN-γ was only seen in samples where CD8 was co-introduced, which suggests that CD8 is essential for the activation of the CD4 T cells through the T37 TCR. These results are in line with previous reported observations by Willemsen *et al.* for virally transduced HLA class I-restricted CD4 T cells (144). They hypothesised that the role of CD8 is not only important in the stabilisation of the TCR:pMHC complex, but also in the subsequent increased activation level by improving the binding kinetics of the complex. Although TCR-redirected CD4 T cells generated by DSE also released granzyme B, the results from the two donors are rather inconclusive. It was already described that specific CD4 cytotoxic lymphocytes show perforin and granzyme B-mediated toxicity in several infectious diseases and malignancies (159). The release of granzyme B in our experiments hints to the fact that, besides its helper role, the cytotoxic capacity of the CD4 T cells can be triggered as well.

It has to be emphasised that the presented results on the activation of the CD4 T cells were only obtained for a small number of replicates and that they should be interpreted with caution. Therefore, future research should start by confirming the results presented in this work with a higher number of independent experiments from multiple donors. Next, it might be interesting to further elucidate the effector functionality of TCR-redirected CD4 T cells through cytokine profiling, including IFN-γ, TNF-α, IL-2, IL-4, IL-5 and IL-10. Direct cytolytic functionality can be assessed through cytotoxicity assays with WT1 peptide-pulsed or full length mRNA-transfected APCs, or by perforin and granzyme B secretion assays. Similar work can be done on the combination of DSE TCR-redirected CD4 and CD8 T cells with HLA-A2-restricted TCRs specific for the same or different target antigens to study their synergistic effect. Alternatively, CD4 T cells can be equipped

with an HLA class-II-restricted TCR in combination with an HLA class-I-restricted CD8 counterpart. Meanwhile, it might be interesting to look into other cryopreservation media to obtain higher yields after freeze-thawing of CD4 T cells and to upscale the DSE protocol in a GMP-compatible fashion for early phase clinical trials in the nearby future.

Conclusion

As most of the obtained results in this thesis should be viewed as preliminary, no definitive conclusions can be made. In summary, our findings confirm that CD4 T cells, especially after expansion, can be effectively equipped with a WT1-specific HLA-A2-restricted TCR with reduced mispairing with endogenous TCR chains through the described double sequential electroporation protocol. The co-expression of the CD8 receptor substantially improves pMHC tetramer binding of the T37 TCR, which suggests CD8-dependency of the studied TCR. Furthermore, analysis of activation surface markers on pre-activated TCR-redirected CD4 T cells shows antigen-specific TCR triggering upon cognate antigen recognition on tumour cells only when CD8 is co-transfected. While results of the IFN-γ and granzyme B secretion of these re-activated CD4 T cells remain preliminary and inconclusive, regarding the need for CD8, it does further support the hypothesis of the cytolytic capacity of CD4 T cells.

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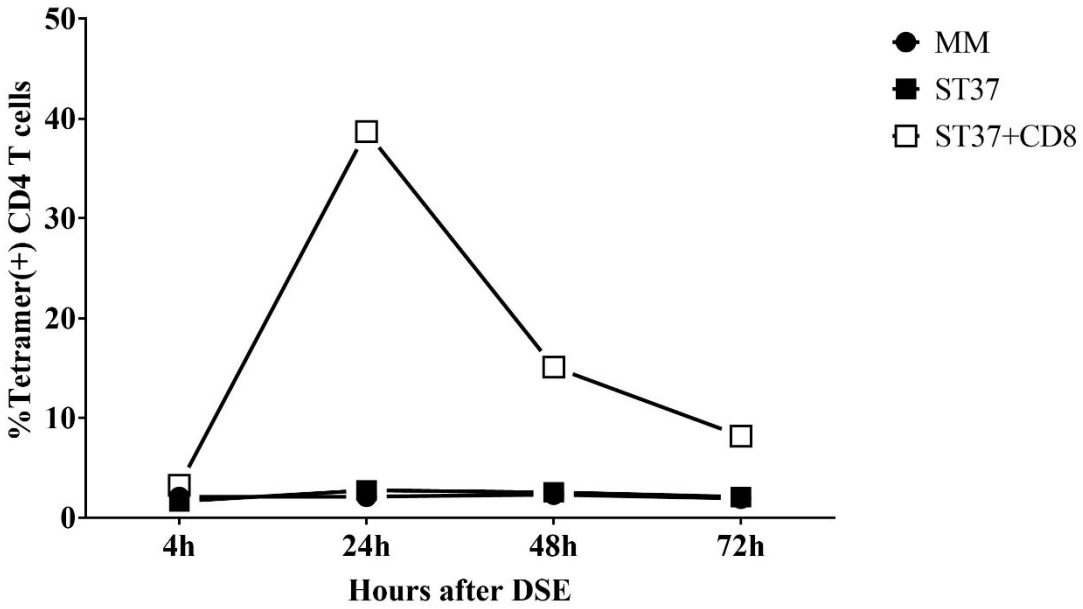
Supplementary data

Appendix A: Cell viability and yield

Supplementary table 1 | Cell viability and yield of CD4 T cells after cryopreservation and DSE.
The percentage of viable cells of the total population and the yield, which is the ratio of viable cells after the treatment over the total amount of cell material before the treatment. Donor 5 was cryopreserved in media containing either FBS or hAB + 10% DMSO. "After DSE" is the mean of all DSE conditions. N = 1 for all donors, except for donor 5 (N = 2). DR = donor; DSE = double sequential electroporation.

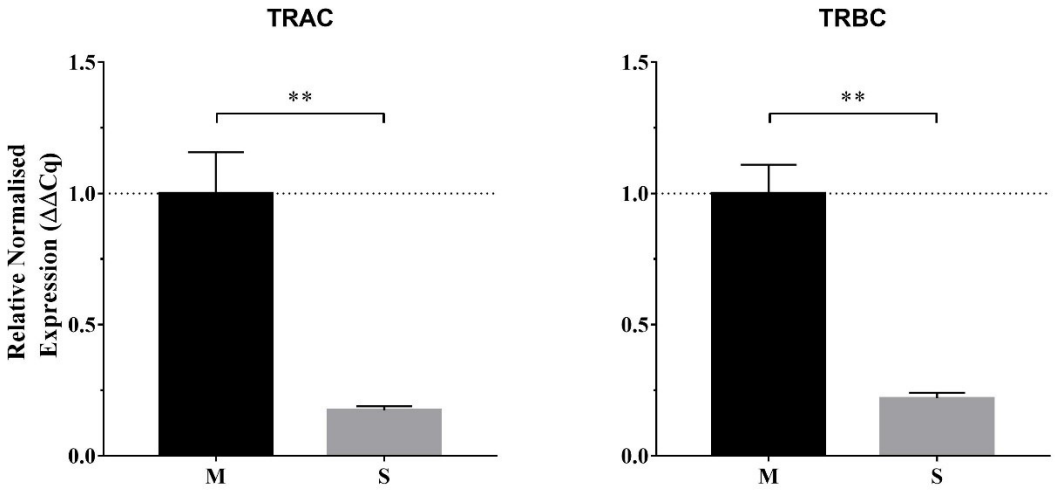
	Viability		Yield	
	After cryopreservation	After DSE	After cryopreservation	After DSE
DR1		93%		49%
DR2		89%		55%
DR3		87%		56%
DR4	74%	85%	38%	38%
DR5 FBS	91%		85%	
DR5 hAB	84%		71%	
DR6	79%	84%	48%	41%
DR8a	81%	89%	61%	58%
DR8b	82%	87%	67%	39%
DR8c	75%		26%	
DR9		89%		58%
DR14FR		88%		43%
DR15FR		91%		55%
DR16		98%		23%
DR17		98%		29%

Appendix B: Kinetics of transgenic TCR surface expression



Supplementary figure 1 | Kinetics of the surface expression of the transgenic TCR in DSE CD4 T cells. DSE CD4 T cells (N = 1) show a peak surface expression of the transgene TCR 24 hours after the second electroporation, similar to previously obtained results for CD8 T cells by Campillo-Davo *et al.* (154). Tetramer staining is only viable when cells are co-transfected with *CD8* mRNA. Electroporation conditions: M = Mock, S = DsiRNA, T37 = T37 *TCR* mRNA, CD8 = *CD8* mRNA.

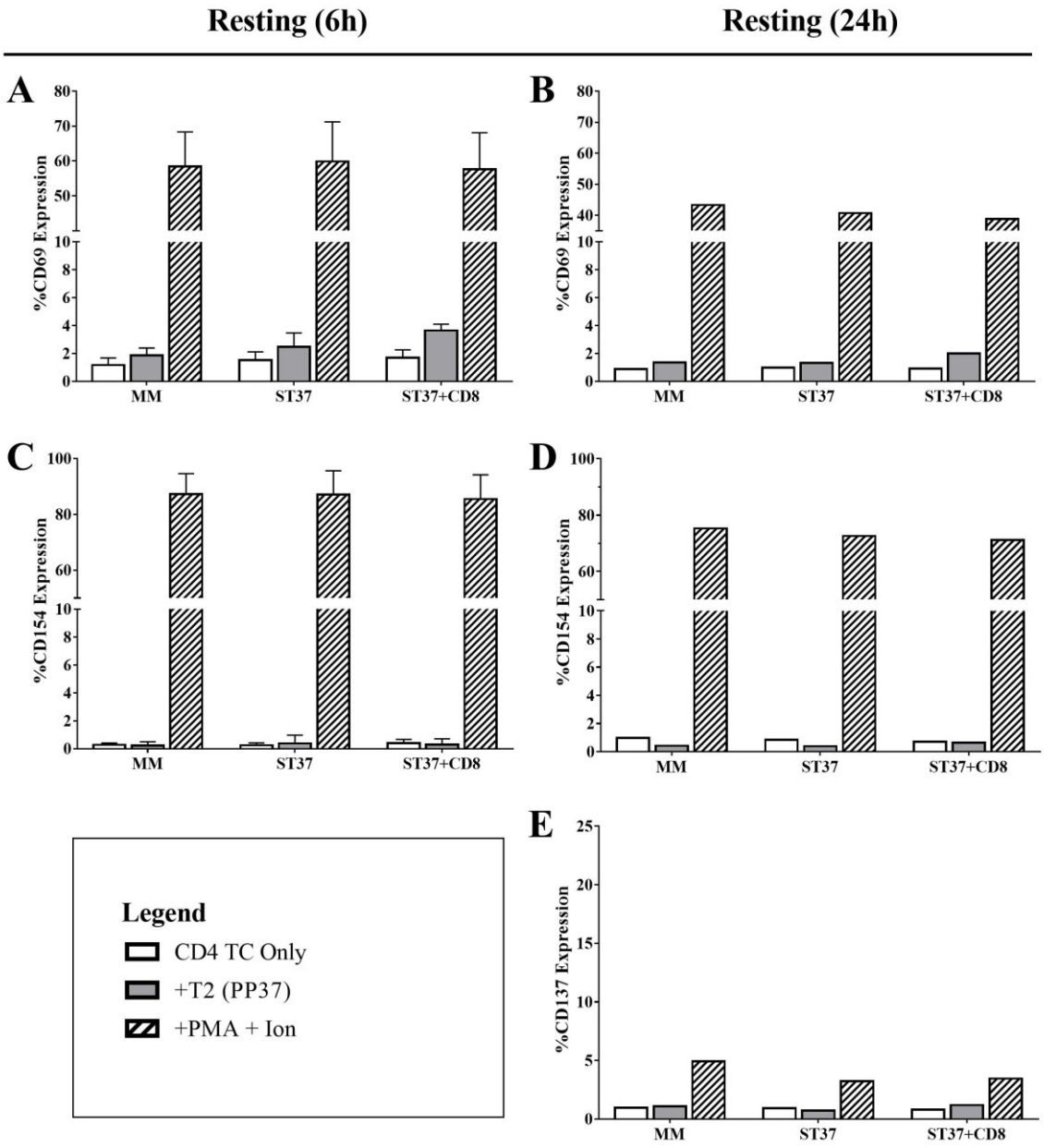
Appendix C: RT-qPCR analysis of pre-activated DSE CD4 T cells



Supplementary figure 2 | RT-qPCR analysis of DsiRNA silencing capacity and T37 *TCR* mRNA transfection efficiency in pre-activated DSE CD4 T cells.

Introduction of DsiRNA targeting the alfa and beta constant regions of the wt *TCR* mRNA (*TRAC* and *TRBC*, respectively) proved to significantly downregulate *TRAC* (left) and *TRBC* (middle) expression in pre-activated DSE CD4 T cells (N = 2). Electroporation conditions: M = Mock, S = DsiRNA.

Appendix D: Activation markers on resting CD4 T cells



Supplementary figure 3 | Activation markers on resting CD4 T cells.

DSE resting CD4 T cells were co-cultured either 6 (N = 2, left) or 24 hours (N = 1, right) with T2 cells on in the presence of PMA and ionomycin. CD69 **(A,B)** and CD154 **(C,D)** expression are best observed at the 6 hour mark, while 24 hours is a recommended time point when assessing CD137 upregulation **(E)**. None of the co-cultures showed a noteworthy upregulation of any activation marker. Electroporation conditions: M = Mock, S = DsiRNA, T37 = T37 *TCR* mRNA, CD8 = *CD8* mRNA. TC = T cells; PP37 = peptide-pulsed with $WT1_{37\text{-}45}$; PMA = phorbol myristate acetate; Ion = ionomycin.

Non-scientific summary

Every year, approximately 500 people are diagnosed with acute myeloid leukaemia (AML) in Belgium alone. AML originates in the bone marrow, where continuously dividing stem cells form specialised blood cells, including red and white blood cells. When mutations occur in the DNA of the stem cells, they can start dividing uncontrollably and will not be able sufficiently differentiate into functional white blood cells. These defective cells will crowd out the healthy cells in the bone marrow and over time spill out into the blood stream.

Chemotherapy is still the standard of care for AML, but despite being effective in the majority of AML patients, many patients relapse within two years because of limited residual cancer cells remained in the body after the treatment. Therefore, it remains essential to search for new treatment approaches that can be used, possibly in combination with or adjuvant to chemotherapy, to completely eliminate all cancerous cells.

Cancer immunotherapy is a heavily researched field of study which attempts to boost the intrinsic power of the immune system to fight off cancer or guide it in the right direction. One method makes use of white blood cells, also called T cells, that can recognise small parts of proteins, or peptides. Most other cells in the body present these peptides on their surface, where they can be recognised by a receptor on the T cell. While CD8 T cells are specialised in killing cells with the peptide that they recognise, CD4 T cells are generally regarded as helpers for the CD8 T cells.

In this master thesis, genetic material was transferred into CD4 T cells by means of an electric shock procedure. This genetic material consisted of two parts: one that largely eliminates the display of the original antigen-recognising receptor on the surface of the CD4 T cell and another part that suits the cell with a new receptor that recognises a leukaemia-derived antigenic peptide. This study showed that the CD4 T cells can be efficiently loaded with the new receptor. Furthermore, initial indications were obtained that they can recognise tumour cells and be activated through this new receptor. Surprisingly, they also suggest that CD4 T cells can show tumour-killing effects, similar to their CD8 relatives. However, further research has to confirm and extend these findings. If successful, this approach might someday be tested on humans and potentially help many people.