Chimeric Antigen Receptor engineered gamma delta T cells for neuroblastoma immunotherapy

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‘I, Anna Capsomidis, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.’

Anna Capsomidis

Abstract

Gamma delta (γδ) T cells are a unique subset of lymphocytes that combine both innate and adaptive immune properties. They are primed for rapid function including tumour cell cytotoxicity, and following activation, have professional antigen presenting function. Chimeric antigen receptors (CARs) are synthetically engineered receptors that combine the specific antigen-binding region of a monoclonal antibody with a signalling domain responsible for T cell activation and cytotoxicity. Given the natural tissue tropism and innate cellular responses of γδ T cells, it was hypothesised that transduction with a CAR would enhance antigen-specific cytotoxicity, whilst maintaining direct antigen presenting function and the ability to migrate towards tumours. Using the tumour antigen GD2 as a model system, we demonstrated that Vδ1 and Vδ2 cells could be activated, propagated and transduced to sufficient number for use in clinical studies in paediatric patients. The addition of GD2-CAR, enhanced γδ T cell innate cytotoxicity through specific killing of GD2-expressing neuroblastoma cell lines. Migration towards tumour cells was not impaired by the presence of the CAR. Following activation, GD2-CAR transduced Vδ2 cells, retained the ability to take up exogenous tumour antigen, and cross-presented processed peptide to responder αβ T cells. This study provides evidence to support the emerging role of CAR γδ T cells as a safe and efficacious immunotherapy for neuroblastoma.

Impact Statement

Neuroblastoma is a rare and aggressive childhood cancer in which tumours arise within sympathetic nervous tissue. Around 100 children are diagnosed with neuroblastoma in the UK each year with approximately half having 'high-risk' disease at the time of diagnosis. These children are treated with intensive and prolonged multi-modal treatment regimes, but despite this, the chance of long-term survival is only 40-50%. For those with relapsed disease, fewer than 10% are alive after 5 years.

Standard therapy for high-risk neuroblastoma usually combines induction chemotherapy, surgery, high-dose chemotherapy with stem cell transplant, radiotherapy, and immunotherapy. These regimes are associated with both acute and long-term toxicities including deafness, renal failure, infertility and secondary malignancies. The development of new treatments that effectively target neuroblastoma cells whilst sparing normal healthy tissues is therefore a research priority.

For resistant cancers or those unsuitable for conventional approaches, harnessing the power and specificity of the immune system is an attractive alternative. The development of targeted, cell-based immunotherapies for childhood malignancies is a rapidly advancing field, with remarkable results already achieved in children with acute lymphoblastic leukaemia. Chimeric antigen receptor (CAR) T cell therapy involves the genetic modification of a patient's own T cells to express a CAR that redirects specificity to a known tumour antigen. CAR T cells have the ability to specifically kill cancer cells and survive long-term providing on-going tumour surveillance.

Despite the impressive results using CAR T cells to treat $CD19^{+}$ haematological malignancies, clinical translation to solid tumour immunotherapy has been hindered by various therapeutic barriers including expansion, persistence, trafficking, and fate within tumours.

The vast majority of CAR T cell engineering approaches have used αβ T cells however the focus of our research, investigates an alternative to conventional CAR T cells using gamma delta (γδ) T cells. γδ T cells comprise 1-10% of circulating T cells and possess many unique advantages due to their innate and adaptive immune properties. When engineered with a CAR, they have the potential for dual antigen cytotoxicity, avoidance of graft-versus-host disease (and hence have potential for an 'off-the-shelf' product) and professional antigen presenting function.

The findings of this thesis demonstrate that γδ T cells can be efficiently transduced to express CAR and propagated *ex vivo* to sufficient number for adoptive transfer using clinical-grade reagents. CAR γδ T cells effectively killed antigen-expressing tumour targets. The most abundant and readily propagated γδ T cell subset, namely Vδ2 cells, had reduced capacity to expand, with a more differentiated and exhausted phenotype. Conversely, the Vδ1 subset were less differentiated and expressed fewer exhaustion markers. This opens up new avenues for scientific investigation; firstly by optimising protocols for CAR Vδ1 expansion using clinical grade-reagents, as these cells may have a greater potential for solid tumour penetration, and secondly by investigating alternative engineering approaches to restore the expansive function of CAR Vδ2 cells. A further advantage over conventional methods, was that CAR γδ T cells retained their ability to take up tumour antigens and cross-presented processed peptide to responder αβ T cells using an *in vitro* melanoma model.

Altogether, the findings presented in this thesis contribute to the accumulating evidence-base and pre-clinical optimisation for the use of γδ CAR T cells in clinical studies.

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First and foremost, I would like to express my sincerest thanks to my primary supervisor Professor John Anderson for giving me the opportunity to undertake this project. He has provided unwavering encouragement, support and guidance at all stages of the project from its conception to completion, and I will be forever grateful for the time and effort he has invested in me. Working in his laboratory striving to improve the outcomes for children with cancer has been an absolute inspiration and privilege. I would also like to extend my thanks to Dr Kenth Gustafsson and Dr Barry Flutter for their additional supervision and scientific direction.

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Abbreviations

αβT	Alpha beta T cell
ADCC	Antibody dependent cell mediated cytotoxicity
AIC	Antibody independent cytotoxicity
AICD	Activation induced cell death
ALK	Anaplastic lymphoma kinase
ALL	Acute lymphoblastic leukaemia
APC	Antigen presenting cell
B7-H3	Cluster of differentiation 276
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CNS	Central nervous system
CRS	Cytokine release syndrome
CSF	Cerebrospinal fluid
CTLA-4	Cytotoxic T lymphocyte associated antigen 4
DC	Dendritic cell
DNA	Deoxyribose nucleic acid
EBV	Epstein Barr virus
ELISA	Enzyme linked immunosorbent assay
E:T	Effector to target ratio
EFS	Event free survival
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
γδT	Gamma delta T cell
GD2	Disialoganglioside, GD2
GMP	Good manufacturing practice
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
ICOS	Inducible costimulator
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
Kb	Kilobases
LAG3	Lymphocyte activated gene-3
MART1	Melanoma Antigen Recognized by T cells 1
MAb	Monoclonal antibody
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MYCN	Amplification of v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homologue (MYCN)
NK	Natural Killer
OS	Overall survival
pAPC	Professional antigen presenting cell
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PD1	Programmed cell death protein 1

PDL1	Programmed death ligand 1
R10	Roswell Park Memorial Institute + 10% FCS
ScFv	Single chain variable fragment
SDF-1	Stromal cell derived factor-1
TAA	Tumour associated antigen
TCR	T cell receptor
TGFβ	TGF-b Transforming growth factor beta
TIL	Tumour infiltrating lymphocyte
Tim3	T cell Ig and mucin domain containing protein-3 TNF Tumour Necrosis Factor
TME	Tumour microenvironment
TNFα	Tumour necrosis factor alpha
Vδ1	Delta-1 T cell
Vδ2	Delta-2 T cell
Vδ3	Delta-3 T cell
WBC	White blood cell

Chapter 1 Introduction

1.1 Neuroblastoma

Neuroblastoma is the most common extracranial solid tumour in the paediatric population affecting approximately 100 children per year in the UK. Most patients are diagnosed before the age of 5 years and the median age at diagnosis is 18 months (1).

Neuroblastoma is an embryonal malignancy derived from neural crest cells. The most common site of tumour development is the adrenal glands but tumours also originate from other locations where sympathetic nervous tissue is present including the paraspinal sympathetic ganglia in the neck, chest, abdomen and pelvis (2).

The term neuroblastoma encompasses a series of neuroblastic tumours including; neuroblastomas, ganglioneuroblastomas and ganglioneuromas. Neuroblastoma is a vastly heterogenous disease and specific clinical and biological factors determine whether the tumour is capable of spontaneous regression or whether tumours metastasise and become refractory to conventional treatment. Age at diagnosis, stage of disease, and the interplay of various molecular, cellular and genetic factors all contribute to neuroblastoma tumour biology and overall survival.

1.1.1 Clinical presentation

The clinical presentation of neuroblastoma is dependent on the site of the primary tumour and presence of metastases. Presentation is therefore varied and can be non-specific. General symptoms include pallor, anaemia, fever, weight loss, anorexia and failure to thrive, however the most common presentation of an adrenal tumour is with an abdominal mass (2). Neuroblastoma can spread by the haematogenous or lymphatic route and by local infiltration. Common sites include the lymph nodes, bone marrow, bone, liver, skin and orbits. Most tumours secrete the metabolites Homovanillic acid

(HVA) and Vanillylmandelic acid (VMA) which can be detected in the urine samples of patients.

1.1.2 Genetics

The majority of neuroblastoma tumours in children are sporadic but 1% have a positive family history of neuroblastic tumours (3). Certain medical conditions have also been linked to a predisposition of developing neuroblastoma including; Hirschsprung disease, congenital central hypoventilation syndrome, and Li-Fraumeni syndrome, an autosomal dominant condition characterised by a TP53 germline mutation (4-6).

No specific environmental aetiological factors have been identified, but the presence of certain oncogenes are associated with its development. Genome-wide association studies (GWAS) have discovered twelve genomic loci that are significantly associated with neuroblastoma formation (7). Although each individual mutation has a relatively small effect on initiating the disease, a combination of several associations can promote malignant transformation.

In addition, certain genes have been shown to have potent oncogenic functions that maintain tumorigenicity in established disease (8) including amplification of v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homologue (MYCN), anaplastic lymphoma kinase (ALK) and paired-like homeobox 2b (PHOX2B) genes. MYCN amplification is associated with the most malignant tumours with poor survival rates (9). It occurs in approximately one quarter of cases and has clinical correlation with aggressive disease.

1.1.3 Risk stratification

Neuroblastoma can be classified into four main risk groups (very low risk, low risk, intermediate risk and high risk) which are dependent on; age at diagnosis, stage of disease (L1, L2, M, MS, defined in Table 1), histology, tumour grade, and presence of certain defined genetic aberrations (namely; MYCN amplification, presence/absence

of 11q aberrations and tumour cell ploidy) (Table 1). The International Neuroblastoma Risk Group (INRG) classification system has defined these groups according to 5-year event free survival (EFS) cut-offs; very low risk >85%, low risk >75 - ≤85%, intermediate risk ≥50% - ≤75%, high risk ≤50% (10). These broad risk groups are further divided into 16 pre-treatment groups to provide an international consensus approach which enables comparison of studies in order to develop the most optimal treatment approaches.

Table 1: International Neuroblastoma Risk Group (INRG) classification scheme [adapted from (10)]

INRG stage	Age (m)	Histology category	Tumour grade	MYCN	11q aberration	Ploidy	Pre-treatment risk group
L1/L2		GN maturing, GNB intermixed					A Very low
L1		Any, except GN maturing, GNB intermixed		NA			B Very low
				Amp			K High
L2	<18	Any, except GN maturing, GNB intermixed		NA	No		D low
					Yes		G Intermediate
	≥18	GNB nodular; neuroblastoma	Differentiating	NA	No		E Low
					Yes		H Intermediate
			Poorly differentiated or undifferentiated	NA			
				Amp			N High
M	<18			NA		Hyperdiploid	F Low
	<12			NA		Diploid	I Intermediate
	12 to <18			NA		Diploid	J Intermediate
	< 18			Amp			O High
	≥ 18						P High
MS	<18			NA	No		C Very low
					Yes		Q High
				Amp			R High

GN, ganglioneuroma; **GNB**, ganglioneuroblastoma; **Amp**, amplified; **NA**, not amplified; **L1**, localized tumour confined to one body compartment and with absence of image-defined risk factors (IDRFs); **L2**, locoregional tumour with presence of one or more IDRFs; **M**, distant metastatic disease (except stage MS); **MS**, metastatic disease confined to skin, liver and/or bone marrow in children < 18 months of age

1.2 Treatment of high-risk neuroblastoma

Whilst the majority of low and intermediate risk group patients have a high chance of cure with chemotherapy, surgery +/- radiotherapy, children with high-risk disease can be difficult to cure (2). According to the International Society of Paediatric Oncology European Neuroblastoma Research Network (SIOPEN) definition, approximately one third of patients are classified as having high-risk disease (any patient with MYCN amplification (other than stage L1) or any patients older than 12 months of age at diagnosis with stage M). In the UK, patients are treated according to trial protocols, for example the SIOPEN HR-NBL-1 trial which has recently closed. Interim recommendations are based on the standard arm of the SIOPEN high-risk neuroblastoma trial, pending results from randomisation arms. This intensive schedule consists of:

- Induction chemotherapy with 'rapid COJEC' followed by collection of peripheral blood stem cells
- Surgery
- Myeloablative therapy; consolidation high dose chemotherapy with busulfan and melphalan followed by re-infusion of peripheral blood stem cells.
- Radiotherapy to the site of the primary tumour
- Differentiation therapy and immunotherapy (dinutuximab beta given by 10 day infusion).

1.2.1 Treatment of relapsed/refractory high-risk neuroblastoma

Patients with relapsed or refractory disease receive individualised treatment and where possible are enrolled in clinical trials. Up to 60% of patients with high-risk neuroblastoma relapse, and these patients remain the greatest challenge. Survival following relapse is particularly poor with overall survival (OS) of 2% (11). These survival statistics are further supported by Basta *et al.* reporting UK data where medium OS post-relapse was 4.5 months (IQR 1.9-11.4) (12). London *et al.* also

reported that patients who relapsed between 6-18 months after diagnosis had the poorest prognosis of all (11).

In the UK difficult cases can be referred for discussion at the National Neuroblastoma Advisory Board for expert opinion. Current options for relapsed disease include an induction chemotherapy phase [which may include enrolment into the BEACON study (13)] followed by consolidation and maintenance, including recruitment to early clinical trials (e.g. when a target has been identified and drug is available) (CCLG).

There is clearly a great unmet need for high-risk relapsed and refractory groups. In order to improve survival it is necessary to provide access to early clinical trials, deliver ethical care, and further develop precision medicine and targeted approaches. Understanding the molecular drivers of cancer and identifying novel targets pave the way forward to the next generation of cancer therapies. Another research priority is to reduce the burden of toxicities by creating therapies that specifically target cancer cells whilst sparing healthy tissues. One such approach is by harnessing the power of the patient's own immune system to specifically target tumour antigens. The development of immune-based treatments for paediatric cancers is a rapidly advancing field [reviewed in (14, 15)] and is explored in more detail in Section 1.3.

1.3 Immunotherapy approaches for neuroblastoma

1.3.1 Neuroblastoma and the immune system

Whilst it is clear that the immune system plays an essential role in cancer surveillance by recognising and eliminating transformed cells, this protective process is hindered by the ability of cancer cells to 'hide' from immune destruction (14). This is recognised as one of the major obstacles for cancer immunotherapy. Hanahan and Weinberg (2011) (16) described "hallmarks" required to facilitate cancer progression including; sustained proliferative signals, evasion of growth suppressors, ability to invade and metastasise, replicative immortality, induction of angiogenesis, resistance to cell death, escape from immune destruction, and deregulation of cellular

metabolism. Borriello *et al.* (17) describe the tumour microenvironment (TME) in neuroblastoma in relation to these "hallmarks". In particular, the authors highlighted that neuroblastoma cells have developed various mechanisms to escape recognition by immune cells by creating a hostile TME. It has been shown that neuroblastoma cell lines and tumour specimens have very low expression of HLA class I (MHC-I), preventing peptide antigen recognition by $CD8^+$ T cells (18, 19). Additionally, MHC-I molecule expression is influenced by pro-inflammatory cytokines including IFNγ (leading to increased expression) (20), and negatively regulated by MYCN amplification, causing downregulation (21). There is also downregulation of NKG2D ligands thereby avoiding innate cytotoxicity induced by infiltrating NK cells (22). The development of an immunosuppressive microenvironment also involves the recruitment of inhibitory tumour associated macrophages and myeloid derived suppressor cells (MDSC). MDSC function by; depleting arginine thereby inhibiting $CD4^+$ and $CD8^+$ (23), inducing T_{reg} cells, and modulating cytokine production of macrophages.

Whilst it is clear that the TME is a "pathologically active niche" (24) with great influence over tumour evolution, as our knowledge of this highly complex process increases, this will hopefully uncover novel approaches for immunotherapy. One of the first attempts to harness the power of the immune system to treat cancer was in 1891 when William Coley injected inactivated Streptococcus pyogenes and Serratia marcescens into patients' tumours in order to initiate a protective immune response (25). He was able to achieve durable responses for several types of cancer using this method coined as "Coley's toxins". Since its concept, cancer immunotherapy has rapidly evolved to either enhance existing anti-tumour immune responses (e.g. cancer vaccines, immune checkpoint blockade) or, specifically empowering the immune system to recognise and eliminate cancer cells (e.g. monoclonal antibodies, chimeric antigen receptor-engineered T cells, T cell receptor-engineered T cells, and tumour infiltrating lymphocytes) (Table 2 & 3). With these recent advances in mind, the current global immunotherapy market has been valued at over 40 billion US dollars, and it has been hypothesised that within 10 years there will be a paradigm shift away

from cytotoxic chemotherapy and that 60% of all cancers will be treated with immunotherapy (25).

Table 2: Classification of therapeutic approaches into tumour antigen targeting and immunomodulating [adapted from (14)]

Tumour antigen targeting	Immunomodulating
Monoclonal antibodies	Cancer vaccines
Bispecific antibodies	Immune check-point inhibitors (CTLA-4, PD-1, PDL-1)
CAR T cells	
TCR-redirected T cells	
Tumour infiltrating lymphocytes	
Viral reactive T cells	
Donor lymphocytes	

IMMUNOTHERAPY	DESCRIPTION	ADVANTAGES	LIMITATIONS
MONOCLONAL ANTIBODIES (MAB)	Bind tumour antigen and augment antibody-dependent cell-mediated cytotoxicity (ADCC) e.g. anti-GD2 (*Ch14.18*)(26) and anti-CD20 (*rituximab*)(27). MAb can also be linked to chemotherapeutics or radionuclides.	'Off the shelf' product Efficacious in Phase III clinical trials	Short half-life, requires repeated administration 'On target, off tumour' side effects Anaphylactoid reactions
BISPECIFIC ANTIBODIES	Simultaneously bind tumour antigen and T-cell e.g. *blinatumomab*(28) binds CD19 and CD3	'Off the shelf' product	Short half-life, requires repeated administration Cytokine release syndrome Potential for 'antigen escape'
CHECKPOINT INHIBITORS	e.g. anti-PD-1 or anti-CTLA-4 (*Ipilimumab*) antibodies block inhibitory immune signals	'Off the shelf' product	Short half-life, requires repeated administration Autoimmunity; e.g. Colitis
TUMOUR VACCINES	Most commonly *ex vivo* production of autologous tumour antigen pulsed dendritic cells for injection	Generation of immunological memory	Patient specific therefore expensive to produce and requires GMP facility
TUMOUR INFILTRATING LYMPHOCYTES (TILS)	T-cells are extracted from the tumour itself and cultured *ex vivo* to large numbers for infusion	Tumour-specific Immunological memory	Patient specific Not reliably produced from all tumour samples Limited study in paediatric patients
VIRAL REACTIVE T-CELLS	T-cells stimulated with viral antigen expressing antigen presenting cells	Immunological memory	Patient specific Small number of cells for infusion
TCR RE-DIRECTED T CELLS	Autologous T-cells are genetically modified with tumour antigen specific T-cell receptors	Immunological memory Directly target tumour antigen Can also target intracellular antigens	Patient specific MHC-restricted Risk of mis-pairing with endogenous TCR Cytokine release syndrome
CAR RE-DIRECTED T CELLS	Autologous T-cells are genetically modified with Chimeric antigen receptors	Immunological memory Can include 'safety switch' MHC-unrestricted Can be engineered with 'costimulatory' domains to enhance efficacy and persistence	Patient specific Can only target known cell surface antigens Cytokine release syndrome 'On-target, Off tumour' side effects Potential for 'antigen escape'

Table 3: Overview of immunotherapy approaches [adapted from (14)]

1.3.2 Anti-GD2 monoclonal antibody

The identification of targetable tumour antigens is fundamental to the success of passive immunotherapy approaches (14). The ideal target should have abundant expression on the surface of tumour cells with no expression on normal tissue to prevent 'on-target, off tumour' toxicities. Despite this, most cancer antigens targeted thus far have in fact had low level expression on normal tissue (26).

GD2 is a disialoganglioside antigen, abundantly expressed on the surface of virtually all human neuroblastoma cells (27) but has limited expression on normal tissues. It is also expressed by Ewing's sarcoma, osteosarcoma, rhabdomyosarcoma, retinoblastoma, melanoma, and small cell lung cancer (28). It is thought to function in the attachment of cancer cells to extracellular matrix proteins (29) and Li *et al.* (30) described how the presence of GD2 is able to suppress immune responses in a murine model. GD2 is expressed at low level on normal foetal and adult tissues, but is restricted to the CNS, peripheral nerves and melanocytes (28).

Several anti-GD2 monoclonal antibodies have been developed for clinical use in recent years. The first antibodies developed [e.g. 3F8 (31)] were murine and associated with the development of human anti-mouse antibodies (HAMA) that compromised their clinical efficacy. This problem was partially overcome through chimerisation which involves fusing the mouse-derived antigen binding domain onto a human IgG constant domain (Figure 1.1). Dinutuximab and dinutuximab beta (produced in different cell lines) combine the variable regions of the original murine IgG3 anti-GD2 monoclonal antibody 14.18 and the constant regions of human IgG1. It has been reported that Ch14.18 has 50-100 x higher antibody dependent cellular cytotoxicity (ADCC) *in vitro* than the murine anti-GD2 monoclonal antibody of IgG2a isotype derived from the same parental hybridoma (32). Humanised anti-GD2 antibodies are also currently being investigated in phase II trials.

Incorporation of dinutuximab beta (Ch14.18) into high-risk trial protocols has resulted in lower recurrence rates and improved 2 year overall survival (1-3), however 5 year survival did not reach statistical significance. The ground breaking randomised study

by Yu *et al.* (33) reported that treatment with dinutuximab combined with IL-2, granulocyte macrophage colony-stimulating factor (GM-CSF) and standard maintenance therapy (isotretinoin) resulted in increased two-year event free survival (EFS) in children with high-risk disease, compared with standard maintenance therapy (isotretinoin) alone (2-year EFS 66 ± 5% vs. 46 ± 5%). Anti-GD2 antibody therapy however is associated with significant toxicities including pain, fever, haemodynamic instability and capillary leak syndrome. Since the closure of the SIOPEN high-risk neuroblastoma trial, (HR-NBL-1) (33) dinutuximab beta has now been approved by the National Institute for Health and Care Excellence (NICE) (34) .

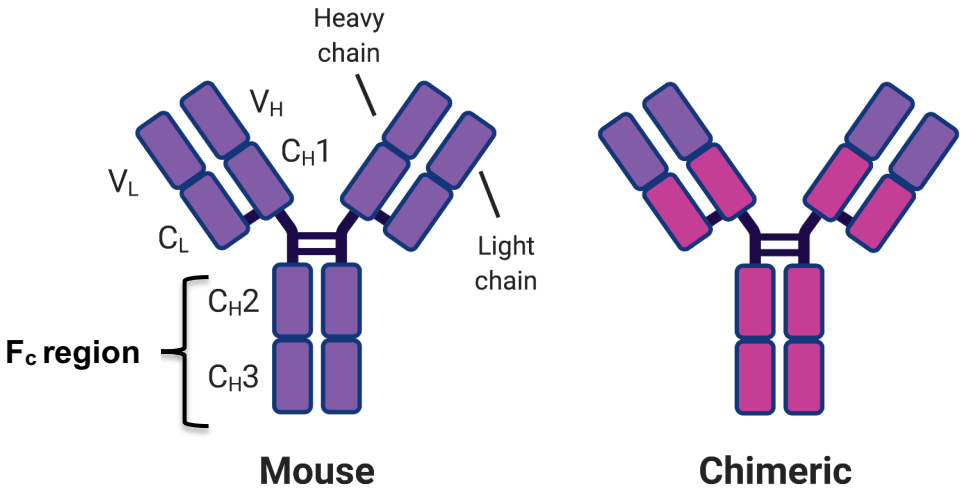


Figure 1.1 Schematic showing the composition of monoclonal antibodies

Immunoglobulin G (IgG) consists of two identical heavy and light chains. The antigen recognition domain consists of variable heavy (V_H) and variable light (V_L) chains containing hypervariable complementarity determining regions (CDRs). The Fragment crystallizable (Fc) region binds to surface Fc receptors on phagocytic and cytotoxic cells to induce complement dependent cytotoxicity (CDC), NK/γδ/myeloid cell mediated antibody dependent cytotoxicity (ADCC), and monocyte mediated antibody dependent cellular phagocytosis (ADCP). Chimeric antibodies, such as dinutuximab/dinutuximab beta, involve a fusion of the variable region derived from a mouse (purple) with human constant regions (pink).

1.3.3 Immune checkpoint blockade

Antibodies have also been engineered to block various immune check-points. PD1 and CTLA4 are examples of inhibitory co-receptors that provide an 'immunological break' to uncontrolled T cell activation. Monoclonal antibodies that target these checkpoints (either their receptor or ligand) can augment existing inhibited immune responses to cancer. PD1 blockade has shown great promise in clinical trials for metastatic melanoma (35) and other adult cancers, and its efficacy is now being tested in paediatric malignancies.

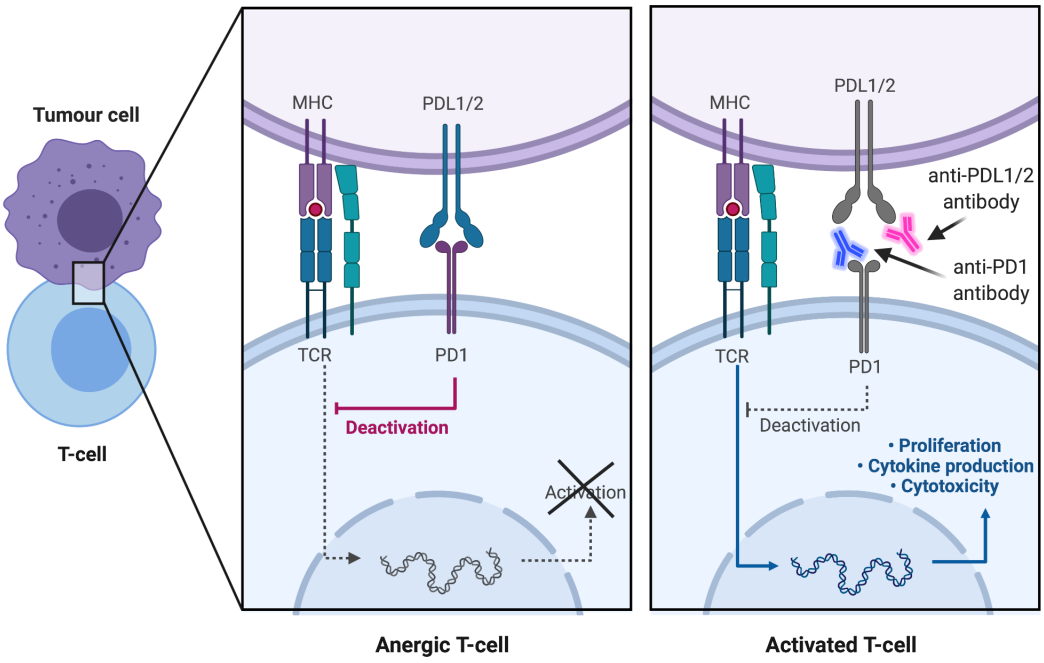


Figure 1.2 Mechanism of action of immune check-point inhibitors

Full T cell activation requires binding of the TCR to tumour antigen in association with MHC (signal 1) together with costimulation from activating costimulatory receptor ligation, including the binding of CD80/86 to CD28 (signal 2). Tumour cells can express PDL1 and PDL2 and engagement of these ligands with PD1 receptors on the surface of T cells inhibits downstream signalling of the TCR by reducing expression and production of cytokines and transcription factors (such as GATA3 and TBET), which are associated with cytotoxic effector function (left box). PD1 antibodies (blue) and PDL1 antibodies (pink) block PD1-PDL1 interaction thereby facilitating T cell activation, proliferation and cytotoxicity (right box). *Image created using BioRender software.*

Paediatric studies have assessed nivolumab (PD1), pembrolizumab (PD1), atezolizumab (PD1), ipilimumab (CTLA4), tremelimumab (CTLA4), durvulmab (PDL1) and avelumab (PDL1) in solid tumours. Sensitivity to PD1 blockade has been shown to be related to high mutational burden (36) thus paediatric malignancies may not be as susceptible to immune checkpoint blockade as some adult cancers (14). It is likely to be the case that paediatric cancers with a higher mutational burden such as glioma and osteosarcoma may respond better to checkpoint blockade and one paediatric study in particular is specifically looking at hypermutant cancers (NCT02992964; The Hospital for Sick Children, Toronto). This is further supported by a report of two paediatric patients with recurrent multifocal glioblastoma multiforme refractory to conventional therapies, who displayed dramatic and durable responses to nivolumab (PD1) (37). Despite overall disappointing results for paediatric solid tumours, it is hypothesised that combining checkpoint blockade with other immunotherapies may lead to more durable responses. The MiNivAN trial is a phase 1 trial currently recruiting patients to study combinational therapy with mIBG, nivolumab and dinutuximab beta (ClinicalTrials.gov identifier NCT02914405).

Whilst dinutuximab beta has significantly impacted survival in children with high-risk neuroblastoma, 50% of patients relapse and 20% are refractory to induction chemotherapy. This highlights a great unmet need for investigating new targeted therapies with greater efficacy, acceptable toxicity profiles and the ability to prevent relapse. One such immunotherapy approach that combines the antigen specificity of a monoclonal antibody with potent cytotoxic T cell function is the adoptive transfer of autologous T cells engineered with chimeric antigen receptors (CAR T cells). Before exploring CAR T cells in further detail (Section 1.9), I will summarise adaptive immune responses with a focus on T cell development, signalling and function.

1.4 T cell immunity

The human immune system is essential for the elimination of pathogens and prevention of disease. It can be broadly classified into two arms; the innate (non-specific) and adaptive (specific) immune systems. The former is responsible for first-

line defence and provides a rapid, general response to pathogen-associated molecular patterns (PAMPs). In contrast, the adaptive immune system is capable of highly specific antigen responses. Lymphocytes are central to this process, namely T and B lymphocytes. B cells mediate humoral immunity primarily through antibody production, whereas T cells mediate cellular immunity through direct contact with their target. T cells may either express either T cell receptor alpha-beta (TCRαβ), or T cell receptor gamma-delta (TCRγδ).

1.5 T cell development

The vast majority of peripheral T cells are αβ T cells containing TCRαβ. A small proportion are γδ T cells, expressing TCRγδ, and this unique subset will be discussed in detail in Section 1.8. αβ T cells can either be 'cytotoxic' that eliminate intracellular pathogens and neoplastic cells ($CD8^+$) or 'helper' ($CD4^+$) cells that are essential moderators of the immune response.

Precursor T cells originate from haemopoietic stem cells in the bone marrow and then migrate to the thymus. The thymus is the site of T cell development where these early thymocyte progenitors (ETP) undergo a series of maturation steps characterised by expression of CD44 (adhesion molecule) and CD25 (interleukin-2 receptor alpha chain) (Figure 1.3). These precursor T cells are known as 'double negatives' (DN) as they lack expression of CD4 or CD8 co-receptors. By the time cells have reached the DN3 stage, cells are committed to either the αβ or γδ lineage. DN3 cells destined to the αβ lineage have successfully rearranged their TCRβ chain and formed a 'pre-TCR' pairing with a pre-TCRα chain and CD3 molecule. Expression of pre-TCR leads to cellular proliferation and differentiation through expression of $CD4^+$ and $CD8^+$ (double positive cells, DP). DP cells produce TCRαβ by rearranging their TCRα chain loci. Cells undergo positive and negative selection though interaction with self-antigen/MHC complex dependent on their affinity. After successful selection, cells downregulate either CD4 or CD8 to become single positive cells and are ready to migrate from the thymus to the peripheral circulation.

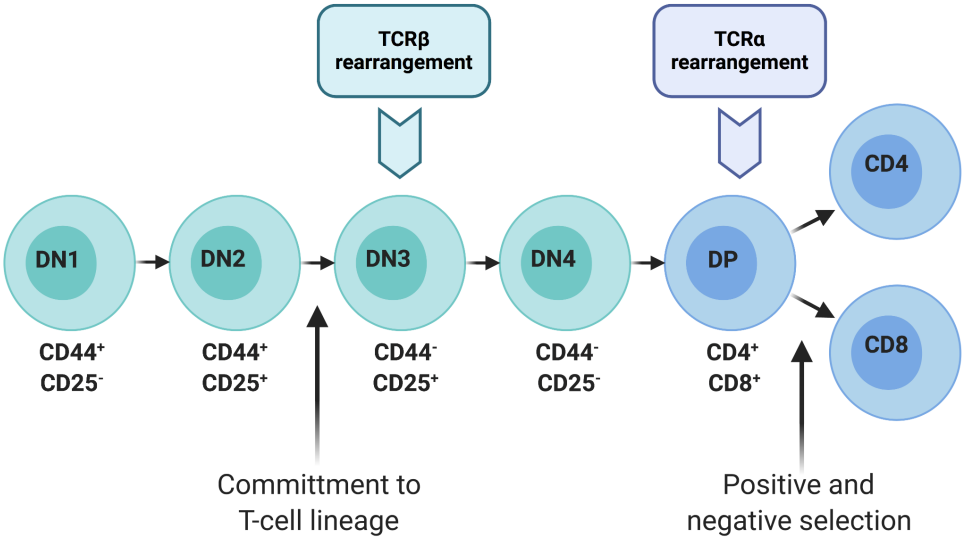


Figure 1.3 T cell development in the thymus

1.6 T cell signalling

The αβ TCR has plays a critical role in the adaptive immune system, by recognising antigen-MHC complex. To form a functional receptor, capable of transmitting intracellular signals leading to T cell activation and proliferation, the TCRαβ heterodimer must associate with a CD3 complex (comprised of invariant γ, δ, ε, and ζ chains). The co-receptors (either CD4 for helper T cells or CD8 for cytotoxic T cells) also contribute to this process by acting as cellular adhesion molecules by binding to their respective MHC molecule thereby stabilising the T cell-antigen presenting cell (APC) interaction.

The intracellular portions of CD3ζ contain 'immunoreceptor tyrosine-based activation motifs' (ITAMs) which become phosphorylated at the tyrosine residues by Src-family protein kinases (namely LcK and Fyn) following engagement of the TCR with its cognate antigen. Phosphorylated ITAMs on CD3ζ act as binding sites for the ζ-chain associated protein kinase 70 (ZAP 70) that when activated induces the phosphorylation of the adaptor proteins LAT (linker for activation of T cells) and

SLP76. This initiates a cascade downstream signalling events resulting in activation of the mitogen-activated protein kinase (MAPK) signalling pathway and Ca^{2+} mobilisation, ultimately resulting in transcriptional and post-translational modifications of the molecules responsible for T cell proliferation and differentiation.

1.7 αβ T cell activation

A key concept in T cell activation is that if signalling occurs solely through the TCR then this can result in anergy or activation induced cell death (AICD). The 2-step model of T cell activation is required for full activation, differentiation, effector function, proliferation and survival (38). Signal 1 involves TCR-peptide-MHC interaction, and signal 2 is the binding of co-signalling receptors (either co-stimulatory or co-inhibitory) to their respective ligands expressed on APCs that direct T cell function and will determine their fate (Figure 1.4). These co-stimulatory receptor-ligand pairs have an essential role in enhancing signalling and promote T cell proliferation, cytokine production and survival. Costimulatory receptors include the Immunoglobulin superfamily (IgSF) (including CD28 and ICOS, which bind to CD80/86 and ICOS-L, respectively), and the Tumour Necrosis Factor receptor superfamily (TNFRSF) (including 41BB, CD27, CD40 and OX40, which bind to 41BB-L, CD70, CD40L and OX40L, respectively). Each of these has varying effects on effector function and memory formation. CD28 interaction with CD80/86 is important for early activation of naïve T cells and is constitutively expressed, where as other are inducible upon activation. Converse to this are co-inhibitory co-signalling receptors including CTLA-4 and PD1 that are expressed on activated T cells which inhibit T cell activation. Defects in these inhibitory mechanism can lead to aberrant immune responses including lymphoproliferation and autoimmunity.

After recognition of peptide-MHC complexes by the TCR, co-signalling receptors co-localise to form the immunological synapse. This is composed of central and peripheral supra-molecular activation clusters (SMACs) which are essential for the spatial organisation of cell surface interactions, cytoplasmic signalling components and scaffolds (39).

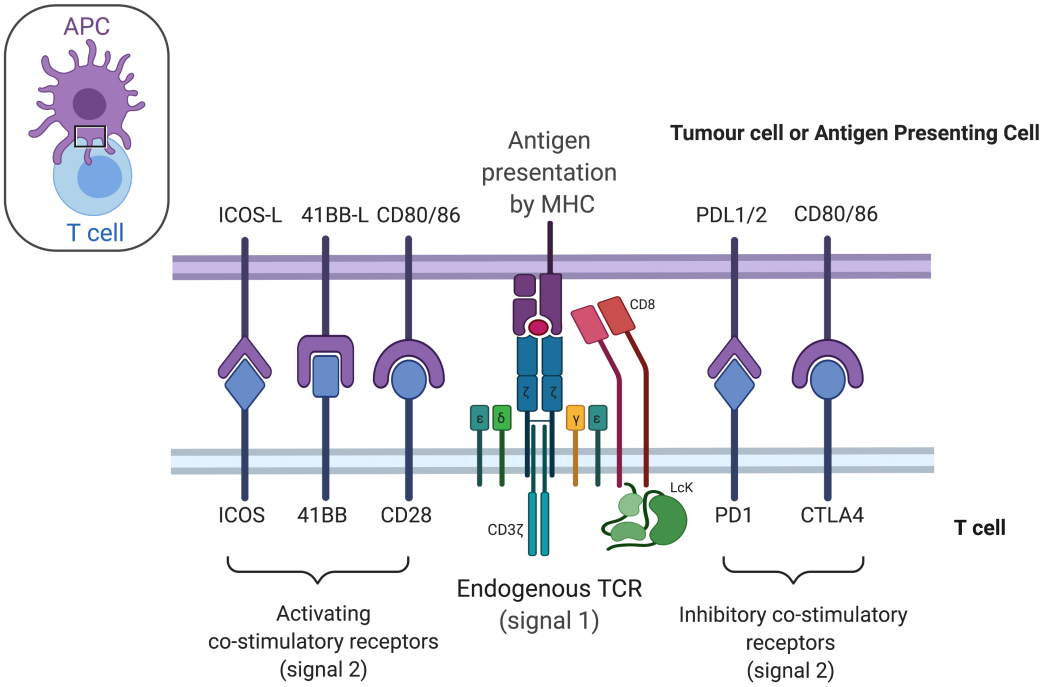


Figure 1.4 The two-signal model of αβ T cell activation

The endogenous T cell receptor (TCR) contains paired α and β chains associated with δ, ε, and γ chains, and signalling ζ chains. Tumour antigen is presented by major histocompatibility complex (MHC) class I or MHC class II which allows recognition by the TCR. The interaction between TCR and peptide-MHC complex is known as signal 1. A second signal is required for full T cell activation provided by activating costimulatory receptors (most prominently; CD28, 41BB, ICOS). CTLA4 and PD1 are inhibitory costimulatory receptors contributing to inhibition of T cell activation. *Image created using BioRender software.*

1.8 γδ T cells

Gamma delta T cells (γδ T cells) comprise 1-10% of circulating lymphocytes (40) and have functional properties advantageous for cancer immunotherapy, including innate and adaptive immune responses (41). γδ T cells can distinguish 'foreign' or transformed cells from healthy cells and, unlike αβ T cells, they are not restricted by the classical MHC pathway, which is major advantage for cancer cell recognition.

Quite remarkably, a recent study by Gentles *et al*. correlated the presence of intra-tumoural γδ T cells with an improved cancer prognosis (42). The authors used a computational approach to infer leukocyte representation in the transcriptomes of more than 5000 tumour samples (although did not include neuroblastoma) and found that infiltrating γδ T cells was the strongest predictor of a favourable outcome. This in itself gives strong rationale for further investigation into their potential for adoptive transfer, tumour homing, and ability to survive within the hostile tumour microenvironment.

γδ T cells were only discovered in the mid-1980s and following this, there have been many questions as to their biological significance with much debate at the biennial International γδ T cell Conference. Whilst some may view that these cells are simply an evolutionary remnant, others strongly believe they play an essential role in natural host defence mechanisms and immune protection against both infections and malignancies (43, 44). Specific to cancer, further evidence for their role in eliminating transformed cells is that tumour infiltrating γδ T cells extracted from melanoma, colorectal and lung samples are cytotoxic *in vitro* (45-47), and patients with acute leukaemia who had increased γδ T cells following allogeneic stem cell transplant had a significant survival advantage (48).

γδ T cells consist of a TCR containing one Vγ (Vγ2-5, 8-9) and one Vδ (Vδ 1-8) chain to form a heterodimer (49), and can be broadly categorised into Vδ1, Vδ2 and Vδ1^{-}/Vδ2^{-} subsets (mostly expressing either Vδ3 or Vδ5) that predominate in humans. γδ T cells expressing the Vδ2 chain paired with Vγ9 (Vγ9Vδ2) are by far the most common in human peripheral blood (50-90%) (known as Vδ2 hereafter). Vδ1 subsets on the other hand are a more heterogeneous population with pairing of Vδ1 with different Vγ chains. These cells are typically concentrated in the skin and mucosa, but can be detected in peripheral blood to varying degrees depending on the individual. Far less is known about Vδ3 (confounded by the lag-time in commercial antibody availability) but they are known to have increased presence in the liver (43).

There is much debate surrounding the process of γδ T cell activation and the identification of naturally occurring ligands. TCR activation mechanisms differ between individual subsets with Vδ2 cells expressing a 'public' TCR repertoire, implying that it is shared between individuals, and Vδ1 cells expressing a largely 'private' TCR repertoire. Vδ2 TCRs therefore have limited variability, and react to a specific set of antigens. γδ T cells containing Vδ1 TCR on the other hand are heterogenous, particularly in early life, and it is not until adulthood that a few distinct clonotypes are selected (50).

γδ T cells exert their cytotoxic function through the release of TNFα, granzymes and perforins. This is in conjunction with the binding of Fas ligand, TNF-related apoptosis inducing ligand (TRAIL) and DNAX accessory molecule 1. CD16 is also expressed on the cell surface and ADCC is reported (40). γδ T cells additionally have a role in immune regulation; they are a source of proinflammatory cytokines including IFNγ, TNFα and IL-17 which are essential for mediating DC, T cell, B cell and stromal cell function.

γδ T cell differentiation into effector memory cells has been described by Dieli and colleagues (51). Antibody staining for CD27 and CD45RA divides subsets into four effector/memory phenotypes; naïve ($CD27^+/CD45RA^+$) [T_{Naive}], central memory ($CD27^+/CD45RA^-$) [T_{CM}], effector memory ($CD27^-/CD45RA^-$) [T_{EM}], and terminally differentiated effector memory ($CD27^-/CD45RA^+$) [T_{EMRA}]. T_{CM} have the highest proliferative potential and express lymph node homing receptors but immediate effector function is more limited, whilst T_{EM} are highly cytotoxic but have lower proliferative capacity (52) (explored further in Section 1.9.2).

1.8.1 Vδ1 cells

γδ bearing the Vδ1 TCR (Vδ1 cells hereafter) constitute approximately 10-30% of total circulating γδ T cells, but are significantly more abundant in epithelial tissues (53). They are of substantial clinical interest due to their naturally less differentiated

memory phenotype (40), reduced susceptibility to activation induced cell death (AICD) (53), and predominance over Vδ2 in tumour samples (45, 54).

Although Vδ1 naturally reside in tissues they can be expanded from peripheral blood using artificial antigen presenting cells (aAPC) (40), concanavalin A (ConA) (55) or anti-CD3 antibody (56). To date, there have been no clinical trials using adoptive Vδ1 cells, and development has been hindered, in part, by the lack of translatable large-scale GMP-approved culture techniques (53). Our group has previously reported that the killing properties of Vδ1 and Vδ2 cells against neuroblastoma targets differ with Vδ1 killing by antibody independent cytotoxicity (AIC) versus predominantly ADCC by Vδ2 cells (40).

1.8.2 Vδ2 cells

γδ T cells of the Vγ9Vδ2 isotype (Vδ2 cells hereafter) are the overwhelmingly expressed subtype in circulating peripheral blood and hence have been studied in the most detail. They can be selectively expanded with ease from PBMC *in vitro* and *in vivo* using hydroxymethyl-but-2-enyl-pyrophosphate (HMBPP) or aminobisphosphonate drugs (such as zoledronate and pamidronate), used in clinical practice to treat osteoporosis (57).

Vδ2 cells are often described as a bridge between the innate and adaptive immune system (58). For their innate functions, they are able to mediate ADCC (40) and phagocytose foreign antigens (59). Adaptive responses include the formation of γδ T subpopulations that develop long-lasting memory analogous to αβ T cells [reviewed in (60)], and their ability to carry out professional antigen presentation (61).

1.8.2.1 Activation

γδ T cells are not major histocompatibility complex (MHC) restricted and recognise intact proteins and non-peptide antigens, in contrast to conventional αβ T cells which

respond only to antigens bound to MHC (62). γδ T cells respond to induced self-antigens that are upregulated secondary to cellular stress, infection or malignant transformation. These naturally induced phosphorylated non-peptide Vδ2 ligands were first reported by Tanaka *et al*. (63) in the context of mycobacterial infection and are commonly known as phosphoantigens (PAg). PAg are a diverse group of naturally occurring and synthetic compounds which have low molecular weight and are produced by microbes and cancers cells. Microbially derived (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) is the most potent activator of Vδ2 cells, and is an immediate precursor in the synthesis of isopentenyl pyrophosphate (IPP) by the non-mevalonate pathway of cholesterol biosynthesis. IPP production can also be enhanced by aminobisphosphonate drugs such zoledronate or pamidronate. Aminobisphosphonates act to inhibit cholesterol synthesis by inhibition of the enzyme farnesyl pyrophosphate in the mevalonate-CoA pathway, leading to accumulation of the by-product, IPP (64).

PAg recognition by Vδ2 cells is required to induce cytotoxic responses of infected and transformed cells. For some time, until very recently, the molecular requirements for PAg binding remained a mystery. The discovery of the role of immunoglobulin superfamily members, butyrophilin 2A1 (BTN2A1) and BTN3A1 have been pivotal to understanding the unique requirements for Vγ9Vδ2 TCR activation (65).

There have been conflicting hypotheses as to how PAg interact with butyrophilins to activate Vδ2 cells (66); the allosteric model and antigen presenting model. The former involves the binding of PAg to the intracellular domain of BTN3A1 which then elicits a change in the extracellular domain either directly or indirectly with the help of another unidentified molecule. The antigen presenting theory involves the direct binding of BTN3A1-PAg complex to the Vδ2 TCR (67, 68), however this model has been challenged by the fact that studies have shown PAg binding to the intracellular 30.2 cytosolic pocket, and additionally that BTN3A1 does not directly engage γδTCR (69). In 2020 this controversy was finally resolved with two studies reporting that BTN2A1 acts as a direct ligand for the Vγ9Vδ2 TCR (binding to Vγ9) and synergises with BTN3A1 to potentiate PAg sensing (70, 71).

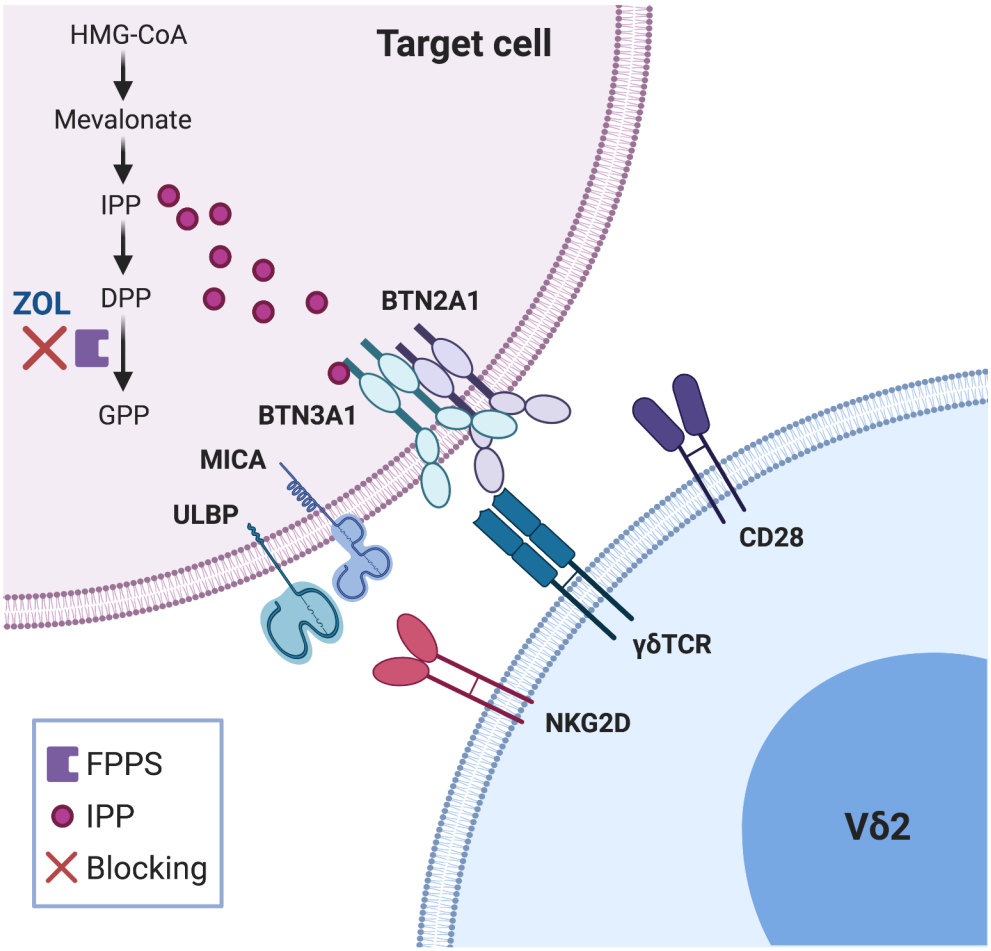


Figure 1.5 Mechanisms of Vδ2 activation

Zoledronate (ZOL) and other bisphosphonate drugs block the enzyme farnesyl pyrophosphate synthase (FPPS) leading to the accumulation of phosphoantigens, including isopentyl pyrophosphate (IPP). Phosphoantigens bind to the intracellular B30.2 domain of BTN3A1 inducing a conformational change. BTN2A1 binds directly to the Vγ9 domain of the Vγ9Vδ2 TCR, and in conjunction with BTN3A1, signals the presence of phosphoantigen to Vδ2 cells. Cell stress and bacterial pathogens induce expression of MHC class I chain-related protein A (MICA) which bind to NKG2D on Vδ2 cells. Costimulatory receptors such as CD28 provide additional costimulatory signals. (DPP, dimethylallyl-pyrophosphate; GPP, geranyl-pyrophosphate). Adapted from (70, 72). *Image created using BioRender software*.

Vγ9Vδ2 cells have endogenous cytotoxicity against various tumours (73). Cytotoxicity is achieved through; the production of cytokines (including tumour necrosis-alpha (TNFα), interferon gamma (IFNγ), IL-4 and IL-10), expression of Fas-L and TRAIL, expression of the Fc receptor CD16 that mediates antibody-dependent cellular cytotoxicity (ADCC), and perforin and granzyme release. Differential cytotoxic function of γδ T cell subsets has been demonstrated and Vδ2 target opsonisation has been shown to augment tumour cell killing (74-79). Specific to neuroblastoma, Fisher *et al*, loaded neuroblastoma cell lines with anti-GD2 antibody (Ch14.18) and demonstrated significantly increased killing of $GD2^+$ cells compared to $GD2^-$ cells.

1.8.2.2 Costimulation

The two-signal model of T cell activation and the role of CD28 costimulation in γδ T cells has been debated with studies reporting paradoxical results (80, 81). A more recent study has demonstrated that CD28 is constitutively expressed and its costimulatory function promotes survival and proliferation, through IL-2 production. Furthermore, CD28 agonists increased γδ T cell proliferation, and conversely this was inhibited by blocking B7 ligands (82). γδ T cells have also been shown to express CD28 ligands including CD80 and CD86 (59) which may allow 'auto' or '*trans*' costimulatory activation of neighbouring γδ cells whereby signal 1 is provided by the TCR or by a chimeric antigen receptor (CAR) [Figure 1.5 (83)].

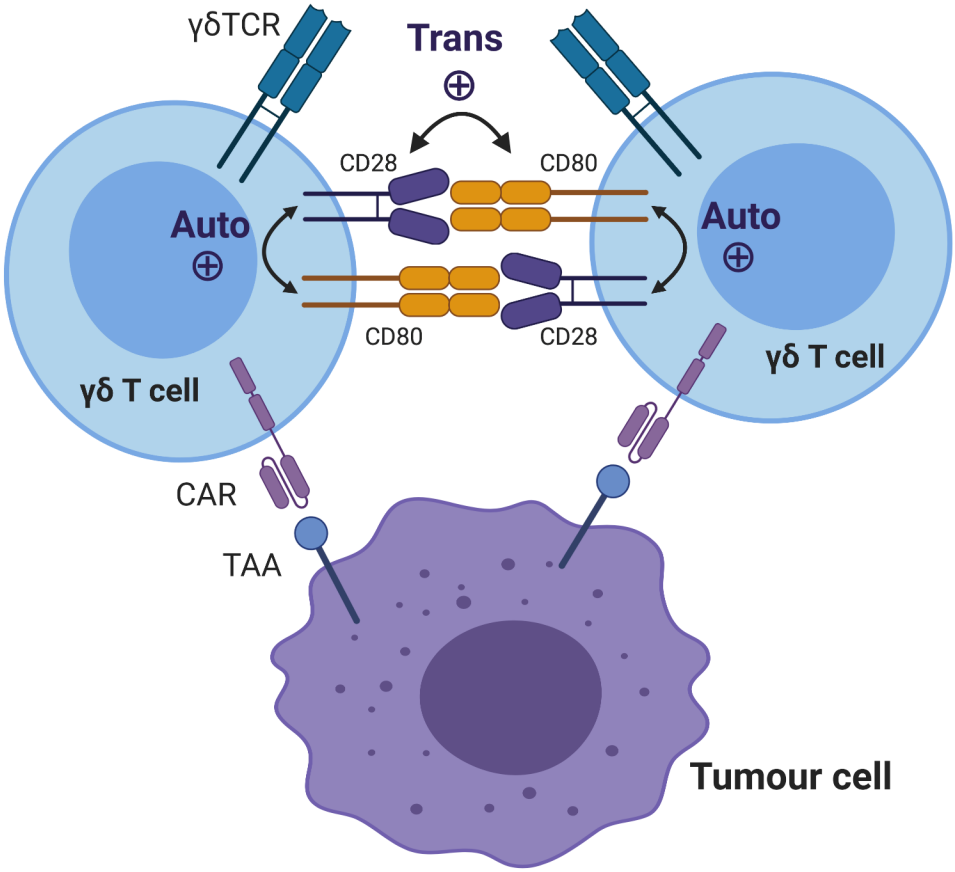


Figure 1.6 Schematic of possible auto and trans-costimulation by γδ T cells

The expression of costimulatory receptor-ligand pairs (e.g.CD28 - CD80/86, 41BB – 41BBL) on the surface of γδ T cells may act to potentiate CAR T cell cytotoxicity through 'auto' and 'trans' costimulation. Concept to provide alternative costimulatory support to CAR T cells proposed by Sadelain *et al*. 2013 (83). CAR; chimeric antigen receptor, TAA; tumour-associated antigen. *Image created using BioRender software*.

1.8.2.3 Antigen Presentation

The ability of γδ T cells to act as professional antigen presenting cells (pAPC) was first described by Brandes and colleagues in 2005 (61). Additionally, γδ T cells specifically of the Vδ2 subtype have been shown to adopt an antigen presenting cell phenotype upon activation with upregulation of classic markers of antigen presentation, costimulation and lymph node-homing (including HLA-DR, CD80, CD86 and CCR7) (59).

To investigate the functional capabilities of γδ T cells, recent studies have shown that Vδ2 cells can take up, process and present peptide antigens to αβ T cells (61, 74, 84), a process known as antigen cross-presentation. Antigen cross-presentation is the complex process of acquiring exogenous antigens and then presenting them on MHC class I molecules to $CD8^+$ T cells. The prototypic pAPC *in vivo* are dendritic cells (DCs) which activate naive $CD8^+$ cells to become cytotoxic effector αβ T cells. Two main intracellular pathways for antigen cross-presentation have been described in DCs (refer to Figure 1.7); the cytosolic and vacuolar pathway [reviewed in (85)]. In the cytosolic pathway, internalised proteins are degraded by the proteosome, and then transported into the endoplasmic reticulum (ER) by transporter associated with antigen processing 1 (TAP1) and 2 (TAP2) before loading onto MHC class I (cytosolic pathway with ER loading). There is also evidence, however, that MHC class I loading can occur in the phagosome (cytosolic pathway with phagosomal loading) (85). The vacuolar pathway involves antigen degradation and MHC class I loading within endosomes/phagosomes.

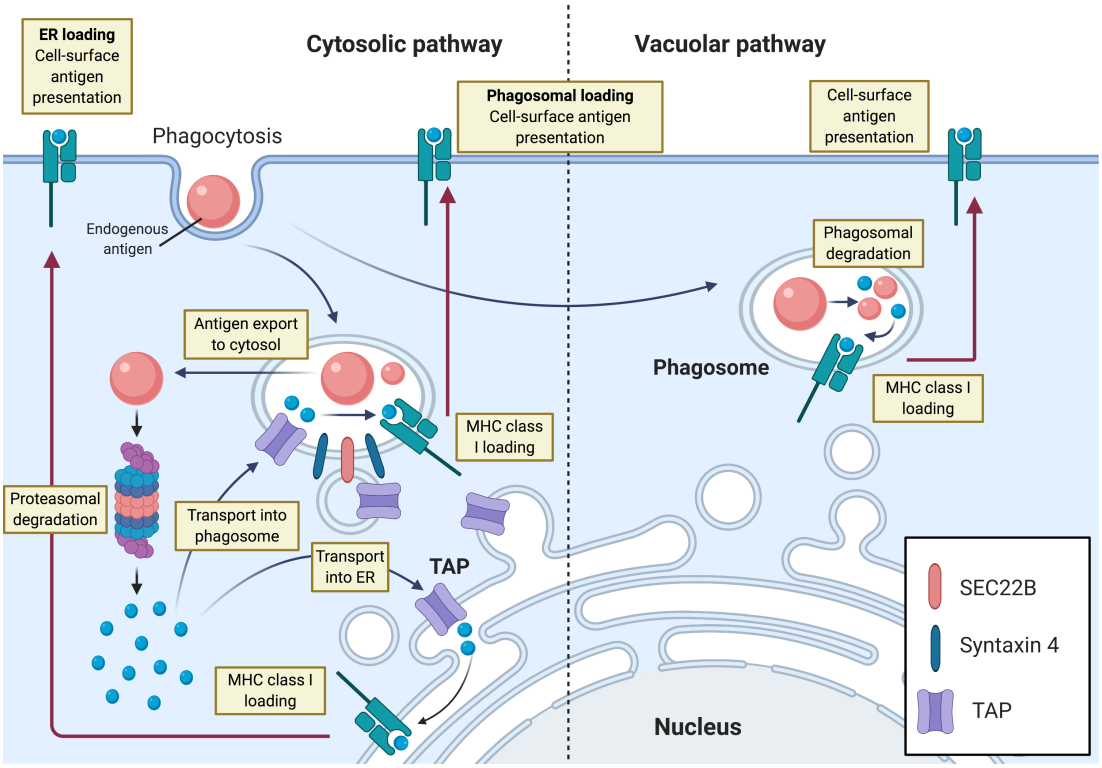


Figure 1.7 Intracellular pathways for antigen cross-presentation in dendritic cells

Antigen cross-presentation allows dendritic cells to process and present exogenous antigens on MHC class I molecules. In the cytosolic pathway (left), exogenous antigen is internalised by phagocytosis, then exported to the cytosol. The antigen is then degraded by the proteasome and the products are be processed either via the cytosolic pathway with ER loading, or cytosolic pathway with phagosomal loading. Alternatively, exogenous antigen can be processed via the vacuolar pathway (right). Image adapted from (85). *Created with BioRender software.*

Cross-presentation was reported firstly in γδ T cells using mycobacterium tuberculosis–purified protein derivative (PPD), and influenza virus-encoded matrix protein (M1) by Brandes *et al.* (84) The authors demonstrated that Vδ2 cells could take up and process these antigens, and consequently induce proliferation of responder $CD8^+$ αβ T cells. To investigate the route of antigen processing by Vδ2 cells, the inhibitors lactacystin and Brefeldin A, were used to target the proteosome and transGolgi network respectively, demonstrating the need for proteosome activity for antigen processing to occur. Although the molecular mechanisms for intracellular antigen processing in Vδ2 are not well defined, a follow up study showed significantly delayed protein degradation and endosomal acidification when compared to monocyte-derived DCs, both of which are favourable conditions for cross-presentation

(86). Interestingly, when changing from an infectious to tumour antigen model, Brandes *et al*., were unable to demonstrate γδ T cell cross presentation using melanoma antigen recognised by T cells-1 (MART1), proposing that this was due to its rapid degradation by the immunoproteosome (61).

The inability of γδ antigen presenting cells (γδ-APC) to cross-present tumour antigens was later contested by Himoudi *et al*. (2012) using paired-box 5 protein (PAX5) and MART-1 (74). The authors reported that in order for cross presentation to occur, a certain threshold of γδ T cell activation must be reached. This 'additional' signal could be produced by CD16 FcγR interaction with surface bound antibody, a requirement termed 'licensing' of γδ T cells for professional antigen presenting function (Figure 1.8). Furthermore, in an infection model, IgG target opsonisation augmented *E. Coli* antigen uptake by Vδ2 cells, which was inhibited by blocking the Vγ9Vδ2 TCR, suggesting a 'licensing' interaction between the TCR and FcγR (59). Translating this to the role of chimeric antigen receptors in γδ T cells, it is hypothesised that provided the correct level of TCR stimulation and costimulation occurs, chimeric antigen receptor-engineered γδ T cells may be sufficiently activated in order to function as pAPCs.

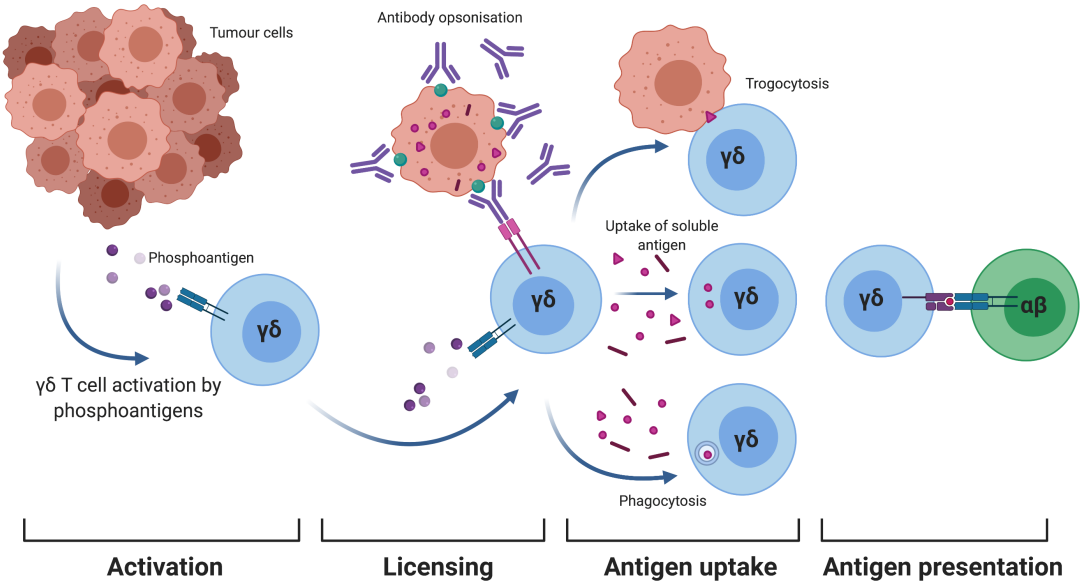


Figure 1.8 Schematic of γδ T cell 'licensing'

γδ T cells are firstly activated by phosphoantigens, then opsonised tumour cells initiate γδ T cell 'licensing' allowing the uptake of extracellular antigen (either by trogocytosis, phagocytosis or soluble uptake) [concept described by Himoudi *et al*., 2013 (74)]. Tumour antigen is then internally processed by γδ T cells and presented on MHC molecules. *Image created using Biorender software.*

1.8.3 Vδ1^{-}/Vδ2^{-} cells

Significantly less is known about γδ T cells expressing Vδ1^{-}/Vδ2^{-} TCRs and their potential role for cancer immunotherapy. Progress in this field has been inhibited somewhat by the delay in commercially available antibodies or reagents to promote their expansion to significant number (including Vδ3). Following γδ T cell expansion with artificial antigen presenting cells, Fisher *et al*. (40) used next generation sequencing to highlight the significant heterogeneity of both gamma and delta chain joining segment and V segment usage. In the same paper it was shown that Vδ1^{-}/Vδ2^{-} γδ T cells have both AIC and ADCC cytotoxic capabilities.

1.9 CAR T cells

1.9.1 CAR T cell design and structure

Chimeric antigen receptors (CAR) have a synthetic modular design that combines the specificity of an antigen-binding ectodomain (most commonly a single-chain variable fragment (ScFv) of a monoclonal antibody) together with a T cell activation endodomain (87). These recombinant receptors contain from their N- to C-terminus 1) an antigen-specific ScFv, 2) an extracellular stalk/spacer region, 3) transmembrane domain, and 4) T cell signalling endodomain(s). Over the last 20 years many novel CAR constructs have been developed that target a wide range of tumour-associated antigens (TAA) expressed on cancer cell surfaces (including proteins, carbohydrates and glycolipids). Binding of a CAR allows the engineered T cell (CAR T cell) to directly kill cancer cells expressing the corresponding TAA, independently of its endogenous TCR specificity. CAR T cells are not restricted by MHC and therefore have the added advantage of being able to target cancer cells that have down-regulated MHC as a mechanism of 'tumour escape' (83). On binding to antigen, CAR T cells are activated resulting in cytotoxicity (e.g. through granzymes and perforins), cytokine release (including IFNγ and TNFα), and proliferation.

The functional properties of CAR are dependent on their specificity, affinity, structure and signalling. The most commonly used antigen-binding structures are ScFv derived from murine-derived monoclonal antibodies (often already in clinical use e.g. Ch14.18). It is also known that CAR-binding is dependent on the distance of the ScFv from the cell surface (linked by an extracellular stalk or hinge-region) for optimal synapse formation (88). There have been many developments and modifications to the design of CARs with regard to their intracellular signalling domain (Figure 1.9). Some of the first CARs contained only CD3ζ (first-generation). First-generation CAR T cells had limited effectiveness and were prone to undergoing anergy (89). They also did not support re-expansion on repeated exposure to antigen encounter (90). Second-generation CARs added a costimulatory molecule (e.g. CD28, 41BB, OX40 or ICOS) to augment CD3ζ signalling. This results in greater signalling strength and persistence

of CAR T cells *in vivo* (91). Third-generation CARs contain three endodomains (e.g. CD3ζ, CD28 and 41BB) and have also been tested with good effect in mouse models (92). More research is required to determine the optimal CAR-conformation to promote function, survival, and re-expansion on repeated antigen exposure, and to avoid T cell exhaustion and adverse patient side-effects resulting from over-activation/cytokine storm (83).

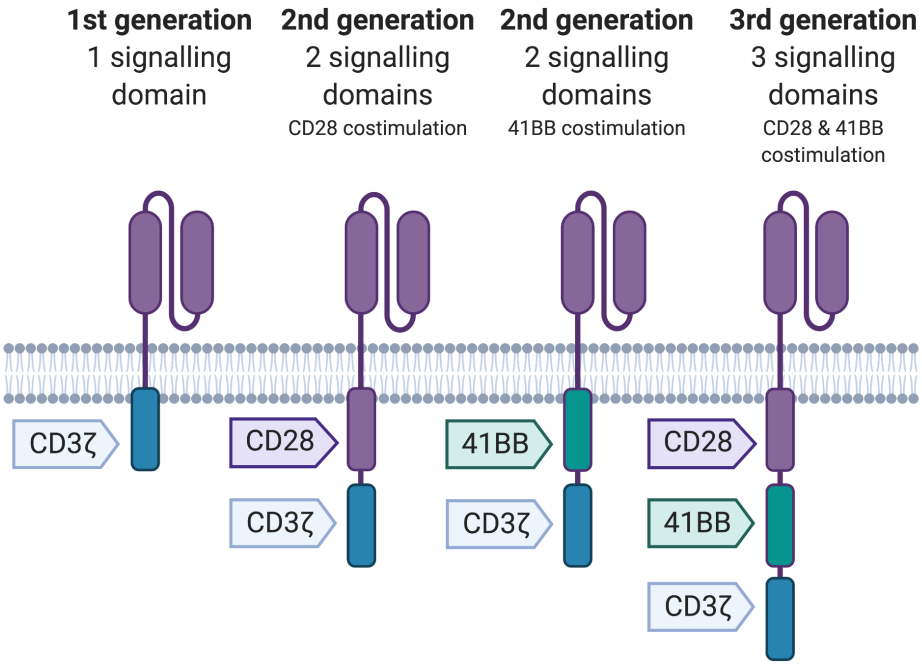


Figure 1.9 Evolution of CAR T cells

First generation CARs contain one signalling domain; CD3ζ. Second generation CARs contain two signalling domains; CD3ζ and one costimulatory domain (e.g. CD28, 41BB, ICOS, OX40). Third generation CARs contain; CD3ζ and two costimulatory domains. Alternative constructs under development include; 4th generation CARs, CARs with suicide switches, dual CARs, and 'armoured' CARs (genetically engineered to secrete cytokines or express ligands to enhance endogenous immune cells). *Created with BioRender.com*

1.9.2 CAR T cell phenotype

The success of adoptively transferred CAR T cells is associated with their ability to survive long-term in the patient (93). The concept of a 'living drug' requires engraftment by CAR T cells enabling them to carry out long-term immunosurveillance. Memory T cell subsets influence effector function and proliferative capacity with more naïve phenotypes having greater therapeutic potential (94, 95).

As shown in Figure 1.10, naïve T cells (T_N) differentiate into central memory (T_{CM}), effector memory (T_{EM}), and effector T cells. Following activation by antigen presenting cells, T_N cells (antigen naïve) proliferate and differentiate into effector T cells. Effector cells home to the required site to exert their cytotoxic function and a small subset of memory T cells are formed, which upon antigen re-exposure undergo rapid re-expansion.

Research groups have demonstrated that both CD4 and CD8 T cells are required in combination to produce the greatest anti-tumour responses (96, 97). Sommermeyer *et al*. analysed CD19 CAR T cells derived from different subsets; $CD4^+/CD8^+$, T_N, T_{CM}, T_{EM} and showed clear differences in their effector functions and proliferative capacity both *in vitro* and *in vivo* (98). The authors reported that $CD8^+$ CAR T cells had higher specific cytotoxicity than $CD4^+$ CAR T cells, but that $CD4^+$ CAR T cells produced more Th1 cytokines in response to antigen recognition (including IFNγ, TNFα and IL-2) and had greater proliferation. $CD4^+$ T_N cells produced the highest levels of cytokines and $CD8^+$ T_N and T_{CM} had the highest level of tumour lysis.

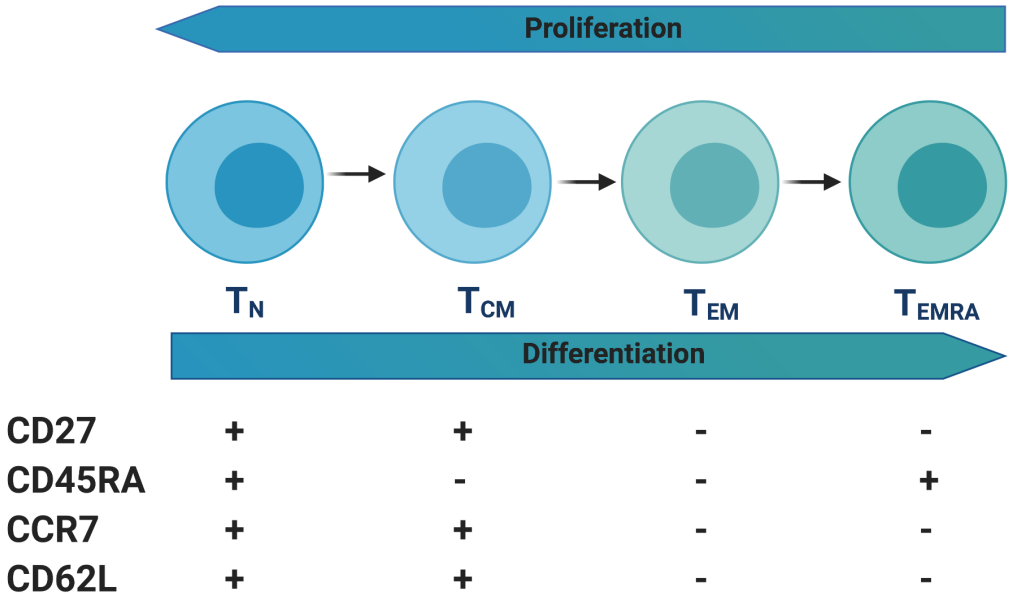


Figure 1.10 CD8 T cell differentiation

T_N, naïve T cells, T_{SCM} stem cell memory, T_{CM} central memory, T_{EM} effector memory, T_{EMRA} terminally differentiated effector memory.

1.9.3 Viral vectors for genetic engineering of CAR T cells

Three main approaches have been used in clinical trials for CAR gene transfer, including both viral and non-viral methods. Gamma retroviral and lentiviral transduction are the commonest method for viral gene transfer, and the sleeping beauty transposon/transposase system is an example of a non-viral plasmid-base method that has been used for CAR T cell manufacture (99).

Gamma retrovirus has been shown to have broad cell tropism, efficient integration and stable gene expression (99). A favourable advantage of using gamma retrovirus is that supernatant containing high-titre gamma retrovirus can be produced by large-scale manufacturer via the expansion of stable producer cells (99). This method is far less labour intensive and more cost efficient than transient transfection methods, used for

lentivirus production which requires 3 or 4 independent plasmids encoding gag-pol-rev, the self-inactivating transfer vector, and the pseudo envelope.

Lentivirus on the other hand, as well as high transduction efficiencies and stable CAR expression, has the additional advantage of allowing efficient gene transfer into non-dividing cells (100-103). In terms of safety, both viral vectors have been designed so they are 'replication incompetent', however there remains a small theoretical risk, during manufacturing or following infusion, that a recombinational event could occur leading to the production of a novel replication-competent virus. To date, however, there is no reported evidence of this having occurred (104). Another potential risk is the induction of insertional oncogenesis and there are case reports of clonal T cell leukaemias resulting from early gene therapy approaches to treat immune deficiencies including X-linked Severe Combined Immunodeficiency Disorder (X-SCID) (105). Again, this has not been reported in patients having received CAR T cells.

The non-viral sleeping beauty (SB) transposon/transposase system requires two DNA plasmids; the first is the transposon that encodes the CAR, and the second is the transposase that permits insertion of the transgene into TA dinucleotide repeats. SB transposon/transposase are then introduced in to T cells by electroporation (99). The main benefits of using the SB transposon/transposase system are the potential for less genotoxicity (106), and that it is not subject to the demanding quality control checks required for viral-based methods.

1.9.4 CAR T cells in clinical trials

Many CAR constructs have now been translated into cellular therapies for both haematological cancers and solid tumours, and have been tested in clinical trials (107). The majority of CAR T cell trials have used autologous peripheral blood mononuclear cells (PBMC), that have been virally transduced and expanded in GMP-laboratories, before re-infusion back into the patient (Figure 1.11). Chemotherapy conditioning of the patient prior to CAR T cell infusion has been demonstrated to enhance function (108). It is thought that the process of lymphodepletion creates extra 'space' for

engraftment and depletes the patient of their own regulatory T cells that may have an inhibitory effect on the infused activated CAR T cells.

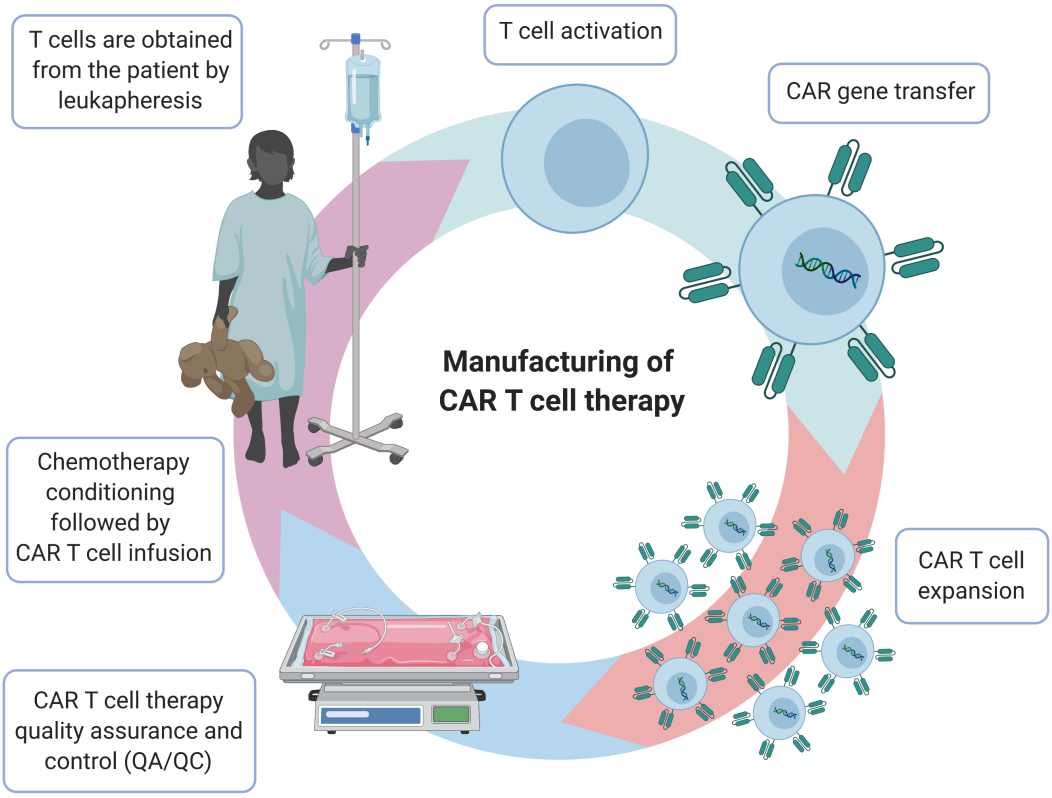


Figure 1.11 CAR T cell therapy manufacture

Created using BioRender software

The most success to date from CAR T cell trials have resulted from targeting CD19 in B cell malignancies (including CLL and ALL) (109-112). A study for relapsed/refractory ALL by Grupp and colleagues (113) reported a 90% complete response rate for 27 patients receiving second-generation CD19 CAR T cells. Sustained remission was demonstrated with a 6-month EFS rate of 67% (95% CI, 51 to 88) and an overall survival rate of 78% (95% CI, 65 to 95) at two years. The main adverse effects were related to 'cytokine storm' affecting all patients to varying degrees, with eight requiring intensive care support. All were successfully managed with IL-6 blockade with tocilizumab.

On commencing this study, there were two CAR T cell trials reported for recurrent/refractory neuroblastoma patients; one targeting L1-CAM (114, 115) and one targeting GD2 (116, 117). The first study by Park *et al.* (2007) was a Phase 1 trial for safety and feasibility where twelve infusions (9 at 10^8 cells/m^2 and 3 at 10^9 cells/m^2) were given to six patients. Metastatic neuroblastoma is known to over-express the L1-cell adhesion molecule (L1-CAM, also known as CD171) and this trial used a 1st generation L1-CAM-CAR with a CD3ζ intracellular signalling domain. There were no reported overt toxicities and no 'off-target' effects resulting from L1-CAM targeting. CAR T cells persisted for up to 7 days in patients with bulky disease, but up to 42 days in those with limited disease. Only one patient out of the six sustained a prolonged survival of 4.5 years.

The second trial by Pule *et al.* (2008) compared EBV-specific cytotoxic lymphocytes engineered with a first-generation GD2-CAR, with cytotoxic lymphocytes lacking viral-specificity engineered with the same CAR (116). It was hypothesised that EBV-specific CAR T cells would have greater tumour cytotoxicity and prolonged survival *in vivo* due to their engagement with professional antigen presenting cells expressing costimulatory ligands. Eleven neuroblastoma patients were treated, with eight having clinically evaluable tumours at the time of infusion. Four out of eight had evidence of tumour necrosis or regression, with one patient sustaining a complete remission. EBV-specific CAR T cells persisted longer (6 weeks compared to 3 weeks for CAR T cells lacking viral specificity). However, a follow up paper showed no difference in the long-term persistence of either cell type (117).

The recent advances in CAR design and delivery pave the way for new clinical trials. Studies using second generation CARs have shown unequivocal responses in haematological malignancies and their efficacy in paediatric solid tumours, including neuroblastoma, are currently being evaluated. There are still many unanswered questions regarding the engineering of CAR T cells for solid tumours including:

- Choice of target antigen to ensure both efficacy and safety

- Optimal delivery of CAR into T cells (retroviral or lentiviral transduction, or alternative methods)
- Choice of costimulatory endodomain(s)
- The importance of other portions of the CAR (including the extracellular spacer/stalk/hinge region) for optimal antigen affinity
- The choice of effector cell (αβ, NK, γδ) or differential T cell subsets (CD4/CD8) and their role in CAR T cell efficacy and persistence (118).
- How to enable T cells to survive and proliferate rather than developing exhaustion/undergoing AICD in the face of ongoing antigenic stimulation.
- How to improve CAR T cell trafficking to the tumour site(s)
- How to overcome the immune suppressive effects of the tumour microenvironment.

1.9.5 CAR T cell related toxicities

Although CAR T cells are potentially curative, they can be associated with severe and life-threatening toxicities (119). Treatment related toxicities vary considerably and are dependent on the CAR T cell construct, cancer type, extent of disease, target antigen, age of the patient, comorbidities and prior therapy. Toxicities can be classified into; 'on target, on tumour' and 'on target, off tumour'. An example of the most serious 'on-target, off tumour' toxicity was in a patient with metastatic colon cancer who received CAR T cells directed against Her2. Soon after infusion, there was acute onset respiratory failure which ultimately led to the patient dying, and this was attributed to CAR-mediated destruction of $Her2^{+}$ lung epithelium (120). This highlights the importance of a clear understanding of tumour antigen expression on normal tissues in order to avoid unforeseen 'off tumour' side effects.

Another important toxicity results from a systemic inflammatory response, known as cytokine release syndrome (CRS). Patients with high tumour burden often experience severe CRS (97), therefore limiting T cell dose, however it may be the case that dividing doses over multiple injections may overcome some of these adverse effects together with optimal supportive care. In the recent ELIANA trial (ClinicalTrials.gov

number, NCT02435849) using CD19-41BBζ CAR T cells in ALL (tisagenlecleucel, Novartis), the remission rate was reported at 81%, however CRS occurred in 77% of patients and neurological events in 40% (121). CRS and neurotoxicity (CAR T cell-related encephalopathy syndrome, CRES) are explored in greater detail in Section 1.9.5.1 and Section 1.9.5.2, respectively.

1.9.5.1 Cytokine release syndrome (CRS)

Cytokine release syndrome (CRS) is an acute inflammatory process resulting from elevated serum cytokines (including IL-6, IFNγ, GM-CSF, IL-15 and IL-8) (122). It is potentially the most serious adverse side effect of CAR T cell administration, and in rare cases can be fatal (123). CRS classically has delayed onset, 1-14 days after CAR T cell infusion corresponding to the peak of CAR T cell activation following antigen encounter. This pattern of clinical features is what has been experienced using CD19 CAR T cells and may differ depending on tumour type, antigen target and CAR structure. Severity of symptoms also appears to correspond with high tumour burden (113, 124), however the degree of CRS does not correlate with tumour responses and clinical outcome.

The clinical features of CRS include fever, hypotension and respiratory compromise. CRS is graded from 1-4, with grade 1 having the mildest symptoms requiring supportive treatment only such as anti-pyretics for fever. Conversely, grade 4 involves life-threatening complications including hypotension with the need for vasopressor drugs and hypoxia necessitating mechanical ventilation (122). The pathogenesis is thought to be mediated not through the release of cytokines by activated CAR T cells, but instead by macrophages producing IL-6, IL-1 and nitric oxide (125). Hence, IL-6 (tocilizumab) blockade can be used to successfully manage severe/life-threatening CRS.

1.9.5.2 CAR T Cell-Related Encephalopathy Syndrome (CRES)

The pathophysiology of CRES is at present not fully understood, but two different theories propose that it results from either diffusion of inflammatory cytokines into the CNS, or caused by disruption of the blood-brain barrier with trafficking of CAR T cells into the CSF (126). The clinical spectrum ranges from mild confusion and language disturbance, to delirium, toxic encephalopathy, seizures, motor weakness and raised intracranial pressure. As the initial presentation in paediatric patients can be subtle, regular neurological assessments are required e.g. CARTOX-10 screening (CAR-T-cell therapy associated TOXicity 10-point neurological assessment) (119). The development of CRES appears to be biphasic, either occurring early soon after CAR T cell infusion correlating with the development of CRS, or alternatively late following CRS resolution. Interestingly IL-6 blockade is often ineffective for treating neurotoxicity (127) and management is patient-specific involving seizure control with anti-epileptic drugs and corticosteroids in the severest of cases.

1.10 Immunotherapy trials involving γδ T cells

There has now been an explosion of published studies on the engineering of CAR αβ T cells for haematological and solid cancers, however using γδ T cells as the effector cell of choice and engineering them with a CAR for adoptive transfer is a relatively new concept. The use of non-engineered γδ T cells has been investigated in phase 1 studies, and has demonstrated the safety of infused cells with varying degrees of efficacy. γδ T cells may be expanded *in vivo* or *ex vivo* with adoptive transfer, and the clinical studies using these two approaches are discussed in Section 1.10.1.1 and Section 1.10.1.2 respectively, before reviewing the available pre-clinical literature on CAR engineered γδ T cells in Section 1.10.2.

1.10.1 'Non-engineered' γδ T cell immunotherapy

Immunotherapy involving γδ T cells has been tested in clinical trials either by giving aminobisphosphonate therapy directly to patients to induce an *in vivo* Vδ2 expansion, or by the adoptive transfer of *in vitro* manufactured cells. There have been four recent comprehensive reviews of γδ T cell trials (128-131) and a summary table of published studies was collated by Hoeres *et al*. (131) classifying studies into two groups; *in vivo* expansion (132-138), or adoptive transfer (139-150), summarised in Table 4.

1.10.1.1 In vivo expansion

The earliest γδ T cell immunotherapy trial, by Wilhelm *et al*. (133) demonstrated the *in vivo* cytotoxicity of Vδ2 cells using pamidronate and IL-2 against non-Hodgkin lymphoma and multiple myeloma. This pioneering study included 19 patients, and although the first patient cohort (n=10) did not show significant γδ T cell activation, the second cohort (n=9) was selected based on γδ T cell response to pamidronate *in vitro*. Subsequently *in vivo* proliferation of Vδ2 was seen in 5 patients, with objective response achieved in 3 patients. Conclusions drawn from this study were that response to treatment could be predicted by Vδ2 cell proliferation *in vitro*, and that treatment was well tolerated and safe. Whilst there have been other supporting studies (observational and phase 1) suggesting a favourable outcome (134, 135, 137), there are also reports of more disappointing results (136, 138) (refer to Table 4 for further trial details).

Combinational treatment with IPH1101 (a BrHPP containing γδ T cell agonist) with the monoclonal antibody rituximab and IL-2 has also been investigated with very promising results (151) in a phase I/IIa study for follicular lymphoma (poster presentation, ASH 2009). A 75% response and 50% CR was reported however these results should be interpreted with great caution as they were presented by the Pharma company (for IPH1101) and never subsequently published in a peer-reviewed journal. No follow up studies have been reported.

Specific to neuroblastoma, circulating γδ T cells are fewer in neuroblastoma patients compared to healthy children (132) but there is demonstrable cytotoxicity towards tumour cells *in vitro* (40). A small phase 1 study conducted by Pressey *et al*. gave 4mg/m^2 zoledronate (ZOL) together with IL-2 to four children with stage IV neuroblastoma that was refractory to conventional treatments. Administration of ZOL led to a 3-10 fold increase in circulating γδ T cells but only restored counts to the lower end of the normal range. Unfortunately, of the four patients enrolled, 1 was withdrawn for abdominal pain of unknown cause, and the other 3 had progressive disease. More positively, in keeping with previous studies in adults, there were no dose limiting toxicities or serious toxicities reported in paediatric patients.

1.10.1.2 Adoptive transfer of non-engineered γδ T cells

Similarly, phase 1 clinical trials using adoptively transferred *ex vivo* expanded γδ T cells have yielded conflicting results. Protocols for the *ex vivo* expansion of γδ T are well established using aminobisphosphonates together with IL-2, and most trials have used repeated doses of IL-2 to drive *in vivo* expansion. As can be seen in Table 4, there have been 12 studies using *ex vivo* expanded cells with 5 out of 8 suggesting a positive anti-tumour effect. Again, the trials had tolerable toxicity profiles but modest efficacy.

Due to the vastly different trial protocols, small sample sizes and variety of cancers studies, γδ T cell immunotherapy trials have not been suitable for statistical comparison. However, it was concluded in a recent systematic review that overall, γδ T cell immunotherapy was superior to second-line treatment in certain cancers (including renal cell carcinoma), with even greater efficacy seen when combined with monoclonal antibody treatment (128).

Table 4: Summary of γδ T cell clinical trials [adapted from (131)]

Reference		Disease	Number of patients	Outcome
In vivo stimulation				
Wilhelm *et al.* **(124)**	2003	Multiple myeloma, lymphoma	19	16% PR, 16% SD
Dieli *et al.* **(125)**	2007	Prostate	18	16% PR, 27% SD
Bennoua *et al.* **(128)**	2010	Renal, gynaecological, gastrointestinal	28	42% SD
Laurent *et al.* **(142)**	2010	Follicular lymphoma	45	26% CR, 18% PD
Meraviglia *et al.* **(126)**	2010	Breast	10	10% PR, 20% SD
Lang *et al.* **(129)**	2011	Renal cell carcinoma	12	16% SD
Kunzmann *et al.* **(127)**	2012	Renal cell carcinoma, melanoma, AML	21	16-42% SD, AML 25% PR
Pressey *et al.* **(123)**	2016	Neuroblastoma	4	25% SD, 75% PD
Adoptive transfer				
Kobayashi *et al.* **(133)**	2007	Renal cell carcinoma	7	Delayed tumour double time in 4/7
Bennouna *et al.* **(130)**	2008	Renal cell carcinoma	10	60% SD
Abe *et al.* **(135)**	2009	Multiple myeloma	6	66% SD
Nakajima *et al.* **(136)**	2010	Lung	10	30% SD
Kobayashi *et al.* **(134)**	2011	Renal cell carcinoma	11	9% CR, 45% SD
Nicol *et al.* **(138)**	2011	Solid tumours	18	16% SD, 16% PR and CR
Noguchi *et al.* **(132)**	2011	Solid tumours	25	12%SD 12% PR
Sakamoto *et al.* **(131)**	2011	Lung	15	40% SD
Cui *et al.* **(140)**	2014	Hepatocellular carcinoma	62	Longer PFS and OS
Wilhelm *et al.* **(137)**	2014	Haematological	4	75% CR
Wada *et al.* **(139)**	2014	Gastric	7	Reduction in ascites in 2/7
Aoki *et al.* **(141)**	2017	Pancreatic	28	Higher recurrence free survival in patients with high GD T cell numbers

Abbreviations: CR, complete remission; OS, overall survival; PD, progressive disease; PFS, progression free survival; PR, partial remission; SD, stable disease.

Whilst there are clearly technical and functional barriers to γδ T cell immunotherapy, further investigation is required to understand why attempts at adoptive transfer have not yielded significant success. There are multiple conceivable explanations that might contribute as to why γδ T therapy has not been efficacious at preventing progressive disease; firstly whether cells are 'hypo-responsive' due to activation-induced anergy or AICD (152), and secondly whether infused γδ T are capable of homing to tumour sites and able to survive within the highly suppressive tumour microenvironment. In their comprehensive review, Presti *et al*. describe the various approaches that have been attempted in order to overcome some of these immunological barriers. This includes combinational therapy with immune check point blockers (such as CTLA-4, PD1 and PDL1), chemotherapy pre-conditioning (153, 154), use of liposomes to improve the biodistribution of aminobisphosphonate drugs (155), bispecific antibodies (156-158) and lastly CARs, the subject of this thesis.

1.10.2 CAR-engineered γδ T cells

As highlighted in Section 1.9.4, there have been unprecedented clinical responses following the adoptive transfer of CAR engineered αβ T cells to treat cancer. Research has focused on using αβ T as the effector cell of choice, however investigation into the properties and efficacy of genetically modified alternative immune cells, including natural killer cells (159-162) and γδ T cells, have shown encouraging results. Due to the promising data reported in pre-clinical studies, a clinical trial using GD2-CAR expressing NKT is currently underway (clinical trial ID NCT03294954).

The literature on CAR transduced γδ T cells is somewhat lagging behind, however there are a handful of pre-clinical studies emphasising their unique potential as an important player in the already crowded immunotherapy field. The first published study reporting the transduction of γδ T cells with CAR was by Rischer and colleagues in 2004 (163) using zoledronate to expand a predominantly Vδ2 population and transduction with first generation CD19ζ or 14.G2aζ (GD2) CAR. The authors reported that Vδ2 could be selectively expanded with zoledronate to large numbers,

and efficiently transduced with CARs using a SFG gammaretroviral vector. CAR^+ Vδ2 upregulated CD69 and secreted IFNγ upon antigen target recognition. Non-transduced Vδ2 cells co-cultured with the same targets had relatively low background IFNγ production (5.7 ± 1.2% non-transduced Vδ2 + $GD2^+$ LAN1 tumour cells, compared to 33 ± 3% for GD2-CAR^+ Vδ2 + $GD2^+$ LAN1). *In vitro* cytotoxicity assays demonstrated antigen specific killing to $CD19^+$ Daudi, Raji and Reh cells by CD19-CAR^+ Vδ2, and GD2-expressing LAN1 and JF cells, by GD2-CAR^+ Vδ2 cells. Background killing by CAR^+ Vδ2s against antigen-negative cell lines remained low.

It was not until 2013 that Deniger *et al*. (164) used feeder cells [artificial antigen presenting cells (aAPC)] to expand a polyclonal repertoire of γδ T cells, and electroporation with sleeping beauty transposon/transposase to engineer Vδ1, Vδ2 and $Vδ1^-/Vδ2^-$ cells with CAR. aAPC used in this paper were modified to express $CD19^+$, $CD64^+$, $CD86^+$, $CD137L^+$, $IL15^+$, and fresh aAPC were added at weekly intervals (165). Using this method, together with cytokine support (IL-2 and IL-21), the authors were able to expand $>10^9$ CAR^+ γδ T cells from starting cells of $<10^6$. Polyclonality of expanded cells was evaluated using digital multiplex assay with Vδ1, Vδ2, Vδ3 and Vγ2, Vγ7, Vγ8, Vγ9, Vγ10 detected. Propagated cells expressed a desirable memory phenotype and homing markers (including CXCR4, CD62L, CCR7). Large numbers of Vδ3 were also preserved.

Cytotoxicity was assessed *in vitro* using chromium release assay and the $CD19^+$ NALM6 leukaemia cell line. γδ T cells expressing CD19-CAR (CD19-CAR^+) had significantly higher cytotoxicity than CD19-CAR^- γδ T cell (p 0.001). In vivo, using a NALM6-eGFF-ffLuc model, the burden of tumour significantly reduced following infusion of CD19-CAR^+ γδ T cells compared to untreated mice, however a non-transduced control was not included in the study.

1.11 Aims & Objectives

The aim of this project is to investigate GD2-directed CAR^+ γδ T cells for neuroblastoma immunotherapy. γδ T cells have variable innate cytotoxicity that can be augmented by either target opsonisation or through the addition of a CAR. γδ T cells have also been shown to have enhanced tissue penetration and antigen-presenting capabilities. It is hypothesised that the unique properties of γδ T cells, bridging the gap between the innate and adaptive immune systems, may confer additional therapeutic advantage over existing αβ T cell approaches, when engineered with a CAR. My objectives are:

- To determine the optimal method of activation, expansion and transduction for the manufacture of CAR γδ T cells suitable for adoptive transfer.

- To characterise and compare CAR γδ and CAR αβ T cells by investigating their functional responses *in vitro* including; phenotype, cytotoxicity, differentiation, proliferation, homing and cytokine secretion.

- To phenotypically characterise CAR γδ T cells for markers consistent with an antigen presenting cell phenotype.

- To study the antigen cross-presentation function of CAR γδ T cells using an *in vitro* model.

Chapter 3 details the experiments investigating methods for γδ T cell activation and transduction with a 2nd generation GD2 CAR. Chapter 4 describes the *in vitro* functional comparisons of different T cell subsets including CAR transduced αβ, Vδ1 and Vδ2 T cells. Lastly in chapter 5, the ability of CAR γδ T cells to cross-present exogenous tumour antigen to αβ T cells is studied, using an *in vitro* model system.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Buffers, media and reagents

Tissue culture reagents were all purchased from Sigma-Aldrich (Dorset, UK). Molecular biology reagents, unless stated otherwise, were purchased from New England Biolabs (UK). Primers and G-blocks were purchased from Integrated DNA Technologies (Glasgow, UK) and peptides were synthesised by ProteoGenix (France).

2.1.2 Proteins

The synthetic peptides ELAGIGILTV ($MART1^{26-35}$) and GHGHSYTTAEELAGIGILTVILGVL ($MART^{16-40}$) were manufactured by ProteoGenix (France).

Table 5: Buffers, media and solutions

BUFFER	CONTENTS/CONCENTRATION
R10 MEDIA	Roswell Park Memorial Institute (RPMI) 1640, 10% heat inactivated fetal calf serum (FCS), 1% penicillin/streptomycin (P/S)
LYSIS BUFFER FOR WESTERN BLOT	150mM NaCl, 50mM Tris Base, 0.05% Sodium dodecyl sulphate, 1% Triton X100
ANTIBODY STAINING BUFFER FOR WESTERN BLOT	5% non-fat milk in TBS-T
BLOCKING BUFFER FOR WESTERN BLOT	5% non-fat milk in TBS
FACS STAINING BUFFER	Phosphate buffered saline (PBS) with 0.5% bovine serum albumin (BSA) and 2.5mM EDTA
LB BROTH	1% typtone, 0.5% yeast extract, 1.0% NaCl, pH 7.0
TBS	50mM Tris base, 150mM NaCl, pH 7.5
TBS-T	50mM Tris base, 150mM NaCl,0.05% Tween-20, pH7.5
TAE BUFFER	40mM Tris-acetate, 1mM EDTA, pH 8.3
MOPS SDS RUNNING BUFFER	50mM MPS, 20mM Tris base, 0.1% SDS, 1mM EDTA, pH 7.7
PROTEIN TRANSFER BUFFER	25mM bicine, 25mM Bis-Tris, 1mM EDTA, pH 7.2

2.1.3 Tumour cell lines

Cell lines were validated to be free from mycoplasma by MycoAlert™ assay (MycoAlert Mycoplasma Detection Kit, Lonza) and confirmed to have genetic identity to the published parental lines by Short Tandem Repeat (STR) analysis. All cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Sigma-Aldrich) plus 10% heat inactivated foetal calf serum (FCS) and 1% penicillin/streptomycin (P/S)

(R10), unless stated otherwise. Human embryonic kidney 293T cells were cultured in Dulbecco's Modified Eagle Medium (Sigma-Aldrich) supplemented with 10% FCS. All cells were incubated in 5% CO2 at 37°C. For adherent cell lines, once 80% confluence was reached, cells were dissociated with 0.05% Trypsin-EDTA (Sigma-Aldrich) and passaged 1:10.

The T cell lymphoblastic leukaemia cell line SupT1 was engineered by a previous member of Professor Anderson's laboratory, to artificially express GD2 by transduction with GD2/GD3 synthase. The cell line SupT1-GD2^{+} was then produced by single cell sorting using a BD FACSAria III cell sorter.

Table 6: Cell lines

CELL LINE	DESCRIPTION	SUPPLIER
SUPT1	T cell lymphoblastic lymphoma cell line	ATCC
SUPT1-GD2	T cell lymphoblastic lymphoma cell line transduced to express GD2	Produced by the Anderson Laboratory
LAN1	Neuroblastoma cell line	ATCC
SK-N-DZ	Neuroblastoma cell line	ATCC
IMR-32	Neuroblastoma cell line	ATCC
MSPES-4	Ewing's sarcoma	Gift from Claudia Rossig (Department of Haematology and Oncology, University Children's Hospital Munster, Munster, Germany).
TC-71	Ewing's sarcoma	Gift from Claudia Rossig
SK-N-SH	Neuroblastoma cell line	ATCC
SK-MEL-28	HLA-A2 negative melanoma cell line	ATCC
NALM-6	B cell lymphoblastic leukaemia cell line	ATCC
HEK293T	Human embryonic kidney cell line	ATCC
AAPC	Derived from K562 human erythro-leukaemia cells and engineered to express 41BBL, CD86 and membrane bound IL15	Gift from Laurence Cooper (Division of Pediatrics, The University of Texas MD Anderson Cancer Center, Houston, Texas).

2.1.4 Primary neuroblastoma cells

Neuroblastoma neurosphere cell lines were established by Jack Barton (Research Technician in Professor Anderson's laboratory). Cell culture supernatant was harvested from confluent flasks and filtered (0.45μm filter, Millipore). Supernatant was stored at -80°C until use.

Table 7: Characteristics of neuroblastoma neurosphere cell lines

	AGE	DIAGNOSIS	BIOPSY	HISTOLOGY	GENETICS
PS1 16s4227	6 days	neuroblastoma	thorax	poorly differentiated	Inconclusive results for 17q imbalance
PS2 16s3383	1 year, 0 months	neuroblastoma	abdomen	undifferentiated	Mycn amplified, 1p/q imbalance, 17q imbalance
PS3 17s0139	9 months	neuroblastoma	neck	poorly differentiated	Low level 2p/q imbalance, inconclusive regarding 17q gain and 11q imbalance

2.1.5 Peripheral blood

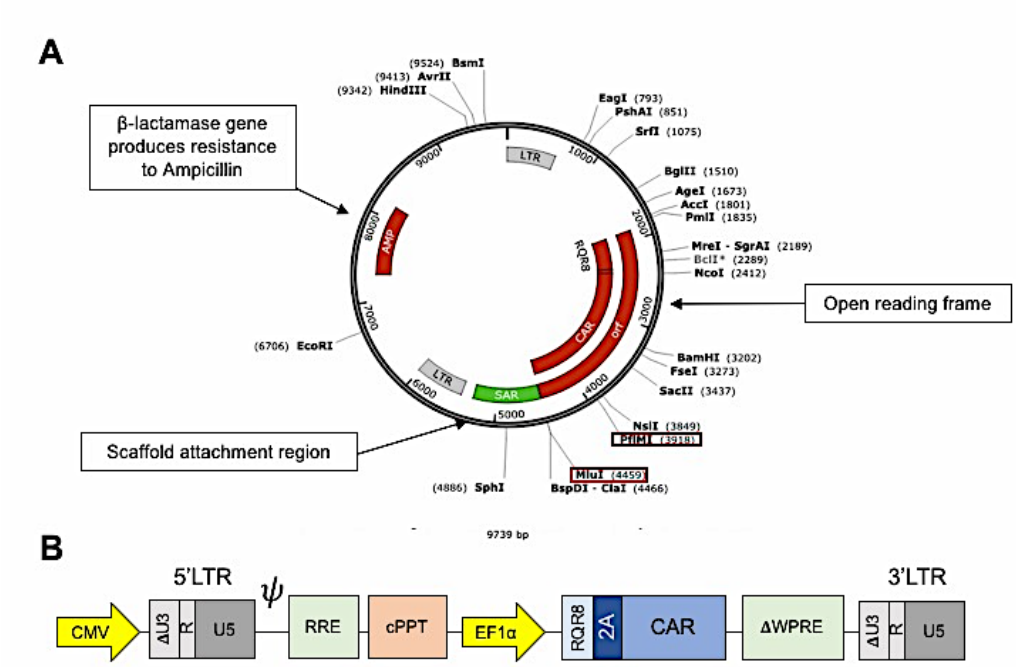
Fresh blood samples were obtained from healthy donors in accordance with protocols approved by the UK Integrated Research Ethics Review, after obtaining informed consent. The UCL Institute of Child Health local guidelines were also adhered to including the maintenance of an anonymised laboratory donor log.

2.2 Methods

2.2.1 Molecular biology techniques

2.2.1.1 GD2-CD28ζ CAR

The clinical-grade SFG retroviral vector SFGmR.RQR8-2A-aGD2huK666-HCH2CH3pvaa-CD28ζ (referred to as GD2-CAR hereafter) was developed by Dr Simon Thomas, Dr Karin Straathof, Prof John Anderson and Dr Martin Pule (UCL Cancer Institute) (unique identifier MP10413) (Figure 2.1). The vector includes two transgenes; a second generation CAR comprising the ScFv from murine anti-GD2 antibody (muK666) that has subsequently been codon optimized and humanized to form huK666. huK666 is fused to IgG Fc region acting as a spacer which is then fused to CD28 transmembrane domain. The CAR endodomains consist of CD28 costimulation and CD3ζ signalling domains. RQR8 suicide gene expressed on the T cell surface allows elimination of CAR T cells by anti-CD20 monoclonal antibody (rituximab) should toxicities occur (166). RQR8 also contains Qbend10 epitope of CD34 to allow detection of transduced CAR^+ cells by flow cytometry (CD34-APC, clone QBend10, R&D systems). A schematic of GD2-CAR viral components and structure is shown in (Figure 2.1).



CMV	CMV transcription promoter
5'LTR	5' Long terminal repeat. This consists of 3 regions; **U3** which functions as a promoter and contains transcriptional enhancers and TATA box, **R** where transcription starts, and **U5** which is involved in reverse transcription.
ψ	MMLV (Maloney murine leukaemia virus) packaging signal
RRE	Rev response element
cPPT	Central polypurine tract (increases transduction)
EF1α	Human elongation factor-1 alpha promoter
RQR8	Suicide/recognition gene
2A	Foot & Mouth 2A virus, cleaved during translation
CAR	Chimeric antigen receptor
ΔWPRE	Mutated woodchuck hepatitis virus post-transcriptional response element
3'LTR	3' Long terminal repeat

Figure 2.1 GD2-28ζ CAR

A, Oncoretroviral SFG vector pseudotyped with an RD114 envelope (MP10413). Highlighted features include long terminal repeats (x2) (LTR), open reading frame (ORF), scaffold attachment region (SAR), and ampicillin resistance (AMP). The restriction sites Pf1MI and MluI are highlighted in red, as corresponding restriction enzymes allow cut and paste of alternate CAR-signalling domains into the ORF. B, linear schematic representation and description of GD2-CAR.

2.2.1.2 GD2-41BBζ CAR

For endodomain comparison experiments it was necessary to clone a GD2-CAR with identical sequence to GD2-CD28ζ CAR but with replacement of CD28ζ with 41BBζ. This GD2-41BBζ plasmid was not available therefore we elected to synthesise it de-novo. The 41BBζ sequence was derived from a CD19-41BBζ CAR and obtained as a 'G-block' (IDT DNA) flanked with restriction sites Pf1MI and MluI. The MP10413 plasmid was 'cut' using the restriction enzymes Pf1MI and Mlu1 (highlighted in red in Figure 2.1), and the new 41BBζ sequence ligated in.

2.2.1.2.1 Digestion of SFG vector or insert using restriction endonucleases

SFG vector (MP10413) (9739 bp) and 41BBζ insert (580 bp) were digested with Pf1MI and MluI restriction endonucleases. 2μg DNA (vector or insert), 2μL Pf1MI, 2μL MluI, 2μL green FD buffer (Thermo Scientific), 2μL Alkaline Phosphatase (Thermo Scientific) (vector only) were made up to a total of 20μL with nuclease free water. This was then incubated for 90 minutes at 37°C. The reaction mixture was separated using 1% agarose gel electrophoresis (100V for 1 hour) stained with ethidium bromide. Bands were then visualised under a blue light transilluminator and carefully excised. DNA was extracted from the gel bands using the Qiaquick gel extraction kit (Qiagen) according to the manufacturer's protocol.

2.2.1.2.2 Ligation

The digested insert and vector were ligated at a 3:1, 5:1 and 7:1 molar ratio, calculated using the following formula:

$$\frac{(\text{kb of insert}) \times (\text{ng of vector})}{(\text{kb vector})} \times \frac{x}{1} = \text{ng of insert needed for a X:1 molar ratio}$$

For ligation, 10µL quick ligase buffer (NEB) and 1µL quick ligase was added to the correctly calculated amounts of vector and insert, and made up to a total reaction volume of 20µL with nuclease-free water. The mixture was incubated at room temperature for 5 minutes before being placed on ice.

2.2.1.2.3 Transformation of E. Coli with plasmid DNA

For the transformation of *E. Coli* with plasmid DNA, 2µL of the ligation mixture was carefully added to a 25µL aliquot of thawed highly efficient NEB5α competent *E. coli* (New England Biolabs). The mixture was incubated on ice for 30 minutes before heat shocking at 42°C for 35 seconds, followed by transfer back to ice for a further 5 minutes.

250µL of SOC media was added and samples were vigorously shaken for 40 minutes at 37°C. Transformed bacteria were then spread onto pre-prepared ampicillin containing (100 µL/ml) agar plates and cultured at 37 °C for 16 hours.

2.2.1.2.4 Mini/Midi plasmid preparation

Five clones from each plate (3:1, 5:1, 7:1) were chosen for mini-culture. The 15 samples were cultured in 3mL LB broth containing ampicillin at 37 °C for 16 hours with vigorous shaking. DNA was prepared using the QIAprep Spin miniprep kit (QIAGEN) according to the manufacturer's protocol. Following digestion with restriction enzymes and running on an agarose gel, correct clones were identified and sent for sequencing. Sequencing results were checked using SnapGene software to ensure the correct sequence alignment.

One correct clone was finally selected to undergo midi-prep. 100µL bacterial culture preparation was added to 100mL ampicillin containing LB broth and cultured at 37°C for 16 hours with vigorous shaking. DNA was prepared using the EndoFree Midi Prep Kit (QIAGEN) according to the manufacturer's instructions. Using a

spectrophotometer, the DNA was quantified and purity checked by achieving an OD 260/280 of between 1.8-2.0.

2.2.2 Cell culture techniques

2.2.2.1 Retroviral production

2.2.2.1.1 Stable transfection

Fresh GD2-CAR retroviral supernatants were collected from stably transfected human embryonic kidney HEK293-based packaging cell line (293Vec-RD114). The 293Vec-RD114 cell line was developed using zeocin and puromycin resistance genes to stably express Moloney murine leukaemia (MLV) gag-pol and RD114 envelope (env) viral proteins and was produced by BioVec Pharma (Canada). Infected 293Vec-RD114 cells were incubated for 48 hours in culture medium (90% DMEM, 10% FCS, 4 mM L-glutamine) at 37 °C and 5% CO2. Following incubation supernatants containing virus particles were snap frozen and stored at -80°C.

2.2.2.1.2 Transient transfection

GD2-28ζ CAR, GD2-41BBζ CAR and MART1 αβ TCR viral supernatant was produced by transient co-transfection of human embryonic kidney derived 293T cells with RD114 envelope protein, gag-pol, and plasmid encoding CAR or TCR. The MART1-TCR plasmid was a kind gift of Dr C. Cohen (National Cancer Institute, USA). $1.5x10^6$ 293T cells were cultured in IMDM supplemented with 10% FCS and 5 mM L-glutamine in 100mm tissue culture plates (Nunclon Delta Surface, Thermo Fisher) until 60% confluency was achieved (approximately 24 hours). For transfection, the reagent GeneJuice (Novagen, Massachusetts, USA) (30μL) was incubated with plain medium (470μL) for 5 minutes, then DNA (12.5μL; 4.75μg RD114 envelope, 4.75μg gag/pol and 3.75μg vector) was added with a further incubation step of 15 minutes. The DNA/GeneJuice solution was then added dropwise to the tissue culture plate with careful agitation to allow uniform distribution. Viral

supernatant was harvested at 48 hours and 72 hours after transfection, then pooled, snap frozen, and stored at -80°C until use.

2.2.2.2 PBMC isolation

Blood samples were collected from healthy laboratory volunteers after obtaining informed consent and in adherence with the Institute of Child Health guidance on the use of blood donations from healthy volunteers for research. Peripheral blood mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (Stemcell Technologies, Cambridge) at 750g for 30 minutes. Following centrifugation, mononuclear cells were carefully aspirated and washed twice in RPMI before cell counting. PBMC were then resuspended at a concentration of $1x10^6$/ml in R10 and plated into a 24-well tissue culture plate

2.2.2.3 Activation of T cells

2.2.2.3.1 Zoledronate (ZOL)

PBMC were resuspended in R10 media supplemented with 100 units/mL IL-2 (Proleukin, Switzerland) and 1 μg/mL zoledronate (Zerlinda 4mg/100 mL, Actavis). Fresh R10 media containing IL-2 (100 U/ml) was replenished every 2-3 days.

2.2.2.3.2 Concanavalin A (ConA)

PBMC were resuspended in R10 media supplemented with 100 units/mL IL-2, 10 ng/mL recombinant IL4 (Cellgenix), and 1 μg/mL Concanavalin A (Sigma-Aldrich). Fresh R10 media containing IL-2 (100 units/ml) and IL-4 (10ng/ml) was replenished every 2-3 days. This expansion protocol was established by Siegers and colleagues (167)

2.2.2.3.3 Artificial Antigen Presenting Cells

Artificial antigen presenting cells (aAPC) were originally developed by Carl June (University of Pennsylvania, US) (165) by engineering human erythro-leukaemia cells (K562) with lentivirus to stably express and secrete costimulatory molecules and cytokines. The aAPC express 41BBL, CD86, CD64 and membrane-bound IL15 (experimental schema is shown in Figure 2.2)

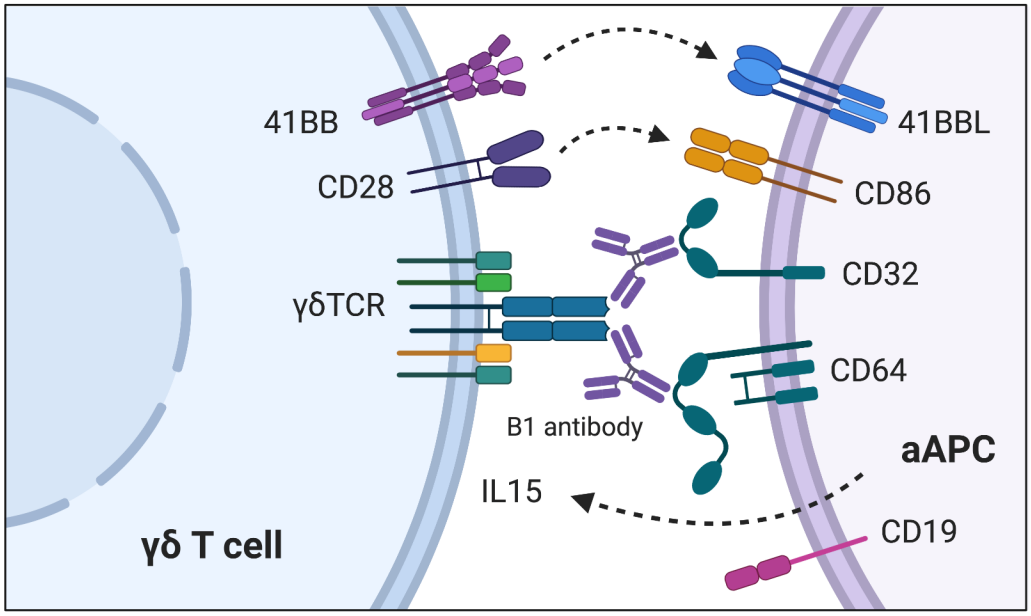


Figure 2.2 Schematic of aAPC- γδ T cell interaction

aAPC have been established from K562 cells and genetically modified to express CD19, 41BBL, CD86, CD32, CD64 and membrane bound IL15. γδ T cells are activated by anti-γδTCR antibody (clone B1) bound to CD32 and CD64 on aAPC. γδ T cell costimulation is provided though, CD28-CD86 and 41BB-41BBL interaction. *Created using BioRender software.*

Although aAPC were initially developed for propagation of αβ T cells, Dr Laurence Cooper (Division of Pediatrics, The University of Texas MD Anderson Cancer Center, Houston, Texas) in collaboration with our own laboratory pioneered a technique for expanding polyclonal γδ cells (40, 164). In brief, following isolation using MACS positive selection with the anti-TCRγδ MicroBead Kit (130-050-701, Miltenyi), co-

cultures of polyclonal γδ T cells were set up with irradiated aAPC (80Gy) coated in anti-γδTCR antibody (LEAF purified B1 antibody, Biolegend) at a ratio of 1:2. Selected γδ T cells were cultured in R10 supplemented with IL-2 (100 units/ml) and IL-21 (60ng/μL) which was replenished every 2-3 days. As reported by Fisher *et al.,* the addition of LEAF purified B1 antibody was found to significantly improve γδ T cell expansion compared to the absence of antibody opsonization (however statistical significance was only achieved after excluding non-responders, defined as a less than three-fold increase over 7 days) (40).

2.2.2.3.4 CD3/CD28 Antibody

αβ T cells were activated and expanded using 1 mg/mL functional grade soluble anti-CD3 antibody (clone OKT3, Miltenyi) and anti-CD28 antibody (clone 15E8, Miltenyi). Fresh R10 media containing IL-2 (100 U/ml) was replenished every 2-3 days. This method of αβ T cell activation was consistent with the Cancer Research UK Phase I trial of anti-GD2 CAR transduced T cells (1RG-CART) in patients with relapsed or refractory neuroblastoma at Great Ormond Street Hospital.

2.2.2.4 Transduction of T cells

Five days following activation with ZOL or ConA, PBMC were transduced in 24 well non-tissue culture plates (FalconTM) with recombinant human fibronectin fragment (RetroNectin®, Takara, Japan) at 5μg/cm2. To each well, 1.5ml of freshly thawed viral supernatant (1:40 dilution) was added to 500μL of viable cells at a concentration of $1x10^6$/ml containing 400 U/ml IL-2. Plates were centrifuged at 1000g for 40 minutes and subsequently incubated at 37°C for 48 hours. Following incubation, cells were transferred to tissue culture-treated plates for the remaining culture period and fresh media containing 100 U/ml IL-2 was replenished every 2-3 days.

For αβ T cell control experiments, CD3/CD28 antibody stimulated cells were transduced 48 hours following activation. The same protocol as for ZOL/ConA was

followed except 500µL of viable cells was added to 1.5ml viral supernatant at a concentration of $6x10^5$/ml.

2.2.3 Cell purification techniques

2.2.3.1 FACS

Thirteen days following initial activation, transduced T cells were sorted into pure populations of CAR^+ αβ and CAR^+ Vδ2 by fluorescence activated cell sorting (FACS) using BD FACSAria flow cytometer. ZOL-activated cells were stained with anti-Vδ2 antibody (Biolegend, clone B6) and QBend10 antibody (Sigma-Aldrich). $Vδ2^+$/ $Qbend10^+$ cells were obtained for further analysis. Transduced CD3/CD28 antibody activated cells were stained with anti-CD56-PE (Biolegend, clone-188) and Qbend10-APC (Sigma-Aldrich, clone 4H11) antibodies. $CD56^-$/ $Qbend10^+$ cells were obtained for further analysis.

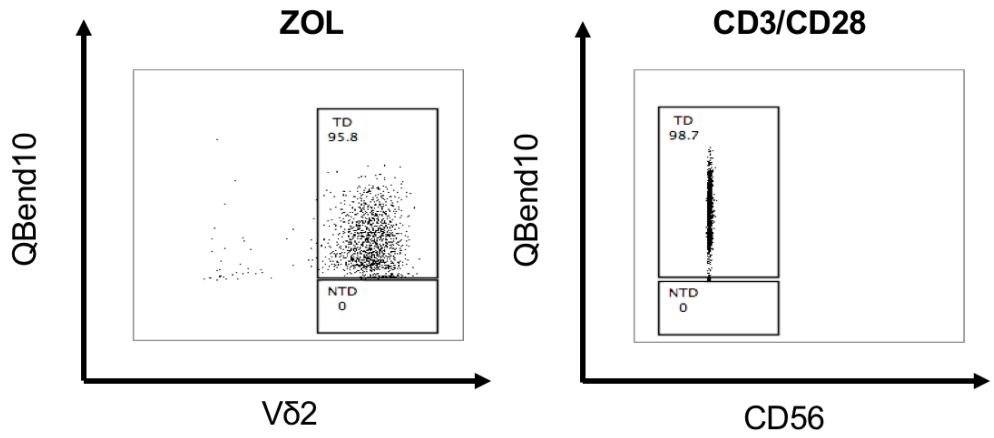


Figure 2.3 Flow cytometry dot plot showing purity check of CAR^+ ($QBend10^+$) Vδ2 and αβ T cells following FACS

2.2.3.2 MACS

Non-transduced control CD3/CD28 antibody activated αβ T cells were depleted of $CD56^+$ cells by CD56 Microbeads according to the manufacturer's instructions (Miltenyi, 130-050-401). Non-transduced ZOL-activated γδ T cells were isolated using the $TCR\gamma/\delta^+$ T Cell Isolation Kit (Miltenyi, 130-092-892).

2.2.4 Labelling with CellTrace™

Following transduction, αβ T cells were cultured for 9 days with 100iU IL-2 before labelling with CellTrace™ (CellTrace™ Violet Cell Proliferation Kit, Invitrogen) according to the manufacturer protocol.

2.2.5 Genetic modification of tumour cell lines

2.2.5.1 Sk-Mel-28-GD2

SK-MEL-28 $GD2^+$ cells were manufactured by transducing SK-MEL-28 melanoma cells (ATCC® HTB-72™, antigen expression HLA A11, A26, B40, DRw4) with a SFG vector (MP9956, SFG.GD3synthase-2A-GD2synthase) to express GD2. Flow cytometric analysis using GD2-PE (Biolegend, clone 14G2a), confirmed virtually all cells stably expressed GD2.

2.2.5.2 NALM-6-GD2-fLuc

GD2 expressing NALM-6 clones (clone #7 and #11) were produced by Rebecca Wallace. The two selected clones were then transduced with VSV-G pseudotyped lentiviral vector particles at a titre of 1.06×10^6 /ml (168, 169). Titres were calculated considering only wells containing 5-30% transduced cells (170). Vector LeGO-Luc2-iV2-$Puro^+$ encoding luciferase and yellow-green fluorescent protein Venus (169, 171) was developed and gifted by Kristoffer Riecken, Laboratory of Prof. Boris Fehse, Research Dept. Cell and Gene Therapy, Department of Stem Cell Transplantation,

UMC Hamburg-Eppendorf, Germany. These cells were then single cell sorted (selecting cells that were double positive for GD2-PE (Biolegend, clone 14G2a), and Venus/YFP, [excitation 515nm, emission 528nm]) using a BD FACS Aria and cultured to confluency. A single clone was then selected for *in vitro* and *in vivo* experiments.

2.2.6 In vitro assays

2.2.6.1 ^{51}Chromium release cytotoxicity assay

In vitro cytotoxicity was assessed using standard 4 hour ^{51}Chromium release assay. Expanded transduced (TD) and non-transduced (NTD) γδ and αβ T cells were used as effector cells and cancer cell lines (LAN1, SK-N-SH, SupT1-GD2, SupT1-ALK) as targets. Targets were labelled with 3.7 MBq $Na_2{}^{51}CrO_4$ (Perkin, Elmer) for 1 hour, with agitation every 15 minutes. Following incubation, targets were washed five times then $5x10^4$ tumour cells added to effector cells at a range of E:T ratios (10:1, 5:1, 2.5:1, 1.25:1) in a Nunc™ 96 well conical bottom plate (Thermo Fisher Scientific) in triplicate. For antibody-dependent cellular cytotoxicity assays human IgG1 anti-GD2 antibody (Ch14:18) was used to opsonise target cells at a concentration of 1μg per $1x10^6$ target cells.

Co-cultures were incubated for 4 hours at 37ºC, following which, 50μL supernatant was harvested and transferred to a high binding isoplate-96 HB (PerkinElmer). 150μL of scintillation solvent (Optiphase Supermix, PerkinElmer) was added to each well and incubated at room temperature overnight. ^{51}Cr release was measured using a 1450 MicroBeta TriLux (PerkinElmer). Target cells in media alone (spontaneous release) and target cells added to 1% TritonX-100 (Sigma Aldrich) (maximum release) allowed for calculation of specific lysis using the following equation:

$$\%\ \text{specific lysis} = \frac{(\text{experimental lysis} - \text{spontaneous lysis})}{(\text{maximum lysis} - \text{spontaneous lysis})} \times 100$$

2.2.6.1.1 γδTCR and NKG2D blocking experiments

Transduced and non-transduced Vδ2 cells were co-incubated for 2 hours with 5μg/ml LEAF-purified anti-γδTCR (Biolegend, clone B1), LEAF-purified anti-NKG2D (Biolegend, clone 1D11) or isotype-matched LEAF purified mouse IgG1κ monoclonal antibody. The blocking properties of anti-γδTCR clone B1 have been described by Correia *et al*. (172).

2.2.6.2 Cytokine production

2.2.6.2.1 Enzyme linked immunosorbent assay

Effector cells ($5x10^5$) were co-cultured with various target cells at a ratio of 1:1 in the presence and absence of IL-2. After 48 hours, supernatant was harvested and analysed for human IFNγ by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (BioLegend, Human IFNγ ELISA MAX™). Target cells were irradiated before use (60 Gy).

2.2.6.2.2 Cytokine bead array

Simultaneous quantification of multiple cytokine concentrations (including IL-2, IL-4, IL-10, IL-17α, TNFα, and IFNγ) in cell culture supernatant was carried out using BD Cytometric Bead Array (BD CBA Human Th1/Th2/Th17 kit, Becton Dickinson Bioscience). $5x10^5$ effector cells were co-cultured with $5x10^6$ irradiated (60 Gy) targets and supernatant harvested after 48 hours. Supernatant was snap frozen and stored at -80ºC until further use. The assay was performed according to the manufacturer's protocol in duplicate and data was analysed using FCAP Array analysis software (Softflow, Inc.).

2.2.6.2.3 ELISpot

Flow sorted CAR^+ γδ T cells were pulsed with S-MART1 or L-MART1 peptide (50μL/ml) for 4 hours at 37°C, then washed twice. $5x10^4$ transduced (MART1 TCR^+) or non-transduced (NT) αβ T cells were plated in triplicate and stimulated overnight with $1x10^4$ peptide loaded CAR^+ γδ T cells on a 96-well ELISpot plate pre-coated with anti-human IFNγ monoclonal antibody (Mabtech 3420-3, Hamburg, Germany). Following overnight incubation, plates were washed then incubated with biotinylated anti-IFNγ monoclonal antibody (1:1000 dilution) (Mabtech 3420-6) for 2 hours. After washing, streptavidin-ALP conjugate (1:1000) (Mabtech 3310-10) was added for 1 hour, then spots developed using AP colour development buffer (Bio-Rad). Plates were left to dry overnight prior to spots being counted on an automated reader (AID ELISpot reader).

2.2.6.3 Proliferation assay

T cells and tumour cells were cultured in 24 well tissue-culture treated plates at an effector: target (E:T) ratio of 1:1. Tumour targets were irradiated (80Gy) prior to co-culture and included SupT1, SupT1-GD2, and LAN1 cell lines. T cells were counted at 48 hours, 72 hours or 7 days using light microscopy with trypan blue exclusion. Where bulk T cell populations were used, T cell subtype proportion was determined by flow cytometry. Where stated, effector cells were pre-labelled with CFSE (CFSE Cell Division Assay Kit, Cayman Chemical) prior to co-culture.

2.2.6.4 Migration assay

Migration assays using a transwell® system were used to determine whether non-transduced and transduced Vδ1, Vδ2 and αβ T cells had improved migration in response to stimulation by tumour cells, tumour cell supernatant or selected chemokines. 10^5 tumour cells (LAN1 and SK-N-SH) in 600μL RPMI/10% FCS media were added to the bottom chamber of a 24-well 6.5mm diameter, 5mm pore transwell chamber (Costar Transwell, Corning, NY) and incubated at 37˚C for 18

hours. The following day 600µL media containing 100ng/ml SDF1 (CXCL12), IP-10 (CXCL10) (R&D Systems, 350-NS-010 / 266-IP-010), or 600µL tumour cell line supernatants or neuroblastoma primary cell supernatants were added to the remaining bottom chambers. $5x10^5$ T cells in 100µL media were then placed into the upper chamber of the transwell plate. For a positive control $5x10^5$ T cells were added directly to the bottom chamber, and for a negative control, media only into the bottom chamber. Plates were then incubated at 37˚C for 4 hours, followed by 10 minutes at 4ºC. Following incubation, all cells in the bottom chamber were harvested and stained with live-dead aqua (LIVE/DEAD® fixable aqua dead cell stain kit, L34957), CD3 (PerCP-Cy5.5), pan αβTCR (BV421), Vδ1 (FITC), Vδ2 (PE) and QBend10 (APC). $1x10^4$ flow cytometric counting beads (Precision Count Beads™, Biolegend, 424902) were added to each sample. Samples were acquired on BD LSRII.

Percentage migration was calculated using the following equation:

$$\%\, migration = \frac{experimental\ control - negative\ control}{positive\ control} \times 100$$

(Where the positive control; $5x10^5$ T cells plated into the bottom well at the start of the assay, and negative control; media only into the bottom well)

2.2.6.5 Western blot

2.2.6.5.1 Preparation and quantification of protein

LAN1, SK-N-DZ, IMR-32 (all neuroblastoma), SK-MEL-28 (melanoma) and SupT1 (lymphoblastic lymphoma) cells were lysed in 200µL lysis buffer (Table 5). 4µL of protease inhibiter (50x) (BD BaculoGold) was added and lysates cleared by centrifugation (18,000g for 5 minutes at 4°C). Supernatant protein quantification was determined by Bradford Assay according to the manufacturers protocol using a spectrophotometer set to 595nm (BioRad Protein Assay Dye Reagent concentrate, Quick Start Bovine Serum Albumin Standard Set). Samples were mixed with 4x laemmli loading buffer (Bio-Rad) (containing 10% β-mercaptoethanol) and denatured

at 95.5°C for 5 minutes before being placed on ice. Cell lysates were stored at -80°C until use.

2.2.6.5.2 Protein separation

Lysate was loaded onto 4-12% Bis-Tris precast gels (NuPAGE, Invitrogen) and separated by SDS-PAGE using Mini Protean III apparatus (BioRad). Gels were electrophoresed in running buffer (Table 5) at 160V for 1 hour.

2.2.6.5.3 Protein transfer

Proteins were then transferred on to a 0.45 μm nitrocellulose membrane (Amersham, Sigma-Aldrich) at 360mA for 1 hour using a BioRad Mini Trans-Blot Electrophoretic Transfer System.

2.2.6.5.4 Western blot

Membranes were washed (TBS-T; 2 x 10 minutes) then blocked for 1 hour at room temperature in blocking buffer (Table 5). Following two further washes (TBS-T; 2 x 10 minutes), blots were incubated overnight at 4°C with human MART1 primary antibody (R&D systems, AF8008) in antibody staining buffer (Table 5). Three further washes (TBS-T; 3 x 10 minutes) were carried out before incubation with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. Blots were again washed (TBS-T; 4 x 10 minutes), then chemiluminescent autoradiography performed to detect the protein bands using Amersham ECL western blotting detection reagent (GE Healthcare)

2.2.7 Flow cytometry

Flow cytometry was performed on a BD LSR II flow cytometer (BD Bioscience) in the UCL Great Ormond Street Institute of Child Health Flow Cytometry Core Facility. Flow cytometry FCS files were imported and analysed using FlowJo v10.1 software

(Tree Star, Inc, Ashland, OR). Compensation was carried out using single-colour controls of either cells or beads (OneComp eBeads, eBioscience). Appropriate isotype controls, fluorescence minus one (FMO), or non-transduced cells were used to validate gating. $1x10^5$ – $1x10^6$ cells were stained with antibodies as detailed in Table 8. Samples were stained for 15 minutes at 4°C and washed twice with FACS buffer. Cells were then resuspended in 100μL fixation buffer (Biolegend) for 20 minutes at room temperature and then washed again before analysis.

General gating strategy involved gating on: 1) FSC-A vs. SSC-A for a lymphocyte gate, 2) FSC-A vs. FSC-W to exclude doublets, 3) FSC-A vs. live/dead to gate on live cells, 4) for lymphocyte subsets; $CD3^+/V\delta1^+$ for Vδ1 cells, $CD3^+/V\delta2^+$ for Vδ2 cells and $CD3^+/\alpha\beta TCR^+$ for αβ T cells. Transduced cells were defined by $QBend10^+$, with a negative gate set by staining non-transduced cells.

Table 8: Antibodies and viability dyes

Specificity	Clone	Isotype	Manufacturer	Fluorochrome
CD3	UCHT1	Mouse IgG2a	Biolegend	PerCP-Cy5.5
Vδ1	REA173	Mouse IgG1	Miltenyi	APC-Vio770
Vδ2	B6	Mouse IgG1	Biolegend	PE
TCRαβ	IP26	Mouse IgG1	Biolegend	BV421
TCRαβ	IP26	Mouse IgG1	Biolegend	PE-Cy7
CD34 QBend10	4H11	Mouse IgG1	Sigma-Aldrich	APC
CD45RA	H100	Mouse IgG2b	Biolegend	PE-Cy7
CD27	O323	Mouse IgG1	Biolegend	BV711
PD1	EH12.2H7	Mouse IgG1	Biolegend	FITC
Tim3	F38-2E2	Mouse IgG1	Biolegend	BV605
HLA-DR	L243	Mouse IgG2a	Biolegend	PE-Cy7
CD86	IT2.2	Mouse IgG2b	Biolegend	BV711
Vβ12	S511	Mouse IgG2b	Abcam	FITC
Fixable viability dye			Life technologies	Aqua
Fixable viability dye			eBioscience	APCeFluor 780

2.3 Statistics

Data was analysed using GraphPad Prism (Version 6.0d). A two-way ANOVA was used for multiple comparisons involving three or more independent variables. A two-

tailed Student's T test was used for comparison of variance between two normally distributed groups. P value of <0.05 was taken to indicate significance (*P < 0.05, **P < 0.005, ***P < 0.001). Mean ± SEM are shown from replicate experiments.

Chapter 3 Manufacture and characterisation of CAR$^+$γδ T cells

3.1 Introduction

As outlined in Chapter 1, there are many published protocols for the activation and transduction of CAR αβ T cells with impressive results demonstrated in clinical trials for paediatric haematological malignancies directed against CD19. CAR αβ T cells are well described in terms of their proliferative capacity, phenotype and function, *in vitro*, *in vivo* and in clinical studies. At the time of commencing this study, there were several reports of non-gene modified γδ T cells for cancer immunotherapy (refer to Table 4) [albeit for adult malignancies, except (132)], however the introduction of an engineered receptor into γδ T cells to direct tumour antigen specificity was a relatively novel concept (163, 164).

As γδ T cells represent a small proportion of the total CD3$^+$ T cell population in peripheral blood, they must be expanded to sufficient number for adoptive T cell transfer. This critical number is yet to be determined given that in CAR αβ T cell trials, it is the proliferative capacity of cells following infusion and on antigen encounter that influences long-lasting efficacy rather than total number of initial CAR T cells infused into the patient. The proliferative capacity of infused cells is dependent on multiple factors including; prior chemotherapy conditioning, antigen density/number of blasts/bulk of disease, and the CAR construct used (including characteristics of the single chain variable fragment, spacer domain, and costimulatory domain). The composition of effector cells (CD4:CD8 ratio) is also important together with their memory phenotype. All of these factors must be carefully balanced with dose-limiting toxicities such as CRS and off-tumour on-target effects.

Clinical trials of CD19 CAR T cells have generally used minimum does of $1x10^6/m^2$ for adoptive transfer, however for solid tumours, this number may need to be

significantly higher depending on the burden of disease [as demonstrated in the tumour infiltrating lymphocyte immunotherapy field, reviewed in (173)].

A number of γδ T cell activation methods have been used by different research groups including artificial antigen presenting cells (aAPC) (40, 164), aminobisphosphonate drugs (57, 163) and concanavalin A (167). Using these different methods we wanted to investigate the most optimal system for γδ T cell expansion for clinical use, and whether activated cells could be transduced with a 2nd generation GD2-CAR.

For αβ T cells, there has been success propagating CAR αβ T cells using GMP-grade aAPC (174), reviewed in (175). Although DCs are the endogenous activators of T cell responses, their potency can vary remarkably and expanding T cells using DCs is a complicated and labour intensive process, requiring numerous cytokines. As a result, more reliable and reproducible methods have been investigated using engineered aAPC. aAPC have been derived from K562 cells (human erythroleukaemic cell line derived from a patient with chronic myelogenous leukaemia), as pioneered by the June laboratory. K562 do not express HLA class I or II molecules and therefore induction of allospecific T cells is avoided. They can also be easily gene modified with stable gene expression. K562 do not express other costimulatory molecules except for CD80 at low level but importantly, they express ICAM-1 and LFT-3 (adhesion molecules) required for immunological synapse formation. K562 aAPC have been extensively studied and can be genetically modified to express other molecules to drive T cell expansion (176, 177). Dr Cooper's group at MD Anderson and the June Laboratory have developed a GMP-grade K562 clone expressing CD64, CD86, 41BBL, CD19 and membrane bound IL-15. Following irradiation these cells have been used to activate and propagate CD19 CAR T cells suitable for adoptive transfer in clinical trials (178). The additional expression of 41BB resulted in cytotoxic T cells with enhanced cytolytic capacity compared to CD28 (179).

A former PhD student in our laboratory, Dr Jonathan Fisher in collaboration with Dr Lawrence Cooper (MD Anderson Cancer Center, The University of Texas), discovered that these same aAPC could be used to expand a full repertoire of γδ T cells

inclusive of Vδ1, Vδ2 and Vδ1$^-$/Vδ2$^-$ cells to large number (more than x1000 fold change) (40, 165). Limitations of this method however should be recognised as it requires; (1) MACS selection on day 1, (2) prolonged culture (28 days), and (3) the addition of irradiated aAPC coated with anti-γδTCR antibody (clone B1) every 7 days (40). Compared to a bead, antibody-based or drug-based approached, manufacturing γδ T cells via the aAPC method incurs greater expense, a prolonged manufacture time (that may simultaneously lead to disease progression in the patient), and increases the potential for contamination (due to the addition of aAPC into sterile culture bags every 7 days, and longer overall culture period).

Simpler, quicker, and more cost efficient γδ T cell expansion methods have been reported in the literature, including using zoledronate (zoledronic acid) to selectively expand Vγ9Vδ2 cells, and Concanavalin A for a polyclonal repertoire, inclusive of Vδ1 subsets (167). These alternative methods were additionally selected for further characterisation and validation as a suitable method of γδ T cell activation.

My second objective was to determine the most favourable method for gamma retroviral transduction with GD2-CAR. On commencing this study, to the best of my knowledge, there were only two published studies investigating γδ T cells engineered with CARs. Rischer *et al*. (2004) (163) used zoledronate to activate γδ T cells followed by retroviral transduction, whereas Deniger *et al*. (2013) (164) used aAPC and non-viral gene transfer using the sleeping beauty system to generate polyclonal γδ T cells with a CD19 specific CAR (further described in Section 1.10.2).

The initial stimulation and time-point for retroviral integration are fundamental to successful gene transfer and CAR expression. As well as the culture methods employed, effectiveness of CARs are also highly dependent on their structure. The optimal choice of costimulatory endodomain for γδ T cells is unknown, and even though CD28 containing CAR are efficacious in αβ T cells (180), this may not be the case for γδ T cells. Lastly, I set out to compare two second generation CAR; GD2-28ζ and GD2-41BBζ, before selecting the most favourable construct for detailed

analysis and efficacy studies (Chapter 4). 41BB in particular was chosen due to its efficacy in 41BB containing CAR αβ T cell trials (181) and based on the available evidence for its role in the activation, expansion and effector functions in γδ T cells (182).

3.1.1 Aims

- To investigate γδ T cell activation methods with expansion to clinically significant number for adoptive transfer in paediatric cancer patients.
- To optimise CAR transduction methods specific to γδ T cells.
- To compare GD2-28ζ and GD2-41BBζ containing CAR constructs in γδ T cells

3.2 Results

3.2.1 Comparison of γδ T cell activation methods

Firstly, it was important to determine the baseline characteristics of healthy donors by evaluating the composition of γδ T cells in peripheral blood. There were significant variations in the total number of γδ T cells and the Vδ1:Vδ2 proportions in the individuals tested. In seven donors, γδ T cell proportion ranged from 0.2 - 2.3% for Vδ1 and 0.8 - 7.0% for Vδ2 (data not shown). A representative flow cytometry dot plot showing the gating strategy and graphical representation is shown Figure 3.1 and Figure 3.2, respectively. Three donors are represented in Figure 3.2 and in 2 out of 3 donors the large majority of γδ T cells are Vδ2. Vδ2 are known to predominate in healthy Caucasians, however certain donors have predominantly Vδ1 γδ T cells as demonstrated in donor 1. Individuals from West Africa have a higher proportion of circulating Vδ1 cells and this is almost certainly due to the exposure to different pathogens (183). For example, cytomegalovirus is linked to the expansion of the Vδ1 cell compartment (184). Flow cytometry gating and classification of γδ T cell subsets or αβ T cells was performed by gating on live/$CD3^+$ cells, then $V\delta1^+$, $V\delta2^+$ or pan-

αβTCR$^+$. Previous experiments I carried out using a pan-γδTCR antibody (clone B1) revealed there was epitope competition between pan-γδTCR$^+$ and Vδ2$^+$ antibodies, therefore requiring an alternative staining approach. In the absence of commercially available Vδ3 antibodies at the time of study, DN/or Vδ1$^-$/Vδ2$^-$ populations were defined by gating on CD3$^+$/αβTCR$^-$/Vδ1$^-$/Vδ2$^-$ cells.

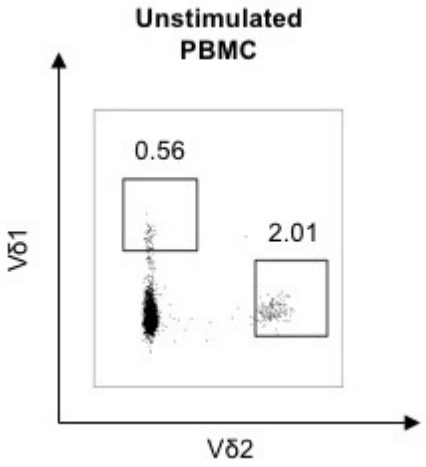


Figure 3.1 Representative FACS plot of Vδ1$^+$ and Vδ2$^+$ γδ T cell proportion in the peripheral blood of a single healthy donor

Cells are gated on live/CD3+ and numbers represent the percentage of Vδ1$^+$ and Vδ2$^+$ present in each selected gate (2.01% Vδ2$^+$ and 0.56% Vδ1$^+$).

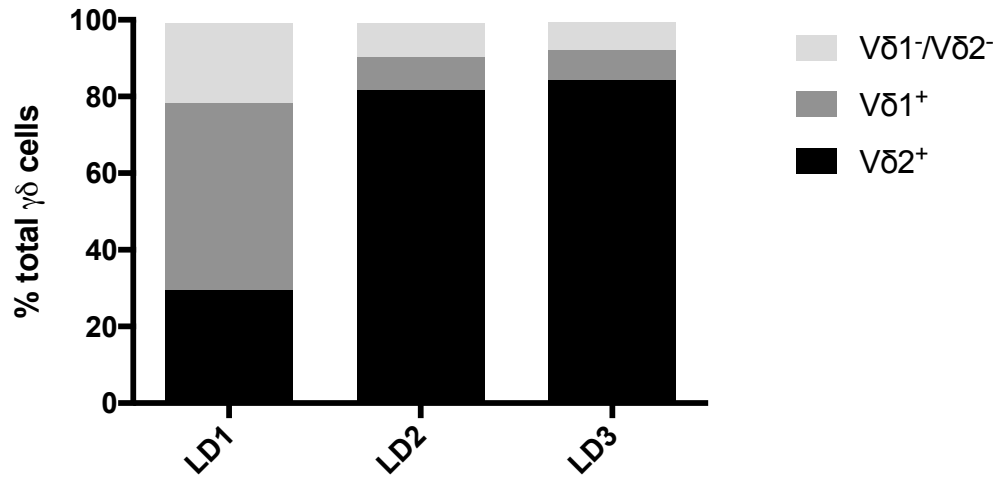


Figure 3.2 Proportion of resting Vδ1$^+$, Vδ2$^+$ and Vδ1$^-$/Vδ2$^-$ subsets in the PBMC of three healthy donors.

PBMC from 3 healthy donors (LD1, LD2, LD3) were stained with CD3, pan-αβ TCR, Vδ1 and Vδ2 antibodies to determine individual subset proportion. Vδ1$^+$ were classified as CD3$^+$/αβ$^-$/Vδ1$^+$, Vδ2$^+$; CD3$^+$/αβ$^-$/Vδ2$^+$, and Vδ1$^-$/Vδ2$^-$; CD3$^+$/αβ$^-$/Vδ1$^-$/Vδ2$^-$. Two donors have predominantly Vδ2$^+$, however LD1 shows a higher proportion of both Vδ1$^+$ and Vδ1$^-$/Vδ2$^-$.

In our laboratory there is a well-established protocol for αβ T cell activation and transduction with a gamma-retrovirus encoding a second generation CAR that is currently being used in the 1RG-CART trial for relapsed/refractory neuroblastoma patients at Great Ormond Street Hospital (ClinicalTrials.gov NCT20261915). This was used as a 'gold-standard' protocol for comparison of γδ T cell expansion and adequate transduction. Two additional methods of γδ T cell activation were chosen following a literature review, using zoledronate (ZOL) or concanavalin A (ConA) in addition to cytokine support, to evaluate potential expansion methods to enable successful retroviral transduction with CAR and production of sufficient cell numbers for adoptive transfer.

As illustrated in Figure 3.3, all three expansion methods (anti-CD3/CD28 antibody, ZOL, and ConA) led to γδ T cell expansion to varying degrees, with highest total cell numbers achieved using ConA. ConA and ZOL led to the preferential expansion of the Vδ2 γδ T cell subset (Figure 3.3). Although the total γδ T cell number was higher for ConA cultures (as demonstrated by the higher fold change), bulk cultures were less

pure with a high proportion of αβ T cells. ConA activation was the only method that gave a mean fold expansion greater than 100 for both Vδ1 and Vδ2 subsets therefore was chosen as a potential activation reagent. Certain donor γδ T cells, however, were non-expanders with ConA.

Figure 3.4 shows representative FACS plots for each activation method. After 13 days, cultured cells have been gated on live/$CD3^+$cells, and plots show the percentage of $V\delta1^+$ and $V\delta2^+$ cells. As can be seen, the proportion of $V\delta1^+$ cells of total $CD3^+$ cells in culture, is relatively small for all culture methods tested.

The reproducibility and purity of a T cell product for patient use is an important factor, firstly for evaluating pre-clinical function, and secondly for predicating its *in vivo* efficacy and safety. ZOL consistently led to the preferential expansion of Vδ2 cells with >80% purity achieved by day 13 of culture (mean 86.48% ± 5.098) (Figure 3.5). Additionally, ZOL has been safely used in adoptive Vδ2 T cell trials previously, whereas ConA is unavailable in GMP grade and is not suitable for patient use. Consequently, I chose ZOL as the preferred reliable activation method for CAR transduction optimisation experiments.

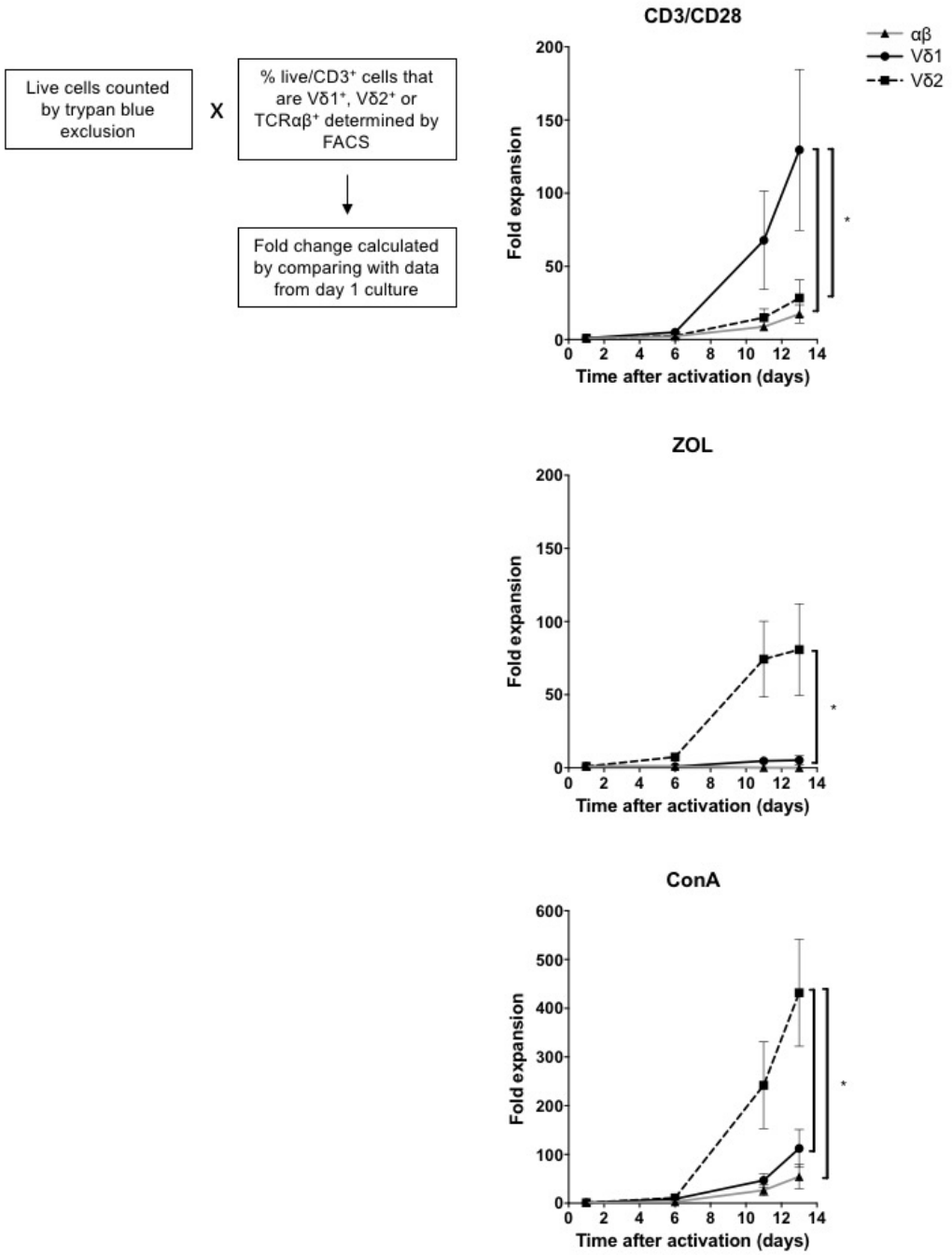


Figure 3.3 αβ, Vδ1, and Vδ2 T cells are successfully expanded from healthy donor PBMC using 3 different activation methods

T cells were activated with either CD3/CD28 antibody, zoledronate (ZOL) or concanavalin A (ConA). Fold expansion of αβ, Vδ1, and Vδ2 T cells was calculated by counting the total number of live cells by trypan blue exclusion and determining the T cell subset proportion by flow cytometry. Data represented as mean ± SEM; 6 individual donors.

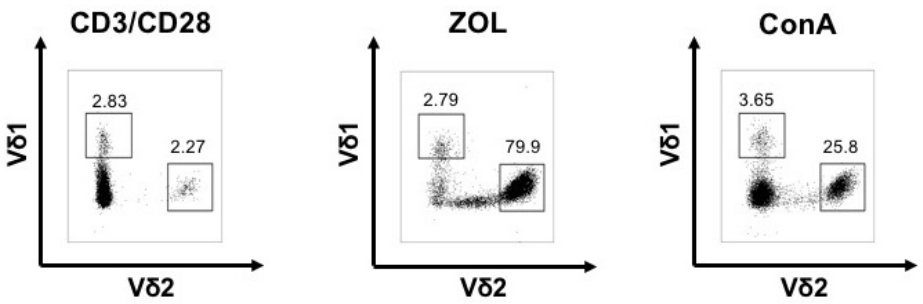


Figure 3.4 Representative FACS plots of expanded T cell subset proportions

Representative flow cytometry dot plots from one healthy donor showing the proportion (%) of Vδ1 and Vδ2 in bulk cultures by day 13 following stimulation with either CD3/CD28 antibody, ZOL, or ConA.

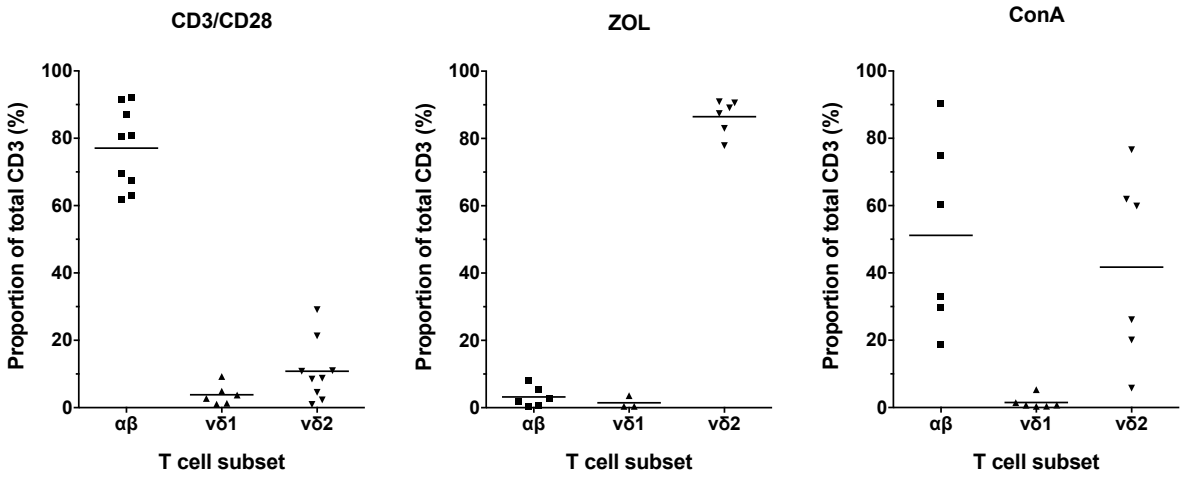


Figure 3.5 αβ, Vδ1 and Vδ2 T cell proportions in day 13 bulk cultures following activation by 3 different methods

T cells were activated with either CD3/CD28 antibody, zoledronate (ZOL) or concanavalin A (ConA) and αβ, Vδ1 and Vδ2 cell proportions within bulk cultures on day 13 were determined by flow cytometry. Percentage T cell subset was calculated by gating on Live/$CD3^+$ cells and either $V\delta1^+$, $V\delta2^+$ or pan-$\alpha\beta TCR^+$. Individual donors are represented and horizontal bar equates to the mean.

3.2.2 γδ T cell transduction with CAR

The CAR construct used in experiments throughout this thesis was developed at the UCL Cancer Institute and is the clinical grade retroviral vector currently being used in the 1RG-CART trial. It consists of two transgenes (described in detail in Section 2.2.1); a second generation GD2-CAR, and marker/suicide gene RQR8 (166). RQR8 contains the target epitope from CD34, that can be identified by anti-CD34 antibody (clone QBend10 conjugated to APC) thereby allowing detection of transduced cells by flow cytometry (% QBend10$^+$ cells). Experiments performed in our laboratory by Senior Post-doctoral Fellow, Dr Barry Flutter and team, have validated that identification of QBend10$^+$ cells by flow cytometry is adequate for defining CAR$^+$ expression. Viral supernatant is easily and economically produced using the stable producer 293Vec-RD114 cell line (as described in Section 2.2.1.1)

Firstly, I sought to establish whether γδ T cells could be efficiently transduced with our GD2-CAR construct following ZOL activation. I noted on preliminary experiments that the transduction efficiency of Vδ2 was lower than that for αβ T cells activated with CD3/CD28 antibody at the same time point. A requirement for successful retroviral transduction is that T cells are actively proliferating for genomic integration to occur. Expansion of Vδ2 T cells with ZOL is a complex and much debated process whereby nitrogen containing zoledronic acid indirectly activates cells by inhibition of farnesyl pyrophosphate synthase leading to intracellular accumulation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These phosphoantigens are then either 'presented' or 'sensed' by butyrophilin molecules to activate γδTCR (66-69). Targeting the optimal time-point for γδ T cell transduction is therefore dependent on the complex interplay of priming APCs [most likely peripheral blood monocytes (185)] and recognition of BTN2A1 by γδTCR leading to activation and proliferation. I hypothesised that as the kinetics of activation and expansion differed between ZOL and CD3/CD28 antibody, it would require a longer initial stimulation culture period prior to transduction for Vδ2. In order to test this, I chose two time points for comparison, day 3 and day 5. Figure 3.6 shows higher transduction

efficiency (TE) for αβ T stimulated with CD3/CD28 antibody, compared to Vδ2 stimulated with ZOL when transduced on day 3. ZOL-activated cells transduced on day 5 had a higher TE than day 3, however this did not reach statistical significance.

The level of CAR^+ expression is an important consideration when developing vectors and T cell expansion protocols for transduction. It is known that in addition to specific antigenic activation, CARs can often undergo antigen-independent activation, known as 'tonic signalling' (186) It is reported that CAR tonic signalling is enhanced by high surface expression and ScFv clustering, and this can lead to CAR T cells having inferior anti-tumour efficacy by increasing differentiation and exhaustion marker expression (187, 188). A transduction efficiency of approximately 30-40% was therefore considered optimal.

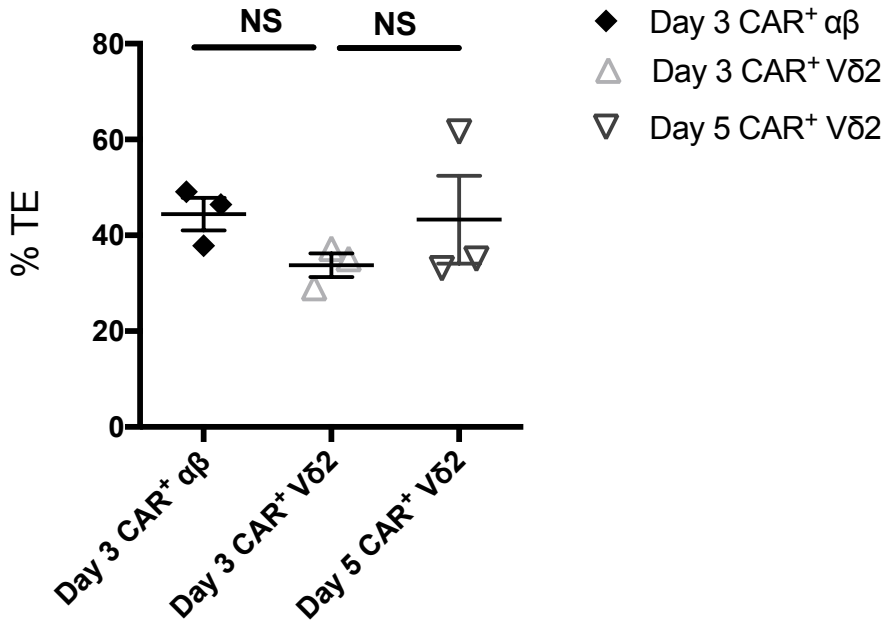


Figure 3.6 Increased CAR expression on Vδ2 following transduction on day 5

ZOL stimulated PBMC were transduced with GD2-CAR on either day 3 or day 5 following activation. Comparison is made to the 'gold standard' GD2-CAR αβ T cell transduction protocol in our laboratory using CD3/CD28 antibody activation and transduction on day 3. Percentage transduction efficiency (%TE) was measured by %QBend10+. Data, mean ± SEM, 3 individual donors.

Following optimisation in Vδ2 cells, I sought to investigate whether Vδ1 cells could also be transduced with CAR following activation with either ConA or CD3/CD28. Retroviral transduction of Vδ1 had not previously been reported. ConA and ZOL activated PBMC were transduced 5 days post stimulation and compared to the standard day 3 αβ T cell transduction protocol as a positive control. As shown in Figure 3.8, transduction efficiency, determined flow cytometrically by identification of QBend10$^+$ cells, was highest for αβ T cells activated with CD3/CD28 antibody (mean 61.57% ± 22.01, n=9). Transduction varied greatly between donors which can probably be explained by the nature, health and proliferation kinetics of T cells affecting genomic integration. The multiplicity of infection (MOI) between experiments was kept consistent by using the same large batch of frozen viral supernatant for serial experiments.

Transduction following activation with ZOL was highest in Vδ2 cells (25.26 ± 17.26, n=8), which is in keeping with the fact that αβ T cells and Vδ1 cells did not expand with ZOL. It should be highlighted that there were two donors who failed to transduce following ZOL stimulation, which was most likely related to the inter-donor variability in the response to aminobisphosphonate treatment.

For transduction using ConA, there was similar transduction efficiency between the T cell subsets (mean 26.02% Vδ2, 32.04% αβT and 32.66% Vδ1), with no statistically significant difference between subsets. It can be concluded that ConA and ZOL can be used to activate and transduce γδ T cells from peripheral blood; ZOL preferentially for Vδ2, and ConA for Vδ1 cells.

Figure 3.7 Gating strategy for CAR$^+$ T cells

Representative FACS plot showing gating strategy for defining CAR+ T cells. An example of Vδ2 cells (pre-gated on live/CD3$^+$ cells) is shown following activation with ZOL, transduction with CAR on day 5, and antibody staining with CD34 (QBend10) for transduction efficiency on day 8. The GD2-CAR construct co-expresses the QBend10 epitope from CD34, allowing detection by flow cytometry (right). Non-transduced cells were also stained with QBend10 antibody to set the negative gate (left). Transduced/CAR$^+$ cells were determined by the percentage of QBend10$^+$ in the T cell population gate compared to non-transduced cells of the same T cell population.

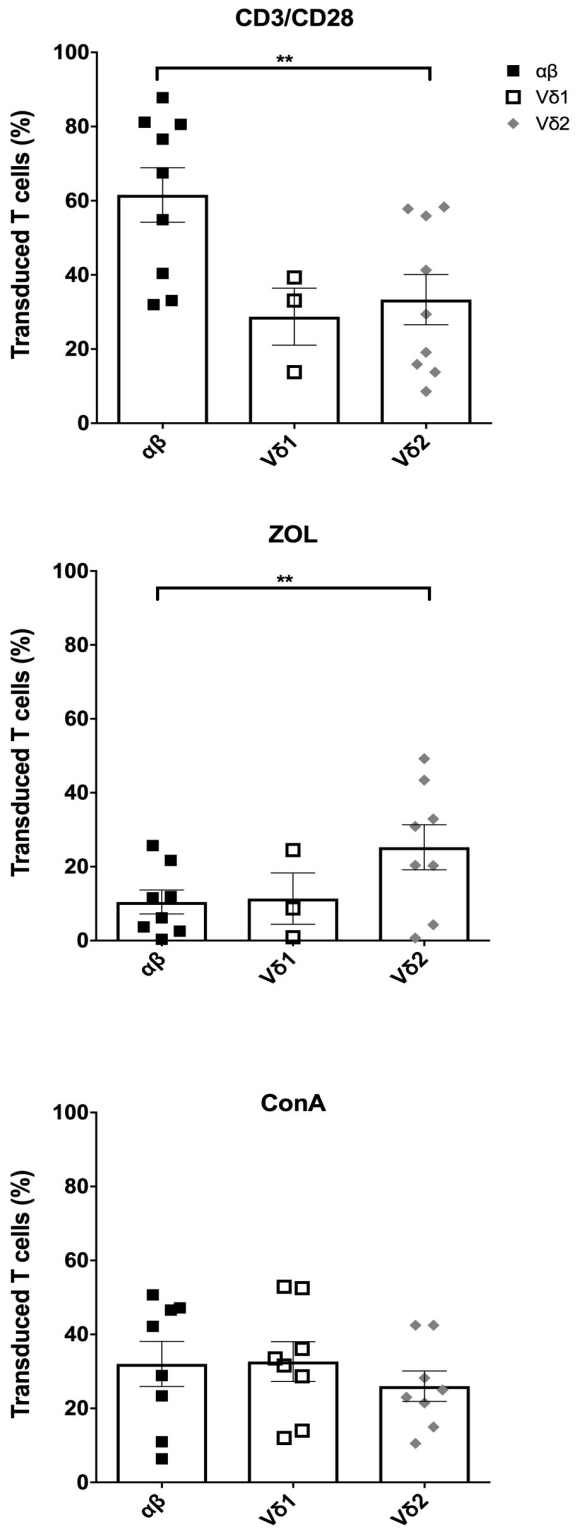


Figure 3.8 Transduction efficiency of T cell subsets using CD3/CD28 antibody ZOL, or ConA

Mean transduction efficiency using CD3/CD28 antibody, ZOL, or ConA activation methods, respectively. Transduced T cells were determined by percentage of T cell population in QBend10^{+} gate. Each data point represents an individual donor and each horizontal line is the mean.

3.2.3 Choice of CAR

Two identical CAR constructs except for their intracellular signalling domains were evaluated; CD28ζ and 41BBζ. The GD2-CD28ζ plasmid (MP10413) was readily available, however a GD2-41BBζ construct with the identical ScFv, spacer region and transmembrane domain had not been generated, therefore I was required to assemble this using the method described in Section 2.2.1.2. This new construct in the same SFG backbone as GD2-CD28ζ was validated by sequencing and the correct alignment was ensured using SnapGene software.

Both CARs are composed of the anti-GD2 ScFv huK666 which is fused to a hinge CH2-CH3 domain of IgG Fc that promotes synapse formation between target antigen and ScFv by enhancing the flexibility of the construct (189). The extracellular domain is fused to the transmembrane domain of CD28 which is a hydrophobic helix and acts to anchor the construct to the cell membrane. As can be seen, the two CARs differ in their endodomains; either containing CD28-CD3ζ or 41BB-CD3ζ (Figure 3.9).

The rationale for choosing to investigate a 41BB costimulatory domain in γδ T cells was based on a convincing body of literature in αβ T cells indicating that 41BBζ containing CARs had different kinetics of anti-tumour activity *in vivo* due to slower initial reactivity but a more sustained proliferation following encounter with target antigen (188).

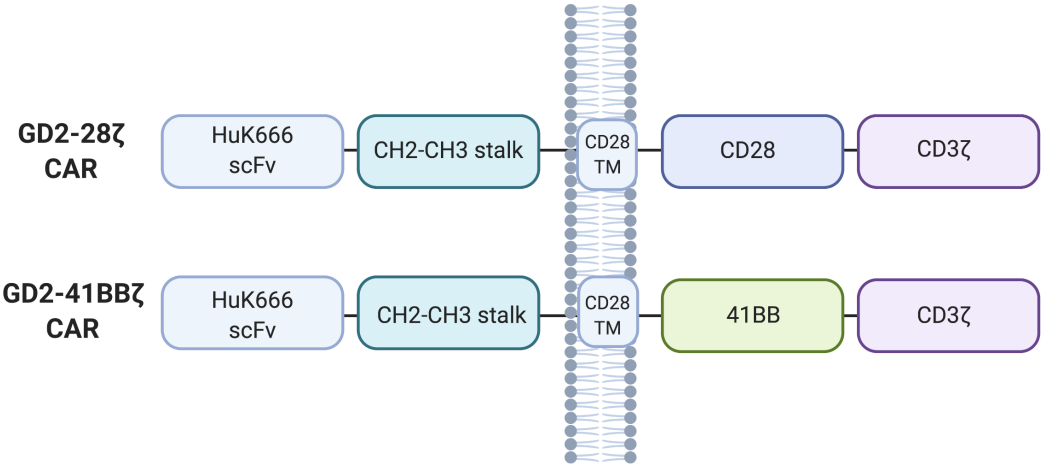


Figure 3.9 Schematic diagram of GD2-28ζ and GD2-41BBζ CAR

Both constructs consist of the huK666 single chain variable fragment (ScFv), a CH2-CH3 spacer region which is then fused to the transmembrane domain of CD28 acting to anchor the receptor to the cell membrane. Constructs differ in their intracellular signalling domains, either containing CD28-CD3ζ or 41BB-CD3ζ.

Using supernatant produced by transient transfection of 293T cells, mean transduction efficiency was higher for 41BBζ containing CAR compared to CD28ζ (19.23% ± 7.689 and 10.07% ± 3.067 respectively, $p>0.05$, $n=3$). It should be noted that viral supernatant was snap frozen before use which may have reduced transduction efficiency compared with fresh supernatant, and that CAR presence was measured by CD34-QBend10 expression (suicide/recognition transgene), rather than the CAR itself. This could have been determined using an anti-Fc antibody that binds to the spacer region of the CAR or an antibody against the ScFv, which was not available at the time.

Anti-tumour activity of the two different CARs was assessed using standard 4-hour Cr^{51} release assay in 3 donors. The target cell line, LAN1 was chosen for its abundant expression of GD2, and specific lysis in response to GD2-CAR αβ T cells. Vδ2 cells were transduced with either GD2-CD28ζ or GD2-41BBζ CAR, and non-transduced Vδ2 served as a negative control. LAN1 tumour cell lysis was observed for both CD28ζ and 41BBζ-containing CARS, however at both 10:1 and 5:1 E:T ratios, cytotoxicity was significantly higher for CD28ζ than 41BBζ (Figure 3.10). This is

despite the higher (but non-significant) transduction efficiency achieved with GD2-41BBζ.

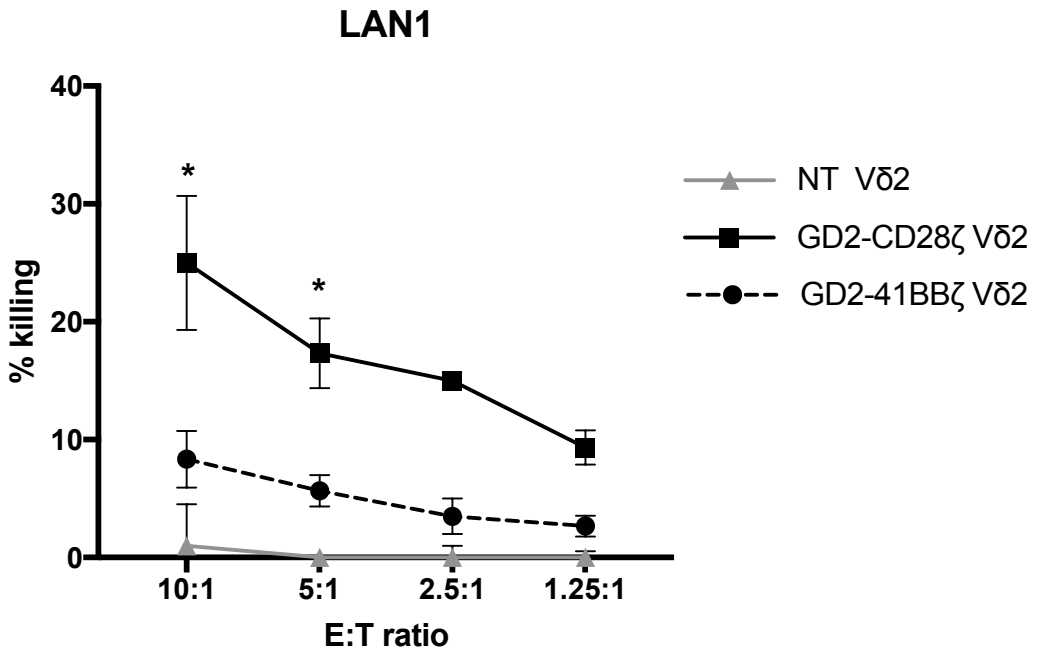


Figure 3.10 ZOL-activated bulk lymphocyte cultures transduced with either GD2-28ζ or GD2-41BBζ demonstrate specific cytotoxicity of GD2$^+$ LAN1 neuroblastoma cells by standard 4-hour ^{51}Chromium release assay.

Data, mean ± SEM, n=3 * $p<0.05$, statistical comparisons were made between GD2-28ζ CAR transduced and GD2-41BBζ CAR transduced cells with a 2-way ANOVA.

The superior short-term cytotoxicity profile of GD2-CD28ζ was mirrored by an experiment to determine 24 hour IFNγ production (Figure 3.11). Vδ2 transduced with either GD2-CD28ζ or GD2-41BBζ were co-cultured with LAN1 cells. After 24 hours supernatant was harvested for quantification of IFNγ by ELISA. GD2-28ζ CAR caused significant production of IFNγ with LAN1 (compared to no target) but this was not the case with GD2-41BBζ CAR.

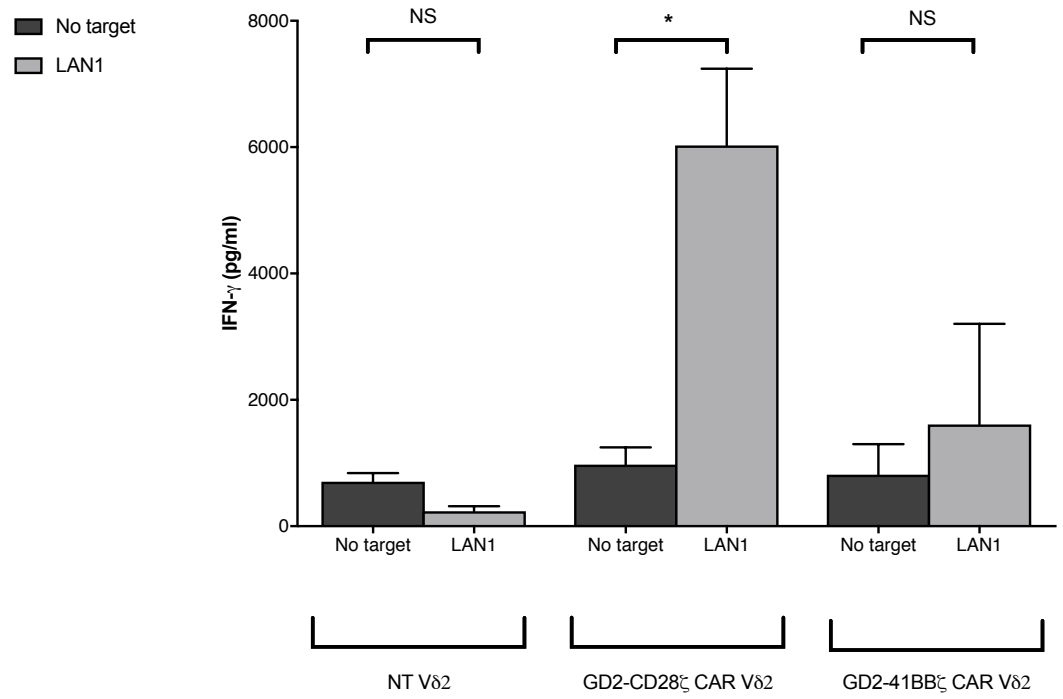


Figure 3.11 IFNγ release by GD2-CD28ζ and GD2-41BBζ CAR transduced Vδ2 on co-culture with LAN1

IFNγ in supernatant was measured by ELISA after 24 hour co-culture at an effector to target ration of 1:1. Graphs show mean and SEM. *$p<0.05$ using paired student t-test. 3 individual donors.

3.3 Discussion

In this chapter, I set out to develop a successful and reproducible method for the expansion and transduction of healthy human γδ T cells with a GD2-specific CAR that would be suitable for use as a therapeutic product for paediatric patients with neuroblastoma. Although it would have been desirable to use blood samples taken from children with neuroblastoma, this was not practical due to the volume of blood required and need for regular samples. Instead I chose to use blood samples from healthy adult donors, which were readily available and in keeping with local ethical approvals. It should be appreciated however that the abundance and function of γδ T cells in healthy adults may be different from samples obtained from children with neuroblastoma, particularly those who have been heavily pre-treated with

chemotherapy. On personal communication with Prof. Anderson, 17 heavily pre-treated patients with neuroblastoma were enrolled in the 1RCART study of GD2-28ζ CAR αβ cells, and the *in vitro* killing, cytokine release and proliferation was compared with 10 healthy donors. For CAR αβ T cells there was no significant difference between the groups, and a suitable CAR T cell product was generated for all patients with neuroblastoma enrolled in the study.

In healthy adult donors, the proportion of Vδ2 largely exceeded Vδ1 in the majority of donors. This is not necessarily representative of paediatric patients with neuroblastoma where the majority of children are diagnosed younger than two years. Vδ1 cells predominate during foetal development and childhood but by adulthood the majority of γδ T cells in peripheral blood are Vγ9Vδ2 cells (190). The finding of Vδ2 exceeding Vδ1 from blood peripheral samples taken from healthy donors in the UK may also differ geographically, for example it is known that in West Africa the Vδ1 population is maintained (twice that of Caucasians) (183).

My first aim in Chapter 3 was to compare three protocols for T cell activation to assess the degree of γδ T cell proliferation, including both Vδ1 and Vδ2 subsets. CD3/CD28 antibody-based activation methods are well established with CD3 providing a strong proliferative signal to the TCR-complex, and CD28 providing the necessary co-stimulation to prevent activated T cells from becoming anergic. Various methods can be used to mimic the cell-cell interaction and immunological synapse required for activation including the use of Fc receptor-bearing accessory cells (such as monocytes) or antibody-coated beads. I chose the former method due to the protocol already being established in our laboratory for CAR T cell clinical trials and also to avoid the requirement for bead removal prior to infusion. Data has since emerged that using an antibody coated bead based approach results in less exhausted and more persistent T cells compared to CD3/CD28 soluble antibody stimulated cells (191). It would be interesting to study whether this difference is observed in γδ T cells.

I demonstrated that all three T cell subsets studied (αβ, Vδ1, Vδ2) proliferated in response to CD3/CD28 antibody, with the highest proliferation seen in Vδ1 cells. The shape of the expansion curves also suggests the potential for proliferation beyond the two weeks tested. The observed Vδ1 proliferation is consistent with the more recent work of Almeida and colleagues (192) who used CD3 soluble antibody together with a clinical grade cytokine cocktail (including IL-4, IFNγ, IL-21, IL-1b and IL-15) to produce Vδ1 cells for immunotherapy of chronic lymphocytic leukaemia (CLL), with impressive levels of Vδ1 proliferation. Crucially the authors additionally demonstrated that the cultured Vδ1 cells express natural cytotoxicity receptors, NKp30 and NKp44, and successfully infiltrated into tumours in a xenograft CLL model. Using bulk CD3/CD28 antibody stimulated cultures would be impractical for a clinical trial evaluating Vδ1 T cell efficacy due to heterogeneous nature of the resulting populations, but a further selection step would significantly increase the proportion of Vδ1 [as reported in (192)].

The second expansion method evaluated the use of zoledronic acid (ZOL). ZOL is a potent bisphosphonate drug that inhibits farnesyl pyrophosphate synthase, the enzyme acting down stream of IPP in the mevalonate pathway. Although initial studies suggested IPP was a natural Vδ2 ligand (193, 194), at the time of starting my PhD studies, data was emerging that a butyrophilin (BTN) 3 family member (BTN3A1) was a critical molecule, essential for Vδ2 TCR activation by IPP (65). More recent data (2020) has indicated the essential role of BTN2A1, when dimerised with BTN3A1, in binding to the framework 4 (FW4) region of Vγ9 chain (70, 71).

As expected, ZOL stimulation resulted in preferential expansion of Vδ2. Despite initial promising Vδ2 proliferative capacity with logarithmic growth between day 6-11, there was an apparent tailing off in culture durations of more than 11 days. This can potentially be explained by higher sensitivity to activation induced cell death using this particular method of stimulation, which was not seen in the other culture methods tested. This phenomenon was also descried by Wang and colleagues, whilst optimising protocols for γδ T cell expansion for cell therapy (195). An alternative

explanation is that the nature of antigenic stimulus with ZOL is qualitatively or quantitatively different to CD3/CD28 since it lacks costimulation. The major benefit of costimulation in αβ T cells has been shown to be the avoidance of AICD leading to greater measured proliferation, rather than cytotoxicity or cytokine response (196)

In contrast, the third method tested used ConA with no apparent slowing of proliferation beyond 11 days of culture. ConA is a mannose/glucose-binding lectin isolated from jack beans and acts as a T cell mitogen by triggering the cross-linking of TCR complexes. This method non-selectively expanded all T cell subsets to large number, although similar to CD3/CD28 antibody, bulk populations were largely αβ T cells and would therefore require a selection step to be clinically relevant. Interestingly, there was a huge amount of donor variability in the response to ConA activation with Vδ2 proportions in bulk cultures ranging from less than 10% to up to 80%. This again could have potential implications if translated to an early phase clinical trial. Although expansion methods using aAPC, ZOL and CD3/CD28 antibody are available commercially in GMP grade, ConA is not. The effect on γδ cells by ConA, however, is an attractive preposition because of its more polyclonal expansion of γδ T cells including the Vδ1 subset (Figure 3.5). ConA has also been shown to have immunomodulatory and autophagic cytotoxicity against hepatoma cells (197) but causes red cell agglutination which could be harmful. Despite ConA not being immediately clinically translatable, proof of concept of its utility could trigger the industrial development of GMP product for clinical translation. High numbers of Vδ1 for clinical use are now potentially achievable through CD3 stimulation, enrichment by cell separation, and addition of a cytokine cocktail during production, as described by Almeida *et al.* (56), (although this work was published after commencing my own study). To help determine whether ConA stimulation has any advantages over CD3 stimulation, it would be important to look at a more detailed immunophenotype and function of γδ T cells comparing the two methods, and some initial data exploring this is included in Chapter 4.

A limitation for the quantification of fold expansion by the chosen method in this study was that it is dependent on manual cell counting using trypan blue exclusion. Caution

should be exercised when interpreting fold-change of Vδ1 cells as they were always a rare population within bulk cultures; a small counting error could translate to a larger error in fold change, due to the difficulty in measuring small populations. This would also account for the wider error bars in the rarer populations. An alternative method to consider for improving accuracy would be to use flow cytometry counting beads (198).

Based on the data, there was better expansion of Vδ1 using CD3/CD28 antibody, that should theoretically expand all subsets. For both Vδ1 and Vδ2, CD3/CD28 antibody and ConA, appeared to expand cells at day 13 and a longer-term experiment would be needed to see if their expansion profiles extend beyond 14 days for the three cell populations. The higher initial expansion rate of Vδ1 compared to Vδ2 using CD3/CD28 antibody can be explained by either Vδ1 having more memory or stem-like properties and are therefore more intrinsically capable for expansion, and/or that Vδ1 are less susceptible to AICD (53) induced by stimulation of the TCR or non-TCR stimulation.

3.3.1 Optimising transduction

It is known that different activation methods and *ex vivo* culture conditions can significantly impact the functional capabilities of effector cells and this is true for αβ CAR T cells. Having shown using CD3/CD28 antibody, ZOL and ConA effectively expands γδ T cells, I was interested to discover which method gives the most optimal level of CAR^+ transduction. In my academic institution there are optimised and established protocols for CAR transduction of αβ T cells. As it was not necessary to repeat this body of work in αβ T cells, I set out to establish an optimal time-course for transduction of Vδ1 and Vδ2 cells using the three methods of activation. I chose to compare two time points, namely day 3 (used in my laboratory's clinical trial protocol) and day 5 (hypothesising that γδ T cells are proliferating at faster rate by day 5 and therefore may transduce with gamma-retrovirus more efficiently). Transduction efficiency of ZOL activated Vδ2 cells was comparable to CD3/CD28 antibody

activated αβ T cells. Although there was no significant difference between the transduction efficiency of ZOL-expanded cells transduced on day 3 and day 5, there was a general trend towards higher transduction and increased total Vδ2 cell number. It would also be interesting to compare even later time points for transduction when γδ T proportion is further enriched in bulk cultures e.g. up to 10 days post-stimulation.

There was wide variability observed between the transduction efficiency of healthy donors using all culture methods. This can be accounted for by differences and unpredictability of donor T cells and this finding has been observed in multiple other CAR T studies. Brentjens *et al*. reported wide differences in T cell fold expansion from 23.6-385 (n=10) with transduction efficiencies ranging from 4-70% (111) and other studies have reported similar wide variability between individual donors (93, 199). Even though CAR^+ expression in γδ T cells was at the lower end, transduction efficiency of both Vδ1 and Vδ2 was still within this generally accepted range. Transduction efficiency was lower for γδ T cells using ZOL and ConA compared to αβ T cells activated with CD3/CD28 antibody, and this might possibly be due to the gentler activation stimulus provided by ConA or ZOL (200). Gamma retroviral transduction was used in this study, however alternative gene transfer methods that do not reply on active cellular division are possible alternatives. This includes lentiviral transduction or RNA electroporation with short-term CAR expression which avoids the safety concerns related to DNA integration into the genome.

I next wanted to investigate methods of γδ T cell expansion that may lead to transduction of the Vδ1 subset for use in functional studies. Both CD3/CD28 antibody and ConA activated Vδ1 cells resulted in CAR transduction, however the proportion of CAR^+ Vδ1 in the bulk population was too small to test their function and efficacy. In order to study this, it was necessary to purify the cells.

3.3.2 γδ T cell costimulation

The choice of costimulatory endodomain has a significant impact on the overall efficacy of CAR αβ T cells. I chose to compare a CD28 and 41BB containing CAR to assess whether there was a difference in the level of *in vitro* cytotoxicity in ZOL expanded γδ T cells. Vδ2 rapidly upregulate 41BB following antigenic stimulation, and in one report this was found to promote proliferation and cytokine production (including IFNγ secretion) (182). Intriguingly, activated Vδ2 also express high levels of 41BBL (201), that may also contribute to γδ T cell activation due to its known reverse signalling ability (202). CD28 is constitutively expressed by Vδ2 and this promotes survival and proliferation by IL-2 production (203). 41BB and CD28 differ structurally and functionally, with the former belonging to the Tumour Necrosis Factor Receptor and latter, the IgG superfamily of coreceptors. CD28 directly associates with protein kinases (e.g. PI3K), whereas 41BB requires adaptor proteins (TRAF2), to link to downstream signalling pathways (80).

There was significantly greater *in vitro* cytotoxicity of CD28 containing CARs compared to 41BB (Figure 3.10). This was surprising given that CAR αβ T cells incorporating CD28 or 41BB domains have shown similar initial response rates in ALL patients (112, 113, 126), and that 41BB containing CARs have superior function in CLL (93), likely due to their superior ability to expand and survive (204). It is reported however, that in short-term killing assays using CD19-CAR αβ cells, there is no significant difference in cytotoxicity when using a first generation or second generation CD28 or 41BB-containing CAR (205). The fact that the generated 41BB CAR in γδ T cells appears to decrease cytotoxicity suggests it may be interfering negatively with CAR function, which would be an unexpected finding. Additional replicates would be needed to test this including inclusion of a first generation and 'mock' construct. To determine if the GD2-41BBζ is interfering negatively it would be necessary to perform signalling experiments in which GD2-28ζ, GD2-41BBζ and GD2-ζ are compared in αβ and γδ T cells, and measuring phosphorylation of intracellular signalling molecules by flow cytometry. This would require inclusion of

an epitope tag into the CAR since there is no antibody against the GD2 ScFv and anti-Fc antibodies are prone to stimulate γδ T cells by crosslinking though the Fc receptors. It remains to be considered that the GD2-41BBζ CAR may not be functional.

On personal communication with a PhD student in our laboratory, Nisansala Dilrukshi Wisidagamage, working with CARs that lack signal 1 (costimulation only CAR) (206), she has observed higher killing in GD2-28 CAR-transduced γδ T cells against antigen negative targets compared to non-transduced γδ T cells (unpublished data). A possible explanation could be that the process of retroviral transduction itself has caused cellular stress resulting in sufficient activation of γδ T cells to enhance innate cytotoxicity to levels higher than the non-transduced controls. In other words, the low level cytotoxicity seen is not specific CAR-dependent killing, but enhanced innate killing by 'activated' γδT. Further investigation is required to decipher whether the GD2-41BBζ is firstly being adequately expressed (by staining for the anti-Fc portion of the CAR, as oppose to simply QBend10 or introduction of an epitope tag), and secondly to determine whether it is functional.

3.3.3 Scale-up for clinical use

One main limitation of using γδ T cells as the effector cell of choice in CAR T cell therapy is the ability of cells to expand to sufficient number for adoptive transfer, particularly in the context of Vδ1 cells. However, as previously discussed, the 'therapeutic' dose of CAR T is dependent on a complex interplay of multiple factors and therefore is likely to vary substantially between individuals. The migratory, proliferative and cytotoxic capacity of infused T cells play an important role in determining dose, therefore the number of Vδ1 and/or Vδ2 will not necessarily match αβ T cells due to their distinguishing properties. A combinational approach with polyclonal CAR γδ T or CAR γδ T plus CAR αβ T cells warrants further investigation for possible synergistic effects *in vivo*. This is further supported by a 1:1 combinational approach of $CD4^+$ and $CD8^+$ CAR T cells which has already proved efficacious (96, 97)

In the context of neuroblastoma, patients undergo leukapheresis to harvest PBMC for *ex vivo* culture and genetic modification. Experience of cell manufacturing for the 1RG-CART trial has been very encouraging where large numbers of αβ T cells ($>1x10^8$) are reliably produced. The patients' white blood cell count and overall health of the cells is important, as this will influence their ability to expand *ex vivo*. Many patients with relapsed/refractory neuroblastoma are profoundly immunosuppressed therefore it is very reassuring that it has been possible to manufacture adequate T cell products for these children. Detailed information as to the optimal CAR T cell dose for paediatric patients will become clearer as the results of worldwide Phase 1 studies currently in progress are reported. Translating this to CAR γδ T cells, I have shown Vδ1 and Vδ2 cells can be expanded >100 fold using ConA and ZOL respectively, which would be sufficient for adoptive cell transfer, and comparable to current αβ T cell approaches.

In summary, this Chapter provides proof of concept that γδ T cells can be sufficiently propagated and retrovirally transduced with a 2nd generation GD2-28ζ CAR with potential for clinical use. This gain of function strategy required further determination of its potential specific advantages over currently available immunotherapies, and this is the focus of investigation in Chapter 4.

Chapter 4 Detailed characterisation of GD2-28ζ CAR$^+$ γδ T cells

4.1 Introduction

In Chapter 3, I determined successful methods for the propagation and transduction of Vδ1 and Vδ2 CAR$^+$ T cells. Following the selection of GD2-28ζ CAR as the construct of choice, I sought to further investigate and characterise transduced γδ T cells with respect to their phenotype, cytotoxicity, cytokine production, proliferative capacity, and ability to migrate to tumours.

As discussed in Section 1.9.2, the phenotype of infused CAR effector cells plays an important role on their ability to kill cancer targets, survive and proliferate. There is a wealth of knowledge in the CD19-directed CAR αβ T cell field, with positive outcomes correlating to high levels of engraftment and long-term persistence of adoptively transferred cells (207). Specific αβ T cell subpopulations with certain memory phenotypes have been associated with the highest therapeutic potential, and combinational therapy with CD4$^+$/CD8$^+$ $T_{N,}$ T_{CM} and T_{EM} confer superior anti-tumour responses (98). For CAR αβ T cells there are many reports detailing specific culture methods for the production of αβ T cell subpopulations with the most desirable characteristics, however for γδ T cells there are still many unanswered questions.

CD8$^+$ CAR T cells are known to have higher tumour lytic ability than CD4$^+$ CAR T cells, but the specific cytotoxicity of CAR$^+$ Vδ1 and Vδ2 subtypes in comparison to CAR αβ T cells has not previously been tested. In addition, I sought to evaluate the cytokine profile of CAR γδ T cells upon antigen encounter, together with their ability to proliferate in response to target recognition. Lastly, the tumour migratory

capabilities of γδ T cells (particularly Vδ1) is a distinguishing property, and I have investigated this further in Section 4.2.8 using transwell assays.

4.1.1 Aims

- To characterise CAR^+ γδ T cells phenotypically.

- To compare CAR^+ γδ T cells to conventional CAR^+ αβ T cells, including cytotoxicity, cytokine production, proliferation and migration.

4.2 Results

4.2.1 γδ T cell activation is associated with costimulatory ligand upregulation

Understanding the dynamic phenotype and function of costimulatory molecule expression on subsets of γδ T cells is imperative to the development of efficacious γδ T cellular therapy. Previous studies have indicated that activated γδ T cells upregulate costimulatory ligands including CD80, CD86, CD40 and CD137L (61, 74). Firstly, I sought to identify which costimulatory receptors and ligands are naturally occurring following alternative methods of activation, and the dynamic patterns of expression on different γδ T cell subsets following prolonged culture. This was of particular interest as published work on minimally stimulated γδ T cells has reported co-expression of cognate costimulatory receptor-ligand pairs (201, 208-210) suggesting the intriguing possibility that γδ T cells are capable of 'auto-' or 'trans-costimulation' (211), or even that they are able to function as combined antigen-presenting/cytotoxic cells.

PBMC were isolated using density gradient separation of whole blood from healthy donors then γδ T cells were then extracted by positive selection using the anti-TCRγδ MicroBead Kit as previously described in Section 2.2.2.3.3. Following isolation, co-cultures of polyclonal γδ T cells were set up with irradiated aAPC coated with anti-

γδTCR antibody (B1) at a ratio of 1:2. Cells were cultured for 28 days with IL-2 and IL-21, and irradiated anti-γδTCR antibody coated aAPC were added every 7 days.

Cells were stained pre-activation and 7, 14, 21 and 28 days post activation to provide phenotypic information on the expression patterns of costimulatory receptor-ligand pairs on $V\delta1^+$, $V\delta2^+$ and $V\delta1^-/V\delta2^-$ subsets. As shown in Figure 4.1 there was a general trend that as costimulatory ligand expression increased following activation, costimulatory receptor expression tended to decrease. In particular there was marked upregulation of CD86 over the 28 day activation period. Although the aAPC method is attractive for propagating polyclonal γδ T cells, for all subsequent experiments outlined in this thesis, ZOL or ConA were used to activate γδ T cells due to challenges achieving sufficient cell number and adequate CAR transduction efficiency using the aAPC method.

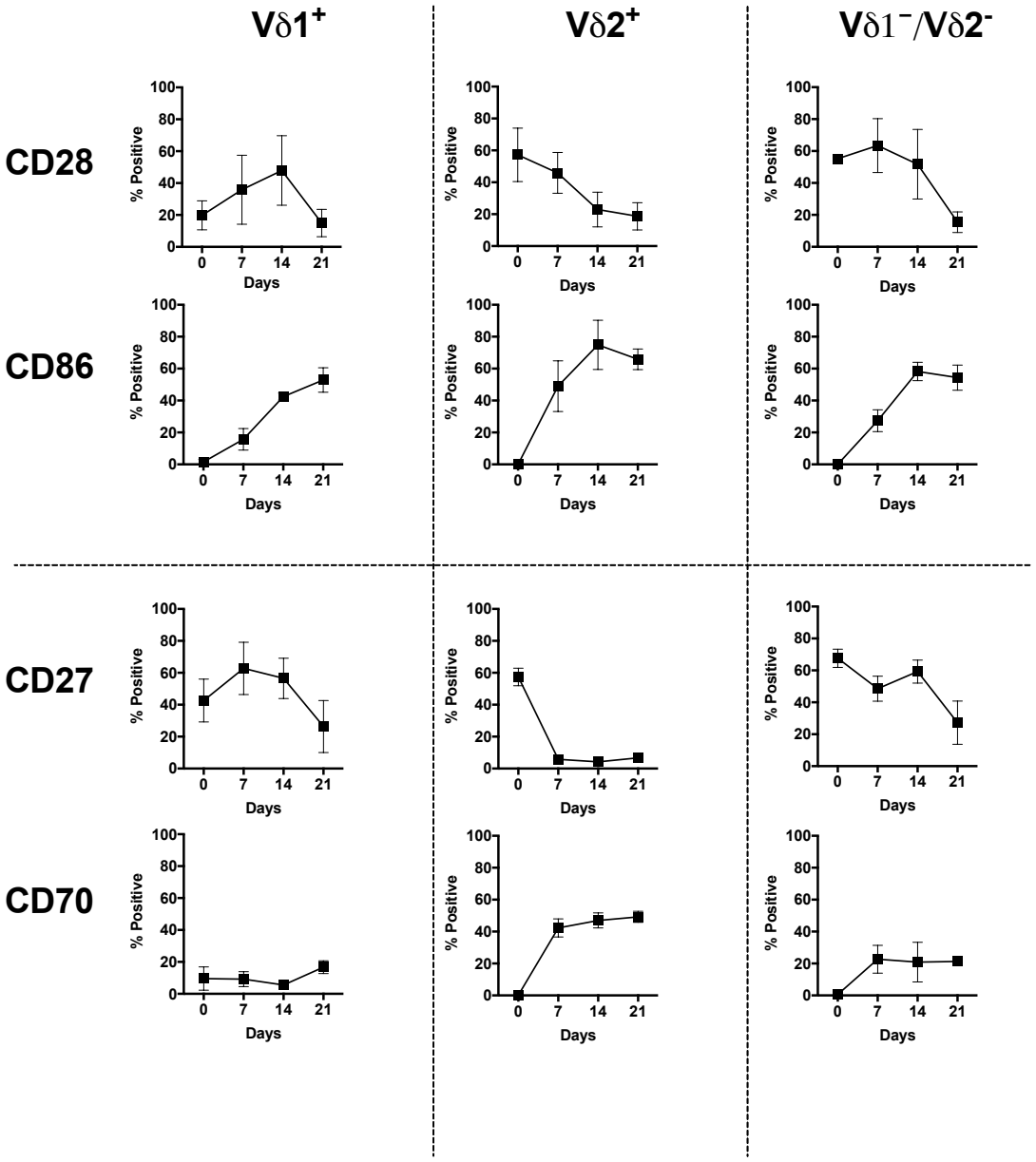


Figure 4.1 Kinetics of costimulatory receptor-ligand pair expression over 3 weeks

Pre-selected polyclonal γδ T cells were activated with aAPC and propagated for 28 days in the presence of IL-2 and IL-21. Expression levels of CD28, CD86, CD27 and CD70 were determined by flow cytometry on day 0, 7, 14, and 21 by gating on individual Vδ1$^+$, Vδ2$^+$ and Vδ1$^-$/Vδ2$^-$ subsets. Mean ± SEM, n=3.

4.2.2 CAR$^+$ Vδ1 cells have a less differentiated memory phenotype than CAR$^+$ αβ and CAR$^+$ Vδ2 T cells

In non-transduced γδ T cells, differentiation into effector memory (T_{EM}) cells upon activation has been described by Dieli *et al*. (51). Antibody staining for CD27 and CD45RA divides subsets into four memory subsets; Naïve, T_N (CD27$^+$/CD45RA$^+$), central memory, T_{CM} (CD27$^+$/CD45RA$^-$), effector memory, T_{EM} (CD27$^-$/CD45RA$^-$), and terminally differentiated cells, T_{EMRA} (CD27$^-$/CD45RA$^+$). T_N and T_{CM} have the highest proliferative potential and express lymph node homing receptors but have a relative lack of immediate effector function, whereas T_{EM} and T_{EMRA} are highly cytotoxic but have lower proliferative capacity (52).

To assess the different memory subsets present in pre-expanded and post-expanded (day13) (CAR$^+$/CAR$^-$) αβ, Vδ1 and Vδ2 cultures, I stained with antibodies for CD27 and CD45RA. Figure 3.1 shows the gating strategy used for defining subsets and quadrant gates were set using FMOs. T cell subsets were pre-gated on live/CD3$^+$ cells and CAR$^+$/CAR$^-$ defined by $^{+/-}$expression of QBend10. The activation method for the expansion of the respective populations was; CD3/CD28 antibody for αβ T cells, ZOL for Vδ2 and ConA for Vδ1.

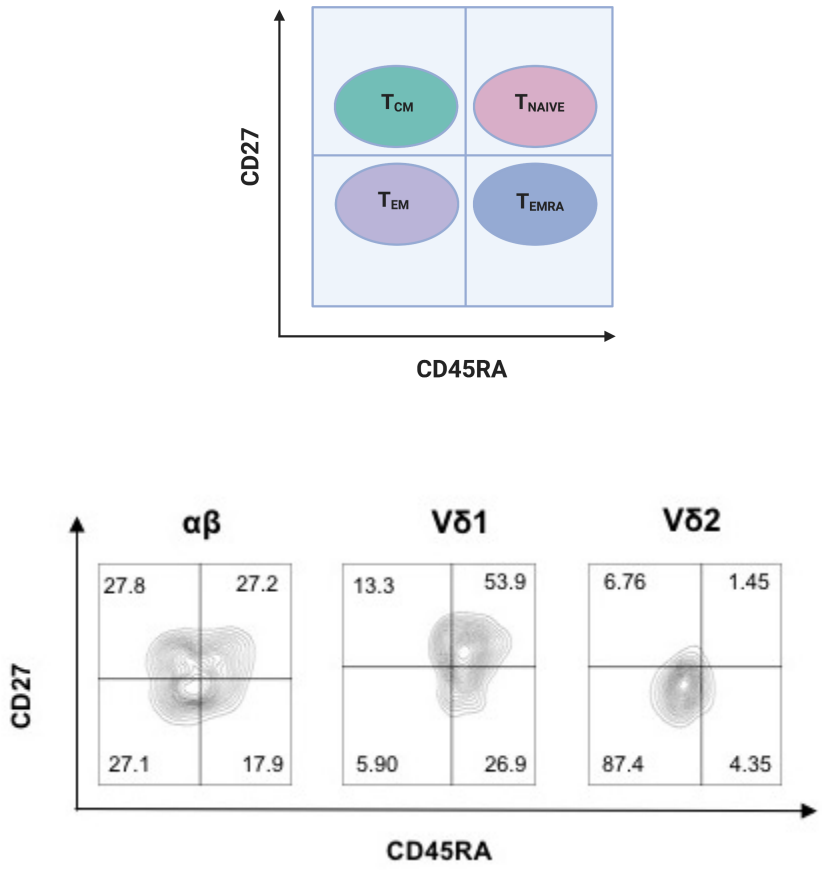


Figure 4.2 Representative FACS plots of T cell subset memory phenotype following activation

Representative flow cytometry contour plots displaying the memory phenotype of CAR^+ αβ, Vδ1 and Vδ2 T cells following 13 day expansion. αβ were activated with CD3/CD28 antibody, Vδ1 with ConA, and Vδ2 with ZOL. T_N, $CD27^+/CD45RA^+$ T_{CM}, $CD27^+/CD45RA^-$ T_{EM}, $CD27^-/CD45RA^-$, T_{EMRA} $CD27^-/CD45RA^+$.

αβ T cells showed a general shift from a predominantly T_N / T_{CM} phenotype to T_{CM} /T_{EM} phenotype at day 13 following activation with CD3/CD28 antibody, and a higher proportion of T_{EMRA} is noted post-expansion (Figure 4.3). In contrast to αβ T cells, a large number of CAR^- and CAR^+ Vδ1 cells maintained a T_N phenotype, which was not affected by prolonged culture or transduction with CAR^+. Interestingly there was also a large number of T_{EMRA} Vδ1 in pre-expanded samples, and following activation and transduction, these Vδ1 T_{EMRA} proportions stayed constant. This finding is consistent with Davey *et al.* who reported that Vδ1 had mixed $CD27^-/CD45RA^+$ and $CD27^+/CD45RA^+$ populations (212).

In contrast Vδ2 cells following activation with ZOL adopted a predominantly T_{EM} phenotype with only a few naïve or T_{CM} cells left in culture for both CAR^+ and CAR^- populations. This is consistent with Dieli *et al*. (213) who reported that Vδ2 were mainly $CD27^-/CD45RA^-$ following activation with ZOL in cancer patients *in vivo*.

Vδ2 cells were found to be more differentiated than Vδ1 and αβ T cells in unstimulated PBMC, which is likely to be a reflection on previous antigenic activation through engagement of the Vγ9Vδ2 TCR. Additional stimulation of the TCR induced by ZOL further differentiates the cells whereas neither ConA stimulation nor CAR expression significantly increase differentiation of Vδ1 cells. Vδ2 cells activated with ConA also showed a similar memory subset distribution to ZOL-activated Vδ2, with the vast majority T_{EM} (data not shown).

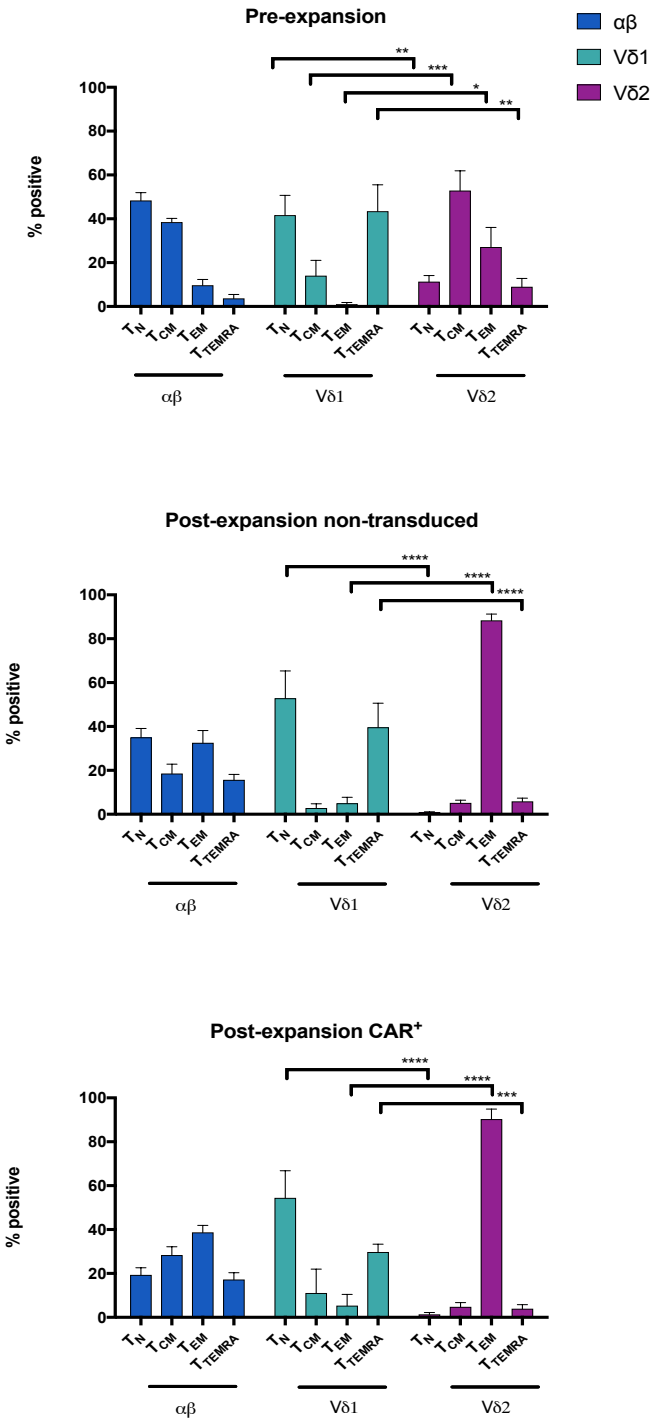


Figure 4.3 Memory phenotype

Memory phenotypes of pre-expansion PBMCs, post-expansion non-transduced cells, and post-expansion CAR^+-transduced cells. αβ were activated with CD3/CD28 antibody, Vδ1 with ConA, and Vδ2 with ZOL. T_N, $CD27^+/CD45RA^+$ T_{CM}, $CD27^+/CD45RA^-$ T_{EM}, $CD27^-/CD45RA^-$, T_{EMRA} $CD27^-/CD45RA^+$. Data represented as mean ± SEM; αβ and Vδ2, n = 6; Vδ1, n = 3. Statistical comparisons were made with a 2-way ANOVA (* $p<0.05$, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$)

4.2.3 CAR$^+$ Vδ1 T cells express significantly fewer exhaustion markers than CAR$^+$ αβ and CAR$^+$ Vδ2 T cells

T cell exhaustion is defined by a pattern of reduced effector function, sustained expression of inhibitory receptors, and a transcriptional state that differs from functional T cells (214). Programmed cell death protein 1 (PD1) and T cell immunoglobulin domain and mucin domain 3 (TIM3) are activation-induced co-inhibitory receptors associated with both T cell activation and exhaustion. Although their expression may transiently increase during T cell activation, it is generally accepted that prolonged high expression, or expression of multiple co-inhibitory receptors is a characteristic of 'exhausted' T cells. More recent understanding points to a PD1 dim/TIM3 negative population of exhaustion precursor cells that are thought to be the population 'reinvigorated' by checkpoint inhibitors. Conversely, PD1 high/TIM3 positive cells are terminally exhausted, have low effector function and cannot be re-invigorated (215).

Despite antigen-induced CAR$^+$ T cell activation and the positive contributory costimulatory effects of CD28 endodomain, chronic antigen exposure may still cause up-regulation of co-inhibitory receptors, that leads to cytotoxic dysfunction, impaired cytokine production and high rates of activation induced cell death. (188, 216). TIM3 and PD1 were chosen as potential markers of exhaustion in γδ T cells based on previous studies (40, 217). The pattern of expression was also deemed important with 'double positive' cells indicating exhaustion, whereas 'single positive' expression may be reflective of either activation or exhaustion.

γδ T cells are thought to express lower levels of PD1 and this was highlighted in a recent study comparing PD1 expression on αβ and Vδ2 T cells following adoptive transfer of mixed populations into NSG mice bearing human B cell lymphomas (218). Vδ2 were found to have significantly lower levels of PD1 expression *in vitro* and a PD1^{low} phenotype was maintained *in vivo*. This is potentially an advantageous

property of γδ T cells, if they are able to evade tumour immunosuppression by avoidance of PD1–PDL1 interaction.

PD1 and TIM3 expression on activated GD2-28ζ CAR^+ γδ T cells is unknown therefore I specifically sought to investigate the role of co-inhibitory receptor expression and whether this was influenced by the chosen activation method, prolonged culture or the process of transduction. Figure 4.4 shows representative FACS plots for the co-expression of PD1 and TIM3 for CAR^+ T cell subsets following 13 day culture. αβ T cells were activated with CD3/CD28 antibody, Vδ1 with ConA and Vδ2 with ZOL. CAR^+ populations were transduced on day 5. Again, quadrant gates were set based on FMO and cells are pre-gated on live/$CD3^+$/CAR^+ cells. Results from three independent donors were tabulated (Figure 4.5). For αβ and Vδ2 cells, transduction with CAR^+, even in the absence of cognate antigen, resulted in statistically significantly higher expression of TIM3 and PD1 compared to non-transduced cells of the same subset (using 2-way ANOVA), however this was not the case for Vδ1. Comparing 'double positive' ($PD1^+$/$TIM3^+$) populations; for αβ T CAR^+ 9.14% ± 2.11 vs. CAR^- 1.84% ± 0.67 (n=6), Vδ1 CAR^+ 5.18% ± 5.18 vs CAR^- 2.09% ± 1.5 (n=4), and Vδ2 CAR^+ 12.79% ± 2.87 vs CAR^- 2.34% ± 0.69.

There are a few possible explanations as to why this phenomenon was observed; firstly, it is likely that there is a degree of tonic signalling by the CAR^+. Long *et al*. (188) reported how CAR structural design can influence the development of αβ T cell exhaustion. They compared GD2-28ζ and CD19-28ζ CARs and found that only GD2-28ζ CAR^+ T cells became exhausted during *ex vivo* expansion; with increased expression of PD1, TIM3 and LAG-3, and upregulation of the transcription factors T-bet and Blimp-1. Western blot using phospho-CD3ζ specific antibodies indicated tonic signalling through the GD2-28ζ, but not CD19-28ζ, CAR. By using CAR-fluorescent protein constructs, the authors discovered that CD19-28ζ CARs had a uniform distribution across the T cell membrane but that GD2-28ζ CARs aggregated in 'punctae' (188). Although the GD2 ScFv used in this study was 14g2a, further investigation is required as to whether the ScFv HuK666 contained in our GD2-28ζ

construct is also causing the same phenomenon; i.e. ScFv clustering leading to CAR^+ signalling. A second explanation is whether the process of genomic integration causes a degree of 'genotoxic' stress leading to self-activation and upregulation of co-inhibitory receptors.

Vδ1 cells had the least 'exhausted' phenotype with little up-regulation of TIM3 and PD1 following transduction compared to CAR^+ αβ T cells and CAR^+ Vδ2 T cells (% double negative populations; CAR^+ αβ 30.50% ± 3.90, CAR^+ Vδ1 70.48% ± 12.40, CAR^+ Vδ2 33.13% ± 7.21). Comparing double negative populations did achieve statistical significance using a 2-way ANOVA (CAR^+ Vδ1 vs CAR^+ Vδ2 $p<0.001$, CAR^+ Vδ1 vs CAR^+ αβ $p<0.001$), however comparing double positive populations did not.

Together these data demonstrate that CAR Vδ1 cells, obtained by ConA activation and expansion, have a more naïve and less exhausted phenotype than conventional CAR αβ T cells which are both desirable properties for immunotherapy .

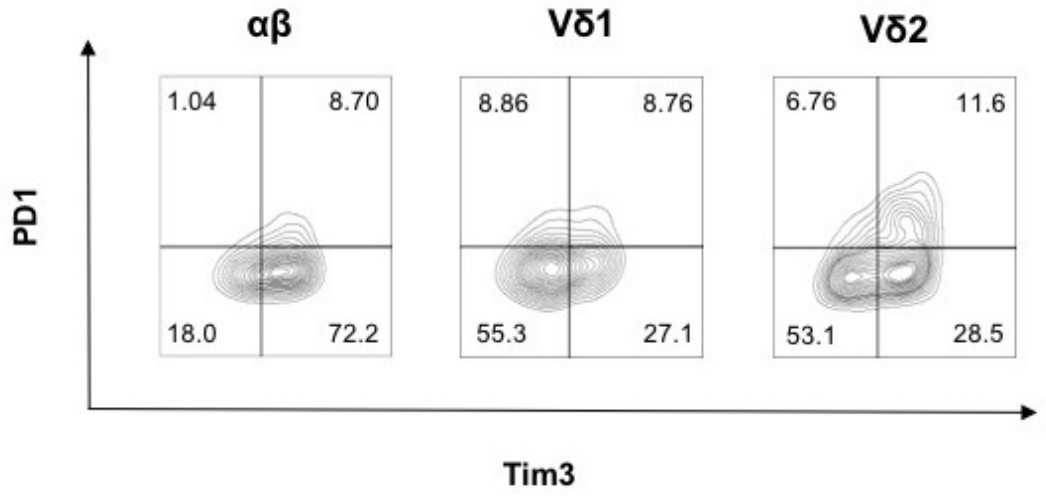


Figure 4.4 Representative FACS plots showing PD1 and Tim3 expression on CAR$^+$ αβ, Vδ1 and Vδ2 T cell subsets

Representative flow cytometry contour plots from a single donor displaying exhaustion marker expression (PD1 and Tim3) on CAR+ αβ, Vδ1 and Vδ2 T cells following 13 day expansion. αβ T cells were activated with CD3/CD28 antibody, Vδ1 with ConA, and Vδ2 with ZOL.

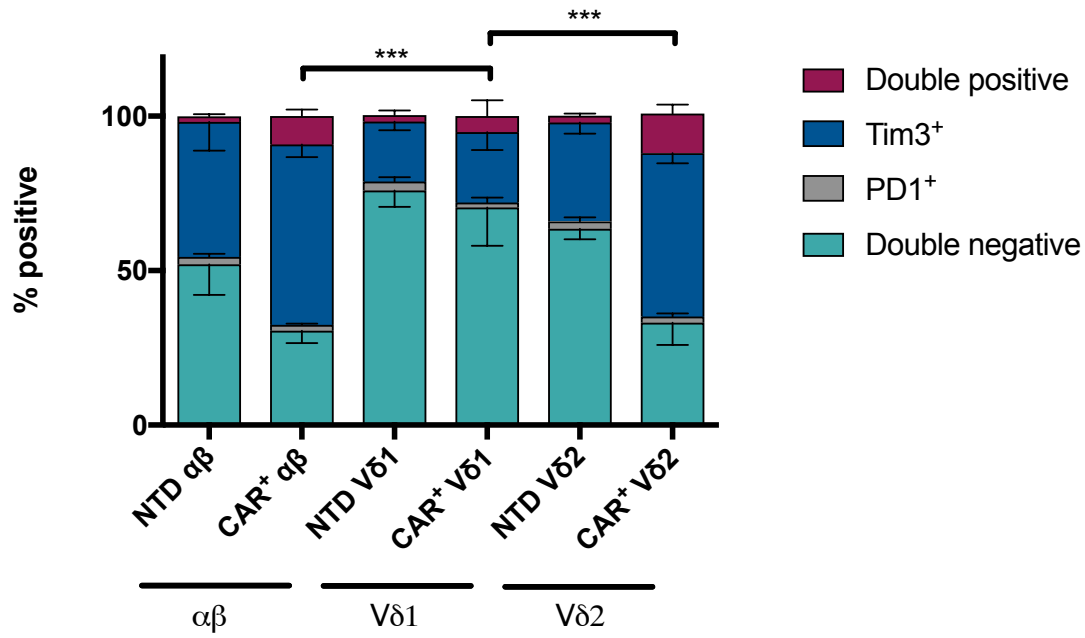


Figure 4.5 Exhaustion marker expression

Expression of PD1 and Tim3 on day 13 post-expanded CAR^+ and CAR^-/non-transduced (NTD) αβ, Vδ1 and Vδ2 cells. αβ T cells were activated with CD3/CD28 antibody, Vδ1 with ConA, and Vδ2 with ZOL. Data represented as mean ± SEM; αβ and Vδ2, n = 6; Vδ1, n = 3; Statistical comparisons were made with 2-way ANOVA (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$). Significant p values shown with brackets compare double negative populations.

4.2.4 Bulk populations of CAR^+ γδ T cells demonstrate antigen specific cytotoxicity

Non-transduced, expanded γδ T cells have been shown to kill a variety of tumours (134, 139, 144, 219, 220), and our group has demonstrated that killing of $GD2^+$ neuroblastoma cell lines is enhanced by the addition of opsonising antibody (40). Susceptibility to killing by Vδ2 cells can also be augmented by target sensitisation with ZOL (221).

We have previously shown that non-transduced aAPC-expanded Vδ1 cells are capable of antibody independent killing of certain $GD2^+$ neuroblastoma cell lines, but that

innate killing by Vδ2 cells is minimal (40). Consequently, I hypothesised that introduction of CAR into Vδ2 T cells would lead to specific killing of $GD2^+$ cell lines without the requirement for prior antibody opsonisation. To investigate the specific killing by CAR^+ Vδ2 cells, I used standard 4 hour 51Chromium release assay. Initially I evaluated the killing properties of these cells using bulk populations of ZOL-activated transduced cells. Tumour targets were chosen for their expression of GD2; for neuroblastoma, $GD2^+$ LAN1 and GD2- SK-N-SH. For a direct comparison of antigen-specific cytotoxicity it was necessary to use the same tumour cell line with or without GD2 expression. Accordingly, a lymphoblastic leukaemia cell line (SupT1) was engineered to express either GD2 or ALK (SupT1-GD2 and SupT1-ALK) (reference cell lines were established by Dr Maria Alonso-Ferrero, Post-doctoral Fellow). Bulk populations of ZOL-activated CAR^+ Vδ2 were cytotoxic towards $GD2^+$ LAN1 and SupT1-GD2 (Figure 4.6). The relatively higher killing seen with SupT1-GD2 compared to LAN1 by CAR^+ Vδ2 cells could be due to differences in the cell line properties and may be related to inhibition by LAN1 cells (although this difference did not achieve statistical significance at any E:T ratio using 2-way ANOVA). An alternative explanation is that differential killing was secondary to disparities in GD2 antigen density expressed on the cell surface. Personal communication with another PhD student in our laboratory (Aysha Patel), who performed antigen quantification experiments, confirmed that SupT1-GD2 had $1x10^6$ GD2 molecules per cell, whereas LAN1 had significantly fewer ($5x10^5$). These experiments were performed using the QIFIKIT that interpolated the MFI of bound GD2 antibody using a calibration curve produced from standardised antibody coated calibration beads. It is most likely, however, that both cell lines express GD2 above the required threshold for activation and effective synapse formation.

Interestingly, it was also observed that the killing of $GD2^-$ targets (SK-N-SH and SupT1-ALK) was higher for SupT1-ALK than for SK-N-SH (although again, this difference was not statistically significant). This could partially be explained by the different susceptibility to innate killing by Vδ2 cells of different tumour cell lines. As reported in the literature, certain cell lines are highly responsive [e.g. Daudi (76)] where as others appear resistant [e.g. neuroblastoma cell lines; Kelly, LAN1, SK-N-

DZ (40)], and the determinants of this innate reactivity are likely to be related to the expression of ligands for innate receptors expressed on γδ cells e.g. NKp30, KIR, DNAM, TRAIL, FAS, NKG2D.

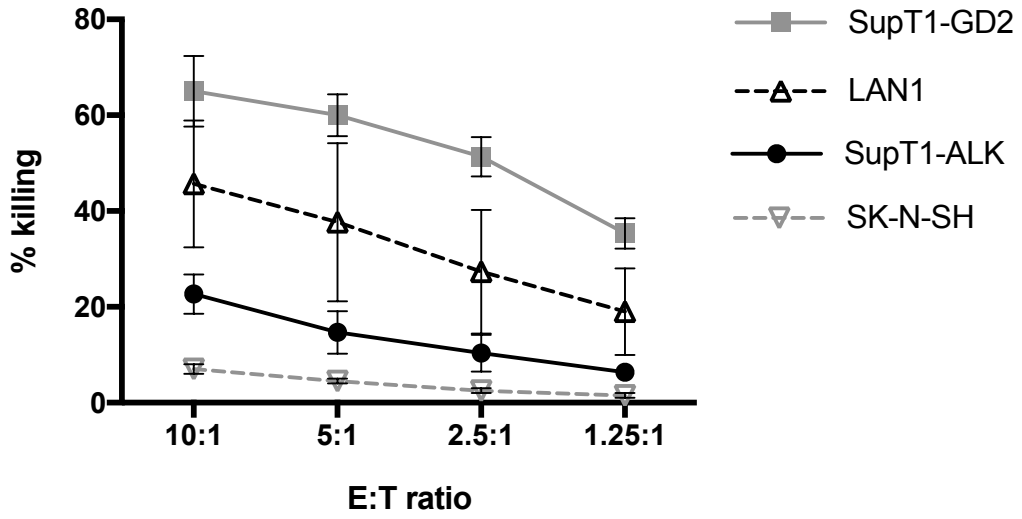


Figure 4.6 Bulk populations of GD2-CAR$^+$ Vδ2 T cells specifically lyse GD2$^+$ tumour targets

Standard 4-hour ^{51}Chromium assay was performed using bulk populations of ZOL-activated GD2-CAR$^+$ transduced Vδ2 cells against GD2$^+$ LAN1 and SupT1-GD2, and GD2$^-$ SK-N-SH and SupT1-ALK as targets.

Following the evaluation of bulk ZOL-activated CAR$^+$ Vδ2 cells, I sought to compare the cytotoxicity of bulk populations of CD3/CD28 antibody, ZOL and ConA-activated cells. As can be seen in Figure 4.7, killing by the three different bulk populations was broadly similar against LAN1. At this point in time, a MSc student (Gabriel Benthall) was evaluating CD3/CD28 antibody and ConA cytotoxicity and this provided a useful comparison. It should be noted however that as these experiments do not have donor matching between the different activation methods, and took place at different time points, caution should be taken when interpreting results. The different E:T ratios used should also be highlighted.

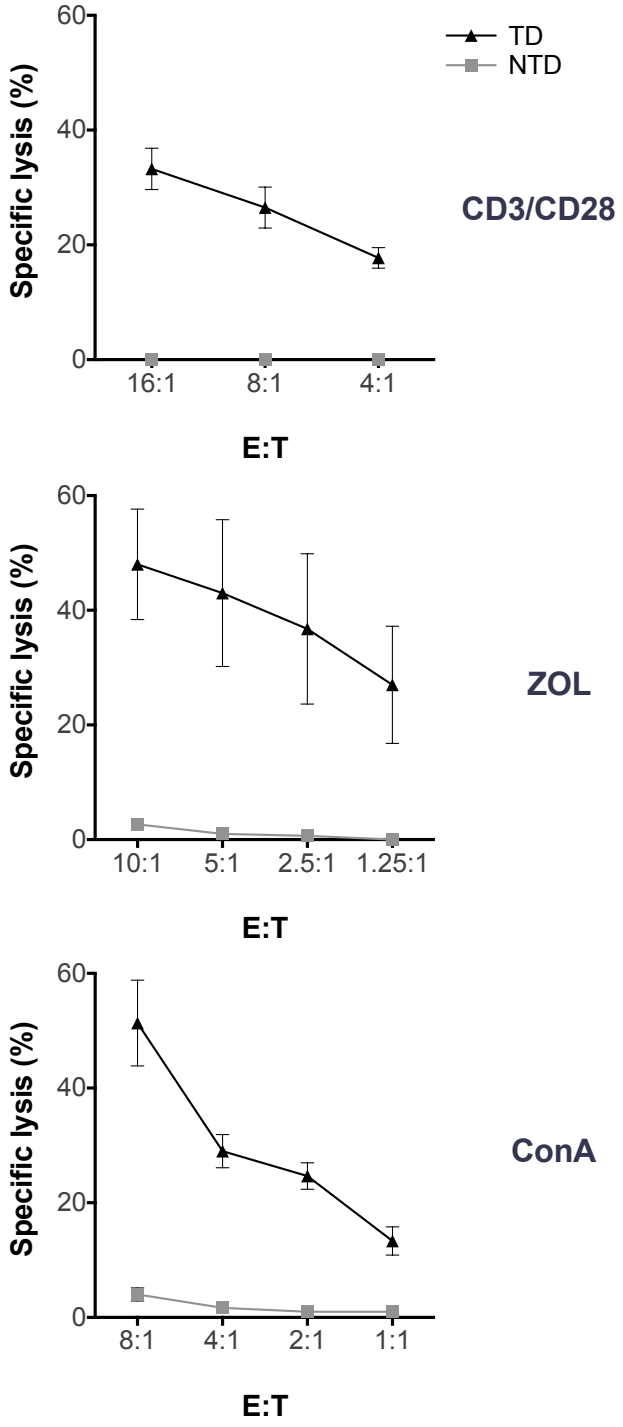


Figure 4.7 Comparison of killing by bulk populations of GD2-28ζ CAR-transduced CD3/CD28 antibody, ZOL, or ConA-activated T cells.

Bulk populations of CD3/CD28 antibody, ZOL or ConA-activated T cells were transduced with GD2-CAR$^+$ (TD) and standard 4-hour ^{51}Chromium assay performed using GD2$^+$ LAN1 cells as targets. Cytotoxicity assay using CD3/CD28 antibody activated T cells was performed by Dr Barry Flutter and ConA-activated cells by MSc student, Gabriel Benthall. Comparison is made to my own data using ZOL-activation.

4.2.5 Purified populations CAR$^+$ αβ, Vδ1 and Vδ2 T cells are each capable of antigen-specific cytotoxicity

Due to bulk populations of CD3/CD28 antibody, ZOL and ConA-activated cells having different T cell subset composition and levels of CAR expression, I wanted to investigate the specific contribution to cytotoxicity by individual T cell subsets. This enabled me to compare the relative short-term antigen-specific cytotoxicity of CAR$^+$ αβ, Vδ1 and Vδ2 T cells to ascertain whether one subset had superiority over another.

Bulk populations of CD3/CD28 and ZOL activated cells were transduced on day 3 and day 5 respectively with GD2-CAR. Cells were cultured for 13 days to achieve sufficient cell number for fluorescent activated cell sorting (FACS) on day 14. Transduced CD3/CD28 antibody-activated cells were stained with CD56 and QBend10 antibodies and CD56$^-$/QBend10$^+$ cells were selected for further analysis. Transduced ZOL-activated cells were stained with Vδ2 and QBend10 and Vδ2$^+$/QBend10$^+$ cells were selected for further analysis. Over 95% purity was achieved using FACS. Non-transduced control CD3/CD28 antibody activated cells were depleted of CD56$^+$ Natural Killer (NK) cells by CD56 MicroBeads, and non-transduced ZOL activated cells were isolated using the TCRγδ isolation kit (refer to 2.2.3.2) .

As shown in Figure 4.8 specific tumour cell lysis by ZOL activated CAR Vδ2 cells were broadly equivalent to that observed with CD3/CD28-activated CAR αβ T cells. Tumour cell killing by non-transduced (NTD) cells of all subsets was minimal. For particular GD2 positive tumour cells (LAN1), and at the highest E:T ratio (10:1), CAR Vδ2 cells had higher specific lysis than CAR αβ T cells (Figure 4.9). This statistical difference was not apparent at lower E:T ratios or against SupT1-GD2. Although CAR Vδ2 may have enhanced cytotoxicity over CAR αβ T cells, it should also be highlighted that FACS sorted αβ T cell populations contained both CD4 and CD8, and one may speculate that different results may have transpired if sorted CAR$^+$ CD4, CD8 and Vδ2 were directly compared.

There was relatively little innate background killing by non-transduced T cells against GD2$^-$ SK-N-SH. For GD2 antigen-negative SupT1-ALK there was higher innate killing by CAR$^+$ Vδ2 compared to non-transduced Vδ2, and CAR$^+$ αβ T cells (Figure 4.8) and again, this may represent an activated or 'licensed' state following CAR genomic integration.

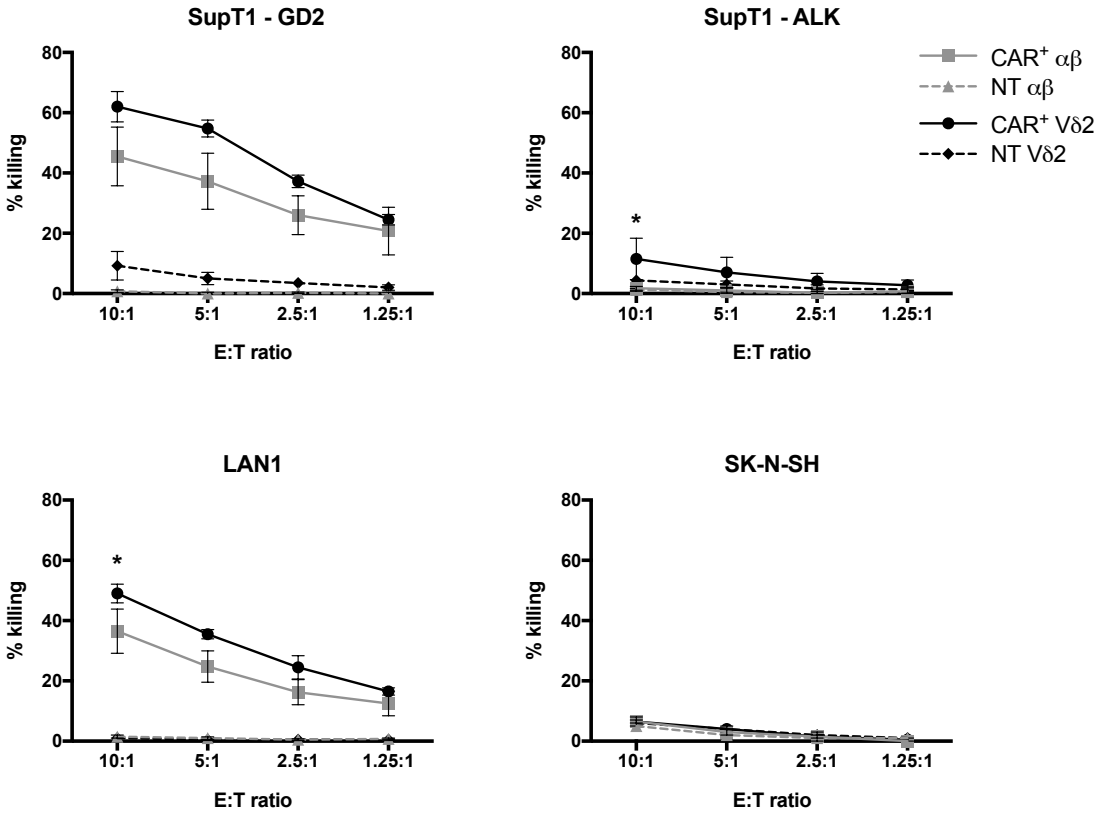


Figure 4.8 Purified FACS sorted populations of CAR$^+$ αβ and Vδ2 T cells are capable of GD2-specific tumour cell lysis

GD2-CAR transduced αβ and Vδ2 were sorted to >95% purity by fluorescent-activated cell sorting. Non-transduced cells underwent magnetic-activated cell sorting (MACS). αβ T cells were stimulated with CD3/CD28 antibody, Vδ2 with ZOL, and cytotoxicity was evaluated in 4 hour ^{51}Cr release assay using GD2$^+$ SupT1-GD2 and LAN1 and GD2$^-$ SupT1-ALK and SK-N-SH. The antigen-specific nature of the cytotoxicity was confirmed by effective killing of SupT1-GD2 with negligible killing of SupT1 transduced with irreverent control antigen (SupT1-ALK). Mean ± SEM, n=4 for SupT1-GD2, SupT1-ALK and LAN1, n=2 for SK-N-SH. Statistical comparisons were made with 2-way ANOVA with Bonferroni's multiple comparisons test (* $p<0.05$) Significant p values shown compare CAR$^+$ αβ and CAR$^+$ Vδ2 populations.

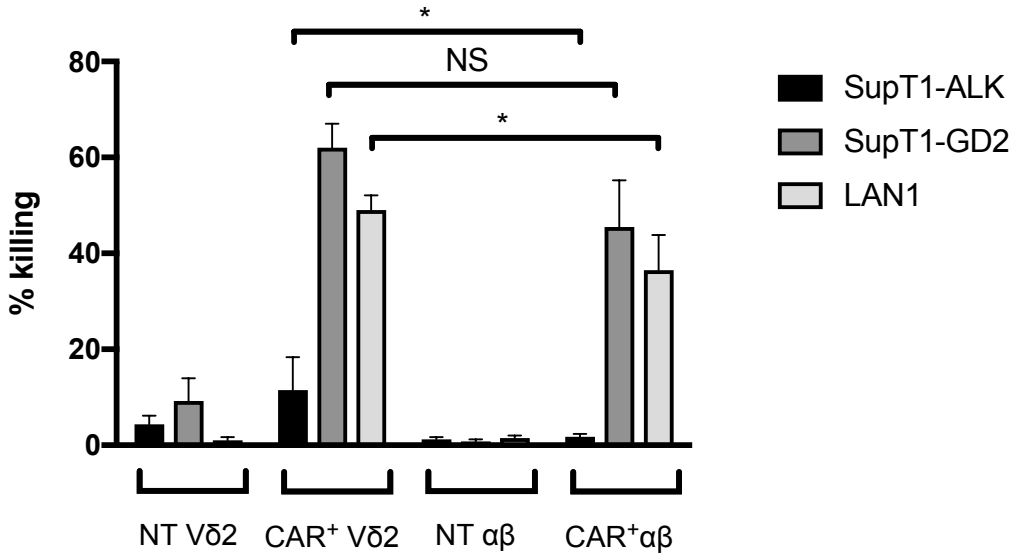


Figure 4.9 Purified CAR αβ and CAR Vδ2 demonstrate antigen specific cytotoxicity against GD2-positive tumour cell lines by ^{51}Chromium release assay at 10:1 effector to target ratio.

Data from Figure 4.8 is presented in bar chart format to directly compare cytotoxicity at 10:1 E:T ratio. Statistical comparisons were made with 2-way ANOVA with Bonferroni's multiple comparisons test (* $p<0.05$), 4 individual donors.

4.2.5.1 Comparison of ADCC and CAR$^+$ mediated killing by Vδ2 cells

ADCC by Vδ2 T cells is a recognised killing mechanism, and hence combinational therapy of GD2 monoclonal antibody with non-transduced Vδ2 cells a valid immunotherapy approach without the need for genetic modification. Consequently, I next compared the cytotoxicity of sorted non-transduced Vδ2 plus Ch14.18 antibody with sorted GD2-CAR$^+$ Vδ2 against GD2$^+$ LAN1 (Figure 4.10). No significant difference was found in the level of cytotoxicity against LAN1 comparing ADCC with CAR-mediated killing. Further discussion of the additional advantages of CAR-transduced Vδ2 is considered in Section 4.3.

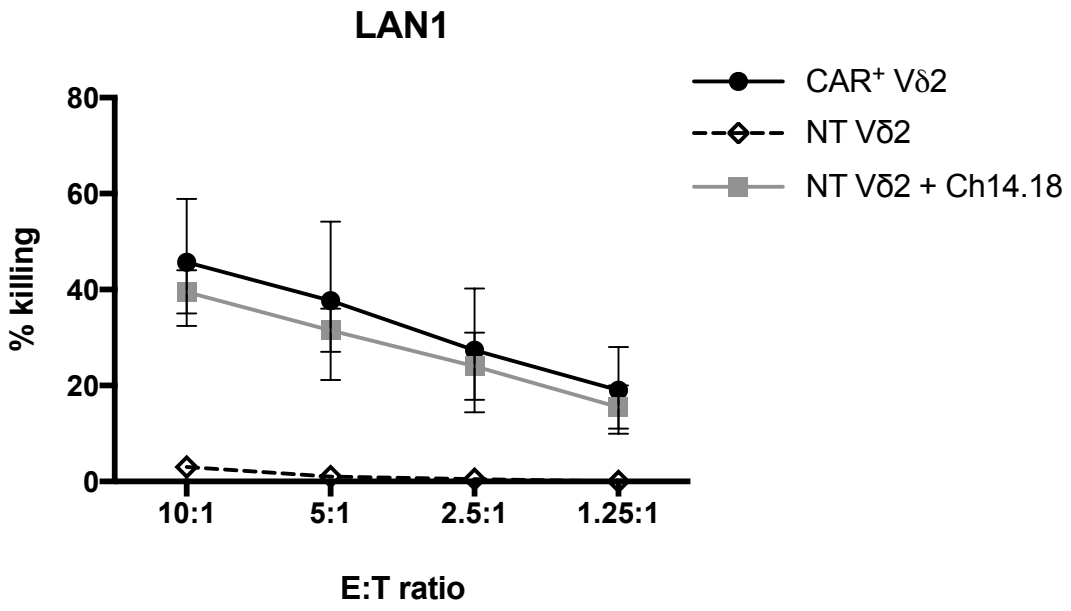


Figure 4.10 Comparison of killing by ZOL-activated CAR$^+$ Vδ2 and non-transduced (NT) Vδ2 in the presence and absence of LAN1 target opsonisation with Ch14.18 antibody.

CAR$^+$ Vδ2 cells were purified by FACS and non-transduced cells by MACS as previously described. Vδ2 cells were activated with ZOL and cytotoxicity was evaluated using standard ^{51}Chromium release assay. ADCC was evaluated by opsonisation of target cells with GD2 antibody (Ch14.18). 3 individual donors.

4.2.5.2 Involvement of γδTCR and NKG2D receptors in CAR-mediated Vδ2 killing

I hypothesised that a possible mechanism for the enhanced killing by CAR$^+$ Vδ2 in 4 hour ^{51}Chromium release assays compared with CAR$^+$ αβ T cells could involve the recruitment of receptors such as the TCR and NKG2D which are known to be important in innate killing by γδ T cells. A key characteristic of γδ T cells that enables them to recognise transformed (including malignant) cells is that they express NK receptors including NKG2D (203). NKG2D ligands (MIC-A, MIC-B and ULBP) are also upregulated in response to cellular stress (222). There is debate whether NKG2D has a primary stimulatory or costimulatory role in activating γδ T cells [reviewed in (81, 223)], therefore I carried out blocking experiments, to investigate whether CAR Vδ2 killing was effected by either NKG2D or γδTCR blockade. CAR$^+$ Vδ2 cells were

FACS sorted, and non-transduced Vδ2 MACS selected as previously described. Effector cells were incubated with either anti-γδTCR, anti-NKG2D or isotype control antibody, and standard 4 hour ^{51}Chromium release assay performed using LAN1 targets. Two donors are represented in Figure 4.11. Antibody blocking did not enhance or reduce CAR Vδ2 cytotoxicity and background killing by non-transduced cells in all groups was minimal. Due to the large numbers of donor cells required, expense of FACS sorting, and initial negative findings, this experiment was carried out as a pilot study with n=2, therefore should be interpreted with caution.

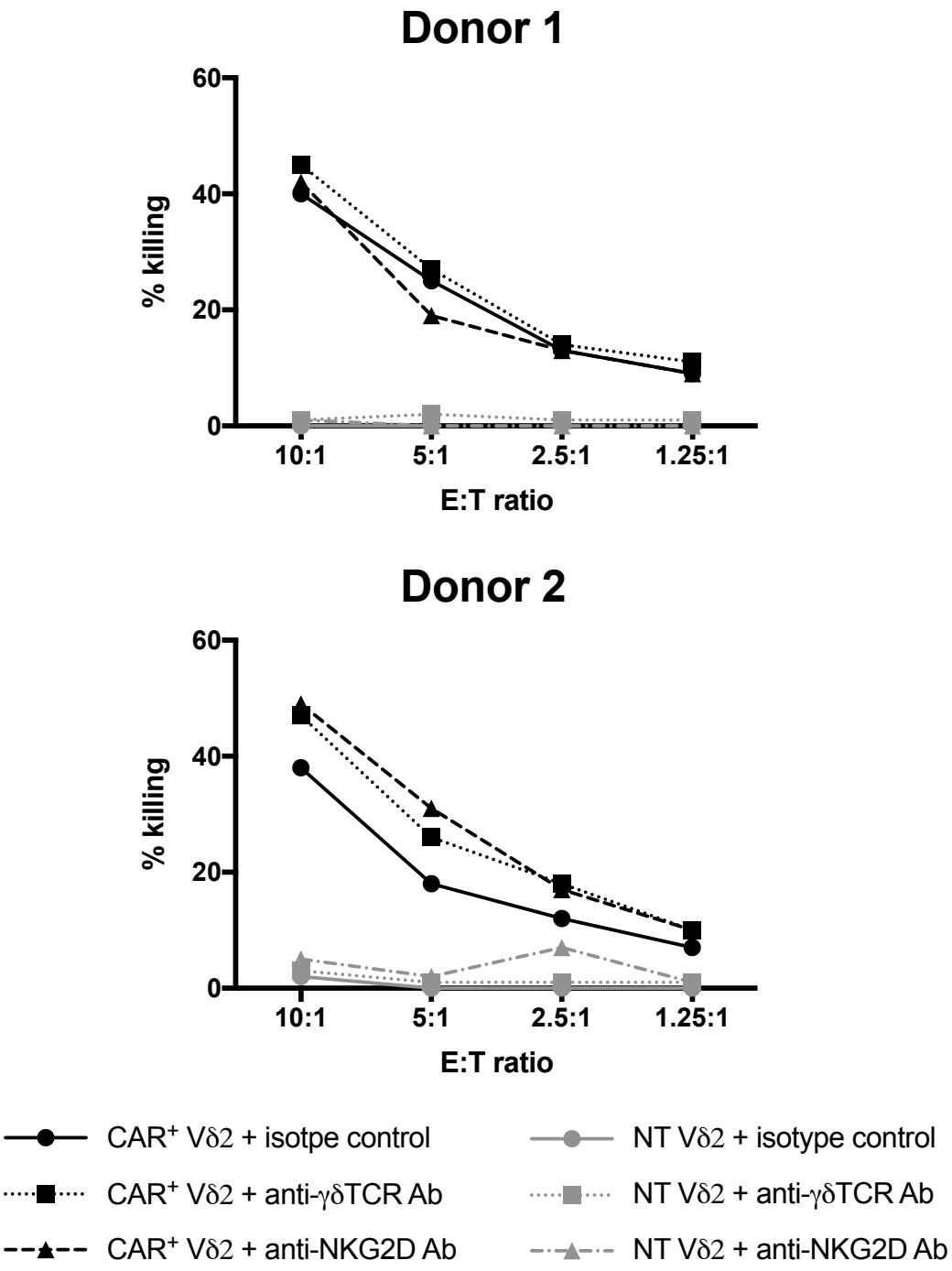


Figure 4.11 TCRγδ and NKG2D blockade

CAR^+ Vδ2 were sorted to >95% purity by FACS and non-transduced (NT) cells underwent MACS. Effector cells were incubated with either γδTCR blocking antibody, NKG2D blocking antibody, or isotype control antibody prior to assessing cytotoxicity in 4-hour Cr^{51} release assay with LAN1 targets. Mean of experimental triplicate shown for each donor tested (donor 1 and donor 2), Pilot study n=2.

4.2.5.3 CAR$^+$ Vδ1 cytotoxicity

Vδ1 cells are an intriguing population with desirable properties including innate killing, enhanced tumour infiltration, and production of anti-tumour cytokines. Deniger *et al*. (164) described the cytotoxicity of a polyclonal γδ T cell repertoire engineered with CAR$^+$, but the individual contribution by γδ T subsets was not established. Following the optimisation of ConA expansion and transduction, we were able to FACS sort pure populations of CAR$^+$ and CAR$^-$ αβ, Vδ1 and Vδ2 for use in cytotoxicity assays. γδ T cells were positively selected using TCRγδ MicroBeads prior to FACS in order to reduce the total cell number for sorting. Cells were then labelled with αβ, Vδ1, Vδ2, and QBend10 before sorting into the respective T cell populations. The following cytotoxicity assay comparing CAR$^+$/transduced (TD) and non-transduced (NTD) subsets was performed in conjunction with Gabriel Benthall (MSc student) (Figure 4.12). Cytotoxicity against LAN1 cells was similar between TD subsets with little background or innate killing by all NTD cells. The lack of innate cytotoxicity by non-transduced Vδ1 was unexpected as these cells are recognised as having powerful tumoricidal activity (53) which is dependent on activation by stress-induced antigens frequently expressed by solid tumours (including MIC-A/B). Vδ1 innate killing of neuroblastoma cell lines has additionally been reported by Fisher *et al*. using aAPC to propagate polyclonal γδ T cells (40), however each cell line is likely to have different sensitivity to innate killing, due to variations in their immunosuppressive properties and innate ligand expression. Different culture methods used to expand cells can also influence Vδ1 differentiation and impact on innate receptor upregulation (e.g. NKp30 and NKp44) (56).

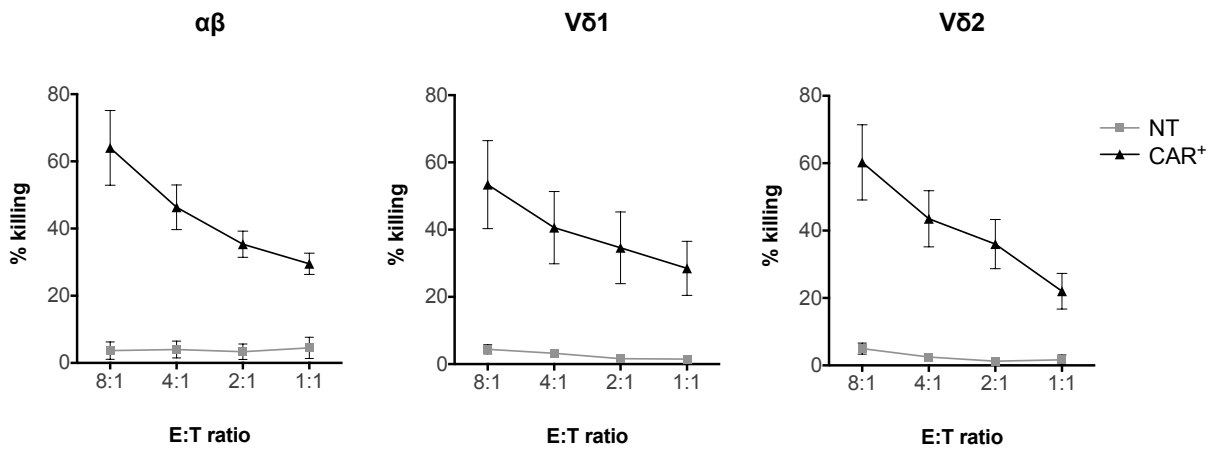


Figure 4.12 Sorted CAR$^+$ αβ, Vδ1 and Vδ2 demonstrate similar antigen specific cytotoxicity against LAN1

Populations of ConA-activated non-transduced (NT) and CAR$^+$ αβ, Vδ1, and Vδ2 T cells were FACS sorted to >95% purity and co-cultured with LAN1 for 4-hr ^{51}Cr release assay. Mean ± SEM; n=3. Experiment performed by Gabriel Benson (MSc student).

4.2.6 CAR$^+$ γδ T cells produce IFNγ and granzyme B in response to engagement with target antigen

CAR$^+$ T cells produce multiple cytokines and chemokines in response to antigen recognition which collectively contribute to the development of potent and long-lasting protection against cancer (93). To investigate the cytokine profile produced by CAR γδ T cells *in vitro*, co-cultures of irradiated tumour targets (LAN1, SupT1, SupT1-GD2) and CAR$^+$ Vδ2 T cells were set up at a 1:1 ratio for 48 hours. Supernatant was harvested and IL-2, IL-4, IL-17, IL-10, IFNγ, TNFα and Granzyme B (GZM-B) measured flow cytometrically by cytokine bead array (Figure 4.13). There was significantly more GZM-B and IFNγ production in CAR αβ T cell cultures compared to CAR γδ T cell cultures with LAN1 (GZM-B mean concentration ± SEM: CAR αβ 4290.77 pg/ml ± 283.28, CAR Vδ2 1699.76 pg/ml ± 326.55, n=3, $p<0.05$) (IFNγ mean concentration ± SEM: CAR αβT 2063.92 pg/ml ± 179.64, CAR Vδ2 540.06 pg/ml ± 200.37, n=3, $p<0.05$). There was also a general trend towards higher IL-2, IL-10, TNFα, IFNγ and GZM-B production by CAR αβ T cells on co-culture

with both GD2 positive tumour targets tested (LAN1 and SupT1-GD2). For GD2 negative targets (SupT1) or cultures containing no targets, αβ T cells appeared to produce substantial GZM-B, however as illustrated in Figure 4.14 there was a single outlier leading to overall skewing of results. Interestingly, CAR αβ produced IL-17 (albeit a very small amount) whereas CAR Vδ2 cells did not, and CAR Vδ2 produced very little IL-2 (Figure 4.13). Neeson *et al*. (224) found that $CD8^+$ T_{EM} cells secrete IFNγ, but have low proliferative response to antigen whereas in contrast T_{CM} secrete IL-2 and proliferate on antigen recognition. The concentration of cytokines produced by CAR αβ T cells is therefore likely to be dependent on their state of differentiation. Referring back to Figure 4.3, and the memory phenotype of CD3/CD28 antibody cultured CAR^+ αβ T cells, there were mixed populations of T_{CM} and T_{EM} which is likely to account for the pattern of cytokine production seen. γδ T cells, in contrast to αβ, are known to produce minimal amounts of IL-2 following activation, and proliferation is dependent on other sources of IL-2 production (such as neighbouring $CD4^+$ cells) (225). This is an important finding as the success of adoptive CAR γδ T cell therapy may be dependent on exogenous administration of IL-2 or combinational therapy with infused 'helper' cells (i.e. combined CAR αβ and γδ T cells).

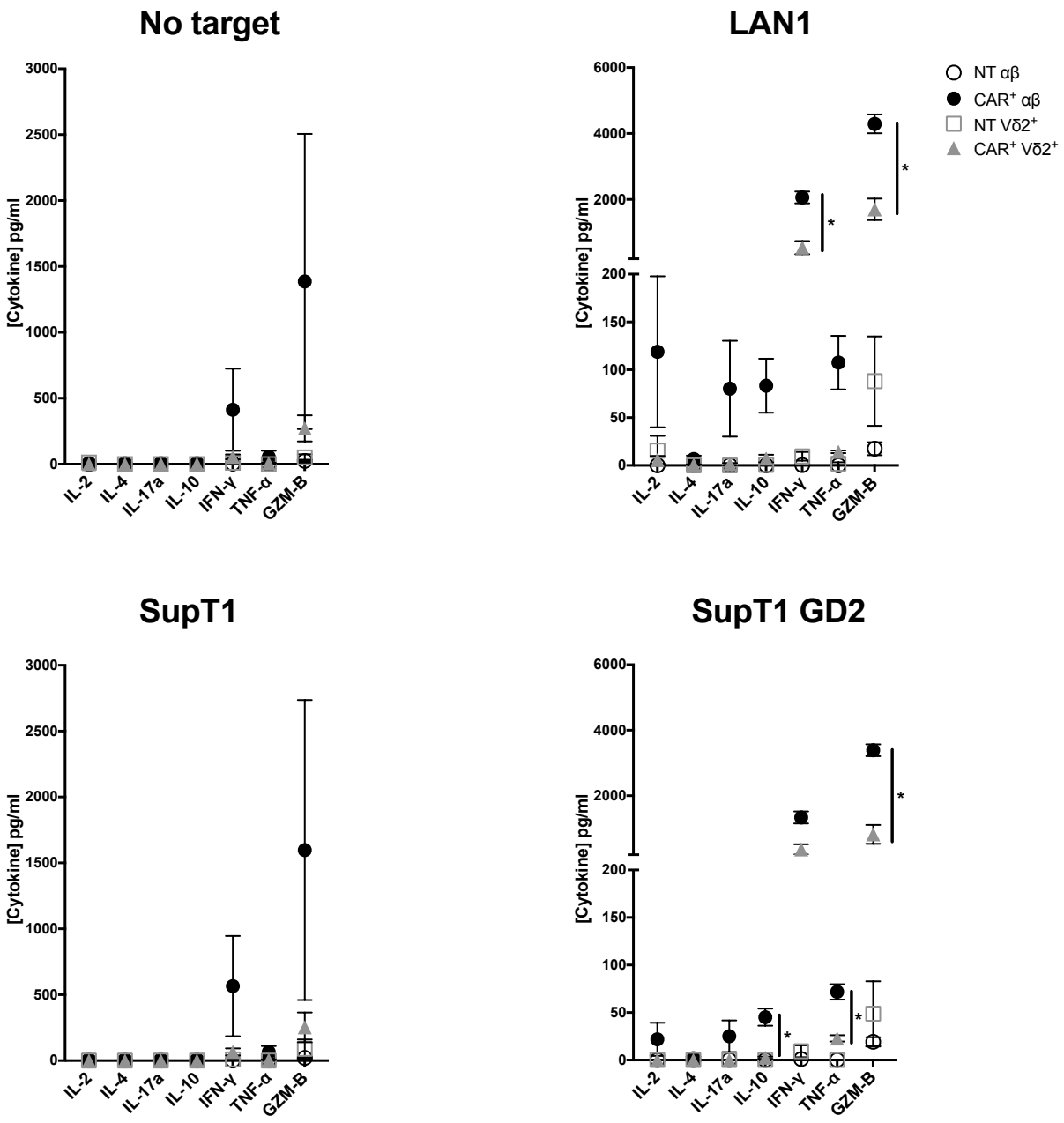


Figure 4.13 Cytokine production by CAR$^{+/-}$ αβ and Vδ2 T cells in the presence of GD2$^{+/-}$ targets

Non-transduced (NT) and CAR^{+} αβ and Vδ2 were co-cultured at a 1:1 ratio with GD2^{-} (SupT1) and GD2^{+} (SupT1 GD2 and LAN1) irradiated target cells. Supernatant was harvested at 48 hours for cytokine quantification by cytokine bead array. Mean ± SEM, n=3. * $P<0.05$ by two-tailed Student's t-test.

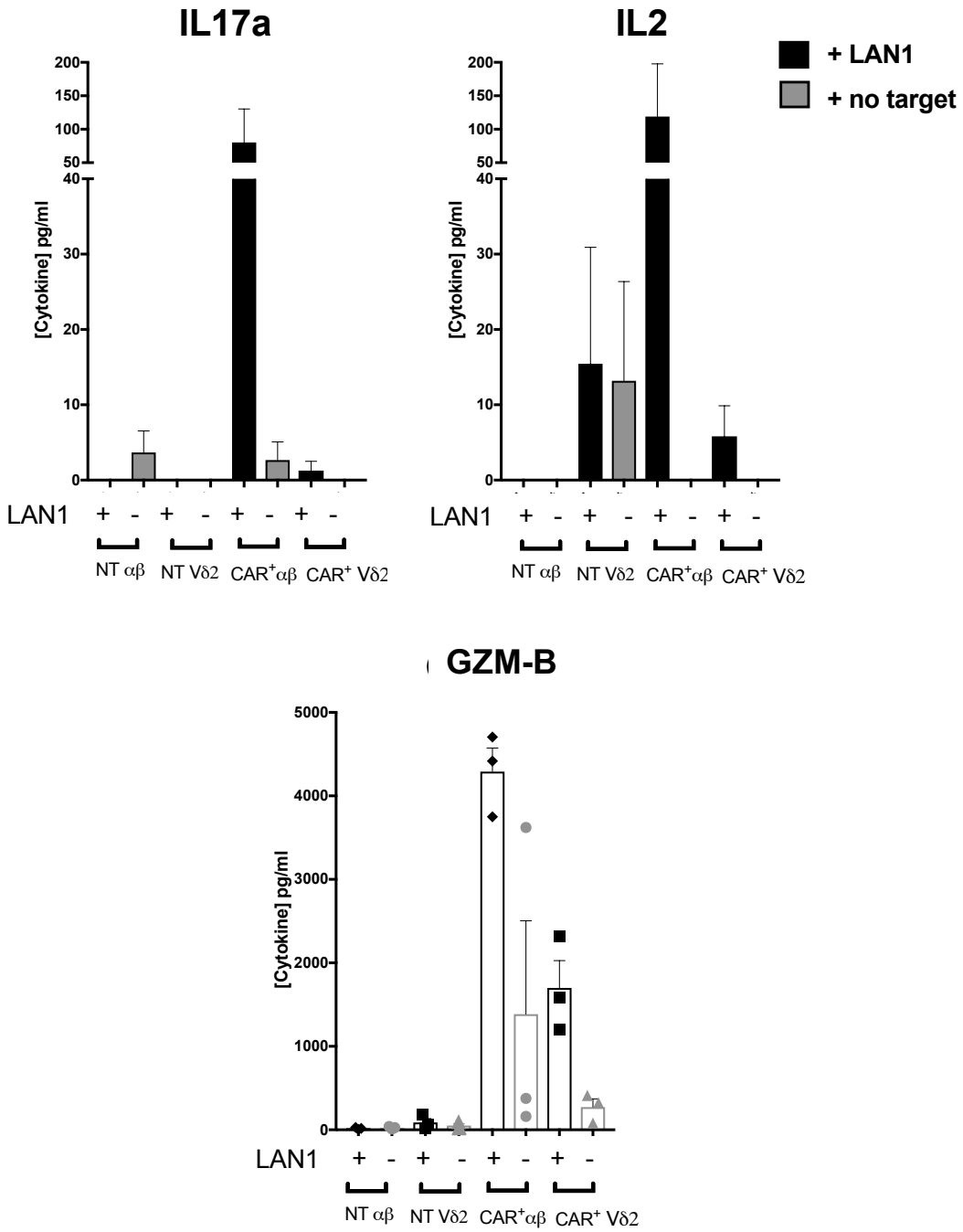


Figure 4.14 Specific cytokines in detail

4.2.7 Expanded CAR$^+$ Vδ2 cells display immediate but not sustained antigen-specific proliferation

The ability of CAR T cells to survive and proliferate following adoptive transfer is paramount to their efficacy. There is a fine balance between inducing a rapid and potent cytotoxic response (with the risk of inducing T cell anergy or AICD), and a more sustained response combined with memory formation. To investigate the proliferative capabilities of CAR Vδ2 cells in response to antigen encounter, I carried out co-culture experiments using irradiated tumour targets. Irradiation prevented outgrowth of the tumour cells over the culture period whilst still providing antigenic stimulation. Antigen-specific CAR Vδ2 proliferation was evaluated using both GD2$^+$ and GD2$^-$ targets.

Day 14 ZOL-activated Vδ2 or CD3/CD28-activated αβ T cells were co-cultured at a 1:1 ratio with irradiated LAN1, SupT1-GD2, SupT1 or no target. Proliferation was calculated by manual cell counting using light microscopy and trypan blue exclusion after 48 hours and at 7 days. Both CAR$^+$ αβ and Vδ2 proliferated in response to GD2-expressing LAN1 and SupT1-GD2 target cells over 48 hours, indicated by increased fold change (Figure 4.15) (mean fold change ± SEM: CAR$^+$ αβ + LAN1: 2.33 ± 0.255 n=3, CAR$^+$ Vδ2 2.173 ± 0.875 n=3). CAR T cells did not proliferate in the absence of targets or GD2$^-$ SupT1 over 48 hours. A significance difference was seen after 7 days with continued proliferation of CAR$^+$ αβ T cells with GD2$^+$ targets, compared to CAR$^+$ Vδ2 T cell contraction (mean fold change ± SEM: CAR$^+$ αβ + LAN1; 4.033 ± 0.285 n=3, CAR$^+$ Vδ2 0.80 ± 0.265, n=3). Interestingly there was some proliferation of non-transduced αβ T cells with SupT1-GD2 (although there is one significant outlier, n=3) and a trend towards increased proliferation by CAR-transduced cells even in the absence of GD2 expressing targets (SupT1 and no target) at 7 days but not 48 hours.

Antigen-specific proliferation was also evaluated using purified FACS sorted CAR$^+$ Vδ2 cells where a mean of 2.74 ± 1.187 fold expansion was observed over 3 days in

CAR^+ Vδ2 cells in the presence of LAN1, whilst there was no increase in cell numbers for non-transduced Vδ2 (1.136 ± 0.105). Both CAR^+ and NT Vδ2 failed to proliferate in the absence of antigen (Figure 4.16).

Although an initial proliferative response was observed in the presence of GD2 antigen for both bulk and purified CAR^+ Vδ2 populations, this was not sustained over the 7 day *in vitro* culture. Possible explanations as to why this occurred in Vδ2 cultures and not αβ T cells, could be due to the dependency on IL-2, where activated T_{CM} αβ T cells are capable of self-production whereas T_{EM} Vδ2 are not. Additionally, Vδ2 are documented to have increased sensitivity to activation induced cell death (226) which is also likely to be affected by the presence of CAR^+.

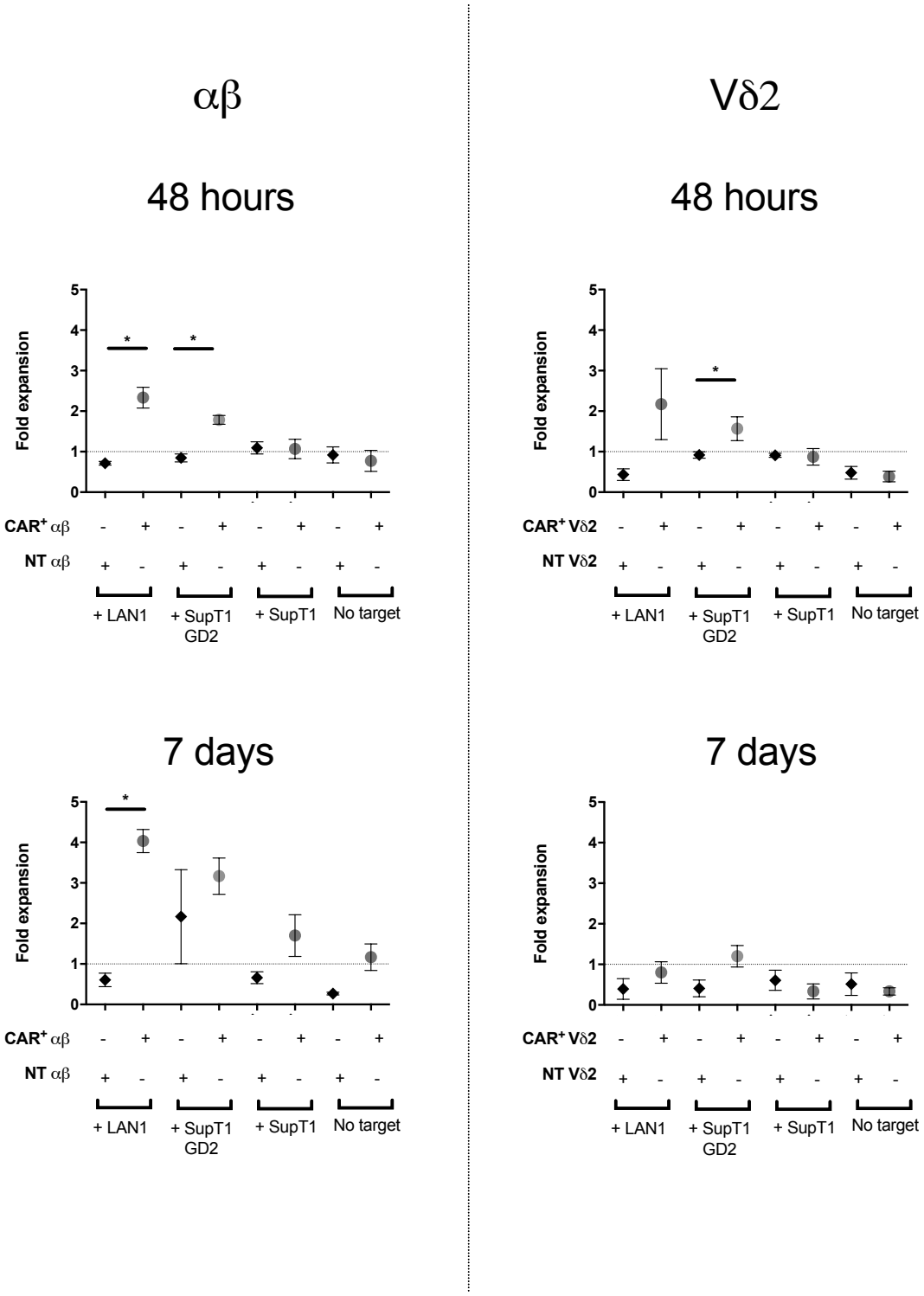


Figure 4.15 CAR Vδ2 T cells show a lack of sustained proliferative response on co-culture with LAN1 targets

Bulk CD3/CD28-expanded αβ T cells and ZOL-expanded Vδ2 were cocultured with irradiated tumour cell lines (LAN1, SupT1-GD2, or SupT1) at a 1:1 ratio. Fold expansion was calculated by counting the total number of live T cells by light microscopy with trypan blue exclusion. Data, mean ± SEM, 3 individual donors. * $P<0.05$ by two-tailed Student's t-test.

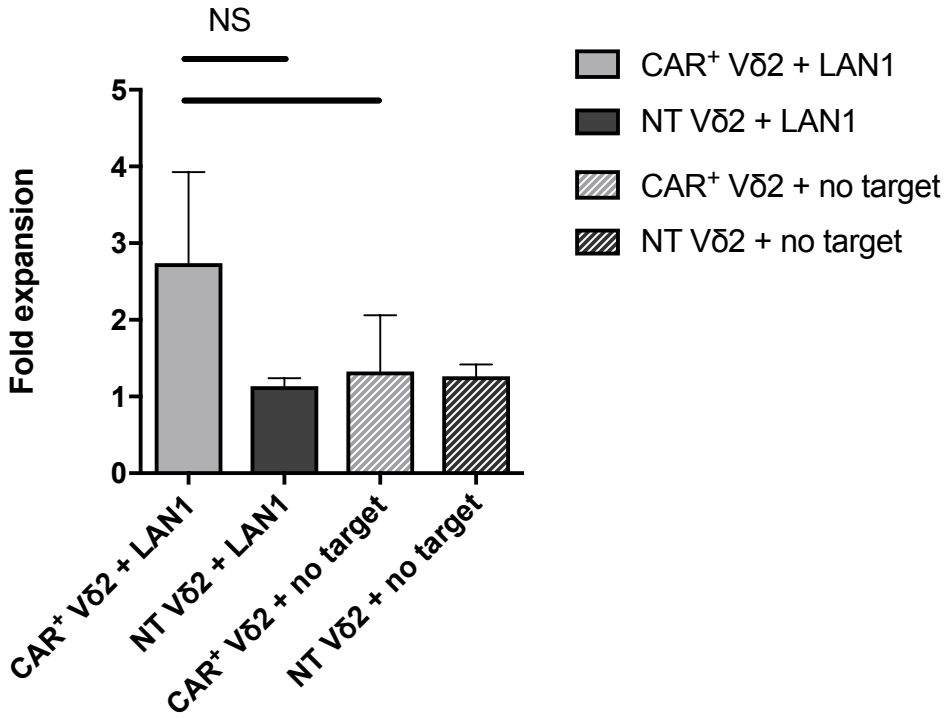


Figure 4.16 72 hour proliferation of FACS purified CAR$^+$ and CAR$^-$ Vδ2 populations in response to GD2 target antigen encounter

ZOL-activated CAR$^+$ and CAR$^-$ (NT) Vδ2 were sorted by FACS (CAR$^+$) or MACS (NT) and co-cultured with irradiated LAN1 cells for 72 hours, in the absence of IL-2. Fold expansion was calculated by trypan blue exclusion. Data, mean ± SEM, n=4. NS P>0.05 by two-tailed Student's t-test.

4.2.8 CAR$^+$ γδ T cells retain capacity to migrate towards tumour cells

γδ T cells are known to have natural tissue residency (in particular Vδ1 cells), and the association of tumour infiltration by γδ T cells correlates with favourable cancer prognosis (42). Consequently, I speculated whether CAR$^+$ γδ T cells might have favourable migration towards antigen-expressing tumour cells. This hypothesis was tested using 4-hour transwell assays to investigate whether GD2-CAR transduced γδ T

cells were capable of migrating towards $GD2^+$ neuroblastoma targets. γδ T cell activation using ConA was selected to allow evaluation and comparison of the migratory properties of αβ, Vδ1 and Vδ2 T cell subsets. Stromal cell-derived factor-1 (SDF-1) was selected as a positive control based on previous reports of its positive transmigratory effect on both Vδ1 and Vδ2 cells (227).

Firstly, I evaluated the background migration due to T cell motility and found that between a mean of 3.88 – 12.55% for individual subsets traverse the wells in the absence of any tumour cells, supernatant or cytokines in the lower chamber (Figure 4.17). Although there appeared to be a trend towards higher motility in non-transduced αβ, Vδ1 and Vδ2 populations compared to their CAR^+ counterpart, this was not statistically significant. Background T cell motility was calculated for each NT and CAR^+ T cell subtype and subtracted from migration in the presence of stimulus to identify stimulus specific migration (Figure 4.18). It was found that all CAR^+ T cell sub-populations had positive mean values for migration towards both LAN1 and SK-N-SH neuroblastoma cell lines and there was no significant different between CAR^+ αβ , Vδ1, and Vδ2 T cells. It appeared that non-transduced αβ and Vδ2 had higher percentage migration than CAR^+ populations, alluding to an inhibitory role of CAR^+, although again this was not statistically significant. Curiously this difference was less pronounced for CAR^+ and NT Vδ1 cells.

There was increased migration by all T cell subsets towards wells containing cultured neuroblastoma cells as opposed to wells containing only their respective supernatant, although this difference was less striking for $GD2^-$ SK-N-SH. This was to be expected and can be partially explained by CAR^+ T cells being activated by $GD2^+$ LAN1 targets leading to increased cytokine and chemokine production.

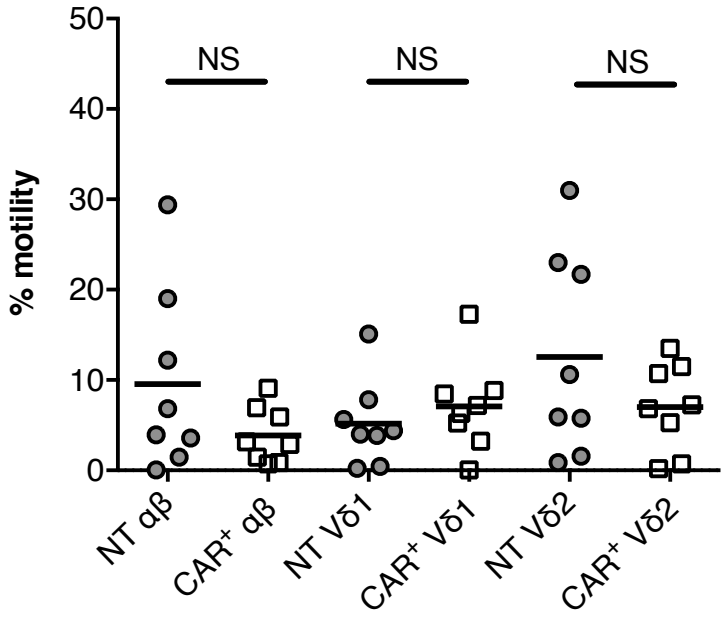


Figure 4.17 T cell motility in the absence of stimulus

Percentage motility of bulk populations of ConA-activated transduced T cells in the absence of stimulus in the lower chamber of a 4-hour transwell migration assay was evaluated. Bulk populations contained both transduced (CAR^+) and non-transduced (NT) αβ Vδ1 and Vδ2. Individual CAR^+/NT T cell subtypes were quantified by flow cytometry using counting beads. NS $P>0.05$ by two-tailed Student's t-test, 8 individual donors.

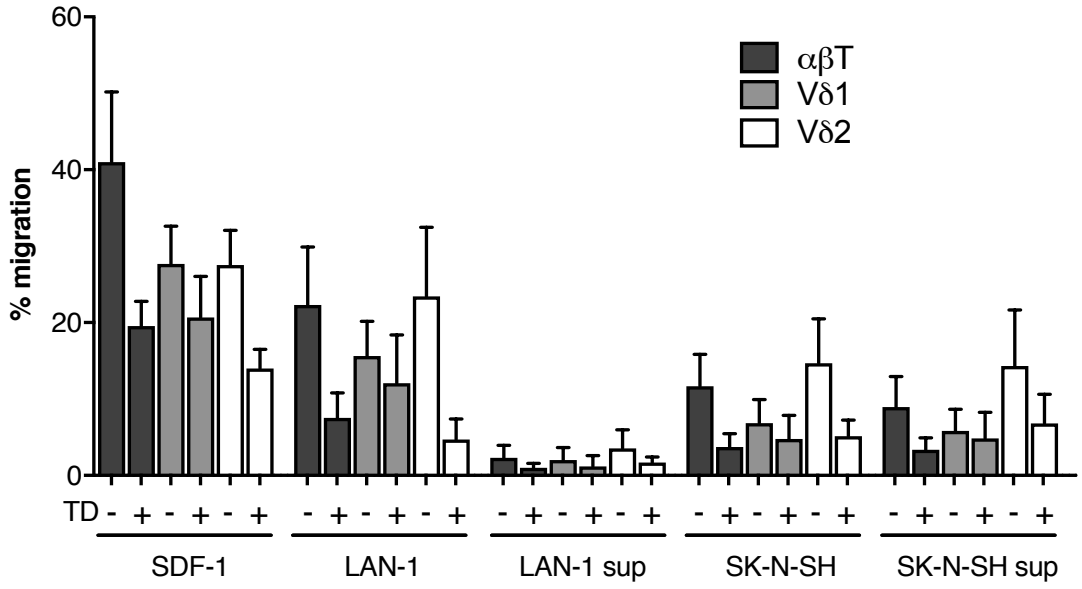


Figure 4.18 Cell line or supernatant dependent migration of CAR$^+$ γδ T cells towards neuroblastoma cell lines and supernatant

CAR T cell migration was assessed by 4-hour transwell migration assay. Bulk populations of ConA activated transduced T cells were investigated for their ability to migrate towards GD2$^+$ LAN1 and GD2$^-$ SK-N-SH neuroblastoma cells or supernatant (sup). Bulk populations used in the chemotaxis assay contained a mixture of both transduced (TD$^+$) and non-transduced (TD$^-$) αβ (dark grey bars), Vδ1 (light grey bars) and Vδ2 (white bars). Individual TD$^+$/TD$^-$ T cell subtypes were quantified by flow cytometry using counting beads. SDF-1 was used as a positive chemokine control. Background motility for each cell subtype was subtracted from migration in the presence of stimulus to identify stimulus-dependent percentage migration. Percentage migration was calculated by (number of migrated cells in the specific condition – number of migrated cells in the negative control for that condition/number of migrated cells in the positive control) x100. Mean ± SEM, 8 individual donors.

Following the use of neuroblastoma cell lines, I next investigated whether supernatant obtained from neuroblastoma neurospheres derived from patient tissue could produce similar responses. Again, there was a parallel trend of higher migration by non-transduced cells compared to CAR$^+$ T cells. There were also marked differences between the three patient samples, with patient 2's supernatant having the highest level of chemo-attraction. This is likely due to the heterogeneity of primary cells used (refer to Table 7).

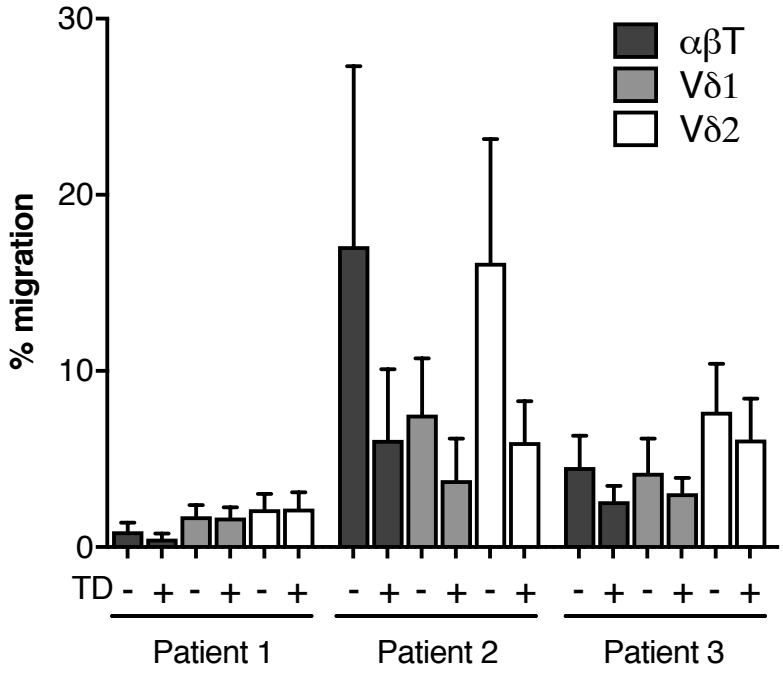


Figure 4.19 Migration of CAR$^+$ γδ T cells towards primary neuroblastoma supernatant.

Percentage T cell migration towards supernatant taken from three neuroblastoma neurosphere lines derived from primary patient tissue. % migration was calculated as previously outlined in Figure 4.18. Mean ± SEM, 8 independent donors.

4.3 Discussion

Our group has previously reported that expanded Vδ1 cells retain innate killing of neuroblastoma cells (40) whilst Vδ2 cells lose cytotoxicity during expansion (40, 59). Our results, however, show negligible innate killing by both Vδ1 and Vδ2 subsets against the LAN1 neuroblastoma cells line, but that this could be augmented by the presence of CAR. A possible explanation for this discrepancy could be due to the different methods used to activate and expand γδ T cells in the two studies.

The key finding of this study was that CAR-dependent cytotoxicity was broadly equivalent in all three T cell subsets studied (αβ, Vδ1, Vδ2). Following FACS purification of individual populations there was a suggestion that CAR Vδ2 cells had higher cytotoxicity than CAR αβ T cells and this reached statistical significance at a

10:1 E:T ratio against LAN1 but was not seen at lower E:T ratios or against other GD2 positive target cells at equivalent ratios.

Expanding CAR αβ T cells from PBMC with soluble CD3/CD28 antibody produces a product with mixed populations of CD4 and CD8 cells. CAR $CD8^+$ T cells use two main cytotoxic pathways; firstly exocytosis of perforin and granzyme containing granules, and secondly expression of TNF-family ligands that induce target cell apoptosis on binding to their respective receptor. It is reported that when engineered with a CAR, both CD4 and CD8 cells are capable of cytolytic degranulation of perforin and granzyme. CD4 T cells express significantly lower amounts of perforin and granzyme compared to CD8 cells, therefore a higher number of cells are required to reach the same level of cytotoxicity or longer time period (i.e. "fast and furious or slow and steady") (228). Additionally it has been shown using time-lapse imaging microscopy in nanowell grids that $CD4^+$ CAR T cells have superior resistance to activation induced cell death compared to $CD8^+$ CAR T cells (181). Despite these differences, fine-tuning the balance between maximising cytotoxicity, reducing adverse patient side-effects, reducing AICD, and increasing persistence appears to work best with a combinational approach with both CD4 and CD8 e.g. at a 1:1 ratio (229).

Limitations of my own cytotoxicity experiments include the fact that αβ T cell populations were mixed and the proportion of CD4/CD8 not quantified. This could have implications for cell lysis in short term chromium release assays if proportions between donor CD8:CD4 varied significantly. As it has been shown that CD4 CAR T cells have lower amounts of granzyme and perforin compared to CD8, they are likely to take longer to achieve comparable cytotoxicity (228, 230)

Another factor is the kinetics of cytotoxicity; it would have been interesting to compare cytotoxicity at more prolonged time points to assess whether CAR γδ T were capable of serial killing, as previously described in CAR αβ T cells (231). Experimentally this could have been carried out by using a flow cytometry based technique using non-

irradiated tumour cells, a live/dead stain and counting beads or a real time killing assay [e.g. xCELLigence assay (232)].

For $CD8^+$ αβ T cells it is known that as the cells become more differentiated, they have reduced capacity for self-renewal and become exhausted. This leads to poor anti-tumour immunity which has been observed in many studies (233-235). One of the main findings concerning γδ T cells was their apparent lack of sustained proliferation following antigen encounter. On meeting target, CAR Vδ2 cells appeared to initially expand but then this effect quickly diminished. Conversely, CAR αβ T cells continued to proliferate in response to antigen. A plausible explanation to account for this finding is that CAR Vδ2 cells were mainly of T_{EM} where as many CAR αβ cells retained a T_N or T_{CM} phenotype. A possible approach to enhancing proliferation of CAR Vδ2 cells would be to investigate alternative culture methods for propagating CAR^+ γδ T cells with a more undifferentiated phenotype, such as the aAPC method used by Deniger and colleagues (164). By electroporating with a CD19 CAR followed by selection for TCRγδ, CD19-CAR^+ γδ T cells demonstrated impressive proliferation with a heterogenous phenotype made up of a mix of naïve, central memory and effector cells. Furthermore, CAR^+ γδ T cells expressed desirable markers for tumour homing including CD62L and CCR7. Interestingly this method avoided direct TCRγδ signalling as propagation was through CD19-CAR engaging with CD19 expressed on the surface of aAPC (see Figure 2.2).

To measure cell number, I used trypan blue exclusion which is a routinely used stain to assess cell viability, however a major limitation of this method is that it is unable to distinguish between apoptotic and necrotic cells. An alternative method would have been to use a flow cytometry based technique such as Annexin V staining together with DAPI or propidium iodide (PI). Annexin V is a probe for phosphatidylserine on the outer membrane of apoptotic cells and can therefore identify programmed cell death at an earlier time point. Conversely DAPI or PI are markers of cell membrane permeability seen in very late apoptotic or necrotic cells. Using this technique it would have been interesting to use sequential staining to evaluate the time-course of cell

death. Alternative methods could be to use Ki67 staining (labelling cells in G1, S, G2 and M phases of the cell cycle) or BrdU used for specific labelling of cells in the S-phase.

Although FACS sorting CAR$^+$ Vδ1 and Vδ2 populations gave pure populations for testing function, this method had a number of limitations. Firstly, it is an expensive method which requires large volumes of fluorescently-labelled antibody and long periods of time on the cell sorter. Only small numbers of cells are acquired from bulk cultures containing the rare population of interest. It is also unknown to what extent the sorting process induces changes in cell function due to shear stress and Binek *et al.* reported it has significant impact on the cellular metabolome (236).

Furthermore, the addition of conjugated antibody for flow sorting could affect the cell's properties and function. For example, an activating antibody applied to the γδ TCR could lead to increased activation or conversely direct the cell towards AICD. It is also unknown whether pure populations may lead to reduced function of cells dependent on their neighbours (e.g. for cytokine production).

Alternative approaches that could be considered include using a flow cytometry based approach using intracellular antibody staining e.g. IFNγ or a CD107a assay. A sequencing based approach could also be used either at a single cell level or for bulk populations. Single cell RNA sequencing of γδ T cells could be used to discover the transcriptional and cytokine signature upon antigenic stimulation, similar to the methods used by Xhangolli *et al.* for CAR αβ T cells (237).

Trafficking of CAR T cells to the tumour site is a stringently controlled and dynamic process [reviewed in (238)]. Tumour infiltrating lymphocytes have high expression CXCR3 and CCR5 and it has been demonstrated that CXCR3-mediated trafficking at the tumour vascular interface is a critical checkpoint to effective T cell-based therapy (239). Integrating chemokine receptors into novel CAR designs has also yielded promising results with enhanced migration and persistence of CAR T cells (240).

Since a major study reported that the amount of γδ T cell infiltrating a tumour was predictive of a favourable patient outcome (42), I wanted to compare the motility and migration of αβ and γδ T cell subsets transduced with CAR, hypothesising that CAR^+ Vδ1 cells may have more favourable migration towards tumour. Results using *in vitro* transwells to assess migration, showed no significant difference between T cell subsets, including those that were CAR^+ or CAR^-. There was a general trend towards reduced migration of CAR^+ T cells of all subsets. This was not the expected result, however should be appreciated in the context of its experimental limitations. Although this method is relatively simple and non-expensive, it is not representative of a more complex mouse model where luciferase luminescent assay can be used to indicate homing to the tumour, or a flow cytometry based approach using fluorescent protein expressing CAR T cells such as GFP-transduced CAR T cells. Another attractive approach would be to use organoids/neurospheres evaluated with video microscopy.

With regard to their cytokine production, pro-inflammatory cytokine release is characteristic of T cell activation including the production of IFNγ and TNFα. The greater production of IL-2 by CAR αβ T cells compared to CAR γδ T cells could in part explain the significantly reduced proliferation seen on antigen encounter. The higher concentration in CAR αβ T cell cultures having paracrine effect is likely to be responsible for their superior survival and proliferation.

IL-17 is a proinflammatory cytokine that contributes to both tumour promotion by facilitating angiogenesis, and tumour elimination by stimulating cytotoxic T cell responses (241). Although research has focused primarily on production by $CD4^+$ T cells (T helper 17 cells), recent studies have investigated IL-17 production by γδ T cells. Various groups have reported that peripheral blood Vδ2 cells contains very few IL-17 producers (<1%) (209, 242), however interestingly Caccamo *et al*. (243) showed that 60-70% of Vδ2 in the CSF of children with bacterial meningitis were $IL\text{-}17^+$. Following analysis of cytokine-containing supernatant following co-culture of CAR^+ Vδ2 with $GD2^+$ targets, I did not identify IL-17 production by γδ T cells, although there was a small amount produced by CAR^+ αβ T cells. The precise significance of IL-17 in the progression of neuroblastoma is unknown, but IL-17 has been found to

promote tumour progression in adult cancers (244). Further insight as to the precise role of IL-17 producing γδ T cells will help guide future immunotherapy strategies targeting this pathway.

Chapter 4 outlines the characteristics of CAR γδ T cells with regard to their cytotoxicity, cytokine production, proliferative capacity and migratory properties. Antigen specific CAR γδ T cell cytotoxicity was achieved comparable to CAR αβ T cells and my next aim was to demonstrate a potential translatable advantage over existing approaches, by investigating whether CAR γδ T cells have capacity for antigen presentation.

Chapter 5 CAR$^+$ γδ T cells and antigen presentation

5.1 Introduction

There is a body of evidence for the role of activated Vδ2 cells as professional antigen presenting cells (pAPC) (61, 74, 84, 245), but it is yet unknown whether CAR$^+$ Vδ2 retain a pAPC phenotype and cross-presentation function following transduction with CAR. There have been conflicting reports regarding the ability of non-transduced Vδ2 cells to cross-present tumour antigens, however building on the theory that Vδ2 cells require a certain threshold of activation to be exceeded (74), it was hypothesised that this could be sufficiently provided by appropriate stimulation/costimulation through a CAR.

The possibility that CAR Vδ2 cells can function as both antigen presenting and cytotoxic effector cells is particularly appealing for cancer immunotherapy. This is potentially biologically plausible given that other immune cells are able to combine innate killing with antigen presentation function e.g. macrophages. Following lysis of tumour cells by CAR mediated killing, it may be the case that CAR$^+$ pAPC Vδ2 can take-up and present tumour antigens to neighbouring αβ T cells, thereby creating long-lasting immunological memory.

A particular obstacle to paediatric solid tumour passive immunotherapy, is that the vast majority of childhood cancers are known to have low immunogenicity (246). The number of neo-antigens expressed by tumours is proportional to the number of nonsynonymous somatic mutations (36, 247), of which paediatric cancers on the whole have fewer than adults (248). This is particularly relevant, and may partially explain why paediatric cancers are less responsive to single-agent checkpoint inhibition (249)

(so-called 'cold' tumours). Based on this knowledge, a well characterised immunogenic tumour antigen was chosen for proof of concept *in vitro* cross-presentation experiments.

Melanoma Antigen Recognized by T cells 1 (MART1, also known as Melan-A) was discovered as a tumour associated antigen recognised by tumour infiltrating lymphocytes of melanoma patients (250). It is a well characterised tumour antigen and has been used in a number of vaccine and TCR transfer clinical trials (251, 252). MART1 was therefore selected as a suitable model antigen for investigating whether CAR transduced Vδ2 cells were capable of cross-presentation based on the promising data in previous studies using non-transduced Vδ2 cells (61). In addition, Himoudi *et al*., (74) found the process of cross-presentation by Vδ2 cells to be tightly regulated with significantly increased stimulation of responder αβ T cells in the presence of opsonised target; a process known as 'γδ T cell licensing'.

A pre-requisite for antigen presentation is the ability for cells to take up exogenous antigen and γδ T cells have been reported to do this by both phagocytosis (253) and trogocytosis (254). Additionally, experiments within Professor Anderson's laboratory using non-transduced Vδ2 (Dr Anne Kramer and Dr Zarah Abeln), demonstrated the phagocytic properties of γδ T cells by internalisation of IgG opsonised 1.0μm beads using ImageStream analysis (59)

5.1.1 Aims

- To establish the pAPC phenotype of CAR^+ Vδ2 cells

- To develop an *in vitro* model for tumour antigen cross-presentation by CAR^+ Vδ2 cells

5.2 Results

5.2.1 CAR$^+$ γδ pAPC phenotype

A hallmark of conventional professional antigen presenting cells (pAPC), such as dendritic cells (DC), is that they characteristically express costimulatory ligands providing 'signal 2' for full T cell activation. Following activation and transduction with CAR, Vδ2 cells acquire similar pAPC markers including CD86 and MHC class II (e.g. HLA-DR).

Figure 5.1 illustrates the dramatic upregulation of CD86 and HLA-DR following propagation with ZOL. 94.133% ± 0.437 of pre-activated Vδ2 were CD86$^-$/HLA$^-$DR$^-$ compared to only 4.187% ± 2.447 and 0.220% ± 0.159 of post-expanded non-transduced and CAR$^+$ Vδ2, respectively. Conversely the vast majority of day 13 expanded cells expressed both CD86 and HLA-DR with significantly higher expression in CAR$^+$ compared to non-transduced subpopulations (mean positive expression ± SEM; day 13 non-transduced Vδ2; 80.533% ± 8.435, day 13 CAR$^+$ Vδ2; 98.533% ± 0.393, $p < 0.01$, n=3). Similar expression levels are reported in response to *E.Coli* activation where sustained high expression of HLA-DR and CD86 was comparable to freshly isolated monocytes (59).

5.2.2 Establishing an *in vitro* model for CAR$^+$ Vδ2 cell cross-presentation

After confirming that CAR$^+$ Vδ2 do indeed adopt an aAPC phenotype following activation, I next investigated whether they could also demonstrate cross-presentation function by establishing a suitable *in vitro* model (Figure 5.2).

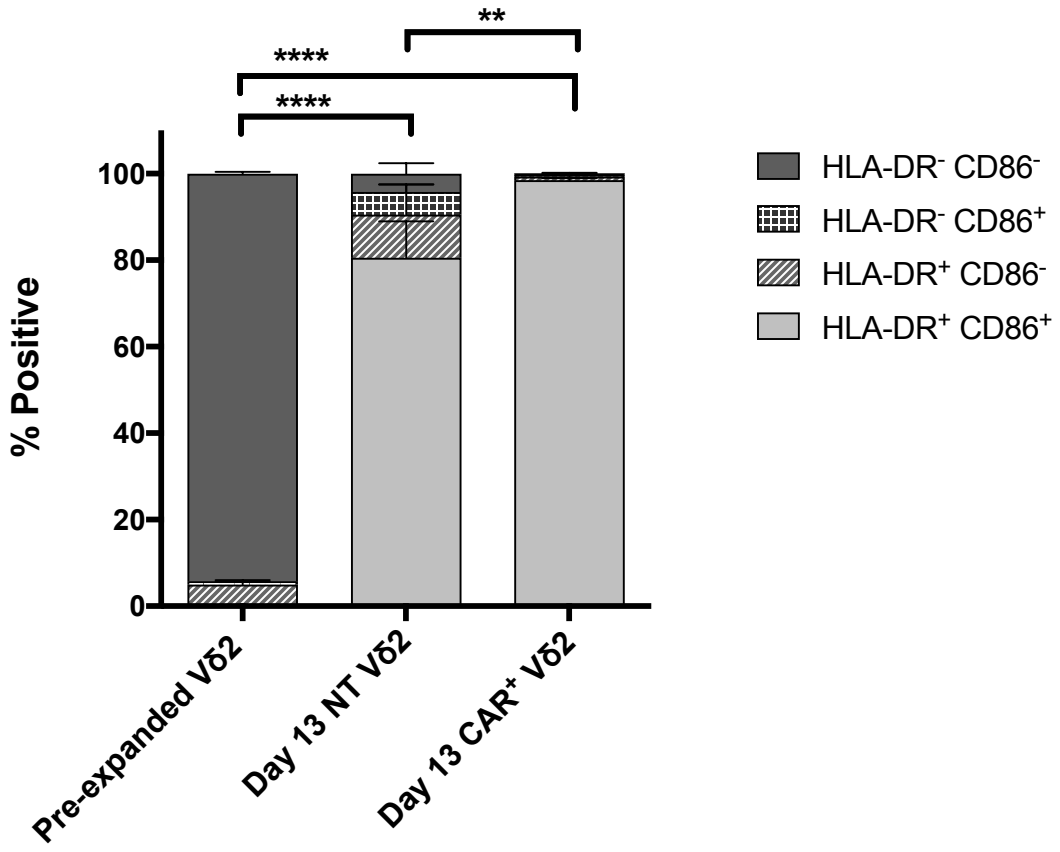


Figure 5.1 CAR$^+$ Vδ2 cells adopt an antigen presenting cell phenotype upon activation

Expression of CD86 and HLA-DR on pre-expanded Vδ2, post-expanded non-transduced (NT) Vδ2, and CAR$^+$ Vδ2 from multiple donors. Vδ2 cells were activated with ZOL and cultured for 14 days with IL-2. Data represented as mean ± SEM, n = 3. Statistical comparisons were made with 2-way ANOVA (* $p<0.05$, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$). Significant p values shown with brackets compare double positive populations.

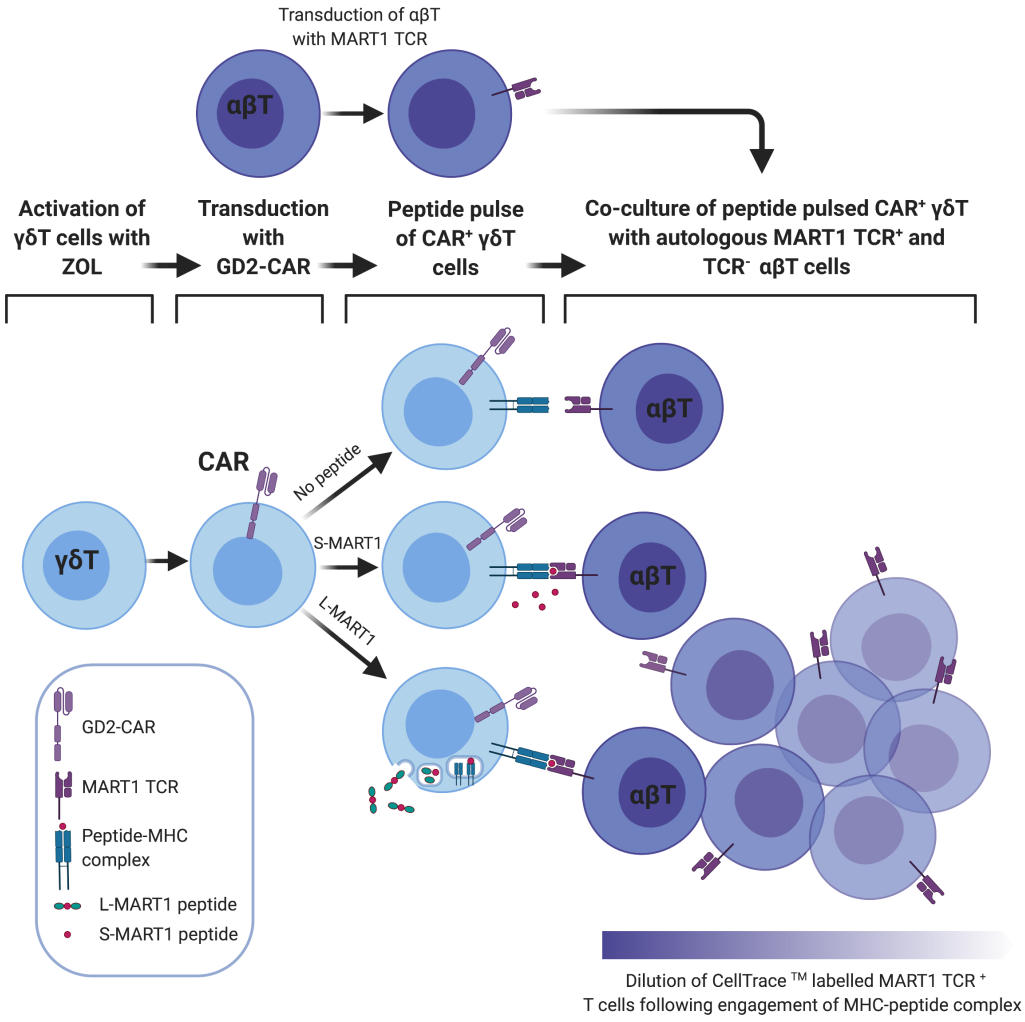


Figure 5.2 Experimental schematic of MART1 peptide cross-presentation by CAR$^+$ Vδ2 T cells

CAR$^+$ Vδ2 cells were pulsed with either no peptide, short peptide (S-MART1) or long peptide (L-MART1). Following antigenic stimulation by peptide-pulsed CAR$^+$ Vδ2 cells, CellTrace (violet) labelled MART1-TCR$^+$ αβ T cells proliferate, resulting in dilution of violet dye. αβ T cells that do not express the specific MART1-TCR$^+$ do not proliferate.

5.2.3 MART1 tumour associated antigen

The requirement for a cross-presentation assay is firstly a source of the antigen. This can either be in the form of a whole protein in purified form or derived from a cell (such as cell lysate) or a long peptide that requires proteasomal processing for MHC class I loading. Secondly, the antigen presenting cell must be of the correct class I type for the proposed short peptide antigen, and lastly the responder cell line is required to have TCR specificity for the short peptide-MHC complex.

I initially considered the potential source of protein for loading and planned to evaluate cross presentation using a MART1 class I restricted peptide derivative. Using a whole cell approach was particularly attractive with a GD2 target cell for specific CAR T cell cytotoxicity combined with a source of exogenous antigen for cross-presentation. I therefore started by searching for a suitable GD2 positive neuroblastoma cell line, that was additionally positive for the chosen target antigen, MART1. Each of these components required generation and/or validation.

5.2.3.1 MART1$^+$ expression in tumour cell lines

Firstly, I identified potential cell lines and confirmed the presence of MART1 using Western blotting. The MART1 gene appears to be highly and selectively expressed in melanoma cells (255), and it has previously been reported that MART1 is not expressed in neuroblastoma cell lines or primary tumours (256). Firstly, I sought to confirm this by western blotting, and as shown in Figure 5.3, the presence of MART1 protein was not detected in any of the neuroblastoma cell lines tested (LAN1, SK-N-DZ and IMR32). The melanoma cell line, SK-MEL-28 was selected as a positive control.

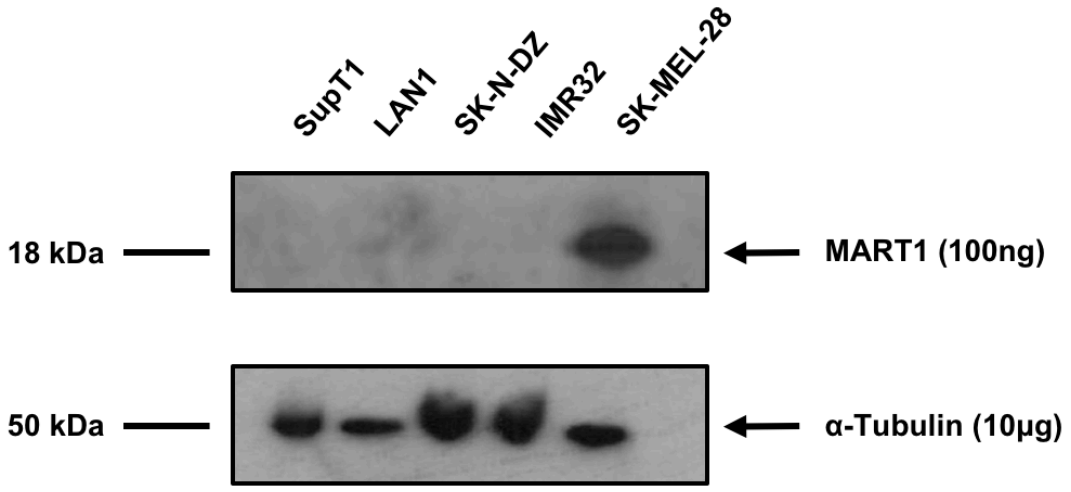


Figure 5.3 Western blot of MART1 expression in neuroblastoma cell lines

Western blot analysis of SK-MEL-28 cell lysate confirms high abundance of MART1 protein (positive control) that is not seen in neuroblastoma cell lysate, LAN1, SK-N-DZ, and IMR32. Bands represent protein detection by MART1 antibody, the size of which correspond to that expected for MART1 protein (18kDa). SupT1 cell lysate was used as a negative control and α-tubulin as a loading control.

Although it would have been preferable to use a neuroblastoma model, the melanoma cell line SK-MEL-28 was selected for use in further experiments, as a source of abundant MART1 antigen. Furthermore, SK-MEL-28 was confirmed by flow cytometry to be $GD2^{dim}$, therefore to enhance its susceptibility to GD2-CAR T cell killing, I transduced this cell line with GD2/3 synthase to provide a new cell line that was $GD2^{bright}$.

5.2.3.2 MART1$^+$ peptides

Synthetically made long peptides have been used in standardised cross-presentation studies involving dendritic cells (257). This complex process is crucially dependent on the composition of the antigen together with its mode of delivery. Long peptides characteristically contain 25-50 amino acids and border the antigenic epitope that is presented by MHC-I. Long peptides cannot be directly loaded onto MHC class I and instead require endocytosis and processing by pAPCs in order to induce cytotoxic T cell responses. The cellular processing mechanisms responsible for cross-presentation

of MART-1 long peptide by human dendritic cells has been described by Menager and colleagues (257). A synthetic peptide derived from the MART1 tumour associated antigen corresponding to position 16 to 40 (L-MART$_{16-40}$) was purchased together with the immunodominant epitope to HLA-A0201 (MART1$_{26-35}$; ELAGIGILTV; S-MART1 hereafter), used as a positive control.

5.2.4 Peptide pulse of CAR$^+$ Vδ2 cells

ZOL activated, CAR$^+$ Vδ2 T cells were cultured for 13 days in the presence of IL-2 as previously described prior to purification by FACS. After 24 hours, purified CAR$^+$ Vδ2 cells were pulsed with synthetic MART1 peptide; either short peptide (S-MART1), long peptide (L-MART1), or no peptide, for 4 hours at 37°C in serum free medium. All peptides were used at a concentration of 5μg/ml. Pulsed Vδ2 cells were the washed twice before use in further assays.

5.2.5 MART1 TCR$^+$ αβ T cell responders

Transduction with a specific MART1 TCR was necessary for naive donor αβ T cells to allow proliferation in response to MART1 antigen presentation by CAR$^+$ Vδ2 cells. This was achieved using a recombinant MART1-reactive HLA-A0201-restricted αβTCR with high affinity specificity for the MART1 immunogenic peptide (MART1$_{26-35}$) (MART1-TCR) (258). This MART1-TCR construct was cloned (258) and kindly gifted by C. Cohen (National Cancer Institute, Clinical Research Center, Bethesda) for use in my experiments.

MART1-TCR$^+$ αβ responder cells were produced from PBMC taken from the same blood draw as that used for the parallel Vδ2 expansion, but frozen until required. On day 5 of the experimental protocol, PBMC were thawed and stimulated with CD3/CD28 antibody, then 48 hours later transduced with MART1-TCR. To quantify proliferation of responder cells, αβ T cells were labelled with CellTrace™. On day 14, peptide pulsed or control CAR Vδ2$^+$ T cells were co-cultured with MART1 TCR$^+$ CellTrace™ labelled αβ T cells at a ratio of 1:3 for 5 days. Proliferation of MART1

TCR$^+$ αβ T cells was evaluated flow cytometrically by gating on MART1 TCR$^+$ αβ cells (Vβ12 chain$^+$) and measuring CellTrace dilution (experimental schemata is illustrated in Figure 5.2 and timeline in Figure 5.4).

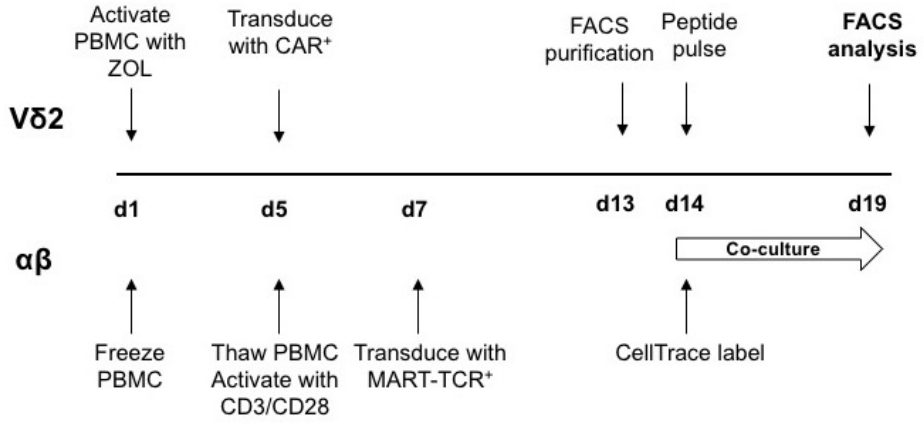


Figure 5.4 Experimental timeline of *in vitro* cross-presentation assay

PBMC from HLA-A2$^+$donors were activated with ZOL or frozen until further use. ZOL-activated Vδ2 were transduced with GD2-CAR$^+$ on day 5 and FACS purified on day 13. CAR$^+$ Vδ2 were pulsed with either synthetic short peptide (S-MART1), long peptide (L-MART1), or no peptide, for 4 hours at 37°C in serum free medium at a concentration of 5μg/ml. MART1-TCR$^+$ αβ responder cells were produced from PBMC taken from the same blood draw as that used for the parallel Vδ2 expansion. On day 5, PBMC were thawed and stimulated with CD3/CD28 antibody, then transduced 48 hours later with MART1-TCR. On day 14 bulk αβ T cells transduced with MART1-TCR were labelled with CellTrace and co-cultured with peptide pulsed CAR$^+$ Vδ2 cells. CellTrace dilution was assessed within the MART1-TCR$^+$ αβ [V-beta 12 (Vβ12) chain] positive and negative gate, distinguishing antigen-specific and non-specific proliferation.

5.2.6 CAR$^+$ Vδ2 cells can cross-present MART1 tumour antigen to responder αβ T cells transduced with a MART1-reactive αβTCR

As shown in Figure 5.5, short peptide (S-MART1) pulsed CAR$^+$ Vδ2 cells led to proliferation of MART1-TCR$^+$ αβ T cells (Vβ12$^+$), but not MART1-TCR$^-$ populations (Vβ12$^-$). Cells were gated on either αβ TCR$^+$/Vβ12$^+$ or αβ TCR$^+$/Vβ12$^-$ and proliferating cells detected by dilution of CellTrace Violet dye. Long peptide pulsing

(L-MART1) led to a modest antigen specific proliferation compared S-MART1 and this was expected given the requirement for L-MART1 endocytosis and intracellular processing.

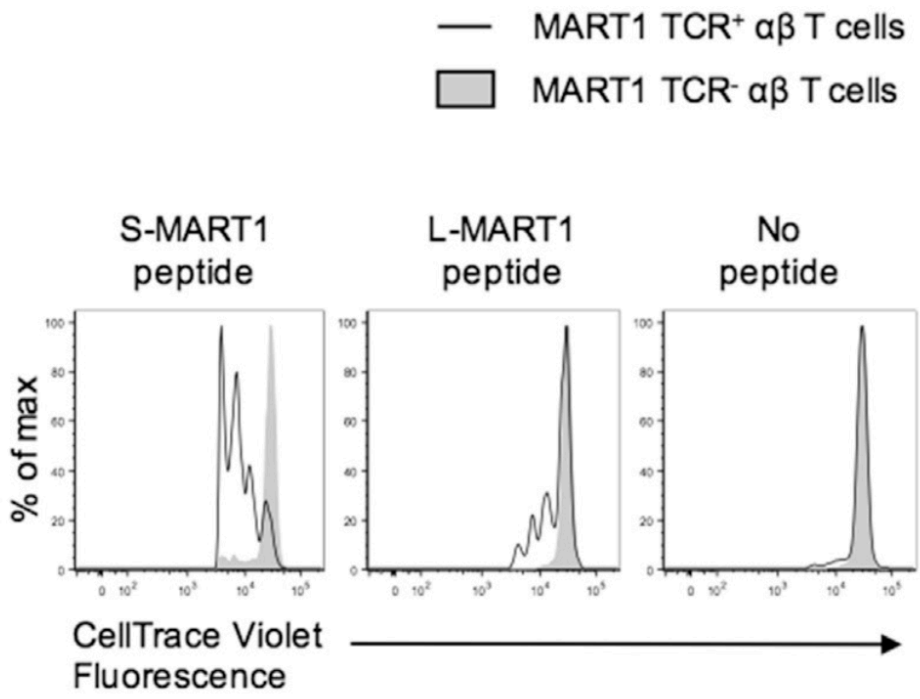


Figure 5.5 Representative flow cytometry histogram showing proliferation of CellTrace labelled MART1 TCR$^+$ αβ T cells in response to co-culture with peptide pulsed CAR$^+$ Vδ2 cells

CAR$^+$ Vδ2 cells were pulsed with either short MART1 peptide (S-MART1), long MART1 peptide (L-MART) or no peptide and then co-cultured with a bulk population of MART1 TCR$^+$ transduced αβ T cells that were pre-labelled with CellTrace Violet dye.

This experiment was replicated in four separate donors with comparable results (Figure 5.6). S-MART1 peptide pulsed CAR$^+$ Vδ2 led to 54.4% ± 13.7% proliferation (mean ± SEM), L-MART1 peptide 14.41% ± 3.85, and no peptide 7.72% ± 2.65. There was a significant difference between the proliferation induced by S-MART and L-MART1 ($p < 0.05$, two-tailed Students t test, n=4). There was also statistical

significance between the proliferation induced by L-MART1 peptide pulsed CAR^+ Vδ2 compared to no peptide ($p < 0.05$). There was no significant statistical difference in the proliferation of MART1-TCR^+ ($V\beta12^+$) compared to MART1-TCR^- ($V\beta12^-$) αβ T cells in response to L-MART1 peptide pulsed CAR^+ Vδ2 ($p = 0.059$, n=4), however this was significant for S-MART1 pulsed CAR^+ Vδ2 ($p < 0.05$, n=4).

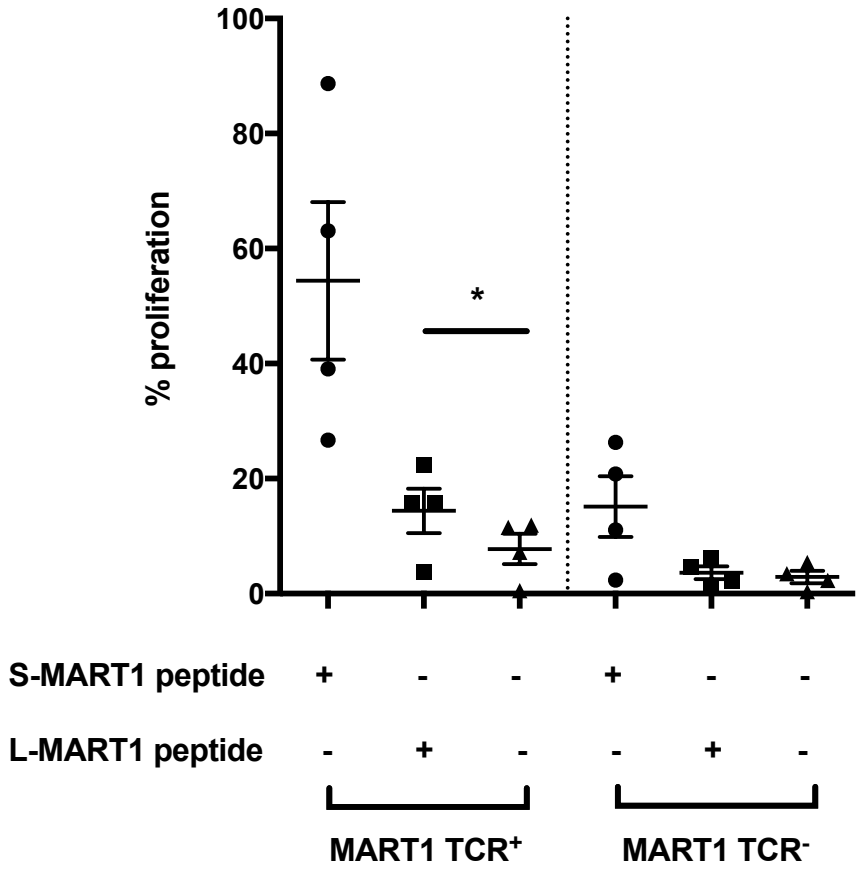


Figure 5.6 CAR^+ Vδ2 cells can cross-present synthetic long MART1 peptide to responder αβ T cells transduced with a recombinant MART1 αβ TCR

CAR^+ Vδ2 cells were co-cultured with CellTrace labelled αβ T responder cells (bulk population containing both $MART1^+$ and $MART1^-$ αβ TCR). $V\beta12^+$ and $V\beta12^-$ responders were gated for analysis. Higher proliferation of Vβ12 negative cells in S-MART1 conditions is probably due to bystander activation by the $V\beta12^+$ responders. The small background proliferation in $V\beta12^+$ cells in the absence of peptide is commonly seen following transduction. Data, horizontal line represents mean value, * P<0.05, statistical comparisons were made with a two-tailed paired Student's t-test (200).

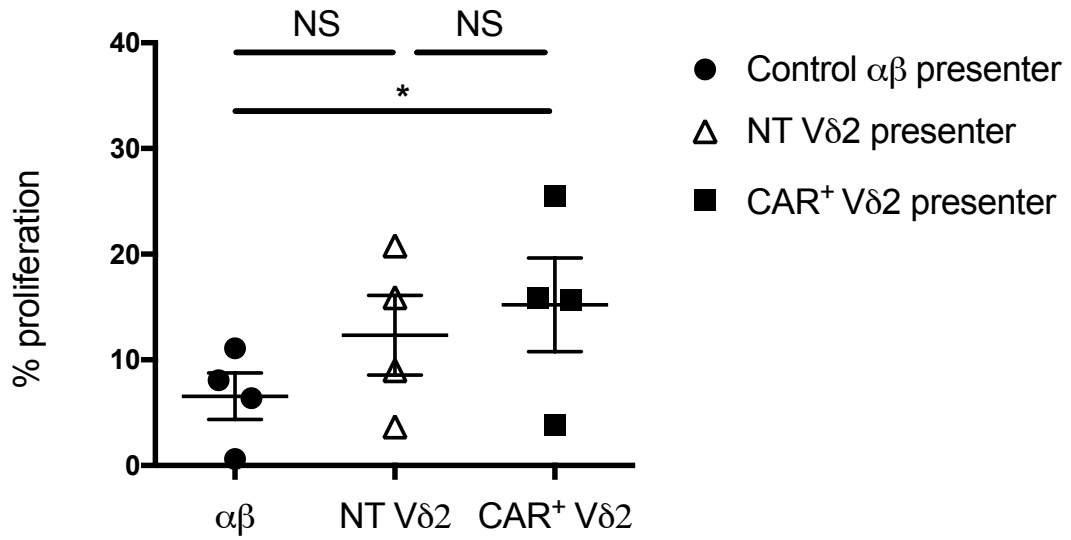


Figure 5.7 Proliferation of MART1-TCR$^+$ αβ T cells in response to L-MART peptide pulsed T cell 'presenters'

CAR$^+$ Vδ2, non-transduced (NT) Vδ2 and αβ (negative control) were pulsed with L-MART1 peptide and co-cultured with CellTrace labelled MART1-TCR$^+$ αβ T cells. Peptide pulsed Vδ2 cells induced a proliferative response unlike peptide pulsed αβ treated identically. Mean ± SEM, n=4. * $P<0.05$, NS $P>0.05$ by two-tailed Student's t-test.

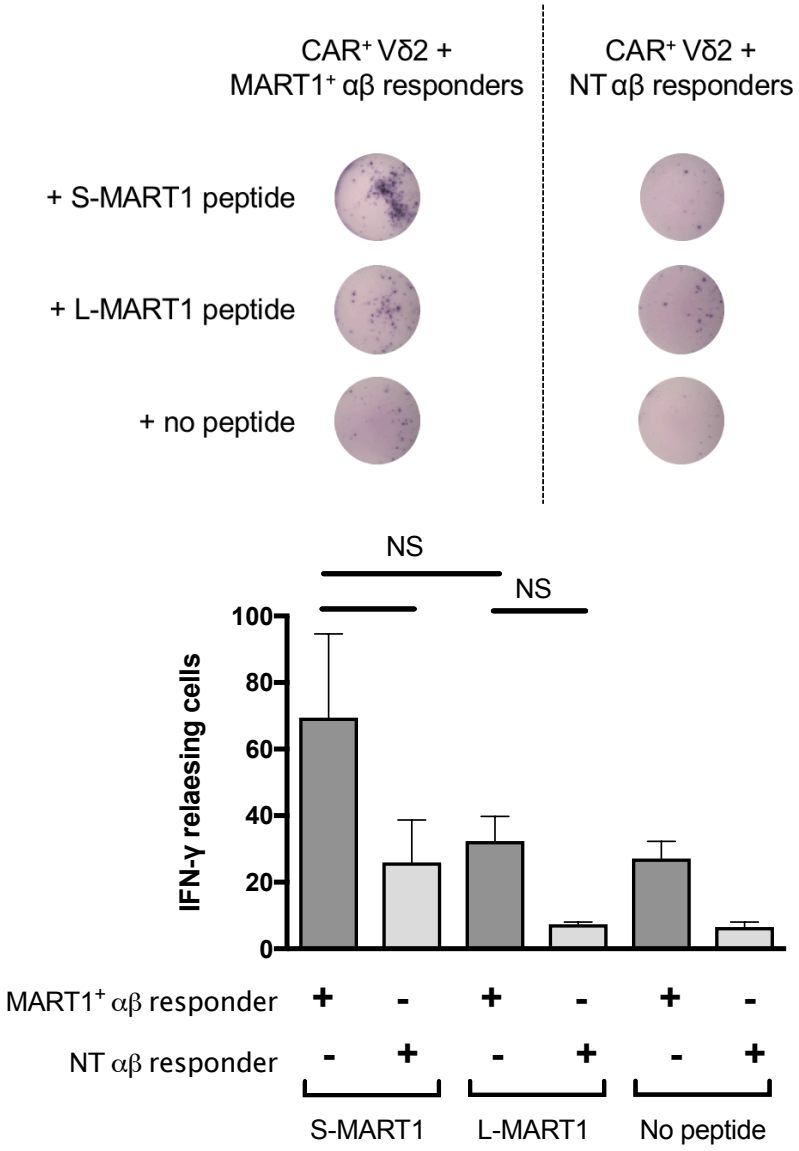


Figure 5.8 ELISpot analysis measuring IFNγ secretion by αβ cells in response to CAR$^+$ Vδ2 cells pulsed with short MART1 peptide (S-MART), long MART1 peptide (L-MART1) or no peptide

1x10^4 peptide-pulsed [S-MART1, L-MART1 or control) CAR$^+$ Vδ2 cells were co-cultured with 5x10^4 αβ responder cells overnight. αβ responder cells were either transduced with MART1 TCR (MART1$^+$) or non-transduced (NT). 3 individual donors.

Lastly an ELISpot was performed to measure release of IFNγ by activated responder cells. Similarly, peptide-pulsed CAR$^+$ Vδ2 cells were washed then co-cultured with either transduced MART1 TCR$^+$ αβ cells or non-transduced αβ cells and repeated in 4 separate donors. As shown in Figure 5.8, the highest number of IFNγ releasing cells

was seen in MART1 TCR$^+$ αβ cells co-cultured with MART1 short peptide pulsed CAR$^+$ Vδ2 cells. No significant difference was seen between MART1$^+$ and MART1$^-$ αβ cells co-cultures with MART1 long peptide pulsed CAR Vδ2, or indeed between MART1$^+$ responders co-cultured with MART1 long peptide or no peptide. There was a general trend towards higher IFNγ release in transduced αβ cells compared to NT, and both transduced and non-transduced αβ in the presence of short peptide. Co-culture of Vδ2 'presenter' and αβ 'responder' was overnight, compared to 5 days in the proliferation assay, which may account for the differences seen. The ratio of cells also differed (1:3 and 1:5).

5.3 Discussion

The aim of this chapter was to investigate whether CAR$^+$ Vδ2 cells retained the capacity for antigen cross-presentation following activation and transduction. There has been scientific debate regarding whether non-transduced Vδ2 are able to cross-present tumour antigen, with only a few groups having studied this, including our own (61, 74). Using a similar experimental design to that previously reported, weak cross-presentation by CAR$^+$ Vδ2 cells was demonstrated however a number of limitations should be recognised.

Brandes *et al.* (61) reported robust CD8$^+$ αβ T cells responses to cross-presenting Vδ2 cells, using mycobacterium tuberculosis purified protein (PPD), and influenza-encoded matrix protein but this effect was not reproduced for experiments using MART1 protein. In order to interpret my own findings, it is necessary to understand some of the differences in methodology and appreciate the possible intracellular signalling pathways to explain why these differences occurred.

Brandes *et al*. similarly designed experiments, where Vδ2 cells were loaded with peptide, washed and then co-cultured with CFSE-labelled responder αβ T cells (αβ

clone rather than TCR transduced cells). Robust activation was seen at a level similar to DCs for mycobacterium tuberculosis purified protein (PPD) and influenza-encoded matrix protein but not MART1. This finding led the authors to further investigate the mechanistic intracellular signalling pathways leading to peptide loading onto MHC class I in Vδ2 cells.

It is known for professional antigen presenting cells such as DCs, that immunogenic amino acids peptides are classically generated by the proteasome. These short peptides are then transported into the endoplasmic reticulum by TAP (transporter for antigen processing) where they are loaded onto MHC class 1 by a multitude of molecular chaperones (259). Proteasomes are large intracellular complexes responsible for degrading proteins, thereby generating antigenic peptides, and are a requirement for cell viability. The 20S proteosome comprises four heptameric rings (2 outer alpha and 2 inner beta rings). Specific inducible subunits (Beta1i, beta 2 i and beta 5i) have been identified that can be induced by IFNγ and TNFα. Complexes containing inducible subunits, or 'immunosubunits' form the immunoproteasome. It has been shown that immunoproteasomes generate more antigenic peptides than standard proteasomes, although protein degradation occurs at the same rate (260)

It was proposed by Brandes *et al.* that the inability of Vδ2 cells to cross-present MART-1 was attributable to differences in expression of the immunoproteosome for DCs and γδ T cells. The authors found that expression of the immunoproteosome was higher in γδ cells than immature DCs and B-cells (determined by expression of B1i by western blot analysis). Using mass spectroscopy to identify the peptide products from proteasome-peptide substrate cultures, the authors concluded that the presence of the immunoproteosome led to absence of MART-$1_{26\text{-}35}$ peptide product. Differences in culturing conditions could lead to differences in whether cells predominantly express standard proteasome or immunoproteosome components. It would have been interesting to compare the relative contributions of each component proteins family to determine which proteasome subtype were predominant in the γδ T cells generated in our laboratory.

A further limitation of the experimental model could be the introduction of 'bystander' effect and it was observed that MART1 TCR$^-$ αβ T cells had higher proliferation in response to S-MART peptide (using CellTrace assay, gating on Vβ12$^-$ cells within a mixed population). However, bystander effect cannot totally account for this difference as this effect was also seen in ELISpot experiments in separately cultured non-transduced αβ T cell populations. This could be attributable to contaminating proteins (from commercially produced and purified peptides) or even due to natural MART-1 autoreactivity, as described by Przybyia *et al*. (261) who discovered a 0.07% natural T cell autoreactivity to melanocyte antigens in healthy donors.

The overall experimental design could have been further optimised by using a 'nonsense' or control peptide condition together with 'no peptide' control. This could have been an alternative long and short peptide tumour antigen such as WT1 or NYESO1. Due to the complexities, limited cell numbers, and cost of purified peptides, there was a limitation to what could be achieved. Similarly, with a DC positive control.

The difference between the proliferation and ELISpot read-outs could be due to the differing co-culture durations for 'presenters' and 'responders' in the two assays. The former used 5 days where as the latter use an overnight co-culture. The timing of the assay is essential as performing the ELISpot too soon or too late may result in missing the cross-presentation signal. In order to investigate this further, a flow cytometry based approach could be used to measure IFNγ at different time points during a prolonged (up to 7 day) co-culture.

The translatable clinical significance of these findings should again be interpreted with caution. Firstly, these are *in vitro* models using an adult cancer model. As previously mentioned paediatric cancers such as neuroblastoma are known to be relatively deficient in neo-antigens reducing the likelihood of antigen cross-presentation by intratumoural γδ T cells. This approach is more likely to be of interest in diseases such as melanoma with high tumour neo-antigens and large numbers of tumour-reactive infiltrating lymphocytes. In conclusion, although weak cross-presentation was

demonstrated this should be interpreted with caution given the experimental design and lack of published evidence by other groups.

Chapter 6 Final discussion and future work

The aim of this thesis was to explore GD2-directed CAR γδ T cells as a potential immunotherapy for paediatric solid tumours, using neuroblastoma as a model system. For children with relapsed high-risk neuroblastoma, prognosis is extremely poor, and the optimum therapy for relapsed disease is not clearly defined. Novel and/or combinational therapies that improve survival and minimise toxic side-effects are desperately required. The tumour antigen, GD2 expressed on the surface of neuroblastoma cells, provides a suitable target antigen for GD2-directed CAR T cells. GD2 has been targeted using monoclonal antibodies in clinical studies and has been shown to be efficacious in the treatment of high-risk neuroblastoma. Clinical experience has also shown that 'on target, off tumour' toxicities are manageable in paediatric patients with good supportive care. Phase 1 trials of GD2-CAR αβ T cells have also demonstrated safety without neurotoxicity.

CAR T cells are a rapidly emerging cancer therapy using the capacity of a patient's own immune system by armouring autologous T cells with a chimeric antigen receptor to target tumour cells. Although impressive results have been observed using CD19-redirected CAR αβ T cells to treat ALL, successful translation to solid tumour immunotherapy, including neuroblastoma, has been hindered by various obstacles including; CAR T cell expansion, tumour trafficking, and their ability to persist within the hostile tumour microenvironment.

At the time of commencing this thesis, CAR T cells for paediatric solid tumours were a relatively new concept, with few published clinical trials demonstrating their efficacy. For neuroblastoma, these early clinical trials showed failure of long-term CAR T cell persistence and limited effectiveness (116). These initial approaches used CAR αβ T cells as the effector cell of choice, based on their proven efficacy against CD19 expressing leukaemias, however in an attempt to overcome some of the specific immunological challenges of solid tumours, various other approaches have been

investigated including novel CAR engineering designs, combinational treatments, and using alternative effector cells with innate properties.

γδ T cells expressing a first generation GD2-directed CAR were first described by Rischer *et al.* in 2004 (163), but since then and up until commencing this thesis, the γδ T cell immunotherapy field had focused predominantly on non-engineered cells for adoptive transfer. Many of these early studies used aminobisphosphonates to propagate populations of Vδ2 cells and this was later followed by a series of publications using new methods for expanding γδ T cells with a broad repertoire of γδTCR subsets. This included the use of ConA (55, 167) and aAPC (40, 164). In 2013, Deniger and colleagues generated CD19-directed CAR γδ T cells by electroporation with CAR followed by negative selection, and expansion using aAPC, IL-2 and IL-21 (164). The resulting population showed a preserved distribution of $V\delta1^+$, $V\delta2^+$ and $V\delta1^-$/ $V\delta2^-$ cells, with mixed memory phenotypes and expression of the lymph node homing markers, CCR7 and CD62L. CD19-CAR^+ γδ T cells generated using this method were efficient at killing $CD19^+$ cell lines, compared to non-transduced/CAR^- γδ T cells.

There remained many unanswered questions regarding the suitability of CAR γδ T cells for adoptive cell transfer, and whether a clinical-grade product could be manufactured to large-scale, with additional therapeutic advantage over existing CAR αβ T cell approaches. Our research group has specifically focused on engineering γδ T cells with CARs in order to redirect their specific cytotoxicity towards GD2, in addition to exploring their unique immunological properties that make these effector cells a potential novel immunotherapy approach.

Evidence to support the hypothesis that CAR γδ T cells may have additional advantages over conventional CAR αβ T cells firstly includes their potential capacity for use as an 'off the shelf' product because, unlike αβ cells, they are not mediators of GvHD (262). It has been shown in recent studies that in HLA-haploidentical haemopoietic stem cell transplant (HSCT) with *ex vivo* αβ T cell depletion, that

patients did not experience visceral or chronic GvHD even in the absence of GvHD prophylaxis (263), and patients with high concentrations of γδ T cells two months post allogeneic HSCT had lower incidence of GvHD (264). Furthermore, in a retrospective study of patients following allogeneic HSCT for ALL, a higher number of γδ T cells in the early post-transplant phase was found to be associated with improved EFS and OS (48), reviewed in (265).

The potential benefit of an 'off the shelf' CAR T therapy is clinically very important, particularly for those patients too unwell to undergo autologous cell harvest or those with rapidly progressive disease. An 'off the shelf' product, without the requirement for further genetic engineering, would overcome the challenges of 'bridging' patients with chemotherapy whilst awaiting autologous CAR T cell manufacturer (which can take up to 4 weeks), and provide an alternative when autologous CAR T cell manufacturing fails. The cost of receiving allogeneic CAR T cells is also likely to be significantly less than the cost of manufacturing autologous CAR T cells for individual patients.

A second advantage for γδ CAR T cells is that have the potential for dual antigen cytotoxicity as they are already primed for innate cytotoxicity. γδ T cells have the ability to recognise molecular patterns associated with malignant transformation and function independently of MHC therefore may have increased efficacy against tumours such as neuroblastoma that express low levels of MHC class I as an immune evasion strategy. By functioning independently of MHC, without the need for prior antigen priming, they may also be better suited for cancers with lower mutational burdens including most paediatric tumours. Thirdly, Vδ1 cells have natural tissue tropism (42) and a large bioinformatic study demonstrated that γδ T cell tumour infiltration, when compared to all other immune cell types studied, was associated with the highest survival (42). Finally, γδ T cells have been shown following activation to acquire a phenotype and functional properties analogous to professional antigen presenting cells (59, 74, 84, 266).

Based on the available literature at the time of writing, the clinical translational potential for CAR γδ T cells for neuroblastoma immunotherapy was investigated. In chapter 3 we hypothesised that following transduction with a CAR, γδ T cells would retain their innate anti-cancer properties as well as acquiring additional antigen-specific toxicity. Following optimisation of expansion and transduction methods specific for γδ T cells, we were able to propagate CAR^+ γδ T cells to significant number with adequate CAR transduction and demonstrate antigen-specific cytotoxicity.

In chapter 4, we further characterised CAR γδ T cells with regard to their cytotoxicity, phenotype, cytokine secretion, and capacity for proliferation and migration. On head-to-head comparison, there was broadly equivalent cytotoxicity demonstrated between CAR^+ γδ and αβ T cells and all subsets studied were able to migrate towards tumour cells *in vitro*. CAR Vδ2 T cells had a more differentiated phenotype with reduced capacity for proliferation compared to CAR αβ T cells. A population of CAR Vδ1 T cells maintained a T naïve phenotype and expressed the fewest exhaustion markers.

Our final approach in chapter 5 was to investigate the capacity of CAR Vδ2 T cells to cross-present exogenous tumour antigen. Although this effect was modest in the experimental model used, this finding opens up a new avenue of research investigating whether CAR γδ T cells have the capacity to sequentially infiltrate the tumour, kill cancer cells, and subsequently through their APC function, cross-present tumour antigen to neighbouring immune cells.

6.1.1 Optimal activation, expansion and transduction for the manufacture of GD2-CAR γδ T cells suitable of adoptive transfer

CAR γδ T cells were expanded from the PBMC of healthy donors using three activation methods; CD3/CD28 antibody, ZOL and ConA. For translation to clinical scale manufacture, ZOL (and other aminobisphosphonate drugs) are immediately suitable for Vδ2 propagation with all reagents available in GMP grade, as previously

described (139, 141, 142, 144-150). For αβ T cell control experiments, the method using CD3/CD28 antibody activation and GD2-CAR transduction is currently being used in a clinical trial at Great Ormond Street Hospital (1RG-CART). Interestingly, this method produced the highest expansion of Vδ1 cells, within the bulk population of predominantly αβ T cells.

The expansion protocol for Vδ1 cells using ConA is currently suitable for use in pre-clinical studies only. Further investigation and development is required for the expansion of enriched Vδ1 cell populations using reagents safe for patient use. The most promising results published by Deniger and colleagues used aAPC, however not all of the reagents used to expand γδ T cells using this method were clinical-grade and the protocol used was complex for clinical scale-up. More recently, Almeida *et al.* have developed and patented an innovative expansion method, using only clinical-grade reagents and without the requirement for feeder cells. This two-stage approach involves a selection by MACS, followed by culture in media containing IL-4, IFNγ, IL-21, IL1β and soluble anti-CD3 antibody (OKT3), with replacement of media every 5-6 days containing IL-15, IFNγ and OKT3 (192). There was up to 2000 fold expansion with enrichment of Vδ1 cells of more than 60%. Results were reproducible using blood from patients with CLL with minimal variation between donors. Protocols are now being established for clinical development in collaboration with bio-pharma companies (both non-engineered and gene-modified) for use in phase 1 studies (examples are GammaDelta Therapeutics and TC BioPharm).

Using the three activation methods studied, Vδ1 and Vδ2 subsets were successfully transduced with a GD2 specific CAR using a maloney murine leukaemia gamma-retrovirus, SFG, pseudotyped with RD114 envelope. The transduction efficiency achieved using ZOL and ConA activation was somewhat lower than that of CD3/CD28 antibody stimulated αβ T cells, but still within acceptable limits for adoptive transfer. Other mechanisms for gene transfer into γδ T cells have also been reported including electroporation of mRNA (164) and lentiviral transduction. Both gamma retroviral and lentiviral transduction methods are being used in CAR T cell clinical trials and when comparisons have been made between the two viral constructs, gammaretroviruses

have conferred higher transduction efficiencies but carry a higher theoretical risk of insertional mutagenesis (267, 268) (although this has been limited to children with SCID, Wiskott-Aldrich and chronic granulomatous disease). Lentiviral integration appears to favour sites away from cellular promoters, whereas gamma-retroviral integrations usually occurs near transcriptional start sites. Electroporation is technically simpler and avoids using viral vectors, thus mitigating the risks of oncogene activation and insertional mutagenesis (269). Although each technique for gene transfer has its own advantages and disadvantages [reviewed in (270)], the main advantage of using lentivirus is that it is possible to transduce less stimulated cells or quiescent cells, which may translate to less differentiated cells, optimal for CAR T cell therapy. Hence lentiviral transduction warrants further investigation in γδ T cells.

The timing of transduction following activation may also influence the capacity of CAR γδ T cells to proliferate, and can affect their phenotype. Although a comparison of day 3 and day 5 transduction was not statistically significant, further investigation using additional longer timepoints and other gene transfer methods would be of value.

Although we chose a CD28 2nd generation CAR, there is increasing knowledge regarding the choice of costimulatory endodomain for optimal CAR T cell phenotype, survival and efficacy. For αβ T cells there have been many innovative engineering approaches to increase tumour specificity and strategies to avoid 'on-target' toxicities. Although for γδ T cells, there are relatively few published studies, Fisher *et al.* designed a chimeric costimulatory receptor containing the endodomain motif from the NKG2D adaptor, DAP10, and found that innate tumour recognition by the Vδ2 receptor in response to phosphoantigens together with costimulation though a GD2-DAP10 CAR, could be used to deliver more specific tumour response. In this 'AND' gate approach to avoid 'on-target, off-tumour' activation , the authors reported enhanced killing of GD2^{+} cell lines but only when the Vγ9Vδ2 TCR was also engaged (206).

6.1.2 Comparison of CAR$^+$ γδ and CAR$^+$ αβ T cell phenotype and functional responses

CAR αβ and CAR γδ T cells were found to have broadly equivalent cytotoxicity *in vitro* against GD2$^+$ neuroblastoma cell lines. To investigate whether γδ T cells had additional advantageous properties over αβ T cells, a series of experiments were performed to compare their proliferative capacity, cytokine production, phenotype and ability to migrate.

A potential significant limitation for translation of CAR Vδ2 T cells to clinical use was their apparent lack of proliferative capacity following antigenic stimulation. The clinically efficacy of CAR T cell therapy is dependent on initial expansion and duration of persistence (271) and for CD19 CAR T cells, long-lasting remissions have correlated with detection of CAR T cells in the patients' peripheral blood, months to years later (113). Although it is generally accepted that CAR T cell persistence is fundamental to achieving lasting remission, this obstacle may be bypassed if an effective E:T ratio can be achieved to eradicate all tumour cells in the first instance (although this approach may be restricted by dose-limiting toxicities). Second or repeated infusions may also be an option, if not restricted by tumour antigen escape [mechanisms reviewed in (272)]. CD19 tumour antigen escape has emerged in the B-ALL field following treatment with CD19 CAR T cells. In a phase 2 trial of tisangenlecleucel (Novartis), of the 16 patients with relapsed disease, 15 had lost expression of CD19. This translates to 15/61 complete responders who later developed CD19 negative relapse (in addition, there were 6 other cases of relapse who were not tested for CD19 expression) (121). This phenomenon is not reported for GD2 antigen which is ubiquitously expressed on neuroblastoma cells, however for other solid tumours, heterogeneity in antigen expression is a major barrier to CAR T cell therapy.

There are various mechanisms to attribute this lack of CAR T cell proliferation and persistence which includes; immune mediated deletion, AICD resulting from IgG

CH_2CH_3 region-derived spacer element of the CAR binding the Fc receptor on innate immune cells (273, 274), and exhaustion of CAR T cells mediated by PD1-PDL1 interactions (275-278). Immunotherapy approaches using Vδ1 are hence particularly appealing given their reported persistence in the circulation, resistance to activation induced cell death (53) and lower expression of exhaustion markers.

The ability of CAR T cells to migrate towards tumour cells was tested using *in vitro* transwell assays. There appeared to be a general trend that CAR transduced T cells migrated less well than their non-transduced counterparts, although this did not reach statistical significance. To further test the hypothesis that γδ T cells have enhanced tumour homing and penetrance, in the first instance, it would be necessary to perform *in vivo* studies. Mouse models studying human γδ T cells have a number of limitations due to significant dissimilarities between human and mouse γδ T cells, including key differences in their ligand specificities. For this reason, it is difficult to model γδ T cell effector functions in transgenic immunocompetent animals, and therefore most studies have used highly immunodeficient mice (e.g. NOD.Cg-Prkdcscid IL-2rgtm1Wjl/SzJ, or NSG mouse) bearing engrafted tumour. Different xenograft models have been established, but none are truly representative of the immunosuppressive tumour microenvironment as there is no interaction with an endogenous immune system. Despite their limitations, xenograft models play an contributing role in demonstrating safety *in vivo* before progressing to phase 1 trials in humans

6.1.3 Cross-presentation by CAR$^+$ γδ T cells

Vδ2 cells transduced with GD2 CAR cross-presented MART1 tumour associated antigen to αβ T cells expressing the HLA-A201-restricted MART1 αβ TCR. The experimental model required the 25 amino acid peptide to be taken up and processed by CAR Vδ2 T cells before eliciting secondary αβ T cell expansion. Although, this provides preliminary results of their *in vitro* functional capabilities, the next important step would be to test other tumour antigens (including tumour cell lysate), and

investigate whether CAR γδ T cells have the ability to cross-present antigens from tumour cells that they themselves have lysed. A model system using GD2 positive/MART1-containing melanoma cells, or alternatively neuroblastoma primary tumours with autologous T cells, could be used to validate this approach.

6.2 Directions for further investigation and clinical translation

This body of work indicates that it is possible to achieve adequate expansion of CAR γδ T cells with enhanced tumour cytotoxicity. Activated CAR Vδ2 cells retain the ability to take up tumour antigens and cross present processed peptide to responder αβ lymphocytes Further work is required to optimise and overcome some of the limitations identified in this study, including CAR γδ T cell proliferation upon secondary antigen encounter and their ability to persist long-term.

The ultimate CAR design has bi/tri-specificity, is engineered for persistence, can overcome the suppressive tumour microenvironment, and ideally can be produced as an 'off the shelf product'. Approaches in αβ T cells have included PD1 inhibition (279), over-expression of chemokine receptors (280, 281), cytokine secreting CARs (282-284), and αβ TCR knock-outs (271). In the already crowded CAR αβ T cell field, γδ T cells have now been marked as an important player with potentially unique capabilities. Further investigation into how improve their efficacy and reduce the burden of toxicities is warranted. In particular, for CAR Vδ2 cells, a combinational approach with PD1 inhibition may be of value, due to their higher expression of exhaustion markers upon antigen stimulation and predisposition to undergo AICD.

In terms of clinical translation, the CAR construct used in this study is also currently being used in a phase 1 trial in αβ T cells at Great Ormond Street Hospital (1RG-

CART) for patients with relapsed or refractory neuroblastoma. Preliminary results (285) have shown that at lower doses ($1x10^7/m^2$), 1RG-CART could not be detected in peripheral blood but for patients treated at higher doses ($1\text{-}10x10^8/m^2$) (n=6) there is limited and transient expansion and transient tumour response in 3/6 (n=6). Importantly, no dose limited toxicities were encountered (Prof Anderson personal communication) and as such, even higher doses are now being investigated. The absence of neurotoxicity using the ScFv Huk666, is an important finding, given that GD2 is expressed on normal neuronal tissue. There is an emerging body of literature demonstrating safety of 14g2a GD2-CARs however, one study using the high-affinity E101K CAR [incorporating a mutated version (E101K) of the anti-GD2 14g2a ScFv that enhances affinity for GD2] reported fatal encephalitis in a pre-clinical model (286) [although the association with on-target off-tumour effect has since been contested (287)].

Vδ1 cells in particular, have distinct properties advantageous for immunotherapy and greater potential for solid tumour penetration. Optimised methods for the clinical scale-up of enriched CAR Vδ1 cells that retain a desired phenotype require further investigation before clinical translation. This could be achieved using the recently published expansion method reported by the Silva-Santos group using OKT3 and cytokine cocktail (192) or even by Vδ1 TCR gene transfer into αβ T cells. There is intense interest in Vδ1 scale-up for immunotherapy and various approaches are currently under investigation by biopharma companies (including Vδ1 cells derived from skin, Gamma Delta Therapeutics).

The bi-specificity of CAR γδ T cells is another desirable characteristic that can also be harnessed to reduce toxicity and/or decrease resistance secondary to antigen downregulation. CARs in αβ T cells have been modified for dual/multi-antigen targeting and combined with Boolean logic gates of 'AND', 'OR' and 'NOT'. Using the innate γδ TCR to recognise transformed cells in combination with CAR overcomes some of the complexities of dual/tandem CAR engineering [reviewed in (288)]. Further work using this approach with costimulatory only receptors in γδ T cells is currently under investigation in our laboratory.

6.3 Final Conclusions

We conclude that γδ CAR T cells, engineered with a 2nd generation GD2-CAR, can be generated in sufficient number for immunotherapy and have potent antigen-dependent cytotoxicity. Their capacity for migration and for uptake and cross presentation of tumour associated antigens marks them out as having potential advantages over conventional αβ CAR T cells, especially in the solid tumour setting.

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