PRE-CLINICAL DEVELOPMENT OF NOVEL ROR1 CHIMERIC ANTIGEN RECEPTOR T CELLS & BISPECIFIC T CELL ENGAGERS

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Declaration

I, Satyen Harish Gohil, 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.

Dedication

To Diya, For Everything.

Acknowledgements

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Abstract

Receptor tyrosine kinase like orphan receptor 1 (ROR1) is an onco-embryonic antigen present on a range of solid and haematological malignancies, including chronic lymphocytic leukaemia. Additionally, limited, low level expression on normal tissues makes it an attractive therapeutic target. Chimeric antigen receptor T cells and Bispecific T Cell Engagers have emerged as exciting immunotherapeutic approaches, utilising the inherent cytotoxic potential of autologous T cells to yield demonstrable benefit for patients. We therefore aimed to generate novel ROR1 CAR T cells and BiTEs.

Following a rat ROR1 immunisation programme, we screened over 150 single cell hybridoma clones to isolate 13 novel antibodies of which 10 bound in a single chain variable fragment format. Iterative optimisation led to two lead candidates that imparted superior cytotoxicity and cytokine secretion. To minimise immunogenicity we screened 50 humanised ROR1 scFv variants and selected a final humanised candidate, hF(1x1), which maintained effector function, specificity and demonstrated broad applicability against a panel of cell lines representing various tumour subtypes.

Focusing specifically on haematological cell lines and CLL, our humanised CAR demonstrates superior cytotoxicity compared to previously reported ROR1 constructs. However, low ROR1 antigen density limits efficacy in B cell malignancies, compared to CD19 CAR T cells and strategies to overcome this are in development.

Our ROR1 BiTE mediates cytotoxicity at low concentrations (ng/ml) and effector to target ratios against cell lines, but in untreated and relapsed CLL patients, inherent T cell dysfunction limits efficacy. This can be overcome with the BTK inhibitor Ibrutinib, with T cells isolated from patients on treatment, showing markedly improved cytotoxicity against autologous CLL cells.

This work has laid the preclinical foundations for translation of these novel agents into clinical trials for a range of tumours including CLL and many of which have high unmet therapeutic need.

Abbreviations

ABC	Antigen binding capacity
ALL	Acute lymphoblastic leukaemia
ASCT	Allogeneic Stem Cell Transplant
BCR	B cell receptor
BCRi	B cell receptor inhibitors
BiTE	Bispecific T Cell Engager
BTK	Bruton's tyrosine kinase
CAR	Chimeric Antigen Receptor
CDR	Complementary determining regions
CEA	Carcinoembryonic antigen
CFSE	Carboxyfluorescein succinimidyl ester
CLL	Chronic lymphocytic leukaemia
CRS	Cytokine release syndrome
DART	Dual affinity retargeting antibodies
DLBCL	Diffuse large B cell lymphoma
ELISA	Enzyme linked immunosorbent assay
EMA	European Medicine Agency
EpCAM	Epithelial cell adhesion molecule
FACS	Fluorescent activated cell sorting
Fab	Fragment antigen-binding
Fc	Fragment crystallisable region
FCR	Fludarabine, Cyclophosphamide, Rituximab
FCS	Foetal calf serum
FMD2A	Foot and mouth disease virus 2A peptide
GD2	Disialoganglioside
GMP	Good manufacturing practice
GvHD	Graft versus host disease
GvL	Graft versus leukaemia/lymphoma
IFNγ	Interferon γ

IHC	Immunohistochemistry
IL-2	Interleukin 2
ip	Intraperitoneally
iv	Intravenous
kDa	kilo Daltons
MCL	Mantle cell lymphoma
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
MRD	Minimal residual disease
mRNA	Messenger RNA
NHL	Non-Hodgkin's lymphoma
NK	Natural killer (cells)
PCP	Planar cell polarity
PDX	Patient derived xenograft
PGK	Phosphoglycerate kinase
PI3K	Phosphoinositide 3-kinase
RACE	Rapid amplification of cDNA ends
ROR1	Receptor tyrosine kinase like orphan receptor 1
ROR2	Receptor tyrosine kinase like orphan receptor 2
SBT	Sleeping beauty transposons
scFv	Single chain variable fragment
SPR	Surface plasmon resonance
TAA	Tumour associated antigen
TCR	T cell receptor
TE	Transduction efficiency
Treg	Regulatory T cell
tEGFR	Truncated epidermal growth factor receptor
WT1	Wilms tumour 1

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1 Introduction

The immune system is intricately related to cancer, playing a vital role in tumour biology, progression and outcomes. Attempts to utilise it therapeutically have faced many hurdles and much debate, but decades of continued research to understand the immunological complexities of cancer, combined with advances in gene therapy has resulted in treatments that demonstrate real promise for patients.

William Coley, a surgeon in the 1890's, utilised Coley's toxin, a mixture of inactivated bacterial species, to trigger immune responses against sarcoma (Coley, 1891). Despite low response rates of 10%, it represented one of the earliest attempts to harness the immune system to target cancer. Following on, Paul Ehrlich in 1909, likened developing neoplasms with invading micro-organisms, to which effective immunity could be mounted (Rhoads, 1954) and these early interventions laid the foundations for immunotherapy.

Initial enthusiasm was muted as tumour cells were thought to be akin to normal tissue and therefore protected by immune tolerance (Burnet and Fenner, 1949, Billingham et al., 1953). This view was challenged, with the realisation that immunocompetent animals treated with chemical carcinogens or tumour cells were protected against subsequent re-challenge and led to the theory of constant immune surveillance (Burnet, 1957, Thomas, 1982). Although paradigm shifting, this was later found to be an overly simplistic approach as the immune system is not solely a passive bystander but plays a pivotal role in tumour evolution. Initial preferential targeting of dominant epitopes, selects for cancer cells better able to evade it, whilst also allowing evolution of cellular strategies to limit tumour antigen expression, such as downregulation of MHC. This immune editing of tumours results in enhanced growth and metastatic potential (Shankaran et al., 2001).

Strategies to utilise the potential of the immune system have made significant progress since Coley's toxin. Although both the innate and adaptive arms are required for effective anti-tumour immunity, $\alpha\beta+$ T cells are the primary focus given their terminal effector status, cytotoxic potential, ability to orchestrate potent immune responses and potential for long lived immunological memory. In keeping with this, T cells are vital for the graft versus leukaemia/lymphoma effect seen with allogeneic bone marrow transplantation (ASCT) and subsequent donor lymphocyte infusions. They mediate the responses to IL-2 therapy and efficacy with PD-1, PDL-1 or CTLA-4 checkpoint inhibition (Rosenberg et al., 1985b, Jenq and van den Brink, 2010, Hodi et al., 2010). Therapeutic responses are dependent on the mobilisation of endogenous polyclonal T cells, whilst more targeted approaches include isolation, expansion and re-infusion of T cells with known specificity for a tumour antigen or through cancer vaccines, which aim to stimulate and expand existing anti-tumour T cells (Rosenberg et al., 1985a, Romero et al., 2016).

A further approach is through genetic engineering of T cells to impart novel specificity. This can be via a new T Cell Receptor (TCR) or artificial chimeric antigen receptor (CAR). A critical limitation of this approach is selection of an appropriate tumour associated antigen (TAA), which should be specific for tumour cells, absent on normal tissues and be stably expressed at a sufficient density to allow for robust immune responses, to minimising antigen escape.

In this regard receptor tyrosine kinase like orphan receptor 1 represents an attractive TAA. It is expressed during embryonic and foetal development but is downregulated by birth and absent from critical organs in adulthood. This differential expression, dependent on developmental stage, is one example of a TAA class. Others include antigens expressed at higher levels on malignant versus normal tissues (such as Wilms tumour 1); those expressed in immune privileged site but also on tumours (such as cancer testis antigens) and TAA resulting from infection with oncogenic viruses (such as human papillomavirus). Another group, and also the largest, are neo-antigens which arise due to the extensive somatic mutation that occurs in the context of cancer cells but are highly patient specific

and cannot be easily targeted with a single novel receptor (Martincorena and Campbell, 2015).

1.1 Receptor Tyrosine Kinase Like Orphan Receptor 1

Receptor tyrosine kinase like orphan receptor 1 (ROR1) and the closely related ROR2, were first identified in 1992, using degenerative primers to screen the SH-SY5Y neuroblastoma cell line for putative tyrosine kinase receptors (Masiakowski and Carroll, 1992). They are both type 1 single pass transmembrane receptors, located on chromosome 1p31-p32 (Reddy et al., 1997) and 9q22 (Oldridge et al., 1999) respectively and are composed of three extracellular domains.

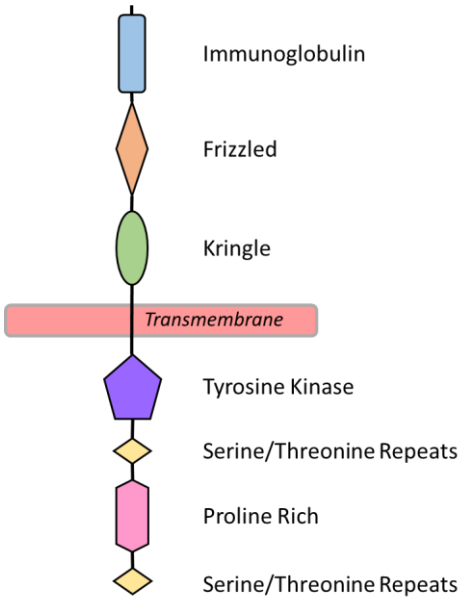


FIGURE 1.1 TOPOGRAPHICAL REPRESENTATION OF ROR1 AND ROR2.

The extracellular domain of ROR1 contains an Immunoglobulin, Frizzles and Kringle Domain. Intracellularly resides a tyrosine kinase domain with a proline rich motif separated by serine/threonine repeats. Adapted from Masiakowski and Carroll, 1982.

An N-terminal Immunoglobulin domain is linked with a Frizzled, cysteine rich domain, followed by the membrane proximal Kringle domain. The intracellular portion contains a tyrosine kinase like domain, along with a proline rich sequence,

the latter flanked by serine threonine repeats. The expected molecular weight is 104 kDa but actual migration is as a 130 kDa protein due to extensive glycosylation, which has a role in signalling (Kaucka et al., 2011). A truncated transcript lacking the extracellular and transmembrane domains has been identified in foetal and adult central nervous tissue, lymphoma and leukaemia cell lines and a subset of solid malignancies, although the precise role for this variant is undefined (Reddy et al., 1996).

The tyrosine kinase domain was initially thought to be capable of auto-phosphorylation, but when introduced into negative cell lines in a purified system, showed no intrinsic kinase activity and this is due to substitutions of key canonical residues (Masiakowski and Carroll, 1992). ROR1 is therefore better considered a pseudokinase, with phosphorylation dependent on cell line specific partner signalling molecules, which are cell type dependent. In chronic lymphocytic leukaemia there is some evidence of constitutive phosphorylation (Gentile et al., 2011, Bainbridge et al., 2014).

The ROR1 family is closely related to the muscle specific tyrosine kinase (MuSK) and tropomyosin receptor kinase (Trk) family and shares many structural features, with the exception of the Kringle domain, which is not seen within any other receptor in the tyrosine kinase family.

There is strong evolutionary conservation with a single homolog in *Caenorhabditis elegans* (CAM-1), which directs migrating cells, orients cell division and controls axon development (Forrester et al., 1999). In *Drosophila melanogaster*, Dnrk and Dror, the homologs of ROR1 and ROR2 respectively, are expressed solely within developing neural tissue (Wilson et al., 1993, Oishi et al., 1997). Homologs have also been detected in *Aplysia californica* (McKay et al., 2001), *Xenopus laevis* (Hikasa et al., 2002) and *Torpedo californica* (Jennings et al., 1993). Expression is seen in neuronal tissue in all cases, where they are postulated to be involved in neurite extension and synapse formation based on in vitro studies (Paganoni and Ferreira, 2005, Paganoni et al., 2010). This led to the synonym of

Neurotrophic Tyrosine Kinase Receptor-Related 1, but this term is no longer widely used.

Analysis of ROR1 in developing murine embryos by in situ hybridisation demonstrate expression in developing brains as would be expected given the above, but also in limbs, especially at the buds. This is in keeping with ROR1 having a role in limb development in other species (Rodriguez-Niedenfuhr et al., 2004). Within the body, ROR1 is expressed in developing lung buds, the aortic arch, heart ventricles, major blood vessels and within areas of the urogenital and gastrointestinal tract (Al-Shawi et al., 2001, Matsuda et al., 2001).

In the first report of ROR1 knockout mice, survival was reported to be less than 24 hours secondary to pulmonary dysfunction and there were no reported gross skeletal morphological abnormalities (Nomi et al., 2001). In contrast ROR2 knockout animals exhibited dwarfism, shortened limbs and tails along with facial abnormalities, ventricular septal defects and respiratory dysfunction (Takeuchi et al., 2000). Mutations in human ROR2 have been identified that account for Robinow and Brachdactyly type 2 syndromes, resulting in similar phenotypic abnormalities; primarily shortened digits and long bones (Schwabe et al., 2000, van Bokhoven et al., 2000). In contrast, no human phenotype or disease has been mapped to mutations within ROR1, suggesting embryonic lethality if absent or mutated.

Re-examination of ROR1 knockout animals by an independent group reported two main differences from the earlier study despite mice being generated through the same mechanism. Firstly, mice survived for more 24 hours, although still died within a few days of birth despite. Secondly, a more focused skeletal examination revealed subtle defects such as fusion of the sternum, moderately shortening of the long bones, cleft in the basosphenoid bone, abnormal development of the C2 vertebral body, kidney defects and enlarged seminal vesicles, which is in keeping with the expression seen in developing murine tissues (Lyashenko et al., 2010).

Combined ROR1 and ROR2 knockouts have a more pronounced phenotype with enhanced sternal defects, dysplasia of the symphyses of bones and complete transposition of the arteries (Nomi et al., 2001).

1.2 ROR1 & Malignancies

ROR1 expression is associated with a range of haematological and solid malignancies making it an attractive therapeutic target. The disease where it is best characterised and most uniformly expressed is chronic lymphocytic leukaemia which I will discuss in greater detail, followed by an examination of its expression within normal human tissues.

1.2.1 Chronic Lymphocytic Leukaemia (CLL)

CLL is the commonest leukaemia in the western world and is a B cell lymphoproliferative disorder characterised by the accumulation of CD5+CD19+ cells in the peripheral blood and secondary lymphoid tissues with resultant peripheral blood lymphocytosis, bone marrow failure, lymphadenopathy, hepatosplenomegaly with or without B symptoms. Associated immune defects result in increased infections, in part, due to hypogammaglobulinaemia but with suboptimal T cell responses also playing a role.

Lymphocytosis, the hallmark of CLL, was initially thought to be due to increased cell survival due to a lack of proliferation seen in vitro and as 99.5% of peripheral blood CLL cells were in the G_0 or G_1 phase of the cell cycle (Andreeff et al., 1980). Heavy water labelling experiments however, show CLL to have a high proliferative capacity, with 10^9 new leukaemic cells generated per day (Messmer et al., 2005), whilst other studies demonstrated markedly reduced telomeres, suggesting multiple cycles of replication (Hultdin et al., 2003). This proliferation occurs within the tumour microenvironment, which plays a pivotal role in the pathogenesis of CLL, as leukaemic cells receive survival and proliferation signals in pseudofollicles

(Herndon et al., 2017). Here they are brought into contact with a range of supporting cells including stromal nurse cells providing favourable cellular interactions and a conducive cytokine milieu (Burger et al., 2000). Signalling through the B cell receptor (BCR) is key to this process and combined with other cell-cell interactions, leads to a cascade of downstream pro-survival and proliferation signals (Herishanu et al., 2013).

The diagnostic criteria of CLL as defined by the International Working Group on CLL (iwCLL) is the presence of greater than $5x10^9$/litre of B lymphocytes in the peripheral blood (with less than 55% pro-lymphocytes), with flow cytometry demonstrating B cell lineage and clonality. CLL shows aberrant expression of the T cell marker CD5 along with the usual B-cell marker CD19, whilst CD20 and surface immunoglobulin levels are lower than on normal B-cells (Hallek et al., 2008).

CLL has a variable clinical course with some patients living with quiescent disease for a number of years, whilst others have a rapidly progressive form of disease requiring urgent therapy. This phenotype is governed by a complex interplay of cytogenetic, molecular and biological aberrations within the leukaemic cells at a clonal and sub-clonal level. The sum of these interactions dictates the biology of the disease, the need and response to treatment as well as long term outcomes. Indeed sub-clones vary over time and with treatment. Massive parallel sequencing has delineated driver mutations and documented clonal evolution patterns (Landau et al., 2015, Landau et al., 2013).

A range of prognostic qualifiers have been developed, beginning with clinical staging systems that assess disease burden and bone marrow failure (Rai et al., 1975, Binet et al., 1981). Despite their age and simplicity, they remain valid tools to stratify patients. More modern approaches assess expression of cell surface and intracellular proteins such as ZAP-70, CD38 and CD49d and of the three CD49d is the most discriminatory (Bulian et al., 2014). Additional prognostic information can be obtained from assessing the molecular signature of the CLL such as the mutation status of the immunoglobulin V gene heavy chain locus, which defines the cell of origin in relation to prior antigen experience (Hamblin et al., 1999, Damle et al.,

1999). This can not only used for prognostication purposes, but has predictive power with regards to response rates to FCR chemotherapy (Thompson et al., 2016).

The advent of high throughout sequencing has revealed a plethora of molecular aberrations that can be used as prognostic markers including mutations affecting the TP53, NOTCH1, SF3B1 and BIRC3 genes, although these are not routinely assessed for in clinical practice (Wang et al., 2011a, Rossi et al., 2012).

At diagnosis, cytogenetic abnormalities occur in 80% of patients and include deletion 13q, deletion 11q and trisomy 12. The key abnormality, and some would argue the most important, is deletion of 17p which encodes the p53 tumour suppressor gene. This is associated with poor risk disease, failure to respond to standard chemotherapy and decreased overall survival (Dohner et al., 2000).

Some patients never require treatment, living with their disease for a number of years, whilst others have a rapidly progressive form requiring prompt therapy. Consensus guidelines developed to guide treatment decisions; Table 1.1 (Hallek et al., 2008).

Treatment Indications
Progressive marrow failure with development of anaemia and/or thrombocytopaenia
Massive (>6cm below costal margin) or progressive symptomatic splenomegaly
Lymph nodes >10cm or progressive lymphadenopathy
Lymphocyte doubling time < 6 months (if starting lymphocyte count >30,000/μl)
Autoimmune anaemia or thrombocytopaenia poorly responsive to standard therapy
Constitutional symptoms - Unintentional weight loss >10% over prior 6 months - ECOG Performance Score 2 or more - Fevers >38 for 2 weeks without evidence of infection - Night sweats > 1 month

TABLE 1.1 IWCLL CRITERIA FOR COMMENCING THERAPY

Treatment for CLL has undergone a revolution with the advent of small molecule inhibitors disrupting BCR signalling, which is vital for CLL survival. They include Ibrutinib; which inhibits Bruton's Tyrosine Kinase (BTK) and Idelalisib; a Phosphoinositide 3-Kinase (PI3K) delta inhibitor. In addition CLL cells have elevated levels of the anti-apoptotic protein BCL2 and Venetoclax, a BH3 mimetic targets this to lead to CLL cell death. These agents have revolutionised the treatment of relapsed/refractory, newly diagnosed and most excitingly 17p deleted patients with significant response rates, which can be maintained for a number of years (Byrd et al., 2013, Byrd et al., 2014, Furman et al., 2014, Roberts et al., 2016).

Prior to the development of these agents the gold standard is the combination of fludarabine, cyclophosphamide and rituximab (FCR) for patients biologically fit enough to receive intensive treatment, termed "go-go" patients (Keating et al., 2005). Additionally, FCR chemotherapy has the advantage of being delivered over approximately 6 months with no need for ongoing treatment. CLL primarily affects the elderly and therefore not all patients would be suitable for FCR. For "slow go" patients, the combination of an anti-CD20 monoclonal antibody such as Rituximab or Obinutuzumab with chlorambucil would be recommended (Goede et al., 2014). For those in between, dose reduced FCR or Rituximab-Bendamustine are other possible options depending on pre-exiting comorbidities and performance status (Eichhorst et al., 2016, Foon et al., 2009). However with the advent of novel agents the boundary between "go-go" and "slow-go" patients has been disrupted due to toxicity profiles that are favourable irrespective of age (Burger et al., 2015b), although these are not completely benign and can have dose limiting toxicities and result in fatalities.

Ibrutinib has been licensed as first line therapy in the Unites States & Europe based on the findings of the RESONATE-2 study which showed superiority over chlorambucil and although these were striking, it did not randomise between comparable arms (Burger et al., 2015b). In the United Kingdom Ibrutinib is authorised in the front line setting for patients with 17p deletion/TP53 mutation or for salvage after failure of at least 1 previous line of therapy. The multi-centre phase III National Cancer Research Institute (NCRI) FLAIR trial is assessing Ibrutinib-

Rituximab against FCR to compare equivalent 1st line regimens for fit non-17p deleted patients. The trial has been expanded to include Ibrutinib-Venetoclax and Ibrutinib monotherapy arms.

One of the major outcomes measures identified with FCR is the attainment of minimal residual disease (MRD), defined as less than 1 CLL cell per 10,000 leucocytes as this is associated with enhanced progression free and overall survival (Kwok et al., 2016). B cell receptor inhibitors (BCRi) by contrast, lead to an initial lymphocytosis, representing translocation of CLL cells from the bone marrow and lymph nodes into the peripheral blood. Unlike traditional chemotherapy which results in rapid tumour de-bulking, the lymphocytosis seen with BCRi can persist for a significant period of time but is not associated with inferior outcomes, with responses improving with time (Woyach et al., 2014). Attainment of MRD negativity is rare with BCRi monotherapy and multiple combination regimens are under investigation to improve this such as with Ibrutinib and Venetoclax, as well as trials looking to stop treatment in those who achieve MRD negativity.

These novel agents are not curative in themselves, with patients required to take continuous treatment, some of which can be associated with dose limiting toxicities requiring cessation. Although patients can be bridged from one novel agent to the next with good responses (Mato et al., 2015), refractory disease still occurs, necessitating novel therapeutic strategies for these high risk patients with unmet need.

In addition, approximately 5% of patients develop a high grade transformation, termed Richter transformation, which is usually a diffuse large B cell (DLBCL) subtype but can rarely transform to a Hodgkin's phenotype. The high grade component is clonally related to the underlying CLL in the majority of cases. Prognosis, is significantly lower than for de novo DLBCL, with the primary treatment objective to consolidate responses with ASCT if fitness allows (Jain and Keating, 2016).

1.2.2 ROR1 and CLL

ROR1 was found to be overexpressed in CLL based on gene expression profiling in two separate studies (Rosenwald et al., 2001, Klein et al., 2001) and confirmed at the mRNA and protein level by a number of groups as demonstrated in Table 1.2. The largest study, analysing over 1500 cases demonstrated expression in 95% of CLL patients, with expression being incorporated into flow cytometry diagnostic panels in some specialised centres (Cui et al., 2016).

ROR1 became a protein of interest in CLL for entirely different reasons following immunotherapy approaches by two different groups. The first investigated infusion of autologous CLL cells transduced to express CD154, as a means of decreasing the immune suppressive nature of CLL and allowing upregulation of costimulatory molecules to promote T cell responses. In this study 3 of the 6 patients developed antibodies against ROR1 and based on this finding, this group went on to develop their own antibody against ROR1, which bound to CLL cells but not normal CD5+ B cells (Fukuda et al., 2008).

The second group used Lenalidomide, an immunomodulatory agent that mediates its anti-leukaemic effect through direct cytotoxicity, modulation of the tumour microenvironment and correction of functional defects in immune cells (Maffei et al., 2016). They demonstrated upregulation of CD154 on CLL cells with subsequent production of anti-ROR1 antibodies (Lapalombella et al., 2010). CD154 upregulation however is not a prerequisite for immune responses as patients can have naturally occurring reactive antibodies and T cells against ROR1 (Hojjat-Farsangi et al., 2015)

Paper	Number of CLL Samples Positive	ROR1 Assessment	Correlation with prognostic markers
(Baskar et al., 2008)	107/107 32/32	mRNA Expression Flow Cytometry	No correlation with ZAP70 No correlation with IgV_H mutational status
(Daneshmanesh et al., 2008)	100/100 18/18	mRNA Western Blotting & Flow Cytometry	No correlation between progressive and non-progressive samples
(Fukuda et al., 2008)	69/69	Flow Cytometry	No correlation with ZAP70
(Hudecek et al., 2010)	8/8 14/14	mRNA Flow Cytometry	
(Li et al., 2010)	7/7	Western Blotting	
(Barna et al., 2011)	16/16 30/30	mRNA Flow Cytometry	
(Shabani et al., 2011)	79/84	mRNA	No correlation with IgV_H mutational status or indolent vs progressive samples
(Uhrmacher et al., 2011)	177/177	Flow Cytometry	No correlation between untreated and treated or with CD38 and ZAP70
(Daneshmanesh et al., 2013)	24/24	Flow Cytometry	% ROR1+ cells increased in 7 patients from Non-Progressive to Progressive Disease
(Hojjat-Farsangi et al., 2013b)	38/38	Western Blotting	
(Janovska et al., 2016)	137/137	Flow Cytometry	
(Cui et al., 2016)	95% of 1568	Flow Cytometry	ROR1High had lower treatment free survival and overall survival ROR1 levels to not change with time or treatment

TABLE 1.2 SUMMARY OF ROR1 EXPRESSION DATA IN CLL.

Although expressed in the majority of CLL patients, there is no evidence to link it to negative prognostic markers such as ZAP70, CD38, Immunoglobulin Heavy Chain mutational status. Furthermore there is no evidence that it varies from

diagnosis through to treatment. A small study in 20 patients suggested that the proportion of ROR1 positive CLL cells was higher in those with progressive disease compared with non-progressive cases (Daneshmanesh et al., 2012). A more recent study has shown higher levels of ROR1 corresponding to a shorter treatment free survival, with $ROR1^{High}$ cases showing enhanced activation of Akt signalling pathway (Cui et al., 2016).This is in keeping with murine studies; when ROR1 was under the control of the IgH promoter to limit expression to the B cell lineage, mice developed a CD5+ lymphoproliferative disorder that could be transmitted to syngeneic animals. The T cell leukaemia 1 (TCL1) transgenic mouse, is frequently used as a mouse model for CLL (Hamblin, 2010) and ROR1xTCL1 mice developed more aggressive disease and potentiation of the Akt signalling pathway (Widhopf et al., 2014).

1.2.2.1 Wnt5a and ROR1

Wnt signalling occurs through two distinct pathways; the canonical pathway, in which signalling stabilises beta-catenin and leads to a range of outputs including cell fate determination, cell renewal and proliferation (Sethi and Vidal-Puig, 2010). In contrast, the non-canonical, planar cell polarity (PCP) pathway is beta-catenin independent and involved in cellular asymmetry and guided cell migration (Witze et al., 2008).

Wnt5a was hypothesised to be the main ligand for ROR1 based on the presence of the cysteine rich domain within the Frizzled portion of the receptor (Saldanha et al., 1998) and there has been an accumulation of evidence supporting a pleiotropic role. CLL cells cultured in the presence of Wnt5a demonstrate enhanced survival (Fukuda et al., 2008) and Wnt5a induces a heterodimer of ROR1 and ROR2 which upregulates CLL motility through activation of Rac1 and RhoA, via the 14-3-3z adaptor protein (Yu et al., 2016, Yu et al., 2017, Sato et al., 2010). In addition, Wnt5a induces ROR1 to complex with haematopoietic-lineage-cell-specific protein 1 (HS1), further enhancing migration of CLL cells (Hasan et al., 2017). There is also

evidence that Wnt5a is produced in an autocrine manner by leukaemic cell with higher Wnt5a levels associated with un-mutated IgV_H samples and earlier time to treatment (Janovska et al., 2016).

1.2.3 ROR1 in other haematological malignancies

ROR1 has been reported in 6-45% of unselected B-ALL patients (Shabani et al., 2007, Shabani et al., 2008, Dave et al., 2012), but is expressed in cases with the t(1;19) translocation. Here, expression is independent of the resulting E2A-PBX1 chimeric transcription factor but due to maturation arrest at an intermediate to late stage of B cell development comparable to normal bone marrow B-cell precursors termed haematogones. These cells are $CD45^{dim}$, CD10+ and CD19+ but also express low levels of ROR1 (Broome et al., 2011, Dave et al., 2012). Within the context of t(1;19) ALL, ROR1 and the pre B cell receptor co-operate to enhance survival such that when pre-BCR signalling is inhibited with dasatinib, there is a reciprocal increase in ROR1 expression mediating enhanced survival (Bicocca et al., 2012).

Other reported lymphoid malignancies expressing ROR1 include Hairy Cell Leukaemia, Mantle Cell Lymphoma (MCL), Marginal Zone Lymphoma, Diffuse Large B Cell Lymphoma and Follicular Lymphoma, although ROR1 levels appear to be lower and with greater variation. Large validation exercises have not been undertaken, thus limiting a true picture to emerge (Hudecek et al., 2010, Barna et al., 2011, Daneshmanesh et al., 2013, Hogfeldt et al., 2013).

ROR1 has also been claimed to be expressed on acute myeloid leukaemia, chronic myeloid leukaemia and multiple myeloma, but this has been reported by single groups, has not been corroborated by others and relied solely on mRNA expression levels with no demonstrated protein expression (Shabani et al., 2011, Daneshmanesh et al., 2013).

1.2.4 ROR1 in solid malignancies

The 4A5 anti-ROR1 antibody developed by Tom Kipps in San Diego demonstrated ROR1 expression in a range of solid and haematological malignancies, specifically: 78/144 (54%) ovarian cancers, 63/110 (57%) colon cancers, 49/64 (77%) lung cancers, 52/58 (90%) lymphomas, 49/55 (89%) skin cancers, 38/48 (83%) pancreatic cancers, 35/48 (73%) testicular cancers, 17/40 (43%) bladder cancers, 28/29 (96%) uterine cancers, 19/21 (90%) prostate cancers, and 10/12 (83%) adrenal cancers (Zhang et al., 2012b).

An assessment by Stanley Riddell's group using the 6D4 antibody, specifically selected and optimised for IHC demonstrated 50% of ovarian cancers to be ROR1 positive (of which 90% of endometreoid adenocarcinomas were positive), 57% of triple negative breast cancers, 42% of lung cancers and 15% of pancreatic cancers, which is considerably lower than the Kipps group.

Within the lung cancer cohort they assessed expression in draining lymph nodes and found only 60% of ROR1 positive primary disease specimens had ROR1 positive metastatic disease (Balakrishnan et al., 2017). Discrepancy between these two studies include the use of differing ROR1 antibody clones, differing IHC protocols and the use of tissue microarrays to assess expression, which can be variable in terms of quality and preparation (Voduc et al., 2008). The Seattle group did use the 4A5 clone as a comparator antibody but claimed it to be suboptimal for IHC analysis.

In addition to these two large studies, other groups have demonstrated expression in a range of malignancies, for example 13/16 renal cell cancer samples showed ROR1 expression at the mRNA level (Rabbani et al., 2010). In breast cancer, expression is seen in 22.4-72.3% of specimens, depending on the study protocol. There is, however, association with higher grade disease, triple negative hormonal

receptor status and metastatic potential secondary to enhanced epithelial-mesenchymal transition and cancer cell survival (Cui et al., 2013b, Chien et al., 2016, Zhang et al., 2012a). Expression has been demonstrated in melanoma cell lines where a dynamic and reciprocal relationship with ROR2 has been reported (Hojjat-Farsangi et al., 2013a, O'Connell et al., 2013, Fernandez et al., 2016).

In ovarian cancer, other groups report 50-55% of cases are ROR1 positive and expression was again associated with lower progression free survival and overall survival (Zhang et al., 2014a, Zhang et al., 2014b). In this setting and unlike melanoma, ROR1 and ROR2 appear to cooperate to bring about a more invasive phenotype and associate with enhanced epithelial-mesenchymal transition; a pre-requisite for metastases (Henry et al., 2015, Henry et al., 2016, Henry et al., 2017).

In lung cancer, there is an association with advanced disease and negative prognostic markers (Yamaguchi et al., 2012, Karachaliou et al., 2014, Liu et al., 2015b, Zheng et al., 2016). Finally neuroblastoma, sarcoma (Potratz et al., 2016, Huang et al., 2015, Elmacken et al., 2015), gastric adenocarcinoma (Chang et al., 2015, Tao et al., 2015) and colorectal carcinoma (Zhou et al., 2017) have also been shown to express ROR1 in variable subsets of tumour samples, but further characterisation of these tumour subtypes is required.

A key finding which highlights the potential of targeting ROR1, is the reported expression on ovarian cancer and glioblastoma stem cells (Zhang et al., 2014b, Jung et al., 2016). These cells are typically more resistant to therapy and form a niche from which relapses occur. Being able to target these cancer initiating cells via ROR1 to enhance anti-tumour responses is an appealing attribute and may lead to more durable responses than with chemotherapy or radiotherapy alone.

1.2.5 ROR1 Expression in healthy normal tissue

No matter how many cancer subtypes express a TAA, its utility depends on normal tissue expression being absent or low. Initial mRNA data for ROR1 demonstrated low level expression on adipocytes, which was subsequently confirmed by flow cytometry but levels were lower compared to CLL cells (Hudecek et al., 2010). ROR1 is also expressed on haematogones in normal marrow, as discussed above. Depletion of these cells does not affect overall bone marrow constitution (Tom Kipps, iwCLL, New York, USA, 2017).

This restricted tissue expression was initially corroborated in a good laboratory practice compliant, tissue cross reactivity IHC study, with no staining seen on normal postpartum tissue using Cirmtuzumab, a high affinity ROR1 antibody. In view of concerns of ROR1 expression on adipocytes, the pancreas and adipose tissue were subjected to detailed scrutiny (Choi et al., 2015a).

These findings were challenged by Balakrishnan et al. who with their ROR1 antibody demonstrated convincing evidence of ROR1 expression in the parathyroid, areas of the gastrointestinal tract, including the stomach and pancreatic islet cells, using both IHC and western blotting (Balakrishnan et al., 2017). Expression on normal tissues has been further confirmed within the Human Protein Atlas datasets (Uhlén et al., 2015) and has realigned our expectations with regards to potential toxicity of targeting ROR1.

1.3 Cellular Immunotherapy

1.3.1 Introduction

The origins of cellular therapy arise from transplantation studies undertaken in the 1950s in which bone marrow derived from foetuses and cadavers were infused into recipients after removal of bone fragments and fat (Thomas et al., 1957). Although it was largely unsuccessful, it was before full knowledge of histocompatibility was elucidated and therefore unsurprising. Even when successful engraftment was achieved, patients often developed fatal graft versus host disease (GvHD). With greater understanding of the basic science that underpinned histocompatibility and better conditioning and immunosuppressive regimens, allogeneic transplantation became more widely used and now is a standard treatment for haematological malignancies.

The potential to generate graft versus leukaemia (GvL) effect was discerned from murine studies (Barnes et al., 1956) but later became apparent in the human setting (Weiden et al., 1979, Weiden et al., 1981). Although many groups had moved to T cell deplete infusions to limit GvHD, this was also associated with a higher risk of disease relapse, confirming T cells' central role in disease control (Marmont et al., 1991). Donor lymphocyte infusions following ASCT, are used a mechanism to control disease relapses through recognition of leukaemia associated antigens or minor histocompatibility mismatches triggering further GvL (Mackinnon et al., 1995).

1.3.2 Allogeneic Stem Cell Transplantation for CLL

ASCT was previously recommended for patients if they achieved no response to intensive therapy, relapsed early after fludarabine containing regimens or had 17p deleted disease. Even in fit patients, myeloablative regimens were associated with significant transplant related complications and mortality and were superseded by reduced intensity conditioning protocols which provided reasonable

overall progression free survival of 50-60% at 2 years with the possibility of achieving further responses with DLI. Despite this, the majority of patients relapse with CLL (Kharfan-Dabaja et al., 2007, Richardson et al., 2013).

With the advent of small molecule inhibitors, the number of allografts being undertaken has substantially decreased and the timing has been deferred until patients have i) failed two lines of conventional therapy and respond to BCR inhibition, ii) have failed front line and BCR inhibition therapy but respond to Venetoclax or iii) have progressive disease or a poor response to Venetoclax (Kharfan-Dabaja et al., 2016). The precise regimen to enable the last cohort of patients to achieve a disease state suitable for transplantation remains challenging.

An alternative to ASCT is autologous transplantation, which utilises high dose therapy with stem cell rescue. This has been assessed in CLL but no clear benefit was demonstrated (Reljic et al., 2015). In addition treatment with Xcellerated T cells, which are autologous T cells ex vivo stimulated and expanded with CD3/CD28 beads before re-infusion into patients, showed no long term benefit (Castro et al., 2004).

1.3.3 TCR Engineered Cells

The native TCR recognises short protein fragments presented in the groove of the major histocompatibility complex. Recognition between a T cell and its cognate MHC:peptide complex leads to activation, proliferation and effector function. Maximal function is dependent on T cells receiving a co-stimulatory signal 2, to provide a positive feedback loop and prevent anergy.

TCR specificity can be redirected by insertion of alternate TCR α and β chains to provide novel T cell specificity against TAA presented by the appropriate MHC. This was first demonstrated with the isolation of the α and β chains from one clone of cytotoxic T cells and transferred to another (Dembic et al., 1986). Proof of concept with this approach has been investigated against a number of antigens in haematological and solid malignancies (Morris and Stauss, 2016) and a recent trial

against NY-ESO-1 demonstrated a 58% overall response rate (Robbins et al., 2015). One of the advantages is that TCR gene therapy is the ability to target intracellular proteins such as Wilms tumour 1 and p53 as well as surface antigens processed by the proteasome and expressed in the context of MHC presentation. Furthermore signalling through the TCR is exquisitely sensitive with 10-100 MHC-peptide complexes able to trigger T cell activation (Valitutti et al., 1995).

Difficulties of TCR gene therapy include the need for correct pairing of alpha and beta chains within T cells, with aberrant cross pairing resulting in potential altered T cell specificity. T cell responses are also dependent on presentation of the peptide fragment on the correct HLA context with the most common being HLA-A*0201 which is present in 40% of the Caucasian population. Despite this, strategies to maximise TCR expression, mute endogenous TCR and enhance signalling have been developed, enhancing the prospect and applicability of TCR engineered T cells (Morris and Stauss, 2016).

1.3.4 Chimeric Antigen Receptor T-Cells

A chimeric antigen receptor (CAR) is composed of an antigen recognition domain fused to intracellular signalling domains which, when introduced and expressed on the surface of T cells allows for MHC independent T cell activation and target cell cytotoxicity. This is important as MHC downregulation is an important evasion mechanism utilised by tumours (Hicklin et al., 1999).

The initial concept of CARs arose when the α and β chains of the TCR were substituted for the heavy and light chain variable regions of antibodies thereby imparting MHC independent antigen recognition (Gross et al., 1989, Goverman et al., 1990). Subsequently the two separate chains were cloned into a single chain variable fragment (scFv) in which the variable regions are linked with a flexible linker sequence, thus maintaining antigen recognition but allowing simpler molecular cloning and transduction (Eshhar et al., 1993).

The antigen recognition scFv, is linked to an extracellular spacer domain, which mediates the distance and flexibility between the CAR on the T cell surface and target cell. This distance can be modified unlike the TCR and peptide-MHC interaction. This distance is critical for effective CAR T cell functioning by allowing appropriate cytotoxic synapse formation, granule secretion and exclusion of inhibitory phosphatases (Hudecek et al., 2013).

The spacer is linked to a transmembrane domain and is linked to intracellular signalling domains, which were initially composed solely of the CD3ζ chain. Although these 1st generation CAR T-cells were able to recognise and kill target cells, long term proliferation was limited (Savoldo et al., 2011).

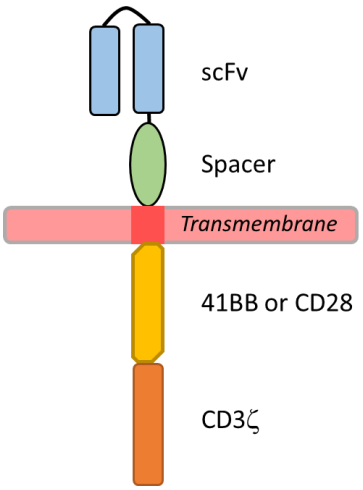


FIGURE 1.2 2ND GENERATION CAR SCHEMATIC

The scFv forms the membrane distal antigen recognition domain and is separated from the T cell membrane by a suitable spacer region. Intracellularly there is a costimulatory domain which is usually CD28 or 41BB intracellular region linked to the CD3ζ intracellular domain.

Physiological signalling requires both TCR (signal 1) and co-stimulation (signal 2) to achieve full T cell activation (Mueller et al., 1989). In CAR format this was reproduced with 2nd generation signalling domains comprising CD3ζ and CD28 or CD3ζ and 41BB fusion proteins, which demonstrate enhanced cytokine secretion, proliferation and most importantly, persistence compared to CD3ζ alone (Maher et al., 2002, Imai et al., 2004). 2nd generation signalling domains remain the most

widely used constructs in clinical trials and the use of CD28 vs 41BB varies by institution and setting. In vitro and in vivo models however, suggest less exhausted T cells with a 41BB-CD3z intracellular chain, whilst CD28 based CAR T cells impart stronger initial cytotoxicity (Long et al., 2015, Cherkassky et al., 2016).

3rd generation signalling domains combine multiple co-stimulatory modules including CD28, 41BB and OX40 with CD3ζ and although these show advantages in pre-clinical settings, clear superiority in clinical trials have yet to be demonstrated (Till et al., 2012, Pule et al., 2005).

Fourth generation CAR T cells are those which in addition to the CAR release soluble pro-inflammatory mediators such as IL-12 (Koneru et al., 2015) or disrupt the tissue micro-environment with release of heparanase, with the aim of promoting tumour infiltration and immune responses, especially against solid malignancies (Caruana et al., 2015). These are just two examples in which CAR T cells can be engineered to enhance their effector function above and beyond the receptor only. Others include manipulation of T cell checkpoints such as secretion of PDL-1 by CAR T cells or expression of a dominant negative PD- 1 receptor to mitigate against exhaustion (Suarez et al., 2016, Cherkassky et al., 2016).

1.3.4.1 CAR T cell Generation

CAR T cell generation is a multistep process, requiring isolation of peripheral blood lymphocytes from patients via leukapheresis, before ex vivo expansion, stimulation and engineering. Once produced, modified cells are cryopreserved until patients have received conditioning chemotherapy, usually with fludarabine and cyclophosphamide, to allow for efficient engraftment (Brentjens et al., 2011).

1.3.4.1.1 Electroporation

mRNA electroporation is a relatively quick and cost effective method to transfect large number of primary cells. Small scale electroporation occurs in cuvettes in which T cells and mRNA encoding the CAR are mixed in a suitable conducive buffer and a set pattern and amplitude of electrical pulses applied. This leads to the formation of pores within the cell membrane, allowing mRNA entry with subsequent transcription and expression. Higher throughput large scale good manufacturing practice (GMP) compliant electroporation occurs in a continuous flow loops, with cells and mRNA passing through a current for a fixed regulated time. This approach has been assessed with mesothelin specific CAR T cells where proof of concept was demonstrated following infusion in 2 patients, one with malignant pleural mesothelioma and the second with pancreatic cancer. A small and transient response was obtained in one patient (Beatty et al., 2014). However, CAR expression is temporary, resulting in significant expenditure and processing for limited efficacy. The rationale however, is that when targeting antigens for the first time, especially those expressed to some degree on normal tissues, transient CAR expression mitigates against significant toxicity due to the short half-life of mRNA and inherently limits CAR expression.

1.3.4.1.2 Sleeping Beauty Transposons (SBT)

SBT is a non-viral electroporation based method which unlike mRNA leads to permanent somatic insertion of the transgene on interest. It relies on insertion of 2 DNA plasmids into cells: the first comprises the transposon, which encodes the CAR sequence sandwiched between inverted repeats and directly repeated DNA sequence motifs and the second is the transposase, which recognises these motifs and translocates the CAR sequence into the host genome (Ivics et al., 1997, Singh et al., 2008). As this technique utilises DNA plasmids there is a considerable cost advantage compared to viral vector based methods, but due to the inherent toxicity of the electroporation process it necessitates significant ex vivo T cell manipulation and processing, including culture on artificial antigen presenting cells, to allow

recovery and expansion. Therefore despite its initial promise, there has been limited uptake and clinical responses (Kebriaei et al., 2016).

1.3.4.1.3 Viral Transduction

Retroviral and lentiviral vectors form the major method by which T cells are engineered. Retroviruses are double stranded RNA viruses that are able to infect dividing cells, following which viral reverse transcriptase generates a DNA template which is inserted into the host genome. The viral genome can be replaced with a transgene of interest (in this case a CAR sequence) to allow permanent modification and expression. Lentiviruses, a subclass of retroviruses, differ in their ability to infect non-dividing cells and have a number of additional genes that regulate viral gene expression and assembly (Mann et al., 1983, Naldini et al., 1996).

Viral vectors need to be able to deliver the transgene into host cells but should be replication incompetent so that new virions are not made. This is achieved primarily by separating the genes needed for viral production *in trans* via separate plasmids.

The transfer plasmid contains the transgene of interest between two long terminal repeats. It also includes a central polypurine tract, psi target site, promoter and woodchuck hepatitis virus post-regulatory element, all of which regulate and enhance transgene expression. One of the helper plasmids contains gag and pol, which is needed for structural proteins and reverse transcriptase respectively, whilst the third plasmid codes for the viral envelope. The most commonly envelope is based on the Vesicular stomatitis virus G (VSVG) glycoprotein, which provides a broad tropism for cells. All three plasmids are required for virus to be made and are transfected together into a packaging cell line, such as HEK-293T. Retroviruses can also be made in specialised helper-free cell lines which constitutively express gag, pol and env requiring only transfection of the transfer plasmid (Elsner and Bohne, 2017).

2nd generation lentiviral vectors are made in a similar fashion requiring three plasmids: a transfer plasmid and 2 helper plasmids, one containing the envelope and the other gag, pol, rev and tat. Rev and tat are additional genes necessary for lentiviral vector generation. To improve safety further and decrease the risk of replication competent virus production. In a third generation system the Rev is placed on an additional plasmid to decrease this risk further. Further safety mechanisms can be introduced by introducing a self-inactivating mutation in the 3' LTR which when transduced is copied to the 5'LTR negates inherent promoter activity (Zufferey et al., 1998).

Concerns with regards to insertional mutagenesis arose following clinical trials with retroviral vectors to treat severe combined immunodeficiency (SCID), due to mutations of the IL-2 receptor γ chain. Although gene therapy was able to restore immunity, 25% of the 20 patients treated developed leukaemia secondary to enhancer mediated mutagenesis (Hacein-Bey-Abina et al., 2003). This led to major investigation of the cause and remedies instituted to prevent this. More recent trials showing no evidence of leukaemia and long term follow up demonstrating safety (Hacein-Bey-Abina et al., 2014, Scholler et al., 2012).

One of the major disadvantages of viral vectors, especially lentiviral vectors is their cost in the clinical grade setting. Manufacture, storage and quality certification can be prohibitively expensive and requires significant infrastructure and capital investment.

1.3.4.2 CD19 CAR T Cells

CAR T cells against a range of haematological and solid malignancy targets have been generated and tested in preclinical studies and transitioned to clinical trials. CD19 however, represents the gold standard by which other studies are assessed, due to early adoption combined with marked clinical success. CD19 expression is highly regulated within the B cell lineage and absent from other tissues making it an attractive target for B cell related malignancies.

CD19 is a 61 kDa protein expressed all stages of B cell development, from pre-B cells onwards until it is lost on plasma cells. It is composed of two immunoglobulin domains separated by a linker region, which is involved with disulphide bonds. It interacts with CD21 and CD81 to regulate B cell signalling through its highly conserved intracellular cytoplasmic tails (Poe et al., 2001).

A number of earlier studies using CD19 CAR T cells laid the foundation for the pivotal publication of 2 papers by the University of Pennsylvania group in 2011 demonstrating efficacy in patients with CLL for CD19 CAR T cells with long term remissions over 2 years in duration and with attainment of MRD (Porter et al., 2011, Kalos et al., 2011). Despite the early reported success of CD19 CAR T cells in CLL, subsequent studies have been less promising and a summary of the clinical trials with CD19 CAR T cells in CLL is shown in Table 1.3.

In view of this some of the focus shifted to ALL and other non-Hodgkin lymphomas, in which higher and deeper response rates have been seen. For example in ALL response rates in the region of 90% have been reported in three centres (Maude et al., 2014, Davila et al., 2014, Turtle et al., 2016).

More recent evidence demonstrates that T cells from patients on Ibrutinib for greater than 1 year show improved transduction, expansion and function in vitro and in vivo (Fraietta et al., 2016a). In addition the Fred Hutchinson group have shown with a defined composition of CD4 and CD8 T cells of 1:1 an overall response rate of 71% was achieved (Turtle et al., 2017). Finally the University of Pennsylvania demonstrate a MRD negativity rate of 89% by multicolour flow cytometry following CAR administration and at least 6 months of therapy with Ibrutinib (Gill et al., 2017). However, such an impressive response rate would be expected given the low burden of disease at the time of CAR T cell infusion combined with the fact that these patients are not as heavily treated as the ones enrolling in earlier clinical trials.

1.3.4.3 Toxicity of CAR T cells

Toxicity from engineered T cell has two forms. The first is 'on target off tumour' toxicity due to expression of the target antigen on normal healthy tissues. For CD19 this is manifested by profound, but non-lethal B cell aplasia due to CD19 expression on normal B cells, which can be managed with intravenous immunoglobulin administration. 'On target off tumour' toxicity can however be severe as seen with the anti-Her2 CAR studies which led to fatal pulmonary toxicity in a patient with colonic cancer due to low levels of Erbb2 on lung epithelial cells. Similarly 3 patients treated with CAR T-cells against carbonic anhydrase IX developed hepatic toxicity due to its expression on bile duct cells (Lamers et al., 2006, Morgan et al., 2010). This concern extends to the whole breadth of gene engineered cells including TCR modified T cells as demonstrated by fatal cardiac toxicity when targeting MAGE-A3 (Cameron et al., 2013).

To try to model this and limit clinical complications, tissue cross reactivity studies and animal models including in non-human primates, form part of the extensive preclinical screening undertaken.

Strategies to mitigate against this include the use of suicide systems in the engineered T cells, as a method by which to purge cells in the event of toxicity. This can be achieved via pharmacological methods, through small dimerisation molecules that trigger apoptosis (Straathof et al., 2005, Di Stasi et al., 2011). Alternatively cell surface proteins can be expressed independent of the CAR, which can be targeted efficiently by monoclonal antibodies. Examples pf these include RQR8 which incorporates the CD20 epitope targeted by Rituximab (Philip et al., 2014) or truncated Epidermal Growth Factor Receptor (tEGFR) which is bound by Cetuximab (Paszkiewicz et al., 2016).

Wu et al. created a system by which the ScFv and extracellular portion are spatially separated from the intracellular signalling domains and only combine in presence of a drug to allow T-cell activation. This provides a dose dependent and tuneable on and off switch (Wu et al., 2015).

The second form of toxicity arises due to tumour killing and resultant rapid expansion of CAR T cells. This manifests primarily as neurological disturbance, pyrexia, elevated inflammatory cytokines and is termed cytokine release syndrome (CRS) in which interleukin 6 (IL-6) plays a central role. Treatment of CRS is supportive in the most part but if severe high dose steroids with or without the anti IL6 antibody, Tocilizumab, which reverses the pro-inflammatory drive. Interestingly IL-6 is not released from the CAR T cell population but from bystander myeloid cells (Barrett et al., 2016).

One of the major complications with regards to CRS includes neurotoxicity, which is thought to represent translocation of highly activated cytotoxic T cells into the cerebrospinal fluid with resultant neuronal irritation and is manifested by seizures, stupor and confusion. Knowledge gathered from numerous trials has led to a suggested management algorithm for the management of CRS incorporating early supportive measures, identification of biomarkers and criteria for therapeutic intervention. There is however some belief that a degree of CRS is required to generate a persistent T cell response (Davila et al., 2014, Lee et al., 2014a, Lee et al., 2014b). CRS has been responsible for patient deaths in clinical trials and therefore close clinical monitoring in centres with experience and facilities is essential for CAR T cell therapy.

Overall CD19 CAR T cells represent an extremely exciting and potent immunotherapeutic with which to target CLL, with the potential for long term clinical remissions.

Publication	CLL Patients	Construct	Conditioning	Cell Dose CAR T cells x10^7/kg	Response
(Brentjens et al., 2011) MSKCC	8	CD19 scFv SJ25C1 Retrovirus CD3/28 Beads 2nd Generation 28z	None or Cyclophosphamide	0.4-3	3xNR, 1xPR, 2xSD, 1xPD and 1xNA
(Porter et al., 2011) (Kalos et al., 2011) UPenn	3	CD19 scFv FMC63 Lentivirus CD3/28 Beads 2nd Generation 41BBz	Pentostatin/Bendamustine +/- Cyclophosphamide	1-1.6	2xCR, 1xPR
(Kochenderfer et al., 2012) NCI	4	CD19 scFv FMC63 Retrovirus CD3 antibody + IL-2 2nd Generation 28z	Fludarabine & Cyclophosphamide	0.3-3	1xSD, 2xPR, 1xCR
(Kochenderfer et al., 2013) NCI	4 Post ASCT	CD19 scFv FMC63 Retrovirus CD3 antibody + IL2 2nd Generation 28z	None	0.4-2.4	2xPD, 1xSD, 1xCR
(Porter et al., 2015) UPenn	14	CD19 scFv FMC63 Lentivirus CD3/28 Beads 2nd Generation 41BBz	Fludarabine/Cyclophosphamide or Pentostatin/Cyclophosphamide or Bendamustine	1.4-110	4xCR, 4xPR, 6xNR CR>4 years in 2 patients

Publication	CLL Patients	Construct	Conditioning	Cell Dose CAR T cells x10^7/kg	Response
(Porter et al., 2014) UPenn	26	CD19 scFv FMC63 Lentivirus CD3/28 Beads 2nd Generation 41BBz	Not specified	5 or 50 in total	5xCR, 4xPR
(Kochenderfer et al., 2015) NCI	4	CD19 scFv FMC63 Retrovirus CD3 antibody + IL2 2nd Generation 28z	Fludarabine and Cyclophosphamide	0.25-0.4	3xCR, 1xPR
(Turtle et al., 2015) Fred Hutch	6	CD19 scFv FMC63 Lentivirus CD3/28 Beads 2nd Generation 41BBz Defined CD4:CD8 of 1:1	Fludarabine and Cyclophosphamide	0.02-2	3xCR, 2xPR, 1xNR
(Turtle et al., 2017) Fred Hutch	24	CD19 scFv FMC63 Lentivirus CD3/28 Beads 2nd Generation 41BBz Defined CD4:CD8 of 1:1	Fludarabine and Cyclophosphamide	0.02-2	17 CR+PR

TABLE 1.3 SUMMARY OF CLINICAL TRIALS UTILISING CD19 CAR T CELLS FOR THE TREATMENT OF CLL.

CR = Complete Response, PR = Partial Response, NR = No Response, NA = Not assessable, SD = Stable Disease. MSKCC = Memorial Sloan Kettering Cancer Centre, UPenn = University of Pennsylvania, NCI = National Cancer Institute, Bethesda, Fred Hutch = Fred Hutchinson Cancer Centre, Seattle.

1.4 Bispecific T Cell Engagers

Conventional antibodies do not recruit or utilise T cells for their cytotoxic action. In order to utilise their potential however, bispecific monoclonal antibodies in which one arm binds a TAA and the other to CD3 were developed to recruit and redirect T cells to target tumour cells. Early attempts at this however demonstrated limited cytotoxicity (Perez et al., 1985, Riethmuller, 2012).

To try and improve efficacy of bispecific antibodies Mack et al. engineered two antigen binding arms as scFvs and placed them as contiguous protein. To allow this both V_H and V_L sequences within a scFv and parallel scFvs were separated by flexible linkers (Figure 1.2). This bispecific antibody construct targeting the 17-1A antigen and CD3, was approximately 55kDa in size, retained specific binding and mediated T cell dependent cytotoxicity greater than the parental bispecific whole antibody (Mack et al., 1995).

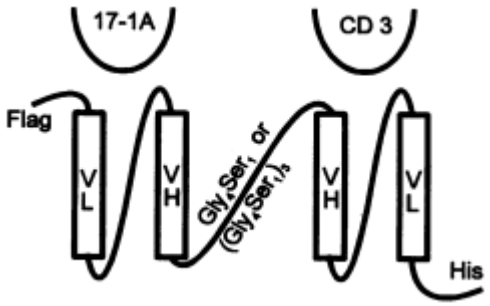


FIGURE 1.3 STRUCTURE OF BISPECIFIC T CELL ENGAGERS.

The first reported Bispecific T cell engager was compose of a scFv targeting the TAA CO17-A along with a CD3 targeting scFv from the TR66 clone. From (Mack et al., 1995).

This led to the eventual generation of a CD19 and CD3 bispecific construct using the same architecture (Loffler et al., 2000), which has subsequently been known by a variety of names including MT103 and AMG103, but most commonly Blinatumomab. The name is derived from **B lin**eage-specific **a**nti-**tu**mour **mo**use **m**onoclonal **a**nti**b**ody and are widely known as BiTEs; Bispecific T cell engagers.

Within this construct the CD19 scFv is thought to derive from HD37 clone and CD3 from the L2K murine antibodies (Nagorsen et al., 2012), although there will have been significant engineering of the parental sequences and the final complete sequence in Blinatumomab is unknown. This uncertainty is compounded in the by differing sequences deposited in different antibody databases.

BiTEs allow for the formation of cytotoxic synapses between T cells and target cells independent of MHC and co-stimulatory molecules (Dreier et al., 2002). The majority of killing is thought to arise from effector and memory T cells as naïve T cells have a higher basal activation threshold. BiTEs lead to upregulation of CD25 and CD69 activation markers, release of cytotoxic granules containing granzyme and perforins, increased cytokine secretion and T cell proliferation leading to target cell death mediated through pore formation as well as triggering of apoptotic pathways (Brandl et al., 2007). In addition BiTEs are able to mediate serial killing of target cells by a single T cell at low concentrations (Hoffmann et al., 2005).

Blinatumomab has been extensively investigated in ALL, with studies demonstrating the potential of treatment in Philadelphia positive and negative ALL (Topp et al., 2014, Martinelli et al., 2017). The first Phase 3 trial using Blinatumomab compared to conventional therapy showed superiority of Blinatumomab with the trial being halted early due to improved survival, although this was only 7.7 vs. 4 months (Kantarjian et al., 2017).

The first phase 1 trial of Blinatumomab was actually in relapsed refractory NHL patients (Bargou et al., 2008, Nagorsen et al., 2012) and further trails have focused on patients with follicular, mantle cell and diffuse large B cell lymphoma, some of which have maintained long term responses despite very low doses of BiTE being administered (Viardot et al., 2016, Goebeler et al., 2016).

Toxicity with Blinatumomab is similar to CAR T cells comprising neurotoxicity thought to be secondary to T cell mediated toxicity through breach of the blood brain barrier. This however can be limited with pre-mediation with dexamethasone, which is a standard part of the treatment protocol. Although the management of

CRS in BiTEs is similar, the short half-life of the molecule in the circulation helps limit the pro-inflammatory drive, on cessation of the infusion.

Both the original CD19xCD3 bispecific and subsequently Blinatumomab have demonstrated in vitro cytotoxicity against CLL, at high target to effector ratios representative of what would occur in patients. However these co-cultures with BiTEs were prolonged and read between 5-7 days, by which time there would have been an inherent decrease in CLL cell viability (Loffler et al., 2003, Wong et al., 2013). In addition although one of the earliest Phase I clinical trials included 2 patients with CLL, substantial Phase II and III trials in CLL are lacking.

1.5 ROR1 Therapeutics

Targeting of ROR1 due to its expression profile is a logical developmental pathway especially as anti-ROR1 antibodies inhibit CLL survival and RNA interference suggested a survival role for ROR1 in cell lines and primary CLL cells (Fukuda et al., 2008, Choudhury et al., 2010).

1.5.1 Antibodies

Cytotoxic antibodies rely on three modes of action for their effect

i) Antibody dependent cellular cytotoxicity (ADCC) which provides an "eat me" signal to the immune system
ii) Complement dependent cytotoxicity (CDC), the terminal phase of which is the generation of a membrane attack complex generating pores in targeted cells
iii) Direct antibody mediated cytotoxicity.

Rituximab, the most widely used antibody in oncology, targets CD20 and improves outcome measures in a range of B cell malignancies. Its ability to efficiently target B cells has also expanded its role into non-malignant conditions. Rituximab works mainly by CDC and newer type II antibodies, such as Obinutuzumab, have been engineered to maximise ADCC and direct cytotoxicity through engineering of the Fc region of the antibody to increase affinity to Fc receptors on macrophages (Boross and Leusen, 2012).

Other antibodies in oncology, such as checkpoint inhibitors and the anti-HER2 antibody trastuzumab, utilise inhibition of signalling as their modus operandi. This is also the main mechanism of action of Cirmtuzumab, the most clinically advanced monoclonal antibody against ROR1. The precursor antibody, D10, was identified from hybridomas due to its ability to inhibit ROR1 mediated signalling, unlike comparator antibodies such as 4A5 and 2A2. Its parental form was a relatively low affinity antibody but it was subject to humanisation and affinity matured. Its final format has shown the ability to inhibit EMT and survival of cancer cells (Zhang et al., 2014b) . A phase I study in 10 patients demonstrated its safety although no overt clinical responses were seen and further clinical trials have been expanded in CLL and initiated in breast cancer (Choi et al., 2015b).

Other groups have developed their own antibodies against ROR1, with a rabbit immunisation programme yielding clones Y31, R11 and R12, which all show minimal CDC. Although the R12 scFv has been utilised in a CAR format, it did demonstrate a degree of ADCC but required a supra-physiologically high concentrations of effector cells to achieve this (Yang et al., 2011).

In comparison, Daneshmanesh et al developed a panel of anti-ROR1 antibodies targeting the three extracellular domains of ROR1 following a mouse immunisation programme with distinct peptides from each domain. They noted direct cytotoxic potential with 5 of their antibodies, 3 of which were also able to induce CDC and one ADCC. The antibodies that bound to more membrane proximal epitopes appeared to show enhanced function (Daneshmanesh et al., 2014, Daneshmanesh et al., 2012). Despite this, no further translational development of these antibodies has been reported to have been undertaken.

1.5.2 Armed Antibodies

In order to improve the cytotoxic potential of antibodies they can be linked with toxins or radioactive nucleotides. A prime example is Brentuximab Vedotin, an antibody drug conjugate which targets CD30, results in internalisation and releases Monomethyl auristatin E, triggering cell death (Francisco et al., 2003). The same strategy has been trialled with Cirmtuzumab but despite initial impressive in vitro functional data, no armed version of the antibody has been planned for assessment (Cui et al., 2013a).

In the similar vein, a Pseudomonal exotoxin was conjugated to the 2A2 ROR1 antibody, which was able to induce CLL and MCL cell death in vitro. This functions to activate phosphatases in B cell malignancies which are present at high levels in inactive forms, thereby triggering apoptosis. Again, despite initial encouraging data, no clinical trials have been planned (Mani et al., 2014, Mani et al., 2015).

1.5.3 Chimeric Antigen Receptor T Cells

The first ROR1 CAR T cell reported utilised the anti-ROR1 2A2 clone and demonstrated toxicity against cell lines and primary CLL cells but spared normal B cells (Hudecek et al., 2010). The same group went onto compare the 2A2 scFv with clone R12 and showed superior cytotoxicity with the latter. They claim this was due to R12 having a higher affinity, but as the epitopes that R12 and 2A2 bind differ, this is not a true comparison. They did however demonstrate the importance of the spacer with the finding that a hinge only spacer, derived from the IgG stalk, provided optimal cytotoxicity (Hudecek et al., 2013).

The R12 CAR therefore became their lead candidate and has been tested in a non-human primates, as it cross reacts with macaque ROR1. Macaques showed no toxicity despite comparable ROR1 expression with humans and high doses of CAR T cells being infused (Berger et al., 2015). This group also demonstrated ROR1 CAR T

cells are able to target breast cancer, neuroblastoma and sarcoma cell lines (Elmacken et al., 2015, Liu et al., 2016). The R12 CAR has subsequently been licensed to Juno therapeutics and is being investigated in a Phase 1 clinical trial for all ROR1 positive malignancies (NCT02706392).

Deniger et al. utilised the sleeping beauty system to engineer T cells expressing and ROR1 CAR utilising the 4A5 scFv clone derived from the Kipps group. Cytotoxic potential was demonstrated against cell lines, but the animal model undertaken required repeated IL-2 administration, which is not usually required with a 2nd generation CAR architecture that was used. This may be secondary to the sleeping beauty manufacturing process which requires significant ex vivo manipulation (Deniger et al., 2015). Sleeping beauty generated ROR1 CAR T cells, based on this construct, were due to be investigated in a clinical trial (NCT02194374) but this study has been withdrawn, citing a lack of available reagent, although the exact rate limiting reagent is not stipulated.

Trial Identification	Reagent	Target	Stage of Development
NCT02222688 University of San Diego	UC-961 (Cirmtuzumab)	Relapsed/Refractory CLL	Recruiting
NCT02860676 University of San Diego	UC-961 (Cirmtuzumab)	Extension study for NCT02222688	Recruiting by invitation only
NCT02776917 University of San Diego	UC-961 (Cirmtuzumab) + Paclitaxel	Locally advanced/metastatic breast cancer unsuitable for resection	Not open
NCT03088878 University of San Diego	UC-961 (Cirmtuzumab) + Ibrutinib	CLL/MCL	Not open
NCT02194374 MD Anderson	Autologous ROR1 CAR T cells 4A5 scFv Sleeping Beauty Modified	CLL	Withdrawn
NCT02706392 Fred Hutchinson/Juno	Autologous ROR1 CAR T cells R12 scFv Lentiviral transduction	CLL MCL ALL Triple negative breast cancer Non-small cell lung cancer	Open

TABLE 1.4 CLINICAL TRIALS WITH ROR1 THERAPEUTICS

A current list of the pending, active and withdrawn clinical trials that target ROR1. From: https://clinicaltrials.gov/ct2/home, as of 28th October 2017.

2 Aims

We hypothesise targeting ROR1 with immunotherapies will allow treatment for a broad range of malignancies, including but not limited to CLL and many with unmet therapeutic need.

Our aim is therefore to identify novel antibodies against ROR1; develop these into best in class CARs and BiTEs and undertake pre-clinical evaluation to generate sufficient data to allow transition to clinical trials.

2.1 Isolate and characterise novel scFv against ROR1

The starting point for CAR or BiTE development is the identification of ROR1 specific scFvs. The first aim is to therefore isolate and characterise novel antibodies against ROR1 following a rat immunisation programme and transition these to a scFv format.

2.2 Develop Chimeric Antigen Receptor T Cells and assess their potential

From the panel of scFv constructs identified, the next aim was to generate a panel of ROR1 CAR T cells, optimise them and select a lead candidate to take forward for more detailed analysis.

2.3 Undertake humanisation of the lead scFv

To limit immune responses directed against a rat derived scFv, the next aim was to undertaken a humanisation programme to identify constructs that maintained or enhanced functional activity, whilst limited immunogenicity.

2.4 Develop Bispecific T Cell Engagers and assess their potential

In conjunction with CAR development we also aimed to generate a ROR1 BITEs and assess their function in a range of malignancies, to diversify immunotherapeutic approaches of targeting ROR1 in addition to CAR T cells.

3 Methods

3.1 Molecular Biology

3.1.1 Transgene Construction

DNA sequences encoding proteins of interest were generated using overlap extension PCR using Phusion DNA polymerase (New England Biolabs). Starting DNA originated from pre-existing plasmid constructs (from Martin Pule). Novel DNA sequences were from G-blocks (Integrated DNA Technologies) or synthesised commercially (Genscript) depending on length and complexity. For commercially sourced DNA sequences, either from G-blocks or plasmids, an initial round of PCR was undertaken to amplify sufficient quantities of DNA for downstream cloning. Codon optimisation for human expression was employed for these constructs.

Were necessary primers were designed to introduce restriction sites at the 5' and 3' positions for downstream cloning. For multiple fragment ligation, amplicons were generated with complementary overhangs. Some sequences required insertion into a subcloning vector for which we used Topo TA (Thermo Fisher).

The PCR cycle for Phusion comprised: 98^{oC} for 2 minutes to denature and activate the polymerase followed by 35 cycles of, 98^{oC} for 40 seconds to denature DNA, 65^{oC} for 40 seconds for annealing, 72^{oC} for 1 minute/kb of amplification. There was a final 10 minutes at 72^{oC}.

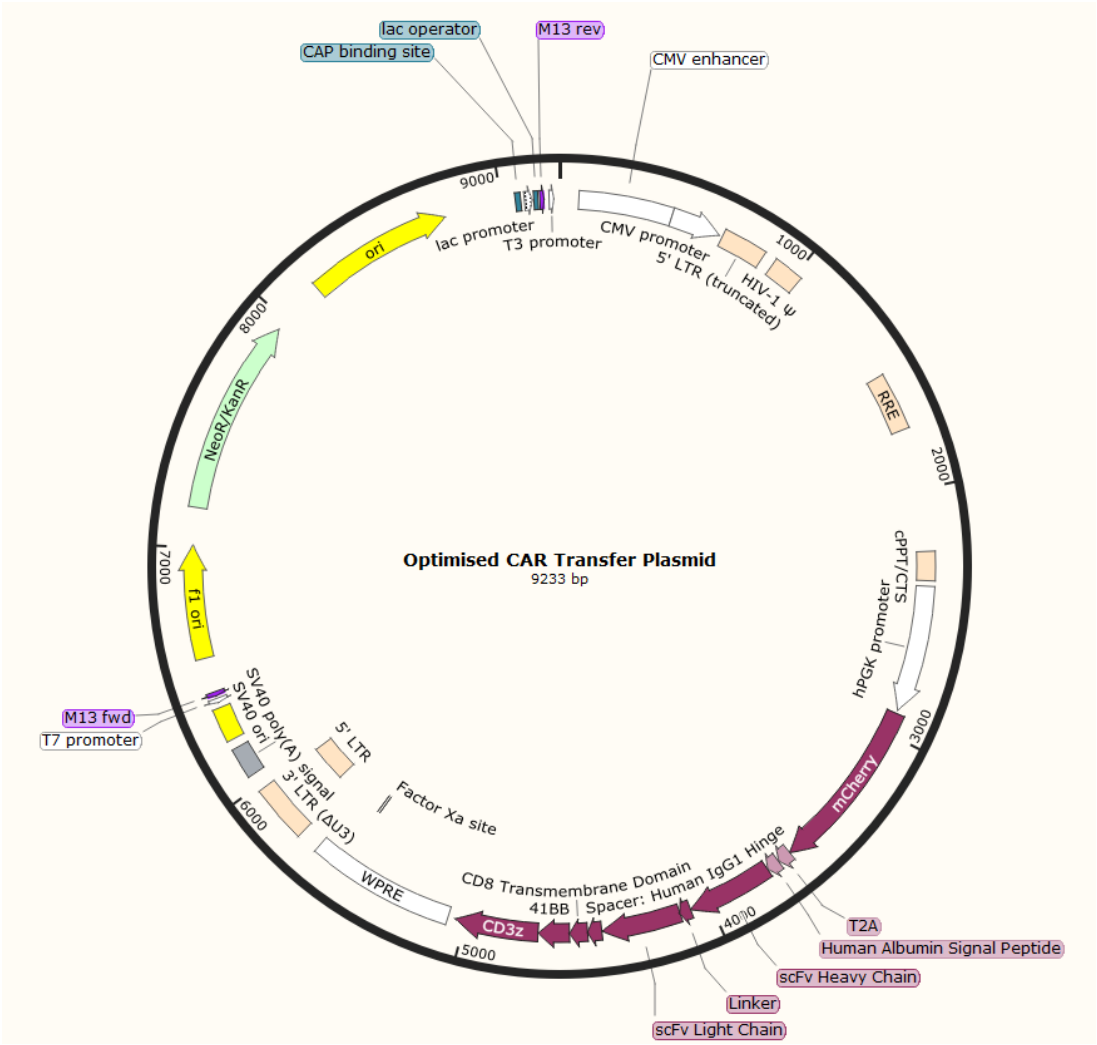


FIGURE 3.1 OPTIMISED TRANSFER PLASMID USED FOR CAR T-CELL PRODUCTION

The CAR sequence of choice was cloned into a 3rd generation lentiviral construct with a human PGK promoter. mCherry was used as a transduction marker and followed by a T2A ribosomal skipping element. The scFv sequence was placed in a heavy chain, linker, light chain format and utilised the hinge spacer from human IgG1. This was followed by a CD8 transmembrane domain with 41BB and CD3ζ intracellular signalling arms. The design was modular such that each component could be switched with simple cut and paste cloning.

3.1.2 Gel Electrophoresis

PCR products and DNA from restriction digests were separated using Tris/Borate/EDTA (TBE) gel electrophoresis and stained with 0.005% Sybrsafe (Thermo Fisher) or 0.01% Ethidium Bromide (Sigma Aldrich). Samples were mixed with a loading dye to aid visualization and deposition into wells. The percentage of TBE used varied from 0.75-2% depending on the size of the expected products. Gels were run at 120V until sufficient separation of DNA had occurred, size was

estimated in comparison with Hyperladder standards (Bioline) and DNA bands corresponding to the correct size were excised with a clean scalpel. Gels were visualised on a transilluminator (Ngene) to avoid ultraviolet degradation.

3.1.3 Gel and PCR Purification

Isolation of DNA from TBE gel fragments as well as purification of PCR products was undertaken with Wizard SV Gel and PCR-Clean Up Kit (Promega) following the manufacturer's instructions. Elution was undertaken with double distilled H_20.

3.1.4 DNA quantification

DNA was assessed using a ND-1000 Nanodrop spectrophotometer (Thermo Fischer) at 260nm to calculate concentration. We also looked at the 260:280nm ratio to gauge quality of the isolated DNA samples for downstream use.

3.1.5 Restriction Digestion

All restriction digests were undertaken using enzymes from New England Biolabs in appropriate buffer. Where double digests required enzymes with incompatible buffers, DNA was subjected to sequential digestion.

5μg of vector backbone was used as starting material in restriction digests. For PCR products all of the DNA obtained following gel purification was used. The final volume for digestion was 100μl and the total enzyme content was 2.5-5% depending on enzymes used.

When using enzymes leaving blunt ends at both sides of the DNA sequence we undertook de-phosphorylation to prevent recircularization of the backbone using Antarctic Phosphatase (New England Biolabs).

3.1.6 DNA ligation

Ligation of DNA with complementary ends was undertaken with Quickligase (New England Biolabs). Digested vector backbone and insert were mixed in a 1:1 ratio in appropriate buffer with 0.5µl of ligase per 10µl reaction. This reaction was left at room temperature for 5 minutes before placing back onto ice and was read for E.coli transformation.

3.1.7 DNA Sequencing

Generated plasmid constructs were sent for Sanger sequencing using appropriate forward and reverse primers (Source Bioscience). Data was analysed using Snapgene Software (GSL Biotech).

3.2 5' Rapid Amplification of cDNA ends (RACE)

Oligoclonal hybridomas were separated into single cell clones either by limited dilution or single cell sorting into 96 well plates and colonies grown until confluent (approximately 2 weeks) with hybridoma cell supplement (Roche). Supernatant was screened against ROR1 positive and negative cell lines to ensure the presence of a specific anti-ROR1 antibody and screened using rat immunoglobulin isotyping ELISA kits (eBioscience or BD Bioscience).

Clones were grown until confluence in 6 well or 10cm plates and then pelleted into RNAlater (Life Technologies) before RNA was extracted using RNA MiniPlus Kit (Qiagen). RNA was reverse transcribed to cDNA using Quantitect Reverse Transcriptase (Qiagen). An aliquot of this cDNA was assessed with GAPDH primers (forward GCCGAGCCACATCGCTCAGA and reverse GAGGCATTGCTGATGATCTTG respectively), which were able to differentiate genomic and cDNA to ensure quality of samples.

cDNA had a poly-C tail added with Terminal Transferase (New England Biolabs) and nested PCR reactions were performed based on the isotyping results

(Phusion: New England Biolabs or Platinum Taq High Fidelity: Life Technologies) to identify the variable regions of the heavy and light chains, using primers specific for light chain isotype and heavy chain isotype.

PCR products were run on a 1% TBE gel and post-stained with Gelstar (Lonza) which provided better resolution of low levels of DNA compared to SybrSafe and Ethidium bromide. Bands of the correct size between 300 and 1000 base pairs were extracted and sent for direct sequencing or inserted into Topo TA or Topo Zero subcloning vectors (Life Technologies) for subsequent sequencing.

To allow us to directly sequence the PCR products, primers were designed such that they bound further within the constant regions and allowed read through of the sequencing reactions without omission of the terminal variable region thereby obviating the need for Topo subcloning.

Primers for 5'RACE	
Description	**DNA Sequence 5' - 3'**
PolyC_Anchor_Outer	ACGGTGCAAACCTTCCTCCAAATCGGG
PolyC_Anchor_Inner	ACGGTGCAAACCTTCCTCCAA
Rat Lambda Outer	GACAGACTCTTCTCCACAGTGTTC
Rat Kappa Outer	CTTGACACTGATGTCTCTGGGATAGAA
Rat Kappa Inner	CACGACTGAGGCACCTCCAGTTGCTAA
Rat IgH Outer	CCCAGACTGCAGGACAGCTGGGAA
Rat IgH Inner	GCTGGACAGGGCTCCAGAGTTCCA
Rat IgM Outer	GTTCTGGTAGTTCCAGGAGAAGGAAA
Rat IgM Inner	CAGGCAGCCCATGGCCACCAAA

Sequence data was compared to the IMGT V-QUEST database of Rat germline immunoglobulin sequences and consensus sequences obtained that were productive and had an in frame signal sequence (Brochet et al., 2008, Alamyar et al., 2012).

Overlap extension primers were designed to amplify the heavy and light chains whilst introducing a GGGGSGGGGSGGGGS linker sequence to generate ScFv constructs. A secreted version of the ScFv was produced by cloning the ScFv sequence in frame with murine IgG2a constant region using NcoI and BamHI sites

(or if needed the compatible BglII or BclI sites) and into the pCCL.PGK lentiviral backbone which included an extracellular spacer, 41BB and CD3ζ using SalI and BamHI sites.

3.3 Bacterial Work

3.3.1 Bacterial transformation

Plasmid amplification was undertaken in C2987H chemically competent DH5α E. coli (New England Biolabs). In isolated cases where enzymes were dam and dcm methyltransferase dependent we utilised the C2925I dam-/dcm- competent E. coli (New England Biolabs).

2µl of Quickligase reaction or 100ng of plasmid for re-transformation was mixed with competent E. coli for 30 minutes on ice before heat shocking at 42^{oC} for 30 seconds. The reaction mixture was returned to ice for 5 minutes before addition of 250µl SOC media (New England Biolabs) and agitated at 37^{oC} for 60 minutes at 220 revolutions per minute before plating onto agar plates with appropriate antibiotic for screening. The antibiotics we used were Ampicillin or Kanamycin depending on the plasmid (Sigma). Plates were incubated at 37^{oC} overnight and single colonies selected and grown in 4 ml of Luria-Bertani (LB) or Terrific broth (TB) overnight with appropriate antibiotic in a rotational shaker.

3.3.2 DNA Isolation from E.Coli Cultures

The method of DNA isolation varied depending on the volume of starting material. For screening purposes we undertook minipreps using Macherey-Nagel miniprep kit with a starting volume of 1.5ml of medium. These were subjected to restriction digest to assess the digest pattern to delineate the correct sequence. For larger quantities of DNA we utilised Macherey-Nagel Midi kits (100ml media), Maxi kits (300ml media) or Qiagen Megaprep Kits (400ml media). DNA was eluted into ddH_2O and quantified as described above. DNA was stored at -20^{oC} until required for use.

3.4 Protein Work

3.4.1 Protein Electrophoresis

For protein separation we utilised the NuPage Novex system. Samples were prepared in a final volume of 60µl with sample reducing reagent and LDS sample buffer. The sample was then boiled at 95^{oC} for 5 minutes. 15µL of each sample was run on a premade 4-12% Tris-Bis gels at 200V, 500mA for 45 minutes on constant voltage mode. A protein standard was incorporated for size estimation (All Novex reagent, Thermo Fisher).

3.4.2 Coomassie staining of protein gels

Following separation gels were stained overnight with Coomassie Blue 0.25% in a rotating agitator. The next day, de-stain was added to remove excess dye and the protein gel photographed. For protein estimate we generated a standard curve using Pierce Bovine Serum Albumin Standards (Thermo Fisher). Protein concentrations were calculated using ImageJ software (Schneider et al., 2012).

3.4.3 Western blotting

Post-separation, protein was transferred from Tris-Bis gels to polyvinylidene difluoride membrane by semi-dry transfer. Sandwich transfer was undertaken at 15V for 15 minutes in transfer buffer (Novex, Thermo Fisher). Following transfer, membranes were blocked with PBS supplemented with 5% milk powder overnight with circular rotation. Staining was undertaken with an anti-His Horseradish Peroxidase antibody (Biolegend) at a concentration of 1:2000 for 1 hour with gentle rotation supplied by rotating plate shaker. The sample was then washed 3 times with PBS supplemented with 0.1% tween for 15 minutes each time. The membrane was developed by addition of Pierce ECL plus Western Blotting Substrate (Thermo Fisher) for 5 minutes. Visualisation of protein bands was undertaken by exposure of X-ray film (Fujifilm) to the membrane for 10-600 seconds depending on signal intensity.

3.4.4 Antibody and fusion Fc protein production

For full length antibodies the light chain sequence was clones in frame with the human kappa light chain constant region in a plasmid co-expressing BFP and in a separate plasmid, the heavy chain in frame with the human IgG1 heavy chain constant region co-expressing GFP (both from Martin Pule). Co-transfection of these into HEK-293T cells liberated antibodies into the supernatant.

For Fc fusion proteins, the ligand of interest (ROR1 or scFv usually) was cloned in frame with the murine IgG2a Fc stalk in the pFUSE-mIgG2a-Fc plasmid (Invivogen, from Martin Pule). As with the antibody, transient transfection of HEK-293T cells led to secretion of Fc fusion proteins into the supernatant which was used for downstream processing.

3.4.5 BiTE purification

3.4.5.1 Screening Purification

When large numbers of BiTEs had to be purified we utilised gravity columns. Supernatant containing BiTE was diluted 1:1 in loading buffer and passed through HiTrap Talon 1ml gravity columns (General Electric), washed and eluted with 3ml of elution buffer containing a final concentration of 150mM Imidazole. Purity and concentration was assessed against a BSA standard in a Coomassie gel. Binding buffer: 50 mM sodium phosphate, 300 mM NaCl1, pH 7.4. Wash buffer: 50 mM sodium phosphate, 300 mM NaCl1, 5 mM imidazole, pH 7.4. Elution buffer: 50 mM sodium phosphate, 300 mM NaCl1, 150 mM imidazole, pH 7.4

3.4.5.2 Large Scale Purification

Media containing BiTE was diluted 1:1 with loading buffer and subjected to fast Protein Liquid Chromatography (FPLC) using 1ml or 5ml HiTrap Talon binding columns with an AKTA Explorer (GE Healthcare Life Sciences). Following loading, washing was undertaken with at least 10 column volumes of wash buffer, elution

was undertaken with a three step process using differential imidazole concentrations and fractionated. Fractions were run on a Coomassie to ascertain BiTE containing aliquots and pooled and subjected to dialysis in Slide-A-Lyzer cassettes (Thermo Fisher) with a molecular cut off of 10 kDa in 5 litres of PBS overnight. Purified, dialysed BiTE was then quantified in A Coomassie gel and 1% BSA added to act as a stabiliser and limit aggregation.

3.4.6 Size Exclusion Chromatography

Purified ROR1 BiTE was subjected to Size exclusion chromatography, undertaken with a Shimadzu Nexera XR HPLC machine and Waters size exclusion chromatography column at Bio-Analysis Centre London, who provided graphical and analytical data with regards to the runs.

3.4.7 Biacore SPR

Surface plasmon resonance was undertaken on a Biacore X100 machine (General Electric). CM5 chips were labelled with mouse capture antibody using the mouse antibody capture kit. Supernatants containing scFvs of interest (with a murine Fc stalk) were then captured on the anti-murine antibodies on the chip. SPR readings were undertaken with dilutions of full length ROR1 conjugated to His (Acro Biosystems). Data was analysed with X100 software to generate kinetics data.

3.4.8 Peptide library ELISA

An ROR1 peptide library for the extracellular domain of ROR1 was generated by Mimotopes. Peptide fragments were composed of an N-terminal biotin, a linker sequence and then 11 amino acids of ROR1, followed by a C-terminal amide group. Each 11 amino acid peptide had 8 amino acids that overlapped the previous peptide whilst introducing 3 new amino acid to its sequence. Lyophilised eROR1 peptides were solubilised in 50% Acetonitrile using a water bath sonicator. Peptides were then diluted in 0.1% Azide in PBS-Tween 20 (PBS-T). Control peptides (Mimotopes)

were treated and processed in the same way. Control antibodies, also provided by Mimotopes, were dissolved in purified water as per manufacturer's instructions.

Briefly, 96-well plates were coated with Neutravidin, blocked with 1% sodium caseinate and washed thoroughly before incubation with the solubilised peptides. After 1 hour of incubation, plates were washed again and incubated overnight with clones SA1 and F. On the next day, plates were washed and incubated for 1 hour with an anti-human IgG antibody conjugated to horseradish peroxidase (HRP). Plates were washed, and antibody signal detected with Tetramethylbenzidine (TMB); the reaction was then stopped with H_2SO_4. Samples were read in a plate reader at a wavelength of 450nm.

3.4.9 Commercial Protein Production

Absolute antibody limited were used as a commercial manufacturing organisation for large scale antibody, BiTE and bispecific scFv-Fc antibody production.

3.5 Tissue Culture

3.5.1 Cell Lines & Culture

Cells lines were obtained from American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), from within institutional banks within the Cancer Institute or University of Verona. Cells were grown in Roswell Park Memorial Institute (RPMI), Dulbecco's Modified Eagle Medium (DMEM) or Iscove's Modified Dulbecco's Medium (IMDM) supplemented with GlutaMAX and 10% South American Origin Foetal Calf Serum (Thermo Fisher). RPMI had HEPES incorporated at a final concentration of 25mM. No antibiotics were used for routine cell culture. Cells were incubated with 5% CO_2 and 37.2^{oC}.

Cell Line	Origin	Media	Nature
HEK293T	ATCC	IMDM	Adherent
SKW6.4	ATCC	RPMI	Suspension
Jeko1	ATCC	PRMI	Suspension
Raji	ATCC	RMPI	Suspension
Kasumi2	DSMZ	RPMI	Suspension
PCL12	DSMZ	RPMI	Suspension
697	DSMZ	RPMI	Suspension
MEC1	University College London	RPMI	Suspension
PANC1	ATCC	RPMI	Adherent
MiaPaCa2	University of Verona	RPMI	Adherent
CFPAC	ATCC	RPMI	Adherent
PSN1	University of Verona	RPMI	Adherent
SUIT2	University of Verona	RPMI	Adherent
MDA-MB-231	University College London	RPMI	Adherent
MCF7	DSMZ	RPMI	Adherent
SKOV3	ATCC	RPMI	Adherent
HOC7	University College London	RPMI	Adherent
HEY	University College London	RPMI	Adherent
SK-Hep-1	University College London	RPMI	Adherent
HUH7	University College London	RPMI	Adherent
U251	University College London	RPMI	Adherent
A72	University College London	RPMI	Adherent
T618A	University College London	RPMI	Adherent
DU145	University College London	RPMI	Adherent
PC3	University College London	RPMI	Adherent

TABLE 3.1 CELL LINES USED WITHIN THIS PROJECT, THEIR PROVENANCE, CULTURE MEDIA AND CHARACTERISTICS

Suspension cell lines were split every 2 to 3 days depending on confluence and diluted 1:10 to 1:20 with fresh media. For adherent cell lines, we washed the adherent layer once with PBS and then added Trypsin-EDTA for 5 minutes at 37^{oC} before adding fresh media with FCS to neutralise the trypsin. Adherent cells were split 1:10 in new culture flasks twice a week.

3.5.2 Transient Transfection

Transient transfection was performed with HEK293T cells in tissue culture dishes or plates using Polyethylenimine (PEI) or GeneJuice (Merck) at the below ratios depending on experimental requirements. HEK293T cells were plates 24 to 48 hours prior to transfection and assessed for optimal confluency. Transfection reagent and serum free media were mixed well and left for 10 minutes before addition of DNA, mixed again and left for 15 minutes before being pipetted onto 293T cells in a drop wise fashion. The mixture was agitated in the plate to ensure optimal distribution and cells returned to the incubator for 3-5 days before cells or supernatant was harvested.

Culture Vessel	PEI/GeneJuice	Serum Free Media	Total DNA
10cm Dish	30µl	470µl	12.5µg
6 well	5µl	95µl	2 µg
12 well	2.5µl	47.5µl	1 µg
24 well	1.25µl	23.75µl	0.5 µg

3.5.3 Stable BiTE producer cell line

Retroviral vectors containing the BiTE sequence of interest along with a GFP reporter gene were produced. HEK293T cells at low levels of confluency were infected in 6 well plates, followed by expansion. GFP positive cells were sorted to

high levels of purity (>99%) and BiTE collected from subsequent passages of stably transduced cells.

3.5.4 Peripheral blood mononuclear cell isolation

Whole blood was obtained by venesection from consenting donors into EDTA containing syringes (final concentration 5mM EDTA). Blood was diluted with RPMI with no FCS in a 1:1 ratio and 25ml of this diluted blood was layered onto 10ml of Ficoll-Paque Plus (General Electric Health Care) in 50ml centrifuge tubes. Tubes were centrifuged at 750G for 40 minutes at room temperature with no acceleration or deceleration. The buffy coat layer was removed using Pasteur pipettes and PBMCs were washed twice with RPMI media with 10% FCS and adjusted to the appropriate concentration.

3.5.5 Magnetic Cell Manipulation

For CAR T cell cultured we removed CD56+ cells comprising NK cells which may lead to false positive non-specific killing in our co-culture assays. This was undertaken with CD56 beads and magnetic columns following the depletion steps. For CLL cell isolation we used a pan CLL negative isolation kit and for isolation of T cells specifically from PBMCs we utilised Pan T cell isolation kit, again a negative selection kit (All Miltenyi Biotec).

3.5.6 Retronectin coating preparation of tissue culture plates

Non tissue culture treated 24 well plates were coated with retronectin (Takara Bio) in preparation for T cell transduction with 500ul of PBS and 4ml of retronectin per well. Plates were sealed with film and left at 4^{oC} for at least 16 hours or until required.

3.6 Viral Vector Production

3.6.1 Retroviral vectors

We utilised the SFG retroviral vector system for use in cell lines which was kindly provided by Martin Pule. RD114-pseudotyped transient retroviral supernatant was generated by triple transfection of 4.69μg Peq-Pam plasmid (GagPol), 3.125μg RDF plasmid (RD114 envelope), and 4.69μg SFG (transfer plasmid), per 10cm plate as above. Supernatant was harvested at 72 hours and used fresh or snap frozen and stored at -80^{oC}.

3.6.2 Lentiviral vectors

For lentiviral vectors we utilised a 3rd generation packaging system developed by Didier Trono through Addgene. This included 3.13μg of pRSV-Rev (contains rev), 4.06 μg of pMDLg/pRRE (contains gag and pol), 2.19μg of pMDG.2 (containing VSVG envelope) and 3.13μg of the transfer plasmid based on a pCCL backbone. HEK293T cells were plated at 2x10^6 cells/10cm plate and 48 hours later the above plasmids were mixed with plain media and GeneJuice. Supernatant containing vectors was harvested 48 hours later and used fresh after filtering through 0.22μm filters or snap frozen.

3.6.3 Primary Cell Transduction

T cells for in vitro and in vivo work were transduced with 3rd generation lentiviral vectors. PBMCs were re-suspended at 2x10^6/ml and seeded at 1ml per 24 well plate and activated with CD3 and CD28 antibodies at 0.5mg/ml (Miltenyi Biotec) or CD3/CD28 Beads (Invitrogen). The next day fresh media was added with IL-2 to a final concentration of 100IU/ml and 6 hours later cells were harvested, counted and re-suspended at 0.6x10^6/ml and 0.5ml was seeded into a 24 well plate pre-coated with retronectin. 1.5ml of lentiviral supernatant was added to each well with fresh IL-2 to a final concentration of 100IU/ml and spun for 40 minutes at

1000G. Two days later cells were harvested, re-suspended at $0.5x10^6$/ml with 50IU/ml of IL-2 and left to expand.

3.7 Flow Cytometry & Sorting

Flow cytometry was undertaken on Becton Dickinson (BD) machines comprising BD Accuri, BD FacsVerse, BD LSR Fortessa or BD Symphony. Cell sorting was undertaken by the UCL Cancer Institute Core Facility on a BD FACS Aria.

Cells of interest were washed with PBS, stained with directly conjugated antibodies +/- viability dye and incubated at 4^{oC} for 30 minutes. Cells were washed again and re-suspended in 500ml of PBS. Isotype controls and non-stained cell populations were included to allow generation of appropriate gates. Where necessary OneComp beads (eBioscience) were used to adjust compensation settings. Data was analysed on FlowJo Software.

For sorting we included Normocin, a broad spectrum antibiotic and antifungal into the media at the time of the sort and for 24-48 hours post sort. Cells were sorted into enriched RPMI media containing 20% FCS and upon healthy recovery of the sorted population the FCS percentage was reduced to 10%.

All antibodies were sourced from Biolegend.

3.7.1 Antigen Binding Capacity measurements

Qifikit beads were used to assess number of target antigens per cell using the manufacturer's instructions (Dako, Agilent Technologies). In brief the cells of interest were incubated with the same concentration of unconjugated murine anti-human ROR1 or CD19 antibodies, washed twice with PBS and then stained with anti-murine Fc conjugated antibody linked to FITC or APC. Calibrated beads with known numbers of binding sites were stained in the same way allowing the construction of a standard curve with mean fluorescence intensity measurements relating to absolute antigens per cell.

3.8 Functional Assays

3.8.1 Chromium Release Assay

Target cells ($1x10^6$) were harvested and centrifuged. The cell pellet was incubated with 3.7 Mega Becquerel of ^{51}Sodium Chromate for one hour, with agitation every 15 minutes, before being washed 3 times with fresh media. 50,000 cells were plated per well in a 96 well plate. Effector cells, comprising CAR T cells, were added at various ratios to a final volume of 200µl per well. Plates were spun at 400G for 5 minutes before incubating at 37^o C for 4 hours. 100µl of supernatant was removed and Chromium release quantified by a Gamma Counter (Perkin Elmer). Maximal lysis was calculated with supernatant with cells incubated with 1% Triton-X.

Chromium release was calculated based on (Experimental Release – Background Release) / (Maximal Release – Background Release) x 100.

3.8.2 Flow Cytometry Based Cytotoxicity Assay

3.8.2.1 Target Cell Preparation

To facilitate a flow cytometry based cytotoxicity assay we found that pre-labelled target cells were optimum to allow accurate delineation of cell number. For cell lines this was primarily achieved by transducing them to express GFP using a retroviral vector with subsequent single cell dilution or flow based cell sorting. For some cell lines and primary CLL samples we labelled cells with Carboxyfluorescein succinimidyl ester (CFSE; CellTrace CFSE Cell Proliferation Kit, Thermo Fisher).

Cells of interest are suspended in 1mL of warm PBS + BSA 0.1%. The working dilution of CFSE in 1mL of warm PBS + BSA 0.1% to give a final concentration of 1µM. Both solutions are mixed in a 1:1 ratio and incubated at 37^{oC} for 10 minutes. The reaction is quenched by adding 5 volumes of cold RPMI and incubate 10 min on

ice. Cells are washed in media for and re-suspended to 0.25x106/ml and 100ml added to each well of a 96 well plate.

3.8.2.2 Effector Cell Preparation

For CAR T cell assays it is vital to normalise expression levels and transduction efficiencies to allow comparable assessment between constructs. Transduction efficiency is assessed the day prior to set up and normalised to the lowest population, through the addition of un-transduced cells. Cells are counted and re-suspended typically at $0.5x10^6$/ml and 100ul added to each 96 well. This gives an effector to target ratio of 2:1 but this can be adjusted depending on the experimental conditions.

3.8.2.3 Assay Conditions

Each condition is undertaken in triplicate to allow statistical analysis to be undertaken. Following mixture of effector and target cells, 96 well plates are spun at 400G for 5 minutes and returned to the incubator overnight. The next day 100μl of supernatant is removed for cytokine ELISA.

A master mix of viability dye (Propidium Iodide from Sigma and Fixable Viability Dye, ebioscience), anti-CD3 antibody (Clone SK7) is added to each well. The master mix also contains Flowcheck Microspheres (Beckman Coulter) to act as an internal control and allow normalisation between individual wells. After 30 minutes at 4^{oC}, the plate is washed and samples transferred to micro-rack FACS tubes and analysed by flow cytometry. The stopping criteria are a 1000 beads per well.

3.8.3 CD107a Degranulation Assay

For CD107a Degranulation Assays we undertook the same process as above with a 2:1 effector to target ratio. At the same time as the assay was established we added 10ml of anti-CD107a antibody (Biolegend or BD Bioscience, Clone H4A3) and

was incubated at 37^{oC}. An hour later we added 2ml of Golgistop (BD Bioscience), a protein transport inhibitor containing Momensin and left to incubate for 2 hours before staining and assessment of CD107a expression on T cells.

3.8.4 MTS Based Cytotoxicity Assay

Co-culture with adherent cells were also undertaken in 96 well plates, containing 1x10^4 target cells, 1x10^4 T cells, and purified BiTE at concentrations of 0.1 ng/mL–1 mg/ml. The effector to target ratio was adjusted based on transduction efficiency and no BiTE was added. Twenty-four hours after the addition of ROR1 BiTE or CD19 BiTE, supernatant was collected for cytokine evaluation. To assess cytotoxicity, we washed the plate of effector cells and used the CellTiter 96 AQueous One Solution Cell Proliferation Assay MTS assay (Promega). This is a colorimetric assay in which the colour change of the media is proportional to the activity and hence survival of cells.

3.8.5 Cytokine ELISA

Interferon-γ and IL2 cytokine levels were quantified using sandwich ELISA (Biolegend) following manufacturers recommendations. For CLL co-cultures we utilised Legendplex Cytokine assay (Biolegend) which is a bead based flow cytometry ELISA assay.

3.9 Immunohistochemistry

All immunohistochemistry was undertaken at the University of Verona by our collaborators, Dr Claudio Sorio and Dr Marzia Vezzalini. The heavy and light chains of our ROR1 scFv were cloned in frame with the murine IgG1 constant and kappa constant regions, respectively, and antibody was produced by Absolute Antibody Ltd. Normal pancreas and pancreatic tissue microarrays were obtained from US-Biomax. Slides were prepared using the standard laboratory protocols. Briefly,

antigen retrieval was undertaken by immersing slides in 0.01 M sodium citrate buffer, pH 6.0 at 95^{oC} for 15 min before cooling and rinsing once with PBS, and then blocked and stained with ROR1 antibody (1:250) in PBS/Tween20, 0.05% BSA, 1% NaN34 mM for 60 min at room temperature. Slides were incubated with the HRP-conjugated secondary, Histofine Simple Stain MAX PO (Nichirei), and developed using Stable DAB Plus (Diagnostic Biosystems).

3.10 Animal Studies

All animal works were performed under the authority of the United Kingdom Home Office Project and Personal License regulations and were compliant with University College London guidelines.

3.10.1 Solid Tumour Studies

3.10.1.1 Intraperitoneal Model

Six- to eight-week-old female Hsd:Athymic Nude-Foxn1 mice (Charles Rivers Laboratories) received 2x10^6 PANC-1 cell transduced to express firefly luciferase or 5x10^6 SKOV3 cells transduced to express firefly luciferase by intraperitoneal injection. Luciferase expression was detected using D-Luciferin (Melford Laboratories), which was injected intraperitoneally (IP) at a dose of 200 mg/mouse. Bioluminescence imaging (BLI) was undertaken using the IVIS Imaging System 100 Series (Perkin Elmer) at multiple time points. Living Image 4.4 software (Perkin Elmer) was used to quantify the BLI signal. T-Cells and BiTE was injected ip at the same time at ratios and concentrations described within the experiments.

3.10.1.2 Subcutaneous Model

5x10^{6}of PANC-1 cells were mixed in an equal volume of Matrigel (Corning) and were injected in the flank of 6–8-week-old Hsd:Athymic Nude-Foxn1. Once the

xenograft were established (minimum size 100 mm^3), mice received $5x10^6$ T cells by single tail vein injection, followed by a daily injection of PBS, ROR1, or CD19 BiTE suspended in 0.1% BSA in PBS mg/kg/mouse). Tumour volume was calculated using the ellipsoidal formula (length × $width^2$)/2.

3.10.2 Haematological Model

6-8 week old Nod-SCID IL2$\gamma^{-/-}$ mice (Charles Rivers Laboratories) were used for in vivo experiments with haematological cell lines. $0.5x10^6$ SKW6.4 or Jeko1 cells were injected into mice via tail vein on Day 0 before being allowed to engraft uniformly. Mice were subsequently randomised and followed by BLI until uniform signals detected. Mice were injected with a single T-cell infusion and BiTE (10μg/kg)/PBS as per the experimental procedure and followed up by BLI expression and survival.

3.10.3 Intravenous injection of mice

Animals were briefly heated in a warming box to 39^{oC} to encourage peripheral vasodilatation. Tail vein cannulation was performed using a 27 gauge needle, followed by intravenous injection of tumour cells (200μl volume). Tumour samples or T-cells were suspended at appropriate concentration in PBS.

3.10.4 Monitoring and euthanasia of experimental animals

Mice were monitored daily for evidence of poor health including poor grooming, hunched posture, piloerection, etc. Animals were culled if found not to be in satisfactory condition, if hind leg paralysis developed, or at pre-specified experimental time points. Termination was by inhalation of escalating concentration of carbon dioxide, with confirmation of death by cervical dislocation.

3.11 Statistical Analysis

Graphs, standard curves and statistical analysis was undertaken using Prism Software v7.03 (Graphpad). Error bars represent standard deviation where depicted. When two groups of variables were being analysed we used the non-paired t-test, when multiple groups were analysed with used either a one way or two way ANOVA with Bonferroni post-test correction depending on the number of variables. A p value <0.05 was taken as significant and where indicated: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

4 Results

4.1 Generation of novel anti-ROR1 scFvs

4.1.1 Introduction

Emil von Behring and Shibasaburo Kitasato were amongst the first to describe the role of passive immunity when they demonstrated serum from diphtheria or tetanus treated animals protected naïve controls (Kaufmann, 2017). In addition, the side chain hypothesis proposed by Paul Ehrling stipulated cells had distinct receptors on the surface, which in reaction with specific targets, were amplified and released into the blood. These studies laid the foundations to the identification and characterisation of antibodies (Winau et al., 2004).

Antibodies were produced for a number of years in in a range of host organisms, but mainly small rodents and rabbits but their polyclonal nature limited their potential. In 1975 lymphocytes from immunised animals were fused with a myeloma cell line, generating a population of immortal monoclonal antibody secreting cells. This ability to generate and subsequently purify a single defined antibody species, revolutionised both science and clinical therapeutics (Kohler and Milstein, 1975).

The basic building block of both CAR T cells and BiTEs is the antigen recognition domain, which is typically composed of an antibody derived scFv but can use naturally occurring ligands (Shaffer et al., 2011).

Existing antibodies against ROR1 have been generated through murine and rabbit immunisation programmes as discussed in section 1.5. We therefore undertook a rat immunisation programme, to increase the likelihood of targeting novel epitopes and broaden the potential antibody repertoire.

4.1.2 Rat Immunisation

Aldevron GmBH undertook an immunisation programme in 3 Wistar rats using their proprietary technology. This involved initial immunisation with a plasmid encoding full length extracellular ROR1, followed by 2 rounds of repeated DNA immunisation using gene gun technology. Genetic immunisation involves delivery of DNA coated on gold micro particles directly into tissue where it is processed into protein, taken up by antigen presenting cells, primarily dendritic cells and presented via MHC, leading to potent cell mediated and humoral immunity (Wolff et al., 1990, Tang et al., 1992, Gurunathan et al., 2000).

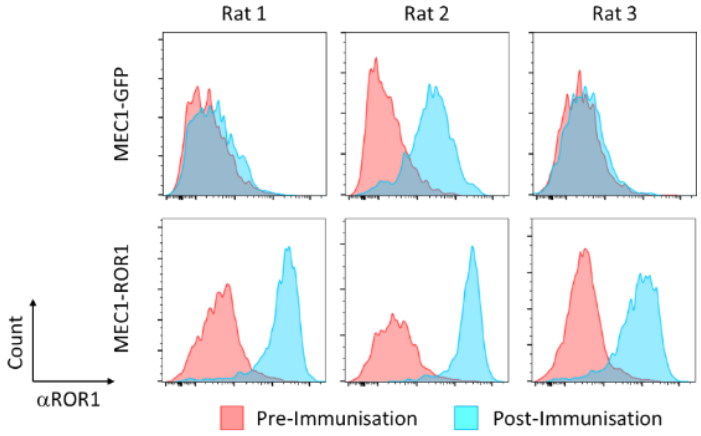


FIGURE 4.1 SEROCONVERSION ASSESSMENT IN IMMUNISED RATS.

The MEC1 cell line is derived from a CLL patient undergoing prolymphocytic transformation but does not constitutively express ROR1 (data not shown). MEC1-GFP cells lines were transduced to express GFP (MEC1-GFP) or full length extracellular ROR1 fused to GFP (MEC1-ROR1). Cells were incubated with pre and post immunisation serum from vaccinated rats followed by incubation with an anti-Rat Fc directed secondary antibody.

Baseline and post-immunisation serum from the three animals was assessed for polyclonal anti-ROR1 antibodies, with rat 1 and 3 demonstrating production of ROR1-specific antibodies (Figure 4.1). Aldevron GmBH subsequently isolated lymph nodes from these animals and resulting lymphocytes were fused with a myeloma cell line resulting in 38 oligoclonal hybridoma clones.

These were transferred to University College London and expanded in culture. Resulting supernatant was screened for antibodies with the ability to differentially bind to a ROR1 positive and negative cell line. Of the 38 clones tested, antibodies against ROR1 were detected in 36, with only clones N and Beta showing no binding (Figure 4.2).

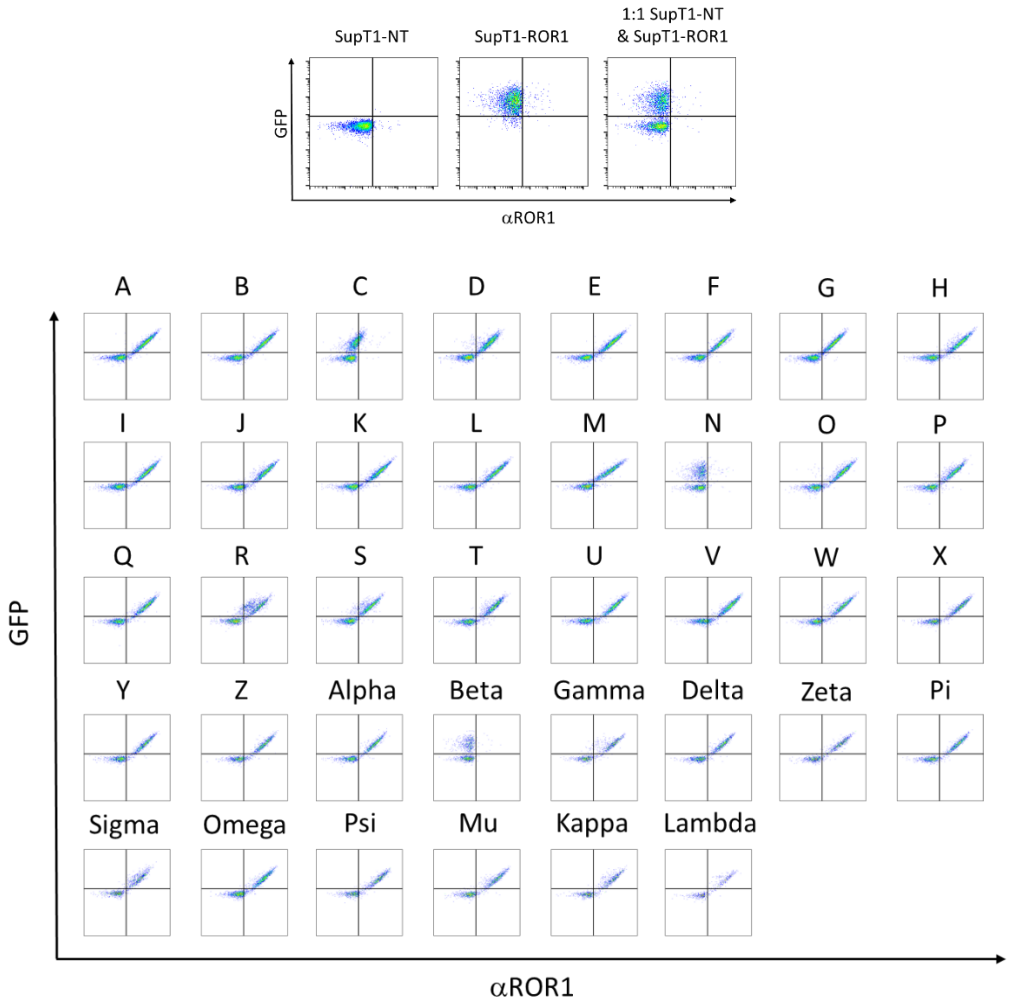


FIGURE 4.2 SCREENING OLIGOCLONAL CLONES.

Non-transduced SupT1 cells (SupT1-NT: GFP negative and ROR1 negative) and SupT1 cells transduced to express ROR1 and GFP (SupT1-ROR1) were mixed in a 1:1 ratio and the two populations differentiated based on GFP expression.

Supernatant from oligoclonal hybridomas was incubated with the mixture of SupT1-NT and SupT1-ROR1 cells, followed by incubation with an anti-Rat Fc directed secondary. A positive shift in the GFP positive population representing the SupT1-ROR1 cells, combined with a lack of a shift in the GFP negative population, demonstrated the presence of a specific anti-ROR1 antibodies.

4.1.3 Derivation of Rat anti-ROR1 antibody sequences

4.1.3.1 Monoclonal hybridoma screening

The 36 oligoclonal hybridomas containing specific anti-ROR1 antibodies were single cell sorted, to yield monoclonal populations. This was essential given that the parental clones may have included multiple clones with differing specificity and therefore AA sequence. The resulting monoclonal hybridoma supernatants were re-screened with ROR1 positive and negative cell lines to determine whether they contained an antibody of interest (Figure 4.3).

Of the multiple single cell clones produced, only 27 of the 36 oligoclonal populations yielded productive monoclonal populations with ROR1 antibodies. The root cause for failure in the remaining 9 clones is unclear as they were subjected to the same methods and processes as the clones which were successfully single cell cloned.

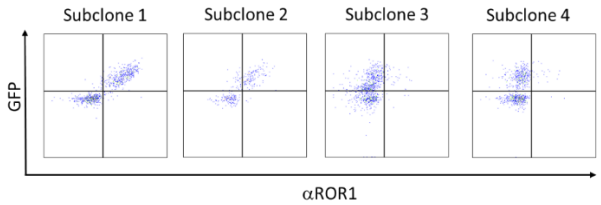


FIGURE 4.3 SCREENING SINGLE CELL CLONES FOR PRESENCE OF ANTI-ROR1 ANTIBODIES

Representative figure: Supernatant from single cell sorted monoclonal cell populations was assessed for the presence of anti-ROR1 antibodies. Two of 4 subclones (1&2) demonstrated presence of anti-ROR1 antibody producing cells and were taken forward to the 5'RACE. Non-binding clones were discarded from further analysis.

4.1.3.2 5' Rapid Amplification of cDNA Ends (5'RACE)

To elucidate the DNA sequence we employed 5'RACE, as depicted in Figure 4.4. This required identification of the light chain (kappa or lambda) and heavy

chain subclass of the parental antibody, which was undertaken using a rat isotyping ELISA kit. This enabled us to select appropriate primers to amplify the variable region of the antibodies using primers targeting sequences within the constant region of the light or heavy chain. The majority were kappa restricted and most frequently utilised the IgG2a and IgG2b heavy chain subclass.

PCR products generated by the 5'RACE were inserted into subcloning vectors and subjected to Sanger sequencing. This was undertaken independently for the light chain and heavy chain for each clone with multiple light chain and heavy chain sequences analysed per clone to identify a consensus sequence. These were compared and assessed within the immunogenetic information system V-Quest (IMGT/V-Quest) database of rat germline sequences to identify productive antibody sequences (Figure 4.5) (Brochet et al., 2008).

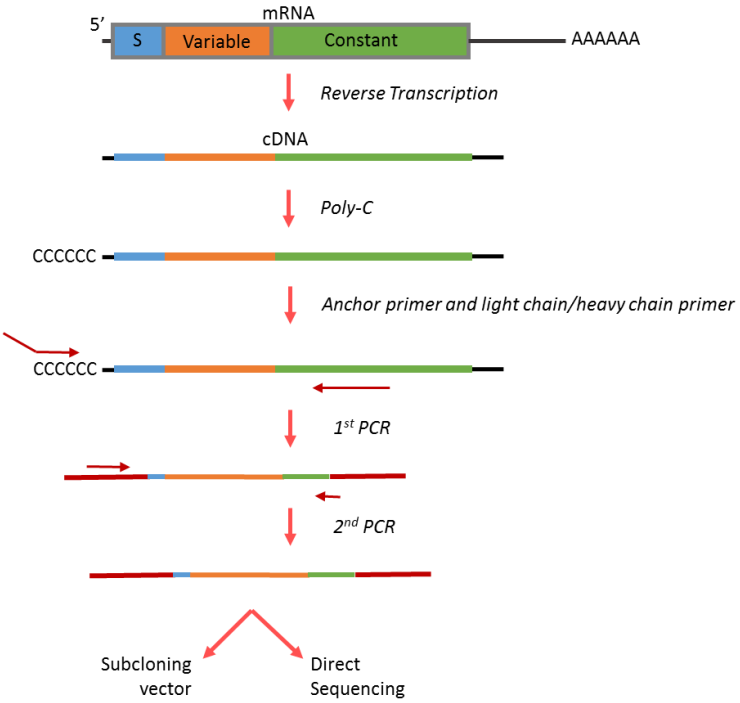


FIGURE 4.4 SCHEMATIC OF 5'RACE

Following mRNA isolation, a high fidelity reverse transcriptase is used to generate a cDNA product. This is subject to a poly-C reaction at the 3' end using terminal transferase, with the resulting poly C tail the backbone for an anchor primer ending in GGG. The other primer is specific for the light chain constant domain or IgG subclass constant domain as identified by isotyping. A nested PCR reaction is then undertaken to amplify the region of interest before being sent for sequencing either directly or via Topo subcloning vectors.

We initially transferred the amplified DNA into Topo subcloning vectors for expansion and sequencing but given the number of clones we were working with, this was a time and resource intensive. PCR products based on the initial primers could not be sent for direct sequencing as the resulting fragments were too short and would not include the entire length of the variable regions. To improve throughput we designed novel light and heavy chain specific primers, which bound further downstream within the constant domain to allow for non-subclass specific primer usage. These new primers were able to amplify cDNA as well as the original primers and when the corresponding DNA was sent for sequencing, it yielded the full variable domain of the antibody.

Sequence number 1: J_Kappa

1 "n" nucleotides have been deleted at the 5' end
from the input sequence

Sequence compared with the rat IG set from the IMGT reference directory

>J_Kappa
tttnttttttttttttttttcccccccccccngggggggggggggggggggggagcat
cctctcttccagcactcagagatggagacagacagactcctgctatgggcactgctgctc
tgggttccaggctccactggtgacattgtcttgacccagtctcctgctttggctgtgtct
gttggccagagggccacaatctcctgtagggccagccaaagtgtcagtatatctagatat
gattttatgcactggtaccaacagaaacccggacagcaacccaaactcctcatctatcgt
gcatccaacctagcatctgggatccctgccaggttcagtggcagtgggtctgggacagac
ttcaccctcaccatcaatcctgtgcaggctgatgatattgcaacctattactgtcagcag
aatagggagtctcctcggacgttcggnggaggcaccaagctggaattgaaacgggctgat
gctgcaccaactgta

Result summary:	Productive IGK rearranged sequence (no stop codon and in-frame junction)		
V-GENE and allele	Ratnor IGKV3S19*01 F	score = 1372	identity = 97,57% (281/288 nt)
J-GENE and allele	Ratnor IGKJ1*01 F	score = 175	identity = 97,22% (35/36 nt)
FR-IMGT lengths, CDR-IMGT lengths and AA JUNCTION	[25.17.36.10]	[10.3.9]	CQQNRESPRTF

FIGURE 4.5 REPRESENTATIVE IMGT V-QUEST OUTPUT

In this example the sequence for light chain clone J, generated through the 5'RACE was inputted into the V-Quest database, revealing a productive IgG kappa rearranged sequence with corresponding homology to a germline rat V gene (highlighted in orange). This was undertaken for all sequencing results obtained.

4.1.3.3 Antibody and scFv production and testing

To assess whether the sequences identified led to productive anti-ROR1 antibodies, the variable regions of the rat light and heavy chains were cloned in frame with a human kappa constant and human IgG1 constant antibody production plasmids respectively. Co-transfection of both plasmids into HEK293T cells resulted in antibody production that was secreted into the supernatant. Supernatant was tested against ROR1 positive and negative cells lines as previously described.

Clone Name of Productive Sequences	Binds as Antibody	Binds as scFv	Clones with Identical Antibody Sequences	IMGT productive sequence but no ROR1 Binding	Unsucessful 5'RACE
Lambda (1)	Yes	No	G (2)	X (3)	M (4)
Omega (1)	Yes	Yes		K (2)	T (1)
Z (1)	Yes	No		L (2)	Zeta (2)
J (8)	Yes	No		Psi (1)	Y (3)
F (1)	Yes	Yes		U (2)	
B (1)	Yes	Yes		Kappa (1)	
A (5)	Yes	Yes	E (1), P (2), Delta (3)		
I (3)	Yes	Yes			
O (2)	Yes	Yes			
Pi (1)	Yes	Yes			
Mu (2)	Yes	Yes			
V (2)	Yes	Yes			
R (3)	Yes	Yes			

TABLE 4.1 SUMMARY OF THE 5'RACE TO IDENTIFY NOVEL ANTI-ROR1 ANTIBODIES

The identity of the solved clones are shown in the first column, along with their ability to bind as antibody or scFv. Clones with identical amino acid composition are highlighted, as well as those which IMGT revealed to have productive sequences but failed to bind to ROR1. The final column identifies clones which were single cell sorted and were shown to have anti-ROR1 antibodies but in which the 5'RACE procedure failed (number of subclones solved per oligoclonal clone)

Over 150 single cell hybridoma clones were subjected to 5'RACE, of which 61 generated PCR bands that we were able to send for sequencing. Of these, 13/27 (48%) of oligoclonal clones were found to have unique anti-ROR1 antibodies based on composition of the complementary determining regions (CDR) of the variable domains, with 4 clones having identical sequences to others. A further 6 clones had productive sequences as identified by IMGT, but the resulting antibodies did not demonstrate binding to ROR1 and 4 clones failed multiple attempts at 5'RACE

despite being single cell sorted and having sufficient levels of cDNA (Table 4.1 and 4.2).

Analysis of the AA sequences showed some to vary by only a few amino acids, such as Clone Z and J. To ensure fidelity of the process, the 5'RACE was repeated to ensure there was no error in the sequencing reads or in the PCR reaction and this confirmed unique sequence identity. As alteration of one amino acid can have dramatic consequences on antibody binding characteristics, they were included for further downstream analysis.

We next generated scFv constructs using overlapping fusion PCR. The sequences were cloned in a heavy chain – linker – light chain format, utilising the GGGGSGGGGSGGGGS motif as linker between heavy and light chain. This was cloned in frame with a signal peptide and murine IgG constant domain allowing for a secreted scFv protein that allowed us to assess binding.

10 of the 13 antibodies bound in scFv format, as assessed by flow cytometry. Modification of the scFv format from heavy-linker-light to light-linker-heavy did not restore binding (data not shown) suggesting conversion from antibody to scFv format abrogated the ability to bind ROR1. Other strategies to try and overcome this limitation include substitution of the linker from $(GGGGS)_3$ to an alternative such the Whitlow linker, which is utilised in the 4A5 scFv format (Whitlow et al., 1993, Deniger et al., 2015).

Heavy Chain	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
Lambda	EVQLQESGPGLVKPAQSLSLTCSVT	GYSITNMYR	WNWIRKFPGNKLEWMGY	INTAGST	DYSPSLRGRVSITGDTSKNQFFLHLTSVTTEDTATYYC	AGFITNPFDF	WGQGVMVTVSS
Omega	EVQVVESGGGLVQPGRSLKLSCVPS	GFTFNNYW	MTWIRQAPGKAPEWVAS	ISNTGGST	FYPDSVRGRFSISRDNTKGTLYLHMTSLRSEDTATYYC	IRNMDA	WGQGTSVTVSS
Z	GKLVESGGGLLKPGGSLKLSCVAS	GFTFDKYW	MHWVRQAPGKGLEWIAE	IEYDGTET	NYAPSIKDRFTISRDNAKNTLYLQMSNVRSEDAATYFC	TTEEMYTTDYYYGFAY	WGQGTLVTVSS
J	DVKLVESGGGLLKPGGSLKLSCVAS	GFSFSKYW	MHWVRQAPGQGLEWIAE	IEYDGTET	NYAPSIKDRFTISRDNAKNTLYLQMSNVRFEDAATYFC	TTEEMHTTDYYYGFAY	WGQGTLVTVSS
F	EVQLVESGGGLVQPGRSLKLSCAAS	GFIFSEHN	MAWVRQAPKKGLEWVAT	ISDDGRNT	YYRDSMRGRFTISRENARSTLYLQLDSLRSEDTATYYC	ASHRYNLFDS	WGQGVMVTVSS
B	DVQLEESGGGLVRPGRSLKLSCADS	GVNFSNRG	MAWVRQAPTKGLEWVAT	ISYDGRII	YYRDSVKGRFSISRENAKSTLYLQMDSLRSEDTATYYC	ARHPIAADWYFDF	WGPGTMVTVSS
A	QVQLQQSGTELVKPASSVRISCKAS	GYTLTTNY	MHWIRQQPGNGLEWIGW	IYPGNGNT	KFNHKFDGRTTLTADKSSSIVYMQLSSLTSEDSAVYFC	ARSDFDY	WGQGVMXTVSS
I	EVQLVESGGGSVQPGRSLKLSCAAS	GFTFSDYN	MAWVRQAPKKGPEWVAT	ITYDVHNA	YYRDSVKGRFTISRDDAKSTLYLQMDSLRSEDTATYFC	ARPGAY	WGQGTLVTVSS
O	QVRLLQSGAALVKPGASVKMSCKAS	GYTFTDYW	MSWVKQSHGKSLEWIGE	IYPNSGAT	NFNEKFKDKATLTVDRSTSTAYMELSRLTSEDSAIYYC	ARGFPNNYLSWFAY	WGQGTLVTVSS
Pi	EVQLVESGGGLVQPGRSLTLSCSAS	GFTFRDYN	MAWVRQAPRKGLEWVAT	ISFDDYNT	YYRDSVKGRFTISRDDAKSTLYLQMDSLRSEDTATYYC	ARPGTY	WGQGTLVTVSS
Mu	QVQLQQSGAELVKPGSSVRISCKAS	GYTITSYD	MHWIKQQPGNGLEGIGW	IHPGNGKI	KYNQKFNGKATLTVDKSSSTAYMQLSSLTSEDSAVYFC	ARGTTRVFPWFAY	WGQGTLVTVSS
V	EVQLVESGGGLVQPGRSLKLSCAAS	GFSFSNYG	MHWIRQAPTKGLEWVAS	ISPTGGNT	YYRDSVKGRFTISRDNTKSTLYLQMDSLRSEDTATYYC	ATDDLYYSGPFAY	WGQGTLVTVSS
R	QIQLVQSGPELKKPGESVKISCKAS	GYTFTNYG	MYWVKQAPGQGLQYMGW	INTETGKP	TYADDFKGRFVFFLETSASTAYLQINNLKNEDMATYFC	AREVKHGLFHWFAY	WGQGTLVTVSS
R12	EQLVESGGRLVTPGGSLTLSCKAS	GFDFSAYY	MSWVRQAPGKGLEWIAT	IYPSSGKT	YYATWVNGRFTISSDNAQNTVDLQMNSLTAADRATYFC	ARDSYADDGALFNI	WGPGTLVTISS
4A5	EVKLVESGGGLVKPGGSLKLSCAAS	GFTFSSYA	MSWVRQIPEKRLEWVAS	ISRGGTT	YYPDSVKGRFTISRDNVRNILYLQMSSLRSEDTAMYYC	GRYDYDGYYAMDY	WGQGTSVTVSS
Light Chain	**FR1**	**CDR1**	**FR2**	**CDR2**	**FR3**	**CDR3**	**FR4**
Lambda	DVVMTQTPVSLPVSLGGQVSISCRSS	QSLEHSNGDTF	LHWYLQKPGQSPRLLIY	RVS	NRFSGVPDRFSGSGSGTDFTLKISRIEPEDLGDYYC	LQSTHFPNT	FGAGTKLELK
Omega	DIQLTQSPSTLSASLGERVTISCRAS	QSISNS	LNWYQQKPDGTVKRLIY	STS	TLESGVPSRFSGSGSGTDFSLSISSLESEDFAMYYC	LQFATYPQVT	FGSGTKLEIK
Z	DIVLTQSPALAVSVGQRATISCRAS	QSVSISRYNF	MHWYQQKPGQQPKLLIY	RAS	NLASGIPARFSGSGSGTDFTLTINPVQADDIATYYC	QQNRESPRT	FGGGTKLELK
J	DIVLTQSPALAVSVGQRATISCRAS	QSVSISRYDF	MHWYQQKPGQQPKLLIY	RAS	NLASGIPARFSGSGSGTDFTLTINPVQADDIATYYC	QQNRESPRT	FGGGTKLELK
F	DIQMTQSPSFLSASVGDRVTINCKAS	QNIDRY	LNWYQQKLGEAPKRLLY	NTN	KLQTGIPSRFSGSGSATDFTLTISSLQPEDFATYFC	LQYNSLPLT	FGSGTKLEIK
B	DIRMTQSPASLSASLGETVTIECLTS	EDIYSD	LAWFQQKPGKSPQLLIY	DAN	SLQNGVPSRFGGCGSGTQYSLQISSLQSEDVATYFC	QQYKNYPPT	FGGGTKLVLK
A	DIQMTQSPSSMSASLGDRVTFTCQAS	QDIGNN	LIWFQQKPGKSPRPLMY	FAT	SLANGVPSRFSGSRSGSDYSLTISSLESEDLADYHC	LQYREYPLT	FGSGTKLDLK
I	DIQLTQSPSSMSASLGDRVSLTCQSS	QGIGKY	LSWYQHKPGKPPKAMIY	YAT	KLADGVPSRFSGSRSGSDFSLTISSLESEDIAIYYC	LQFDDYPWT	FGGGTKLELK
O	DIVLTQSPALAVSLEQRVTIACKTS	QNVDNHGISY	MHWYQQKSGQEPKLLIY	EGS	NLAVGIPARFSGSGSGTDFTLTIDPVEADDIETYYC	QQSKDDPRT	FGGGTKLELK
Pi	DIQLTQSPSSLSASLGDRVSLTCQSS	QGIGKY	LSWFQHKPGKPPKPVIN	YAT	NLADGVPSRFSGRRSGSDFSLTISSLESEDTAIYYC	LQFDDFRWT	VGGGTKLELK
Mu	QFTLTQPKSVSGSLRSTITIPCERS	SGDIGDNY	VSWYQQHLGRPPINVIY	ADD	QRPSEVSDRFSGSIDSSSNSASLTITNLQMDDEADYFC	QSFDSNFDIPV	FGGGTKLTVL
V	DIKMTQSPSFLSASVGDRVTINCKAS	QNITRF	LNWYQQELGEAPTLLIY	NTN	NLQTGIPSRFSGSGSGTDFTLTISSLQPEDVATYFC	LQHGSRPRT	FGGGTKLELK
R	QFTLTQPKSVSGSLRSTITIPCERS	SGDIGDSY	VSWYQQHLGRPPINVIY	ADD	QRPSEVSDRFSGSIDSSSNSASLTITNLQMDDEADYFC	QSYDRNVDFNTV	FGGGTKVTVL
R12	ELVLTQSPSVSAALGSPAKITCTLS	SAHKTDT	IDWYQQLQGEAPRYLMQ	VQSDGSY	TKRPGVPDRFSGSSSGADRYLIIPSVQADDEADYYC	GADYIGGYV	FGGGTQLTVT
4A5	DIKMTQSPSSMYASLGERVTITCKAS	PDINSY	LSWFQQKPGKSPKTLIY	RAN	RLVDGVPSRFSGGGSGQDYSLTINSLEYEDMGIYYC	LQYDEFPYT	FGGGTKLEMK

TABLE 4.2 AMINO ACID SEQUENCE OF 13 NOVEL ANTI-ROR1 ANTIBODIES

The final consensus amino acid sequences of the heavy and light chains of the 13 novel anti-ROR1 antibodies are provided and we have also included the sequences for the R12 and 4A5 antibodies. Complementary determining regions (CDR) and framework regions (FR) were identified as per IMGT.

4.1.4 Domain binding characterisation

To characterise which of the three extracellular domains of ROR1 specific scFvs bound, we generated a panel of SupT1 based cell lines expressing truncated forms of ROR1 (Figure 4.6). Screening our scFvs against this panel demonstrated 8 bound solely to the membrane distal immunoglobulin domain, whilst clone F was unique in its binding to the frizzled domain. Clone V bound only when both immunoglobulin and frizzled domains were present, suggesting the conformation of the mature protein generated the target epitope. We also assessed the binding profile of the previously described R12 and 4A5 scFvs generated by other groups, demonstrating they both bound the immunoglobulin domain.

We further screened the original oligoclonal hybridoma supernatants against this panel of cell lines. Of the 36 clones with anti-ROR1 antibodies, only 2 bound the Frizzled domain (Clones F and U), one bound the Immunoglobulin-Frizzled domain (Clone V), whilst the remaining 33 were all directed against the Immunoglobulin domain.

To better understand this bias we undertook a multi-protein alignment of human, murine, rabbit and rat ROR1 using deposited sequences, to assess for differential amino acid usage in the extracellular domains. The region with the highest sequence variation occurred within the immunoglobulin domain, thus providing an explanation of the preferential targeting of this domain. Within the frizzled domain there were two amino acid substitutions between human and rat ROR1 and only one in the Kringle domain (Figure 4.7).

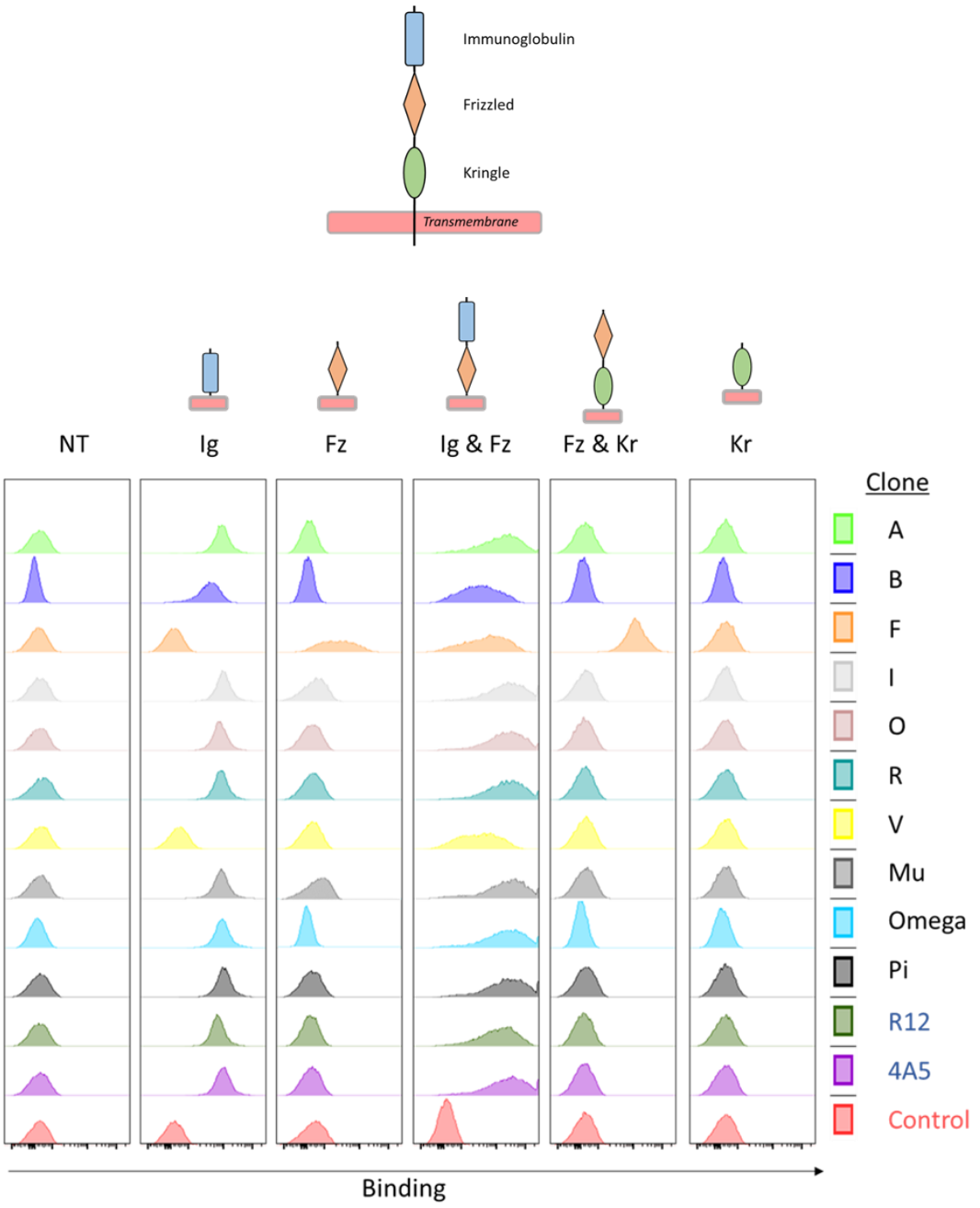


FIGURE 4.6 SCFV DOMAIN MAPPING

The extracellular domains of ROR1 are shown in the schematic (Ig = Immunoglobulin, Fz = Frizzled, Kr = Kringle). Supernatant containing scFvs fused the murine IgG2a constant domain was incubated with SupT1-NT (ROR1 negative) or a panel of SupT1 cell lines transduced to express various truncated extracellular domains. Binding was assessed with an anti-murine Fc conjugated secondary antibody. In addition to the 10 scFv clones we identified, we also included the R12 and 4A5 scFvs reported by other groups. With the exception of clone F, which binds the frizzled domain and clone V, which binds only the Ig-Fz protein, all other clones bound the immunoglobulin domain.

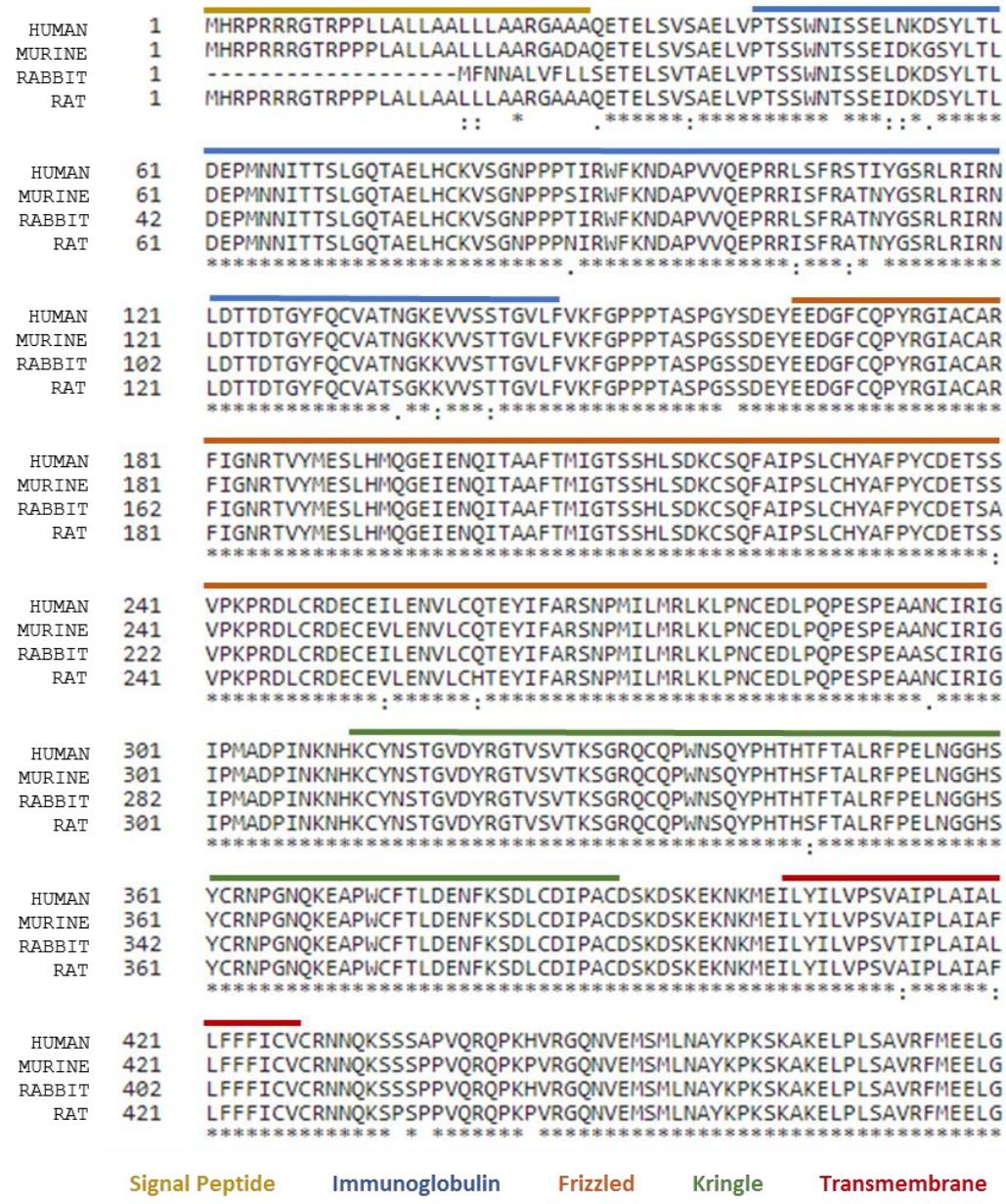


FIGURE 4.7 INTERSPECIES ROR1 ALIGNMENT

Human, murine, rabbit and rat ROR1 protein sequences were aligned using Uniprot web based software (http://www.uniprot.org/align/). The signal peptide, transmembrane and three extracellular domains are highlighted. Variation between species is highlighted. Uniprot accession numbers: Human (Q01973), Murine (Q9Z139) and Rabbit (G1U5L1). For rat ROR1 we used NCBI reference sequence NP_001102141.1 as the corresponding Uniprot sequence was only partially complete.

4.1.5 Section Discussion

Within this section of work we have demonstrated how from 38 oligoclonal hybridomas we arrived to 13 novel anti-ROR1 antibodies and 10 scFvs. These form the antigen recognition domain critical for the next phase of this project.

At the outset of the project we were informed that the oligoclonal hybridomas contained a mixture of clones, with the possibility of each having multiple antibodies of differing specificity and therefore AA sequence. However, our 5'RACE analysis found this to be an incorrect assumption, with all of the subclones demonstrating a single antibody sequence. Had we had access to this information at the beginning it would have significantly reduced the amount of clones that were screened.

Characterisation revealed the majority of antibodies targeted the membrane distal immunoglobulin domain, which is in keeping with the lower homology between human and rat ROR1 in this region. We identified 2 oligoclonal clones with anti-frizzled domain antibodies (Clone F and U) but were only able to solve 1 using 5'RACE, which was disappointing as an additional unique binder against the frizzled domain would have allowed for an interesting functional assessment, especially had there been substantial differences in affinity.

We found no binders against the kringle domain, likely due to the high homology of this region between all species. Daneshmanesh et al. have however developed a kringle specific anti-ROR1 antibody, although their strategy involved using domain specific vaccination compared to use of full length protein that we undertook (Daneshmanesh et al., 2012). The same group also found that naturally occurring antibodies against ROR1 preferentially targeted the Kringle domain in CLL patient samples, and although these were able to mediate some in vitro cytotoxicity, they were not associated with clinically relevant side effects (Hojjat-Farsangi et al., 2015).

Mice are by far the commonest laboratory animal used to generate therapeutic antibodies, although rats and rabbits can also be used. As others groups utilised murine and rabbit based immunisation programmes we utilised rats in an attempt to generate novel antibodies. Indeed despite strong similarities across species, there exists unique amino acid differences between human and rat ROR1 that are not seen in murine or rabbit ROR1, increasing the likelihood of novel antibody identification.

The method I utilised to identify antibodies, based on screening hybridomas and solving antibody sequences by 5'RACE is a well-established pathway in antibody discovery. We were able to solve 47% (17/36) of the clones of which 4 were identical antibody sequences. Although 19 clones remain unsolved, on balance it was felt progressing the 10 scFvs we had already identified to the next stage of work took priority over continuing to solve hybridoma clones.

An alternative strategy to solve these would be the use of next generation sequencing to generate consensus light and heavy chain variable sequences without the need for manual 5'RACE. This would, if optimised, allow for a high throughout and mass resolution of the antibody repertoire and has been utilised by other groups (Fischer, 2011).

The use of phage display technology to isolate and characterise novel antibodies is standard practice. Bacteriophages are viruses which infect bacteria and are composed of an extracellular protein core that can be engineered to express Fab or scFv molecules. Phages expressing the antibody fragment of interest could be selected for through serial panning rounds to lead to a final cohort of antibodies which are subsequently sequenced (McCafferty et al., 1990). Phage display libraries can be generated from naïve or immunised cell populations including with a human repertoire to abrogate the need for downstream humanisation of the sequence. Libraries can include huge numbers of potential binders, with Iontas, a commercial antibody discovery company having a humanised library with 40 billion distinct binders (Iontas, 2017).

For this process, the variable light and heavy chains are cloned into the extracellular protein coat of the bacteriophage, which subsequently expresses the

scFv on its surface. The next stage involves screening the phages for specific binding to the target antigen; unbound phages are washed off, whilst phages which bind are enriched. This process is repeated to yield a selection that is highly specific for the antigen in question and from which the sequence can be identified in a high throughput automated manner. An added advantage of this process is that the phage display antibodies in a scFv format, which is the format required for CAR and BITE production. Screening scFvs at the outset, rather than antibodies requiring conversion to scFv would avoid the loss of binding demonstrated with 3 of the isolated clones. This process also leads to access to antibodies with a much broader range of affinities, up to and including into the femtomolar range, unlike that obtained by traditional immunisation programmes. In keeping with this all of the antibodies we isolated had affinities in the nM range as assessed by Biacore analysis (K_D: 1.5-5.5nM).

ROR1 Antibody	K_D, M
Z	2.037E-9
Lambda	2.450E-9
Omega	2.614E-9
A	1.809E-9
B	1.505E-9
F	5.460E-9
J	3.351E-9
I	2.436E-9
Pi	1.979E-9
O	3.501E-9

TABLE 4.3 AFFINITY CONSTANTS FOR 10 OF THE IDENTIFIED ROR1 ANTIBODIES.

Antibodies for above clones were generated with a human Fc stalk and affinity assessed by Biacore SPR analysis (Data generated by Solange Paredes-Mosscosso).

Despite being unable to solve all of the hybridomas clones, we generated sufficient number of scFvs to allow us to transition onto the next phase of the project and begin construction of ROR1 CARs and BiTEs.

4.2 ROR1 Chimeric Antigen Receptor T Cells

4.2.1 Introduction

The basic structure of a chimeric antigen receptor was depicted in Figure 1.2, with the scFvs identified forming the membrane distal antigen recognition domain. We first needed to formalise the viral vector and intracellular signalling domains to utilise for our functional assessment. Given the success of the University of Pennsylvania group with lentiviral vectors using a 2nd generation CAR with 41BB as the co-stimulatory domain along with CD3ζ, we sought to use the same configuration as the starting point for our CAR studies (Porter et al., 2011, Kalos et al., 2011).

The overall aim of this section of work was to narrow the 10 scFvs identified above to a final lead scFv as a CAR T cell clinical therapeutic.

4.2.2 Initial Assessment

4.2.2.1 Cytotoxicity

The scFvs along with their native signal peptide were cloned in frame to a 3rd generation lentiviral vector backbone, downstream of a phosphoglycerate kinase (PGK) promoter. T cells were transduced using lentiviral vectors to generate ROR1 CAR T cells with a CD8α spacer domain and 41BB and CD3ζ signalling domains (Figure 4.8A).

Expression of CARs on the T cell surface was assessed by binding to recombinant ROR1, for which we utilised in house generated full length ROR1 protein fused to a murine IgG stalk (Figure 4.8B). Function of the ROR1 CAR T cells was typically assessed in a flow cytometry based cytotoxicity assay in which T cells

and target cells were co-cultured for 24 hours before assessment of target cell survival.

We utilised the ROR1 negative T-lymphoblastic leukaemia, SupT1 cell line (Smith et al., 1984) and SupT1 cells transduced to express ROR1 as negative and positive controls (SupT1-NT and SupT1-ROR1 respectively) (Figures 4.8C-D). We also included ROR1 CAR T cells utilising the previously published R12 scFv sequence as a comparator (Berger et al., 2015, Hudecek et al., 2013). Constructs demonstrated minimal off target cytotoxicity against SupT1-NT cells, whilst all constructs generated marked cytotoxicity of SupT1-ROR1 cells, without clear superiority for one scFv in particular (Figure 4.8E&F).

SupT1-ROR1 cells express ROR1 at high levels due to expression being mediated through retroviral transduction (Figure 4.8C). We therefore assessed cytotoxicity against cells which constitutively expresses ROR1 at more clinically relevant levels and chose the EBV transformed SKW6.4, B lymphoblastoid cell line (Saiki and Ralph, 1983)(Figure 4.8G). For this work we also included a CD19 CAR as comparator, utilising the fmc63 scFv as per the University of Pennsylvania group (Khalil et al., 2016).

Unlike the cytotoxicity with SupT1-ROR1 cells, in which the normalised survival range was 1.4-20.2% between scFvs, in the assay with SKW6.4 cells, there was much more variable cytotoxicity, despite the same effector to target ratio being used, with normalised survival from 8.4% to 108.2% (Figure 4.8H). Despite the minimal cytotoxicity seen with some CAR T cell constructs, we knew the CAR T cells were active as the same transgenic T cells were able to kill the SupT1-ROR1 population.

Inter-clone variation for some of the constructs, namely clones Omega, A and B, was likely due to using different donors between experiments with subsequent variation in donor T cell fitness, CD8:CD4 ratio and transduction efficiency. Despite this, there was a clear superiority demonstrated for CD19, with mean target cell survival of 2.17% (n=2 donors) compared to median survival across all ROR1 scFv clones of 85.1% (Range 8.4-108.2%). The one exception was ROR1 CAR T cells expressing the clone F scFv, with mean survival of 8.7% (n=6 donors).

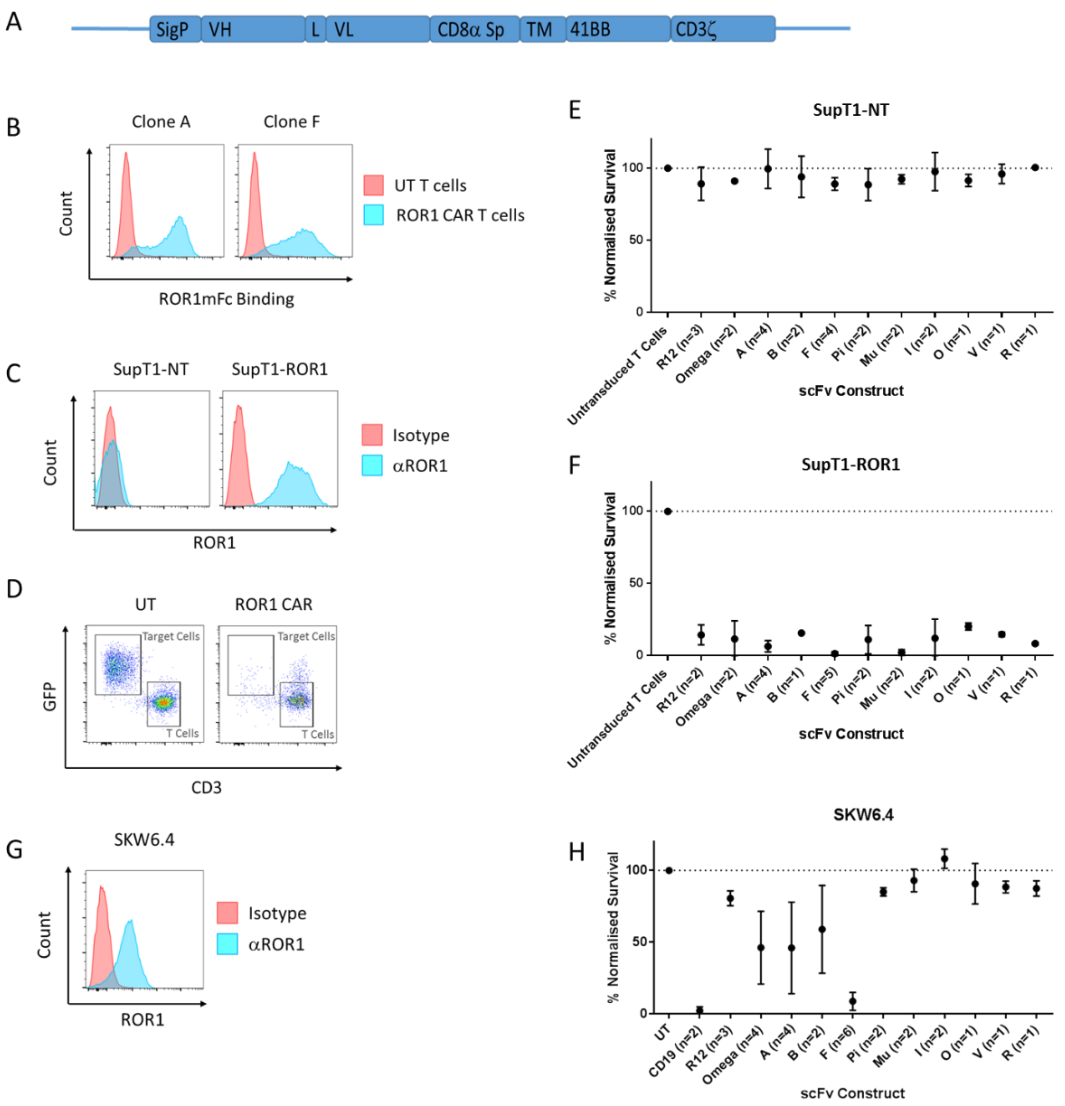


FIGURE 4.8 INITIAL ASSESSMENT OF ROR1 CAR T CELLS EXPRESSING SCFVS IDENTIFIED THROUGH HYBRIDOMA SCREENING

A. Schematic representation of CAR structure: The signal peptide (SigP) and scFv sequence comprising V_H-Linker (L)-V_L sequences were cloned in frame with a CD8α spacer, transmembrane domain (TM) and 41BB and CD3ζ intracellular signalling elements. B. Transduction was assessed by incubating ROR1 CAR T cells with ROR1 protein fused to a murine Fc stalk (ROR1mFc) followed by staining with anti-murine Fc secondary conjugated to AlexaFluor647. C. SupT1-NT and SupT1-ROR1 cells were stained with isotype control or anti-ROR1 antibody (Clone 2A2) to demonstrate ROR1 surface expression. D. Representative flow cytometry plots demonstrating cytotoxicity assay: Target cells (GFP+CD3-) and T cells (GFP-CD3+) were co-cultured for 24 hours and viable cells assessed compared to un-transduced (UT) T cells. E&F. Cumulative cytotoxicity of our 10 novel and the previously published R12 scFv based ROR1 CAR T cell constructs against SupT1-NT and SupT1-ROR1 cell lines at an effector to target ratio of 2:1 following 24 hours of co-culture (n=number of individual donors assessed). G. ROR1 expression on the SKW6.4 cell line. H. Cumulative cytotoxicity against SKW6.4 cell line with inclusion of a CD19 CAR T cell control arm comprising the fmc63 scFv.

4.2.2.2 CD107a Degranulation

As part of the initial assessment we also analysed CD107a degranulation of CAR T cells when cultured with SupT1-NT, SupT1-ROR1 and SKW6.4 cell lines. CD107a or lysosomal associated membrane protein 1 (LAMP1) is a component of the lysosomal membrane which form intracellular vesicles. Upon T and NK cell activation these cytotoxic vesicles, which contain granzyme and perforins, translocate to the cell surface, allowing CD107a expression to be detected.

All CAR T cell constructs led to CD107a degranulation in response to SupT1-ROR1 cell lines, with mean CD107a expression in ROR1 CAR T cells of 31.4% compared with 1.33% un-transduced T cells. However clone F showed higher CD107a degranulation in this context at 54.3% compared to the next ROR1 CAR construct at 29.5% (clone I).

This was further highlighted at lower antigen density with SKW6.4 cells, as only clone F CAR T cells resulted in marked CD107a degranulation at 4 hours; 47.2% of T cells were CD107a positive, compared with an average of 14.2% for the other clones and 4.08% for un-transduced T cells (Figure 4.9). The corresponding cytotoxicity assessment at 24 hours using the same T cells plated at the same time, correlated with CD107a expression for all cell lines.

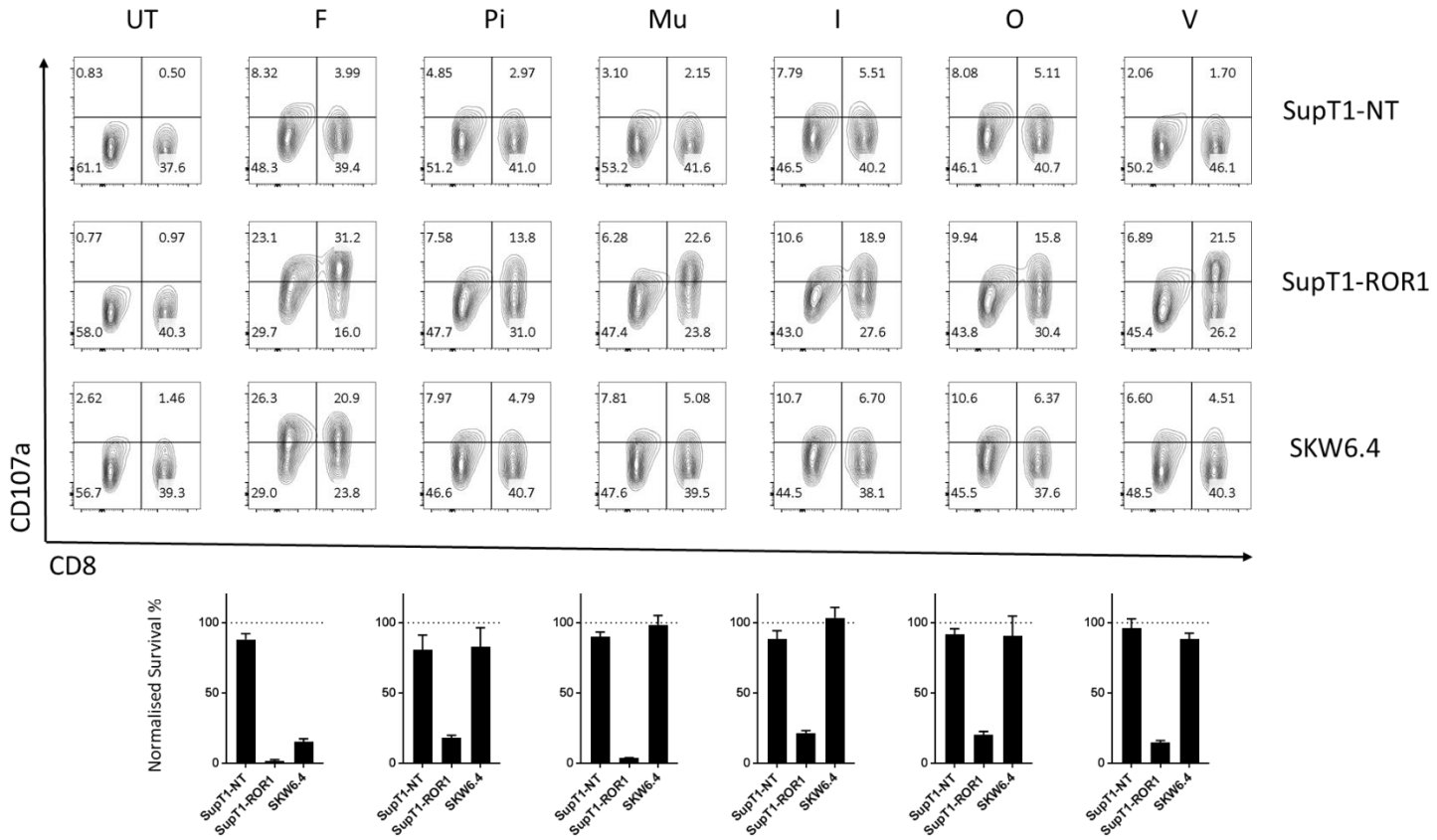


FIGURE 4.9 CD107A DEGRANULATION ASSAY

SupT1-NT, SupT1-ROR1 and SKW6.4 cells were co-cultured with Un-transduced (UT) or ROR1 CAR T cells expressing clone F, Pi, Mu, I, O or V scFvs (representative for other immunoglobulin constructs). Co-cultures were undertaken at a 2:1 effector to target ratio for 4 hours and resulting T cell degranulation assessed via detection of surface CD107a by flow cytometry. Corresponding cytotoxicity at 24 hours for the identical cultures represented as histograms under each scFv construct. Although significant CD107a degranulation was seen with all constructs against SupT1-ROR1, only clone F led to an increase against SKW6.4 cells.

4.2.3 Extracellular Spacer Optimisation

From this initial analysis, clone F emerged as the lead scFv, however as it was the only scFv that bound to the frizzled domain we investigated whether modification of the extracellular spacer, which modulates the distance between CAR T cell and target cell, would enhance effector function.

We selected a panel of extracellular spacers that generated a range of inter-cellular distances between target and effector T cells. The largest was the HCH_2CH_3 spacer comprising the hinge and constant region of the human IgG1 heavy chain (239 AA). The next was the CD8α stalk which was used in our initial assessment (81 AA). The third was solely the hinge region of human IgG1 (19 AA) and the final spacer was a short 15 AA sequence that comprised the $(GGGGS)_3$ linker motif (Figure 4.10A).

To allow for assessment of spacer function in relation to ROR1 binding domain we selected clone F as the frizzled binder and selected Clone A to be representative for immunoglobulin domain binding scFvs (Figure 4.10B). CAR T cells with these 4 spacer iterations were generated for each clone A and F and assessed in a cytotoxicity assay against SupT1-ROR1 and SKW6.4 cells with corresponding analysis of CD107a degranulation (Figure 4.10C). The spacer made a significant difference in terms of cytotoxicity and T cell degranulation. Overall, the hinge spacer provided optimal cytotoxicity for both clone A and F, against SupT1-ROR1 (<1% survival) and SKW6.4 (3.1% survival) cell lines.

We noted a reciprocal relationship between the scFv binding domain, spacer length and cytotoxicity. For clone A, the longer spacers, both HCH_2CH_3 and CD8α resulted in lower cytotoxicity compared to the shorter Hinge and Linker spacers (80% and 45.8% vs 3.6% and 6.7% respectively for SKW6.4). In comparison, cytotoxicity with clone F improved with shortening the length up to the Hinge spacer, but further reduction resulted in a dramatic loss of function (2.5% vs 69.9% for Hinge vs Linker spacer).

The Hinge and Linker spacer differ by only 4 amino acids suggesting that the four extra AA modulates a critical distance for clone F and its epitope. An alternative is that the linker spacer does not allow accurate scFv presentation but this can be discounted as it allowed clone A CAR T cells to function well.

In the context of TCR and peptide:MHC interaction the optimal distance excludes negative inhibitory phosphatases such as CD45 and CD148 and this may not occur with a suboptimal spacer length. Therefore a similar situation likely exists with CAR T cells such that too long a spacer increases the inter-cellular distance allowing these inhibitory signalling motifs to dominate the immunological synapse, whilst too short results in suboptimal scFv and antigen binding (Srivastava and Riddell, 2015).

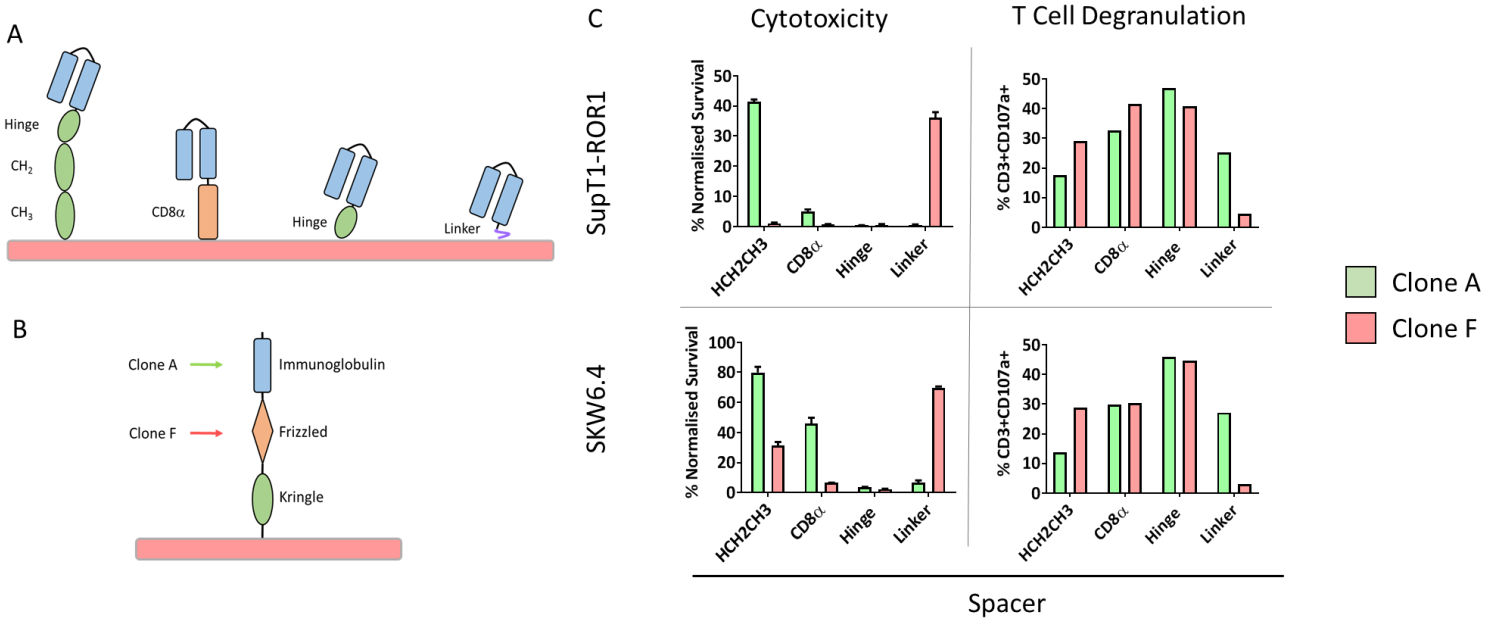


FIGURE 4.10 EXTRACELLULAR SPACER OPTIMISATION

A. Schematic of the different extracellular spacers constructed which include the HCH_2CH_3 domain of human IgG1 heavy chain, the CD8α extracellular stalk, the hinge region only of the IgG1 stalk and a short flexible linker. B. Schematic of binding domains for clone A (Immunoglobulin) and clone F (Frizzled). C. CAR T cells expressing clone A or F scFvs with all of the spacer domain configurations were generated and co-cultured with SupT1-ROR1 and SKW6.4 target cells. Cytotoxicity and CD107a T cell degranulation was assessed at 24 and 4 hours respectively.

To confirm which CAR structure to take forward, we repeated this experiment with the leading spacers for each scFv. For clone A we selected the hinge and linker spacer, whilst for clone F, we used the CD8α and hinge spacer. We focused on the SKW6.4 cell line as this represented a more challenging target and used lower effector to target ratios to highlight functional differences between the spacers. This confirmed that the hinge spacer afforded significant benefit for clone A over the linker spacer and for clone F over the CD8α spacer (Figure 4.11).

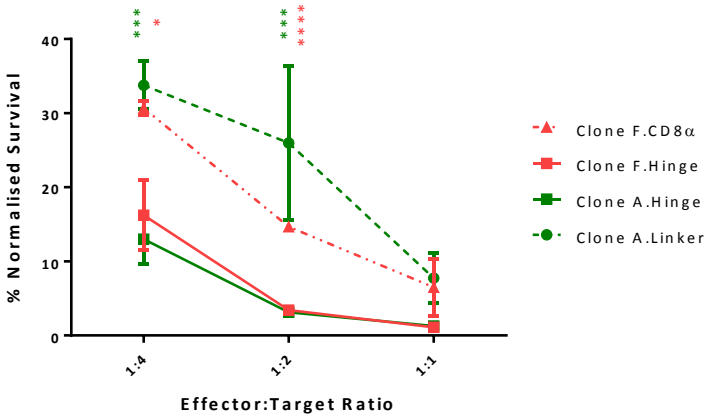


FIGURE 4.11 HINGE SPACERS PROVIDE SUPERIOR CYTOTOXICITY AGAINST SKW6.4 CELLS

CAR T cells expressing the clone A scFv with a hinge spacer or linker spacer or clone F scFv with the CD8α or hinge spacer were co-cultured with SKW6.4 cells at varying effector to target ratios for 24 hours in a standard cytotoxicity assay. There was significantly higher cytotoxicity with the hinge constructs irrespective of scFv. Clone A hinge vs linker (p=0.004 and 0.002 at 1:4 and 1:2 ratio respectively) and clone F hinge vs CD8α spacer (p=0.01 and p=0.00004 at 1:4 and 1:2 ratio respectively). No significant difference was seen between clones A & F hinge constructs at the 1:1 ratio. Statistical analysis: unpaired t-test.

4.2.4 Assessment with Hinge Spacer

Having demonstrated the hinge spacer provided optimal cytotoxicity for both Immunoglobulin and Frizzled binding scFvs, we substituted the CD8α spacer for the hinge spacer for all 10 scFvs in the CAR format. Repeat assessment with the hinge spacer demonstrated improved cytotoxicity compared with CD8α spacer (Figure 4.12).

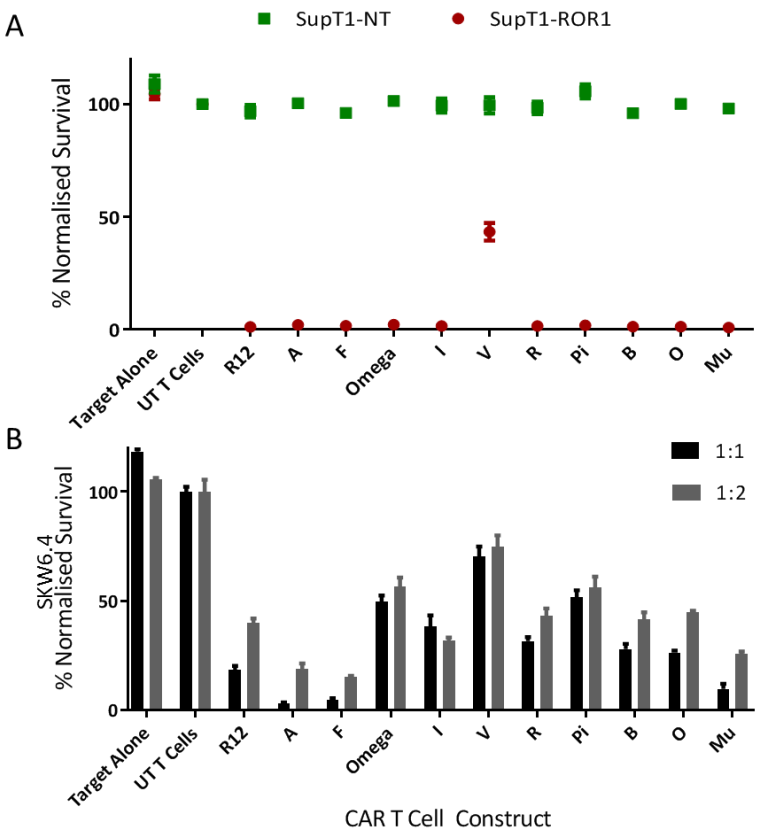


FIGURE 4.12 CYTOTOXICITY WITH HINGE SPACER DOMAIN.

ROR1 CAR T cells were generated for all 10 scFvs we had identified as well as the previously published R12 scFv but in which the CD8α spacer has been replaced by the hinge spacer. CAR T cells were co-cultured with A. SupT1-NT and SupT1-ROR1 cells in a 2:1 effector to target ratio. At 24 hours no off target cytotoxicity was seen against SupT1-NT cell line compared to almost complete eradication of SupT1-ROR1 cells, with the exception of clone V. B. Cytotoxicity against SKW6.4 cells at a 1:1 and 1:2 effector to target ratio showed more variable cytotoxicity with clones A, F and Mu demonstrating the highest levels of cytotoxicity.

As before the SupT1-ROR1 cell line was highly sensitive to CAR T cell mediated killing with almost complete eradication of target cells. The one exception was with clone V which showed incomplete killing of SupT1-ROR1 cells, suggesting the unique epitope it binds to limits cytotoxicity in this format. It is possible that alternate spacers may improve function of clone V CAR T cells, although this was not assessed (Figure 4.12A).

When we assessed CAR T cells with a hinge spacer against the SKW6.4 cells, we saw improved killing compared to the corresponding constructs with a CD8α spacer. Clones A, F and Mu provided the highest levels of cytotoxicity (at 1:1

effector to target ratio normalised survival of 3.14%, 4.8% and 9.85% respectively) (Figure 4.12B).

In view of this, repeat assessment was undertaken using T cells comprising these scFvs against SKW6.4 cells (Figure 4.13A) and also primary CLL cells (Figure 4.13B). Cytotoxicity was similar and not statistically different between constructs, but cytokine secretion in CLL cells, was significantly enhanced with clone A and clone F co-cultures compared to those with clone Mu. These constructs were therefore selected to take forward for more detailed analysis.

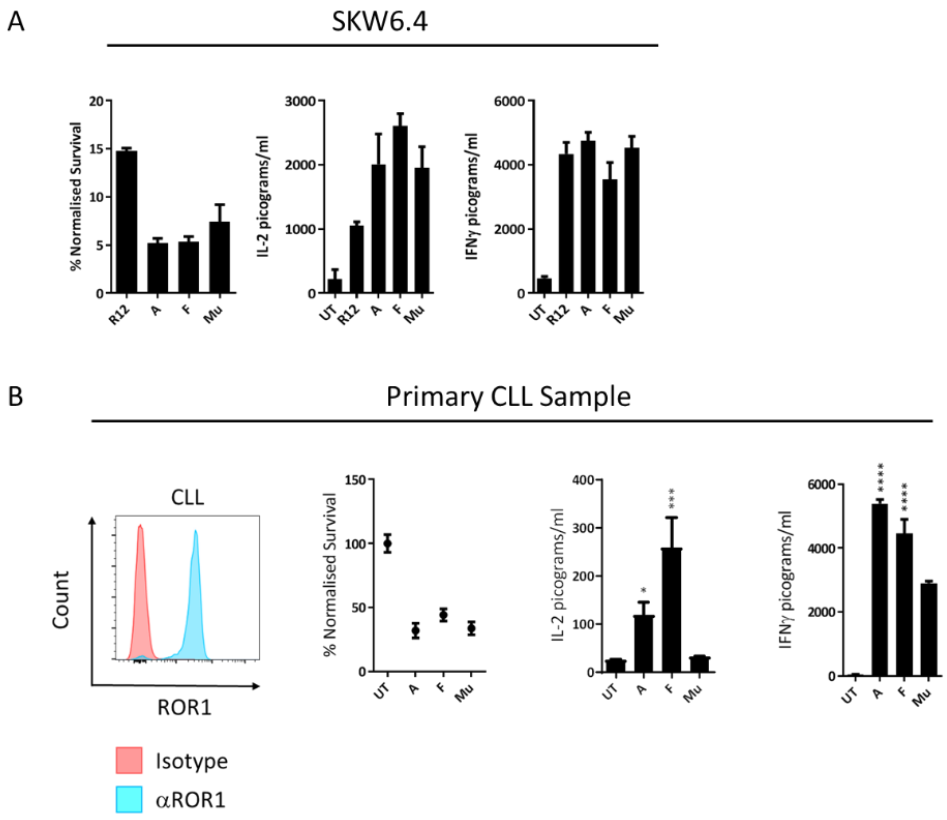


FIGURE 4.13 LEAD CONSTRUCT ASSESSMENT

A. CAR T cells expressing the clone A, clone F and clone Mu scFvs were co-cultured with SKW6.4 cell line in a 1:1 effector to target ratio and cytotoxicity, IL-2 and IFNγ cytokine secretion assessed at 24 hours. B. Healthy donor T cells were transduced to generate clone A, F and Mu CAR T cells and cultured against primary CLL cells. Flow cytometry plots demonstrate ROR1 expression on CLL cells. Cytotoxicity at 24 hours at 2:1 effector to target ratio demonstrates significant cytotoxicity with all constructs compared to un-transduced T cells. IL-2 and IFNγ cytokine secretion at 24 hours was also assessed. Statistical analysis: One way ANOVA with Mu CAR T cells as control arm comparator. Figure representative of 2 independent experiments.

4.2.5 CAR construct optimisation

When using shorter spacer sequences, such as the hinge or linker, the apparent transduction efficiency (TE) as determined by binding to ROR1mFc was consistently lower than with longer spacers (Figure 4.14A). For clone A, the CD8α spacer routinely gave apparent transduction efficiencies in the region of 70-80%, but this dropped to 10-20% with the linker spacer. We hypothesised this was an artefactual due to steric hindrance and inefficient binding of ROR1mFc to the scFv when located more membrane proximally in a CAR format, as opposed to actually representing true TE.

To better define this we transferred the CAR sequences into a bicistronic construct which included the blue fluorescent protein (BFP) gene separated by the foot and mouth disease virus 2A peptide (FMD2A). The former acted as an independent and inherent transduction marker, whilst the latter is a self-cleaving small peptide allowing equimolar expression of both transgenes from a single construct (Figure 4.14B). Transduction efficiencies were comparable with these constructs when assessing BFP expression but shorter spacers showed significantly lower ROR1mFc binding (Figure 4.14C) confirming our earlier hypothesis.

Accurate assessment of TE provides critical information with regards to the fidelity of vector production, T cell transduction, functional readouts and subsequent data interpretation. To overcome this limitation, we assessed whether an anti-rat Fab' specific antibody directed against the framework regions of the clone A and F scFv would provide an accurate measure of CAR expression. However, we were only able to detect expression of the clone F scFv but not clone A on the surface of transduced T cells, thereby limiting the utility of this approach (Figure 4.14D).

In parallel, we also assessed whether CAR expression could be enhanced by substitution of the parental signal peptide from the rat hybridoma library to the human albumin or IL-2 signal peptides. We found that for a given level of transduction as measured by BFP expression the signal peptide had no bearing on

CAR expression. This provided valuable information with respect to the design of our subsequent lentiviral vectors.

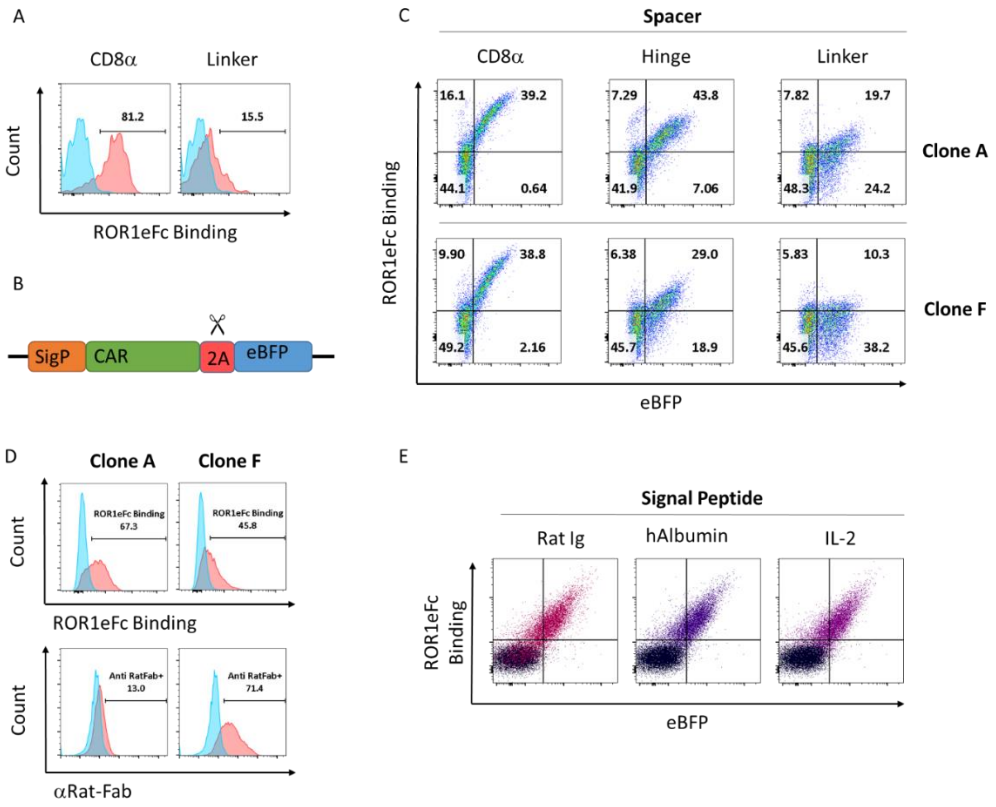


FIGURE 4.14 CAR DETECTION AND SIGNAL PEPTIDE OPTIMISATION

A. Flow cytometry plots demonstrating lower levels of ROR1eFc expression with linker spacer compared to CD8α spacer despite using identically synthesises viral vectors, representative of multiple repeats. B. Schematic of a bicistronic plasmid in which the CAR is separated from the BFP open reading frame by a FMD2A skipping element. C. CAR T cells were generated for clones A & F with the CD8α, hinge and linker spacer, all of which included the BFP marker gene. Apparent transduction, as measured by ROR1mFc binding was lower with shorter spacer domains despite equivalent expression of BFP. D. Assessment with anti-Rat Fab directed antibody to detect CAR expression compared with ROR1mFc binding demonstrated ability to detect clone F but not clone A scFv E. Assessment of the parental rat derived antibody signal peptide with the albumin or IL-2 signal peptides showing identical levels of expression irrespective of signal peptide.

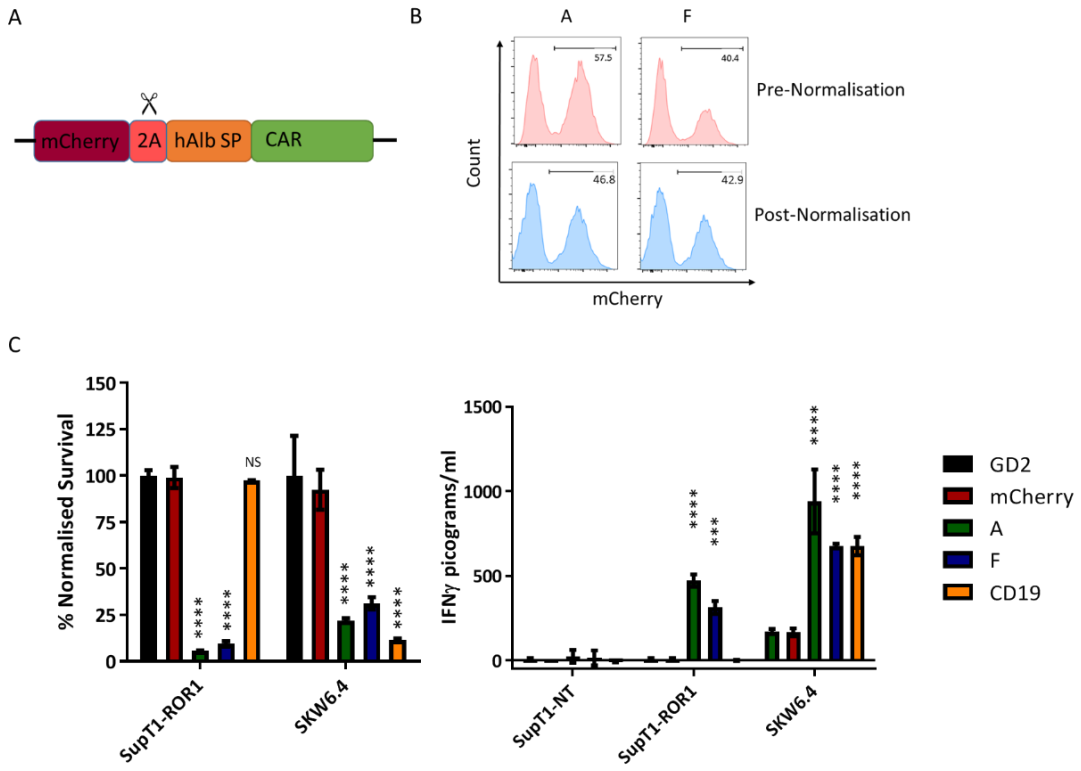


FIGURE 4.15 NORMALISED CO-CULTURE

A. Schematic of optimised CAR transgene used for functional assessment. The CAR sequence comprising scFv, spacer domain and intracellular signalling domain lies in frame with the human albumin signal peptide which is within a bicistronic construct separated by the FMD2A peptide allowing expression of CAR and mCherry in a 1:1 ratio. B. Example of normalisation of CAR populations: CAR T cells for Clone A and F were generated and transduction efficiency assessed by mCherry expression. Post transduction there was a difference in transduced T cells within the bulk population (57.5 vs 40.4%). Un-transduced T cells were added back resulting in more comparable populations (46.8 vs 42.9%) C. Normalised co-culture against SKW6.4 cells demonstrates significant cytotoxicity compared with mCherry alone cells for SupT1-ROR1 and SKW6.4 cell lines with corresponding IFNγ secretion. Statistical Analysis: One way ANOVA with GD2 CAR T cells as the control group.

We therefore generated a bicistronic construct in which the CAR and fluorescent protein mCherry (Shaner et al., 2004), were separated by a FMD2A peptide. All constructs utilised the human albumin signal peptide due to ease of cloning and restriction site usage. This also removed the rat derived signal peptide, limiting immunogenicity. Inclusion of mCherry allowed for a simple flow cytometry based method to delineate transduction efficiency without affecting T cell function. It also allowed for normalisation of different CAR populations to enable a more

meaningful comparisons between different constructs, by decreasing bias introduced by variation in transduction efficiencies. We also incorporated the previously published anti-disialoganglioside (GD2) scFv (Pule et al., 2008) into this backbone to act as a more suitable and representative negative control as opposed to un-transduced T cells. GD2 is expressed on a range of tumours with neuroectodermal origin and GD2 CAR T cells are being developed for neuroblastoma (Pule et al., 2008)

4.2.6 Jeko1 Model

We next assessed the potential of clone A and F CAR T cells against the Jeko1 cell line, which was originally derived from a patient with mantle cell lymphoma (Jeon et al., 1998). This cell line expresses ROR1 at higher levels compared to SKW6.4 cells, although ROR1 expression is still lower than CD19 (Figure 4.16A). We also included fmc63 CD19 CAR T cells within this comparison to provide an insight into functional differences of targeting these common haematological antigens. Our control was CAR T cells expressing a GD2 specific scFv. In a standard co-culture assay cytotoxicity was seen with CD19 fmc63 as well as ROR1 clone A and clone F CAR T cells with similar IFNγ secretion. However IL-2 secretion was significantly enhanced with CD19 CAR T cell co-cultures above that for the comparator ROR1 CAR cultures (Mean IL-2 10807pg/ml for CD19 vs 1532 and 2812 for clones A and F respectively, $p<0.001$) .

We next undertook a murine model based on the published work of the Riddell group (Hudecek et al., 2013). Mice were injected with firefly luciferase transduced Jeko1 cells and allowed to establish before treatment with CAR T-cells. Control T cells expressed an anti-GD2 scFv based CAR as a negative control. There was a clear reduction in the tumour burden in mice treated with Clone A and F CAR T cells, although disease was still present at Day 21. In comparison the CD19 CAR treated animals were virtually tumour free as assessed by bioluminescence imaging at the same time point. We noted a spontaneous decrease in bioluminescence

signal from day 17 onwards in the control animals and this is in keeping with previous reports with the Jeko1 model (Hudecek et al., 2013).

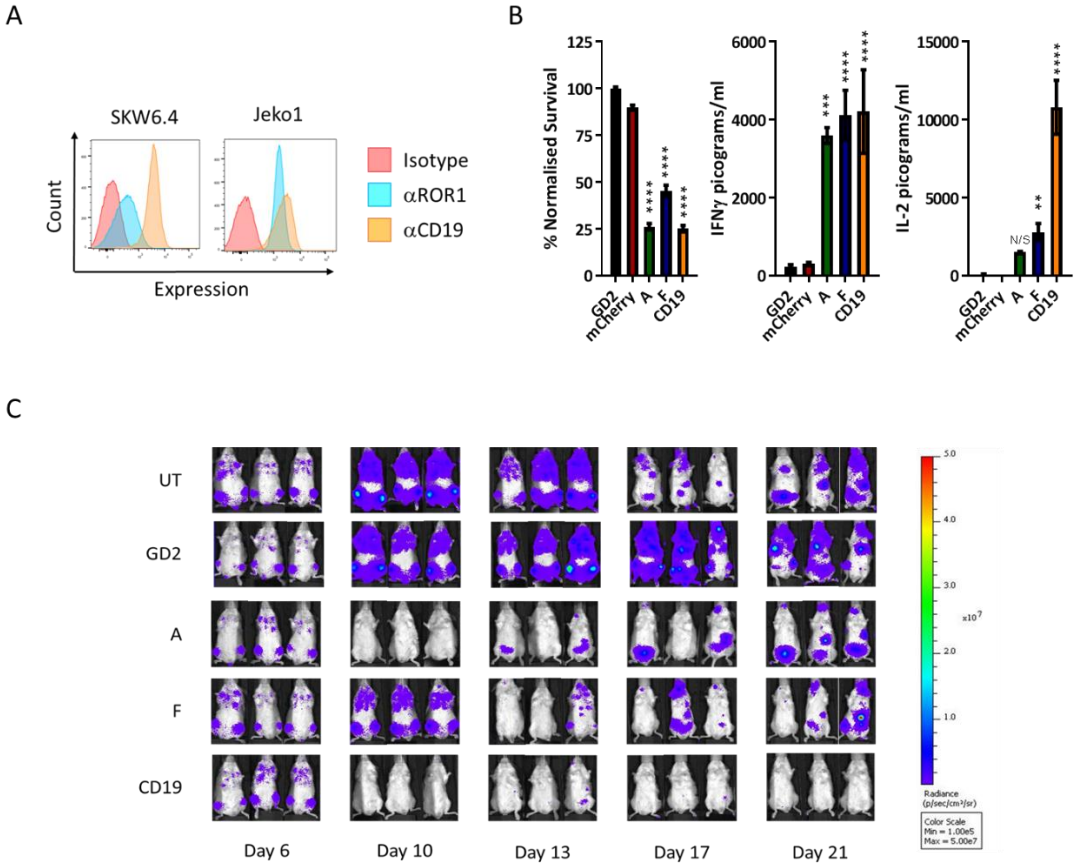


FIGURE 4.16 IN VITRO AND IN VIVO JEKO1 MODEL WITH ROR1 AND CD19 CAR T CELLS

A. Comparison of CD19 and ROR1 expression in SKW6.4 and Jeko1 cell lines. B. Co-culture of Jeko1 cells with CAR T cells expressing the CD19fmc63, Clone A and Clone F scFv at 2:1 effector to target ratio, resulted in significant cytotoxicity and cytokine secretion compared to T cells transduced to express just mCherry. Statistical analysis: One way ANOVA with GD2 as the comparator arm .C. Mouse model. Nod SCID gamma-/- mice were injected with $0.5x10^6$ Jeko1 cells via tail vein transduced to express firefly luciferase and allowed to engraft. On Day 6 mice were treated with $4x10^6$ GD2, Clone A, Clone F or CD19 CAR T cells. The GD2 CAR was used as a negative control and CD19 CAR as a positive control. Mice treated with clone A and clone F CAR T cells demonstrated lower tumour burden compared to control mice at day 21, however mice treated with CD19 CAR T cells demonstrated almost complete loss of bioluminescence signal.

4.2.7 ROR1 CAR T cells demonstrate cytotoxicity against a range of solid tumour cell lines

As discussed earlier, a wide variety of cancer subtypes express ROR1 and co-culture of clone A or F CAR T cells with the pancreatic adenocarcinoma PANC-1 cell line demonstrated T cell activation as evidenced by clustering and expansion. This was not seen with CD19 CAR T or un-transduced T cells under the same conditions (Figure 4.17).

We therefore assessed whether they could target a panel of solid tumour cell lines and included pancreatic adenocarcinoma (PANC1), breast cancer (MDA-MB-231), ovarian cancer (SKOV-3, HOC7 & HEY), hepatic origin cancers (SK-Hep-1 & HUH7), glioblastoma (U251 & A172), melanoma (T168A) and prostate cancer (DU145 & PC3) in the analysis. Cytotoxicity was seen with clone A and F CAR T cells but not control CD19 CAR T cells. It did not however correlate with ROR1 expression, implying intrinsic cell line mediators modulate susceptibility. In keeping with this cytokine secretion was variable between cell lines, despite being cultured with identical CAR T cells from the same donor. Only co-cultures with PANC1, DU145, PC3 and MDA-MB-2331 cell lines showed marked IFNγ and IL-2 secretion (Figure 4.18). Although clone A based CAR T cells tended to lead to higher rates of cytotoxicity compared with Clone F, this was not statistically significant and so we took both clone A and F forward for humanisation.

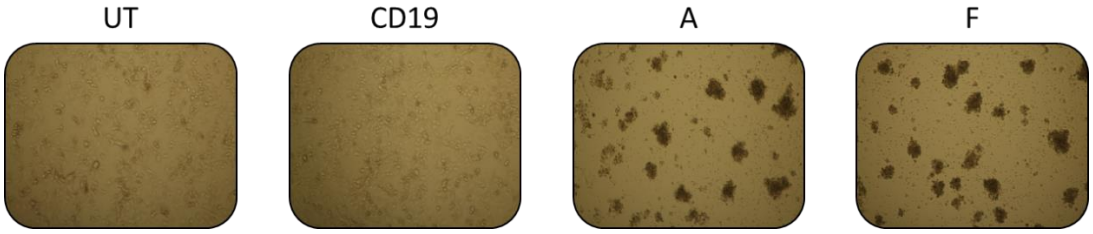


FIGURE 4.17 CLONE A AND F CAR T CELLS SHOW ACTIVATION IN CO-CULTURES WITH ROR1 POSITIVE TARGETS

Representative photographs of co-cultures between un-transduced (UT) and CD19, clone A and clone F CAR T-cells with PANC-1 cell lines. Clone A and F T cells demonstrate expansion and clustering with loss of background PANC-1 cells unlike CD19 and UT controls.

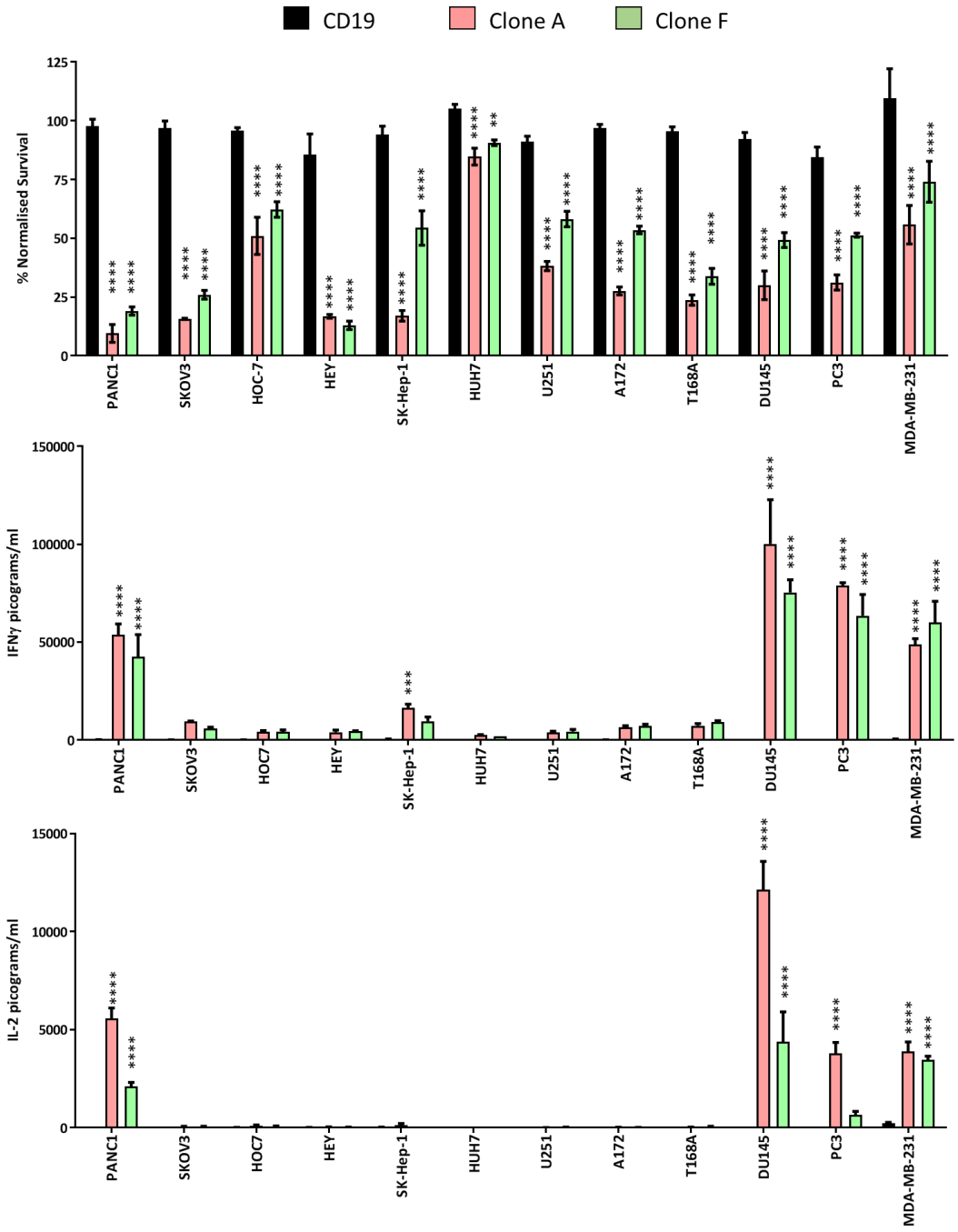


FIGURE 4.18 ROR1 CAR T CELLS MEDIATE CYTOTOXICITY AGAINST A PANEL OF SOLID TUMOUR CELL LINES.

Solid tumour cell lines expressing ROR1 representing pancreatic, ovarian, hepatic, glioblastoma, melanoma, prostate, and breast cancer were co-cultured with ROR1 CAR T cells at 8:1 effector to target ratio and survival assessed via a MTS assay at 24 hours in triplicate. ROR1 CAR T cells mediated significant cytotoxicity against all cell lines irrespective of ROR1 expression. Cytokine secretion did not correlate with killing with only PANC1, DU145, PC3 and MDA-MD-231 cells demonstrating significant increase.

4.2.8 Epitope Discovery

To better characterise clone A and F, we wanted to delineate the exact epitope of ROR1 which they bound, whilst ensuring no cross reactivity to ROR2.

4.2.8.1 Clone A and F do not cross react with human ROR2

Human ROR1 shares 58% homology with ROR2 (Figure 4.19A) and to ensure there was no unintended non-specific binding we transduced the SupT1 cell line to express human ROR2 (SupT1-ROR2). Using clone A and F scFv-mIgG fusion proteins we confirmed no cross reactive binding by flow cytometry (Figure 4.19B).

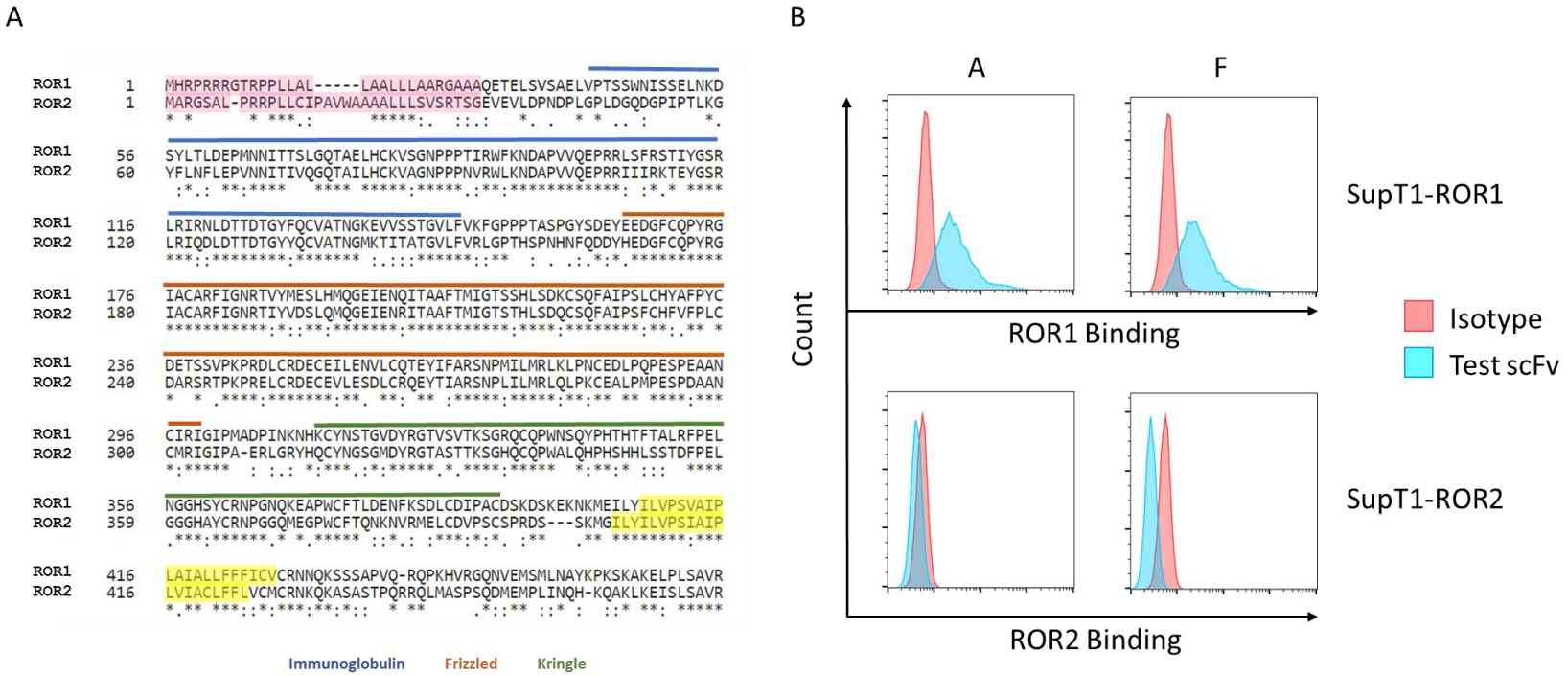


FIGURE 4.19 ASSESSMENT OF ROR2 BINDING

A. Alignment of human ROR1 and ROR2 with the signal peptide highlighted by shaded red and transmembrane domain by shaded yellow: Uniprot accessions Q01973 and Q01974 respectively. B. We generated a SupT1 cell line expressing ROR2 (SupT1-ROR2) in addition to our established SupT1-ROR1 cell line. Clone A and F scFvs were fused to murine IgG stalk and produced, followed by incubation with either SupT1-ROR1 or SupT1-ROR2 cells. Binding was detected with an anti-murine IgG antibody but neither clone A or F bound to SupT1-ROR2 cells.

4.2.8.2 Epitope mapping

To better characterise the exact epitopes targeted we panned the extracellular domain of ROR1 with clone A and F antibodies. This was undertaken with a panel of ninety 11 amino acid peptide fragments representing the immunoglobulin and frizzled domains of ROR1. Protein fragments overlapped by 8 amino acids in order to take into account conformational change and maximise potential epitope expression, to allow detection of binding with clone A and F antibodies.

For clone A the ELISA readings were highest for protein fragments at the beginning of the immunoglobulin domain, just after the signal peptide (Figure 4.20A). In keeping with this a SupT1 cell line transduced to express a truncated immunoglobulin domain demonstrated loss of binding with clone A, although both competitor R12 and 4A5 antibodies were still able to bind (data not shown). This provided evidence that clone A bound an epitope distinct from other reported anti-immunoglobulin domain ROR1 antibodies.

Within the region highlighted there was a single AA at position 48 that differed between human and rat ROR1, which was isoleucine and threonine respectively. To confirm that clone A bound to this epitope, we next generated a panel of SupT1-ROR1 cell lines with single amino acids substitutions at positions 47, 48 and 50. Substitution of isoleucine at position 48 of ROR1 resulted in complete loss of binding with clone A. The adjacent asparagine at position 47 was also required for binding but was not as critical. This was in comparison with the serine at positon 50, modification of which did not disrupt binding.

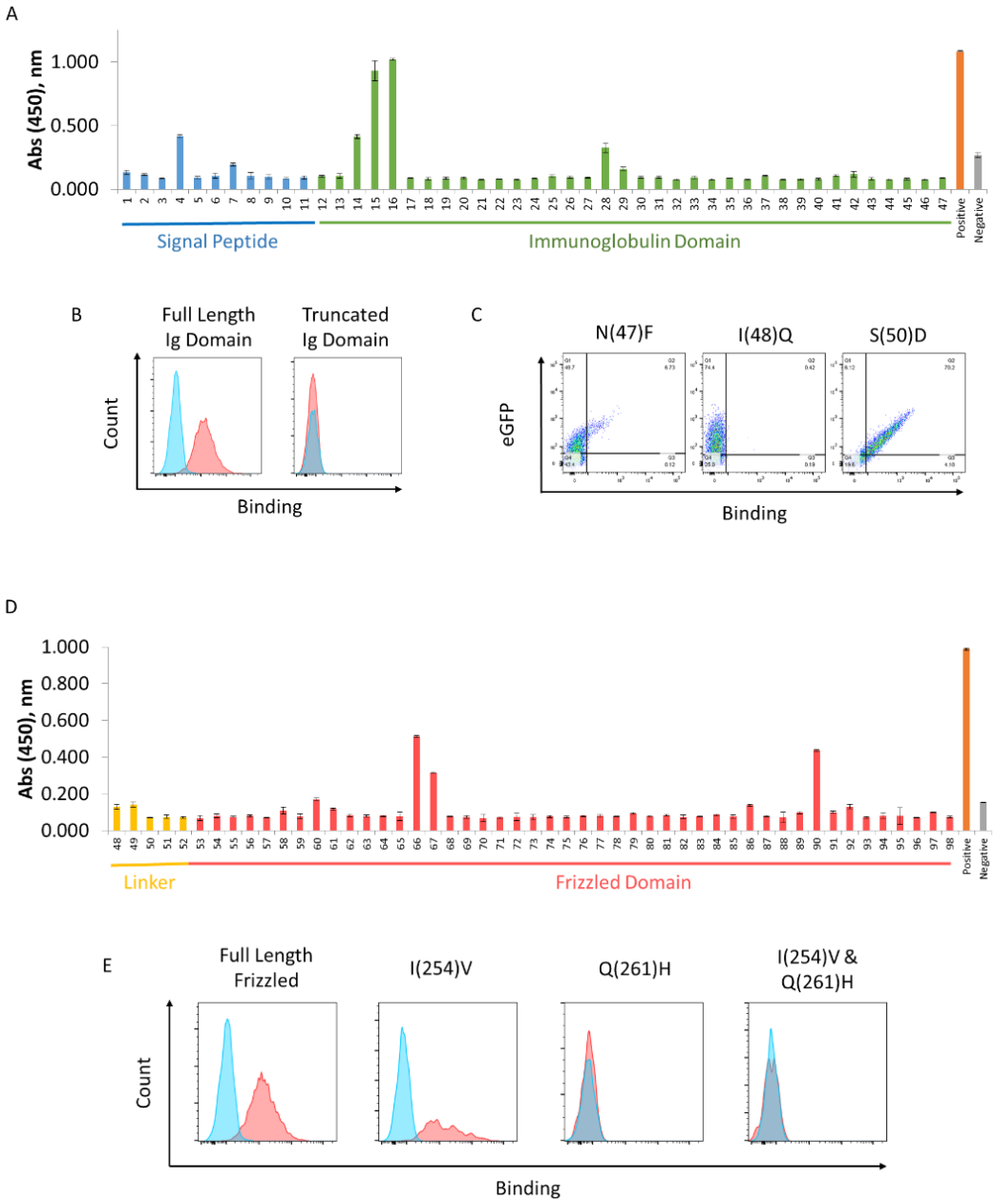


FIGURE 4.20 EPITOPE MAPPING

A. 11 amino acid peptide fragments corresponding to the signal peptide and immunoglobulin domain of ROR1 were panned with a clone A antibody with a human IgG1 stalk. Binding was detected using anti-human HRP conjugated secondary. Peptide fragments 14, 15 and 16 demonstrating a positive signal. B. SupT1 cells transduced to express a truncated ROR1 protein show no binding with a Clone A antibodies compared to full length ROR1. C. Single amino acid substitutions within human ROR1 were undertaken at positions 47, 48 and 50. These modified proteins were transduced into SupT1 cells and demonstrated the binding epitope focused on isoleucine at positon 48. D. 11 amino acid peptide fragments corresponding to the linker between the Immunoglobulin domain and frizzled domain as well as the entire frizzled domain were panned with a Clone F antibody, as above. A positive signal was detected with peptide fragments 66 and 67 and 90. Differences in amino acid sequences between human and rat was only seen within peptide fragment 90. E. Single amino acid substitutions at position 254, 261 or both of ROR1 were undertaken and SupT1 cells transduced to express these modified ROR1 proteins. Modification of glutamine at position 261 showed complete loss of binding to Clone F, highlighting its target epitope.

We undertook the same process for Clone F, with the ELISA highlighting potential binding sites within two regions of the frizzled protein. The first was with peptides 66 and 67, but as there was complete homology between human and rat ROR1 within the amino acid sequence represented by these fragments, this was felt to be a false positive. By comparison, peptide 90 corresponded to ILENVLCQ in humans and VLENVLCH in rats, and contained the 2 AA different between human and rat. We therefore created 3 SupT1-ROR1 clones with substitutions within these amino acids and found that glutamine at position 261.

We had therefore identified the epitopes targeted by both scFvs of interest.

4.2.9 Humanisation

One of the rationales for targeting ROR1 as opposed to CD19, is sparing of the normal ROR1 negative B cell population. However at the same time, continued presence of normal CD19+ B cells allows for immune responses directed against a rat derived scFv. This has been seen with murine scFvs and have led to clinically significant outcomes, including anaphylaxis with mRNA modified mesothelin CAR T cells (Maus et al., 2013) or antibody responses, with α-folate receptor or carbonic anhydrase IX specific CAR T cells (Lamers et al., 2006, Kershaw et al., 2006). T cell mediated immune responses are also possible due to cross presentation of components of the CAR on MHC.

CD19 CAR T cells by comparison, inherently neutralise the risk of antibody based immune responses by eradicating the normal B cell population, with B cell recurrence associated with a higher risk of relapse.

Humanisation of the antigen binding arms of antibodies was first described in 1986 when the complementary determining regions of the B1-8 murine antibody were grafted onto the framework region of a human antibody, whilst preserving the binding characteristics (Jones et al., 1986).

We attempted to undertake this in house and grafted CDR from clone F onto a homologous human framework identified by IgBLAST software (Ye et al., 2013), but the resulting humanised scFv lost its ability to bind ROR1 (data not shown). We therefore outsourced sequence design to Genscript Limited, who provided 5 potential humanised sequences for both the heavy and light chains for clones A and F, resulting in potentially 25 novel humanised scFvs for each clone.

4.2.9.1 Generation and screening of humanised scFv constructs

To screen which of the humanised scFvs retained binding we cloned all 50 constructs in a secreted scFv format fused to a murine IgG stalk. We chose to screen scFvs rather than full length antibodies as this was the final configuration we required for the CAR structure. All 50 scFv constructs were tested against SupT1 cells expressing either the Ig domain (to screen for Clone A) or Fz domain (for Clone F). 3 of 25 scFvs bound for clone F, and in all cases the scFv utilised humanised light chain 1, suggesting the other light chains disrupted antigen binding. For Clone A there were 17 binders which utilised a more varied heavy and light chain mix, but all constructs incorporating heavy chain 3 and light chain 1 demonstrating binding to ROR1.

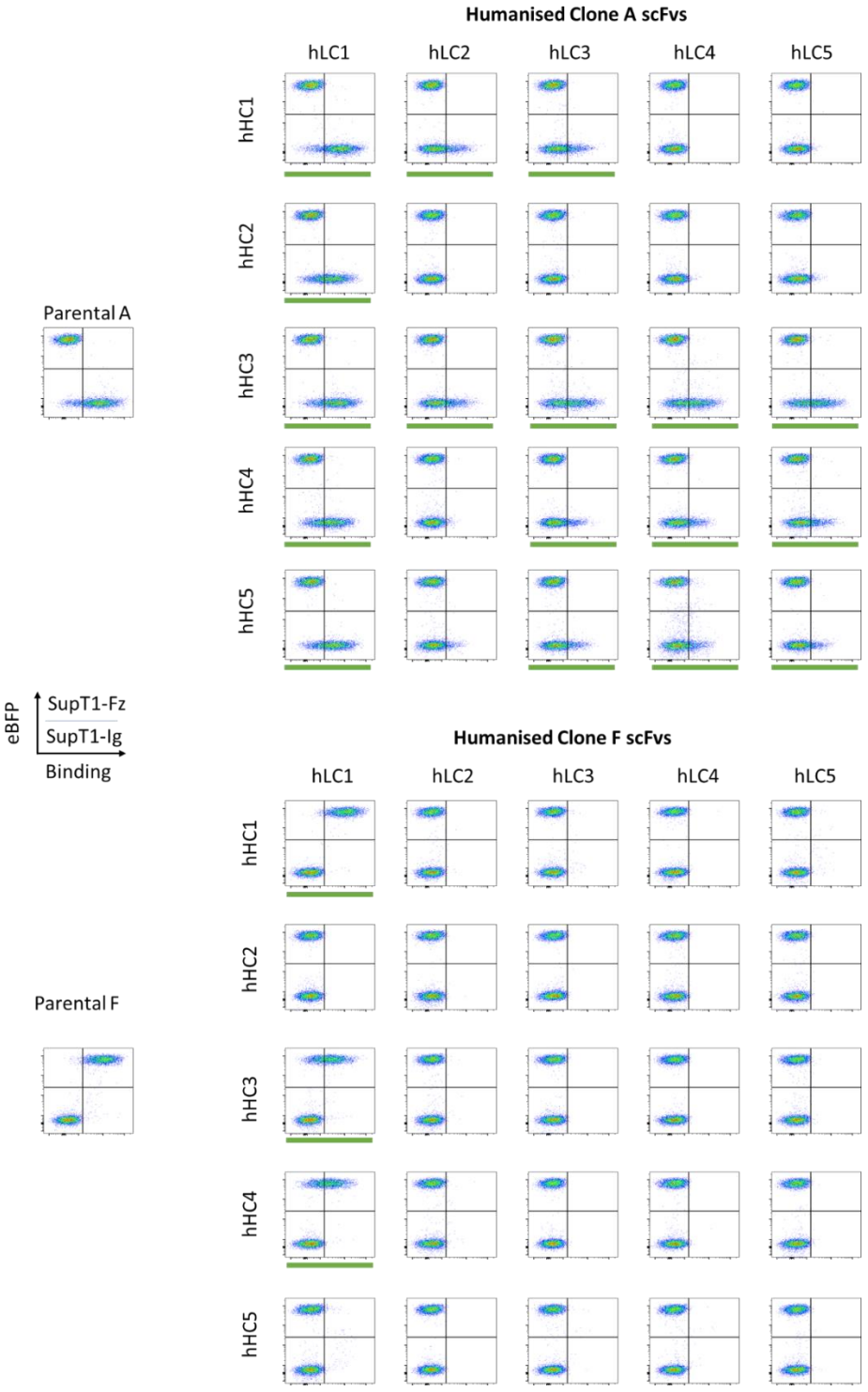


FIGURE 4.21 SCREENING OF HUMANISED SCFV

5 humanised heavy and 5 light chain sequences were designed by Genscript resulting in 25 potential scFvs each for clone A and F. All 50 scFvs were generated by Phusion PCR from G-blocks and cloned in frame with a murine IgG stalk. Secreted scFv-Fc was produced in HEK293T cells and supernatant screened against SupT1 cell lines expressing either the immunoglobulin domain (BFP negative) or frizzled domain (BFP positive). Binding was detected with an anti-murine IgG secondary. Of the potential 25 binders, 3 humanised scFvs for clone F and 17 for clone A bound their respective target. scFv. Heavy chain/Light Chain combinations that bound are highlighted with green underscore.

4.2.9.2 Functional screening of humanised CAR constructs

As binding does not necessarily correlate with function, the 20 scFv sequences that bound for both clone A and F were cloned into our CAR backbone. Initial assessment was based on CD107a degranulation, with all 3 humanised Clone F constructs being comparable to the parental F. For clone A however, only constructs incorporating humanised light chain 1 afforded CD107a degranulation against target cells, implying the framework sequence imparted by this variant contributed to the stability and/or affinity of the scFv and its subsequent interaction with ROR1 (Figure 4.22).

Importantly we saw higher levels of background CD107a degranulation with certain clone A constructs (1x1, 1x3 and 4x1) when co-cultured with the ROR1 negative SupT1-NT cell line. To further investigate this we assessed cytotoxicity with humanised clone A and F constructs. For clone F, cytotoxicity and cytokine secretion was similar between parental and humanised constructs, although there was a trend for higher IL-2 secretion with the humanised construct.

In comparison for clone A constructs, we saw non-specific cytotoxicity against SupT1-NT cells, especially with (3x1) and (4x1). This was accompanied by significantly enhanced IFNγ secretion, irrespective of ROR1 expression (Figure 4.22).

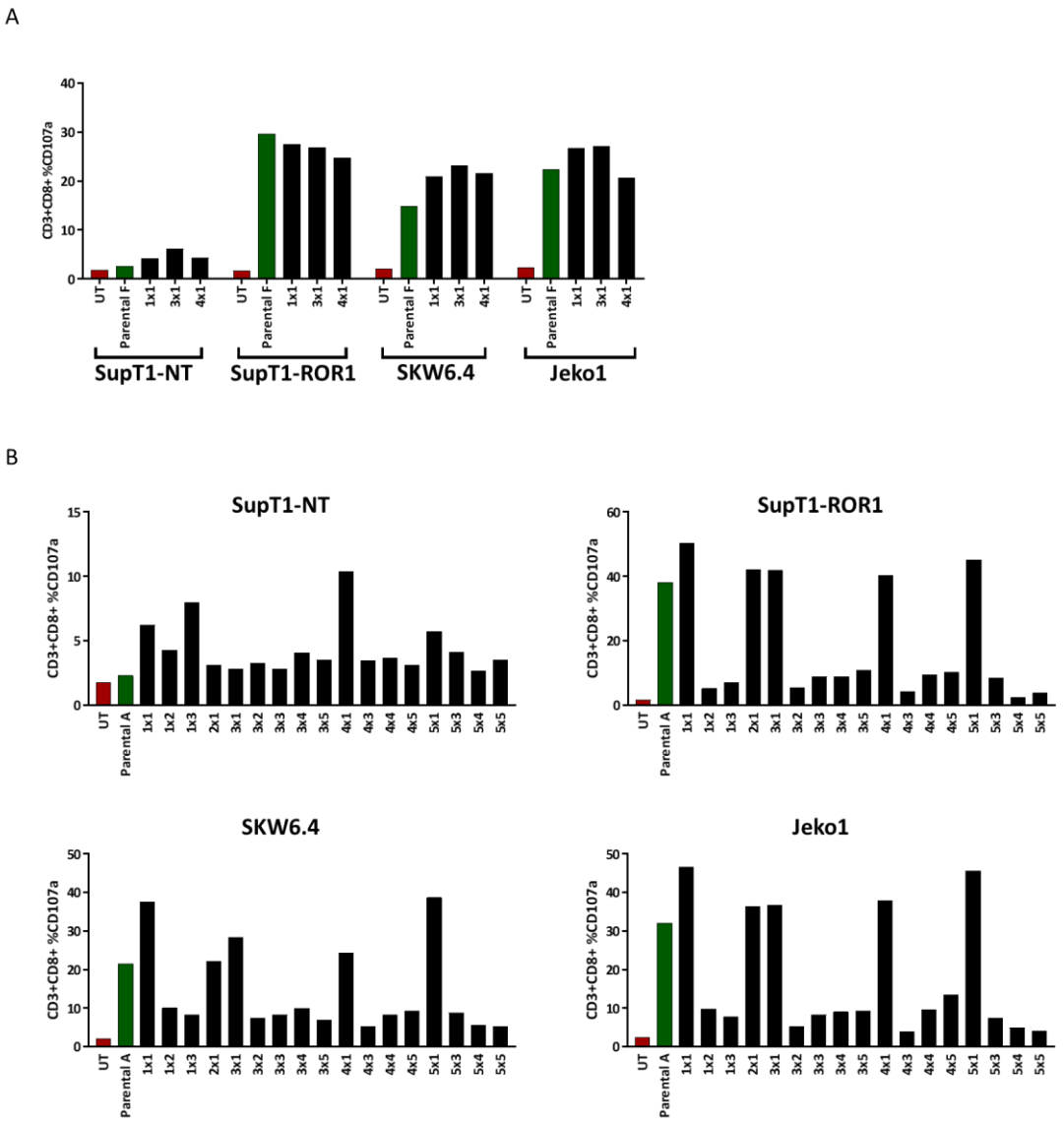


FIGURE 4.22 CD107A DEGRANULATION SCREEN OF HUMANISED BINDERS.

ScFvs identified to bind ROR1 were cloned into a CAR format and CAR T cells generated and cultured with target cells for 4 hours before assessment of CD107a expression by flow cytometry. Humanised constructs labelled heavy chain number x light chain number. A. CD107a degranulation with humanised clone F scFvs was similar to the parental scFv. B. Humanised clone A constructs demonstrated variation in degranulation with only those incorporating the humanised light chain 1 showing comparable CD107a expression to the parental construct. We noted higher background CD107a degranulation with humanised constructs (1x1), (1x3) and (4x1).

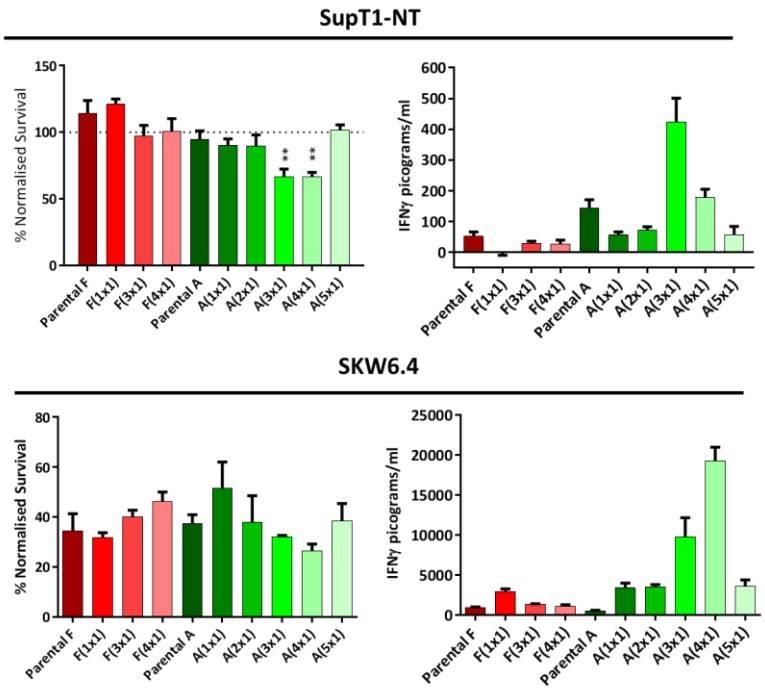


FIGURE 4.23 CYTOTOXICITY AND IFN SECRETION OF LEAD HUMANISED CONSTRUCTS

CAR T cells expressing humanised scFvs for Clone F (shaded red) or clone A (shaded green) were cultured with SupT1-NT or SupT1-ROR1 at a 2:1 effector to target ratio and cytotoxicity assessed at 24 hours. Humanised clone F constructs showed no significant cytotoxicity against SupT1-NT cell lines and similar cytotoxicity against SupT1-ROR1. For Clone A constructs non-specific cytotoxicity was seen with A(3x1) and A(4x1) with corresponding non-specific IFNγ secretion. Representative of 2 independent experiments.

We next assessed cytotoxicity in other ROR1 negative cell lines to better interpret the CD107a results. The K562 cell line, which was originally derived from a patient with chronic myeloid leukaemia (Lozzio and Lozzio, 1975), is ROR1 negative. Despite this, co-culture with all humanised clone A CAR T cell constructs resulted in cytotoxicity at 24 and 96 hours (Figure 4.24A-B).

We expanded upon this finding and used A(1x1) as a representative humanised clone A CAR T cell against the ROR1 negative MCF7 breast cancer cell line, which also demonstrated non-specific cytotoxicity and IFNγ secretion (Figure 4.24C-D).

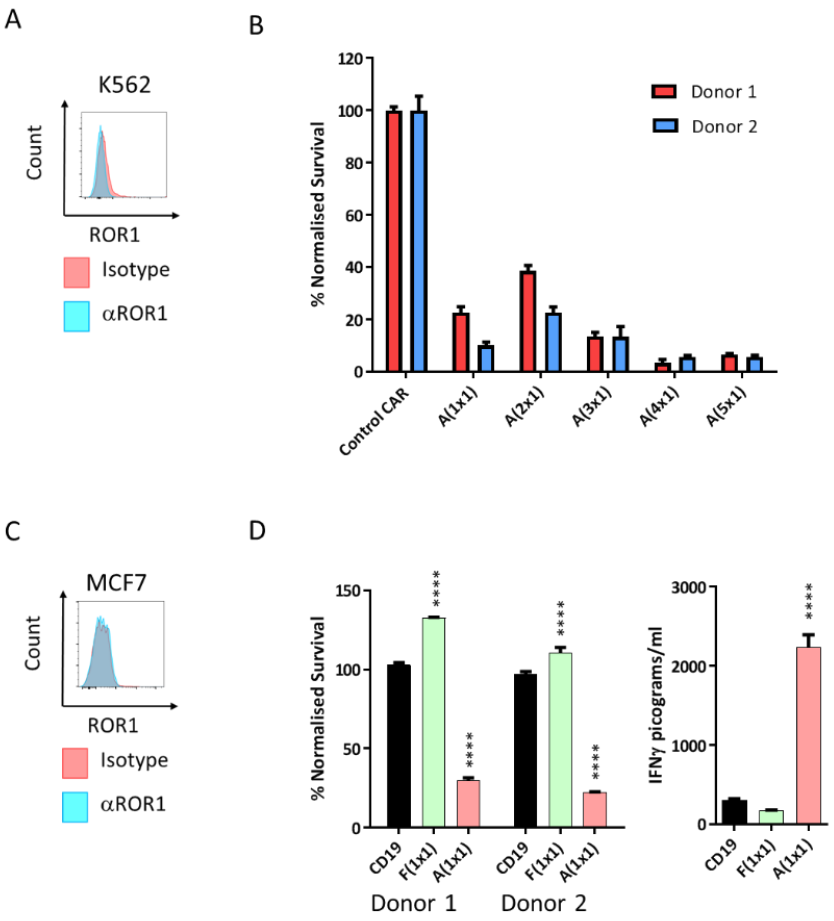


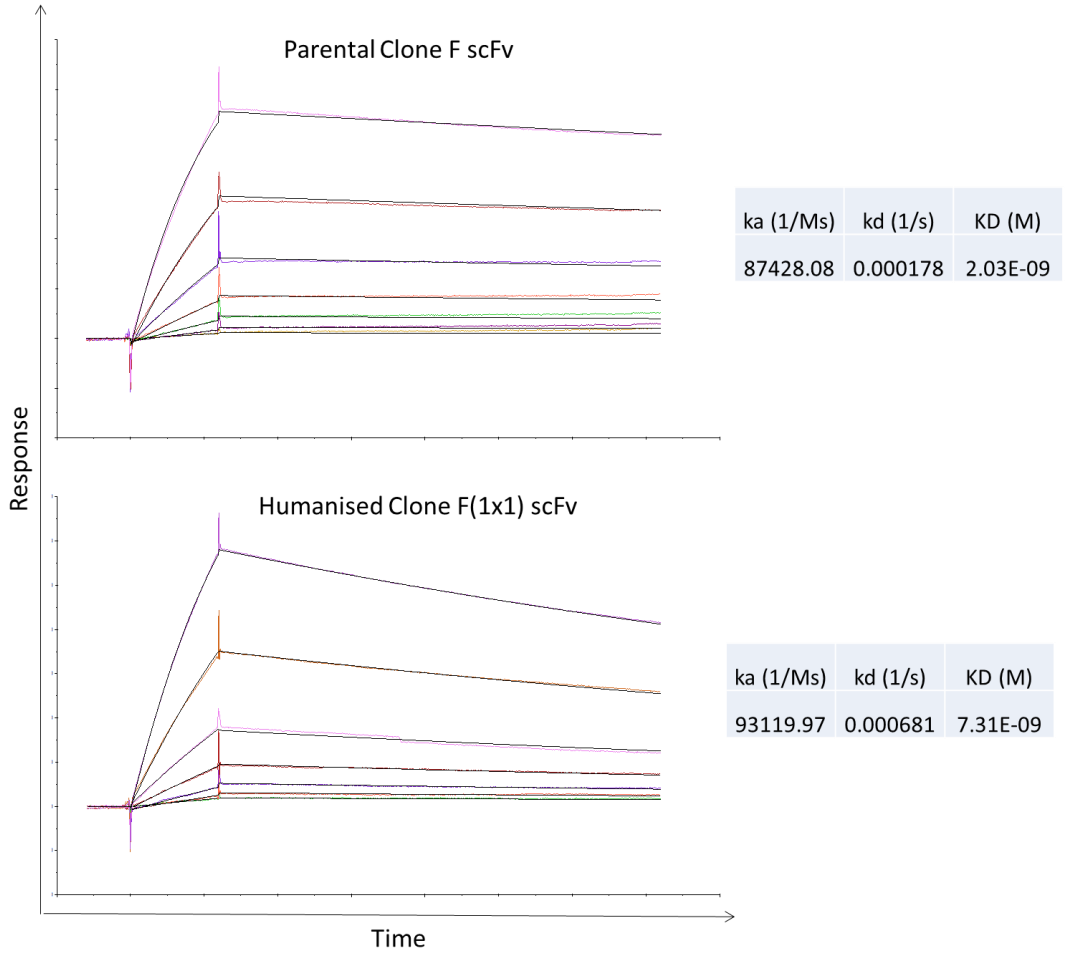
FIGURE 4.24 CLONE A MEDIATES NON-SPECIFIC KILLING OF ROR1 NEGATIVE TARGET CELLS

A. The K562 chronic myeloid leukaemia cell line does not express ROR1. B. ROR1 CAR T cells expressing humanised clone A scFvs were co-cultured with K562 cells with resultant cytotoxicity at 96 hours compared to a CD19 CAR control construct, in 2 independent donors. Effector to target ratio 2:1. C. The MCF7 breast cancer cell line lacks ROR1. D. Co-culture of MCF7 cells with CAR T cells expressing the humanised clone A construct 1x1 resulted in significant cytotoxicity at 24 hours based on a MTS assay with corresponding IFNγ secretion. This was not seen with the humanised F construct 1x1. Statistical analysis: One way ANOVA with CD19 CAR T cells as control arm.

The non-specific killing seen with clone A could have been a result of the humanisation process. We re-assessed parental clone A CAR T cells with K562 and MCF7 cell lines but still demonstrated off target toxicity (data not shown), suggesting inherent non-specific binding with this scFv. This had not envisaged based on our earlier screen with SupT1-NT cell lines and in hindsight, a more detailed cross-examination with a panel of ROR1 negative cell lines, much like that undertaken with panel of solid tumour cell lines, may have detected this at an earlier stage.

These findings led us excluding clone A constructs from our future work plan and promoted humanised clone F(1x1) to the lead construct, to be taken forward. Further analysis with CAR T cells expressing F(1x1) did not show any evidence of spontaneous cytokine secretion in resting cultures, nor any features of tonic T cell activation or exhaustion as measured by PD-1 expression compared to un-transduced or CD19 CAR T cells (data not shown).

4.2.9.3 Affinity of parental and humanised clone F scFv



ka (1/Ms)	kd (1/s)	KD (M)
87428.08	0.000178	2.03E-09

ka (1/Ms)	kd (1/s)	KD (M)
93119.97	0.000681	7.31E-09

FIGURE 4.25 SURFACE PLASMON RESONANCE TRACES FOR PARENTAL CLONE F AND HUMANISED F(1X1) CONSTRUCT

Affinity of clone F and humanised hF(1x1) was undertaken with a Biacore X100. ScFv-Fc was immobilised on a CM5 chip using a mouse antibody capture kit and traces obtained by passing across varying concentrations of ROR1-His (1.5625nM to 100nM). ka = association rate constant, kd = dissociation rate constant and K_D = equilibrium dissociation constant.

To better characterise our lead and parental clone F scFv we undertook surface plasmon resonance to define affinity constants for their interaction with ROR1. This was especially true as modification of the framework region can affect affinity. The reported K_D for parental F was $2.03x10^{-9}$ M compared with $7.31x10^{-9}$ M for the humanised scFv. This alteration in affinity did not result in differential cytotoxicity but may have accounted for the slightly higher IL-2 secretion seen with the humanised construct. Although not undertaken SPR analysis of hF(3x1) and hF(4x1) would have demonstrated whether their inferiority with respect to hF(1x1) was secondary to differences in affinity.

4.2.9.4 Assessment of lead humanised F, hF(1x1), construct against a panel of haematological cell lines

Having selected our lead construct, we next assessed its cytotoxic potential along with IFNγ and IL-2 secretion against a range of haematological cell lines constitutively expressing ROR1. Within this screen we compared CAR T cells expressing the hF(1x1) construct with previously published R12 and 4A5 scFv constructs. To allow direct comparison, all constructs shared the same lentiviral vector backbone including signal peptide, hinge spacer domain and 41BB-CD3ζ intracellular signalling domains. As before we included the fmc63 CD19 CAR as control as haematological malignancies can be targeted by both ROR1 and CD19 CAR T cells. The CD19 construct was identical to the ROR1 CAR construct except it contained the CD8α spacer.

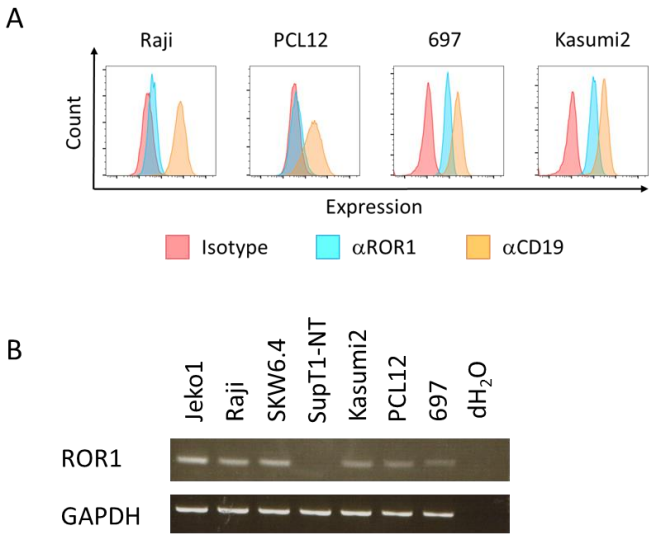


FIGURE 4.26 EXTENDED PANEL OF ROR1 POSITIVE CELLS

A. ROR1 and CD19 expression for Raji, PCL12, 697 and Kasumi2 cell lines was undertaken by flow cytometry, compared to an isotype control. B. RT-PCR using ROR1 and GAPDH specific primers in the above cell lines as well as for Jeko1, SKW6.4 and SupT1-NT.

Our panel included Kasumi2 and 697 ALL cell lines; Raji, Burkitt's lymphoma cell line and PCL12, a CD5+ EBV transformed CLL cell line along with SKW6.4 cells that we had used from the outset of the project.

PCL12 and Raji cells demonstrated only a minor shift by flow cytometry (Figure 4.24A) and to confirm this expression we undertook RT-PCR demonstrating a band for ROR1 at the correct size. This was especially important for the Raji cell line, as it had previously been reported to be ROR1 negative (Baskar et al., 2012), but in keeping with our findings a an independent group has also reported Raji cells to be ROR1 positive (Li et al., 2013)

With regards to ROR1 CAR T cells, across 2 donors, hF(1x1) led to significantly higher cytotoxicity against SKW6.4, Raji, PCL12 and Kasumi2 cells compared to both R12 and 4A5. Against 697 cells cytotoxicity with hF(1x1) was comparable with R12 but still superior to 4A5. IFNγ secretion was variable between donors and ROR1 CAR T cell constructs, however, IL-2 was consistently higher with hF(1x1) over R12 or 4A5.

Inclusion of CD19 CAR T cells allowed a more complete comparison and demonstrated both cytotoxicity and cytokine secretion was superior with CD19 CAR T cells as opposed to ROR1 CAR T cells when targeting haematological cell lines that express both antigens, although at markedly different levels.

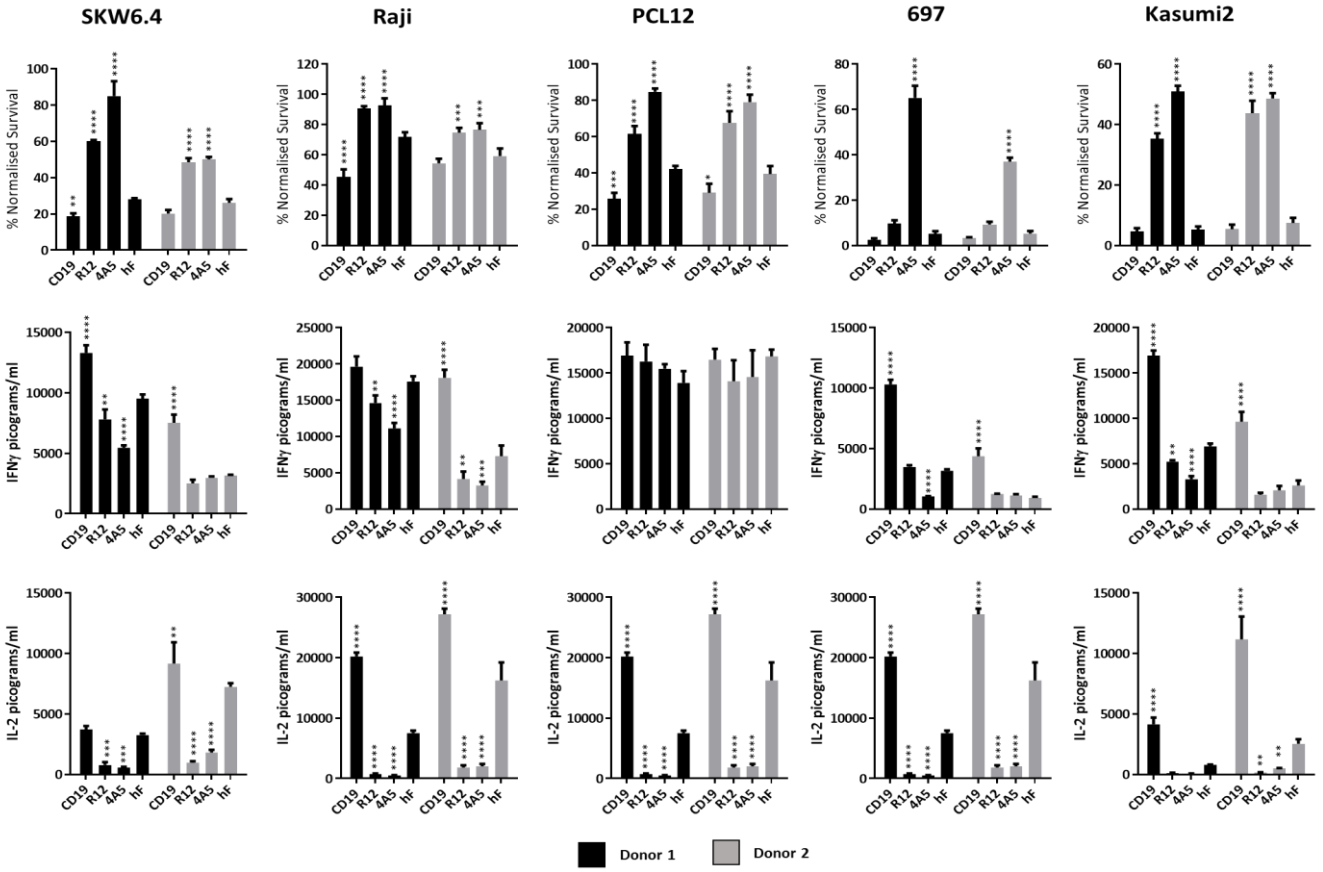


FIGURE 4.27 CYTOTOXICITY AND CYTOKINE SECRETION WITH ROR1 CAR T CELL AND CD19 CAR T CELLS AGAINST A PANEL OF HAEMATOLOGICAL CELL LINES.

CAR T cells expressing the CD19 fmc63 scFv or ROR1 CAR constructs expressing either the R12, 4A5 of hF scFv were generated for 2 donors, normalised based on mCherry expression and cultured with a panel of haematological cell lines (SKW6.4, Raji, PCL12, 697 and Kasumi2) at a 2:1 effector to target ratio and cytotoxicity and IFNγ and IL-2 secretion assessed at 24 hours. Statistical analysis undertaken with two way ANOVA with hF as the comparator arm.

4.2.10 Cytotoxicity against primary CLL cells

We next tested cytotoxicity against primary patient samples. Although ROR1 is expressed on CLL, ALL and MCL, it is best described in CLL cells where it is expressed in greater than 95% of patients (Cui et al., 2016). To better understand our target we assessed the antigen density of ROR1 and CD19 in 20 CLL patients. Median antigen binding capacity (ABC) for ROR1 was 2304 molecules/cell with a range of 800-4828, whilst CD19 was expressed at significantly higher levels: median 12583 molecules/cell (Range 5894-23652). This level of ROR1 expression is in keeping with a more limited assessment undertaken in only 5 CLL samples (Baskar et al., 2008). Our calculated CD19 antigen density was also comparable with other published series in CLL (Ginaldi et al., 1998, Cabezudo et al., 1999).

We next co-cultured primary CLL cells from 6 patients with CAR T cells generated from healthy donors expressing the CD19 fmc63 scFv or the ROR1 R12, 4A5 or hF constructs. In keeping with the cell line data, hF(1x1) provided higher levels of cytotoxicity compared to R12 and 4A5 but was inferior to CD19 CAR T cells.

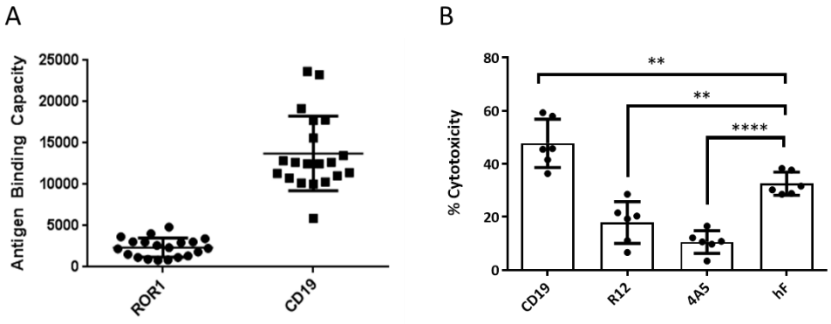


FIGURE 4.28 ROR1 AND CD19 ANTIGEN DENSITY ON PRIMARY CLL CELLS AND CYTOTOXICITY

A. Antigen binding capacity assessment in 20 primary CLL samples demonstrated a log lower expression of ROR1 compared to CD19. B. CAR T cells expressing the CD19 fmc63 scFv or ROR1 R12 4A5 and hF(1x1) scFvs were co-cultured with 6 CLL samples. hF(1x1) resulted in higher cytotoxicity compared to R12 and 4A5, whilst CD19 showed enhanced cytotoxicity. Statistical analysis: one way ANOVA. Representative of 2 independent experiments.

4.2.11 3rd Generation and costimulatory CAR T Cells

We hypothesised that the low antigen density of ROR1 on cell lines and CLL cells contributed to reduced effector function in comparison to CD19. In an attempt to enhance function we generated a panel of alternative intracellular signalling domains. This included addition of either CD28 or OX40, both of which are involved with T cell co-stimulation and activation in a third generation CAR format. We also generated a further two variants, one with an addition 41BB domain and one with a further 41BB and CD3z domain (Figure 4.27A).

Despite these additional intracellular signalling modules there was no clear improvement in cytotoxicity or cytokine secretion compared to the original 2nd generation construct (Figure 4.27B). Although we did not compare these constructs with CD19 CAR T cells or use primary CLL cells for cytotoxicity, the lack of enhanced cytotoxicity or cytokine secretion, especially IL-2 suggested that these would be no better than the original 2nd generation construct.

As an alternative, T cells can be made to express 2 or more distinct CARs on their surface, either through multiple vectors or within a single vector. CoCAR constructs were initially designed to limit off target toxicity by delivering the CD3ζ through one CAR and co-stimulatory signal 41BB or CD28 through a second CAR (Kloss et al., 2013). We modified this approach by leaving the ROR1 scFv coupled with both 41BB and CD3ζ but introduced a CD19 CAR that provided additional CD28 stimulation (Figure 4.27B).

The aim of this approach was to lower the activation threshold and nullify the limitation of low ROR1 density. There was no off target toxicity against SupT1-NT cells and showed comparable killing of SupT1-ROR1 cells in a 4 hour chromium cytotoxicity assay. However, co-culture of the CoCAR construct with SupT1-CD19 cells demonstrated high levels of cytotoxicity, which was not initially envisaged. We hypothesised that CD28 stimulation through a CD19 CAR would not lead to killing without a corresponding CD3ζ signal. Possible reasons for cytotoxicity of CD19

targets with a CD19-CD28 CoCAR include cross pairing of the intracellular signalling domains such that CD19 CAR co-opts the ROR1 CAR CD3ζ domain, which we can investigate with the use of a non-signalling CD3ζ. Alternatively aberrant clustering, induced by multiple CARs on the T cell surface may lead to non-specific activation via tonic signalling and exhaustion (Long et al., 2015).

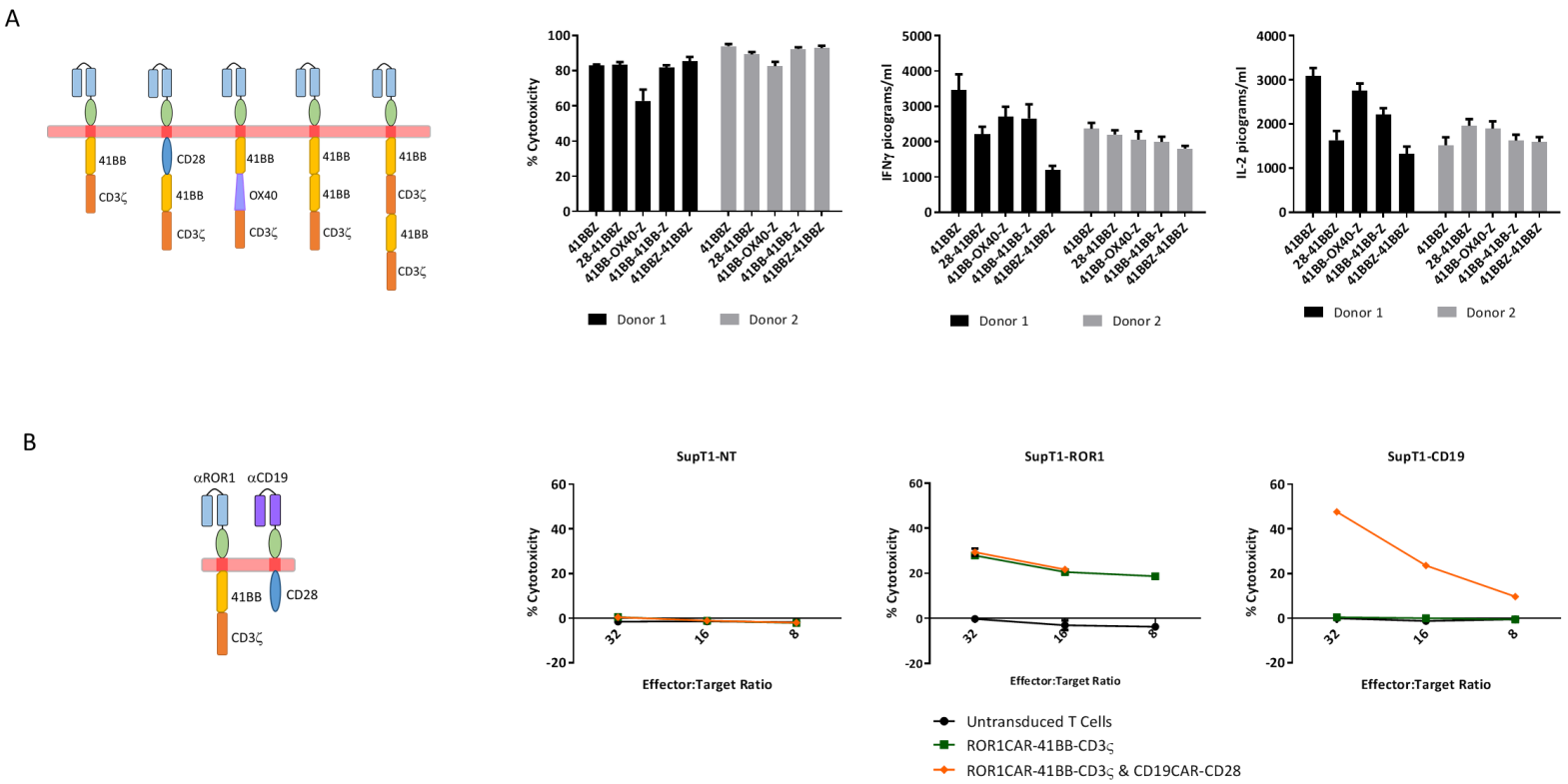


FIGURE 4.29 3RD GENERATION SIGNALLING DOMAINS AND COCAR CONSTRUCTS DO NOT ENHANCE ROR1 CAR T CELL FUNCTION

A. We generated a panel of 3rd generation signalling domains that included CD28 or OX40 in addition to 41BB-CD3ζ. Alternative constructs included an addition 41BB or 41BB-CD3ζ domain. ROR1 CAR T cells with the hF scFv were generated and co-cultured with SKW6.4 cells at a 2:1 effector to target ratio and cytotoxicity and cytokine secretion assessed at 24 hours. There was no significant different in cytotoxicity between the 2nd generation construct and 3rd generation signalling domains. B. CoCAR T cells expressing a 2nd generation hF based ROR1 CAR and 2nd CD19 fmc63 CAR signalling to CD28 were generated and compared with traditional ROR1 CAR T cells. A 4 hour chromium release assay demonstrated comparable cytotoxicity of SupT1-ROR1 cells with both constructs but high levels of CD19 toxicity with the CoCAR construct.

4.2.12 CAR Section Discussion

Within this section of work we detailed how starting from 10 scFvs we reached our final humanised hF(1x1) construct, with the aim of incorporation into CAR T cells for the treatment of ROR1 positive malignancies, specifically CLL.

4.2.12.1 ScFv selection and comparison

Initial screening of the different scFvs followed by iterative optimisation of spacer length allowed us to compare different ROR1 scFvs. The extracellular spacer was a key factor in improving efficacy of CAR T cells, with the 19 AA hinge spacer of human IgG1 leading to optimal cytotoxicity irrespective whether the scFv bound to the immunoglobulin or frizzled domain of ROR1. The importance of spacer length has also been demonstrated for a range of other scFvs including the R12 scFv that also targets ROR1 (James et al., 2008, Hudecek et al., 2013).

The introduction mCherry, as a reliable and easy to detect transduction marker allowed us to undertake accurate comparison between constructs. Although this was suitable for pre-clinical assessment, it is unsuitable for clinical translation due to immunogenicity. Other strategies to allow accurate assessment of TE, include the inclusion RQR8 or tEGFR, both of which can be stained for with monoclonal antibodies. An added functionality of these marker proteins is that they can also be targeted as suicide system for therapeutic purging of CAR T cells as a strategy to minimise toxicity (Philip et al., 2014, Paszkiewicz et al., 2016).

An alternative is the inclusion of a protein tag within the structure of the CAR, such as StrepTagII, although this inherently increases immunogenicity and is therefore unsuitable for clinical use (Liu et al., 2016).

To allow for accurate detection of transduced cells in vitro and for future clinical trials we have embarked on the generation of a monoclonal type 2 anti-idiotype antibody through collaboration with BioRad Limited, that specifically

detects humanised clone F on the surface of T cells, irrespective of whether it has bound to ROR1 (Jena et al., 2013).

4.2.12.2 Humanisation as a mechanism to decrease immunogenicity and enhance persistence.

Our 2 lead candidates from initial selection, clones A and F, were subjected to a humanisation process to decrease the risk of immune responses directed against the rat derived scFv. These can be antibody mediated resulting in decreased persistence of transgenic T cells (Lamers et al., 2006, Kershaw et al., 2006), especially when there is retention of the normal B cell population as occurs with the majority of CAR T cells not targeting CD19 (Berger et al., 2015). Persistence can also be limited by T cell immune responses, even for CD19 CAR T cells (Turtle et al., 2016).

Humanisation is therefore an integral component of CAR design and in keeping with this, humanised CD19 CAR T cells have led to clinical responses in patients previously treated with murine CD19 CAR T cells who had lost expression of transduced cells (Maude et al., 2016a, Maude et al., 2016b). More recent humanisation strategies aim to minimise immunogenicity further by modification of the of the intracellular signalling domains, such as the fusion site between 41BB and CD3ζ, due to potential presentation on MHC class I (Sommermeyer et al., 2017).

The innate arm of the immune system can also limit persistence, as CAR T cells incorporating a full IgG stalk spacer, bind to Fc γ receptors on monocytes and NK cells. This however can be abrogated by introducing mutations residues amino acids within the IgG required for FcγR engagement (Hombach et al., 2010), although this is not seen with the hinge spacer we have selected.

Humanisation itself does not guarantee a lack of immunogenicity. To model this CAR constructs can be screened against a large panel of donor derived dendritic, B and T cells to assess for immune responses in the context of a wide

range of MHC classes, such as that provided by Lonza through their Epibase in vitro platform.

4.2.12.3 hF(1x1) provides superior cytotoxicity of low density antigen targets compared to R12 and 4A5 CAR T cells

Comparison of our hF(1x1) scFv demonstrated superior cytotoxicity against a panel of haematologically derived cell lines and primary CLL cells compared with comparator non humanised ROR1 CAR T cells (R12 and 4A5), with the added advantage of having a humanised binder.

The affinity of hF and 4A5 scFvs are similar (K_D 7nM and 4nM respectively) and thus the difference in effector function is likely due to the epitope-spacer dynamic. The K_D value for the R12 Fab is 0.56 nM but is unknown for R12 in the scFv format, thus not allowing a direct comparison. Nevertheless, hF(1x1) imparted superior cytotoxicity and cytokine secretion compared to R12. Overall I believe that the superior efficacy seen with hF(1x1) was in part due to it targeting the more membrane proximal frizzled domain rather than the affinity of the scFv per se, although generation of affinity matured or limited variants would allow for interesting functional assessments to be made.

A consequence of the humanisation process is the potential alteration of scFv affinity, due to modification in the framework region. Our humanised hF(1x1) construct showed higher affinity compared to the parental rat scFv (7nM vs 2nM) and this difference may have mediated higher levels of IL-2 secretion we saw.

Affinity can have significant consequences on CAR T cell function. For example, with EGFRvIII CAR T cells, higher affinity variants induce greater IFNγ and IL-2 secretion, as well as being able to successfully target lower antigen density cells (Liu et al., 2015a). In keeping with this, and in relation to ROR1, the high affinity R12 demonstrated tumour eradication in vitro and in vivo compared to lower affinity 2A2 bearing CAR T cells (Hudecek et al., 2013). There is however a limit to affinity, as excessively high affinity levels can lead to activation induced cell death and

decreased effector function (Watanabe et al., 2014). This finding is in contrast to TCR gene modified cells in which the converse is true, with high affinity TCR peptide interactions limiting killing of low peptide targets and increasing the risk of non-specific activation (Thomas et al., 2011).

One of the major limitations of this aspect of the project was the relatively narrow assessment of effector function, as only cytotoxicity and cytokine secretion at 24 hours was assessed. Prolonged co-cultures, in vitro re-challenge, assessment of T cell proliferation and exhaustion profiles as well as wider cytokine secretion are vital in understanding difference in biology.

Moreover more complex in vivo models may allow for difference in survival, disease burden and T cell expansion to be better defined, especially between the parental and humanised F scFv, thereby assessing the wider functional consequences of the higher IL-2 secretion seen.

Analysis was abbreviated in part, based on the comparison of ROR1 and CD19 CAR T cells, focusing our efforts on improving ROR1 CAR T cell function before more detailed assessment.

4.2.12.4 Antigen density limits efficacy of ROR1 CAR T cells in comparison with CD19 for the treatment of CLL

Our primary focus was the development of ROR1 CAR T cells as a therapeutic for patients with CLL. One of the challenges is the low antigen density of ROR1, especially compared with CD19. Therefore, although hF(1x1) was the leading ROR1 CAR construct against haematological cell lines and CLL cells, it was inferior to a CD19 CAR T cells, which represents the gold standard in current clinical trials.

This corroborates the work of others and highlights that although cytotoxicity can be preserved at a low antigen density, it nevertheless limits the necessary cytokine production and proliferation required to support prolonged tumour eradication. For example, for a CD20 CAR encompassing a CD28 and CD3ζ

intracellular signalling domains, approximately 200 molecules/target was needed for lysis but around 2000 needed for cytokine production (Watanabe et al., 2015). This has been confirmed by a group targeting CD123 who found similar findings, such that approximately 1600 CD123 molecules were able to lead to target cell killing and cytokine secretion, but approximately 5000 were needed for robust proliferation (Arcangeli et al., 2017) and unsurprisingly a similar finding occurs with normal TCR signalling (Valitutti et al., 1996). Given that the mean ROR1 antigen density on CLL cells is approximately 2000 molecules, this presents a limitation with standard 2nd generation CAR T cells.

One of the theoretical advantages of targeting ROR1 as opposed to CD19 is the sparing of the normal cell population, thus limiting hypogammaglobulinaemia secondary to B cell aplasia. Despite initial concerns, this has not emerged as a limiting factor with CD19 CAR T cell therapy, as routine infusion of intravenous immunoglobulin minimises associated risks. In addition for those patients who achieve prolonged disease eradication, the inclusion of suicide systems allow for purging of the transduced T cell population and reversal of B cell aplasia (Paszkiewicz et al., 2016).

A potential contributing factor to the success of CD19 CAR T cell therapy may be due to the continued production of CD19+ B cells within the marrow. These act to stimulate and promote ongoing turnover of CD19 CAR T cells, thus enhancing persistence. In comparison, ROR1 CAR T cells would not receive this continued drive for proliferation within the marrow and is unlikely to receive this from normal tissue expressing ROR1.

This brings us to the inherent paradox of targeting ROR1 with CAR T cells. We require them to effectively target low levels of ROR1 on CLL but this increases the inherent risk of targeting normal tissues such as pancreatic islets cells, areas of the gastrointestinal tract and parathyroid cells which also express ROR1 at low levels. Indeed given that R12 based CAR T cells become activated and release IFNγ

in co-cultures with adipocytes and pancreatic islet cells (Balakrishnan et al., 2017), we would expect a similar finding with hF(1x1) CAR T cells.

ROR1 CAR T cells expressing the R12 CAR showed safety in non-human primates, despite them having a similar expression profile of ROR1 with no gastrointestinal or pancreatic toxicity noted suggesting ROR1 expression at these sites may be shielded from circulating CAR T cells, although whether this would still be the case following a systemic pro-inflammatory state such as cytokine release syndrome is unknown (Berger et al., 2015).

CRS is a limiting feature of CD19 CAR T cell therapy, with sometimes fatal consequences, although algorithms to guide its management have been refined with increasing knowledge of the biology and kinetics driving it (Teachey et al., 2016, Lee et al., 2014b). ROR1 therapy, secondary to lower antigen density may limit severe CRS, thereby improving safety for patients with CLL, the majority of whom are elderly and have co-morbidities, thus allowing expansion of CAR T cell therapy. This has to be balanced against the slower rate of killing and cytokine secretion, which increases risk of T cell exhaustion, due to chronic stimulation through the CAR, a feature common in chronic viral infections and cancer (Wherry and Kurachi, 2015).

4.2.12.5 Strategies to enhance ROR1 CAR T cell

Despite the above, ROR1 remains an attractive target for CLL. One possible method to circumvent limitation of low antigen density, would be to pharmacologically increase ROR1 levels, thus modulating enhanced cytotoxicity. Proof of this approach comes from evidence with decitabine, which increases CD33 expression on AML blasts, sensitising them to a monoclonal antibody (Vasu et al., 2016). Dasatinib has been shown to increases ROR1 gene expression in t(1;19) cell lines (Bicocca et al., 2012), but we found no increase in ROR1 in CLL cells cultured with dasatinib. Other platforms screening large numbers of drug candidates to assess for enhanced ROR1 expression may yield an alternative candidate.

In an attempt to enhance the inherent function of hF(1x1) CAR T cells we assessed a variety of 3rd generation signalling domains, but did not see convincing evidence of improved function. This is in comparison to 3rd generation CD19 CAR constructs where they have demonstrated higher cytokine secretion and persistence (Pule et al., 2005). Whether this is also due to antigen density or the specific combination of scFv epitope and affinity remains unknown but requires further validation. Although 2nd generation CAR constructs remain the gold standard in clinical trials, investigation of third generation constructs with a CD19 targeting scFv are underway (Tang et al., 2016). The most interesting is the SAGAN trial which undertakes co-infusion of 2nd generation and 3rd generation CAR T cells, with an aim to map fate and persistence in a range of lymphoid malignancies including ALL, NHL and CLL (NCT01853631).

A further method to try and improve ROR1 CAR T cell function was the introduction of a costimulatory CAR to lower the activation threshold. Assessment of this was terminated due to unintended cytotoxicity against CD19+ROR1- targets, which was not the aim of when solely supplying a CD28 signal through the CoCAR.

Had this shown more promise, an additional advantage of utilising the CoCAR was that co-stimulation through CD19 may allow the CAR T cell population to be preserved, even in the absence of ROR1 targets in the bone marrow, leading to prolonged persistence.

We are currently refining this approach with the substitution of the CD19 with a CD5 CoCAR, taking advantage of the aberrant CD5 expression on CLL cells. As part of this we have undertaken modification of the spacer and transmembrane domain to limit cytotoxicity to solely ROR1+CD5+ CLL cells and not normal CD5+ T cells. Proof of using CD5 as a target comes from the treatment of T cell leukaemia/lymphoma and concerns with regards to elimination of the entire normal and CAR T cell population was not realised as fratricide was limited, although it is yet to be tested in clinical trials (Mamonkin et al., 2015).

The use of multiple CAR on the surface of the same T cell can be manipulated in a variety of ways using Boolean logic. For example the use of 2 CARS targeting different antigens on the surface of tumour cells, with both leading to a full activation signal is an example of an 'OR' gate that can be used to limit antigen negative escape (Zah et al., 2016). As described by Kloss et al. this can be modified to be an 'AND' gate require the presence of both antigen to fully activate T cells, thus acting as a safety system (Kloss et al., 2013). The costimulatory CAR can be switched to having an inhibitory effect such that T cell activation by normal tissues is limited even in the presence of a tumour antigen (Fedorov et al., 2013).

Other strategies to enhance CAR T cells is through the release of pro-inflammatory cytokines to enhance function (Koneru et al., 2015), accessory trans co-stimulation through 41BBL (Zhao et al., 2015) and intrinsic PDL-1 checkpoint inhibition (Cherkassky et al., 2016). More advanced gene editing has allowed expression of the CAR to be driven by the TCR α locus as opposed to a constitutive promoter, further enhancing CAR T cell function by allowing physiological recycling of the CAR on and off the cell surface, thereby limiting tonic signalling and preventing exhaustion (Eyquem et al., 2017).

Furthermore, the entire design and manufacture process can be manipulated to enhance CAR T cell function. For example cells manufactured with CD3/CD28 activation beads demonstrate superior function compared to those activated with anti-CD3 antibodies and IL-2 (Barrett et al., 2014), although this has been further refined to demonstrate limited stimulation leads to a superior CAR T cell product compared to continuous stimulation (Kagoya et al., 2017). Additionally, the use of IL-7 and IL-15 improve T cell fitness, by increasing the percentage of T memory stem cells (Xu et al., 2014). Other strategies include the selection and transduction of specific T cell subsets with high effector and proliferation potential, such as memory T cells (Berger et al., 2008, Wang et al., 2011b), to the use of defined subsets of CD4 and CD8 T cells to generate a more consistent T cell product between patients (Sommermeyer et al., 2015). These manufacturing variations have to be balanced against the need for extra manipulation of cell fractions in a GMP environment, with associated costs and longer turnaround times.

Within the clinical trial setting, optimisation of conditioning regimens, management of CRS (Ruella et al., 2016, Lee et al., 2014b, Lee et al., 2014a) and combinational therapy or small molecule inhibitors are allowing for enhanced response rates. Given the success of checkpoint inhibitors with solid malignancies, natural synergy between these and CAR T cells are being exploited with co-administration (Chong et al., 2017). CAR R cells can also be manipulated to secrete checkpoint inhibitors (Li et al., 2017b) or through CRISPR/Cas9 mediated disruption of the PD-1 locus within T cells (Rupp et al., 2017).

All of the above strategies could be utilised to enhance ROR1 CAR T cell therapy, but it is unclear whether these modifications would make them comparable to existing 2nd generation CD19 CAR T cells, which themselves can be improved with these modifications.

This does raise the question as to whether there is a role for ROR1 CAR T cells for CLL. However given that response rates for CLL are lower than for ALL (Fraietta et al., 2016b); development of ROR1 CAR T cells as a therapeutic remains a valid aim, with optimisation beyond the traditional 2nd/3rd generation format required. Additionally, patients who fail CD19 CAR therapy, through a variety of escape mechanisms including alternative splicing (Sotillo et al., 2015), differentiation to other phenotypes (Gardner et al., 2016, Evans et al., 2015) or loss of persistence, may benefit with ROR1 CAR T cell challenger.

Although we have focused on CLL, ROR1 is expressed on Mantle Cell and ALL, but characterisation has not been as detailed as CLL. In ALL, its expression is limited to 5% of patients with t(1;19) translocation, but this cohort tend to do well with intensive chemotherapy, limiting their potential.

Furthermore, a key advantage of developing ROR1 CAR T cells is that it is not limited to haematological malignancies and the ideal therapeutic setting may therefore be in solid cancers, where CD19 is not expressed. We have demonstrated proof of concept with this approach against a panel of solid tumour cell lines and have expanded this more recently to focus on neuroblastoma cell lines with the aim

of undertaking assessment against primary human tumour samples via patient derived xenograft (PDX) modelling.

Despite this, demonstrating efficacy with CAR T cell therapy against solid malignancies has been more difficult, despite a range of target antigens being investigated (Gilham et al., 2012). There is however some evidence of progress, such as with the IL-12 receptor α2 CAR for glioblastoma (Brown et al., 2016), but more in order to achieve success, the immunosuppressive tumour microenvironment in solid malignancies has to be overcome. At a starting point however, transition of our hF(1x1) ROR1 CAR, in a standard 2nd generation format, for the treatment of high risk tumours with unmet need (such as pancreatic cancer) would allow us to demonstrate initial safety and feasibility, which can be built upon with novel CAR architectures, advanced genetic engineering and combinational therapy.

4.3 ROR1 Bispecific T Cell Engager

4.3.1 Introduction

The basic structure of a BiTE comprises two single chain variable fragments joined in tandem with a flexible linker. We consulted the IMGT database of antibodies and utilised the deposited sequence for Blinatumomab as the basic structure of our ROR1 BiTE (Lefranc et al., 1999). This comprises the fmc63 CD19 scFv in a light chain-linker-heavy chain format, followed by the OKT3 CD3 scFv in a heavy chain-linker-light chain format. We substituted the CD19 scFv for our panel of ROR1 scFvs and utilised the same CD3 scFv. The OKT3 CD3 antibody was the first monoclonal antibody licensed for use in humans to prevent transplant rejection and is a potent activator of T cells (Smith, 1996). It is unlikely this is the actual sequence of Blinatumomab used clinically, which has never been published in any scientific papers and in hidden within the patent filings made by Micromet and subsequently Amgen.

FIGURE 4.30 BLINATUMOMAB SEQUENCE AS PER IMGT

The amino acid sequence and structure of Blinatumomab as deposited with IMGT. The CD19 scFv is joined to the CD3 scFv with a GGGGS linker. It also comprises a hexa-histidine tag to allow for purification.

4.3.2 Construct and initial generation

We generated ROR1 BiTEs through overlapping PCR using G-blocks that were codon optimised. As the structure of our scFvs was heavy chain-linker-light chain we maintained this in the BiTE configuration. We also maintained the GGGGSGGGGGSGGGGS linkers between the heavy and light chains of the scFv but wobbled them to limit the potential of homologous recombination in viral vectors. As with the IMGT sequence of Blinatumomab we maintained the GGGGS linker between the CD19 and CD3 scFvs.

In keeping with other reported BiTEs we included a hexa-histidine tag at the N terminus of the BiTE to allow for detection and purification (Figure 4.25A). This did not affect binding compared to BiTE without a hexa-histidine tag (data not shown).

The construct was transferred into a retroviral vector and BiTE produced in HEK-293T cells through either transient transfection or retroviral transduction to generate a stable producer cell line. Supernatant containing either ROR1 or CD19 BiTE, demonstrated binding to target cells and T cells (Figure 4.25B) and cytotoxicity against ROR1 target cells in the presence of effector T cells (Figure 4.25C), although this was only a crude assessment as BiTE was not quantified or purified in these samples.

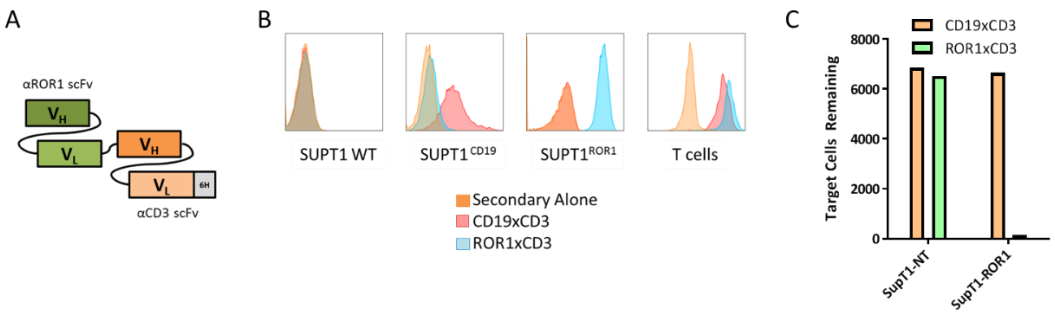


FIGURE 4.31 STRUCTURE, BINDING AND INITIAL CYTOTOXICITY OF OUR ROR1 BITE

A. The basic schematic of our ROR1 BiTE construct which comprises an ROR1 scFv in tandem with a CD3 scFv with an N terminal hexa-histidine tag. B. Supernatant from HEK-293T transduced cells comprising either CD19 BiTE (CD19xCD3) or a ROR1 BiTE (ROR1xCD3) was incubated with target cells. This was followed by incubation with an anti-His secondary and assessment by flow cytometry. SupT1-WT cells, which lack CD19, ROR1 and CD3 showed no binding with either CD19 or ROR1 BiTE. SupT1-CD19 and SupT1-ROR1 cells showed specific binding to the CD19 and ROR1 BiTE respectively. T cells bound both CD19 and ROR1 BiTEs through CD3. C. Supernatant from CD19 BiTE or ROR1 BiTE producing HEK-293T cells was cultured with SupT1-ROR1 cells with PMBCs in a 1:1 effector to target ratio. ROR1 BiTE mediated significant cytotoxicity of target cells compared to CD19 BiTE.

4.3.3 BiTE Purification & Characterisation

We next focused on developing a method to purify and characterise the produced BiTEs to allow for direct comparison based on equivalent concentrations. Our final protocol utilised FPLC HiTrap TALON binding columns. These contain a mixture of agarose beads with immobilised cobalt which binds to the hexa-histidine tag and provided superior purification compared to nickel based columns. Elution was undertaken using an Imidazole gradient. The resulting elution fractions contained two peaks, the first of which corresponded to non-specific protein binding at low concentrations of Imidazole whilst the second peak contained the BiTE, as demonstrated by Coomassie stain of eluted fractions. The corresponding Western blot demonstrates a high degree of purify, although this was under reducing conditions. The purified BiTEs were quantified using a standard curve generated with dilutions of a BSA protein standard and BiTEs were stored at 4^{oC}, with 1% BSA. Storage at lower temperatures or without of BSA as stabiliser, resulted in loss of function, likely secondary to aggregation.

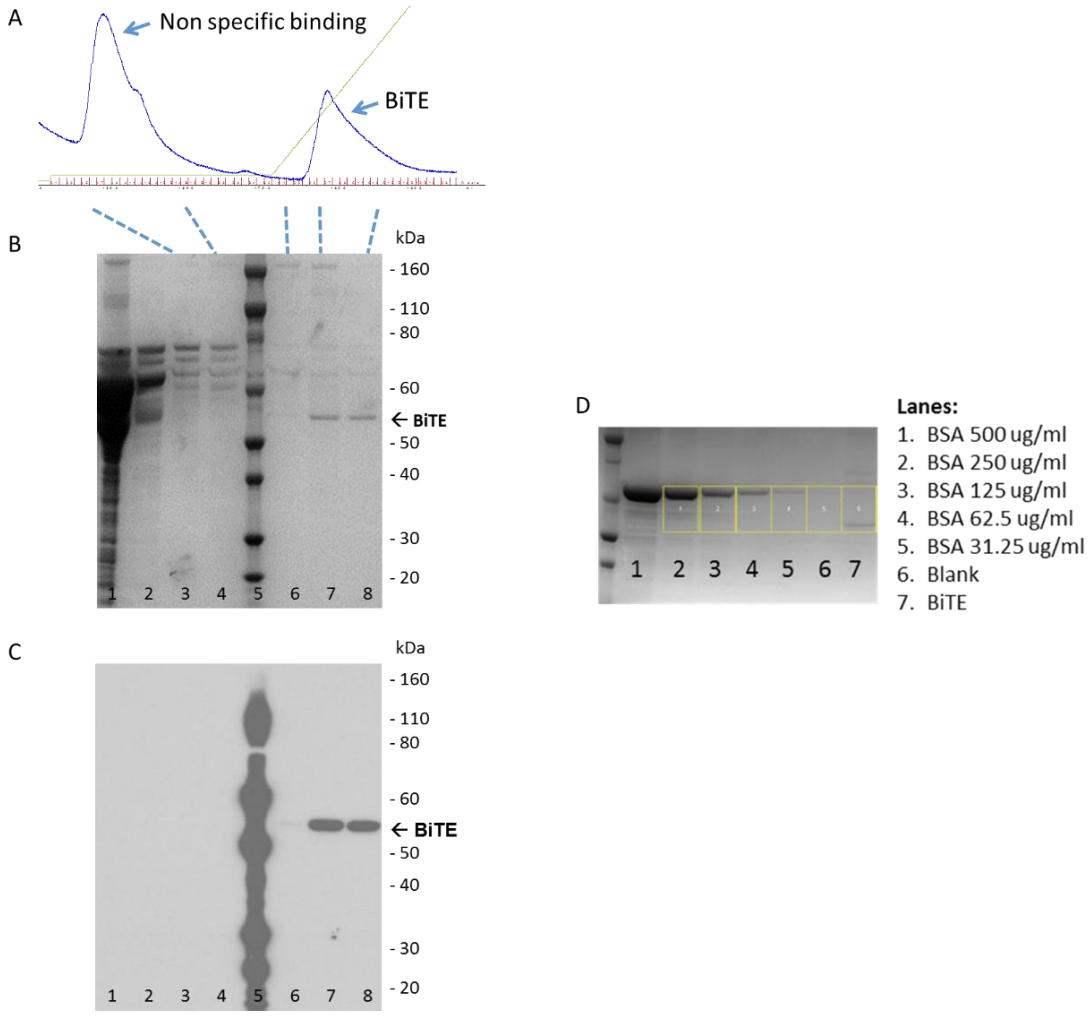


FIGURE 4.32 BITE PURIFICATION AND QUANTIFICATION

Supernatant from HEK-293T cells producing BiTE was passed through a HisTALON column using an AKTA FPLC system with elution via an Imidazole gradient.

A. UV chromatography (blue line) of the elution phase of purification with two peaks. The first corresponds to elution of non-speciifc binding proteins, whilst the second conatins the BiTE. The green line corresponds to the Imidazole concentraion and dashed red lines at the botton the fractions collected. B. Coomassie stain demonstrating that the first peak is non-specific protein eluted at a lower imidazole concentration, whilst the second peak corresponds to fractions with BiTE. C. Western blot using an anti-His-HRP secondary demonstrates His portein at the expected size with significant purity. D. Quantification was based on the generation of a BSA protein standard curve as analysed on a Coomasie using ImageJ software to generate a standard curve.

4.3.4 ROR1 BiTE binding epitope dictates effector function

Once we were able to reliably purify and quantify BiTE across multiple runs, we were in a position to compare BiTEs with different ROR1 scFvs. We found that against a panel of pancreatic adenocarcinoma cell lines, a ROR1 BiTE targeting the frizzled domain (Clone F, as used in the CAR) provided enhanced killing at lower concentrations compared to BiTEs targeting the immunoglobulin domain: At 0.1μg/ml cytotoxicity with clone F was 23.5% and 4.2% for MiaPaCa2 and SUIT2 cells respectively with clone F compared with 56.4 and71.5% for clone omega (Figure 4.31).

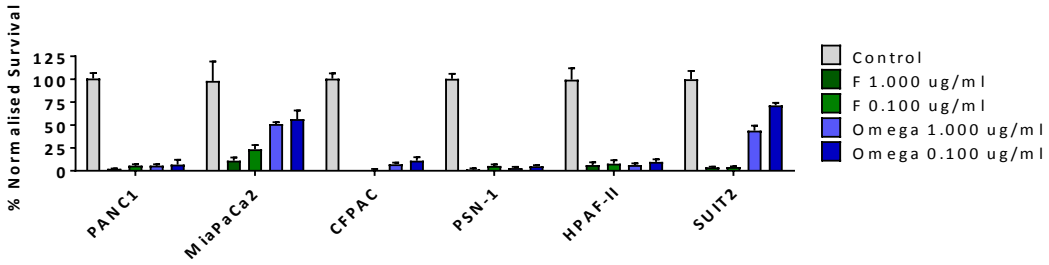


FIGURE 4.33 SCREENING ROR1 BITE BASED ON TARGET EPITOPE

A panel of pancreatic adenocarcinoma cell lines were cultured with peripheral blood mononuclear cells at a 1:1 T cell to target ratio in the presence of a frizzled targeting BITE (F) or an Immunoglobulin targeting BiTE (Omega) at 1ug/ml or 0.1ug/ml. Cytotoxicity between F and Omega BiTEs was similar for Panc1, CFPAC1, PSN-I and HPAF-II cell lines but against MiaPaCa2 and SUIT2 we saw superior killing with F BiTE. Representative of 2 independent experiments.

BiTEs bring T cells and target cells into close proximity to allow for MHC independent activation. The inter-cellular distance between T cell and target cell is vital for effector function, akin to that seen with CAR T cells and physiological TCR signalling. The importance of this aspect of immunotherapy has also been demonstrated for both BiTEs and bispecific antibodies (Bluemel et al., 2010, Li et al., 2017a). We therefore selected clone F as our lead BiTE, in keeping with the scFv chosen for the CAR.

4.3.5 ROR1 BiTE Mediates toxicity against multiple solid tumour cell lines

At this stage the primary focus of the ROR1 CAR project was haematological malignancies. To diversify the range of potential therapeutic targets to include non CD19 targets, we chose to focus our BiTE characterisation on solid tumours.

4.3.5.1 Pancreatic Adenocarcinoma

Pancreatic adenocarcinoma is one of the leading causes of cancer deaths with overall 5 year survival of less than 10%, despite significant investment in novel therapies. Major advances have been lacking in part due to late presentation, inherent resistance to chemotherapy and a lack of targets amenable to therapeutic intervention. In addition there is a clear lack of efficacy with immune checkpoint inhibitors (Kleeff et al., 2016).

A proportion of pancreatic cancers express ROR1 and in order to assess whether our ROR1 BiTE could be used as a potential therapeutic, we assessed function against the PANC1 cell line, which expresses ROR1 (Lieber et al., 1975).

Co-culture of T cell and 1μg/ml ROR1 BiTE with PANC1 cells results in significant cytotoxicity at 24 hours compared to T cell cultured with a CD19 BiTE, with resulting T cell clustering and proliferation. This was also associated with significant IL-2 and IFNγ release.

BiTEs are active at extremely low concentrations and we therefore assessed cytotoxicity against a panel of pancreatic adenocarcinoma cell lines from 1000ng/ml to 0.1ng/ml. There was variation in target cell susceptibility highlighted by the difference between PANC1 and MiaPaCa2 cell lines, which cannot be explained by target cell expression. We nevertheless demonstrated cytotoxicity at 0.1ng/ml of BiTE compared to control cultures with no BiTE across the panel of cell lines.

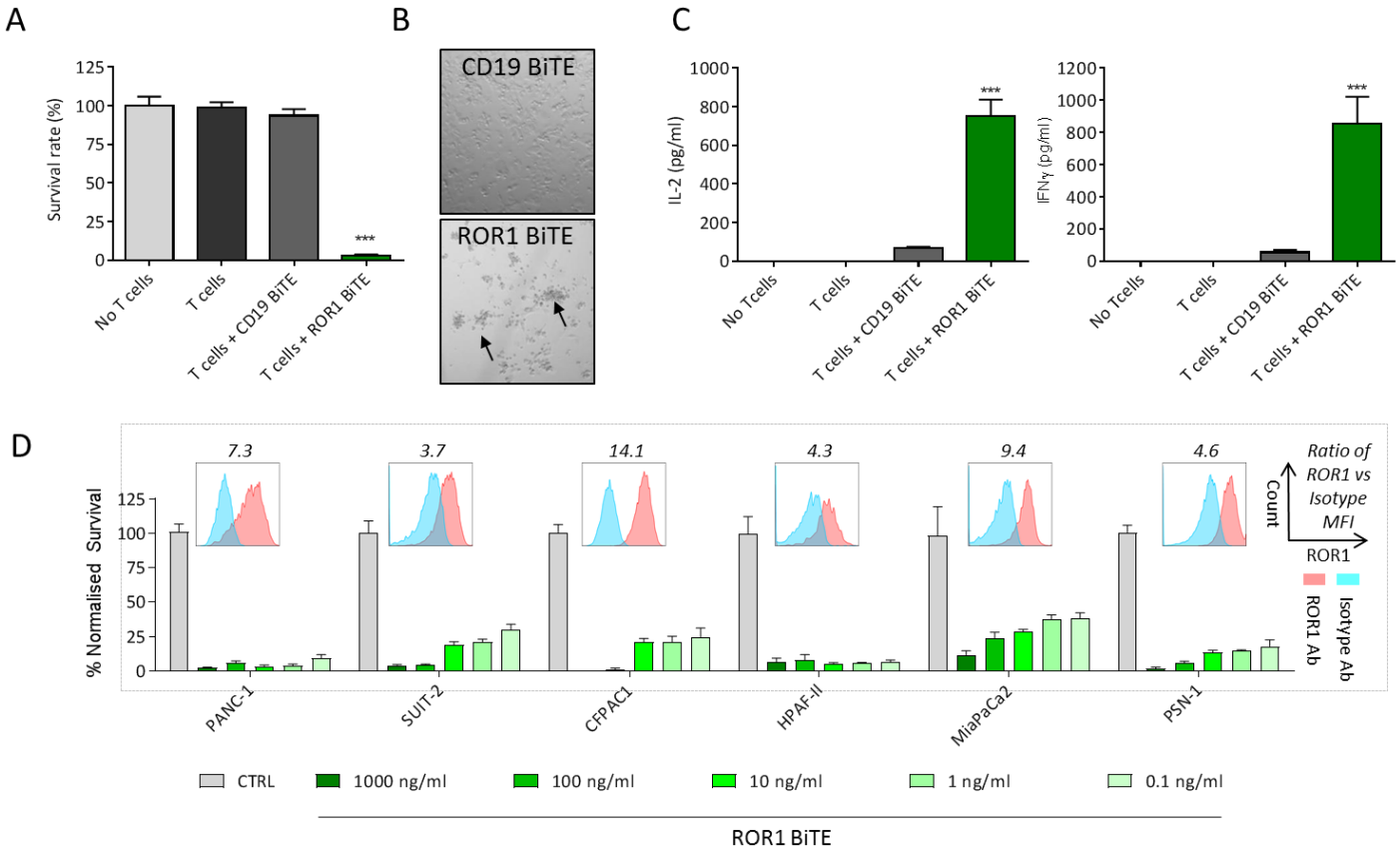


FIGURE 4.34 ROR1 BITE MEDIATES CYTOTOXICITY AGAINST A PANEL OF PANCREATIC ADENOCARCINOMA CELL LINES.

PANC1 cells cultured with T cells and ROR1 BiTE in a 1:1 effector to target ratio demonstrates A. significant toxicity compared to control BiTE. B. It is associated with T cell clustering and results in C. significant IL-2 and IFNγ secretion. D. A panel of pancreatic adenocarcinoma were cultured with T cells and ROR1 BiTE from 1000ng/ml to 0.1ng/ml, demonstrating cytotoxicity at low concentrations, but which did not correlate ROR1 expression levels (inset flow cytometry plots).

4.3.5.1.1 In vivo evaluation of ROR1 BiTE against PANC1 cell lines

We next undertook two distinct mouse models. In the first mice were injected intraperitoneally with PANC1 cells and human T cells along with CD19 BiTE or ROR1 BiTE. BiTE treatment was administered for 5 days intraperitoneally, with ROR1 BiTE treated animals showing a reduction in the bioluminescence signal at 1:4 and 1:2 target to effector ratio, compared with CD19 BiTE treated animals (Figure 4.33A).

We next undertook a more challenging subcutaneous model in which PANC1 tumours were established in the flank of mice until average volume were 100mm^3. Control mice were treated with a single infusion of T cells and 7 days of PBS or CD19 BiTE and compared with mice treated with ROR1 BiTE. There was a significant decrease in tumour size in ROR1 BiTE treated animals compared to control mice cohorts. At a longer follow up at day 28, ROR1 BiTE treated mice had smaller tumours due to a reduced growth rate.

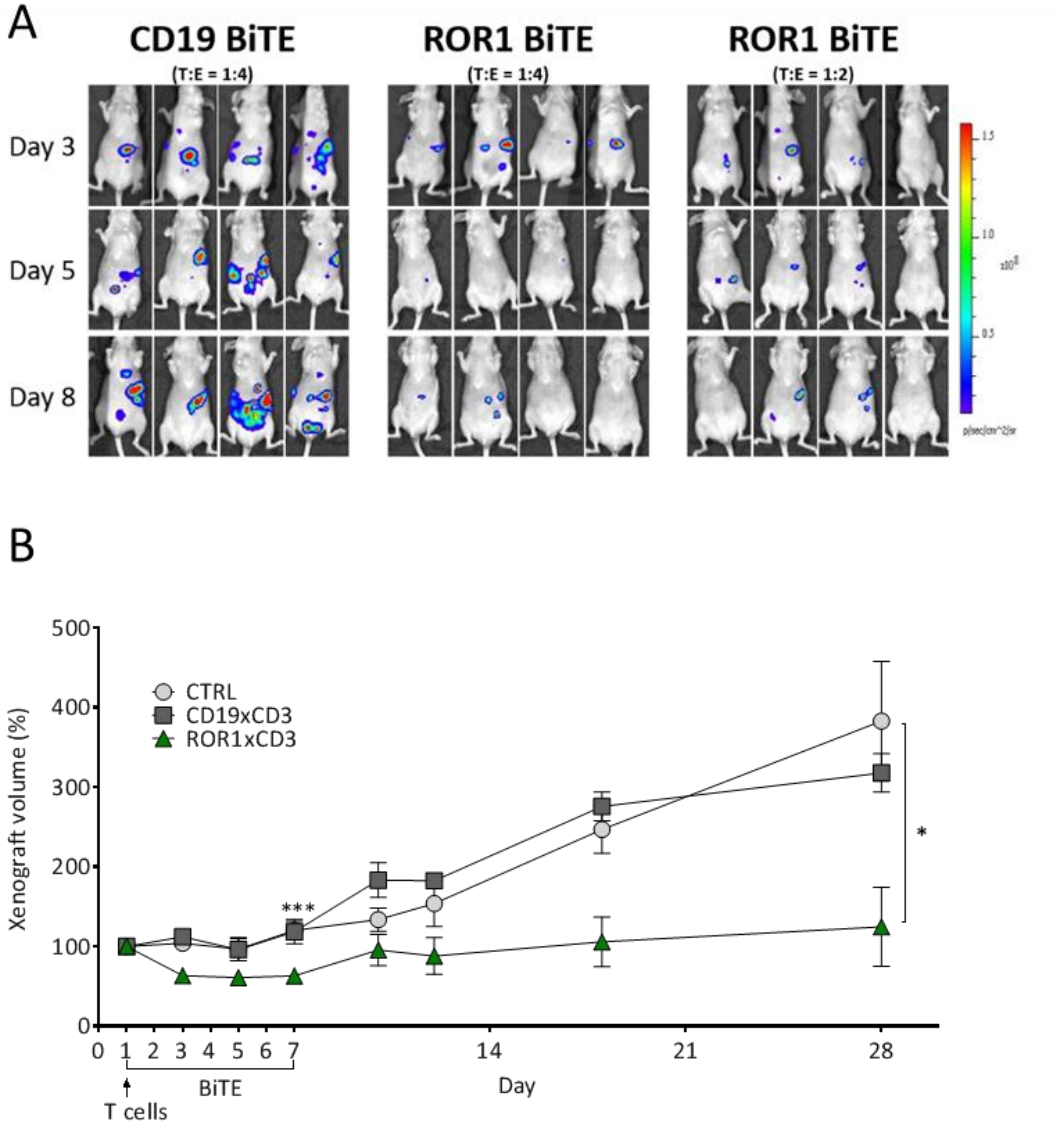


FIGURE 4.35 ROR1 BITE REDUCES TUMOUR BURDEN IN MURINE MODELS OF PANCREATIC ADENOCARCINOMA

A. Intraperitoneal Engraftment: 2×10^6 PANC-1 luciferase cells were administered per athymic nude mouse followed by a single dose of purified human T cells injection intraperitoneally (8×10^6 T cells for the CD19 BiTE group; 8×10^6 and 4×10^6 T cells for the ROR1 BiTE groups to yield 1:4 and 1:2 target to effector ratio). BiTEs were injected daily at 10 µg/kg/mouse for 5 days and PANC-1 engraftment was assessed by in vivo bioluminescent imaging (BLI) with decreased BLI signals at day 8.

B. Established xenograft model: PANC-1 cell lines (5×10^6) were injected in the right flank of immunocompromised athymic nude mice and xenografts were established to a minimum size of 100 mm^3. Mice then received a single intravenous injection of purified T cells (5×10^6) and were treated with ROR1 BiTE, CD19 BiTE or PBS at 10 µg/kg/day intravenously daily for 7 days and the size of the tumour measured by a caliper. Follow-up of these animals at day 28 showed that ROR1 BiTE treated mice had lower tumour volumes compared with the control cohorts despite no additional ROR1 BiTE administration.

4.3.5.2 ROR1 Mediates cytotoxicity against a broad range of solid tumour cell lines

Having established proof of concept against pancreatic adenocarcinoma we next assessed breast and ovarian cancer cell lines. Although treatments for both of these has advanced, patients with resistant and metastatic disease have an unmet therapeutic need and may benefit from ROR1 targeted therapy.

We therefore assessed ROR1 expression in breast (MDA-MB-231 and MCF7) and ovarian (SKOV-3, HOC-7 and HEY) cell lines. We took advantage of the lack of expression of ROR1 on MCF7 cell lines to act as a control for non-specific activation and killing. Our ROR1 BiTE mediated significant toxicity of MDA-MB-231 cell line with resulting cytokine secretion but not the MCF7 cell line. Importantly, there was no significant cytokine secretion above background with co-culture of T cells and ROR1 BiTE in the absence of the ROR1 on the cell surface. Cytotoxicity and IFNγ and IL-2 secretion was also seen against SKOV3, HOC7 and HEY cell lines. An in vivo model using SKOV3 cells expressing firefly luciferase, injected intraperitoneally with T cells and ROR1 BiTE, demonstrated the potential to reduce disease burden in mice.

We next assessed cytotoxicity with ROR1 BiTE against hepatic origin cancers (SK-Hep-1 and HUH7), glioblastoma (U251 and A172), melanoma (T618A) and prostate cancer (DU145 and PC-3) and demonstrated significant cytotoxicity compared with CD19 BITE. As before target cell killing was associated with IFNγ and IL-2 secretion, but this was variable and non-related to ROR1 expression, as seen with CAR T cells.

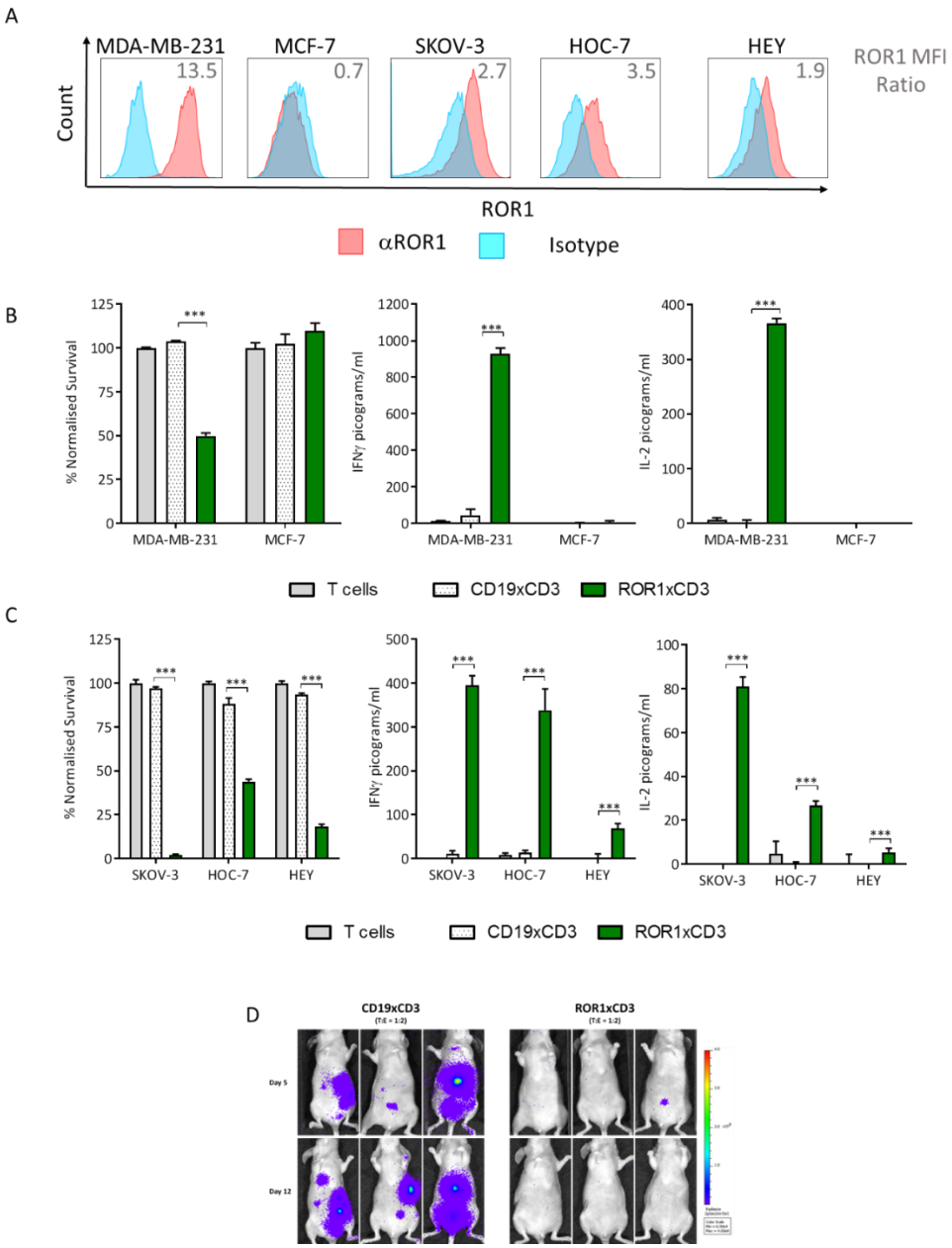


FIGURE 4.36 ROR1 BITE PROVIDES IN VITRO AND IN VIVO SPECIFIC CYTOTOXICITY AGAINST BREAST AND OVARIAN CANCER CELL LINES.

A. Flow cytometry staining of ROR1 on MDA-MB-231, MCF7 breast cancer cell lines and SKOV-3, HOC-7 and HEY ovarian cancer cell lines, compared with isotype control. Values represent fold increase in MFI value. B. Cytotoxicity and cytokine secretion against ROR1 positive MDA-MB-231 but not ROR1 negative MCF7 cell lines as assessed by a cell viability assay using 1 µg/mL ROR1 BiTE at 24 h compared with CD19 BiTE (1:1 Effector: Target ratio). C. Cytotoxicity and IFNγ secretion of ovarian cancer cell lines as assessed by a cell viability MTS assay using 1 µg/mL ROR1 BiTE at 24 h compared with CD19 BiTE. D. 5×10^6 SKOV-3 cells expressing firefly luciferase per mouse were administered intraperitoneally, followed by a single dose of 10×10^6 human T cells. BiTEs were injected daily at 10 µg/kg/mouse for 5 days. SKOV3.Luc engraftment was assessed by BLI.

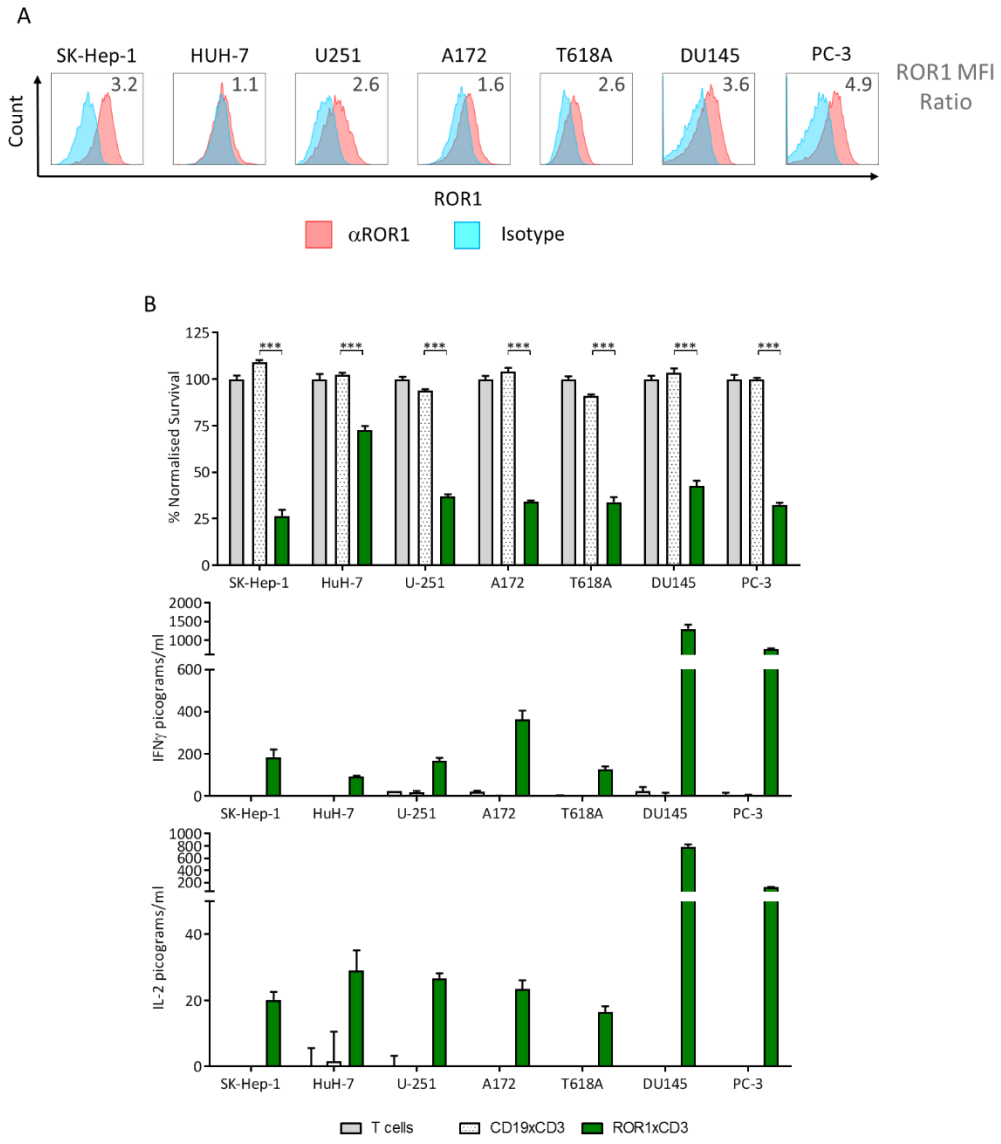


FIGURE 4.37 ROR1 BITE ENABLES TARGETING OF A RANGE OF TUMOUR SUBTYPES.

A. ROR1 expression was assessed on SK-Hep1, HUH7, U251, A172, T618A, DU145 and PC-3 cell lines representative of hepatic, glioblastoma, melanoma and prostate cancer; values within plots indicate fold increase of MFI compared to isotype control. B. Significant cytotoxicity against ROR1 positive cell lines compared with control CD19 BiTE (1:1 effector to target ratio; 1μg/ml BiTE).

4.3.6 Humanisation of our ROR1 BiTE

4.3.6.1 ROR1 scFv humanisation

The next step, as with the CAR, was to undertake humanisation of the ROR1 BiTE. Given that we were using clone F as our scFv we substituted the parental rat sequence with the 3 humanised binders identified earlier.

We confirmed that the hF(1x1) provided superior cytotoxicity compared to the other humanised constructs. This was not apparent at high concentration of BiTE (from 1000 to 100ng/ml) but became pronounced at 1ng/ml where mean survival for parental F and F(1x1) was 5.3% and 9.7% respectively compared with 78.4% and 105.8% for F(3x1) and F(4x1).

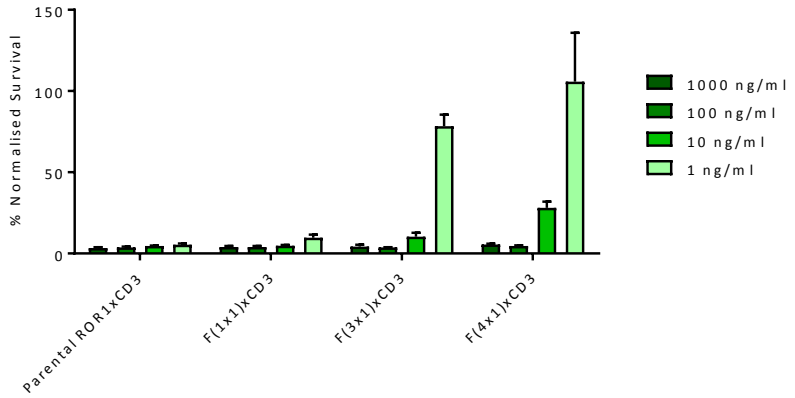


FIGURE 4.38 ASSESSMENT OF BITE CONSTRUCTS WITH HUMANISED AROR1 SCFV

The humanised clone F scFv constructs F(1x1), F(3x1) and F(4x1) were cloned into the ROR1 BiTE backbone with OKT3 CD3 scFv and produced in HEK293T cells followed by purification with HiTrap TALON gravity columns. Concentrations were normalised and cytotoxicity assessed against SKW6.4 cell line with T cell to target ratio of 1:1. The construct with humanised F(1x1) provided similar cytotoxicity to the parental clone, unlike clones F(3x1) and F(4x1), which although retained function at BiTE concentrations up to 100ng/ml, lost function at further dilutions.

4.3.6.2 CD3 scFv Humanisation

To maximise the utility of humanised the ROR1 scFv we next undertook humanisation of the CD3 scFv to further decrease immunogenicity. Genscript provided 5 humanised heavy and 5 humanised light chains based on the parental OKT3 antibody sequence. These were combined in a scFv format and 12 of the 25 constructs retained binding to CD3 in a secreted scFv format, as previously demonstrated (data not shown).

All 12 of these CD3 binders were transitioned into our BiTE format with hF(1x1) as the partner scFv. Supernatants were purified using gravity columns and concentrations normalised as before. Cytotoxicity was similar with all of these constructs compared to the parental OKT3 scFv for SKW6.4 and PANC1 cell lines. However, higher cytokine secretion was consistently seen with humanised constructs that included the light chain 5 sequence CD3 sequence (corresponding to nx5). Overall, the ROR1 BiTE containing the CD3(3x5) scFv resulted in higher cytokine secretion and was selected as our lead humanised CD3 binder.

The fully humanised ROR1 BiTE hF(1x1) and CD3(3x5), showed a similar binding profile to the parental construct. In an in vivo intraperitoneal model, it retained effector function as before.

Compared to the prior PANC1 in vivo intraperitoneal model, there superior tumour eradication was seen as assessed by bioluminescence imaging. However direct comparison was not possible as mice were treated with 3 injections of T cells and weekly BiTE compared with a single T cell administration and 5 consecutive days of BiTE in order to demonstrate the potential for enhanced tumour eradication, using a treatment regimen that would be more clinically relevant that just a single administration of BiTE.

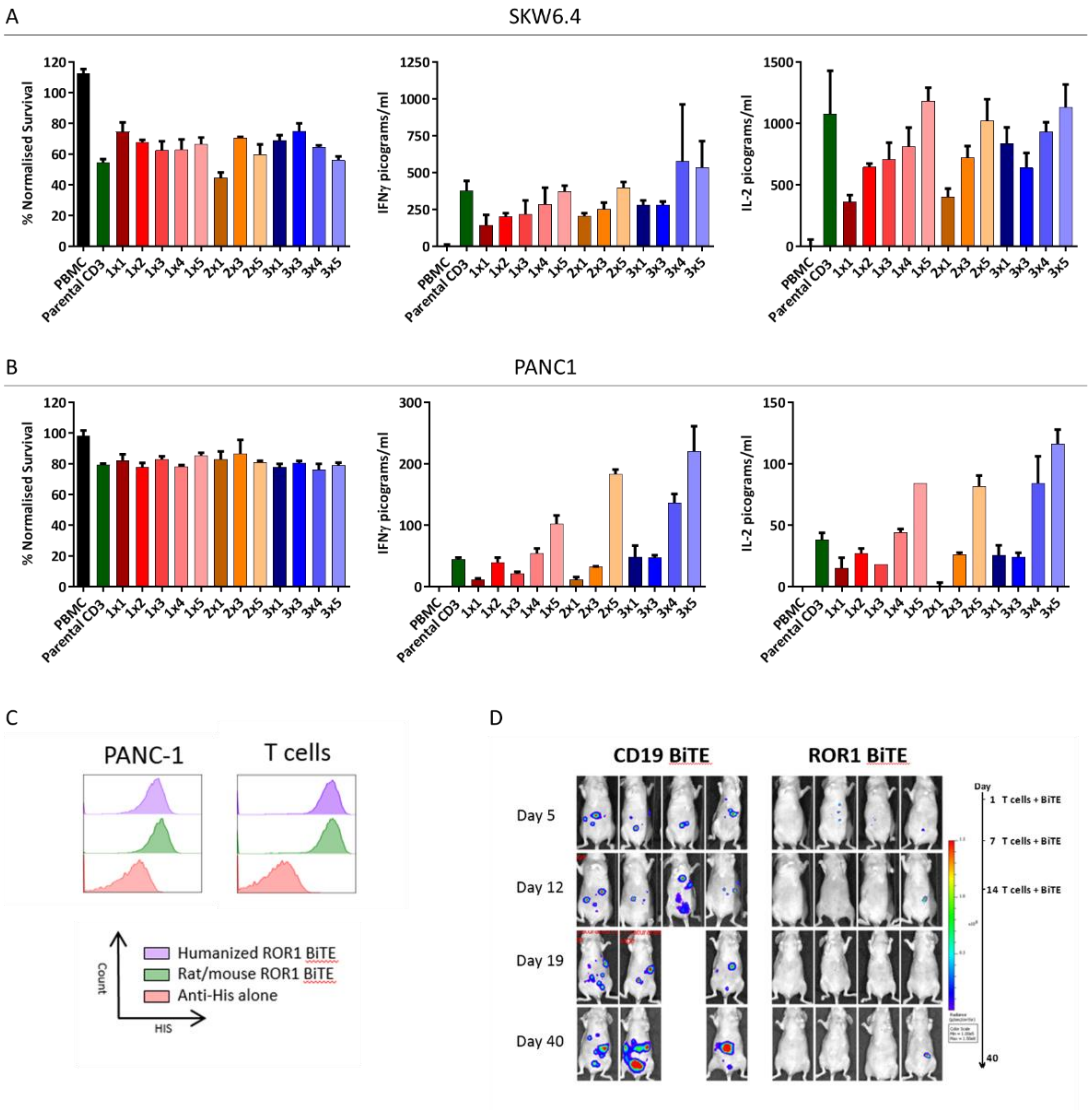


FIGURE 4.39 FUNCTIONAL ASSESSMENT OF HUMANISED CD3 SCFVS IN A FULLY HUMANISED ROR1 BITE

12 humanised CD3 scFvs were combined with our humanised hF(1x1) scFv to generate a panel of fully humanised ROR1 BiTEs. Following purification the concentration of BiTE was normalised to 5.5ng/ml and functional differences with regards to cytotoxicity and cytokine secretion assessed with SKW6.4 (A) and PANC1 (B) cell lines. Humanised constructs with light chain 5 showed higher cytokine secretion, with CD3(3x5) showing consistently higher secretion. C. Binding of the fully humanised hF(1x1)xCD3(3x5) BiTE was comparable with the non-humanised BiTE by flow cytometry at identical concentrations. D. Mice received 2×10^6 PANC-1.Luc cells/mouse intraperitoneally followed by three doses of purified human T cells (4×10^6). Humanised BiTE was injected once per week at 10 µg/kg/mouse and PANC-1.Luc engraftment was assessed by in vivo bioluminescent imaging.

4.3.6.3 Size Exclusion High Pressure Liquid Chromatography

Coomassie and western blotting demonstrated a single band at the correct size for the BiTE. Although this may represent a pure product, the reducing conditions means that we are unable comment on the formation of BiTE aggregates which can due to interactions of the scFv arms (Gil and Schrum, 2013).

To elucidate whether this was an issue, we undertook size exclusion high pressure liquid chromatography, through Bio-Analysis Centre, London. This demonstrated, at the highest concentrations of 10ug/ml there was a small peak suggestive of aggregation (Figure 4.40, depicted by * and enlarged view underneath), but this corresponded to less than 5% of the total BiTE. In addition, the main BiTE peak was preceded by a non-specific double peak. Here, the chromatogram trace for the BiTE runs overlapped with that of buffer alone and hence was a buffer artefact as opposed to aggregation.

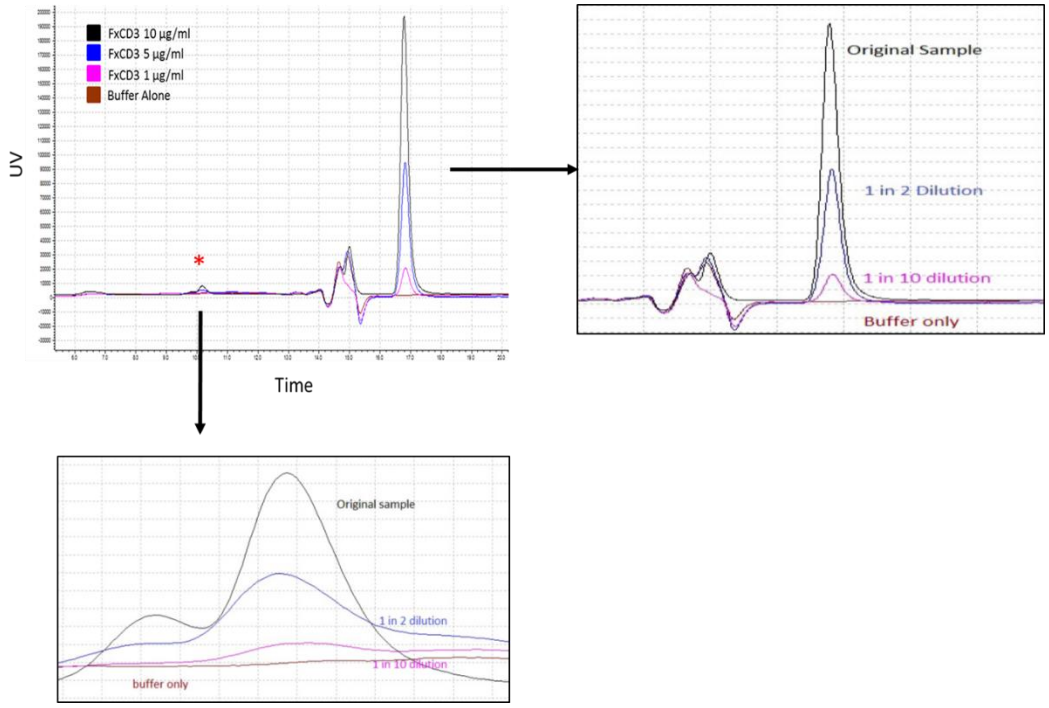


FIGURE 4.40 SIZE EXCLUSION CHROMATOGRAPHY DEMONSTRATES NO SIGNIFICANT AGGREGATION OF OUR ROR1 BITE

Purified fully humanised ROR1 BiTE was subjected to size exclusion HPLC at 10, 5 and 1 µg/ml and compared with buffer alone. There was a small aggregation peak (* and magnified in the lower panel, which corresponded to <5% of total protein at 10µg/ml. Assessment of the double peak before the main BiTE peak (magnified figure) overlap with the buffer alone, confirming this is not due to aggregation.

4.3.7 BiTE mediates significant cytotoxicity against haematological cell lines

Having demonstrated the potential to target a range of solid malignancy cell lines we next assessed function against haematological cell line. We confirmed cytotoxicity at low concentrations against SKW6.4, Jeko1 and Kasumi2 cell lines (Normalised survival 9.7%, 20.1% and 31.3% at 1ng/ml respectively).

In an in vivo model, Nod-SCID IL2$\gamma^{-/-}$ mice received SKW6.4 cells intravenously and were monitored until systemic disease established. On day 4 they were treated with a single dose of T cells and 5 days of ROR1 BiTE or control PBS. ROR1 BiTE treated animals demonstrated a significant survival advantage (Log Rank test, p=0.025).

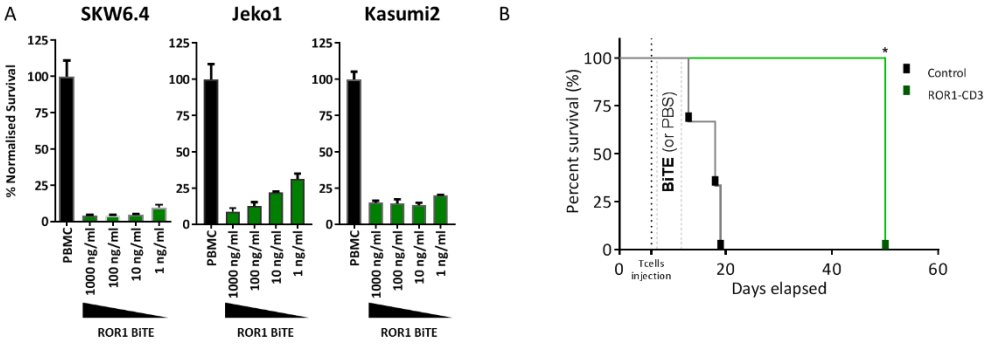


FIGURE 4.41 ROR1 BITE EVALUATION IN HAEMATOLOGICAL CELL LINES.

A. Co-culture of target cell lines with T-cells at a 3:1 effector to target ratio with ROR1 BiTE at various concentrations leads to significant cytotoxicity of SKW6.4, Jeko1 and Kasumi2 cell lines at 24 hours based on a FACS based killing assay. Data representative of at least 2 independent experiments. B. ROR1 BiTE improves survival in an in vivo model of disseminated B-cell leukaemia. $0.5x10^6$ SKW6.4 cells were engrafted in NOD SCID gamma mice for 4 days before infusion with 10^7 T cells and 5 days of ROR1 BiTE or PBS. Mice treated with ROR1 BITE had significantly enhanced survival compared to control mice (p=0.025, n=6 in total).

4.3.8 hF(1x1) mediates cytotoxicity in a BiTE format but not as a bispecific antibody.

BiTEs are small 55kDa proteins, freely filtered by the kidneys and therefore have a short half-life necessitating continuous infusions. Bispecific antibodies aim to harness the dual targeting properties of BITEs but in a conventional antibody format with an Fc stalk. These do however, rely on the correct pairing of heavy and light chain arms to ensure preserved and specific antigen binding, which can be a limiting feature of manufacture and purification.

A number of full bispecific antibodies in this format are being tested in clinical trials including Catumaxomab, which binds EpCAM and CD3 (Strohlein and Heiss, 2010, Heiss et al., 2010) and Lymphomun, which binds CD20 and CD3 (Schuster et al., 2015). These classify themselves as trifunctional antibodies as the Fc stalk allows recruitment of FcR bearing effector cells. Bispecific antibody manufacture has been made more commercially viable through the knobs-in-hole modification, which introduced mutations into each heavy chain CH3 domain to force hetero-dimerisation of the correct arms (Ridgway et al., 1996).

A simpler construct is the scFv-Fc format, here the individual scFvs are continuous with heavy chain CH2 and CH3 domains. Dimerisation occurs through disulphide binds between the heavy chains as well as through use of knobs in hole modification of the CH3 domain (Figure 4.42). The final scFv-Fc bispecific antibody has a molecular weight of 110kDa as opposed to 55kDa for the BiTE and 150kDa for a full antibody format.

We assessed whether the humanised scFvs we had generated for ROR1 and CD3 would function as a scFv-Fc. This was produced, purified and characterised by Absolute Antibody Limited. Functional comparison between the BiTE and the bispecific scFv-Fc antibody demonstrated that although the BiTE mediated significant cytotoxicity of SKW6.4 and Kasumi2 cell lines as previously, the bispecific scFv-Fc showed no appreciable cytotoxicity at the same molar concentrations, despite both arms binding to their respective targets (Figure 4.42). We hypothesise

that the bispecific scFv-Fc format inhibits T cell and target cell interactions due to inappropriate inter-cellular distance or secondary to a lack of flexibility that allows optimal binding to both antigens.

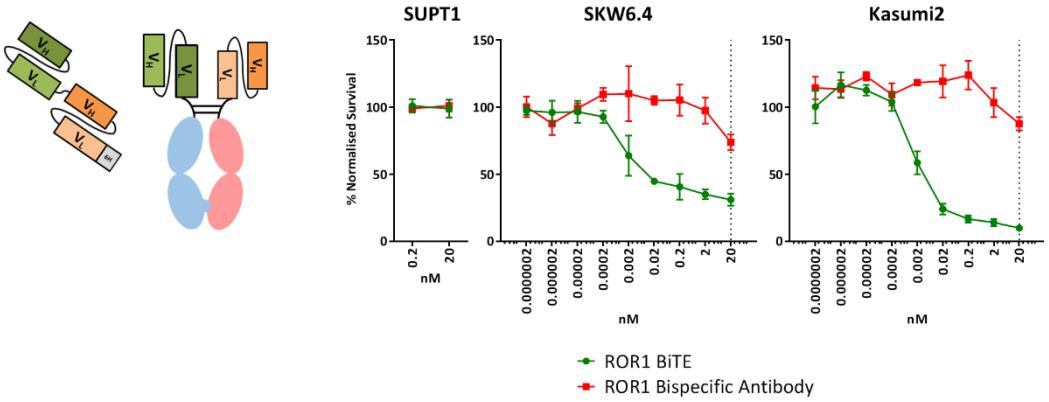


FIGURE 4.42 ROR1 BITE PROVIDES ENHANCED CYTOTOXICITY COMPARED WITH A ROR1 BISPECIFIC ANTIBODY IN THE SCFV-FC FORMAT

Structure of conventional ROR1 BiTE and the corresponding scFv-Fc bispecific antibody which utilises the same scFv sequences. Association of heterodimers occurs through disulphide bonds and knobs in hole modification of the CH3 domain. No off target cell killing was seen with ROR1 BiTE or bispecific antibody against SupT1 cells. ROR1 BiTE but not bispecific antibody mediated cytotoxicity of SKW6.4 and Kasumi2 cell lines with an IC50 of 0.002nM.Representative of 2 independent experiments.

4.3.9 Ibrutinib enhances ROR1 BiTE mediated cytotoxicity against primary CLL

We next assessed the potential of our ROR1 BiTE to target primary CLL cells, which provides a more challenging target compared to cell lines. Initial assessment with allogeneic healthy donor T cells, co-cultured with CLL cells in the presence of 1μg/ml ROR1 BiTE resulted in decreased CLL viability at 24 hours, with normalised survival of 32.3% (Figure 4.43A).

In comparison, when we used T cells isolated from CLL patients, we saw no cytotoxicity at 24 hours under identical conditions (Figure 4.43B). In these experiments, T cells were from patients with untreated CLL or from

relapsed/refractory patients with relatively high circulating lymphocyte counts, although we adjusted for this by using a constant effector to target ratio.

We hypothesised that the lack of ROR1 BiTE mediated killing was due to inherent T cell dysfunction. In CLL, this is multifactorial in origin and includes abnormal T cell synapse formation, increased levels of inhibitory signalling molecules and an exhausted phenotype (Riches et al., 2013, Ramsay et al., 2008, Brusa et al., 2013). It also explains the higher risk of infections and secondary malignancies (Morrison, 2009, Tsimberidou et al., 2009).

Ibrutinib, a first in class Bruton's Tyrosine Kinase (BTK) inhibitor, demonstrates impressive efficacy in front line, relapsed/refractory and p53 deleted patients (Byrd et al., 2014, O'Brien et al., 2014, Burger et al., 2015a). In addition to the direct anti-leukemic effect mediated by BTK inhibition, it modulates the tumour micro-environment, skews T cells to a Th1 phenotype, reduces PD-1 expression and reverses pseudo-exhaustion (Podhorecka et al., 2017, Yin et al., 2017, Niemann et al., 2016, Natarajan et al., 2016a, Natarajan et al., 2016b, Long et al., 2017). This may be in part due to the off target effects of Ibrutinib on other kinases, as the above T cell modulation is less apparent with the much more BTK specific Acalabrutinib (Long et al., 2017).

We therefore isolated T cells from patients receiving Ibrutinib for at least 3 months and cultured them with autologous CLL cells. This demonstrated significantly enhanced cytotoxicity compared to non-Ibrutinib treated patients (survival rate 43% vs 85% respectively, p=0.012) (Figure 2C). This cohort included patients who had failed multiple previous lines of therapy as those with 17p deletions. Ibrutinib treatment was associated with enhanced secretion of a range of pro-inflammatory T cell mediators including TNFα, IFNγ, Granzyme A and perforin (Figure 2D).

To ensure this effect was not secondary to enhanced CLL sensitivity, we analysed matched samples from the same patient pre and post Ibrutinib treatment. Post Ibrutinib T cells were able to kill leukaemic cells irrespective of whether the

CLL cells had been exposed to Ibrutinib, confirming improved T cell function was driving force of improved BiTE function (Figure 4.41E).

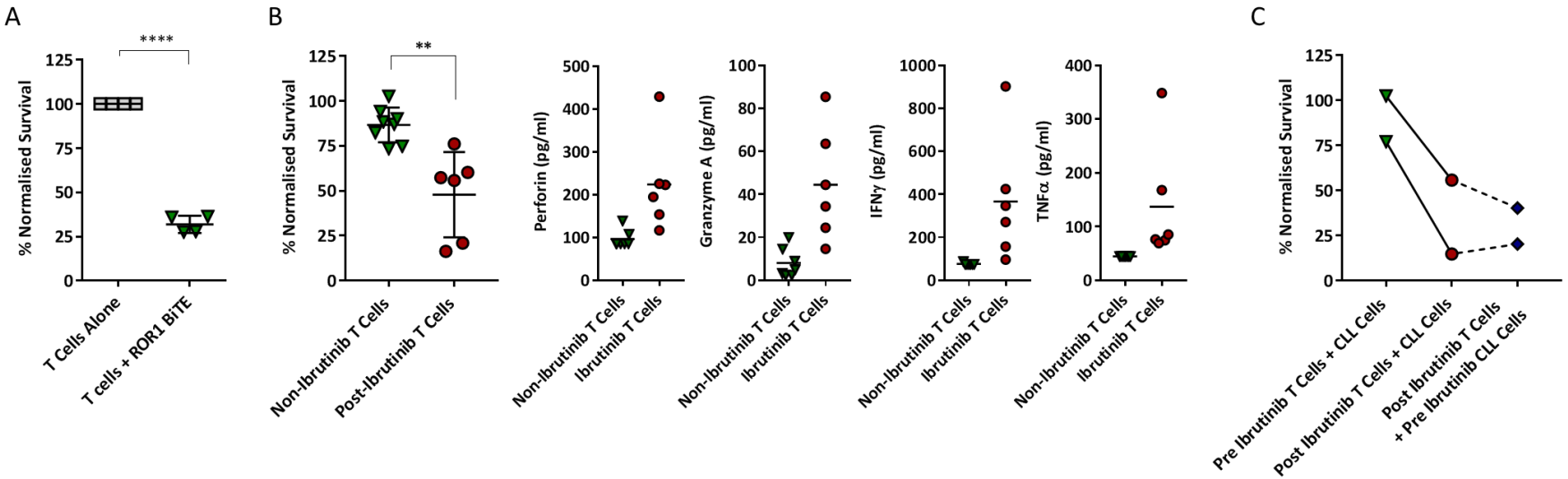


FIGURE 4.43 IBRUTINIB PRE-TREATMENT ENHANCES ROR1-BITE MEDIATED CYTOTOXICITY AND CYTOKINE SECRETION AGAINST PRIMARY CLL CELLS

A. Co-culture of primary CLL cells (n=4) with allogeneic healthy donor T-cells (3:1 effector to target ratio) and ROR1-BiTE at 1μg/ml results in marked cytotoxicity ($p<0.0001$ One sample t-test). Data representative of 3 independent experiments. B. T cells isolated from non-Ibrutinib treated patients showed minimal cytotoxicity when co-cultured with ROR1-BITE and autologous CLL cells (n=9) whilst T-cells isolated from patients on Ibrutinib and co-cultured with autologous CLL cells and ROR1 BiTE showed markedly improved cytotoxicity (n=6, $p=0.012$) and perforin, granzyme A, IFNγ and TNFα secretion. C. Matched pre and post Ibrutinib T-cells from the same patient were co-cultured with CLL cell isolated at the same time point. Ibrutinib treatment significantly improves ROR1 BiTE mediated cytotoxicity irrespective of the CLL cells exposure to Ibrutinib.

The above experiments were undertaken with high effector to target ratio of 3:1 which was fixed to allow for comparison between patients. This is certainly higher than would be encountered in patients and to better define if our ROR1 BiTE retained function with a more physiological T cell and target cell numbers we isolated PBMCs from a patient with CLL but did not undertake T cell and CLL separation. Despite the patient being on Ibrutinib for over 6 months 47.4% of PBMCs were CD19+CD5+ leukaemic cells and expressed weak levels of ROR1. CD3+ T cells accounted for 26.58% of the total. Addition of ROR1 BiTE to cultures without further manipulation demonstrated dose dependent cytotoxicity at 24 hours. Although preliminary, this has to be further corroborated with a larger cohort of samples from patients on Ibrutinib, with a more focused assessment of T cell phenotype.

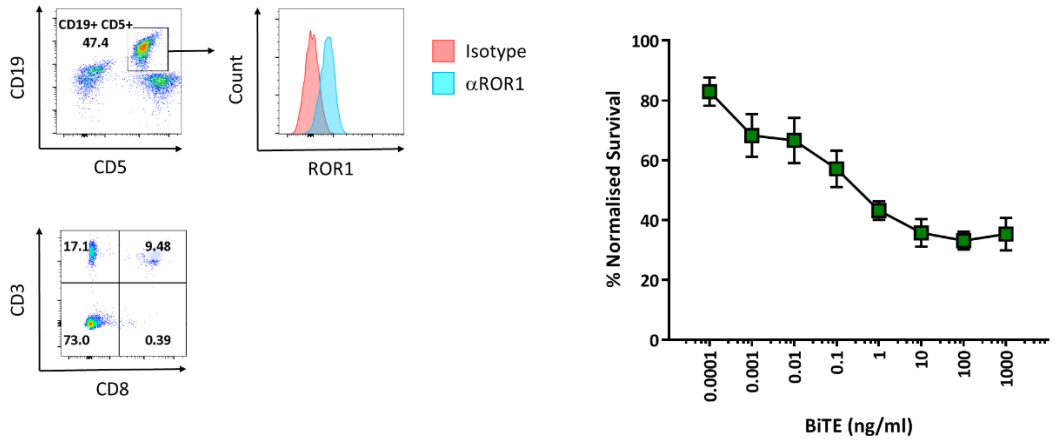


FIGURE 4.44 ROR1-BITE RETAINS FUNCTION AT PHYSIOLOGICAL A EFFECTOR TO TARGET RATIOS

PBMCs from a patient with CLL who had been on Ibrutinib for greater than 6 months were isolated and characterised and demonstrated 47.4% residual CLL at this time point (CD19+CD5+ cells), which were weakly ROR1 positive. CD3+ T cells accounted for 25% of the total PBMC population with 17.1% CD4+ and 9.5% CD8+. Addition of ROR1 BiTE from 1000ng/ml to 0.0001ng/ml demonstrated effective cytotoxicity at a T cell to CLL

Given the immunosuppressive nature of the CLL, especially in untreated patients or those with high burden relapsed disease, the use of Ibrutinib to de-bulk disease and reset T cell function allows a rationale context to maximise efficacy with BiTE therapy. In addition, although we had planned to undertake a comparison

of Blinatumomab against our humanised ROR1 BITE but due to limited access have been unable to undertake this yet.

4.3.10 Immunohistochemistry (IHC) using clone F antibody

Targeting of ROR1 with an affinity matured antibody has been proven safe in patients with CLL and ROR1 CAR T cells incorporating the R12 scFv showed no toxicity in non-human primates. The R12 scFv has been transitioned to a clinical trial targeting a range of malignancies, including CLL.

Given the unique nature of our scFv in terms of the epitope it binds, it is essential we exclude non-specific binding. We therefore undertook immunohistochemistry in collaboration with Dr Claudio Sorio at the University of Verona, who has provided the images for this section of the thesis.

A full conventional antibody with the same variable regions as in the humanised hF(1x1) scFv, as used in both the CAR and BiTE, was generated by Absolute Antibody Limited with a murine IgG1 Fc stalk. IHC against a panel of normal tissues as well as pancreatic cancer was undertaken with this but was limited by quality of the tissue microarrays. We nevertheless demonstrated strong staining in pancreatic cancer samples, which was not seen in normal pancreas, with the exception of pancreatic islet cells, as previously reported (Balakrishnan et al., 2017). Extended staining against normal human tissues comprising brain, kidney, heart, liver and lung showed no staining but some staining was seen in stomach tissue, which is again in keeping with Balakrishnan et al.

In order to progress this we are in discussion with Propath limited to undertake a tissue cross reactivity study with our ROR1 antibody, comprising hF(1x1) to better define any non-specific binding.

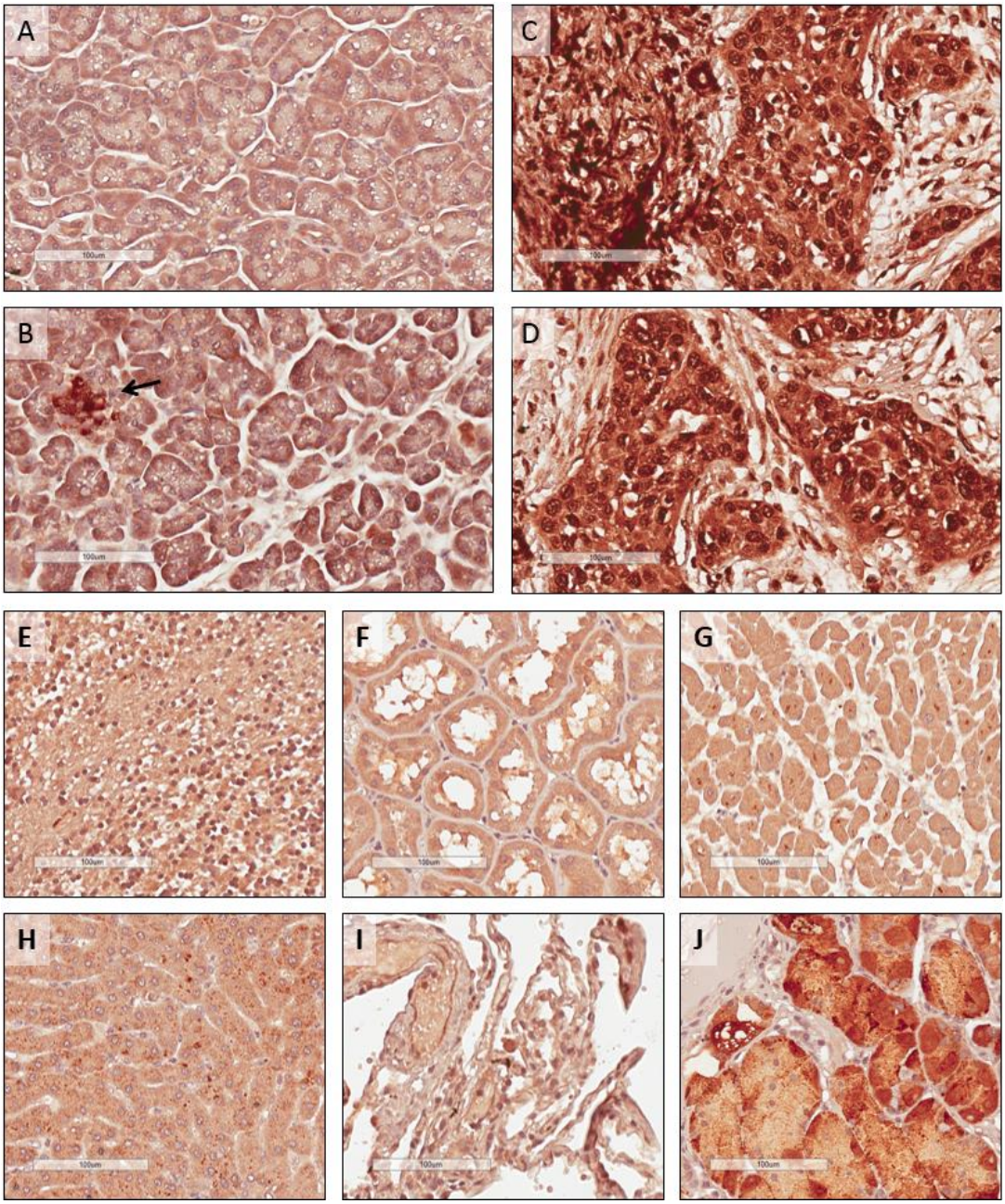


FIGURE 4.45 IMMUNOHISTOCHEMISTRY STAINING OF HUMAN TISSUES.

Tissue microarrays were stained with anti-human ROR1 antibody with the same specificity and antigen binding arms as the BiTE. (A) and (B) Normal pancreas, with pancreatic islet cells highlighted (arrow); (C) and (D) pancreatic cancer from two independent patients; (E) normal brain; (F) normal kidney; (G) normal heart; (H) normal liver; (I) normal lung, and (J) normal stomach. Scale bars: 100 µm.

4.3.11 BiTE Section Discussion

4.3.11.1 ROR1 BiTE generation and assessment

Within this section of work we have detailed how we have generated a ROR1 BiTE for the treatment of solid and haematological malignancies. It functions both at a low concentrations and at a low effector to target ratio, leading to cytotoxicity and resultant pro-inflammatory cytokine release in vitro and in mouse models. We developed a simple FPLC protocol using an imidazole gradient resulting in reproducible purification process with the final product not demonstrating significant levels of aggregation by size exclusion HPLC, a pre-requisite for clinical translation.

As per CAR T cells, the distance between target and effector cell, as mediated by the BiTE, is critical for optimal effector function. A BiTE targeting the frizzled domain demonstrated superior cytotoxicity than those targeting the more membrane distal immunoglobulin domain. The rat immunisation programme did not unfortunately generate any antibodies specific for the kringle domain, although it would have been of interest to assess the function of a BiTE targeting this most membrane proximal domain.

Other strategies to enhance BiTE function include modification of the linker between each scFv arms. We utilised the GGGGS linker but we have seen improved function with other BiTE constructs with longer linkers, such as $(GGGGS)_3$ (data not shown) and deserves consideration.

We substituted the parental rat scFv with the humanised binders generated earlier and demonstrated the hF(1x1) provided comparable cytotoxicity, even at low concentrations. This was therefore selected as the humanised scFv for the ROR1 arm of the BiTE and it is likely that the distance we generated between effector and target cell was key to efficacy.

To fully humanise the BiTE, we tested a panel of 25 humanised CD3 scFv sequences based on OKT3, of which 12 bound. Head to head assessment led to a lead candidate, CD3(3x5), which showed comparable cytotoxicity but enhanced

cytokine secretion. This fully humanised BiTE retained in vivo function in mice, but one limitation of this statement, was the lack of comparison between the fully humanised BiTE and the BiTE containing the murine OKT3 scFv. Biacore SPR assessment of these different CD3 scFvs would also add valuable information as to whether the humanisation process had modified the affinity and accounted for the higher cytokine secretion seen.

We utilised the OKT3 scFv based on the availability of the sequence, the lack of intellectual property restriction associated with its use and the ability to activate T cells. It is likely that the final Blinatumomab construct does not utilise the OKT3 sequence, but one which balances affinity with T cell activation. An early report with a CD19 BiTE, which may not be representative of Blinatumomab demonstrated a relatively low affinity for CD3 and a higher affinity for CD19 (2.6×10^{-7} M and 1.49×10^{-9} M respectively as assessed by flow cytometry Scratchard analysis rather than SPR (Dreier et al., 2002). It is thought that the lower affinity of the CD3 arm allows T cells to receive serial triggering of CD3, thus leading to activation. It may also explain how a single T cell can engage multiple target cells with BiTEs (Hoffmann et al., 2005).

Our humanised scFv has an affinity of 7nM, which is comparable to the affinity of the anti-CD19 fmc63 scFv of around 5nM (Sommermeyer et al., 2017), although both of these are much lower than that reported for Blinatumomab. To assess whether increasing the affinity of hF(1x1) would allow enhanced function we require a panel of antibodies that target the same epitope, maintain specificity and have a higher affinity. This can be achieved by screening phage display libraries against the known epitope of clone F or undertaking error prone PCR or alanine substitution to generate a high affinity variant although the latter two options have no guarantee of success and have to be screened to ensure no loss of specificity.

A further iteration would be the identification of a panel of novel CD3 scFvs through phage display, which are able to activate T cells and have differing affinities to assess the optimal configuration of ROR1 and CD3 scFv with in vitro and in vivo function and specificity. Getting these correct is important, as a bispecific antibody targeting C-type lectin-like molecule-1 and CD3 demonstrated higher levels of in

vitro cytotoxicity with a very high affinity CD3ε binder, but significant spontaneous T cell activation and resultant toxicity in non-human primates (Leong et al., 2017).

Our BiTE demonstrated no autonomous activation and cytokine secretion of T cells in in vitro in cultures. Importantly we saw no toxicity associated with human T cell and ROR1 BiTE administration in mice, despite high doses of both being used and the ability of clone F to cross react with murine ROR1 based on the target epitope being shared between humans and mice.

4.3.11.2 Other Bispecific ROR1 Formats

One of the limitations of BiTEs is their relatively short half-life, necessitating continuous infusions. This however, introduces an inherent safety advantage in that ceasing infusion limits adverse events due to rapid clearance of circulating BiTE. Modification of the BiTE through pegylation or addition of albumin binding moieties are strategies to increase the half-life (Kontermann, 2009). Another strategy is the use of bispecific antibodies, which incorporate an Fc stalk and also bring into play FcR receptor bearing immune cells such as NK cells.

We assessed whether our humanised hF(1x1) and CD3(3x5) scFvs function in a more antibody like configuration as a scFv-Fc. Although binding to each target antigen was retained, the scFv-Fc format did not show demonstrable cytotoxicity against cell lines. There are a number of potential causes for this, including the distance introduced on the scFv-Fc format creates a suboptimal distance between target and effector cell, or alternatively the presence of the Fc domain inhibits interactions between the cells. Substitution of the Frizzled binding clone F with one of the Immunoglobulin binding constructs, may have led to enhanced function in this format but was not tested due to the complexity of manufacture of these antibodies.

A range of other antibody constructs have been designed to bring T cells and target cells in close proximity and include **d**ual **a**ffinity **re**t**a**rgeting antibodies

(DARTs) (Johnson et al., 2010). DARTs are similar to BiTEs but differ in that the variable domains of heavy and light chains of the 2 antigen binding arms are on 2 separate polypeptide chains which interact covalently, with further stabilisation through a C terminal disulphide bridge (Figure 4.46) (Rader, 2011).

A CD19 DART has demonstrated superiority over a corresponding CD19 BiTE in vitro (Moore et al., 2011) but this has not been transitioned to clinical trials. A CD123 DART, has demonstrated in vivo eradication of AML blasts in mice, eradication of CD123+ cells in non-human primates (Chichili et al., 2015) and has been safety administered in a Phase I clinical trial (NCT02152956). The DART format was created to overcome limitations of commercialising BiTEs due to patents filed by Micromet, which was subsequently acquired by Amgen.

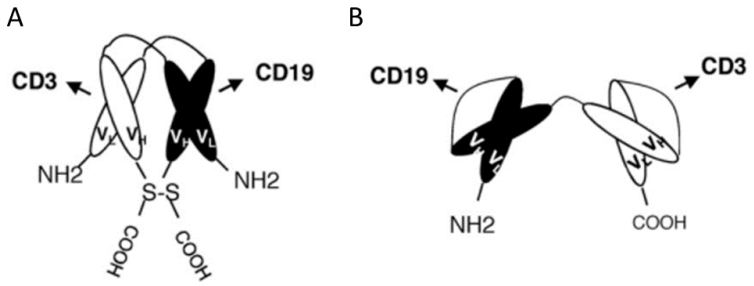


FIGURE 4.46 SCHEMATIC OF DART VS BITE

A. Schematic of a CD19 DART, in which two separate proteins interact through covalent hetero-dimerisation to generate a bispecific binding protein in comparison with B. A CD19 BiTE which is composed of a single protein chain. From (Moore et al., 2011).

A ROR1 DART, combining an ROR1 scFv with a CD3 scFv along with a Fc domain to extend half-life has been generated by Macrogenics and demonstrates in vitro and in vivo efficacy across a range of tumour subtypes including against the PC3 cell line we used in our solid malignancy panel (Barat et al., 2016). Direct comparison of our BiTE and the DART is difficult, due to differences in the scFvs, additional Fc domain and the cell lines used. However, as BiTEs and DARTs are similar in size and structure, combined with our finding that targeting frizzled give

superior cytotoxicity a comparison of the R12 DART and hF(1x1) DART would make an interesting comparison.

4.3.11.3 ROR1 BiTE mediates cytotoxicity against CLL in Ibrutinib treated patients

Assessment of primary CLL cells is difficult due to inherent cell death ex vivo, especially in the absence of feeder cells and exogenous cytokines that provide a favourable microenvironment (Collins et al., 1989, Burger et al., 2000).

An initial CD19 BiTE and subsequently clinical grade Blinatumomab have both been tested against primary CLL (Wong et al., 2013, Loffler et al., 2003). Within these assays BiTE was added to peripheral blood mononuclear cells containing both CLL and effector cells, at effector to target ratios comparable to peripheral blood. Functional cytotoxicity readouts were undertaken 5-10 days later, thus representing prolonged co-cultures. We assume earlier time points were not reported because evidence of cell death would have been limited. This would have been because of a disadvantageous effector to target ratio combined with impaired T cell function. Therefore, although these studies demonstrated cytotoxicity, these results have to be taken in relation to the high rates of spontaneous cell death at this time point, especially as the mechanism of BiTE activation and resultant cytotoxicity is rapid (Dreier et al., 2002).

We chose to assess purified T cells and CLL cells in a fixed ratio to overcome variability introduced by differing effector to target rations, although we note that the purification process may have affected T cell function. We demonstrated healthy donor T cells were able to mediate cytotoxicity against CLL cells but T cells from patients were not. Although these patients had CLL, the majority were Binet stage A patients and in otherwise good general health implying a direct role of the immunosuppressive nature of CLL in limiting ROR1 BiTE mediated function (Riches et al., 2013, Ramsay et al., 2008, Brusa et al., 2013). We next demonstrated that Ibrutinib treatment normalises T cell function in terms of BiTE mediated cytotoxicity and cytokine secretion, with this effect being T cell dependent. We initially using a

higher effector to target ratio of 3:1 to make this assessment comparable with the non-Ibrutinib samples but we have also demonstrated retained effector function at physiological levels of CLL and T cells in a patient pre-treated with Ibrutinib. This is being expanded upon in a wider cohort of Ibrutinib treated patients to ensure reproducibility.

Although Ibrutinib monotherapy has resulted in improved outcomes for relapsed/refractory and newly diagnosed patients, with prolonged responses for a number of years, it rarely leads to disease eradication in the form of MRD negativity. This is not associated with a poor responses, as disease burden can improve slowly with time (Barr et al., 2016).

This provides insight into the optimal sequencing of BiTE therapy for CLL. Based on the above ROR1 BITE administration in untreated patients or those with high disease burden with suboptimal T cell function will lead to inadequate responses, whilst treatment following Ibrutinib would be the ideal context to utilise improved T cell function to eradicate residual disease and aim for a MRD negative status. In addition, regulatory T cell numbers correlate with function of Blinatumomab in ALL, with a cut off of 8.525% FoxP3+ T cells discriminating responders from non-responders (Duell et al., 2017) and Ibrutinib decreases the ratio of Treg cells in CLL patients (Long et al., 2017). A study investigating combined use of Ibrutinib and Blinatumomab in relapsed/refractory ALL is ongoing (NCT02997761).

Ibrutinib is not the only pharmacological agent that can reverse T cell dysfunction in CLL, with lenalidomide also demonstrating repair of the immunological synapse and ability to mount immune responses (Ramsay et al., 2008, Lapalombella et al., 2010) Investigation of T cell function with ROR1 BiTE in this patient cohort is also of interest, although high rates of toxicity with its use in CLL patients may preclude future combination therapy with BiTEs (Chanan-Khan et al., 2017)

Beyond CLL, Ibrutinib is being investigated in solid malignancies as it inhibits mast cells, decreases fibrosis and alters the stroma in pancreatic cancer with beneficial effect in mouse models (Masso-Valles et al., 2015). A trial of Ibrutinib in

combination with chemotherapy for pancreatic cancer is recruiting patients currently (NCT02562898) and could be combined with ROR1-BITE therapy in the future.

Overall, the use of ROR1 (or CD19) BiTE post Ibrutinib represents the clinical context best suited to test their efficacy within CLL and other haematological malignancies.

4.3.11.4 ROR1 BiTE for solid malignancy

We have demonstrated that our ROR1 BiTE can mediate cytotoxicity of a range of solid tumour cell lines including pancreatic cancer, breast cancer and ovarian cancer. We demonstrate in vivo ability to reduce disease burden and limit tumour growth but at a fixed and relatively high BiTE dose. These models could be further refined with the use of lower concentrations to delineate dose response assessment.

Nevertheless, the combined data provides proof of concept of this approach and is in keeping with other BiTEs that target solid tumour antigens including EpCAM (English et al., 2015, Cioffi et al., 2012, Salnikov et al., 2009), CEA (Osada et al., 2010) and PSMA (Friedrich et al., 2012).

Despite this, efficacy of BiTEs in solid malignancies has been lacking even when safely administered and tolerated, such as with the CEA BiTE (Pishvaian et al., 2016). Interestingly, with regards to EpCAM, Catumaxomab a trifunctional, full bispecific antibody has been licensed for the treatment of malignant ascites (Berek et al., 2014), whilst Solitomab, the EpCAM BiTE equivalent, has had development paused. It is the context of ascites and intra-peritoneal treatment that is of interest as detectable responses against solid lesions have not been seen. BiTEs also have a theoretical advantage of better tumour penetration given their smaller size compared to antibodies, although this has not been conclusively demonstrated.

As with CAR T cells, BiTE function can be enhanced with checkpoint inhibitors, as demonstrated with a CEA targeting BiTE (Osada et al., 2015). In addition PDL-1

upregulation has been seen as an escape mechanism following Blinatumomab (Köhnke et al., 2015), with non-responders having higher levels of PD-1 (Duell et al., 2017). A number of clinical trials with checkpoint inhibition with Blinatumomab are underway (NCT03160079 & NCT03160079).

A potential method to circumvent current limitations is through the synergistic use of ionising radiation, to induce immune cell infiltration, reprogramme the tumour micro-environment and allow enhanced function with immunotherapeutics, including BiTEs and CAR T cells (Weichselbaum et al., 2017).

Other methods to enhance BiTEs include the transduction of T cells to deliver BiTE to tumour targets, with the rationale of the BiTE simulating both the transduced T cell and bystander T cells, whilst also driving enhanced mRNA expression of the BITE following activation (Velasquez et al., 2016, Iwahori et al., 2015). Oncolytic viruses can also be modified to deliver BiTEs to combine viral induced cell damage with local production of BITE (Freedman et al., 2017).

Taken together, our ROR1 BiTE represents a novel therapeutic for a range of solid tumour subtypes, but to ensure successful clinical translation, the context in which they are used is equally important. The FDA approval of Catumaxomab for malignant ascites, provides an interesting therapeutic indication in which to possibly test our ROR1 BiTE. More widely for solid tumours in general, combination with other modalities and agents will likely be needed to realise their potential but in the interim a phase I trial to assess safety and feasibility is the next step for targeting solid malignancies.

5 Overall Discussion & Future Work

ROR1 is an attractive therapeutic target due to its expression haematological and solid malignancies, making it a rationale choice for CAR T cell and BiTE therapy. More importantly ROR1 expression in solid tumours correlates with aggressive disease and an invasive phenotype and thus self-selects for high risk patients, who have limited therapeutic options. Expression on a cancer stem cell like cells, in ovarian cancer and glioblastoma, add to its appeal and is in keeping with its embryonic nature.

From an initial hybridoma library we have undertaken extensive pre-clinical evaluation of a novel humanised ROR1 CAR T cell and BiTE constructs to generate lead versions for each format. Within the CAR domain, this competes with the R12 CAR which is already being assessed in clinical trials, but ours has the advantage of being humanised as well as demonstrating enhanced cytotoxicity and cytokine secretion against a panel of cell lines. The different ROR1 domain hF(1x1) binds to along with the above functional differences warrant assessment in clinical trials to assess for safety and efficacy.

In addition our BiTE is the first published bispecific against ROR1 that functions as low effector to target ratios and low concentrations and similarly deserves assessment in clinical trials (Gohil et al., 2017).

These also compete with Cirmtuzumab, which is being investigated in CLL, with planned expansion in trials with Ibrutinib and paclitaxel combination therapy. The low antigen density on CLL, however precludes utility of using cytotoxic antibodies for CLL. As part of our screening process we assessed the ability our 13 ROR1 antibodies to mediate ADCC, CDC, direct cytotoxicity or internalisation but saw no evidence of this, thus highlighting a limitation of their use in an unarmed format (data not shown).

5.1 BiTE vs CAR T Cells

Although both CAR T cells and BiTEs utilise the potential of endogenous T cells, direct comparison between them is fraught with difficulty because of the different mechanisms by which they function. Despite this, a study using the same scFv in a BiTE and CAR format, demonstrated CAR T cells, specifically CD8+ subtype, have a greater sensitivity to low antigen targets compared with the corresponding BiTE (Stone et al., 2012). However this study had a number of limitations: firstly the affinity of both scFvs was not defined and both CAR and BiTE construct were not optimised in terms of extracellular spacer and affinity. In keeping with this a BiTE targeting the intracellular oncoprotein WT1, with a TCR mimic antibody converted into a scFv format, is able to recognise ultra-low copies of the antigen on target cells due to enhanced affinity for the target (Dao et al., 2015).

Proof of concept with both CAR T cells and BiTEs has been undertaken by targeting CD19 in haematological malignancies, with significant success leading to FDA approval of both. CAR T cells have seen major commercial interest with the formation of a large number of pharmaceutical companies keen to maximise on their potential. The eventual aim being incorporation of CAR T cell therapy as a standard of care, which is likely given current response rates and progress within the field. In relation to BiTEs, a number of companies and academic groups are developing differing bispecific T cell engaging antibodies, such as DARTs, to challenge the patent portfolio of Amgen, which can only expand their clinical use and offer wider therapy options.

Despite the potential and success of CAR T cells there are significant logistical considerations associated with their wider use. T cell engineering requires large quantities of validated viral vectors, with associated production and qualification costs, especially for those based on a lentiviral platform as a stable producer cell line is not a viable option. In addition, the need to undertake leukapheresis can only

be undertaken at specialist units and manufacturing at central sites has associated shipping and processing requirements and costs. Novartis, in conjunction with the University of Pennsylvania have gone some way in demonstrating the feasibility of this approach with the ELIANA trial, which included central manufacturing unit, with worldwide shipping not seeming to affect response rates (Buechner et al., 2017). The cost of their CAR T cell product, known as Tisagenlecleucel (CTL019) is approximately $475,000 per infusion, whilst Yescarta, the CD19 CAR generated by Kite Pharma costs $373,000. This compares with $178,000/year for Blinatumomab when initially launched.

Apart from lower costs, BITEs are suitable for large scale batch production allowing generation of an off the shelf product which is not patient specific. There is no need for associated T cell isolation, vector production and manipulation, therefore reduces costs and expands potential therapy.

CAR T cells raise the possibility of achieving long term disease control following a single infusion of T cells, due to persistence of the CAR T cell population. Although BiTEs can induce remission and MRD negativity, CAR T cells appear to provide a more durable response.

One common feature of both is the risk of CRS, which can be fatal and requires close clinical monitoring and early intervention with steroids, tocilizumab and multi-organ support. One benefit of BiTEs in this regard is their short half-life, with cessation of the infusion leading to rapid decrease in circulating BiTEs. Steroids are routinely used with BiTE administration to prevent CRS, with minimal loss of effector function but are not typically used with CAR T cells due to limiting effects on efficacy and persistence of the transduced population.

One current limitation of both CAR T cell and BiTEs is the disappointing responses seen with solid malignancies. Targeting ROR1 would allow for critical analysis of targeting the same antigen in haematological and solid malignancies to better define tumour and T cell factors that mediate response or resistance and initial assessment in phase 1 clinical trials to assess safety can be then built upon using additional synergistic therapies.

5.2 Further Preclinical Assessment

5.2.1 Immunohistochemistry

Although the expression of ROR1 has been documented, we need to ensure tissue binding with our scFv is as expected, which is especially true given our experience with clone A. To better address this we are in discussion with Propath Limited, to undertake a GLP qualified and FDA and EMEA compliant tissue cross reactivity study with both humanised hF(1x1) and CD3(3x5) as full antibodies. This comprises two phases, an initial optimisation phase to ensure reproducible staining and detection with the antibody followed by staining of good quality tissue sections and independent histopathology review to determine specificity.

For the EMEA the following tissues are required: Tonsil, thymus, lymph node, Bone marrow, blood cells, Lung, liver, kidney, bladder, spleen, stomach including underlying smooth muscle, intestine, pancreas, parotid, thyroid, parathyroid, adrenal, pituitary; brain, peripheral nerve, heart, striated muscle, ovary, testis, Skin and blood vessels (EMA/CHMP/BWP/532517/2008) and FDA panel which includes all target organs/tissues, bone, bone marrow, liver, spleen, adrenal, kidney, lung, heart, urinary bladder, gallbladder, thyroid, brain, gonads, gastrointestinal tract and adjacent organs of interest (FDA, 1997).

In addition to the above we are in collaboration with the Royal Marsden NHS Trust and St Jude Children's Hospital to undertake IHC on a range of haematological and solid malignancies respectively to add to the body of evidence with regards to ROR1 expression, especially with our antibody.

5.2.2 Murine and Non-Human Primate Studies

5.2.2.1 Patient Derived Xenografts

Our in vivo experiments were undertaken with cell lines, which may not necessarily represent the complexities of patient samples. This can be overcome in part by utilising primary human tumour samples which can be engrafted directly into animals which may better represent the original tumours (Siolas and Hannon, 2013). These do have their own inherent complexities and limitations (Stewart et al., 2017) but in vivo assessment with patient derived xenograft models would provide more robust evidence of the potential of ROR1 therapeutics both CAR T cells and BITES. We have investigated the use of these via the Jackson Laboratory PDX depository, EurOPDX and through ongoing collaborations.

5.2.2.2 Toxicology Assessment

The expression of ROR1 within the parathyroid, pancreatic islet cells and areas of the gastrointestinal tract is of concern and requires careful consideration and detailed assessment to demonstrate safety before translation to clinical trials.

Although ROR1 in developing mouse embryos has been assessed, its expression in adult mice is unknown. We plan to undertaken RT-PCR and western blotting from adult murine tissue to qualify murine ROR1 expression. If confirmed, we would substitute the CD3 scFv with an anti-murine CD3 capable of activating murine T cells and infuse this murine ROR1 BiTE into immunocompetent mice to assess toxicity. This would take advantage of the ability of clone F to bind to murine ROR1 as the target epitope is conserved between mice and humans.

The preclinical assessment of Blinatumomab utilised a similar approach with a murine specific CD3. The exact sequence of the anti-murine CD3 Amgen utilised is unknown, but was reported to have a similar affinity to that found in

Blinatumomab. Despite thus, administration of our BITE with high doses of human T cells demonstrated no toxicity in terms of behaviour, weight loss, skin changes or distress. An alternative to this, provided we demonstrate murine ROR1 expression, is the repopulation of Nod-SCID IL2$\gamma^{-/-}$ mice with a human haematopoietic system, in which we would simply infuse ROR1 BiTE to assess potential toxicity (Ishikawa et al., 2005).

Cirmtuzumab and R12 CAR T cells have been tested in non-human primates and the lack of toxicity in these studies is at odds with expression data and in vitro toxicity against adipocytes. This may be because Cirmtuzumab primarily functions by inhibition of signalling as opposed to mediating cytotoxicity, which therefore does not provide proof of safety with CAR or BiTE therapies. Furthermore CAR T cell administration in primates may drive only low level activation due to limited expression and therefore insufficient to generate a sufficiently pro-inflammatory state to drive expansion and subsequent toxicity, that may be encountered in patients with significant disease burden. The ROR1 CAR T cell clinical trial already recruiting patients will provide further safety information in this regard.

For the ROR1 BiTE, there is the added complication that the OKT3 CD3 scFv does not react with all non-human primate species, specifically Rhesus macaque, therefore limiting utility of testing in this group of animals. One potential non-human primate model to use is the Chimpanzee, in which OKT3 shows T cell activation, as was undertaken with Blinatumomab, but restrictions on testing in chimpanzees, along with the limited information that may be gleaned, negates the utility of this approach.

5.3 Manufacture

5.3.1 CAR T Cells

CAR T cells require viral vector production incorporating the transgene of interest which can be undertaken with academic partners (for example the viral

vector facility at King's College London), or through a range of commercial suppliers. In addition to the cost, it is the time scale required for production and qualification. Following this, any potential trial requires a manufacturing facility that is able to generate CAR T cells from the starting leukapheresis product and requires careful process development and qualification of standard operating protocols including for T cell activation, transduction, cell splitting, freezing and quality release criteria. In this regard a number of scale up runs would need to have been undertaken to test the processes and screen for any deficiencies. This production process may also be disease specific, as in CLL, large number of contaminating leukaemic cells may require alteration in methodologies which have to be properly accounted for. To start this process, we have successfully applied for and obtained NHS ethical approval to undertake leukapheresis from CLL patients to develop and validate a robust production process for CAR T cells pending confirmation of the final retroviral construct.

5.3.2 ROR1 BiTE

Once we are satisfied that we have the optimum scFv configuration based on affinity of the ROR1 and CD3 arm we can proceed to manufacture of the BiTE. Blinatumomab is produced in CHO cells before purification and lyophilisation, the latter to ensure stability and limit aggregation as per the EMEA assessment report (EMEA/H/C/003731/0000). Although we have included a hexa-histidine tag in our BiTE, there is no mention of this for Blinatumomab in the EMEA filing. We have explored a range of manufacturing options for the ROR1 BiTE from transient transfection to generation of a stable producer cell line in conjunction with NHS Blood and Transplant. We are currently developing a GMP compliant purification process utilising the His tag followed by downstream processing to place the BiTE into an optimised buffer followed by assessment of stability and aggregation over time. This will be undertaken in line with the EMA guidance (EMA/CHMP/BWP/532517/2008), which covers production, specification and characterisation of monoclonal antibodies.

5.4 Concluding Remarks

We have described how from an initial rat immunisation we have developed ROR1 CAR T cells and ROR1 BiTEs, both of which demonstrate efficacy in proof of principle assessments. CAR T cells and BiTEs gave unique advantages and limitations and it is likely that they will both need to be combined with other agents, such as checkpoint inhibitors to maximise their clinical efficacy, especially for solid tumours, as well as selecting the right patient group and providing treatment within the correct context.

Despite their appeal, careful consideration has to be made of normal ROR1 expression on healthy tissue to mitigate against on target off tumour toxicity. In this regards initial assessment with BiTEs to demonstrate safety of targeting ROR1 with our scFv, in a system which is cheaper and easier to translate to clinical trials, combined with the ability to start at exceedingly low concentrations is appealing. Additionally, this makes use of the inherent short half-life of BiTEs to minimise toxicity and allow gradual dose escalation to assess for safety.

Following on from this and provided there are no limiting toxicities, we would be much more confident of the ability to safely administer ROR1 CAR T cells, with the aim of generating long lived immune responses.

Overall the ROR1 BiTEs and CAR T cells we have generated warrant assessment in high risk patients with unmet therapeutic need and strategies to realise this in phase I clinical trials are underway.

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