Title of Thesis:

Development and Clinical Implementation of Next-Generation CAR-T cell Manufacture Methodology

Dr Maeve O'Reilly

UCL

MD (Res)

Maeve O'Reilly MD (Res)

'I, Maeve O'Reilly , 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.'

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Abstract

Maeve O'Reilly MD (Res)

Chimeric Antigen Receptor (CAR) T-cell therapies for haematological malignancy have revolutionised the treatment of patients with relapsed/refractory B-cell cancers. The scope of these therapies in other haematological and even solid cancers holds much promise for the future.

However, there are several major bottlenecks for delivery of these novel therapies: availability of academic clinical trials for CAR T-cell therapies is limited in the UK and in particular, the paucity of manufacturing slots means that the majority of potentially eligible patients cannot access these therapies. Streamlining the manufacturing process and pipeline to enhance manufacturing capability and scalability would begin to address this challenging issue. Secondly, a large proportion of patients referred for consideration of autologous CAR T-cell therapy are heavily pre-treated with chemotherapy and their T-cell populations for harvest are often numerically (and in some cases functionally) compromised. Referring patients early, prior to several lines of toxic chemotherapy would be optimal, but this is logistically challenging. Another approach would be to review apheresis practice and work to enhance T-cell collection at the front end.

Lastly, these new therapies are associated with a constellation of unique potential side effects. The clinical delivery of bespoke gene-modified T-cell therapies in routine Haemato-Oncology Units requires a paradigm shift in medical, nursing, pharmacy and stem cell lab care pathways.

This project undertakes to address these issues through the initiation and delivery of an academic study of autologous CD19 CAR T-cells for relapsed/refractory acute lymphoblastic leukaemia (B-ALL). This project will (1) investigate and establish a streamlined manufacturing platform for CAR T-cell manufacture for the UCL CAR T-cell programme; (2) explore the single-centre apheresis practice at UCLH to define a model/guideline for successful CAR T-cell apheresis and (3) to begin to establish the clinical infrastructure required to deliver these therapies 'routinely' at a large central London teaching hospital.

Impact Statement

The findings and insights of this thesis will have an impact on the field of T-cell Immunotherapy, both within academia at UCL and beyond. Through the completion of this project, we have firstly and most importantly demonstrated the feasibility of closed, semi-automated manufacture of CAR-T cells using the CliniMACS Prodigy®. We have demonstrated that the Prodigy® system represents a less labour-intensive and more cost-effective and scalable mode of manufacture. These results have prompted a change from the conventional open "manual" process to the Prodigy® system for all of our current and prospective academic CAR-T trials at UCL. This represents a major shift in practice. Through the completion of this project, we have demonstrated that a university institution with limited clean room

infrastructure (relative to a commercial company) can establish a scalable and decentralised manufacturing hub, thereby expanding our portfolio of early phase clinical studies, facilitating rapid translation of science to the bedside and increasing the availability of these therapies to patients at a lower cost. The insights from this project also has implications for commercial companies, companies who are driven by profit margins and achieving economies of scale. As the field of T-cell immunotherapy moves beyond haematological malignancies and demand accelerates, an optimised automated manufacturing platform becomes a prerequisite for successful delivery.

Secondly, our review of apheresis practice at UCLH represents a stepping-stone to streamlining our service for future patients. We have devised an algorithm using pre-procedure ALC and a CD3+ target yield in an attempt to personalise this procedure, hoping to minimise the risk of a failed harvest for the few, whilst perhaps shortening the apheresis procedure for many. This algorithm requires prospective validation but has the potential to overhaul our current practice. If validated in a larger cohort of patients, sharing this knowledge with other CAR-T centres via peer-reviewed publication would increase its impact.

Finally, the establishment of an Immunotherapy service in a large teaching hospital requires a paradigm shift in medical, nursing, pharmacy and stem cell laboratory care pathways and logistics. As one of the leading immunotherapy units in the UK, this work provides a guide for other prospective centres on the core requirements and the challenges of the implementation of such a service.

Acknowledgements

Maeve O'Reilly MD (Res)

A very special mention for my Dad, my biggest and unconditional lifelong supporter, and my two brothers, Michael and David.

A final thank you to the patients and their families, our UCLH trials team and all the UCLH staff who have helped us build and deliver our exciting new Immunotherapy service.

Abbreviations

AABB	American Association of Blood Banks
AC	Anticoagulant
AC	Ambulatory care
ACDA	Anticoagulant Citrate Dextrose Solution A
AITL	Angioimmunoblastic T-cell lymphoma
ALC	Absolute Lymphocyte Count
ALCL	Anaplastic large cell lymphoma
ALL	Acute Lymphoblastic leukaemia
ASBMT	American Society of Bone Marrow Transplantation
ASTCT	American Society for Transplantation and Cellular Therapy
ATIMP	Advanced Therapeutic Investigational Medicinal Product
AUC	Area under the curve
BBB	Blood brain barrier
BM	Bone marrow
BNP	b natriuretic peptide
BMR	Batch Manufacturing Record
BV	Blood volume
CAR	Chimeric antigen receptor
CCGTT	Centre for Cell, Gene and Tissue Therapeutics
CE	Collection Efficiency
CIBMTR	Centre for international blood and marrow transplant research
CLL	Chronic Lymphocytic leukaemia
CNS	Central Nervous System
COA	Certificate of analysis

CPF	Clinical practice facilitator
CR	Complete response
CRF	Controlled rate freezer
CRi	Complete response with incomplete count recovery
CRS	Cytokine Release Syndrome
CSF	Cerebrospinal fluid
CTA	Clinical Trial Authorisation
CTC	Clinical trials centre
CTP	Cellular Therapy Product
CV	Coefficient of variation
DLBCL	Diffuse Large B Cell Lymphoma
DLI	Donor Lymphocyte Infusion
DMSO	Dimethyl sulfoxide
EBMT	European Society of Bone Marrow Transplantation
EC	European Commission
ECG	Electrocardiogram
ECOG	Eastern cooperative oncology group
ED	Emergency department
EGFRVIII	Epidermal Growth Factor Receptor Variant III
EM	Environmental Monitoring
EMA	European Medicines Agency
ERS	Electronic Record System
FACS	Fluorescence-activated cell sorting
FACT	Foundation for the Accreditation of Cellular Therapy
FAP	Fibroblast activation protein
FBC	Full blood count
FBK	FACS-based killing
FCS	Fetal calf serum
FDA	Food and Drug Administration
GCP	Good clinical practice
GDP	Good Distribution Practice
GMM	Genetically modified material

GMP	Good Manufacturing Practice
GOSH	Great Ormond Street Hospital
HCV	Hepatitis C virus
HD	Healthy Donor
HIV	Human immunodeficiency virus
HLA	Human Leucocyte Antigen
HLH	Haemophagocytic lymphohistiocytosis
HSA	Human Serum Albumin
HSCT	Haematopoietic Stem Cell Transplant
HTA	Human Tissue Authority
ICANS	Immune Effector Cell-Associated Neurotoxicity Syndrome
ICH	Institute of Child Health
IDM	Infectious Disease Markers
IEC	Immune Effector Cell
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
IMPD	Investigational medicinal product dossier
IND	Investigational New Drug
ISCT	International Society of Cellular Therapy
ITU	Intensive care unit
IVIG	Intravenous immunoglobulin
JACIE	Joint Accreditation Committee ISCT Europe and EMBT
LN	Liquid Nitrogen
mAbs	Monoclonal antibodies
MAS	Macrophage activation syndrome
MDS	Myelodysplastic syndrome
MDT	Multidisciplinary Team
MGUS	Monoclonal gammopathy of undetermined significance
MHC	Major Histocompatibility Complex
MHRA	Medicines and Healthcare Regulatory Agency
MM	Multiple Myeloma
MNC	Mononuclear Cells

MOI	Multiplicity of Infection
MRD	Minimal residual disease
MSC	Microbiological safety cabinet
MUGA	Multiple gated acquisition scan
NHL	Non-Hodgkins Lymphoma
NHS	National health service
NICE	National Institute for Health and Care Excellence
NIHR	National Institute for Health Research
NMDA	N-methyl-D-aspartate
NK	Natural Killer
NT	Non-transduced
NY-ESO-1	New York esophageal squamous cell carcinoma-1
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCNSL	Primary CNS lymphoma
PCP	Pneumocytis carinii pneumonia
PCR	Polymerase chain reaction
PD	Process development
PET	Positron emission tomography
Ph	Philadelphia chromosome
PI	Principal investigator
PIS	Patient information sheet
PK	Pharmacokinetics
PM	Patient-derived material
PMBCL	Primary Mediastinal B cell Lymphoma
PRO	Patient reported outcomes
PSF	Product Specification File
PTCL	Peripheral T cell lymphoma
QC	Quality Control
QM	Quality manager
QOL	Quality of life
QP	Qualified Person

RCV	Replication Competent Virus
RE	Relative error
REMS	Risk evaluation mitigation strategy
RN	Retronectin
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SD	Standard deviation
SOP	Standard Operating Procedure
TBV	Total blood volume
TCI	"to come in"
TCR	T- Cell Receptor
TCT	T-Cell Transduction
TIL	Tumour Infiltrating Lymphocytes
tFL	Transformed Follicular Lymphoma
TKI	Tyrosine kinase inhibitor
TLS	Tumour lysis syndrome
TNF	Tumour Necrosis Factor
TRBC1	T-cell receptor beta constant-1
TSE	Transmissible Spongiform Encephalopathy
UC	Ultracentrifuge
UCLH	University College London Hospital
URS	User requirement specification
US	Ultrasound
WT	Wild type

CHAPTER 1:

1 INTRODUCTION

1.1 Context of the project

Autologous Chimeric Antigen Receptor (CAR) T-cell therapies for haematological malignancy have revolutionised the treatment of patients with relapsed and refractory B-cell cancers. The scope of these therapies to reach into other haematological and even solid cancers holds much promise for the future.

However, there are several major bottlenecks for delivery of these novel therapies: the availability of academic clinical trials for CAR T-cell therapies is limited in the UK and in particular, the paucity of manufacturing slots means that the majority of potentially eligible UK patients cannot access these therapies. Streamlining the manufacturing process and pipeline to enhance manufacturing capability and scalability to patient need would begin to address this challenging issue. Secondly, a large proportion of patients referred for consideration of autologous CAR T-cell therapy are heavily pre-treated with chemotherapy and for this reason their T-cell populations for harvest are often numerically (and in some cases functionally) compromised. Clearly, referring patients early, prior to several lines of toxic chemotherapy would be optimal, but this is logistically challenging. Another approach would be to review apheresis practice and work to enhance T-cell collection at the front end.

Lastly, these new therapies are associated with a constellation of potential side effects that are not shared by other conventional therapies. Indeed, the clinical delivery and administration of bespoke gene-modified T-cell therapies in routine Haemato-Oncology Units requires a paradigm shift in medical, nursing, pharmacy and stem cell lab care pathways and logistics.

This project undertakes to address the above issues through the initiation and delivery of an academic study of autologous CD19 CAR T-cells for relapsed/refractory acute lymphoblastic leukaemia (B-ALL). This project will (1) investigate and establish a streamlined manufacturing method and platform for CAR T-cell manufacture for the UCL CAR T-cell programme; (2) explore the single-centre apheresis practice at University College London Hospital (UCLH) to define a model/guideline for successful CAR T-cell apheresis and (3) to begin to establish the clinical infrastructure required to deliver these therapies 'routinely' at a large central London teaching hospital.

1.2 The history of T-cell immunotherapy for cancer

T-cell mediated cancer immunotherapy represents an exciting era of medicine, holding the potential to revolutionise cancer therapeutics. However, the concept of cancer immunotherapy, namely utilising the patient or donor immune system to combat cancer is not a new one. Nobel prize winner, Dr Edward Donnall Thomas, performed the first successful bone marrow transplants, thereby validating the concept of graft-versus-tumour effect (1). First used in 1990 and now widely used for relapsed haematological disease post haematopoietic stem cell transplant (HSCT), donor lymphocyte infusions (DLI) have consolidated the theory of the potency of the graft-versus-tumour effect (2,3). This paved the way for another avenue of research, the passive administration of anti-tumour T cells, termed adoptive T- cell therapy.

Research pioneered by Steven Rosenberg at the National Cancer Institute (NCI) led to the first-in-human studies of tumour-infiltrating lymphocytes (TILs) in patients with metastatic melanoma in the 1980's, with objective response rates of 55% (albeit of short duration) (4). TILs are unmodified T cells harvested from the patient's tumour, expanded ex-vivo in the presence of interleukin (IL)-2 and returned to the patients (+/- systemic IL-2). Following on from this early work, lymphodepletion regimens were optimised to include fludarabine and cyclophosphamide, agents thought to facilitate T-cell engraftment by depleting both regulatory T -cells and host cytokine sinks and increasing levels of homeostatic cytokines such as IL-7 and IL-15 (5,6) with higher rates of sustained responses (7). The field of TILs has since expanded to include other solid tumour indications such as renal, lung, breast, cervical and ovarian cancers (8–12) and is evolving to TILs targeting unique mutations expressed in individual tumours (clonal neoantigens) (13,14). Limitations of TIL therapy which have precluded its widespread usage to date include the need for surgical tissue, inter-patient variability in degree of lymphocytic infiltration of tumour and the complex manufacturing protocols required to generate potentially therapeutic doses. These cells also depend on the endogenous T-cell receptor (TCR) for antigen recognition and are therefore MHC-restricted. The affinity of the TCR for cancer targets can limit activation and cytotoxicity potential of tumour-specific T-cells (15,16).

Non-genetically modified adoptive T-cell therapies for cancer such as TILs have been followed closely by trials using TCR gene-engineered T-cells in melanoma and many solid malignancies. The T cells are engineered to express an exogenous TCR. These studies have proven that engineered T cells can target and elicit responses in some patients with metastatic disease, albeit with severe on-target, off-tumour toxicity in some cases (17–20). The most promising class of targets are cancer germline antigens, such as NY-ESO-1. These are expressed during fetal development and then re-expressed on cancers later in life. NY-ESO-1 is expressed on melanoma and solid tumours such as myeloma, lung, sarcoma and ovarian with demonstrable efficacy in the absence of severe toxicity in clinical studies to date (21,22). Similar to TIL therapy, use of TCR-engineered T-cells is limited by a long and laborious

manufacture process, MHC-restriction, variable antigen expression and affinity (23). The stimulation of the TCR despite low antigen expression is thought to be a prerequisite for the efficacy of TCR gene therapy. However, use of TCRs with artificially modified high affinities has been limited by loss of specificity, increased reactivity against cells that lack the relevant antigen and disruption of serial TCR signalling (and hence T cell activation at low antigen concentrations). One approach to address this has been to increase the surface expression of the TCR on engineered T cells (by substituting amino acid residues in the TCR variable domain) without altering the affinity, with subsequent enhanced T cell proliferation, cytokine production and cytotoxicity (24).

1.3 Chimeric Antigen Receptor (CAR) T-cells

1.3.1 Chimeric Antigen Receptor (CAR) T-cell design

CARs are synthetic receptors that can be incorporated into human T cells, thereby redirecting antigen specificity. They are composed of an extracellular binding domain (typically a single chain variable fragment against the target antigen), a hinge region, a transmembrane domain and one or more intracellular signalling domains. A CAR can be generated against any cell surface antigen (to date, CARs cannot target intra-cellular antigens, unlike TILs and TCR-engineered T cells). Antigen binding triggers CAR T-cell activation via the intracellular signalling domain and this activation is independent of MHC (in contrast to TILs and TCR-engineered T-cells). Kuwana and Eschaar in the late 1980's were the first to demonstrate that such synthetic receptor molecules could trigger T cell activation independent of the MHC (25,26). Preliminary human studies of T-cells genetically modified with CARs were safely performed in patients with human immunodeficiency virus (HIV) infection (27). Autologous CD4 and CD8 T cells were retrovirally transduced with a HIV-targeting gene (CD4 zeta) with the aim of MHC-unrestricted HIV-specific targeting by both CD4 and CD8 T cells. Long-term persistence of these cells has been reported in some patients (28).

CAR-T cells targeting CD19 in haematological malignancies have emerged as the most successful of CARs. "First generation" CARs comprised of a single chain variable fragment (scFv) linked to a simple CD3 zeta signalling domain. Till et al demonstrated the feasibility and safety but modest efficacy of first generation CD20 CARs in patients with NHL (29). These findings of limited efficacy and poor persistence in vivo in patients prompted further research endeavour. A significant breakthrough was achieved when researchers proposed that CAR signalling should mirror that of innate TCR signalling. Innate T-cell activation is a 2-step process. Signal 1 is generated by the binding of the TCR to its specific antigen in association with MHC. Signal 2 is transmitted through an aligned co-stimulatory receptor such as CD28 which initiates full T-cell activation and proliferation. CD28 is thought to play a pivotal

role in T-cell activation, both directly and indirectly, by stimulating the expression of other co-stimuli and cytokines, modulation of the cell cycle, cell differentiation and survival (Figure 1) (30). Additional molecules such as ICOS, 4-1BB and OX40 on the T-cell interact with respective ligands found on antigen-presenting cells (APCs), further modulating T-cell and B-cell response (30) (Figure 2).
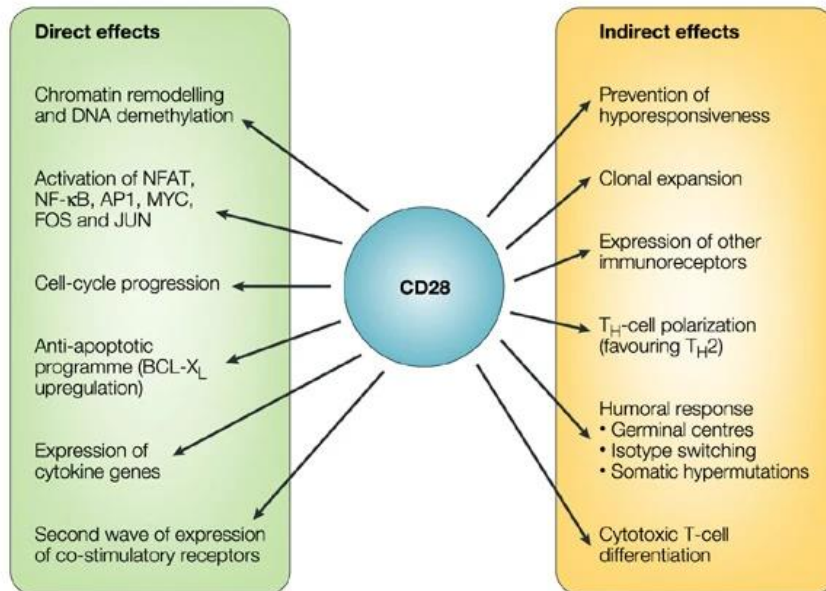
Nature Reviews | Immunology

Figure 1: Direct and indirect effects of CD28 in promoting full T-cell activation (30)
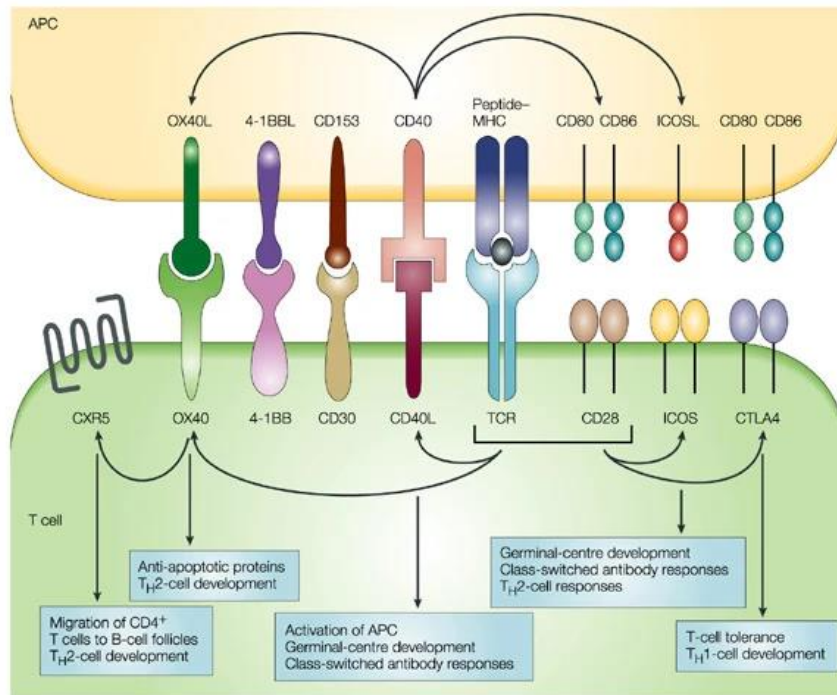
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Figure 2: Other co-stimuli enhanced by CD28 signalling (30)

 In redesigning more complex CAR constructs, this time with an scFv linked to a CD3 zeta signalling domain fused to a co-stimulatory domain derived from e.g. CD28 or 41BB, researchers were able to recapitulate native T-cell signalling systems in an attempt to improve upon the first generation CARs described above. The fusion of a single co-stimulatory signal onto the CAR construct creates a new class of CARs, referred to as 'second generation' CARs. Seminal work was done by the team at Baylor, led by Barbara Savoldo who demonstrated the superiority of second generation relative to first generation CD19 CAR constructs (31). Patients with B-cell lymphomas were simultaneously infused with first and second generation autologous CD19 CARs. CAR-T cell expansion and persistence was monitored by PCR specific for both transgenes with second generation constructs shown to be vastly superior in both respects. The most widely used second generation constructs use a CD28 or 4-1BB co-stimulatory domain. The selection of a specific of co-stimulatory domain is thought to reprogram T-cell metabolism on activation of the CAR. CD28 constructs have been shown to favour rapid differentiation to an effector-memory phenotype and enhance mechanisms such as glycolic metabolism, potentially at the cost of cell persistence. In contrast, 4-1BB constructs appear to skew T cell differentiation to a central memory phenotype and promote mechanisms such as oxidative metabolism and mitochondrial biogenesis. This may explain why 4-1BB CARs can be slower to eradicate tumours but with superior persistence (and comparable outcomes over time) (32,33). Phosphorylation events on

CD28 and 4-1BB CAR-T cell activation have also been analysed by mass spectrometry. Stronger and faster protein phosphorylation was initiated by CD28 CAR activation with downstream transcriptional changes and enhanced expression of molecules such as granzyme B, TNF-alpha and IFN-gamma, supportive of the effector phenotype hypothesis. Conversely, 4-1BB CARs activation promoted the expression of memory T cell genes (34). The relative efficacy of CD28 and 4-1BB CARS in CD19-positive malignancies is discussed in Section 1.3.3.1 and 1.3.3.2. The use of co-stimulatory domains other than CD28 and 4-1BB have yet to be evaluated in clinical trials.

In an attempt to harness the preferential effector phenotype of CD28 and memory phenotype of 4-1BB constructs, third generation CARs have been designed to include two co-stimulatory domains in one receptor. The constructs of first, second and third generation CARs are illustrated in Figure 3 (35). There is conflicting preclinical data on the relative efficacy of third versus secondary generation CARs with reports of inferior (36), superior (37) and comparable efficacy (38). The addition of a second co-stimulatory domain adds further complexity to CAR design, the functionality potentially compromised by the proximity of the domain to the cell membrane (39). To date, there have been few clinical studies of 3rd generation CD19 CARs in man, all with small numbers of patients (40–42). No head to head comparison with a 2nd generation CAR has been performed.

Fourth generation CARs, also known as TRUCKs or "armoured CARs" have been devised with the aim of overcoming the immunosuppressive tumour microenvironment, particularly in solid malignancies. Engineered to include an additional transgene encoding for an inducible cytokine (or other immune-stimulating mechanism), these constructs facilitate local and targeted delivery (of cytokines or other mechanism), thereby potentially avoiding systemic side effects. Examples are demonstrated in Figure 4. Armoured CARs with inducible IL-18 have been shown to be superior to 2nd generation constructs in mouse models in terms of proliferation, persistence and anti-tumour activity (43). In another example, constitutive expression of CD40 ligand, normally expressed on activated T-cells, binds to CD40 ligand on B-cells and macrophages in the tumour microenvironment and has been shown to potentiate cytotoxicity of CD19 CARs in mice (44). Use of CAR-T cells that secrete PD-1 blocking scFv prolonged survival in mice in both haematological and solid tumour models, suggesting that this mechanism of local delivery may augment the efficacy of CAR-T and bystander T-cells at the tumour site (45).
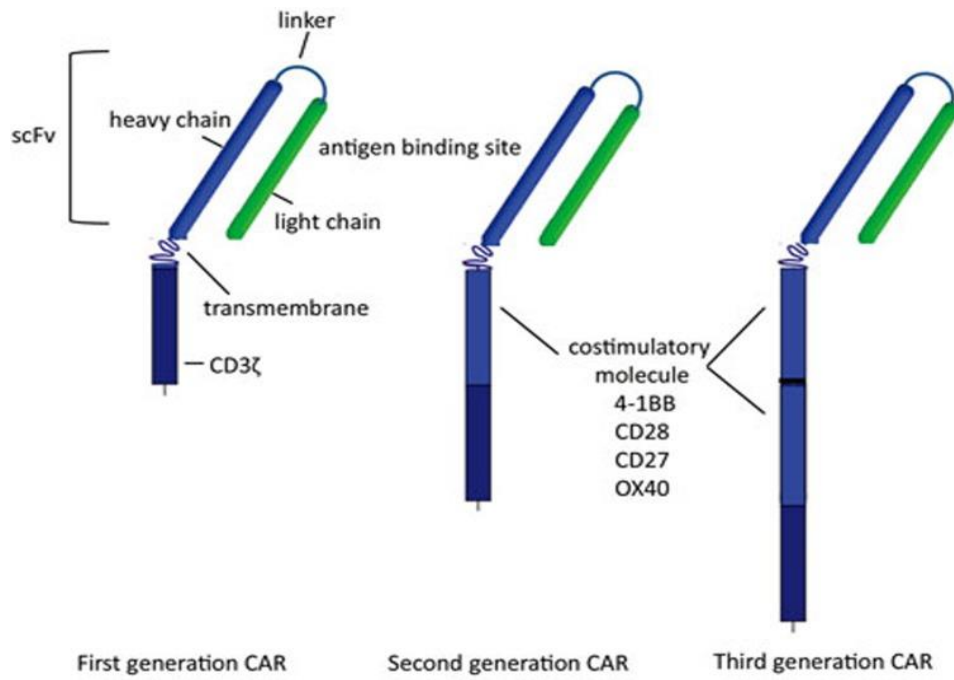
Figure 3: First, second and third generation CAR constructs (35)
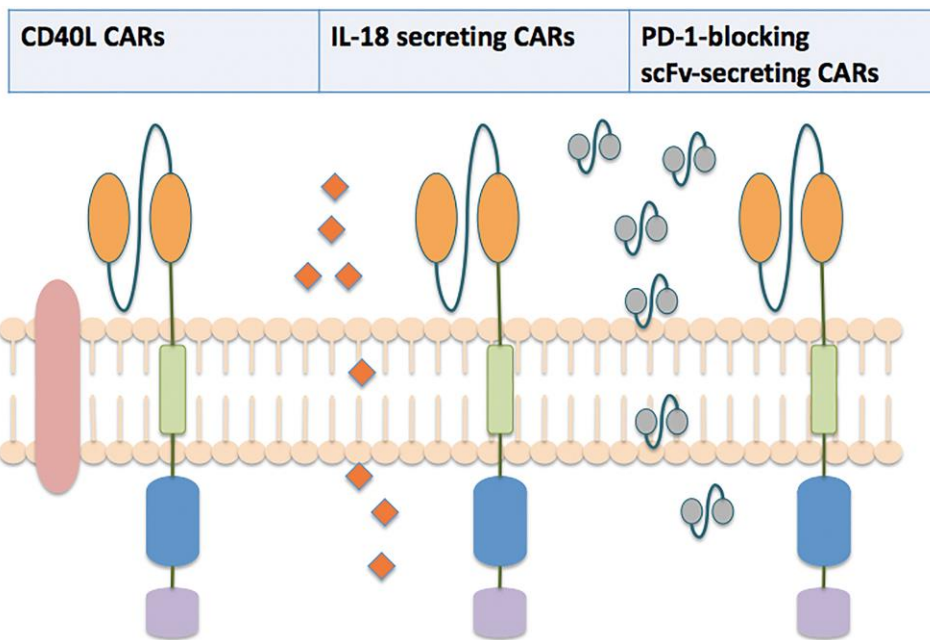
Figure 4: Examples of armoured CARs (46)

1.3.2   Chimeric Antigen Receptor (CAR) T-cell pharmacokinetics

The CAR construct must reach and engage with the antigen, proliferate and kill the tumour cell. For a durable response, the cells must persist (duration likely dependent on the clinical indication) and form memory. T-cell kinetics vary considerably between CAR constructs and individual patients. Unlike traditional drugs which have a defined chemical composition, engineered T-cell products vary hugely in their composition (quantitatively and qualitatively) and are characterised by in vivo expansion and often downstream cell exhaustion, senescence and death. The pharmacokinetics (PK) of CAR-T cells usually encompasses peak CAR-T expansion (or AUC) and persistence. Looking at data generated from larger clinical trials, responses to the licensed CD28 CAR in B-NHL, axicabtagene ciloleucel (Axicel, Gilead), correlated with in vivo CAR expansion (AUC over 5 times higher in responders) (47). Similarly, CAR-T expansion correlated with clinical response in patients with B-ALL and CLL receiving the 4-1BB CAR, Tisagenlecleucel (Tisagen, Novartis) (48). In contrast, CAR-T expansion was 6-fold lower in DLBCL patients treated with Tisagen (possibly related to T-cell trafficking to nodal and extra-nodal sites or intrinsic T cell factors) and did not correlate with clinical efficacy (49,50). Whilst the exact duration is unknown, persistence of CAR-T cells is a prerequisite for a durable clinical response. Constructs with 4-1BB have been shown to persist for longer than those with a CD28 co-stimulatory domain, CTL019 transgene detectable up to 780 and 693 days in B-ALL (48) and DLBCL (49) patients respectively.

It is now clear that cell differentiation status of adoptively transferred cells and immunological memory are of huge significance where anti-tumour activity and cell persistence are concerned. There is particular interest in T-cells with a central memory (Tcm) or a stem cell memory phenotype (Tscm), the latter characterised by the ability to self-renew and further differentiate into effector and memory populations (51,52). Data from mouse models also suggests that Tscm populations transduced with CD19 CARs have enhanced anti-tumour activity (53,54). The vast majority of clinical studies do not pre-select autologous T cells on the basis of differentiation, therefore potentially harvesting T- cells at a later stage of maturation, cells which may have adequate effector function but are unable to form memory and persist.

As discussed in Section 1.2, the optimisation of lymphodepleting (LD) regimens in the TIL studies facilitated T-cell engraftment and led to higher rates of sustained responses (7). Lymphodepleting agents are thought to facilitate T-cell engraftment by depleting both regulatory T-cells and host cytokine sinks and increasing levels of homeostatic cytokines such as IL-7 and IL-15 (5,6). Brentjens et al looked at low and high dose cyclophosphamide LD regimens in B-ALL with improved survival in the latter group (55). The landmark study by Turtle et al demonstrated that the addition of fludarabine to a cyclophosphamide-only LD regimen impacted on CAR T expansion, persistence and clinical response (56). There are no large randomised studies directly comparing different lymphodepletion regimens

(dosing, timing, drugs) and huge heterogeneity is noted in clinical studies (57). In the JULIET Phase 2 clinical trial, 20% of patients of patients received Bendamustine as part of the LD regimen (58). This agent has also been explored with anti-CD30 CARs in Hodgkin's lymphoma where clinical benefit was only noted in combination with fludarabine (59). Alemtuzumab, a monoclonal antibody against CD52, is a potent lymphodepleting agent, under investigation in predominantly in "off the shelf" CAR T studies. Further research is needed to define the optimal LD regimen for each disease indication.

1.3.3 CAR T-cell clinical trials to date in haemato-oncology and solid tumours

Following on from successful pre-clinical validation and early clinical experience in the US, second generation CAR therapy has been the subject of numerous academic and commercial clinical trials in patients with cancer. Most clinical experience with CAR T-cells is in a subset of haematological cancers, namely B-cell leukaemia and lymphoma. B-cell cancers (and indeed native, healthy B-cells) express high levels of the cell surface protein, CD19. CD19 is in some ways the perfect CAR T-cell target due to its high cell surface expression on malignant B-cells and critically in its limited expression on other tissues of non-B-cell lineage. This latter element limits the risks of off-target toxicity which could severely limit the application of CAR-T cells targeting CD19. CD19 is a type 1 transmembrane protein, encoded by the CD19 gene on the short arm of chromosome 16, and is a member of the immunoglobulin super-family. It is expressed on early pre-B cells (from heavy chain rearrangement) until plasma cell differentiation. Structurally, it consists of 2 C2-type Ig-like domains (extracellularly), a transmembrane domain and a cytoplasmic domain which contains multiple tyrosine residues, of which Y391, Y482 and Y513 are thought to be the most important for functionality (Figure 5) (60). The primary function of CD19 is to establish intrinsic B-cell signalling thresholds. It does this by modulating B-cell receptor signalling, both amplifying and attenuating the signal (61). Additionally, the pivotal role of CD19 in B-cell development and differentiation is highlighted by findings in CD19 deficient mice, including hypo-responsiveness to transmembrane signals, hypogammaglobulinaemia and impaired T-cell dependent antibody responses (61,62).

The ideal target for a CAR should only be expressed on cancer cells (or surrounding stroma) and not on normal tissue. Unfortunately, few (if any) targets are as specific as CD19. Ablation of normal B-cells by CD19-targeting CARs is considered a tolerable on-target off-tumour side effect. Preclinical animal models are often an unsatisfactory platform to assess for off-tumour toxicity as tissue distribution of the antigen may be species-specific. Additionally, many of the scFv ectodomains in mouse models may not recognise the non-human components of the targeted antigen. The majority of targets tested to date are expressed in low levels on normal tissues (HER2, GD2) or are expressed solely by a specific cell lineage (CD19, CD20). Efforts to improve the efficacy and safety of CARs with regard

to antigen selection include selection of lineage-associated rather than lineage-specific antigens, hence avoiding the ablation of an entire cell lineage. An example of this is targeting TRBC1 in T-cell lymphomas (63). Targeting multiple antigens to reduce the risk of antigen escape is another strategy under investigation by several research groups (Section 1.3.3.1). Critical for tumour growth, the tumour stroma is deemed more genetically stable than the cancers cells themselves and preclinical studies targeting fibroblast activation protein (FAP) or vascular endothelial growth factor receptor (VEGFR)-2, either in alone or in combination with tumour targets, has been published (64,65). Other targets will be discussed in disease-specific sections below.
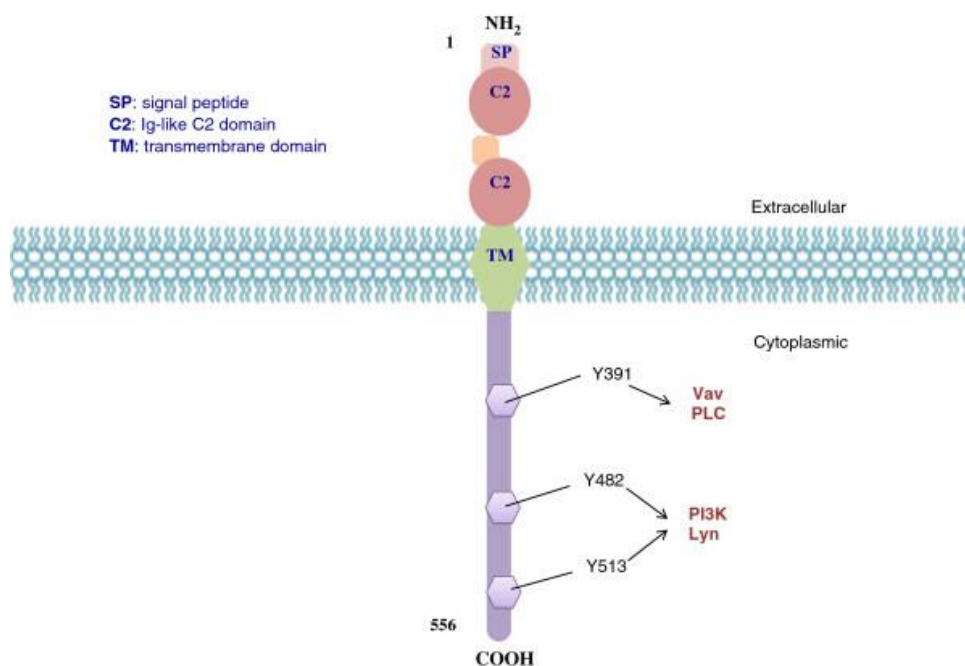
Figure 5: CD19 molecular structure (60)

1.3.3.1 B-acute lymphoblastic leukaemia (B-ALL)

While the 5-year event-free survival (EFS) for children and young adults diagnosed with B-ALL is approaching 90% (UKALL2003 study) (66), outcomes remain poor for certain high risk groups including those who are refractory to induction or have a persistent minimal residual disease (MRD) signal (67). Up to 20% of children with B-ALL will ultimately relapse with a long-term disease free survival of 40-50% (despite allogeneic transplant) (68,69). Relapse post allograft confers a very poor prognosis, with a small minority of patients suitable for a second allograft (70,71). Options for the majority of patients who are unsuitable for a second allograft were few and none were curative. Second generation CD19-targeting CARs have demonstrated remarkable clinical efficacy in the treatment of relapsed and refractory paediatric B-ALL, including relapse post bone marrow transplant (72–76) . As

discussed in Section 1.3.3, CD19 is expressed on all B-cells, including leukaemic blasts in B-ALL and malignant B-cell lymphoma cells. Remission rates of >80% have been reported in cohorts of paediatric patients with high risk relapsed and refractory B-ALL, sustained responses of approximately 50% at 12 months reported in the Eliana Phase 2 clinical trial of Tisagen in children and young adults (76) (Figure 6). On the basis of this Phase 2 clinical trial, the FDA and EMA approved Tisagen (Kymriah, Novartis), in August 2017 and August 2018 respectively, for the treatment of relapsed or refractory B-ALL after 2 or more lines of standard therapy in patients up to the age of 25 years. Real-world data from the Centre for International Blood and Marrow Transplant Research (CIBMTR) registry was presented at the ASH 2019 Annual Meeting and reported a CR rate of 88%. At a median follow up of 5.8 months (range 2.6-16.9), the 6 month duration of response (DOR), EFS and OS was 77%, 68% and 94% respectively (77).

Many different CAR constructs are currently under investigation in paediatric ALL. The ZUMA-4 study is assessing the use of Axicel, a CD28 CAR licensed for B-cell lymphomas, in paediatric and young adult B-ALL with CR and CRi (CR with incomplete count recovery) rates ranging from 64-100% with different dosing regimens (78). The focus in this field has now shifted to designing safer and more efficacious CARs, Ghorashian et al demonstrating that a low affinity CD19 CAR with a faster off rate may have greater proliferative and cytotoxic potential in vivo, without compromising safety (79).

Loss of surface of expression of CD19 or 'antigen escape' is thought to account for 10-30% of relapses after CAR-T cell therapy in B-ALL (73,80). Mechanisms of CD19 negative escape include alternative exon splicing of CD19 gene which leads to the expression of variants lacking the domains required for recognition by CAR-T cells (80), emergence of CD19 negative clones (81) or lineage switch (82,83). To counteract the risk of antigen loss, bispecific CARs (CD19/CD22) or sequential CD19 and CD22 CAR infusions are being explored. Wang et al administered sequential autologous CD19 and CD22 CARs to 89 adult and paediatric patients with B-ALL or B-NHL. With a median follow up of 14 months, only 1 patient suffered antigen loss-related relapse (84) suggesting that this strategy may reduce the risk of antigen escape. At a median follow-up of 9.5months, a bispecific CD19/CD22 CAR administered to 12 paediatric and adult patients with B-ALL was not associated with antigen escape. All relapses (n=3) retained expression of CD19 and CD22 suggestive of loss of persistence (85). Amrolia et al at ASH 2019 reported their updated results on the AMELIA study, a bispecific CD19/CD22 CAR. Again, antigen positive relapse due to loss of CAR-T persistence was the more common cause of disease recurrence (86).
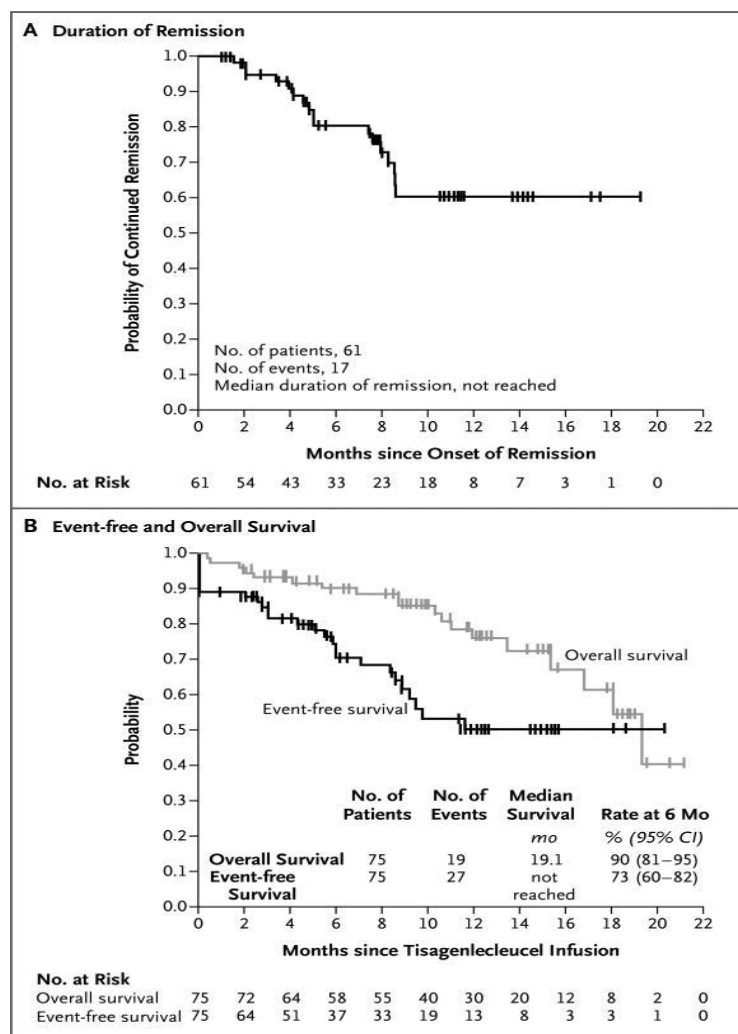
Figure 6: Duration of remission, EFS and OS with Kymriah in Eliana study (76)

In contrast to paediatrics, adults with newly diagnosed B-ALL have a 30% chance of long-term disease-free survival. While 90% of patients may achieve an initial complete remission (CR) with induction chemotherapy, up to 50% will relapse. Achieving a second CR followed by an allogeneic stem cell transplant offers the best chance at cure for the subset of suitable patients. Considering rates of transplant-related mortality and disease relapse, less than one third of patients can expect long-term disease-free survival post allograft (87,88). As with paediatrics, relapse post allograft confers a very poor prognosis (89). Encouraged by the efficacy of CD19-targeting CARs in paediatric disease, adult studies have followed suit but as yet there is no licensed product. While high initial CR rates can be achieved in adults, treatment outcomes in adult ALL have been compromised somewhat by toxicities in an older more vulnerable patient cohort, particularly in those with a higher disease burden (56,90,91). Tisagen, licensed for paediatric and young adult ALL, was administered to 35 adults aged 20 -70 years with a low dose, high dose and a fractionated high dose regimen. CR rates ranged from 33% (low dose) to 90% (fractionated high dose). There was a 50% mortality rate (CRS and sepsis) in the cohort of

patients who received a single high dose infusion (n=6). Toxicity in the fractionated high dose protocol was more manageable (mortality 5%) with a 2 year OS and EFS of 73% and 49.5% respectively (90). Axicel is also being explored in adult ALL in the ZUMA-3 study (92). Toxicity rates in adult ALL CAR studies have prompted fractionated and adjusted dosing regimens. The adult ALL study at UCL, ALLCAR19, uses fractionated cell dosing, adjusted for disease burden. Again, using a low affinity CAR with a fast "off rate", deep remissions can be achieved with seemingly lower toxicity in the small numbers of patients treated to date (93).

1.3.3.2 B-Non-Hodgkin's Lymphoma (B-NHL)

Diffuse large B-cell lymphoma (DLBCL) is the most common form of NHL. Standard first line treatment is RCHOP (rituximab, cyclophosphamide, hydroxydaunorubicin, vincristine and prednisolone) chemoimmunotherapy, curative in up to 60% of patients (94). For younger fitter patients with relapsed or refractory disease, salvage chemotherapy followed by autologous stem cell transplant (ASCT) is the standard second line therapy. However, 50% of patients who relapse after or are refractory to first line therapy are ineligible for ASCT and 50% of patients will relapse after ASCT (95). The SCHOLAR-1 study (96), a pooled retrospective analysis of 636 patients with refractory DLBCL, reported response rates of 26% (CR rate of 7%) and a median OS rate of 6.3 months in this patient cohort, highlighting an area of unmet need where alternative salvage therapies are required.

The advent of CD19 CARs in B-cell lymphoma has been transformative. CD19-targeting CARs can elicit prolonged durable responses in a subset of patients with relapsed and/or refractory high-grade B-cell lymphoma. Phase 2 clinical trials of second generation CD19 CARs in high grade B-cell lymphomas such DLBCL, transformed follicular lymphoma (tFL) and primary mediastinal B-cell lymphoma (PMBCL) have demonstrated overall response rates (ORR) of 52-83% with approximately 40% of patients in CR at 2 years post therapy (47,50,97). On the basis of the JULIET (Tisagen) and ZUMA-1 (Axicel) Phase 2 clinical trials, the FDA and EMA approved both Tisagen (May and August 2018 respectively) and Axicel (October 2017 and August 2018 respectively) for high grade B-cell lymphoma patients who have relapsed after or are refractory to two lines of standard chemotherapy treatment. Real-world experience of Tisagen in 47 patients with DLBCL, presented at ASH 2019, reports an ORR and CR rate of 60% and 38% respectively at a median follow-up of 5.8 months (range 0.9-8.9) (98). Larger datasets from the US are available to assess the real world efficacy of Axicel (n=295) with an ORR and CR rate of 70% and 52% respectively from the CIBMTR registry (99). A further retrospective study of commercial Axicel (n=275), inclusive of 129 (43%) who did not meet ZUMA-1 eligibility pre-apheresis on the basis of co-morbidities, confirms a high ORR of 82% and a CR rate of 64%. Median DOR was not reached and median PFS and 12-month PFS were 8.3 months

and 47% respectively (100). In contrast with US datasets, the UK real-world experience of Axicel and Tisagen has been collated prospectively (and nationally) using centrally-reviewed selection criteria on an intention to treat basis (101,102). The French have also presented their real-world data (103,104). These datasets serve to highlight differences in data collection and reporting, patient selection and turnaround times/funding streams, all of which may potentially impact on reported success rates. Comparison between Tisagen and Axicel, reported retrospectively (105), is difficult to interpret due to different patient populations, use of / exclusion of bridging and poorly standardised toxicity grading systems, although Tisagen is generally thought to be less toxic. Toxicities of both agents are discussed in Section 1.6.7.

Lisocabtagene maraleucel (liso-cel, JCAR017), is another 2nd generation CD19 CAR with a 4-1BB co-stimulatory domain that is under investigation in high grade B-cell lymphoma including DLBCL, PMBCL, grade 3B FL and mantle cell lymphoma (MCL). In a cohort of 268 patients in the TRANSCEND study, an ORR and CR rate of 73% and 53% respectively was achieved (106), with a favourable toxicity profile allowing the potential for outpatient administration in select patients (107). In contrast to currently licensed products, transduced CD4 and CD8 vials are administered separately in the ratio of 1:1. An application for approval was submitted to the FDA in December 2019.

MCL is another CD19-expressing NHL that seems set to benefit from the cell therapy advances made in other B-cell malignancies, KTE-X19 achieving an ORR, CR and 12-month PFS rate of 93%, 67% and 61% respectively in high risk patients (108). Submission to the FDA is expected imminently.

As discussed in Section 1.3.3.1, antigen loss is also thought to be a prominent cause of relapse in B-NHL. It is often more difficult to diagnose as many patients may not have a repeat biopsy to confirm disease recurrence in the setting of PET positivity post therapy. Use of sequential CD19 and CD22 CARs (84), CD19/CD22 (109) and CD19/CD20 (110) bispecific CARs are under investigation.

1.3.3.3 Chronic Lymphocytic Leukaemia (CLL)

Between 2015 and 2017, 3824 people were diagnosed with CLL in the UK, 41% of cases aged ≥75 years (111). The majority of patients will have indolent disease and are managed with "watch and wait" strategies but a small proportion of patients will have a rapidly progressive and refractory clinical phenotype. CAR-T cell therapy in CLL faces challenges, mainly the inherent T cell defects, both quantitative and qualitative, which may account for the lower CR rates in CLL with CAR-T cell therapy relative to other B-cell malignancies. Brentjens et al were the first group to explore the use of CD19-targeting CARs in CLL, infusing 8 patients with a second generation construct (74). CTL019 was studied in heavily pre-treated patients with an ORR and CR rate of 57% and 29% respectively (112). A

further dose optimisation study of CTL019 suggested greater efficacy at higher ($5x10^8$) versus lower ($5x10^7$) CAR-T cell doses without additional toxicity in 38 infused patients. The achievement of a CR was associated with prolongation of survival (not reached in those with a CR versus 64 months with no CR) (113). Liso-cel has also been studied in high risk CLL patients who failed previous ibrutinib therapy with CR rates ranging from 21-46%. (114,115). At a median follow up of 9 months, 78% of responders remained progression-free (115). Ibrutinib may have a role prior to or concurrently with CAR-T cell therapy in CLL patients. This agent is thought to improve T-cell function by the inhibition of IL-2 inducible T-cell kinase with downstream effects on CAR-T expansion and inhibition of PD-1 expression. In mouse models, CAR-T cells administered concurrently with ibrutinib demonstrated superior engraftment and anti-tumour activity (116). Abrogation of proinflammatory cytokines and potentially lower rates of CRS (117) is another reason for the addition of ibrutinib to CAR-T clinical trials in CLL. Preliminary results in small numbers of patients have suggested lower rates of grade 3 CRS (0% vs 26%) and higher response rates (88% vs 56%) in the ibrutinib arm (118).

1.3.3.4 Multiple Myeloma (MM)

The treatment landscape for multiple myeloma (MM), a predominantly incurable clonal plasma cell disorder, has been transformed in the last 15 years with the advent of novel agents such as proteasome inhibitors, immunomodulatory drugs and monoclonal antibodies. The use of these agents, usually in combination, has dramatically increased the depth of response with studies showing that MRD negativity is associated with a longer PFS and possibly OS (119). OS has increased to 7-10 years (120) and the focus has shifted gradually to managing short and long-term toxicities of therapy and maintaining quality of life. Malignant plasma cells are usually CD19 negative. The expression of CD19 on myeloma stem cells has been purported but not proven. CLT019 was studied in combination with high dose melphalan and a second autologous transplant with modest efficacy (2/10 achieved improved PFS relative to first autograft) (121). B-cell maturation antigen (BCMA), expressed on plasma cells but not on other essential tissues, is a member of the TNF receptor family and has a role in the regulation of B-cell maturation. Several BCMA-targeting CARs are under investigation. The LEGEND-2 study demonstrated an ORR of 88% and CR of 68% with a median PFS of 15.1 months (median follow up 8 months) in 57 Chinese patients treated with a BCMA CAR with a 4-1BB co-stimulatory domain (122). The CARTITUDE-1 study in the US will further evaluate this construct and preliminary results were presented at ASH Annual Meeting in December 2019 (123). Idecabtagene vicleucel (ide-cel) is another BCMA CAR with a 4-1BB co-stimulatory domain that has demonstrated high MRD negative remission rates (45%) in a heavily pre-treated patient population and is expected to be submitted for FDA approval in the near future (124).

As with CD19 CARs, downregulation and loss of BCMA is thought to account for a significant proportion of disease relapses post therapy. Strategies to overcome this include targeting both CD19 and BCMA (125) (thereby also targeting myeloma stem cells), targeting both BCMA and TACI in the APRIL study (126,127) and the concurrent use of a gamma secretase inhibitor (to increase the expression of BCMA on plasma cells) (128). The success of Daratumumab (a humanised monoclonal antibody against CD38) in MM has engendered interest in the CD38 antigen and preliminary results are available for a bispecific CAR targeting both BCMA and CD38 demonstrating high ORR and CR rates (129). SLAMF7, also known as CS1, is expressed on normal and neoplastic plasma cells (and NK cells) and number of trials are underway (NCT03710421, 03778346). GPRC5D, expressed on CD138+ myeloma cells and independent of BCMA expression, is another target of interest (130).

1.3.3.5 Other haematological malignancies

Classical Hodgkin's lymphoma (CHL) is a curable disease for the majority of patients with a 5-year survival of 86% (131). Approximately 25% of patients will be refractory or relapse after initial chemotherapy. The standard of care for these patients is salvage chemotherapy followed by an ASCT with an expectation of a durable response in 50% of patients if an adequate remission is achieved pre-ASCT (CR or near CR) (132,133). With the advent of novel therapies such as brentuximab vedotin and checkpoint inhibition, outcomes at second relapse have improved. The clinical success of the anti-CD30 antibody drug conjugate, Brentuximab, and the expression of CD30 by Reed-Sternberg cells has generated interest in CD30-targeting CAR-T cells for relapsed CHL. Preliminary results of efficacy in a very small number of patients have been published with CR rates of 75% (6/8) (134). There is concern that fratricide (killing of CAR-expressing cells by each other) may compromise efficacy as CD30 is also expressed on activated T-cells.

Acute myeloid leukaemia (AML) has a 5-year survival of 40% in patients diagnosed under the age of 60 years (135). It has not benefited from a wealth of novel agents conferring a survival advantage in other haematological malignancies. Progress in the AML CAR-T field has been complicated by the lack of an AML-specific antigen, many of which are expressed on normal haematopoietic stem cells. The use of the anti-CD33 antibody drug conjugate, gemtuzumab oozogamicin, in AML has generated interest in the use of CD33 CARs (136). As a target, CD123 (a transmembrane alpha chain of IL-3 receptor) is expressed on the majority of AML cells but use of CD123 CARs has been complicated by the on-target off-tumour effect of eradicating normal myelopoieis, suggesting that options such as "biodegradable CARs" modified with messenger RNA, an inducible suicide system or a rescue allograft should be considered (137). Other targets such as CD117 and NKG2D are under evaluation (138,139).

T-cell NHLs are a heterogeneous group of diseases, of which peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS), anaplastic large cell lymphoma (ALCL), angioimmunoblastic T-cell lymphoma (AITL) and cutaneous T-cell lymphoma are the most common. Historically, T-cell lymphomas are associated with a worse overall prognosis relative to B-cell counterparts. Less than one third of patients with PTCL-NOS survive long-term (140). Other than Brentuximab vedotin for those with CD30-expressing disease, approved therapies for relapsed or refractory disease demonstrate only modest efficacy. Designing CARs for T-lineage malignancies is complicated by the lack of a tumour-specific antigen. Unlike B-cell aplasia, T-cell aplasia is considered a potentially life-threatening on-target off-tumour side effect. Strategies to overcome this include targeting selected T-cell subsets, short-lived CAR-T cells and incorporation of an inducible suicide system. Maciocia et al have designed a construct that targets either TRBC1 or TRBC2 based on the knowledge that malignant T-cells are clonally restricted, thereby preserving an adequate number of normal T-cells (63). Secondly, fratricide compromising efficacy is a concern. Possible solutions to this include gene editing to remove the relevant antigen on the CAR-T surface, targeting downregulated antigens or using NK CARs (141).

1.3.3.6 Solid tumours

The transition of CAR-T cells into the solid tumour field represents a significant challenge. Breast and lung cancer, the most common cancers worldwide, accounted for 24.6% of all cancers diagnosed in 2018 (both sexes) (142). With regard to global cancer incidence, Non-Hodgkin's lymphoma and leukaemia are ranked in 11th and 13th place respectively. Therefore, the race for success in solid tumours, comparable to CD19 CARs in haematological malignancies, is well underway with large pharmaceutical companies keen to invest huge sums of money in the research and development of CAR-T cell immunotherapy for solid cancers. Progress to date has been hampered by a number of factors. Firstly, there is a paucity of tumour-specific targets in solid malignances. For example, CD19 in B-ALL and DLBCL is seen as an "expendable" antigen. While it is expressed on normal B-cells, B-cell aplasia post CAR therapy can be abrogated with intravenous immunoglobulins, if required in the setting of recurrent infections. The most studied tumour targets in solid tumours to date include EGFRvIII for glioblastoma, GD2 for neuroblastoma and mesothelin for a variety of epithelial cancers (143). Shared antigens between tumour and normal tissue have resulted in on-target off-tumour toxicity in normal organs (144,145). With the exception of CAR-T cells targeting GD2 in neuroblastoma (146,147), CAR-T cells in solid tumours have demonstrated limited efficacy to date. Response is also thought to be hampered by physical barriers and poor trafficking to and persistence of the cells at the tumour site (148,149). Fourth-generation CARs designed with the purpose of augmenting CAR functionality and overcoming barriers in the tumour microenvironment are discussed in Section 1.3.1.

1.3.4   The Future

Despite the success of this therapy in haematological malignancies to date, many challenges remain. While initial response rates in CD19+ malignancies are high, relapses are common. The optimal CAR construct for engraftment and persistence (and safety) has yet to be defined, as described in Section 8.5.1.2. Failure of engraftment and loss of persistence (particularly in B-ALL) often leads to disease recurrence. Understanding mechanisms of relapse, including both antigen negative and positive relapse, is paramount for prolonging the duration of response and disease eradication (Section 8.5.1). Strategies to enhance the efficacy, safety and persistence of CAR-T cells is also discussed in Section 8.5.1.

The future of this therapy will also encompass the exploration of alternative sources of cells, namely allogenic CARs or other "off the shelf" alternatives. This will circumvent the fixed patient variables that may compromise T-cell fitness and hence CAR-T cell functionality, namely the nature of the underlying disease, patient age and previous treatments (Section 8.2.5.2).

It goes without saying that expanding the indications beyond haematological malignancy to solid tumours is a huge hurdle in the coming decades.

1.4 Chimeric Antigen Receptor (CAR) T-cell manufacture in academic centres

Translational research in T-cell immunotherapy is progressing rapidly at a pace that far exceeds our ability to deliver these products to the bedside in a timely manner. Barriers to effective and efficient translation to the clinic include complex manufacturing protocols, infrastructural deficiencies (clean room laboratories, apheresis units, stem cell laboratories), staff training, high costs and hugely burdensome regulatory and Good Manufacturing Practice (GMP) requirements (150–152). Partnership between the pharmaceutical industry and academia with subsequent commercialisation of CAR-T cell immunotherapy is already in motion. Novartis pharmaceuticals and Kite/Gilead have paired with University of Pennsylvania and NCI, respectively. To date academic centres not affiliated with commercial companies have been running small scale studies with the limited infrastructure at hand, presenting logistical and financial challenges. A framework for the large-scale clinical implementation and provision of these therapies must be established.

1.4.1 What is cGMP and why is it important for CAR T-cell manufacture?

Good Manufacturing Practice (GMP) guidance, mandated and governed by the FDA in the US and EMA in the EU, is a quality system that covers all areas of pharmaceutical production to ensure a tightly controlled, standardised, reproducible and auditable process with the release of a safe, sterile and (potentially) potent/effective product (150). There is variability between FDA and EMA requirements and even between member states within the EU.

In the US, ATIMPs for Phase 1 studies are manufactured under Investigational New Drug (IND) approval. The IND trial authorisation is evidence of compliance with US FDA GMP standards (153). Phase 1 studies are exempt from the GMP requirements laid out in Title 21 of Code of Federal Regulations 211 unless or until they are used in phase 2 studies or later (154). A concise summary of what is required is detailed in "CGMP for Phase 1 Investigational Drugs" (155). The Chemistry, Manufacturing and Control (CMC) section is a component of the IND application and mandates details on the facility, starting materials, QC testing and product management (156).

In the EU, the requirements for ATMPs are well established with regulations specifically for ATIMPs (REGULATION (EC) No 1394/2007) and clinical trials of medicinal products for human use (157). Directives such as Directive 2004/23/EC, 2006/17/EC and 2006/86/EC and Tissues and Cells Directive also provide a framework for ATMPs. Directive 2009/120/EC and the EC-issued "Guidelines on Good Manufacturing Practice specific to Advanced Therapy Medicinal Products" were devised with a view to addressing the complexity and risks of ATIMP manufacture and highlighting the importance of a functioning quality system (158). CAR-T cells are classified as ATMPs under the Directive 2004/23/EC. In contrast to the US, manufacturing authorisation and clinical trial authorisation (CTA) are required for all stages of clinical trial development (phase 2 or 3 only in the US). Applications for CTA are submitted to the competent authority in individual member states and requirements may differ within EU member states. Table 1 displays the regulatory requirements for ATIMPs in US, EU and Canada (153). These standards are then laid out in controlled documents issued by the relevant manufacturing facility (standard operating procedures, SOPs and forms) and cover the following areas: personnel, quality control (QC) and qualified person (QP), facilities and equipment, control of components, manufacturing and records, assays for release testing and stability, packaging, labelling and distribution. These variables are summarised in Table 2 (150,159). Given that UCL are currently under the auspices of the EU, I will focus on EU GMP guidelines for the remainder of this chapter. The European Commission (EC) issued "Guidelines on Good Manufacturing Practice specific to Advanced Therapy Medicinal Products" (158) in November 2017 which addresses the requirements in the aforementioned areas relating to ATIMP manufacture and is discussed in more detail below.

	Description	Distinguishing features	GMP Inspections	International relevance
Canada (Health Canada)	Cell therapy products (CTPs) must follow GMP requirements

Flexible risk-based approach is taken (as required by technology)

More stringent GMP approach throughout clinical trial phases | Establishment license not strictly required for any phase of clinical trial, strategically necessary by Phase 3 | Reviewed by Biologics and Genetic Therapies Directorate (pre-market review group). Clinical trial application for all phases of clinical trial | No explicit evidence for meeting GMP is provided by Health Canada

Implicit evidence of meeting GMP standards is provided in a clinal trial "No Objection Letter" |
| **United States (FDA)** | CTP must follow GMP requirements

FDA relies on pre-market regulatory professionals to assess Phase 1 products during IND submission reviews. Phase 2/3 require manufacturing site registration | Establishment licence required for manufacture phase 2 + 3 clinical trial products | Phase 1 clinical trial products reviewed under IND submissions by CBER* (FDA)

Establishment registered with FDA, listed on DECRS** | An establishment registration from US FDA indicated GMP compliance for phase 2 or 3 studies (but not phase 1) |
| **European Union (EMA)** | Requirements for ATMPs well established (see above) | Manufacturing authorisation required for all stages of clinical trial development | Inspections by a competent authority/QP of a member state | A QP site declaration indicates the site complies with EU GMP for all phases of study |

Table 1: Brief description of regulatory requirements for ATIMPs in Canada, US and EU

*Centre for Biologics Evaluation and Research (CBER)

**Drug Establishments Current Registration Site (DECRS)

1.4.2 The tenets of cGMP and critical components of a cGMP facility

As outlined in Table 2 below, cGMP relies upon the presence of well-trained personnel, quality control and qualified person oversight of all elements, the establishment and maintenance of appropriate facilities and equipment, excellent record-keeping and audit trail, fit-for-purpose release assays to ensure potency and sterility of products for patients and robust labelling and good distribution practice (150,152,158).

Area	GMP requirements and associated documents/systems
Personnel	• Adequate training, education and regular reassessment
QC/QP function	• Review of incoming materials
• Review of manufacturing protocols and testing procedures

	• Final product release • Investigation of non-conformances and complaints
Control of components	• Identification and traceability of all components used in manufacturing • Handling and control of components • Acceptance criteria for all components
Facilities and equipment	• Maintenance and certification of equipment • Cleanroom environment- air handling, temperature and lighting control, appropriate environmental monitoring/investigation of deviations • Assessment of contamination and cross-contamination risk
Recordkeeping	• Batch manufacturing records • Consumables/reagents records • Sterility, environmental monitoring and release testing results • Equipment maintenance/servicing/calibration records • Record of product storage, location and distribution • Training records for all staff • Non-conformance records • QC and QP reports
Assays for release testing and stability (specific to individual products)	• Assays to assess identity and purity • Potency assay, if available • Assays to assess stability over time
Packaging, labelling and distribution	• Packaging to ensure no leakage/damage/contamination • Labelling with appropriate identifiers to ensure correct allocation • Traceability of final product

Table 2: GMP requirements for ATIMP product manufacture

1.4.2.1 The tenets of cGMP: Personnel

Each manufacturing team must have an adequate number of appropriately qualified staff with the relevant practical experience and a clear understanding of responsibilities. Training on the principles of GMP, aseptic manufacturing, high risk products, testing and traceability of the product should be provided at initial induction. Given the likelihood staff will be handling "genetically modified organisms" in the field of ATIMPs, risks of viral vectors, cross-contamination and potential environmental impact should be discussed. Participation in a process simulation test (media fill) is recommended prior to participation in aseptic manufacturing. Staff must also demonstrate competence at gowning (annual reassessment). Training records must be regularly updated. Gowning stipulations are dependent on the laboratory grade. EC guidelines (158) make the following recommendations:

D grade: "Hair and, where relevant, beard and moustache should be covered. A general protective suit and appropriate shoes or overshoes should be worn."

C grade: "Hair and where relevant beard and moustache should be covered. A single or two-piece trouser suit gathered at the wrists and with high neck and appropriate shoes or overshoes should be worn. They should shed virtually no fibres or particulate matter"

A/B grade: "Sterile headgear should totally enclose hair and, where relevant, beard and moustache; it should be tucked into the neck of the suit; a sterile face mask and sterile eye coverings should be worn to prevent the shedding of droplets and particles. Appropriate sterilised, non-powdered rubber or plastic gloves and sterilised or disinfected footwear should be worn. Trouser legs should be tucked inside the footwear and garment sleeves into the gloves. The protective clothing should shed virtually no fibres or particulate matter and retain particles shed by the body"

1.4.2.2 The tenets of cGMP: QP/QC function

The head of QC is involved in all decisions affecting the quality of the product (158), including:

- Incoming reagents and starting material
- Testing of the final product (including outsourcing)
- Supervision of retention samples (if applicable)
- Stability testing
- Staff training
- Equipment maintenance.

All documents relating to above should be readily accessible. The QP responsible for ATIMPs should have the relevant training in this field (cell and tissue biology, biotechnology, characterisation and potency assays) and a detailed knowledge of the manufacturing process for which they are taking responsibility (158). He/she must ensure that each batch meets the GMP regulatory requirements and the requirements of the Product Specification File (PSF, CCGTT) or CTA in relation to:

- All manufacturing steps (including controls and testing)
- Raw materials and starting material
- Verification of donor and recipient match
- Quality of final product excipients
- Transmissible Spongiform Encephalopathy (TSE) status, viral and microbial safety of the final product is in line with CTA
- QC checks on the final product meet the release criteria

- Review of non-conformances and impact on product quality

- Appropriate storage and transport conditions

- GMP requirements/regulations and product specification in the destination country (if being exported)

1.4.2.3 The tenets of cGMP: Facilities

The clean room facilities must conform to GMP standards (158). The premises must be adequately controlled to ensure an aseptic environment. In accordance with EC guidelines and ISO 146441-1, the airborne particle concentration and microbial load cut-off is dependent on the laboratory grade and is displayed in Table 3 and Table 4, respectively.

Grade	Recommended maximum limits for particles $\geqq 0.5\ \mu m/m^3$		Recommended maximum limits for particles $\geqq 5\ \mu m/m^3$	
	in operation	at rest	in operation	at rest
A	3 520	3 520	20*	20*
B	352 000	3 520	2 900	29
C	3 520 000	352 000	29 000	2 900
D	Set a limit based on the risk assessment	3 520 000	Set a limit based on the risk assessment	29 000

* Due to limitations of monitoring equipment a value of 20 has been retained. Frequent sustained recoveries below that value should also trigger an investigation.

Table 3: Airborne particle cut-off (non-viable contamination) on basis of laboratory grade (158)

Grade	Air sample cfu/m3	Settle plates (diameter 90mm) cfu/4 hours*	Contact plates (diameter 55 mm) cfu/plate	glove print 5fingers cfu/glove
A**	< 1	< 1	< 1	< 1
B	10	5	5	5
C	100	50	25	-
D	200	100	50	-

* Individual settle plates may be exposed for less than 4 hours. Where settle plates are exposed for less than 4 hours the limits in the table should still be used. Settle plates should be exposed for the duration of critical operations and changed as required after 4 hours.

** It should be noted that for grade A the expected result should be 0 cfu recovered; any recovery of 1 cfu or greater should result in an investigation.

Table 4: Limits for microbial contamination (viable contamination) for each laboratory grade (158)

Environmental monitoring (EM) is the method whereby contamination control measures can be assessed. It encompasses viable/non-viable contamination, temperature control and air pressure differentials. Viable contamination is assessed using settle plates, contact plates and finger dab plates within grade A and lower grade environments. The identification of growth of colonies on a

contact/settle plate/finger dab plate should prompt species identification and assessment of impact on product sterility. Non-viable contamination is assessed using airborne particle counter systems. Designated cut-offs are tabulated above. Air pressure control is also an essential component of contamination prevention. Fundamentally, air pressure in the area of higher cleanliness should have a higher positive pressure relative to the area of lower cleanliness with a recommended pressure gradient of 10-15kPa (158).

1.4.2.4 The tenets of cGMP: Equipment

The pieces of equipment commonly required for GMP CAR T-cell manufacture, both open and closed processes, are summarised in Figure 27 and Table 10 (closed process) . Equipment must be validated, be appropriately calibrated and maintained and not represent any hazard to the product (adequate cleaning/storage) (158).

1.4.2.5 The tenets of cGMP: Record keeping

Meticulous documentation is an essential component of a GMP quality system. Primarily, the purpose of this recordkeeping is to monitor and record all components which may compromise the integrity of the process and the quality of the product and to ensure traceability of raw materials/starting material and final product (158). Unambiguous step by step guidance for staff is delineated in standard operating procedure (SOP) documents which are either facility or team specific. The second important component is the retention of relevant results and reports. Data integrity must be maintained by ensuring adequate controls are in place to prevent loss/ accidental damage and unauthorised access or manipulation (keys, personal passwords).

In relation to starting material and raw materials, the following details must be recorded: description, origin (especially if animal/human origin), quality requirements, acceptance criteria, storage and transport conditions, instructions for sampling and testing. Each step of the manufacturing process (BMR, staff and associated training records, reagents/consumables, EM, equipment, deviations, QC and QP reports) must be clear and legible. With regard to the final product, release criteria should be clearly stipulated in the PSF and available to the QP at the time of batch release. Particular attention should be paid to the name/identification of the product, qualitative and quantitative requirements and associated acceptance criteria, shelf life, storage and transport conditions and labelling and packaging (traceability) (158).

1.4.2.6 The tenets of cGMP: Assays for release testing and stability

Release of an ATIMP batch is based on the final product meeting all criteria listed in the Certificate of Analysis (CoA) (158). This controlled document lists the individual tests, test methods, result that must be achieved and actual result obtained for each product and is specific to an individual trial. Broadly speaking, release criteria for ATIMPs usually include viability, cell count, cell identity, purity, sterility and transduction efficiency (if applicable).

Viability is assessed immediately prior to cryopreservation, usually with flow cytometry analysis for 7-aminoactinomycin D (7-AAD) or an exclusion dye such as Trypan Blue. A cut-off of 70% is universally accepted. Identity and purity of the final product is assessed by flow cytometry. Subtyping of the final cell formulation will confirm the presence or absence of contaminating cells. Sterility using the blood culture system such as BD Bactec is used to confirm the final product is microbiologically sterile. Endotoxin and mycoplasma testing are also recommended (158). Potency assays are required only for Phase 3 clinical studies. Options to demonstrate potency of a product include incubation of the CAR-T cell product with CD19-expressing cell lines and evidence of cytokine release on incubation with the relevant antigen.

Robust stability data for each product in specified storage conditions, outlined in the CTA is usually not available at the start of the clinical study but accrued over time. "Ongoing stability programs" should be in place to ensure that the product remains in specification for its shelf life (158).

1.4.2.7 The tenets of cGMP: Packaging, labelling and distribution

ATIMPs should be appropriately packaged, in line with the CTA or marketing authorisation, to ensure safe transit and no leakage or potential for contamination. The quality of the product should be maintained during storage and shipping and inspected for leaks on receipt at stem cell laboratory and prior to infusion. Labelling of ATIMPs must comply with the guidance of Regulation (EU) No 536/2014 (158). Transport conditions should be clearly documented and confirmed to be adequate (temperature-monitored, appropriate container). These conditions are known to have a direct impact on T-cell quality. However, compliance with these conditions surprisingly falls outside the scope of GMP and are not deemed to be the responsibility of the manufacturer (unless contracted) (158).

Outsourcing of tasks such as shipping to other trained personnel should give special consideration to prevention of cross-contamination and maintenance of traceability. In the case of imported ATIMPs the

transport conditions will be checked by the QP prior to batch release to ensure they confirm to the CTA or marketing authorisation.

1.4.3   Current methodologies of CAR T-cell manufacturing to cGMP

CAR T-cell manufacture is a specialised, labour-intensive multi-step process. A typical process comprises the following steps: T-cell isolation, activation, transduction, expansion and cryopreservation (Figure 7) (152). Manufacturing to cGMP requires highly skilled operators and costly clean room infrastructure (159). This process is performed in accordance with GMP standards. Duration ranges from 7-22 days with a mean of 12 days (160), with a general trend towards a shorter process by many research groups.
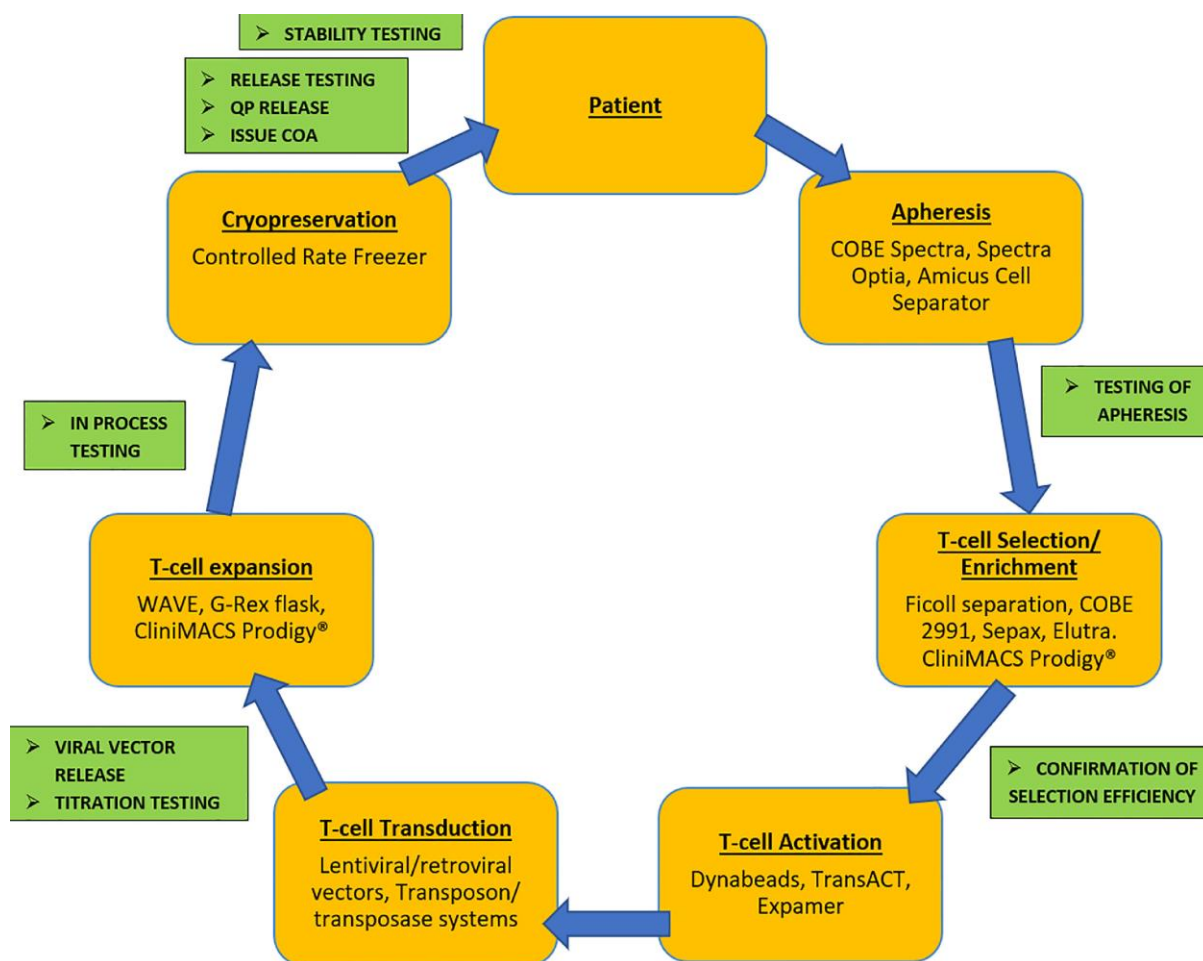
Figure 7: Flowchart of standard elements of CAR-T manufacture (152)

1.4.3.1 cGMP CAR T-cell manufacture: T-cell activation- current practices

T-cell activation is critical to CAR T cell transduction efficiency. It is achieved in vitro using antigen presenting technologies to deliver concurrent TCR and costimulatory signals. The primary signal via the TCR (signal 1) and the secondary co-stimulatory signal (signal 2) via CD28, 4-1BB or OX40 are required for activation (161). Choice of activation method varies widely between centres and clinical studies. Cell-based T-cell activation using artificial antigen-presenting cells (such as dendritic cells, K-562 derived cells) that are HLA-matched and conform to GMP standards are complex (162). This has been surpassed by antibody-based and bead-based T-cell activation. Antibody-coated nanobeads such as MACS GMP TransAct™ (Miltenyi BioTec), a polymeric biodegradable nanomatrix impregnated with anti-CD3/CD28 mAbs are biodegradable and do not require formal bead removal. There are no commercially available assays to determine the presence/absence of residual TransAct™ in the final CAR T cell product, but the Product Information File reports that excess TransAct™ is depleted by culture wash and media exchange steps built into the T Cell Transduction (TCT) protocols. TransAct™ has been shown to be comparable to Dynabeads (Dynal) (163), super-paramagnetic anti-CD3/CD28 antibody coated beads (CTL019, licenced as Kymriah). Validated quality control testing must be performed to ensure removal of Dynabeads from the product prior to infusion. Magnetic bead removal in the GMP clean room is labour-intensive and associated with significant cell loss. This has prompted exploration of non-bead-based activation reagents. An example is Expamer™ (Juno Therapeutics), a soluble T cell activation reagent comprising a streptactin backbone associated with low affinity anti-CD3/CD28 Fab fragments. This is depleted from the T-cell culture by washing steps (164,165).

1.4.3.2 cGMP CAR T-cell manufacture: T-cell transduction- current practices

Transduction is the process whereby a segment of foreign DNA or a foreign gene are transferred into the host cell genome by a vector, viral or otherwise. The introduction of a CAR gene into T-lymphocytes can be achieved using viral and non-viral delivery systems. Retroviral and lentiviral vectors are the most common gene delivery methods used in CAR T cell manufacture, accounting for 41% and 54% respectively of all manufactured products (164). Transduction efficiency requires T-cell activation for gene transfer, particularly when using retroviral vectors (can only transduce dividing cells).

Modified wild type (WT) lentiviruses are vehicles for the delivery, stable integration and expression of cloned genes into cells. The vector must behave as a lentiviral genome to allow it to pass as a virus from the producer cell line, and so contains regions of WT lentiviral genome 'in cis' for incorporation into a viral particle. This includes the packaging sequence (to ensure capsidation of vector RNA into virions), the tRNA binding site (to allow reverse transcription of vector RNA into DNA in the target cell), long terminal repeat (LTR) sequences that permit 'jumping' of reverse transcriptase between RNA strands

during DNA synthesis, and other sequences necessary for integration of vector DNA into the host cell chromosome. Vector genomes do not require that the viral structural proteins gag, pol and env be retained, with the result that non-viral genes can be cloned into the remaining space.

Viral vector manufacture is a multi-step quality-controlled process. 293T cells from a master cell bank are thawed and expanded for 2 weeks in multi-layer cell factories until it reaches the optimal sub-confluent density for DNA plasmid transfection. Multiple cell factories were transfected with the lentiviral vector packaging system comprising of helper plasmids and transfer vector plasmid (all self-inactivating) by methods such as the calcium phosphate method. Viral vector is harvested into sub-batches 72 hours following transfection and undergoes purification and sterile filtration prior to cryopreservation and QC testing (166). GMP viral vector manufacturing facilities in the UK are answerable to the MHRA, in the US to the FDA (166,167).

Viral vectors are extremely expensive due to the labour-intensive manufacture methodology, the requirement for clean room infrastructure and the extensive safety testing needed for vector release. In an attempt to reduce costs, retroviral vectors can be produced using stable packaging cell lines (166). Equivalent stable lentiviral packaging cell lines are in development (168).

Potential risks from retro- and lentiviral vectors include insertional mutagenesis and the generation of replication competent virus (RCV). To date, insertional mutagenesis has never been reported in the CAR field but has been reported in the use of gene therapy for immunodeficiency syndromes (169,170). Strategies to minimise the risk of viral recombination and RCV generation include 'in cis' delivery of the required plasmids for transient transfection of producer cell lines (often HEK293 cells) (171).

Non-viral gene delivery with 'Sleeping Beauty'(SB) (172) or PiggyBac transposon/transposase (173–175) technology is considerably cheaper than viral vector. There is a risk of insertional mutagenesis due to the random nature of gene integration but is thought to be lower than with viral vector. Disadvantages include low gene transfer efficiency and the requirement for prolonged in vitro culture to generate clinically relevant cell numbers (176,177). From a clinical risk perspective, the MHRA stipulate that patients who are received a genetically modified product are followed up for 15 years to monitor for emergence of RCV and where indicated, for integration site analysis.

1.4.3.3 cGMP CAR T-cell manufacture: T-cell expansion- current practices

T-cells are expanded in functionally closed culture systems following gene transfer to reach the cell numbers required for clinical application. A variety of methods can be used including T-flasks, plates or culture bags and bioreactors such as the G-Rex flask (Wilson Wolf Manufacturing), the WAVE Bioreactor (GE Life Systems) and the CliniMACS Prodigy® (Miltenyi BioTec). T flasks are

impractical for large scale work as they are labour intensive and require multiple open handling steps in safety cabinets (178). Static culture bags can be welded together via sterile tubing and offer a semi-closed culture system, but media exchange is a manual process and is not easily scalable. Bioreactors offer several advantages: the G-Rex flask (Figure 8) is a closed system cylindrical vessel with optimised gas exchange and sampling options and permits rapid expansion of cells in an incubator (179).

Figure 8: G Rex flask Wilson Wolf

Figure 9: WAVE Bioreactor GE Healthcare Life Sciences

The GE WAVE rocking bioreactor (Figure 9) is an automated, closed culture system comprising a gas permeable culture bag (Cellbag®, GE Healthcare Life Sciences) placed on a rocking platform. Inbuilt sensors for mass flow, pH, gas pressure and concentrations of CO_2 and O_2 allow automated perfusion and gas exchange with the effect of reducing manual labour and media consumption (180,181). Problems with the WAVE include lack of scalability (one product per bioreactor), cost and potential biological implications such as a reported skew towards CD4+ T cell expansion (180).

The choice of culture medium varies between studies and there is a move towards the use of serum free formulations such as X-VIVO 10 (Lonza). Cytokine support with Interleukin 2 (IL-2) or IL-7 plus IL-15 is centre-dependent. From a practical perspective, it is critical for any CAR T cell manufacturing group to secure their supply chain and to be able to validate alternative GMP compliant reagents and consumables to protect their process.

1.4.4   cGMP CAR T-cell manufacture: Increasing automation

From a quality control perspective and to truly enable scalability of autologous CAR T-cell production, the GMP compliant semi-automated, fully closed CliniMACS Prodigy® is an attractive option. The closed system removes the requirement for high classification clean rooms, reduces the risk of product

contamination through minimal operator handling steps and is less labour intensive for staff. The Prodigy® permits T-cell selection, activation, transduction, expansion, sampling and harvesting on a single-use tubing set (TS520) according to a programmable activity matrix (178) and delivers CAR T-cells of similar phenotype and function to those generated by the other methods (182) at cell numbers required for clinical trial application.

The single -use disposable tubing set, inserted into the 24 valves of the device consists of a series input and output lines for sample loading, attachment of buffer, media and reagents, drainage of waste and in-process testing. There is a magnetic separation column and a CentriCult chamber for cell enrichment and cell centrifugation/cultivation respectively (37°C/5% CO2) (183) (Figure 10). Pre-selected T-cell subpopulations (e.g. CD62L, or CD4 and CD8) or unselected whole leukapheresis can be used as starting material and T-cell expansion can exceed 30-fold after 14 days of culture. Evaluation of the potency and toxicity of CAR T-cells manufactured in this way is being prospectively evaluated in clinical studies.

Images of the chamber can be captured in-process using the integrated microscope camera. The CliniMACS Prodigy® has an increasing range of clinical applications. Feasibility has been demonstrated for the generation of CMV-specific T-cells (184), monocyte derived dendritic cells (185), expansion of natural killer cells (186) and isolation of CD34+ stem cells (187,188) and macrophages (189). The use of this device for more complex manufacturing protocols including T-cell enrichment or selection of specific T-cell subset (190) and the generation of CD19/20 CAR-T cells from fresh and frozen healthy donor (HD) material has been demonstrated by several groups (182,191–194). Members of our group have previously demonstrated the feasibility of manufacturing functional CD19 CAR-T cells from HD material using the Prodigy system (195). However, the bulk of the clinical demand will be for autologous CD19 CAR-T products. Until earlier this year there was a paucity of data supporting the use of the CliniMACS Prodigy® in patient-derived material (PM) (191,196). Castella et al now report the successful use of the Prodigy system to generate 28 autologous products for patients with B-ALL, B-NHL and CLL (197). As yet there is no data comparing manual and semi-automated manufacturing methodologies in PM. Other biotech companies are developing alternative proprietary automated cell manufacturing platforms as alternatives to the Prodigy and to help academia and pharma to meet the high demand for products.

Figure 10: Image of CliniMACS Prodigy ® (183)

1.4.4.1 cGMP CAR T-cell manufacture: T-cell cryopreservation- current practices

Cryopreservation of CAR T-cell products prior to infusion is standard practice. Advantages include flexible scheduling of patient infusions and time to complete extended quality control tests required for QP review and release. Validated cryopreservation is essential for large scale centralised manufacture. From a quality perspective, this step is critical, as suboptimal cryopreservation can lead to reduced cell numbers, impaired viability and altered cell phenotype and function (198). Most CAR T-cell manufacturing facilities use cryopreservation methods developed for hematopoietic cells, namely resuspension in isotonic buffer containing 10% dimethyl sulfoxide (DMSO), transfer to a controlled-rate freezer and longer-term storage in vapour-phase liquid nitrogen (199). CD3+ T-cells cryopreserved in this way retain viability (post-thaw recovery between 50-90%) (200) with maintained CAR T-cell phenotype and function (201). Lower DMSO concentrations (between 5 to 10%) can be used with extracellular protective agents such as human serum albumin (HSA), plasma, serum, and solutions of hydroxyethyl starch (HES). GMP compliant commercial formulations are also available including CryoStor®, pre-formulated with DMSO in final concentrations of 2%, 5%, and 10%. For clinical use, the DMSO content of the product should not exceed 1g/kg as per consensus of the European society of Bone Marrow Transplantation (EBMT) and the American Association of Blood Banks (AABB) (202).

Some groups passively freeze CAR T-cell products in a -80oC mechanical freezer (203). Increasingly, CAR-T cell cryopreservation is performed in a programmable controlled rate freezer (CRF) which delivers a reduction in temperature of 1ºC per minute to avoid formation of intracellular ice crystals. Once sufficiently dehydrated, cells can be rapidly cooled to the final storage temperature and transferred to vapour-phase liquid nitrogen (LN) for storage at temperatures below -150ºC (204). From a quality perspective, advantages of the CRF include consistency of freeze, versatility of programming and data traceability for individual products. Disadvantages include the high costs associated with the large volumes of liquid nitrogen required to run the device. New mechanical CRFs (e.g. Asymptotes) are less expensive to run and overcome several safety issues related to the use of liquid nitrogen in enclosed spaces.

Stability testing of individual products should be performed to ensure preserved viability, expression of CAR transgene and functional capability (cytotoxicity or cytokine release data) over time according to validated assays. Prior to infusion, cryopreserved products are retrieved from vapour-phase LN and are transported under principles of good distribution practice (GDP) to hospital sites in temperature-logged, validated LN dry shippers by contracted couriers.

1.5 Chimeric Antigen Receptor (CAR) T-cell apheresis practice

1.5.1   Chimeric Antigen Receptor (CAR) T-cell apheresis practice: Background

The starting material for the manufacture of CAR-T cells is CD3+ T-lymphocytes derived from unstimulated leukapheresis of patients (autologous) or healthy donors (allogeneic). Despite the presumed importance of this starting material, there is no international guidance on the optimal peripheral blood absolute lymphocyte count (ALC) for collection, the optimal number of target cells for manufacture, the removal of contaminating myeloid cells or standardised quality testing of the collected material. The low risk process of apheresis involves the removal of whole blood, separation of the components by centrifugation, removal of the peripheral blood mononuclear cells (PBMCs) and then return of the remaining components to the patient or donor (205,206). Special precautions are required in the paediatric population (207,208). The Spectra Optia (TerumoBCT Inc.) (Figure 11) and the Amicus Cell Separator (Fenwal Inc./Fresenius Kabi AG) are currently approved for this process. The COBE Spectra (TerumoBCT Inc.), the device with the most literature supporting its use (209–211), is no longer approved for cell harvest for T-cell immunotherapy. Despite advances, including increasing automation of the process, inter-donor and intra-donor variability in collection efficiency (CE) persist (212).

Figure 11: Spectra Optia Apheresis System by Terumo BCT

1.5.2   Chimeric Antigen Receptor (CAR) T-cell apheresis practice: Target cells

CD3+ lymphocytes, the target cells for collection, are found in the mononuclear cell layer (Figure 12 (213). There are obvious differences between healthy allogeneic donor and autologous patient-derived material, the latter likely compromised by disease and prior cytotoxic therapy. Recognised risk factors for low CD3+ cell counts in patient-derived starting material include a low proportion of lymphocytes or CD3+ cells, a high proportion of NK cells and high proportions of circulating blasts in the peripheral blood. With a minimum cut-off of 0.6×10^9 and an optimal cut-off of 2×10^9 CD3+ cells, Allen et al reported minimum and optimal yields of 97% and 77% respectively (209). This study, using the COBE Spectra, included 59 paediatric and young adult patients with CD19 and/or CD22-expressing B-cell malignancies and 12 patients with GD2-expressing solid tumours. A more recent study (210) also using the COBE Spectra, with a similar patient demographic (n=99) demonstrated feasibility of apheresis and successful manufacture (96%) with a collection target of 1×10^9 total MNCs/kg and a minimum ALC cut-off of 0.1×10^9 /L. Data is also emerging on the use of the Spectra Optia for T-cell harvest in heavily

pre-treated lymphopenic paediatric patients (214). The minimum ALC and CD3+ count pre-collection varies between studies, Novartis stipulating an ALC > 0.5 x 10^9/L and absolute CD3+ count >0.15 x 10^9/L. In the setting of lymphopenia, the mononuclear cell layer is narrow. This may impact on the red blood cell-white blood cell interface on the machine, complicating a pure lymphocyte yield (213). Until the publications by Tuazon et al (211) and Korell et al (215), there was minimal data on MNC collections for downstream CAR-T cell manufacture in older adults with advanced B-cell malignancies, particularly using the Spectra Optia. Tuazon et al (n=92) identify advancing age, a diagnosis of ALL and a high pre-apheresis platelet count as potential predictors for a CE < 40% using the Spectra Optia (n=21) or the COBE Spectra (n=71). Korell et al demonstrate the feasibility of using the Spectra Optia for T-cell harvest in 41 adults with B-NHL or B-ALL for currently licensed products (n=41).
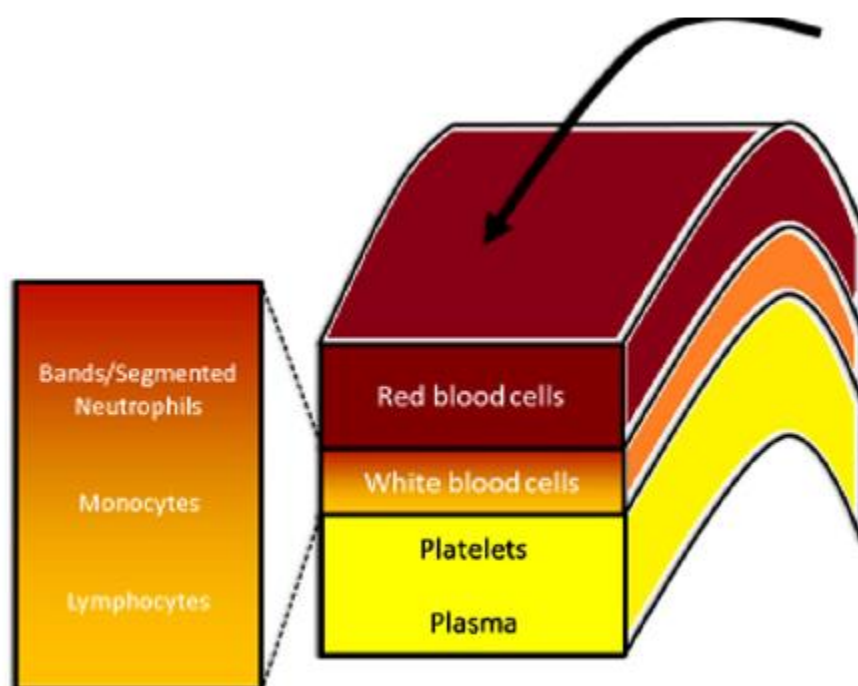
Figure 12: Peripheral blood separation via leukapheresis (213)

1.5.3   Chimeric Antigen Receptor (CAR) T-cell apheresis practice: Contaminating cells

Contamination of apheresate with cells of myeloid lineage, namely monocytes, which also reside in the mononuclear cell layer, has been shown to yield lower numbers of transduced cells and limit T-cell expansion (216–218). Separation of monocytes from lymphocytes prior to commencing T-cell culture can be performed by GMP grade immunomagnetic selection beads (Miltenyi Biotec). Additionally, the

process of elutriation separates cell components on the basis of size (and density), thereby isolating lymphocytes from monocytes (219,220). This has been incorporated successfully into manufacturing protocols in some centres (221). Spectra Optia by TerumoBCT also uses a colour coding system to facilitate isolation of specific portions of the MNC layer (212,222). Contamination of cell concentrates with red cells, platelets and anticoagulant, thought to impact on T-cell activation (223) can be circumvented with the use of cell washing (Lovo Cell Processing System, COBE 2991 Cell Processor, (TerumoBCT Inc)), elutriation (Elutra Cell Separation System, TerumoBCT Inc) and separation methods such as ficolling (Sepax II, Biosafe) and ficoll-free (X-Lab, Thermogenesis/Cesca). The use of this equipment is centre and trial dependent, adding further to the variability of starting material and precluding direct comparisons.

1.5.4   Chimeric Antigen Receptor (CAR) T-cell apheresis practice: Collection protocols

While the COBE Spectra requires intermittent manual intervention, the Spectra Optia is based on counterflow centrifugation elutriation technique: MNC and platelets are extracted by centrifugation from anticoagulated whole blood into a secondary chamber, the platelets removed and returned to the donor. The target MNCs are then transferred from the secondary chamber into a collection bag using collection port which collects the cells on the basis of target optical density and by flushing the chamber periodically with plasma. The frequency of flushing is dependent on the detection of RBC in the collection line (224,225). T-cell collection using the Spectra Optia can be performed using the MNC or continuous MNC (CMNC) protocols. The MNC protocol exerts a high centrifugal force to whole anticoagulated blood entering the separation channel, with a resultant "interface layer". This interface layer contains all leucocytes and (most) platelets. The cells are then transferred to a secondary chamber, platelets extracted by elutriation and returned to the donor. The CMNC protocol exerts a lower centrifugal force, platelets remain suspended in plasma and do not require a second separation step. Both protocols use an automated interface management system, capable of identifying the interface layer and extracting based on target optical density (225). In healthy donor T-cell apheresis for DLI, the CMNC protocol has been shown to be superior in terms of lower blood volumes processed and shorter procedure time (225).

At UCLH the Spectra Optia (TerumoBCT inc) CMNC protocol is used for all T-cell harvests. Improved efficiency and comparable safety in the collection of mononuclear cells for cellular therapy (relative to the COBE Spectra) has been demonstrated in a paediatric cohort (226). No parallel comparison has been performed in an adult population. Apheresis protocols for T-cell immunotherapy are drafted at an institutional level.

1.5.5   Chimeric Antigen Receptor (CAR) T-cell apheresis practice: The future

The future of T-cell harvest for CAR-T cell manufacture will be approached from several different starting points. The advent of allogenic "off the shelf" CAR-T cell products (Section 8.2.5.2), albeit with their own risks, will circumvent the known qualitative and quantitative limitations of patient-derived starting material. These limitations are often related to fixed patient variables such as age, underlying diagnosis and lines of previous therapy. Ex-vivo processing to optimise autologous collections is another avenue under exploration (Section 8.5.1.1). Thirdly, the timing of MNC collections, namely moving the apheresis process to earlier in the treatment pathway, could potentially preserve T-cell quantity and quality for downstream manufacture. The key will be to determine which variables in the starting material have an impact on CAR-T cell product efficacy and persistence (Section 8.3.4.1).

1.6 CAR T-cell clinical service development: Current paradigms

Memorial Sloan Kettering Cancer Centre (MSKCC) have defined the essential components required for the clinical implementation of commercial and clinical trial CAR-T cell products (227,228). This has prompted other centres to publish their approach to establishing a CAR-T service (229). The majority of centres delivering these therapies are FACT-JACIE accredited and leverage the infrastructure already in place for haematopoietic stem cell transplantation (HSCT) to integrate these therapies (230,231). HSCT units are equipped to support regulatory, cellular processing, quality, data management and clinical care delivery systems required to integrate these therapies. This allows the centre to capitalise on optimised workflow systems. Whether integration into the HSCT, disease-specific or independent immunotherapy service is deemed the most appropriate approach for an individual centre (232), robust infrastructure, extensive administrative and multi-disciplinary co-ordination and communication is required to manage the complex scheduling logistics, maintain the chain of identity and deliver safe clinical care. The mode in which the clinical infrastructure is established is defined at a local level.

An example 8-step work-flow model based on that proposed by MSKCC (228) is detailed below.

1.6.1   CAR T-cell clinical service development: Patient intake

This initial step at MSKCC is led by administrative, nursing and financial staff. Pre-screening is co-ordinated by a clinical nurse co-ordinator. Review of full patients history including pathology reports, imaging and CD19 status of the tumour at the pre-screening phase can expedite treatment for patients, CAR-T cell therapy or further chemotherapy (227).

While not applicable for clinical trial products, policy for reimbursement for licensed CAR-T cell products may differ between member states. It is recommended that the efficacy and safety data is reviewed and a care value assessment made (230). A survey by ASBMT reported that licenced CAR-T cell products required approval by an "institutional committee" in 60% of cases. In the majority of cases, this was a HSCT committee (230).

1.6.2   CAR T-cell clinical service development: CAR-T consultation Service

At MSKCC this consultation service is spearheaded by CAR-T cell physicians (certified CAR physicians), nurses and social workers. Eligibility for commercial and trial products, fitness for therapy and disease burden is assessed. The patients are discussed at a weekly MDT meeting attended by CAR and disease-specific physicians.

ASBMT recommends that each patient's clinical case is discussed at a MDT meeting at an institutional level to review eligibility and to ensure there is a consensus on the choice of CAR-T cell product (230). A survey by the Chronic Malignancies Working Party of EBMT highlights the lack of consensus amongst physicians in relation to CAR-T cell therapy exclusion criteria (231), particularly regarding prior allograft, autoimmune disease and a history of malignancy.

1.6.3   CAR T-cell clinical service development: Cell collection, shipping and receipt

T-cell apheresis is discussed in Section 1.5. Feasibility of cell collection and CAR-T cell manufacture has been demonstrated with lymphocyte counts as low as 0.1×10^9/L in paediatric and young adult patients (210). Apheresis unit and stem cell laboratories must have appropriate capacity and training. Priority is given to maintaining the chain of identity of cells. Cellular processes must be in line with FACT/JACIE guidelines and manufacturer's instructions.

Most centres use the infrastructure in place for HSCT to integrate these therapies. This avoids system redundancies and allows centres to capitalise on optimised work-flow systems (230). Eligibility for

apheresis is defined and assessed locally. Most respondents in the EBMT survey agreed that a poor performance status (ECOG>2) and a history significant cardiac disease (Class III or IV congestive cardiac failure, poorly controlled angina, myocardial infarction or ventricular arrhythmia within 6 months) should preclude apheresis (231).

1.6.4   CAR T-cell clinical service development: Bridging Strategy

Bridging period can be 1-2 months depending on cell expansion rates, treatment planning and insurance authorisation (US). Close communication between the manufacturing facility, CAR-T cell centre and primary haematologist in the referring centre allows for treatment planning post cell harvest. Evaluation of pace of disease progression and expected date of infusion is paramount. Patients must be counselled regarding the risk of manufacturing failure. Bridging chemotherapy aims to maximise disease control whilst minimising toxicity and regimens can be highly variable (227). The use of radiation as a bridging strategy has been explored in small numbers of patients with no demonstrable adverse effect on CAR-T toxicity profiles (233–238). This is an attractive option to obtain disease control whilst avoiding systemic side effects.

1.6.5   CAR T-cell clinical service development: Lymphodepletion

Hospital pharmacy plays an integral role and conditioning regimen order sets for fludarabine and cyclophosphamide (specific for individual commercial products) must be incorporated into chemotherapy protocols. Given this is considered a low toxicity regimen, administration of lymphodepleting chemotherapy in the outpatient setting is appropriate based on patient risk assessment and is facilitated by 41.9% of centres in the ASBMT survey (230). Most investigators consider poor performance status (ECOG>2) as an contraindication to commencing lymphodepletion (231).

1.6.6   CAR T-cell clinical service development: CAR-T cell infusion

SOPs for documentation and verification of infusion (in line with FACT/JACIE) with involvement of hospital pharmacy must be established. Documents (SOPs and infusion records) already in place in HSCT units, can serve as a foundation for the development of ATIMP-specific documents relating to cell thaw and infusion. Up to 77% of CAR-T cell infusions are administered in the inpatient setting (230). Site of infusion must take disease burden, patient co-morbidities and product-specific toxicity

profiles into account (high rates of early toxicity with Axicel (47)). Outpatient administration and management is an option in selected centres with careful patient selection. The patient must be reviewed on the day of infusion to ensure that they are fit to proceed. The communication pathway between the treating physicians, stem cell laboratory and infusion team must be established locally. At MSKCC the patients are consented specifically for the CAR-T infusion (228).

1.6.7   CAR T-cell clinical service development: Post Infusion Care and Toxicity management

Anticipated patient volumes, average length of stay (often stipulated in trial protocol) and toxicity rates must be considered for service planning (staffing, inpatient capacity including ITU).

Known toxicities of CD19 directed therapy include cytokine release syndrome (CRS), neurotoxicity, HLH/MAS, tumour lysis syndrome, cytopenia, B-cell aplasia and infection (239). CRS is supraphysiological immune system activation characterised by fever and increased production and release of cytokines (effector cytokines and those associated with macrophage activation) including interferon gamma, TNFa, IL2 IL6, IL8 and IL10 Often the first clinical sign of CRS is high fever, often ≥40 °C. This may progress to life-threatening hypoxia, shock and end organ dysfunction. The risk and incidence of CRS (35-93%) varies depending on the CAR-T cell product used, the nature and burden of underlying disease (especially in B-ALL), the CAR-T cell dose and the presence of concomitant infection (240).

After infusion, CAR-T cells home to the bone marrow, lymph nodes and tissues, where they recognize the relevant antigen (CD19 in B-ALL and DLBCL), are activated and proliferate rapidly. This usually correlates with CAR-T expansion in the peripheral blood (241,242).

The understanding of the pathophysiology of CRS and neurotoxicity continues to evolve, with many mechanisms not yet fully elucidated. Data using xenogeneic mouse models suggests that monocytes and macrophages play a key role in the development of both CRS and neurotoxicity (243–245). Macrophages appear to communicate with activated CAR-Ts either via costimulatory receptors or T cell–derived cytokines. This leads to further activation of macrophages, which then secrete chemokines, cytokines and inducible nitric oxide synthase, which further activate the CAR-T cells and recruit innate immune cells exacerbating the inflammatory response. A schematic representation of this process is displayed in Figure 13 (245).
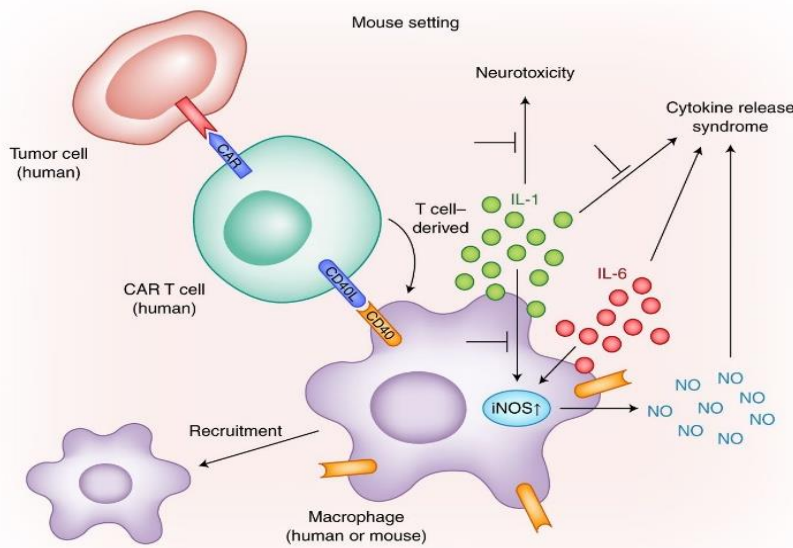
Figure 13: Suggested pathophysiology for cytokine release syndrome and neurotoxicity (245)

Other suggested mechanisms for CAR-T related neurotoxicity, a clinical syndrome which may range from a tremor and mild expressive dysphasia to global aphasia, cerebral oedema and seizures, include endothelial activation and blood brain barrier disruption (246), elevated levels of NMDA receptor agonist in the cerebrospinal fluid (247) or simply diffusion of inflammatory cytokines into the central nervous system (CNS) with a picture consistent with pan-encephalitis (248). The Phase II ROCKET trial (NCT02535364), sponsored by Juno Therapeutics, was terminated early following the death of 5 patients from cerebral oedema, deemed attributable to rapid CAR T-cell expansion and a surge in IL-15 (249,250). This prompted huge safety concerns and vigilance from practicing centres with regard to toxicity management. A uniform grading system has been devised by the American Society for Transplantation and Cellular Therapy (ASTCT) in an attempt to harmonise CRS and neurotoxicity grading systems across centres and studies internationally (251). Guidance on the management of these toxicities has been published (240) and institution-specific standard operating procedure is applied.

Management guidelines for CRS and neurotoxicity are usually drafted locally based on expert recommendations (240), must be readily available and close communication with intensive care and neurology physicians maintained. In US centres FDA-mandated training for physicians, nurses and pharmacy with an associated knowledge assessment as part of Risk Evaluation Mitigation Strategy (REMS) is performed (228). This may then be audited by manufacturing companies.

Tocilizumab is a humanised monoclonal antibody that blocks the IL-6 receptor, initially approved in rheumatoid arthritis (252) and now licenced for the treatment of CRS. FDA-approved dosing is 8mg/kg (max 800mg) for adults, 12mg/kg if <30kg, doses shown to rapidly reverse CRS (73,239,241). Side

effects include hepatoxicity and cytopenias. However, the main limitation of Tocilizumab is its inability to cross the blood brain barrier (BBB). Blockage of the IL-6 receptor has been shown to increase levels of IL-6 in serum (253), theoretically leading to the passive diffusion of IL-6 into the CNS and potentially increasing the risk of neurotoxicity. Stocks of IL-6 blocking agents such as tocilizumab must be readily available on the ward, for infusion within 2 hours as per REMS requirements.

Siltuximab is an anti-IL-6 monoclonal antibody (human-murine) which binds to IL-6 with high affinity (240) and is approved for the treatment of multicentric Castleman's disease. Given it binds to IL-6 in the circulation, it prevents transit of IL-6 into the CNS, a potential advantage over tocilizumab. It is not yet licenced for CRS and prospective studies are required to compare its relative efficacy with tocilizumab. Dosing is 11mg/kg over one hour. At the current time this agent is considered second or third line for the treatment of CRS (230).

Anakinra is an IL-1 receptor antagonist, currently licenced for rheumatoid arthritis, systemic juvenile idiopathic arthritis, adult onset Still's disease and cryopyrin-associated periodic syndromes. It is recommended (off license) for secondary haemophagocytic lymphohistiocytosis and macrophage activation syndrome (including post CAR-T cell therapy). In mouse models there is evidence to suggest that IL-1 release proceeds IL-6 release and may initiate IL-6 release. Use of this agent in CRS (not yet approved) may potentially halt the inflammatory cascade at an earlier stage (245). This concept has prompted the use of prophylactic anakinra in patient receiving CD19-targeting CAR-T cell therapy (NCT04359784, NCT04150913, NCT04148430).

Corticosteroids are reserved for second line after tocilizumab in the treatment of CRS. This is due to the concern that they may hamper the persistence and efficacy of the infused cells (unsupported by clinical data). Precise dosing and schedule are debated, with the majority of centres using dexamethasone (due to better CNS penetration) (254) and reserving the use of methylprednisolone for more severe cases (230).

Corticosteroids are the mainstay of therapy for ICANS. Anti-IL-6 therapy is given in the setting of concurrent CRS. There is no international consensus on seizure prophylaxis. As it is not possible to predict which patients will develop ICANS, seizure prophylaxis is recommended in patients receiving products known to cause ICANS (240,255). Almost two thirds of respondents prescribe seizure prophylaxis in the ASBMT survey, levetiracetam the agent of choice given its favourable side effect and interaction profile and lack of effect on cytokine levels (230,256). Most centres continue this agent for 30-60 days (230).

Experimental therapies: Given the significant morbidity (and known mortality) associated with CAR-T cell mediated neurotoxicity, there is a clinical need for effective prophylactic and/or therapeutic treatments. Other agents under investigation include defibrotide (NCT03954106) and the anti-human GM-CSF monoclonal antibody, lenzilumab (NCT04314843). There may be a role for agents such as

itacitinib (Janus associated kinase antagonist) (257) and dasatinib in CRS. Dasatinib inhibits lymphocyte-specific protein tyrosine kinase and has been shown to rapidly and reversibly inhibit CAR-T cells (258,259).

1.6.8   CAR T-cell clinical service development: Follow-up for response and toxicity

The role of the dedicated CAR T-cell clinic: transition to the outpatient setting should be streamlined with an appropriate pathway (disease-specific or CAR team) and documentation to support this. Outside of clinical trials, most centres have not yet established a dedicated long-term follow up model (231). The MHRA and FDA stipulate 15 years of follow-up for recipients of a genetically modified product, aiming to capture potential secondary malignancies and insertional mutagenesis. "Late effects" clinics within HSCT services could provide a model of care for CAR-T patients. Whilst shared care with referring centres may be possible for licenced products, specific testing may only be available centrally (CAR-T cell persistence).

Disease response evaluation: treatment response assessed at month 1. Data from small studies have not demonstrated a survival difference between ALL patients who achieved a MRD negative remission post CAR and did or did not proceed to a bone marrow transplant (91). However, the decision to proceed with a bone marrow transplant in patients with B-ALL in remission post CAR should be considered on a case by case basis pending a prospective randomised study of "CAR only" versus "bridge to allograft".

Late cytopenia: Grade 3 or 4 cytopenia beyond day 30 can occur in up to 30% of patients, requiring growth factor and/or red cell and platelet support (47,50). These cytopenias are potentially attributable to CAR activity, which is thought to suppress haematopoiesis with subsequent bone marrow hypocellularity (260). In another study of ALL, NHL and CLL patients led by the Fred Hutchinson Cancer Research Centre, prolonged significant cytopenias were noted in 16% of patients of 15.2 to 21.7 months duration (261). Anti-infective prophylaxis to include anti-bacterial and anti-fungal cover is recommended during periods of prolonged neutropenia. Anti-viral and pneumocytis jiroveci pneumonia prophylaxis should be considered for up to a year in these patients (260).

Hypogammaglobulinaemia and B-cell aplasia: may precede and is expected post CAR-T cell therapy (73,261,262) but may eventually resolve. In a study by Hill et al, 17 of 82 patients (21%) had B-cell recovery by day 90 (262). In the updated Zuma-1 data, recovery of B-cell function was detected in patients with ongoing clinical responses in 17%, 61% and 75% of patients (n=33) at 3, 9 and 12 months respectively (262), suggesting that persistence of functional CAR-T cells is not be a prerequisite for sustained responses in B-cell lymphoma. Children may be more susceptible than adults (boosted by

infection or re-vaccination) to symptomatic hypogammaglobulinaemia post CAR-T cell therapy due to the relative deficiency of established plasma cell clones (263). CD19 expression wanes as B-cells terminally differentiate into CD138+ CD38+ plasma cells, suggesting that eradication of CD19+ clones may not deplete the entire plasma cell department, maintaining some memory antibody responses (264). The use of prophylactic intravenous immunoglobulin (IVIG) post CAR-T cell therapy is not supported by any randomised clinical trials. The decision to administer IVIG is centre-dependent, some choosing to administer empirically to all patients with IgG<400mg/dL and others selecting patients with IgG<400mg/dL and recurrent bacterial infections. A guidance document has recently been published (not evidence based) (265).

Rates of infection in 133 adult patients (median age 54 years) with ALL, NHL and CLL within 28 days of CAR-T cell infusion have been reported at 23% (infection density of 1.19 per 100 days at risk) with lower rates from day 29-90 (infection density 0.67 per 100 days at risk). In the first 28 days, bacterial infections were more common (17%) with a higher prevalence of viral infections from day 29-90. Overall, 41% of infections in the first 90 days were considered severe, 6% life threatening (262). Park et al report rates of infection of 42% in adult ALL patients in the first 30 days post CAR. Again, bacterial infection predominated in the early post treatment phase, with higher rates of viral infection (mainly respiratory viruses) between day 31 and 180 (91). Higher grades of CRS conferred a greater risk of infection, particularly bacterial infections (91,262). Rates of infection presenting beyond day 90 in 54 patients with ALL, NHL and CLL were 61% (infection density 0.55 per 100 days at risk), predominantly upper and lower respiratory tract infections. Microbiological isolates were obtained in 24% of infections events (60% bacterial, 31% viral and 9% fungal) (261).

Infections post CAR-T cell therapy are multifactorial; pre-existing immunosuppression due to previous cytotoxic therapy or HSCT, poor marrow reserve, concomitant disease, lymphodepletion with secondary cytopenias and mucosal disruption, CRS as an independent risk factor and associated immunosuppressive treatments (tocilizumab, steroids) may contribute. Choice and duration of anti-bacterial, anti-viral, PCP and anti-fungal prophylaxis is not evidence-based and is decided at an institutional level. Low CD4 counts have been demonstrated at 1 year post Axicel therapy (median 155cells/ul) (266). There is some guidance in this field (267). Similarly, with re-vaccination programmes post CAR, data is extrapolated from other patient cohorts (HSCT, recipients of Rituximab) in the absence of formalised guidance. Recipient of Rituximab have been shown to mount antibody responses 6 months post therapy, despite the absence of peripherally detected B-cells (268). Antibody responses post CD19 CAR-T cells may be elicited by long-lived CD19 negative plasma cells (269). Guidelines should be formalised at an institutional level, taking account of available expert guidance (267,270)

Second cancers: retroviral and lentiviral vectors harbour the risk of replication competency and insertional mutagenesis (oncogenic activation). Given the short follow-up to date, the risk of secondary malignancies cannot be estimated. Many of these patients have received multiple lines of previous therapy, including HSCT, conferring a malignancy risk above the baseline population risk. A subsequent malignancy rate of 15% (non-melanomatous skin cancer, melanoma, MM, MDS) was reported in one study in a heavily pre-treated patient cohort, many of whom had pre-existing risk factors (cytogenetic abnormalities, MGUS, previous HSCT) (261).

No cases of T-cell malignancy post CAR-T cell infusion or replication-competent retro/lentivirus in T-cell products have been reported to date. One case of a transduced leukaemia blasts with a resultant resistant ALL has been published (271).

1.6.9   CAR T-cell clinical service development: Financial, regulatory and reporting requirements

Financial requirements: billing and reimbursement protocols in the US are managed by administrative personnel. The MHRA and FDA require 15 years of follow-up for recipients of genetically modified products, primarily to assess for insertional mutagenesis and secondary malignancies. Data managers are required to collate outcome and adverse event data, the latter also reportable to the FDA or the manufacturer.

The 8-step workflow model based on the model proposed by MSKCC captures the essential components of the pathway of a potential CAR-T cell patient and is transferrable to cellular therapy centres in the EU (with the exception of billing/insurance and financial requirements).

Regulatory requirement: FACT and JACIEs: while regulatory bodies such as the FDA and EMA are responsible for the approval and regulation of these therapies, the Foundation for Accreditation of Cellular Therapy (FACT) and Joint Accreditation Committee ISCT-Europe & EBMT (JACIE) stipulate standards specific to cellular therapy processes. FACT was established in 1996 by the ASBMT and the International Society of Cellular Therapy (ISCT) with the goal of formalising evidence-based guidance in bone marrow transplantation and cellular therapy practice. FACT provide a voluntary accreditation program (3-yearly inspections) for HSCT units and Umbilical Cord Banks (UCB) with 90% of eligible US centres holding accreditation (272). Building on the standards and practice established for HSCT units, FACT have formulated an accreditation program and guidance specific to Immune Effector Cells (IEC). IECs are defined as "cells used to modulate, elicit, or mitigate an immune response for therapeutic intent". This is inclusive of dendritic, natural killer, T or B cells. Similar to guidelines in the Common Standards, focusing on donor workup, apheresis collection, labelling, storage, documentation, and product administration (273), the IEC-specific standards give particular attention to **(1)** Chain of

custody of cells (particularly given manufacture may be performed by a 3rd party) **(2)** management of CRS including training of relevant staff **(3)** co-ordination with and education of other subspecialist teams, with rapid escalation of care if required and finally, **(4)** data collection on safety, efficacy and outcomes (272).

JACIE is Europe's only official accreditation body in the field of HSCT and cellular therapy and collaborates with FACT to establish international guidance. In order to administer licensed CAR-T cell products in the UK, hospitals have to be JACIE accredited and assigned as a designated ATIMP centre by NHS England. Similar to FACT, JACIE stipulate standards on quality management systems, staff training and education, toxicity management plans and communication pathways with relevant subspecialties.

1.7 Aims of the project

This project undertakes to address the bottlenecks and limitations of CAR T-cell delivery in the academic setting. The focus will be to:

(1) investigate and establish a streamlined manufacturing method and platform for CAR T-cell manufacture for the UCL CAR T-cell programme with a focus on autologous products for patients with B-ALL (the ALLCAR19 study).

(2) explore the single-centre apheresis practice at University College London Hospital (UCLH) to define a model/guideline for successful CAR T-cell apheresis and

(3) to begin to establish the clinical infrastructure required to deliver these therapies 'routinely' at a large central London teaching hospital. These lessons will be directly applied through an NIHR-sponsored clinical trial, ALLCAR19.

1.7.1   The ALLCAR19 clinical study

ALLCAR19 is a 20-patient open label single site Phase I study of autologous CD19 CAR T-cells for relapsed/refractory B-ALL (NCT02935257) with GMP manufacturing of CAR T cells in an academic cGMP facility affiliated to UCL. Current manufacture methodologies are outlined in Section 1.4.3. T-cells are genetically modified with a lentiviral vector coding for a second generation CD19 CAR derived from the CAT13.1E10 hybridoma, (CATCAR, Figure 14). The CD19 binding domain has a faster binding off-rate relative to other CD19 CARs, a characteristic which will hopefully translate into a slower tempo of activity and longer engraftment leading to lesser toxicity and a more durable response.
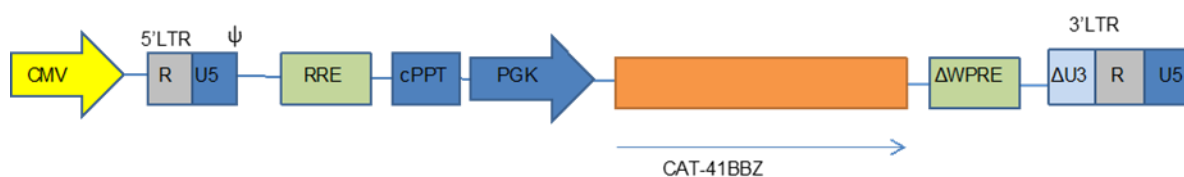
Figure 14: Map of CAT CAR vector

On the UCL CAR T-cell programme the open Dynabead process is in use as per University of Pennsylvania and has been validated in the context of the CARPALL trial (trials.gov. NCT02443831) for paediatric patients with B-ALL. Initial attempts to use this method for the ALLCAR19 adult B-ALL study were challenging due to the huge workforce, clean room time and QC efforts required to produce and release each product. Furthermore, the multiple user-dependent steps lend itself to the process itself being variable and error-prone. In an attempt to standardise and simplify the manufacture method to improve standardisation and scalability, we initiated this research project to investigate a semi-automated, closed manufacture system in place of the conventional open Dynabead based process. A central project goal is to validate and optimise a Miltenyi CliniMACS Prodigy® based cell manufacture process for the generation of autologous CD19 CAR-T cells for use on the ALLCAR19 study.

1.7.2   Apheresis practice at UCLH

In accordance with local practice at UCLH, a standard 2 x blood volume (BV) apheresis is performed on the Spectra Optia, unless absolute lymphocytes count (ALC) is <0.5 x 10^9/L, prompting escalation to a 2.5 BV harvest. However, a personalised approach is preferred, to identify higher risk patients, thereby shortening and prolonging procedure time as appropriate, to improve the patient experience and to increase the capacity of our unit. This approach must include variables that are readily available in the clinic for ease of use. In our experience in adult patients with advanced B-cell malignancies enrolled on the academic CAR-T cell studies at UCLH, a target of 1.5 x 10^9 CD3+ cells is more than sufficient to proceed with cell manufacture and cryopreserve excess material (to be used in the event of a manufacturing failure).

The aim was to devise an algorithm to be used prospectively at UCLH, incorporating peripheral blood (PB) ALC (x 10^9/L), a CD3+ cell target of 1.5 x 10^9 and litres of blood to be processed.

1.7.3 Clinical infrastructure for CAR-T cell therapy at UCLH

With an optimised CAR-T cell manufacturing process at UCL and parallel NICE approval of two CD19 CAR-T cell products for relapsed/refractory high-grade B cell lymphoma (Tisagen and Axicel) and B-ALL (Tisagen, under the age of 25 years), a significant increase in patient numbers was projected at UCLH. Dedicated streamlined T-cell Immunotherapy infrastructure was required to integrate these therapies. Embedding the UCL CAR Program within the HSCT service was deemed to be the most appropriate and efficient approach, thereby capitalising on optimised workflow systems and reducing system redundancies. UCLH has a large HSCT unit, performing over 100 allografts per year and has the infrastructure to support the regulatory, cellular processing and clinical care delivery systems required for CAR-T cell therapy.

The final aim of this project was to begin to establish and to facilitate the development of the clinical infrastructure required to implement these therapies safely and efficiently.

CHAPTER 2:

2 MATERIALS AND METHODS

2.1 Viral Vector

2.1.1 Lentiviral vector production and concentration

The viral vector used in the process development step of this project was prepared in Martin Pule's laboratory. The transfer plasmid was generated by sub-cloning the CATCAR sequence into a third-generation self-inactivating (SIN) pCCL.PGK lentiviral transfer vector to create the pCCL.PGK.αCD19CAT-41BBζ vector (cloned by one of Martin Pule's research team). The virus was produced by transient triple transfection of HEK 293T cells with the transfer plasmid and 3 helper plasmids (pMDLg/p; pMD.G; pRSV/Rev).

293Ts were plated at a concentration of 1.5- 2x10^6 cells in complete IMDM (Lonza, 10% FCS (Labtech)+1% GlutaMAX (Gibco)) per 10cm plate to ensure 70-85% confluency was reached at 48 hours. Plates were transfected by mixing 470ul plain IMDM with 30ul gene juice per plate. The mixture was incubated at room temperature (RT) for 5 minutes before the addition of 3.13ug of transfer vector, 4.06ul of pMDLg/pRRE, 3.13ul of RSV-rev and 2.19ul of pMD.G2 per plate. The mixture was incubated for a further 15 minutes at room temperature and 500ul was added dropwise per plate. Plates were incubated until harvest at 48 hours post transfection. Virus supernatants were pooled, aliquoted and snap frozen in a dry ice and ethanol bath. Supernatants were stored at -80ºC.

As we were targeting a specific MOI, some virus was concentrated and titered.

The ultracentrifuge (UC) was cooled to 4°C on the day of the 48-hour harvest. The vector was harvested into 50ml falcons and spun at 1000g for 5 minutes before transfer into UC tubes and balanced buckets in the UC. Post ultracentrifugation, the concentrated vector was then aliquoted for freezing by discarding the supernatant, resuspending with OptiMEM and resting each tube on ice (60 minutes) before harvesting the supernatant from the tubes and snap freezing in dry ice and ethanol bath and storing at -80°C.

2.1.2 Viral Vector titration

Following concentration, the vector was titred on 293T cells (decreasing volume of supernatant are used to transduce a fixed volume of adherent cells) and healthy donor T-cells. The titre is defined as the

number of cells transduced per ml of viral supernatant. Relative titre is more accurate than absolute titre and titration should always be performed relative to a virus of known concentration.

Viral Vector titration using 293T cells

The titre of the concentrated virus was determined by flow cytometry by analysing CAR expression on 293Ts following transduction. $1x10^5$ 293T-cells were plated per well of a 12 well plate in 1ml complete IMDM supplemented with 5ug/ml polybrene (Millipore). Plated cells were incubated for 4 hours. Vector samples are thawed and serially diluted in cold complete IMDM for both control and reference vector aliquots before adding to the 293T cells. Plates were incubated for 72 hours and CAR expression was determined using flow cytometry. Only transduction efficiencies between 5-25% CAR+ cells were used to determine the titre using the formula below.

$$\text{Titre}\left(\frac{\text{IU}}{\text{ml}}\right) = \frac{\text{no. of cells at transduction x} \dfrac{\text{\% CAR positive cells}}{100}}{\text{End diluted vector volume (ml)}}$$

Viral Vector titration using PBMCs

Healthy donor blood (2 donors) was collected into EDTA blood tubes. PBMCs were isolated using density gradient centrifugation. Whole blood was diluted at a 1:1 ratio in plain RPMI (pRPMI) and layered on Ficoll® Paque (GE Healthcare). Tubes were spun at 750G for 40 minutes with no break and acceleration to generate plasma, PBMC, Ficoll and granulocyte/erythrocyte gradients. PBMCs were carefully collected and washed twice, once in pRPMI and once in complete RPMI (cRPMI, RPMI 1640; Lonza +10% FBS) prior to use. PBMCS were then plated at $2x10^6$ cells/ml in cRPMI with 0.5μg/ml anti-CD3 (Miltenyi Biotec) and 0.5μg/ml anti-CD28 (Miltenyi Biotec). Cells were incubated for 18 hours followed by the addition of IL-2 (GenScript, 100 IU/ml) and a further 6-hour incubation. The thawed vector (control and aliquot for titration) were diluted in cRPMI for the relevant MOI conditions (MOI 1.25, MOI 2.5, MOI 5), plated onto Retronectin-treated (Clontech, Takara Bio) non-tissue culture plates and stored at 4°C for 30 minutes. The PBMCs were resuspended at $6x10^5$ cells/mL with 400 IU IL-2/ml and 500ul of this mix was added to each well of the vector-coated Retronectin-treated plate. The plated were centrifuged at 1000g for 40 minutes and incubated for 72 hours. On day 4 the cells were harvested from the Retronectin plate, washed with cRPMI and supplemented with 100IU/ml of IL-2. On day 7 CAR expression on the vector-transduced PMBCs and controls was assessed by flow cytometry.

2.1.3 Free Viral Vector Assay

The purpose of this assay was to determine the infectivity risk of intermediate and final products generated using the CliniMACS Prodigy. Supernatants of cell suspension (2mls) were collected during a validation process for ALLCAR19 (vector BR-VVX-106) on day 0 (before transduction, hence non-transduced cells), days 4, 6 and 8. Supernatant was frozen at -80°C. The collected supernatants were thawed and run in parallel at the end of the process to assess vector titre.

293T cells were seeded at $1x10^5$ cells per well in 1ml IMDM (Lonza) +10% FBS (Labtech) per well, incubated for 6 hours, after which polybrene (Millipore, UK) at 5ug/ml is added to each well. The supernatants collected on day 0, 4, 6 and 8 of process were thawed and 200µl is added to each well. Cells were incubated for 60-72 hours and then harvested using Cell Dissociation Solution (Sigma-Aldrich). Cells were primary stained for the transgene CAT19CAR using recombinant CD19-rbIgG1Fc fusion protein secreted from a producer cell line (termed MP21502) and secondary stained using goat anti-rabbit IgG-PE (111-116-144, R-Phycoerythrin Affini PureF(ab')₂ Fragment Goat Anti-Rabbit IgG (H+L); 1Jackson Immuno Research Inc). Viability was assessed using FVD-eFluor506 (65-0866, Fixable Viability Dye eFluor® 506, Invitrogen; Thermo Fisher Scientific) as per table below. Percentage transduction of cellular supernatant from day 0, 4 , 6 and 8 of the manufacturing process was then determined by flow cytometry

Primary Stain	Secondary Stain
CD19-rbIgG1Fc (fusion protein secreted from a producer cell line (MP21502), that binds specifically to the CAT19CAR transgene).	Goat anti-rabbit IgG-PE (for detection of CD19-rbIgG1Fc)
	FVD-eFluor 506 (Fixable Viability Dye eFluor® 506)

Table 5: Staining panel for residual ALLCAR19 viral vector

2.2 Tissue culture and cell lines

HEK 293T cells are derived from human embryonic kidney cells and are characterised by their ease of transfection and high transgene expression. Optimal culture conditions involved 1:4 passage every 2-3 days in IMDM (Sigma Aldrich), supplemented with 10% heat-inactivated (FCS) (Labtech), 2mM L-glutamine (Gibco), 100U/mL penicillin and 100µg/mL streptomycin (Gibco).

Suspension cells used included SUPT1 T lymphoblasts derived from a child with T-cell lymphoblastic leukaemia. SUPT1 CD19 cells, transduced to express the CD19 antigen and RAJI B lymphocytes derived from a child with Burkitt's lymphoma. All cells were cultured in T75cm^2 flasks containing RPMI medium (Lonza), supplemented with 10% FCS and 1% glutamax (Gibco) (cRPMI) in a humified incubator at 37°C and 5%CO$_2$.

2.3 T-cell isolation and transduction

Prior to the validation of our CAR-T manufacturing process in autologous patient-derived material on the CliniMACS Prodigy®, patient-derived leukapheresis (diagnosis of B-ALL) was used to generate CD19 CAR-T cells in the process development (PD) laboratory. In order to emulate the "open" (manual) and "closed" (Prodigy) process in the PD laboratory, the following conditions were tested in one donor with parallel non-transduced conditions.

	T-cell Immunomagnetic Selection	**Activation**	**Cytokines**
Condition 1 (Simulating new closed Prodigy® Process)	Yes	TransAct	IL-7 and IL-15
Condition 2 (Simulating open manual process)	No	DynaBeads	No cytokines

2.3.1   CD4/CD8 T-cell selection, activation and lentiviral transduction

To emulate the cGMP Prodigy CAR T-cell manufacturing process, a pure population of T-cells was isolated from the leukapheresis using the Pan T-cell isolation kit (Miltenyi Biotec) following the manufacturer's instructions. T-cells were re-suspended at 1x10^6/ml in TexMACs medium (Miltenyi Biotec) supplemented with 3% human serum (Sigma Aldrich) and 10ng/ml IL7/IL15 (cTexMACS) (Miltenyi Biotech). Cells were activated for 24 hours by adding 10μl/ml of the TransAct (Miltenyi Biotec) reagent. To mimic the cGMP "open" Dynabead CAR-T manufacturing process at GOSH, unselected T-cells were resuspended at 3:1 bead: cell ratio (bead concentration 4x10^8 beads/ml) and plated at 1x10^6 /ml in X-vivo (Lonza) supplemented with 5% Human AB serum.

The T-cells were transduced on retronectin coated plates 24 hours post activation (TransAct and Dynabeads). The concentrated lentivirus was mixed with cTexMACS media to attain a |MOI of 3.5 per well to be transduced. 1ml of this mix was added to the coated 24 well plates. Activated T-cells were resuspended in cTexMACS such that 3×10^5 cells were added to each transduction well in 500ul. Plates were spun at 1000G for 40 minutes prior to incubation for 72 hours. PBMCs were harvested and maintained at 1×10^6/ml in cTexMACS and expanded for 4 days. On day 8, cells for condition 1 and negative controls were harvested and assessed by flow cytometry. For condition 2, Dynabeads were removed on day 7 using Dynamag and cells (and controls) were incubated overnight prior to flow cytometry.

2.4 Flow cytometric analysis

Fluorescence Activated Flow Cytometry (FACS) for the PD work on healthy donor/patient material was performed at the UCL CI on the BD LSR Fortessa (BD, San Jose, CA) or the MACSQuant® Analyser 10 (Miltenyi Biotec). FACS for the GMP-compliant validation processes was performed at the CCGTT on the BD Celesta (BD, San Jose, CA).

Analysis was done with FloJo 10 software (Tree Star Inc., Ashland OR).

2.4.1 Trucount™ analysis

Cell counting for the release assay validation and Prodigy manufactures was performed using Trucount™ tubes (BD). CD3, CD45 and 7-AAD (5uL of each) were added to cells (0.5×10^6) in the Trucount™ tube and incubated for 20 minutes at RT in the dark, resuspended in PBS and run on the flow cytometer. The number of live T-cells was established using the following algorithm.

Calculation of cell count using Trucount™
Bead events: _____
Gated events: _____
Absolute number of CD3$^+$ CD45$^+$ viable cells per µL =
$$\frac{CD3^+/CD45^+ \text{viable events}}{\text{Bead events}} \times \frac{\text{Known number of beads}}{\text{test volume (µL)}}$$ $$\times \text{dilution factor} = _____$$

2.4.2 Transduction efficiency for ALLCAR19

Cell surface staining to assess transduction efficiency was determined on final day of process using CD45, CD3 and CAR stain. Staining for CAT CAR consisted of the addition of 5uL of human Fc block (BD, Biosciences) to cells (0.5×10^6) and incubation for 10 minutes in the dark at RT. The cells were then incubated with anti-idiotype for 20 minutes in the dark at RT. An anti-idiotype antibody is an antibody which binds to the variable part of another antibody including the unique antigen binding site (a combination of epitopes). The secondary stains were added (Table 6) (anti-rat antibody conjugated to PE, CD3, CD45 and viability) and cells were incubated in the dark for 20 minutes. Cells were resuspended in PBS prior to flow cytometry.

Reagent	Conjugate	Clone	Manufacturer	Volume (uL)
Anti-human CD3	APC-Cy7	UCHT1	Biolegend	5
Anti-human CD45	FITC	2D1	BD	5
Human Fc gamma block	NA	NA	BD	5
Anti-rat Ig	PE	Polyclonal	Biolegend	5
7AAD	NA	NA	Miltenyi	5
Anti-CAT idiotype	NA	NA	Evitria	1

Table 6: CATCAR transduction efficiency flow staining panel

2.4.3 Extended phenotyping

Cellular composition, T-cell maturation and exhaustion profiles of starting material and final products and non-transduced controls were analysed on day 0 (before selection) and on final day of process for CD19 CAR-T cells generated in the PD laboratory as per Section 2.3 and GMP-grade products at the CCGTT (Section 2.8). For the cellular composition, maturation and exhaustion panels, cells are prepared as per Section 2.4.2 (Fc block and anti-idiotype staining, incubation and wash) followed by 20 minutes of incubation at RT in the dark with secondary antibody panels displayed in Table 7, 8 and 9 respectively. Composition panel includes fluorescent antibodies against CD45, CD3 (T-cells), CD16 and CD56 (NK cells), CD19 and CD10 (B-cells/blasts) and CD14 (monocytes). CD45RA and CCR7 expression was used to differentiate naïve (Tn, CD45RA+/CCR7+), central memory (Tcm, CD45RA-/CCR7+, effector memory (Tem, CD45RA-/CCR7-) and terminally differentiated (Teff, CD45RA+/CCR7-) T-cells. T-cell exhaustion was assessed by labelling cells with fluorescent antibodies against CD4, CD8, PD-1 and Tim-3. 7-Amino-Actinomycin-D (7-AAD) was used to exclude dead cells unless stated otherwise.

Maeve O'Reilly MD (Res)

Reagent	Conjugate	Clone	Manufacturer	Vol per sample (uL)
Anti-human CD3	APC-Cy7	UCHT1	Biolegend	5
Anti-human CD45	VioBlue	REA747	Miltenyi	2
Anti-human CD16	VioGreen	REA423	Miltenyi	2
Anti-human CD56	BV510	HCD56	Biolegend	5
Anti-human CD19	FITC	HIB19	BD	5
Anti-human CD10	FITC	SN5C	Invitrogen	5
Anti-human CD14	APC	REA599	Miltenyi	2
7-AAD	NA	NA	Miltenyi	2
Diluted anti-rat	PE	Polyclonal	Biolegend	5

Table 7: Cellular composition flow staining panel

Reagent	Conjugate	Clone	Manufacturer	Vol per sample (uL)
Anti-human CD3	APC-Cy7	UCHT1	Biolegend	5
Anti-human CD45RA	FITC	HI100	eBiosciences	2
Anti-human CD4	VioGreen	MT466	Miltenyi	2
Anti-human CD8	PB	SK1	Biolegend	5
Anti-human CCR7	APC	GO43H7	Biolegend	5
7-AAD	NA	NA	Miltenyi	2
Diluted anti-rat	PE	Polyclonal	Biolegend	5

Table 8: T-cell maturation flow staining panel

Reagent	Conjugate	Clone	Manufacturer	Vol per sample (uL)
Anti-human CD4	FITC	RPAT4	Biolegend	5
Anti-human CD8	APC	SK1	Biolegend	5
Tim-3	BV500	F382E2	Biolegend	5
PD-1	BV450	EH122H7	Biolegend	5
Viability e780			eBiosciences	0.5
Diluted anti-rat Ig	PE	Polyclonal	Biolegend	5

Table 9: T-cell exhaustion flow staining panel

2.4.4   Cell sorting

Electronic cell sorting was performed on the BD FACS Aria (BD, San Jose, CA) as part of the release assay validation for ALLCAR19. CATCAR CD19 CAR-T cells, generated as described in Section 2.1.2 from healthy donor PBMCs were harvested, resuspended in sterile PBS and stained as per Table 6 (with negative controls) to determine transduction efficiency. Once transduction efficiency is confirmed, cells were transferred to sterile closed FACS tubes at a concentration of 1 x 10^7/ml for sorting on the BD FACS Aria. Sorted cells were collected in tubes containing complete RPMI/normacin (to prevent contamination from sorting).

Following the sort, cell count and purity were assessed. The cells were resuspended at 1.5–2 x 10^6/ml in complete warm RPMI /IL-2/ 1x Normacin and incubated prior to use as per Section 2.9.2.

2.5 Cell-based assays

Flow cytometry based killing assay (FBK)

FBK assays were performed on the products generated on the CliniMACS Prodigy® (Process B). CD19CAR-expressing T cells and NT PBMCS from the same patient were used as effectors. SupT1-NT, SupT1 CD19 and Raji cells were used as GFP-expressing target cell lines. Transduction efficiency on transduced cells was determined by flow cytometry as per Table 6. NT PBMCs were thawed and NK depleted using anti-CD56 magnetic beads (Miltenyi Biotec) to avoid NK-mediated non-specific killing. Cells including CAR-T, NTs, SupT1-NT, SupT1 CD19 and Raji cells were each resuspended in 100ul of complete RPMI at $5x10^5$/ml (1:1 effector to target ratio) before addition to the wells (96 well V-bottom plate Nunc, Thermofisher) in triplicate and incubation for 24 hours and 48 hours. The supernatant was harvested at 24 hours and frozen at -80°C for cytokine analysis (Section 2.6). At 24- and 48-hours cultures were stained with Phycoerythrin-conjugated anti-CD3 antibody and eFluor 506 fixable viability dye (eBioscience). CountBright™ beads (Invitrogen™) were added to each tube to allow absolute determination of target cells numbers. Samples were run on BD FACS Fortessa (BD Biosciences) and analysed using FlowJo v. 10 analysis software. Effector cells were identified as the CD3+GFP- population while target cells were identified as CD19+GFP+ (SupT1-CD19 and Raji cells) or as CD19-GPF+ (SupT1 NT).

CD19-specific cytotoxicity was determined by the number of live target cells remaining in the co-cultures with CD19 CAR-T cells, normalised by the numbers of live targets in the co-cultures with non-transduced T-cells.

2.6 Cytokine assays

Bead Immunoassay

CD19 CAR-T cells generated from Process B were co-cultured with CD19 expressing cell lines (Raji, SupT1-CD19 and SupT1-NT) in a cytotoxicity assay as described in Section 2.5 for 24 hours. 100µl supernatants from these cultures were harvested and analysed for cytokines IL6, IL2 and INFγ, using LEGENDplex™ bead-based immunoassay kit (Biolegend), according to the manufacturer's instructions. Samples were run on LSR Fortessa (BD Biosciences) and the concentration of each analyse extrapolated based on a standard curve using the LEGENDplex™ data analysis software.

2.7 cGMP Methods: 'introduction of a new process'

To facilitate the introduction of the new ALLCAR19 manufacturing process at the CCGTT, a change control document was prepared. A change control is a systematic approach to the introduction of a new process/equipment or a change to an existing process. The change control document for the introduction of the TCT process using the CliniMACS Prodigy® for the ALLCAR19 clinical trial described the rationale, the changes planned, the risks (and mitigating factors) of the new process to the patient, staff, laboratory, product quality and product release. Once the change control document was approved, a qualification and validation (Q+V) protocol was drafted and process validation could commence. The purpose of the Q+V document was to describe the validation process in detail, including the manufacturing schedule, the acceptance criteria and analytical methods for the success of each validation run, in-process controls and the equipment/facilities to be used. The aim of process validation is to demonstrate that a specific manufacturing process can consistently generate results meeting the acceptance criteria outlined in the validation protocol and relevant trial IMPD (despite accepted variability of starting material). Three process validations were performed (Section 2.7.5). All aspects of the process validations (training, reagents, consumables, staff, laboratory sterility, environmental monitoring etc) are in accordance with GMP guidelines (158). On completion of validation, a validation report was prepared.

2.7.1   cGMP practical training to deliver CAR T-cells for clinical trials

In line with initiating manufacturing capability at the CCGTT, a new cGMP manufacturing team was established. Training was split into 2 sections:

(1) CCGTT specific/cGMP training to access and safely use the clean rooms

(2) specific training related to CAR T-cell manufacture.

The latter training and guidance documentation were drafted by team members with experience in manufacturing gene-modified T-cell therapies. Training time to functionality was approximately 4-6 months. Process-specific SOPs (drafted by our team) were assessed at a team level while designated facility-specific SOPs on GMP systems (Table 2) were assessed by the CCGTT quality team. Prior to authorisation to commence aseptic manufacturing, each team member had to perform a "Broth transfer test". This test is a requirement for all new starters and every 6 months for trained clean room technicians and assesses aseptic technique in a grade A environment, negative blood culture bottles after 10 days of incubation denoting a pass. Competence in gowning, environmental monitoring and cleaning of equipment was demonstrated for each staff member.

Validation of the CliniMACS Prodigy for the ALLCAR19 clinical trial was performed by 4 staff members. As our team has expanded, team members are now allocated specific responsibilities (flow cytometry, incoming materials, QC). More experienced team members are given the opportunity to lead on a product manufacture. A weekly, minuted team meeting has been established to discuss upcoming manufactures, non-conformances, updating of controlled documents and stock requirements.

2.7.2   Equipment for ALLCAR19

The primary device for our validations and subsequent CAR-T manufacture was the CliniMACS Prodigy®. This is discussed in detail in Section 1.4.4. The room in which a closed system is used for CAR-T manufacture must be at least a grade D (158). To facilitate the introduction of the device to the clean rooms, a user requirement specification (URS) document was prepared. Without this formal document, new devices cannot enter the clean room. This document was reviewed by the facilities manager and once the requirements around calibration, maintenance and servicing are known to the clean room management team, they then take responsibility for the kit and police the subsequent servicing, maintenance. The URS document outlines the following:

(a) intended use: generation of CAR-T cells for UCL academic trials
(b) cleaning: wiped with 70% sterile IMS, disposable tubing set
(c) equipment-specific operational requirements: power supply (110 – 230V, must be earthed), gas supply for CO_2 and compressed air, software version 1.2 or later, room temperature 15-25°C, humidity 10-75%, flat stable surface capable of supporting 100kg and free of vibrations.
(d) and service support: 3-year service contract from Miltenyi Biotech (maintenance, repair and technical support)

To ensure compliance with URS and GMP requirements, additional qualifications were performed to determine performance of the new equipment. This included Installation Qualification (IQ) and

Operational Qualification (OQ). IQ verifies the appropriate calibration and installation of the machine and associated connections (CO2 and compressed air) in line with the URS. The OQ assesses the ability of the device to operate as designed. The IQ/OQ was performed by a Miltenyi Biotec engineer. A process qualification (PQ) was then performed to assess the ability of the device to operate consistently for its intended purpose (specific manufacturing process). A simulated ALLCAR19 manufacturing run was performed as part of this PQ.

A list of the equipment used for the ALLCAR19 project in the clean rooms is outlined below in Table 10.

Equipment Type	Supplier
CliniMACS Prodigy® and inbuilt Tube Sealer	Miltenyi Biotec GmbH
PocH-100i cell counter	Sysmex
BD Celesta	BD Biosciences
MACSQuant CCGTT	Miltenyi Biotec GmbH
Plasmatherm (D2)	Barkey
Class II microbiological safety cabinet	Jencons-pls
Bag centrifuge in D lab	Thermo Scientific
Bag centrifuge in B lab	Hettich
TSCD-II sterile tubing welder	Terumo BCT Ltd
Incubator	RS Biotech
Monitored Fridge in D2	Lec Technical
Multivac Vacuum chamber	Jencons-pls
Kryo 560-16	Planer plc
Digital balance	Sartorius
PlasmaPress	Baxter

Table 10: Equipment required for ALLCAR19

2.7.3   cGMP specific documents for ALLCAR19

On establishment of our new manufacturing team, our first step was to draft process specific BMRs, SOPs and forms. The initial drafts were prepared prior to our first validation run on the CliniMACS Prodigy® and subsequently modified and optimised as the validations progressed. The final validation run for each clinical study was performed with authorised BMR and SOPs in place. The core

manufacturing documents (SOPs, forms and worksheets) prepared for ALLCAR19 are displayed in Table 11. The BMR for each process was filed in a designated folder with all associated forms, results and reports. All folders are held in a locked cabinet in an office with a coded door. Folders for incoming materials and critical starting materials such as vector are also stored here. Access to this office is limited to CCGTT staff and research teams. Access to the locked cabinet is restricted to our manufacturing team as per EU guidance.

CCGTT Documents for ALLCAR19
BMR
ALLCAR19
SOPs
T-Cell Transduction Prodigy Procedure
CAT CAR Release Flow Assays
Associated Trial- Specific Controlled Documents
ALLCAR19 Index Form
CAT CAR Transduction Efficiency and Trucount Worksheet
CAT CAR T-cell Subsets Worksheet
Certificate of Analysis
QP Certification for Batch Release
Associated Trial-Specific Uncontrolled Documents
ALLCAR19 Product Specification File
Non-Trial Specific Controlled Documents
Staff Plan
Pre-Production Checklist
Stock Cards
Cryopreservation Worksheet
Trucount Worksheet

Table 11: List of documents drafted by manufacturing team at CCGTT for ALLCAR19

2.7.4   EM for validations/product manufacture for ALLCAR19

To conform with in-process environmental monitoring requirements (during process validations and CTP manufacture), in a grade A environment our team prepared settle plates, contact plates (cabinet/sleeve) and finger dab plates (A and B operator) for each process. Airborne particle counting was performed for each process in a grade A environment. Confirmation of adequate air pressure differentials was recorded in the BMR prior to entering the laboratory. All results were recorded in the BMR prior to QC and QP review. Weekly and monthly EM is performed in each laboratory independent

of in-process testing. The frequency of this testing is dependent on laboratory grade. Competence in EM monitoring was a prerequisite for all team members.

2.7.5 Qualification and validation of ALLCAR19 CAR-T cell manufacture

Three validation runs of the TCT protocol (with CD4/CD8 selection) for generation of CD19CAT-41BBζ (CATCAR) T cells for use in ALLCAR19 clinical trial using the Miltenyi CliniMACS Prodigy® System were performed. Fresh (n=2) or frozen (n=1) non-mobilised excess leukapheresate from three patients with B-ALL registered on the UCL autologous CD19 CAR-T cells study ALLCAR19 (NCT02935257) was used to develop our process and validate the use of the CliniMACS Prodigy® for the production of autologous CD19 CAR-T cells, first of which also served as a PQ (manual manufactures were performed in parallel as a comparison according to GMP standards using fresh material from the same patients (n=3) as per Section 2.8). The TCT process is described in detail in section 2.8.2 and Figure 15. A material transfer agreement was in place between the Sponsor (UCL), the CCGTT and Pule labs for use of this material at the CCGTT. Evidence of patient/donor consent for use of this material is held by the Sponsor and a copy kept in the relevant BMR.

As per MHRA requirements, a medium fill test was performed initially, replacing all reagents with broth, to confirm the sterility of our process. Sterility was assessed by inoculation of BACTEC bottles with broth at the beginning and end of the run (before cryopreservation). The final cell products obtained in the three validation runs were assessed according to the release criteria outlined in the Q+V protocol and displayed in Table 12.

	TEST	MODE OF TESTING	ACCEPTANCE CRITERIA
MEDIUM FILL TEST	**Sterility** (Day 0, 8)	BacTec	No growth
PQ/PV RUNS	**Cell Count**	Flow cytometry-based Trucount™ CD45+CD3+	Absolute number between $\geq$ 5×10^6 (min) and $\leq 5 \times 10^8$ (max) CD45+CD3+CAR+ cells
	Cell viability	Trucount™ / Flow cytometry for CD45, CD3 and 7AAD	$\geq$ 70% Viability (pre-cryopreservation) of CD45+CD3+ T-cells
	Cell Transduction	CAR transduction efficiency by flow	$\geq$ 15% CAR+ cells (Live CD3+/CD45+T cells)

	Max. volume of individual ATIMP dose	Not applicable	<12ml/kg patient body weight*
	Sterility: Endotoxin	Kinetic Turbidimetric	$\leq$ 2EU/ml
	Sterility: Microbiology	BACTEC culture system	No growth
	Sterility: Mycoplasma	qPCR	None detected
RESIDUAL FREE VIRAL PARTICLES	**Infectivity risk of final product**	293T cells transduction	$\leq$ 1% transduced cells

Table 12: QC tests and acceptance criteria at Day 0 and Day 8 of the TCT process and on product thaw

*to ensure DMSO dose is <1g/kg

2.7.6   QC/QP role for ALLCAR19

Given the products generated during our validation of the CliniMACs Prodigy® were, by definition, not released, the QP at the CCGTT had no role in batch release until our IMPD amendment was approved by the MHRA to commence manufacture for ALLCAR19. However, QP approval of the ALLCAR19 change control was required prior to the implementation of the new process. The final (3rd) validation run was also performed on approved, issued and version-controlled SOPs and BMR.

 The head of QC oversaw the incoming materials and facilitated outsourcing on the testing of our final products for the validations (mycoplasma and endotoxin). At the CCGTT a QC review and QP product release date is requested on day zero of each manufacture. The BMR, associated documents and reports is reviewed by the head of QC for 5 days prior to the scheduled QP release date. On the release of the product, the QP release documents are sent to the clinical trial sponsor via encrypted email. Our turnaround time for product release on receipt of a fresh leukapheresis is 23 days.

2.8 cGMP methods: 'manufacturing'

2.8.1   "Open" CAR-T manufacturing process (Process A)

The open or manual CAR-T manufacturing process was performed at Institute of Child Health (ICH) by Great Ormond Street Hospital (GOSH) GMP team. Media was prepared in a grade A Isolator using XVivo™ 15 (Lonza) supplemented with 5% heat-inactivated Human AB Serum (Life Science Production). Cytokine support (100 IU/ml IL-2 Proleukin®, Novartis) was only used in the context of

poor cell expansion. Process buffer was prepared simultaneously using CliniMACS PBS/ethylene-diamine-tetra acetic acid (EDTA) (Miltenyi Biotec) supplemented with 0.5% human albumin solution (HAS) (Zenalb, Bio Products Laboratory). Total white cell and T-lymphocyte count by CD45+ and CD3+ TruCount (BD Biosciences) was obtained for the leukapheresate. PBMCs within the leukapheresate were then stimulated with human T cell CD3/CD28 Dynabeads™ (CTS™, GIBCO™) at a beads: cells ratio of 3:1 in a grade A environment and cultured overnight in cell differentiation bags (MACS, Miltenyi) in an incubator (37°C, 5% CO2). Two cell differentiation bags (MACS, Miltenyi) were then coated with Retronectin® (TAKARA BIO) in a grade A environment and the cells were transferred to the Retronectin® coated bags for the transduction process. The lentiviral vector was added to the cells at a MOI of 5 (based on CD3+ cell count) and the cells were incubated (37°C, 5% CO2). Sterility of cells and all reagents was tested using blood culture bottles (BacTecs, BD). The cells were washed and re-suspended in complete media at a target concentration of up to 1×10^6/ml.

These cells were then transferred to a Xuri™ Wave Bioreactor system (GE Healthcare) (Grade B or C environment) and expanded for up to 4 days. During the expansion phase, cell count and viability were assessed daily (Sysmex; Trypan Blue, Sigma) to determine and maintain the cell concentration of 0.5 to 1×10^6/ml. The maximum capacity of the Wave Bioreactor is 1000ml exceeding which, the 'Perfusion' was enabled which allowed the removal of a defined volume of media from the Wave Bioreactor through the 'Waste Port' and addition of fresh media through the 'Feed Port' at regular intervals. On day 7 of the process, the cells were harvested from the Wave Bioreactor by the welding of transfer bags (Terumo).

Using the CTS™ DynaMag™ Magnet, the CD3/CD28 Dynabeads™ were removed from the cells by passing the cell suspension over the Magnet and collecting the de-beaded cells in an attached collection bag (Terumo). The bead removal process was performed in grade B or C laboratory. Once the cells were collected, they were then transferred to a grade A environment for sterility testing, viability and cell counts (Sysmex analyser, Trypan Blue, Sigma). The cells were rested for 18-24 hours in an incubator at 37°C, 5% CO2 before proceeding to the cryopreservation step.

On the final day of the process (day 8), the cells were washed by centrifugation and harvested using process buffer supplemented with 0.5% HAS. Flow cytometry analysis (BD, FACS Canto) was performed to determine the transduction efficiency, viability and CD3+ cell counts. The cells were then formulated in a final composition of process buffer with 4% HAS and 7.5% DMSO (Origen Biomedical) at a density of 10×10^6 CD45+ cells/ml prior to cryopreservation in respective cell doses in a controlled rate freezer (Kryo 560-16, Planer) and transfer to LN storage.

2.8.2 "Closed" CAR-T manufacturing process (Process B)

The semi-automated (closed) TCT process on the CliniMACS Prodigy performed by our UCL GMP team at the CCGTT. TS520 tubing set and TCT process software were used for all 3 CD19 CAR-T cell manufactures on the CliniMACS Prodigy® in a grade D clean room (Figure 15).

Prior to the arrival of the leukapheresis, process buffer, CliniMACS® PBS/EDTA (Miltenyi Biotec), supplemented with 0.5% HAS (Zenalb®, Bio Products Laboratory) and TexsMACS GMP media (Miltenyi Biotec) supplemented with 3% heat-inactivated AB serum (Life Science Production) and 10ng/ml of recombinant human (IL)-7 and recombinant human IL-15 (Miltenyi Biotec) were prepared in a grade A environment in a grade B laboratory. Frozen leukapheresis was thawed in a plasmatherm (Barkey), washed with TexsMACS media supplemented with 10% AB serum and benzonase (50U/ml, Merck Millipore) and incubated overnight in TexsMACS media supplemented with 3% AB serum. Samples for microbiological testing were taken at scheduled timepoints throughout the process.

Fresh or frozen leukapheresis was resuspended in process buffer prior to sample loading onto the CliniMACS Prodigy® by sterile welding. Total white cell and T-lymphocyte count by CD45+ and CD3+ Trucount™ (BD Biosciences) and percentage of target cells (CD4+, CD8+) were recorded prior to CD4 and CD8 T-cell enrichment using immunomagnetic beads (Miltenyi Biotec). The maximum capacity of the separation column is 3×10^9 target cells. Post cell separation the CD3+ cell concentration was recorded in the application programme prior to cell cultivation. The cells were activated with TransAct® (polymeric nanomatrix conjugated to recombinant humanised CD3 and CD28 agonist, Miltenyi Biotec).

Twenty-four hours post activation, the cells are transduced with a lentiviral vector. Transfer of required vector units/volume (based on agreed MOI) to a transfer bag was performed in a grade A environment in a B laboratory (dedicated to the handling of genetically modified material) prior to connection to the CliniMACS Prodigy® by sterile welding.

To remove residual activation reagent, cells were washed on day 4 post activation using the automatic Culture Wash and then expanded until day 8 of manufacture in fresh media supplemented with IL-7 and IL-15 and human serum (3%). The conditions of the semi-automated runs were defined using the "activity matrix". This encompasses automatic feeding, media exchange and agitation of cells. In-process QC testing (cell count, viability) was performed on day 4 and day 6 using an automated haematology analyser (Poch-100i, Sysmex) and CD45+/CD3+ Trucount™.

On day 8, the cells were harvested using process buffer supplemented with 0.5% HAS, then formulated in a final composition of process buffer with 4% HAS and 7.5% DMSO (Origen Biomedical) at a density of 10×10^6 CD45+ cells/ml prior to cryopreservation in a CRF (Kryo 560-16, Planer) and transfer to LN storage.

2.8.3   Comparison of "open" and closed" CAR-T cell manufacture

For the "open" and "closed" process we compared process-related variables such as starting cell number, vector usage, cell expansion, transduction efficiency, final product composition, viability and CAR-T cell numbers. Number of GMP-trained staff and hours of work by day of process (including hours in grade A environment) for open and semi-automated processes were calculated in real time. An approximate cost analysis for both methodologies was performed, including consumables, reagents and vector. Items of equipment required for both processes were also recorded.
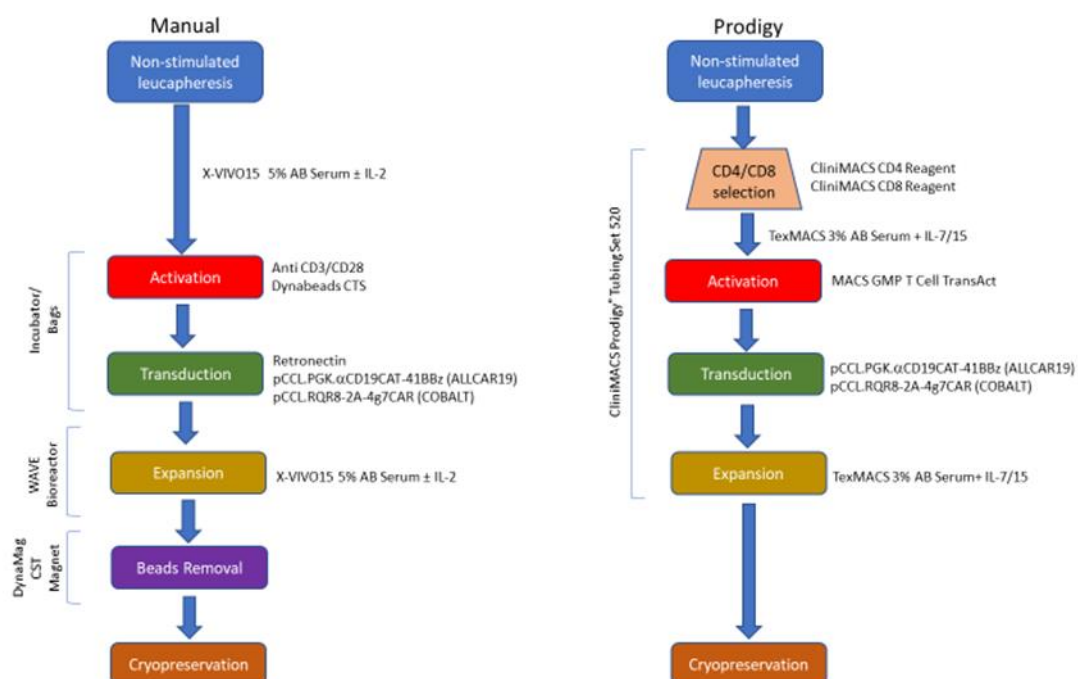
Figure 15: Comparison of manual and semi-automated manufacturing of CD19 CAR-T cells

2.8.4   Packaging, labelling and distribution of ALLCAR19 ATIMPS

Miltenyi CryoMACS bags (and associated overwraps) are the chosen bags for cryostorage at the CCGTT and have been validated in-house. All leukapheresate and final CAR-T products were stored in temperature-monitored vapour phase LN dewars between -130°C and -196°C. The final products were labelled with MHRA-approved trial-specific labels are stored in a dewar dedicated to genetically modified (GM) material. All labels have 3 patient identifiers, manufacturing batch ID, date of cryopreservation/expiry, recommended transport and storage temperature and cell dose (Figure 16). The labels are checked and signed by a second member of the manufacturing team. On a date arranged

with the stem cell laboratory at UCLH, the cells for clinical use are removed from the GM dewar, inspected for leaks and shipped in a temperature-monitored cryoshipper to the hospital site.

(a)

(b)

Figure 16: Example ALLCAR19 secondary (a) and primary (b) labels

2.9 Release flow assay validation for ALLCAR19

2.9.1 Release criteria for ALLCAR19

Release assays for CAR T-cells are described in Section 1.4.2.6. For a Phase I clinical study, we were mandated to confirm basic phenotype, purity and sterility. Release assays must be reproducible, sensitive and specific. The release criteria for ALLCAR19 are displayed in Table 13. Endotoxin and mycoplasma testing were outsourced to NHS Blood Transfusion in Scotland and Minerva Analytics (LAGeSO accredited) in Germany, respectively. Endotoxin testing is performed via kinetic turbidimetric method, as described in the European Pharmacopoeia Monograph 2.6.14 (274). Test

samples were validated by NHSBT at serial 1 in 2 dilutions ranging from 1:2 to 1:200. All samples were spiked with 0.1 Endotoxin units (EU)/ml and the endotoxin concentration was measured to assess the recovery of the spike. At a 1:100 dilution of test sample, the spike recovery was 97% (a minimum spike recovery of 75% is indicative of consistent future assay performance). Screening for mycoplasma is by PCR for 16s RNA. Cell culture specimens are spiked with a known level of Mycoplasma hominis/ bovis/fermentans/genitalium and penetrans DNA which must test positive for validation (performed by Minerva). A semi-nested assay targeting the Bacterial 16S genus is performed and this method can detect as low as 10 copies/ml. BD Bactec bottles (CE marked) were incubated for 10 days to assess for sterility.

Release Criterion	Specification
Sterility	No growth after 10 days incubation
Endotoxin	<2EU/ml
Mycoplasma	None detected
Minimum cell dose	$\geq$5 x 10^6 CAR-T cells
Transduction Efficiency	$\geq$15% CAR+ cells (CD3+/CD45+ cells)
Viability	$\geq$70% pre-cryopreservation
Max volume of ATIMP dose	$\leq$12ml/kg

Table 13: Release criteria ALLCAR19

2.9.2   Validation of release flow for ALLCAR19

We devised a flow-cytometric assay that would allow us to confirm CART-cell numbers from 100% transduction down to below 3.125% (well below the 15% transduction efficiency limit set for product release on ALLCAR19). CE marked BD Trucount™ cell counting tubes were also validated to ensure the results were consistent between samples and between users.

The following parameters were validated:

(1) Transduction Efficiency

(2) CD3 quantification by Trucount

Full validation of the ALLCAR19 flow release assays required the large volume manufacture of an ALLCAR19 CAR T-cell product according to the research grade T-cell activation and transduction methods outlined in Section 2.1.2 using healthy donor starting material. Evidence of consent for use of this material is held by Pule labs. The lentiviral vector (pCCL.PGK-aCD19CAT-41BBζ) for this purpose was manufactured, concentrated and titred in the PD lab as per Section 2.1. Once the cells were

made in bulk, a sterile flow sort for CAR+ cells was performed (Section 2.4.4) and the sterile, pure CAR T-cells were spiked at known concentrations into autologous CD3 (non-CAR) cells using serial dilutions. This gave us CAR expression of 100%, 50%, 25%, 12.5%, 6.25%, 3.1% and 0%.

Accurate (viable) CD3/CD45+ cell counts of CAR and NT T-cells was determined by Trucount™. Samples were stained as per Section 2.4.1 and run in triplicate for each dilution (with exception of sample A1-5 for Trucount due to insufficient sample) to demonstrate inter-sample accuracy and consistency. To assess CAR expression post thaw, two frozen vials from a previous ALLCAR19 manufacture were thawed at 1-2 weekly intervals. The samples are stained as per Table 6 to determine persistence of CAR expression and viability post thaw.

The assays were performed on both the cGMP BD Celesta (serial number 466034400067) and the back-up MACSQuant (serial number S/N 2558) cytometers. As per cGMP practice, the BD Celesta is under a regular service/maintenance under contract from BD and has daily QC checks (CS&T beads) prior to use.

The acceptance criteria for the assay were based on specificity (background), accuracy, within-range precision and dynamic range and lower limits of quantification.

FACS Assay	Principle	Antibodies
CD3 Trucount	Determine CD3 absolute count	CD3 APC-Cy7 CD45 FITC 7-AAD
Transduction Efficiency	Determine % CD3+ Determine % CD45+ Determine % transduction of viable CD45+/CD3+ cells	**Primary Layer:** FC gamma block Anti-CAT CAR idiotype **Secondary Layer:** CD45 FITC CD3 APC-Cy7 Anti-Rat IgG-PE 7AAD

Table 14: Flow cytometry staining for ALLCAR19 release flow validation

2.10   Optimisation of Apheresis

2.10.1 Patient data collection

Data on all T-cell harvests (for subsequent CAR-T cell manufacture) from August 2016 to November 2018 was collated retrospectively at UCLH using medical records and electronic databases as part of a UCLH Trust registered service evaluation and clinical audit. The donors were registered on 6 different

CAR-T cell studies and provided informed consent. With the exception of one trial, all were designed for adult patients. Diagnoses included B-ALL, DLBCL and MM. T-cell expansion was not assessed prior to enrolment.

2.10.2 Apheresis procedure data collection

All T-cell harvests (CMNC programme) were performed on the Spectra Optia by a total of eleven experienced operators in a dedicated 8-bedded apheresis unit according to local SOPs and the manufacturer's instructions. Collection rate, anticoagulant (AC, ACDA) infusion rate and AC: Blood ratio were programmed at 0.8ml/min, 0.9mls/min/kg and 13.5:1 respectively. Blood primes were not required, given this was almost exclusively an adult cohort. An ALC of <0.5 x 10^9/L prompted escalation from a standard 2 x BV harvest to a 2.5 x BV harvest. The Spectra Optia used a formula based on sex, height and weight to estimate total blood volume (TBV). Femoral vascular catheters were inserted pre-procedure if vascular access was deemed by the apheresis team to be unsatisfactory. A vascular access assessment was performed prior to each procedure. All patients provided written informed consent for the procedure. Adverse events were recorded in clinical notes or on the relevant apheresis database. The patients must be afebrile with no evidence of active severe infection at the time of the procedure.

Collection efficiency (CE) is an objective quality parameter which is used to assess the ability of a harvest procedure to extract the target cells from the donor circulation. It is expressed as a percentage and considers the total number of target cells that were available for collection in the donor, the total number of target cells ultimately collected and the volume of blood processed.

CE was calculated using the following algorithm:

CE2% = Total targeted cells collected in product / (pre- [targeted cells] * total blood processed)

2.10.3 Laboratory workup pre and post apheresis

A peripheral blood full blood count (FBC) (Sysmex XN-350) with automated differential was performed on each patient pre and post apheresis. Manual differential counts and flow cytometry to detect circulating disease was performed as indicated. FBC (Sysmex XN-350), CD3% and cell counts (MACSQuant Analyzer 10) was performed on the apheresis product. Post apheresis CD3 counts were not performed routinely and were therefore not available for analysis.

2.10.4 CD3+ targets

In our experience in adult patients with advanced B-cell malignancies (using our validated method of manufacture for UCL academic studies), a target of 1.5×10^9 CD3+ cells is more than sufficient to proceed with cell manufacture and cryopreserve excess material (to be used in the event of a manufacturing failure). To allow for cell losses secondary to thawing and T-cell enrichment, an absolute minimum CD3+ cell count of 0.6×10^9/L pre-cryopreservation is considered acceptable (no excess in event of manufacturing failure).

Based on the data collected on 34 autologous MNC collections using the Spectra Optia, we devised an algorithm to be validated prospectively at UCLH based on PB ALC ($\times 10^9$/L), a CD3+ cell target of 1.5×10^9 and litres of blood to be processed. A customized prediction algorithm to determine the blood volume required for each patient to achieve the target CD3+ dose was established using variables from the linear regression of pre-apheresis CD3+ cell count and the collected concentration of CD3+ cells per volume of blood processed. This linear regression was described by the following equation:

CD3+/Litres of whole blood = a x CD3+ pre-count +b

a= slope b=intercept

The slope represents the correlation coefficient, namely how the variance in the independent variable (peripheral blood CD3+ pre-count or ALC) can be used to predict the dependent variable (CD3+ count per litre of blood processed). The intercept is the point on the y axis where the regression line intercepts the y axis, namely the CD3+ cells collected with the CD3+ count is zero.

R2 represents the coefficient of determination and indicates how closely the regression line approximates the real data points. It tells us the proportion of variance in the dependent variable that is predictable from the independent variable. An R2 of 1.0 is a perfect fit.

2.10.5 CAR-T cell manufacture

Downstream CAR-T cell manufacture was performed by manual (n=13) or semi-automated (n=27) methodology. The manual method comprised of T-cell activation with Dynabeads™, transduction with a lentiviral vector, expansion in a WAVE Bioreactor, bead removal and cryopreservation. In 2018, all manufacturing was transitioned to a semi-automated GMP-compliant cell processing device, the CliniMACS Prodigy®, with an amended, optimised and scalable protocol. T cells were enriched using CD4 and CD8 immunomagnetic beads, activated with TransAct™, transduced with a lentiviral vector, expanded with IL-7/IL-15 cytokine support in a closed system and finally cryopreserved.

Successful manufacture was defined as meeting trial cell dose requirements.

2.11   Statistical analysis

Statistical analysis for the comparison of manual and semi-automated process was performed using GraphPad Prism version 7.0. Unpaired parametric t-tests, one or two-way ANOVAs were used. Descriptive statistics and statistical comparisons for the apheresis project were performed using Stata software version 12.0 with the assistance of Terumo. Relationships between apheresis variables were determined by linear regression analyses for parametric variables. Mann-Whitney test was used for non-parametric variables.

2.12   Ethical Approval and Consent

Each patient provided written informed consent for storage and use of excess apheresis material in accordance with Declaration of Helsinki when they consented to the ALLCAR19 clinical trial. All leukapheresis donors provided written informed consent for the collection of apheresis material.

2.13   My Contribution

I was employed as a Clinical Research Fellow with the UCL CAR-T cell team from August 2017 until October 2019. My contribution to this project in reference to the Methods and Results is as follows:

Process Development

Viral Vector work

Lentiviral vector production, concentration and titration and free viral vector assays:

I performed these experiments with the assistance of my research colleague Juliana Dias Alves Pinto.

Cell-based assays

I performed these experiments with the assistance of my research colleagues Ketki Vispute and Juliana Dias Alves Pinto.

Maeve O'Reilly MD (Res)

Preclinical Optimisation of the ALLCAR19 manufacture method

I performed this experiment with the assistance of my research colleague Juliana Dias Alves Pinto. I independently ran the flow cytometry and analysed the data in Flojo and GraphPad Prism version 7.0.

GMP

For this project I completed full-time GMP training at the CCGTT over a 6-9 month period and became an independent GMP-compliant CAR-T cell manufacturer. Our GMP team at the CCGTT consisted of Juliana Dias Alves Pinto and I (full-time), Claire Roddie and Ketki Vispute (part-time). I have since manufactured and released 8 GMP-compliant CAR-T cell products for 3 UCL CAR-T clinical trials.

With regard to my specific contribution to the introduction and validation of the ALLCAR19 clinical trial at the CCGTT:

Documents

To proceed with this project, I drafted many iterations of the ALLCAR19 BMR, SOPs, change controls, qualification and validation protocols, certificate of analysis, labels and worksheets (full list in Table 11).

Process B/Prodigy Cell manufacture

As part of a small team , I manufactured and cryopreserved all 3 autologous products using the CliniMACS Prodigy® (as per Section 2.8.2).

Flow Cytometry

I performed the flow cytometry (Trucount/transduction efficiency/extended phenotyping) independently using the BD Celesta and MACSQuant Analyzer®. I can compensate and trouble-shoot both machines. I analysed all data using independently using Flojo software and prepared the graphs in GraphPad Prism version 7.0 with the assistance of Juliana Dias Alves Pinto.

Release Flow Assay validation

I performed this experiment, flow and analysis with the assistance of my GMP team (3 staff).

Apheresis Project

Data Collection

I collected all data independently for this project.

Flow cytometry

I independently analysed the relevant leukapheresis subtyping using Flojo software

Statistical analysis

The statistical analysis for this project was performed with assistance from Terumo.

Clinical Service Development

Patient care

I participated in the weekly Immunotherapy clinic, screening referrals, selecting patients and exploring bridging options. I am a sub-investigator on all of our academic CAR-T trials.

Documents

I drafted our institutional SOP for the management CRS, ICANS and long-term follow-up with input from my colleagues. I attended the JACIE meetings to prepare for our accreditation.

Education

I delivered education sessions on CAR-T cell therapy and its complications to many departments across UCLH. This included sub-specialities whose help we had enlisted (ITU, neurology, infectious disease, cardiology, psychological support) and ancillary departments that we needed to operate efficiently (apheresis, pharmacy, infusion team, bed managers, administrators etc).

T-cell meeting

I presented at this meeting on a fortnightly/monthly basis. This meeting has since expanded to include our solid tumour colleagues.

Much of the clinical service development at UCLH has involved streamlining communication, referral, admission and discharge pathways. I have participated in this as part of my team (by designing referral templates, mailing lists, checklists, proformas etc)

CHAPTER 3:

3 PRECLINICAL OPTIMISATION OF THE ALLCAR19 MANUFACTURE METHOD

3.1 Introduction

Prior to validation of the CliniMACs Prodigy® for the generation of autologous CD19 CAR-T cells, patient-derived leukapheresis was used to generate CD19 CAR-T cells in the PD laboratory as per Section 2.3. The purpose of this small-scale experiment was to compare conditions simulating the new closed Prodigy® and open manual Dynabead process in terms of cell expansion, transduction efficiency and extended phenotyping. Each condition (below) had a parallel non-transduced condition.

	T-cell Immunomagnetic Selection	Activation	Cytokines
Condition 1 (Simulating new closed Prodigy® Process)	Yes	TransAct	IL-7 and IL-15
Condition 2 (Simulating open manual process)	No	DynaBeads	No cytokines

3.2 Results

3.2.1 Composition and extended phenotyping of starting material

Patient-derived leukapheresis was thawed on day -1, rested overnight and flow cytometry including subtyping, maturation and exhaustion panels were performed as per Section 2.4.3. The composition of the starting material (prior to CD4 and CD8+ T cell selection) is displayed in Table 15. The maturation profile of live single CD3+ cells demonstrated a central memory population of 26.8% (Figure 17). CD3+ cells in the starting material were minimally exhausted as shown in Figure 18.

Cellular Composition of Starting Material*	Process Development ALL Sample
Lymphocytes (CD45+/CD3+) (% Live cells)	75.3
Monocytes(CD45+/CD14+) (% Live cells)	19.1
NK (CD45+/CD56/16+) (% Live cells)	5
NKT (CD45+/CD3+/CD56/16+) (% Live cells)	1.7
CD10/19+ cells (B cells/blasts) (% Live cells)	0
CD4 (% of CD3+ cells)	84.2
CD8 (% of CD3+ cells)	12.3

Table 15: Composition of ALL PD sample by flow cytometry

*Numbers expressed as a percentage of live single CD45+ cells
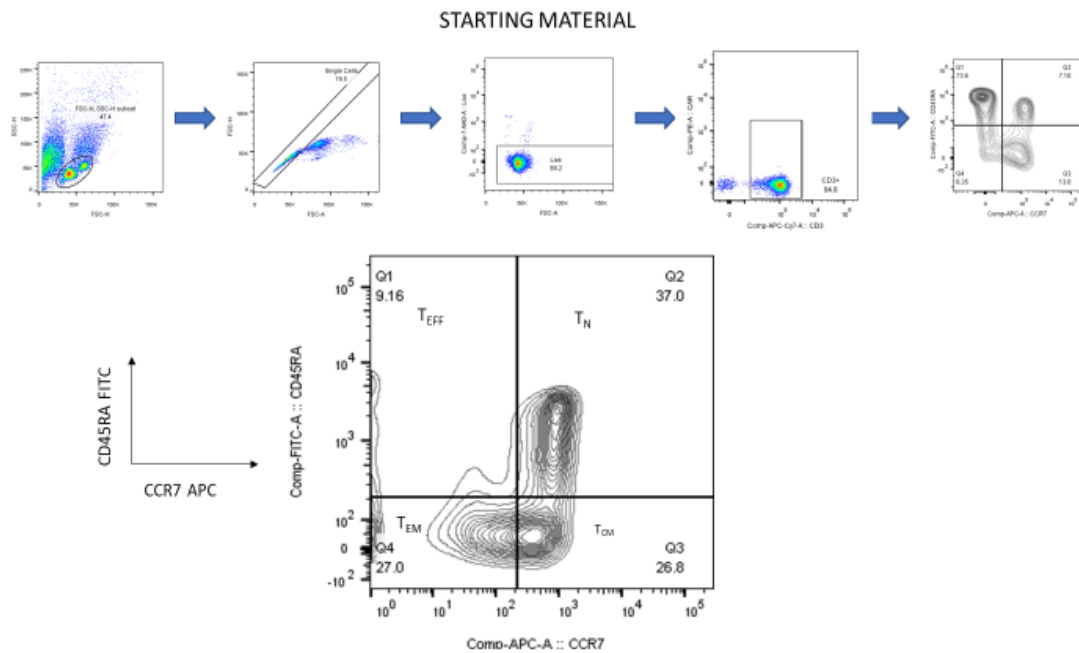
Figure 17: Maturation profile of live single CD3+ cells based on expression of CD45RA and CCR7

(The top panels show the gating strategy for analysis of starting material. Subsets were defined as: naïve T-cells (T_N: CD3+CCR7+CD45RA+), central-memory T-cells (T_{CM}: CD3+CCR7+CD45RA-), effector-memory T-cells (T_{EM}: CD3+CCR7-CD45RA-) or effector T-cells (T_{EFF}: CD3+CCR7-CD45RA+).
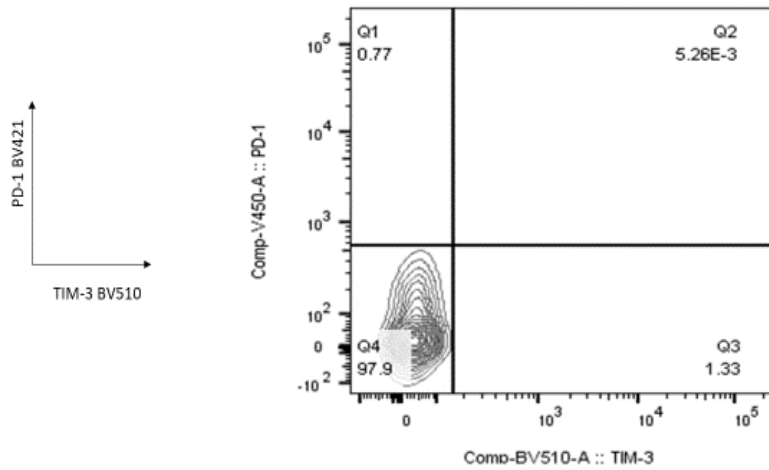
Figure 18: Exhaustion profile of CD3+ T cells in thawed leukapheresis based on expression of TIM-3 and PD-1

3.2.2   Cell expansion and final CAR-T cell products for Condition 1 and Condition 2

Fold expansion for Condition 1 and 2 is displayed in Figure 19. Condition 1, simulating the Prodigy® process demonstrated a 15- fold expansion by day 8 relative to a 3.2-fold expansion for Condition 2 (simulating the open manual process).
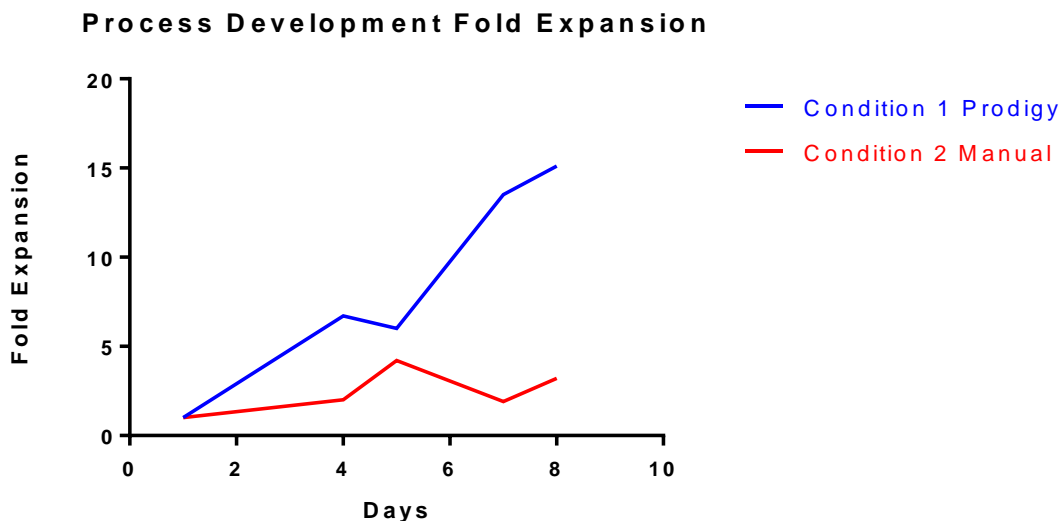
Figure 19: Comparison of fold expansion for Condition 1 and 2 during the 7 days after lentiviral transduction

(For Condition 2, day 7 cell count was performed after the bead removal stage. WBC counts were performed using a Sysmex hematological counter.

The transduction efficiency for Condition 1 and 2 is displayed in Figure 20 and was 60.1% and 76.8% respectively (viability>95% for both conditions). By day 8, Condition 1 had generated 97.4 x 10^6 total CAR-T cells compared with 20.7 x 10^6 total CAR-T cells for Condition 2.
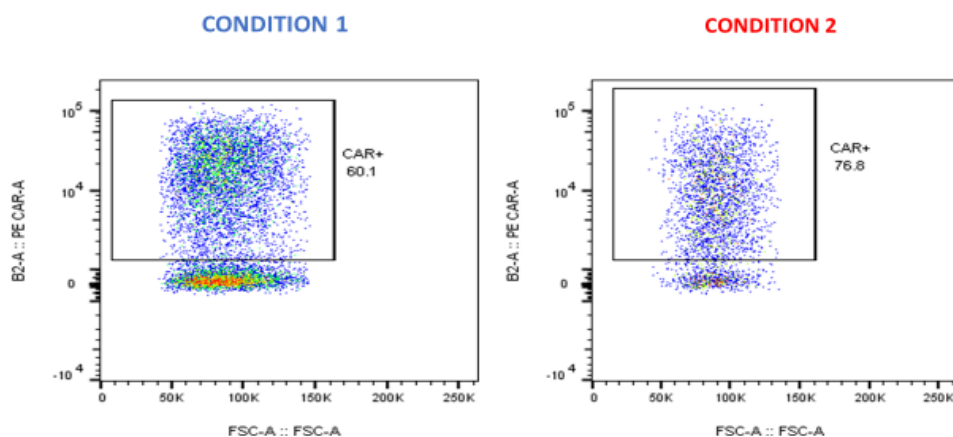
Figure 20: Transduction efficiency of live single CD3+ T cells in Condition 1 and 2

The maturation profile of CD3+ CAR-T cells for Condition 1 and 2 is displayed in Figure 21 (gated on Day 0 CD3+ NT cells). CARs generated via Condition 1 and Condition 2 were enriched with cells of a central memory phenotype by day 8 (range 39.1-68.2%). Both conditions generated minimally exhausted CARs in the final products (Figure 22, gated on Day 0 CD3+ NT cells).
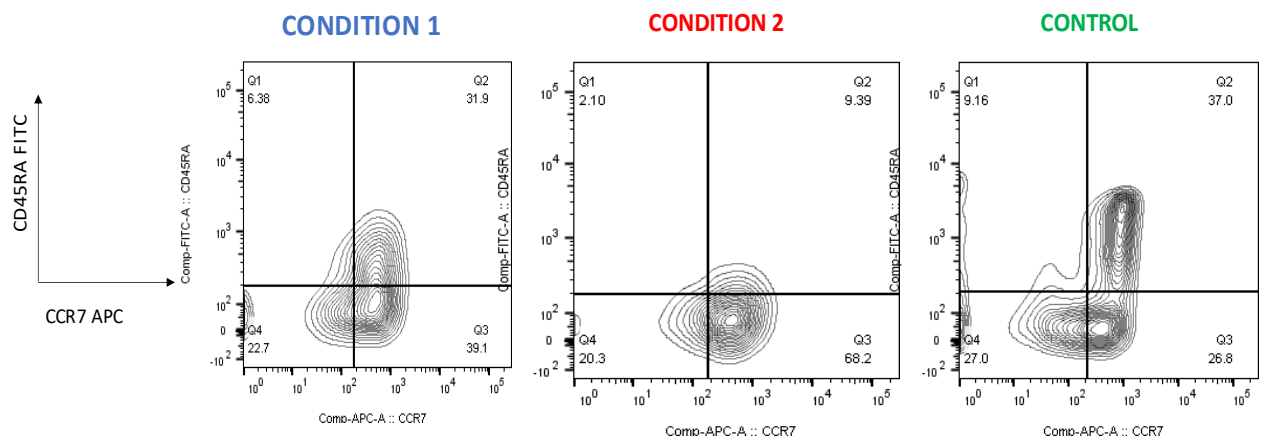
Figure 21: Maturation profile of CD3+ CARs for Condition 1 and 2 based on expression of CD45RA and CCR7

(The control displays the maturation profile of CD3+ T cells in the thawed leukapheresis based on expression of CD45RA and CCR7 (also shown in Figure 17))
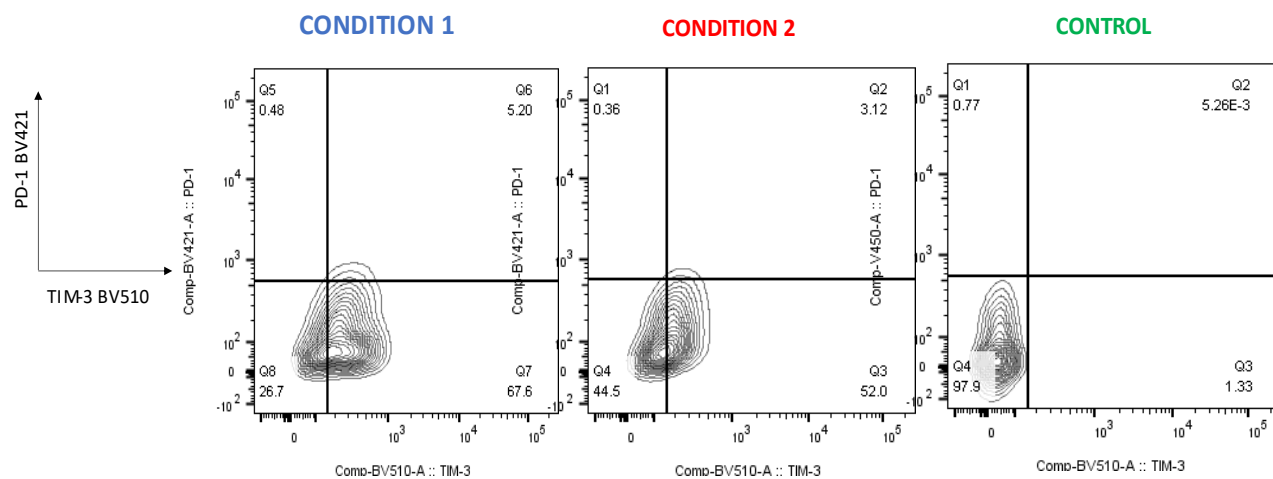
Figure 22: : Exhaustion profile of CD3+ CARs for Condition 1 and 2 based on expression of Tim-3 and PD-1

(The control displays the exhaustion profile of CD3+ T cells in the thawed leukapheresis based on expression of TIM-3 and PD-1 (also shown in Figure 18))

3.3 Discussion

This small-scale experiment in one donor with B-ALL demonstrated that our new proposed process using the CliniMACS Prodigy®, simulated in Condition 1 in our PD laboratory (T-cell selection, TransAct™ for T-cell activation with IL-7/IL-15 cytokine support) was worth transitioning to the GMP clean rooms. This process demonstrated superior T-cell expansion, higher absolute CAR-T cell numbers and a high percentage of CARs with a central memory and a minimally exhausted phenotype. This supported our decision to proceed with validation of the TCT process using the CliniMACS Prodigy®.

The limitations of this small-scale experiment are acknowledged. Due to clinical urgency (patients awaiting therapy), a single comparator experiment was performed prior to proceeding to the validation of the CliniMACS Prodigy®. Standard practice would encompass the use of 3 donors and/or replication of this experiment in triplicate. Functional testing of the CAR T cells generated by both processes was not performed. This is also acknowledged as a limitation of this experiment.

CHAPTER 4:

4 CGMP CAR T-CELL MANUFACTURE: MANUAL VERSUS SEMI-AUTOMATED

4.1 Introduction

The overarching aim was to optimise and streamline autologous CAR-T cell manufacturing at UCL for the ALLCAR19 study to permit scaling of manufacture to meet patient need. CAR T-cell manufacture is a specialised, labour-intensive multi-step process. A typical process comprises the following steps: T-cell isolation, activation, transduction, expansion and cryopreservation. Manufacturing to cGMP requires highly skilled operators and costly clean room infrastructure. Current manual manufacturing methodologies employed on the UCL CAR T-cell programme include multiple open handling steps as part of an open/Dynabead process (Section 2.8.1) with long hours in a grade A cGMP environment and significant use of high-level clean room resources. Closed-process manufacture as an alternative to a manual process would be highly desirable. Per product, it could reduce the requirements for clean room time, reduce the number of highly trained operators required for each manufacture making the process more cost- and time-effective. Closed processing also represents a lower risk of microbiological contamination. This project proposes to transfer this manufacture process onto the semi-automated, closed Miltenyi CliniMACS Prodigy® system. A summary of Process A (open, manual) and Process B (closed, semi-automated) processes are described in detail in Section 2.8 and Figure 15.

Fresh (n=2) or frozen (n=1) non-mobilised excess leukapheresate from three patients with B-ALL registered on the UCL autologous CD19 CAR-T cells study, ALLCAR19, was used to develop and verify the robustness of the CliniMACS Prodigy® system (process validation). Manual manufactures were performed in parallel according to GMP standards using fresh material from the same patients (n=3).

4.2 Aims of this chapter

1   Validating semi-automated CAR-T cell manufacture on the CliniMACS Prodigy®
   - Compare process-related variables such as starting cell number, vector usage, cell expansion, transduction efficiency, final product composition, viability and CAR-T cell numbers to those obtained with the conventional Dynabead process.

- Compare number of staff members required, total staff hours, grade A time, equipment and costs (approximate) to those of the conventional Dynabead process.

2   Contaminating cells in the starting material, particularly monocytes, can have a detrimental effect on downstream CAR-T cell manufacture (216–218). Circulating leukaemic blasts may also be harvested at the time of leukapheresis in patients with a significant burden of disease. Therefore, T-cell enrichment of the starting material for CD4+ and CD8+ cells using immunomagnetic beads (Miltenyi Biotec) will be validated on the Prodigy® system, thereby removing contaminating cells prior to the initiation of cell culture. Selection for CD4+ and CD8+ cells also facilitates a degree of standardisation of starting material.

3   No cytokines (or IL-2 if poor expansion) in our manual process will be switched to IL-7 and IL-15. This is based on the evidence that the use of IL7 and IL-15 appears to enrich the memory T-cell compartment (central memory and stem cell memory), a T cell compartment thought to improve persistence and anti-tumour activity in vivo. This has been validated previously by several groups (275–279).

4   To reduce the high costs associated with the use of high vector volumes, a lower MOI of viral vector will be used.

4.3 Results: Parallel run outcomes

4.3.1   Composition and extended phenotyping of starting material

Cellular composition of the starting material for the three manufactures is displayed in Table 16. Median percentage of CD3+ cells in leukapheresis was 58.4% (range 50.3-63.5). There was a predominance of CD4+ T-cells in the apheresis products with a mean CD4: CD8 of 1.5:1 (range 0.43-3:1). For process B, median efficiency of CD4 and CD8 immunomagnetic selection was 95.7% (range 91.6-97.8%) . The maturation and exhaustion profiles for the starting material is demonstrated in Figure 23, 29 and 30. The leukapheresis had a variable maturation profile with a T_{CM} population varying from 11.9-25.4%.

Cellular Composition of Starting Material*	ALL-01	ALL-02	ALL-03
Lymphocytes (CD45+/CD3+) (% Live cells)	63.5	50.3	58.4
Monocytes(CD45+/CD14+) (% Live cells)	14.6	20.9	14.9
NK (CD45+/CD56/16+) (% Live cells)	1.38	22.8	17.0
NKT (CD45+/CD3+/CD56/16+) (% Live cells)	0.0	4.6	7.9
CD10/19+ cells (B cells/blasts) (% Live cells)	0.25	1.2	0.07
CD4 (% of CD3+ cells)	25.5	50.6	64.6
CD8 (% of CD3+ cells)	60.3	42.7	21.9

Table 16: Cellular composition of starting material for patients with ALL (n=3)

*Numbers expressed as a percentage of live single CD45+ cells
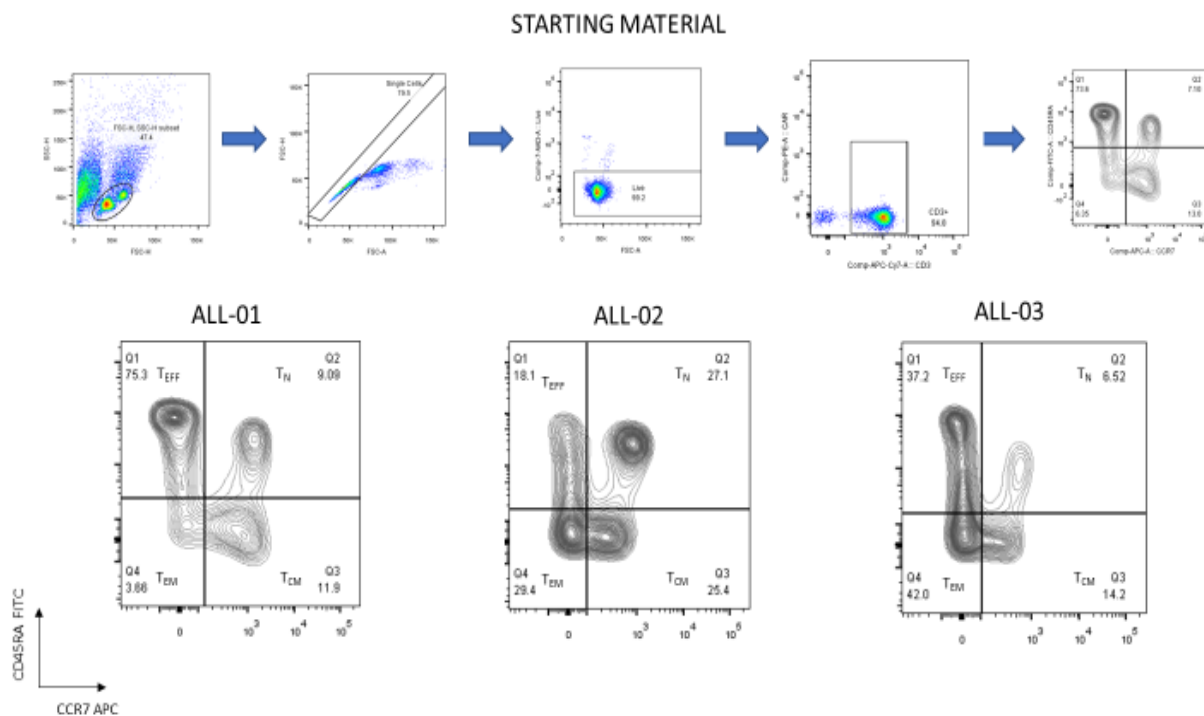
Figure 23: Maturation profile of total T-cells in starting material based on expression of CD45RA and CCR7

The top panels in Figure 23 show the gating strategy for analysis of starting material. Subsets were defined as: naïve T-cells (T_N: CD3+CCR7+CD45RA+), central-memory T-cells (T_{CM}: CD3+CCR7+CD45RA-), effector-memory T-cells (T_{EM}: CD3+CCR7-CD45RA-) or effector T-cells (T_{EFF}: CD3+CCR7-CD45RA+).

4.3.2   Cell expansion during manufacture for "open" and "closed" process

Cell count by Sysmex and/or TruCount (CD3+) was taken during cell manufacture for Process A and B. For Process A, the starting cell number for transduction was fixed at 500 x 10^6 lymphocytes. Median starting cell number for process B was 246 x 10^6 CD3+ cells (range 164-348 x 10^6). Given consistent cell expansion using the Prodigy system, our manufacturing team confidently reduced starting CD3+ cell numbers as the validations progressed to 70-100 x 10^6 CD3+ T-cells. Figure 24 shows the fold expansion after lentiviral transduction for both methodologies. With process B, a mean 10-fold expansion was consistently achieved by day 8, despite the variability of the starting material. In contrast, expansion in Process A was significantly lower: of the 3 manufactures, 1 failed to generate the target CD19 CAR T-cell dose for the trial.
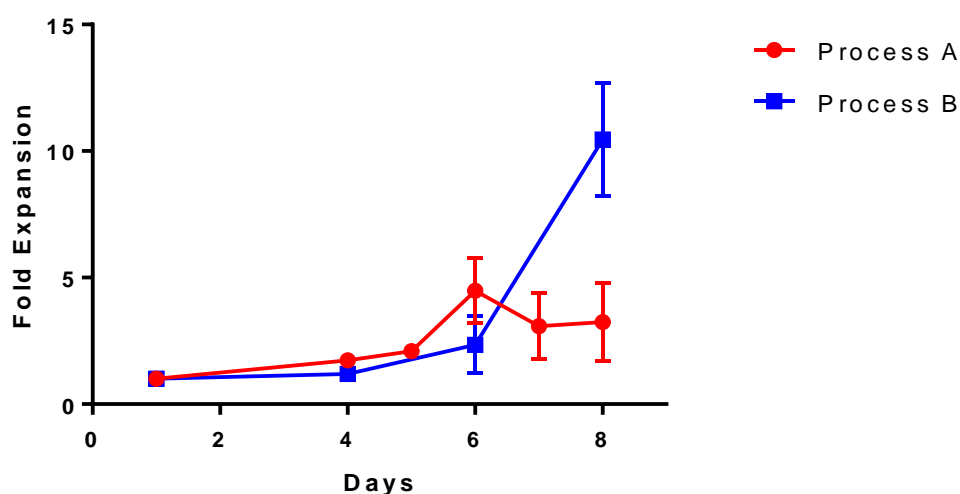
Figure 24: Comparison of fold expansion for processes A and B during the 7 days after lentiviral transduction

(For Process A, day 7 cell count was performed after the bead removal stage. WBC counts were performed at each manufacturing site using a Sysmex hematological counter and/or TruCount. Data shows mean ± SD for 3 manufactures with each protocol)

4.3.3 Transduction Efficiency for "open" and "closed" process

The vector for the ALLCAR19 clinical trial (pCCL.PGK.αCD19CAT-41BBζ) is discussed in Section 2.1. For Process A, MOI was fixed at 5. To validate a lower MOI and conserve vector, we explored a MOI of 2.5 in Process B. Transduction efficiency and MOI for all three manual and semi-automated manufactures is displayed in Table 17 and Figure 25. All products manufactured by Process A and B achieved the release criteria of 15% CAT CAR+ T-cells. Process B used 3-4-fold less vector and a reduced MOI, with no significant impact on the transduction efficiency of the final product.
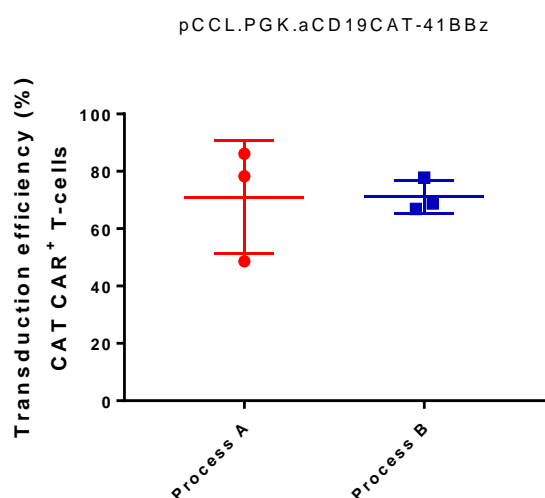
Figure 25: Transduction efficiency of the final products transduced with the CD19CAT-41BBz vector

(Transduction efficiency of the final products transduced with the CD19CAT-41BBz vector and stained for CAT CAR expression with an anti-idiotype antibody. Results were obtained by flow cytometry analysis of a fresh sample of each final product, following the Standard Operational Procedures at each manufacturing site. Results are expressed as mean± SD of 3 manufactures with each protocol)

4.3.4 Final products generated by "open" and "closed" process

4.3.4.1 Composition of the final products (Process A+B)

Figure 26 demonstrates the viability (A), the absolute number of live CAR T-cells (B) and percentage of CD4+ and CD8+ cells (C) in each final product generated by Process A and B. Cellular composition and transduction efficiency of the three products is displayed in Table 17. Process B produced remarkably consistent results. The final products demonstrated high viability (>99%) with a consistent yield of at least 1.1×10^9 live CAR-T cells (median 1.5, range 1.1- 1.71×10^9). Process A was highly

variable in terms of viability, transduction efficiency and final CAR-T cell numbers (median 1.72 x10^9, range 0.05-2.2 x 10^9) with notably poor expansion in 1 of the 3 patients (failed to achieve target cell dose for trial). All 3 products generated by Process A and B were sterile, negative for mycoplasma spp and Endotoxin.

With both manufacturing methods, the final products were enriched in CD4$^+$ T-cells. The mean CD4:CD8 ratio (of total T-cells) was 2.9:1 (range 1.2:1-4.6:1) and 5:1 (range 3.4:1-7.2:1) for Process A and B respectively (p=0.24). Mean transduction efficiency of CD4+ and CD8+ cells was 76.9% (range 64.6-93.5%) and 56.8% (range 50.1-68.2%) respectively (data only available for Process B).
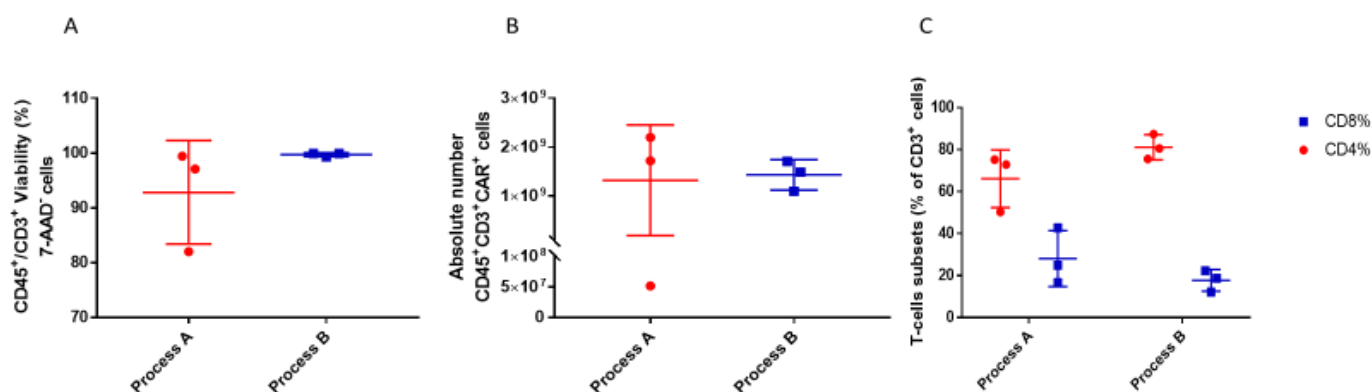
Figure 26: Viability, absolute number of CAR-T cells and % of CD4 and CD8 of final products generated with Process A and B.

Viability determined by % of 7AAD- cells (A), absolute number of CAR+CD3+CD45+ cells (B) and % of CD4 and CD8 (C) of the final products generated with Process A and Process B. All results were obtained by flow cytometry analysis of a fresh sample of the final products, in accordance with local Standard Operational Procedures. Data shows mean ± SD for 3 manufactures with each protocol.

Cellular Composition Final Product	Manual Process A			Prodigy Process B		
	ALL01	ALL02	ALL03	ALL01	ALL02	ALL03
Lymphocytes (CD45+/CD3+) (%Live cells)	95.70	94.60	87.80	98.6	99.10	97.60
NK (CD45+/CD56/16+) (% Live cells)	0.29	1.21	3.84	0.12	0.15	0.12
NKT(CD45+/CD3+/CD56/16+) (% Live cells)	0.05	0.37	0.71	1.16	1.08	0.86
CD10/19+ cells (% Live cells)	0	0.30	0	0.06	0.03	0.09
Viability (7-AAD-) (% CD45+/CD3+)	97.1	99.4	82	99.9	99.9	99.3
Total CAR+ cells (x10^9)	1.72	2.2	0.05	1.5	1.1	1.71
Transduction efficiency (% CD3+ live cells)	78.3	86.1	48.6	68.8	66.9	77.8
Vector	CAT	CAT	CAT	CAT	CAT	CAT
MOI	5	5	5	2.5	5	2.5
Vector Units (x10^6)	2500	2500	2500	870	820	615

Table 17: Cellular composition of final products (n=3) by manual (Process A) and semi-automated (Process B) methods

4.3.4.2 Maturation of final products (Process B)

Although the starting leukapheresis had a highly variable maturation profile, the final products generated on the CliniMACS Prodigy® were enriched with cells of a central memory phenotype (median 40.6%, range 16.9-55.7%) (Figure 27 and 28).
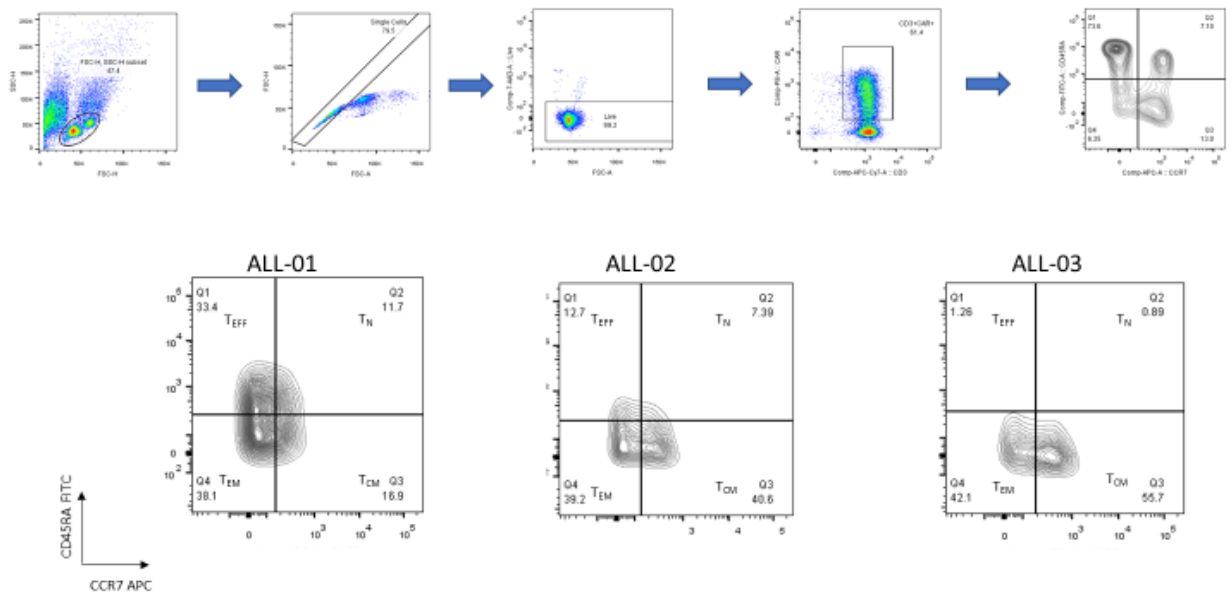
Figure 27: Maturation profile of CD19 CAR+ T-cells based on expression of CD45RA and CCR7 by flow cytometry

(The top panels show the gating strategy for analysis of final cell product. Subsets were defined as: naïve T-cells (T_N: CD3+CCR7+CD45RA+), central-memory T-cells (T_{CM}: CD3+CCR7+CD45RA-), effector-memory T-cells (T_{EM}: CD3+CCR7-CD45RA-) or effector T-cells (T_{EFF}: CD3+CCR7-CD45RA+). Final CD19 CAR+ T-cells products were consistently enriched in T_{CM} subsets)
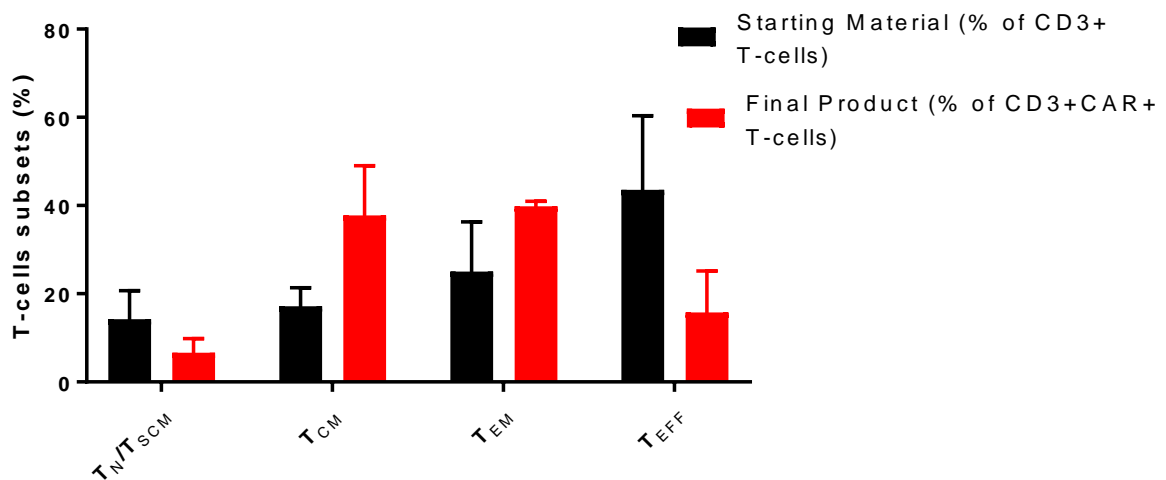
Figure 28: Maturation profile of total T-cells in starting material and final CAR+ T-cells (Process B) based on expression of CD45RA and CCR7 by flow cytometry

(Subsets were defined as: naïve T-cells (T_N: CD3+CCR7+CD45RA+), central-memory T-cells (T_{CM}: CD3+CCR7+CD45RA-), effector-memory T-cells (T_{EM}: CD3+CCR7-CD45RA-) or effector T-cells (T_{EFF}: CD3+CCR7-CD45RA+). Results represent mean ± SD of the 3 manufactures)

Figure 28 compares the T-cell memory subsets in the patients starting material relative to the CAR-T cell memory subsets in the final products generated by the CliniMACs Prodigy® (Process B). As noted above there is enrichment of the central memory population.

4.3.4.3 Exhaustion of final products (Process B)

Exhaustion of CD4+ and CD8 T cells in the starting material and final products (generated by Process B) based on expression of PD-1 and Tim-3 by flow cytometry is displayed in Figure 29 and 30 respectively. For both CD4+ and CD8+ cells, the expression of PD-1 and Tim-3 on CAR-T cells and total T-cells is reduced in the final products relative to the starting material. This may be consistent with cell death, namely death of exhausted cells and survival of more robust T-cells as the manufacture progresses and in the final products. Results represent mean ± SD of the 3 manufactures.
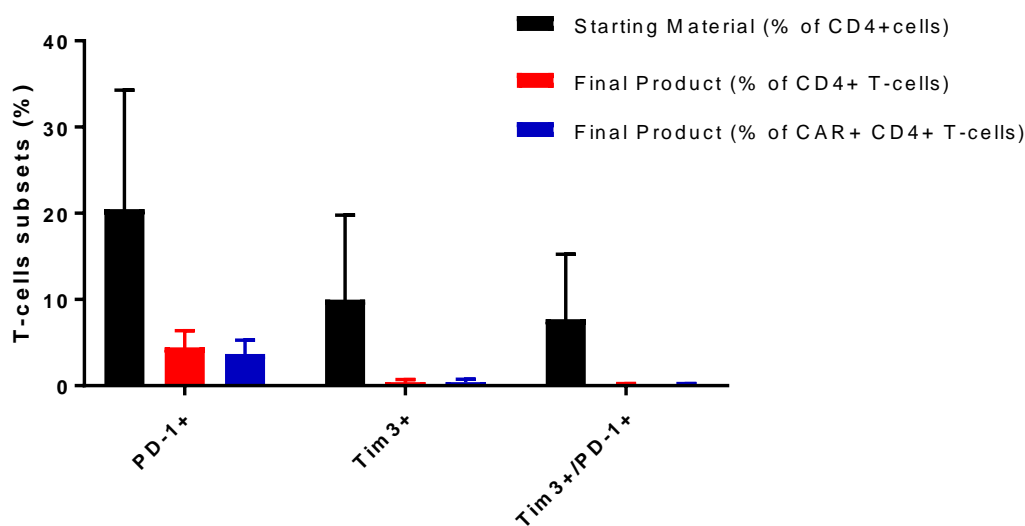
Figure 29: **Exhaustion profile of CD4+ T-cells in starting material and CD4+ T cells and CAR+ T-cells in final product (Process B)**
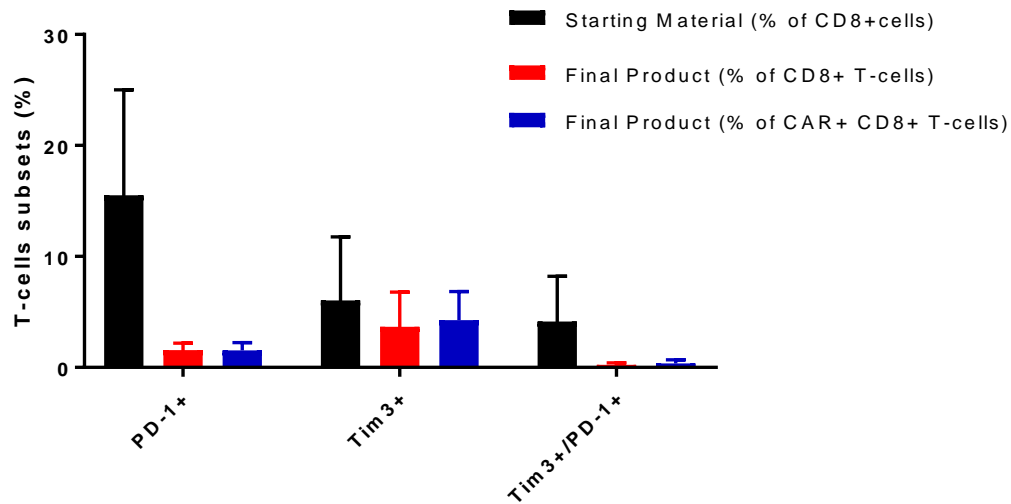
Figure 30: Exhaustion profile of CD8+ T-cells in starting material and CD8+ T cells and CAR+ T-cells in final product (Process B)

4.3.4.4 Functional analysis of CD19 CAR-T cells (Process B)

CAR-T cells manufactured via process B were co-cultured against CD19-expressing cell lines (Supt1-CD19 and RAJI) as described Section 2.5. Figure 31 demonstrates the lysis of CD19 expressing SupT1 CD19 and RAJI target cells (ET ratio 1:1) in co-cultures at 24 hours and 48 hours. Non-specific lysis is noted at 48 hours.

Furthermore, the CAR-T cells also secreted cytokines (IFNγ and IL2) in response to co-culture with the CD19 expressing target cells at 24 hours (Figure 32). IL2 secretion by CAR-T cells was significantly higher compared to the non-transduced controls (P=0.04).
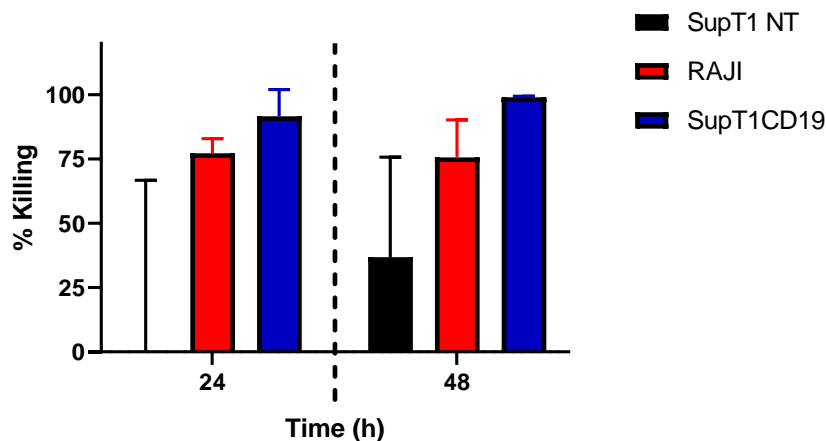
Figure 31: %killing by CD19 CAR-T cells manufactured via process B in response to co-culture with CD19 expressing cell lines Supt1-CD19 and RAJ

(Results represent mean +/- SD for 3 products)
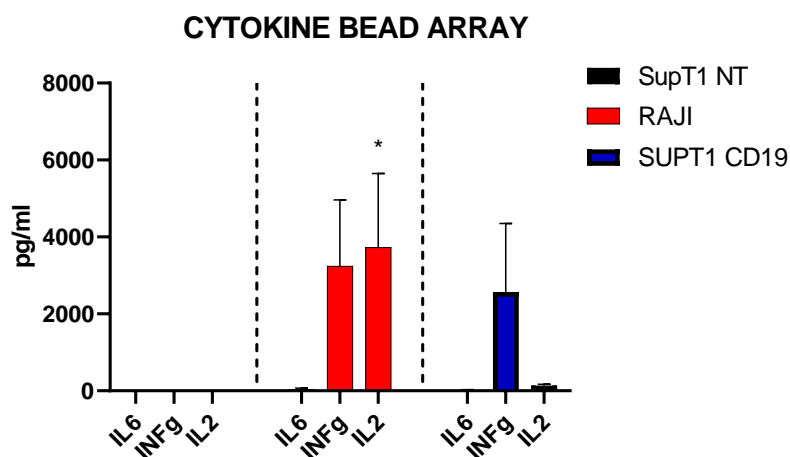
Figure 32: Cytokine secretion in co-culture supernatants was assessed at 24 hours (Process B)

(Results represent mean +/- SD for 3 products)

4.3.4.5 Comparison of manual and semi-automated process by ALLCAR19 release criteria

The three autologous CD19 CAR-T cell products generated by the A and B manufacturing methodologies were compared in terms of ALLCAR19 release criteria (Table 13). While all 3 products generated via manual and semi-automated methods met the release criteria for the trial, one product generated via the manual method (ALL03) met the minimum dose for batch release but failed to meet

the trial dose (Table 18). The minimum dose stipulated for release is $\geq 5 \times 10^6$ CAR-T cells, the trial dose is 410 x 10^6 CAR-T cells and the manual manufacture for ALL03 generated 50 x 10^6 CAR-T cells.

Release Criteria ALLCAR19

Release Criterion	Specification	Manual			Prodigy		
		ALL01	ALL02	ALL03	ALL01	ALL02	ALL03
Sterility	No growth after 10 days incubation	✓	✓	✓	✓	✓	✓
Endotoxin	<2EU/ml	✓	✓	✓	✓	✓	✓
Mycoplasma	None detected	✓	✓	✓	✓	✓	✓
Minimum cell dose	$\geq 5 \times 10^6$ CAR-T cells	✓	✓	✓	✓	✓	✓
Transduction Efficiency	≥15% CAR+ cells (CD3+/CD45+ cells)	✓	✓	✓	✓	✓	✓
Viability	≥70% pre-freeze	✓	✓	✓	✓	✓	✓
Max volume of ATIMP dose (DMSO exposure)	≤12ml/kg	✓	✓	✓	✓	✓	✓

Table 18: ALLCAR19 release criteria by manual and semi-automated methods for each product

(ALL03 met the minimum dose criteria for release but failed to meet the trial dose)

4.3.5   Comparison of resources for "open" and "closed" process

4.3.5.1 Use of staff resources and cGMP clean room time

Figure 33 shows a comparison of the reagents, consumables, equipment, grade A hours, total hand-on hours and staff required for each step of the two processes. For Process A (manual), 7 out of the 9 manufacturing steps were labour-intensive and conducted in Grade A cGMP conditions, requiring at least 2 highly trained staff members (media and buffer preparation, cell activation, transduction, transfer to Wave Bioreactor, sampling, magnetic bead removal and final product cryopreservation). For Process B (Prodigy), of the 9 days of manufacture, only 3 steps (4 steps if frozen material) required Grade A cGMP conditions (media and buffer preparation, thawing and washing of leukapheresis, preparation of vector and final product cryopreservation). Although the overall cost of consumables was similar between the two methods, Process B was considered more cost-effective due to a reduction in the use of costly clean room facilities (including Grade A time), total staff hours, vector cost per manufacture and equipment requirements. A 72% reduction in Grade A time (8 hours versus 29 hours) and 56% reduction of total hands-on time (41 hours versus 93 hours) is achieved with a semi-automated process in comparison to the manual protocol. This significantly reduces the costs associated with staff and their training requirements, clean room rental, gowning and environmental monitoring. The CliniMACS Prodigy® also replaces several items of critical equipment, including incubators, WAVE bioreactors

and the DynaMag™ Magnet, reducing floorspace requirements, cleaning, maintenance and servicing costs.

		Reagent Preparation	CD4/CD8 selection	Set up/ Activation	Transduction	Transfer to Wave Bioreactor	Expansion	Beads Removal	Cryopreservation
Process A	Grade A Hands-on Staff no.	5 hours 6 Hours 2	N/A N/A N/A	4 Hours 5 Hours 3	3 hours 3 Hours 2	4 Hours 6 hours 3	3 hours 3 hours 2	5 hours 6 Hours 3	5 Hours 6 Hours 3
	Additional reagents/ consumables	N/A	N/A	- Dynabeads - Cultivation Bags	- Cultivation Bags - Retronectin - Consumables for cell wash/feed - Higher volume of vector required (3-4 fold)	- WAVE bags - 2L X-VIVO15 - MACS GMP Recombinant Human IL-2 (if applicable)	-2L X-VIVO15 -MACS GMP Recombinant Human IL-2 (if applicable	-CliniMACS PBS/EDTA+HAS -X-VIVO15 -MACS GMP Recombinant Human IL-2 (if applicable	- Consumables for cell washing and sampling
	Equipment required	PlasmaTherm	N/A	- Incubator	- Incubator	- Incubator - WAVE Bioreactor	- WAVE Bioreactor	- DynaMag CST Magnet - WAVE Bioreactor	- CRF
Process B	Grade A Hands-on Staff	4 hours 4 hours 2	N/A 5 hours 2	N/A 1 hour 2	1 hour 1 hour 2	N/A N/A N/A	N/A 2 hours 2	N/A N/A N/A	3 hours 5 hours 3
	Additional reagents/ consumables	N/A	- CliniMACS CD4 Reagent - CliniMACS CD8 Reagent - TS 520 Tubing Set	- MACS GMP T Cell TransAct	N/A	N/A	- 2.5L TexMACS 3% AB serum - MACS GMP Recombinant Human IL-15 - MACS GMP Recombinant Human IL-7	N/A	N/A (Cell wash and harvest performed by the Prodigy)
	Equipment required	- Sterile Tubing Welder	- CliniMACS Prodigy	- CliniMACS Prodigy - Sterile Tubing Welder	- CliniMACS Prodigy - Sterile Tubing Welder	N/A	- CliniMACS Prodigy - Sterile Tubing Welder	N/A	- CRF

Figure 33: Comparison of the reagents, consumables, equipment, grade A hours, total hand-on hours and staff required for each step of the two processes

4.3.5.2 Use of vector

Considering the production of cGMP lentiviral vector is the most expensive component of the CAR-T manufacturing process, conservation of vector with Process B represented the greatest cost saving in this comparison study. Based on an approximate cost of £300,000 for a 25ml batch of lentiviral vector, use of 2500 IU to transduce 500×10^6 lymphocytes (fixed for process A) for one manual manufacture at a MOI of 5 equates to approximately £26,000 (based on titer of 1.13×10^9 IU/ml and 2.2ml of vector per manufacture).

At the CCGTT, in our hands, we have validated starting cell numbers (CD3+) of 70-100 x 10^6 (data not shown) .Given this project has also validated an MOI of 2.5 (Section 4.3.4), our current practice at the

CCGTT is to commence cell culture with as few as 100 x10^6 lymphocytes using the Prodigy system and with a MOI as low as 2.5 (CAT CAR vector). Using the same titer, one manufacture using the Prodigy system equates to 250 IU of vector (221 ul) at a cost of approximately £2600, conferring vector cost savings of up to £23,400 per manufacture.

4.3.6 Residual vector test (Process B)

Residual viral vector was assessed (BR-VVX-106) by obtaining peri-process supernatant samples at Day 0, 4, 6 and 8 of the second ALLCAR19 validation process (ALL-02) (Section 2.1.3). This was to determine the viral vector infectivity at the different stages of the Prodigy-based CAR-T cell manufacture process. Percentage transduction of the supernatant was assessed on HEK293T cells by recombinant CD19-rbIgG1Fc/-anti-rabbit IgG-PE staining and flow cytometry.

Supernatant harvested at Day 6 and Day 8 shows minimal/absent residual viral vector infectivity (Table 19) and as such, cells coming off the Prodigy process after Day 6 can be handled in clean rooms outside of a GM-specific laboratory as required.

Transduction Efficiency ALL02 supernatant (%)			
Day 0	Day 4	Day 6	Day 8
0	29.65	0.75	0.15

Table 19: Residual viral vector infectivity ALLCAR19

4.3.7 Submission of IMPD to MHRA

To change manufacturing methodology and site of manufacture, a major amendment was submitted to the MHRA in April 2018. This was approved in June 2018. Following this amendment, all ALLCAR19 CAR-T cell products have been manufactured using the CliniMACS Prodigy® at the CCGTT. To submit these amendments, regulatory documents were drafted and edited by the manufacturing team, submitted to the Clinical Trials Centre (CTC) for review and ultimately submitted to the MHRA.

4.4 cGMP manufacturing: Conclusions

Either cryopreserved or fresh leukapheresis from three patients with B-ALL were obtained with consent and processed, both via the conventional manual open Dynabead process and the closed, semi-automated CliniMACS Prodigy®. It is acknowledged that a direct comparison of 2 methodologies should use solely fresh or frozen material from the same patients and ideally performed by the same manufacturing team within a designated laboratory. Indeed, to tease out the relative impact of individual variables and reagents on the success of the process, many conditions are warranted (examples displayed below). Such a comparison would however have been prohibitively expensive and time-consuming (patients awaiting therapy).

	T-cell Immunomagnetic Selection	Media	Activation	Cytokines
Condition 1 **(Simulating new closed Prodigy® Process)**	Yes	TexMACS	TransAct	IL-7 and IL-15
Condition 2 **(Simulating open manual process)**	No	X-VIVO	DynaBeads	No cytokines
Condition 3	No	TexMACS	Dynabeads	IL-7 and IL-15
Condition 4	Yes	TexMACS	TransAct	No cytokines
Condition 5	Yes	X-VIVO	Dynabeads	No cytokines
Condition 6	No	TexMACS	TransAct	IL-7 and IL-15

Therefore, three runs were successfully completed by both methodologies as detailed in 2.8.1 and 2.8.2 (Condition 1 and 2). FACS analysis of each final sample was performed. The number of CD45+/CD3+/CAR+ on day 8 of the Prodigy® manufactures was sufficient for cryopreservation of all maximum trial doses. One product (ALL03) generated via the manual method met the minimum dose for release but failed to meet the trial dose. The minimum criteria for viability and transduction efficiency were met on all runs and all products were sterile with no mycoplasma or endotoxin detectable. Residual free viral particles at different stages of the Prodigy® process was also assessed, to determine infectivity risk of intermediate and final products. No viral vector infectivity was detected by day 6, indicating the cell product imposes minimal risk to the CCGTT and other users/products from this stage of manufacture.

While both methods successfully generated 3 autologous CD19 CAR-T cell products, the CliniMACS Prodigy® produced remarkably consistent results in terms of viability (>99%), cell expansion, transduction efficiency and CAR-T cell yield. The manual process was much more variable in this respect. Standardisation of the CAR-T manufacturing process with consistent results, irrespective of the starting material is highly desirable.

Extended phenotyping (maturation and exhaustion) and functionality testing (FBK and cytokine release) of the final products was only available for the products generated via the Prodigy® system (due to lower cell expansion on the manual method, no excess material was available for testing: all cells were diverted to the patient product and the MHRA mandated retention vials). These results demonstrated minimally exhausted CAR-T cell products enriched with central memory T-cell populations, capable of killing CD19+ cells in vitro and stimulating cytokine release.

The closed, semi-automated Prodigy® system is a more time-efficient mode of manufacture, with significant reductions in total clean room time, grade A time, staff resources and equipment requirements. Use of lower vector volumes translate into a significant cost saving. The use of this platform lends itself to manufacture at scale, a huge advantage given the complexity of this manufacturing process. This project confirms that the TCT process using the CliniMACS Prodigy® is safe and efficient for manufacture of CAT CAR T cell products.

The effect of mode of manufacture on CD4 and CD8 T-cell subsets in the final products and the clinical implications of this remain unclear. The mean CD4:CD8 ratio in the starting material was 1.5:1 (range 0.43-3:1). The mean CD4:CD8 ratio of total T-cells in the final products was 2.9:1 (range 1.2-4.6:1) and 5:1 (range 3.4-7.2:1) for manual and Prodigy processes respectively (p=0.24). Total T-cells was chosen as the comparator as transduction efficiency of CD4 and CD8 T-cell subsets was not available for the manual process. The mean CD4:CD8 ratio for total CAR-T cells generated on the CliniMACS Prodigy® was 8.2:1 (range 4.9-13.3:1). Mean transduction efficiency of CD4+ and CD8+ cells was 76.9% (range 64.6-93.5%) and 56.8% (range 50.1-68.2%) respectively (data only available for Prodigy® process). Castella et al report the largest dataset of autologous products (n=27) generated on the Prodigy System. CD4+ cells expanded relative to CD8+ cells but only in patients with a CD4/CD8 ratio of <1 in the starting material. By comparison, the proportion of CD4+cells tended to decrease in patients with a CD4/CD8 ratio of >1 in the starting material. The transduction efficiency of CD4+cells was significantly higher than CD8+cells. Interestingly, in smaller scale experiments, Castella et al demonstrated that cytokines and transduction had no effect on the CD4:CD8 ratio but that CD4+ and CD8+ T-cell numbers equilibrated somewhat in prolonged culture (197). A non-statistically significant expansion of CD4+ CARs relative to CD8+ CARs has been noted previously with the CliniMACS Prodigy® using autologous PB in children with B-ALL (n=4) (196). Preferential expansion of CD8+ CAR-T cells has been demonstrated using healthy donor material on the Prodigy® system (192,280).

There are many potential causes for an abnormal CD4:CD8 ratio in the starting material and final products. CD4 and CD8 T-cell subsets are known to differ between patients and healthy donors, Sommermeyer et al demonstrating that patients with B-cell malignancies are more likely to have a higher proportion of CD8+ T-cells and therefore a CD4/CD8 ratio < 1 (281). Intensive chemotherapy is also known to disrupt the CD4:CD8 ratio and CD4+ T-cells take longer to recover to baseline levels due to age-dependent decline in thymopoiesis (282). Additionally, Gomes-Silva et al demonstrated preferential expansion of CD4+ T-cells with a 4-1BB relative to a CD28 co-stimulatory domain (283). Through this set of validations, we have measured the CD4:CD8 ratios of total T-cells in starting material and final products and the relative transduction efficiency of CD4 and CD8 T cells. It highlights the variability in composition of the starting material and final products and in relative expansion of CD4 and CD8 T-cells as the manufacture progresses. Expansion of CD4 and CD8 T-cells during manufacture may be determined by the CD4/CD8 ratios in the starting material (as proposed by Castella et al). This was observed using ALL01 (but not ALL-02 or ALL-03) starting material during our process validations. We will evaluate this in an ongoing way through the clinical study. Again, the higher transduction efficiency of CD4+ cells is noted. While CD8+ CAR-T cells have been shown to have superior cytotoxic activity, CD4+ CAR-T cells produce more Th1 cytokines and may have superior proliferative potential (281). CD4+ and CD8+ CAR-T cells have been demonstrated to have synergistic activity in mouse models (281,284,285) highlighting that both subsets play an important role in T-cell immunotherapy.

Data on in vivo expansion of the CAR T-cell subsets in treated patients is keenly awaited to determine whether this has an impact on CAR T-cell efficacy and/or toxicity.

CHAPTER 5

5 CGMP FLOW CYTOMETRY RELEASE ASSAY FOR ALLCAR19

5.1 Introduction

The EC guidelines on GMP medicinal products for human use do not offer formal guidance on the validation of release flow assays for exploratory phase studies (sterility and microbial assays should be validated however). Validation of all analytic assays for batch release is expected for pivotal clinical trials (158). Flow cytometric methods for drug products can be challenging to validate due to cell type in question, number of reagents used and potential lack of reference material. O'Hara et al address these issues in their recommendations for validation of flow cytometric testing for drug products (286).

Accuracy is defined as the degree to which the result of a measurement, calculation, or specification conforms to the correct value or a standard. No formal guidelines exist as to what accuracy is desired for a bioanalytic assay. However, O'Hara et al suggest an acceptable level of accuracy ±20% of nominal value for assays used for drug development. Precision refers to the closeness of two or more measurements to each other, denoted in this case by the coefficient of variation (CV). The CV is a measure of relative variability. It is the ratio of the standard deviation to the mean. The within-run CV value should not exceed 20% (286).

5.2 Aims of this chapter

Manufacture of anti-CD19 CAR T-cells for the ALLCAR19 trial involves lentiviral transduction of T cells to achieve cell surface expression of CAT CAR (minimum of 15% stipulated for release). As part of the release criteria assessment, this construct is detected with a specific primary anti-CAT idiotype antibody followed by a PE-conjugated secondary stain, directed against the Fc-fragment of the anti-idiotype primary.

The aim of this validation was to determine the specificity, accuracy and within-run precision (across serial dilutions) of the release flow assays for anti-CD19 CAT CAR T-cells generated using the Prodigy® on 2 separate flow cytometers (primary instrument (BD FACSCelesta™) and back-up instrument (Miltenyi MACSQuant®). The following 3 flow cytometry parameters were validated by the GMP team.

- Transduction efficiency
- CD3+ Trucount™

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Samples were generated through serial dilution of CAT CAR+ flow-sorted cells into autologous non-transduced (NT) T-cells (Section 2.9.2) across a range of dilutions from 100% to 3.1%. Samples for Trucount™ and transduction efficiency analysis were stained in triplicate (with exception of A1-A5 Trucount due to insufficient sample) as per Section 2.4.1 and Table 6 respectively. Acquisition was performed on two different devices, BD FACSCelesta™ Flow Cytometer and MACSQuant® Flow Cytometer.

5.3 Results

5.3.1   Transduction efficiency flow validation

Tables 20 and 21 demonstrates the results of the transduction efficiency validation for ALLCAR19. Samples A0 (NT cells) to A6 (100% CAR) were run in triplicate on the BD FACS Celesta™ and MACSQuant® Flow Cytometer. Mean CAR percentage, SD, CV and RE was calculated for each dilution. Fluorescence minus one (FMO) samples are used as NT controls for each condition (A0-A6) and record background events (7-AAD-negative CD45+ CD3+ CAT+ cells in NT negative controls). FMO results are then subtracted from each reading to record true positive events (CAR-FMO).

For each dilution, acceptance criteria outlined in Table 25 are met. Results are also summarised further in Table 25.

Sample	BD FACSCelesta™							CV ≤20%	RE ≤±20%
	% CAR[1]	FMO[2]	% CAR-FMO	Mean % CAR	SD[3]	CV[4] (%)	RE[5] (%)		
A0 0%	0.1	0.1	0.0	0.0	0.1				
	0.1		0.0						
	0.2		0.1						
A1 3.125%	3.3		3.2	3.3	0.1	3.0	5.6	Yes	Yes
	3.4		3.3						
	3.5	0.1	3.4						
A2 6.25%	6.2		6.1	6.2	0.2	3.2	-0.8	Yes	Yes
	6.5		6.4						
	6.3	0.1	6.2						
	13.6	0.0	13.6	13.3	0.4	3.0	6.4		

Sample	% CAR	FMO	% CAR-FMO	Mean % CAR	SD	CV (%)	RE (%)	CV ≤20%	RE ≤ ±20%
A3 12.5%	12.9		12.9					**Yes**	**Yes**
	13.4		13.4						
A4 25%	25.8		25.7	25.9	0.2	0.8	3.6	**Yes**	**Yes**
	26.0		25.9						
	26.1	0.1	26.0						
A5 50%	53.0		52.9	52.5	0.4	0.8	5.0	**Yes**	**Yes**
	52.7		52.6						
	52.2	0.1	52.1						
A6 100%	99.6		99.6	99.6	0.1	0.1	-0.4	**Yes**	**Yes**
	99.5		99.5						
	99.6	0	99.6						

Table 20: Transgene stain results for A0-A6 (in triplicate) for ALLCAR19 transduction efficiency assay (BD Celesta)

[1] % of CAT CAR-PE$^+$ cells gated from CD45$^+$/CD3$^+$/7-AAD$^-$ cells

[2] % of PE$^+$ cells gated from CD45$^+$/CD3$^+$/7-AAD$^-$ cells on samples with no anti-idiotype stain

[3] SD = Standard error

[4] CV= Coefficient of variation = $100 \times SD \div mean$

[5] RE= Relative error = $100 \times (measured - spiked) \div spiked$

	Miltenyi MACSQuant®							CV ≤20%	RE ≤ ±20%
Sample	% CAR[1]	FMO[2]	% CAR-FMO	Mean % CAR	SD[3]	CV[4] (%)	RE[5] (%)		
A0 0%	0.31	0.4	-0.1	-0.1	0.1				
	0.17		-0.2						
	0.25		-0.1						
A1 3.125%	3.23	0.2	3.1	3.2	0.1	3.1	2.4	**Yes**	**Yes**
	3.27		3.1						
	3.41		3.3						
A2 6.25%	5.95	0.3	5.7	5.9	0.2	3.4	-5.6	**Yes**	**Yes**
	6.29		6.0						
	6.18		5.9						
A3 12.5%	12.9	0.1	12.8	13.1	0.6	4.6	4.8		

	13.8		13.7					**Yes**	**Yes**
	12.8		12.7						
A4 25%	26.4	0.1	26.3					**Yes**	**Yes**
	26.6		26.5	26.4	0.2	0.8	5.6		
	26.7		26.6						
A5 50%	52.2	0.2	52.1					**Yes**	**Yes**
	53.6		53.5	52.4	0.9	1.7	4.8		
	51.8		51.7						
A6 100%	99.5	0.1	99.4					**Yes**	**Yes**
	99.7		99.6	99.6	0.2	0.2	-0.4		
	99.8		99.7						

Table 21: Transgene stain results for A0-A6 (in triplicate) for ALLCAR19 transduction efficiency assay (MACSQuant®)

[1] % of CAT CAR-PE$^+$ cells gated from CD45$^+$/CD3$^+$/7-AAD$^-$ cells

[2] % of PE$^+$ cells gated from CD45$^+$/CD3$^+$/7-AAD$^-$ cells on samples with no anti-idiotype stain

[3] SD = Standard error

[4] CV= Coefficient of variation = $100 \times SD \div mean$

[5] RE= Relative error = $100 \times (measured - spiked) \div spiked$

Further validation of this assay comprised a sample re-test at an interval to confirm consistency of performance. Cells were stained on the last day of the manufacture process (day 8) prior to cryopreservation and the assay was repeated on two samples post thaw. Post thaw samples results (Table 22) were comparable to those obtained on the fresh product on the last day of manufacture (relative error < 20%).

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Sample	Date	BD FACSCelesta™		MACSQuant®	
		Transduction efficiency (%)	RE (%)[1]	Transduction efficiency (%)	RE (%)[1]
ALL-03 Final ATIMP (Manufacture day 8)	16/03/2018	77.8	N/A	79.2	N/A
ALL-03 QC Sample post thaw	26/03/2018	73.8	-5.14	74.2	-6.3
ALL-03 QC Sample post thaw	9/5/2018	83.0	6.68	79.7	0.63

[1]RE= Relative error = $100 \times$ (% measured - % Final ATIMP) ÷ % Final ATIMP, N/A not applicable

Table 22: Transgene stain results for post thaw for ALLCAR19 assay validation

5.3.2 Trucount™ validation

Final cells suspensions were generated at 1×10^6 CD45+/CD3+/7-AAD- cells/mL and A0 and A6 dilution was run in triplicate (insufficient sample for A1-A5). Mean cell count, SD, CV and RE was calculated for the 2 dilutions. For each dilution, acceptance criteria outlined in Table 25 are met. An example gating strategy for the use of Trucount™ beads is demonstrated in Figure 34. Calculation of cell numbers using Trucount™ is described in Section 2.4.1.
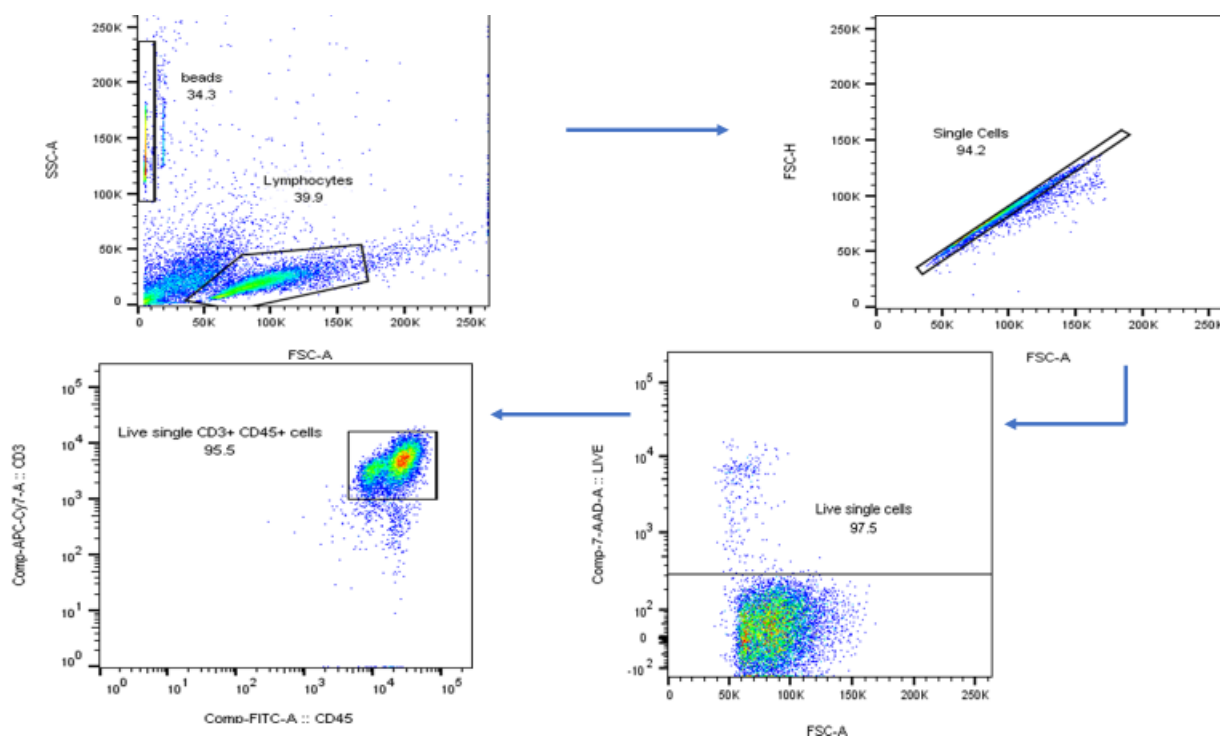
Figure 34: Example gating strategy for calculation of T-cell numbers using Trucount™

	TRUCOUNT™ STAIN - Samples at 1 x 10^6 cells/ mL					CV ≤20%	RE ≤ ±20%
	BD FACSCelesta™						
	Counts (x10^6/mL)	Mean (x10^6/mL)	SD[1]	CV[2] (%)	RE[3](%)		
A0	1.06	1.04	0.03	2.88	4	Yes	Yes
	1.01						
	1.04						
A1	1.10	1.10			10		Yes
A2	1.07	1.07			7		Yes
A3	1.08	1.08			8		Yes
A4	1.08	1.08			8		Yes
A5	1.10	1.10			10		Yes
A6	1.09	1.07	0.03	2.80	7	Yes	Yes
	1.03						
	1.08						

Table 23: Trucount™ results (CD45+/CD3+) for A0 to A6 (in triplicate for A0 and A6) for ALLCAR19 release flow assay validation (BD Celesta)

[1]SD = Standard error

[2] CV= Coefficient of variation = $100 \times SD \div mean$

[3]RE= Relative error = $100 \times (measured - spiked) \div spiked$

TRUCOUNT™ STAIN - Samples at 1 x 10^6 cells/ mL							
	MACSQuant®					**CV** $\leq 20\%$	**RE** $\leq \pm 20\%$
	Counts (x10^6/mL)	**Mean (x10^6/mL)**	**SD[1]**	**CV[2] (%)**	**RE[3](%)**		
A0	0.92	0.96	0.05	5.20	-4	**Yes**	**Yes**
	1.02						
	0.93						
A1	1.04	1.04			4		**Yes**
A2	0.90	0.90			-10		**Yes**
A3	1.04	1.04			4		**Yes**
A4	1.03	1.03			3		**Yes**
A5	1.10	1.10			10		**Yes**
A6	0.96	0.96	0.01	1.04	-4	**Yes**	**Yes**
	0.96						
	0.95						

Table 24: Trucount™ results (CD45+/CD3+) for A0 to A6 (in triplicate for A0 and A6) for ALLCAR19 release flow assay validation (MACSQuant®)

[1]SD = Standard error

[2] CV= Coefficient of variation = $100 \times SD \div mean$

[3]RE= Relative error = $100 \times$ (measured - spiked) $\div$ spiked

5.4 Conclusions

Validation results are summarized in Table 25. The minimum criteria for specificity, accuracy, within run precision and dynamic range were met for all samples. These results confirm that the staining method and analysis protocol described by ALLCAR19 Release Flow Assay SOP are sufficient for assessment of transduction efficiency and absolute CD45$^+$/CD3$^+$ live cells count and therefore adequate assays for release of CAT CAR T cells products. Additionally, the satisfactory results obtained on these tests for both machines, validates the use of BD FACSCelesta™ Flow as the primary instrument for product release flow cytometry assays and MACSQuant® Flow Cytometer as a back-up machine.

Critical Step / Evaluation criteria	Acceptance Criteria	Validation Results		Final Result
		Celesta	MACSQuant	
Specificity (background)	Sample A0 % < 2 standard deviations sample A1 value.	A0 = 0 0 SD	A0 = 0.1 1 SD	PASS
Accuracy	Relative Error (RE): ≤ ±20% for sample A1-A6.	-0.4-6.4% CAR Stain	-0.4-5.6% CAR Stain	PASS
		4-10% Trucount*	3-10% Trucount*	
Within run-precision	CV ≤20% comparing samples A1-A6 within assay	0.1-3.2% CAR Stain	0.2-4.6% CAR Stain	PASS
		2.80-2.88% Trucount*	1.04-5.2% Trucount*	
Dynamic range and LLOQ	Acceptance criteria for accuracy and precision met for samples A1-A6 i.e. samples containing 3.1% - 100% transduced cells	PASS	PASS	PASS
CAR expression post thaw	RE=100 x (% measured - % CAR expression on day of cryopreservation) / % CAR expression on day of cryopreservation should be ≤ ±20%	-5.1-6.7%	-6.3-0.6%	PASS

Table 25: Acceptance criteria and ALLCAR19 flow validation results

*Samples A0 and A6

CHAPTER 6:

6 CGMP CAR T-CELL MANUFACTURE: OPTIMISING APHERESIS

6.1 Introduction

CD3+ lymphocytes are the target cells for collection for CAR-T cell manufacture (Section 1.5). The majority of the published data on T-cell harvest for immunotherapy relates to paediatric and young adult populations (Section 1.5.2) but the bulk of demand is in older patients. Cell collection can be a challenging task in a heavily pre-treated and heterogeneous cohort of patients. There is evidence to suggest that the quality of the starting material can impact on clinical responses (Section 8.3.4.1). However, there is no international guidance and limited published literature on the optimal timing of the procedure, the optimal ALC or number of target cells for manufacture with each commercial company and academic unit stipulating their own requirements. With a review of practice at UCLH, we sought to optimise this critical step in older adults with advanced B-cell malignancies for our prospective academic CAR-T clinical trials at UCL.

6.2 Aims of this chapter

As discussed in Section 1.5, there are a range of apheresis platforms in clinical use. However, there has been a shift in the field towards increased use of the Terumo Spectra Optia device due to perceived advantages of automation (Section 1.5.4). The Spectra Optia has superseded the COBE Spectra in our apheresis unit at UCLH and we have focussed our analysis on this device with the aim of devising an algorithm/model to guide T-cell collection for CAR manufacture in adult patients with advanced B-cell malignancies.

In our experience in adult patients with advanced B-cell malignancies enrolled on academic CAR-T cell studies at UCLH, a target of 1.5×10^9 CD3+ cells is sufficient to proceed with cell manufacture on the Prodigy® system and for cryopreservation of back-up material to be used in the event of a manufacturing failure. As an example, for comparison Kite Gilead request 2×10^9 MNCs as a target for downstream commercial Axicel manufacture.

In accordance with local practice at UCLH, a standard 2 x blood volume (BV) apheresis is performed on the Spectra Optia, unless ALC is $<0.5 \times 10^9$/L, prompting escalation to a 2.5 BV harvest. Our rate of failure of T-cell harvest (and cell manufacture) is extremely low. However, a personalised approach

is preferred for such a heterogeneous cohort, to identify higher risk patients, thereby shortening and prolonging procedure time (as appropriate) to achieve a target CD3+ count. We wished to devise an algorithm to be used prospectively at UCLH, to enable individualised calculation of the required blood volume to be processed on the Spectra Optia to achieve the proposed CD3+ target of 1.5×10^9 using only the patient peripheral blood ALC (x 10^9/L). We chose variables that are readily available in the clinic for ease of use.

A shorter procedure will undoubtedly improve the patient experience and also increase the capacity of our apheresis department. By reducing the future burden of CD3+ count testing, we may also reduce the workload in our stem cell laboratory. The ultimate goal is to allow scaling of the service to meet patient need.

6.3 Results

6.3.1   Patient cohort

From August 2016 to November 2018, 40 patients underwent 42 apheresis procedures using the Spectra Optia at UCLH. Two patients had a second apheresis procedure, one due to bacterial contamination of the initial material and the other due to an electrical fault complicating the first cell manufacture.   The majority of the patients were male (68%). Of the 40 patients, 4 (10%) were healthy donors who donated as part of our allogeneic CAR-T cell trial. The remaining donors (n=36, 90%) attended for autologous donation with the following diagnoses: B-ALL n=14 (35%), DLBCL n=14 (35%), MM n=8 (20%). Two patients had circulating disease at the time of donation. Four donations were excluded from the analysis, one patient with B-ALL (inaccurate CE), two patients with DLBCL (no CD3+ pre-count n=1, no record of blood volume processed n=1) and one patient with MM (no record of blood volume processed). Therefore, 34 autologous donations and 4 allogeneic donations were available for analysis.

Patient disease demographics are summarised in Table 26.Median age and range for B-ALL, DLBCL and MM was 30 years (16-63), 49 years (28-61) and 60 years (45-61) respectively. These were heavily pre-treated patient populations, 48% had a previous haematopoietic stem cell transplant (HSCT, n=12) or autologous stem cell transplant (ASCT, n=7). Pre-collection blood counts and apheresis product yields are displayed in Table 27. Six patients (17%) had an ALC <0.5 x 10^9/L (range 0.2-0.49 x 10^9/L) at the time of donation.

Diagnoses			
	B-ALL N=14	Lymphoma N=14	Myeloma N=8
Gender			
Male	10	10	5
Female	4	4	3
Age (yrs)			
Mean	36	46	57
Median	30	49	60
Range	16-63	28-61	45-61
Weight (kg)			
Mean	78.4	81.2	78
Median	78	79.5	78.5
Range	49-109	55-98.8	62.6-95.8
Previous lines of therapy (excluding transplant) (n)			
Mean	3	4	5
Median	3	3	5
Range	2-6	2-6	4-7
Previous Transplant (n)			
HSCT	12	0	0
ASCT	0	2	5

Table 26: Patient demographics at time of autologous donation

6.3.2   Apheresis procedures

Poor venous access prompted the insertion of a femoral vascular catheter in 12 cases (29%). The remaining harvests were collected via peripheral access. The most frequent complication associated with the procedure was self-reported tingling/paraesthesia (21.4%, n=9). One patient developed hypotension and required intravenous fluids. Another patient was admitted overnight for investigation

and management of fevers that developed during the procedure. The overall complication rate was 26%, (n=11).

The complication rate with the Spectra Optia in this study is above that reported for apheresis for CAR-T cell production in the literature to date. Published complication rates range from 0-15% (209,210,287). However, 2 of these studies used the COBE Spectra and administered prophylactic intravenous (IV) calcium gluconate throughout the procedure. The only study comparing the COBE Spectra and Spectra Optia for T-cell harvest for CAR-T cell manufacture (paediatric) also administered prophylactic IV calcium to maintain serum levels (226). At UCLH, prophylactic IV calcium is not used, which may account for the higher than average reported rates. Self-reported paraesthesia is also presumed to be ACDA toxicity and treated empirically until symptoms resolve. Serum calcium is checked pre-procedure and is only repeated during the harvest if symptoms persist or worsen. In addition, this is a small dataset and our patients are empowered to report their symptoms. All reported symptoms were mild.

6.3.3   Apheresis products

Apheresis product yields by disease are summarised in Table 27. All patients had between 2 and 2.5 estimated blood volumes processed. The Spectra Optia uses a formula based on sex, height and weight to estimate total blood volume (TBV). Mean haematocrit percentage in the final products ranged from 2-3% for all groups which is in line with general guidance (288).

We observed a wide range in blood volumes processed, even within disease groups. This likely reflects the variability in patient weight (Table 26). While the numbers are too small to assess significance (n=4), healthy donors had a higher median ALC pre-collection, a higher median collection efficiency and CD3+ cell yield relative to autologous donors.

A target collection efficiency (CE2%) of >40% was sought, in line with other centres and published guidance (ref).

Variable	ALL N=13*	Lymphoma N=12*	Myeloma N=7* (9 procedures)	Healthy Donor N=4
Blood volume processed (mls)				
Mean	10284	10192	9461	8696
Median	10204	10264	11106	9440
Range	6526-13820	7197-13347	4064-11854	5776-11282
Pre-collection FBC, Median (range)				
WCC x 10^9/L	4.5** (1.6-9.8)	5.7 (1.6-10.4)	3.0 (2.1-5.3)	6.7 (5.1-8.8)
Lymphocytes x 10^9/L	0.8** (0.4-2.5)	0.8 (0.2-2.6)	1.4 (0.6-1.9)	1.8 (1.6-2.0)
CD3 % of WCC	14.3 (5.1-73.2)	14.2 (3.0-47.7)	20.9 (11.9-40.3)	15.2 (10.3-15.8)
Granulocytes x 10^9/L	1.8 (0.3-7.4)	3.4 (0.9-8.8)	1.6 (1.0-2.8)	4.3 (3.3-6.7)
Monocytes x 10^9/L	0.7 (0.04-1.4)	0.8 (0.3-1.4)	0.4 (0.04-1)	0.4 (0.1-0.7)
Platelets x 10^9/L	66 (17-339)	227 (115-444)	58 (141-316)	236 (147-285)
HCT %	32.1 (22.1-37.6)	34.3 (28.5-42.2)	32.1 (26.3-36)	41.1 (31.6-45.4)
Apheresis Yield, Median (range)				
WCC x 10^9/L	55 (27-192)	62 (24-107)	56 (41-146)	69 (58-170)
HCT %	3 (1-4)	2 (2-5)	3 (2-5)	2.5 (2-4)
Platelet (x10^9/L)	480 (120-3420)	2185 (820-4780)	1070 (220-1870)	1725 (1390-2670)

Lymphocytes %	69.2 (36.6-98.2)	51.8 (22.6-74.2)	68.6 (37.2-85.4)	70.2 (64.3-73.8)
CD3%	33.3 (19.9-79.3)	41.2 (20.3-59.4)	42.3 (20.6-72.5)	46.3 (43.6-54.7)
Monocytes %	25.4 (8.5-46.7)	39.9 (16.3-65.1)	22.3 (1.3-46.3)	19.2 (10.8-27.2)
Granulocyte %	5.7 (3-21.8)	6.2 (2.6-59.2)	8.9 (2.5-52.5)	8.4 (3.6-24.9)
Collection Efficiency (CE2%)	61 (31.2-103.1)	60.4 (32.1-96.6)	59.7 (46.3-73.2)	68.1 (50.2-100.1)
Total lymphocytes x 10^9	6.5 (2.3-22)	5.8 (0.9-10.8)	5.0 (3.6-9.1)	7.8 (4.5-17.2)
CD3+ cells collected/L ($x10^8$)	4.6 (1.1-8.1)	4.5 (1.1-8.6)	3.4 (2.4-7.5)	5.5 (5.0-11.3)
Total CD3+ cells collected ($x10^9$)	4.3 (1.0-7.5)	5.0 (0.9-8.6)	3.7 (2.7-5.4)	5.2 (3.0-12.7)
Target reached? (n, %) (CD3+ target of 1.5 $x10^9$)	12 (92%)	10 (83%)	7 (100%)	4 (100%)

Table 27: Apheresis collection parameters, pre-collection FBC and apheresis product yields for all donations

*B-ALL excluded n=1 (inaccurate CE), DLBCL excluded n=2 (no CD3+ precount n=1, no record of blood volume processed n=1), MM excluded n=1 (no record of blood volume processed).

**2 patients with circulating blasts excluded from median ALC calculations

6.3.4   Collection efficiency (CE) and CD3+ yield for autologous donors

Median CD3 CE2% for B-ALL, DLBCL and MM was 61% (range 31.2-103.1), 60.4% (32.1-96.6) and 59.7% (range 46.3-73.2) respectively. There was no statistically significant difference between CE2% for B-ALL, DLBCL and MM or between patients with central or peripheral venous access. A CE2% ≥40% is generally considered acceptable for cell harvest based on published literature (211). Four patients had a CE2% <40% (range 31.2-38.3%). Tuazon et al identified increasing age, a diagnosis of ALL and higher pre-apheresis platelet counts as risk factors for a low CE with MNC collections (211).

These variables were not applicable to our 4 patients albeit we accept that patient numbers are small. Mean age and platelet count of these four patients (male n=2, female n=2) with ALL (n=2) and DLBCL (n=2) was 39.5 years (range 16-57) and 239 x 10^9/L (range 66-339) respectively. Chen et al identified a low pre-collection Hb on multivariate analysis as potential risk factor for a low CE in MNC collections, patients with a Hb <10g/dl having a significantly lower CE than those with a Hb 10-12g/dl or >12g/dl (289). Our 4 patients had a mean Hb of 10.3g/dl (range 79-13.6). Two patients had a Hb <10g/dl which may have contributed to the CE<40% (B-ALL n=1, DLBCL n=1). Regardless of the CE, all four patients reached the CD3+ cell target of 1.5x10^9/L and had a successful manufacture.

Median CD3+ yield for B-ALL, DLBCL and MM was 4.3 x 10^9 (range 1.0-7.5), 5.0 x 10^9 (range 0.9-8.6) and 3.7 x 10^9 (range 2.7-5.4) respectively. Three patients (8.8%) harvested fewer CD3+ cells than the target of 1.5 x 10^9 (B-ALL n=1, DLBCL n=2). While these 3 patients had a PB ALC<0.5 x 10^9/L (median 0.23, range 0.18-0.42 x 10^9/L), only 2/3 patients had a 2.5 TBV harvest in line with our institutional SOP. The third patients had a 2.0 TBV harvest but the reason for this was not documented in the clinical notes. We have now implemented an apheresis prescription to stipulate harvest parameters based on lymphocyte counts on the day of the procedure.

Apheresis CE2% was acceptable at >40% in all 3 cases, but PB CD3 pre-counts were the lowest of the whole cohort (mean 0.15 x 10^6/ml, range 0.11-0.18 x 10^6/ml) and less than 10L of blood was processed during each procedure. Allen et al have previously recommended that a minimum of 10L should be processed based on their review of suboptimal harvests with the COBE Spectra (290). This suggests that the collection target may have been reached if a larger volume of blood had been processed. It also emphasises the importance of personalising the collection on the basis of patients' cell counts.

We recognise from the published literature that a high proportion of NK cells and blasts and low circulating numbers of CD3+ cells in the PB pre-harvest correlate with poor CD3 yields in the final apheresate (290). Subtyping of peripheral blood was only available for one patient who failed to reach the CD3+ cell target (DLBCL n=1). This demonstrated a CD3+ and NK percentage of 8.5% and 53% respectively (of live single CD45+ cells). Subtyping of apheresis products was available for 15 patients (healthy donor n=3, DLBCL n=-3 and B-ALL n=9) including 2 of the patients who failed to reach the CD3+ target (B-ALL n=1, DLBCL n=1). Mean percentage of NK cells and monocytes (of live single CD45+ cells) in the apheresis material for donors who reached target (n=13) was 8.3% (range 0.02-26.6%) and 7.0% (range 0-26.2%). The two patients who failed to reach the CD3+ target had a NK count of 17.8% (DLBCL) and 67.1% (B-ALL) and a monocyte count of 39.9% (DLBCL) and 26.7% (B-ALL) respectively. No blasts were detected. While the numbers may be too small to draw conclusions, we can say that two of the patients who failed to reach the CD3+ harvest target had higher proportions of NK cells and monocytes in their apheresis material relative to mean proportions of those who reached the target.

Regardless of the cell yield, all three products were manufactured successfully, but more fundamental questions around the in vivo impact of products manufactured in the event of poor CD3 yield from harvest is of interest.

6.3.5 Correlation between CD3+ yield, CD3+ pre-count and ALC

In practice, the stem cell lab run a pre-harvest CD3+ count on every pre-harvest FBC. We hypothesised that if ALC was a sufficient surrogate for CD3 quantitation (flow cytometric method), this would save staff time and resource per harvest. In our analysis the correlation between CD3+ pre-count and ALC pre-count was 0.74 (Figure 35) which suggests that we could potentially move towards ALC in lieu of CD3+ pre-count. The limitations here include a correlation coefficient of <1 and this may reflect the small total number of patients. This is being evaluated in a larger cohort currently.

$$y = 0.6934x - 0.0407$$
$$R^2 = 0.7407$$
$$p < 0.0001$$
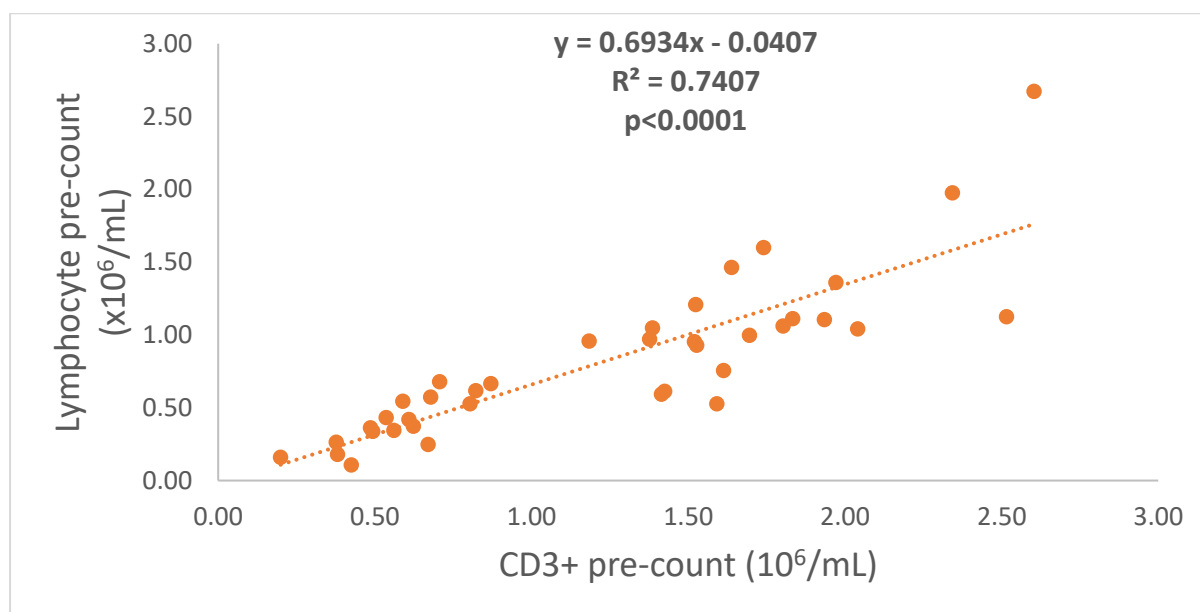
Figure 35: Correlation between CD3+ pre-count (x10^6/ml) and ALC (x10^6/mL)

Allen at al have previously used CD3+ pre-count to predict T-cell yield during harvest using the COBE Spectra with a correlation coefficient of 0.74 (290). Figure 36 demonstrates the correlation between CD3+ pre-count and CD3+ cell yield per litre of blood processed for all patient donations (n=34) (correlation coefficient 0.67). This data indicates a moderate correlation between CD3+ pre-count and CD3+ yield per litre of blood processed. Recognising the need to streamline our system to continue to improve scalability and minimise the workload for both the apheresis department and our stem cell laboratory, we wished to identify whether ALC (rather than CD3) could be used to predict yield. ALC

is readily available in the clinic with a rapid turnaround time. The correlation coefficient of ALC as the predictor of CD3 yield per litre of blood processed is 0.62 (Figure 37). This aligns nicely with the CD3+ pre-count data and suggests that ALC may be an adequate surrogate for CD3 pre-count pending prospective validation.

Given both of these correlation coefficients are less than 1 and the patient cohort is small, our algorithm will be validated prospectively. If we are successful in validating this algorithm, we will consider its application in UCL academic CAR-T studies going forward.

If we replace the CD3+ count pre-harvest with ALC (pending prospective validation), can we also replace the CD3+ count on the apheresis product with pre-harvest ALC, thereby further reducing the workload for our stem cell laboratory staff post procedure? Can we extrapolate the total CD3+ cell yield from the pre-harvest ALC?. Figure 38 demonstrates that the correlation between pre-harvest ALC and total CD3+ cell yield is relatively weak (0.42). Based on this patient cohort, it is therefore not possible to extrapolate total CD3+ yield based on PB ALC in clinic pre-procedure.
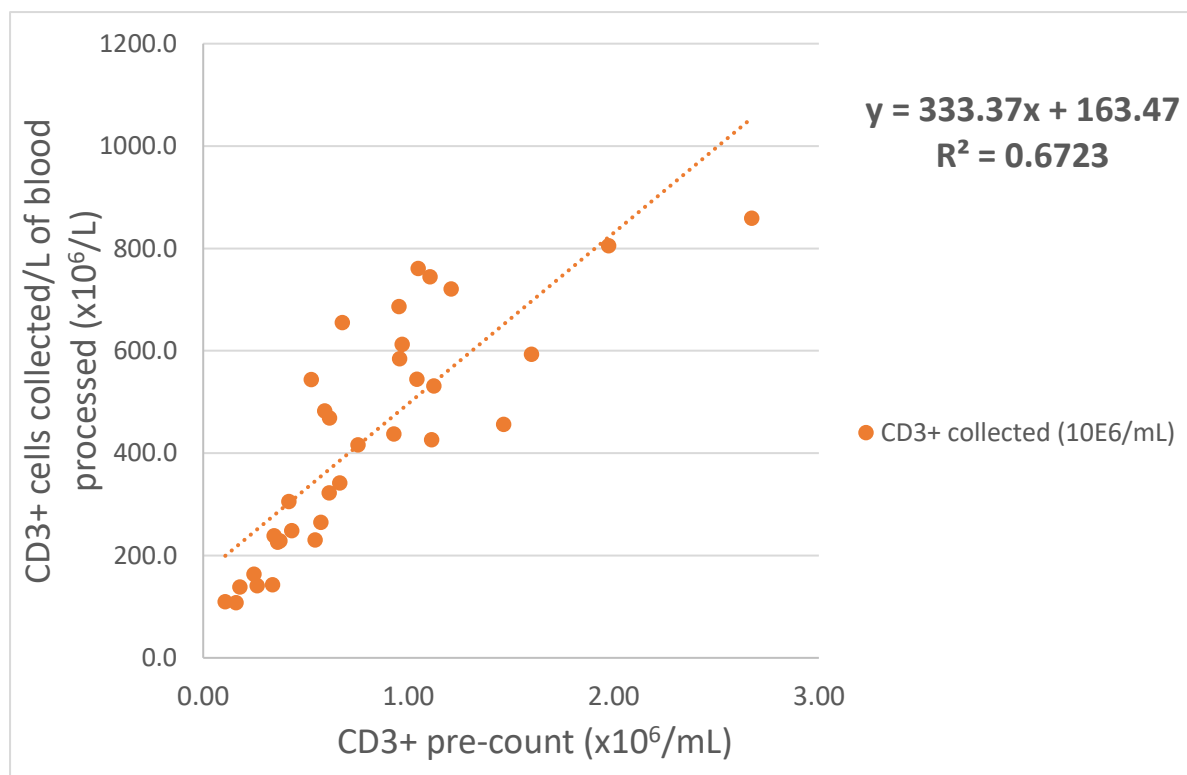
Figure 36: Correlation between CD3+ pre-count (x10⁶/ml) and CD3+ cells collected per litre of blood processed (x10⁶/L) for 34 donations (excludes healthy donor)
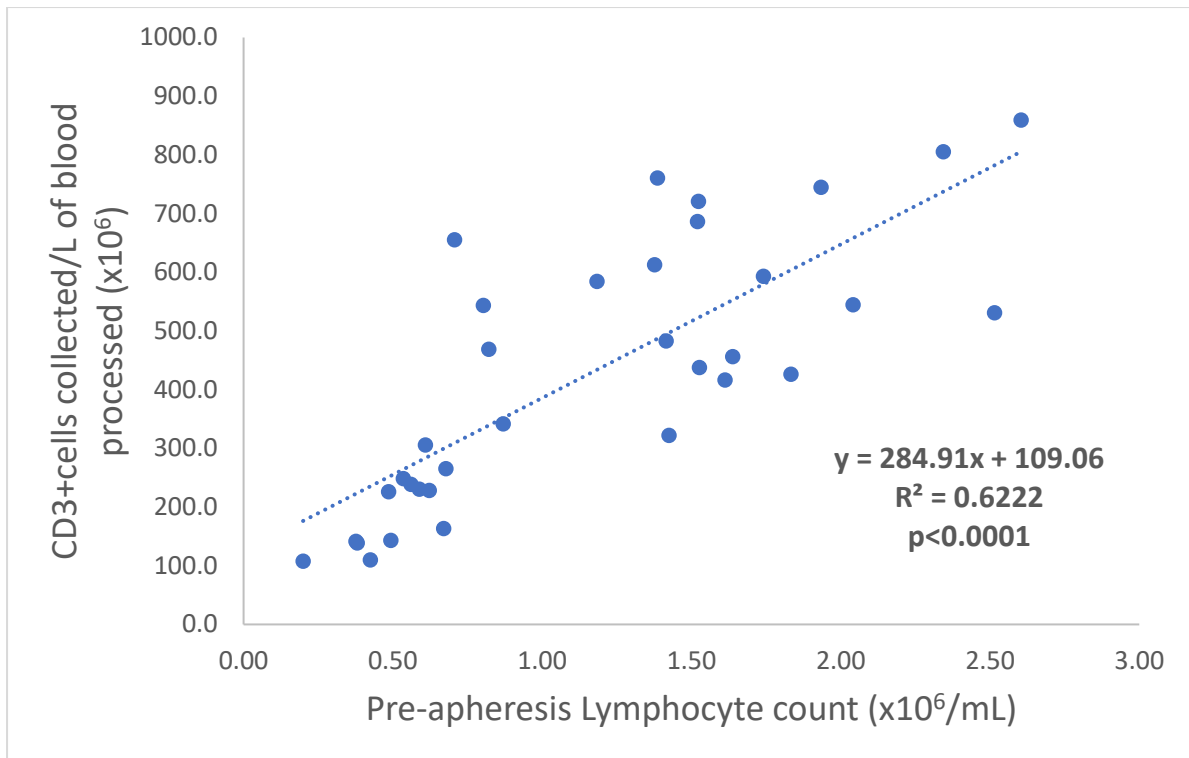
Figure 37: Correlation between pre-apheresis lymphocyte count (x10⁶/ml) and CD3+ cells collected per litre of blood processed (x10⁶)
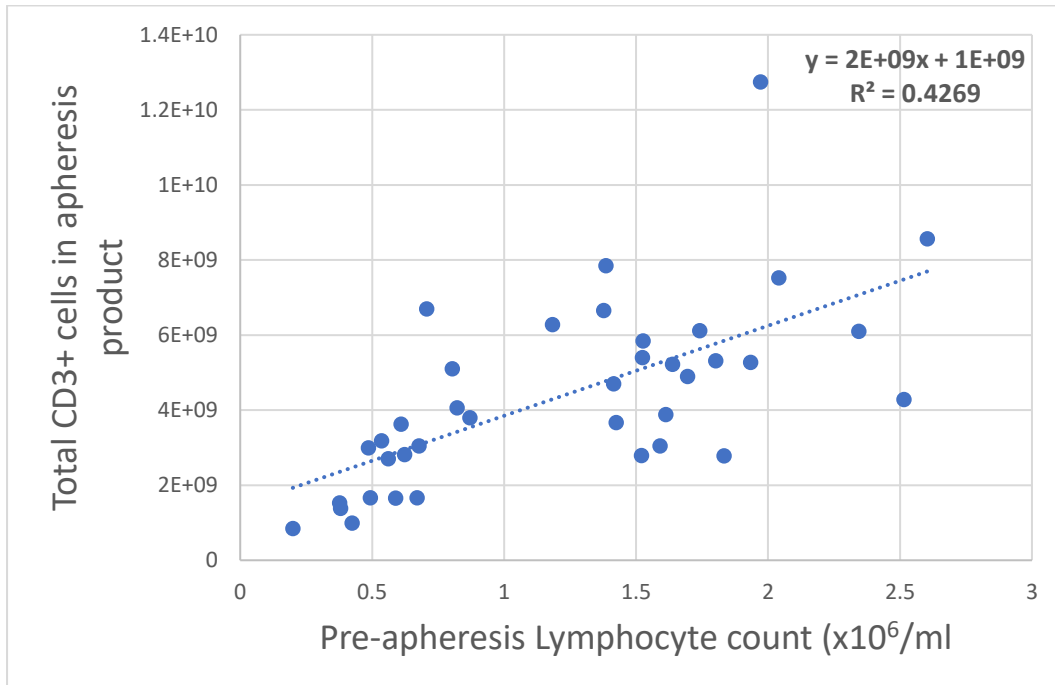
Figure 38: Correlation between pre-apheresis lymphocyte count (x10⁶/ml) and total CD3+ cells collected

6.3.6   CAR-T cell manufacture

In our experience, the Spectra Optia can successfully deliver adequate CD3+ T-cell harvests for CAR T-cell manufacture. Of the 42 T-cell harvests conducted (including 2 repeat procedures as described in section 6.3.1), 40 CAR-T products were successfully manufactured and met the dose requirements for QP release.

Of those, 5/40 (12.5%, DLBCL n=4, B-ALL n=1) met the minimum dose for release but did not meet the trial protocol dose. Two of these lymphoma patients were excluded from the analysis in Table 27 due to lack of CD3+ precount (n=1) and record of blood volume processed (n=1), precluding relevant calculations. Mean CE and CD3+ cell counts were available for 3 of the 5 patients and were 65.4% (range 57.7-76.1%) and 3.4×10^9 CD3+ cells (range 3-4 x 10^9) respectively, indicative of good quality harvests that exceeded the target CD3+ cell yield. This would suggest that CAR T-cell manufacturing feasibility is dependent upon several factors of which starting CD3+ T-cell numbers is just a single component.

6.4 Optimising apheresis: Conclusions

We retrospectively reviewed our single centre experience of 40 MNC harvests with a particular focus on patient/autologous MNC harvests (34/40) conducted on the Spectra Optia (TerumoBCT Inc) for CAR-T cell manufacture in adults with heavily pre-treated B-ALL, DLBCL and MM (Table 26).This adds to the limited pre-existing data on such harvests in adults with advanced B-cell malignancies using this device (211,215).

The inherent variability of patient-derived starting material represents a challenge to apheresis technicians and manufacturers. Underlying disease and prior lymphotoxic therapy can complicate the collection of a sufficient quantity of CD3+ target cells. Manufacturing failures, whilst uncommon, do occur. Allen et al identified low proportions of circulating lymphocytes or CD3+ cells, a high proportion of NK cells and circulating blasts as potentially contributory factors in patients who failed to reach their centre's designated CD3+ cell target of 2×10^9 total(209). Three of our patients (8.8%%) failed to harvest the proposed UCLH target of 1.5×10^9 CD3+ cells. These three patients had the lowest CD3+ pre-count of the patient cohort and may have benefited from a prolonged or potentially a second day of harvest. Only four (11.8%) of our patients (B-ALL n=2, DLBCL n=2, M: F 1:1) had a CE<40%. We

cannot identify or corroborate predictive factors in this small cohort as previously reported by Tuazon et al (211) but 50% of these patients had a Hb of <10g/dl at the time of harvest, a risk factor previously reported (289).

All 40 of the CAR-T manufactures met the QP dose requirements for release. A proportion (12.5%) failed to meet the dose requirements for trial. This underlines the fact that CAR manufacturing is complex and is dependent on several factors including but not limited to CD3 T-cells counts obtained from apheresis.

A simple and effective protocol would be desirable to streamline and at the same time try to personalise this particularly important initial step of CAR-T cell manufacture. Based on our experience at UCLH, the vast majority of T-cell apheresis procedures using a target BV of 2-2.5 effectively capture the required CD3+ T-cell target for manufacture. However, our prerogative was to identify ways in which the procedure could be streamlined for stem cell lab and shortened (where possible) for the apheresis department so we could more efficiently use the service and scale apheresis to meet demand. Attempts to minimise/remove the requirement for pre- and post-apheresis flow cytometric evaluation of CD3 and the ability to shorten apheresis times to 1 rather than 2/more blood volumes may be beneficial to the running of the department and enhance access of CAR-T slots to more patients.

This analysis indicates that not all patients made target dose and these could have been easily identified pre-procedure on the basis of ALC in starting FBC/sysmex. We propose that for this population, longer collection times or 2nd day collections may be of benefit, but this would need evaluation in a prospective manner. We have devised an algorithm using pre-procedure ALC and a CD3+ harvest target of 1.5 x 10^9 total in an attempt to personalise this procedure, hoping that this can minimise the risk of poor harvest for the few, whilst perhaps shortening the apheresis procedure for many. Validating this algorithm prospectively, we aim to extrapolate the volume of blood to be processed in litres, thereby shortening procedure time if not required and prolonging the procedure for patients at higher risk of failure. The PB ALC is readily available in clinic, facilitating a simple calculation. Allen et al devised a similar calculation using pre-apheresis CD3+ cell count and a target CD3+ of 2 x 10^9(209).

Our algorithm has limitations.

1. Firstly, and most importantly, this algorithm has been devised using data from a small number of patients and be should be validated in larger prospective patient cohorts. Correlation coefficient with current patient numbers is 0.62 (moderate correlation).

2. Secondly, circulating disease may falsely elevate the ALC. In the literature the incidence of circulating blasts at the time of cell harvest ranges from 4% to 34% (210,211,290). The presence of circulating blasts is thought to reduce the CD3+ yield (290). The percentage of blasts in the apheresis material has been shown to be concordant with the

peripheral blood or enriched in a minority of cases (290). A spuriously high ALC on initial FBC or a high-risk patient should prompt further investigation with a blood film and/or flow cytometry. In this instance, a similar algorithm using CD3+ pre-count could be used (Figure 36). T-cell selection to remove contaminating cells prior to cell manufacture is discussed in Section 1.5.3 and 8.5.1.1.

3. Thirdly, it does not account for lymphocyte recruitment. Better recognised in mobilised regimens, this phenomenon is also a feature of unstimulated T-cell harvests (225,291). It relates to the release of CD3+ target cells from the lymphatic system into the peripheral blood during the process of apheresis. Therefore, the CD3+ cell yield may far exceed the figures predicted by the CD3+ pre-count, skewing our data. In our cohort, there was no statistically significant difference in recruitment by disease. In the absence of post-procedural CD3+ cell counts, recruitment was calculated using post apheresis ALC.

Lymphocyte recruitment is calculated as follows:

[(total collected target cells) + (post circulating target cells x patient total blood volume)] / [pre circulating target cells x patient total blood volume]

4. Finally, absolute numbers of target cells aside, little is known about the impact of fixed pre-apheresis patient variables (age, diagnosis, prior therapies) on T-cell functionality, differentiation and exhaustion status (Section 8.3.4.1). These factors likely affect proliferation and potentially cytotoxicity and persistence of these cells in vivo.

Until validated in a larger cohort, we plan to gather data prospectively in our CAR-T clinical trial patients. Feasibility of cell manufacture with 1.5×10^9 CD3+ cells will be under regular review.

CHAPTER 7

7 CAR-T CLINICAL SERVICE DEVELOPMENT TO FACILITATE DELIVERY OF TRIALS

7.1 Introduction

UCLH registered its first patient on a CAR-T clinical trial in August 2016. Since then, the immunotherapy service has expanded to include a large portfolio of academic and commercial CAR-T studies with a consequent surge in patient numbers. Figure 39 demonstrates the trend in academic, commercial and licenced CAR-T cell infusions at UCLH since the establishment of the clinical service in 2016 (until May 2020). In preparation for the influx of CAR-T patients with the subsequent high demand on institutional resources, a framework to streamline the referral and treatment pathway was required. Embedding the UCL CAR Program within the well-established haematopoietic stem cell transplant (HSCT) service at UCLH was deemed to be the most appropriate and efficient approach. UCLH HSCT unit has the infrastructure to support the regulatory, cellular processing and clinical care delivery systems required for CAR-T cell therapy.
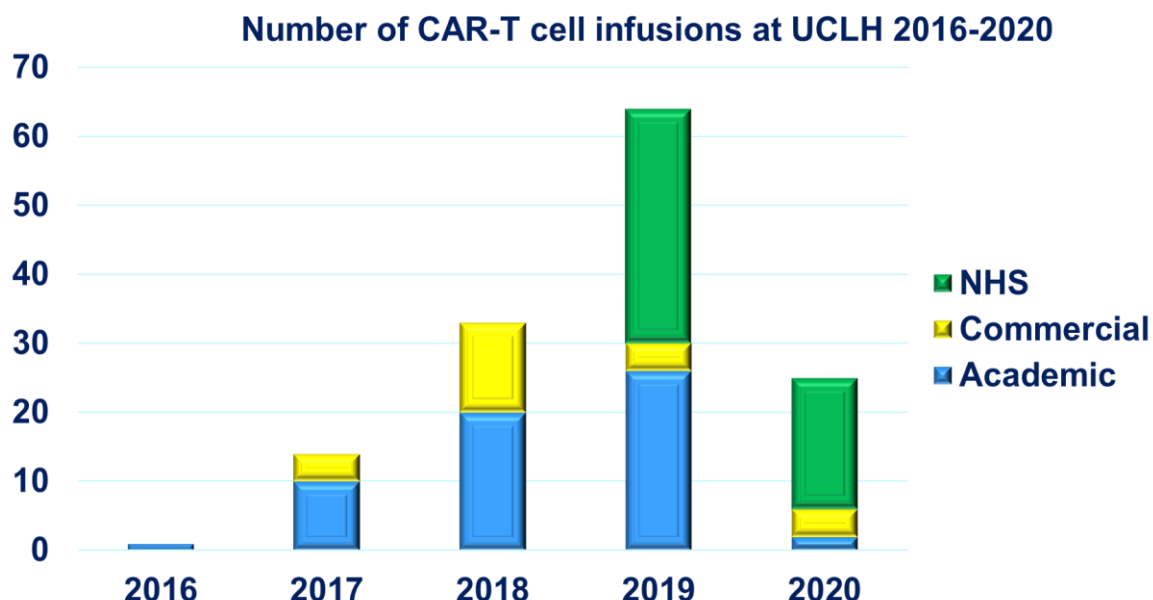
Figure 39: Number of CAR-T cell infusions at UCLH from 2016 to May 2020 (academic, commercial, NHS)

7.2 Aims of this chapter

Workflow model systems have been proposed by other centres, both from a medical and nursing prospective (227,228,232). The essential components of the UCLH CAR-T workflow model that needed to be implemented include:

(1) Immunotherapy clinic
(2) Patient Optimisation
(3) Cell harvest
(4) Admission for lymphodepleting chemotherapy and cell infusion
(5) Post-infusion care
(6) Long-term follow-up

This chapter discusses the aforementioned components of a functioning immunotherapy service, required for the provision of CAR-T therapy trials. Aside from expansion of pre-existing departments such as apheresis and the stem cell laboratory, JACIE accreditation requirements also dictated unique infrastructural needs.

7.3 Immunotherapy clinic

A once weekly immunotherapy clinic was established in the MacMillan Cancer Centre (MCC) to facilitate the influx of CAR-T trial referrals from UCLH and other centres across the UK. The clinic was manned by an Immunotherapy consultant, 1-2 clinical research fellows and 1-2 clinical trial practitioners. The clinic was established as a branch of the HSCT outpatient department. The service then expanded to include all referrals for NHS-Approved CAR-T cell products with plans to include commercial CAR-T studies and cellular therapy trials for solid tumours. The subsequent inclusion of patients referred for NHS CAR led to the allocation of 2 clinical nurse specialists to aid with the significant increase in patient numbers.

7.3.1 Patient referral

Referrals to the immunotherapy clinic for UCL CAR-T academic studies and for NHS-approved CAR-T cell products have a diagnosis of B-ALL or high-grade B-cell lymphoma (DLBCL, PMBCL or tFL).

On receipt, the referrals are screened by the immunotherapy team. Previous imaging and histology with relevant reports are retrieved. Multi-disciplinary team (MDT) discussion with disease-specific physicians is scheduled (if required). Patient Information Sheets (PIS) are forwarded to potential trial-eligible patients via email prior to their clinic visit to facilitate consent on the day of attendance.

Patients with B-ALL or high-grade B-cell lymphoma, referred for one of the licensed CAR T-cell products must be approved by the National CAR T Clinical Panel. This panel, established to ensure equitable access and eligibility criteria are fulfilled, convene weekly via teleconference. Each designated ATIMP centre has a panel representative, supported by clinical experts, patient representatives and a panel lead.

7.3.2   First consultation

At the first consultation, the patient's treatment pathway, complications to date and medical history are reviewed. Therapeutic options, including but not limited to CAR-T cell therapy are discussed with patient and family. CAR-T cell mechanism of action, short and long-term toxicities and outcome data are discussed. Specific risks such as CRS and ICANS are highlighted, considering the incidence and severity with particular CAR-T products. A UCLH Trust document is given to the patient and includes a description of CAR-T cell therapy, screening, the apheresis procedure, lymphodepleting chemotherapy, the CAR-T cell infusion and potential toxicities, follow-up post discharge and contact details for our clinical trials team.

Once consent for trial is obtained, trial screening can commence. Bloods (full blood count, renal, liver, bone profile, lymphocyte subsets, immunoglobulins and blood group), virology (Hepatitis BsAg, Hepatitis BcAb, anti-HCV, HIV 1+2, HTLV-1, syphilis serology), electrocardiogram (ECG) and vascular assessment to determine suitability for apheresis via peripheral line are performed. Contraceptive choices are clearly documented in the medical notes. All potential patients are advised to avoid conception for 12 months post therapy, using at least one method of highly effective contraception. Additional screening investigations include a cerebrospinal fluid (CSF) assessment to ensure absence of CNS disease in B-ALL patients, an echocardiogram or MUGA to confirm adequate cardiac function and demonstration of CD19 expression on leukaemic blasts or malignant B-cell lymphoma histology. Full diagnostic and relapse bone marrow and CSF reports are reviewed (flow cytometry reports, flow plots to depict percentage of blasts expressing CD19, cytogenetic and minimal residual disease (MRD) markers and testing location, if applicable). Trial referral forms are regularly updated to capture this information.

7.3.3   Patient selection

Patient selection is a pivotal component of the immunotherapy clinic. The typical patient referred for assessment has relapsed and/or refractory disease despite multiple lines of standard therapy, often including HSCT. An important focus is to consider performance status, cumulative treatment burden, active co-morbidities and infections that may preclude treatment. Assessment of pace, burden and site of disease is paramount. Time to complete screening, registration, apheresis and cell manufacture is

 factored into the decision to recruit a patient and patients should be counselled regarding the risk of rapid disease progression which may exclude them from treatment. Most studies stipulate a performance score of 0 or 1 prior to recruitment and infusion. Patient selection for UCL academic studies is decided by the Immunotherapy consultant and CAR-T clinical fellows.

7.3.4   Bridging chemotherapy

Chemotherapy/radiotherapy is ideally avoided prior to cell harvest unless urgent treatment is required. Pulses of steroids (with appropriate anti-infective and tumour lysis syndrome prophylaxis) are considered in patients with symptomatic or accelerated disease, provided the steroids are stopped 7 days prior to harvest. Options for effective bridging therapy post apheresis can be limited in patients with relapsed/refractory disease despite multiple lines of non-cross-reacting chemotherapy. At UCLH, intensely myelosuppressive regimens (such as FLAG-IDA) are generally avoided as bridging in the ALL population. This is based on the low success rates, prolonged recovery times and the potential toxicity including infection in a heavily pre-treated patient population with poor marrow reserve, particularly post allograft. Targeted therapies such as Inotuzumab ozogamicin (humanised monoclonal antibody against CD22 linked to a cytotoxic agent) can achieve deep sustained remissions in patients with CD22 expressing disease (292), albeit with its own unique toxicity profile. Blinatumomab, a bispecific T-cell engager antibody (BiTE) against CD19/CD3, is generally avoided given it also targets CD19 with a theoretical higher risk of CD19 negative relapse post CAR. The majority of the Philadelphia (Ph) negative ALL patients receive a maintenance-like regimen such as weekly/fortnightly vincristine and dexamethasone with intra-thecal CNS prophylaxis. Ph+ ALL patients are checked for resistance mutations and prescribed a tyrosine kinase inhibitor (TKI), considering the risk profile of individual agents and previous TKI use.

Bridging therapy for high grade B-NHL patients is discussed at the MDT, individualised and usually facilitated by the referring centre. It can be a difficult balance between achieving adequate disease control and maintaining fitness for therapy, fitness which is often compromised by more intensive

salvage regimens. Those with known chemo-refractory disease may benefit from radiotherapy (Section 7.3.4) or novel antibody-based regimens such as Rituximab-Bendamustine and Polatuzumab (293). Higher pre-treatment tumour bulk has been associated with inferior outcomes with Axicel (294). Disease progression during cell manufacture (often despite bridging) is recognised as one of the most common reasons for failure to get to cells.

7.3.5   Optimisation of patients pre-CAR-T cell therapy

As documented in the literature (50,76) a significant proportion of patients recruited to CAR-T studies will not receive therapy. Reasons cited include disease progression, infectious complications and failed CAR-T cell manufacture. This is independent of patients who fail trial screening, the numbers of which may or may not be reported in trial publications. Appropriate patient selection is paramount. However, despite rigorous patient assessment and good compliance with anti-infective prophylaxis, this vulnerable patient cohort may not respond to salvage therapy and succumb to infection or rapidly progressive disease.

7.3.5.1 Optimisation of patients pre-CAR-T cell therapy: Infection prophylaxis

ALL patients with active disease or receiving bridging chemotherapy are prescribed anti-viral, pneumocystis pneumonia (PCP) and anti-fungal prophylaxis with the aim of preventing opportunistic infections. Aciclovir 200mg three times daily is the standard anti-viral prophylaxis. Choice of PCP and fungal cover is dependent on infection history, renal/liver function, peripheral blood cytopenias (relevant to co-trimoxazole) and allergy history. Patients with high grade lymphoma are commenced on anti-viral and PCP prophylaxis. The use of anti-fungal cover is dependent on bridging protocol, cytopenias and steroid use. We enlist the help of our Infectious Disease and Microbiology consultation service in complex cases. Infection history and records of resistant organisms are requested in preparation for admission. Referring centres and patients are advised to be vigilant regarding infection and compliant with prophylactic medication. We encourage early contact should the patient become unwell or require admission to their local hospital.

7.3.5.2 Optimisation of patients pre-CAR-T cell therapy: Cardiology

Specialist cardiology advice is sought if patients have a history of cardiac disease. While there is limited evidence on the cardiac consequences of CAR-T cell therapy in particular adult cardiac risk groups (mainly as these patients are excluded from clinical trials), the cardiac complications of CRS are well recognised (239,240). Alvi et al reported a cardiovascular (CV) event rate of 12% in 137 patients (88% B-NHL) who received CAR-T cell therapy (CV death n=6, decompensated heart failure n=6, arrythmia n=5) and suggested elevated baseline troponin levels and severity of CRS correlate with worse CV outcomes (295). Other groups have suggested that hypertensive patients are at higher risk of CV toxicity (296). In paediatric cohorts, echocardiographic abnormalities were found in 41% of patients with CRS requiring inotropic support, many of which persisted on discharge. A blast count of >25% and pre-existing cardiac disease increased the risk of hypotension requiring inotropic support (297).

At UCLH a cardiac screening form is completed on the day of initial assessment (to include previous cumulative anthracycline exposure) and rapid review by our cardiologist (with special interest in immunotherapy) is arranged, if required. An echocardiogram or MUGA is standard screening investigation for our clinical studies.

7.3.5.3 Optimisation of patients pre-CAR-T cell therapy: Psychological support

It is well recognised that haematological malignancies impact adversely on quality of life (QoL) (298,299). This is true of patients receiving chemotherapy and long-term survivors (300,301) suggesting that QoL data should also be captured for CAR patients during and after therapy. Dedicated QoL surveys have not been devised for CAR patients, clinical studies using an array of survey methods across different timescales to gather patient-reported outcomes (PROs) (302).

Psychological support may be of benefit to CAR-T cell patients before and after therapy. This cohort of patients have usually exhausted other potentially curative treatment options and can be a highly anxious treatment group with fear of the side effects of therapy and cancer recurrence at the forefront. With the help of MacMillan Support in the MCC, UCLH can offer counselling sessions and support for patients and their carers. CAR-T patients have a dedicated section of the unit (timed with the Immunotherapy clinic) to meet the counsellors and other CAR-T cell patients (if they wish). QoL data will be collected prospectively with patient consent.

7.4 T-Cell apheresis and stem cell laboratory units

The process of apheresis is discussed in Section 1.5. UCLH has an established 8-bedded apheresis unit and a stem cell laboratory, both pivotal departments for the CAR service. These units, established as part of the HSCT service, had to increase capacity to accommodate the surge in CAR-T patient numbers. While local T-cell apheresis protocols are followed regardless of the CAR-T product, commercial companies and clinical trials have specific requirements (cell numbers, labelling and packaging, cell receipt and distribution and product return) that must be adhered to. Staff education and training to facilitate a streamlined service, maintaining chain of custody and conforming to JACIE standards at all stages is paramount.

The feasibility of the service also requires communication between many different departments; the CAR-T medical and nursing team, inpatient BMT team, apheresis staff, stem cell laboratory staff, the manufacturing team/company and bed managers to plan for apheresis, manufacture, product receipt and admission. At UCLH the apheresis, stem cell laboratory, medical and nursing staff use a specific database to approve cell harvest, incoming cells, lymphodepletion and product release.

7.5 Admission for lymphodepleting chemotherapy and cell infusion

Patients are reviewed in the Immunotherapy clinic 1 week and 2-3 days prior to admission. This is to pre-emptively address disease-associated or infectious complications which may compromise or delay therapy. Pre-admission checklists have been drafted to confirm relevant tests have been performed and reviewed. This includes echocardiogram and formal glomerular filtration rate (if applicable), CNS imaging (if applicable), re-staging investigations (PET scan, BM if applicable), blood status forms, consent for lymphodepletion, allocation of specific chemotherapy protocol, pregnancy test (if applicable). Patients are again counselled about the treatment pathway and potential toxicities. The decision to admit to the ambulatory care (AC) unit is dependent on patient performance status, disease burden and AC-specific eligibility criteria. Pharmacy, BMT inpatient team, stem cell laboratory and cell infusion team are informed regarding upcoming admissions in a weekly email.

The cell infusion is led by one of the stem cell nurses, trained specifically in the administration of CAR-T cells by Clinical Practice Facilitators (CPFs) at UCLH. There is a medical and trials team presence at the infusion of all trial CAR-T cell products. Batch Release Certificates (BRC) are reviewed at the bedside to as an additional identity check with labels on the cell bag. Trial -associated paperwork is completed by the trials team at the bedside; time product removed from shipper, time for cell thaw and time to cell infusion. All CAR-T cell products are administered with a non-filtered giving set in line with our local SOP.

7.6 Post-infusion care

Institutional guidelines have been drafted for the investigation and management of CRS and ICANS (Section 1.6.7). In line with the publication of the ASTCT guidelines on CAR-T related toxicity (251), CRS and ICANS SOPs were updated to incorporate the changes. ASTCT guidance was drafted with the aim of harmonizing toxicity grading across clinical studies and trial sites internationally. Tocilizumab is readily available in the fridge on designated wards. Trial-specific protocols are also referenced for grading and toxicity guidance. Education of staff has been prioritised to optimise patient care post-infusion, with regular scheduled sessions for junior and senior haematology trainees, nursing staff, stem cell infusion team, ICU outreach teams and psychological support teams. Smart links have been established on our electronic health record system (EHRS) for the bedside grading of CRS and ICANs. In accordance with JACIE accreditation for cellular therapy and to build expertise, UCLH has a designated neurology and ITU physician.

7.7 Post Discharge

Patients are followed up closely in the immunotherapy clinic on discharge. Written information is provided to all patients at the initial visit ('CAR T-cell welcome pack') with contact details of relevant staff and emergency contact details. All patients should carry the UCLH CAR T-cell contact card in case of emergencies and an irradiated blood products card given the exposure to Fludarabine (purine analogue). The point of contact for trials patients is the UCLH T-cell trials team and the CAR-T CNS for patients receiving NHS CAR T-cells. Communication with the referring team is important to ensure they are aware of the potential signs and symptoms of CAR-T related toxicity. The patients are counselled in detail regarding early contact with the trials team/medical services should they become unwell. Patients who receive NHS-approved CAR-T cells must remain within one hour of the hospital for 28 days post cell infusion.

7.7.1 Post Discharge: Prevention of infection

A supportive care guidance document to reduce the risk of infection post therapy was drafted at an institutional level. This details optimal choice and duration of anti-viral, PCP and anti-fungal cover. Aciclovir 200mg three times daily is our standard prophylaxis for herpes reactivation. Co-trimoxazole 960mg twice daily three days per week is our first line for PCP and toxoplasmosis prophylaxis.

Pentamidine nebuliser is administered monthly for patients who have had insufficient count recovery (neutrophils must be >1 x 10^9/L and platelets >100 x 10^9/L unsupported). Both anti-viral and PCP cover are continued for at least 3 months post therapy and until CD4 counts are >0.2 x10^9/L. A recent review of CD4 counts post Axicel demonstrated a median CD4 count of 155 cells/ul (range 33-269) in patients at 1 year post treatment (266). These agents are continued for longer periods in delayed neutropenia. Itraconazole 200mg twice daily is our prophylactic anti-fungal agent of choice in the setting of late neutropenia. GSCF support is administered as required. Some guidance has been published on anti-infection prophylaxis (267). Delayed cytopenias and the incidence of infection post CAR is discussed in Section 1.6.8 (91,261,262).

 Hypogammaglobulinaemia, universal amongst patients who respond to CD19 CAR-T cells can be abrogated with monthly intravenous immunoglobulin infusions, if clinically indicated (Section 1.6.8). In the absence of randomised controlled trials, use of IVIG is centre-dependent. A recent guidance document has been published (265). At UCLH, patients are screened for hypogammaglobulinaemia and recovery of B-cell function at scheduled trial timepoints. Prophylactic IVIG is commenced in patients with hypogammaglobulinaemia and recurrent bacterial infections. While trough levels are monitored, response to IVIG is primarily assessed clinically. There is minimal evidence or guidance on revaccination programmes post CAR-T therapy. Many centres devise their own protocols to facilitate this. At UCLH, we consider revaccination at 6-9 months post therapy. Pneumococcal serology is assessed at month 6 followed by a vaccination response assessment. Antibody responses post CD19 CAR-T cells may be elicited by long-lived CD19 negative plasma cells (269). Guidelines should be formalised at an institutional level, taking account of available guidance and opinion (267,270)

7.7.2   Post Discharge: Psychological Support

As discussed in Section 7.3.5.3, MacMillan Cancer Support offer free counselling sessions, advice on financial assistance and the opportunity to meet other CAR-T cells patients and survivors in the post treatment phase. A dedicated area and time have been assigned for CAR-T patients and their families to meet the Support volunteers and counsellors. Fear of cancer recurrence is well-recognised in cancer survivors and can negatively impact on quality of life (303). Financial distress due to travel expenses and loss of earnings due to illness can exacerbate psychosocial stress (304). Dedicated QoL surveys have not been devised for CAR patients, clinical studies using an array of survey methods across different timescales to gather patient-reported outcomes (PROs) (302).

7.7.3   Post Discharge: Long-term follow up

The MHRA and FDA advocate 15 years of follow up for the recipients of GM products. Potential risks include replication-competent viral vectors, emergence of secondary malignancy, insertional mutagenesis and autoimmune disease. A SOP providing guidance on long term follow up was prepared. If it is not specified in the trial protocol or the patients are not linked to a specific long-term follow up study, this document serves as a guide to staff on patient monitoring beyond the initial treatment phase. It focuses specifically on outpatient clinical assessments, ethically-approved testing and storage of follow up samples with patient consent. Table 28 lists the investigations that should be performed on follow up outpatient visits. These investigations may include disease re-staging (BM/PET/LN biopsy), CAR-T cell persistence and detection of replication-competent virus (replication competent lentivirus or replication competent retrovirus) and insertional mutagenesis. Should a secondary malignancy/disorder occur (suspected to be related to the CAR-T cell product), specialist advice should be sought and appropriate investigations instigated. Given the novelty of this therapy and as new data emerges, the analysis of stored samples at future timepoints may include flow cytometry, PCR, immunohistochemistry, single cell genomics/transcriptomics and chip cytometry.

ASSESSMENTS/TESTS	TIME FROM INFUSION OF CAR-T CELLS				
	M1-3	M3-12	Year 2	Year 3-4	Year 5-15
	Monthly	3-monthly	4-monthly	6-monthly	Annually
Disease Reassessment					
Bone marrow Assessment (if applicable)	X	X			
PET scan (if applicable)	X	X			
Blood samples					
CAR transgene persistence[1]	X	X	X	X	X

RCL or RCR detection[2]		X	X[2]	X[2]	X[2]
Insertional mutagenesis[3]		X	X	X	X
Pregnancy test[4]		X			
Other[5]	X	X	X	X	X

Table 28: List of investigations at scheduled timepoints post CAR-T cell infusion and long-term follow-up

1. **CAR transgene persistence:** Analysis of blood samples (qPCR/flow cytometry) to cease when two negative results recorded at least 3 months apart and at least 12 months from first CAR-T cell infusion. Sample collection and storage should continue thereafter (in the absence of disease progression).

2. **RCL and RCR detection:** Storage of blood samples (qPCR) every 3 months for 12 months after first CAR-T cell infusion. If results of 2 consecutive tests are negative 12 months post first CAR-T cell infusion, collection and storage of samples can continue annually (for future analysis, if required).

3. **Insertional mutagenesis:** Blood samples will be collected and stored. Insertion site analysis will be performed in cases where insertional mutagenesis is considered a potential cause or contributor (new malignancy, neurological/ haematological condition or other).

4. **Pregnancy test:** Serum or urine beta-human chorionic gonadotropin (HCG) will be checked in women of childbearing potential every 3 months for the first 12 months after CAR-T cell infusion (with approval/consent)

5. **Other:** Blood samples to assess for end-organ toxicity. This may include immunoglobulin levels, serology, vaccination response assessment, lymphocyte subsets, bNP, troponins, FBC, inflammatory markers, renal, liver and bone profile.

7.8 T-cell meeting

To coincide with the establishment of the cellular immunotherapy service, a T-cell MDT meeting was scheduled every fortnight. Via this platform, all new patient referrals for CAR-T therapy, patient follow-up, treatment-related toxicities and imminent admissions are discussed. Service planning, staffing and logistical issues relating to service provision are also addressed. Following the approval of Tisagen and Axicel by NICE, patients for CAR-T cells on the NHS are also discussed. The meeting is attended by the Immunotherapy Team (Immunotherapy consultants, clinical fellows, clinical trial practitioners, clinical nurse specialists, data managers), immunotherapy lead for pharmacy, apheresis and stem cell laboratory and lymphoma and myeloma consultants/principal investigators with an interest in cellular therapy. The meeting is also attended by clinical oncologists with a special interest in immunotherapy

(principal investigators of cellular therapy trials). The introduction of a new cellular therapy trial must be discussed at this meeting prior to approval/implementation. Every 6 months an audit of morbidity and mortality is presented at this T-cell meeting. Attendance is recorded at every meeting in accordance with JACIE requirements.

7.9 JACIE accreditation

JACIE is the only European accreditation body for haematopoietic stem cell transplantation and cellular therapy (Section 1.6.9). In order to administer licenced CAR-T cell products in the UK, hospitals must be JACIE-accredited.

7.9.1   JACIE accreditation: Expression of Interest

Firstly, an expression of interest form was submitted by UCLH to JACIE. This form details specific information on the infrastructure in place (quality management, regulatory, apheresis, cellular processing, clinical and pharmacy) and the centre's previous CAR experience.

7.9.2   JACIE accreditation: Pre-inspection documents

Prior to inspection, a list of pre-inspection documents must be compiled ready for review.

Quality documents, policies and guidelines	- Quality management plan (full document detailing IEC use) - All IEC-related SOPs (including toxicity management) - Guidelines (communication, patient monitoring/transfer) - Nursing guidelines (management of complications, respiratory, cardiac, renal and liver dysfunction, TLS, anaphylaxis, neutropenic fever and pain)
Clinical Monitoring and outcome review	- Criteria for safety and efficacy with review of outcomes - Endpoints approved by clinical programme director
Third party agreements	Service level agreements for provider of products/manufacturer

Training and education	• Documentation of training of clinical programme director and physicians caring for CAR-T cell patients (topic, type and dates) • Evidence of training of clinical programme director and physicians caring for CAR-T cell patients on CRS, ICANS, end-organ toxicities, TLS and evaluation of outcomes
Nurses	• Nursing policy on staffing (number of nurses per patient, numbers of relief/temporary and rotational staff and those with specialist qualification in haematology/oncology)
Pharmacists	• Documentation of procedure for receipt of IEC • Documentation of training of pharmacists caring for CAR-T cell patients (topic, type and dates)

UCLH was awarded JACIE accreditation for cellular therapy in June 2018.

7.10   CAR-T Clinical Service Development: Conclusions

Each centre must establish their own work-flow model for the integration of CAR-T cell therapy. At UCLH the immunotherapy service was embedded within the HSCT department, a department equipped to build upon pre-existing infrastructure to meet JACIE accreditation requirements for cell harvest, cellular processing, quality systems, regulation, data reporting and safe inpatient care. Logistically, provision of these therapies requires a cohesive multi-disciplinary effort across departments encompassing medical (including ITU and neurology), nursing, trials, stem cell laboratory, apheresis, pharmacy, administrative, data management, bed management and educational staff. From initial consultation of the patient to assessment in the long-term follow-up clinic, efficient and streamlined communication between the relevant departments is paramount for scheduling, maintaining the chain of identity of cells, service planning and safe clinical care. Building links with other subspecialties such as cardiology, infectious disease, microbiology and psychological support has helped to optimise our patients pre and post therapy.

Challenges of service development have included:

(1) **Staffing**

Rapid expansion of the immunotherapy service on NICE approval of Tisagen and Axicel led to increased pressure on existing staffing resources, both in the immunotherapy, BMT and wider nursing teams. Recruitment of a new Immunotherapy consultant, 2 clinical fellows, a CNS team and administrative support has allowed for increased capacity for both NHS CAR-T cell products and upcoming academic and commercial T-cell studies. Estimated projected patient numbers in the coming years will facilitate service planning.

Medical Teams

The number of CAR-T inpatients, cared for by allograft team, have surged in recent months. A dedicated CAR inpatient fellow has been allocated to the ward to co-ordinate the care of these patients.

Nursing Teams

Three new members of the lymphoma CNS team have been allocated to co-ordinate the care of the NHS CAR patients. The UCL clinical trials team also have a nurse to assist with patient care.

Apheresis and stem cell laboratory staff

These departments have expanded to include new team members, facilitating service provision.

Administrative Support

Scheduling of appointments and co-ordination of re-staging investigations is led by the administrative staff. This team also play a pivotal role in interdepartmental communication pathways to facilitate service planning.

Data managers

Data managers for clinical trials are responsible for collating regulatory and monitoring data. Staff resources are dependent on predicted upcoming workload.

Pharmacy

A dedicated T-cell Immunotherapy pharmacist is responsible for preparing and authorising lymphodepletion order sets, screening chemotherapy and supportive care prescriptions.

Infusion team

As a large HSCT unit, UCLH has a large team of stem cell nurses. To facilitate the administration of CAR-T cells, these nurses received additional training in accordance with the

SOP on administration of ATIMPs. Trained nurses were then added to a log of CAR-T cell stem cell nurses.

(2) Capacity

Inpatients

Inpatient burden has increased significantly with the establishment of this service. Attempts to address this include maximising the use of AC for outpatient administration of lymphodepletion, facilitating early discharge in well patients and optimising patient care (prevention of infection). The concept of "pre-habilitation" may be considered with the help of our physiotherapy colleagues. A monthly admission list is circulated to the relevant departments, facilitating service planning.

Outpatients

Capacity in the outpatient clinic (clinic rooms) has increased to accommodate the volume of referrals, patient follow-up post discharge and late effects clinic.

Apheresis and Stem Cell Laboratory

Staff have been recruited as required. Evening and weekend apheresis slots may be an option pending the volume of future patients.

Ancillary Departments

Echocardiograms, PET/MRI scans, bone marrow examinations, vascular catheters and central access put pressure on the respective departments. Reservation of appointment slots several weeks in advance, pre-empting difficult cases and allocation of designated slots for the CAR service has addressed capacity issues.

(3) Education

The implementation of the CAR service at UCLH has required regular and repeated training of medical and nursing staff on the complications of therapy and their respective management. Teaching has extended from the most junior members of the medical and nursing teams to senior ITU physicians. SOPs on toxicity and supportive care management constitute mandatory training for haematology trainees and consultants. Podcasts and webinars have been circulated with the aim of capturing the attention of the maximum number of staff members. Continued education of our staff and colleagues will be a goal of the service.

(4) Communication

Establishing streamlined communication pathways between medical (inpatient and outpatient), nursing, apheresis, stem cell laboratory, pharmacy, infusion, administrative and bed management staff has been a challenge. This high-risk patient cohort require urgent therapy and the pathway must be expedited to facilitate an admission on the earliest possible date. Unfortunately, patients are often deferred due to clinical progression (requiring alternate therapy or palliation) and infection, rendering service planning a difficult feat. Maintaining the lines of communication between the relevant departments is paramount for effective service provision. Optimisation of the service also requires collation of feedback from respective departments and establishing pathways to pre-empt and circumvent these challenges.

(5) Academic and Commercial CAR T programme

The advent of the licensed CAR T cell products in B-ALL and high-grade B-cell lymphoma has presented challenges to the academic programme, a programme primarily focused on the delivery of novel Phase 1 clinical trials. Commercial products have allowed our centre to deliver this therapy at a pace we could never have achieved as an academic institution. However, it has prompted us to shift our focus to patients with an unmet clinical need (e.g CNS lymphoma, T-cell malignancies) and enter the field of solid tumours.

CHAPTER 8

8 DISCUSSION AND CONCLUSIONS

8.1 Introduction

Autologous CAR T-cell therapies have now emerged as standard of care for relapsed and/or refractory B-ALL (up to age 25 years) and high-grade B-cell lymphomas, with much promise in other haematological malignancies and solid tumours. However, there are a number of challenges precluding the widespread availability of these therapies in the UK (152,160). Infrastructural, financial, GMP and regulatory hurdles preclude rapid transition of this technology to the clinic and limit capacity to a few select centres. In the academic setting, there are limited CAR-T clinical trials, hampering patient access to these therapies. Academic studies (that are open) are limited by a paucity of manufacturing slots. Optimising the manufacturing process to enable scalability is the first step towards meeting patient demand. A second challenge is successful autologous cell harvest in a heavily pre-treated and lymphopenic patient cohort. Failure to achieve adequate cell numbers may result in manufacturing failures. Harvesting patients earlier in the treatment pathway or adopting a personalised approach to this important step may address this challenging issue. Thirdly, the safe treatment of this unique patient cohort and implementation of these therapies requires a cohesive multi-disciplinary approach and a shift in pre-established clinical care delivery systems; medical, nursing, pharmacy and cellular processing.

The main goal of this project was to investigate and establish a streamlined manufacturing method and platform for CAR T-cell manufacture for the UCL CAR T-cell programme with a focus on autologous products for patients with B-ALL (the ALLCAR19 study). We successfully demonstrated that the closed, semi-automated CliniMACS Prodigy system is a more robust, standardised, time-efficient and scalable mode of manufacture with significant reductions in clean room resources (and hence cost) relative to the open and conventional manual process. Through the completion of this project, we have demonstrated that a university institution with limited clean room infrastructure (relative to a commercial company) can establish a scalable and decentralised manufacturing hub, thereby expanding our portfolio of early phase clinical studies, facilitating rapid translation of science to the bedside and increasing the availability of these therapies to patients at a lower cost.

Secondly, we aimed to explore the single-centre apheresis practice at UCLH to define a model/guideline for successful CAR T-cell apheresis. Based on our experience at UCLH, we have devised an algorithm using peripheral blood ALC and a CD3+ target of 1.5 x 10^9 to extrapolate the volume of blood to be

processed in litres. This algorithm requires prospective validation but has the potential to overhaul our current practice. If validated in a larger cohort of patients, sharing this knowledge with other CAR-T centres via peer-reviewed publication would increase its impact.

Lastly, we aimed to begin to establish the clinical infrastructure required to deliver these therapies at a large central London teaching hospital. At UCLH the immunotherapy service was embedded within the HSCT department, a department equipped to build upon pre-existing infrastructure to meet JACIE accreditation requirements for cell harvest, cellular processing, quality systems, regulation, data reporting and safe inpatient care. As one of the leading immunotherapy units in the UK, this work provides a guide for other prospective centres on the core requirements and the challenges of the implementation of such a service.

8.2 Optimisation of a new method of manufacture

8.2.1 Pre-clinical optimisation of new process

Our small-scale experiment using one patient donor with B-ALL demonstrated that Condition 1 simulating the Prodigy® process was superior in terms of T-cell expansion and final CAR-T cell numbers. Viability, CAR-T T-cell differentiation status and markers of exhaustion were comparable between the two conditions. These results supported the transition of our process to the GMP clean rooms for further validation (despite the acknowledged limitations of this small-scale experiment).

8.2.2 Optimisation of CAR-T manufacture using the CliniMACS Prodigy®

While the use of the Prodigy system for the generation of CAR-T cells from healthy donor material has been validated by several groups (191,192,194,195), there is a paucity of data supporting its use in patient-derived material (191,196,197) and no data comparing manual and semi-automated manufacturing methodologies in patient-derived material. Either cryopreserved or fresh apheresis material from three patients with B-ALL were processed, both via the open Dynabead process and the closed, semi-automated CliniMACS Prodigy®.

8.2.3 Comparison of "open" and "closed" process

8.2.3.1 Final products

While both methods successfully generated 3 sterile autologous CD19 CAR-T cell products, the CliniMACS Prodigy produced remarkably consistent results in terms of viability (>99%), cell expansion (mean of 10 fold by day 8, range 6.9-14.6), transduction efficiency (median 68.8%, range

66.9-77.8%) and CAR-T cell yield (median 1.5 x 10^9, range 1.1- 1.71 x 10^9) . The "open" process was highly variable in terms of viability (median 97.1%, range 82-99.4%) expansion (mean of 3 fold by day 8, range 0.2-5.1), transduction efficiency (median 78.3%, range 48.6-86.1%) and final CAR-T cell numbers (median 1.72 x 10^9, range 0.05-2.2 x 10^9) with notably poor expansion in 1 of the 3 patients (failed to achieve target cell dose for trial). The additional step on the Prodigy® system of high purity T-cell enrichment using CD4 and CD8 immunomagnetic beads removed contaminating non-target cells and allowed for a degree of standardisation of cell composition prior to the commencement of cell culture.

Although the starting leukapheresis had a highly variable maturation profile, the final products generated on the CliniMACS Prodigy® were enriched with cells of a central memory phenotype (median 40.6%, range 16.9-55.7%). Expression of PD-1 and Tim-3 on CD4+ and CD8+ T cells in the final products (generated by Prodigy® system) was reduced relative to the starting material. This may be consistent with cell death, namely death of exhausted cells and survival of more robust T-cells as the manufacture progresses. CAR-T cells manufactured on the CliniMACS Prodigy® demonstrated lysis and secreted cytokines when co-cultured against CD19-expressing cell lines (Supt1-CD19 and RAJI).

8.2.3.2 Time and cost

Although the overall cost of consumables is similar between the two methods, the Prodigy® system was considered more cost-effective due to a reduction in the use of costly clean room facilities, total staff hours (41 hours versus 93 hours), grade A time (8 hours versus 29 hours), vector cost per manufacture (£2600 versus £26000, based on MOI of 2.5 and starting cell number of 100 x 10^6 lymphocytes) and equipment requirements (Figure 33). A full cost analysis of the use of the Prodigy® system in a German academic setting has reported cost per product of £33,000-60,000 depending on number of machines per clean room and vector usage (305). Zhang et al costed their TCT Prodigy process at $25,000 per product (192).

8.2.3.3 Limitations

Extended phenotyping (maturation and exhaustion) and functionality testing (FBK and cytokine release) of the final products was only available for the products generated via the Prodigy® system. While these results demonstrated minimally exhausted CAR-T cell products enriched with central memory T-cell populations, capable of killing CD19+ cells in vitro and stimulating cytokine release, due to a lack of excess material, we were unable to compare these parameters with those for the manual process. Therefore, we cannot comment on the effect of IL7/15 (Prodigy®) on CAR-T differentiation status relative to no cytokines (manual), impact of mode of manufacture on cell exhaustion or the relative potency of the final products.

8.2.4   Final conclusions

A robust, scalable and cost-effective manufacturing platform that will consistently generate a sterile, potent and viable product at the target dose, despite huge inter-donor variability, is the holy grail of CAR-T cell manufacture. Our results demonstrate that the CliniMACS Prodigy® represents a more robust, standardised, cost-effective and scalable mode of CAR-T cell manufacture relative to the manual method. Our group at UCL have presented preliminary in vivo efficacy of autologous CAR-T cells generated using the Prodigy system in B-ALL (93) and 3 UCL studies using this device have recently completed recruitment (NCT02443831, NCT02431988, NCT02935257). Further evidence to support the clinical efficacy and persistence of these cells relative to those generated by other methods is eagerly awaited.

8.2.5   Future directions in CAR-T cell manufacture

8.2.5.1 Centralised and decentralised CAR-T cell manufacture

Commercial CAR-T cell products such as Axicel and Tisagen are manufactured in large facilities in El Segundo, California (Axicel) and Morris Plains, New Jersey (Tisagen) with plans to extend manufacturing capacity to hubs in Europe. Academic institutions are primarily running small facilities with relatively little infrastructure. Partnerships between academia and commercial companies such as between University of Pennsylvania and Novartis have allowed for greater patient access to these novel products.

Decentralised manufacture, namely splitting manufacture into various locations, may increase patient access, support local jobs and expertise and save on distribution costs but is complicated by maintenance of process quality standards across sites and achieving economies of scale (306). Automation of the CAR-T manufacturing process may offset some of the costs of decentralisation, reduce inter-site and inter-product variability and enable global dissemination of these therapies. Harrison et al discuss the automated culture platforms that may be adapted for the manufacture of cell and gene therapies and propose a labour model for decentralised manufacture (Figure 40) (306). The CliniMACS Prodigy® is one such semi-automated device. The Octane Cocoon™ is another example of an all-in-one automated culture system which may be suitable for decentralised manufacture. Capable of parallel production of multiple products, this may have a competitive advantage over the CliniMACS Prodigy®. In its patent Shi et al describe 40-60 fold cell expansion over 10-14 days with a viability of 97% and transduction efficiency of 65% (307). There is no clinical data supporting its use at the present time.

The direction of travel in the field of CAR-T manufacture will be largely determined by the advent of an "off the shelf" CAR-T cell product (Section 8.2.5.2). A safe and effective "off the shelf" product will further encourage manufacture at scale, facilitating the production of multiple patient products in one run. An automated stirred-tank bioreactor system (ambr® 250) to facilitate large-scale expansion of potential future allogeneic "off the shelf" CAR-T products is one of the many systems under investigation (308).

Hub Facility Labour

- Hub contains the senior organisational oversight roles which give strategic insight
- Decentralised senior roles feed in tactical management information
- Administration e.g. sales, accounts centralised to minimise duplication of roles
- Competitive advantage cost savings from centralising technical roles such as IT and maintenance
- Centralisation of QC and QP have the potential to deliver the biggest cost savings but are the most radical proposal

Roving Labour

- Individuals or teams are multi-directional to fill roles as required
- Potential roles include maintenance, QC, QP, technical or operators
- Potential for poor worker satisfaction and high consulting salary rates
- No "talent pooling" benefits
- Decision-making is spread out and able to influence at a local site level and organisational level
- Roles are less planned and reactive in nature
- Allows "parachuting in" expertise at required sites without taking on commensurate salary liability

Decentralised Site Labour

- Small teams each with desired "skills pool"
- Contains operators and technical staff but may outsource QP, QC, administration and senior roles
- Finely balanced skill requirements are not resilient to staff shortages
- Recruiting staff to some locations may be challenging which affects operational readiness
- Potential for poor worker satisfaction and high consulting salary rates
- Reduced "talent pooling" benefits
- Decision-making is spread out and able to influence at a local site level and organisational level

Figure 40: Models of labour allocation for decentralised manufacture (306)

8.2.5.2 "Off the shelf" CAR-T cell products

The routine application of "off the shelf" or universal CAR-T cells would represent a major advance in the field of CAR T-cell manufacture. They are an attractive option due to their ready availability, (facilitating treatment of patients with rapidly progressive disease), use of healthy donor material (potentially overcoming quantitative and qualitative challenges of autologous material) and use of one donor to generate products for many patients (no inter-product variability and minimal risk of manufacturing failure), conferring a huge cost and scalability benefit. The key barriers to the widespread use of "off the shelf" CAR-T cells include rejection of the incoming cells by the recipient immune system (mediated by Class 1 MHC on the donor T cells) and the presence of the TCR on donor T cells, eliciting graft versus host disease in an unmatched recipient (309).

Genome engineering tools have been used to address the latter issue, to knock out the TCR. Nucleases perform like a "molecular scissors", editing the genome by targeting specific DNA sequences and cutting it using a nuclease domain. The MD Anderson group generated zinc finger nucleases (ZFN), eliminating expression of the endogenous TCR (310). Transcription activator-like effector nucleases (TALEN), disrupting the TCR alpha and CD52 genes (311), have been used to generate the UCART19 product, currently under investigation in the PALL (NCT02808442) and CALM (NCT02746952) trials in paediatric and adult B-ALL respectively. Preliminary data has been published (312,313). Clustered regularly interspaced short palindromic repeats (CRISPR/Cas9), RNA-guided DNA targeting technology (314) has also been used to generate off the shelf CAR-T cells, again by disrupting the TCR (315,316). MSK have devised a technique which incorporates the CAR construct into the TRAC locus, simultaneously preventing TCR expression and introducing a CAR that is under control of the TRAC locus, allowing uniform expression, enhanced potency and prevention of tonic signalling (315). However, current gene editing techniques do not reach 100% knock out efficiency and despite additional depletion and selection steps, the risk of GVHD, triggered by a very small number of TCR-positive cells persists.

Persistence of TCR-negative off the shelf CAR-T cells in the body may be hampered by recipient T-cells recognising non-self HLA, leading to responses of short duration. Aside from lymphodepletion to eradicate the patient's T-cell compartment (transiently), approaches to address this include use of ZFN and CRISPR systems to remove HLA molecules from the CAR-T cells (317–319), avoiding T-cell mediated rejection. However, activated T-cells may also express HLA Class II and lack of self-antigens on incoming CAR T-cells would render them susceptible to NK-mediated lysis. Enforced expression of other HLA molecules such as HLA-E and HLA-G may circumvent this issue (317,320). While gene editing of CAR-T cells represents the most promising approach to universal products, in vivo safety and efficacy of these cells is lacking and must be assessed in early phase studies. Off target cleavage and new translocations can occur. Researchers must be vigilant regarding the oncogenic potential of

these highly modified cells. Gene editing may also complicate the manufacturing process with regard to conforming to GMP standards and optimising clinical grade expansion protocols.

Another approach to allogenic CAR-T cells is to harvest the patient's original stem cell donor (if relapse post HSCT) provided there is no active or significant history of GVHD. Overall, with appropriate patient selection this strategy has been associated with a low risk of GVHD (321). A study of allogeneic CD19 CARs post HSCT has completed recruitment at UCL (NCT02893189) and preliminary data has been presented (322). Alternative effector cells such as virus-specific T-cells, NK cells, NK-T cells, γδ T cells and induced pluripotent stem cells (iPSCs) are under investigation. Virus-specific T-cells, most commonly used post HSCT to treat viral infections, have been shown to elicit minimal GVHD despite HLA mismatches. Transduction of allogeneic virus-specific T-cells with a CD19 CAR have demonstrated responses in B-cell lymphoma, albeit in small numbers with lower response rates than expected in this patient cohort. No GVHD was reported (323). NK cells do not require HLA matching, supported by the safe administration of haploidentical NK cells to patients with a variety of cancers (324). There are a number of early phase studies assessing the efficacy of NK CARs targeting antigens such as CD19 (NCT02892695), CD33 (NCT02944162) and CD7 (NCT02742727). With regard to clinical data published to date, NKs harvested from cord blood and transduced with a CD19 CAR have demonstrated some efficacy in patients with CD19+ lymphoid malignancies (325). NK-T cells (326), γδ T cells (327) and induced pluripotent stem cells (iPSCs) (328) are being explored as other "off the shelf" CAR options. A bank of iPSCs of common HLA types can be generated with the capacity to self-renew and facilitate indefinite use. As cells are generated from one clonal pluripotent cell line, the downstream CAR-T cells would be homogenous. Safety and efficacy data for iPSC CARs is lacking.

8.3 Optimisation of T-cell apheresis

8.3.1 Optimisation of T-cell apheresis at UCLH

There is no international guidance on the ALC cut off or number of CD3+ cells required for CAR-T cell manufacture, most academic centres and commercial companies stipulating ALC cut offs or CD3+ numbers based on their respective validated manufacturing process. The COBE Spectra is no longer approved for use, superseded by the Spectra Optia, with even less data supporting its use for downstream CAR-T manufacture, particularly in adult patients (211,215). As the bulk of the demand for autologous CAR-T products will be for adult patients, we retrospectively reviewed 34 autologous MNC harvests in adults with heavily pre-treated B-ALL, DLBCL and MM, for downstream CAR-T cell manufacture, using the Spectra Optia at UCLH.

We sought to personalise and streamline the procedure of T-cell harvest for this heterogeneous cohort of patients with a view to increasing the capacity in our unit (apheresis and stem cell laboratory) to meet clinical demand and improve the patient experience. We devised an algorithm to be validated prospectively at UCLH, to enable individualised calculation of the required blood volume to be processed on the Spectra Optia to achieve the proposed CD3+ target of 1.5 x10^9 using only the patient peripheral blood ALC (x 10^9/L).

8.3.2   Utility and limitations of T-cell apheresis algorithm

The correlation between pre-harvest ALC and CD3+ cells per litre of blood processed was deemed to be moderate in this cohort of patients (coefficient 0.62).Three of our patients (8.8%) failed to reach the target of 1.5 x 10^9, all easily identified as high risk based on ALC and CD3+ pre-count. Our numbers are too small to draw firm conclusions on the failure to reach target in these 3 patients but they may have benefited from a prolonged or second day of harvest and 2/3 of the patients (data available n=2) had a relatively higher proportion of NK cells in their apheresis products, similar findings in the peripheral blood previously identified as a risk factor for low CD3+ cell yield (confirmed in 1/3 patients) (290). At present, our algorithm has a number of limitations including the small number of patients in this study with a moderate correlation coefficient, the potential for circulating disease to falsely elevate the ALC, the inability to account for lymphocyte recruitment (225,291) and T-lymphocyte functional capacity with these variables. This algorithm, if validated prospectively, will only be applicable to our academic CAR-T clinical trial patients given each commercial company have different requirements for their respective starting material.

8.3.3   Final conclusions

As the number of CAR-T clinical trials and disease indications for therapy continue expand at UCLH (and internationally), approaches to optimise and streamline our service have become increasingly important. The starting material for subsequent downstream CAR-T cell manufacture likely has huge implications on the quality of the CAR product (Section 8.3.4.1). While natural variability in starting material is part of the complexity of these products and we may not be able to control qualitative variables of the cell yield (as of the present time), ensuring we have a sufficient quantity for patients at high-risk of failure and shortening the procedure for those at low risk, would allow us to make the best use of our resources to meet demand, increase capacity and improve the patient experience. To validate this algorithm, we plan to gather data prospectively in a larger cohort of CAR-T clinical trial patients. Feasibility of cell manufacture with 1.5 x 10^9 CD3+ cells will be under regular review.

We will review our strategy for subtyping of peripheral blood pre-harvest to establish the proportion of circulating NK cells, in an attempt to further identify patients at higher risk of failure. Anaemic patients also appear to be higher risk of a low CE. This mechanism is incompletely understood as haemoglobin should not affect the separation of components of the MNC layer and is not a recognised risk factor with peripheral blood stem cell harvests (329). CE has been reported to be higher in patients with a higher Hb regardless of diagnosis (289). Hb may therefore simply represent a surrogate marker for MNC reserve in the extravascular compartment and may reflect disease activity (289). These patients will also be considered higher risk in our prospective review.

8.3.4   Future directions in T-cell apheresis

8.3.4.1 Starting material and T-cell dysfunction

At the present time, the starting material validated by most groups for CAR-T cell manufacture is autologous CD3+ T-cells obtained by leukapheresis. Potential developments in the autologous field include the acquisition of CD3+ T-cells from as little as 100mls of PB (196) and isolation of T-cells from autologous cryopreserved stem cell units (330). Harvesting T-cells earlier in the treatment pathway, prior to the administration of lymphotoxic chemotherapy, may facilitate the collection of healthier apheresate (quantitatively and qualitatively). The use of CAR-T cells after failure of first-line therapy is under investigation in a number of CAR-T cell studies in high-grade B-cell lymphoma using Axicel (NCT03391466, ZUMA-7) and liso-cel products (NCT03575351, NCT03483103) and in B-ALL with Tisagen (NCT03876769) . Harvesting autologous T-cells upfront at the point of diagnosis or after the failure of first line therapy for storage and downstream use (should further lines of therapy fail) is another avenue yet to be explored. Apheresis, stem cell laboratory, storage and funding capacity and potentially an unnecessary intervention in a significant number of patients would be complicating factors. This approach would require validation in a clinical study. While upfront and earlier harvest may surmount the impact of lymphotoxic chemotherapy on T-cell number and function, it will not overcome fixed patient variables.

There are a number of such variables to consider. The bulk of the demand for autologous products is for adults, median age at diagnosis of NHL at 67 years (331). Older age has been demonstrated to effect CD8+ T-cell proliferation and senescence (332). T-cell dysfunction in cancer patients may also be present at a much earlier stage in tumorigenesis. Scheitinger et al suggest that CD8+ T-cell dysfunction is antigen-driven and enters an irreversible phase in established tumours (333). T-cell dysfunction may also be an inherent component of certain haematological conditions. It is a known risk factor for B-cell lymphoma (334), supported by the development of post-transplant lymphoproliferative disorders (T-

cell depletion to prevent graft rejection) and the increased risk of B-cell lymphoma in HIV patients (335). Das et al demonstrated deficits in naïve T-cells in many paediatric cancers (particularly solid) at diagnosis, further depleted by cumulative cycles of chemotherapy (336). CLL is another condition characterised by T-cell defects, particularly in advanced disease (337). Fraietta et al analysed apheresate and CAR-T cell products of CLL patients with responding and non-responding disease. Expansion during manufacture predicted response and correlated with expansion in vivo, suggesting the T-cell product rather than disease-related factors was influencing the clinical outcome. Gene expression profiling of the CAR-T cell products in responding and non-responding patients demonstrated an early memory and exhausted/differentiated phenotype respectively (338). This group also identified a subset of T-cells in the apheresate associated with a better clinical response (CD27+CD45RO-CD8+ T cells) (338). Hoffman et al demonstrated (albeit in small numbers) that even untreated CLL patients generated a more differentiated product (CD45RA+CCR7-) than healthy donors (CD45RA+CCR7+) (339), again highlighting the impact of underlying disease on T-cell function in CLL.

Can we further modify patient-derived starting material to overcome fixed patient variables and/or preferentially generate a particular subset of CAR-T cells known to correlate with clinical efficacy? We recognise that the use of IL-7 and IL-15 cytokine support during cell manufacture can enrich the T-cell memory compartment (central memory and stem cell memory), a T cell compartment thought to improve persistence and anti-tumour activity in vivo (275–279).While there are many barriers to the use of healthy donor universal T-cells (Section 8.2.5.2) and data from Fraietta and Hoffman et al has yet to be reproduced in diseases other than CLL, screening and selection of healthy donors (including those with a high frequency of early memory T-cells) is also an attractive future strategy to counteract fixed patients variables.

8.4 Clinical service development for provision of academic CAR-T cell therapies

Establishment and integration of a CAR-T work-flow model is determined at an institutional level. At UCLH, leveraging the clinical, regulatory, cell harvest, cellular processing, data management and quality infrastructure in place for HSCT allowed for the rapid expansion of our service. Regardless of the mode of implementation, provision of these therapies requires a cohesive MDT effort across departments encompassing medical (including ITU and neurology), nursing, trials, stem cell laboratory, apheresis, pharmacy, administrative, data management, bed management and educational staff. Efficient and streamlined communication between the relevant departments is paramount for scheduling, maintaining the chain of identity of cells, service planning and safe clinical care. Building links with other subspecialties such as cardiology, infectious disease, microbiology and psychological support has helped to optimise our patients pre and post therapy.

8.4.1   Final conclusions and future directions in clinical service development

As the complexity of these products increases, the relevant teams (medical, nursing, apheresis, stem cell laboratory, pharmacy, educational and trials) will have to evolve to accommodate and ensure the delivery of safe clinical care. Within haematology, licensing of CAR-T cell products beyond B-ALL and high-grade B-cell lymphoma to diseases such as MM and MCL will result in another surge in clinical demand, prompting review of service delivery, communication pathways and capacity. Early phase studies in solid tumours (TCR-engineered T-cell, TIL and CAR products) will have unique and often yet to be determined toxicity profiles. These studies should be delivered in specialist centres experienced in the provision of cellular therapy. At UCLH, the admission and clinical care pathways for patients enrolled on solid tumour studies is provided by the Immunotherapy and HSCT teams, with specialist input from oncology disease-specific services. Demand for efficacious cellular therapy for solid tumours will increase exponentially in the coming years, the race the emulate the success of CD19-targeting CARs well underway.

Establishing the place of CAR-T cell therapy in the disease pathway and the patients mostly likely to derive long-term benefit are important questions for randomised controlled trials, the outcome of which will likely map future work-flow models.

8.5 Future directions in CAR-T cell therapy

8.5.1   Understanding CAR-T cell failure and strategies to improve CAR-T cell efficacy

Approximately 10-20% of patients will have no response to CD19-targeting CAR-T cell therapy (47,76). Of those who achieve an initial remission, 30-50% of patients will relapse, usually within 12 months of infusion (47,76,91). Failure of CAR-T expansion/persistence and loss or alteration of the target antigen are the most commonly cited causes of CAR-T cell failure.

8.5.1.1 Loss of CAR-T persistence

Loss of CAR-T persistence or failure of CAR-T cell expansion often accounts for early relapse of B-ALL, a disease that seems to require a more prolonged duration of CAR-T cell mediated immunosurveillance relative to B-cell lymphoma. It is well recognised that lymphodepletion facilitates

T-cell engraftment and superior persistence (Section 1.2). However, persistence of cells continues to vary between and within clinical trials and further research is needed to define the optimal regimen.

The facets of a CAR-T cell that determine its persistence are yet to be elucidated. The impact of disease and treatment-related T-cell dysfunction on *ex vivo* and *in vivo* T-cell expansion is discussed in Section 8.3.4.1. Avenues to optimise T-cell function at the front end include an earlier T-cell harvest, optimised CAR-T manufacturing protocols to remove contaminating cells, selection of T-cells with an earlier memory phenotype and use of healthy donor starting material (Section 8.2.5.2). Contaminating cells such as blasts and myeloid-derived suppressor cells are known to hamper T-cell growth (217,218,340) and potentially trigger relapse or confer resistance to therapy as evidenced by the transduction of a single leukaemic B-cell (271). One group have demonstrated superior *in vivo* expansion and higher transduction efficiency with more efficient T-cell selection using CD4 and CD8 immunomagnetic beads (341). Selection of T-cells with central memory or stem cell memory phenotype prior to activation (190,342) or skewing of T-cells towards this phenotype during the manufacturing process using IL-7 and IL-15 (343) are under exploration. A subset of T-cells with a memory phenotype in the starting material have been shown to correlate with response in CLL populations (338). Gardner et al have demonstrated higher expression of LAG-3 and TIM -3 by flow cytometry in CD4+ and CD8+ CAR T-cells expanding *in vivo* in patients with non-responsive B-ALL (relative to those with responding disease). As demonstrated previously non-responsive patients had lower peak CAR-T cell numbers. When comparing the starting material for both patients groups, patients with an inferior response had a higher percentage of PD-1 and LAG-3-expressing CD8+ T cells (PD-1 for CD4+ only) (344).

The use of agents such as ibrutinib (116,117,345) to augment T-cell functionality and persistence is also of great interest (Section 1.3.3.3). In addition, phosphatidylinositol 3-kinase (PI3K) inhibitors (such as idelalisib) have been shown in mouse models to preserve a less differentiated T-cell state with superior expansion and cytotoxic activity (346). Zheng et al have demonstrated that tonic signalling through CAR CD3z immunoreceptor tyrosine-based activation motif (ITAM) may hamper CAR persistence, a pathway disrupted by PI3K inhibitors. Again, the PI3K inhibitor promoted a less differentiated T cell state (347). PD-1 inhibitors have generated interest in the CAR-T cell field given their potential synergistic role in maintaining CAR persistence *in vivo*. PD-1 expression on CAR-T cells has been shown to increase from the point of infusion to the point of peak expansion (348) and pre-clinical data supports PD-1 inhibition as a potential strategy to augment efficacy and persistence (349,350). Preliminary clinical data supports this (351) with many studies underway.

Immunological rejection is a recognised phenomenon, particularly in patients exposed to repeated infusions. The scFv of the CAR construct in most clinical studies is murine in origin. Turtle et al demonstrated the presence CAR-specific T cell responses and identified immunogenic epitopes within the murine FMC63 scFV in five patients who received a second infusion with no associated *in vivo*

expansion (56). This has generated interest in humanised CAR constructs with pre-clinical studies demonstrating enhanced persistence, less tonic signalling and exhaustion (352–354). Clinical responses have been reported in small numbers of heavily pre-treated patients, including those with relapses post murine-derived CAR-T cells (355).

In some instances, transient CAR-T cell persistence may be preferable. This may include when investigating novel targets with a high risk of on-tumour off-target toxicity. Transient persistence can be achieved by transfection of T-cells with mRNA encoding the CAR. mRNA cannot integrate into the host genome, unlike a viral vector which permanently integrates the CAR. This strategy may require multiple doses to achieve a therapeutic dose and can be complicated by immune-mediated rejection and anaphylaxis (356). There are many studies underway investigating the use of mRNA CARs including in solid tumour models (357–359). Other methods of regulating CAR-T cell persistence for the purposes of safety are discussed in Section 8.5.3.

8.5.1.2 CAR-T cell design

The contribution of CAR design (namely the antigen-binding domain, hinge region and transmembrane domain) to CAR-T cell efficacy, persistence and safety is likely highly significant but definitive data is lacking in this field. There are no standardised tests to assess potency (360) and products have not been directly compared. Defining reliable criteria to determine CAR-T cell potency is a challenge for the future.

The impact of the co-stimulatory domains such as CD28 and 4-1BB on T-cell expansion and persistence is well-recognised, CD28 CARs eliciting rapid expansion with an effector phenotype but shorter persistence and 4-1BB CARs tending to skew T-cells towards a memory phenotype with slower expansion and potentially superior persistence. No head to head comparison has been performed. The evidence supporting each generation of CAR and co-stimulatory domain are discussed in detail in Section 1.3.1. Other than 3rd generation CARs with two co-stimulatory domains combining the effector function of CD28 CARs with the apparent superior memory function of 4-1BB CARs, another strategy to modulate CAR activation has been proposed by Feucht et al. This group calibrated the activation potential of a CD28 CAR model by designing a CAR with a single immunoreceptor tyrosine-based activation motif (ITAM), thereby modulating effector and memory function and facilitating increased persistence of the CD28 CAR (361). There is also a role for targeted genomic integration of the CAR, as evidenced by TET2 disruption in a patient with CLL. This resulted in enhanced potency and a central memory phenotype CAR-T cell (362). Similarly, integration of the CAR into the TRAC locus using CRISPR–Cas9 demonstrated more uniform CAR expression, superior cytotoxic activity and less differentiation and exhaustion in mouse models (relative to conventionally transduced T cells) (315).

A more in depth understanding of the factors that determine CAR potency and those that predict clinical responsiveness combined with advances in gene editing technologies will likely guide future CAR design.

8.5.1.3 Antigen escape

Antigen escape is discussed in Section 1.3.3.1. Loss of surface of expression of CD19 or 'antigen escape' is thought to account for 10-30% of relapses after CAR-T cell therapy in B-ALL (73,80). Mechanisms of CD19 negative escape include alternative exon splicing of CD19 gene which leads to the expression of variants lacking the domains required for recognition by CAR-T cells (80), emergence of CD19 negative clones (81) or lineage switch (82,83). Molecular work-up of a case of CD19- relapse post blinatumomab identified defective CD19 membrane export in the post-endoplasmic reticulum compartment as the mode of resistance (363). Exploration of other mechanisms of CD19 antigen loss, identifying patients at higher risk and reducing this risk are challenges for the future.

Aside from theoretically higher risk patients, such as those who have detectable CD19- clones by flow cytometry at screening and those previously treated with blinatumomab, we do not have validated predictors for antigen negative relapse. More complex flow cytometric methods, minimal residual disease monitoring and sequencing may be helpful in identifying patients with pre-treatment subclones, who may not be good candidates for CARs targeting a single antigen. Gardner et al report persistent disease identified on next generation sequencing in a significant proportion of ALL patients (67.5%) with MRD negative remissions at month 1, of whom 65% achieved a molecular CR at month 2 (364). The presence of persistent disease by sequencing may identify patients at higher risk of relapse.

It is recognised that complete antigen loss is not a prerequisite for relapse. Diminution of cell surface expression has been shown to trigger relapse with CD22 CARs, with interpatient variability in the threshold antigen density associated with this relapse. The authors postulate that downregulation of CD22 was due to post-transcriptional modification as they did not identify genomic alterations, altered isoform expression, changes in gene copy number or diminished RNA expression (365). Understanding the role of antigen density thresholds in anti-tumour responses and the mechanisms of escape will be pivotal to the design of future CAR constructs and clinical studies.

Tumour heterogeneity may also account for a proportion of relapses. While CD19 is thought to be ubiquitously expressed on B-cells, CD19- subclones may be present at diagnosis (366), rare cases of CD19 partial expression/negativity in B-ALL have been reported (367) and malignant B-cell

progenitors may be CD19- (368). The data to support dual-targeting CARs is discussed in Section 1.3.3.1. There are challenges to achieving optimal functionality of these complex multi-targeted constructs, constructs that can target both antigens simultaneously and with equivalent capacity (369).

8.5.2 Hurdles for solid tumours

As discussed in Section 1.3.3.6, CAR-T cells have demonstrated minimal efficacy in solid tumours. In clinical trials targeting antigens such as mesothelin, PSMA and ERBB2, best responses have been stable disease in 24-67% of patients (370–372). The greatest challenge is the identification of tumour-specific targets. This dramatically increases the risk of "on target, off tumour toxicity". Combinatorial antigen recognition approaches may promote selective eradication of malignant cells (373).

The immunosuppressive microenvironment is also thought to hamper T-cell efficacy and gene engineering strategies are likely advance the field in this respect. Tumour stromal cells produce molecules such as TGF-beta, IL-10 and indoleamine-2,3-dioxygenase which can suppress the effector T-cell response (374). Expression of a TGF-beta dominant negative receptor in T cells has been shown to increase T-cell functionality and the anti-tumour response (375). Incorporating the use of checkpoint blockade, either via the use of 4th generation CARs (Section 1.3) or systemic co-administration has the potential to augment the endogenous T-cell response and is of particular relevance in solid tumours, where upregulation of immune checkpoint ligands such as PD-L1 is well recognised. IL-12 may be a valuable asset in the solid tumour field. Fourth generation CARs are designed to secrete IL-12 into the tumour on activation and trigger an innate immune response against malignant cells (376). IL-12 also has an inhibitory effect on regulatory T-cells and myeloid suppressor cells (377,378). The challenge of CAR-T-cell migration into the tumour may be overcome by co-expression of chemokine receptors such as CCR4 or CXCR2 (379,380).

8.5.3 Improving safety

While CD19-targeting CAR-T cells are associated with a low treatment-related mortality, there is significant associated morbidity. CRS and ICANS (Section 1.6.7) can occur in up to 90% and 64% of patients respectively (47). Given that the bulk of the demand is for older patients, strategies to ameliorate toxicity will be the focus of future studies. Simple risk-adapted approaches such as fractionated and lower CAR-T dosing for patients with a higher disease burden have been shown to dramatically reduce mortality in adult ALL (90). Consideration of CARs with a lower toxicity profile

in more vulnerable patients is also of importance (Liso-cel is marketed as an outpatient CAR therapy (107)). Active infection should be a contraindication for cell infusion, given the associated inflammation can increase the mortality risk of CRS (90). Tocilizumab and corticosteroids are the mainstay of CRS and ICANs management and many other therapeutic interventions are under investigation (Section 1.6.7). The optimal timing for the use of reversal agents such as tocilizumab and corticosteroids is delineated in expert guidance (255,260). Some groups have explored earlier use of tocilizumab and steroids in small numbers of patients with good effect (381) but concerns regarding prophylactic tocilizumab increasing the risk of neurotoxicity (382) and steroids impacting on long-term outcome persist (383).

As CARs move beyond CD19+ malignancies and into the solid tumour field, assessing the risk and burden of toxicity becomes more complex. There is growing interest in gene engineering strategies to mitigate this risk. Safety or suicide switches can be incorporated into the CAR construct and activated by certain pharmaceutical agents. Examples include inducible caspase 9 system (384,385), CD20 targeted by Rituximab (386), HSV thymidine kinase targeted by ganciclovir (387) and epidermal growth factor receptor targeted by cetuximab (388).

Maeve O'Reilly MD (Res)

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Maeve O'Reilly MD (Res)