British Pharmacopoeia

Consultation

British Pharmacopoeia public consultation for draft guidance for T cell and NK cell characterisation assays

Consultation period 10 February 2023 to 1 May 2023

Medicines & Healthcare products Regulatory Agency

British Pharmacopoeia

1. Patients, standards, and innovation

The quality of a medicine is critical to ensuring its safety and efficacy, and therefore the medicine's suitability for patients. Pharmacopoeial standards are part of an interlinked system, together with good practice guidelines and regulatory assessment, that form a foundation to ensuring medicines are of an acceptable quality. Additionally, standards have a place in supporting and enabling innovation through the availability of consistent and widely applicable quality requirements. Innovation in the field of medicines and healthcare has the potential to support patients throughout the world to live longer, healthier, and happier lives.

In recognition of the increasingly important role of biological medicines to healthcare worldwide, the Medicines and Healthcare products Regulatory Agency (MHRA) has developed and implemented a Strategy for pharmacopoeial public quality standards for biological medicines.[1] This strategy, adopted following consultation with stakeholders, laid out a vision of working collaboratively to explore and develop new standard setting approaches for biological medicines. It included a commitment to investigate and take forward standard setting opportunities for innovative Advanced Therapy Medicinal Products (ATMPs).

ATMPs have the potential to be transformative to patients and healthcare globally. However, development, characterisation, and production of these innovative medicines is challenging due to their high complexity, their product specificity, and the still-emerging technologies that support them. Publications such as the Advanced Therapies Manufacturing Taskforce Action Plan,[2] the Medicines Manufacturing Industry Partnership's Manufacturing Vision for UK Pharma[3] and stakeholder feedback have emphasised the important role that standards can have in the development of these medicines. This includes a focus on the value of widely applicable standards that could support knowledge building and facilitate analytics and characterisation.

This draft guidance was written by experts in the ATMP community to support those involved in the development of analytical methods throughout the product lifecycle, and therefore contribute to the quality assurance of innovative medicines for patients.

The MHRA and British Pharmacopoeia would like to recognise and thank the numerous experts in the BP's Working Party for ATMPs that have contributed to the development of this text. The work has been supported by a joint-staff secondment scheme between the BP and the UK's Cell and Gene Therapy Catapult.[4]

2. The draft document

As part of the MHRA strategy for the creation of pharmacopoeial public quality standards for biological medicines, the British Pharmacopoeia Working Party for ATMPs, established in March 2020, has engaged with groups across the cell and gene therapy community to develop

[1] https://www.gov.uk/government/consultations/strategy-for-pharmacopoeial-public-quality-standards-for-biological-medicines

[2] http://www.abpi.org.uk/publications/advanced-therapies-manufacturing-action-plan/

[3] https://www.abpi.org.uk/publications/manufacturing-vision-for-uk-pharma-future-proofing-the-uk-through-an-aligned-technology-and-innovation-road-map/

[4] https://ct.catapult.org.uk/

non-mandatory guidance for key analytical technologies to ensure quality throughout the product lifecycle. The working party has developed two sets of guidance to support ATMP development across a wide range of organisations, laboratory settings, and therapy types. As such, the guidance is product-agnostic and does not provide a step-by-step protocol, nor constitute a prerequisite for product acceptance, but instead offers measures to ensure the production of robust, comparable, and reproducible data within and across organisations.

The ATMP industry continues to grow rapidly worldwide, with increasingly sophisticated scientific discoveries being translated into therapies. There are a variety of challenges in characterising these experimental living medicines. Any CGT product must be characterised in terms of identity, purity and potency and the choice of, and route to, validation of these assays largely lies with the developer and manufacturer. Establishing robust potency assays grows in importance throughout the development of a CGT product and becomes critical in the later clinical stages. As products move towards pivotal clinical trials and licensure, establishing the mechanism of action of the product becomes critical. This requires the potency assays to yield rich data which informs the interpretation of the outcomes in vivo, whether in models or in early human trials.

The draft guidance for T cell and NK cell characterisation assays is intended to be helpful for several reasons:

- Standardised approaches to T cell and NK cell characterization, help to ensure that results from different laboratories are comparable and reliable.
- Provide a framework of considerations for validation of T cell and NK cell assays, which helps to ensure that the assays have the necessary sensitivity, specificity, and reproducibility to produce meaningful results.
- Helps to ensure that T cell and NK cell assays are performed with consistent quality, as they provide recommendations for how to perform the assays, what reagents and materials to use, and how to interpret the results.
- Helps to inform patient diagnosis, treatment, and monitoring, leading to improved patient care. The draft guidance offers information around setting rational levels of cytotoxic function for product release, general methods, and starting materials.

The draft T cell and NK cell characterisation assays best practice guidance is included as Annex 1 to this document.

3. How to contribute

The draft guidance for T cell and NK cell characterisation assays will be posted online for public consultation for a period of two months. During this time, we are asking stakeholders to complete and return the response document, available on our website, to BiolStandards@mhra.gov.uk.

When reviewing the guidance, you may want to consider the following points:

- Do you agree with the technical recommendations made in the document?
- Are the key methods for particle characterisation covered?
- Are there any aspects which you think are missing from the document?
- Is there any terminology within the document that you think needs to be more clearly defined?
- Is the document understandable and are recommendations clear and unambiguous?
- Could the format/style of the guidance be improved?

In addition to the request for technical comments, the response form includes more general questions around the value of the guidelines and other work within the area of ATMPs where standards and standardisation could add value. This information will be used to help the BP to understand and prioritise future work related to ATMPs.

4. Confidentiality and Freedom of Information

Information we receive, including personal information, may be published, or disclosed in accordance with the access to information regimes (primarily the Freedom of Information Act 2000 (FOIA), the Data Protection Act 1998 (DPA) and the Environmental Information Regulations 2004).

Please let us know if you would like any information you provide to be treated in confidence, and please indicate any commercial sensitivities. We will maintain that confidence and resist disclosure under the access to information regimes where possible and in compliance with our legal obligations. We will also consult you and seek your views before any information you provided is disclosed.

British Pharmacopoeia

Annex 1 - Draft guidance: T cell and NK cell characterisation assays

Medicines & Healthcare products Regulatory Agency

Contents

0. Abbreviations

ADCC	Antibody Dependent Cellular Cytotoxicity
ADV	Adenovirus
ALC	Absolute Lymphocyte Count
ALL	Acute Lymphoblastic Leukaemia
AO	Acridine Orange
ATCC	American Type Culture Collection
ATIMP	Advanced Therapy Investigational Medicinal Product
ATMP	Advanced Therapy Medicinal Product
AUC	Area Under Curve
BASO	Basophils
CAR	Chimeric Antigen Receptor
CBC	Complete Blood Count
CDER	Centre for Drug Evaluation and Research
CFR	Code of Federal Regulations
CLL	Chronic Lymphocytic Leukaemia
CM	Culture Medium
CMIA	Chemiluminescent Microparticle Immunoassay
CMV	Cytomegalovirus
CRS	Cytokine Release Syndrome
CSF	Cerebrospinal Fluid
CV	Coefficient of variation
DLBCL	Diffuse Large B-cell Lymphoma
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EBV	Epstein–Barr virus
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EOS	Eosinophils
FCM	Fluorescence-Activated Cell Sorting
FBS	Foetal Bovine Serum
FCS	Foetal Calf Serum
FDA	Food and Drug Administration
FIO	For Information Only
FITC	Fluorescein Isothiocyanate
FMO	Fluorescence Minus One
FRET	Förster Resonance Energy Transfer
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
HCT	Haematocrit Level
HLA	Human Leukocyte Antigens

HTA	Human Tissue Authority
ICH	The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IND	Investigational New Drug
IVD	In Vitro Diagnostic
LDH	Lactate Dehydrogenase
LLOQ	Lower Limit of Quantification
LOD	Limit of Detection
LXM	Lymphocytes
MHC	Major Histocompatibility Complex
MNC	Mononuclear Cell
NAT	Nucleic Acid Testing
NCR	Natural Cytotoxicity Receptors
NEU	Neutrophils
NGS	Next Generation Sequencing
NK	Natural Killer
OCR	Oxygen Consumption Rate
OOS	Out of Specification
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
PLT	Platelets
PMA	Phorbol 12-myristate 13-acetate
iPSC	Induced Pluripotent Stem Cells
QC	Quality Control
RBC	Red Blood Cells
RLU	Relative Light Unit
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
SBT	Sequencing Based Typing
SOT	Solid Organ Transplant
SRC	Spare Respiratory Capacity
SSOP	Sequence Specific Oligonucleotide Probe
SSP	Sequence Specific Primer
TAA	Tumour Associated Antigens
TCR	T Cell Receptor
TNC	Total Nucleated Cell Count
ULOQ	Upper Limit of Quantification
USP	United States Pharmacopoeia
VCN	Vector Copy Number
WBC	White Blood Cells

1. Introduction

The Cell and Gene Therapy (CGT) industry continues to grow rapidly worldwide, with increasingly sophisticated scientific discoveries being translated into therapies. There are a variety of challenges in characterizing these experimental living medicines. Any CGT product must be characterized in terms of identity, purity and potency and the choice of, and route to, validation of these assays largely lies with the developer and manufacturer. Establishing robust potency assays grows in importance throughout the development of a CGT product and becomes critical in the later clinical stages. As products move towards pivotal clinical trials and licensure, establishing the mechanism of action of the product becomes critical. This requires the potency assays to yield rich data which informs the interpretation of the outcomes in vivo, whether in models or in early human trials.

The flexible nature of assay development has led to variability in the sets of assays designed to capture key product characteristics. This is driven by a variety of factors at different developmental stages. These include speed to initial clinical trial; the complexity of the product; and the ability to qualify and validate the assays used. These factors can drive developers to use the simplest assays, however, the potency of any cellular product is likely to be multi-factorial and may require several assays to compile the appropriate data. In this guidance we concentrate on potency assays for T cell and NK cell CGT products. This encompasses antigen-specific T cells, genetically modified T cells, such as CAR-T cells and NK cells. One factor which may slow the adoption of multiple and complex assays may be confusion between the requirement to qualify and/or validate the potency assays. Qualification is a function of good laboratory practice (GLP) and ensures that the assay and equipment is suitable and effective within the context of the intended purpose. Validation in this context goes much further than the reliable performance of the assay but requires more extensive data collection and correlation with intended outcomes for the use of the product *inter alia.* In this guidance we aim to capture the elements of qualification and validation which are currently in use for the different assays to guide the reader to what might be most suitable for their own products.

2. Assays Common to NK and T cell

2.1 Minimum data set T cells

The human immune system is comprised to two main types of T cells, CD4+ T cells and CD8+ T cells, both of which are also CD3+. They are a type of leukocyte that originate in the bone marrow and mature in the thymus. In the thymus, the T cells replicate and differentiate into various subtypes, mainly helper and regulatory which are majority CD4+ and cytotoxic and memory which are usually CD8+. There are various subtypes of CD4+ T helper cells including: Th1 – responsible for the inflammatory response, key for defence against intracellular bacteria, viruses, and cancer, Th2 – vital for extracellular pathogens, Th17 – involved in gut pathogens and mucosal barriers, T follicular helper (Tfh) – coordinate with B cells in the adaptive immune response, Th22 – usually involved with anti-inflammatory responses. CD8 T cells have also been categorised into the following: T naïve (Tn), T stem cell memory (Tscm), T central memory (Tcm), T effector memory (Tem) and T effector (Teff). Memory CD4 T cells have also been associated with a variety of phenotypes (Figure 1). Tn have the ability to differentiate into any of the other subtypes and traffic across the lymphoid tissues. Tscm can differentiate and self-renew, Tcm's can persist long term within tissues but have limited cytotoxic function and have a preference to traffic to secondary lymphoid tissues. Tem cells exhibit cytolytic activity and express chemokine receptors and adhesion molecules necessary for trafficking to peripheral tissues. Tem usually terminates into Teff. Teff are responsible for vital anti-viral and tumour activities but are terminally differentiated and usually suffer from exhaustion after

prolonged antigen stimulation and hence have limited *in vivo* expansion and persistence capabilities.

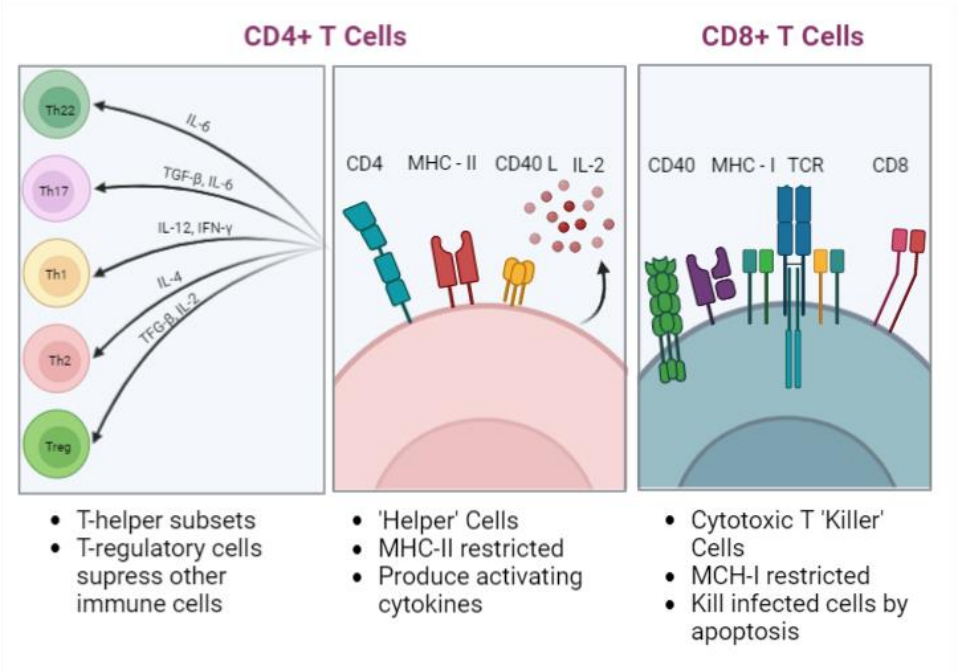

Figure 1. CD4 and CD8 T Cells

Genetically modified (GM) T cells, including CAR T cells, allogenic T cells and T cell receptor T cells (TCR T cells) have demonstrated significant advances in the treatment of malignant tumours. In the scope of this report, it can be assumed that most characterisation assays can be used for both types of T cell treatments, unless otherwise stated.

T cell characterisation is vital prior to its use as a therapeutic product (Figure 2). The most used assays are as follows:

- Flow analysis for surface and intracellular marker expression
- Viability
- Gene expression
- Functional assays
- ELISpot
- ELISA
- ICS
- Cytotoxicity
- Proliferation/Suppression
- Assessment of transduction efficiency and VCN for GM T cells
- Monitoring of GM T-cell persistence following administration

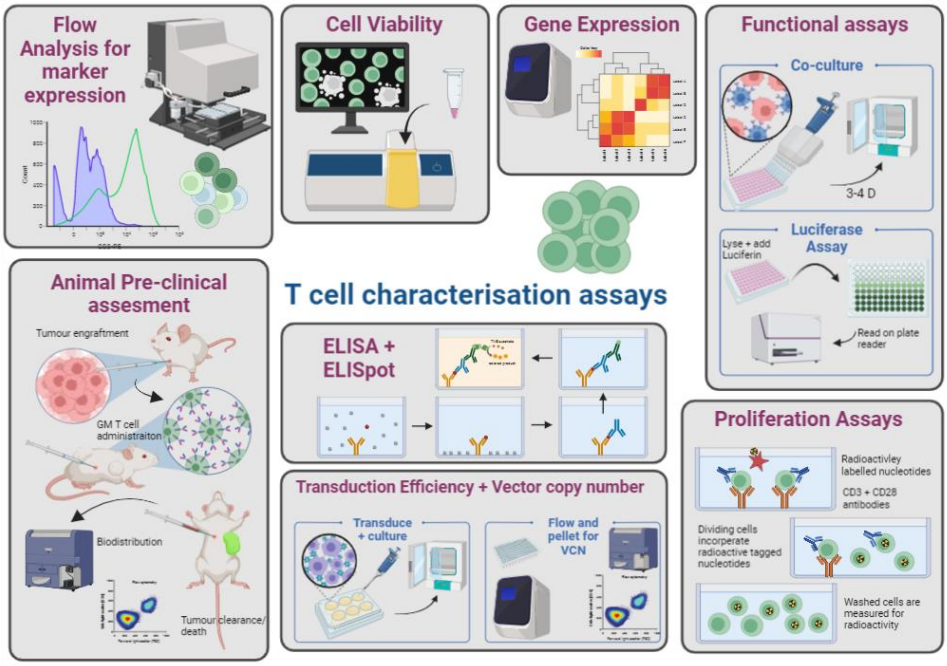

Figure 2. T Cell Characterisation Methods

2.2 Flow Cytometry

The application of flow cytometry is imperative for characterising the T cell product. It allows the detection of specific surface receptors and intracellular markers that help define the T cell product. It is used to calculate the proportion of T cells that express the CAR or the specific T cell receptor, in addition to the proportion of CD8/CD4 T cells and other non-T cells that could be regarded as contaminants. In more recent years, identifying the specific subtypes of the T cells, especially the CD8+ T cells has become more prominent as increasing number of studies are associating early memory phenotype T cells with better clinical outcomes. There is no consensus on the surface makers used to identify each of the subtypes, but the most used markers are given in table 1.

Marker	Tn	Tscm	Tcm	Tem	Teff
CD45RA	+	+	-	-	+
CD45RO	-	+	+	+	-
CCR7	+	+	+	-	-
CD62L	+	+	+	-	-
CD27	+	+	+	+/-	-
CD95	-	+	+	+	+
CD28	+	+	+	+/-	-
CD57	-	-	-	+	+

Table 1 Common surface markers used to identify cell subtype

The same process is used to identify the various CD4 subtypes and other cells that may be present in the culture, such as NK cells, macrophages, and B cells. Flow cytometry also allows gating to assess the proportion of the CAR positive T cells that are also CD8 T cells with cytotoxic markers. This could be used to assess clinical effectiveness.

2.3 Viability

The absolute number of live cells in the T cell product after the generation process is an important factor. Some T cell processes may take 14-28 days before the product is ready for

infusion into the patient. As such, assessing cell viability at different stages of the process is important. Viability assays contain a specially designed reagent that determines viability based on cellular membrane integrity (membrane integrity dyes), cellular function such as enzymatic activity (enzyme activity substrates), or metabolic activity (metabolic activity reagents) and can be measured using fluorescence microscopy, flow cytometry and microplate readers.

2.4 Functional assays

One of the most important characteristics of a T cell product that should be assessed is cytotoxic function; the ability of the T cells to specifically target and kill the tumour cells. This is commonly carried out by measuring the activation of the T cells in response to antigen stimulation through the upregulation of various surface markers (CD69, CD25) using flow cytometry and the release of key cytokines such as IFNγ, IL2, or Granzyme B. This can be done using ELISA and ELISpot (neither identify the phenotype of cytokine-producing cells) or intracellular cytokine staining and cytokine bead arrays, both of which are flow cytometry-based methods. Proliferation of T cells post antigen stimulation is also assessed using internal cellular dyes and tracked using flow cytometry. In addition, the gold standard of assessing function are *in vitro* cytotoxic assays which involve co-culturing the T cells with the target tumour cells that express the specific antigen. The extent of killing of the tumour cells can be measured using a range of methods that assess loss of membrane integrity such as MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) uptake, chromium release, lactate dehydrogenase activity and luciferase activity (which requires a luciferase expressing tumour target cell line).

2.5 Gene expression

Transcriptomic analysis allows a more detailed characterisation of the T cell product but may not be a requirement when submitting licensing application/variations to regulators. Gene expression assessment could be useful for process changes and demonstrating a thorough analysis of the novel T cell product compared to the original process. Single cell RNA sequencing has been used to define T cell clonality at the stage of identification of specific transcriptomic pathways associated with CAR T cell function.[1] Other analyses identified important transcription factors (TOX, TOX2 and NR4A) involved in regulating T cell exhaustion which could be modified to help improve effector function of the T cell product.[2] Another study confirmed that if c-Jun, an AP-1 family transcription factor associated with productive T cell activation, is overexpressed, the GM T cells become resistant to exhaustion and hence have better longer lasting cytotoxic function both *in vitro* and *in vivo*.[3] Taken together, gene expression analysis of highly functioning GM T cells could lead to the identification of key factors that could be used in developing a more effective GM T cell product. Gene expression analysis is also used to confirm that the GM T cells are what they are suggested to be from other forms of characterisation, such as flow cytometry.

2.6 Assessment of transduction efficiency and VCN for GM T cells

Flow cytometry can be used to stain for the CAR or TCR to assess the transduction efficiency of the process. This quantitates the amount of CAR/TCR that is expressed on the cell surface. A specific antibody should be used when detecting the CAR/TCR, however a single-chain fragment variable can also be used. Single-chain fragment variables tend to have higher non-specific staining / binding and may require additional staining optimisation. In addition to measuring the level of transduction using the quantification of the surface expression of the receptor, vector copy number (VCN) is also used to measure the average number a vector has been integrated within each cell, which is also used to assess the risk of insertional mutagenesis. Viral integration into the host genome poses a risk of altering the normal expression of cellular genes and should be addressed as a safety parameter. Both the ability

for the vector to enter and integrate into the cell and the extent at which it is expressed and presented on the cell surface are part of the transduction process. VCN is usually measured using quantifiable polymerase chain reaction using primers to detect the transgene and a housekeeping gene which is used to normalise the number of transgene copies per cell. Using the percentage of cells that express the transgene, the VCN per CAR/TCR expressing cell can be calculated. VCN as a function of CAR-expressing cells will provide a more accurate representation of the VCN in transduced cells, thereby presenting a more precise representation of product risk for insertional mutagenesis.

2.7 Monitoring of GM T-cell persistence following administration

Once the GM T cell product has been introduced into the patient, it is common practice to assess the amount of the GM T cells over prolonged periods of time. This is usually done by drawing blood or collecting tissue samples, isolating the T cells, and subsequently staining them for the CAR or TCR to identify those that have been GM. VCN as described above could also be used to identify GM T cells from blood or tissue.

2.8 Challenges of common assays

One of the biggest challenges across the field is the limited ability to compare data sets, different laboratories use varying criteria to characterise the GM T cell product. Not all studies investigate the varying phenotype of their product, and when phenotypic analysis is carried out there are discrepancies between the markers used to identify each subtype. CD45RA and CD45RO are used interchangeably however they do not follow a similar expression pattern in differentiating T cells, CCR7 and CD62L are also used, however CCR7 has been shown to be variable in certain cases. The methodology for staining is also different between studies which may result in an over representation of one marker compared to another study. Controls and gating strategies used for the identification of each population may differ. It should be noted that extra consideration should be taken when comparing studies and drawing out conclusions between the T cell phenotype and the correlated outcome.

Similar concerns are found with the *in vitro* functional assays carried out. The variability between assays here is even greater due to the number of factors that differ. These include:

- Tumour target cells.
- Level of antigen expression within the target cells.
- Other cell subtypes included in the assays such as CD4 T cells which affect both CD8 T cell function and exhibit individual cytotoxic function.
- Controls used and the choice and read out of the assay itself.

Different tumour cell lines express the TAA at varying levels which will in turn affect the extent of activation of the CAR T cells and hence subsequent function. The same is true for not assessing or normalising to the amount of the TAA expression on the same tumour target cells. Contaminating subtypes of cells within the assay can skew data as NK cells, CD4 T cells and macrophages can all affect the viability of the target cell. The function observed may not be solely due to the GM T cells and they can also alter the GM T cell function itself. The controls used in an assay are also imperative, whether it is using untransduced T cells to show background non-specific killing or using a target cell line with low or no expression of TAA to confirm the specifics of the GM T cell killing. The length of time of the assays and the effector target ratio (E:T) are also factors to be considered as having a high E:T ratio could bias the positive results, similar with extending the duration of the co-cultures. Additionally, the sensitivity of detection of viability or cell death varies between the assays; some assays based on CD107a expression for cytotoxicity versus others that use membrane integrity such as MTT or mitochondrial fitness to assess viability. Limited studies have carried out side by side

comparisons of these assays, therefore care should be taken when comparing the functional extent of GM T cell products.

There are currently no standardisation protocols for the starting material used with regards to product characterisation and use in clinic. Donor variability results in additional complexity when comparing studies, as the T cells and other cell subpopulations may have varying effects on transduction efficiency and cytotoxic function on the GM T cell product. Further investigation is required to fully understand the role of the other cell types and whether an additional purification step is required for final stage use of the GM T cells.

Despite the significant advances in developing animal models to investigate the *in vivo* effects of a GM T cell product there are still several challenges to be overcome. The fundamental issue is the mouse models may not accurately replicate what occurs within humans, for example, mice are unable to mimic CRS which is one of the largest concerns with the use of GM T cell products in the clinic. Mouse CAR T cells also have a lower length of persistence compared to human CAR T cells and are also more prone to activation induced cell death.[4] Human xenograft mouse models though they allow for the assessment of human CAR T cells against human tumour cells, it is limited in understanding the role of or effect on other immune cell types during the process in addition to the effects the CAR T cells have on healthy human tissues. In a case where the addition of various cytokines were used to augment CAR T cell function, the cytokine effect on bystander cells such as NK cells would not be observed and may have a negative effect when used in humans.[5] In addition to the high cost of running non-human primate studies, the lack of tumour cells in the animals and the small numbers used per group in a study limits the possible range of responses that would be observed in human studies.

These are all considerations to be taken when carrying out functional and characterisation studies of GM T cell products and when analysing and comparing data between studies.

3. NK cells as immunotherapeutic ATMPs

3.1 Introduction

Since the initial discovery of natural killer (NK) cells in the mid-1970s, their ability to target and lyse “abnormal” cells without prior sensitisation has been recognised and, increasingly, valued therapeutically. Our understanding of human NK cell biology has advanced dramatically over the past 30 years, particularly as we have understood the fundamental differences between murine and human NK cells.

In humans, NK cells represent approximately 5-15% of circulating lymphocytes. They are considerably fewer in number than T cells, but their lack of clonal restriction means that, potentially, every NK cell can recognise a tumour or infected cell in contrast to the very rare clonally reactive T cell. NK cells have a complex array of activating receptors which bind to cell surface antigens upregulated by stress signals such as viral infection or malignant transformation. These are termed “natural cytotoxicity receptors” (NCRs) and their ligands include heat shock proteins such as MICA/B and ULBPs. In addition, most human NK cells express the receptor for IgG Fc, CD16, which triggers lysis in an antibody-dependent manner. Unlike T cells, NK cells also express inhibitory ligands, and the outcome of an interaction with a tumour or virally infected cell is determined by the balance of inhibitory and activating signals. It is important to note that signalling via CD16 overcomes all inhibitory signalling. Manipulation of this balance of signals in favour of activation is central to most NK immunotherapies.

The first inhibitory ligands identified on NK cells were receptors for MHC. These fall into two families, the immunoglobulin-like receptors known as Killer Immunoglobulin-like Receptors (KIRs) and the C-type lectin receptor, NKG2A. KIRs bind to MHC class I ligands while NKG2A binds to HLA-E. Expression of relevant MHC Class I and or HLA-E at sufficient density on a cell inhibits NK cell activation and thus increases the threshold of activating signals required to trigger NK cell mediated lysis or cytokine secretion.

Downregulation of cell surface MHC expression is common among virally infected and cancer cells, thus increasing their likelihood of activating resting NK cells but lack of MHC expression alone is not adequate to trigger NK cell activity; the NK cell must receive adequate activating signals as well. NK killing of the MHC class I deficient cell line K562 is dependent upon activating signals through CD2 on the NK cell; blockade of CD2 on the NK cell or of its ligand, CD15, on the K562 cell abrogated lysis despite the absence of MHC class I on the target cell. Blockade of NK inhibition in the absence of NK activation is unable to trigger NK cell-mediated lysis. Furthermore it has been shown that resting NK cells require at least two activating signals on an MHC class I deficient cell to initiate lysis or cytokine secretion.[6] Thus, for the purposes of immunotherapy, we can consider resting NK cells as requiring multiple activating signals to exceed a threshold whilst recognising that the level of the threshold for activation is raised by the presence of inhibitory signals.

The earliest trials of NK immunotherapy used ex-vivo activated autologous NK cells in conjunction with IL-2 infusions and, latterly as a consolidation for high dose chemotherapy, with autologous peripheral blood stem cell transplantation. Infusions of activated NK cells were generally well tolerated across all these trials in haematological malignancies and solid tumours, although cytokine release syndrome associated with the concomitant IL-2 was a recurring problem. None of the trials showed significant clinical benefit and this was attributed to the innate inhibition of autologous NK cells by low level MHC expression on the cancer cells.

An obvious solution to target cell mediated NK inhibition through MHC expression was the use of allogeneic NK cells which lack inhibitory ligands for the patient's MHC haplotype. Individual inhibitory KIRs are restricted to specific HLA alleles, and it is usually possible to find a haploidentical relative to a patient or an unrelated volunteer donor who lacks multiple HLA ligands for the inhibitory KIR expressed on the therapeutic NK cells. Whilst this won't abrogate all target cell mediated inhibition, it does lower the threshold of activation signals required to initiate lysis. Since the early 2000s, most NK cell immunotherapy trials have used allogeneic NK cells; either from related donors or unrelated cord blood/adult blood/iPSC donations.

Like autologous NK immunotherapies, allogeneic NK cells, even when activated, have been very well tolerated with very few adverse events reported. Unlike allogeneic T cells, NK cells do not mediate graft versus-host disease (GvHD) and do not secrete inflammatory cytokines at concentrations associated with cytokine release syndrome. Whilst adoptive NK cells don't engraft and have short survival times *in vivo*, equally they seem not to invoke an allogeneic rejection response either. This has led to the current enthusiasm for allogeneic NK cells to be used as off-the-shelf treatments for patients, irrespective of HLA-matching.

Allogeneic NK cell immunotherapies are now in widespread development, both unmodified and as genetically modified cells carrying CARs or other potentiating moieties. Multiple sources are being used as starting materials including volunteer adult donor peripheral blood or apheresates, umbilical cord blood haematopoietic stem cells, umbilical cord blood NK cells and iPSC-derived lymphoid progenitor cells.

The manufacturing processes involved differ substantially depending upon starting material and hence in process QC requirements differ too. At one extreme, the donor is a HLA-mismatched related or unrelated volunteer donor, specific to the patient and a single batch run manufactures a drug product for a single patient, with little or no ex-vivo expansion. NK cell products derived from non-directed allogeneic donor blood or apheresates or cord blood starting material may produce several tens of doses after the expansion phase in manufacture but there is a limit to the degree of expansion without loss of function. At the other end of the spectrum are iPSC-derived NK cells where a master cell bank of clonal iPSC is derived from a human donor which are then expanded and differentiated to CD34+ iPSC-derived haematopoietic progenitor cells which are banked. Seed stocks from the iPSC-HPC are then used to manufacture batches of NK cells by further ex-vivo differentiation/expansion. Genetic modification of each of these allogeneic NK cell types is common and the point at which the gene insertion or deletion occurs is dependent upon the developer.

3.2 Minimum data set for identification of NK Cells

3.2.1 Identity

Human NK cells are generally defined as CD56+ve and CD3-ve as determined by flow cytometry. This combination of two well characterised surface molecules for which there are a host of suitable qualified monoclonal antibodies provides a reliable and robust method for identification and enumeration. In vitro diagnostic (IVD) grade reagents are available for both anti-CD3 and anti-CD56 from multiple suppliers and should be sued where possible. Human cells with “NK properties” are described in the literature which lack CD56, but these are rare and are not in development as NK cell immunotherapies. CD56+ cells which co-express CD3 are termed NKT cells and recognise target cells through different receptors. They share some functions with NK cells but are substantially different and are not in such widespread development for immunotherapy at present.

Human NK cells are often considered as two major subsets based on the intensity of expression of CD56. High expression of CD56, so called $CD56^{bright}$ NK are poorly cytotoxic in vitro and are associated with secretion of cytokines such as interferon gamma and tumour necrosis factor. The second, $CD56^{dim}$, subset contains the cytotoxic NK cells and is the larger of the two groups in normal peripheral blood and these cells are predominantly CD16+ve. CD16 is the common FcGamma receptor and mediates antibody dependent cellular cytotoxicity (ADCC). Activation of NK cells through NCRs leads to synthesis and secretion of a matrix metalloprotease, ADAM17, which cleaves CD16 from the surface and prevents inadvertent ADCC. This shedding of CD16 is one of the earliest markers of NK cell activation and may be used to distinguish activated NK which are capable of lytic function from exhausted NK which have killed target cells and can no longer function. It is important to note that the presence of “CD16-ve” NK cells is not necessarily an indicator of NK cell dysfunction; it could reflect the presence of activated NK cells, and this is important when phenotyping NK cell products which may have been expanded or activated during manufacture.

Natural cytotoxicity receptors	**CD**	**Known ligand**
2B4	CD244	CD48
α-integrin		vascular endothelial growth factor
CD2	CD2	CD15 & CD58
CD94/NKG2C		HLA-E
DNAM1	CD266	nectin2 (CD112), PVR (CD155)

FcyRIII	CD16	IgG
NKp30	CD337	B7-H6, BHG6/ BAT3, galectin
NKp44	CD336	MLL5-Nidogen-1, PDGF-DD, PCNA
NKp46	CD335	viral HA and HN, properdin
NKG2D	CD314	MICA, MICB, ULBPs
TLR3/9		microbial constituents, CpGs

Killer Immunoglobulin-like activating receptors	**CD**	**Known ligand**
KIR3DS2		HLA-C C1, HLA-A* 11:01
KIR2DS4		HLA-F, HLA-C, HLA-A* 11
KIR2DS5		HLA-C
KIR3DS1	CD158b	HLA-C2
KIR2DL4	CD158d	HLA-G

Inhibitory receptors	**CD**	**Known ligand**
ILT2/LIR-1	CD85J	HLA-G
KIR2DL1, DL2, DL3	CD158a,b	HLA-C, HLA-B
KIR3DL1, DL2	CD158e,k	HLA-A, -B or -F
LAG-3		MHC class II
LAIR-1	CD305	collagen
NKG2A	CD159a/ CD94	HLA-E
PD-1	CD279	PD-L1, -L2, CD273
Siglec 7	CD328	ganglioside DSGb5
TIGIT		PVR (CD155, CD274), nectin2 (CD112), nectin4, CD113
TIM3		galectin-9, HMGB1, CEACAM1

Table 2 Receptors and their known ligands

When characterising NK cells, it is helpful to breakdown the identity markers with respect to function such as activation, inhibition, maturation, chemokine receptors and checkpoints. See Figures 3 and 4.

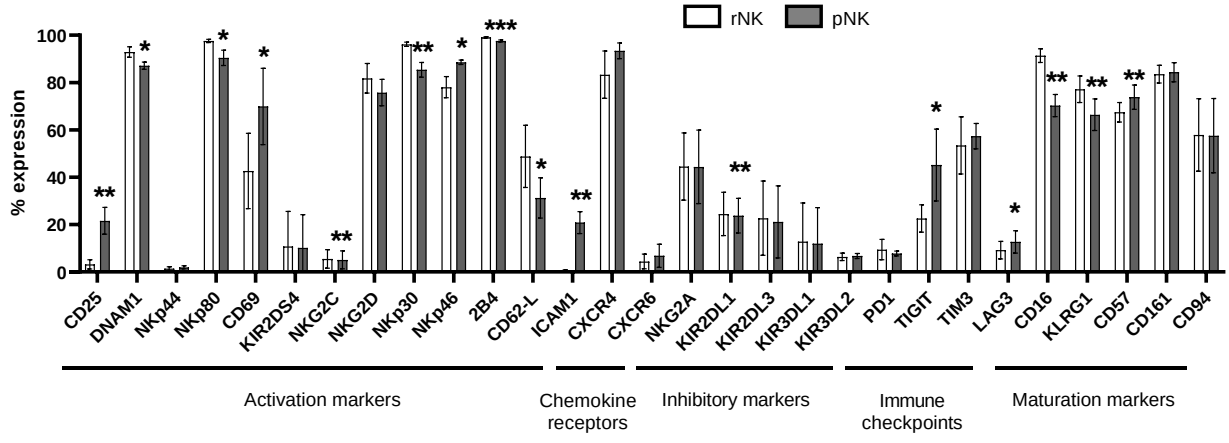

Figure 3. A typical comparison of resting human NK (rNK) cells directly analysed from adult peripheral blood compared to the same cells after overnight priming (pNK)

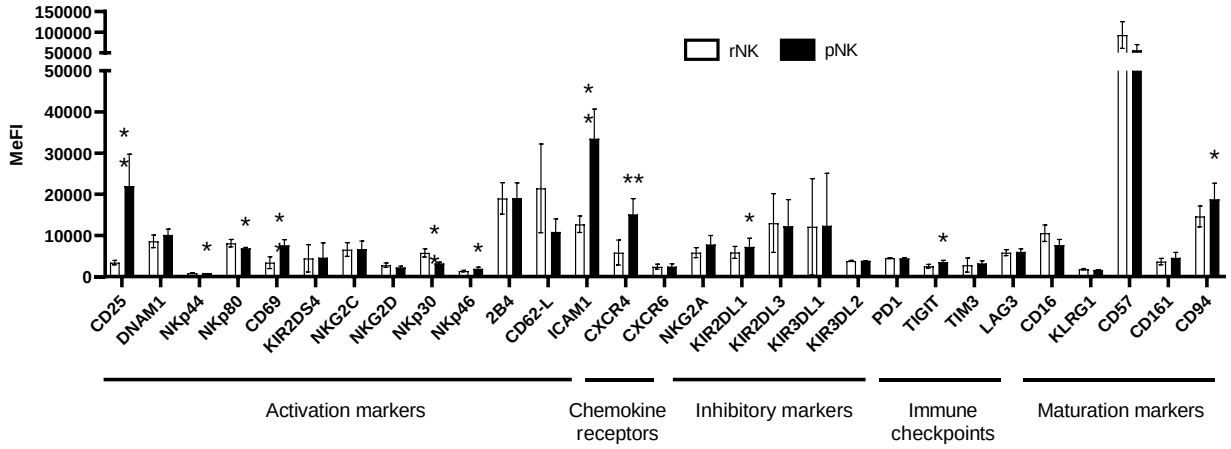

Figure 4. It is important to note that the intensity of expression of markers may also be important in defining a drug product compared to the starting material of even the drug substance stage and here, the relative fluorescence intensity (median channel fluorescence or absolute number of molecules) is the relevant analyte.

Immature NK cells – The intensity of expression of CD56 has been used to define the maturation state of NK cells but, in the context of allogeneic immunotherapy products derived from iPSC or cord blood, a better marker combination uses CD11b and CD27. Immature NK cells are CD27+/CD11b-ve and transition through a dual positive stage to a mature phenotype of CD56+/CD11b+/CD3-ve/CD27-ve.

Mature NK – These cells lack CD27 and will express one or more inhibitory markers from the panel above. CD57 has been used to identify terminally differentiated cytotoxic NK cells but it remains expressed after cytolytic exhaustion so is an unreliable marker for potential function. NK cells can also be characterised based on their expression of NCRs, but these do not identify different subsets, rather the target ligands which can activate them. Over 80% of peripheral blood NK cells express NKG2D which is the C-type lectin receptor for MICA/B and the ULBPs. This is the predominant activating ligand for tumour cell lysis. It is expressed as a homodimer in contrast to the other NKG2 receptors which each dimerise with CD94. NKG2A, C and E dimerise with CD94 and bind to HLA-E. NKG2A contains an intracellular ITIM and inhibits NK function whereas both NKG2C and E are associated with DAP12 and function as activating ligands. NKG2C is of relevance as it appears to identify a functional state of NK cells termed “memory like” or “adaptive” and these cells appear to have greater lytic activity to tumour cells and be resistant to KIR-induced suppression.

Activated NK cells – The earliest surface marker of NK cell activation is the shedding of CD16 following release of ADAM17. The reason for this shedding is unclear although it results in an NK cell which can no longer be triggered by antibody binding and is thus committed to function via NCR ligation. NK cells evolved prior to the adaptive immune response and NK cells isolated from invertebrates, which lack adaptive immunity, do not express CD16. The intracellular domain of CD16 in human NK cells is tightly linked to CD3z and it may be that the shedding of CD16 is important since it releases intracellular CD3z to bind to NK cell co-stimulatory molecule like CD2 which require CD3z for downstream signalling.

Following CD16 shedding, NK cell activation leads to CD69 expression and CD25 upregulation. The role of CD69 remains unknown but CD25 is the intermediate affinity IL-2

receptor (IL2Ra) and its expression on NK cells increases their affinity for IL-2. This drives proliferation and cytolytic function in vitro and in vivo. NK cell activation also increases expression of adhesion molecules such as ICAM-1 although this is an unreliable marker of NK cell activation due to the heterogeneity of expression.

3.3 General methods for NK-cell characterisation

Flow analysis for surface marker expression and semi-quantitation and NK cell enumeration by single platform technology.

As with all flow cytometric analyses, the choice of fluorochrome and often choice of monoclonal antibody clone may affect the result, as will changing the cytometer which is used. These parameters must be considered when designing and qualifying assays for in-process controls and release criteria. Since there is no standard NK cell against which reagents can be assessed, a reproducible assay, typically with an intra-assay CV of <5% and an inter-assay CV of <15% is required.

The design of an immunophenotyping panel is always a trade-off of the antibodies and fluorochromes available but it is advised to start with the most weakly expressed molecules and those where a change in expression level is a critical attribute Aim to use the most fluorescently efficient conjugates for these. Typically, phycoerythrin or allophycocyanin and their derivatives show excellent signal to noise ratio compared to less efficient fluorochromes like FITC. Since the expression of CD56 is low on mature, functional NK cells, it is advisable to use a high efficiency fluorochrome such as PE or APC in any panel, see figure 5.

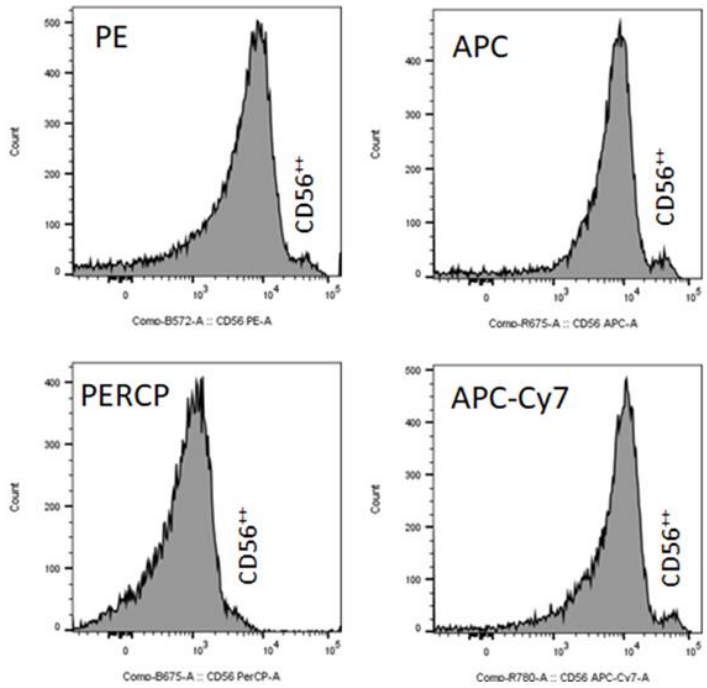

Figure 5. CD56 expressed in different fluorochromes.

In contrast, CD3 is always expressed at high density on T cells and NKT cells and its role in NK cell characterisation is as an exclusion marker. It is usually a good idea to assign the fluorochrome with the poorest signal to noise ratio to CD3 (e.g., FITC) or, due to the easy availability of conjugated antibodies to CD3, to assign the more esoteric fluorochromes (e.g., Pacific Blue) since they are unlikely to be available for the rarer NK cell markers. Once an

understanding of the S:N ratio of different fluorochromes is known, degrees of fluorescent overlap with other fluorescent channels you plan to use can be checked. Construct a checkerboard of dual markers to compare the results of each critical marker in the presence of others. When a working panel has been established, samples of cells with each marker singly can be labelled. Compare the “percent positive” and “median channel fluorescence” from the cells labelled with the single marker against the same cells labelled with all the other markers in the panel but lacking the marker of interest – known as fluorescence minus one (FMO). The FMO reagents should all test negative for the test marker each time. Finally, you can mix all the antibodies of interest and compare each analyte in the mixed panel with the result from the same analyte used alone.

For a typical procedure for enumeration and subset analysis of NK cells in peripheral blood and in NK cell drug product refer to appendix 1.

3.4 Functional assays

3.4.1 Cytokine secretion

Like all lymphocytes, cytokine secretion is an important function which is often very relevant to measure in the characterisation or donor NK cells and NK cells within a drug product. Cytokine secretion assays are not specific to NK cells and those described above for assessment of T cells and T cell subsets should be used for NK cell analyses. These include ELISpot, ELISA, Intra cytoplasmic cytokine section (ICS) by flow cytometry, Luminex and others.

NK cells can secrete a host of cytokines but the most relevant are: IFN-a, IFN-g, TNF-a, GM-CSF, IL1b, IL-8, IL-10, IL-13 as well as important chemokines such as MIP1-a, MIP1-b, RANTES, CCL1, CCL2, CCL3, CCL4, CCL5 and CXCL8. Plainly, with a specific research question in mind or a particular function required of an NK drug product, it is possible to limit the number of cytokines/chemokines to be analysed. In such cases, a simple ELISA or limited ICS can be used. For screening NK cell function, however, a broad-spectrum approach is a powerful and cost-effective option. In the example below, a 30-analyte broad-spectrum assay to measure the *in vivo* response to an NK cell activating agent is shown. The assay in figure 6 allowed the identification of the key nine cytokines which were upregulated out of the wide panel of 30.

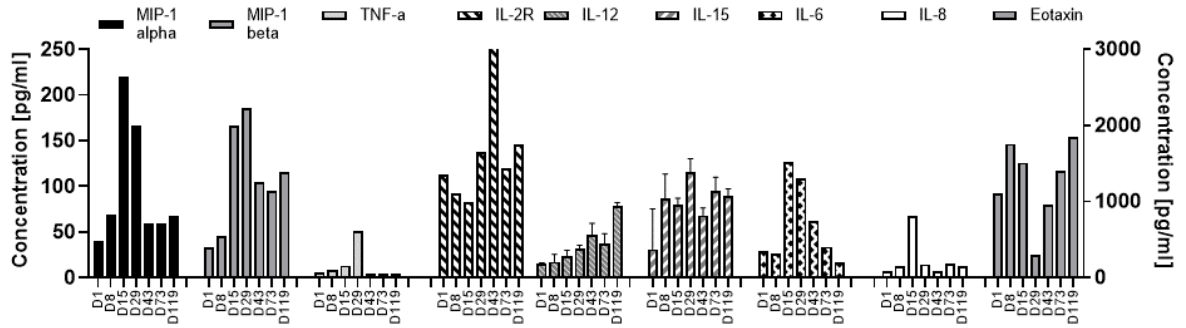

Figure 6. A 30-analyte broad-spectrum assay to measure the in vivo response to an NK cell activating agent is shown.

3.4.2 Cytotoxicity

Probably the most common and most important aspect of NK cell function is cytotoxicity. The traditional radiometric assay using ^{51}Cr is no longer in use given the hazards involved and the complexity of management of purchasing, storage, and disposal of radiochemical substance. This assay relied upon the loading of target cells with ^{51}Cr sodium chromate which was retained within live cells and only released into the supernatant as cells died. The assay required the measurement of "spontaneous release" of ^{51}Cr as measured by a gamma-counter from control cells which were not incubated with the effector NK cells, and "total release" from target cells lysed with water. Percent-specific lysis was calculated from the straight line plotted between "spontaneous" and "total". This assay is a measure of target cell membrane integrity and most current NK killing assays rely upon the same measurement in a non-radiometric test. Membrane integrity can be measured by egress of a naturally occurring cellular enzyme such as LDH or of a loaded reagent such as CFSE. Typically, egress assays use a plate-based spectrophotometer or spectrofluorometer and calculate the specific lysis using the same type of formula as conventional ^{51}Cr-release assays.

3.4.3 Challenges

NK cell biology and the clinical application of NK cells are fast moving fields with constant advances in NK cell subset definitions and the complexity of memory-like NK cell definitions. Conventional flow cytometric analyses are adequate at present with the condition that fluorochrome choice for each surface antigen is critical, and panels for identity and purity need very careful validation. The use of Cytof cytometers is increasing for in depth, highly multiparametric analysis of NK cells and other cell populations. The advantage of this technique is the specificity of mass spectrometry signal from each metal label which removes the risk of spectral overlap of conventional fluorometric cytometry. However, few reagents are available for NK phenotyping at present and thus conjugation must be performed in-house prior to these experiments which renders them difficult to qualify or validate. There are no IVD qualified reagents for Cytof cytometers so they must be regarded as a research tool at present, rather than potential tools for in-process and release QC testing.

Another challenge remains the choice of appropriate cytotoxicity assay and target cells. Flow cytometric assays in which the absolute number of remaining live cells is used as the indicator are robust, cheap, and reproducible. It is important to consider the best cell line to use as a target and to optimise the number of population doublings since thawing or splitting prior to the assay. Aim to split the target cells (or replate them if adherent) on the day before the planned assay, so that they are in exponential growth phase on the day of the assay. Assay standardisation remains difficult due to the need for a stable target cell line(s) and a control donor NK cell population as the internal assay control. Human NK cells do not recover well from cryopreservation, so a donor NK pool is difficult to maintain, but not impossible. Unlike T cells, NK cell function is not HLA-restricted and there is little evidence of NK:NK allorecognition, so pools of NK cells from multiple donors are possible. This presents a way to develop a standard control NK population for functional assays but, inevitably, requires a development strategy and a process to move to a new pooled donor standard prior to the complete depletion of the current donor pool.

One aspect which was not discussed above was the possible need to conduct functional assays in hypoxic conditions if studying NK responses to solid tumours since the tumour microenvironment is considerably more hypoxic than peripheral blood or conventional laboratory incubators with 5% CO_2. This may be something to be considered depending upon the product in development and its intended use.

4. Setting rational levels of cytotoxic function for product release and potential efficacy

4.1 T cells

Cytolytic activity is a major function that requires measurement through several assays to understand efficacy. Assays will be product and cell type (NK and T cell) dependent. This section will focus on killing assays: flow cytometry, chromium, and cytokine assays: secretion and intracellular FCM (e.g., memory cells) (FDA guidance – Potency Tests for Cellular and Gene Therapy Products). A sensitive and reproducible cytotoxicity assay that collectively reflects these functions is an essential requirement for translation of these cellular therapeutic agents.

4.2 Chromium (^{51}Cr) release cytotoxicity assay

Historically this assay was considered the gold standard for assessing cell-mediated cytotoxicity. It relies on passive internalisation and binding of ^{51}Cr by target cells from sodium chromate. Lysis of the target cells by the by effector killer cells leads to the release of the radioactive probe into the cell culture supernatant, which can be detected by a gamma-counter. However as more laboratories limit their use of radioactivity, there has been a switch to non-radioactive methods.

Cell-surface markers can be used to define cell subsets based on lineage and developmental stage, as well as function when they are labelled with fluorochrome-conjugated antibodies and analysed by flow cytometry. These surface markers have different forms and functions and include receptors for both soluble and cell-bound ligands, ion channels, glycoproteins, phospholipids and more. For example, CD4 is a surface marker for T helper cells that can be further differentiated based on expression of other chemokine receptors and cluster of differentiation (CD) markers. Live cells stained with antibodies can be sorted based on unique staining patterns.

Cytometry, in its purest form, is the measurement of cell characteristics, which can include cell size, cell count, cell cycle and more. This technique allows analysts to get highly specific information about individual cells. Some flow cytometry assays look at recovery rather than killing (note there are some FCM based assays that look at dead cells rather than recover). These assays work by placing a defined number of cells in a co-culture which have effector and target cells. How many cells survive the assay shows your recovery therefore indicating level of killing. Other assays look directly at killing by labelling target cells with a fluorescence dye (e.g., CFSE) and then co-culturing with effector cells at fixed ratios and after a fixed period of time (e.g., 4 hours) assaying cell death by the use of makers such as 7-AAD or propidium iodide.

Cytokine release assays which are performed for characterisation and release e.g., Ella is a widely used platform for the quantification of soluble biomarkers. It is a very robust platform with high precision, accuracy, and reproducibility. However, within the ATMP sector there are challenges with the use of cytokine release assays for drug product release. One to note is there is a degree of inherent variability within the cytokine release methods. It can be difficult to define which cytokine is linked to cytotoxicity (e.g., granzyme B, TNF alpha, ILT2, IFN gamma). This is due to cytokine release upon targeted or non-targeted cell death. Due to this inherent variability, large pools of data must be analysed to identify key cytokines. As part of the assay the fold increase background and positive control levels to identify the ratio between the two. Again, due to targeted and non-targeted cell death this can influence the fold increase observed. Flow Cytometry methods alone cannot be used to assess drug

product potency. However, assessing cellular phenotypes of the product can be useful. Some challenges for these methods are reviewed below.

4.3 Challenges setting rational levels of cytotoxic function

Several key challenges will be explored in this section for the killing and cytokine assays mentioned.

^{51}Cr method is limited to its semiquantitative nature and low sensitivity and is technically challenging in terms of repeated stimulation of effector cells, which might distort the actual behaviour of cells from their original state. Many laboratories are not able to use radioactivity for safety or licensing reasons which is why many labs have moved to FCM based assays

4.3.1 Interpretation of killing and cytokine assays

Flow cytometry analyses events (total number of events recorded). Number of washing steps that are performed as part of test method vary, this could remove cells and/or remove effector cells which in turn alter killing results. For example, if a 96 well plate had samples prepared 12 times per column, there is a potential to lose cells 12 times which could impact results across those well plates.

How the data is analysed for total number of events can be difficult. This is because there is no standardised way of interpreting the data. For example, if there is a target-only well and then a co-culture well, to calculate the killing you would calculate the difference between these two. However, another way is identifying if there is any killing and not quantifying this activity but reporting as present/absent. For example, if you measure CD19 CAR expression this can be either quantifiable or simple detection. This is a challenge characterisation phase.

4.3.2 Data analysis

Performing manual gating on samples. If a drug product is not very potent, the target and effector cells will look integrated. To better define those populations, it is good practice to have a gating guide with as many examples as possible to remove subjective interpretations between analysts. Population percentage is mainly looked at when investigating an Out of Specification (OOS) test result for flow cytometry.

4.3.3 Cytokine assay challenges

There is inherent variability with the test methods. It may be difficult to define which population is responsible for killing (e.g., granzyme B, TNF alpha, IL2, IFN gamma). Large pools of data would be needed. Interpret fold increase, background levels and test results.

4.3.4 FCM – population percentage

A challenge with killing assays is the specificity for target cells versus bi-standards. It is important that the assay measures specificity of the product, mainly for safety reasons, because if not specific, the product could kill everything. Therefore, it is important to have controls (i.e. the cells must be specific to reduce off target cells) in place to determine if the cytotoxicity is specific and therefore the assay must detect this. Killing assays must have specific targets and must identify non-known targets. For example, wild type (contains target antigen) and knock out (no antigen attached to the CAR) cells. The target is RAJI cell lines. The challenge is understanding how cell growth could impact results. For example, cell growth rate should be well balanced, there are difficulties if cell line growth is going too slow versus too fast. This must be considered during method development.

For the assays mentioned, use of materials is a key challenge. It is important to understand the quantity of cells used and whether they are truly representative of the entire product. This

is important due to *in vitro* versus *in vivo* representation. Is the assay able to show how close the effectors are to your targets within the body? Is the concentration within the body being represented as part of your test method? Controls used in a test method are important as they show the system has worked. A key challenge in the industry is if a positive control fails but the sample passes, is this considered a true failure? Therefore, several factors should be considered for the control used. Controls in place when manufactured to GMP, how were the controls vialled versus final product when they are frozen. It has been recognised there is no standardised control for these types of assays. At a minimum there should be a negative control (one that shouldn't kill/secrete cytokine and acts as baseline and a positive control that is known to kill/secrete cytokines). These controls may be transduced/untransduced cells or may be cell lines that acts as assays control only and do not reflect the manufacture process.

For cell-based assays accuracy should be determined. This is the most difficult requirement to cover because there may need to be qualified material which during development is not always available. A challenge with cytokine expression is defining the cytokine that is representative of the biologic response.

Suggesting best practice for the use and assessment of cytotoxic function as release criteria for ATIMPs is challenging as there is often limited material to assay. In addition, although cytotoxic function may corelate with clinical outcomes as most ATIMPs are not pure populations, it is always possible that a small number of uncharacterised cells are the ones that expand and provide clinical efficacy. It is important to separate analysis done for information and assay development from those done as part of in process testing that form stop/go criteria or product release testing. For in process testing and release testing, the criteria set should be those that indicate quality, safety, and likely efficacy. As the EMA Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials - Scientific guideline advises characterisation of the product during development should help establish appropriate assays to determine quality, safety and potency assays that should be relevant to the believed biological mechanism of action. *In vitro*, animal models and clinical trials corelate with human *in vivo* efficacy and if they are consistent with presumed mode of biological activity, can be used to analyse products. As the brief discussion below highlights, these assays are dependent on both the cell type (T or NK cells) and the antigen (tumour, viral, other) being targeted.

Different cells secrete diffident cytokines. The relevance of cytokines in activating the immune system is dependent on the target being recognised. There is a balance between cytokines produced that assist with tumour clearance and cytokines that can cause disease (e.g., cytokine release syndrome). In a recent review on CAR T cells[7] highlight that clinical response correlates with persistence of CAR T cells with a memory phenotype without exhaustion of the cells. The review also discussed that efficacy of cells may be dependent on cytokines and that the relevant cytokine(s) may be tumour/antigen dependent. For example, CAR T cells against solid tumours seem to be more efficacious if they express IL-17A, IFNg, IL-2, TNFa and IL-22. Another review[8] of NK-CAR T cells shows the importance of IL-15 in murine model systems and highlights that some of the new NK-CAR trials transduce NK cells with both the appropriate CAR construct and a construct expressing IL-15; this is primarily to support NK expansion but IL-15 may also have a direct clinical role too. NK cytokine secretion correlating with efficacy is different from T cells in that the efficacious NK cells secrete IFNg, IL-3 and GMCSF.[9] CAR T cells classically express high levels of IFNg, IL-6, IL-2 and TNFa. Modified a 2nd generation CAR's hinge region and showed decreased secretion of IFNg, IL-6, IL-2 and TNFa while being as effective as unmodified 2nd generation CAR at reducing tumour burden in a murine model.[10] This suggests that these cytokines may not be essential for effectiveness,

and it is known that IL-6 is a major inducer of cytokine release syndrome. IFNg expression is assayed in viral specific cells such as EBV CTLs as a measure of activity.[11] In addition, anti-viral CTLs may be selected because they express IFNg post antigen (e.g., adenoviral) stimulation.[12] Using literature and preclinical studies (in vitro and animal studies where appropriate), researchers should identify the cytokines most relevant to the ATIMP being manufactured (e.g., IFNg for anti-viral CTLs). Where cell banks or multiple doses of a product are made, particularly allogenic products where there is a risk of GvHD and the benefit of GvHL, performing assays to demonstrate the cytotoxicity/function of the cells should be undertaken. For bespoke autologous products this may not be possible as there may not be sufficient material; however, the risks are also lower for these products as there is no risk of GvHD.

Assays for information only (FIO) should be indicated as such. These assays are used to gather information and characterise the cells potentially being given and should be corelated with other in vitro assays and in vivo model studies. Ideally, they should be as comprehensive as possible to gather the most data. They may be run during trials but need to be clearly identified as FIO. The products will be released regardless of FIO results. If an assay and its results are essential for ensuring a safe, effective product then it must be a release criteria test and not FIO. Assays for in process testing or release, should be based on developmental studies and previous FIO to identify the most suitable cytokines/assays for measurements. These should be based on cell type (T or NK) are well as the antigen target (virus, solid tumour, blood cell e.g., CD19). They must also correlate with efficacy in vitro and in vivo model systems. They should be biologically plausible and reflect the believed mode of action of the cells. Process and release testing need to be completed in relevant time frames (in process testing in hours and release testing hours to days depending on whether it is a fresh or frozen product). Cytokine analysis may be possible in these time scales, however cytotoxic assays may not be possible. In addition, it's always possible for a small population of cells not detected by these assays to be the key functional component of the cell product, and thus for this cell type not to be assessed. Assays undertaken for release testing must be standardised so that they are reproducible and can be undertaken by any suitably trained individual. In summary, FIO and developmental assays help establish the appropriate in process and release testing which must be based upon cell type, target and believed mechanism of action.

4.4 NK cell cytotoxicity

An alternative measurement of membrane integrity is the ability to prevent ingress of a non-cell permeable "viability" dye such as 7-AAD or propidium iodide. These dye ingress assays typically use flow cytometry to analyse the degree of target cell killing. Both assays tend to measure percent lysis at a single point in time, typically after four hours. This works very well with dye egress assays as all the dye released from dead cells accumulates in the wells until analysis. With dye ingress assays, only the cells which remain intact can be analysed; cells which have died and broken up appear as debris in the flow cytometric plots. To overcome this, it is better to measure the "absolute number of live cells" in the control tubes and in the test tubes to calculate the percent specific lysis. Intra-assay CVs of <10% across triplicate samples can be achieved readily with these assays. There is no need to create a "total" lysis sample although a positive control using a known functional NK cell sample is very valuable and essential when you get to the stage of assay qualification or validation. A typical protocol for a flow cytometric dye ingress assay is detailed in the Annex.

An example of a typical flow cytometric cytotoxicity assay is shown in figure 7. The target cells are readily identified as the right-hand plot show the gated PKH-67+ target cells as FSC-H versus To-Pro expression and it is evident that the target cells are viable by dye exclusion.

The lower plots are the same but show the K562 target cells in the presence of NK cells at an E:T ratio of 5:1. The PKH-67+ target cells are readily apparent and, when plotted onto the FSC vs To-pro plot, show three populations: Live cells within the “live” gate and a population of FSC^{low}/$To\text{-}Pro^{wk}$ cells which are apoptotic and then a larger population of FSC^{low}/$To\text{-}Pro^{hi}$ which are late stage apoptosis/necrotic cells. Both samples contain the same number of PKH-67+ target cells but the K562 alone sample contains 48660 live K562 in contrast to the 5266 live K562 in the test sample. The specific lysis = $\left(\frac{48660-5266}{48660}\right) * 100 = 89.2\%$.

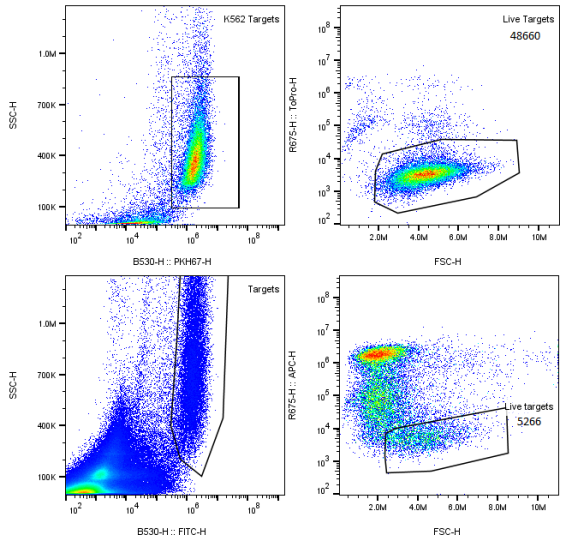

Figure 7. An example of a typical flow cytometric cytotoxicity assay. The upper two plots show K562 target cells alone after a four-hour incubation. The left-hand plot shows the PKH-67 fluorescent membrane dye expression on the x-axis (log) and the SSC-H on the y-axis (linear).

A popular alternative to measuring target cell lysis is the measurement of surface expression of LAMP-1 (CD107a) on NK cells after co-culture with target cells. LAMP-1 is normally present on the inner membrane of perforin-containing intracellular vesicles. As the NK cell releases perforin upon binding to a target cell, the intracellular membrane becomes incorporated into the NK cell membrane and LAMP-1 is apparent on the NK cell surface. The timing of LAMP-1 expression during the NK:target cell interaction is variable so it is essential that the anti-CD107a antibody is present within the co-culture during the whole period of analysis and, after 1 hour, you should add Monensin and return the cell suspension to the incubator for another three hours. Upon completion of the co-culture, the samples are labelled with anti-CD3 and anti-CD56 and analysed by flow cytometry to determine the percentage of CD3-/CD56+ NK cells which have upregulated LAMP-1 (CD107a) which is compared to that of NK cells incubated in the absence of target cells.

Whilst this is widely assumed to measure NK degranulation, it is known that NK cell activation with PMA/ionomycin or by membranes of tumour cells can induce CD107a expression without detectable release of granzymes or perforin so it may be better to regard this assay as a measure of NK cell activation.

A typical CD107a NK cell killing assay is shown in figure 8. The plots show the gating strategy of NK cells by morphology (FSC-H vs SSC-H) and then selection of live NK cells by exclusion of the viability dye, Zombie yellow. Finally, the NK cells are selected based on CD56 expression level, $CD56^{dim}$ in red and $CD56^{bright}$ in blue.

The lower series of plots shows the same NK cells after four hours of co-incubation with K562 target cells as an E:T ratio of 2:1. The same gating strategy excludes K562 and cell debris and shows significant increase in the frequency of CD107a+ NK cells (15.12%) and the restriction of this to the red CD56^{dim} subpopulation.

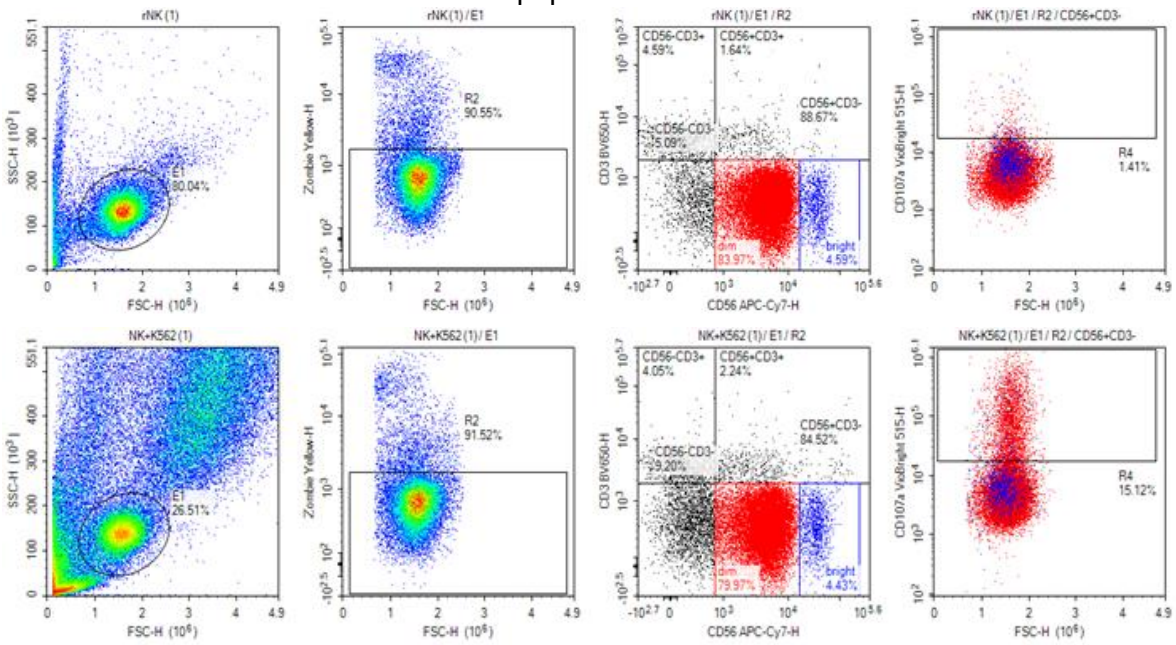

Figure 8. A typical CD107a NK cell killing assay. The upper panel shows resting NK cells express alone after four hours of incubation with a small number of CD107a+ve cells (1.41%). The final plot shows an overlay of the two NK subpopulations and the frequency of CD107a+ cells.

More recently, dynamic cell killing assays have become available. These devices use different technologies to measure cell killing and, unlike the assays described above, can measure target cell lysis over extended periods of time, during which the target cells are able to proliferate. This dynamic aspect to the assay can provide valuable data to help understand the relationship between NK cell killing and tumour growth kinetics.

In both systems, the target cells are loaded into cell culture plates and growth is established, typically for 24 hours. NK cells are added to wells at chosen E:T ratios and the plates are returned to the devices and incubated for up to 120 hours at 37˚C/5%CO_2. The data are acquired at predetermined time intervals; typically, every 15 minutes. Both devices can capture images of the co-cultures as well as enumerating the number of live cells, see figure 9 for an electrical impedance-based assay.

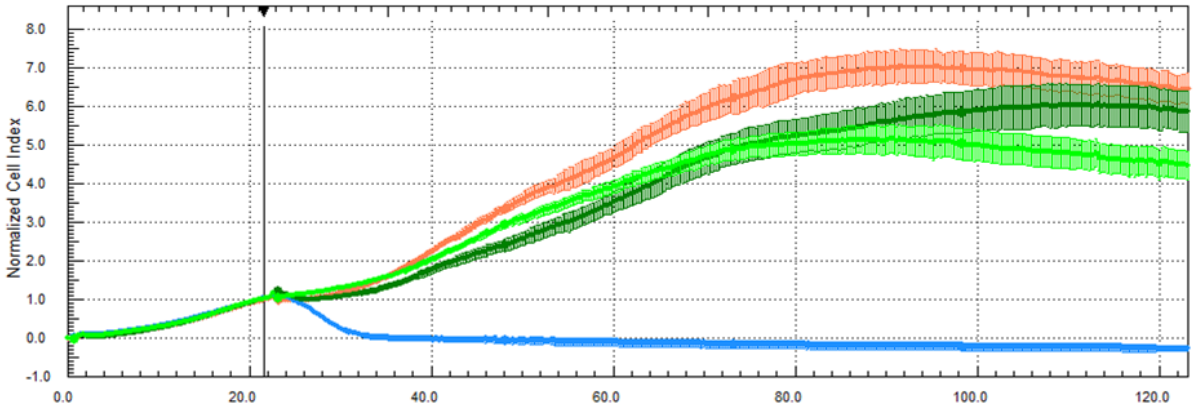

Figure 9. A typical cell index plot from an electrical impedance-based assay.

The target cells in this case were SKOV3 ovarian cancer cells and the graph shows the growth of the SKOV3 cells over the initial 24h prior to the addition of NK cells activated with IL15 as a positive control (blue line) and resting NK cells from the same donor (dark green) compared to NK cells activated with an experimental priming agent (light green). The SKOV3 target cells alone are shown in the orange line. The number of live cells in each well was recorded every 15 minutes and each was run in triplicate. The lines show the mean cell index at each timepoint and the standard deviation.

From figure 9, it can be deduced that IL15 activated NK cells from this donor were highly potent and induced rapid and total killing of SKOV3 within 12 hours. The resting NK cells induced some target cell killing between 60-100 hours whereas those treated with the experimental activating agent followed a similar early dynamic but retained the ability to lyse SKOV3 even at 120 hours of co-culture.

The absolute amount of target cell lysis in each condition is represented by the area under the curve (AUC) and this can be calculated to give a “relative degree of lysis”, see figure 10.

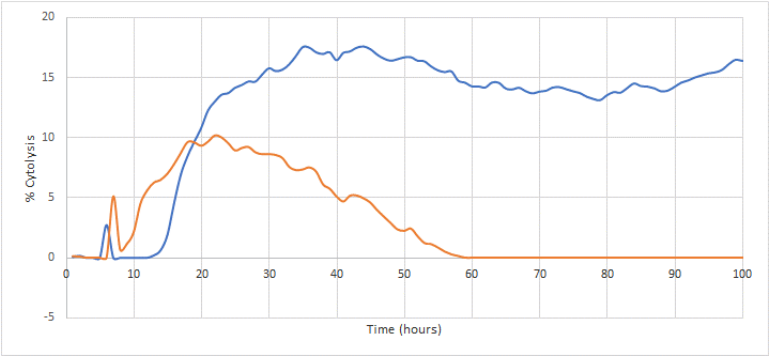

Figure 10. In this plot the resting NK lysis is shown in orange and the experimentally activated NK cells are represented by the blue line.

The use of “percent lysis” would be misleading as the duration of lytic effect is different in the two conditions. The area under the curve of the resting NK killing is 73.7 AU compared to 627 AU under the experimentally activated NK cells, i.e. a 750% increase in cytolysis.

4.4.1 Target cell conjugation and avidity

For NK cells to lyse a target cell it must first form an adequate immune synapse with the target cell. Although immune synapses are well documented, the specific nature of the molecules involved in stabilising the NK:target cell interaction are not fully known. The synapse certainly consists of combinations of activating and inhibitory ligands plus adhesion molecules and may include checkpoint inhibitors in some circumstances. the constituents of an NK:target cell synapse were first delineated in 2002[13] and subsequent groups have looked at specific constituents such as NKp46.[14] The NK-costimulatory molecule, CD2, is probably the best measure of the synapse formation since it parallels the reorganisation of filamentous actin which is critical for synapse formation.[15] The photomicrograph in figure 11 shows the capping of the CD2 molecules at the synapse with the tumour cell.

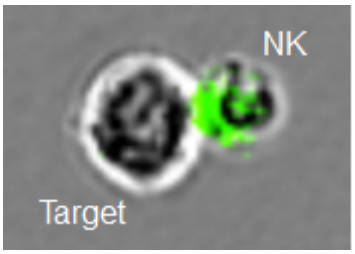

Figure 11. Photomicrograph shows the capping of the CD2 molecules at the synapse with the tumour cell.

The simplest and most widely used method to enumerate the frequency of NK:target cell conjugation is flow cytometric analysis of co-incubated NK and target cells where each is pre-labelled with a fluorescent membrane marker.

Since conjugation is an essential step in NK function, it may be possible to use an assay such as this to show increased conjugate formation to a standardised tumour cell line (e.g., K562 as “NK-sensitive” and RAJI as “NK resistant”). This can be used as a potency assay for NK cells expanded and activated during a manufacturing process.

The strength of the immune synapse is termed the “avidity” of the interaction and, recently, the Z-Movi™ from Lumicks has been shown to be a useful tool for the measurement of the strength of binding. The device consists of coated glass microscope slides which can bind target cells of choice, these can then be imaged by the device. NK cells are fluorescently labelled and added at concentrations of choice and co-incubated with the slide-bound effector cells for the desired duration. Two acoustic forces are then applied across the slide at 90^{o} angles. The amplitude of these forces is then slowly increased and, as it rises, they will disrupt the bound NK cells; the amount of force required being an indicator of the strength of the synapse, i.e. the avidity. The release of the NK cells is captured visually by the device and the percentage which remains bound as the force (measured in picojoules) increases is recorded and plotted.

One company is already using the Z-movi to screen suitable NK cell donor products for optimal avidity for subsequent generation of an allogeneic NK cell product and CART developers are using it to choose the optimal CAR construct so it is likely that this will migrate to selection of CARs for NK cells.

Z-movi can be used as a potency assay for activated NK cells by determining a “minimum percentage increase in avidity” required for an NK cell to gain the ability to lyse a previously resistant target cell. An example is shown below where the avidity of resting NK and NK cells

activated with IL2 (LAK-2) and IL-15 (LAK-15) to NK-resistant SKOV3 cells has been analysed. Resting NK cells bind very weakly to SKOV3; over 90% of NK:tumour cell conjugates are disrupted with 100 pN of force. IL-2 and IL-15 activated NK cells bind more avidly. The greatest differential appears to be around 200 pN. The right-hand graph in figure 12 shows the relationship between avidity at 200 pN and target cell lysis taken from a parallel flow cytometric killing assay.

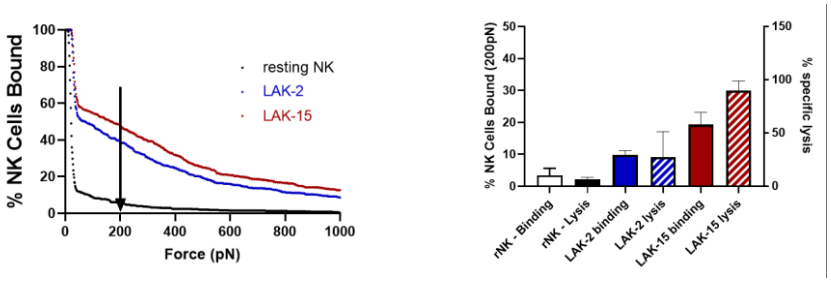

Figure 12. The relationship between avidity at 200 pN and target cell lysis taken from a parallel flow cytometric killing assay.

5. Common assays for manufacturing release and patient monitoring

5.1 QC testing

To facilitate product release and ensure patient safety, several assays must be carried out during the manufacturing process as well as on the final product. Typically, these are divided into safety assays/tests and functional assays/tests specific to the therapeutic product to define efficacy and quality; each of these two categories of assays/tests will be considered below.

5.1.1 Safety testing

Testing may be required at different points during manufacture to ensure product safety. The level of safety testing required will be driven by the level of risk identified during the risk assessment phase of product development and during engineering runs, aimed at fine-tuning the manufacturing process. Typical safety tests for autologous and allogeneic cell therapy products will include endotoxin, sterility, and mycoplasma. Additional safety tests are required where cells have undergone genetic modification. Below are some considerations when developing a testing schedule:

- Open processing vs closed processing – if open, consider additional sterility testing at critical points.
- Purchased reagents vs prepared in house – if prepared in house, additional sterility and endotoxin testing may be required before use.
- Length of culture period – as time in culture increases so does the risk of mycoplasma contamination.
- Final product presentation – fresh vs frozen; where the product must be administered as a fresh formulation, some tests, such as mycoplasma, which take between 4-6 weeks to complete, may not be carried out or will be carried out but reported retrospectively.

5.1.2 Sterility testing

Sterility testing for cell therapy products is essential for product release. Sterility testing should be carried out in line with Appendix XVI E. Microbiological Examination of Cell-based Preparations and Appendix XVI A. Test for Sterility.

5.1.3 Endotoxin testing

Endotoxin testing must be carried using validated methods, compliant with Appendix XIV C. Endotoxin testing may be carried out on reagents ahead of the manufacturing process, as well as on in-process sample though this is not a requirement. As with sterility testing, where volume is very limited and taking cells from the final product may have a detrimental impact on patient treatment, options such as using culture supernatant prior to final formulation can be risk assessed as either an alternative to final product or as an adjunct to a non-compliant volume.

5.1.4 Mycoplasma

Whilst not a common occurrence, testing for mycoplasma remains a key assay for manufacturing release of product. Mycoplasma testing should be carried out in compliance with Appendix XVI B. Microbiological Examination of Non-sterile Products section 3. The gold standard assays remain the culture method and indicator cell culture method, however, molecular methods are replacing these gold standard methods. Molecular methods must meet the same limit of detection as the current gold standard method – 10 CFU/ml. A same time turnaround using molecular methods is a significant improvement on the indicator cell method, which takes up to 28 days to complete and could therefore be a rate-limiting step in product release.

5.1.5 Virology

For donor/patient starting material, stringent virology testing will be required in line with. The HTA requires donors/patients to be tested for HIV 1 and 2, hepatitis B (HBsAg and anti-HBc), hepatitis C (anti-HCV-ab) and syphilis. Additional markers such as CMV, West Nile Virus and HTLV-I/II may be required depending on the donor/patient's history. Repeat testing or NAT testing is also required as detailed in the HTA's Guide.

Virology testing may also be repeated at the end of the culture period where it is necessary to confirm the status of a donor to ensure compatibility with a patient (e.g., CMV).

5.1.6 Retroviral/lentiviral safety [Replication competence]

Where T-cells have been modified using retroviruses or lentiviruses, testing is required to detect the presence of replication competent virus vectors. Whilst virus vectors are manufactured to be replication incompetent, it is possible for some competent viral vectors to be present in the stock material at levels below the detection limit for the assay.

Current FDA guidance Testing of Retroviral Vector-Based Human Gene Therapy Products for Replications Competent Retrovirus During Product Manufacture and Patient Follow-up recommends that sufficient amount of vector is tested to demonstrated that the vector contains <1 RCR per patient dose as well as all retroviral vector transduced cells products be tested for RCR, regardless for the culture time of these products. Typically, this testing is 1% of final vector or 10^6 of pooled vector producing cells or ex vivo transduced cells.

If there is accumulated manufacturing and clinical experience that demonstrates that a transduced cell product is consistently RCR-negative, this data can be provided to support reduction or elimination of testing ex vivo genetically modified cells for RCR as a product moves towards commercialisation.

There has also been an acceptance of alternative methods being deemed appropriate for lot testing of ex vivo transduced cells in lieu of culture based methods, particularly when there are time constraints present. As such, PCR-based assays are deemed acceptable in place of the long culture-based methods.

5.2 Quality and efficacy/potency testing

As with other cell therapies, there are quality parameters T-cell and NK cell drug products which must be defined to ensure patient safety and product efficacy. Given the complexity of some of the processes used to derive T-cell and NK cell therapies, there will be many assays used to assess not only the quality of the product but also its efficacy/potency. Demonstrating ATMP potency remains one of the greatest challenges in the field as no one assay can provide a readout which directly links to in vivo efficacy. Table 3 provides some parameters for consideration as well as the approach to assess these parameters (please note - this is not an exhaustive list).

	Parameter	Approach	Standard/guidance
Purity	Visual appearance (colour / opacity)	Visual assessment	
	Visible particulates	Visual assessment	FDA guidance - Inspection of Injectable Products for Visible Particulates Guidance for Industry (Draft Dec-2021)
	Impurities	Flow cytometry	
	Phenotypic profile	Flow cytometry	EP 2.7.23 / 2.7.24 ATMP flow cytometry guidance
	Residual vector	PCR/culture-based methods	Considerations for the Development of Chimeric Antigen Receptor (CAR) T Cell Products (Draft – Mar-2022) Testing of Retroviral Vector-Based Human Gene Therapy Products for Replication Competent Retrovirus During Product Manufacture and Patient Follow-up (Jan-2020)
Potency	Phenotypic profile	Flow cytometry	EP 2.7.23 / 2.7.24 ATMP flow cytometry guidance Considerations for the Development of Chimeric Antigen Receptor (CAR) T Cell Products (Draft – Mar-2022)
	Transgene/gene modification expression (Vector Copy Number)	PCR/flow cytometry	Considerations for the Development of Chimeric Antigen Receptor (CAR) T Cell Products (Draft – Mar-2022)
	Cell viability	Flow cytometry	
	Cytokine secretion	ELISA	
	Cell killing	See section 2.3	
Identity	Genetic identity (STR analysis, HLA typing)	PCR, qPCR, Next Generation Sequencing	Considerations for the Development of Chimeric Antigen Receptor (CAR) T Cell Products (Draft – Mar-2022)

Table 3. Parameters for consideration as well as the approach to assess these parameters.

Without extensive QC testing, it is not possible to proceed to product release. However, the extent of testing should be proportionate to the level of risk the product may pose to the patient. Consideration must also be given to the overall “QC tax” as discussed below, which in turn must not have a detrimental impact on patients’ access to the therapy.

5.3 "QC tax"

Whilst it is essential to test every batch of product to its fullest extent to ensure patient safety and product efficacy, this must be balanced against the impact taking final product will have on the overall availability of the product for patients. Approaches to reduce or manage the QC tax on final product batches is recognised across different guidance documents relating to ATMPs – see PICS guidelines and Annex 19 of Part 4 EU guidance.

5.3.1 Routine QC testing

QC sampling is necessary to test product batches for release against the agreed product specification. As mentioned in the section above, samples may be taken at different points during the manufacturing process, especially at points which have been assessed as carrying increased risk. To ensure appropriate sampling and testing are carried out, it is essential to generate a QC sampling plan; this plan should be developed in the early stages of process development to assess the QC tax on the process. Where the QC tax is significant, this is the time at which to determine, using a risk-based approach, which samples may be removed, switched for an alternative (supernatant vs cellular material) or a reduced volume taken. Examples of this have been mentioned in the sections on sterility and mycoplasma testing, where there is scope to use material from the manufacturing process as a surrogate for testing final product in compliant volumes. The intended outcome of QC sampling and testing is not only to ensure the product is safe but also to be able to investigate the process should something go wrong such as positive sterility in the final drug product; with the correct sampling strategy it should be possible to determine at what point in the manufacturing process the issue arose.

5.3.2 Reference and retention samples

Over and above standard sampling for QC testing for batch release, additional reference and retention samples must also be taken; a reference sample is a sample of a batch of starting material, packaging material or finished product which is stored for the purpose of being analysed should the need arise. The reference sample should not be used for routine testing. A retention sample is a sample of a fully packaged unit from a batch of finished product; this sample is stored for identification purposes. Where the sample point for reference and retention samples is the same, the samples may be interchangeable. Sufficient reference material should be retained to carry out two full sets of analytical tests. The guidance documents referenced above provide in depth information around reference and retention samples to be taken during the manufacturing process, whilst considering the need to minimise the QC tax throughout the process.

There are various types of assays used for manufacturing release of cell products as well as patient monitoring. These include safety assays (bioburden, endotoxin, sterility) and assays measuring the strength and function of the product (e.g., potency). Ideally it would be possible to use the same assay for testing both the cell product and patient samples throughout the monitoring period, but there are challenges with this. The purpose of this section it to explore the challenges shared across methods as well as delving into specific challenges relating to individual assays.

When testing cell products, one of the key considerations is whether the product is autologous or allogenic. Table 4 highlights key differences between these products and how this can present differing challenges for each product type.

Autologous cells	**Allogeneic cells**
Cells come from patient and so are limited in number.	Cells are manufactured in large batches from unrelated donor tissues used for many patients "off the shelf".

Key focus on minimising losses through testing to preserve as much product for the patient as possible. It is expected that in some cases sample volume will be less than optimum but to minimise sample usage washes, supernatants and leftovers from the manufacturing process tubing etc. can be used.	Higher testing volumes can be accommodated due to larger batch sizes although there is still a consideration for analytical volumes.
Cell product generation can be on site at clinic or hospital although some manufacturing can occur at a manufacturing site involving transport of the patient cells between sites.	Cell product generation occurs in a manufacturing facility potentially in a different country to the patients. Consideration to shipping processes needs to be undertaken to maintain the quality and traceability of product while as it moves between locations.
Time limited to manufacture and test before being infused back into patient.	More time available for extensive product testing prior to being released for patient use.
Patient cells show more variation from batch to batch. These are also diseased cells which inherently are more variable and less robust than healthy donor cells. This can cause challenges in the analytics caused by this variation. Controls play a very important role in ensuring the accuracy of data generated.	Less variation seen as each batch used for many patients. Analytical data can be less variable and therefore easier to qualify/validate the assays.
Difficult to use automation due to time constraints. As each batch is one patient who is awaiting treatment, delays due to use of analytics are not sensible or feasible leading to analytics being performed on single batches as soon as testing is required.	Automation can be used for batch testing and increase throughput. This is due to there being a much bigger timeline between the generation of the product and the administration to the patient. As such, multiple batches can be tested together to make use of automation and increase efficiency of testing processes.
Autologous cells may be fresh from the patient being treated and modified prior to returning to the patient. This means that timing in critical and often testing such as sterility testing occurs simultaneously with the processing. This is an accepted risk.	Testing can be completed in advance of any release and the product stored frozen making the supply chain to the patient more manageable. There is less risk involved in the completion of all testing well in advance of the release and first patient treatment with once setting of testing being sufficient to treat many patients.

Table 4. Key differences between Allogeneic and Autologous products and how this can present differing challenges for each product type.

Potency testing is one of the most challenging analytics to be undertaken on a cell product, particularly given that it incredibly challenging to test true potency within an analytical method on cell product. The most typical approach taken it to use a surrogate method looking at markers of potency such as protein expression. Typically flow cytometry is used for this type of assay and the British Pharmacopoeia ATMP Guidance Application of Flow Cytometry provides a lot of guidance on how to approach the development and qualification/validation activities.

6. In process control versus final identity and potency

Ensuring potency, identity, or safety ahead of the completion of manufacturing can have a positive impact by reducing costs. Progress over the past decades have allowed process measurements such as pH, temperature, or pressure but also measurements related to the biological, physical, and chemical attributes of the materials being processed. These measurements can be:

- **At-line**: measurement where the sample is removed, isolated from, and analysed near the process stream.
- **On-line**: Measurement where the sample is diverted from the manufacturing process and may be returned to the process stream.
- **In-line**: Measurement where the sample is not removed from the process stream and can be invasive or non-invasive.

However, implementation of process analytical technologies (PATs) for this purpose is still in early phases of development (PAT — A Framework for Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance).

Implementation of PATs could help to predict the outcome of a manufacturing process (e.g., by analysing specific cytokines) or alert if actions are needed to ensure the process is successful (e.g., indicate if a specific metabolite is below the desired concentration to take the required action). However, this is dependant of the type of ATMP being manufactured (autologous vs allogeneic), length of the manufacturing process (e.g., 3 days vs. 14 days) and the initial considerations taken during process development. The competent authority should be consulted before implementation of these type of analysis to fulfil regulatory compliance and start from the analytical method development stage.

There are three main analytical method stages:

Assay development - this is an optimization phase where several parameters are evaluated to detect the analyte/s of interest. It is recommended to optimise the following parameters at the initial stage to ensure that the method would be suitable for validation at a later stage:

- Reference standards
- Critical reagents
- Calibration curve
- Quality control samples
- Selectivity and specificity
- Sensitivity
- Accuracy
- Precision
- Recovery
- Stability of the analyte in the matrix.

The sponsors should consider those parameters to avoid new assay development and comparability studies between methods before assay validation. Some of the parameters to be evaluated could be challenging such as reference standards. These are not always commercially available and could be a potential challenge. In this scenario a fully characterised qualified batch (representative of the intended final product) could be and justified.

Assay qualification - once the assay is developed and optimized, the next phase would be to qualify the assay. This step aims to ensure that the assay is fit for purpose providing confidence in the result. It is generally accepted that:

- Assay qualification is performed to assess and determine the performance of the assay evaluating parameters such as specificity, linearity, precision (repeatability and intermediate precision) and accuracy – range (ULOQ, LLOQ and LOD) or interference can also be evaluated.
- Assay qualification has no pre-determined acceptance criteria or performance specifications that must be met for the parameters evaluated although, based on the intended application, there may be performance capability requirements.
- A method cannot fail qualification, it can (and should) be reoptimized until it can achieve the required performance. If it cannot achieve the required performance, it should be rejected for the application.

Assay validation - once the assay is qualified, the sponsor should have confidence on the performance of the assay and the outcome of the results and should be able to start the validation of the analytical procedure. Current guidelines are available (ICH Q2 R1 Validation of Analytical Procedures) and the sponsor should consider them from the development stage.

The validation exercise should be based on a pre-defined protocol where the experimental work is described and acceptance criteria for each of the parameters assessed are defined. The acceptance criteria for each parameter and test can vary as it will depend on the analyte of interest and type of procedure used to measure the analyte (e.g., evaluate cytokine expression levels through flow cytometry, ELISA or Luminex/MSD technologies). However, the sponsor is encouraged to assess which technologies they have access to and how they can reduce sources of variability (use of qualified reagents, calibrated equipment, experience operators. US Pharmacopoeia (USP) provides guidance or recommendations on the type of analysis that could be considered and performed when validating biological assays <1033>. In addition, the EMA Guideline on bioanalytical assay validation focussed on chromatographic and ligand binding assays offers the expected acceptance criterion for the validation parameters for this type of methods. This guideline describes when partial validation or cross validation should be carried out in addition to the full validation of an analytical method. A comparison between FDA and EAEU requirements (See Table 5) has been performed by providing additional support to the sponsor to evaluate the acceptance criteria expected for these type of methods.[16]

Parameter	FDA Requirements	EAEU requirements
Calibration curve Linearity	**Elements**	
	A blank [no analyte, no internal standard (IS)], a zero calibrator (blank plus IS), and at least six non-zero calibrator levels covering the quantitation range, including LLOQ in every run; All blanks and calibrators should be in the same matrix as the study samples; The concentration-response relationship should be fit with the simplest regression model.	Blank sample (treated biological sample without analyte or IS), zero calibrator (treated biological sample with IS) and at least six different calibrator concentrations; Repeat analysis is allowed for each calibration standard; Use of a blank sample of the same variety that will be obtained in the study; Use of a dependence that simply and reliably allows the analytical signal response to be described as a function of the analyte concentration.
	Acceptance criteria	
	Non-zero calibrators should be ± 15% of nominal (theoretical) concentrations, except at LLOQ where the calibrator should be ± 20% of the nominal concentrations in each validation run; 75% and a minimum of six non-zero calibrator levels should meet the above criteria in each validation run.	Experimentally calculated calibrator concentrations should be 15% of the nominal values (except at LLOQ, for which these values can be 20%); 75% of calibrators at 6 different concentrations; If the aforementioned repetitions are used, 50% of test samples for each calibrator concentration should meet the criteria (within 15% or 20% at LLOQ).
Quality controls (QCs)	**Elements**	Refer to Accuracy and Precision. Stability
	Runs to assess accuracy and precision: Four QCS, including LLOQ, low (L: defined as three times the LLOQ), mid (M: defined as mid-range), and high (H: defined as high range) from at least five replicates in at least three runs. Other validation runs: L, M and H QCs in duplicate.	
	Acceptance criteria	
	Refer to Accuracy and Precision Runs, Other Validation Runs, and Stability Evaluations.	
Selectivity	**Elements**	
	Analyse blank samples of the appropriate biological matrix from at least six individual sources.	At least six different sources of the corresponding blanks without analyte are used (with experimental confirmation). A lower number of sources is allowed for rare varieties of biological samples.

	Acceptance criteria	
	Blank and zero calibrators should be free of interference at the retention times of the analyte(s) and the IS; Spiked samples should be ± 20% at LLOQ; The IS response in the blank should not exceed 5% of the average IS responses of the calibrators and QCs.	The response at LLOQ is ≤ 20% for the analyte; 5%, for the IS.
Specificity	**Elements**	See Selectivity above. Selectivity studies can in some cases require studies of the impacts of active-pharmaceutical-ingredient metabolites and degradation products formed during sample preparation and medications used simultaneously.
	The method specificity should be assessed for interference by cross-reacting molecules, concomitant medications, bio-transformed species, etc.	
	Acceptance criteria	
	See Selectivity above.	
Carryover	**Elements**	
	The impact of carryover on the accuracy of the study sample concentrations should be assessed.	The impact of carryover must be assessed by adding blank samples after samples with high concentrations or calibrators of the upper limit of quantitation.
	Acceptance criteria	
	Carryover should not exceed 20% of LLOQ.	Carryover in a blank sample after a standard with high concentration should not exceed 20% of the LLOQ; for the IS, 5%.
Sensitivity/LLOQ	**Elements**	
	The lowest nonzero standard on the calibration curve defines the sensitivity (LLOQ).	The LLOQ is considered the lowest calibrator.
	Acceptance criteria	
	The analyte response at LLOQ should be ≥ 5 times the analyte response of the zero calibrator; The accuracy should be± 20% of nominal concentration (from ≥ 5 replicates in at least three runs); The precision should be ±20% CV (from ≥ 5 replicates in at least three runs).	The LLOQ analyte signal should be at least five times greater than the blank signal; Acceptable accuracy and precision; The LLOQ must be adapted to the expected concentrations and study aim (e.g., LLOQ in bioequivalence studies should not be greater than 5% of Cmax (minimal Cmax from the entire cohort).
	Elements	

Accuracy and Precision	Accuracy and precision should be established with at least three independent runs, four QC levels per run (LLOQ, L, M, H QC), and ≥ 5 replicates per QC level.	Three runs on at least two different days; Within-run analysis of at least five samples of the same concentration for at least four different concentrations within the method range. Recommended concentrations: LLOQ, triple the LLOQ (L), ~30 – 50% of the upper limit of determined concentrations (M), at least 75% of the upper limit of determined concentrations (H).
	Acceptance criteria	
	For accuracy and precision, the run should meet the calibration curve acceptance criteria and include the LLOQ calibrator. This run has no QC acceptance criteria. **Accuracy: Within-run and between runs** ±15% of nominal concentrations; except 20% at LLOQ. **Precision: Within-run and between runs** ±15% CV, except 20% CV at LLOQ.	**Accuracy: Within-run and between runs** ±15% of nominal except ±20% at LLOQ. **Precision: Within-run and between runs** ±15% CV except ±20% CV at LLOQ.
Other Validation Runs	**Elements**	
	≥3 QC levels (L, M, H) in at least duplicates in each run.	
	Run acceptance criteria	
	Meet the calibration acceptance criteria ≥ 67% of QCs should be ±15% of the nominal (theoretical) values, ≥ 50% of QCs per level should be ±15% of their nominal concentrations.	N/A
Recovery	**Elements**	
	Extracted samples at L, M, and H QC concentrations versus extracts of blanks spiked with the analyte post extraction (at L, M, and H).	N/A
Matrix effect		**Elements**

		At least six blank series from different sources are used for MS methods, measurements made at L and H QC; Alternate version (if the main approach is unsuitable): assess response variance between series using at least six matrix series in which analyte is added at L and H QC concentrations; Less than six different matrix series are allowed if the matrix has limited availability; however, such an approach must be justified; Assessment of nonstandard samples is also recommended (e.g., hyperlipidaemic plasma or plasma from blood after haemolysis, samples from special patient groups).
		Acceptance criteria
		±15% CV of matrix factor normalized to IS; ±15% CV for series (alternate version).
Stability	**Elements**	
	For auto-sampler, bench-top, extract, freeze-thaw, stock solution and long-term stability, perform at least three replicates at L and H QC concentrations.	Used for L and H QC samples; Stability of stock and working analyte and IS solutions, freeze-thaw, short-term stability at room temperature or benchtop temperature, long-term stability; Extract in autosampler, if necessary.
	Acceptance criteria	
	The accuracy (% nominal) at each level should be±15%.	Accuracy for each concentration (at M level) should be ±15% of nominal.
Dilution	**Elements**	
	QCs for planned dilutions, 5 replicates per dilution factor: Accuracy: ±15% of nominal concentrations; Precision: ±15% CV..	Analyte at concentrations above ULOQ, diluted blank (at least five determinations for each dilution).
		Acceptance criteria
		Accuracy and precision of 15%.
Repeat analysis	No re-analysis of individual calibrators and QCs is permitted.	

Table 5. Bioanalytical method parameters and Acceptance Criteria: Comparison of FDA and EAEU requirements[16]

Specification criteria for each parameter detailed in the validation protocol must be met by every validation run for the validation study to pass and for the method to be considered validated. If a validation run fails, the root cause should be investigated and resolved before the method can be considered fully validated.

Complete assay validation is required for lot release assays, raw materials, in process testing, residual testing, stability and GLP studies. Therefore, it is recommended that the sponsor approaches assay development with the requirements for assay validation, considering the suitability of the selected assay for its intended purpose.

Table 6 aims to support the sampling strategy of in-process control samples needed to establish a correlation between initial manufacturing process steps and final product characterization as a starting point to predict batch manufacturing outcome.

Parameter	**In process**	**Final ID**	**Final Potency**
Cell count	X		X
Viability	X		X
Transgene presence	X (After transduction – depends on process length)	X	
VCN	(Sample collection)	X	
Transduction efficiency (PCR)		X	
Transduction efficiency (Prot)		X	
Appearance		X	
Purity (immunophenotype specific to mechanism of action)	X	X	
Characterization - Activation - Therapeutic cell subpopulation (effector/memory) - Secondary elements - Metabolites	X	X	
Impurities	X	X	
Potency/Potency panel	(Sample collection)		X
Safety?			

Table 6. Sampling strategy of in-process control samples

7. T cell assays

7.1 Memory status and safety vs efficacy

Over the last few years, increasing amounts of studies suggest that CAR T cells with an early memory phenotype have been associated with better clinical outcomes. A recent study[17] showed that there was a correlation between the proportions of naïve/stem cell memory (Tn/Tscm) and the ability of CD4 and CD8 T cells to proliferate. With regards to early clinical responses, progression free survival and overall survival positively correlated with increased numbers of naïve/stem cell memory CD8 T cells that have a higher proliferative capacity. Interestingly, the effects were opposite with the CD4 T cells. An earlier study[18] demonstrated that patients infused with a CD19 CAR had *in vivo* CAR T cells expansion that was positively correlated with amount of the CD8(+) CD45RA(+) CCR7(+) T cell subset present, usually defined as the stem cell memory T cells. In their pre-clinical studies, the higher the levels of the stem cell memory T cells, the higher the cytotoxic function observed. A thorough analysis

on the CAR T cells from patients with remission and those that did not respond to the treatment, in order to better understand the clinical successes was performed.[19] There was an enrichment of T cell memory linked genes such as STAT3/IL6 in CAR T cells from complete responding patients and an up regulation of genes associated with T cell effector differentiation, exhaustion, apoptosis, and glycolytic metabolism in non-responders. This was also supported by the increased numbers of the memory like T cells in those patients with sustained remission. A study[20] showed that predefining the T cell subsets can result in a more even level of cytotoxicity compared to random numbers of the various T cell groups. They found that cytotoxic function was significantly enhanced when deriving CD8 CAR T cells from the central memory and naïve subset when compared to the effector memory subset. Another study[21] which investigated the transcriptomic profile of 71 patient's T cells prior to CD19-CAR T cell manufacturing. The main findings were that long term CAR T cell persistence was positively correlated with naïve and early memory T cells, an increase in apoptotic signalling pathways in T effector and effector memory subsets and that LEF1 and TCF7 are imperative in maintaining naïve and early memory phenotypes. These are a few studies that highlight the positive effects of an early stem cell memory phenotype and better clinical outcomes. Additional studies further support the benefits of a CAR T cell product that have an early memory phenotype.[22]

There are increasing numbers of studies that support the correlation of early memory T cell phenotype and improved efficacy.[23] This emphasises the importance of defining the CAR T cell product before use in the clinic. Another vital property of a CAR T cell product is its efficacy. Despite the obvious nature of its importance, the ability to define the efficacy of a CAR T cell product by phenotype is less clear. CAR T cell efficacy can be divided into three main sections: 1. *In vitro* 2. *In vivo* within animals (pre-clinical) 3. *In vivo* (humans). To date, neither the *in vitro* nor the pre-clinical efficacy data guarantee the actual *in vivo* efficacy once administered within patients. As such, the current standard is to show adequate *in vitro* and pre-clinical efficacy before the product is allowed in clinical trials. *In vitro* efficacy is measured using various cytotoxic assays as described in detail in section 3.4.2. The overall aim of these assays is to assess how well the CAR T cells can specifically kill the target cells, usually tumour cells that express the antigen the CAR recognises. A CAR T cell will be defined as having *in vitro* efficacy if it can specifically kill more antigen expressing cells than the background non-specific killing abilities of T cells. Following this, the CAR T cells may be administered to animal models that have had the human tumour cells introduced into the animal and tumour clearance and animal survival are used as the measure of success and CAR T cell efficacy. CAR T cells with pre-clinical efficacy can clear the tumour within the animal model and usually result in the long-term survival of the animal. Finally, the *in vivo* assessment of efficacy is measured by tumour control, patient survival and levels of remission. *In vivo* efficacy is defined by the proportion of patients that respond to the treatment through either complete or partial remission versus the non-responders depending on the type of cancer being treated. This is usually compared to current standard of care. For example, for a CD19-CAR product, any less than 70% 1/13 complete response rate would be considered unsuccessful. However, in a solid tumour setting, 10% response rate would be a positive outcome if there are no other treatment options. The efficacy of a CAR T cell product has multiple levels which should be evaluated individually and in the right order, which in turn allows its use in phase II/III trials where its final efficacy will be assessed and used to determine if it is a suitable product to use in therapy.

Taken together, the composition of the phenotype of the CAR T cell product has a direct effect on its overall efficacy. However, additional investigation is required to assess the relationship between the T cell phenotype and subsequent safety profiles. Another vital aspect of a CAR T cell product is its safety. The two main safety concerns associated with CAR T cells is

cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS). There have also been reports of on-target/off-tumour toxicity and healthy tissue damage due to other cell types expressing the CAR antigen. 30 to 100% of patients are affected by CRS, with 10-30% reporting grade 3 levels or higher.[24] In most cases, CRS onset occurs for 1 to 10 days within the first 14 days post infusion with rare carses of delayed onset reported.[25] CRS is defined by a fever of over 38°C, hypoxemia and haemodynamic instability; whereby the severity is measured according to the American Society for Transplantation and Cellular Therapy consensus criteria. High grade CRS is usually associated with the tumour burden, CAR T cell dose and concurrent infection. CRS is caused by the activation of the CAR T cells resulting in effector cytokine release which in turn triggers pro-inflammatory cytokine release. 20-60% of the CD19-CAR T patients suffered from ICANS, with 12-30% grade 3 or higher. ICANS usually occurs within 3-5 days from infusion or with CRS and 10% of patients exhibit delayed ICANS. ICANS symptoms include confusion, agitation, tremors, and seizures. Other highly occurring symptoms are hesitant speech, dysphasia, and deterioration of handwriting. The pathophysiology of ICANS has been suggested to result from the combination of inflammatory cytokines that increase the vascular permeability; activation of the endothelial layer which in turn breaks down the blood-brain barrier and increased cerebrospinal fluid (CSF) cytokines. Other complications include cardiovascular toxicities which in 10 to 20% of patients, prolonged cytopenia's and hypogammaglobulinemia.

7.2 Challenges related to T cell assays

Different laboratories define memory/naïve/effector/central stem cell memory T cells differently which limits comparability between studies. For CD4+ T cells numerous markers have been used alongside CD45RA+ to distinguish naïve and effector T cells including CD31, CD27, CD62L, CCR7 and CD28. In many clinical trials the focus is on cytotoxic CD8 T cells. Naïve (CD27+, CD28+, CCR7+, CD45RA+), effector RA+ (CD27-CD28-CCR7-CD45RA+), effector memory (CD27-CD28-CCR7-CD45RA-) and central memory CD8+ T cells (CD27+CD28+CCR7+CD45RA-) have been defined.[26] Other markers that have been used include using CD45RO and CD127 (interleukin 7 receptor alpha chain) which is used to identify memory precursor cells. One study[19] showed that increased CD8+CD27+CD45RO- cells correlated with sustained remission of CLL. Another study[27] use CD62L and CD45RA to define naïve (CD45RA+CD62L+, central memory (CD62L+, CD45RA-), effector (CD62L-Cd45RA-) and effector memory (CD62L-CD45RA+); they also show prior to stimulation and transduction (fresh from the donor) the cells were approximately 70% naïve phenotype and these switched to approximately 30% central memory during the transduction process. This pool of transduced cells effectively killed target cells and cured their mouse model system. However, this highlights the issue: regardless of the T cell phenotype, most trials are not sorting for pure T cell populations so whether analysed *in vitro*, in animal models or in clinical trials, there is a correlation of phenotype with effectiveness but little absolute proof of it. When more functional analysis assays such as cytokine secretion, intracellular cytokine analysis or upregulation of activation markers such as CD69, HLA DR, CD154 (CD40L), CD138 are performed, these indicate *in vitro* function rather than *in vivo* efficacy. Immunophenotyping assays help characterise the product during the manufacture process and/or as release criteria as an indication of probable efficacy.

With ATIMPs there is a balance between risk of the therapy and the benefit for the patient. Historically the early trials of gene therapy for rare diseases and early CAR T cell therapy were performed in patients who had few other treatment options, and the benefit far exceeded the risk as the patient were likely to die without the ATIMP treatment. Autologous products have a different risk profile as they do not cause graft vs host disease, compared to allogeneic products. Allogeneic products frequently have more comprehensive characterisation as many

doses are made in a single batch from a healthy donor so there may be more cells available for quality analysis. As ATIMPs are used earlier in the treatment cycle, more allogenic products are used and in patients where there are other therapeutic options available, the risk benefit analysis changes. Immunophenotyping to characterise the memory status of the cells being infused is one component of reducing risk.

8. Variability in starting material carry-through to product characterization

In this section considerations are made about factors affecting starting material quality, sources of material variability, and their impact on process performance and, as a result, on the final product.

Best practice guidance is proposed to mitigate such variability and increase the chance of success in T-cell therapy manufacture.

This section focusses on:

- Technologies commonly used for pre-apheresis donor screening.
- Methods used for starting material release and characterisation testing.
- Assays used for characterisation and testing of the selected cells.

8.2 Challenges of starting material heterogeneity and variability

One of the key technical challenges in T-cell product manufacturing is associated with starting material heterogeneity and donor-to-donor / patient-to-patient variability. This can significantly affect the manufacturing process consistency between batches and across indications. It is, therefore, important to acknowledge and understand the source of variability to develop more robust and / or tailored manufacturing processes and control strategies for these innovative therapies.

There are many factors affecting the apheresis material quality that must be accounted for. Some of these variables can be mitigated by developing and implementing centre-to-centre standardised collection and post-collection procedures.[28] The key challenge remains to manage the variability derived from those factors inherent to patient / donor specificity, acknowledging that some of them might remain uncontrolled.

The purpose of mononuclear cell (MNC) collection via apheresis for a T-cell product manufacture, is to reduce unwanted cell populations (e.g., red blood cells, granulocytes, platelets). However, it should be noted that the mononuclear cell apheresis will always be a direct reflection of the cell populations circulating in the patient / donor at the time of collection.[29] As a result, since the apheresis devices are unable to differentiate between white cell subsets, a collection may result in a heterogenous mixture consisting of mononuclear cells, granulocytes and / or even circulating malignant cells (relevant to autologous therapies).[28]

Multiple studies have shown the heterogeneous and variable composition of the apheresis collections amongst donations, between healthy donors and patients, and across disease indications (for autologous therapies). This includes differences in total cell count, absolute lymphocyte count (ALC), CD4+:CD8+ T-cell ratio, and overall T-cell fitness and differentiation status, which in turn may influence process performance,[30, 31] characterised leukapheresis material, selected T-cells and the resulting CAR+ T-cell products for patient samples obtained from liso-cel clinical trials (NCT02631044 and NCT03331198). The data generated showed high variability of ALC and CD3+ T cells in leukapheresis material between patients and across disease indications, as well as substantial variation in cell composition (T cells, B cells, and

monocytes) both within and across disease indications. CD4+:CD8+ T cell ratio is another source of leukapheresis variability (wide range observed) between patients, with an increased CD4+:CD8+ T-cell ratio observed in patients with B-cell malignancies.

Similarly, inter-patient / inter-donor variability and impact of the clinical indication was demonstrated in where leukapheresis materials collected from healthy donors as well as lymphoma and acute lymphoblastic leukaemia (ALL) patients were analysed (Table).[31]

Characteristics	**Healthy donors**	**Lymphoma**	**ALL**
	n = 30	*n* = 32	*n* = 6
Total nucleated cells ($x10^8$), median (range)	149.0 (66.4–392.7)	100.4 (9.3–340.5)	62.5 (19.7–156.0)
Total CD3+ cell count ($x10^8$), median (range)	72.0 (4.1–185.9)	41.0 (4.2–231.8)	26.0 (4.0–68.0)
Haematocrit (%), median (range)	3.9 (1.8–7.4)	2.6 (1.3–7.4)	2.5 (1.1–3.3)
Monocytes (%), median (range)	N/A	24.7 (8.1–53.8)	14.7 (6.2–33.0)
Platelets ($x10^8$), median (range)	987.0 (418.0–7551.0)	1088.0 (147.0–3120.0)	615.5 (170.0–1310.0)
Viability (%), median (range)	99.8 (99.6–100)	99.9 (99.6–100)	99.8 (99.6–99.9)

Table 7. Leukapheresis product characteristics variability amongst donations, between healthy donors and patients, and across clinical indications[31]

Moreover, as far as autologous therapies are concerned, patient disease status at the time of leukapheresis collection might significantly affect the quality of collected materials. Enrichment for MNCs does not remove circulating malignant cells, and, in some occasions, collection of an insufficient number of benign T cells can result in failure to achieve the targeted therapeutic dose.[28] The Proceedings of a Workshop held in 2018 on “Exploring Sources of Variability Related to the Clinical Translation of Regenerative Engineering Products”,[29] reported that blood counts of peripheral blood drawn on the day of apheresis collection varied with the clinical indication. It was noted that patients with leukaemia tended to have lymphocytosis (increased lymphocytes), and patients with lymphoma tended to have lymphopenia (low levels of lymphocytes), consistent with the two diseases. This was aligned with an analysis of the impact of clinical indication on manufacturing success, suggesting that the lowest success rate is associated with manufacturing products from cells from lymphoma patients, and may be, therefore, indication specific.

The presence of circulating malignant cells in the apheresis collection may also result in the risk (although very rare) of an unintentional transduction of the residual malignant cells during the manufacture of genetically modified T-cell therapies, with potentially serious safety implications.[32]

8.3 Material attributes and end-product potency

Several clinical studies have indicated that the increasing successful outcomes in patients treated with CAR-T immunotherapies is strictly associated with the level of CAR T cell expansion early after infusion, while long-term persistence is required to prevent relapses.[30] However, this success is accompanied with many challenges including limited T cell ability to

expand and persist after infusion due to the tumour microenvironment.[33] The complex structure composed of immunosuppressive cells embedded in a modified extracellular matrix (ECM), contributes to a failure to clear antigen and leads to the dysfunction of T cells after reinfusion. Moreover, the phenotypic composition of T cells isolated from patients and in the infused products, and differences in chemotherapy regimens administered to the patients determine CAR–T cell expansion and persistence in vivo. Some studies have shown that the persistence of CAR-T cells is correlated with the presence of less differentiated T-cell subsets within the infusion product.[34] For instance, failure of manufacture or functionality of CAR T cells from patients with acute lymphoblastic leukaemia (ALL) and non-Hodgkin lymphoma was correlated with the selective depletion of early lineage cells during each cycle of chemotherapy[35] another group reported a patient's response to CD19 CAR was associated with the percentage of CD8+CD45RO–CD27+ in the leukapheresis sample in chronic lymphocytic leukaemia (CLL),[19] confirming published *in vitro* and *in vivo* studies showing that increased frequencies of naive and memory T cells in starting material corresponds with more naive-like and central memory-like CAR T-cell phenotypes in the final product.

8.4 Impurities derived from SM and their impact on final product

Apheresis collection is a method currently used in nearly all CAR T cell trials as the starting material for the CAR T cell manufacturing process. Specific seeding concentrations are required for successful expansion of T cells. However, the patient-to-patient variability in T cell numbers can lead to collect an insufficient number of T cells and thus to failure to meet the targeted therapeutic dose. One study showed that the targeted therapeutic dose (0.3×10^6-3.0×10^6/kg) was not reached in 31% of the cases when the CD3+ cells were lower than 2×10^9 in the stating material.[36] Therefore, the need to set a T-cell threshold based on target doses, apheresis devices and manufacturing process should be included for consistency and predictability of the outcome.

In addition, the cell composition of the apheresis can strongly impact the manufacturing process. Unwanted cell populations create difficulties for T-cell purification, proliferation, functionality, and viability. For instance, the presence of activated granulocytes has multiple impacts on T cell proliferation, activation, and cytokine production by affecting the viability and function of T cells. Activated platelets can bind other cells and create clumps, altering cell density and affecting the efficiency of the density gradient separation. Monocytes may also affect the T cell proliferation by adhering to the cell culture bags and phagocyting the magnetic beads used for the cGMP large-scale CAR T cell manufacturing platform. Wang *et al*. suggest that CD14+ content should be monitored in apheresis products, and that monocyte depletion must be included in the manufacturing process when more than 40% of CD14+ cells are contained in apheresis products to avoid non-specific phagocytosis of the magnetic beads used to activate CD3+ T cells.[37]

8.5 HLA typing – Allogeneic T cell therapies

All donor material should be HLA typed to avoid antibody mismatches. When TCR's are knocked out, it is still necessary to know original HLA type.

8.5.1 Viral specific T cells – Allogeneic T cell therapies

Other than to reduce starting material variability, donor screening prior to apheresis collection is recommended in the context of adoptive T cell therapies requiring T cells derived from seropositive donors.[38] Allogeneic hematopoietic stem cell transplantation and solid organ transplantation (SOT) expose patients to a marked immunosuppression either by T-cell depleting the graft intended for transplantation (with the former therapy), or by assuming life-long immunosuppressive medications (with the latter therapy). Both approaches are aimed to

prevent the occurrence of graft-versus-host disease (GvHD) after transplantation. The compromised T-cell compartment may easily expose the patient to viral opportunistic infections, such as Epstein–Barr virus (EBV), Cytomegalovirus (CMV), and adenovirus (ADV), amongst the most common. Adoptive transfer of virus-specific T cells represents an attractive therapeutic strategy to restore the antiviral immunity in the transplant recipients.

The manufacturing techniques of these virus-specific T-cell products have evolved over the years and an option may consist in the selection of viral-antigen specific T cells from the donated material, stimulation, expansion, and infusion into the patient. For this purpose, seropositivity of the donor for a specific viral antigen is a requirement to optimise the efficacy of the resulting T-cell therapy.[39]

Commonly used serological tests for donor plasma screening are enzyme-linked immunosorbent assays (ELISA) for immunoglobulin class G (IgG) antibodies against cytomegalovirus (CMV), Epstein-Barr virus (EBV) or adenovirus (ADV). However, one study, (Sukdolak 2013) recorded over 10% false positive results amongst the donors tested with the standard ELISA technique. In this scenario, seronegative was confirmed by a Western Blot-based serology test. Therefore, the use of multiple orthogonal methods may be advisable.

In-depth screening (characterisation) of potential T-cell donors may further increase the chance of success for adoptive T cell therapies (Sukdolak 2013). This screening includes characterisation of viral specific T cells by IFN-γ enzyme-linked immunospot (ELISpot) at first, followed by detailed phenotypic analysis by flow cytometry using peptide-major histocompatibility complex (pMHC) multimer staining (for details on the flow cytometry technique, please refer to Advanced Therapy Medicinal Products Guidance – Flow Cytometry, and functional analysis using IFN-γ cytokine secretion assay.

ELISA method - The method consists in a chemiluminescent microparticle immunoassay (CMIA). It is designed to qualitatively detect and semi-quantitatively determine IgG antibodies against a specific viral antigen in the donor plasma. Antigen-coated paramagnetic microparticles are incubated with the donor sample to allow for binding of human IgGs with the microparticles. This is followed by the addition of the anti-human IgG acridinium-labelled conjugate to produce a chemiluminescent reaction. This is measured in relative light unit (RLU). Seropositivity or seronegativity are confirmed by comparing the RLU in the reaction with the previous calibration.

Western Blot method - This technique allows for qualitative detection of viral specific IgGs with high specificity and sensitivity. It is designed as a confirmatory assay following screening assays, such as ELISA. The test uses recombinant virus specific antigens separated by electrophoresis and transferred onto a nitrocellulose membrane. The strip is incubated with the prepared donor plasma sample, followed by addition of peroxidase-conjugated anti-human IgG antibodies. After addition of a colouring solution, colour bands will appear where the IgG antibodies have bound.

ELISpot method - According to the ELISpot method as described in,[40] donor-derived peripheral blood mononuclear cells (PBMCs) are incubated (stimulated) overnight with the respective viral specific peptide (e.g., ppCMV*pp65*, ppCMV*IE-1*, ppEBV*EBNA-1*, ppEBV*LMP2A*, ppEBV*BZLF1*, ppADV5*hexon*). Negative controls are incubated in the absence of viral specific peptides. PBMCs stimulated at high concentration of CMV, EBV, and flu virus control peptide pool serve as positive controls and are analysed for system suitability purposes.

Following incubation, IFN-γ secretion is detected using biotin-conjugated antihuman IFN-γ antibodies and streptavidin-alkaline phosphatase and revealed by 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT Liquid Substrate). Spots are counted using

ImmunoScan Core Analyser and respective software. Means of duplicate wells are calculated and expressed as spots per well (spw). Donors are identified as: high responders (≥50 spw), low responders (10 spw to 50 spw), and non-responders (<10 spw).

Cytokine secretion assay - In the cytokine secretion assay, donor-derived PBMCs are incubated for four hours with the respective viral specific peptide (e.g., ppCMV*pp65*, ppEBV*BZLF1*, ppADV5*hexon*) to trigger the intracellular production of IFN-γ by memory lymphoid cells with peptide specificity. Cells are then labelled with the IFN-γ catch reagent to coat cells with an IFN-γ antibody, followed by addition of a second PE labelled IFN-γ antibody (enrichment reagent) enhancing the signal. Anti-CD8 and anti-CD4 monoclonal antibodies are also added, and the samples analysed by flow cytometry, aimed to quantify the frequencies of IFN-γ+/CD3+, IFN-γ+/CD8+ and IFN-γ+/CD4+ T cell populations. After confirmation of HLA and serology typing, a donor is chosen based on the IFN-γ+/CD3+ T cell frequency (i.e., >0.01% IFN-γ+/CD3+ T cells) obtained by IFN-γ secretion assay following stimulation with the viral specific peptide.[40]

8.5 Starting material release and characterisation testing

Considerations for the Development of Chimeric Antigen Receptor (CAR) T Cell Products recommends establishing acceptance criteria related to minimum cell number, viability, and percent CD3+ cells for the leukapheresis material, to increase the probability of manufacturing success of T cell-based therapies. However, further characterisation of the starting material is strongly advised to allow identification and, where possible, control of the material attributes influencing the downstream manufacturing steps as well as affecting the final product quality / functionality. This additional characterisation may include testing for percent and absolute number of CD4+ and CD8+ T cells, NK cells, monocytes, B cells.

With respect to CD19 CAR T cell therapies, it has been reported that the use of a SM characterised by terminally differentiated cells resulted in products with limited capacity to replicate and ability to evolve to long-lived memory cells. Additionally, flow analyses conducted on the SM derived from paediatric R/R ALL patients showed a skewing towards more differentiated effector cells in their T cell repertoires, compared to heathy donors. This is likely the result of chronic chemotherapy-induced lymphopenia.

It has been reported that patient's response to a CD19 CAR T cell therapy was linked to high frequency of CD27+ / CD45RO- / CD8+ T cells in the SM, confirming that less differentiated cells in the SM have more efficient engraftment compared to more differentiated cells.[19] Additionally, it has been demonstrated that high frequencies of inhibitory receptors (e.g., LAG3 and PD-1) on the SM T cells, combined with a reduced capacity to secrete TNF-α may be used to prospectively identify patients at higher risk to experience therapeutic failure and for whom alternative therapies may be more beneficial.[41]

8.6 Cell count and viability

In this section examples of assays commonly used for leukapheresis starting material testing for release into manufacture and / or characterisation are provided and described.

8.6.1 Dual-fluorescence method

The use of acridine orange (AO) and propidium iodide (PI) dyes is recommended for accurate viability analysis of primary cells, such as PBMCs, in heterogeneous samples, i.e., containing debris and unwanted non-nucleated cell types, such as red blood cells. AO and PI are nuclear staining dyes, i.e., they bind to the nucleic acid. AO is permeable to both live and dead cells and stains all nucleated cells, generating a green fluorescence. PI enters cells with damaged membranes and, therefore, stains all dead nucleated cells, generating a red fluorescence. Cells stained with both dyes, fluoresce red due to Förster resonance energy transfer (FRET),

so all live nucleated cells fluoresce green, and all dead nucleated cells fluoresce red. Since mature mammalian red blood cells do not contain nuclei, only mononuclear cells produce a fluorescent signal. As a result, there is no need to lyse red blood cells, eliminating an extra sample preparation step. There are various automated cell counters able to rapidly provide cell counting and viability information of cell suspensions, which remove operator variability.

8.6.2 Flow cytometry with enumeration beads with vital dye staining

The flow cytometry method with vital dye staining is commonly used to measure cell viability in cell suspensions. As mentioned above, the PI can enter cells with a damaged membrane and will therefore stain dead cells. It binds to the double stranded DNA by intercalating between base pairs. PI is excited at 488 nm and emits at a maximum wavelength of 617 nm. Therefore, this dye can be used in combination with other fluorochromes excited at the same 488 nm wavelength. Details on the method and best practice guidance are provided in Advanced Therapy Medicinal Products Guidance – Flow Cytometry.

8.6.3 Automated haematology analysers (blood cell counters)

Haematology analysers are used to perform a complete blood count (CBC), including red blood cells (RBC), white blood cells (WBC), haemoglobin, platelets (PLT), and haematocrit level (HCT). Depending on the type of analyser, additional blood parameters may be measured, such as neutrophils (NEU), lymphocytes (LXM), monocytes (MONO), eosinophils (EOS), basophils (BASO). They work by using multiple techniques or a combination of flow cytometry, spectrophotometry, and electrical impedance (Coulter's principle).

The 3-part differential cell counter to measure the size and volume of a cell. The principle is based on the detection and measurement of changes in electrical resistance produced by a particle (cell) suspended in a conductive electrolyte solution. This type of analyser can identify 3 types of WBCs: NEU, LXM and MONO.

The more advanced 5-part differential cell counter combines the flow cytometry technique with the electric impedance, allowing for assessment of the granularity (inner complexity) and size of the cells. This type of analyser can discriminate the 5 major WBC subpopulations (NEU, LXM, MONO, EOS, BASO).

8.6.4 Cell composition

Flow cytometry methods are usually used to assess the cellular composition and memory phenotypes of the apheresis starting material. Commonly evaluated subpopulations (markers) include CD3+, CD4+ T cells, CD8+ T cells, CD3– / CD56+ / CD16– NK cells, CD19+ B cells and CD14+ monocytes.[42] Additionally, memory differentiation can be assessed through evaluation of CCR7 / CD45RA and CD27 / CD28 subpopulations.[30]

8.6.5 Define a target cell number threshold for successful manufacture

The apheresis will always be a direct reflection of the cell populations circulating in the patient / donor at the time of collection, including the concentration of circulating T cells. In turn, the T-cell yield collected by apheresis represents a critical parameter for the manufacturing success of a T cell-based therapy. Therefore, it is recommended to set a minimum target cell number to accept the apheresis material for manufacture. A low T-cell number in the starting material could detrimentally impact the manufacture since specific seeding concentrations are needed to allow for successful T cell expansion. Additionally, loss of target cells may occur during the various processing steps ahead of the cell culture, resulting in failure to achieve the final target yield.[28]

Other factors to be considered when establishing acceptable thresholds for target cells include the target therapeutic dose, target patient population (paediatrics *vs* adults), intended clinical indication. Therefore, criteria may need to be set (and justified) on a case-by-case basis.

Throughout the clinical development, as more process knowledge and product understanding are gained, critical material attributes for the leukapheresis should be revisited and established based on clinical samples retrospectively analysed.

For some commercial products a minimum total nucleated cell count (TNC) and CD3+ T cell number in the leukapheresis material have been established, requiring TNC ≥ $20x10^8$ and CD3+ cells ≥ $10x10^8$ to start the manufacture of the therapy for the treatment of R/R ALL in children and young adults as well as for R/R DLBCL in adults.[31, 43]

Seattle Children's Hospital set a target of > $1x10^9$ total MNCs for the apheresis collected from paediatric and young adult patients enrolled in the NCT02028455 and NCT02311621 clinical trials. Whilst no criterion was set for CD3+ cells in the apheresis collection, a retrospective analysis showed that CAR-T cell manufacture was successful even with low numbers of CD3+ cells in the peripheral blood measured before apheresis (i.e., < 150 CD3+ cells/µL).[44]

However, in the three paediatric clinical trials NCT01593696, NCT02315612, and NCT02107963, an acceptance criterion was set for the target CD3+ cells in the apheresis material with a minimum of $0.6x10^9$ CD3+ cells (required for successful cell growth or transduction efficiency) and a target of $2×10^9$ CD3+ cells (to allow for cryopreservation of extra cells if needed for a second cell culture).[36]

8.6.6 Impurities characterisation and threshold: RBC, NK cells, and neutrophiles

Developing scalable manufacturing processes to ensure the desired final product quality is dependent on the quality of the starting materials. It is recommended to characterize all the components present in the starting material including the target cells along with other nontarget cells. Phenotypic and/or genotypic profiles of the cellular components should be addressed by using relevant identity markers and a purification process should be in place to reduce impurities.

Flow cytometry methods can be used to assess cellular composition, memory phenotypes and cell proliferation.

8.7 Selected cells (i.e., CD3+ cells, antigen-specific T cells) characterisation:

8.7.1 Metabolism profile - Activation/exhaustion

Upon stimulation of antigen receptors, T cells undergo metabolic reprogramming to fulfil their bioenergetic, biosynthetic and redox demands. Mitochondrial metabolic profile switch from catabolic to anabolic metabolism allowing T cells to differentiate into diverse functional subsets and to mount a robust, antigen specific adaptive immunity against invading pathogens and tumour. However, chronic exposure to tumour microenvironment and chemotherapy impairs mitochondrial function, including spare respiratory capacity (SRC), resulting in aberrant metabolic reprogramming upon stimulation of T cells. SRC is the energy reserve of the cells available to produce energy in response to increased stress or work and as such is associated with cellular survival. Therefore, SRC represents a functional parameter to evaluate the quality of the cells and the success of CAR T-cell therapy. SRC is determined by this calculation: Maximal oxygen consumption rate (OCR) / (Basal OCR *100). OCR of cells is used as a parameter to study mitochondrial function as well as a marker of factors triggering the switch from healthy oxidative phosphorylation to aerobic glycolysis in cancer cells. Oxygen

consumption is traditionally measured by phosphorescent oxygen probe in living cells able to be quenched by oxygen.

8.7.2 Cell subset composition (ratio)

8.7.2.1 Cell phenotype expression – Exhaustion and Inhibiting Inflammation

Flow cytometry methods are usually used to assess the cellular composition of exhausted markers such as programmed cell death protein 1 (PD1) and cytotoxic T lymphocyte antigen 4 (CTLA4) inhibit effector function upon several mechanisms that regulate T cell function by:

- Binding target receptors or ligands (CD80/CD86) and/or preventing the optimal formation of micro clusters and lipid rafts.
- Modulation of intracellular mediators, which can cause local and transient intracellular attenuation of positive signals from activating receptors such as the TCR and co-stimulatory receptors.
- The induction of inhibitory genes.

Typically, the higher the number of inhibitory receptors co-expressed by exhausted T cells, the more severe the exhaustion

9. GM cell assays

9.1 Vector potency testing in cells

Potency is defined as "the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result." (21 CFR 600.3(s)). This essentially means that a potency assay must be a quantitative measure of a product's biological activity, ideally measuring a specific response in a disease-relevant system if possible.

For cell products, there is an expectation for potency testing not only on the final cell product manufactured but also on the viral vectors used in the manufacture as a raw material. The aim of testing the viral vector is to ensure consistent manufacture of viral vector with a sufficient potency to induce the expected biological effect as well as to highlight batches that do not meet this before it is utilised for transducing patient or donor cells. Transgene expression alone as a measure of potency may be sufficient to support early-phase IND studies: however, additional measures of biological potency will likely be requested for clinical study(s) intended to provide primary evidence of effectiveness to support a marketing application. Additionally, it is recommended that vector lot release testing include assays to determine the vector concentration that can be used to normalise the amount of vector used for transduction during CART T cell manufacturing.

A typical approach to vector potency testing is to transduce a permissible T cell line, activate the transduced cells through incubation and then measure a response, either their killing ability through a killing assay or a surrogate such as the release of cytokine by ELISA. For the measurement of the release of cytokines, there are a variety of commercially available ELISAs that can be used to measure cytokine concentration within supernatant but staining for activation markers can also be utilised and quantified by flow cytometry.

9.2 Challenges related to GM cell assays

9.3 Cell line types

One of the major challenges of setting up a viral vector potency assay is how to develop a suitable assay that is as relevant to the disease system while being robust and consistent

enough to provide data to identify if batches manufactured are of sufficient quality. The most relevant assay would utilise patient or donor cells, but these are naturally highly variable which is not conducive to a robust consistent assay. Given that potency testing will be conducted again on the cell product, with the main aim of viral vector potency testing to evaluate consistency of each batch manufactured against set specification, the general preference is to utilise commercially available immortalised cell lines where cellular variation can be reduced to a minimum. Cell banks can be generated ensuring continuity of testing over the course of many years and therefore batches manufactured, allowing tighter specifications to be set and met with each batch tested.

Appendix 1 Protocols

NK cell immunophenotyping and absolute count

STARTING MATERIALS:

1 ml of fresh blood samples collected into an anticoagulant EDTA (Pink Cap EDTA tubes)
Or
$1x10^6$ Freshly isolated PBMCs
Or
$1x10^6$ of suspension of cells from the drug product (or intermediate)

CONSUMABLES & REAGENTS LIST:

CONSUMABLES/ REAGENTS	**SUPPLIER/ MANUFACTURER**	**CATALOGUE No**
Trucount Tubes	BD Biosciences	340334
CD56 APC-Cy7 [clone: HCD56]	BioLegend	318332
CD3 BV650 [clone: SK7]	BD Biosciences	563999
CD69 APC [clone: FN50]	ThermoFisher	MA1-10274
NKG2D PE [clone: 1D11]	BioLegend	320806
CD16 VioBlue [clone: VEP13]	Miltenyi	130-099-080
NKp46 AF488 [clone: 9E2]	BioLegend	331938
Zombie Yellow Fixable Viability Kit	BioLegend	423103
BD FCM lysing solution (10X)	BD Biosciences	349202
UltraComp eBeads™ Compensation Beads	Invitrogen by Thermo Fisher Scientific	01-2222-41
Staining buffer: Phosphate-buffered saline (PBS) without calcium chloride and magnesium chloride with 2% FBS		
Heat-inactivated fetal bovine serum (FBS)	Gibco	10500064
Deionized water		
Phosphate-buffered saline (PBS) without calcium chloride and magnesium chloride	MERCK LIFE SCIENCE UK LTD	D8537-6X500ML

5ml sterile round bottom tubes	Falcon	352054
Sterile Eppendorf Safe-Lock Tubes 2.0 ml	Eppendorf	0030121597
10/20µl Sterile Filter Pipette Tip	Starlab UK Ltd	S1120-3810
200µl Sterile Filter Pipette Tip	Starlab UK Ltd	S1120-8810
1000µl Sterile Filter Pipette Tip	Starlab UK Ltd	S1122-1830

EQUIPMENT
Centrifuge
Pipetboy
Single channel pipettes
Vortex
Class II Microbiological safety cabinet
Flow Cytometer

PROCEDURE

A) Compensation using UltraComp eBeads:

1) Label FCM tubes for each fluorochrome that will be used in the experiment.
2) Add 5ul of each mAbs to the tubes.
3) Mix beads by vigorously inverting at least 10 times or pulse-vortexing and add 1drop of beads to each tube.
4) Mix well by flicking, inverting vigorously, or pulse vortexing.
5) Incubate 15min, 4°C, in the dark.
6) After incubation time wash samples using 2ml Staining Buffer (300g, 5min, RT).
7) Add 200ul of Staining Buffer, mix briefly by flicking or pulse vortexing before analysis and perform automated compensation.

B) Absolute NK count in peripheral blood sample procedure using Trucount Tubes:

1) Pipette 5 µL of each of the appropriate monoclonal antibody reagent just above the stainless steel retainer. Do not touch the pellet.
2) Pipette 50 µL of well-mixed, anticoagulated whole EDTA blood onto the side of the tube just above the retainer (use reverse pipetting technique, avoid smearing blood down the side of the tube. If whole blood remains on the side of the tube, it will not be stained with the reagent).
3) Cap the tube and vortex gently to mix. Incubate for 15 min. in the dark at room temperature.
4) Dilute the 10X concentrate BD FCM lysing solution 1:10 with room temperature deionized water (The prepared solution is stable for 1 month when stored in a glass or high density polyethylene container at room temperature).
5) Add 450µl 1X BD FCM lysing solution to the tube.
6) Cap the tube and vortex gently to mix. Incubate for 15 minutes in the dark at room temperature. The sample is now ready to be analysed on the Flow Cytometer (Vortex the samples thoroughly at low speed to resuspend beads and reduce cell aggregation before running them on the flow cytometer). The compensation should be performed firstly (Procedure A).
7) Set up the stopping gate for acquisition “NK cells: CD56+CD3-“as 50,000 events.

8) Obtain the absolute count of the cell population (A), by dividing the number of NK cell events (CD56+ CD3-) (X) by the number of bead events (Y), and then multiplying by the BD Trucount bead concentration (N/V, where N = number of beads per test* and V = test volume). A = X/Y × N/V (*this value is found on the BD Trucount tube foil pouch label and might vary from lot to lot).

C) Phenotyping PBMCs or drug product/intermedicate procedure for checking expression of markers of interest:

1) Perform this procedure for rNK (Day 1) and TpNK (Day 2).
2) Run compensation using UltraComp eBeads described above (Procedure A.)
3) Aliquot sample containing $1x10^6$ of PBMCs from experimental conditions: before (rNK, Day 1) and after priming (TpNK, Day 2) to the 5ml FCM tubes at least in duplicate and prepare one additional sample for unstained control;
4) Live cells detection:
   a) For reconstitution, pre-warm the kit to room temperature; add 100 µl of DMSO to one vial of Zombie Yellow™ dye and mix until fully dissolved.
   b) Wash cells using 2ml of PBS (300g, 5min, RT).
   c) Dilute Zombie Yellow dye 1:100 in PBS and resuspend cells pellet in diluted 100ul Zombie Yellow solution.
   d) Incubate the cells for 15-30min at room temperature, in the dark.
   e) Wash one time with 2ml of Staining Buffer.
   f) Continue phenotyping with antibody staining procedure as desired.
5) Sample phenotyping:
   a) Resuspend cell pellet in 100µl of staining buffer;
   b) Prepare Mastermix of mAbs (*Table 1.*) into sterile Eppendorf*:*

Name of mAbs	**Volume per test (according to manufacturer's instruction)**	**x8**
CD56	5µl	40 µl
CD3	5µl	40 µl
CD69	10µl	80 µl
NKG2D	5µl	40 µl
CD16	10µl	80 µl
Nkp46	5µl	40 µl

Mastermix of mAbs.

   c) Add to each sample Mastermix in volume 40 µl.
   d) Incubate samples 15min, 4°C, in the dark;
   e) After incubation time wash samples using 2ml of Staining Buffer (300g, 5min, RT);
   f) Discard supernatant and add 200ul of Staining Buffer;
   g) Analyse samples using Flow Cytometer;
   h) Perform compensation procedure before running experimental samples (Procedure A.).
   i) Run unstained sample as a control;
   j) Set up the stopping gate for acquisition "NK cells: CD56+CD3-"as 20,000 events

Typical procedure for measurement of NK cell cytotoxicity by LDH release:

NK cell cytotoxicity assay by LDH release

STARTING MATERIALS:

1 ml of fresh blood samples collected into an anticoagulant EDTA (Pink Cap EDTA tubes)
Or
$1x10^6$ Freshly isolated PBMCs
Or
$1x10^6$ of suspension of cells from the drug product (or intermediate)

Set up the cytotoxicity assay as described below (NK killing assay by flow cytometry) but ensure that you include a "total lysis" control containing target cells alone. Upon conclusion of the co-culture with the target cells, centrifuge each to a pellet (300g / 5min) and collect the supernatants. To determine the maximal LDH release, add a 10% Triton-X lysis solution to the "total lysis" control and centrifuge to remove debris.

The supernatant will contain varying amounts of target cell derived LDH and it will be proportionate to the percentage of dead cells. Supernatants can be frozen for batch analysis or analysed immediately using one of the many commercially available LDH assay kits.

Appendix 2 Animal pre-clinical assessment

Animal models are the last stage of GM T cell characterisation prior to use in human studies. *In vivo* animal assessments provide useful proof of concept data for cytotoxic anti-tumour function despite the limitations. The main limitations include inaccurate modelling of the human immune responses in animals, species specificity of the CAR T cells and the tumour targets and xenogeneic graft versus host response. The most used mouse models include syngeneic, xenograft, transgenic, and humanized.[45] Syngeneic also known as immunocompetent allograft mouse models use mouse derived CAR T cells, antigens and tumour cells which allow investigation of the CAR T cell properties and activity in a functional immune system. This can result in the identification of any on target off tumour toxicity and other possible risks. Another model is the human xenograft mouse, whereby human CAR T cells and tumour cells are injected into an immunocompromised mouse, commonly NSG. The advantage of this model over the syngeneic model is that it allows the examination of human CAR T cells against human tumours and may be more representative of what could happen in patients. Immunocompetent transgenic mice are also used, though less frequently, for the observation of safety issues. Mice are made to express a human tumour associated antigens (TAA), to assess the levels of on target off tumour effects in healthy tissues that also express the TAA. These models used mouse CAR T cells; the mice have normal immune systems but investigate human TAA specific CAR constructs. Humanized transgenic mouse models use mice that are immunocompromised and implanted with human immune cells and investigate the effects of human CAR T cells with human tumours. This model attempts to develop a more complete representation of what could occur in patients. Finally, primate models have also been utilised to assess CAR T cell function and side effects, as their immune system more closely mimics the human immune system, especially for recapitulating clinical toxicity.

Limitations of animal models of human NK function:

Human NK cells are substantially different from those of in-bred rodents such as laboratory mice. Mice lack most of the NCRs expressed on human NK cells (NKp30, NKp44 and NKp80) and express a family of inhibitory markers, Ly49, which are absent from human NK cells, although share similar function with the inhibitory KIR molecules expressed on human NK cells. Furthermore, human IL-15 is an obligate survival cytokine for human NK cells, and it cannot be replaced with murine IL-15. In the absence of human IL-15, murine models can only be used for short term experiments over periods of hours. Regular administration of human IL-15 is possible but, since IL-15 is an activating cytokine for human NK cells, it renders it difficult to assess the true physiological nature of the experiment.

The transgenic huIL-15/NOG mouse was described recently and is now available from commercial suppliers of laboratory mice. This removes the need for daily IL-15 injections and may be a better model of *in vivo* NK function, but there is a potential limitation of relative lack of sequence homology between human and murine adhesion molecules. The growth of a model human tumour cell line in a laboratory mouse is likely to create a proinflammatory environment which potentiates the trafficking of human NK cells to the tumour site which is independent of the physiological mechanisms of true NK cell ingress into a tumour site. Thus, although widely used and occasionally required by regulatory agencies, it must be remembered that murine pre-clinical models of efficacy and particularly safety, are unreliable and need cautious interpretation.

Appendix 3

Best Practice Guidelines Common to NK and T cells

With regards to the use of T cell products in therapy it is essential to ensure a consensus characterisation protocol for both the phenotype of the T cells and their subsequent function. This would include defining the panel of surface markers and/or intracellular proteins that would indicate the phenotype of the T cells and the gating strategies used to define each of the subsets. In addition, specific factors when carrying out *in vitro* cytotoxicity assays should also be defined, such as: effector: target ratio, length of the assay, the read out and the controls required to show specific killing. The use of mouse and other animal models should also be better defined, such as: limiting the dosing strategy, using a similar model of efficacy (survival versus tumour clearance) and defining the tumour burden prior to treatment.

Best Practice Guidance *NK Cells*

NK cell identity by flow cytometry

Minimum markers should be CD3-ve/CD56+ with the condition that the fluorochrome used for the anti-CD56 antibody is a high efficiency reagent such as PE, APC, APC-Cy7. Any anti-CD56 reagent must be able to detect CD56dim cells as a discrete subset from the non-NK cells in the mix. CD19+ B cells never express CD56 so these can be used as a control population in a suspension of peripheral blood mononuclear cells which are dual labelled with CD19 and the CD56 reagent of choice. All CD19+/CD3- B cells should be clearly separated from the CD56dim/CD3- NK cells (use any fluorochrome for the CD3 and CD19 antibodies which is spatially separated from the fluorochrome used for the anti-CD56 reagent). CD16 is an important NK cell marker but is shared with many other cell types and should only be used in conjunction with anti-CD56. Combined reagents in which the anti-CD16 and anti-CD56 antibodies share the same fluorochrome are available but should not be used for NK cell identity assays because CD56-ve NK cells are much rarer than CD16+/CD56- cells which are not NK cells. Use of this combination reagent risks including CD16+ monocytes in the total NK cell population. A more complete identity panel would include CD45+ and a viability dye.

NK cell purity by flow cytometry

This depends entirely upon the expected impurities of the analyte. The possible impurities in a donor NK cell apheresis sample will be other peripheral blood mononuclear cells (T cells, B cells monocytes, gamma-delta T cells and NKT cells), all of which can be identified by a combination of anti-CD3, anti-CD14 and anti-CD19 sharing a single fluorochrome in a classical “dump channel”.

When manufacturing an allogeneic NK product for adoptive immunotherapy, the critical contaminant is allogeneic T cells since these can mediate transfusion graft-versus-host disease which is invariably fatal. The maximum number of contaminating T cells which can be tolerated will be dependent on numerous clinical factors, including the intensity/type of immunodepleting conditioning therapy of the patient and the degree of HLA mismatch between donor and patient. In trials of adoptive therapy with NK cells from HLA-haplo-identical donors, the maximum level of donor T cells in the drug product is usually limited to fewer than $1x10^4$ T cells/kg recipient weight.

If the analyte is an NK cell drug product derived from an induced pluripotent stem cell, the important impurity will be non-differentiated iPSC and the suitable panel of reagents will be very different.

NK cell activation markers

CD69 is a reliable marker of NK cell activation and appears within 60 minutes of priming. Upregulation of the IL-2 receptor-a, CD25, is an early marker of NK cell activation whilst CD16 shedding is another common event associated with NK cell activation by tumour cells and is mediated by the rapid secretion of proteolytic ADAM17 which cleaves the transmembrane domain of CD16.

CD107a is also expressed after PMA/ionomycin or cytokine stimulation and is increasingly viewed as an activation marker.

NK cell maturation markers

CD16 is commonly used as a marker of maturation on resting NK cells since it is largely absent from $CD56^{high}$, immature NK cells yet are present at high frequency and density on $CD56^{dim}$ cells. CD57 (HNK-1) is an antigen which is shared with a subset of CD8+ T cells but, on CD56+/CD3- NK cells, it is a reliably marker of terminal maturation. In patients with chronic viral infections, it has been shown to be a marker of functional exhaustion.

NK cell enumeration

In common with all other cell types, NK cell enumeration should be performed by a single platform method. Either volumetric flow cytometry or by use of an internal bead standard. The calculation of absolute number of NK cells by combination of “percent positive NK cells” by flow cytometry and “absolute lymphocyte” count by some other cell counter is not recommended.

NK cell cytotoxicity

Any of the assays described above are reliable and reproducible. You should aim for an intra-assay CV of <15% and an inter-assay CV of <30% for a single donor. There is great heterogeneity of NK cell function between healthy individuals, even to a single target cell line so do not expect reproducibility between donor samples.

Best practice guidelines T cell only

Ideally there should be consensus markers for defining memory and effector T cell populations. CD45RA is essential and is included in most schemes. CD28 and CD138 (41BB) should not be used as these are being incorporated as co-stimulatory markers in many fourth/fifth generation CAR constructs. It is important to distinguish assays being done for information and those being undertaken as manufacturing stop/go steps or release criteria. As discussed in a recent review of paediatric CAR T cells,[46] although central memory T cell are associated with longer persistence and effector memory T cells with shorter persistence but possibly higher initial cytotoxic activity, it is unclear which results in a better clinical outcome; not only tumour clearance but avoidance of cytokine release syndrome. As mentioned above, most CAR T cell products are impure products containing a mixture of memory, effector, and naïve T cells. It is unclear what the optimal dose of T cells is as even a few T cells may rapidly expand in this presence of antigen.

Although there are unknowns about T memory phenotype and clinical outcomes including optimal T cell dosing which may be antigen dependent, it is important to standardize markers and include CD45RA so that comparative data can be generated. Characterisation of the product infused is essential for understanding the product mechanism of action.

The role of pre-apheresis screening of patients

Mitigation of starting material variability – Autologous and allogeneic T cell therapies

Considering that mononuclear cell apheresis will directly reflect the cell populations circulating in the patient / donor at the time of collection, a standardised pre-apheresis screening of patients may be a valid tool to mitigate starting material variability and increase collection efficiency. Therefore, it is good practice to establish some inclusion criteria to increase the probability of success for collection and following manufacture, e.g., directly measuring the circulating target CD3+ cell number by flow cytometry, or, a surrogate marker, such as absolute lymphocyte count (ALC).

Some CAR-T cell trial sponsors have started defining an ALC threshold as inclusion criterion for enrolment into the trials. Amongst these, the Seattle Children's Hospital, Sponsor of the CD19 CAR T cell therapy trial (NCT02028455) for the treatment of paediatric ALL, has set the ALC at a minimum of 100 cells/µL for inclusion in the trial.[28] For the Tisagenlecleucel (Kymriah) clinical trials, an ALC threshold was set at 500 cells/µL, with a minimum CD3+ cell count of 150 cells/µL.[47]

Some publications[31, 48] have reported that sufficient apheresis CD3+ cells yield for successful CAR-T cell manufacture is achievable also for patients with low peripheral blood ALC (e.g., with ALC < 100/µL), in which case a greater processed blood volume may be required. Whilst this conclusion warrants further investigation, the University Hospital of Heidelberg has implemented[31] a clinical check-up performed before leukapheresis according to standardised procedures to increase the probability of CAR-T cell manufacturing success. This check-up includes screening for infectious disease markers as well as blood count (lymphocyte count included) and immunophenotyping by flow cytometry for CD4+ and CD19+ cells (for details on the flow cytometry technique, please refer to Advanced Therapy Medicinal Products Guidance – Flow Cytometry. According to this pre-apheresis screening procedure, the collected data would feed into an algorithm developed by the University group, based on leukocyte and lymphocyte count, to instruct on the optimum apheresis parameters to select (e.g., volume of blood to be processed to reach an acceptable number of CD3+ cells in the apheresis) and increase the chance of a successful collection prior to CAR-T cell manufacture. Additional exclusion criteria are accounted for, resulting in a possible cancellation or delayed collection.

Therefore, attention should be taken when setting acceptance criteria for inclusion into the trial. Often, these therapies (either autologous or allogeneic) may represent the last resort for some patients following failure of front-line treatments. Therefore, on some occasions, e.g., for patients with very low lymphocyte counts, proceeding with multiple apheresis (where feasible) to achieve the acceptable number of target cells in the collection may be a better solution opposed to depriving patients of this treatment opportunity.

Additional considerations should be made for autologous therapies about the target population, specifically whether adults or paediatric patients are subject to leukapheresis.

Paediatrics are more sensitive to the apheresis procedure compared to adults, considering the smaller total blood volume and their susceptibility to hypocalcaemia and hypothermia during the collection process, requiring slower apheresis rates and smaller processed blood volumes[47, 49]. Apheresis is generally more efficient at lower peripheral leukocyte counts and efficiency will decrease the longer the processing takes. Whilst in adults this may not be a major factor, it is instead for small children. As a result, a successful apheresis in children will require a higher peripheral blood ALC to start from compared to normal-sized adults.[50]

Best practice GM Cells only

Testing controls

All potency assays are multi-step complex assays involving not only the transduction of a relevant cell line but then typically supernatant harvesting and downstream end-point testing of these supernatants to produce the reportable result. It is therefore essential to implement sufficient controls through the assay to quantify any known variation and trend this over time to increase the confidence in any test sample data produced.

Controls included should cover both positive and negative controls which allow evaluation of not only potential points of contamination but also highlight instances of suboptimal performance or increase variation which could negatively impact any test results reported.

For all cell-based assays, a representative sample or reference standard should be included from the transductions through to any endpoint measurement. This would be a well characterised sample that has been proven to give a consistent result that can be trended and even have specific acceptance criteria. There is a lot of discussion about controls specific for flow cytometry is described in the Advanced Therapy Medicinal Products Guidance – Flow Cytometry.

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