Sheffield Hallam University

Functionality of stored cryopreserved lymphocytes and preservation of antigen-specific responses

DAY, Victoria Zoe

Available from the Sheffield Hallam University Research Archive (SHURA) at:

https://shura.shu.ac.uk/33762/

A Sheffield Hallam University thesis

This thesis is protected by copyright which belongs to the author.

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author.

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given.

Please visit https://shura.shu.ac.uk/33762/ and http://shura.shu.ac.uk/information.html for further details about copyright and re-use permissions.

Functionality of Stored Cryopreserved Lymphocytes and Preservation of Antigen-Specific Responses

Victoria Zoe Day

A doctoral project report submitted in partial fulfilment of the requirements of the Sheffield Hallam University for the degree of Doctor of Professional Studies

May 2023

Candidate Declaration

I hereby declare that:

1. I have not been enrolled for another award of the University, or other academic or professional organisation, whilst undertaking my research degree.

2. None of the material contained in the thesis has been used in any other submission for an academic award.
3. I am aware of and understand the University's policy on plagiarism and certify that this thesis is my own work. The use of all published or other sources of material consulted have been properly and fully acknowledged. The data presented in this thesis was obtained in experimental work carried out by myself and my colleagues in NHSBT, Barnsley. I played a major role in the preparation and execution of the experiment, and the data analysis and interpretation are entirely my own work. Any contributions from colleagues in the collaboration, such as diagrams or calibrations, are explicitly referenced in the text.
4. The work undertaken towards the thesis has been conducted in accordance with the SHU Principles of Integrity in Research and the SHU Research Ethics Policy.

The word count of the thesis is **51952**.

Name	Victoria Day
Award	Doctorate of Professional Studies
Date of Submission	25th May 2023
Faculty	Health and Wellbeing
Director(s) of Studies	Dr. S. Haywood-Small & Prof. N Woodroofe

Table of Contents

Abstract

Donor lymphocyte infusions (DLI) are routinely used as second line treatment to protect against relapse of haematological malignancies following haematopoietic stem cell transplant (HSCT). Lymphocyte collections are increasingly being used as starting materials for chimaeric antigen receptor-T (CAR-T) manufacture. Current protocols for storage and cryopreservation of lymphocytes are based on those developed for CD34+ HSC grafts and may not be appropriate for lymphocytes. As clinical use of lymphocyte products increases, there is a pressing need to identify cryopreservation and storage protocols that will optimise lymphocyte recovery and function when cells are stored or shipped prior to use. This study investigated the effects of storage time at 4°C prior to cryopreservation on post-thaw recovery and functionality of T lymphocytes from non-mobilised and granulocyte-colony stimulating factor (G-CSF) mobilised peripheral blood.

Non-mobilised lymphocytes were extracted from sixteen apheresis cones from platelet donations and purified by density gradient separation. Lymphocytes exposed to G-CSF were obtained from the residual quality control samples from nine G-CSF mobilised apheresis harvests from volunteer donors. Lymphocyte preparations were phenotyped by flow cytometry and the total number of viable T cells present determined. Cells were stored for either 24, 24-48 or 48-72 hours at 4°C prior to rate-controlled cryopreservation. Following a minimum of 7 days storage, cells were thawed and stimulated with CD3/CD28 activation beads for 4, 24 and 72 hours followed by flow cytometric assessment of CD25 and CD69 expression on CD4+ and CD8+ cells. Post-thaw recovery of viable T cells from each cryopreserved sample was calculated. Expression of CD25/CD69 and CD3+ viability and recovery were compared for cells cryopreserved after the different times in storage.

The results demonstrated a significant loss of viable T cells after cryopreservation that became greater over time from collection to cryopreservation. If cryopreserved within 24 hours of collection, means of 77% of non-mobilised and 57% of mobilised CD3+ cells were recovered post thaw. After >48 hours, the mean recovery dropped to 28% and 15% respectively. Expression of activation markers after stimulation appeared unaffected by length of time to cryopreservation in both sample groups indicating that T cells that survived the freeze thaw process were functionally unharmed. Exposure to G-CSF did not affect T cell responses to stimulation.

These findings demonstrate that current protocols for storing and handling lymphocytes require modification to optimise recovery of T cells post thaw. Further study on a larger sample group, is required to determine optimum protocols. As functionality post thaw was unaffected by time to cryopreservation or G-CSF stimulation, this positive finding indicates current freezing protocols do not influence lymphocyte functionality which will be advantageous for future therapies using T cells.

Abbreviations

7-AAD	7-Amino-Actinomycin D
ACD-A	Anticoagulant Citrate Dextrose solution A
ANOVA	Analysis of Variance
BM	Bone Marrow
BMT	Bone Marrow Transplant
BSBMT	British Society for Bone Marrow Transplant
CAR-T	Chimaeric Antigen Receptor -T
CFSE	5,6-Carboxy Fluorescein diacetate Succinimidyl Ester
CFU-GM	Colony Forming Unit – Granulocytic Myeloid
CMT	Cellular and Molecular Therapy
CMV	Cytomegalovirus
Cy7	Cyanine-7
CS&T	Cytometer Set-up and Tracking
D1	Day 1
D2	Day 2
D3	Day 3
DLI	Donor Lymphocyte Infusion
DMSO	Dimethyl Sulphoxide
EBMT	European Society for Bone Marrow Transplant
EBV	Epstein Barr Virus
ELISPOT	Enzyme Linked Immunospot assay
EMS	Environmental Monitoring System
FACT	Foundation for the Accreditation of Cell Therapy
FBC	Full Blood Count
FITC	Fluorescein isothiocyanate
FSC	Forward Scatter
G-CSF	Granulocyte-Colony Stimulating Factor
GvL	Graft versus Leukaemia
GvHD	Graft versus Host Disease

HAS	Human Albumin Serum
HIV	Human Immunodeficiency Virus
HLA	Human Leucocyte Antigen
HSC	Haematopoietic Stem Cell
HSCT	Haematopoietic stem cell transplant
HPC	Haematopoietic progenitor cells
HPC-A	Haematopoietic progenitor cells - Apheresis
HTA	Human Tissue Authority
ICC	Intracellular Cytokine
ICS	Intracellular Cytokine Staining
IFNɣ	Interferon gamma
IL2	Interleukin 2
ISCT	International Society for Cellular Therapy
JACIE	Joint Accreditation Committee of the International Society for Cellular Therapy and the European Society for Blood and Marrow Transplantation
LPA	Lymphocyte Proliferation Assay
MHC	Major Histocompatibility Complex
MNCs	Mononuclear Cells
MSC	Microbiological Safety Cabinet
NHS	National Health Service
NHSBT	National Health Service Blood and Transplant
NK	Natural Killer
PBMC	Peripheral Blood Mononuclear Cells
PBSC	Peripheral Blood Stem Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerthyrin
PHA	Phytohaemagglutinin
PQC	Performance Quality Control

PWM	Pokeweed Mitogen
QC	Quality Control
RBC	Red Blood Cell
RT	Room Temperature
SCD	Sterile Connecting Device
SSC	Side Scatter
TB	Tuberculosis
TCR	T Cell Receptor
Th	T helper cell
TNFα	Tumour Necrosis Factor alpha
TNC	Total Nucleated Cells
Treg	Regulatory T cell
VZV	Varicella Zoster Virus
WBC	White Blood Cell

List of Tables

List of Figures

List of Appendices

Acknowledgements

I would like to acknowledge my supervisors, Dr. Sarah Haywood-Small and Professor Nicola Woodroofe for their unfailing support and advice throughout the DProf programme, and without whose encouragement I would never have completed this thesis. I would also like to acknowledge my workplace supervisor, Dr. John Girdlestone for his expert advice and guidance.

I would particularly like to thank Paulina Nowosiad from the Advanced Therapy Unit at NHSBT Barnsley for her expert advice and help with the flow cytometry. I would also like to thank all my colleagues in the Advanced Therapies Unit at NHSBT Barnsley, for their help in processing samples and support throughout, and without whom it would not have been possible to complete this project.

On a personal note, I owe a huge thank you to Warwick Brown for his endless patience and encouragement, and for putting up with me throughout a difficult period.

1 Chapter 1 Introduction

1.1 Haematopoietic Stem Cell Transplantation

1.1.1 Historical Perspectives

Allogeneic haematopoietic stem cell transplant (HSCT) is the current standard of care for treatment of chemotherapy resistant haematological malignancies, primarily leukaemia and lymphoma. HSCT is not a new treatment – the first successful bone marrow transplant (BMT) was performed by Dr E. Donnell Thomas in New York, United States of America (USA) in 1956 using bone marrow from an identical twin to treat a child with leukaemia. By the mid 1980's bone marrow transplant had become a routine treatment in Europe and the USA (Bortin *et al.* 1992). In essence, the principle and practice of BMT has changed very little in the subsequent six decades (Barrett and Craddock, 2020).

During the transplant process, the recipient bone marrow is ablated with chemotherapeutic agents and radiotherapy to destroy all the marrow cells, both normal and malignant. The empty marrow is then repopulated by HSC collected from a matched healthy individual and the donor cells are introduced into the recipient via intravenous infusion. Haematopoietic stem cells (HSC) then migrate to the recipient bone marrow, where they engraft and reconstitute the bone marrow environment. Transplanted cells can create sustained multilineage haematopoiesis (Servais *et al.* 2013, Locatelli *et al.* 2014), producing the full spectrum of normal blood cells of donor origin within the recipient bone marrow (Figure 1.1). The common lymphoid progenitor cells give rise to the cells of the adaptive immune response, B and T lymphocytes whereas the common myeloid progenitor cells give rise to cells of the innate immune system and specifically

macrophages which are the key antigen presenting cell, linking the innate and adaptive immune system responses to foreign antigens.

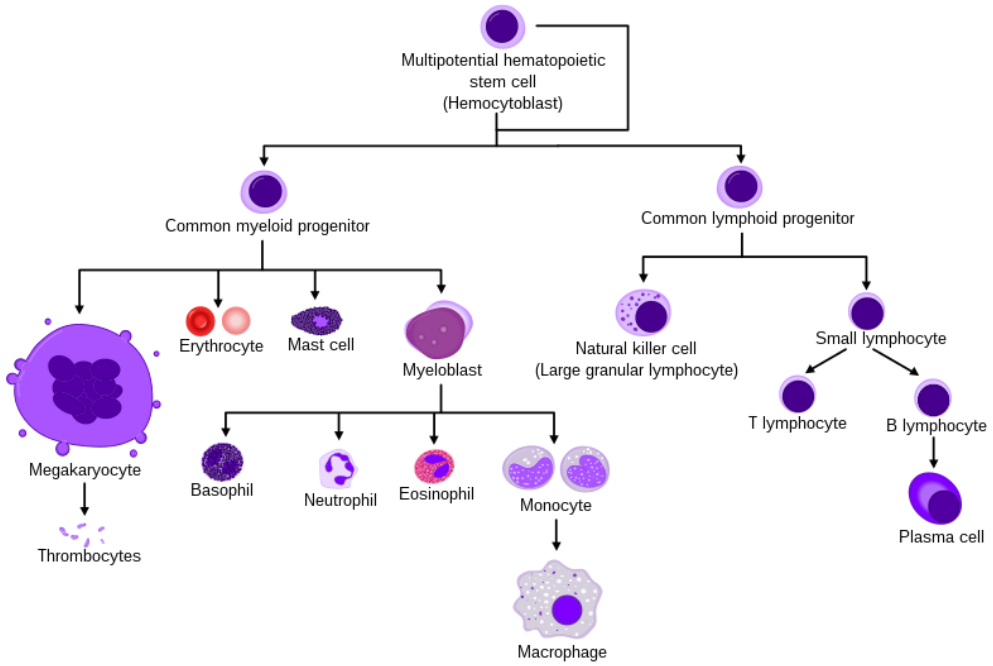


Figure 1.1: Image: Haematopoiesis (human) diagram (Rad and Häggström, 2009)

1.1.2 Bone Marrow as a Cell Source

Historically, the cell source used for HSCT was bone marrow. During a bone marrow harvest procedure, the donor cells are collected via a long, wide gauge needle inserted directly into the ileac crest of the pelvis under general anaesthetic. Syringes of blood are withdrawn from the marrow space containing the resident bone marrow cells as well as circulating blood cells until an adequate number of human stem cells (HSTs) to reconstitute the recipient's immune system have been collected. Multiple aspirations are required and there may be several puncture sites as up to 1200mL of marrow may be required to achieve a suitable cell dose for an adult recipient.

The mean CD34+ cell dose collected from bone marrow harvest is approximately 2-3 x 10^6/Kg recipient weight (Korbling and Anderlini, 2001, Anasetti *et al.* 2012). Studies on patient outcome from patients transplanted with PBSC have shown that an optimal number of >5 x 10^6 CD34+ cells/Kg recipient weight has been identified as able to provide rapid and sustained engraftment (Pierelli *et al.* 2012, Remberger *et al.* 2020). The lower CD34+ cell dose in bone marrow transplants contributes to longer engraftment time; mean neutrophil and platelet engraftment has been shown to be 5-7 days longer for patients transplanted with bone marrow compared to PBSC, which results in longer hospital stay and increased risk of complications (Anasetti *et al.* 2012).

The harvest procedure of necessity requires a hospital admission and a minimum of a one-night stay. The procedure can be painful for the donor and can result in anaemia through blood loss if a high-volume collection is needed. The collected bone marrow contains not only HSTs but high numbers of mature leukocytes, platelets and red cells which are not required for transplant and can cause problems during storage and infusion of the marrow. If the marrow is infused into the recipient with no further manipulation, then donor and recipient must be blood group compatible as a marrow harvest of sufficient volume to provide a good cell dose will also contain a sufficient red cell volume to cause a severe transfusion reaction where the recipient has antibodies to donor red cell antigens. Mature granulocytes can also cause problems in unmanipulated grafts as they have a short half-life with around 85% cell death in culture after 72 hours (Colotta *et al.* 1992). Unmanipulated marrow that is stored or shipped prior to infusion may therefore contain a high number of dead granulocytes. Release of cytotoxic chemicals and free DNA from the dead cells can cause bystander damage to the viable HSCs in the graft and adversely affect its ability to engraft (Agashe *et al*. 2017).

1.1.3 Manipulation of Bone Marrow Graft

It is possible to manipulate a bone marrow graft to remove or reduce the number of red cells and granulocytes within it, thereby reducing the risks of transfusion reaction or cell death. However, these are specialist procedures that require the use of complex cell separation equipment and highly trained operators. In addition, to manipulating the graft, the collecting system must be accessed, allowing the potential for contamination during the manipulation. Any intervention where the collection system containing the product is opened, must be performed in a clean room as the Human Tissue Authority (HTA) require all open interventions to be performed in a sterile environment, if regulatory compliance is to be maintained. Not all cell therapy laboratories have access to a clean room, or the specialist equipment required and so are unable to perform these manipulations. In addition to the difficulties posed by the operational requirements, *ex-vivo* manipulation of a graft carries substantial risks of unacceptable cell loss as well as contamination of the graft (Kim-Wanner *et al.* 2017).

1.1.4 Apheresis Procedures

These disadvantages of bone marrow as a cell source for transplant provided an incentive for clinicians to find an alternative cell source. Peripheral blood as a source of stem cells has mostly replaced the need for bone marrow collections, since the mid-1990s. In this process, leukocytes can be collected from peripheral blood using a cell separator during a procedure known as apheresis. The cell separator is a programmable machine that can be set to collect a specific fraction of the peripheral blood (white blood cells (WBC), red blood cells (RBC), platelets, plasma) as required and return the remainder to the circulation. HSCs are found within the mononuclear fraction of the blood, but under normal circumstances are retained in the bone marrow until they reach

maturity making them inaccessible to collection by apheresis. To facilitate mobilisation of immature blood cells from the marrow compartment, donors are administered with granulocyte-colony stimulating factor (G-CSF) (Stoikou *et al*. 2020).

1.1.5 Granulocyte-Colony Stimulating Factor

G-CSF is a cytokine which stimulates granulocyte proliferation. It is produced in response to inflammatory stimuli by many different tissues and its release into the bloodstream stimulates increased neutrophil proliferation and mobilisation from the bone marrow (Molyneaux *et al.* 1990). Administration of G-CSF to healthy donors was found to increase the number of haematopoietic progenitor cells in the peripheral blood, making it an ideal mobilising agent and has been in routine use for this purpose for the last 3 decades (Bendall, and Bradstock, 2014, Stoikou *et al.* 2020). Unlike bone marrow harvests, apheresis procedures are minimally invasive and do not require donor hospital admission. Collected cell numbers are usually far higher, making it a better option for adult recipients, and red cell contamination is usually below the threshold for initiating a transfusion reaction. Probably because of the higher cell number, the risk of graft failure for transplants performed using mobilised peripheral blood stem cells (PBSC) has been shown to be approximately a third of that for transplants using BM (Passweg *et al.* 2011, Olsson *et al.* 2015).

1.1.6 HSCT transplants performed in the United Kingdom

In 2020, 1596 allogeneic HSCT transplants were performed in the UK. The most common diagnosis of the patients treated was myelodysplasic syndrome/acute myeloid leukaemia which comprised 45% of the total. Other acute leukaemias, lymphomas and aplastic anaemias comprised the majority of the remaining 55%. 1223 (77%) of the transplants performed in the UK utilised donor cells procured by apheresis (source:

British Society of Blood and Marrow Transplantation and Cellular Therapy https://bsbmtct.org/activity/2020/).

As seen from the British Society of Blood and Marrow Transplantation (BSBMT) data, apheresis collections are now the preferred option in the UK, both because there is reduced impact on the donor and because the number of cells that can be harvested is higher, which enables satisfactory transplant doses to be achieved for larger recipients. In most cases, the donor cells are infused into the recipient with little or no laboratory manipulation.

1.1.7 Clinical Role of Human Leukocyte Antigen (HLA) in Relation to HSCT Transplants

The human major histocompatibility complex (MHC), also known as human leukocyte antigen (HLA) complex, is a specific group of molecules expressed on the cell surface that are crucial for the recognition of self and non-self, molecules by the immune system (Bertaina and Andrani, 2018). Graft-versus-host disease (GvHD) is a potentially serious complication of allogeneic stem cell transplantation and reduced-intensity allogeneic stem cell transplantation, which occurs when the transplanted donor immune system recognises the recipient as non-self and mounts an immune response against recipient cells. Initially transplants were only performed between identical twins or siblings to avoid the complication of life-threatening GvHD. GvHD occurs when the donor derived alloreactive T cells within the graft recognise the recipient cells as non-self and create an immune response against them. When the donor and recipient are MHC mismatched, a large proportion of the donor T cells in the graft will target host derived peptides in the context of recipient MHC. This results in selective expansion of T cells with TCRs that have a high affinity for host MHC molecules or peptides presented in their context.

Unmanipulated HSC grafts usually contain a high number of T cells and GvHD in the context of HSCT can be difficult to control. Skin, liver and intestine are the major targets of HSCT related GvHD, but niche forming cells in the bone marrow can also be affected, leading to long term deficits in haematopoiesis or immune function (Szyska and Na, 2016).

The first successful transplant between HLA matched unrelated individuals was performed in 1968 on a child with severe combined immune deficiency (Gatti *et al*. 1968). HLA matching of donor and recipient significantly reduces the risk of GvHD and allows transplant of HSC from unrelated donors. Current HLA matching transplant practice is to match donor and recipient at 5 loci: HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLADQB1 (Little, *et al.* British Society of Histocompatibilty and Immunogenetics Guideline 2021) to minimise the likelihood of either graft rejection or GvHD. However, the likelihood of a full HLA match is only 25% for those recipients who have full siblings and not all patients have siblings. To increase access to suitable donors for patients without a suitable relative, registries of healthy volunteers willing to donate to another individual have been created.

The Anthony Nolan Trust was the first donor bone marrow registry and was set up in the UK in 1974. Other worldwide registries soon followed. Formation of the donor registries has increased donor availability to the point where 40-70% of recipients are able to find a donor somewhere in the world (Little*, et al*., 2021) but this has had the knock-on effect of markedly increasing the time that grafts spend in transit. Grafts are routinely collected on one continent for end use on another and frequently spend up to 48 hours in transit before receipt at the transplant centre. Largely because of the requirement to

cryopreserve HSC during the Covid pandemic, there is now substantial evidence that shipment of HSC followed by cryopreservation does not adversely impact patient engraftment (Hamadani *et al.,* 2020, Thibideaux *et al.,* 2023). However, in the case of lymphocytes, there is evidence that shipment at 4°C can impair both recovery and functionality (Johnson *et al.,* 2022, Jerram *et al*., 2021) indicating that even where storage and shipment take place under tightly controlled conditions, extended shipment times could potentially have adverse consequences for the viability and functionality of lymphocyte grafts.

1.1.8 Infusion of Donor Lymphocytes

Not all tumour cells are destroyed by the pre-transplant conditioning regime and for the long-term outcome to be successful, the transplanted immune system must be able to recognise and target any remaining tumour cells – in what is known as the Graft Versus Leukaemia (GvL) effect. In some patients, tumour cell recognition and destruction by the transplanted cells is not completely effective and one of the main causes of death following HSCT is relapse of the original disease. Lymphocytes from the donor graft are responsible not only for GvHD but also for initiation of GvL. Since the 1990s, infusion of donor lymphocytes (DLI) from the original donor has been used to induce a GVL effect. They can be used either prospectively to treat falling donor chimerism, as evidenced by re-emergence of recipient origin leukocytes with no evidence of relapse, or they can be used reactively to treat re-emergence of disease (Mackinnon *et al*. 1995, de Vos *et al.,* 2019).

Use of DLI has been shown to provide a degree of protection against relapse, and in some cases to dramatically improve long term survival (Schmid *et al.* 2007, Eefting *et al*. 2014), particularly in the case of patients with chronic myeloid leukaemia (Kolb 2008, Ye

et al. 2022). The precise mechanism by which DLI provide protection against relapse is not yet fully established and the clinical results obtained have been found to be extremely variable, but there are indications that the infused lymphocytes are able to stimulate marrow infiltrating CD8+ cells and reverse the T cell exhaustion produced by a state of chronic inflammation, enabling the host to mount an effective immune response against the tumour (Bachireddy *et al*. 2014, *Yi et al*. 2010, Zhou *et al.* 2020).

DLI are collected from the peripheral blood of the healthy HSC donor, either at the time of the original transplant or later once a need for them has been identified. The cells are then analysed by flow cytometry and divided into aliquots based on the number of CD3+ lymphocytes present. These aliquots may then be given fresh to the recipient or frozen for future use. The standard protocol for treatment with DLI is to give escalating doses of CD3+ cells over time (Mackinnon *et al*. 1995) until either the recipient marrow stabilises at 100% donor cells with no disease markers detected by cytogenetic or molecular methods, or until GvHD is induced. Induction of mild (Grade I-II) GvHD has been shown to be essential to produce long term remission (Itonaga *et al.* 2013, Rozmus *et al.* 2022) and patients who never get GvHD have a higher relapse rate than those that do.

Clinical practice in the South Yorkshire region, in common with most transplant units worldwide, is to collect DLI pre-emptively and cryopreserve them for future requirements. The cells are donated by volunteer donors (related or unrelated) who may be unable or unwilling to provide cell donations on repeated occasions. If the original donor can no longer donate cells (for whatever reason) then there are few remaining treatment options for a patient with incipient relapse. Re-transplant with a

new donor may be the only curative therapy and outcomes are generally poor (Clinical commissioning policy: second allogeneic haematopoietic cell transplant for relapsed disease all ages 2017). Even if the donor is available to provide further collections, the time taken to arrange a donation and the cost of each procurement make it impractical to collect only at need. Given the high relapse rate in allogeneic transplants, it is therefore the preferred option to cryopreserve and store multiple escalating doses when the donor is available, even if there is no clinical need at the time, rather than run the risk of donor unavailability when needed. As a result of this practice, the cells may be stored for months or even years before they are used.

The cost of procuring cells from a donor registry is particularly high, with costs for procurement of a donor from the USA at approximately £30,000 per donation at the time of writing. To save costs, some transplant laboratories routinely utilise surplus cells from mobilised Haematopoietic Progenitor Cell - Apheresis (HPC-A) donations as donor lymphocytes. This saves them the cost of a second procurement if the patient relapses as the cells are already banked. In the South Yorkshire region, clinical practice is to use a maximum of 5 x 10^6 CD34+/Kg body weight for transplant. Allogeneic products containing more than this cell dose are therefore split in the laboratory to create a transplant dose which is issued to the patient and the remainder which are cryopreserved as escalating DLI doses for use against future relapse.

1.2 Cryopreserved DLI Products

1.2.1 Cryopreservation of DLI protocol

Cryopreservation of DLI products is carried out using the same protocols as used for HPC. That these protocols are effective for HPCs is well established as outcome data in the form of patient engraftment and survival can easily be assessed (Tricot *et al.* 1995).

Although there are no infallible *in vitro* tests for the potency of cryopreserved HPC, the ability of the graft to form blood cell colonies as assessed by the colony forming units-granulocyte macrophage (CFU-GM) assay has been shown to correlate well with engraftment (Rowley *et al.* 1987). The CFU-GM assay is currently the standard used worldwide to predict the efficacy of products that have been in long term storage and is mandated as an investigative tool in the 8th edition standards produced by the Foundation For the Accreditation of Cell Therapy (FACT) and spectrum vi, the Joint Accreditation Committee of the International Society for Cellular Therapy (ISCT) and the European Society for Blood and Marrow Transplantation (EBMT). It is performed on a stored pilot vial representative cellular product, or occasionally on a cryopreserved bag of cells, if there are enough to permit sacrifice of a single bag. The decision to use the cryopreserved product is made based on the results obtained from this assay. In clinical practice within NHSBT, the absolute number of viable CD34+ cells in a product (assessed by flow cytometry) is a release criteria for frozen HSC products where there may have been a processing error or that have spent >5 years in liquid nitrogen storage.

Viable cell dose calculation is performed on these cells and the data backed up with CFU-GM assays. Cell viability is assessed using exclusion staining with 7-Aminoactinomycin D (7-AAD). 7-AAD is a dye that stains nuclear material, often binding to double stranded DNA. 7-AAD is excluded from healthy cells as it is unable to pass through intact cell membranes. Cells with membrane damage will allow 7-AAD into the nucleus and cells will fluoresce at 648nm. The intensity of the staining increases as damaged cells take up more dye, making it possible to separate the healthy cell populations from those with membrane damage (Schmid *et al*. 1992). See Figure 1.2.

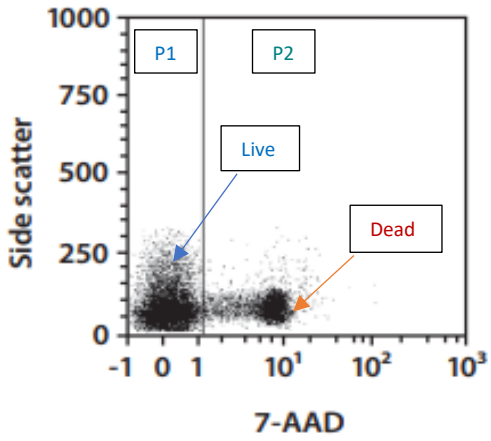


Figure 1.2: Flow cytometry dot plot of live/dead gating with 7-AAD. Two distinct cell populations are visible. Dead cells in region P2 are shown staining positive for 7-AAD while healthy cells have not taken up the dye and are shown in region P1. Image adapted from Adan, A., *et al.* (2017).

There is emerging evidence that lymphocyte cell numbers and activity may be adversely affected by cryopreservation and long-term storage in nitrogen (Li *et al.* 2022, Pi *et al*. 2020), but there is currently no easily available assay to determine the viability and functionality of the stored lymphocytes. Outcome data is difficult to interpret or standardise, as patient response to infusion of DLI is extremely variable. The cells are aliquoted on a crude CD3+ cell analysis with no further phenotyping to identify CD3+ subsets. In the absence of a suitable test for functionality, the only test of the quality of the cell product currently in routine use in most laboratories is assessment of membrane integrity using DNA staining with either trypan blue or 7AAD as performed for CD34+ cells.

However, unlike CD34+ cells there is no *in vitro* test data to confirm the results. Membrane integrity assays are usually performed prior to cryopreservation and provide

an indication of the general condition of the product on arrival at the processing laboratory. Membrane integrity assays can also be performed on a thawed sample of cryopreserved cells to give an indication of how well they have survived the cryopreservation process. However, this assay may detect gross damage to cell membranes but does not provide any indication of the functionality of the cells. There is some evidence, particularly in the case of lymphocytes, that they may appear normal in trypan blue or 7-AAD assays but are apoptotic and unable to respond to stimuli (Sparrow *et al.* 2006). Although calculation of the number of viable CD3+ cells remaining in a DLI frozen product could be performed and used as a release criterion, in practice this is rarely done, largely because of the absence of a suitable *in vitro* test of activation potential to confirm the results.

1.2.2 Cryopreserved DLI Products in the South Yorkshire Region

In the absence of an easily available assay to demonstrate cell viability, clinical practice in the South Yorkshire region is therefore to issue the cells for patient use with no further testing, even after protracted storage. The standard protocol in the region is to freeze multiple doses starting with a dose of 5 x 10^5 CD3+cells/Kg body weight and increasing at half log intervals to a maximum dose of 1 x 10^8 CD3+cells/Kg. The frozen DLI are administered to the patient following the dose escalation procedure described by Mackinnon in 1995. Clinical efficacy of the infused product is measured by assessment of donor chimerism by polymerase chain reaction (PCR) (Bader *et al.* 2005, Tozzo *et al.* 2021).

A completely successful transplant will result in full donor chimerism where 100% of the bone marrow derived cells are of donor origin. Re-emergence of bone marrow derived cells of recipient origin can be an indicator of incipient relapse and in the South Yorkshire

region, donor chimerism is closely monitored for up to 2 years post-transplant. If the percentage of nucleated blood cells derived from the donor drops below 95% then DLI are infused, if available. Chimerism is retested at 2-week intervals post infusion. If there is no clinical response to the lowest cell dose after a period of 6 weeks, as measured by an increase in donor chimerism, then the next dose is infused. The treatment can be continued until either donor chimerism returns to 100% or GvHD is induced. The absence of a detectable clinical response is not investigated partly due to the lack of a suitable assay to provide assurance of the cell viability and partly due to the inherent variability of individual responses (Tozzo *et al.* 2021).

As a result, it is not known whether a poor response by a patient to a product is caused by damage sustained by the cells during storage and cryopreservation or by patient associated factors. Current practice for preparation and storage of DLI is a pragmatic approach designed to minimise costs to the transplant unit and maximise the number of cells stored. It is not based on scientific findings and raises questions about best practice which requires further investigation.

1.3 Impact of Cryopreservation on Lymphocyte function

In 1979, Merker *et al*. reported that freezing peripheral blood lymphocytes did not affect their capacity to form rosettes in a mixed lymphocyte reaction. Subsequently there have been some published studies describing no impact of cryopreservation and storage on the lymphocyte's phenotype or ability to respond to stimuli. Sambor *et al*. (2014) studied frozen samples of 131 leukapheresis products (95 from HIV negative and from 36 HIV positive individuals found that over a cryopreservation period of 7 years, T cell subsets were able to respond to viral antigens Epstein Barr Virus (EBV), Cytomegalus Virus

(CMV), influenza and HIV-1). Viability, recovery, and functionality were consistently recovered from cryopreserved samples from the same donor. The cells in this study were held in liquid nitrogen, but the cryopreservation and thawing methods were not detailed. A comprehensive study by Weinberg *et al.* in 2009 found good correlation between pre- and post-freeze lymphocyte proliferation assays (LPAs), on cells from 104 individuals (49 HIV infected and 55 uninfected). The LPA response to Candida, Tetanus toxin and pokeweed mitogen (PWM) were assessed at 8 hours post collection, after cryopreservation in 10% dimethyl sulphoxide (DMSO) and storage in liquid nitrogen. LPA responses to all the antigens were conserved over cryopreservation and storage for a period of up to 15 months in liquid nitrogen. Weinberg *et al.* 2009 did also report that CD45R0 and CD62L markers, found on naïve T cells (Courville and Lawrence, 2021, Foster *et al.* 2004) were diminished in cryopreserved cells, but there was no demonstrable effect on function. Reduced post-thaw expression of CD62L in Tregs was also reported by Florek *et al.* (2015). In this case, however, Treg function was found to be compromised and they were unable to protect against GvHD in a murine model.

There are reports of cryopreservation having an adverse effect on lymphocyte function. Owen *et al.* (2007) found that the ability of CD4+ cells from HIV infected individuals to produce IFNγ was reduced after >1 year in storage. In 2017, Ford *et al*. also reported a post-thaw reduction in the number of IFNγ producing CD4+ cells derived from peripheral blood mononuclear cells (PBMC) drawn from donors in a in a malaria vaccine trial. Both these results corroborate those reported by Keane *et al*. (2015) who compared leucocyte mitochondrial responses in cells stored in liquid nitrogen for up to 3 months. They found no deficit in mitochondrial responses on storage of up to 1 month, but thereafter found decreasing basal respiration and ATP. However, the cells used in this

study were not specifically MNCs and so the results may not translate across all leucocyte populations. Tompa *et. al* (2018) reported that cryopreservation of lymphocytes from 50 healthy blood donors did not affect the percentage of Treg or memory T cells present post thaw, but it did reduce the numbers of naïve CD4+ and CD8+ cells. Li *et al*. (2021) reported reduction of naïve CD8+ cells (although not CD4+) in cryopreserved PBMC from 57 healthy donors. Li *et al*. also reported loss of Tregs as reported by Florek *et al*. (2015) and Weinberg *et al*. (2009).

Although some studies on natural killer (NK) cells have found no difference in activity pre and post cryopreservation (Pollara *et al.* 2011) there is some convincing evidence differences in activity between cryopreserved and fresh NK cells. Reduced numbers of CD16+CD56dim cells were found in thawed samples compared to fresh, and the level of background CD107a degranulation as higher in samples that had just been thawed (Marti *et al*. 1993, Mata *et al*. 2014). Schafer *et al.* (2020) also found reduced post-thaw numbers of CD56+ cells in a study of 77 cryopreserved mobilised apheresis products. This result is contradicted by a small study by Anderson *et al.* (2019) who reported no difference in the expression of CD4, CD8, CD19 or CD56 in thawed samples from 12 healthy donors. Although Anderson *et al*. found no difference in phenotype post thaw, they did report increased cytokine production in stimulated samples.

In 2020, Schafer *et al.* also reported significant reduction in T cell proliferation ability post-thaw, as did Worsham *et al* (2017) when DLI collected from 12 donors were cryopreserved in 10% DMSO. Worsham *et al*. (2017) compared proliferation and cytokine secretion between paired samples of cells cryopreserved in CryoStor® CS5, which contains 5% DMSO, with those cryopreserved in 10% DMSO. They reported that

cells preserved in CryoStor® CS5 had better preserved proliferation and inflammatory cytokine response compared to those cryopreserved in 10% DMSO. In contrast, in 2019, Panch *et al*. reported that although CD3+ viability was reduced at 48 hours in culture when thawed lymphocytes were used as starting materials for Chimeric Antigen Receptor T cell (CAR-T) manufacture compared to fresh cells, T cell phenotype and expansion in culture was unaffected. The persistence *in vivo* and clinical effectiveness of the manufactured products were also unaffected.

The data reviewed here show that although there is evidence indicating that cryopreservation has no adverse impact on the ability of lymphocytes to respond to stimuli, there are many papers that demonstrate impairment. A large range of freezing and sample handling techniques have been employed by the various groups, and it is possible that reported results, particularly in older studies may have been affected by poor handling. For example, a study from 2000 by Weinberg *et al.* showed that PBMC viability on thawed samples was higher in laboratories with experienced staff. Admittedly the sample number in this study was small (only 54 samples from 27 laboratories) but it gives an indication of how much operator expertise could affect results.

The standard of older published work on cryopreservation is variable overall and the array of different methods employed to achieve the same ends has muddied the waters and led to directly conflicting results. Many of the studies cited are large multicentre trials where the quality of the work produced will have been hard to monitor (Weinberg *et al.* 2000, 2009 and 2010) because of the large number of operator dependant

variables. Some of them (Bourguignon *et al*. 2014, Anderson *et. al* 2019) are reports on very small sample numbers.

Many of the studies do not detail their methods for storage and cryopreservation in the first place. The cryoprotectant used to cryopreserve the cells may well have an impact on both the phenotype and functionality of the thawed cells and incorrect conclusions may be drawn by comparing publications where the where the cryoprotectant media is not stated. 10% DMSO is the most commonly used cryoprotectant for clinical HSC and DLI products (Worsham *et al*. 2017) and there is current debate about its suitability for cryopreservation of lymphocytes. There have been conflicting reports about post-thaw lymphocyte functionality after cryopreservation in 10% DMSO and 5% DMSO. For example, Juhl *et al*. (2021), found more consistent proliferation and mixed lymphocyte reactions in cells cryopreserved in 10% DMSO compared to 5%, whereas Worsham *et al*. found that proliferation ability and inflammatory responses were more severely impaired after cryopreservation in 10% DMSO. Pi *et al*. (2020), reported no differential impairment in recovery or function of either CD4+ or CD8+ cells from 10 healthy volunteers cryopreserved in 10% DMSO or in DMSO free cryoprotectants.

As a part of their 2009 study, Weinberg *et al*. also compared different methods of thawing cryopreserved cells and demonstrated that a fast thaw followed by dropwise addition of wash medium significantly increased cell recovery as measured by trypan blue exclusion, a result that corresponds with the data produced by Disis *et al.* (2006) and Honge *et al*. (2017) demonstrating superior cell viability when thawed cells are diluted dropwise with warmed medium. Honge *et al*. cryopreserved MNC derived from peripheral blood samples from 20 healthy blood donors. Cells were thawed in parallel

and resuspended in media at 4°C, RT and 37°C. The thawed samples were then analysed by flow cytometry and the recovery of viable CD45+ cells assessed by 7-AAD exclusion was calculated. The poorest recovery of viable CD45+ cells was found when cells were resuspended in cold media; there was no significant difference found between media at RT or 37°C. This finding potentially has implications for NHSBT practice, as current SOPs require dropwise dilution of thawed cells in media at 4°C.

Mata *et al.* (2014) showed that resting the thawed cells for at least 5 hours after thawing and washing restored activity in chromium release assays and CD107a degranulation assays to pre-freeze levels. This finding conflicts with the results obtained by Bourguignon *et al*. (2014), where resting post thaw dramatically reduced the recovery of CD8+ cells, and with Germann *et al.* (2013) who demonstrated marked impairment in viability and activity in PBMCs rested overnight post thaw, compared to those challenged with CMV antigen immediately post thaw.

1.4 Cryopreservation methods and storage conditions

Several different cryopreservation methodologies are routinely used in the literature. Some studies used passive techniques, where samples are placed in an insulated box in an electric freezer overnight for up to 24 hours and frozen to -70°C or -80°C and some used rate programmable freezers. Samples that have been passively frozen are variably stored at temperatures ranging from -70-80°C or transferred to liquid nitrogen for ongoing storage. Work by Smith *et al.* (2007) and Weinberg (2010) indicate that storage at -80°C may be associated with poor viability and reduced function, simply because the cells are likely to be subject to temperature fluctuation when in this type of storage.

Programmable freezers use liquid nitrogen to take the sample temperatures down to temperatures below -130°C. On completion of the freezing programme, the samples are then transferred to liquid nitrogen for long term storage. Programmable freezing protocols have been showed to produce a better outcome in terms of cell viability and ability to respond to stimuli (Buhl *et al.* 2012, Koryakina *et al.* 2014).

The wide variation in cryopreservation practice, particularly as described in older published papers, are the primary focus of most publications on cryopreservation and post-cryopreservation storage contributes to the fact that the available literature cannot provide a conclusive answer to the question of whether there is any detectable adverse effect on the cell following storage and/or cryopreservation.

Although the 2009 study by Weinberg showed no significant difference in LPA between cells stored at -70°C and those stored in liquid nitrogen, in 2010 Weinberg *et al.* published a smaller study that showed impaired ELISPOT responses to CMVpp65 antigen for cells stored at -70°C compared to those stored in liquid nitrogen, an impairment which increased with length of storage at this temperature. This result may be explained by examining the data from a comprehensive study by Smith *et al.* (2007). Smith investigated the effects of different sub-optimal storage conditions: -20°C, cycling between -130°C and -70°C or storage in liquid nitrogen (control group). They examined CD4+ cell responses to Varicella Zoster Virus (VZV), a multiple antigen peptide pool and PHA by ELISPOT assay and staining with apoptotic markers. They found marked and significant impairment of the response to stimuli (particularly apparent with the response to PHA) after three cycles of storage temperature fluctuation or after a few hours at -20°C. Unusually, they found that the results improved after >4 cycles of storage

temperature changes between -130°C and -70°C. Staining with Annexin V demonstrated a marked increase in the apoptotic population under poor storage conditions. This study was conducted by using multiple samples from only two donors, but the results reached statistical significance for all parameters studied.

A study by Germann *et al.* (2013) examined the effects of undisturbed storage in liquid nitrogen compared with cycling between -102°C and -135°C on paired samples from 10 healthy donors. They demonstrated conclusively that thawed samples that had cycled between temperatures had reduced viability assessed by trypan blue, and a reduced response to CMV peptide as assessed by production of IFNγ using ELISPOT. Results from cells stored in steady state liquid nitrogen were comparable to fresh cells based on trypan blue exclusion and IFNγ measurement. Similarly, recently published work by the Lonza Group AG showed that lymphocyte responses as assessed by carboxyfluorescein diacetate succinimidyl ester (CFSE) and cytokine secretion were unaffected by cryopreservation that took place <24 hours post collection (Dunnick *et al.* 2022).

1.5 Storage Prior to Cryopreservation

The question as to whether reports of alteration in T cell response is caused by the freeze/thaw process itself, poor sample handling or by storage prior to cryopreservation is not fully answered by the available literature. A case for storage prior to cryopreservation having an adverse impact on lymphocyte viability/functionality was made by Bull *et al*. (2007). In a study on samples taken from 11 healthy donors, they found an 8% loss of viability in peripheral blood cells frozen at 24 hours as opposed to freezing 8 hours post collection. They also found 32% reduction in recovery and 36-56% reduction in interferon gamma (IFNγ) production by enzyme linked immunosorbent spot assays (ELISPOT) if the cells were held for 24 hours prior to cryopreservation. It must be

noted that in this study, cells were stored at room temperature (RT) prior to cryopreservation and the results may have been affected by granulocyte breakdown during storage. Also in 2007, Kierstead *et al*. reported that reducing storage time of PBMC to <12 hours prior to freezing was associated with a marked increase in IFNγ ELISPOT responses and a 3-fold improvement in T cell responses to an adenovirus-based HIV vaccine. In 2009, Chiva-Blanch *et al.* investigated the effect of 24-hour storage followed by cryopreservation on the expression of P-glycoprotein or Rhodamine 123 efflux. They found no significant difference between the behaviour of lymphocytes from 12 healthy volunteers when assayed fresh, fresh frozen, held for 24 hours or frozen after 24 hours. Chiva-Blanch also studied lymphocyte apoptotic markers, instead of trypan blue exclusion and found no significant differences in the Annexin V or 7-AAD staining between the groups, indicating that storage conditions prior to freezing did not push cells into apoptosis. This study remains the only publication that reported the effect of pre-cryopreservation storage for more than a few hours. Unfortunately, it does not detail the exact length of storage, or the storage conditions used.

As a result of this work, larger studies have been conducted by several groups. Olemukan *et al.* (2010) examined the phenotype and response of HIV infected cryopreserved PBMCs from 59 donors over 3 years and reported freezing within 8 hours of collection ensured a stable and continued response over time. However, they did not assess the potential impact of a longer time to cryopreservation by testing after 8 hours. The same applies to the large multi-centre study by Sarzotti-Kelsoe *et al*. (2014) where cell viability was assessed on samples taken from several hundred HIV and autoimmune patients at 3 different study sites. It has been assumed that the good recoveries and viabilities obtained were due to the short time from collection to freezing, but no functional

testing was carried out. In a small study of HIV CD8+ lymphocytes from 22 individuals, Bourguignon *et al.* (2014) reported reduced recovery of cells if they were stored for >7 hours prior to cryopreservation, but on further investigation, this effect is marginal and not statistically significant. Weinberg (2010) also states that in their 2009 study that storage for >8 hours prior to cryopreservation reduced recovery and viability of cells, but this is not reported in the 2009 paper. Similarly, Olson *et al.* (2011) reported loss of lymphocyte viability and function in samples drawn from 250 healthy donors if the samples were shipped at temperatures <22°C for >8 hours. More recently, studies on PBMCs isolated from donor blood samples have shown no impact on thawed lymphocyte phenotype (Anderson *et al*. 2019) or function after storage for up to 24 hours at 4°C prior to cryopreservation (Honge *et al*. 2017) or alternatively have found significant decreased numbers of viable CD4+ lymphocytes compared to samples stored at RT after storage for up to 24 hours (Jerram *et al.* 2021).

Other papers do not specify whether or for how long, or under what conditions cells were held prior to cryopreservation so it is difficult to draw any conclusions. The available data is limited and storage times and conditions are largely not described. Most of the relevant publications are old, reflecting the fact that clinical practice was established many years ago and there has been little incentive to investigate possible improvements until recently. It must also be stressed that most published studies relate to PBMCs derived from peripheral blood samples stored in blood tubes, not bags as is the case for clinical transplant products and therefore the obtained results cannot be fully applied to clinical products.

What published work there is on clinical products focusses on storage times of up to 24 hours (only the study by Chiva-Blanch in 2009 investigated the effects of storage for >24 hours), when in clinical practice in the South Yorkshire region, lymphocytes are almost always at least 24 hours old when they are cryopreserved and sometimes considerably older. The question of whether extended storage prior to cryopreservation impacts on lymphocyte responsiveness remains unanswered.

A definitive answer is now even more urgently required due to changes in clinical practice and adoption of CAR-T therapies. CAR-T are anti-cancer cellular therapeutic products manufactured from peripheral blood T cells. The T cell receptor is genetically modified *ex-vivo* to bind to tumour specific markers. In the last 2-3 years CAR-T therapies targeting CD19 or CD20 on B lymphocytes have moved from 4th line treatment to 2nd line treatment for B cell leukaemia and lymphoma. CAR-T cells are prepared from lymphocytes harvested by apheresis. All CAR-T products currently licensed in the UK are autologous products so must be collected from the patient, wherever they are located and shipped to the manufacturing facility, which may be some distance away.

Cells are subject to a manufacturing process that takes 12-14 days, and the cellular starting material must be sufficiently viable to survive the manipulation and expand to produce a clinical dose. Given the uncertainty around lymphocyte responsiveness after extended storage prior to cryopreservation, some manufacturers prefer to cryopreserve the starting material immediately after collection and ship frozen cells to the manufacturing site, accepting the risk of a catastrophic failure during cryopreservation (KYMRIAH® Novartis Europharm). Others ship the starting material unfrozen at 4°C (YESCARTA®, Kite Pharma) and start the manufacturing process within 24 hours of

collection which, although achievable in many cases, limits the accessibility of the therapy to patients located within shipping distance of the manufacturing facility and places great strain on the logistics infrastructure. Products for both HSCT and CAR-T production are uniformly shipped at 4°C which contradicts Jerram's 2021 finding that thawed PBMCs from donor blood samples fare better if stored at 37°C prior to cryopreservation. In 2022 Johnson *et al*. compared the responses of PBMCs derived from 8 healthy blood donors against *S. aureus* after shipment at ambient temperature for up to 48 hours. They reported that lymphocyte phenotype and function was conserved for up to 24 hours storage but a decrease in the inflammatory response was seen after 48 hours. Again, this study was performed on peripheral blood collected in tubes and cannot be directly extrapolated to apply to products shipped in bags, but it does have implications for current shipping and storage practices.

1.6 G-CSF mobilised harvests

As previously discussed, to save the transplant centre the cost of procuring another donation, DLI are often prepared from surplus HPC, apheresis cells that are not required for the initial transplant. As these are mobilised cells collected by apheresis, they have of necessity been exposed to G-CSF during the collection process. It is known that G-CSF can inhibit T-lymphocyte function, reducing levels of some adhesion factors and pushing naïve CD4+ and CD8+ T cells into a state of anergy (Vasconcelos *et al.* 2003, Chang *et al*. 2009). G-CSF has been shown to induce tolerance and suppression of lymphocyte function and expansion (Mielcarek *et al*. 1998, Joshii *et al*. 2001, Rutella 2007) and post-transplant administration of G-CSF has been shown to impair immune recovery in hapolidentical transplants (Volpi *et al.* 2001). There is significant evidence that G-CSF has anti-inflammatory and anti-apoptotic properties (Boneberg *et al.* 2002, Katoda *et al*.

2012, Wright *et al.* 2017, Modi *et al*. 2020). The immune modulating effect of G-CSF therefore raises the question of whether the DLI prepared from G-CSF mobilised products will be clinically effective as the lymphocytes may be less responsive than lymphocytes collected from non-mobilised donors. The transplant centre may be guilty of a false economy in funding preparation and long-term storage of such cells. Clinical opinion in the UK as to the benefit of DLIs prepared from mobilised products is currently split. In the Yorkshire region alone, the South Yorkshire transplant teams take the view that if excess mobilised cells are available then they are used as DLI, while in West Yorkshire they are not used and non-mobilised collections are commissioned at need. Outcome from both the South and West Yorkshire centres working with NHSBT meets BSBMT benchmarks (confidential data – cannot be shared) so little can be gleaned from the clinical data.

Contaminating granulocytes do not survive storage or freeze/thaw procedures well. As G-CSF stimulates granulocyte proliferation, HSC products mobilised with G-CSF, may contain large numbers of contaminating granulocytes (Vasconcelos *et al*. 2003) which will rapidly become apoptotic if the products are stored for any length of time, or when they are cryopreserved. The Spectra Optia apheresis devices currently in use at NHSBT apheresis collection centres are programmed to collect only the MNC fraction, reducing but not eradicating granulocyte contamination in apheresis products (Lopez-Pereira *et al.* 2020). However, despite recent technological advances such as the Optia cMNC programme, granulocyte counts in apheresis products remain high and investigators intending to study PBMCs from mobilised subjects need to be aware of the potential problems they can cause. Dead granulocytes release cytoplasmic and nuclear material into the local environment. Decay products from dead granulocytes can cause death of

other cell types via bystander damage, leading to reduction in reported cell numbers and viability. They can also physically interfere with assays by causing cell clumping that can block some analytical instruments (Agashe *et al.* 2017).

Flow cytometers where cells are projected through a narrow aperture are particularly vulnerable to blockage and results from time sensitive tests can be lost if this happens. Treatment of the thawed samples with DNAse may make it easier to analyse the cells (Garcia-Pineres *et al*. 2006) but does not reflect the situation *in vivo*. It is possible to remove granulocytes by density separation, but this contributes further variables and may damage the ability of MNCs to respond functionally. Density gradient separation may damage or activate some cell types but using whole blood may suppress the activation of others. Each sample type has its own pitfalls and optimisation of sample preparation is required.

1.7 Laboratory measurement of lymphocyte activation

Lymphocyte activation in response to stimulus can be measured in a variety of ways; cell expansion, cytokine production, or expression of cell surface markers associated with activation. Historically, the gold standard by which cell division has been measured in the laboratory was by tritiated thymidine (Taylor *et al.* 1956). Dividing cells incorporate the radioisotope into their DNA and the radio-signal can then be detected by scintillation counting. Signal strength is proportional the amount of cell division. However, the use of radioisotopes has health and safety implications and other ways of monitoring cell division have been adopted. Flow cytometric approaches can capture cell division utilising the fluorescent dye CFSE. This dye penetrates cell membranes and binds to cytoplasmic proteins. When analysed by flow cytometry, steady state cells absorb the dye and give a single highly fluorescent peak. As cells divide, the stain is incorporated

into the next generation of cells and the fluorescence decreases with each division as the dye is diluted (Lyons and Parish, 1994). CFSE has been shown to be capable of demonstrating cell division in activated lymphocytes (Lyons 2000, Dunnick *et al*. 2022). Although CFSE is easy to use and has been shown be sensitive and consistent, it does have some disadvantages. Fluorescein is one of the most used conjugates for commercial flow cytometry reagents. Using CFSE in a multi parameter analysis, therefore prevents the researcher from using some of the best and most easily available reagents particularly if using an instrument not equipped with a violet laser (Quah and Parish, 2012).

Analysis of cell phenotype and number together with their ability to produce cytokines are essential for the assessment of the ability of the lymphocyte response to stimulus. The principal target cytokines in published papers are IFNγ, tumour necrosis factor alpha (TNFα), and interleukin 2 (IL2), although tests can be developed for any cytokine for which there is a monoclonal antibody (Huang *et al*. 2015).

Cytokine production can be analysed as they are secreted extracellularly by enzyme linked immunosorbent assays (ELISAs) or by ELISPOT assays (Mobs and Schmidt 2016), and commercial kits are available for most cytokines for either technology. Alternatively intracellular cytokine (ICC), production can be assessed (Garibay-Escobar *et al*. 2003). ICC assays have the advantage over ELISPOT in that the cells producing the cytokines can be characterised by cell surface markers simultaneously (Sambor *et al*. 2014, Munier *et al.* 2009). In Garibay-Escobar's initial publication, cells from healthy controls and patients with chronic tuberculosis were stimulated with PHA and /or recombinant IL2. The study showed that stimulated lymphocytes, upregulated the markers CD25 and

CD69 (CD69 only being present on activated cells). CD3+CD69+ cells produced IL2, and IFNγ whereas CD3+CD25+ cells produced IL2.

CD25 and CD69 have been well characterised and have been used in numerous studies to assess T lymphocyte activation, making them good candidate markers for use in this current study. See Figure 1.3.

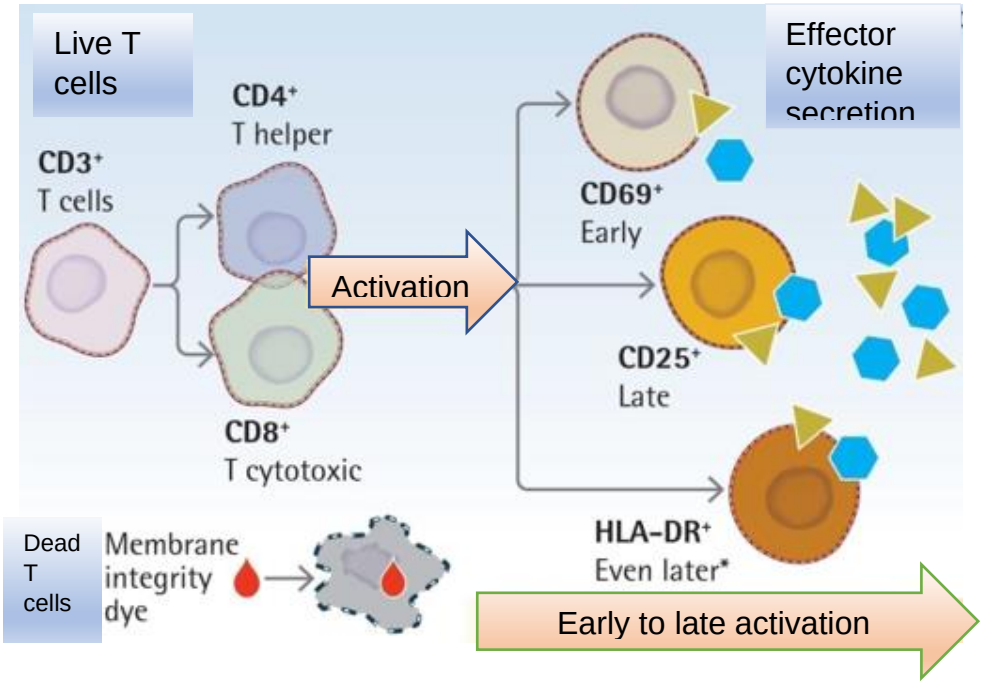


Figure 1.3: Schematic of early and late phase T cell activation markers showing early activation (CD69), mid-stage activation (CD25) and late activation (HLA-DR). Image adapted from Sartorius https://intellicyt.com/. Downloaded 23/03/23.

The first marker to be upregulated after stimulation is CD69 (Bajnok *et al.* 2017). CD69 is an inducible type II C-Lectin receptor expressed on the cell surface after T cell receptor (TCR)/CD3 engagement. It is expressed at very low levels on resting T cells (Garibay-Escobar *et al.* 2003) and has been shown to upregulate on most lymphocyte and NK cells

between 3-12 hours after activation, remaining elevated for up to 24 hours and then declining (Reddy *et al.* 2004). Surprisingly, it may also have a role in regulating the immune responses of T helper cells (TH1 and TH17) cells (Cibrian, Sanchez-Madrid, 2017).

CD25 is the α chain of the trimeric IL2 receptor. It is normally expressed at low levels on the surface of regulatory and resting memory phenotypes (McHugh *et al.* 2002) and is upregulated on the surface of activated T cells, approximately 24 hours after TCR/CD3 engagement. Levels remain elevated for some days (Caruso *et al.* 1997). See Figure 1.4.

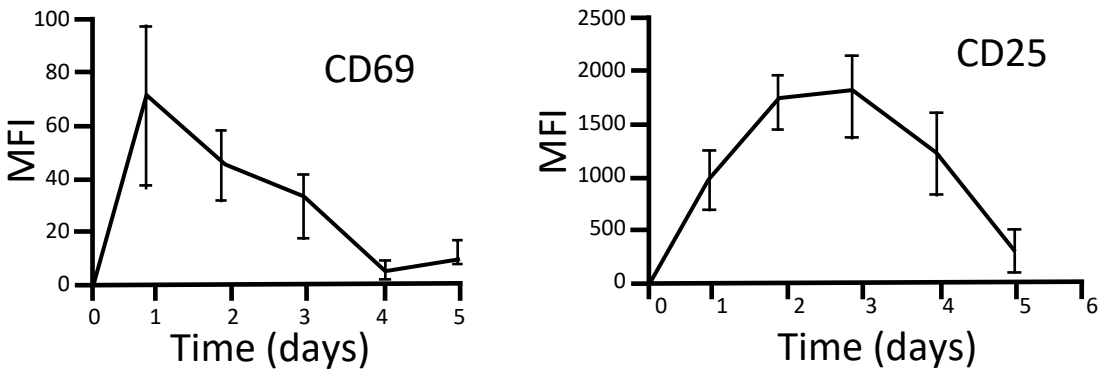


Figure 1.4: Timelines for CD25 and CD69 expression on T lymphocytes after stimulation showing mean fluorescence intensity (MFI) peak at 24 hours for CD69 and 48-72 hours for CD25.
Image adapted from Teixero *et al*. (2009).

1.8 Stimulation and Culture Conditions

It is clear from the various reports in the literature that the strength of the lymphocyte response to stimulation varies considerably depending on the nature of the antigen. The most used antigen is PHA which elicits a strong response under most conditions, and it was used in several of the papers reviewed here. Smith *et al*. (2007) demonstrated that

the PHA response in an LPA was tenfold stronger than the response against CMV antigen, as measured by ICC assays. The strength of the result did not compromise sensitivity. Several studies have used viral or fungal antigens in LPAs, as many of them relate to vaccine research, and responses to these are harder to measure as they are generally weaker but are still capable of attaining statistical significance under test conditions (Weinberg 2000, 2009, 2010, Sambor *et al*.2014, Smith *et al.* 2015). Other studies have successfully used tuberculosis (TB) antigens, pertussis toxin and a variety of bacterial antigens. It is also possible to use manufactured CD3/CD28 stimulation beads (Ruitenberg *et al*. 2011, da Silva Ferreira *et al.* 2015) which circumvent the problems posed to the researcher caused by the requirement to hold pathogenic organisms/toxins in a licensed cell therapy manufacturing unit such as at NHSBT. The beads are well defined and are manufactured to good manufacturing practice (GMP) standard which eliminates problems related to inter-batch variability. They have been shown to induce reliable activation comparable with that generated by PHA (Jiao *et al*. 2019). The disadvantage of using CD3/CD28 stimulation beads is that they block the CD3 site on the cell so analysis of CD3 positive cell numbers is no longer possible by flow cytometry.

1.9 Current protocols for sample handling / storage or cryopreservation on lymphocytes

In the South Yorkshire region, 85% of DLI are prepared from surplus HPC-A. Although some are collected in Sheffield for related recipients, many other products are collected by donor registries and may originate from collection centres both within and outside the UK. Typically, a donor apheresis collection is booked over two days. If sufficient cells for transplant are collected in a single day, cells will be shipped the day following

collection and will arrive approximately 24 hours after they have been collected for a UK collection or 36 hours after a European/USA collection. If the collection requires 2 days to obtain sufficient cells for transplant, the cells will be shipped on the day of the second collection. In practice, this means that cells from donor registries are always a minimum of 24 hours old on receipt in the laboratory and may well be significantly older. Cells from overseas collection centres and 2-day collections typically arrive out of working hours (usually between 7pm and 1am) and because of overnight staffing constraints, are not processed at NHSBT until the following day.

On arrival in the laboratory, priority is always given to preparation of the portion of cells that will be issued for transplant, as these are the cells on which the patient's life depends. In an NHS environment where staffing resources are limited, this may mean that for products arriving mid-afternoon, the donor lymphocyte cryopreservation is frequently held over to the following day while priority is given to preparation issue of the transplant dose. The result of this practice is that if cells have already been in transit for 24-48 hours to reach the laboratory, they can be approaching 72 hours by the time they are cryopreserved. 72 hours post end of collection is the maximum permitted time for cells to be stored prior to cryopreservation according to NHSBT protocols. This time limit was determined as the maximum time whereby the benefit of keeping cells fresh outweighs the benefit of freezing them (Pamphillon and Mijovic 2007). It has subsequently been adopted as a worldwide standard.

Work leading to this prescribed time limit was however done on CD34+ HPCs not lymphocytes although it is applied to them equally. Non-mobilised lymphocyte collections are received more rapidly as they are only ever booked as a 1-day collection

and are shipped immediately after completion of collection. Even those coming from the USA are usually received within 24 hours, although if they arrive late in the working day, staffing constraints in the evenings are such that they may not be cryopreserved until the following day by which time they may be up to 36 hours old.

There are two distinct points to be addressed concerning storage and cryopreservation of DLI preparations. Lymphocytes are often stored/and or cryopreserved for use in ongoing studies, as well as for therapeutic use in transplantation. Most of the published data relates to sample storage for functional assays in autoimmune disease or immunodeficiencies. The effect of storage prior to cryopreservation has been little studied, but there is a reasonable amount of published work on the effect of cryopreservation itself on lymphocytes.

1.10 Study Aims and Objectives

The aim of the study was to provide a definitive answer to the questions about the responsiveness of lymphocytes that have been stored at 4°C for up to 72 hours prior to cryopreservation, as is current clinical practice in the Yorkshire region. Tightly controlled and monitored conditions for storage and cryopreservation were applied according to NHSBT protocols to ensure that the data produced was not affected by the sample handling discrepancies commonly seen in the literature. This was determined by three key objectives:

i) Cells destined for use as DLI were cryopreserved immediately on receipt in the laboratory and additional samples were stored for up to 72 hours at 4°C to assess any impact of storage, prior to freezing, on lymphocyte responsiveness.

ii) Lymphocytes from G-CSF mobilised harvests as well as those that originated from non-mobilised harvests were assessed under the same conditions to determine any differences in response to stimulation.

iii) In addition, the study aimed to develop an *in vitro* test of lymphocyte functionality that could be used within NHSBT laboratories to confirm fitness for use of clinical DLI products. If suitable, this test will then be further used to prospectively test the responsiveness of clinical products in long term storage and the results used to inform clinical practice within the NHS as to the cost-effectiveness of cryopreservation and long-term storage of DLI.

2 Chapter 2 Materials and Methods

2.1 Project Overview

The aim of this research was to investigate differences in the response of cryopreserved human CD3+ lymphocytes to activation after thawing, compared with fresh lymphocyte preparations. Lymphocytes were isolated from peripheral blood, stored for up to 72 hours at 4°C and then cryopreserved. Cryopreserved lymphocytes were subsequently thawed and stimulated with beads coated with anti-CD3 and anti-CD28 to stimulate T cells in a manner that partially mimics stimulation by antigen-presenting cells. Phenotyping by flow cytometry for lymphocyte subsets and activation markers was performed prior to cryopreservation as well as after thawing, at 4-, 24- and 72-hours post thaw and following activation.

The lymphocytes used in the study were isolated from:

i) The apheresis cones of healthy platelet donors.

ii) Apheresis harnesses of healthy donors undergoing collection of G-CSF mobilised peripheral blood stem cells.

iii) Samples taken for quality control analysis from G-CSF mobilised apheresis products collected from healthy HSC donors.

Samples were collected and analysed over the period February 2022 – March 2023. Colleagues from the Advanced Therapy Team at NHSBT Barnsley helped with preparing and running samples on the flow cytometer.

2.2 Research Ethics Overview

The apheresis cones and harnesses used in the study were collected via the NHSBT non-clinical issue system, which is in place to provide researchers with anonymised human

material from volunteer donors. The materials are available for projects meeting criteria for either service development or ethically approved research and are made available on successful application to NHSBT. The researcher must demonstrate either approval from an HRA Research Ethics Committee or a completed HRA online research tool indicating that ethical approval is not required. This study did not require ethical approval as it qualified as service development, as determined by use of the NHS HRA tool (Appendix 1). Materials provided by NHSBT for non-clinical use are appropriately consented and supply complies with all statutory and regulatory obligations including (but not limited to) the Human Tissue Act (2004) and associated Codes of Practice and Standards. Materials supplied are NOT consented for use in animal models or in studies where genetic analysis may establish the identity of the donor. The applicant must work in a facility with appropriate HTA licenses for the type of study to be performed. Once the scope of the research had been reviewed and accepted by the internal R&D committee, a non-clinical issue account is authorised for use only in the stated study. In this case, an application was made for supply of materials for the study in April 2022 and the application was accepted in May 2022. Material is supplied on receipt of a written application by the researcher (see Appendix 2). No information about the donor, including age or sex is available to the researcher.

The samples studied from apheresis products were from healthy volunteer donors. All donors who have products collected by NHSBT provide consent for the use of samples of fresh or frozen blood or product for testing relevant to the quality of their stored cells. This information is captured in Parts 1 and 2 on FRM1570 Consent for Testing Storage and Discard of Stem Cells or Lymphocytes (see Appendix 3).

2.3 Lymphocyte preparations

2.3.1 Sample numbers

Sample numbers used in the study were determined through review of published studies. Sample numbers in reported literature were found to be highly variable depending on study design. High sample numbers ranging from 50 to several hundred were seen in large multicentre studies primarily focussed on HIV research or tissue banking (Bourguinon *et al.* 2014, Sambor *et al.* 2014, Smith *et al.* 2007). Sample numbers were far lower in those studies with a similar design to the proposed study: *in vitro* studies on human samples with a focus on lymphocyte cryopreservation, storage or activation (Boudreaux *et al.* 2019, Buhl *et al.* 2012, Garibay-Escobar 2003). Sample numbers in these type of studies ranged from as few as 6 (Kumar and Meneghal, 2022) to 20 (Bajnok *et al.* 2017). Due to the scarcity of the starting materials, after consultation with the Sheffield Hallam University statistician, it was initially decided that a sample size of 15 would be used as this number fell within the mid-range seen in the literature.

2.3.2 Apheresis cones

Apheresis cones are waste products produced from the apheresis harnesses used in collection of platelets from healthy donors on a cell separator. During the collection procedure, cells are trapped in this part of the apheresis set and can be retrieved aseptically in the laboratory. Apheresis cones were selected as starting materials for this study as they are rich sources of nucleated cells collected from healthy donors. Lymphocyte numbers of up to 1 x 10^9 can be isolated from a single cone, and the characteristics of the lymphocytes isolated from them have been shown to be equivalent to those isolated from peripheral blood (Boudreaux *et al.* 2019). Both the cell concentration and their characteristics were ideal for the purpose of this study. Cones

are readily available to researchers within NHSBT and can be ordered to arrive within specified timeframes which allowed cell analysis in a timely manner. As the donors are healthy, functionality of the cells derived from the cones was expected to fall within the normal range.

One of the aims of this study was to compare the response of lymphocytes collected by apheresis that have been exposed to G-CSF to those that have not, after cryopreservation and thawing following the sample handling procedures used for clinical products within NHSBT (see Appendix 4 for full list of NHSBT Standard Operating Procedures and controlled documents followed in the study). It was therefore essential to ensure that there was no possibility that the cones were obtained from a donor who had recently been exposed to G-CSF as either donor or patient. Although some platelet donors are registered on the stem cell donor registries (Anthony Nolan, British Bone Marrow Registry), anyone who has donated blood or bone marrow is excluded from any kind of blood component donation for a minimum period of 6 months. People who have been treated for cancer are also excluded from being platelet donors. Therefore, it was certain that the cone cells used had not been exposed to G-CSF and were suitable for the non-G-CSF arm of the study. Apheresis cones were collected during the afternoon session at NHSBT collection centres in Leeds and Sheffield following SOP412 – Trima Apheresis Procedures and shipped to the study site as soon as they were available, arriving between 17.00 - 19.00. They were placed into temperature controlled and monitored storage at 4°C immediately on receipt at NHSBT and stored overnight. The cones were processed the following morning, to ensure that the Day 1 cells were cryopreserved within 24 hours of collection. Cones that had been delayed in transit and unable to be processed within 24 hours of collection were omitted from the study.

2.3.3 Apheresis harnesses

After an apheresis collection, the used harness contains large numbers of cells that cannot be extracted for clinical use but can be aseptically collected in a laboratory. They are typically used as a source of $CD34^+$ mobilised peripheral blood stem cells for research applications but all leukocyte sub-types are present. The cells of interest in this study were the lymphocytes that had been exposed to G-CSF during the mobilisation process. The apheresis harnesses were from G-CSF mobilised peripheral blood collections performed at the NHSBT Therapeutic Apheresis centres in Leeds and Sheffield. The collections were performed in the morning and the harnesses were shipped to the study site as soon as they were available, arriving at approximately 14.00. They were stored overnight at 4°C as in section 2.3.2 above. Harnesses that were delayed in transit and unable to be processed within 24 hours of collection were omitted from the study.

2.3.4 Mobilised cells from apheresis collections

Mobilised apheresis products contain very large numbers of cells. A nucleated cell count of >200 x 10^6/mL is normally expected in products collected at NBSBT apheresis units. 1.5mL sample of product therefore contained a minimum of 300 x 10^6 nucleated cells which was sufficient to perform all the testing required for the current study. On receipt of the product in the Cellular and Molecular Therapy (CMT) laboratory, 5-6mL of product is routinely removed for quality control testing, including sterility, full blood count and flow cytometric analysis of CD34+ cells. The cells used in this study were taken from the subsample removed for quality control testing and did not therefore impact on the recipient. The samples used in this study were from mobilised products collected from Antony Nolan Centre donors at the NHSBT Therapeutic Apheresis centre in Sheffield. Samples were received immediately post collection, diluted to a volume of 10mL with

4.5% Huma Albumen Serum (HAS), stored overnight at 4°C and used for the study within 24 hours of collection. Samples from products with a nucleated cell count of <200 x 10^6/mL were not used as they did not contain sufficient cells to complete all experimental conditions.

2.4 Key Technology

All instruments used in this study were in routine use within NHSBT laboratory at the time and were calibrated and maintained according to the manufacturers' specifications. Control testing to ensure that the equipment was functioning to specification was performed daily for those items of equipment requiring daily validation. Controls were run on the equipment, prior to starting work on the study samples. If the controls failed, study samples were not analysed until satisfactory corrective action had been taken and control testing had passed specification.

2.4.1 Equipment used in this study

Refer to Table 2.1 below.

Equipment	Make/Model	Used for
Haematology analyser	Sysmex XS1000i	Full blood counts on samples, Total nucleated cells
Programmable rate-controlled freezer	Planer Kryo 560-16	Rate controlled cryopreservation of samples
Vapour phase nitrogen storage vessel	MVE XLC 1400	Vapour phase storage of cryopreserved samples
Sterile Connecting Device	Terumo TSCDII	Aseptic connections
Heat sealer	Lyungberg & Kogel Biosealer CR4	Sealing lines connecting bags/other consumables
CO_2 incubator	PhCBI Incusafe	CO_2 controlled culture system
Environmental monitoring system	Pharmagraph	Monitoring of environmental conditions (temperature, CO_2 concentration)

Table 2.1 Key equipment used in the study.

2.4.2 Flow cytometer

The standard methods for measurement of T cell immune responses, include ELISPOT, intracellular cytokine staining (ICS) assay, tetramer assay and flow cytometry. In this study, cell phenotyping by flow cytometry was selected as the appropriate tool for assessing cellular responsiveness, as it has been demonstrated to be a powerful technique for functional T cell analysis (Mousset *et al.* 2019). Developed in 1968 by Wolfgang Gohde, flow cytometry is an analytical methodology whereby individual cells expressing specific markers can be accurately counted. Fluorescent markers are attached to individual cells using monoclonal antibodies targeting extracellular or intracellular structures. The cells are passed through a laser in a unicellular stream and if a fluorescent dye is attached to the cells a flash of light is emitted. The light emitted is passed through a series of filters and mirrors before it is captured in a photomultiplier tube. A detector in front of the light beam measures light scattered forward (forward scatter (FS)) and detectors to the side of the beam measure light scattered sideways (sideways scatter (SS)). FS correlates with cell size and SS with cell granularity, allowing cells to be characterised on the basis of FS/SS patterns (Macey 2007). Staining of cell surface markers with fluorescent dyes enables the cells to be further characterised based on both morphology and the light emission from the cell specific markers expressed. See Figure. 2.1.

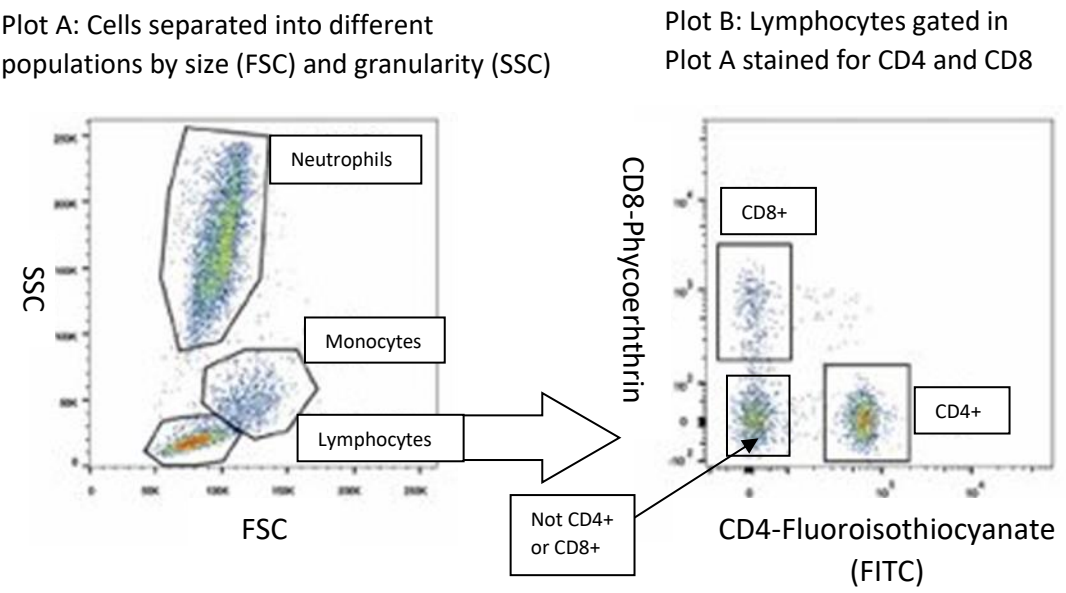


Figure 2.1: Flow cytometry dot plot of leukocytes (Plot A) Forward scatter (FSC) versus side scatter (SSC) (Plot A) and CD4-FITC versus CD8-PE (Plot B). *Each* dot represents a single cell analysed by the flow cytometer. The characteristic position of different cell populations is determined by differences in cell size and granularity. After gating on the lymphocyte population, cells stained positive for CD4-FITC (516nm) and CD8-PE (574nm) can be identified. Cells not stained with either CD4 or CD8 are shown close to the axes. Image adapted from Riley, R. and Idowu, M. Principles and Applications of Flow Cytometry (2012).

Flow cytometry was used as the primary method for cell phenotyping and enumeration. The flow cytometer used in this study was a FACSLyric™ (Becton Dickinson, Wokingham, UK). It is a two-laser instrument equipped with a blue laser (488nm) and a red laser (460nm). It can analyse up to a total of 6 colours (4 on the blue laser and 2 on the red laser). The instrument was used in conjunction with TruCount™ Tubes (Becton Dickinson, Wokingham, UK) which allowed measurement of absolute cell counts.

2.4.3 Set up of the flow cytometric protocols: Colour Compensation

Spectral overlap is the phenomenon produced when emissions from one fluorochrome are detected in channels designed for a different fluorochrome. It occurs because most fluorochromes have broad emission spectra and although the band-pass filters within

the instrument are designed to select the appropriate excitation and emission ranges for specific fluorophores, the trailing edge of emission from one fluorochrome may be detected as a false positive signal by the detector for another. For example, as seen in Figure 2.3, although FITC peak emission is at 516nm, it continues to emit light at up to 600nm and spills over into the PE detector with peak emission at 574nm.

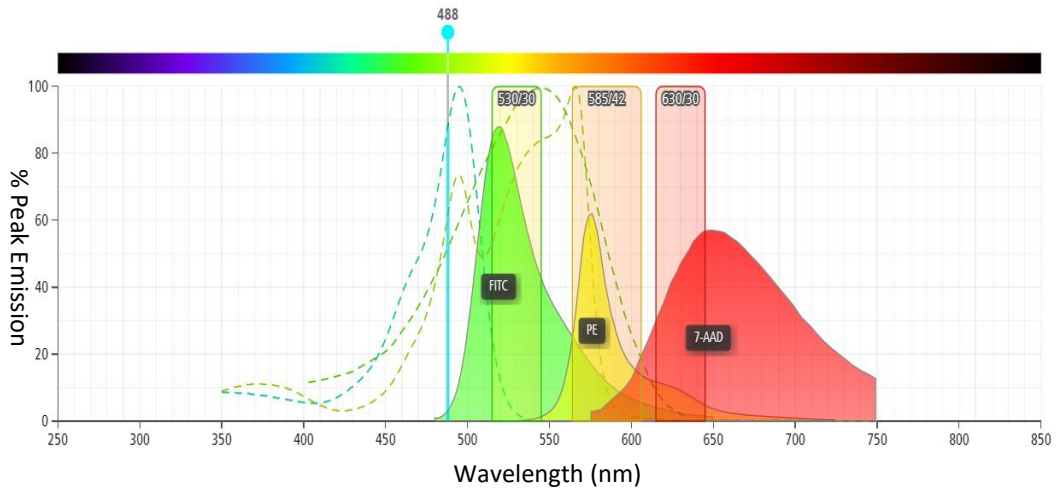


Figure 2.2: Demonstration of spectral emission overlap between Fluoroisothiocyanate (FITC), Phycoerythrin (PE) and 7-amino-actinomycin D (7-AAD) when excited by a 488nm blue laser. Image generated from BD Biosciences Spectrum Viewer.

Colour compensation can be used to reduce the impact of spectral overlap by preventing the detection of the unwanted overlap signal. However, some fluorochromes combinations have such severe spectral overlap that they cannot be compensated for and therefore cannot be used together. Selection of appropriate fluorophores is therefore a critical part of study design (McKinnon 2018). As the study samples were

analysed on a 2 laser Becton Dickinson FACSLyric™, advice about suitable fluorochromes taken from the Becton Dickinson technical expert to ensure those selected could be adequately compensated on the instrument (Table 2.2).

Fluorochrome used in study	Excitation wavelength (nm)	Emission wavelength (nm)
Phycoerythrin Cyanine 7(PECy7)	566	778
Fluorescein 5-isothiocyanate (FITC)	491	516
Allophycocyanin-Cy7 (APC-Cy7)	754	779
Phycoerythrin (PE)-Cy7	566	574
Phycoerythrin (PE)	566	574
Allophycocyanin (APC	651	660
Allophycocyanin (APC)	651	660
Fluorescein 5-isothiocyanate (FITC)	491	516
Phycoerythrin (PE)	566	574
Phycoerythrin (PE)	566	574
Phycoerythrin Cyanine 7(PECy7)	566	788
7-aminoactinomycin-D	546	647

Table 2.2 Fluorochromes used in the study; selected to minimise spectral overlap (from BD Biosciences Fluorochrome Reference Chart).

2.4.4 Panel 1: Lymphocyte Subsets

Panel 1 was designed to provide information about the phenotype of the starting material. It included cell markers commonly used to characterise lymphocytes (Wang *et al.* 2017) and fluorochromes that were compatible with the FACSLyric™ (Omana-Zapata *et al.* 2019). Human peripheral blood samples are inherently variable and to be able to fully understand any observed experimental effects it was necessary to understand the cellular content of the starting material. Differences in the responses of cells from

different donors may be a direct result of the presence of different cell populations and totally unrelated to experimental conditions. To this end, a standard lymphocyte phenotyping panel was used to determine both the absolute number and percentage of cells staining positive for CD3, CD4, CD19, CD25, CD56 and CD16. This enabled characterisation and enumeration of T and B lymphocytes, Tregs, and NK cells. Panels designed using these markers were initially defined several years ago (Maecker *et al.* 2007, Wullner *et al.* 2010, Mata *et al.* 2014) but the basic elements of the phenotype are still included in more recent and complex studies (Blache *et al.* 2021) indicating that they were still relevant and a suitable panel to use in this study.

The antibodies used are detailed Table 2.3.

Target Cell Population	**CD Markers Defining Population**	**Antibodies**
Nucleated Cells	CD45	Anti-CD45-FITC
Viable cells	None – DNA binding	7-AAD
T Lymphocytes	CD3	Anti-CD3-PE-Cy7
Th Lymphocytes	CD3+CD4+	Anti-CD3-PE-Cy7 Anti-CD4-APC-Cy7
Cytotoxic T Lymphocytes	CD3+CD4-	Anti-CD3-PE-Cy7 Anti-CD4-APC-Cy7
B Lymphocytes	CD19+	Anti-CD19-APC
Natural Killer Cells	CD3-CD16+ and or CD56+	Anti-CD3-PE-Cy7 Anti-CD16/56-PE
Monocytes	CD16+	Anti-CD16-PE

Table 2.3 Antibodies used in Panel 1.

Fresh pre-cryopreservation samples were tested with this panel after density separation and washing (cones and harnesses) or dilution (apheresis product).

Panel 1 gating hierarchy

The gating strategy is shown in Figure 2.3. Cells were identified as events that were not excluded as either Beads or Debris as shown in Plot 2. Events characterised as Cells were classified as viable or non-viable according to 7-AAD absorption (Plot 3). Viable Cells were then gated against CD45/SSC to identify cell populations by morphology (Plot 4). Although T lymphocytes require the presence of monocytes to respond to antigen (Mata *et al.* 2014), the classical monocyte marker CD14 could not be included in the panel due to lack of available fluorescence channels on the flow cytometer used for the study. Although most monocytes are also CD16+ (Kapellos *et al.* 2019), the limitations of the instrument prevented the use of anti-CD16 for monocyte identification. Monocytes were therefore identified morphologically by FS/SC. Viable monocytes were identified by morphology in Plot 4 (Monos) and backgated into Histogram 1 to confirm their scatter pattern remained as expected for monocytes (Monos2). Viable lymphocytes were identified by morphology (Plot 4) and then gated against CD3, CD4, CD19 and CD16/CD56 to identify Th cells, cytotoxic T cells and NK cells as shown in Plots 5, 6 and 7. Due to the limitations of the flow cytometer it was not possible to use anti-CD8 in Panel 1. CD8+ cells were therefore identified as CD3+CD4- lymphocytes. The validity of this approach was tested by comparison of the number of CD3+CD4- events/µL from Panel 1 against the number of CD3+CD8+ events from Panel 3 where it was possible to include anti-CD8.

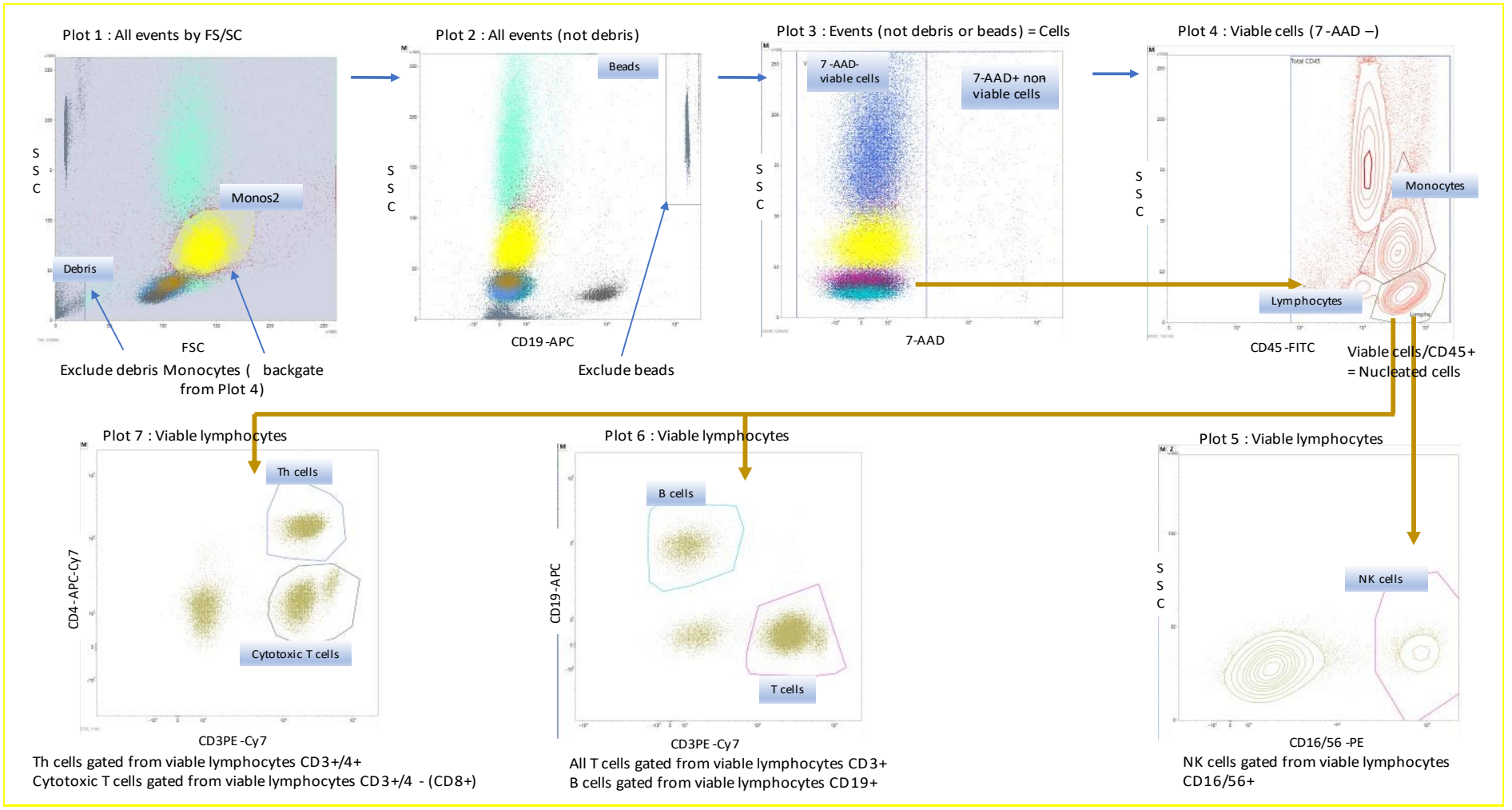


Figure 2.3: Panel 1 gating hierarchy. Debris and Beads excluded as shown on Plots 1 and 2. Cells identified as all events not excluded as Beads/Debris. Cell events staining 7-AAD+ excluded as non-viable as shown on Plot 3. Viable cells then gated by CD45/SSC to identify populations by morphology as shown on Plot 4. Viable lymphocytes further characterised as: CD3+CD4+ Th and CD3+CD4- Cytotoxic T cell populations (Plot 7); CD3-CD19+ B lymphocytes (Plot 6); NK cells CD16/CD56+ (Plot 5). Monocytes identified by morphology on CD45/SSC on Plot 4and back gated onto FS/SSC plot 1 (Monos2) to confirm the population.

2.4.5 Panel 2: Regulatory T Cells

Panel 2 was designed to identify regulatory T cells (Tregs), naïve and memory T cells that could potentially affect the responsiveness of the lymphocytes in the starting product. Tregs are a distinct population of CD4+ T cells which co-express high levels of CD25 (McHugh *et al.* 2002, Sjaasted *et al.* 2021). They are implicated in the dampening down of the allo-response and achievement of tolerance. It can therefore be postulated that DLI products containing high levels of T regs, could be less effective at inducing GvL than those with lower numbers. As the study compared the responses of lymphocytes from different donors, the number of T regs in each starting product could potentially have affected the experimental results. T Regs were identified as CD4+CD25+CD127Lo (Wang *et al.* 2017, Sjaasted *et al.* 2021). CD4+CD25+CD127hi was used to define activated effector T cells (Simonetta *et al.* 2010). CD45RA was used to discriminate between naïve T cells (CD4+CD45RA+) that have been shown to give rise to T cells that maintain their Treg expression when expanded (Tian *et al.* 2017) and CD8+CD45RA- memory cells (Toma *et al.*2022). As described in Panel 1, CD8+ lymphocytes were again identified as CD3+CD4-.

Fresh pre-cryopreservation samples were tested with this panel after density separation and washing (cones and harnesses) or dilution (apheresis product).

The antibodies used are detailed in Table 2.4.

Target Cell Population	CD Markers Defining Population	Antibodies
T Lymphocytes	CD3	Anti-CD3-FITC
T Helper Lymphocytes	CD3+CD4+	Anti-CD3-FITC Anti-CD4-APC-Cy7
Cytotoxic T Lymphocytes	CD3+CD4-	Anti-CD3-FITC Anti-CD4-APC-Cy7
T Reg	CD3+CD4+CD25+CD127Lo	Anti-CD3-FITC Anti-CD4-APC-Cy7 Anti-CD25-APC Anti-CD127-PE-Cy7
T effector (activated)	CD3+CD4+CD25+CD127Hi	Anti-CD3-FITC Anti-CD4-APC-Cy7 Anti-CD25-APC Anti-CD127-PE-Cy7
T central/effector memory cell	CD3+CD4+CD25-CD127+	Anti-CD3-FITC Anti-CD4-APC-Cy7 Anti-CD127-PE-Cy7
Naïve T Helper Lymphocytes (non-memory phenotype) Some effector memory phenotype	CD3+CD4+CD45RA+	Anti-CD3-FITC Anti-CD4-APC-Cy7 Anti-CD45-RA-FITC
Cytotoxic T Lymphocyte (Memory phenotype)	CD3+CD4-CD45RA+	Anti-CD3-FITC Anti-CD45RA-FITC Anti-CD4-APC-Cy7

Table 2.4 Antibodies used in Panel 2.

Panel 2 gating hierarchy (see Figure 2.4)

Beads and debris were excluded as described for Panel 1 in section 2.7.3. Lymphocytes were identified by morphology using FSC/SSC (Plot 1). Total lymphocytes were classified as viable or non-viable according to 7-AAD absorption (Plot 3). Viable T lymphocytes were then identified as CD3+ (Plot 4). Th cells were identified as CD3+4+ and cytotoxic T cells as CD3+CD4- (Plot 5). Treg cells were identified as CD4+ cells staining CD25+CD127Lo (Plot 6). Activated effector T cells were identified as CD4+ cells staining CD25+CD127Hi (Plot 6).

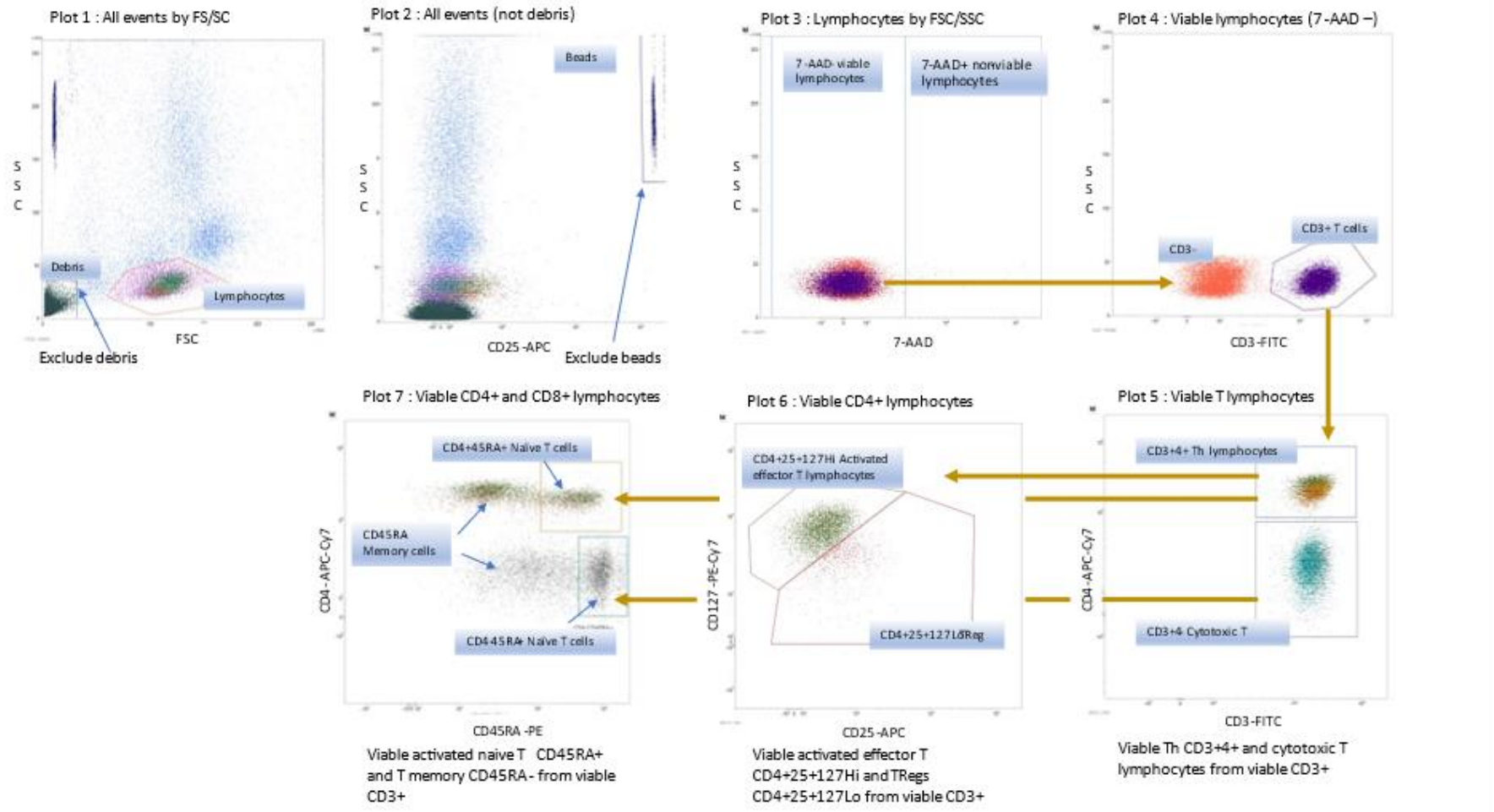


Figure 2.4: Panel 2 gating hierarchy. Debris and Beads excluded as shown on Plots 1 and 2. Lymphocytes identified by morphology on Plot 1. Lymphocyte events staining 7-AAD+ excluded as non-viable as shown on Plot 3. Viable T lymphocytes identified by CD3/SSC as shown on Plot 4. Viable T lymphocytes further characterised as: CD3+CD4+ Th and CD3+CD4- Cytotoxic T cell populations (Plot 5); CD4+25+CD127Hi activated effector T and CD4+CD25+CD127Lo Tregs (Plot 6) ; CD3+CD45RA+ naïve T cells and CD3+CD8+ memory T cells (Plot 7).

2.4.6 Panel 3: Activated T Cells

Panel 3 was designed to demonstrate the ability of the lymphocytes to activate in response to antigen. CD25 and CD69 were used to demonstrate early and middle activation markers, as discussed in introduction section 1.5.

Fresh pre-cryopreserved samples were tested with this panel after density separation and washing (cones and harnesses) or dilution (apheresis product). Post-cryopreservation samples were tested immediately post thaw and at 4, 24 and 72 hours in culture. The antibodies used are detailed in Table 2.5.

Target Cell Population	CD Markers Defining Population	Antibodies
T Lymphocytes	CD3	Anti-CD3-FITC
T Helper Lymphocytes	CD3+CD4+	Anti-CD3-FITC Anti-CD4-APC-Cy7
Cytotoxic T Lymphocytes	CD3+CD8+	Anti-CD3-FITC Anti-CD8-PE-Cy7
Activated T Helper Lymphocytes (early phase)	CD3+CD4+CD69+	Anti-CD3-FITC Anti-CD4-APC-Cy7 Anti-CD69-PE
Activated Cyotoxic T Lymphocytes (early phase)	CD3+CD8+CD69+	Anti-CD3-FITC Anti-CD8-PE-Cy7 Anti-CD69-PE
Activated T Helper Lymphocytes (late phase)	CD3+CD4+CD69+	Anti-CD3-FITC Anti-CD4-APC-Cy7 Anti-CD25-APC
Activated Cytotoxic T Lymphocytes (late phase)	CD3+CD8+CD69+	Anti-CD3-FITC Anti-CD8-PE-Cy7 Anti-CD25-APC

Table 2.5 Antibodies used in Panel 3

Panel 3 gating hierarchy (see Figure 2.5 below)

Beads and debris were excluded as described for Panel 1 in section 2.7.3. Remaining events were characterised by morphology using FSC/SSC (Plot 3). Events identified as lymphocytes were then gated against CD3+ to define T lymphocytes (Plot 4). T lymphocytes were classified as viable or non-viable according to 7-AAD absorption (Plot 5). Viable Th cells were identified as CD3+4+ and cytotoxic T cells as CD3+CD4- (Plot 6). Activated Th and cytotoxic T cells were identified by gating against CD25 and CD69 (Plots CD3+ lymphocytes from Plot 4 were gated against CD4 and 7-AAD to allow independent identification of viable Th (CD3+CD4+7-AAD-) and cytotoxic T cells (CD3+CD4-7-AAD-) in Plots 9 and 10.

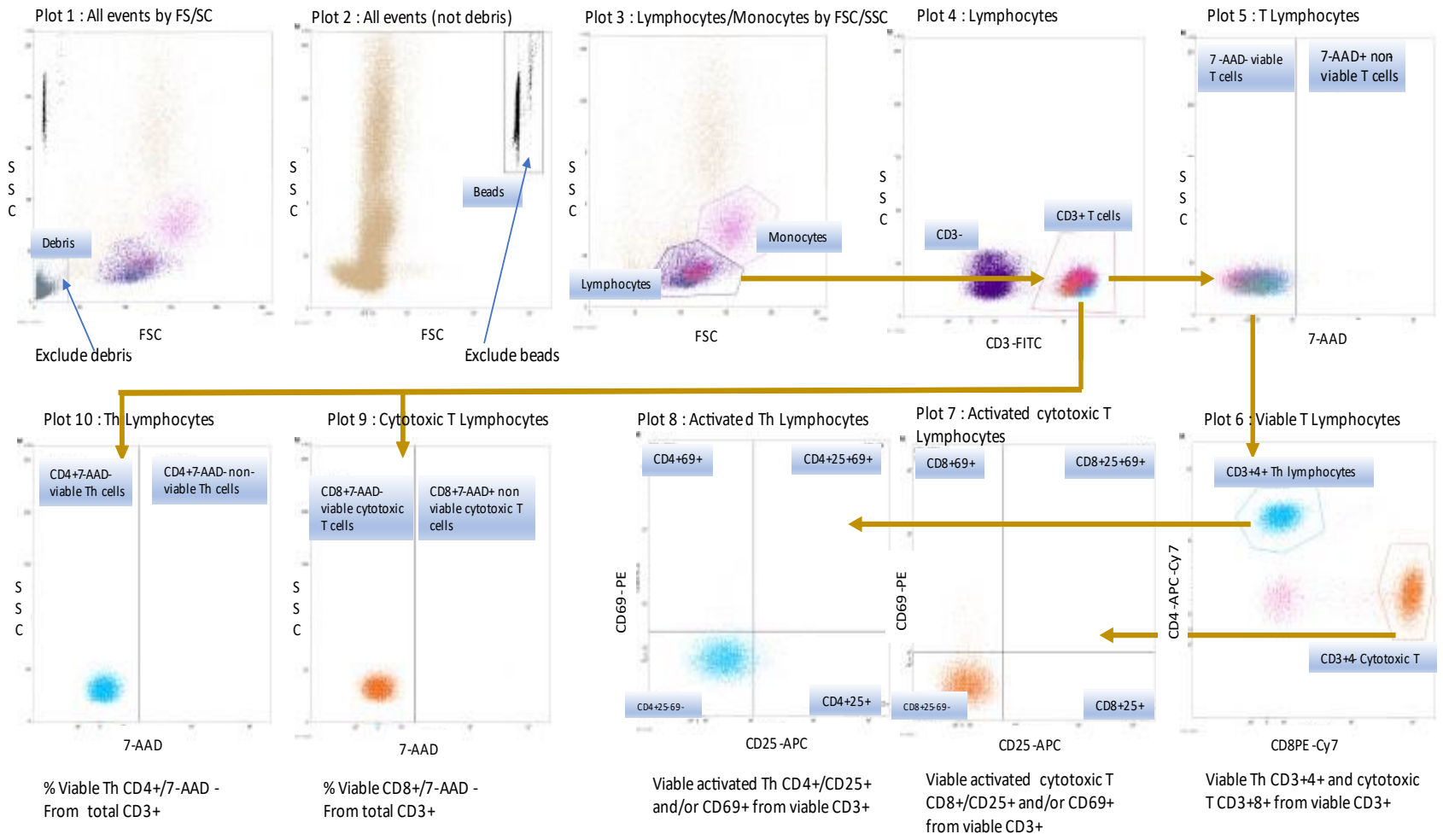


Figure 2.5: Panel 3 gating hierarchy. Debris and Beads excluded as shown on Plots 1 and 2. Lymphocytes identified by morphology on Plot 3. T lymphocytes identified by CD3/SSC as shown on Plot 4. T lymphocyte events staining 7-AAD+ excluded as non-viable as shown on Plot 5. Viable T lymphocytes further characterised as: CD3+CD4+ Th and CD3+CD8+ Cytotoxic T cell populations (Plot 6); Activation of Th and cytotoxic T lymphocytes determined by expression of CD25 and CD69 (Plots 7 and 8). Specific viabilities of Th and cytotoxic T lymphocyte populations determined by staining with 7-AAD (Plots 9 and 10).

2.4.7 Cell Viability

7-Aminoactinomycin D (7-AAD) was included in all 3 panels as a marker for cell viability. Cells that stained positive were excluded from absolute cell number calculations and the percentage viability of all cell phenotypes was calculated. Although this study was not at clinical scale, calculation of the absolute number of viable cells at each stage provided valuable information about how well cells survived over storage, cryopreservation and culture.

2.5 Key Consumables

2.5.1 Key consumables list

Key consumables used in the study are listed in Table 2.6. For a full list of all consumables used, see Appendix 5.

Consumable	Product	Manufacturer
Density gradient separation medium 1.077g/cm^3	Lympholyte®-H	CedarLane Labs, Burlington, Canada
Dulbecco's Phosphate Buffered Saline	Dulbeccos Phosphate Buffered Saline 500mL	Stem cell Technologies, Vancouver, Canada
Density separation tubes	SepMate™ tubes	Stem cell Technologies, Vancouver, Canada
6mL dockable syringe	RF-T15 6mL syringe	OriGen Biomedical, Austin, TX USA
Lymphocyte culture medium	Immunocult™-XF T Cell Expansion Medium 10981	Stem Cell Technologies Vancouver, Canada
4.5% Human Albumin Solution (HAS)	Zenalb 4.5%	BioProducts Laboratory Ltd. Elstree, UK
Interleukin 2 (IL2)	Recombinant IL2 (CHO expressed)	Stem cell Technologies, Vancouver, Canada
CD3/38 activation beads	Immunocult™ Human CD3/CD28 T Cell Activator	Stem Cell Technologies, Vancouver, Canada
24 well plates	Sarstedt 83.3922.500	Sartstedt AG & Co KG, Numbrecht, Germany
6 well plates	Stem Cell Technologies 38016	Stem Cell Technologies Vancouver, Canada
Dimethyl Sulphoxide (DMSO)	Cryosure 50mL	WAK-Chemie Medical GmbH, Steinbach, Germany
CryoMacs freezing bags	Cryo 50/250	Miltenyi Biotec, Bergisch Gladbach, Germany
600mL transfer pack	Transfer pack with coupler – 600mL	Fresenius Kabi UK, Runcorn, UK
Absolute count tubes	TruCount™ Tubes	Becton Dickinson UK, Winnersh Triangle, UK

Table 2.6 Key consumables.

2.5.2 Flow cytometry antibody list

All antibodies were supplied by Becton Dickinson UK Ltd. Some of the antibodies used in the study were already in routine use at the study site at the time the study was performed. To minimise the financial impact on the department, these were also used for the study and the remaining antibodies selected following advice from Becton Dickinson.

Antibody	Clone
Anti-CD3-Phycoerythrin -Cyanine 7 (PE-Cy7)	UCHT1
Anti-CD3-Fluorescein isothiocyanate (FITC)	HITC3α
Anti-CD4-Allophycocyanin- Cyanine-7 (APC-Cy7)	RPA-T4
Anti-CD8- Phycoerythrin -Cyanine 7 (PE-Cy7)	HIT8A
Anti-CD16/56- Phycoerythrin (PE)	B159/3G8
Anti-CD19- Allophycocyanin (APC)	HIB19
Anti-CD25- Allophycocyanin (APC)	MA251
Anti-CD45- Fluorescein isothiocyanate (FITC)	HI30
Anti-CD45-RA- Fluorescein isothiocyanate (FITC)	HI100
Anti-CD69- Phycoerythrin (PE)	FN50
Anti-CD127- Phycoerythrin -Cyanine 7 (PE-Cy7)	HIL-7R-M21
7-Aminoactinomycin-D (7-AAD)	Not Applicable

Table 2.7 Antibody clones used.

2.6 Methods: Sample Preparation

2.6.1 Aseptic technique

All open processing steps were performed in a Microbiological Safety Cabinet (MSC) to prevent contamination during cell manipulation. The MSC was located in a dedicated development space where the was no risk of crossover contamination from any other materials in use in the department. All operators who performed this procedure were

qualified in aseptic technique in accordance with NHSBT policies and standard operating procedures. To comply with NHSBT policy, operators must complete bi-annual broth simulation exercises, whereby they perform a dummy cell process using tryptone soya agar broth instead of cellular products. At the end of processing, the broth is sampled and tested for bacterial and fungal contamination. Operators must demonstrate a zero contamination rate, proving that they are able to perform open manipulation of cellular products without introducing microbiological contamination. For this reason, it was not deemed necessary to perform microbiological culture assays on the study samples.

2.6.2 Preparation of starting cell preparations

All work was carried out in a temperature-controlled laboratory with a set point of 21°C. Room temperature (RT) in this case was therefore defined at 21°C.

i) Apheresis cones

The cones were removed from 4°C storage and placed in the MSC at room temperature. The lines on each end of the cone were cut with sterile scissors and the cone placed over a sterile tube to allow the cells to drain into the tube by gravity. Aseptic removal of the cells from the cones took from 5-10 minutes. The collected cells were then diluted with an equal volume of 2% HAS in PBS for density gradient separation.

ii) Apheresis harnesses

The harness was suspended within the MSC so that gravity allowed the cells trapped within the set to drain into an empty bag docked onto the harness using a sterile connecting device (SCD). To remove the last cells trapped within the set. A syringe was filled with 50mL of 2% HAS in PBS and attached to a port on the harness and the HAS/saline flushed through the harness. The extracted cells were then transferred to 50mL conical tubes for density gradient separation.

iii) Apheresis samples

Samples were removed aseptically from the apheresis pack by connecting a dockable 6mL syringe to the line on the pack using a sterile connecting device (SCD). 6mL of cells were drawn up into the syringe. 4mL was aliquoted for sterility testing. The remaining 2mL was assigned for routine quality control (QC) testing (cell counting and flow cytometry for CD34+ cells). After satisfactory completion of QC tests any remaining sample was reserved for the study. The volume remaining ranged from 1-1.5mL depending on the volume used for testing. The reserved cells were held in the dockable syringe pending the results of the QC analysis. When released for the study, the syringe was transferred to the MSC and the cell sample dispensed into a 15mL conical tube. 4.5% HAS was added to the cells to a final volume of 10mL.

2.6.3 Mononuclear cell preparation

The density separation medium used was Lympholyte®-H. Following the manufacturer's instructions, the medium was shaken vigorously and left to stand in the dark at RT until all the bubbles had dispersed before it was used.

i) Apheresis Cones

The mononuclear cells were separated from the diluted cell suspension using the SepMate™ system (Miltenyi Biotech, UK). The diluted cells were added to a SepMate™ tube filled with Lympholyte®-H density gradient medium and centrifuged in a temperature-controlled centrifuge: 1000g for 10 minutes at 20°C. After centrifugation, the SepMate™ tubes were transferred to the MSC. The supernatant was discarded and the enriched MNC layer at the interface was carefully removed and placed in a clean 50mL conical tube. MNC layers from multiple tubes were pooled into a single tube to a maximum volume of 20mL. The cells were washed 3 times by adding 2% HAS/PBS to

make a total volume of 50mL, followed by centrifugation at 400g for 10 minutes at 20°C. After washing the cell pellet was resuspended in 4.5% HAS to a final volume of 10mL at RT. The HAS was added slowly to the cell pellet with constant gentle mixing to avoid osmotic shock.

ii) Apheresis harnesses

The volume of starting material retrieved from the harnesses was much greater than that retrieved from the cones (>100mL as compared to <30mL for the cones) making it impractical to use SepMate™ tubes. The apheresis cells were therefore layered straight onto Lympholyte®-H dispensed into a 50mL conical tube. The ratio of cells to Lympholyte®-H to was 2:1. The maximum volume used in each tube was therefore 15mL of Lympholyte®-H and 30mL of cells. The number of tubes required to perform the separation was determined by the starting sample volume. The tubes were centrifuged at 800g for 20 minutes at 20°C. After centrifugation, the tubes were transferred back into the MSC. The supernatant was discarded and the MNC layer was carefully removed into a clean 50mL conical tube. MNC layers from multiple tubes were pooled into a single tube to a maximum volume of 20mL. As before, the number of tubes required was dependant on the starting volume. The tubes containing the MNC layer were then topped up with RT PBS to a volume of 50mL. The tubes were centrifuged at 500g for 5 minutes at 20°C. The supernatant was discarded, and the cell pellets were pooled, if required, and resuspended in 4.5% HAS at RT as described above.

iii) Apheresis samples

The cell samples from the apheresis products were not prepared by density separation for two reasons: 1) they were collected as the mononuclear fraction by a programmable cell separator (Spectra Optia) and did not contain sufficient contaminating red cells or

granulocytes to make density separation required and 2) to minimise any patient impact, cell numbers taken from the product were the minimum that could be used for the study and potential cell losses during the separation procedure could have rendered the samples unusable.

2.6.4 Cell Counts

A 500µL sample of the prepared resuspended cells was removed using a sterile pipette. A cell count was then performed on a using a Sysmex 1000i haematology analyser. The number of nucleated cells present in the MNC preparation was then calculated:

$$WBC \times 10^6/mL \times Volume\ (10mL) = TNC \times 10^6$$

The study required a minimum of 300 x 10^6 nucleated cells. Starting products that did not meet this criterion were discarded. If the product contained sufficient cells to perform all study conditions, the initial flow cytometry panels to establish cell phenotype and activation marker expression were performed to establish the baseline characteristics of the individual sample.

2.7 Methods: Flow Cytometry

2.7.1 Analyser set up and sample preparation

On each day that the BD™ FACSLyric™ was used, performance quality control (PQC) was performed using BD® Cytometer Set & Tracking (CS&T) beads. The CS&T beads are used to quality control the instrument optics, electronics, and fluidics. The instrument measures position and fluorescence intensity of the beads against the target profile for the bead lot to confirm sensitivity and reproducibility for the instrument. In addition, performance of PQC optimises colour compensation for the assays to be used each day.

The BD™ FACSLyric™ provides an automated report of the PQC result and the instrument was only used to analyse study samples if a 'PASS' result was achieved.

100µLwas removed from the sample used to perform the WBC analysis. The 100µL sample was diluted in PBS (BD FACSflow™) to a WBC concentration of 10-20 x 10^6/mL. This was the optimum cell concentration range for sample analysis on the FACSLyric™. A Full Blood Count (FBC) was then performed on the diluted sample using the Sysmex 1000i to confirm the accuracy of the dilution.

BD Trucount™ tubes were used in the study to enable generation of absolute cell counts. A lyse no-wash protocol was employed to ensure that the BD Trucount™ beads were conserved in the tubes. Lysing solution was made freshly each day. 1mL of FACS™ Lysing Solution 10X Concentrate was added to 9mL of sterile distilled water in a sterile conical tube. The manufacturer specification for the products states that BD FACS™ Lysing Solution is compatible with both wash and no-wash protocols.

Antibodies were dispensed into BD Trucount™ tubes at volumes according to the manufacturers' recommendation for the number of cells to be stained:

Panel 1: 20µl 7-AAD, 5µl anti-CD3-PE-Cy7, 5µl anti-CD4-APC-Cy7, 20µl anti-CD45-FITC, 20µl anti-CD19-APC, 20µL anti-CD16/56-PE.

Panel 2: 20ul 7-AAD, 20µL anti-CD3-FITC, 20µL anti-CD25-APC, 5µl anti-CD4-APC-Cy7, 20µL anti-CD45RA-PECy7, 5µL anti-CD127-PE-Cy7.

Panel 3: 20µl 7-AAD, 20µL anti-CD3-FITC, 5µL anti-CD4-APC-Cy7, 5µL anti-CD8-PeCy7, 20µL anti-CD25-APC, 20µL anti-CD69-PE.

50µL of the diluted cell suspension was added to each tube by reverse pipetting. The samples were mixed manually by gentle shaking of the tubes and incubated for 15 minutes in the dark at 21°C. After this time, 1mL of FACSLyse was added to the samples

and these were incubated for a further 5 minutes at 21°C. Samples were resuspended manually by gently shaking the tubes and run immediately on the FACSLyric™. The instrument stop condition for all 3 panels was set as either 100,000 events or 180 seconds data acquisition to ensure sufficient events were acquired. Protocols for the different antibody panels used were stored on the FACSLyric™ to ensure that testing was performed under the same conditions on each occasion. To allow for the inherent variability of biological samples, the gates set on the assays were not fixed. They could be moved by the operator to ensure the relevant cell populations were included. All results were reviewed by an expert user and adjusted where necessary prior to authorising the results.

2.8 Method: Storage and cryopreservation

Storage and cryopreservation conditions were designed to replicate those routinely in use in NHSBT laboratories. Cells were stored under the same conditions as clinical products in storage devices monitored by an independent Environmental Monitoring System (EMS). Cryopreservation was performed following NHSBT's in house validated procedures for cryopreservation of human cells for infusion (Appendix 4). The prepared cell products were split into three aliquots (by volume) for cryopreservation on Days 1, 2 and 3. The volume of each aliquot was used to calculate the absolute cell numbers contained within it. There were minor variations in the aliquot volumes resulting in the different pre-freeze cell counts in seen in Tables 4.2, 4.3, 4.4 and 4.5.

The washed cells were transferred to a breathable 600mL transfer pack (Fresenius Kabi UK) and the volume of the cells adjusted to a minimum of 20mL. The maximum permitted nucleated cell concentration in the transfer pack was 100 x 10^6/mL and samples with cells at higher concentrations were diluted to reduce the count to <100 x

10^6/mL. The cell bag was then placed at 4°C until the temperature of the product reached NHSBT specification of between 2-8°C.

A cryoprotectant mixture of 20% DMSO (CryoSure, WakChemie Medical GmbH) in 4.5% HAS was prepared. 80mL of 4.5% HAS at 4°C was dispensed into a CryoMACS® 250 PVC free cryobag (Miltenyi Biotec) via needle and syringe. The CryoMACS bag was placed on gel pack pre-cooled to 4°C and 20mL of DMSO was added slowly with constant mixing. The prepared cryoprotectant was then cooled to 4°C before use.

The cell product was A needle and syringe were used to transfer one third of the cell product to a CryoMacs® 50 cryobag. The bag was labelled with an identifying number, date of collection and Day 1 Cryopreservation. The bag was placed on a gel pack pre-cooled to 4°C and an equal volume of the cryoprotectant added via needle and syringe. The cryoprotectant was added slowly with constant mixing. A 1mL sample of the cell/cryoprotectant mixture was transferred via needle and syringe to a 1.8mL vial suitable for cryogenics (Nunc® CryoTube®). The bag and vial were immediately transferred to a Kryo 560-16 rate-controlled freezer (Planer Ltd.) and cryopreserved using the routine NHSBT freezing programme. Rate controlled cryopreservation allows the freezer protocol to be designed for the type of product being cryopreserved and has been demonstrated to be optimal for cryopreservation of human blood cells (Buhl *et al.* 2012). Temperature sensors inside the freezer control the chamber temperature to the programmed temperature profile. In this study an additional sensor was placed next to the bag inside the freezing cassette to provide assurance that the sample temperature matched the programmed chamber temperature (see Figure 2.6 below). The freezing programme used in the study has been in use for cryopreservation of clinical products

for more than 20 years and there is a wealth of internal clinical data available to demonstrate its suitability.

Programme steps:

Hold 4°C 5 minutes.

Ramp 1: -1°C/minute to -30°C.

Ramp 2: -2°C/minute to -60°C.

Ramp 3: -20°C/minute to -180°C.

Hold -180°C until unloaded into vapour phase nitrogen immediately on completion of programme.

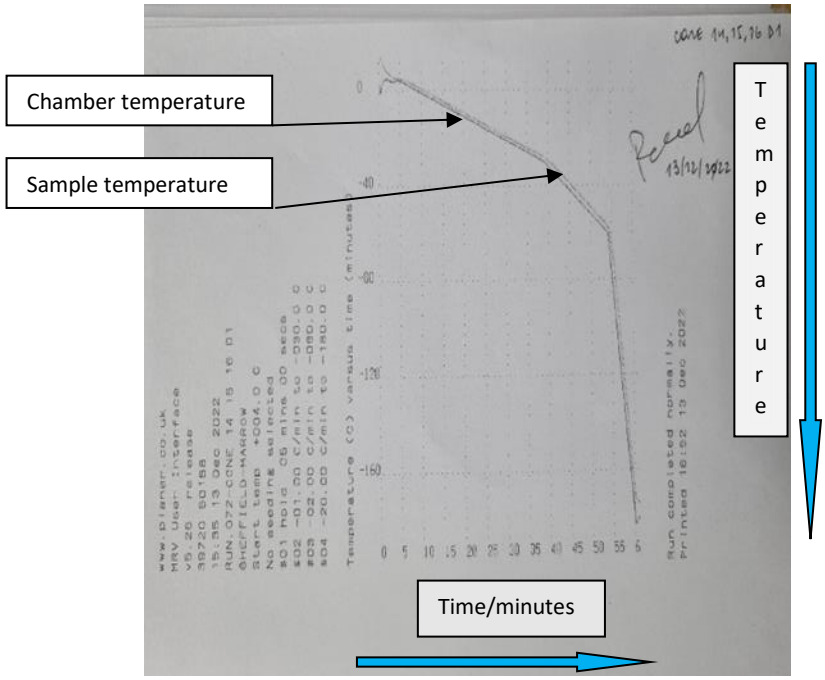


Figure 2.6: Trace from programmable freezer. Showing close alignment between chamber temperature and sample temperature.

To avoid membrane damage caused by prolonged exposure to DMSO prior to freezing (de Menorval *et al.* 2012), the maximum time between the first addition of cold cryoprotectant to starting the rate-controlled freezer was <20 minutes. After

cryopreservation, the cells were immediately transferred to vapour phase nitrogen storage for a minimum of 7 days before thawing and stimulation. The storage temperature was maintained below -150°C and was monitored by an EMS system. Frozen samples where storage temperatures did not meet this specification were excluded from the study.

The remaining cells were stored at 4°C for 24 hours. Exactly 24 hours after the Day 1 cryopreservation, a second aliquot of the stored cells was cryopreserved following the same protocol. This was the Day 2 Cryopreservation. 48 hours after Day 1 cryopreservation, the final aliquot was cryopreserved. This was the Day 3 cryopreservation.

2.9 Methods: Stimulation

The cryopreserved bag was removed from the vapour phase nitrogen storage and thawed rapidly in a water bath at 37°C. The cells were then transferred to an MSC, and all work was then performed in the MSC to minimise contamination. The thawed cells were transferred from the cryobag to a sterile 50mL conical tube via a needle and syringe. A 250µL sample was removed from the thawed cells for FBC/Panel 3 flow cytometry to establish the number of viable cells present immediately post thaw. The cell sample was diluted dropwise 1: 5 with BD Pharmingen™ Stain Buffer (BSA) (Becton Dickinson UK) at 21°C prior to FBC and flow cytometric testing. The remaining cells were diluted with an equal volume of ImmunoCult™ -XF T (Stem Cell Technologies) at 21°C. The media was added dropwise to the thawed cells to minimise osmotic shock as described by Disis *et al.* (2006). The resuspended cells were centrifuged at 300g for 5 minutes at RT. The supernatant was removed, and the cell pellet was resuspended in 10mL of 4.5% (v/v) HAS. A further wash was performed as before. The washed cell pellet

was resuspended in ImmunoCult™ -XF T (Stem Cell Technologies) at RT to an approximate cell concentration of 10 x 10^6/mL, calculated from the WBC on the sample taken immediately post thaw. A 100µL sample was taken from the resuspended cells and FBC/Panel 3 flow cytometry was performed on this sample to establish the number of viable CD3+ cells/mL.

Culture medium was prepared by adding 10mL of 4.5% HAS to 190mL of ImmunoCult™ -XF T in a sterile bottle. 100mL of prepared media was transferred to a second sterile bottle and 100µL of IL2 at a concentration of 0.1mg/mL added to produce an IL2 concentration of 0.1µg/mL. Initially cells (cone samples 1-10) were seeded into a 6 well plate (Stem Cell Technologies) at a density of 1 x 10^6 viable CD3+/mL. 3 x 10^6 viable cells were seeded into each of 3 wells of a 6 well plate, and the total volume of the well, adjusted to 3mL with the prepared medium. However, the samples from prepared apheresis harnesses did not yield sufficient cells to seed 3 x 10^6 viable CD3+ into each well. To reduce the number of cells required for the assay, Cones 11-16, apheresis harness cells and samples from mobilised products were seeded into a 24 well plate (Sarsted AG & Co KG). 1 x 10^6 viable CD3+ were seeded into 3 wells of the plate and the volume of the well, adjusted to 1mL with the prepared medium. The seeding density remained at 1 x 10^6/mL. The selection of 1 x 10^6/mL as optimum seeding density was based on work previously performed within NHSBT on T cell activation assays (Kumar, and Meneghal, 2022).

Well 1: Control sample -Cells + Media

Well 2: Control for experimental effect due to the presence of IL2 alone -Cells + Media supplemented with Human Recombinant IL2 *E.coli* expressed (Stem Cell Technologies)

Well 3: Test sample - Media supplemented with Human Recombinant IL2 *E.coli* expressed (Stem Cell Technologies) and Immunocult™ Human CD3/CD28 T Cell Activator (Stem Cell Technologies).

The sample plate was incubated for exactly 4 hours in a 37°C humidified incubator at 5% CO_2/95% air. After 4 hours, a 250µL sample was aseptically removed from each well. The contents of the cells were mixed gently using a pipette prior to sampling to ensure that the sample taken was representative. An FBC and flow cytometric Panel 3 were performed. Sampling and Panel 3 flow cytometry was repeated at 24 and 72 hours. A summary of the study design is shown in Figure 2.7.

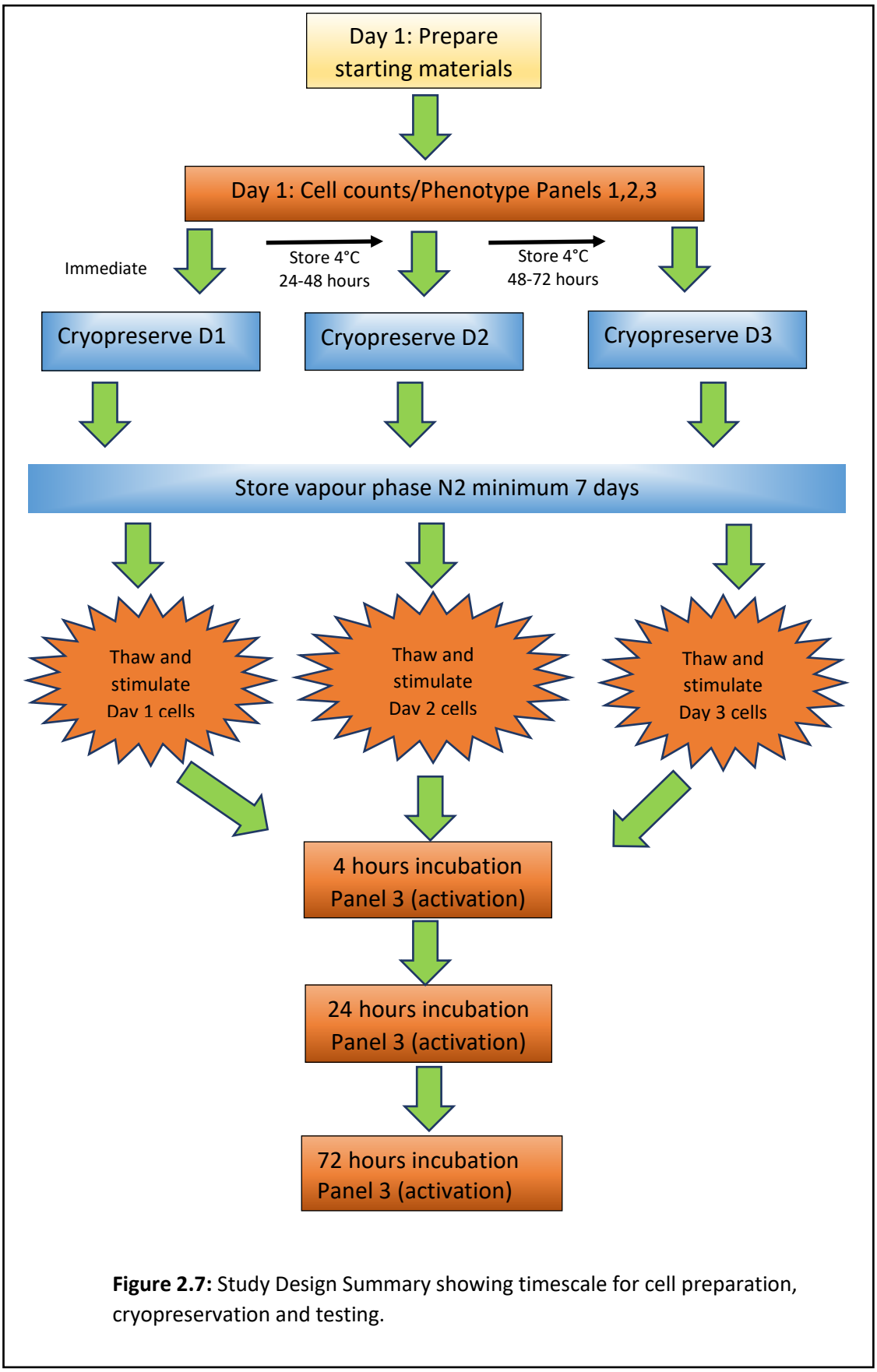


Figure 2.7: Study Design Summary showing timescale for cell preparation, cryopreservation and testing.

2.10 Data Collection and Statistical Analysis

The raw flow cytometry data was exported as csv files from the FACSLyric™ into Microsoft Excel spreadsheets. Calculations of absolute cell counts were performed within the Excel spreadsheets. The data was then transferred into GraphPad Prism version 9.5 for statistical analysis.

As it had not been possible to use anti-CD8 in Panels 1 and 2 because of the lack of available fluorescence channels on the FACSLyric™, the validity of identifying CD8+ T cells as those staining CD3+4- was confirmed by comparison of the numbers of CD3+4- cells/mL from Panel 2 with the number of CD3+8+ cells/mL from Panel 3. The analysis was performed on the same sample, taken prior to cryopreservation and the values obtained confirmed using Wilcoxon rank test. A non-parametric test was used on this occasion as QQ plotting showed that these data did not follow a normal distribution. No significant difference was found between the two methodologies (Appendix 6).

Advice on statistical analysis was taken from Ellen Marshall, statistician at Sheffield Hallam University. Following this advice samples with missing data sets were excluded from the analysis. As the distribution of the samples under test was unknown and biological samples cannot be assumed to have a normal distribution, a Shapiro-Wilk test for normal distribution was applied to all paired comparisons prior to selecting the method of analysis. The Shapiro-Wilk test has been shown to be the most appropriate for small samples numbers ($n<50$) (Mishra *et al.* 2019). The data was confirmed to have a normal distribution, therefore paired or unpaired t-tests were used to interrogate the data for differences between two groups. To reduce the likelihood of detecting false experimental effects, False Discovery Rate (FDR) correction factor was applied to all the

p-values obtained using t-tests (Benjamini and Hochberg 1995). All reported p values are FDR corrected.

Analysis of variance (ANOVA) was used to interrogate the data for comparisons between multiple groups of the same sample size, using Tukey's multiple comparison test as a post-hoc test to confirm significant differences in the means. Residuals for all analyses were plotted on QQ plots to confirm normality for each ANOVA and therefore validity of the test. Where sample groups were the same size ANOVA was performed using a repeated measures model Where it was not possible to perform repeated measures ANOVA because of differences in the sample sizes between the experimental groups, a mixed model was used. All hypothesis tests were two-sided, with a significance level of $p < 0.05$.

3 Chapter 3 Phenotyping Results for Cryopreserved Cells

3.1 Background

The cells prepared from the apheresis cones, apheresis samples and apheresis harness starting materials were phenotyped prior to cryopreservation to determine the cell populations and numbers present.

The aim of the study was to determine relationships, if present, between the phenotype of the starting cell population, pre-cryopreservation, and their recovery and activation rates post-thaw. It was therefore important to establish the initial phenotype of the cell populations within the different groups. Any differences between the groups were determined by Statistical analyses.

As described in Chapter 2, each sample was tested with 3 different flow cytometric panels to establish the numbers of the following cell types:

i) Granulocytes
ii) T, B and NK lymphocytes
iii) Naïve/memory phenotype T cells
iv) Tregs
v) Activated T cells

The viability of CD45+ cells in the prepared starting material was tested by staining with 7-AAD to determine whether samples used in the study were in good condition prior to cryopreservation. Samples that had been damaged by poor handling or storage were not included in the cryopreservation study as it would have been impossible to detect experimental effect.

3.2 Study samples

Cells derived from sixteen apheresis cones, nine apheresis samples and five apheresis harnesses were phenotyped for the study. Sufficient lymphocyte numbers were retrieved from the cones and the apheresis samples to fulfil the cryopreservation experimental conditions for the study. The apheresis harnesses unfortunately did not yield sufficient lymphocytes to fulfil the study conditions and their use was discontinued after 5 harnesses had been processed. The viability and 3-part differential results for these harnesses are presented in this chapter as they offer some degree of explanation for the failure to recover sufficient cells, but as the samples were not used in the study the results are shown for information only. As only five harnesses were processed, the number of harness samples was insufficient to provide robust evidence about their cell content and they were excluded from statistical analysis. The harness recovery results are discussed fully in Chapter 4.

Due to an error setting up the T lymphocyte subset panel (Panel 2) on the FACSLyric™ which initially went unnoticed, the flow cytometer did not acquire sufficient TruCount™ bead events from cones 1-7 for the T cell subset data to be valid. The number of bead events acquired for these samples was <200 when the manufacturers recommended minimum for validity is 1000 bead events. The results obtained on these samples were therefore excluded from the analysis as they were almost certainly inaccurate. The error was discovered after all the bags from both the cone and apheresis sample groups had already been thawed, making it impossible to repeat the tests.

To obtain data on the number of Tregs, naïve and memory phenotype lymphocytes present in cones 1-7, the Day 1 cryopreserved pilot vials were thawed and analysed with Panel 2. The pilot vial for Cone 2 could not be located. However, the effect of

cryopreservation itself on the cells stored in vials could have altered the cell populations present and the results obtained on the seven thawed samples could not have been considered as reflective of the pre-cryopreserved sample. To better understand the impact of cryopreservation on the cells stored in vials, the cryopreserved vials from all sixteen cones and nine apheresis samples were located and thawed. The vials were thawed rapidly in a water bath at 37°C and diluted in cell culture media at 21˚C following the same procedure used for thawing the bags. T cell subsets were successfully analysed on the stored pilot cryovials from all samples.

3.3 Data analysis

To confirm that the lymphocyte subset data obtained followed a normal distribution pattern, a Shapiro-Wilk test and QQ plots were performed on all the subsets tested using GraphPad Prism. All the subsets studied were found to be normally distributed, enabling the use of parametric testing. The data generated from the cone, apheresis and harness (where applicable) sample groups were compared. The viability data between the groups was compared using a one-way ANOVA. As lymphocyte subsets were only analysed for cone and apheresis samples, student t-tests for independent samples were used to compare the cell populations derived from the different groups. These populations were also compared to reference ranges for peripheral blood from healthy donors that were available.

Student t-tests for paired samples were applied to the pre-cryopreserved and thawed samples from each group to assess the impact of cryopreservation.

3.4 Cell viability from Panel 1

CD45+ viability testing by exclusion with 7-AAD was performed on the cone and harness samples after density gradient separation had been completed but prior to

cryopreservation. Apheresis samples were not separated by density gradient and were tested after dilution for cryopreservation. The viability results for all sample types are shown in Table 3.1.

Cones (n=16)		**Apheresis Samples (n=9)**		**Apheresis Harnesses (n=5)**	
Sample Number	CD45+ viability (%)	Sample Number	CD45+ viability (%)	Sample Number	CD45+ viability (%)
Cone 1	97.59	APH01	98.72	HAR 1	87.30
Cone 2	96.91	APH02	97.27	HAR 2	91.55
Cone 3	92.27	APH03	99.12	HAR 3	94.20
Cone 4	98.50	APH04	99.20	HAR 4	96.23
Cone 5	94.35	APH05	99.39	HAR 5	96.82
Cone 6	98.50	APH06	99.12		
Cone 7	93.88	APH07	97.91		
Cone 8	98.00	APH08	97.90		
Cone 9	96.43	APH09	98.13		
Cone 10	97.51				
Cone 11	95.09				
Cone 12	97.20				
Cone 13	98.39				
Cone 14	97.26				
Cone 15	97.64				
Cone 16	97.63				
Mean	**96.70**		**98.53**		**93.22**
Median	**97.38**		**98.72**		**94.20**
Range	**92.27-98.39**		**97.27-99.39**		**87.30-98.82**

Table 3.1 Percentage Viability of CD45+ cells determined by staining with 7-AAD for cone, apheresis and harness samples.

The mean viability of the cone derived cells was slightly lower (cones = 96.70%; apheresis samples = 98.53%) and the range slightly greater than that of the apheresis samples (92.27-98.39 vs 97.27-99.39), which probably reflected the fact that the cone cells had been subject to physical trauma in the form of density gradient separation.

Analysis using one-way ANOVA for multiple comparisons did not demonstrate a significant difference between the cone and apheresis sample viabilities (p=0.104). Harness samples had the lowest mean viability (93.22%) and this difference was statistically significant (p= 0.0079 for cones and 0.0003 for apheresis samples), although the harness sample numbers were so small that the poor viability found in HAR1 (87.3%) was sufficient to affect the mean and no reliance can be placed on this result (Figure 3.1).

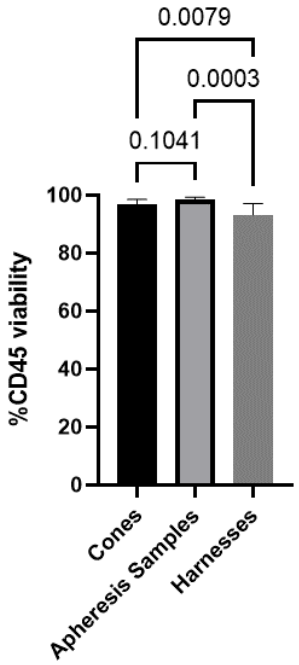


Figure 3.1: Comparison of pre-cryopreservation viability cells from 16 cone samples (C1-16), 9 apheresis samples (APH1-9) and 5 harness samples (HAR1-5) prior to cryopreservation. Viability assessed by flow cytometry exclusion staining with 7-AAD of cellular events identified by CD45/SSC. Means shown +/- SD. Statistical analysis by one-way ANOVA, all p values shown.

3.5 WBC Differential counts of cell starting material from Panel 1

Lymphocytes and monocytes were identified by flow cytometric morphology using CD45+/SSC gating. The lymphocyte population was further characterised with lymphocyte specific markers (see section 3.5). As described in Chapter 2, monocytes

were identified by morphology only due to the lack of available fluorescence channels on the FACSLyric™ to enable use of a monocyte specific marker. Specific markers for granulocytes were not used in the study and granulocytes were therefore identified as CD45+ cellular events not classified as either lymphocytes or monocytes. The differential results are shown in Table 3.2.

Cones n=16				Apheresis Samples n= 9				Apheresis Harnesses n=5			
Sample Number	Lymphocyte (%)	Monocyte (%)	Granulocyte (%)	Sample Number	Lymphocyte (%)	Monocyte (%)	Granulocyte (%)	Sample Number	Lymphocyte (%)	Monocyte (%)	Granulocyte (%)
Cone 1	59.46	31.30	9.24	APH01	32.34	28.22	39.44	HAR 1	11.36	6.21	82.43
Cone 2	63.56	34.15	2.29	APH02	47.75	27.91	24.34	HAR 2	13.22	4.19	82.58
Cone 3	70.18	24.76	5.05	APH03	45.24	27.62	27.13	HAR 3	24.05	14.62	61.33
Cone 4	75.93	12.01	12.06	APH04	55.29	29.09	15.62	HAR 4	23.25	30.72	46.02
Cone 5	49.00	50.11	0.89	APH05	35.76	23.45	40.79	HAR 5	28.67	24.63	46.70
Cone 6	66.85	27.70	5.45	APH06	40.67	29.80	29.53				
Cone 7	64.01	33.78	2.21	APH07	28.72	38.35	32.94				
Cone 8	70.17	27.84	1.99	APH08	47.40	28.99	23.61				
Cone 9	52.04	42.09	5.87	APH09	35.66	32.19	32.15				
Cone 10	60.20	38.23	1.57								
Cone 11	52.63	39.14	8.23								
Cone 12	65.45	24.78	9.77								
Cone 13	61.15	29.34	9.51								
Cone 14	59.20	32.43	8.37								
Cone 15	64.19	24.28	11.54								
Cone 16	68.24	25.31	6.46								
Mean	**62.47**	**31.08**	**6.28**		**40.98**	**29.51**	**29.51**		**19.07**	**15.15**	**63.49**
Median	**63.56**	**31.30**	**6.46**		**40.82**	**28.99**	**29.53**		**23.25**	**14.89**	**62.41**
Range	**49.00-75.93**	**12.01-50.11**	**0.89-12.06**		**28.72-47.75**	**23.45-32.19**	**15.62-39.44**		**11.36-28.67**	**6.21-30.72**	**47.02-82.58**

Table 3.2 Three-part white blood cell differential of prepared cones after density separation and apheresis product samples taken from the product bag determined by flow cytometry using CD45/SSC. Lymphocytes/monocytes/ granulocytes calculated as % of all viable CD45+ cellular events.

The cells from cone samples were prepared by density gradient separation and as a result would be expected to have low levels of granulocyte contamination. Mean granulocyte content in the prepared cone samples was found to be 6.25% and ranged from 0.89 – 12.06% of viable CD45+ cells.

The apheresis samples, not having undergone density gradient separation, had a higher level of granulocyte contamination than the cones; mean = 29.51% and range = 16.62-39.44%. APH1 and APH5 had the highest granulocyte content; 39.44% and 40.79% respectively. APH4 had the lowest number of granulocytes at 15.62%.

The cells retrieved from the apheresis harnesses largely failed to separate on density gradient separation. The mean granulocyte content of the prepared harness cells was 63.29%, and HAR 1 and 2 had granulocyte content of >80% after separation. Attempts to improve this outcome by repeating the separation process resulted in unsustainably high cell losses and were therefore abandoned.

Graphical representation of the white blood cell differentials is shown in Figure 3.2 but statistical comparison of the 3 experimental groups was not undertaken as the results could not be expected to be the same, due to the differences in cell preparation methods.

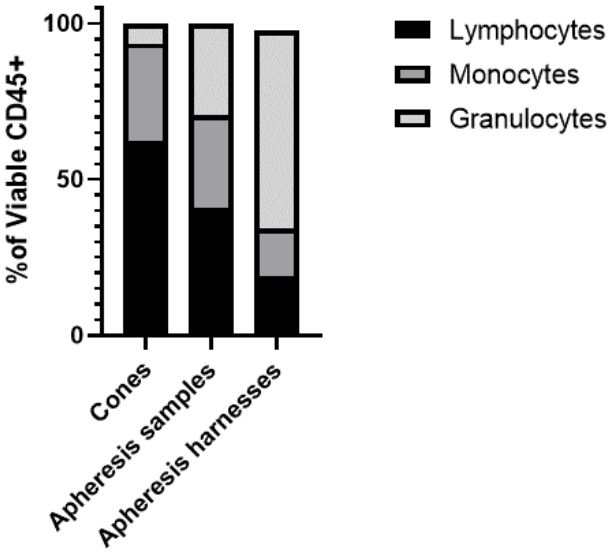


Figure 3.2: Graphical representation of the mean 3-part differentials for prepared pre-cryopreservation cone samples C1-16), apheresis samples (APH1-9) and harness samples (HAR1-5) performed by flow cytometry. Cellular events identified by CD45/SSC. CD45+ events subtyped into lymphocytes, monocytes and granulocytes by degree of side scatter.

3.6 Discussion of viability and WBC Differential of cell starting materials from Panel 1

Viability

The NHSBT specification for CD45+ viability on a fresh product as assayed using 7-AAD is >90% (SPN256). All starting materials, except for HAR1, met this specification and would have been regarded as fit for clinical use. Cone 3 had a lower viability than the other samples but on investigation, no factor was found that indicated poor handling. It had been received in the same consignment as Cone 4 which had a post-separation viability of 98.5% and there had been no difficulties extracting the cells from the cone, or during density gradient separation. Although the viability was lower than the mean, as Cone 3 met NHSBT specification, it remained in the study.

The cells originating from apheresis harnesses had a slightly lower viability than the cells originating from cone or apheresis samples, probably because of the additional physical trauma undergone removing the cells from the harness, combined with the high numbers of granulocytes present. It was possible that the cells extracted from the harnesses had been damaged during MNC apheresis, which can take up to 5 hours and processes 2-3 blood volumes (Davis *et al.* 2016) as opposed to the 40-minute Trima platelet collection process. However, both mean (98.53%) and median (98.72%) viability of the apheresis samples were the highest of the experimental groups, ruling out the possibility of adverse impact on the cells derived from the Optia apheresis MNC collection itself.

Good cell density separation was achieved on all the cone samples, and sufficient lymphocytes were recovered to fulfil the study conditions. There was a wide range of viable cells recovered within the populations identified as lymphocytes or monocytes. Cone 4 was found to be 75.93% lymphocytes and Cone 5: 49.00% lymphocytes. The best explanation for the wide variation in lymphocyte content of the prepared cones is that they are directly related to the lymphocyte count of the donor prior to donation. Platelet donors can donate several times a year and there is emerging evidence that frequent platelet donation can result in lymphopoenia in the donor (Zhao *et al*. 2020). It was therefore possible that Cone 5 was collected from a frequent donor with lower peripheral blood lymphocytes than the Cone 4 donor.

The apheresis samples displayed surprisingly low variation in cellular composition, given that the Spectra Optia cell separator, although programmed to maximise collection of mononuclear cells (Davis *et al.* 2016), can only collect the cells that are present in the

donor at the time and donor responses to G-CSF can be highly individual. The mean MNC content was 70% with only APH1 and 4 below this level. The finding confirmed that NHSBT apheresis settings deliver accurate MNC products in line with published data (Davis *et al*. 2016).

The samples derived from apheresis harnesses performed extremely poorly after density gradient separation. The cells retrieved from the apheresis harnesses all had starting granulocyte contents of 50-80% and lymphocyte contents ranging from 10-20%, as determined by FBC on the Sysmex 1000i haematology analyser prior to separation. Differential counts performed on immature blasts or G-CSF mobilised cells are not completely accurate for this type of analyser (Herklotz and Huber, 2001) so the differential results obtained can only be regarded as indicative of cell content, but they do provide valuable information. As the harnesses had to be flushed to retrieve all available cells, the volume obtained from each harness was high, ranging from 100-150mL. It is probable that the combined factors of high volume and high starting granulocyte content prevented good separation. As the cell losses on some separations were high (80% of starting cells were lost from HAR 1) and the starting lymphocyte numbers were low in most cases, it was not deemed worthwhile to repeat the separations.

3.7 Lymphocyte subset phenotypes from Panel 1

The viable lymphocyte populations from the sixteen cone and nine apheresis samples were identified on CD45/SSC. CD45+ cells staining 7-AAD+ were excluded from analysis. The lymphocytes were further classified as T, B and NK cells using the markers CD3/4/8 (T cells) CD19 (B cells) and CD16/56 (NK cells) as described in Chapter 2. The percentage of viable lymphocytes categorised as T, B and NK cells was then calculated for both

sample groups. The harnesses performed so poorly, and in some cases contained so few lymphocytes that they were not further phenotyped. The results from individual samples are shown in Tables 3.3 and 3.4.

Sample Number n=16	CD3+ All T cells (%)	CD3+CD4+ Th cells % of CD3+	CD3+CD8+ Cytotoxic T % of CD3+	CD4:8 Ratio	CD19+ B Cells (%)	CD16/56+ NK cells (%)
Cone 1	74.63	54.91	45.09	1.22	9.57	13.19
Cone 2	72.14	63.64	36.36	1.75	11.18	15.51
Cone 3	62.68	50.32	49.68	1.01	12.46	22.18
Cone 4	72.25	56.73	43.27	1.31	13.97	11.45
Cone 5	55.12	77.10	22.90	3.37	19.15	21.48
Cone 6	56.92	65.54	34.46	1.90	23.83	17.65
Cone 7	74.70	68.43	31.57	2.17	14.55	8.90
Cone 8	67.62	56.51	43.49	1.30	15.37	19.46
Cone 9	64.00	76.23	23.77	3.21	12.28	18.80
Cone 10	51.85	60.38	39.62	1.52	13.11	33.99
Cone 11	69.55	77.76	22.24	3.50	15.92	11.54
Cone 12	77.92	71.93	28.07	2.56	7.82	10.62
Cone 13	79.33	51.15	48.85	1.05	10.39	6.81
Cone 14	78.81	57.52	42.48	1.35	12.20	3.95
Cone 15	65.82	71.33	28.67	2.49	18.91	11.91
Cone 16	74.10	67.47	32.53	2.07	15.05	7.95
Mean	**68.59**	**64.18**	**35.82**	**1.99**	**14.11**	**14.71**
Median	**70.85**	**64.59**	**35.41**	**1.83**	**13.54**	**12.55**
Range	**51.85-79.33**	**51.15-77.76**	**22.24-49.68**	**1.01-3.50**	**7.82-23.93**	**3.95-33.99**
Ref. Range*	**53.0-83.0**	**NA**	**NA**	**0.9-5.0**	**5.0-21.0**	**5.0-32.0**

**Reference range for German healthy blood donors from Zhang et al. 2016*

Table 3.3 T/B/NK lymphocyte content of prepared cones after density separation determined by flow cytometry. T/B/NK content was calculated as a % of all viable lymphocytes identified by morphology using CD45/SSC. CD4+ and CD8+ lymphocytes calculated as a % of viable CD3+ lymphocytes. NA - Not available.

Sample Number n=9	%CD3+ All T cells	%CD3+CD4+ Th cells % of CD3+	%CD3+CD8+ Cytotoxic T % of CD3+	CD4:8 Ratio	%CD19+ B Cells	%CD16/56+ NK cells
APH01	71.98	46.38	53.62	0.86	16.76	9.31
APH02	78.88	69.89	30.11	2.32	11.00	7.80
APH03	76.06	64.51	35.49	1.82	13.74	11.08
APH04	67.46	62.51	37.49	1.67	15.59	20.99
APH05	77.70	57.31	42.69	1.34	12.40	6.80
APH06	78.44	57.10	42.90	1.33	10.34	7.35
APH07	72.97	58.21	41.79	1.39	18.70	6.07
APH08	67.86	64.25	35.75	1.80	17.46	9.77
APH09	66.89	57.06	42.94	1.33	23.51	7.99
Mean	**73.14**	**59.69**	**40.31**	**1.54**	**15.50**	**9.68**
Median	**72.97**	**58.21**	**41.79**	**1.39**	**15.59**	**7.99**
Range	**66.89-78.88**	**46.38-64.51**	**30.11-53.62**	**0.86-2.32**	**10.34-23.51**	**6.07-20.99**
Ref. Range	**53.0-83.0**	**NA**	**NA**	**0.9-5.0**	**5.0-21.0**	**5.0-32.0**

**Reference range for German healthy blood donors from Zhang et al. 2016*

Table 3.4 T/B/NK lymphocyte content of apheresis product samples taken from the product bag determined by flow cytometry. T/B/NK content was calculated as a % of all viable lymphocytes identified by morphology using CD45/SSC. CD4+ and CD8+ lymphocytes calculated as a % of viable CD3+ lymphocytes. NA - not available.

The phenotypes of the lymphocyte subsets from separated cone sample cells had high inter-sample variability for all studied subsets (Figure 3.3) but were in line with the wide ranges found by other researchers establishing reference ranges for peripheral blood in different populations (Choi *et al.*, 2014, Zhang 2016 *et al.*). With the exception of Cone 10, which had slightly elevated levels of NK cells (33.99%), the mean percentage of the lymphocyte population for all cone samples staining CD3+, CD19+ and CD16/CD56+ fell within the normal range for Caucasian peripheral blood samples as established by Zhang *et al*. (2016), despite the fact that the samples had been retrieved from an apheresis set and had then undergone density gradient separation.

The inter-sample variability in the apheresis samples was lower than that seen in the cone samples (Figure 3.3), again confirming the accuracy of the MNC collection procedures as performed at NHSBT. The mean percentage of lymphocytes staining for CD3+, CD19+ and CD16/CD56+ in the apheresis samples were also in line with the published data (Choi *et al.*, 2014, Zhang 2016 *et al.*) for normal peripheral blood samples.

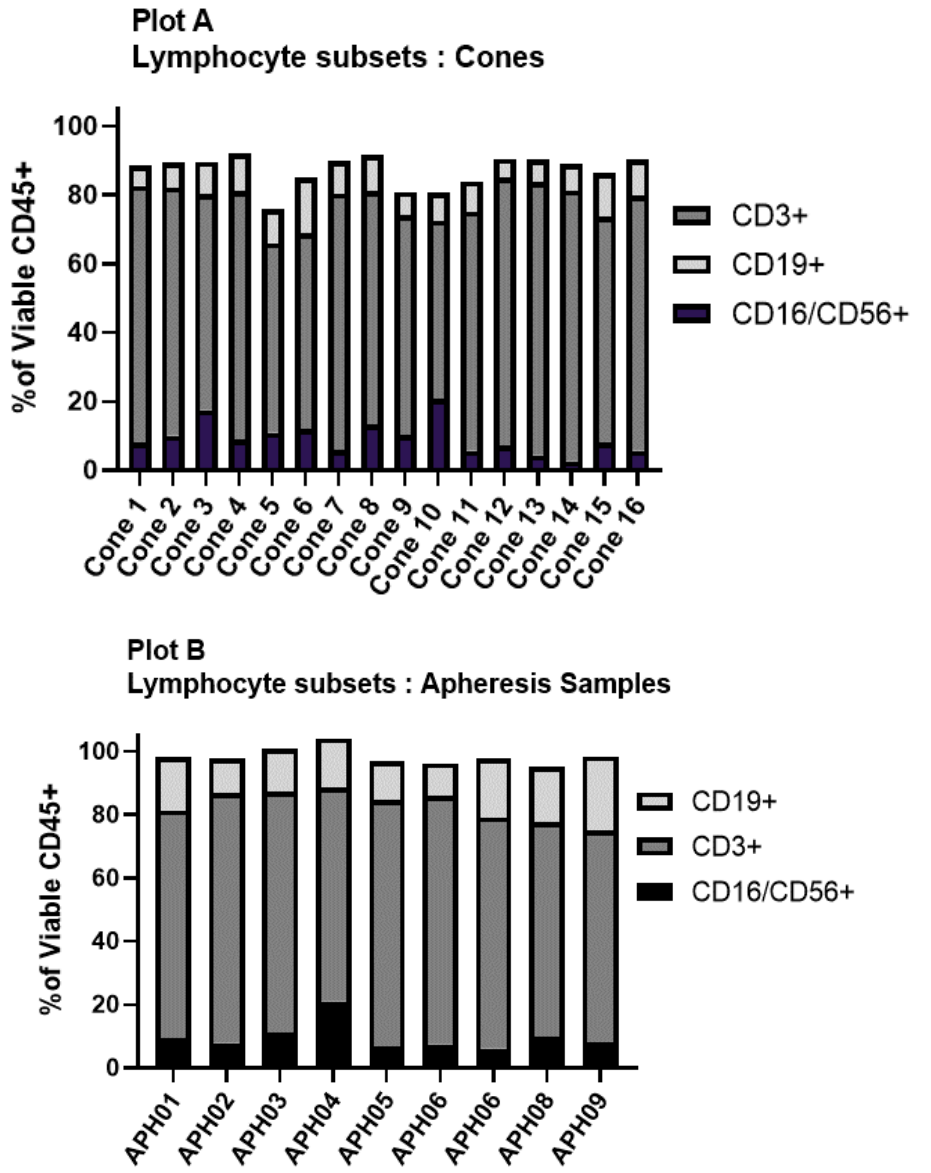


Figure 3.3: Graphical representation of lymphocyte subsets for cone samples C1-16 (Plot A) and apheresis samples APH1-9 (Plot B) as determined by flow cytometry pre-cryopreservation. Lymphocytes identified by morphology on CD45/SSC and subtyped as staining with CD19+ (B cells), CD3+ (T cells) and CD16/CD56+ (NK cells). Subsets shown as a percentage of viable lymphocytes. Viability determined by dye exclusion staining with 7-AAD.

The range of CD4 and CD8 positive cells was again wider in the cone samples than in the apheresis samples (Figure 3.4). The CD4:CD8 ratio ranged from 1.01 – 3.50 in the cones and from 0.86 – 2.32 in the apheresis samples. All samples from both groups fell withing

the normal range of 0.9-5.0 as published by Zhang *et al.*, (2016) although APH1 was found to have a CD4:CD8 ratio of 0.86 which was at the bottom of this range.

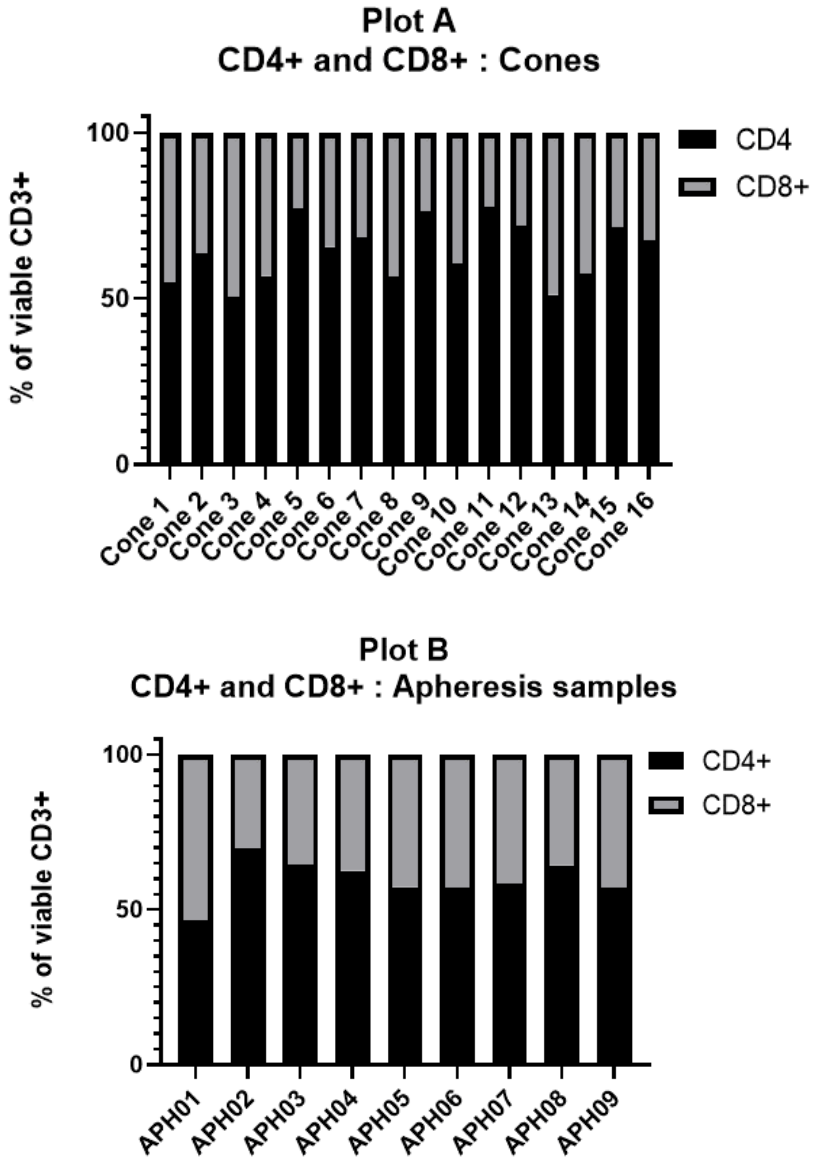


Figure 3.4: Graphical representation of CD4:8 expression on viable CD3+ cells from cone samples C1-16 (Plot A) and apheresis samples APH1-9 (Plot B) as determined by flow cytometry pre-cryopreservation. Lymphocytes identified by morphology on CD45/SSC. Lymphocytes staining positive for CD3+ subtyped as staining CD3+CD4+ (Th cells) and CD3+CD4- (cytotoxic T cells). Viability determined by dye exclusion staining with 7-AAD.

Although most results from both sample groups fell within the range for normal peripheral blood in German donors as reported by Zhang *et al.* in 2016, the percentage

of CD3, CD19 and CD16/CD56 lymphocytes and the CD4:8 ratio was compared between groups to determine if there were any significant differences between the cell populations derived from cones and apheresis samples. The expression of each marker in the two groups was tested using two-way ANOVA. No significant differences in expression of any of the markers studied were found between sample groups; p = 0.4595 (CD3), 0.4738 (CD4), 0.4738 (CD8), >0.9999 (CD4:CD8), 0.9965 (CD19), 0.3414 (CD19/CD56) (see Figure 3.5).

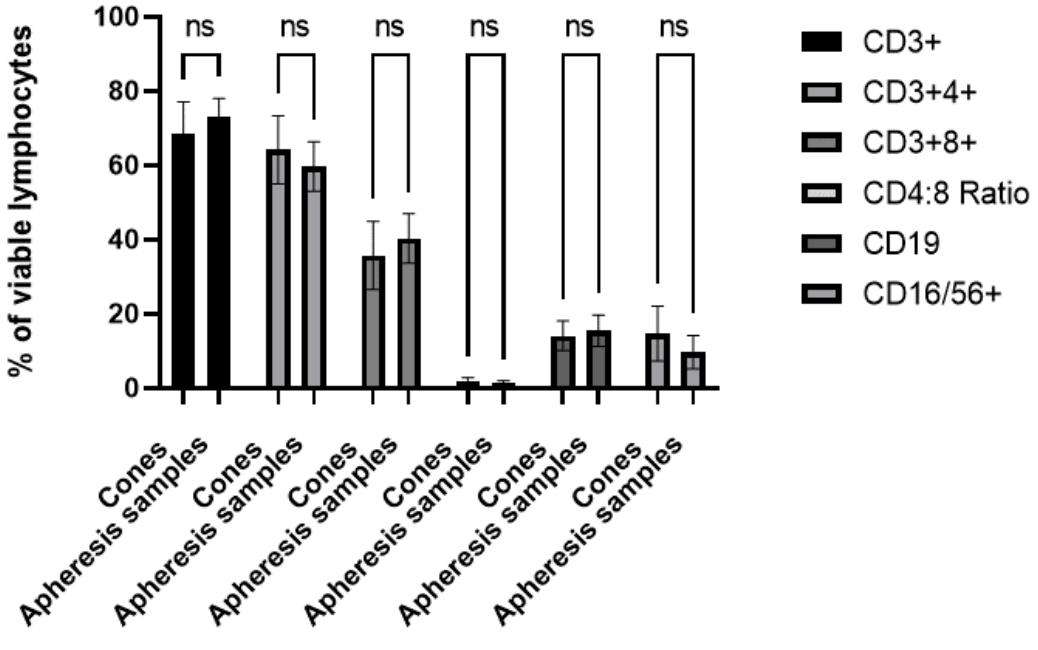


Figure 3.5: Statistical comparison of lymphocyte subsets on cells derived from cone C1-16 and apheresis samples APH1-9 determined by flow cytometry pre-cryopreservation. T (CD3+), B (CD19+), NK (CD16+/CD56+) and CD4:8 ratio compared between groups. ns – not significant by two-way ANOVA. Means shown +/- SD.

3.8 Panel 2: Treg, Naïve and Memory Phenotypes

As described in Chapter 2, lymphocytes from both cones and apheresis samples were identified from morphology by FSC/SSC flow cytometry. Lymphocytes staining positive for the viability marker, 7-AAD, were excluded from the analysis as non-viable. Viable lymphocytes staining CD3+ were further sub-classified to identify Tregs (CD4+CD25+CD127Lo), activated effector T cells (CD4+CD25dimCD127Hi), naïve T cells (CD4+CD45RA+/CD8+CD45RA+) and memory T cells (CD4+CD45RA-/CD8+CD45RA-). It was not possible to use anti-CD8 in the panel due to the lack of available fluorescence channels. CD8+ cells were therefore identified as CD3+CD4- lymphocytes.

The percentage of viable CD3+ lymphocytes classified as Treg, effector, memory and naïve phenotypes was calculated and the two sample groups compared. As explained in section 3.2, the thawed pilot vials from all samples were also tested with the T cell subset panel. The T subset results from the thawed vials were compared with the results from the pre-cryopreservation samples for cones 8-16 and all the apheresis samples and are shown in Tables 3.5 and 3.6.

Sample Number n=8	Fresh Treg	Thawed Treg	Fresh activated T effector	Thawed activated T effector	Fresh naïve T	Thawed naïve T	Fresh naïve Th	Thawed naïve Th	Fresh naive cytotoxic T	Thawed naïve cytotoxic T	Fresh T memory	Thawed T memory	Fresh T effector	Thawed T effector
	%CD4+CD25+ of viable CD3+		%CD4+CD127+CD25dim of viable CD3+		%CD45RA+ of viable CD3+		%CD4+CD45RA+ of viable CD3+		%CD8+CD45RA+ of viable CD3+		%CD4+CD45RA- of viable CD3+		%CD8+CD45RA- of viable CD3+	
Cone 1	**	1.43	**	46.69	**	54.30	**	23.89	**	30.40	**	24.44	**	21.25
Cone 2	**	**	**	**	**	**	**	**	**	**	**	**	**	**
Cone 3	**	1.39	**	31.78	**	25.71	**	8.62	**	17.09	**	24.71	**	49.41
Cone 4	**	1.04	**	46.11	**	49.63	**	20.48	**	29.16	**	27.21	**	22.85
Cone 5	**	3.34	**	71.12	**	21.13	**	16.83	**	4.30	**	58.76	**	19.99
Cone 6	**	3.58	**	59.40	**	33.94	**	21.28	**	12.65	**	42.63	**	23.07
Cone 7	**	3.53	**	65.55	**	46.11	**	35.33	**	10.78	**	34.59	**	19.06
Cone 8	3.64	1.22	52.77	42.73	51.93	53.63	26.09	21.25	25.84	32.38	30.98	23.52	16.89	22.88
Cone 9	4.38	1.65	71.50	42.68	57.74	36.15	48.85	19.55	8.89	16.61	27.80	26.10	14.32	37.78
Cone 10	8.98	1.06	51.48	36.60	32.34	28.80	18.08	9.81	14.26	18.99	42.96	27.93	24.58	43.02
Cone 11	7.2	2.8	68.77	56.10	40.15	25.87	34.73	18.21	5.42	7.65	41.49	41.10	18.38	32.78
Cone 12	5.29	2.12	66.01	59.55	40.67	34.01	27.03	19.23	13.64	14.79	44.47	42.74	14.86	23.13
Cone 13	3.45	1.51	46.95	37.36	42.52	36.38	22.99	15.53	19.54	20.85	27.37	23.61	30.15	39.95
Cone 14	4.82	0.94	52.97	32.16	45.10	43.94	18.12	7.60	26.98	36.34	39.89	25.68	14.85	30.17
Cone 15	8.43	3.69	62.27	57.78	48.36	36.71	30.39	17.62	17.97	19.09	40.65	44.25	10.81	18.61
Cone 16	2.81	0.63	65.16	40.25	42.89	37.73	23.67	10.67	19.22	27.06	44.30	30.37	12.58	31.60
Mean	**5.44**	**1.74**	**59.76**	**45.02**	**44.63**	**37.02**	**27.77**	**15.50**	**16.86**	**21.53**	**37.77**	**31.70**	**17.49**	**31.10**
Median	**4.82**	**1.51**	**62.27**	**42.68**	**42.89**	**36.38**	**26.09**	**17.62**	**17.97**	**19.09**	**40.65**	**27.93**	**14.86**	**31.60**
Range	**2.81-8.98**	**0.63-3.69**	**46.95-71.50**	**32.16-57.78**	**32.34-57.74**	**25.87-53.63**	**18.12-48.45**	**7.60-21.25**	**5.42-26.98**	**7.65-36.34**	**27.37-44.47**	**23.52-44.25**	**10.81-24.58**	**18.61-43.02**

** Not tested

Table 3.5 Treg, Effector, Naïve and Memory cell phenotypes shown as a % of viable CD3+ lymphocytes from fresh cone samples pre-cryopreservation and thawed samples post cryopreservation. Thawed results for cones 1-7 (**) were not included in the analysis and are shown for information only.

Sample Number n=9	Fresh Treg	Thawed Treg	Fresh activated T effector	Thawed activated T effector	Fresh naïve T	Thawed naïve T	Fresh naïve Th	Thawed naïve Th	Fresh naive cytotoxic T	Thawed naïve cytotoxic T	Fresh T memory	Thawed T memory	Fresh T memory/ effector	Thawed T memory effector
	%CD4+CD25+ of viable CD3+		%CD4+CD127+CD25dim of viable CD3+		%CD45RA+ of viable CD3+		%CD4+CD45RA+ of viable CD3+		%CD8+CD45RA+ of viable CD3+		%CD4+CD45RA- of viable CD3+		%CD8+CD45RA- of viable CD3+	
APH01	4.11	3.90	42.52	42.05	44.27	44.59	16.66	16.59	27.61	28.01	29.97	29.42	25.66	25.65
APH02	4.59	4.23	64.64	65.26	63.41	62.47	43.18	42.43	20.24	20.04	26.34	27.13	9.93	10.11
APH03	5.25	4.49	59.66	60.38	38.41	37.84	23.25	23.50	15.16	14.34	41.35	41.42	20.16	20.33
APH04	4.05	5.79	59.45	60.39	47.32	50.65	27.42	29.66	19.90	20.98	36.10	36.49	16.57	12.55
APH05	4.63	4.83	55.15	51.33	33.97	32.76	24.42	21.89	9.55	10.88	35.63	34.32	30.35	32.80
APH06	3.48	4.25	54.19	51.15	45.61	45.03	25.51	24.25	20.11	20.78	32.38	31.16	21.80	23.82
APH07	6.66	3.07	51.49	52.57	52.17	50.17	35.29	32.16	16.88	18.01	23.15	23.39	24.60	26.44
APH08	7.51	4.57	57.37	59.97	50.84	49.67	27.22	25.91	23.62	23.77	37.14	38.58	11.85	11.78
APH09	6.83	4.02	50.46	47.36	71.28	69.84	35.91	30.95	35.38	38.89	21.22	20.52	7.36	9.58
Mean	**5.23**	**4.35**	**54.99**	**54.50**	**49.70**	**49.22**	**28.76**	**27.48**	**20.94**	**21.74**	**31.47**	**31.38**	**18.70**	**19.23**
Median	**4.93**	**4.25**	**55.15**	**52.57**	**47.32**	**49.67**	**27.22**	**25.91**	**20.11**	**20.78**	**32.38**	**31.16**	**20.16**	**20.33**
Range	**3.48-7.51**	**3.07-5.79**	**42.52-64.64**	**42.95-65.26**	**33.97-71.28**	**32.76-69.84**	**16.66-43.18**	**16.59-42.43**	**9.55-35.38**	**10.88-38.89**	**21.22-41.35**	**20.52-41.42**	**7.36-30.35**	**9.58-32.80**

Table 3.6 Treg, Effector, Naïve and Memory cell phenotypes shown as a % of viable CD3+ lymphocytes from fresh apheresis samples pre-cryopreservation and thawed samples post cryopreservation.

While reference ranges for Treg populations in healthy donors have been established (Niu *et al.* 2020, Garcia-Prat *et al.* 2019), reference ranges for the prevalence of naïve and memory T cells have not, making it difficult to assess the observed phenotypes against the published literature. The reference range for Tregs established by Niu *et al.* (2020) for healthy adults in a Chinese population was 2.17-7.94% of CD3+. T cell subsets are known to vary slightly with population demographic (Zhang *et al.* 2016) but the differences are small, and the reference range established by Niu *et al.* provides a suitable guide for the study samples. The mean percentage of Tregs for the study samples in both groups were very similar; mean cone Treg = 5.44% of viable CD3+ and mean apheresis sample Treg = 5.23% of viable CD3+. Both means fell within the normal range. The Treg content of all individual samples in both groups also fell withing the normal range, except for Cones 10 and 15 which exceeded it slightly (8.98% and 8.43% respectively). See Figure 3.6.

A wider range of results was seen for effector T cells in the pre-cryopreservation samples, particularly in the cone samples (range 46.95-71.50%) although the means were similar; 59.76% for cones and 54.99% for apheresis samples. The apheresis samples were again very consistent, except for APH1 which had 42.52% effector T cells while all other samples ranged between 51-65% (Figure 3.6).

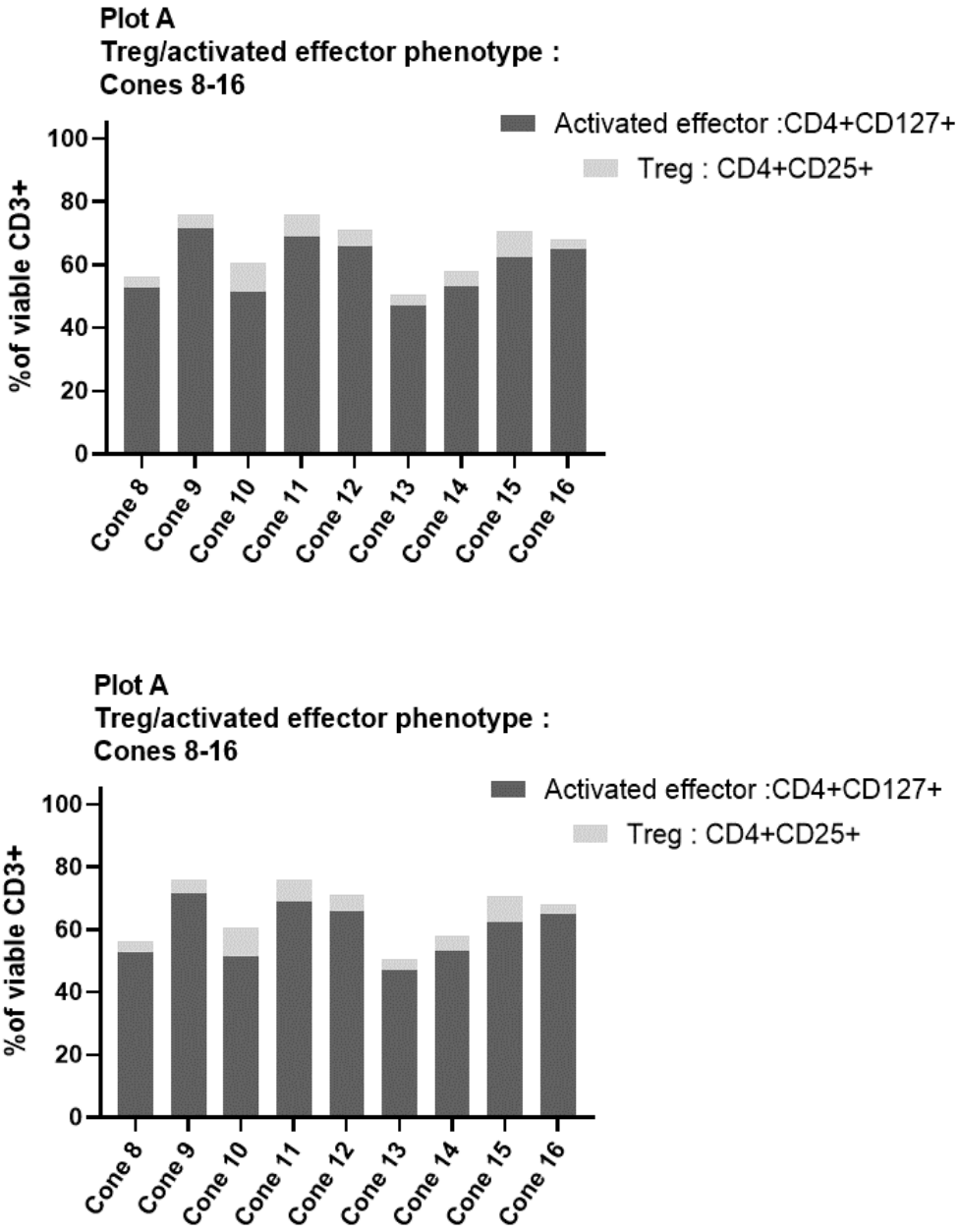


Figure 3.6: Graphical representation of proportion of total viable CD3+ lymphocytes typed as Treg (CD4+CD25+CD127Lo) and activated effector cells (CD4+CD127HiCD25Dim in lymphocytes derived from cone samples C8-16 (Plot A) and apheresis samples APH1-9 (Plot B) determined by flow cytometry pre-cryopreservation. Viability determined by dye exclusion staining with 7-AAD.

Naïve T cells were identified as those expressing CD45RA. CD45RA- T cells were identified as Memory T cells. In the absence of established reference ranges for expression of naïve and memory phenotypes, it is difficult to know whether the results obtained in the study are consistent with normal blood samples, but it seems probable that they are, given that all the other parameters tested fell within normal ranges. In both sample groups the split between naïve and memory phenotypes was similar; the mean naïve population for cones was 44.6% and for apheresis samples it was 49.7% (see Figure 3.7). The cones appeared to have a more consistent split between memory and naïve phenotypes, but the ranges seen in these populations did not appear to differ substantially from the ranges seen in the other cell populations studied.

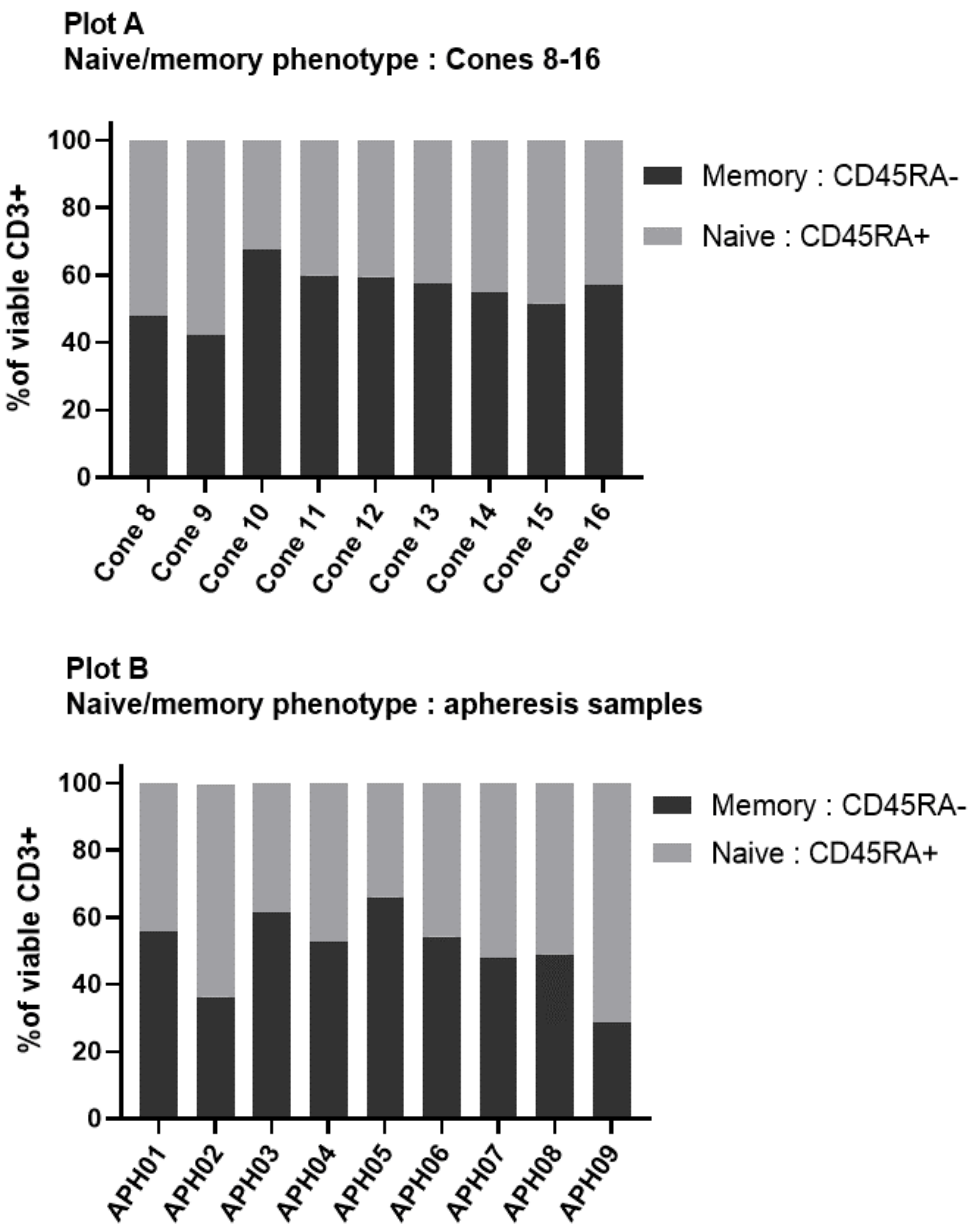


Figure 3.7: Graphical representation of proportion of viable CD3+ lymphocytes typed as naive (CD45RA+) and memory (CD45RA-) in lymphocytes derived from cones C11-16 (Plot A) and apheresis samples APH1-9(Plot B) pre-cryopreservation determined by flow cytometry. Viability determined by dye exclusion staining with 7-AAD.

Garcia-Prat *et al.* (2018) published a study of the range of naïve versus memory phenotypes in paediatric subject's peripheral blood. The oldest study group age range was 12-18 years, and as the naïve/memory ratio was found to decrease with age, the

results are not completely applicable to the adult donor population. However, they do serve as guidance for the normality of the results obtained in the study.

The ranges of naïve/memory types as a % of the CD3+ lymphocyte population established in 12–18-year-olds was as shown in Table 3.7.

Naïve and Memory T Cell Phenotypes as a Percentage of Total CD3+				
T cell population	CD markers used to define population	Reference mean and range	Study mean and range; Cone Samples	Study mean and range; Apheresis Samples
Naïve T cell	CD4+CD45RA+	44 (31-57)	28	29 (17-43)
	CD8+CD45RA+	21 (10-27)	17	20 (10-35)
Memory T cell	CD4+CD45RA-	30 (16-56)	38	31 (21-41)
	CD8+CD45RA-	55 (28-31)	55	50 (7-30)

Table 3.7 Proportion of viable T cells classified as naïve or memory phenotypes. Study results compared to reference range established by Garcia-Prat *et al.* (2018)

The samples in both study groups had a lower proportion of naïve T cells, particularly in the CD4+ population, than found in paediatric subjects which is explained by the fact that the study samples were all from adult donors. Overall, the cones had a slightly lower ratio of naïve: memory cells, seen in both CD4+ and CD8+ populations, although it was not found to be statistically significant when tested using two-way ANOVA. This finding may be explained by the fact that donor panels are now only recruiting volunteer HSC donors under the age of 30 while platelet donors may donate up to 65 years of age. Although the donors in the study were anonymous, it is probable that the apheresis donors were younger than the platelet donors.

To determine whether the T cell subset population differed between the cones and the apheresis samples, the prevalence of Tregs (CD4+CD25+CD127Lo), activated effector T

cells (CD4+CD127+), Naïve (CD45RA+) and Memory cells (CD45RA-) were compared between the two groups using two-way ANOVA. No significant difference was found between the any of the T cell subsets studied; p=>0.9999 (Treg), p=0.3734 (activated effector T cell), p=0.9994 (naïve CD4+), p= 0.5559 (naive CD8+), p= 0.1159 (memory CD4+), p=0.9981 (memory CD8+). See Figure 3.8.

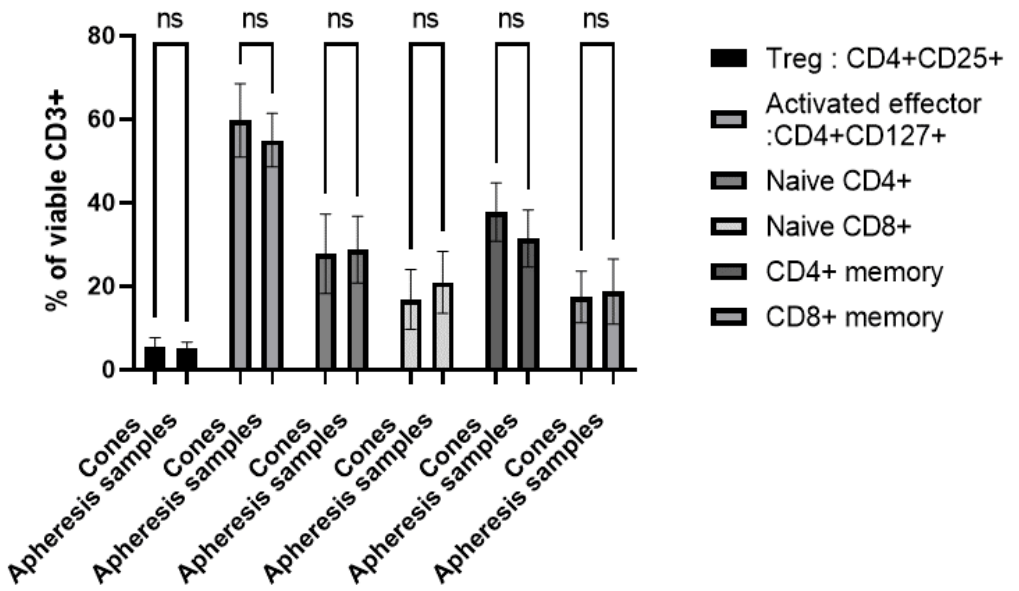


Figure 3.8: Statistical comparison of extended T cell subsets in lymphocytes derived from cone samples C8-16 and apheresis samples APH1-9 pre cryopreservation. ns – not significant by two-way ANOVA. Means shown +/- SD.

3.9 Cryopreserved samples

The CD3+CD4+ and CD3+CD8+ cell results for fresh pre-cryopreservation samples and thawed samples are shown in Tables 3.8 and 3.9 below. The T lymphocyte subsets in the fresh samples pre-cryopreservation and the thawed sample results are shown in Tables 3.5 and 3.6 above.

Sample Number n=8	Fresh CD3+CD4+ Th cells % of CD3+	*Thawed CD3+CD4+ Th cells % of CD3+*	Fresh %CD3+CD8+ Cytotoxic T % of CD3+	*Thawed %CD3+CD8+ Cytotoxic T % of CD3+*	Fresh CD4:CD8 Ratio	*Thawed CD4:CD8 Ratio*
Cone 1	**	*48.33*	**	*51.65*	**	*0.94*
Cone 2	**	**	**	**	**	**
Cone 3	**	*33.33*	**	*66.5*	**	*0.50*
Cone 4	**	*47.69*	**	*52.01*	**	*0.92*
Cone 5	**	*75.59*	**	*24.29*	**	*3.11*
Cone 6	**	*63.92*	**	*35.73*	**	*1.79*
Cone 7	**	*69.92*	**	*29.83*	**	*2.34*
Cone 8	57.07	*44.77*	42.73	*55.26*	1.34	*0.81*
Cone 9	76.65	*45.64*	23.21	*54.38*	3.30	*0.84*
Cone 10	61.04	*37.74*	38.84	*62.01*	1.57	*0.61*
Cone 11	76.22	*59.31*	23.81	*40.43*	3.20	*1.47*
Cone 12	71.5	*61.97*	28.5	*37.92*	2.51	*1.63*
Cone 13	50.36	*39.14*	49.68	*60.8*	1.01	*0.64*
Cone 14	58.01	*33.28*	41.83	*66.5*	1.39	*0.50*
Cone 15	71.04	*61.87*	28.79	*37.7*	2.47	*1.64*
Cone 16	67.97	*41.04*	31.8	*58.65*	2.14	*0.70*
Mean	**65.54**	***47.20***	**34.35**	***52.63***	**2.10**	***0.98***
Median	**67.97**	***44.77***	**31.80**	***55.26***	**2.14**	***0.81***
Range	**50.36-72.22**	***37.74-61.97***	**23.21-49.68**	***37.70-66.50***	**1.01-3.30**	***0.50-1.64***

***No thawed data available for Cone 2 as pilot vial could not be located*

Table 3.8 CD4+ and CD8+ as a percentage of all viable CD3+ cells pre-and post-cryopreservation. cones 8-16. Mean, median and range shown for cones 8-16. Thawed results for cones 1-7 included in table for information only.

Sample Number n=9	Fresh CD3+CD4+ Th cells % of CD3+	*Thawed CD3+CD4+ Th cells % of CD3+*	Fresh %CD3+CD8+ Cytotoxic T % of CD3+	*Thawed %CD3+CD8+ Cytotoxic T % of CD3+*	Fresh CD4:CD8 Ratio	*Thawed CD4:CD8 Ratio*
APH01	46.63	*46.01*	53.26	*53.99*	0.88	*0.85*
APH02	69.51	*69.56*	30.17	*30.44*	2.30	*2.28*
APH03	64.59	*64.92*	35.32	*35.08*	1.83	*1.85*
APH04	63.52	*66.15*	36.47	*33.85*	1.74	*1.95*
APH05	60.05	*56.21*	39.95	*43.79*	1.50	*1.28*
APH06	57.89	*55.41*	42.11	*44.59*	1.37	*1.24*
APH07	58.44	*55.55*	41.56	*44.45*	1.41	*1.25*
APH08	64.36	*64.49*	35.64	*35.51*	1.81	*1.82*
APH09	57.13	*51.48*	42.87	*48.52*	1.33	*1.06*
Mean	**60.23**	***58.86***	**39.71**	***41.14***	**1.57**	***1.51***
Median	**60.05**	***56.21***	**39.95**	***43.79***	**1.50**	***1.28***
Range	**46.63-69.51**	***46.01-69.56***	**30.17-53.26**	***30.44-48.52***	**0.88-2.30**	***0.85-2.28***

Table 3.9 CD4+ and CD8+ as a percentage of all viable CD3+ cells for apheresis samples pre-and post-cryopreservation

Cryopreservation had a significant impact on all the CD3+ cell populations studied that were derived from cones (Figure 3.9). Paired t-tests were used to determine the statistical significance of the observed alteration in T cell populations pre-cryopreservation and post-thaw. CD4+ lymphocytes were more severely impacted than CD8+ lymphocytes. The mean %CD4+ dropped from 65.54% to 47.20% post thaw ($p=0.00165$), while the mean %CD8+ rose from 34.35% to 52.63% ($p=0.000177$). The mean CD4:CD8 ratio dropped accordingly from 2.10 to 0.98 ($p=0.000874$). The bottom of the normal range for CD4:CD8 ratio is 0.9 (Zhang *et al.* 2016) and although the mean thawed results for the cones remained within it, the individual results showed that Cones 8, 9, 10, 13, 14 and 16 all had CD4:CD8 ratios of <0.9. Of those compared, only cones C11, C12 and C15 maintained a normal CD4:CD8 ratio post cryopreservation.

Although not included in the analysis due to lack of a pre-cryopreservation comparator, cones C1, C3 and C4 were also found to have a low CD4:CD8 ratio.

This effect was not seen in the apheresis samples, where CD4+ and CD8+ cells appeared to be not impacted by cryopreservation and thawing (Figure 3.9). The mean pre-cryopreservation CD4:CD8 ratio was 1.57 dropping slightly to 1.51 post thaw. No significant difference was found between the pre-cryopreservation and post-thaw results (Figure 3.9). Of the samples studied, only APH9 showed any evidence of loss of CD4+ cells. In this sample the CD4:CD8 ratio dropped from 1.33 to 1.06. APH1 had a CD4:CD8 ratio just below normal range (0.88) pre-cryopreservation but was found not to be impacted after thawing (0.85).

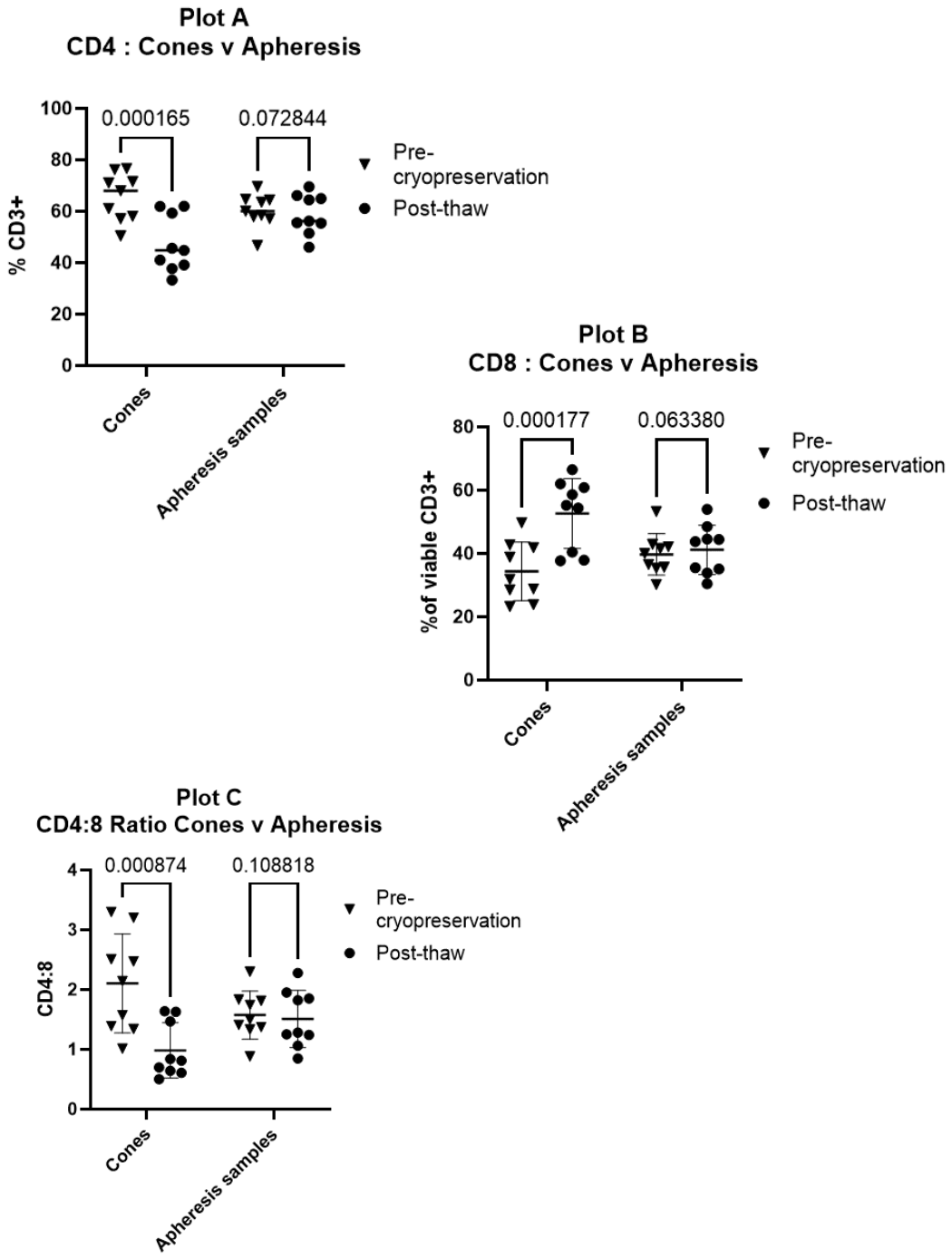
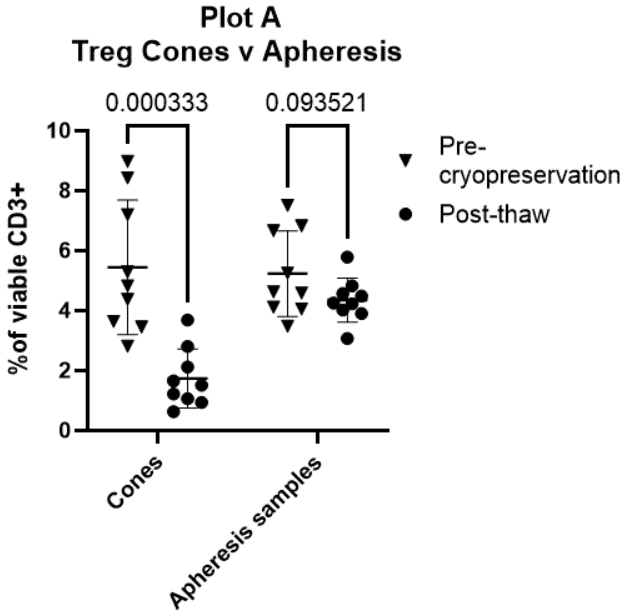


Figure 3.9: Comparison of CD4 and CD8 expression in cones and apheresis samples pre-cryopreservation and post thaw using paired t-test for each sample group. CD4:CD8 expression on viable CD3+ cells from cone samples C8-16 (Plot A) and apheresis samples APH1-9 (Plot B) as determined by flow cytometry pre-cryopreservation. Lymphocytes identified by morphology on CD45/SSC. Lymphocytes staining positive for CD3+ subtyped as staining CD3+CD4+ (Th cells) and CD3+CD4- (cytotoxic T cells). Viability determined by dye exclusion staining with 7-AAD. Means shown +/-SD. All p-values shown.

Cells expressing a Treg phenotype were found to be significantly reduced in the thawed cone samples (Figure 3.10). The mean proportion of CD3+ identified as Tregs in samples C8-16 dropped from 5.44% in the fresh sample to 1.74% in the thawed samples ($p=0.0003$). The mean Treg proportion in apheresis samples APH1-9 was also slightly reduced from 5.23% to 4.35% of CD3+ but this result was not significant ($p=0.093521$). Pre-cryopreservation Treg numbers were found to have a broad range, particularly in the cone samples where the range dropped from 2.81-8.98 to 0.63-3.69. Without exception, fewer Tregs were identified in the thawed cone samples than in the pre-cryopreservation samples, for most samples. The same picture was not true for the apheresis samples where, although the mean proportion of Tregs dropped slightly, individual samples were found that had either increased or decreased numbers post-thaw.



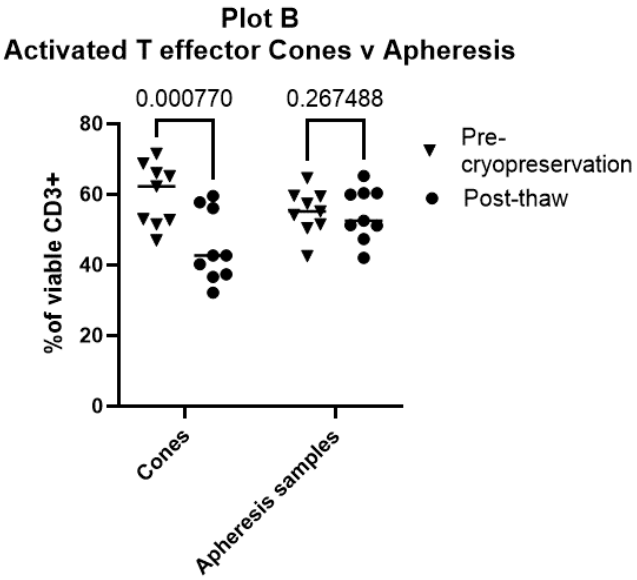


Figure 3.10: Comparison of Treg and activated effector cells populations derived from cone samples C8-16 (Plot A) and apheresis samples APH1-9 (plot B) pre-cryopreservation and post thaw using paired t-tests for each sample group. Proportion of total viable CD3+ lymphocytes typed as Treg (CD4+CD25+CD127Lo) and activated effector cells (CD4+CD127HiCD25Dim) determined by flow cytometry. Viability determined by dye exclusion staining with 7-AAD.Means shown +/-SD. All p-values shown.

A slight reduction in the proportion of naïve T cells post-thaw was seen again in cone samples only. The mean percentage of naïve cells dropped from 44.6% to 37.02%, thereby increasing the proportion of T cells with a memory phenotype. The difference

was significant (p= 0.0255) and was derived from a reduction in the numbers of CD4+CD45RA+ cells. The CD8+CD45RA+ cell numbers were unaffected by cryopreservation and thawing. The proportions of naïve or memory phenotype T cells from apheresis samples were completely unaffected by cryopreservation and thawing (p= 0.3911)

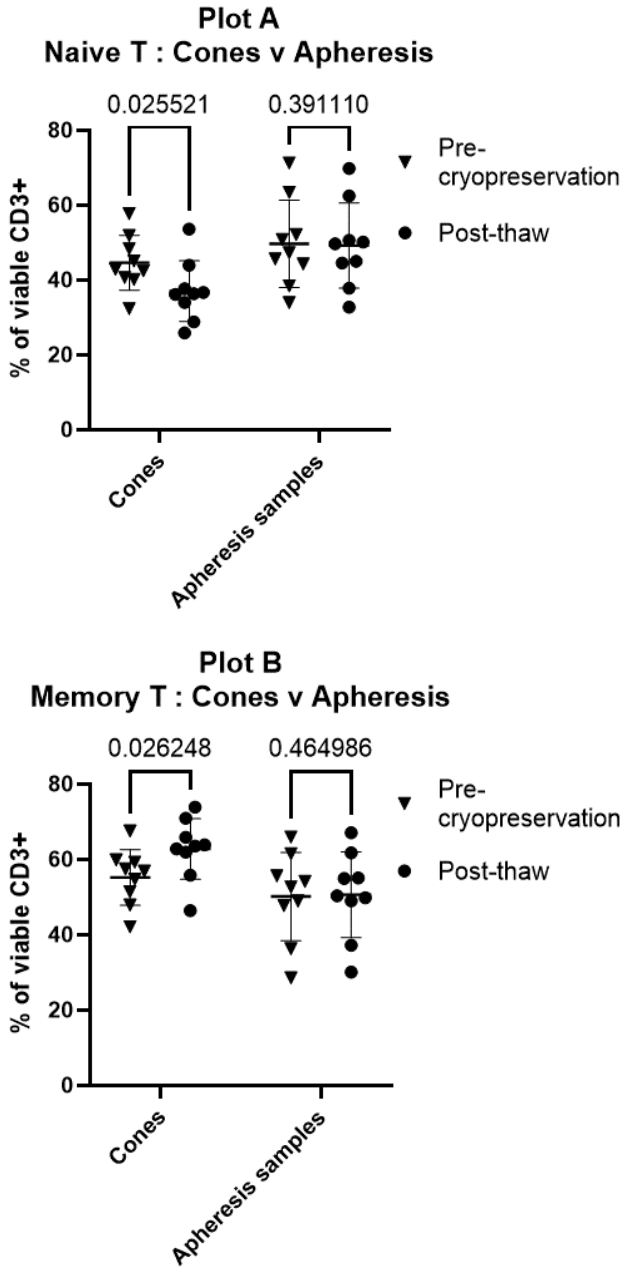


Figure 3.11: Comparison of Naïve and Memory populations phenotypes in cones and apheresis samples pre-cryopreservation and post thaw using paired t-tests for each sample group. Populations derived from cone samples C8-16 (Plot A) and apheresis samples APH1-9 (plot B) pre-cryopreservation and post thaw. Proportion of total viable CD3+ lymphocytes typed as naïve (CD4+45RA+/CD8+45RA+) and memory (CD4+45RA-/CD8+45RA-) determined by flow cytometry. Viability determined by dye exclusion staining with 7-AAD. Means shown +/-SD. All p-values shown.

3.10 Discussion of the lymphocyte subsets and T cell subset results

The results obtained for all cell populations indicated that the cell separation methods used did not impact specific lymphocyte populations. The phenotype of lymphocytes separated manually using density gradient media or using automated programming on the Spectra Optia, did not differ significantly from each other and the samples used were still reflective of those found in normal blood. The findings from the cone derived samples confirmed those of Boudreaux *et al.* (2019) who reported that lymphocytes extracted from leukocyte cones have the same phenotype and function as those from normal peripheral blood. Both sample types were collected using Anticoagulant Citrate Dextrose solution A (ACD-A) thereby eliminating any effect on phenotype that could potentially have been caused by use of different anticoagulants. The samples were all stored overnight before being phenotyped which could potentially have affected the phenotype of the stored cells. However, a 2018 study of 50 samples from healthy blood donors by Tompa *et al.* found no differences in expressed lymphocyte cell surface markers in cells stored for 4 or 24 hours, indicating that the phenotypes reported on the stored cells in this study were representative of the cells at collection.

Any effect seen in behaviour at activation/stimulation could therefore be attributed to experimental effect, rather than skewing of the starting population.

There were some marginal outliers from established ranges; Cone 6 and APH09 had B cell content that narrowly exceeded the normal range and Cone 10 had NK cell content that narrowly exceeded the normal range. Reported normal ranges identified in healthy donors are wide and that they vary with age, sex and ethnicity (Choi *et al*. 2014, Zhang *et al*. 2016 Garcia-Prat *et al*.2018, Omana-Zapata *et al*. 2019). The donors in this study were anonymous, making it impossible to relate any findings to individual donor

characteristics. In addition, classification by flow cytometry is affected by multiple variables: analyser type, gating strategy and operator expertise to name but a few (van der Strate *et al.* 2017). It was therefore not deemed necessary to further investigate the outlying results.

The results of the comparison of CD3+ cell subtypes in thawed vials compared to the pre-freeze results showed a marked impact in the case of lymphocytes derived from cones only. The apheresis samples maintained their pre-cryopreservation profile, while the cone samples showed a significant loss of CD4+ cells, a finding that was particularly marked in Tregs. It was possible that the alteration in observed T cell phenotype was derived from the fact that the freezing protocol in use was one optimised for bags, not vials (Hunt, 2019, Meneghal *et al*., 2020) and the sub-optimal cryopreservation conditions could have contributed to the death of some cell populations in the vials. However, it would be expected that both samples from apheresis and cones would be equally impacted which was not the case here. In fact, given the poor survival of granulocytes during cryopreservation in 10% DMSO (Vian and Higgins, 2014), it would be expected that the samples from apheresis, containing up to 30% granulocytes, would have shown higher overall cell losses than the density gradient separated cone cells. There is some evidence that storage in the presence of granulocytes can adversely impact the number of detectable CD4+ cells. A multicentre study published by Agashe *et al.* in 2017 provided some evidence that granulocyte contamination of stored samples can lead to a reduction of identified CD4+ events after 6-18 hours storage. The cones were stored overnight prior to density gradient separation and potentially could have been damaged by the presence of granulocytes at this stage, leading to a higher loss of CD4+ cells during the cryopreservation process. However, Agashe's study related to cells

that had been shipped at 20°C to different study sites, while the samples in this study were stored at 4°C. The apheresis samples in our study were also stored overnight prior to cryopreservation for an average period of 18 hours, and there was no identifiable impact on the number of CD4+ events post-thaw in this group. In addition, both groups of samples demonstrated high CD3+ cell viability when tested prior to cryopreservation, and the phenotypes of both groups fell within the normal ranges at this point.

A decrease in the CD4+ cell population, and particularly Tregs, in thawed cells as seen in this study has been reported by several researchers, although others have reported no impact on prevalence of lymphocyte subsets at all (Anderson *et al*. 2019, Tompa *et al*. 2018). Li *et al*. (2022) reported a deficit in CD8+ events with CD4+ events unaffected by cryopreservation. In 2017, Ford *et al.* reported differential loss of the CD4+ compartment in a study of cryopreserved paired donor PBMC samples. Both a decrease in numbers and loss of function after cryopreservation and thawing has been reported in Tregs derived from PBMC (Florek *et al*. 2015, Weiner *et al*. 2015), although the study by Tompa *et al*. (2018) reported no deficit in the percentage of CD3+ cells staining CD4+CD25+ in PBMCs isolated from whole blood by Ficoll separation after cryopreservation. Tregs represent a small fraction of total CD3+ in normal blood and inaccuracy of flow cytometry increases with low numbers of events so it is possible that the deficit seen in this study, or the lack of deficit seen in Tompa *et al*.'s study, may be related to the relatively low number of Treg events analysed. In this study, the mean number of events classified as Treg was 330 for cone samples but 906 for apheresis samples indicating the results for apheresis samples would be more accurate (Lambert *et a*l., 2020). However, flow cytometric analysis for rare cell populations has been shown to be reliable where the number of target events acquired is >100 (Lambert *et al.,* 2020,

Macey, 2007), so the results found here probably demonstrate a real effect, particularly when related to the reduction in CD4+ T cell numbers was seen across the spectrum of subsets.

Tompa *et al*. (2018) also found no impact on the ratio of naïve:memory phenotypes pre- and post- cryopreservation. Our study showed a small reduction in the number of naïve CD4+ cells after thaw which is compatible with the findings of Courville and Lawrence (2020) who demonstrated a reduction in the numbers of naïve phenotypes after cryopreservation and thawing.

The thawed T cell extended subset results presented in this study were obtained from pilot cryovials. These were thawed only when it was discovered that the results from Cones 1-7 were not valid because of the flow cytometer set-up error. The samples cryopreserved in bags were not tested for T cell subsets when thawed for activation, so it is not possible to know whether the effect of cryopreservation and thawing found in the study was caused by the cryovials themselves or whether it was also present in the bags. Most of published literature also refers to lymphocytes cryopreserved in vials as bags are expensive and require specialist facilities to cryopreserve and store them. One study by Schafer *et al.* in 2020 did examine the phenotype and function of thawed lymphocytes from mobilised apheresis collections. They reported no alteration in the ratio of CD4+ to CD8+ cells post-thaw, which corroborates the findings for apheresis samples from our study, but they did not phenotype the thawed cells further. The absolute numbers of viable CD3+ lymphocytes pre-freeze and post-thaw could not be compared using Panel 2 as it was not set up to gather this information. However, when the bags themselves were thawed for activation, the absolute numbers of viable CD3+

lymphocytes still present after thaw were calculated and compared to the number present pre-cryopreservation. These results are fully discussed in Chapter 3, but to summarise, viable CD3+ cell losses of between 10-80% were seen under the different study conditions, indicating that the reduction of some cell populations seen in the thawed samples was at least in part, reciprocated in the bags. Further study will be required to address this issue.

3.11 Chapter 3: Summary and Key Findings

1. Insufficient cells were retrieved from the apheresis harnesses to fulfil the experimental criteria. It is recommended that researchers doing similar work do not attempt to use these as a starting material.

2. No statistically significant differences in lymphocyte phenotype (B, T, NK, Treg, naïve and memory phenotype) were found between the cells derived from the cone and apheresis samples, although the CD3+:CD8+ ratio found in apheresis samples was lower (1.54) than that found in cones (1.99). The lymphocyte phenotypes seen in both sample groups fell within normal ranges reported in the literature.

3. Comparison of T cell subsets from the pre-freeze cone samples with cells cryopreserved in vials demonstrated statistically significant loss of CD4+ T cells with a 50% reduction in CD4+:CD8+ ratio from 2.1 to 0.98. Tregs were reduced from 5.44% of viable CD3+ to 1.74% and a reduction in CD3+ cells expressing a naïve phenotype from 44.6% to 37.0% was also seen. In contrast, cells derived from apheresis samples appeared to be unaffected by cryopreservation. The CD4+:CD8+ ratio, and percentage of naïve T cells remained unchanged post-thaw.

4 Chapter 4 Results – Total Nucleated and Viable CD3+ Cell Recoveries Post-Cryopreservation

4.1 Background

To determine whether the cell numbers in the product prior to cryopreservation were maintained over the cryopreservation and thawing process, the numbers of viable CD3+ and TNC (both viable and non-viable) in the thawed products were calculated. Cryopreserved DLI products are issued for clinical use based on the cell numbers present in the product prior to cryopreservation.

It was therefore an important aim of the study to determine the impact of both the cryopreservation itself, and of extended storage time pre-cryopreservation on the viable cell numbers remaining in the thawed product. This information could then be used to determine the accuracy of the information about the product supplied to the clinicians when the cells are requested for transplant, and the potential requirement for further testing. It could also be used to establish a recommended maximum pre-cryopreservation storage time for lymphocyte products.

4.2 Summary of the study methods

Cells from sixteen cone samples, nine apheresis samples and three harnesses were cryopreserved in 10% DMSO following standard NHSBT cryopreservation protocols. The cells were cryopreserved in CyoMACS® freezing bags (Miltenyi Biotec) at a WBC concentration ranging from 10-20 x 10^6/mL. The bags were frozen using a Planer Kryo 560-16 rate-controlled freezer and transferred to storage in vapour phase nitrogen immediately after completion of the programme. The cells were stored for a minimum of 7 days in vapour phase nitrogen before thawing for stimulation.

The bags were removed from vapor phase storage and thawed rapidly in a water bath at 37˚C. As soon as the contents were thawed, a sample was removed for FBC and flow cytometric analysis to enable calculation of the TNC and viable CD3+ cell recovery immediately post thaw. These samples were diluted dropwise in media at 21˚C and used for both flow cytometry analysis and FBC. The total number of nucleated cells recovered from each product bag was calculated using the WBC from the Sysmex 1000i haematology analyser and the product volume as recorded on the product worksheet. The percentage recovery after thaw was then calculated from the number of cells present in the bags prior to cryopreservation.

$$\% \text{ TNC thaw recovery} = \frac{\text{WBC} \times 10^6/\text{mL post-thaw} \times \text{bag volume(mL)}}{\text{WBC} \times 10^6/\text{mL pre-thaw} \times \text{bag volume(mL)}}$$

Similarly, the total number of viable CD3+ cells in each product bag were calculated using the viable CD3+/mL produced by the FACSLyric™ and the product volume.

$$\% \text{ viable CD3+ thaw recovery} = \frac{\text{Viable CD3+} \times 10^6/\text{mL post-thaw} \times \text{bag volume(mL)}}{\text{Viable CD3+} \times 10^6/\text{mL pre-thaw} \times \text{bag volume(mL)}}$$

The results for each sample were plotted individually, allowing for investigation of anomalous results.

Although no microbiology testing was performed on any of the samples used in the study, no evidence of infection as indicated by clumping or severe cell loss was evident in any of the thawed samples.

4.3 Data analysis

Recovery and viability data generated from D1, D2 and D3 cryopreservation samples was analysed to compare outcome between the different days of cryopreservation within the sample groups using one-way ANOVA. The outcome between sample groups was compared using two-way ANOVA. QQ plots were generated to confirm normality of distribution for all tests and results accepted only if a normal distribution was confirmed. All tests performed were found to have a normal distribution, confirming the validity of the approach. On the advice of the Sheffield Hallam University statistician, samples with missing data sets were excluded from the analysis.

4.4 Summary of the study samples

4.4.1 Apheresis cones

Sixteen apheresis cones were tested in the study. Sufficient cells were yielded from all 16 cones to cryopreserve cells under all study conditions.

D1 and D2 bags were cryopreserved from cones C3 and C4, but due to an oversight in the laboratory, the D3 bags were not cryopreserved. The data generated from the D1 and D2 bags was analysed.

The Panel 3 flow cytometry for C10 on D2 and D3 was performed on incorrect samples and the CD3+ viability and viable CD3+/mL results from this sample were therefore excluded from the analysis.

4.4.2 Apheresis samples

Nine apheresis samples were tested in the study. Eight of the nine apheresis samples contained sufficient cells to cryopreserve all study conditions. APH04 did not contain sufficient cells to cryopreserve in bags but was cryopreserved in 1mL vials on D1, D2 and D3. The RCF programme used was designed to maximise recovery of cells cryopreserved

in bags and it is known that cell recovery is lower for cells cryopreserved in vials as compared to bags (Hunt, 2019, Meneghal *et al.*, 2020). As a result, the cell viability and recovery data from the vials were excluded from the analysis.

4.4.3 Apheresis harnesses

Five apheresis harnesses were tested in the study. Unfortunately, the number of nucleated cells that could be extracted from the harnesses was far lower than expected – the harnesses yielded between 30-50% of the cell numbers retrieved from the apheresis cones. Density gradient separation further reduced the numbers of available cells to the point where insufficient cells were retrieved from 4 of the 5 apheresis harnesses tested to fulfil the study conditions.

Harness 1

Only 19% of the nucleated cells extracted from the set were still present after density gradient separation and 82.43% of the remaining cells were identified as granulocytes by flow cytometry (See Chapter 3). Only 3.8 x 10^6 viable CD3+ cells were identified, which was insufficient for the study. The harness sample was discarded.

Harness 2

Although recovery of nucleated cells after density gradient separation was 89%, 82.58% of the recovered cells were identified as granulocytes by flow cytometry (see Chapter 3). Possibly because of the high granulocyte content, the cells extracted from the set clumped during overnight storage prior to D1 cryopreservation. A total of 36.8 x 10^6 viable CD3+ remained and D1, Day 2 and D3 bags were cryopreserved. However, when the Day 1 bag was thawed, the recovery of viable CD3+ cells was extremely poor (6.91%).

The total number of viable CD3+ was 8.38 x 10^5. They were plated but failed to expand in culture.

Harness 3

Although recovery of nucleated cells after density gradient separation was 47%, the MNC extracted from the set clumped during overnight storage prior to D1 cryopreservation. A total of 8.83 x 10^6 viable CD3+ remained and D1 and D2 bags were cryopreserved. However, when the D1 bag was thawed, the recovery of viable CD3+ cells was poor (20.48%). The total number of viable CD3+ cells was 9.03 x 10^5. They were plated but failed to expand in culture.

Harness 5

38% of the nucleated cells were recovered post density gradient separation and there were sufficient CD3+ cells (66 x10^6) to cryopreserve on D1 and D2. However, when the bags were thawed, the cells clumped so severely that it was not possible to recover sufficient cells for the study. The samples were discarded.

Harness 4

Harness 4 alone yielded sufficient cells to adequately fulfil all study conditions. Cell recoveries and viabilities for all 3 days were calculated for this harness (shown in Tables 4.2 and 4.4 below). However, the results from harness 4 were not included in the data analysis as it was the only representative of the sample type. It was concluded that apheresis harnesses did not routinely contain sufficient cells to fulfil the study conditions and their use was discontinued after the first 5 had been processed.

Harness Number n=5	Pre-Freeze D1 CD3+ viability	Thawed CD3+ viability Day 1 (%)	Thawed CD3+ Recovery Day 1 (%)	TNC Recovery Day 1 (%)	Thawed CD3+ viability Day 2 (%)	Thawed CD3+ Recovery Day 2 (%)	TNC Recovery Day 2 (%)	Thawed CD3+ viability Day 3 (%)	Thawed CD3+ Recovery Day 3 (%)	TNC Recovery Day 3 (%)
HAR 1	99.6	CLUMPED PRE-CRYOPRESERVATION – NOT CRYOPRESERVED								
HAR 2	99.4		6.9	36.4	INSUFFICIENT CELLS FOR D2/D3 CRYOPRESERVATION					
HAR 3	98.4		20.5	23.7	INSUFFICIENT CELLS FOR D2/D3 CRYOPRESERVATION					
HAR 4	97.7	38.6	5.4	85.5	31.1	4.1	56.1	15.4	2.2	58.5
HAR 5	99.9	CLUMPED ON THAWING – UNABLE TO TEST								
Range	97.7-99.9		5-4–20.5	23.7-85.5						

Table 4.1 Nucleated cell count recovery, thawed CD3+ viability and viable CD3+ recovery pre- and post-freeze from apheresis harnesses 1-5; showing %recovery of nucleated cells in thawed bags cryopreserved on Day 1 (harnesses 2,3,4), Day 2 (harness 4 only) and Day 3 (harness 4 only*).*

4.5 Discussion of harness failures

All the harnesses had good CD45+ and CD3+ viability on arrival in the laboratory, but all contained very low total numbers of lymphocytes. This finding is compatible with reported MNC collection efficiency of up to 60% for Optia MNC harvests (Davis *et al.* 2016) – most circulating lymphocytes had been collected into the product bag and therefore did not remain in the set to be extracted for the study. The harnesses were separated using Lympholyte in 50mL tubes as opposed to SepMate™ tubes which were used for the cone samples. There is some evidence that recovery of MNC is superior in SepMate™ tubes (Grievink *et al.* 2016) and it is therefore possible that the method of density separation contributed to the poor MNC recovery from the harnesses. However, in this case, the granulocyte contamination in the cells retrieved from the harnesses was so overwhelming that even after density separation, >60% of the cells present were still

granulocytes (See Chapter 3). As discussed in Chapter 1 granulocytes have a short half-life (Colotta *et al.* 1992, Klinkmann *et al.* 2021), surviving 24-48 hours in storage. Degradation of granulocytes during storage and cryopreservation releases cytotoxic materials into the surrounding cells causing bystander damage and cell death (Vemeren *et al.* 2021). Bystander damage from granulocyte degradation was the most likely cause of the poor CD3+ cell recoveries post-thaw and the sample clumping both pre-and post-cryopreservation.

Harnesses 2 and 3 did contain enough viable CD3+ to cryopreserve a single bag on D1 but when they were thawed the total number of viable CD3+ cells recovered was $<1 \times 10^6$ which was insufficient to be plated for culture. Notwithstanding the poor recovery, the cells from harnesses 2 and 3 were plated at a reduced concentration but failed to expand in culture. After 72 hours in culture only 2-4% of the viable CD3+ cells that had been plated could be detected, indicating that they had died during the incubation period.

It was not possible to draw any conclusions from the very limited recovery results obtained.

4.6 Total nucleated cells recoveries

Although the WBC from the haematology analyser includes dead/apoptotic cells as well as viable cells, the total number of cells recovered can still be used as an indicator of the efficacy of the freezing process. The results for cone and apheresis sample groups are shown in Tables 4.2 – 4.3.

	Total Nucleated Cells x 10^6								
Sample Number n=16	Pre-Freeze D1*	Thawed D1	Recovery D1 (%)	Pre-Freeze D2*	Thawed D2	Recovery D2 (%)	Pre-freeze D3*	Thawed D3	Recovery D3 (%)
Cone 1	148.0	117.6	79.0	165.0	70.6	43.0	165.0	73.5	44.5
Cone 2	500.0	515.2	100.0	643.0	331.4	51.8	643.0	366.7	57.0
Cone 3	160.0	156.3	97.7	160.0	240.0	100.0	**	**	**
Cone 4	139.0	108.7	78.2	139.0	109.6	78.8	**	**	**
Cone 5	237.0	205.8	84.0	237.0	231.0	97.5	237.0	186.2	78.6
Cone 6	261.0	226.5	87.0	261.0	183.0	70.4	261.0	202.2	77.5
Cone 7	111.0	92.4	83.0	111.0	82.1	74.0	111.0	75.0	67.7
Cone 8	625.0	432.6	69.2	625.0	415.2	66.4	625.0	367.0	58.8
Cone 9	357.0	300.0	84.0	357.0	291.6	81.7	357.0	330.5	92.6
Cone 10	479.7	477.6	99.6	479.7	427.7	89.2	479.7	415.4	86.6
Cone 11	610.0	534.3	87.5	610.0	585.0	95.9	610.0	583.7	95.7
Cone 12	568.0	564.0	99.3	568.0	588.0	100.0	610.0	547.2	89.7
Cone 13	487.0	456.0	93.6	487.0	489.0	100.0	487.0	501.0	100.0
Cone 14	483.0	419.0	86.6	483.0	365.0	75.6	483.0	388.0	80.3
Cone 15	398.0	436.0	100.0	423.0	291.0	68.8	373.0	299.0	80.2
Cone 16	702.7	475.2	67.6	703.0	462.0	65.7	703.0	480.7	68.3
Mean			**87.3**			**78.7**			**77.0**
Median			**86.8**			**77.2**			**79.4**
Range			**67.6-100.0**			**43.0 – 100.0**			**44.5-100.0**

** *Samples not cryopreserved*

Table 4.2 Nucleated cell counts pre- and post-freeze from apheresis cones 1-16; showing %recovery of nucleated cells in thawed bags cryopreserved on D1, D2 and D3.

*Pre-freeze viable TNC numbers calculated from the WBC count of the prepared starting material x volume of the aliquot prepared for cryopreservation. TNC recovery values were calculated as a percentage of the pre-freeze TNC number originally aliquoted into the bags.

	Total Nucleated Cells x 10^6								
Sample Number n=8	Pre-Freeze D1*	Thawed D1	% Recovery D1 (%)	Pre-Freeze D2*	Thawed D2	% Recovery D2 (%)	Pre-Freeze D3*	Thawed D3	Recovery D3 (%)
APH 1	111.7	97.5	87.3	111.7	88.32	79.1	111.7	99.8	80.4
APH 2	132.3	87.4	66.0	133.7	99.36	74.3	135.2	79.1	58.5
APH 3	156.2	127.4	81.6	156.2	122.88	78.7	147.5	109.9	74.5
APH 4 **	55.3	26.0	47.0	55.3	22.00	39.8	55.3	26.0	47.0
APH 5	171.9	132.0	76.8	171.9	121.60	70.7	171.9	126.4	73.5
APH 6	153.1	124.5	81.3	153.1	114.75	74.9	163.4	118.4	72.5
APH 7	208.7	123.6	59.2	226.1	127.40	56.3	191.3	99.0	51.7
APH 8	122.7	77.0	62.7	116.3	81.00	69.7	122.7	88.4	72.0
APH9	171.9	161.0	93.6	171.9	140.70	81.8	171.9	161.0	93.6
Mean			**78.3**			**77.4**			**71.1**
Median			**76.8**			**74.3**			**72.5**
Range			**59.2-93.6**			**56.3-81.8**			**51.7-93.6**

***Insufficient cells to freeze in bags so sample was frozen in vials. Post thaw viabilities and recoveries were excluded from the analysis as not representative.*

Table 4.3 Nucleated cell counts pre- and post-freeze from apheresis samples 1-8; showing %recovery of nucleated cells in thawed bags cryopreserved on D1, D2 and D3. *Pre-freeze TNC numbers calculated from the WBC count of the prepared starting material x volume of aliquot prepared for cryopreservation. TNC recovery calculated as a percentage of the pre-freeze TNC number originally aliquoted into the bags.

<u>Cone sample cell recovery</u>

The TNC recovery data for cone samples was found to be consistent across all three days. The mean recovery on D1 was 87.3%; D2 78.7%, and D3 77.0%. Although recovery did drop with increasing time to cryopreservation the difference was not found to be significant using one-way ANOVA (Figure 4.1). The difference in recovery between D1 and D3 almost achieved significance (p=0.0589), however, examination of the individual data for the cones indicated that the difference in recovery was attributable to a wider range of recovery results in D2 and D3 samples rather than a consistent decrease in cell numbers (Figure 4.1) and was affected by two outliers: Cone 1 and Cone 2. Cone 1

showed unusually poor TNC recovery on both D2 and D3 (43.0% and 43.5%), and Cone 2 showed poor recovery on D2 only (57.0%). If the results from Cones 1 and 2 were excluded from analysis, no difference in TNC recovery between D1, D2 and D3 cryopreservation was observed. The viable CD3+ recovery from Cones 1 and 2 was good on both D2 and D3, although the CD3+ viability on D3 was low for both samples. The granulocyte content of Cone 1 was 9.24% and Cone 2 was 2.29%, which was insufficiently high to account for the cell losses seen. Cones 1 and 2 were the first samples processed in the study so the most likely explanation of the low TNC recovery on D2 and D3 was laboratory error.

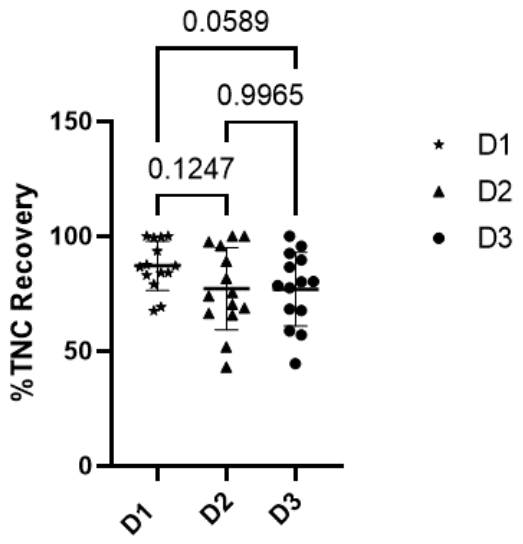


Figure 4.1: Calculated TNC recovery for cells derived from cone samples cryopreserved D1, D2 and D3 as determined by WBC performed on sample immediately post thaw. APH4 shown as outlier on D1 and D2 as cryopreserved in vial. Means shown +/-SD from cone samples 1-16. Statistical analysis by one-way ANOVA, all p values shown. Samples with missing data sets excluded.

Apheresis samples

For apheresis samples, the TNC recovery overall was slightly lower than for cone cells and showed a decrease in cell recovery with increasing time to cryopreservation; 78.3%

D1, 77.4% D2, and 71.1% D3. There was again wide variation in the results obtained. Recovery ranged from 51.7-93.6 but it did not follow an observable pattern (Figure 4.2). The difference in TNC recovery between D1, D2 and D3 recovery was not significant in the case of apheresis samples (see Figure 4.2). APH4 was cryopreserved in vials and was excluded from analysis. It is shown as an outlier on D1 and D2.

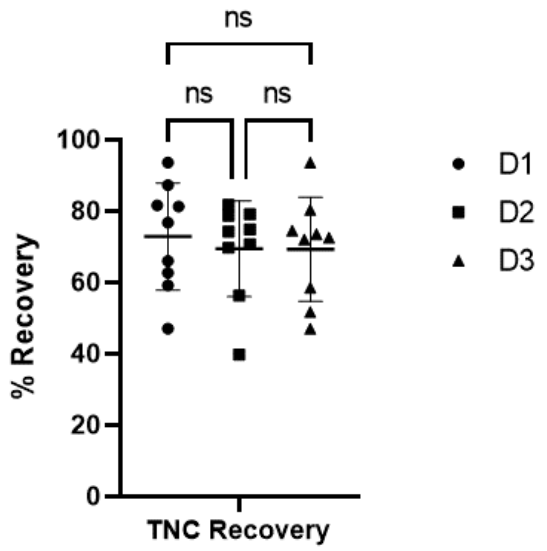


Figure 4.2: Calculated TNC recovery for cells derived from apheresis samples cryopreserved D1, D2 and D3 as determined by WBC performed on sample immediately post thaw tested. APH4 shown as outlier on D1 and D2 as cryopreserved in vial. Means shown +/-SD from apheresis samples 1-9. ns- not significant by one-way ANOVA. APH4 excluded from analysis.

The mean recoveries for both apheresis cones and samples were very similar and although the mean recovery for apheresis samples was lower than that for cone samples on all 3 days of cryopreservation, no significant difference was found between them using two-way AVOVA; p= 0.1359 D1. p= 0.6652 D2, p=0.8276 D3 (see Figure 4.3).

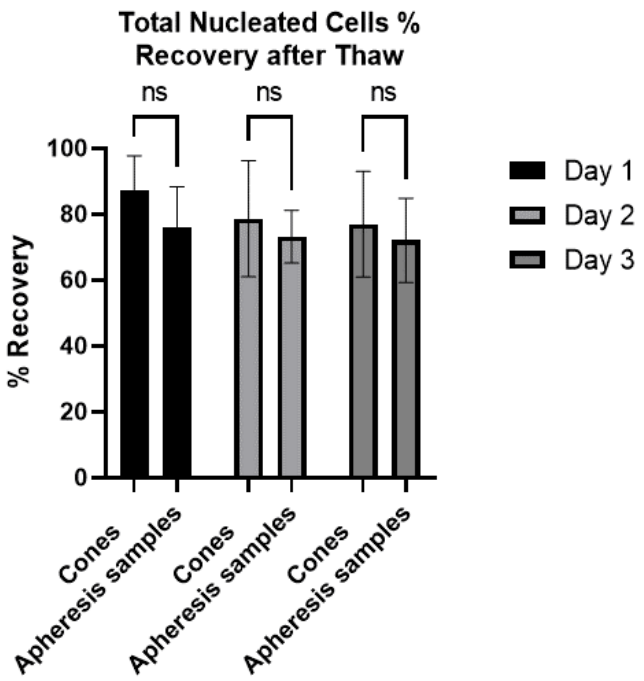


Figure 4.3: Statistical comparison of calculated %TNC recovery for cones C1-16 and apheresis samples APH1-9 cryopreserved on D1, D2 and D3 as determined by WBC performed on sample immediately after thaw. Means shown +/-SD. ns-not significant by two-way ANOVA.

4.7 CD3+ Cells: recoveries and viabilities

CD3+ viability was calculated as the percentage of total CD3+ cells detected by flow cytometry. It was calculated both on the prepared sample prior to Day 1 cryopreservation or further storage and from a sample taken immediately after thawing the cryopreserved bags, prior to washing or resuspension.

Viability was assessed for CD3+ cells only using 7-AAD. Good discrimination was shown between populations staining 7-AAD+ and 7-AAD- in all test samples immediately post thaw (see Figure 4.4 below).

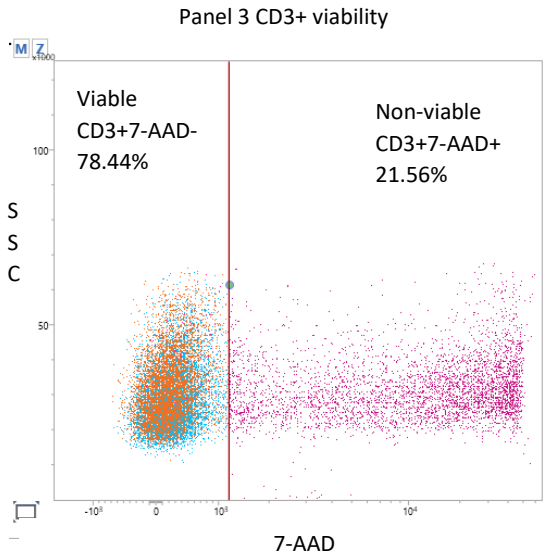


Figure 4.4: Histogram taken from FACSLyric™ showing typical 7-AAD dye exclusion staining of CD3+ cells in a thawed sample. Clear discrimination shown between viable CD3+7-AAD- cells and non-viable CD3+7-AAD+ cells.

4.7.1 CD3+ cell viability pre-cryopreservation and post-thaw

The viability of CD3+ cells from all sample types was close to 100% as assessed on the prepared sample prior to Day 1 cryopreservation or continued storage. Mean pre-cryopreservation CD3+ viability was for Cones = 98.6%, harnesses = 99.0% and apheresis samples = 99.7% (See Tables 4.4 – 4.5 below).

Sample Number n=16	Pre-freeze CD3+ Viability (%)	Viable CD3+ Cells x 10^6 Pre-Freeze D1*	Thawed CD3+ Viability D1 (%)	Total CD3+ Cells x 10^6 Thawed D1	Total CD3+ Cells Recovery D1 (%)	Viable CD3+ Cells Recovery D1 (%)	Viable CD3+ Cells x 10^6 Pre-Freeze D2*	Thawed CD3+ Viability D2 (%)	Total CD3+ Cells x 10^6 Thawed D2	Total CD3+ Cells % Recovery D2 (%)	Viable CD3+ Cells Recovery D2 (%)	Viable CD3+ Cells x 10^6 Pre-Freeze D3*	Thawed CD3+ Viability D3 (%)	Total CD3+ Cells x 10^6 Thawed D3	Total CD3+ Cells Recovery D3 (%)	Viable CD3+ Cells Recovery D3 (%)
Cone 1	99.5	39.3	70.9	55.3	100.0	99.8	45.9	77.2	28.6	62.3	48.1	45.9	27.4	36.1	78.8	21.6
Cone 2	98.7	62.0	92.7	66.6	100.0	99.6	79.7	55.9	97.4	100.0	68.3	79.7	30.0	52.0	65.2	19.6
Cone 3	87.4	111.7	89.6	83.6	75.8	67.1	111.7	77.8	53.0	47.5	36.9	**	**	**	**	**
Cone 4	99.2	95.0	87.7	76.0	80.0	70.2	95.0	77.5	55.6	58.5	45.3	**	**	**	**	**
Cone 5	99.0	62.7	88.3	84.4	100.0	100.0	62.7	74.6	59.9	95.6	71.3	62.7	34.3	51.8	82.6	28.4
Cone 6	99.2	97.0	87.1	61.8	63.7	55.5	90.5	43.6	67.2	74.2	32.4	97.0	76.0	76.5	79.9	60.0
Cone 7	99.4	51.3	91.7	39.4	76.7	70.3	51.3	77.7	34.6	67.3	52.3	51.3	39.1	28.3	57.0	21.5
Cone 8	99.5	268.0	83.1	204.0	76.1	63.3	268.0	79.1	193.7	72.3	57.2	268.0	60.2	176.0	65.7	39.5
Cone 9	99.5	108.8	81.5	104.9	96.4	78.6	108.8	79.2	110.2	100.0	80.2	108.8	72.7	63.6	58.4	42.5
Cone 10	99.3	184.4	88.7	130.7	70.9	62.9	153.7	***	***	***	***	153.7	***	***	***	***
Cone 11	98.5	219.5	63.7	123.2	56.1	35.8	219.5	59.2	114.1	52.0	30.8	219.5	46.9	94.4	43.0	20.2
Cone 12	99.7	258.3	73.2	208.6	80.7	59.1	258.3	66.7	204.5	79.2	52.8	258.3	53.5	146.6	56.8	30.4
Cone 13	99.9	236.5	74.7	168.2	71.1	53.1	236.5	66.0	149.6	63.3	41.7	236.5	62.9	144.0	60.9	38.3
Cone 14	99.9	244.7	72.6	146.8	60.0	43.6	244.7	64.3	113.2	46.3	29.7	244.7	39.4	71.0	29.0	11.4
Cone 15	99.8	172.6	70.0	128.0	74.2	51.9	183.3	39.8	50.0	27.3	10.9	161.8	30.2	40.7	25.1	7.6
Cone 16	99.8	368.4	76.7	204.8	55.6	42.6	368.4	71.8	166.3	45.2	32.4	368.4	66.3	155.5	42.2	28.0
Mean	**98.6**		**80.8**		**77.3**	**65.8**		**67.4**		**66.0**	**46.0**		**49.1**		**57.3**	**28.4**
Median	**99.5**		**82.3**		**76.0**	**63.1**		**71.8**		**63.3**	**45.3**		**46.9**		**58.4**	**28.0**
Range	**87.4-99.9**		**63.7-92.7**		**55.6-100.0**	**35.8-100.0**		**39.8-79.2**		**27.3-100.0**	**10.9-80.2**		**27.4-76.0**		**25.1-82.6**	**7.6-60.0**

***Samples not cryopreserved *** Incorrect samples tested – results not valid*

Table 4.4 Viable CD3+ cell counts pre- and post-freeze from Cones 1-16; showing %recovery of viable CD3+ cells in thawed bags cryopreserved on D1, D2 and D3. *Pre-freeze CD3+ cell numbers calculated from the CD3+ cell count of the prepared starting material x volume of aliquot prepared for cryopreservation. CD3+ cell recovery calculated as a percentage of the pre-freeze CD3+ cell number originally aliquoted into the bags.

Sample Number n=9	Pre-freeze CD3+ Viability (%)	Viable CD3+ Cells x 10^6 Pre-Freeze D1*	Thawed CD3+ Viability D1 (%)	Total CD3+ Cells x 10^6 Thawed D1	Total CD3+ Cells Recovery D1 (%)	Viable CD3+ Cells Recovery D1 (%)	Viable CD3+ Cells x 10^6 Pre-Freeze D2*	Thawed CD3+ Viability D2 (%)	Total CD3+ Cells x 10^6 Thawed D2	Total CD3+ Cells % Recovery D2 (%)	Viable CD3+ Cells Recovery D2 (%)	Viable CD3+ Cells x 10^6 Pre-Freeze D3*	Thawed CD3+ Viability D3 (%)	Total CD3+ Cells x 10^6 Thawed D3	Total CD3+ Cells Recovery D3 (%)	Viable CD3+ Cells Recovery D3 (%)
APH 1	99.2	24.8	66.2	13.0	52.2	34.6	24.8	54.4	11.7	47.1	25.6	24.8	38.0	10.6	42.6	16.2
APH 2	99.9	39.2	79.4	34.6	88.2	70.0	39.2	62.2	28.0	71.4	44.4	39.2	33.9	18.1	46.3	15.7
APH 3	99.9	41.3	88.4	29.6	71.8	63.4	41.3	92.9	33.8	82.0	76.2	39.0	54.6	17.2	44.0	24.1
APH 4 **	99.8	16.2	94.4	7.6	47.1	44.5	16.2	84.1	6.2	38.3	32.2	16.2	66.2	5.4	33.3	22.1
APH 5	99.7	39.9	83.7	30.9	77.4	64.8	39.9	66.8	25.1	63.0	42.0	39.9	36.6	14.1	35.3	12.9
APH 6	99.9	44.3	76.3	30.2	68.0	51.9	44.3	61.0	23.4	52.8	32.2	45.6	42.3	17.4	38.2	16.2
APH 7	99.8	38.9	52.8	9.3	23.9	16.8	42.1	45.5	10.9	25.8	14.5	35.6	31.8	6.1	17.3	6.6
APH 8	99.9	34.6	44.4	10.6	30.8	15.8	31.6	46.7	11.6	36.8	21.0	38.6	30.4	8.3	21.4	7.5
APH9	99.8	50.1	62.5	22.3	44.5	27.8	50.1	66.9	21.0	42.0	28.0	50.1	47.8	16.9	33.8	16.2
Mean	**99.8**		**69.2**		**57.1**	**43.3**		**62.0**		**52.6**	**35.1**		**39.4**		**34.9**	**15.3**
Median	**99.8**		**71.3**		**60.1**	**44.5**		**61.6**		**49.9**	**32.2**		**37.3**		**36.7**	**16.2**
Range	**99.2-99.8**		**44.4-94.4**		**23.9-88.2**	**16.8-70.0**		**41.8-92.9**		**36.8-82.0**	**14.5-76.2**		**30.4-66.2**		**17.3-46.3**	**7.5-24.1**

** *Insufficient cells to freeze in bags so sample was frozen in vials. Post thaw viabilities and recoveries were excluded from the analysis as not representative.*

Table 4.5 Viable CD3+ cell counts pre- and post-freeze from Apheresis Samples 1-9; showing %recovery of total and viable CD3+ cells in thawed bags cryopreserved on D1, D2 and D3. *Pre-freeze CD3+ cell numbers calculated from the CD3+ cell count of the prepared starting material x volume of aliquot prepared for cryopreservation. CD3+ cell recovery calculated as a percentage of the pre-freeze CD3+ cell number originally aliquoted into the bags.

The mean post-thaw CD3+ viability for the cone cells and apheresis samples cryopreserved on D1 was 80.8% for cone samples and 69.2% for apheresis samples. The D2 and D3 thawed viabilities however were significantly lower for both cone samples; 67.4% D2, 49.1% D3 (Figure 4.5) and apheresis samples; 62.0% D2 and 39.4% D3 (Figure 4.6). Examination of the individual data points showed that the reduced mean D2 and D3 viabilities in cone samples resulted from an increased range in viability with some samples (cones C6, C9 and C16) maintaining thawed viability of >65% on D3 while others dropped to <35% (cones C1, C2, C3 and C15).

The same finding was not present in the apheresis samples where the viability range reduced from 44.4 – 94.4% on D1 to 30.6 - 66.2% on D3. The overall lower mean viability seen in apheresis samples, was attributable to APH7 and 8, which were found to have lower than average CD3+ viability on all days of cryopreservation. The effect was particularly apparent on D1 where both samples had viabilities substantially less than the mean. No laboratory error was found that could account for the poor viability, but it was noted that the TNC recovery for both these samples was also lower than the mean with the except for APH8 D3 which was equivalent to the mean.

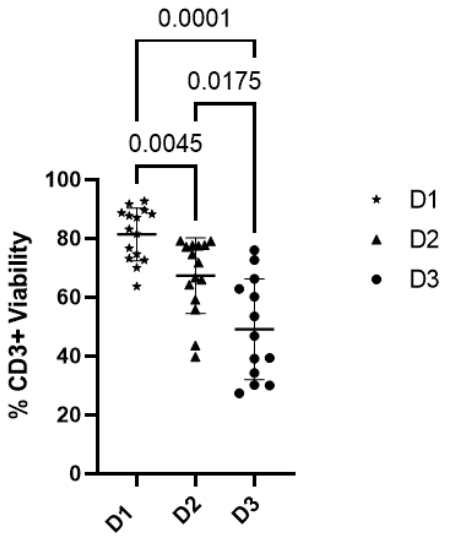


Figure 4.5: Post-thaw CD3+ viability for cone samples cryopreserved on D1, D2 and D3.CD3+ viability as determined flow cytometrically by 7AAD exclusion staining. Means shown +/-SD from cone samples C1-16. p values shown from one-way ANOVA. Samples with missing data sets excluded from analysis.

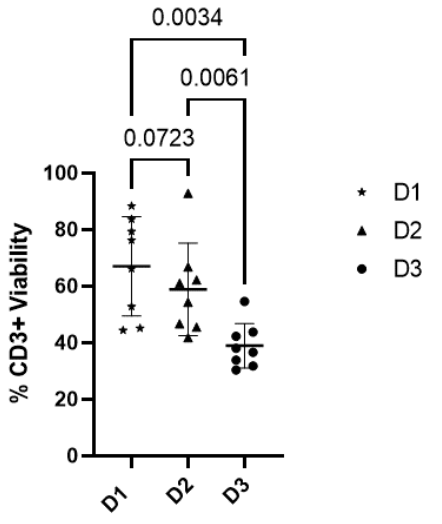


Figure 4.6: Post-thaw CD3+ viability for apheresis samples cryopreserved on D1, D2 and D3. CD3+ viability as determined flow cytometrically by 7AAD exclusion staining. Means shown +/-SD from apheresis samples APH1-9. p values shown from one-way ANOVA. APH4 excluded.

On comparison of the thawed CD3+ cell viability between cone and apheresis samples cryopreserved on D1, D2 and D3, although the mean viability for apheresis samples was lower than that for cones on each day, the difference was not statistically significant

when analysed by two-way ANOVA. p = 0.4373 D1, p= 0.9576 D2, p=0.6392 D3. See Figure 4.7.

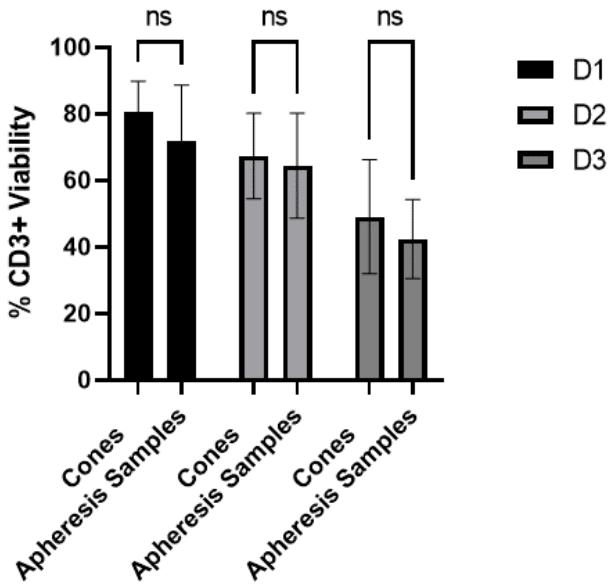


Figure 4.7: Comparison of post-thaw CD3+ viability of cells derived from cone samples C1-16 and apheresis samples APH1-9 cryopreserved on D1, D2 and D3. CD3+ viability as determined flow cytometrically by 7AAD exclusion staining. Means shown +/-SD. ns - not significant by two-way ANOVA. Samples with missing data sets excluded.

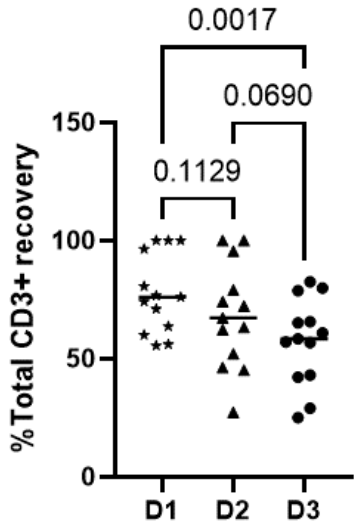
4.7.2 CD3+ cell recovery post-thaw

Total numbers of CD3+ cells both viable and non-viable as defined by dye exclusion staining with 7-AAD, present in the thawed bags were calculated for each product. There was evidence of CD3+ cell loss after cryopreservation and thawing in both cone and apheresis derived samples and total CD3+ cell recovery rates were lower than TNC recovery for both sample groups.

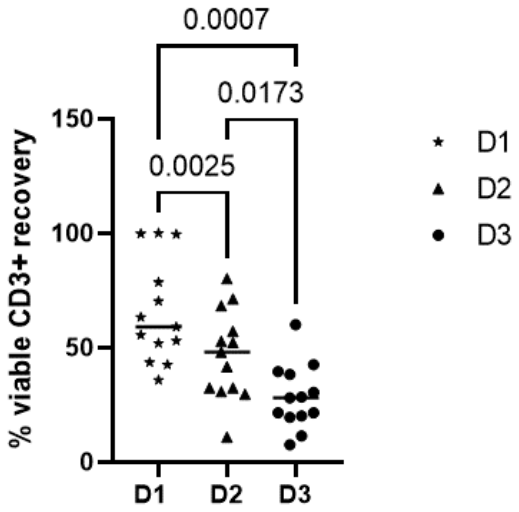
<u>Cones</u>

Mean total CD3+ recoveries were found to be 77.3% for cones samples cryopreserved on D1, D2 and D3 recoveries were lower; 66.0% D2 and 57.3% D3 (Figure 4.8). When viable CD3+ cell numbers were calculated, recovery was found to be lower. 65.0% of

viable CD3+ cells were recovered from cone samples cryopreserved on D1, 46.0% from D2 samples and only 28.4% of viable CD3+ cells recovered from samples cryopreserved on D3. Although the total CD3+ recovery was reduced from D1 to D2 and from D2 to D3, only the reduction in CD3+ cells recovered from D1 to D3 achieved statistical significance when analysed by one-way ANOVA ($p=0.0017$) (Figure 4.8A). Although the range of data generated for viable CD3+ recovery increased slightly from D1 to D2/D3, the significant decrease in recovery seen in D2 and D3 cone samples was not generated solely by outliers as was the case for TNC recovery. The reduction in viable CD3+ cell recovery was statistically significant from D1 to D2 ($p=0.0025$), D2 to D3 ($p=0.0173$) and D1 to D3 ($p=0.0007$) (Figure 4.8B). The D2 and D3 CD3+ results for Cone 10 were excluded from analysis as on investigation of anomalous results for these samples, it was found that the wrong samples had been tested.



4.8A Total CD3+ cells: CD3+7-AAD+/-



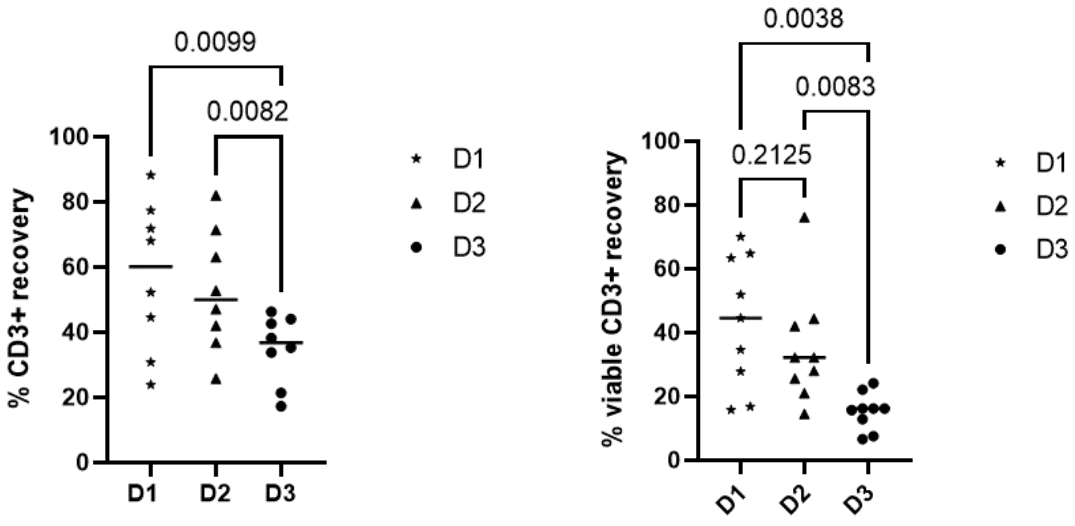
4.8B Viable CD3+ cells: CD3+7-AAD-

Figure 4.8: Calculated post-thaw CD3+ recovery from thawed product bags derived from cone samples C1-16 cryopreserved on D1, D2 and D3. Total CD3+ cell numbers as determined flow cytometrically by staining with anti-CD3, using TruCount™ tubes to generate absolute cell counts. Viability assessed by dye exclusion staining with 7-AAD. Means shown +/-SD. p values shown from one-way ANOVA. Samples with missing data sets excluded.

Apheresis Samples

The CD3+ cell recovery found in thawed product bags derived from apheresis samples was substantially lower than that found in bags derived from cone samples. The mean recovery dropped significantly between days of cryopreservation, falling from 57.1% on D1, to 52.6% on D2 and 34.9% on D3. Unlike cones, the range of recovery results decreased with increased time to cryopreservation; the D3 results appearing more homogenous than the D1 results (See Figure 4.9). The reduction in recovered CD3+ cells was found to be statistically significant on all 3 days when analysed by one-way ANOVA: D1 to D2 p= 0.0045, D2 to D3 p= 0.0175 and D1 to D3 p= 0.0001.

The number of viable CD3+ cells recovered from the thawed apheresis samples was extremely poor. The mean viable CD3+ recovery from bags cryopreserved on D1 was 43.3%, on D2 35.1% and on D3 only 15.3%.



4.9A Total CD3+ cells: CD3+7-AAD+/-

4.9B Viable CD3+ cells: CD3+7-

Figure 4.9: Calculated post-thaw CD3+ recovery from thawed product bags derived from apheresis samples APH1-9 cryopreserved on D1, D2 and D3. Total CD3+ cell numbers as determined flow cytometrically by staining with anti-CD3, using TruCount™ tubes to generate absolute cell counts. Viability assessed by dye exclusion staining with 7-AAD. Means shown +/-SD. p values shown from one-way ANOVA. APH4 excluded.

Although no significant difference had been found between the thawed CD3+ viability of cells derived from cone and apheresis samples on any day of cryopreservation, there was a lower total CD3+ cell recovery in apheresis samples compared to cone samples on all 3 days of cryopreservation (see Figure 4.10 below). The difference was significant on D3 only (p= 0.0063). indicating higher cell losses during storage and cryopreservation in the apheresis samples.

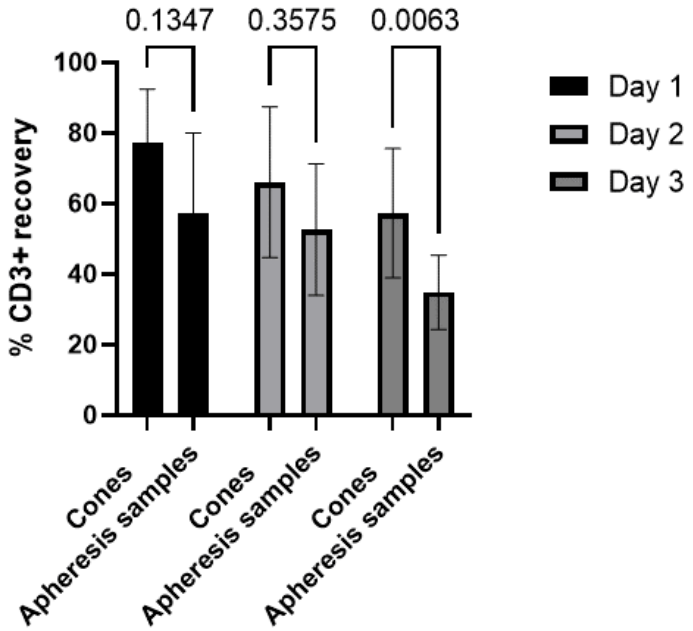


Figure 4.10: Comparison of calculated total thawed CD3+ recovery from thawed product bags for cells derived from cones and apheresis samples cryopreserved on D1, D2 and D3 by two-way ANOVA. Total CD3+ cell numbers as determined flow cytometrically by staining with anti-CD3, using TruCount™ tubes to generate absolute cell counts. Means shown +/-SD from samples C1-16 and APH1-9. p values shown from two-way ANOVA. Samples with missing data sets and APH4 excluded.

Recovery of viable CD3+ cells was found to be similarly lower in apheresis derived samples compared to cone derived samples on all 3 days of cryopreservation (see Figure 4.11). In the case of viable CD3+ cell recovery the difference in recovery was significant on D1 (p= 0.0400) and on D3 (p= 0.0408), although not D2 (p= 0.3312).

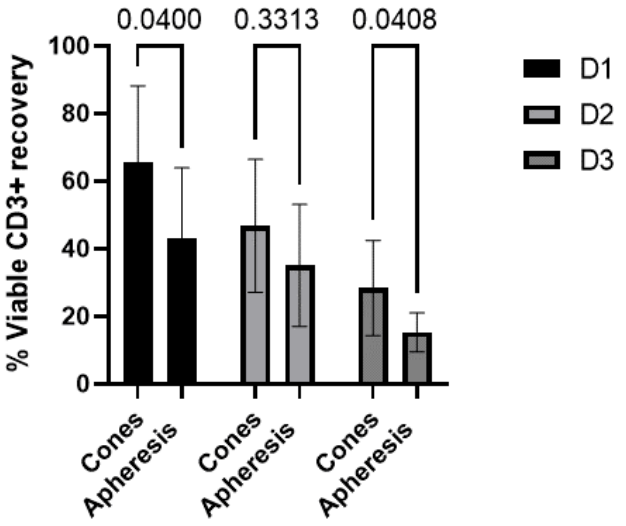


Figure 4.11: Comparison of calculated viable thawed CD3+ recovery from thawed product bags for cells derived from cones and apheresis samples cryopreserved on D1, D2 and D3 by two-way ANOVA. Total CD3+ cell numbers as determined flow cytometrically by staining with anti-CD3, using TruCount™ tubes to generate absolute cell counts. Viability assessed by dye exclusion staining with 7-AAD. Means shown +/- SD from samples C1-16 and APH1-9. p values shown from two-way ANOVA. Samples with missing data sets and APH4 excluded.

4.8 Discussion of the results for total nucleated cell recoveries

Total nucleated cell (TNC) viability post-thaw was not assessed by flow cytometry. The nucleated cell recovery reported was calculated from all intact cells detected by the Sysmex 1000i haematology analyser. The overall TNC recoveries were high in both sample groups on all 3 days of cryopreservation, remaining above 70% even on D3 cryopreservation. The TNC recovery was slightly higher in cone derived samples on all 3 days compared to apheresis derived samples, which probably reflected increased cell death associated with the higher granulocyte content of the apheresis samples; mean granulocyte contamination in cone samples was 6.28% compared to 29.51% in apheresis derived samples. This difference did not reach statistical significance on any day of

cryopreservation. Given the significantly higher granulocyte contamination in the apheresis samples, the difference in TNC recovery both between sample groups and between days of cryopreservation was less than might have been expected, given that granulocytes degrade rapidly during storage (Colotta *et al*., 1992, Klinkmann *et al*., 2021, Vemeren *et al*., 2021). Although nucleated cell recovery after cryopreservation and thawing is not a figure commonly reported in the literature, the recovery of both sample types was in the study was similar to that found by Germann *et al.* (2016) who reported 82% recovery of MNC immediately post thaw, and Steininger *et al.* (2013) who reported a TNC recovery of 81% in thawed apheresis samples from 12 volunteer donors. As the calculation of TNC recovery is based on all intact cellular events detected by the analyser, including cells that are apoptotic or already dead, it is not a clinically useful statistic, but does provide information about the suitability of the sample handling and cryopreservation techniques employed. The fact that >80% of nucleated cells were recovered after cryopreservation on D1 indicated that the samples had not been mistreated during the process.

4.9 Discussion of the results for CD3+ cell viability and recovery

Published literature has to date focussed on the effects of cryopreservation and low temperature storage on lymphocytes. The effect of extended storage pre-cryopreservation has not been a priority. In addition, recovery of viable lymphocytes is a parameter that is rarely reported in the literature at all. As a result, only one report of either thawed viability or recovery was be found in the literature where lymphocytes had been stored for >24 hours prior to cryopreservation (Fisher *et al.,* 2014), and in this study, it was not made clear exactly how long the products had been stored for prior to cryopreservation. Most studies focus only on viability/response to stimulation or the

presence of cell populations as a percentage of the whole, which allows the researcher to assess the differential impact of cryopreservation and thawing on specific phenotypes but does not allow us to know how many cells of which phenotype have failed to survive the process. It was therefore difficult to assess the results obtained in the study against published work.

CD3+ thawed viabilities

The thawed viability for CD3+ cells derived from cone samples and frozen on D1 (80.8%) was broadly in line with MNC and CD3+ post-thaw viability reported in other published works (Li *et al*., 2022, da Silva Ferreira *et al.,* 2015) although higher viabilities have also been reported. Honge *et al.* (2017) reported mean thawed PBMC viability of 92%. The thawed CD3+ viability for apheresis samples was lower than that for the cone derived cells (69.2%), although the result was not statistically significant between the two sample types. The thawed CD3+ viabilities decreased significantly with increased length of storage prior to cryopreservation in both sample groups, dropping to 49.1% for cone derived CD3+ cells and 38.9% for apheresis derived CD3+ cells by D3.

Most of the published literature refers to the thawed viability of MNCs derived from density gradient separation of whole blood and it was not possible to find any comparable results on the study of apheresis products. Of those studies that reported CD3+ recovery post thaw in apheresis products (Fisher *et al.,* 2014, Stroncek *et al.,* 2011, Steininger *et al*., 2013), none reported the actual CD3+ thawed viability so it was not possible to comment further on the results obtained. Schafer et al. (2020) reported a mean thawed cell viability of 51% from 77 G-CSF mobilised apheresis products, but

unfortunately the publication does state whether this is CD45+ cell viability or T cell viability.

Thawed CD3+ viability was maintained above the NHSBT threshold for release of 70% CD3+ viability for cells derived from cones cryopreserved on D1 and D2. CD3+ cells derived from apheresis almost met this criterion for cells cryopreserved on D1 only. Apheresis derived CD3+ cells cryopreserved on D2 and D3, and cone derived cells cryopreserved on D3 failed to meet it by a substantial margin. If found in a clinical product these results would have initiated an investigation into the suitability of the product for clinical use, but as stated in Chapter 1, thaw viability testing on clinical products is rarely performed. In the light of the study results, consideration should be given to introducing release testing for lymphocyte products, but further work will first be required to consolidate the study findings.

CD3+ thawed cell recoveries

The total number of CD3+ cells found in the thawed products in both sample groups was lower than the numbers cryopreserved, indicating a loss of some CD3+ cells during the cryopreservation and thawing process. For cells derived from cones the total CD3+ recovery was 77% on D1, indicating a CD3+ cell loss of 23%. The viable CD3+ cell recovery was lower still at 65.8%. Recovery of both total and viable CD3+ cells decreased with time to cryopreservation and the discrepancy between total and viable cell numbers increased, indicating that not only were a higher proportion of cells destroyed by cryopreservation and thawing if stored for a longer period prior to cryopreservation, but that a higher proportion of those remaining suffered membrane damage that rendered them non-viable although still detectable by flow cytometry. The total recovered CD3+

cells on D3 was 57.3% but only half of these were still viable (28.4%). Overall, the recovery results obtained for Cones 1-10 showed a slightly higher degree of variability than those for Cones 11-16 and the mean recovery was higher (Figure 4.11) This was probably because these were the first samples tested and the process was still being optimised.

For cells derived from apheresis products, the picture was worse, with significantly lower numbers of total and viable CD3+ cells detectable on D1 (57.1%; 43.3% respectively). These figures dropped to 39.4% by D2 and 15.3% by D3.

What published data on CD3+ cell recovery there is relates to CD3+ cell recoveries in cryopreserved apheresis samples, from both mobilised and non-mobilised donors. The total CD3+ cell recovery results obtained in this study from cone derived samples were in line with the total CD3+ cell recoveries reported by Stroncek *et al*. (2011) who reported a 76% recovery of total CD3+ in a study of 311 cryopreserved non-mobilised harvests and Fisher *et al.* (2014) who reported 66.6% total recovery of CD3+ from 14 cryopreserved apheresis donations from volunteer unrelated donors. Fisher *et al*. also reported that the CD3+ cell recovery from related donors was significantly higher than that from unrelated donors. They postulated that this was because cells from unrelated donors had been shipped to the investigating site and were therefore older than the related donor cells when cryopreserved. These investigators did not report viable CD3+ cell recovery. Steininger *et al.* (2013) reported 81.5% recovery of viable CD3+ cells in a study of 12 cryopreserved non-mobilised apheresis collections which was significantly higher than the results found here. The results obtained from the CD3+ derived from mobilised apheresis samples were significantly lower than the published results for non-

mobilised products. In 2014, Fisher did compare CD3+ recovery between mobilised and non-mobilised products from related donors only and found there to be no difference, which was not the case in this study where the recovery from mobilised products was significantly poorer. However, it must also be borne in mind that cells derived from apheresis cones are not identical to cells from non-mobilised apheresis collections and may behave differently. However, all investigators report very wide ranges in results, a finding which was also seen in this study. Given the small number of apheresis samples tested in this study, it is possible that the poor viability outcomes from APH7 and APH8 skewed the results for the apheresis group and resulted in a significant difference being found between the cone and apheresis derived samples. Further testing with larger sample numbers is required to fully answer this question. Interestingly, the one sample that was cryopreserved in vials as opposed to bags showed higher thawed viability than the cells cryopreserved in bags but similar overall viable cell recovery, indicating that there was a higher loss of CD3+ cells in vials as compared to bags. This finding was in keeping with findings that rate controlled freezing programmes designed to be effective for bags are unsuitable for cryovials (Hunt, 2019, Meneghal *et al*. 2020).

Although the comparison between the non-mobilised cone derived cells and the mobilised apheresis derived cells may require further work, it was clear that increasing time in storage prior to cryopreservation has an adverse impact on thawed cell numbers in both study groups. However, it can clearly be seen from the results of this study that the number of viable CD3+ cells that can be retrieved from a cryopreserved product derived from cone samples drops to less than a third of the cell numbers originally cryopreserved (28.4%) if the cells are held for 48-72 hours prior to cryopreservation. For cells derived from mobilised apheresis harvests the outcome of storage pre-

cryopreservation was even more marked; only 15.3% of cells originally cryopreserved on D3 were still present and viable when thawed. NHSBT does not use calculated viable cell recovery as a release criterion, possibly because it is difficult to know what a suitable cut-off point would be, given the lack of published material on the subject, but in the light of the study results reported here, consideration should be given to introducing such a specification.

4.10 Chapter 4: Summary and Key Findings

1. The percentage recovery of total nucleated cells from the thawed products was reduced slightly from cryopreservation on D1 to cryopreservation on D3 for cells derived from both cone and apheresis samples. The reduced recovery was not statistically significant in either sample group with no significant differences seen between the sample groups.

2. The thawed CD3+ cell viability, as determined by dye exclusion staining, showed a statistically significant reduction with increased time to cryopreservation in both sample groups, dropping from 77% for cone and 69% for apheresis derived samples on D1 to 49% and 39% respectively on D3. CD3+ cells derived from cone samples had higher thawed viability than those derived from apheresis samples cryopreserved on D1, D2 and D3 but this difference was not statistically significant.

3. Recovery of non-viable and viable CD3+ cells from the thawed products also dropped sharply with increasing time to cryopreservation. Recovery of viable CD3+ dropped from 66% for cone and 43% for apheresis derived samples on D1 to 46% and 35% on D2, and just 28% and 15% respectively on D3. Higher cell numbers were recovered from cone

compared to apheresis derived cell products on all 3 days of cryopreservation and this difference was statistically significant.

Summary : The findings indicated that the practice of storing cells for up to 72 hours prior to cryopreservation could have a serious adverse impact on their quality and efficacy following transplantation. This was particularly the case for apheresis derived products. The reported cell doses in cryopreserved products, based on the pre-cryopreservation CD3+ cell numbers is therefore likely to be completely inaccurate.

5 Chapter 5 Activation Results

5.1 Background

The aim of this study was to investigate the functional responses of lymphocytes to stimulation after storage and cryopreservation and identify the optimum way to handle and cryopreserve them in to preserve their functionality. Current clinical practice in our region permits a storage period for cells intended for use as DLI at 4°C of up to 72 hours prior to cryopreservation. It is not known if this storage period generates any deficits in the lymphocyte response to stimulation, as there is no suitable test available to assess functionality for lymphocytes associated either with extended storage at 4°C prior to cryopreservation, or with exposure to G-CSF. To this end, a functional assay was designed that could be easily applied to pilot vials from clinical products.

5.2 Study samples

Cells from sixteen cone samples and nine apheresis samples were cryopreserved in 10% DMSO following standard NHSBT cryopreservation protocols. The cells were cryopreserved in CyoMACS® freezing bags (Miltenyi Biotec) at a WBC concentration ranging from 10-20 x 10^6/mL. The bags were frozen using a Planer Kryo 560-16 rate-controlled freezer and transferred to storage in vapour phase nitrogen immediately after completion of the programme. The cells were stored for a minimum of 7 days in vapour phase nitrogen before thawing for stimulation.

The bags were removed from vapor phase storage and thawed rapidly in a water bath at 37°C. As soon as the contents were thawed, a sample was removed for FBC flow cytometric analysis to enable calculation of the TNC and viable CD3+ cell recovery immediately post thaw. The remainder of cells from the thawed bags were then slowly resuspended in warmed media and washed to remove DMSO. A sample of the washed

cells was taken for further flow cytometric analysis to determine the number of viable CD3+ cells present for seeding. Cells were seeded into plates at a concentration of 1 x 10^6 viable CD3+cells/mL. Each sample was seeded into 3 wells as follows:

i) Media only (ImmunoCult XF-T, STEMCELL Technologies).
ii) IL2 (STEMCELL Technologies) + media to control for the effect of IL2 alone on the cells.
iii) IL2 + CD3/CD28 activation beads (STEMCELL Technologies) + media.

The plated cells were incubated in a temperature-controlled CO_2 incubator. Samples were aseptically taken from the wells to test for viable numbers of CD4+/CD8+ lymphocytes/mL and for the presence of the early and late activation markers CD25 and CD69 by flow cytometry at 4, 24, and 72 hours in culture.

Activated lymphocytes were identified as CD4+ or CD8+ lymphocytes that also expressed CD25 and/or CD69 following the gating strategy as described in Chapter 2.

It was not possible to perform absolute CD3+ counts on the stimulated samples because the CD3/CD28 activation beads block the CD3 antibody binding site on the cells. Therefore, the absolute number of viable cells/mL that were either CD4+ or CD8+ was used to calculate the number of viable lymphocytes present at 4, 24 and 72 hours in culture for the control, IL2 and stimulated sample groups. The number of viable CD4+/CD8+ cells present at these timepoints was compared to demonstrate the level of either expansion or cell death in culture in each of the three sample groups.

The percentage of viable CD4+ and CD8+ lymphocytes expressing CD25 and or CD69 was determined at each time point. The percentage expression at the different time points was then compared to the expression of the same markers after thawing and washing

but prior to culture to enable any changes in cell expression levels to be plotted against time for each sample group.

5.3 Data Analysis

Data analysis was performed using two-way ANOVA. Residuals were plotted on a QQ plot to check for normal distribution. All the data analysed was found to be normally distributed and alternative analyses for non-parametric data were not required. The expansion and activation results for lymphocytes cryopreserved on D1, D2 and D3 were compared to determine if time to cryopreservation had an impact on lymphocyte response. The results from the cones were then further compared with the results from the apheresis samples to determine if there was a difference between the two sample groups. Samples with missing data sets were excluded from the analysis on the advice of the Sheffield Hallam University statistician.

5.4 Viable CD3+ Cell Expansion on Stimulation

The viability of the stimulated CD4+ and CD8+ cells was assessed by staining with 7-AAD at each sampling time point (Table 5.1).

	Mean viability in culture (%)					
Day cryopreserved	D1		D2		D3	
Cell type	CD4+	CD8+	CD4+	CD8+	CD4+	CD8+
Cones C1-16						
4 hours	61.32	61.82	51.95	54.27	44.59	43.30
24 hours	64.94	58.28	57.70	53.07	50.01	36.65
72 hours	71.99	66.14	73.64	63.47	54.34	41.02
Cones C11-16						
4 hours	90.04	86.38	81.67	75.38	72.08	69.11
24 hours	81.67	75.38	84.95	77.14	65.17	53.27
72 hours	93.05	89.27	87.45	77.14	79.74	64.30
Apheresis APH1-9						
4 hours	80.46	79.93	81.74	79.71	72.71	69.32
24 hours	72.23	67.03	77.24	73.39	77.24	73.39
72 hours	81.56	76.59	89.97	90.14	89.48	88.72

Table 5.1 Mean viability of stimulated CD4+ and CD8+ cells sampled from culture at 4, 24 and 72 hours. Determined by 7-AAD dye exclusion staining.

Cells from cones 1-10 should have been seeded at 1x 10^6 viable CD3+ cells/well. However, on analysis of the number of viable CD3+ cells at 4, 24 and 72 hours in culture, it became apparent that there had been an error in seeding density. C1 was seeded at a very high density and C2-10 had been seeded at a lower density than that required. The median number of viable CD4+/CD8+ cells at 4 hours culture for C1-10 was found to be 0.373 x 10^6/mL while the median number in C11-16 was 0.901 x 10^6/mL at the same time point (see Table 5.2.). Probably because of the sub-optimal seeding density, the cells did not expand in culture as well as those from cones 11-16. The expansion results from C1-10 and C11-16 were therefore analysed both independently and pooled. No significant differences were found between the numbers of viable CD4+/CD8+ cells in the control condition (media only) and those cultured in IL2 (media + IL2) at any time

point, indicating that any experimental effects observed were due to stimulation with CD3/28 activation beads, not due to the presence of IL2 in the medium.

5.4.1 Time in Culture: 4 hours

Cone Samples

4H	Total Viable CD4+ and CD8+ cells/mL								
Cone Samples	Control			IL2			Stimulated		
n=16	D1	D2	D3	D1	D2	D3	D1	D2	D3
C1	2.37E+06	1.25E+06	2.24E+05	2.41E+06	1.09E+06	1.88E+05	2.32E+06	8.38E+05	8.11E+04
C2	3.22E+05	1.39E+05	1.06E+05	2.03E+05	1.88E+05	6.16E+04	1.30E+05	7.05E+04	5.73E+04
C3	2.61E+05	1.97E+05	**	2.30E+05	1.67E+05	**	1.91E+05	1.65E+05	**
C4	1.29E+05	1.65E+05	**	1.40E+05	3.08E+05	**	1.36E+05	1.56E+05	**
C5	5.02E+04	8.63E+04	1.41E+04	8.19E+04	8.48E+04	1.32E+04	6.21E+04	7.19E+04	1.33E+04
C6	1.26E+05	1.13E+05	1.24E+05	1.65E+05	1.02E+05	1.29E+05	1.16E+05	1.04E+05	1.42E+05
C7	2.35E+05	1.78E+05	2.52E+04	2.06E+05	2.19E+05	8.71E+03	2.08E+05	1.95E+05	5.17E+03
C8	9.97E+04	1.24E+05	4.56E+04	1.10E+05	1.13E+05	2.99E+04	8.37E+04	1.11E+05	2.14E+04
C9	8.08E+03	5.28E+03	1.93E+05	7.88E+03	6.66E+03	1.69E+05	7.21E+03	3.81E+03	1.70E+05
C10	6.73E+04	2.82E+04	5.15E+04	6.86E+04	2.25E+04	5.09E+04	6.07E+04	2.46E+04	4.71E+04
C11	8.50E+05	8.40E+05	6.77E+05	8.41E+05	7.86E+05	7.88E+05	7.60E+05	7.45E+05	5.78E+05
C12	9.70E+05	8.19E+05	5.54E+05	9.75E+05	8.77E+05	5.99E+05	9.93E+05	8.82E+05	5.91E+05
C13	8.05E+05	7.71E+05	6.31E+05	8.03E+05	8.01E+05	7.45E+05	7.19E+05	6.13E+05	5.73E+05
C14	8.11E+05	5.84E+05	5.85E+05	8.10E+05	6.29E+05	6.45E+05	6.18E+05	4.48E+05	5.12E+05
C15	7.97E+05	8.25E+05	6.02E+05	8.35E+05	7.83E+05	6.63E+05	6.88E+05	7.08E+05	5.99E+05
C16	9.30E+05	9.56E+05	7.75E+05	9.50E+05	8.96E+05	8.19E+05	7.98E+05	7.13E+05	6.94E+05
Mean	**5.52E+05**	**4.43E+05**	**3.29E+05**	**5.52E+05**	**4.42E+05**	**3.51E+05**	**4.93E+05**	**3.66E+05**	**2.92E+05**
Median	**2.91E+05**	**1.88E+05**	**2.08E+05**	**2.18E+05**	**2.64E+05**	**1.78E+05**	**2.00E+05**	**1.80E+05**	**1.56E+05**

*** Samples not cryopreserved*

Table 5.2 Viable CD4+/CD8+ cells/mL in control, IL2 and stimulated cone samples cryopreserved on D1, D2 and D3 after 4 hours in culture.

At 4 hours in culture, it was possible to detect some differences in the numbers of viable CD3+ cells in the stimulated groups as compared to the control groups. As can be seen in Table 5.2, because of the errors in seeding density, the range of results from C1-10 was very wide, and cell numbers were low, making it impossible to detect any

experimental effect in this group and masking effects seen in C11-16 when the data was pooled.

In C11-16, the data range was much tighter and a decrease in the numbers of viable CD4+/CD8+ cells in the stimulated group as compared with the control groups was seen on all three days of cryopreservation, although this result was not statistically significant. Cells cryopreserved on D1 maintained viable cell numbers close to those seeded after 4 hours in culture but viable cell numbers in the control, IL2 and stimulated groups were found to decrease steadily with increasing time to cryopreservation. This difference was statistically significant when tested by two-way ANOVA in the case of control D1 to D3 ($p=0.0058$), D2 to D3 ($p=0.0269$); IL2 D1 to D3 (0.0473) and stimulated D1 to D3 ($p=0.0362$) of cryopreservation (see Figure 5.1). The mean number of viable CD4+/CD8+ cells seen in the control conditions did not alter, indicating that the difference between the groups resulted from expansion in the stimulated cell group rather than cell death in the control groups. The day of cryopreservation did not impact viable CD3+ cell numbers in short term culture. There was no significant difference found in the mean number of CD3+ cells/mL seen on D1 (0.493×10^6), D2 (0.366×10^6) and D3 (0.292×10^6) in the stimulated samples.

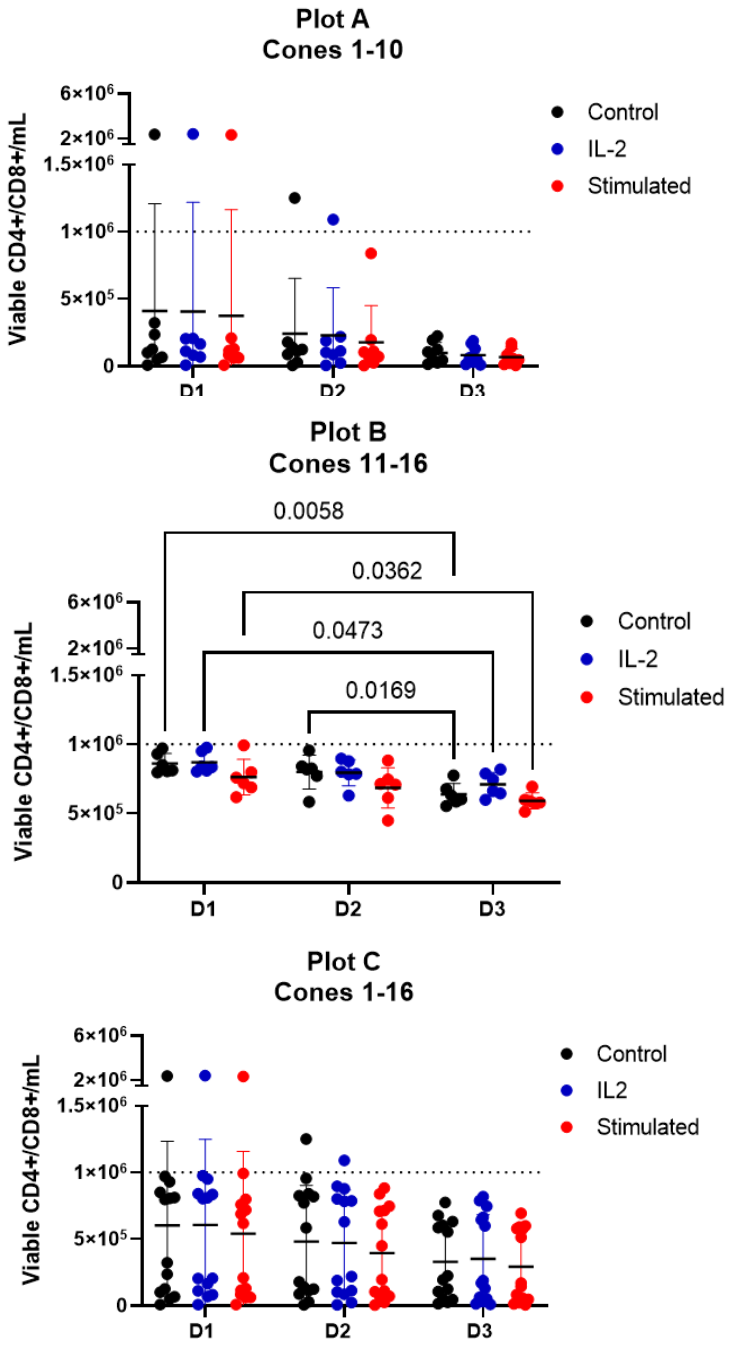


Figure 5.1: Viable CD4+/CD8+cells/mL after 4 hours in culture determined by flow cytometry using Trucount™ tubes in control, IL2 only and stimulated cells from cone samples cryopreserved on D1, D2 and D3. CD4+ and CD8+ cells identified by additional staining of CD3+ cell population. Viability of identified CD4+ and CD8+ cells assessed by dye exclusion staining with 7-AAD. Number of viable CD4+cells /mL and CD8+cells/mL was calculated and combined to create a single value. Results calculated for cone samples C1-10 (Plot A), cone samples C11-16 (Plot B) and cone samples C1-16 (Plot C). p values from two-way ANOVA. Only p values <0.05 are shown. Means shown +/- SD.

Apheresis Samples

4H	Total Viable CD4+ and CD8+cells/mL								
Apheresis Samples	**Control**			**IL2**			**Stimulated**		
n=9	**D1**	**D2**	**D3**	**D1**	**D2**	**D3**	**D1**	**D2**	**D3**
APH1	6.57E+05	9.27E+05	**	5.95E+05	7.62E+05	5.66E+05	4.47E+05	6.70E+05	5.61E+05
APH2	7.96E+05	9.18E+05	7.18E+05	6.99E+05	8.30E+05	**	5.47E+05	7.44E+05	4.67E+05
APH3	7.16E+05	8.22E+05	7.58E+05	7.26E+05	7.17E+05	6.50E+05	5.87E+05	5.11E+05	5.64E+05
APH4	6.99E+05	6.52E+05	6.61E+05	6.63E+05	6.73E+05	6.55E+05	5.32E+05	5.71E+05	5.82E+05
APH5	8.40E+05	8.70E+05	6.91E+05	7.78E+05	7.38E+05	6.63E+05	7.45E+05	6.37E+05	5.63E+05
APH6	6.95E+05	6.61E+05	6.45E+05	7.15E+05	6.92E+05	6.51E+05	5.28E+05	4.87E+05	5.95E+05
APH7	8.33E+05	9.84E+05	8.51E+05	8.42E+05	1.04E+06	8.61E+05	6.36E+05	8.88E+05	7.50E+05
APH8	1.06E+06	1.07E+06	9.22E+05	1.11E+06	1.15E+06	1.01E+06	1.03E+06	7.60E+05	7.81E+05
APH9	3.18E+05	4.03E+05	2.71E+05	3.58E+05	4.42E+05	2.25E+05	3.11E+05	3.32E+05	1.96E+05
Mean	**7.35E+05**	**8.12E+05**	**6.90E+05**	**7.21E+05**	**7.82E+05**	**6.60E+05**	**5.96E+05**	**6.22E+05**	**5.62E+05**
Median	**7.16E+05**	**8.70E+05**	**7.04E+05**	**7.15E+05**	**7.38E+05**	**6.53E+05**	**5.47E+05**	**6.37E+05**	**5.64E+05**

*** Not tested*

Table 5.3 Viable CD4+/CD8+ cells/mL in control, IL2 and stimulated apheresis samples after 4 hours in culture.

Lymphocytes derived from apheresis samples showed a decrease in viable cell numbers from those originally seeded in the control, IL2 and stimulated groups for those cryopreserved on all three days (Table 5.3). The reduction in cell numbers from D1 to D3 and D2 to D3 in the IL2 group and from D2 to D3 in the control group attained statistical significance when tested using two-way ANOVA (IL2 D1-D3 p= 0.0315, D2-D3 p= 0.0056; control D1-D3 p=0.0062) (Figure 5.2). Mean cell numbers in the stimulated groups were lower than those seen in the control and IL2 groups on all three days, although this difference was not significant. The mean number of viable CD4+/CD8+ cells in the stimulated group dropped to approximately 50% of the cells seeded within the first four hours of culture, for cells cryopreserved on D1, D2 and D3. The mean number of viable cells/mL in the control, IL2 and stimulated groups was not affected by day of

cryopreservation at this time point. Mean viability of CD4+ and CD8+ cells as tested with 7-AAD dye exclusion in the stimulated group was found to be high on all three days of cryopreservation, indicating that the reduction in cell numbers was due to loss of cells. Very low viable CD3+ cell numbers were found in the APH9 sample cryopreserved on D1, D2 and D3 in the control, IL2 and stimulated conditions. It was possible that a seeding error had occurred on all three samples, although APH9 was the last sample analysed and experimental design was well established at this point making it unlikely that such an error had occurred. However, the thawed viability and viable CD3+ recovery for APH9 were withing the normal experimental range (see Chapter 4) and the D1 control sample in culture had a CD3+ cell viability of 90.66% when tested at 4 hours, making it unlikely that the sample was damaged during processing. This left seeding error as the most probable explanation.

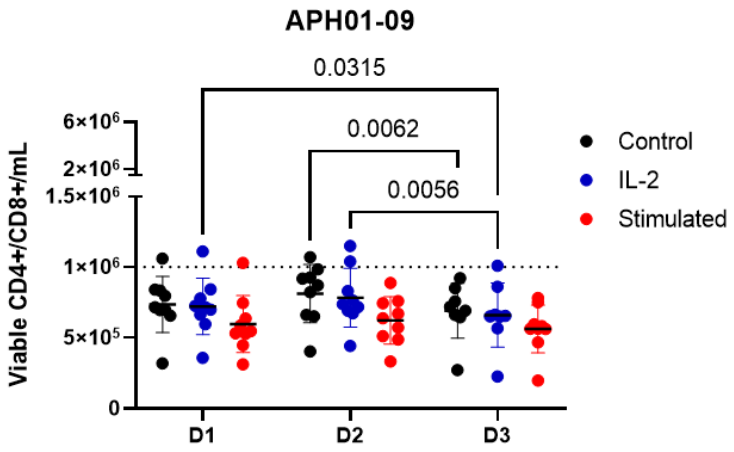


Figure 5.2: Viable CD4+/CD8+ cells/mL after 4 hours in culture determined by flow cytometry using Trucount™ tubes in control, IL2 only and stimulated apheresis samples cryopreserved on D1, D2 and D3. CD4+ and CD8+cells identified by additional staining of CD3+ cell population. Viability of identified CD4+ and CD8+ cells assessed by dye exclusion staining with 7-AAD. The number of viable CD4+ cells/mL and CD8+cells/mL was calculated and combined to create a single value. Results calculated for APH1-9. p values from two-way ANOVA. Only p values <0.05 are shown. Means shown +/- SD.

5.4.2 Time in culture time: 24 hours

At 24 hours incubation, there was more evidence of cell death in all experimental groups with CD3+ numbers dropping below those seeded in all cone and apheresis groups, particularly those cryopreserved on D3 (Tables 5.4 and 5.5). The percentage of viable CD4+ and CD8+ cells in the stimulated culture remained high (Table 5.1).

Cone Samples

24H	Total Viable CD4+ and CD8+cells/mL								
Cone Samples	Control			IL2			Stimulated		
n=16	D1	D2	D3	D1	D2	D3	D1	D2	D3
C1	1.73E+06	8.62E+05	5.15E+04	1.79E+06	8.68E+05	5.52E+04	1.57E+06	7.40E+05	4.78E+04
C2	2.77E+05	2.22E+05	7.64E+03	2.53E+05	8.41E+04	3.86E+03	2.15E+05	6.01E+04	4.21E+03
C3	2.55E+05	1.80E+05	**	2.71E+05	1.75E+05	**	1.90E+05	1.07E+05	**
C4	1.29E+05	1.72E+05	**	1.15E+05	1.62E+05	**	9.99E+04	1.45E+05	**
C5	9.43E+04	5.54E+04	5.31E+03	8.31E+04	5.20E+04	5.91E+04	6.33E+04	2.92E+04	4.73E+04
C6	1.61E+05	1.01E+05	1.11E+05	1.59E+05	1.01E+05	1.33E+05	9.34E+04	8.25E+04	1.01E+05
C7	2.25E+05	1.98E+05	6.68E+03	2.37E+05	1.77E+05	6.69E+03	1.81E+05	1.85E+05	1.30E+03
C8	8.38E+04	1.11E+05	6.81E+04	1.03E+05	1.34E+05	5.76E+04	8.57E+04	8.70E+04	5.15E+04
C9	3.13E+04	1.17E+05	1.81E+05	2.92E+04	1.17E+05	1.79E+05	2.82E+04	5.40E+04	1.59E+05
C10	6.06E+04	1.66E+04	3.29E+04	6.01E+04	1.13E+04	3.32E+04	4.77E+04	1.54E+04	3.61E+04
C11	6.58E+05	6.18E+05	5.46E+05	6.45E+05	6.14E+05	5.70E+05	5.26E+05	5.32E+05	4.25E+05
C12	7.71E+05	6.39E+05	4.17E+05	8.10E+05	6.15E+05	4.27E+05	7.04E+05	6.94E+05	3.80E+05
C13	8.06E+05	6.40E+05	4.30E+05	8.04E+05	7.04E+05	5.12E+05	5.88E+05	5.86E+05	4.23E+05
C14	6.19E+05	3.49E+05	2.92E+05	6.07E+05	3.79E+05	2.86E+05	4.89E+05	3.03E+05	2.62E+05
C15	6.68E+05	6.30E+05	4.48E+05	6.42E+05	5.62E+05	4.08E+05	5.27E+05	4.98E+05	3.60E+05
C16	7.98E+05	7.22E+05	4.56E+05	8.06E+05	7.12E+05	5.19E+05	7.23E+05	5.46E+05	4.29E+05
Mean	**4.61E+05**	**3.52E+05**	**2.18E+05**	**4.63E+05**	**3.42E+05**	**2.32E+05**	**3.83E+05**	**2.92E+05**	**1.95E+05**
Median	**2.66E+05**	**2.10E+05**	**1.46E+05**	**2.62E+05**	**1.76E+05**	**1.56E+05**	**2.02E+05**	**1.65E+05**	**1.30E+05**

***** Samples not cryopreserved***

Table 5.4 Viable CD4+/CD8+ cells/mL in control, IL2 and stimulated cone samples after 24 hours in culture.

In C1-10, gradual cell loss could be seen from increasing time to cryopreservation D1 to D3 in all three experimental groups. Mean overall numbers of viable cone derived cells/mL remaining in the control group were 0.33 x 10^6 on D1, 0.21 x 10^6 on D2, and

0.06 x 10^6 on D3. Cell numbers in the stimulated group were even lower; D1 = 0.29 x 10^6, D2 = 0.16 x 10^6, D3 = 0.06 x 10^6 but overall cell numbers were so low, particularly in the D3 samples that it was not possible to draw any significant conclusions. Statistical analysis using two-way ANOVA returned no significant results in the C1-10 group (Figure 5.3). The same picture of gradual cell loss with increasing time to cryopreservation was also found in C11-16. However, in this group, the data was better controlled and statistical analysis returned some significant results. In C11-16, the number of viable cells still detectable had dropped below those seeded by 24 hours in culture in the control, IL2 and stimulated groups. The mean viable cell numbers/mL were highest in samples that had been cryopreserved on D1 (mean control = 0.798 x 10^6; mean IL2 = 0.719 x 10^6; mean stimulated = 0.593 10^6 dropping to; mean control = 0.456 x 10^6; mean IL2 = 5.97 x 10^6; mean stimulated = 0.380 x 10^6 by cryopreservation on D3 (see Table 5.4). The viable CD4+/CD8+ cell numbers were found to be significantly reduced in cells cryopreserved on D3 compared to D1 (p= 0.0039) (Figure 5.3) although not between cryopreservation on D1 to D2 or between D2 to D3. The number of viable cells was found to be lower in the control group cryopreserved on D2 (p= 0.0475) and D3 compared to D1 (0.0023). In the IL2 group, the number of viable cells was found to be reduced in cells cryopreserved on D2 (p=0.0238) and D3 (p= 0.0252). No difference was found between the number of viable CD3+ cells in the stimulated group compared to either control group on any day of cryopreservation.

When C1-16 were analysed together, the reduction in cell numbers seen in C11-16 between samples cryopreserved on the different days was masked and the only significant difference remaining was seen the control group between cells cryopreserved on D2 to D3 (p=0.0393) (Figure 5.3).

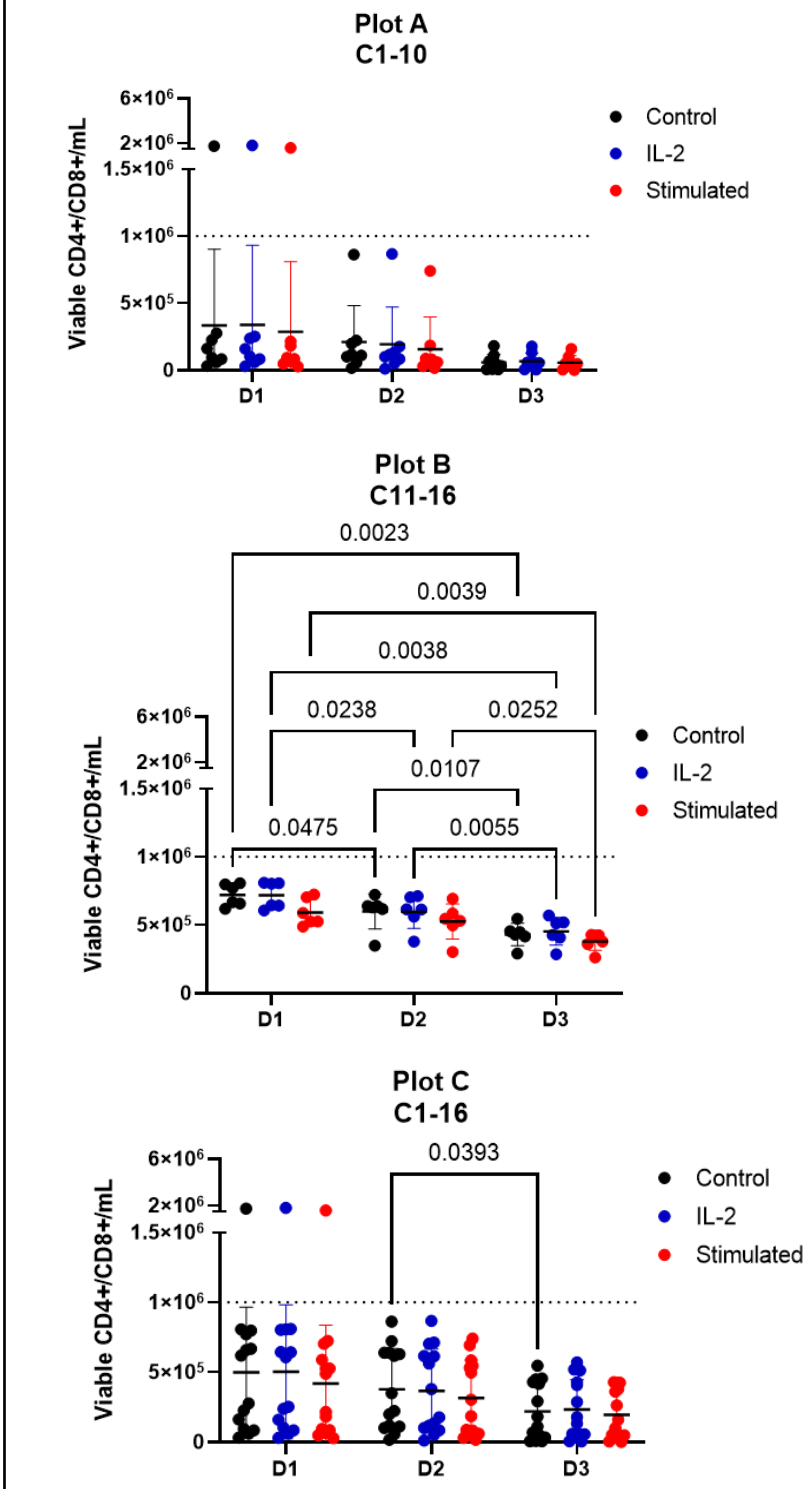


Figure 5.3: Viable CD4+/CD8+ cells/mL after 24 hours in culture determined by flow cytometry using Trucount™ tubes in control, IL2 only and stimulated cells from cone samples cryopreserved on D1, D2 and D3. CD4+ and CD8+ cells identified by additional staining of CD3+ cell population. Viability of identified CD4+ and CD8+ cells assessed by dye exclusion staining with 7-AAD. Number of viable CD4+cells /mL and CD8+cells/mL was calculated and combined to create a single value. Results calculated for cone samples C1-10 (Plot A), cone samples C11-16 (Plot B) and cone samples C1-16 (Plot C). p values from two-way ANOVA. Only p values <0.05 are shown. Means shown +/- SD.

Apheresis Samples

24H	Total Viable CD4+ and CD8+ cells/mL								
Apheresis Samples	Control			IL2			Stimulated		
n=9	D1	D2	D3	D1	D2	D3	D1	D2	D3
APH1	4.03E+05	7.57E+05	5.85E+05	4.17E+05	8.15E+05	6.38E+05	3.16E+05	6.18E+05	4.65E+05
APH2	6.32E+05	8.36E+05	5.89E+05	5.30E+05	7.85E+05	5.78E+05	2.92E+05	5.97E+05	4.08E+05
APH3	2.99E+05	4.56E+05	4.83E+05	2.34E+05	3.47E+05	4.25E+05	1.78E+05	2.93E+05	2.91E+05
APH4	3.62E+05	4.68E+05	4.88E+05	3.16E+05	2.89E+05	4.91E+05	1.88E+05	1.91E+05	1.88E+05
APH5	5.70E+05	6.16E+05	4.72E+05	4.16E+05	5.20E+05	3.52E+05	4.54E+05	3.69E+05	3.75E+05
APH6	1.45E+05	3.03E+05	4.23E+05	1.34E+05	3.99E+05	4.82E+05	8.40E+04	3.20E+05	2.46E+05
APH7	5.02E+05	8.69E+05	7.15E+05	4.50E+05	7.67E+05	7.79E+05	3.57E+05	5.57E+05	4.73E+05
APH8	6.73E+05	1.01E+06	7.54E+05	7.22E+05	1.08E+06	8.62E+05	2.81E+05	4.23E+05	4.67E+05
APH9	2.66E+05	6.80E+05	1.96E+05	2.78E+05	3.70E+05	2.00E+05	2.60E+05	6.39E+05	1.48E+05
Mean	**4.28E+05**	**6.66E+05**	**5.23E+05**	**3.89E+05**	**5.97E+05**	**5.34E+05**	**2.68E+05**	**4.45E+05**	**3.40E+05**
Median	**4.03E+05**	**6.80E+05**	**4.88E+05**	**4.16E+05**	**5.20E+05**	**4.91E+05**	**2.81E+05**	**4.23E+05**	**3.75E+05**

Table 5.5 Viable CD4+/CD8+ cells/mL in control, IL2 and stimulated apheresis samples after 24 hours in culture.

Similarly to the cone derived samples, the number of viable CD4+/CD8+ cells present in the apheresis derived samples dropped below those seeded in control, IL2 and stimulated conditions at 24 hours in culture on D1, D2 and D3 of cryopreservation. However, in contrast to the cone derived cells, the apheresis derived cells with the lowest numbers of viable cells were found to be those cryopreserved on D1 (Table 5.5). The mean numbers of viable apheresis derived cells/mL remaining in the control group were 0.428 x 10^6 on D1, 0.666 x 10^6 on D2, and 0.523 x 10^6 on D3. In the IL2 group the numbers were very similar to those in the control group. Again, the lowest cell numbers overall were found in the stimulated group; D1 = 0.268 x 10^6; D2 = 0.445 x 10^6; D3 = 0.340 x 10^6. When tested with analysis by two-way ANOVA, no significant differences were found in the stimulated groups cryopreserved on the different days, although there were significantly lower numbers of cells in the stimulated group compared to the

control and IL2 groups for cells cryopreserved on D3 only (p=0.0033 and 0.0326 respectively). In the control and IL2 groups, significantly higher cell numbers were found in samples cryopreserved on D2 compared to D1 (p=0.0102 and 0.0353 respectively), although the mean numbers were still lower than those initially seeded, indicating that the higher numbers resulted from reduced cell death in samples cryopreserved on D2 (Figure 5.4). High cell numbers were found in APH9 in the control group and stimulated groups on D2 cryopreservation only; APH9 D2 control = 0.680 CD4+/CD8+ cells x 10^6/mL compared to 0.266 x 10^6/mL and 0.196 x 10^6/mL on D1 and D3 respectively. APH9 D2 stimulated = 0.639 x10^6/mL compared to 0.260 x10^6/mL and 0.148 x10^6/mL on D1 and D3 respectively. The APH9 IL2 sample was found to have a similar number of viable CD4+CD8+ cells on all 3 days (D1= 0.278 x10^6/mL, D2 = 0.370 x10^6/mL, D3 = 0.20 x10^6/mL) which suggests laboratory error may have played a role in the control and stimulated groups. However, after examination of the data, no error was identified, and no alternative explanation could be found for this result. It therefore remained in the analysis.

Although some individual donor samples maintained their cell numbers close to those seeded in D1, D2 and D3 control samples (APH1, APH2, APH7), the remaining samples did not. There was evidence of cell death, not expansion at this time point. Viability of CD4+ and CD8+ cells in the stimulated group remained high (Table 5.1).

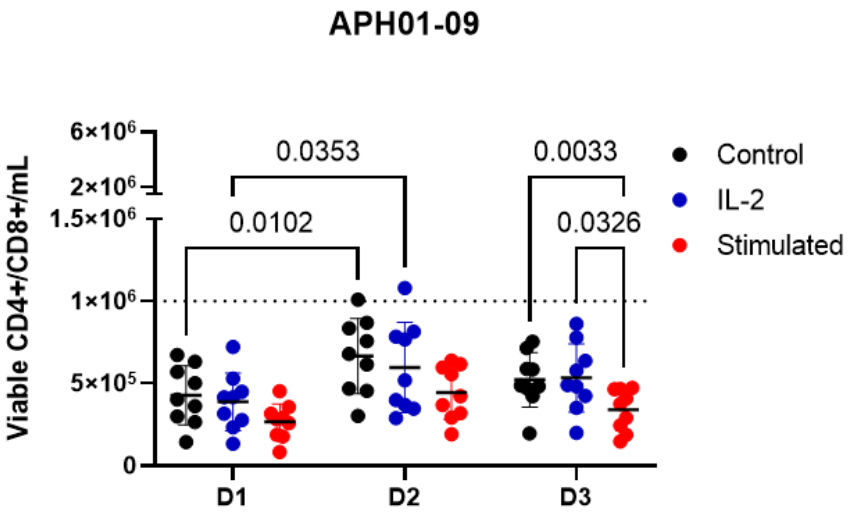


Figure 5.4: Viable CD4+/CD8+ cells/mL after 24 hours in culture determined by flow cytometry using Trucount™ tubes in control, IL2 only and stimulated apheresis samples cryopreserved on D1, D2 and D3. CD4+ and CD8+cells identified by additional staining of CD3+ cell population. Viability of identified CD4+ and CD8+ cells assessed by dye exclusion staining with 7-AAD. The number of viable CD4+ cells/mL and CD8+cells/mL was calculated and combined to create a single value. Results calculated for APH1-9. p values from two-way ANOVA. Only p values <0.05 are shown. Means shown +/- SD.

5.4.3 Culture time: 72 hours

Cone Samples

72H	Total Viable CD4+ and CD8+ cells/mL								
Cone Samples	Control			IL2			Stimulated		
n=16	D1	D2	D3	D1	D2	D3	D1	D2	D3
C1	1.77E+06	8.15E+05	3.91E+04	1.59E+06	7.26E+05	3.95E+04	2.23E+06	7.61E+05	2.90E+04
C2	3.57E+05	8.18E+04	1.17E+03	3.05E+05	5.44E+04	7.84E+03	3.83E+05	7.92E+04	2.95E+03
C3	2.52E+05	1.89E+05	**	2.42E+05	1.72E+05	**	3.16E+05	1.97E+05	**
C4	1.27E+05	1.98E+05	**	1.44E+05	1.88E+05	**	1.48E+05	1.98E+05	**
C5	7.93E+04	5.78E+04	7.04E+03	8.54E+04	6.38E+04	1.14E+04	1.49E+05	1.05E+05	8.53E+03
C6	1.51E+05	1.03E+05	1.88E+05	1.75E+05	9.77E+04	1.32E+05	3.47E+05	2.10E+05	2.12E+05
C7	2.39E+05	1.76E+05	8.96E+02	2.98E+05	2.35E+05	3.20E+03	3.88E+05	3.10E+05	4.91E+03
C8	1.91E+04	1.50E+04	4.44E+04	8.61E+03	1.31E+04	4.39E+04	5.38E+03	1.98E+04	2.19E+04
C9	3.11E+04	1.20E+05	9.17E+04	2.81E+04	1.02E+05	1.06E+05	2.93E+04	7.43E+04	1.07E+05
C10	2.45E+04	1.14E+04	2.86E+04	3.29E+04	1.27E+04	3.55E+04	3.61E+04	1.16E+04	3.30E+04
C11	6.44E+05	4.89E+05	3.53E+05	6.04E+05	4.90E+05	3.42E+05	8.33E+05	6.49E+05	3.98E+05
C12	7.64E+05	5.44E+05	2.98E+05	7.39E+05	5.43E+05	3.43E+05	1.08E+06	1.10E+06	6.21E+05
C13	6.83E+05	5.96E+05	3.98E+05	6.75E+05	6.21E+05	4.74E+05	1.24E+06	8.57E+05	6.37E+05
C14	5.34E+05	2.90E+05	2.84E+05	5.74E+05	3.52E+05	3.03E+05	7.87E+05	6.69E+05	4.07E+05
C15	7.06E+05	6.20E+05	4.32E+05	6.73E+05	5.76E+05	4.14E+05	1.12E+06	9.87E+05	6.30E+05
C16	7.82E+05	6.11E+05	3.99E+05	7.56E+05	6.06E+05	4.35E+05	1.08E+06	8.69E+05	6.55E+05
Mean	**4.48E+05**	**3.07E+05**	**1.83E+05**	**4.33E+05**	**3.03E+05**	**1.92E+05**	**6.36E+05**	**4.44E+05**	**2.69E+05**
Median	**3.04E+05**	**1.93E+05**	**1.40E+05**	**3.01E+05**	**2.11E+05**	**1.19E+05**	**3.85E+05**	**2.60E+05**	**1.60E+05**

******* *Samples not cryopreserved*

Table 5.6 Viable CD4+/CD8+ cells/mL in control, IL2 and stimulated cone samples after 72 hours in culture.

After 72 hours incubation, no experimental effect was observed on cells in C1-10. Almost no viable cells were detectable in any group cryopreserved on D3 after collection (Table 5.6). Examining the data generated from C11-16, there was an observable reduction in viable cell numbers in both the control groups which increased with length of time to cryopreservation.

For cones 11-16 significant expansion of the stimulated cell group as compared to the control and IL2 groups was seen at 72 hours culture for cells cryopreserved on D1 and D2. The mean number of viable CD4+/CD8+ cells/mL in this group had returned to that originally seeded (mean viable cells/mL D1 = 1.02 $x10^6$/mL; D2 = 0.855 $x10^6$/mL). Cells cryopreserved on D3 did less well and although the mean cell numbers increased slightly from 24 hours culture from0.380 $x10^6$ /mL to 0.558 $x10^6$/mL mL) there was little evidence of cell expansion. Mean cell numbers in the control and IL2 groups remained very similar to those present at 24 hours culture (Table 5.6) with significantly lower viable cell numbers present in the samples cryopreserved on D2 and D3 compared to D1 (Figure 5.5). The increased cell numbers seen in samples cryopreserved on D1 compared to D3, and between D2 and D3 were statistically significant; D1 to D3 $p<=0.0001$ and D2-D3 $p= 0.0020$ (Figure 5.5).

When the data from C1-10 and C11-16 was pooled, some statistical significance was lost. However, it was still clear that CD3+ cells derived from cones expanded best when cryopreserved on D1 (D1-D3 $p=0.0331$; D2-D3 $p= 0.0083$) with decreasing viable cell numbers seen in samples cryopreserved on D2 and poor expansion seen in samples cryopreserved on D3 (Figure 5.5).

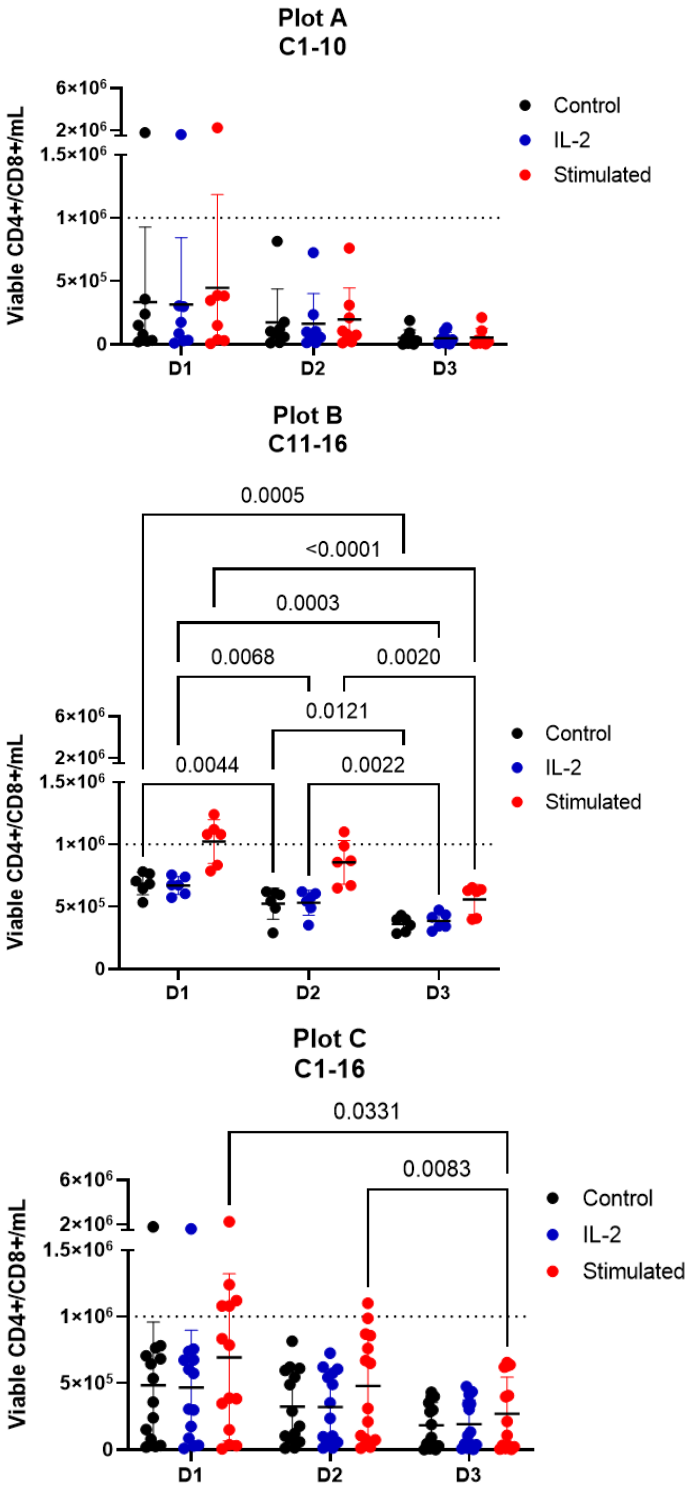


Figure 5.5: Viable CD4+/CD8+/mL after 72 hours in culture determined by flow cytometry using Trucount™ tubes in control, IL2 only and stimulated cells from cone samples cryopreserved on D1, D2 and D3. CD4+ and CD8+ cells identified by additional staining of CD3+ cell population. Viability of identified CD4+ and CD8+ cells assessed by dye exclusion staining with 7-AAD. Number of viable CD4+cells /mL and CD8+cells/mL was calculated and combined to create a single value. Results calculated for cone samples C1-10 (Plot A), cone samples C11-16 (Plot B) and cone samples C1-16 (Plot C). p values from two-way ANOVA. Only p values <0.05 are shown. Means shown +/- SD.

Apheresis Samples

72H	Total Viable CD4+ and CD8+cells/mL								
Apheresis Samples	Control			IL2			Stimulated		
n=9	D1	D2	D3	D1	D2	D3	D1	D2	D3
APH1	3.66E+05	7.26E+05	5.38E+05	3.80E+05	7.35E+05	6.04E+05	4.22E+05	5.93E+05	4.56E+05
APH2	1.81E+05	7.14E+05	5.19E+05	1.55E+05	7.36E+05	5.33E+05	6.34E+04	1.46E+06	1.14E+06
APH3	3.02E+05	4.93E+05	6.05E+05	2.20E+05	6.08E+05	6.36E+05	4.83E+05	8.77E+05	7.94E+05
APH4	4.72E+05	6.43E+05	6.68E+05	4.91E+05	5.93E+05	5.26E+05	7.88E+05	2.06E+06	1.84E+06
APH5	6.12E+05	6.37E+05	5.43E+05	6.36E+05	6.47E+05	6.16E+05	7.61E+05	6.64E+05	3.92E+05
APH6	1.97E+05	5.06E+05	5.92E+05	1.84E+05	5.22E+05	5.82E+05	3.94E+05	1.23E+06	7.59E+05
APH7	4.46E+05	8.62E+05	7.62E+05	4.66E+05	7.97E+05	7.59E+05	4.89E+05	6.46E+05	4.27E+05
APH8	6.31E+05	7.54E+05	8.66E+05	7.22E+05	9.60E+05	8.73E+05	4.98E+05	8.28E+05	7.95E+05
APH9	1.32E+05	2.31E+05	1.37E+05	1.55E+05	2.55E+05	1.42E+05	3.81E+05	4.07E+05	1.63E+05
Mean	**3.71E+05**	**6.19E+05**	**5.81E+05**	**3.79E+05**	**6.50E+05**	**5.86E+05**	**4.75E+05**	**9.73E+05**	**7.52E+05**
Median	**3.66E+05**	**6.43E+05**	**5.92E+05**	**3.80E+05**	**6.47E+05**	**6.04E+05**	**4.83E+05**	**8.28E+05**	**7.59E+05**

Table 5.7 Viable CD4+/CD8+cells/mL in control, IL2 and stimulated apheresis samples after 72 hours in culture.

The results for apheresis derived cells were less uniform that those seen in the cone derived cells. Although significant differences in viable cell numbers were seen between each of the three days of cryopreservation, the data was very wide ranging and was therefore influenced by outlying samples. APH2 and APH4 both expanded substantially, with the cell numbers in APH4 reaching double the number seeded. Other samples, APH1, APH5 and APH7, did not respond well and viable cell numbers remained below those seeded. APH9 did not respond at all and remained an outlier. Although there was clear evidence of cellular expansion in all samples except APH9 from the numbers present at 24 hours in culture, the mean number of viable CD4+ and CD8+ cells in culture

did not quite attain the original number of cells seeded, reaching a maximum of 0.973 x 10^6/mL for cells cryopreserved on D2.

Overall CD4+/CD8+ cells derived from apheresis samples were found to expand most effectively when cryopreserved on D2. Cells cryopreserved on D1 contained the lowest number of viable CD3+ cells after 72 hours in culture; the mean number of viable CD4+/8+cells/mL found in samples cryopreserved on D1 was found to be 0.475 x 10^6. Samples cryopreserved on D2 contained the highest cell numbers; median viable cell/mL = 0.973 x 10^6, dropping back to 0.75^{56} for samples cryopreserved on D3 (Table 5.7). The decrease in cell numbers from D2-D3 was statistically significant (p= 0.0147) but not the increase in numbers from D1-D2 (p= 0.2601). The difference between the control conditions and the stimulated condition was not statistically significant on any day of cryopreservation. Significantly increased cell numbers were seen in the control and IL2 groups cryopreserved on D1 compared to D2 (control D1-D2 p= 0.0315; IL2 D1-D2 = 0.0254), as was seen at 24 hours in culture (Figure 5.6).

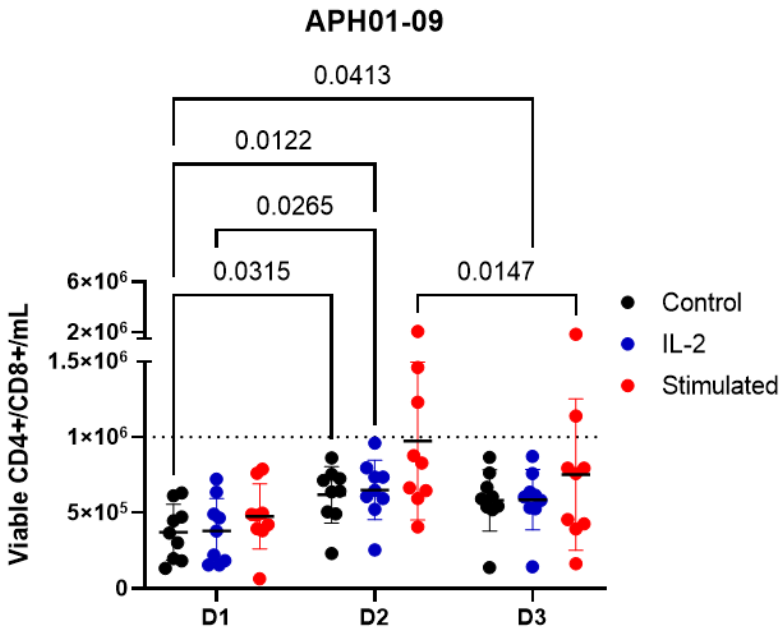


Figure 5.6: Viable CD4+/CD8+/mL after 72 hours in culture determined by flow cytometry using Trucount™ tubes in control, IL2 only and stimulated apheresis samples cryopreserved on D1, D2 and D3. CD4+ and CD8+cells identified by additional staining of the CD3+ cell population. Viability of identified CD4+ and CD8+ cells assessed by dye exclusion staining with 7-AAD. The number of viable CD4+ cells/mL and CD8+cells/mL was calculated and combined to create a single value. Results calculated for APH1-9. p values from two-way ANOVA. Only p values <0.05 are shown. Means shown +/- SD.

5.4.4 **Summary of Expansion**

To summarise, cone and apheresis derived CD4+/CD8+ cells behaved completely differently when stimulated and cultured after cryopreservation and thawing. Cone derived cells expanded best in culture when frozen within 24 hours of collection (D1) with a gradual reduction in viable cell numbers seen as the length of time to cryopreservation increased (D2-D3) (p=<0.0001 at 72 hours culture) (Figure 5.5).

Apheresis derived cells expanded best in culture when held for 24-48 hours in storage at 4°C prior to cryopreservation (p=0.0024 D2-D3; p= 0.0589 D1-D2). Surprisingly, cells held in storage for 48-72 hours (D3) expanded better than cells cryopreserved within 24

hours of collection (D1) although this difference was not significant (Figure 5.6). The findings from the viable CD3+ cell numbers determined in all experimental conditions are summarised in Figure 5.7.

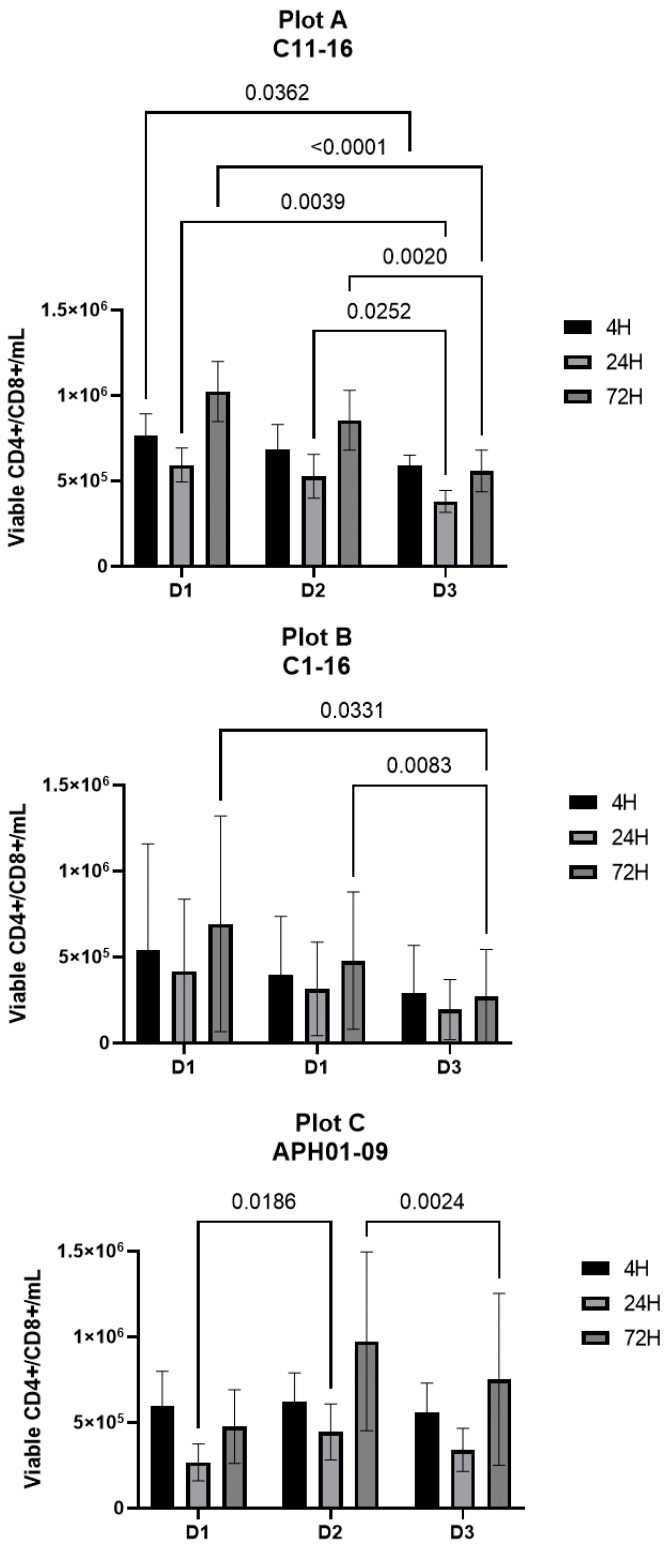


Figure 5.7: Viable CD4+/CD8+cells/mL after 4, 24 and 72 hours in culture determined using Trucount™ tubes for stimulated cone and apheresis samples cryopreserved on D1, D2 and D3. CD4+ and CD8+ cells identified by additional staining of CD3+cell population. Viability of identified CD4+ and CD8+ cells was assessed by dye exclusion staining with 7-AAD. Number of viable CD4+cells/mL and CD8+cells/mL calculated and combined to create a single value. Results calculated for cones C11-16 (Plot A), cones C1016 (Plot B) and APH1-9 (plot C). p values from two-way ANOVA. Only p values <0.05 are shown. Means shown +/- SD.

5.5 Activation Markers

5.5.1 Pre-cryopreservation expression of CD25 and CD69 cell markers

The samples were stained for activation markers CD25 and CD69 using Panel 3 when prepared for cryopreservation and again after thawing and washing, prior to seeding into plates for stimulation. CD25 and CD69 were found to be expressed at low levels in all pre-cryopreservation samples, except for C1 in which 18.48% of CD4+ cells also expressed CD25. As cones C1-7 failed the T cell subset panel, it was not known what the pre-cryopreservation numbers of Tregs were in C1 but the percentage of CD3+ cells that were CD4+25+ after thaw of a pilot vial was found to be only 1.43%. After thawing and washing only 3.1% of CD4+/CD8+ cells were found to express CD25; it is therefore likely that the C1 CD25 pre-cryopreservation result was erroneous, but it remained in the analysis as it could not be proved to be so. Baseline CD25+ expression in the rest of the prepared cone and apheresis cell samples pre-cryopreservation, was found to correspond to that obtained from the T cell subsets performed using Panel 2 and discussed in Chapter 3. The mean number of CD4+ cells expressing CD25 and/or CD69 was found to be 6.88% in cone samples and 4.37% in apheresis samples. The mean number of CD8+ cells expressing CD25 and/or CD69 was found to be 5.16% in cone samples and 4.52% in apheresis samples. See Tables 5.8 and 5.9.

	Percentage of CD4+ cells expressing CD25 and CD69					Percentage of CD8+ expressing CD25 and CD69				
Sample Number n=16	**CD4+CD25-CD69- (%)**	**CD4+CD25+ (%)**	**CD4+CD69+ (%)**	**CD4+CD25+ CD69+ (%)**	**Total activated CD4+ (%)**	**CD8+CD25-CD69- (%)**	**CD8+CD25+ (%)**	**CD8+CD69+ (%)**	**CD8+CD25+ CD69+ (%)**	**Total activated CD8+ (%)**
Cone 1	81.2	18.48	0.24	0.08	18.8	93.59	4.61	1.73	0.07	6.41
Cone 2	96.94	2.77	0.26	0.03	3.06	96.39	0.33	3.29	0	3.61
Cone 3	94.79	4.07	1.09	0.05	5.21	97.21	0.77	2	0.01	2.79
Cone 4	96.14	2.63	1.09	0.14	3.86	96.31	0.79	2.88	0.02	3.69
Cone 5	90.67	5.87	3.23	0.23	9.33	90.72	3.55	5.29	0.44	9.28
Cone 6	91.06	6.31	2.41	0.21	8.94	90.5	6.55	2.84	0.11	9.5
Cone 7	94.74	4.5	0.7	0.06	5.26	95.7	2.38	1.82	0.1	4.3
Cone 8	94.45	4.77	0.72	0.05	5.55	96.03	0.79	3.16	0.01	3.97
Cone 9	96.47	2.98	0.51	0.04	3.53	96.52	1.15	2.28	0.05	3.48
Cone 10	90.97	7.91	0.94	0.19	9.03	89.31	1.28	9.3	0.11	10.69
Cone 11	91.13	7.56	1.17	0.13	8.87	96.57	0.48	2.9	0.05	3.43
Cone 12	91.73	6.39	1.73	0.15	8.27	96.75	0.52	2.73	0	3.25
Cone 13	90.85	8.5	0.58	0.07	9.15	91.28	0.93	7.77	0.01	8.72
Cone 14	96.97	2.61	0.4	0.02	3.03	97.33	0.49	2.16	0.01	2.67
Cone 15	93.45	5.85	0.67	0.03	6.55	95.93	1.89	2.16	0.02	4.07
Cone 16	98.38	0.93	0.67	0.02	1.62	97.36	0.94	1.65	0.05	2.64
Mean	**93.12**	**5.76**	**1.03**	**0.09**	**6.88**	**94.84**	**1.72**	**3.37**	**0.07**	**5.16**
Median	**93.95**	**5.31**	**0.71**	**0.07**	**6.05**	**96.17**	**0.94**	**2.79**	**0.04**	**3.83**
Range	**81.20-98.38**	**0.93-18.48**	**0.40-3.23**	**0.02-0.23**	**18.8-1.62**	**89.31-97.36**	**0.33-4.61**	**1.65-3.29**	**0-0.44**	**2.64-10.69**

Table 5.8 CD25 and CD69 expression on viable CD4+ and CD8+ lymphocytes in prepared cone samples pre-cryopreservation.

	Percentage of CD4+ cells expressing CD25 and CD69					Percentage of CD8+ expressing CD25 and CD69				
Sample Number n=9	**CD4+CD25-CD69- (%)**	**CD4+CD25+ (%)**	**CD4+CD69+ (%)**	**CD4+CD25+ CD69+ (%)**	**Total activated CD4+ (%)**	**CD8+CD25-CD69- (%)**	**CD8+CD25+ (%)**	**CD8+CD69+ (%)**	**CD8+CD25+ CD69+ (%)**	**Total activated CD8+ (%)**
APH01	94.10	4.15	1.72	0.03	5.90	96.32	0.13	3.53	0.02	3.68
APO02	96.04	3.64	0.32	0.00	3.96	97.64	0	2.36	0.00	2.36
APH03	98.03	1.30	0.67	0.00	1.97	96.28	0.75	2.97	0.00	3.72
APH04	95.78	3.75	0.42	0.05	4.22	96.79	0.43	2.77	0.00	3.21
APH05	92.87	5.5	1.39	0.24	7.13	91.83	1.38	6.64	0.15	8.17
APH06	94.24	4.16	1.35	0.25	5.76	94.84	0.88	4.27	0.02	5.16
APH07	96.92	2.26	0.80	0.02	3.08	92.10	0.20	7.70	0.00	7.90
APH08	97.23	1.57	1.18	0.02	2.77	97.01	0.39	2.60	0.00	2.99
APH09	95.44	3.50	1.00	0.07	4.56	96.49	0.19	3.32	0.00	3.51
Mean	**95.63**	**3.31**	**0.98**	**0.08**	**4.37**	**95.48**	**0.48**	**4.02**	**0.02**	**4.52**
Median	**95.78**	**3.64**	**1.00**	**0.03**	**4.22**	**96.32**	**0.39**	**3.32**	**0.00**	**3.68**
Range	**92.87-98.03**	**1.30-4.16**	**0.32-1.78**	**0-0.25**	**1.97-5.90**	**91.83-97.64**	**0-1.38**	**2.36-7.70**	**0-0,15**	**2.36-7.90**

Table 5.9 CD25 and CD69 expression on viable CD4+ and CD8+ lymphocytes in prepared apheresis samples pre-cryopreservation.

5.5.2 Post thaw expression of CD25 and CD69 on CD4+ and CD8+ lymphocytes

When thawed and washed for stimulation, the percentage of CD4+ and CD8+ cells expressing CD25 and/or CD69 present in the washed samples was found to be reduced from that found in pre-cryopreservation tests with the mean percentage CD4+CD25+/CD69+ and CD8+CD25+/CD69+dropping gradually from D1 to D3 reaching 3.71% and 3.39% respectively in cone samples on D3 and 2.21% and 2.83% in apheresis samples on D3 (see Table 5.10). This finding corresponded with the observed loss of Tregs in the thawed samples as discussed in Chapter 3.

	Percentage of CD4+ cells expressing CD25 and CD69				**Percentage of CD8+ expressing CD25 and CD69**			
Sample numbers Cones n=16 Apheresis n= 9	**CD4+CD25+ (%)**	**CD4+CD69+ (%)**	**CD4+CD25+CD69+ (%)**	**Total activated CD4+ (%)**	**CD8+CD25+ (%)**	**CD8+CD69+ (%)**	**CD8+CD25+CD69+** (%)	**Total activated CD8+** (%)
Cones 1-16 Pre-Cryopreservation	**5.76**	**1.03**	**0.09**	**6.88**	**1.72**	**3.37**	**0.07**	**5.16**
Cones 1-16 Thawed D1	5.46	0.73	0.08	6.27	1.01	3.04	0.04	4.09
Cones 1-16 Thawed D 2	3.56	0.58	0.09	4.24	1.07	3.33	0.11	4.50
Cones 1-16 Thawed D3	2.66	1.03	0.03	3.71	0.94	2.42	0.03	3.39
APH Pre-Cryopreservation	**3.31**	**0.98**	**0.08**	**4.37**	**0.48**	**4.02**	**0.02**	**4.52**
APH Thawed D1	1.60	0.79	0.02	2.41	0.44	3.31	0.02	3.77
APH Thawed D2	1.59	0.73	0.03	2.35	0.33	2.92	0.01	3.26
APH Thawed D3	1.28	0.91	0.02	2.21	0.27	2.55	0.01	2.83

Table 5.10 Reduction of cell activation markers CD25 and CD69 on CD4+ and CD8+ cells detected on thawed samples frozen on D1, D2 and D3.

Event numbers in all tests were very low, particularly for CD69+ cells, so although the difference between fresh and thawed samples attained statistical significance using 2-way ANOVA to compare the sample groups (Figure 5.8), too much importance should not be attached to these results.

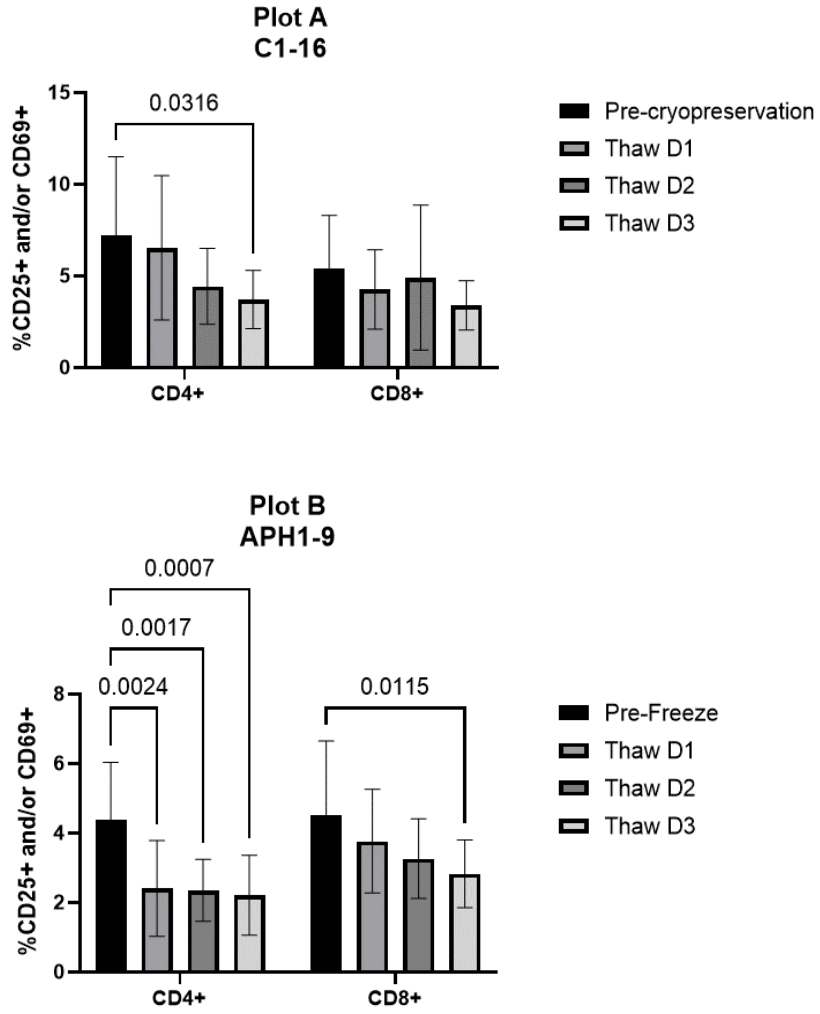


Figure 5.8: Statistical comparison of CD25 and CD69 expression on lymphocytes derived from cone and apheresis samples pre-cryopreservation and immediately post-thaw (prior to culture) on D1, D2 and D3 of cryopreservation. CD25 and CD69 expression assessed by flow cytometry as a percentage of viable CD4+ and CD8+ lymphocytes. Means +/-SD shown from samples C1-16 (Plot A) and APH1-9 (Plot B). p values from two-way ANOVA. Only p values <0.05 shown.

5.5.3 Expression of CD25 and CD69 in culture

For this study, upregulation of CD25 and CD69 expression was regarded as evidence of activation (Motamedi *et al.* 2016, Bajnok *et al.* 2017, Texeiro *et al.* 2009). The percentage of viable CD4+ and CD8+ cells expressing CD25 and/or CD69 was calculated for the

samples removed from culture at 4, 24, and 72 hours. The mean expression of CD25 and CD69 in the control, IL2 and stimulated groups at the time points sampled is shown in Tables 5.11 and 5.12. Gating was established on the control sample and that gate maintained for both the IL2 and stimulated samples to ensure that reported upregulation was a result of the experimental conditions (Figures 5.9 and 5.10).

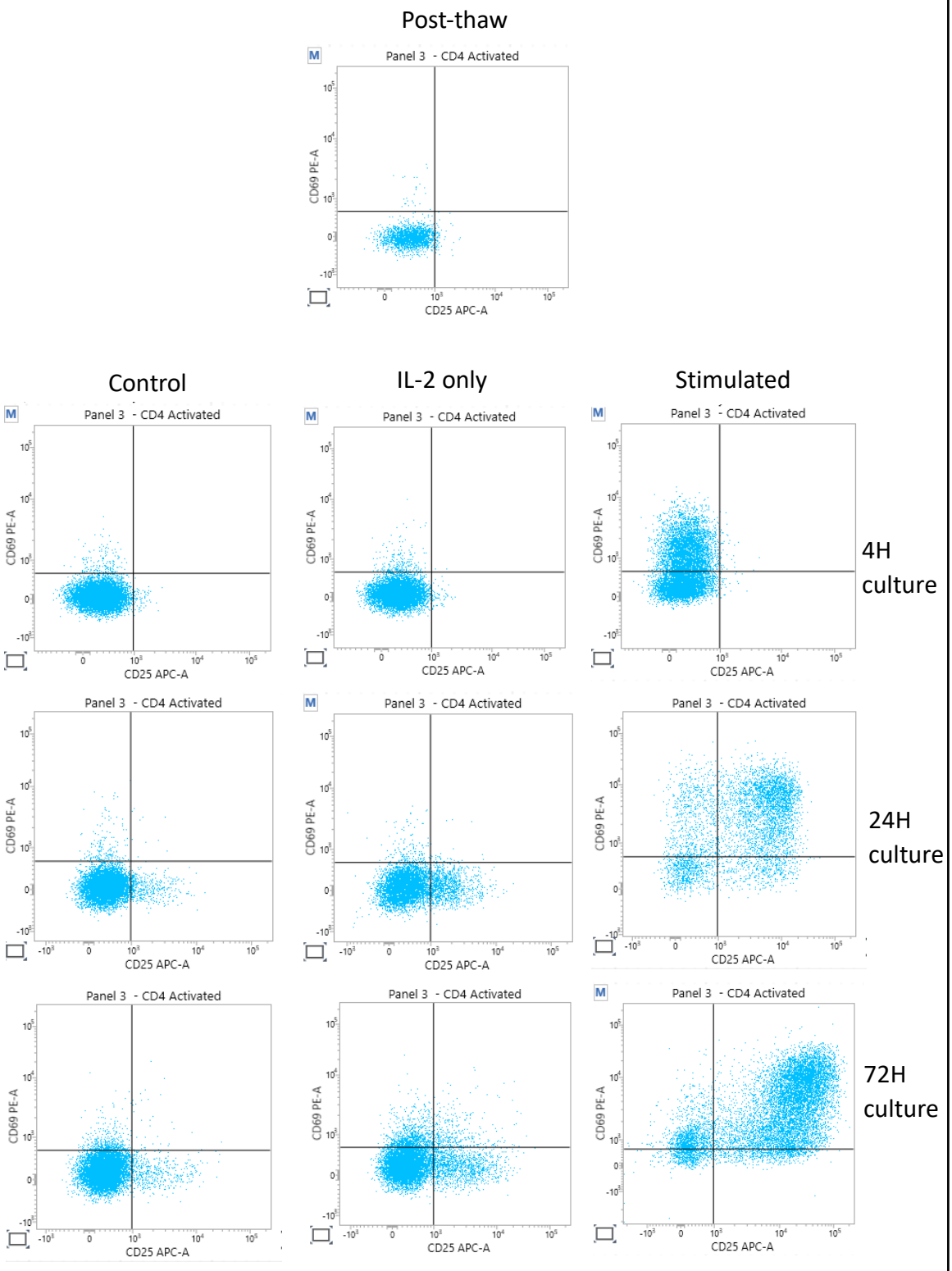


Figure 5.9 Identification of CD4+CD25+CD69+ cell population in post-thaw, control, IL2 and stimulated samples by flow cytometry. Samples tested immediately post-thaw, and at 4, 24 and 72 hours in culture. Viable CD4+cells identified by dye exclusion staining with 7-AAD stained with anti-CD25-APC and anti-CD69-PE. Gating set on control sample and conserved in IL2 and stimulated samples.

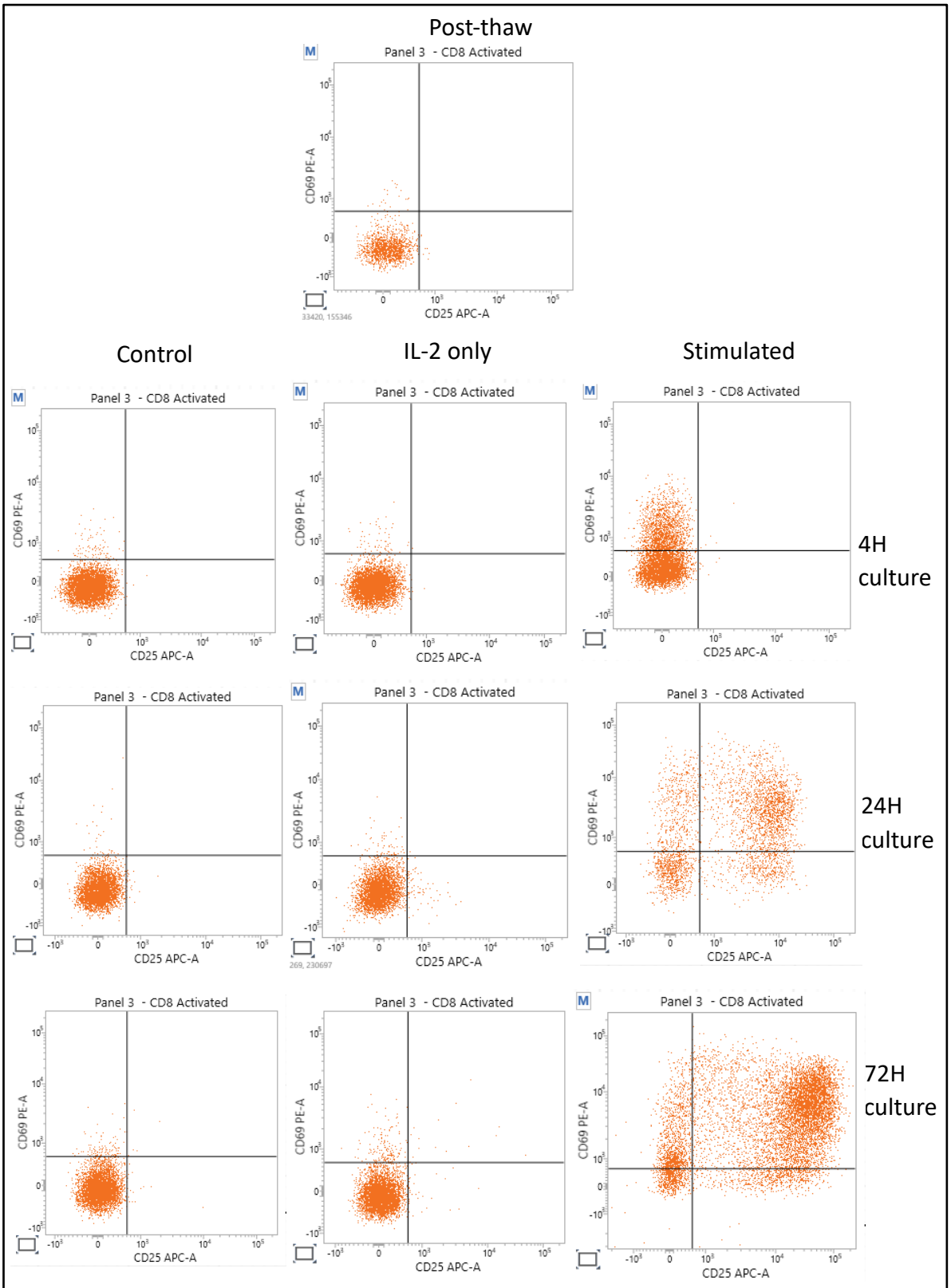


Figure 5.10 Identification of CD8+CD25+CD69+ cell population in post-thaw, control, IL2 and stimulated samples by flow cytometry. Samples tested immediately post-thaw, and at 4, 24 and 72 hours in culture. Viable CD8+cells identified by dye exclusion staining with 7-AAD stained with anti-CD25-APC and anti-CD69-PE. Gating set on control sample and conserved in IL2 and stimulated samples.

Cone samples

The mean CD25 and CD69 expression for viable CD4+ and CD8+ lymphocytes found in the cone derived samples is summarised in Table 5.11. Due to the seeding density errors on samples from C1-10, flow cytometric event numbers acquired in these samples were sub-optimal. The potential inaccuracy in reporting caused by the low numbers of events acquired was compounded by the fact that the mean CD3+ cell viability of the control samples from C1-10 at 4 hours in culture was found to be only 35.64% and as a result <2000 viable CD3+ events had been acquired in these samples. Although the percentage cell viability improved slightly over time in culture, event numbers acquired by flow cytometry remained low so the reported results must be treated with caution.

After 4 hours in culture, the mean CD3+ cell viability found in control samples from C11-16, which had been seeded at the correct density, was found to be 79.15% and viable CD3+ event numbers exceeded 10,000. The 4-hour results from cones C1-16 and from C11-16 were therefore analysed independently (Figure 5.11).

Day of Cryopreservation									
Cones 1-16 n=16	**D1**	**D2**	**D3**	**D1**	**D2**	**D3**	**D1**	**D2**	**D3**
Incubation Time (hours)	**4**			**24**			**72**		
%CD4+CD25+	2.28	1.41	0.80	15.34	12.55	11.35	38.97	38.95	39.62
%CD4+CD69+	40.95	39.01	33.94	15.23	15.84	23.61	6.49	6.20	9.71
%CD4+CD25+CD69+	1.76	1.16	0.97	31.29	32.07	29.79	34.53	37.47	27.28
%Total activated CD4+	**44.99**	**41.58**	**35.70**	**61.86**	**60.46**	**64.75**	**79.99**	**82.62**	**76.61**
%CD8+CD25+	0.34	0.28	0.14	11.30	8.49	7.27	30.10	30.73	29.39
%CD8+CD69+	43.16	39.94	29.88	23.16	24.89	25.51	15.01	15.85	17.19
%CD8+CD25+CD69+	0.51	0.37	0.59	22.89	22.87	18.44	28.62	31.94	27.13
%Total activated CD8+	**44.00**	**40.59**	**30.62**	**57.35**	**56.25**	**51.21**	**73.72**	**78.52**	**73.72**

Table 5.11 Mean CD25 and CD69 expression on CD4+ and CD8+ lymphocytes from Cones 1-16 cryopreserved on D1, D2 and D3.

Cone samples after 4 hours in culture:

Both CD4+ and CD8+ cells derived from cone samples showed high levels of activation after 4 hours in culture (Figure 5.11). Activation, as identified by upregulated expression of CD25 and/or CD69 in the stimulated samples was significantly higher than that seen in the control samples when tested using two-way ANOVA. This finding was seen in both C1-16 (D1 CD4 p=0.0012, D1 CD8 p=0.0280, D2 CD4 p=0.0002, D2 CD8 p=0.0040, D3 CD4+ p=0.0023, D3 CD8+ p=0.0033) and C11-16 (D1 CD4 p=0.0107, D1 CD8 p=0.0229, D2 CD4 p=0.0224, D2 CD8 p=0.0219, D3 CD4+ p=0.0008, D3 CD8+ p=0.0370) indicating that the high activation level found was not an error created by low event numbers in C1-10 (Figure 5.11). No significant differences were seen between the control and the IL2 groups indicating that the effect was caused by the activation beads. The mean percentage of both activated CD4+ and CD8+ cells decreased slightly with increased time

to cryopreservation; D2 CD4+ = 41.58%; D2 CD8+ = 40.59%, D3 CD4+ = 35.70%, D3 CD8+ = 30.62%. However, these differences did not attain statistical significance in either group when analysed using two-way ANOVA (Figure 5.11).

The high level of activation after 4 hours in culture was attributable almost entirely to raised CD69 expression on both CD4+ and CD8+ cells. As expected, CD25 expression was very low at this time point. The maximum expression of CD25 after 4 hours in culture was 2.28% of CD4+ cells and 0.34% of CD8+ cells. The highest CD69+ expression was seen in cells cryopreserved on D1 and was similar on both CD4+ and CD8+ cells; 40.95% CD4+69+ and 44.00% CD8+69%. Very low numbers of CD4+ or CD8+ cells staining positive for both CD25 and CD69 (<2%) were seen at this time point.

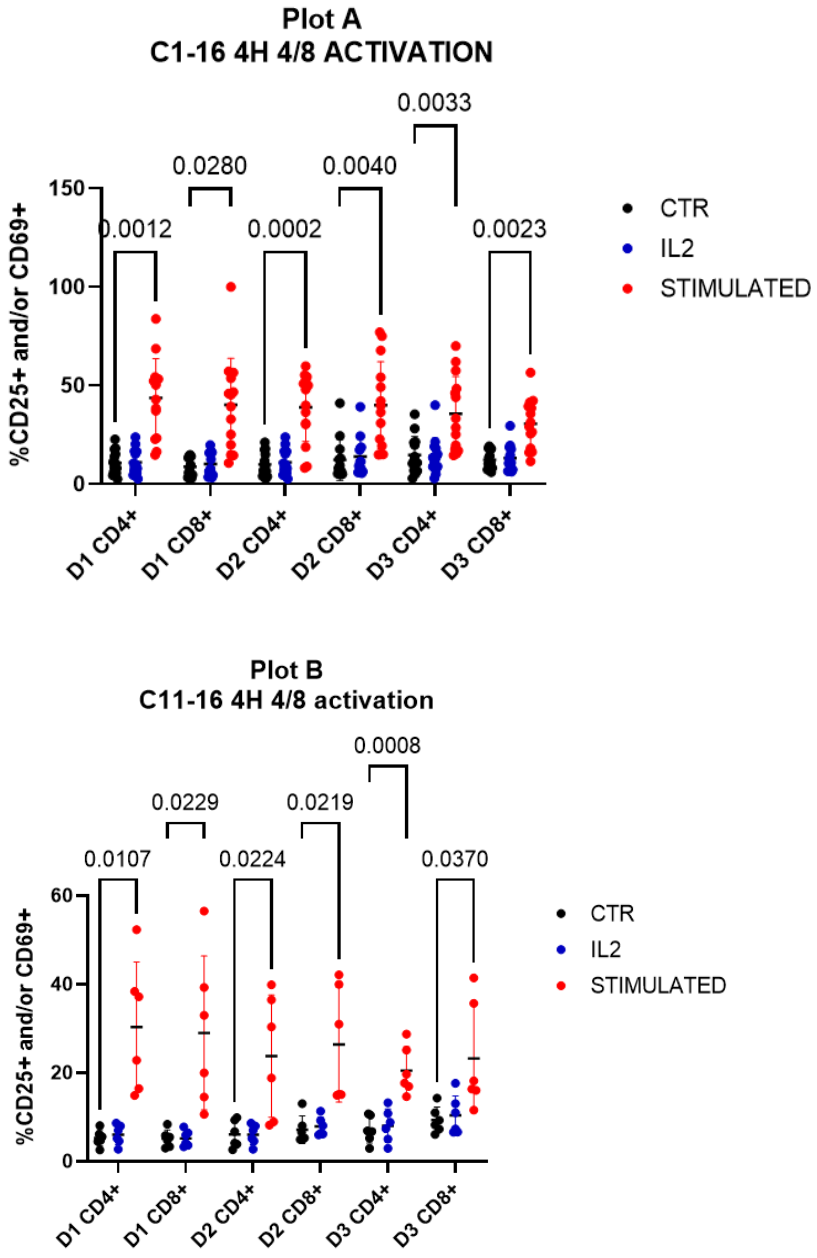


Figure 5.11: Comparison of activated viable CD4+ and CD8+ lymphocytes in cells derived from cone samples after 4 hours in culture determined by flow cytometry in control, IL2 and stimulated groups. Activated lymphocytes classified as CD25+, CD69+ or CD25+CD69+ cells. Viability of CD4+ and CD8+ cells determined by dye exclusion staining with 7-AAD. Cone samples C1-16 (Plot A). Cone samples C11-16 shown separately (Plot B). p values from two-way ANOVA. Only p values <0.05 shown. Means shown +/-SD.

Cone Samples after 24 – 72 hours in culture:

The percentage of activated CD4+ and CD8+ cells increased with time in culture in cells cryopreserved on all 3 days. The expression of CD25/CD69 was significantly higher in the stimulated group as compared to the control or IL2 groups for all parameters studied when tested by two way-ANOVA. Slightly higher numbers of CD4+ cells expressing activation markers were seen after 24 hours in culture in the IL2 group compared to the control on D1 ($p=0.0108$) and D2 ($p=0.0009$) of cryopreservation, and after 72 hours of culture in D1 cells only ($p=0.0017$) indicating low level activation of some CD4+ cells in response to IL2 alone (Figure 5.12). However, the differences were small and not seen in all study conditions. Mean values for CD25/CD69 expression on cells cryopreserved on D1 were, CD4+ = 61.86%; CD8+ = 57.35%; on D2 CD4+ = 61.86%. CD8+ = 57.35%; and on D3 CD4+ = 79.99%, CD8+ = 73.72% (Table 5.11). This was due to increased expression of CD25 during the longer period in culture. CD69 expression gradually decreased with time in culture on both CD4+ and CD8+ cells, although expression on CD8+ cells remained slightly higher than that on CD4+ cells. CD69 expression on CD4+ cells was also retained slightly longer on cells cryopreserved on D3 when compared to D1 and D2; D1 6.49%; D2 6.20%; D3 9.71%.

Mean CD25 expression increased on both CD4+ and CD8+ cells from <2.3% on CD4+ cells and <0.5% on CD8+ cells at 4 hours culture. Cells cryopreserved on D1 CD4+ = 38.97%, CD8+ =30.1%; on D2 CD4+ =38.95, CD8+ = 30.73%; and on D3 CD4+ =39.62%, CD8+ =29.39% at 72 hours culture (Table 5.11). CD25+ expression was consistent on cells cryopreserved on D1, D2 and D3.

CD4+ and CD8+ cells staining dual positive for both CD25+and CD69+ increased from <2% of the total at 4 hours incubation to approximately 30% of the total by 72 hours in culture. The percentage of dual positive cells was consistently lower in CD8+ cells compared to CD4+. At 24 hours in culture the mean percentage of CD25+CD69+cells cryopreserved on D1 was CD4+ =31.29%, CD8+ = 22.89%; on D2 CD4+ = 32.01%, CD8+ = 22.78%, and on D3 CD4+ = 29.79%, CD8+ = 18.44%. At 72 hours culture CD25/CD69 expression for cells cryopreserved on D1 was, CD4+ =34.53%, CD8+ = 28.62%; on D2 CD4+ = 37.47%, CD8+ = 31.94% and on D3 CD4+ = 27.28%, CD8+ = 27.13% (Table 5.11). There were no statistically significant differences in expression of activation markers at 72 hours in culture between days of cryopreservation when tested using two-way ANOVA (Figure 5.13).

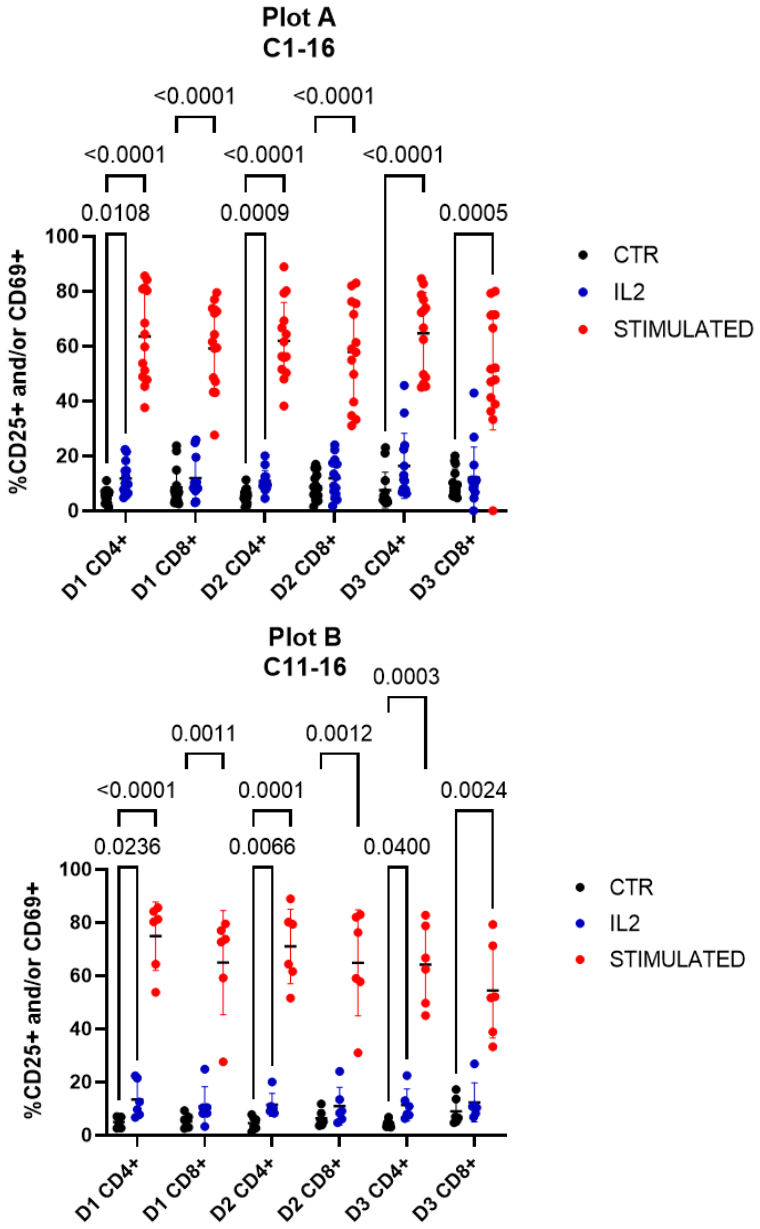


Figure 5.12: Comparison of activated viable CD4+ and CD8+ lymphocytes in cells derived from cone samples after 24 hours culture determined by flow cytometry in control, IL2 and stimulated groups. Activated lymphocytes classified as CD25+, CD69+ or CD25+CD69+ cells. Viability of CD4+ and CD8+ cells determined by dye exclusion staining with 7-AAD. Cone samples C1-16 (Plot A). Cone samples C11-16 shown separately (Plot B). p values from two-way ANOVA. Only p values <0.05 shown. Means shown +/-SD.

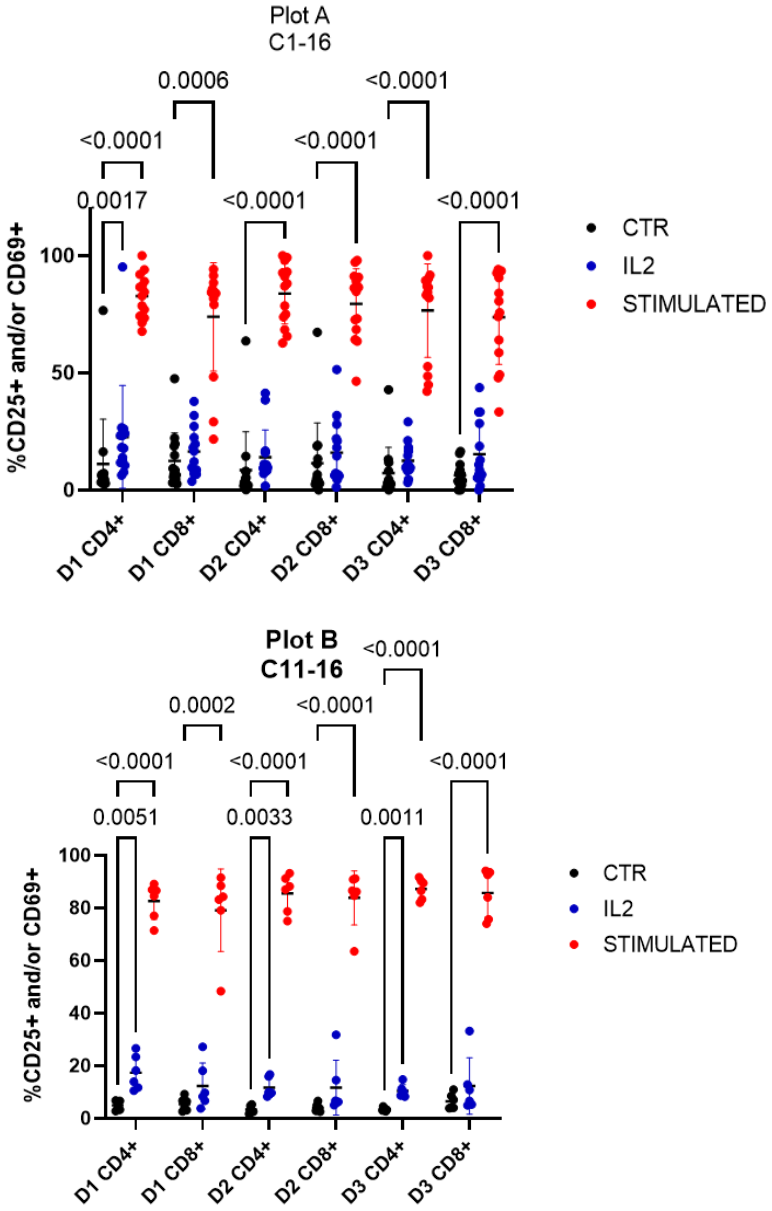


Figure 5.13: Comparison of activated viable CD4+ and CD8+ lymphocytes in cells derived from cone samples after 72 hours culture determined by flow cytometry in control, IL2 and stimulated groups. Activated lymphocytes classified as CD25+, CD69+ or CD25+CD69+ cells. Viability of CD4+ and CD8+ cells determined by dye exclusion staining with 7-AAD. Cone samples C1-16 (Plot A). Cone samples C11-16 shown separately (Plot B). p values from two-way ANOVA. Only p values <0.05 shown. Means shown +/-SD.

5.5.4 Activation markers: apheresis samples

The mean CD25 and CD69 expression for viable CD4+ and CD8+ lymphocytes found in the apheresis derived samples is summarised in Table 5.12. The apheresis derived samples were seeded at the correct density and sufficient viable CD3+ events were

acquired on all samples to ensure the accuracy of the data. The mean viability of the apheresis derived cells after 4 hours culture was 76.15%. This result was similar to that seen in cones C11-16, suggesting that the low 4-hour viability found in C1-10 was probably related to the low seeding density.

Apheresis samples 1-9 n=9	D1	D2	D3	D1	D2	D3	D1	D2	D3
Incubation Time (hours)	4			24			72		
%CD4+CD25+	0.79	0.83	0.67	12.81	11.11	7.06	17.74	18.92	13.38
%CD4+CD69+	30.51	42.51	45.60	10.84	11.42	14.09	4.49	3.54	2.84
%CD4+CD25+CD69+	0.55	0.86	0.84	50.69	64.14	63.92	62.33	71.13	76.46
%Total activated CD4+	**31.85**	**44.19**	**47.12**	**74.35**	**86.67**	**85.07**	**84.56**	**93.60**	**92.68**
%CD8+CD25+	0.17	0.08	0.09	18.01	14.78	9.79	24.79	31.71	22.24
%CD8+CD69+	22.24	34.94	42.85	9.90	14.49	18.01	6.54	6.02	9.78
%CD8+CD25+CD69+	0.12	0.13	0.27	37.87	50.38	51.42	55.93	54.74	57.02
%Total activated CD8+	**22.54**	**35.15**	**43.21**	**65.79**	**79.65**	**79.22**	**87.26**	**92.47**	**89.04**

Table 5.12 Mean CD25 and CD69 expression on CD4+ and CD8+ lymphocytes from apheresis samples APH1-9 cryopreserved on D1, D2 and D3 at 4, 24 and 72 hours in culture.

Apheresis Samples after 4 Hours in Culture

As was found in the cone derived samples, both CD4+ and CD8+ cells derived from apheresis samples showed high levels of activation after 4 hours in culture (Figure 5.14). Activation, as identified by upregulated expression of CD25 and/or CD69 in the stimulated cell samples was significantly higher than that seen in the control samples when tested using two-way ANOVA for all samples tested. (D1 CD4+ p=0.0006, D1 CD8+ p=0.0030, D2 CD4+ p=<0.0001, D2 CD8+ p=<0.0001, D3 CD4+ p=0.0018, D3 CD8+ p=0.0007). No significant differences were seen between the control and the IL2 groups indicating that the effect was experimental in origin. The mean percentage of both

activated CD4+ and CD8+ cells increased slightly with increased time to cryopreservation. For cells cryopreserved on D1, CD4+=31.85%, CD8+ = 22.54%; on D2 CD4+ = 44.19%, D2 CD8+ = 35.15%; and D3 CD4+ = 47.12%, D3 CD8+ = 43.21%. The increase in the percentage of activated CD8+ cells between cells cryopreserved on D1 and D3 was the only finding that was statistically significant (p=0.0027) when analysed using two-way ANOVA (Figure 5.14).

Again, the high level of cell activation after 4 hours in culture was attributable almost entirely to raised CD69 expression on both CD4+ and CD8+ cells. CD25 expression was expressed on <1% of CD4+ and CD8+ cells at this time point. The highest CD69+ expression was seen in cells cryopreserved on D3 and was higher on CD4+ cells compared to CD8+ cells; CD4+CD69+ = 47.12%; CD8+CD69+ = 43.21% (Table 5.12).

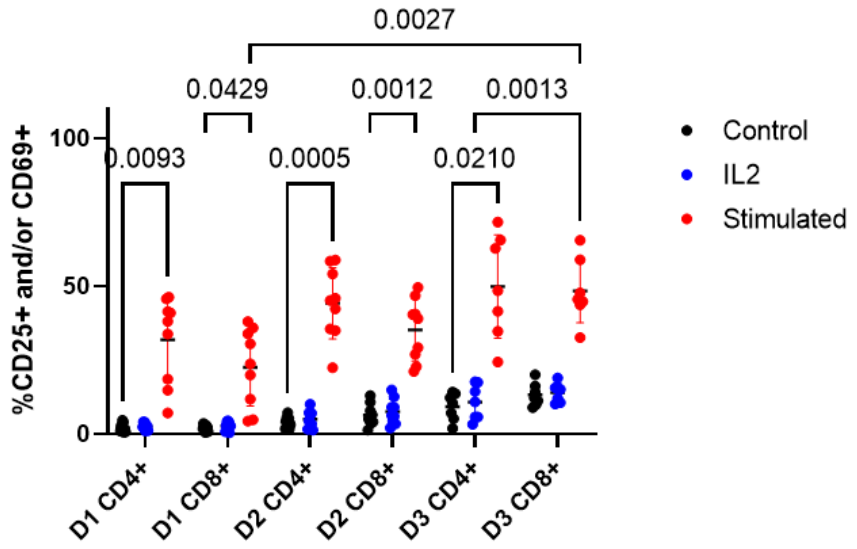


Figure 5.14: Comparison of activated viable CD4+ and CD8+ lymphocytes in cells derived from apheresis samples APH1-9 after 4 hours culture determined by flow cytometry in control IL2 and stimulated groups. Activated lymphocytes classified as CD25+, CD69+ or CD25+CD69 cells. Viability of CD4+ and CD8+ cells determined by dye exclusion staining with 7-AAD. p values from two-way ANOVA. Only p values <0.05 shown. Means shown +/-SD.

Apheresis samples after 24 – 72 hours in culture

The percentage of activated CD4+ and CD8+ cells increased rapidly with time in culture for cells cryopreserved on all 3 days. The expression of CD25/CD69 was significantly higher in the stimulated group as compared to the control or IL2 groups for all parameters studied when tested by two way-ANOVA. Activation levels for both CD4+ and CD8+ cells were similar at both the 24- and 72-hour time points. Mean total activation at 24 hours in culture for cells cryopreserved on D1 was found to be: CD4+ = 74.35%, CD8+ = 65.79%; on D2 CD4+ = 86.67%, CD8+ = 79.65%; on D3 CD4+ = 85.07%, CD8+ = 79.22% (Figure 5.15). No significant differences were found between these days when analysed using two-way ANOVA. A small but significant increase in expression of activation markers on CD4+ cells in the IL2 group was seen on all three days of cryopreservation at 24 hours culture (D1 p=0.0499), D2 p= 0.0009, D3 p= 0.0028) and 72 hours culture (D1 p= 0.0008, D2 p= 0.0105, D3 p= 0.024) (Figure 5.16). This finding was not seen in CD8+ cells at any time point. See Figures 5.15 and 5.16.

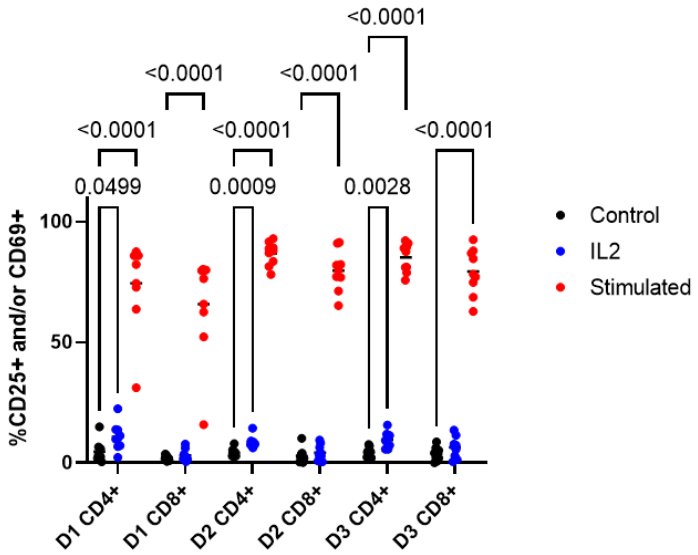


Figure 5.15: Comparison of activated viable CD4+ and CD8+ lymphocytes in cells derived from apheresis samples APH1-9 after 24 hours culture determined by flow cytometry in control IL2 and stimulated groups. Activated lymphocytes classified as CD25+, CD69+ or CD25+CD69+ cells. Viability of CD4+ and CD8+ cells determined by dye exclusion staining with 7-AAD. p values from two-way ANOVA. Only p values <0.05 shown. Means shown +/-SD.

Mean cell activation at 72 hours in culture was found to be extremely high, with almost all viable CD4+ and CD8+ cells expressing CD25 and/or CD69. For cells cryopreserved on D1; CD4+ = 84.56, CD8+ = 87.26%; on D2 CD4+ = 93.60%, CD8+ = 92.47%; on D3 CD4+ = 92.68%, CD8+ = 89.04% (Table 5.12). Although the mean cell activation seen on cells cryopreserved on D2 and D3 was higher than that seen on cells cryopreserved on D1 at both 24 and 72 hours in culture, the difference was not statistically significant at either time point (Figure 5.16).

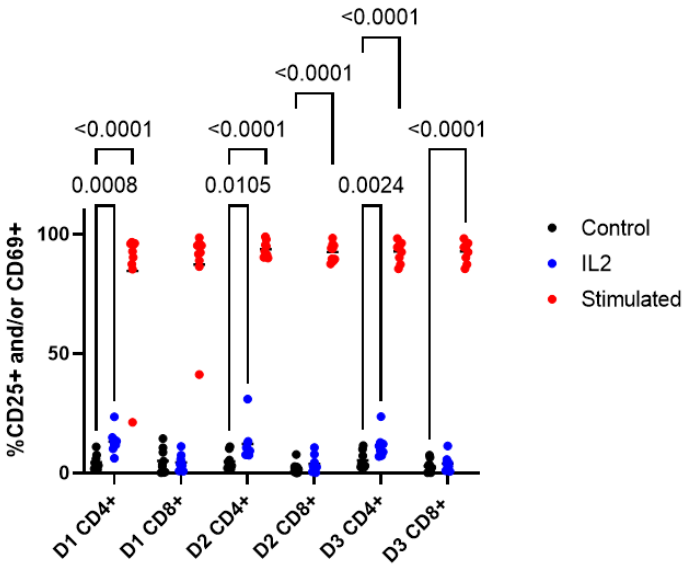


Figure 5.16: Comparison of activated viable CD4+ and CD8+ lymphocytes in cells derived from apheresis samples APH1-9 after 72 hours culture determined by flow cytometry in control IL2 and stimulated groups. Activated lymphocytes classified as CD25+, CD69+ or CD25+CD69+ cells. Viability of CD4+ and CD8+ cells determined by dye exclusion staining with 7-AAD. p values from two-way ANOVA. Only p values <0.05 shown. Means shown +/-SD.

The increased level of activation was due to raised expression of CD25 during the longer period in culture. The percentage of cells expressing CD69 only, gradually decreased with time in culture on both CD4+ and CD8+ cells, although CD69 expression on CD8+ cells remained slightly higher than that on CD4+ cells throughout. (Table 5.12).

Mean CD25 expression increased on both CD4+ and CD8+ cells from <2.3% on CD4+ cells and <0.5% on CD8+ cells at 4 hours culture to D1 CD4+ = 38.97%, D1 CD8+ =30.1%; D2+ CD4+ =38.95, D2 CD8+ = 30.73%; D3 CD4+ =39.62%, D3 CD8+ =29.39% at 72 hours culture (Table 5.12). CD25+ expression was consistent across days of cryopreservation.

The number of CD4+ and CD8+ cells dual staining for CD25 and CD69 increased with length of time culture. Mean numbers of dual positives were <1% of the total after 4 hours but increased rapidly over 24-48 hours. At 24 hours the mean percentage of cells staining CD25+69+ cryopreserved on D1 was CD4+ = 50.69%, CD8+ = 55.93%; on D2

CD4+ = 64.14%, CD8+ = 54.74%; on D3 CD4+ = 63.92%, CD8+ = 57.02%. At 72 hours the mean percentage staining CD25+CD69+ reached on D1 CD4+ = 62.33%, CD8+ = 55.93%; on D2 CD4+=71.13 %, CD8+ = 54.74%; and on D3 CD4+= 76.46%, CD8+ = 57.02% (Table 5.12). There was a higher percentage of CD4+CD25+CD69+ at both 24 and 72 hours in culture for cryopreserved on D2 and D3 compared to D1. The percentage of CD8+CD25+CD69+ was not affected by day of cryopreservation.

5.5.5 Comparison of CD25 and CD69 expression on lymphocytes derived from cone and apheresis samples

CD4+ and CD8+ lymphocytes derived from cone samples were found to have similar levels of CD25 and CD69 expression on all days of cryopreservation. This was the case at all time points in culture. Apheresis samples, in contrast had weakest expression of activation markers in cells cryopreserved on D1 at all time points tested, although the difference was only significant in the case of CD8+ cells at 4 hours in culture. When the two data sets were compared using two-way ANOVA, no significant differences were found between the groups cryopreserved on D1, although both CD4+ and CD8+ lymphocytes derived from apheresis samples expressed higher levels of activation markers at 24 and 72 hours in culture (Figure 5.17).

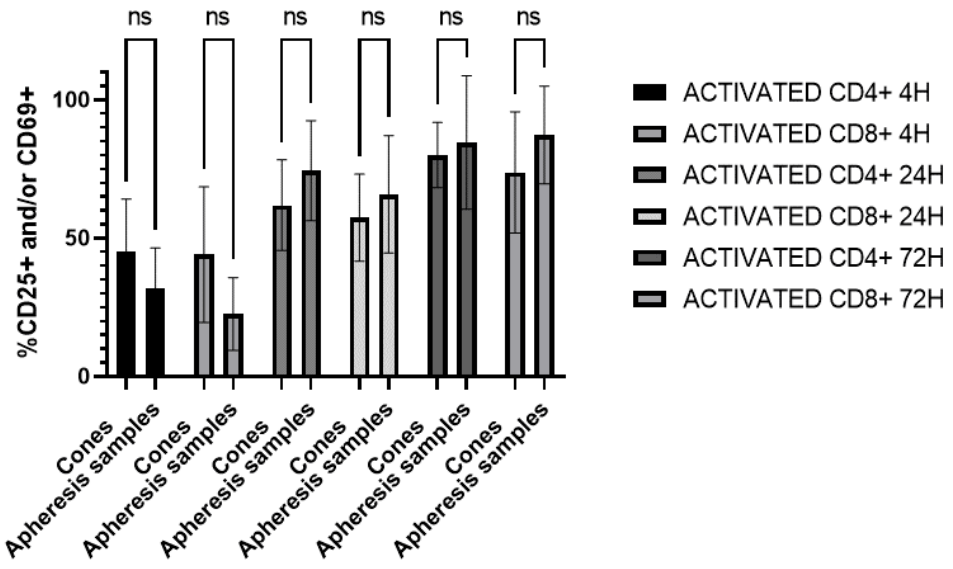


Figure 5.17: Statistical comparison of activated CD4+ and CD8+ lymphocytes derived from cone samples C1-16 and apheresis samples APH1-9 cryopreserved on D1. Percentage of viable CD4+ and CD8+ lymphocytes expressing CD25 and/or CD69 at 4 hours, 24 hours and 72 hours of culture determined by flow cytometry. Viability determined by dye exclusion staining with 7-AAD. ns – not significant by two-way ANOVA. Means shown +/-SD.

On D2 of cryopreservation, cells derived from apheresis samples were found to have a significantly higher percentage of activated CD4+ and CD8+ at 24 (CD4+ p= <0.0001; CD8+ p= 0.0011) and 72 hours (CD4+ p=0.0225; CD8+ p= 0.0109) in culture than cells derived from cone samples (Figure 5.18).

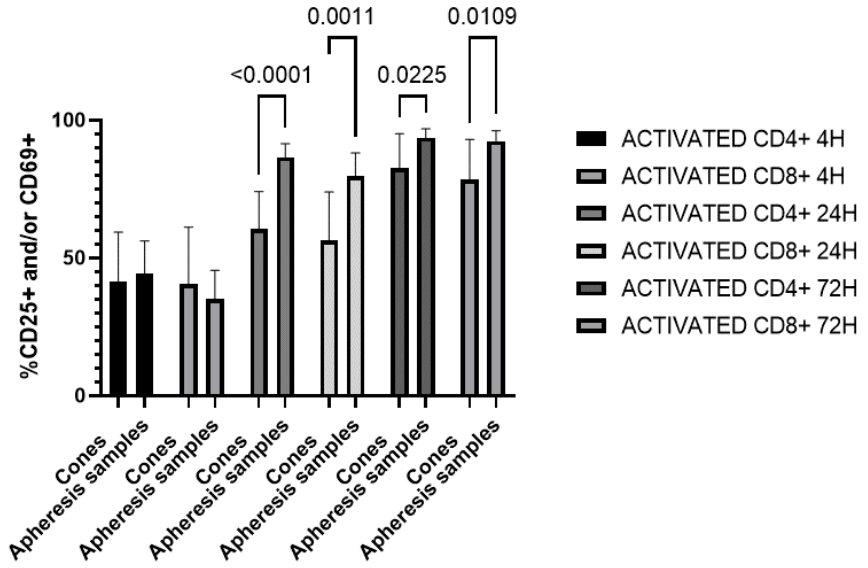


Figure 5.18: Statistical comparison of activated CD4+ and CD8+ lymphocytes derived from cone samples C1-16 and apheresis samples APH1-9 cryopreserved on D2. Percentage of viable CD4+ and CD8+ lymphocytes expressing CD25 and/or CD69 at 4 hours, 24 hours and 72 hours of culture determined by flow cytometry. Viability determined by dye exclusion staining with 7-AAD. p values from two-way ANOVA. Only p values <0.05 are shown. Means shown +/-SD.

For cells cryopreserved on D3, although lymphocytes derived from apheresis samples had a higher level of activation than those derived from cones at all time points, the difference was only significant at 24 hours in culture (Figure 5.19).

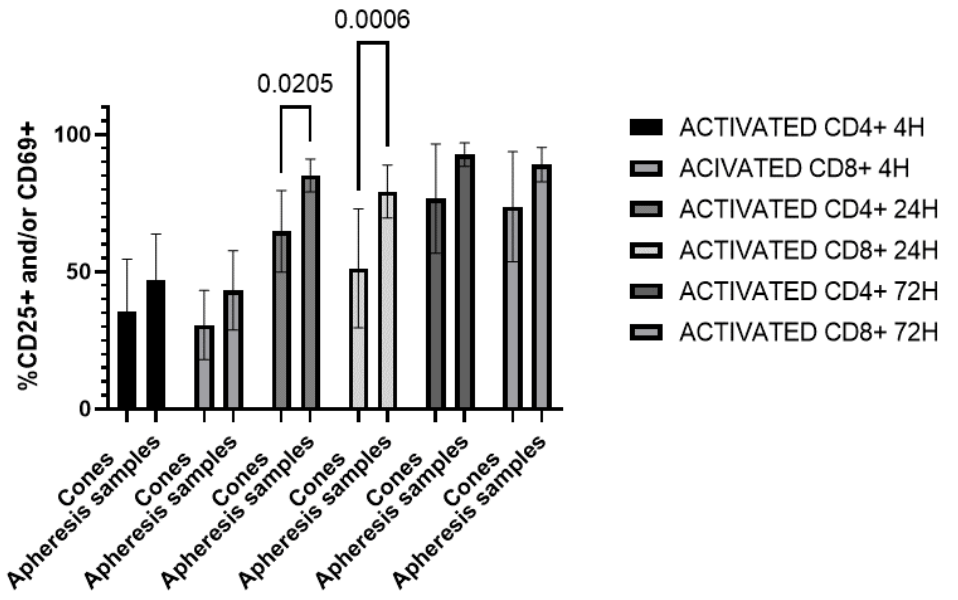


Figure 5.19: Statistical comparison of activated CD4+ and CD8+ lymphocytes derived from cone samples C1-16 and apheresis samples APH1-9 cryopreserved on D3. Percentage of viable CD4+ and CD8+ lymphocytes expressing CD25 and/or CD69 at 4, 24 and 72 hours in culture determined by flow cytometry. Viability determined by dye exclusion staining with 7-AAD. p values from two-way ANOVA. Only p values <0.05 are shown. Means shown +/-SD.

5.6 Discussion of the CD3+ expansion and activation

5.6.1 Errors and anomalies

The poor seeding density seen in cells retrieved from cones 1-10 was almost certainly an error generated by miscalculation of viable/non-viable CD3+ cells. The low CD3+ viability seen in the control samples at 4-hours in culture indicated that dead cells had been included in the seeding calculation. Clearly initial viable cell numbers in these samples were so low that the cells were unable to respond to stimulation and gradually died in culture. However, although our internal data indicated an optimum CD3+ seeding density of 1 x 10^6/mL for stimulation and culture, there are publications indicating that concentrations as low as 1 x 10^5/mL produce satisfactory results (Juhl *et al*. 2021). There may be other unidentified sample handling factors related to our inexperience with the

laboratory techniques employed which reduced the ability of the cells to response. Results from this group were included in the overall analysis, but the results from C11-16 were analysed separately to determine whether experimental effects present in correctly seeded samples were being masked by the poor results from C1-10.

5.6.2 Upregulation of activation markers in response to stimulation

When correctly seeded, cells derived from cone cells were consistent in their response to stimulation and responded equally well to stimulation regardless of the day of cryopreservation. By 72 hours in culture both CD4+ and CD8+ lymphocytes derived from cone cells expressed activation markers on over 70% of viable cells. The apheresis derived lymphocytes also showed very high levels of activation regardless of the day of cryopreservation, although the percentage of both activated CD4+ and CD8+ lymphocytes was slightly higher in samples cryopreserved on D2 and D3 compared to (93.6% of CD4+ and 92.5% of CD8+ on D2 compared to 84.6% and 87.3% in D1). This finding was not statistically significant. Mean activation in the apheresis derived cells was uniformly higher than in the cone derived cells at all time points sampled. The high levels of responsiveness seen are consistent with the numerous studies on cellular responses obtained from cryopreserved lymphocytes post thaw (Tompa *et al*. 2018, Keane *et al*. 1025, Da Silva Ferreira *et al*. 2015, Sambor *et al*. 2014 Weinberg *et al.* 2009). However, as none of these studies examined the effect of storage of lymphocytes for >24 hours prior to cryopreservation, it is not possible to compare this aspect of the results to the published literature.

Aside from the overall percentage activation shown, both cone and apheresis samples demonstrated extremely rapid upregulation of CD69, with between 30-40% of CD4+ and CD8+ expressing CD69 after four hours in culture. The percentage of cells expressing

CD69 only tailed off in culture to <10% of CD4+ in both cone and apheresis groups and to <10% of apheresis derived CD8+ by 72 hours culture. CD69 expression in CD8+ cone cells remained at 15-17% after 72 hours. This finding was consistent with published work demonstrating upregulation of CD69 within 1-3 hours of stimulation followed by a decline after 24 hours but remaining elevated for up to 5 days (Texeiro et al. 2009, Cibrian & Sanchez-Madrid, 2017, Bajnok et al. 2017). CD25+ expression in both sample groups also followed the pattern described in the literature, showing rapid upregulation from 24 hours in culture, reaching a peak at 72 hours (Texeiro et al. 2009).

However, there was a difference between the cone derived and apheresis derived groups in the number of cells expressing CD25 only compared to those that expressed both CD25 and CD69 at 72 hours in culture. 30% of CD4+ cells derived from cones were CD25+CD69+ at 72 hours compared with 62-75% of apheresis derived CD4+. Similarly, 30% of CD8+ cells derived from cone cells were CD25+69+ at 72 hours in culture compared with 55-57% of apheresis derived CD8+. 40% of cone derived CD4+ cells expressed CD25 only compared to 15-17% of apheresis derived CD4+ cells. There was no difference in CD25 expression in CD8+ cells. These findings indicate that CD69 remained upregulated on the apheresis derived cells, particularly CD4+, longer than it did on the cone derived cells. Given that the lymphocyte starting populations were almost identical in phenotype, it is difficult to explain the findings. Possibly the contaminating monocytes in the apheresis group contributed to the increased upregulation of CD69 in this group. However, this theory does not accord with recent publications showing that lymphocyte activation by CD3/CD28 beads can be reduced in the presence of monocytes as monocytes can block the activation beads (Wang *et al*. 2022, Noakes *et al.* 2021), and that lymphocyte activation is more effective if monocytes

are depleted. Possibly the difference seen on our study related to the relatively small number of samples tested and the wide variability seen on individual donor response to stimulation (Noakes *et al.* 2021, Fisher *et al*. 2014). Further study with increased samples numbers will be required to confirm this finding.

5.6.3 Absolute numbers of cells in culture

Cells were seeded at a viable CD3+ cell concentration of 1 x 10^6/mL but analysis of the absolute counts from the samples taken during culture was performed on viable CD4+ and CD8+ cells because the CD3/CD28 activation beads block the CD3 antibody binding site. This approach meant that the numbers seeded at the start and the numbers counted during culture were measured slightly differently. However, as CD4+ and CD8+ lymphocytes make up the vast majority of total CD3+ cells (Choi *et al*. 2014, Zhang *et al*. 2016, Garcia-Prat *et al.* 2019), this approach was valid. As discussed in section 5.6.3, the cells in culture from both sample groups responded well to stimulation and there was no significant difference in expression of activation markers between cells cryopreserved at different time points. Cells present in culture responded well but significant differences were found in the number of cells present both between sample groups and between days of cryopreservation.

Viable CD4+ and CD8+ cell numbers dropped below those seeded in both apheresis and cone derived cells, as culture progressed, and the effect was more pronounced in the stimulated groups than in the control and IL2 groups. This confirmed the findings of Jiao *et al*. (2019), who found significantly increased levels of apoptosis in T cells stimulated with CD3/CD28 activation beads compared to an unstimulated control group, and Planch *et al*. (2019) who reported that viable cell CD3+ cell numbers in CAR-T culture significantly decreased after 48 hours. The viability of the cells in culture remained high

in all sample groups, indicating that the missing cells had been destroyed. The cells did expand in culture, but only in the case of cone derived cells C11-16 cryopreserved on D1 (1.02 x 10^6/mL) and apheresis derived cells cryopreserved on D2 (0.973 x 10^6/mL) did the cell numbers regain or exceed those originally seeded. Cone cells cryopreserved on D2 reached a concentration of 0.855 x10^6/mL and apheresis samples cryopreserved on D3 reached a concentration of 0.752 x 10^6/mL.

The findings of the study regarding cone derived cells corroborated findings from those researchers who have looked at this effect. However, the finding that apheresis derived cells cryopreserved 24-48 hours and 48-72 hours after collection showed significantly better survival and expansion in culture than those cryopreserved <24 hours post collection was novel. It had been assumed that degradation of residual granulocytes during extended storage would adversely impact the ability of lymphocytes to respond to stimulus (Agashe *et el.* 2017), but this appeared not to be the case. Possibly the granulocytes in the apheresis products were still present in the cells cryopreserved on D1 and then were able to supress the lymphocyte response after thawing (Johnson *et al*. 2022). Stroncek *et al*. (2016) reported that the presence of contaminating myeloid cells reduced the expansion of lymphocytes in CAR-T manufacturing, which corresponds with the findings in our study. Granulocytes in the products that had been stored for a longer period may have died prior to cryopreservation (Colotta *et al.* 1992) and were therefore not able to affect the lymphocyte response. No assessment was made of residual granulocyte contamination post thaw, so it is not possible to answer this question, although this can be addressed in future work.

5.7 Chapter 5: Summary and Key Findings

1. Cone derived samples: An increased time to cryopreservation was found to have an adverse impact on the viability and expansion capability of lymphocytes derived from cone cells. Both CD4+ and CD8+ lymphocytes derived from cone cells were found to have the highest viability in culture and best cell expansion after stimulation when cryopreserved on D1. Cells cryopreserved on D2 also showed good viability and expansion and did not differ significantly from those cryopreserved on D1. Cells cryopreserved on D3 were found to have significantly lower viability and poorer expansion than those cryopreserved on D1 or D2. Expression of CD69 on stimulated cells was induced more rapidly on cells cryopreserved on D1 and D2 (approximately 40% CD69+ after 4 hours) with lower expression (approximately 30%) in cells cryopreserved on D3. Induction of CD25 did not appear to be affected by time to cryopreservation and after 72 hours incubation, the percentage of both CD4+ and CD8+ cells expressing CD25 and/or CD69 was similar for cells cryopreserved on D1, D2, and D3 (80% of CD4+ and 75% of CD8+).

2. Apheresis derived samples: Lymphocytes derived from apheresis samples behaved very differently to those derived from cone cells. Increased time to cryopreservation was not found to adversely impact viability or expansion after stimulation. Viability of both CD4+ and CD8+ cells in culture increased slightly for cells cryopreserved on D2 and D3 compared to those cryopreserved on D1 and the cells cryopreserved on D2 showed the best expansion, followed by D3. Cells cryopreserved on D1 had significantly poorer expansion than those cryopreserved on D2 or D3. Expression of CD69 on stimulated cells was induced more rapidly on cells cryopreserved on D3 (46% of CD4+ and CD8+ after 4 hours) with lowest expression (30% of CD4+ and 22% of CD8+) in cells cryopreserved on

D1. Induction of CD25 did not appear to be affected by time to cryopreservation. After 72 hours incubation, the percentage of CD4+ expressing CD25 and/or CD69 was highest for cells cryopreserved on D2 (94%) and D3 (93%) and lowest on those cryopreserved on D1 (84%). No difference was observed in activation markers on CD8+ cells cryopreserved on D1, D2 or D3.

Summary: The results from Chapter 5 clearly demonstrated that non-mobilised cone derived lymphocytes respond best to stimulation when cryopreserved within 24 hours of collection. The results from Chapter 4 also demonstrated the highest recovery of viable CD3+ cells from products cryopreserved on D1. It is therefore, recommended that these products are cryopreserved within 24 hours of collection where possible, and at the very latest within 48 hours of collection.

The findings were less clear for the mobilised apheresis derived lymphocytes, as expansion after stimulation was significantly poorer for cells cryopreserved on D1 compared to D2 and D3. However, the results from Chapter 4 demonstrated the best recovery of viable cells cryopreserved on D1 and very poor recovery of viable CD3+ cells from products cryopreserved on D3, so the recommendation is that apheresis products destined for use as DLI are cryopreserved within 48 hours of collection.

6 Chapter 6 Discussion

6.1 Overview and Study Aims

This research study examined the effect of storage time at 4°C prior to cryopreservation on the recovery and responsiveness to stimulation of viable T lymphocytes after thawing. Lymphocytes derived from non-mobilised apheresis cones and from G-CSF mobilised apheresis harvests were cryopreserved after storage for <24, 24-48 and 48-72 hours. The cryopreserved cells were then thawed out and functional assays performed *in vitro*. The study was designed to mimic as closely as possible the storage and cryopreservation protocols in place for clinical products processed within NHSBT, so that any findings could be applied to clinical practice. Donor lymphocytes for patient use are collected by apheresis either prospectively, or at need, and cryopreserved as aliquots of CD3+ cells. The cells are then stored in vapour phase nitrogen until required to treat re-emergence of disease in transplant by initiating GvL, thereby preventing full blown relapse. The products may be stored for a period of years before use, and as there is no suitable release test to assure quality, they are issued to the patient based on the numbers of viable CD3+ cells that were obtained when the fresh product was first received in the laboratory. In routine clinical practice in the Yorkshire region, for operational and staffing reasons, lymphocyte products destined for use as DLI for are frequently stored at 4°C for over 48 hours before being cryopreserved. There is evidence that prolonged cold storage of lymphocytes can adversely impact T lymphocyte recovery and function (Johnson *et al.,* 2022, Jerram *et al*. 2021), so the principal aims of the study were:

i) To determine whether local practice of extended storage at 4°C could impair the ability of lymphocytes to respond to stimuli, thereby reducing their clinical effectiveness and putting patients at increased risk of relapse.

ii) To make recommendations as to the optimum length of storage time to preserve the lymphocyte response.

Storage and handling of lymphocytes is becoming an increasingly important topic in the field of haematology/oncology as the use of CAR-T therapies to treat malignant disease increases. The starting material for all CAR-T therapies currently authorised for use in Europe and the USA are autologous lymphocytes collected by apheresis. These products have of necessity to be shipped from the collection centre to the manufacturing hub, which may be some distance away, and it is critical that they are handled in such a way as to maximise their ability to expand *in vitro* during manufacture.

The second aim of the study was to determine whether lymphocytes from surplus portions of G-CSF mobilised HSC, which are not required for transplant are suitable for use as DLI. It is routine clinical practice to cryopreserve aliquots of mobilised HSC as DLI products to save the cost of a second donor procurement. Given the known immunomodulating effects, both of G-CSF itself (Boneberg *et al.* 2002, Modi *et al*. 2020, Engelmann *et al*. 2022) and of contaminating granulocytes (Stroncek *et al*. 2016, Johnson *et al*. 2022) that are present in HSC products, there has always been a question as to the responsiveness of these lymphocytes and whether using them for DLI is a false economy.

6.2 Key Findings

6.2.1 Phenotypes of the starting cell preparations

The lymphocyte subsets of both cone and apheresis derived lymphocytes were very similar and pre-cryopreservation samples from both fell within the normal ranges

described for peripheral blood lymphocytes. However, phenotypes of individual samples were highly variable and as the sample numbers tested were limited it was difficult to draw conclusions about cellular responsiveness. When T cell subsets from the pre- and post-cryopreservation samples were compared, the samples derived from cone samples showed alterations in phenotype typical of those reported in the published literature; reduced numbers of naïve T cells, Tregs and a reduced CD4:CD8 ratio (Jerram *et al*. 2021, Li *et al.* 2022, Florek *et al*. 2015, Tompa *et al.* 2018) while those from apheresis derived samples appeared completely unaffected by the process. This was an unexpected finding which has not previously been reported and further study with a larger sample group is required to confirm it.

6.2.2 CD3+ cell viability and recovery post-thaw

The CD3+ viability and recovery post-thaw was significantly affected by time to cryopreservation in both cone and apheresis derived samples. Cells cryopreserved within 24 hours of collection (D1) contained the highest numbers of viable cells when thawed, and cells that had been held for over 48 hours (D3) prior to cryopreservation fared extremely badly. Only 15% of the viable T cells originally cryopreserved could be detected in thawed apheresis derived cells cryopreserved on D3. The recovery of viable T cells from the G-CSF mobilised apheresis group was consistently poorer than that from the non-mobilised cone group but this finding was significant only for cells cryopreserved on D3. The reduction in recovery and viability seen with increased time in storage corroborated the findings of other researchers that T lymphocyte viability, recovery and responsiveness can be reduced by storage at 4°C for more than a few hours (Bull *et al*. 2007, Kierstead *et al.* 2007, Olsson *et al*. 2011, Fisher *et al.* 2014, Jerram *et al*. 2021, Johnson *et al.* 2022).

Sample numbers in the study were small and the overall results could have been influenced by the poor performance of individual samples, but the trend was clear for all samples in both study groups.

6.2.3 T cell response to stimulus after thaw

Substantial T cell death was found when cells were seeded into culture, and a gradual decline in numbers of viable CD4+ and CD8+ cells was seen in non-mobilised cone derived cells until they had reached 72 hours in culture at which point significant expansion of both CD4+ and CD8+ cells could be seen in samples cryopreserved on D1 and D2 only. Cone derived CD4+ and CD8+ cells cryopreserved on D3 did not expand. Similarly, there was evidence of CD4+ and CD8+ cell death in samples derived from G-CSF mobilised apheresis derived samples which gradually worsened with time in culture up to the 72-hour point, at which evidence of expansion was seen. Unlike the cone derived cells, no expansion was seen in cells cryopreserved on D1, with the best result seen in those cells cryopreserved on D2, followed by those cryopreserved on D3. Both CD4+ and CD8+ lymphocytes from mobilised and non-mobilised sources responded well to stimulus with CD3/28 activation beads. No significant differences were seen between the different sample groups or between samples cryopreserved on different days.

6.2.4 Conclusions

Although surviving T cells in culture from both mobilised apheresis and non-mobilised cone samples demonstrated no impairment in upregulation of activation markers related to pre-cryopreservation storage time, post thaw recoveries of viable CD3+ cells and their ability to expand in culture were significantly affected.

Non-mobilised cone derived cells clearly showed both poorer recovery and response when measured by CD3+ cell expansion as pre-cryopreservation storage time increased.

Cells cryopreserved within 24 hours of collection showed good recovery of viable CD3+ on thawing (77%) and satisfactory expansion in culture with maximum viable CD3+ cell numbers reaching 1.02 x 10^6/mL. Non-mobilised cells cryopreserved 24-48 hours after collection showed acceptable viable CD3+ recovery (67%) and some expansion in culture; maximum viable CD3+ cell number reaching 0.855 x 10^6/mL. Cells cryopreserved after 48 hours storage had the poorest viable CD3+ recovery (57%) and did not expand in culture. The study findings clearly showed that cell numbers and function of T cells from non-mobilised products were best preserved when cryopreserved within 24 hours of collection, but that acceptable cell numbers and function were maintained for up to 48 hours.

Recovery of viable CD3+ cells from G-CSF mobilised apheresis derived cells was poorer than that from cone cells and worsened significantly as time to cryopreservation increased. Cells cryopreserved within 24 hours of collection were found to have 57% recovery of viable CD3+ cells after thaw, but they did not expand in culture: maximum viable CD3+ cell number reaching only 0.475 x 10^6/mL. In contrast, cells cryopreserved 24-48 hours after collection had poor CD3+ cell recovery after thaw (35%) but expanded well in culture; maximum viable CD3+ cell number reaching 0.973 x 10^6/mL. Cells cryopreserved >48 hours after collection had a very poor viable CD3+ cell recovery (15%), although they did expand in culture; maximum viable CD3+ cell number reaching 0.752 x 10^6/mL. The study findings demonstrated a trade-off between recovery and expansion of viable CD3+ cells from mobilised products cryopreserved <24 hours and 24-48 hours post collection. As a result, storage times of up to 48 hours may adequately preserve T cell numbers and function in this group, but the finding requires further investigation to confirm it.

6.3 Limitations of the study

The study was performed in a busy operational laboratory which had just been equipped with a new flow cytometer. The operator was relatively inexperienced in the use of this instrument which resulted in programming errors. As a result, the first ten cone samples were poorly seeded, and the initial T cell subset panel was incorrectly performed on the first seven cone samples. Availability of source cellular materials were limited, and insufficient cell numbers were available to cryopreserve more than one bag per day per sample. This meant that in the event of an error thawing or seeding cells from a bag, that there was no option to repeat that part of the study. In addition, the study design was such that it took approximately three weeks to gather all the data from one sample, which left little time to obtain further samples in the event of a failure. As a result, the potentially inaccurate results generated from the first ten cone samples, combined with the highly individual responses seen in all data sets meant that further study will be required to confirm the findings from the stimulation and expansion study. Improved training and better allocation of time to perform the analysis will enable better management of future work.

The primary investigative tool used in the study was flow cytometry. One of the limitations associated with this technology is that is that it provides information about cell phenotype but cannot provide information about the functionality of the cells. The study investigated upregulation of CD25 and CD69 as these are the two most cited activation markers (Motamedi *et al.,* 2016, Schwab *et al.,* 2019). Due to budgetary and time constraints, cytokine release assays or CFSE assays, which have been used by other researchers to provide conclusive evidence of cellular function post cryopreservation

and thawing (Li et al. 2022, Boudreaux et al 2019, Juhl *et al.,* 2021), were not performed here.

Cell viability in this study was assessed by dye exclusion staining with 7-AAD, which differentiates between those cells which have sustained serious membrane damage from those that have not. 7-AAD cannot detect those cells that have intact membranes but are apoptotic (Duggleby *et al.* 2012). One of the key measures used throughout was the absolute cell number/mL of viable CD3+, CD4+ and CD8+ cells. It is therefore highly likely that some of the cells classified as 'viable' by dye exclusion were apoptotic, and as such the viable cell numbers in this study were overreported.

The study investigated the recovery and response of CD3+ cells after storage and cryopreservation but did not investigate the effect of the 4°C storage period itself. Cell recoveries and stimulation assays were performed on the prepared cells prior to storage but not repeated each day prior to cryopreservation. As a result, it is not known what, in any, deficit may have already been present when the cells were cryopreserved.

The study aimed, as far as possible to replicate cryopreservation and storage conditions for real clinical products. However, the fact remains that in the case of cone derived lymphocytes, the cells used were similar but not the same as MNCs collected by apheresis for use either as DLI or CAR-T starting materials. The observed phenotypes of the cone derived cells after density gradient separation indicated that they were in every respect identical to those that are collected by apheresis, but in the absence of functional assays and a suitable comparator group this cannot be assumed.

The study was small, and a wide range of CD3+ recovery results were seen in between the individual samples. This is a finding seen in other studies, particularly those on G-

CSF mobilised apheresis products, and it has been suggested that it is related to donor factors (Fisher *et al.* 2014, Schafer *et al.* 2020) but it makes it difficult to draw robust conclusions from a study of this size. Further study on larger sample group is required to confirm the findings.

Finally, the study was performed *in-vitro* using artificial means of stimulation and it is not possible to speculate whether the lymphocyte response to stimulation would be the same *in-vivo.* Evidence from CAR-T manufacturing suggests that although cryopreservation accelerates apoptosis and cell death in culture, the persistence and clinical effectiveness of the manufactured cells *in vivo* is not affected (Panch *et al.* 2019). Further study using clinical samples and patient follow up data would be required.

6.4 Recommendations for future research and changes in practice

6.4.1 Cryoprotectant formulation

The cells used in this study were cryopreserved in 4.5% HAS and 10% DMSO following standard NHSBT cryopreservation protocols which were originally designed and validated to maximise recovery of CD34+ cells. However, there is considerable evidence that 10% DMSO is not the best cryoprotectant for lymphocytes, and that both cell recovery and functionality is better conserved if the DMSO concentration is reduced to 5% (Worsham *et al.,* 2017, Fisher *et al.,* 2014, Abzari *et al.,* 2019). Several other studies reporting adverse impact on lymphocyte recovery or functionality post-thaw, also used 10% DMSO as a cryoprotectant (Ford *et al.* 2017, Schafer *et al*. 2020). It has been postulated that high DMSO concentration may selectively deplete alloreactive T cells which could explain the higher relapse rates seen by some groups in cryopreserved HSCT during the Covid era (Guo *et al*. 2023). There are equally some studies reporting either no impact of cryopreservation in 10% DMSO (Pi *et al*. 2020) or a positive benefit (Juhl *et*

al. 2021). However, it is known that DMSO can cause side effects in recipients and is toxic to thawed cells if they are maintained at temperatures above freezing for any length of time (De Abreu Costa *et al.* 2017) and as a result, NHSBT has been considering reducing the DMSO percentage in cryoprotectant medium from 10% to 5%. The very poor recovery of viable CD3+ cells found here, particularly in samples that had been stored for longer periods clearly indicates that further study into formulation of cryoprotectant media used for lymphocytes is urgently required.

6.4.2 Functional assays

The assay used in this study was intended to be a simple method to assess lymphocyte function. Although the assay studied provided information about expression of activation cell markers on the T cells surviving in culture, it was not able to discriminate between samples cryopreserved on different days, despite the heavy cell losses seen when viable CD3+ cells were calculated post-thaw. It is therefore recommended that NHSBT investigate additional assays that may be able to detect the numbers of functionally impaired or apoptotic cells both pre-cryopreservation and post-thaw. The ideal assay for this purpose is one that can be performed in <24 hours, so that the laboratory can decide about the fate of a product before it is either issued to a patient or has started an expensive manufacturing run. STAT-5 phosphorylation (Bitar *et al*. 2019) and Annexin V assays (Duggleby *et al.,* 2012) should be investigated as more sensitive detection methods for apoptosis than the dye exclusion assays currently in use. CFSE expansion and cytokine secretion assays can then be combined with cell surface activation markers to provide a more comprehensive view of functional capability.

6.4.3 Storage conditions

Lymphocytes are stored and shipped at 4°C largely because this is the temperature used for HSC products for transplant. However, there is a body of evidence to show that lymphocyte function can be impaired by storage at 4°C and that for optimal recovery, storage at RT or above is recommended (Johnson *et al*., 2022, Jerram *et al.,* 2021). NHSBT should therefore investigate the functionality and recovery of lymphocytes stored at different temperatures both pre-and post-cryopreservation using the assays described in section 6.4.2, as it is quite probable that storage conditions suitable for HSC products are unsuitable for lymphocyte products.

Finally, NHSBT operating procedures for thawing cells for CFU-GM and viability assays specify rapid thawing and dropwise resuspension of cells in 4.5% HAS at 4°C. As all publications about frozen PBMC thawing recommend thawing into warmed media (Disis *et al*. 2006, Honge *et al.* 2017 Li *et al*. 2022, Baboo *et al.* 2019). NHSBT will need to consider investigating the appropriateness of this technique both for HSC samples and for lymphocyte samples. As the release criteria for issue of a thawed product within NHSBT is a viability of 70% and viable cell recovery is not calculated, it is possible that poorer thawed viabilities/recoveries resulting from defective technique have had no clinical impact and have therefore passed unnoticed. In any case, in clinical practice, thawed viability testing is almost exclusively performed on CD34+ cells, not lymphocytes so there is little internal data. Further study is required to confirm whether a change to procedure is required.

The results of this study, particularly for apheresis derived samples, showed wide individual variation and further samples should be tested to confirm their reproducibility. In addition, it may be possible to test samples from existing stored

products if these are no longer required for clinical use. As time from collection to cryopreservation is documented in the product records, it will be possible to categorise these samples by length of time to cryopreservation and compare the responses of the cells to stimulation on this basis. This additional work will serve the dual purpose of expanding the data from the existing study and confirming the accuracy (or not) of the reported cell doses sent to clinicians.

In summary, extended storage beyond 48 hours at 4°C impairs the ability of T lymphocytes to respond to stimuli, thereby reducing their clinical effectiveness and putting patients at increased risk of relapse. This study, although small, suggests that the optimum length of storage time to preserve the lymphocyte response is <48 hours and ideally <24hours, particularly if the cells are from non-mobilised collections.

In practice cryopreservation of DLIs as soon as they are received in the laboratory could present a serious staffing problem. Ensuring that DLI products received in routine working hours are cryopreserved as soon as they arrive is achievable, and staff will be made aware of the potential impact of not doing so. However, as discussed in Chapter 1, allogeneic products for transplant are often received out of routine working hours at NHSBT Barnsley and are therefore stored at 4°C until the morning when staff are available to cryopreserve them. Under the current operating model, if immediate cryopreservation was required, this work would fall to the single on-call member of staff. Cryopreservation of these products is a complex process requiring two members of staff, so it is currently not possible to delegate the entire process to the on-call operator. Increasing out of hours staffing to cover this activity will be an unpopular move with

those who will be required to participate in it as well as the cost implications and thus should not be undertaken without careful review of the potential benefits.

7 References

Abazari, A., Stevens, S. D., B.J. Hawkins, B. J., Mathew , A., Castelli, L. (2019). Process optimization for CD4+ and CD8+ T cell formulation and cryopreservation. *Cytotherapy*, **21** (5), S21. https://doi.org/10.1016/j.jcyt.2019.03.383

de Abreu Costa, L., Henrique Fernandes Ottoni, M., Dos Santos, M.G., Meireles, A.B., Gomes de Almeida, V., de Fátima Pereira, W., Alves de Avelar-Freitas, B., Eustáquio Alvim Brito-Melo, G. (2017). Dimethyl Sulfoxide (DMSO) Decreases Cell Proliferation and TNF-α, IFN-γ, and IL-2 Cytokines Production in Cultures of Peripheral Blood Lymphocytes. *Molecules*, **22** (11), 1789. doi: 10.3390/molecules22111789. PMID: 29125561; PMCID: PMC6150313.

Adan, A., Alizada, G., Kiraz, Y., Bara, N. Y., Nalban, T. A. (2017). Flow cytometry: basic principles and applications. *Crit Rev Biotechnol*. **37**(2):163-176. doi: 10.3109/07388551.2015.1128876. Epub 2016 Jan 14. PMID: 26767547.

Agashe, C., Chiang, D., Grishin, A., Masilamani, M., Jones, S.M., Wood, R.A., Sicherer, S.H., Burks, A.W., Leung, D.Y.M., Dawson, P., Sampson, H.A., Berin, M.C. (2017). Impact of granulocyte contamination on PBMC integrity of shipped blood samples: Implications for multi-center studies monitoring regulatory T cells. *J Immunol Methods*, **449**,23-27. doi: 10.1016/j.jim.2017.06.004. Epub 2017 Jun 16. PMID: 28629732; PMCID: PMC5573627.

Alencar, S., Garnica, M., Luiz, R., Nogueira, C., Borojevic, R., Maiolino, A., Dutra, H. (2010).

Cryopreservation of peripheral blood stem cell: the influence of cell concentration on cellular and hematopoietic recovery. *Transfusion*, **50** (11), 2402-2412. https://doi.org/10.1111/j.1537-2995.2010.02743.x

Anderson, J., Toh, Z-Q., Reitsma, A., Do, L., Nathanielsz, J., Licciardi, P. (2019). Effect of peripheral blood mononuclear cell cryopreservation on innate and adaptive immune responses. *Journal of Immunological Methods*, **465**, 61-66, ISSN 0022-1759. https://doi.org/10.1016/j.jim.2018.11.006.

Anasetti, C., Logan, B.R., Lee, S. J., Waller, E. K., Weisdorf, D.J., Wingard, J. R., Cutler, C. S.,Westervelt, P., Woolfrey, A.,Couban, S., Ehninger, G., Johnston, L., Maziarz, R.T., Pulsipher, M. A., Porter, D. L., Mineishi, S., McCarty, J. M., Khan, S. P., Anderlini, P., Bensinger, W.I., Leitman, S.F., Rowley, S.D., Bredeson, C., Carter, S.L., Horowitz, M. M., Confer, D.L. (2012). Peripheral-Blood Stem Cells versus Bone Marrow from Unrelated Donors. *New England Journal of Medicine,* **367** (16), 1487-1496. doi: 10.1056/NEJMoa1203517 https://doi.org/10.1056/NEJMoa1203517

Baboo, J., Kilbride, P., Delahaye, M., Milne, S., Fonseca, F., Blanco, M., Meneghel, J., Nancekievill, A., Gaddum, N., Morris, G.J. (2019). The Impact of Varying Cooling and Thawing Rates on the Quality of Cryopreserved Human Peripheral Blood T Cells. *Sci Rep*., **9** (1):3417. doi: 10.1038/s41598-019-39957-x. PMID: 30833714; PMCID: PMC6399228.

Bachireddy, P., Hainz, U., Rooney, M., et al. (2014). Reversal of in situ T-cell exhaustion during effective human antileukemia responses to donor lymphocyte infusion. *Blood*, **123**, (9), 1412-1421.

Bader, P., Niethammer, D., Willasch, A. Kreyenberg, H., Klingebiel T. (2005). How and when should we monitor chimerism after allogeneic stem cell transplantation? *Bone Marrow Transplant* **35**, 107–119. https://doi.org/10.1038/sj.bmt.1704715

Bajnok, A., Ivanova, M., Rigó, J., Jr, & Toldi, G. (2017). The Distribution of Activation Markers and Selectins on Peripheral T Lymphocytes in Preeclampsia. *Mediators of inflammation*, 8045161. https://doi.org/10.1155/2017/8045161

Barrett, J., Craddock, C. (2020). Bone marrow transplantation in the United Kingdom – past, present and future. *British Journal of Haematology,* **191** (4) 612-616. https://doi.org/10.1111/bjh.1715

Bendall, I., Bradstock, K., (2014) G-CSF: From granulopoietic stimulant to bone marrow stem cell mobilizing agent. Cytokine & Growth Factor Reviews: 25 (4), 355-367. https://doi.org/10.1016/j.cytogfr.2014.07.011

Benjamini Y, Hochberg Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Statist Soc B*. **57**(1):289–300.

Bertaina, A., Andrani, M. (2018). Major Histocompatibility Complex and Hematopoietic Stem Cell Transplantation: Beyond the Classical HLA Polymorphism. *Int J Mol Sci*., **19**(2):621. doi: 10.3390/ijms19020621. PMID: 29470425; PMCID: PMC5855843.

Bitar, M., Boldt, A., Freitag, M-T., Gruhn, B., Kohl, U., Sack, U. (2019). Evaluating STAT5 phosphorylation as a mean to assess T cell proliferation. *Front. Immunol*., **10** – 2019. https://doi.org/10.3389/fimmu.2019.00722

Blache, U., Weiss, R., Boldt, A., et al. (2021). Advanced flow cytometry assays for immune monitoring of CAR-T cell applications. *Front. Immunol*. **12.** DOI=10.3389/fimmu.2021.658314.

Boneberg, E.M., Hartung, T. (2002). Molecular aspects of anti-inflammatory action of G-CSF. *Inflamm. res*. **51**, 119–128.https://doi.org/10.1007/PL00000283

Bortin, M.M., Horowitz, M.M., Gale, R.P., Barrett, A.J., Champlin, R.E., Dicke, K.A., Gluckman, E., Kolb, H.J., Marmont, A.M., Mrsic, M., et al. (1992). Changing trends in allogeneic bone marrow transplantation for leukemia in the 1980s. *JAMA,* **268**(5):607-12. PMID: 1321298.

Boudreaux, C., Carli, C., Lamarche, C., (2019). Leukoreduction system chambers are a reliable source for the manufacturing of T-cell therapeutics. *Transfusion*, **59**, 1300-1311.

Bourguignon, P., Clément, F., Renaud, F.,Le Bras, V., Koutsoukos, M., Burny, W., Moris, P., Lorin, C., Collard, A., Leroux-Roels, G., Roman, F., Janssens, M.,Vandekerckhove, L. (2014). Processing of blood samples influences PBMC viability and outcome of cell-mediated immune responses in antiretroviral therapy-naïve HIV-1-infected patients. *Journal of immunological methods*, **414**, 1-10.

Buhl, T., Legler, T. J., Rosenberger, A., Schardt, A., Schön, M. P., Haenssle, H.A. (2012). Controlled-rate freezer cryopreservation of highly concentrated peripheral blood mononuclear cells results in higher cell yields and superior autologous T-cell stimulation for dendritic cell-based immunotherapy. *Cancer Immunol Immunother*, **61**(11):2021-31. doi: 10.1007/s00262-012-1262-0. Epub 2012 Apr 22. PMID: 22527251; PMCID: PMC3493671.

Bull, M., Lee, D., Stucky, J., Chiu, Y. L., Rubin, A., Horton, H., McElrath, M.J. (2007). Defining blood processing parameters for optimal detection of cryopreserved antigen-specific responses for HIV vaccine trials. *Journal of immunological methods,* **322** (1–2), 57-69. doi: 10.1016/j.jim.2007.02.003. Epub 2007 Feb 28. PMID: 17382342; PMCID: PMC1904432

Chang, Y-J., Zhaou, Y-X., Huo, M-R., Huang, X-J. (2009). Expression profiles of adhesion molecules on naïve T cells in bone marrow grafts of healthy donors treated with granulocyte colony-stimulating factor. *Transplant immunology,* **21** (4), 228-233.

Chiva-Blanch, G., Giménez-Bonafe, P., Llaudó, I., Torras, J., Cruzado, J. M., Pétriz, J., Castaño, E., Franquesa, M., Tortosa, A., Herrero-Fresneda, I., Rama, I., Bestard, O., Grinyó, J. M., & LLoberas, N. (2009). Different storing and processing conditions of human lymphocytes do not alter P-glycoprotein rhodamine 123 efflux. *Journal of pharmacy and pharmaceutical science*, **12***,* (3), 357-366.

Cibrián, D., Sánchez-Madrid, F., (2017). CD69: from activation marker to metabolic gatekeeper. *Eur J Immunol*, **47**(6):946-953. doi:10.1002/eji.201646837.

Choi, J., Lee, S., J., Lee, Y., A., Maeng, H., G., Lee, J., K., Kang, Y., W. (2014). Reference values for peripheral blood lymphocyte subsets in a healthy Korean population. *Immune Netw*. **14**(6), 289-95. doi: 10.4110/in.2014.14.6.289. Epub 2014 Dec 22. PMID: 25550695; PMCID: PMC4275386.

Colotta, F., Re, F., Polentarutti, N., Sozzani, S., Mantovani, A. (1992). Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. *Blood*, **80**(8), 2012-20. PMID: 1382715.

Courville, E.L., Lawrence, M. G. (2021). Characteristic CD45RA/CD45RO maturation pattern by flow cytometry associated with the CD45 C77G polymorphism. *Cytometry*, **100**, 602– 605. https://doi.org/10.1002/cyto.b.21993

Davis, O., Blunk, B., Loos, T., Cox, T. (2016). Analysis of Spectra Optia MNC procedures: reviewing data to improve process performance. *Transplantation and Cellular Therapy*, **22** (3), S275. DOI: https://doi.org/10.1016/j.bbmt.2015.11.716

Disis, M. L., dela Rosa, C., Goodell, V., Kuan, L. Y., Chang, J. C., Kuus-Reichel, K., Clay, T. M., Kim Lyerly, H., Bhatia, S., Ghanekar, S. A., Maino, V.C., Maecker, H. T. (2006). Maximizing the retention of antigen specific lymphocyte function after cryopreservation. *J Immunol Methods,* **308**(1-2):13-8. doi: 10.1016/j.jim.2005.09.011. Epub 2005 Oct 21. PMID: 16337957.

Duggleby, R., Querol, S., Davy, R., et al. (2012). Flow cytometric assessment of apoptotic CD34+ cells by Annexin V labelling may improve prediction of cord blood potency for engraftment. *Transfusion*, **52**, 549-559.

Dunham, R. M., Cervasi, B., Brenchley, J. M., Albrecht, H., Weintrob, A., Sumpter, B., Engram, J., Gordon, S., Klatt, N. R., Frank, I., Sodora, D. L., Douek, D. C., Paiardini, M., Silvestri, G. (2008). CD127 and CD25 expression defines CD4+ T cell subsets that are differentially depleted during HIV infection. *J. Immunol.***180** (8), 5582-92. doi: 10.4049/jimmunol.180.8.5582. PMID: 18390743; PMCID: PMC2683429.

Dunnick, K., M., DeOca, K., Menezes, A., Ly, N., Sharma, A. (2022). Whitepaper_The-Freshness-of-Cryopreserved-PBMCs_Lonza.

Eefting, M., von dem Borne, P., de Wreede, L., et al. (2014) Intentional donor lymphocyte-induced limited graft versus host disease is essential for long-term survival of relapsed acute myeloid leukaemia after allogeneic stem cell transplantation. *Haemtologica*, **99** (4,) 751-758

Engelmann, C., Berg, T. (2022). G-CSF treatment in decompensated liver disease: a double-edged sword? *Hepatol Int* **16**, 979–982. https://doi.org/10.1007/s12072-022-10379-8

Fisher, V., Khuu, H., David-Ocampo, V., Byrne, K., Pavletic, S., Bishop, M., Fowler, D.H., Barrett, A.J., Stroncek, D.F. (2014). Analysis of the recovery of cryopreserved and thawed CD34+ and CD3+ cells collected for hematopoietic transplantation. *Transfusion*, **54** (4),1088-92. doi: 10.1111/trf.12428. Epub 2013 Oct 10. PMID: 24117879; PMCID: PMC3983171.

Florek, M., Schneidawind, D., Pierini, A., Baker, J., Armstrong, R., Pan, Y., Leveson-Gower, D., Negrin, R., Meyer, E. (2015). Freeze and Thaw of CD4+CD25+Foxp3+ Regulatory T Cells Results in Loss of CD62L Expression and a Reduced Capacity to Protect against Graft-versus-Host Disease. *PLoS One*, **10** (12): e0145763. doi: 10.1371/journal.pone.0145763. PMID: 26693907; PMCID: PMC4691201.

Ford, T., Wenden, C., Mbekeani, A., Dally, L., Cox, J.H., Morin, M., Winstone, N., Hill, A.V.S., Gilmour, J., Ewer, K. J. (2017) Cryopreservation-related loss of antigen-specific IFNγ producing CD4+ T-cells can skew immunogenicity data in vaccine trials: Lessons from a malaria vaccine trial substudy. *Vaccine*, **35**(15):1898-1906. doi: 10.1016/j.vaccine.2017.02.038. Epub 2017 Mar 9. PMID: 28285985; PMCID: PMC5387668.

Foster, A., Marangelo, M., Sartor, M., Alexander, S., Hu, M., Bradstock, K., Gottlieb, D. (2004). Human CD62L- memory T cells are less responsive to alloantigen stimulation than CD62L+ naïve T cells: potential for adoptive immunotherapy and allodepletion. *Blood*, **104** (8), 2403-2409.

García-Piñeres, A.J., Hildesheim, A., Williams, M., Trivett, M., Strobl, S., Pinto, L. A. (2006). DNAse treatment following thawing of cryopreserved PBMC is a procedure suitable for lymphocyte functional studies. *Journal of immunological methods,* **313** (1-2), 209-213.

Garcia-Prat, M., Álvarez-Sierra, D., Aguiló-Cucurull, A., Salgado-Perandrés, S., Sara Briongos-Sebastian, S., Franco-Jarava,C., Martin-Nalda, A., Colobran,R., Montserrat, I., Hernandez-Gonzales, M., Pujol-Borell, R., Soler-Palacin, P., Martiez-Gallo, M. (2019). Extended immunophenotyping reference values in a healthy pediatric population. *Cytometry Part B*, **96** (3) 223-233. https://doi.org/10.1002/cyto.b.21728

Garibay-Escobar, A., et al. (2003). Integrated measurements by flow cytometry of the cytokines IL-2, IFN-γ, IL-12, TNF-α and functional evaluation of their receptors in human blood. *Journal of immunological methods,* **280** (1-2), 73-88.

Gatti, R., Meuwissen, H., Allen, H.,Hong, R. (1968). Good immunological reconstitution of sex-linked lymphopenic immunological deficiency. *Lancet*, **292**, 1366-1369.

Germann, A., Oh, Y-J.,Schmidt, T.,Schön, U., Zimmermann, H., von Briesenet, H. (2013). Temperature fluctuations during temperature cryopreservation reduce PBMC recovery, viability and T-cell function. *Cryobiology*, **67,** 193-2000.

Gniadek, T.J., Garritsen, H.S.P., Stroncek, D., Szczepiorkowski, Z.M., McKenna, D.H. (2017). Biomedical Excellence for Safer Transfusion (BEST) Collaborative. Optimal Storage Conditions for Apheresis Research (OSCAR): a Biomedical Excellence for Safer Transfusion (BEST) Collaborative study. *Transfusion,* **58** (2), 461-469. doi: 10.1111/trf.14429.

Grievink, H., Luisman, T., Kluft, C., Moeland, M., Malone, K. (2016). Comparison of three isolation techniques for human peripheral blood mononuclear cells: cell recovery and viability, population, composition, and cell functionality. *Biopreservation and Biobanking*, **14** (5), 410-415. http://doi.org/10.1089/bio.2015.0104

Guo, M., Liu, J., Clark, P. et al. (2023). Cryopreserved versus fresh peripheral blood allogeneic stem cell transplantation outcomes in patients receiving post-transplant cyclophosphamide for graft-versus-host prophylaxis during the COVID-19 pandemic: a single center experience. *Int J Hematol* **117**, 428–437. https://doi.org/10.1007/s12185-022-03493-8

Herklotz, R., Huber, A. R. (2001). Precision and accuracy of the leukocyte differential on the Sysmex XE2100. *Sysmex Journal International*, **11** (1), 8-21.

Hamadani, M., Zhang, M-J., Tang, X-Y., Fei, M., Brunstein, C., Chhabra, S., D'Souza, A., Milano, F., Phelan, R., Saber, W., Shaw, B. E., Weisdorf, D., Devine, S. M., M. Horowitz, M. M. (2020). Graft Cryopreservation Does Not Impact Overall Survival after Allogeneic Hematopoietic Cell Transplantation Using Post-Transplantation Cyclophosphamide for Graft-versus-Host Disease Prophylaxis. Biology of Blood and Marrow Transplantation, **26**, (7), 1312-1317. ISSN 1083-8791, https://doi.org/10.1016/j.bbmt.2020.04.001.

Hønge, B.L., Petersen, M.S., Olesen, R., Møller, B.K., Erikstrup, C. (2017) Optimizing recovery of frozen human peripheral blood mononuclear cells for flow cytometry. *PLoS ONE* **12**(11): e0187440. https://doi.org/10.1371/journal.pone.0187440

Huang, J., Ehrnfeldt, C., Paulie, S., Zuber, B., Ahlborg, S. (2015). ELISpot and ELISA analyses of human IL-21-secreting cells: Impact of blocking IL-21 interaction with cellular receptors. *Journal of immunological methods,* **417** (0), 60-66.

Hunt, C., J. (2019). Technical Considerations in the Freezing, Low-Temperature Storage and Thawing of Stem Cells for Cellular Therapies. *Transfus Med Hemother*., **46** (3),134-150. doi: 10.1159/000497289. Epub 2019 Mar 28. PMID: 31244583; PMCID: PMC6558338.

Itonaga, H., Tsushima, H., Taguchi, J., et al. (2013). Treatment of relapsed adult T-cell leukaemia after allogeneic haematopoietic stem cell transplantation: the Nagasaki Transplant Group experience. *Blood*, **121** (1), 219-225.

Jerram, A., Guy, T.V., Beutler, L., Gunasegaran, B, Sluyter, R., Fazekas, de St., Groth, B., McGuire, H.M. (2021). Effects of storage time and temperature on highly multiparametric flow analysis of peripheral blood samples; implications for clinical trial samples. *Biosci Rep*., **41** (2): BSR20203827. doi: 10.1042/BSR20203827. PMID: 33600563; PMCID: PMC7921292.

Jiao, J., Zhao, X., Hou, R., et al. (2019). Comparison of two commonly used methods for stimulating T cells. *Biotechnol Lett*. Dec; **41**(12):1361-1371. doi: 10.1007/s10529-019-02743-w. Epub 2019 Oct 21. PMID: 31631231.

Johnson, R.K., Overlee, B.L., Sagen, J.A. *et al.* (2022). Peripheral blood mononuclear cell phenotype and function are maintained after overnight shipping of whole blood. *Sci Rep* **12**, 19920. https://doi.org/10.1038/s41598-022-24550-6

Joshii, S., Lynch, J., Pavletic, S., et al. (2001). Decreased immune functions of blood cells following mobilisation with granulocyte colony stimulating factor: association with donor characteristics. *Blood*, **98**, 1963-1970.

Juhl, M., Christensen, J., Pedersen, A., Kastrup, J., Ekblond, A. (2021). Cryopreservation of peripheral blood mononuclear cells for use in proliferation assays: First step towards potency assays. *J. Immunol. Meth*. **244**, 112897. https://doi.org/10.1016/j.jim.2020.112897.

Kadota, R., Koda, M., Kawabe, J., Hashimoto, M., Nishio, Y., Mannoji, C., Miyashita, T., Furuya, T., Okawa, A., Takahashi, K., Yamazaki, M. (2012). Granulocyte Colony-Stimulating Factor (G-CSF) Protects Oligpdendrocyte and Promotes Hindlimb Functional Recovery after Spinal Cord Injury in Rats. *PLoS ONE* **7**(11): e50391. https://doi.org/10.1371/journal.pone.0050391

Kapellos, T. S., Bonaguro, L., Gemünd, I., Reusch, N., Saglam, A., Hinkley, E. R., Schultze, J. L. (2019). Human Monocyte Subsets and Phenotypes in Major Chronic Inflammatory Diseases. *Frontiers in Immunology*, **10** 1664-3224. https://www.frontiersin.org/articles/10.3389/fimmu.2019.02035.

Keane, K., Calton, E., Cruzat, V., et al. (2015). The impact of cryopreservation on human peripheral blood leucocyte bioenergetics. *Clinical science*, **128** (10), 723-733.

Kierstead, L. S., Dubey, S., Meyer, B., Tobery, T. W., Mogg, R., Fernandez, V.R., Long, R., Guan, L., Gaunt, C., Collins, K., Sykes, K.J., Mehrotra, D.V., Chirmule, N., Shiver, J.W., Casimiro, D.R. (2007). Enhanced rates and magnitude of immune responses detected against an HIV vaccine: effect of using an optimised process for isolating PBMC. *AIDS research and human retroviruses*, **23**, (1), 86-92.

Kim-Wanner, S.Z., Bug, G., Steinmann, J. *et al.* (2017). Erythrocyte depletion from bone marrow: performance evaluation after 50 clinical-scale depletions with Spectra Optia BMC. *J Transl Med* **15**, 174 (2017). https://doi.org/10.1186/s12967-017-1277-6

Klinkmann, G., Doss, F., Goudeva, L., Doss, S., Blasczyk, R., Milej, M., Koch, S., Mitzner, S., Altrichter, J. (2021) Prolonged storage of purified granulocyte concentrates: Introduction of a new purification method. *Transfusion*, **62** (1) 194-204. https://doi.org/10.1111/trf.16732

Kolb, H. (2015). Graft versus leukaemia effects of transplantation and donor lymphocytes. *Blood*, **112** (12) 4371-4383.

Korbling, M., Anderlini, P. (2001). Peripheral blood stem cell versus bone marrow allotransplantation: does the source of hematopoietic stem cells matter? *Blood*, **98** (10), 2900–2908. https://doi.org/10.1182/blood.V98.10.2900

Korngold, R., Sprent, J. (1978). Lethal graft-versus-host disease after bone marrow transplantation across minor histocompatibility barriers in mice. Prevention by removing mature T cells from marrow. *J Exp Med*., **148**:1687–98. 10.1084/jem.148.6.1687.

Koryakina, A., Frey, E. and Bruegger, P. (2014). Cryopreservation of human monocytes for pharmacopeial monocyte activation test. *Journal of immunological methods,* **405,** 181-191.

Kumar, S., Meneghal, J. (2022). Optimising and standardising the cryopreservation of cellular samples: new data and insights from the UKs advanced therapy treatment centres network programme. *Cell and Gene Therapy Insights*, **8** (6): 967-976. DOI :10.18609/cgti.2022.144

Lambert, C., Yanikkaya, D. G., Keller, T., Preijers, F., Psarra, K., Schiemann, M., Özçürümez, M., Sack, U. (2020). Flow Cytometric Analyses of Lymphocyte Markers in Immune Oncology: A Comprehensive Guidance for Validation Practice According to Laws and Standards. *Frontiers in Immunology*. **11**. DOI=10.3389/fimmu.2020.02169

Li, B., Yang, C., Jia, G. *et al.,* (2022). Comprehensive evaluation of the effects of long-term cryopreservation on peripheral blood mononuclear cells using flow cytometry. *BMC Immunol.* **23**, (30). https://doi.org/10.1186/s12865-022-00505-4

Little, A.M., Akbarzad-Yousefi, A., Anand, A., Diaz Burlinson, N., Dunn, P.P.J., Evseeva, I., Latham, K., Poulton, K., Railton, D., Vivers, S., Wright, P.A. (2021). BSHI guideline: HLA matching and donor selection for haematopoietic progenitor cell transplantation. *Int J Immunogenet*. ;**48** (2):75-109. doi: 10.1111/iji.12527. Epub 2021 Feb 10. PMID: 33565720.

Locatelli, F., Lucarelli, B., Merli, P. (2014). Current and future approaches to treat graft failure after allogeneic hematopoietic stem cell transplantation. *Expert Opin Pharmacother*.,**15**,23–36.

López-Pereira, P., Sola, A., Vicuña A., Cámara Montejano, C., Muñoz, C., Alegre, A., Aguado, B. (2020). Retrospective comparison between COBE SPECTRA and SPECTRA OPTIA apheresis systems for hematopoietic progenitor cells collection for autologous and allogeneic transplantation in a single center. *J Clin Apher*. **35**(5):453-459. doi: 10.1002/jca.21826. Epub 2020 Aug 15. PMID: 32798328.

Lyons, A. B. (2000). Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution. *Journal of immunological methods,* **243** (1–2), 147-154.

Lyons, A. B., Parish, C. R. (1994). Determination of lymphocyte division by flow cytometry. *Journal of Immunological Methods*, **171**, 131-137

Ma, Q., Wang, Y., Lo, A., Gomes, E., Junghans, R. P. (2010). Cell Density Plays a Critical Role in Ex Vivo Expansion of T Cells for Adoptive Immunotherapy. *Journal of Biomedicine and Biotechnology.* **Volume 2010**, Article ID 386545 https://doi.org/10.1155/2010/386545

Mackinnon, S., Papadopoulos, E.B., Carabasi, M. H., et al. (1995). Adoptive immunotherapy evaluating escalating doses of donor leukocytes for relapse of chronic myeloid leukemia after bone marrow transplantation: separation of graft-versus-leukemia responses from graft-versus-host disease. *Blood,* **86** (4,1261-8).

Macey, M. G. (2007). Principles of Flow Cytometry. In: Macey, M.G. (eds) Flow Cytometry. Humana Press. https://doi.org/10.1007/978-1-59745-451-3_1

Maecker, H., T. (2009). Multiparameter flow cytometry monitoring of T cell responses. *Methods Mol. Biol*. **485**, 375-91. doi: 10.1007/978-1-59745-170-3_25. PMID: 19020838; PMCID: PMC4436148.

Marits, P., Wikström, A.C., Popadic, D., Winqvist, O., Thunberg, S., (2014). Evaluation of T and B lymphocyte function in clinical practice using a flow cytometry based proliferation assay. *Clin Immunol.,***153** (2):332-42. doi: 10.1016/j.clim.2014.05.010. Epub 2014 Jun 6. PMID: 24909732.

Marti, F., Miralles, A., Peiro, M., Amill, B., de Dalmases, C., Pinol, G., Rueda, F., Garcia, J. (1993). Differential effect of cryopreservation on natural killer cell and lymphokine-activated killer cell activities. *Transfusion,* **33** (8), 651-655.

Mata, M. M., Mahmoud, F., Sowell, R. T., Baum, L. L. (2014). Effects of cryopreservation on effector cells for antibody dependent cell-mediated cytotoxicity (ADCC) and natural killer (NK) cell activity in 51Cr-release and CD107a assays. *Journal of immunological methods,* **406,** 1-9.

McHugh, R., Whitters, M., Picarillo, C., et al. (2002). CD4+25+ immunoregulatory T cells: gene analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity*, **16** (2), 311-323. DOI : https://doi.org/10.1016/S1074-7613(02)002280-7

McKinnon KM. (2018). Multiparameter Conventional Flow Cytometry. *Methods Mol Biol.* **1678**, 139-150. doi: 10.1007/978-1-4939-7346-0_8. PMID: 29071679; PMCID: PMC8054300.

Meneghel, J., Kilbride, P., Morris, G. (2020). Cryopreservation as a Key Element in the Successful Delivery of Cell-Based Therapies—A Review. *Front. Med*., Sec. Gene and Cell Therapy, Volume **7** – 2020. https://doi.org/10.3389/fmed.2020.592242

de Ménorval, M. A., Mir, L., M., Fernández, M. L., Reigada, R. (2012). Effects of dimethyl sulfoxide in cholesterol-containing lipid membranes: a comparative study of experiments in silico and with cells. PLoS One, 7(7), e41733. doi: 10.1371/journal.pone.0041733. Epub 2012 Jul 25. PMID: 22848583; PMCID: PMC3404987.

Merker, R., I. Check, I., Hunter, R.L. (1979). Use of cryopreserved cells in quality control of human lymphocyte assays: analysis of variation and limits of reproducibility in long-term replicate studies. *Clinical. Experimental. Immunology*, **1**, 116.

Mishra, P., Pandey, C., Singh, U., Gupta, A., Sahu, C., Kashri, A. (2019). Descriptive Statistics and Normality Tests for Statistical Data. *Ann Card Anaesth*. **22** (1), 67–72. doi: 10.4103/aca.ACA_157_18

Möbs, C., Schmidt, T. (2016). Research Techniques Made Simple: Monitoring of T-Cell Subsets using the ELISPOT Assay. Journal of Investigative Dermatology, 136, (6), e55-e59. ISSN 0022-202X. https://doi.org/10.1016/j.jid.2016.04.009.

Molineux, G., Pojda, Z., Hampson, I., N., Lord, B. I., Dexter, T.,M. (1990) Transplantation potential of peripheral blood stem cells induced by granulocyte colony-stimulating factor. *Blood,* **76**, 2153–8.

Modi, J., Menzie-Suderam, J., Xu, H., et al. (2020). Mode of action of granulocyte-colony stimulating factor (G-CSF) as a novel therapy for stroke in a mouse model. *J Biomed Sci*. **27**:1-19.

Mousset, C., Hobo, W., Woestenenk, R., Preijers, F., Dolstra, H., van de Waart, A. (2019). Comprehensive phenotyping of T cells using flow cytometry. *Cytometry Part A*. **95** (6) : 586-694

Motamedi, M., Xu, L., Elahi, S. (2016). Correlation of transferrin receptor (CD71) with Ki67 expression on stimulated human and mouse T cells: The kinetics of expression of T cell activation markers. *J. Immunol. Methods*. **437**, 43-52. doi: 10.1016/j.jim.2016.08.002. Epub 2016 Aug 20. PMID: 27555239.

Munier, C. M. L., Zaunders, J. J., Ip, S., Cooper, D. A., Kelleher, A. D. (2009). A culture amplified multi-parametric intracellular cytokine assay (CAMP-ICC) for enhanced detection of antigen specific T-cell responses. *Journal of immunological methods,* **345** (1–2), 1-16.

Niu, H-Q., Zhao, X-C., Li, W., Xie, J-F., Liu, X-Q., Luo, J., Zhao, W-P., Li, X-F. (2020. Characteristics and reference ranges of CD4+T cell subpopulations among healthy adult Han Chinese in Shanxi Province, North China. *BMC Immunology*, **21**, Article number: 44.

Noakes, E., Peticone, C., Kotsopoulou, E., Bracewell, D., G. (2021). Enriching leukapheresis improves T cell activation and transduction efficiency during CAR T processing. *Molecular Therapy Methods and Clinical Development*, **20,** 675-687. DOI: https://doi.org/10.1016/j.omtm.2021.02.002

Olemukan, R. E., Eller, L. A, Ouma, B. J., Etonu, B., Erima, S., Naluyima, P., Kyabaggu, D., Cox, J. H., Sandberg, J.K., Wabwire-Mangen, F., Michael, N. L., Robb, M. L., de Souza, M. S., Eller, M. A. (2010). Quality monitoring of HIV-1-infected and uninfected peripheral blood mononuclear cell samples in a resource-limited setting. *Clin Vaccine Immunol*. **17** (6):910-8. doi: 10.1128/CVI.00492-09. Epub 2010 Mar 3. PMID: 20200187; PMCID: PMC2884426.

Olson, W.C., Smolkin, M.E., Farris, E.M., Fink, R.J., Czarkowski, A.R., Fink, J.H.et al. (2011) Shipping blood to a central laboratory in multicenter clinical trials: effect of ambient temperature on specimen temperature, and effects of temperature on mononuclear cell yield, viability and immunologic function. *J. Transl. Med.* **9**, 26 10.1186/1479-5876-9-26.

Olsson, R.F., Logan, B.R., Chaudhury, S., Zhu, X., Akpek, G., Bolwell, B.J., Bredeson, C.N., Dvorak, C.C., Gupta, V., Ho, V.T., Lazarus, H.M., Marks, D.I., Ringdén, O.T., Pasquini, M.C., Schriber, J.R., Cooke, K.R. (2015). Primary graft failure after myeloablative allogeneic hematopoietic cell transplantation for hematologic malignancies. *Leukemia*, **29**(8):1754-62. doi: 10.1038/leu.2015.75. Epub 2015 Mar 16. PMID: 25772027; PMCID: PMC4527886.

Omana-Zapata, I., Mutschmann, C., Schmitz, J., et al., (2019). Accurate and reproducible enumeration of T-B- and NK lymphocytes using the BD FACSLyric™ 10 colour system. A multisite clinical evaluation. *PLOS ONE* **14** (1) e211207. https://doi.org/10.1371/journal.pone.0211207

Owen, R. E., Sinclair, E., Emu, B., Heitman, J.W., Hirschkorn, D.F., Epling, C. L., Tan, Q. X., Custer, B, Harris, J. M., Jacobson, M. A., McCune, J.M., Martin, J. N., Hecht, F.M., Deeks, S. G., Norris, P. J. (2007). Loss of T cell responses following long-term cryopreservation. *Journal of immunological methods,* **326** (1–2), 93-115. doi: 10.1016/j.jim.2007.07.012

Pamphilon, D., Mijovic, A. (2007). Storage of hemopoietic stem cells. *Asian J. Transfusion Science*. **1**(2):71-6. doi: 10.4103/0973-6247.33848. PMID: 21938237; PMCID: PMC3168124.

Passweg, J.R., Zhang, M.J., Rocha, V., Kan, F., Champlin, R.E., Isola, L.M., Gee, A.P., Gibson, J., Laughlin, M.J., Lazarus, H.M., Loren, A., Marks, D.I., Gratwohl, A., Eapen, M. (2011). Donor characteristics affecting graft failure, graft-versus-host disease, and survival after unrelated donor transplantation with reduced-intensity conditioning for hematologic malignancies. *Biol Blood Marrow Transplant,* **17**(12):1869-73. doi: 10.1016/j.bbmt.2011.07.008. Epub 2011 Jul 20. PMID: 21771571; PMCID: PMC3217167.

Panch, S.R., Srivastava, S.K., Elavia, N., McManus, A., Liu, S., Jin, P., Highfill, S.L., Li, X., Dagur, P., Kochenderfer, J.N., Fry, T.J., Mackall, C.L., Lee, D., Shah, N.N., Stroncek, D.F. (2019). Effect of Cryopreservation on Autologous Chimeric Antigen Receptor T Cell Characteristics. *Mol Ther*., **27**(7), 1275-1285. doi: 10.1016/j.ymthe.2019.05.015. Epub 2019 May 30. PMID: 31178392; PMCID: PMC6612799.

Pi, C.H., Hornberger, K., Dosa, P., Hubel, A. (2020). Understanding the freezing responses of T cells and other subsets of human peripheral blood mononuclear cells using DSMO-free cryoprotectants. *Cytotherapy*, **22** (5):291-300. doi: 10.1016/j.jcyt.2020.01.013. Epub 2020 Mar 25. PMID: 32220549; PMCID: PMC7682753

Pierelli, L., Perseghin, P., Marchetti, M., et al. (2012). Best practice for peripheral blood progenitor cell mobilization and collection in adults and children: results of a Società Italiana Di Emaferesi e Manipolazione Cellulare (SIDEM) and Gruppo Italiano Trapianto Midollo Osseo (GITMO) consensus process. *Transfusion*. **52**(4):893–905.

Quah, B.J., Parish, C.R., (2012). New and improved methods for measuring lymphocyte proliferation in vitro and in vivo using CFCE-like fluorescent dyes. *Journal of immunological methods*, **379**, 1-14.

Rad, A., Häggström M. (2009). Haematopoiesis (human) diagram.png CC-BY-SA 3.0 license.
https://commons.wikimedia.org/wiki/File:Hematopoiesis_(human)_diagram_en.svg
Downloaded 01/05/2023

Reddy, M., Eirikis, E., Davis, C., Davis, H. M., & Prabhakar, U., (2004) Comparative analysis of lymphocyte activation marker expression and cytokine secretion profile in stimulated human peripheral blood mononuclear cell cultures: an in vitro model to monitor cellular immune function. *Journal of Immunological Methods*, **293**, (1-2), 127–142.

Remberger, M., Grønvold, B., Ali, M., Mattsson, J., Egeland, T., Lundin, K.U., Myhre, A., Abrahamsen, I., Heldal, D., Dybedal, I., Tjønnfjord, G.E., Gedde-Dahl, T., Fløisand, Y. (2020). The $CD34^+$ Cell Dose Matters in Hematopoietic Stem Cell Transplantation with Peripheral Blood Stem Cells from Sibling Donors. *Clin Hematol Int*. **4** (2), 74-81. doi: 10.2991/chi.d.200221.001. PMID: 34595446; PMCID: PMC8432346.

Rowley, S. D., Zuehlsdorf, M., Braine, H.G., et al. (1987). CFU-GM content of bone marrow graft correlates with time to hematologic reconstitution following autologous bone marrow transplantation with 4-hydroperoxycyclophosphamide-purged bone marrow. *Blood,* **70** (1) 271-275. PubMed PMID: 3297203.

Rozmus, J., Bhatt, S. T., Buxbaum, N. P., Cuvelier, G.D. E., Li, A. M., Kitko, C. L., Schultz, K. R. (2022). Is It Possible to Separate the Graft-Versus-Leukemia (GVL) effect against B cell acute lymphoblastic leukemia from graft-versus-host disease (GVHD) after hematopoietic cell transplant? *Frontiers in Pediatrics*, **10.** https://www.frontiersin.org/articles/10.3389/fped.2022.796994

Ruitenberg, J. J., Boyce, C., Hingorani, R., Outnam, A., Ghanekar, S. A. (2011). Rapid assessment of in vitro expanded human regulatory T cell function. *Journal of immunological methods,* **372** (1-2), 95-106.

Rutella, S. (2007). Granulocyte colony stimulating factor for the induction of T-cell tolerance. *Transplantation*, **84** (suppl), S26-30.

Sambor, A., Garcia, A., Berrong, M., Pickeral, J., Brown, S., Rountree, W., Sanchez, A., Pollara, J., Frahm, N., Keinonen, S., Kijak, G. H., Roederer, M., Levine, G., D'Souza, M. P., Jaimes, M., Koup, R., Denny, T., Cox, J., Ferrari, G. (2014). Establishment and maintenance of a PBMC repository for functional cellular studies in support of clinical vaccine trials. *Journal of immunological methods,* **409** (0), 107-116. doi: 10.1016/j.jim.2014.04.005. Epub 2014 Apr 29. PMID: 24787274; PMCID: PMC4152919.

Sarzotti-Kelsoe, M., Needham, L.K., Rountree, W., Bainbridge, J., Gray, C.M., Fiscus, S.A., Ferrari, G., Stevens, W.S., Stager, S.L., Binz, W., Louzao, R., Long, K.O., Mokgotho, P., Moodley, N., Mackay, M., Kerkau, M., McMillion, T., Kirchherr, J., Soderberg, K.A., Haynes, B.F., Denny, T.N. (2014). The Center for HIV/AIDS Vaccine Immunology (CHAVI) multi-site quality assurance program for cryopreserved human peripheral blood mononuclear cells*. J Immunol Methods*. **409**,21-30. doi: 10.1016/j.jim.2014.05.013. Epub 2014 Jun 5. PMID: 24910414; PMCID: PMC4138272.

Schäfer, A.K., Waterhouse, M., Follo, M., Duque-Afonso, J., Duyster, J., Bertz, H., Finke, J. (2020). Phenotypical and functional analysis of donor lymphocyte infusion products after long-term cryopreservation. *Transfus Apher Sci*., **59** (1):102594. doi: 10.1016/j.transci.2019.06.022. Epub 2019 Jul 10. PMID: 31303510.

Schmid, I. et al. (1992) Dead cell discrimination with 7-amino-actinomycin D in combination with dual color immunofluorescence in single laser flow cytometry. *Cytometry* **13** (2): 204–208.

Schwab, L., Michel, G., Bein, G., Hackstein, H., (2019). CD71 surface analysis of T cells: a simple alternative for extracorporeal photophoresis quality control. *Vox Sanguinis*. **115,** 81-93.

Servais, S., Beguin, Y., Baron, F. (2013). Emerging drugs for prevention of graft failure after allogeneic hematopoietic stem cell transplantation. *Expert Opin Emerg Drugs*, **18**,173–92.

Shevyrev, D., and Tereshchenko, V., (2020). Treg Heterogeneity, Function, and Homeostasis. *Front. Immunol*. **10**:3100. doi: 10.3389/fimmu.2019.03100.

Da Silva Ferreira, V., Mesquita, F., Mesquita, D., Andrade, L., (2015). The effects of freeze/thawing on the function and phenotype of CD4+ lymphocyte subsets in normal individuals and patients with systemic lupus erythromatosus. *Cryobiology*, **71** (3), 507-510.

Simonetta, F., Chiali, A., Cordier, C., Urrutia, A., Girault, I., Bloquet, S., Tanchot, C., Bourgeois. C. (2010). Increased CD127 expression on activated FOXP3+CD4+ regulatory T cells. *Eur J Immunol*. **40** (9), 2528-38. doi: 10.1002/eji.201040531. PMID: 20690182.

Smith, J. G., Joseph, H. R., Green, T., Field, J., Wooters, M., Kaufhold, R., Antonello, J., Caulfield, M. J. (2007). Establishing acceptance criteria for cell-mediated-immunity assays using frozen peripheral blood mononuclear cells stored under optimal and suboptimal conditions. *Clinical and vaccine immunology,* **14** (5), 527-537.

Smith, S., Smits, K., Joosten, S., et al. (2015). Intracellular cytokine staining and flow cytometry: considerations for application in clinical trials of novel tuberculosis vaccines. *PloS One*, **10** (9), e0138042

Sparrow, R.L., Komodromou, H., Tippett, E. et al. (2006). Apoptotic lymphocytes and CD34+ cells in cryopreserved cord blood detected by the fluorescent vital dye SYTO 16 and correlation of L-selectin (CD62L) expression. *Bone marrow transplantation*, **38,** 61-67

Steininger, P.A., Smith, R., Geier, C., Zimmermann, R., Eckstein, R. and Strasser, E.F., (2013). Leukapheresis in non–cytokine-stimulated donors with a new apheresis system: first-time collection results and evaluation of subsequent cryopreservation. *Transfusion*, ***53***(4), pp.747-756.

Stoikou, M., van Breda, S.V., Schäfer, G., Vokalova, L., Giaglis, S., Plattner, A., Infanti, L., Holbro, A., Hahn, S., Rossi, S. W., Buser, A. (2020). G-CSF Infusion for Stem Cell Mobilization Transiently Increases Serum Cell-Free DNA and Protease Concentrations. *Frontiers in Medicine,* **7** https://www.frontiersin.org/articles/10.3389/fmed.2020.00155

van der Strate, B., Longdin, R., Geerlings, M., Bachmayer, N., Cavallin, C., Litwin, V., Patel, M., Passe-Coutrin, W., Schoelch, C., Companjen, A., Scheel Fjording, M. (2017). Best practices in performing flow cytometry in a regulated environment: feedback from experience within the European Bioanalysis Forum. *Bioanalysis* **9** (16), white paper. https://doi.org/10.4155/bio-2017-0093

Stroncek, D.F., Ren, J., Lee, D.W., Minh Tran, M., Frodigh, S. E., Sabatino, M., Khuu, H., Merchant, M. S., Mackall, C. L. (2016). Myeloid cells in peripheral blood mononuclear cell concentrates inhibit the expansion of chimeric antigen receptor T cells. *Cytotherapy*, **18**, (7), 893-901. ISSN 1465-3249. https://doi.org/10.1016/j.jcyt.2016.04.003.

Stroncek, D. F., Xing, L., Chau, Q., Zia, N., McKelvy, A., Pracht, L., Sabatino, M., Jin, P. (2011). Stability of cryopreserved white blood cells (WBCs) prepared for donor WBC infusions. *Transfusion*, **51** (12), 2647-2645. https://doi.org/10.1111/j.1537-2995.2011.03210.x

Szyska, M., Na, I., (2016). Bone Marrow GvHD after Allogeneic Hematopoietic Stem Cell Transplantation. *Front. Immunol*., Mar 30;**7**:118. doi: 10.3389/fimmu.2016.00118. PMID: 27066008; PMCID: PMC4811960.

Taylor, H., Woods, P.S., Hughes, W.L., (1956). The organisation and duplication of chromosomes as revealed by autoradiographic studies using tritium-labelled thymidine. *Proc. Natl. Acad. Sci. U.S.A*., **43**, 122-128.

Texeiro, E., Daniels, M., Hamilton, S., Schrum, A., Bragado, R., Jameson, S., Palmer, E. (2009). Different T cell receptor signals determine CD8+ memory versus effector development. *Science* **323** (5913), 502-505. https://doi.org/10.1126/science.1163612.

Thibideaux, S., Coble, D., Day, V., Magali, J., Fontaine, M. G., Gardiner, N., Geach, T., Schwarz, J., Vasovic, L., Wyre, R., Girdlestone, J., Biomedical Excellence for Safer Transfusions (BEST) Collaborative. (2023). Assessing deviations for HPCs obtained during COVID-19 (ADHOC): Evaluating impact of the COVID-19 pandemic on cellular therapy products and processes, the BEST collaborative study. Transfusion, 63 (4), 782-790. https://doi.org/10.1111/trf.17311.

Tian, Y., Babor, M., Lane, J., Schulten, V., Patil, V., Seumois, G., Rosales, S., Zheng, F., Picarda, G., Burel, J., Zapardiel-Gonzalo, J., Tennekoon, R., Da Silva, A. Premawansa, A., Premawansa, G., Wijewickrama, A., Greenbaum, J., Vijayanand, P., Wieskopf, D., Sette, A., Peters, B. (2017). Unique phenotypes and clonal expansions of human CD4 effector memory T cells re-expressing CD45RA. *Nat Commun.,* **8**, 1473. https://doi.org/10.1038/s41467-017-01728-5

Toma, G., Lemnia, I. M., Karapetian, E., Grosse, I., Seliger, B. (2022). Transcriptional analysis of total CD8+ T cells and CD8+45RA- memory T cells from young and old healthy blood donors. *Front. Immunol*. **13**:806906. Doi:10.3889/fimmu.2022.806906

Tompa, A., Nilsson-Bowers, A., Faresjo, M., (2018). Subsets of $CD4^{+}$, $CD8^{+}$ and $CD25^{hi}$ lymphocytes are in general not influenced by isolation and long term cryopreservation. *J. Immunol*, **201** (6), 1799-1809. https://doi.org/10.4049/jimmunol.1701409

Tozzo, P., Delicati A, Zambello R, Caenazzo L. (2021). Chimerism Monitoring Techniques after Hematopoietic Stem Cell Transplantation: An Overview of the Last 15 Years of Innovations. *Diagnostics (Basel),* **11**(4):621. doi: 10.3390/diagnostics11040621. PMID: 33808342; PMCID: PMC8065704.

Tricot, G., Jagganth, S., Vesole, D., et al. (1995). Peripheral blood stem cell transplants for multiple myeloma: identification of favorable variables for rapid engraftment in 225 patients. *Blood*, **85** (2) 588-596.

Trück, J., Mitchell, R., Thompson, A.J., Morales-Aza, B., Clutterbuck, E.A., Kelly, D.F., Finn, A., Pollard, A.J. (2014). Effect of cryopreservation of peripheral blood mononuclear cells (PBMCs) on the variability of an antigen-specific memory B cell ELISpot. *Hum Vaccin Immunother*., **10**(8):2490-6. doi: 10.4161/hv.29318. PMID: 25424961; PMCID: PMC4896800.

Vasconcelos, Z.F., Santos, B.M., Costa, E.S., Lima, M., Tabak, D.G., Bouzas, L.F., Azevedo, W.M., Barcinski, M.A., Bonomo, A. (2003). T-lymphocyte function from peripheral blood stem-cell donors is inhibited by activated granulocytes. *Cytotherapy,* **5** (4), 336-45. doi: 10.1080/14653240310002252. PMID: 12944240.

Vermeren, S., Elks, P. M., Ellett, F. (2021). Neutrophil Functions in Host Immunity, Inflammation and Tissue Repair. *Frontiers in Immunology*, **12**. DOI=10.3389/fimmu.2021.810346 https://www.frontiersin.org/articles/10.3389/fimmu.2021.810346

Vian, A., M., Higgins, A., Z. (2014). Membrane permeability of the human granulocyte to water, dimethyl sulfoxide, glycerol, propylene glycol and ethylene glycol. *Cryobiology,* **68** (1), 35-42. https://doi.org/10.1016/j.cryobiol.2013.11.004

Volpi, I., Perruccio, K., Tosti, A., Capanni, M., Ruggeri, L., Posati, S., Aversa, F., Tabilio, A., Romani, L., Martelli, M.F., Velardi, A (2110). Postgrafting administration of granulocyte colony-stimulating factor impairs functional immune recovery in recipients of human leukocyte antigen haplotype-mismatched hematopoietic transplants. *Blood*, **97**(8), 2514-21. doi: 10.1182/blood.v97.8.2514. PMID: 11290617.

de Vos, J., Baudoux, E, Bay, J. O, Calmels, B., Cras, A., El Cheikh, J., Guerout-Verite, M.A., Lacassagne, M.N., Lamure, S., Letellier, C., Menard, A,L,, Daguindau, E., Poiré, X., Yakoub-Agha, I., Guillaume, T. Injections de lymphocytes du donneur (DLI) : recommandations de la Société francophone de greffe de moelle et de thérapie cellulaire (SFGM-TC) [Donor Lymphocyte Infusions (DLI): Guidelines from the Francophone Society of Bone Marrow Transplantation and Cellular Therapy (SFGM-TC)].(2019). *Bull Cancer*., **106** (1S): S35-S39. French. doi: 10.1016/j.bulcan.2018.10.002. Epub 2018 Dec 20. PMID: 30580911.

Wada, F., Jo, T., Arai, Y. *et al.* T-cell counts in peripheral blood at leukapheresis predict responses to subsequent CAR-T cell therapy. *Sci Rep* **12**, 18696 (2022). https://doi.org/10.1038/s41598-022-23589-9

Wang, W., Xiang, H-P., Wang, H-P., Zhu, L-X., Geng, X-P. (2017). CD4+CD25+CD127high cells as a negative predictor of multiple organ failure in acute pancreatitis. *World Journal of Emergency Surgery*, **12** (7). https://doi.org/10.1186/s13017-017-0116-7

Wang, H., Tsao, ST., Gu, M. *et al.* (2022). A simple and effective method to purify and activate T cells for successful generation of chimeric antigen receptor T (CAR-T) cells from patients with high monocyte count. *J Transl Med* **20**, (608). https://doi.org/10.1186/s12967-022-03833-6

Weinberg, A., Zhang, L., Brown, D., et al. (2000). Viability and functional activity of cryopreserved mononuclear cells. *Clinical and diagnostic laboratory immunology*, **7** (4), 714-716.

Weinberg, A., Song, L.Y., Wilkening, C.L., Fenton, T., Hural, J., Louzao, R., Ferrarim G., Etter, P.E., Berrong, M., Canniff, J.D., Carter, D., Defawe, O.D., Garcia, A., Garrelts, T.L., Gelman, R., Lambrecht, L.K., Pahwa, S., Pilakka-Kanthikeel, S., Shugarts, D.L., Tustin, N.B. (2010). Optimization of storage and shipment of cryopreserved peripheral blood mononuclear cells from HIV-infected and uninfected individuals for ELISPOT assays. *J Immunol Methods*., **363**(1), 42-50. doi: 10.1016/j.jim.2010.09.032. Epub 2010 Oct 1. PMID: 20888337; PMCID: PMC3068047.

Weinberg, A., Song, L.Y., Wilkening, C.L., Sevin, A., Blais, B., Louzao, R., Stein, D., Defechereux, P., Durand, D., Riedel, E., Raftery, N., Jesser, R., Brown, B., Keller, M.F., Dickover, R., McFarland, E., Fenton, T. (2009). Pediatric ACTG Cryopreservation Working Group. Optimization and limitations of use of cryopreserved peripheral blood mononuclear cells for functional and phenotypic T-cell characterization. *Clin Vaccine Immunol*., **16**(8):1176-86. doi: 10.1128/CVI.00342-08. Epub 2009 Jun 10. PMID: 19515870; PMCID: PMC2725535.

Weiner, J., Duran-Struuck, R., Zitsman, J., Buhler, L., Sondermeijer, H., McMurchy, A., Levings, M., Sykes, M., Griesemer, A. (2015). Restimulation After Cryopreservation and Thawing Preserves the Phenotype and Function of Expanded Baboon Regulatory T Cells. *Transplantation Direct,* **1**(1), 1-7. DOI: 10.1097/TXD.0000000000000511

Worsham, D. N., Reems, J. A., Szczepiorkowski, Z. M., McKenna, D. H., Leemhuis, T., Mathew, A. J., Cancelas, J. A. (2017). Biomedical Excellence for Safer Transfusion (BEST) Collaborative Group. Clinical methods of cryopreservation for donor lymphocyte infusions vary in their ability to preserve functional T-cell subpopulations. *Transfusion*, **57**(6), 1555-1565. doi: 10.1111/trf.14112. Epub 2017 Apr 28. PMID: 28453219.

Wright, C. R., Ward, A. C., Russell, A. P., (2017). Granulocyte colony-stimulating factor and its potential application for skeletal muscle repair and regeneration. *Mediators Inflamm*. **2017,** 1-9.

Wullner, D., Zhou, L., Bramhall, E., Kuck, A., Goletz, T.J., Swanson, S., Chirmule, N., Jawa, V. (2010). Considerations for optimization and validation of an in vitro PBMC derived T cell assay for immunogenicity prediction of biotherapeutics. *Clin Immunol*.,**137**(1):5-14. doi: 10.1016/j.clim.2010.06.018. Epub 2010 Aug 13. PMID: 20708973.

Ye, Y., Yang, L., Yuan, X., He, H., and Yi, L. (2022). Optimization of Donor Lymphocyte Infusion for AML Relapse After Allo-HCT in the Era of New Drugs and Cell Engineering. *Front. Oncol.,* **11**. https://doi.org/10.3389/fonc.2021.790299

Yi, J., Cox, M., Zajac, J., (2010). T cell exhaustion; characteristics, causes and conversion. *Immunology*, **129** (4), 474-481.

Zhang, K., Wang, F., Zhang, M., Cao, X., Yang, S., Jia, S., Wang, L., Luo, J., Deng, S., Ming Chen, M. (2016). Reference ranges of lymphocyte subsets balanced for age and gender from a population of healthy adults in Chongqing District of China. *Clinical Cytometry*, **90** (6), 538-542. https://doi.org/10.1002/cyto.b.21323

Zhao, J., Gabriel, E., Norda, R., Höglund, P., Baden, L., Diedrich, B., Marits, P., Enoksson, S., Gansner, J., Kaufman, R., Dickman, P., Edgren, G. (2020). Frequent platelet donation is associated with lymphopenia and risk of infections: A nationwide cohort study. *Transfusion,* **61** (2), 464-473.
https://doi.org/10.1111/trf.16175

Zhou, M., Sacirbegovic, F., Zhao, K. *et al.* (2020). T cell exhaustion and a failure in antigen presentation drive resistance to the graft-versus-leukemia effect. *Nat Commun* **11**, 4227. https://doi.org/10.1038/s41467-020-17991-y

8 Websites

BD Biosciences Fluorochrome Reference Chart. Downloaded 28/02/2022.

https://www.bd.com/resource.aspx?IDX=17753

BD Biosciences Spectrum Viewer. Downloaded 11/02/23.

https://www.bdbiosciences.com/en-gb/resources/bd-spectrum-viewer

British Society of Blood and Marrow Transplantation and Cellular Therapy.

https://bsbmtct.org/activity/2020/

Clinical commissioning policy: second allogeneic haematopoietic cell transplant for relapsed disease all ages 2017.

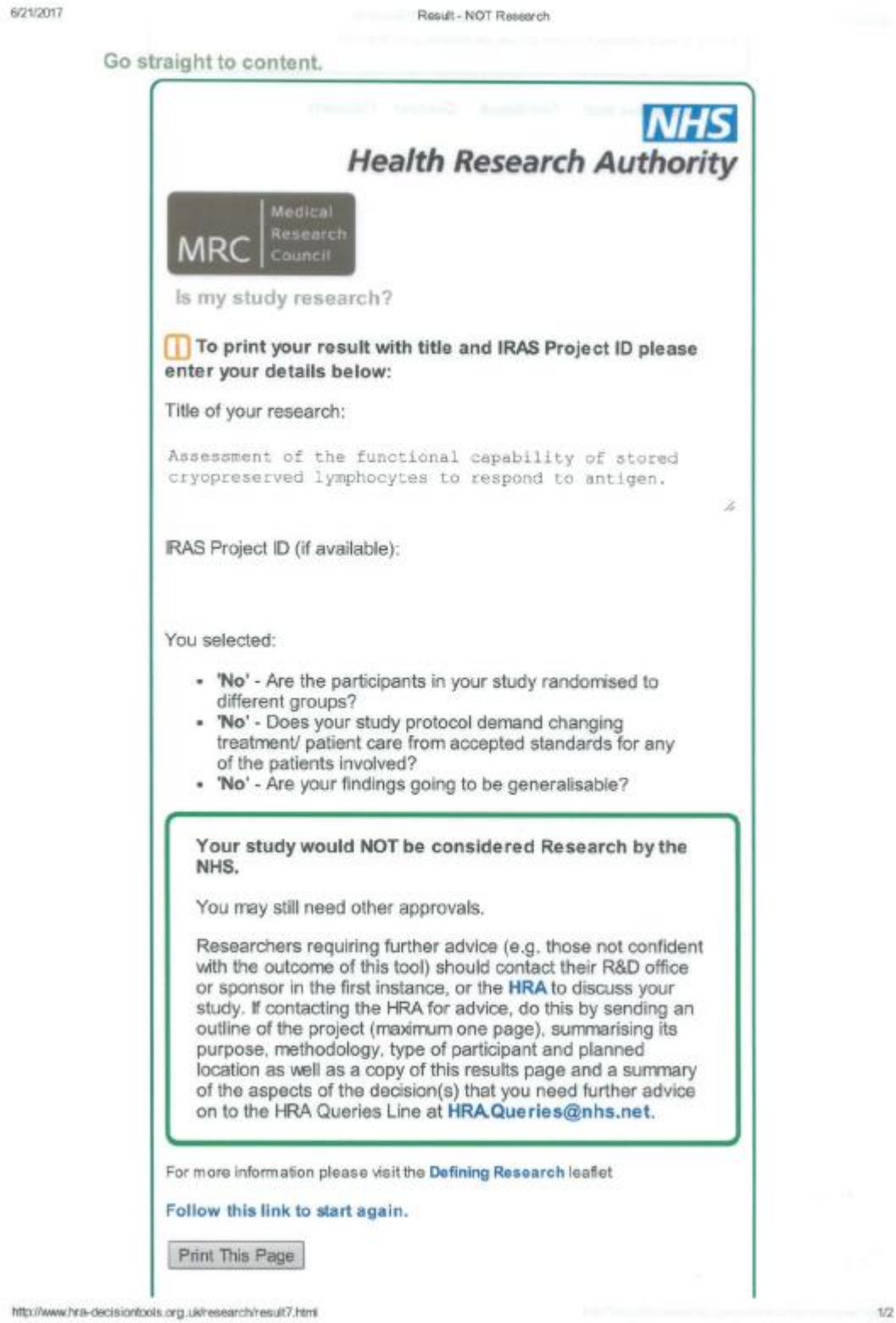
https://www.england.nhs.uk/wp-content/uploads/2017/02/clin-comms-policy-16068p.pdf

FACT/JACIE international standards for haematopoietic cellular therapy product collection processing and administration eights edition version 8.2 December 2021.

https://www.ebmt.org/sites/default/files/2021-05/STS_5_2_041_FACT-JACIE%20Standards%20Eighth%20Edition_R1_05172021_for%20web.pdf

9 Appendices

Appendix 1: HRA research tool



6/21/2017 Result - NOT Research

Go straight to content.

NHS
Health Research Authority

MRC Medical Research Council

Is my study research?

To print your result with title and IRAS Project ID please enter your details below:

Title of your research:

Assessment of the functional capability of stored cryopreserved lymphocytes to respond to antigen.

IRAS Project ID (if available):

You selected:

- **'No'** - Are the participants in your study randomised to different groups?
- **'No'** - Does your study protocol demand changing treatment/ patient care from accepted standards for any of the patients involved?
- **'No'** - Are your findings going to be generalisable?

Your study would NOT be considered Research by the NHS.

You may still need other approvals.

Researchers requiring further advice (e.g. those not confident with the outcome of this tool) should contact their R&D office or sponsor in the first instance, or the HRA to discuss your study. If contacting the HRA for advice, do this by sending an outline of the project (maximum one page), summarising its purpose, methodology, type of participant and planned location as well as a copy of this results page and a summary of the aspects of the decision(s) that you need further advice on to the HRA Queries Line at HRA.Queries@nhs.net.

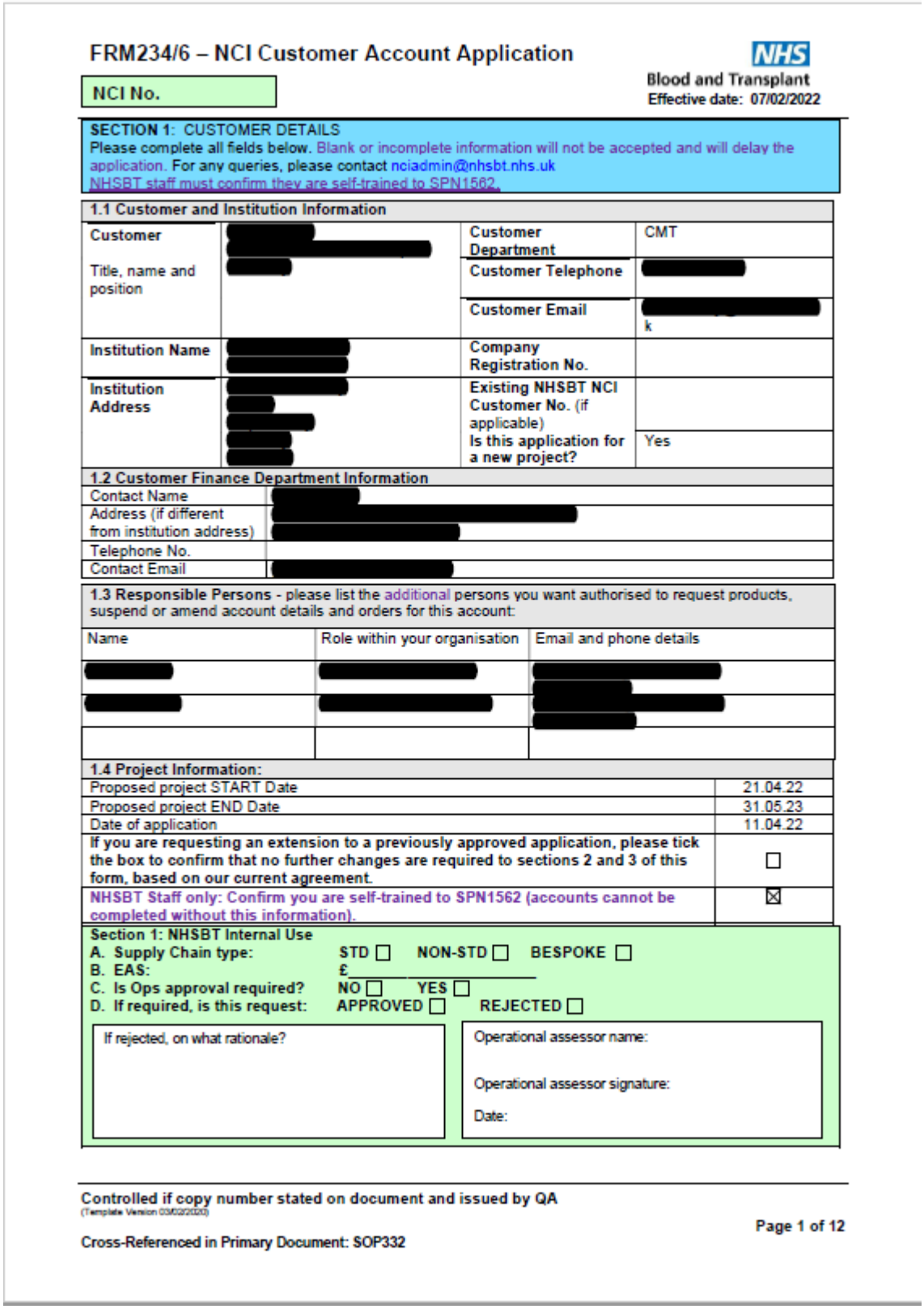
For more information please visit the Defining Research leaflet

Follow this link to start again.

Print This Page

http://www.hra-decisiontools.org.uk/research/result7.html 1/2

Appendix 2: Non-Clinical Issue account acceptance



FRM234/6 – NCI Customer Account Application

NHS Blood and Transplant
Effective date: 07/02/2022

NCI No.

SECTION 1: CUSTOMER DETAILS
Please complete all fields below. Blank or incomplete information will not be accepted and will delay the application. For any queries, please contact nciadmin@nhsbt.nhs.uk
NHSBT staff must confirm they are self-trained to SPN1562.

1.1 Customer and Institution Information

Customer Title, name and position	[redacted]	**Customer Department**	CMT
		Customer Telephone	[redacted]
		Customer Email	[redacted] k
Institution Name	[redacted]	**Company Registration No.**	
Institution Address	[redacted]	**Existing NHSBT NCI Customer No.** (if applicable)	
		Is this application for a new project?	Yes

1.2 Customer Finance Department Information

Contact Name	[redacted]
Address (if different from institution address)	[redacted]
Telephone No.	
Contact Email	[redacted]

1.3 Responsible Persons - please list the additional persons you want authorised to request products, suspend or amend account details and orders for this account:

Name	Role within your organisation	Email and phone details
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]

1.4 Project Information:

Proposed project START Date	21.04.22
Proposed project END Date	31.05.23
Date of application	11.04.22
If you are requesting an extension to a previously approved application, please tick the box to confirm that no further changes are required to sections 2 and 3 of this form, based on our current agreement.	☐
NHSBT Staff only: Confirm you are self-trained to SPN1562 (accounts cannot be completed without this information).	☒

Section 1: NHSBT Internal Use

A. Supply Chain type: STD ☐ NON-STD ☐ BESPOKE ☐
B. EAS: £_____
C. Is Ops approval required? NO ☐ YES ☐
D. If required, is this request: APPROVED ☐ REJECTED ☐

If rejected, on what rationale?

Operational assessor name:

Operational assessor signature:

Date:

Controlled if copy number stated on document and issued by QA
(Template Version 03/02/2020)

Page 1 of 12

Cross-Referenced in Primary Document: SOP332

SECTION 2: PURPOSE, INTENDED USES AND SUPPORTING INFORMATION
Please complete all fields below. Incomplete information or unchecked boxes will not be accepted and will delay the application. For any queries, please contact nciadmin@nhsbt.nhs.uk

2.1 PURPOSE: Please confirm the purpose for which materials are required (you may tick more than one)	Yes	No
a. Medical research and development	☐	☒
b. Validation/development of new products including components therapeutic products and processing/manufacturing methods	☐	☒
c. *In vitro* diagnostic test / process validation and/or laboratory quality control	☒	☐
d. Education and training	☐	☒
e. Production or manufacture of biochemical assay (including, but not restricted to, the production or manufacture of a reagent or reagents)	☐	☒

2.2 INTENDED USE: Please answer ALL questions (a)-(f) below. Where you answer "yes" to any question, please provide brief details in the box provided.	Yes	No
(a) EXPORT: Do you intend to use material and/or data derived from materials supplied by NHSBT outside of England, Wales or Northern Ireland? Details	☐ Please answer 2.2(b)	☒ Please answer 2.2(b)
(b) THIRD PARTIES: Do you intend to pass on materials supplied by NHSBT to any 3rd party in whole or part for any reason including quality control? Details	☐ Please answer 2.2(c)	☒ Please answer 2.2(c)
(c) DNA/RNA: Do you intend to undertake any RNA/DNA analysis of materials supplied? Details *Note: NHSBT permits limited DNA/RNA analysis of gene expression and of specific genes and proteins.*	☐ Please answer 2.2(d)	☒ Please answer 2.2(e)
(d) DNA/RNA: Is analysis likely to establish the identity of the donor of the material? Details *For office use: Requires CARE assessment if yes*	☐ Please answer 2.2(e)	☒ Please answer 2.2(e)
(e) Animal Models: Will materials supplied be used in animal models? Details *For office use: Requires CARE assessment*	☐ Please answer 2.2(f)	☒ Please answer 2.2(f)
(f) THERAPEUTIC APPLICATION: Do you require clinical-specification material for a clinical use (*e.g. for administration to a person/participant in a clinical trial/as starting material for a cellular therapy*)? Please summarise trial purpose/outputs and confirm any MHRA (or equivalent) trial approval. (i) Approval body / (ii) Approval reference *For office use: Requires CARE assessment*	☐ Please answer (i) / (ii)	☒ Please go to 2.3 Please go to 2.3

2.3 SUPPORTING INFORMATION: Please use the space below to explain exactly how you intend to use the material requested, any tests undertaken and what happens to the results of those tests.

- R&D users are required to include a copy of the research proposal abstract.
- Clinical Trial users are required to include a copy of the trial approval or application
- For multiple projects please contact nciadmin@nhsbt.nhs.uk for presentation requirements.
- Do you intend to test for any of the following: Syphilis, Hepatitis B virus (HBV), Human immunodeficiency virus (HIV), Hepatitis C virus (HCV), Hepatitis E Virus (HEV) or Human T-lymphotropic virus (HTLV)

In vitro assessment of the functional capability of lymphocytes that have been stored prior to cryopreservation to respond to antigen
Site: CMT Laboratory, NHSBT Barnsley.
Performed by [redacted]
DEFINATIONS/ACRONYMS
•CRF Control Rate Freezer
DLI - Donor Lymphocyte Infusion
MNC - Mononuclear Cell
•LN2 Liquid Nitrogen
•HAS Human Albumin Solution
•HPC(A)Haematopoietic Progenitor Cell-Apheresis
G-CSF Granulocyte Colony Stimulating Factor
•LAFLaminar Air Flow cabinet
•SCD Sterile Connecting Device
•CMT - Cellular and Molecular Therapy
•FBC Full Blood Count
•FCS Fetal Calf Serum
•PBS Phosphate Buffer Saline
•IL-2 Interleukin 2
•SOP Standard Operating Procedure
•FRM Form
•DAT Data sheet
•cGMP Current Good Manufacturing Practice
•PPE ersonal Protective Equipment

INTRODUCTION
Donor lymphocytes (DLI) from healthy individuals are routinely cryopreserved and stored for varying lengths of time before being reinfused into bone marrow transplant recipients to target and eradicate leukaemic relapse post transplant. It is assumed that the stored cells maintain their ability to respond to antigen over the freezing/storage process and that they will be effective clinically. However, the effect of storage and cryopreservation on lymphocyte function is currently poorly understood. In addition, donor lymphocytes are often prepared from excess cells intended for initial transplant and been exposed to granulocyte-colony stimulating Factor (G-CSF) during the collection process. G-CSF is known to have an immune modulation effect on lymphocyte activity but to date here have been no published studies comparing the responses of stored cryopreserved lymphocytes that have been exposed to G-CSF with those that have not.

AIMS and OBJECTIVES

Aim 1 - Compare the in vitro responses of thawed cryopreserved lymphocytes that were stored for >24 hours prior to cryopreservation with those that were frozen immediately to stimulation with the plant lectin Phytohaemagglutinin (PHA) or CD3/28 beads. The following flow cytometric tests will be used :

- Intracellular cytokine production (IL2/ TNFα/IFN-γ)
- Cell surface marker expression (CD3/4/8/14/19/25/56/69) by flow cytometry
- Apoptosis markers

Aim 2 - Compare the in vitro responses of lymphocytes that were mobilised with G-CSF with those that were not under the same conditions as above.

Aim 3 – Compare the in vitro responses of lymphocytes collected from male and female donors under the same conditions as above (if possible)

MATERIAL and EQUIPMENTS
Reagents

•DMSO
Ficol
•Human Serum Albumin
•Liquid Nitrogen
•Fluorescently conjugated antibodies to cell surface markers
•7-Amino actinomycin viability dye (7-AAD)
•CD-Chex plus
•CD3/CD28 beads for T cell activation
•Trucount tubes
•Distilled water
•Lysing solution
•RPMI cell culture medium
•PBS
•Fetal Calf Serum
•Recombinant IL-2 for cell culture
Equipment's and Accessories
•LN2 Control Rate Freezer
•CryoMACS bags and Cryovials
•Cassettes
•SCD
•Lyric Flow cytometer
•CO2 incubator
•Biosafety Cabinet
•Haematology analyser
•Pipettes (10-1000 µl) and pipette tips
•Tube sealer
•Balance
•Vacuum packer
•Thermal printer
•Water bath

ANALYTICAL METHODS and REFERNCE DOCUMENTS
Leukocyte cones from mobilised and non mobilised apheresis donors will be received (FRM3242) and a mononuclear fraction prepared by Ficol-density separation. The separated MNCs will then be phenotyped by flow cytometry for lymphocyte specific and activation cell surface markers.
The MNC will then be resuspended in 4.5% HAS at WBC of <100 x 10^6/mL. The MNC willthen be split into 3 equal fractions for cryopreservation.
Fraction 1 : Cryopreserved within 24 hours of collection
Fraction 2 : Stored for 24-48 hours at 4C and then cryopreserved
Fraction 3 : Stored for 48-72 hours at 4C and then cryopreserved
The MNCs will be cryopreserved in accordance with the Standardised Cryopreservation Protocol (SOP2050). A inge bag and two x 2 ml cryovials will be cryopreserved using VFQ and Planner CRF (SOP5171) using the Sheffield BMT profile.
Bags and cryovials will be labelled with the following information: collection location, date of collection, date of cryopreservation, mobilised/unmobilised and unique number from 01 to 20 to create a unique sample ID. As an example, mobilised cells collected in Sheffield on 10/02/22 and cryopreserved on 11/02/22 would be labelled SH10/02/22- 11/02/22MOB1
Bags and vials will be stored for a minimum of 1 week in LN2 vat for future analysis.
Thawing
Wearing appropriate PPE the frozen MNC will be removed the Cryo Bag/cryovial from the -150 Cryovat and immediately thawed in according to the Standardised Thawing Protocol using water bath at 37°C.
T-cell stimulation and proliferation assay

MNCs will be incubated with CD3/CD28 beads for T cell activation and proliferation.
Flow cytometry analysis
Flow cytometry for activation markers will be performed on the cryopreserved samples after 3 days in cuture.
Results will be compared with the pre-cryopreservation testing.

Virology Testing

Please make clear if you intend to test for any of the following: Syphilis, Hepatitis B virus (HBV), Human immunodeficiency virus (HIV), Hepatitis C virus (HCV), Hepatitis E Virus (HEV) & Human T-lymphotropic virus (HTLV)? Yes ☐ No ☒

If yes, please provide details of the tests you will perform:
Details

Please confirm that you understand and agree to notify NHSBT of any positive results from the virology screens stipulated above including any information NHSBT requires about the testing process, protocol or reagents used:
Yes ☒ No ☐

2.4 Ethics – please tick one of the following options			
☐	I am undertaking research work which **DOES REQUIRE** Research Ethics Committee (REC) approval		
	☐ I have received REC approval	REC Number	*NOTE: You are required to submit a copy of your REC Approval with your*
		Approval body	

		Approval start date Approval end date	*application. NHSBT will reject applications that are not accompanied by your REC approval*
	☐ I am requesting NHSBT Cord Bank to extend its Tissue Bank Ethical Approval	*NOTE: This ONLY applies for applications requesting cord blood products (listed in Appendix A). If your application is approved by an internal NHSBT committee this option will allow you to carry out your research without needing to have separate REC approval* **ACTION: Now go to Section 3**	
☐	I am undertaking research work which **DOES NOT** require Research Ethics Committee (REC) approval	*NOTE: NHSBT will **only** accept written output from the HRA toolkit (hra.nhs.uk) as evidence that REC approval is NOT required. You are required to provide this confirmation with your application. NHSBT will reject applications that are not accompanied by this evidence* **ACTION: Now go to Section 3**	
☒	I am not undertaking research work	*NOTE: Applicants are advised to refer to the HRA toolkit (hra.nhs.uk)* **ACTION: Now go to Section 3**	

SECTION 3: COMPONENTS AND MATERIALS

3.1 Materials: The materials available from NHSBT are sourced from a range of internal departments listed in Appendices A-H. Each of these departments is required by NHSBT to undertake a review of your application and assess whether, or not, we are able to supply materials requested for your proposed uses.

To help this assessment, please indicate in the table below the Appendices from which you are requesting material/s. As required, you may tick more than one Appendix. For any queries, please contact nciadmin@nhsbt.nhs.uk

	TICK BELOW
APPENDIX A: BLOOD and BLOOD COMPONENTS	☒
APPENDIX B: COVID-19 CONVALESCENT PLASMA	☐
APPENDIX C: CORD BLOOD	☐
APPENDIX D: THERAPEUTIC APHERESIS SERVICES	☒
APPENDIX E: PATIENT SAMPLES FROM RED CELL IMMUNOHAEMATOLOGY (RCI)	☐
APPENDIX F: PATIENT SAMPLES FROM IBGRL*	☐
APPENDIX G: CELLULAR AND MOLECULAR THERAPIES	☐
APPENDIX H: MICROBIOLOGY SERVICES SURVEILLANCE (MSS)	☐
APPENDIX I: HISTOCOMPATIBILITY AND IMMUNOGENETICS (H&I)	☐
APPENDIX J: TISSUES	☐

*International Blood Group Reference Laboratory

3.2 List of materials that may be requested from NHSBT to support non-clinical work:
For the each of the materials you are requesting in Appendices A-H, please indicate:
(a) the number of units you require and
(b) your anticipated order frequency (e.g. daily, weekly, monthly, 6-monthly etc)

NOTE: No charge is made by NHSBT for the donated material. NHSBT is mandated to recover the costs of issue, retrieval and making components and materials available. Details will be provided when an application has been reviewed and approved by NHSBT.

APPENDIX A: BLOOD and BLOOD COMPONENTS				
Item Code	Product	Volume per unit	Number of units required	Frequency (weekly, monthly etc)
NC01	OTC Serum AB (rarely available)	200ml		
NC02	OTC Serum non AB	200ml		
NC04	Cryo depleted Plasma	200ml		
NC05	Plasma	250ml		
NC07	Buffy Coats	50ml		
NC08	Buffy Coat Residue			
NC09	Expired Platelets			
NC12	Neonatal Expired Platelets			
NC13	Untested Whole Blood	485ml approx.		
NC15	Research Red Cells	200ml		
NC16	Expired Red Cells	200ml		
NC18	Random Donor Samples (EDTA Tube)	1 tube (6ml approx.)		
NC19	Cryo – Single Donor Unit			
NC20	Research Platelets	1 unit		
NC22	Random Donor Samples	1 deep well micro plate		
NC24	Leukocyte Cone	1	2	weekly
NC26	Rare Donor Sample	1		
NC50	Clinical Spec Research Red Cells	220–340ml approx.		
NC52	Clinical Spec Res Whole Blood	485ml		
NC54	Clinical Spec Res Plasma			
NC55	Cryo 1 - Clinical Spec Res Cryo Pooled			
NC56	Clinical Spec Research Platelets Pooled			
NC58	Clinical Spec Research Platelets Apheresis			

APPENDIX B: COVID-19 CONVALESCENT PLASMA			
	Volume of sample required ml	Number of samples required	Frequency (weekly, monthly etc)
Plasma – small volume donor samples of convalescent plasma with detectable COVID Antibodies.			
Plasma – 150ml + unit of convalescent plasma with detectable COVID Antibodies (typically 150-280ml typical)			
For all of the above please note that: • Convalescent Plasma is a limited resource. We aim to provide samples with microlitre volumes. • COVID antibody titre levels are derived from Euroimmune Assay. • Low titre (1.1-3) medium titre (4-9) high titre (10+)			

APPENDIX C: CORD BLOOD			
Product		**Number of units required**	**Frequency (weekly, monthly etc)**
Fresh Cord blood unit			
Fresh Cord Blood Red Cells - minimum volume 20mls			
Fresh volume reduced Cord blood unit			
Fresh Cord Plasma			
Frozen Cord blood unit <2.0x10^6 Total CD34+ve cells			
Frozen Cord blood unit 2.0-3.99x10^6 Total CD34+ve cells			
Frozen Cord blood unit >4.0x10^6 Total CD34+ve cells			
Fresh Cord Tissue as a starting material in a clinical trial or as starting material for an ATMP to be issued under the 'specials' scheme*			
Fresh Cord Blood as a starting material in a clinical trial			
Placenta and Cord tissue for R&D -please specify requirements on section 2.3.			
CORD BLOOD ADDITIONAL PROCESSING / TESTING			
Maternal Infectious Disease Marker Testing***	☐	ABO/Rh***	☐
CBU Plasma Infectious Disease Marker Testing****	☐	Maternal Samples****	☐
Full Blood Count***	☐	Bacteriology / Fungal Screening***	☐
HLA (Class I ABC Class II DRB1)***	☐	CFU Culture****	☐
Frozen CBU Plasma Sample 2ml**	☐	Frozen CBU Whole Blood Sample 2ml**	☐
CD34 Count***	☐		

*Fresh CBU is issued from Colindale (London) Tuesday – Friday after 8am. All Fresh CBUs undergo mandatory testing for IDM markers and will be added to all requests. The NHS Cord Bank is unable to guarantee the availability and supply of CBUs. Frozen CBU is shipped from Filton.
**Available addition with Frozen units only
***Available addition with Fresh units only
**** Available addition on both Fresh and Frozen units

APPENDIX D: THERAPEUTIC APHERESIS			
Product – typically supplied untested unless specified.	**Volume per unit**	**Number of units required**	**Frequency (weekly, monthly etc)**
Plasma: residual from single patient plasmapheresis	2 litres approx.		
Used CD34 therapeutic Harness. Single patient NOTE: Patients are virology tested (covering HIV, HCV, HBV and syphilis) prior to PBSC collection.	1	2	weekly
Red cells, residual from single patient exchange	2 litres+		
White cells – residual from single patient exchange	2 litres		
Harness & Column: single patient low density lipids	1		
Harness: residual blood following from ECP procedures	1		
Platelets: residual material from Platelet depletion procedure, single patient NOTE: There is usually less than 1 unit a year available	2 litres approx.		

APPENDIX E: PATIENT SAMPLES from RED CELL IMMUNOHAEMATOLOGY (RCI)			
Condition - typically 0.5ml – 5ml of frozen archive samples from patients with the following conditions	**Volume of sample required ml**	**Number of samples required**	**Frequency (weekly, monthly etc)**
Haemolytic Disease of the Foetus & Newborn (HDFN)			
Sickle Cell disease			
Thalassaemia			
Paroxysmal Nocturnal Haemoglobinuria (PNH)			
Paroxysmal Cold Haemoglobinuria (PCH)			
Myodyspasia			
Auto Immune Haemolytic Anaemia (AIHA)			
Cold Haemolytic Disease (CHAD)			
Determination of Feto-Maternal Haemorrhage (FMH)			
Drug associated AIHA			
Ante natal samples			
For all of the above please note that: • RCI are unable to detail volumes, specificities or strength (titre/quantification value) of each type in advance but will confirm details and availability on application, • Samples can only be released when the minimum RCI retention period has expired, therefore RCI cannot guarantee the availability of any sample, • Patient samples supplied may have been initially sampled and tested in RCI laboratories, therefore volumes of materials cannot be guaranteed.			

APPENDIX F: PATIENT SAMPLES from IBGRL *(International Blood Group Reference Laboratory)*			
Typically 0.5ml – 2ml of frozen archive plasma samples or 0.1ml – 0.5ml of frozen archive red cell samples from patients referred for antibody or phenotype investigation	**Volume of sample required ml**	**Number of samples required**	**Frequency (weekly, monthly etc)**
Antibody investigation – rare antibodies please specify			
Cross matching investigation- rare phenotype please specify			
DNA from samples referred for blood group genotyping			
For all of the above please note that specific requirements should be detailed in section 2.3: • We are unable to detail volumes, specificities or strength (titre/quantification value) of each type in advance but will confirm details and availability on application, • We cannot guarantee the availability of any sample, • Patient samples supplied may have been initially sampled and tested in NHSBT laboratories, therefore volumes of materials cannot be guaranteed.			

APPENDIX G: CELLULAR AND MOLECULAR THERAPY PRODUCTS			
Product	**Volume per unit**	**Number of units required**	**Frequency (weekly, monthly etc)**
HPC(A) (Haemopoietic progenitor cells - Apheresis)	100ml		
NOTE: All materials are supplied cryopreserved and availability cannot be guaranteed These products are mobilised products from patients where there is no longer a clinical need for storage For any other CMT product or sample type please detail your requirements in section 2.3			

APPENDIX H: MICROBIOLOGY SERVICES SURVEILLANCE (MSS)				
Product	Positive Marker	Volume per unit	Number of units required	Frequency (weekly, monthly etc)
Marker Positive Plasma Unit	(See Section 2.3)	250-270ml		

APPENDIX I: HISTOCOMPATIBILITY AND IMMUNOGENETICS (H&I)			
Product	Volume per unit	Number of units required	Frequency (weekly, monthly etc)
PI (platelet immunology)			
GI (Granulocyte immunology)			

NOTE: Typically, H&I offer Sera to EQA schemes with the following specificities:

- *HLA*
- *HPA*
- *HNA Ab +ve and –ve*

If you have a requirement for this material type for an EQA scheme or NHSBT use, please use the free text box on Section 2.3 to detail your exact requirements for H&I material.

APPENDIX J: TISSUE SERVICES *NOTE: Tendons are supplied either decontaminated or irradiated. Please specify at time of ordering*				
Item Code	Product (Tissue Services)	Volume per unit	Number of units required	Frequency (weekly, monthly etc)
TP2003	Dried washed irradiated cancellous cubes	10x10x10mm (Pack 5)		
TP2005	Dried washed irradiated cancellous chips	6x6x30mm (Pack 5)		
TP2006	Dried washed irradiated tricortical wedge	30x15mm		
TP2007	Washed irradiated humeral shaft	1		
TP2008	Frozen washed irradiated humeral head	1		
TP2011	Frozen washed irradiated cortical strut	Small 15cm		
TP2012	Frozen washed irradiated cortical strut	Medm 19cm		
TP2013	Frozen washed irradiated cortical strut	Large 24cm		
TP2014	Freeze-dried washed irradiated cortical strut	Small 15cm		
TP2015	Freeze-dried washed irradiated cortical strut	Medium 19cm		
TP2016	Freeze-dried washed irradiated cortical strut	Large 24cm		
TP1001	Fresh frozen femoral head	Minimum 50g		
TP1002	Fresh frozen femoral heads	Small		
TP1003	Irradiated fresh frozen femoral head	Minimum 50g		
TP1004	Washed irradiated femoral head	Whole		
TP1005	Frozen washed irradiated femoral head	Half		
TP1006	Freeze-dried washed irradiated femoral head	Whole		
TP1007	Freeze-dried washed irradiated femoral head	Half		
TP1008	Freeze-dried washed irradiated femoral head	Slice		
TP3001	Irradiated ground cancellous/cortical mix	35cc		
TP3002	Freeze-dried irradiated ground cancellous/cortical mix	70cc		
TP3003	Freeze-dried washed irradiated cancellous/cortical - coarse	15cc		

APPENDIX J: TISSUE SERVICES

NOTE: Tendons are supplied either decontaminated or irradiated. Please specify at time of ordering

Item Code	Product (Tissue Services)	Volume per unit	Number of units required	Frequency (weekly, monthly etc)
TP3004	Freeze-dried washed irradiated cancellous/cortical - coarse	35cc		
TP3005	Dried washed irradiated cancellous/cortical - medium	15cc		
TP3006	Freeze - dried washed irradiated cancellous/cortical - medium	35cc		
TP3007	Dried washed irradiated cancellous/cortical --- fine	15cc		
TP3008	Dried washed irradiated cancellous/cortical --- fine	35cc		
TP4001	Osteochondral cryopreserved whole patella	1		
TP4008	Osteochondral cryopreserved femoral condyle left lateral	1		
TP4009	Osteochondral cryopreserved femoral condyle right lateral	1		
TP4010	Osteochondral cryopreserved proximal tibia left lateral	1		
TP4011	Osteochondral cryopreserved proximal tibia right lateral	1		
TP4012	Osteochondral cryopreserved femoral condyle left medial	1		
TP4013	Osteochondral cryopreserved femoral condyle right medial	1		
TP4014	Osteochondral cryopreserved proximal tibia left medial	1		
TP4015	Osteochondral cryopreserved proximal tibia right medial	1		
TP5009	Putty	1cc		
TP5010	Putty	5cc		
TP5011	Putty	10cc		
TP5012	Paste	1cc		
TP5013	Paste	5cc		
TP5014	Paste	10cc		
TP5015	Powder	10cc		
TP2010	Washed irradiated hemi-pelvis	1		
TP4002	Frozen washed irradiated proximal femur right	1		
TP4003	Frozen washed irradiated proximal femur left	1		
TP4004	Frozen washed irradiated distal femur left	1		
TP4005	Washed irradiated distal femur right	1		
TP4006	Washed irradiated proximal tibia left	1		
TP4007	Frozen washed irradiated proximal tibia right	1		
TP4019	Frozen washed irradiated proximal humerus left	1		
TP4020	Frozen washed irradiated proximal humerus	1		
TP7001	Cryopreserved aortic valve	1		
TP7002	Cryopreserved pulmonary valve	1		
TP7003	Cryopreserved non-valved aortic conduit	1		
TP7004	Cryopreserved non-valved pulmonary conduit	1		
TP7005	Cryopreserved superfacial femoral artery	Per cm		
TP7006	Pericardium	Patch small		
TP7007	Pericardium	Patch med[m]		
TP7008	Pericardium	Patch large		

APPENDIX J: TISSUE SERVICES

NOTE: Tendons are supplied either decontaminated or irradiated. Please specify at time of ordering

Item Code	Product (Tissue Services)	Volume per unit	Number of units required	Frequency (weekly, monthly etc)
TP7010	Cryopreserved Pericardium	1		
TPAdmin	Heart admin fee	1		
TP6001	Frozen whole achilles with bone block	>16cm		
TP6002	Frozen whole patella tendon – with bone block	1		
TP6003	Frozen whole patella tendon – with pre-shaped bone block	1		
TP6004	Frozen whole semitendinosus long	>27cm		
TP6005	Frozen whole semitendinosus medium	20-27cm		
TP6006	Frozen whole semitendinosus short	<20cm		
TP6015	Frozen whole extensor mechanism - custom	1		
TP6019	Frozen whole achilles with bone clock	<16cm		
TP6020	Frozen whole tibialis anterior long	>35cm		
TP6021	Frozen whole tibialis anterior medium	30-35cm		
TP6022	Frozen whole tibialis anterior short	<30cm		
TP6016-18	Meniscus is available either right or left and in a range of sizes. Please contact Customer Care. *NOTE: Tendons are supplied either decontaminated or irradiated. Please specify at time of ordering.*			
TP6016	Cryopreserved meniscus whole	1		
TP6017	Cryopreserved meniscus medial	1		
TP6018	Cryopreserved meniscus lateral	1		
TP9001	Frozen amniotic membrane	2x2cm		
TP9002	Frozen amniotic membrane	3x3cm		
TP9003	Amniotic membrane	5x5cm		
TP8006	dCELL Dermis® Human dermis small	3x3cm		
TP8007	dCELL Dermis® Human dermis medium	5x5cm		
TP8008	dCELL Dermis® Human dermis large	5x10cm		
TP8001	Cryopreserved split skin large pack	Minimum 330 CM2		
TP8003	Irradiated split skin large pack	Minimum 330 CM2		

Cost recovery will include next day delivery by 1pm for tissue products

NHS
Blood and Transplant

Part Academic Block
John Radcliffe Hospital
Headley Way
Headington
Oxford
OX3 9BQ

Tel: 01865 381066
www.nhsbt.nhs.uk

Sent via email to: [redacted]

Date: 26/05/2022

Dear Customer

Re: Non Clinical Issue Account – Request approved

Thank you for your recent application, which has been reviewed by NHSBT.

I am pleased to inform you that your application has been approved; a copy of this is enclosed for your records.

NHSBT is able to make these products available to you thanks to the generous actions of our donors. I have set out a few important pieces of information below to help ensure that we are able to meet your needs and to protect the interest of NHSBT and our valued donors.

I have also enclosed a Non Clinical Component Request form. You will need to complete one of these each time you request products.

Internal users are required to read SPN1562 before requesting NCI products.

Customer and Agreements Information:
Your customer account number is: D086

Our Agreement to supply you with products starts on **26/05/2022.**
Our Agreement ends on **31/05/2023** after which time, supply will stop.

If you wish NHSBT to consider an extension to this supply, we ask that you submit an application approximately 3 months before the end date to request continuation of materials from us. This will be subject to our assessment process.

Ordering Process:

- Each request must contain an ORDER NUMBER. This is a purchase order number raised within your organisation that will be used by NHSBT to raise an invoice. Purchase Orders must be sent to [redacted]

- **For Blood and Blood Components (Appendix A):**

Please email your request form to your assigned Hospital Services Department on: [redacted]

- **For Convalescent COVID Samples (Appendix B)**
  Please email your COBA Request Form to: [redacted]

- **For Cord Blood and Cord Tissue (Appendix C):**
  Please place your orders via https://www.smartsurvey.co.uk/s/4HF67/ or email your request form to: cordblood.orders@nhsbt.nhs.uk
  Office: [redacted] Laboratory: [redacted]

- **For Tissue (Appendix F):**
  Please email your request form to: [redacted]

Important information on the products and limitation of use:

1. The only products which may be ordered against this request are:

   - Leukocyte Cone

   These will be charged according to the published price list applicable at the time. A copy of the current pricelist is enclosed.

2. You may only use the products supplied for the use that has been approved. If you require any different products or if the intended use is not that stated on the original request form a new application form must be completed and authorised before any of these products will be supplied.

3. The products may only be used by, or on behalf of the persons that are named in your application.

4. The use of the supplied products by others and/or for uses not stated in your application is strictly prohibited and may result in immediate cessation of supply.

5. Products will be charged per item with a charge of £3.99 towards the cost of packaging per request.

   If you require the products to be delivered this will have to be agreed with the supply centre and an ad hoc delivery charge applied. Your supply centre can advise you of this cost.

6. Where credit is given, payment is due within 30 days from the date of the invoice. Failure to adhere to these terms may affect the continuation of supply of the product.

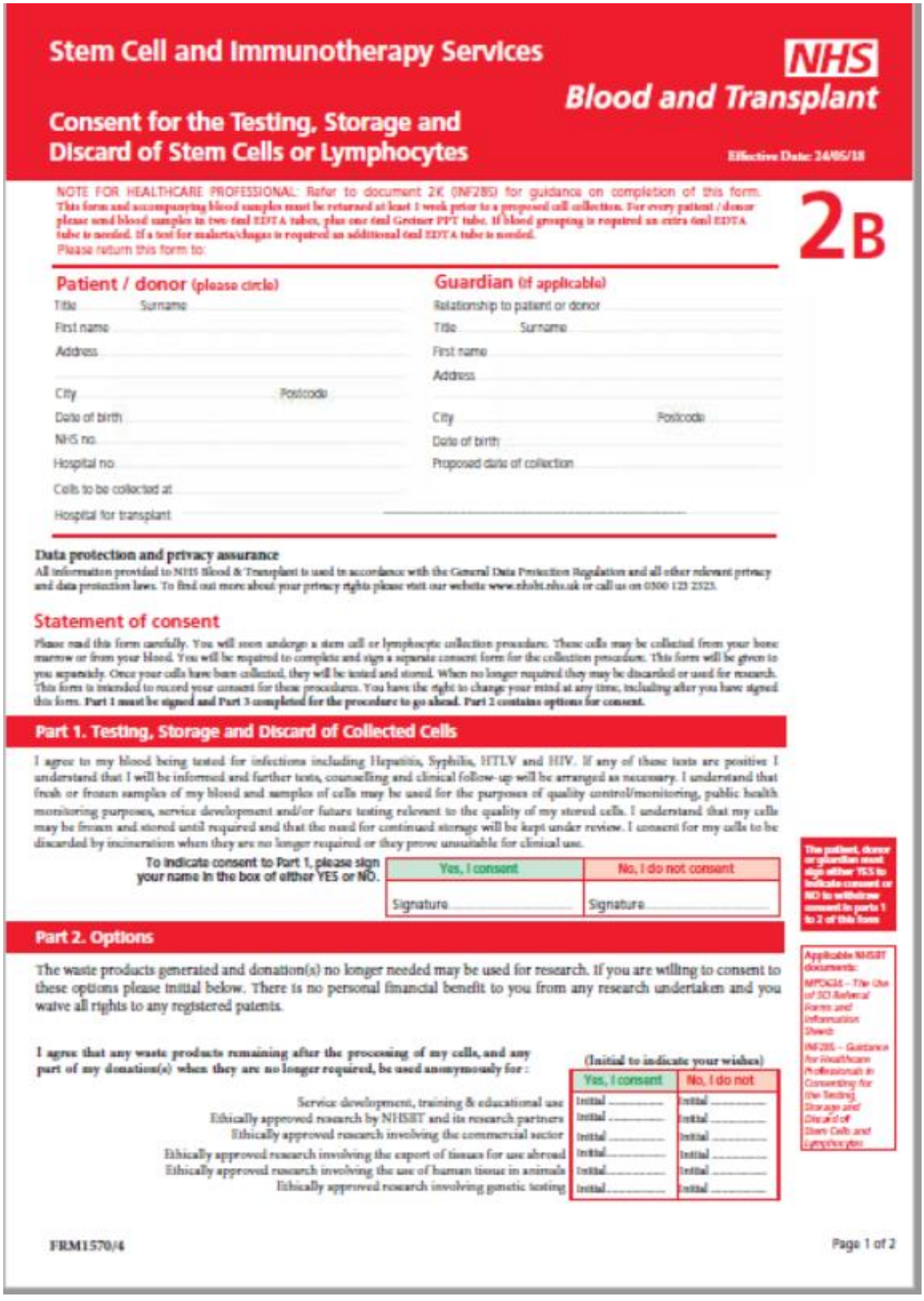
NHSBT looks forward to supporting your work. In the event of a query regarding supply, please contact me on [redacted]

Yours sincerely,

[redacted]
NCI Sales and Administration
nciadmin@nhsbt.nhs.uk

Your customer feedback regarding any element of your experiences with this service can be sent to NCIadmin@nhsbt.nhs.uk or my line manager [redacted] Where an issue has not been resolved, please go to: https://hospital.blood.co.uk/commercial-and-customer-service/complaints-compliments-and-feedback/

Appendix 3: FRM1570 Consent for Testing Storage and Discard of Stem Cells of Lymphocytes



Stem Cell and Immunotherapy Services

NHS Blood and Transplant

Consent for the Testing, Storage and Discard of Stem Cells or Lymphocytes

Effective Date: 24/05/18

2B

NOTE FOR HEALTHCARE PROFESSIONAL: Refer to document 2K (INF285) for guidance on completion of this form. This form and accompanying blood samples must be returned at least 1 week prior to a proposed cell collection. For every patient / donor please send blood samples in two 6ml EDTA tubes, plus one 6ml Gentner PPT tube. If blood grouping is required an extra 6ml EDTA tube is needed. If a test for malaria/chagas is required an additional 6ml EDTA tube is needed.

Please return this form to:

Patient / donor (please circle)

Title Surname

First name

Address

City Postcode

Date of birth

NHS no.

Hospital no.

Cells to be collected at

Hospital for transplant

Guardian (if applicable)

Relationship to patient or donor

Title Surname

First name

Address

City Postcode

Date of birth

Proposed date of collection

Data protection and privacy assurance

All information provided to NHS Blood & Transplant is used in accordance with the General Data Protection Regulation and all other relevant privacy and data protection laws. To find out more about your privacy rights please visit our website www.nhsbt.nhs.uk or call us on 0300 123 2323.

Statement of consent

Please read this form carefully. You will soon undergo a stem cell or lymphocyte collection procedure. These cells may be collected from your bone marrow or from your blood. You will be required to complete and sign a separate consent form for the collection procedure. This form will be given to you separately. Once your cells have been collected, they will be tested and stored. When no longer required they may be discarded or used for research. This form is intended to record your consent for these procedures. You have the right to change your mind at any time, including after you have signed this form. **Part 1 must be signed and Part 3 completed for the procedure to go ahead. Part 2 contains options for consent.**

Part 1. Testing, Storage and Discard of Collected Cells

I agree to my blood being tested for infections including Hepatitis, Syphilis, HTLV and HIV. If any of these tests are positive I understand that I will be informed and further tests, counselling and clinical follow-up will be arranged as necessary. I understand that fresh or frozen samples of my blood and samples of cells may be used for the purposes of quality control/monitoring, public health monitoring purposes, service development and/or future testing relevant to the quality of my stored cells. I understand that my cells may be frozen and stored until required and that the need for continued storage will be kept under review. I consent for my cells to be discarded by incineration when they are no longer required or they prove unsuitable for clinical use.

To indicate consent to Part 1, please sign your name in the box of either YES or NO.

Yes, I consent	No, I do not consent
Signature	Signature

The patient, donor or guardian must sign either YES to indicate consent or NO to withdraw consent in parts 1 to 2 of this form

Part 2. Options

The waste products generated and donation(s) no longer needed may be used for research. If you are willing to consent to these options please initial below. There is no personal financial benefit to you from any research undertaken and you waive all rights to any registered patents.

I agree that any waste products remaining after the processing of my cells, and any part of my donation(s) when they are no longer required, be used anonymously for :

	(Initial to indicate your wishes) Yes, I consent	No, I do not
Service development, training & educational use	Initial	Initial
Ethically approved research by NHSBT and its research partners	Initial	Initial
Ethically approved research involving the commercial sector	Initial	Initial
Ethically approved research involving the export of tissues for use abroad	Initial	Initial
Ethically approved research involving the use of human tissue in animals	Initial	Initial
Ethically approved research involving genetic testing	Initial	Initial

Applicable NHSBT documents:

MPDGSS – The Use of SCI Referral Forms and Information Sheets

INF285 – Guidance for Healthcare Professionals in Consenting for the Testing, Storage and Discard of Stem Cells and Lymphocytes

FRM1570/4

Page 1 of 2

Stem Cell and Immunotherapy Services

2B – continued

Name	Date of birth

Consent for the Testing, Storage and Discard of Stem Cells or Lymphocytes

Part 3. Signatures

To be completed by the patient, donor or guardian

I confirm that I have read and signed the above sections. I have received and understood sufficient information to give informed consent.

Name (print) ..... Signature ..... Date .....

To be completed by the Healthcare Professional with appropriate knowledge of the proposed procedures

I confirm that I have counselled and consented the patient, donor or guardian in accordance with NHSBT guidance document 2K (INF285) and the Department of Health *Reference Guide to Consent for Examination or Treatment*. I have discussed the nature of the proposed procedures and have discussed any particular concerns of the patient, donor or guardian. I have read and applied the HTA's codes of practice on the 'Donation of Allogeneic Bone Marrow and Peripheral Blood Stem Cells for Transplantation' and on 'consent'. I confirm that I have emphasised:

- the rationale for the stem cell or lymphocyte collection and its potential therapeutic benefits;
- the need for microbiology testing;
- storage issues, including the need for discard and the use of stem cells for research, service development and education.

I have provided the patient, donor or guardian with the following leaflets and tapes:

.....

.....

.....

Name (print) ..... Signature .....

Job title ..... Date .....

To be completed by the Interpreter (where appropriate)

I have interpreted the information above to the patient, donor or guardian to the best of my ability and in a way in which I believe he/she can understand.

Name (print) ..... Signature .....

Job title ..... Date .....

Important notes (tick if applicable)

Patient, donor or guardian has consented to participation in a clinical trial ☐

Patient, donor or guardian has withdrawn consent ☐

Additional information .....

.....

.....

Appendix 4: List of NHSBT Standard Operating Procedures used in the study

NHSBT does not routinely distribute copies of procedures to third parties, however copies of specific documents can be provided on request.

Document Number	Document Title
SOP412	Trima Apheresis Procedures
SOP2300	Thawing ampoules for viability and colony forming unit (CFU) assay
SOP2606	Processing and Cryopreservation of Cells by Closed Process
SOP5171	Cryopreservation Using a Planer Controlled Rate Freezer
SPN256	Process Specification for Stem Cell and Immunotherapy Products

Appendix 5: Detailed consumable list

Item	Code	Manufacturer
50mL Falcon Tubes	KDB326	Greiner Bio-One, Stonehouse, UK.
Falcon 50ml conical tube, sterile bagged	352070	Corning, Corning, USA
5mL Serological Pipette	86.1253.001	Sarstedt Ltd. Leicester, UK
25mL Serological Pipette	86.1685.001	Sarstedt Ltd. Leicester, UK
Spike Injection Port	4500069	OriGen Biomedical, Austin, TX USA
Falcon tubes 5ml with lids	352054	Fisher Scientific, Loughborough, UK
BD CD25 (IL-2 Receptor)	555434	Becton Dickinson UK, Winnersh Triangle, UK
BD CD69 (Very Early Activation Antigen)	555531	Becton Dickinson UK, Winnersh Triangle, UK
BD CD3	555339	Becton Dickinson UK, Winnersh Triangle, UK
BD CD4	557871	Becton Dickinson UK, Winnersh Triangle, UK
BD CD8	566858	Becton Dickinson UK, Winnersh Triangle, UK
BD CD19	555415	Becton Dickinson UK, Winnersh Triangle, UK
BD CD45	555482	Becton Dickinson UK, Winnersh Triangle, UK
BD CD45RA	555489	Becton Dickinson UK, Winnersh Triangle, UK
BD CD3	555336	Becton Dickinson UK, Winnersh Triangle, UK
BD CD16/56	561904	Becton Dickinson UK, Winnersh Triangle, UK
CD127 (IL-7 Receptor α chain)	560822	Becton Dickinson UK, Winnersh Triangle, UK
BD 7-AAD	559925	Becton Dickinson UK, Winnersh Triangle, UK
Pipette Tips 100-1000uL (96 per box)	LW6475S	Alpha Laboratories, Eastleigh, UK

Item	Code	Manufacturer
Pipette Tips 1-200uL (96 per box)	LW6360	Alpha Laboratories, Eastleigh, UK
Pipette Tips 0.1-10uL (96 per box)	ZS1020S	Alpha Laboratories, Eastleigh, UK
19G Needle	FTR586	Becton Dickinson UK, Winnersh Triangle, UK
10mL Serol. Pipette	734-1738	Sarstedt Ltd. Leicester, UK
50mL Serol. Pipette	734-1740	Corning, Corning, USA
Air Inlet	FSB545	Codan Ltd., Wokingham, UK
50mL Syringe	FWC408	Becton Dickinson UK, Winnersh Triangle, UK
BD Pharmingen Stain Buffer (FBS)	554656	Becton Dickinson UK, Winnersh Triangle, UK
SepMate™-50 100 pack IVD	85450	Stem Cell Technologies Vancouver, Canada
500ml Storage Bottles	430282	Corning, Corning, USA
Dulbecco's Phosphate Buffered Saline	Dulbecco's Phosphate Buffered Saline 500mL	Stem cell Technologies, Vancouver, Canada
6mL dockable syringe	RF-T15 6mL syringe	OriGen Biomedical, Austin, TX USA
Lymphocyte culture medium	Immunocult™-XF T Cell Expansion Medium 10981	Stem Cell Technologies Vancouver, Canada
4.5% Human Albumin Solution (HAS)	Zenalb 4.5%	BioProducts Laboratory Ltd. Elstree, UK
Interleukin 2 (IL2)	Recombinant IL2 (CHO expressed)	Stem cell Technologies, Vancouver, Canada
CD3/38 activation beads	Immunocult™ Human CD3/CD28 T Cell Activator	Stem Cell Technologies, Vancouver, Canada
24 well plates	Sarstedt 83.3922.500	Sartstedt AG & Co KG, Numbrecht, Germany
6 well plates	Stem Cell Technologies 38016	Stem Cell Technologies Vancouver, Canada

Item	Code	Manufacturer
Dimethyl Sulphoxide (DMSO)	Cryosure 50mL	WAK-Chemie Medical GmbH, Steinbach, Germany
CryoMacs freezing bags	Cryo 50/250	Miltenyi Biotec, Bergisch Gladbach, Germany
600mL transfer pack	Transfer pack with coupler – 600mL	Fresenius Kabi UK, Runcorn, UK
Absolute count tubes	TruCount™ Tubes	Becton Dickinson UK, Winnersh Triangle, UK

Appendix 6: Statistical comparison of CD4- cells from Panel 2 with CD8+ from Panel 3

	CD3+4- x 106/mL from P2 compared to CD3+8+ from P3														
	C1	**C2**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**	**C11**	**C12**	**C13**	**C14**	**C15**	**C16**
P1	2.55	3.03	1.07	4.68	0.83	4.23	3.57	1.02	1.77	1.31	2.07	3.42	1.74	0.89	1.95
P3	2.52	2.93	2.00	3.58	0.83	4.47	3.07	1.20	1.61	2.44	3.54	3.61	1.81	0.90	2.05

Wilcoxon matched pairs rank test (GraphPad Prism 9.5)

	Wilcoxon test	
4	vs.	vs.
5	Column A	P2
6		
7	**Wilcoxon matched-pairs signed rank**	
8	P value	0.3822
9	Exact or approximate P value?	Exact
10	P value summary	ns
11	Significantly different (P < 0.05)?	No
12	One- or two-tailed P value?	Two-tailed
13	Sum of positive, negative ranks	67.00 , -38.00
14	Sum of signed ranks (W)	29.00
15	Number of pairs	15
16	Number of ties (ignored)	1
17		
18	**Median of differences**	
19	Median	0.03000
20		
21	**How effective was the pairing?**	
22	rs (Spearman)	0.9143
23	P value (one tailed)	<0.0001
24	P value summary	****
25	Was the pairing significantly effective'	Yes