ANTHONY NOLAN
and
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Cell Separation and Cryopreservation of Cord Blood Fractions for Immunotherapeutic Applications

by

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Abstract

In 2008 Anthony Nolan opened the UKs first public umbilical cord blood (CB) bank (Anthony Nolan Cell Therapy Centre, ANCTC) in which CB is stored for haematopoietic stem cell (HSC) transplantation. Due to strict quality thresholds, the majority of units are not suitable for transplantation. Therefore, ANCTC aims to create a Biobank allowing these units to be stored for other purposes. To ensure cell products retained high levels of viability and potency, this study optimised banking processes starting with assessing the effects of transport conditions.It is essential that units are of the highest possible quality upon arrival at ANCTC, yet there is no consensus as to the optimal transport conditions of HSCs. Therefore, different fresh storage temperatures and the effect of delaying cryopreservation was assessed on three sources of HSCs. Cells were found to be better maintained at refrigerated temperatures and to avoid significant losses in potency, delays in cryopreservation should be minimised to <24 hours for bone marrow and <48 hours for CB and mobilised peripheral blood stem cells. Based upon these observations, ANCTC maintains all fresh samples at refrigerated temperatures and aims to cryopreserve them within 24 hours of collection.

Banking cells involves cryopreservation and potentially long term storage of samples, however, suboptimal cryopreservation conditions can result in reduced cell viability. Cryoprotectants are used to reduce damage during the freeze and thaw stages of cryopreservation but they have been linked to toxic effects observed in cells. Dimethyl sulphoxide (DMSO) was found to exhibit a dose-dependent toxic effect on CB and optimal concentrations were found to be between 7.5% and 10% (v/v). This study also highlights the importance of minimising exposure to DMSO to <1hour prior to freezing and <30 minutes post-thaw. In addition, the presence of 1% (w/v) dextran-40 in the cryoprotective solution was found to be crucial to maintaining cell potency.

The Biobank would require the storage of specific pure cell populations. Tregs are vital for the homeostasis of the immune system and have the potential to be used therapeutically in autoimmunity or transplantation, thus making these cells an ideal candidate for the Biobank. CB Tregs were found to have reduced suppressive abilities compared

to their adult counterparts, due to lower levels of FoxP3 intensity and CD39 expression. However, their higher frequency and a more defined $CD25^+$ population facilitates the isolation process. Two banking strategies were assessed, using both research and GMP grade methods. Firstly banking an isolated pure Treg population which, post-thaw, the cells maintained their phenotype but viability and suppressive ability was reduced. Alternatively, mononuclear cells were banked and Tregs isolated post-thaw. This strategy resulted in comparable isolation yields and purities compared to fresh cells, however, improved viabilities and higher suppressive abilities were observed compared to the cryopreserved pure Treg samples ($p=0.0012$). Therefore, this banking strategy was found to be more efficient.

Overall, this study has optimised banking procedures from the transport of fresh samples to isolation and cryopreservation of pure cell populations. Therefore, this study has laid the foundations for the creation of a Biobank within the ANCTC allowing the distribution of cell products not only for research purposes, but also potentially for their use in immunotherapeutic interventions.

I wish to dedicate this thesis to my parents,

for their love and endless support

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I would like to take this opportunity to express my utmost appreciation and gratitude all those who made this thesis possible.

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LIST OF ABBREVIATIONS

§	Section
4C	Refrigerated temperature (4-8°C)
20C	Room temperature (19-22°C)
7AAD	7-Aminoactinomycin
AnnV	Annexin V
ANCTC	Anthony Nolan Cell Therapy Centre
BFU-E	Burst-Forming Unit-Erythroid
BM	Bone marrow
CB	Cord blood
CBMC	Cord blood mononuclear cells
CCR4	C-C chemokine receptor type 4
CFSC	Carboxyfluorescein succinimidyl ester
CFU	Colony Forming Unit
CFU-E	Colony Forming Unit-Eythrocyte
CFU-GEMM	Colony Forming Unit-Granulocyte,Erythrocyte,Monocyte,Megakaryocyte
CFU-GM	Colony Forming Unit-Granulocyte,Monocyte
CPD	Citrate phosphate dextrose
CRF	Control rate freezer
DMSO	Dimethyl sulphoxide
DNase	Deoxyribonuclease
FACT Netcord	Foundation for the Accreditation of Cellular Therapy-Netcord
FACS	Fluorescent activated cell sorting
Ficoll	Ficoll density gradient cell separation method
FoxP3	Forkhead box P3
FSC	Forward scatter
GMP	Good manufacturing practice
Gr	Granulocyte

GvHD	Graft versus host disease
GvL	Graft versus leukaemia
HCT	Haematocrit
HLA	Human leukocyte antigen
HPC	Haematopoietic progenitor cell
HSC	Haematopoietic stem cell
HSCT	Haematopoietic stem cell transplant
ICOS	Inducible T-cell costimulator
ISHAGE	International Society of Haematology and Graft Engineering
MACS	Magnetic activated cell sorting
MB	Maternal blood
MBio	Miltenyi Biotec MidiMACS
MFI	Median Fluorescence Intensity
MHC	Major histocompatibility complex
MNC	Mononuclear cells
Mr Frosty	Nalgene Mr Frosty freezing container
PB	Peripheral blood
PBSC	Peripheral blood stem cells
RBC	Red blood cells
SCT	Stem Cell Technology EasySep
SSC	Side scatter
TCD	Total containment device
TCR	T cell receptor
TNC	Total nucleated cells
Treg	T regulatory cells
WB	Whole blood

CHAPTER 1

INTRODUCTION

1.1 Haemopoiesis

Blood is highly regenerative; in an adult approximately one trillion (10^{12}) cells are generated daily.[1] Haemopoiesis (from the Greek $\alpha\tilde{\imath}\mu\alpha$ (haima): blood and $\pi o\iota\epsilon\tilde{\imath}\upsilon$ (poiein): to make) is the process in which blood cells are formed. This process is continuous and responds rapidly to haemorrhage, infection or hypoxia.[2] The location of haemopoiesis changes during foetal development; initially in the foetal yolk sac, six weeks after conception it moves to the foetal liver and spleen, after six months and onwards haemopoiesis occurs in the bone marrow (BM).[3]

The haematopoietic system can be divided into four sections: haematopoietic stem cells (HSC), progenitor cells (HPC), precursor cells and fully differentiated cells with specialised functions. During haemopoeisis HSCs divide, one daughter cell remains a HSC, whilst the other daughter cell becomes a multipotent HPC committed to differentiation along a specific lineage. Haemopoiesis continues along one of two lineages, myeloid or lymphoid (shown in Figure 1.1).

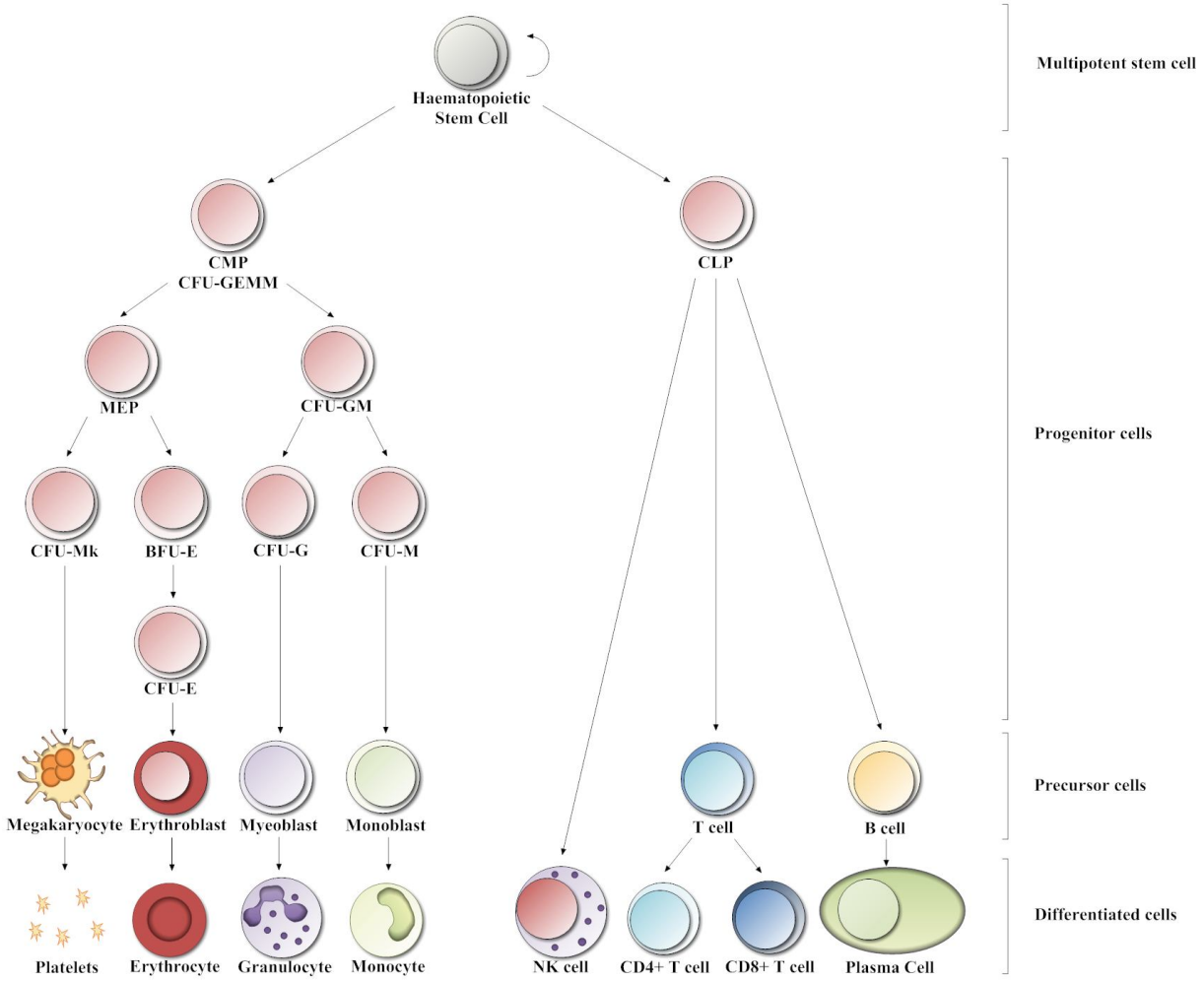

FIGURE 1.1: Overview of haemopoiesis through the common myeloid and lymphoid progenitors. CMP, common myeloid progenitor; CFU-GEMM, granulocyte-erythrocyte-macrophage-megakaryocyte colony forming unit; MEP, megakaryocyte-erythroid progenitor; CFU-Mk, megakaryocyte colony forming unit; BFU-E, erythrocyte burst forming unit; CFU-E, erythrocyte colony forming unit; CFU-GM, granulocyte-macrophage colony forming unit; CFU-G, granulocyte colony forming unit; CFU-M, macrophage colony forming unit; CLP, common lymphoid progenitor; NK, natural killer cell. Figure created using SmartDraw and adapted from Stirewalt *et al.*[4]

1. Myeloid lineage

   The common myeloid progenitor (CMP) is a granulocyte-erythrocyte-macrophage-megakaryocyte colony forming unit (CFU-GEMM) which differentiate into a range of different cells such as megakaryocytes which produce platelets; anucleated erythrocytes; monocytes which circulate the peripheral blood producing macrophages and dentritic cells at the site of infection, and granulocytes, a collection of granulocytic cells with a lobed nucleus.

Granulocytes consist of: short lived phagocytic neutrophils which are the most abundant leucocyte (50 to 60%); eosinophils which have cytotoxic properties particularly against parasites (1-3% of leucocytes); basophils which play a role in the inflammatory response and are the least common leucocyte (<2%); and mast cells which function in a similar manner to basophils and play an important role in the development of allergy.

2. Lymphoid lineage

   The common lymphoid progenitor (CLP) differentiate into natural killer (NK) cells which are cytotoxic cells essential for innate immunity, B cells which mature in the BM and on activation form plasma cells which secrete antibodies and T cells which mature in the thymus into $CD8^+$ cytotoxic T cells or $CD4^+$ helper cells with the ability to differentiate further upon activation (described in §1.6.2.3).

1.2 Haematopoietic stem and progenitor cells

The concept of stem cells arose from studies by Till and McCullogh on the irradiation of BM in mice.[5] Stem cells are defined as undifferentiated cells with the capacity to divide and self-renew indefinitely. More explicitly, upon cell division stem cells produce an identical daughter stem cell (self-renewal) and a progenitor cell with a more limited differentiation potential. Whereas cells cultured *in vitro* can divide only a finite number of times (40-60 times depending on telomere length) prior to senescence,[6] stem cells are able to divide indefinitely due to the expression of telomerase.[7] Stem cells can be classified depending on the numbers of different cells they can differentiate into. This is defined as cell potency and stem cells can be divided into four different groups.

1. Totipotency

   These are the most versatile of the different stem cells and only include the fertilised egg (zygote) and the first few divisions in embryonic development. These cells have the potential to divide and differentiate into any cell type found within the body including extra-embryonic tissues such as the placenta. Beyond the first few divisions the cells specialise into pluripotent stem cells.

2. Pluripotency

   Like totipotent stem cells, these cells can give rise to all three germ layers (endoderm, mesoderm and ectoderm) and therefore, can form any cell found within the body, however, they do not have to potential to differentiate into the cells required to form the placenta and are therefore, not totipotent.

3. Multipotency

   Descendants of pluripotent cells, these cells are less plastic and can only differentiate in to cells along a certain lineage, for example HSCs which produce all the different types of blood cells.

4. Unipotency

   These cells have to ability to differentiate into only one specific cell type and are often referred to as progenitor cells. For example the erythroid progenitor cells (BFU-E and CFU-E) have only the potential to differentiate into erythrocytes.

1.2.1 Characterisation of HSC and HPCs

The cluster of differentiation (CD) are a series of protein molecules that are used as markers to identify specific cell populations. CD molecules are often functional and can be receptors or ligands or involved in cell adhesion and homing.[8,9] No single marker for identifying all HSCs has been identified. This is made difficult as markers are gained and lost as the cell differentiates. CD34 was the first marker to be identified on HSCs and is currently the most commonly used marker for their identification and during enrichment protocols.[10] CD34 is believed to play a role as an adhesion molecule through the attachment of HSCs to the BM extracellular matrix or to stromal cells.[11] The $CD34^+$ population include the majority of cells with the ability to repopulate the haematopoietic system, all of the long-term culture initiating cells (LTC-ICs) and many of the Colony Forming Units (CFUs).[10] This $CD34^+$ population contains a mixture of HSCs and HPCs which can be differentiated by their different levels of expression of other markers. There is however, evidence of primitive $CD34^-$ cells with HSC properties which can generate $CD34^+$ *in vitro* and *in vivo*.[12,13] These $CD34^-$ cells can be further characterised through

expression of CD133 and absence of CD38 and lineage-specific markers. CD133 (AC133) expression patterns in HSCs overlap with the expression of CD34; CD133 is expressed on the majority of $CD34^+$ cells including the more primitive stem cells and the $CD34^-$ HSCs.[14,15] HSC and HPCs do not express the markers associated with differentiated blood cells (lineage markers or lin). These markers can include CD2, CD3, CD4, CD7, CD8, CD10, CD11b, CD14, CD19, CD20, CD56, CD235a and identify T cells, B cells, NK cells, monocytes and granulocytes.

1.2.2 Sources of HSCs

1.2.2.1 Bone Marrow as a source of HSCs

In an adult human haemopoiesis occurs within the bone marrow (BM), this is a soft spongy highly vascularised tissue located in the interior of bones. There are two types of BM; the medulla ossium rubra (red marrow) in which the haematopoietic cells reside and medulla ossium flava (yellow marrow) which consists mainly of fat cells. At birth all BM consists of red marrow, the proportion of yellow marrow increases with age and the ratio between the two is roughly half and half in adults. However, at times of severe blood loss, the yellow marrow can convert back to red marrow to increase blood cell production through haemopoesis. Schofield in 1978 proposed the concept of a stem cell niche within the BM, this is compromised of stromal cells which associate with the HSCs and provide specific signals that help maintain their function.[16] The location of the HSCs within the niche remains controversial and it has been suggested that there is more than one niche between which the HSCs migrate between in response to injury or certain migration signals.[17]

HSC harvests from BM are performed under general anaesthetic, the cells are taken from the red marrow in the iliac crest of the pelvis. The needle can be repositioned after each aspiration in order to isolate the required number of cells. CD34 has been found to be expressed in 1-4% of the nucleated cells extracted from this site.[10]

1.2.2.2 Peripheral blood as a source of HSCs

In response to specific signals HSCs can migrate from the BM to the blood stream (mobilisation) and back to the BM (homing). CD34 is expressed in approximately 0.06% of nucleated cells found within the blood periphery.[18] However, it is possible to mobilise HSCs from the BM into the peripheral system. This is achieved through administering the cytokine granulocyte colony-stimulating factor (G-CSF) over three or four days. This increases the percentage of $CD34^+$ cells to >1%, these cells can then be harvested by leukapheresis.

1.2.2.3 Cord blood as a source of HSCs

CB has been found to contain similar CFU-GM numbers compared to BM and is considered to be a good source of HSC and HPCs.[19] CB can be collected either *in utero* (during the third stage of labour prior to delivery of the placenta) or *ex utero* from vaginal or caesarean deliveries. A large needle is inserted into the umbilical vein and the blood drains into an attached collection bag containing anticoagulant. The procedure is non-invasive and there is no harm to the mother or baby. From each placenta a volume of ∼100ml can be collected. As the volume of blood collected is limited to what remains in the placenta and cord after it has been clamped, CB collections tend to yield lower cell counts compared to BM and PBSC collections.

1.3 HSC Transplants

HSC transplants (HSCT) have routinely been used to reconstitute the haematopoietic system after high dose chemotherapy.[20,21] Patient conditioning treatments originally involved high doses of chemotherapy and total body irradiation leading to BM ablation destroying tumour cells and creating space in the stem cell niches within the BM for the transplanted HSCs to home to. Since a graft-versus-leukemia (GvL) effect was observed during autologous transplants,[22] the intensity of the pre-transplantation conditioning has been reduced. Levels are high enough to avoid rejection of the transplanted cells,

but allow the GvL effect to eliminate the tumour cells. This new conditioning regime may lead to higher rates of relapse, but the mortality associated with transplantations has been reduced significantly.[23]

The first HSCTs were reported in 1959 in which two patients with leukaemia received BM cells from identical twins.[24] HSCTs are currently used to treat a range of malignant haematological diseases and congenital BM disorders. According to the Worldwide Network for Blood and Marrow Transplantation (WBMT) the one millionth HSCT was performed in December 2012.[25]

HSCTs can be performed using cells from the patient (autologous) or from a donor (allogeneic). Autologous transplants involves the harvesting of HSCs from the patient's own BM or PBSCs; these cells are cryopreserved and stored until the patient completes the pre-transplantation conditioning regime, at which point the cells are thawed and re-infused. Allogeneic transplants require a volunteer to donate their HSCs to a patient. High levels of human leukocyte antigen (HLA) matching is required between donor and patient to avoid graft-versus-host-disease (GvHD). As all the HLA genes are located on one chromosome there is a 25% chance a sibling will be a match, therefore, many patients will not find a suitable donor within their family. In the UK alone there are currently 1,800 patients searching for a match.[26] The first successful HSCT from an unrelated donor occurred in 1973 in New York. This led to the creation of the first register of people willing to donate their HSCs to strangers in 1974 called the Anthony Nolan Bone Marrow Register*. There are currently over 22,000,000 donors from 72 different registers across 52 countries registered with the Bone Marrow Donors Worldwide (BMDW).[27] With these large numbers of volunteers willing to donate, caucasians have a 60% chance of finding a donor with suitable levels of HLA matching.[28] However, patients with uncommon HLAs or those from ethic minorities have a reduced chance of finding a suitable donor. In these cases, a haploidentical sibling or mismatched unrelated donor may be used. These transplantations are associated with an increased risk of GvHD and samples are

*Anthony Nolan was born in 1971 with the rare condition Wiskott-Aldrich syndrome for which the only cure was HSCT. With no suitable match found within his family his mother, Shirley Nolan, established the first BM register which she named after her son. Sadly a suitable donor could not be found for Anthony Nolan who died in 1979 aged eight.

often depleted of T cells prior to infusion leading to increase risk of graft rejection, and disease relapse as the GvL effect is reduced.[29,30]

The European Group for Blood and Marrow Transplantation (EBMT) conducted a survey between 1990 and 2010 in 654 transplant centres.[31] Over this time period, 30,012 patients received HSCTs, 59% were autologous and 41% allogeneic. The EBMT reported that the overall survival 5 years after HSCT is approximately 53%, and 44% after 10 years.

1.3.1 Graft-versus-host-disease

GvHD is a major complication that can arise during HSCT. GvHD is initiated when donor T cells raise an immunological response against the host and is a significant cause of mortality and morbidity following allogeneic HSCTs. GvHD occurs in approximately 60% fully HLA matched HSCTs,[28] but the frequency and severity increases greatly if there is an HLA mismatch between donor and host.[32,33] There are two forms of GvHD with distinct clinical manifestations:

1. Acute GvHD

   Usually observed within 100 days after transplantation as the HSCs engraft, but can occur later if a reduced intensity conditioning regime is used prior to HSCT. Acute GvHD (aGvHD) targets the skin resulting in a maculopapular rash, liver, gastrointestinal tract and occasionally the eye and oral mucosa.[34] Severity of aGvHD is graded (0-IV) based on factors such as the levels of bilirubin and diarrhoea and the percentage of the body surface covered by the rash.[35] Patients with grade I-II aGvHD have a long term survival rate of >80% patients with grade III or IV aGVHD tend to have poor outcomes with long term survival probability of 30% and <5% respectively.[36] Patients are treated with a combination of immunosuppressive drugs such as cyclosporine and methotrexate.[37] Approximately 50% of patients with aGvHD will also develop chronic GvHD (cGvHD).[38]

2. Chronic GvHD

   Occurring more than 100 days post-HSCT, there may be some overlap period between aGvHD and cGvHD occurrence. However, cGvHD is a distinct syndrome with features similar to autoimmune disorders and can develop even when the patient did not have aGvHD. Many organs can be affected by cGvHD including the skin, liver, eyes, gastrointestinal tract and the lungs. cGVHD is a major cause of long term morbidity and mortality in patients and has a significant effect on the quality of life after HSCT. However, cGvHD can also lead to an increase in GvL. As in aGvHD, cGvHD is also treated with immunosuppressive drugs, but these drugs are required for much longer periods of time extending over months to years.

1.3.2 The use of CB in HSCT

The use of CB to treat cancer was first reported by Ende in 1963. The patient suffered from lymphangiosarcoma and had previously been treated with surgery, chemotherapy and radiation. With no further treatment options, 17 CB units were infused (untested for HLA compatibility) with the hope that 'youth factors' could inhibit the sarcomas. Although the patient initially showed improvement, she died within a year of the transplant.[39] In 1966 a patient with myelogenous leukaemia was given 500ml CB, assessment of the patients blood after the transplant discovered only the donors cells remained, which without the knowledge that CB contained HSCs was not thought to be feasible.[40] CB was used to treat leukaemia again in 1970 on a 16 year boy with acute lymphoblastic leukaemia. The patient received eight CB units untested for HLA compatibility. The patient also received continued treatments with chemotherapy and remained in remission for nearly one year.[41]

In 1989 it was discovered that CB was a source of CFU-GM, BFU-E and CFU-GEMM and could be used as an alternative to BM for HSCT.[19] The first successful cord blood transplantation was performed the same year in Paris by Gluckman *et al*:[42] Haematopoietic reconstitution was achieved in a 5 year old boy with Fanconi anaemia through a cord blood transplant from his HLA-identical sister. This patient remained in remission

and is still alive today. In 1996, the first unrelated mismatched CB transplantation was performed in a child[43] and in an adult.[44] Since then thousands of HSCTs using CB have been performed.[45]

The main advantage of using CB over adult sources of HSCs is the almost immediate availability of a unit, due to CB banks which store samples at cryogenic temperatures. On average the transplant centre can receive a CB unit 25-36 days earlier than a donation from an adult donor.[46] These units are HLA typed and tested for bacterial and viral contaminations prior to freezing. Therefore, CB units are available when the patient requires them without inconveniencing a donor. A reduction in the frequency and severity of GvHD has been noted when using CB for transplants rather than adult sources of HSCs. However, the GvL effect remains. This allows units with only partial HLA matching of 4/6 to be tolerated after transplantation, making it more likely to find a suitable match for a patient.[47] Other advantages include the ease of procurement with no risk to the donor, a reduced likelihood of viral infections being transmitted and no need to maintain databases of potential donors who may become untraceable or unable to donate for medical or personal reasons.

The $CD34^+$ cell count of a unit being transplanted has been linked to time to engraftment.[48] Therefore, as CB units contain lower cell doses compared to BM and PBSC, the major disadvantage of using CB in transplants are the increase in time to platelet and neutrophil engraftment.[49] In an attempt to overcome this, two CB units from different donors can be simultaneously infused into a patient.[50,51] Engraftment is usually achieved by only one infused unit, the reason why one CB unit dominates over the other is unknown.[52] However, there is one reported case of long term haematopoietic reconstitution with substantial contributions from both CB units infused.[53]

1.4 CB banking

In 2009 it was reported that over 20,000 CB transplants have been performed[45] and there are currently over 600,000 CB units in the BMDW database from 47 CB banks in 33 countries.[27] The first CB bank (CBB) was created in New York in 1993.[54,55] This

involves the long term storage of CB samples under cryogenic temperatures. Broxmeyer *et al* reported that to avoid cell losses, in CB which already has reduced counts compared to BM, samples should be cryopreserved as whole samples.[19] However, Rubinstein *et al* suggest reducing the volume and increasing the cell concentration would allow for more efficient storage and avoid the high costs for liquid nitrogen usage.[55] This group developed a protocol involving the addition of hydroxyethyl starch (HES) to the blood causing the erythrocytes form a rouleaux which along with some plasma can be removed after centrifugation to reach the required volume.

Two types of CB banks exist; private banks in which the stored CB is for exclusive use by the family who pay for the service, or public banks in which CB samples are stored with the potential to be released to any patient that requires them. The odds of requiring an autologous transplant has been reported to be between 1 in 1000 and 1 in 200,000.[56] In addition to this, autologous transplants are often unsuitable as most conditions that require a transplant would already exist in the patient's CB unit. Therefore, many physicians who perform paediatric HSCTs recommend against the private storage of cord blood for autologous use.[57]

1.4.1 CB bank regulations

Three European Union directives (2004/23/EC,[58] 2006/17/EC[59] and 2006/86/EC[60]) were established to ensure the quality and safety of human tissues and cells is maintained from collection to their use in therapy. The relevant countries have added these directives to national legislation, designating competent authorities to implement them.[61] Within the UK, the collection and banking of CB (regarded as a tissue) is regulated by the Human Tissue Authority (HTA) under the Human Tissue (Quality and Safety for Human Application) Regulations 2007.[62] The HTA has produced guidance documents for the procurement, processing, storage and distribution of CB.[63] This document states that processing procedures must be validated either by the establishment or based on data from published studies and these validated methods must be documented in Standard Operating Procedures (SOPs).

CB banks also have the opportunity for voluntary accreditation programs by adhering to standards developed by consensus of world-renowned experts within the field. The Foundation for the Accreditation of Cellular Therapy (FACT) is a non-profit corporation co-founded by the International Society for Cellular Therapy (ISCT) and the American Society of Blood and Marrow Transplantation (ASBMT). FACT establishes standards for high quality medical and laboratory practice in cellular therapies and promotes continued progress within this field. In 1997, FACT collaborated with The International NetCord Foundation (NetCord), a non-profit international organisation of the largest CB banks, and together published the first NetCord-FACT International Standards for Cord Blood Collection, Processing, Testing, Banking, Selection and Release with new editions published approximately every three years. These NetCord-FACT standards are universally recognised by transplant programs and are based upon published medical evidence whenever possible. When published data is not available, requirements are based upon accepted scientific theory.

1.4.1.1 Good Manufacturing Practice

Good Manufacturing Practice (GMP) systems ensure products are consistently produced and controlled in accordance to relevant quality standards.

Within the EU, there are two directives describing the principles and guidelines of GMP for medicinal products: 2003/94/EC applies to medicinal products for human use[64] and Directive 91/412/EEC for veterinary use.[65] GMP guidelines are not a series of instructions on how to manufacture products, instead they are a series of general principles which must be applied to the manufacturing process. GMP guidelines are regularly revised and therefore, GMP is often referred to as current GMP or cGMP.

GMP covers all aspects of both production and quality control. Production should be clearly defined, controlled and performed in accordance with pre-established instructions and procedures. These procedures should be validated to ensure consistency and compliance with pre-defined specifications and critical process regularly re-validated.

GMP facilities are regularly inspected by competent authorities using Quality Risk Management principles. Failure to comply with GMP standards whilst making a medical product can result in product recall, fines and even custodial sentences.

1.4.2 Biobank

CB is being increasingly used over BM and PBSC in haematopoietic stem cell transplants. Once considered to be a waste product from birth, CB is now collected from the umbilical cord and placenta and sent to cord blood banks where it is processed (involving a volume reduction procedure), cryopreserved and stored until it is required for a transplant. One such cord blood bank was established at Nottingham Trent University by Anthony Nolan, the charity that established the worlds first bone marrow register. Anthony Nolan's Cord Blood Bank (Anthony Nolan Cell Therapy Centre, ANCTC) has instigated high quality thresholds for CB units in order to be banked for potential HSCT. Examples of these quality thresholds include a minimum total nucleated cell (TNC) count of 1.2×10^9 and a minimum $CD34^+$ count of 3.2×10^6 cells. Units that do not meet these requirements can be used for other purposes including research. Anthony Nolan aims to create a research grade Biobank within the CB bank where the research grade units can be cryopreserved and stored either as whole blood, isolated CB mononuclear cells (CBMC) or pure isolated cell types. These CB cellular fractions can be stored until they are required by researchers for their projects.

1.5 Cryopreservation

A Biobank requires the cryopreservation and long term storage of samples. Successful cryopreservation of mammalian cells was first documented by Polge *et al* in 1949 when they successfully revived bull spermatozoa which had been frozen in glycerol.[66] The techniques used in 1949 were used again by Barnes *et al* in 1955 in the cryopreservation of mouse spleens which upon thawing were still viable and functional and could regenerate the haematopoietic system in irradiated mice.[67] Over the years the techniques of HPC

cryopreservation were further developed through the assessment of different cooling and thawing rates and the use of different cryoprotectants.[68–79]

1.5.1 Risk to the cells during cryopreservation

During the process of cryopreservation the highest risk to the cells are during freezing and thawing; no further damage should occur during the storage period if the temperatures are low enough to halt cell metabolism.[80,81] This can be achieved through storing the cells below temperatures of -130°C which has been shown to solidify the storage medium sufficiently to halt metabolism.[82] As a result of this, lower storage temperatures of -196°C have resulted in higher post-thaw recoveries than samples stored at -70°C.[83,84] Despite the DNA-repair system being inactive, background radiation does not appear to have any effect on cells undergoing long term storage.[85]

1.5.1.1 Risk to the cells during freezing

The rate of cooling during freezing can have a significant impact on cells (Figure 1.2). Cooling the cells slowly favours extracellular ice formation where thermal exchange with the freezing chamber is faster and the likelihood of a nucleation point is higher.[82,86] The removal of liquid water results in an increasing salt concentration in the extracellular space, this increases the osmolality and pulls the water out of the cells resulting in their shrinkage.[87] However, cells can be cooled too slowly; if the cells are maintained at sub-zero temperatures before freezing they are exposed to high concentrations of solutes for prolonged periods of time. This can cause the cells to become dehydrated and shrink to the point of damaging their membranes.[88] This is known as the solutions effect[87,89] and leaves the cells more susceptible to thermal shock and mechanical stress caused by ice formation.[87,90,91] If cells are cooled rapidly, ice crystals will form in both the extra- and intra-cellular regions as insufficient water is removed. This intracellular ice damages the internal structure of the cells and membrane leading to cell death.[92] A cooling rate of 1-3°C minute^{-1} has been shown to be optimal when freezing BM.[93]

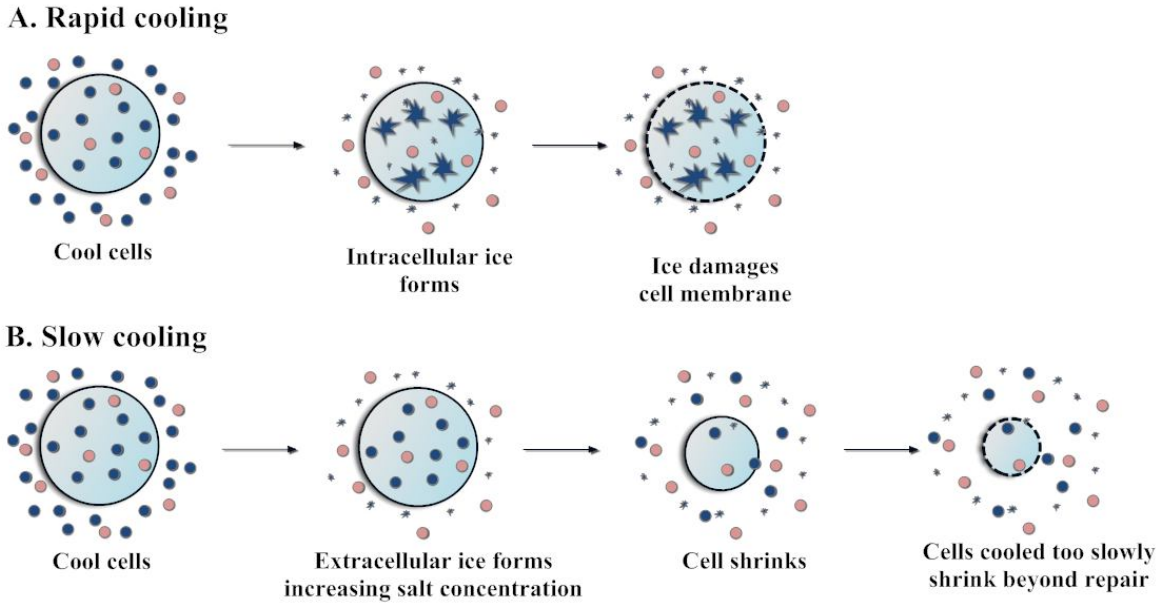

FIGURE 1.2: The effect of water on cells during cryopreservation: Freezing (A) Rapid cooling of cells results in the formation of ice in the extra- and intra-cellular regions. Intracellular ice crystals cause damage to the membrane resulting in cell lysis. (B) Slow cooling of the cells results in the formation of extracellular ice increasing extracellular solute concentration leading to efflux of water and cell shrinkage. Diagram created using SmartDraw.

The duration of the phase transition, when the water solidifies releasing its latent heat, correlates with cell mortality.[72,94] This is an important step of the freezing process which needs to be controlled, especially when freezing larger samples.[86] The first controlled rate freezer (CRF) apparatus in which samples are placed inside a chamber and liquid nitrogen pumped in intermittently to reach the required temperature was developed by Hill *et al.*[95] The use of a CRF ensures the freezing conditions are reproducible through a series of defined temperature changes (the freezing protocol currently used at ANCTC is shown in Figure 1.3). The chamber of the CRF (represented by the red line in the graph) is cooled at a rate of 1°C minute^{-1} until a temperature of -10°C is reached, at this point the heat of fusion is liberated from the sample. To compensate for the release of heat and avoid rewarming the sample (represented by the green line) the temperature of the chamber is reduced by -20°C minute^{-1} reaching -50°C and then rewarmed back to -18°C to prevent the sample from cooling too quickly once the latent heat has been compensated for. The temperature of the sample is decreased further and at an increasing faster rate down to the target temperature of -120°C. At this point the

sample is removed from the chamber of the CRF and transferred to the vapour phase of liquid nitrogen storage tanks (maintained at <-170°C).

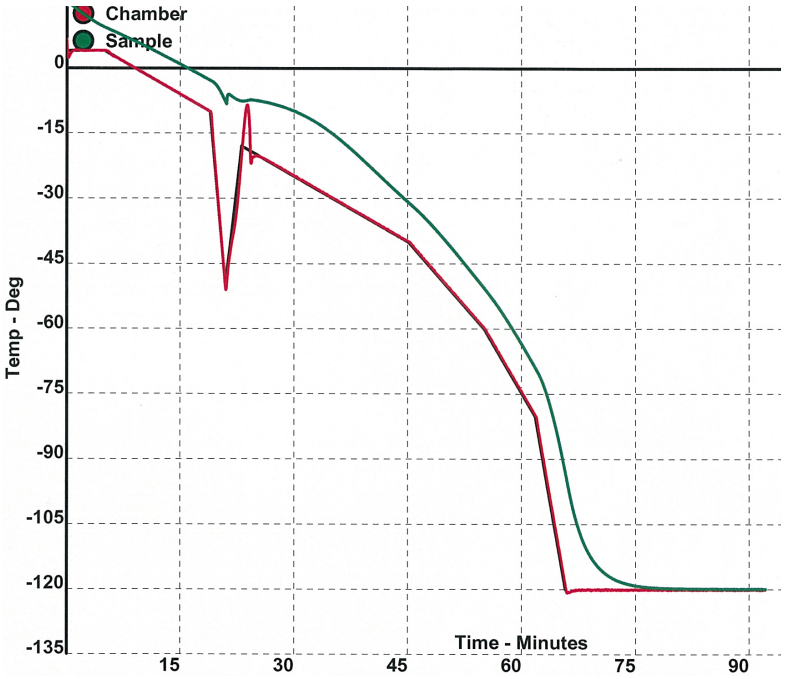

FIGURE 1.3: Control Rate Freezer (CRF) freezing program used at ANCTC

1.5.1.2 Risk to the cells during thawing

The speed of thawing of a sample has also been shown to be critical to the viability of cells (Figure 1.4). A rapid thaw, involving immersing the sample into a water bath at 37°C, has been shown to be less damaging to cells as ice crystals are thawed quickly preventing them from joining into larger crystals leading to structural disruption.[74] As the ice crystals melt forming liquid water, the salt concentration in the extracellular region is reduced resulting in an influx of water into the cells causing them to swell which can lead to lysis.

1.5.2 Cryoprotectants

Cryoprotective agents are used during cryopreservation to prevent the damage caused by the formation of ice and by changes in osmotic pressure.

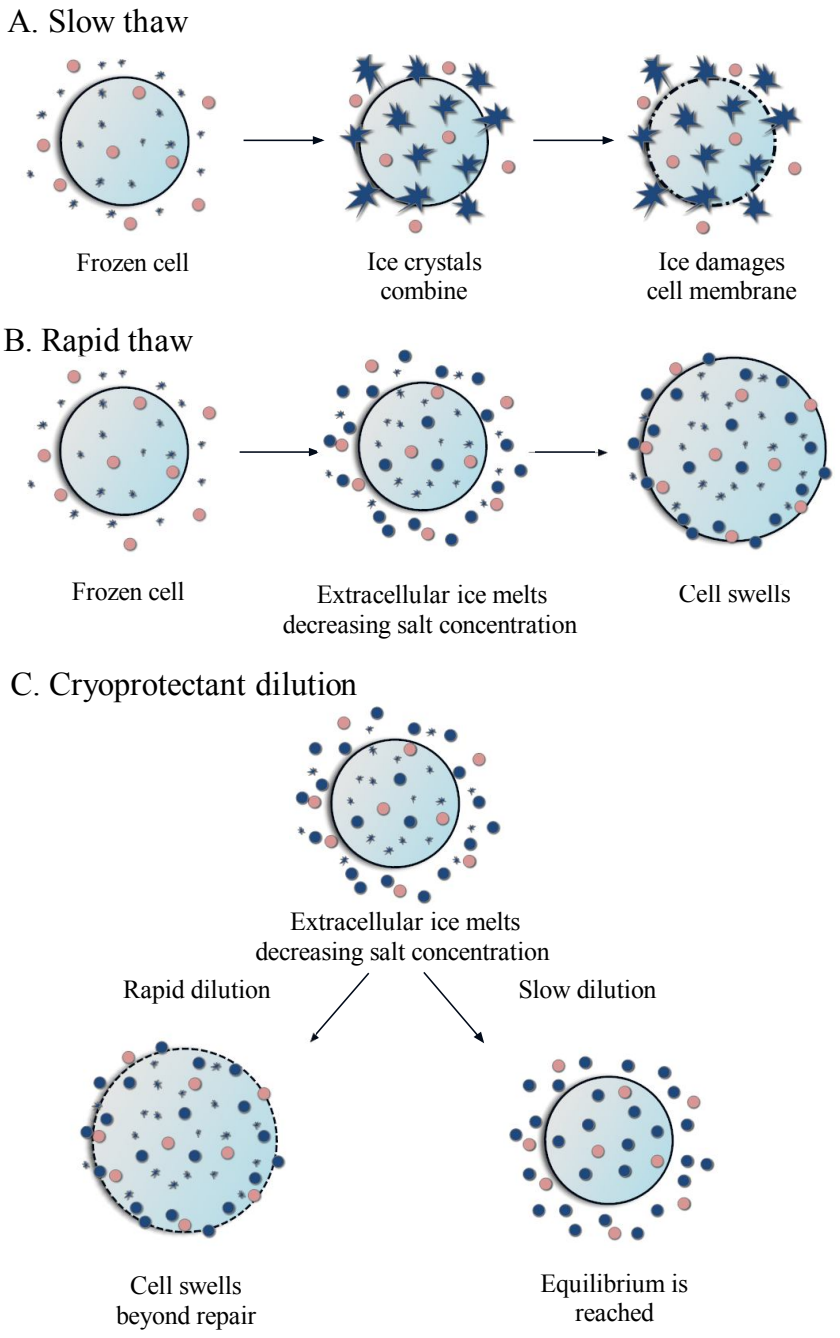

FIGURE 1.4: The effect of water on cells during cryopreservation: Thawing. (A) Slow thawing of cells leads to ice crystals combining into larger crystals causing membrane damage. (B) As the ice melts the salt concentration in the extracellular portion of the sample decreases leading to the influx of water causing the cell to swell potentially to the point of damage. (C) Rapid dilution of the cryoprotectant post-thaw exacerbates the osmotic pressure, whereas slow dilution allows equilibrium to be reached. Diagram created using SmartDraw.

There are two types of cryoprotectants:

1. Intracellular or penetrating cryoprotectants - penetrate the cell preventing formation of ice crystals within the cell (e.g. glycerol, dimethyl sulphoxide (DMSO)).

2. Extracellular or non-penetrating cryoprotectants - work by further increasing the solute concentration in the extracellular region to cause further removal of water from the cells and reduce the possibility of intracellular ice formation[96] (e.g. hydroxyethyl starch (HES) and dextran).

1.5.2.1 The use of DMSO in cryopreservation

Initially glycerol was used as a cryoprotectant before DMSO was introduced as an alternative option[97,98] and shown to be superior in the cryopreservation of HPCs.[99,100]

DMSO is a naturally occurring polar aprotic solvent which acts by forming hydrogen bonds with extracellular water, thereby slowing the formation of ice crystals and reducing the osmotic pressure and solution effect.[101] The ease at which a cryoprotectant can pass through the cell membrane is important in cryopreservation. Cryoprotectants that pass through the membrane slowly (e.g. glycerol) or rapidly (e.g. DMSO) bind to the water molecules in the cytoplasm blocking the efflux of water preventing cellular dehydration and shrinkage.[102,103] These cryoprotectants can also prevent the formation of intracellular ice by reducing the probability of its nucleation.[96] It has also been suggested that DMSO works by stabilising the tertiary structure of protein and lipid complexes.[72,104] The ability of a cryoprotectant to penetrate the membrane is also important during the thawing of a sample. The melting of the extracellular ice crystals causes an osmotic gradient, water enters the cell and the cryoprotectant needs to diffuse out of the cell rapidly to avoid the cell from swelling.

1.5.2.1.1 Toxic effects of DMSO in patients

DMSO is believed to have dose-dependent toxic effects and has been linked to adverse reactions during HSCT.[105–107] Some of the minor side effects from transplantation such

as nausea and abdominal cramps are believed to be caused by the intravenous infusion of a chilled sample.[105] A survey assessing current practices in the use of DMSO at transplant centres found a wide range of different concentrations were used (2.2-20%), with a trend of higher incidence and severity of adverse reactions in patients who had been infused with larger doses of DMSO.[102] DMSO and its metabolites dimethyl sulfide and dimethyl sulfone have been found in the plasma of patients for up to 48 hours after infusion.[108,109] These compounds have been linked to side effects including cardiovascular complications,[106,110,111] respiratory problems,[112,113] anaphylactic reaction,[102] renal dysfunction[114] and neurological toxicity.[115–118] It has also been observed that the odour of DMSO from patients has caused headaches and gastrointestinal reactions in oncology nurses.[109] Toren *et al* suggested DMSO may have some anti-tumour effects upon infusion by inducing the differentiation of malignant cells,[119] however there has never been any further support for this concept. Despite the removal of DMSO prior to infusion, adverse reactions can still occur.[120] Therefore, other factors must contribute to the adverse reaction and in this regard the number of granulocytes infused has been found to correlate with the level of severity of the adverse reactions.[121]

1.5.2.1.2 Toxic effects of DMSO to the cells

As well as toxicity in patients, DMSO may also cause cellular injury. Douay *et al* report a progressive loss of CFU in BM after exposure to 10% (v/v) DMSO at 4°C with a recovery of 24% after 30 minutes decreasing further to 10% after 3 hours.[122] However, Rowley *et al* found no toxic effects of 10% DMSO to BM HPCs at 4°C or 37°C, although they observed a loss in HPCs when higher concentrations of cryoprotectant were used or the exposure time extended.[123] Branch *et al* found no significant losses in CFU when BM remained in 8% (v/v) DMSO for up to 2 hours after thawing.[124]

DMSO has been shown to induce apoptosis by interacting with programmed cell death receptors and their ligands.[125,126]Another suggested mechanism of DMSO toxicity is via denaturation of proteins caused by the formation of hydrogen bonds with the cryoprotectant,[127] in particular with fructose dehydrogenase resulting in the impairment of glycolysis.[128]

The effects of freezing and thawing may not affect all cells equally, as $CD34^+$ cells have been shown to be more resistant to the damage caused by cryopreservation than other nucleated cells.[129] It has also been observed that within the $CD34^+$ population the $CD38^-$ early progenitor cells demonstrate higher resistant to the effects of cryopreservation than more mature $CD38^+$ cells.[130]

Current CB cryopreservation protocols were established to maintain HPC viability. Granulocytes are much more susceptible to cooling and freezing injury compared to other cells and are not preserved efficiently under these conditions. Post-thaw they lyse releasing nucleoprotein and lysosomal enzymes which promotes clumping of the sample.[131,132] Different freezing protocols have been developed in order to successfully cryopreserve granulocytes these involve combining DMSO with HES as cryoprotectants.[133,134]

Also, the toxic effects of DMSO may not be equal across different cells, as Douay *et al* found higher levels of toxicity in the more mature BM precursor cells[122] and Hiroshi Ara *et al* also found the more mature BFU-E to be more sensitive than CFU-E.[135]

The speed of DMSO addition and removal is believed to be a contributing factor to cell death, as rapid addition may cause the cells to shrink below their critical minimal volume due to an abrupt increase in osmotic pressure.[136,137] In order to avoid damage caused by osmotic shock and the latent heat released, slow addition of a pre-cooled DMSO solution has been suggested.[55,122] However, this has been contradicted by Meyer *et al* who report improved post-thaw quality after 'fast' addition of DMSO to CBMC.[138] In contrast, Radke *et al* found no difference in the percentage of apoptotic $CD34^+$ cells when comparing DMSO addition speeds in fresh CB.[139]

1.5.2.1.3 DMSO concentrations

A survey to EBMT (European Society for Bone and Marrow Transplantation) centres on the various uses of DMSO for stem cell transplantation showed that a wide range of concentrations were used when cryopreserving cells (2.2-20% v/v)with 10% being the most commonly used.[102] As DMSO has been shown to be toxic to patients in a dose related manner, the use of lower concentrations to prevent this has been suggested.[105–107]

However, this will also have an effect on the ability to cryopreserve viable cells and CFUs.

It has been reported that the use of 5% (v/v) DMSO is the optimal concentration and results in higher post-thaw CFU recoveries.[140–142] However, other groups have found no significant difference in viable cell and CFU recovery between the use of 5% or 10%.[143–145] The effect of a reduction in DMSO concentration on transplant outcomes was evaluated by Akkök *et al*, and it was reported that the concentration of DMSO is independent of the time to neutrophil and platelet engraftment.[146] In addition, the proportion of adverse reactions has been found to be higher when using 10% than 5% (19.1% versus 6.8% respectively).[143]

7.5% (v/v)DMSO has been reported to be the optimal concentration for CFU recovery in PBSC samples, despite the TNC recovery being higher when using 10%.[147] Donaldson *et al* also report 7.5% (v/v) DMSO to be optimal concentration for cryopreservation with higher $CD34^+$ recoveries. They observed 5% to be as effective as 10%, and concentrations below resulted in very low recoveries of $CD34^+$.[148] However, concentrations as low as 2.2% have been shown to engraft successfully in patients undergoing HSC transplants, although the post-thaw cell recoveries are not reported.[149]

Combinations of cryoprotectants have also been used to reduce the concentration of DMSO required. Adding the non-penetrating cryoprotectant HES along with DMSO has been shown to reduce the post-thaw clumping caused by granulocytes.[150] In PBSC samples, the use of DMSO and HES together has been shown to result in similar[151] if not higher[152,153] CFU recoveries. However, there is no apparent advantage in combining the use of HES with DMSO when cryopreserving CB.[148]

1.5.2.1.4 Removal of DMSO post-thaw

Due to the toxic effects observed to cells and patients, the removal of DMSO through a wash procedure has been suggested. This involves a slow dropwise dilution with an equal volume of a dextran-40 and albumin solution to reduce osmolality (Figure 1.4C).[55,154]

Washing DMSO from samples post-thaw is a time consuming procedure and can be technically difficult with large cell losses reported.[55,155] Therefore, automated cell washing devices have been developed to remove DMSO post-thaw under a closed system and these include the Cytomate,[156] SEPAX[157] and Cobe.[158]

However, washing and spinning of thawed BM has been shown to result in cell clumping leading to losses.[159] To avoid clumping of the cells post-thaw, DNase is often added to the washing solution to break down free DNA and disperse any clumps.[148,160,161] However, Bayer *et al* however found that DNase did not help to prevent clumping.[162]

There are contradicting reports on the effect that washing samples has on engraftment speed; some report no significant difference between washed and unwashed samples,[163,164] Kurtberg *et al* found washing samples facilitated engraftment speed[43] and Akkok *et al* found washing led to an increase in platelet engraftment by two days and recommend washing to be necessary only for patients with a high risk of DMSO toxicity.[165] Therefore, some transplant centres will directly infuse thawed HPCs into a patient[86] despite documented evidence of toxic effects caused by infused DMSO,[166] and others administer the samples in fractions to prevent toxicity.[167]

1.5.2.2 The use of dextran-40 in cryopreservation

Dextran is a complex branched polysaccharide comprised of glucose units of varying lengths. Dextran has multiple applications including its use in surgery,[168] vaccines,[169] ophthalmology[170] and as a blood volume expander where it can be used to replace lost blood when a donor is not available.[171]

Dextran is also used as a non penetrating cryoprotectant. Studies assessing the use of dextran when cryopreserving erythrocytes observed that the optimal concentration was dependent upon the rate of cooling during freezing, with 25-40% (w/v) dextran being optimal for a drop of 1°C second^{-1} whereas 15-21% (w/v) was found to be optimal for a cooling rate of 4-8°C second^{-1}.[172–174]

Rubinstein *et al* state that in preliminary experiments the presence of 1% (w/v) dextran-40, in combination with 10% (v/v) DMSO, improved the post-thaw viability of CB

leucocytes by 3-10%.[55] Based on this article, Dextran-40 has been commonly used in combination with DMSO when cryopreserving CB. However, this data has never been published.

Available is multiple molecular weights up to 2,000 kDa, larger dextran molecules of 60 kDa or more are poorly excreted from the kidney and remain in the blood until they are metabolised into glucose and water.[175] Although 70% of dextran-40 (molecular weight: 40 kDa) is excreted by the kidney within 24 hours of intravenous infusion, it has still been linked to cases of toxicity during HSCT.[176,177] Therefore, it has been suggested that dextran-40 should be removed from the cryoprotective solution or that alternative cryoprotectants should be considered.[176]

1.6 An overview of immunology

The role of the immune system is to protect from infectious microorganisms though the recognition of pathogens and then react to eliminate them. The immune system is composed of two components; the innate and adaptive response. The innate system is non-specific, fast acting, but short-lived. In contrast, the adaptive system is highly specific and generates immune memory which improves the capacity of the host to eliminate subsequent infections from the same pathogenic challenge.

1.6.1 Innate immunity

Innate immunity provides the first line of defence against pathogens and consists of physical barriers (epithelial surfaces including the skin) and chemical substances (for example the low pH within the stomach or microbial substances such as lysozyme in saliva). Considering the human body is exposed to high numbers of potentially infectious agents, yet rates of infection are low, it can be assumed that these barriers prevent the vast majority of pathogens from entering the body. Once an infectious agent passes into the tissues, it is immediately recognised by phagocytes such as the macrophages, neutrophils and dendritic cells (DC) which engulf and destroy the invading microorganism, along with natural killer (NK) cells, through cytotoxic actions.[178] Activation of these cells

releases cytokines (such as IFN-γ, TNF-α, IL-1, IL-6, IL-8 and IL-12) which initiates inflammation.[178] Inflammation is characterised by calor (heat), rubor (redness), tumor (swelling) and dolor (pain). These changes are due to the increased rate of blood flow to the site and an accumulation of fluid, proteins and more immune cells from the blood. An important component of the innate system are the Toll-like receptors (TLR) found on phagocytic cells.[179] Activation of the TLR stimulates the production of cytokines, such as TNF-α and the expression of the co-stimulatory molecules B7.1 (CD80) and B7.2 (CD86), thereby facilitating the adaptive response.[178]

1.6.2 Adaptive immunity

The innate immune system is non-specific, defending only against pathogens with certain molecular patterns that are not found on host cells, and its ability to defend against the same pathogen repeatedly is unaltered. In contrast, the adaptive immune system, although slower to respond, leads to a long-lasting memory of specific pathogens and allows more rapid protection against repeat encounters.[180,181] Induction of adaptive immunity begins when an antigen presenting cell (APC), such as a DC, ingests a pathogen and displays small fragments of it's peptides on the major histocompatibility complex (MHC) on the cell surface.[182,183] This activates the APC which up-regulates its expression of co-stimulatory molecules and migrates to the lymph node, where it may come into contact with lymphocytes. The adaptive system can be separated into two parts, humoral or cell-mediated immunity which are performed by B cells and T cells respectively.

1.6.2.1 Humoral response

The humoral response involves B cells, so called because they are produced and mature in the bone marrow. naïve B cells circulate the secondary lymphoid tissues and peripheral blood where they are activated after encountering a specific pathogen and a helper-T cell activated by the same antigen.[184] Upon activation, B cells mature into plasma cells which secrete large quantities of antibodies or immunoglobulins (Ig) with a unique structure specific to the presented antigen.[185] The antibodies circulate the bloodstream

until they come into contact with the antigen that stimulated their production. Antibody binding inactivates viruses and marks pathogens, recruiting other cells to destroy them. During activation memory B cells are also produced which have the ability of rapid clonal expansion if the host is re-exposed to the same pathogen.[185]

1.6.2.2 Cell mediated response

The cell-mediated response involves T cells which are produced in the bone marrow, but mature in thymus. These naïve cells circulate between the blood and lymphoid periphery until they come into contact with an APC displaying their specific antigen. The T cell recognises the MHC presentation of the antigen through the T cell receptor (TCR). In addition to the interaction of the TCR with the MHC, a second interaction is required between CD28 on the T cell and B7.1 (CD80) or B7.2 (CD86) on the APC (TCR from a $CD4^+$ cell and MHC class II binding shown in Figure 1.5).[186] If only the TCR-MHC interaction occurs the cell becomes unresponsive and potentially anergic.[187] Upon encounter with their antigen and binding of the co-stimulatory molecules, the cell is activated and proliferation induced, thereby generating a population of antigen-specific effector T (Teff) cells. $CD8^+$ T cells are activated through the MHC class I molecules to produce cytotoxic T cells (CTL) which induce apoptosis in their target cells through the release of the lytic enzyme granzyme and perforin. $CD4^+$ cells are activated through MHC class II molecules and differentiate into different subsets with different functions depending on the cytokines secreted by the APC.[188,189]

Major histocompatibility complex

The major histocompatibility complex (MHC) was first discovered during the rejection of transplanted tumours.[191,192] In humans these genes are called human leucocyte antigen (HLA) and are situated on the short arm of chromosome 6 (6p21.3).[193] This contains the genes for MHC class I (HLA-A, -B and C) and class II (HLA-DR, -DP and -DQ). These two classes are composed of distinct subunits which fold to have similar 3D structures. Both MHC class I and II molecules consist of two polypeptide chains forming four subunits; three of the MHC class I subunits are formed from an α chain and the

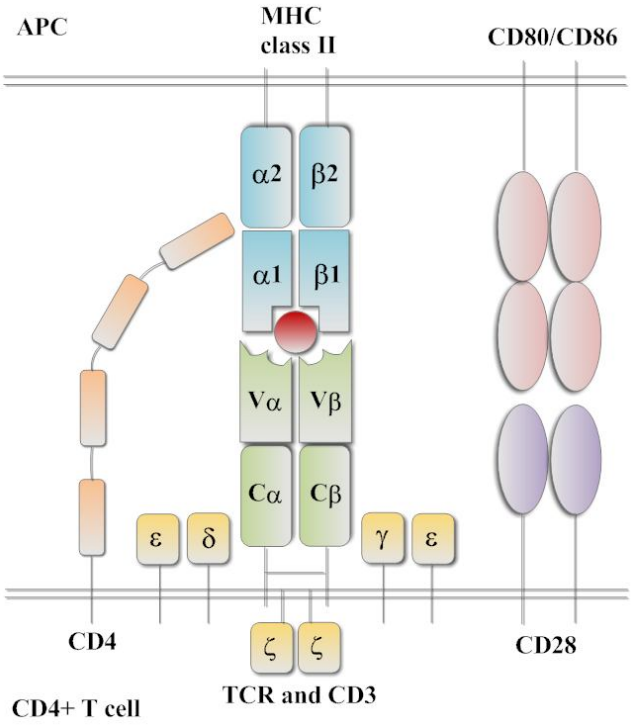

FIGURE 1.5: Binding of the TCR complex on a $CD4^+$ cell to the MHC class II molecule with co-stimulation by CD28 to B7. Figure created using SmartDraw and adapted from Janeway *et al.*[190]

remaining subunit a β_2-microglobulin.[194,195] The α_1 and α_2 subunits form the cleft and binding site for the antigen whilst the α_3 subunit spans the membrane. The MHC class II molecule also consists of an α and a β chain folding to form four subunits.[196] The antigen binding cleft is formed of subunits α_1 and β_1 whilst α_2 and β_2 both span the membrane.

The different classes present antigens to different T cells and have different levels of expression on different cells. MHC class I binds to peptides from the cytosol and presents them to $CD8^+$ T cells which when activated, have the ability to kill any cell recognised. These peptides are usually from viruses that have replicated in the cell and so MHC class I molecules are expressed on all nucleated cells. MHC class II molecules presents peptides, that have been internalised and degraded within vesicles to $CD4^+$ T cells and are expressed in cells that participate in the immune response.[197]

T cell receptor

The antigens presented by APCs on the MHC molecules are recognised by T cells via the T cell receptor (TCR). The TCR on the majority of T cells is composed of an α and β chain[†] linked by a disulphide bond and similar in structure to the Fab fragment of an Ig molecule.[200] The two chains of a TCR consist of a constant (C) region which spans the membrane and a variable (V) amino-terminal region. The ability of a T cell to recognise a specific antigen and the wide variety of antigens recognised by different T cells lies within the formation of the V region. The genes that encode this region are split into different segments; V, D and J gene segments.[201] Whilst the T cell is maturing in the thymus, these genes rearrange themselves creating a variety of different TCRs making each one unique and able to bind to a specific peptide on a given MHC molecule.[202,203] A failure to rearrange these genes, such as in SCID (severe combined immunodeficiency) mice, removes the adaptive immune system and is fatal.[204]

The TCR recognises the antigen, but is unable to signal this to the cell, it is therefore, associated with the CD3 complex consisting of γ, δ, ϵ and ζ chains.[200] Each CD3 chain contains an intracellular immunoreceptor tyrosine-based activation motif (ITAM) which is phosphorylated upon antigen recognition by the tyrosine kinases Fyn and Lck, initiating a signalling pathway leading to activation of the T cell.

1.6.2.3 Activated CD4$^+$ cell lineages

Once activated, the CD4$^+$ T cells differentiate into different subsets depending on the type of antigen presented, the cytokines secreted by the APC and the strength of the TCR-MHC interaction (Figure 1.6).[188,189] If high levels of IL-12 are secreted, Th1 cells are generated through the transcription factors STAT4 (signal transducer and activator of transcription) and T-bet.[205] These cells produce high levels of IFN-γ, TNF-β and IL-2 and play a role in activation of macrophages and development of CTLs.[206,207] In the presence of IL-4, the activated CD4$^+$ cell differentiates into Th2 cells requiring the activation of GATA3 and STAT6. Th2 cells secrete IL-4, IL-5, IL-9 and IL-13

[†]The TCR on a small subset of T cells present at epithelial surfaces consists of γ and δ chains. These $\gamma\delta$-cells are predominantly CD4$^-$CD8$^-$ and can act as part of both the innate and adaptive immune response with the capacity to function as APCs whilst also having anti-tumour properties.[198,199]

supporting the development of the humoral response by stimulating B cells to differentiate into plasma cells and produce antibodies.[205,208] Th17 cells are distinguished by their production of IL-17 and have a specialised developmental pathway, which is distinct from Th1 or Th2 cells. The combination of TGF-β and IL-6 induce the differentiation of Th17 cells through the activation of STAT3.[209,210] This in turn induces the expression of the transcription factor retinoic acid-related orphan receptor gamma t (RORγt).[209,210] Once induced, Th17 effector function and phenotype is maintained by the presence of IL-23.[209] As well as IL-17, Th17 cells also secrete IL-21, IL-22 and, in humans, IL-26.[211,212] Through these cytokines, Th17 cells play a role in the defence against extracellular pathogens.[211,212] Differentiation of follicular helper T (Tfh) cells requires IL-21 and the transcription factor Bcl-6.[213] These cells secrete IL-21 and regulates B cell maturation.[214] $CD4^+$ T cell activation with high levels of TGF-β secretion leads to the differentiation of an induced T regulatory (iTreg) population[215–217] (discussed further in §1.7.3.1).

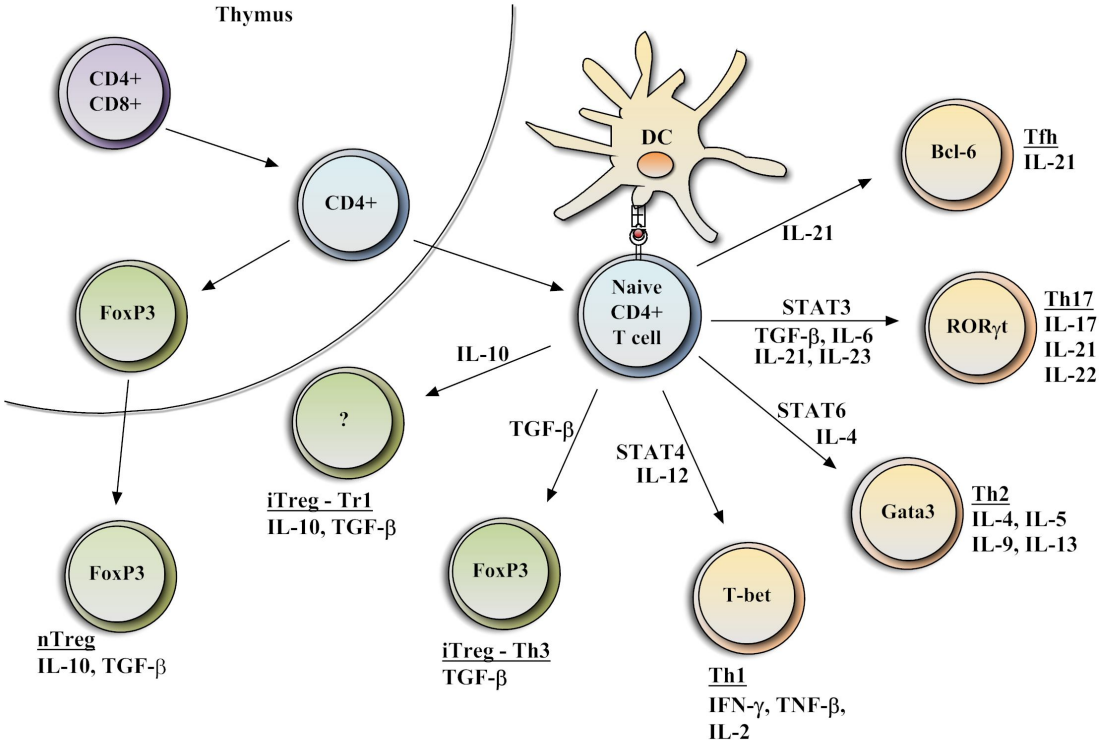

FIGURE 1.6: After activation via the TCR $CD4^+$ cells mature into Th1, Th2, Th17, Tfh or iTregs depending on the type of antigen presented, the cytokines present and the strength of the TCR-MHC interaction. Figure created using SmartDraw and adapted from O'Shea *et al.*[218]

1.6.3 Immunological tolerance

In the 1900's Ehrlich and Morgenroth proposed that the immune system, whilst able to mount a response against an antigen, has a mechanism of avoiding reactivity to itself.[219]

Lymphocytes with receptors that strongly bind self antigens are removed before developing into immunocompetent cells. This occurs as they mature in the bone marrow and thymus for B and T cells respectively. Mechanisms for negative selection include induction of anergy and deletion via apoptosis.

Tolerance through anergy

Tolerance can be achieved through the functional inactivation of T cells whilst maintaining their viability, this is termed anergy. There are two mechanisms of initiating anergy; clonal anergy and adaptive tolerance.[220] Clonal anergy is induced through incomplete activation of a T cell by antigen recognition minus the co-stimulation from the APC required. This leads to downregulation of IL-2 preventing the cells from proliferating. This is observed mostly in T cells which have been previously activated and can be reversed by IL-2 signalling. Adaptive tolerance occurs mostly in naïve T cells and can be induced in the presence of a persistent antigen. Adaptive tolerance differs from clonal anergy in that as well as reduced IL-2 production, other cytokines such as IL-4 and IFN-γ are down regulated and the anergic state is maintained through persistence of the antigen, and is not reversed by IL-2.[221]

Tolerance through deletion

In response to a pathogen, T cells are activated, undergo proliferation and differentiate to form Teff cells. Once the infection has been cleared, large numbers of activated Teff cells remain which are no longer needed. To prevent the accumulation of these potentially harmful cells, they are removed through apoptosis.[222] Apoptosis can be initiated through different mechanisms, such as through the activation of caspase-8 via increased expression of Fas and its ligand FasL.[223] Another pathway leading to the onset of apoptosis involves the pro-apoptotic protein Bim, which via the release of cytochrome

c from the mitochondria, leads to the activation of caspase-9.[224,225] Mice deficient for both Fas and Bim accumulate T cells and develop autoimmunity,[226,227] thereby suggesting thaat these two pathways play important roles in lymphocyte homeostasis.

Dominant tolerance

The presence of a specific T cell population can actively suppress the actions of other immunological cells through a variety of mechanisms.[228] This population is known as regulatory T cells (Tregs) and are the cells chosen to focus on within this project when banking a pure isolated cell population.

1.7 Regulatory T cells

The concept of a cell capable of exerting suppressive activity was first suggested in 1970. Gershon and Kondo demonstrated that lymphocytes derived from the thymus of mice could prevent specific immune responses against exogenous antigens, they named these cells 'suppressor cells'.[229] However, due to a lack of reliable molecular techniques at the time, these suppressor T cells could not be further characterised and so their existence was questioned.[230] Suppressor cells were observed again in a series of experiments in which high dose UVB exposure generated suppressor T lymphocytes in the spleen and lymph nodes of mice.[231–233] However, it was Sakaguchi *et al* in 1995 who were the first to identify these cells by demonstrating that a population of $CD4^+$ cells co-expressing the IL-2 receptor α chain (CD25) had a suppressive function in mice.[234] These cells, renamed regulatory T cells (Tregs), constituted approximately 5-10% of peripheral $CD4^+$ T cells and were found to be suppressive, but anergic.[235,236] A similar population was identified in humans in 2001,[237–242] and over the years it has become clear that Tregs are critical in human immunological homeostasis via their ability to control autoimmune responses.[243–247]

1.7.1 Development of Tregs

Multi-organ autoimmunity can be induced in mice through neonatal thymectomy, and the adoptive transfer of $CD4^+CD25^+$ cells from normal mice can prevent autoimmune disease.[248–250] This, combined with the observation that the depletion of $CD4^+CD25^+$ cells in mice results in short term autoimmunity, led to the conclusion that Tregs develop in the thymus.

In humans, it is believed that Hassall's corpuscles, concentric epithelial cells found within the medulla of the thymus, contribute to Treg development by expressing thymic stromal lymphopoietin (TSLP). This activates dendritic cells which, in turn, induce the differentiation of thymic $CD4^+$ T cells into $CD4^+CD25^+$ regulatory cells.[251,252]

1.7.2 Phenotype of Tregs

The Treg population in mice has been well defined as being $CD4^+CD25^+FoxP3^+$. Phenotyping human Tregs has proved to be difficult, as it has been found that these markers are not specific to a regulatory population.[253–258] The human Treg population has been found to be heterogeneous in both phenotype and functional ability.

1.7.2.1 CD25

IL-2 has a range of effects on T cells leading to proliferation, differentiation, cytokine production and even immune tolerance.[259,260] Tregs do not secrete IL-2, but do express the IL-2 receptor. The IL-2 receptor consists of three subunits, an α chain (CD25), β chain (CD122) and the common cytokine receptor γ chain (CD132). Of the three subunits, only CD25 is specific to the IL-2 receptor, whereas CD122 and CD132 form parts of other cytokine receptors.[261–263] Once bound, the IL-2-IL-2R complex is rapidly internalised, the CD25 section is recycled and returned to the cell surface, whereas IL-2, CD122 and CD132 are degraded in the lysosomal compartment.[264] The $CD25^+$ population in mice has been found to contribute to the maintenance of self tolerance via the down regulation of immune responses to self and non-self antibodies in an antibody

non-specific manner.[234] However, CD25 is up-regulated on activated T cells, and in adult humans a large population of $CD4^+CD25^+$ memory cells can be observed.[265] Only cells with the highest CD25 expression ($CD25^{high}$) have been shown to have suppressive abilities and can therefore, be considered regulatory.[242] $CD4^+CD25^{high}$ T cells are also enriched for many other surface markers, although none have been found to be unique to Tregs. Therefore, a combination of CD25 expression with other markers, linked to Tregs and suppressive function, is used for the identification of Tregs.

1.7.2.2 FoxP3

FoxP3 (forkhead box P3) is a member of the forkhead/winged-helix family of transcriptional regulators.[266] The forkhead box is a DNA-binding motif which allows FoxP3 to regulate expression of the genes controlling cell proliferation and differentiation.[267]

Mutations in FoxP3 are associated with 'Scurfy' in mice[268–270] and immunodysregulation, polyendocinopathy and enteropathy X-linked (IPEX) syndrome in humans.[271] This mutation changes the amino-acid sequence in the DNA-binding domain of the protein,[272–274] leading to dysfunction of Tregs and therefore, dysregulation of the immune system and subsequent autoimmunity. This disorder presents in the first few months of life and frequently results in death within two years. Mice that over express the FoxP3 gene have been shown to produce fewer mature T cells with reduced functional capacities and are unable to mount an effective humoral response.[275] This, combined with the development of autoimmunity in FoxP3 deficiency, suggests that this transcriptional regulator is essential for normal immune homeostasis.[276]

FoxP3 has been described as a master switch in Treg differentiation and function.[267,277] FoxP3 can block the transcription factors NF-κB,[278] NFAT,[279] HAT/HDAC[280] and AML-1/Runx1[281] which, through transcribing the cytokines IL-2 and IFN-γ, play important roles in the activation and differentiation of T cells.[282,283] The presence of FoxP3 not only blocks these transcription factors, but also overrides them to increase the transcription of molecules associated with Tregs such as CD25.[284]

FoxP3 is highly expressed in $CD25^+$ Tregs and is currently considered to be the most specific marker, as its expression has been found to correlate with suppressive function.[267] However, not all $FoxP3^+$ cells possess suppressive abilities.[258] FoxP3 expression can be induced through TCR activation and low levels of expression have also been found in non-suppressive $CD4^+CD25^-$ cells.[244,285] FoxP3 is localised to the nucleus and so its identification requires fixing the cells followed by permeabilisation making functional analysis impossible if this marker is used for isolating the cells.

1.7.2.3 CD127

The IL-7 receptor is a cell surface protein consisting of two subunits; an α chain (CD127) and a γ chain (CD132) which is shared by other cytokine receptors (receptors for IL-2, IL-4, IL-9, IL-15 and IL-21).[261–263] CD127 plays a role in the proliferation and differentiation of T cells and is expressed by many cells including mature T cells, B cell progenitors, monocytes and thymocytes. Levels of CD127 expression have been found to inversely correlate with FoxP3 and suppressive function.[286,287] Therefore, CD127 has been accepted as an alternative Treg marker. As CD127 is a cell surface marker, it provides an alternative to FoxP3 for identifying and isolating Tregs. However, it has been demonstrated that Tregs up-regulate CD127 upon *in vitro* and *in vivo* activation.[288] Furthermore, this marker can be down-regulated during the early stages of T cell activation and therefore, some non-suppressive $FoxP3^{low}CD45^+$ T cells express low levels of CD127.[258,289]

1.7.2.4 Other markers associated with Treg function

The markers mentioned above are commonly used to identify Tregs. There are several other markers that have been linked to Tregs found in mice and humans. A brief description of a few of these markers is included below.

1.7.2.4.1 CD39

Extracellular ATP concentrations in healthy tissues are regulated by ATP/ADPases.[290,291] Intracellular ATP concentrations are maintained at high levels of 3-5mM.[292] If a cell is lysed or the membrane damaged large quantities of ATP are leaked into the plasma. Extracellular ATP binds to the P2X7 and P2Y2 receptors promoting leucocyte recruitment and release of proinflammatory cytokines such as IL-1β.[293]

CD39, also known as ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1), was originally considered to be a B cell activation marker, but has been shown to be expressed by other cells such as dendritic cells and subsets of T cells.[294] CD39 hydrolyses ATP and ADP to AMP,[290,295] and the removal of extracellular ATP results in an anti-inflammatory effect. The AMP produced by CD39 can be further metabolised by CD73 to produce adenosine which, when bound to its A2A receptor, exhibits immunosuppressive effects on $CD4^+$ and $CD8^+$ T cells by increasing intracellular cyclic AMP leading to the inhibition of IFN-γ and IL-2 production.[296–298]

CD39 has been found to be expressed in virtually all mouse $CD25^+$ and $FoxP3^+$ cells.[299] The same group also report the majority of these Tregs co-expressed CD73, although this was not found to correlate with FoxP3 expression. The Tregs of $CD39^{-/-}$ mice have reduced *in vivo* suppressive abilities and *in vivo* are unable to prevent rejection of an allograft.[297] Through these studies, it is clear that CD39 and CD73 play an important immunoregulatory role in mice. In adult humans, the role of CD39 is not so well defined and it is found to be expressed on only a subset of Tregs.[299] $CD39^+$ Tregs possess an activated memory phenotype and co-express CD45RO and CCR6.[299] This subpopulation has been shown to suppress production of the pro-inflammatory cytokine IL-17 whilst $CD39^-$ cells produce IL-17.[300]

1.7.2.4.2 ICOS

Lymphocytes require two activation signals, the first is through interaction of the T cell receptor (TCR) with major histocompatibility class antigens (MHC) on an antigen

presenting cell (APC). The second activation signal is involves the interaction of a member of the CD28 family with a B7 protein presented by the APC. This second signal prevents the lymphocyte from undergoing apoptosis or becoming anergic.

Inducible costimulator (ICOS or CD278) is a member of the CD28 receptor family and plays a role in T cell activation and survival.[301] Although ICOS is expressed in the majority of murine Tregs,[302] human Tregs can be divided into two subsets based on ICOS expression. Expression of ICOS is up-regulated upon antigen encounter and it is therefore, expressed on most $CD45RO^+$ cells,[303] whereas $ICOS^-$ cells can be $CD45RO^+$ or possess a more naïve $CD45RO^-$ phenotype.[304] $ICOS^+$ and $ICOS^-$ Treg subsets have been found to suppress using different mechanisms.[304] $ICOS^-$ Tregs express TGF-β (suppresses T cell function) and are only able to suppress through cell-cell contact.[304] The $ICOS^+$ Treg subset also produce TGF-β, but at lower quantities than $ICOS^-$ cells.[304] $ICOS^+$ cells also suppress APC function through the production of IL-10,[305] and they share features with Tr1, Th1 and Th17 cells by expressing IL-17 and INF-γ.[306]

1.7.2.4.3 CTLA-4

Cytotoxic T lymphocyte antigen 4 (CTLA-4 or CD152) a cell surface marker, is expressed by $CD4^+$ and $CD8^+$ T cells.[307] The structure of CTLA-4 is closely related to CD28[308] and so these receptors compete for B7 binding (CD80 and CD86) on the APC.[309,310] Binding of CTLA-4 instead of CD28 to the B7 ligands results in an opposing signal; although CD28 binding leads to the activation of T cells, CTLA inhibits IL-2 secretion and cell proliferation resulting in an immunosuppressive effect.[311] Although CTLA-4 functions at the cell surface, its expression is a dynamic process and it cycles between the surface and intracellular vesicles, thereby regulating its immunosuppressive effects.[312] Therefore, the detection of CTLA-4 is often achieved using intracellular staining which, as for FoxP3, limits it usefulness for the isolation of functional cells.

1.7.2.5 Expression of homing receptors by Tregs

Tregs are believed to suppress through multiple mechanisms, many of these would require close contact with the target cells. Therefore, Tregs need to migrate from the circulatory system into specific tissues.[313]

1.7.2.5.1 CCR4

Chemokines are a group of cytokines that can be divided into four subgroups based on the arrangement of the N-terminal cysteine residues: C, CC, CXC and CX3C. Chemokines play an important role in the homing and migration of leucocytes. T cells with different functions express different sets of trafficking receptors in order to ensure that they migrate to the appropriate tissue.[314]

C-C chemokine receptor type 4 (CCR4 or CD194) attracts T cells to non-lymphoid tissues such as the skin and lung.[315–317] Adult Tregs have been shown to express more CCR4 compared with $CD25^-$ T cells,[318–320] and FoxP3 transduced $CD4^+CD25^-$ T cells have been shown to upregulate CCR4 and acquire immunoregulatory abilities, suggesting a relationship between CCR4 and suppressive function.[277] The more mature memory Tregs with a $CD45RA^-$ phenotype have been shown to express higher levels of CCR4 than the more naïve $CD45RA^+$ subset.[319,321]

1.7.2.5.2 CD62L

CD62L (L-selectin) is a lymphoid tissue T cell homing receptor which is required for migration into the secondary lymphoid tissues.[322] The T cells that express CD62L have been found to possess the naïve phenotype $CD45RA^+/RO^-$. After T cell receptor engagement, $CD62L^+$ is down-regulated and the cells adopt a memory phenotype ($CD45RA^-/RO^+$).[323]

CD62L deficient mice show a significant reduction of the number of lymphocytes localised to the lymph nodes, confirming the role L-selectin plays in the homing to lymphoid tissues.[324] CD62L interacts with peripheral node addressin (PNAd) on the high endothelial venules (HEV), and this initiates cell rolling.[323,325] This activates lymphocyte function associated antigen 1 (LFA-1) via a G-protein-coupled receptor.[326,327] The activated LFA-1 binds to adhesion molecules (ICAM-1) on the HEV allowing the cell to migrate into the lymph node (Figure 1.7).[328]

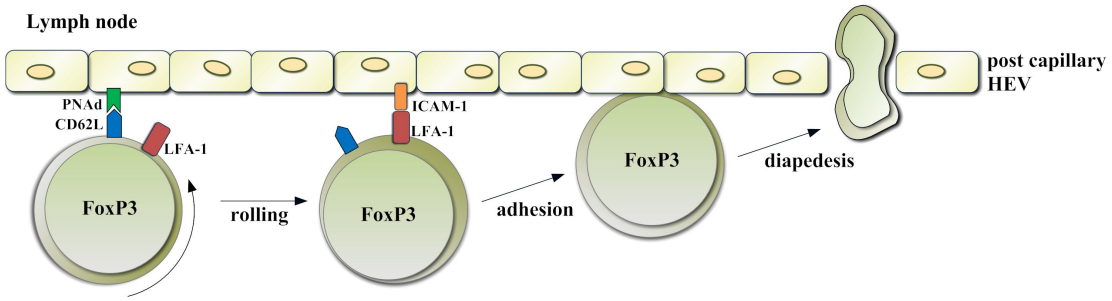

FIGURE 1.7: Mechanisms of cell homing from the periphery into lymph nodes via CD62L. Rolling of the cell is initiated when CD62L interacts with peripheral node addressin (PNAd). Lymphocyte function associated antigen 1 (LFA-1) binds with adhesion molecules (ICAM-1) to mediate firm adhesion to the epithelial surface and allow diapedesis or migration of the cell through the epithelial cells. Figure created using SmartDraw and adapted from Lewis *et al.*[329]

Both $CD62L^+$ and $CD62L^-$ Treg subsets have been shown to be anergic and equally suppressive *in vitro* upon polyclonal[330,331] and allogeneic stimulation.[322] Also both subsets have been reported to be equally protective in a mouse model of colitis.[332] However, only the $CD62L^+$ Treg population is able to delay the onset of diabetes in NOD/SCID (non-obese diabetic/severe combined immunodeficiency) mice[333] and only these cells can protect mice against GvHD.[322] Since only the $CD62L^-$ donor T cells induce GvHD,[334,335] it has been suggested that this homing receptor is required to enter the secondary lymphoid organs in order to localise the priming site of the alloreactive $CD4^+$ T cells.[322]

1.7.3 Types of Tregs

The Tregs described so far develop in the thymus[336] and are known as naturally occurring regulatory T cells (nTregs). Additional suppressor populations including induced T

regulatory cells (iTreg) which develop in the peripheral blood[215] and $CD8^+$ suppressor cells have been identified.[337]

1.7.3.1 Induced Tregs

Cells that develop from $CD4^+FoxP3^-$ T cells in the periphery are known as adaptive or induced Tregs (iTreg). These cells can be induced *in vitro* from $CD4^+CD25^-$ T cells in the presence of IL-2 and TGF-β.[215–217] The production of iTregs has been found to be completely independent of thymus derived nTregs, as mice devoid of nTregs are still able to generate iTregs.[338] Although nTregs and iTregs express CD25, FoxP3, GITR and CTLA-4, the higher levels of Helios and Neurophilin-1 expression by nTregs allows these cells to be identified using flow cytometry.[338]

The iTreg population contains Type 1 regulatory (Tr1) and T helper 3 (Th3) suppressive cells. The *in vitro* induction of Tr1 cells was first described by Groux *et al* as a consequence of stimulating naïve CD4 T cells with high levels of IL-10.[215] These cells were found to mostly produce IL-10 and TGF-β, with which they are able to suppress Th1 and Th2 cells.[339] The suppressive role of Tr1 cells has found to be non-overlapping, but complementary to nTregs.[340] T helper 3 cells can be generated by the addition of TGF-β to naïve T cells.[341] These cells exert suppressive effects over Th1 and Th2 cells via the production and secretion of TGF-β.[342] The differences between nTregs and iTregs are summarised in Table 1.1.

1.7.3.2 CD8 regulatory T cells

$CD8^+$ suppressive cells display many similarities to their $CD4^+$ counterparts; in that they express CD25 and FoxP3 and consist of a heterogeneous population.[356] These cells have been found to suppress using similar mechanisms as CD4 Tregs and their presence is believed to be complementary to these cells.[357,358]

TABLE 1.1: Characteristics of nTregs and iTregs

Properities	nTreg	iTreg - Tr1	iTreg - Th3
Site of maturation	Thymus[336]	Periphery[215]	Periphery[215]
Required for activation	Costimulation[343]	IL-10[215]	TGF-β[341]
Markers	FoxP3^{+}[267] Neurophilin-1^{+}[338] Helios^{+}[338] CD49d^{-}[344]	FoxP3^{-}[345] Neurophilin-1^{-}[338] Helios^{-}[338] CD49b^{+}[346]	FoxP3^{+}[347] Neurophilin-1^{-}[338] Helios^{-}[338]
Mechanism	Secretion of IL-10,[348] TGF-β[349] and IL-35[350] Metabolic disruption[351] Cytolysis[352, 353] Inhibition of DC[354]	Secretion of IL-10 and TGF-β[339]	Secretion of TGF-β[342]
Target cells	APC and Teff[355]	Teff[339]	Teff[342]

1.7.4 Mechanism of suppression

Tregs remain anergic after TCR stimulation, even with the addition of co-stimulatory signals by APC.[359] Only in the presence of high doses of IL-2 in combination with TCR stimulation will Tregs proliferate.[360] TCR stimulation is required in order for Tregs to become functional.[343] This has been shown through the ability of fixed Tregs cells to assert their suppressive effects if they had been activated before fixation.[361] Once activated, these cells suppress in an antigen non-specific manner[331] and therefore, do not have to be viable to mediate suppression.[362] Tregs have been shown to suppress using a variety of different mechanisms (shown in Figure 1.8) which can be grouped into four different 'modes of action'.[355]

1. Secretion by inhibitory cytokines

   (a) IL-10

   Interleukin-10 (also known as human cytokine synthesis inhibitory factor) has been suggested to have an important role in controlling inflammation by suppressing proinflammatory cytokines, chemokines and adhesion molecules.[348]

IL-10 functions by suppressing the expression of proinflammatory cytokines (such as IL-1 and TNF), chemokines that are involved in recruiting Teff and by inhibiting the expression of costimulatory molecules and MHC class II antigens in APCs.[348]

(b) TGF-β

Transforming growth factor β can be soluble or membrane bound. TGF-β has been shown to inhibit the proliferation of T cells by inhibiting the production of IL-2 and IFN-γ[349,363] and also affects the differentiation of naïve $CD4^+$ cells.[364] TGF-β null mice have increased expression of MHC class I and II molecules and an increase in inflammation in multiple organs which leads to death within three weeks of birth, suggesting that this cytokine plays a role in controlling the expression of both MHC classes.[365]

(c) IL-35

A member of the IL-12 family, is expressed by resting and activated Tregs but not Teff cells.[350] IL-35 has been shown to suppress naïve $CD4^+$ T proliferation *in vitro* and in addition, a loss of IL-35 expression leads to reduced suppression *in vivo*.[350]

2. Suppression by metabolic disruption

(a) IL-2

IL-2 is essential for proliferation and differentiation of T cells.[366] $FoxP3^+$ cells are unable to produce IL-2, suggesting that Tregs with their high expression of CD25 can deplete the IL-2 from the local environment, thus depriving nearby target cells from this cytokine.[351] This theory has been further developed by Pandiyan *et al* who suggest that apoptosis can be induced by Tregs through deprivation of cytokines such as IL-2.[367]

(b) CD39

As described in §1.7.2.4.1, expression of CD39 can hydrolyse extracellular ATP and ADP to AMP, thereby removing a proinflammatory signal.[290,295] Extracellular AMP can be further metabolised by CD73 to produce adenosine which binds to the A_{2A} receptor and inhibits proinflammatory cytokine

production.[297,368,369] The binding of adenosine to the receptor not only suppresses the function of the target cells, but also through the inhibition of IL-6 and secretion of TGF-β, leads to the generation of induced Tregs.[370]

(c) cAMP

The generation of adenosine can also increase cyclic adenosine monophosphate (cAMP) levels. cAMP can be transferred to Teff cells through GAP junctions.[371] Raised levels of cAMP leads to inhibition of IL-2 and IFN-γ and the subsequent proliferation of $CD4^+$ T cells.[372]

3. Suppression by cytolysis

Tregs may also act as cytotoxic cells and exert their suppressive function by releasing cytotoxic granules. Cytotoxic granules contain the pore-forming protein perforin and granzymes (part of the serene protease) which directly kill effector cells by initiating apoptosis.[373] Activated nTreg cells have been shown to express predominantly granzyme A,[374] whereas iTregs generated from human $CD4^+$ T cells via CD3 and CD46 in the presence of IL-2 express granzyme B.[375] Both types of Tregs exhibit cytotoxic effects on $CD4^+$ and $CD8^+$ T cells, monocytes and dendritic cells in a perforin-dependent but fasL-independent manner.[375]

4. Targeting the APC

(a) CTLA-4

Competes with CD28 and binds to CD80 and CD86 on DCs activating indolamine 2,3-dioxygenase (IDO) which catalyses the degradation of tryptophan, depleting the stores needed for Teff cell proliferation, into kynurenine which induces apoptosis in the Teff cells.[376–378]

(b) LAG3

Lymphocyte activation gene 3 (LAG3) is a CD4 homologue that binds MHC class II molecules expressed by immature DCs. This engages a Immune-receptor-Tyrosine-based-Activation-Motif (ITAM) which induces an inhibitory signalling pathway and suppresses the maturation and immunostimulatory capacity of the DC.[354]

(c) Neurophilin-1

Promotes long interactions between Tregs and immature DCs, thereby blocking their maturation.[379]

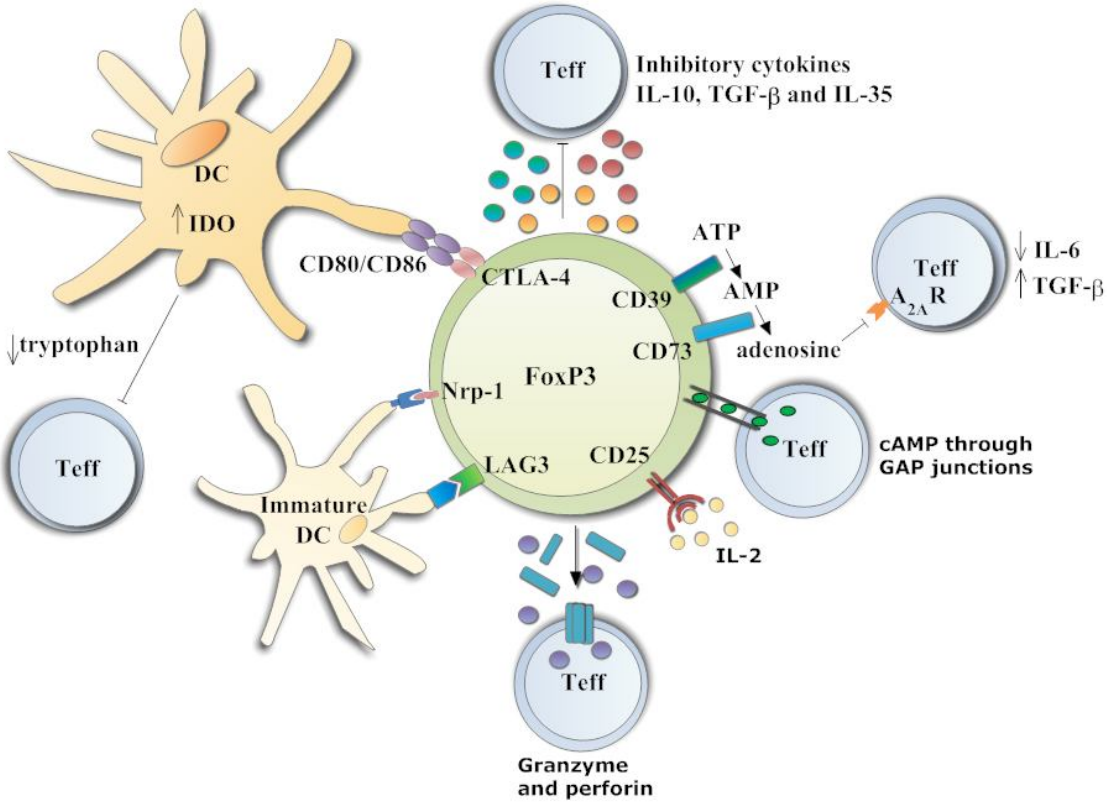

FIGURE 1.8: Mechanisms of immune suppression by Tregs through the release of inhibitory cytokines, disrupting metabolic activity, cytolysis and by targeting the APC. Figure created using SmartDraw and adapted from Vignali *et al.*[355]

There is much debate on the mechanisms of Treg suppression with many contradictory reports. However, as Tregs are able suppress a variety of immune cells in various tissue sites, in all likeliness there is not one single mechanism through which Tregs function. The mechanism of suppression is probably dependent on factors such as the site of the immune reaction as well as the type and activation status of the target cell and the Treg itself.

1.7.5 Use of Tregs in cell therapy

The importance of Tregs in both mice[248,249,351] and humans[237,238,242] has been well documented. These cells have been shown to play an important role in controlling

autoimmunity, tumour immunity, transplantation tolerance, allergy and microbial immunity.[243,336,380] It is no surprise that these cells have been targeted for immunotherapy in the treatment for many immune disorders, however, this has proved to be challenging. In order to activate their immunosuppressive functions, Tregs require stimulation through their TCR. As there is no known method of stimulating Tregs without activating other T cells it is not possible to administer the activation signal directly into the patient. Alternatively adoptive cell therapy techniques can be employed in which the Tregs are isolated from a patient, activated *ex vivo* before reinfusion.[381] This also allows functional assessment of the cells prior to re-administration reducing the risk of adverse reactions.

1.7.5.1 Tregs in autoimmunity

Autoimmunity compromises a large group of diseases characterised by a lack of self tolerance and has been shown to affect ∼8% of the human population.[382] Autoimmune diseases can be caused by defective Tregs, for example in the case of IPEX syndrome which is caused by mutations in the FoxP3 gene.[383] Although HSCT is currently the only curative option, a recent study reported that $CD4^+$ T cells isolated from a patient could be converted into functional Tregs after lentivirus-mediated FoxP3 gene transfer and therefore, potentially provide an alternative approach for the treatment of this disease in the future.[384]

Type 1 diabetes mellitus (T1D) results from the immune system destroying the insulin producing β-cells in the pancreas.[385] There is no consensus on the effect Tregs have on T1D, some report that the frequency of these cells is altered,[386] whilst others link a reduction in suppressive ability with this autoimmune disease.[387] Tregs have been used in the treatment of T1D in NOD mice, with the adoptive transfer of Tregs slowing the progression of diabetes and in some cases reversing the onset of this disease.[333,388] There is an ongoing clinical trial assessing the safety of infusing different doses of expanded Tregs in patients T1D whilst also looking into the effects these cells have on insulin β-cell function.[389]

Systemic lupus erythemathosus (SLE) is a chronic autoimmune disease that can affect almost any organ system. The majority of studies on Treg frequency within SLE patients report a decrease in this cell population,[390–392] however, there are also reports indicating an increase in Treg numbers.[393,394] There are several possible reasons for these discrepancies: Firstly, the disease activity in a patient varies between an active state and phases of remission, these fluctuations can alter cytokine release and the distribution of immune cells. Secondly, some of these studies identify Tregs using FoxP3, whereas others rely on the $CD25^{high}$ phenotype. The lack of a specific Treg marker is particularly problematic in SLE sufferers as a non suppressive $CD4^{+}FoxP3^{+}CD25^{low}$ T cell population has been identified which is not found in healthy humans.[395,396] *Ex vivo* expansion of Tregs isolated from SLE patients restored their functional capabilities and suggests that these cells have potential for the treatment of SLE.[397]

1.7.5.2 Tregs in alloimmunity

Allorecognition occurs after transplantation when the host's immune system reacts to MHC complexes from the donor.[398] Three pathways of allorecognition have been described: direct, indirect and semi-direct. The direct pathway occurs when the host T cell recognises the donor MHC molecules displayed on the transplanted cells. In the indirect pathway, the donor MHC molecules are processed and presented by the host APCs. The semi-direct pathway involves the transfer of intact MHC molecules from the donor to the host's APCs.[399] The mechanism for this is not fully elucidated, but it is believed to involve cell-cell contact[400] or their release within exosomes which can be internalised by APCs.[401]

1.7.5.2.1 Solid organ transplant

As with HSCT, if there is histoincompatibility between host and donor after a solid organ transplant an immune response is elicited. Rejection of a graft can occur at different stages: hyperacute, acute and chronic rejection. Hyperacute rejection usually occurs within the first few hours of the transplant. This type of rejection is humorally

mediated and occurs if the patient has pre-existing antibodies specific to the donor antigens. This type of rejection presents itself so quickly because vascularisation of the graft is destroyed by the activation of the complement system leading to thrombosis in the capillaries.[398] Acute rejection is initiated by a T cell response and develops between one week and six months of transplantation. This type of rejection is associated with the direct allorecognition pathway and rapidly elicits an immune response as the antigen is presented by APCs within the organ. All transplants will have some level of acute rejection unless the donor is a perfect HLA match for the patient. This type of rejection results in an increase in the risk of developing chronic rejection. Chronic rejection occurs months or years after the transplant and is the major cause of organ failure. This type of rejection can be humoral or cell-mediated and is associated with the indirect allorecognition pathway. This requires more time to elicit an immune response, as it requires the capturing and processing of the antigen. Chronic rejection is caused by inflammatory vascular injury to the organ. This is characterised by accelerated atherosclerosis of the blood vessels accompanied by collagen production by fibroblasts resulting in scarring and atropy.

To prevent immunological rejection of the transplanted organ, patients often require long-term immunosuppression which can leave them susceptible to microbial pathogens and tumour formation. Infection and cancer are major contributors to mortalilty post-transplant.[402,403] Currently clinical trials are being performed in patients which have previously undergone a liver transplant. These patients are being treated with several courses of 1 $\times 10^6$ Treg cells kg^{-1} whilst their immunosuppressive drug treatment is withdrawn.[404] Another current clinical trial involves kidney[405] transplant patients in which immunosuppressive drugs are administered with or without Tregs and both groups monitored for three years.

1.7.5.2.2 HSCT

Allogeneic HSCT are routinely performed to treat blood disorders and cancers. The most frequent complication of allogeneic HSCT is GvHD and occurs in 30-70% of patients.[406] Pathophysiology of aGvHD has been described in three phases.[407] The first

phase (conditioning or afferent phase) begins with damage to the host's tissue during the conditioning regime. Damage occurs mainly in the liver and intestinal mucosa caused by the release of inflammatory cytokines such as IL-1, TNF-α and IFN-γ, and the up-regulation of the major histocompatibility complex (MHC) on APCs. In the second phase (induction and expansion phase) the donor T cells are activated by the inflammatory cytokines and APCs. These T cells proliferate and differentiate into effector cells. In the third and final phase (effector phase), the activated T cells induce cytotoxicity, Fas-FasL-mediated apoptosis and cytolysis mediated by granzyme B and perforin. This results in damage to the host's tissue and the characteristic features of aGvHD.

Lethally irradiated mice that received a Treg depleted HSCT have been shown to exhibit increased severity of GvHD following a donor leukocyte infusion (DLI) compared to mice that received a whole BM transplant.[408] This, combined with several studies showing that the adoptive transfer of Tregs ameliorates GvHD in mice undergoing HSCT[409–411] whilst maintaining the GvL effect,[412] suggests these cells have the potential to be used therapeutically to prevent or reduce the severity of this disease.

There are conflicting reports on the frequency of Tregs in humans suffering from GvHD. Magenau *et al* report a 40% reduction in patients with GvHD compared to those without, and found the frequency of Tregs inversely correlated with the severity of GvHD.[413] In contrast, Clarke *et al* report elevated levels of Tregs in GvHD sufferers, but with reduced CD62L expression compared to patients without GvHD.[414]

A CB derived Treg dose escalation study has been performed in patients undergoing a double CB HSCT.[415] Treg doses of 1 to 30×10^5 cells kg^{-1} were infused one day after the transplant with no toxicity and a reduction in the incidence of grade II-IV aGVHD compared to historical controls observed. There are other ongoing clinical trials assessing the feasibility of using Tregs against GvHD, including a phase I trial in which *ex vivo* expanded donor Tregs will be infused 2 days prior to allogeneic HSCT,[416] and a phase II trial in which donor Tregs and rapamycin will be administered to patients already suffering from aGVHD.[417]

1.7.6 Treg Banking

In some cases it may be convenient to isolate Tregs sometime prior to their use therapeutically. In these situations, it would be beneficial to cryopreserve these cells whilst ensuring suppressive function remains intact.

1.7.6.1 Isolation of Tregs

Tregs for clinical use need banking procedures that comply with good manufacturing practice (GMP). The isolation process should be performed in a closed and sterile system using clinical grade CE marked reagents.

Tregs are a rare population (5-10% of $CD4^+$ T cells) and large numbers of cells would be required for therapeutic applications. Therefore, post-isolation expansion, involving culturing cells with anti-CD3, anti-CD28 and high doses of IL-2, is often needed to reach the cell doses required for clinical purposes.[411] Although approaches used during expansion tend to favour the proliferation of non suppressive T cells, Rapamycin can be added during expansion in order to favour the proliferation of Tregs.[418] Therefore, in order for expansion to be successful, the starting population must be of high purity. The peripheral blood from adult humans can contain large numbers of activated $CD25^+$ T cells which do not possess immunosuppressive abilities.[265] As Tregs are often isolated based on CD25 expression, low purities with high numbers of contaminating $CD25^+CD127^+$ cells are often observed.[419]

The purity of these cells is also particularly important when treating patients with autoimmune diseases, as it would be detrimental to put alloreactive cells into patients already suffering from immunological imbalance.[420] Purity may not be such a problem if infusing during a HSCT, where Tregs can be used to prevent or reduce the severity of GvHD; in this setting the infusion of non-regulatory T cells may aid in the GvL effect.[421]

GMP grade Treg isolation has previously been performed on leukapheresis samples resulting in low $CD4^+CD25^{high}$ cells purities of 40-60%.[422] Expansion of these samples

found the small number of contaminating B cells (1-3%) increased 10-fold after expansion. Impurities did not improve despite the addition of three consecutive CD25 enrichment procedures.[423] In an attempt to improve purities of these samples, negative selection or depletion of cytotoxic T cells and B cells (using CD8 and CD19 respectively) have been performed prior to CD25 enrichment.[419,424] Although this improved $CD25^+$ purity to 80-95%, the percentage of $CD25^{high}$ remained low (30-60%) with high numbers of contaminating $CD127^+$ cells (20-30%). Peters *et al* improved Treg purity by depleting $CD127^+$ cells, however, as clinical grade CD127 microBeads are not available these cells were depleted using fluorescent activated cell sorting (FACS).[419]

1.7.6.2 Cryopreservation of Tregs

In some cases it may be convenient to isolate Tregs some time prior to their use therapeutically, in these situations it would be beneficial to cryopreserve these cells. However, it is therefore, important to ensure that these cryopreserved cells will maintain their immunosuppressive capabilities upon thawing.

It has been reported that cryopreserving adult PBMCs results in a significant reduction in the frequency of Tregs cells after thawing (reduced from 5.58% of $CD4^+$ cells to 3.57%, p=0.0016).[425] Peters *et al* cryopreserved Tregs isolated from leukapheresis products and found them to have reduced post-thaw viabilities of 70-80%, with decreased suppressive abilities. However, they observe these suppressive abilities could be restored following expansion. Alternatively the cells maintained suppressive abilities if expanded prior to cryopreservation.[419]

1.8 Rationale and outline of this study

Approximately 20% of CB units arriving at the ANCTC meet the criteria to be banked for HSCT use. However, samples that are unsuitable for this purpose (usually due to low cell numbers or medical exclusions) can be used for other purposes such as research. To avoid discarding these units, Anthony Nolan aims to create a Biobank within the ANCTC, storing units either as whole blood, isolated CB mononuclear cells (CBMC) or pure cell populations. This study aims to improve the protocols used within the Biobank ensuring that they are efficient, reproducible and result in a cellular product that maintains the highest possible level of viability and potency. This will be achieved by breaking the banking processes down into sections: transportation of the sample from maternity to ANCTC, the isolation of CBMC, cryopreservation of cells and finally assessing the ability to bank a pure cell population. The outline of the study is shown in Figure 1.9

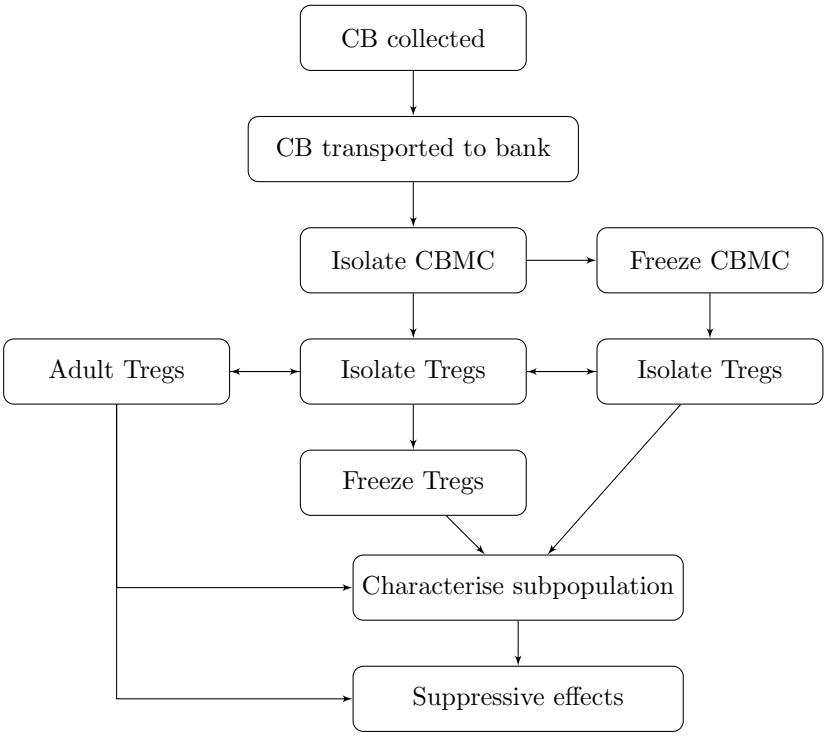

FIGURE 1.9: Outline of this study to improve CB cell isolation and cryopreservation techniques in the creation of a Biobank at ANCTC

The maternities collecting CB for the ANCTC are spread across the UK resulting in a delay to processing and cryopreservation due to transportation of the samples. There is currently no consensus for optimum fresh storage conditions for CB and it is vital that the samples are of the highest possible quality on arrival. Therefore, the first area of investigation is to assess the effects of temperature on the fresh storage of CB, in addition to other sources of HPCs, and to ascertain the effects of delaying cryopreservation. This part of the study will be of benefit to samples designated for the Biobank as well as those cryopreserved for the HSCT bank, where any improvements in quality can lead to improvements in transplantation outcomes.

Isolation of CBMCs is often required for research and is the first step towards pure cell isolations. Cells are currently separated through the ficoll density gradient protocol, a time consuming process with high user variability. The company Biosafe has developed an alternative approach to CBMC isolation. As with the ficoll density gradient protocol this new method also exploits differences in cell density, but within a closed system, and without needing to add density gradient media thereby reducing the risk of contamination. Within this study, the cell fractions obtained using this new protocol will be compared against ficoll-derived MNCs to determine if this method is a suitable alternative.

The Biobank will require long term storage of cellular fractions; this will be achieved through cryopreservation. Cryopreservation of cells can lead to a reduction in viability and functional capabilities post-thaw, particularly due to toxicity from cryoprotectants such as DMSO. Therefore, it is important to optimise the protocols in order to maintain cell potency. To address this, the toxic effects of DMSO will be assessed at different stages of cryopreservation before determining the optimal concentration of both DMSO and dextran-40 within the cryoprotective solution. In addition, the effect of the speed of addition of the cryoprotective solution will be addressed.

Finally, the Biobank will require the ability to store frozen samples of pure cell populations with the potential to be distributed as clinical grade products for therapy or for research and developmental purposes. Tregs play an important role in the homeostasis

of the immune system by suppressing the proliferation of other lymphocytes and therefore, have the potential to be used therapeutically in autoimmunity or transplantation. These features, in addition to their tendency to protect tumours, also make these cells an interesting target for researchers and are therefore, are an ideal candidate for the Biobank. However, therapeutic application requires a large number of Tregs with high purity and these cells are rare and potentially difficult to obtain from adult donors. To begin with, the phenotype and suppressive ability of CB derived Tregs will be compared to their adult counterparts. If CB is found to be a suitable source of these cells, isolation and cryopreservation protocols (both research grade and GMP compliant) will be developed whilst ensuring cell viability and potency is maintained.

CHAPTER 2

MATERIALS AND METHODS

2.1 Laboratory Consumables and Equipment

2.1.1 Reagents and a list of producers

Reagents were stored as per manufacturer's instructions and used before the expiry date.

Culture Media	**Supplier**
RPMI	Lonza
Methocult GF H84434	Stem Cell Technologies
Methocult GF H84444	Stem Cell Technologies

Culture Media Supplements	**Supplier**
DNase-1	Calbiochem
Human AB serum	Lonza
IL-2	Prospec
Iscove's modified Dulbecco's medium (IMDM)	Stem Cell Technologies
L-glutamine	Lonza
Magnesium chloride ($MgCl_2$)	BDH, VWR International Ltd
Penicillin-streptomycin solution	Sigma
Purified NA/LE mouse anti-human CD3	BD Pharmingen
Purified NA/LE mouse anti-human CD28	BD Pharmingen
Sodium citrate	BDH, VWR International Ltd

Chemical Reagents	**Supplier**
^{3}H-thymidine	Perkin-Elmer
Acetic acid	Fisher Scientific
Annexin V binding buffer	BD Pharmingen
Carboxyfluorescein succinimidyl ester (CFSE)	Invitrogen
CliniMACS buffer	Miltenyi Biotec GmbH
Dextran-40	Origen Biomedical
Dimethyl sulphoxide (DMSO) solution	Origen Biomedical
DMSO/dextran syringes	Origen Biomedical
Ethanol	Shield Medicare
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Ficoll-Paque Premium	GE Healthcare
Fixation/Permeabilization concentrate	eBioscience
Fixation/Permeabilization diluent	eBioscience
Isopropanol	Fisher Scientific
Liquid nitrogen	Cryoservice
Microscint-O scintillation fluid	Perkin-Elmer
Perfect count microspheres	Cytognos
Permeabilization buffer	eBioscience
Pharm Lyse	BD Biosciences
Phosphate buffered saline (PBS)	Lonza
Presept	Johnson & Johnson
RoboSep buffer	Stem Cell Technologies
Sodium azide (NaN_3)	BDH, VWR International Ltd
Trypan blue	Sigma-Aldrich

Immunochemical Reagents	**Supplier**
7AAD	BD Pharmingen
Annexin V	BD Pharmingen
Mouse anti-human CD3 Pacific blue	Invitrogen
Mouse anti-human CD3 PE	BD Pharmingen
Mouse anti-human CD3 PerCP	Biolegend
Mouse anti-human CD4 APC	Biolegend
Mouse anti-human CD4 Pacific orange	Invitrogen
Mouse anti-human CD4 PerCP	BD Bioscience
Mouse anti-human CD25 APC-Cy7	Biolegend
Mouse anti-human CD25 PE	BD Biosciences
Mouse anti-human CD25 PE-CF594	BD Horizon
Mouse anti-human CD34 PE	BD Pharmingen
Mouse anti-human CD39 PE	BD Pharmingen
Mouse anti-human CD45 APC	BD Pharmingen

Immunochemical Reagents continued	**Supplier**
Mouse anti-human CD45 APC-Cy7	Biolegend
Mouse anti-human CD45 FITC	BD Pharmingen
Mouse anti-human CD49d PerCP-Cy5.5	Biolegend
Mouse anti-human CD62L APC-Cy7	Biolegend
Mouse anti-human CD69 Alexa Fluor 700	BD Pharmingen
Mouse anti-human CD127 FITC	BD Pharmingen
Mouse anti-human CD127 PE-Cy7	eBioscience
Mouse anti-human CD133 PE	Miltenyi Biotec GmbH
Mouse anti-human CD194 (CCR4) PerCP-Cy5.5	BD Pharmingen
Mouse anti-human CD278 (ICOS) PE-Cy7	eBioscience
Rat anti-human FoxP3 APC	eBiosciences
Mouse IgG1,κ Alexa Fluor 700 isotype control	BD Pharmingen
Mouse IgG1,κ APC isotype control	BD Pharmingen
Mouse IgG1,κ APC-Cy7 isotype control	Biolegend
Mouse IgG1,κ FITC isotype control	BD Pharmingen
Mouse IgG1,κ PE isotype control	BD Pharmingen
Mouse IgG1,κ PE-CF594 isotype control	BD Horizon
Mouse IgG1,κ PE-Cy7 isotype control	eBiosciences
Mouse IgG1,κ PerCP-Cy5.5 isotype control	BD Pharmingen
Mouse IgG1,κ PerCP-Cy5.5 isotype control	Biolegend
Mouse IgG2a Pacific Blue isotype control	Invitrogen
Mouse IgG2a Pacific Orange isotype control	Invitrogen
Mouse IgG2b,κ APC isotype control	Biolegend
Mouse IgG2b,κ PE isotype control	BD Pharmingen
Rat IgG2a,κ APC	eBiosciences
Compensation beads	BD Biosciences
CliniMACS CD4 reagent	Miltenyi Biotec GmbH
CliniMACS CD25 reagent	Miltenyi Biotec GmbH
CD133 MicroBead kit	Miltenyi Biotec GmbH
EasySep CD25 positive selection kit	Stem Cell Technologies

2.1.2 Equipment

Disposable equipment and plasticware	**Supplier**
0.2μm filters	Fisher Scientific
40μm cell strainer	BD Falcon
6-well flat bottomed plates	Sarstedt
24-well plates	Millipore
96-well U bottomed plates	Nunc
96-well plate harvester filters	Perkin-Elmer
5ml Polystyrene round bottom tube	BD Falcon
14ml Polystyrene round bottom tube	BD Falcon
150ml Transfer bag	Miltenyi Biotec GmbH
AK-100	Biosafe
Blood transfusion filter	Miltenyi Biotec GmbH
Centrifugation tubes (15ml, 50ml)	Sarstedt
CliniMACS TS-600 tubing set	Miltenyi Biotec GmbH
Cord blood collection bags containing 25ml CPD	Grifols or Macopharma
Cryopreservation bags	Biosafe
Cryovials	StarLabs
Eppendorf tubes	Sigma-Aldrich
Filter tips (10μl, 20μl, 200μl, 1ml)	StarLabs
Leucosep tubes	Greiner Bio One
MACS pre-separation filters	Miltenyi Biotec GmbH
MACS separation columns LD columns	Miltenyi Biotec GmbH
MACS separation columns LS columns	Miltenyi Biotec GmbH
MACS separation columns MS columns	Miltenyi Biotec GmbH
Needles	BD Microlance
Needles blunt ended	BD Microlance
Pasteur pipettes	Fisher Scientific
SEPAX CS490 kits	Biosafe
SEPAX CS530.4 eMNC kits	Biosafe
SEPAX CS900 kits	Biosafe
SepMate tubes	Stem Cell Technologies
Seriological pipettes (5ml, 10ml, 25ml)	Sarstedt
Transwell-96 permeable support with 0.4μm pore	Sigma-Aldrich
Universal tubes	Fisher

Equipment	**Supplier**
-80°C Freezer	Sanyo
Biofuge 13, microcentrifuge	Heraeus Instruments
CliniMACS	Miltenyi Biotec GmbH
CO_2 incubator MCO-18AIC	Panasonic
Controlled rate freezer (CRF)	Planar
Coolmix	Biosafe
Coulter AcT 5diff CP counter	Beckman Coulter
Cryocart	Chart
EasySep magnet	Stem Cell Technologies
FACSCalibur flow cytometer	BD
FACSCanto flow cytometer	BD
Gallios flow cytometer	Beckman Coulter
Haemocytometer	Hawksley
Mars Flow hood	Scanlaf
Microplate scintillation counter	Packard
Microscope	Olympus BH-2
Microscope, Inverted	Olympus CKX41
MidiMACS separator	Miltenyi Biotec GmbH
MiniMACS separator	Miltenyi Biotec GmbH
MoFlow XDP cell sorter	Beckman Coulter
Nalgene Mr Frosty	Thermo Fischer Scientific
Nitrogen storage tanks	Chart
Rotine 420R Centrifuge	Hettich Zentrifugen
SEPAX	Biosafe
Syringe pump	WPI SP120P2
SYSMEX XE-2100	SYSMEX
TCD blade wafers	Link Medical
Top count scintillation counter	Packard
Total containment device (TCD)	Link Medical
Transport boxes	Sarsted
Tube sealer	Sebra
Waterbath GD120	Grant

Software	**Supplier**
CellQuest Pro	BD Biosciences
FACSDiva	BD Biosciences
FlowJo	Tree Star
Gallios	Beckman Coulter
InStat	GraphPad Prism
SmartDraw	SmartDraw, LLC
Summit	Beckman Coulter

2.1.3 Buffers

2.1.3.1 Buffers for tissue culture

White cell counting solution
0.6% (v/v) acetic acid
PBS

Trypan blue solution
0.1% (v/v) Trypan blue
PBS

Freezing solution
50% (v/v) DMSO
5% (w/v) Dextran-40
PBS

Freezing media
1% (w/v) Dextran-40
10% (v/v) DMSO
Human AB serum

Thawing solution
7.5% (w/v) Dextran-40
5% (v/v) Human AB serum
0.63% (w/v) Sodium citrate
5 mmol L^{-1} $MgCl_2$
1000 IU ml^{-1} DNase-1

MACS buffer
1mM EDTA
1% (v/v) Human AB serum
PBS

Culture media
2.5% (v/v) Human AB Serum
500 mM L-glutamine
1% (v/v) Penicillin-streptomycin solution
RPMI

Treg suppression assay media
1 μg ml^{-1} CD3
2 μg ml^{-1} CD28
200 IU IL-2
Culture media

2.1.3.2 Buffers for flow cytometry analysis

FACS buffer
1% (v/v) human AB serum
0.1% (w/v) NaN_3
PBS

FACS lysing solution
1% (v/v) Pharm lyse
Distilled water

Annexin V binding buffer
1% (v/v) Annexin V binding buffer
Distilled water

CFSE staining buffer
5% (v/v) Human AB serum
PBS

Fixation/permeabilisation solution
25% (v/v) Fix/Permeabilization concentrate
75% (v/v) Fix/Permeabilization diluent

Permeabilisation wash buffer
1% (v/v) Permeabilization buffer
Distilled water

2.2 Sample collection and transport to the Cell Therapy Centre

2.2.1 Ethical approval

The use of bone marrow and mobilised peripheral blood samples was approved by the Trent Ethics Committee and authorised by Nottingham University Hospital (REC reference number: 09/H0405/15). CB units collected for the ANCTC are consented for research use if they are not suitable for HSCTs (REC reference number: 10/H0405/27). All samples were obtained with written, informed consent and samples were stored in accordance with the Human Tissue Act 2008.

2.2.2 Umbilical cord blood collection

CB was collected *ex utero* by trained midwives. The umbilical vein was punctured and CB collected by gravity into collection bags containing 25ml citrate-phosphate-dextrose. Throughout this procedure, the delivery of the baby and the timing of umbilical cord clamping was not affected in anyway.

2.2.3 Bone marrow collection

BM samples were collected from donors (one autologous and nine allogeneic) from City Hospital, Nottingham or University College Hospital, London. The anticoagulant used was acid citrate dextrose (ACD-A), with added Heparin if the samples showed any signs of clotting during the harvest. The cells were filtered to remove bone fragments and a 10ml sample from each harvest was used in this study.

2.2.4 PBSC collection

Mobilised peripheral blood samples were collected from patients at City Hospital, Nottingham. Donors underwent a stem cell mobilisation regime using granulocyte-colony

stimulating factor (G-CSF) involving daily subcutaneous administration of Lenograstim (the doses given are shown in Table 2.1). PBSC harvest commenced when peripheral blood $CD34^+$ count $\geq 10/\mu$l. Harvest was performed using the Mononuclear Cell Collection program on a Spectra Optia Apheresis system. ACD-A was used to prevent coagulation of the sample. If the required cell dose was not met, the donor was given a further 526mcg Lenograstin and the harvest was repeated the following day. Due to ethical reasons, when samples were collected for this study, 1ml was taken from the donor's PBSC sample and diluted to 10ml with autologous plasma to ensure the patient received their full dose.

TABLE 2.1: Daily doses of Lenograstim administered to mobilise stem cells from BM to periphery

Patient weight (kg)	Daily dose (mcg day)
<55	526
55-80	789
81-110	1052
>110	1315

2.2.5 Transport

CB samples are transported in a cooled environment (4-15°C) from the maternities to the Cell Therapy Centre on a daily basis. Between collection and transportation, samples were maintained at refrigerated temperatures (2-8°C). After collection of BM and PBSC, immediate transport was arranged to the Anthony Nolan Cell Therapy Centre using a cooled environment (4-15°C).

2.3 Fresh storage, cryopreservation and thawing

2.3.1 Fresh storage

Unless otherwise stated, samples were maintained at refrigerated temperatures (4-8°C) without agitation.

2.3.2 Freezing whole blood or CBMC

2.3.2.1 Freezing methods - Controlled Rate Freezer (CRF)

Cryopreservation bags containing 20ml sample were chilled using a Coolmix device set at 4°C and mixed for 10 minutes. A pre-chilled DMSO/dextran-40 syringe was attached to the bag and using a syringe pump, 5ml of the solution was added over a 10 minute period (unless stated otherwise, see §2.5.4) on the Coolmix to give a final DMSO and dextran-40 concentration of 10% (v/v) and 1% (w/v) respectively (unless stated otherwise, see §2.5.5).

The bags were frozen to -120°C in a controlled rate freezer (CRF) (see Table 2.2 and Figure 1.3 for the freezing program) and transferred via a Cryocart to the vapour phase of liquid nitrogen storage tanks.

Table 2.2: Control Rate Freezer (CRF) freezing program used at ANCTC

Temperature change (°C min^{-1})	Target temperature (°C)
0.0	4.0
-1.0	-10.0
-20.0	-50.0
+15.0	-18.0
-1.0	-40.0
-2.0	-60.0
-3.0	-80.0
-10.0	-120.0

2.3.2.2 Freezing methods - Nalgene Mr Frosty freezing container

Samples to be frozen in cryovials were cooled for 10 minutes at refrigerated temperature before the addition of chilled freezing solution, resulting in a final concentration of 10% (v/v) DMSO and 1% (w/v) dextran-40 (unless stated otherwise, see §2.5.1). The cells were frozen overnight in a Nalgene Mr Frosty freezing container containing isopropanol in a -80°C freezer before being transferred to the vapour phase of liquid nitrogen tanks.

2.3.3 Thawing protocol

Samples were maintained for a minimum of 24 hours in liquid nitrogen tanks. The sample to be thawed was removed from the liquid nitrogen tanks and rapidly thawed in a 37°C waterbath. The sample was then assessed for cell recoveries and viability.

2.3.3.1 Wash protocol

In some cases the samples were washed post-thaw in order to remove DMSO. After thawing the sample in the water bath, an equal volume of chilled thawing solution was added dropwise. The sample was further diluted by the addition of culture medium and the cells sedimented at 450×g for 20 minutes at 4°C. The supernatant was carefully removed and the pellet treated for 2 minutes with 1-2 volumes of DNase (10^6 ml^{-1}). The sample was then washed and resuspended in culture media.

2.4 CBMC isolation

2.4.1 Ficoll-Paque Premium density gradient separation

Blood sample and Ficoll-Paque Premium were warmed to room temperature. 15-30ml of blood was carefully layered on top of 15ml Ficoll-Paque Premium in 50ml centrifugation tubes. The tubes were centrifuged at 400×g for 30 minutes at 20°C with the brakes switched off. After centrifugation, the plasma layer was removed followed by the

mononuclear cell layer which was washed twice with PBS before being resuspended in 20ml autologous plasma.

2.4.1.1 Leucosep density gradient separation

Blood sample and Ficoll-Paque Premium were warmed to room temperature. 15ml Ficoll-Paque Premium was added to the Leucosep tube. To pass the separation medium through the porous barrier, before the addition of blood, the tubes were centrifuged at 1000×g for 30 seconds at 20°C. After ensuring the separation media is below the porous barrier, 15-30ml of blood sample was poured on top of the barrier and the Leucosep tubes were centrifuged at 1000×g for 10 minutes at room temperature with the brakes switched off. 20ml plasma was removed from the tubes before pouring off the remaining sample into a 50ml centrifugation tube, the porous barrier avoids recontamination with granulocytes and erythrocytes. The cells were washed twice with PBS and resuspend with 20ml autologous plasma.

2.4.1.2 SepMate density gradient separation

Blood sample and Ficoll-Paque Premium were warmed to room temperature. 15ml Ficoll-Paque Premium was added to the Sepmate tube by passing it through the hole in the centre of the insert. 15-30ml of blood was pippetted down the side of the tube and spun at 1200×g for 10 minutes with the brake on. The top layer poured off and cells washed twice with PBS.

2.4.2 eMNC processing

CB units were processed using the eMNC program on a SEPAX machine. After removal of 1ml of blood for initial analysis, the blood bag was attached to a CS530.4 SEPAX kit using a Total Containment Device (TCD). The blood bag and CS530.4 kit was placed on the SEPAX device and the eMNC program initiated. When complete, the kit was taken from the SEPAX and the final product bag sealed and removed. 300μl of the final sample was taken from the final product bag for post-process analysis and the remaining

sample was either cryopreserved, as described in §2.3.2.1, or CD133 cells were isolated as described in §2.8.1.1

2.5 DMSO toxicity assessment

2.5.1 DMSO toxicity in fresh samples

CBMC units were split in to five aliquots, cooled to 4°C and an equal volume of chilled freezing solution was added slowly to give final concentrations of: 0% (v/v) DMSO, 1% (w/v) dextran-40; 10% (v/v) DMSO, 0% (w/v) dextran-40; 10% (v/v) DMSO, 1% (w/v) dextran-40; 20% (v/v) DMSO, 1% (w/v) dextran-40; 40% (v/v) DMSO, 1% (w/v) dextran-40. Numbers of viable cells were assessed immediately (time point zero, t=0) for each aliquot by flow cytometry (protocol described in §2.7.3.1 and 2.7.3.2) and the functional ability of the HPCs was assessed using the colony forming unit (CFU) assay (protocol described in §2.9). The remaining volume for each aliquot was split in two, with half stored at room temperature (19-22°C) and half at refrigerated temperature (4-8°C). After set time points (1, 2 and 24 hours) the quality of the cells was reassessed for each concentration at each temperature.

2.5.2 DMSO toxicity when delaying cryopreservation

CBMCs from 11 samples were split into two fractions. DMSO and dextran-40 was added to one fraction reaching a final concentration of 10% (v/v) and 1% (w/v) respectively. A 1ml aliquot (t=0) was immediately removed from this fraction and frozen according to §2.3.2.2. The other fraction was maintained without DMSO and dextran-40 during this stage (control sample). The remaining volumes were split again with half maintained at room temperature and the other half refrigerated. At set time points after freezing the t=0 sample (15 minutes, 30 minutes, 60 minutes, 120 minutes and 24 hours) an aliquot was taken from each of the four groups and frozen (10% (v/v) DMSO 1% (w/v) dextran added to the control cells before freezing). After a minimum of 24 hours in the vapour

phase of the liquid nitrogen tank, the samples were thawed rapidly in a 37°C waterbath and the quality of the cells reassessed.

2.5.3 DMSO toxicity in thawed CB

CBMCs from 10 CB units were isolated, the cell potency assessed and the samples were cryopreserved with a final concentration of 10% (v/v) DMSO 1% (w/v) dextran in a controlled rate freezer. After a minimum of 24 hours in the nitrogen tank the samples were thawed in a 37°C water bath and split into three aliquots. The first was left unmanipulated, designated 'not washed' and assessed for cell potency immediately, one sample was washed to remove all DMSO using the protocol in §2.3.3.1 and designated 'wash'. The final section was diluted 1:1 by the drop-wise addition of thaw media to reduce the DMSO concentration by half, this fraction was designated 'diluted'. All three fractions were maintained at room temperature and cell viability was assessed at t=0 (immediately post-thaw after any manipulations), 30 minutes, 1 hour, 2 hours, 24 hours and 48 hours after t=0.

2.5.4 DMSO addition speed

CBMCs were resuspended to 40ml in autologous plasma, split and placed in in cryopreservation bags which were chilled using a Coolmix device set at 4°C and mixed for 10 minutes. A DMSO/dextran-40 syringe was attached to each bag and using a syringe pump, 5ml of the solution was added to one sample at a rate of 0.5ml minute^{-1}, and to another sample at a rate of 10ml minute^{-1} on the Coolmix to give a final DMSO and dextran-40 concentration of 10% (v/v) and 1% (w/v) respectively. The bags were then frozen to -120°C in a controlled rate freezer (see §2.3.2.1 for the freezing protocol) and cell viability reassessed upon thawing.

2.5.5 Optimal concentration of DMSO

Cell potency of fresh CBMC samples was assessed. The samples were frozen using a CRF with different DMSO final concentrations of 10%, 7.5%, 5%, 2.5% or 0% (v/v)

(10 CB units were frozen with each concentration of DMSO). The samples were thawed rapidly in a water bath at 37°C and cell potency reassessed immediately without any post-thaw manipulations.

2.6 Cell counts

2.6.1 Manual counts

Red cells were lysed prior to counting through 1 in 4 dilution of the blood sample in the white cell counting solution. A haemocytometer was used to enumerate the cells.

To assess viability, an equal volume of the lysed red cell suspension was diluted with Trypan blue solution allowing identification of the dead cells which could then be excluded from the cell count.

2.6.2 Automated counts

Leukocyte counts were performed using either a Beckman Coulter AcT 5diff CP Counter or SYSMEX XE-2100. These instruments quantitate a range of parameters including leucocytes (which can be further broken down into granulocytes, lymphocytes and monocytes), erythrocytes and the haematocrit.

2.7 Flow cytometry

Flow cytometry was used to identify, enumerate and assess viability of different populations of cells expressing specific markers. This technique involves directing a laser beam onto a stream of liquid containing cells in single file. Upon contact with a cell, the beam of light is scattered onto a series of detectors. The forward scatter (FSC) detector is in line with the original source of light and detects the light that is deflected by the cell which is directly proportional to its size. The side scatter (SSC) detector is perpendicular to the source of light, it receives the light deflected by the internal components of

the cell (such as the nucleus), therefore, detecting the level of granularity and structural complexity within the cell.

Antibodies with a fluorochrome attached can be bound to the cells surface markers or internally. When a light beam of a particular wavelength hits the fluorochrome it becomes excited causing a release of energy in the form of light at a wavelength specific to that particular fluorochrome. This emitted light can then be detected by other detectors through a series of filters and mirrors allowing the identification of a bound antibody to a particular cell.

Different flow cytometers were used during this project, a list of instruments with their laser and filter specifications are included in Table 2.3. Each flow cytometer was calibrated daily according to the manufacturer's guidlines and the calibration beads specific to that machine.

TABLE 2.3: Flow Cytometers used throughout project

Machine	Company	Lasers	Filters	Software
FACSCalibur	BD	blue laser (488nm) red laser (635nm)	530/30, 585/42, 670LP 661/16	CellQuest Pro
FACSCanto	BD	blue laser (488nm) red laser (633nm)	530/30, 585/42, 670LP, 780/60 660/20, 780/60	Diva
Gallios	Beckman Coulter	blue laser (488nm) red laser (638nm) violet laser (410nm)	525/40, 575/30, 620/30, 696/30, 755LP 660/20, 725/20, 755LP 450/40, 550/40	Gallios Software
MoFlo XDP	Beckman Coulter	blue laser (488nm) red laser (635nm) violet laser (405nm)	530/40, 580/30, 613/30, 670/30, 740LP 670/30, 720/45, 780/40 450/65, 560/40	Summit Software

2.7.1 Compensation and use of isotype controls

The light emitted by the fluorochromes covers a range of wavelengths, and there can be an overlap between different fluorochromes leading to some spill-over as the light is picked up by other detectors. To remove this spectral overlap the signal can be

subtracted from the unwanted detectors, a process known as compensation. Compensation was achieved using compensation beads which were either stained with each of the individual fluorochromes or their isotopes. This allowed identification of the positive and negative populations for each marker and the subtraction of peaks picked up by undesired detectors. Compensation was performed manually on the FACSCalibur, FACSCanto and MoFlo machines and using the automated protocol on the Gallios.

Isotype controls were used at identical concentrations and staining conditions as the antibodies in order to confirm positive detection rather that non-specific binding. The isotype of each primary antibody used is listed in Table 2.4.

TABLE 2.4: List of antibodies and concentrations used for flow cytometry analysis

Antibody	Fluorochrome	Company	Clone	Isotype	Concentration (μg/μl)
CD3	Pacific Blue	Invitrogen	S4.1	Ms IgG_{2a}	0.60
CD3	PE	BD Pharmingen	UCHT1	Ms IgG_1,κ	1.00
CD3	PerCP	Biolegend	SK7	Ms IgG_1,κ	0.25
CD4	APC	Biolegend	OKT4	Ms IgG_{2b},κ	0.25
CD4	Pacific Orange	Invitrogen	S3.5	Ms IgG_{2a}	0.60
CD25	APC-Cy7	Biolegend	BC96	Ms IgG_1,κ	0.25
CD25	PE	BD Bioscience	2A3	Ms IgG_1,κ	0.03
CD25	PE-CF594	BD Horizon	M-A251	Ms IgG_1,κ	0.25
CD34	PE	BD Pharmingen	581	Ms IgG_1,κ	0.06
CD39	PE	BD Pharmingen	TU66	Ms $IgG2_{2b},\kappa$	0.19
CD45	APC	BD Pharmingen	HI30	Ms IgG_1,κ	0.06
CD45	APC-Cy7	Biolegend	HI30	Ms IgG_1,κ	0.50
CD45	FITC	BD Pharmingen	HI30	Ms IgG_1,κ	0.04
CD62L	APC-Cy7	Biolegend	DREG-56	Ms IgG_1,κ	0.30
CD69	Alexa Fluor 700	BD Pharmingen	FN50	Ms IgG_1,κ	1.00
CD127	FITC	BD Pharmingen	HIL-7R-M21	Ms IgG_1,κ	0.38
CD127	PE-Cy7	eBiosciences	eBioRDR5	Ms IgG_1,κ	0.50
CD194 (CCR4)	PerCP-Cy5.5	BD Pharmingen	1G1	Ms IgG_1,κ	1.00
CD278 (ICOS)	PE-Cy7	eBiosciences	ISA-3	Ms IgG_1,κ	0.13
FoxP3	APC	eBiosciences	PCH101	Rat IgG_{2a},κ	0.50
Annexin V	FITC	BD Pharmingen	N/A	N/A	0.05

2.7.2 Titrations of antibodies

The antibodies were titrated in order to avoid wasting reagents whilst maintaining the optimal separation between positive and negative populations. 1×10^6 cells were stained with a range of concentrations of antibody. The population of interest for each antibody dilution was observed by gating on the lymphocytes through their FSC and SSC properties. The positive and negative peaks were gated and the median fluorescence intensity (MFI) and standard deviation (SD) were calculated for each peak. The stain index (SI) calculated with the following formula:

$$SI = \frac{(MFI_{pos} - MFI_{neg})}{SD_{neg}}$$

The dilution with the highest SI was taken as the optimal antibody concentration. A list of antibodies used and their optimal concentration are shown in Table 2.4.

2.7.3 Haematopoietic progenitor cell analysis

2.7.3.1 ISHAGE CD34$^+$ enumeration

CD45$^+$ and CD34$^+$ cell enumeration was assessed based on the International Society of Haematotherapy and Graft Engineering (ISHAGE) guidelines.[426,427] For this, 0.6×10^6 cells were stained with anti-CD45 fluorescein isothiocyanate (FITC) and CD34 phycoerythrin (PE) conjugated antibodies for 15 minutes at 4°C. Erythrocytes were removed by incubating the cells with FACS lysing solution for 15 minutes in the dark (at room temperature). Viability was assessed by the exclusion of cells containing 7AAD. Perfect count beads were used for enumeration, 2000 bead events were analysed for each sample. The formula to calculate the absolute cell count is shown below:

$$absolute\ cell\ count = \frac{number\ of\ cells\ gated}{number\ of\ beads\ gated} \times \frac{number\ of\ beads\ per\ test}{sample\ test\ volume}$$

Flow cytometry was performed on either a BD FACSCalibur or FACSCanto and analysed using CellQuest Pro or FlowJo. The gating strategy for cell enumeration is shown in Figure 2.1.

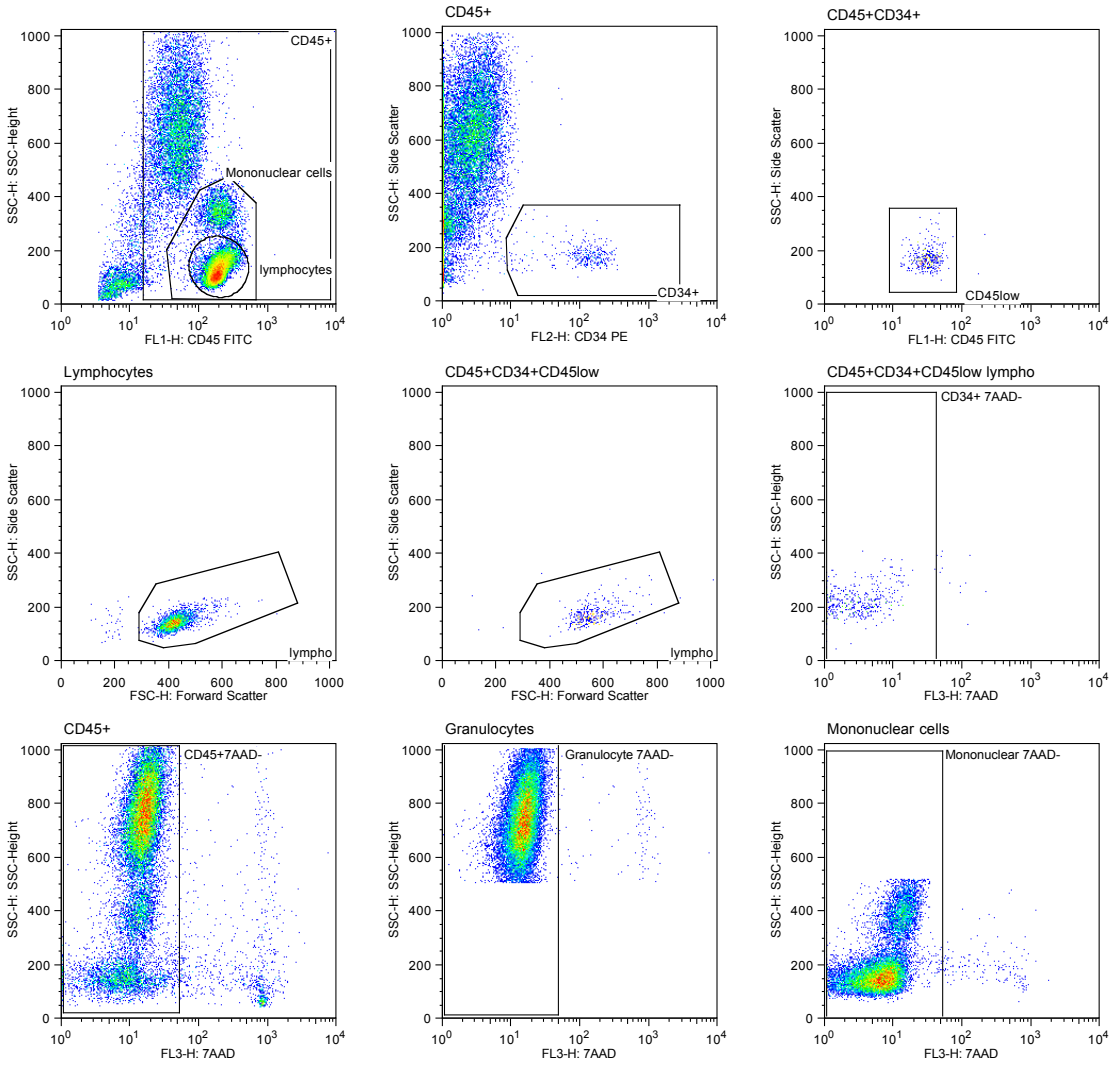

FIGURE 2.1: Flow cytometry gating strategy: HPC enumeration. HPC cells were identified using the following criteria: low CD45 expression, high CD34 expression and a FSC and SSC similar to that of lymphocyte cells (low SSC and low/medium FSC). Viability was assessed through the exclusion of cells stained with the 7AAD dye.

2.7.3.2 Apoptosis assessment

The 7AAD dye, used in the standard enumeration of HPCs, penetrates cells where the membrane is no longer intact. Therefore, only cells undergoing late apoptosis or necrosis are identified using this dye. In order to get a more accurate viability assessment

Annexin V was used to identify early-stage apoptotic cells by staining surface exposed phosphatidylserine.

Apoptosis was assessed using an adaption of the ISHAGE guidelines.[428] For this, 0.6×10^6 cells were stained with anti-CD45 allophycocyanin (APC) and CD34 PE conjugated antibodies and the erythrocytes lysed with using FACS lysing solution. The cells were washed and resuspended in Annexin V binding buffer and incubated with 7AAD and Annexin V. Flow cytometry was performed on a BD FACSCalibur or FACSCanto and analysed using CellQuest Pro or FlowJo. The gating strategy for viable cell assessment is shown in Figure 2.2.

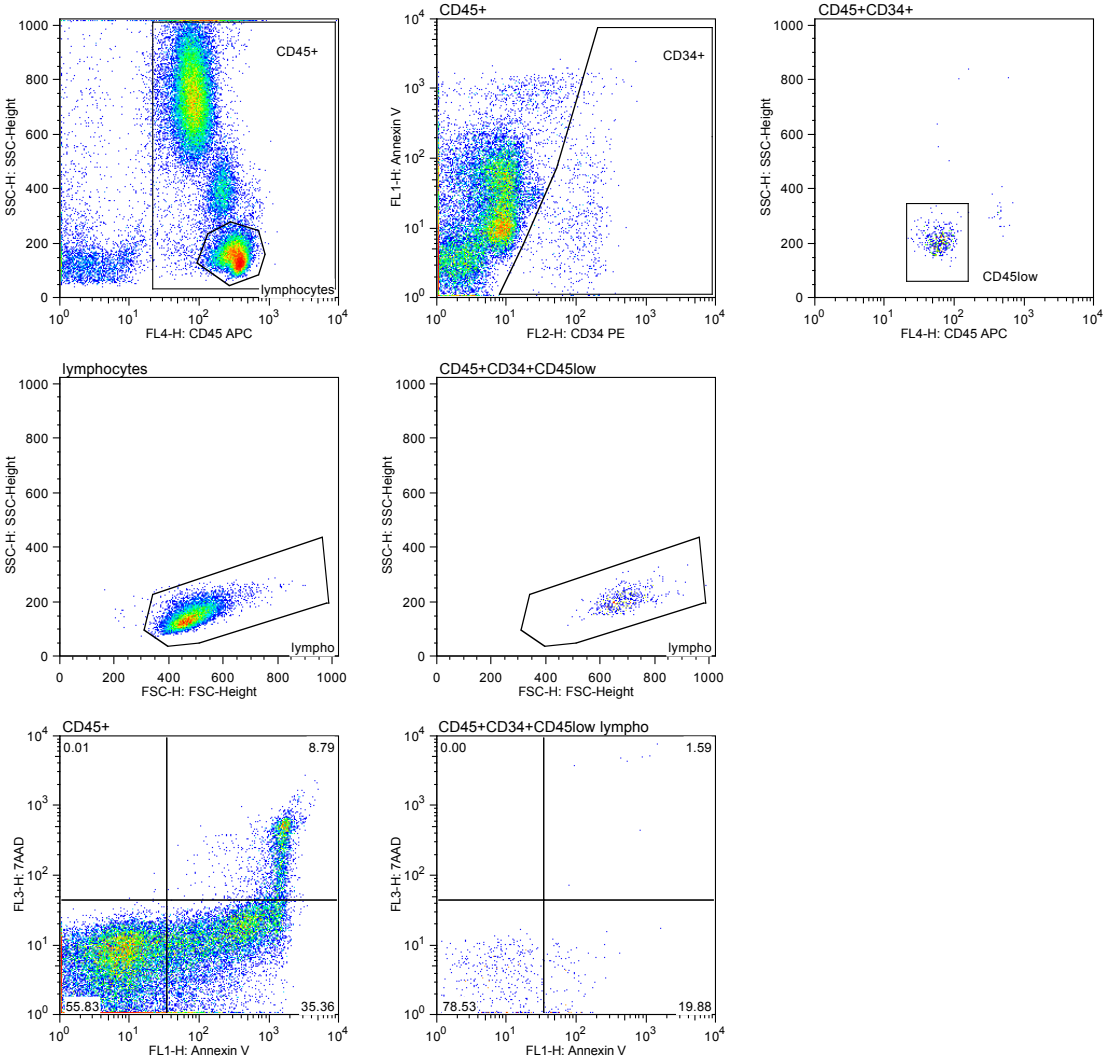

FIGURE 2.2: Flow cytometry gating strategy: HPC viability. HPC cells were identified using a slightly modified version of the HPC enumeration strategy (see Figure 2.1) before identifying the cells stained with 7AAD and/or Annexin V.

Enumeration of AnnV$^-$ viable cells was calculated by running this method in parallel with the standard enumeration protocol (§2.7.3.1) and applying the following formula:

$$viable\ cell\ count = absolute\ cell\ count \times \frac{CD34^+ AnnV^-\ gated}{CD34^+\ cells\ gated}$$

Where the *absolute cell count* was calculated during the standard enumeration protocol.

2.7.4 T regulatory cell analysis

2.7.4.1 Treg cell surface staining

0.5×10^6 cells were incubated with a previously prepared antibody cocktail (containing the conjugated antibodies for the cell surface markers of interest) for 15 minutes at 4°C. Following this, the cells were treating with FACS lysing solution for 15 minutes at room temperature. Samples were washed twice and resuspended in FACS buffer before flow cytometry assessment.

2.7.4.2 FoxP3 staining

Staining for intracellular FoxP3 requires a different protocol in which the cells are fixed and permeabilised to allow the dye to reach the nucleus. Intracellular staining was performed as recommended by the manufacturer (eBioscience). Cells were stained for surface antigens as described in §2.7.4.1. After washing off excess antibody, the cells were vortexed in order to ensure that the pellet had completely dissociated. The cells were then resuspended in 1ml fixation/permeabilisation solution and vortexed again. After 1 hour incubation at 4°C, the cells were washed twice using permeabilisation wash buffer. The cells were resuspended in 100μl permeabilisation wash buffer containing the FoxP3 APC antibody or isotype control. After incubating the cells for 1 hour at 4°C, the cells were washed twice using permeabilisation wash buffer and resuspended in FACS buffer before flow cytometry assessment using a FACSCanto or Gallios machine. The gating strategy for identifying Tregs is shown in Figure 2.3.

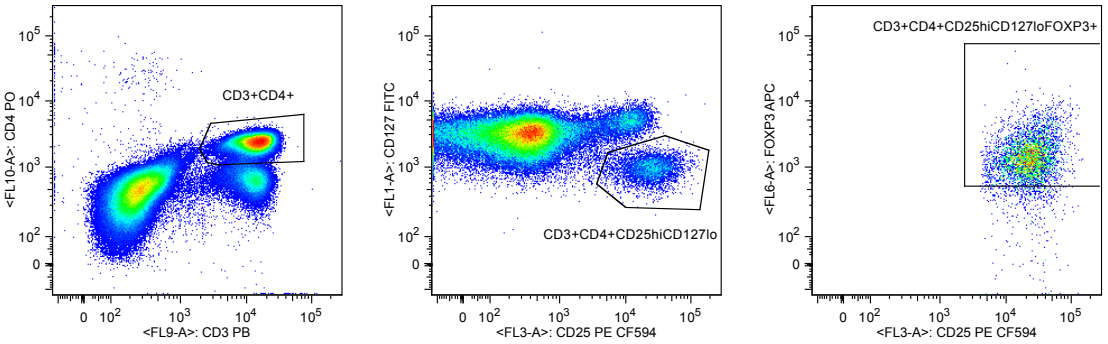

FIGURE 2.3: Gating strategy identifying T regulatory cells through the expression of $CD3^{+}CD4^{+}CD25^{high}CD127^{low}FoxP3^{+}$.

2.7.4.3 Treg viability assessment

Percentages of apoptotic and necrotic cells were assessed on Tregs using a protocol based on the CD34 viability assessment described in §2.7.3.2. For this, 0.5×10^{6} cells were stained with CD45 APC-Cy7, CD4 APC, CD25 PE and CD127 PE-Cy7 for 15 minutes at 4°C. Following this the cells were washed and resuspended in Annexin V binding buffer and incubated for 15 minutes with 7AAD and Annexin V at room temperature.

2.8 Cell isolation

2.8.1 Magnetic Activated Cell Sorting (MACS)

Magnetic activated cell sorting (MACS), developed by Miltenyi Biotec, involves labelling cells with antibodies attached to magnetic MicroBeads. The cell suspension is passed through a MACS column containing ferromagnetic spheres placed in a magnetic field. The labelled cells remain within the column allowing unlabelled cells to flow through and be collected (negative selection or depletion). After washing the labelled cells can be eluted from the column and collected (positive selection).

2.8.1.1 CD133$^+$ isolation using Miltenyi Biotec protocol

CBMCs were resuspended in 300μl MACS buffer per 10^8 cells. 100μl FcR blocking reagent followed by the same volume of CD133 Microbeads was added per 10^8 cells. The cells were mixed well and incubated for 30 minutes at refrigerated temperatures. After incubation, any unbound reagent was washed off in 20× their volume of MACS buffer and centrifuged at 300×g for 10 minutes before being resuspended in 2ml MACS buffer.

An LS column (capacity of up to 10^8 labelled cells or 2×10^9 total cells) was placed in a MidiMACS magnet with a pre-separation filter on top of the column. The column and filter was rinsed with 3ml MACS buffer. The cell suspension was added to the filter 1ml at a time allowing the first 1ml to enter the column before adding the next. The buffer dripping through the column contains the negative fraction and was collected. The column was washed 4× with 3ml MACS buffer. When the washing stage was completed, the column was carefully removed from the magnet and the positive cells eluted with 2× 5ml MACS buffer using the plunger provided with the column. The positive fraction was centrifuged at 300×g for 5 minutes and the pellet resuspended MACS buffer.

To increase the purity, the positive fraction was passed through a second column. When using a MS column (capacity of 10^7 labelled cells or 2×10^8 total cells) the cells were resuspended in 500μl MACS buffer. The MS column was placed in the MiniMACS magnet and rinsed with 1.5ml MACS buffer. The cells were added to the column 500μl at a time and the negative fraction washed through with 4× 500μl MACS buffer. The positive fraction was eluted with 2× 1ml MACS buffer and the cells centrifuged at 300 ×g for 5 minutes.

2.8.1.2 CD4$^+$ enrichment using Miltenyi Biotec MidiMACS

CBMCs were resuspended in 300μl MACS buffer per 10^8 cells. 100μl CD4 Microbeads was added per 10^8 cells. The cells were mixed well and incubated for 30 minutes at refrigerated temperatures. After incubation, any unbound reagent was washed off in

20× their volume of MACS buffer and centrifuged at 300×g for 10 minutes before being resuspended in 2ml MACS buffer.

$CD4^+$ cells were enriched by passing the sample through a single LS column as described in §2.8.1.1.

2.8.1.3 $CD25^+$ isolation using Miltenyi Biotec MidiMACS

$CD25^+$ isolations were performed according to the protocol suggested by Figueroa *et al.*[429] Briefly, CBMCs were resuspended at 300×10^6 cells ml^{-1} in MACS buffer, 4% (v/v) CD25 reagent was added and the cells incubated for 15 minutes at room temperature. The cells were washed and passed through two columns as described in §2.8.1.1.

2.8.1.4 $CD25^+$ isolation using Stem Cell Technology EasySep

CBMCs were resuspended at 1×10^8 cells ml^{-1} in RoboSep buffer in a 14ml polystyrene round bottom tube. The EasySep CD25 Positive Selection Cocktail was added to the cells at 50μl ml^{-1} cells, mixed and incubated for 15 minutes at room temperature. EasySep Magnetic Nanoparticles were mixed using a pipette, added at 50μl ml^{-1} cells and incubated at room temperature for 10 minutes. The volume of cells was made up to 10ml with RoboSep buffer, mixed and the tube placed in the EasySep magnet for 5 minutes. To remove unwanted cells, the EasySep magnet and tube are inverted for 3 seconds pouring off the supernatant fraction. The tube was removed from the magnet and the remaining cells resuspended in 10ml RoboSep buffer. The tube was placed back in the magnet and the separation step repeated a further 3 times leaving the isolated cells within the tube.

2.8.1.5 CliniMACS

4 to 6 CB samples were pooled into one collection bag and mixed well. The volume of the blood was reduced to 100ml using the volume reduction program on a SEPAX device in conjunction with a CS-490 kit. CBMCs were isolated from the sample using

the Density Gradient protocol on the SEPAX with a CS-900 kit which had 90ml ficoll injected into the output bag.

CBMC cells were resuspended at 300×10^6 cells ml^{-1} in MACS buffer and 4% (v/v) CD25 reagent added. The cells were incubated at room temperature for 15 minutes before washing in MACS buffer and resuspended in 75ml MACS buffer and transferred to a 150ml bag. A CliniMACS TS-600 tubing set was prepared, a 1L CliniMACS buffer bag containing 1% (v/v) AB serum was spiked onto the kit and a 150ml transfer bag was attached onto the output line. Enrichment protocol 3.2 was initiated on the CliniMACS and the sample bag attached when directed.

2.8.2 Fluorescent Activated Cell Sorting (FACS)

Fluorescent Activated Cell Sorting (FACS) is a specialised type of flow cytometry that allows the separation of specific cell populations based on their binding to antibodies attached to a fluorochrome. As with flow cytometry, cells are passed in single file through a laser beam and a series of detectors receive the light emitted by the fluorochrome. The flow of cells is then broken down into droplets containing a single cell which is directed into different tubes using an electrical charge

The $CD4^+$ population was enriched from CBMCs as in §2.8.1.2. The $CD4^+$ cells were resuspended at a concentration of 300×10^6 cells ml^{-1} in FACS buffer and incubated with either 15% (v/v) CD25 PE or 5% (v/v) CD25 APC-Cy7 and 15% (v/v) CD39 PE for 15 minutes at refrigerated temperatures. Excess antibody was washed off before the cells were reuspended at 20×10^6 cells ml^{-1} and passed through a 40μm filter. Sorting was performed on a Beckman Coulter MoFlo (laser and filter specifications shown in Table 2.3) under a high purity setting with the drop envelope set to 1. Sorted cells were gated on lymphocytes and $CD25^+$ expression with doublets excluded, these cells were collected in a tube containing chilled human AB serum.

2.9 Haematopoietic progenitor functional assay

The Colony-Forming Unit (CFU) assay is an *in vitro* assay based upon the ability of HPCs within a semi-solid media to proliferate and differentiate into colonies in response to cytokines. The colonies formed are enumerated and characterised according to their morphology. The CFU assay was performed using Methocult-4434. After flow cytometry analysis, 450 $CD34^+$ cells were seeded into 3ml Methocult and the sample volume made up to 300μl with Iscove's Modified Dulbecco's Medium. Following thorough mixing, 1.1ml was plated in duplicate. After 14 days incubation at 37°C in humidified air and 5% (v/v) CO_2, CFU colonies were scored by examination under an inverted microscope.

2.10 Treg Functional assessment

The functional capacity of Tregs are assessed by their ability to suppress the proliferation of activated $CD25^-$ cells. Within this project, the level of $CD25^-$ proliferation is measured either through the uptake of tritiated thymidine or by observing the number of cell divisions using flow cytometry and carboxyfluorescein succinimidyl ester (CFSE).

2.10.1 $CD25^-$ cells for proliferation

Tregs have been shown to be equally suppressive of autologous and allogeneic $CD25^-$ cells.[430–432] Therefore, throughout this project, the $CD25^-$ cells used to assess Treg suppression were from the same adult donor. A large batch of $CD25^-$ cells were produced which were aliquotted, cryopreserved and a single vial was thawed as required. This ensured similar levels of proliferation and Treg contamination with the $CD25^-$ fraction in all experiments.

2.10.1.1 Depletion of CD25

Adult mononuclear cells (PBMCs) were isolated using the protocol described in §2.4.1. $CD25^+$ cells were depleted using the Miltenyi Biotec MidiMACS system, cells were

resuspended at 100×10^6 cells ml^{-1} in MACS buffer, 10% (v/v) CD25 reagent was added and the cells incubated for 15 minutes at room temperature. The cells were washed and passed through two columns as described in §2.8.1.1. The CD25$^-$ fraction was resuspended in AB serum at a concentration of 10×10^6 cells ml^{-1} and aliquotted into cryovials (1ml or 10×10^6 cells per vial). The cells were cooled and DMSO and dextran-40 were added slowly to reach final concentrations of 10% and 1% respectively. Vials were frozen as described in §2.3.2.2 and thawed as described in §2.3.3. Post-thaw total nucleated cell (TNC) recovery and CD45$^+$ cell viability is shown in Figure 2.4.

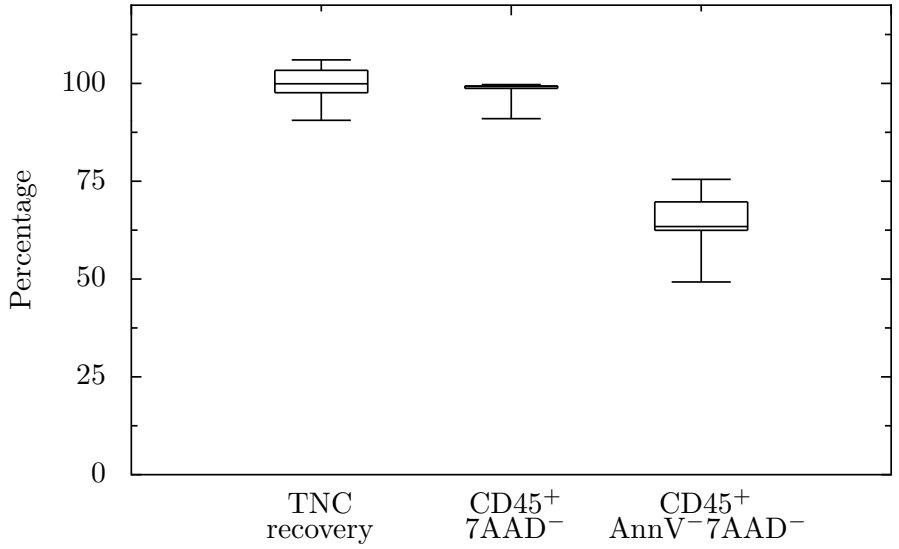

FIGURE 2.4: Total nucleated cell recovery (TNC) and viability (assessed by 7AAD alone or 7AAD and AnnV) of thawed CD25$^-$ cells used in the assessment of the suppression functions of Tregs (n=25). Data presented as a box and whisker plot showing medians, quartiles and ranges.

2.10.1.2 Optimisation of proliferation

To initiate proliferation in the CD25$^-$ cells, anti-CD3 and anti-CD28 are added to the culture media. These antibodies were titrated to ensure the optimum level of proliferation was achieved. When culturing 0.1×10^6 CD25$^-$ fresh cells per well the optimum concentrations were found to be 1μg ml^{-1} for anti-CD3 monoclonal antibody (mAb) and 2μg ml^{-1} for anti-CD28 mAb, shown in Figure 2.5A.

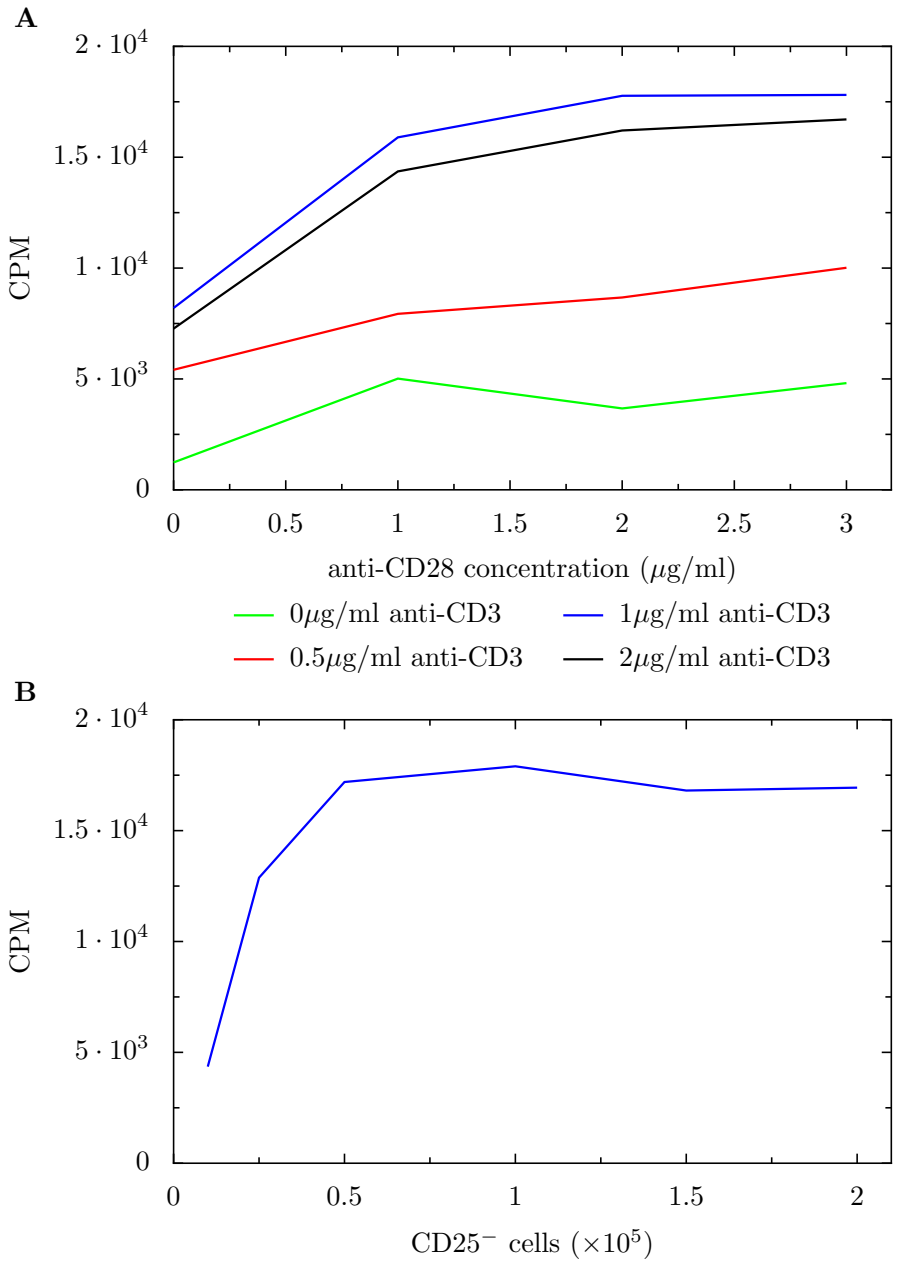

FIGURE 2.5: Optimisation of the CD25$^-$ cell proliferation assay used in the assessment of the suppression functions of Tregs. (A) anti-CD3 mAb and anti-CD28 mAb titration and (B) CD25$^-$ cell titration when using 1μg ml$^-$1 anti-CD3 mAb and 2μg ml$^-$1 anti-CD28 mAb (due to variability between experiments, data shown is representative of 3 repeats)

To determine the optimal proliferation conditions in the thawed $CD25^-$ cells they were split into two post-thaw, one half was washed (as described in §2.3.3.1), whereas the other half was not washed prior to culturing. Although the washed cells had a higher viability of 93.6% compared to the unwashed cells (85.6%), both fractions showed similar levels of proliferation (CPM of 32,565 and 34,465 for washed and unwashed respectively). Therefore, the wash step was found to be unnecessary for the thawed $CD25^-$ cells. To ensure the optimal number of $CD25^-$ cells were used in the proliferation assay, a range of cell concentrations were cultured with 1μg ml^{-1} anti-CD3 and 2μg ml^{-1} anti-CD28 mAbs. As shown in Figure 2.5B, 1×10^5 cell were found to be optimal for the proliferation assessment.

2.10.2 Assessment using uptake of ^{3}H-thymidine

A vial of $CD25^-$ PBMCs were thawed according to §2.3.3 and resuspended to 2×10^6 cells ml^{-1} in culture medium. 50μl of the $CD25^-$ cell suspension was added to each required well of a 96-well plate (total of 0.1×10^6 cells per well). The isolated Tregs were added at a range of $CD25^-$:$CD25^+$ ratios (1:1 to 1:20 $CD25^+$:$CD25^-$). Tregs were not added to one set of $CD25^-$ cells in order to assess the level of proliferation in these cells and therefore, the ability of Tregs to suppress when cultured together. Each well was made up to 100μl with culture medium. To initiate proliferation in the $CD25^-$ population, anti-CD3 mAb and anti-CD28 mAb were added to reach concentrations of 1μg ml^{-1} and 2μg ml^{-1} respectively in each well. Each combination was performed in triplicate and all wells had a final volume of 200μl. As controls $CD25^-$ cells were cultured alone without the anti-CD3 mAb and anti-CD28 mAb stimulation to assess background levels of proliferation. Also Tregs were cultured alone with anti-CD3 mAb and anti-CD28 mAb to ensure these cells remained anergic under these conditions.

Plates were incubated at 37°C and 5% (v/v) CO_2 for 4 days before the addition of 20μl of 0.37MBq ml^{-1} ^{3}H-thymidine, plates were then incubated for a further 16-18 hours. The cells were harvested onto Unifilter GF/C plates and the levels of ^{3}H-thymidine uptake assessed using a Packard Top Count Scintillation Counter.

Results were expressed as mean counts per minute (CPM) and the percentage suppression by the Tregs calculated using the following formula:

$$percentage\ suppression = 1 - (\frac{CPM_{CD25^{-}CD25^{+}\ together}}{CPM_{CD25^{-}\ alone}}) \times 100$$

2.10.3 Assessment using CFSE

Carboxyfluorescein succinimidyl ester (CFSE) is a fluorescent dye which is converted from the more permeable, carboxyfluorescein diacetate succinimidyl ester within the cytoplasm of the cell. As CFSE is less permeable it remains within the cell and during proliferation is partitioned equally amongst daughter cells upon each division. CFSE can be detected on a flow cytometer, with daughter cells showing peaks with increasingly lower intensities of expression, the area of each peak represents the numbers of cell at that stage of the cycle. Thawed $CD25^{-}$ PBMCs were stained with CFSE according to the protocol suggested by Quah *et al.*[433] Cells were resuspended in 1ml CFSE staining buffer in a non-wetted tube. The tube was laid horizontally and 110μl CFSE staining buffer was placed on the non-wetted portion at the top of the tube. 1.1μl CFSE (dissolved in DMSO to a concentration 5mM as according to manufacturer's instructions) was resuspended in the 110μl CFSE staining buffer at the top of the tube. The tube was inverted and vortexed immediately in order to ensure uniform distribution of CFSE amongst the cells. Cells were maintained at room temperature for 5 minutes and washed three times with 10× volume of CFSE staining buffer. Cells were resuspended to 2×10^{6} cells ml^{-1} in culture medium and suppression assays were performed in triplicate in 96 well plates as described in §2.10.2.

After four days the wells from each combination were combined, washed in FACS buffer and the cells stained with APC-conjugated anti-CD4 mAb for 15 minutes at 4°C. 7AAD was added to cells prior to analysis using a FACSCanto.

The percentage suppression by the Tregs was calculated using the following formula.

$$percentage\ suppression = \frac{\%\ CFSE\ proliferating\ cells_{CD25\text{-}CD25+\ together}}{\%\ CFSE\ proliferating\ cells_{CD25\text{-}\ alone}} \times 100$$

2.10.4 Transwell assay

96 well transwell plates with 0.4μm pore inserts were used to assess if Treg suppression occurred through a cell-cell contact dependent manner. As in the standard suppression assay (described in §2.10.2), 0.1×10^6 thawed $CD25^-$ cells were added per well, 0.05×10^6 Tregs were placed in either the upper chamber or lower well along with the $CD25^-$ cells ($CD25^+$:$CD25^-$ ratio of 1:2, combinations performed in triplicate). 1μg ml^{-1} anti-CD3 and 2μg ml^{-1} anti-CD28 were added and all wells were made up to 200μl. The plate was incubated for 4 days before ^{3}H-thymidine addition and plate harvesting performed as described in §2.10.2.

2.11 Statistical analysis

Results are expressed as mean ± standard deviation. Due to low numbers of replicates the data generated did not follow a normal (Gaussian) distribution, this was confirmed using the Kolmogorov-Smirnov Test. Therefore, when comparing paired samples, the Wilcoxon Matched Pairs Test (comparing two samples) and Friedman Test followed by Dunn's Multiple Comparison Test (comparing three samples) was used. For unpaired samples, the Mann Whitney U Test and Kruskal-Wallis Test (followed by Dunn's Multiple Comparison Test) were used for the comparison of two and three or more samples respectively. In any given test $p<0.05$ was considered to be statistically significant. Statistical analysis was performed using GraphPad InStat.

Graphs were created using Python and the PyX package. Statistically significant results are noted within graphs using the following symbols: *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

CHAPTER 3

TRANSPORTING CORD BLOOD TO THE BANK

3.1 Introduction

Haematopoietic progenitor cell (HPC) transplants are routinely used to restore the haematopoietic system after high dose chemotherapy.[20,21] HPCs can be obtained from three different sources: bone marrow (BM), mobilised peripheral blood (PBSC) and umbilical cord blood (CB). Although HPC grafts can be infused after a short storage period post-collection they are frequently cryopreserved, such as in autologous transplantation and in the case of some allogeneic procedures, particularly when CB samples are used. A delay between harvest of the cells and their cryopreservation is becoming usual as processing laboratories can be some distance from the collection centres. Jansen *et al* reported that the median transport time for HPC products collected in Europe to the USA is 37 hours (range 19-50 hours).[434] Worryingly, it has also been reported that PBSC and BM products with an increased transport time can lead to slower platelet engraftment,[435–437] suggesting a potential impact of the conservation conditions on the transplantation outcomes. Optimal fresh storage conditions have not as yet been established and the current literature remains inconclusive; according to current literature CB, PBSC and BM all have optimal storage temperatures of either 4°C or 25°C depending upon which group conducted the study and over what time period (Table 3.1).[19,54,438–457]

TABLE 3.1: Literature review of HSC fresh storage

Group	HSC Source	Effect of storage temperature	Effect of storage time
Rubinstein *et al.*[54]	CB	$CD34^+$ cell recovery highest when maintained at ambient temperatures prior to processing	Not assessed
Campos *et al.*[438]	CB	Significant decrease when maintaining cells at 4°C prior to freezing	No significant losses pre- or post-thaw observed for samples kept for 24 hours at room temperature.
Moldenhauer *et al.*[439]	CB	MNC and $CD34^+$ recoveries decrease when stored at 4°C compared to room temperature	After 24 hours CFUs decreased for both temperatures. After 72 hours CFUs decreased further for samples at 4°C but increased above the 24 hour levels for samples at room temperature
Broxmeyer *et al.*[19]	CB	CFU recoveries maintained when stored at 4°C and 25°C but not 37°C	Progenitor cells remain functionally viable for 3 days at 4°C and 25°C
Shlebak *et al.*[440]	CB	No difference in 4°C or 25°C, assessed up to 9 hours	Processing should be done in under 6 hours, definitely before 9 hours
Louis *et al.*[441]	CB	20°C storage resulted in reduced MNC recovery and $CD45^+$ viability compared to 4°C	72 hour storage at 20°C led to reduced MNC and $CD34^+$ recovery. No significant loss at 4°C after 72 hours
Hubel *et al.*[442–444]	CB	Storage preferable at 4°C which resulted in higher MNC counts	Can be stored for 24 hours before losses in pre- and post-thaw MNC and CFU-GM observed
Tsagias *et al.*[445]	CB	Reduced MNC, $CD34^+$ and CFU when stored at 25°C compared to 4°C	Recoveries reduced to ∼60% and ∼80% for 25°C and 4°C respectively after 24 hours and ∼20% and ∼60% for 25°C and 4°C respectively after 72 hours
Solomon *et al.*[446]	CB	Higher TNC, viable CD34 and CFU recovery observed at lower temperatures	Cells can be stored >80 hours at 4°C, 48 hours at 25°C and 12 hours at 37°C before significant losses were observed
Kurtz *et al.*[447]	CB	Not assessed. Maintained at 1-6°C	No loss in $CD34^+$ cells 3 days after collection

Continued from Table 3.1

Group	HSC Source	Effect of storage temperature	Effect of storage time
Mangalik *et al.*[448]	BM	Not assessed. Maintained at 4°C	Only minor CFU losses observed when stored up to five days
Michalova *et al.*[449,450]	BM	Viability maintained for longer at cooler temperatures	Cells remained viable for two days at 37°C and four days at 4°C
Kohsaki *et al.*[451]	BM	Not assessed. Maintained at 4°C	BFU-E recovery was reduced to 84% after 2 days, 44% after 4 days and 12% after 7 days
Lasky *et al.*[452]	BM	$CD34^+$ viability and CFU recovery declined rapidly when stored at 37°C. Higher recoveries when cells maintained at 4°C compared to 22°C	Poor CFU recovery after 7 days. Cell viabilities maintained for 3 days at 4°C and 22°C
Antonenas *et al.*[453]	BM	No losses between 4°C and room temperature over 72 hours. 37°C storage removed all viable $CD34^+$ cells	Numbers of cells recovered reduced as time progressed
	PBSC	Significantly greater loss of $CD34^+$ cells when maintained at room temperature. Storage at 37°C removed all viable cells	Numbers of cells recovered reduced as time progressed. Higher losses in allogeneic rather than autologous samples
Ruiz-Argüelles *et al.*[454]	PBSC	Not assessed. Maintained at 4°C	MNC viability remained over 90% over 72 hours
Jansen *et al.*[455]	PBSC	Cell viability decreased as temperature increased with significant losses in CFU-GM observed at 17°C and 22°C	Cell viability decreased over 96 hours, significant CFU-GM losses observed after 24 hours and 72 hours for samples at 22°C and 4°C respectively.
Moroff *et al.*[456]	PBSC	Not assessed. Maintained at 1-6°C	Samples can be maintained for 3 days without losses in pre- or post-thaw cell counts and viability
Petzer *et al.*[457]	PBSC	Higher CFU recoveries were obtained when storing at 4°C compared to room temperature	Cells may be stored overnight (16-20 hours) at 4°C without significant losses in CFU

Quality parameters used when assessing the potency of these stem cell products usually consist of identifying the numbers of viable cells assessed using 7AAD and numbers of colony forming units (CFUs). In this study post-thaw CFUs are focussed on as a main graft potency surrogate, as this has been found to be the best current predictive marker of engraftment with a minimum of 12.0 $\times 10^4$ CFU-GM kg^{-1} required for rapid neutrophil and platelet engraftment.[458–460] No other studies have evaluated all three sources of HPCs in the same laboratory with the same quality parameters. This is becoming particularly important when more centralised facilities for cell processing are being developed. This chapter investigates the effect of temperature on the fresh storage of CB, BM and PBSC; to determine the maximum time these cells can be stored before losing potency and to determine the effects that different storage conditions have on the ability to freeze and thaw the cells.

3.2 Results

Ten CB, eleven PBSC and ten BM samples were used in this study. A schematic representation of the experimental protocol can be found in Figure 3.1. Within 4 hours of collection, samples were split into 1ml aliquots. One was assessed immediately and cryopreserved (designated t=0), whilst the others were split with three stored at room temperature (19-22°C [20°C]) and the remaining three at refrigerated temperatures (4-8°C [4°C]). At set time points (24, 48, and 72 hours from t=0), an aliquot was taken from each storage condition and assessed. The remaining sample was cryopreserved and reassessed upon thawing.

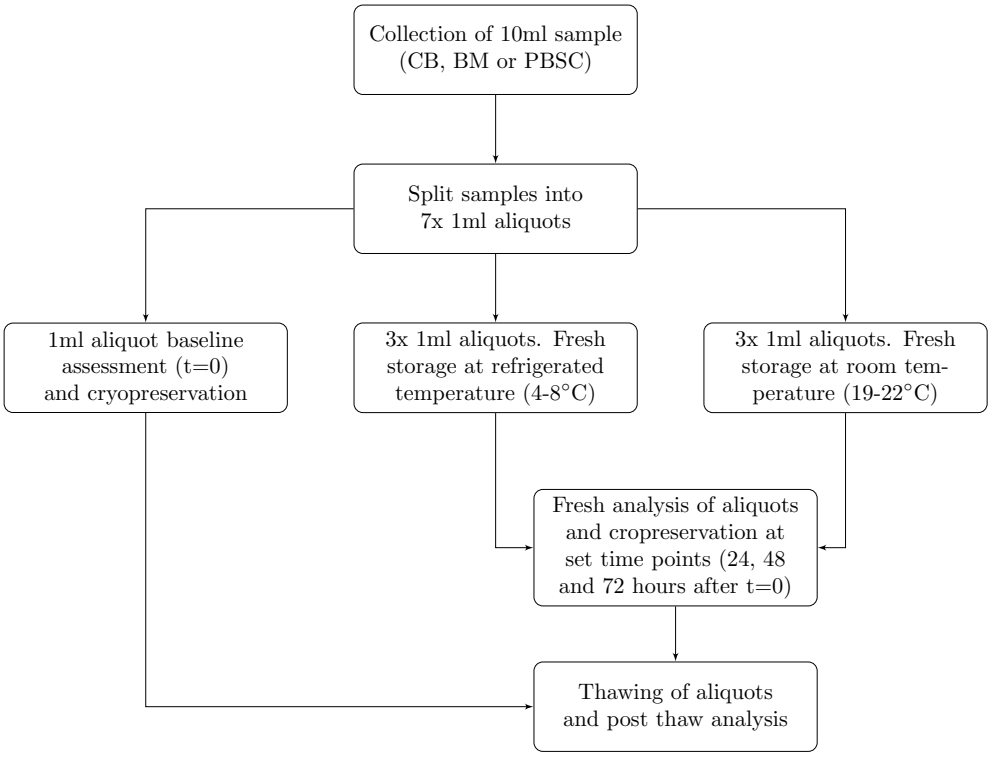

FIGURE 3.1: Schematic representation of the experimental protocol used to assess the effects of fresh storage conditions on CB, BM and PBSC samples

The mean viable nucleated and $CD34^+$ cell counts assessed by flow cytometry and CFUs after collection (t=0) are shown in Table 3.2 and the mean viable cells counts after cryopreservation at t=0 are shown in Table 3.3. At the time of collection the cell concentration varied between samples and was 12.93 ± 7.02 x$10^6\mu l^{-1}$ for CB, 20.88 ± 6.25 x$10^6\mu l^{-1}$ for PBSC and 29.70 ± 18.86 x$10^6\mu l^{-1}$ for BM. The viability of the

samples, assessed using 7AAD, gave viabilities of 95.67 ± 8.48%, 96.74 ± 3.78% and 94.63 ± 3.07% for CB, PBSC and BM samples respectively.

TABLE 3.2: Mean viable cell and CFU count per micro litre in fresh CB, PBSC and BM samples assessed at t=0

		$CD45^+7AAD^-$ cell count ($x10^3$ μl^{-1})	$CD34^+7AAD^-$ cell count (μl^{-1})	CFU count (μl^{-1})
CB	Mean ± SD (Range)	12.21 ± 6.58 (5.83-25.85)	74.99 ± 115.50 (11.03-392.30)	14.16 ± 10.33 (4.58-41.33)
PBSC	Mean ± SD (Range)	21.10 ± 6.64 (9.48-32.60)	234.46 ± 325.55 (75.71-1205.60)	41.35 ± 43.93 (7.50-168.00)
BM	Mean ± SD (Range)	27.97 ± 17.26 (8.48-66.50)	484.29 ± 453.89 (93.87-1427.20)	104.91 ± 102.53 (16.50-297.75)

TABLE 3.3: Mean viable cell and CFU count per micro litre in post-thaw CB, PBSC and BM samples after cryopreservation at t=0

		$CD45^+7AAD^-$ cell count ($x10^3$ μl^{-1})	$CD34^+7AAD^-$ cell count (μl^{-1})	CFU count (μl^{-1})
CB	Mean ± SD (Range)	10.55 ± 6.96 (3.38-23.63)	64.95 ± 119.58 (8.01-401.76)	7.98 ± 8.16 (1.56-30.00)
PBSC	Mean ± SD (Range)	18.62 ± 5.46 (6.99-28.32)	208.84 ± 278.36 (56.96-1026.19)	15.92 ± 9.93 (6.25-33.75)
BM	Mean ± SD (Range)	12.70 ± 10.77 (2.42-41.43)	252.98 ± 258.00 (41.99-900.00)	83.18 ± 98.89 (2.50-273.75)

3.2.1 The effect of storage temperature and time on $CD45^+7AAD^-$ cells

To elucidate the optimum storage condition for the three sources of HPCs, numbers of viable $CD45^+$ cells assessed by 7AAD were monitored and the effects of the two temperatures compared, the results are shown in Figure 3.2. In CB the $CD45^+7AAD^-$ cell count shows a significant difference between the two storage temperatures after 72 hours with recoveries of 86.78 ± 11.64% at 20°C and 94.14 ± 9.57% at 4°C (p=0.0004). This significant decline at 72 hours was also observed in viable granulocytes (97.54 ± 16.29% and 76.79 ± 23.71% for 4°C and 20°C respectively, p=0.02) and mononuclear cells (99.41

± 8.86% and 90.68 ± 9.33% for 4°C and 20°C respectively, p=0.0008) identified by flow cytometry through CD45 expression and side scatter. No statistical difference was observed between the two fresh storage temperatures over the 72 hour time period in the post-thaw analysis. The mean PBSC $CD45^{+}7AAD^{-}$ cell count also showed a difference between the two storage temperatures after 72 hours (p=0.03). Post-thaw cells stored at 20°C for 24 hours prior to cryopreservation resulted in a higher recovery of $CD45^{+}7AAD^{-}$ (p=0.009), however, after 48 hours, cells stored at 4°C exhibited higher recovery over 20°C and became significantly greater after 72 hours (p=0.02). Although no significant difference was identified in BM $CD45^{+}7AAD^{-}$ cells, a trend to higher recoveries after storage at 4°C was observed in fresh samples. With a recovery after 72 hours of 77.55 ± 15.62% at 20°C and 97.24 ± 40.65% at 4°C (p=0.24).

All three sample types maintained at 4°C in fresh, and prior to cryopreservation resulted in higher $CD45^{+}7AAD^{-}$ recoveries post-thaw. As a result of this, the effects of prolonged storage time were assessed in samples maintained at 4°C. CB $CD45^{+}7AAD^{-}$ cell counts show a gradual decrease in the samples over the 72 hours time period, but were not significant (p=0.08 for fresh and p=0.58 for thawed). However, the granulocyte subpopulation declined by a significant amount after 72 hours (recovery of 76.79 ± 23.71%, p=0.02). Mean PBSC $CD45^{+}7AAD^{-}$ cell count decreased by a significant amount after 48 hours of 4°C storage (89.75 ± 13.32% recovery p=0.03). This loss was observed earlier in thawed samples and resulted in a significant decrease after 24 hours, with a recovery of 91.39 ± 10.53% (p=0.04). This decrease was observed in the granulocyte cell subpopulation (recovery of 74.76 ± 16.36%, p=0.01). BM $CD45^{+}7AAD^{-}$ recovery resulted in no significant difference after 72 hours of fresh storage. However, when assessed post-thaw, a significant loss in $CD45^{+}7AAD^{-}$ cells was detected after 72 hours with a recovery of 68.66 ± 26.85% (p=0.005). This decrease was also observed in the mononuclear cell subpopulation with a recovery of 70.55 ± 29.93% (p=0.02).

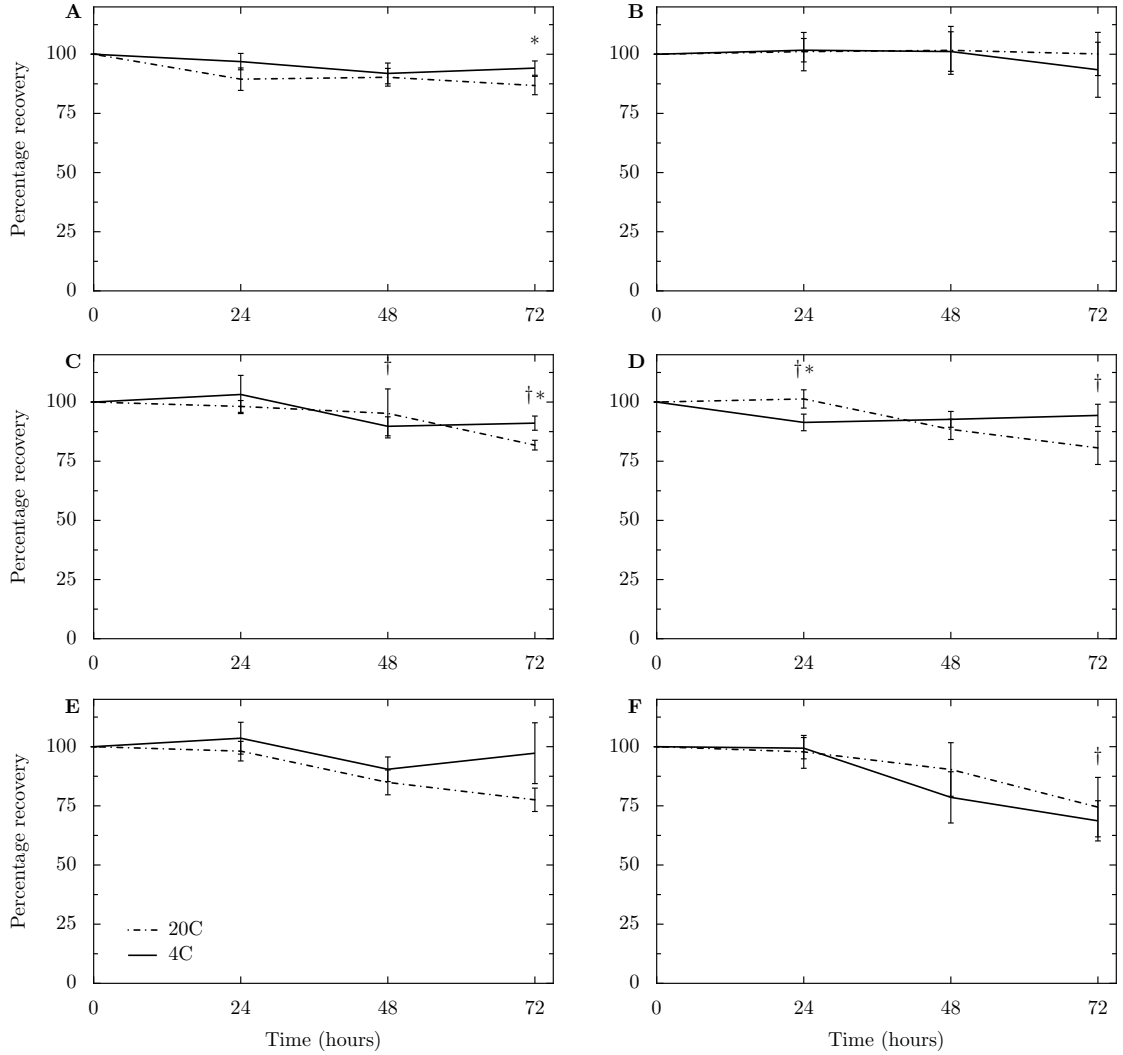

FIGURE 3.2: Mean $CD45^{+}7AAD^{-}$ recovery in HPC products during prolonged fresh storage at different temperatures and the effects of prolonged storage before cryopreservation in (A) fresh CB cells, (B) post-thaw CB cells, (C) fresh PBSCs, (D) post-thaw PBSCs, (E) fresh BM cells, and (F) post-thaw BM cells (n=10 for CB and BM and n=11 for PBSC). Error bars correspond to the SEM. * $p<0.05$ between storage temperatures. † $p<0.05$ in cells stored at refrigerated temperatures compared to t=0.

3.2.2 The effect of storage temperature and time on $CD34^+7AAD^-$ cells

Temperature assessment of $CD34^+7AAD^-$ in Figure 3.3, showed a trend towards higher recoveries in fresh CB samples stored at 4°C. However, a significant difference was observed in the thawed samples after 48 hours storage prior to cryopreservation (74.95 ± 24.89% recovery after 20°C storage against 90.14 ± 21.34% after 4°C storage, p=0.046). An increased loss of $CD34^+7AAD^-$ cells was observed in PBSC samples after 72 hours in cells stored at 20°C over 4°C, both in fresh and thawed samples (p=0.01 for fresh samples and p=0.005 post-thaw). Although no significant difference was observed between BM samples stored at 20°C or 4°C throughout the 72 hours, those at 4°C exhibited a higher mean recovery.

When assessing the effects of time on the HPC sources at 4°C, CB showed a decrease in the fresh $CD34^+7AAD^-$ cell counts in the first 24 hours after collection (recovery of 81.82 ± 19.24%, p=0.03). A significant reduction was also observed after 72 hours in fresh samples as well as in the thawed samples (86.27 ± 16.39% p=0.03 for fresh and 86.16 ± 18.29%, p=0.04 for thawed samples). A significant reduction in PBSC $CD34^+7AAD^-$ cells was observed after 48 hours in both fresh and thawed samples (90.03 ± 14.84% fresh recovery p=0.05, 86.80 ± 14.13% thaw recovery p=0.005). This significant difference continued to samples stored for 72 hours prior to cryopreservation (p=0.002). In BM a significant loss was observed after 48 hours fresh storage at 4°C (recovery of 85.58 ± 17.02%, p=0.03) and later in the thawed samples after 72 hours (recovery of 70.20 ± 24.46%, p=0.003).

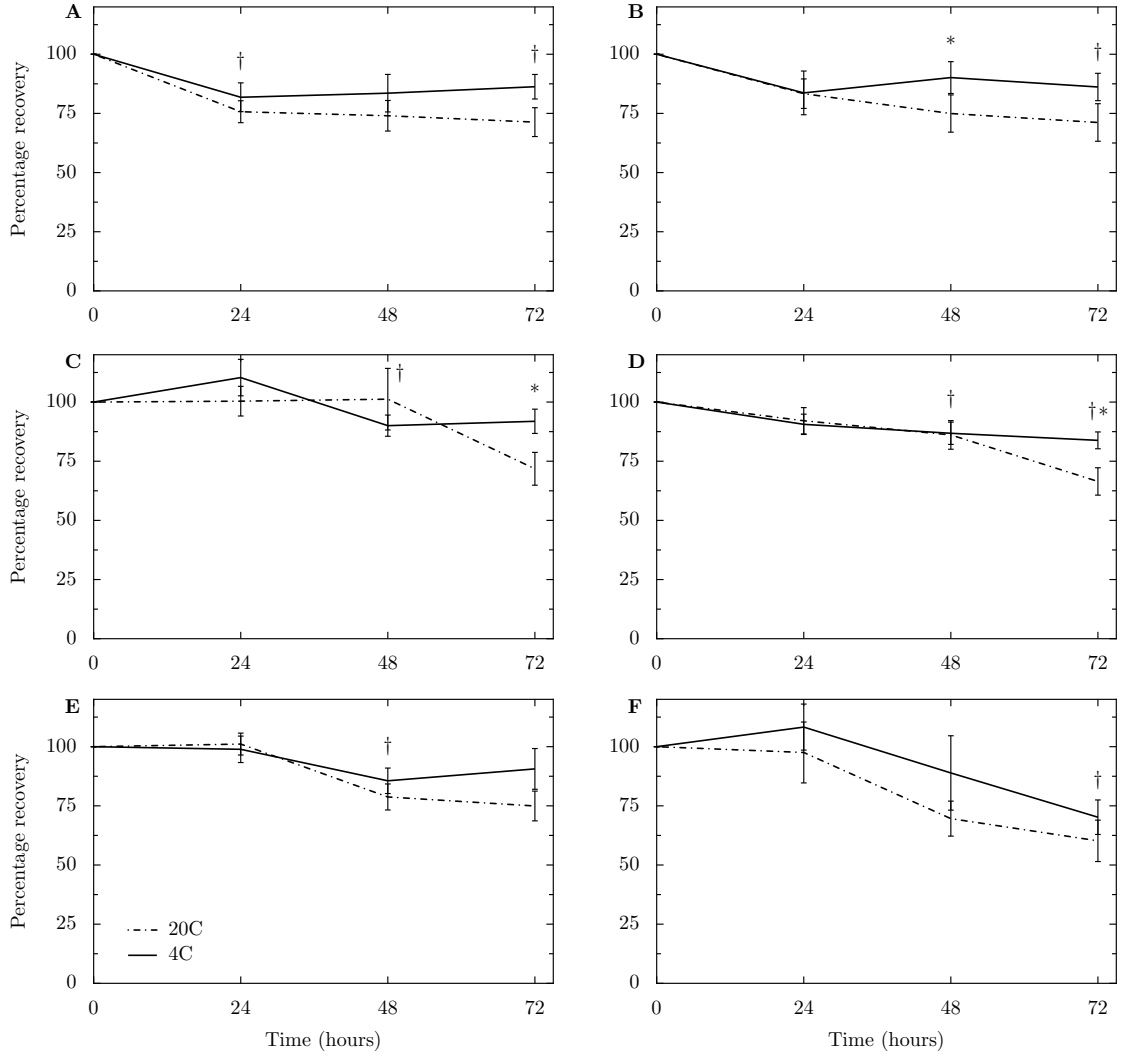

FIGURE 3.3: Mean $CD34^{+}7AAD^{-}$ recovery in HPC products during prolonged fresh storage at different temperatures and the effects of prolonged storage before cryopreservation in (A) fresh CB cells, (B) post-thaw CB cells, (C) fresh PBSCs, (D) post-thaw PBSCs, (E) fresh BM cells, and (F) post-thaw BM cells (n=10 for CB and BM and n=11 for PBSC). Error bars correspond to the SEM. * $p<0.05$ between storage temperatures. † $p<0.05$ in cells stored at refrigerated temperatures compared to t=0.

3.2.3 The effect of storage temperature and time on $CD34^+AnnV^-7AAD^-$ cells

The use of 7AAD to assess cell viability is common in CB banks, however, this stain only detects necrotic and late apoptotic cells. Consequently Annexin V was used to identify early apoptotic cells giving an improved viability assessment, which has been found in a previous study to correlate with CFU.[428] The assessment of fresh storage temperature on $CD34^+AnnV^-7AAD^-$ cells is shown in Figure 3.4.

In the CB samples, a trend to higher mean recoveries was observed when cells were stored at 4°C. As with the $CD34^+7AAD^-$ enumeration, these differences were not found to be significant. Lower $CD34^+AnnV^-7AAD^-$ counts were observed in PBSC samples stored at 20°C after 72 hours in both the pre- and post-thaw assessment (p=0.0151 and p=0.047 pre- and post-thaw respectively). As in the CB analysis, no significant difference was observed between the two storage temperatures over 72 hours for BM samples (76.59 ± 41.67% and 81.45± 36.56% recovery after 72 hours for 4°C and 20°C respectively).

Storing CB at 4°C for up to 72 hours prior to cryopreservation did not result in a significantly reduced $CD34^+AnnV^-7AAD^-$ cell recovery (87.16 ± 7.03% and 89.97 ± 18.32% for fresh and post-thaw analysis respectively). Higher numbers of $CD34^+AnnV^-7AAD^-$ cells were lost in the PBSC samples with a mean recovery of 73.42 ± 11.67%, p=0.0395 after 48 hours, which was further reduced to 63.72 ± 18.01%, p=0.0097 after 72 hours. Delaying cryopreservation of BM for up to 72 hours at 4°C did not result in significantly reduced $CD34^+AnnV^-7AAD^-$ recovery in the pre- and post-thaw assessment (76.59 ± 41.67% and 76.00 ± 48.65% for fresh and post-thaw analysis respectively).

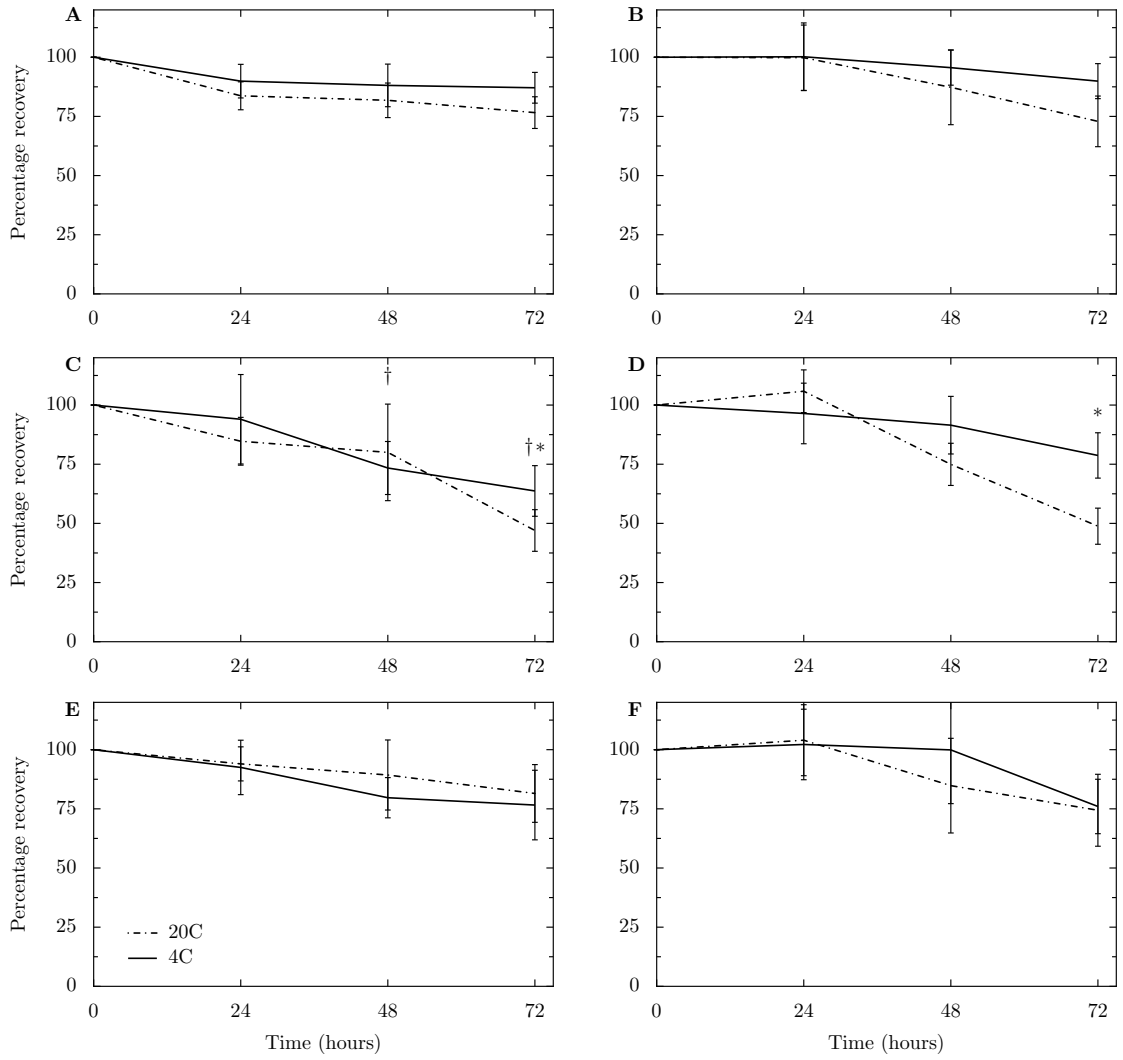

FIGURE 3.4: Mean $CD34^{+}AnnV^{-}$ $7AAD^{-}$ recovery in HPC products during prolonged fresh storage at different temperatures and the effects of prolonged storage before cryopreservation in (A) fresh CB cells, (B) post-thaw CB cells, (C) fresh PBSCs, (D) post-thaw PBSCs, (E) fresh BM cells, and (F) post-thaw BM cells (n=10 for CB and BM and n=11 for PBSC). Error bars correspond to the SEM. * $p<0.05$ between storage temperatures. † $p<0.05$ in cells stored at refrigerated temperatures compared to t=0.

3.2.4 The effect of storage temperature and time on CFU assessment

CFU is currently considered to be the best predictor of engraftment and therefore, its assessment is the best way of assessing the potency of HPC samples.[458–460] CFU analysis was performed on all aliquots, and photographs of CFU dishes from a CB sample are shown in Figure 3.5. Comparing the two storage temperatures on the CFU recovery of fresh HPC products a trend of higher recoveries when stored at 4°C was observed, data shown in Figure 3.6. However, in the post-thaw samples, the difference was shown to be significant. In CB a significant difference was observed after 72 hours (recovery of 38.50 ± 31.65% and 54.12 ± 34.2% for 20°C and 4°C respectively p=0.02), 48 hours for PBSC (45.56 ± 20.23% for 20°C against 67.34 ± 26.19% for 4°C p=0.01) and 24 hours for BM samples (40.51 ± 11.52% for 20°C against 63.60 ± 28.03% for 4°C p=0.02). This suggests that 4°C is the optimum temperature to maintain all three HPC samples after collection.

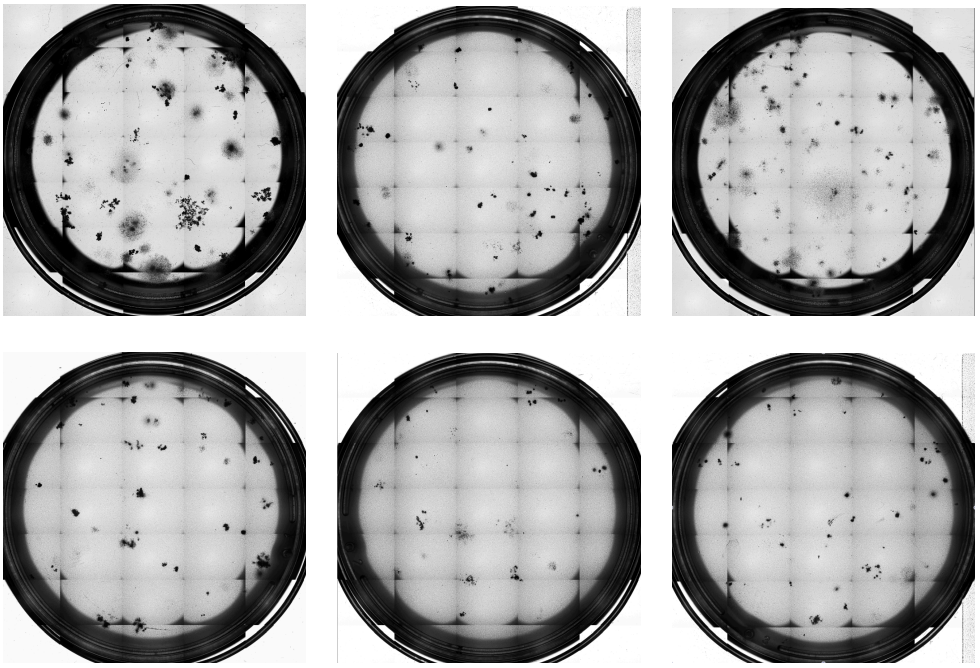

FIGURE 3.5: Photographs of CFU dishes from a CB sample taken during the assessment of the effects of fresh storage conditions when delaying cryopreservation. CFU dishes from a fresh (top row) and thawed (bottom row) CB sample at t=0 (left column) or 72 hours after storage at 20°C (middle column) or 4°C (right column). Dishes shown are representative of 10 CB samples. Photographs taken and stitched together using a StemVision (Stem Cell Technologies).

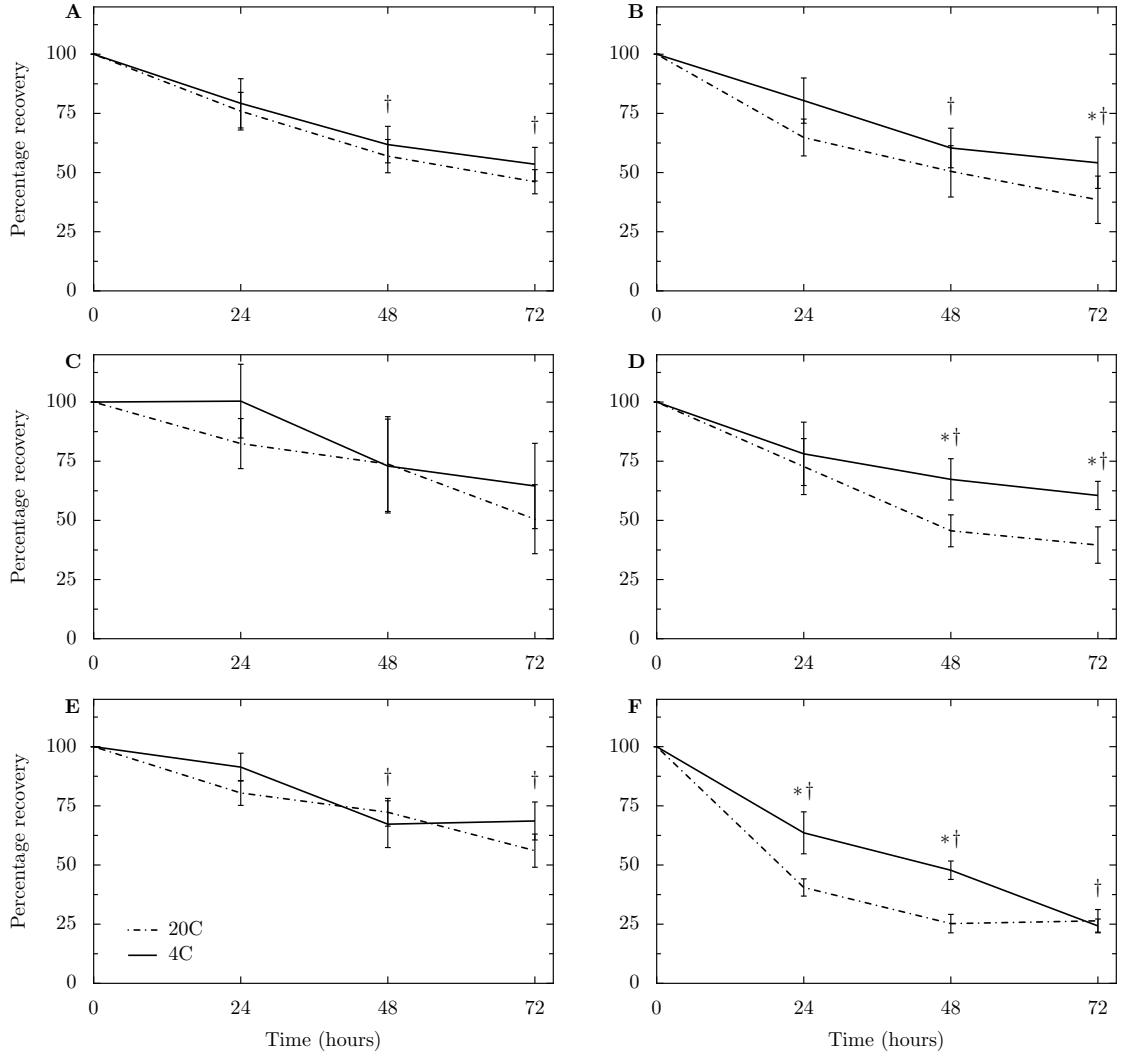

FIGURE 3.6: Mean CFU recovery in HPC products during prolonged fresh storage at different temperatures and the effects of prolonged storage before cryopreservation in (A) fresh CB cells, (B) post-thaw CB cells, (C) fresh PBSCs, (D) post-thaw PBSCs, (E) fresh BM cells, and (F) post-thaw BM cells (n=10 for CB and BM and n=11 for PBSC). Error bars correspond to the SEM. * $p<0.05$ between storage temperatures. † $p<0.05$ in cells stored at refrigerated temperatures compared to t=0.

For samples stored at 4°C, time had a continuous influence on the potency of the HPCs both in the fresh and post-thaw data. A statistically significant reduction in CFUs from those at t=0 in CB samples stored at 4°C was observed after 48 hours in both fresh (61.82 ± 24.36% recovery, p=0.0008) and thawed samples (60.40 ± 26.42% recovery, p=0.001). Although a decline was noted, no significant loss of CFU was observed in fresh PBSC samples stored at 4°C. However, cryopreservation after 48 hours fresh storage resulted in a thaw CFU recovery of 67.34 ± 26.19% compared to those frozen at t=0 (p=0.006). BM samples resulted in a significant reduction in CFUs after 48 hours 4°C storage (67.25 ± 31.25% recovery compared to fresh t=0 p=0.009). In contrast a significant reduction was observed after 24 hours in the post-thaw samples (63.60 ± 28.03% recovery compared to thaw t=0 p=0.003). Table 3.4 summarises the main results regarding banking showing the CFU percentage losses after delaying cryopreservation.

TABLE 3.4: Mean post-thaw CFU loss in CB, PBSC and BM after prolonged storage at refrigerated temperatures prior to cryopreservation. Percentages indicate mean recovery ± s.d. and p values indicate differences in cells stored at refrigerated temperatures for up to 72 hours prior to cryopreservation compared to those frozen at t=0

	24 hours	48 hours	72 hours
CB	19.59 ± 30.24%	39.62 ± 26.42%	45.88 ± 34.22%
p value	0.071	0.001	0.002
PBSC	21.88 ± 40.15%	32.66 ± 26.19%	39.46 ± 17.86%
p value	0.141	0.006	0.0002
BM	36.40 ± 28.03%	52.21 ± 11.68%	75.74 ± 8.73%
p value	0.003	$9.1\text{x}10^{-7}$	$5.1\text{x}10^{-9}$

3.3 Discussion

These studies are the first to identify the effects of time and different storage temperatures prior to cryopreservation on the viability and CFU capacity of cells of all three sources of HPCs. Higher recoveries were obtained, in all cell types assessed, for samples maintained at refrigerated temperatures prior to cryopreservation. The difference between the two storage temperatures was significant in $CD45^+$ cells after 72 hours for PBSC samples; in $CD34^+$ cells after 48 hours for CB and 72 hours for PBSC; and in CFUs after 24 hours for BM, 48 hours for PBSC and 72 hours for CB. The assessment of time showed continuous reductions in the numbers of viable cells and CFUs pre- and post-thaw, however, there were slight differences between the sources of HPCs. CB post-thaw viable $CD45^+$ cell recoveries showed a gradual, but not significant decline over the 72 hours, however, PBSC and BM samples gave a significant difference compared to samples frozen at t=0 after delays of 24 hours (p=0.04) and 72 hours (p=0.005) respectively. The effect of an increased delay prior to cryopreservation on viable $CD34^+$ cell recovery in PBSC samples resulted in a significant difference after 48 hours (p=0.005), a significant loss occurred later in CB and BM samples at 72 hours (p=0.04 and 0.003 respectively). CFU recovery declined significantly after a 24 hour delay prior to cryopreservation in BM samples and after a 48 hour delay in CB (p=0.001) and PBSC samples (p=0.003). However, although losses in CFUs were not significant in CB and PBSC samples until 48 hours, 19.59% of CFUs were lost in CB and 21.88% lost in PBSC samples after a delay of 24 hours. CB units are routinely frozen and banked prior to transplantation; this data suggests that units should be maintained at refrigerated temperatures and cryopreserved as soon after collection as possible (preferably within the first 24 hours, but no later than 48 hours) in order to retain the highest possible potency of the cells. This range is in support of the current recommendations of FACT Netcord standards[461] and in agreement with other studies.[441–445,462,463] The PBSC results confirm earlier reports that the viability of the cells within samples are maintained best when stored at refrigerated temperatures.[455–457,464,465] The fresh BM results are consistent with those obtained by other groups.[451–453] However, upon thawing, BM samples resulted in low viable cell and CFU recoveries, suggesting it is better to maintain BM

samples in a fresh state for up to 72 hours than to cryopreserve. In this study cells were stored in 1ml aliquots in cryovials to compare different conditions on the same sample. However, in this experimental set up, the cryopreservation protocol is not consistent with that used in clinical processing in which larger volumes are cryopreserved with the use of controlled rate freezers. Although comparing these two methods of cryopreservation in CB samples resulted in 7.27% fewer CFUs recovered when freezing small volumes (data shown in §5.2.2), we still consider the pattern of recoveries to be representative. In conclusion, this study highlights the importance of minimising the delay between collection of a sample and cryopreservation or infusion into a patient. Thawed cell products resulted in significantly decreased CFU recoveries when cryopreservation had been delayed for 24 hours in BM samples and 48 hours for both CB and PBSC samples. As the CFU dose given to a patient is the best current predictive marker of engraftment,[458–460] this study shows that delays in cryopreservation can increase risk for an unsuccessful transplant.

Based on these observations, CB samples are transported to the ANCTC in a cooled environment using a validated transport box that maintains a temperature range of 4°C to 15°C. If this temperature range is not met during transportation, samples are processed as normal but with additional viability tests post-thaw to ensure the cells maintained an acceptable level of potency. The ANCTC now also aims to process and cryopreserve all CB units designated for HSCT banking within 24 hours of collection. However, this is not always possible due to the high costs of courier runs between the maternity and ANCTC which cannot occur more than once per day. Therefore, to avoid the CFU losses observed after 48 hours ANCTC has set a maximum time for cryopreservation to 36 hours after collection. Any CB samples that cannot be cryopreserved within this timeframe are downgraded to research use only but can still be stored within the Biobank.

CHAPTER 4

ISOLATION OF CBMC

4.1 Introduction

The collected volume of cord blood (CB) is highly variable; since August 2012 the average volume received at the Anthony Nolan Cell Therapy Centre (ANCTC) is 97.36ml (range 14.32ml to 297.30ml, n=7195). For the efficient storage of a large number of different units in cryotanks, each one has its volume reduced to 21ml, regardless of the cell density, and is stored at 25ml (after the removal of a final sample for analysis and addition of 5ml cryoprotectant solution). The CB units designated for potential haematopoietic stem cell transplantation (HSCT) use undergo volume reduction by SEPAX (produced by Biosafe).[466] The samples that are unsuitable for HSCT and designated for the Biobank will also require volume reduction in order to fit within the cryotanks.

A complete description of the SEPAX machine is beyond the scope of this work. Briefly, this is a fully automated closed system which removes a proportion of the erythrocytes and plasma, reducing the sample to the required volume for storage. The input blood bag is attached to a single use, sterile tubing set consisting of separation chamber and output bags for the different fractions connected by a series of tubes and valves (Figure 4.1). The separation chamber is a large syringe which when placed inside the SEPAX device rotates along its vertical axis applying centrifugal forces on the blood sample. This

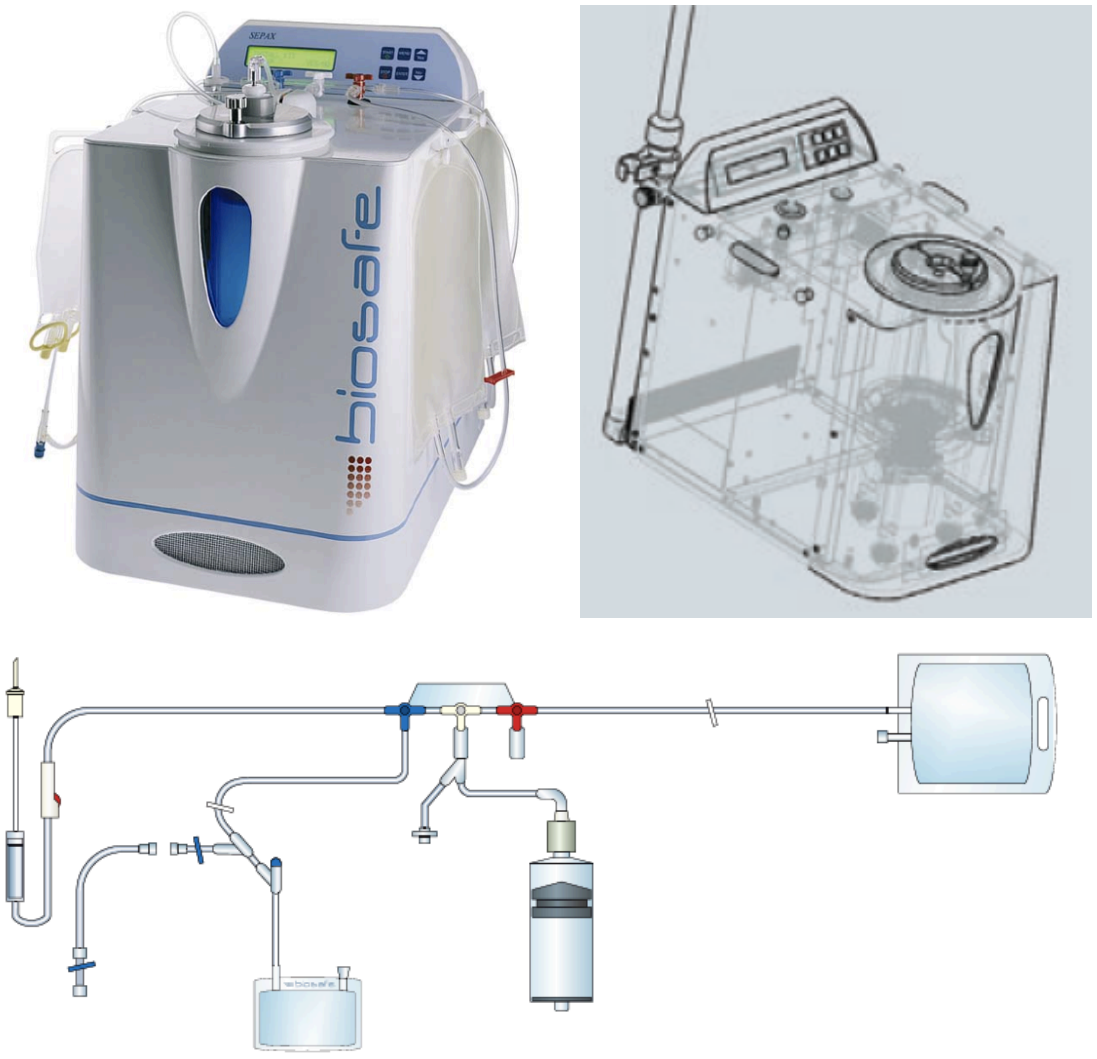

FIGURE 4.1: Biosafe's SEPAX device and CS540 processing kit. Images from http://www.biosafe.ch

separates the sample into three fractions according to cell density; plasma collects down the centre of the tube and denser erythrocytes are pushed to the outer wall leaving the buffy coat to collect in the middle (Figure 4.2). After centrifugation, the blood is pushed out of the separation chamber, plasma first followed by the buffy coat and finally the erythrocytes. As the fractions are pushed into the tubing, they pass through an optical sensor which detects the cellular content through differences in light absorbance, and directs the various fractions to their designated collection bag through a series of valves.

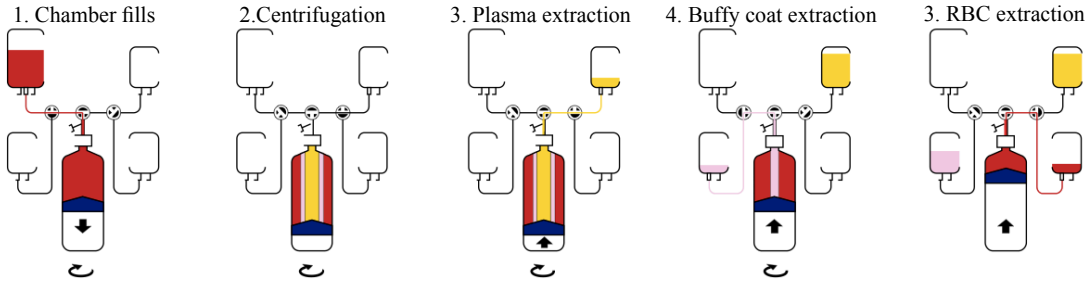

FIGURE 4.2: The process of cell separation through centrifugation by Biosafe's SEPAX device and tubing sets. Images adapted from http://www.biosafe.ch

The isolation of mononuclear cells (MNC) is often required for their use in research and cell therapy. A common procedure for obtaining mononuclear cells (MNC) is the Ficoll Density Gradient Method (Ficoll).[467–469] This involves the careful layering of blood on top of Ficoll, a polysaccharide with a specific density, followed by centrifuging the blood and Ficoll at a low speed. Upon contact with the Ficoll, the erythrocytes aggregate and form a rouleaux* increasing their density. During centrifugation the denser red blood cells (RBC) and granulocytes (Gr) sediment allowing easy removal of the MNCs. There are several shortcomings with this procedure; the lack of processing reproducibility, high user variability, the open nature of the system and the time consuming layering of the blood on top of the Ficoll. If the layer between Ficoll and blood is not clear and distinct prior to centrifugation, the MNC layer not form after centrifugation and cells will be lost. Therefore, care must be taken when layering the blood on top of the Ficoll, and when removing the MNC layer post-centrifugation, in order not to mix the layers. This can take some time and requires a certain level of skill leading to low processing reproducibility and high user variability. This process requires the addition of Ficoll and is performed in 50ml tubes increasing the risk of contamination.

Biosafe has developed a protocol, named eMNC, using a SEPAX machine that would be a potential alternative to the Ficoll method. Volume reduction processes involving the SEPAX use sealed, sterile tubing sets consisting of a chamber for centrifugation and various output bags for the different fractions all connected by a series of tubes and valves. This tubing set ensures the samples maintain their sterility. The eMNC

*Due to their flat discoid shape erythrocytes can stack together forming long chains which resemble a stack of coins. These structures are called rouleaux and can be induced by resuspending the cells in macromolecules such as dextran and Ficoll.[470]

process aims to separate the MNC fraction through centrifugation alone without the need for a density gradient media, such as Ficoll. As the sample is pushed back out of the centrifugation chamber, it passes through an optical sensor which identifies the cellular content, and a series of valves direct each fraction to a designated collection bag. Removing the need to add Ficoll further minimises the risk of contamination. Although the new protocol takes roughly the same time as the Ficoll method, another potential advantage is that it is completely automated, removing user variability and allowing a technician to be free to perform other duties.

As part of a collaboration with Biosafe, the aim of this study is to assess if this new protocol can be used as an alternative to the Ficoll method. To achieve this the eMNC products must have a final volume of 21ml with similar or improved MNC recovery, granulocyte and erythrocyte depletion compared to the Ficoll method. A maximum final haematocrit[†] limit of <5% has been set as a target for erythrocyte depletion.

[†]the percentage volume that the erythrocytes occupy within the blood sample

4.2 Results

4.2.1 eMNC

CBMCs (cord blood mononuclear cells) were isolated using either the new eMNC program on a SEPAX machine or with the Ficoll method. Data were reported back to Biosafe who made alterations to the eMNC program and sent updated versions back for testing.

Three versions of the eMNC protocol released by Biosafe were assessed and compared to 116 CB units processed with Ficoll (versions 1 and 4 were not released for testing). The CB samples processed using the eMNC protocol had a mean initial volume of 75.72ml (range 55.13 to 100.59ml). The eMNC program was successfully able to reduce these volumes to a mean of 21.18ml (range of 14.79 to 22.47%). The first version of eMNC tested (version 2) showed a greater total nucleated cell (TNC) recovery of 46.07 ± 26.09% compared to 33.70 ± 10.98% in the units processed using Ficoll. This higher TNC recovery was due to not as a result of an increase in MNC recovery (46.14 ± 18.73% for eMNC against 46.26 ± 18.28% for Ficoll), but due to the higher granulocyte recovery (32.06 ± 28.47% for eMNC against 11.88 ± 10.57% for Ficoll, p=0.00748). Version 2 also shows a higher erythrocyte (RBC) recovery for the eMNC product (6.60 ± 3.97% for eMNC against 0.46 ± 0.72% for Ficoll, p<0.0001). The visual appearance of the eMNC product also confirms higher levels of erythrocyte contamination when compared to a product processed with Ficoll. The median post-process haematocrit level (HCT) of the eMNC product is 8.44 ± 0.05% compared to 0.30 ± 0.86% for the Ficoll products (p<0.0001). Version 3 (the second to be assessed) had alterations to the program in an attempt to improve the granulocyte and erythrocyte depletion. The products from this version had improved MNC recovery of 58.69%, but similarly poor granulocyte and erythrocyte recoveries of 32.15 ± 23.97% and 7.73 ± 4.94% respectively. Changes made to the final version released for testing (version 5) included an update in the tubing set kit test prior to the process, the erythrocytes were redirected to a separate bag rather than remaining in the separation chamber at the end of the run and also improvements were made to the 'tube cleaning' with the aim to reduce erythrocyte contaminants. This

version showed improved granulocyte depletion (recovery of 21.02 ± 10.34%), but a high erythrocyte recovery of 8.96 ± 2.44% with a HCT of 11.88 ± 0.03% which is still much higher then the 5% limit initially set. Viability assessment of both Ficoll and the three eMNC versions tested all resulted in high levels of $CD45^{+}7AAD^{-}$ (>99%).

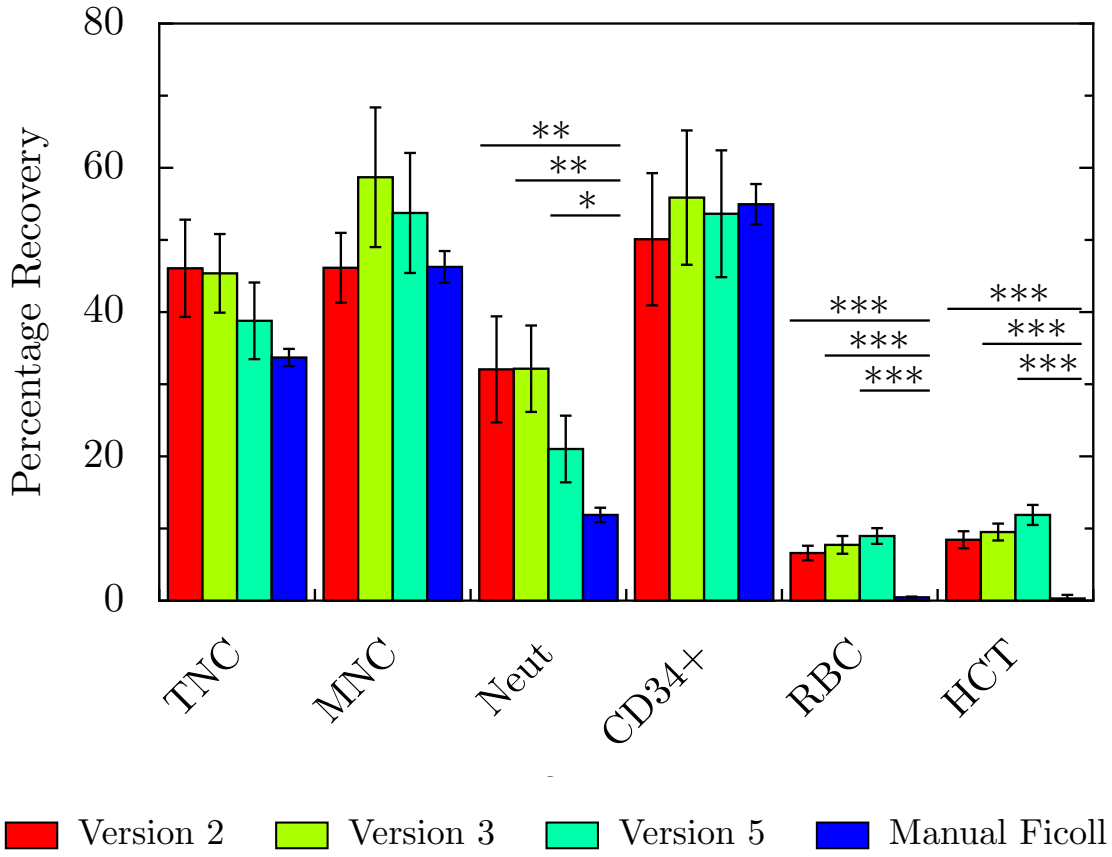

FIGURE 4.3: Isolation of CBMCs: eMNC versus manual ficoll - cell products. Comparing the three versions of the eMNC program against manual Ficoll in the isolation of mononuclear cells from CB (n=14 for version 2, n=16 for version 3, n=5 for version 5 and n=116 for Ficoll). Error bars correspond to the SEM.
*p<0.05, **p<0.01 and ***p<0.001 compared to the Ficoll method

4.2.1.1 Cryopreserving eMNC products

The ability to cryopreserve the eMNC products was assessed and the cell recoveries and viability compared to post-thaw Ficoll products (Figure 4.4). Lower TNC, $CD34^{+}$ cell and CFU recoveries were observed post-thaw in the eMNC products compared to samples processed using Ficoll. The eMNC units also had lower viabilities (assessed using 7AAD and AnnV), however, these differences were not found to be significant.

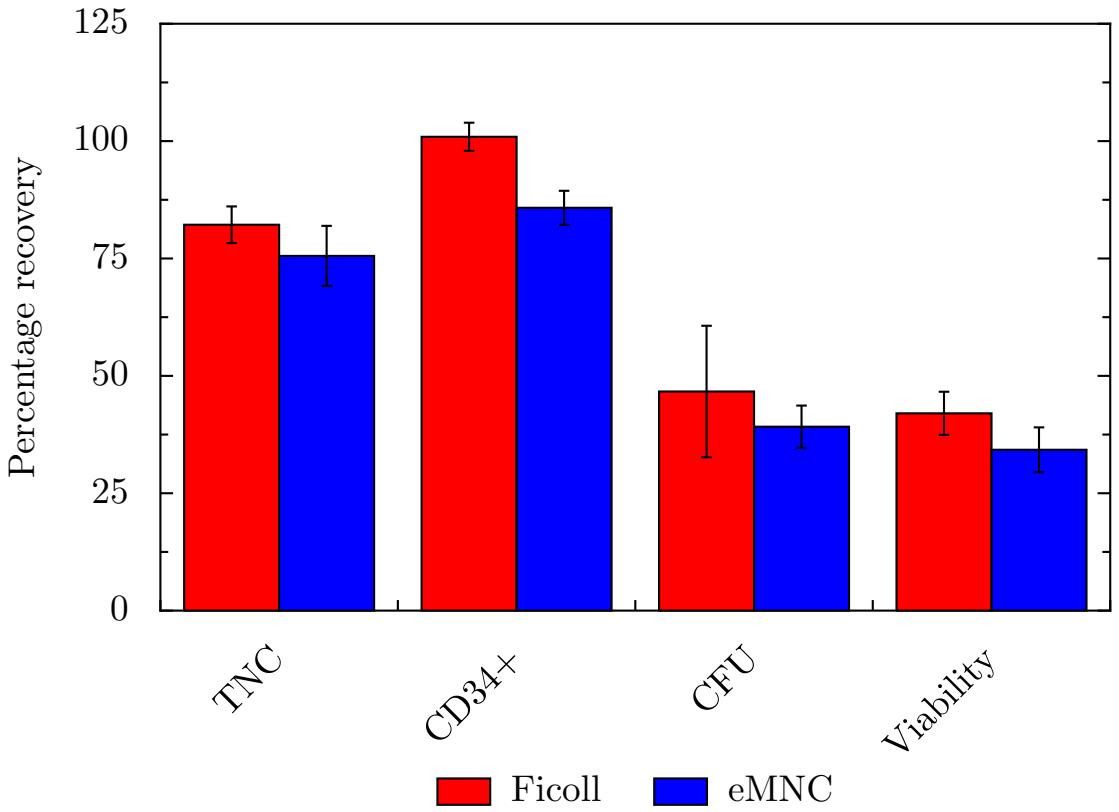

FIGURE 4.4: Isolation of CBMCs: eMNC versus manual ficoll - post-thaw cell recoveries and viability. Comparing post-thaw cell recovery and viability of eMNC version 5 (n=4) and Ficoll (n=4) processed products. Error bars correspond to the SEM.

4.2.1.2 Cell isolation from eMNC products

MNC enrichment procedures are commonly performed prior to isolating pure cell populations. Therefore, the ability to isolate $CD133^+$ cells from eMNC products was compared to isolations from cells processed using the Ficoll method. Seven $CD133^+$ cell isolations were performed, three of which were on units processed by eMNC and the other four were on units processed using Ficoll. As shown in Figure 4.5, the isolations from the eMNC products resulted in $CD133^+$ fractions with lower purity compared to Ficoll samples (77.97 ± 8.03% and 88.98 ± 7.60% for eMNC and Ficoll respectively, p=0.0106). Isolations using eMNC products also resulted in lower levels of $CD133^+$ cell recovery (36.6 ± 10.00% and 44.84 ± 12.28% for eMNC and Ficoll respectively). Similar $CD133^+$ viability and CFU recoveries were observed for both MNC isolation methods.

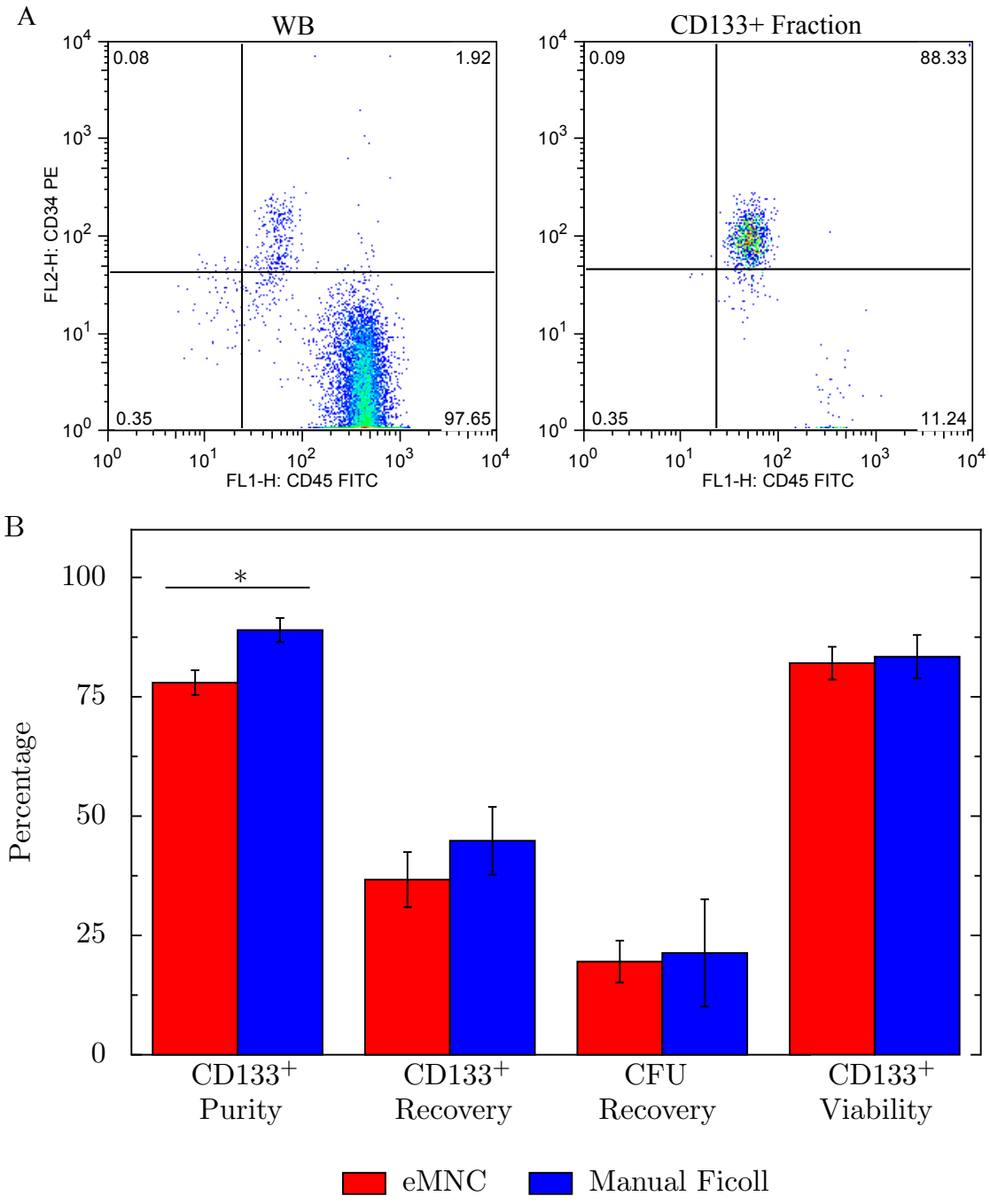

FIGURE 4.5: Isolation of CBMCs: eMNC versus manual ficoll - $CD133^+$ cell isolation. (A) Dot plot showing $CD133^+$ cells in whole blood and post-isolation. Data shown rare epresentative of 4 samples. (B) Comparing the products from $CD133^+$ isolations from CBMCs isolated using Ficoll (n=4) or the eMNC version 5 program (n=3). Error bars correspond to the SEM. * denotes a $p<0.05$, ** denotes a $p<0.01$ and *** denotes a $p<0.001$

4.2.2 Optimising Ficoll density gradient protocol

The eMNC separation protocol was found not to be a suitable alternative to Ficoll due to high levels of contaminating erythrocytes and granulocytes in the cell product. Therefore, to improve Biobanking processes, the Ficoll method currently used at ANCTC (as described in §2.4.1) was further optimised for CB samples.

4.2.2.1 Diluting blood before layering

Prior to isolating MNCs using Ficoll, blood is often diluted 1:1 or 1:2 with PBS. This reduces the density of the blood making layering over Ficoll easier. Dilution also reduces the cell concentration preventing MNCs from becoming trapped as the erythrocytes aggregate and fall to the bottom of the tube. The need to dilute CB prior to MNC enrichment was assessed. By removing the dilution step fewer tubes would be required, speeding up the process and reducing the cost of reagents and consumables. To assess the feasibility of using whole blood CB samples were split into two fractions, one of which was diluted 1:1 with PBS, the other fraction left undiluted. Ficoll separation was performed on both fractions and the cell products compared (results shown in Figure 4.6). Dilution of CB prior to MNC isolation resulted in an increase in TNC recovery compared to undiluted samples. However, no difference in the MNC (lymphocyte and monocyte populations) recovery was observed. The increase in TNC recovery was found to be due to lower levels of granulocyte depletion (granulocyte recovery of 30.59 $\pm$ 16.42 and 18.44 $\pm$ 19.46 for diluted and undiluted samples respectively p=0.0195). Both methods resulted in high levels of erythrocyte depletion with low final haematocrit levels of <1%.

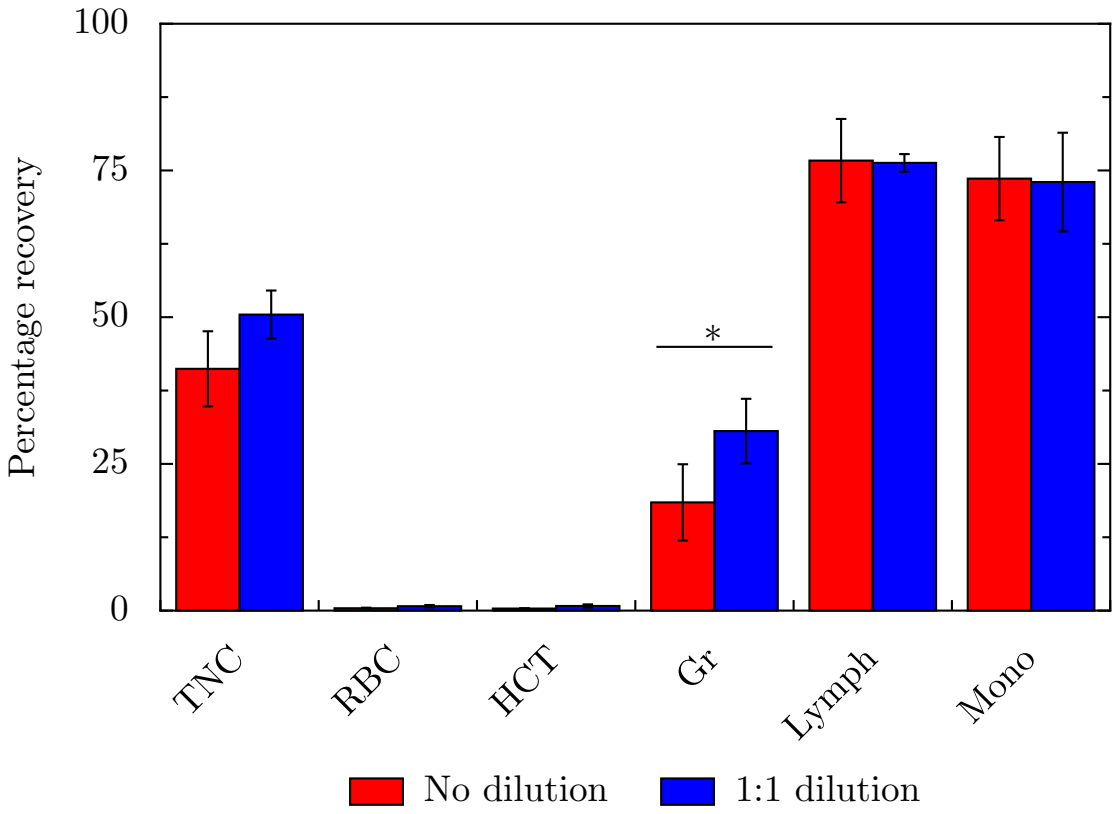

FIGURE 4.6: Assessing the effects of diluting blood prior to CBMC isolation using the Ficoll Density Gradient Protocol (n=10). Error bars correspond to the SEM. * denotes a $p<0.05$, ** denotes a $p<0.01$ and *** denotes a $p<0.001$

4.2.2.2 Specialised tubes developed for Ficoll processing

Specialised tubes have been developed to improve the Ficoll density gradient method. Greiner Bio-One have developed the Leucosep tube and Stem Cell Technologies developed the Sepmate tube. These tubes are similar to the 'standard' 50ml Falcon tubes except the addition of a barrier separating Ficoll from the blood. This reduces the time required for the Ficoll process (shown in Figure 4.7)

The Leucosep tubes contain a porous barrier which allows Ficoll to pass through into the lower chamber during a short centrifugation step. Ficoll passes into the lower chamber of a Sepmate tube through a small opening in the barrier. Blood can be poured directly on top of the barrier without the risk of mixing with the Ficoll. During centrifugation the barriers allow erythrocytes to pass through and remain in the lower chamber. As the erythrocytes cannot remix with the MNCs in the top chamber, the brakes can be applied, reducing the time for centrifugation. The cells remaining in the top chamber containing

the MNCs are poured off and washed. The cell products isolated using Leucosep and Sepmate tubes were compared to standard tubes without the barrier (Figure 4.8).

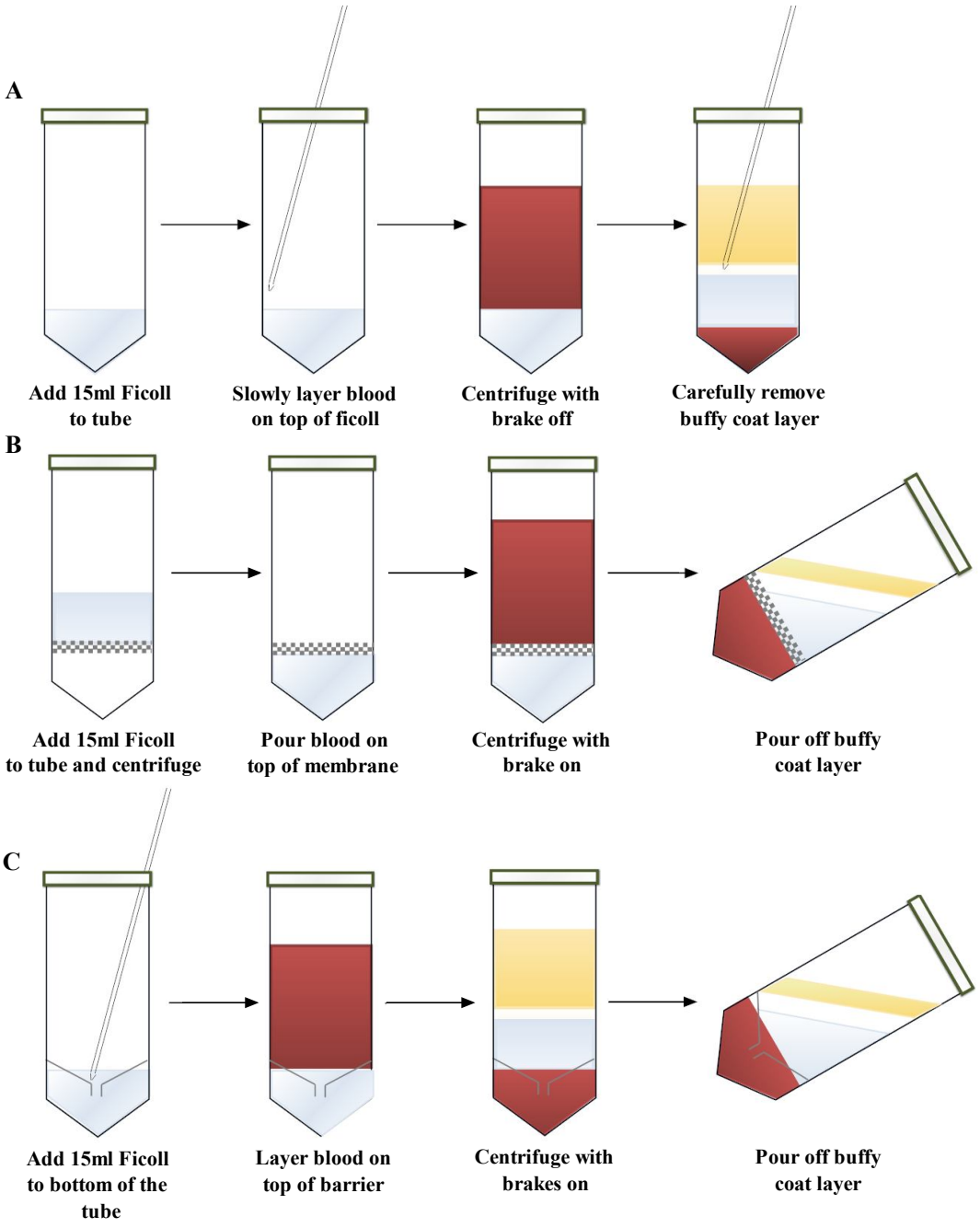

FIGURE 4.7: Ficoll Density Gradient Protocol using different tubes: (A) Standard Falcon tubes, (B) Leucosep tubes or (C) Sepmate tubes. Diagram created using SmartDraw.

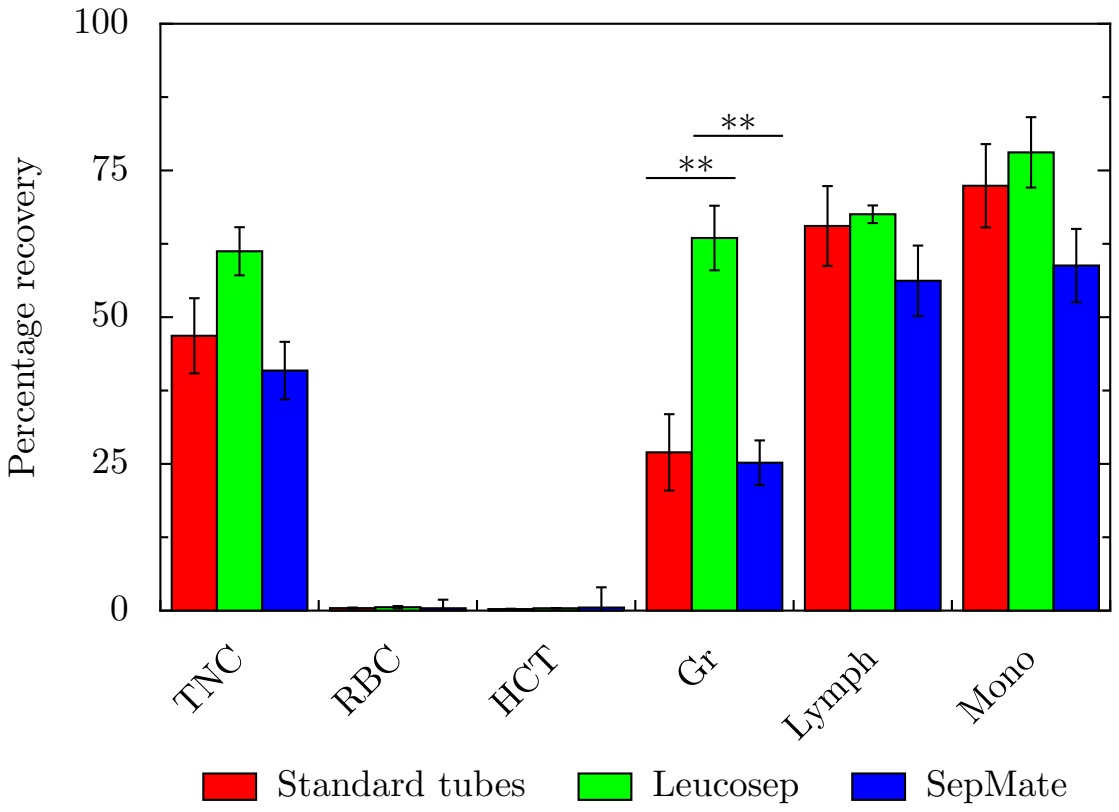

FIGURE 4.8: Ficoll Density Gradient Protocol using different tubes: Mean cell recoveries after mononuclear cell isolation using either Leucosep, SepMate or standard Falcon tubes (n=5). Error bars correspond to the SEM. * denotes a $p<0.05$, ** denotes a $p<0.01$ and *** denotes a $p<0.001$

High levels of erythrocyte depletion was achieved using all three types of tubes with a final haematocrit level of <1%. The TNC recovery was highest for Leucosep tubes due to slightly increased lymphocyte and monocyte cell recovery, but also the high granulocyte recovery of 63.49 ± 35.8% compared to 25.24 ± 10.07% for the Sepmate tubes (p=0.0061) and 26.97 ± 13.5% for standard tubes (p=0.0029). The Sepmate tube resulted in similar levels of granulocyte depletion compared to standard tubes, however, the recovery of MNCs was slightly reduced. Therefore, the standard tubes proved to be the most efficient when isolating MNCs using Ficoll.

4.3 Discussion

The eMNC protocol was successfully able to reduce the volume of a CB unit to 21ml allowing easier storage within the nitrogen cryotanks. The units processed by eMNC resulted in a cell product with higher MNC recoveries and CD34 recoveries that are comparable to units processed by Ficoll. The viabilities of the cells (assessed by 7AAD) were comparable to units processed by Ficoll. However, the eMNC protocol consistently resulted in a cell product with a lower depletion of granulocyte and erythrocytes and with a final haematocrit above the 5% target. Post-thaw analysis of units processed by eMNC and Ficoll resulted in slightly lower cell recoveries, viability and lower CFUs. This is potentially due to the higher levels of contaminating granulocytes (discussed further in Chapter 5).

The HPC isolations performed on both eMNC and Ficoll products resulted in a CD133 fraction with lower cell recovery, purity (p=0.0106) and viability possibly due to the erythrocyte contaminants which increase the difficulty of the handling of the cells. For example when washing the cells the high levels of erythrocytes result in an unstable/loose pellet after centrifugation. The Ficoll method uses the differences in the cell's density to separate the MNC layer. During centrifugation, the denser aggregated red blood cells and granulocytes will pass through the Ficoll towards the bottom of the tube, resulting in the MNC and plasma layers remaining above the Ficoll. As the Ficoll separates the erythrocytes and granulocytes from the MNCs, the required layer can be removed avoiding contamination from any unwanted cells. The eMNC process does not have the advantage of this separating layer and therefore, it may remain more difficult to avoid contamination from unwanted cells.

The introduction of the eMNC protocol has many advantages including the lack of user variability, it is a 'walk away' process leaving technicians free to perform other duties. There is also a reduced risk of contamination as the sample remains within a closed sterile tubing set without the need to add a separation media such as Ficoll. However, no contamination was observed in either the eMNC or Ficoll samples suggesting that although the risk is higher when using the Ficoll procedure, as long as correct techniques

are applied the risk is minimal. When considering the eMNC as an alternative to the Ficoll method, the low levels of granulocyte and erythrocyte depletion mean this is not currently feasible and further improvements would need to be made.

As the eMNC was not found to be a suitable replacement, the ability to further optimise the Ficoll protocol used at the ANCTC was assessed. Blood is often diluted prior to being layered over Ficoll, this should minimise the risk of trapping MNCs within the erythrocyte aggregates preventing a loss in MNC recovery.[469] However, dilution of blood samples increases the number of tubes needed to perform the procedure resulting in increased expense and processing time. There is also the risk of a reduced MNC recovery if the cells of interest are spread over several tubes. Therefore, the Ficoll procedure was performed on diluted and undiluted CB samples and the products compared. Similar erythrocyte depletion was observed with both products resulting in low haematocrit levels of <1%. However, higher TNC recovery was observed in samples diluted prior to isolation which was found to be due to lower levels of granulocyte depletion (p=0.0195). These data suggests that diluting CB prior to layering over Ficoll is not only unnecessary, but also results in poorer granulocyte depletion.

Several specialised tubes for Ficoll processing have been developed. These involve the use of permeable barriers which prevent the blood from mixing with the Ficoll during the layering stage and also allow rapid removal of the MNC layer after centrifugation. Two such tubes (Leucosep and Sepmate) were tested and compared to the standard Falcon tube without a barrier. These tubes reduced the time for MNC enrichment from ∼45 minutes to ∼15 minutes. All tubes tested resulted in high levels of erythrocyte depletion. However, the cells isolated using the Leucosep tubes had higher levels of granulocyte contamination (p=0.0029 and p=0.0061 compared to the standard Falcon and Sepmate tubes respectively). This suggests that the granulocytes were not able to pass through the permeable barrier of the Leucosep tube during centrifugation. Using the Sepmate tubes resulted in a MNC fraction with similar levels of granulocyte depletion, but a slightly reduced lymphocyte and monocyte recovery compared to the standard Falcon tubes. Therefore, the cheaper, standard Falcon tubes remain optimal

for Ficoll processing, as they resulted in cell products with high MNC recovery and granulocyte depletion.

Based on these observations, the Ficoll processing performed at the ANCTC Biobank will be carried out on undiluted, whole CB using the standard Falcon tubes. This will ensure high levels of erythrocyte and granulocyte depletion are achieved whilst minimising the cost and time required for this cell separation protocol.

CHAPTER 5

OPTIMISING THE CRYOPRESERVATION OF CORD BLOOD MONONUCLEAR CELLS

5.1 Introduction

A CB Biobank would involve cryopreservation and potentially long term storage of samples until their use is required. Therefore, the next stage of the Biobank development would be to improve on the methods used in cryopreservation and thawing of the CB cells.

There is a risk of damage to the cells during freezing and thawing stages of cryopreservation and suboptimal conditions can result in reduced cell viability. The cause of the damage to the cells is due to changes in osmotic pressure as ice forms in the extracellular space, resulting in a reduction of water between cells leading to an increasing concentration of the solutes.[82,86] This leads to shrinkage of the cells as water exits to compensate for the rising solute concentration.[87] If the cells are maintained at low temperatures for too long before freezing the cells can shrink to the point of irreversibly damaging their membranes.[88] Whilst frozen there should be no further risk to the cells provided they are cooled low enough to block cell metabolism.[80,81] During thawing of the cells the ice crystals melt reducing the concentration of solutes in the extracellular space, there

is then an influx of water into the cell, counterbalancing the osmotic gradient, resulting in the cells swelling potentially to the point of bursting. Therefore, it is crucial to optimise cryopreservation protocols in order to maintain the highest possible number of functional HPCs.

Dimethyl sulphoxide (DMSO) is a commonly used cryoprotectant which protects the cells from the changes in osmotic pressure by slowing the formation of ice crystals in the extracellular compartment, and by preventing the formation of ice crystals within the cell which can disrupt the membrane.[101] Currently the cryoprotectant freezing mix, added to the cells prior to freezing, contains DMSO and dextran-40, which functions as an osmotic stabiliser. The cryoprotectants are added to the samples resulting in a final concentration of 10% (v/v) DMSO and 1% (w/v) dextran-40.[55,86,471] However, there is evidence to suggest that DMSO may be toxic to cells if added in high concentrations or for prolonged periods.[122] Reports have been published suggesting that lower concentrations of DMSO result in higher viable cell recoveries post thaw.[140,141,148,153,472] Different concentrations of DMSO will be compared in the cryopreservation of CBMCs to identify the optimum freezing media. Furthermore, the toxic effects of DMSO will also be assessed by storing cells in different concentrations at different temperatures over 24 hours. This will ensure viable cells are not lost in the time between DMSO addition and the starting of the CRF.

This part of the study aims to optimise the cryopreservation conditions for CBMCs. This will be achieved through assessing the quality of the cells through viable cell and CFU recoveries throughout the cryopreservation processes. Also, by assessing the toxic effects of DMSO and dextran-40 on cells, identifying the optimal concentration of these chemicals, as well as investigating the effect of DMSO addition speed and its removal post-thaw.

5.2 Results

5.2.1 Freezing whole CB v. CBMC

Different cells may require different cryopreservation protocols. Compared to mononuclear cells, granulocytes are more susceptible to thermal shock during freezing and thawing, and poor cryopreservation of these cells may have a detrimental effect on the rest of the sample due to the release of nucleoproteins and lysosomal enzymes which can lead to cell clumping.[473,474] Therefore, the post-thaw cellular recoveries were compared when cryopreserving whole CB versus granulocyte-depleted CBMCs using either the controlled rate freezer (CRF) or a Nalgene Mr Frosty.

Both freezing methods resulted in higher viable cell recoveries in the granulocyte depleted CBMC samples. When using the CRF (Figure 5.1A), significantly higher percentages of total nucleated cells (TNC) were observed in CBMC samples (71.74 ± 9.36% when freezing whole CB, 85.84 ± 10.35% when freezing CBMC, p=0.0077), $CD45^+7AAD^-$ (63.90 ± 8.27% for CB, 85.94 ± 12.47% for CBMC, p=0.0009), viable mononuclear cells (MNC $7AAD^-$) (75.80 ± 9.78% for CB, 89.83 ± 15.02% for CBMC, p=0.0434), $CD34^+7AAD^-$ (58.09 ± 19.92% for CB, 88.02 ± 19.99% for CBMC, p=0.0062) and $CD34^+AnnV^-$ (34.69 ± 12.58% for CB, 60.45 ± 19.15% for CBMC, p=0.0085). A higher CFU recovery was also observed when freezing CBMC, although the difference was not significant (57.89 ± 28.40% for CB and 76.49 ± 22.40% for CBMC).

Whilst using the Mr Frosty to freeze whole CB and CBMC samples (Figure 5.1B) significant differences were observed for TNC (71.66 ± 5.64% using the CRF, 84.25 ± 9.19% for CBMC, p=0.0021), $CD45^+7AAD^-$ (59.49 ± 9.78% for CB, 77.39 ± 11.01% for CBMC, p=0.0031) and $CD34^+7AAD^-$ (50.50 ± 14.34% for CB, 72.14 ± 12.77% for CBMC, p=0.0031). Although higher recoveries of MNC, $CD34^+AnnV^-$ and CFUs were observed for CBMCs, the differences were not significant (MNC $7AAD^-$ recoveries of 72.90 ± 11.65% for CB, 81.85 ± 13.27% for CBMC $CD34^+AnnV^-$ recoveries of 23.39 ± 13.71% for CB, 37.73 ± 16.94% for CBMC and CFU recoveries of 50.62 ± 18.88% for CB, 53.04 ± 4.29% for CBMC).

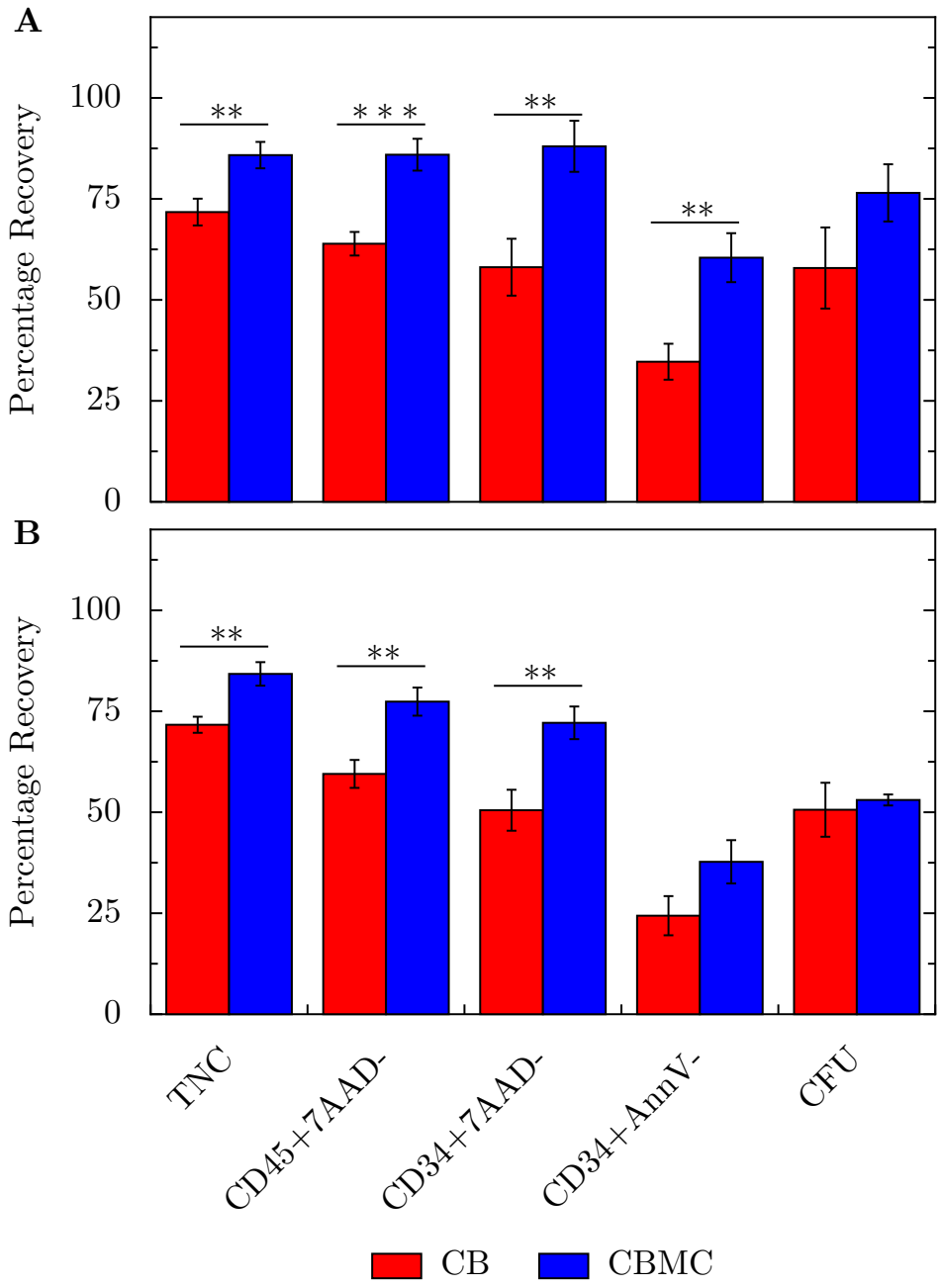

FIGURE 5.1: A comparison of post-thaw recoveries when freezing whole CB v. CBMC (A) in a CRF or (B) Nalgene Mr Frosty. (n=8 for CB and n=10 for CBMC) Error bars correspond to the SEM. *p<0.05, **p<0.01 and ***p<0.001

5.2.2 Freezing methods - CRF v. Mr Frosty

Two different methods of freezing cells were employed during this project. Larger samples of 20ml were cryopreserved in cryobags in a controlled rate freezer (CRF) with a set cooling program (Figure 1.3). Smaller samples (up to 2ml) were frozen in cryovials using a Nalgene Mr Frosty in an -80°C freezer, which cools at 1°C minute^{-1} before being transferred to liquid nitrogen tanks. Both freezing protocols were compared when cryopreserving whole CB (Figure 5.2A) and CBMC samples (Figure 5.2B).

When freezing whole CB higher recoveries were observed when using the CRF, although the differences between the two methods were not significant (TNC recovery of 71.74 ± 9.36% when using the CRF and 71.66 ± 5.64% when using the Mr Frosty, CD45$^+$7AAD$^-$ recovery of 63.90 ± 8.27% using the CRF and 59.49 ± 9.78% using the Mr Frosty, CD34$^+$7AAD$^-$ recovery of 58.09 ± 19.92% using the CRF and 50.50 ± 14.34% using the Mr Frosty, CD34$^+$AnnV$^-$ recovery of 34.69 ± 12.58% using the CRF and 24.39 ± 13.71% using the Mr Frosty and CFU recovery of 57.89 ± 28.40% using the CRF and 50.62 ± 18.88% using the Mr Frosty).

As with freezing whole CB, higher recoveries were observed in CBMC samples when using the CRF for TNC (85.84 ± 10.35% using the CRF, 84.25 ± 9.19% using the Mr Frosty, not significant), CD45$^+$7AAD$^-$ (85.94 ± 12.47% using the CRF, 77.39 ± 11.01% using the Mr Frosty p=0.002), CD34$^+$7AAD$^-$ (88.02 ± 19.99% using the CRF, 72.14 ± 12.77% using the Mr Frosty p=0.0039), CD34$^+$AnnV$^-$ (60.45 ± 19.15% using the CRF, 37.73 ± 16.94% using the Mr Frosty p=0.0039) and CFU (76.49 ± 22.40% using the CRF, 53.04 ± 4.29% using the Mr Frosty p=NS).

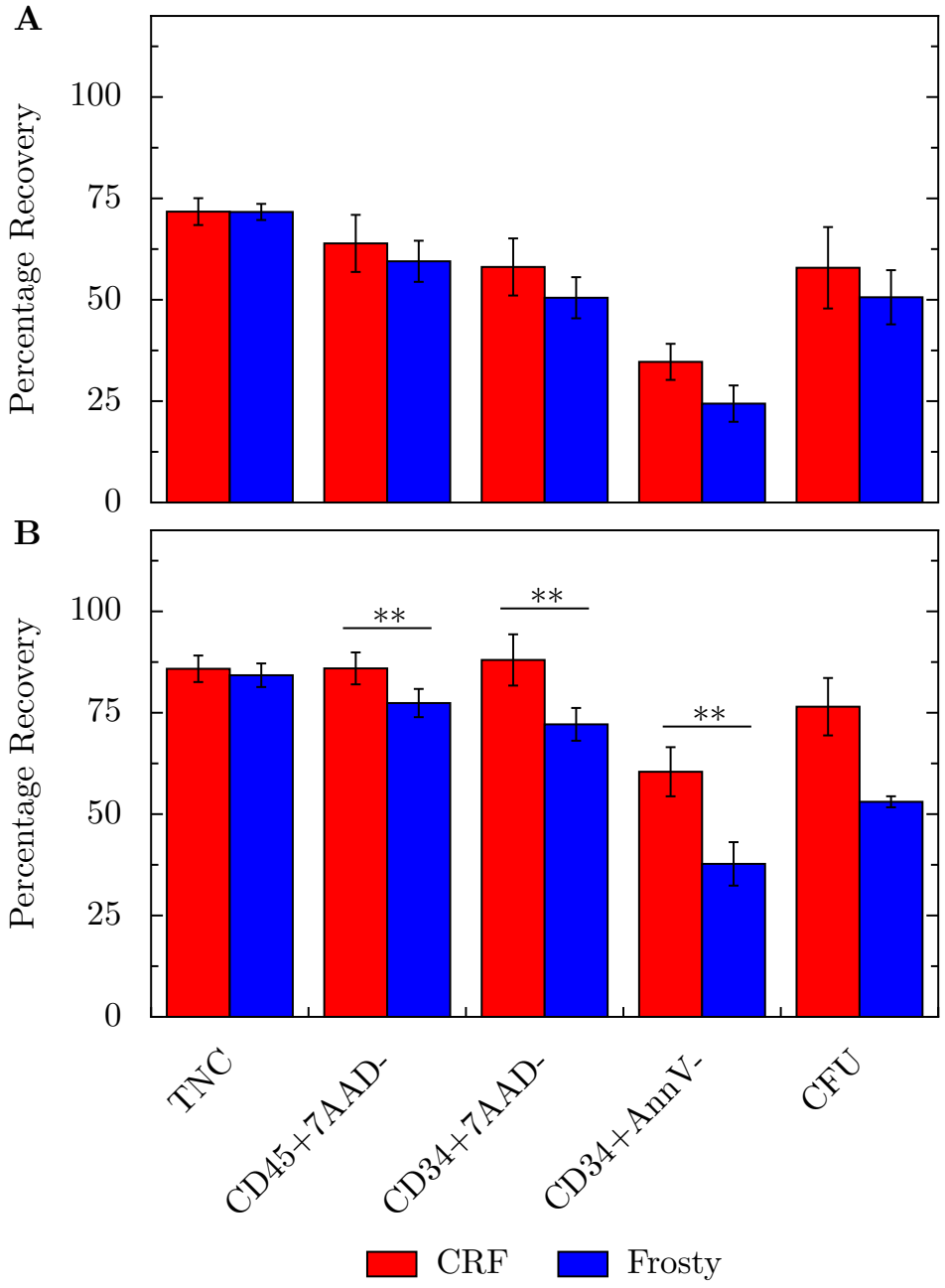

FIGURE 5.2: A comparison of post-thaw recoveries from the two freezing protocols (CRF or Mr Frosty) when freezing (A) whole CB and (B) CBMCs. (n=8 for CB and n=10 for CBMC) Error bars correspond to the SEM. *p<0.05 and **p<0.01

5.2.3 Resuspension media - plasma concentration

Throughout this project the mononuclear cell samples have been resuspended in 100% (v/v) autologous plasma post-isolation. Cryopreserving CBMCs in autologous plasma was assessed and compared to cells resuspended in a lower concentration of 20% (v/v) plasma and RPMI. Reducing the final concentration of plasma to 20% (v/v) before freezing (Figure 5.3) resulted in similar post-thaw cell recoveries compared to 100% (v/v) plasma. Therefore, for the rest of this project 100% (v/v) plasma was used when resuspending CBMCs prior to cryopreservation.

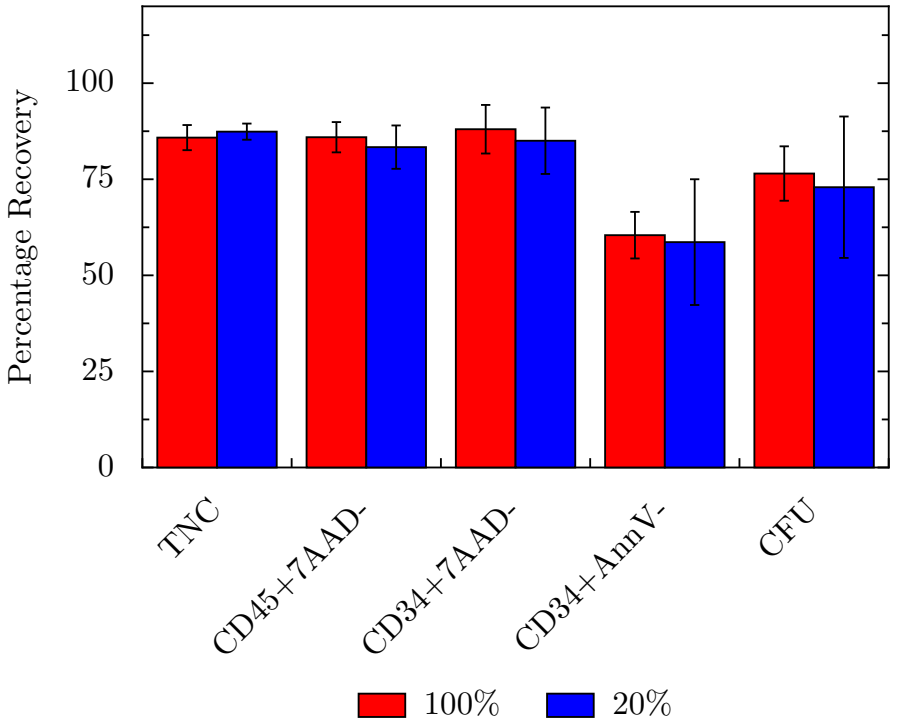

FIGURE 5.3: The effect on cell recovery when resuspending CBMCs in different concentrations of plasma prior to cryopreservation (n=7 for 20% (v/v) and n=10 for 100% (v/v)) Error bars correspond to the SEM.

5.2.4 Optimal dextran-40 concentration

Dextran-40 is often added to the sample along with the cryoprotectant to reach a final concentration of 1% (w/v). The effect of the presence of dextran-40 in the cryopreservation solution was assessed. First the toxic effect of dextran-40 on CB was assessed

by maintaining fresh CBMCs in 1% (w/v) dextran-40 for 24 hours. This was shown to have little effect on cell count, viability and on CFU recovery (Figure 5.4A).

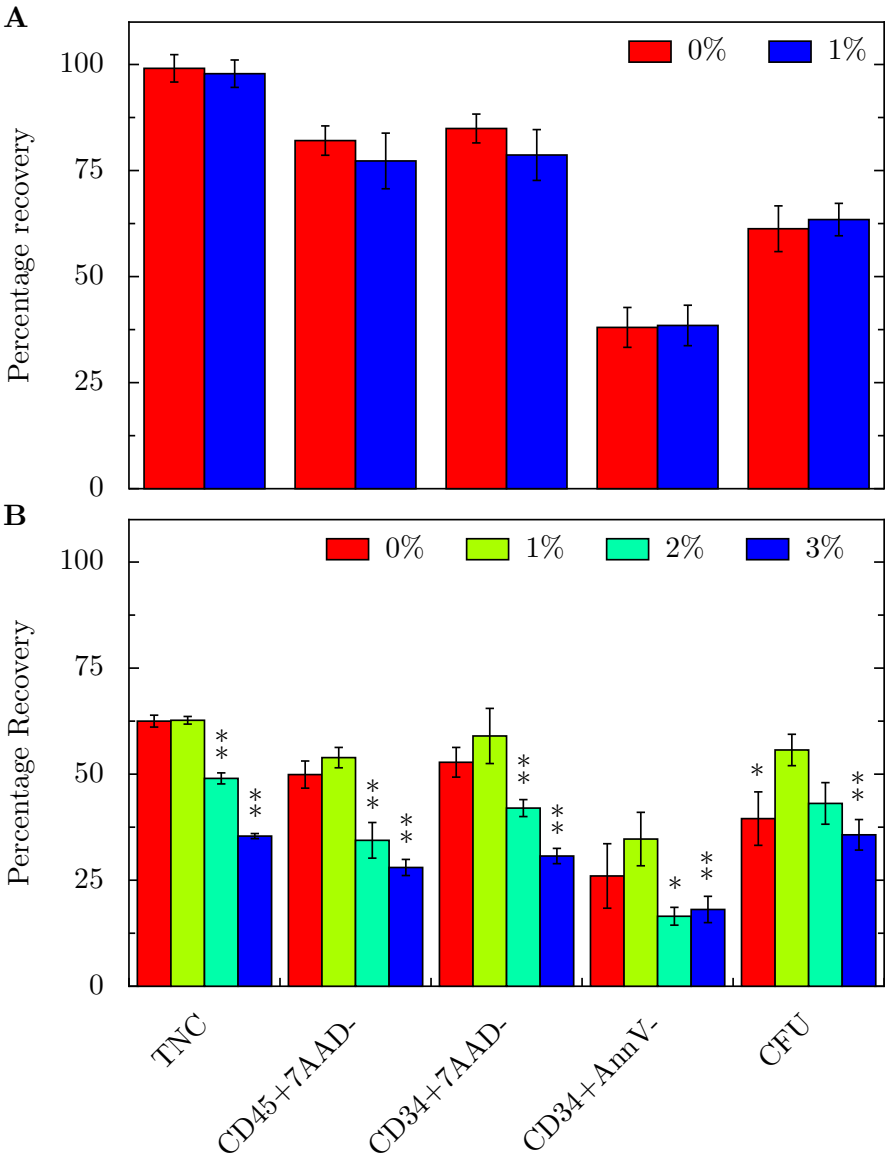

FIGURE 5.4: The effect of dextran-40 on CBMCs. (A) Mean cell and CFU recoveries when fresh CBMC samples were stored with or without 1% (w/v) dextran-40 for 24 hours (n=10). (B) Mean post-thaw cell and CFU recoveries when CBMC samples were cryopreserved with a range of dextran-40 concentrations (from 0% to 3% (w/v)) (n=10). Error bars correspond to the SEM. *p<0.05, **p<0.01 and ***p<0.001 compared to samples in 1% (w/v) dextran-40

The optimal dextran-40 concentration was assessed when cryopreserving CBMCs. The concentration of dextran-40 was shown to be critical (Figure 5.4B). Compared to 1% (w/v) dextran-40, 0% resulted in similar nucleated cell recoveries, but lower recoveries when assessing viable cells and a significantly lower CFU recovery (p=0.0391). Cryopreserving cells with 2% (w/v) dextran-40 resulted in lower recoveries of TNC (p=0.0078),

$CD45^+7AAD^-$ (p=0.0078), $CD34^+7AAD^-$ (p=0.0078), $CD34^+AnnV^-$ (p=0.0156) and CFU (not significant) compared to using 1% (w/v). Increasing the concentration further to 3% (w/v) also resulted in significantly lower viable and functional cell recoveries (TNC p=0.0078, $CD45^+7AAD^-$ p=0.0078, $CD34^+7AAD^-$ p=0.0078, $CD34^+AnnV^-$ p=0.0078 and CFU p=0.0078). Therefore, for the rest of this project, the DMSO freezing solution added to cells prior to freezing contained dextran-40 to reach a final concentration of 1% (w/v).

5.2.5 Toxic effects of DMSO

The effects of DMSO on CB cells through the different stages of cryopreservation was assessed. First, the effect of different concentrations in fresh CB over 24 hours at either refrigerated or room temperature (protocol summary shown in Figure 5.5A). Then, the effects of delaying cryopreservation after DMSO addition (Figure 5.5B), followed by the effect of DMSO post-thaw looking into the benefits of washing to completely remove DMSO or dilution to reduce its post-thaw concentration (Figure 5.5C). The effects of adding DMSO at different speeds to the cells post-thaw and finally assessing the optimal DMSO concentration for cryopreserving CB samples.

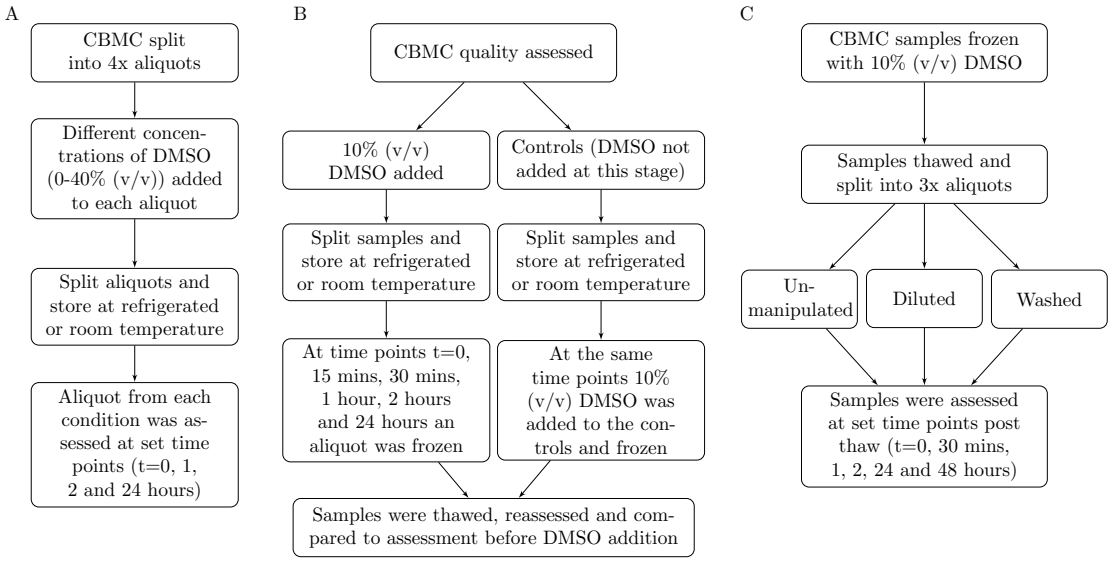

FIGURE 5.5: Flow chart of the protocols used to assess DMSO toxicity at different stages of cryopreservation

5.2.5.1 In fresh CB

The effects of different concentrations of DMSO on fresh CBMC samples maintained at refrigerated temperatures is shown in Figure 5.6. Increasing the concentration of DMSO resulted in lower numbers of viable cells and CFUs recovered showing a dose related toxic effect. This is particularly apparent in the CFUs and Annexin V analysis of the $CD34^+$ cells; concentrations of 40% (v/v) DMSO resulted in an immediate loss of $CD34^+AnnV^-$ cells with a recovery of 1.75 ± 2.23% (p=0.0002) and a CFU recovery of 3.75 ± 8.00% (p=0.0313) compared to samples without DMSO at time point zero. Recoveries declined further over 24 hours to 0.64 ± 1.17% (p=0.0039) and 1.70 ± 1.29% (p=0.002) for Annexin V and CFU analysis respectively.

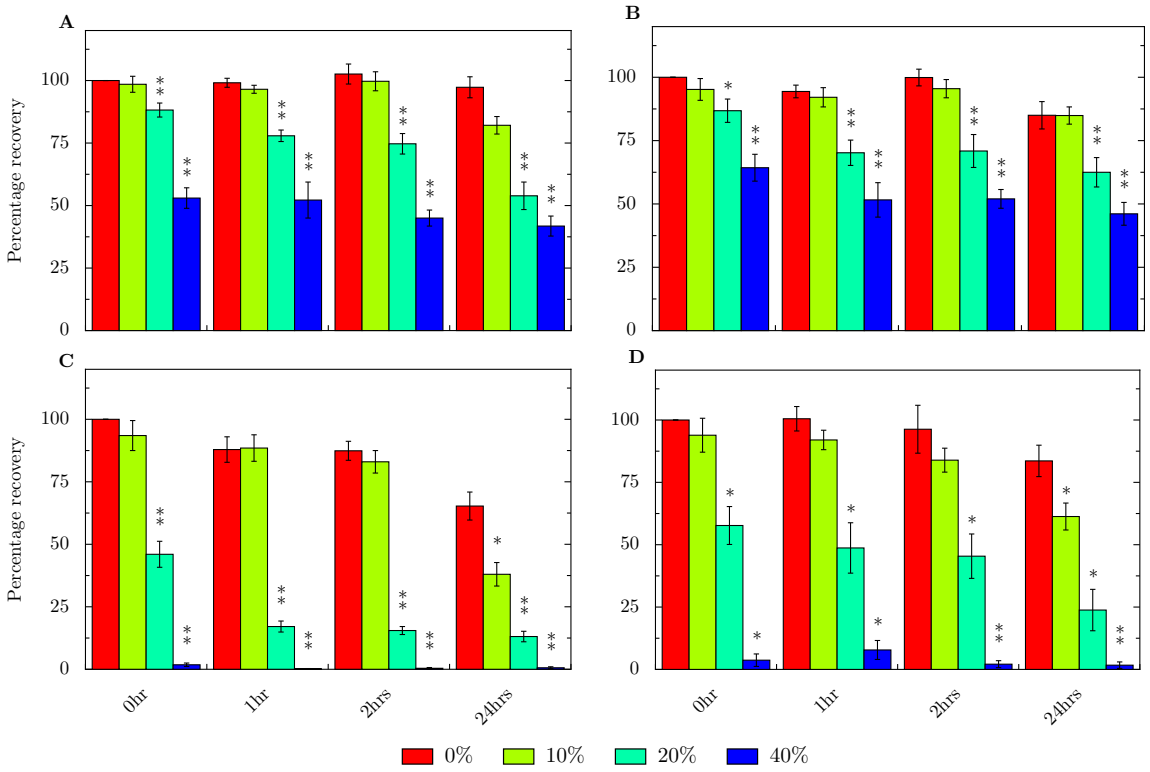

FIGURE 5.6: Mean (A) $CD45^+7AAD^-$, (B) $CD34^+7AAD^-$, (C) $CD34^+AnnV^-$ and (D) CFU recovery in CBMC samples after fresh storage at 4°C, for up to 24 hours, with a range of DMSO concentrations from %0 to 40% (v/v) (n=10). Error bars correspond to the SEM. *p<0.05, **p<0.01 and ***p<0.001 compared to 0% DMSO at the same time point

The addition of 20% (v/v) DMSO resulted in a recovery of 43.03 ± 16.35% (p=0.002) of $CD34^+AnnV^-$ cells at time point zero and a CFU recovery of 57.68 ± 23.94% (p=0.0313). After 24 hours of storage recoveries were reduced to 13.14 ± 6.71% (p=0.0039) for Annexin V analysis and 23.76 ± 26.37% (p=0.0313) for CFU analysis.

Samples stored with 10% (v/v) DMSO (the concentration commonly used during cryopreservation) resulted in no significant difference, compared to samples with 0% (v/v) DMSO, when assessed immediately after addition with average viable cell recoveries of 98.53 ± 10.18% for $CD45^+7AAD^-$ cells, 95.18 ± 13.57% for $CD34^+7AAD^-$ cells, 93.52 ± 19.00% for $CD34^+$Annexin V^- cells and 93.86 ± 21.37% for the CFUs. The mean viable cell and CFU recoveries were assessed after 1, 2 and 24 hours of storage with 10% (v/v) DMSO (data shown in Table 5.1). With 10% (v/v) DMSO fresh CBMC samples showed no significant reduction in recovery for up to 2 hours compared to the assessment at time point zero and also when compared to the samples stored without DMSO for the same length of time. When reassessed at 24 hours after addition, samples with 10% (v/v) DMSO had significantly reduced recoveries compared to samples stored without DMSO for 24 hours (Figure 5.6) and compared to assessment immediately after DMSO addition (Table 5.1).

TABLE 5.1: Mean viable cell and CFU recovery after fresh storage of CBMCs with 10% (v/v) DMSO at refrigerated temperatures (n=10). Percentages indicate mean recovery ± s.d. and p values indicate differences at the same DMSO concentration compared to t=0 NS indicates a non significant result

Storage time	$CD45^+$ $7AAD^-$	$CD34^+$ $7AAD^-$	$CD34^+$ Annexin V^-	CFU
1 hour	96.55 ± 4.91%	92.12 ± 11.96%	88.52 ± 16.78%	91.97 ± 12.22%
p value	NS	NS	NS	NS
2 hours	99.72 ± 12.03%	95.5 ± 11.42%	82.96 ± 14.31%	83.92 ± 15.33%
p value	NS	NS	NS	NS
24 hours	82.06 ± 10.94%	84.91 ± 10.75%	38.02 ± 14.86%	61.28 ± 17.05%
p value	0.0039	0.0098	0.0078	0.0313

The effects of two different temperatures (refrigerated (4°C) and room (20°C) temperature) on the samples stored for 24 hours with 10% (v/v) DMSO was assessed. As shown in Figure 5.7, there was no significant differences between the temperatures in the mean recovery of viable cells and CFUs.

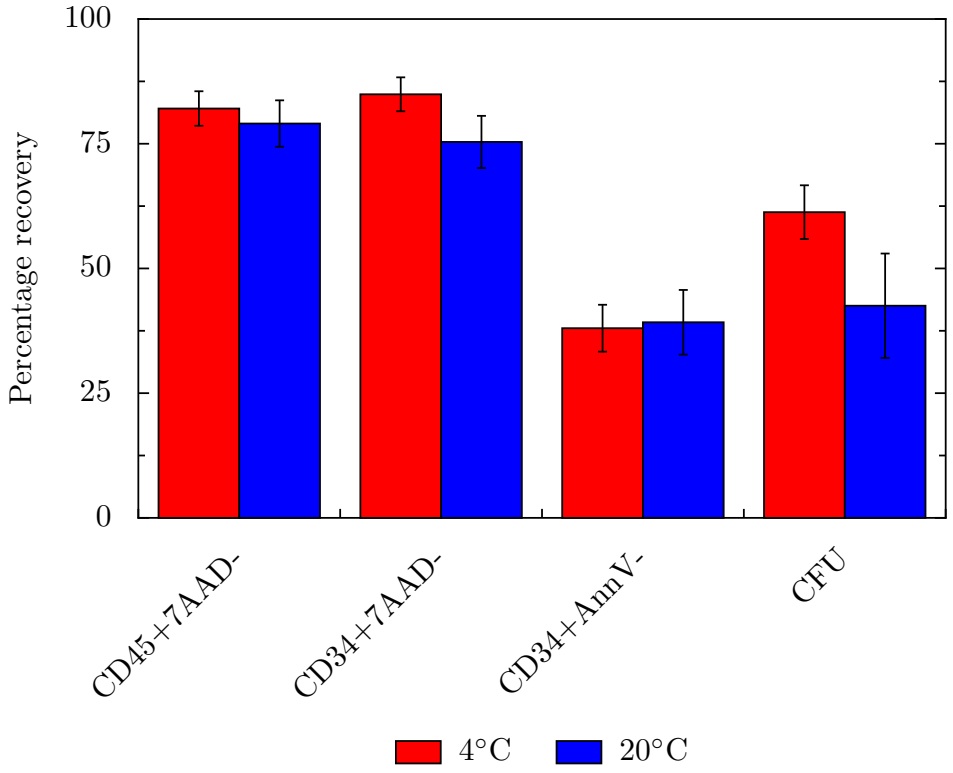

FIGURE 5.7: Mean viable cell and CFU recovery in fresh CBMC samples stored with 10% (v/v) DMSO for 24 hours at either refrigerated (4°C) or room (20°C) temperatures (n=10). Error bars correspond to the SEM.

5.2.5.2 Delaying cryopreservation of CB

The effects on CBMCs of delaying freezing after addition of 10% (v/v) DMSO were assessed (Figure 5.8). As shown in Figure 5.5B, CBMC samples were split into two fractions; DMSO was added to one half whilst the other was left without DMSO (control fraction). At set time points (0 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours and 24 hours after DMSO addition) an aliquot was taken from each fraction and frozen (DMSO was added to the control just before freezing).

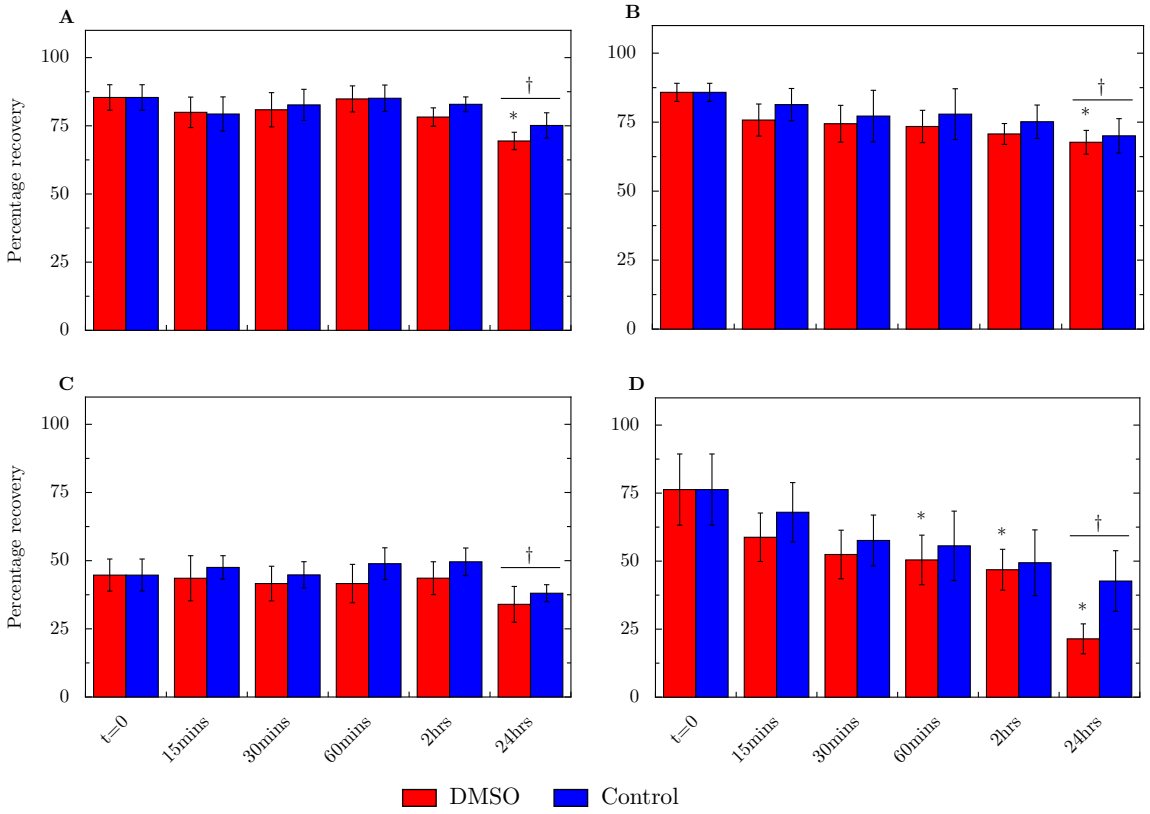

FIGURE 5.8: Mean (A) $CD45^+7AAD^-$, (B) $CD34^+7AAD^-$, (C) $CD34^+AnnV^-$ and (D) CFU recovery when delaying cryopreservation of CBMCs for up to 24 hours after addition of 10% (v/v) DMSO (n=10). Error bars correspond to the SEM. †p<0.05 between samples with DMSO and control at the same time point and *p<0.05 compared to t=0

As with the assessment of DMSO toxicity in fresh CBMC samples, significant losses in post-thaw viable cell recoveries were not observed until samples had been stored with 10% (v/v) DMSO for 24 hours prior to freezing compared to those frozen immediately ($CD45^+7AAD^-$ 69.43 ± 10.62% p=0.0098 and $CD34^+7AAD^-$ 67.71 ± 15.54% p=0.0195. A noticeable decline in CFU recovery was observed when delaying freezing for 15 minutes, however the loss was not significantly lower until after a delay of 1 hour (50.44 ± 21.02% p=0.0098).

No significant difference was found in post-thaw cell recovery between the samples stored with DMSO for up to 2 hours prior to cryopreservation compared to the controls (stored for the same time period, but without DMSO). However, samples that had been stored in 10% (v/v) DMSO for 24 hours before being cryopreserved resulted in lower recoveries compared to the control samples; $CD45^+7AAD^-$ (69.43 ± 10.62% with DMSO, 75.12 ±

12.57% control p=0.0098), $CD34^+7AAD^-$ (67.71 ± 15.54% with DMSO, 81.04 ± 18.56% control p=0.0195), $CD34^+AnnV^-$ (38.06 ± 16.68% with DMSO, 33.99 ± 19.67% control p=0.0098), and CFU (21.44 ± 17.08% with DMSO, 42.68 ± 31.96% control p=0.002).

The effect of storing samples with or without 10% (v/v) DMSO at different temperatures (room or refrigerated) prior to cryopreservation was assessed (Figure 5.9). Post-thaw analysis showed significantly lower recoveries for samples containing DMSO stored at room temperature, but only after delaying cryopreservation for 24 hours ($CD45^+7AAD^-$ p=0.0098 and $CD34^+AnnV^-$ p=0.001).

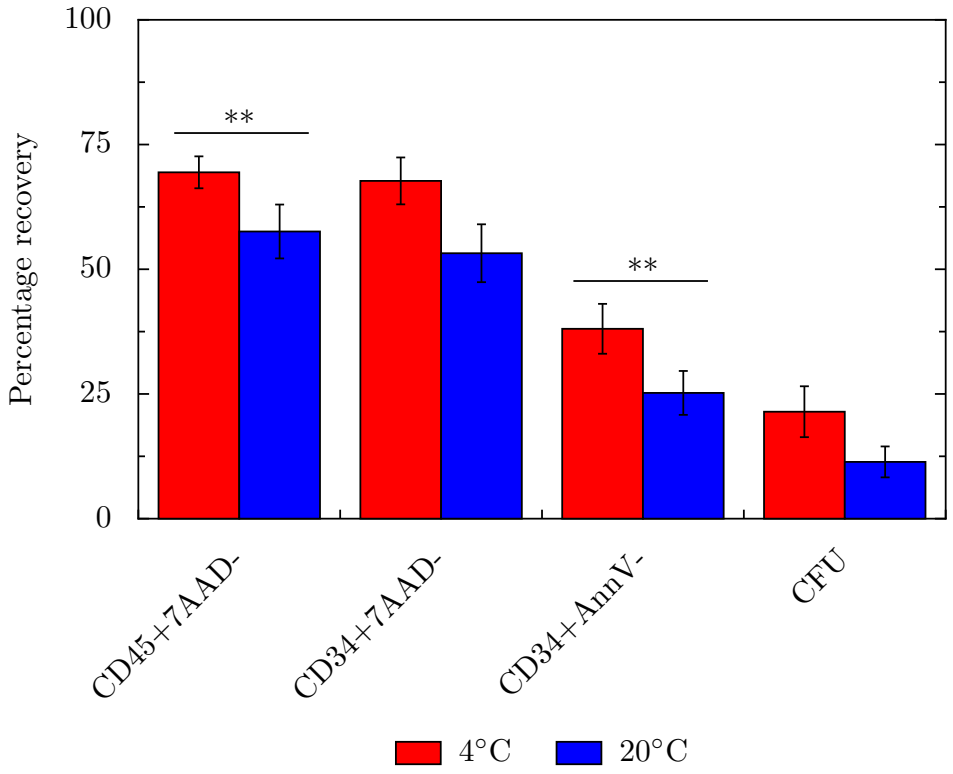

FIGURE 5.9: Mean post-thaw viable cell and CFU recovery in fresh CBMC samples stored with 10% (v/v) DMSO for 24 hours at either refrigerated (4°C) or room (20°C) temperatures prior to freezing (n=10). Error bars correspond to the SEM. * denotes a p<0.05

5.2.5.3 Effect of DMSO post-thaw

In the assessment of the toxic effects of DMSO post thaw, CBMCs were cryopreserved with 10% (v/v) DMSO, thawed and split into three samples; un-manipulated (DMSO concentration maintained at 10%), diluted (gradual addition of thawing solution to reduce DMSO concentration to 5%) and washed (dilution followed by centrifugation to

remove DMSO before resuspending the cells in culture media). Cell potency was compared over a period of 48 hours post-thaw. The t=0 assessment (assessed immediately after any post thaw manipulations) resulted in lower cell counts in the washed samples due to some of the cells being lost during the procedure (mean $CD45^+$ cell counts of $13.26 \pm 4.36 \times 10^6$ ml^{-1} for un-manipulated cells, $14.08 \pm 7.58 \times 10^6$ ml^{-1} for diluted and $11.44 \pm 3.42 \times 10^6$ ml^{-1} for washed samples). However, in order to assess the effects of DMSO on post-thaw samples, the cell recoveries were normalised against t=0 for each subsequent time point (Figure 5.10). After the wash losses had been taken into account, the washed samples retained higher recoveries over the 48 hours compared to diluted samples and both retained higher recoveries compared to the un-manipulated samples. Significant differences were observed between the three post-thaw procedures in CFUs after 2 hours (p=0.0130) and in the viable cells after 24 hours (p=0.0476 for $CD45^+7AAD^-$, p=0.0476 for $CD34^+7AAD^-$ and p=0.0307 for $CD34^+AnnV^-$).

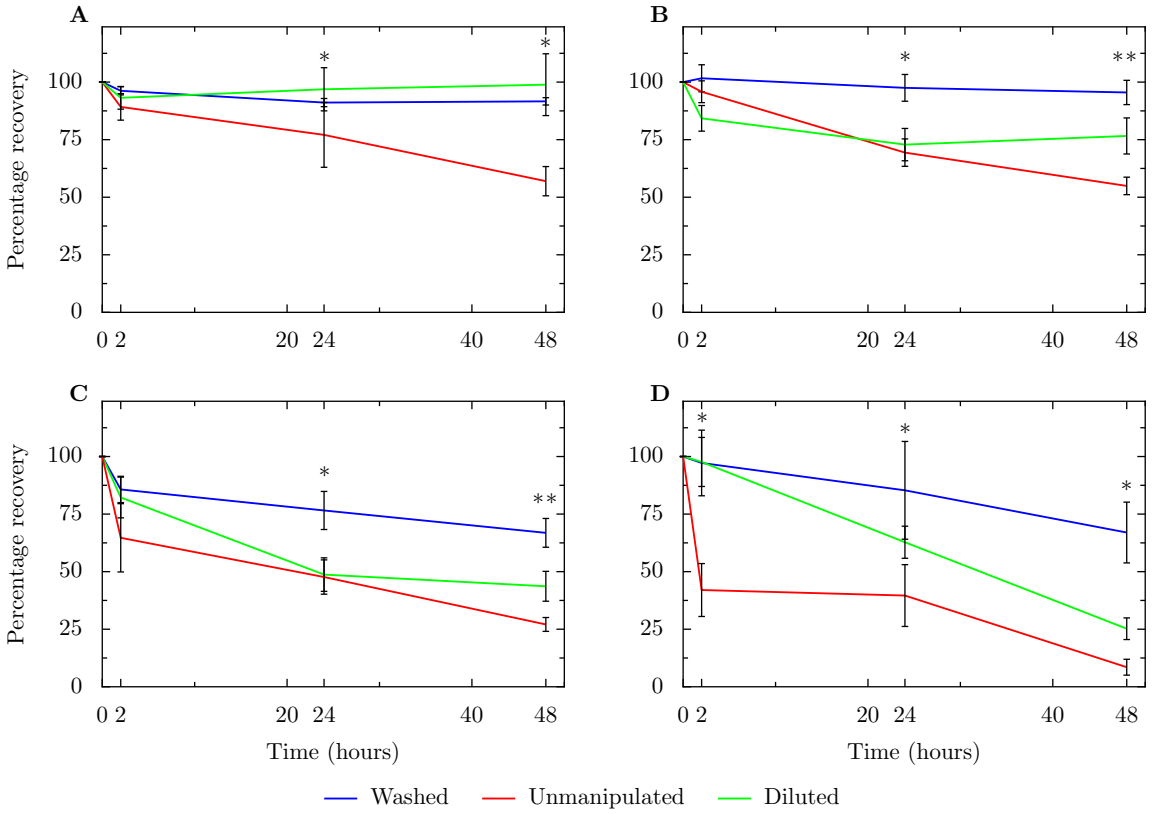

FIGURE 5.10: Mean (A) $CD45^+7AAD^-$, (B) $CD34^+7AAD^-$, (C) $CD34^+AnnV^-$ and (D) CFU recovery in post-thaw CBMC samples that had either been left un-manipulated, diluted 1:1 or washed to remove all DMSO (n=10). Error bars correspond to the SEM. *p<0.05, **p<0.01 and ***p<0.001 between different protocols

The numbers of viable cells and CFUs identified decreased over time for all the post-thaw protocols, with significant losses compared to recoveries at time point 0 after 2 hours for un-manipulated samples, 24 hours for diluted samples and 48 hours for washed samples.

To assess at what time point within 2 hours of thawing that the un-manipulated cells lost significant numbers of viable cells and CFUs this experiment was repeated with recoveries assessed 30 minutes, 1 hour and 2 hours after thawing. Figure 5.11 shows the significant losses occurred after 1 hour of thawing ($CD45^{+}7AAD^{-}$ recovery of 91.08 ± 8.28% p=0.0059, $CD34^{+}AnnV^{-}$ recovery of 81.38 ± 21.98% p=0.0371 and CFU recovery of 69.71 ± 32.31% p=0.0313). The time points at which significant loses were observed in the different parameters is summarised in Table 5.2.

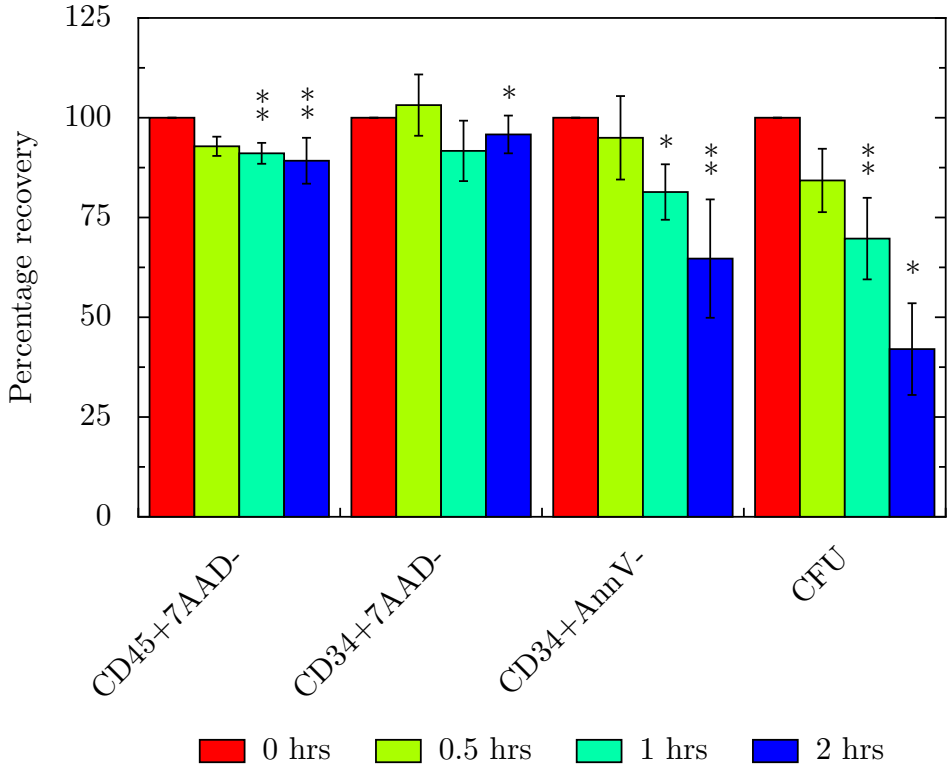

FIGURE 5.11: Mean viable cell and CFU recovery in post thaw CBMC samples that have been left un-manipulated for up to 2 hours (n=10). Error bars correspond to the SEM. *p<0.05 and **p<0.01 compared to recoveries 0 hours post-thaw

TABLE 5.2: Mean viable cell and CFU recoveries when significant losses were observed post-thaw in samples which have either been left un-manipulated, diluted 1:1 or washed to remove DMSO

Post thaw protocol		$CD45^+$ $7AAD^-$	$CD34^+$ $7AAD^-$	$CD34^+$ Annexin V^-	CFU
Un-manipulated	Time point	1 hour	2 hours	1 hour	1 hour
	% recovery	91.08 ± 14.18	95.81 ± 11.63	81.38 ± 19.90	69.71 ± 32.31
	p value	0.0059	0.002	0.0371	0.0313
Diluted	Time point	24 hours	24 hours	24 hours	24 hours
	% recovery	96.88 ± 16.95	72.85 ± 14.86	48.72 ± 20.59	62.77 ± 21.25
	p value	0.002	0.0078	0.0039	0.0313
Washed	Time point	48 hours	48 hours	48 hours	48 hours
	% recovery	91.64 ± 7.20	95.50 ± 12.33	66.85 ± 32.79	66.99 ± 24.80
	p value	0.002	NS	0.001	0.0451

5.2.5.4 Optimal DMSO concentration

In spite of the low levels of toxicity found when using 10% (v/v) DMSO to cryopreserve CB samples, the effects of reducing the concentration further when freezing was assessed. Different CBMC samples were cryopreserved with a range of DMSO concentrations (0-10%) and their post-thaw cell viability and CFU recoveries compared. The mean leukocyte count (WBC) of post-thaw CBMCs gave similar counts for all concentrations of DMSO (Figure 5.12), however this is a total cell count without any indication of viability. 7AAD exclusion shows a decline in viable $CD45^+$ and $CD34^+$ cell recovery as the concentration of DMSO is reduced with those frozen with 5% (v/v) DMSO and less giving a significantly lower recovery compared to those frozen with 10% (p=0.0226 for both $CD45^+7AAD^-$ and $CD34^+7AAD^-$ when 5% DMSO was used).

However, the use of Annexin V to exclude apoptotic cells from the viable $CD34^+$ count resulted in the measurement of higher recoveries (although not significantly higher than 10%) when a concentration of 7.5% (v/v) DMSO was used (76.42 ± 38.30% with 7.5% (v/v) DMSO and 65.10 ± 20.65% with 10% (v/v) DMSO). This increase in HPC potency after cryopreservation with 7.5% (v/v) DMSO was confirmed by the CFU assay (78.10 ± 37.60% with 7.5% (v/v) DMSO and 60.38 ± 32.13% with 10% (v/v) DMSO) with

significantly low recoveries when 2.5% (34.20 ± 18.94% p=0.0047) and 0% (30.07 ± 28.69% p=0.0011) was used.

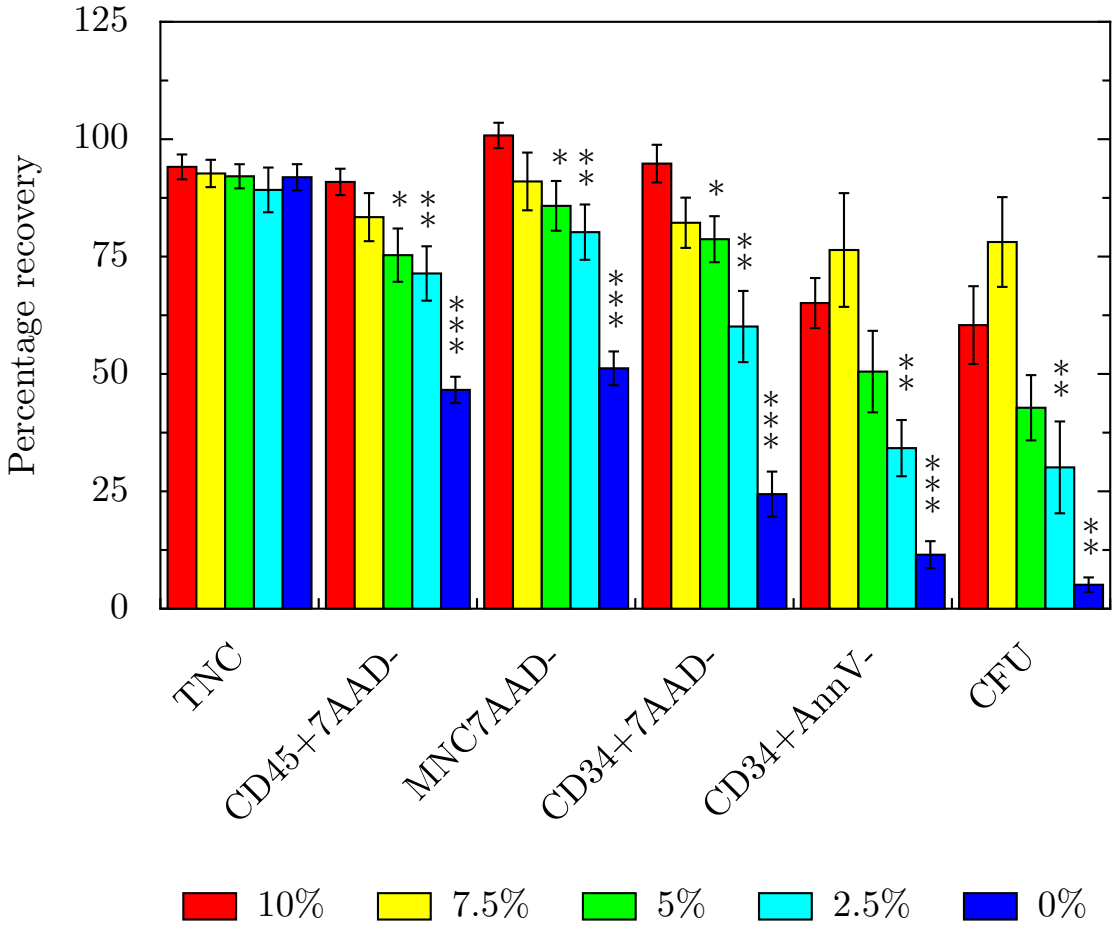

FIGURE 5.12: Mean post-thaw cell and CFU recoveries when CBMC samples were cryopreserved with a range of DMSO concentrations (from 0% to 10%) (n=10). Error bars correspond to the SEM. *p<0.05, **p<0.01 and ***p<0.001 compared to recoveries when 10% (v/v) DMSO was used

5.2.6 Cryoprotective solution addition speed

DMSO is commonly added to the cells in a slow drop-by-drop fashion or by syringe pump at a rate of 0.5ml minute^{-1}. The effect of adding the DMSO at a faster rate of 10ml minute^{-1} was assessed and the post-thaw viable cell and CFU recoveries compared to the current protocol. Mononuclear cells were mixed, split into two freezing bags, pre-cooled to 4°C on a Biosafe Coolmix before the DMSO was added at the different rates. The two bags were frozen using a CRF and transferred to the gas phase of a liquid

nitrogen tank for a minimum of 24 hours. The bags were thawed at the same time and the recovery of viable and functional cells assessed. Figure 5.13 shows no significant differences between the two DMSO addition rates in the recoveries of viable $CD45^+$ and $CD34^+$ when assessed using 7AAD and Annexin V and also similar recoveries of CFUs post-thaw.

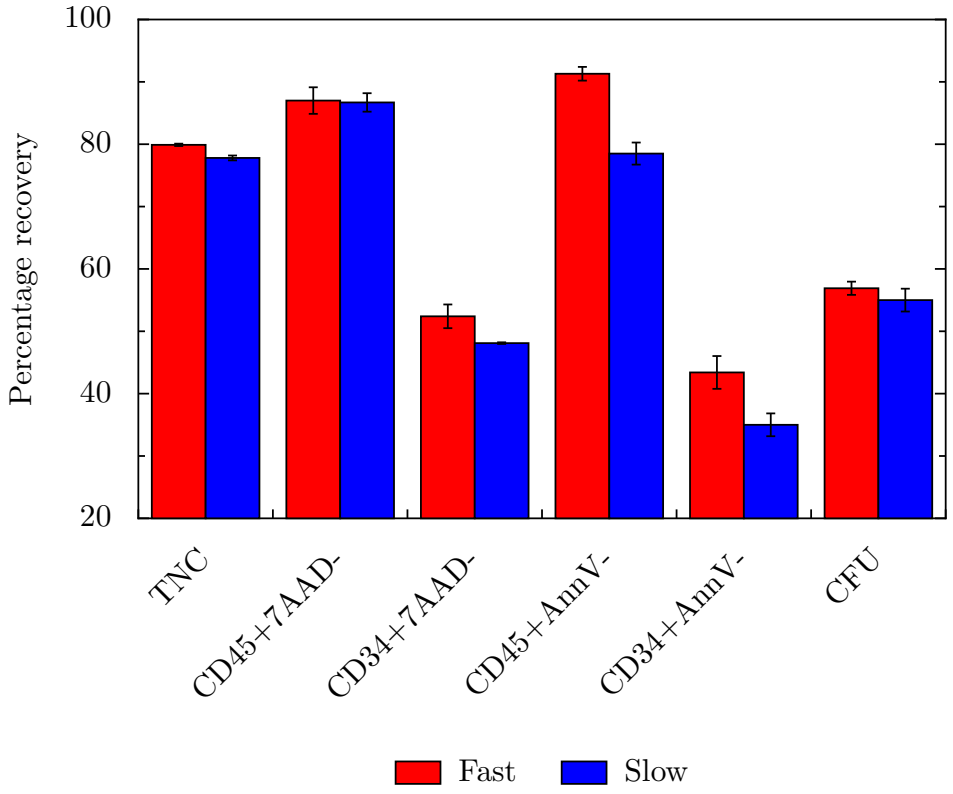

FIGURE 5.13: Mean post-thaw viable cell and CFU recovery when 10% (v/v) DMSO is added at 0.5ml $minute^{-1}$ (slow) or 10ml $minute^{-1}$ (fast) prior to freezing (n=10). Error bars correspond to the SEM.

5.3 Discussion

This Chapter looks into optimising the cryopreservation procedures for CB samples by identifying the different stages of the process that can have a negative impact on the cells.

Different cells within CB have different levels of tolerance to the osmotic stress during the freeze and thaw stages of cryopreservation. Polymorphonuclear cells such as granulocytes are known to require different cryopreservation conditions.[133,474,475] Consequently the ability to cryopreserve whole CB versus granulocyte depleted CBMC samples was assessed. TNC and $CD45^+$ recoveries were found to be significantly lower in the whole CB samples, but also the viable MNC and $CD34^+$ recoveries were reduced when compared to CBMC samples. This could be due to the release of lysosomal enzymes and nucleoproteins as the granulocytes lyse which is known to have a negative impact on other cells and cause clumping.[131,132,134] However, no post-thaw cell clumping or gel formation was observed in the whole CB samples.

Two freezing methods have been used throughout this project, freezing larger samples of 20ml using a controlled rate freezer (CRF) and smaller samples of 1ml using a Nalgene Mr Frosty freezing container. When comparing these two methods, higher recoveries were obtained when using the CRF, this was particularly apparent when cryopreserving CBMC cells where significant differences were detected when during the viable CD45 and CD34 cell assessment. At the freezing point of the samples, when the water crystallises, heat is released which can have deleterious effects of the viability of the cells. Using a CRF the temperature changes can be pre-programmed in order to compensate for the latent heat released. However, this is not possible in a Mr Frosty, which cools at a constant rate of $1°C\ minute^{-1}$, although as the samples in the Mr Frosty are a much smaller volume the quantity and duration of the liberated heat is reduced.

It was originally believed that the higher the concentration of a cryoprotectant, the higher its cryoprotective ability.[148] However, this study has demonstrated a concentration dependant toxicity of DMSO to cells within fresh CB. High concentrations of 40% (v/v) DMSO removed almost all viable and functional HPCs with a CFU recovery of 3.75

± 8.00% when assessed immediately after the addition of the cryoprotectant. Baxter and Lathe suggest DMSO may impair glycolysis by binding to fructose diphosphatase deactivating it.[128] Fahy suggested another more general hypothesis involving the denaturation of all proteins by the interaction of DMSO with their hydrophobic residues.[127] However, at concentrations as high as 40% the loss of viable cells could in part be due to the heat released upon DMSO addition, despite attempts to maintain samples at 4°C. The addition of 10% (v/v) DMSO (the concentration commonly used in cryopreservation) to fresh CBMCs did not result in significant viable $CD45^+$ or $CD34^+$ cell losses, when assessed using 7AAD of Annexin V, for up to 2 hours at either refrigerated or room temperature. After 24 hours incubation of fresh CB with 10% (v/v) DMSO significant losses were observed in $CD45^+7AAD^-$ (p=0.0039), $CD34^+7AAD^-$ (p=0.0039), $CD34^+AnnV^-$ (p=0.0039) and the CFU assay (p=0.002) compared to samples stored without DMSO for the same time period. It was also not until assessment at this time point that the samples with 10% (v/v) DMSO also showed significant losses compared to the assessment immediately after DMSO addition ($CD45^+7AAD^-$ p=0.0039, $CD34^+7AAD^-$ p=0.0098, $CD34^+AnnV^-$ p=0.0078 and the CFU assay p=0.0313). Therefore, our study builds on the work of Rowley *et al*[123] who observed that HPCs can tolerate 10% (v/v) DMSO for up to an hour, but refute that of Douay *et al* who found a 24% CFU recovery after 30 minutes of exposure to 10% (v/v) DMSO at 4°C reducing to 10% after 3 hours.[122]

The effects of DMSO exposure may be different in cryopreserved samples as the concentrations of solutes, such as DMSO, increases upon the formation of ice.[476] Therefore, the effect of delaying cryopreservation after the addition of DMSO was assessed. Compared to samples frozen immediately after DMSO addition, significant losses in viable cell recovery were observed after 24 hours in analysis of CD45 and CD34 (p=0.0098 and 0.0195 respectively), but earlier for CFU recovered after a 1 hour delay (p=0.0098). Samples in which cryopreservation was delayed for 2 hours after 10% (v/v) DMSO addition showed no significant losses in viable cells or CFUs compared to control samples stored for the same time period but with DMSO added just before freezing. However, as with the fresh samples, lower viable $CD45^+7AAD^-$ (p=0.0098), $CD34^+7AAD^-$ (p=0.0195), $CD34^+AnnV^-$ (p=0.0098) cell and CFU (p=0.002) recoveries were observed compared to control samples when cryopreservation was delayed for 24 hours. Therefore, the HPCs

were found to be tolerant of the increase in DMSO concentration during the freezing process perhaps due to the cooler temperatures. This tolerance to concentrations of 10% can also be demonstrated by the ability of cryopreserved HPCs to consistently engraft in patients.

It has been recommended that cells are maintained at chilled temperatures after DMSO addition in order to minimise the toxic effects of DMSO.[55,122] However, no significant difference was observed in fresh CBMC samples stored with 10% (v/v) DMSO at refrigerated or room temperature for up to 24 hours, thereby supporting the work of Rowley *et al* who report no toxic effects of DMSO to bone marrow cells at 37°C.[123] However, losses were noted in samples maintained in 10% (v/v) DMSO at room temperature for 24 hours prior to freezing compared to those at refrigerated temperatures ($CD45^+7AAD^-$ p=0.0098 and $CD34^+AnnV^-$ p=0.001).

CB is commonly washed post-thaw in order to remove DMSO and avoid its toxic effects to cells and the adverse effects noted in patients upon infusion.[55,105–107,161] A hyperosmolar solution is added to the cells in order to decrease the risk of osmotic shock after the intracellular ice melts; this is followed by a washing centrifugation step removing DMSO. However, a washing step can lead to cell losses, in this study an average of 13.7% of $CD45^+$ cells were lost compared to the un-manipulated samples. It is crucial to avoid this loss, as there is already a low cell dose associated with CB. After the initial losses had been taken into account, the washed samples retained higher recoveries of viable and functional HPCs over the 48 hours compared to the diluted and un-manipulated samples. A significant reduction in CFU was observed 1 hour after thawing in the un-manipulated samples (69.71 ± 32.31%, p=0.00313), after 24 hours the diluted samples (62.77 ± 21.25%, p=0.0313), and after 48 hours in the washed samples (69.99 ± 24.80%, p=0.0451). Therefore, the most suitable post-thaw procedure to obtain highest levels of viable cells depends upon the length of time expected before infusion can be achieved; within 1 hour the samples do not require any manipulation, within 2 hours it is better to reduce the DMSO concentration through dilution, if more than 2 hours then all DMSO should be removed through washing.

The CB bank guidelines for cryopreservation are based upon the protocols used in freezing BM or PBSC samples in which 10% (v/v) DMSO is used as the cryoprotectant.[477,478] Although this study has shown that this concentration of DMSO has limited toxic effects on cells within CB for up to 2 hours before freezing it is still linked to complications in patients after transplantation. As the toxic effects post infusion has been linked to the dose, the symptoms should decrease as concentration and volume of DMSO decreases. Therefore, the effect of reducing the concentration of DMSO during cryopreservation of CB was assessed. Although there are studies suggesting 5% (v/v) DMSO is a viable option[141,148] significant losses were found in $CD45^+7AAD^-$ and $CD34^+7AAD^-$ cells at this concentration compared to samples frozen with 10%. Cryopreservation in 7.5% (v/v) DMSO was found to result in higher CFUs recovered compared to samples frozen in 10%. However, this higher recovery was not found to be significant suggesting cryopreserving CB within a range of 7.5-10% (v/v) DMSO would be acceptable.

Dextran-40 is a polysaccharide comprising of multiple glucose units with a molecular weight of 40 kDa. Due to its high molecular weight, dextran-40 is a non-penetrating cryoprotectant and can aid cells during both the freezing and thawing stages by maintaining the osmotic balance. In 1995 Rubinstein *et al* published an article stating that they had observed in preliminary experiments an improvement in post-thaw viability of CB leucocytes by 3-10% when 1% (w/v) dextran-40 was used in combination with 10% (v/v) DMSO.[55] Although this data was never published, many CB banks have added 1% (w/v) dextran-40 to their cryoprotective solution. However, the introduction of dextran-40 to the cryoprotective solution has coincided with an increase of severe CB infusion reactions.[176] Therefore, this study is the first to show the effect on both cell viability and potency when cryopreserving CB with a range concentrations of dextran-40 in combination with 10% (v/v) DMSO. The presence of dextran-40 was shown to be crucial as removing it resulted in lower post thaw recoveries of $CD45^+7AAD^-$, $CD34^+7AAD^-$, $CD34^+AnnV^-$ and CFUs (p=0.0391) compared to freezing with 1%. Increasing the concentration to 2% significantly reduced the recoveries of TNC (p=0.0078), $CD45^+7AAD^-$ (p=0.0078), $CD34^+7AAD^-$ (p=0.0078) and $CD34^+AnnV^-$ (p=0.0156). 2% (w/v) dextran also reduced the CFU recovery but the reduction was not significant until the

concentration was increased to 3% (p=0.0078). This confirmed that the optimal concentration of dextran-40 when cryopreserving CBMCs is 1%.

Current CB banking protocols suggest the cryopreservation solution containing DMSO be chilled to 4°C and added at a slow rate to prevent sudden changes in osmotic gradient.[55] However, a comparison of two different addition speeds (slow rate of 0.5ml minute^{-1} and faster rate of 10ml minute^{-1}) resulted in no significant difference in post-thaw viable cell and CFU recoveries. This furthers the work of Radke *et al* who found that prompt addition of DMSO did not alter the percentage of apoptotic cells in fresh samples,[139] however, unlike in this study, they did not confirm the effect post-thaw. This observation could lead to positive changes in cryopreservation protocols, as a faster rate of cryoprotectant addition would allow samples to be transferred to a CRF sooner, thereby, reducing DMSO exposure time.

In conclusion, a dose-dependant toxicity of DMSO to cells within CB was observed. When freezing CBMC samples, short term exposure of up to 1 hour to 10% (v/v) DMSO prior to freezing results in no significant losses in viable or functional HPCs, however significant losses are observed when increasing the exposure time or concentration. The optimum concentration of DMSO and dextran-40 in the cryoprotectant solution when cryopreserve CB was found to be 7.5-10% (v/v) and 1% (w/v) respectively. In order to avoid toxic effects of DMSO post-thaw, the procedures taken should depend upon the time until infusion; within 1 hour leave samples un-manipulated (to avoid any losses through washing), up to 2 hours it is better to dilute to reduce the DMSO concentration by half, if more than 2 hours then the samples should be washed to remove all cryoprotectant.

CHAPTER 6

BANKING A PURE CELL POPULATION

6.1 Introduction

Chapters 3 to 5 describe the optimisation of the processes involved in transportation of CB to the bank, isolation of CBMC and their subsequent cryopreservation whilst ensuring the maximum numbers of viable and functional cells are maintained. This will form the start of the Biobank. The next stage will be to allow the Biobank to store and distribute frozen samples of pure populations of a particular cell type. This will involve developing isolation techniques for a specific cell, ensuring production of a pure population that can be cryopreserved and thawed whilst maintaining its potency. In this project one particular cell type found within CB has been chosen for investigation, that of regulatory T cells (Tregs).

Tregs play an important role in the regulation of the immune system by suppressing the proliferation of other lymphocytes.[234,237–241,243–247] Due to this suppressive quality Tregs are a potential source of treatment for many immune disorders.[430,431,479] However, therapeutic application in humans requires large numbers of Tregs with high purity.[415,480] Unfortunately these cells are rare (5-10% of peripheral $CD4^+$ T cells) and potentially difficult to obtain as a functional product with high levels of purity.[419]

One obstacle in isolating Tregs is the absence of specific markers; the Treg population in adults has been found to be heterogeneous both phenotypically and functionally. Generally, Tregs are considered to be $CD4^+CD25^{high}FoxP3^+CD127^{low}$. However, these markers are not unique to these cells.[253–258] CD25, the α chain of the IL-2 receptor, is up-regulated on activated T cells resulting in a large population of $CD25^+$ cells with the adult $CD4^+$ population that do not have suppressive capabilities,[265] therefore, only cells with the highest expression of this marker are considered regulatory.[242] FoxP3 is currently considered to be the most specific marker, it is highly expressed in $CD25^+$ Tregs and has been linked to suppressive ability.[267] However, low levels of this intracellular marker have been observed in non-regulatory $CD4^+CD25^-$ cells.[244,285] The expression of FoxP3 in regulatory cells has been found to inversely correlate with expression of CD127, the α chain of the IL-7 receptor.[286,287] However, it has been observed that upon activation Tregs can up-regulate expression of this marker[288] and CD127 has been found to be down-regulated in some non suppressive cells.[258,289]

Although FoxP3 is currently the most accepted marker for Treg identification, its detection involves fixing and permeabilising cells and it is therefore, unsuitable for isolating viable cells. The lack of a unique cell-surface Treg marker makes it difficult to isolate a pure population. Most strategies involving adult blood consist of multiple steps starting with depletion of B cells and cytotoxic T cells prior to isolating the CD25 population.[419,422–424] Although these strategies have resulted in high $CD25^+$ purities (80-95%), these contain a large proportion of non-regulatory $CD25^+CD127^+$ cells (20-30%).[419] In contrast to adult Tregs, CB contains fewer activated cells and the $CD25^+$ cells forms a well defined population consisting of regulatory cells, facilitating isolations and often only one CD25 enrichment step is required, as described by Figueroa *et al.*[429]

The first stage in improving Treg isolation is to further characterise these cells, comparing CB cells to those from adult sources. Once Tregs can be identified, preferably by a single specific marker or by a combination of cell surface markers, the processes involved in isolation and cryopreservation can be optimised ensuring these cells retain their functional potency post-thaw and therefore, are suitable for banking.

6.2 Results

This Chapter aims to improve CB Treg banking procedures. First, by further characterising the phenotype and functional differences between Tregs from CB against adult blood. This is followed by assessing different isolation techniques for research grade Tregs, the ability to cryopreserve these cells, and finally GMP grade isolations.

6.2.1 Comparing CB v. adult Tregs

This section aims to compare differences between Tregs from cord blood (CB), adult peripheral blood (PB) and peripheral blood from the pregnant mothers (MB). This will be assessed first through their expression of different markers followed by differences in their functional abilities.

6.2.1.1 Characterisation of CB v. adult Tregs

Analysis of CB lymphocytes show a mean expression of CD25 in 7.27 ± 2.13% of the $CD4^+$ cells (Figure 6.1A), whereas higher numbers were observed in the adult blood samples (28.90 ± 15.00% and 59.30 ± 11.20% for PB and MB respectively). The CB $CD25^+$ cells can be observed as one distinct population, however, within the adult samples two subpopulations were observed (Figure 6.1B); a small population with high intensity of CD25 ($CD25^{high}$, 3.42 ± 2.07% and 4.15 ± 1.21% of $CD4^+$ cells in PB and MB respectively) and a larger population with intermediate levels of CD25 expression ($CD25^{int}$, 37.50 ± 9.01% and 52.60 ± 10.70% of $CD4^+$ cells in PB and MB respectively).

FoxP3 is considered to be a reliable marker for identifying Tregs,[238] as it has been shown to correlate with their suppressive function.[336,481,482] Intracellular staining techniques were employed to analyse FoxP3 in the $CD25^+$ populations (Figure 6.1C). In the CB samples, 76.71 ± 14.86% of $CD25^+$ cells expressed FoxP3. Comparing the two $CD25^+$ subgroups in the adult samples, a higher percentage of cells expressed FoxP3 in the $CD25^{high}$ population (81.91 ± 9.05% against 3.82 ± 2.04%, p=0.0078 and 78.24 ± 15.04% against 2.73 ± 1.29%, p=0.0039 for PB and MB respectively) with a higher

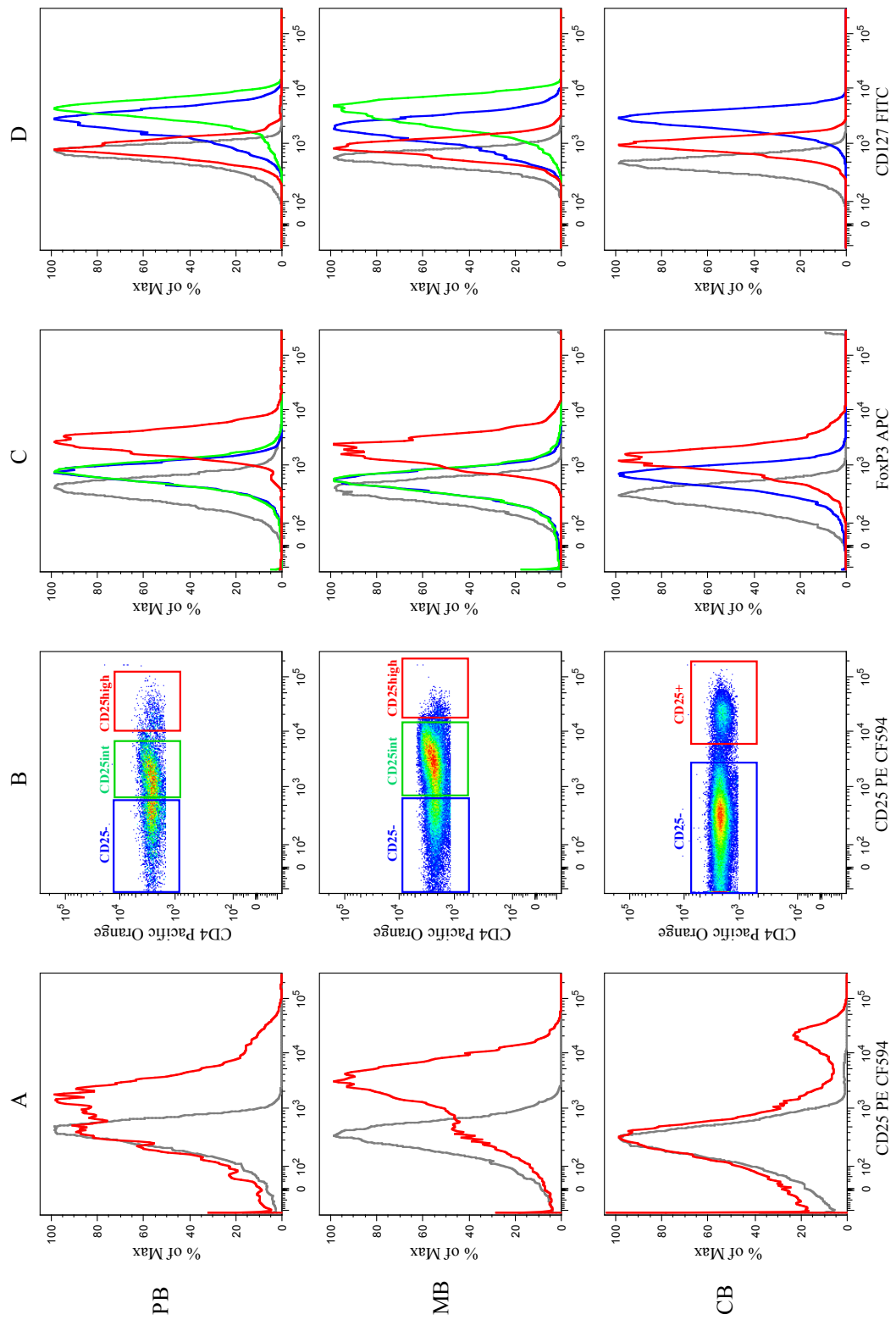

FIGURE 6.1: Comparison of CD25, FoxP3 and CD127 expression in adult and CB samples. (A) CD25 expression in adult peripheral blood (PB), maternal blood (MB) and cord blood (CB) $CD4^+$ cells compared to the isotype control (grey line). (B) Dot plots show presence of a $CD25^{int}$ population in PB and not in CB. Treg markers in $CD4^+CD25^+$ lymphocytes: Histograms show levels of expression of (C) FoxP3 and (D) CD127 in PB and CB. Red line represents $CD25^{high}$ (PB) or $CD25^+$ (CB), green line represents $CD25^{int}$ (PB only), blue line represents $CD25^-$ and grey line represents isotype control. Results shown are representative of 16 adult (7 PB and 9 MB) and 12 CB experiments.

intensity than that of the CB CD25$^+$ cells (MFI=1466 for adult CD25^{high} and 1027 for CB CD25$^+$). Whereas the CD25^{int} subpopulation showed similar FoxP3 intensities as the CD25$^-$ cells.

CD127 expression has been found to inversely correlate with FoxP3 and therefore, suppressive function.[286,287] The levels of CD127 expression in CB, PB and MB are shown in Figure 6.1D. A mean of 78.65 ± 15.03% of CB CD25$^+$ cells were found to express low levels of CD127. As with the FoxP3 analysis of the adult cells, CD127^{low} was observed in the CD25^{high} rather than the CD25^{int} population (89.15 ± 3.15% against 4.08 ± 1.39%, p=0.0078 and 84.33 ± 7.20% against 2.55 ± 1.03%, p=0.0039 for PB and MB respectively). The higher levels of FoxP3 and reduced CD127 expression in the CD25^{high} group suggest that this subpopulation of adult CD25$^+$ T cells have suppressive abilities and therefore, are considered to be Tregs.

The percentage of Tregs (identified with the following phenotype CD25$^+$FoxP3$^+$CD127^{low}) in CD4$^+$ T cells was assessed (Figure 6.2) and found to be higher in MB than PB (3.41 ± 1.26% and 2.33 ± 1.16% for MB and PB respectively), but highest in CB samples (5.67 ± 2.07%). However, these differences were not found to be statistically significant.

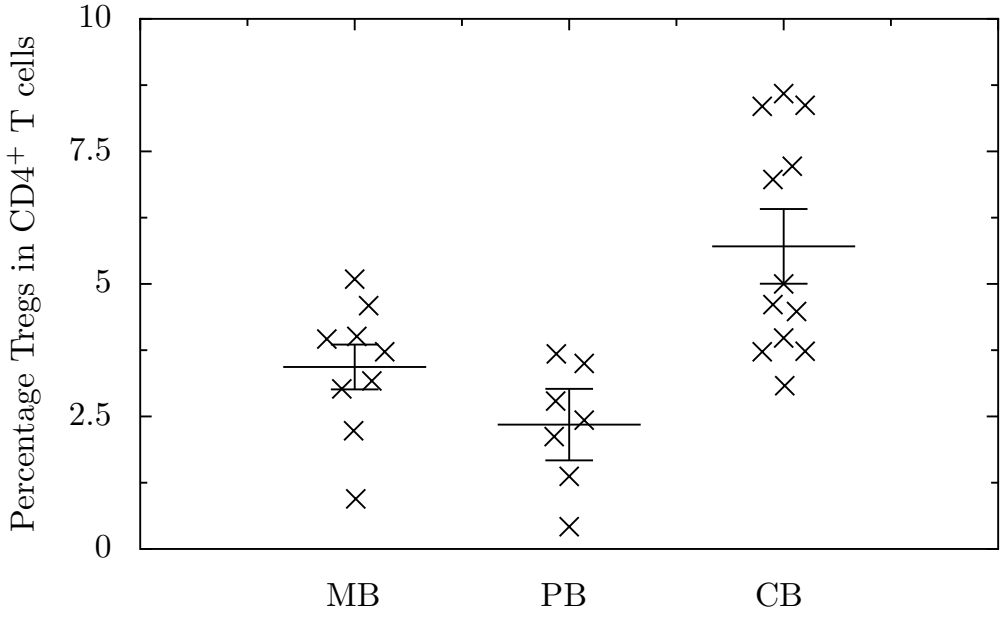

FIGURE 6.2: Mean percentage of Tregs (CD25^{high}FoxP3$^+$CD127^{low}) in CD4$^+$ lymphocytes from MB (n=9), PB (n=7) and CB (n=12) samples. Error bars correspond to the SEM.

To further characterise the phenotype of $CD3^+CD4^+CD25^+FoxP3^+CD127^{low}$ T cells from PB, MB and CB whole blood samples, cells were stained with additional Treg-associated markers and their levels of expression assessed and compared (Figure 6.3).

Levels of the homing receptors CCR4 (homes to the skin and lung) and CD62L (homes to secondary lymphoid tissues) were assessed in the three sources of Tregs. CCR4 expression was found to be significantly lower in CB Tregs (40.51 ± 11.21%) compared to those from MB (68.24 ± 6.97%, p<0.0002) and PB (74.98 ± 9.88%, p=0.0002). Although MB $CD25^{high}$ cells had lower levels of expression of CCR4 than those from PB, the differences were not found to be statistically significant. Levels of CD62L expression in Tregs was lowest at 77.48 ± 16.03% in the PB samples, 82.90 ± 3.76% in MB and highest levels were observed in CB (87.79 ± 4.85%).

CD39, an ectoenzyme, has immunoregulatory properties through the hydrolysis of proinflammatory extracellular ATP to AMP[290,295] which can be further converted by CD73 to the immunosuppressive adenosine.[297] CD39 expression was found to be highest at 59.13 ± 8.64% in PB samples, reduced to 47.18 ± 17.03% in MB and significantly lower in CB (20.19 ± 11.99%, p=0.0015 and p=0.0008 compared to PB and MB respectively). Differences between MB and PB CD39 expression were not found to be statistically significant. Flow cytometry analysis of the $CD4^+CD25^{+/high}$ populations reveal that the CD39 subset is mostly expressed in the $FoxP3^+$ cells with only 4.31 ± 1.25%, 1.61±2.27 and 1.11 ± 2.19% CD39 expression in $FoxP3^-$ cells for PB, MB and CB respectively (Figure 6.4). Intensity of FoxP3 expression was found to be significantly higher in $CD39^+$ compared to the $CD39^-$ subpopulation (MFI=1455 ± 645 for $CD39^+$ and 914 ± 366 for $CD39^-$, p<0.0001).

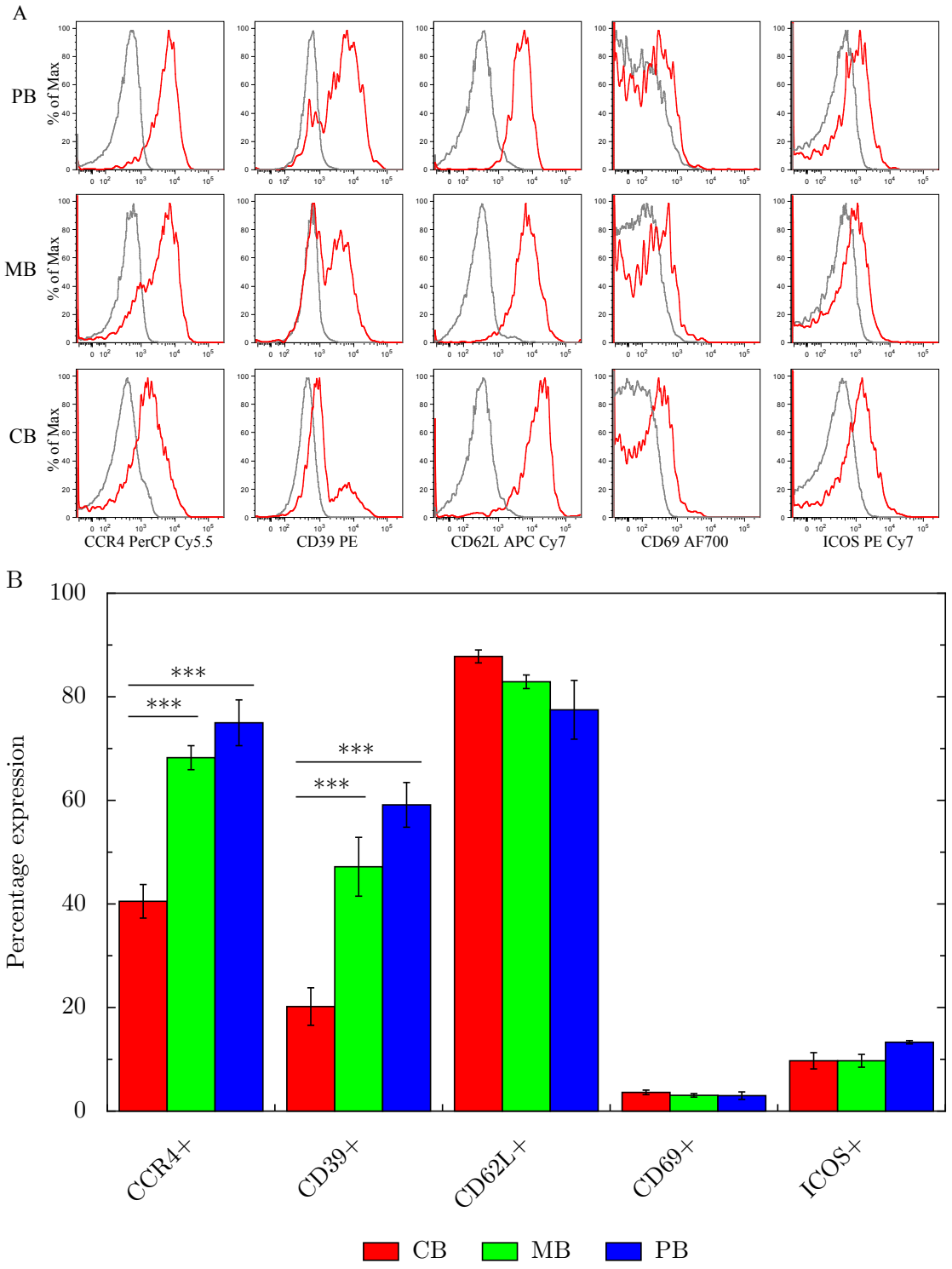

FIGURE 6.3: Further characterisation of Tregs within CB, MB and PB. (A) Histograms showing levels of expression of CCR4, CD39, CD62L, CD69 and ICOS in PB and MB CD25^{high} and CB CD25^{+} compared to the isotype (grey line). Data shown are representative of 4 PB, 9 MB and 11 CB samples. (B) Mean percentage expression of these cell surface markers in CD4^{+}CD25^{+}CD127^{low}FoxP3^{+} in CB (n=11), PB (n=4) and MB (n=9) samples. Error bars correspond to the SEM. *p<0.05, **p<0.01 and ***p<0.001 between CB, MB and PB samples

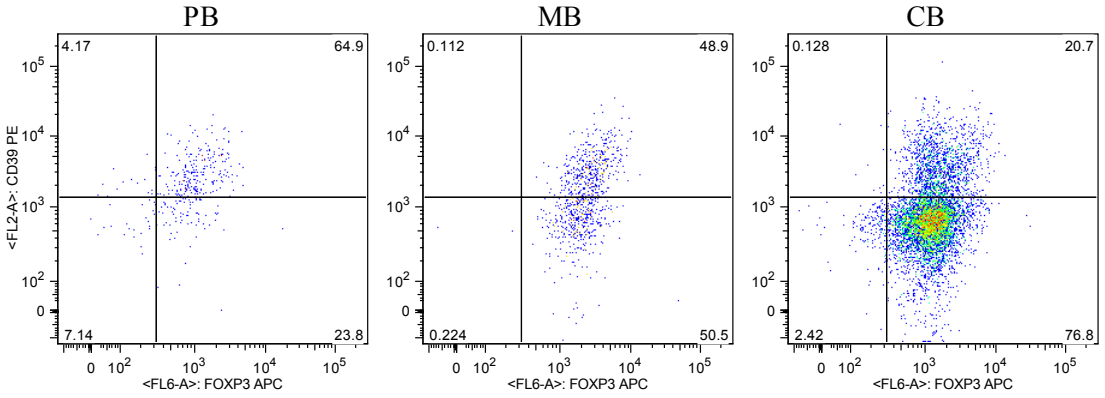

FIGURE 6.4: Levels of CD39 and FoxP3 expression in $CD4^+CD25^{high}$ adult cells (PB and MB) and $CD4^+CD25^+$ CB cells. Data shown are representative of 4 PB, 9 MB and 11 CB samples.

Inducible costimulator (ICOS), a member of the CD28 receptor family, plays a role in T cell activation and survival.[301] ICOS is upregulated upon antigen encounter and identifies memory-like Tregs.[304] Similar levels of ICOS expression were observed in the different sources of Tregs (9.72 ± 5.19% for CB, 9.59 ± 13.30% for MB and 13.30 ± 0.65 for PB%) with equal levels of CD69 (early activation marker) expression (∼3%).

6.2.1.2 Functional ability of CB v. adult Tregs

The suppressive abilities of Tregs derived from CB were compared to those from PB samples. $CD25^+$ Tregs were isolated using a MACS system with a mean $CD25^+CD127^{low}$ purity within $CD4^+$ cells of 95.1 ± 4.68% and 95.4 ± 1.41% for CB and PB respectively. These cells were co-cultured at different ratios with $CD25^-$ cells, stimulated with soluble anti-CD3 ($1\mu g\ ml^{-1}$), and anti-CD28 ($2\mu g\ ml^{-1}$) to initiate proliferation. Changes in proliferation of the $CD25^-$ cells was assessed by uptake of tritiated thymidine (Figure 6.5) or through changes in CFSE intensity (Figure 6.6). In order to compare the suppressive abilities of Tregs from CB and PB samples, the $CD25^-$ cells used in all experiments were from the same batch of PB cells (described in §2.10.1).

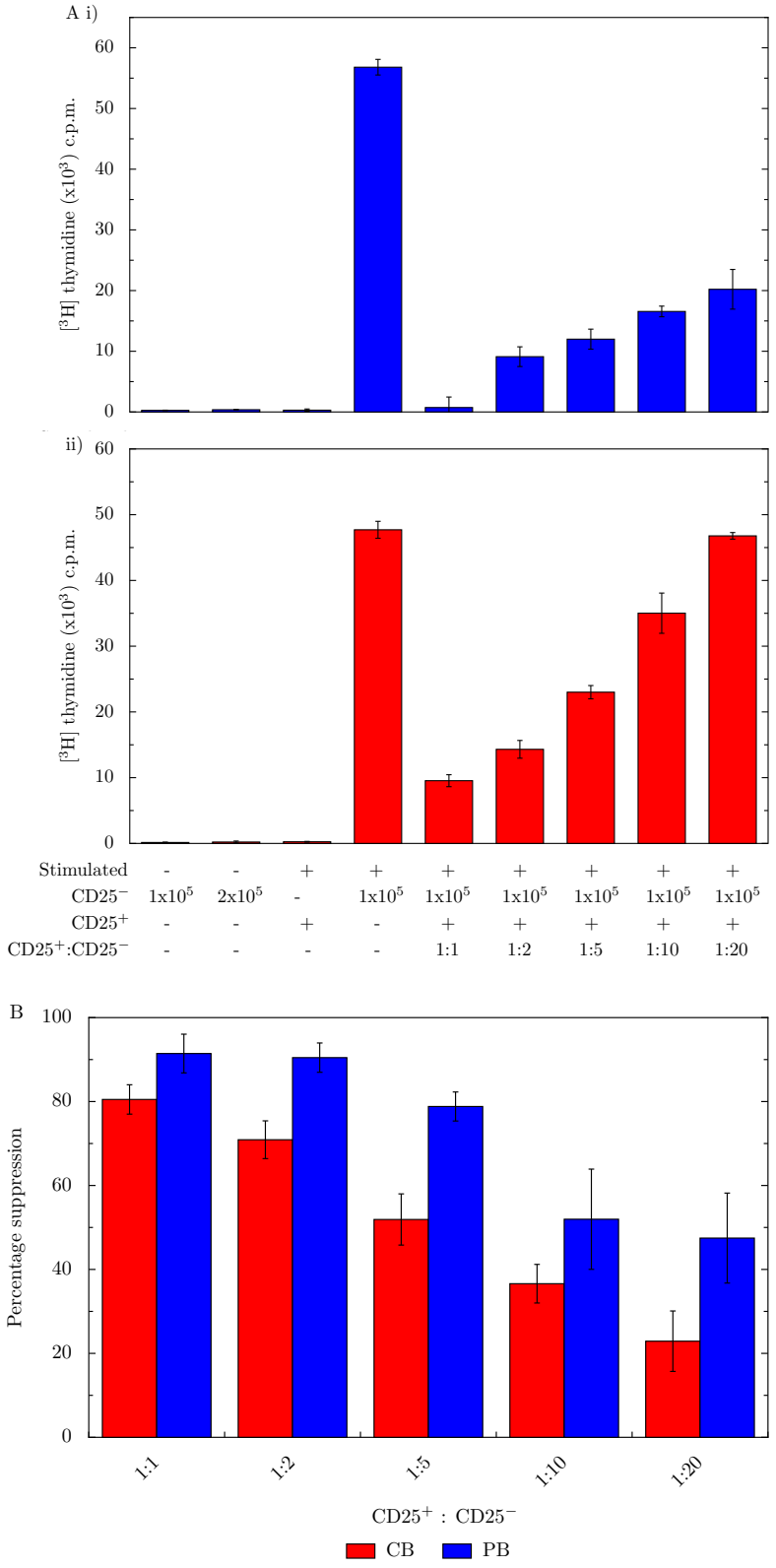

FIGURE 6.5: Comparing suppression of proliferating CD25$^-$ cells by CB and PB Tregs: Assessed through the uptake of tritiated thymidine. (A) Thymidine uptake of CD25$^-$ cells when co-cultured with (i) PB or (ii) CB Treg under the indicated conditions. Data shown are representative of 4 PB and 14 CB samples. (B) Mean percentage suppression of proliferating CD25$^-$ cells by different ratios of CB (n=14) and PB (n=4) Tregs.

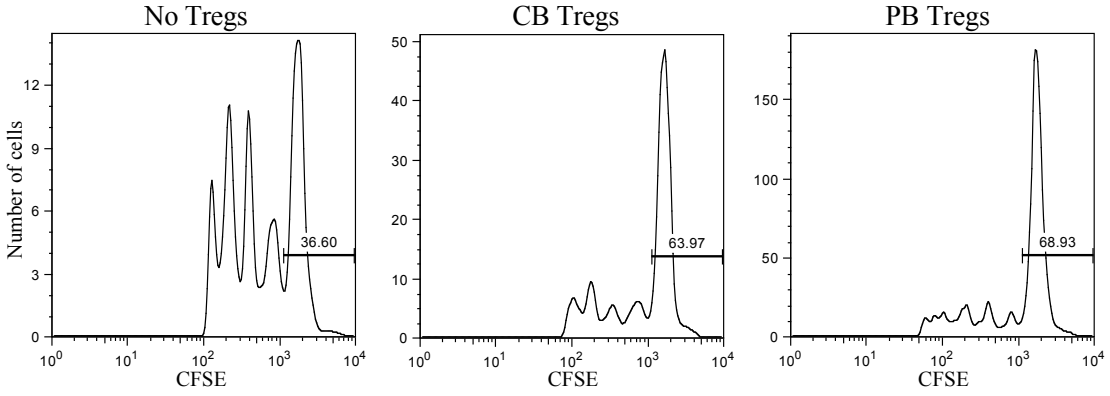

FIGURE 6.6: Comparing suppression of proliferating CD25$^-$ cells by CB and PB Tregs: Assessed using CFSE and flow cytometry. CFSE stained CD25$^-$ cells stimulated with or without the presence of Tregs (Treg:Teff ratio of 1:2). Data shown are representative of CB (n=5) and PB (n=3) samples

Consistently high levels of [^{3}H] thymidine uptake were observed for the stimulated CD25$^-$ cells (Figure 6.5A), with negligible levels for unstimulated cells. The CD25$^+$ cells from both PB and CB did not proliferate when stimulated and were therefore, shown to be in an anergic state. The CD25$^+$ cells, from both PB and CB, suppressed the proliferation of the CD25$^-$ cells in a dose dependant fashion. PB Tregs were found to have higher levels of suppressive function for all CD25$^+$:CD25$^-$ ratios compared to CB Tregs (Figure 6.5B), however, the differences between the two Treg sources were not found to be statistically significant.

It has been suggested that after activation through the TCR, Tregs require cell-cell contact in order to suppress effector cell proliferation.[351] Therefore, the suppressive abilities of CB and PB Tregs through cell-cell contact or soluble factors was assessed using transwell plates (Figure 6.7). It was observed that both CB and PB Tregs were able to suppress proliferating CD25$^-$ cells when mixed or separated by the transwell membrane.

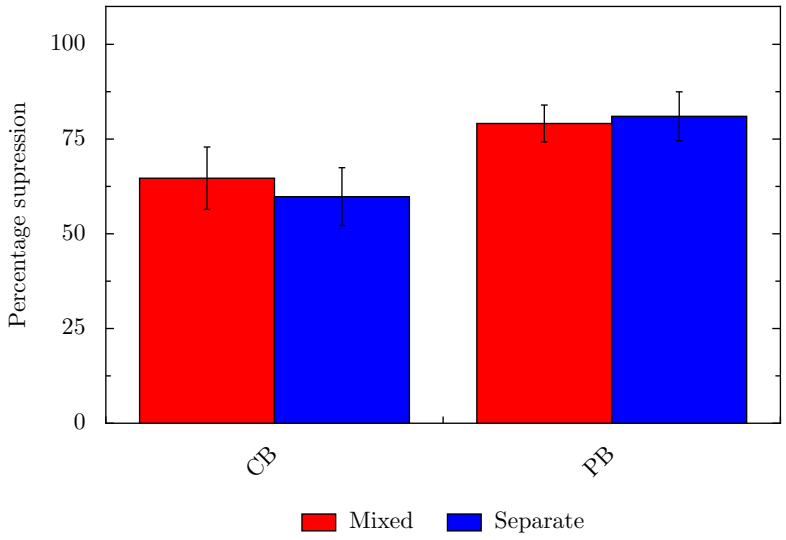

FIGURE 6.7: Comparing suppression of proliferating CD25$^-$ cells by CB and PB Tregs: Using a transwell system to assess cell-cell contact dependence or independence. Mean suppression of Teff by CB (n=5) or PB (n=3) Tregs (Treg:Teff ratio of 1:2) when cultured together or separated by a transwell membrane with pore size of 0.4μm. Error bars correspond to the SEM.

6.2.2 Isolation of Tregs

The practical use of Tregs required their isolation from whole blood samples. Unfortunately these cells are rare and potentially difficult to obtain as a pure and functional product.[419] Although FoxP3 is not unique to Tregs it is considered to be the most specific marker in their identification.[238] However, this is an intracellular marker and requires fixing and permeabilisation of the cells, therefore, the use of FoxP3 for isolations is unfeasible. CD25 is commonly used when isolating Tregs, however, CD25 is up-regulated as T cells become activated,[265] therefore, using this marker alone in the isolation of adult Tregs can result in low purities. To combat this, CD127, which has been shown to inversely correlate with FoxP3 expression,[286,287] is often used in combination with CD25$^+$ to improve the purity of Treg isolations. As shown in section 6.2.1.1, there are fewer activated CD25 cells in CB and a high proportion of the CD25$^+$ population express FoxP3 and low levels of CD127. Therefore, this marker alone can be employed to isolate pure populations of Tregs from CB.[429]

In order to create a Biobank Bank of pure cell populations, different methods of isolating Tregs from CB samples were tested using expression of CD25 to separate cells. These methods can be grouped into two different techniques:

1. Magnetic Activated Cell Sorting (MACS)

   Involves attaching an antibody, labelled with a magnetic bead, to the cells and separating labelled cells using a magnetic field. Within this study two different MACS techniques from two companies were employed:

   (a) Miltenyi Biotec MidiMACS system (MBio)

   Miltenyi Biotec developed the MACS technique which involves passing the labelled cells through a column, containing ferromagnetic spheres, within a magnetic field. The labelled cells remain within the column allowing the unlabelled cells to pass through. Any remaining unlabelled cells can then be washed and the labelled cells eluted from the column by the removal of the magnetic field.

   (b) Stem Cell Technologies EasySep system (SCT)

   The method developed by SCT uses a similar concept to MBio, but instead of passing cells through a column, the sample remains in a tube placed within a magnetic field and the unlabelled cells are poured off.

2. Fluorescence Activated Cell Sorting (FACS)

   A specialised type of flow cytometry, separates specific cell populations based on their binding to antibodies attached to a fluorochrome. A rare target population within a large sample can extend sorting times. To avoid this, the $CD4^+$ cells were first enriched using MACS followed by $CD25^+$ isolation using the Beckman Coulter MoFlo cell sorter.

These three different isolation procedures were used to separate Tregs from other CB cells based on their expression of $CD25^+$. Total nucleated cell (TNC) recovery (assessed as the percentage recovery of nucleated cells from the pre-isolation nucleated cell count) from the starting cell count was lowest when using the cell sorter ($0.08 \pm 0.04\%$ (range

0.06 to 0.11%)) and MBio (0.09 ± 0.04% (range 0.05 to 0.14%)) protocols compared to SCT (0.35 ± 0.13% (range 0.23 to 0.52%) p=0.00787 compared to both flow cytometric cell sorting and MBio). The $CD25^+$ cell yield (assessed as the percentage of $CD25^+$ cells recovered from the pre-isolation $CD25^+$ cell count) was also highest in the SCT samples (33.59 ± 16.91%), compared to 8.37 ± 5.10% (p=0.0087) for samples isolated using the MBio protocol and 4.87 ± 0.57% for flow cytometric cell sorting).

The purity of the Tregs, their viability and their suppressive ability were also compared between the three methods (Figure 6.8). Flow cytometric cell sorting resulted in the highest purity of $CD4^+$ cells in the $CD45^+$ population of 90.95 ± 4.60% compared to 82.07 ± 5.03% when using the MBio method (p=N.S.), and lowest when using the SCT method (66.96 ± 11.85%, p<0.01 compared to sorted cells and p=0.03 compared to MBio). Flow cytometry analysis of the isolated products (Figure 6.9) shows the presence of contaminating monocytes (observed in the SSC v. FSC plots) and B cells (identified as $CD3^-CD4^-$ lymphocytes) in the SCT isolations. However, the identity of these contaminating cells was not confirmed by further staining (for example CD14 or CD19 staining to identify monocytes and B cells respectively). CD25 purity within the $CD45^+$ population was again highest in the flow cytometric cell sorted samples with a mean of 89.90 ± 5.94% compared to 81.73 ± 4.94% for MBio (p=N.S.) and 66.54 ± 11.70% for SCT isolations (p<0.01 compared to sorted cells and p=0.03 compared to MBio). Cell sorting also resulted in a higher (although not significantly so) proportion of $FoxP3^+$ within the $CD4^+CD25^+$ population. However, these isolated cells had a slightly lower $CD25^+$ purity within the $CD4^+$ cells and lower $CD127^{low}$ purity within the $CD25^+$ population compared to the MACS protocols. Similar levels of $CD39^+$ expression was observed between the cells isolated from all three protocols.

Isolating Tregs using the MACS methods resulted in similar viabilities (86.91 ± 3.10% and 89.52 ± 3.50% for the MBio and SCT protocols respectively) compared to a reduced viability of 75.25 ± 4.46% when using the flow cytometric cell sorter (p=N.S.).

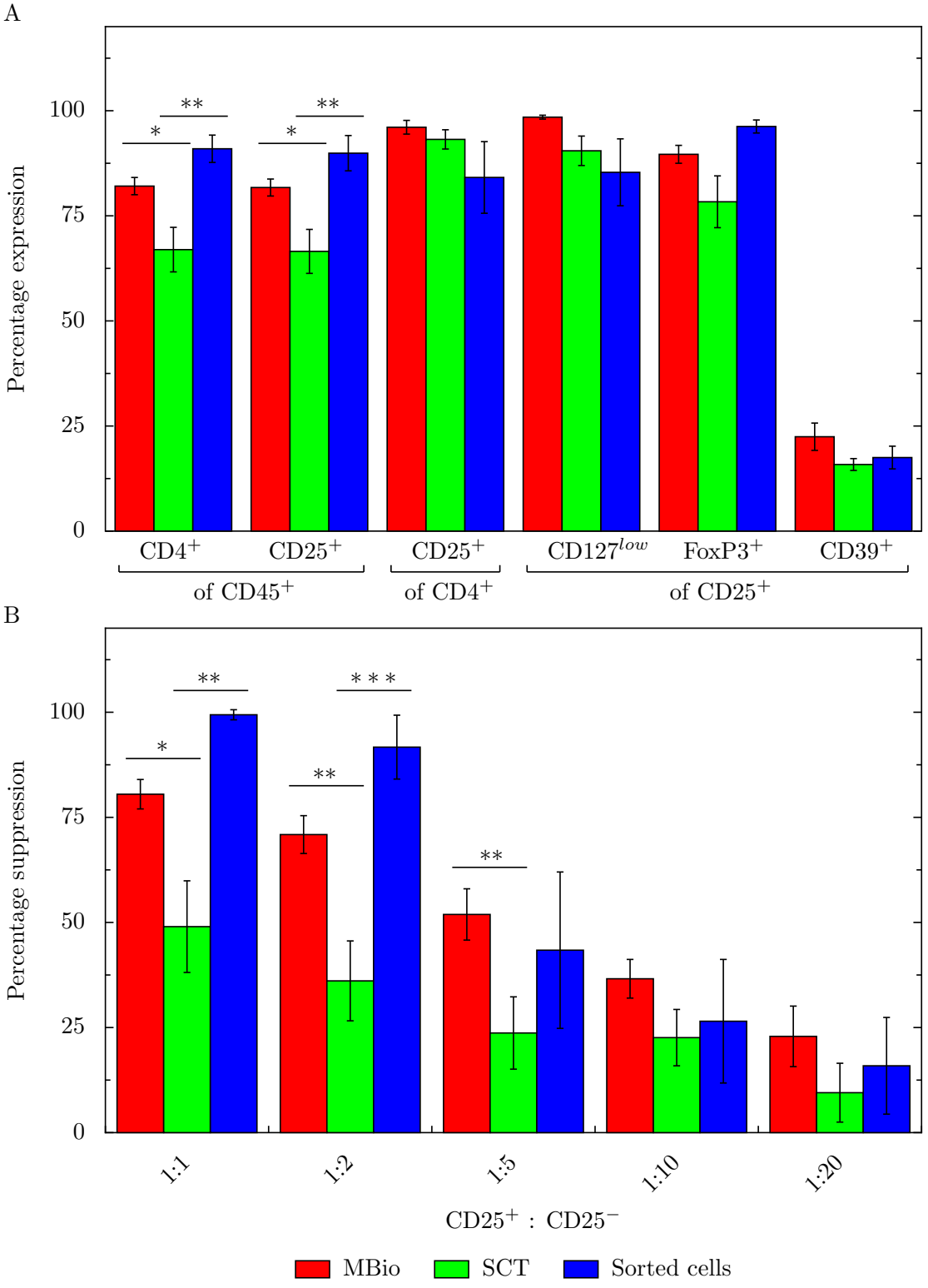

FIGURE 6.8: Comparison of Treg purity and suppressive abilities when using different isolation protocols. (A) Assessment of post-isolation purity through mean percentage expression of Treg markers (B) suppressive effects of isolated cells from each separation method (MBio n=14, SCT n=7 and flow cytometric cell sorting n=3). Error bars correspond to the SEM. *p<0.05, **p<0.01 and ***p<0.001 between isolation protocols

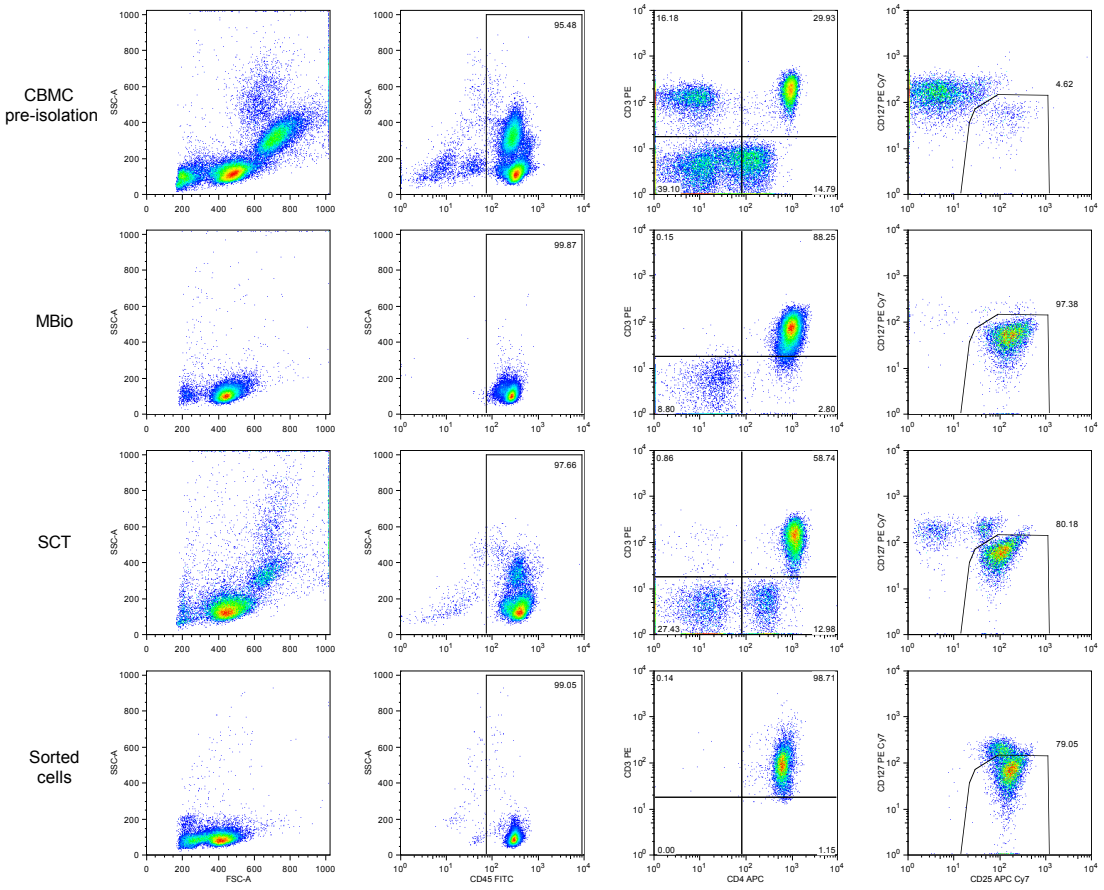

FIGURE 6.9: Comparison of Treg purity when using different isolation protocols: Flow cytometry dot plots showing purity of Tregs pre- and post- CD25 isolation (data are representative of MBio n=14, SCT n=7 and flow cytometric cell sorting n=3 samples).

The suppressive abilities of the cells isolated using the different methods are shown in Figure 6.8B. When using a higher proportion of Tregs to $CD25^-$ cells in the suppression assay, a greater difference is observed between the methods. At a Treg:Teff ratio of 1:1 the mean percentage suppression by the flow cytometric cell sorted samples was highest at 99.37 ± 0.32% followed by 80.5 ± 13.16% for the MBio samples and 49.03 ± 28.92% for the SCT cells (p=0.0028). At a ratio of 1:5 and below, the MBio samples had greater suppressive abilities (although not significantly higher) compared to the two other methods. A high correlation was observed between the purity of Tregs isolated using the various methods and their suppressive capabilities (r^2=0.59; p=0.0003, shown in Figure 6.10).

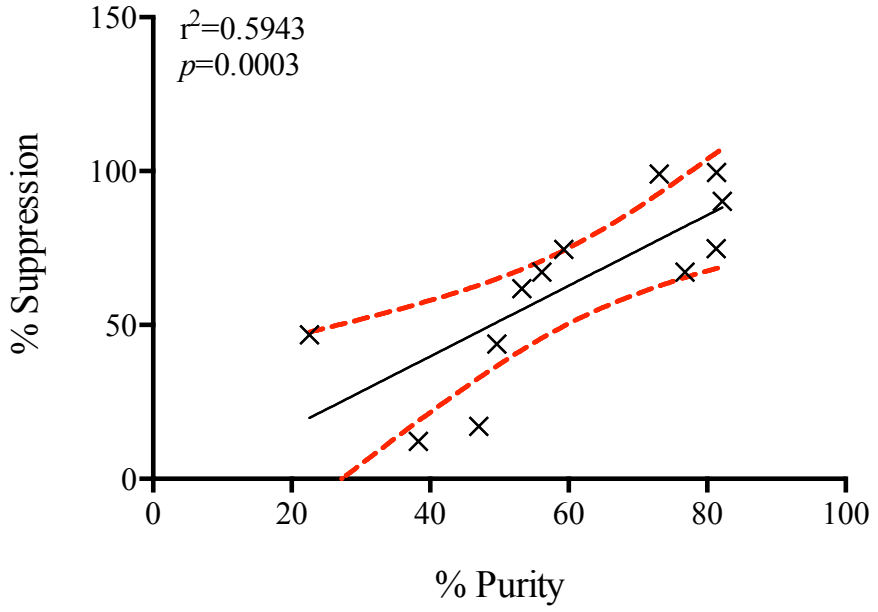

FIGURE 6.10: Correlation between percentage Treg purity (percentage $CD4^+CD25^+CD127^{low}$ within the $CD45^+$ fraction) with percentage suppression (at a Treg:$CD25^-$ ratio of 1:2) (n=12). Red dotted lines correspond to the 95% CI, analysis performed using Spearman's nonparametric correlation.

6.2.2.1 Characterisation of the positive and negative fractions post-isolation

Isolation of $CD25^+$ cells using Miltenyi Biotec's MidiMACS system (MBio) removed almost all $CD25^+$ cells from the negative fraction with an average contamination of 0.21 ± 0.02% $CD4^+$ cells, p=0.0278 (Figure 6.11A). The positive fraction also contained the majority of $FoxP3^+$ and $CD127^{low}$ cells (p=0.0278).

The distribution of cells expressing the markers tested in section 6.2.1.1 was assessed in the positive and negative fractions of MBio isolations, shown in Figure 6.11B. It can be observed that almost all the $CCR4^+$ and $CD39^+$ cells are found in the $CD25^+$ fraction (56.67 ± 6.45% and 1.35 ± 0.57% CCR4 expression in the positive and negative fractions respectively, p=0.0278 and 20.80 ± 9.05% against 1.74 ± 2.01% CD39 expression, p=0.027). Higher level of ICOS expression were also found in the positive fraction (7.85 ± 1.37% in $CD25^+$ versus 1.72 ± 1.67% in $CD25^-$ and CD69 was also increased (6.89 ± 2.65% versus 0.97 ± 0.37%). High levels of CD62L were observed in all fractions (83.11 ± 8.28% CD62L expression in whole CB, 87.62 ± 12.91% in $CD25^+$ fraction and 84.78 ± 8.14% in the $CD25^-$ fraction).

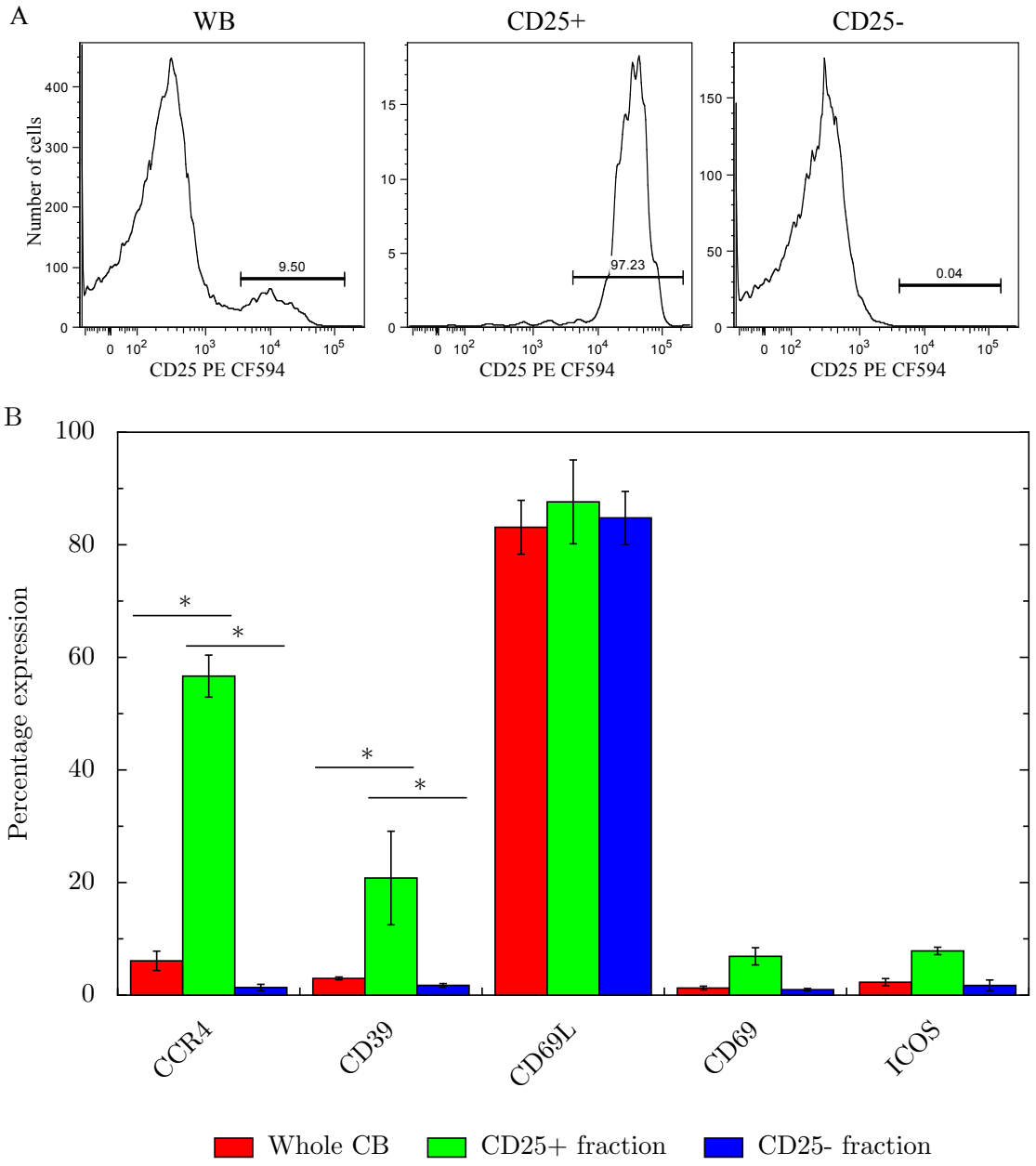

FIGURE 6.11: Characterisation of post-isolation positive and negative fractions: (A) Levels of CD25 expression in whole CB, the positive and negative fraction post-isolation, representative of 14 samples (B) Mean percentage cells expressing CCR4, CD39, CD62L, CD69 and ICOS in $CD4^+$ cells from whole CB, CD25 positive and negative fractions (n=14).

6.2.2.2 Isolation of $CD25^+CD39^{+/-}$ subpopulations

The Tregs from PB samples were found to contain higher levels of CD39 expression (shown in Figure 6.3). To assess if this marker is important for Treg suppressive function, and perhaps contributing to the higher suppression observed by the adult cells, CB

cells were enriched for $CD4^+$ (using MBio) prior to isolating the $CD25^+CD39^+$ and $CD25^+CD39^-$ subpopulations through cell sorting.

Figure 6.12A shows the purity of these isolations, with the CD39 positive fraction expressing $CD25^+CD39^+$ in $75.63 \pm 5.99\%$ of its cells and a mean contamination of $CD39^-$ cells in $14.88 \pm 9.66\%$ of the population (p=0.0039).

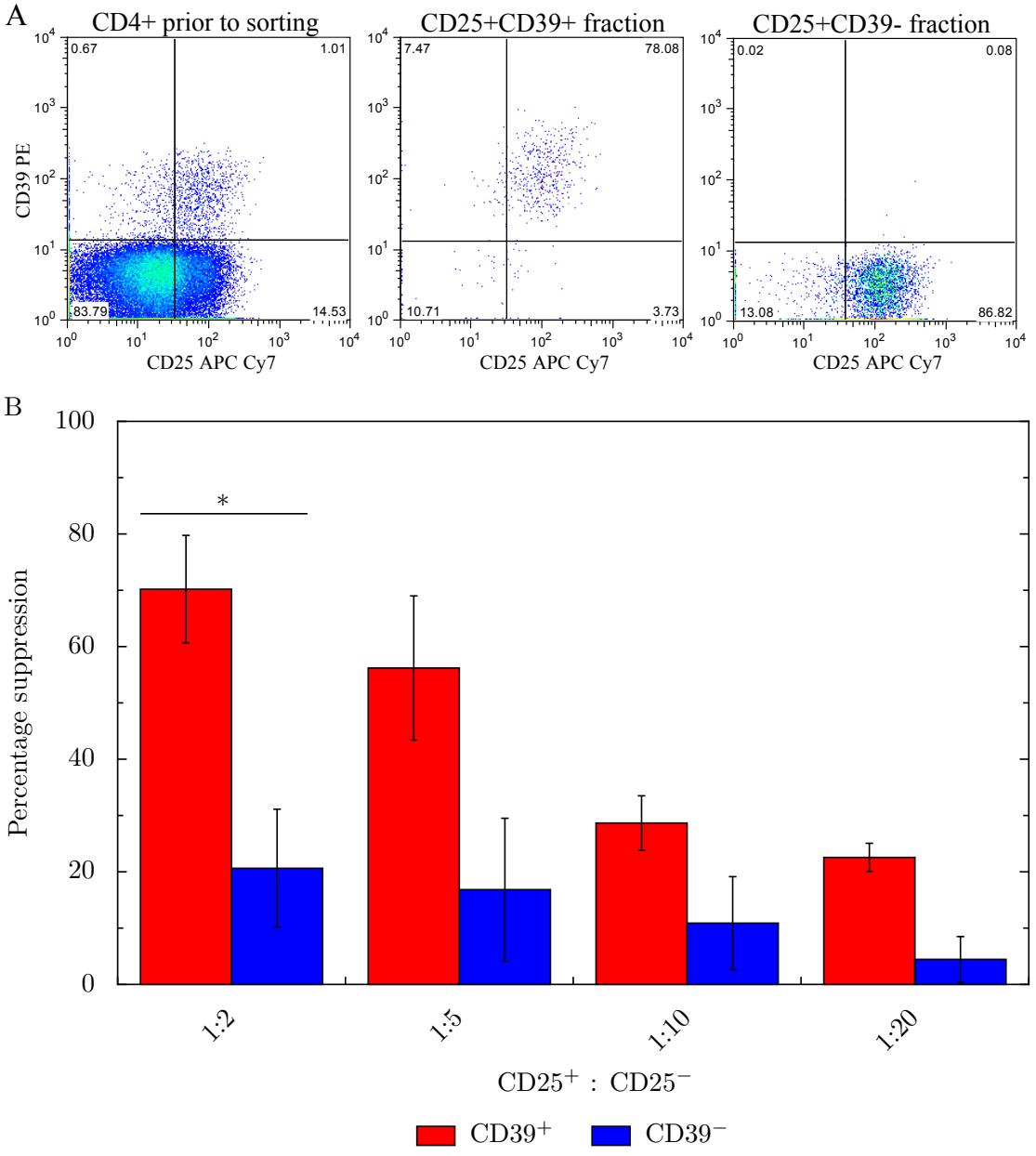

FIGURE 6.12: Comparison of the suppressive abilities of $CD25^+CD39^+$ and $CD25^+CD39^-$ subpopulations (A) $CD25^+CD39^+$ and $CD25^+CD39^-$ subpopulations were isolated from enriched $CD4^+$ cells, data are representative of three samples. (B) suppressive effects of isolated subgroups (n=3). Error bars correspond to the SEM.

High levels of CD39 depletion were observed in the $CD25^+CD39^-$ population (88.43 ± 4.01%) with low levels of $CD39^+$ contaminating $CD39^+$ in 2.44 ± 4.16% of cells (p=0.0022). However, despite pre-enriching the CD4 population prior to sorting, the isolation procedure took an extended amount of time and the isolated cells were found to have low viabilities of 59.33 ± 29.21% and 50.77 ± 37.68% for $CD25^+CD39^+$ and $CD25^+CD39^-$ populations respectively.

The $CD25^+CD39^+$ population was found to have higher levels of suppression of proliferating $CD25^-$ cells than the $CD25^+CD39^-$ population (Figure 6.12 B) with a significant difference observed at a ratio of 1:2 (p=0.0341).

6.2.3 Cryopreservation and thawing of Tregs

There are two options for the cryopreservation and storage of Tregs:

1. Cryopreservation of isolated pure populations of Tregs
2. Isolation of Tregs from cryopreserved CBMCs

6.2.3.1 Cryopreservation of isolated pure Treg population

Tregs were isolated, using the MBio protocol, with an average purity of 87.03 ± 8.11% $CD25^+$ cells within the $CD45^+$ population, and frozen at a concentration of 2.0×10^6 cells ml^{-1} in AB serum and 10% (v/v) DMSO 1% (w/v) dextran. Post-thaw yields of 82.84 ± 10.39% for TNC and 76.33 ± 12.40% for $CD25^+$ cells were observed. Thawing these cells exhibited a reduced viability of 76.46 ± 15.00% ($AnnV^-7AAD^-$) and an increase of 14.84% in the percentage of apoptotic cells ($AnnV^+7AAD^-$). The thawed cells were found to maintain their levels of expression of CD4 (83.32 ± 8.23% of $CD45^+$ cells), CD25 (98.28 ± 0.70% of $CD4^+$ and 81.74 ± 8.07% of $CD45^+$ cells), CD127 (98.90± 0.42% of $CD25^+$ cells), Figure 6.13A)

The suppressive abilities of the thawed Tregs, shown in Figure 6.8B, were found to be reduced compared to the pre-freeze analysis. At a $CD25^+$:$CD25^-$ ratio of 1:2 the

suppression induced by thawed cells was 58.70 ± 23.91% compared to 82.07 ± 10.72% pre-freeze (p=0.0313).

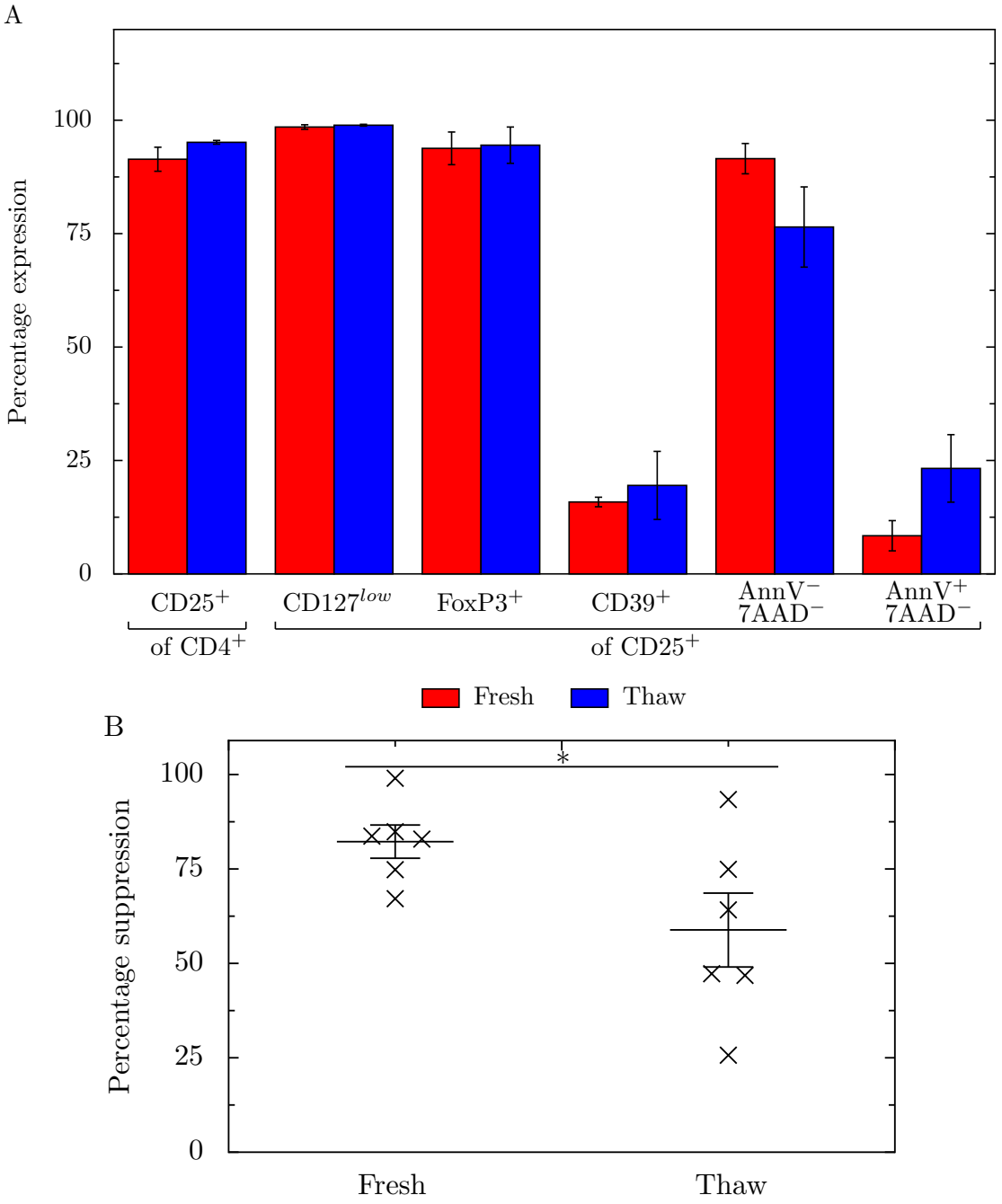

FIGURE 6.13: Comparison of Treg purity and suppressive abilities pre- and post-thaw. (A) Mean percentage expression of Treg markers in pre- and post-thaw isolated CD25$^+$ cells (B) suppressive effects of pre-freeze and post-thaw isolated cells at a CD25$^+$:CD25$^-$ of 1:2 (n=6). Error bars correspond to the SEM. *p<0.05 between isolation protocols

6.2.3.2 Isolation of Tregs from cryopreserved CBMC

The ability to isolate Tregs from thawed CBMC samples was assessed; first by determining the effects of freezing Tregs within the CBMC fraction, followed by the potential to isolate these cells post-thaw.

6.2.3.2.1 Effects of freezing WB/CBMC on Treg frequency

The effects of cryopreservation on the frequency of the Treg population within either whole blood (WB) or CB mononuclear cell fractions (CBMC) samples was assessed by comparing pre-freeze and post-thaw cell counts measured using flow cytometry.

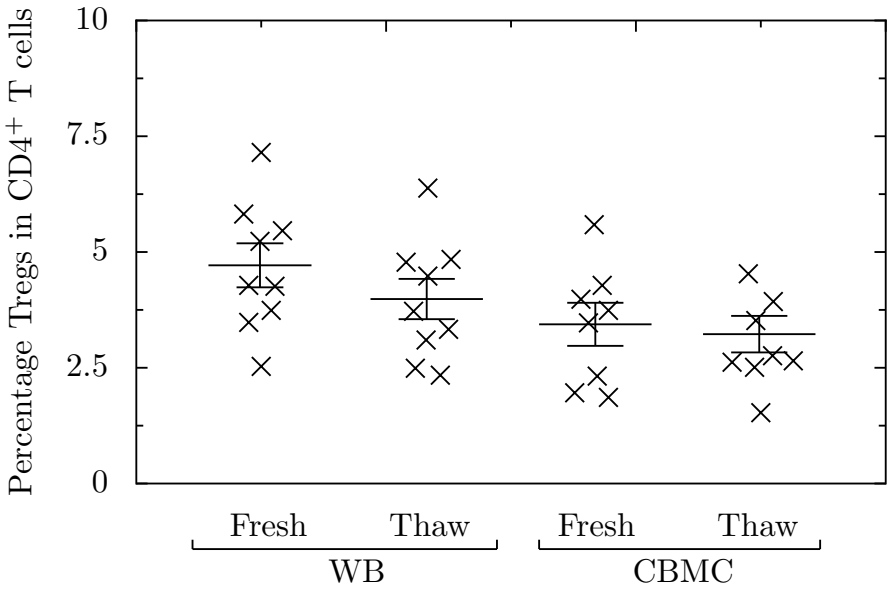

FIGURE 6.14: Frequency of Tregs in $CD4^+$ T cells from fresh and thawed WB (n=9) and CBMC (n=8) samples. Error bars correspond to the SEM.

Compared to the fresh analysis, the mean proportion of Tregs ($CD25^+CD127^{low}$) in $CD4^+$ T cells was reduced in the post-thaw samples for both WB and in CBMC (Figure 6.14). For WB the proportion was reduced from $4.66 \pm 1.40\%$ in freshly isolated samples to $3.94 \pm 1.30\%$ in the post-thaw analysis and CBMC samples from $3.40 \pm 1.28\%$ in fresh to $3.01 \pm 0.94\%$ post-thaw, however, these reductions were not found to be statistically significant.

Using Spearman's Test, a strong correlation was observed between percentages of Tregs found in fresh and thawed WB (r^2=0.9666, p=0.0002) and CBMC (r^2 =0.7931, p=0.0046) samples (Figure 6.15). This suggests that the thaw method used results in a consistent cell product.

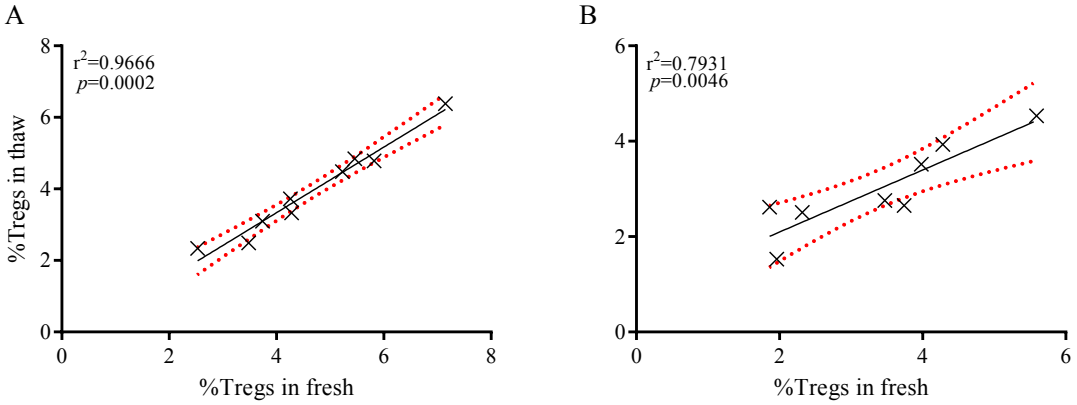

FIGURE 6.15: Correlation between percentage of Tregs ($CD25^+FoxP3^+CD127^{low}$) from $CD4^+$ T cells in fresh and frozen (A) WB (n=9) and (B) CBMC (n=8) samples. Red dotted lines correspond to the 95% CI, analysis performed using Spearman's nonparametric correlation.

Viability assessment of the Tregs in pre- and post-cryopreserved samples was performed using Annexin V to identify apoptotic cells and 7AAD to exclude cells with disrupted membranes. A reduction of 9.87% in the mean percentage of viable cells and an increase in apoptosis of 9.40% was observed after thawing in the WB samples (Table 6.1), whereas in the CBMC samples similar levels of viable and apoptotic cells were observed between fresh and thawed cells.

TABLE 6.1: Mean percentage of viable and apoptotic Tregs in fresh and thawed WB (n=9) or CBMC (n=8) samples

Sample fraction	Fresh or thaw	% Viable Tregs	% Apoptotic Tregs
WB	Fresh	93.4 ± 2.10%	6.52 ± 1.99%
	Thaw	83.53 ± 7.72%	15.92 ± 7.41%
CBMC	Fresh	85.17 ± 3.21%	14.13 ± 2.54%
	Thaw	88.69 ± 2.90%	11.27 ± 2.54%

6.2.3.2.2 Isolation of Tregs from cryopreserved CBMC

Another Treg banking option involves the storage of cryopreserved CBMC followed by CD25 isolation after thawing. CBMC samples were cryopreserved in 10% (v/v) DMSO 1% (w/v) dextran using a controlled rate freezer (CRF) and stored for a minimum of 24 hours. The samples were thawed and washed to remove DMSO and maintain the osmotic balance. $CD25^+$ cells were then isolated using either the MBio or SCT method.

A recovery of 0.17 ± 0.05% TNC and 6.10 ± 2.48% $CD25^+$ was observed when isolating Tregs from thawed CBMC using the MBio protocol. Higher recoveries were obtained using the SCT protocol (0.61 ± 0.08% TNC and 15.14 ± 4.15% CD25 recoveries, p=0.0223). The isolated cells had similar viabilities of 93.45 ± 1.98% and 90.13 ± 13.02% for MBio and SCT respectively. Apoptosis was observed in 6.27 ± 2.01% of MBio isolated cells and 6.75 ± 8.01% SCT cells. As with the fresh isolations, the MBio method resulted in higher purities of $CD25^+$ cells in the $CD45^+$ population (92.14 ± 4.28% and 86.40 ± 5.63% for MBio and SCT respectively, Figure 6.16A). Both methods had high levels of FoxP3 and $CD127^{low}$ expression in the $CD25^+$ cells. Due to the increase in purity, the MBio isolated cells had significantly higher suppressive abilities across a range of $CD25^+$:$CD25^-$ ratios compared to cells isolated using the SCT protocol (Figure 6.16B).

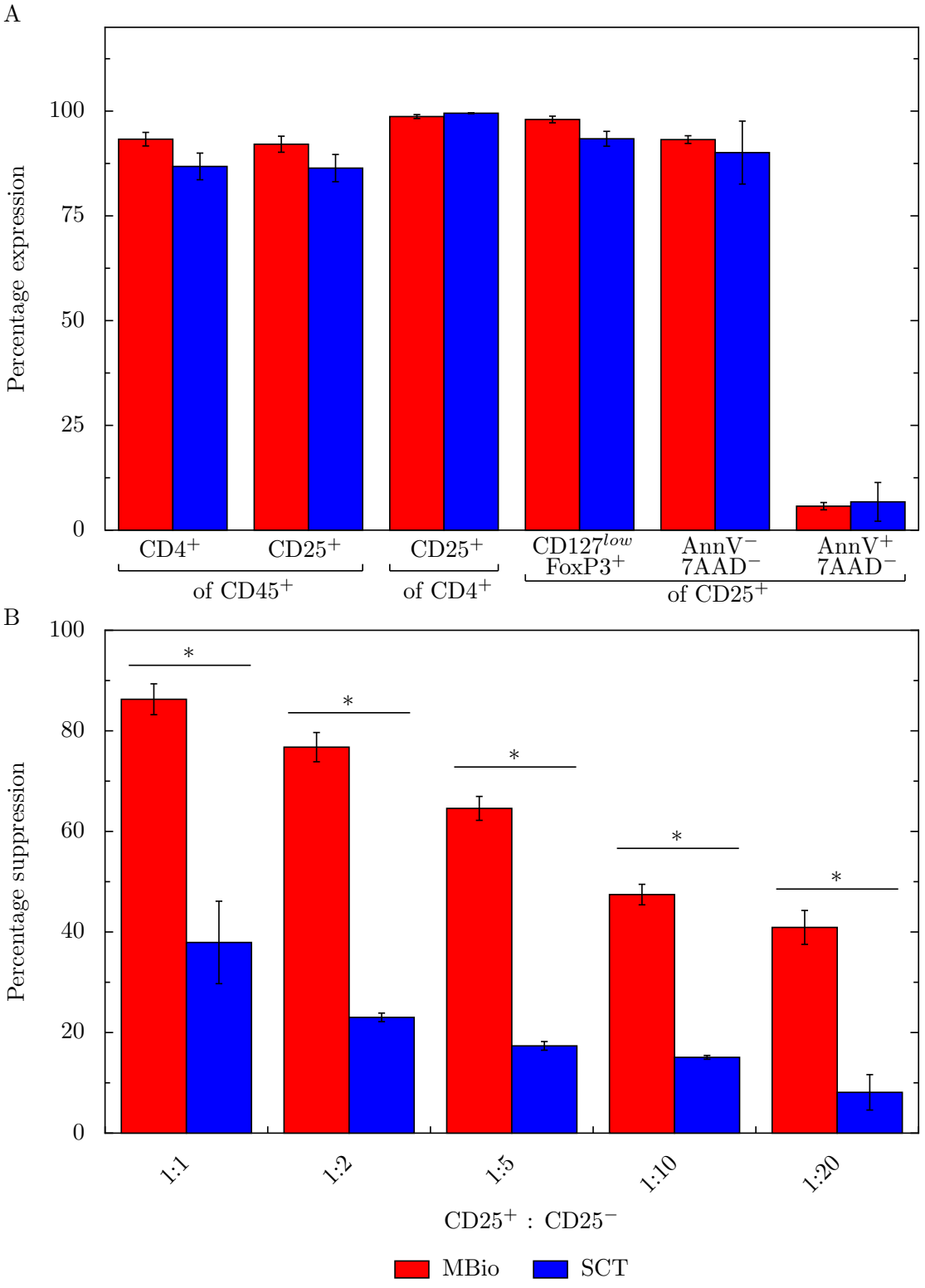

FIGURE 6.16: Comparison of Treg purity and suppressive abilities when using different isolation protocols on thawed CBMC samples. (A) Assessment of post-isolation purity on the basis of mean percentage expression of Treg markers (B) suppressive effects of isolated cells from each separation method (MBio n=5 and SCT n=3). Error bars correspond to the SEM. *p<0.05 between isolation protocols.

6.2.3.3 Treg banking: pure population or isolation from thawed CBMC?

The two different methods of banking Tregs have been assessed (cryopreservation of isolated pure Treg populations and isolation of cells from cryopreserved CBMCs).

Comparing the thawed $CD25^+$ populations, isolated using the MBio protocol, resulted in similar TNC recoveries from the starting number in the fresh WB samples (0.04 ± 0.02% for frozen CBMC and 0.03 ± 0.01% for thawed CD25, shown in Figure 6.17A). Higher CD25 yields from the initial fresh WB count were observed when isolating from thawed CBMCs (1.61 ± 0.24% and 1.09 ± 0.09% for thawed CBMC and thawed CD25 respectively, p=0.0049). These two methods resulted in $CD25^+$ cells with similar purities and levels of both FoxP3 and $CD127^{low}$ expression (Figure 6.17B). However, the cells isolated from thawed CBMC had higher viabilities than the thawed pure cell populations (93.14% against 76.46%) and lower levels of apoptotic cells (5.43% against 23.25%), shown in Figure 6.17C. The suppressive abilities of these cells were assessed at Treg:$CD25^-$ ratio of 1:2 and the thawed CBMC isolations resulted in higher suppression than the thawed $CD25^+$ cells (76.76% against 58.70%, p=0.0012 shown in Figure 6.17D).

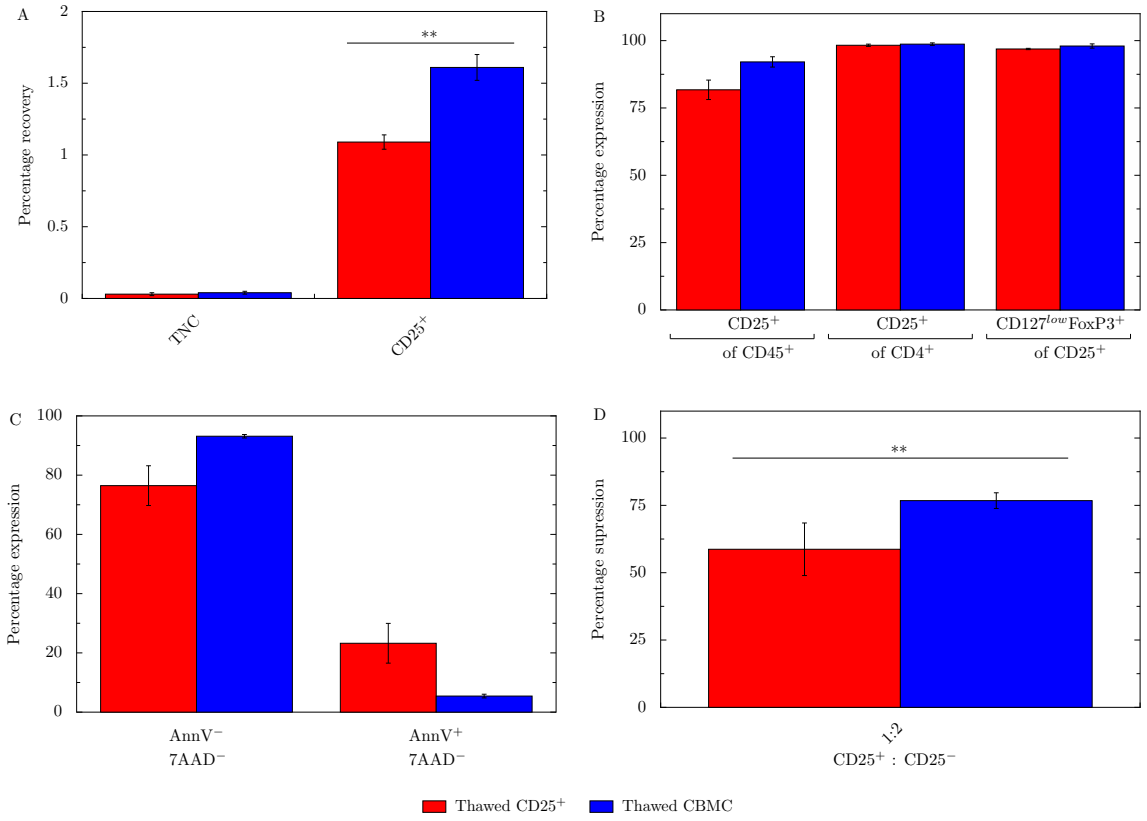

FIGURE 6.17: Comparison of cryopreserved $CD25^+$ cells (n=6) against cells isolated from thawed CBMC samples (n=5) (A) TNC and $CD25^+$ recovery from initial fresh whole blood samples, (B) purity of post-thaw isolated samples, (C) cell viabilities and levels of apoptosis (D) suppressive effects of Tregs. Error bars correspond to the SEM. $^{**}p<0.01$ compared to 0% DMSO at the same time point

6.2.4 GMP grade isolations and banking

For their use in therapeutic applications, Tregs need to be isolated with high levels of purity under GMP grade conditions. To achieve this, cells will be isolated using clinical grade reagents (such as the CE marked CD25 microBeads) and performed under aseptic techniques through the use of closed systems (CBMC separations using a SEPAX and CD25 enrichment using a CliniMACS device).

Four to six cord blood units were pooled, resulting in a mean volume of 546.67ml. These pooled units underwent a volume reduction protocol and gradient separation using ficoll on a SEPAX machine to isolate CBMCs. The cells were stained for CD25 using clinical grade microBeads and the labelled cells were isolated using the enrichment 3.2 protocol on a CliniMACS.

The initial pooled samples contained a mean TNC count of 52.05×10^8 cells with a mean $CD25^+$ cell count of 1.04×10^8. Volume reduction of these samples using a SEPAX machine resulted in a mean TNC recovery of 95.35% (49.63×10^8 cells). The density gradient cell separation protocol on the SEPAX reduced the TNC count to 19.39×10^8 (recovery of 37.24%) through the removal of granulocytes and reduced the $CD25^+$ cell count to 0.52×10^8 (recovery of 50.06%).

Isolating $CD25^+$ cells from fresh CBMC using a CliniMACS resulted in a TNC yield of 0.13 ± 0.07% and CD25 recovery of 5.08 ± 2.97%. These cells had high levels of CD25 purity with a mean of 76.84 ± 8.63% in the $CD45^+$ population (Figure 6.18A). These cells expressed high levels of FoxP3 (82.88 ± 15.23%) and $CD127^{low}$ (90.00 ± 4.97%) with a mean viability of 94.82 ± 3.31% and 4.46 ± 3.09% apoptotic cells. The isolated cells from pooled units suppressed in a dose-dependant manner (Figure 6.18B) with levels similar to to the single MBio isolations in Figure 6.8B.

In some cases it may be convenient to isolate Tregs some time prior to their use therapeutically, in these situations it would be beneficial to cryopreserve these cells whilst ensuring suppressive function remains intact. Cryopreserving these GMP grade isolated cells resulted in post-thaw recoveries of 67.81 ± 13.45% for TNC and 68.24 ± 22.68% for CD25. Compared to the pre-freeze assessment, these thawed cells had lower viabilities of 73.70 ± 8.75% (p=0.06) and higher levels of apoptosis (21.72 ± 4.98% of cells were apoptotic (p=0.0079). The thawed cells maintained their levels of purity (75.82 ± 13.83% CD25 expression in the $CD45^+$ cells), but had slightly reduced levels of suppression (shown in Figure 6.18).

As with the smaller scale research grade Treg isolations (§6.2.3.2.2), the ability to isolate functional cells from thawed CBMC samples was assessed using GMP grade protocols. Cryopreserved CBMC samples were thawed, washed using a SEPAX and pooled (4-6 units per experiment) resulting in mean TNC and $CD25^+$ cell counts of 16.76×10^8 and 0.68×10^8 cells respectively. The pooled samples were washed to remove DMSO (post-wash mean TNC count of 13.21×10^8 with a recovery of 78.82%) and labelled with clinical grade CD25 microBeads. Isolating $CD25^+$ cells from thawed CBMC using a CliniMACS resulted in a recovery of 0.29 ± 0.22% of the post-thaw TNC and 4.04 ± 0.96% of the

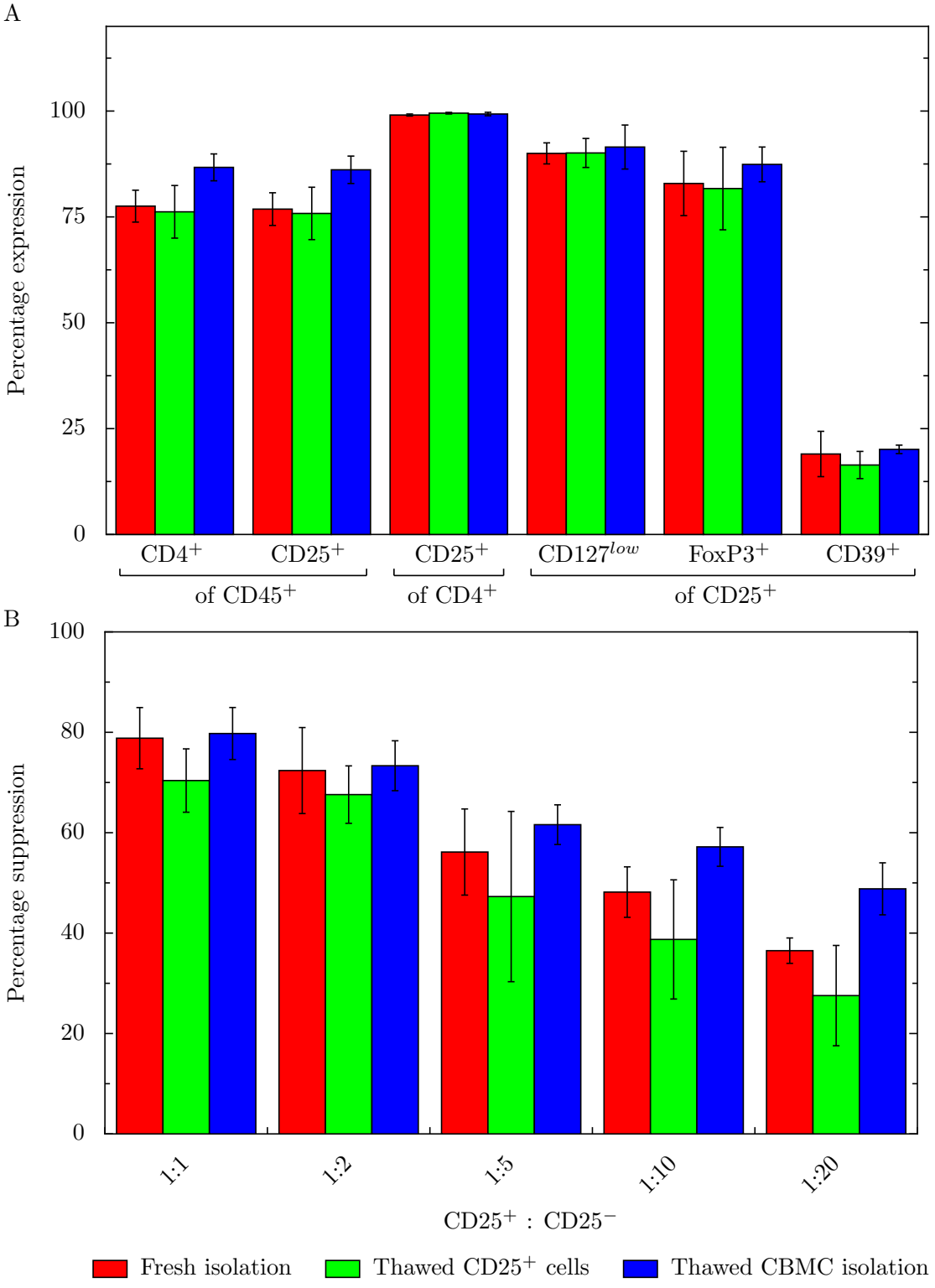

FIGURE 6.18: GMP grade isolations on fresh samples (n=5), cryopreserved isolated $CD25^+$ cells (n=5) and cells isolated from thawed CBMC (n=4). (A) Assessment of post-isolation purity through mean percentage expression of Treg markers (B) suppressive effects of isolated cells from each banking method. Error bars correspond to the SEM.

initial $CD25^+$ population. These isolated cells had viabilities of 95.83 ± 1.62% and 3.47 ± 1.61% were apoptotic. These cells had slightly elevated CD25 purity in the $CD45^+$ population compared to the fresh isolations (86.13 ± 6.48%) and similar levels of FoxP3 and CD127 expression (shown in Figure 6.18A). The suppressive capabilities of the cells isolated from thawed CBMC were similar to those from fresh isolations at higher $CD25^+$:$CD25^-$ ratios, but at ratios of 1:5 to 1:20 these cells were found to be more suppressive, however, the differences were not found to be statistically significant (shown in Figure 6.18B).

6.2.4.1 GMP Treg banking: pure population or isolation from thawed CBMC?

From the fresh WB samples a slightly lower TNC recovery was observed in the thawed $CD25^+$ cells (0.07 ± 0.04%) compared to the cells isolated from thawed CBMC (0.08 ± 0.06%). A similar pattern was noted when assessing the CD25 yield (1.12 ± 0.68% thawed CD25 recovery against 1.22 ± 0.36% thawed CBMC recovery). The $CD25^+$ cells isolated from thawed CBMC also had higher viabilities and lower levels of apoptosis compared to the thawed $CD25^+$ cells (p=0.0159 for both viable and apoptotic cells).

The same pattern of higher viabilities and functional abilities in the $CD25^+$ cells isolated from thawed CBMCs over the cryopreserved pure $CD25^+$ cell population was observed in the research grade MBio isolations shown in §6.2.3.3.

6.3 Discussion

This Chapter aimed to improve isolation and cryopreservation techniques for a pure cell population from CB allowing their long term storage within the Biobank. Tregs were chosen as these cells are crucial for the homeostasis of the immune system through the suppression of immune cells and have therapeutic applications during transplants and in the treatment of autoimmunity.

Tregs from CB and PB sources are known to have different properties. Therefore, the first aim of this Chapter was to assess the differences in phenotype and functional activity between Tregs from these two sources to ensure the CB cells are a suitable product for the Biobank. A difference in CD25 expression was observed between CB and adult samples with a larger proportion of $CD4^+$ cells expressing the CD25 marker in the adult samples. Within these adult $CD25^+$ populations two subsets were observed; a larger $CD25^{int}$ and smaller $CD25^{high}$ (p=0.0001). However, only the $CD25^{high}$ cells showed high levels of expression of the intracellular marker FoxP3 (p<0.001) and low levels of the cell surface marker CD127 (p=0.002) compared to $CD25^{int}$, suggesting that these cells possess a regulatory phenotype. This correlates with Baecher-Allen *et al* who demonstrated that the $CD25^{high}$, but not $CD25^{int}$, population was able to suppress proliferating T cells *in vitro*.[242] The $CD25^{int}$ population is believed to be recently activated or memory T cells and have been found to express CD45RO and the $CD25^-$ population are naïve or resting T cells.[238,483] As antigen stimulation is scarce in CB, the $CD25^{int}$ or memory population is much smaller, therefore, a more distinct $CD25^+$ population with high expression can be observed. This population was found to poses a regulatory phenotype through expression of low levels of CD127 and high levels of FoxP3, although the intensity of this intracellular marker was reduced compared to adult $CD25^{high}$ cells. It has been shown that naïve $CD45RA^+$ Tregs have a lower FoxP3 intensity than memory $CD45RO^+$ cells,[484] therefore, the lower levels of intensity observed in this study could be linked to the lower levels of $CD45RO^+$ cells found in CB.[485]

Due to high intracellular ATP concentrations,[292] if a cell is damaged large amounts of ATP are released inducing a proinflammatory response.[486,487] CD39 plays an immunoregulatory role through the removal of extracellular ATP, converting it to AMP thereby preventing inflammation.[290,295] CD39 is constitutively expressed in mouse $CD25^+$ cells but only in a subset of human adult Tregs.[299] In this study the majority of $FoxP3^+$ cells expressed CD39, these $CD39^+$ cells were also found to have a higher intensity of FoxP3 expression than the $CD39^-$ Treg subpopulation ($p<0.0001$). However, lower levels of CD39 expression in the Tregs were observed in CB compared to PB ($p=0.0015$) and MB ($p=0.0008$). Borsellino *et al* observed a much larger variation of $CD39^+$ expression in adult $FoxP3^+$ cells (2-60%). They further characterised this population and discovered they had an activated or memory-like phenotype through the expression of the memory markers CD45RO, CCR6 and HLA-DR.[299] The co-expression of activated/memory markers with CD39 accounts for the reduced levels in CB where cells tend to posses a more naïve phenotype.[485]

As cells mature they can change their expression of specific homing receptors.[321] This study has shown the more naïve CB Tregs express higher levels of the lymphoid tissue homing receptor CD62L than the PB cells which tend to contain a larger memory population. In other studies both the $CD4^+CD25^+CD62L^+$ and $CD4^+CD25^+CD62L^-$ subpopulations have been shown to be anergic, to suppress proliferating cells *in vitro*[330,331,333] and can also prevent adoptive transfer of colitis in mouse models.[332] However, only the $CD4^+CD25^+$ $CD62L^+$ population have been shown to protect mice from diabetes[333] and lethal GvHD.[322] Donor $CD62L^-$ T cells do not cause GvHD in mice,[334,335] suggesting this marker may be necessary to enter the site where the allo-reactive T cells cause GVHD.[322] Higher levels of expression of CCR4, which attracts cells to non lymphoid or intestinal tissues,[318] were observed in the PB samples compared to CB ($p=0.0002$). It has been previously reported that expression of these two different trafficking receptors are linked to CD45RA/RO expression.[319] This confirms that the more naïve $CD45RA^+$ CB cells express higher levels of CD62L, whilst the adult cells with a larger population expressing the memory phenotype of $CD45RO^+$ express higher levels of CCR4. This switch in trafficking receptor expression is believed to occur sometime between 18 months

and 3 years of life.[321] Therefore, the reduced CCR4 and increased CD62L expression in CB Tregs demonstrates a more naïve phenotype compared to their adult counterparts.

The presence of persistent paternal antigens poses a unique challenge to the maternal immune system during pregnancy and the mechanism by which the maternal immune system tolerates the foetus is not fully understood. One proposed mechanism is through an increase production of Tregs by both the mother and foetus[243] and low levels of these cells during pregnancy have been linked to preeclampsia[488,489] and recurrent miscarriage.[490–492] Conflicting observations in the frequency of Tregs in the periphery during pregnancy and postpartum have been noted. It has been reported by different groups that frequency of maternal peripheral Tregs increases dramatically in early pregnancy[493,494] and the up-regulation of these cells has been linked to the increasing oestrogen concentrations in both mice[495] and humans.[496] These groups also report that peripheral Treg frequency remains high throughout pregnancy but declines dramatically during labour, although, frequency still remains higher than before the pregnancy.[493,494] However, it has also been reported that the frequency of peripheral Tregs in maternal blood does not increase, instead Tregs are found to be enriched at the maternal-foetal interface,[490,497,498] particularly at the decidua parietalis.[499,500]

In this study although a higher frequency of $CD25^{+}FoxP3^{+}CD127^{low}$ cells within the $CD4^{+}$ population were observed in MB samples compared to the non-pregnant PB group, the difference was not found to be statistically significant. A higher frequency within the periphery during pregnancy could leave both the mother and foetus vulnerable to infection due to the non-specific nature of Treg suppression. Therefore, an increase in Tregs at the maternal-foetal interface rather than in the periphery would promote tolerance without the risk of infection.

The homing marker CCR4 attracts cells to non-lymphoid tissues, such as the skin and lung.[315–317] Within this study, CCR4 was also found to be highly expressed on MB peripheral Tregs. The expression of this homing marker could aid migration of these cells to the maternal-foetal interface through its ligand CCL17, which has been found to be highly expressed in the decidua.[501] The presence of this ligand has also been shown to be important in the migration of $CCR4^{+}$ Th2 cells to the decidua.[501]

A comparison of the suppressive capabilities of Tregs isolated from CB or PB was performed. Wing *et al* reported CB Tregs suppressed $CD25^-$ cells stimulated with myelin oligodendrocyte glycoprotein (MOG), but not staphylococcal enterotoxin B (SEB), whereas PB Tregs suppressed both.[253] Whereas Fujimaki *et al* reported CB Tregs not only showed no suppressive capability when stimulated with anti-CD3/anti-CD28 beads but they proliferated themselves and were therefore, not in an anergic state.[502] Differences in alloreactivity of $CD25^-$ cells from CB and PB have been observed due to an increased frequency of memory cells in PB compared to the more naïve CB cells. This leads to a lower threshold of activation and therefore faster and higher levels of proliferation.[503,504] As third party Tregs have been shown to be equally suppressive as those from the same donor,[430–432] all suppression assays were performed using thawed $CD25^-$ cells from the same PB sample. This allows comparison of Treg suppressive abilities from different samples by ensuring similar levels of proliferation and Treg contamination with the $CD25^-$ fraction. Tregs were isolated with similar $CD25^+CD127^-$ purities of 95.1% and 95.4% from CB and PB respectively. Isolated Tregs, from both sources, were shown to be anergic, as culturing them with anti-CD3 and anti-CD28 did not result in an increase in proliferation as detected by tritiated thymidine uptake. Co-culturing Tregs with stimulated $CD25^-$ cells resulted in lower levels of suppression from the CB Tregs across a range of $CD25^+$:$CD25^-$ ratios. A lower intensity of FoxP3 expression was observed in the CB $CD25^+$ cells compared to those from the adult $CD25^{high}$ population which could account for their reduced suppressive capabilities. Adult $CD25^{high}$ cells were also found to have higher levels of CD39 expression compared to CB cells. Cells expressing this marker were found to have higher levels of intensity of FoxP3 than $CD25^+CD39^-$ cells ($p<0.0001$). It was also observed that the $CD25^+CD39^+$ population, isolated from CB, showed higher suppressive abilities than those that were $CD25^+CD39^-$ ($p=0.0341$). Reduced levels of $CD39^+$ Tregs have been observed in patients with multiple sclerosis and patients with renal allograft rejection, whilst no differences in $CD39^-$ levels were observed.[300,505] This suggests that CD39 plays a regulatory role and may serve as a more specific marker for Tregs. Therefore, the higher levels of CD39 noted in adult Tregs could lead towards their higher suppressive abilities.

There are many reports suggesting that Treg suppression is dependant on cell-cell contact.[351] However, Collison *et al* reported that cell contact was only critical in activating the suppressive function of Tregs and that once activated they could suppress in a contact independent manner.[506] The manner of which Tregs suppress (whether contact dependent or independent) was compared between adult and CB Tregs. Using transwell plates, Tregs stimulated with soluble anti-CD3 and anti-CD28 were either cultured with the target $CD25^-$ cells together in the lower chamber or separated by the transwell membrane. Similar levels of suppression were observed, in both CB and PB samples, between Tregs cultured with the target $CD25^-$ or separated by the transwell membrane. This suggests that Tregs from both sources are able to suppress in a contact independent manner. This result is controversial as the majority of studies suggest the function of nTregs are contact dependent.[351,359] However, there are a number of studies that report Tregs suppress though the release of suppressive cytokines.[507,508] The data obtained in this study could be followed up by assessing if the suppression is due to the presence of suppressive cytokines, such as IL-10 and TGF-β, through experiments that block these cytokines.

Despite their reduced suppressive activity compared to PB cells, CB Tregs are still an attractive product for the Biobank due to their higher frequency and more defined $CD25^+$ population which would facilitate the isolation process. In this study the efficiency of two different isolation techniques were assessed; MACS (of which two protocols were tested) and cell sorting. Comparing the MACS protocols, higher TNC and $CD25^+$ cell recoveries were obtained through the SCT isolations (p=0.0079 and p=0.0087 for TNC and CD25 respectively). Very few $CD25^+$ cells were observed within the negative fraction of MBio isolations, therefore, differences in cell yield may be due to the different techniques of retaining the labelled cells. In the SCT protocol the cells are held within a magnetic field surrounding a tube whilst the unlabelled cells are poured away, whereas in the MBio method the cells are passed through a column where some may remain despite repeated purging after washing the unlabelled cells off. The cells isolated using the SCT protocol had lower CD4 (p=0.03) and CD25 (p=0.03) purities within the CD45 population. SCT cells also had lower expression of FoxP3 and $CD127^{low}$ within the $CD25^+$ population than the MBio cells. Contaminating cells were identified as monocytes and

B cells. These reduced purities resulted in significantly lower suppressive abilities from the SCT isolated cells (at Treg:$CD25^-$ ratios of 1:1 p=0.0109, 1:2 p=0.0025 and 1:5 p=0.0083).

MACS techniques enrich the positively labelled population regardless of expression levels, therefore, an advantage of sorting cells is the ability to gate and isolate the populations of interest. This is particularly useful when isolating Tregs as the $CD25^{high}$ population can be targeted specifically. Compared to the MBio technique, the cell sorting protocol resulted in slightly lower TNC and $CD25^+$cell recoveries. However, the cell sorted isolations had higher CD4 and CD25 purities within the CD45 population and higher suppressive capabilities, however, these differences were not found to be significant. One disadvantage of using cell sorting to separate rare populations is the amount of time it can take to sort through a large sample, this leaves cells exposed to the FACS sheath fluid for long periods of time reducing viability. In an attempt to reduce sorting time, the cells were enriched for CD4 using MACS prior to CD25 isolation. This would account for the higher CD4 purities compared to the single isolation step MBio protocol. Within the $CD4^+$ cells, the cell sorted isolations resulted in a slightly lower mean $CD25^+$ purity compared to MBio. Despite attempts to reduce the sorting time, the sorted cells had lower viabilities (mean of 75.25%) compared to MBio (86.91%).

Overall, the MBio protocol has been shown to be the most efficient. These isolated cells had high Treg purities of 81.73% $CD25^+$ within $CD45^+$ cells (these purities were significantly higher than SCT) and expressed high levels of FoxP3 and low levels of CD127 (89.63% of $CD25^+$ cells were $FoxP3^+$ and 98.47% were $CD127^{low}$). High Treg purities were observed in one separation step from CB with no further purification needed, unlike isolations from PB which can require CD8, CD19 and CD127 depletion depending on the level of purity needed.[419] For example, high levels of purity are essential when expanding cells as the conditions used tend to favour proliferation of non suppressive T cells.[418] These cells had high levels of viability 86.91% and significantly higher levels of suppression than SCT isolations. Although these assessments were lower than those observed when using the flow cytometric cell sorting protocol, the differences were not found to be significant between these two isolation methods. The flow cytometric cell

sorted isolations required two stages; CD4 enrichment by MACS followed by CD25 isolation. This results in a long process putting the cells under strain and resulted in lower viabilities compared to MBio (which required only one isolation step). Therefore, the MBio protocol was found to be the most efficient for Treg isolation.

Due to the high levels of purity, viability and suppressive capabilities of the MBio isolated cells, this method was used prior to cryopreserving $CD25^+$ cells. Cryopreserving the isolated $CD25^+$ cells resulted in a mean post-thaw TNC yield of 82.84%. These cells maintained their expression levels of CD4, CD25, FoxP3, CD127 and CD39. The $CD25^+$ cells had significantly lower suppressive abilities post-thaw compared to the fresh analysis (p=0.0313). Peters *et al* reported that cryopreserved adult Tregs required expansion post-thaw in order to regain functional capabilities.[419] This post-thaw expansion was found to be unnecessary by Mavin *et al* as thawed Tregs were able to suppress $CD8^+$ cells in an MLR as efficiently as the pre-thaw assessment and were able to protect GvHD target tissue.[509] However, the reduced suppressive ability of the thawed CB Tregs in this study is likely to be due to the reduction of the percentage of viable cells (76.46%) and increase in apoptosis (23.25%).

Cryopreservation of WB or CBMC resulted in a reduction of Treg frequency of $CD4^+$ T cells from 4.66% to 3.94% and 3.40% to 3.01% in WB and CBMC respectively. This had only been previously reported in PB studies by Elkord *et al* who found the frequency of PB Tregs to be significantly reduced post-thaw.[425] In this study although post-thaw CB Treg frequency was reduced, it was not found to be significantly lower than the pre-freeze levels. The significant reduction that Elkord *et al* reported could be in-part due to their freezing protocols which involved resuspending PBMCs directly into FBS containing 10% (v/v) DMSO rather than resuspending the cells before the slow addition of pre-cooled DMSO. Viability assessment of these samples showed a reduction in the percentage of viable cells in the post-thaw WB samples, whereas in the CBMC samples similar levels of viable and apoptotic cells were observed between fresh and thawed cells. A similar pattern was observed in Chapter 6 (§5.2.1) where numbers of viable $CD45^+$ and $CD34^+$ cells were lower when cryopreserving WB rather than the granulocyte depleted CBMCs.

As the frequency of Tregs in cryopreserved CBMCs were conserved post-thaw, the ability to isolate Tregs from these samples was assessed using the MBio and SCT protocols. As with the fresh isolation, the SCT method resulted in higher yields of TNC and CD25 compared to MBio (p=0.0223). However, unlike the fresh isolations, the two methods resulted in cell products with comparable purities. Despite the similarities in purity, the suppressive abilities of the SCT isolation were significantly reduced compared to MBio (at $CD25^+$:$CD25^-$ of 1:1 p=0.014, 1:2 p=0.0104, 1:5 p=0.0137, 1:10 p=0.0285 and 1:20 p=0.0285).

Biobanking requires potentially long term storage of samples, this can be achieved through cryopreservation. Within this study two banking options were assessed. The first involved isolation of CD25 from fresh cells and their subsequent cryopreservation. Upon thawing these cells maintained their expression of Treg markers CD25, FoxP3 and low levels of CD127. However, losses in cell viability and suppressive potency (p=0.0313) were observed. The alternative approach to Treg banking involves maintaining the cells as part of the CBMC fraction during cryopreservation and isolating the cells of interest post-thaw. Isolating Tregs from thawed CBMC resulted in lower TNC and $CD25^+$ cell recoveries from the starting fresh WB sample compared to fresh isolations (p=0.0238 and p=0.0341 for TNC and CD25 respectively). However, calculating the TNC yield from the CBMC fraction prior to isolation results in higher recoveries in the thawed isolations (p=0.0238). Therefore, the differences in recovery from the fresh WB samples occur during the cryopreservation of CBMC (p=0.0476). Compared to fresh isolations, higher purities of CD4 (p=0.0043) and CD25 (p=0.0087) in the leucocytes were observed, this in combination with higher viabilities led to higher levels of suppression of proliferating $CD25^-$ cells. Therefore, once the cell losses associated with the cryopreservation of CBMCs were taken into account, the use of thawed CBMCs was found to be an efficient isolation strategy to obtain a pure, functional Treg population. Between the two types of thawed Tregs, the cells maintained as CBMCs during cryopreservation resulted in higher $CD25^+$ cell recovery (p=0.0049). These cells also had higher viabilities, less apoptotic cells and therefore, higher levels of suppression (p=0.0012). Therefore, the most efficient CB Treg banking procedure involves the storage of CBMCs followed by a $CD25^+$ cell

isolation post-thaw, unless a set cell dose is required in which case cryopreservation of pre-isolated Tregs may provide a better predictor of post-thaw cell dose.

The TNC yield from GMP grade fresh CD25 isolations was found to be slightly higher than the research grade MBio isolations (0.13% and 0.09% for CliniMACS and MBio respectively). However, the percentage of $CD25^+$ cells in the $CD45^+$ population was reduced (76.84% versus 81.73%). This led to a lower $CD25^+$ recovery of 5.08% (compared to 8.37% for MBio isolations). Despite the lower levels of CD25 purity, these cells had high levels of FoxP3 expression (82.88% of $CD25^+$ cells), high viability (94.8%) and were found to be as equally suppressive as the MBio cells.

The two banking options, performed on the MBio isolations, were also assessed on the GMP grade separations and a similar pattern was observed. Although cryopreserving the isolated $CD25^+$ population resulted in a reduction in viability (p=0.06) and slightly lower suppression post-thaw, they did not require expansion as recommended for cryopreserved adult Tregs by Peters *et al.*[419] As observed in the MBio isolations, GMP isolation of $CD25^+$ cells from thawed CBMC resulted in higher viabilities (p=0.0159) and higher suppressive abilities than the cryopreserved $CD25^+$ population.

In summary, CB Tregs showed phenotypic and functional differences to their adult counterparts. The $CD25^+$ population within the $CD4^+$ cells appear as a distinct population in CB of which the majority co-express FoxP3 and low levels of CD127. The $CD25^+$ population is much larger in adult $CD4^+$ cells but mostly consists of activated non suppressive T cells, only the $CD25^{high}$ cells had a regulatory phenotype ($FoxP3^+$ $CD127^{low}$). CB Tregs were found to be less suppressive *in vitro* than adult cells, this is in part could be due to the differences in intensity of FoxP3 and levels of CD39 expression observed. Due to the lower levels of activated non suppressive $CD25^+$ cells, CB Tregs can be isolated from mononuclear cells in a single step, with no further purification as required by adult Tregs. These cells can be banked, either as research or clinical grade products, as a pure isolated population. Alternatively, Tregs can be cryopreserved as part of the CBMC fraction and isolated post-thaw. Post-thaw, using either banking procedure, these cells retained high levels of CD25 and FoxP3 expression. However, cells isolated

from thawed CBMC samples had higher viabilities and suppressive capabilities than those cryopreserved as a pure population.

CHAPTER 7

DISCUSSION

Anthony Nolan Cell Therapy Centre (ANCTC) currently receives an average of 393 cord blood (CB) units to the bank every month (calculated from the last 10 months: April 2013 to January 2014). Of these units, an average of only 22.54% were found to be suitable for potential use in haematopoietic stem cell transplants (HSCT) and banked for that purpose. The majority of samples are considered to be unsuitable due to the quality thresholds put in place at ANCTC such as a minimum total nucleated cell (TNC) count of 1.2×10^9 and a minimum $CD34^+$ cell count of 3.2×10^6. The consent obtained from the mother prior to collection allows for these samples to be used for other purposes such as research. Therefore, instead of discarding these samples ANCTC aims to create a Biobank storing CB either as whole blood, mononuclear cells (CBMC) or pure cell fractions allowing the distribution of these samples to researchers around the globe. This study aimed to aid ANCTC in creating this Biobank through the optimisation of the procedures required for the isolation and storage of CB fractions. For the purpose of this study, the procedures were broken down into four sections; transportation of fresh CB from the collection site to the bank, CBMC isolation, cryopreservation of CBMC and the isolation and banking of pure cell populations. Throughout each section the quality of the cells were monitored to ensure they maintained viability and functional abilities throughout the whole banking process from collection to thawing.

ANCTC has different maternities collecting CB samples situated across the UK. Due to transportation times there is a delay between the collection of a sample in the maternity and its cryopreservation at the bank. To ensure the CB samples arrive at the bank with the highest level of potency, Chapter 3 assessed the impact of two different storage temperatures on fresh CB, the effects of time on cell potency and the effects of fresh storage on the recovery of cells post-thaw after delaying cryopreservation. This section of the study is of benefit to both the Biobank and the HSCT bank but also assessed the effects of fresh storage prior to cryopreservation on two other sources of haematopoietic stem cells (HSC): bone marrow (BM), and mobilised peripheral blood stem cells (PBSC). Due to differences between studies in the storage temperatures tested and in the methods of assessing cell potency, there is no consensus in the literature regarding the optimal fresh storage conditions for the three sources of HSCs. For example, regarding fresh storage temperature many studies indicate CB should be maintained at refrigerated temperatures in order to avoid significant reductions in cell recoveries.[441–446] In contrast there are studies reporting that cell recovery is optimum at ambient temperatures,[54,438,439] whereas other studies report no significant difference in cell recovery if cells are stored at 4°C or 25°C, although losses are reported at 37°C.[19,440] Similarly for BM, most studies report higher recoveries whilst stored at refrigerated temperatures[449,450,452] however, Antonenas *et al* found no difference in $CD34^+$ cells over 72 hours between storage at 4°C or room temperature.[453] PBSC samples are reported to have higher cell viabilities when stored at cooler temperatures.[453,455,457] As discussed in Chapter three the effects of two different storage temperatures (refrigerated temperatures, 4-8°C and room temperature, 19-22°C) were assessed on fresh CB, PBSC and BM and the effects these temperatures had prior to cryopreservation. Viable $CD45^+$ (assessed by exclusion of $7AAD^+$ cells) and $CD34^+$ cell (assessed using 7AAD and Annexin V) in addition to CFU recoveries were assessed. Higher recoveries were noted in samples maintained at refrigerated compared to room temperature with significant differences observed in $CD45^+7AAD^-$ cell recovery after 72 hours in CB and PBSC; in $CD34^+7AAD^-$ after 48 hours in CB and 72 hours in PBSC and BM; and significant differences in CFU recovery after 48 hours in CB and BM.

As refrigerated temperatures were found to be optimal for maintaining viable and functional cells, the effect of fresh storage time and delaying cryopreservation was analysed for samples stored at this temperature for up to 72 hours. Continuous reductions in viable cell and CFU recoveries were observed in the HPC samples as time progressed. Compared to t=0, pre-freeze analysis of CB samples showed significant reductions after 24 hours in $CD34^{+}7AAD^{-}$ cells and after 48 hours in CFU recovery. Significant losses in viable $CD45^{+}$ and $CD34^{+}$ cells were observed after 48 hours of fresh storage of PBSC. BM samples also lost significant numbers of viable $CD34^{+}$ and CFU after 48 hours.

Post-thaw analysis highlighted a loss in viable and functional cells when cryopreservation was delayed. For CB samples significant losses were observed, compared to cryopreservation at t=0, when freezing had been delayed for 48 hours in CFU recovery and in $CD34^{+}7AAD^{-}$ cells after 72 hours. This is in agreement with studies reported by Campos *et al*[438] and Hubel *et al*[442–444] who report that cryopreservation of CB can be delayed for 24 hours with minimal losses in progenitor cells but refutes other studies in which no significant losses were observed for as long as 72 hours.[441,446,447] Significant post-thaw losses were observed in viable $CD34^{+}$ cells and CFUs after 48 hours in PBSC samples contradicting other groups who report PBSC samples maintained viable cell and CFU recoveries for as long as 72 hours after collection.[454,455] In the assessment of BM samples, significant losses in post-thaw CFU recovery were observed after a 24 hour delay to cryopreservation with viable CD45 and CD34 losses not observed until a delay of 72 hours.

Based on these data, samples sent to the ANCTC are now maintained in a cooled environment within the temperature range of 2°C to 15°C. If this temperature range is not met during transportation, samples are processed as normal but with additional viability tests post-thaw to ensure the cells maintained an acceptable level of potency. The ANCTC aims to process and cryopreserve all CB units designated for HSCT banking within 24 hours of collection. However, this is not always possible as samples are collected 24 hours a day but to avoid high costs, courier runs between the maternity and ANCTC occur once a day. Therefore, to avoid the CFU losses observed after 48 hours ANCTC

has set a maximum time for cryopreservation to 36 hours after collection; samples that cannot be cryopreserved within this timeframe can still be stored in the Biobank.

The isolation of CBMCs is often the first step in many experiments and when isolating pure cell populations. The Biobank aims to store some CB samples as CBMC fractions, therefore, the second area of investigation was to optimise the CBMC isolation protocol employed at ANCTC. A common procedure for isolating CBMCs is through the Ficoll Density Gradient Method (ficoll).[467–469] This involves layering blood over ficoll, a polysaccharide with a specific density, followed by centrifugation resulting in the separating of different fractions of the blood according to their density. This is a time consuming process with low reproducibility and high user variability. Biosafe, the company that produces the SEPAX machines routinely used at the ANCTC to reduce the volume of CB samples designated for HSCT banking, developed a program (eMNC) on the SEPAX device with the aim of isolating CBMCs and replacing the ficoll protocol. This new protocol would separate the CBMC cells based on their density through centrifugation without the need to add density gradient media. The eMNC protocol is fully automated, therefore, the low reproducibility and high user variability associated with ficoll should be reduced whilst freeing the technician to perform other duties. Another potential advantage of this new method is a reduced risk of contamination as the separation is performed within a sealed sterile tubing set without the need to add exogenous material such as the density gradient media. As part of a collaboration with Biosafe the cell fractions obtained from this new protocol were compared with ficoll-derived CBMCs to assess if this method can be optimised and become a replacement for ficoll based isolations. Three different versions of the eMNC program were assessed. Compared to ficoll-derived CBMCs the cell products had similar mononuclear cell recoveries but lower levels of granulocyte and erythrocyte depletion. Isolation using ficoll separates cells due to their density with larger granulocytes and aggregated erythrocytes falling below the layer of ficoll whilst the cells of interest remain above the density gradient media. As the eMNC does not have this layer of ficoll, the cells of interest remain directly on top of the unwanted denser cells, this makes it difficult to remove the CBMCs whilst avoiding contaminating cells. Low granulocyte depletion led to reduced post-thaw viabilities when cryopreserving the cell fractions isolated using eMNC. CBMC enrichment

is often the first step taken when isolating pure cell populations, therefore, the ability to isolate $CD133^+$ cells from eMNC and ficoll products were compared. The CD133 fraction isolated from the eMNC-derived CBMC samples had lower cell recoveries, purity and viabilities. This was due to difficulties during the isolation such as formation of a loose pellets after centrifugation and trapping other cells within the separation column resulting from the contaminating erythrocytes. Therefore, despite attempts by Biosafe to optimise the program, the eMNC cellular products were inferior to those using ficoll. As the new protocol was not found to be a suitable replacement to the ficoll method, Biosafe decided to discontinue the development of this program.

As the ficoll procedure remains the most efficient method of isolating CBMCs at the ANCTC, attempts were made to further optimise the process. The first of which assessed if it was necessary to dilute the blood prior to layering over ficoll. This is often performed to reduce the density of the blood and cell concentration facilitating the layering step and preventing mononuclear cells from becoming trapped amongst the aggregating erythrocytes.[469] Lower levels of granulocyte depletion were observed when samples were diluted prior to separation. Therefore, leaving samples undiluted was beneficial to CBMC isolation whilst also reducing the number of tubes required, making the process quicker to perform and more economical. The ficoll process performed at ANCTC uses standard 50ml conical bottom tubes, specialised tubes have been developed to facilitate the ficoll procedure such as the Leucosep and Sepmate tubes. These tubes have a physical barrier separating the ficoll from the blood allowing easier layering and removal of the desired cellular fraction post-centrifugation. The CBMC products obtained when using these two tubes were tested against that from the standard tube without the barrier. The CBMC product obtained from the standard tube had lower levels of granulocyte contamination than the Leucosep tubes and higher mononuclear cell recoveries compared to the SepMate tubes. Therefore, the optimal ficoll procedure for CBMC isolation from involves undiluted CB with the use of the more economical standard tubes.

The Biobank involves cryopreservation and potentially long term storage of CB fractions until they are required. Therefore, the third stage of the Biobank development, as described in Chapter 5, was to improve on the methods used during the freezing

and thawing of the cells. Cryopreservation of cells requires a cryoprotectant to prevent damage to the membrane through the formation of ice crystals and changes in osmotic pressure.[101] Dimethyl sulphoxide (DMSO) is commonly used as a cryoprotectant when freezing the different sources of HSCs and can be used at a range of concentrations from 2.2 to 20%.[102] Higher concentrations may increase the cryoprotective effect,[148] however, there is conflicting evidence as to whether DMSO is toxic to cells[122] or not.[123,124] In this study the toxicity of DMSO was assessed at three different stages of cryopreservation: when added to fresh CB, if freezing is delayed after DMSO addition and in post-thaw samples. A dose dependant toxicity of DMSO to fresh CB cells was observed with the addition of 40% removing almost all viable and functional cells immediately after addition. Possible mechanisms of DMSO toxicity include the denaturation of proteins caused by hydrogen bond formation with the cryoprotectant[127,128] or through the induction of apoptosis by interacting with programmed cell death receptors and their ligands.[125,126] However, losses of viable cells may also be due to the exothermic reaction between DMSO and water, a large increase in temperature was noted when high concentrations were added despite attempts to maintain cells at 4°C. The addition of DMSO to a final concentration of 10% (v/v) was not found to have any significant effects on cell viability or functional abilities for up to 2 hours in fresh CBMC samples or post-thaw when freezing had been delayed for up to 1 hour after addition of the cryoprotectant. This furthers the studies by Rowley *et al* who report HPCs can tolerate 10% (v/v) DMSO for up to an hour[123] but disagrees with Douay *et al* who found significantly reduced CFU recoveries after 30 minutes of exposure.[122] In order to reduce any toxic effects of DMSO, it has been suggested to maintain samples at refrigerated temperatures.[55,122] However, in this study no significant differences were observed between CBMC samples with 10% (v/v) DMSO stored at refrigerated or room temperature for up to 24 hours. Post-thaw DMSO is commonly washed from samples to avoid its toxic effects to cells already weakened by the cryopreservation process.[55,105–107,161] However, washing cells can be time consuming and can result in poor cell recoveries as clumping can occur during centrifugation.[55,155,159] To prevent this, DNase is often added to the wash buffers.[148,160,161] Within this study washing CBMC samples post-thaw resulted in an average loss of 13.7% $CD45^+$ cells. Once these losses had been taken into account,

CBMC samples that were un-manipulated post-thaw and remained in 10% (v/v) DMSO, resulted in significant reductions in CFUs after one hour. These losses occurred at a faster rate than reported by Regan *et al* who observe the recovery of CFUs to be stable for up to 2 hours after thawing.[510] However, a recent study by Huang et al reports significant CFU losses occurred as soon as 30 minutes post-thaw.[511] Dilution of thawed CBMC samples, reducing the DMSO concentration to 5% prevented significant CFU losses until 24 hours whereas the complete removal of DMSO delayed the observation of significant CFU losses until 48 hours post-thaw.

A wide range of DMSO concentrations are used to cryopreserved HSCs for transplantation (from 2.2 to 20%) with 10% the most commonly used.[102] As DMSO has been shown to be toxic to cells as well as patients[105–107] in a dose related manner, various studies suggest lowering the concentrations to 5%.[140–142] However, lowering concentrations will also have an effect on the ability to cryopreserve viable cells and CFUs. Despite minimal cell toxicity observed at DMSO concentrations of 10%, the effect of further reducing the concentration was assessed. Although 5% has been reported to be an alternative concentration when cryopreserving CB,[140] significantly reduced viable $CD45^+$ and $CD34^+$ cell recoveries were observed in this study. The optimum DMSO concentration was found to be within the range of 7.5% to 10%. This range of DMSO concentrations has also been reported to be optimal for the cryopreservation of PBSC samples.[147]

Previously, only an observation from preliminary experiments had been reported stating that 1% (w/v) dextran-40 in combination with 10% (v/v) DMSO results in a moderate improvement in post-thaw leucocyte viability,[55] however this data has never been published. Based on this statement, many CB banks have added dextran-40 to their cryoprotective solutions. This study is the first to show that the presence of dextran-40 not only results in an increase in leucocyte viability but also better preserves the viability and potency of CD34+ HPCs. This study has also confirmed that 1% (w/v) dextran-40 is the optimal concentration when cryopreserving CB cells in 10% DMSO.

The Biobank requires the ability to store pure cell fractions isolated from CB. For the purposes of this study regulatory T cells (Tregs) were chosen. These cells play a vital role in maintaining homeostasis of the immune system through the promotion

of immunological tolerance.[243–247] Due to their immuno-suppressive capabilities, these cells have the potential to be used therapeutically to prevent unwanted immunological reactions such as in autoimmune diseases[389] and allogeneic transplants.[404,417]

Isolation strategies for Tregs are often based on their expression of the α chain of the IL-2 receptor (CD25). This marker is also upregulated in activated and memory T cell populations. Therefore, adult peripheral blood (PB) samples contain a high frequency of $CD25^+$ cells but only those expressing the highest levels co-express FoxP3 and can be considered regulatory. This leads to a practical problem when isolating Tregs from PB as further isolation steps are required to remove the non-regulatory contaminating $CD25^{int}$ cells. CB cells have been shown, in this study and others,[483,512] to have a more defined $CD25^+$ regulatory population due to the decreased opportunity for CB T cells to become activated through encountering antigens. This allows for easier isolation of these cells however, it has been suggested that CB-derived Tregs are not mature enough to be suppressive.[513] Therefore, when assessing the feasibility of banking CB Tregs at ANCTC, the first aim is to compare these cells against their adult counterparts to ensure they possess a regulatory phenotype and function.

The frequency of Tregs (identified with the phenotype $CD25^+FoxP3^+CD127^{low}$) within $CD4^+$ T cells was compared between CB and PB samples. Higher levels of 5.67% of CB $CD4^+$ cells co-expressed the Treg markers compared to 2.33% in PB. This is in agreement with Wing *et al* and Godfrey*et al* who also observed higher frequencies within CB.[483,512]

CB and PB Tregs were compared for expression of antigens associated with suppressive cell function. Differences were observed in the expression of CCR4, which is involved with homing of cells to non-lymphoid tissues. Higher levels of CCR4 expression were observed in PB Tregs compared to CB cells (p=0.0002). Expression of this marker has been linked to the activation status of a cell and memory cells, expressing $CD45RO^+$, tend to have higher levels of this homing marker.[319,321] Since PB Tregs have been shown to be predominantly $CD45RO^+$[514] this could account for their increased levels of CCR4 compared to CB cells which instead possess a naïve $CD45RA^+$ phenotype.[483]

Differences in CD39 expression were also observed between CB and PB Tregs with higher levels found in adult cells (p=0.0015). This marker has been linked to the suppressive

function of Tregs through the conversion of pro-inflammatory extracellular ATP into AMP.[290,295] As with CCR4 this marker has been linked to a memory phenotype of CD45RO, CCR6 and HLA-DR[299] which could explain why lower levels were observed in CB Tregs. In order to assess if this marker played a regulatory role in CB Tregs, the $CD39^+$ and $CD39^-$ subpopulations were isolated from $CD25^+$ CB samples. Higher intensity of FoxP3 expression was observed in the $CD39^+$ subpopulation along with higher levels of suppression (p=0.0341 at a Treg:$CD25^-$ ratio of 1:1). This confirms that CD39 is linked to the suppressive function of CB Tregs.

The immuno-suppressive capabilities of Tregs isolated from CB and PB sources were compared. Cells from both sources were found to be anergic when stimulated with anti-CD3 and anti-CD28, confirming the observations made by other groups.[236,359] When co-cultured with stimulated $CD25^-$ cells, PB Tregs were found to be more efficient at suppressing proliferation (although differences were not found to be significant). As previously mentioned, lower intensity of FoxP3 and lower levels of CD39 expression were observed in CB Tregs. These two markers are linked with regulatory function and could, in part, explain why CB Tregs showed lower levels of suppression.

Despite their lower levels of suppression compared to PB Tregs, CB cells are still an attractive cellular population for the Biobank due to their higher frequency within $CD4^+$ cells and more defined CD25 population. As Tregs are isolated based on CD25 expression, separation from CBMCs can be achieved in a single CD25 enrichment step[429] as opposed to the problems with contaminating activated and memory $CD25^+$ cells when isolating from adult sources which require further isolation steps to be removed.[419] Within this study, three different isolation techniques were compared; flow cytometric cell sorting and two MACS methods (MBio and SCT). A comparison of the two MACS methods resulted in lower yields for MBio (p=0.0087) however, the cells isolated had higher levels of purity (p=0.03) and suppressive capabilities (p=0.0028). Isolation of CD25 using flow cytometric cell sorting resulted in cells with equally high levels of purity compared to the MBio protocol but these cells had higher suppressive capabilities despite lower viabilities. To avoid extended sorting times which can reduce viabilities further, $CD4^+$ cells were isolated from CBMCs prior to flow cytometric cell sorting

$CD25^+$ cell separation. Therefore, although these isolated Tregs had higher levels of suppression than those isolated using MBio, it was decided that this MACS method was the most efficient. An alternative Treg isolation approach is through the depletion of $CD49d^+$ and $CD127^+$ cells from a $CD4^+$ enriched population.[515] This leads to the isolation of 'untouched' Tregs which would be desirable for immunotherapeutic applications. This isolation strategy was not assessed during this study but should be considered in future investigations at ANCTC.

Two strategies for banking Tregs were assessed; cryopreservation and banking of isolated pure population or banking of CBMCs with a view to isolate the cells of interest post-thaw. Cryopreserving isolated Tregs resulted in high cell recovery post-thaw. However, a reduction in viability led to reduced suppressive ability compared to pre-freeze assessment (p=0.0313). The assessment of the effects of cryopreservation on Tregs could be furthered by assessing if the mechanism of suppression changes post-thaw through transwell assays or blocking cytokines. The cryopreservation of adult mononuclear cells has been shown to result in a significant reduction of Treg frequency in $CD4^+$ T cells post-thaw.[425] However, only a slight reduction was observed when cryopreserving CBMCs (reduced from 3.94% pre-freeze to 3.01% post-thaw). As the loss during CBMC cryopreservation was low, the ability to isolate Tregs post-thaw was assessed. $CD25^+$ cells were isolated from thawed CBMCs with similar purities to those observed during fresh isolations. Higher viabilities and suppressive capabilities were observed compared to post-thaw pure $CD25^+$ pops (p=0.0012 at a Treg:$CD25^-$ ratio of 1:2). Therefore, the most efficient banking procedure for Tregs was found to be through the cryopreservation and storage of CBMCs with the cells of interest isolated post-thaw.

For their use in therapeutic applications Tregs would need to be isolated under GMP grade conditions. Therefore, the two banking options were assessed using CE marked reagents and a CliniMACS cell separation device. The fresh isolation of Tregs using the CliniMACS resulted in product with lower levels of purity compared to research grade MBio protocol but these cells were found to be equally suppressive. As observed in the research grade isolations, cryopreservation of these cells led to lower post-thaw viabilities and suppressive capabilities compared to cells isolated from thawed CBMCs.

Therefore, Tregs can be stored within the CBMC fraction in the Biobank and isolated post-thaw using research or GMP grade protocols resulting in a cell product with high purity, viability and suppressive abilities.

For their use therapeutically, large numbers of Tregs would be required. As only a small number can be obtained from an individual CB unit, to reach the doses required these cells are often expanded using anti-CD3, anti-CD28 and high doses of IL-2. Tregs are capable of expansion after cryopreservation, as demonstrated by Peters *et al* who restored the suppressive activity of post-thaw PB cells.[419] Therefore, assessing the ability to expand post-thaw CB-derived Tregs or to cryopreserve expanded cells whilst ensuring the cells maintain suppressive phenotype and activity could be assessed at ANCTC.

7.1 Further work

In this study Tregs were focussed upon when considering banking of specific cell population. However, the Biobank would require the storage of different cell populations found within CB, for instance HPCs and natural killer (NK) cells.

The potential to bank HPCs was touched upon in Chapter 4 when CD133 cells were isolated from CBMCs resulting in purities of 88.98%. However, the effect of cryopreserving these cells was not assessed in this study.

NK cells are cytotoxic lymphocytes, which form part of the innate immune system, responding rapidly to virally infected and tumour cells. These cells are defined as $CD3^{-}CD56^{+}$ cells and comprise 15-30% of lymphocytes in CB.[516,517] These cells were named natural killer as it was initially believed that they did not require activation to become functional.[518] These cells recognise tumour and infected cells through different mechanisms such as the down regulation of MHC class I molecules on target cells[519] or the upregulation of NKG2D ligands on distressed cells.[520] NK cells mediate their cytotoxic effects through the release of cytoplasmic granules, such as perforin and granzymes[521] or are able to mediate apoptosis through the death receptor.[522] NK cells also recruit other immune cells through the secretion of cytokines such as IFN-γ and

TNF-α.[523] Due to the increase in incidence of tumour resistance to drugs and radiation therapy, investigations into the use of immunotherapies are increasing. NK cells have been shown to kill leukaemia cells *ex vivo*[524] and are believed to be the main effector cell of the graft-versus-leukaemia (GvL) effect observed post-HSCT. Current clinical trials involving NK cells are assessing the tolerable does (from 1×10^6 to 1×10^8 cells) that can be administered after HSCT[525,526] whilst assessing any improvement in the GvL and graft-versus-host-disease (GvHD) effect.[527] These cells can be isolated through positive selection of CD56 cells or preferentially untouched NK cells can be obtained through the depletion of T cells, B cells, dendritic cells, stem cells, monocytes, granulocytes and erythroid cells using a cocktail of magnetic bead-labelled antibodies.

7.2 Concluding remarks

In summary, this study has laid the foundations for the creation of a Biobank at ANCTC. This has been achieved by optimising the different stages of the banking process, starting with the effects of transport conditions and delaying cryopreservation. No other study has evaluated all three sources of HPCs in the same lab, with the same quality variables. This is becoming increasingly important as more centralised facilities for cell processing are being developed. Within this study, it was observed that HPC products are better maintained at refrigerated temperatures prior to cryopreservation, as well as highlighting the importance of minimising delays to freezing in order to avoid losses in cell potency. Cryopreservation is an important part of biobanking and allows the long term storage of cells. However, suboptimal cryopreservation techniques can result in a loss of cell viability and functionality. This study assessed the toxic effects of the cryoprotectant DMSO at different stages of cryopreservation and it was observed that when handled correctly the detrimental effects of DMSO to the cells can be minimised allowing high post-thaw recovery of potent cells. In addition, this is the first study to show that the presence of 1% (w/v) dextran-40 in the cryoprotectant solution is crucial to maintaining CB cell potency post-thaw. Finally, Tregs were chosen to assess the ability of the Biobank to store specific cell populations. Comparing CB Tregs to their adult counterparts, a reduced suppressive ability was observed in CB cells which also

exhibited decreased FoxP3 intensity and lower levels of CD39 expression. However, a higher frequency of Tregs was noted in CB samples with fewer contaminating $CD25^+$ non-Treg cells facilitating the isolation process. Two banking strategies were assessed, the more efficient protocol was found to be isolating Tregs from banked CBMCs rather than banking pure Treg populations. These banking processes were adapted to become GMP compliant allowing distribution of these cell products not only for research purposes but also potentially for their use in immunotherapeutic interventions.

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Communications resulting from this study

Publications

Avoiding room temperature storage and delayed cryopreservation provide better post-thaw potency in hematopoietic progenitor cell grafts. **L.J. Fry**, S.G. Querol, S.G. Gomez, M. Green, S. Anderson, J. Horder, S. McArdle, R. Rees, and J.A. Madrigal. *Transfusion*, 53(8):1834 - 1842, 2013

In preparation

Assessing the toxic effects of DMSO on cord blood to determine exposure time limits and the optimum concentration for cryopreservation.

Abstracts

Impact of time and temperature on cord blood graft potency after thawing. **L.J. Fry**, S.G. Gomez, S.G. Querol, S. Mcardle, R. Rees and A. Madrigal. *World Cord Blood Congress*. Rome, Italy. November 2011

Presentations

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