Impact of storage and cryoprotectants on the function of cord blood hematopoietic stem cells

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ABSTRACT

Cord blood (CB) has emerged as a significant source of hematopoietic stem cells (HSC) for transplantation. Large distances between collection and processing sites combined with staff availability can lead to long processing delays of CB unit (CBU). Standard agencies limit CBU storage at room temperature (RT) to a maximum of 48 hours from collection to freezing. Slow-engraftment and graft failure are major issues related to CB transplantation. I hypothesized that prolonged storage at RT reduces the engraftment activities of CBU due to the loss in HSC numbers. I set to test my hypothesis by performing serial and limiting-dilution transplantation assays in immunodeficient mice. My results showed that the engraftment activity of CBU was significantly perturbed by prolonged storage (>40 hours) at RT. In line with my hypothesis, the transplantation assays suggested that the engraftment deficit originates from loss in HSC numbers. My findings provide results for CB banks to make an informed decision on how long CBU can be stored at RT before processing.

Conversely, CBU must be cryopreserved before use, and loss of function can occur due to osmotic shock and mechanical damage from uncontrolled ice-crystal growth (ice-recrystallization) during freezing and thawing. Current cyroprotectants like dimethyl-sulfoxide fail to inhibit ice-recrystallization. However, a novel class of small ice-recrystallization inhibitor (IRI) molecules (N-aryl-D-aldonamides) have been developed. I hypothesized that supplementation of cryopreservation solution with IRIs will improve the post-thaw viability and engraftment activity of CBU. Herein, I identified two IRIs (IRI 2 and IRI 6) that improved the post-thaw recovery of hematopoietic clonogenic and multipotent progenitors. Moreover, supplementation of CB graft with IRI 2 was beneficial to engraftment and had no negative impact on the differentiation and self-renewal activities

of HSCs. Taken together, my results demonstrate for the first time that IRI may be beneficial to the engraftment activity of HSC graft and support further investigation.

To

My Parents

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DECLARATION

I, Suria Jahan, hereby declare that the work presented in this thesis is my own research work. Wherever contributions of others are involved, every effort has been made to indicate that clearly, with due reference to the literature, and acknowledgement of collaborative research.

Suria Jahan
3rd October 2019, Ottawa, ON Canada.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATION

ALDH	Aldehyde dehydrogenase
ANOVA	Analysis of variance
APC	Allophycocyanine
BM	Bone marrow
CB	Cord blood
CBS	Canadian Blood Services
CBU	Cord blood unit
CD	Cluster of differentiation
CFU	Colony forming unit
CIHR	Canadian Institutes of Health and Research
CLA	Cutaneous lymphocyte antigen
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CPA	Cryoprotectant
CXCR4	C-X-C chemokine receptor type 4
DMSO	Dimethyl sulfoxide
EG	Ethylene glycol
ELDA	Extreme limiting dilution analysis
EPC	Endothelial progenitor cells
EPCR	Endothelial protein C receptor
EryP	Erythroid progenitor
EtOH	Ethyl alcohol
FACT	Foundation for the Accreditation of Cellular Therapy
FITC	Fuorescein isothiocyanate
G-CSF	Granulocyte - colony stimulating factor
GM	Granulocyte macrophage
GMP	Granulocyte monocyte progenitor
GTP	Guanosine triphosphate
GVHD	Graft versus host disease
HES	Hydroxyethyl starch
HLA	Human leukocyte antigen

HSC	Hematopoietic stem cell
HSCT	HSC transplantation
HSPC	Hematopoietic stem and progenitor cell
IIF	Intracellular ice formation
IRI	Ice recrystallization inhibitor
LDA	Limiting dilution transplantation assay
LMPP	Lymphoid-primed multipotent progenitors
MSC	Mesenchymal stem/stromal cell
MEM	Minimum Essential Medium
MEP	Megakaryocyte erythroid progenitors
MGS	Mean grain size
MNC	Mono nuclear cell
MPP	Multipotent progenitor
NK	Natural killer
NMR	Nuclear magnetic resonance
NOD	Nonobese diabetic
NSG	NOD SCID gamma mouse
PBS	Phosphate buffered saline
PBSC	Peripheral blood stem cell
PE	Phycoerythrin
PEG	Polyethylene glycol
PEP	Platelet engrafting progenitor
PG	Propylene glycol
PSGL-1	P-selectin glycoprotein ligand-1
PVP	polyvinyl pyrrolidone
RBC	Red blood cell
ROS	Reactive oxygen species
RT	Room temperature
SCID	Severe combined immune deficient
SEM	Standard error of the mean
SRC	SCID repopulating cells
TNC	Total nucleated cell
TNF	Tumor necrosis factor

CHAPTER 1
INTRODUCTION

1.1. Hematopoiesis and hematopoietic stem cell (HSC)

1.1.1. Hematopoiesis

Hematopoiesis is a continuous process of formation and development of various types of blood cells with divergent functions such as red blood cells (RBC), platelets, neutrophils and lymphoid cells to name a few [1]. Enormous numbers of adult blood cells are constantly regenerated throughout the life from HSC through a series of progenitor stages. Hematopoiesis is organized as a cellular hierarchy of progenitor cells with various potential all derived from a common precursor cell, the HSC. HSCs and all stem cells are functionally defined by two key properties - self-renewal and multilineage differentiation [2].

1.1.2. Hematopoietic stem and multipotent compartment

Extensive experimentation with mice has enabled scientists to improve our understanding of hematopoiesis and most precisely the stem cell compartment. Over the years, hematopoiesis has been described as a cellular hierarchy maintained by self-renewing HSCs that reside at the apex of its pyramidal structure [3, 4]. Functional studies revealed that the regenerative potential of HSCs could be assayed with *in vivo* repopulation assays which established the existence of multipotential HSCs [5]. Accessibility, robust functional

assays, well-established prospective isolation and successful clinical application have made haematopoiesis one of the best-established mammalian stem cell systems.

In all models of hematopoiesis, HSCs undergo long-term self-renewal while giving rise to cells of all the blood lineages. Under the classical model, this self-renewing capacity of HSC is progressively lost as they transit into short-term self-renewing and multipotent progenitor (MPP) states which give rise to progenitors that initiate the myeloid and lymphoid branches [6]. From MPPs, the common lineages for myelopoiesis (common myeloid progenitor - CMP) and lymphopoiesis (common lymphoid progenitor - CLP) are derived. The oligopotent CMPs undergo further restriction into bivalent Granulocyte monocyte progenitor (GMPs) that give rise to granulocytes/monocytes and Megakaryocyte erythroid progenitors (MEP) that go on to make platelets/RBCs (Figure 1A, Figure 1B) [7, 8].

Several independent studies have documented the developmental scheme of HSC, as it gives rise to a series of progenitor cell intermediates that undergo a gradual fate restriction. A complex roadmap persists in the lineage relationships between stem cells, progenitors and mature cells during the developmental transitions based on molecular basis. Woolthuis, C.M., *et al.* reported that heterogeneity within the HSC population is related to lineage potential, including a megakaryocyte-biased HSC that directly gives rise to megakaryocyte progenitors and bypasses classical intermediate commitment stages, including the CMP and MEP (Figure 1C) [9].

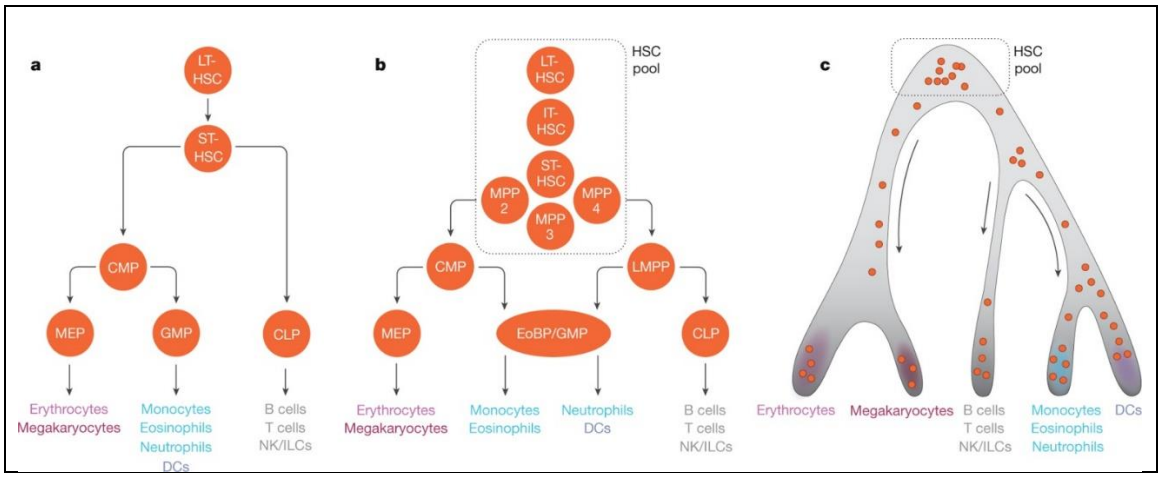

Figure 1: Models of the hematopoietic hierarchy.

(a) Classical model of the hematopoietic hierarchy with a strict separation between the myeloid and lymphoid branches as the first step in lineage commitment downstream of the HSC. (b) New findings showed that the HSC pool is now accepted to be more heterogeneous both in terms of self-renewal and differentiation properties, the myeloid and lymphoid branches remain associated further down in the hierarchy via the lymphoid-primed multipotential progenitor (LMPP) population. (c) From 2016 onwards, single-cell transcriptomic snapshots indicate a continuum of differentiation. Each red dot represents a single cell and its localization along a differentiation trajectory. (Figure adapted from E Laurenti & B Göttgens, 2018 [10])

Notta, *et al.* showed a hierarchy mainly composed of two-tiers in adults. The top-tier contains multipotent cells such as HSCs and MPPs and a bottom-tier is composed of committed unipotent progenitors. They have reported that, the origins of the megakaryocyte (Mk) lineage branch change from human fetal liver (FL) to adult bone marrow (BM). Also in FL, Mk progenitors were enriched but not restricted to the stem cell compartment whereas in BM, the Mk lineage was closely tied to the fate of multipotent cells (Figure 2) [11].

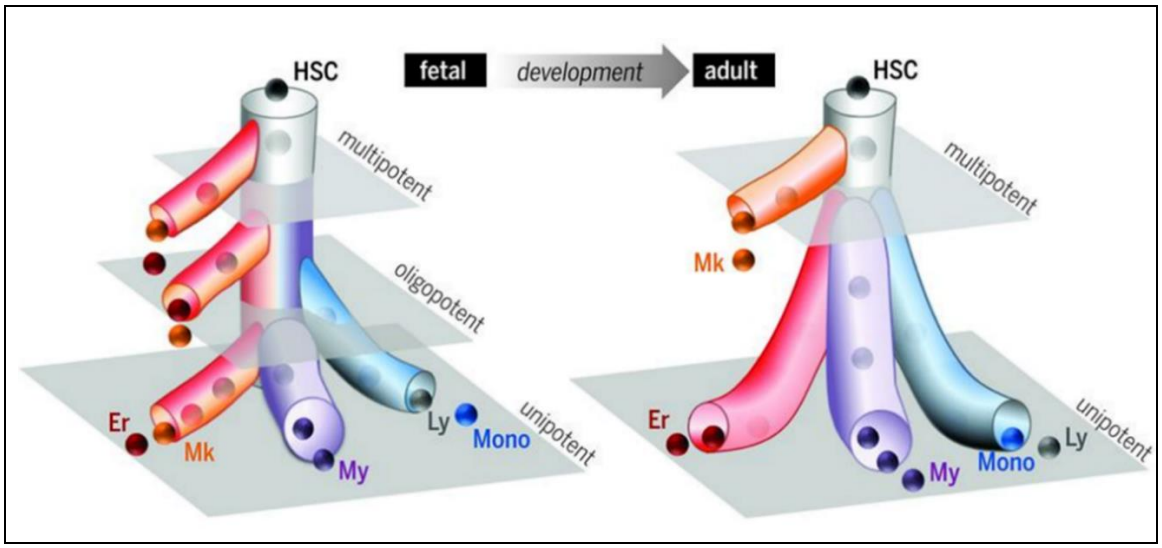

Figure 2: Redefined model of human blood development

The redefined model envisions a developmental shift in the progenitor cell architecture resulting in a two-tier hierarchy by adulthood. (Figure adapted from Notta, *et al.* 2016 [11])

Moreover, researchers around the world are still interested to understand the network of ongoing developmental processes in which a single lineage program eventually becomes dominant while all others are repressed. Such research will provide insight on cellular roadmap that specifies lineage relationships between stem, progenitor and mature cells of normal development of hematopoiesis in future.

1.1.3. HSC Markers

A major challenge for stem cell researchers is the identification and isolation of HSC from larger pools of cells. It is difficult to identify HSC population as they are rare, look like many other blood or BM cells, and no single antigen can identify HSC. It is estimated that HSC represent approximately 1 in 10,000-15,000 cells of the BM and 1 in 100,000 nucleated cells in the blood [12]. Therefore, techniques for isolation and identification of HSCs are often dependent on the detection of cell surface markers and cluster of differentiation (CD) antigens. CD34 is known to be one of the best markers for human hematopoietic stem and progenitor cells (HSPCs). CD34 is a transmembrane

phosphoglycoprotein [13] which is highly expressed on most human HSPCs, but absent on mature blood cells. This marker is also expressed by other cells like endothelial progenitor cells, endothelial cells of blood vessels, etc. [14]. Expression of CD34 on the cell surface of multipotent hematopoietic progenitors and its progressive down regulation on more mature cells suggest that it can play role in the maintenance of the undifferentiated HSPCs [15]. CD34 has been hypothesized also to have a role in enhancing cell proliferation [16]. The stage-specific expression of CD34 suggests that CD34 is potentially involved in regulating the early phases of developmental hematopoiesis based on its higher level of expression on the earliest HSCs [17, 18]. Human HSCs are enriched in $Lin^{-}CD34^{+}CD38^{-}$ population of cord blood (CB) and BM and also express another antigen, CD90 [19]. Ishikawa, *et al.* reported that transplantation of $Lin^{-}CD34^{+}CD38^{-}$ human CB cells into sub-lethally irradiated mice showed multi-lineage engraftment [20]. Baum, *et al.* identified a rare set of CD90/Thy-$1^{+}CD34^{+}$ human fetal BM cells that is highly enriched for pluripotent progenitor activities (myeloerythroid, B-lymphoid and T-lymphoid system) and long-term culture initiating ability [21]. Majeti *el al.* eventually identified the multilineage compartment and long-term HSC by the addition of the CD90 and CD45RA antigens. With such combination, long-term HSC were identified by the profile Lin^{-} $CD34^{+}$ $CD38^{-}$ $CD90^{+}$ $CD45RA^{-}$ [19]. MPPs were distinguished from HSCs by the lack of CD90 expression and functionally by the loss of self-renewal properties [19]. Notta, *et al.* reported that HSCs are further enriched by the expression of CD49f (~1 in 10) and MPPs can be identified by loss of CD49f expression (Figure 3) [22].

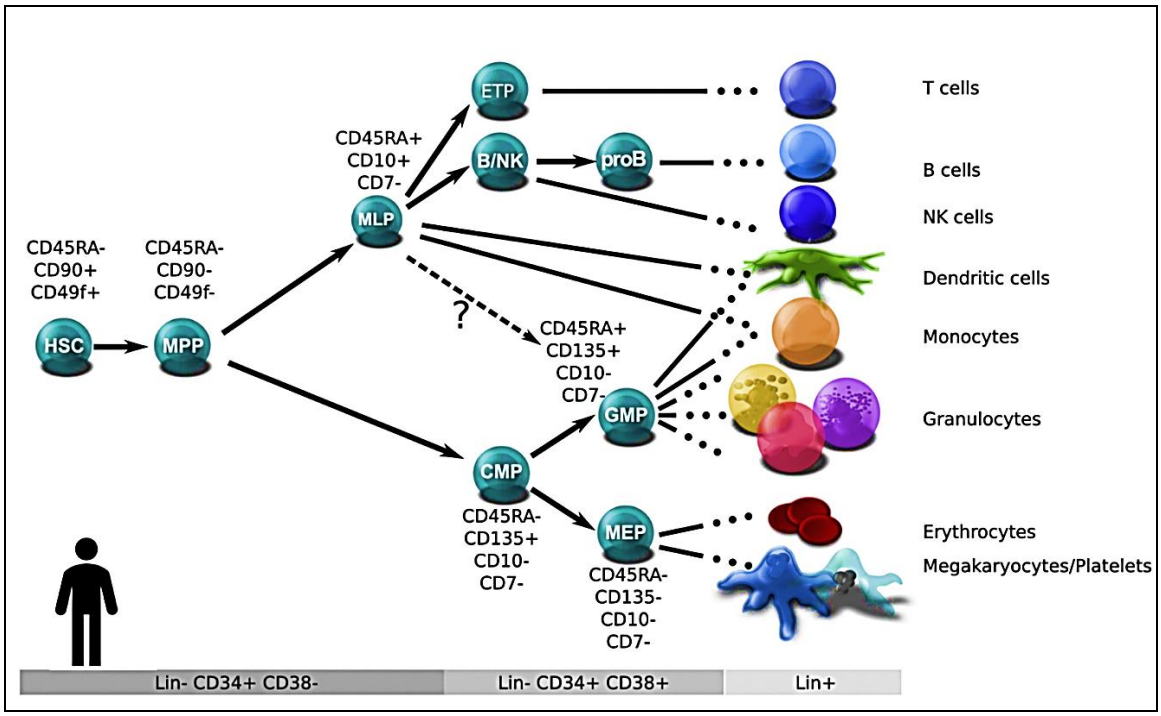

Figure 3: Lineage determination in the adult human hematopoietic hierarchy

The major classes of stem and progenitor cells are defined by cell surface phenotypes, which are listed next to each population and in the grey bars below each schematic. Terminally differentiated cells are shown on the right, and inferred lineage relationships are depicted with arrows. HSCs are defined by the expression of CD49f and other markers. MPPs can be identified by the loss of CD49f expression. (Figure adapted from Doulatov S., *et al.* [23]).

Furthermore, Doulatov, *et al.* identified seven distinct progenitor sub-fractions of Lin−CD34$^+$ cell–enriched populations on the basis of their expression of different cell surface markers CD45RA, CD135 (Flt3), CD7, CD10, CD38 and CD90 [24]. Based on their study, HSC as CD34$^+$CD38$^-$CD90$^+$CD45RA$^-$Flt3$^+$CD7$^-$CD10$^-$, MPP (CD34$^+$CD38$^-$CD90$^-$CD45RA$^-$Flt3$^+$CD7$^-$CD10$^-$), MLP7$^-$ (CD34$^+$CD38$^-$CD90^{neg-lo}CD45RA$^+$Flt3$^+$CD7$^-$CD10$^+$), MLP7$^+$ (CD34$^+$CD38$^-$CD90^{neg-lo}CD45RA$^+$Flt3$^+$CD7$^+$CD10$^+$), CMP (CD34$^+$CD38$^+$CD90$^-$CD45RA$^-$Flt3$^+$CD7$^-$CD10$^-$), GMP (CD34$^+$CD38$^+$CD90$^-$CD45RA$^+$Flt3$^+$CD7$^-$CD10$^-$), MEP (CD34$^+$CD38$^+$CD90$^-$CD45RA$^-$Flt3$^-$CD7$^-$CD10$^-$) and B-NK (CD34$^+$CD38$^+$CD90$^-$CD45RA$^+$Flt3$^+$CD7$^-$CD10$^+$) human progenitors were separated with distinct functionality. Recently, Fares, *et al.* showed that Endothelial protein C receptor

(EPCR/CD201) is a reliable marker of human CB HSC for both uncultured cells and cultured cells especially expanded with UM171 (a pyrimidoindole derivative previously shown to expand CB HSCs) [25]. It was reported that EPCR expression defines a subset of HSCs with a more primitive phenotype ($CD34^{+}CD38^{-}CD49f^{med}CD90^{+}CD133^{+}$) [26]. Another way of enriching HSC has been based on their high dye efflux capacity, in so called side population (e.g. Hoechst, rhodamine) [27]. Cell surface marker and dye efflux-based techniques can also be combined to achieve a higher enrichment level of HSC [22].

1.1.4. HSC transplantation (HSCT) - a curative treatment option

HSCT has become a curative treatment option to treat different blood related malignancies and deficiencies. Especially it helps to restore the stem cells when the BM get damaged by disease, chemotherapy (chemo) or radiation. [28]. There are two types of HSCT, allogeneic and autologous. In autologous transplantation, the patient's own cells are used while for allogeneic transplantation cells are used from a donor. In allogeneic transplantation, Human leukocyte antigen (HLA) typing is used to find matching donors for patients. HLA proteins or markers are found on most cells, and immune system uses these markers to recognize which cells belong to body and which do not. HLA mismatch has been associated with graft failure, delayed immune reconstitution, graft versus host disease (GVHD) and mortality [29]. HSCT procedure can also be called BM transplant, a peripheral blood stem cells (PBSC) transplant or a CB transplant depending on the source of HSC. HSCT was first conceived with BM more than 60 years ago and since then multiple changes in clinical practices have been incorporated to get better outcome from HSCT. This still remains a complex procedure to cure the underlying disease and prevent relapse especially for patients with severe complications [30]. Researchers around the world today are not only

focused to cure different types of blood cancer, but they are also trying to improve the transplant methods and reduce the undesirable risks.

1.1.5. Principal sources of HSCs

Around 45,000–50,000 HSCTs are performed each year for the treatment of lymphoma, leukemia, immune deficiency illnesses, hemoglobinopathies, and for other blood related malignancies [31]. HSC products for autologous or allogeneic transplantation are available from BM, mobilized peripheral blood and CB (Figure 4). Selection of HSC source depends on the donor availability and transplantation indication.

BM has been the first source of HSC used in transplantation which was introduced by E. D. Thomas in 1963 [32]. BM harvest is a highly invasive procedure since it is taken from the posterior iliac crests and for that, epidural or general anesthesia is needed (Figure 4). To ensure the sterility of the BM, a closed-circuit system of aspiration is needed. The collected BM can be infused immediately, or it can be stored at 4 °C for 24 hours [33, 34].

The most popular source of HSC nowadays are PBSCs, first introduced in 1981 [35]. PBSCs transplantation is associated with quick engraftment compared to BM-derived stem cells transplantation. As PBSCs are found in lower numbers in blood, multiple apheresis are required to collect adequate amount of PBSCs (Figure 4) [36]. The number of leukapheresis can be reduced to one or two times by mobilization of HSCs to the peripheral blood with granulocyte - colony stimulating factor (G-CSF, 6 μg/kg/day) treatment [37]. Stimulation of healthy donors with G-CSF treatment is not considered for significant side effects but rare occurrence of nontraumatic splenic rupture do occur [38, 39]. The apheresis machine separates the nucleated cells from blood and the remaining blood components are then re-introduced back into the bloodstream of the donor. The HSC-enriched cells are filtered, stored in bags, and frozen until the patient is ready for transplantation [34, 36].

Finally, the third most common sources of HSC is umbilical CB, which is discussed in detail in the next section.

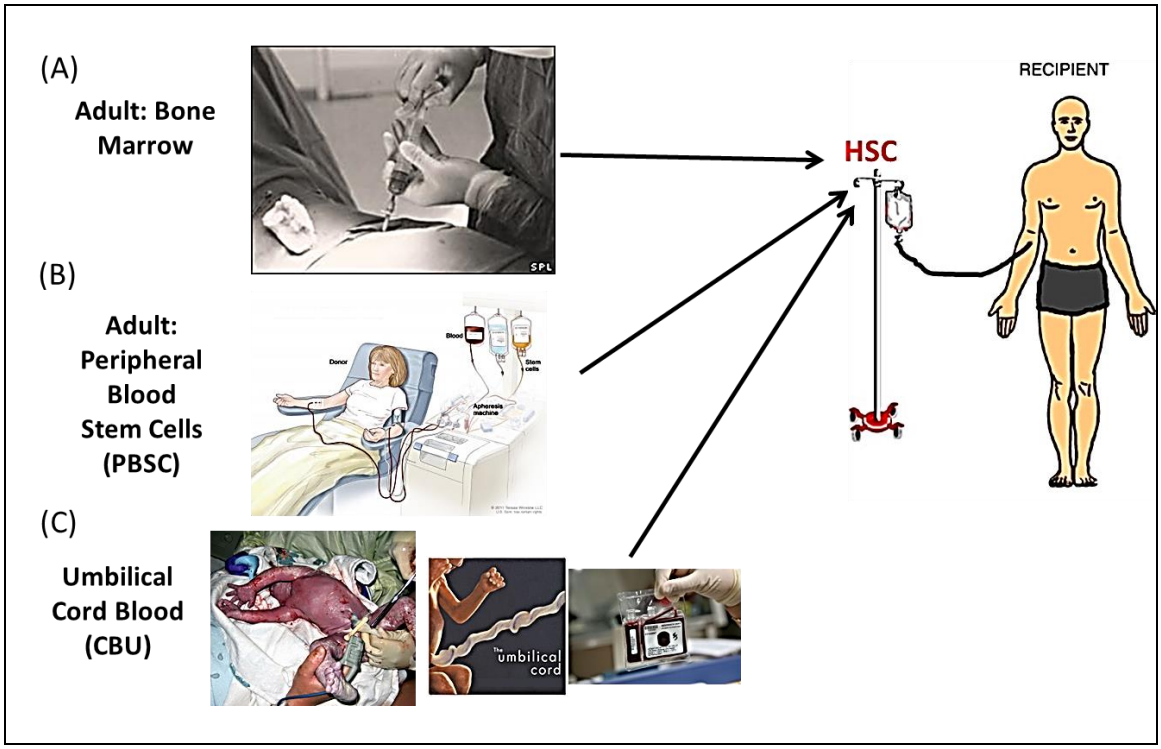

Figure 4: Different sources of HSCs

(A) Harvest of HSC from BM. (B) Collection of HSCs from peripheral blood and (C) Umbilical CB collection and use as a source of HSC.

1.2. Umbilical CB

CB unit (CBU), which historically was considered a waste and simply discarded, can be collected shortly after birth and has a great capacity to reconstitute the hematopoietic system (Figure 4). In 1974, it was shown that the cells of CB have the ability to grow hematopoietic colonies [40, 41]. By 1983, the concept of using CB as an alternative source of HSC for transplant had been proposed [42].The first successful CB transplantation was performed in a 5-year-old boy with Fanconi's anemia in 1988 [43]. Wang, J.C., *et al.* reported through limiting dilution transplantation analysis that CB has a higher frequency of HSC than BM or peripheral blood [44] . CB has become an important source of HSC for allogeneic HSCT when HLA-matched sibling and unrelated donors are unavailable. CB

cells are relatively immunologically naive, which allows for multiple-antigen mismatches, though engraftment and the survival outcomes positively correlate with HLA matching [45]. Usually CB should meet a match of at least 4/6 HLA matching for HLA-A, -B at the antigen level, and HLA-DRB1 at the allelic level [46]. The influence of HLA disparities on the outcome of CBU transplantation is controversial and Gluckman, *et al.* showed that the higher the number of cells, the lower the number of HLA disparities, which is good for better engraftment but the higher the number of HLA disparities, the higher the chance of acute GVHD [47]. A haploidentical donor matches usually 50% of the recipient's HLA and donor may be the recipient's parent, sibling or child [48-50]. The development of haplo transplants in recent years has gained significant interest with the introduction of new GVHD strategies such as T cell depletion with high $CD34^+$ doses to overcome risk of graft failure. Also, haplo-cord transplant reported faster immune reconstitution with rapid B-cell and delayed T-cell recovery [49, 51].

However, CB transplantation remains a curative therapy for patients with leukemia, lymphoma, myeloma, myeloproliferative disorders, and etc. Around 40–70 mL of fetal CB can be collected immediately after the cord is clamped and cut. Prior to collection of CB, the cord is wiped with alcohol or betadine to ensure sterility of the collection. The placenta is separated, and CB is collected into a sterile donor blood collection system with pre-anticoagulated (Citrate phosphate dextrose) collection kits [52]. The proliferative capacity is higher in HSPCs isolated from CB compared to those obtained from adult BM, with better replating potential and larger *in vitro* colonies. In addition, nearly one quarter of the total number of CB derived progenitors are multipotent, but in adult marrow, the percentage of multipotent progenitors is only 2–3% [53]. Based on combined effect of HLA and TNC, 5/6 match CBUs with pre-cryopreservation TNC doses 2.5 $x10^7$/ kg would lead to overall survival above 50% and for a similar survival rate, a higher TNC dose would be

required (>5 X 10^7/kg) when 4/6 match grafts were used [54]. Besides Sobol et al, reported that $CD34^+$ cell dose ($\geq 1.5 \times 10^5$/kg) helps to predict faster engraftment and can be useful for graft selection [55]. However, the success of CB HSC transplant depends on the cell dose [56] and one of the main challenges of CB bank is to conserve high cell yields during processing to ensure a high rate of usage of cryopreserved CBUs.

1.2.1. Public banking of CBU

Public banking of CB was initiated in 1992 at the New York Blood Center (New York, NY, USA) [57, 58]. CB banking is related to processing, testing, cryopreservation, storage, listing, selection, reservation, release, and distribution of CBUs intended for HSCT. The clinical outcomes after CBU transplantations are influenced by the number of cells available in the CBU. Typical CB bank inventory contains units with a median count of post processing total nucleated cells (TNC) at 1.04 X 10^9, but the median post-TNC count of units selected for transplantation from the National Marrow Donor Program, Be the Match Registry is 1.76 X 10^9 cells [59]. In Canada, Canadian Blood Services (CBS) began as a national public CB bank in 2013 with an aim to store high quality CBUs to meet the increasing demand [60]. The CBS banking threshold for Caucasian is $\geq$ 1.5 x10^9 TNC and for non-Caucasian is $\geq$ 1.25 x10^9 TNC for a single CBU [61]. To characterize and collect the CB derived cellular therapy products consistently, CB banking industry is eager to participate in the development of standardized procedures. Most of the CB banks follow the international standards for CB collection, banking and release. The NETCord-FACT provides such standard manual, which is an outcome of collaboration between the International NetCord foundation (NetCord) and the foundation for the accreditation of cellular therapy (FACT). NetCord is an association of CB banks founded in 1997, to promote the highest quality in CB products to balance the global supply and demand for

CB and to encourage and facilitate the use of CB transplants by promoting laboratory and clinical research and providing professional and public education. FACT was founded in 1996 by the American society for blood and marrow transplantation (ASBMT) and the international society for cellular therapy (ISCT). These Standards are designed to provide guidelines for CB Banks to support the procedures related to CB donor management, collection, processing, testing, cryopreservation, storage, listing, search, selection, reservation, release, distribution, etc. and to balance the global supply and demand for high quality CBU [62]. The development of these standards ensures that the quality of CBUs from different CB banks is more constant. Also, the purpose of such manual is to provide guidance to applicants for accreditation and to on site inspectors.

1.2.2. Advantages and disadvantages of CB derived HSCT

The advantage of CB as a source of HSC is that it can be easily extracted, resulting in no risk or pain for both the mother and the baby. The other advantages of CB HSC include ease of procurement (few weeks), less stringent requirements for HLA matching, reduced GVHD disease compared to other stem cell sources, and improved access to transplant, especially for ethnic population [63]. Moreover, CB is capable of giving rise to other cells. Such as, mesenchymal stem/stromal cells (MSCs) are identified after *in vitro* culture of CB mononuclear cells and endothelial progenitor cells (EPC) are detected in culture of CB mononuclear or $CD34^{+}$ cells. This advantage of CB makes it a good substitute source for tissue engineering in the field of regenerative medicine [52, 64]. The difficulty related to CB derived HSCs is that, it takes usually long time to engraft which can be the reason for high risk of infection in patient. Prolonged periods of neutropenia, thrombocytopenia and slow recovery of the immune system are main issues associated with CB transplants. The platelet engraftment is usually a month longer and 1-2 weeks longer for neutrophil in CB

transplant compared to PBSC and BM transplants [65, 66]. CB might not contain sufficient numbers of cells to engraft and the success of CB engraftment is related to CB banking processes [67, 68]. Usually, ~ 2-3 x10^8 TNC can be harvested from one CBU, while the recommended minimum total number of TNC for HSCT to an adult is 2.5 x10^7/ kg [69, 70]. Approximately 10–20% of patients receiving an unrelated CB transplantation, fail to engraft [65, 71].

1.2.3. Approaches to overcome shortcomings of CB transplantation

CBUs with lower TNC numbers are associated with higher risk of graft failure, delayed hematopoietic engraftment and delayed immune reconstitution. In case, several CBUs are available with similar number of TNC and HLA match grades, the best CBU should be selected based on the highest content of colony forming cells and $CD34^+$ cells as studies have revealed that units with the highest colony forming cells and $CD34^+$ cells are preferred for optimal engraftment [72, 73]. Selection of CBUs with sufficient TNC number is challenging for adult patients and a single CBU transplantation is usually not sufficient to provide enough cells. Double CBU transplantation is required for adults to get the minimum threshold of cells required for the procedure though the slow engraftment remains an issue. Moreover, double CBU transplantation did not show any advantage over single CBU transplantation as similar outcome was observed in both single and double CBU transplantation [74-76]. Also, reduced platelet recovery and higher GVHD risk were reported after double unit CB transplantation [74].

Conversely, several strategies are also under investigation to improve CB transplantation and engraftment. Clinical trials in recent years have demonstrated that short term engraftment of neutrophil and to a lesser extent platelet can be significantly improved with *ex vivo* expanded CB HSC. Moreover, long-term engraftment from expanded HSC is

possible if the expanded cells are co-transplanted with their non-expanded lymphocyte fractions. Various cell culture protocols have been reported such as expansion in co-culture with MSCs [77] or expansion with notch ligand Delta 1 [78] or NiCord (nicotinamide) [79] or with stem cell agonist molecules such as, Stem Regenin 1 (SR1) [80] or pyrimido-indole derivative (UM171) [81]. A complementary strategy is to improve homing of HSC. For example, this has been achieved with prostaglandin E2 transient treatment which up regulates cell surface receptor of C-X-C chemokine receptor type 4 (CXCR4) (a hepta helical receptor coupled to heterotrimeric guanosine triphosphate (GTP) binding proteins that plays a role in homing of HSC) [82, 83].

Researchers are also pursuing various efforts to find alternative solution to maintain or improve the quality of CBU to achieve better outcome from CB transplantation. The reconstitution ability of CB cells might be lost during different stages of collection, handling, processing, manufacturing, storage, shipment, etc. [68]. The development and validation of new potency assays for the evaluation of CBUs, along with the improvement of factors associated with the maintenance of CB quality during collection, processing, storage, etc. are very fundamental research, which can support to reduce the CB transplantation related shortcomings.

1.2.4. Factors affecting the quality of CB HSCs

Loss of reconstitution activity of CB derived HSCs may take place during CB collection, processing, storage, shipment and infusion [68]. To avoid this, precise protocols need to be in place and followed during all processing stages to maintain the quality of CBU and to maximize outcomes of CB transplantation. Many clinical reasons can be responsible for the failure of engraftment of CB transplantation including potency [84, 85]. Cell potency is related to the ability of HSPC to carry out their normal function and undergo self-renewal

and differentiate into other cell types as required [86]. Engraftment failure is often related to improper potency of the stem cell graft. The potency of a CBU can be hampered during the manufacturing process, i.e. CB cells can be stressed during processing, cryopreservation, and thawing. The cell dose (i.e. TNC) is correlated highly with the engraftment outcome, and therefore it is necessary to obtain CBUs with sufficient TNCs [45, 47]. Maternal, neonatal, collection, manufacturing and storage factors can influence the CB cell content of CBUs. Some factors are stated here which can affect the CB cell or quality of CBU, either directly or partially.

1.2.4.1. The impact of maternal and infant factors on CB quality

Allan, *et al.* reported that during collection of CB, delayed cord clamping is associated with reduced volume and TNC count of units collected [60]. The exogenous factor like, volume collected from CBU is very important in the settings of making quick decisions whether banks should invest in the processing and analysis of collected units. As collected volume correlates well with hematopoietic measures of CBU quality such as TNC count and $CD34^+$ cell number [87, 88]. Interestingly, higher birth weight is associated with greater CB collection volume and can be considered as an initial criterion for the selection of CB donors to increase efficiencies of CB cells [87]. Solves, *et al.* reported that there is a relationship between placental weight and CB volume that independently influence TNC, $CD34^+$ cells and colony forming unit (CFU) content [89]. Page, *et al.* reported that younger gestational age (34-37 weeks), Caucasian race of the infant, shorter time to processing (<10 hours), higher birth weight (>3500 g), and larger collection volumes (>80 mL) positively influence the potency of the CBUs [84]. There are conflicting results based on maternal age. Studies revealed that younger mothers had a higher $CD34^+$ cell concentration and a larger placenta has a higher volume of CB [90]. With the increase in mother's age, HSC numbers considerably decreased in CBU and it was also reported that TNC counts are

significantly increased in CBU from women older than 25 and 20–37 years as compared to other age groups [90-92]. But some studies also reported that maternal age has no effect on CB [93, 94]. Mohyeddin Bonab, *et al.* reported, vaginal delivery and heavier babies as the dominant factors that are significantly associated with increased TNC of CBU [92]. CB collection either by *in utero* or *ex utero* method showed comparable TNC, $CD34^+$ cell, and CFU count but bacterial contamination, low volume, clotting, and delay until processing were usually higher with the *in utero* collection method [95].

1.2.4.2. Impact of processing on CB quality

After collection, CB is shipped from the collection center to a CB bank for processing. The distance between CB collection and processing centers can vary significantly. Therefore, it is crucial to set the maximum allowed time and the shipping temperature between collection to start of the processing and cryopreservation of CBU, to minimize the loss of viability and functionality of CB cells. The current practice at the national public CB bank is to store CB in gas-permeable blood bags at room temperature (RT) (22 ± 4°C) for up to 48 hours prior to cryopreservation [96-98]. The impact of storage at RT on CBU will be discussed further below.

Conversely, efficient CB processing method must be used to maximize cell recoveries and high viability. Plasma and RBC depletion also referred to as volume reduction, has been adopted worldwide for CB processing. The Rubinstein protocol [99] described the procedure of volume reduction in which, CBUs are processed by hydroxyethyl starch (HES) in order to save storage space and storage cost. There are different separation methods e.g. plasma depletion, density gradient, Hetastarch and a novel method known as PrepaCyte-CB. Basford, *et al.* showed that PrepaCyte-CB was the most flexible method with the highest clonogenic potential after processing and more significantly after

cryopreservation compared to other methods listed above [100]. In many CB banks, volume reduction of CBU is performed using HES and the Sepax (Biosafe) automated cell processing system. Post-processing HES and non-HES results were comparable, but non-HES method resulted in a significantly lower post-thaw recovery of viable $CD34^+$ cells as measured by 7-aminoactinomycin D (7-AAD) and AnnexinV staining when compared with HES reduction method [101]. Furthermore, cryopreservation and cryoprotectants (CPAs) are other important factors that affect the quality of CB HSC during storage of the CBU. Indeed, cryopreservation and thawing exacerbate cell lesions increasing apoptosis and necrosis [102-104]. This topic will be discussed in a separate section below.

1.2.4.3. Impact of storage on CB quality and potency

In Canada, CBS accepts CBUs collected at locations up to 3500 km away from processing centers which can result in extended processing delays (>40 hours). Ideally, CB should be processed shortly after collection, but long delays are common due to logistical difficulties. Optimization of condition to store CBU before processing is an important issue for banking and transplantation. Therefore, determining the maximum allowed time that might pass between collection to start of processing and cryopreservation, without compromising the CB quality is very crucial. Standard agencies limit CB storage to a maximum of 48 hours from collection to freezing at a temperature range of 4-22 °C [96-98] but this topic remains controversial.

Biochemical cell lesions acquired during storage leads to cell loss due to apoptosis. Schwandt, *et al.* suggested that delays between collection and cryopreservation should be minimized as extended time reduces the number of viable cells and CFUs before/after cryopreservation. They have also reported that cryopreservation is preferable within the first 24 hours, and no later than 48 hours, to retain the maximum possible potency [98].

Several studies that measured viability of CB cells by dye exclusion (e.g. 7-AAD) reported that over 85% of $CD45^+$ cells remain viable when stored at RT for up to 48 hours [105-111]. However, Pamphilon, *et al*. showed that prolonged storage at RT has detrimental effect on viability, function and recovery of CB $CD45^+$ cells, $CD45^+CD34^+$ cells and HSPC as detected by CFU assay [109, 112]. Moreover, Fry, *et al.* showed that viability of $CD45^+$ cell decreased 13% upon storage at RT in comparison to immediately processed CBU with a reduced functional capacity of progenitors [113]. Certainly, a great deal of controversy exists between studies due to differences in experimental settings as for example, viability assessment based on DNA dye staining alone fails to detect apoptotic cells which are known to be functionally inactive [105, 107-110, 114]. Duggleby, *et al*. reported that apoptotic ($CD34^+$ annexin V^+) cells were found within the 7AAD-gated population and non-apoptotic ($CD34^+$ annexinV^-) cells correlated with better CFUs [115]. Also, other studies revealed that storage prior to processing influences membrane reorganization and loss of phospholipid in different cells of blood (e.g. RBC, dendritic cells) [116, 117]. It is also known that $CD34^+$ cells that have initiated early stages of apoptosis as indicated by their binding to annexin V, are devoid of engraftment activity [103].

One of the main challenges of CB banks is to conserve high cell yields during processing as the success of HSC transplantation is largely dictated by the cell dose [118]. Whether processing delays and storage condition contribute to the slower engraftment of CBU is unclear. Fact is that, the impact of storage on the function and loss of HSCs are poorly described as most of the published studies used *in vitro* assays which do not directly measure the activity of HSC [119, 120]. But it is likely HSPCs acquire cell injuries during storage which perhaps reduce their therapeutic potency. In line with this, Guttridge *et al.* showed that the post-thaw viability of $CD45^+$ cells and $CD34^+$ cells were inferior to that measured prior to cryopreservation, which further supports that cryopreservation and

thawing exacerbates cell storage lesions leading to increased apoptosis and necrosis [102, 103, 115, 121]. Moreover, studies in the context of BM and PBSC transplantation revealed that increased transport time can be associated with delayed engraftment of platelets [122, 123].

Yang, et al, reported that post-thaw TNC counts will be lower than prefreeze counts because neutrophils do not survive the thaw [124]. Roya, et al, showed a negative correlation between the content in neutrophil and post-thaw $CD45^+$ cell viability in CB products [125] as neutrophils poorly survive the stress of cryopreservation and thawing processes. Also same group showed that neutrophils are highly necrotic in post-thaw and that interfere with the viability of CB cells as this population most severely damaged by cryopreservation and thawing [126]. However, the storage of CB products are better maintained at refrigerated temperatures before cryopreservation to retain the highest possible potency of the cells [110, 113]. Louis, *et al.* suggested that, if CB is needed to store before processing and cryopreservation, it is preferable to store the CBU at 4°C [112].

Furthermore, reactive oxygen species (ROS) is a by-product of mitochondrial oxidative phosphorylation (OxPhos) and causes damage to cell structures [127]. Studies reported that very low level of intracellular ROS in HSCs are essential to maintain HSC quiescence and excessive ROS generation induces apoptotic cell death in HSCs [128-133]. In addition, an inflammatory cytokine, tumor necrosis factor (TNF)-α promotes ROS production in many cell types including stem and progenitors [134, 135]. Impaired engraftment of HSC was also reported as a result of TNF-α mediated increased ROS levels in murine system [135]. The link between TNF-α and ROS-mediated cell lesion due to storage of CBU at RT is unclear at this time and needs to be explored further.

Louis, *et al.* investigated the impact of 72 hours storage at RT on long-term HSCs activity and showed that *in vitro* assays poorly correlated with *in vivo* results and that CB stored at RT had lost more than ~90% of their engraftment activity compared to other CBU processed immediately. While this study brings light on the negative impact of processing delay on engraftment, there were several limitations, such as exclusion of a split design and, the threshold storage time point of 48 hours for non-related donation was not investigated [112]. Also, their experimental design did not allow them to quantify the loss of engraftment activity because they did not use limiting dilution transplantation assay (LDA) and, the impact on HSC self-renewal activity was not tested with secondary transplants. Lastly, published data regarding optimum storage time is inconsistent and a better understanding of the influence of RT on CB quality during storage is crucial. Therefore, it is very important to investigate the maximal allowable storage time of CBU prior processing (≤ 48 hours at RT [96-98]) to ensure the quality of CB product. Herein, I have investigated this shortcoming in **chapter 2** and also investigated the impact of 31 hours processing delay on CBU since 90% of CBUs at the CBS are processed within 31 hours (**Appendix 8.3**).

1.2.5. Challenges of CBU collection and banking to ensure product quality

Collection and banking of CB for transplantation has grown in numbers over the past years [136]. Due to the high cost of CB storage and the direct relationship between the numbers of cells in a CBU with the outcomes of transplantation, it is very important for CB banks to maintain high TNC, to improve the quality of the product in order to provide the best service. The impact of extrinsic factors (e.g. time from collection to processing, collected CB volume, etc.) and intrinsic factors (e.g. TNC count and $CD34^+$ cell counts) need careful consideration in order to focus the banking on high quality/potent CBU. For this reason,

only CBUs with at least 1.5×10^9 TNC (or 1.3×10^9 for ethnic minorities) are considered for banking at CBS [61]. Donor screening, collection and transport, processing, testing, freezing and distribution must be standardized and should meet international thresholds of quality which are ensured by accreditation and compliance with nationally and internationally recognized regulatory bodies [52, 61]. Moreover, reliable and reproducible $CD34^+$ cell count and CFU assay testing must be achieved and standardized within and between banks. Indeed, optimal strategies to guide public CB banks in the selection of collected units for banking still remain an active debate [102]. Parameters that are associated with graft adequacy are summarized in next section.

1.3. Parameters associated with graft quality and potency

Measures of graft adequacy is important before a CBU can be considered for both banking and CB transplantation. Several variables related to donor dependent, collection procedures, transportation of CB to the processing laboratory, processing procedures, etc. can be optimized to increase the yield of quality product. But besides these, it is also important to assess the efficacy of CBU or product quality/potency with reliable, standardized and validated assays to track the impact of different variables related to the final product potency. Key parameters associated with graft quality measurement and assays related to the evaluation of CB quality or potency are mentioned below.

1.3.1. Total volume collected

Wen, S.H., *et al.* and Jaime-Perez, J.C. reported that collected volume of CBU correlates finely with the hematopoietic measures of the quality of the unit, e.g. TNC count and $CD34^+$ cell number. Such parameter can aid banks to decide which units to exclude without performing additional expensive characterization [87, 88]. The volume of a collected CBU is a very simple but reliable measure without any major inter observer variability thus, it

remains as an important and cost-effective parameter to estimate the HSPC content and hence blood cell forming potential of a CBU.

1.3.2. TNC count

Generally TNC is the most reported and standard parameter in addition to the collected volume of the CBU [45, 47]. Both TNC and $CD34^+$ cell counts are important parameters which are associated with graft competence. Studies reported that the success of engraftment and survival of patients after CB transplantation is highly dependent on either the number of infused TNCs or the number of $CD34^+$ cells per kilogram of the body weight of the recipient [38, 54, 56, 69]. Processing and cryopreservation methods can also impact the TNC content of CBU and these methods differ considerably between CB banks. Several studies have reported a strong correlation between TNC counts and engraftment activity of CBU [45]. Currently, TNC count is one of the most standardized and reproducible measurements of the CBU cell dose available at the time of graft selection to define the CB potency.

1.3.3. Viability assay

Studies showed that the viability of $CD34^+$ cells are overestimated with dye exclusion measurements (e.g. 7-AAD), which is the main technique used by CB banks around the globe to qualify CBU. 7-AAD [137, 138] penetrates the damaged membranes of dead cells only, Annexin V [139, 140] stains phosphatidylserine which is translocated to the outer membrane in apoptotic cells. The impact of storage was investigated using more sensitive techniques such as Annexin V binding assay that detects apoptotic cells [103, 115, 141]. They showed that roughly one of every three $CD45^+CD34^+$ cells can stain positive with Annexin V. Radke, *et al.* [142] and Schwandt, *et al.* [101] revealed that the flow cytometric

assessment of early apoptotic and necrotic cells by staining with Annexin V and 7-AAD respectively, is a feasible method for predicting the amount of CFUs.

1.3.4. $CD34^+$ cell count

$CD34^+$ cell counts have been correlated with the rate of engraftment after CBU transplantation [73]. The technique of $CD34^+$ cell enumeration can differ between banks/processing centers if the enumeration of $CD34^+$ cell is not performed using a single platform flow cytometry in accordance with International Society of Hematopoietic and Graft Engineering guidelines [143]. Therefore, quality of banking is not possible to judge by processing recovery rate of $CD34^+$ cells primarily, since enumeration of $CD34^+$ cells shows large variation between banks [144]. The content of post-processing $CD34^+$ cells should be determined, as it is a good reference for engraftment and potency of CBU. The post-thaw viability and concentration of $CD34^+$ cells can reveal the unexpected cell loss that could occur during cryopreservation [145]. Guttridge, *et al.* also reported that the post-thaw viability of $CD34^+$ cells decreased as a function of storage time due to the increased number of apoptotic $CD34^+$ cells [141]. Therefore, $CD34^+$ cell content after processing the CBU is supportive to measure the graft adequency. In addition, there are two methodologies which can be used to enumerate $CD34^+$ cells. One is the single platform method, where a known number of fluorescent beads are added to the sample and the other is dual platform method, in which data from a conventional hematology analyzer and a flow cytometer is combined [146, 147]. The single platform method is easier to use than the dual platform method and mostly adopted now but Naithani R., *et al.* showed that both methods are effective for $CD34^+$ cell count and results were comparable between the two methods [148].

1.3.5. CFU assay

The CFU assay is one of the best indicators of engraftment potential of grafts and it is regarded as the gold standard for potency assay for HSC grafts [72, 149]. CFU assay is accomplished by plating cells in semisolid methylcellulose with appropriate media supplements and hematopoietic growth factors [150]. The number of CFU-Granulocyte macrophage (GM) has been reported to be associated with the transplantation outcome of HSCs from autologous BM and allogeneic transplantation of CB [72, 151-153]. Furthermore, it has been reported that successfully transplanted CBUs had a higher number of CFU GM progenitor [150]. Based on this fundamental information, the loss of activity of progenitors of CBU can be assessed. The shortcomings of CFU assay are related to the variation in colony count between individuals, long period of culture (2 weeks) and requirement for well trained technicians.

1.3.6. Aldehyde dehydrogenase (ALDH) activity

Intracellular enzyme ALDH plays an important role in retinol metabolism and it is expressed to a relative extent in primitive hematopoietic cells in a number of species [154]. This enzyme is responsible for the oxidation of aldehydes to carboxylic acids, which are also metabolized by muscle and heart [155]. ALDH is highly expressed in HSC and progenitor enriched subpopulations ($ALDH^+$ cell or $ALDH^{bright(br)}$) [156-159]. $ALDH^{br}$ populations isolated from CBU are highly enriched for CFUs compared to $ALDH^{dim}$ cells [160]. More importantly, Storm, *et al.* showed that most of the $CD34^+$ cells with short and long-term engraftment activity are found within the $ALDH^{br}$ subpopulation [156]. Furthermore, purified $ALDH^{br}$ human CB progenitors transplanted into immune-deficient mice exhibited robust lymphoid and myeloid engraftment [161]. Consistent with the link between ALDH activity and engraftment, ALDH based assay has recently been shown to

be a useful potency assay for CBUs [162]. Therefore, this assay can be used to monitor the frequency of HSPC with short and long-term reconstitution activities in CBU, and based on this, I have used the assay in the current study to test the potency of stored CBU.

1.3.7. Combining Factors in a "Scoring" System

It is clear that the selection of a quality CB product depends on several factors. Therefore, it is important to study the role of combining several factors to optimize the selection method of CBU for transplantation. Page, *et al*. reported that the hazard ratios for pre- and post- cryopreservation graft viabilities for TNC, $CD34^+$ cells, CFUs, mononuclear cells (MNC) and volume were collected and calculated in terms of their correlation with neutrophil engraftment. The magnitude of the hazard ratios was used to develop a scoring system which was fascinating [163]. Further study is required to examine the application of such scoring systems for selecting a quality CBU to get better outcome after transplantation.

1.3.8. Other stem and progenitor cell assays

1.3.8.1. Long-term culture initiating cell (LTC-IC) assay

Sutherland, *et al*. showed that the presence of most primitive cells capable of sustained repopulation of hematopoiesis *in vivo* can be measured by *in vitro* LTC-IC assay [164]. This assay measures the frequency of progenitors with self-renewal and differentiation activities [165, 166]. Using this assay, it is possible to determine the loss of frequency of multipotent progenitors due to different factors that can potentially affect the CB potency. Furthermore, this assay might be helpful to correlate with *in vivo* data to predict the engraftment loss of HSC.

1.3.8.2. Xenotransplantation assay

The life-long blood cell production is driven by the action of a small number of multipotent HSCs with long-term self-renewal potential, and in order to investigate this activity, the best available tools are transplantation assays. Xenotransplantation systems have been used to study the ability of human cells to initiate and maintain the hematopoietic system *in vivo* over thirty years. Severe combined immunodeficiency (SCID) mouse is a very useful model to perform transplantation. It is the gold standard in human transplantation for the validation of biomarkers of hematopoiesis and to demonstrate the presence of HSC in cells of interest [167]. In such models, HSCs are referred to as SCID repopulating cells (SRC) [168, 169]. The Non-obese diabetic (NOD)-SCID xenotransplantation model has been used as recipient to identify and study long-term SRC in CB, BM, and growth factor mobilized PBSC transplantation. The NOD-SCID mouse strain is deficient in B cells and T cells but can develop functional natural killer (NK) cells [167, 170-172]. The latter led to development of new mouse strains with more severe immune deficiency to get higher efficiency for transplantation in human HSPCs studies. As IL-2Rγ-chain deficiency blocks NK cell development and results in additional defects in innate immunity, by combining the IL-2Rγ^{null} gene with conventional SCID and Rag1/2^{null} mice, NOD-SCID gamma (NSG) (NOD/LtSz-SCIDIl2rg-/-) mouse was developed [168]. To measure functional HSCs, the best method is to transplant cells serially in immune deficient mice (i.e. serial transplantation assay) [173] and to enumerate HSCs, LDA is widely used [174].

1.3.8.3. Serial transplantations assay

Long-term HSC can be distinguished from multipotent progenitors and short-term HSC based on their engraftment kinetics, long-term engraftment, and ability to repopulate secondary recipients. Hogan, C.J., *et al.* showed that progenitors support high levels of human cell engraftment within 4–12 weeks but not within 16-20 weeks of post-

transplantation compared to HSC recipient mice, whereas HSC supports engraftment well past 16-20 weeks [167]. Notta, *et al*. applied secondary transplant in xenograft model as a benchmark to distinguish the long-term HSCs from multipotent progenitors [22]. Therefore, the effect of different factors like processing delay, storage of CBU, cryopreservation method etc. on the function of HSC can be investigated by serial transplantation assay.

1.3.8.4. Limiting dilution transplantation assay

It is important to be able to measure the number of HSCs accurately in HSC graft samples. LDA method can be used to measure the frequency of HSCs [174]. Deleterious effect of processing delay (i.e. storage at RT) or other factors on CB quality before processing and after cryopreservation can be assessed by measuring the frequency of both SRC and platelet engrafting progenitors using LDA. LDA has been used to estimate HSC frequency for a long time, but LDA was only recently used to estimate the frequency of platelet engrafting progenitors. Indeed, Cheung, A.M., *et al.* showed that the platelet response was dose-dependent which made the use of LDA applicable for this response [119]. In LDA, the frequency of both SRC and platelet engrafting progenitor can be determined by Poisson statistic (regression analysis). Cell dose is plotted against the percentage (%) of negative response in the different cell dose groups tested, and the Poisson statistic stipulates the frequency of the response of interest equal to the dose that provide 37% negative response [175].

The biological properties of the product can be reflected into a potency assay [85]. Of note, many of the assays (like, LTC-IC, cell growth, ALDH activity) to measure the potency of CB product have not been used till date to investigate the impact of storage on the quality of CBU in CB bank. Indeed, inadequate potency assay may be indirectly responsible for

the transplantation of poor CBU that may result in slow engraftment and even engraftment failure. Therefore, CB banks need to set a functional assay for their product, which should be done post-processing and post-thaw (on segment) and, in the context of a quality monitoring program.

1.4. Cryopreservation

Cellular injuries take place during freezing and thawing steps of cryopreservation for tissues and cells [176]. Cooling rates that are either too high or too low can kill cells [177] and affect the CB quality. To get high recovery of functionally viable cells, it is crucial to develop a successful cell cryopreservation strategy, from the selection of the CPA [178] to the end step of cryopreservation process.

Cryopreservation is a process that involves the use of very low temperatures to preserve structurally intact cells and tissues for extended period of time. At temperatures close to -196 °C, all biochemical processes are effectively stopped, allowing storage for prolonged periods [179, 180]. However, freezing is fatal to most living organisms due to the intra- and extracellular ice crystals formation, and also results in changes to the chemical setting of cells that in turn lead to cellular mechanical constraints and injury [181]. The cryobiological response and cryosurvival during the freezing and thawing cycle vary between cell types. Frozen specimens if cryopreserved properly, may remain in a state of suspended cellular metabolism indefinitely, and can be thawed as needed. Due to the rapid development of therapies for the clinical treatment of diseases in the area of transfusion and transplantation, it is crucial to preserve these cellular therapy products by cryopreservation [182]. Cryopreservation protocols have been extensively studied and optimized depending on cell type. Technologies for the cryopreservation of cells or tissues are constantly improving. During cryopreservation process, cooling and warming rates, use

of different types of CPA and their concentrations are very important factors. Cryo-injury is experienced as a result of cooling, storage and thawing of the sample. Cooling and warming rates and CPAs are exploited to minimize this damage [177]. Unfortunately, current clinically-used CPAs exhibit some cytotoxicity. Furthermore, they do not control ice growth or recrystallization which is a major cause of cryo-injury. Cryopreservation of grafts allows HSC to be stored and transported from the site of processing to the site of clinical use. The best cryopreservation results are obtained through research-based approaches that are adjusted for the specific purpose of cryopreservation.

1.4.1. Mechanisms of cryoinjury

The major steps in cryopreservation involve (1) addition of a CPA with cells or tissues before cooling; (2) cooling rate of the cells or tissues to a low temperature i.e. sub-zero temperatures (-80 °C or -196 °C) and its storage; (3) warming of the cells or tissues; and in some instances a fourth step includes removal of the CPA from the cells or tissues after thawing [183]. At any of these steps, cellular injury can impact the viability and/or functionality. The length of time between the addition of the CPA and cooling of the sample is critical and is minimized due to the toxicity of the CPA. After thawing and prior to transfusion or transplantation, the CPA may be removed in order to minimize the side-effects upon administration of the product. The process of CPA removal often involves a series of washing and centrifugation steps. Yang, *et al.* has confirmed that removal of dimethyl sulfoxide (DMSO) through several washing steps decrease the recovery of $CD34^+$ cells [145]. The cryo-injury of cells is best explained by Mazur's two factor hypothesis [179], which will be presented in the section below.

1.4.1.1. Cryo-injury during cooling process

At -5°C, the extracellular solution and cells become super cooled but remain unfrozen. Approximately, between -5°C and -15°C, ice nucleates in the extracellular medium but the cells' contents remain unfrozen and super cooled. Due to the extracellular ice formation solutes are excluded from the ice which cause an increase in solute concentration state in the extracellular space. At this stage, water comes out from the cell osmotically and freezes externally as the system attempts to attain equilibrium [179]. Cryoinjury subsequently depends on the freezing rate applied and described well by Mazur's two factor hypothesis. If cells become cooled too rapidly, the intracellular water of the cell will not be lost quickly enough to maintain the equilibrium. Due to the rapid drop of temperature the intracellular water freezes and intracellular ice formation (IIF) occurs. The later leads to membrane damage [179, 184]. In addition, recrystallization of intracellular ice during thawing can cause mechanical damage to the cells. Intracellular ice can be harmless depending on the amount of ice formed, the location and the mechanism of formation. Conversely, if cooling rate is very slow, the cells will lose water rapidly and will face severely dehydrated state and do not freeze intracellularly. This severe loss of volume not only disrupts the membrane but exposes the cell to very high solute concentrations causing cell injury. This state of cell is known as solution effect [179]. Therefore, optimal cooling rate is required to prevent IIF and to avoid excessive cell shrinkage to overcome the solution effect (Figure 5). There is an optimal cooling rate for each cell type which depends on the cells' membrane permeability to water and solutes. This was experimentally confirmed by Mazur in 1984 [179]. Cooling protocols (e.g. 1–5 °C/min) seem to be the consensus among most laboratories for cryopreservation of CB cells as it allows the cells to gradually dehydrate in response to a growing ice phase and increasing extracellular solute concentration [185].

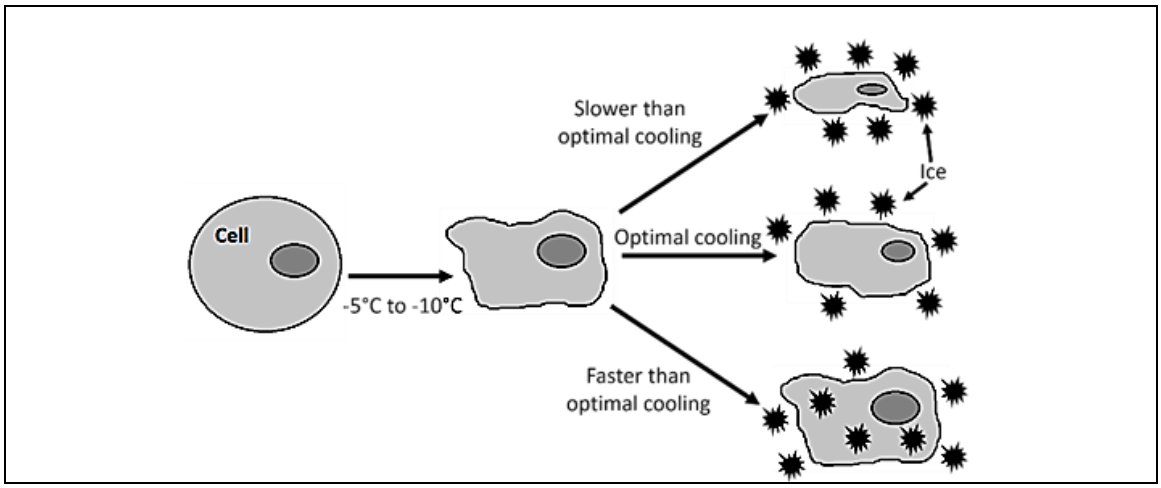

Figure 5: Schematic drawing of physical events in cells during freezing (Figure adapted from Gao, D., *et al.* 2000 [179]).

1.4.1.2. Cryo-injury associated with the warming process

Cells can experience cryo-injury during warming and thawing process. Thawing rate whether slow or fast can cause mechanical damage to the cell, and cryo-injury experienced during warming and thawing can involve recrystallization of ice. If thawing rate is too slow, intra and/or extracellular ice crystals will recrystallize into larger ice crystals which are responsible for the rupture of cell membranes and result in mechanical damage to the cells leading to necrosis. Therefore, it is important to perform rapid thawing of the cells in order to minimize this ice recrystallization formation. However, it is not possible to eliminate ice recrystallization fully and such type of cryo-injury is associated with reduced post-thaw viabilities in many cell types [179]. CB cell thawing at a rate of approximately 100 °C/min utilizing a 37 °C water bath results in the best post-thaw recovery and viability of cells [186].

1.4.2. CPAs and their mechanisms of action

Cryo-injury can be reduced using optimal freezing and thawing rates, but post-thaw cell viabilities are still needed to be improved. Therefore, cell cryopreservation requires an optimal cooling rate and the presence of CPA, which reduces cell shrinkage during

dehydration and inhibits IIF [187]. Typical CPAs are low molecular weight organic compounds that effectively penetrate into the cells and prevent the IIF, which include glycerol, DMSO, ethylene glycol (EG) and propylene glycol (PG) [188]. The permeability of the cell membrane to water and CPAs is an important factor determining the recovery of HSCs following cryopreservation. CPA used for cell preservation are usually divided into two classes: intracellular/permeating CPAs and non-permeating/extracellular CPAs [189].

Intracellular CPAs, DMSO and glycerol can cross the cell membrane and protect cells from cryoinjury associated with slow cooling rates by preserving cell volumes and reducing "solution effects". During slow cooling, cells experience lethally high solute concentrations. Penetrating CPAs effectively dilute these solutes and therefore reduce the temperature at which the critical salt concentration is reached. At the same time, penetrating CPAs prevent excessive cell shrinkage by replacing water within the cell [177, 179, 182, 189]. Extracellular CPAs do not penetrate the cell membrane and function by stabilizing cell membranes. This type of CPA mainly forms a shield around the cell, thus minimizing the effects of dehydration due to the freezing process. Non-permeating CPAs include HES, polyvinyl pyrrolidone (PVP), polyethylene glycol (PEG), trehalose, sucrose and dextran. They are usually used when fast cooling rates are utilized because they increase the osmolality of the extracellular solution and accelerate dehydration [177, 179, 182, 189]. Besides these, direct inhibition of ice crystal formation and application of antioxidants and other compounds can be used to reduce cell death from apoptosis during freezing and thawing cycle [190, 191]. Wu L.K., *et al.* reported that supplementation of CPA solution with mono and disaccharides are beneficial as they have the ability to inhibit ice recrystallization to improve post-thaw viability of $CD34^+$ cells in cryopreserved CBU [192]. Changing ice pattern formation can influence the freezing response. As a result,

molecules that alter the freezing of water might play a vital role in cryopreservation. Several small-molecules, i.e. ice recrystallization inhibitor (IRIs) have been demonstrated as effective CPAs for HSCs [192, 193].

1.4.2.1. Cryopreservation of CB HSCs and its importance in regenerative medicine

Proper cryopreservation of cells is the best way to improve reproducibility of potency between grafts and to protect important stocks of cells that may be difficult to replace. Cell-based therapeutics emerged as a critical aspect of modern healthcare in regenerative medicine and transfusion. Loss of cell viability after cryopreservation can reduce the potency of HSC transplant. Most importantly, HSCT success correlates with the cell dose and potency [118, 149] of the graft. The role of HSCT in the treatment of hematologic and non-hematologic malignancies is rapidly expanding. CBU is a great source of HSCs, widely used in transplantation in which hematopoietic reconstitution is crucial. Viability and functional integrity of CB should be maintained using the advanced cryopreservation technique to ensure that these cells can be used for transplantation in future. In some situations, fresh stem cells can be used for allogeneic transplants if the cells are transplanted within 72 hours of harvest. However, virtually all autologous and allogeneic transplants require cryopreservation [177]. The cryopreservation process is important for all types of stem cell collections, but perhaps it is particularly critical for CB HSC as the HSC from CB, harvested at the time of birth and can be stored in public CB banks for an unknown recipient for an indeterminate time span. However, increased rate of graft failure or delayed platelet and neutrophil recovery are major life-threatening issues associated with CB transplants. Slower engraftment is partially due to the significant reduction of neutrophil and platelet-engrafting HSPC in CBU vs. adult stem cell grafts [119]. Reduced potency directly correlates to the risk of graft failure [120] and potency can be decreased as a result

of freezing and thawing [149]. Also, ice recrystallization during freezing and thawing can influence the post-thaw cell viability and functionality. Cryopreservation processing that yields consistently high recovery of functionally viable cells is important for the successful future use of CB HSC. Taken together, the major issues requiring improvement in regards to CB HSCs cryopreservation are, i) maximization of post-thaw viability and recovery of CB cells and, ii) improvement of post-thaw recovery of progenitor and HSC potencies to overcome engraftment failure. The IRIs currently represent an excellent strategy to improve post-thaw recovery of CB cells while maintaining significantly greater HSC potency [192].

1.4.2.2. CPA for HSC grafts

DMSO is the permeable CPA of choice for all grafts. DMSO is available in clinical grade preparations and is known to be much more permeable to most cells than glycerol. Clinical protocols for cryopreservation of CB HSCs use 10% DMSO as the CPA solution. Rubenstein, *et al.* and Hunt, *et al.* reported that using 10% DMSO can maintain the membrane integrity and CFU recovery [99, 194, 195]. Meyer, *et al.* also reported optimal results with 10% DMSO as measured by $CD34^+$ cell recovery and CFU analysis [196]. While 10% DMSO solution is very effective and maintains high levels of cell viability, there are significant drawbacks associated with the use of DMSO [197]. The most important is toxicity [198]. DMSO has been shown to have adverse effects on gastrointestinal, renal, hepatic, central nervous, cardiovascular and respiratory systems. Several side effects have been associated with transfusion of DMSO into patients including headache, nausea, vomiting, hypertension, anaphylactic shock, and many others [177]. Generally, DMSO has been thought to exert chemical toxic effects on cells resulting in loss of proliferative potential of HPCs [199]. Therefore, due to toxic effects of DMSO some precautions are required during cryopreservation process. To avoid toxicity to the cells

being cryopreserved, Rubinstein, *et al.* [99] reported the addition of DMSO to the cells for up to 15 minutes and in a final concentration of 10%. In addition, it has been found that the degree of toxicity is directly related to the amount of DMSO transfused into the patient. Therefore, efforts to reduce toxicity include the removal of DMSO prior to transfusion [177]. This process is expensive, time consuming, and results in cell loss. Removal of DMSO by centrifugation was reported with decreased viability and recovery of $CD34^+$ cells in cryopreserved CBU [145]. Alternatively, graft can be diluted to simplify the processing and avoid cell losses [200]. In my work, I adopted the same thaw protocol developed in our lab for the CB bank, which involves two dilution steps to provide time to adjust to the change in osmolarity and avoid excessive cell swelling that occur upon rapid dilution [126].

While DMSO is very efficient for cryopreservation, there is much room for improvements. Indeed, up to 30% of $CD34^+$ cells were reported to be apoptotic in post-thaw, and this correlated with a reduced ability to engraft [103, 201]. Approximately 20% of patients receiving a CB transplant will fail to engraft, in part due to the inadequate potency of the unit as a result of cryopreservation [149]. Moreover, Sasnoor, *et al.* have found that cryopreservation impairs the growth factor responsiveness of HSPC, and this correlated to a reduced ability to proliferate and differentiate post-thaw [202-204].

1.4.2.3. Innovation of small molecules to inhibit ice recrystallization

The main purpose to use different CPAs (permeable and non-permeable) in combination or alone is to reduce cryo-injury and improve post-thaw recovery. Different CPAs reduce cryo-injury and improve post-thaw recovery by diluting intracellular solutes, preserving cell membrane volumes, and stabilizing cell membranes. However, they are unable to control ice growth and recrystallization which is a major source of cryo-injury. To

overcome this, cells are typically subjected to very rapid thawing conditions in order to minimize this ice recrystallization [177]. But it is not possible to completely eliminate ice recrystallization by employing rapid thawing conditions. However, compounds (IRI) with the ability to inhibit ice recrystallization were shown to be effective CPAs for RBCs [193].

These IRI compounds represent a new class of CPA with a novel mechanism of action, non-toxic and capable of controlling ice recrystallization which might help to improve the current cryopreservation protocols [192, 193]. The IRI molecules can be used to inhibit the ice recrystallization during freezing and thawing by adding them with conventional CPA. Extensive research has been going on to discover a better CPA by using different concentrations of DMSO in combination with other available potential CPAs such as EG, I,2-PG, starches and sucrose for better cell viability and stem cell content [178].

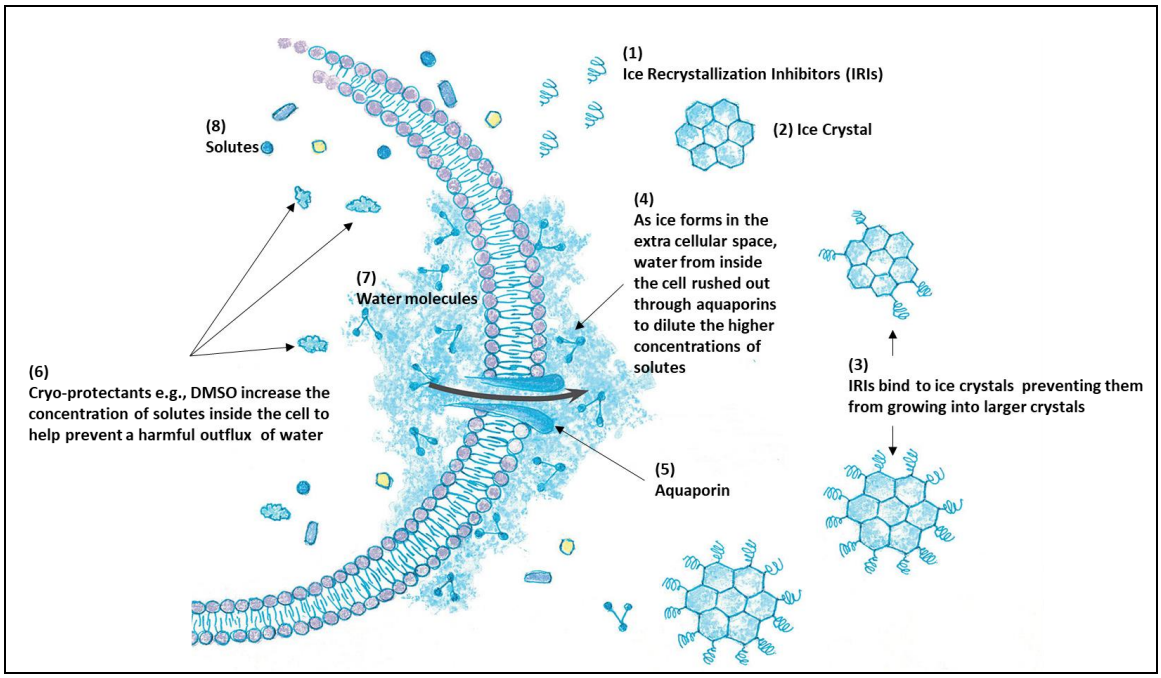

Figure 6: Activity of IRI and DMSO during cryopreservation

However, the conventional CPAs such as, DMSO and glycerol function by different mechanisms and do not control ice recrystallization (Figure 6).Therefore, based on Briard, J.G., *et al.* [193] published work related to using novel small molecule IRIs to control the growth of ice during freezing and inhibition of ice recrystallization in RBC, can be

proposed for HSC cryopreservation with the aim of minimizing cellular damage during cryopreservation and maintaining HSC functionality. Herein, I have investigated the effect of N-aryl-D-aldonamides, IRI small molecules in post-thaw progenitor recovery chapter 3, and the effect of IRI 2 (N-(2-fluorophenyl)-D-gluconamide) on HSC and progenitor engraftment activity in chapter 4.

1.5. Rationale, hypothesis and objectives

Large distances between collection and processing sites combined with staff availability can result in long processing delays of CBU. Accreditation agencies have allowed storage of CBUs at RT for up to 48 hours for non-related collection which roughly correspond to a maximum of 43 hours of storage and it is up to the bank to validate the temperature of storage that maintains the quality and potency of CBU. However, such storage at RT contributes or not to the slower engraftment of CB remains unclear. Therefore, in current study, the impact of storage at RT on the engraftment activities of CBU will be investigated using the best available and most recognized murine transplantation models to challenge this historical shortcoming.

Hypothesis 1:

Extended storage at RT before cryopreservation can interfere with the potency of CB grafts and contribute to the slow engraftment or engraftment failure due to a significant loss of HSC and its activity.

Objective 1.1: Test the impact of storage for 40 hours at RT on CBU viability and potency.

Objective 1.2: Test the impact of storage for 40 hours at RT on CBU engraftment activity using serial and LDA transplantation models.

Objective 1.3: Test the impact of storage for 28 hours at RT on CBU viability and potency.

Moreover, loss of function in cryopreserved cells occurs following cryoinjuries due to osmotic shock and mechanical damage from uncontrolled ice crystal growth (i.e. ice recrystallization) during freezing and thawing. Cryoinjuries adversely affect the engraftment activity of HSC. Such outcome is clinically significant for CB transplantation where doses of HSC and progenitors are limiting, and engraftment is typically delayed. Ben, *et al*. developed novel small molecules (N-aryl-D-gluconamides) with IRI activity, where other conventional CPAs (like; DMSO and glycerol) function by different mechanisms but can't reduce or control the ice recrystallization. In this study, the role of these novel small molecules will be tested as additives to the conventional 10% DMSO CPA solution to determine whether they can improve the post-thaw recovery of progenitors and most importantly, the engraftment activity of CBUs.

Hypothesis 2:

Supplementation of IRI with the conventional CPA during cryopreservation will improve the post-thaw CB quality and engraftment activity.

Objective 2.1: Test the effect of IRIs on post-thaw CB quality.

Objective 2.2: Test the effect of IRI 2 (N-(2-fluorophenyl)-D-gluconamide) on the engraftment activity of CB HSC using the serial transplantation assay.

CHAPTER 2
LONG PROCESSING DELAY IMPAIRS THE ENGRAFTMENT ACTIVITY OF CBU[1]

2.1. Summary

Background: Maximizing the quality and potency of CBU is a common goal of public CB banks. Low transplant cell dose in CB transplantation is regarded as the principal culprit for the slower engraftment but other factors such as processing delay may be at play. CB banks can store CBU at RT up to 48 hours before cryopreservation. However, the impact of processing delay on HSC function is unclear. We hypothesized that prolong storage at RT reduces the engraftment activities of CBU due to a loss in HSC numbers.

Study Design and Methods: CBU were processed shortly after collection or after storage at RT. The impact of storage on engraftment activity was investigated in a series of xenotransplantation experiments.

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[1] Based on a manuscript soon to be submitted by Suria Jahan, Roya Pasha, Emily Doxtator, Javed K. Manesia, Mike Halpenny and Nicolas Pineault. First author Suria Jahan participated in all experimental designs, CB processing, in vitro assays, mice transplantation assays, data analysis and wrote the manuscript. Roya Pasha assisted in some of the in vitro assays and in mice sample processing. Emily Doxtator and Javed K. Manesia have participated in some mice sample processing. Mike Halpenny provided some guidance and reviewed the manuscript. Nicolas Pineault has supervised the project and edited the manuscript.

Results: Prolonged storage negatively impacted platelet and leucocyte engraftment, and significantly reduced human BM chimerism in primary and secondary transplants. Our results suggest that the reduction in engraftment activity originates from a loss in the net number of stem and progenitors. Further investigation revealed that extended storage at RT induces significant biochemical changes in CBU that are consistent with loss in engraftment activity. This included increases in TNF-α, increased in ROS levels and, the down regulation of the homing receptor CXCR4.

Discussion: Altogether, these results stress the importance of rapid processing of CBU when stored at RT to minimize losses in engraftment activity.

2.2. Introduction

Umbilical CB collection is a useful means to collect HSC graft for future use for patients with complex or rare HLA requirements. As such, CBS public CB bank preferentially banks CBU from minorities or mixed background.

The primary criteria used for the selection of similarly matched CBU is the TNC content, since this parameter correlates strongly with engraftment [205]. A major variable identified as working against cell recovery and graft quality is the time between collection and processing, referred herein as processing delay or storage [106, 107, 110, 113, 122, 206-208]. CB banks can store CBU at RT before processing for lengthy period as long as CBU are cryopreserved within 48 hours post-collection (NETCord-FACT standard D3.2.6 [209]). Delays in processing does occur and is attributed mostly to large distances between some collection sites and the processing facility (e.g. 4,000 km between Vancouver and Ottawa' processing centre).

A retrospective analysis of 815 donations at the CBS public CB bank revealed a negative correlation between full processing time and CB cell viability ($R=0.74$, $p<0.0001$).

Moreover, storage at RT has also been reported to lead to losses in the recovery of viable $CD45^+$ cells up to 25% [113]. The impact on $CD34^+$ cells and CFU progenitors is somewhat conflicting [109, 110, 112, 113], though some have reported losses for $CD34^+$ cells of 15-30% [113, 121] and CFU[109, 110, 113] associated mainly with storage at RT. Losses in cell yield is especially critical for CB, since the starting cell content in this graft source is far smaller than an apheresis grafts, and that CB transplantations are associated with slower engraftment kinetics of platelets and neutrophils [210].

TNC and $CD34^+$ cell counts may not represent accurate measure of engraftment potential for CBU stored for prolonged periods of time prior to processing [112]. Only two studies addressed the impact of pre-processing storage on the engraftment activity of CBU using immunodeficient mice. It was reported that the proportion of annexin V^+ $CD34^+$ cells increases over storage time [121], and that the engraftment activity of CBU was inversely correlated with the level of annexin V staining[103]. Conversely, CBU stored for 3 days at RT (maximal allowed storage time for related collection[209]) were found to have little engraftment activity whereas other CBUs stored at 4°C produced engraftment similar to baseline[112]. However, the impact of long storage time (e.g. ~40 hours) more likely to be encountered in public CB Bank has never been addressed. In addition, the molecular mechanisms responsible for the loss of CB stem and progenitors is unclear, though it is likely due to cell storage lesions [211].

Here, we sought to fill the gap in knowledge about the impact of pre-processing delay or storage at RT has on the engraftment activity of CBU. Toward this, CBU were processed following common banking procedures and transplanted with minimal manipulation post-thaw in NOD/SCID/IL-2Rγc-/- (NSG) mice to recapitulate transplant settings. Our findings show that long storage (>40 hours) at RT before processing is detrimental to the engraftment activity of CBU, but the engraftment deficit can be avoided if CBU are

processed within 31 hours post-collection. In addition, we present evidences that increases in TNF-α levels during storage may initiate increases in ROS levels in CB cells that may in turn explain the loss in stem and progenitor function during storage.

2.3. Materials and Methods

2.3.1. CBU processing, cryopreservation

Human CBUs (n=7) collected less than 12 hours and containing a minimum of 500 million TNC were obtained from the CBS CB for Research Program after obtaining institutional review board (REB PROTOCOL REFERENCE # 2013.016 - Hematopoietic stem cells and transfusion medicine) approval and written informed consent. CBUs that were excluded from banking due to TNC count inferior to $1.3X10^9$ were assigned for developmental work. CBU were split in half with one half processed right away (baseline control) and the second after a storage period at RT (Figure 7). Storage was done in the original gas-permeable collection bag left in validated thermally-insulated blood transportation box designed to maintain a range of 15-25°C (NETCord-FACT Standard C7.5). Buffy coat preparation was done by adding Hespan (6% Hetastarch in 0.9% Sodium Chloride, B. Braun Medical Inc., Irvine, CA, USA). Hespan was added at a volume corresponding to 20% of the CBU. The mixture was gently agitated and incubated at RT for 90 minutes (min). The leukorich plasma (i.e. buffy coat) was collected and rinsed cells were resuspended in plasma. The buffy coats were cooled to 4°C after which a cryosolution (55% DMSO with dextran-40, Akron Biotech, Boca Raton, FL, USA) was slowly added to a final concentration of DMSO and dextran of 10% and 1% respectively. Buffy coats were split into 1-mL aliquots in cryovials and frozen at ~-1°C/min in alcohol-based device (Mr. Frosty, Thermo Fisher Scientific, Waltham, MA, USA) overnight at -80°C [126] and then transferred to the liquid nitrogen drawer tank until use.

2.3.2. Thawing of aliquoted CBUs

Aliquoted CBUs (1 ml) were thawed by holding the cryovial in 37°C water bath for approximately 60-70 seconds. Next, the contents were mixed with RT diluent consisting of 4% human albumin (HA) (Grifols Ltd., Barcelona, Spain) in PlasmaLyte-A (Baxter, Deerfield, IL, USA) in a stepwise fashion. Initially 1.5 mL diluent was added to thawed 1 mL CB aliquot, and cells were allowed to equilibrate at RT for 15 min, followed by an addition of 2.5 mL of diluent and additional 15 min incubation (referred to a total as 30 min) [126]. Samples were rinsed (300g/10 mins) and resuspended in 1 mL of Iscove's Modified Dulbecco's Medium (IMDM) (Life Technologies) with 2% Fetal Bovine Serum (FBS) (HyClone, Thermo Fisher Scientific) solution. TNC density was measured manually with hemocytometer or using a pocH-100i hematology analyzer (Sysmex, Ontario, Canada).

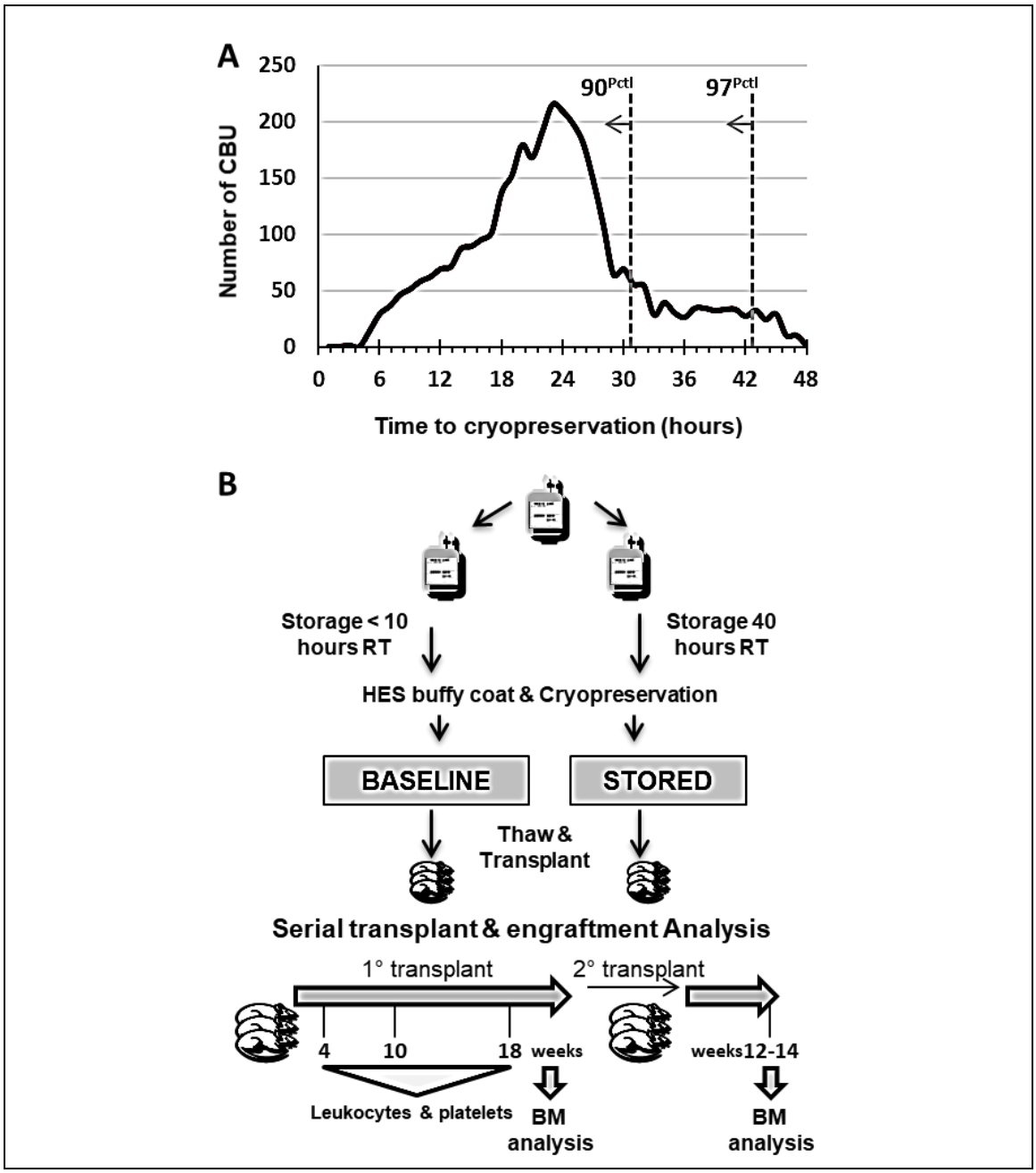

Figure 7: Time from collection to cryopreservation for CBU and experimental design overview.

A) Distribution of elapsed time from collection to freezing for 3,360 CBU processed at the CBS public CB Bank from 2015 to 2019. The time to cryopreservation between collection and cryopreservation studied herein are indicated by dashed lines (stored at RT for 40 and 28 hours before processing, respectively). B) Overview of the experimental design and serial transplantation assay used to address the impact of processing delay (40 hours storage at RT) on the engraftment activity of CBU.

2.3.3. Flow cytometry analysis

Flow cytometry was carried out using a FACS-Attune (Thermo Fisher) [212]. Dead cell and debris were gated out by the forward and side-scatter and/or Sytox-AAD staining (Life Technologies). Compensation was carried out with fluorescent labelled microbeads (Molecular Probes- Thermo Fisher Scientific Inc.) and fluorescent intensity minus one stained samples were used as control to set quadrants and gates. An analysis of the $CD34^+$ cell percentage of each sample was performed using dual platform; cell counts done with pocH-100i hematology analyzer and flow cytometry analysis of CD34 and CD45 cell frequencies with the help of CD34/CD45/7-AAD-protocol based on ISAGE gating strategy [213]. Unless specified otherwise, all antibodies were purchased from Becton Dickinson Pharmingen (Mississauga, Ontario, Canada). Utilized volumes of antibodies are indicated below in the Table 1 and were determined by titration on target cells. Antibodies were added to CB sample containing approx. 1.5-2.0 X 10^6 cell in 100 µL and incubated for 30 min at 4°C in the dark. CB cells were lysed adding 1 mL of lysis buffer (BD Biosciences) followed by an additional incubation for 10 min in the dark at RT. Next, cells were pelleted (250 X g /6 min) and suspended in in IMDM with 2% FBS solution or in annexin V binding buffer (for viability assay) followed by staining with annexin V Alexa 488 and a dead cell stain kit (SYTOX AADvanced, Thermo Fisher Scientific) following the manufacturer's instructions.

Table 1: Antibodies used for flow cytometry

Antibody	Clone	Conjugation	Quantity used per 100 µL cell suspension
CD19*	HIb19	phycoerythrine (PE)	5 µL
CD14	M5E2	PE	5 µL
CD45	HI30	Allophycocyanine (APC)	3 µL
CD45	HI30	Fuorescein isothiocyanate (FITC)	3 µL
CD33	WM53	PE	3 µL
CD34	581	PE	5 µL
CXCR4	12G5	APC	5 µL
CLA**	HECA-452	FITC	5 µL
CD3	UCHT1	APC	3 µL
CD56	NCAM16.2	PE	5 µL
Human CD41a	HIP8	APC	1 µL
Mouse CD41	MWReg30	FITC	3 µL

* Cluster of differentiation (CD), ** Cutaneous lymphocyte antigen (CLA)

2.3.4. ALDH assay

ALDH activity assay was done following manufacturer instructions (StemCell Technologies, Vancouver, Canada) using flow cytometry. Non-toxic substrate for ALDH can freely diffuse into intact, viable cells and the BODIPY aminoacetaldehyde is converted to the fluorescent product BODIPY aminoacetate by ALDH activity. In short, post-thaw CB samples (both baseline and stored) containing ~2.0 X 10^6 cells were stained with antibodies (CD45 and CD34) and incubated for 30 min in dark at 4°C. CB cells were lysed adding 1 mL of lysis buffer followed by an additional incubation for 10 min in the dark at RT and resuspended cell in aldefluor assay buffer. Finally 5 µL of the activated ALDEFLUOR™ Reagent per mL of sample were added. The negative stain control consisted of stained samples treated with diethylamino-benzaldehyde (DEAB). Both test and control samples were incubated for 50 min at 37°C. All samples were centrifuged (8 min at 1280 rpm) and cell pellets were resuspended in 0.5 mL of ALDEFLUOR™ Assay Buffer. Finally, SytoxAAD was added to 500 µL of cell suspension and incubated for 10 min at RT and run by FACS.

2.3.5. Oxidative stress detection

ROS were measured using Cell ROX deep red (Thermo Fisher Scientific) by flow cytometry. First red blood cells were removed from sample containing ~1.5 X 10^6 CB cells, by addition of 1 ml 1X BD Lysis buffer followed by 10 min RT incubation in dark, and cells pelleted at 300 X g for 8 minutes for both baseline and stored. Cells were resuspended in IMDM with 2% FBS solution and cells were then stained with CD34 (5µL/100 µL cells) and CD45 (3µL/100 µL cells) antibodies and Cell Rox reagent (1000 nM). Incubate the samples at 37°C for 45 min and in the last 15 min of incubation Sytox diluted in PBS (10X) was added at 3ul/100 µL cells. Tert-butyl hydroperoxide (TBHP) was used to induce oxidative stress (positive control) and N-acetylcysteine (NAC) was used to increase the antioxidant capability of the cell (negative control).

2.3.6. Enzyme linked immunosorbent assay (ELISA)

TNF-α levels were measured in plasma for three independent CBUs following manufacturer' instructions (Thermo Fisher Scientific). In short, plasma preparation was performed by mixing Hespan, at a volume corresponding to 20% of the CBU followed by an incubation at RT for 90 min. Plasma was collected carefully leaving RBC and buffy coat layers. Collected plasma from CBUs less than 15 hrs post collection was considered as "baseline" plasma and from same units 48 hrs later collected plasma was "stored" plasma. The plasma was aliquoted and kept at -80°C until use. TNF-α levels were measured in plasma samples post-thaw. Standard curve was performed in duplicate and samples were performed in triplicate. For standard curve the absorbances were obtained for different concentrations over the range of 0−1000 pg/mL human TNF- α. Read the absorbance at 450 nm. Read the plate within 2 hours after adding the stop Solution.

2.3.7. CFU assay

Post-thaw CB cells (40,000 to 50,000) or murine pre-freeze BM cells (40,000) after RBC lysis were plated per plate in MethoCult H4434 (StemCell Technologies) in duplicate and incubated for 14 days in humidified atmosphere 5% CO_2 at 37°C. Colonies were scored manually according to standard morphological classification.

2.3.8. Transplantation of CB cells in NSG mice

Mice work was preapproved by the University of Ottawa animal care committee in accordance with the standards of the Canadian Council on Animal Care and with the Animal for Research Act. The protocol "OPTIMIZATION OF CORD BLOOD STEM CELL TRANSPLANTATION TO ACCELERATE HEMATOPOIETIC RECOVERY" has been given the number BMl-141. Thawed CB TNC were incubated before injection with OKT3 antibody (1 μL of 1mg/ml OKT-3 solution/ $1X10^6$ TNC) (BioXCell, NH, USA) for 20 mins at 4°C to prevent GVHD and OKT3 antibody was diluted in phosphate buffered saline (PBS) with 2% FBS [214]. Eight to twelve weeks old female NOD.Cg-PrkdcSCID Il2rgtm1Wjl/SzJ (NSG, Jackson Laboratory, Bar Harbor, Maine, USA) mice were irradiated (300 cGy 137Cs, Gammacell Exactor 40, Best Thetratonics, Ottawa, ON, Canada) and transplanted i.v. with CB TNC at indicated cell doses. Secondary transplants were transplanted with 85% of the BM collected from primary recipients. All transplants were age and sex-matched and each cohort contained 3-7 mice. For the limiting dilution assay (LDA), baseline and stored mice group received net doses of TNC indicated in Table 2. Irradiated (1500 cGy) helper cells were added to keep the overall cell dose similar to that of the highest transplanted cell dose group.

Table 2: Cell transplantation doses and engraftment outcomes.

Group	Dose TNC (post-thaw)	Long-term PEP [1]		SRC[2]		
		No. recipients positive/total	1/PEP	No. recipients positive/total	1/ SRC	95% CI
CBU 2						
Baseline	25000	4/4	1/55542	3/4	1/15534	5748 - 41981
	50000	2/3		3/3		
	200000	1/2		2/2		
Stored	25000	2/4	1/144920	1/4	1/78836	29944 - 207558
	50000	0/3		1/3		
	200000	2/3		3/3		
p value			NS		0.025	
CBU 3						
Baseline	4000	3/3	1/73458	1/3	1/79010	27981 - 223103
	7000	1/2		1/2		
	150000	0/1		1/1		
	300000	2/2		2/2		
Stored	4000	1/3	1/469717	1/3	1/113038	33550 - 380852
	7000	0/1		1/1		
	150000	0/2		1/2		
	300000	NA		NA		
p value			0.048		NS	

[1]mouse were deemed positive if hPLT level was ≥ 20 hPLT/µL for CBU 2 and if, hPLT level was ≥ 10 hPLT/µL in CBU 3, see Figure 12A for total PEP, [2]mouse were deemed positive if %hCD45+ > 1.0% in CBU 2 and %hCD45+ > 0.06% in CBU 3, see Figure 12B for total SRC.

2.3.9. Human engraftment analyses

Human platelet (hPLT) engraftment analyses were done short-term (4 weeks) and long-term (>14 weeks) after transplantation was done as recently described [215]. Human myeloid (CD45-APC/CD33-PE) and lymphoid (CD45-APC-CD19-PE) leucocytes were analysed from the plasma-poor fraction. Long-term BM engraftment analysis was performed past 18 weeks as indicated. The frequencies of long-term SCID repopulating cells or platelet engrafting progenitors [119] were calculated using the extreme limiting dilution analysis (ELDA) application [216] as indicated in Table 2. Since the engraftment levels between both CBU tested differed largely, different engraftment cut-off had to be used to score mice as positive or negative for human engraftment for each CBU (Table 2).

Each experiment contained negative engraftment control mice injected with PBS. The cut-off point or threshold to consider the mice as positive or negative for engraftment was determined based on each experiment's control group. This approach was performed to avoid the batch effect. The threshold value was set in such a way, so that the control mice was not considered as positive due to the background effect. Also, the baseline or stored mice value that was similar to control mice was considered as negative response. The net number of SCID repopulating cells or platelet engrafting progenitors or platelet engrafting activity were calculated by multiplying the frequency of SCID repopulating cells (or platelet engrafting progenitors) by the net number of TNC recovered per CBU halves post-thaw.

2.3.10. Statistical analysis

Statistical analyses were performed using GraphPad InStat v3.00 and Prism 7 (GraphPad, La Jolla, CA, USA). Unless stated otherwise, mean ± standard error of the mean (SEM) presented, with "n" indicating number of biological replicates (i.e. independent CBU tested). The number of mice per arm and per group are indicated in the figure legends. Significant differences were determined by paired student-t-test for in vitro assays and Mann-Whitney or two-way analysis of variance (ANOVA) tests for engraftment data as indicated.

2.4. Results

2.4.1. Overview of the experimental design

Storage at RT is currently in used by most public CB banks including those in operations in Canada. At the CBS, the mean time between collection and freezing time from 2015 to 2019 for CBU was just under 23 hours (Figure 7A). The experimental design used to test

the impact of storage on the engraftment activity of CBU is presented in Figure 7B. Freshly collected CBU were split in two fractions upon arrival; the first half was processed immediately and served as baseline control (stored at RT for 10.2 ± 1.2 hours, frozen by 13.8 ± 2 hours post-collection). The second half was processed after a 40-hour storage period at RT (stored group, stored for 40.3 ± 0.2 hours, frozen by 43.7 ± 0.2 hours). This delay was selected as it is close to the maximum time allowed so that CBU be cryopreserved by 48 hours post-collection (FACT standard D3.2.6 [209]).

2.4.2. Impact of processing delay on in vitro measures of CBU quality and potency

Viability analysis and potency assays are done by all FACT-accredited CB banks on CBU post-processing and post-thaw to ensure quality and potency. We investigated if the viability assay, ALDH assay and CFU assay would predict a decrease in engraftment activity associated with 44-hours processing.

The net number of TNC was found unchanged between both groups, and all CBU samples (n=7) from both groups passed the minimal threshold for post-thaw cell viability based on DNA-dye exclusion assay for both $CD45^+$ cells (≥40 %) and $CD34^+$ cells (≥ 70%) (data not shown). Annexin V and Sytox staining confirmed that viability of TNC and $CD45^+$ cells were slightly lower in the stored group due to a significant increase in the presence of necrotic cells in stored samples (Figure 8A). In contrast, a slight increase in the proportion of viable $CD34^+$ cells were observed in stored samples.

The frequencies and total number of $CD45^+$ALDH bright and $CD34^+$ALDH bright cells did not differ significantly between both groups (Figure 8B). The only noted difference was that the proportion of ALDH bright cells within the $CD34^+$ subset was reduced in the stored group vs. baseline (mean 80 ±3% vs. 73 ±2%, p=0.04). The net yields of total CFU

and CFU-GEMM were significantly reduced in stored samples (Figure 8C). The later may be of functional consequence since the content in CFU in CBU graft has previously been shown to correlate with engraftment in clinic settings [149, 217].

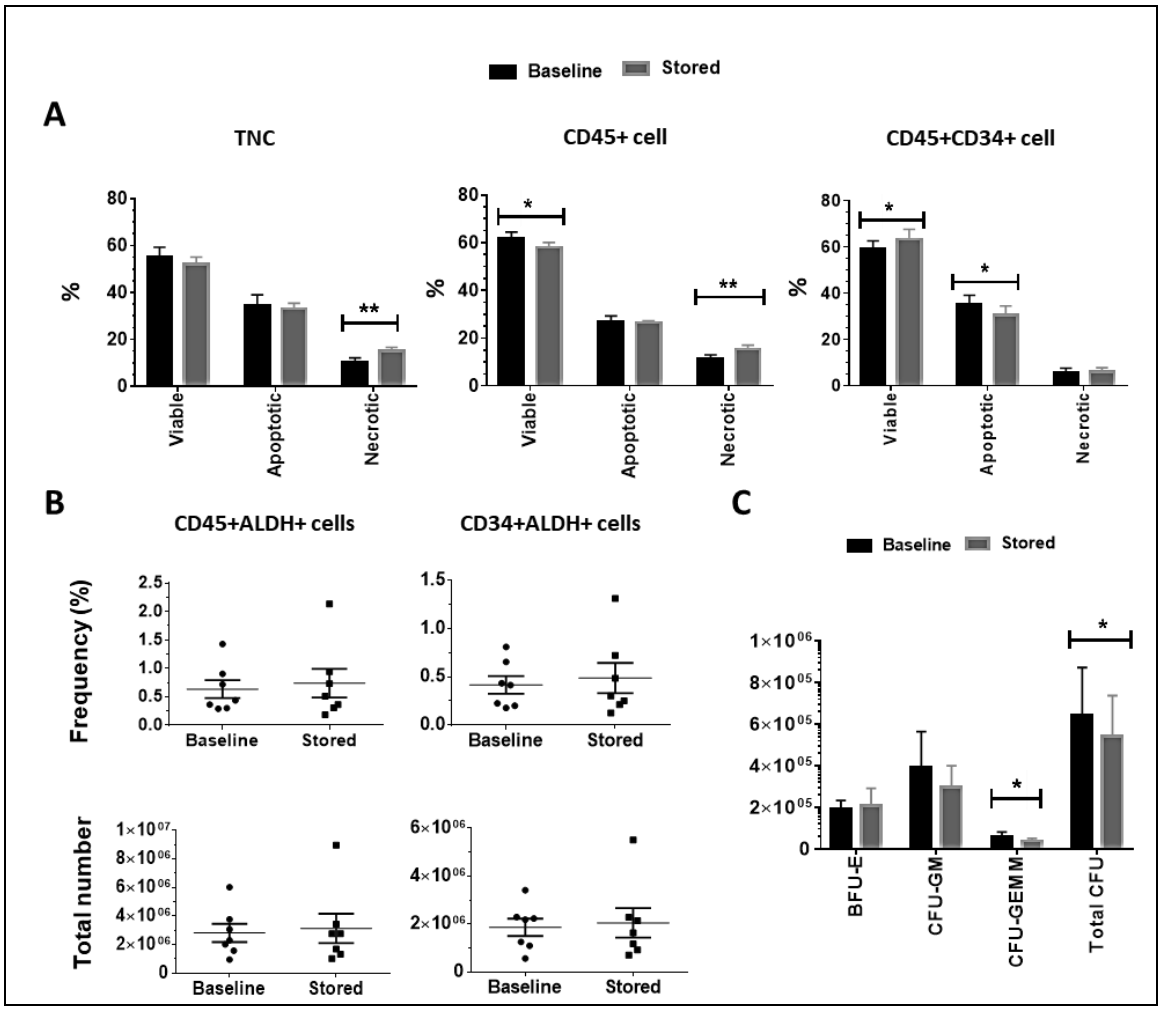

Figure 8: Impact of 40 hours of storage at RT and processing delay on post thaw CB quality.

A) Viability analyses of CBU post-thaw using sytox-AAD and annexin V staining in indicated cell subpopulations. B) Frequency and total number of ALDH subpopulations in CBU post-thaw. C) Net number of CFU progenitors measured in CBU post-thaw. Results presented as mean ± SEM for baseline and stored groups (n=7). * P≤0.05 paired t-test.

2.4.3. Extended storage at RT before processing of CBU is associated with reduced platelet and leucocyte engraftment

The serial transplantation assay was used to assess function and potency of the CB HSC present in CBU from baseline and stored groups (Figure 7B). Thawed CBU aliquots were

transplanted after minimal processing to closely mimic transplant practices. To allow for a fair comparison between both groups and because storage at RT has been reported to decrease cell viability [113], we set out to transplant doses of TNC that contained 7,500 viable CB $CD34^+$, defined herein as annexin Vneg $CD34^+$ SSCLow cells. This staining was done to capture early apoptotic $CD34^+$ cells which are not detected by DNA-based viability dyes and which are known to be devoid of engraftment activity [103]. The post-thaw cell viabilities and cell doses transplanted for each group for all 3 CBU tested are summarised in Table 3.

Table 3: Characteristic of the CBU tested and cell dose transplanted in baseline and stored groups for 44 hours processing delay (40 hours storage at RT).

CBU	**Sample**	**CD34+ (%)**	**Viability (%)[1]**		**Dose TNC (post-thaw)**	
			TNC	CD34+	$x10^6$/mice[2]	$x10^7$/kg
CBU1	Baseline	0.40	94.1	54.6	1.87	9.08
	Stored	0.37	93.0	45.2	2.02	9.80
CBU2	Baseline	0.31	68.9	77.2	2.43	11.80
	Stored	0.73	67.3	87.4	1.02	4.98
CBU3	Baseline	0.61	73.3	30.3	1.24	6.00
	Stored	1.02	73.3	35.1	0.73	3.56
Mean (SD)	Baseline	0.44 (0.2)	78.8 (13.4)	54.1 (23.5)	1.9 (0.6)	9.0 (2.9)
Mean (SD)	Stored	0.71 (0.3)	77.9 (13.5)	55.9 (27.8)	1.3 (0.7)	6.1 (3.3)

[1] Based on annexinV staining, 2 net number of TNC that contains 7,500 annexinVneg CD34+ cells. No significant differences revealed by paired t-test.

Circulating human platelet (hPLT) and leucocyte were measured to track engraftment in the periphery (Figure 9, Figure 10). The kinetics of hPLT levels for the CBU tested are presented in Figure 9A; consistent with previous studies [218, 219], hPLT levels fluctuated highly within groups of humanized NSG mice and between time points. Moreover, hPLT level kinetics varied between the 3 donors tested which reflects the usual variation observed between primary human samples (Figure 9A). Nonetheless, a clear trend of reduced hPLT levels were observed for the 3 CBU tested at most time points in recipients of stored CBU

(Figure 9B). The deficit in engraftment was also seen for leucocytes (Figure 10), levels in baseline groups were frequently superior to that measured in stored groups. The overall reduction in $CD45^+$ leucocytes was due to reductions in both $CD33^+$ neutrophils and/or $CD19^+$ B cells (Figure 10). These results indicate that engraftment in the periphery was lower when CBUs were processed after 40 hours delay with RT storage.

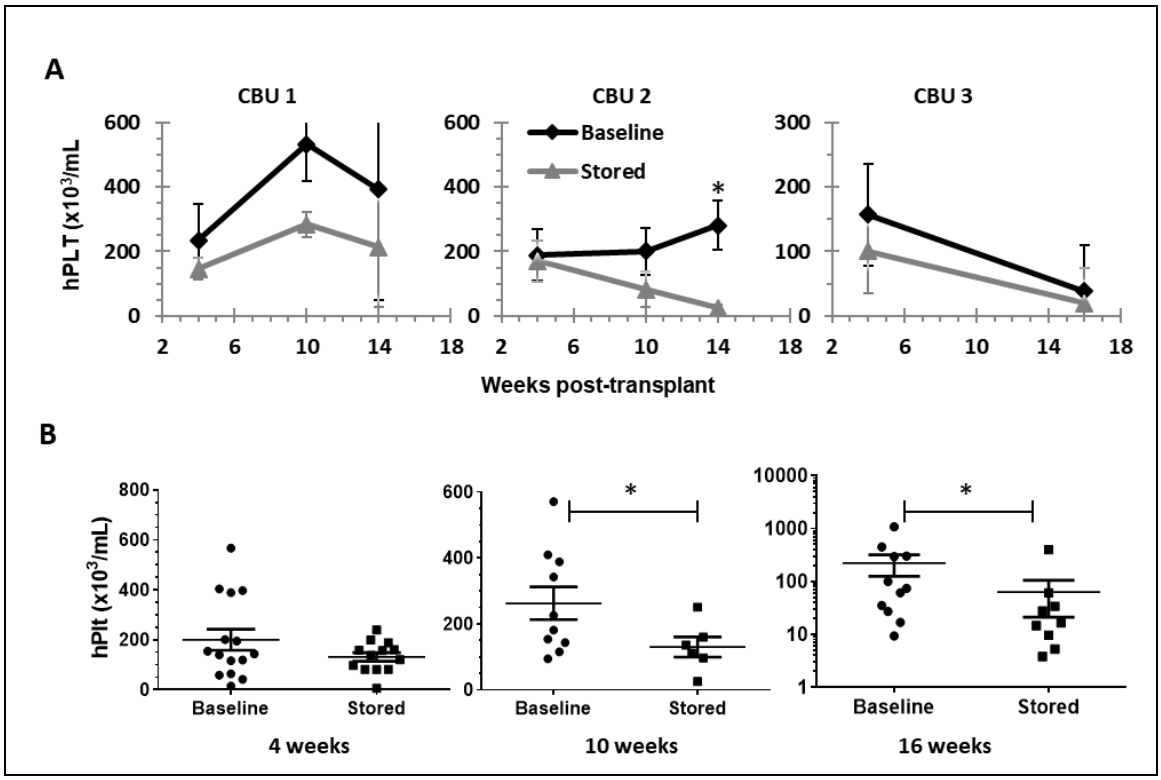

Figure 9: Impact of processing delay on the thrombopoietic activity of CBU.

A) Platelet engraftment kinetics in NSG mice injected with CB cells from baseline or stored CBU half. Levels of human platelets shown as a function of time post-transplantation for 3 CBU tested (mean ± SEM, 3-5 mice per group per CBU). B) Engraftment level of hPLT in baseline and stored group as a function of time post-transplant (mean ± SEM, 9-11 mice per group, n=3). Each symbol corresponds to an individual mouse. The horizontal line and error bars represent the mean ± SEM. Significant differences were determined by Mann-Whitney test * $P \leq 0.05$. NS; not significant.

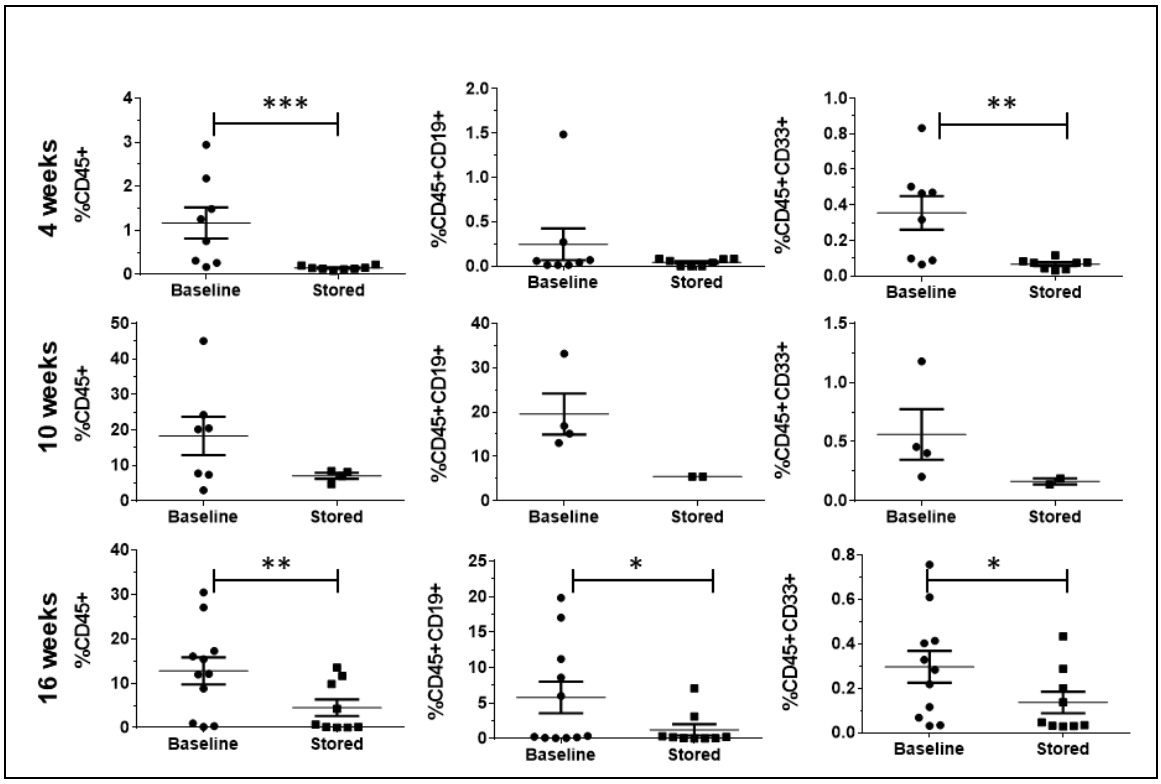

Figure 10: Impact of processing delay on leucocyte engraftment.

A) Percentages of human $CD45^+$ cell, $CD45^+CD19^+$ B cell and $CD45^+CD33^+$-neutrophils at indicated time point in baseline and stored group. Each symbol corresponds to an individual mouse. The horizontal line and error bars represent the mean ± SEM (9-11 mice per group, n=3). Significant differences were determined by Mann-Whitney test * $P \leq 0.05$.

2.4.4. Prolonged processing delay impairs the BM engraftment activity of CBU

Primary recipients were sacrificed at week 18 to investigate the capacity of HSC in the CBUs to support long-term BM engraftment. Human $CD45^+$ cell chimerism was reduced by 2-fold in recipients of the stored CBU (Figure 11A). However, all lympho-myeloid lineages investigated were present in CBU stored cohorts (Figure 11A and Figure 32 in Appendix 8.1) indicating that the engraftment deficit was mostly quantitative in nature. Lineages investigated included all three major lymphoid lineages ($CD19^+$ B-, $CD3^+$ T- and $CD56^+$ NK- cells), $CD14^+$ monocytes-enriched cells, $CD33^+$ myeloid cells, $CD45^-CD41^+$ megakaryocytes and $CD34^+$ stem and progenitor-enriched cells (Figure 11A). Moreover, most lineages were present at similar distributions within the human BM cells in both

groups except for a reduction in the proportion of T cells in stored group for reasons that is unclear at this time (Figure 11B). A 2-fold reduction in the net number of human progenitors (CFU) per mice was also detected in the stored cohort though the difference was short of being significant (Figure 11C).

Serial transplantation remains the best tool to assess the self-renewal activity of long-term HSC. Human BM engraftment in secondary mice was investigated 12-14 weeks post-transplant, for a net cumulative transplant period of 28-30 weeks for each graft. The gap in human chimerism between the baseline and stored groups was exacerbated in the secondary transplants with a mean 10-fold reduction in human $CD45^+$ cells in the stored cohorts vs. baseline (Figure 11D).

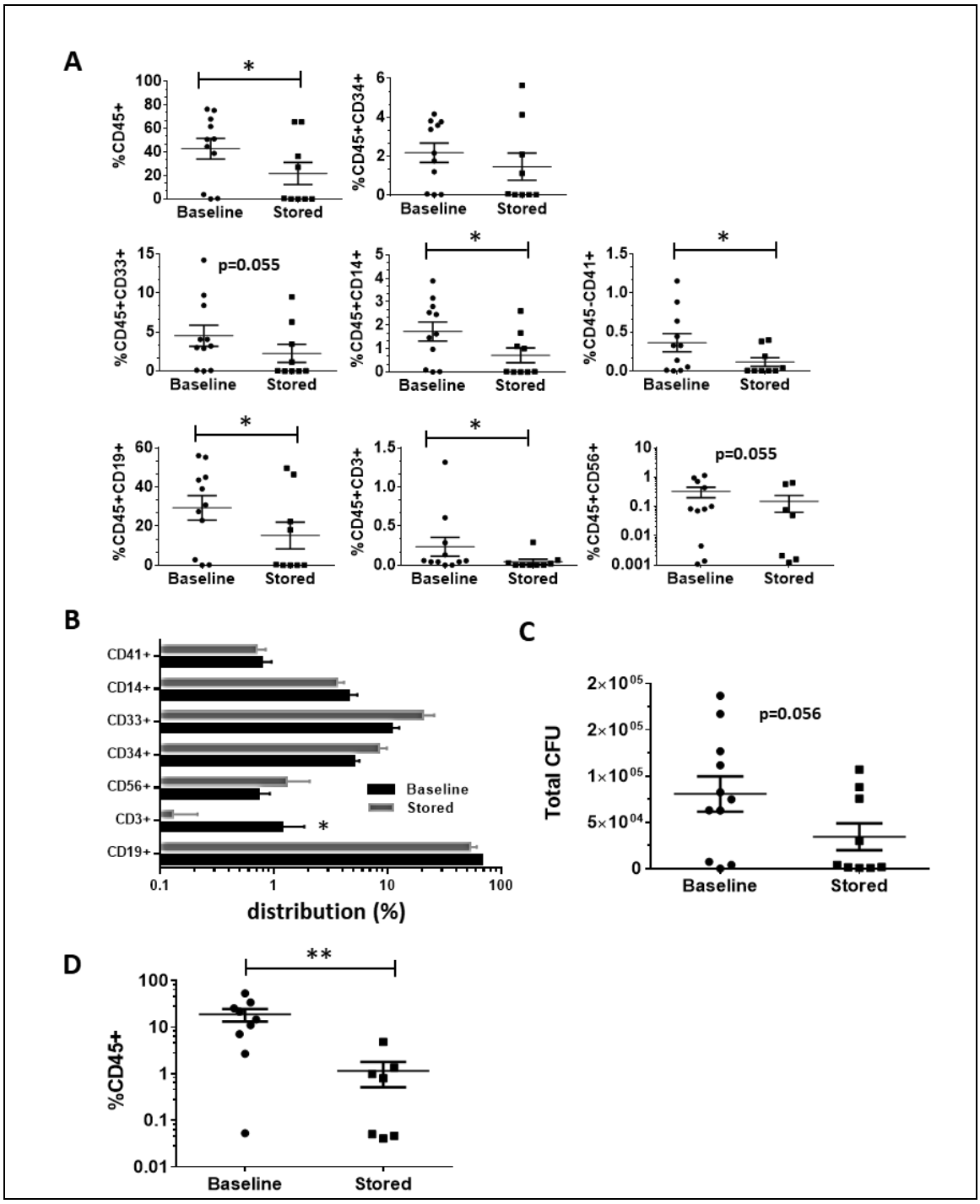

Figure 11: Impact of processing delay on human BM reconstitution.

A) Multilineage human BM engraftment analysis of primary transplants 18 weeks post-transplantation. Each symbol corresponds to a mouse. The horizontal line and error bars represent the mean ± SEM of 9-11 mice per group (n=3). B) Lineage distribution of human BM engraftment in primary recipients (mean ± SEM). C) Total number of human CFU progenitors in the BM of primary recipients (mean ± SEM). D) Human $CD45^+$ BM cell engraftment in secondary recipients. Each symbol corresponds to a mouse. The horizontal line and error bars represent the mean ± SEM (7-9 mice per group, n=3). Significant differences were determined by Mann-Whitney test.

2.4.5. Extended processing delay leads to a loss in engrafting stem and progenitors

The serial transplantation results provided evidences that prolonged storage at RT of CBU can lead to loss of CB HSC function. This phenomenon could be explained either due to a net loss in HSC numbers, and/or due to impaired function of the HSC compartment (e.g. self-renewal impairment). To discriminate between both possibilities, LDA was used with two of CBU tested beforehand to estimate the loss in engraftment activity associated with processing delay. For these experiments, similar doses of CB TNC were transplanted for both groups and the engraftment outcomes are summarized in supplementary Table 2.

The estimated net number of HSC and progenitor cells providing long-term platelet engrafting progenitors and SCID repopulating cells are presented in Figure 12. In line with the reduction in platelet levels, the net number of platelet engrafting activity was significantly reduced in the two CBU tested (Figure 12A). Similarly, the net number of SCID repopulating cells measured at 22-weeks was also reduced in both CBU though the difference between both groups was not significant (Figure 12B). Together these results are consistent with the concept that prolonged storage at RT before processing leads to a deficit in engraftment activity likely due to losses in long-term engrafting stem and progenitor cell numbers.

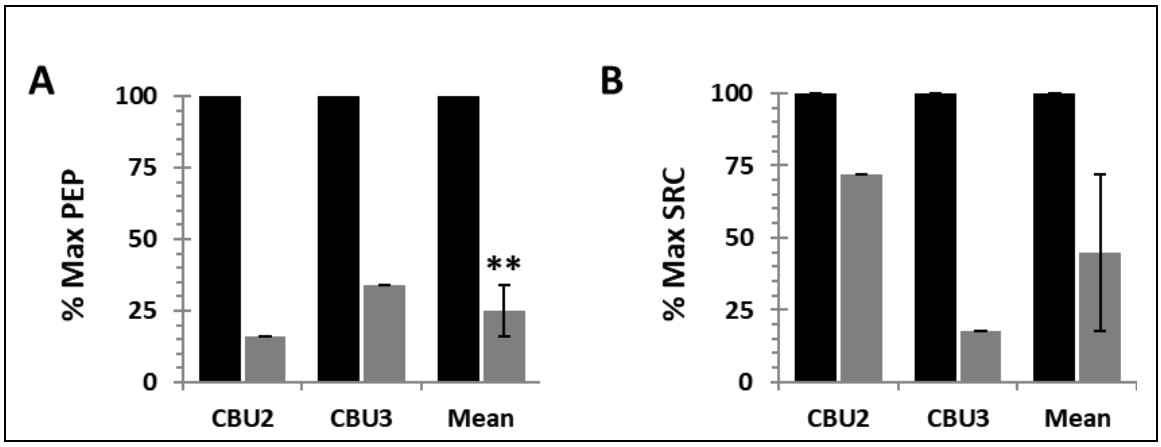

Figure 12: Extended storage at RT reduces the number of engrafting progenitors.

Percentage of maximum numbers of long-term platelet engrafting progenitor (PEP) (A) and long-term SRC (B) measured in baseline and stored groups. Results presented for individual CBU (CBU2 and CBU3) tested and the mean ± SD (n=2). The net number of PEP or SRC were normalized to those calculated for the baseline which was arbitrary set at 100%. ** P≤0.01 paired t-test between stored and baseline groups.

2.4.6. Storage of CBU for 40 hours at RT impacts CXCR4 expression and results in accumulation of ROS

Modulation of the homing efficiency of stem and progenitors has major impact on engraftment outcomes. Homing is mediated in part by the expression of cell surface receptor, such as CXCR4 and fucosylated P-selectin glycoprotein ligand-1 (PSGL-1); increased expression of CXCR4 and increased fucosylation of PSGL-1 (detected herein with CLA antibody) are associated with improved homing and better CB engraftment [220, 221]. The frequency of CLA^+CD34^+, $CXCR4^+CD34^+$ and CLA^+ $CXCR4^+CD34^+$ cells in samples post-thaw were not different between groups (data not shown). Similarly, the proportion of $CD34^+$ cells that were CLA^+ was unchanged by storage at RT. However, a small but significant reduction in the proportion of $CD34^+$ cells that co-expressed CXCR4 was detected (Figure 13A).

The function of HSC has been shown to be affected by ROS though some controversy exist as some papers suggest that high ROS levels in HSC is associated with loss of engraftment function [133], while others reported that ROS positive cells post-thaw have greater CFU

activity and accelerate engraftment [222]. ROS levels were first investigated in CB samples post-thaw. Contrary to our expectations, ROS levels were significantly lower in stored samples when compared to baseline in both $CD45^+$ and $CD34^+$ cells (Figure 13B). We hypothesized that ROS positive cells in stored samples maybe lost during the freezing and thawing process. In line with this, the frequencies of ROS positive CB cells was significantly greater in stored samples in both $CD45^+$ and $CD34^+$ cells post processing before freezing (Figure 13C). TNF-α has been reported to induce ROS in several cell type including stem and progenitor cells [134]. TNF-α levels were measured in baseline and stored samples post processing; increased concentrations of TNF-α of 1.8-, 3.7- and 5.2-folds were observed in stored samples (mean of 19 ± 2 pg/mL in baseline Vs. 66 ± 14 pg/mL in stored, $p=0.04$, $n=3$). Together, these results provide a glimpse of some of the molecular changes that can occur in CB progenitors during storage that may lead to losses in engraftment activity.

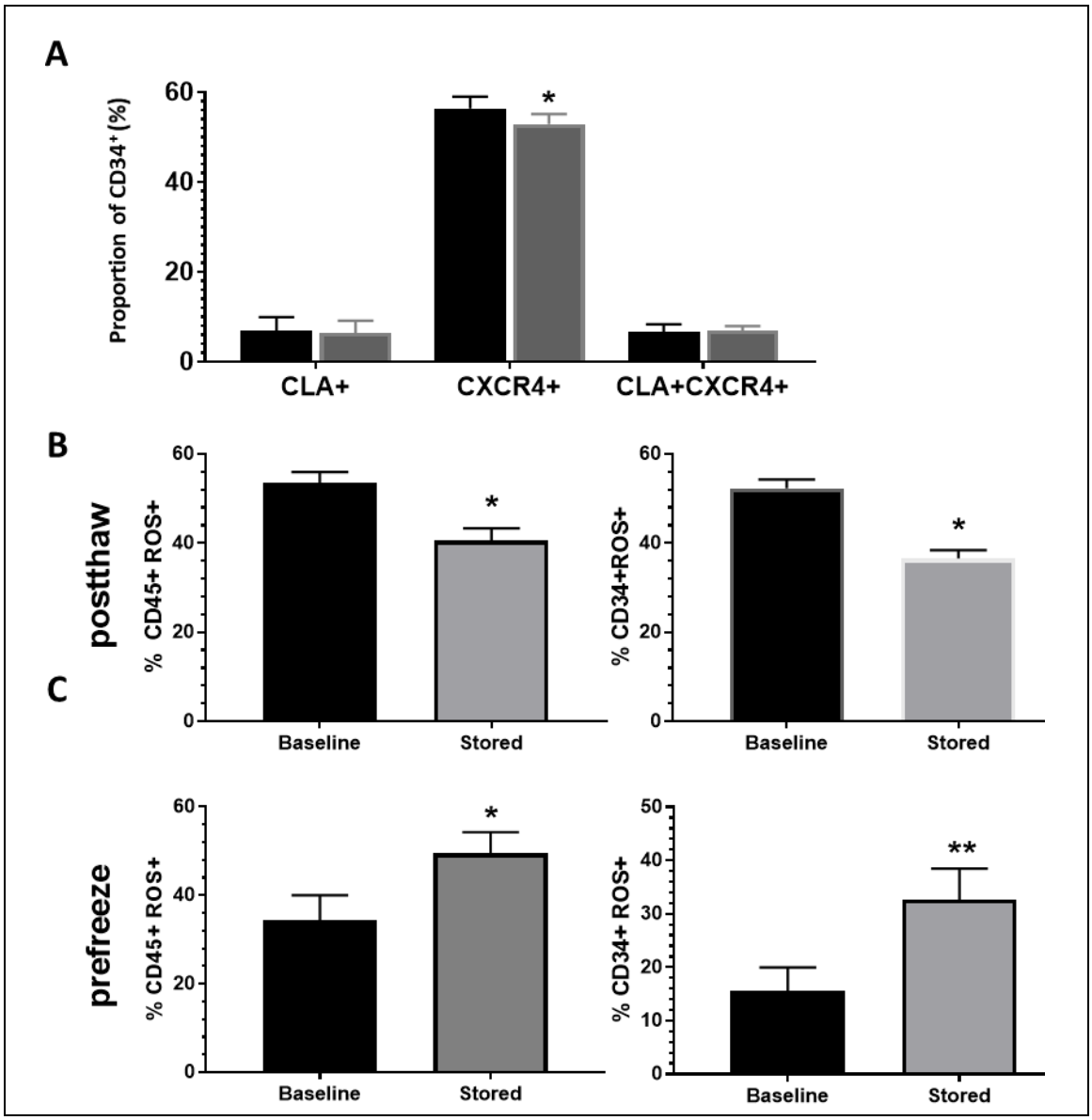

Figure 13: Extended storage of CB CBU at RT results in reduced CXCR4 expression and ROS accumulation.

A) Proportion of CB $CD34^+$ cells that co-expressed CLA, CXCR4 and CLA and CXCR4 in baseline (black bars) and 40 hours stored (grey bars) samples (mean ± SD, n=3). B-C) Percentages of ROS^+ $CD45^+$ and $CD34^+$ cells in baseline (black bars) and 40 hours stored (grey bars) samples post-thaw (panel B) or fresh post processing (panel C) (mean ± SD, n=4). * P<0.05, **<0.01 paired t-test.

2.5. Discussion

A major issue of CB transplantation is the slow hematopoietic recovery, highlighted by long delays in platelet and neutrophil engraftment. As such it is critical to maximize cell recovery and to ensure that stem cells and progenitors be functional post-thaw to preserve

the engraftment activity of CBU. Extended processing delay is one of several variables known to reduce post-processing cell recovery [106, 110, 112, 113, 121, 208]. In the present study, the impact of storage on the engraftment activity of CBU was thoroughly investigated and, potential biochemical changes leading to loss in engraftment were explored.

The engraftment activity of CBU was significantly diminished if CBU were stored for 40 hours at RT before processing. This was established first with the serial transplantation assay; clear reductions in platelet and leucocyte levels were evident short term after transplantation that lasted the entire duration of the experiments. The reduction in engraftment in the periphery was the results of reduced BM engraftment in primary (2-fold) recipients. The engraftment deficiency was more striking in secondary recipients (10-fold). The latter is a clear sign of reduced long-term HSC function in primary CB grafts, likely due to reduced numbers rather than impaired self-renewal activity. Indeed, complimentary results with the LDA supports this assumption as two out of two CBU tested showed engraftment reductions in platelet engrafting activity and SCID repopulating cells, though the low sample size did not provide sufficient power to achieve significant differences for the SCID repopulating cells. One limitation of the current study is that only 3 CBUs were tested in transplant models due in part to difficulties in getting appropriate CBU. However, in support of our results, the engraftment deficit was observed in all CBU tested in both transplant models and, using two different cell dose transplantation schemes (viable $CD34^+$ cells and net TNC). Furthermore, our results are partly supported by work of Louis, *et al*. in which CBUs stored for 3 days at RT had lost most of their engraftment activity [112]. Of note, the loss in engraftment activity associated with long storage (≥ 40 hours) at RT was avoided by limiting storage at RT to 28 hours for full processing by 31 hours post-collection (Appendix 8.3).

How CBU quality should be measured is a constant debate among scientists, cell bankers and transplant specialists. Herein based on annexinV staining, we found that the post-thaw viability of CB TNC and $CD45^+$ cells were slightly reduced after long storage [121], while the viability of $CD34^+$ cells was not but rather slightly increased. The latter is however consistent with 3 studies that reported slight increases or no reduction in the recovery of $CD34^+$ cells following storage at RT [109, 110, 114]. Currently, the majority of banks and transplant centres use the CFU assay as potency assay, and a strong correlation between CFU content and engraftment has been established in clinic [149, 217]. On the other hand, the ALDH assay is an upcoming assay that may one day complement or perhaps replace the CFU assay [156, 223]. Both assays were applied to our samples; in contrast to the reduction seen with the CFU assay, the net number of ALDH bright cells was not significantly different between baseline and 40-hours stored samples, though a significant reduction in the proportion of ALDH bright cells was observed in the $CD34^+$ subset. Hence, in this study the CFU assay predicted best the loss in engraftment activity associated with extended storage at RT.

Our study also provides first insights into the molecular mechanisms that may be responsible for the loss of HSC and progenitors during storage of CB graft. Certainly, the accumulation of cell storage lesions as previously reported in other blood products are likely at play [211]. In line with this, we observed an increase in ROS positive cells in CBU stored for 40 hours at RT before cryopreservation. Our results suggest that the rise of ROS positive CB cells from stored CBU samples is perhaps due to the release of TNF-α in CBU during storage. Indeed, TNF-α is an inflammatory cytokine known to promote ROS production in many cell types including stem and progenitors [134, 135]. TNF-α has also been reported to promote differentiation of HSC [134] and to negatively impact HSC function in transplant experiments [133, 135, 224]. Particularly fitting to our results, TNF-

α mediated increase in ROS levels in murine HSC was shown to impair HSC reconstitution function, including reductions in secondary engraftment [135]. Taken together, this suggest that ROS accumulation due to the release of TNF-α may be partially responsible for the loss in stem and progenitor activity during storage at RT. Whether the addition of anti-oxidant (or TNF inhibitor) post-collection can protect stem and progenitors from ROS-mediated cell lesions during storage will be worth pursing in future work [135].

In addition, we investigated whether cell surface receptors known to be implicated in the trafficking of stem and progenitors to the BM could be affected by long storage. We found a slight reduction in the frequency of $CD34^+$ cells that co-expressed CXCR4, the receptor for the chemokine SDF-1. Increases in CXCR4 expression has been shown to improve homing to the BM and improve engraftment [220]. Its currently unclear whether the observed small reduction in CXCR4 contributes or not to the observed loss of engraftment.

In conclusions, results from our study demonstrate that long (≥ 40 hours) processing delays can lead to loss in engraftment activity when CBU are stored at RT. Therefore, if long storage length is required, hypothermic storage is recommended as it improve cell recovery [109, 110] and may help to preserve the engraftment activity [112]. Moreover, we present clear indications that prolonged storage at RT results in biochemical changes in CBU and CB cells that may explain the loss in stem and progenitor cell activity. Future studies will explore whether modulation of these pathways can prevent losses in HSC activity.

2.6. Acknowledgement

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government of Canada and as a condition of this support, this article must contain the statement “The views expressed herein do not necessarily represent the views of the federal government”. N. Pineault is an NBF Scholar.

CHAPTER 3
SMALL-MOLECULE ICE RECRYSTALLIZATION INHIBITORS IMPROVE THE POST-THAW FUNCTION OF HEMATOPOIETIC STEM AND PROGENITOR CELLS[2]

Article
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Small-Molecule Ice Recrystallization Inhibitors Improve the Post-Thaw Function of Hematopoietic Stem and Progenitor Cells

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[2]Based on an article "Small-Molecule Ice Recrystallization Inhibitors Improve the Post-Thaw Function of Hematopoietic Stem and Progenitor Cells" published in ACS Omega on November 28, 2016. This project was done in full collaboration with synthetic organic and biological chemistry lab of Dr. Robert N. Ben at University of Ottawa. Jennie G. Briard has synthesized and developed the IRI molecules, conducted experimental design, prepared cryosolutions, analysis of the results and wrote the manuscript. Suria Jahan has participated in experimental design, performed work presented in Figure 23 and Table 4 on viability analysis and assisted J. Briard for result interpretation and manuscript preparation that relates to hematopoietic assays. Priya Chandran has participated in progenitor assays. Robert N. Ben was the principal supervisor for this work with assistance from Nicolas Pineault and David Allan that jointly supervised some aspect of the project. Robert N. Ben and Nicolas Pineault edited the manuscript.

3.1. Summary

The success of HSCT depends in part on the number and the quality of cells transplanted. Cryoinjuries during freezing and thawing reduce the ability of hematopoietic stem and progenitor cells (HSPCs) to proliferate and differentiate after thawing. Up to 20% of the patients undergoing umbilical CB transplant experience delayed or failed engraftment, likely because of the inadequate hematopoietic potency of the unit. Therefore, the optimization of cryopreservation protocols, with an emphasis on the preservation of HSPCs, is an important issue. Current protocols typically utilize a 10% DMSO CPA solution. This solution ensures 70−80% post-thaw cell viability by diluting intra-cellular solutes and maintaining the cell volume during cryopreservation. However, this solution fails to fully protect HSPCs, resulting in the loss of potency. Therefore, a new class of CPAs (N-aryl-D-aldonamides) was designed and assessed for the ability to inhibit ice recrystallization and to protect HSPCs against cryoinjury. Several highly active IRI were discovered. When used as additives to the conventional CPA solution, these nontoxic small molecules improved the preservation of functionally divergent hematopoietic progenitors in the CFU and long-term culture- initiating cell assays. By contrast, structurally similar compounds that did not inhibit ice recrystallization failed to improve the post-thaw recovery of myeloid progenitors. Together, these results demonstrate that the supplementation of cryopreservation solution with compounds capable of controlling ice recrystallization increases the post-thaw function and potency of HSPCs in CBU. This increase may translate into reduced risk of engraftment failure and allow for greater use of cryopreserved CBUs.

3.2. Introduction

Transplantation of HSC and HSPC products offers life-saving treatment for patients suffering from hematological cancers and non-malignant hematological diseases such as hemoglobinopathies, immuno- deficiency syndromes, and inherited metabolic disorders [225, 226]. Moreover, HSPCs are increasingly used for novel and emerging regenerative therapies, including the treatment of neurological disorders, cardiac disease, and diabetes [227]. In comparison to BM or mobilized peripheral blood, HSCT using umbilical CB can be associated with delayed neutrophil and platelet engraftment due to the limited volume that can be collected and the resulting reduced dosage of cells [228]. However, CB offers several advantages such as ready availability, reduced risk of GVHD, negligible risk to donor, and less stringent HLA matching requirements [229, 230].

Although stem cell grafts from adult donors are collected and infused as quickly as possible without cryopreservation, Umbilical CB are processed and stored at −196 °C for future use [177]. Moreover, in the case of autologous hematopoietic trans- plantation, patients' cells are collected, cryopreserved, and reinfused following the conditioning treatment to rescue haematopoiesis [177]. Cryopreservation introduces several issues that may compromise the success of transplantation. First, HSPCs are cryopreserved using the CPA, DMSO [99]. The DMSO present in the thawed HSPC product has been shown to have adverse effects on the gastrointestinal, renal, hepatic, central nervous, cardiovascular, and respiratory systems [177, 197, 231-233]. In an effort to reduce toxicity, the removal of DMSO before transfusion has been employed for patients at risk with pre-existing conditions [234-236]. However, Yang, *et al.,* have demonstrated that removing DMSO through repeated washing decreases viability and recovery of CB $CD34^+$ cells [145]. The second issue with cryopreservation is that high levels of apoptotic $CD34^+$ cells (up to 30%) are observed following cryopreservation, and this is correlated with a reduced ability to engraft [103,

145, 201, 237]. Additionally, Sasnoor, *et al.* have found that cryopreservation impairs growth factor responsiveness, and this is correlated with a reduced ability to proliferate and differentiate post-thaw [202-204].

Recently, Ben, *et al.* reported that the supplementation of the CPA solution with mono- and disaccharides possessing the ability to inhibit ice recrystallization improved the post-thaw viability of $CD34^+$ cells from cryopreserved CBU [192]. However, the success of HSPC transplantation is directly correlated with both the number and the quality of cells transplanted [174, 238-244]. A recent study correlated prefreeze and post-thaw cell characteristics to determine the best indicators for successful engraftment and concluded that the CFU dose was the best indicator for successful engraftment [149]. Therefore, we sought to assess the post-thaw clonogenic potential of HSPCs cryopreserved with small molecules capable of controlling ice growth or recrystallization. In this report, a new class of novel small molecules, N-aryl-D- aldonamides (Figure 14), was rationally designed and assessed for their ability to inhibit ice recrystallization. Several very effective IRIs were discovered. When used as additives to the conventional 10% DMSO CPA solution, these nontoxic small molecules improved the post-thaw recovery from hematopoietic progenitors from cryopreserved Umbilical CBUs.

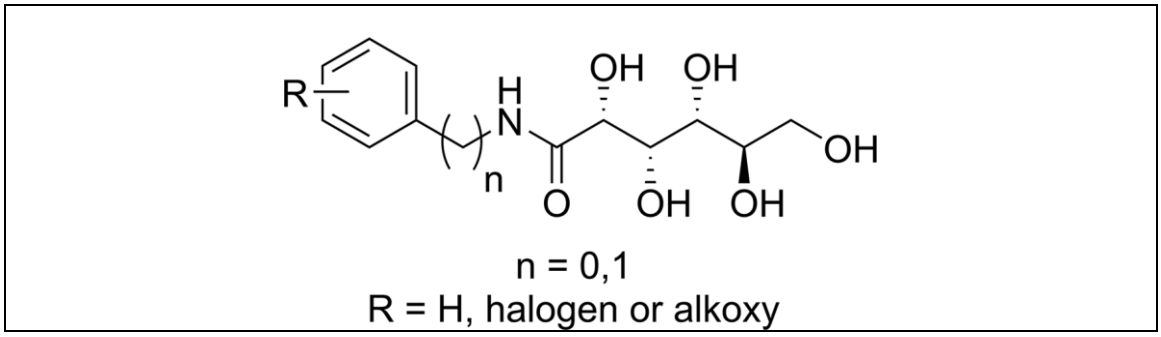

Figure 14: General structure of N-aryl-D-aldonamides

3.3. Results and discussion

3.3.1. Ice Recrystallization Inhibition Activity of N-Aryl- D-aldonamides

A series of N-aryl-D-aldonamides were synthesized and assessed for their ability to inhibit ice recrystallization using the splat-cooling assay (Figure 15). Briefly, compounds were dissolved at 22 mM in PBS, except for compound 2, which was insoluble at 22 mM and assessed at 11 mM. A 10 μL droplet of this solution was dropped onto a precooled (−80 °C) block of polished aluminum to form a frozen wafer, which was then held at −6.4°C for 30 min. The wafer was then photographed, and the ice crystal sizes were calculated using ImageJ analysis software (see appendix 8.2 for details). The inhibitory activity is represented as a percentage of mean grain size (MGS) relative to a PBS positive control for ice recrystallization. As such, a smaller percentage represents a more highly active molecule (i.e., smaller ice crystals in the sample).

Compound 1 was moderately active with a MGS of 65% when compared with the PBS control. However, the addition of a fluorine atom at position 2 on the aromatic ring (compound 2) resulted in a highly active IRI with a MGS of 3% (Figure 15). Interestingly, a fluorine substituent in the para-position (3) abolished much of this activity (MGS of 90%), whereas a chlorine substituent in the para-position (4) partially restored the lost IRI activity. Conversely, when this chlorine substituent was positioned in the ortho-position (5), the compound was inactive. Interestingly, the introduction of a methoxy functional group in the para-position (6) resulted in a very effective IRI with a MGS of 4%, whereas substitution with the same functional group in the ortho-position (7) abolished this activity (Figure 15). Compound 8 with a carbon linker between the aryl ring and the amide bond possessed an IRI activity of approximately 40%. Compound 9, having two fluorine substituents in positions 2 and 6 of the aromatic ring, was a highly IRI active molecule with

a MGS of 13%. However, when the fluorine substituents were in positions 3 and 5 of the aromatic ring (10), the molecule was inactive (Figure 15). In summary, these results show that IRI molecules 2 and 6 are the most potent IRI compounds identified in this directed screen followed by 4 and 9. Conversely, structurally similar compounds 3, 5, 7, and 10 were inactive IRIs that were utilized as controls for the IRI active molecules.

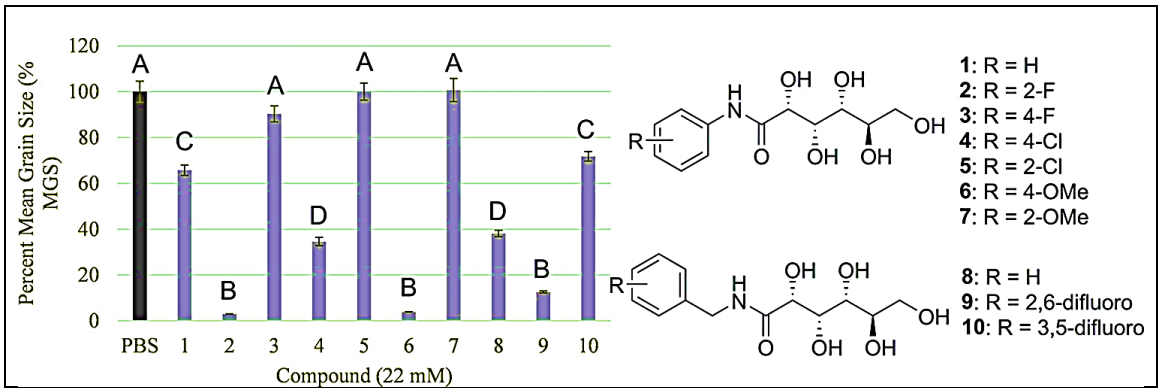

Figure 15: Structures and IRI activities of N-aryl-D-aldonamides 1−10

Compounds were assessed at 22 mM except for compound 2, which was insoluble at 22 mM and tested at 11 mM. IRI activity is expressed as a percentage of MGS relative to a PBS positive control for ice recrystallization. Error bars are reported as the SEM. Statistical significance ($p < 0.05$) was assessed using a one-way ANOVA with a Tukey multiple comparison test. Compounds with similar activities ($p > 0.05$) are marked with the same letter above the bars in the graph, and statistical differences ($p < 0.05$) are indicated by the use of different letters above the bars.

3.3.2. Impact of IRIs on the Post-thaw Viability of CBU CD34$^+$ Cells from Leukocyte Concentrates

In the clinic, leukocyte concentrates (i.e., buffy coat) consisting of TNCs containing myeloid and lymphoid cells and HSPCs are isolated from units by RBC depletion using HES. CB LCs were extracted from the CBU, and the total number of viable CD34$^+$ cells was quantified by flow cytometry using the single-platform ISHAGE method for CD34 cell enumeration. Small-scale cryopreservation experiments were designed to carefully mimic clinical preservation protocols (DMSO and dextran in 0.9% saline) to effectively compare the cryopreservation success of HSPCs that are cryopreserved with IRI active molecules [99].

We hypothesized that by supplementing this CPA solution with IRIs, the concentration of DMSO could be reduced without influencing post-thaw cell viability. Therefore, the LCs were cryopreserved with final DMSO concentrations of 0, 2, 5, and 10% DMSO (using a 1:5 dilution with the LC). These CPA solutions were also supplemented with IRI active compounds 2, 4, 6, and 9. The CPA solution was added to LCs (50 000 $CD34^+$ cells/mL) and then cooled at a rate of 1 °C/min to −80 °C before storage in a liquid nitrogen Dewar flask for 48 h. The samples were then thawed in a 37 °C water bath and diluted, and the post-thaw viability was assessed by flow cytometry using 7-AAD. As shown in Figure 16, there is no increase in the $CD34^+$ cell viability upon supplementation of the 0 and 2% DMSO CPA solution with IRIs. In fact, in most instances, there are statistically significant decreases in the post-thaw viability. When 5% DMSO is used, there is no difference in the post-thaw viability with the IRIs. The same results were obtained with 10% DMSO with the exception of a slight reduction in the viability with 9. These results suggested a cytotoxic effect by IRIs. However, when the IRI compounds were assessed for cytotoxicity using the MTT assay (details in appendix 8.2), compounds 2, 4, 6, and 9 were found to be less cytotoxic at the concentrations utilized when compared with 10% DMSO (Figure 17).

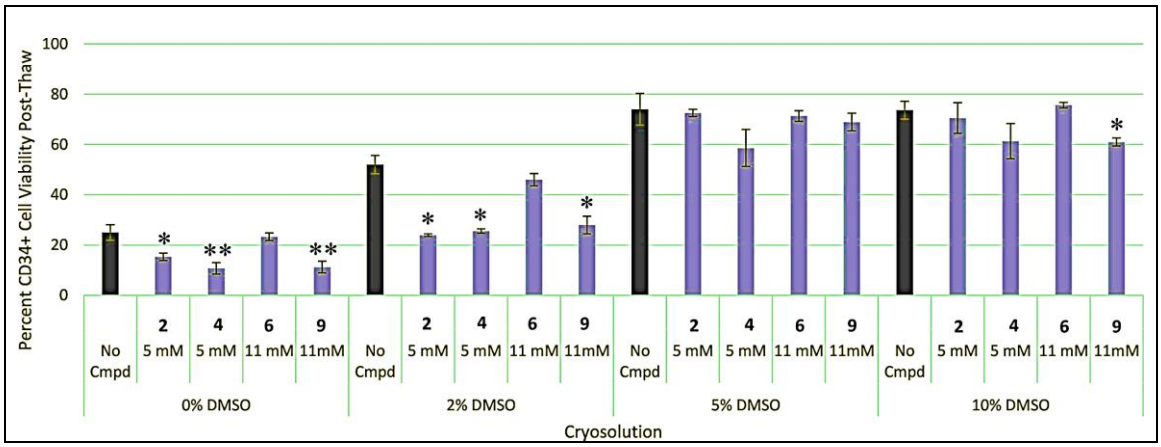

Figure 16: Post-thaw viability of $CD34^+$ cells in LCs cryopreserved in various concentrations of DMSO and IRI active compounds 2, 4, 6, and 9.

Mean ± SEM presented of 3−7 independent experiments. Viability was assessed by flow cytometry using the ISHAGE-gating strategy with 7-AAD. Statistical significance, marked by asterisks, was assessed using ANOVA with a Dunnett's test for comparison to the control (no compound) with a 95% (*) or 99% (**) confidence level.

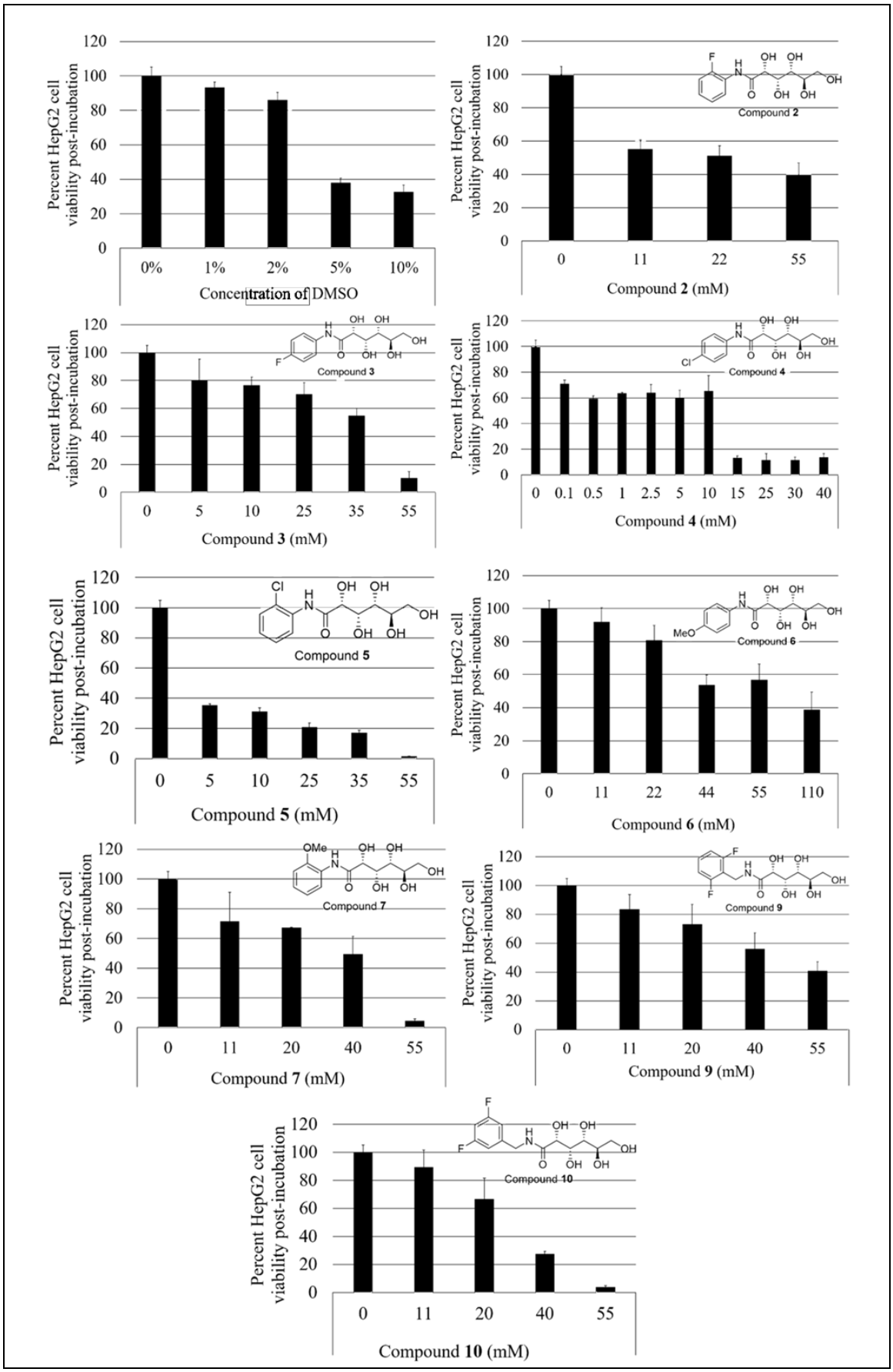

Figure 17: Cytotoxicity of DMSO and N-aryl-aldonamides accessed by the MTT assay.

3.3.3. Impact of IRIs on the Post-Thaw Viability of CBU Committed and Multipotent Progenitors

The success of HSPC transplantation is directly correlated with both the number and quality of cells transplanted [174, 238-244]. The only test capable of identifying the presence of functional HSC is the *in vivo* transplantation assay [245, 246]. This assay measures the ability of transplanted HSPCs (or LCs) to reconstitute the entire blood forming system of an ablated host [245, 246]. In this assay, hematopoietic reconstitution is measured in the peripheral blood samples at ≥4 months post-transplantation [245, 246]. This time consuming and expensive assay is not ideal for predicting the engraftment of banked CBUs. Therefore, transplant centers typically select HLA- matched units based on the TNC count, the number of $CD34^+$ cells, and the number of committed progenitors detected using the CFU assay in the banked units [149]. Importantly, patients who fail to engraft will often engraft successfully when another unit is given, indicating that conventional methods of graft selection are imperfect and do not discriminate units that will be unlikely to engraft [149]. Up to 20% of the patients receiving a CB transplant will fail to fully engraft, in part because of inadequate potency of the unit [45, 65, 247, 248].

A recent study correlated prefreeze and post-thaw cell characteristics to determine the best indicators for successful engraftment and concluded that the CFU (committed progenitor) dose was the best indicator for successful engraftment [149]. Typical post-thaw recoveries of TNCs of a cryopreserved CBU were >80%, but the yield of colonies obtained in CFU assays after thawing was only 21.2% [149]. On the basis of this measure of graft adequacy, only 2.8% of the inventory at a large CB bank would provide an adequate dose to ensure timely engraftment for a patient >50 kg [149]. To overcome this significant limitation, two or three separate umbilical CBUs are often transplanted in adult recipients to obtain a sufficient dose [249]. This is, however, very costly and can lead to increased incidence of

GVHD. The reduction in post-thaw CFU yields may reflect the damage to the unit during cryopreservation, shipping, and thawing. Therefore, the optimization of cryopreservation protocols for HSPCs, with an emphasis on the preservation of clonogenic CFU potential, is an important issue for optimizing storage in CB banks.

Given that the CFU assay is one of the best indicators of engraftment potential [72, 149], we sought to determine whether IRIs could improve the post-thaw recovery of committed progenitors of cryopreserved LCs. IRIs 2, 4, 6, and 9 were selected because of their high IRI activity and their low cytotoxicity levels (Figure 17). First, we tested whether the addition of IRIs 2, 4, 6, and 9 at concentrations lower than those used to assess IRI activity could improve the post-thaw recovery of committed progenitors. The increase in the number of committed progenitors in thawed LCs was observed for most committed progenitors, but the differences versus control (no compound) were not significant (Figure 18).

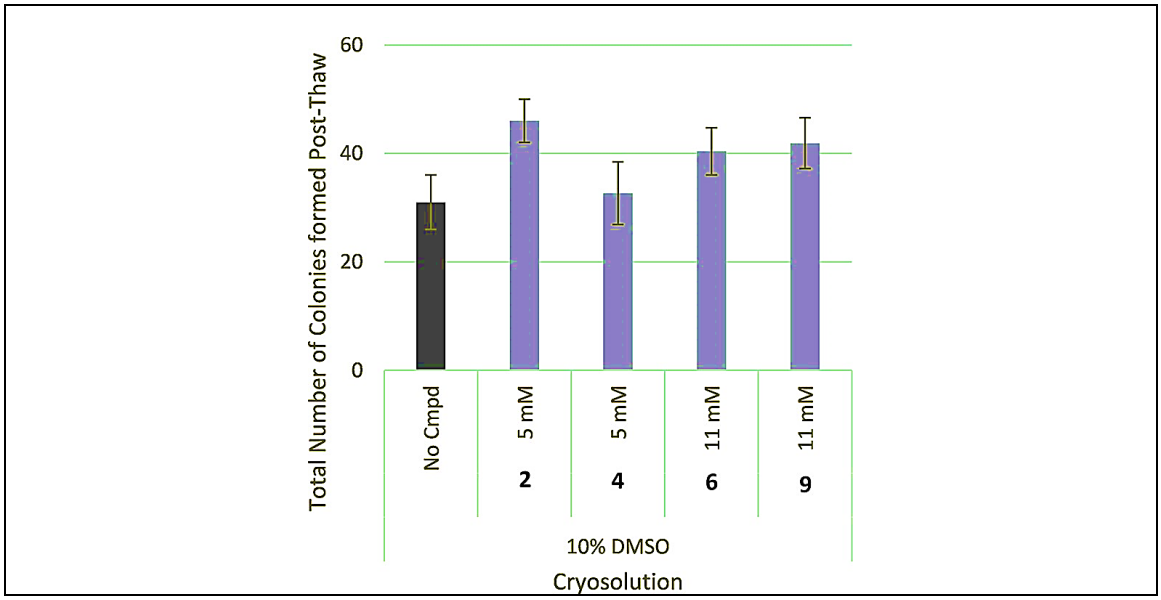

Figure 18: Total number of colonies formed (per 80 µL thawed LC) post-thaw after cryopreservation of LCs in 10% DMSO supplemented with IRI active molecules.

Mean ± SEM presented of 6−10 independent experiments. There is no statistical significance ($p > 0.05$) as assessed using ANOVA with a Tukey multiple comparison test.

A concentration scan was performed to identify the optimal concentration for each IRI. Remarkably, significant increase ($p < 0.001$, 0.01, or 0.05) in the number of progenitors in a dose dependent manner was obtained when the standard CPA solution was supplemented with IRI active molecules (Figure 19). Compound 2 with the highest IRI activity was most effective at 12.5 and 25 mM ($p < 0.05$ and 0.01), producing 2-fold increases in the number of committed progenitor cells when compared with the control. Compound 4 with a moderate IRI activity did not produce significantly more colonies than the control ($p > 0.05$). Compound 6 with an IRI activity equivalent to compound 2 produced the most colonies out of the compounds tested at 27.5 mM ($p < 0.05$), more than 2.4 times greater than the 10% DMSO CPA solution. Finally, compound 9 with the third highest IRI activity promoted progenitor recovery slightly lower than compounds 2 and 6.

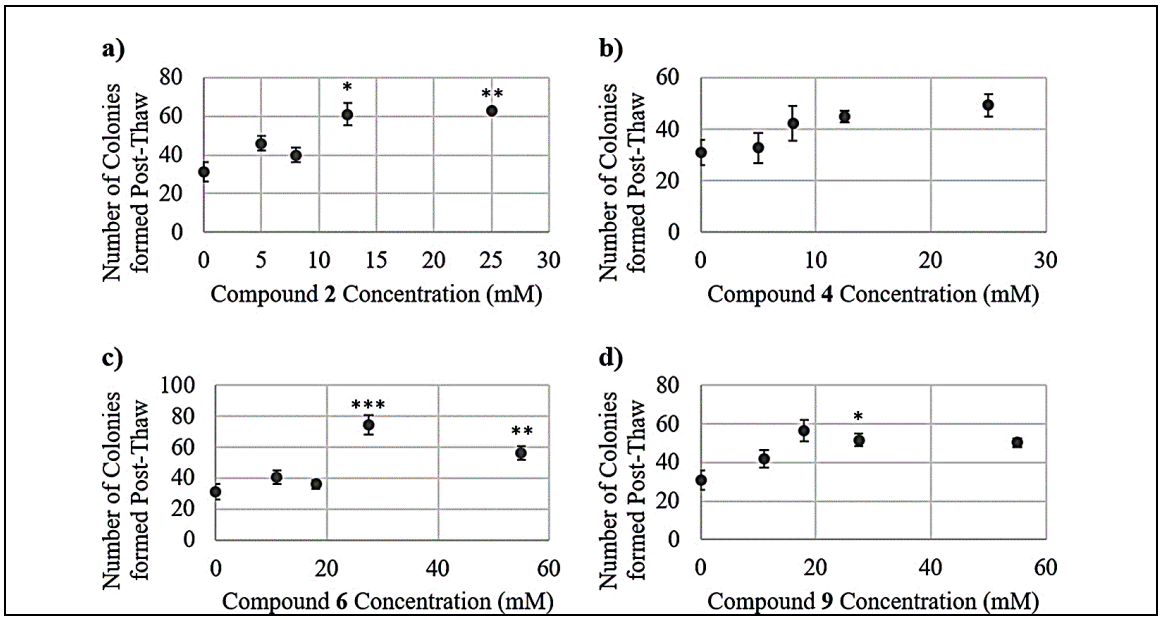

Figure 19: Dose response for the total number of colonies formed (per 80 µL thawed LC) post-thaw after cryopreservation.

Dose response for the total number of colonies formed (per 80 µL thawed LC) post-thaw after cryopreservation of LCs with 10% DMSO supplemented with various concentrations of (A) compound 2, (B) compound 4, (C) compound 6, or (D) compound 9. Mean ± SEM presented of 2−10 independent experiments. Statistical significance, marked by asterisks, was assessed using ANOVA with a Dunnett's test for comparison to the control (no compound) with a 95% (*), 99% (**), or 99.9% (***) confidence level.

There was no significant increase in the post-thaw viability of $CD34^+$ cells when compared with the standard 10% DMSO CPA solution in each case (Figure 20). In fact, the percentages of post-thaw $CD34^+$ cell viability were poorly correlated with colony formation ($R^2 = 0.20$, data not shown). These results further suggest that the post-thaw viability is not an accurate measure of cryopreservation success and CB potency. However, although there were no differences in the percentages of post-thaw $CD34^+$ cell viability, the net recovery of viable $CD34^+$ cells was significantly greater with IRI 2 at 25 mM with a 1.6-fold increase when compared with the control ($p < 0.0001$) and IRI 9 at 18 and 55 mM, with a 1.4-fold increase when compared with the control ($p < 0.05$ and 0.01, Figure 21).

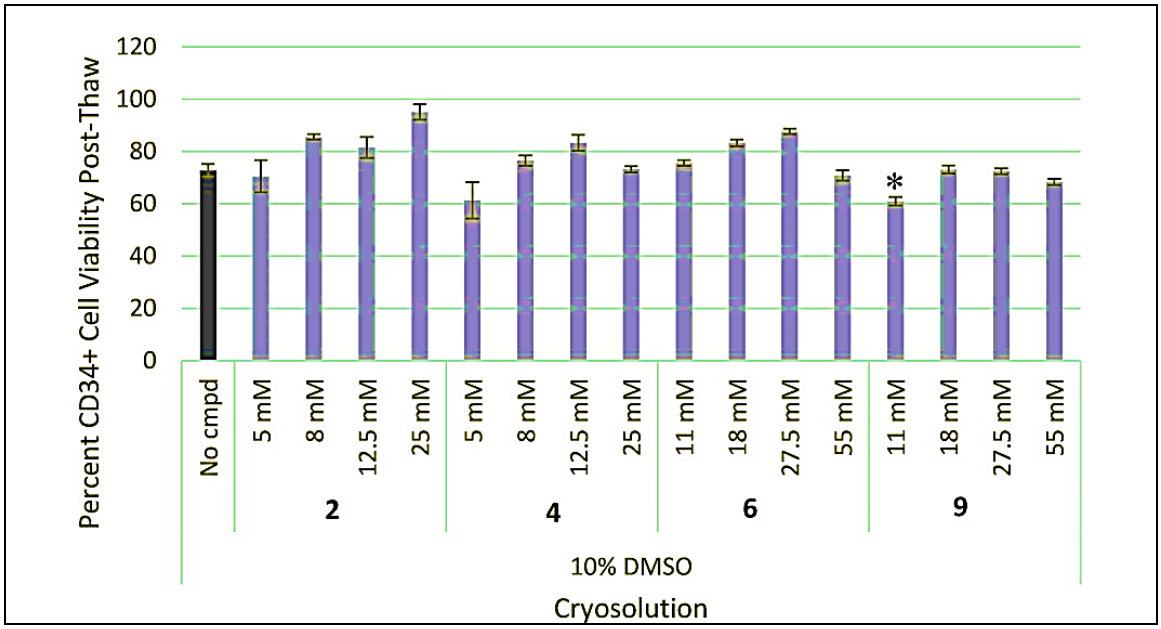

Figure 20: Post-thaw viability of $CD34^+$ cells.

Post-thaw viability of $CD34^+$ cells from LCs cryopreserved in 10% DMSO supplemented with IRI active compounds 2, 4, 6 and 9 at various concentrations. Mean ± SEM presented of 3-7 independent experiments. Statistical significance, marked by asterisks, was assessed using one-way ANOVA with a Dunnett's test for comparison to the control (no cmpd) with a 95% (*) confidence level.

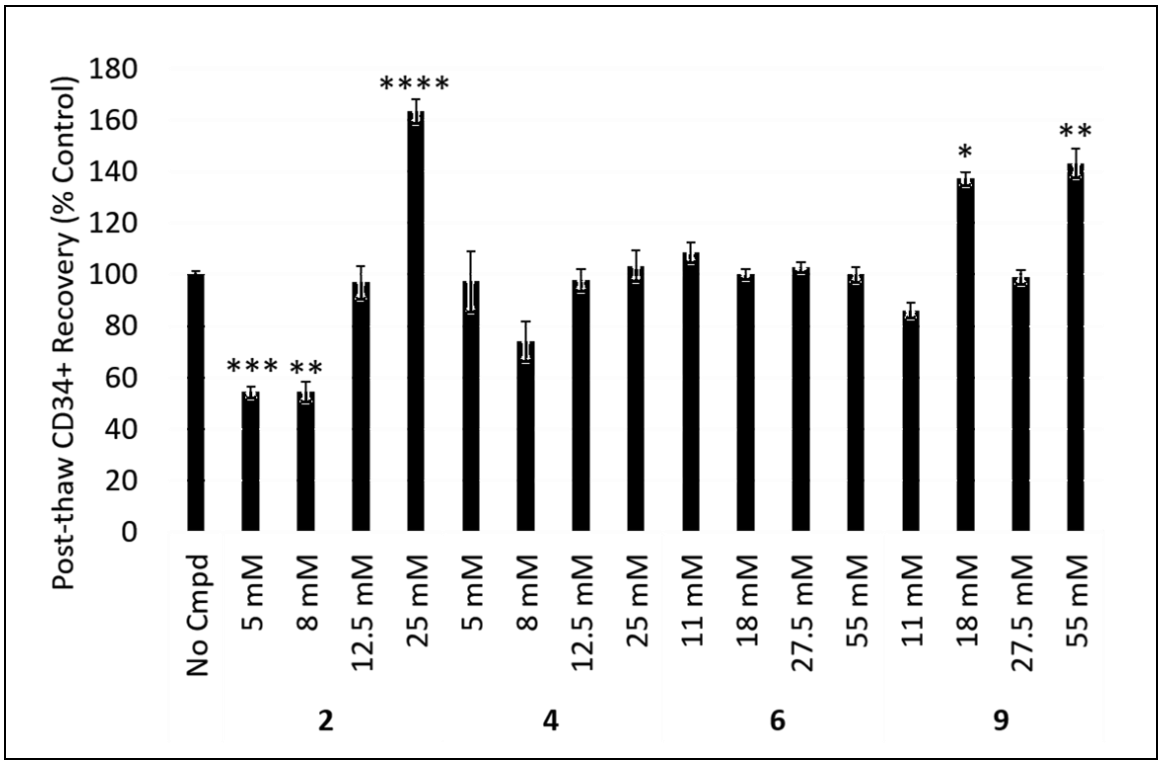

Figure 21: Mean ± SEM of yield of $CD34^+$ cells recovered (n=2-7).

Total number of $CD34^+$ cells were normalized in each experiment to the number obtained in DMSO control (no cmpd) which was arbitrarily set as 100%.

The number of CFU-GMs has been reported to be associated with the transplantation outcome of HSPCs from autologous BM and CB [72, 151-153, 250, 251]. Furthermore, it has been reported that successfully transplanted umbilical CBUs had a higher number of CFU-GMs than those that were not transplanted [150]. LCs supplemented with 6 or 9 resulted in a significant increase ($p < 0.0001$ and 0.05) in CFU-GEMM formation post-thaw (Table 4). Furthermore, IRI 6 increased the post-thaw yield of CFU-GM colonies (Table 4).

Table 4: Number of CFU-GM and CFU-GEMM Formed Post-Thaw and Proportion (Mean % ± SD) of Viable, Necrotic, and Apoptotic CD34+ Cells Measured in LCs Post-Thaw

CPA solution	CFU-GMs[a]	CFU-GEMMs[a]	% viable CD34+[b]	% necrotic CD34+[b]	% apoptotic CD34+[b]
10% DMSO (no cmpd)	7 (±1.5)	3 (±1.0)	65.0 (±5.2)	12.2 (±10.3)	22.9 (±5.1)
10% DMSO + 2 (12.5 mM)	9 (±2.5)	0 (±0.0)	70.3 (±12.0)	9.2 (±9.6)	20.5 (±2.3)
10% DMSO + 2 (25 mM)	11 (±1.1)	2 (±0.7)	69.5 (±10.6)	13.0 (±4.0)	17.6 (±14.6)
10% DMSO + 6 (27.5 mM)	14 (±0.7)[c]	16 (±2.5)[c]	67.3 (±13.2)	14.0 (±9.2)	18.7 (±4.0)
10% DMSO + 9 (18 mM)	11 (±0.7)	8 (±0.4)[c]	70.7 (±7.5)	10.0 (±9.6)	19.3 (±2.1)

[a]Mean ± SEM presented of 4−10 independent experiments. The number of CFU-GM/GEMM (per 80 μL thawed LC) formed post-thaw. [b]Mean ± SD presented of two independent experiments. Proportion of viable (Sytox− Annexin−), necrotic ($Sytox^+$ $Annexin^+$), and apoptotic ($Sytox^-$ Annexin V^+) $CD34^+$ cells presented. [c]Significant difference ($p < 0.05$) compared with 10% DMSO (no compound) as assessed using Student's t- test.

IRIs 2 and 6 were the most effective inhibitors of ice recrystallization and supported the largest increase in the number of post-thaw committed progenitors and $CD34^+$ cell numbers, whereas the least IRI active compound (4) produced a similar number of progenitors as the DMSO control. To investigate whether this protective effect correlated with the ability to control ice growth and recrystallization, LCs were cryopreserved with structurally similar compounds (3, 5, 7, and 10) that did not exhibit an IRI activity. As expected, these compounds had very little effect on the $CD34^+$ post-thaw viability (Figure 22A) and the total number of colonies formed in the CFU assay (Figure 22B). These results suggest that the compounds capable of controlling ice and recrystallization have a

protective effect on the ability of CB CD34$^+$ cells and committed progenitors to proliferate and differentiate post- thaw.

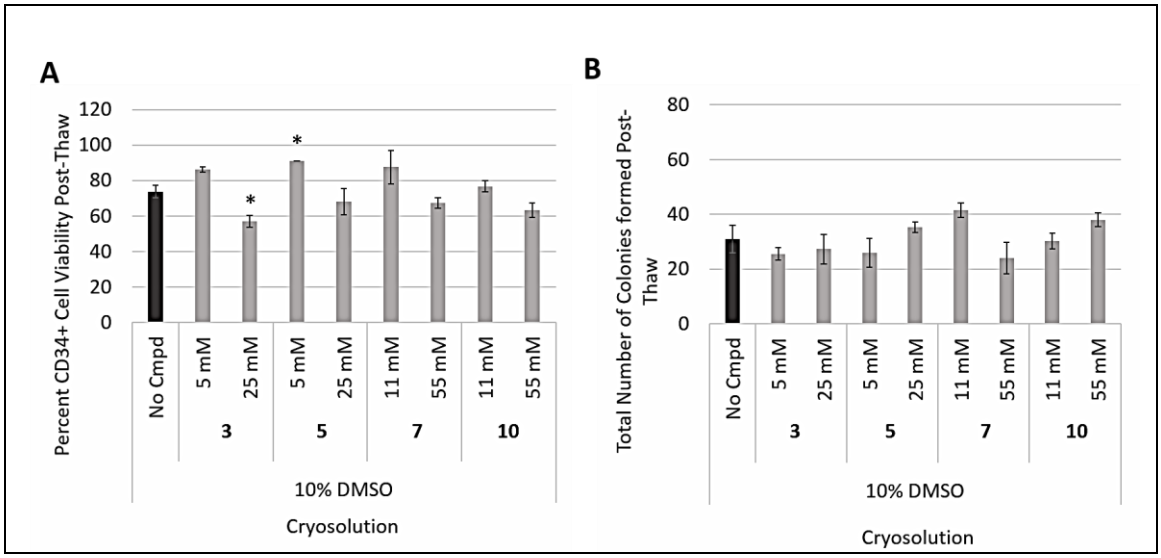

Figure 22: Effect of non-IRI compounds on the viability and committed progenitors in post-thaw LCs

(A) Post-thaw viability of CD34$^+$ cells from LCs cryopreserved in 10% DMSO supplemented with non- active compounds 3, 5, 7 and 10 at max solubility concentration and lowest concentration. Mean ± SEM presented of 3-7 independent experiments. Statistical significance, marked by asterisks, was assessed using one-way ANOVA with a Dunnett's post-test for comparison to the control (10% DMSO) with a 95% (*) confidence level. (B) Total number of colonies formed post-thaw after cryopreservation of LCs in 10% DMSO supplemented with non-active molecules at max and min concentration. Mean ± SEM presented of 4-10 independent experiments. There is no statistical significance ($p > 0.05$) as assessed by one-way ANOVA with a Tukey multiple comparison test.

The increased recovery of CFU indicated that IRIs 2, 6, and 9 improve the post-thaw functional ability of progenitors. To further investigate this cryoprotective effect of IRIs on more immature progenitors, we measured the frequency of multi- potent progenitors in CB LCs supplemented with the most effective IRIs (2, 6) using the stringent LTC-IC assay. This assay measures the frequency of progenitors with self-renewal and differentiation activities significantly superior to progenitors detected using the CFU assay [165]. The frequency of LTC-IC in supplemented LCs was measured using limiting dilution analysis (LDA, Figure 23A). The frequencies of LTC-IC were increased up to 2-fold in post-thaw LCs supplemented with IRIs 2 (25 mM, $p < 0.05$) and 6 (27.5 mM, $p > 0.05$, Figure 23B).

Moreover, the total number of LTC-ICs recovered was always greater in the cryopreserved samples containing IRIs (Figure 23C), resulting in a 2.25-fold increase in LTC-IC yields versus control (Figure 23D).

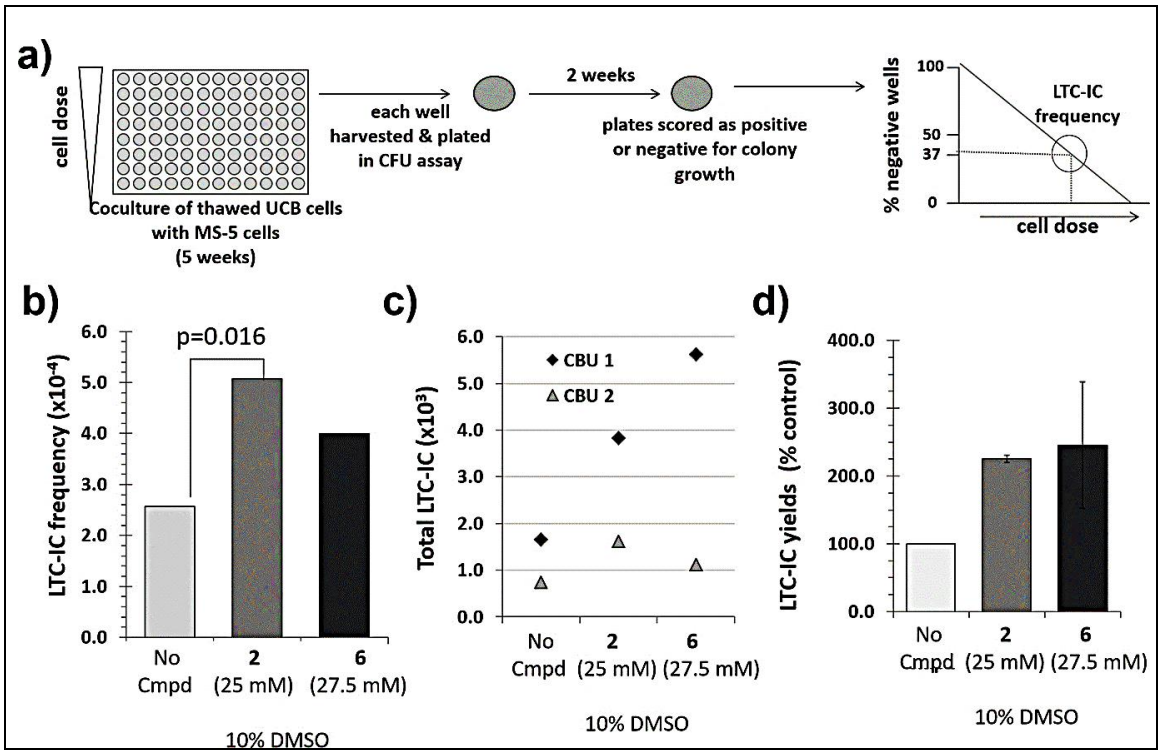

Figure 23: Frequency and the total number of LTC-ICs in post-thaw LCs after cryopreservation with 10% DMSO supplemented with IRIs 2 (25 mM) and 6 (27.5 mM).

A) Overview of LDA used to measure the frequency of LTC-ICs in LCs. (B) Mean frequency of LTC-ICs estimated using ELDA (n = 2). (C) Total number of LTC-ICs obtained per thawed UCB vial for two units tested. The total number of LTC-ICs for each experiment was obtained by multiplying the measured TNC count with the frequency of LTC-ICs for each individual treatment. Each unit is indicated by different symbols. (D) Mean ± SEM of the yield of LTC-ICs recovered (n = 2). Total number of LTC-ICs was normalized in each experiment to the total number obtained in the DMSO control [i.e., LTC-IC yield = (total LTC-IC with sample)/(total LTC-IC with no compound control) × 100%].

Interestingly, the most effective IRIs in this study, 2 and 6, provided the greatest yield of committed progenitors and multipotent progenitors post-thaw. Moreover, the cryoprotective ability of the IRIs 2 and 6 was extended to more immature progenitors. Indeed, the frequency of multipotent progenitors in CB LCs was significantly increased 2.5- and 3.2-fold over 10% DMSO. Moreover, the total yield of multipotent progenitors

was increased 2-fold over control. Importantly, the preservation of progenitor function during cryopreservation was confirmed to be a result of the molecules' ability to inhibit ice recrystallization. Structurally similar compounds devoid of IRI activity failed to improve the recovery of myeloid progenitors. Taken together, these results demonstrate that IRI activity during freezing and thawing of CB LCs protects both committed and immature progenitors against cryo-injury that lead to a significant loss in CB potency.

Finally, we investigated the impact of IRIs 2, 6, and 9 on $CD34^+$ cell integrity and viability post-thaw using Annexin V staining, which detects apoptotic cells. Although no significant differences were identified, the proportion of viable $CD34^+$ cells tended to be higher for all IRI-cryoprotected samples tested when compared with the DMSO control. The results suggest that this may be the result of decreased loss in $CD34^+$ cells to apoptosis and/or necrosis, although these results will need to be confirmed and assessed further in future work. This could be significant because several studies have indicated that $CD34^+$ cells can exhibit high levels of apoptosis post-thaw and as a result significant cell loss is experienced post-thaw [103, 252-254]. Furthermore, it has been shown that CB $CD34^+$ cells with signs of apoptosis (Annexin V^+) fail to engraft when transplanted into immunodeficient mice [103].

3.3.4. Ice Recrystallization Inhibition Activity of N-Aryl- aldonamides in the Presence of CB Cells

The IRI activity of compounds 1−10 was measured utilizing PBS solutions containing 22 mM of compound. Although this is our standard method for measuring the IRI activity of newly synthesized molecules, the CPA solutions were composed of IRI dissolved in 0.9% saline/0.5% dextran (from Leuconostoc spp., Mr ≈ 40 000, Sigma) in water at concentrations different from 22 mM and in the presence of plasma and 10% DMSO. When

assessed in PBS, compounds 2 and 6 were strong IRIs, whereas compounds 3 and 7 were inactive (Figure 15). We predicted that IRI active compounds would retain their IRI activity in the presence of LCs and ice crystals should be noticeably smaller in size in the presence of an active IRI. Thus, solutions of LCs with active and inactive IRI compounds were assessed for ice recrystallization inhibition activity (Figure 24). The 10% DMSO was excluded from the solutions because, in practice, concentrations above 6% (v/v) are very difficult to use in this assay and produce inconsistent results because of large portions of unfrozen solution in the ice wafer [255]. Compared with PBS (Figure 24, panel A), blood plasma alone possesses remarkable IRI activity, with a MGS of 13% (Figure 24, panel B). This is not surprising as plasma contains many different lipids, carbohydrates, proteins, and salts. LCs with saline/dextran (CPA solution without DMSO or IRI) are less active (24% MGS) when compared with plasma alone that can be attributed to the dilution of plasma with saline/dextran (Figure 24, panel C). LCs in plasma and saline/ dextran supplemented with compounds 2 (25 mM) and 6 (27.5 mM) show increased IRI activity when compared with LCs in plasma and saline/dextran alone with activities of 9 and 3%, respectively (Figure 24, panels D and E). By contrast, ineffective IRIs 3 (25 mM) and 7 (55 mM) that are structurally similar to 2 and 6, respectively, had IRI activities of 18 and 17%, respectively (Figure 24, panels F and G). These results obtained in LCs confirm the previous results of IRI activity and support the concept that IRI active compounds 2 and 6 are potent inhibitors of ice recrystallization in CB cells during cryopreservation.

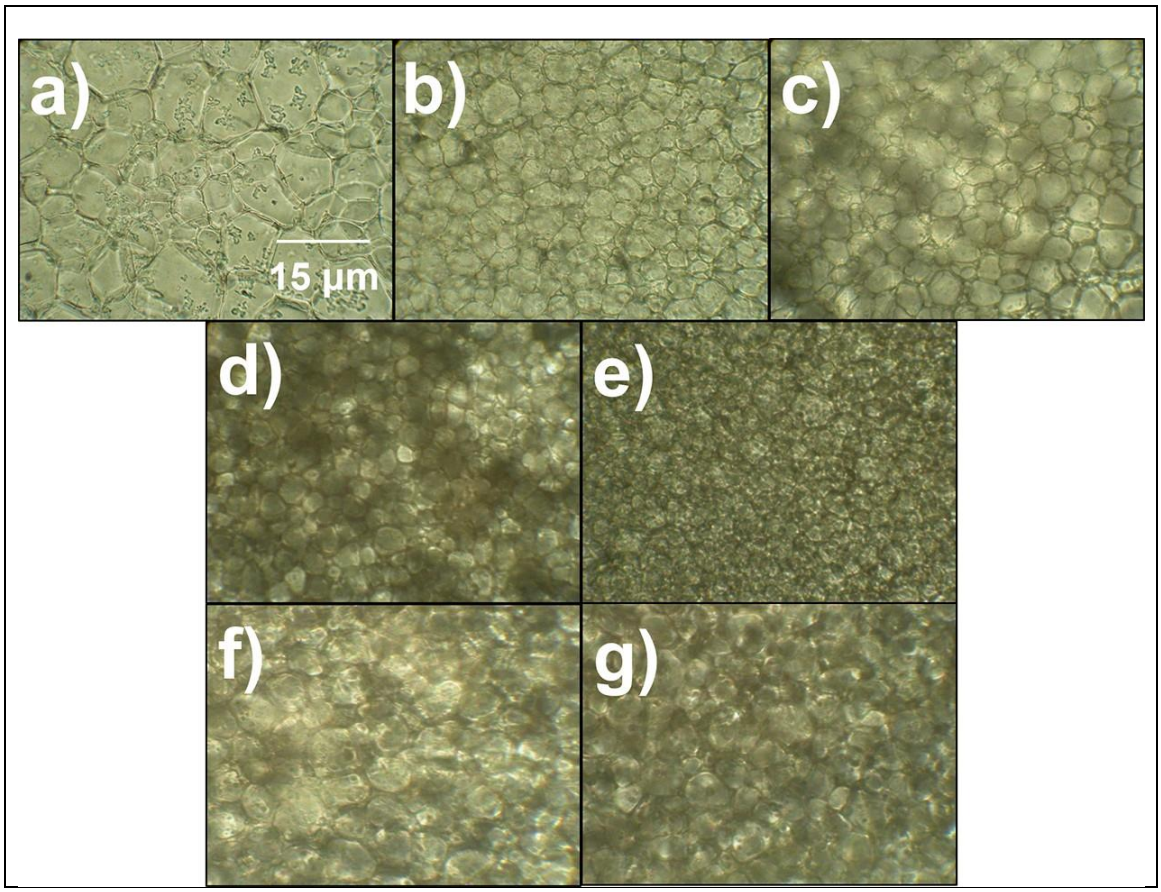

Figure 24: Images of ice crystals when LCs are in the presence of IRI active compounds after 30 min of ice recrystallization (at −6.4 °C).

A) PBS positive control for ice recrystallization. (B) Blood plasma. (C) LCs diluted with saline/dextran. (D) LCs in saline/dextran supplemented with 2 (25 mM). (E) LCs in saline/dextran supplemented with 6 (27.5 mM). (F) LCs in saline/dextran supplemented with 3 (25 mM). (G) LCs in saline/ dextran supplemented with 7 (55 mM).

In summary, this study identified a series of small molecules capable of controlling ice growth and recrystallization. Compounds 2 and 6 with the most potent IRI activity significantly improved the post-thaw recovery of committed progenitors and multipotent progenitors when supplemented with the standard CPA solution in LCs. These results together with the strong correlation between engraftment and net TNC and progenitor content (i.e., dose) reported in clinical studies support the hypothesis that the use of IRIs during cryopreservation could improve the engraftment capacity of CB. Future work will aim to test this hypothesis by performing the transplantation of IRI-cryopreserved CB cells into immunodeficient mice. Moreover, the capacity of these IRIs to protect other cellular

products such as mesenchymal stromal cells will also need to be investigated. In conclusion, this study demonstrates that the inhibition of ice recrystallization during freezing and thawing through the addition of IRIs significantly improves the post-thaw potency of the cryopreserved CB.

3.4. Experimental section

3.4.1. Splat-Cooling Assay for Ice Recrystallization Inhibition Activity

The assessment of IRI activity has been previously described. Briefly, IRI activity is determined using a method termed “splat-cooling” assay [256]. In this method, an analyte is dissolved in PBS, and a 10 μL droplet of this solution is dropped from a micropipette through a 2 m high plastic tube (10 cm in diameter) onto a block of polished aluminum precooled to approximately −80 °C. The droplet freezes instantly on the polished aluminum block. The resulting wafer is carefully removed from the surface of the block and transferred to a cryostage held at −6.4 °C for annealing. After a period of 30 min, the wafer is photographed between crossed polarizing filters using a digital camera (Nikon CoolPix 5000) fitted to the microscope. A total of three images are taken from each wafer, and this process is repeated with two additional wafers. During flash freezing, ice crystals spontaneously nucleate from the super cooled solution. These initial crystals are relatively homogeneous in size and quite small. During the annealing cycle, recrystallization occurs, resulting in a dramatic increase in the ice crystal size. Image analysis of the ice wafers was performed using domain recognition software. IRI activity is reported as a percentage of MGS of ice crystals in the presence of the solute when compared with the MGS of ice crystals in a control solution of PBS.

3.4.2. Collection and Processing of Human Umbilical

Umbilical CB was collected following healthy term delivery and informed consent from mothers, in accordance with institutional approval from the Research Ethics Board of The Ottawa Hospital (protocol 2006460-01H). The CBU was processed within 48 h of collection. The CBU was diluted with 6% hetastarch (Hespan) to obtain a final concentration of 1% hetastarch and incubated for 10 min at RT [8]. The tubes were then centrifuged at 50g at 10 °C for 15−20 min. For total volumes up to 30 mL, 15−17 min centrifugation was carried out, and for total volumes of 30−35 mL, 17−20 min centrifugation was carried out. The supernatant and buffy coat (plasma and leukocytes) were removed carefully (not collecting RBCs) from the tubes and were collected in a 50 mL Falcon tube. This tube was centrifuged at 400g and 10 °C for 10 min to pellet cells. The plasma was removed and kept on ice. The cell pellet was resuspended and combined in plasma to a total volume of 20 mL (LC). The LC was kept on ice for the duration of its use. Total mononuclear ($CD45^+$) and $CD34^+$ cell concentrations and viability were determined using flow cytometry.

3.4.3. Cryopreservation of $CD34^+$ Cells

Aliquots of LC containing 50 000 $CD34^+$ cells were added to 2.0 mL cryovials. Cryovials were centrifuged at 400g and 10 °C for 5 min, the supernatant was removed, the plasma was added, and the cells were suspended by pipetting. Pre-made CPA solutions were added, and the cells were mixed by pipetting. For specific volumes of plasma and CPA solutions, see appendix 8.2. Cryovials were placed in a Mr. Frosty rate-controlled freezing container, which was then placed in a −80 °C freezer for 24 h. After 24 h, the cryovials were transferred to a liquid nitrogen Dewar flask for storage until thawed for analysis.

3.4.4. Post-Thaw Recovery and Viability of $CD34^+$ and $CD45^+$ Cells from LCs

Flow cytometry analysis was performed using the ISHAGE-gating strategy (International Society of Hematotherapy and Graft Engineering) for $CD45^+$ and $CD34^+$ cell measurements [213]. Frozen LC samples were thawed in a 37 °C water bath and analyzed without washing. In short, the LC was diluted with 10-fold Dulbecco's phosphate buffered saline (DPBS), 200 µL was stained with CD45 FITC and CD34 phycoerythrin (PE) antibodies, and the mixture was incubated in the dark at RT. 7-AAD was added and incubated for 5 min. Finally, 20 µL of Countbright counting beads (Life Technologies) was added, and the suspension was diluted to 1 mL with 1× RBC lysis buffer (Bio Legend). Samples were analyzed using a Beckman Coulter Gallios flow cytometer. The proportions of viable, apoptotic, and necrotic cells were measured by staining LC with CD34-PE, CD45RA-allophyco- cyanin (APC), Annexin V-FITC, and Sytox following the manufacturer's directions (Life Technologies).

3.4.5. CFU and Long-Term Culture-Initiating Cell (LTC-IC) Assays

Cryovials were thawed in a 37 °C water bath and diluted with 900 µL of Iscove's modified Dulbecco's media (IMDM) (10% fetal bovine serum (FBS), 1% penicillin/ streptomycin). Cryovials were placed on ice until washed. The samples were mixed by pipetting, and 80 µL was transferred to a 15 mL Falcon tube. IMDM (10% FBS; 5 mL) was added, and this mixture was centrifuged at 1100 rpm for 6 min. The supernatant was removed by aspiration, and the cell pellet was resuspended in 1 mL of IMDM (2% FBS). The suspension was mixed by pipetting. 150 µL of this cell suspension was added to 3 mL of Methocult media, and plating was performed as previously described [257]. Colonies were counted and scored at day 14 according to the Standardized Guide provided by manufacturer. LDA was used to measure the frequency of LTC- IC in thawed cryovials by

plating decreasing doses of CB. TNCs (250, 600, 1200, 3000, 6000, 9000, 18 000, and 30 000) were co-cultured for 5 weeks with the stromal cell line MS-5 on 0.1% gelatin precoated 96-well flat bottom culture plates in a human long-term culture medium (Myelocult MS 300) with hydrocortisone. Half medium change was carried out weekly. After 5 weeks, the entire well content was transferred to 1 mL of methylcellulose (MethoCult H4434), and the plates were scored for colony growth or not after 2 weeks. Each dose was tested in 4−9 replicates. LDA was carried out using the ELDA web-based application [216]. All reagents for progenitor assays were from StemCell Technologies.

CHAPTER 4
INHIBITING ICE RECRYSTALLIZATION DURING CRYOPRESERVATION OF CORD BLOOD GRAFTS IMPROVES PLATELET ENGRAFTMENT[3]

[3]Based on an article "Inhibition of ice recrystallization during cryopreservation of cord blood grafts improves platelet engraftment" published in Transfusion on March 18, 2020. This project was done in full collaboration with synthetic organic and biological chemistry lab of Dr. Robert N. Ben at University of Ottawa. First author Suria Jahan has participated in all experimental design, CB processing, in vitro assays, mice transplantation assays, data analysis and wrote the manuscript. Madeleine K. Adam has synthesized the IRI molecules, prepared cryosolutions, took part in some of the in vitro assays and edited the manuscript. Javed K. Manesia and Emily Doxtator have participated in some in vitro assays and mice sample processing. Both Nicolas Pineault and Robert N. Ben jointly supervised the whole project and edited the manuscript.

4.1. Summary

Background: Platelet engraftment following cord blood (CB) transplantation remains a significant hurdle to this day. The uncontrolled growth of ice, a process referred to as ice recrystallization, is one of several mechanisms that lead to cell loss and decreased potency during freezing and thawing. We hypothesized that reducing cell damage induced by ice recrystallization in CB units (CBU) would reduce losses of stem and progenitor cells and therefore improve engraftment. We previously demonstrated that the ice recrystallization inhibitor (IRI), N-(2-fluorophenyl)-D-gluconamide (IRI 2), increases the post-thaw recovery of CB progenitors. Herein, we set out to ascertain whether IRI 2 can enhance platelet and bone marrow (BM) engraftment activity of HSCs in cryopreserved CBU using a serial transplantation model.

Study Design and Methods: CBU were processed following standard volume/red cell reduction procedure and portions frozen with DMSO supplemented or not with IRI 2. Thawed CB samples were serially transplanted into immunodeficient mice.

Results: Our results show that supplementation of DMSO with IRI 2 had several beneficial effects. Specifically, higher levels of human platelets were observed in the peripheral blood ($p<0.05$, $n=4$) upon transplant of CBUs preserved with the IRIs. In addition, human BM chimerism and the number of human CFU progenitors in the BM were superior in IRI 2 recipients compared to DMSO recipients. Moreover, IRI 2 had no negative impact on the multilineage differentiation and self-renewal activities of HSCs.

Discussion: Taken together, these results demonstrate that supplementation of a hematopoietic graft with IRI can improve the post-thaw engraftment activities of HSC.

4.2. Introduction

Cryopreservation is the most reliable means of conserving HSC grafts for transplantation. However, even using conventional cryopreservation protocols and cell-permeating cryoprotectants such as DMSO or glycerol, significant cellular damage occurs, and this decreases the post-thaw viability and often impairs the functional capacity of cells [115, 258, 259]. This happens in part, as a result of the uncontrolled growth of ice (recrystallization) in the sample during the freezing and thawing [260-262]. While conventional cryoprotectants can increase post-thaw functionality, unlike IRIs they function via different mechanisms do not effectively control the growth of ice [260]. Thus, technologies that inhibit ice recrystallization and increase post-thaw viability and functionality, constitute an important opportunity to improve cryopreservation. Improvements in cryopreservation processes are urgently required for the clinical translation and full market deployment of cellular and regenerative therapies.

Umbilical cord blood (CB) stem cells have emerged as an alternative source of HSC for allogeneic transplantation since the first CB transplantation was successfully performed in 1988 [43]. Minimizing cell loss during the processing of CB units (CBU), cryopreservation and the thawing of samples is critical, since the starting cell content is far smaller compared to apheresis grafts. Moreover, significant improvements in engraftment and overall survival have been achieved with CB transplantation, such as disease free survival and reduced incidence of chronic graft-versus-host disease have been reported with CB over matched unrelated donor [263]. However, one significant disadvantage that remains with CB transplantation is the very slow engraftment kinetics of platelets, with a median in engraftment of 43 days versus 28 days for matched unrelated donors. Neutrophil engraftment is also slightly slower but not as slow as with platelets [263]. Since the success of transplantation and engraftment is correlated with both the number and the quality of

cells transplanted, improving the cryopreservation of CB grafts is a method to improve the outcome of the transplantation [9-11].

Previous studies have revealed that cryopreservation is associated with a significant level of apoptotic $CD34^+$ cells and this may contribute to poor engraftment [103, 115, 264] and reduced ability to proliferate and differentiate post-thaw [202, 203]. Optimization of cryopreservation protocols to maintain the quality of HSCs is an important challenge that has been poorly investigated. In our opinion, this represents an excellent opportunity to demonstrate the *in vivo* application of small molecule IRIs as cryoprotectants. The standard cryoprotective agent for HSC grafts is dimethyl sulfoxide (DMSO) [265, 266]. However, DMSO is unable to control ice growth and recrystallization during the freezing and thawing procedures and this is a major source of cryoinjury. A handful of studies have reported alternate cryoprotectants such as ethylene glycol, propylene glycol, and sugars but have yet to be adopted by researchers and clinicians [189, 202, 266-269].

The Ben laboratory has been designing and synthesizing small molecule IRIs and has demonstrated their advantages in many *in vitro* cellular systems [260, 270-274]. The structure of aldonamide derived IRI has been shown in Figure 25. IRI molecules inhibit the process of ice recrystallization and thus protect HSC and progenitors against cryoinjuries during freezing and thawing**.** In fact, supplementation of a cryo-medium with an IRI prior to freezing can increase the post-thaw viability and function of various cell types including red blood cells and stem cell grafts **[271**, **275]**. Indeed, carbohydrates with potent IRI activity were shown to increase the yield of non-apoptotic cells and hematopoietic progenitors post-thaw [276]. Moreover, we recently demonstrated IRI 2 (*N*-(2-fluorophenyl)-D-gluconamide) also improved the post-thaw recovery of progenitors in CBUs when used in combination with DMSO and dextran. Thus, IRIs show interesting potential as effective cryoprotectants for the long-term storage of CBUs**.** Of the four

previously reported IRIs, (IRI 2) was very effective in improving the post-thaw recovery of colony forming unit (CFU) progenitors and that of multipotent progenitors measured using the long-term culture-initiating cell assay [271]. However, the ability of IRI 2 to improve the engraftment activity of a stem cell graft has never been investigated to date using a murine transplant model.

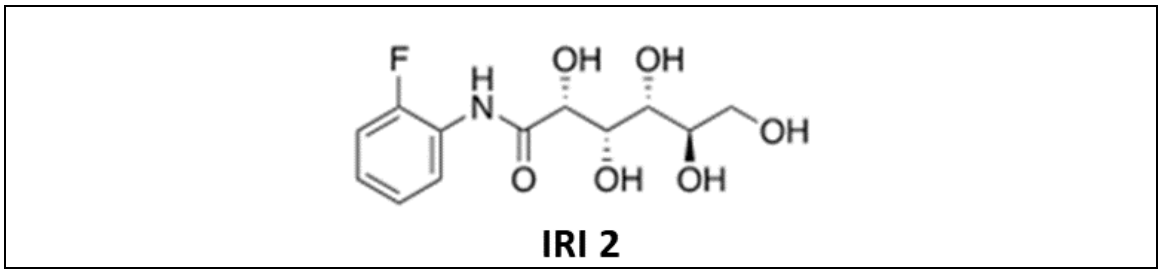

Figure 25: Structure of the aldonamide-based IRI 2.

In this study, we set out to define the impact of IRI **2** on the stem cell activity of CBU. This was achieved by using **a** serial transplantation assay to investigate the differentiation and self-renewal activities of HSCs. Our findings show that IRI **2** is non-toxic and well tolerated by hematopoietic cells and progenitors. More importantly, CB cells cryopreserved with DMSO supplemented with IRI **2** sustained superior platelet engraftment along with enhanced long-term bone marrow (BM) engraftment than those cryopreserved with DMSO alone. In addition, HSC key properties, self-renewal and multilineage differentiation, were unaffected by IRI **2**.

4.3. Materials and Methods

4.3.1. Cryosolutions

Dimethyl sulfoxide cryosolutions were prepared by diluting dimethyl sulfoxide (Sigma, D8418, Oakville, Ontario, Canada) with autoclaved distilled water containing 0.9% saline and 5% w/v dextran (Sigma, 31389, Oakville, Ontario, Canada). Cryosolutions containing *N*-(2-fluorophenyl)-D-gluconamide (IRI **2**) were prepared by dissolving the appropriate amount of lyophilized ice recrystallization inhibitor in a prepared 10% DMSO solution

(0.9% saline with 5% w/v dextran). IRI **2** was used at 10 or 25 mM as indicated based on dose response experiments on post-thaw $CD34^+$ cell viability and CFU analyses [271].

4.3.2. Synthesis of N-(2-fluorophenyl)-D-gluconamide

All chemical reagents were purchased from commercial sources (2-fluoroaniline and gluconolactone from Sigma-Aldrich; acetic acid from Fisher) and used without further purification. The synthesis of *N*-(2-fluorophenyl)-D-gluconamide was adapted from that previously described and characterization was consistent [271]. In short, 2-fluoroaniline (3.2 mL, 33 mmol) was added to a stirring suspension of D-(+)-gluconic acid δ-lactone (2.00 g, 11.2 mmol) in glacial acetic acid (20 mL). The mixture was stirred at 100 °C for 2.5 hours before being cooled to room temperature. The reaction was monitored by thin layer chromatorgraphy (TLC) analysis using ethyl acetate:methanol (8:2) with product R_f = 0.25. After evaporation of solvent, the resulting brown crude product was recrystallized twice from boiling 99% ethanol to yield a white crystalline solid (1.51 g, 47% yield). NMR spectra were recorded at ambient temperature on a Bruker AVANCE 400 MHz spectrometer (Bruker, Madison, WI). Low resolution mass spectrometry was performed at the John L. Holmes Mass Spectrometry Facility at the University of Ottawa where measurements were performed on a Micromass Q-TOF mass spectrometer (Waters Corporation, Milford, MA) in positive electrospray ionization mode (ESI+).

4.3.3. Hep G2 toxicity assay

Cell viability was measured with resazurin to assess the toxicity of IRI **2** (0-25 mM) on Hep G2 cells following manufacturer recommendations. In brief, the human hepatic Hep G2 cells were cultured at 37 °C and 5% CO_2 in Minimum Essential Medium (MEM) (Sigma) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% non-essential amino acids, and 0.1% 1M sodium pyruvate. Hep G2 cells were cultured

with or without IRI **2** and 30 µL 0.01% resazurin and incubated for 24 hours at 37 °C and 5% CO_2 prior to recording fluorescence measurements on a SpectraMax plate reader. Hep G2 cells in MEM and cells subjected to 1% TritonX-100 served as the controls.

4.3.4. CBU processing and cryopreservation

A total of 9 CBUs were obtained from the Canadian Blood Services' Cord Blood for Research Program after ethic board approval. All mothers gave informed consent for the donations to be used for research and development. Each CBU had a minimum volume of 50 mL and a total nucleated cell (TNC) count greater than 0.9 $x10^9$ but inferior to 1.5$x10^9$. Upon arrival, CBUs were processed to prepare the leukorich plasma (i.e. buffy coat). TNCs were either cryopreserved with 10% DMSO (control) or cryopreserved with 10**%** DMSO supplemented with IRI **2** at 10 or 25 mM (Figure 26). Buffy coat preparation was done by adding Hespan (6% Hetastarch in 0.9% Sodium Chloride, B. Braun Medical Inc., Irvine, CA, USA) solution corresponding to 20% of the CBU volume in the 50 mL conical tube containing CBU. After addition of Hespan, the mixture was gently agitated and incubated at RT for 90 minutes. The buffy coat was collected and centrifuged at 450g for 10 minutes. The cell pellets were resuspended in plasma up to 10 mL, kept at 4 °C, and a TNC count was performed using a hematology analyzer (pocH-100i, Sysmex Corporation, Ontario, Canada). After aliquoting into cryovials and another centrifugation, the cell pellets were resuspended in the appropriate cryosolutions (10% DMSO or 10**%** DMSO with IRI **2**). Samples were frozen at ~ -1 °C/min in a series of 1 mL cryovials using an isopropyl alcohol-based device following manufacturer instructions (Mr. Frosty, Thermofisher Scientific, Waltham, MA, USA). After being cooled to -80 °C, frozen cryovials then transferred and were maintained in a liquid nitrogen storage tank until use.

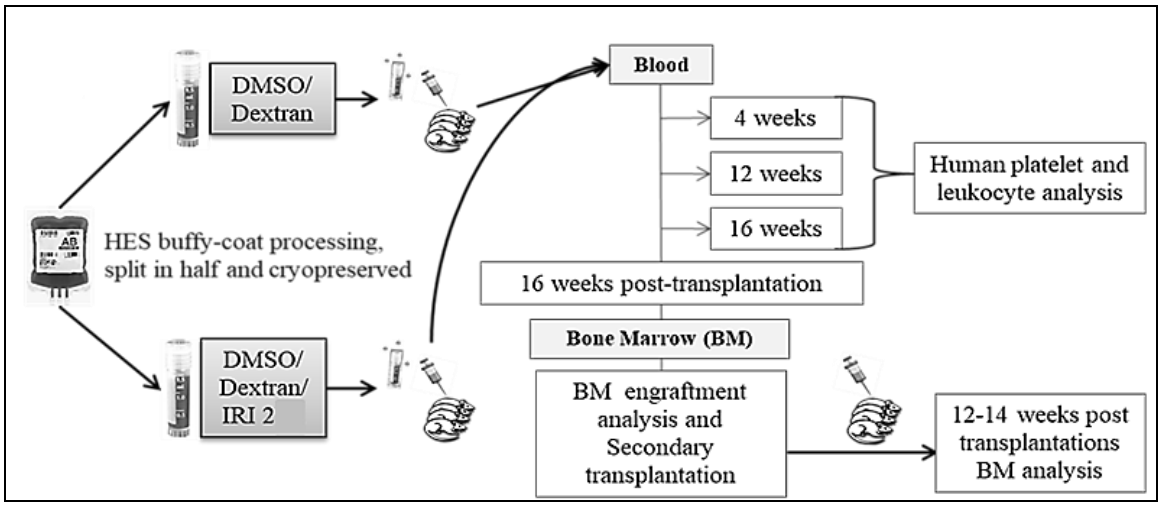

Figure 26: Experimental design used to address the impact of IRI 2 on the engraftment activity of CBU.

CBU were volume and red cell reduced using hetastarch (HES) and the leucocyte-enriched buffy coats were cryopreserved with DMSO and dextran supplemented or not with IRI 2

4.3.5. Thawing of CBU

Buffy coat aliquots were thawed following a thaw and dilute procedure optimized for CBUs at the Canadian Blood Services [26]. In short, 1 mL aliquots were rapidly thawed in 37°C water bath, then diluted 5-fold with a thaw solution (4% human albumin (Grifols Ltd, Barcelona, Spain) in PlasmaLyte-A (Baxter, Deerfield, IL, USA) solution) in a two-step process that included a 15 min. osmotic equilibration period. Thawed samples were then centrifuged (300g for 10 min.) and cells were resuspended in 2 mL of Iscove's Modified Dulbecco's Medium (Life Technologies) with 2% Fetal Bovine Serum (HyClone, Thermo Fisher Scientific) solution. TNC density was measured using a pocH-100i hematology analyzer (Sysmex, Ontario, Canada).

4.3.6. Flow cytometry analysis

FACS-Attune flow cytometer was used to analyze CB cells, platelet, leucocyte and bone marrow (BM) preparations (Thermo Fisher) [212]. Compensation was carried out with fluorescent labeled microbeads (Molecular Probes- Thermo Fisher Scientific Inc.) and

fluorescent intensity minus one stained sample were used as control to set quadrants and gates. Dead cell and debris were gated out by the forward and side-scatter and/or Sytox-AAD staining (Life Technologies). For viability analysis, 1.0 x 10^6 nucleated cells were treated with 2 mL ammonium chloride-based lysis solution (BD Biosciences) to lyse the red cells. Next, cells were pelleted (250 xg /8 min.) and suspended in annexin V binding buffer (Life Technologies, ThermoFisher Scientific) followed by staining with CD34-PE, CD38-PECy-7, CD45-APC antibodies (BD Biosciences, San Jose, CA, USA) and annexin V Alexa488 and Sytox® AADvanced™ following manufacturer instructions. Sample were kept on ice after further dilution with annexin V binding buffer and data was acquired within 1 hour.

4.3.7. CFU assay

CFU assay was performed by plating various doses of CB TNC or murine BM cells (40,000-50,000 per plate) in methylcellulose-based media (MethoCult H4434, StemCell Technologies) in duplicate and incubated in humidified atmosphere (5% CO_2) at 37 °C. Colonies were scored by microscope after 2 weeks of culture.

4.3.8. Transplantation of CB cells in NSG mice

Mice work was preapproved by the University of Ottawa animal care committee in accordance with the standards of the Canadian Council on Animal Care and with the Animal for Research Act. CB TNCs were incubated for 20 mins at 4°C with OKT-3 antibody (1 µL of 1mg/mL OKT-3 solution/ 1x10^6 TNC) (BioXCell, NH, USA) before transplantation to prevent graft versus host disease [214]. Eight to ten week old NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG, Jackson Laboratory, Bar Harbor, Maine, USA) mice were irradiated (300 cGy ^{137}Cs, Gammacell Exactor 40, Best Thetratonics, Ottawa, ON, Canada) and transplanted intravenously (i.v.) with CB TNCs at doses indicated in Table 5.

Secondary recipients were transplanted with 85% of the BM collected from individual primary recipients. A total of 4 CBUs (CBU #1-4) were tested in primary transplants (5 mice/group/CBU) and, 3 CBUs (CBU #1-3) were tested in secondary transplants (3-4 mice/group/CBU). All transplant groups were age- and sex-matched.

Table 5: Characteristic of the CBUs tested in transplant assays and cell doses.

			Viability (%)*		Dose TNC
CBU	**Sample**	**Frequency $CD34^+$ (%)**	**TNC**	**$CD34^+$ cells**	**$x10^6$/mice****
CBU 1	DMSO	0.29	61.1	70.5	2.00
	IRI **2**	0.28	64.6	70.1	2.00
CBU 2	DMSO	0.72	50.9	65.6	1.50
	IRI **2**	0.65	47.6	67.9	1.50
CBU 3	DMSO	0.57	43.6	69.5	2.00
	IRI **2**	0.59	41.3	59.6	2.00
CBU 4	DMSO	0.20	56.6	56.4	3.00
	IRI **2**	0.23	51.8	56.7	3.00
Mean (SD)	DMSO	0.45 (0.2)	53.0 (7.6)	65.5 (6.4)	2.1 (0.6)
Mean (SD)	IRI **2**	0.44 (0.2)	51.3 (9.9)	63.6 (6.4)	2.1 (0.6)

* Based on annexin V staining, ** net number of TNC. No significant differences detected.

4.3.9. Human engraftment analyses

Human platelet (hPLT) engraftment analyses were done short-term (ST, 4 weeks) and long-term (LT, >14 weeks) after transplantation in two steps [218]; the concentration of murine PLT was first measured by staining diluted blood with rat anti-mCD41-FITC, and the proportions of human PLT (hPLT) and murine PLT were determined in platelet-rich plasma (400,000 total platelet events acquired) stained with species specific antibodies (hCD41-APC, mCD41-FITC, Becton Dickinson Pharmingen). Human myeloid (CD45-APC/CD33-PE) and lymphoid (CD45-APC-CD19-PE) leucocytes were analyzed from the plasma-poor fraction. LT BM engraftment analysis was performed past 16 weeks as indicated. BM cells were harvested from hind legs, red blood cells lysed with ammonium

chloride (Stem Cell Technologies) and analyzed by cytometry using antibodies indicated above. The antibodies used included CD19- phycoerythrine (PE), CD14-PE, CD45-allophycocyanine (APC), CD33-PE, CD34-PE, CD3-APC human CD41a (GPIIb)-FITC, CD56-PE, CD45-FITC (Becton Dickinson Pharmingen, Mississauga, Ontario, Canada).

4.3.10. Statistical analysis

Engraftment results obtained with CBU #1-3 (25 mM IRI **2**) and CBU #4 (10 mM IRI **2**) were analyzed together since the two IRI **2** doses produced similar outcomes when compared to their respective DMSO controls. hPLT and CFU engraftment data were analyzed as followed; data was log-transformed before comparison between the two groups, significant difference between the DMSO and IRI **2** mice groups for platelets, leucocyte and CFU levels were determined with a mixed model analysis, donor random effects were modeled to control potential clustering problem. Paired student-t-test between groups was done for 2 groups comparison of in vitro data. P values ≤ 0.05 were considered significant. Statistical analyses were performed using the statistical software SAS/STAT 9.3 (SAS Institute Inc., Cary, NC) and Graph-Pad Prism version 7 (GraphPad Software, San Diego, CA).

4.4. Results

4.4.1. IRI 2 is not cytotoxic and is well tolerated by hematopoietic progenitors

Cryoprotectants can be toxic to cells due to their physicochemical properties. To assess the cytotoxic effect of IRI **2**, cell viability was performed using resazurin with the human hepatic cell line Hep G2. Exposure to IRI **2** for 24 hours was well tolerated by Hep G2 cells and showed very little toxicity (Figure 27A). The cytotoxic effect of IRI **2** on human hematopoietic progenitors was assessed with the CFU assay. CB cells were first incubated

with or without IRI **2** at 25 mM (concentration based on previous work [271]) for an hour at 4 °C after which cells were placed in culture for the CFU assay. CB cells incubated in plasma and DMSO served as controls. In line with the Hep G2 cell results, IRI **2** did not significantly perturb the clonogenic properties of fresh CB cells (Figure 27B).

The impact of IRI **2** on the viability of the CBU post-thaw was investigated by annexin V and DNA staining. No significant differences between IRI **2** and DMSO groups were observed for the viability of TNC, $CD45^+$ cells and $CD45^+CD34^+$ cell subpopulations. Comparable results were also observed for the proportion of apoptotic and necrotic cells (Figure 27C). Taken together, these results showed that IRI **2** has low cytotoxicity and that is well tolerated by CB cells and progenitors.

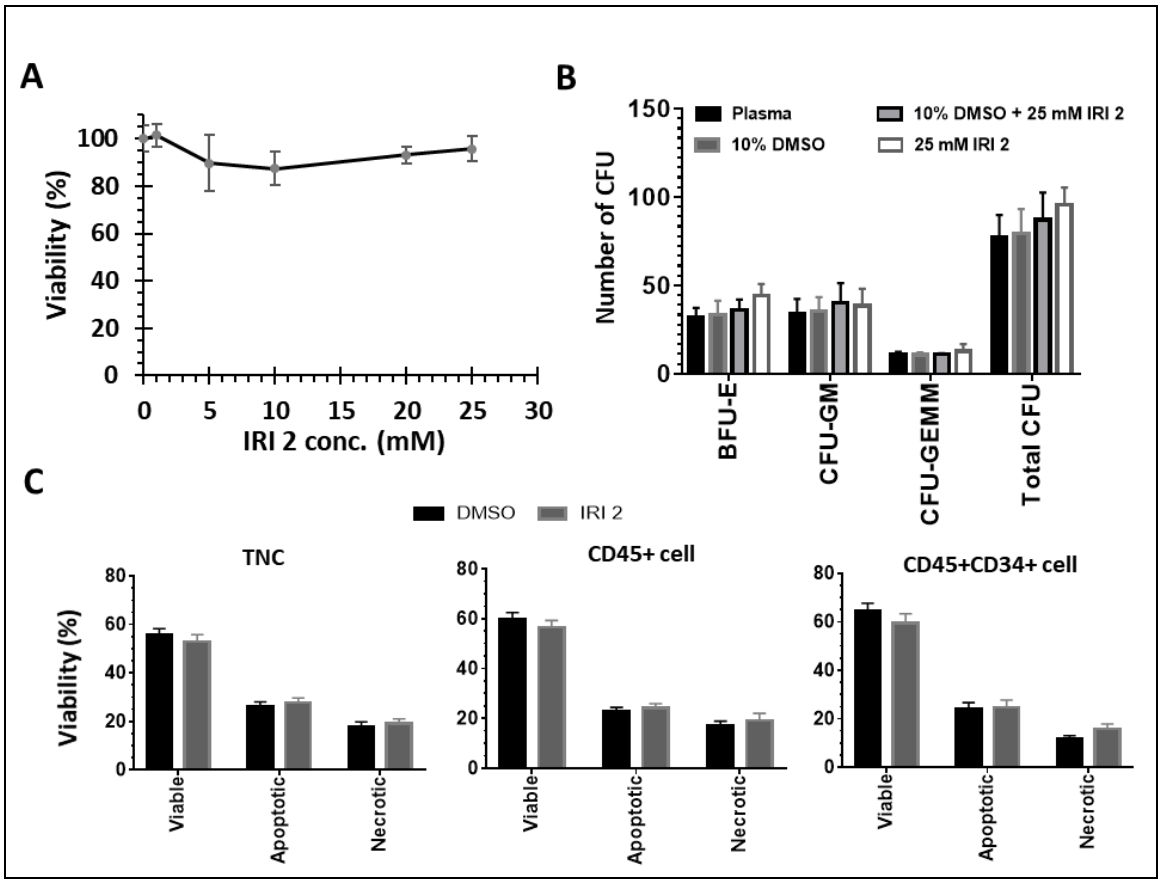

Figure 27: Cell viability in the presence of IRI 2.

(A) Viability of Hep G2 cells as a function of IRI **2** concentration. IRI **2** was well tolerated by Hep G2 cells and showed very little toxicity after 24 hours of incubation with IRI **2**, up to near its maximum solubility (mean ± SD, n = 2-4). **(B)** Number of CFU present in fresh CB graft exposed to DMSO and IRI **2** (25 mM) for 1 hour. Net number of colonies per 40,000 CB TNC presented (mean ± SEM, n=3). Burst forming unit-erythroid (BFU-E), CFU-granulocyte-monocyte (CFU-GM), CFU-Granulocyte erythrocyte monocyte and megakaryocyte (CFU-GEMM). (**C**) Viability analyses of indicated CB cell subpopulations in CBU post-thaw using annexin V and sytox-AAD staining (mean ± SEM, n=5).

4.4.2. Experimental design used to assess impact of IRI 2 on HSC engraftment activity

The experimental design used to test the impact of IRI **2** on the engraftment activity of CBU is presented in Figure 26. In short, freshly collected CBUs were processed following common banking procedures (i.e. buffy coat preparation with volume and red cell reduction) and CB TNC were split in equal portions. One portion was cryopreserved with 10% DMSO, 0.9% saline and 5% w/v dextran and served as baseline control (i.e. DMSO

group). Others were cryopreserved with the same cryoprotectants as the control but also supplemented with the IRI **2** at either 25 mM (CBU #1-3) or 10 mM (CBU #4). The engraftment activities of CBUs were investigated in NSG mice with the serial transplantation assay (Figure 26). Results obtained with both concentrations of IRI **2** were analysed together as indicated in the statistical analysis section. The doses of TNC and the post-thaw viabilities of $CD45^+$ and $CD34^+$ cells for the four CBUs tested are summarized in Table 5.

4.4.3. Impact of IRI 2 on the platelet and leucocyte engraftment

Platelet engraftment in NSG mice was characterized by tracking human platelets (hPLT) in the peripheral blood at 4, 11 and 15 weeks post-transplantation by flow cytometry analysis (Figure 28A). hPLT levels were greater at 4 weeks post-transplantation in the IRI **2** cohort than that observed in the control group though the difference was not significant (Figure 28B, $p=0.17$). The increased level of hPLT in the IRI **2** cohorts was even more pronounced with time, being 3.7- and 2.0-fold greater at 11 and 15 weeks post-transplantation (Figure 28B, $p<0.001$ and $p<0.05$). In line with this, combined analysis of the entire hPLT data set confirmed that the levels of hPLT were superior in IRI **2** mice (Figure 28C, $p<0.001$). Conversely, the overall levels of human $CD45^+$ leucocytes in the periphery at the 3-time points investigated were not significantly different between the control and IRI **2** group (Table 6). Myeloid ($CD45^+CD33^+$) and B-cell ($CD45^+CD19^+$) lineages were also detected in both two mice groups (Figure 29). The only significant difference was a slight increase in the frequency of human myeloid $CD45^+CD33^+$ leucocytes in IRI **2** cohort 15 weeks post-transplantation (mean 0.80% ± 0.78 (SD) vs. 0.59% ± 0.59 for IRI **2** and control groups, $p<0.05$).

Table 6: Frequency of human CD45^{+} leucocytes in mice groups at indicated time point

	Week 4	Week 11	Week 15	Cumulative
DMSO	2.5 ± 0.9	9.3 ± 4.3	14.7 ± 5.5	9.2 ± 5.1
IRI 2	2.1 ± 0.9	12.4 ± 4.8	17.1 ± 7.0	9.5 ± 6.4
p values*	0.064	0.272	0.281	0.193

Mean ± SEM, 17-19 mice total per group, n=4. [1] p values determined by mixed model analysis.

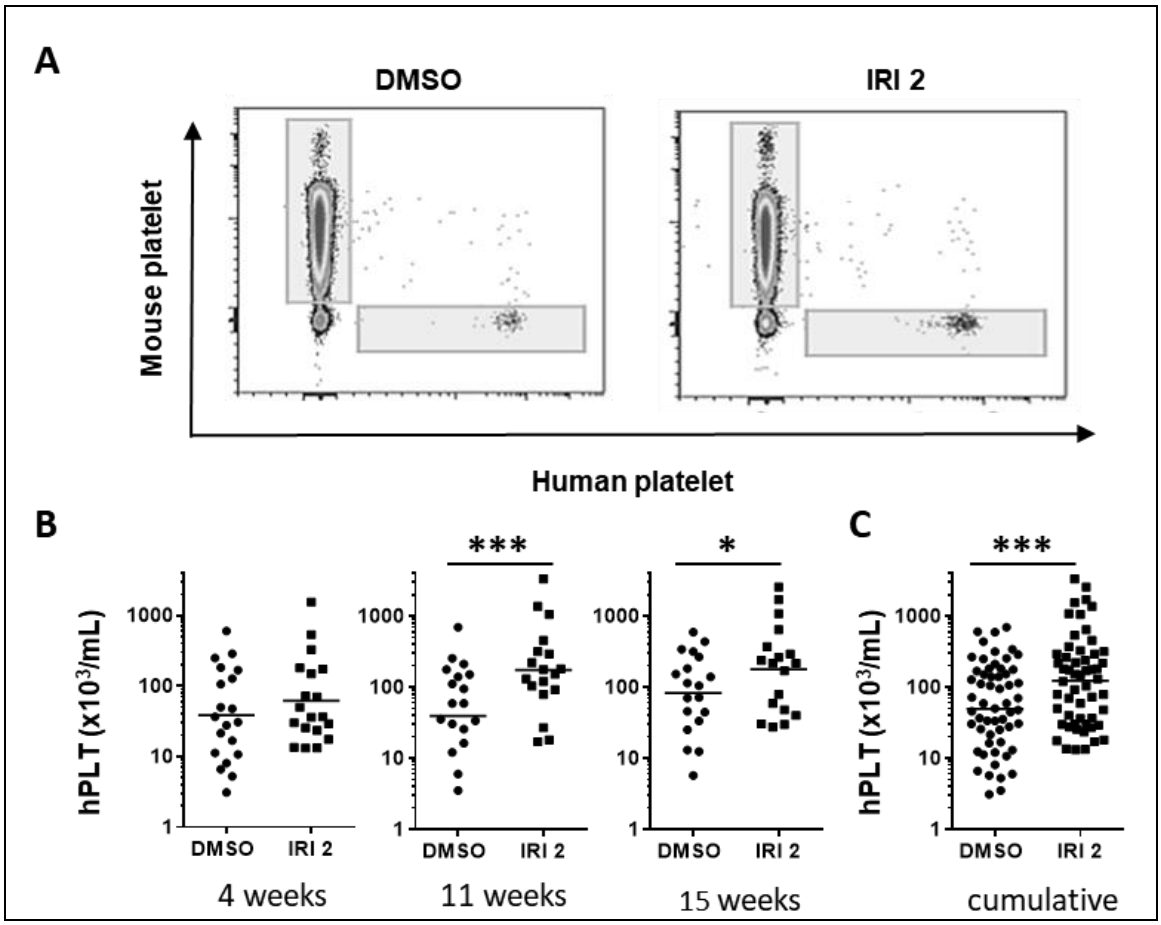

Figure 28: Impact of IRI 2 on the platelet engraftment activity of CBU grafts.

(A) Representative flow cytometry analysis of murine and human platelets in NSG mice transplanted with CBU cryopreserved with DMSO supplemented or not with IRI **2**. (**B)** Engraftment level of hPLT in DMSO and IRI **2** group as a function of time post-transplant (**C)** Overall level of hPLTs in DMSO and IRI **2** recipients. Overall difference for all platelet data was performed by fitting a mixed model with time (week 4, 11 and 15) adjusted in the model. For panel B and C, each symbol corresponds to actual level for each mouse and the line to the geometric mean (17-19 mice total per group, n=4, * $p<0.05$, *** $p<0.001$).

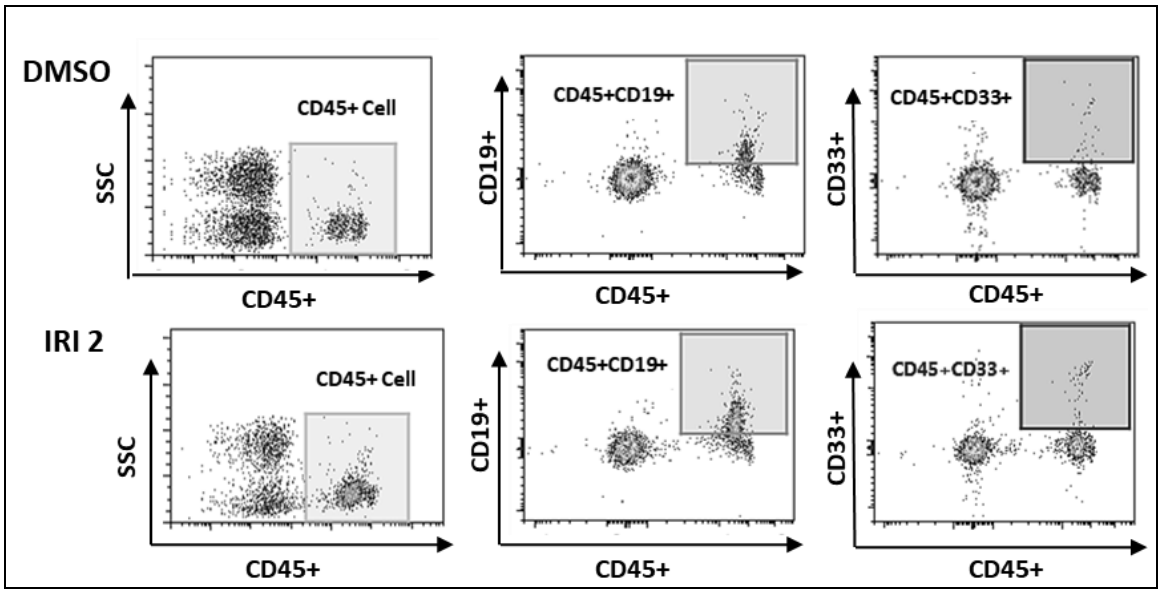

Figure 29: Detection of human leucocyte in humanized mice.

Representative flow cytometry analysis of human $CD45^+$ leucocytes, $CD45^+CD33^+$ myeloid cells and $CD45^+CD19^+$ B-cells in NSG mice transplanted with CBU cryopreserved with DMSO supplemented or not with IRI **2**.

4.4.4. Impact of IRI 2 on BM engraftment activity of HSCs in CBU

Previous results showed that complementation of DMSO with IRI **2** improves hPLT engraftment. Next, we investigated the capacity of IRI **2** treated CBU to support long-term engraftment by analyzing human BM engraftment 16 weeks after transplantation (Figure 30A). CBUs treated with IRI **2** showed an improvement in overall human chimerism detected by the pan-hematopoietic marker CD45 (Figure 30A). Furthermore, the net number of human progenitors detected in the BM of the IRI **2** mice cohort were significantly superior to that measured in the control DMSO litermate. These included the net numbers of CFU-Granulocyte Monocyte (GM), CFU-Granulocyte erythrocyte monocyte and megakaryocyte (CFU-GEMM) and total CFU (Figure 30B). Multilineage analysis was carried out to investigate whether IRI **2** has any undesirable impact on the differentiation activity of HSC from the treated CBU. Normal lineage differentiation was observed for the human BM cells derived from the IRI **2** treated CBU as indicated by

similar pattern of myeloid and lymphoid lineage distribution in both mice groups (Figure 30C). Representative multi-lineage flow cytometry analysis are presented in Figure 31.

BM from primary recipients were then transplanted into secondary mice to test the impact of IRI **2** on the self-renewal activity of HSC with human BM engraftment analyzed 12-14 weeks post-transplantation in secondary recipients. As shown in Figure 30D, human BM engraftment in secondary IRI **2** mice were not significantly different between both groups ($p=0.37$).

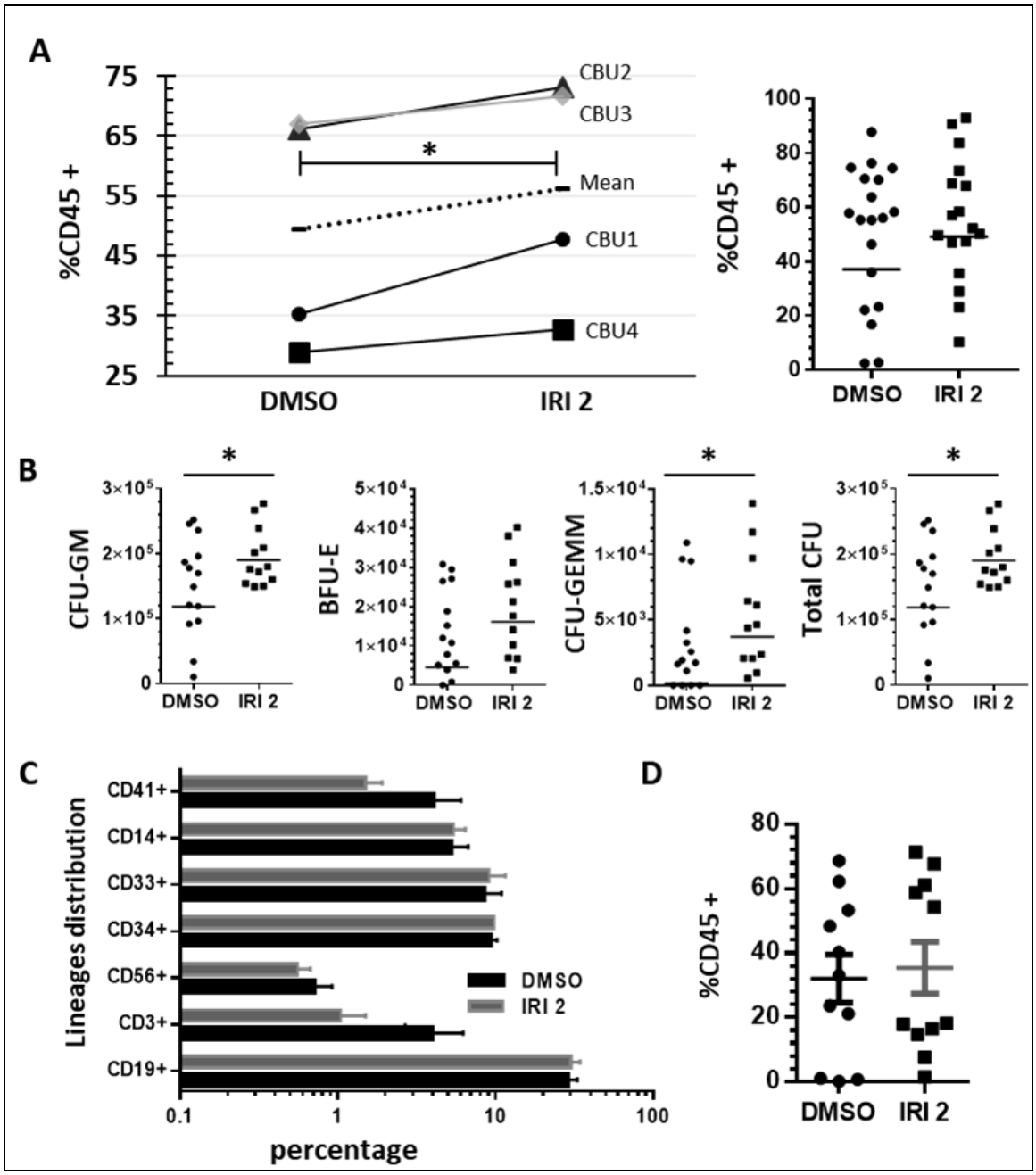

Figure 30: Impact of IRI 2 on Long-Term BM engraftment activity of CBUs.

A) Frequency of $CD45^+$ BM cells in primary mice 16 weeks post-transplantation. The left graph summarizes the mean engraftment for each CBU tested (the dash line presents the mean engraftment for each group, * $p<0.05$ paired t-test). The right graph presents the engraftment level for all individual mice (17-19 mice total per group, n=4) and geometric mean (line). **B)** Net number of human progenitors measured in the BM of primary DMSO and IRI **2** recipients (geometric mean (line), 11 mice per group, n=3). **C)** Lineage distribution of human BM engraftment in primary recipients (mean ± SEM, n=4). **D)** Human BM engraftment in secondary transplants (mean ± SEM, n=3).

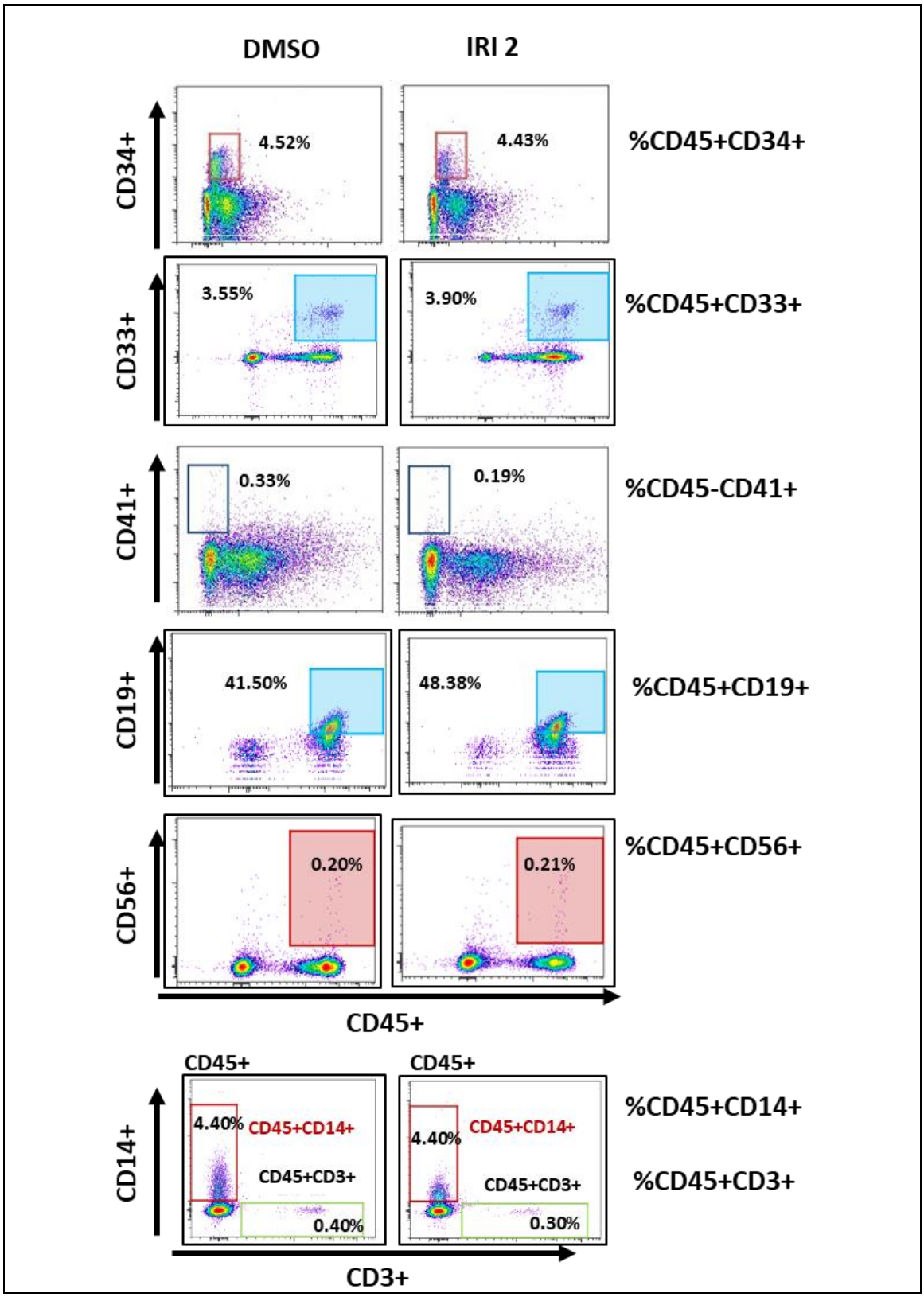

Figure 31: Multilineage BM engraftment

Representative analyses of long-term human BM lympho-myeloid engraftment in mice transplanted with DMSO or IRI 2 cryoprotected CB cells.

4.5. Discussion

A large multicenter study has recently confirmed that CBU is an excellent source of HSC for leukemic pediatric patients without a matched sibling donor as indicated by overall survival and lowest incidence of chronic graft-versus host disease [263]. However, platelet engraftment remains lengthy and the limited number of cells available per unit remains a limitation. Consequently, frequent platelet transfusions are required to avoid serious or life-threatening bleeding related to thrombocytopenia. Since, the speed of engraftment is largely dictated by the dose of stem and progenitor cells transplanted [149, 238, 277], we hypothesized that improving the cryopreservation of CBU may improve ensuing engraftment. Herein, we tested for the first time to our knowledge the impact of a novel class of cryoprotectant, ice recrystallization inhibitor (IRI), on the engraftment activity of CBU using xenograft transplantations.

Cryoprotectants are indispensable to limit irreversible damages to cells during the freezing and thawing periods that would otherwise comprise their viability and function. The development of an efficient method for the optimal cryopreservation of HSCs from CBUs is critical for maintaining the maximum engraftment potential of CBUs in both public and private CB banks. IRIs are an emerging class of cryoprotectants that can prevent cryoinjuries and may one day benefit stem cells and new cellular therapy products. Our previous study had evaluated several new IRIs and had identified IRI **2** (*N*-(2-fluorophenyl)-D-gluconamide) as a promising compound. It was shown to efficiently control ice recrystallization, an activity that correlated with better post-thaw recovery of clonogenic and multipotent progenitors when supplemented with DMSO [271].

The low cytotoxicity profile of IRI **2** on Hep G2 cells and hematopoietic stem and progenitors in combination with the compound's capacity to increase the post-thaw

recovery of progenitors [271] made it an ideal IRI candidate to test in the first transplant assay. The engraftment data showed that the cryopreservation of CBUs with IRI **2** enhanced the engraftment activity of CBU. This was first evident by the increased levels of hPLT in IRI **2** transplants at both short and long-term post-transplantation. In contrast, human leucocytes were present at similar levels in both mice groups. This difference may be the results of the differences in lifespan of the human platelets and leucocytes in mice, as hPLT have a very short half-life in mice due to phagocytosis (~ 12 hours [278]) whereas lymphoid cells circulate for weeks to months [279].

HSC activity can only be assessed in transplant models over a long-period of time and with serial transplantation providing definite insight into long-term HSC [23]. In this work, the long-term engraftment in primary transplant was measured at week 16 and it revealed that human $CD45^+$ chimerism was higher in the IRI **2** treated CBU recipients. In addition, the number of human hematopoietic progenitors (CFU-GM, CFU-GEMM and CFU-total) was raised over that measured in DMSO control group. Our transplant results also indicate that long-term HSCs are well preserved in the presence of IRI **2**. Indeed, no significant differences were observed for the lineage differentiation of human HSC into stem and progenitors ($CD34^+$), myeloid ($CD33^+$, $CD14^+$, $CD41^+$), and lymphoid ($CD19^+$, $CD3^+$, $CD56^+$) subsets and, the level of human engraftment in secondary recipients was similar between both mice groups.

In summary, our data showed that IRI **2** is well tolerated and not toxic to Hep G2 cells and hematopoietic progenitors, and that cryopreservation of CB grafts with IRI **2** as an additive to the conventional freezing medium maintains the stem cell properties of CB HSC. More importantly, IRI **2**-protected grafts had improved platelet and BM engraftment in primary recipients. This study provides the first evidence that IRI could play a significant role in future clinical applications by reducing damages associated with ice recrystallization

during freezing and thawing processes. IRI **2** is currently under investigation as a new cryoprotectant for many other cell types important for applications in regenerative medicine and cellular therapies. Furthermore, pharmacokinetic and toxicological studies are underway, and these will validate the development potential of IRI **2**.

4.6. Acknowledgement

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CHAPTER 5
GENERAL DISCUSSION

CB can be selected as an alternative for patients who need allogeneic stem cell transplantation but don't have a well-matched donor among family members or general population [280]. However, CB transplantation is associated with delayed engraftment of neutrophils, and especially of platelets [65, 66]. Furthermore, the lower number of hematopoietic progenitors and HSCs in CBU compared with BM or mobilized PBSC sources translates into increased risk of graft failure and delayed hematopoietic engraftment [118, 119]. The quality of the CB product is a function of the collection, subsequent storage conditions (length and temperature), processing of the unit, cryopreservation and thawing methods. As the number of TNC infused into the recipient correlates highly with the speed of engraftment, it is therefore necessary to obtain CBUs with high TNCs. CB banks must conserve high cell yields during collection, storage and processing to maximize their product quality. Standard agencies like, AABB and Net-Cord-FACT limit CB storage to a maximum of 48 hours from collection to freezing at a temperature range of 4-22 °C [96, 97], and it is up to the bank to validate the temperature of storage that maintains quality and potency. Whether storage of CBU at RT before processing contributes to slower engraftment remained unclear prior to my work, as most studies used *in vitro* assays.

In addition, CB bank must conserve CBU for years until their use, which is accomplished by cryopreservation. Cryopreservation method that yields consistent high recovery of functionally viable cells is crucial for future usage. Cryopreservation protocols for HSC remained relatively unchanged since the first CB banking program started in 1992 [99]. Optimization of cryopreservation protocol is needed to improve the post-thaw recovery, viability of CB cell and to reduce the DMSO related toxicity. The success of HSC transplantation is correlated with the cryopreservation procedure as both number of TNC and the quality of cells transplanted can be affected by cryopreservation method. Therefore, improving the cryopreservation method of CB grafts will be supportive to improve the outcome of the transplantation [188, 253, 254]. Cryoinjury caused by the formation of ice crystals can be minimized using CPA that can inhibit the ice recrystallization during freezing and thawing process. An emerging new class of CPA are small IRIs, **which can** inhibit the process of ice recrystallization and thus protect cells against cryoinjury.

Hence, with the long-term objective to provide results that can be used to improve the quality of banked CBUs, I investigated in this dissertation, the influence of storage at RT prior to cryopreservation on the quality and potency of CBU and, tested new CPAs to improve the potency of the CBU post-thaw.

5.1. The influence of RT storage on the potency of CBU post-thaw

Several aspects of CB collection and processing are controversial topics and numerous studies have been carried out with the aim to identify the factors that influence the recovery of CB cell from a CBU. Many studies have shown that prolonged storage at RT has adverse effect on the viability, function and recovery of CBU derived HSCs and progenitors [109, 112, 113]. But the impact of storage on the function and loss of HSCs remains poorly described as most of the studies used *in vitro* assays which do not measure the activity of

HSC [119, 120]. Therefore, optimization of storage time and condition to store the CBU before processing are important issues for both banking and transplantation, since the starting cell content is far smaller in CBU compared to apheresis grafts. Here, I sought to fill the knowledge gap about the impact of pre-processing delays and storage at RT on the engraftment activity of CBU. In my work, I chose to focus my efforts on studying CB samples post-thaw since it better reflects clinical settings given that all CBUs go through cryopreservation and thawing prior to transplantation.

In chapter 2, my results from in vitro assays showed that the post-thaw viability of CB TNC and $CD45^+$ cells were slightly reduced after long storage ($\geq$ 40 hours) of CBU at RT [141]. Nonetheless, all CBUs tested passed the minimal Net-Cord threshold for viability (over 70% for $CD34^+$ cells, and over 40% for $CD45^+$ cells) as well as potency, since all CB samples produced colonies in the CFU assay. However, the increased frequency of apoptotic cells (Table 3) in stored samples was consistent with the decline in engraftment outcome and the results support the study of Shim, *et al*. that showed annexin V^+ cells had lack of engraftment activity [103]. In contrast, a slight increase in the proportion of viable $CD34^+$ cells were observed in stored samples which was a surprise, as opposite results were expected. However, other studies reported either similar results or simply no reduction in the recovery of $CD34^+$ cells following storage at RT [109, 110, 114], which indicated that $CD34^+$ cells were largely intact upon storage at RT. Furthermore, Majka, et al, reported that at RT, $CD34^+$ cells produced antiapoptotic factors in combination with cytokines that promoted cell maintenance in an autocrine/paracrine fashion [281]. However, the net yields of total CFU and CFU-GEMM were significantly reduced in stored samples. On the other hand, the net number of $ALDH^{br}$ cells was not significantly different between baseline and 40-hours stored samples. This result was contrary to my expectations since a previous study by Shoulars, *et al*. reported that $ALDH^{br}$ cells in CBU correlate well with

CFUs [162]. However, this could be due to the low sample size of my study (n=7) and/or the usual variation between CBUs. The results also confirm that it is very hard to find an *in vitro* assay that can predict or reflects changes in the engraftment activity of CBU. However, the CFU assay predicted some loss in engraftment activity. This is certainly consistent with the strong correlation between CFU content and engraftment that has been established in clinic [149].

My main hypothesis was that extended processing delay with storage at RT is one of the several variables which reduces the post-thaw potency of CB HSC. I tested and confirmed this hypothesis using two xenotransplantation assays; the serial transplantation and LDA transplantation assays. NSG mice were used due to their longer lifespan that supports long-term engraftment and also supports superior engraftment capabilities than NOD-SCID mice [168]. The serial transplantation remains the best experimental tool for the assessment of long-term repopulating HSC [22] and the LDA is used to estimate the frequency of CB HSC [174] associated with processing delay.

The thawed CBU aliquots were stained to capture apoptotic and necrotic $CD34^+$ cell to determine the doses of nucleated cell to be injected that would contain ~7500 viable $CD34^+$ cells (Table 3) for both baseline and stored groups. This dose was selected to avoid engraftment saturation and was based on a pilot transplantation assay with OKT-3 treated CB cells, an assay which I set up in our lab. This scheme was selected to ensure a fair comparison between both groups, but no strategy is without its issues. In this case, the net number of TNC varied between each group. On the other hand, I chose to transplant similar dose of TNC for both groups for the LDA transplantation assay (Table 2). This way both cell dose strategies were used to understand the effect of prolonged storage (>40 hours) at RT on the engraftment capacity of CBU. Importantly, both models provided similar results reinforcing the validity of my findings and conclusions.

My results revealed that the engraftment activity of CBU was significantly reduced following storage at RT and it was first observed with the serial transplantation assay. All 3 CBUs tested showed reductions in platelet and leukocyte levels at most time points in stored group when compared to baseline. Remarkably, the significant reduction of $CD45^+$ leukocytes in both short and long-term was the result of reductions in both myeloid and B-cells. These results were the consequence of reductions in BM engraftment in primary $CD45^+$ cell transplants. These results are partially supported by Louis, *et al*. where they reported that CBUs stored for 3 days at RT almost lost their engraftment activity [112]. In line with this, the frequencies of lymphoid and myeloid cells in the BM also showed some reductions in the stored group though all the differences were not significant. Nonetheless, the multi-lineage engraftment activity of HSC was not impacted by the RT storage as all lineages were present and for most at normal distribution. Strikingly, the BM engraftment in secondary recipients was more perturbed in stored group ($p<0.01$).

Furthermore, whether this impaired HSC activity for stored samples was the result of reduced number of HSC due to storage was further investigated using LDA transplantation assay. The complimentary results revealed large reductions in the net number of human platelet engrafting progenitors in stored units ($p=0.005$) and in the net number of SCID repopulating cells ($p=0.20$), though the low sample size did not provide enough power to achieve significant difference for the SCID repopulating cells. Taken together, my results with the secondary transplants coupled with those from the LDA assay suggest that prolonged storage at RT leads to the engraftment deficit due to the loss of long-term engrafting stem and progenitor cell numbers.

There are however some limitations to my study. One limitation is, it's not possible to tell apart the impact of the length (40 hours) and the RT storage on the deficit in engraftment activities as both variables were tested together. Another limitation is that only 3 CBUs

were tested in transplant models. Several factors were responsible for the latter. First, there were significant delays in acquiring CBU ≤ 12 hours from the time of collection to the arrival of the CBU at Canadian Blood Service. In addition, xenotransplants are expensive to obtain and to maintain which limited the number of mice available for my work. However, in support of my results, the engraftment deficit was observed in all CBUs tested and in both transplant models. Moreover, and as indicated above, I used two different cell dose strategies, and both procedures showed similar results, which reinforce that extended delays reduce the post-thaw potency of CB HSC. Furthermore, reduction in long-term BM engraftment was also observed with a fourth donor used to test the effect of the small molecule (UM171) on CBU upon storage at RT (Appendix 8.4). My results are also partly supported by the work of Louis, *et al*. [112].

A retrospective analysis of over 3,000 CBUs processed at the Canadian Blood Services CB bank (2015-2019) revealed that 90% of CBUs are cryopreserved by 31 hours. Following that, I wanted to test this length of storage at RT on HSC activity. Therefore, I investigated whether CBUs half stored at RT and fully processed by 31 hours (28 hours of storage at RT) would exhibit similar engraftment deficits as seen previously with the longer storage (≥ 40 hours, RT) (Appendix 8.3). The viability of TNC and $CD45^+$ cells was similar between baseline and stored groups, and no significant differences in the CFU content was detected between groups. Only a small reduction in the proportion and net number of $CD34^+$ $ALDH^{br}$ cells was observed in the stored group. The engraftment activity of both CBU groups was also highly similar since no differences were observed for platelet and leucocyte levels and none also for the long-term human BM engraftment done at 25 weeks post transplantation. Based on these results, CBUs processed within 31 hours have engraftment activities comparable to the baseline. My results suggest that the majority of the CBUs within the CBS' public CB bank inventory is of good quality and potency.

The molecular mechanisms or factors which are responsible for the engraftment deficit induced by prolonged RT storage still need to be explored further. Studies reported that biochemical signals upon different conditions (hyperthermia, radiation, toxins, hypoxia, etc.) lead to cell lesions through different intra/extra cellular pathways and eventually cause cell death [282-284]. Conversely, the inflammatory cytokine TNF-α can be produced by different cells like macrophages, monocytes, endothelial cells, neutrophils, etc. and play a role in a variety of functions [285] upon different physiological responses and reactions. TNF-α is also known to promote ROS production in many cell types including stem and progenitors [212, 214]. Studies revealed that it plays a role in the differentiation of HSC [212] and can impact negatively the function of HSC in transplantation model [152, 214, 215]. Based on that, I investigated the effect of extended storage (≥ 40 hours) at RT on biochemical changes in CBU. Interestingly, my results revealed that several biochemical changes occurring in CBU stored at RT, some of which are consistent with losses in engraftment or HSC activity. This included increase in TNF-α and ROS level post-processing and, the down regulation of the homing receptor CXCR4 in stored samples. However, conclusive evidence of the functional impact of these changes is lacking and these will need to be explored further.

On the other hand, small molecules have now been identified that can promote *ex vivo* HSC expansion such as SR1 [65] and UM171 [66]. UM171 was also reported to reduce apoptosis without affecting the proliferation of the HSCs [286]. Based on that, I investigated whether UM171 can prevent the loss in viability and engraftment activity associated with storage at RT for 40-44 hours (Appendix 8.4). The results showed that cell viability was unchanged by the addition of UM171, and no significant differences was observed between groups for $ALDH^{br}$ cells either. Likewise, the net number of progenitors recovered were similar, though a tendency of increased CFU was seen with UM171

samples at 35 and 50 nM ($p>0.05$). Furthermore, preliminary *in vivo* data revealed no differences in long-term human BM chimerism between the 4 groups. Based on these results, it appears that UM171 is not detrimental to CB cell during storage of CBU at RT but whether UM171 can provide protective effect to HSC remains unclear as additional experiments will be required to determine this.

5.2. Testing new CPAs to improve the post-thaw potency of CBU

The standard CPA that is most widely used for the cryopreservation of HSC graft is DMSO at 10% final concentration. Due to the indications of cellular toxicity and patient-related side effects, there is an increasing demand for reducing the DMSO concentration or using alternative CPAs for cryopreservation [197, 265, 266]. Disaccharides like trehalose and sucrose have been currently under investigation to be used as natural source of CPA [287]. Extensive research has been going on to discover better CPAs by using different concentrations of DMSO in combination with potential CPAs such as EG, PG, starches and sucrose that could improve cell viability and stem cell recovery [189, 202, 266-269]. However, loss of function in cryopreserved cells occurs following cryoinjuries due to osmotic shock and mechanical damage from uncontrolled ice recrystallization during freezing and thawing [288]. These cryoinjuries likely adversely affect the engraftment activity of HSC. Such outcome may be clinically significant for CB transplantation where doses of HSC and progenitors are already limiting, and engraftment is typically delayed. Therefore, improving the use of CPAs, to minimize the re-crystallization of ice is crucial factor in the field of cryo-storage of CB cell. The current CPA, DMSO is unable to control ice growth and recrystallization during the freezing and thawing procedures, which is a major source of cryoinjury. The Ben laboratory has been designing and synthesizing small IRI molecules and has demonstrated their advantages in many *in vitro* cellular systems

[192, 193, 201, 289, 290] but the capacity of IRI to improve the engraftment activity of HSC graft had never been investigated. Hence, I collaborated with the group of Dr. Robert Ben to explore whether small IRIs could improve the post-thaw recovery of progenitors and the engraftment activity of CBUs.

Towards this, I investigated whether the addition of aryl-alditols to 10% DMSO (conventional) could improve the protection of CB progenitors against cryoinjury. In a first set of experiments CB cells were cryopreserved with different concentrations of DMSO (0, 2, 5, and 10%) supplemented with IRI active compounds 2, 4, 6, and 9 (Chapter 3). IRI 2 and 6 supplemented with 5% and 10% DMSO showed comparable results in post-thaw viability of $CD34^+$ cells vs control. As previously indicated, the CFU assay is the best indicator of engraftment potential [149] and reductions in post-thaw CFU yields may reflect damages to the CBU during cryopreservation and thawing. The results showed a trend of higher number of clonogenic progenitors in thawed IRI samples vs control. Among all, compounds 2 and 6 (with the highest IRI activity) showed 2-fold increase in the number of progenitor cells vs. control. To investigate the cryoprotective effect of IRIs further, I measured the frequency of multipotent progenitors in CBU supplemented with the IRIs 2 and 6 using the stringent limiting dilution LTC-IC assay. The frequency of multipotent progenitors was increased 2.5-fold for sample cryopreserved with IRI 2 and 3.2-fold for sample cryopreserved with IRI 6 over DMSO control. Taken together, my results revealed that controlling ice growth and recrystallization with some IRIs can improve the post-thaw recovery of progenitors.

Next, I have investigated whether supplementation of the conventional 10% DMSO CPA solution with IRI 2 (chapter 4) would have a beneficial impact on the engraftment activity of CBU. I decided to focus on IRI 2 as it's protective activity on progenitors was more reliable than IRI 6, which was also tested in a single unit (Appendix 8.5). IRI 2 was well

tolerated by CB progenitor cells which was first observed by the clonogenic properties of fresh CB TNC incubated for an hour with IRI 2 (chapter 4). Likewise, there was no significant differences between IRI 2 and DMSO groups for cell viability of $CD45^+$ cells and $CD45^+CD34^+$ cell subpopulations, and for the frequencies and net numbers (data not shown) of $CD45^+ALDH^{br}$ and $CD34^+ALDH^{br}$ cells post-thaw.

The transplantation data showed that the hPLT levels were increased in IRI 2 transplants at both short and long-term post-transplantation. In line with this, combined analysis of the entire hPLT data set confirmed that the levels of hPLT were superior in IRI 2 cohort tested for four donors. Surprisingly, the same trend was not observed for human leucocyte, which were mostly similar between both groups. This difference between the leucocyte and platelets may be the results of the differences in lifespan of the different cell types [278, 279]. The long-term human BM analysis revealed that human $CD45^+$ chimerism and the number of human progenitors were both superior in the IRI 2 group. Conversely, the level of human BM engraftment in secondary recipients was similar between both mice groups. My results demonstrate that HSC are well preserved in the presence of IRI 2. Also, HSC preserved with IRI 2, maintained their key stem cells properties of multilineage differentiation and self-renewal. An optimal CPA should provide protective function to cells of interest during cryopreservation and it should be non-toxic [291].

In addition, IRI 6 showed very similar results to those previously seen with IRI 2. Indeed, no significant differences in viability and $ALDH^{br}$ cells for CBU cryopreserved with DMSO and IRI 6 was observed (Appendix 8.5). While IRI 6 had no significant impact on hPlt and leukocyte engraftment, the long-term BM analysis revealed that human $CD45^+$ cell engraftment was slightly higher in IRI 6 vs. DMSO, though the difference was not significant. As for IRI 2, the multilineage differentiation capacity of CB HSC protected with IRI 6 was also unaffected. Furthermore, the level of human progenitors (i.e. CFU) in

the primary recipients, and levels of engraftment in secondary recipients appeared higher in IRI 6 though the differences were not significant.

As indicated before, IRI had not been investigated in an HSC transplant setting. Based on the protective effect on progenitors, I expected IRI 2 to provide a greater impact on the engraftment levels in mice. However, the cell dose used might have been too high at times which resulted in very high level of engraftment in both groups. Another issue related to variations in engraftment level within the same group [292] from same donor and variation between independent donors. Hence, all the observations of the study represent the underlying phenomenon and due to the presence of variations, the errors are distributed unequally which resulted in less power for IRI 2 treated sample here.

Taken together, my results demonstrate that supplementation of CB grafts with IRI 2 and IRI 6 is well tolerated by hematopoietic progenitors and not deleterious to HSC. Importantly, IRI can improve the engraftment properties of CB grafts (hPlt and BM) which demonstrate their protective effects on HSC as CPA. Also, the nearly similar post-thaw functionality and engraftment outcome for IRI 2 and IRI 6 indicate that these molecules likely protect HSC by similar mechanisms during freezing and thawing.

CHAPTER 6
CONCLUSION

Conditions to maintain hematopoietic graft before cryopreservation remain controversial. Processing delay prior to cryopreservation should be minimized to avoid significant losses in cell potency. Many banks receive CBUs from distant sites and therefore, delays in processing due to various logistical reasons such as staff availability, distance between collection and processing sites, unexpected shipping delays, etc. might persist. It is critical to maximize cell recovery and to ensure that stem cells and progenitors be functional post-thaw to preserve the engraftment activity of CBU. My present study on impact of storage at RT on HSC has fulfilled an important gap in our understanding of this topic on CBU quality and potency since storage at RT is a common practice in the field of CB banking. Taken together, my data demonstrate that storage for periods longer than 40 hours should be avoided as it translates in a loss in HSC numbers leading to reduced engraftment activity that could further delay engraftment. CBU should be processed and cryopreserved shortly after collecting, ideally within 31 hours as this processing length was associated with engraftment activity comparable to baseline. In addition, the CFU assay remains the best predictor for loss of engraftment. Subsequently, if longer storage period is required, I recommend the storage of CBU at 4 °C as it improves cell recovery [109, 110] and might help to preserve the engraftment activity as reported by Louis, *et al*. [112]. Moreover, banks should consider opening collection sites that are close to their processing laboratories

and the courier schedules should be adjusted to transport collected CBUs back to the processing laboratory as quickly as possible. I believe that these results will be of great importance to researchers, clinicians and decision makers working in the field of stem cell banking and transplantation.

The other work of my dissertation was focused on finding new alternative for cryopreservation of CBU. In my work, I focused on small IRI molecules tested as an additive to conventional CPA (i.e. 10% DMSO, dextran-40). The results from my study demonstrate that supplementation of hematopoietic graft with IRIs can improve the post-thaw recoveries of progenitors and engraftment activities. It also showed that IRI 2 and IRI 6 have no deleterious effect on HSC. My work also provides strong evidence that IRI 2 is a new CPA that could play a significant role in future clinical applications possibly by reducing cryoinjuries associated with ice recrystallization during cryopreservation.

CHAPTER 7
FUTURE PERSPECTIVE

CB is a useful source of HSC for patients that lack matched donors, but complications related to CB transplantation are well known [119]. My study has revealed that prolonged storage (≥ 40 hours) at RT before processing of CBU, is deleterious to engraftment activity. However, further investigations could look at differentiating the impact of the length (i.e. 40 hours) and the storage temperature, as previously done by Louis, *et al.* for related donation (3 days storage). I would expect that engraftment deficit to be much lower if CBUs were stored at 4 °C at different time length.

Moreover, my results present evidence of increased TNF-α levels during storage of CBU which may initiate increases of ROS level in CB cells during storage, and perhaps the decline in potency due to cell lesion. TNF-α and ROS may directly be responsible for the loss of HSC [134, 135] but it needs to be firmly demonstrated. This could be tested by the addition of anti-oxidants such as superoxide dismutase (SOD)/peroxidase/glutathione (GSH) /vitamin C/Vitamin E to the CBU during storage, as anti-oxidants maintain normal ROS level by keeping the redox balance and cellular component integrity [293]. Later engraftment levels of treated and non-treated CB grafts can be compared to baseline in NSG mice. Furthermore, addition of TNF inhibitor to the CBU during storage, to determine whether it is responsible for the rise in ROS and, to protect stem and progenitors from ROS-mediated cell lesions will be worth pursing as future work [135]. Moreover, ROS levels at

28 hours could be investigated as CBU processed within 31 hours (Appendix 8.3) showed comparable engraftment activity.

These results may provide new avenue to improve the stability of HSC graft before processing. Loss of HSC due to apoptosis during storage might follow one of two mechanisms, either intrinsic (mitochondrial pathway) or extrinsic pathways (death receptor e.g. Fas, TNF receptor i.e. TNFR1). In mitochondrial pathway, pro-apoptotic and protective molecules play a role to regulate mitochondrial permeability and it's activated by most cellular stresses. B-cell lymphoma (Bcl-2) protein prevents apoptosis by blocking the release of cytochrome c from the mitochondrion. On the other hand, in extrinsic pathway/death receptors are members of the TNF receptor family (e.g. TNFR1 receptor and TNF-related apoptosis-inducing ligand i.e. TRAIL) and related protein called Fas (CD95) contain a death domain which needs to be activated to execute apoptosis [284, 294, 295]. Furthermore, the mitochondrial membrane permeability is usually governed by the Bcl-2 family proteins which can be either pro-apoptotic (Bcl-10, Bax, Bak, etc.) or anti-apoptotic (Bcl-2, Bcl-x, Bcl-XL, etc.) [284]. Thus, investigation of the impact of RT storage on the expression levels of pro and anti-apoptotic proteins in stored $CD34^+$ cell would provide some insights and potential correlation between oxidative stresses, cell injuries and cell loss by apoptosis.

The use of small molecules known as agonist or enhancer of HSC expansion e.g. UM171 [81] or SR1 [80] or both in combination have never been investigated as potential protective molecules in the context of storage post-collection of CBU. My preliminary data showed that, UM171 is not detrimental to CB cell during storage at RT. Supplementation of UM171 or SR1 or both in combination, to CBU during storage at RT could be investigated in future as a potential HSC preservative outside their normal use.

On the other hand, follow-up investigations are required to study the role of small IRI molecules compared to conventional CPA for CB graft. Additional donor should be tested for IRI 6. Combining two or more functionally divergent IRI molecules could be tested for CBU, and IRI could be further investigated for other sources of HSC (e.g. PBSC and BM) and for different types of stem cell (e.g. mesenchymal). Conversely, whether IRI can allow the use of lower concentration of DMSO needs to be investigated further. Moreover, the influence of IRI on cryoinjuries normally associated with ice recrystallization during freezing and thawing processes in CB cells is an area still open for investigation, and it would be interesting to explore in the near future. In addition, molecules that have been tested in this thesis are first generation IRI molecules. New derivatives from IRI 2 and IRI 6 (designed at Ben's lab) might have improved solubility and/or stability due to their structures, which can be investigated in future for better protective activity than their parent structures. On the whole, these diverse approaches will be helpful to understand better the conditions to preserve CB grafts and to support the transplantation and other associated clinical applications.

CHAPTER 8
APPENDIX

8.1. Chapter 2: Extended processing delay impairs the engraftment activity of CBUs

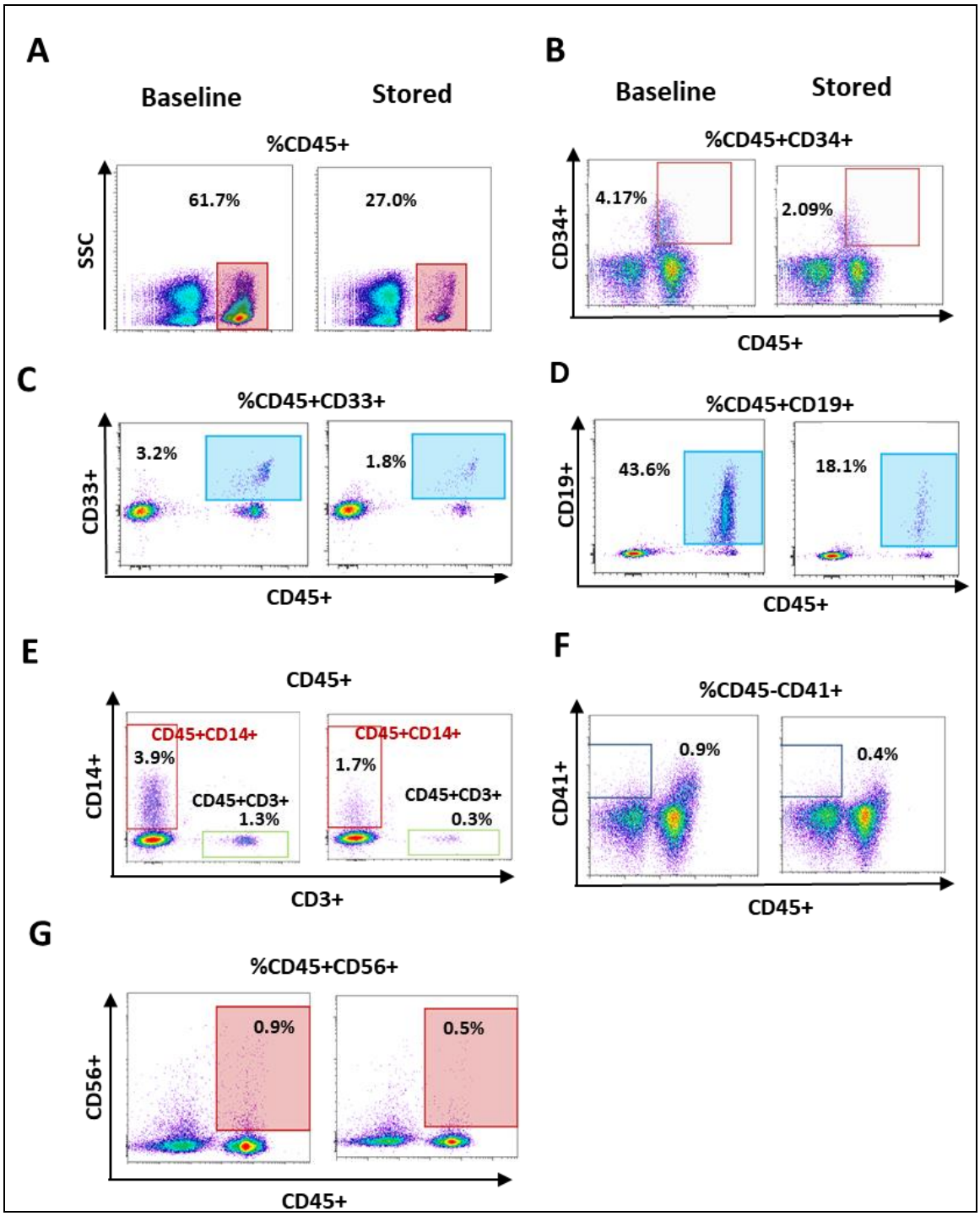

Figure 32: Representative of myeloid and lymphoid flow cytometric analyses of human BM engraftment in mice.

(A) $CD45^+$ cell, (B) $CD45^+CD34^+$ cell, (C) $CD45^+CD33^+$ cell, (D) $CD45^+CD19^+$ cell, (E) $CD45^+CD14^+$ cell and $CD45^+CD3^+$ cell, (F) $CD45^-CD41^+$ cell and (G) $CD45^+$ $CD56^+$ cell. Representative results for each group presented.

8.2. Chapter 3: Small-molecule ice recrystallization inhibitors improve the post- thaw function of hematopoietic stem and progenitor cells

8.2.1. General Experimental

Anhydrous reactions were performed under a positive pressure of dry argon in flame-dried glassware. Air or moisture-sensitive reagents and anhydrous solvents were transferred with oven dried syringes. Reactions were monitored using analytical thin layer chromatography with 0.2 mm pre-coated silica gel aluminum plates 60 F254 (E. Merck). Components were visualized by illumination with a short-wavelength (254 nm) ultra-violet light and/or staining (ceric ammonium molybdate or orcinol/sulphuric acid stain solution). ^{1}H (300, 400 or 500 MHz) and ^{13}C NMR (100 or 125 MHz) spectra were recorded on a Bruker Avance 400 or Bruker Avance 500. Deuterated DMSO (DMSO- d_6) was used as NMR solvents. Chemical shifts are reported in ppm using the solvent residual peak as an internal standard. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; quint, quintet; m, multiplet and br, broad. Low resolution mass spectrometry was performed on a Micromass Quatro-LC Electrospray spectrometer with a pump rate of 20 μL/min using electrospray ionization (ESI).

8.2.2. Synthesis of Aryl-Alditols

8.2.2.1. N-phenyl-D-gluconamide (1)

To a solution of D-gluconic acid-*d*-lactone (0.50 g, 2.81 mmol) in MeOH (10 mL) was added aniline (0.26 mL, 2.81 mmol). The mixture was stirred under reflux for 48 hours. The solvent was evaporated, and the residue was recrystallized in Ethyl alcohol (EtOH) to afford 1 as a pale brown solid (87%). Characterization data is consistent with that of previously reported data.[1,2] ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.68 (s, 1H), 6.86 (d, J = 8.06 Hz, 2H), 6.56 (t, J = 8.06 Hz, 2H), 6.21 (t, J = 8.06 Hz, 1H), 4.86 (d, J = 5.29 Hz, 1H), 3.76 (d, J = 5.18 Hz, 1H), 3.70-3.72 (m, 2H), 3.53 (t, J = 5.87 Hz, 1H), 5.33 (t, J = 4.83

Hz, 1H), 3.16-3.19 (m, 1H), 2.73-2.78 (m, 1H), 2.68 (t, J = 2.65 Hz, 2H), 2.52-2.58 (m, 1H). ^{13}C NMR (400 MHz, DMSO-d_6): δ 171.5, 138.5, 128.6, 123.4, 119.5, 74.2, 72.3, 71.5, 70.3, 63.3. LRMS (ESI): *m/z* calcd. for $C_{12}H_{18}NO_6$ $[M + H]^+$ 272.1, found 272.2.

8.2.2.2. N-(2-fluorophenyl)-D-gluconamide (2)

To a solution of D-gluconic acid-d-lactone (0.50 g, 2.81 mmol) in acetic acid (5 mL) was added 2-fluoroaniline (0.81 mL, 8.42 mmol). The mixture was stirred under reflux for 1 hour. The crude product was precipitated with hexanes and filtered to obtain a dark brown sludge. The solid was recrystallized in EtOH to afford 2 as a white powder (33%). Characterization data is consistent with that of previously reported data. [2] ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.21 (br. s, 1H), 8.13 (td, J = 7.87 Hz, 2.05 Hz, 1H), 7.31-7.26 (m, 1H), 7.20-712 (m, 2H), 5.95 (d, J = 5.96 Hz, 1H), 4.67 (d, J = 6.94 Hz, 1H), 4.62 (dd, J = 8.44 Hz, 5.36 Hz, 2H), 4.38 (t, J = 5.72 Hz, 1H), 4.24 (dd, J = 4.90 Hz, 3.35 Hz, 1H), 4.04-4.01 (m, 1H), 3.62-3.49 (m, 3H), 3.42-3.33 (m, 1H). ^{13}C NMR (400 MHz, DMSO-d_6): δ 171.5, 152.5 (d, $J_{C,F}$ = 241.58 Hz), 125.9 (d, $J_{C,F}$ = 10.71 Hz), 124.7 (d, $J_{C,F}$ = 7.29 Hz), 124.5 (d, $J_{C,F}$ = 3.36 Hz), 122.0, 115.2 (d, $J_{C,F}$ = 18.84 Hz), 73.9, 72.2, 71.6, 70.2, 63.3. ^{19}F NMR (300 MHz, DMSO-d6): δ -128.8. LRMS (ESI): *m/z* calcd. for $C_{12}H_{15}FNO_6$ $[M-H]^-$ 288.1, found 288.1

8.2.2.3. N-(4-fluorophenyl)-D-gluconamide (3)

To a solution of D-gluconic acid-*d*-lactone (0.20 g, 1.12 mmol) in MeOH (10 mL) was added 4-fluoroaniline (0.11 mL, 1.12 mmol). The mixture was stirred under reflux for 48 hours. The solvent was evaporated, and the residue was recrystallized in EtOH to afford 3 as a white powder (30%). Characterization data is consistent with that of previously reported data. [2] ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.80 (s, 1H), 6.90 (q, J = 5.47 Hz, 2H), 6.30 (t, J = 9.24 Hz, 2H), 4.86 (d, J = 4.98 Hz, 1H), 3.76 (d, J = 5.35, 1H), 3.69-3.72 (m,

2H), 3.53 (t, J = 5.95 Hz, 1H), 3.33 (t, J = 4.98 Hz, 1H), 3.16-3.19 (m, 1H), 2.73-2.77 (m, 1H), 2.67-2.69 (m, 2H), 2.53-2.58 (m, 1H). ^{13}C NMR (400 MHz, DMSO-d_6): δ 171.5, 158.1 (d, $J_{C,F}$ = 239.94 Hz), 135.0 (d, $J_{C,F}$ = 2.20 Hz), 121.4 (d, $J_{C,F}$ = 7.72 Hz), 115.1 (d, $J_{C,F}$ = 21.99 Hz), 74.2, 72.2, 71.5, 70.3, 63.3.^{19}F NMR (400 MHz, DMSO-d_6): δ -119.4. LRMS (ESI): *m/z* calcd. for $C_{12}H_{16}FNaNO_6$ $[M+Na]^+$ 312.3; found 312.2.

8.2.2.4. N-(4-chlorophenyl)-D-gluconamide (4)

To a solution of D-gluconic acid-*d*-lactone (0.20 g, 1.12 mmol) in acetic acid (5 mL) was added 4-chloroaniline (0.12 mL, 1.12 mmol). The mixture was stirred under reflux for 2 hours. The crude product was precipitated with hexanes, filtered and crude solid was recrystallized in EtOH to afford 4 as white crystals (180mg, 52%). Characterization data is consistent with that of previously reported data.[2] ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.7 (s, 1H), 7.8 (d, J = 9.1 Hz, 2H), 7.35 (d, J = 8.8 Hz, 2H), 5.71 (d, J = 5.3 Hz, 1H), 4.59 (d, J = 4.9 Hz, 1H), 4.55-4.53 (m, 2H), 4.36 (t, J = 5.7 Hz, 1H), 4.18 (dd, J = 5.1, 3.7 Hz, 1H), 4.02-3.99 (m, 1H), 3.61-3.57 (m, 1H), 3.52-3.51 (m, 2H), 3.42-3.36 (m, 1H). ^{13}C NMR (400 MHz, DMSO-d_6): δ 171.8, 137.6, 128.4, 126.9, 121.2, 74.2, 72.2, 71.5, 70.3, 63.3. LRMS (ESI): *m/z* calcd. for $C_{12}H_{16}ClNaNO_6$ $[M+Na]^+$ 328.7, found 328.0.

8.2.2.5. N-(2-chlorophenyl)-D-gluconamide (5)

To a solution of D-gluconic acid-*d*-lactone (0.20 g, 1.12 mmol) in acetic acid (5 mL) was added 2-chloroaniline (0.12 mL, 1.12 mmol). The mixture was stirred under reflux for 2 hours. The crude product was precipitated with hexanes, filtered and crude solid was recrystallized in EtOH to afford 5 as white crystals (10 mg, 3%). Characterization data is consistent with that of previously reported data.[2] ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.40 (s, 1H), 8.34 (dd, J = 1.61, 8.29 Hz, 1H), 7.52 (dd, J = 1.52, 7.97 Hz, 1H), 7.35 (dt, J = 1.58, 7.84 Hz, 1H), 7.13 (dt, J = 1.63, 7.71 Hz, 1H), 6.14 (d, J = 4.72 Hz, 1H), 4.7 (d, J =

7.36 Hz, 1H), 4.65 (dd, *J* = 2.77, 5.96 Hz, 2H), 4.39 (t, *J* = 5.66 Hz, 1H), 4.26 (dd, *J* = 3.07, 4.75 Hz, 1H), 4.05 (td, *J* = 3.0, 7.54 Hz, 1H), 3.48-3.61 (m, 3H), 3.37-3.43 (m, 1H). ^{13}C NMR (400 MHz, DMSO-d_6): δ 171.5, 134.4, 129.2, 127.8, 124.7, 122.3, 120.8, 74.0, 72.2, 71.6, 70.2, 63.2. LRMS (ESI): *m/z* calcd. for $C_{12}H_{16}ClNaNO_6$ $[M+Na]^+$ 328.7, found 328.0.

8.2.2.6. N-(4-methoxyphenyl)-D-gluconamide (6)

To a solution of D-gluconic acid-*d*-lactone (0.20 g, 1.12 mmol) in MeOH (15 mL) was added p-anisidine (0.14 g, 1.12 mmol). The mixture was stirred under reflux for 24 hours. The solvent was evaporated, and the residue was recrystallized in EtOH to afford 6 as white crystals (21%). Characterization data is consistent with that previously published.[2,3] ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.41 (s, 1H), 7.61 (d, *J* = 9.28 Hz, 2H), 6.86 (d, *J* = 9.00 Hz, 2H), 5.65 (d, *J* = 5.32 Hz, 1H), 4.58 (d, *J* = 5.10 Hz, 1H), 4.53-4.51 (m, 2H), 4.36 (t, *J* = 5.85 Hz, 1H), 4.14 (dd, *J* = 4.10 Hz, 5.18 Hz, 1H), 4.01-3.99 (m, 1H), 3.72 (s, 3H), 3.61-3.56 (m, 1H), 3.51-3.49 (m, 2H), 3.41-3.36 (m, 1H). ^{13}C NMR (400 MHz, DMSO-d_6): δ 171.0, 155.3, 131.7, 121.0, 113.7, 74.1, 72.3, 71.5, 70.3, 63.3, 55.1. LRMS (ESI): *m/z* calcd. for $C_{13}H_{18}NO_7$ $[M-H]^-$ 300.1, found 300.1.

8.2.2.7. N-(2-methoxyphenyl)-D-gluconamide (7)

To a solution of D-gluconic acid-*d*-lactone (0.10 g, 0.56 mmol) in MeOH (10 mL) was added o-anisidine (0.07 g, 0.56 mmol). The mixture was stirred under reflux for 24 hours. The solvent was evaporated, and the residue was recrystallized in EtOH to afford 7 as white crystals (65%). Characterization data is consistent with that of previously reported data.2 1H NMR (400 MHz; DMSO-d_6): δ 9.25 (s, 1H), 8.33 (d, *J* = 8.0 Hz, 1H), 7.07-7.05 (m, 2H), 6.95-6.90 (m, 1H), 6.03 (d, *J* = 4.56 Hz, 1H), 4.64-4.60 (m, 3H), 4.38 (t, *J* = 5.57 Hz, 1H), 4.20 (t, *J* = 3.36 Hz, 1H), 4.05-4.03 (m, 1H), 3.86 (s, 3H), 3.61-3.50 (m, 3H), 3.43-3.39 (m, 1H). ^{13}C NMR (400 MHz; DMSO-d_6): 170.9, 147.7, 127.1, 123.5, 120.5, 118.1,

110.8, 74.2, 72.5, 71.6, 70.1, 63.3, 55.8. LRMS (ESI): *m/z* calcd. For $C_{13}H_{19}NaNO_7$ $[M+Na]^+$ 324.1, found 324.0.

8.2.2.8. N-benzyl-D-gluconamide (8)

To a solution of D-gluconic acid-*d*-lactone (0.89 g, 5.02 mmol) in MeOH (30 mL) was added benzylamine (0.69 mL, 5.02 mmol). The mixture was stirred under reflux for 48 hours. The solvent was evaporated, and the residue was recrystallized in EtOH to afford 8 as white crystals (97%). Characterization data is consistent with that of previously reported data.[1,2] ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.17 (t, J = 6.36 Hz, 1H), 7.21-7.3 (m, 5H), 5.46 (d, J = 5.14 Hz, 1H), 4.58 (d, J = 4.77 Hz, 1H), 4.52 (d, J = 5.39, 1H), 4.47, (d, J = 7.71 Hz, 1H), 4.36 (t, J = 5.75 Hz, 1H), 4.31 (d, J = 6.49 Hz, 2H), 4.07 (t, J = 4.53 Hz, 1H), 3.96-3.98 (m, 1H), 3.56-3.58 (m, 1H), 3.51 (s, 2H), 3.36-3.39 (m, 1H). ^{13}C NMR (400 MHz, DMSO-d_6): δ 172.6, 139.6, 128.1, 127.1, 126.6, 73.7, 72.5, 71.6, 70.1, 63.4, 41.8. LRMS (ESI): *m/z* calcd. for $C_{13}H_{20}NO_6$ $[M+H]^+$ 286.1, found 286.1.

8.2.2.9. N-(2,6-difluorobenzyl)-D-gluconamide (9)

To a solution of D-gluconic acid-*d*-lactone (0.20 g, 1.12 mmol) in MeOH (15 mL) was added 2,6-difluoroaniline (0.13 mL, 1.12 mmol). The mixture was stirred under reflux for 24 hours. The solvent was evaporated, and the residue was recrystallized in EtOH to afford 9 as white crystals (89%). Characterization data is consistent with that of previously reported data.[2] ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.89 (t, J = 5.73 Hz, 1H), 7.33-7.43 (m, 1H), 7.07 (t, J = 8.09 Hz, 2H), 5.35 (d, J = 5.32 Hz, 1H), 4.53 (d, J = 5.00 Hz, 1H), 4.38-4.48 (m, 3H), 4.27-4.34 (m, 1H), 4.00 (dd, J = 5.18 Hz, 3.77 Hz, 1H), 3.87-3.91 (m, 1H), 3.53-3.59 (m, 1H), 3.44-3.45 (m, 2H), 3.35-3.39 (m, 1H). ^{13}C NMR (400 MHz, DMSO-d_6): δ 172.6, 167.7 (d, $J_{C,F}$ = 8.41 Hz), 160.3 (d, $J_{C,F}$ = 8.18 Hz), 130.3 (t, $J_{C,F}$ =10.37 Hz), 114.7 (t, $J_{C,F}$ = 19.56 Hz), 112.0 (dd, $J_{C,F}$ = 18.85 Hz, 6.51 Hz), 74.0, 72.8, 72.0, 70.6, 63.8.

^{19}F NMR (300 MHz, DMSO-d_6): δ -114.5. LRMS (ESI): *m/z* calcd. for $C_{13}H_{17}F_2KNO_6$ $[M+K]^+$ 360.1, found 360.1.

8.2.2.10. N-(3,5-difluorobenzyl)-D-gluconamide (10)

To a solution of D-gluconic acid-*d*-lactone (0.20 g, 1.12 mmol) in MeOH (10 mL) was added 3,5-difluorobenzylamine (0.15 mL, 1.12 mmol). The mixture was stirred under reflux for 48 hours. The solvent was evaporated, and the residue was recrystallized in EtOH to afford 10 as a white powder (87%). Characterization data is consistent with that of previously reported data.[2] ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.35 (t, J = 6.25 Hz, 1H), 6.70-7.07 (m, 3H), 5.56 (d, J = 4.92 Hz, 1H), 4.60 (d, J = 4.52 Hz, 1H), 4.56 (d, J = 5.32 Hz, 1H), 4.51 (d, J = 7.31 Hz, 1H), 4.35-4.40 (m, 2H), 4.25-4.30 (m, 1H), 4.09 (t, J = 4.26 Hz, 1H), 3.96-3.98 (m, 1H), 3.56-3.60 (m, 1H), 3.50 (s, 2H), 3.36-3.39 (m, 1H). ^{13}C NMR (400 MHz, DMSO-d_6): δ 173.0, 162.4 (d, $J_{C,F}$ = 246.05 Hz), 162.3 (d, $J_{C,F}$ = 245.67 Hz), 144.7 (t, $J_{C,F}$ = 8.79 Hz), 109.9 (dd, $J_{C,F}$ = 24.77 Hz, 6.37 Hz), 101.9 (t, $J_{C,F}$ = 25.98 Hz), 74.0, 72.6, 71.6, 70.2, 63.4, 41.2. ^{19}F NMR (300 MHz, DMSO-d_6): δ -110.4. LRMS (ESI): *m/z* calcd. for $C_{13}H_{17}NO_6F_2$ $[M + H+]^+$ 322.1, found 322.2.

8.2.3. Cryosolutions

Cryosolutions were prepared in distilled autoclaved water supplemented with 0.9% saline and 5% dextran for cryopreservation experiments and diluted with different volumes of plasma in cryovial depending on desired concentration of IRI. Compounds 2, 3, 6 and 7 were prepared at 25 mM and compounds 1, 4, 5 and 8 were prepared at 55 mM. 100, 50, 33 or 20 µL of 25 mM 2, 3, 6 or 7 was added to cryovials containing 50,000 CD34+ cells suspended in 0, 50, 67, or 80 µL of plasma, respectively, for a final IRI concentration of 25, 12.5, 8 or 5 mM, respectively. 100, 50, 33 or 20 µL of 55 mM 1, 4, 5 or 8 was added to

cryovials containing 50,000 $CD34^+$ cells suspended in 0, 50, 67, or 80 µL of plasma, respectively, for a final IRI concentration of 55, 22.5, 18 or 11 mM, respectively.

8.2.4. MTT assay with HepG2 cells

The MTT assay was performed as described previously using HepG2 cells.4–6 HepG2 cells were plated in 96-well plates and allowed to reach confluency. Cells were then treated with 100 µL of MEM media supplemented with the compound of interest at various concentrations and incubated at 37°C for 16 h with 5% CO2. Cells incubated with MEM without supplement were used as a negative control, and cells supplemented with 1% Triton-X were used as a positive control. Following incubation, the supplemented media was removed and 200 µL of fresh media and 50 µL of MTT solution (5 mg/mL) in Hank's balanced salt solution (HBSS) were added and the plates were incubated at 37°C with 5% CO2 for 3 h. The plates were then centrifuged, the media aspirated and 200 µL of MTT solubilization solution (10% Triton X-100, 0.1 N HCl in isopropanol) was added to each well. The plates were incubated at RT in the dark for 2-4 h and the absorbance of each well was then read at a wavelength of 570 nm with a multi-well plate reader (AD 34°C Absorbance Detector, Beckman Coulter, Inc., Mississauga, ON). Each experiment was repeated on three separate plates in 10 consecutive wells for each condition. The average absorbance was calculated and compared to the control. Viability was reported as a percentage of the control.

8.3. Impact of CBU stored at RT and cryopreservation within 31 hours post-collection on HSC engraftment activity

Several aspects of CB collection and processing are controversial topics and optimization of condition to store the CBU before processing is an important issue for banking and transplantation. However, CB transplantation is associated with slow platelet and neutrophil engraftment [65, 66]. Maximization of the quality of CB product is a continuous process to work with. Standard agencies limit CB storage to a maximum of 48 hours from collection to freezing at RT [96-98]. In chapter 2, results from my study demonstrate that long (≥ 40 hours) processing delays can lead to loss in engraftment activity when CBU are stored at RT. The delay associated with transport time and processing reduce the CBU quality, consequently the time of storage prior to cryopreservation need to be reduced. A retrospective analysis of 3,359 CBU processed at the CBS CB bank (2015-2019) revealed that 90% of CBU are cryopreserved by 31 hours (Figure 7). Hence, I investigated whether CBU half stored at RT and fully processed by 31 hours (28 hours of storage at RT) would exhibit similar engraftment deficits as seen previously with the longer storage (≥ 40 hours).

8.3.1. Hypothesis

The loss in engraftment activity associated with long storage (≥ 40 hours) at RT can be avoided by limiting storage at RT to 28 hours for full processing by 31 hours post-collection.

8.3.2. Objectives

- Measure the viability and $ALDH^{br}$ cells and CFU assay of HSPC
- Measure the engraftment activity of CBU stored at RT and cryopreserved by 31 hours post-collection.

8.3.3. Method

CBU were processed following common banking procedures. Freshly collected CBU were split in two fractions upon arrival; the first half was processed immediately and served as baseline control (stored at RT for 9.9 ± 3.9 hours, frozen by 13.1 ± 4 hours post collection). The second half was processed after a (~31 hour) storage period at RT (stored group, stored for 28 ± 0.1 hours, frozen by 31 ± 0.2 hours). CBUs (n=3) were processed with hetastarch and cryopreserved as buffy coats. Thawed cells were tested for viability (annexin v, sytox-7AAD), ALDH activity, and CFU assay and also transplanted into NSG mice as stated in materials and methods section under Chapter 2.

In this series of transplants, 2 million TNC were transplanted in both groups (viability and cell dose indicated in Table 7 in Appendix 8.3.3). Long-term BM engraftment was investigated 25 weeks post-transplantation; in baseline and 31 hours stored groups and no secondary transplants were carried out for this set of experiments.

Table 7: Characteristic of the CBU tested and cell dose transplanted in baseline and stored groups for 31 hours processing delay (28 hours storage at RT).

CBU	**Sample**	**Viability (%)[1]**			**Dose TNC (Post-thaw)**	
		Frequency $CD34^+$ (%)	TNC	CD34+ cells	$x10^6$/mice[2]	$x10^7$/kg
CBU4	Baseline	0.40	78.69	72.11	2.00	9.71
	Stored	0.33	92.63	73.26	2.00	9.71
CBU5	Baseline	0.22	93.28	60.87	2.00	9.71
	Stored	0.19	87.77	69.47	2.00	9.71
CBU6	Baseline	0.31	95.61	57.93	2.00	9.71
	Stored	0.26	94.39	59.93	2.00	9.71
Mean (SD)	Baseline	0.31 (0.1)	89.2 (9.2)	63.6 (7.5)	2.0 (0.0)	9.7 (0.0)
Mean (SD)	Stored	0.26 (0.1)	91.6 (3.4)	67.6 (6.9)	2.0 (0.0)	9.7 (0.0)
P value		0.05	0.72	0.24	NA	NA

[1] based on annexinV staining, 2net number of TNC

8.3.4. Results and conclusion

I performed the same in vitro quality and potency assays on CBU processed by 31 hours. Viability of TNC and $CD45^+$ cells were similar between both groups, except for a small rise in the proportion of viable $CD34^+$ cells in the stored group (Figure 33A). There were no significant differences in the net numbers of viable cells (Figure 33B). With these 3 CBU tested, a small but significant reduction in the frequency and net number of $CD34^+$ ALDH bright cells was observed in the stored group (Figure 33C). However, no differences in the CFU content was detected (Figure 33D).

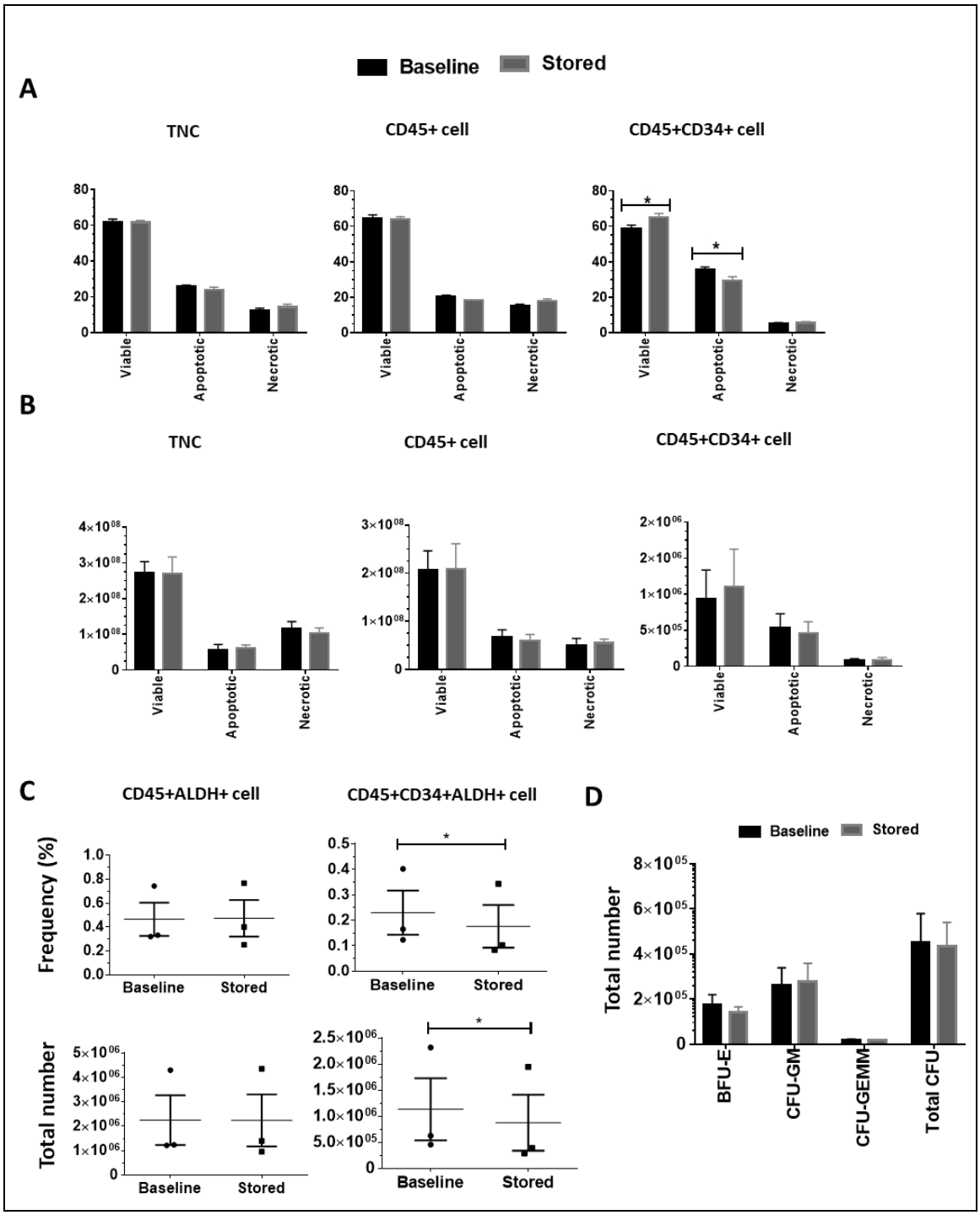

Figure 33: Post-thaw CB quality of CBU stored for 31 hours at RT before processing and cryopreservation.

A-B) Proportions and net number of viable, apoptotic and necrotic cells for indicated cell subpopulations. C) Frequency and total number of ALDH subpopulations in CBU post-thaw. D) Net number of CFU progenitors measured in CBU post-thaw. Results presented as mean ± SEM for baseline and 31 hours stored groups (n=3). P values were calculated by paired t-test.

Platelet and leucocyte engraftment at 4 weeks after transplantation were not significantly different between the baseline and 31 hours stored groups, and similar observations were made at 17 weeks (Figure 34 and Figure 35).

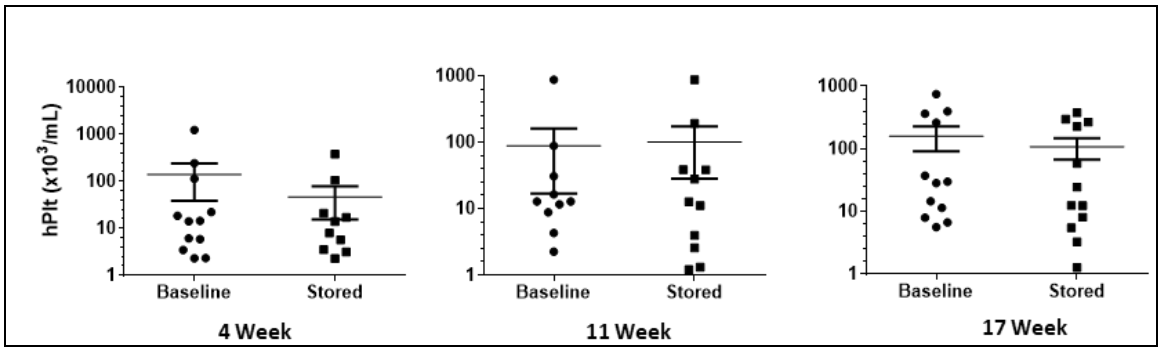

Figure 34: Impact of 31 hours processing delay on the thrombopoietic activity of CBU.

Human platelet levels in baseline and stored group as a function of time post-transplantation (mean ± SEM, 12 mice per group, n=3). Each symbol corresponds to an individual mouse, while the horizontal line and error bars represent the mean ± SEM. Significant differences were determined by Mann-Whitney test * P≤0.05.

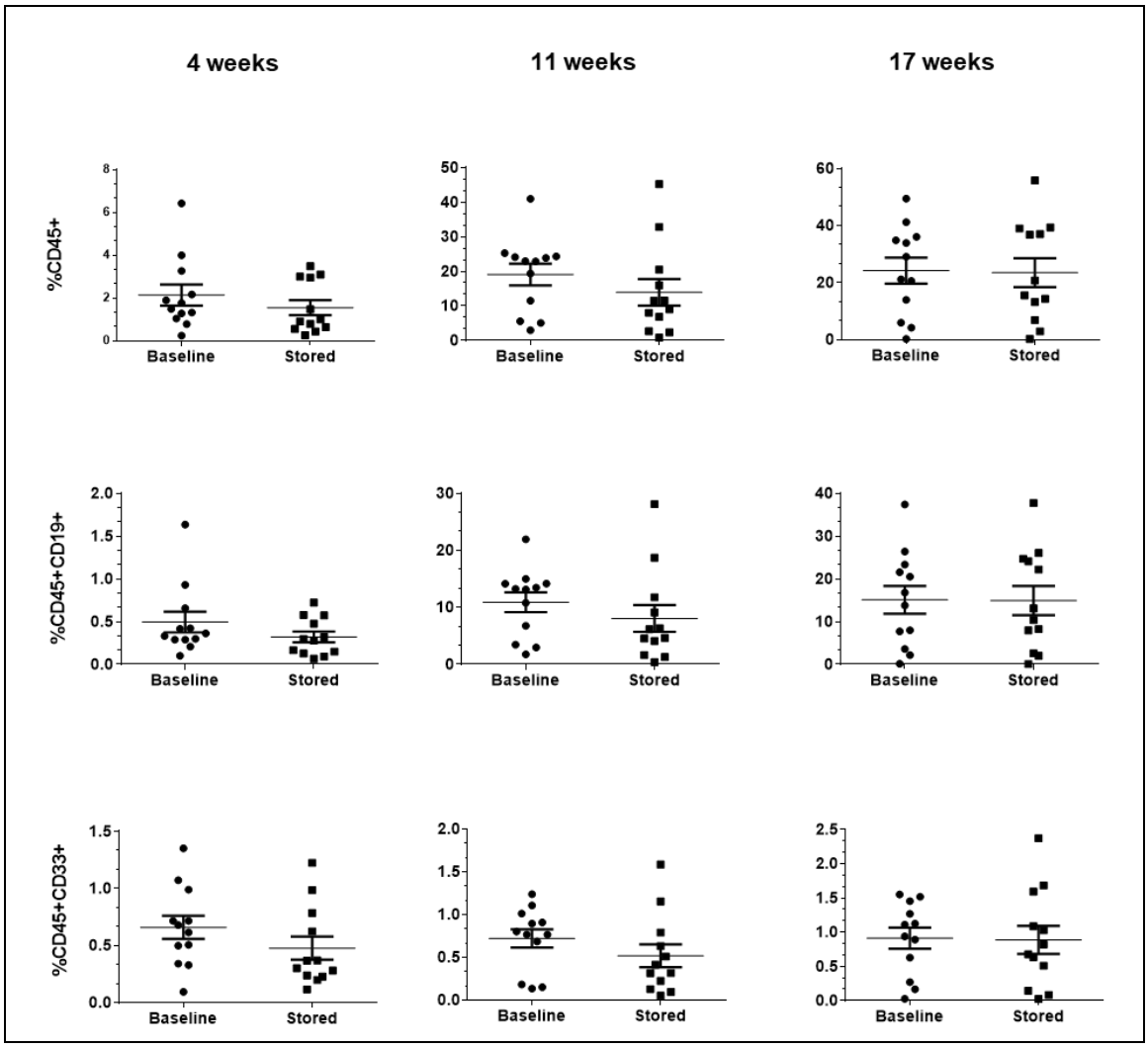

Figure 35: Impact of 31 hours processing delay on leucocyte engraftment.

A) Human $CD45^+$ cell, $CD45^+CD19^+$ B cell and $CD45^+CD33^+$-myeloid engraftment level at indicated time point in baseline and stored group (mean ± SEM, 12 mice per group, n=3). Each symbol corresponds to an individual mouse. The horizontal line and error bars represent the mean ± SEM. Significant differences were determined by Mann-Whitney test * $P \leq 0.05$.

Long-term BM engraftment was investigated in all mice 25 weeks post-transplantation; human BM engraftment in baseline and the 31 hours stored groups were comparable (Figure 36A). Moreover, there was no significant difference in all myeloid and lymphoid lineages between groups (Figure 36A). Furthermore, the distributions of human engraftment (Figure 36B) and net content of human CFU (Figure 36C) were similar in both mice cohorts as well. In light of these comparable engraftment results, no secondary transplants were carried out.

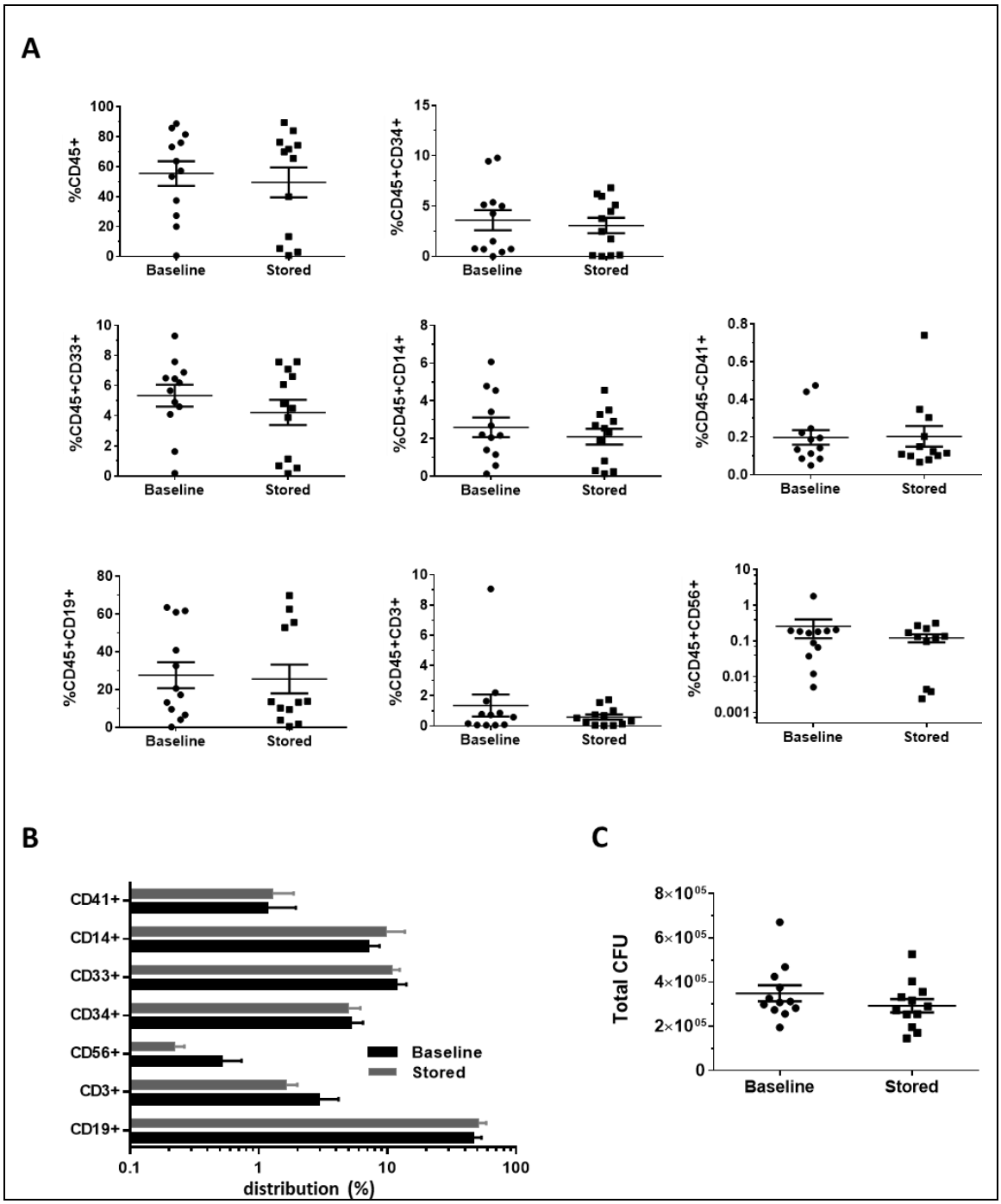

Figure 36: Impact of 31 hours processing delay on human BM reconstitution.

A) Multilineage human BM engraftment analysis of recipients 25 weeks post-transplantation. Each symbol corresponds to a mouse. The horizontal line and error bars represent the mean ± SEM of 12 mice per group (n=3). B) Lineage distribution of human BM engraftment in recipients (mean ± SEM). C) Total number of human CFU progenitors in the BM of recipients (mean ± SEM). Significant differences were determined by Mann-Whitney test * $P \leq 0.05$ for all panels except panel B which was done by two-way ANOVA.

The extended storage (i.e. ≥ 40 hours) at RT before processing negatively impacted platelet and leucocyte engraftment and the results from long-term BM chimerism was significantly reduced. In addition, the deficit in BM engraftment was exacerbated in secondary transplants indicating that storage (i.e. ≥ 40 hours) at RT before processing could contribute and impair slower engraftment kinetics (see in chapter 2, Figure 9, Figure 10 and Figure 11). In contrast, first the same in vitro quality and potency assays performed on CBU processed by 31 hours and no significant difference between baseline and stored group for viability of TNC and $CD45^+$ cells, except for a small rise in the proportion of viable $CD34^+$ cells in the stored group. A small but significant reduction in the frequency and net number of $CD34^+$ ALDH bright cells was observed in the stored group but no significant difference in the CFU content was detected. In line with that, CBU processed within 31 hours had engraftment activities comparable to the baseline group. The results from these *in vivo* data recommend limiting to 31 hours for full processing of CBU from time of collection to cryopreservation if units are stored at RT.

8.4. Impact of UM171 on CBU stored at RT for 40-44 hours

There are several small molecules which have been used to promote and maintain the expansion of HSC in culture. Pyrimidoindole derivative UM171 showed advantageous effect on expansion of HSC in *ex vivo* with functionally validated long-term *in vivo* repopulating capability but how or which pathway(s) UM171 involves to maintain HSC in vitro are still unknown [81]. In CB expansion cultures, UM171 support to reduce apoptosis without affecting the proliferation of the HSCs [286]. One of the main challenges of CB banks is to improve the quality of their conserved CBUs. It is not always possible to process CBU immediately after collection due to logistical difficulties and prolonged storage at RT has adverse effect on the viability, function and recovery of CB cells including $CD34^+$ cells and progenitors, which may limit its therapeutic potency [109, 112]. Though UM171, is one of the most potent small molecules enhancer of HSC expansion, no study has investigated the effect of UM171 on CB storage at RT before processing and cryopreservation (i.e., to combine UM171 with CB during storage at RT until processing to see its effect on the quality of CBU). In this study I wanted to investigate whether CB stored at RT for 43-44 hours combined with UM171 can reduce the detrimental effect of storage on CB cell to improve or maintain the quality of the CBU.

8.4.1. Hypothesis

Storage of CBU with UM171 at RT before processing will improve the post-thaw CB quality.

8.4.2. Objectives

- Measure the viability and $ALDH^{br}$ cells and CFU assay of HSPC
- Measure the engraftment activity of CBU stored with UM171

8.4.3. Method

CBU were processed with hetastarch and cryopreserved as buffy coats. For that CBUs were split in seven parts, one part was processed and cryopreserved immediately (baseline <22 hours) and the others after 43-44 hours storage at RT with UM171 at different concentrations (stored) or without UM171 (no treatment) (Figure 37). CBUs were stored with UM171 at different concentrations (20 nM, 35 nM, 50 nM, 70 nM and 100 nM) to observe the dose dependent effects of UM171 on CB cell. Thawed cells were tested for viability (annexin v, sytox-7AAD), ALDH activity, CFU assay and also transplanted into NSG mice as indicated in materials and methods in Chapter 2 unless stated otherwise.

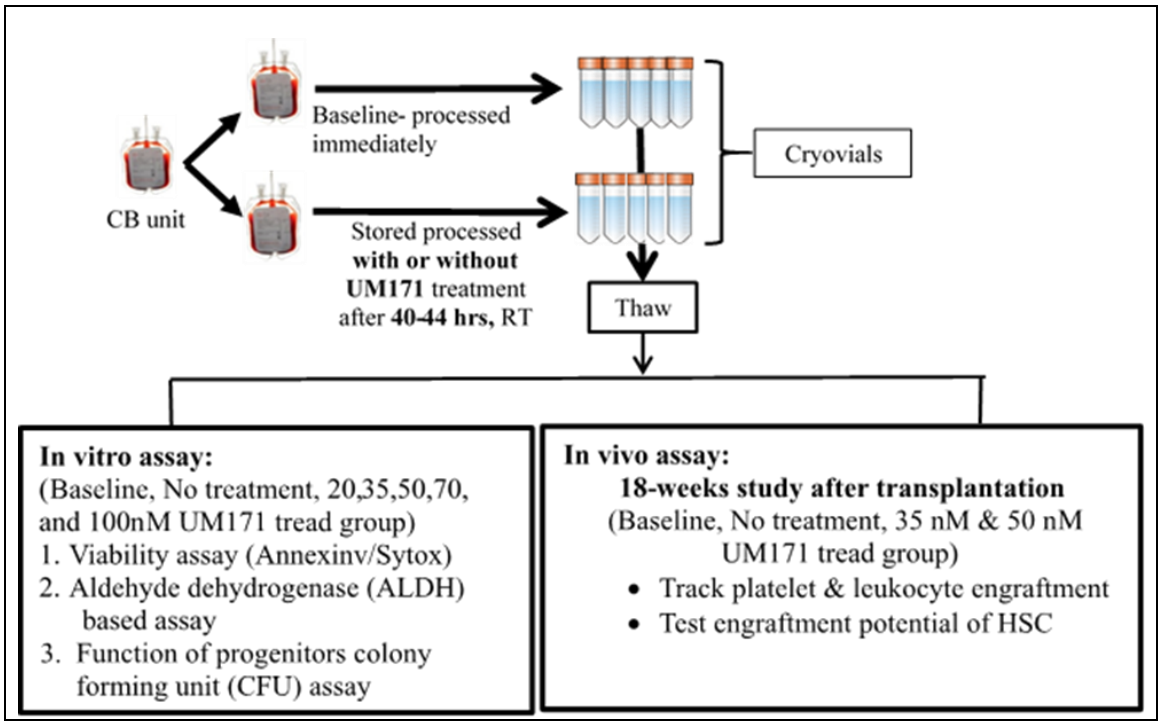

Figure 37: Overview of the study design of processing and post-thaw assays for CBU stored with UM171

8.4.4. Results and conclusion

The viability assay (n=5) showed that the frequency of viable TNC, $CD45^+$ cells and $CD45^+$ $CD34^+$ cells were unchanged by addition of UM171 vs. control (Figure 38A). Moreover, there was no notable difference for the frequency of $ALDH^{br}$ cells between groups for both

$CD45^+$ $ALDH^{br}$ and $CD34^+$ $ALDH^{br}$ cells (Figure 38B). Similarly, no significant differences were observed between stored and control group for the net number of progenitors recovered post-thaw (n=5) (Figure 38C).

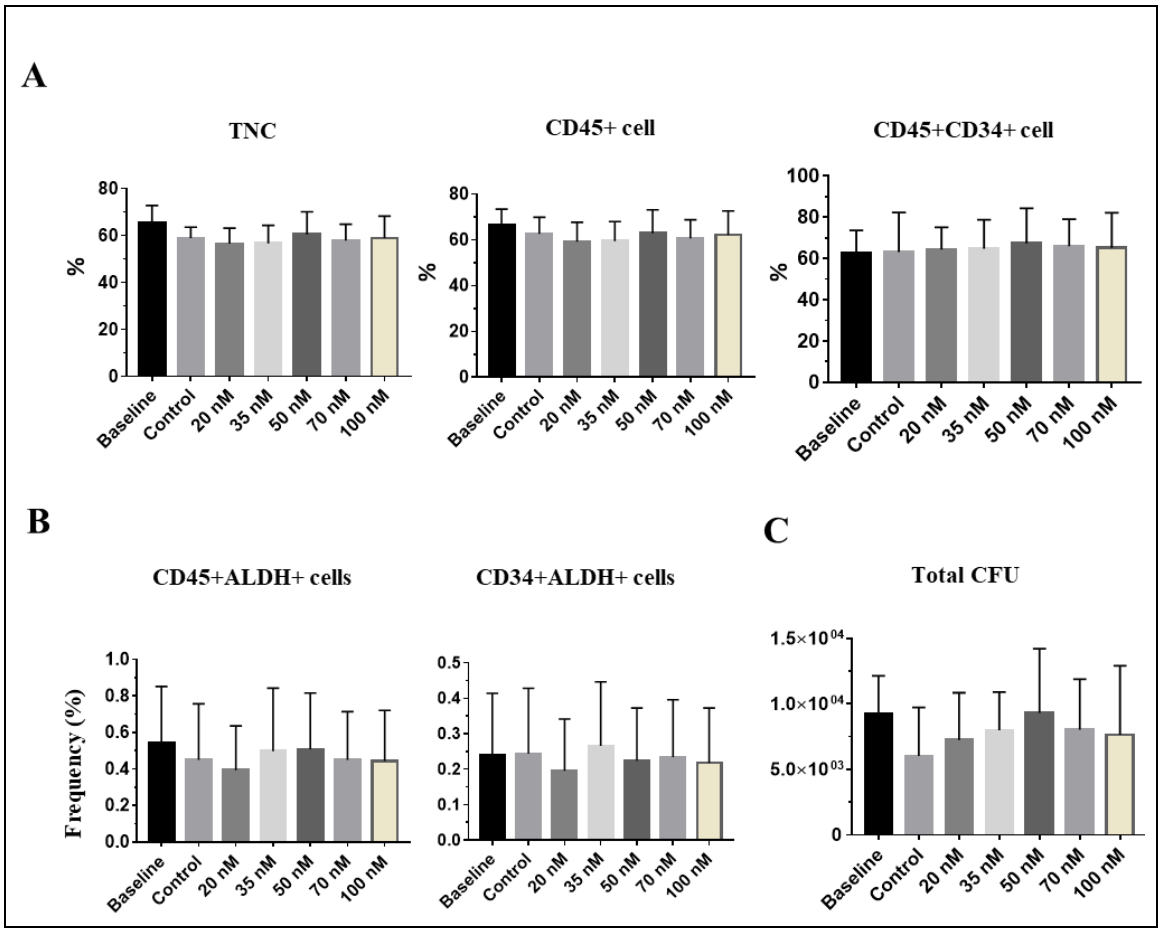

Figure 38: Impact of processing delay on CB cells treated with or without UM171

Results represent the summary of the proportion of (A) viable TNC, $CD45^+$ cell and $CD45^+$ $CD34^+$ cells, (B) frequency of $CD45^+$ $ALDH^{br}$ cells and $CD34^+$ $ALDH^{br}$ cells, (c) number of total colonies count of CB cell (45K per plate seeded) for baseline, stored with or without UM171 (no treatment or control) samples. Results presented and summarized based on mean ±SEM; n=5. Significant differences were determined by one-way ANOVA, $p>0.05$.

Though the results were not significant, it appears that CB cell stored at RT with UM171 at 50 nM showed a trend of better CFU count over no treatment (Control), and similarly higher $CD34^+ALDH^{br}$ cells. However, these results need to be confirmed with more donors.

I had the opportunity to test the impact of UM171 on engraftment activity of CB stored at RT. For this, four groups of mice were transplanted (baseline, control, 35 nM and 50 nM) with 2x10^6 TNCs (OKT-3 treated antibody). Engraftment in the periphery was analysed 4-17 weeks post transplantation; hPlt levels were generally very low in all 4 groups and not significantly different (data not shown). Also, the leukocyte engraftment (%$CD45^+$ cell) was similar between the 4 mice groups at all-time points (Figure 39).

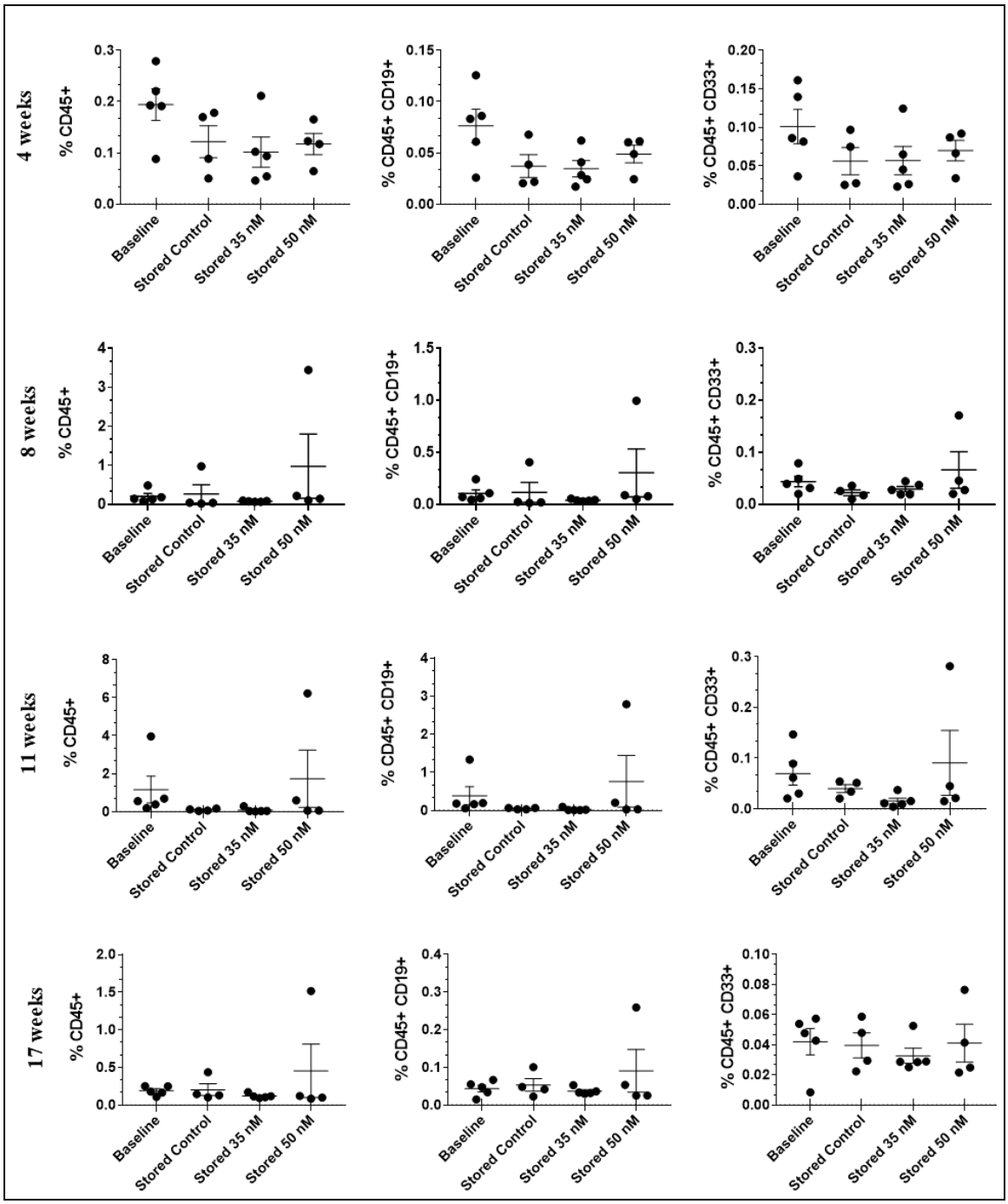

Figure 39: Impact of processing delay on leukocyte engraftment properties of CBU stored at RT with or without UM171.

Results presented and summarized based on mean ± SEM; donor =1, at different time points in mice transplanted with baseline, no treatment (control) and stored samples. (4-5 mice per group). Results presented and summarized based on mean ± SEM; donor =1. Significant differences were determined by one-way ANOVA, $p>0.05$.

Long-term BM analysis was performed 18 weeks post-transplantation. No significant differences in human chimerism between the 4 groups were detected and, both myeloid

and lymphoid lineages were detected in all groups (Figure 40). Of note, the mice available for this assay were not optimal as they were more than 15 weeks old male mice which explains the lower engraftment observed.

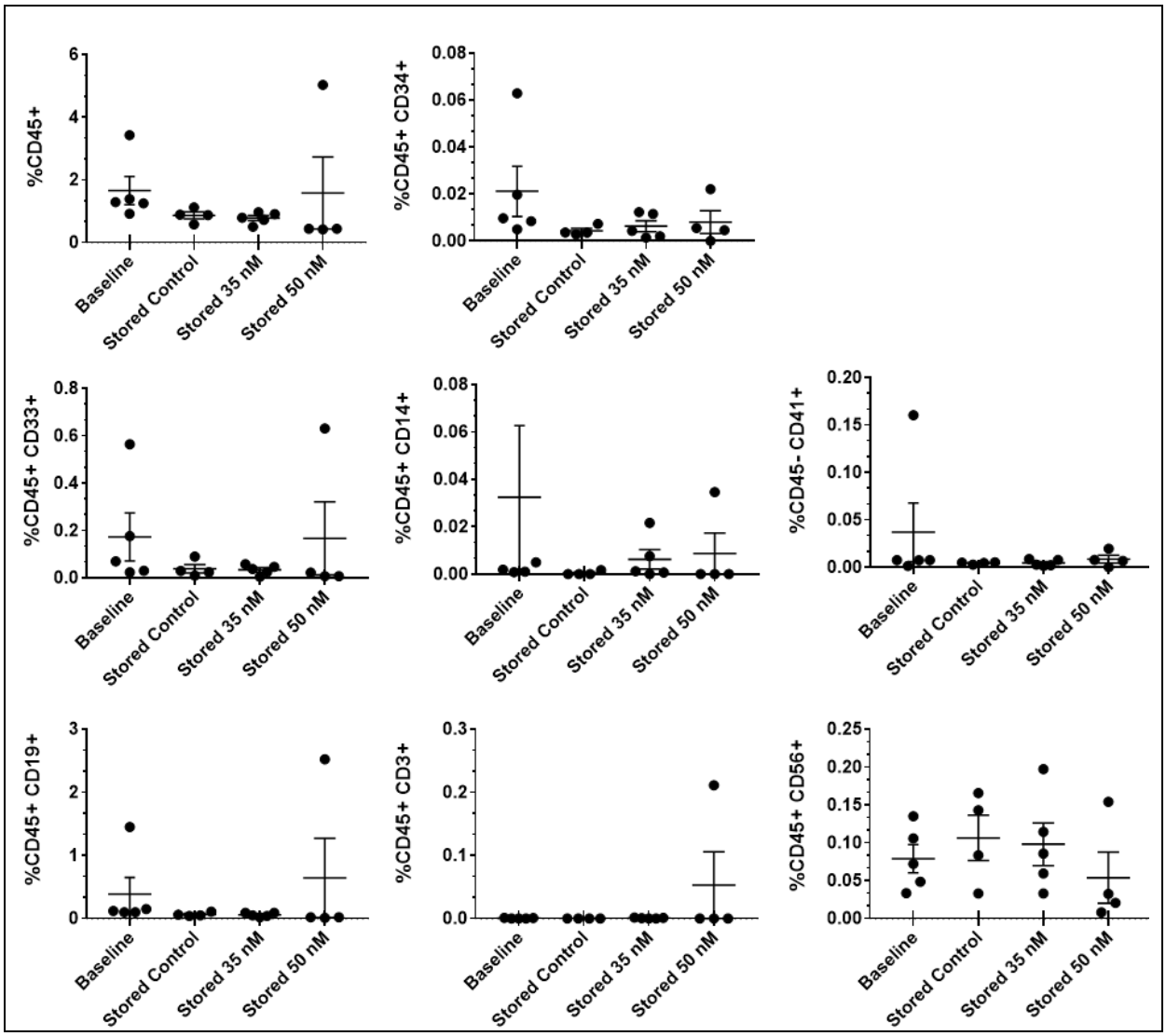

Figure 40: Human BM engraftment in primary transplants

Human BM engraftment analysis in primary recipients (mean ± SEM, n=1 donor, 4-5 mice per group). Significant differences were determined by one-way ANOVA, $p>0.05$.

These preliminary data suggest that, UM171 is not detrimental to CB cell during storage of CBU at RT. Whether UM171 (possibly at 35 or 50 nM) may protect HSC and progenitor and lead to better engraftment will need to be confirmed in additional assays.

8.5. Impact of IRI N-(4-methoxyphenyl)-D-gluconamide (IRI 6) on post-thaw CB quality and engraftment activity

Briard, J.G., *et al.* showed that a series of small molecules are capable of controlling ice growth and recrystallization. N-(4-methoxyphenyl)-D-gluconamide (IRI 6, 27.5 mM) was one of those molecules with potent IRI activity and showed significantly improved post-thaw recovery of committed progenitors and multipotent progenitors in CBU when supplemented with the standard CPA solution [271]. In line with this finding, I wanted to test whether the addition of IRI 6 to inhibit the ice recrystallization during freezing and thawing can improve the engraftment activity of CB HSC in cryopreserved CBU.

8.5.1. Hypothesis

Supplementation of IRI 6 with the conventional CPA (10% DMSO) during cryopreservation can improve the post-thaw CB quality.

8.5.2. Objectives

- Measure the viability and $ALDH^{br}$ cells and CFU assay of HSPC in post-thaw.
- Measure the engraftment activity of CBU cryopreserved with IRI 6.

8.5.3. Method

CBUs were processed with hetastarch and cryopreserved as buffy coats with 10% DMSO (control) or 10% DMSO with IRI 6 (IRI 6). Thawed cells were tested for viability (annexin v, sytox-7AAD), ALDH activity, and CFU assay and also transplanted into NSG mice as stated in materials and methods section under Chapter 4.

8.5.4. Results and conclusion

The viability assay (n=3) showed no significant difference in viable TNC, $CD45^+$ cells and $CD45^+$ $CD34^+$ cells for CBU cryopreserved with DMSO and IRI 6 (Figure 41A). Also, no

significant difference was observed for the frequencies of $CD45^+$ and $CD34^+$ $ALDH^{br}$ cells (Figure 41B) and in colony number for progenitors recovery post-thaw (n=3) between DMSO and IRI 6 group (Figure 41C). In line with this, the clonogenic properties of fresh CB TNC incubated for an hour at 4°C with IRI 6 (Figure 42) was comparable with control and DMSO suggesting that the IRI 6 is well tolerated by CB progenitor cells.

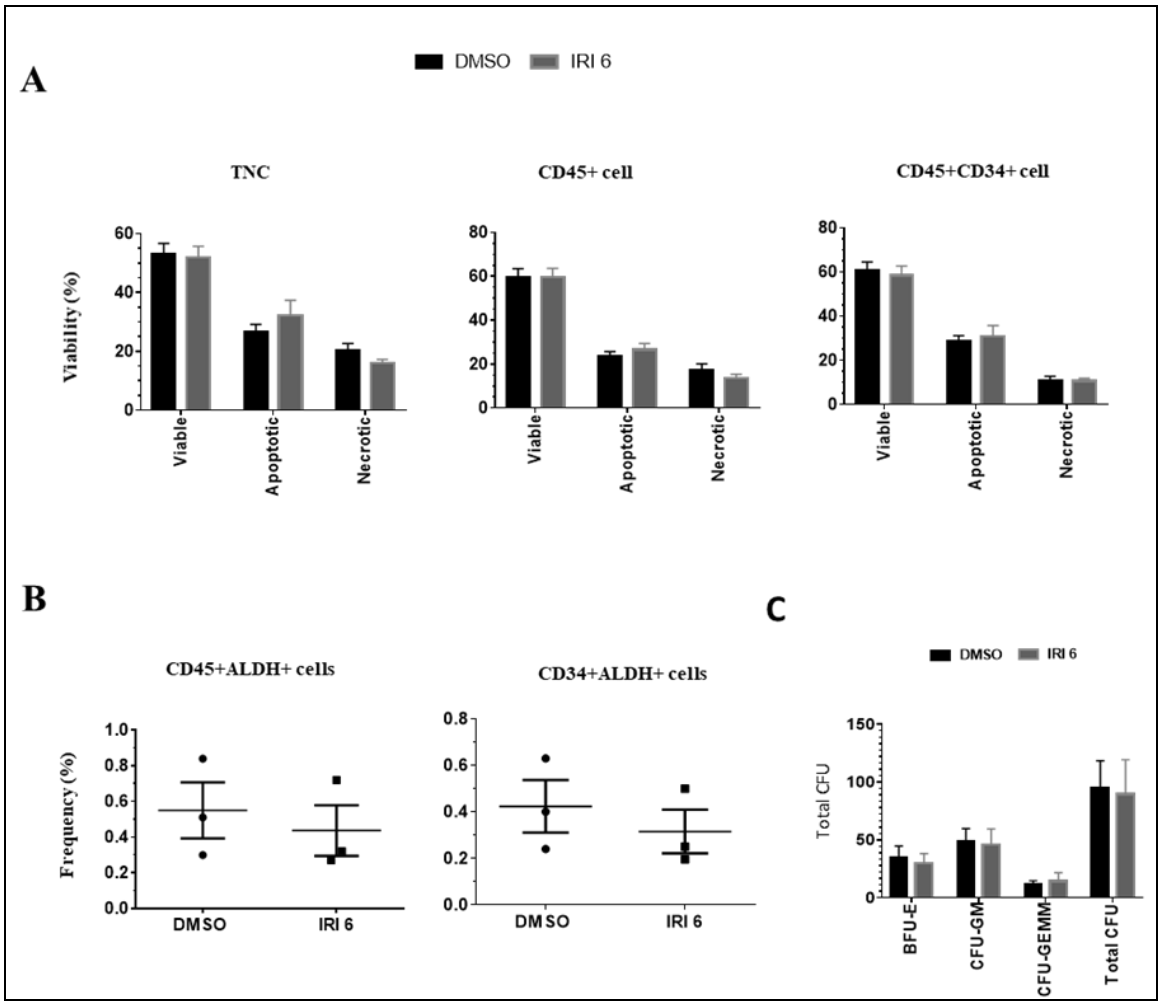

Figure 41: Impact of IRI 6 on post thaw CB quality

A) Viability analyses of CBU post-thaw using sytox-AAD and annexin V staining in indicated cell subpopulations (n=3). B) Frequency of $ALDH^{br}$ subpopulations in CBU post-thaw (n=3). C) Number of total colonies count of CB cell (45K per plate seeded) (n=3). Results presented as mean ± SEM for DMSO and IRI 6 groups. Significant differences were determined by two-way ANOVA for panel A. Panel B and C were done by student paired t-test, $p>0.05$.

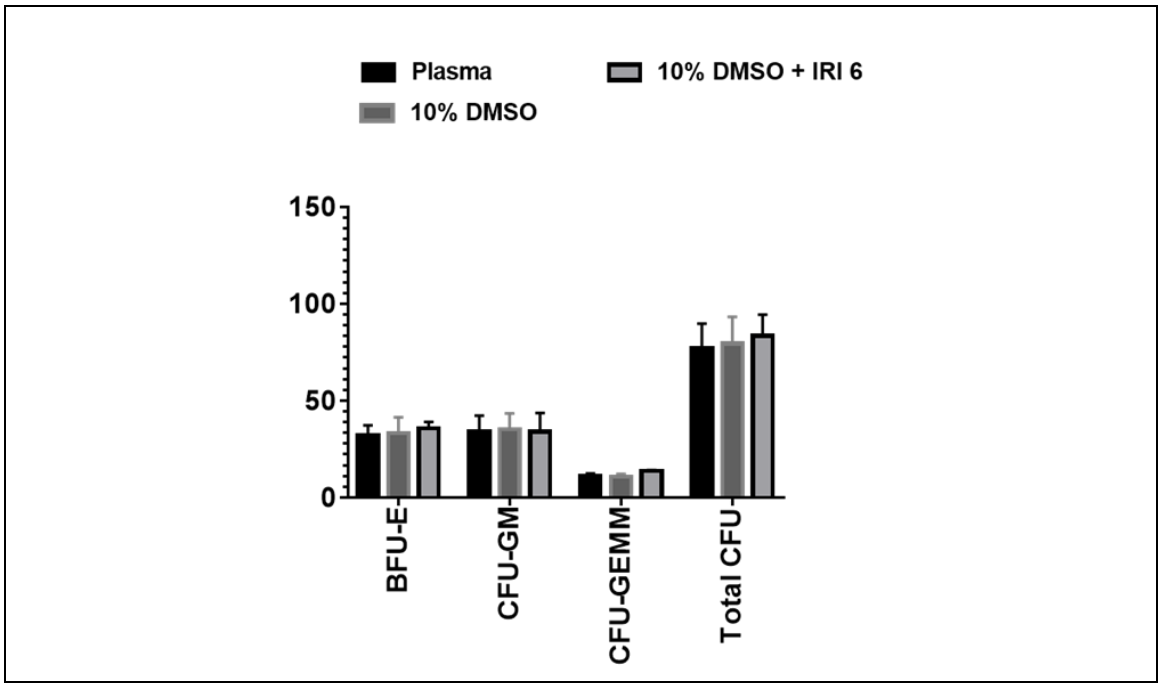

Figure 42: Cytotoxic effect of IRI 6

Number of CFU present in fresh CB graft exposed to DMSO and IRI 6. Number of colonies per 40,000 CB TNC presented (mean ± SEM, n=3). Burst forming unit-erythroid (BFU-E), CFU-granulocyte-monocyte (CFU-GM), CFU-Granulocyte erythrocyte monocyte and megakaryocyte (CFU-GEMM). $p>0.05$, significant differences were determined by 2-way ANOVA.

Human engraftment in NSG mice was first characterized by tracking platelets and leucocytes in the periphery short and long-term after transplantation (Figure 43, Figure 44). The complementation of DMSO with IRI 6 had no significant effect on hPlt engraftment in NSG mice (Figure 43).

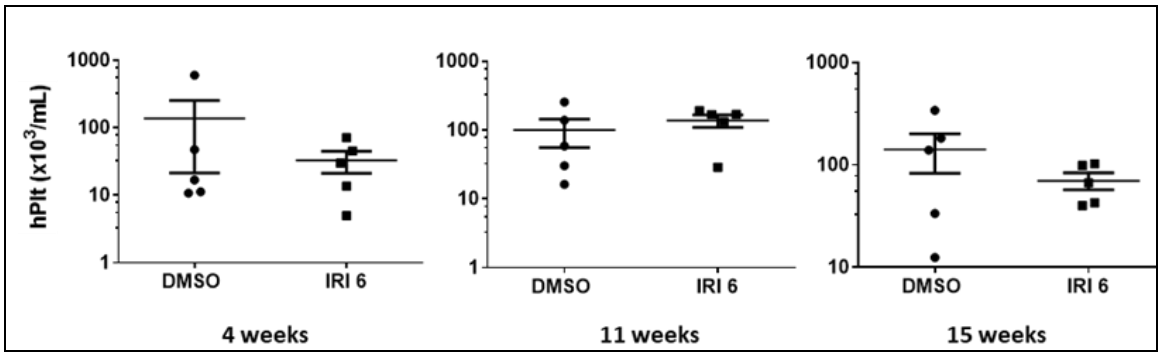

Figure 43: Impact of IRI 6 on the thrombopoietic activity of CBU.

Platelet engraftment in NSG mice injected with CB cells cryopreserved with DMSO or IRI 6. Levels of human platelets shown for one donor. Each symbol corresponds to an individual mouse. The horizontal line and error bars represent the mean ± SEM (5 mice per group). $p>0.05$, significant differences were determined by Mann-Whitney test.

On the other hand, the levels of human leukocytes between both groups were also similar at all-time point tested (Figure 44). Further investigation of the circulating human cells revealed that IRI 6 grafts were able to contribute equally well to myeloid ($CD45^+CD33^+$) and B cell ($CD45^+CD19^+$) lineages.

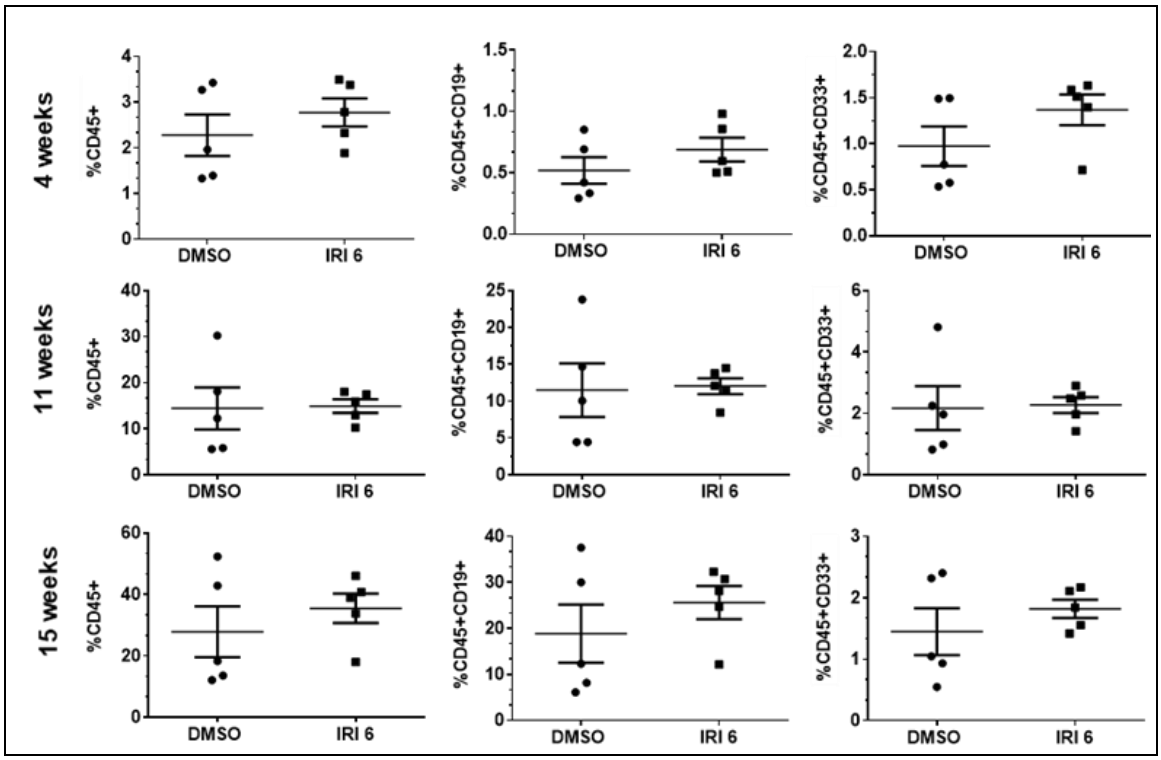

Figure 44: Impact of IRI 6 on leucocyte engraftment properties of CBU.

Leukocytes engraftment in NSG mice injected with CB cells cryopreserved with DMSO or IRI 6. Human $CD45^+$ cell, $CD45^+CD19^+$ B cell and $CD45^+CD33^+$-myeloid engraftment level at indicated time point in DMSO and IRI 6 group. Each symbol corresponds to an individual mouse. The horizontal line and error bars represent the mean ± SEM (5 mice per group for single donor). Significant differences were determined by Mann-Whitney test.

Long-term BM analysis was performed 16 weeks post-transplantation. $CD45^+$ cell engraftment was slightly higher in IRI 6 mice when compared to DMSO though the difference was not significant (66.24 ± 4.6% vs. IRI 6 74.24 ± 2.11% $CD45^+$ cells, p=0.08, Figure 45). Myeloid, B cell, NK cell and T cell also showed higher frequencies (%) in IRI 6 group compared to DMSO in primary recipient of BM analysis though not all differences were significant (Figure 45A-B). The lympho-myeloid distribution analyses of the BM revealed that IRI 6 did not perturb the differentiation potential of human HSC (Figure 45C). Interestingly, the level of human progenitors (i.e. CFU) in the primary recipients, and levels of engraftment in secondary mice appeared higher in IRI 6 recipients though the results failed to be significant (Figure 45D-E, p>0.05).

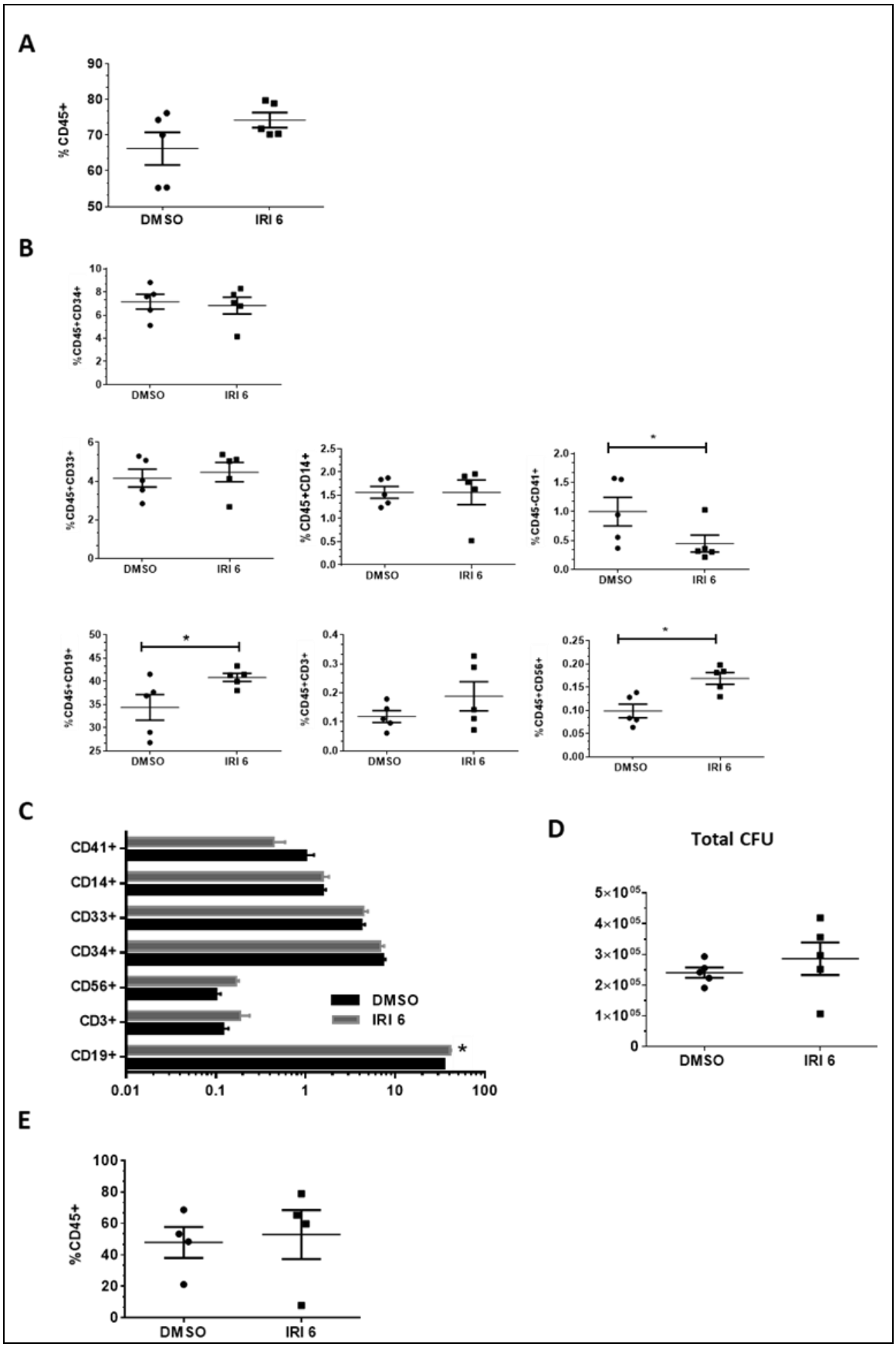

Figure 45: Impact of IRI 6 on human BM reconstitution.

A) Human BM engraftment analysis of primary transplants 16 weeks post-transplantation. Each symbol corresponds to a mouse. The horizontal line and error bars represent the mean ± SEM of 5 mice per group (n=1). B) Multilineage human BM engraftment analysis of primary transplants. C) Lineage distribution of human BM engraftment in primary recipients (mean ± SEM). D) Total number of human CFU progenitors in the BM of primary recipients (mean ± SEM). E) Human $CD45^+$ BM cell engraftment in secondary recipients. Each symbol corresponds to a mouse. The horizontal line and error bars represent the mean ± SEM (4 mice per group, n=1). Significant differences were determined by Mann-Whitney test for A, B and E panels, panel D was done by student paired t-test and panel C which was done by two-way ANOVA.

Taken together, these preliminary results indicate, that supplementation of hematopoietic graft with IRI 6 is not deleterious to HSC and, perhaps beneficial to the engraftment activities. However, these results will need to be confirmed in additional experiments.

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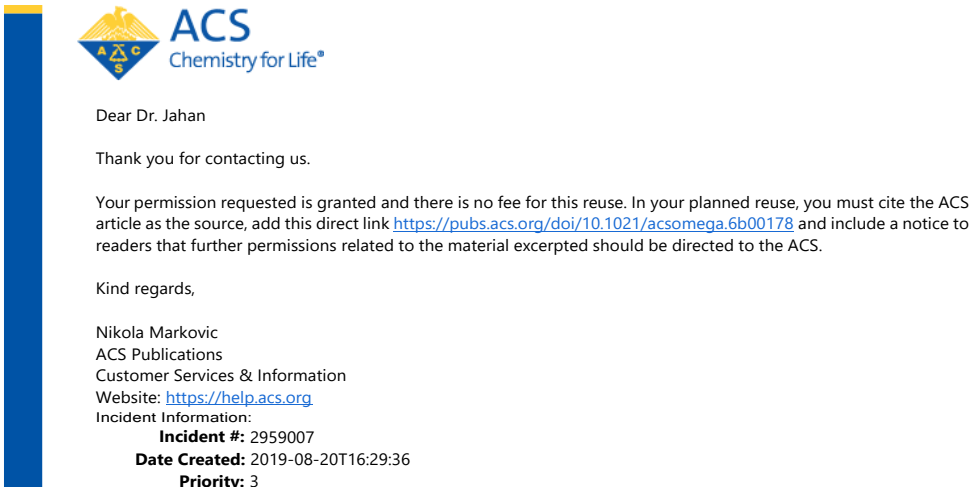

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