New Approaches to Evaluate the Immune and Endothelial Modulation Potential of Stored Red Blood Cell Concentrates

by

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Abstract

Red blood cell (RBC) transfusion is the most common treatment for anemia caused by blood loss due to hemorrhage or other pathological conditions. Despite being a life-saving therapy, RBC transfusion it is often associated with undesirable effects ranging from hemolytic and non-hemolytic transfusion reactions resulting from ABO mis-matching to infection transmission and transfusion related immune modulation (TRIM). Although the mechanisms involved in TRIM are multifactorial, previous studies have concentrated on the effect of RBC hypothermic storage lesion (HSL) on RBC quality and patient transfusion outcomes. The progressive biophysical and biochemical changes RBCs undergo during storage (HSL) has led to the controversial discussions as to whether transfusion of fresh versus old RBCs would alleviate the effects of TRIM. Recently, several retrospective studies have examined the clinical effects of sex-mismatched transfusions and the role of donor characteristics on patient post-transfusion outcomes. Although these studies suggest that donor age and/or sex influence transfusion outcomes in critically ill, there is a significant void in the *in-vitro* studies exploring plausible mechanisms for this effect.

The overall objective of this thesis was to evaluate the role of donor age and sex on the immune and endothelial modulation potential of stored red blood cell concentrate (RCC) supernatants *in-vitro*. To achieve this objective, two novel bioassays to evaluate of RBC products were established. The monocyte monolayer assay (MMA) is a compatibility testing method for evaluating the clinical significance of RBC alloantibodies. Time-consuming monocyte isolation procedures and requirement for fresh monocytes have limited application of the MMA. This thesis assessed the viability and functional properties of monocytes from pooled cryopreserved

buffy-coat (BC)-derived peripheral blood mononuclear cells for MMA application. Results showed comparable viability and phagocytosis ability of anti-D sensitized RBCs by pooled cryopreserved monocytes from BCs with fresh peripheral blood monocytes supporting its utility in assessing the clinical significance of RBC alloantibodies as well as the evaluation of the immune modulatory activity of stored RCCs *in-vitro*. In addition, the feasibility using the trans-epithelial electrical resistance (TEER) assay, a non-invasive real-time technique for monitoring cells in culture while using human umbilical vein endothelial cells (HUVECs) as a model for human endothelium was established. TEER assay showed that RBC HSL negatively impacts endothelial permeability *in-vitro*.

Evaluation of the impact of donor age and sex on the immune modulation potential of stored RCC supernatants showed that RCC supernatants from fresh blood products (day 7) collected from blood donors ≥60-years old (-yo) enhanced the expression of pro-inflammatory cell adhesion molecules (CAMs) by HUVECs and had enhanced disruptive effect on HUVEC integrity compared to younger donors (≤30-yo). Additionally, a consistent trend towards lower pro-inflammatory activity following treatment with male ≤30-yo RCC supernatants was observed, as demonstrated by lower expression of CAMs throughout storage, lower disruptive effect on HUVEC integrity as well as reduced cytokines release by monocytes and HUVECs compared to the other three donor groups. The work presented in this thesis confirm that donor characteristics affect RBC quality parameters and influence the endothelial and immune modulation potential of RCC supernatants and, suggest that closer attention should be paid to RBC donor age and sex in immunomodulatory studies and their potential role in adverse transfusion outcomes. This

thesis also presented tools that may be exploited to study mechanisms regulating permeability or activation of the endothelium as well as innate immune activation post-transfusion.

Preface

This thesis is an original work by Betty Jepchirchir Kipkeu. Experimental work in which this thesis is part of was performed following the ethics approval granted by the University of Alberta and Canadian Blood Services (CBS) Research Ethics Boards (protocol number Pro00059754 and 2015.032 respectively) under the project title "Role of blood component manufacturing on microvesicle-induced transfusion-related immune modulation". This work received funding from the CBS Intramural Grant program (grant number 2015IG-JA), funded by the federal government (Health Canada) and the provincial and territorial ministries of health. The views herein do not necessarily reflect the views of CBS, or the federal, provincial and territorial governments of Canada.

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Dedication

To Mum, Eliseba Cheptarus Yego and Dad, Joseph Kipkeu Yego. Your prayers, love and support made me the woman I am today. I hope this thesis makes you proud.

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List of Abbreviations

AC	alternating current
ACD	acid citrate-dextrose
ANOVA	analysis of variance
APC	allophycocyanin
APCs	antigen presenting cells
AS	additive solutions
ATP	adenosine triphosphate
B 3	band 3
BC	buffy coat
BSA	bovine serum albumin
CAM	cell adhesion molecule
CBS	Canadian Blood Services
CI	cell index
CO_2	carbon dioxide
CPD	citrate-phosphate-dextrose
CPDA	citrate-phosphate-dextrose-adenine
DMSO	dimethyl-sulfoxide
DNA	deoxyribonucleic acid
EBM-2	endothelial basal medium-2
ECIS	electric cell-substrate impedance sensing
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGM-2	endothelial growth medium-2
EVs	extracellular vesicles
FBS	fetal bovine serum
FDA	Food and Drug Administration
FGF-2	fibroblast growth factor-2
FITC	fluorescein isothiocyanate

G-CSF	granulocyte colony-stimulating factor
Glut 1	glucose transporter 1
GM-CSF	granulocyte-macrophage colony-stimulating factor
GSH	reduced glutathione
H	hour
Hb	hemoglobin
Hb_S	supernatant hemoglobin
Hb_T	total hemoglobin
Hct	hematocrit
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HiCN	cyanmethemoglobin
HLA	human leukocyte antigen
HS	hypothermic storage
HSL	hypothermic storage lesion
HUVECs	human umbilical vein endothelial cells
ICAM	intravascular cell adhesion molecule
IFN	interferon
IL	interleukin
K+	potassium ion
LPS	lipopolysaccharide
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCP	monocyte chemoattractant protein
MCV	mean corpuscular volume
MDC	macrophage-derived chemokine
MIP	macrophage inflammatory protein
MMA	monocyte monolayer assay
Na+	sodium ion
NADH	nicotinamide adenine dinucleotide
NCI	normalized cell index

NetCAD	network center for applied development
NO	nitric oxide
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PE	phycoerythrin
PS	phosphatidylserine
RA	rheumatoid arthritis
RBCs	red blood cells
RCCs	red blood cell concentrates
RCF	red cell filtration
RCTs	randomized controlled trials
RES	reticuloendothelial system
RNA	ribonucleic acid
RT	room temperature
RTCA	real-time cell analysis
RT-CES	real time cell electronic sensing
SAG	saline-adenine-glucose
SAGM	saline-adenine-glucose-mannitol
TEER	Trans-epithelial electrical resistance
TGF	transforming growth factor
TNF-α	tumor necrosis factor-alpha
TRALI	transfusion-related acute lung injury
TRIM	transfusion-related-immune-modulation
USA	United States of America
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
WB	whole blood
WBF	whole blood filtration
YO	years old
2,3-DPG	2,3-diphosphoglycerate

Chapter 1

Introduction

1.1 Red blood cells

Red blood cells (RBCs), also known as erythrocytes, are the most abundant cell type found in human blood. RBCs are small (~ 7 – 8 μM in diameter), anucleate and biconcave in shape [1]. In adults, RBCs are produced in the bone marrow under the control of the hormone erythropoietin and released into circulation as reticulocytes, enucleate cells containing residual ribonucleic acid (RNA) [2, 3]. Reticulocytes lose their nucleus and mature as they pass through splenic vessels [2, 4]. It is estimated that the life span of mature RBC is 110 – 120 days with most of this time being spent traversing the capillaries of the microcirculation [5, 6]

Although the primary function of RBCs is to supply oxygen to tissues, RBCs also contribute to the regulation of vascular tone, vascular antioxidant systems as well as immune regulation [2, 5]. The efficiency of RBCs as oxygen carriers is attributed to its biconcave shape, thought to be maintained by the plasma membrane in association with the cytoskeletal support [1, 6, 7]. This adaptation enables RBCs to reversibly change their shape to allow movement through capillaries with diameter smaller than that of RBCs (microcirculation) [7]. RBC plasma membrane is composed of membrane lipids, membrane proteins and skeletal proteins [6]. The lipid bilayer is made up of equal proportions by weight of cholesterol and phospholipids [6]. The phospholipids are asymmetrically distributed between the inner and the outer leaflet of the plasma membrane [6]. Phosphatidylcholine and sphingomyelin are located in the outer leaflet while phosphatidylethanolamine and phosphatidylserine (PS) are restricted to the inner leaflet [6]. It has been suggested that several energy dependent/independent phospholipid transport proteins maintain phospholipid bilayer asymmetry. For instance, 'Flippases' move phospholipids from the

outer to the inner leaflet whereas 'Floppases' does the opposite against concentration gradient in an energy dependent manner [6]. 'Scramblases' on the other hand move phospholipids bi-directionally down their concentration gradient in an energy dependent manner [6]. Over 50 RBC transmembrane proteins have been identified and characterized. Transport proteins include Band 3 (B3, anion transporter), Glut 1(glucose transporter 1), Kidd antigen protein (urea transporter) among others [6]. Intracellular cell adhesion molecule (ICAM)-4 is involved in the interaction of RBCs and integrins [6]. The RBC skeletal framework is composed of spectrin-based protein including β-spectrin, actin, protein 4.1R, adducing, tropomyosin, adducing and tropomodulin [6]. These proteins form a long filamentous spectrin that is responsible for regulating RBC membrane mechanical stability and deformability [1, 6].

Enclosed in RBC phospholipid bilayer is a special protein known as hemoglobin, a tetramer made up of two alpha and two beta globin chains [2-5, 8]. Each chain encloses one of the four heme groups that carry oxygen in the complete tetramer [2-5, 8]. Like any other living cell, energy is essential for RBC *in-vivo* function [9]. RBCs respire anaerobically through the glycolytic pathway (Embeden-Meyerhof Pathway) where, energy in the form of adenosine triphosphate (ATP) is produced from the conversion of one glucose molecule to pyruvate [2-4, 8, 9]. The ATP generated plays numerous roles including maintenance of glycolysis, electrolyte balance between plasma and RBC cytoplasm, synthesis of glutathione (anti-oxidant), maintenance of hemoglobin's iron in reduced ferrous state and preservation of membrane phospholipid asymmetry among other functions [9]. At the end of their life span, RBCs are removed from the circulation by phagocytic cells of the reticuloendothelial system (RES) mainly located in the spleen, bone marrow and liver [2-4]. Two mechanisms have been proposed to explain clearance of senescent RBCs. It has been

shown that RBCs lose ~ 20 % of its hemoglobin content in the course of their life span because of membrane loss through vesiculation [10] This results in a loss of membrane phospholipid asymmetry leading to exposure of PS, a signal for the removal of RBCs by macrophages [5, 11]. Alternatively, progressive oxidative damage to B3 proteins results in clustering of B3 exofacial domain [5]. This increases the affinity of B3 to naturally occurring anti-B3 autoantibodies, which activates the complement system [5].

1.2 Brief history of RBC transfusion and biobanking

RBC transfusion is the most widely accepted life-saving treatment for patients suffering from anemia caused by hemorrhage due to injury, surgery, malignancy and other hematological conditions [12]. Currently, RCCs are the most transfused blood component with over 1.1 million units being transfused in Canada each year [12]. The advent of blood transfusion can be traced back to the 1600s when William Harvey, a British physician first discovered blood circulation [13]. In 1668, Jan Swammerdam, a Dutch biologist and microscopist observed RBCs for the first time [6, 13, 14]. Initial blood transfusion experiments were performed on dogs and lambs, but it was not until 1818 when an obstetrician James Blundell successfully performed human to human blood transfusion as a treatment for post-partum hemorrhage [14, 15]. Since blood was found to coagulate as soon as it left the body, initial blood transfusion had to be done directly from the donor and the recipient [14, 15]. The discovery of anticoagulant sodium citrate in 1914 by Adolf Hustin meant that blood could be stored under refrigeration for days, creating the possibility of separation of blood donors and recipients in time and space [3, 14-16].

In 1915, Rous and Turner developed a preservative solution composed of citrate (anticoagulant) and glucose (nutrient source), allowing the storage of blood kept on ice for a few weeks post-collection [3, 14-19]. While using the citrate-glucose solution formulated by Rous and Turner, Robertson in 1918 documented the transfusion of preserved blood that had been kept for up to 26 days [19]. This led to the eventual establishment of the first blood bank in France during the first world war [19]. In an attempt to reduce bacterial contamination in blood products, the United States Army Corps began autoclaving citrate contained in glass bottles before addition of blood [20]. They chose citrate alone because it was found that glucose caramelized upon heating [20]. However, Loutit and Mollison solved this problem when the discovered that by maintaining the pH of citrate-glucose below 5.8, the solution could be autoclaved without caramelization of glucose [20].

In 1962, it was discovered that the addition of adenine to RBCs could slow the loss of ATP and cell viability associated with storage of RBCs [20, 21]. Current storage solutions such as citrate-phosphate-dextrose/adenine (CPD/A) represent modifications to the initial storage solution developed by Rous and Turner and can preserve blood for up to three weeks [3, 14]. In the early 1980s, additive solutions were invented in an attempt to solve the problem of slower blood flow caused by high viscosity (high hematocrit) of packed RBCs [22]. The first commercial additive solution was developed in Sweden and was named SAG (saline adenine glucose) [22]. However, there was still concerns of increased hemolysis in the last two weeks of storage while using SAG [22]. This led to the addition 525 mg of mannitol to SAG and renaming it to SAGM [22]. SAGM is the main additive solution used in Canada while Adsol (AS-1), Nutricel (AS-3) and Optisol (AS-5) have been licenced for use in the USA and Europe [20]. The use of additive solutions

extends the shelf life of RCCs to a maximum of six weeks without a significant loss in cell viability [20].

1.3 RCC manufacturing and storage

The invention of plastic blood-storage bags in the 1950s played a significant role towards modern blood banking and, was a big step from the storage of blood in glass containers during the First World War [3, 20]. The main advantages that came with the use of plastic blood-storage bags was that they were light in weight, resistant to breakage and provided a closed environment devoid of microbial contamination [20]. They also provided a continuous closed system to produce blood components including RBCs, plasma and platelets from a single donation. Transfusion practice has largely moved away from the transfusion of whole blood units to the transfusion of individual blood components depending on the medical needs of the patient.

In North America, three methods are commonly used in the production of RBC units. First is the apheresis method (mainly in the United States of America) that relies on technology to selectively collect RBCs from donor blood while returning the rest of the blood back to the circulation [23, 24]. Because it is automated, this method has been shown to result in RBC products with consistent quality and quantity [23-25]. The buffy coat method, also known as red cell filtration (RCF) method and whole blood filtration method (WBF) are the two main methods used in producing RBC units from anticoagulated whole blood (WB) in Canada [26]. The RCF method results in the production of a RBC and a buffy coat unit from a single WB donation. Here, 450 ± 30 mL of WB is collected into plastic blood-storage bags containing 70 mL of CPD anticoagulant followed by a rapid cooling to room temperature (RT, 18-24 ˚C). The unit is held

overnight at RT then centrifuged the following day at 3000 – 3500 x g for 11 minutes. Following centrifugation, blood components are separated by a semi-automated device into satellite bags connected at the top (plasma) or bottom (RBCs) of the primary bag leaving the buffy coat in the original bag [25-27]. About 110 mL of SAGM is then added to RBCs and leucoreduced by filtration at RT. With the WBF method, 450 ± 30 mL of WB is collected into plastic blood-storage bags containing 70 mL of CPD anticoagulant and cooled to 1 – 6 ˚C followed by leukoreduction within 72 hours post-collection. The unit is then centrifuged at higher speed (>4500 x g) for 6 – 8 minutes and a semi-automated extraction device used to separate plasma from RBCs. About 110 mL of SAGM is finally added to the RBCs [25]. Both the RCF and WBF methods yield red blood cell concentrates (RCCs) with a volume and hematocrit range of 250 – 350 mL and 55 – 65 % respectively.

1.3.1 Factors influencing RCC product characteristics

RCCs collected from healthy donors have long been regarded as equivalent in terms of quality and efficacy. This supports the standard issue and use of RCCs to patients, without much consideration to the RCC manufacturing method or donor characteristics such as age or sex. It has become known that even RCC from the same donor manufactured using two different methods result in significantly different product quality [25]. The RCF and WBF manufacturing methods used in Canada have been shown to result in RCC products differing in a number of aspects [25]. For instance, RCC produced using the WBF method have been shown to have higher unit volume accounted by more residual plasma compared to RCC produced via RCF method [25, 28]. Similarly, RCF RCCs have been shown to present with lower storage hemolysis, lower water

permeability and larger EVs compared to WBF RCCs [26, 29, 30]. Further, studies have demonstrated that different RCC processing methods result in products with varying free DNA, mitochondrial DNA as well as different EV composition [31, 32].

The second and perhaps the most important factor influencing RCC product quality is donor characteristics. Donor characteristics encompasses a wide range of biological and environmental differences within donor population including age, sex, race, ethnicity, inheritable genetic conditions such as sickle cell, as well as donor lifestyle [30]. RCCs from male donors have been shown to have higher hematocrit, total hemoglobin and hemolysis levels compared to RCC from female donors [29, 33], while significantly lower hemolysis in units from premenopausal female donors when compared to donations from other donor populations has been reported [29, 34]. A number of theories have been proposed to explain this trend, based on known physiological differences between aging male and female blood donors and their circulating RBCs [34-36]. Testosterone has been shown to promote erythropoiesis [37] which could explain the increased hematocrit and hemoglobin levels observed in male donors compared to female donors [38]. Alternatively, the monthly blood loss in premenopausal female donor results in a younger population of circulating RBCs, which are less susceptible to stress and hemolysis during storage [36]. Further, It has been suggested that a decrease in the surface area to cell volume ratio of circulating RBCs results in an increase in the osmotic fragility of circulating RBCs in older individuals [39] although, this finding was challenged by a study that found no significant difference in osmotic fragility in RBCs from older versus young donors [38].

Taken together, donor characteristics and RCC manufacturing method have been shown to be the key influencers of pre-storage RBC quality and could determine the extent of RBC HSL. Therefore, understanding how donor characteristics, RCC manufacturing method and HS duration interact *in-vitro* is a step towards elucidating the potential impact of RCC quality on patient outcomes.

1.3.2 RBC Hypothermic Storage Lesion (HSL)

Preservation of functionally viable RBCs is necessary for clinical transfusion practice. RCCs suspended in additive solutions are normally stored under refrigeration at 1 – 6 ˚C for a maximum of six weeks [3]. This form of biopreservation is also referred to as hypothermic storage (HS), conditions in which the temperature is lower than the normal physiological temperature but higher than the freezing point of the suspending medium [3]. HS reduces the metabolic processes in RBCs to a minimum resulting in the conservation of energy as well as reduction in waste accumulation, which can cause injury in the absence of *in-vivo* detoxification mechanisms [3, 8]. However, in an *ex-vivo* environment, RBC undergo numerous biophysical and biochemical changes collectively referred to as hypothermic storage lesion (HSL) [3, 8]. Common characterization of RBC HSL and its effects on RBC integrity and function are described below.

As HS progresses, RBCs lose part of their plasma membrane as extracellular vesicles (EVS) changing their shape from a biconcave disc to spheroechinocytes which have limited ability to move through microcirculation [16]. EVs are heterogeneous populations of phospholipid vesicles released into the blood by erythrocytes, leukocytes, platelets, or endothelial cells, under physiological or pathological conditions (activation, stress, apoptosis or necrosis) [40, 41]. Under

normal physiological conditions, microvesiculation serves to remove damaged materials such as oxidized proteins and senescence molecules including PS thus, postponing the removal of RBCs *in-vivo* [41-45]. RBC EV generation under blood banking conditions has been shown to increase with storage. Research has shown that EVs from stored RCCs contain a substantial amount of hemoglobin that serves as nitric oxide scavenger, resulting in interruption of vascular homeostasis [46, 47]. Pro-inflammatory effects of EVs have been shown by their ability to induce cytokine and chemokine production by monocytes *in-vitro* [40]. In addition, EVs express phosphatidylserine (PS), a phospholipid membrane component with pro-coagulant activity and the number of EVs expressing PS has been shown to increase with storage duration (3). RBC microvesiculation also leads to redistribution and loss of phospholipids occurs leading to expression of PS, a marker of senescence, leading to increased RBC destruction and decreased survival post transfusion [48, 49]. Significant elevation of free hemoglobin and extracellular potassium (K^+) through hemolysis has also been shown to escalate RBC exposure of PS, aiding further in early removal of RBCs from circulation [11, 25, 49, 50]. The alterations in RBC membrane has been shown to be a result of decreasing pH and ATP concentration during storage, important factors regulating RBC membrane symmetry [49]. Flippases activity has been shown to decreased with increasing RBC HS duration and could only be restored if intracellular ATP and pH is corrected [48]. Recently, a study showed that RBC water permeability significantly increases with duration of HS, which could have a negative implication on the ability of RBCs to withstand osmotic stress [51]. The changes in RBC shape during HS equals to loss of RBC deformability and could result to poor microcirculatory flow post-transfusion and reduced survival *in-vivo* [48]

In addition to RBC membrane biophysical changes, RBCs undergo a numerous biochemical change when stored ex-vivo for prolonged periods of time. RBC energy metabolism has been shown to be significantly affected by HS [52, 53]. The constant decrease in RBC intracellular ATP during HS causes numerous disruptions to metabolic processes including the maintenance of the sodium (Na^+)/K^+ pump and glucose transport [54]. The Na^+/K^+ pump is an ATP-driven process located in the cell membrane and is responsible for maintaining the osmotic balance between intra- and extra-cellular environment [54]. An osmotic imbalance in RBC during HS storage culminates in leakage of intracellular K^+ and subsequent accumulation in the suspending medium. 2,3-diphosphoglycerate (2,3-DPG) is a metabolite and an allosteric modifier of hemoglobin and its role is to regulate oxygen affinity of hemoglobin [53, 54]. Similarly, it has been shown that 2,3-DPG depletion usually occurs within the first two weeks of HS. Because of 2,3-DPG depletion, the hemoglobin oxygen affinity increases which in turn decreases RBC oxygen delivery ability. Although 2,3-DPG is normally restored within 72 hours of transfusion, the immediate efficacy of transfused RBCs remains in question [55, 56]. In addition, studies have shown that RBCs are able to release nitric oxide (NO) under acidic and hypoxic conditions [57]. NO is important for the oxygen-dependent regulation of blood flow due to its vasodilator effect on blood vessels [57, 58]. The ability of RBCs to generate NO has been shown to decline during HS and may result in reduced oxygen delivery [57].

As oxygen transporters, RBCs are constantly subjected to an enormous amount of oxidative stress [53]. A secondary consequence of ATP depletion is hemoglobin oxidation [53]. Hb oxidative damage seems to be a result of the progressive aging of RBCs and depletion of endogenous antioxidants. For instance, reduced glutathione (GSH) is essential for the

neutralization hydrogen peroxide to water and oxygen molecules by glutathione peroxidase as well as protection of hemoglobin from oxidative damage [59]. The oxidation of hemoglobin causes disulphide crosslinks between globin chains and distorts hemoglobin structure and could potentially result in hemoglobin-induced membrane damage [8, 57].

Since the primary aim of RBC transfusion is to restore tissue oxygenation in anemic patients, efficacy of RCC transfusion is measured by its ability to increase and sustain plasma hemoglobin concentration in individuals. Although the functionality of stored RBCs post-transfusion remains understudied, there is a consensus that RBC HSL affect RBC function post-transfusion [55, 56]. For instance, 2,3-DPG depletion results in a left-shift of the oxyhemoglobin dissociation curve leading to reduced oxygen delivery [60]. Further, RBC rheology (study of flow properties of RBCs) have been shown to change during HS of RBCs [60]. The ability of RBCs to flow through the circulatory system depends on the aggregation and adhesion to the endothelium which, have been shown to increase with HS [60]. As a result, these changes could result in vascular occlusion with potential increase in intravascular pressure [60]. The effect RBC HSL can also be mirrored by the loss of approximately 25 % of transfused RBCs within 24 hours of transfusion [60].

1.4 RBC transfusion induced immune modulation (TRIM)

Current Canadian standards on the quality of RBCs for transfusion include the assessment of percent hemolysis (must not exceed 0.8 %) as well as post-transfusion recovery (75 % of transfused RBCs must remain in the circulation 24 hours post-transfusion) [60]. These standards however do not address many of the other effects that HSL has on RBC quality, which could have

negative effects after transfusion. Although lifesaving, RCC transfusion has been shown to alter the immune system in a variety of ways leading to immune suppression, activation or pro-inflammatory responses [61, 62]. In fact, evidence suggest that RCC transfusion is associated with increased risks of severe complications and mortality in critically ill patients, the same patient population likely to benefit the most from RBC transfusion [63, 64]. This phenomenon has been termed transfusion related immune modulation (TRIM) [61, 62]. In recent years, restrictive transfusion strategies have been adopted with the intention of reducing the number of patients receiving blood transfusion therefore, avoiding harmful effects associated with blood transfusion [65].

While the exact mechanisms of TRIM remain unclear, many studies have evaluated the effect of donor characteristics, RCC manufacturing method and HS length individually or upon interaction with each other on the overall immune modulation effect of RCCs. Previously, studies have shown improved graft survival in patients who received allogeneic blood transfusion prior to undergoing renal transplant [66, 67]. To explain this effect, it was suggested that donor antigen presenting cells might lose the potential to induce co-stimulation if stored hypothermically. This results in failure of naive T cells to mount an immune response despite the presence of foreign antigens thus, promoting immunosuppression [68]

Several retrospective studies evaluating the impact of donor factors on patient outcomes have so far provided important information on TRIM. Reports indicate an increase in patient mortality following transfusion of RCCs from female donors compared with male donors [69-73]. Contrary to these findings, one French study showed no increase in mortality in male recipients

receiving RCCs from female donors [74]. Transfusion related acute lung injury (TRALI) is currently the leading cause of transfusion related mortality characterized by onset of lung injury within six hours of transfusion and has been strongly associated with receiving plasma products from female donors [12]. This effect has been attributed to the presence of human leukocyte antigens (HLA) in transfused blood components. In addition, heritable genetic conditions and donor ethnicity influence the ability of stored RCCs to withstand osmotic and oxidative damage resulting in increased hemolysis [38, 75]. The implication is that a patient receiving such unit could be exposed to significant amount of free hemoglobin which, has been shown to cause multiple organ failure [76].

Even though research has shown that RCC manufacturing method significantly affect RCC product quality, their impact on patient outcome is yet to be investigated in detail. A Canadian study showed that transfusion of ≤ 8 days old WBF RCCs was associated with a higher hazard ratio for mortality compared with RCF RCCs [77]. In fact, it has been suspected that the volume of residual plasma in WBF RCC could be the reason behind occurrence of TRALI in some patients receiving RCC transfusion [78]. The amount and composition of residual leukocytes, platelets and plasma differ with RCC manufacturing method and have been suggested to be the origin of mitochondrial and cellular DNA, which are believed to be mediators of inflammation and endothelial damage [31, 33]. Recently, evidence suggest that EVs contained in RCC products contribute to immunomodulatory effects by priming and activating neutrophils as well as inducing pro-inflammatory cytokine production by monocytes [79-82].

Furthermore, transfusion of RCC stored for longer periods has been shown to contribute to significant immune modulation effects. In a murine model, the concentration of non-transferrin bound hemoglobin was shown to increase upon clearance of old RBCs by macrophages of the reticuloendothelial system [83]. One study demonstrated that circulating non-transferrin bound hemoglobin appeared to encourage the growth of pathogenic bacteria *in-vitro* [84]. In humans, evidence suggest that the pro-inflammatory effect of plasma hemoglobin and heme *in-vivo* involve the consumption of nitric oxide (NO), an important modulator of vascular tone [55, 85-87] resulting in endothelial dysfunction. Reduction in RBC deformability as well as an increase in RBC aggregation and adhesion during HS have all been shown to reduce microvascular flow and increase inflammation involving the endothelium [55].

1.5 Approaches to the investigation of TRIM: past, present and future

As previously mentioned, previous literature suggests that the interaction between blood donor characteristics, manufacturing methods and HS length likely affect RCC product quality and, influence patient outcome post-transfusion. Like many studies, the use of experimental animal models is believed to be the ideal means to examine the effect of stored blood products on transfusion outcomes [88, 89]. In general, previous studies addressing the impact of fresh versus old RBC transfusion seem to agree with the idea that old RBCs are pro-inflammatory and contribute to significant adverse outcomes in animal models. This was shown by the inability to restore microcirculatory perfusion [88], occurrences of intravascular hemolysis, hypertension and vascular injuries [89] as well as inability to reduce infarct size [89] following transfusion of old RBCs. However, studies using animal models demonstrates fundamental similarities and

differences between human RBCs and those of other species. For instance, one study showed that murine RCCs exhibited similar but accelerated aging process compared to human RCCs [90].

Stemming from the effect of HSL on RBC quality was the potential of adverse transfusion outcomes following transfusion of old versus fresh RBCs in human. To study this effect, several randomized controlled trials (RCTs) were conducted around the globe. The "Age of Red Blood Cell in Premature Infants" (Canada), "Age of Blood Evaluation" (Canada and Europe), " Red Cell Duration Study" (USA), and "informing fresh versus old red cell management" (Australia, Canada, Israel and USA) RCTs did not show improvement in neonatal morbidities, decrease in mortality in critically ill patients, decline in multiple organ dysfunction score or decrease in in-hospital mortality respectively, after transfusion of fresh RBCs compared to old or standard issue RBC units [91-94]. Although the issue of blood storage duration and patient outcome is still controversial, focus has shifted to the contribution of donor characteristics specifically, age and sex to patient transfusion outcomes. Majority of the studies in this area have been performed retrospectively by using data from clinical databases and linking RCC products with patient outcomes [95]. Findings show a link between donor-recipient sex mis-matching [69-72, 95] and/or age of the blood donor [71] with an increased risk of mortality. In many of these studies, interpretation of clinical outcomes have been limited by numerous confounding factors including the statistical model used, the unequal number of RCC units transfused in the patient population and differences in RCC manufacturing methods [96, 97].

Even though retrospective or interventional studies are likely to yield important insights regarding the immune modulation potential of stored RCCs, there is a significant gap in *in-vitro*

studies evaluating differences in RCC products in relation to TRIM as well as investigating the mechanisms of TRIM. The innate immune system is an important effector of immune responses following RCC transfusion [98]. Innate immune system is composed of phagocytic cells (monocytes and macrophages) and proteins including complement and cytokines which, are genetically programmed to recognize and eliminate molecular patterns associated with invading pathogenic microorganisms [98]. Since monocytes are a key component of the innate immune system, several monocyte culture models have been developed in the past to study innate immune responses. The monocyte monolayer assay (MMA) is an *in-vitro* bioassay that has been used for decades in the evaluation of the clinical significance of RBC alloantibodies [61]. The main shortcoming of the MMA is the need for fresh monocytes isolated from the fresh peripheral blood of healthy individuals, adding to the technical and logistical challenges involved carrying out the assay. In previous studies, innate immune function in transfused patients was evaluated by the ability of monocytes to secrete tumor necrosis factor-alpha (TNF-α) following stimulation with lipopolysaccharide (LPS) *in-vitro* [61, 99]. As monocytes have been shown to secrete a wide range of cytokines, the understanding of the innate immune system in response to RBC transfusion is limited by the assessment of only one or a few cytokine markers.

In addition to the limited understanding of the innate cellular responses, there is a need for a better understanding of the role of the endothelium in TRIM. For a long time, the endothelium has been assumed to be a passive structure whose sole function is to line the vasculature. Current knowledge is that the endothelium has important roles in the immune system and cardiovascular function. As evidenced by studies evaluating the effect of RBC transfusion on endothelial function, the potential harm of stored RCC products remain

understudied [100, 101]. Upon activation, as in the case of inflammation, endothelial cells express cell adhesion molecules (CAMs) that aid in trapping, adhesion and extravasation of leukocytes into inflamed tissues [102, 103]. They also secrete cytokines that aid in the recruitment of leukocytes into inflamed tissues [104]. At the same time, the permeability of the endothelial barrier increases to allow movement of leukocytes, water and nutrients into the extravascular compartment [102, 103]. These markers of inflammation or endothelial activation could provide vital information on the mechanisms modulating endothelial function following transfusion while using previously tested endothelial models *in-vitro*. For instance, commercially available human umbilical vein endothelial cells (HUVECs) provide a convenient and reliable platform for studying human endothelium physiology and pathophysiology *in-vitro* [104, 105]. Advancement in technology has also made it possible for real-time analysis of endothelial cell cultures in a label-free manner while using a real time cell analyzer (RTCA) [106]. This tool has a potential application in transfusion medicine as its high throughput could allow simultaneous comparison of blood products and, could contribute to a better understanding of the mechanisms of TRIM [106, 107].

1.6 Thesis approach

In consideration of the current literature demonstrating that RBC transfusion is associated with adverse patient outcome, there is a need for more studies on the mechanisms involved, which requires an understanding of the fundamental differences in RCCs as contributed by donor characteristics, manufacturing method and HS. Scrutiny of the literature indicates that there are

limited studies employing *in-vitro* bioassays as a means of evaluating the immune modulation potential of stored RCCs with specific focus on the innate immune system and the endothelium.

The core of this thesis will be based on two areas. First is the development of two *in-vitro* bioassays for use in transfusion medicine. The MMA assay involves the culture of monocytes in monolayers, which are then used to test phagocytic responses of opsonized RBCs [108] as well as to evaluate the innate immune function in terms of cytokine secretion [104]. For decades, this assay has been performed using fresh monocytes isolated from fresh peripheral blood [108]. Owing to the challenges associated with getting donors to donate blood each time the assay is performed, this thesis aims to develop a method to cryopreserve monocytes and exploit the use of pooled cryopreserved monocytes isolated from buffy coats (BCs) as a novel source of the monocytes. In addition to the MMA, the feasibility of using the trans-epithelial electrical resistance (TEER) assay will be established. TEER assay is a non-invasive real-time technique for monitoring cells in culture with a wide range of applications in drug development and cancer research but novel to transfusion medicine. While using HUVECs as a model of human endothelium, it may be possible to evaluate the potential effect of RCCs on endothelial function and provide further insights into the interaction between HSL, donor factors and TRIM.

1.7 Thesis hypotheses and objectives

This thesis will test the following hypotheses: (1) monocytes from pooled cryopreserved BC peripheral blood mononuclear cells (PBMCs) in MMA assays will show similar viability and function to monocytes from pooled fresh BC PBMCs as well as monocytes from pooled fresh PB PBMCs in terms of the ability to phagocytose sensitized RBCs and secrete cytokines/chemokines

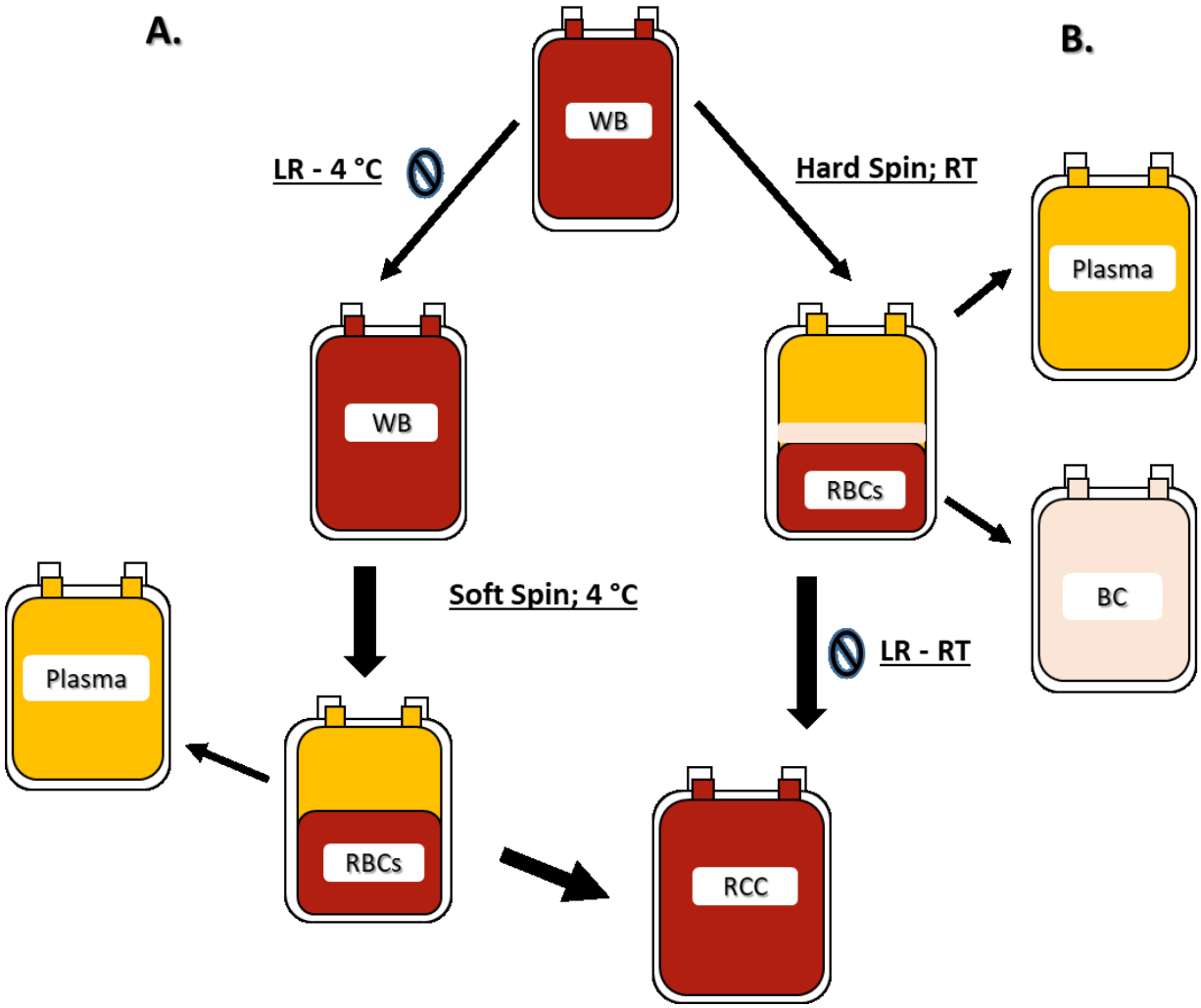
upon stimulation with bacterial endotoxins *in-vitro*; (2) RBC HSL negatively impacts endothelial permeability *in-vitro* as demonstrated by TEER assay; (3) Blood donor age and sex influence RCC quality parameters and significantly influence the immune modulation potential of RCC supernatants *in-vitro.* The main objective of this thesis is to develop bioassays for use in the evaluation of the contribution of donor characteristics to the immune modulation effects of stored RCCs. This thesis is composed of three experimental studies corresponding to three specific research aims (SRAs):

SRA – 1: To develop and assess the utility and efficacy of pooled cryopreserved BC-derived monocytes for MMA application (Chapter 2)

SRA – 2: To establish essential elements of TEER measurements for use in assessing the potential of stored RBC supernatants to modulate endothelial permeability (Chapter 3)

SRA – 3: To investigate the impact of donor age and sex on RCC characteristics and the effect of RCC supernatants on the innate activation and endothelial function (Chapter 4)

Figure 1.1: Diagrammatic illustration of blood component manufacturing methods: whole blood filtration (A) and red cell filtration (B) processing methods. WB = whole blood, LR = leukoreduction and RT = room temperature.



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Chapter 2

Evaluation of the functional properties of cryopreserved buffy coat-derived monocytes for monocyte monolayer assay[1]

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2.1 Introduction

Red blood cell (RBC) transfusion is a lifesaving therapy for patients suffering from anemia [1]. This treatment often comes with the risk of alloimmunization that could result in the occurrence of a clinically significant antibody, leading to a hemolytic transfusion reaction (HTR) and hemolytic disease of the fetus and newborn (HDFN) [1, 2]. A clinically significant antibody is one that causes reduced survival of transfused RBCs [1-4]. Monocytes are a key component of the innate immune system and their central role in antigen presentation, inflammation and phagocytosis has been associated with the pathophysiology of HTR and HDFN [1, 2, 4]. Monocyte monolayer assay (MMA) is an *in-vitro* assay that has been used clinically to predict the survival of RBCs in alloimmunized patients [3, 5-7]. Early reports by Hunt and colleagues [8] , Schanfield and colleagues [2] and Nance and colleagues [7] employed the MMA assay for the detection and determination of *in-vitro* RBC alloantibody clinical significance [2, 7, 8]. MMA utility has evolved over the years to include the examination of hemolysis associated with intravenous immunoglobulin (IVIG) administration and to evaluate receptor-mediated phagocytosis [9, 10].

Selection of RBCs for alloimmunized patients involves choosing a unit of blood that is serologically compatible with the recipient as shown by indirect antiglobulin test (IAT) [11]. A study by Noumsi and coworkers demonstrated the value of MMA as a cross-match tool as it would allow the transfusion of antigen positive RBCs without adverse transfusion outcomes and, could translate to better transfusion support for alloimmunized patients [6].

In previous reports, monocytes for MMA have been isolated from fresh peripheral blood (PB) of healthy, allogeneic or autologous donors, and challenged with sensitized RBCs

immediately after isolation [3, 5, 9]. The use of autologous monocytes from patients ensures that the MMA closely predicts transfusion outcomes as it has been shown that monocytes from different healthy individuals posses variable degree of activity [5, 6, 12]. Due to the logistical constraints that come with the utility of autologous monocytes, the use of fresh allogenic monocytes for clinical MMA remains common practice with the belief that the use of healthy donor monocytes does not significantly affect the ability of the MMA to predict the clinical significance of alloantibodies in question; although, as mentioned, this has been challenged [5, 12, 13]. Some laboratories that may perform the MMA require multiple tubes of PB (10-30 mL) to obtain enough monocytes for the MMA. This can be difficult to achieve in both autologous and allogeneic donation processes due to time-consuming donor recruitment procedures and consent requirements.

Due to the challenges associated with the use of freshly isolated PB monocytes in MMA, this study proposes the use of monocytes from pooled cryopreserved buffy coat (BC) peripheral blood mononuclear cells (PBMCs) in the MMA as an alternative to fresh PB monocytes. BCs are an enriched leukocyte suspension obtained from centrifugation of whole blood (WB) and are usually a waste product of the blood component manufacturing processes [14]. Thus, BCs are readily available in the blood manufacturing facilities therefore avoiding the need to recruit separate donors specifically for the MMA assay. This will allow the possibility of performing hundreds of MMA tests from only a few PBMC isolation procedures. In addition, pooling PBMCs from multiple BC donors can control for donor variation in the allogeneic MMA when using single donors.

Cryopreservation is the storage of cells suspended in cryoprotective agents such as dimethyl sulfoxide (DMSO) and glycerol at extremely low temperature, allowing most of the biochemical reactions within the cells to be preserved [15]. Cryopreserved cells can be stored and used for years, even decades without any significant loss in viability or cell function [15]. The hypothesis of this study is that monocytes from pooled cryopreserved BC PBMCs (MCBCs) in MMA assays will perform similarly to monocytes from pooled fresh BC PBMCs (MFBCs) as well as monocytes from pooled fresh PB PBMCs (MFPBs) in terms of the ability to phagocytose sensitized RBCs *in-vitro* and show similar ability to secrete cytokines and chemokines upon stimulation with bacterial lipopolysaccharides (LPS). Additionally, current study aims to evaluate the potential use of MCBCs in assessing clinical significance of RBC alloantibodies.

2.2 Materials and methods

2.2.1 Buffy coats

Both the Canadian Blood Services (CBS) and University of Alberta Research Ethics Boards (protocol number 2015.032 and Pro00059754 respectively) granted ethics approval for the study. Three ABO/Rh matched BC components (48 h post-collection) were obtained from CBS, Edmonton Centre, Canada. BCs had been processed from WB of healthy donors as previously described [16]. Donors provided a signed, informed consent at the time of donation. Briefly, WB was collected into bags containing citrate phosphate dextrose (CPD) (MacoPharma LQT7291 LX leucoflex-LCR-Diamond) and held overnight at room temperature (RT) [16-18]. Units were centrifuged the following day and the three components (platelet-poor plasma, BC and RBC) were collected into separate satellite bags attached to the top and bottom of the primary

collection bag with the aid of a semi-automated extraction device, according to CBS standard operating procedures[17, 18].

2.2.2 Peripheral blood

Three ABO/Rh matched donors were recruited for the study and approximately 15 mL of PB was drawn from each individual by venipuncture into EDTA vacutainer tubes (BD, Franklin, NJ, USA). Samples were processed within 2 h of collection.

2.2.3 PBMCs isolation

Each BC unit was gently mixed, then diluted (1:1) with RPMI-1640 (contains 20 mmol/L HEPES, L-glutamine, without NaHCO3 - Sigma-Aldrich, Oakville, ON) plus 10 % fetal bovine serum (FBS, Sigma-Aldrich, Oakville, ON). Aliquots from each donor were used to obtain initial total nucleated cell counts (Coulter AcT, Beckman Coulter, NY, USA). From each aliquot, thin blood smears were prepared on glass slides and stained with Hema 3 stains (Fisher Scientific, Kalamazoo, MI, USA) for microscopic analysis of white blood cell differential counts. All BC units were processed individually and PBMCs pooled before cryopreservation. Histopaque-1077 density gradient (15 mL, RT, density 1.077 g/mL, Sigma-Aldrich, Oakville, ON) was dispensed into 50 mL centrifuge tubes and 15-17 mL of the diluted BC was layered on top. The tubes were then centrifuged (700 × g, RT, 40 min, Eppendorf 5810 R, Hampton NH, USA) and the PBMC layer (made up of lymphocytes and monocytes) from each BC unit was evaluated for isolation recovery. The PBMCs were then pooled and washed three times with 30 mL RPMI + 10 % FBS (400 × g, RT, 7 min, Sigma-Aldrich, Oakville, ON) to remove any residual blood cells and assessed for percent

recovery after washing. The procedure for PBMC isolation from fresh PB was similar to the procedure used to isolate BC PBMCs except, PB were not diluted prior to isolation process as performed as described previously [5]. BC and PB units tested were all from different healthy individuals. Monocytes from pooled BC PBMCs were tested fresh and post cryopreservation.

2.2.4 PBMC cryopreservation, thawing and viability assessment

Cryopreservation media containing 20 % DMSO (Fisher Scientific, NJ, USA), 40 % RPMI-1640 and 40 % FBS (Sigma-Aldrich, Oakville, ON) was cooled on ice and added (1:1) to PBMCs to a final concentration of 20 $\times 10^6$ cells/mL. Aliquots were dispensed into sterile cryovials and left to equilibrate with cryoprotective solution on ice for 1 h, followed by overnight storage in a -80 °C freezer and finally liquid nitrogen storage. PBMCs were quickly thawed in a 37 °C water bath, diluted in nine volumes of warm culture media (37 °C) and washed twice (550 × g, 7 min). Cells in each tube were re-suspended in 3 mL culture media and aliquots taken to determine percentage recovery post-wash. A Trypan Blue (GIBCO/ Invitrogen Life Technologies) exclusion assay and a haemocytometer (Fisher Scientific, NJ, USA) was used to determine the membrane integrity of the isolated and thawed PBMCs.

2.2.5 Monocyte functional assessment

Group O positive red cell concentrate (RCC) units were obtained from Canadian Blood Services' Network for Applied Development (netCAD) in Vancouver, Canada. All RCC units had been processed from WB of healthy donors as previously described [16-18]. Scianna-2 (Sc2) positive RBCs were cryopreserved as pellets in a solution of dextrose/sucrose/NaCl and K2

EDTA/NaOH and CPDA-1 (CBS Diagnostic Services, Vancouver). Two RBC units were randomly selected and tested against anti-AnWj or anti-Jra, as these antigens occur at high frequencies in each population [19, 20]. RBCs were sensitized by addition of equal volume of anti-D (human polyclonal IgG, Gamma Biologicals/Immucor, a kind gift from Marilyn Moulds), or four volumes of patient serum containing either anti-Sc2, anti-AnWj or anti-Jra alloantibodies, confirmed by the Canadian Blood Services National Reference Laboratory, followed by 1 h incubation (37 °C, 5 % CO_2). Sensitized cells were then washed with PBS three times and re-suspended in culture media (~5 % sensitized RBCs suspension). Portions of the sensitized RBCs were used to perform an IAT [11]. Monocytes were also incubated with 10 µg/ mL LPS suspensions from *E. coli* (serotype 055; B5, Sigma-Aldrich, Oakville, ON) which, act as an external signal for cell activation resulting in cytokine production from PBMCs [21-23]. The culture media used in the experiments was used as a negative control.

2.2.6 Sources of alloantibodies[2]

Serum were obtained from patients known to contain anti-Sc2, anti-AnWj or anti-Jra alloantibodies [24, 25]. Patients had presented with symptoms of transfusion reactions detailed in Table 2.3.

2.2.7 Monocyte monolayer assay[3]

MMA was performed as previously described [5, 6, 12]. Briefly, monocyte monolayers were prepared on 22 × 22 mm glass coverslips (Fisher Scientific, NJ, USA) treated with Poly-L-Lysine hydro-bromide (PLL) solution. Coverslips were placed in 35 mm culture dishes (Stem Cell

[2,3]Dr. Branch (CBS Toronto) and Mr. Christopher Ward (Associate Professor, MLS) provided alloantibodies and anti-human globulin respectively used in this study.

Technologies, Vancouver, Ca.) before 1 mL of 2.0 $\times 10^6$/mL intact PBMC suspension was added. Culture dishes were incubated (37 °C, 5 % CO_2) for 1 h to allow for monocyte adherence and the coverslips were then washed three times with PBS at 37 °C to remove any non-adherent cells (such as lymphocytes). Monocyte monolayers were incubated with either 1 mL of anti-D-sensitized RBCs or 400 µL of either anti-Sc2, anti-AnWj or anti-Jra-sensitized RBCs, 1 mL LPS suspension or 1 mL culture media and incubated (37 °C, 5 % CO_2) for 2 h. Negative controls included monocyte monolayers incubated with unsensitized RBCs, as well as the culture media. Cell culture supernatants (anti-D-sensitized RBCs, LPS and culture media conditions) were transferred into labelled micro-tubes and spun (10000 × g, 5 min) to obtain cell-free supernatants and stored at -80 ˚C for subsequent cytokine analysis. Stained coverslips (Hema 3 stains, Fisher Scientific, Kalamazoo, MI, USA) were examined microscopically for adherence and phagocytosis. Phagocytosis index (PI) was determined as the number of fully phagocytosed RBCs per 100 monocytes. Multiple phagocytosis (%) was defined as the proportion of monocytes that ingested more than one RBC compared to those that ingested a single RBC. Experiments involving anti-D-sensitized RBCs, LPS and culture media conditions were performed in triplicates on three different days using MFBCs, MCBCs and MFPBs (n=9).

2.2.8 Cytokine quantification[4]

Luminex technology using MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panels (EMD Millipore, Toronto, ON, Canada) was used to assess the concentration of 40 cytokines/chemokines present in monocyte culture supernatants. The samples were brought to RT and added to 96-well plates. Commercially available bead conjugated with specific capture

[4]Multiplex cytokine analysis was performed by Dr. Trang Duong (Hospital for Sick Children, Toronto).

antibody was then added to each well and the plate incubated overnight at 4 ˚C. The wells were washed and incubated with biotin-labelled secondary antibody for 1 hour and further incubated with streptavidin-phycoerythrin for 30 min. The samples were analyzed using Luminex laser-based fluorescent analytical test instrumentation and the cytokine/chemokine concentrations determined from standard curves prepared on each plate (expressed as picogram per milliliter - pg/mL). The cytokine/chemokine panel consisted of interleukin (IL)-1 receptor antagonist (RA), IL-1 alpha (α), IL-1 beta (β), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, interferon gamma (IFN-γ), IFN-α2, eotaxin, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein (MCP)-1, MCP-3, macrophage inflammatory protein (MIP)-1α, MIP-1β, tumor necrosis factor (TNF)-α and TNF-β, fibroblast growth factor (FGF)-2, transforming growth factor (TGF)-α, macrophage-derived chemokine (MDC), fractalkine, sCD40L, Flt-3L, GRO (growth-relate oncogene), IFN-inducible protein-10 (IP-10), and RANTES (regulated on activation normal T cell expressed and secreted). The assay was performed following manufacturer's instructions as previously described [23, 26].

2.2.9 Statistical analyses

One-way ANOVA (SPSS 23.0 software, IBM, Armonk, NY) with Tukey Post-hoc analysis was used to compare differences in phagocytosis and cytokine secretion in MFBCs, MCBCs and MFPBs. Mann-Whitney U Test was used to compare the cell recoveries post isolation and wash

as well as PBMC membrane integrity. Data were expressed as mean ± standard deviation (SD). A $p < 0.05$ was considered statistically significant.

2.3 Results

2.3.1 PBMC recovery and purity after isolation

The PBMC, lymphocytes, monocytes, neutrophils, RBC and platelet recoveries post-isolation and post-wash are shown in Table 2.1. Cell isolation recoveries (%) were calculated from the number of cells in the sample after isolation or washing divided by the number of cells present before isolation/washing. The isolation recovery rate for PBMCs (p=0.024), lymphocytes (p=0.024) and monocytes (p=0.047) were significantly different between fresh BC and fresh PB (Table 1). The average monocyte ratio increased from 7 ± 2 % and 8 ± 3 % before PBMC isolation to 29 ± 5 % and 30 ± 3 % after isolation in fresh BC and fresh PB respectively. This proportion further increased to 93 ± 2 % and 95 ± 2 % upon adhesion onto PLL-treated coverslips and culture in fresh BC and fresh PB respectively.

2.3.2 Influence of cryopreservation on cell count, viability and purity

Following cryopreservation, thawing and washing, 84.8 ± 5.1 % of the PBMCs were recovered from cryopreserved BCs. This is comparable to the results obtained from fresh BC and fresh PB after a three-step wash (Table 2.1). According to Trypan Blue Exclusion assay, pooled cryopreserved BC PBMCs showed 95.2 ± 1.2 % membrane integrity while 100 % of the pooled PBMCs isolated from fresh BC and fresh PB were found to be intact. There was no statistically significant difference in PBMC post-thaw recovery or membrane integrity between groups.

Examination of the effect of cryopreservation storage time showed that there was no statistically significant difference in the phagocytosis ability between MCBCs stored for three weeks and those stored for over 6 months (<1 month, PI = 78 ± 2 and >6 months, PI = 75 ± 4, n = 3). PBMC membrane integrity over time was consistent (<1 month = 94.1 %, >6 months = 95.5 %).

2.3.3 Influence of cryopreservation on monocyte function

2.3.3.1 Phagocytosis

For anti-D-sensitised RBCs, MCBCs resulted in a PI of 80 ± 6 compared to MFBCs (PI; 77 ± 11) and MFPBs (PI; 82 ± 10, Figure 2.1). A hundred monocytes that ingested RBCs were counted; 49 ± 10 % of the monocytes ingested more than one RBC for MCBCs. This is comparable to 51 ± 4% for MFBCs and 59 ± 6 % for MFPBs (Figure 2.1). The negative control PI (un-sensitized RBCs along with culture media) was zero. There was no statistically significant difference in the phagocytosis ability/characteristics between MCBCs, MFBCs and MFPBs. Anti-D sensitized RBCs consistently resulted in a strong (4+) IAT reactivity. Studies have generally indicated a requirement of R2R2 phenotyped cells for use as positive controls as this is the phenotype with the greatest expression of D-antigen on the surface of the RBCs for sensitization [5, 9, 10]. For all our experiments, group O Rh positive RBCs from donor units were used as positive controls. All positive controls had a high PI (> 60) and were considered valid. When MCBCs were challenged with previously established clinically significant alloantibody-sensitized RBCs (Table 2.3), the results were consistent with previous findings: an average PI of 9 ± 2 for anti-Sc2, 64 ± 5 and 56 ± 5 for anti-AnWj and 11 ± 3 and 22 ± 10 for anti-Jra-sensitized RBCs. Alloantibodies tested showed a weak (1+) IAT reactivity.

2.3.3.2 Cytokine and chemokine secretion

Release of 40 cytokines and chemokines in LPS-activated MCBCs, MFBCs and MFPBs were assessed; 12 were detected in considerable concentrations (Table 2.2). Treatment with LPS resulted in a significant increase in TNF-α, IL-1β, IL-6, IL-8, MIP-α ($p < 0.01$), MIP-β and GRO ($p < 0.05$) secretion from MCBCs compared to both MFBCs and MFPBs. TNF-α secretion was also significantly increased in MFBCs compared to MFPBs ($p < 0.05$). IL-1RA was significantly elevated in MCBCs compared to MFPBs. Treatment with anti-D-sensitized RBCs resulted in a significant increase in IL-8, MIP-α and IL-1RA secretion from MCBCs compared to both MFBCs and MFPBs ($p <0.05$). Further, MCP-1 secretion was significantly higher in MCBCs and MFBCs for both the anti-D-sensitized RBC and LPS treatment groups compared to MFPBs ($p < 0.05$). IL-8 was significantly increased in both MFBCs and MCBCs compared to MFPBs ($p < 0.05$). GRO levels were significantly higher in both MFBCs and MCBCs compared to MFPBs ($p<0.05$). Fractalkine levels was slightly raised in MCBCs compared to both MFBCs and MFPBs ($p<0.05$). There was a statistically significant increase in RANTES secretion in MFBCs in the anti-D-sensitized-RBCs treatment compared to MCBCs ($p < 0.05$). Eotaxin was detected only in the RBC treatment group though no significant difference between groups was observed.

2.4 Discussion

Clinically significant antibodies are frequently associated with HTR and HDFN [3, 6, 11]. The need for specific antigen negative RBCs to meet the compatibility criteria may be difficult to meet and brings forth logistical constraints to the blood bank systems [27-29]. Flickinger [28] showed that 14.5 % of requests for rare RBCs remains partially filled or unfilled over a 18 month

period. Furthermore, Seltsam and coworkers pointed out that in central Europe, transfusion support for one third of patients with RBC alloantibody to a high incidence antigens was unsatisfactory [29]. MMA has proven a useful *in-vitro* technique, which may be applied as a complementary cross-match tool for patients with unusual RBC antibody presentation to predict clinical outcomes and improve transfusion support for these patients [2-6].

However, practical restrictions of the MMA assay have limited its usefulness in both clinical and research applications. The most critical factor is the requirement for isolation of PBMCs from fresh PB and the repeated use of density gradients to isolate the PBMCs, which brings up many logistical issues with donor recruitment and tissue culture expertise of staff. Allogeneic MMA approaches also suffer from logistical problems of space and time limitations as well as inter-donor variability. BCs are an enriched leukocyte suspension not normally utilized for clinical transfusion purposes; thus, are discarded after blood component production processes [14]. They provide an advantage of large-scale isolation of PBMCs from several BC units [14, 30]. In this study, variability due to donor factors was effectively reduced as each batch of pooled PBMCs was composed of three donors. The isolation protocol utilized in this study successfully produced a large quantity of PBMCs that was comparable to other density gradient separation techniques [31, 32]. We set our isolation protocol acceptability criteria to obtain limits of < 15 %, < 5 % and < 5 % platelet, RBC and neutrophil contamination respectively, which was consistently achieved throughout this project (Table 2.1). Washing of PBMCs to remove other blood cells is a critical step as it often leads to loss of cells [14]. After a three-step wash, over 80 % of the PBMCs from both BC and PB sources were recovered (Table 2.1). Studies have shown that the temperature at which the blood samples are held before the assay performance influences cell

aggregation making it difficult to obtain pure isolates [33]. This study was constrained by the CBS processes, so that the BC units us were held at RT for 48 h prior to PBMC isolation. As there was some inter-donor variability, not appearing to be consistent with donor age, sex or blood group, all BC units were processed individually and the PBMCs pooled post-isolation. This could be the reason behind lower cell recoveries obtained from BCs compared to PB. The membrane integrity of pooled PBMCs from fresh BC and fresh PB were comparable indicating that the blood component manufacturing process did not adversely affect PBMC viability.

Cryopreservation of PBMCs offers an attractive advantage of long-term storage, consistent results and standardized experimental conditions [15, 34-36]. It is widely known that cryopreservation potentially causes damage to cell membranes and adversely affect cell viability and function [33]. However, a carefully designed cryopreservation protocol can minimize these damages [36]. Currently, numerous clinical studies routinely use cryopreserved PBMCs to investigate immune response to infectious diseases, clinical vaccine development as well as development of immune based therapies [36-38]. Results from this study are agreeable with published data showing that over 90 % of cryopreserved PBMCs maintained intact cell membranes post-thaw, independent of the storage time, creating a novel utility for unused BC units often discarded during blood component production process [14, 32, 35, 39]. The isolation of PBMCs from three BC units (~30 mL each) resulted in approximately 0.5×10^9 PBMCs, which translates in the average monocyte ratio increasing from 7 ± 2 % to 29 ± 5 % before and after PBMC isolation respectively. The pooling, freezing and thawing of PBMCs from three BC units result in about 200 mL of PBMC cell suspension at a concentration of 2.0×10^6 cells/mL, which provides enough post-thaw recovery of PBMCs to perform approximately 200 MMA assays.

In addition to PBMC recovery and viability, other functional aspects of MCBCs, MFBCs and MFPBs were evaluated; the ability to phagocytose anti-D-sensitized RBCs *in-vitro*. Analysis of the phagocytic responses showed that MCBCs phagocytosed comparable numbers of sensitized RBCs as MFBCs and MFPBs, providing evidence that cryopreservation did not significantly alter monocyte function (Figure 2.1). There are conflicting reports on whether monocytes retain their phagocytosis ability after cryopreservation and thawing [34]. One study showed a comparable phagocytic function between fresh and cryopreserved monocytes; another showed that 25-30 % of monocyte phagocytosis ability is lost following cryopreservation [14, 35]. However, current study provide evidence that the approach of using DMSO and a slow rate of cooling maintain monocyte viability and function for use in an MMA.

As the number of RBCs phagocytosed by each monocyte solely determines PI, presence of a few hyperactive monocytes could falsely increase the PI thus, misrepresenting the clinical importance of an alloantibody [10]. It was therefore essential to examine the phagocytosis characteristics exhibited by MCBCs, MFBCs and MFPBs. Results showed that there was no statistically significant difference in the number of monocytes that phagocytosed single versus multiple RBCs between groups, indicating that monocytes phagocytosis function was not impacted by the pooling, freezing and thawing processes. Positive controls for each experimental condition were performed using anti-D-sensitized group O Rh positive RBCs that were obtained from RCC units from multiple healthy donors. Prior to conducting this study, the use of more readily-available O positive, Rh unphenotyped RBC for MMA assay was investigated as it would alleviate the need for finding a R2R2 donor for each experiment or, for the additional procedures of cryopreserving and preparing such control RBCs. Using the same PI acceptability criteria for

R2R2 positive controls as in previous studies [10, 12]. It was determined that all O Rh positive cells from the RCCs used in this study showed high rates of opsonisation (IAT = 4+) and did not affect phagocytosis by the monocytes (PI > 60), and therefore could be deemed appropriate and valid positive controls. This further facilitates the implementation and interpretation of this assay, as the quantitative limits to control for the inter-donor variability on phagocytosis in the MMA were put in place.

Further, this study provides evidence establishing the suitability of MCBCs for use in evaluating clinical significance of human alloantibodies. As summarized in Table 2.3, the three alloantibodies studied in this report were originally tested by MMA using fresh autologous or allogeneic monocytes which indicated that the anti-Sc2 [24], –AnWj (Branch, DR; unpublished results) and –Jr^a [25] alloantibodies showed a positive MMA reactivity (PI > 5). In addition to these findings, several other case reports support this assessment [19, 20, 40, 41]. RBCs expressing Sc2 antigens are extremely rare; as are the antibodies against them [41, 42]. The frequency of the Sc2 antigen is less than 1 % in the general population, appearing to be more common in a consanguineous Canadian Mennonite population and remaining absent from populations of African or Native American descent [42]. It is for this reason that the MMA was performed using cryopreserved Sc2 positive RBCs. In contrast, AnWj and Jra RBC antigens are high frequency antigens, which make antibodies against these antigens very rare [19, 20, 25, 40, 41]. These alloantibodies are of IgG type and may be formed during pregnancy or post-transfusion [19, 20, 24, 25, 40, 41]. Results show a positive MMA reactivity for these antibodies which correlated well with the patient's clinical history and MMA that was performed using the same patients' sera but with fresh autologous or allogenic monocytes [24, 25, 40]. Also, MMA showed very high levels of

phagocytosis of anti-AnWj-sensitized RBCs despite an IAT result showing that only a small amount (1+) of AnWj antibodies were bound to the RBCs. This phenomenon has been described in the past where studies have shown that the strength of opsonisation does not necessarily correlate with significant monocyte interactions leading to phagocytosis [6, 10, 12].

In addition to phagocytic assessment, this study provides important new information on the function of MCBCs; cytokine release, which may be important in investigation of immunomodulatory mechanisms of allosensitized RBCs role in pathophysiology. When stimulated, monocytes release low molecular mass glycoproteins known as cytokines [21]. They have multifunctional pro- and anti-inflammatory roles in inflammation, immunity against infectious agents as well as regulation of various biologic systems [21, 43-46]. As a measure of the innate immunocompetence, the ability of monocytes to secrete TNF-α in response to LPS stimulation is commonly assessed [21, 43, 47-49]. The current study evaluated a comprehensive panel of cytokines/chemokines in addition to TNF-α, in a paired experimental design not previously reported involving MFBCs, MCBCs and MFPBs. Interestingly, it was found that MCBCs when stimulated with LPS, secreted higher amounts of TNF-α, IL-8, IL-1β, IL-6, MIP-1α, MIP-1β and GRO compared to both MFBCs and MFPBs (Table 2.2). Abnormal cytokine production has been shown to play a role in the pathogenesis of various disorders [21]. For instance, IL-6, IL-1 and TNF-α are multifunctional pro-inflammatory cytokines involved in pathogenesis of rheumatoid arthritis (RA) and other autoimmune conditions [22, 43-45] . In RA, IL-1 and TNF-α inhibit bone synthesis by activating osteoclasts while IL-6 induce polyclonal activation of B-cells resulting in production of rheumatoid factor [43]. TNF-α also has cytocidal effect on tumor cells [43]. B-chemokines including MIP-α, MIP-β and RANTES are potent chemo-attractants for

mononuclear cells in acute parasitic infections [50, 51] leading to tissue destruction. IL-1RA is a naturally occurring cytokine that competitively blocks the binding of IL-1α and IL-1β to IL-1 receptors therefore reducing the severity of inflammatory conditions [52]. IL-10 on the other hand exhibit robust inhibitory effects on monocyte cytokine production both in-vivo and in-vitro [53]. IL-8 is produced a variety of immune cells including monocytes, macrophages, neutrophils and eosinophils and primarily serves as a chemoattractant for neutrophils to the site of chronic inflammation [22]. The observation that cryopreserved monocytes or PBMCs exhibit enhanced cytokine production has been previously described. For example, a study showed that cryopreserved PBMCs produced higher levels of TNF-α, IFN-γ and IL-2 than fresh PBMCs [21]. Similarly, Venkataraman showed that cryopreservation induced elevated IL-6 and TNF-α production, cytokines whose major source upon LPS stimulation are monocytes [46, 54]. These reports are supported by another study showing that cryopreservation induced spontaneous release of IL-6, IL-10, IL-12, IL-13 and IFN-γ from PBMCs [37]. The increased cytokine release from cryopreserved PBMCs has been attributed to the cryopreservation effects, as many cryoprotectants such as DMSO used in this study, are membrane permeable and osmotically active compounds, augmented by the physical processes of freezing and thawing, which could lead to cell pre-activation [15, 37, 55]. It has also been suggested that cryopreservation alters the cell membrane and cytoskeleton and may alter the genes involved in cytokine secretion leading to poor or over-expression of different markers [37]. Irrespective of the mechanism by which cytokine secretion is altered in cryopreserved PBMCs, which requires further investigation, cytokines did not appear to influence phagocytic responses of MCBCs evaluated in this study.

2.5 Conclusion

This described study compared the viability, phagocytosis and cytokine secretion in MFBCs, MCBCs and MFPBs. First, it provides detailed information on the isolation, pooling and cryopreservation of PBMCs from a novel source, usually discarded during blood component manufacturing. Results show that phagocytosis ability of MCBCs was not significantly affected by cryopreservation, although there were some influences on cytokine production. Secondly, this study provide evidence for the use of allogeneic MCBCs in an MMA for evaluating the clinical significance of alloantibodies previously implicated in transfusion-related reactions. Thirdly, the study provides new information on the influence of cryopreservation on monocyte cytokine/chemokine secretion that could significantly affect cytokine data while using cryopreserved cells in clinical studies. The adoption of the use of MCBCs in clinical MMA can reduce the amount of time needed to perform frequent isolations and minimize donor variability thus allowing for analysis that is more consistent while creating utility for unused BC units. In Chapter 4 of this thesis, the use of pooled cryopreserved monocytes from BCs will be used in the MMA to evaluate the influence of donor factors (age and sex) on the immune modulation potential of stored RCC supernatants *in-vitro.*

Table 2.1 - Cell recovery (%) after Histopaque isolation procedure.

Source of blood	PBMC recovery after isolation (%)	Lymphocyte recovery after isolation (%)	Monocyte recovery after isolation (%)	Neutrophil recovery after isolation (%)	PBMC recovery after wash (%)	RBC recovery after wash (%)	Platelet recovery after wash (%)
BC	67.4 ± 6.3⁂	71.8 ± 4⁂	72.8 ± 3.8⁂	3 ± 2	86.9 ± 6.7	0.4 ± 0.1	5.4 ± 2
PB	75.8 ± 7.7	76.7 ± 3.3	78.1 ± 6	0	85.9 ± 3.1	0.06 ± 0.02	6.2 ± 2.4

Data represent mean ± SD (n = 9). The values in columns 2-5 are the averages from nine donor blood processed in three different experiments from both fresh BC and fresh PB. The values in columns 6-8 are the averages obtained after pooling the PBMCs from three donors in each experiment. ⁂ = Significant difference BC vs PB sources (p<0.05).

Table 2.2 - Cytokines/chemokines (pg/mL) secreted by monocytes derived from pooled fresh BC, cryopreserved (frozen) BC and fresh PBMCs.

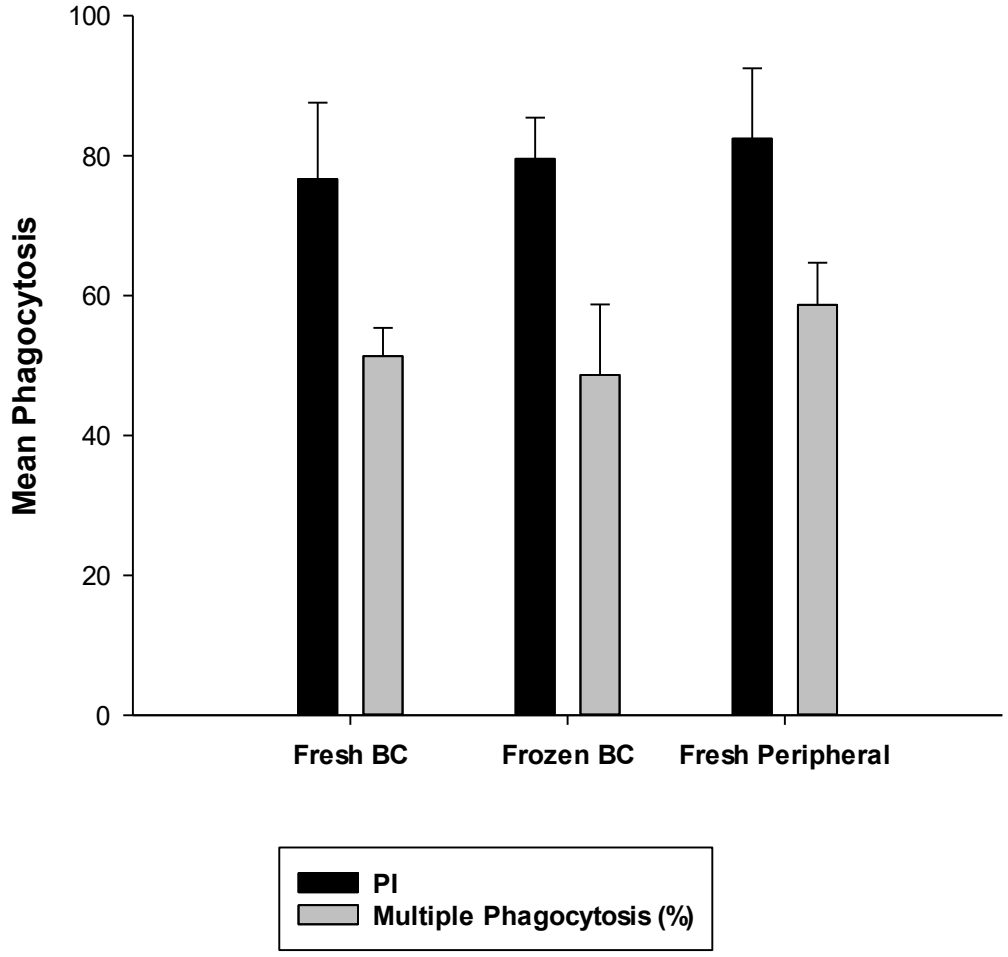
	Anti-D-sensitized RBCs Treatment			LPS Treatment			Negative Control		
Cytokine/chemokine	**Fresh BC**	**Frozen BC**	**Fresh PBMCs**	**Fresh BC**	**Frozen BC**	**Fresh PBMCs**	**Fresh BC**	**Frozen BC**	**Fresh PBMCs**
TNF-α	4.3 ± 2.6	10.9 ± 4.6	10.6 ± 10.1	222.7 ± 94.2†	535.3 ± 86.0‡§	80.6 ± 36.8	1.0 ± 1.0	0.8 ± 0.2	0.7 ± 0.5
IL-6	<OOR	3.6 ±3.9	2.7 ± 2.2	64.6 ± 35.4	197.7 ± 32.4‡§	35.7 ±15.4	<OOR	<OOR	<OOR
IL-8	154.0 ± 103	342.6 ± 84.6‡§	61.9 ± 30.6	1272.5 ± 925.1	3376.8 ± 693.0‡§	286.6 ± 65.6	47.4 ± 19.4†	104.3 ± 32.2‡§	6.5 ± 4.8
GRO	14.0 ± 6.2	25.8 ± 9.0	13.7 ± 9.9	414.5 ± 195.5	1010.0 ± 359.4‡§	86.8 ± 15.1	11.0± 6.7†	12.3 ± 5.4 §	3.2±2.2
IL-1ra	4.0 ± 2.4	10.3 ± 3.8 ‡§	0.8 ± 0.9	3.9 ± 2.0	8.0±2.6§	1.3 ± 1.1	0.9± 0.8	1.6 ± 0.4	0.6 ± 0.8
IL-1β	1.4 ± 1.8	1.0 ± 0.6	0.2 ± 0.4	8.5 ± 3.0	38.4± 5.3‡§	3.5 ± 1.5	0.1± 0.2	0.3 ± 0.2	0.1±0.3
Eotaxin	10.2 ± 4.5	13.4 ± 3.0	10.4 ± 5.8	<OOR	<OOR	<OOR	<OOR	<OOR	<OOR
MIP-1α	18.8 ± 11.0	50.1 ± 28.6‡§	19.7 ± 15.5	235.9 ± 95.7	556.6 ± 106.4‡§	110.1 ± 37.9	1.6 ± 1.0	2.1 ± 0.6	1.5 ± 0.7
MIP-1β	22.0 ± 18.3	26.6 ± 13.1	22.1 ± 16.5	230.5 ± 104.1	418.5 ± 65.5‡§	127.2 ± 57.5	2.4 ± 2.5	3.7 ± 2.5	0.7 ± 1.5
MCP-1	50.2 ± 22.3†	70.6 ± 14.3§	26.8 ± 3.9	25.1 ± 16.4†	30.0 ± 5.2§	7.2 ± 5.2	7.3 ± 4.4	14.8 ± 11.1	7.3 ± 6.9
Fractalkine	3.6 ± 2.8	3.7 ± 2.0	2.8 ± 2.7	6.4 ± 2.0	3.3 ± 3.5	2.7 ± 2.4	0.7 ± 1.0	4.2 ± 1.5‡§	2.1 ± 1.6
RANTES	175.5 ± 41.0‡	113.0 ± 12.9	153.9 ± 50.0	255.4 ± 70.6	95.6 ± 21.6	283.9 ± 232.4	213.4 ± 49.7‡	77.0 ± 43.7	203.2 ± 132.5

Data represent mean ± SD (n = 9). Monocytes from pooled PBMCs were incubated with either anti-D-sensitized RBCs, LPS or culture media (negative control) for 2 h. † = Significant difference fresh BC versus fresh PBMCs, ‡ = Significant difference fresh BC versus frozen BC and § = Significant difference frozen BC versus fresh PBMCs (p<0.05). <OOR = out of range below.

Table 2.3 - Reported clinical significance of alloantibodies tested in this study. Alloantibodies were all originally tested in MMA by Dr. Donald R. Branch using freshly obtained autologous or healthy donor monocytes.

Alloantibody	**Clinical Significance**
Anti-Jra	**HDN**: Fetal hemoglobin at 27 weeks gestation 4.8 g/dL, 4 intrauterine transfusions with Jr(a-) blood 2 weeks apart, baby born by C-section at 35 weeks, Jr(a+) by genotyping; DAT positive; bilirubin increased from 59 to 109 umol/L[25]
Anti-Sc^2	**HTR**: Rigors, nausea, abdominal pain and fever (39.4 ^oC) after 75 mL of donor blood (Sc2+)[24]
Anti-AnWj	**HTR:** Repeated transfusion reactions that included fever and rigors with small volumes of blood transfused and only a 1+ serologic incompatibility. Hemoglobin dropped from 9.2 g/dL to 5.4 g/dL despite multiple transfusions (Donald R. Branch, personal communication)

Figure 2.1 - Comparison of the phagocytosis indices (PI) and multiple phagocytosis (%) between monocytes derived from pooled fresh BC, cryopreserved (frozen) BC and fresh PBMCs incubated for 2 h with anti-D-sensitized RBCs (n = 9).



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Chapter 3

Trans-epithelial electrical resistance (TEER) assay for the evaluation of RCC-induced endothelial modulation

3.1 Introduction

As previously reviewed in Chapter 1, red blood cells (RBCs) under hypothermic storage (HS) undergo a wide range of biophysical and biochemical changes collectively referred to as hypothermic storage lesion (HSL), including the increased release of extracellular vesicles (EVs) from the cell membrane, cell-free hemoglobin, damaged proteins and lipids and an increase in hemolysis with HS length [1, 2]. These changes have been shown to reduce RBC oxygen-carrying capacity and influence transfusion outcomes by modulating the immune system of transfused patients [1-5]. However, little remains to be known about the effect of stored red blood cell concentrates (RCCs) on the endothelium both *in-vitro* and *in-vivo*, demonstrating a need for improved methods that focus not only on RBC metabolism, but also on more complex interaction of transfused RBCs on the endothelium and immune system of transfused patients.

The human endothelium is a single, yet biologically active layer of cells lining the vasculature including veins, arteries and capillaries [6]. Endothelial cells are connected to each other by tight and adherens junctions, multi-protein complexes that seal the spaces between the cells [6-8]. Under normal physiological conditions, the endothelium forms a physical barrier between blood and the surrounding tissues (interstitial tissues) by maintaining a low and selective permeability to fluids and solutes [7-9]. Two main ways by which cells, fluids and solutes cross the endothelium have been demonstrated: paracellular/intercellular (between cells) and transcellular (through cells) [6]. Overall, vascular permeability is a result of a number of processes that either increase or decrease endothelial barrier function including normal physiological process (extravasation of leukocytes into tissues, maintenance or repair of the vasculature and

formation of new blood vessels) as well as pathological processes (inflammation, acute lung injury and tumorigenesis) [8, 10].

There has been an increasing interest in the study of the physiology and pathophysiology of human endothelium through the years. Consequently, several *in-vitro* techniques have been developed to assess endothelial barrier integrity [11-15]. Earlier models relied on cells grown on permeable supports and endothelial permeability assessed by measuring the movement of radioactively-labelled or dye-labelled hydrophilic substances such as glucose [11-13, 15]. These techniques offered the advantage of accessibility to the apical and basolateral compartments of the cell monolayers therefore mimicking the normal physiology of the endothelium [15]. However, the use of tracer compounds have been limited by their instability and the requirement for special handling and storage precautions [15]. In addition, they are less sensitive and can interfere with endothelial barrier integrity, making it impossible to monitor cells in culture over time [12, 15].

Significant development in the studies of the endothelial system occurred in 1984, when Ivar Giaever and Charles Keese developed a non-invasive impedance-based method for the *in-vitro* monitoring of mammalian fibroblasts grown on evaporated gold electrodes onto which an alternating current (AC) was applied [16]. In this study, they found the total impedance of the system to be reflective of the changes in morphology, motion and density of the cells over time [16-19]. Impedance-based monitoring of cells has also been used to investigate electric properties of cell membrane as well as to understand mechanisms of cell-substrate attachment [18, 19]. To date, impedance-based technologies has been employed to monitor other adherent

cell types representing various human physiological models such as the human vein, blood-brain barrier and gastrointestinal tract [15, 20-22].

Trans-epithelial electrical resistance (TEER) is the measurement of electrical resistance across an endothelial cell monolayer [15]. The basic principle is that when endothelial cells are grown to confluency, they form intact cell-to-cell junctions that are able to resist the passage of electrical current [15]. A recent method for TEER measurement is the real-time cell analysis (RTCA) using platforms such as electric cell-substrate impedance sensing (ECIS) or real time cell electronic sensing (RT-CES) [23-27]. In these platforms, cells are grown on multi-well culture plates coated with gold-film electrodes and impedance monitored in real-time by an RTCA analyzer [23-28]. The cells adhere to non-porous surfaces (no basolateral fluid compartment available); therefore, there is no possibility of performing experiments that require transport of substances across the cell monolayer [23-29]. TEER measurements using RTCA platforms offers numerous advantages over other conventional methods. In general, it is a highly sensitive non-invasive method that can be used to monitor cells though various phases of growth and differentiation [12, 15, 27].

RTCA using impedance biosensors has had wide application in recent years. To mention a few, RTCA has been used to identify and profile cytotoxicity of chemicals associated with environmental and human health hazards [23, 27-31]. In cancer research, it is commonly used to study the metastases of various human cancers [26, 32, 33]. In drug development research, RTCA has proven a useful tool for investigating the potential side effects of newly developed drugs to the endothelium [9, 13, 29]. The opportunity for RTCA application in transfusion and blood

banking setting is evident. As the need for blood products (RCCs, plasma and platelets) continue to increase globally, measures are constantly being applied to ensure demand is met [34]. This includes the development and introduction of new blood product manufacturing methods, expansion of donor catchment areas as well as the biopreservation of blood products to allow for a longer shelf-life [34]. As a result, recent concern has been the impact transfusion of fresh versus old RBCs on transfusion outcomes [1, 35-37]. This is a concern because as RBCs are held in storage longer, so is the progression of HSL [1, 36, 37]. And since an intact endothelium is important for normal functioning of body tissues [7, 12], it is necessary to investigate the potential of this valuable treatment in modulating the barrier integrity and immune system of the recipient resulting in increased morbidity or mortality.

Recently, few studies have applied RTCA to evaluate the effect of platelets on the stability of the endothelium *in-vitro* [24, 25]. In these studies, platelet products from different donors that had been stored for up to 5 days at different temperature were tested. It was shown that TEER decreased (increased paracellular permeability) as the length of platelet storage increased and showed that fresh platelets (day 1) from different donors produced more variable TEER measurements than day 5 platelets [24, 25]. A similar study showed that EVs from aged platelets impair microvascular pulmonary endothelial cell barrier function [37]. Further, TEER measurement has been used to evaluate the potential benefit of different formulations of plasma products that could play a significant role in overcoming the logistical challenges of using fresh frozen plasma. These studies demonstrated that spray-dried and lyophilized plasma reduces pulmonary vascular permeability and inflammation in hemorrhagic shock [38, 39].

Considering the association between RBC transfusion and TRIM, the aim of the current study was to adapt the TEER assay in transfusion and biobanking as an additional tool for evaluating the potential of stored RCC supernatants to modulate endothelial permeability in-*vitro*. To do this, basic elements of the assay were tested and established using human umbilical vein endothelial cells (HUVECs) as a model for human endothelium [40]. The hypothesis was that RBC HSL negatively impacts endothelial permeability *in-vitro* as demonstrated by TEER assay.

3.2 Materials and Methods

3.2.1 Blood collection, manufacturing and preparation of RCC supernatants

Both the Canadian Blood Services (CBS) and University of Alberta Research Ethics Boards (protocol number 2015.032 and Pro00059754 respectively) granted ethics approval for the study. Three saline-adenine-glucose-mannitol (SAGM) RCCs used in this study were processed from whole blood using BC method at the Canadian Blood Services' Network Center for Applied Development (netCAD) in Vancouver, as previously described in Chapter 1 (Figure 1.1) [41, 42]. RBC units were stored at 1 – 6 ˚C for 42 days and sampled aseptically at day 7, 21 and 42 of storage. For sampling, units were mixed by gentle massage and five inversions and, 5 mL sample drawn through a sampling site coupler (4C2405, Fenwal) using an 18-gauge needle attached to a 10 mL syringe. RBC samples were centrifuged (2200 × g, 4 ºC, 10 min, Eppendorf 5810 R, Hampton NH, USA) to obtain cell free supernatants. All supernatants were stored in -80 ˚C for 1 - 3 months and thawed at room temperature before use in experiments.

3.2.2 HUVECs culture

HUVECs (C2519A, Lonza Group Ltd., Walkersville, MD, USA) were purchased as pooled primary cells frozen at passage 1 and grown as described previously [43]. HUVECs at passage 1 were sub-cultured for 4 - 5 days in endothelial growth medium-2 (EGM-2, Lonza) and passage 2 cells cryopreserved in cryopreservation media containing EGM-2, 10 % fetal bovine serum (FBS, Sigma-Aldrich, Oakville, ON, Canada) and 10 % dimethyl-sulfoxide (DMSO, Fisher Scientific, NJ, USA). Passage 2 HUVECs were thawed in 37 ˚C water bath, diluted in 3 ml EGM, and seeded in T25/75 culture flasks (Corning Incorporated, Corning, NY). Cells were grown for 4 - 5 days while culture media was changed every other day until the cells attained a surface coverage of 80 %. HUVECs were then treated with Trypsin-EDTA at room temperature (RT, 2 - 3 min, Lonza) for cell harvesting, followed by addition of trypsin neutralizing solution and centrifugation (1000 RPM, RT, 5 min, Eppendorf 5810 R, Hampton NH, USA). HUVECs were resuspended in EGM-2 and cell counts obtained (Beckman Coulter, Mississauga, ON, Canada). HUVECs suspensions were used in experiments within 1 hour.

3.2.3 Real time cell electronic sensing (RT-CES) system[1]

RT-CES system (ACEA Bioscience, San Diego, CA) uses microelectronic biosensor technology to analyze cellular events in a real-time, non-invasive and label-free manner [21, 30, 44]. The system was composed of four parts: the RT-CES device station (workstation); the RT-CES analyzer**;** RT-CES control unit (Computer) and 96-well culture plate (E-Plate, Figure 3.1) [23]. The E-plate, coated with gold microelectrode sensors, was connected with the workstation from

[1]The author would like to acknowledge Drs. Xing Fang Li and Chris Le, (Division of Analytical and Environmental Toxicology) for providing access and use of the RT-CES system, and Dr. Birget Moe for precious assistance and training in performing RTCA.

inside the cell culture incubator. The workstation was connected to the RT-CES analyzer via a cable for impedance analysis. The data from the each well was exported to the computer, which uses the RT-CES software to perform various analysis. Briefly, cells seeded into an E-plate are exposed to a constant electric current at the cell-electrode interphase and the change in impedance in each well is measured by the RT-CES analyzer at three different frequencies 10 kHz, 25 kHz, 50 kHz. The impedance measurement (Z) is converted by the system into resistance (R), a frequency-dependent parameter, using the equation below:

$$Z = R + jx$$

Where, X is the reactance and j the square root of -1. R-values are then converted to cell index (CI); a unitless parameter calculated using the following equation [23, 29, 30, 45, 46],

$$CI = \max_{i = 1,\ldots, N} \left[\frac{R_{cell}(f_i)}{R_o(f_i)} - 1 \right]$$

R_o (f_i) represent the resistance of the microelectrode/solution interface without cell attachment and R_{cell} (f_i) represent resistance of the microelectrodes when cells are attached [30, 46]. When applicable, CI values were normalized at the time of treatments (performed by RT-CES software). At the chosen time, all values were set to NCI of 1.0 (100 % values) as this provides an equal platform to compare the effect of treatments [44]. Therefore, an increase or decrease in NCI directly relates to changes in cell attachment, spreading, morphology and/or density in response to a treatment [21, 29, 30, 44, 46].

3.2.4 Determination of HUVECs seeding density

A 96-well E-plate (ACEA Bioscience, San Diego, CA) was pre-treated with 20 µg/mL fibronectin (F1141, Sigma-Aldrich, Oakville, ON, Canada). To determine the optimal cell seeding density for TEER assay using the RT-CES system, HUVECs suspensions were prepared at concentration of between 10000 to 60000 cells/mL. To begin, 50 µL of EGM-2 was dispensed into each well of the E-plate and baseline impedance measured (background check). HUVECs were thoroughly mixed and a multi-channel pipette used to dispense 100 µL of HUVECs suspensions into each well corresponding to the concentration tested (8 wells/concentration). HUVECs were grown for 7 days (37 °C, 95 % humidity, 5 % CO_2) and culture media changed every other day (< 48 h, final volume of 200 µL). The plate was returned into the workstation and impedance measurements set for every 1 h. Data was displayed on the computer as a graph of CI vs time. The parameter of interest was the time it took for cells monolayers to reach confluency (peak CI).

3.2.5 Determination of TEER assay controls

HUVECs were seeded into the E-plate at a concentration of 3000 cell/well and allowed to grow until the impedance curve plateaued. Triton-X-100 and disodium-ethylenediaminetetraacetic acid (disodium-EDTA, Sigma-Aldrich, Oakville, ON, Canada) were tested as potential positive controls for TEER assay. Triton X is a powerful detergent that causes cell lysis even in small concentration [9, 47] while disodium-EDTA is a powerful chelating agent agent that makes calcium unavailable for use in the formation of adherens junctions that bind endothelial cell together [9, 48]. When used at the right concentration, the treatment effect of these two agents can be seen as a drastic drop in CI thus indicating a loss in cell-cell and/or cell-

substrate attachment resulting in an increase in paracellular permeability [9]. Triton-X solutions at concentration of 0.1 %, 0.5 % and 1 % v/v (n = 8) and disodium-EDTA solutions at concentration of 0.5 %, 2 % and 5 % v/v (n = 12) were prepared in endothelial basal media (EBM-2) at RT. HUVECs monolayers in the wells were treated with controls after several hours of the impedance curve flattening off. Impedance was measured at 1 h interval throughout the experiment. HUVECs monolayers treated with EGM-2 served as negative controls.

3.2.6 Determination of RCC supernatant concentration for TEER assay

HUVECs were seeded into the E-plate at a concentration of 3000 cell/well and grown until the impedance curve plateaued. Day 7 supernatants from three RCC units were thawed and used to prepare three concentrations of RBC supernatants in EBM-2 (undiluted, 1:2 and 1:5 dilutions). HUVECs monolayers were treated with the supernatants (10 wells/concentration) and monitored for changes in TEER every 1 h for 48 h.

3.2.7 Influence of RCC supernatants on endothelial permeability: effect of storage lesion

HUVECs were seeded into the E-plate at a concentration of 3000 cell/well and grown until the impedance curve plateaued. Three RCC supernatants from day 7, 21 and 42 of storage were thawed and each sample diluted (2:3) in EBM-2. HUVECs were treated (8 well/treatment) and impedance measurements taken every 1 hr for 48 h.

3.2.8 Statistical analyses

The number of experimental /treatment conditions as well as the number of wells available for use in each E-plate determined the sample size in each experiment. A minimum of

three replicates for each treatment condition was performed in all experiments. Mixed between-within subject analysis of variance (mixed design ANOVA, SPSS 25.0 software, IBM, Armonk, NY) with Tukey post-hoc analysis was used to compare the treatment effects on endothelial permeability as well as evaluate the interaction between treatment and time effects. Data were expressed as mean ± standard error of the mean (SEM). A p value less than 0.05 was considered statistically significant.

3.3 Results

3.3.1 Optimal HUVECs seeding density

Figure 3.2 shows a graph of CI versus time (h) for HUVECs seeded into E-plates at 1000 - 6000 cells/well. Optimal attachment and growth of HUVECs were achieved through the coating of the culture wells with fibronectin as indicated by the attainment of consistently high peak CI (>8) in all experiments (Figure 3.2 - 3.6). The CI represented the quantitative measure of the growth status in terms of surface coverage as well as the strength of cell attachment to each other as well as to the substrate at any given time point [9]. Overall, HUVECs showed similar growth characteristics irrespective of the initial seeding density. The time after which the cells attained a peak CI corresponded to the start of the plateau phase. HUVECs seeded at 1000, 2000 and 3000 cells/well took longer time to attain confluency (peak CI of 9.8 ± 0.2 at 108 h, 10.0 ± 0.4 at 96 h and 9.9 ± 0.3 at 91 h respectively) compared to HUVECs seeded at 4000, 5000 and 6000 cells/well (peak CI of 9.8 ± 0.3 at 78 h, 9.2 ± 0.3 at 52 h and 8.7 ± 0.3 at 48 h respectively). The time to confluency for HUVECs in TEER assay was determined to be approximately 60 - 100 hours, corresponding to a seeding concentration of 2000 – 4000 cell/well.

3.3.2 The effect of positive control treatments on TEER

The untreated HUVECs monolayers consistently maintained high CI values (Figure 3.3, Figure 3.4). Study showed that untreated HUVEC monolayers maintain a stable CI for more than 150 hours without significant drop in CI. Treatment of HUVEC monolayers with 0.5 % and 1 % Triton X-100 as well as 5 % disodium-EDTA resulted in an immediate drop in TEER to zero within the first hour. Treatment with 0.1 % Triton X-100 resulted in statistically significant decrease in TEER compared to the sham treated negative control (culture media) at 6, 12 and 24 h post-treatment ($p<0.0001$). The interaction between treatment and time effects for Triton X-100 were non-significant (Figure 3.3). Treatment of HUVEC monolayers with 0.5 % and 2 % disodium-EDTA resulted in statistically significant decrease in TEER in a concentration dependent manner at 6, 12 and 24 h post-treatment ($p<0.0001$, Figure 3.4). There was a statistically significant interaction between treatment and time effect ($p<0.0001$).

3.3.3 Dose response effect of RBC supernatants on endothelial permeability

The average NCI for the negative control remained consistently high over time (Figure 3.5). There was no statistically significant effect in TEER for HUVEC monolayers treated with 1:5 dilution of RCC supernatant versus the negative control at 6 h, 12 h, 24h and 48 h post-treatment. Treatment with 1:2 dilution of RCC supernatant did not result in statistically significant decrease in endothelial permeability compared to treatment with 1:5 dilution of RCC supernatant at all time points except at 48 h ($p<0.01$). Treatment with 1:2 dilution of RBC supernatants resulted in statistically significant increase in endothelial permeability compared to the negative control at 12h ($p=0.0017$), 24 h ($p<0.0001$) and 48 h ($p<0.0001$) post-treatment. Treatment with undiluted

RCC supernatants resulted in statistically significant increase in endothelial permeability compared to all other treatments at all time points (except against the positive control at 6 h $p<0.001$). Analysis showed a statistically significant interaction between treatment and time effects ($p<0.0001$).

3.3.4 Influence of RCC supernatants on endothelial permeability: effect of HSL

The average NCI for the negative control remained consistently high over time (Fig 3.6). Paired multiple comparison showed that day 42 RBC supernatant treatment resulted in statistically significant higher NCI compared to treatment with both the culture media (negative control, $p=0.02$) and day 7 RBC supernatants ($p<0.01$) at 6 h. Treatment with day 7 RBC supernatants resulted in statistically significant increase in endothelial permeability compared to the negative control ($p<0.01$), day 21 ($p=0.019$) and day 42 ($p<0.01$) RBC supernatant treatments at 12 h. Similarly, treatment with day 7 RBC supernatants resulted in statistically significant increase in endothelial permeability compared to treatment with day 42 RBC supernatant treatment at 24 h ($p=0.034$). Results indicate a statistically significant increase in endothelial permeability following treatment of HUVEC monolayers day 42 RCC supernatants compared to treatment with both day 21 and day 7 RCC supernatants at 48 h ($p<0.001$). In addition, treatment of HUVEC monolayers with day 21 versus day 7 RCC supernatants resulted in a statistically significant increase in endothelial permeability at 48 h post-treatment ($p<0.01$). The treatment and time interaction was found to be statistically significant ($p<0.001$).

To summarize the key results of this study, the optimum seeding density was found to be 2000 – 4000 cells/well. In addition, the concentrations of Triton X and disodium-EDTA all resulted

in a statistically significant increase in endothelial permeability in a concentration dependent manner. 1:2 but not 1:5 dilution of RCC supernatants resulted in moderate but statistically significant increase in endothelial permeability whereas undiluted RCC supernatants resulted in the most damaging effects compared to all treatment (including positive control). Based on these results it was decided that future TEER experiment would be conducted using 3000 cells/well as the seeding density while 0.5 % v/v disodium-EDTA was selected as a positive control treatment. It was further decided that 2:3 dilution of RCC supernatants would be used to test the effect of stored RCC supernatants as described in Section 3.3.4 as well in experiments described in Chapter 4.

3.4 Discussion

Studies show that RBC units meant for transfusion differ greatly from each other when various quality aspects are considered [34, 49]. This is partly due to unique donor characteristics as well as the cumulative effect of manufacturing processes and HS [49]. The endothelium had been thought in the past to be an inert anatomical structure and, only performed the role of a semi-permeable barrier between blood and tissues [50]. It has now been appreciated that it plays a crucial role in the immune system as they express various receptors and are able to bind to immune cells as well as secrete cytokines and chemokines *in-vivo* [6-8, 50]. Since an intact endothelial barrier is essential for the overall functioning of the immune system, the current study sought to employ TEER measurement as viable indicator in assessing the potential of stored RCC supernatants to modulate endothelial permeability *in-vitro*.

Little is known about the role of the initial seeding density of cells on the growth and functionality of cell lines. One study showed that increasing the seeding density decreases the population doubling time of cancer cell lines and that cells seeded at a lower density tend to produce a normal growth curve than those seeded at a higher densities [51]. To establish the optimum conditions for running a TEER assay, it was essential to determine a suitable initial seeding density of the chosen cell line. HUVECs were chosen as a model for human endothelium because they can be grown to a monolayer and are able to express majority of the junctional protein complexes found in a normal human endothelium [9]. Results indicate that the initial seeding density only determines the time it takes for HUVECs to reach confluency and does not significantly influence the highest attainable CI (cell line specific characteristic) [44]. Therefore, the decision on the best seeding density had to take into consideration the balance between time and space available for cell growth. In the current study, a seeding density of 3000 cells/well was chosen, as it would allow the HUVECs time to mature within the space available. Although growth curves in Figure 3.2 are in agreement with the literature that typical HUVECs growth curve is composed of a lag phase (cell attachment), exponential phase (rapid cell proliferation) and a plateau phase (confluent monolayer), a conspicuous decrease in CI can be seen for HUVECs seeded at 6000 cells/well after 90 hours of culture, pointing out the importance of choosing an appropriate seeding density [44].

In addition to establishing an optimum density for seeding HUVECs, two compounds were tested for their potential use as positive controls in TEER assay. Triton X-100 is a laboratory grade detergent routinely used to lyse cells to extract proteins and organelles as well as to permeabilize living cells [47]. This study shows that Triton X-100 was instantly lethal to HUVECs at a

concentration of above 0.1 % v/v (Figure 3.3). This is consistent with previous studies showing 0.1 % Triton X-100 is enough to lyse cells leading to the loss of attachment to the extracellular matrix resulting in the drastic drop in TEER [9]. On the other hand, Disodium-EDTA is chelating agent with both industrial and medicinal uses [48]. In the current study, EDTA is specifically important because of its calcium chelation properties. Cadherins are calcium-dependent cell adhesion molecules (CAMs) that are essential for the formation of adherens junctions that bind endothelial cells together [52]. As such, treatment of HUVECs monolayers with EDTA interferes with the connection between cells leading to increased endothelial permeability. Although Triton X-100 and disodium EDTA possess different mechanism of action regarding the modulation of the TEER, they are both toxic to HUVECs at high concentrations and vice versa. In further studies, disodium-EDTA was used as positive control because its mode of action relates more to the interference of the endothelial barrier integrity.

Further, this study sought to evaluate whether HUVECs monolayer would show a dose response when treated with various dilutions of stored RBC supernatants. Figure 3.5 show that treatment with undiluted RBC supernatants resulted in significant increase in endothelial permeability compared to diluted supernatants. This can be explained that dilution reduces the concentration of EVs, cytokines and other bioactive compounds present in supernatants thus alleviating the adverse effects on the endothelial barrier integrity. However, the fact that 1:2 versus 1:5 dilution of RBC supernatants did not show a significant TEER reduction could imply that other factors other than the simple act of dilution played a role. One possible explanation is that there could be an interaction between the diluent (EBM) and RBC supernatants that results in significant lose in potency of the diluted RBC supernatants. This finding could also imply that a

there is certain minimum dosage or concentration of RCC treatment below which endothelial permeability is not significantly altered. From a transfusion point of view, it could also shed light on how massive transfusion negatively affect the endothelial function.

Considering that RBCs are transfused directly into the vein of the recipient, it plausible to assume that the endothelium would be the primary casualty of any potential adverse effect of RBC transfusion. An alteration of endothelial barrier function can result from the action of inflammatory cytokines, pathogens or activated blood cells leading to complex pathophysiology such as edema, lowered microcirculatory perfusion or worse, organ dysfunction and systemic shock [6-9]. TEER assay was used to assess the effect of HSL on RBCs and to test the hypothesis that the RBC HS length contribute to modulation of endothelial permeability *in-vitro*. As expected, the measurement of TEER was sensitive enough to show the difference in RBC stored for 7, 21 and 42 days (Figure 3.6). The effect of HSL on RBC quality has been implicated in endothelial dysfunction following transfusion of old RCCs [2, 53, 54]. It has been suggested that increase in RBC hemolysis during HS result in accumulation of free and EV-enclosed hemoglobin that scavenge nitric oxide (NO), an important regulator of vascular tone [10]. The reduction in NO bioavailability leads to increased vasospasm and vasoconstriction and, has been implicated in worse cardiovascular outcomes in some patients [2, 10]. Similarly, heme has been shown to stimulate the expression of cell adhesion molecule (CAMs) on endothelial cells, which is a sign of endothelial activation and inflammation, facilitating recruitment and extravasation of leukocytes to extravascular environment [55-57]. In addition to the potential effect of hemoglobin and its derivatives, previous study suggested that EVs from residual leukocytes and platelets were responsible for the pro-inflammatory effect of fresh RCCs whereas EV accumulating during HS

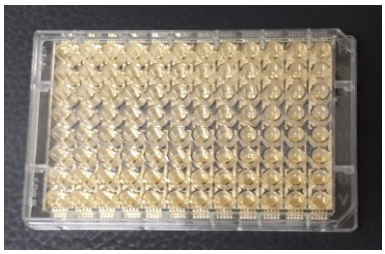
could be responsible for the immune modulatory effects of old RCCs [58]. Figure 3.6 shows that at 48 hours post-treatment, endothelial permeability was directly influenced by the RCC storage length, which is consistent with progression of HSL [35].

3.5 Conclusion

This described study tested the basic elements that are essential for running a TEER assay for transfusion and biobanking application. TEER assay showed adequate sensitivity to various control and RBC supernatant treatments supporting its potential use in assessing the role of RBC HSL in immune modulation. One limitation of the current study is that other factors that could influence reproducibility of the assay such as day/time of running experiments, different batches of HUVECs and the length of RBC supernatant storage were not investigated. This aspect was limited by availability of RCC samples for testing. Regardless, this assay provides a unique opportunity to evaluate the immune modulation potential of not only RBC products, but also platelet and plasma products by their ability to alter endothelial permeability *in-vitro*. It offers the benefit of monitoring behavior of cells in a real-time and label-free manner for extended period while comparing different treatment conditions. In chapter 4, the TEER assay will be used to assess the contribution of other factors such as donor age and sex to the immune modulation potential of stored RCC supernatants.

Figure 3.1 - Components of the xCELLigence real-time cell analysis system (ACEA Biosciences) include the 96-well E-plate, RT-CES workstation (holds the 96-well E-plate - B), an RT-CES analyzer and a RT-CES control unit (C). Pictures taken by Betty Kipkeu.

A)



96-well E-plate

B)

C)

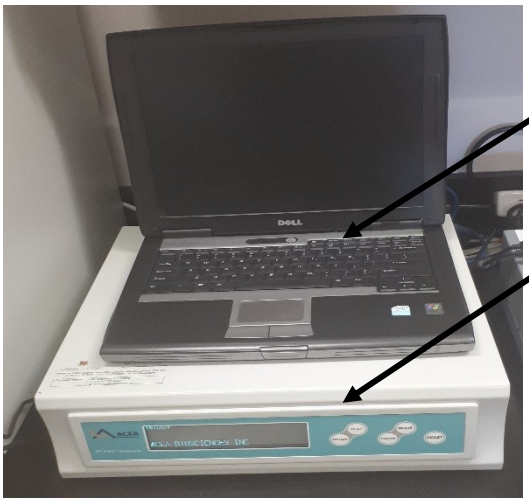


Figure 3.2 – Cell indices versus time showing the growth curves of HUVECs that were grown to confluency from initial seeding densities of between 1000 to 6000 cells/well (n = 8). The arrows show the time and effect of media change during the experiments.

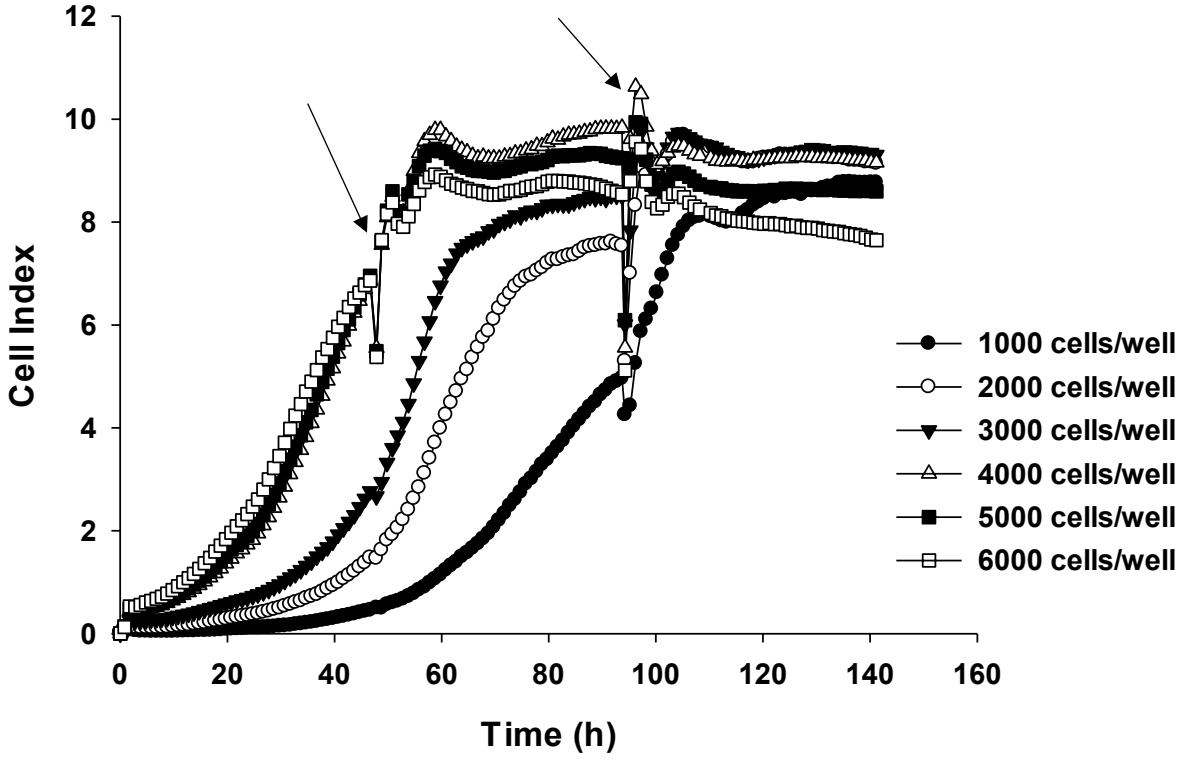


Figure 3.3 - Normalized cell indices versus time showing the treatment effect of various concentration of Triton X-100 on HUVEC TEER. Data represent the mean ± SEM (n = 8).

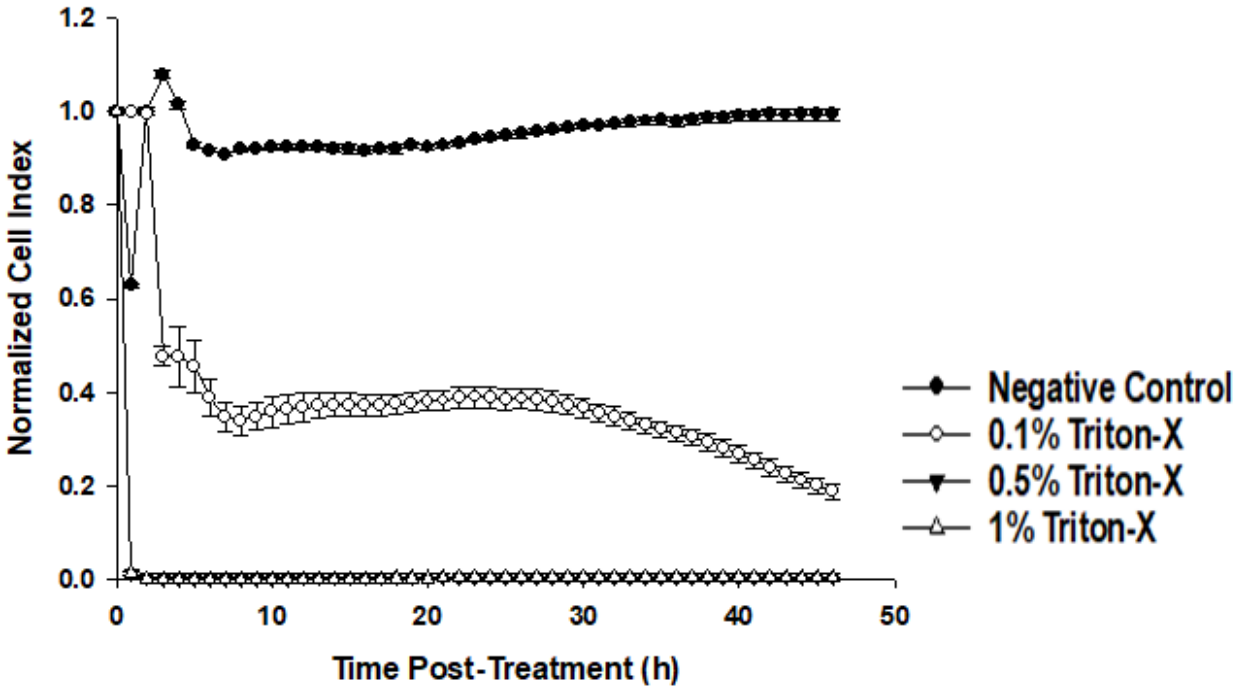


Figure 3.4 - Normalized cell indices versus time showing the treatment effect of various concentration of disodium -EDTA on HUVEC TEER. Data represent the mean ± SEM (n = 12).

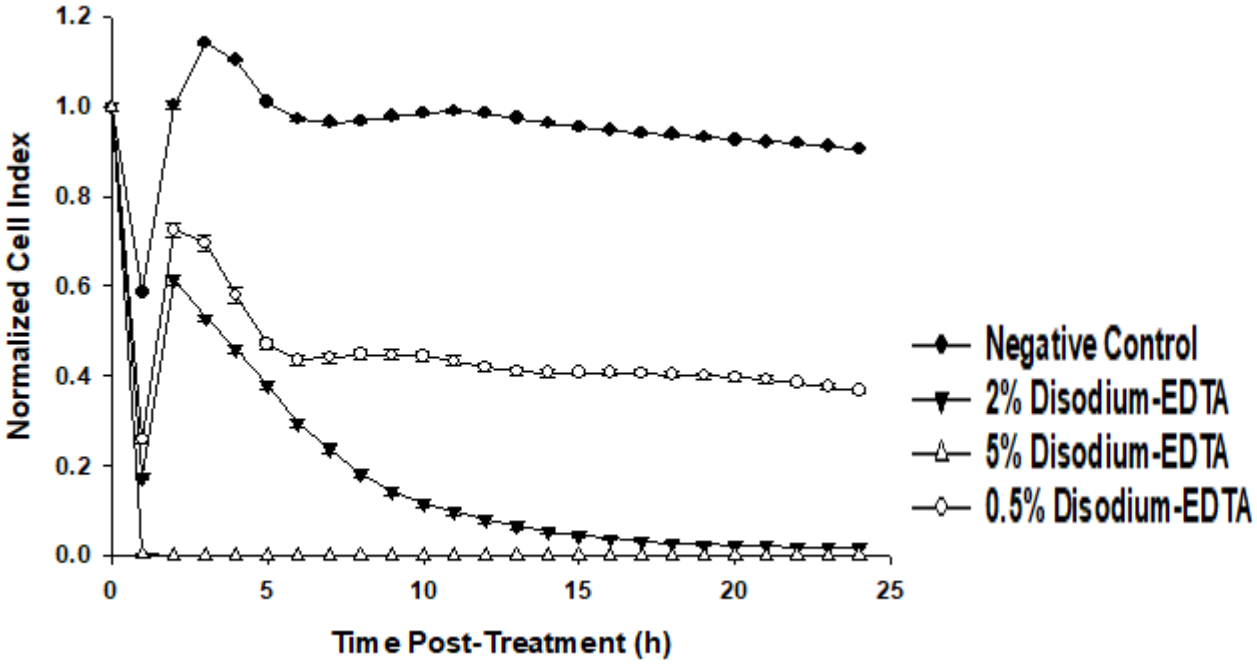


Figure 3.5 - Normalized cell indices versus time showing changes in endothelial permeability for HUVECs monolayers treated with undiluted, 1:2 and 1:5 dilution of RCC supernatants. Data represent the mean ± SEM (n = 10).

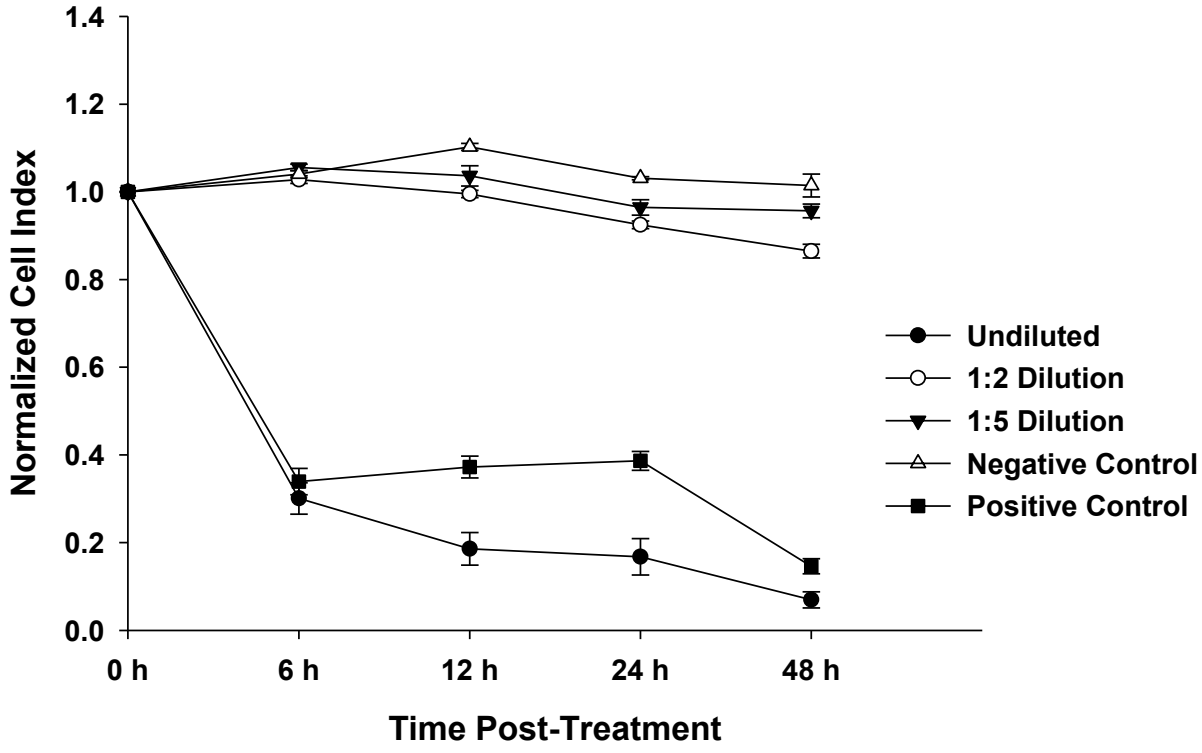
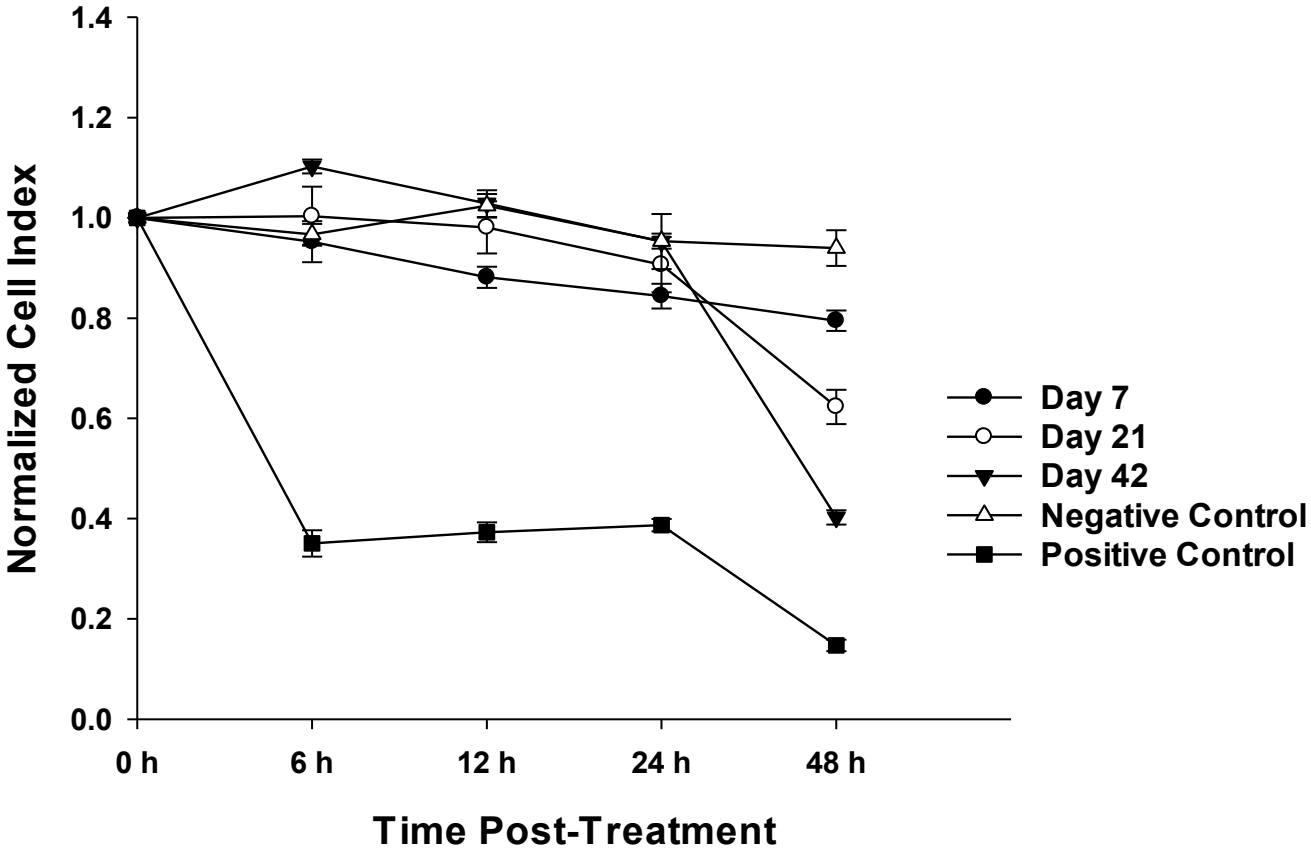


Figure 3.6 - Normalized cell indices versus time showing the influence of RBC supernatants stored for 7, 21 or 42 days on HUVEC endothelial permeability. Data represent the mean ± SEM (n = 3).



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Chapter 4

Evaluation of the contribution of donor age and sex to the immune and endothelial modulation potential of stored RCC supernatants[1]

[1]This chapter with minor modifications has been published as : Kipkeu BJ, Almizraq RJ, Branch DR, Acker JP, Holovati JL. Red Cell Supernatant Effects on Endothelial Cell Function and Innate Immune Activation is Influenced by Donor Age and Sex. 2018. Available at: https://onlinelibrary.wiley.com/doi/10.1111/voxs.12472.

4.1 Introduction

In Canada, red blood cells (RBCs) destined for transfusion are routinely assessed for storage hemolysis (should not exceed 0.8 % at the end of 42 days of storage) as well as post-transfusion recovery (75 % of transfused RBCs must remain in the circulation 24 hours post-transfusion) [1-4]. This is to ensure that RBCs meet the standards for safety and efficacy like any other drug [4, 5]. Although majority of RBC products meet the above criteria at any point in hypothermic storage (HS), differences in various in-vitro quality aspects is evident [1, 5]. For instance, differences in RBC storage hemolysis has been attributed to unique donor characteristics such as age, sex, ethnicity, and heritable genetic traits [6-8]. These genetic variations among humans could significantly influence the extent of hypothermic storage lesion (HSL) resulting in RBC products with different *in-vitro* quality described in Chapter 1 [3, 6-11].

A current concern in transfusion medicine is the association between donor factors and adverse patient clinical outcomes following blood transfusions. A widely documented adverse clinical transfusion outcome is the occurrence of transfusion associated acute lung injury (TRALI) resulting from transfusion of plasma products from female donors [12-14]. Retrospective studies have shown that donor-recipient sex mismatch [13, 15-18] and/or age [18] of the blood donor is associated with an increased risk of mortality. In one study, it was found that receiving a red cell concentrate (RCC) product from young (**<** 45 years) female blood donor was associated with up to an 8 % increase in the risk of death for each unit transfused compared to receiving an RCC transfusion from a male door [15]. The findings of this study was however challenged by Edgren *et al.* (2017) who found no association between donor age or sex with patient survival and, pointed out that results from Chase *et al* was significantly influenced by non-adjustment for

confounding factors including the number of transfused units [19, 20]. As this observation has not been seen in retrospective analysis of French or Scandinavian patient cohorts [20-22], it has been proposed that other factors such as differences in the blood product manufacturing method or donor demographics may be responsible [23].

RCC transfusion has also been shown to modulate the recipient immune response in what has now been termed as transfusion related immune modulation (TRIM) [12, 24]. Forms of TRIM encompass immune suppression (i.e. reduced chances of allograft rejection in kidney transplants) [25, 26] and immune activation or pro-inflammatory responses (i.e. increased bacterial infection recurrence and cancer metastases) [26-28]. While a reduction in the quality characteristics of RCC as a result of donor characteristics, component manufacturing method or HS may contribute to TRIM, the mechanisms involved in TRIM are multifactorial [24]. Current TRIM studies focus on the influence of RCC transfusion on the vascular endothelium and the immune system as a whole. As an important component of the immune system, the vascular endothelium can be activated by cytokines or endotoxins in inflamed tissues resulting in changes in the expression of endothelial cell adhesion molecules (CAMs) which permit the adhesion and migration of circulating leukocytes [29, 30]. Changes in RBC characteristics and the presence of extracellular vesicles (EVs), free hemoglobin, vasoactive lipids, cytokines and other biologically active agents in stored RCCs has been shown to contribute to endothelial dysfunction [31, 32]. For instance, the increased accumulation of free hemoglobin in stored RCC scavenge nitric oxide (essential for maintenance of vascular tone) and could result in endothelial dysfunction [9, 10, 33, 34] while changes in red cell EVs are capable of inducing production of pro-inflammatory cytokines and

chemokines in peripheral blood mononuclear cells (PBMCs) [35-38] and stimulate T cell proliferation *in vitro* [39]

As donor characteristics play a significant role in pre-storage and post-storage quality of RCC units, these differences could have potent immunomodulatory and vascular disruptive effects. Thus, the aim of this study aims was to investigate the impact of donor age and sex on RCC characteristics as well as the effect of RCC supernatants on the vascular endothelium and the immune system. Previously developed models of the endothelium and the innate immunity were employed to assess the release of cytokines from monocytes and HUVECs, expression of cell adhesion molecules (CAM) by HUVECs as well as the modulation of endothelial permeability following treatment with RCC supernatants *in-vitro*.

4.2 Materials and methods

Figure 4.1 shows the study design for this chapter. All samples were tested in replicates.

4.2.1 Manufacturing of RBC components and preparation of RCC supernatants

Both the Canadian Blood Services (CBS) and University of Alberta Research Ethics Boards (protocol number 2015.032 and Pro00059754 respectively) granted ethics approval for this study. RCC and buffy coat (BC) components were manufactured from whole blood using the BC method (netCAD, Vancouver) as described in Chapter 2 (Section 2.2.1). Sixteen individual RCC units in saline-adenine-glucose-mannitol (SAGM) additive solution were randomly selected into four groups based on donor age and sex namely, male ≤ 30 years old (yo) (n = 4), male ≥ 60-yo (n = 4), female ≤ 30-yo (n = 4) and female ≥ 60-yo (n = 4). RBC units were stored at 1 – 6 ºC for 42

days and sampled aseptically at day 7, 21 and 42 of storage as described in Chapter 3 (Section 3.2.1). Three ABO/Rh matched BC components (48 h post-collection) were held at room temperature (RT) and used in the isolation of peripheral blood mononuclear cells (PBMCs) for MMA assay as described in in Chapter 2 (Section 2.2.3). Cell-free RCC supernatants were prepared as described in Chapter 3 (Section 3.2.1). All supernatants were stored in -80 ºC for 2 - 8 months and were thawed at RT before use in experiments.

4.2.2 Assessment of RBC *in-vitro* quality

4.2.2.1 Hemolysis

The breakdown of RBC cell membrane leads to the release of free hemoglobin (Hb) into the solution in which RBCs are suspended. The amount of free Hb was measured spectrophotometrically using the cyanmethemoglobin (Drabkin's) method as previously described [1]. To obtain the total Hb (Hb_T), each RBC sample was mixed by inversion and 5 µL of RBCs mixed with 1 mL Drabkin's solution (containing 0.61 mM potassium ferricyanide, 0.77 mM potassium cyanide, 1.03 mM sodium dihydrogen phosphate and 0.1 % Triton X-100, Sigma-Aldrich, Oakville, ON, Canada). For supernatant Hb (Hb_S), The remaining sample was centrifuged (2200 × *g*, 10 min, 4°C) and 40 µL of the supernatant mixed with 1 mL of Drabkin's reagent. Samples were incubated in the dark at RT for at least five minutes. Reactions involves the conversion of Hb to methemoglobin by potassium ferricyanide followed by conversion of methemoglobin to cyanmethemoglobin (HiCn) by potassium cyanide. The absorbance of HiCn was measured using a spectrophotometer (SPECTRAmax PLUS 384, Molecular Devices

Corporation, Sunnyvale, CA, USA) at 540 nm wavelength and this absorbance was directly proportional to the Hb concentration in the suspension, calculated following the equation below:

$$C = \frac{A540 \; x \; M \; x \; F}{\varepsilon 540 \; x \; l \; x \; 1000} \qquad \textbf{Eqn. 4.1}$$

Where, C = concentration of Hb (g/L), A540 = absorbance of the solution at 540 nm, M = molecular mass of Hb monomer (16114.5 mg/mmol), F = dilution factor, ε540 = extinction coefficient of HiCN at 540 nm (11.0 cm-1·mM-1) and l = light path in cm. Hematocrit (Hct) was determined visually after the centrifugation of RBC-filled capillary tubes (14,850 × *g*, RT, Haematokrit 2010, Andreas Hettich GmbH & Co., Tuttlingen, Germany) as previously described [1]. Finally, percent hemolysis was calculated as a ratio of Hb_S to Hb_T adjusted for Hct using the equation below:

$$\textbf{Hemolysis (\%)} = \{(1 - \textbf{Hct}) \times \textbf{Hb}_S \textbf{ (g/L)}\} \; / \; \textbf{Hb}_T \textbf{ (g/L) x 100} \qquad \textbf{Eqn. 4.2}$$

Three-level hemoglobin control (low, medium and high ranges) were used as a standard for HbT (Stanbio Laboratory, Boerne, TX) as previously described [1].

4.2.2.2 Hematological indices

An automated hematological analyzer (Coulter AcT 8, Beckman Coulter, CA USA) was used to assess RBC indices as previously described [1]. The RBC indices included the Hb concentration, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular

hemoglobin concentration (MCHC). MCV is the average volume of individual RBCs and is expressed in femtolitre (fL). MCH is the average weight of hemoglobin in individual RBCs, expressed in picograms (pg) and is calculated by dividing Hb concentration (g/L) by RBC count (RBCs/L). MCHC, expressed in grams/decilitre (g/dL), is the average amount of hemoglobin (hemoglobin concentration) per RBC. MCHC is calculated by dividing Hb concentration (g/L) by the Hct. The analyzer has a sensing zone consisting of an aperture placed between two electrodes and a current path introduced by a low-concentration electrolyte. As a cell passes through the aperture, a volume of electrolyte-containing solution equivalent to the immersed volume of the cell is displaced from the sensing zone causing a short-term change in impedance across the aperture. This impedance can be measured as a voltage or current pulse and is proportional to the volume of the detected cell. The number of pulses detected by the equipment determines the number/concentration of cells in a sample. The equipment uses the HiCn method to determine the HbT concentration.

4.2.3 Treatment of monocytes with RCC supernatants

PBMCs were isolated from three BCs, pooled, cryopreserved in 20 % dimethyl sulfoxide (DMSO, Sigma-Aldrich, Oakville, ON, Canada) and stored in liquid nitrogen (-196 ˚C) as described in Chapter 2 (Section 2.2.3). The PBMCs had been stored for 1 year (-196 ˚C) the time of use in experiments. Monocyte monolayers from cryopreserved BC PBMCs for monocyte monolayer assay (MMA) were prepared as described in Chapter 2 (Section 2.2.7). Monocytes were treated with 1 mL of day 7, day 21 or day 42 RCC supernatants (20 % v/v in culture media) followed by 4-h incubation (37 °C, 5 % CO_2). The 20 % v/v of RCC supernatants chosen was an approximate

volume to weitht ratio of a 20 mL/kg RCC transfusion [40]. To evaluate the phagocytic function of the cryopreserved BC-derived monocytes, monocyte monolayers were incubated for 2 h with 1mL of 5 % anti-D-sensitized group O Rh positive RBCs. Coverslips were washed in physiological buffered saline (PBS), stained (Hema 3 stains, Fisher Scientific, Kalamazoo, MI, USA) and examined microscopically for phagocytosis. The phagocytosis index (PI) was determined as the number of fully phagocytosed RBCs per 100 monocytes. Positive control for cytokine/chemokine secretion included monocytes incubated with 1 mL of 10 µg/ mL lipopolysaccharide (LPS) suspensions from *E. coli* (serotype 055; B5, Sigma-Aldrich, Oakville, ON, Canada). Monocytes incubated with 1 mL culture media served as negative controls. Monocyte cell culture supernatants were prepared for cytokine analysis as described in chapter 2 (Section 2.2.7).

4.2.4 Treatment of human vein endothelial cells (HUVECs) with RCC supernatants

HUVECs were cultured from primary cell (C2519A, Lonza Group Ltd., Walkersville, MD) as previously described in Chapter 3 (Section 3.2.2) [41, 42]. Following trypsinization, HUVECs (passage 2) at a concentration of 0.2 $\times 10^6$ cells/mL were seeded into 12-well flat-bottom culture plates and incubated for 24 h (37 °C, 5 % CO_2) in endothelial growth medium-2 (EGM-2, CC- 3162, Lonza Group Ltd., Walkersville, MD, USA). The media was then replaced with endothelial basal medium (EBM, CC- 3156, Lonza Group Ltd., Walkersville, MD, USA) + 1 % fetal bovine serum-FBS (F1051, Sigma-Aldrich, Oakville, ON, Canada) followed by 24 h incubation. HUVECs were treated with day 7, day 21 or day 42 RCC supernatants (20 % v/v in EBM + 1 % FBS) for 24 h. Positive control for cytokine/chemokine secretion as well as the expression of cell adhesion molecules (CAMs) included HUVECs incubated with 20 µg/ mL LPS suspensions from *E. coli* (serotype 055;

B5, Sigma-Aldrich, Oakville, ON, Canada). HUVECs incubated with EBM + 1 % FBS served as the negative controls. HUVECs cell culture supernatants were prepared for subsequent cytokine analysis as described in Chapter 2 (Section 2.2.7).

4.2.5 Flow cytometry analysis of the expression of CAMs by HUVECs

Analysis of the expression of CAMs by HUVECs was performed as previously described [42]. HUVECs monolayers were washed with PBS and harvested using StemPro Accutase (A1110501, Gibco by Life Technologies, Grand Island, NY, USA). HUVECS were washed with staining buffer (0.1 % bovine serum albumin in PBS - Sigma-Aldrich, Oakville, ON, Canada). HUVECs were stained with commercial antibodies (BD Pharmingen, San Diego, CA, USA) against three endothelial CAMs namely cluster of differentiation- 31 (CD-31; endothelial cell marker), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin. The antibodies included fluorescein isothiocyanate (FITC) anti-human CD31 (CD31), phycoerythrin (PE) anti-human CD106 antibody (VCAM-1) and allophycocyanin (APC) anti-human CD-62E (E-selectin). To control for non-specific binding of the antibodies, commercial mouse IgG1 isotype controls for FITC, PE and APC labeled antibodies (BD Pharmingen, San Diego, CA, USA) were used. Cells were incubated in the dark for 15 min at RT, washed once in staining buffer and fixed with 10 % of 36-38 % formaldehyde (F8775, Sigma-Aldrich, Oakville, ON, Canada) in PBS. Fixed cells were analyzed the following day using a FACSCanto II flow cytometer and FACSDiva computer software (BD Biosciences, San Jose, CA, USA). For each sample, 10000 CD-31 positive cells were analyzed for the percent expression of both VCAM-1 and E-selectin as previously described [42].

4.2.6 Cytokine/chemokine analysis

Luminex technology using MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panels (EMD Millipore, Toronto, ON, Canada) was used to assess the concentration of 10 cytokines/chemokines secreted into monocyte and HUVECs culture supernatants as previously described in Chapter 2 (Section 2.2.8) [42, 43]. The cytokines/chemokines included granulocyte-monocyte-colony-stimulating factor (GM-CSF), interferon-gamma (IFN-γ), interleukin (IL)-1-beta (IL-1β), IL-4, IL-6, IL-8, IL-10, IL-12p70, tumor necrosis factor-alpha (TNF-α), and monocyte chemoattractant protein -1 (MCP-1).

4.2.7 Trans-epithelial electrical resistance (TEER) assay

HUVECs were cultured from primary cell (C2519A, Lonza Group Ltd., Walkersville, MD) as previously described in Chapter 3 (Section 3.2.2) [41, 42] while TEER assay was performed using passage 2 HUVECs as described in Chapter 3 (Section 3.2.7.). To assess the influence of RCC supernatants on endothelial permeability, confluent HUVECs monolayers were treated with 200 µL of day 7, day 21 or day 42 RCC supernatants (2:3 dilution in EBM, 4 replicates for each sample) and impedance measured hourly for 48 h. HUVECs monolayers incubated with EGM-2 served as negative controls while those treated with 0.5 % v/v disodium-EDTA were used as positive controls.

4.2.8 Statistical analyses

A non-parametric one-way analysis of variance (Kruskal-Wallis *H* Test, SPSS 25.0 software, IBM, Armonk, NY) was used for pair-wise comparison of the expression (%) of VCAM-1 and E-

selectin by HUVECs, cytokine/chemokine secretion by HUVECs and monocytes based on donor group at different testing point as well as compare the effect of storage length on within each donor group. Mixed between-within subject analysis of variance (mixed design ANOVA, SPSS 25.0 software, IBM, Armonk, NY) with Tukey post-hoc analysis was used to compare the treatment effects on endothelial permeability as well as evaluate the interaction between treatment and time effects. Data were expressed as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM). A p value less than 0.05 was considered statistically significant.

4.3 Results

4.3.1 In-vitro quality of stored RBCs

Percent hemolysis and RBC hematological indices for RCC units obtained from male ≤ 30-yo, male ≥60-yo, female ≤ 30-yo and female ≥ 60-yo are shown in Table 4.1. Results shows no statistically significant difference in hemolysis between groups throughout storage. However, there was a non-statistically significant trend towards increased hemolysis as the storage length increased. All RCC units maintained acceptable hemolysis levels of below 0.8 % at the end of 42-day storage (Canadian Standards Association). Female donors presented with lower, although non-statistically significant hemoglobin values compared to male donors day 7 and day 21. Male donors presented with statistically higher hemoglobin values at day 42 compared to female donors ($p<0.05$). All RCC units showed hematological indices values within the normal reference range for healthy donors [1].

4.3.2 Flow cytometry analysis of the expression of CAMs by HUVECs: effect of donor age and sex

Results from flow cytometry analysis of CAMs expression on HUVECs are shown in Table 4.2. Treatment with the stored RCCs resulted in an increase in the expression of VCAM-1 compared to the negative control for all donor groups ($p<0.05$). There was a statistically significant increase in the expression of VCAM-1 by HUVECs treated with supernatants from male ≥ 60-yo versus younger donor RCCs ($p=0.021$ male ≤30-yo; $p=0.043$ female ≤30-yo) RCCs at day 7. Similarly, treatment with RCC supernatants from female ≥60-yo resulted in a significant increase in expression of VCAM-1 compared to the younger donor RCCs at day 7 ($p=0.021$ male ≤30-yo; $p=0.043$ female ≤30-yo). Treatment with day 21 RCC supernatants from male ≤30-yo resulted in significantly lower expression of VCAM-1 compared to treatment with RCC supernatants from all other donor groups ($p=0.021$ female ≤ 30-yo; $p=0.029$ female ≥60-yo; $p=0.047$ male ≥60-yo). There was a statistically significant decrease in the expression of VCAM-1 following treatment of HUVECs with RCC supernatants from male ≤30-yo versus the rest of donor groups ($p=0.043$ male ≤30-yo; $p=0.033$ female ≤ 30-yo; $p=0.021$ female ≥60-yo) at day 42. There was no statistically significant difference in the expression of E-selectin between groups at day 7 and day 21 although, male ≤30-yo RCC supernatants stimulated the least expression of E-selectin compared to all other donor groups. At day 42, treatment of HUVECs with male ≤30-yo RCC supernatants resulted in significantly lower expression of E-selectin compared to older donors ($p=0.043$ male ≥60-yo; $P=0.021$ female ≥60-yo).

4.3.3 Flow cytometry analysis of the expression of CAMs by HUVECs; effect of HS length

Analysis of the influence of RCC storage length shows a statistically significant decrease in the expression of VCAM-1 from HUVECs following treatment with RCC supernatants from all donor groups at day 42 compared to day 7 ($p<0.05$) and at day 21 compared to day 7 (except for female ≤30-yo, $p<0.05$). There was no consistent increase or decrease in the HUVECs expression of E-selectin following treatment with RCC supernatants from all donor groups as a function of RCC storage duration (Table 4.2).

4.3.4 Influence of RCC supernatants on endothelial permeability: effect of donor age and sex

Treatment with RCC supernatants from all donor groups resulted in statistically significant increase in endothelial permeability compared to the negative control ($p<0.0001$, Figure 4.1, Table 4.3). Treatment of HUVECs with day 7, day 21 and day 42 RCC supernatants from female donors resulted in statistically significant increase in endothelial permeability compared to treatment with male ≤30-yo RCC supernatants at all time points ($p<0.01$). Treatment of HUVECs with day 7, day 21 and day 42 RCC supernatants from male ≥60-yo donors resulted in statistically significant increase in endothelial permeability compared to treatment with male ≤30-yo RCC supernatants at all time points (except for 12-h post-treatment, $p<0.01$). There was a statistically significant increase in endothelial permeability following treatment with day 21 and day 42 RCC supernatants from male ≥60-yo versus female ≤30-yo RCC supernatants ($p<0.01$). Treatment of HUVECs with day 21 and day 42 RCC supernatants from female ≥60-yo donors resulted in statistically significant increase in endothelial permeability compared to treatment with female

≤30-yo RCC supernatants at 6 h and 12 h post-treatment ($p<0.01$). Overall, treatment of HUVECs monolayers with male ≤30-yo RCC supernatants had the least effect on endothelial permeability.

4.3.5 Influence of RCC supernatants on endothelial permeability: effect of HS length

Statistical analysis of the TEER data show a statistically significant interaction between treatments and time (Table 4.3). This was shown by the consistent increase in endothelial permeability with time post-treatment. Treatment with day 42 RCC supernatants from all donor groups (except male ≤30-yo) resulted in increased endothelial permeability compared to treatment with day 7 RCC supernatants at 6 h and 12 h post-treatment ($p<0.05$).

4.3.6 Monocytes cytokine/chemokine secretion: effect of donor age and sex

The viability of pooled cryopreserved PBMCs from BCs was 94 % ± 2 while monocytes incubated with anti-D-sensitized RBCs resulted in a PI of 74 ± 4. Release of 10 cytokines/chemokines in monocytes treated with RCC supernatants from male ≤30-yo, male ≥60-yo, female ≤30-yo and female ≥60-yo were assessed; 4 were below the detection limit for the assay (Table 4.4). There was no statistically significant difference in secretion of IL-8 and MCP-1 in monocytes treated with RCC supernatants from all donor groups at day 7. Treatment with male ≥60-yo RCC supernatants resulted in significantly elevated secretion of IL-1β, IL-6 and TNF-α compared to treatment with male ≤30-yo RCC supernatants at day 7 ($p<0.05$). There was a statistically significant increase in monocyte secretion of IL-6 and TNF-α following treatment with female ≤30-yo versus male ≤30-yo and male ≥60-yo versus female ≥60-yo RCC supernatants at day 7 ($p<0.05$). Treatment of monocytes with RCC supernatants from all donor groups did not

result in significant secretion of IL-1β, IL-6 and IL-10 (below detection limit) at day 21 as well as IL-6 and IL-10 at day 42 (Table 4.4). There was no statistically significant difference in secretion of TNF-α in monocytes treated with RCC supernatants from all donor groups at day 21. Treatment of monocytes with RCC supernatants from female donors resulted in statistically significant increase in IL-8 and MCP-1 compared to treatment with male ≤30-yo RCC supernatants at day 21 ($p<0.05$). There was a statistically significant increase in monocyte secretion of MCP-1 following treatment with male ≥60-yo versus male ≤30-yo RCC supernatants at day 21 ($p<0.05$). There was no statistically significant difference in secretion of IL-1β, TNF-α and MCP-1 in monocytes following treatment with RCC supernatants from all donor groups at day 42. There was a statistically significant increase in monocyte secretion of IL-8 following treatment with male ≥60-yo versus female ≤30-yo RCC supernatants at day 42 ($p<0.05$).

4.3.7 Monocytes cytokine/chemokine secretion: effect of HS length

There was no statistically significant difference in secretion of IL-1β in monocytes treated with RCC supernatants from all donor groups at day 7 versus day 42 (Table 4.5). Treatment of monocytes with RCC supernatants from all donor groups resulted in statistically significant decrease in IL-8 and MCP-1 at day 21 and day 42 compared to day 7 ($p<0.05$). There was a statistically significant decrease in monocyte secretion of TNF-α following treatment with RCC supernatants from all donor groups at day 21 compared to day 7 ($p<0.05$) Treatment of monocytes with male ≥60-yo and female ≤30-yo RCC supernatants resulted in statistically significant decrease in TNF-α at day 42 versus day 7 ($p<0.05$).

4.3.8 HUVECs cytokine/chemokine secretion: effect of donor age and sex

Release of 10 cytokines/chemokines in HUVECs treated with RCC supernatants from male ≤30-yo, male ≥60-yo, female ≤30-yo and female ≥60-yo were assessed; 6 were below the detection limit for the assay (Table 4.4). Treatment with RCC supernatants from female ≥60-yo resulted in statistically significant increase in secretion of GM-CSF, IL-6, IL-8 and MCP-1 in HUVECs compared to treatment with male ≤30-yo RCC supernatants at day 7 ($p<0.05$). There was a statistically significant increase in secretion of IL-6 and IL-8 in HUVECs treated with male ≥60-yo versus male ≤30-yo RCC supernatants at day 7 ($p<0.05$). Significantly elevated secretion of GM-CSF, IL-6 and MCP-1 in HUVECs was observed following treatment with female ≥60-yo versus male ≤30-yo RCC supernatants at day 21($p<0.05$). There was a statistically significant increase in secretion of IL-6 and IL-8 in HUVECs treated with male ≥60-yo versus male ≤30-yo RCC supernatants at day 21 ($p<0.05$). However, HUVECs treated with male ≤30-yo versus male ≥60-yo RCC supernatants secreted significantly higher MCP-1 at day 21 ($p<0.05$). Treatment with RCC supernatants from male ≥60-yo resulted in statistically significant increase in secretion of IL-8 in HUVECs compared to treatment with female ≤30-yo RCC supernatants at day 21 ($p<0.05$). There was no statistically significant difference in the secretion of MCP-1 in HUVECs following treatment with day 42 RCC supernatants from all donor groups. Significantly elevated secretion of GM-CSF and IL-8 in HUVECs was observed following treatment with female ≥60-yo versus male ≤30-yo RCC supernatants at day 42 ($p<0.05$). There was a statistically significant increase in secretion of IL-6 in HUVECs treated with male ≥60-yo versus male ≤30-yo RCC supernatants at day 42 ($p<0.05$). Treatment with RCC supernatants from donors ≥60-yo resulted in statistically

significant increase in IL-8 secretion in HUVECs compared to treatment with RCC supernatants from donors ≤30-yo ($p<0.05$).

4.3.9 HUVECs cytokine/chemokine secretion: effect of HS length

There was no statistically significant difference in secretion of GM-CSF in HUVECs treated with day 7, day 21 or day 42 RCC supernatants within each donor group (Table 4.5). Treatment with RCC supernatants from male ≤30-yo resulted in statistically significant increase in IL-6 secretion in HUVECs at day 42 compared to day 7 ($p<0.05$). Treatment with day 21 and day 42 RCC supernatants from male ≤30-yo resulted in statistically significant increase in MCP-1 secretion in HUVECs compared to day 7 ($p<0.05$). There was no statistically significant difference in secretion of IL-8 in HUVECs following treatment with day 7, day 21 or day 42 RCC supernatants from male ≤30-yo. Treatment with day 42 RCC supernatants from male ≥60-yo resulted in statistically significant increase in HUVECs secretion of IL-6, IL-8 and MCP-1 compared to treatment with day 7 RCC supernatants ($p<0.05$). There was no statistically significant difference in secretion of GM-CSF, IL-6, IL-8 and MCP-1 in HUVECs following treatment with day 7, day 21 or day 42 RCC supernatants from female ≤30-yo. There was no statistically significant difference in secretion of MCP-1 in HUVECs following treatment with day 7, day 21 or day 42 RCC supernatants from female ≥30-yo. Treatment with day 42 RCC supernatants from female ≥60-yo resulted in statistically significant increase in HUVECs secretion of IL-6 and IL-8 compared to treatment with day 7 RCC supernatants; however, there was a reduction in IL-8 secretion in HUVECs following treatment with day 21 compared to day 7 and day 42 RCC supernatants ($p<0.05$).

4.4 Discussion

Even though many studies have demonstrated that donor age and sex significantly influence the pre-storage and post-storage quality of RBC products, it remains unknown whether these differences could translate to distinct *in-vitro* immune profiles or transfusion outcomes in patients. Using an endothelial and innate immunity model systems, this study evaluated the effect of similarly produced RCC supernatants obtained from donors of different age and sex to stimulate the expression of CAMs, modulate endothelial integrity, or stimulate cytokine secretion in monocytes and HUVECs *in-vitro*.

Besides forming a physical barrier between blood and surrounding tissue, the endothelium is an important component of the immune system [44]. Upon activation by cytokines or endotoxins, endothelial cells (ECs) express CAMs that aid in the adhesion of circulating white blood cells (WBCs) allowing their migration into inflamed tissues [29, 30, 44, 45]. Inflammation is generally a protective immunological process aimed at clearance of an infectious agent from the body [29]. However, overexpression of CAMs in healthy tissues can be harmful and could weaken the immune system of an individual [29]. In this study, two endothelial CAMs that play crucial roles in inflammation were assessed. E-selectin (CD-62E) aids in the interaction between endothelial cells and neutrophils while VCAM-1 (CD 106) is involved in monocyte trafficking [29, 30, 44]. Results from this study show a heightened expression of VCAM-1 from HUVECs incubated with day 7 RCC supernatants compared to treatment with day 42 RCC supernatants (Table 4.2). Similarly, while employing MMA as a tool to evaluate the effect of stored RCC supernatants on innate immune function, this study showed an increased pro-

inflammatory cytokine release from monocytes incubated with day 7 compared to day 21 and day 42 RCC supernatants (Table 4.4). These results suggest that fresh RCC supernatants are more pro-inflammatory than supernatants from old RCC products. These results could support reports from recent studies showing that fresh RCC transfusion could result in worse transfusion outcomes in some patients. For instance, one study found a 2-fold increase in mortality for patients receiving fresh versus old RCCs although, it was presented that results of the study largely depended on the statistical model used [46]. Similarly, recent studies show an increased secretion of IL-8 from monocytes co-cultured with fresh (≤7 days) supernatants whole blood filtered (WBF) RCC products [40] while transfusion fresh WBF RCCs was associated with higher in-hospital mortality compared with mid-aged BC RCC products [47]. Cytokines are low molecular mass glycoproteins that participate in inflammation and immunity against infectious agents as well as the regulation of various biologic systems [48]. As previously described in Chapter 2 (Section 2.4), cytokines have numerous pro- or anti-inflammatory functions [48]. Abnormal cytokine production has been associated with pathogenesis of various disorders and its levels has been used clinically to assess the severity and prognosis of inflammation in critically ill patients [40, 48]. Previous studies have demonstrated enhanced CAMs expression on ECs and cytokine release from monocytes following exposure to cytokines [49]. Therefore, this study postulates that residual WBCs and platelets actively secrete cytokines into RCC supernatants during storage. This hypothesis is consistent with results from a study that showed that tumor necrosis factor-alpha (TNF-α) [50] and RANTES (regulated upon activation, normal T cell expressed and secreted) were elevated in fresh RCCs and were thought to promote the progression of cancer following transfusion of fresh RCCs [51].

On the other hand, results from the endothelial model used in this study indicate that old RCC (Day 42) had more disruptive effect on endothelial integrity and stimulated increased cytokine release compared to fresh (Day 7) RCC supernatants. These results are in agreement with the literature suggesting that old RBCs are more immunomodulatory than fresh RCCs [9, 11, 52]. The finding that day 21 RCC supernatants stimulated the least cytokine/chemokine concentration from monocytes (Table 4.4) suggest that opposing factors could modulate immune stimulatory potential of RCC supernatants during HS, including differing concentrations of residual cell, soluble factors, EVs and free hemoglobin at different points in HS. In one study, it was shown that the concentration of TNF-α was highest at day 1 and declined significantly by day 10 of storage in RCCs while those of IL-1 and IL-8 constantly increased with HS [50]. These cytokines are all highly potent stimulators of the immune system and the endothelium and could be responsible for part of immune modulation effects seen in some patients post-transfusion [40, 50]. In addition, a recent study showed that RBCs harbor a number of cytokines which are released upon hemolysis [53]. This could explain the increase in cytokine concentration in RCC supernatants during HS resulting from increased RBC breakdown [43, 50, 51].

The second key finding of this study regards to the effect of donor age and sex on the immune and endothelial modulation of RCC supernatants. This study shows that RCC supernatants from fresh blood products (7 d) collected from older blood donors (≥60-yo) enhance the expression of pro-inflammatory CAMs (Table 4.2) and have enhanced disruptive effect on endothelial integrity (Table 4.3 and Figure 4.1) compared to younger donors (≤30-yo). Additionally, a consistent trend towards lower pro-inflammatory activity following treatment with male ≤30-yo RCC supernatants was observed. This was demonstrated by lower expression

of CAMs throughout storage (Table 4.2), higher NCI values (Table 4.3) as well as reduced cytokines release by monocytes and HUVECs (Table 4.4 and Table 4.5) compared to the other three donor groups. Since donor age is independently associated with increased hemolysis as found in previous studies [6, 54], we postulate that free hemoglobin and heme present at higher amounts in RCC supernatants from older donors is responsible for the heightened endothelial activation that was observed (Table 4.2). Heme has been shown to stimulate the expression of intracellular cell adhesion molecule-1(ICAM-1), VAM-1 and E-selectin from HUVECs *in vitro* [55, 56]. The pro-inflammatory effect of plasma hemoglobin and heme *in vivo* has been shown to involve the consumption of nitric oxide (NO), an important modulator of vascular tone [55, 56]. In summary, it appears that the immunogenicity of stored RCC products cannot be attributed to only one factor but, could be a combination of many. While studies suggest that residual platelets or WBCs are responsible for some of RCC immune modulatory effects [50, 51], the composition of RCC supernatants in terms of cytokines or other soluble mediators could play a significant role and, could explain lower immunogenicity in young male donors although, this aspect was beyond the scope of this study.

Considering the contradicting evidence regarding the immune modulation potential of stored RCCs, it has been suggested that the heterogeneous presentation of TRIM may be similar to RBC alloimmunization, where immunologic consequences of transfusion are highly host and/or clinical context specific [24]. Previously, it has been shown that donor antigen presenting cells (APCs) may lose the potential to induce co-stimulation if stored hypothermically. This results in failure of naive T cells to mount an immune response despite the presence of foreign antigens thus, promoting immunosuppression [57]. With regard to donor characteristics, RCC from male

donors in this study were found have higher hematocrit, total hemoglobin and hemolysis levels compared to RCC from female donors (Table 1) [6, 54]. Multiple groups have reported significantly lower hemolysis in units from premenopausal female donors when compared to donations from other donor populations [6, 7, 54]. In an attempt to explain these differences in RCC product quality, a number of theories have been suggested based on known physiological differences between aging male and female blood donors and their circulating RBCs [7, 58, 59]. Testosterone has been shown to promote erythropoiesis [7], which could explain the increased hematocrit and hemoglobin levels observed in male donors compared to female donors, although this study did find an obvious differences in RBC quality parameters between donor groups to support this theory [6]. Alternatively, it has been suggested that monthly blood loss in premenopausal female donor results in a younger population of circulating RBCs, which are less susceptible to stress and hemolysis during storage [23, 54, 59]. As evidenced by this list of contrasting observations and ideas, it is difficult to come to a consensus on the mechanisms behind the influence of donor sex on RBC storage quality.

4.5 Conclusion

While the majority of the current retrospective studies examining the clinical effects of sex-mismatched transfusions and the role of donor factors focus on the impact of stored RCC products on patient transfusion outcomes, there is a gap in the *in vitro* studies exploring plausible mechanisms for this effect. So far, no study has evaluated the influence of donor age and sex on the immunomodulatory activity of stored RCCs with a specific interest on endothelial function. This study is limited in two ways. First, donor characteristics including, mean age, number of

donations, frequency, hemoglobin or donor weight at time of donation were not provided. Second, the direct mechanisms of RCC-induced endothelial activation or permeability were not studied. However, this study presents tools that offer the possibility of a better understanding of the mechanisms regulating permeability or activation of the endothelium and the innate immune system post-transfusion. Further, the results of this study suggest that closer attention should be paid to the age and sex of the donors providing the RCC products for immunomodulatory studies and the impact donor factors could have in adverse transfusion reactions.

Table 4.1: Hemolysis and hematological indices values for RBC units obtained from four donor groups. RCC units were tested at day 7, day 21 and day 42 of storage. Data represent mean ± SD (n = 4).

Treatment Group	Hct (%)	HbT(g/L)	HbS (g/L)	Hemolysis (%)	MCV (fL)	MCH (pg)	MCHC (g/L)
Day 7							
Male ≤ 30	60.0 ± 1.4	181.7 ± 8.8	0.56 ± 0.08	0.12 ± 0.01	88.1 ± 6.2	28.9 ± 3.2	326 ± 8.2
Male ≥ 60	60.0 ± 1.9	183.4 ± 5.8	0.66 ± 0.17	0.15 ± 0.03	91.2 ± 5.2	29.4 ± 1.3	323 ± 9.8
Female ≤ 30	59.0 ± 2.4	177.3 ± 6	0.57 ± 0.11	0.13 ± 0.01	95.2 ± 2.5	29.8 ± 2.1	318 ± 6.4
Female ≥ 60	59.0 ± 0.8	180 ± 10. 6	0.56 ± 0.07	0.13 ± 0.01	96.6 ± 2.9	30.6 ± 1.2	320 ± 7.3
Day 21							
Male ≤ 30	60.0 ± 1.2	183.5 ± 4.7	0.74 ± 0.05	0.16 ± 0.02	87.2 ± 7.4	28.9 ± 3.1	328 ± 6.4
Male ≥ 60	61.0 ± 1.3	184.0 ± 3.2	0.84 ± 0.12	0.18 ± 0.03	91.7 ± 4.0	29.6 ± 2.0	323 ± 7.7
Female ≤ 30	59.0 ± 1	176.7 ± 5.6	0.72 ± 0.13	0.16 ± 0.03	95.1 ± 0.9	29.1 ± 1.4	316 ± 3.1
Female ≥ 60	58 ± 2.1	182.4 ± 6.7	0.63 ± 0.1	0.16 ± 0.04	94.1 ± 2.3	28.1 ± 2.4	312 ± 7.1
Day 42							
Male ≤ 30	60.0 ± 1.5	185.0 ± 7.1	1.25 ± 0.25	0.27 ± 0.05	88.8 ± 6.8	28.5 ± 2.8	321 ± 7.2
Male ≥ 60	60.0 ± 0.6	186.8 ± 6.7	1.22 ± 0.08	0.27 ± 0.02	93.9 ± 4.0	29.7 ± 1.8	316 ± 7.7
Female ≤ 30	59.0 ± 1	174.3 ± 3.9*	0.82 ± 0.12	0.20 ± 0.02	96.1 ± 1.4	25.6 ± 2.1	305 ± 8.1
Female ≥ 60	58.0 ± 2.0	178.2 ± 2.9*	0.85 ± 0.15	0.20 ± 0.03	97.7 ± 2.5	27.2 ± 3.1	308 ± 6.1

* = Significant difference compared to male donor groups ($p<0.05$).

Table 4.2 - Expression of cell adhesion molecules (CAMs) on HUVECs treated with RCC supernatants at day 7, day 21 and day 42 of storage. Expression scores (%) were normalized to the negative control at each testing point.

Treatment Group	Male ≤ 30	Male ≥ 60	Female ≤ 30	Female ≥ 60	Positive Control
VCAM- 1					
Day 7	14.3 ± 2.4	22.2 ± 2.7^{*1,3}	15.35 ± 4	20.8 ± 1.4^{*1,3}	37.5 ± 2.8
Day 21	3.7 ± 2.1^{*a}	9.6 ± 3.2^{*1a}	10.2 ± 1.1^{*1}	8.3 ± 0.6^{*1,3a}	27.5 ± 0.5
Day 42	3.3 ± 1.7^{*a}	7.3 ± 2.6^{*1a}	8.1 ± 1.6^{*1a}	7 ± 0.5^{*1ab}	19.1 ± 1
E-selectin					
Day 7	1.4 ± 0.6	3.2 ± 1.9	2.4 ± 1.3	1.6 ± 0.6	3.8 ± 1.2
Day 21	1.8 ± 0.8	3.7 ± 2.5	1.5 ± 0.9	2.5 ± 0.8	3.7 ± 0.7
Day 42	1.5 ± 0.3	2.6 ± 1.4^{*1}	1.6 ± 0.6	2.3 ± 0.3^{*1}	2.9 ± 0.07

Data represents the mean ± SD (n=4). *1 = significant difference compared to male ≤30-yo, *2 = significant difference compared to male ≥60-yo, *3 = significant difference compared to female ≤30-yo at each testing point. *a = Significant difference compared to day 7 and *b = significant difference compared to day 21 within each donor group ($p<0.05$).

Table 4.3 – Normalized cell indices of RCC supernatant-treated HUVEC at defined post-treatment times.

Treatment Group			Post-Treatment Time (h) 6	12	24	48
Female	≤ 30-yo	Day 7	0.64 ± 0.08^{*1}	0.46 ± 0.06^{*1}	0.31 ± 0.03^{*1}	0.10 ± 0.01^{*1}
		Day 21	0.53 ± 0.07^{*1a}	0.39 ± 0.03^{*1}	0.26 ± 0.02^{*1}	0.15 ± 0.02^{*1}
		Day 42	0.55 ± 0.12^{*1a}	0.32 ± 0.12^{*1a}	0.17 ± 0.087^{*1a}	0.10 ± 0.07^{*1}
	≥ 60-yo	Day 7	0.81 ± 0.10^{*1,2,3}	0.47 ± 0.02^{*1,2}	0.29 ± 0.01^{*1,2}	0.19 ± 0.02^{*1}
		Day 21	0.30 ± 0.02^{*1,3a}	0.20 ± 0.02^{*1,3a}	0.08 ± 0.01^{*1,3a}	0.02 ± 0.01^{*1a}
		Day 42	0.41 ± 0.17^{*1,3a}	0.24 ± 0.02^{*1,3a}	0.1 ± 0.02^{*1a}	0.05 ± 0.02^{*1a}
Male	≤ 30-yo	Day 7	0.91 ± 0.04	0.62 ± 0.02	0.50 ± 0.03	0.40 ± 0.04
		Day 21	0.86 ± 0.09	0.73 ± 0.13^{*a}	0.52 ± 0.07	0.37 ± 0.06
		Day 42	0.89 ± 0.09	0.62 ± 0.17^{*b}	0.32 ± 0.08^{*ab}	0.22 ± 0.05^{*ab}
	≥ 60-yo	Day 7	0.73 ± 0.01^{*1,3}	0.64 ± 0.03^{*3}	0.37 ± 0.06^{*1}	0.17 ± 0.10^{*1}
		Day 21	0.32 ± 0.02^{*1,3a}	0.22 ± 0.03^{*1,3a}	0.09 ± 0.02^{*1,3a}	0.02 ± 0.01^{*1,3a}
		Day 42	0.42 ± 0.16^{*1,3ab}	0.25 ± 0.04^{*1,3a}	0.11 ± 0.02^{*1ab}	0.05 ± 0.01^{*1,3a}
Negative control		Day 7	1.0 ± 0.02	1.04 ± 0.01	1.04 ± 0.01	0.96 ± 0.01
		Day 21	1.0 ± 0.02	1.05 ± 0.02	1.0 ± 0.01	0.96 ± 0.03
		Day 42	1.0 ± 0.01	1.1 ± 0.0	1.0 ± 0.01	0.93 ± 0.01
Positive control		Day 7	0.25 ± 0.01	0.23 ± 0.02	0.17 ± 0.03	0.02 ± 0.01
		Day 21	0.24 ± 0.01	0.21 ± 0.02	0.13 ± 0.03	0.02 ± 0.01
		Day 42	0.26 ± 0.01	0.23 ± 0.03	0.12 ± 0.05	0.02 ± 0.01

Data represents the mean ± SD (n = 4). *1 = significant difference compared to male ≤30-yo, *2 = significant difference compared to male ≥60-yo, *3 = significant difference compared to female ≤30-yo at each testing point. *a = Significant difference compared to day 7 and *b = significant difference compared to day 21 within each donor group ($p<0.01$).

Table 4.4 - Cytokines/chemokines (pg/mL) secreted by monocytes following incubation with RCC supernatants.

Treatment Group	IL-1β	IL-6	IL-8	IL-10	TNF-α	MCP-1
Day 7						
Male ≤ 30	1.86 ± 1.3	3.48 ± 2.3	585.3 ± 162.9	1.61 ± 0.3	10.96 ± 5	529.7 ± 165.8
Male ≥ 60	6.94 ± 1.9*1	17.37 ± 6.1*1	1078 ± 733.3	2.63 ± 0.8	37.01 ± 10.3*1	637.2 ± 59.3
Female ≤30	5.49 ± 2.8	11.99 ± 4.9*1	994.1 ± 478.4	2.3 ± 0.7	28.72 ± 10.6*1	607 ± 322.3
Female ≥ 60	3.19 ± 2.9	5.43 ± 4.8*2,3	572.2 ± 66.6	1.78 ± 1.1	15.76 ± 9.6*3	544.4 ± 189.5
Negative Control	1.44 ± 1.4	3.58 ± 2.9	255.4 ± 13.41	0.84 ± 0.2	8.19 ± 5.4	69.46 ± 21.6
Positive Control	1065 ± 304.7	582.3 ± 124.3	1266 ± 153.7	12.43 ± 5.1	1872 ± 194	110 ± 23.3
Day 21						
Male ≤ 30	<OOR	<OOR	99.08 ± 33.4*a	<OOR	2.32 ± 1.3*a	84.02 ± 12*a
Male ≥ 60	<OOR	<OOR	137.2 ± 31.5*a	<OOR	2.62 ± 1.3*a	137.3 ± 17.7*1a
Female ≤30	<OOR	<OOR	203 ± 49.6*1a	<OOR	5.69 ± 5.8*a	152.6 ± 70.9*1a
Female ≥ 60	<OOR	<OOR	145.3 ± 21.7*1a	<OOR	1.61 ± 0.3*a	133.4 ± 23.3*1a
Negative Control	1.22 ± 1.4	3.93 ± 2.2	178.2 ± 35.4	0.98 ± 0.3	11.7 ± 0.4	65.05 ± 12.8
Positive Control	201.6 ± 54.8	505.7 ± 125.1	1480 ± 501.8	1.68 ± 0.8	877.8 ± 140	142.98 ± 10.8
Day 42						
Male ≤ 30	1.56 ± 1	<OOR	141.5 ± 83.2*a	<OOR	7.16 ± 5.7	115.1 ± 38.1*a
Male ≥ 60	2.34 ± 1	<OOR	169.2 ± 30.48*a	<OOR	10.35 ± 2.6*a	141.2 ±10.54*a
Female ≤30	1.29 ± 1.1	<OOR	103.2 ± 30.5*1,2a	<OOR	6.08 ± 3.2*a	137.9 ± 27.2*a
Female ≥ 60	2.76 ± 2.6	<OOR	147 ± 44.4*a	<OOR	13.44 ± 10.6*b	157.3± 42.61*a
Negative Control	2.23 ± 0.9	4.59 ± 2.2	175 ± 31.4	0.45 ± 0.1	17.41 ± 5.3	72.15 ± 12.6
Positive Control	390 ± 66.7	880.7 ± 203.8	1801 ± 165	4.94 ± 1.2	1454 ± 261	78.28 ± 19

Data represent mean ± SD (n = 4). *1 = significant difference compared to male ≤30-yo, *2 = significant difference compared to male ≥60-yo, *3 = significant difference compared to female ≤30-yo at each testing point. *a = Significant difference compared to day 7 and *b = significant difference compared to day 21 within each donor group ($p<0.05$). <OOR = out of range below.

Table 4.5 - Cytokines/chemokines (pg/mL) secreted by HUVECs following incubation with RCC supernatants.

Treatment Group	GM-CSF	IL-6	IL-8	MCP-1
Day 7				
Male ≤ 30	1.49 ± 1.5	90.87 ± 12.3	525.82 ± 158.4	4722.2 ± 1168.2
Male ≥ 60	4.12 ± 3.9	156 ± 29^{*1}	970.03 ± 212.7^{*1}	4337.1 ± 2380
Female ≤30	2.84 ± 2.5	137.18 ± 59.6	932.63 ± 561.3	8518.4 ± 3984.9
Female ≥ 60	4.37 ± 1.3^{*1}	203 ± 29.9^{*1}	1420.8 ± 254.1^{*1,2}	9898.7 ± 4358.2^{*1}
Negative Control	OOR<	58.39 ± 2.7	191.32 ± 4.2	3715.4 ± 832.2
Positive Control	230 ± 30.8	810.86 ± 52.2	349 ± 126.6	13577 ± 5893.1
Day 21				
Male ≤ 30	1.79 ± 2.2	83.85 ± 28.4	549.05 ± 214.9	11197 ± 3439.8^{*a}
Male ≥ 60	4.75 ± 4.1	194.49 ± 23.5^{*1}	1501.8 ± 445.1^{*1,3}	6535.2 ± 2655.3^{*1}
Female ≤30	2.96 ± 3.2	143.88 ± 68.4	687.6 ± 265.3^{*2}	7666.3 ± 3530.9
Female ≥ 60	5.38 ± 1.8^{*1}	208.73 ± 38.6^{*1}	857.77 ± 327.1	6202 ± 2530^{*1}
Negative Control	OOR<	58.27 ± 4.5	146.4 ± 26.8	3185 ± 688.77
Positive Control	139.58 ± 5.8	600.27 ± 50.6	1245.2 ± 279 .89	5376.9 ± 3400
Day 42				
Male ≤ 30	3.1 ± 3.8	190.69 ± 62.3^{*ab}	568.71 ± 116.9	8989.8 ± 2046.4^{*a}
Male ≥ 60	6.58 ± 5.3	301.92 ± 41^{*1ab}	2874.3 ± 1285.7^{*1,3a}	7511 ± 968.2^{*a}
Female ≤30	3.05 ± 3.8	193.98 ± 85*	904.27 ± 334.7	5984.3 ± 2392.2
Female ≥ 60	5.83 ± 2.5^{*1}	287.86 ± 69.5^{*a}	3529.1 ± 1589.9^{*1,3a}	7393.7 ± 1387.8
Negative Control	OOR<	60 ± 8.4	173.58 ± 19.8	4028.3 ± 709.6
Positive Control	151 ± 11.5	689.99 ± 73	11244 ± 1224.1	7770.6 ± 2741.2

Data represent mean ± SD (n = 4). *1 = significant difference compared to male ≤30-yo, *2 = significant difference compared to male ≥60-yo, *3 = significant difference compared to female ≤30-yo at each testing point. *a = Significant difference compared to day 7 and *b = significant difference compared to day 21 within each donor group ($p<0.05$). <OOR = out of range below.

Figure 4.1 - Flow chart illustrating the study design for Chapter 4. All samples were tested in replicates.

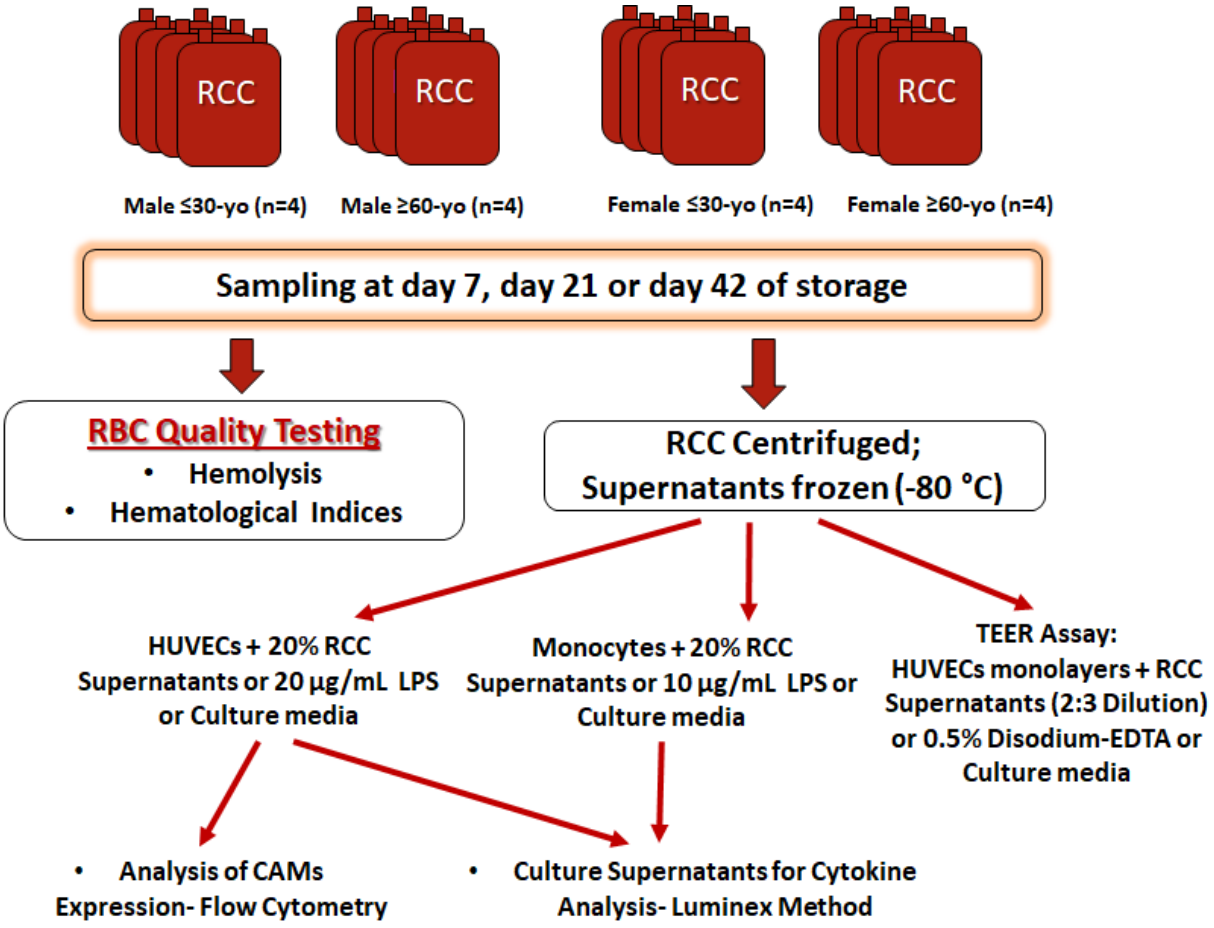
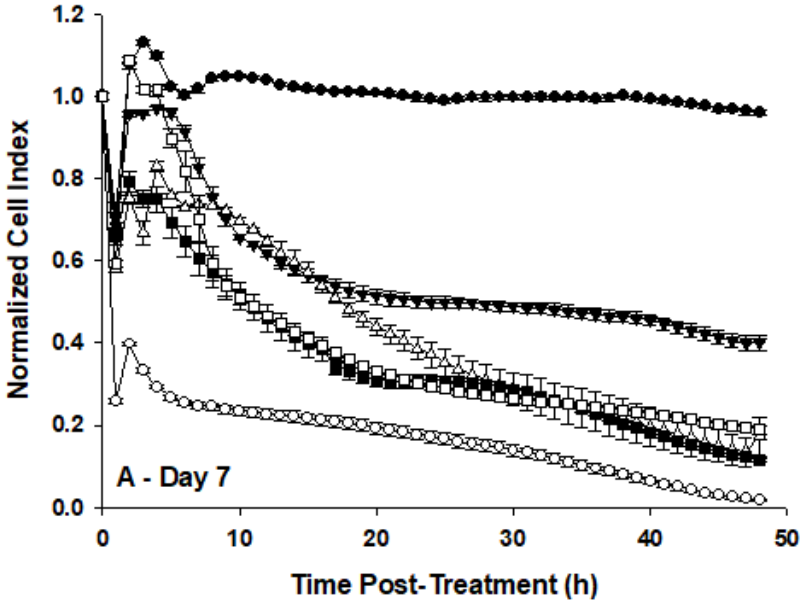
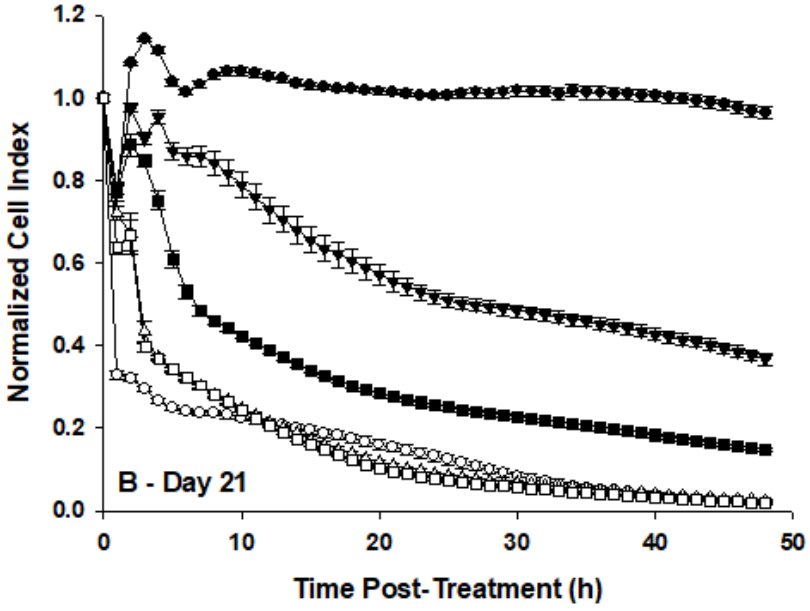
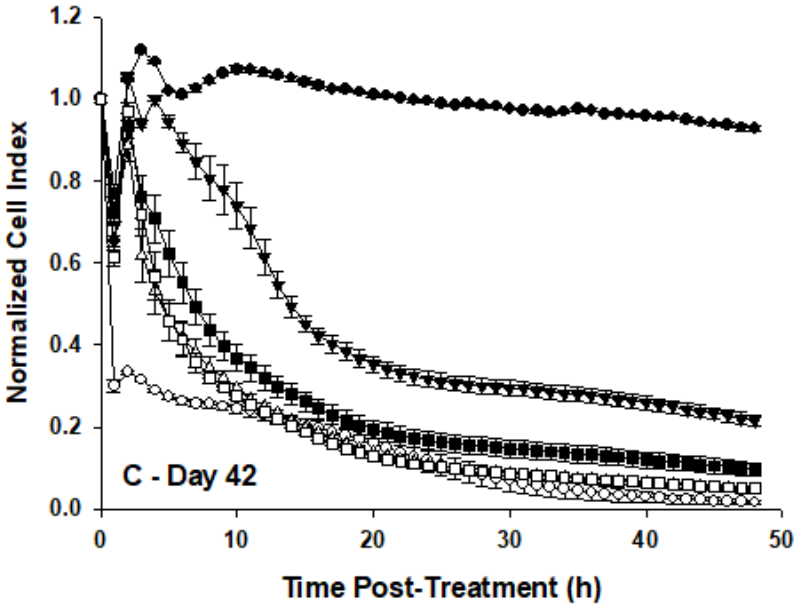


Figure 4.2 - Normalized cell indices versus time showing changes in TEER following treatment with RCC supernatants from different donor groups at day 7 (A), day 21 (B) and day 42 (C) of storage. —●— = negative control, —○— = positive control —▼— = male ≤ 30-yo, —△— = male ≥ 60-yo, —■— = female ≤ 30-yo and —□— = female ≥60-yo. Data represent mean ± SEM (n = 4).




Normalized Cell Index
1.2
1.0
0.8
0.6
0.4
0.2
0.0
B - Day 21
0
10
20
30
40
50
Time Post-Treatment (h)


Normalized Cell Index
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C - Day 42
0
10
20
30
40
50
Time Post-Treatment (h)

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Chapter 5

General Discussions and Conclusions

5.1 Review of thesis objectives

The life-saving benefit of red blood cell (RBC) transfusion is heavily dependent on the *ex-vivo* preservation of RBCs while maintaining the *in-vivo* RBC functional viability [1-3]. Although the current RBC biopreservation techniques have significantly improved the safety of RBC transfusion, RBCs continue to be negatively affected by hypothermic storage lesion (HSL) [1-3]. HSL is a myriad of biomechanical and biochemical changes that RBCs undergo during hypothermic storage (HS) [1-3]. These changes include membrane alteration that result in RBC shape change, decreased deformability, increased microvesiculation as well as increased hemolysis with HS length [1-4]. Collectively, RBC HSL has been associated with adverse patient outcomes including transfusion reactions and transfusion related immune modulation (TRIM) [5-8]. This has led many to question the efficacy of RBC transfusion to improve patient outcome, as it has been shown to result to worse patient outcomes in some critically ill patients [9-11]. Recent addition to the complexity of HSL is the reports of the impact of donor characteristics such as age and sex as well as RBC manufacturing method on the immunomodulatory effects of stored RBCs [12-14]. This idea has drastically changed the current perception that all RBC products are equivalent regarding the quality and efficacy post-transfusion. In consideration of the current controversies in the literature regarding the association of donor factors and patient outcome, this thesis sought to evaluate the role of donor age and sex to the immune modulation effect of red cell concentrates (RCCs). To achieve the overall objective of the thesis, two assays novel to evaluation of RBCs in relation to immune modulation were established.

The first bioassay I established and validated in our lab for investigating RBC HSL and TRIM was the monocyte monolayer assay (MMA – Chapter 2). MMA is an in-vitro assay that is currently used to test clinical significance of RBC alloantibodies [15, 16]. Recently, the assay was shown to be a reliable tool for selecting RBC products for alloimmunized patient presenting with antibodies to high frequency antigens [17]. The MMA is however limited by the requirement for fresh monocytes isolated from fresh peripheral blood [17]. Since performing MMA is a long and tedious procedure, isolation of fresh peripheral blood mononuclear cells (PBMCs) each time the assay is conducted will create additional time and technical challenges. The experimental objective for Chapter 2 therefore was to evaluate and compare the functional properties of pooled cryopreserved buffy coat (BC)-derived monocytes to both pooled fresh peripheral blood monocytes and pooled fresh BC- derived monocytes. Monocytes from the three sources were all tested for their viability, ability to phagocytose anti-D-sensitized RBCs as well as secrete cytokines/chemokines upon stimulation with bacterial lipopolysaccharide (LPS). The results from this study showed a comparable viability and phagocytosis ability of pooled monocytes from all sources. However, monocytes from pooled cryopreserved BC PBMCs showed significantly heightened cytokine/chemokine secretion compared to monocyte from the other two sources. This finding was surprising, warranting further investigation, although, some studies have previously demonstrated an increased cytokine secretion from PBMCs and monocytes, which has been hypothesized to be the effect of cryoprotectants as well as the process of freezing that result in cell activation [18-20]. As the phagocytosis ability of cryopreserved BC-derived monocytes did not appear to be affected by the process of cryopreservation, the secondary objective of Chapter 2 was to test the potential of cryopreserved BC-derived monocytes for use

in evaluating clinical significance of RBC alloantibodies *in-vitro*. MMA showed anti- Jr^a, -Scianna-2 and anti-AnWj to be clinically significant (all resulted in phagocytosis index of >5) again supporting its potential use in the MMA.

Since the first contact of transfused RBCs is with the endothelium, the harmful effect of RBC transfusion could have significant impact on endothelial function. As such, I adopted the trans-epithelial electrical resistance (TEER) assay discussed in Chapter 3 to test the potential of RCC supernatants ability to modulate endothelial permeability. TEER assay employs a microelectronic biosensor technology to monitor cells in culture in a real-time, non-invasive and label-free manner [21, 22]. In this chapter, human umbilical vein endothelial cells (HUVECs) were used as an *in-vitro* model for human endothelium. TEER assay involves culture of cells over a period of time on multi-well electrode coated culture plates. Throughout the experiment, electrical impedance across the growing HUVECs monolayers were measured as cell index (CI) until the cells reached confluency. At this point HUVECs monolayers were exposed to treatments including culture media (negative control), Triton X and disodium-ethylenediaminetetraacetic acid (positive control) or RCC supernatants. A decrease in CI in TEER assay corresponds to an increase in endothelial paracellular permeability in response to treatment. Results showed that TEER assay showed sensitivity to various concentration of positive control and showed the ability of stored RCC supernatants to alter endothelial permeability. The complete validation of the TEER assay was limited by availability of RCC samples for testing, warranting further investigation, as the effects of time as well tech to tech variability on the assay performance were not evaluated. However, based on the results, I felt confident that the most important elements of the assay were established, supporting its use in Chapter 4 experiments.

The final step in this thesis was to employ the MMA and TEER assay to evaluate the immune and endothelial modulation potential of stored RCC supernatants. The original aim of this chapter was to assess the contribution of each of the RCC fractions (cells, EV-containing supernatants and EV-poor supernatants). Because of the challenges in obtaining RCCs from the defined donor groups (Chapter 4), I decided to collect and freeze RCC supernatants from all units and test them all at the same time. This decision immensely reduced some confounding factors such as day-to-day variation in conducting several experiments using different lots of reagents and culture media. The experimental objective for Chapter 4 was to evaluate the effect of donor age and sex on RBC quality parameters as well as the ability of RCC supernatants to stimulate the expression of cell adhesion molecules (CAMs) from HUVECs, cytokine/chemokine secretion from monocytes and HUVECs as well as modulate HUVEC permeability *in-vitro*. Results from this study showed a trend towards lower hemoglobin values in female donors compared to male donors which was expected due to biological differences such as monthly loss of blood in women through menstruation. As difference in hemolysis levels has been shown to be lower in female donors compared to male donors, This study did not find a statistically significant difference in RBC storage hemolysis between groups, contrary to other published literature [23]. A potential contribution to this finding was the small sample size in the study which, was limited by availability of blood donors. Results also demonstrated that overall, treatment with RCC supernatants from male ≤ 30-yo donors induced lower HUVEC expression of VCAM-1, lower monocyte and HUVECs cytokine/chemokine release and showed the least effect on endothelial permeability. The results of this study supported my hypothesis that blood donor age and sex

influence RCC quality parameters and significantly influence the immune modulation potential of RCC supernatants *in-vitro*.

5.2 Contribution to science and future direction

The contribution of my thesis to science is based on two main aspects;

1) Providing possible solutions to existing challenges facing the evaluation of the clinical impact of RBC transfusion through bioassay development and utilization (Chapter 2 and Chapter 3).

2) providing insights on the contribution of donor age and sex to the overall immune and endothelial modulation effect of stored RCC supernatants (Chapter 4).

To expound on the first point, the current MMA clinical practice requires one to isolate monocytes from 20 – 30 mL of fresh peripheral blood from a single donor each time the assay is performed. As the assay is increasingly gaining attention for its use in selecting RBCs for alloimmunized patients, the requirement for fresh monocytes could limit its utilization. My thesis offers an alternative to fresh monocytes; pooled cryopreserved BC-derived monocytes. Since many BC components remain unused after blood component production and are usually discarded, BCs are an excellent, easily available and rich source of PBMCs. In addition, the idea of pooling PBMCs before cryopreservation can control for donor variability associated with using monocytes from single donors each time. Results from this thesis (Chapter 2), supports the clinical application of the MMA as a valuable tool to provide transfusion support for alloimmunized patients as it would allow performance of MMA that is consistent manner while creating utility for unused BCs. As an extension from my work, the Canadian Blood Services

clinical leadership led by DR. Gwen Clarke intends to adopt the use of pooled cryopreserved monocytes from BCs in the clinical MMA for the rare blood type program. This will alleviate the practical restriction of using freshly isolated monocytes and increase utility of the MMA and will ultimately improve transfusion support for alloimmunized patients as well as patients with rare blood types. In addition, the MMA may be key for identification of any new alloantibodies to guide future clinical transfusion practice. MMA using pooled cryopreserved BC-derived monocytes may also aid in the evaluation of the innate immune response to transfusion in terms of activation and cytokine secretion, which, has been shown to be an important marker of inflammation severity following transfusion.

Likewise, the TEER assay is an important tool that has practical potential for evaluating the effects of transfused RBCs on the endothelium. With a wide application across different fields, the TEER assay remain underutilized in transfusion medicine. Recently, it has been used to evaluate the effect of platelet products on the endothelium [24, 25]. However, no study has used the technique for testing RBC products, reiterating a need for its exploitation in RBC immune modulation studies. My study provides important direction towards TEER adoption in the blood banking setting. For instance, scientists from the University of British Columbia, Canada recently found out that a certain gut enzyme could be used to cleave RBC blood group antigens therefore turning any blood type into universal type O blood, that can be transfused to any patient (information retrieved from; science.ubc.ca). Although manipulation of blood could hold the key to eliminate the need of cross-matching donor blood with recipient serum, it is not yet known whether the process will affect RCC characteristics or the immune modulation potential of the

stored RCCs. TEER may be useful in testing the effects of any novel manipulation of RBC products on endothelial permeability therefore, ensuring safe transfusion practice.

As the controversies surrounding the association of donor factors and patient transfusion outcome continue, a disconnect between patient outcomes as shown by retrospective studies and *in-vitro* studies evaluating difference in RBC products or investigating the potential mechanisms behind TRIM is evident. My thesis (Chapter 4), which shows that indeed, there is an association between donor age and sex with immune stimulatory effect of RCC supernatants contributes to the body of knowledge by pointing out that close attention should be paid to the age and sex of blood donors, as they could have a significant effect on patient outcome. This study points out that the immune modulation effect of RCC products are not equivalent and, that RCCs from some donor groups have less immunogenic and endothelial modulation effects. If confirmed with additional studies, this may lead to a recommendation to select RCC for transfusion to specific patient population such as the critically ill, based on observed immune modulation effects.

The work presented in this thesis is limited in two main ways. First, the complete validation of the TEER assay presented in Chapter 2 was not performed as this aspect was limited by availability of RCC samples for testing. Owing to its usefulness, future validation of the assay including establishing the coefficient of variation between experiments run on different days and by different individuals is required to maximize its potential in transfusion medicine. Secondly, work presented in Chapter 4 does not provide explanation behind the immune modulation effect of RCC supernatants in the different donor groups, as this was beyond the scope of the MSc

thesis. Nonetheless, I present novel tools that could be used in the future to investigate mechanisms underlying TRIM occurrences *in-vitro*. I hope that my work will be applied to other clinically relevant cells, guide future research and advance transfusion medicine towards better and safer transfusion products to improve Canadian patients lives.

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