# The Role of Extracellular Vesicles and Red Blood Cell Manufacturing Methods on Transfusion-Related Immunomodulation

by

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## Abstract

Transfusion of red blood concentrates (RCCs) is a lifesaving procedure. Nonetheless, a number of studies have revealed that transfusion of RCC products is still associated with increased risk of serious clinical outcomes. Additionally, studies demonstrating the deleterious consequences of transfusion-related immunomodulation have had conflicting results. While many previous studies have focused on accumulation of potentially harmful immunomodulatory mediators during RCC storage, recent randomized clinical trials have failed to demonstrate benefit with fresh RCC transfusion in critically ill or hospitalized patients. Noteworthy, it has been suggested that RCC manufacturing methods, which are rarely accounted for in interventional trials, may have confounded these results. In addition, the presence of extracellular vesicles (EVs) in RCC product is an important factor that has emerged as a potential mediator of the immunomodulatory activity post transfusion. Therefore, this research has focused on investigating the impact of different manufacturing methods and extracellular vesicles on immunomodulatory activity of RCC *in vitro* as an approach to minimize or eliminate the parameters responsible for poorer clinical outcomes.

This thesis tested the hypothesis that non-RBC generated vesicles in RCC are potent mediators of RCC immunomodulatory activity *in vitro*, and the characteristics of these vesicles are influenced by method of blood component manufacturing and length of RCC hypothermic storage. Investigations were conducted at several levels, from detecting and characterizing extracellular vesicles in RCC products using different approaches, to assessing the immunomodulatory activity that EVs play *in vitro* as a function of blood manufacturing method and storage duration.

Despite the progress that has been made to understand EVs, the technical limitations and lack of standardization of procedures used in EVs characterization likely contribute to the considerable variability in the reported literature. As the biological complexity of EVs creates excessive challenges and difficulties in detecting, and characterizing these EVs, most studies do not take into account the heterogeneity of EVs in the RCC products in terms of concentration, content, size, and phenotype (cell of origin). Therefore, for this research different detection methods were used, including flow cytometry, dynamic light scattering and the novel tunable resistive pulse sensing technique to characterize the diversity of EVs in the nano and sub-micron range. Results of this work have verified that RCCs contain a mixed population of EVs and not all EVs in RCC are solely from the constituent RBCs.

Furthermore, assessing the impact of the blood component manufacturing methods on quality characteristics of stored RCCs showed that blood manufacturing methods significantly influence the immunomodulatory effects of RCC supernatant on monocytes *in vitro* and significantly affect RBC and non-RBC EV characteristics throughout storage. Collectively these differences have the potential to impact quality and safety of RBC products. The work presented here is among the first to document a functional consequence related to RCC quality measures and EV characteristics that result from different blood component manufacturing methods. In addition, this work showed that automated washing of RCCs products can reduce the immunomodulatory activity associated RCC supernatants *in vitro*.

This work provides a better understanding of the issues that exist with current blood products, as an aim to improve the blood component manufacturing processes and the quality of the stored RCCs. This research contributes to our understanding of the complex relationship between the storage duration, blood manufacturing method, what is in the blood bags, and

transfusion-related immunomodulation. Herein a novel and strong scientific foundation for the role of blood manufacturing methods and RCC EVs in immunomodulation is discussed. The tools and methods used in this work can be used in the future studies to identify the specific factors or mediators associated with transfusion-related immunomodulation.

## Preface

This thesis is an original work by Ruqayyah Jassim Almizraq. The research project, of which this thesis is a part, received research ethic approval from the University of Alberta Research Ethics Board, Project Name "Role of blood component manufacturing on red cell damage and changes in white blood cells, No. Pro00059754". It, also, received approval from the Canadian Blood Services Ethics Board, Project Name "Role of blood component manufacturing on red cell damage and changes in white blood cells, No. 2015.032"

In **Chapter 2**, the protocol for the dynamic light scattering (Zetasizer) was developed with the help of PhD student, Dr. Luciana da Silveira Cavalcante. Data was collected by myself.

In **Chapter 3,** data collection for the quality parameters (ATP, 2,3-DPG, deformability, hemolysis and hematological indices) was performed with the technical support of Anita Howell, Tracey Turner, Angela Hill and April Xu (Centre of Innovation, Canadian Blood Services). The remaining data was collected by myself.

Data preformed in **Chapter 4** in this thesis forms part of a research collaboration, led by Dr. Jason Acker (Professor at University of Alberta) with Drs. Jennifer A. Muszynski (Nationwide Children's Hospital, Columbus, OH, USA), Philip J. Norris (University of California, Blood Systems Research Institute, San Francisco, USA), and Nicole Juffermans (Academic Medical Center, Amsterdam, the Netherlands). Cytokine data for the monocytes assay was collected by Somaang Menocha (Nationwide Children's Hospital, Columbus, OH, USA). I collect the data for EV cell of origin in Dr. Norris Lab (Blood Systems Research Institute, San Francisco) with technical help from Heather Inglis. The pulmonary cell assay was performed by PhD student (Mathijs R. Wirtz, Academic Medical Center, University of

Amsterdam, Amsterdam, the Netherlands). Note that the pulmonary cell assay is part of another PhD thesis and therefore, is not be included in this thesis, but used to support the discussion in Chapter 4. Potassium supernatants were analyzed by laboratory services at University of Alberta Hospital. Data collection for hemolysis was performed with technical help of Tracey Turner (Centre of Innovation, Canadian Blood Services). Samples for residual WBCs were sent to Canadian Blood Services National Testing Lab for data collection. The remaining data was collected by myself.

Cytokine analysis in **Chapter 5** was done in collaboration with Dr. Donald Branch at Canadian Blood Services in Toronto. Multiplex Cytokine analysis was performed by Dr. Trang Duong from the Hospital for Sick Children Research Institute in Toronto. The data collection for the MMA and HUVEC assay was performed with the help of Betty Kipkeu (MSc student, Laboratory Medicine and Pathology, University of Alberta). Data for EV cell of origin was collected in Dr. Norris Lab (Blood Systems Research Institute, San Francisco) by Dylan Hampton. Samples for residual WBCs were sent to Canadian Blood Services Brampton Testing Lab for data collection. The remaining data was collected by myself.

The experimental designs in **Chapters 2** were developed by myself. The experimental designs in **Chapters 3** and **4** was developed with the assistance of Dr. Acker and Anita Howell, as well as our collaborators. The experimental design in **Chapter 5** was developed by myself.

The data analysis in **Chapters 2, 3, 4** and **5** and concluding **chapter 6** are my original work, as well as the literature review in **Chapter 1**. Dr. Qi-long Yi (Canadian Blood Services statistician) assisted with statistical analysis for **Chapter 3** and **4**.

**Chapter 1** of this thesis has been published as review paper as Almizraq RJ, Seghatchian J, Acker JP. Extracellular vesicles in transfusion-related immunomodulation and the role of blood component manufacturing. Transfusion and Apheresis Science. 2016;55(3):281-291. Dr. Seghatchian contributed to the editing of the manuscript. Dr. Acker was the supervisory author and was involved with concept formation and manuscript editing.

**Chapter 2** has been published as Almizraq R.J., Seghatchian J., Holovati J.L., Acker J.P. Extracellular vesicle characteristics in stored red blood cell concentrates are influenced by the method of detection. Transfusion and Apheresis Science. 2017;56(2):254-260. I was responsible for the data collection and analysis as well as the manuscript composition. Dr. Holovati and Dr. Seghatchian contributed to manuscript review. Dr. Acker was the supervisory author and was involved with concept formation, data analysis and manuscript editing.

**Chapter 3** of this thesis has been published in two manuscripts. The first paper of this chapter has been published as Almizraq, R.J., Holovati, J.L., Acker, J.P. Characteristics of extracellular vesicles in red blood concentrates change with storage time and blood manufacturing method. Transfusion Medicine and Hemotherapy. 2018; 45 (3):185-193. I was responsible for the data collection and analysis as well as the manuscript composition. Dr. Holovati contributed to concept formation and manuscript review. Dr. Acker was the supervisory author and was involved with concept formation, data analysis and manuscript editing. Portion of this chapter (extracellular vesicles and quality parameters data) was also published as Acker, J.P., Almizraq, R.J., Millar, D. and Maurer-Spurej, E. Screening of red blood cells for extracellular vesicle content as a product quality indicator. Transfusion. 2018:58(3); 2217-2226. This second publication is part of a research collaboration, led by Dr. Jason Acker (Professor at University of Alberta) with Dr. Elisabeth Maurer-Spurej (University

of British Columbia). Dr. Acker was the first author responsible the manuscript composition. I was responsible for the data collection and analysis as well as the manuscript composition relating to extracellular vesicles and red blood cell quality control parameters. Daniel Millar and Dr. Maurer-Spurej were responsible for the rest of data collection, analysis and manuscript editing. Dr. Maurer-Spurej was involved with concept formation and manuscript editing.

**Chapter 4** has been published as Almizraq R.J., Norris .PJ., Inglis H., Menocha S., Wirtz M.R., Juffermans N., Pandey S., Spinella P.C., Acker J.P., and Muszynski J.A. Blood manufacturing methods affect red blood cell product characteristics and immunomodulatory activity. Blood Advances. 2018; 2(18):2296-2306. Heather Inglis, Somaang Menocha and I performed the experiments; Ms Pandey, Dr. Norris and Dr. Acker sourced the products used in this study; I analyzed the results with the help of Drs. Norris, Juffermans, Spinella, Acker, and Muszynski. Drs. Norris, Juffermans, Spinella, Acker, Muszynski and I designed the research and prepared the manuscript.

Part of the project in **Chapter 4** has been submitted for publication in 2019 as *Wirtz M.R, *Almizraq R.J., Weber N.C., Inglis H.C., Norris P.J., Pandey.S, Spinella P.C., Muszynski J.A., Acker J.P, Juffermans N.P.. Red blood cell manufacturing methods impact lung injury in a model of mechanical ventilation. *Wirtz M.R (first author, a PhD student at the University of Amsterdam) and I (first Co-author) contributed equally to this manuscript. Wirtz M.R, Inglis H.C, Pandey.S, and I performed the experiments; Ms Pandey, Dr. Norris and Dr. Acker sourced the products used in this study; Wirtz M.R, Weber N.C, and I analyzed results and Wirtz M.R made the figures; Wirtz M.R, Drs. Norris, Spinella, Muszynski, Acker, Juffermans and I designed the research and wrote the paper. Note that only the methods and data related to extracellular vesicles and quality parameters data were included in **Chapter 4** and the rest the

methods and data (lung model) from this manuscript is not included in my thesis, as it will be part of another student's PhD thesis. However, part of the discussion of this publication was used to support the findings in **Chapter 4** and develop the rationale for **Chapter 5.**

"*﴿وَمَا أُوتِيتُمْ مِنَ الْعِلْمِ إِلَّا قَلِيلًا﴾"

**"*You have been given of knowledge nothing except a little*"***

I dedicate this thesis to The Great God "Allah"… Thank you for the guidance, strength, and the power of mind to reach such success and honor

***And***

I dedicate my humble effort to my beloved brother, Nassir Almizraq. If it was not his great support to come to Canada, none of this would have been happened.

***Forever Grateful and Thankful!***

* *Quran surah Al-Isra 85 (QS 17: 85) in Arabic and English translation*

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*"Coming together is a beginning, staying together is progress, and working together is success."*
*– Henry Ford*

The success of my thesis required a great deal of guidance, assistance, and support from several individuals, and the completing of my dissertation and writing the finishing touch of it today would not have been possible without their support and advice. Therefore, I would like to express my sincere gratitude to the following people, who contributed in one way or another to the completion of this thesis.

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## List of Abbreviations:

| | |
|---|---|
| ABT | allogenic blood transfusion |
| ACD | acid citrate-dextrose |
| ACP | automated cell processor |
| AD | apheresis derived |
| ANOVA | analysis of variance |
| APC | allophycocyanin |
| ASs | additive solutions |
| ATP | adenosine triphosphate |
| BC | buffy coat |
| BSA | bovine serum albumin |
| CAMs | cell adhesion molecules |
| CBS | Canadian Blood Services |
| CD40L | CD40 ligand |
| CPC | Carboxylate polystyrene calibration particles |
| CPD | citrate-phosphate-dextrose |
| CPDA-1 | citrate-phosphate-dextrose-adenine-1 |
| CSA | Canadian Standards Association |
| DAMPs | damage-associated molecular patterns |
| DLS | dynamic light scattering |
| DMSO | dimethyl sulfoxide |
| DPG | diphosphoglycerate |
| EBM-2 | endothelial basal medium-2 |

| | |
|---|---|
| EDTA | ethylenediaminetetraacetate |
| EImax | maximum elongation index |
| E-selectin | endothelial-leukocyte adhesion molecule-1 |
| EVs | extracellular vesicles |
| FBS | fetal bovine serum |
| FC | Flow cytometer |
| FITC | fluorescein isothiocyanate |
| FSC | forward scatter |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| Hb | hemoglobin |
| Hct | hematocrit |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HiCN | cyanmethemoglobin |
| HLA | human leukocyte antigen |
| HS | hypothermic storage |
| HUVECs | human umbilical vein endothelial cells |
| ICAM-1 | intercellular adhesion molecule-1 |
| IFN | interferon |
| IgG | immunoglobulin G |
| IL | interleukin |
| ILVs | intraluminal vesicles |
| K+ | potassium |
| KEI | shear stress required to achieve half of the EImax |

| | |
|---|---|
| LORCA | laser-assisted optical rotational cell analyzer |
| LPS | lipopolysaccharide |
| MCF | mean corpuscular fragility |
| MCH | mean corpuscular hemoglobin |
| MCHC | mean corpuscular hemoglobin concentration |
| MCP | monocyte chemoattractant protein |
| MCV | mean corpuscular volume |
| MFI | mean fluoresce intensity |
| miRNA | microRNAs |
| MMA | monocyte monolayer assay |
| MPs | microparticles |
| mtDNA | mitochondrial DNA |
| MVBs | multivesicular bodies |
| MVs | microvesicles |
| NADH | nicotinamide adenine dinucleotide |
| netCAD | Network Centre for Applied Development |
| NO | nitric oxide |
| NP | nanopores |
| PBMCs | peripheral blood mononuclear cells |
| PBS | phosphate-buffered saline |
| PLT-EVs | platelet-EVs |
| PRP | platelet-rich plasma |
| PS | phosphatidylserine |

| | |
|---|---|
| RBCs | red blood cells |
| RCCs | red blood concentrates |
| RCF | Red cell filtered |
| RCTs | randomized controlled trials |
| RK | Reagent kit |
| RMP | red blood cell microparticle |
| SAGM | saline-adenine-glucose-mannitol |
| SHb | supernatant hemoglobin |
| SNARE proteins | soluble N-ethylmaleimide-sensitive factor attachment protein receptors |
| SSC | side scatter |
| TACO | transfusion associated circulatory overload |
| TGF | transforming growth factor |
| THb | total hemoglobin |
| TNF-α | tumor necrosis factor alpha |
| TRALI | transfusion-related acute lung injury |
| TRIM | transfusion-related immunomodulation |
| TRPS | tunable resistive pulse sensing |
| VCAM-1 | vascular cell adhesion molecule-1 |
| WB | whole blood |
| WBC | white blood cell |
| WBD | whole blood derived |
| WBF | whole blood filtration |

# Chapter 1*

# Introduction

* Aversion of this chapter has been published as a review paper as Almizraq RJ, Seghatchian J, Acker JP. Extracellular vesicles in transfusion-related immunomodulation and the role of blood component manufacturing. Transfusion and Apheresis Science. 2016;55(3):281-291.

## 1.1.THE RISKS ASSOCIATED WITH LIFE-SAVING BLOOD TRANSFUSIONS

Transfusion of red blood cell concentrates (RCCs) is a necessary, lifesaving medical intervention. RCCs are given to increase oxygen delivery to tissues in clinical situations where the circulating RBC level is low (anaemia). Approximately 1.2 million RCCs are collected and transfused each year in Canada[1,2], and more than 90 million units are transfused globally[3]. RCCs are used to treat patients in a wide variety of medical and surgical interventions. Approximately 30% of critical care patients, and more than 50% of cardiac surgery patients will receive blood products during their hospital stay [4,5]. Unfortunately, like any medical therapy blood transfusion comes with risks.

As a global industry committed to improving the safety of blood products, significant efforts have been made to reduce the infectious risks associated with blood transfusion. For example, the occurrence of transfusion-related infections is now very low (approximately 1 in 8 million for HIV, 1 in 6.7 million for Hepatitis C and 1 in 1.7 million for Hepatitis B).[6] With the risk of transfusion-transmitted disease significantly reduced, efforts are now focusing on several immune and non-immune transfusion adverse events such as acute and delayed hemolytic reactions, Transfusion Related Acute Lung Injury (TRALI), Transfusion Associated Circulatory Overload (TACO), and hypotensive reactions that have been associated with increased mortality and morbidity in transfusion recipients (summarized in[7,8]).

There is an emerging interest in the risks posed by the ability for blood transfusion to modulate the immune system of recipients. Transfusion Related Immunomodulation (TRIM) has been implicated in adverse clinical outcomes such as increased infection, acceleration of cancer growth, multiple organ dysfunction and short-term mortality after transfusion[9,10]. Purported mechanisms for TRIM include the release of immunosuppressive prostaglandins,

activation of T lymphocytes by exosomes, inhibition of cytokine production (IL-2), suppression of monocytes and cytotoxic T-cells and increase in T-cell suppressor activity[9,11,12]. A large number of observational trials have suggested the RBC transfusions may be associated with increased morbidity and mortality[8], with several studies attempting to demonstrate the deleterious consequences of transfusion induced immune suppression with conflicting results.[13-17] While efforts to understand the biological mechanisms responsible for TRIM are underway, and clinical studies to examine the outcomes associated with immunomodulation continue, the role that blood component manufacturing has on the cell and cell-free components within blood components is rarely appreciated. This article will review the current evidence for a role for extracellular vesicles (EVs) in transfusion-related immunomodulation and will discuss the impact that different methods used to collect, manufacture and store blood have on the composition and characteristics of EVs in RCCs.

## 1.2.EXTRACELLULAR VESICLES - WHAT ARE THEY?

### 1.2.1. A Definition of Extracellular Vesicles

The first discovery of extracellular vesicles was in 1964 when Chargaff and West[18,19] identified "subcellular factors" in cell-free plasma and showed that these factors played a role in blood clotting. In 1967, Wolf[20] confirmed the presence of these subcellular factors using electron microscopy when he was studying the "platelet dust" that was known to be shed by platelets during storage[19,21]. In most recent reviews, EVs are classified based on the mechanism of formation and the biophysical properties of the vesicles[22]. Accordingly, EVs can be categorized into two major types: exosomes and microvesicles[19].

### 1.2.2. Mechanism of Microvesiculation

Microvesiculation is a controlled process by which EVs or, membranous vesicles are formed and released *in vivo* and *in vitro* by cells in response to a variety of conditions and stimuli including hypoxia, oxidative stress and shear stress[23-26]. Cells can release a mixed population of EVs which are heterogeneous submicron-sized vesicles surrounding by a phospholipid bilayer and contain proteins, lipids, and variety of genetic molecules[27-31]. Although the term cell-derived vesicles or EVs is usually used when referring to the exosomes and/or microvesicles[32,33], this is dependent on the formation, function, cell of origin, and characterization.

### 2.2.1. Exosome Formation

Exosomes are released by many types of cells and they existing in most, if not all, of the biological fluids including, but not limited to saliva, urine, milk, blood, seminal and cerebrospinal fluid[19,33]. There are two general pathways or mechanisms for exosome formation [19,24,33-35]. The first one is the classical mechanism where the exosomes originate as a consequence of multivesicular bodies (MVBs) fusing with the plasma membrane. Briefly, this process starts by inward budding after a selection of proteins and lipids to form vesicles inside the intraluminal vesicles (ILVs). The late endosomes accumulate inside the cell and are termed MVBs. Subsequently, these MVBs could fuse with the lysosomes or fuse with the plasma membrane. The fusion between the limiting membrane of the MVBs with the plasma membrane results in the release of the exosomes into the extracellular space. Protein sorting is one of the main reasons for MVBs formation and the release of vesicles which allows the maintenance of specific proteins in the plasma membrane or the elimination of others[28,36]. It has been shown that activated platelets undergo exocytosis or the release of exosomes not only via MVBs but

also by the fusion of platelet α-granules[24]. Additionally, it has been shown that the MVBs serve in an intermediate stage of α-granules production[37,38]. For a further understanding of the platelet secretory system, the formation, characterization, and secretion mechanisms of granules is reviewed by Koseoglu et al[25].

In 2006, Booth et al[34] described a direct exosome formation pathway. They show that the exosome formation as presented by Denzer et al[24] and Stoorvogel et al[35] is not the only pathway that exists in cells. By studying Jurkat T-cells, Booth et al. showed that these cells have endosome-like domains of plasma membrane and from these domains the cell can directly bud and release exosomes which share similar functions as the other extracellular vesicles. Although other cells, such as erythroleukemia cell lines, have been shown to form exosomes directly, the range of cells that may directly release exosomes are still not yet known[19]. Therefore, additional investigations are required to define cells types which are capable of directly release these exosomes under different conditions and to determine what influences this specific type of formation.

It has been demonstrated that these two types of exosomes (formed by the classical and the direct pathway) are very similar in diameter, density and surface markers which make it very difficult to distinguish between them[19]. This has made the identification and the biogenesis classification of EVs even more complex and challenging. Studies to discriminate these exosomes from the other EVs and to eliminate the confusion in the classification are needed.

**1.2.2.2.Microvesicles (MVs) Formation**

In general, the formation of microvesicles or microparticles involves an outward shedding of the plasma membrane when the cell undergoes cytoskeletal re-organization and loses asymmetrical distribution of lipids within the plasma membrane[22,39]. Typically, under

resting conditions, phosphatidylserine (PS) is located in the inner leaflet of the plasma membrane[39]. The asymmetric distribution of PS is maintained by three different enzymes: flippases, floppases and scramblases[39]. The inward (flip) and outward (flop) PS translocations are ATP-dependent while the movement of PS between both membrane leaflets (scramblases) is ATP-independent[39]. However, when the cell undergo simulation, injury or apoptosis, the floppase can mediate the translocation of the PS to the outer leaflet of the plasma membrane. Loss of lipid asymmetric distribution can rapidly occur when calcium influx inhibits the activity of the flippase allowing for translocation of phospholipids[39].

Based on this principle of formation it is expected that all microvesicles express PS on their surfaces but this is not always the case. It has been shown that some endothelial-derived microvesicles are PS-negative when labelled with Annexin-V[39,40]. Therefore, the expression of PS on microvesicles is still controversial[39]. However, in order to understand these types of matters and to examine the logic behind the EVs formation, it is important to look at the factors mediating the microvesiculation.

#### 1.2.2.3. Regulation of Microvesiculation

Microvesiculation will vary under different situations[24,39]. While the mechanisms underlying vesiculation are not well established, a number of studies highlight several factors that can enhance or diminish this process[33,35,41]. For instance, it has been shown that changes in intracellular calcium can trigger the release of MVs[33,41,42]. In addition, the fusion of MVBs with the plasma membrane, the last step of exosome secretion, may involve an interaction between specific SNARE proteins (soluble N-ethylmaleimide-sensitive factor attachment protein receptors)[24,33]. From a blood storage point of view, it is known that several changes occur to stored blood which can trigger and induce microvesiculation. For example, prolonged storage

of red blood cells, leads to an increase in lipid peroxidation and protein oxidation as a result of oxidative stress which can contribute to the formation of MVs[43]. Moreover, the depletion of ATP and the elevation of intracellular calcium concentration during *ex vivo* storage can enhance microvesiculation[44]. Consequently, these changes reduce the quality of the cells and increase number of PS-positive MVs which may lead to numerous adverse clinical outcomes post transfusion as these MVs circulate in the blood stream[45].

It is recognized that the EVs in the red cell concentrate contain a mixture of microvesicles and exosomes[46]. However, as erythrocytes are able to produce MVs but lack the capacity to release exosomes[47,48], there has been some difficult in attributing adverse clinical events directly to the red blood cell MVs. Therefore, an accurate classification of the EVs is critical in studying the potential effects of each type of EVs on the quality of the blood products. However, it is important to mention that the formation of EVs is not always bad. Reticulocytes, an immature erythrocytes, form and release exosomes during maturation as a means to clear some membrane proteins such as the transferrin receptor[23,24,35,49]. This demonstrate that exosomes can mediate the clearance of cellular waste and are involve in cell hemostasis[32,50].

Even though there are vast studies that explain the formation of EVs, there is a lack of knowledge on the molecular mechanisms of microvesiculation[51]. Perfect understanding of the biology of microvesiculation is important to understand the advantages and prevent the adverse effects of these vesicles.

### 1.2.3. Characterization of Extracellular Vesicles

In addition to the differences in biogenesis, EVs vary in other properties such as size, composition and biomarkers. While exosomes are generally small spherical vesicles (~50-100 nm), the microvesicle or microparticles are morphologically more heterogonous (~50-1000

nm)[32,51-54]. The biophysical and biochemical parameters of these vesicles reflect their biogenesis and cell sources. EVs hold specific biomarkers that have been shown to differ from cell to cell and reflect the content and the surface markers of their cell origin[55-57]. Accordingly, these different makers can be used to characterize EVs. For example, exosomes can be characterized by the proteins that are associated with the fusion and the sorting process such as the GTPases, annexins and flotillin. Taking cell markers into consideration, red blood cell MVs can be identified by the presence of glycophorin A[58]. However, Choi et al.[57] pointed out that several studies report different findings and significant variances in EV protein compositions, which are likely due to the differences in the techniques used for isolation, purification and characterization.

### 1.2.4. Limitation in Classification and Characterization

Although numerous reviews and articles significantly contribute to our understanding of EVs and their possibility in mediating physiological and pathological processes, some important problems require our consideration. The first matter is associated with the confusion in the terminology used to name the membranous vesicles[59]. The practice is to name the EVs based on their cell of origin (e.g. platelet-derived vesicles, red blood cell-derived vesicles, and leukocyte-derived vesicles) while recently an argument was made to name the vesicles based on the mechanism of formation (exosome, microvesicles, and apoptotic bodies) in order to overcome problems with the preparation of the vesicles[59-61]. The second challenge in classifying EVs has to do with the different methods used in their isolation and purification. Isolating vesicles from plasm without protein contamination or aggregation is still a major challenge[59,62]. The third challenge is with the inconsistency used to classify EVs according to size. For example some studies define the size range for the exosome from 50-100 nm[63] while

others give a range from 40-150 nm[32]. These data suggest that the biological complexity of EVs including the variation in morphology, size, composition, cellular source and mechanism of formation, can contribute to the technical challenges and difficulties in isolating, detecting, and characterizing EVs[19,56]. Although there are a wide variety of methods and techniques being used to purify, identify, quantify and characterize EVs, there are several limitations associated with these techniques[27,28,30,56]. There is a significant need for standardized methods and improvements in technologies to detect and characterize EVs.

### 1.2.5. Function of Extracellular Vesicles

Extracellular vesicles are gaining significant attention in the literature due to their potential role as biomarkers for disease, their use as delivery systems, and their role in various biological and pathological processes[53,64-68]. Over the last decade, it has been shown that EVs act as vehicles for the transport of specific cell components including lipids, proteins and functional nucleic acid (such as RNA molecules) from a donor cell to recipient or target cell[64,68-71]. The delivery of the EV cargo can result in modification or modulation of the recipient cell function[54,64,69]. This procedure was shown in studies[69,72] which detected a high amount of microRNAs (miRNAs) inside EVs that were not only transferred to the target cells but also translated into proteins. The data suggests that EVs have the ability to modulate functional genetic information through a novel mechanism of cell to cell communication or intercellular exchange[69]. The promise of this discovery has led to a significant growth in the study of EVs as drug delivery vehicles to promote therapeutic activity, such as gene therapy, and to reduce adverse pharmaceutical side-effects[54,64,68,69,72]. It is important to mention that both types of EVs, microvesicles and exosomes, have shown the capacity to deliver molecular cargo from one cell to another[69,72-74]. Nevertheless, the term “exosomes” is used more than “microvesicles” in this

approach, which could be due to the fact that exosomes have been the EVs most extensively studied [68,75]. However, most of the studies use the term EVs because the terminology and classification is not standardized yet as previously mentioned[54,60].

EVs are an attractive candidate as therapeutic, drug delivery vehicles and as diagnostic markers due to their natural occurrence and ability to carry and transfer molecular cargo to target cells[54,64,69,71,72]. Extracellular vesicles as natural delivery system may be beneficial to eliminate or reduce the challenges associated with the current strategies of drug delivery such as liposomes, the synthetic delivery system[64,69]. One of the major advantages of using EVs over liposomes is the complexity of surface compositions of EVs which can lead to the effective transfer of the cargo and efficient fusion or internalization with the recipient cell[64,67]. In addition, EVs express complement regulators and self–markers to avoid recognition and clearance by phagocytes which can enhance their stability in the circulation[64,76]. Subsequently, EVs can circulate for longer time and transfer their cargo to the recipient cell even over long distances[64]. Regardless of these advantages and promising results, there are issues and challenges that need to be addressed before using them in clinical situations[66,67,71]. For instance, there is a general lack of understanding of the complicated roles and the related mechanisms of EVs in health and disease[64,66,71,77]. Moreover, there are difficulties in identifying the appropriate method to load particular types of cargo into EVs[67,71].

Cell to cell communication, an important process in multicellular organisms, is known to be achieved by either a direct contact between cells or by transfer of cell-secreted molecules such as growth factor, cytokines and chemokines[78-82]. Recently, EVs have been shown to be important mediators of cell-cell communication[32,59,60,63,83]. Extracellular vesicles can facilitate signal transduction in a paracrine and autocrine manner[60,78,84]. Studies have demonstrated

several possible mechanisms of effective interaction between EVs and target cells to deliver the cargo which include; fusion with the plasma membrane, binding to the recipient cell surface, and endocytosis or internalization[24,33,79,85]. However, these mechanisms are not well understood,[79] and additional investigations and studies are required in this field.

The participation of EVs in transferring genetic information is one of their most interesting functions[54,79,80]. As previously mentioned, cells release EVs which can contain miRNAs into body fluids to communicate with target cells[69,79,80]. MicroRNAs are small non-coding RNAs that serve as regulatory molecules in various biological/pathological conditions such as inflammation and immune response[79,86,87]. Since the discovery of a role for miRNAs in immunomodulation, a number of studies have identified unique miRNAs that can regulate the immune response either by inhibition or promotion of transcription in immune cells[79]. These miRNA can be found in the extracellular environment bound to high-density lipoprotein, or packed into EVs to protect these miRNAs from degradation and facilitate effective transportation and cell to cell communication[78,79]. While several studies have provided evidence that EVs can transfer active molecules and mediate intercellular signalling or exchange, the mechanisms of molecule sorting into EVs and the exact cell signalling pathways are far from being understood[33,78,79].

## 1.3. EXTRACELLULAR VESICLES AND TRANSFUSION-RELATED IMMUNOMODULATION

### 1.3.1. Transfusion-Related Immunomodulation

Transfusion-related immunomodulation has been suggested as one mechanism to explain the adverse clinical outcomes, such as infection, multi-organ dysfunction and mortality,

that have been associated with blood transfusion[88-90]. The TRIM effect of allogenic blood transfusion has been known since 1970 when whole blood transfusions were shown to enhanced graft survival after kidney transplantation[91]. Although the exact mechanisms of TRIM are still not resolved[92], some possible mechanisms include: the suppression of cytotoxic cell and monocyte/macrophage activity, proliferation of suppressor T-cell activity, and inhibition of interleukin-2, and activation of T-cell by exosomes[46,88,90,93,94]. These mechanisms have been suggested to be a result of the infusion of active allogenic leucocytes which down regulate the immunity of recipients, and/or the infusion of soluble mediators such as soluble human leukocyte antigen (HLA) peptides, histamine, proinflammatory cytokines, CD40 ligand (CD40L or CD154), free hemoglobin, or EVs which have been shown to accumulate during the storage[88,93-96]. However, further studies, are necessary to identify the exact causes and triggers for TRIM and to understand the mechanisms associated with these clinical implications.

### 1.3.2. Immunomodulatory Effects of Extracellular Vesicles

Recently, the immunomodulatory potential of EVs in the blood products has emerged as an important focus of studies in transfusion medicine[63,97,98]. It has been shown that EVs may play a significant role in mediating immunomodulatory effects[46,99-101]. For example, it has been found that EVs in the RCC units, which accumulate during storage, contribute to neutrophil priming and activation and thus promote the inflammatory response in the transfused patient with older blood[98,101]. Moreover, it has been suggested that EVs transfer genetic information such as miRNA, which may play an important role in regulating the immune system[84,102]. In addition, platelet EVs can be messengers that may affect recipient immunity[79,84]. It has been revealed that platelet EVs expressing CD40L can deliver signals to B cells to simulate immunoglobulin G (IgG) production and recruit adaptive immune response in support $CD4^+$ T

cell[84,103,104]. This indirect interaction between EVs and T cells was shown by Danesh et al[46] who suggest that blockade of CD40L can prevent an increase in T-cell proliferation. Furthermore, there are several other ways that EVs influence inflammation or coagulation processes such as the presence of PS on their surface which can trigger the production of the tissue factor[19,82,105]. A number of studies have shown other effects of EV on the immune system including their ability to enhance production of chemokines and cytokines, stimulate the proliferation of T cell, and induce tumor necrosis factor (TNF-a) production by monocytes[46,106]. However, the role of EVs in RCCs on TRIM is not firmly established as shown by the work of Muszynski et al[107], who suggest that the effect of stored RCCs on the immunosuppression of monocyte is due to protein-bound RNAs and not EVs in RCC units.

The discrepancy in the potential mechanisms observed among studies can be due to a number of factors including differences in the blood products studied, techniques, tools, and different manipulation processes. Zhang et al[63] reviewed the potential immune modulatory roles of the EVs from immune cells and non-immune cells. They suggest that there can be different effects from EVs on the immune system depending on different factors such as the EV cell of origin, the class of the EVs, as well as the type of EVs isolation methods used. Studies examining the role of EVs on TRIM are often difficult to evaluate as there is significant confusion or over generalization in terminology that is used to describe what is being examined [59,78,84] rather than appreciating the role for each type of EVs individually. For instance, Danesh et al[46] indicated that RCC EVs as small as 200 nm and positive for CD63 marker are exosomes but not microvesicles and they (the exosomes) are the type of EVs that induce cytokines TNF-a secretion in monocyte. According to their study, they excluded the microvesicles while several studies prove that microvesicles, including erythrocyte EVs, can be in the range of 200

nm[32,53,63,105,108]. In order to better understand TRIM, efforts need to be taken to characterize the phenotype of all EVs within the population and to understand the role that each has on the biological effect being studied. Understanding the immunomodulative action of each type of EVs in RCC units can be a great help to understand their potential roles adverse transfusion outcomes.

### 1.3.3. A Role for Hemoglobin-Bound EVs in TRIM

Erythrocyte membrane, hemoglobin, and cellular energetic are the fundamental components required for effective oxygen transportation to the tissue[109]. As the erythrocyte ages under the hypothermic storage conditions, the metabolic components are depleted, resulting in a changes to the cell membrane which target the erythrocytes for destruction[110,111]. The disruption of the erythrocyte membrane leads to the formation of extracellular vesicles (EVs) and the release of hemoglobin, as free hemoglobin and EV-bound hemoglobin, into the surrounding environment[111,112]. In circulation, cell-free hemoglobin and hemoglobin-bound EVs react with the vasodilator nitric oxide (NO), an important molecule which modulates blood flow, much faster than intact RBCs. This results in an increase in NO consumption which will induce vasoconstrictions, hypertension, vascular injury, and may enhance inflammation post transfusion[110,111,113]. Transfusion of stored RCCs or supernatant from stored RCCs can inactivate endothelial NO, induce systematic hypertension and may partly participate in multiple organ dysfunction and mortality, especially when massive transfusion is required[113-116].

Soluble and EV-bound hemoglobin are present in the RCCs, and the amount of the EV-bound hemoglobin can exceed the amount of cell-free hemoglobin[117]. EV-bound hemoglobin is much more effective that free hemoglobin at removing NO from circulation as EV-bound

hemoglobin is not cleared by haptoglobin following transfusion[111]. Transfusion of stored RCCs can cause systematic inflammatory activation via the mononuclear phagocyte system, and this is associated with membrane-encapsulated hemoglobin[118]. Moreover, it has been shown that EVs in stored RCC units can promote inflammatory chemokines bioactivity upon interaction with platelets in vivo[106]. In order to understand the potential roles of EVs on the deleterious clinical events after transfusion of RCCs, it is important to determine the role that EVs and hemoglobin-bound EVs may have on TRIM.

## 1.4. RED BLOOD CELL CONCENTRATES AND MANUFACTURING METHODS: ARE ALL RED BLOOD CELL CONCENTRATES EQUIVALENT?

### 1.4.1. Whole Blood Processing Methods and RCC Storage Affect Quality

While RCCs are stored in the blood bank refrigerator, they are still metabolically active and as a result undergo progressive storage age-related changes. This "hypothermic storage lesion" includes oxidation of cellular structures and membrane vesiculation resulting in loss of membrane flexibility, and depletion of other key metabolites such as ATP and the hemoglobin regulator 2,3-DPG[119,120]. This current knowledge of the red cell storage lesion has been used to propose biological pathways to explain mechanisms whereby older blood could contribute to patient morbidity and mortality. Several biological pathways have been suggested, including: the role of microvesicles in stored blood in the pathogenesis of thrombosis, inflammation and responses to pathogens[121]; activation of platelets in the stored RCC product that could cause platelet-white blood cell aggregate complexes with procoagulant activity[122]; deformability changes that result in red cells becoming entrapped in the spleen[123]; decreased blood flow because of rigidity of transfused red cells, and decreased oxygen delivery as microvesicle-

entrapped hemoglobin and free plasma hemoglobin serve as potent scavengers of nitric oxide once a patient is transfused[110]. The possible impact of the duration of RCC storage prior to transfusion on outcome has been tested in a number of large, international, randomized controlled trials (RCTs)[124-127], but other factors may also contribute to the "storage lesion" and related transfusion outcomes.

One element that is emerging as an important mediator of "what's in the bag" of transfused RCCs is the manufacturing methods that are used to separate the RBCs, platelets and plasma from whole blood. The method used to separate blood components from whole blood[128-131], the storage solutions used[132-134] and other factors such as pre-storage leukoreduction[135] have all been shown to affect the characteristics of transfused products and may influence quality. Variation in blood products also arises from normal biological differences in the donor population[134,136]. Therefore, it has been very difficult to achieve any level of global, or even national, standardization of blood products, which has confounded current clinical and laboratory based studies aimed at examining transfusion reactions[137].

By examining the characteristics of RCCs produced in Canada, we have shown a lack of equivalency across the red cell products distributed for transfusion[138-140]. Similar studies in the US have evaluated RCCs prepared from whole blood donations using the platelet rich plasma method for separation and RBCs collected through an automated apheresis process (both methods not currently used in Canada)[141]. Collectively, these studies showed differences in the levels of hemolysis, potassium, cytokine and microparticle levels, oxidative stress, oxygen carrying capacity, deformability, and residual plasma, platelet and leukocyte concentrations. It is no longer appropriate to consider all RCCs used in transfusion as being equivalent.

While it is well established that differences in the methods used to manufacture RCCs can have an impact on the in vitro quality characteristics, there is limited data to show that these differences have any clinical relevance. In a recently published retrospective study, the method of whole blood processing was shown to be associated with in hospital mortality of transfused adults [142]. Patients who received fresh RCCs (≤ 7 days of storage) that were prepared by a whole blood filtration, top / top manufacturing method were associated with a higher risk of in-hospital mortality than was transfusion with mid-age RBCs (stored 8-35 days) prepared by the red cell filtration, top / bottom method. This work is significant in that it suggests that blood component manufacturing is important and that different clinical outcomes may be determined by the method used to collect, store and manufacture the blood components.

### 1.4.2. Extracellular Vesicles and Different Blood Component Manufacturing Methods

An increase in EVs with storage has been identified as a significant indicator of storage lesion[143-146]. The number of EVs in a RCC will vary during the storage depending on the storage solution that is used. For example, a higher number of EVs has been observed in RCC with SAGM (saline-adenine-glucose-mannitol) in comparison to RCC with AS-1(Additive solution-1)[138]. In addition, the populations of EVs in stored RCCs is heterogeneous in terms of size, concentration, composition and cell of origin and will depend on the component preparation method that is used[138,140,147,148]. Leukoreduced RCCs have much lower concentrations of platelet- and leukocyte derived EVs that non-leukoreduced products[148]. Manufacturing processes known to affect the level of hemolysis in the RCCs, such as centrifugation force, hold time and temperatures before component separation and component extraction have been associated with an increase in EVs in the RCC. In addition, EV accumulation during storage has been shown to be significantly different among different blood donors with the impact of

donor age, sex and donation frequency being potential factors[44,115,134,149]. Therefore, it is important to study how blood component manufacturing can affect the composition of the RCC and the potential role that each fraction in RCCs can have on the biological activity of the products.

When we consider a RCC, there are three distinct fractions that are influenced by the blood component-manufacturing environment. They are: 1. *Cellular Fraction*. This fraction contains the targeted red blood cells. Irrespective of the method used for RCC production, RBCs predominate in this fraction throughout the product storage duration. Depending on the method used for RCC production, the concentration of non-RBC cells (ie. platelets, leukocytes, endothelial cells) in this fraction will vary. 2. *Extracellular Vesicles*. This fraction contains all of the EVs found in the blood of healthy donors that are not removed during component production, as well as those EVs formed during production and RCC storage. It has been shown that the phenotype of the EVs in RCCs will dramatically change during storage as cells age in the blood bag and undergo apoptosis and necrosis[120,150,151]. It has been shown that characteristics of this fraction will vary with the method of RCC production. For example, pre-storage leukoreduction not only removes leukocytes but also platelet-derived EVs in the RCCs[152], with the timing of the filtration and the product filtered (i.e. whole blood vs buffy coat depleted blood) influencing the EVs in the RCC[153]. 3. *Storage solution/medium*. This fraction contains what remains from RCCs after fractions 1 and 2 are extracted. It can also be seen as the "vesicle-depleted supernatant". It contains water-soluble cellular by-products (i.e. hemoglobin, potassium, hydrophilic lipids) that accumulate in RCCs as a result of the storage lesions. The composition of this fraction changes throughout storage and is heavily influenced by the cell fraction released due to oxidative stress and membrane lysis.

Each fraction present in a RCC can contribute uniquely to transfusion-associated immune activation and vascular reactivity. These include but are not limited to immune activation by Hb and free heme[154], NO scavenging by free and membrane-bound Hb[110], immunomodulation by cytokines released by "contaminating" WBCs and platelets[155], increased phosphatidylserine (PS) exposure-mediated adhesion to the endothelium[156], pro-coagulatory activity by EVs expressing PS[45,157], immune activation by EV carrying complement and immunoglobulins[158], and potentiation of pro-inflammatory effects by water-soluble oxidized products of arachidonic acid[159]. Complicating our ability to understand the role that each factor may have in specific transfusion-associated reactions is the underlying pathology of the patients receiving the blood products[160,161]. Understanding the individual contributions of each fraction to immune activation or suppression will greatly help in understanding the role that blood component manufacturing plays in this multifactorial environment.

### 1.4.3. Can Manufacturing Method Be Used to Reduce TRIM?

Several approaches have been introduced to improve the quality of the stored blood and to reduce post-transfusion adverse outcomes[15,162-164]. Leukoreduction is one of the practical strategies to improve the RCC quality by removing 99.9% of leukocytes and platelets[15]. Leukoreduction can reduce the quantity of the non-RBC EVs and may reduce the bioactivity of these EVs when taking the method of production into consideration. However, leukoreduction mitigates, but does not abrogates the immunomodulatory effects of transfusion[15]. For that reason, washing of RCC to remove the soluble mediators/factors and reduce the bioactivity of the stored RCC, regardless of the manufacturing method, maybe an effective additional procedure to reduce the immunomodulatory effect of transfusion[15,162,165]. Enhanced leukoreduction and washing of RCCs may lead to reductions in the incidence of TRIM.

## 1.5. CONCLUSION

Transfusion of RCCs is a lifesaving procedure. However, several studies have shown that RCC transfusion is associated with an increased risk of serious and lethal adverse clinical outcomes. Data is emerging which suggests that extracellular vesicles in stored RCCs may have a role in mediating the immunomodulatory effects of RCC transfusion. However, RCC units have been shown to contain a mixed population of EVs and not all EVs in RCC are solely from the constituent RBCs. The concentration of the different EVs (the RBC EVs and the non-RBC EVs), their composition, as well as their effects on the quality of the products vary depending on the manufacturing methods used to produce the RCC units. The influence of the different component manufacturing methods on RCC fractions, including non-RBC cells and non-RBC EVs, which may contribute to TRIM, are only now beginning to be examined. With improvements in blood component manufacturing technologies and processes, the impact that EVs have on post-transfusion outcomes may be reduced or eliminated. Studies are necessary to better understand the important donor, manufacturing, storage and recipient factors affecting the role of EVs on patient outcomes.

## 1.6. THESIS APPROACHES

Transfusion of RCCs is a lifesaving procedure. However, several studies have shown that RCC transfusion is associated with an increased risk of serious and lethal adverse clinical outcomes. Data is emerging which suggests that extracellular vesicles in stored RCCs may have a role in mediating the immunomodulatory effects of RCC transfusion. However, RCC units have been shown to contain a mixed population of EVs and not all EVs in RCC are solely from

the constituent RBCs. The concentration of the different EVs (the RBC EVs and the non-RBC EVs), their composition, as well as their effects on the quality of the products vary depending on the manufacturing methods used to produce the RCC units. The influence of the different component manufacturing methods on RCC fractions, including non-RBC cells and non-RBC EVs, which may contribute to TRIM, are only now beginning to be examined. With improvements in blood component manufacturing technologies and processes, the impact that EVs have on post-transfusion outcomes may be reduced or eliminated. Studies are necessary to better understand the important donor, manufacturing, storage and recipient factors affecting the role of EVs on patient outcomes. Therefore, this research will focus on investigating the role RCC-derived EVs and blood component manufacturing methods on the immunomodulatory activity of hypothermically stored RCCs.

The first part of this thesis focuses on detecting and identifying "what is in the bags" of differently manufactured RCCs and highlighting the presence of non-RBC fractions in the RCC products including residual cells and cell-derived EVs. The second part of this thesis was conducted to understand the role that cell-derived EVs and blood manufacturing methods may play in the quality and immunomodulatory activity of RCC products with an attempt to relate this knowledge to the development of better manufacture processing, preservation and transfusion. Thus, the last part of this research focuses on identifying strategies that may eliminate the parameters responsible for poorer clinical outcomes.

## 1.7. HYPOTHESIS AND THESIS OBJECTIVES

This thesis will test the hypothesis that non-RBC generated vesicles in RCC are potent mediators in RCC immunomodulatory activity in vitro, and the characteristics of these vesicles

are influenced by method of blood component manufacturing and length of RCC hypothermic storage.

The thesis consists of experimental studies with four specific research aims (SRAs):

**SRA1:** Detect and characterize extracellular vesicles in stored red blood cell concentrate using different method of detection **(Chapter 2)**.

**SRA2:** Examine the impact of blood component manufacturing on extracellular vesicles and red blood cell quality control parameters during hypothermic storage **(Chapter 3)**.

**SRA3:** Assess the influence of blood manufacturing methods on red blood cell product characteristics, extracellular vesicles subtypes, and immunomodulatory activity **(Chapter 4)**.

**SRA4:** Investigate the effect of washing on extracellular vesicle and the immunomodulatory activity of stored red blood cell concentrate **(Chapter 5)**.

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# Chapter 2*

## Extracellular Vesicle Characteristics in Stored Red Blood Cell Concentrates Are Influenced by Method of Detection

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## 2.1. INTRODUCTION

Heterogeneous populations of extracellular vesicles (EVs) have been shown to be present in and accumulate in red blood cell concentrates (RCCs) throughout storage and can be an indicator of red blood cell damage[1-5]. Extracellular vesicles, including exosome and microvesicles/microparticles (MVs/MPs), are submicron-sized vesicles (50 nm to 1000 nm in diameter) released *in vitro* and *in vivo* from many types of cells[6-9]. Extracellular vesicles have recently gained considerable attention due to their roles in numerous biological processes[10-13]. Extracellular vesicles contain various bioactive molecules (proteins, lipids, and nucleic acids such microRNAs)[12,14-18]. Recently, it has been shown that EVs, both MVs and exosome, are key mediators of intercellular signaling and communication[14,16,18-20]. The biological complexity of EVs including the variation in morphology, size, composition, cellular source and the biogenesis, create excessive challenges and technical difficulties in detecting, quantifying and size profiling EVs[6,21-23]. It has been shown that different EV characteristics can be observed when a sample is prepared or analyzed with different procedures or techniques[6,24,25]. Of note, most studies examining the biological implications of RBC EVs do not take into account the heterogeneity of EVs in the RCC products in terms of concentration, content, size, and phenotype.

Even though there are a wide variety of methods and techniques being used to detect and characterize EVs in blood products, there are several limitations associated with these methods[6,24-26]. For instance, flow cytometry (FC), is the most common optical method used to identify, quantify and characterize the EVs as it is readily available to research groups[11,13,26,27]. Nonetheless, one of the major limitations of standard FC is the lower limit of detection as most flow cytometers are unable to detect EVs less than 300 nm[7,11,13,26]. Notably, several studies

have shown considerable attention to the importance of small EVs (exosomes) in cell-cell communication, cell signalling by participating in antigen presentation, and their potential roles in immunomodulation [15,28,29]. Therefore, improved instrumentation and techniques have become available to allow the characterization of small EVs[6,11,30,31].

Nano-sizing instruments can be used to determine a wide size range of nano-sized particles by measuring the Brownian motion of particles in a sample using the dynamic light scattering (DLS). Dynamic light scattering is simple to apply and it can be used to accurately determine the size distribution of monodisperse populations of particles ranging from 1 nm to 6 μm[26]. This technique enables the detection of the small particle that cannot be detected by FC. However, measuring polydisperse or heterogeneous populations, such as EVs from body fluids, becomes problematic because there is a tendency to bias results toward the detection of larger particles[7,26]. Furthermore, absolute concentrations of EVs cannot be determined by DLS techniques[26]. A tunable resistive pulse sensing (TRPS) technology can be used to determine the size and concentration of small particles[6,22]. The TRPS technology uses nanopore electrical impedance to achieve single-molecule detection that can be used to determine the concentration and size profile of a wide range of particles in a sample [22]. DLS and TRPS techniques provide the opportunity to resolve nanoscale EVs in blood products.

The aim of this study was to evaluate the advantages and disadvantages of using three different techniques (TRPS, FC, and DLS) to characterize EVs in stored RCCs.

## 2.2. MATERIALS AND METHODS

### 2.2.1. Blood Collection

Whole blood was collected and RCCs were produced according to standard operating procedures at Canadian Blood Services. Briefly, whole blood units were collected from eligible donors into Top-and-Bottom blood collection packs *(LCRD quadruple T/B CPD/SAGM 500 mL Bactivam ITL, LQT7291LX, MacoPharma, Mouvaux, France)* and processed using the Top-Bottom filtration system (n=3). As previously explained [32,33], approximately 480 mL of whole blood was collected in 70 mL of citrate-phosphate-dextrose (CPD)-anticoagulant. Units were held overnight and centrifuged to separate the blood components. The extracted red blood cells were suspended in about 110 mL of saline-adenine-glucose-mannitol (SAGM) within 24 hours of stop-bleeding time, and the RCC units were luekoreduced by filtration at room temperature. All units were then stored at 1-6 °C for up to 43 days.

### 2.2.2. Sampling and Study Design

Sampling was performed using a validated technique as previously described [34,35]. At each testing point (day 3, 7, 21, and 42) units were gently massaged and thoroughly mixed by inversion and 12 mL of RBCs was aseptically drawn from each bag into 15 mL conical tubes using a sampling site coupler and an 18-gauge needle that attached to a 25 mL syringe. Samples were centrifuged at 2200 x g for 10 min at 4 °C (Eppendorf 5810R). The supernatant of each sample was equally distributed into three 1.5 mL microtubes for triplicate measurements as illustrated in the experimental design **Figure 2.1**. Prepared RCC supernatants were used for EVs characterization with TRPS, FC, and DLS methods to reduce variability in sample preparation across the methods. The microparticle size and concentration by the TRPS were measured on day 3, 7, 21, and 42. Since the TRPS technique is very time consuming, the microparticle count by flow cytometry and sizing by DLS were completed on the following

days; day 4, 8, 22, 43. Each sample (n=3) was run in triplicate in order to reduce sample variability, to verify results and generate acceptable data.

### 2.2.3. Flow Cytometry Characterization of RCC EVs*

The RBC microparticle (RMPs) count was measured based on a flow cytometry flow rate technique as previously published[4] with some modifications. Flow cytometry is the most common and ideal method used to quantify particles based on their phenotype[36,37]. The flow cytometer principle allows for the measurement of the properties of fluorescently labelled cells or particles in a suspension as they pass in a single file in front of a laser permitting the order to be detected, counted, and characterized[38]. The light from a laser is either scattered or absorbed (fluorescence), and the detectors placed in flow cytometer, such as forward scatter (FSC) and side scatter (SSC), collect and measure the scattered and fluorescent light. Characterization of the particles is done by their size which correlate with the FSC light and by their relative granularity which is shown by the scattered light [36,37]. In this study, RCC supernatants (40 µL) were diluted with 1x phosphate buffered saline (PBS; pH 7.4) then incubated with a fluorescein isothiocyanate (FITC) anti-human CD235a antibody (Invitrogen, MHGLA01) for 15 min in the dark at room temperature (20-25 °C). For nonspecific antibody binding, an isotype control (FITC mouse IgG1, k isotype control; Invitrogen, Life Technologies, Frederick, USA) was used. Frozen RBCs (-80 °C) served as positive controls for RBC microvesiculation. Buffer with FITC-CD235a antibody but no RBCs or supernatant was used as the negative control. All samples were filtered using 12 x 75 culture tubes with filter tops (0.2 µm filter top tubes, VWR, Cat. No. CA28143-315). Prepared samples were run on a bench-top digital flow cytometer

* The author would like to acknowledge Dr. Aja Rieger (Flow Cytometry Core Manager, Faculty of Medicine and Dentistry, University of Alberta) for the training in flow cytometer.

(LSR-Fortessa X-20, BD Biosciences, San Jose, USA), which was equipped with 5 separated lasers (375 nm, 405 nm, 488 nm, 561 nm, and 633 nm) and the system operates using BD FACSDiva 8.0.1 software (BD Biosciences). Latex beads 1.0 µm in diameter (Bangs Laboratories, USA) were used to generate a gate around the desired population of microparticles to further classify them based on their size and only microparticles ≤ 1.0 µm in diameter were analyzed. FSC and SSC of the flow cytometer, measured on a logarithmic scale, was used to distinguish between RBC and RMPs populations. For this study only RBC-EVs/RMPs events were counted, as only particles positive for glycophorin A-FITC and less than 1.01 µm in size as shown on a FSC-SSC dot plot were detected. TruCOUNT beads (BD Bioscience, Mississauga, ON) were used to determine the absolute number of RMPs/µL. Absolute numbers of RMPs/µL were calculated using the following equation:

$$Absolute\ count\ of\ microparticles\ per\ \mu L =$$

$$\frac{Number\ RMPs\ (\text{CD235a positive})\ events}{Number\ \ TruCOUNT\ beads\ gated\ events}\ x\ \frac{Number\ of\ TruCount\ Bead\ per\ tube}{Suspension\ Volume}\ x\ dilution\ factor$$

Eq.2.1

Where:

*Number of RMPs events* = the number of FITC-CD235a/Glycophorin A positive events in the red blood cell microparticle gate

*Number of TruCOUNT beads per tube* = the bead count from the TruCOUNT package for the specific lot number used

*Number of TruCOUNT beads even*t = the number of events in the TruCOUNT bead gate

*Suspension volume* = the volume of buffer used to suspend the TruCOUNT beads, and

*Dilution factor* = determined from the ratio of buffer and Fluorochromes added to the RCC supernatant sample

### 2.2.4. Dynamic Light Scattering/Zetasizer*

As the flow cytometer lacks the ability to resolve nanoparticles (e.g. EV < 200nm), we used a dynamic light scattering instrument, as DLS is the most widely used technique for the nanoparticles size measurements. The mean size (nm in diameter) of EV in RCC supernatant was determined using Zetasizer Nano S (Malvern Instruments Ltd, Worcestershire, UK) as previously described[39]. The DLS/Zetasizer Nano system measures the speed of particles that move by Brownian motion and correlates this to the size of the particles, where small particles move quickly and large particles move slowly. A leaser provides the light source to illuminate the sample particles and a detector is used to measure the intensity fluctuations of the scattered light by the different size of particles[40,41]. Uniform polystyrene microspheres of 120 nm, 200 nm, and 400 nm diameter (Bangs Laboratories, USA) in a 0.01% concentration in PBS were used as standards. Supernatants (300 µL) were diluted with PBS (600 µL) and analyzed with the Zetasizer immediately using sample refractive index of 1.43 (phospholipid liposomes). Samples were allowed to equilibrate for 2 min at 25 °C. The dispersant refractive index value was 1.33 (water). The size of the observed EVs populations was determined by Z-average size and polydispersity index (PdI).

### 2.2.5. Tunable Resistive Pulse Sensing Assessment

* The author would like to acknowledge Dr. Luciana da Silveira Cavalcante (Laboratory Medicine and Pathology, University of Alberta) for the training and technical help with DLS.

To be able to quantify and distinguish the different subpopulations of EVs based on their size profile, the number and size characterization of EVs were measured using a tunable resistive pulse sensing instrument (qNano system; IZON Science Ltd, Christchurch, New Zealand). The tunable resistive pulse sensing technology uses the Coulter principle on nanoscale and allows for single- molecules detection through a nano-sized pore[22,42-44]. In TRPS, a nanopore separates two types of fluid cells; 1) the lower fluid, which is filled with electrolyte solution (particle-free) and 2) the upper fluid which is filled with the sample of interest. When a voltage is applied across the fluid cell via silver-silver chloride electrodes, ions move between the electrodes through nanopore and create a baseline current. As a particle passes through the nanopore, it displaces a volume of electrolyte causing a temporary increase in the resistance and corresponding decrease in the measured current. A temporary alternation in the established current detected allows for the sizing and counting of particles in electrolyte solution. Each particle or nanoparticle passing through the nanopore creates a blockade event "resistive pulse" which is displayed in real time. The magnitude of this blockade event is proportional to the volume of the particles[44]. Size profiling of sample particles (unknown particles diameter) can be accomplished by comparing the blockade events produced by the sample particles with the blockade events produced by calibration particles (known diameter)[22,43,44]. In this study, two different nanopores (NP200 and NP400, IZON Science Ltd) were used in this study to target EVs in the size range of 100 nm to 1000 nm using a standard stretch range (43-47 mm). Carboxylate polystyrene calibration particles (CPC200 and CPC500; IZON Science Ltd.) were used with the NP 200 and NP400 nanopores respectively for optimization and to insure an accurate size and concentration measurements. The size profile of EVs in RCC supernatant samples was performed by comparing the resistive pulses (blockades) of the EVs (unknown

diameter) with the resistive pulses resulting from measurement of calibration particles (known diameter). Both CPC200 and CPC500 were suspended in Solution A (Fluid Cell Electrolyte, IZON Reagent kit, RK1, IZON Science Ltd) according to the target particle concentration as recommended by the manufacture. Supernatant samples were initially diluted with Solution Q (qEV Electrolyte, IZON Science Ltd) at 1:1 dilution and the sample dilution adjusted as required to target a particle rate of 1000-2000/min. Samples were filtered with a 0.80 μm syringe filter and/or 0.45 μm syringe filter before being analysed with NP400/NP200 respectively as recommending by the manufacturer. All calibration and sample measurements were run under the same conditions as recommended by the manufacture and at least 1000 particles were recoded with two different pressures. A minimum of 2 pA difference between the two pressures was applied with the standard pressure range (1 unit=1 mbar). The resolution range of NP200 with CPC200 was set to detect EVs < 200 nm, while NP400 with CPC500 was set to detect EV ≥ 200 nm. Data obtained were analyzed using Izon Control Suite software (Izon Control Suite Version 3.2.2.268, Izon Science Ltd).

### 2.2.6. Statistical Analysis

Data analysis was performed using statistical computer software (IBM SPSS Statistics 23.0, Armonk, NY, USA). Analysis of variance (ANOVAs) was used to identify significant differences within the storage period for each assay. ANOVAs followed by a Scheffe post hoc test were used to evaluate any significance among pairwise comparisons of testing time points during the storage time. Probability (*P*) values less than 0.05 was considered significant. Correlational analysis was performed using SigmaPlot 13.0 (Systat Software Inc.)

## 2.3. RESULTS

### 2.3.1. Flow Cytometry

Fluorescence based flow cytometry was used to identify and quantify microparticles in RCC supernatant samples. Flow cytometric analysis showed that the absolute number of RMPs/μL (CD235a+) increased gradually during hypothermic storage (**Figure 2.2**). In comparison to day 4 of storage, a statistical significant increase in the number of RMPs/μL was detected on day 22 and day 43 ($p < 0.0001$), but there was no significant difference in the number of RMPs/μL on day 8 when compared with day 4 of storage ($p = 0.979$).

### 2.3.2. Dynamic Light Scattering

A Malvern Zetasizer nano-system was used to determine a wide size range of nano-sized particles in RCC supernatant samples using dynamic light scattering. Dynamic light scattering analysis of RCC supernatants showed that the zeta-average size of EVs (d.nm) changed during storage time (**Figure 2.3**). A significant increase in the zeta-average size of EVs was identified on day 8, 22 and day 43 in comparison to the initial testing point (day 4; $p < 0.05$). Results show that the average size of EVs increased from less than 100 nm on day 4 to about 200 nm on day 43 of storage.

### 2.3.3. Tunable Resistive Pulse Sensing

An IZON Science Ltd qNano using TRPS technology was used to characterize the micro- and nano-particles in RCCs. Results from the qNano show an increase in the concentration of EVs (EVs/mL) during RCC storage (**Figure 2.4**). Further characterization of EVs based on their size showed that the concentration of the EVs < 200 nm significantly increased throughout storage ($p < 0.05$; **Figure 2.4A**). In addition, a significant increase in the number of EVs ≥ 200

nm was observed on day 21 and day 42 of storage in comparison to day 3 ($p<0.001$), but no statistically significant differences were observed on day 7 in comparison to day 3 of storage ($p=0.931$; **Figure 2.4B**). Notably, overall qNano data indicated that the concentration of small EVs is greater than the larger EVs at all of the testing points during storage (**Figure 2.5**). Furthermore, in comparison to day 3 of storage, the size profile of EVs obtained using two different nanopores to cover a wider dynamic analysis range showed a statistical significant decrease in the size of EVs on day 7, 21 and 42 ($p<0.001$; **Table 2.1**).

### 2.3.4. Correlations

To assess the relationships between the concentration (EVs/mL) of EVs from FC and TRPS/qNano, exploratory correlational analyses were performed. As illustrated in **Figure 2.6A**, a very week correlation between the concentration (EV/mL) of EVs measured by FC and qNano/NP200 ($R^2=0.008$). When the concentration EVs $\geq$ 200 nm measured by qNano/NP400 compared with EVs measured by FC, a moderately positive correlation was identified ($R^2$ = 0.29, **Figure 2.6B**). However, a weak correlation was identified between the EVs concentration measured by FC and total concentration of EVs measured by qNano using NP200 and NP400 (**Figure 2.6C**), suggesting that the EVs data are not comparable between these two techniques due to the different resolution of methods used to detect the particles of interest.

Correlation analyses were also executed to evaluate the relationships between the size of EVs from DLS/Zetasizer and TRPS/qNano (**Figure 2.7**). Moderate correlations were observed between z-average sizes of EVs measured by DLS and the size profile of individual population of EVs obtained by qNano using two different nanopores (NP200/small EVs, **Figure 2.7A**; NP400/large EVs, **Figure 2.7B**). However, correlation analysis revealed that there was a strong relationship between the z-average sizes of EVs measured by DLS and the average size of

overall EVs measured by qNano (**Figure 2.7C**). This data suggest that DLS is able to resolve a wide range of particles, including particles < 200 nm, as does the qNano system. However, DLS reports only average size of all particles in a suspension and lacks the ability to report an accurate size profile or concentration of subpopulations of particles.

## 2.4. DISCUSSION

Throughout the last decades, EVs have gained great consideration due to their potential roles in various biological and pathological processes[9,23,30,45-48]. However, limitations associated with the current technologies used to characterize EVs have hampered the standardization of EVs in blood products[20,27]. This study shows that the characterization of EV present in stored RCC products is significantly influenced by the method of detection used.

Quantitative analysis using the flow cytometer and a TRPS device showed an increase in the number of EVs during hypothermic storage (**Figure 2.2 and 2.4**). However, when compared with the TRPS device, we found that FC was unable to identify and quantify EVs ≥ 200 nm in size. This is consistent with previous studies that stated the lack of current flow cytometers to resolve small EVs[7,11,26,49,50]. In addition, correlation analysis was performed to evaluate the relationship between number of EVs obtained by TRPS and by FC methods throughout storage (**Figure 2.6**). Moderate positive correlation was identified between FC and qNano (NP400) EVs concentration, while very weak correlations observed with the qNano (NP200). Noteworthy, results obtained from qNano techniques showed that the majority of EVs (60-90 %), generated during the storage were between 90 nm to 200 nm in size (**Figure 2.5**). Knowing that RCCs

contain a heterogeneous population of EVs that change during storage [29], it is likely that not all EVs in this study are entirely generated by RBCs. As a result, the observed increase in EVs can be due to changes in the RBC-EV and non-RBC EV (i.e. EVs from platelets and white blood cells) populations. Therefore, it is critical to further examine these small EVs and evaluate their potential roles in adverse clinical outcomes post-transfusion.

A comparison of the TRPS and DLS techniques demonstrate considerable differences in the EVs size profile during storage. While the qNano measurements showed a significant decrease in the mean size of EVs, the Zetasizer/DLS showed a significant increase in the average size of EVs during storage. DLS assesses the size distribution of particles in the suspension when they scatter the laser light under the Brownian motion [40,41]. However, it has been indicated that measuring poly-disperse or heterogeneous populations with the DLS system can be problematic as it is biased towards larger particles present in the solution[7,26]. In addition, unlike the TRPS technique, DLS is unable to distinguish between different subpopulations of EVs as it reports only the average size of the measured particles. Therefore, when correlation analysis was performed to assess the relationship between the size of EVs detected during storage by the qNano and Zetasizer/DLS (**Figure 2.7**), a moderate to weak correlation was observed (NP200; $R^2 = 0.29$), (NP400; $R^2 = 0.26$). However, the correlation between the average size of EVs detected during storage by the qNano and Zetasizer/DLS showed a very strong positive correlation ($R^2 = 0.95$).

This study clearly showed that the EVs data varied across the detection methods and the data cannot be directly compared due to the differences in the measurement capabilities of each technique. In addition, the scope of this study was not to select the best method(s) to characterize EVs in stored RCCs, which is difficult to do in the absence of a reference method.

This study further stresses the importance of developing reference method(s) to establish a standardization and to make it possible to compare published data relating to this important parameters.

As each technique provides certain benefits and limitations, it is important to understand these before selecting a specific method to evaluate the characteristics of EVs in stored RCCs. Characterizing the size, concentration and phenotype of EVs in stored RCCs can be used to better understand the donor, manufacturing and storage factors that influence patient outcomes. Similarly, by understanding the characteristics of the EVs in a sample, we may begin to gain a better appreciation of the role that different subpopulations of EVs may be playing in their complex biological activity. For this reason, it may be necessary to employing multiple methods to examine EVs to more fully understand the complexity of factors affected EVs in stored RCCs.

## 2.5. CONCLUSION

This study provides evidence that quantitative or qualitative evaluation of the EVs present in RCCs will be dependent on the detection method used and the testing points examined during storage. When compared with TRPS technology, FC detects only fluorescently labelled EVs ≥ 200 nm and DLS reports only the average size, not the actual size, of EVs. Despite the progress that has been made to understand EVs, the technical limitations and lack of standardization of procedures used in EVs characterization likely contribute to the considerable variability in the reported literature[20,27]. In addition, this study demonstrates that the majority of EVs detected during early storage of RCC are small EVs or exosome-size vesicles which may not be solely generated form RBCs. It is therefore important that further studies on EVs in stored RCCs utilize

multiple techniques to characterize EVs if we are to advance our understanding of the role that EVs have in adverse transfusion events and post-transfusion outcomes.

**FIGURE AND TABLE:**

**Figure 2.1:** Experimental design for the characterization EVs in stored RCCs using three different techniques: Flow cytometer, dynamic light scatting (DLS), and tunable resistive pulse sensing (TRPS).

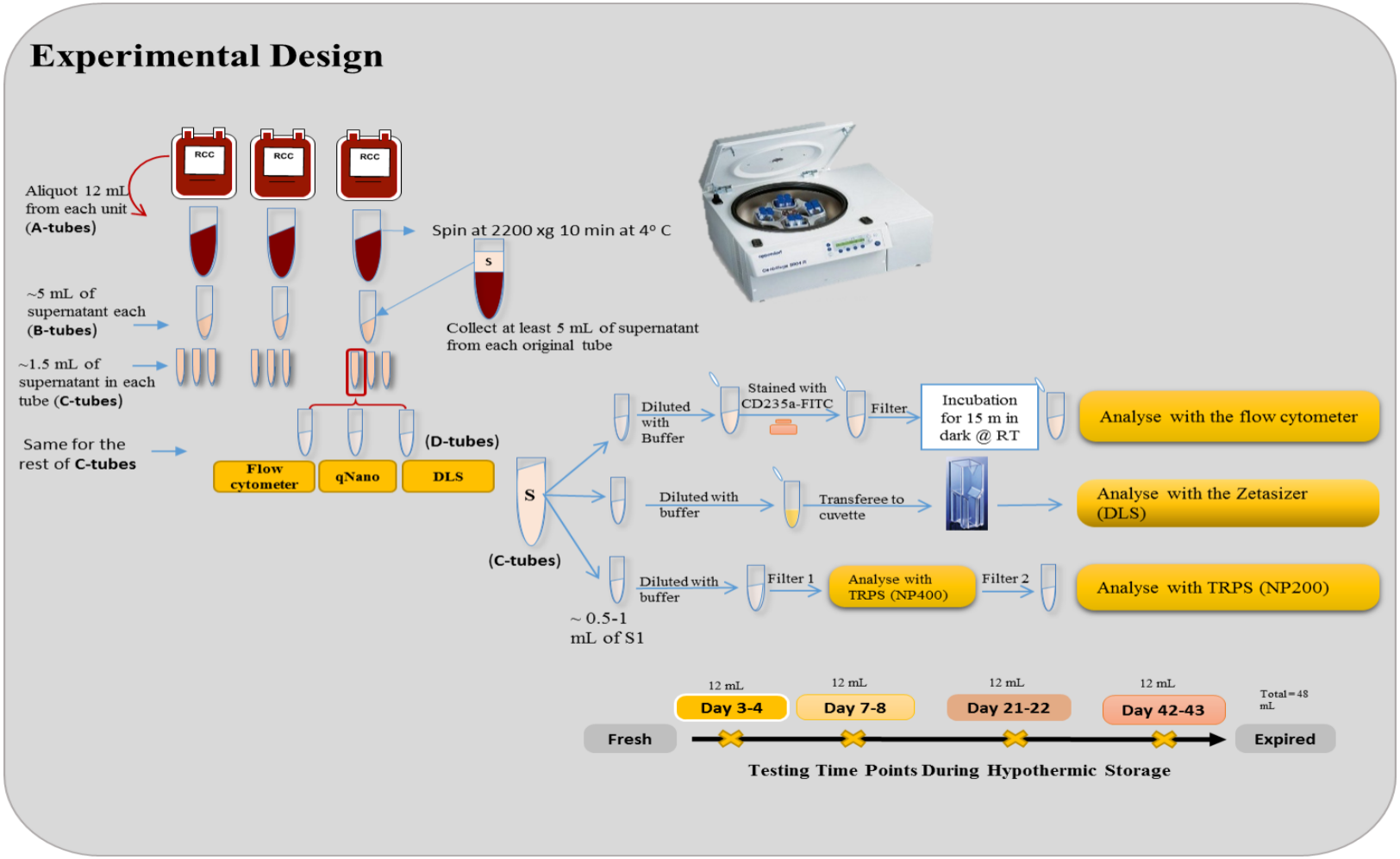

**Figure 2.2:** Flow cytometry - Absolute number of RBC microparticles/µL in up to 43 d stored RCCs (n=3, in triplicate measurements). Data are reported as mean ± 1SD. *Significant results ($p < 0.0001$) in comparison to day 4 values.

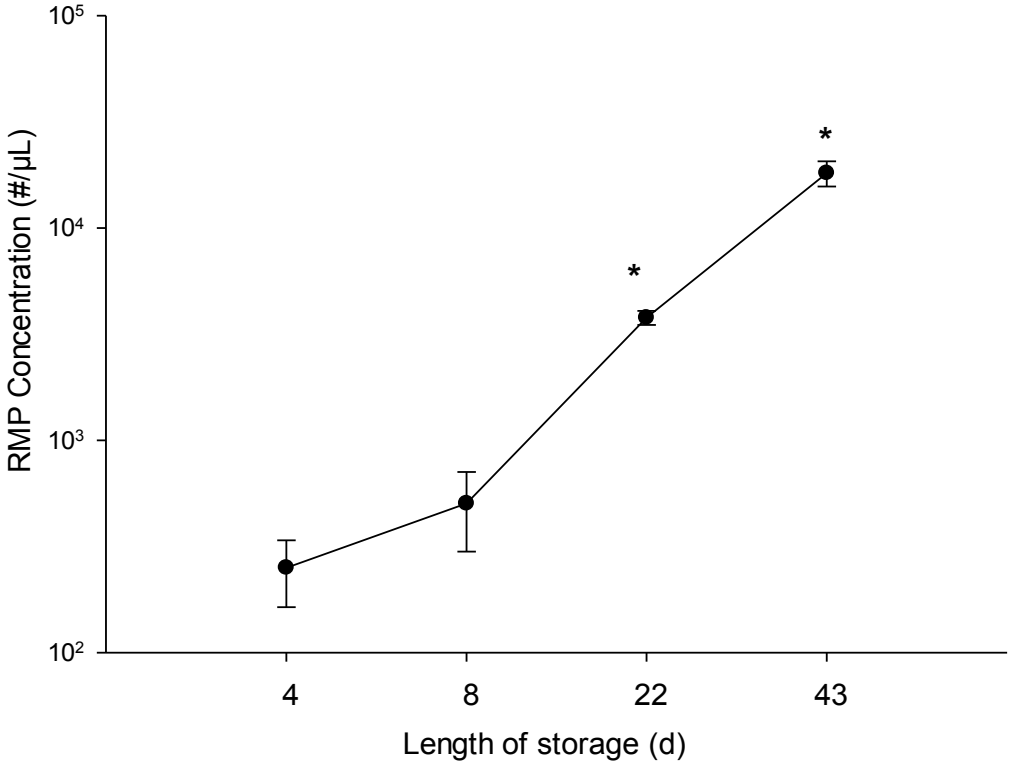

**Figure 2.3:** Dynamic light scattering assessment of RCC during storage for up to 43 d. The average size of EVs (d. nm) is reported (mean ± 1SD). *Significant results ($p < 0.05$) in comparison to day 4 values.

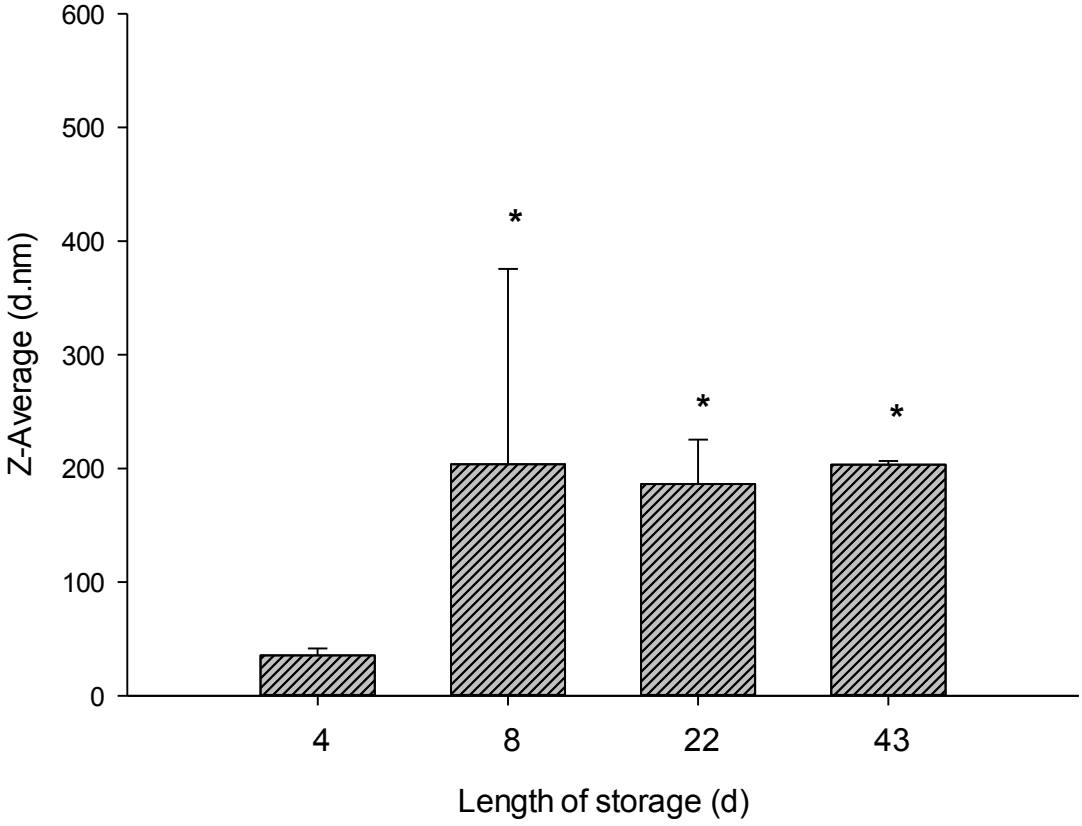

**Figure 2.4:** TRPS assessment of the concentration of EVs/mL in RCC stored for up to 42 analyzed using the IZON qNano system with NP400 (A) and NP200 (B). Data are reported as mean ± 1SD. *Significant results ($p < 0.05$) in comparison to day 3 values.

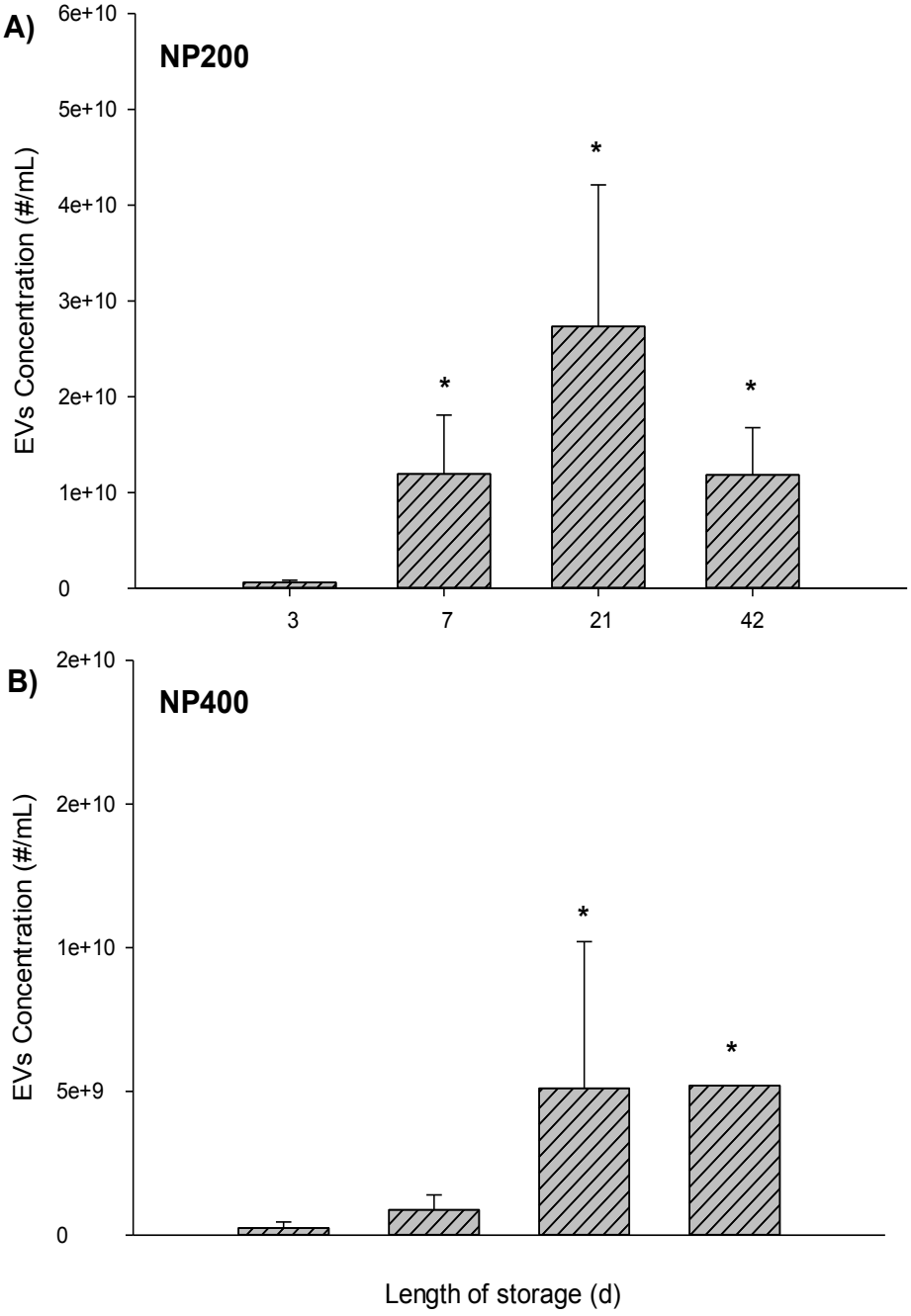

**Figure 2.5:** Concentration (mL) vs size (nm) histogram of extracellular vesicles in RCCs stored for up to 42 days as measured by the TRPS (qNano system). NP200 (Black), NP400 (Gray).

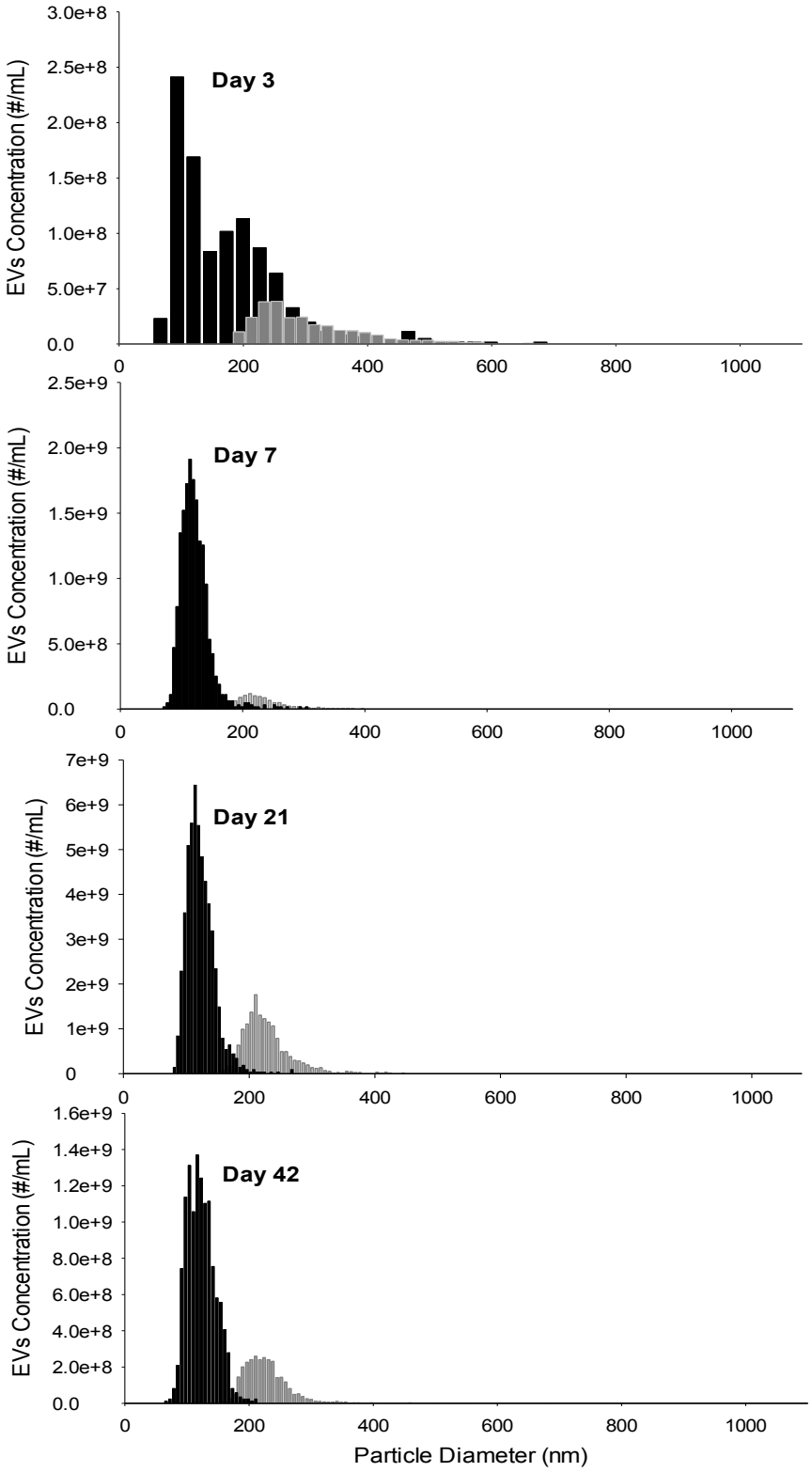

**Figure 2.6:** Relationship between the concentration (EVs/mL) of EVs from FC and TRPS (qNano); (A), small EVs (NP200), (B) large EVs (NP400), (C) total EVs (NP400 and NP200) in stored RCCs.

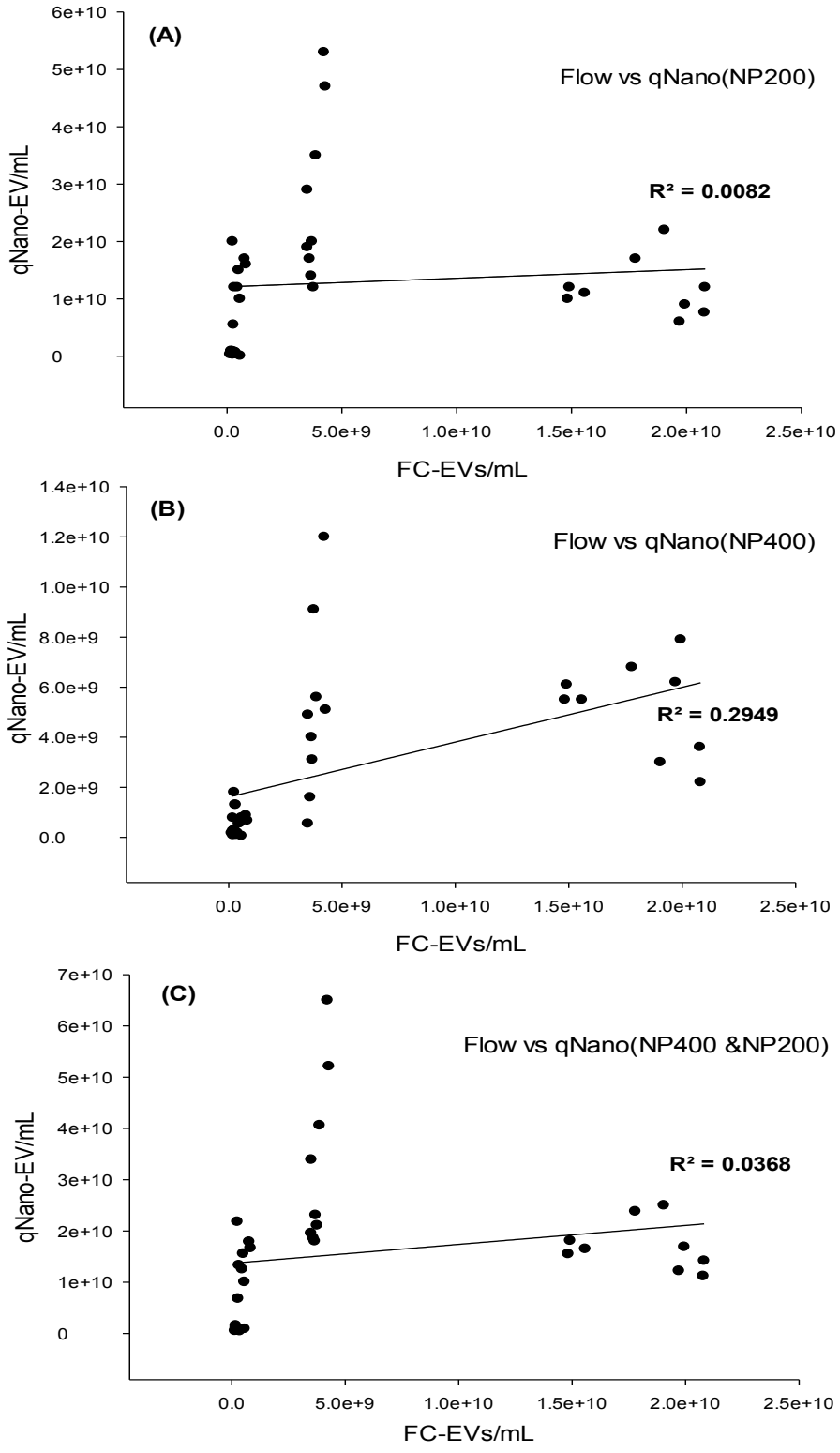

**Figure 2.7:** Relationship between the size (nm) of EVs from DLS (Malvern Zetasizer) and TRPS (qNano); (A), small EVs (NP200), (B) large EVs (NP400), (C) total EVs (NP400 and NP200) in stored RCCs.

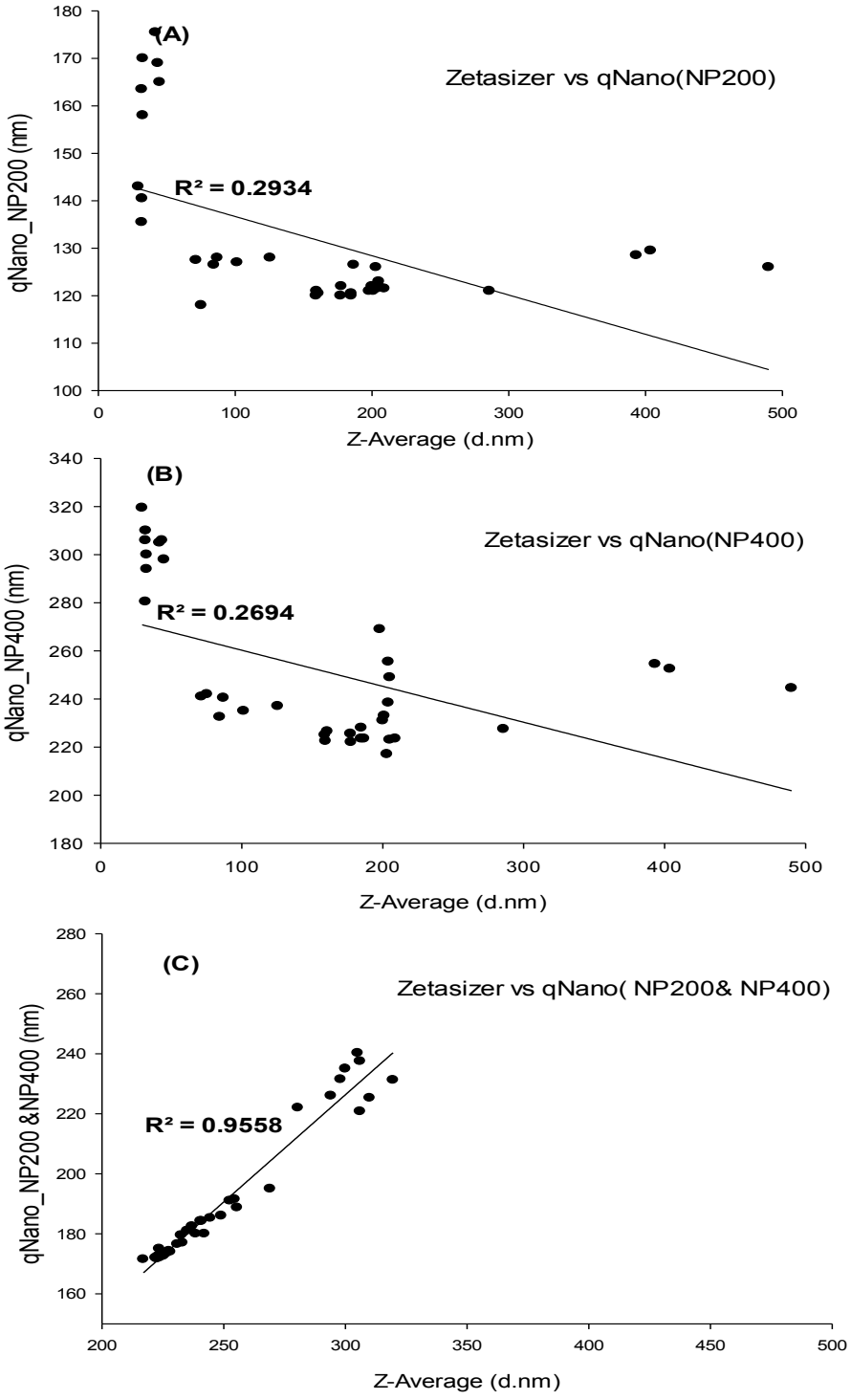

**Table 2.1:** Size of EVs from TRPS (qNano) measurements (Median ± 95% Cl)**.**

**qNano EVs size (nm) in diameter**

| | **Day 3** | **Day 7** | **Day 21** | **Day 42** |
|---|---|---|---|---|
| | Median ± 95 % Cl | Median ± 95 % Cl | Median ± 95 % Cl | Median ± 95 % Cl |
| NP200 | 163.5±11.2 | 127.5±2.6 | 120.5±1.6* | 122±1.2* |
| NP400 | 305.0±8.4 | 241.0±1.7 | 225.0±1.7* | 233±13.1* |

*Significant results ($p < 0.05$) in comparison to day 3

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# Chapter 3*

# Characteristics of Extracellular Vesicles in Red Blood Concentrates Change with Storage Time and Blood Manufacturing Method

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## 3.1. INTRODUCTION:

Red blood cell concentrates (RCCs) are used worldwide as a transfusion therapy product [1,2]. The quality control of the blood components is vital during processing and storage for the patient safety [3]. Although several accomplishments have been made to maintain the integrity and function of blood cells and to improve the quality of stored blood components for better transfusion outcomes [4-8], a number of studies have revealed that transfusion of red blood cell (RBC) products is still associated with increased risk of adverse clinical events [9-15]. Many recent studies have focused on effects of storage duration on the quality of blood products and their clinical consequences [10,11,13,16,17]. However, the quality of the RCC products can depend on several other factors such as donor-to-donor variability, variation in the quality testing methods, and blood manufacturing methods [1,2,18,19]. Although the processing of the blood components is controlled [3], the RCCs issued for transfusion are not manufactured or treated equally and the variations between RCCs produced by different manufacturing methods are not well appreciated. In Canada, the blood processing methods include red cell filtered (RCF, Top-and-Bottom) and whole blood filtered (WBF, Top-and-Top) RCCs [18]. Variations between differently manufactured RCCs have been observed in many studies, such as differences in levels of hemoglobin, hematocrit, and cytokines, all of which have been shown to influence the quality profiles of the transfused products [6,20-22].

Extracellular vesicles (EVs) vary in RCCs and plasma components depending on the blood processing method used [22-25]. Blood products can contain a mixed population of EVs, including exosome and microvesicles, which are heterogeneous submicron-sized vesicles surrounding by a phospholipid bilayer and contain proteins, lipid, and a variety of RNA molecules [26-30]. The presence of EVs in stored RCC products, which accumulate during storage, has been identified

as a significant indicator of the storage lesion [4,5,31,32]. Based on current knowledge, it has been suggested that EVs in stored blood are associated with a number of adverse outcomes such as neutrophil activation and promote an inflammatory response in the recipients of older blood [33-36]. Thus, EVs are potentially important in the quality of the blood products, *in vitro* and *in vivo*, and clearly require further investigations. Noteworthy, the heterogeneity of EVs such as their size, concentration, content and phenotype, is not well-considered in most of the studies investigating EVs in RCC products. These characteristics of EVs, particularly the small EVs/exosomes (50-200 nm in size), in stored blood components are not often reported due to the technical challenges in detecting these heterogeneous submicron-sized particles [37]. Although the flow cytometer is the most common technique used to identify and quantify EVs in suspension [29,38,39], EVs less than 300 nm are undetectable by this method [28,29,38]. Accurately distinguishing between diverse subpopulations of EVs in differently manufacturing RCCs is not usually considered in RBC storage studies. The aim of this study was to assess the impact of different blood manufacturing methods and duration of hypothermic storage (HS) on the subpopulations of extracellular vesicles in relation to other commonly evaluated *in vitro* quality parameters of RCC products.

## 3.2.MATERIALS AND METHODS

### 3.2.1. Blood Collection and Sampling

Whole blood was collected and RCCs were produced according to standard operating procedures at Canadian Blood Services (CBS). Briefly, whole blood units (n = 24) were collected from eligible donors and manufactured using either a red cell filtration [RCF;

top/bottom, n=12] or a whole blood filteration [WBF; top/top, n=12] as previously described[22,40].

During the RCF procedure, as described in Chapter 2, whole blood collected with 70 mL of citrate-phosphate-dextrose (CPD)-anticoagulant were rapidly cooled to 18-24 ºC and held overnight. Products were then centrifuged at 3493 × g for 11 min to separate the blood components (plasma, RBCs, and buffy coat) and saline-adenine-glucose-mannitol (SAGM) was added to the extracted RCCs. The RCC units were leukoreduced by filtration at room temperature within 24 hours of stop-bleed time. For the WBF units, whole blood was collected with 70 mL of CPD-anticoagulant, cooled (1-6 ºC) and leukoreduced by filtration within 48 hour of stop-bleed time before being separated. Filtered units were then centrifuged at 4552 × *g* for 6 min to separate the blood components (plasma and RBCs only). An automated extractor (Compomat G4) was used to extract plasma and then SAGM is added to RCCs. All RCC units produced with both manufacturing methods were stored at 1-6 ℃ for up to 43 days.

Red blood concentrate sampling was performed three times during the storage using a validated technique as previously described in Chapter 2. At each testing point (day 7, 21, and 42), 14 mL of RBCs was aseptically drawn from each bag into 15 mL conical tubes. For each sample, 10 mL was centrifuged at 2200 x g for 10 min at 4 °C (Eppendorf 5810R) and the supernatant was collected for analysis using the tunable resistive pulse sensing (TRPS) technology (qNano system; IZON Science Ltd, Christchurch, New Zealand). The remaining 4 mL of RBCs were used for *in vitro* quality assessments (hemolysis, spun hematocrit, deformability, ATP, 2,3-DPG, RBC hematologic indices) and flow cytometry.

### *3.2.2. In Vitro* Assessment of RCC Units*

*Hemolysis*

Hemolysis measurement is one of the most important and useful determinants of blood product quality[41,42]. Hemolysis signifies the disruption of the intact red blood cell (RBC) membrane which results in the release of hemoglobin (Hb) and in this study hemolysis was determined as previously described [6,31,43] using a Drabkin's-based spectrophotometric method, which is considered the gold standard method. For hematocrit (Hct), RCCs were aspirated into self-sealing hematocrit capillary tube and read visually after centrifugation for 5 min in a Hct centrifuge (Hettich Haematokrit Centrifuge Type 2010, Tuttlingen, Germany). Total hemoglobin (THb) was determined by diluting RBCs 1:200 in Drabkin's reagent (0.61 mmol/L potassium ferricyanide, 0.77 mmol/L potassium cyanide, 1.03 mmol/L potassium dihydrogen phosphate, and 0.1% Triton X-100). Supernatant hemoglobin (SHb) was determined by diluting the supernatant 1:12.5 in Drabkin's reagent and incubated in the dark for at least 5 minutes at room temperature. Two-hundred µL of each sample was transferred into a flat bottom microplate (Corning Life Science, USA) and the absorbance was measured spectrophotometrically at 540 nm using a microplate reader (SpectraMax 384 Plus, Molecular Devices Corp., Sunnyvale, CA). Commercial tri-level hemoglobin controls (low, medium and high ranges) were used as controls for total hemoglobin (Stanbio Laboratory, Boerne, TX). Hemolysis is then calculated using the hematocrit (Hct), the amount of SHb and total Hb (THb) in the sample using the following equation[42]:

$$\text{Hemolysis (\%)} = [(100\text{-Hct}) \times \text{SHb}]/ \text{THb} \qquad \text{Eq. 3.1}$$

* The author acknowledge Anita Howell and Tracey Turner (Centre for Innovation, Canadian Blood Services) and for their technical help with the RCC in vitro assessments.

Where:

Hct = hematocrit (%)

SHb = supernatant hemoglobin (g/L)

THb = total hemoglobin (g/L)

*RBC Deformability*

RBC deformability was measured using a laser-assisted optical rotational cell analyzer (Mechatronics, Zwaage, Netherlands) as previously described [44,45]. For this study, RBCs were diluted 1:100 in polyvinylpyrrolidone and subjected to shear stresses ranging from 0.95 to 30 Pa at a temperature of 37 ºC. The diffraction pattern produced by the scatter of a laser beam at each stress was collected and consequently plotted as a deformability curve. RBC deformability curves were linearized via the Eadie-Hofstee technique, as previously publised[44], to obtain two RBC deformation kinetic parameter ($EI_{max}$ and $K_{EI}$). $EI_{max,}$ a measure of deformability, can be defined as the maximum elongation index predicted at an infinite shear stress while $K_{EI}$, a measure of rigidity, can be defined as the shear stress required to achieve half of $EI_{max}$. All values are expressed as means ± standard error (SEM).

*RBC Hematological Indices*

Hematological indices, including RBC count, the mean corpuscular volume (MCV), mean corpuscular Hgb (MCH), mean corpuscular Hb concentration (MCHC), Hb, and Hct were determined using a hematological analyzer (Coulter Automated Cell Counter; Coulter AcT, Beckman Coulter, New York, NY)[45]. The RCC sample was mixed thoroughly by inversion and 12 µL was aspirated in open mode by the Coulter AcT 8 Hematology Analyzer. Samples were diluted inside the instrument, as described in the operator's manual (AcT Series Analyzer, 2010). RBC count (RBC/L) and MCV (fL) was determined based on changes in electrical impedance

as RBCs in suspension pass through the coulter aperture. Hct was calculated by the following equation:

$$\text{Hct} = (\text{RBC count} * \text{MCV}) / 10 \qquad \text{Eq. 3.2}$$

THb (g/L) was quantified by the system using a modified cyanmethemoglobin method. MCH is the average weight of Hb in in individual RBC, expressed in units picograms (pg) and calculated by dividing Hb concentration (g/L) by RBC count (RBCs/L). MCHC was expressed in grams/decilitre (g/L), calculated by dividing Hb concentration by the Hct.

*Adenosine Triphosphate*

Adenosine triphosphate (ATP) concentration of RCC samples was assessed spectrophotometrically using a commercially available kit and controls (DiaSys Diagnostic Systems GmbH, Holzheim, Germany), as previously described[6,31,45]. The RBC samples were added to 10% trichloroacetic acid, vortexed and placed on ice. The supernatants were then combined with substrates (glucose, and NAD+) and enzymes (hexokinase and glucose-6-phosphate dehydrogenase) required for the enzymatic reaction to occur. ATP from the sample is used in two chemical reactions, which ultimately convert glucose into 6-phosphogluconate and NADH. The resulting amount of NADH produced, which is proportional to the amount of ATP within the sample, was measured spectrophotometrically at 340 nm. The amount of ATP in the sample was calculated as µmol/dL using the following equation:

$$\text{ATP (µmol/dL)} = \frac{\Delta A \; x \; V \; x \; F \; x \; 100}{\varepsilon 340 \; x \; v \; x \; d} \qquad \text{Eq.3.3}$$

Where:

ΔA= (absorbance of the samples solution at 340 nm)-(absorbance of the blank at 340 nm),

V= total volume of the sample,

F=dilution factor of the sample preparation,

ε= extinction coefficient of NADH (6.3 at 340 nm),

v =sample volume used in ATP assay, and

d= light path (cm)

The amount of ATP in the sample was normalized using the total Hb concentration (μmol/g Hb).

*2,3-diphosphoglycerate*

Determination of 2,3-diphosphoglycerate (2,3-DPG) was performed using a commercial kit according to manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany) and normalized using the total Hb concentration as previously described[6]. RCC sample was diluted (1:5) with in 0.6 mol/L perchloric acid on ice then centrifuged to obtain a clear supernatant. Supernatant was diluted (8:1) with 2.5 mol/L potassium carbonate and incubated for 1 h on ice to neutralize the perchloric acid then centrifuged to obtain the supernatant. The extracted supernatant was used measure the 2,3-DPG from RBCs using a commercial kit according to manufacturer's instructions. The 2,3-DPG from RBCs is determined from a series of six chemical reactions which result in the production of glycerol-3-phosphate and the depletion of two NADH molecules per each 2,3-DPG molecule. NADH absorbance was measured at 340 nm on the SPECTRAmax PLUS 384 (Molecular Devices Corporation). The concentration of 2,3-DPG in the test sample, which is indirectly related to the amount of NADH remaining after the reaction, was calculated using SoftMax Pro software (Molecular Devices Corporation) according to the equation below:

$$2,3-\text{DPG (mmolL)} = \frac{\Delta A \; x \; V \; x \; F}{2 \; x \; \varepsilon 340 \; x \; v \; x \; 1} \qquad \text{Eq.3.4}$$

Where:

ΔA= (absorbance of the sample) – (absorbance of the blank) at 340 nm

Vs = sample volume used in the assay (µL)

F= dilution factor for sample

ε340= extinction coefficient of NADH at 340 nm (6.3 cm-1·mM-1)

L = light path (cm)

VT = total reaction volume (µL)

Distilled water was used as a blank and an in-house 2,3-DPG standard (2.5 mmol/L) prepared from (2,3-diphospho-d-glyceric acid pentasodium salt; Sigma-Aldrich, St. Louis, USA) was used as an assay control. The 2,3-DPG concentration was normalized using the Hb content of each sample and reported as µmol/g Hb.

All *in vitro* quality parameters were assessed on day 7 (fresh) and day 42 (expiry). The microparticle count by flow cytometry was measured on day 7, 21, 42. The extracellular vesicle size and concentration by the TRPS were completed on day 8, 22, and 43.

### 3.2.3. Extracellular Vesicles Characterization

#### *3.2.3.1. Flow Cytometry Assay for extracellular Vesicle Quantification*

The RBC microparticles (RMPs) count was measured based on a flow cytometry flow rate technique as previously published[31,37] and described in Chapter 2 with some modifications for the purpose of this study. In this study, the total RCC sample was used instead of the RCC supernatant as in Chapter 2 because the centrifugation force used to obtain RCC supernatant may pull down some RCC-EVs, such as RBC microparticles. Therefore, it was important for

the study to measure total RMPs to better correlate the total number of RMPs in the RCC products with the RBC *in vitro* quality parameters. In addition, the expression of phosphatidylserine (PS), which has been shown to contribute in RBC membrane lesion and microvesiculation during HS[31], was measured for further characterization of the surface of RMPs and assessment of the quality of RCC products. Therefore, two markers were used in this study: fluorescein isothiocyanate (FITC) anti-human CD235a antibody (MHGLA01, Invitrogen, Life Technologies, ON, Canada) which was used as a marker for RBCs and RMPs, and APC Annexin V (BD PharMingen, San Jose, CA) which was used to measure the exposure of phosphatidylserine (PS) on the particles. RBCs (5 µL) were diluted with 1X PBS then incubated with (FITC) anti-human CD235a antibody and APC Annexin V for 15 min in the dark at room temperature. Prepared samples were run on a bench-top digital flow cytometer (LSR-Fortessa X-20, BD Biosciences, San Jose, USA) and analyzed using BD FACSDiva 8.0.1 software (BD Biosciences, USA) as previously described in Chapter 2 (Section 2.2.3).

#### *3.2.3.2. TRPS Assay for Extracellular Vesicle Concentration and Size-profiling*

Quantification and size characterization of EVs in RCCs were measured using a tunable resistive pulse sensing instrument (qNano system; IZON Science Ltd) as previously described in Chapter 2 (Section 2.2.5).

### 3.2.4. Statistical Analysis

Statistical analysis was completed using computer software (IBM SPSS Statistics 23.0, Armonk, NY, USA). Analysis of variance (ANOVAs) followed by a Tukey post hoc test was used to identify significant differences within the storage period for EVs assays and to evaluate any significance amongst pairwise comparisons of testing time points during the storage time.

Using Univariate ANOVAs followed by Bonferroni test, interaction between the blood manufacturing methods and storage time was examined to determine if manufacturing method type effect varies with storage time. Paired t tests were used to identify significant differences between the testing time points (days 7 and 42) for the *in vitro* quality parameters. Correlational analysis was performed using SigmaPlot 13.0 (Systat Software Inc.). Probability (p) values less than 0.05 were considered significant.

## 3.3. RESULTS:

### 3.3.1. Flow Cytometer: RBC Microparticles and Phosphatidylserine Expression Change with Storage Duration and Blood Manufacturing Methods

Flow cytometric analysis showed that the absolute number of $CD235^+$ microparticles/μL gradually increased throughout the storage period for both blood processing methods, RCF and WBF (**Figure 3.1A**). In both blood manufacturing methods, a significant increase in the number of RMPs/μL was observed on day 21 (RCF; $p = 0.003$, WBF; $p = 0.017$) and day 42 (RCF and WBF; $p < 0.001$) of HS in comparison to day 7. Early in storage (day 7), a significant difference in the number of RMPs/μL between the RCF and WBF methods was seen ($p = 0.033$). Further flow cytometric investigation revealed differences between the RCF and WBF methods in the percentage of RMPs expressing PS on day 7 ($p < 0.001$) and day 42 ($p = 0.010$). While there was no significant change in the percentage of RMPs expressing PS observed during storage with RCF-RCCs, a significant decrease in the percentage RMP-PS was detected on day 21 and day 42 ($p <0.001$) with WBF-RCCs. Further analysis of RMPs showed significant decreases in the median fluorescence intensities (MFIs) for the expression of PS on RMPs on day 42 of storage (**Figure 3.1C**) for both blood manufacturing products (RCF; $p = 0.035$, WBF; $p <$

0.001). Noteworthy, throughout HS, MFIs of RMP-PS for RCF samples was significantly lower than WBF samples ($p < 0.001$, **Figure 3.1C**).

### 3.3.2. TRPS: Extracellular Vesicles in Stored RCCs Differ Between the Blood Processing Methods

The TRPS analysis showed that heterogeneous submicron-sized vesicles are present in RCC products and accumulate during storage (**Figure 3.2** and **Figure 3.3**). Although the total concertation of EV/mL increased significantly during storage in RCF and WBF units, RCCs produced by WBF contained greater numbers of EVs in comparison to RCF (**Figure 3.3C**). Extracellular vesicles in the stored RCC products were further categorized based on their size as either small EVs (EVs < 200 nm) or large EVs (EVs ≥ 200 nm). This characterization shows that the concentration of small EVs was higher in WBF units in comparison to the RCF (**Figure 3.3**), and a significant difference between the two methods was observed again on day 43 of storage ($p = 0.0015$, **Figure 3.3A**). Statistically significant differences between the blood processing methods in the number of EV/MPs ≥ 200 nm were identified only on day 22 of storage ($p = 0.0017$, **Figure 3.3B**). In addition, EVs size profiling showed significant differences between RCF and WBF products on day 22 and 43 of storage ($p < 0.01$, **Table 3.1**).

### 3.3.3. RBC *in Vitro* Quality Parameters are Affected by Blood Manufacturing Methods and Storage Time

Significant differences were observed between RCF and WBF methods in the level of ATP at the end of HS time (day 42; $p = 0.0102$**, Table 3.2**). In addition, there was a statistical significant decrease in the levels of ATP and 2,3-DPG on day 42 of storage compared to day 7 ($p < 0.001$).

In both RCF and WBF manufacturing methods, MCV slightly increased while MCHC significantly decreased throughout storage ($p < 0.001$, **Table 3.2**). Furthermore, the MCHC was significantly higher in WBF method in comparison to the RCF method early in the storage ($p = 0.0162$) and at expiry ($p = 0.0496$). However, there was no significant change detected in MCH during storage or between processing methods.

There was a significant increase in percent hemolysis during storage in both RCC products (RCF and WBF; $p < 0.001$, **Table 3.2**). Notably, on day 42, WBF method showed greater percent hemolysis in comparison to the RCF method ($p = 0.005$). Nevertheless, all samples fell within the acceptable level of hemolysis (less than 0.8 %) required by to the Canadian Standards Association [46,47].

Red blood cell deformability measurements showed slight but statistically significant differences throughout storage in RCF and WBF methods (**Table 3.2**). $El_{max}$, which is a measure maximum elongation index of RBC, decreased significantly at day 42 in comparison to day 7 of storage in RCF ($p < 0.001$) and WBF method ($p = 0.039$). Although RCCs produced by the RCF method were associated with lower $El_{max}$ at expiry ($0.50 \pm 0.01$) in comparison to WBF units ($0.52 \pm 0.02$), no statistical significant differences were identified between the two groups ($p = 0.095$). In addition, there were no significant difference in $K_{EL}$, which is a measure of RBC rigidity, between the two processing methods at early storage ($p = 0.452$) or at expiry ($p = 0.215$).

The relationships between the microvesiculation and *in vitro* quality parameters throughout the HS period were further investigated for both RCF and WBF RCCs (**Figure 3.4**). Moderate negative correlations were identified between number of EVs and level of ATP in both manufacturing methods (RCF: $R^2 = 0.562$, WBF: $R^2 = 0.562$) (**Figure 3.4A**). Additionally,

while less strong negative correlations were observed between total number of EVs and $EI_{max}$ was observed with RCF units ($R^2 = 0.456$), even weaker correlations were identified with WBF units ($R^2 = 0.346$) (**Figure 3.4B**). Correlation analysis also showed a moderately positive relationship between EVs concentration and percent hemolysis in both processing methods (RCF: $R^2 = 0.443$, WBF: $R^2 = 0.478$) (**Figure 3.4C**).

## 3.4. DISCUSSION

Although regulatory standards are applied to the processing and storage of RCCs to ensure the safety of the blood products, a number of product quality characteristics have been shown to be impacted by the manufacturing method and hypothermic storage [3,22,31,48]. Currently, the role that the storage duration may play on the quality of blood products and transfusion outcomes has been the focus of several studies [22,35]. Variability exists within the blood products themselves [48,49], which may contribute to the storage lesions and adverse clinical outcomes. The blood component manufacturing processes and donor characteristics have emerged as major elements to explain some of the variability and conflicting clinical observations detected amongst blood products [22,48]. Here, we add to the current understanding on the impact of blood manufacturing methods and hypothermic storage duration on the characteristics of different subpopulations of EVs and *in vitro* quality parameters of RCCs produced by RCF and WBF processing methods. Significant differences in extracellular vesicles subpopulations and concentrations as well as cellular quality parameters were observed amongst RCCs products throughout storage time.

Data from TRPS showed that the total number of EVs increase significantly during HS in both methods ($p<0.05$) and WBF contained the highest number of EVs in comparison to RCF which is consistent with other studies by our group [19,22,31]. It has been suggested that the characteristics of EVs can vary among the blood products due to the variation in the blood processing methods [50,51]. Bakkour *et al.* [19] highlighted some feasible reasons to explain the variation in EVs characteristics between RCF and WBF RCCs including variability in the temperature and the lengths of preprocessing storage time as well as leukoreduction technique. For instance, WBF filtration occurs before centrifugation and component separation while for RCF, leukoreduction occurs after these processes. Taking into account that the leukoreduction removes not only leukocytes but also platelets and platelet-derived EVs from RCCs [52], and the buffy-coat removal improves the efficacy of pre-storage leukoreduction [53], it would be logical to predict that the lower EVs count in RCF in comparison to WBF RCC (**Figure 3.1**) may be due to the leukoreduction process. This is consistent with recent reports showing that RCF units have lower concentrations of platelet-EVs and WBC-EVs when compared with other blood manufacturing methods, including the WBF method[54] . This also can explain our novel data captured by the TRPS which showed that the concentration of small EVs/ exosomes (< 200 nm) was greater in WBF units in comparison to the RCF with significant differences between the two methods on day 43 of storage ($p=0.001$). It has been previously shown that RCF RCCs contain fewer residual cells (platelet and white blood cells) in comparison to WBF RCCs as the RCF method removes the majority of the undesirable cells during the preparation of the buffy coat and before the leukoreduction [19]. In view of that, it is also more likely that WBF RCCs contain more small EVs such as platelet-derived EVs and WBC-derived EVs in comparison to RCF RCCs. However, it is important to mention that identifying the cell of origin of these small

EVs (< 200 nm) detected in this study is yet to be elucidated, especially that most of the studies investigating EVs in RCC products account only for the microparticles that are characterised by the flow cytometer which lacks the ability to resolve the small EVs. Recently, great attention has been directed to the role of the "contaminating" residual cells in RCCs, but the role of the EVs derived from these residual cells, which likely reflecting the same role as their cell of origin, is still narrowly considered. Therefore, further studies are required to investigate the cell of origin of these EVs, particularly the small EVS, and their potential role on the quality of the products as well as their immunomodulatory effects post transfusion.

In addition to the EVs data from TRPS, FC analysis showed that the concentration RBC (CD235+) derived MPs significantly increased during the storage period in both manufacturing methods. It has been suggested that RBC membrane changes occur *ex vivo,* and there is a reduction in ATP and an increase in hemolysis, which are clearly associated with RBC membrane changes and microvesiculation [31,50,55-57]. This supports the findings of this study where a positive correlation were observed between EVs and hemolysis, and a negative correlation with the level of ATP and deformability parameters ($El_{max}$). Therefore, we hypothesize that the EV profile differences detected in the first week of storage are likely to be attributed to the cellular lesions induced by the variation in the blood processing methods, while the differences observed after the first week, toward the end of storage, are more likely reflecting the storage-related impacts [22].

In this study, assessing the RBC metabolism including ATP and 2,3-DPG showed significant depletion of these key metabolites by the end of storage (**Table 3.2**). Depletion of ATP has been correlated with the *in vivo* survival of RBCs post transfusion and the loss of 2,3-DPG from RBCs can impaired oxygen transporting capacity[58]. Notably, ATP concentrations

was significantly lower in the WBF (1.79 ± 0.44) in comparison to RCF units (2.42 ± 0.64) on day 42 of storage. The level of ATP in the majority of WBF RCCs fell below the minimum recommended range of 2.3 to 2.7 µmol/g Hb, which has been shown to correlate with 75% survival of transfused RBCs 24 hours post-transfusion[59]. However, as has been recently shown in metabolomics, lipidomic and proteomic studies, the hypothermic storage lesion involves many more changes to RBC metabolism that simply depletion of ATP and 2,3 DPG. Further studies are required to investigate the clinical impact of metabolic changes to stored RCCs.

Additionally, WBF RCCs showed stronger correlation between EVs and hemolysis throughout HS in comparison to RCF RCCs. Furthermore, FC data also revealed that the RMPs and the percent of these RMPs expressing PS, a procoagualent factor, were significantly lower in RCF in comparison to WBF RCCs. Increasing the number of RMPs, which contain hemoglobin and expressing PS, along with free hemoglobin from hemolysis, are more likely to influence recipient immune response post transfusion [22]. For example, it has been shown that RMPs containing hemoglobin have the ability to scavenge nitric oxide with the potential of reducing its bioavailability in post transfusion resulting in impaired vascular function [60]. Moreover, Camus and his group revealed that the RMPs carrying heme are cytotoxic as they can induce oxidative stress and apoptosis by activating the production of the endothelial reactive oxygen species [61]. Noteworthy, recent study by Danesh *et al.*[62] showed that the EVs concertation in stored leukoreduced RCCs was largely due to the increase in both RBC (CD235+) derived EVs and platelet ($CD41a^{+}$) derived EVs. They also demonstrated that leukoreduced RCCs contain small EVs/exosomes that are positive for CD63 marker and these exosomes have the capability to induce cytokine TNF-a secretion in monocytes. However, the accurate size, concentration and as well as the phenotype of these exosomes were not revealed.

The results of this study highlight the differences between the differently manufactured RCC products in term of the size and concentration of EVs, especially small EVs. However, the differences in the final manufactured products may not only influence the quality of the blood products but also may influence patients clinical outcomes [6]. Therefore, more studies are required to examine the potential adverse clinical outcomes of the EVs found within RCCs produced by different manufacturing methods in order to provide better blood products in clinical care. However, it is important to mention that it is not yet clear whether these findings and differences observed are due to the differences in manufacturing methods or a contribution of the manufacturing with other variables such as donor characteristics. Although donor factors such as sex and age may influence RCC products during storage [48], the main focus of this project was to understand the role of manufacturing process and storage duration on the quality of RCC products. Further comprehensive studies are needed to understand the role of donor factors, storage duration, and blood manufacturing processes on the patient outcomes.

## 3.5. CONCLUSION

Here we show that heterogeneous submicron-sized vesicles are present in RCC products and the diverse populations of EVs is dependent on the blood manufacturing method. RCCs produced by WBF contained greater numbers of EVs in comparison to RCF, particularly due to the higher concentration of small EVs/exosomes in WBF. This study also showed that the size-profile and concentration of EVs is in a dynamic state of change throughout the RCC hypothermic storage. Differences in the final manufactured products may not only influence the quality of the blood products but also may affect patients' clinical outcomes. Therefore, further investigations to improve our understanding of the factors or processes that might be causing

the variation amongst blood products is warranted in order to develop better strategies to minimise the risk associated with the transfusion of RCCs produced by different blood manufacturing methods, and to ensure better quality products are provided in to patients.

**TABLES:**

**Table 3.1:** Modal size of EVs from qNano measurements (Mean ± SD). (*) Significant results ($p < 0.05$) in comparison to day 8 values. (†) Significant results ($p < 0.05$) in comparison to WBF method.

| Methods | EVs Size (nm) | Day 8 | Day 22 | Day 43 |
|---|---|---|---|---|
| **RCF method** | | | | |
| | *EVs < 200* | 180 ± 7.2 | 186 ± 6.0† | 187 ± 6.7†* |
| | *EVs ≥200* | 211 ± 15.8 | 205 ± 1.4† | 208 ± 3.4† |
| **WBF method** | | | | |
| | *EVs < 200* | 177 ± 7.9 | 168 ± 12.5 | 168 ± 6.6 |
| | *EVs ≥ 200* | 214 ± 11.5 | 219 ± 5.7 | 218 ± 6.2 |

**Table 3.2:** Hemorheology and metabolism parameters for RCCs stored for up to 42 days (mean ± SD). (*) Significant results ($p < 0.05$) in comparison to day 7 values. (†) Significant results ($p < 0.05$) in comparison to WBF method.

| | RCF (Top/Bottom) | | WBF (Top/Top) | |
|---|---|---|---|---|
| **Methods** | **Fresh** | **Expired** | **Fresh** | **Expired** |
| **Metabolism** | | | | |
| ATP (µmol/g Hgb) | 4.25 ± 0.63 | 2.42 ± 0.64*† | 3.90 ± 0.62 | 1.79 ± 0.44* |
| 2,3 DPG (µmol/g Hgb) | 5.10 ± 2.98 | 0.00 * *(BDL)* | 5.49 ± 2.10 | 0.00 **(BDL)* |
| **Hematologic Indices** | | | | |
| MCV (fL) | 93.16 ± 4.15 | 96.93 ± 4.04 | 91.89 ± 3.95 | 94.78 ± 4.18 |
| MCH (pg) | 29.73 ± 1.57 | 29.55 ± 1.15 | 29.98 ± 1.43 | 29.34 ± 1.33 |
| MCHC (g/L) | 319.08 ± 6.20 † | 304.83 ± 5.34*† | 326.42 ± 7.54 | 309.67 ± 6.04* |
| **Hemolysis (%)** | 0.09 ± 0.02 | 0.22 ± 0.05*† | 0.13 ± 0.06 | 0.35 ± 0.15* |
| **Deformability** | | | | |
| Elmax | 0.53 ± 0.01 | 0.50 ± 0.01* | 0.53 ±0.01 | 0.52 ± 0.02* |
| KEl | 1.43 ± 0.13 | 1.41 ± 0.18 | 1.52 ± 0.26 | 1.56 ± 0.31 |

Data are reported as mean ± SD *BDL= Below Detection Limit*

**FIGURES:**

**Figure 3.1:** Flow cytometer results shown the absolute number of $CD235^{+}$ microparticles/µL (A), the percent RMP-PS, and MFI of PS on RMPs in stored RCC products (RCF and WBF). Data are reported as mean ± 1SD. *Significant results ($p < 0.05$) in comparison to Day 7 values. (δ) Significant results ($p < 0.05$) in comparison to WBF method.

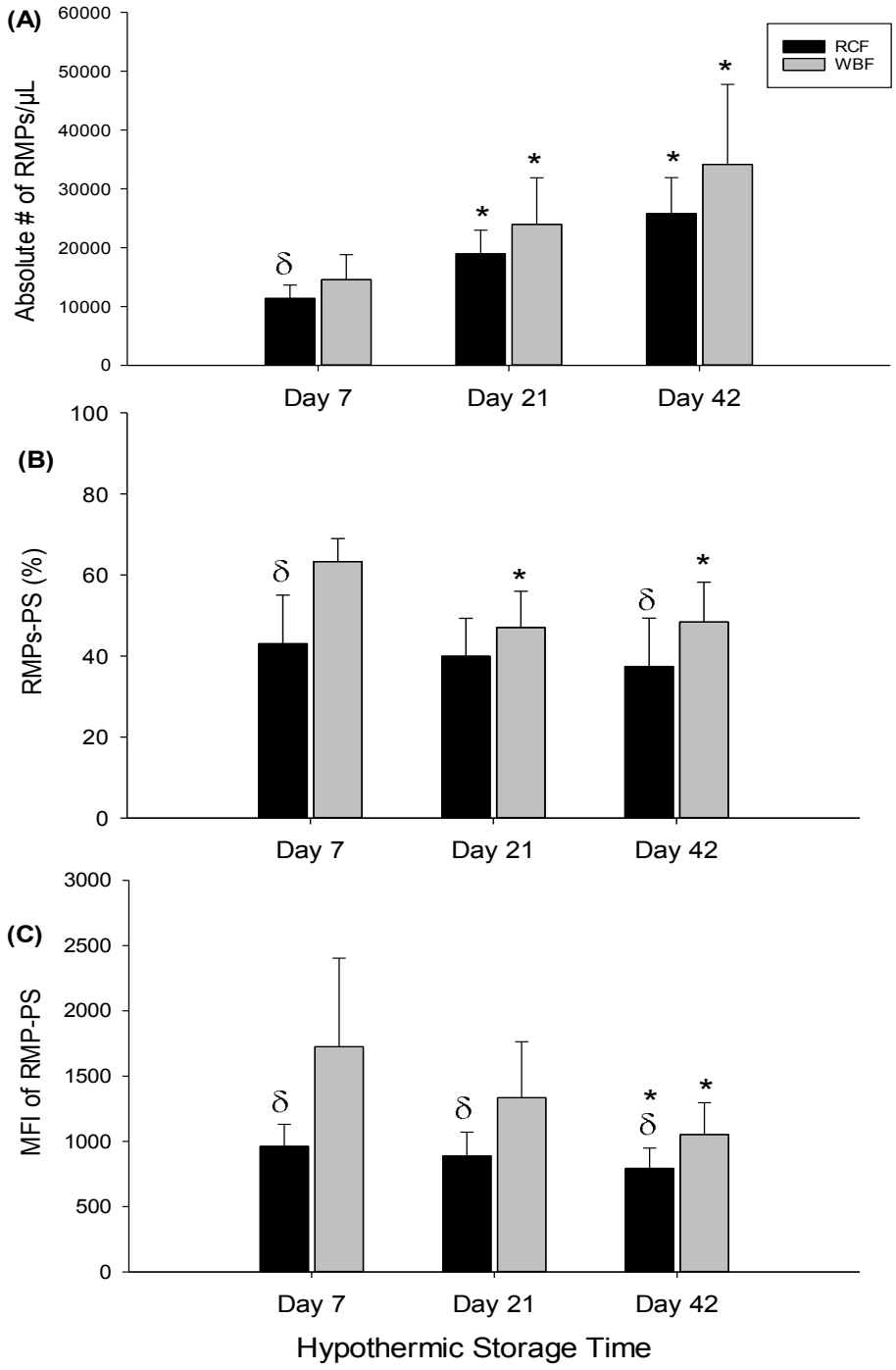


**Figure 3.2:** Representative concentration (#/mL) versus size (nm) histograms of extracellular vesicles in hypothermically stored RCCs (WBF; A and RCF; B) up to 43 days as measured by the TRPS.

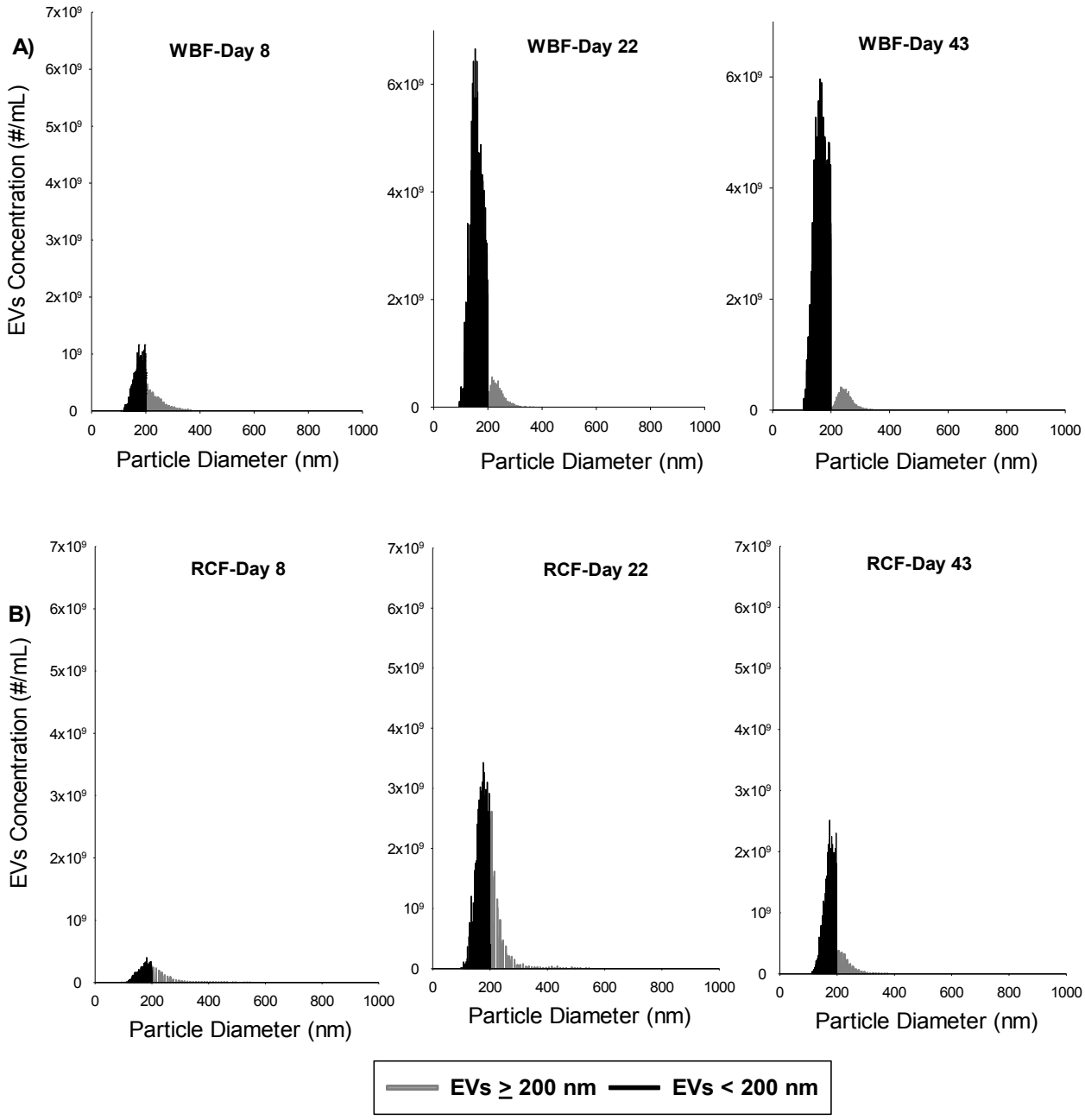
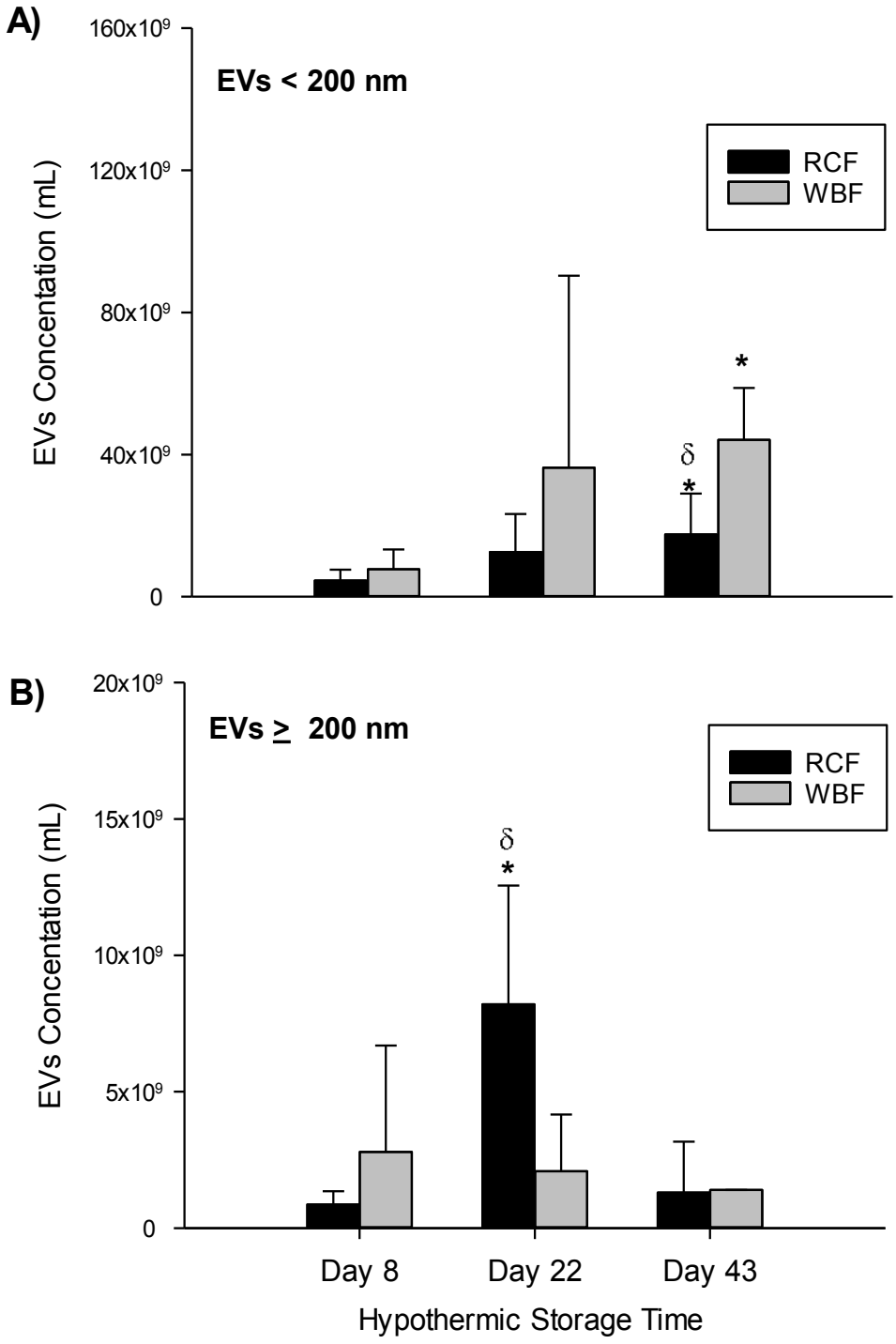


**Figure 3.3:** Concentration of EVs/mL in RCC products stored for up to 43 d analyzed by the TRPS system; (A) EVs < 200 nm using NP200 , (B) EVs ≥ 200 nm using NP400, and (C) total EVs using NP200 and NP400. Data are reported as mean ± SD. Significant results ($p < 0.05$) in comparison to day 8 values (*) or in comparison to B2 method (δ).



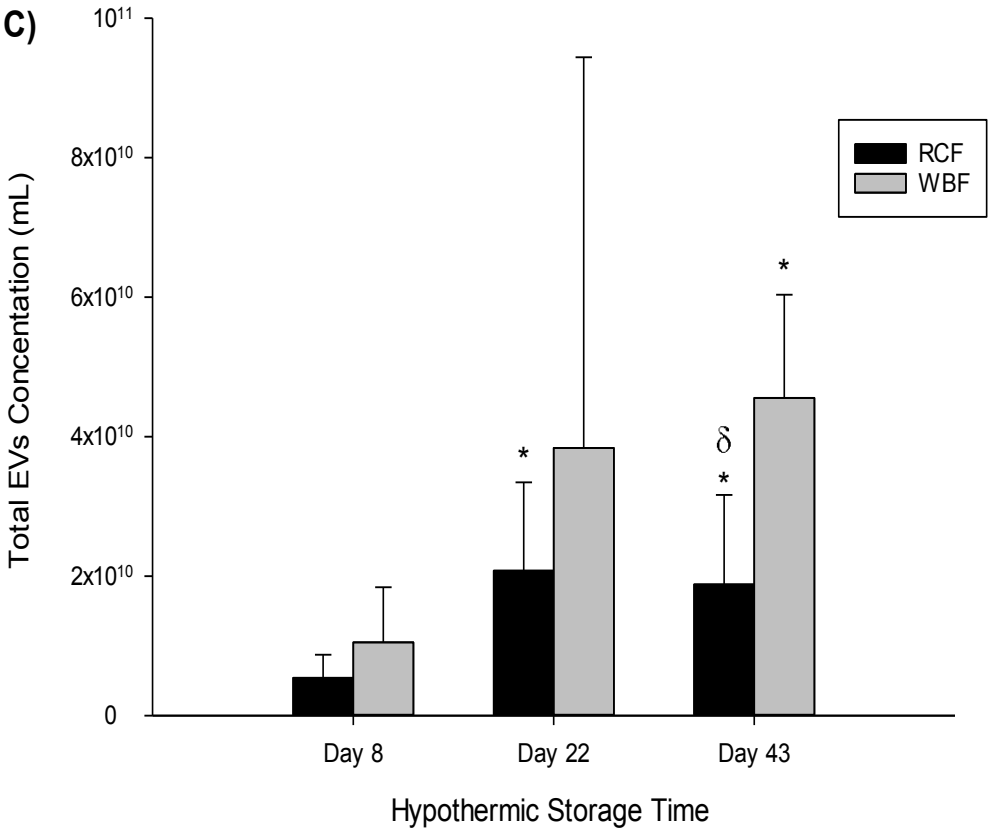
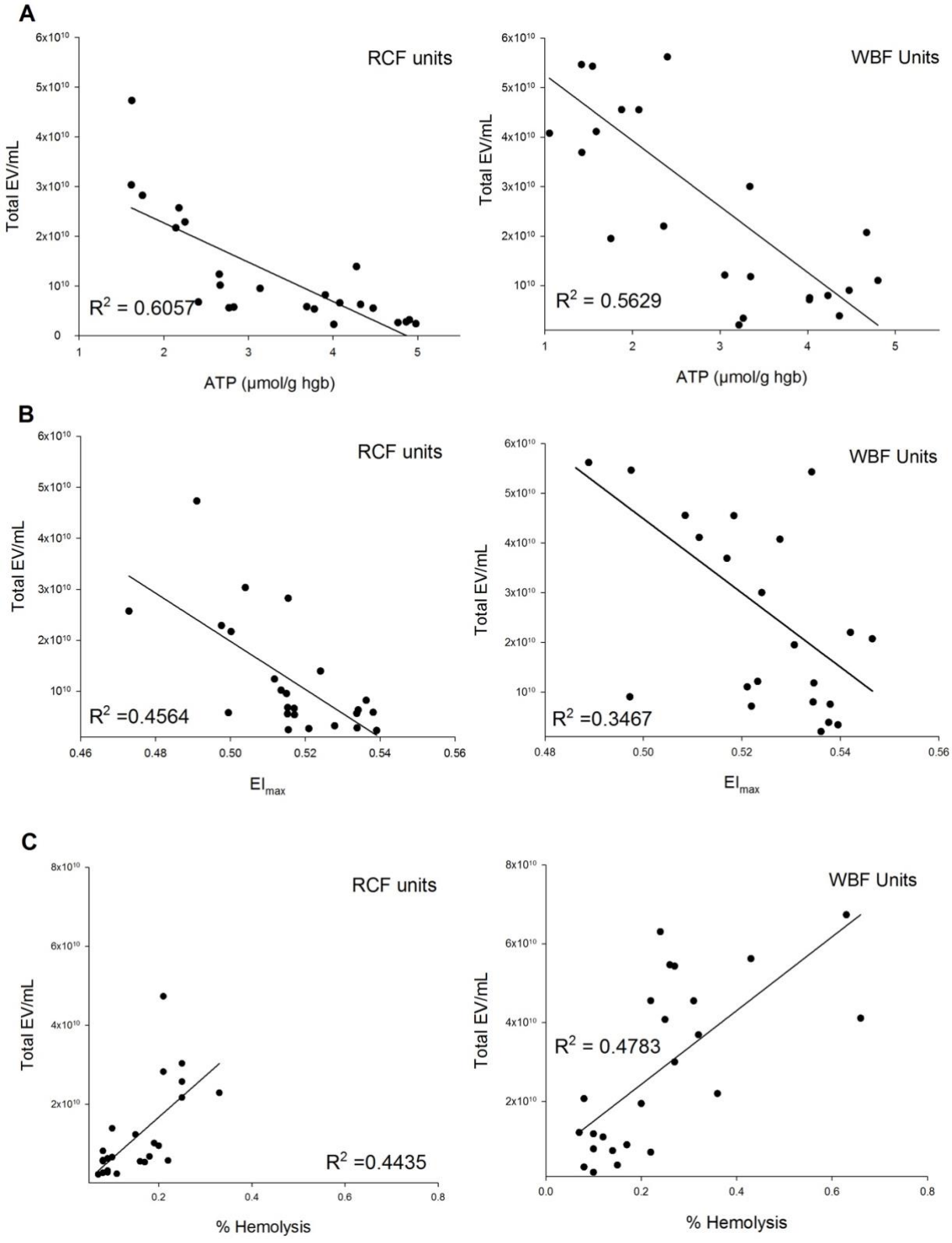

C)
$10^{11}$
$8x10^{10}$
$6x10^{10}$
$4x10^{10}$
$2x10^{10}$
0
Total EVs Concentation (mL)
RCF
WBF
*
*
δ
*
Day 8
Day 22
Day 43
Hypothermic Storage Time

**Figure 3.4:** Relationship between the total concentrations of EVs (EVs/mL) using TRPS and; (A), ATP, (B) $El_{max}$, (C) % hemolysis of stored RCCs (RCF and WBF method).



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# Chapter 4*

## Blood Manufacturing Methods Affect Red Blood Cell Product Characteristics and Immunomodulatory Activity

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## 4.1. INTRODUCTION

Red blood cell transfusion remains common, particularly in critically ill patients[1-3]. However, transfusion of red cell concentrates (RCC) is independently associated with increased risks of nosocomial infection, organ dysfunction, and death[4-6]. Transfusion-related immunomodulation (TRIM) includes both immunosuppressive and inflammatory effects that may in part explain increased risks in patients who receive blood transfusions[7-11]. Mechanisms of adverse effects related to red cell transfusion remain uncertain, though RCCs contain a host of biologically active mediators, in both soluble and cell-associated forms, which may contribute to organ dysfunction via alterations in recipient inflammation and immune cell function[7,12-14]. While many previous studies have focused on accumulation of potentially harmful immunomodulatory mediators during RCC storage[8,15,16], recent randomized clinical trials have failed to demonstrate benefit with fresh RCC transfusion in critically ill or hospitalized patients[17], thus calling into question the clinical relevance of storage-related TRIM effects. Noteworthy, it has been suggested that RCC manufacturing methods, which are rarely accounted for in interventional trials, may have confounded these results[18,19]. Differences in blood component manufacturing methods and RCC characteristics across clinical trial sites may mask the effect of RCC storage duration on patient outcomes[18,19]. Indeed, in a large Canadian registry study comparing the whole blood filtration method to the red cell filtration method for RCC product preparation, transfusion with fresh whole blood filtered red cells was independently associated with in-hospital mortality[20]. Differences in blood component manufacturing methods may result in significant differences in potential immunomodulatory mediators, such as intracellular factors released by hemolysis, residual platelets and leukocytes,

and extracellular vesicles (EVs), and may play a significant role in post-transfusion immunomodulatory effects[21-24].

In addition, the presence of EVs in RCC product is an important factor that has emerged as a potential mediator of the immunomodulatory activity post transfusion [25-27]. EVs, heterogeneous submicron-sized vesicles, are produced and released by many types of cells[26,28]. However, most studies do not take into account the heterogeneity of EVs in RCCs in terms of the size, phenotype /cell of origin, composition and surface biomarkers. As these EVs, which accumulate in RCC during storage, can differ in terms of their biogenesis and biophysical properties[29] and can be influenced by different blood manufacturing methods, their immunomodulatory activity may vary as well. Thus, the aim of this study was to investigate the impact of different manufacturing methods on RCC characteristics, including hemolysis, residual cell counts, and extracellular vesicles; and on immunomodulatory activity of RCC supernatants on monocyte function.

## 4.2. MATERIALS AND METHODS

### 4.2.1. Blood Collection and Manufacturing

All blood donors provided signed, informed consent at the time of donation. Whole blood was collected from healthy donors and RCCs (n = 32) were produced using four different blood manufacturing methods[30] (8 units per method). Whole blood filtered (WBF) and red cell filtered (RCF) were collected by Canadian Blood Services while apheresis derived (AD) and whole blood derived (WBD) were collected by Blood Systems Inc. in the United States.

*Whole Blood Filtration Method*: whole blood was collected into blood collection sets (DQE 7292LX, Leucoflex MTL1 quadruple Top/Top system, MacoPharma) processed using the whole blood filtration method as described in Chapter 3 (Section 3.2.1).

*Red Cell Filtration Method*: Whole blood was collected into blood collection sets (LQT 7292LX Leucoflex LCR-Diamond quadruple Top/Bottom system, MacoPharma) and processed using the Red Cell Filtered method as described in Chapter 2 (Section 2.2.1).

*Apheresis Derived Methods;* RCCs collected using apheresis cell separators (Trima Aceel® Apheresis System, Terumo BCT; Software 6.0.6; Trima Accel 80500 kit) with 70 mL of anticoagulant Citrate Dextrose Solution, Solution A (ACD-A) *and* 200 mL Additive *Solution* (*AS-3).* After collection, *RCC* units were filtered at room temperature.

*Whole Blood Derived Method:* Whole blood was collected into blood collection sets (Fenwal 4R1587P Flex Triple, WB 500 ml) with 70 mL of citrate-phosphate-dextrose (CPD)-anticoagulant. WB units were centrifuged at 5895 x*g* for 8 min at 1–6 °C. Plasma was extracted, the RCC was retained in the original bag, and 110 mL of Additive *Solution* (AS-1) was added.

### 4.2.2. Shipping, Storage and Sampling*

Using packing configurations designed to maintain RCC at an appropriate temperature (1-10 °C), RCC units were shipped to the Canadian Blood Services laboratory in Edmonton, AB, Canada. All shipments arrived within 24 h of being packed and RCCs were stored between 1-6 °C in a monitored refrigerator for up to 42 days. RCC sampling (25% of the unit volume) was

* The author acknowledge Anita Howell, Tracey Turner, Angela Hill, April Xu (Centre for Innovation, Canadian Blood Services) and Dr. Luciana da Silveira Cavalcante (Department of Laboratory Medicine and Pathology, University of Alberta) for their technical support.

performed once on day 5 (fresh) and once on day 42 (expiry) post-collection using a validated sampling technique as previously described in Chapter 2. An aliquot (5 mL of RCC) of each day 5 sample was used for residual cell counting and *in vitro* quality parameter testing. The remaining RCC samples were centrifuged at 1000 x *g* for 10 min at 4 °C (Eppendorf 5810R) to separate cells from supernatant. Supernatant was collected and transferred to cryovials and frozen at ≤ 65 °C. One frozen supernatant aliquot from each unit (fresh and expiry) was used at Canadian Blood Services for *in vitro* quality assessments and to measure EV concentration and size profile by qNano. Additional frozen supernatant aliquots from each unit (fresh and expiry) were shipped on dry ice to two centers for additional analyses: 1) Blood Systems Research Institute (San Francisco, CA) to determine the cell of origin of EVs by flow cytometry; and 2) The Research Institute at Nationwide Children's Hospital (Columbus, OH) for monocyte co-culture testing. All testing was performed on the day 5 and day 42 aliquots, except residual cell counts, which were only measured on day 5 (**Figure 4.2.**).

### 4.2.3. *In Vitro* Quality Assessment of RCC Units

*Hemolysis*

Hemolysis was determined using a Drabkin's-based spectrophotometric method as previously described in Chapter 3 (section 3.2.2).

*Supernatant Potassium*[*]

Supernatant samples were sent to an accredited laboratory (Alberta Health Services, Edmonton, Alberta, Canada) for analysis on an automated chemistry analyzer (DXC800 System, Beckman Coulter, Inc., Fullerton, CA) to measure supernatant potassium

[*] Potassium supernatants were analyzed by Laboratory Services at the University of Alberta Hospital.

concentrations as described previously[31]. To measure potassium concentrations, RCC supernatant was mixed with a buffered solution, which is used to establish a constant activity coefficient for potassium ions and calibrating the electrode to concentration values. Potassium ion concentration is then determined by indirect potentiometry utilizing a potassium ion selective electrode in conjunction with a sodium reference electrode in the analyzer.

### 4.2.4. Residual Cell Counts*

Red cell concentrate samples were sent to the Canadian Blood Services National Testing Laboratory (Ottawa, ON, Canada) to determine residual white blood cell (WBC) levels using flow cytometry as previously described[22]. White cell fixative (50 µL) was added to the RCC sample (450 µL) in a labelled microcontainer dry $K_2$ EDTA tube and sent to the laboratory for testing. As per the manufacturer's instructions, a WBC enumeration kit (LeukoSure, Beckman Coulter, Mississauga, Ontario, Canada) was used to determine the absolute WBC counts by flow cytometry. Briefly, lyse reagent from the LeukoSure kit were added to the RCC sample to lyse the RBCs and permeabilize WBCs. the samples then stained using the LeukoSure stain reagent and incubated for 15 minutes at room temperature in the dark. Thus, the nucleated cells (WBCs) in the sample emit fluorescence in proportion to their DNA content while the mature RBCs and platelet, which do not contain DNA, are not detected[22]. Standardized LeukoSure fluorospheres were added just before analysing the sample on the flow cytometer[22].

Residual platelet counts were also measured by the flow cytometer using lineage-specific monoclonal antibodies as described previously[23,24] with some modifications. Briefly, RCCs (100 µL) were diluted with buffer (1X PBS) and 5 µL of the fluorescently labeled monoclonal

* Residual white blood cells were analyzed by the Canadian Blood Services National Testing Laboratory (Ottawa, ON, Canada)

antibodies (PerCP/Cy5.5 anti-human CD41a antibody; BD Biosciences) were added to identify platelets. Commercial isotype control (PerCP/Cy5.5 mouse IgG1, Isotype control; BD Biosciences) was used as a negative control. As previously described in Chapter 2 (section 2.2.3), after 15 min of incubation in the dark at room temperature, prepared samples were run on a bench-top digital flow cytometer (LSR-Fortessa X-20, BD Biosciences). TruCOUNT beads (BD Bioscience, Mississauga, ON) used to determine the absolute number of platelets/μL. Results were analyzed using BD FACSDiva 8.0.1 software (BD Biosciences)

### 4.2.5. Extracellular Vesicle Characterization

#### *4.2.5.1. QNano Assay for Extracellular Vesicle Concentration and Size-profiling*

Quantification and size characterization of EVs in RCCs were measured using a tunable resistive pulse sensing instrument (TRPS/qNano system; IZON Science Ltd) as previously described in Chapter 2 (Section 2.2.5) with some modification due to the updated IZON Control Suite software to version 3.3 and the change in the reagent kit by the manufacturer. Therefore, supernatant samples and calibration particles were diluted with electrolyte solution (Measurement Electrolyte, IZON Reagent kit, RK1) before being analyzed with the new IZON Control Suite software to obtain the data.

#### *4.2.5.2.Flow Cytometry Assay for Extracellular Vesicle Phenotyping and Quantification*[*]

EV phenotyping was performed using a modified flow cytometer assay as previously described in detail[32,33]. While in Chapter 2 and Chapter 3 we measured only RBC-EVs/RMPs, for the purpose of this study, the flow cytometer assay was further modified to measure other

[*] The author would like to acknowledge Heather Inglis (Blood Systems Research Institute, San Francisco, USA) for training and her help with the modified flow cytometry method to measure extracellular vesicle subtypes.

phenotypes of EVs. In this study, 20 µL of the supernatant of each RCC product was stained with the following linage-specific monoclonal antibodies to identify the cell of origin of EVs: CD41a-PerCP-Cy5.5, CD142-APC, CD66b-PE, CD144-BV421, CD235a-FITC, CD3-FITC, and CD14-PE-Cy7 (Biolegend), and CD16-ECD, CD19-PerCP-Cy5.5, and CD62P-APC (BD Biosciences). Stained samples were incubated in the dark for 30 min at room temperature (20-25 °C), diluted in 1 x PBS and acquired on LSR II (BD Biosciences) benchtop flow cytometer equipped with 3-laser (20 mW Coherent Sapphire 488 nm blue, 25 mW Coherent Vioflame 405 nm violet, and 17 mW JDS Uniphase HeNe 633 nm red) was used. BD FACS Diva 6 software (BD Biosciences). Sufficient events were collected to provide approximately ≥ 5,000 gated EV events. An APC Anti-Mouse Bead Kit (Life Technologies) was used to set the compensation with the single-stained compensation control. Small size beads range from 0.2 µm to 1 µm (Megmix-Plus SSC beads, Biocytex) were used to generate the EV gate and to further classify them based on their size (only EVs ≤ 1.0 µm in diameter were analyzed). BD TruCOUNT tubes (BD Biosciences) were used to obtain the absolute number of EVs/µL. Data were analyzed using FlowJo version10.

### 4.2.6. Monocyte Co-Culture Experiment*

#### *4.2.6.1.Blood Collection and Monocyte Isolation*

Monocytes were isolated from whole blood of eight healthy adult donors as previously described[34,35]. For this study, up to 120 mL of blood was drawn in ethylenediaminetetraacetate (EDTA) coated blood collection tubes (Becton Dickinson, Franklin Lakes, NJ) for monocyte isolation. Monocytes were isolated within 30 minutes after blood draw and were used

* Monocyte co-culture experiment was performed by Somaang Menocha from the Research Institute at Nationwide Children's Hospital in Columbus, Ohio.

immediately in co-culture models. Cell isolation and stimulation studies were all performed using aseptic techniques to ensure sterility in a tissue-culture hood. Monocytes were isolated as previously described [34,35]. Briefly, whole blood was diluted 1:1 in PBS, and peripheral blood mononuclear cells were then collected by density gradient centrifugation using Lymphocyte Separation Medium (Mediatech, Manassas, VA). Monocytes were further purified by positive selection using CD14 magnetic beads (Miltenyi Biotec, Auburn, CA) and were re-suspended in complete tissue culture media (RPMI 1640 + 10% fetal bovine serum + 1% penicillin/streptomycin). Percent purity using this method is at least 98% as previously reported[36].

#### *4.2.6.2.Monocyte* in vitro *Transfusion Model and Cytokine Measurements*

Monocytes isolated from whole blood of eight healthy adult donors were used immediately in co-culture models. The monocyte co-culture model was adapted from our previously published *in vitro* transfusion model[34,35]. For each experimental replicate, $1\times10^6$ healthy adult monocytes were plated on 12-well tissue culture plates in complete tissue culture media with 20% by volume RCC supernatants or complete tissue culture media only as control for 4 hours at 37 °C in 5% $CO_2$ incubator. The 20% by volume of RCC supernatant was chosen to approximate the volume ratio of a 20 mL/kg RBC transfusion. After this incubation, cells were either unstimulated or stimulated with 1 ng/mL of lipopolysaccharides (LPS) from *Salmonella enterica* serotype abortus equii (Sigma, St Louis, MO) for 4 h. Stimulated (to evaluate LPS-induced cytokines) and unstimulated monocyte wells (incubated with RCC supernatant or control medium but without the addition of LPS to evaluate cytokine production without LPS) were incubated under the same conditions for the same durations. Cell supernatants from LPS-stimulated and unstimulated monocytes from each well were collected and stored at -80 °C for

batch analysis. Pro-inflammatory cytokines TNF-α, IL-1β, and IL-8 and the anti-inflammatory cytokine IL-10 were quantified by chemiluminescence using the IMMULITE 1000 automated chemiluminometer (Siemens Healthcare Diagnostics, Los Angeles, CA). All experimental replicates were performed using different healthy adult monocyte donors and different RCC units. Endotoxin and pyrogen-free reagents and labware were used for all experiments. Cytokines data were normalized to % of control to reduce the degree of inter-individual variability in baseline monocyte response.

### 4.2.7. Statistical Analysis*

For the monocyte co-culture experiment, comparisons between RCC product groups were analyzed using analysis of variance (ANOVA) with Dunnett's post-test for multiple comparisons. All statistical analyses were performed using Prism 7.00 (GraphPad Inc.). For EV characterization, statistical analysis was completed using SPSS (IBM SPSS Statistics 23.0). ANOVAs followed by a Tukey post hoc test was used to identify significant differences within the storage period for EVs assays and to evaluate any significance among pairwise comparisons of testing time points during the storage time. Paired t tests were used to identify significant differences between the testing time points (days 7 and 42). Pearson correlation coefficient and associated p-value were calculated between EVs and cytokines for all of the RCC units and for each blood manufacturing method. Linear model analysis was performed to test the significant of the correlations between the manufacturing methods. Probability (p) values less than 0.05 were considered significant throughout the study.

* The author acknowledge Dr. Qi-long Yi, Canadian Blood Services statistician, for assistance with data analysis

## 4.3. RESULTS:

### 4.3.1. *In Vitro* Quality Parameters:

***Residual Cells Count***

While all of the blood manufacturing methods had similar level of residual WBCs, quantities of residual platelets differed among the products based on the processing methods used (**Figure 4.3A and 4.3B**).

***Supernatant Potassium and Percent Hemolysis***

As expected, there was a significant increase in hemolysis during storage in all of the RCC products (**Figure 4.4A**), with no significant differences among manufacturing methods of the expired units. However among day 5 units, AD RCCs demonstrated greater hemolysis compared to RCF (p=0.006) and WBF (p=0.025) units. Supernatant potassium also increased over storage time in all of the RCC products, with no significant differences among manufacturing methods at day 42 (**Figure 4.4B**). On day 5 of storage, supernatant potassium was significantly higher in WBF units compared to RCF (p= 0.024) and WBD (p= 0.008) RCCs (**Figure 4.4B**).

### 4.3.2. Characterization of EVs Populations by Tunable Resistive Pulse Sensing:

There was an increase in the total number of EVs (EVs/mL) on day 42 in comparison to day 5 of storage in all blood manufacturing methods. In addition, the number of small EVs/exosomes (< 200 nm**)** was greater than large EVs (≥ 200 nm) in all of the products on day 5 and 42 **(Figure** 4.**5A and 4.5B**). Notably, the highest level of EVs < 200 nm were in AD units which were significantly different from WBD on day 5 (p = 0.0115) as well as WBD and RCF on day 42 (p=0.0050 and p=0.0083 respectively), **Figure 4.5A**. No statistical significant differences among the blood products was observed with larger EVs (EVs ≥ 200 nm) except on day 42 between AD and WBF RCCs (p=0.0106**, Figure 4.5B**). Furthermore, the size profile of

EVs showed significant differences in the EVs size-profile among all RBC products ($p<0.05$). On day 5 of storage, WBF-RCCs had a different EVs size-profile (smaller EVs, 91.1 ± 6.8 nm) in comparison to apheresis-RCCs (125.4 ± 37.0 nm), ($p=0.009$). On day 42 of storage, the mean of small EVs (< 200 nm) apheresis and WBF RCCs was lower compared to RCF and WBD products ($p<0.05$) (data not shown).

### 4.3.3. EVs Quantification and Cells of Origin by Flow Cytometry:

Across all groups, EV counts measured by flow cytometry were orders of magnitude lower than those measured by TRPS, suggesting that flow cytometric analyses may have missed some of the smaller EV. Consistent with the TRPS data, flow cytometry results showed significant increase in the number of total EVs (EVs/μL) on day 42 of hypothermic storage in all of blood manufacturing methods ($p<0.05$) compared to day 5 (**Figure 4.5C**). Among day 5 supernatants, RCF units had the lowest total EV and platelet-derived EV concentrations (**Figure 4.5C and Figure 4.5C E**), while AD units had the highest RBC-derived EV concentrations (**Figure 4.5D**). Among day 42 supernatants, RBC-derived EV concentrations were highest in WBD supernatants (**Figure 4.5D**), while RCF supernatants again demonstrated the lowest concentration of platelet-derived EV (**Figure 4.5E**). In addition, RCF units contained the smallest concentration of WBC-EVs (CD3+, CD14+, CD16+, CD19+, CD66b+) compared to the other RCC products (**Figure 4.5F and Figure 4.6).**

### 4.3.4. Monocyte Co-culture

The immunomodulatory effects of RCC supernatants on monocytes were mixed and differed by manufacturing method. Regardless of storage duration, AD and WBD RCC supernatants were immunosuppressive with respect to TNFα production in response to LPS (**Figure 4.7A**). Meanwhile, day 42 WBD supernatants produced more IL-8 in the absence of LPS (**Figure 4.8**),

suggesting a mixed immunosuppressive and inflammatory response to WBD RCC at day 42. Exposure to day 5 WBF RCC supernatant resulted in increased LPS-induced IL-1β production (**Figure 4.7B**) and higher IL-8 in the absence of LPS (**Figure 4.8**) suggesting an augmented inflammatory response to *fresh* WBF RCC. Monocyte LPS-induced IL-10 and IL-8 production did not differ from controls for any of the RCC supernatants evaluated (**Figure 4.7B and Figure 4.7D**).

### 4.3.5. Correlations between Residual Cells, EVs and cytokine production

Exploratory correlational analyses were performed to assess the relationships between monocyte function and the amount of residual cells with all of the RCC products **(Figure 4.9).** Significant and clear negative correlations were identified between residual platelet count and LPS - induced pro-inflammatory cytokine production: TNF-α (r =0.543, p=0.002), LPS IL-8 (r=0.507, p=0.005), suggesting that higher residual platelet counts are associated with immunosuppressive activity (**Figure 4.9: A and C**). Similarly, residual platelet count was negatively correlated with IL-8 production in the absence of LPS, again suggesting a potentially anti-inflammatory phenotype (r=0.550, p=0.003)] (**Figure 4.9D***)*. Conversely, there were no strong correlations identified between residual WBCs and monocyte cytokine production, although the correlation between residual WBCs and LPS-induced IL-10 was statistically significant (p= 0.043, r= 0.378) (**Figure 4.9E-H**).

Additional correlation analyses were executed to evaluate the relationships between the monocyte function and cell-derived EVs. For fresh RCC products, no significant correlation was found between cytokine production and platelet-EVs, RBC-EVs or total WBC-EVs (**Table 4.3a**). However, as presented in **Table 4.3a**, significant moderate negative correlations were identified between LPS-induced TNF-α and B cell-derived and monocyte derived-EVs

[$CD19^+$EVs (0.437, p=0.017), $CD16^+$EVs (0.467, p=0.010)] in fresh products. Likewise, LPS-induced IL-10 significantly and negatively correlated with B cell-derived, monocyte-derived, and T cell-derived EVs [$CD19^+$EVs (r=0.513, p=0.004), $CD16^+$EVs (r=0.499, p=0.005) and $CD3^+$EVs (r=0.379, p=0.042)].

At day 42 of storage, there was a significant negative correlation between platelet-EVs and LPS-induced TNF-α (r=0.352, p=0.048; **Table 4.3b**). In the absence of LPS stimulation, a clear positive correlation was identified between IL-8 and total WBC-EVs as well as CD14+ monocyte-EVs (r=0.570, p=0.001; r=0.610, p=0.0004, respectively; **(Table 4.3b**).

## 4.4. DISCUSSION

In this study, different manufacturing methods influenced the quality control parameters and EVs characteristics of RCC products and were associated with differential immunomodulatory activity *in vitro*. Our findings are in agreement with previously published studies documenting differences in RCC quality measures, including levels of hemolysis, potassium, deformability, and residual plasma, platelet and leukocyte concentrations, and EV quantities across manufacturing method[30,37-39]. It is no longer appropriate to consider all RCCs used in transfusion as being equivalent. The current study is among the first to document a potential functional consequence related to these differences.

While factors associated with TRIM are yet to be fully elucidated, studies have suggested that the infusion of damaged or active cells, and/or foreign antigens/mediators in both soluble and cell-associated forms, are potential immunomodulatory mediators that are strongly associated with TRIM[7,12-14]. Several studies have shown that RCC products contain residual

cells and accumulate cell-derived factors in the supernatant during storage, such as EVs, which have been shown to have proinflammatory and immunosuppressive potential[30,39-42]. For instance, in previous publication by Danesh *et al.* in 2014[43], the authors demonstrated proinflammatory effects, including increased release of proinflammatory cytokines from monocytes after incubation with exosomes (small EVs) isolated from RCCs, suggesting that RCC may contribute to TRIM. Conversely, in other previous work of our group, supernatants from leukoreduced stored RCCs that had been depleted of EVs suppress monocyte function *in vitro* and extracellular protein-bound RNAs, such as microRNA, were implicated as a potential soluble mediator of immunosuppression[35].

In this study, an immunosuppressive effect was identified with AD and WBD RCC supernatants as shown by the significant reduction in the release of the inflammatory cytokine (TNF-α) by monocytes in response to LPS-stimulation. TNF-α is an important cytokine in immune activation and anti-microbial immunity[44-46]. In clinical studies, low whole blood TNF-α production in response to LPS is a reproducible marker of immune suppression in critically ill patients, associated with risks of nosocomial infection, prolonged organ dysfunction, and death[47-49]. Our findings are in agreement with previous studies reporting similar immunosuppressive activity of WBD RCC products[34,50].

In our exploratory analyses relating immunomodulatory activity to cell-derived EVs, a statistically significant correlation was identified in this study between platelet-derived EVs and the suppressed LPS-induced TNF-α production in RCCs at expiry. Similar to what was observed for residual platelets, there was a negative correlation between platelet-EVs and LPS-induced TNF-α production, suggesting that platelet-derived EVs correlated with immunosuppressive activity. Since neither residual cells nor EV population correlations perfectly explain the mixed

immunomodulatory effects observed with different blood manufacturing methods, it is likely that other mediator(s) in the supernatant of the blood products might play an important role in these effects. Although the focus of this study was not to analyze the soluble immunomodulator factors in the blood product supernatant, the immunomodulatory roles of several soluble mediators, including platelet-derived mediators, has been examined. For instance, Perros *et al.* 2015[51] showed that supernatant from platelet concentrate co-cultured with dendritic cells resulted in significant immunosuppression as evidenced by downregulated IL-12, IL-6, IL-1α and TNF-α. It has been indicated that this could be due to soluble mediators present in the supernatant such as histamine, platelet factor 4(PF4), and sCD40L that can regulate the expression and the production of cytokines and chemokines[51]. Furthermore, Ando *et al.*[52] revealed that platelets upon stimulation secret suppressive soluble factors, more likely to be protein(s), which may downregulate the macrophage responses without direct cell-cell contact. Recent work from our group showed that platelet-EVs induced TGF-β secretion without inducing proinflammatory cytokines in EV-exposed monocytes[53].

Interestingly, our study failed to identify significant correlations between RBC-EVs and monocyte cytokine production across manufacture methods for either fresh RCC or RCC at expiry, consistent with our recent publication measuring effects of RBC-EVs on monocyte activation[53]. Previous studies suggest an immunosuppressive role of RBC-EVs. Sadallah *et al.*[54] observed a significant reduction in the release of LPS-induced inflammatory cytokines (TNF-α, and IL-8) in the presence of exosomes derived from isolated erythrocytes. They postulated that the immunosuppressive effects could be due to phosphatidylserine (PS) expressed on the surface of the RBC-EVs which has been shown to down regulate the immune response. It has been also suggested that the RBC-EVs react with Toll-like receptors (TLR) and

down regulate their ability to activate the macrophage in the presence of LPS stimulation[54]. Whether transfusion of these EVs within the RCC product may account for some of reported immunosuppressive activity associated with transfusion remains uncertain and requires further investigations.

While an immunosuppressive effect was observed with the supernatant from AD and WBD RCCs, supernatants from fresh WBF units resulted in significantly higher inflammatory cytokine (IL-8) production from the unstimulated monocyte model in comparison to controls. IL-8 is a very important mediator and regulator of the innate immune response[55]. It is also believed to be a valuable diagnostic tool as it has been used along with other cytokines, such as IL-6, to determine the severity of inflammation in the body before death[55,56]. Interestingly, fresh WBF units, which were associated with higher IL-8 production in the absence of LPS, were shown to have lower residual platelets. At the same time, RCC that resulted in monocyte IL-8 expression similar to control values had higher residual platelet counts, suggesting that perhaps residual platelets may blunt inflammatory effects of other mediators in this model. Therefore, the effect of residual platelets on immunomodulatory activity and patient clinical outcomes is worth additional examination.

The augmented inflammatory responses associated with *"fresh"* but not *"expired"* WBF products is a novel finding that could provide a biological mechanism for the data recently published by Heddle *et al.* 2016.[20] In that registry study, transfusion of fresh (≤7 days of storage) WBF was associated with higher in-hospital mortality compared to the mid-age (8-35 days) of the reference group (RCF RCCs). Collective our work suggests that storage duration and blood manufacturing method used to produce the blood components could both affect

patient clinical outcomes. However, additional investigation is warranted to validate and explain these findings, and to identify the causative factors associated with these outcomes.

Important to mention, our study has limitations. This study focused on the cytokine production of monocytes due to its clinical relevance especially in critical ill patients. However, the immunomodulatory effects RCC supernatant on other immune cell types or on other measures of the monocyte function may be different. Similarly, *in vitro* models may not reflect the complexity of the biological system *in vivo* and the interactions between immune and non-immune cells, endothelial cells, and microenvironment, which all may influence host response to transfusion. Furthermore, in this study we examined only fresh (day 5) and expired (day 42) RCC supernatant because it covers the storage time range for RCC transfusion, but earlier points such as day 0 or day 1 may better reflecting the influence of manufacturing methods without a storage effect. In addition, we did not measure the effect of the EV-free supernatant or the potential soluble immunomediators in the supernatant; it is likely that these factors might play an important role in the mixed immunomodulatory effects observed with different blood manufacturing methods. We view these as important future studies. Furthermore, we centrifuged the blood product to collect the supernatant for testing and it is possible that the centrifugation may generate more EVs in the supernatant and may release the cargo of some cells or particles, which may affect the final results of this study. Moreover, not all EVs in this study were categorized based on their cell of origin given the small EVs/exosomes that were detected by the TRPS technique but were not identified by the flow cytometer. Thus, the exploratory correlation analysis relating immunomodulatory activity to cell-derived EVs did not include *all* EVs, but rather those large enough to be detected on the flow cytometer (>100-150 nm). Furthermore, our correlation analysis preformed here was an exploratory correlation

only, and we did not correct for multiple comparisons in the correlations due to hypothesis generating exploratory data. Additionally, it is important to mention that it is not yet clear whether these findings and differences observed are due to the differences in manufacturing methods or due to other variables such as donor characteristics. Although donor factors such as sex and age may influence RCC products during storage[57], the main focus of this project was to investigate the effect of different manufacturing methods on RCC characteristics and immunomodulatory effects on monocyte activity.

## 4.5. CONCLUSION

In conclusion, this study shows that blood manufacturing methods significantly influence the immunomodulatory effects of RCC supernatant on monocytes *in vitro* and significantly affect RBC and non-RBC EVs characteristics throughout storage, which have the potential to impact quality and safety of RBC products. Effects were largely independent of storage duration, suggesting that the differences observed between RCC manufacturing methods may account for differences in studies examining clinical effects of RCCs storage duration, particularly within international multi-center studies. Results warrant further examination of their potential immunomodulatory effects and clinical consequences.

**FIGURES:**

**Figure 4.1:** Visual Abstract

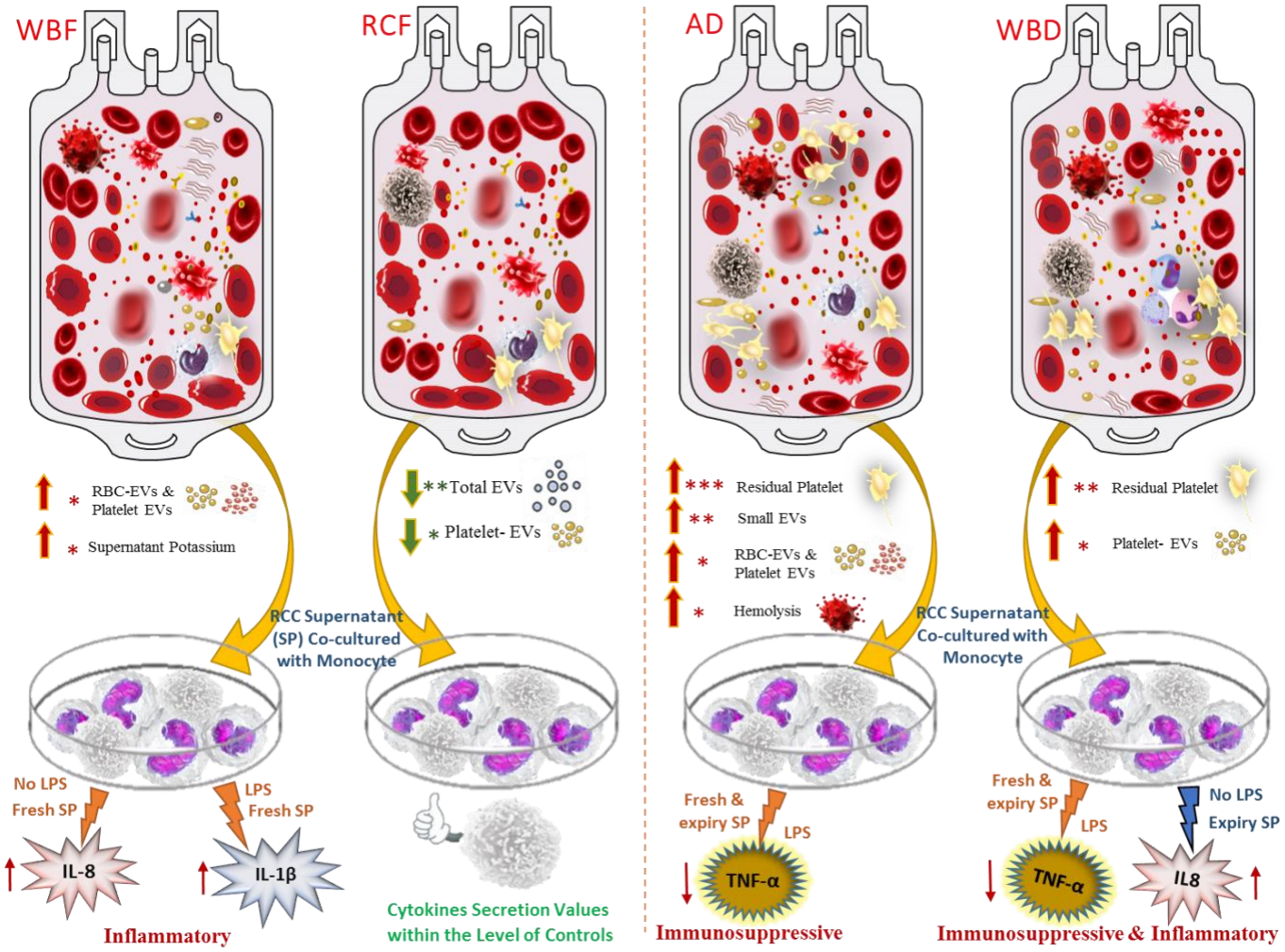

**Figure 4.2***:* Overview of experimental methods.

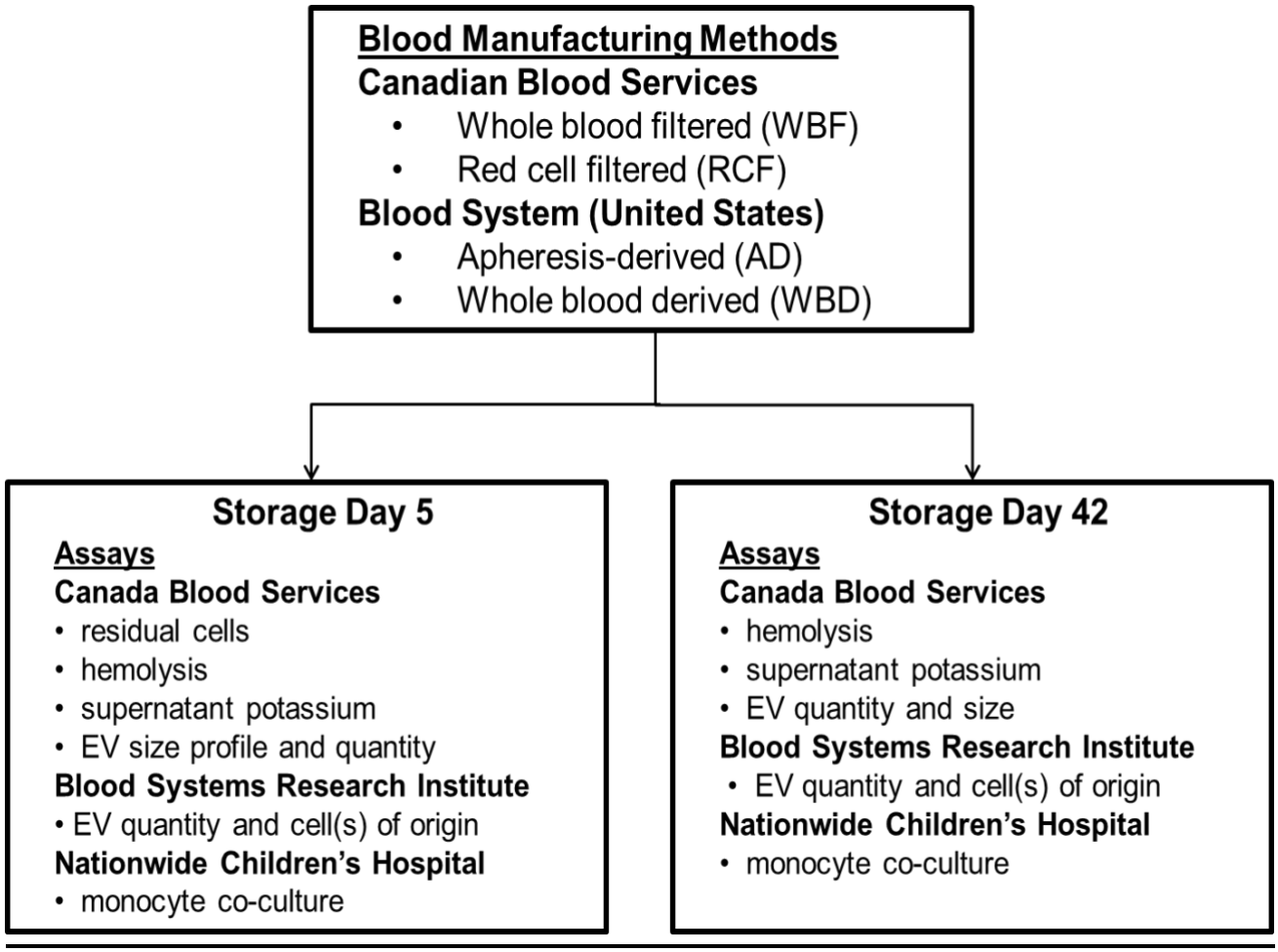

**Figure 4.3:** Residual white blood cell (WBC) and platelet counts in RCCs produced by different manufacturing methods as measured on day 5 of storage. Data reported as scatter dot plots with mean and standard deviation.

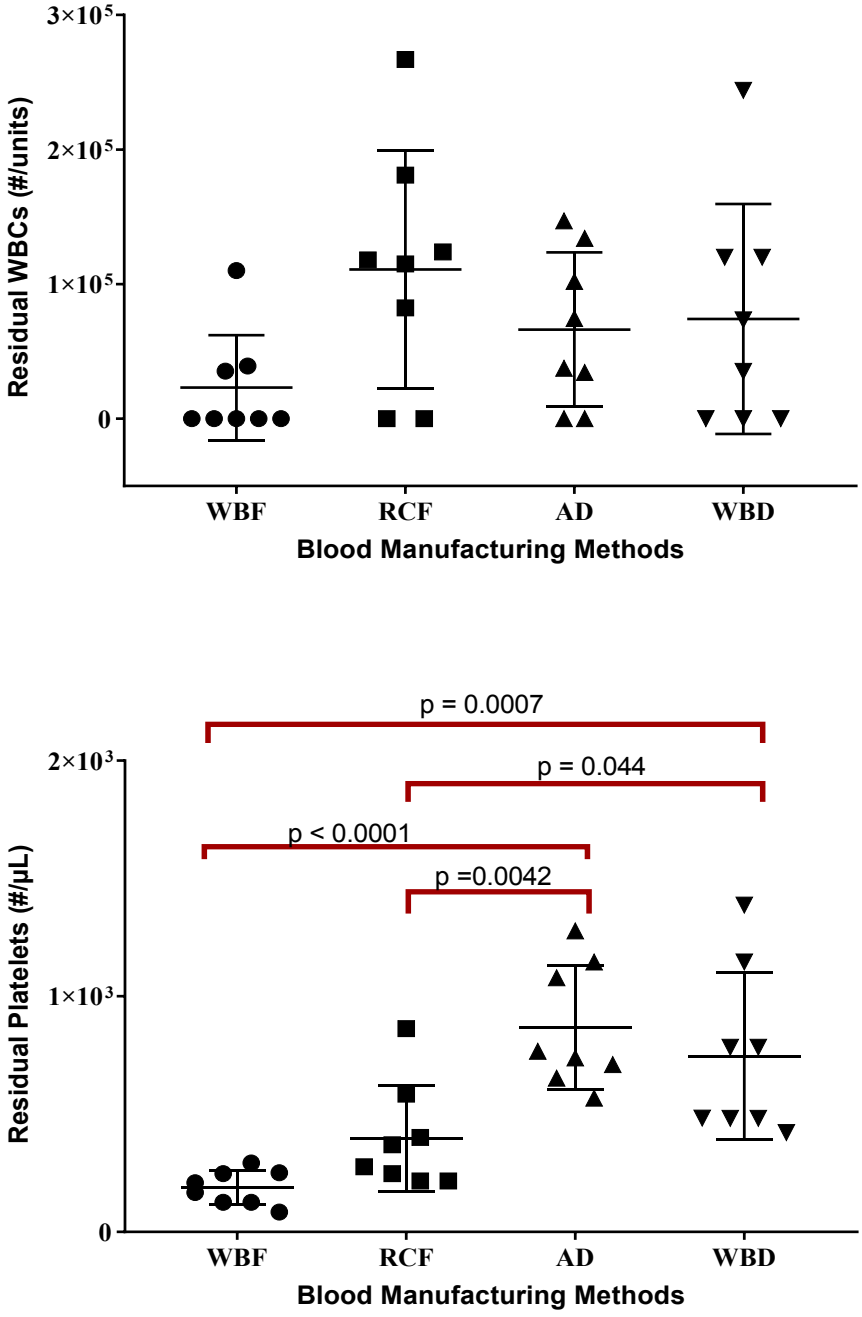

**Figure 4.4:** Hemolysis and supernatant $K^+$ of differently manufactured RCC products. Dot plots display: (A) percent hemolysis and (B) level of supernatant $K^+$ on day 5 (fresh/unfilled) and day 42 (expired/gray filed) of stored and differently manufactured RCC products. Data reported as scatter dot plots with mean and standard deviation. *Significant results ($p < 0.05$) in comparison to day 5 values. (δ) Indicates significant difference ($p < 0.05$) compared to the noted blood manufacturing methods.

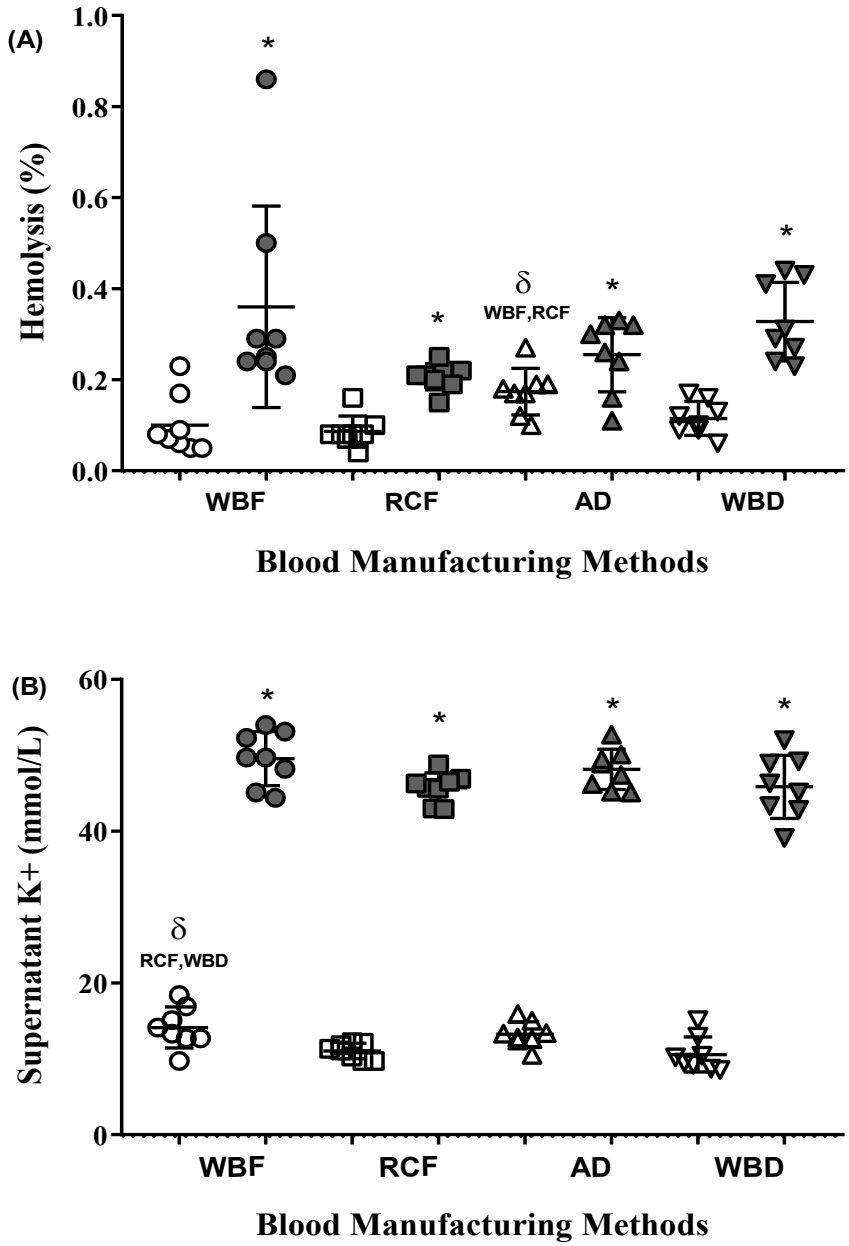

**Figure 4.5:** Concentration of EVs and their subpopulations in RCC products stored for up to 42 d analyzed by the TRPS system and flow cytometry system. (A) EVs < 200 nm, (B) EVs ≥ 200 nm, (C) total EVs, (D) RBC-EVs, (E) Platelet-EVs and (F) WBC-EVs (WBC-EVs = CD19+, CD14+, CD16+, CD3+, and CD66b+ EVs combined together. Data are reported as mean ± SD. (*) Significant results ($p < 0.05$) in comparison to day 5 values. (δ) Indicates significant difference ($p < 0.05$) compared to the noted blood manufacturing methods (n=8 per blood manufacturing method).

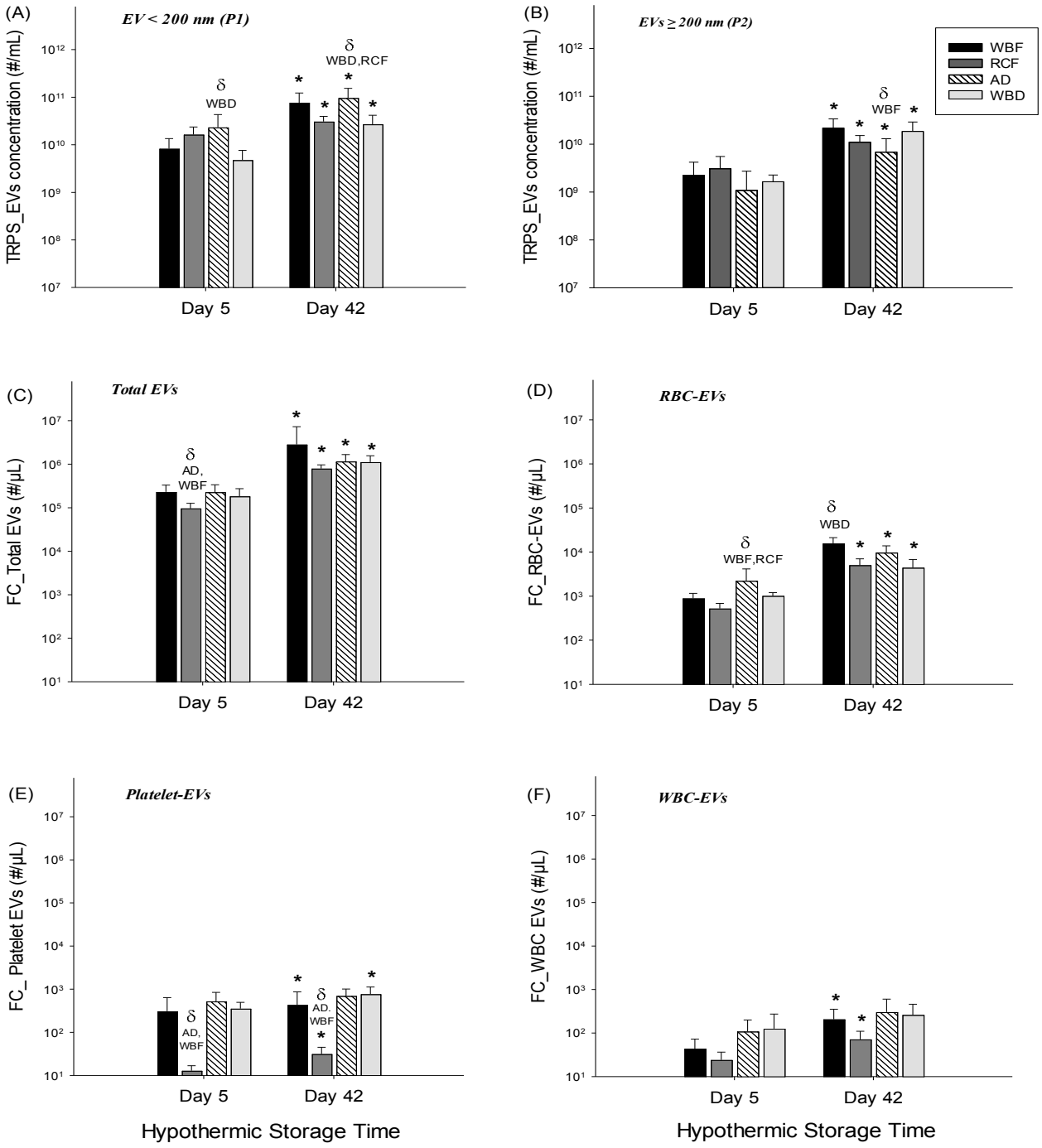

**Figure 4.6:** Number of EVs based on their cell of origin for all manufacturing methods during hypothermic storage. Data reported as scatter dot plots with mean and standard deviation (day 5/ green and day 42/red) in four differently manufactured RCC products (WBF, RCF, AD, and WBD). *Significant results ($p < 0.05$) in comparison to day 5 values. (δ) Indicates significant differences ($p < 0.05$) blood manufacturing methods.

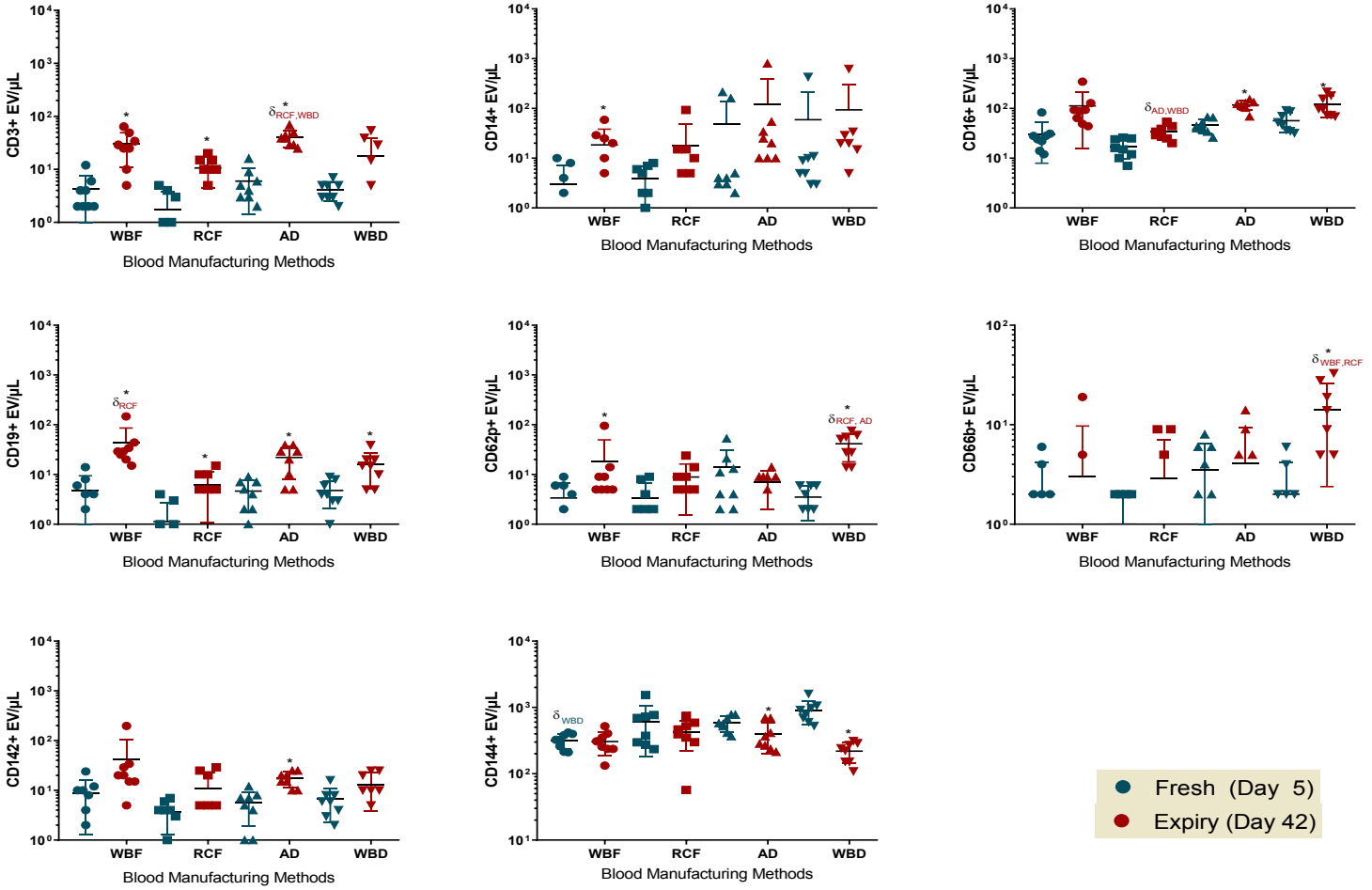

**Figure 4.7:** Monocyte LPS-induced cytokine production [(A) TNF-α, (B) IL-10, (C) IL-1β, and (D) IL-8] following exposure of RCC supernatant from different RCC manufacturing methods at fresh (day 5/unfiled) and at expiry (day 42/gray). *Significant level in comparison to control (*P<0.05; **P<0.01; ***P<0.001).

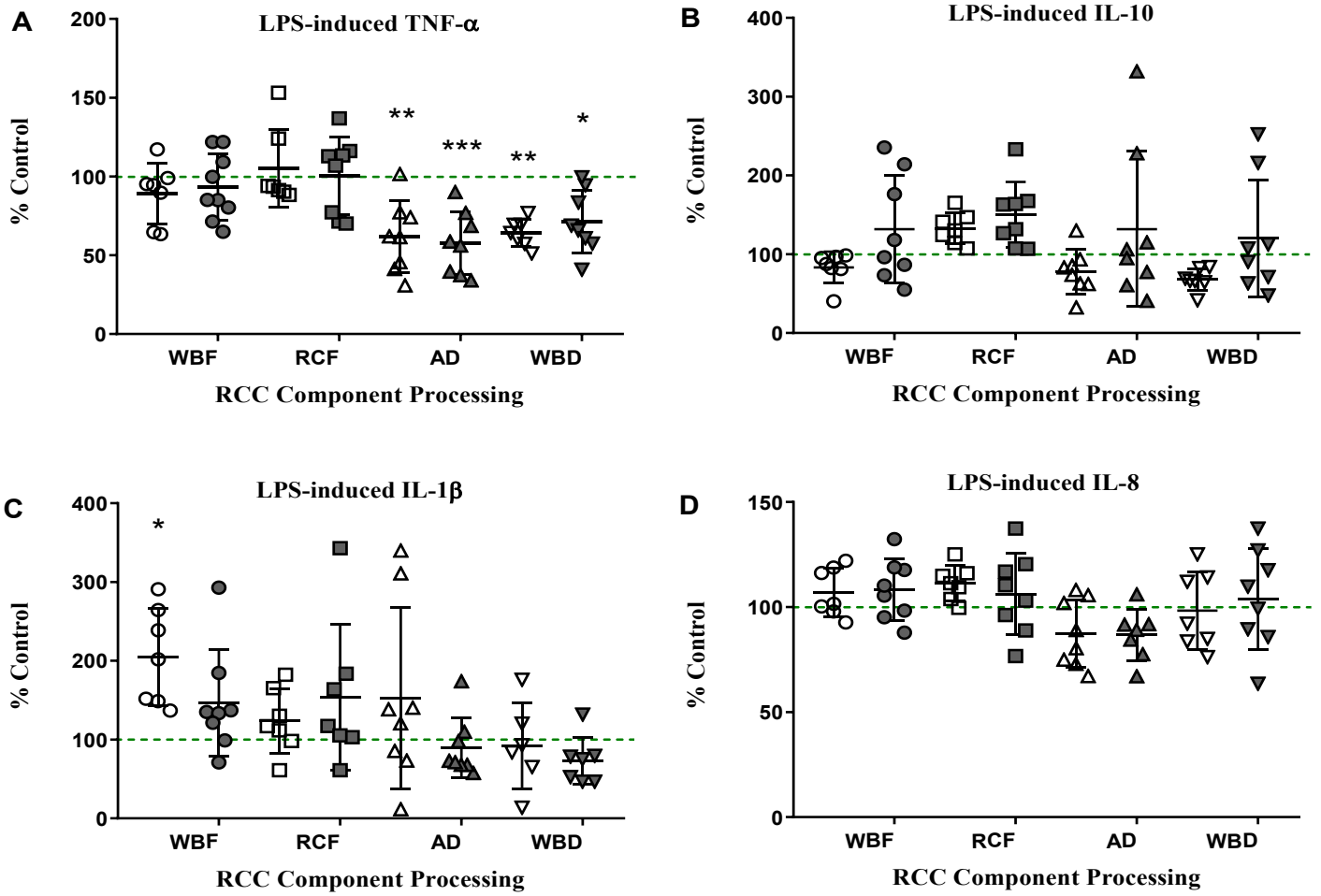

**Figure 4.8:** Monocyte IL-8 production in the absence of LPS stimulation and following exposure to RCC supernatant from different RCC manufacturing methods at fresh (day 5/unfiled) and at expiry (day 42/gray). *Significant level in comparison to control (***P<0.001; ****P<0.0001).

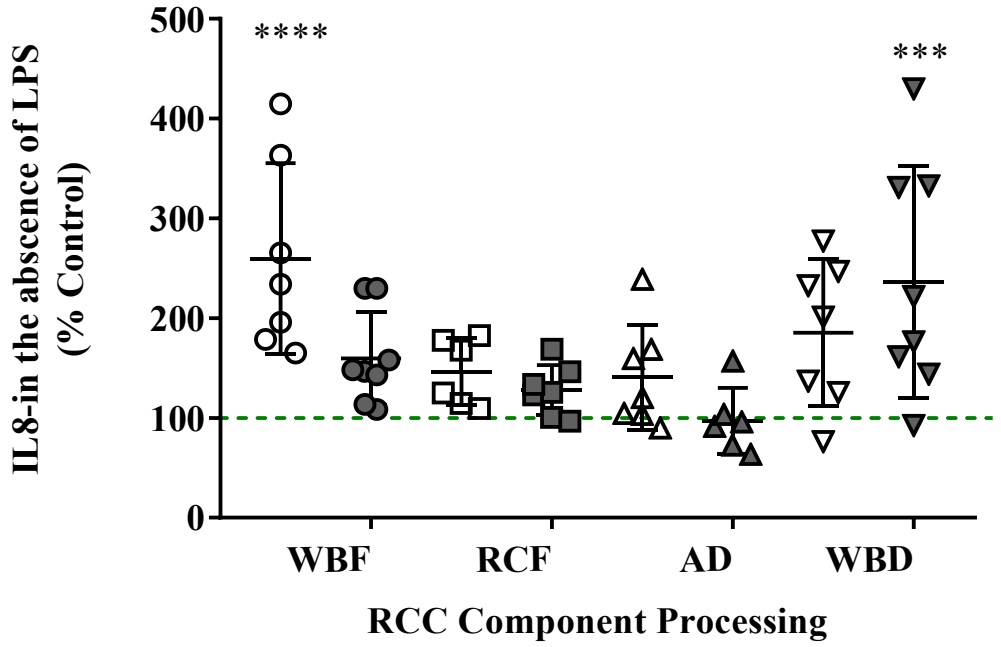

**Figure 4.9*:*** Overall correlations between the level of residual cells and monocyte cytokines production for all manufacturing methods [WBF (grey), RCF (green), AD (red), and WBD (blue)].

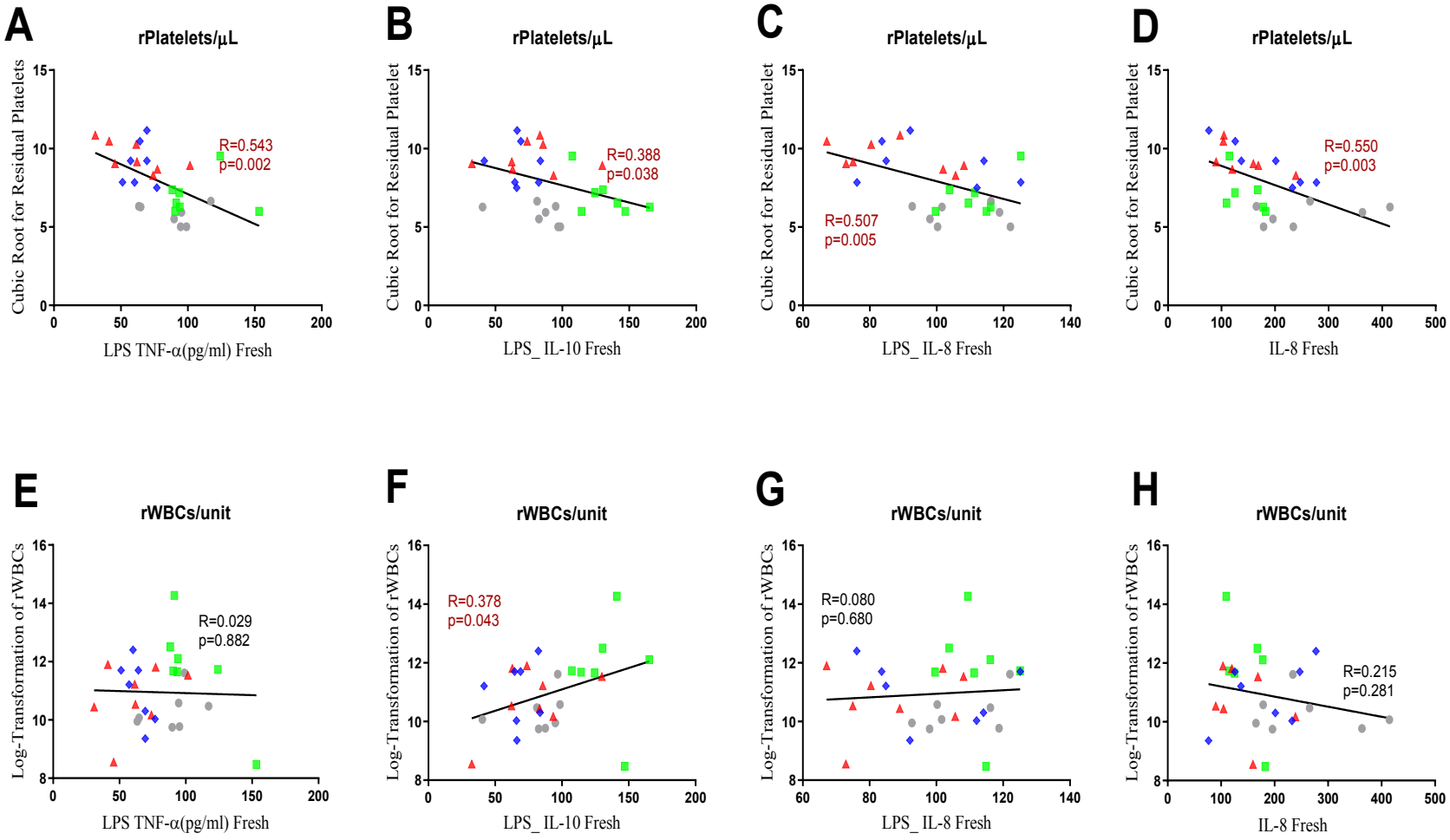

**Table 4.1:** Controls (raw data) for monocyte co-culture experiment used to normalized the cytokines data to "% of control" to reduce the degree of inter-individual variability in baseline monocyte response.

| Experiment ID number | LPS TNF (pg/ml) | LPS IL 10 (pg/ml) | LPS IL-1β (pg/ml) | LPS IL 8 (pg/ml) | no LPS IL8 (pg/ml) |
|---|---|---|---|---|---|
| **1** | 11205.50 | 62.95 | 178.50 | 123464.00 | 437.00 |
| **2** | 28607.30 | 75.60 | 105.67 | 117177.00 | 573.00 |
| **3** | 18919.00 | 33.50 | 100.00 | 101763.00 | 411.50 |
| **4** | 26191.50 | 90.70 | 74.10 | 147964.50 | 138.00 |
| **5** | 23091.50 | 108.00 | 108.00 | 130995.00 | 234.00 |
| **6** | 17766.00 | 51.00 | 275.50 | 99134.00 | 4086.50 |
| **7** | 18766.50 | 30.90 | 204.50 | 141275.50 | 25534.00 |
| **8** | 27678.00 | 107.00 | 105.50 | 183519.00 | 15929.50 |

**Table 4.2:** Modal size of EVs (nm) in diameter from TRPS measurements (Mean ± SD). (*) Significant results ($p < 0.05$) in comparison to day 5 values. (†) Indicates significant differences ($p < 0.05$) blood manufacturing methods.

| Methods | EVs Size | Day 5 | Day 42 |
|---|---|---|---|
| **1. WBF** | *EVs < 200 nm* | 91.1 ± 6.8†3 | 141.4 ± 14.8* †2,4 |
| | *EVs≥ 200 nm* | 247.5 ± 25.8†2,3,4 | 257.5 ± 14.6 |
| **2. RCF** | *EVs < 200 nm* | 117.5 ± 8.9 | 175.4 ± 9.1* |
| | *EVs≥ 200 nm* | 237.3 ± 5.9 | 237.7 ± 4.4 |
| **3. Apheresis** | *EVs < 200 nm* | 125.4 ± 37.0 | 135.1± 41.2†2,4 |
| | *EVs≥ 200 nm* | 243.8± 4.3 | 239.0 ± 5.4* †2,4 |
| **4. WBD** | *EVs < 200 nm* | 104.9 ± 3.3 | 181.8 ± 5.0* |
| | *EVs≥ 200 nm* | 216.6 ± 3.1†1,2,3 | 231.6 ± 4.6* |

**Table 4.3*:*** Overall Pearson correlation coefficient was calculated between EVs based on their cell of origin and cytokines expression for **all** RCC units

Table 4.3a: Pearson correlation between EVs and Cytokines: **Fresh Products**

| EVs Markers/Cell of Origin | Fresh: correlation (p-value) | | | | |
|---|---|---|---|---|---|
| | **LPS TNF** | **LPS IL-8** | **LPS IL-10** | **LPS IL-1 Beta** | **IL 8** |
| **CD41a+ (Platelet)-EVs+** | -0.2432 (0.2037 ) | -0.2832 (0.1365 ) | -0.2895 (0.1276 ) | -0.1116 (0.5717 ) | 0.1559 (0.4375 ) |
| **CD235a+ (Erythrocyte )-EVs** | -0.0473 (0.8076 ) | -0.0969 (0.6169 ) | 0.0017 (0.9930 ) | 0.0941 (0.6339 ) | -0.1078 (0.5923 ) |
| **CD144+ (Endothelial)-EVs** | -0.1566 (0.4172 ) | -0.0213 (0.9125 ) | -0.0438 (0.8216 ) | -0.3346 (0.0818 ) | -0.1421 (0.4795 ) |
| **CD66b+ (Granulocyte)-EVs** | -0.2074 (0.2804 ) | -0.0704 (0.7165 ) | -0.0842 (0.6642 ) | -0.1624 (0.4090 ) | 0.1677 (0.4031 ) |
| **CD19+ (B-lymphocyte)-EVs** | **-0.4378 (0.0175 )** | -0.3113 (0.1002 ) | **-0.5133 (0.0044 )** | -0.3035 (0.1164 ) | 0.1571 (0.4339 ) |
| **CD14+ (Monocyte)-EVs** | -0.1968 (0.3063 ) | -0.0087 (0.9644 ) | -0.0666 (0.7316 ) | -0.2717 (0.1620 ) | -0.0101 (0.9603 ) |
| **CD16+(NK/Active Monocyte)-EVs** | **-0.4678 (0.0105 )** | -0.2412 (0.2075 ) | **-0.4990 (0.0059 )** | -0.3585 (0.0610 ) | 0.0958 (0.6345 ) |
| **CD3+ (T lymphocyte)-EVs** | -0.3498 (0.0629 ) | -0.2573 (0.1779 ) | **-0.3792 (0.0425 )** | -0.1809 (0.3569 ) | 0.0751 (0.7096 ) |
| **CD142+(Tissue Factor)-EVs** | **-0.4359 (0.0181 )** | -0.3474 (0.0648 ) | -0.1520 (0.4313 ) | 0.0632 (0.7494 ) | 0.1538 (0.4439 ) |
| **Total WBCs-EVs*** | -0.322 (0.088) | -0.088 (0.659) | -0.211 (0.271) | -0.354 (0.064) | 0.024 (0.906) |

*WBC = $CD19^+$, $CD14^+$, $CD16^+$, $CD3^+$, and $CD66b^+$ EVs combined together.

Table 4.3b: Pearson correlation between EVs and Cytokines: **Expiry Products**

| | Expiry: correlation (p-value) | | | | |
|---|---|---|---|---|---|
| | **LPS TNF** | **LPS IL-8** | **LPS IL-10** | **LPS IL-1 Beta** | **IL 8** |
| **CD41a+ (Platelet)-EVs** | **-0.3522 (0.0481 )** | -0.1707 (0.3585 ) | -0.1214 (0.5081 ) | -0.2096 (0.2662 ) | 0.2064 (0.2827 ) |
| **CD235a+ (Erythrocyte )-EVs** | 0.0175 (0.9245 ) | -0.0135 (0.9425 ) | -0.0355 (0.8471 ) | 0.0638 (0.7378 ) | 0.1089 (0.5738 ) |
| **CD144+ (Endothelial)-EVs** | 0.1328 (0.4688 ) | -0.2048 (0.2692 ) | **0.4465 (0.0104 )** | 0.2226 (0.2371 ) | -0.3168 (0.0940 ) |
| **CD66b+ (Granulocyte)-EVs** | -0.2486 (0.1701 ) | -0.3200 (0.0792 ) | -0.0926 (0.6141 ) | 0.0868 (0.6483 ) | 0.1924 (0.3174 ) |
| **CD19+ (B-lymphocyte)-EVs** | 0.0076 (0.9670 ) | 0.0175 (0.9255 ) | -0.0932 (0.6117 ) | 0.0004 (0.9984 ) | 0.2064 (0.2828 ) |
| **CD14+ (Monocyte)-EVs** | -0.1669 (0.3612 ) | 0.0984 (0.5983 ) | **0.3690 (0.0377 )** | -0.2113 (0.2622 ) | **0.6102 (0.0004 )** |
| **CD16+(NK/Active Monocyte)-EVs** | -0.2149 (0.2375 ) | -0.1500 (0.4205 ) | -0.0889 (0.6284 ) | -0.1786 (0.3449 ) | 0.1014 (0.6006 ) |
| **CD3+ (T lymphocyte)-EVs** | -0.0919 (0.6169 ) | -0.2547 (0.1668 ) | 0.0924 (0.6150 ) | -0.1344 (0.4788 ) | -0.0744 (0.7014 ) |
| **CD142+(Tissue Factor)-EVs** | 0.0029 (0.9876 ) | 0.0012 (0.9949 ) | -0.0353 (0.8481 ) | 0.0663 (0.7277 ) | 0.1727 (0.3703 ) |
| **Total WBCs-EVs*** | -0.228 (0.210) | 0.000 (1.000) | -0.275 (0.127) | -0.245 (0.191) | **0.570 (0.001)** |

***WBC = $CD19^+$, $CD14^+$, $CD16^+$, $CD3^+$, and $CD66b^+$ EVs combined together.

**Table 4.4**: Pearson correlation between EVs and Cytokines for **each** RCC manufacturing method.

Table 4.4a: Pearson correlation between EVs and Cytokines: **Fresh**

| EVs Markers/Cell of Origin | Manufacturing Methods | Fresh: correlation (p-value) | | | | |
|---|---|---|---|---|---|---|
| | | **LPS TNF** | **LPS IL-8** | **LPS IL-10** | **LPS IL-1 Beta** | **IL 8** |
| **CD41a+ (Platelet)-EVs+** | WBF | -0.0693 (0.8826 ) | 0.2230 (0.6307 ) | 0.0899 (0.8480 ) | -0.4014 (0.3721 ) | 0.4127 (0.3575 ) |
| | RCF | 0.4729 (0.2839 ) | 0.1558 (0.7388 ) | 0.3279 (0.4727 ) | -0.2578 (0.5767 ) | 0.0325 (0.9513 ) |
| | AD | **0.7601 (0.0286 )** | 0.3126 (0.4509 ) | 0.6544 (0.0783 ) | -0.1619 (0.7018 ) | -0.0024 (0.9960 ) |
| | WBD | -0.0089 (0.9848 ) | **-0.8175 (0.0247 )** | -0.2049 (0.6594 ) | -0.5966 (0.2113 ) | -0.2111 (0.6495 ) |
| | P_value * | 0.1835 | 0.5025 | 0.4682 | 0.7865 | 0.7604 |
| **CD235a+ (Erythrocyte )-EVs** | WBF | 0.3862 (0.3922 ) | 0.6691 (0.1002 ) | -0.0049 (0.9918 ) | -0.3445 (0.4493 ) | 0.3758 (0.4061 ) |
| | RCF | -0.0827 (0.8602 ) | 0.2741 (0.5520 ) | 0.6379 (0.1232 ) | 0.2784 (0.5455 ) | 0.3324 (0.5198 ) |
| | AD | **0.7127 (0.0472 )** | 0.5312 (0.1755 ) | 0.6393 (0.0878 ) | 0.1179 (0.7810 ) | 0.0624 (0.8942 ) |
| | WBD | 0.3800 (0.4005 ) | -0.4115 (0.3590 ) | 0.3702 (0.4137 ) | 0.2716 (0.6026 ) | 0.0203 (0.9655 ) |
| | P_value * | **0.0201** | 0.1938 | 0.1351 | 0.9822 | 0.997 |
| **CD144+ (Endothelial)-EVs** | WBF | -0.6422 (0.1199 ) | 0.0109 (0.9815 ) | -0.2504 (0.5882 ) | 0.0410 (0.9305 ) | 0.4798 (0.2759 ) |
| | RCF | 0.1570 (0.7367 ) | 0.2248 (0.6279 ) | 0.1452 (0.7560 ) | -0.7369 (0.0588 ) | -0.5103 (0.3010 ) |
| | AD | -0.1296 (0.7597 ) | 0.1490 (0.7247 ) | -0.0795 (0.8515 ) | 0.1487 (0.7254 ) | 0.4327 (0.3322 ) |
| | WBD | 0.5520 (0.1989 ) | 0.1394 (0.7657 ) | 0.6413 (0.1206 ) | 0.1347 (0.7992 ) | 0.2038 (0.6612 ) |
| | P_value * | 0.4152 | 0.9269 | 0.3552 | 0.0575 | 0.3042 |
| **CD66b+ (Granulocyte)-EVs** | WBF | 0.1345 (0.7738 ) | 0.5441 (0.2068 ) | 0.4870 (0.2677 ) | -0.0028 (0.9953 ) | 0.1274 (0.7855 ) |
| | RCF | 0.1425 (0.7605 ) | -0.1467 (0.7537 ) | 0.3069 (0.5032 ) | 0.2254 (0.6271 ) | 0.5115 (0.2997 ) |
| | AD | -0.1906 (0.6512 ) | -0.2115 (0.6151 ) | 0.0350 (0.9345 ) | -0.6485 (0.0820 ) | 0.4470 (0.3146 ) |

| | Methods | LPS TNF | LPS IL-8 | LPS IL-10 | LPS IL-1 Beta | IL 8 |
|---|---|---|---|---|---|---|
| | WBD | 0.6795 (0.0931 ) | 0.5975 (0.1566 ) | 0.4157 (0.3536 ) | **0.8330 (0.0395 )** | 0.4627 (0.2958 ) |
| | P_value * | 0.43 | 0.3461 | 0.7468 | **0.0491** | 0.7010 |
| **CD19+ (B-lymphocyte)-EVs** | WBF | -0.2164 (0.6412 ) | -0.1872 (0.6877 ) | -0.1511 (0.7463 ) | -0.3535 (0.4366 ) | 0.3228 (0.4801 ) |
| | RCF | 0.5252 (0.2261 ) | 0.7841 (0.0369 ) | -0.5476 (0.2033 ) | -0.3006 (0.5124 ) | -0.4301 (0.3946 ) |
| | AD | -0.5797 (0.1320 ) | -0.5081 (0.1986 ) | -0.3472 (0.3995 ) | -0.5689 (0.1411 ) | 0.2285 (0.6222 ) |
| | WBD | 0.3567 (0.4322 ) | 0.1894 (0.6842 ) | 0.3053 (0.5055 ) | -0.0145 (0.9783 ) | 0.0845 (0.8571 ) |
| | P_value * | 0.2665 | 0.3393 | 0.7161 | 0.9323 | 0.8801 |
| **CD14+ (Monocyte)-EVs** | WBF | 0.0318 (0.9460 ) | 0.5810 (0.1713 ) | -0.0572 (0.9030 ) | 0.1813 (0.6972 ) | 0.2673 (0.5623 ) |
| | RCF | 0.3748 (0.4074 ) | 0.3849 (0.3939 ) | 0.4523 (0.3082 ) | 0.0212 (0.9640 ) | 0.2544 (0.6266 ) |
| | AD | -0.1692 (0.6887 ) | -0.1419 (0.7375 ) | 0.1001 (0.8136 ) | -0.4545 (0.2578 ) | 0.2708 (0.5569 ) |
| | WBD | 0.2797 (0.5436 ) | 0.3741 (0.4083 ) | 0.5094 (0.2429 ) | -0.0742 (0.8888 ) | 0.0838 (0.8583 ) |
| | P_value * | 0.6918 | 0.601 | 0.261 | 0.9572 | 0.9642 |
| **CD16+(NK/Active Monocyte)-EVs** | WBF | 0.1617 (0.7291 ) | 0.4878 (0.2667 ) | 0.0866 (0.8536 ) | -0.4852 (0.2697 ) | 0.4969 (0.2565 ) |
| | RCF | 0.4498 (0.3112 ) | 0.0640 (0.8916 ) | 0.2019 (0.6642 ) | -0.1174 (0.8020 ) | 0.2146 (0.6830 ) |
| | AD | -0.2150 (0.6091 ) | -0.5212 (0.1853 ) | 0.0763 (0.8576 ) | -0.4832 (0.2252 ) | -0.4102 (0.3607 ) |
| | WBD | -0.7178 (0.0693 ) | 0.1896 (0.6839 ) | -0.6791 (0.0934 ) | -0.3538 (0.4915 ) | -0.0129 (0.9781 ) |
| | P_value * | 0.117 | 0.3385 | 0.1976 | 0.7407 | 0.5940 |
| **CD3+ (T lymphocyte)-EVs** | WBF | 0.2508 (0.5874 ) | 0.5329 (0.2181 ) | 0.2299 (0.6200 ) | -0.1449 (0.7565 ) | 0.2614 (0.5712 ) |
| | RCF | 0.2832 (0.5383 ) | 0.1411 (0.7628 ) | 0.3677 (0.4171 ) | 0.1630 (0.7270 ) | 0.3531 (0.4923 ) |
| | AD | -0.2894 (0.4868 ) | -0.3269 (0.4293 ) | -0.4631 (0.2479 ) | -0.3664 (0.3720 ) | 0.3416 (0.4534 ) |
| | WBD | 0.2891 (0.5295 ) | 0.7200 (0.0681 ) | 0.2972 (0.5174 ) | 0.2859 (0.5829 ) | 0.0935 (0.8420 ) |
| | P_value* | 0.6139 | 0.3507 | 0.286 | 0.7398 | 0.7284 |

| EVs Markers/Cell of Origin | Manufacturing Methods | Fresh: correlation (p-value) | | | | |
|---|---|---|---|---|---|---|
| | | **LPS TNF** | **LPS IL-8** | **LPS IL-10** | **LPS IL-1 Beta** | **IL 8** |
| **CD142+(Tissue Factor)-EVs** | WBF | -0.3825 (0.3970 ) | -0.6268 (0.1320 ) | 0.3858 (0.3927 ) | **0.8114 (0.0267 )** | -0.7206 (0.0677 ) |
| | RCF | **-0.7770 (0.0398 )** | -0.6787 (0.0937 ) | 0.0395 (0.9330 ) | 0.0336 (0.9430 ) | -0.1539 (0.7710 ) |
| | AD | -0.3452 (0.4024 ) | -0.2398 (0.5673 ) | 0.2103 (0.6172 ) | -0.4057 (0.3187 ) | 0.2982 (0.5160 ) |
| | WBD | -0.2271 (0.6244 ) | -0.1750 (0.7075 ) | 0.3828 (0.3967 ) | 0.2635 (0.6138 ) | **0.8137 (0.0260 )** |
| | P_value * | 0.9804 | 0.5485 | 0.7827 | 0.0603 | **0.0072** |

Table 4.4b: Pearson correlation between EVs and Cytokines: **Expiry**

| EVs Markers/Cell of Origin | Manufacturing Methods | Expiry: correlation (p-value) | | | | |
|---|---|---|---|---|---|---|
| | | **LPS TNF** | **LPS IL-8** | **LPS IL-10** | **LPS IL-1 Beta** | **IL 8** |
| **CD41a+ (Platelet)-EVs** | | | | | | |
| | WBF | -0.2338 (0.5773 ) | -0.3304 (0.4242 ) | -0.1695 (0.6883 ) | 0.0424 (0.9207 ) | 0.4923 (0.2152 ) |
| | RCF | 0.0096 (0.9821 ) | 0.2295 (0.5846 ) | -0.1434 (0.7347 ) | 0.6543 (0.1108 ) | 0.6530 (0.1118 ) |
| | AD | 0.3151 (0.4471 ) | -0.7000 (0.0799 ) | 0.0766 (0.8570 ) | **0.7377 (0.0367 )** | -0.0762 (0.8859 ) |
| | WBD | 0.3179 (0.4429 ) | 0.4635 (0.2474 ) | 0.5009 (0.2061 ) | 0.4392 (0.3242 ) | -0.2242 (0.5935 ) |
| | P_value * | 0.6034 | 0.1114 | 0.5274 | 0.294 | 0.3899 |
| **CD235a+ (Erythrocyte )-EVs** | | | | | | |
| | WBF | -0.0713 (0.8667 ) | -0.1721 (0.6835 ) | -0.1991 (0.6364 ) | -0.1390 (0.7428 ) | 0.6275 (0.0958 ) |
| | RCF | -0.1381 (0.7443 ) | -0.3326 (0.4208 ) | 0.2640 (0.5275 ) | -0.5336 (0.2173 ) | -0.6010 (0.1535 ) |
| | AD | -0.3123 (0.4514 ) | 0.4722 (0.2846 ) | 0.1937 (0.6458 ) | 0.2313 (0.5816 ) | 0.5073 (0.3044 ) |
| | WBD | -0.6183 (0.1023 ) | -0.5983 (0.1171 ) | -0.3592 (0.3822 ) | -0.1434 (0.7591 ) | 0.0367 (0.9312 ) |
| | P_value * | 0.9956 | 0.8939 | 0.8418 | 0.9629 | **0.024** |
| **CD144+ (Endothelial)-EVs** | | | | | | |
| | WBF | -0.0376 (0.9295 ) | -0.3914 (0.3376 ) | 0.1399 (0.7410 ) | 0.2266 (0.5894 ) | 0.3878 (0.3425 ) |
| | RCF | 0.0974 (0.8186 ) | -0.1883 (0.6551 ) | 0.5922 (0.1220 ) | -0.1237 (0.7916 ) | **-0.8133 (0.0261 )** |
| | AD | 0.2995 (0.4711 ) | -0.3870 (0.3910 ) | 0.5916 (0.1224 ) | 0.4122 (0.3102 ) | -0.2377 (0.6501 ) |
| | WBD | -0.2831 (0.4968 ) | 0.1155 (0.7853 ) | -0.1936 (0.6460 ) | -0.1260 (0.7878 ) | 0.0021 (0.9961 ) |
| | P_value * | 0.8324 | 0.6774 | 0.2274 | 0.5498 | **0.0487** |
| **CD66b+ (Granulocyte)-EVs** | WBF | -0.3527 (0.3915 ) | -0.2908 (0.4847 ) | -0.1484 (0.7257 ) | 0.8410 (0.0089 ) | -0.1177 (0.7814 ) |
| | RCF | -0.6580 (0.0761 ) | -0.3577 (0.3843 ) | -0.3944 (0.3336 ) | 0.3314 (0.4678 ) | 0.0028 (0.9953 ) |

| | Manufacturing Methods | LPS TNF | LPS IL-8 | LPS IL-10 | LPS IL-1 Beta | IL 8 |
|---|---|---|---|---|---|---|
| | AD | 0.3292 (0.4259 ) | -0.3527 (0.4378 ) | 0.4993 (0.2078 ) | 0.3443 (0.4037 ) | -0.0639 (0.9043 ) |
| | WBD | -0.1332 (0.7533 ) | -0.5328 (0.1740 ) | 0.0340 (0.9364 ) | 0.3694 (0.4148 ) | -0.2745 (0.5105 ) |
| | P_value * | 0.7133 | 0.7927 | 0.7824 | 0.4372 | 0.993 |
| | P_value * | 0.7133 | 0.7927 | 0.7824 | 0.4372 | 0.993 |
| **CD19+ (B-lymphocyte)-EVs** | WBF | -0.0505 (0.9054 ) | -0.1555 (0.7131 ) | -0.1560 (0.7121 ) | -0.0968 (0.8196 ) | 0.6140 (0.1054 ) |
| | RCF | 0.6227 (0.0992 ) | 0.6636 (0.0728 ) | 0.4514 (0.2616 ) | -0.1052 (0.8224 ) | -0.4852 (0.2698 ) |
| | AD | 0.3731 (0.3626 ) | -0.0545 (0.9076 ) | -0.0326 (0.9389 ) | 0.1567 (0.7110 ) | -0.3034 (0.5589 ) |
| | WBD | -0.1665 (0.6936 ) | 0.1766 (0.6757 ) | -0.4371 (0.2788 ) | -0.5999 (0.1545 ) | 0.4653 (0.2453 ) |
| | P_value * | 0.9292 | 0.8826 | 0.9021 | 0.9049 | 0.059 |
| | P_value * | 0.9292 | 0.8826 | 0.9021 | 0.9049 | 0.059 |
| **CD14+ (Monocyte)-EVs** | WBF | 0.2686 (0.5201 ) | **0.7864 (0.0206 )** | 0.1654 (0.6956 ) | 0.0591 (0.8895 ) | 0.3372 (0.4140 ) |
| | RCF | -0.3954 (0.3323 ) | -0.6149 (0.1047 ) | 0.0241 (0.9549 ) | -0.0356 (0.9396 ) | 0.1305 (0.7803 ) |
| | AD | 0.1994 (0.6358 ) | 0.0812 (0.8626 ) | **0.8173 (0.0132 )** | -0.1534 (0.7168 ) | **0.8384 (0.0371 )** |
| | WBD | -0.2182 (0.6037 ) | 0.5618 (0.1473 ) | -0.0568 (0.8937 ) | -0.4006 (0.3732 ) | 0.6570 (0.0767 ) |
| | P_value * | 0.7783 | 0.6067 | 0.0615 | 0.6778 | 0.5547 |
| | P_value * | 0.7783 | 0.6067 | 0.0615 | 0.6778 | 0.5547 |
| **CD16+(NK/Active Monocyte)-EVs** | WBF | 0.0489 (0.9084 ) | -0.0783 (0.8537 ) | -0.1658 (0.6948 ) | -0.2041 (0.6278 ) | 0.5963 (0.1187 ) |
| | RCF | -0.2937 (0.4801 ) | -0.3281 (0.4275 ) | -0.3853 (0.3459 ) | 0.1563 (0.7379 ) | 0.5775 (0.1745 ) |
| | AD | 0.4036 (0.3214 ) | -0.0056 (0.9905 ) | 0.3353 (0.4168 ) | 0.4735 (0.2360 ) | 0.1523 (0.7733 ) |
| | WBD | 0.1822 (0.6659 ) | -0.1637 (0.6986 ) | 0.3611 (0.3795 ) | 0.6251 (0.1333 ) | -0.5581 (0.1505 ) |
| | P_value * | 0.9673 | 0.9968 | 0.7073 | 0.3676 | **0.0358** |
| | P_value * | 0.9673 | 0.9968 | 0.7073 | 0.3676 | **0.0358** |
| **CD3+ (T lymphocyte)-EVs** | WBF | 0.6338 (0.0915 ) | 0.3643 (0.3750 ) | 0.1738 (0.6806 ) | -0.2602 (0.5336 ) | 0.3559 (0.3869 ) |

| | Manufacturing Methods | LPS TNF | LPS IL-8 | LPS IL-10 | LPS IL-1 Beta | IL 8 |
|---|---|---|---|---|---|---|
| **CD3+ (T lymphocyte)-EVs** | RCF | 0.1411 (0.7389 ) | -0.1052 (0.8042 ) | 0.4584 (0.2533 ) | 0.1263 (0.7872 ) | 0.4775 (0.2785 ) |
| | AD | 0.5198 (0.1867 ) | -0.3374 (0.4592 ) | 0.6793 (0.0639 ) | 0.2305 (0.5829 ) | 0.0716 (0.8928 ) |
| | WBD | 0.0056 (0.9895 ) | -0.3128 (0.4506 ) | -0.5711 (0.1393 ) | -0.3469 (0.4459 ) | -0.0503 (0.9058 ) |
| | P_value* | 0.3832 | 0.4771 | 0.1004 | 0.626 | 0.7349 |
| | P_value* | 0.3832 | 0.4771 | 0.1004 | 0.626 | 0.7349 |
| **CD142+(Tissue Factor)-EVs** | WBF | -0.1230 (0.7716 ) | -0.2751 (0.5096 ) | -0.1047 (0.8050 ) | -0.0789 (0.8526 ) | 0.5602 (0.1487 ) |
| | RCF | 0.4134 (0.3087 ) | 0.4749 (0.2343 ) | -0.0857 (0.8401 ) | 0.3364 (0.4606 ) | -0.1654 (0.7230 ) |
| | AD | -0.0420 (0.9213 ) | 0.5375 (0.2134 ) | 0.5131 (0.1934 ) | -0.1080 (0.7990 ) | 0.6043 (0.2039 ) |
| | WBD | -0.6996 (0.0534 ) | -0.0985 (0.8165 ) | -0.6676 (0.0704 ) | -0.6314 (0.1283 ) | 0.4598 (0.2516 ) |
| | P_value * | 0.8868 | 0.5922 | 0.9301 | 0.9457 | 0.0969 |

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# Chapter 5

## Washing Red Blood Cell Products Modifies Non-Red Blood Cell Vesicles and Immunomodulatory Activity Red Cell Concentrate Supernatants

## 5.1. INTRODUCTION

In medicine, red cell concentrate (RCC) are one of the most commonly used transfusion products and a life-saving therapy [1,2]. An increased risk associated with transfusion of RCCs, which include immune and nonimmune adverse reactions[3-9], has been an area of interest and controversy for over 20 years. Although RCC products have been reported to modulate or alter immunity[10-15], the causative fraction(s) associated with the immunomodulation has not been determined. While the exact mechanisms of the augmented immunosuppression or proinflammatory effects associated with RCC transfusion are not defined, studies have suggested that the infusion of active cell-associated forms and/or soluble mediators such as cytokines, bioactive lipids, hemoglobin, and extracellular vesicles (EVs), are potential immunomodulatory mediators[8,16-19]. Studies have been focusing on demonstrating the associations between the soluble mediators and adverse clinical outcomes[5,6,20,21].

Recently, EVs have emerged as key indicators and potential potent mediators of immunity[13,22,23]. It has been found that EVs in stored RCC units, which accumulate during storage, contribute to neutrophil priming and activation and thus promote an inflammatory response in the transfused patient[13,24]. Moreover, it has been emphasized that the different effects of the EVs on the blood recipient's immune system depends on different factors such as the EVs cell of origin, the characteristics of the EVs, as well as the type of EV isolation methods[23]. Notably, it has been suggested that non-RBC EVs and soluble mediators are the responsible agents that induce immune response *in vitro*[25,26]. In the previous chapters (Chapter 2, 3 and 4), we showed that EVs from leukoreduced and stored RCCs are heterogonous in quantity, size and cell of origin. In addition, we observed that EVs, depending on their phenotype, are associated with RCC immunomodulatory activity (as illustrated in chapter 4).

Studies suggest that washing RCCs to remove soluble mediators and/or inflammatory components, may reduce the immunomodulatory activity and other adverse reactions associated with blood components and may lead to better post-transfusion clinical outcomes[19,27-30]. Although washing of RCC product is well established, the influence of washing on the quality of RCC products can differ depending on several factors including but not limited to the type of wash (manual, automated)[31], washing system (open or closed system)[32], the time of storage (pre and post wash)[30,33] and the additive solution used for post wash storage[31,33]. While manual washing with an open systems can limit the shelf life (post wash storage duration) to 24 hours, several centers including our group, have shown that the using a "closed" system and an automated cell processor (ACP) such as ACP 215, can extend the expiry time of washed RCCs[31,32]. In addition, the RBC *in vitro* quality characteristics have been assessed following washing with the ACP 215 and using different additive solutions and different pre/post washing storage time. It has been showed that a maximum of 14 days prewash storage and 7 days postwash is optimal for RCC stored in saline-adenine-glucose-mannitol (SAGM) additive solution[33].

This study tested the hypothesis that the non-RBC generated vesicles in RCC are potent mediators of RCC pro-inflammatory activity in vitro, and washing RCCs with the ACP 215 automated cell processor reduces these vesicles, and subsequently decreases the inflammatory activity of RCCs. To investigate that, the effect of washing on RBC quality parameters, residual cells, EVs characteristics in two different prewash (2 days and 14 days) and postwash (24 hours and 7 days) storage time following an optimal condition of washing were examined. Monocyte-cytokine production and human umbilical vein endothelial cell (HUVEC) adhesion molecule were also used to assess the immunomodulatory activity of RCCs. Once the effects of washing

were determined, the result of washed and unwashed RCC were compared to determine the potent factor(s) associated with the RCC immunomodulatory activity. We also investigated whether spiking platelet-EVs, which were shown to be associated with immunomodulatory activity in Chapter 4, in washed RCC supernatant induce pro- or anti-inflammatory response in monocytes and HUVECs.

## 5.2. MATERIAL AND METHODS

### 5.2.1. RBC collection and Manufacturing

All blood donors provided signed, informed consent at the time of donation. Whole blood was collected from healthy donors and RCCs (n = 16) were produced using a Whole Blood Filtration Method[34] (WBF; top/top). Briefly, whole blood was collected into blood collection sets (DQE 7292LX, Leucoflex MTL1 quadruple Top/Top system, MacoPharma) with 70 mL citrate-phosphate-dextrose (CPD)-anticoagulant and processed using the whole blood filtration method. After collection, whole blood was cooled (1-6 $^{o}$C) and leukoreduced by filtration in the refrigerator within 24 hour of stop-bleeding time before being separated. Filtered units were then centrifuged at $4552 \times g$ for 6 min to separate the blood components. An automated extractor (Compomat G4, Fresenius-Kabi) was used to extract plasma and saline-adenine-glucose-mannitol (SAGM) was added to RCC units. All units were then stored at 1-6 °C.

### 5.2.2. Study Design and Sample Processing

A pool-and-split experimental design was used for the 16 units to produce 4 equivalent RBC units in each of the following experimental groups based on pre-wash storage time: washed day 2 post collection, unwashed day 2 post collection, washed day 14 post collection, unwashed day 14 post collection. After pooling, units were split back into the original stored containers. The

unwashed RCC units served as controls. Washed units were sampled immediately post wash to calculate the percent recovery. Then, each experimental group was sampled 24 hours post wash and 7 days post wash for testing as illustrated in **Figure 5.1**.

Red blood concentrate sampling was performed three times during the storage using a validated technique as previously described [33,35]. At each testing point (pre-wash baseline testing, 24 hours post-wash , and 7 days post-wash), an appropriate amount of RBCs was aseptically drawn from each bag into pre-labelled conical tubes. For baseline testing, 3 ml of RBCs was drawn and used to measure in vitro quality (spun hematocrit, hemolysis and RBC hematologic indices) for all units. For 24 h and 7 days post wash testing points, 18 ml of RBCs was drawn and 4 mL was used to measure in vitro quality and residual cells count. The remaining (14 mL) was centrifuged at 2200 x g for 10 min at 4 °C (Eppendorf 5810R) and the supernatant was collected for EVs phenotyping and quantification by flow cytometry, characterization of EVs by a tunable resistive pulse sensing (TRPS) technology, and assessment of immunomodulatory activity of RCC supernatants by quantifying monocyte cytokine production capacity and expression of HUVEC adhesion molecules in an *in vitro* model.

On day 7 post wash, the rest of the RCC units were centrifuged at 2200 x g for 10 min at 4 °C and the supernatant was collected to examine the effect of platelet-EVs on the *in vitro* immunomodulatory activity of supernatants.

### 5.2.3. Washing of RCCs Using the ACP 215*

An automated closed system cell processor (the ACP 215 from Haemonetics Corporation, Braintree, MA) was used to wash the RCC units at different storage time for the assessment of

* The author would like to acknowledge Tracey Turner (Centre of Innovation, Canadian Blood Services) for the training in ACP 215.

RCCs and EVs characteristics and the immunomodulatory activity of the RCC supernatant. RCCs were washed on day 2 or day 14 post-collection using the ACP 215 following the manufacturing recommended protocol (Haemonetics sample standard operating procedure - cell wash protocol TRN-SOP-100007-30) as previously described[33,36]. Briefly, a Haemonetics cell washing disposable set was loaded onto the ACP 215 and the set was connected to room temperature wash solution (0.9% saline–0.2% dextrose) and an additive solution (SAGM) using a spike and luer lock connector, respectively. The RCC unit then was sterilely connected to the cell washing disposable set using a sterile docking/connecting device (CompoDock, Fresenius-Kabi AG). The measured weight of the unit were entered into the cell processor modifiable settings and the appropriate hematocrit (Hct) value was used based on the weight of the RCC units according to the manufacturer's standard operating manual. A Hct of 55% was used for units with a weight ≤ 270 g and a Hct of 65% was used for units with a weight > 270 g. The RCC unit was then automatically washed with the ACP 215 using saline–dextrose and the final product re-suspended in SAGM. The entire unit was washed by the ACP 215 in one round of four wash cycles using a total of 920 mL of saline-dextrose when RCCs weighing ≤ 270 g while two rounds of four wash cycles were used with a total of 1840 mL of saline-dextrose for RCCs weighing > 270 g. Percent recovery was measured post wash and the washed RCC was immediately stored at 1–6 °C until testing. Pre-washing and post-washing weight were measured for each unit and RBC recovery was calculated for each washed unit using its weight and the Hct results using the following formula:

$$\text{RBC recovery (\%)} = \frac{[(post-wash\ weight\ (g) \times post-wash\ Hct\ (l/l)]}{[(pre-\ wash\ weight\ (g) \times pre-\ wash\ Hct\ (l/l))]}\ x100 \qquad \text{Eq.5.1}$$

### 5.2.4. In Vitro Quality Assessment of RCC Units

Percent hemolysis, total hemoglobin (THb), supernatant hemoglobin (SHb), Hct, RCC hematological indices and residual cell counts were measured as previously described in chapter 3, section 3.2.2. Residual cell counts were measured as described in chapter 4 section 4.2.4.

### 5.2.5. Extracellular Vesicle Characterization

#### *Tunable Resistive Pulse Sensing Assay for the* Characterization EV Populations

Quantification and size characterization of the heterogeneous population of EVs in RCCs units were assessed by a tunable resistive pulse sensing instrument (TRPS/qNano system; IZON Science Ltd) as described in the previous chapter using the new IZON Control Suite software Version 3.3 (Chapter 4, section 4.2.5.1).

#### *Flow Cytometry Assay for Extracellular Vesicle Phenotyping and Quantification*[*]

Identification and quantification of EVs phenotype were assessed based on a flow cytometry flow rate technique as previously described in Chapter 4 (Chapter 4, section 4.2.5.2).

### 5.2.6. Generation of Pure Platelet Extracellular Vesicle and Spiking Process

Preparation and isolation of pure platelet-EVs were performed as previously described[37] with some modifications. Briefly, an apheresis platelet unit was washed manually with 0.9% saline–0.2% dextrose solution and stored at 22-25 °C on the agitator/shaker for 5 days to generate platelet-EVs (PLT-EVs). Pure platelet-EVs were then isolated from the stored platelets unit by differential centrifugations as follows. Stored platelets were sampled into collection

[*] Flow cytometer analysis was performed by Dylan Hampton from Blood Systems Research Institute in San Francisco

tubes and centrifuged at 1000 g (10 min, 22 °C) to remove cells from the plasma. Separated cell-free plasma was then centrifuged at 13,000 g (10 min, 4 °C) to obtain platelet-free plasma. Collected platelet-free plasma was then diluted with 1x phosphate buffered saline (PBS) in 1:5 ratio and centrifuged at 100,000g for 1 h at 4 °C. Supernatant was removed by aspiration and EVs pellet was suspended in SAGM then stored at -80 °C for the spiking immunomodulatory assays. The isolated EVs in suspension were analyzed with TRPS for total EVs concentration and size profiling. In addition, the EV suspension was analyzed with flow cytometer to measure PLT-EVs concentration and to determine purity of isolation.

On day 7 post washing of each experimental group (RCC washed on day 2 and RCC washed on day 14), 1 mL of washed supernatant was spiked with 17.5 µL of PBS (sham control) or 17.5 µL of the isolated PLT-EVs (~430,000 platelet microparticle/mL). The amount of PLT-EVs used for spiking was chosen based on the detected amount of these EVs in the unwashed WBF-RCCs as measured by the flow cytometer in our previous study[38].

### 5.2.7. Monocyte in vitro Transfusion Model and Monocyte Co-Culture Experiment*

#### *5.2.7.1.Peripheral Blood Mononuclear Cells (PBMCs) Isolation*

Three buffy coat (BC) components from healthy adult donors were obtained from netCAD (Vancouver, Canada) and used to isolated PBMCs as previously described[39]. Each of the BC product was mixed and diluted (1:1) with RBMI 1640 (contains 20 mmol/L HEPES, l-glutamine, without NaHCO3) and 10% fetal bovine serum (FBS). Fifteen mL of the diluted BC was layered on top of an equal volume of room temperature Histopaque-1077 density gradient (density 1.077 g/mL) and centrifuged (700 × g, 20 °C, 40 min, acceleration= 3, breaks=0,

* The author would like to acknowledge Betty Kipkeu (MSc student, Laboratory Medicine and Pathology, University of Alberta) for the training and technical help with the monocyte assay.

Eppendorf 5810R) to separate the PBMC layer (lymphocytes and monocytes) by a density gradient centrifugation. PBMCs were collected from the three BC units, pooled, and washed three times with 30 mL of RPMI plus 10% FBS (400 × g, 20 $^{o}$C, 7 min) to remove any residual cells. PBMCs were then suspended in 37 $^{o}$C RPMI and cell viability was determined by the trypan blue exclusion method to adjust the cell suspension to 20 x $10^{6}$/mL before being cryopreserved [cryopreservation medium; 20% DMSO (dimethyl sulfoxide), 40% RPMI 1640, and 40% FBS] and stored in liquid nitrogen (-196 °C) until use in the monocyte monolayer assay (MMA).

#### *5.2.7.2. Treatment of Monocytes with Prepared RCC Supernatants and Isolated Extracellular Vesicles*

The monocyte monolayer assay used in this study was adapted from *Branch and colleagues*[40,41] and performed as previously described[39,40,42,43]. To prepare the monocyte monolayers for this study, 22 x 22 mm glass coverslips treated with poly-L-lysine solution (Sigma Sigma-Aldrich, Oakville, ON, Canada) were placed in 35-mm culture dishes (Stem Cell Technologies). One ml (2.0 ×$10^{6}$/mL) of the prepared PBMCs suspension were then placed on each of these coverslips and incubated for one hour at 37 $^{o}$C with 5% $CO_2$ to allow the monocytes to adhere to the coverslips. The non-adherent cells were removed by washing the coverslips three times with 1 mL of 37 °C PBS. The positive control for the assay was prepared using O positive RBCs which were washed three times with PBS and incubated with equal volume of IgG anti-D (The anti-D used in this study was provided by Dr. Branch, CBS Toronto) for 1 h at 37 °C. One ml of the anti-D-sensitized RBCs were added to each coverslip and incubated for 2 h (37 °C, 5% $CO_2$) to serve as positive controls to evaluate the phagocytic function of monocytes. Monocyte monolayers were incubated (4 h, 37 °C, 5% CO2) with 1 mL

of 10 μg/ml lipopolysaccharide (LPS) suspensions from E. coli (serotype 055; B5, Sigma-Aldrich) and served as a positive control for the cytokines/chemokines assay. The monocyte monolayers were incubated with 1 ml of culture medium for 2 h (for the phagocytosis assay) and 4 h (for the cytokines/chemokines assay) at 37 °C, 5% $CO_2$ and served as a negative control. For the washing study, monocytes were incubated for 4 h with either 1 ml of supernatants from washed or unwashed units (20% v/v in culture media) from each category; 24 hours post d 2 wash, 24 hours post d 14 wash, 7 days post d 2 wash, and 7 days post d 14 wash). The 20% by volume of RCC supernatant was chosen to approximate the volume ratio of a 20 mL/kg RBC transfusion. For the spiking study, monocyte monolayers were incubated for 4 hours with 1 mL of either d2-washed RCC supernatants spiked with PBS (Sham control), d 14-washed RCC supernatants spiked with PBS, d2-washed RCC supernatants spiked with PLT-EVs, d 14-washed RCC supernatants spiked with PLT-EVs, or pure PLT-EVs (20% v/v in culture media).

The supernatants of the monocyte co-culture experiment were then harvested, centrifuged at 10,000 x g for 5 min to obtain a cell-free supernatant, and frozen at -80 °C for cytokines analysis. To assess the phagocytic function of monocyte, coverslips were washed three time with PBS to remove the non-phagocytosed RBCs then stained (Hema 3 stains, Fisher Scientific, Kalamazoo, MI, USA). The stained coverslips were then mounted to a microscopic slides and examined microscopically to determine the phagocytosis index (the number of fully phagocytosed RBCs per 100 monocytes).

#### *5.2.7.3.Multiplex Cytokine/Chemokine Analysis*[*]

[*] Multiplex Cytokine analysis was performed by Dr. Trang Duong from The Hospital for Sick Children Research Institute in Toronto.

Luminex xMAP (multi-analyte profiling) technology is a new technology that allows detection and quantitation of multiple cytokines and chemokines from a single sample using proprietary beads sets which can be recognized and measured by a flow cytometry-based instrument[44,45]. Luminex technology with MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panels (EMD Millipore, Toronto, ON, Canada) was used in this study to measure 10 cytokines/chemokines secreted in monocyte culture supernatants. The cytokines/chemokine panel included tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin (IL)-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, monocyte chemo-attractant protein -1 (MCP-1), and granulocyte-monocyte-colony-stimulating factor (GM-CSF). The analysis of this assay was performed according to the manufactures instructions as previously described[44]. Briefly, supernatants were thawed and equilibrated at room temperature before being added to 96-well plate (all samples were measured in duplicate). A mixture of antibody-coated beads/microspheres were added to the plate to be incubated with a standard and each supernatant sample to allow the beads to capture the analytes in the test sample. Once the beads capture the analytes, the wells were washed and incubated with biotin-labelled secondary antibody for 1 hour and further incubated with streptavidin-phycoerythrin for 30 min to complete the reaction on each microspheres. Samples were then analyzed with the Luminex insterument where microspheres were distinguished based on spectral properties of the beads and the fluorescent signals. The unknown concentrations of one or more analytes (cytokine/chemokine) in the sample were then determined based on standards of known analyte concentration (standard/calibration curves) prepared for each plate[44].

### 5.2.8. Human Umbilical Vein Endothelial cells (HUVECs) Co-Culture Experiment*

#### *5.2.8.1. Preparation of HUVEC* Culture

Cryopreserved HUVECs (C2519A, Lonza Group Ltd., Walkersville, MD, USA) were purchased as pooled primary cells (≥ 500,000 cells/cryovial) and cultured as described previously[43,46]. The cells was thawed at 37 $^{o}$C at the water bath and the cells were then cultured as a monolayer in tissue/T75 culture flasks (Corning Incorporated, Corning, NY, USA) with prepared endothelial growth medium-2 (EGM-2; endothelial basal media (EBM-2) supplemented with a bullet kit (LONZA®, CC-3162) until confluent (~80% coverage). Cells were then harvested and transferred to 12-well flat-bottomed culture plates and the EGM-2 was replaced with basal media (EBM-2) contaning1% foetal bovine serum (FBS) 24 h prior to the incubation with RCC supernatant or EVs.

#### *5.2.8.2. Treatment of HUVEC with Prepared RCC Supernatants and Isolated Extracellular Vesicle*

HUVECs were incubated with controls, RCC supernatants or isolated EVs (20% v/v in EBM-2 + 1% FBS) at 37 °C, 5% $CO_2$ for 24 hours. HUVECs incubated with a 20 µg/ml LPS solution from E. coli (serotype 055; B5, Sigma-Aldrich, St. Louis, USA) served as positive control while HUVECs incubated with EBM-2 + 1% FBS served as negative controls for the expression of CAMs. After the 24 hours incubation, the treated HUVECs cells were harvested using StemPro Accutase (A1110501, Gibco by Life Technologies, Grand Island, NY, USA) for the adhesion molecules analysis using the flow cytometry.

#### *5.2.8.3. Flow Cytometry Analysis of the Expression of HUVEC Adhesion Molecules*

* The author would like to acknowledge Betty Kipkeu (MSc student, Laboratory Medicine and Pathology, University of Alberta) for the training and technical help with HUVEC culture

Analysis of cell adhesion molecules (CAMs) expressed on HUVECs was preformed as previously described[47]. The harvested HUVEC post co-culture were washed with staining buffer (0·1% bovine serum albumin in 1X PBS). HUVECs were then stained with 5 µL of commercial antibodies (BD Pharmingen, San diago, CA, USA) which targeted three endothelial CAMs: fluorescein isothiocyanate (FITC) anti-human CD31 which was used as a marker for endothelial cells, phycoerythrin (PE) anti-human CD106 antibody and allophycocyanin (APC) anti-human CD-62E were used to measure the exposure of vascular cell adhesion molecule (VCAM)-1 and E-selectin respectively. Commercial mouse IgG1 isotype controls for FITC, PE and APC labelled antibodies (BD Pharmingen) were used to account for non-specific binding of the antibodies. Stained samples were incubated for 15 min in the dark at room temperature before being analyzed using a FACSCanto II flow cytometer and FACSDiva computer software (BD Biosciences).

### 5.2.9. Statistical Analysis

Statistical analysis was completed using SPSS (IBM SPSS Statistics 25.0) and Prism 8.00 (GraphPad Inc.). Paired t-tests were used to identify significant differences between washed vs unwashed RCCs values and between the different testing time points (24 h and 7 day). For the spiking study, analysis of variance (ANOVA) followed by a Tukey post hoc test was used to identify significant differences within the groups and to evaluate any significance amongst pairwise comparisons of the differentially treated groups at each storage time. Pearson correlation coefficient and associated p-value were calculated between EVs and cytokines or between EVs and HUVEC adhesion molecules for all of the washed and the unwashed RCCs. Linear model analysis was performed to test the significant of the correlations between the

groups. Data were expressed as mean ± standard deviation. Probability (p) values less than 0.05 were considered significant throughout the study.

## 5.3.RESULTS

### 5.3.1. In Vitro Quality Parameters

***Acceptable Criteria and Baseline Testing***

All units used in this study met the Canadian Standards Association (CSA) standard criteria[48], where hemolysis must be ≤ 0.8 % of the RBC mass, Hct ≤ 0.8 L/L, and Hb should be ≥ 35 g/unit in 90% of units tested. The baseline testing showed that all unit exceeded the CSA criteria with 100% of the units having a total average of hemolysis of 0.25 % ± 0.08 %, Hct of 0.58 ± 0.4 L/L, and the level of Hb was 55 ± 4.95 g/unit. In addition, all washed unit met the CSA standard for RBC recovery (≥ 80%) with 100 % of the washed units (n=8) having a mean RBC recovery of 98 ± 2 %.

***In Vitro Quality Measurements of Washed and Unwashed Units***

The Drabkin-based spectrophotometric method used to determine hemolysis showed that all stored RCC units of all experimental groups (washed and unwashed RCCs) were within the acceptable limits according to the CSA criteria. **Figure 5.2A** illustrates that RCCs washed on day 2 and stored for 24 hours were significantly lower in hemolysis (0.25 % ± 0.01 %) in comparison to the control of the correspondent group (unwashed day 2-stored for 24 h; 0.33 % ± 0.05 %), p=0.038. However, there was no statistical significant difference observed between washed and unwashed RCCs for the rest of the testing points. Noteworthy, washing on day 14 had a lower affect on RCCs hemolysis in comparison to washing on day 2, where the mean hemolysis ranged from (0.25 % ± 0.01 %; 24 h-postwash) to (0.35 % ± 0.06 %; 7 d-postwash)

for group washed on day 2 in compared to (0.19 % ± 0.10 %; 24 h-post wash) to (0.19 % ± 0.03 % ; 7 d-post wash) of RCC washed on day 14. The storage effect on the washed RCC was clearly observed with the RCC units washed on day 2 where a significant increase in the level of hemolysis was detected on day 7 postwash compared to 24 h postwash ($p=0.041$).

As expected, spun Hcts were significantly lower in washed RCCs (total average of 0.54 ± 0.2 L/L) versus unwashed RCCs (0.62 ± 0.1 L/L) in all experimental groups ($p<0.05$) as shown in **Figure 5.2B**. In addition, all experimental groups showed that the SHb was lower in washed RCCs compared to unwashed units except the group washed on day 2 and stored for 7 days (**Figure 5.2C**), which correlated with the level of hemolysis of the same group (**Figure 5.2A**). On the other hand, no significant difference was observed between or within the experimental groups in the level of total Hb (**Figure 5.2D**).

Data obtained from the hematology analyzer showed that there were significant differences in the RBC hematological parameters between the experimental groups (Table 5.1). On day 7 postwash, MCV was significantly higher than 24 h postwash test in all groups except group washed on day 14 ($p<0.01$). Statistically significant differences between washed and unwashed RCC were observed in the MCH ($p=0.006$) and MCHC ($p=0.010$) with the group washed on day 14 and stored for 24 h compared to the unwashed control of correspondent group.

### 5.3.2. Washing With ACP 215 Was Not Sufficient to Remove Residual Cells from RCCs

Flow cytometry data for residual cells showed that there no significant differences between the washed or unwashed RCC products in term of the residual platelet counts (**Figure 5.3A**). Although the residual platelet count was slightly higher after two weeks of storage, no statistical difference between the experimental groups was observed. As illustrated in **Figure 5.3B**, storage time resulted in a decrease in the amount of rWBCs in unwashed RCCs. However, there

was no significant difference between the washed RCC groups. Residual WBCs were considerably lower only with the group washed on day 2 and stored for 24 h in compared to unwashed RCC of correspondent group.

### 5.3.3. EV Concentration and Size-profiling by Tunable Resistive Pulse Sensing

As expected, the unwashed RCCs showed that the number of EVs (EVs/mL) increased during the storage time (**Figure 5.4)**. In addition, **Figure 5.4** shows that washing RCCs with the ACP 215 reduced the concentration of small (EVs < 200 nm; **Figure 5.4A**) and large (EVs ≥ 200 nm; **Figure 5.4B**) EVs in comparison to unwashed RCCs. For small EVs/exosome, a significant difference between washed and unwashed RCCs was observed with the group washed on day 2 and stored for 24 h ($p<0.001$) as well as the group washed on day 14 and stored for 7 days ($p=0.022$). Noteworthy, TRPS data show that as the length of the postwash storage period increased, there was also an increase in number of EVs in both populations (small and large EVs). A significant difference between 7 d storage postwash versus 24 h storage was observed with group washed on day 14 (small EVs, $p=0.040$; large EVs, $p=0.025$).

### 5.3.4. EV Quantification and Cells of Origin by Flow Cytometry

Flow cytometer data shows an increase in the total number of EVs (EVs/μL) with longer storage duration in both washed and unwashed RCCs (**Figure 5.5A**). The measured EV count was significantly higher in day 7 postwash samples compared to their respective 24 hours postwash values for both washed groups (washed d2, $p=0.032$; washed d14, $p=0.003$). Additionally, irrespective of washing time, the number of total EVs was lower when measured 24 h postwash compared to unwashed RCCs of the same corresponding group. As illustrated in **Figure 5.5.B**, unwashed RCC showed a significant increase in the number of RBC-EVs ($CD235a^+$ EVs) during storage ($p<0.05$). RCCs washed on day 14 and stored for 24 h had

significantly lower concentrations of RBC-EVs compared to their unwashed controls (p=0.048). However, the concentration of RBC-EVs in RCCs washed on day 2 and stored for 7 days remained unchanged from their respective 24 h values (p=0.11). Groups washed on day 14 had significantly higher RBC-EVs concentrations on day 7 compared to 24 h values (p<001). In addition, the FC data showed that washing with the ACP 215 led to a significant reduction in the number of platelet-EVs ($CD41a^+$ EVs) compared to the unwashed RCCs of the same corresponding groups, irrespective of washing and storage time (p<0.05) as demonstrated in **Figure 5.5C**. Furthermore, the concentration of platelet-EVs of the washed RCCs was increased again after 7 days of postwash storage, and a significant increase was observed with the group washed on day 14 (p=0.027) compared to 24 h values. Irrespective of the postwash storage time, the amount of total WBC-EVs was significantly lower in the group washed in day 14 compared to their respective unwashed controls (**Figure 5.5D)**. Regarding the subtypes of WBC-EVs, only $CD16^+$(NK/Active Monocyte)-EVs and $CD19^+$(B-lymphocyte)-EVs showed a significant difference between the unwashed versus washed on day 14 groups **(Figure 5.6.d and Figure 5.6.f).** RCC washed on day 14 and stored for 24 h had lower concentrations of $CD144^+$ (endothelial)-EVs in comparison to unwashed RCC of the same corresponding group. In addition, the number of tissue factor ($CD142^+$)-EVs was significantly lower in washed RCC after 24 hours postwash storage compared to their respective unwashed groups **(Figure 5.6.h).**

### 5.3.5. Monocyte Co-culture Experiments and Cytokine Release

Release of 10 cytokines and chemokines from the monocyte was assessed for the washing and the spiking project. Four (MCP-1, IL-8, TNF-α and IL-10) were within the range of detection and six (IL-1β, IL-4, IL-6, IL-12p70, IFN-γ, GM-CSF) were below/out of the detection rage (**Table 5.2).** Evaluating the phagocytosis ability/phagocytic function of the

cultured monocyte resulted in phagocytic indexes (PI) finding of zero for the phagocytosis negative controls and above 65 % for the phagocytosis positive controls, suggesting that the viability and functionality of monocytes were maintained during the culturing.

***Effect of Washing on Cytokines Release by Monocyte***

The inflammatory activity of RCC supernatants on monocytes was reduced by the washing of RCCs with the ACP 215 as evidenced by significantly reduced production of the inflammatory chemokines, MPC-1 and IL-8, compared to unwashed RCCs (**Figure 5.7A&B, Table 5.2**). While IL-8 and MCP-1 production were significantly higher than controls after the exposure to unwashed RCC supernatant ($p<0.05$), IL-8 and MCP-1 production did not differ from controls when exposed to any of the washed RCC supernatants regardless of pre and post washing storage time. In addition, exposure to unwashed RCC supernatant resulted in an increase in the inflammatory cytokine, TNFα, compared to controls. However, production of TNFα was found to be absent or lower than the limit detection in the washed RCCs. As indicated in **Table 5.2,** the anti-inflammatory activity of unwashed RCCs was detectable only with 24 h testing points as evidenced by increased production of IL-10, which was below the detection limit for the rest of the experimental groups.

***Effect of Spiking on Cytokines Release by Monocyte***

Stimulation of monocytes with platelet-derived EVs led to an increase in the production of inflammatory chemokines/cytokines compared to controls. In comparison to negative controls, a significant increase in the production of IL-8 **(Figure 5.7C)** and MCP-1**(Figure 5.7D)** was detected in the group washed on day 14 and spiked with platelet-EVs ($p=0.002$). Although exposure to sham control of d14-washed group resulted in increased IL-8 production ($228 \pm 59$) compared to negative control, no statistical differences was observed ($p=0.076$). Noteworthy,

there was no significant difference between platelet-EVs spiked and sham control in the d14-washed group, suggesting that other soluble mediators may remained in the washed RCCs and play a role in elevating IL-8 during postwash storage. On the other hand, there was a significant difference in the level of MCP-1 between d14-washed/platelet-EVs spiked supernatant and negative controls, as well as their respective sham control ($p<0.001$). Notably, when monocytes were stimulated with platelet-EVs alone, IL-8 and MCP-1 secretion was significantly elevated ($p<0.0001$) and reached similar or higher levels than what was observed with unwashed RCCs. In addition, increase in the production of the inflammatory cytokine, TNFα and the anti-inflammatory cytokine, IL-10 by monocytes following the exposure of the isolated platelet-EVs (**Table 5.2B)** suggest that platelet-EVs or other mediators, such as platelet factors or proteins, play a role in inducing dual pro-inflammatory and anti-inflammatory effects.

### 5.3.6. Expression of HUVECs Adhesion Molecules

***Effect of Washing on HUVEC Adhesion Molecules***

The expression of VCAM-1 and E-selectin by HUVECs was significantly altered following the incubation with washed RCCs in comparison to unwashed RCCs. In comparison to control, the expression of VCAM-1 was significantly higher when HUVECs were exposed to unwashed RCC supernatant ($P<0.0001$) while expression of VCAM-1 did not differ from controls when HUVEC were exposed to any of the washed RCC supernatants, irrespective of the experimental groups **(Figure 5.8A).** On the other hand, HUVEC incubated with washed RCCs had significantly higher expression of E-selectin compared to controls ($p<0.0001$) and the expression of E-selectin was elevated more with prolonged prewash and postwash storage time **(Figure 5.8B).** A significant increase in the E-selectin expression was observed only when

HUVEC were incubated with unwashed RCCs in the first and last testing point (24 h- unwashed d 2 and 7 d-unwashed d 14, $p<0.0001$).

***Effect of Spiking on HUVEC Adhesion Molecules***

A significant increase in VCAM-1 expression was detected when HUVECs were incubated with the d 14-washed/platelet-EVs spiked RCC supernatant in comparison to negative control ($p=0.024$, **Figure 5.8C**) and sham control of the correspondent group ($p=0.006$). However, no significant increase in the level of VCAM-1 was observed with the spiked RCC supernatant that was washed on day 2. Notably, in HUVECs simulated with platelet-EVs alone, the VCAM-1 expression was significantly elevated ($p<0.0001$) as observed with unwashed RCCs. **Figure 5.8D** illustrates that washed and spiked RCC significantly stimulated HUVECs E-selectin expression similar to what was observed with the washed RCC in the absence of platelet-EVs spiking ($P<0.5$). Moreover, the data show that there is no significant differences between platelet-EVs spiked-RCC supernatant and their respective sham controls. Furthermore, HUVEC E-selectin expression was not statistically different compered to control when exposed to the isolated platelet-EVs alone ($p=0.059$), suggesting that platelet-EVs may not be involved in the expression of E-selectin by HUVEC.

### 5.3.7. Correlations Analysis

***Correlations between EVs and Cytokine Production***

Exploratory correlational analyses were performed to assess the relationships between the monocyte function and cell-derived EVs **(Figure 5.9)**. Significant and clearly positive correlations were identified between platelet-EV concentration and the pro-inflammatory chemokines production: MCP-1 ($r = 0.735$, $P <0.0001$) and IL-8 ($r = 0.486$, $P=0.005$),

suggesting that higher concentrations of platelet-EVs in WBF RCC supernatant are associated with proinflammatory activity. Conversely, there were no strong or significant correlations identified between RBC-EVs and monocyte cytokine production. While there were no significant correlations identified between WBC-EVs and IL-8 production, the correlation between WBC-EVs and MCP-1 was statistically significant ($p= 0.007$, $r = 0.468$).

***Correlations between EVs and Adhesion Molecules***

Exploratory correlational analyses were also performed to evaluate the relationships between the expression of HUVEC adhesion molecules and cell-derived EVs **(Figure 5.10)**. Significant and strongly positive correlations were identified between the amount of platelet-EV and VCAM-1 ($r = 0.672$, $P <0.0001$) while a negative correlation was identified with E-selectin expression ($r = 0.534$, $P=0.001$). In contrast, no strong or significant correlations were identified between RBC-EVs and the expression of HUVEC adhesion molecules. There were no strong or significant correlations identified between E-selectin expression and WBC-EVs. However, WBC-EVs significantly and positively correlated with VCAM-1 expression ($r = 0.495$, $P=0.003$).

## 5.4.DISCUSSION

The washing of RCC has long been performed to remove or reduce Hb, plasma, plasma proteins or lipids-based immunomodulatory substances, and level of potassium in order to improve clinical outcomes for specific patients such as those who are sensitive to potassium, IgA-deficient, require multiple transfusions, have antibodies against plasma proteins, or have experienced transfusion reactions[31,49-51]. However, little is known about the effect of washing

on other immunomodulatory mediators in RCCs such as EVs and residual cells. In addition, the mechanism in which RCC products can trigger or simulate an immune response in transfusion patients remains largely unexplored. In contrast with traditional measures of washed RCCs, the data in this study reveal that washing RCC with the ACP 215 was not sufficient to remove residual cells, but it was effective at reducing the number of EVs, particularly non-RBC EVs, and attenuating the pro-inflammatory activity RCCs supernatants.

In term of RBC *in vitro* quality parameters, this work demonstrates that all washed RCC units from all experimental groups were within the acceptable regulatory limits for hemolysis, hematocrit, and hemoglobin, which were consistent with previously published studies[31,33,49]. Noteworthy, hematologic measures indicate that when RCC are washed late in storage (day 14 post collection), there is no significant difference in hemolysis or the level of SHb between 24 h and 7 days postwash storage. However, RCCs washed on day 2 post collection showed an increase in hemolysis and SHb in day 7 postwash compared to 24 h postwash values. We postulate that blood processing and manufacturing may induce short-term stress to the RBCs, and with washing we remove most of the fragile cells and reduce SHb (as observed with 24 hours post day 2-wash, **Figure 5.3A and B**). However, the fragile cells that remain postwash, which are more susceptible to haemolysis[30], result in a higher hemolysis and SHb with longer postwash storage. Thus, this data suggest that postwash storage needs to be carefully considered when fresh RCCs are washed as the increased hemolysis and SHb may result in worsened transfusion outcomes, which is in agreement with previously published washing data[30].

Even though washed RCCs met all regulatory standards, it is clear that supernatants from RCC washed with the ACP 215 cause a significant elevation in the expression of E-selectin on HUVECs. E-selectin, an endothelial-specific adhesion molecule, is known to play an important

role in endothelial-leukocyte interactions and is induced on the endothelium following inflammatory stimulation[52]. It has been also shown that the platelet-specific chemokine (platelet factor 4; PF4) released during platelet activation, is able to increase E-selectin expression on endothelial cells via stimulation of nuclear factor-κB[53]. As mechanical washing can strongly stress and activate platelets[54], we postulate that the residual platelet detected in RCC products of this study, which remain even after washing, are more likely activated by the washing process. If this is true, these activated residual platelets may released PF4 that subsequently stimulates E-selectin expression on HUVECs incubated with washed RCC supernatants. Noteworthy, the data showed that as the pre and postwash storage period increased, there was also an increase in the E-selectin expression on HUVCs incubated with supernatants from washed RCCs, suggesting that the effect of washing on E-selectin expression is also time dependent. However, additional studies are needed to test these assumptions.

On the other hand, while the supernatants from unwashed RCCs strongly stimulate the expression of VCAM-1 on HUVECs, RCC washing was effective in reducing the expression of VCAM-1 on HUVECs to the same level of negative controls. VCAM-1, a vascular cell adhesion molecule-1 best known for its binding functions (binds to α4β1-integrin) and ability to mediate the adhesion of leukocyte to vascular endothelium[55]. Expression of VCAM-1 on endothelial cells has been shown to be stimulated by cytokines[56,57], heme[58], or platelet-derived EVs[59,60]. Although heme and cytokines were not measured in our RCC products, our data revealed that the number platelet-EVs in RCCs strongly and significantly correlated with the expression of VCAM-1 when HUVECs were incubated with washed or unwashed RCC supernatants. Higher concentrations of platelet-EVs were associated with increased expression of VCAM-1. Platelet-EVs contain considerable amount of the proinflammatory cytokine, RANTES, which can

activate endothelial cells and enhance leukocyte recruitment[60,61]. Reducing platelet-EVs by washing RCC showed a significant reduction in the VCAM-1, irrespective of the washed experimental groups.

In addition, monocytes exposed to washed-RCCs supernatant resulted in reduced inflammatory cytokine production, which was significantly high or higher with unwashed RCC exposure compared to controls. Monocytes play a central role in the innate immune response and their ability to produce cytokines is a fundamental measure of their immune functions[25]. Imbalanced production of pro-inflammatory or anti-inflammatory cytokines has been shown to be associated with adverse outcomes[25,62-64]. In this study, monocytes exposed to unwashed RCC supernatant revealed augmented inflammatory cytokine and chemokines production. This data is in agreement with what we observed in Chapter 4 where RCC supernatants produced from *fresh* WBF units resulted in significantly higher IL-8 production in the non-LPS stimulated monocyte model in comparison to controls. While the level of TNFα for fresh WBF supernatant was out of the detection range in the previous chapter, using the Luminex technology for this study instead of the IMMULITE 1000 automated chemiluminometer allowed us to observe a significant increase in the release of this inflammatory cytokine (TNF-α) by monocyte when incubated with unwashed RCC supernatant. Additionally, the expanded cytokine/chemokine panel in this study further highlighted the proinflammatory activity of WBF RCC supernatant as shown by the significant increase in the release of chemoattractant chemokines (CCL2 / MCP-1) by monocytes. Given the important roles that these cytokines and chemokines play in the immune system, imbalanced production of these immune mediators could significantly impact patient clinical outcomes[65]. Inflammatory activities, including increased inflammatory cytokine production and neutrophil activation, associated with RCC transfusion have been

reported in several studies[13-15]. For instance, Dani et al 2017[15] showed an increase in the proinflammatory cytokine, such as IL-1β, IL-6, IL-8, TNF-α, IFN-γ, IL-17, MCP-1, and an increase in adhesion molecules including ICAM-1 and VCAM-1 in infants who receive RCC transfusions[15], which support the findings observed in this study. In addition, Belizaire et al 2012[13] suggest in their study that RCC-derived microparticles are a major contributor to the neutrophil priming, activation and the inflammatory response observed in patients who received older RCCs. While the exact mechanisms responsible for the undesirable proinflammatory effects of RCC products is still unresolved, this study has show0n that washing RCC is an effective method to reduce these effects. Moreover, our exploratory correlational analyses showed a very clear positive and significant correlation between platelet-derived EV and the proinflammatory chemokines; IL-8 and MCP-1. Additionally, we observed a moderate but significant correlation between total number of WBC-derived EVs and MCP-1. While no significant correlation was seen between RBC-EVs and monocyte functions, these correlation analyses suggest that perhaps both platelet-EV and WBC-EVs may play a significant role in the augmented inflammatory effects observed in this model. Therefore, the effect of these non-RBC EVs on immunomodulatory activity at the bedside is worth further investigations.

Nonetheless, it is important to mention that spiking the washed-RCC groups with platelet-EVs only resulted in a significant increase in the expression of VCAM-1, and production of IL8 and MCP-1 in the RCC group washed on day 14, but not with the group washed on day 2. In addition, the increased expression of VCAM-1 on HUVECs and production of inflammatory cytokines by monocytes associated with exposure to washed day 14/platelet-EVs spiked group was lower than what were observed with the unwashed RCCs. One may speculate that other mediators in RCC, not only the platelet-EVs, play a role in the elevated expression of VCAM-

1 on HUVECs and production of inflammatory cytokines. However, it should be noted that stimulating HUVECs and monocytes only with platelet-EVs significantly induced VCAM-1, IL8 and MCP-1, similar to the levels of unwashed RCC products. These data indicate that platelet-EVs may indeed play a major role in the inflammatory phenotype observed. While there are several possibilities for the lower response of HUVECs or monocytes with respect to the expression of VCAM-1 and cytokines production following washing and spiking with platelet-EVs, we postulate that the quantity of spiked platelet-EVs and the source of platelet-EVs isolation are two major limitations effecting these data. The estimated amount of spiked platelet-EVs was calculated based on the flow cytometer phenotype assay. Due to limitations in the resolution of particles < 200 nm by flow cytometer, a significant amount of small platelet-EVs (platelet exosomes) may not have been detected and included in the calculation for the spiking project. Although TRPS data showed that the number of small EVs/exosomes (< 200 nm) was significantly higher than large microparticle (≥ 200 nm), the cell of origin of these exosomes was resolvable. Since a precise and accurate method to quantify and identify the cell of origin of exosomes is not available, this limitation may resulted in an inaccurate estimation for number of platelet-EVs required in the spiking and may account for the differences in the results observed.

In addition, platelet-EVs used for the spiking study were isolated from platelet concentrate units, not WBF RCCs to obtain sufficient amount of pure platelet-EVs. Therefore, the properties of isolated platelet-EVs from platelet concentrates may account for some of the differences in the inflammatory phenotype observed in this study. Furthermore, on day 7 post washing, supernatants from washed RCCs were frozen for the use in the spiking study. This step may have impacted what remains in the supernatant postwash such as residual cells and other RCC

substances. This may have blunted the proinflammatory effects of the spiked platelet-EVs. Noteworthy, washed RCCs were shown to accumulate more EVs with increased postwash storage duration, regardless of their phenotype and irrespective of the experimental group, which could be due to the generation of these EVs by RBC and the residual cells (platelet and WBCs) that remain post washing. Notably, on day 7 postwash, the total number of these EVs was higher in the RCCs washed day 14 compared to the units washed on day 2 (**Figure 5.4 and 5.5**). The different quantities of the residual cells, EVs, or other substances remaining in the supernatant depends on the experimental group may explain the difference in the spiking data for the group washed on day 2 versus the group washed on day 14. While measuring the other soluble mediators in RCC was beyond the scope of this study, their role in immunomodulation has been reported[25,65,66]. Thus, the efficacy of washing RCC products to reduce immunomodulatory activity presented in this study may not be solely attributed to reducing non-RBC EVs but a combination of multiple factors/mediators that were not measured in this study. Therefore, additional investigations are needed to address these limitations and to translate these in vitro results to in vivo and clinical outcomes.

## 5.5.CONCLUSION

Together the results from monocyte and HUVECs models used in this study indicate that RCCs produced by WBF method are inflammatory, which is consistent with our finding in Chapter 4. Washing these RCCs with the ACP 215 decreased the inflammatory phenotype observed. The data from this chapter further suggests that the non-RBC EVs, particularly platelet-EVs, is associated with the inflammatory phenotype observed with WBF RCCs and reducing these vesicles by washing, can attenuate the immunomodulatory activity of the

examined RCCs. While additional investigations are required to validate these findings and to further address the limitations, implementation of washing processes for the RCC may improve transfusion outcomes.

Noteworthy, while the data of this chapter suggests that platelet-derived EVs correlate with inflammatory activity, the results of the previous chapter suggest that platelet-EVs correlate with immunosuppressive activity. Taking in consideration the controversial reports regarding CD41a-expressing EVs on immune activity[37,67-70], the data from this thesis further suggest that the same type of EVs may bias toward inflammatory or immunosuppressive activity depending on the co-stimulatory factors, which in our study were blood manufacturing methods and storage duration. Therefore, the immune heterogeneity of EVs in RCCs needs to be more carefully considered when evaluating the immunomodulatory activity of blood products.

Overall, the finding of this chapter when considering the data from the previous chapters of this thesis, strongly support our overall hypothesis that non-RBC generated vesicles in RCC are potent mediators in RCC immunomodulatory activity *in vitro*, and the characteristics of these vesicles are influenced by method of blood component manufacturing and length of RCC hypothermic storage. This work suggests that EVs can be a novel biological indicator for quality of RCC products, which may reflect the efficacy and safety of the blood product, and contribute to transfusion-related immunomodulation.

**Figure 5.1:** Experimental design for washing and spiking study.

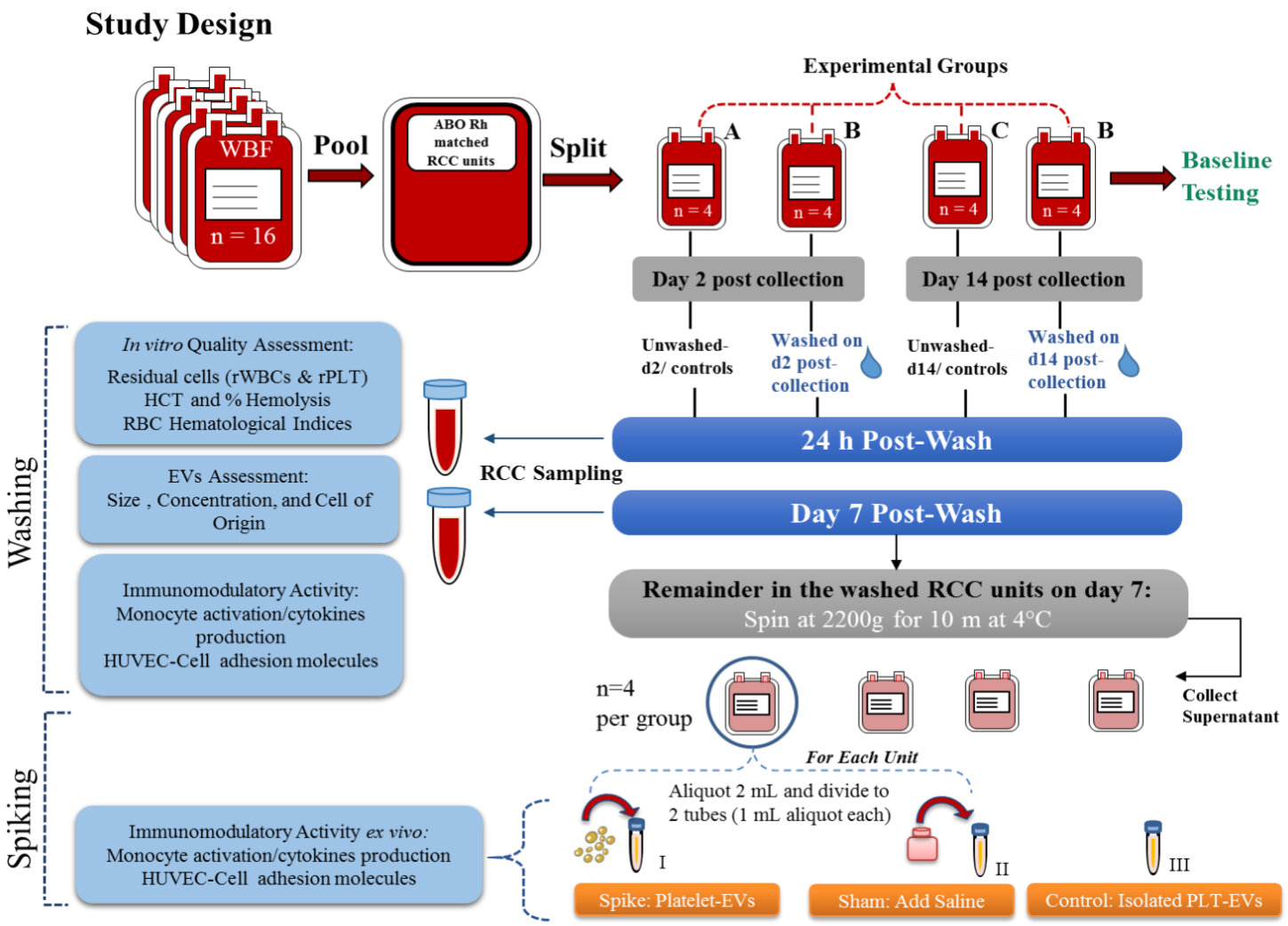

**Figure 5.2:** RBC *in vitro* quality parameters. Dot plots display hemolysis (A) and spun Hct (B), supernatant Hb (C), and total Hb (D) for RCCs for unwashed (filed/Red) and washed (unfiled/blue) units. Data reported as scatter with mean and standard deviation
*a (P <0.05); significant results compared with **unwashed** sample of the correspondent group
*b (P <0.05); significant difference in comparison with **24 h** values of the correspondent group
*c (P <0.05); significant difference between washed/unwashed **day 2** vs washed/unwashed **day 14** of the correspondent group

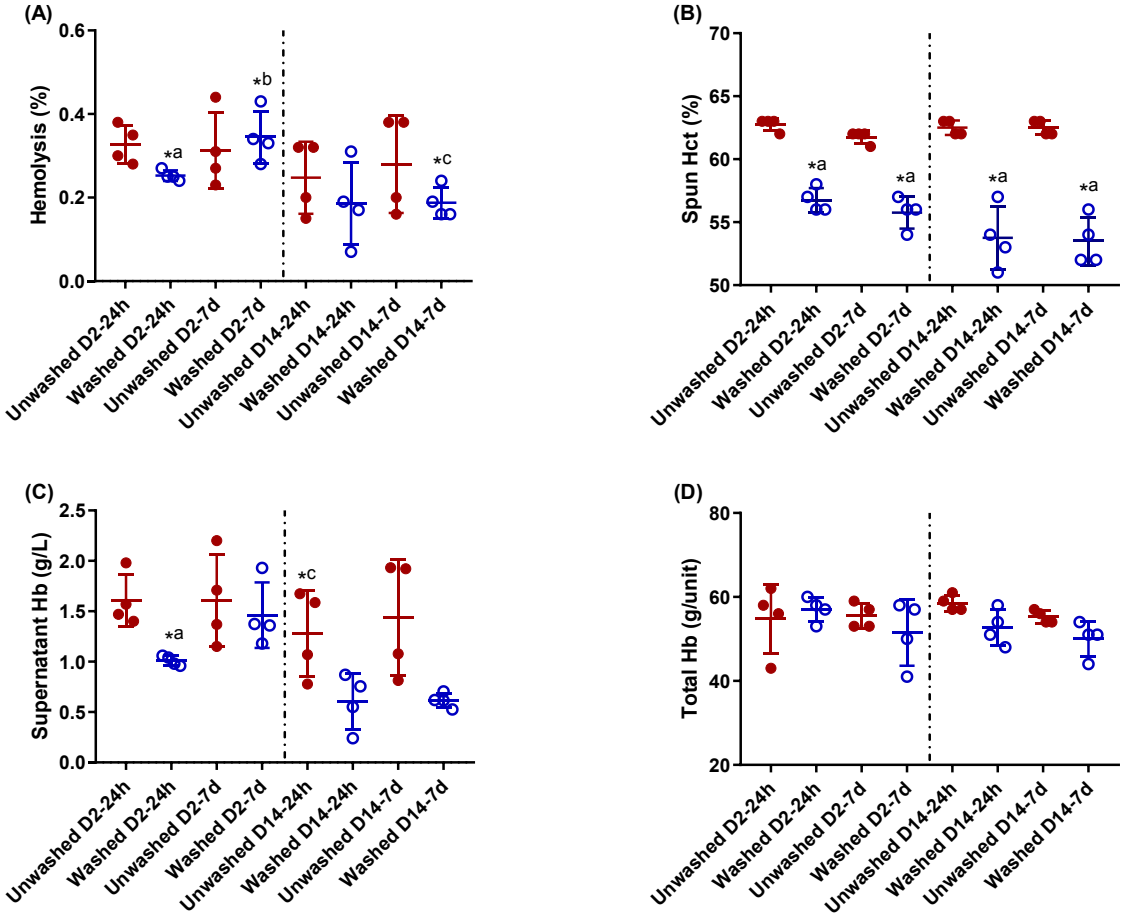

**Figure 5.3:** Residual white blood cell (WBC) and platelet counts in RCCs for unwashed (filed/Red) and washed (unfiled/blue) units. Data reported as scatter dot plots with mean and standard deviation

*a (P <0.05); significant results compared with **unwashed** sample of the correspondent group

*b (P <0.05); significant difference in comparison with **24 h** values of the correspondent group

*c (P <0.05); significant difference between washed/unwashed **day 2** vs washed/unwashed **day 14** of the correspondent group

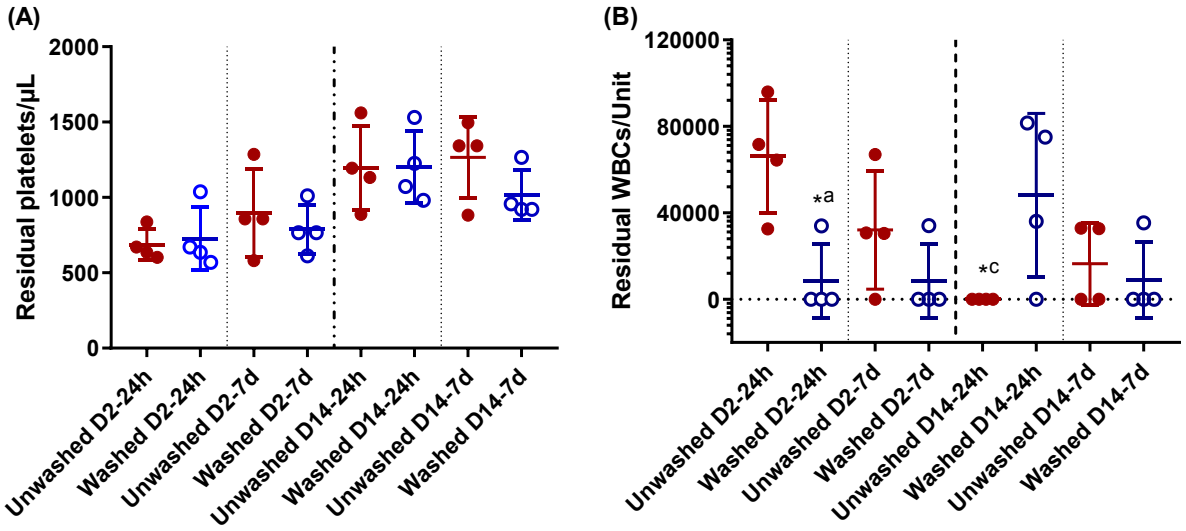

**Figure 5.4:** Concentration of EVs (EVs/mL) in RCC products analyzed by the TRPS system. (A) EVs < 200 nm, (B) EVs ≥ 200 nm. Data reported as scatter dot plots with mean and standard deviation for unwashed (filed/Red) and washed (unfiled/blue) units.
*a (P <0.05); significant results compared with **unwashed** sample of the correspondent group
*b (P <0.05); significant difference in comparison with **24 h** values of the correspondent group
*c (P <0.05); significant difference between washed/unwashed **day 2** vs washed/unwashed **day 14** of the correspondent group

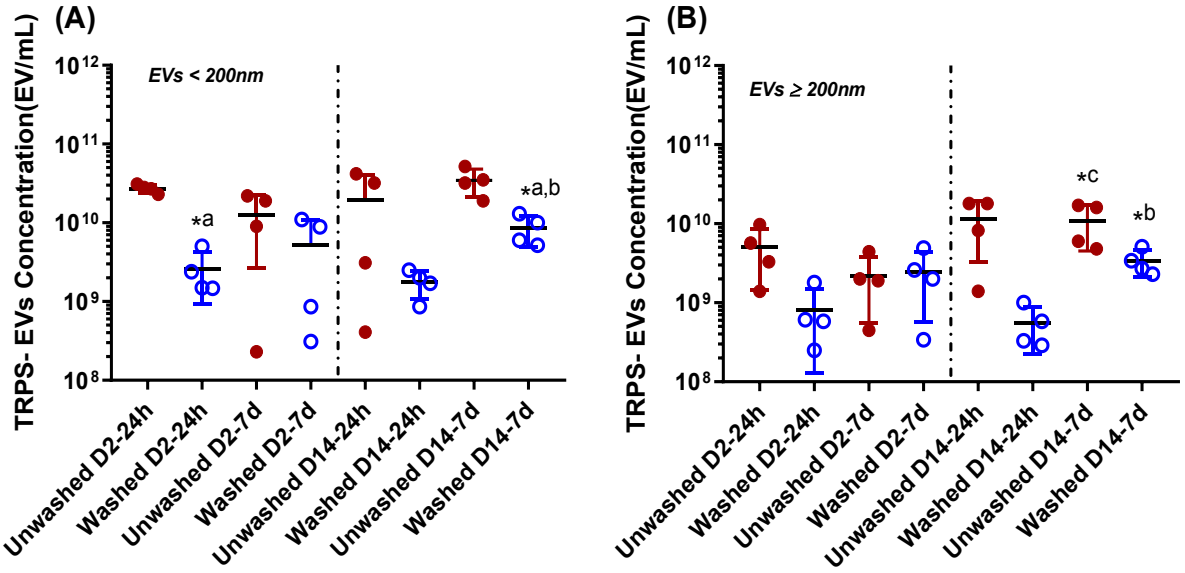

**Figure 5.5:** Concentration of EVs (EVs/µL) subpopulation in RCC products analyzed by flow cytometer. (A) Total EVs, (B) RBC-EVs, (C) Platelet-EVs and (D) WBC-EVs [WBC = CD19+, CD14+, CD16+, CD3+, and CD66b+ EVs combined together]. Data reported as scatter dot plots with mean and standard deviation.
*a (P <0.05); significant results compared with **unwashed** sample of the correspondent group
*b (P <0.05); significant difference in comparison with **24 h** values of the correspondent group
*c (P <0.05); significant difference between washed/unwashed **day 2** vs washed/unwashed **day 14** of the correspondent group

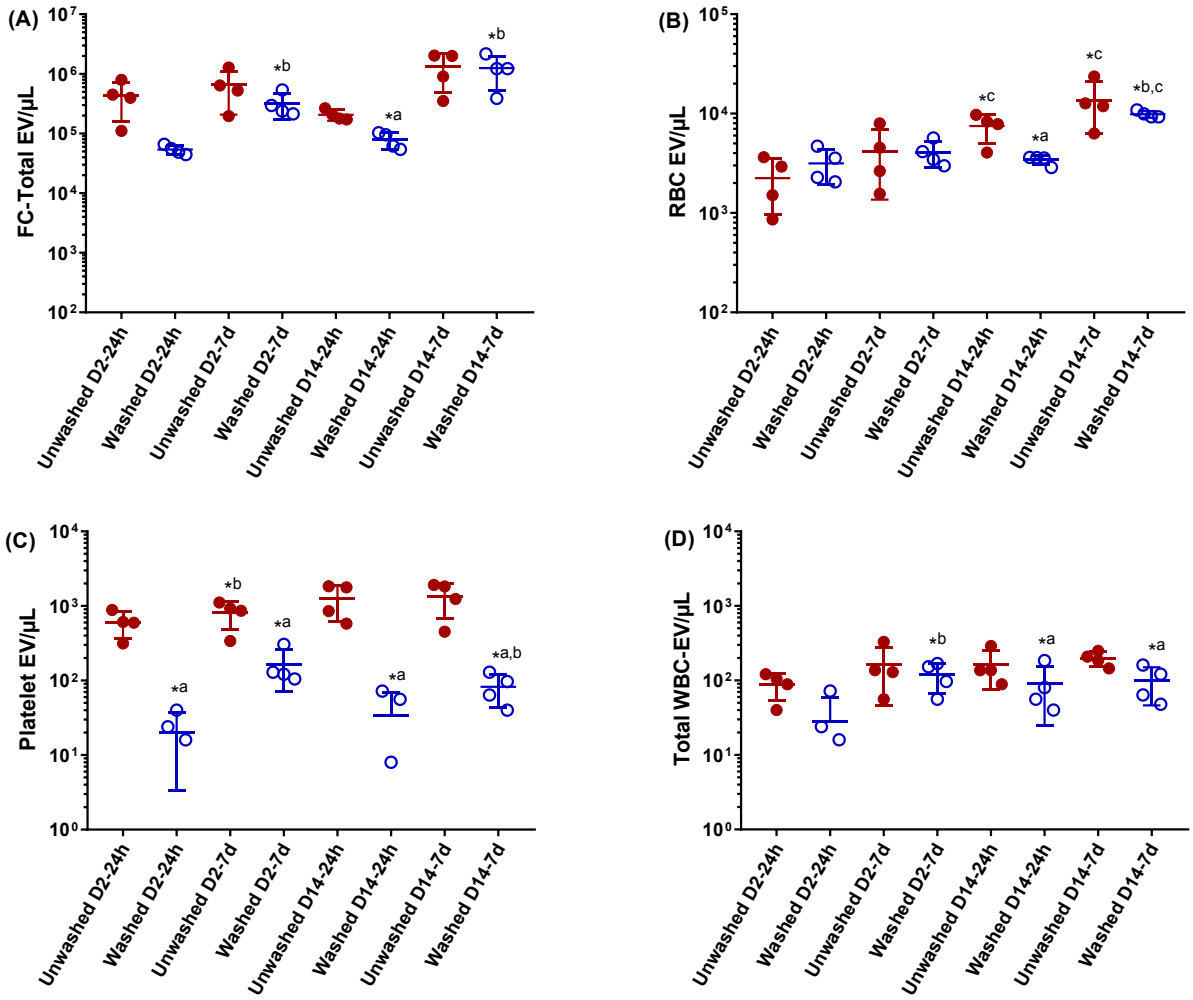

**Figure 5.6:** Concentration (EVs/µL) of subtype of WBC-derived ($CD19^+$, $CD14^+$, $CD16^+$ $CD3^+$ and $CD66b^+$) EVs, tissue factor ($CD142^+$)-EVs, P-selectin ($CD62p^+$)-EVs and endothelial ($CD144^+$)-EVs using the flow cytometer. Data reported as scatter dot plots with mean and standard deviation. *($P < 0.05$); significant results compared with unwashed sample of the correspondent group.

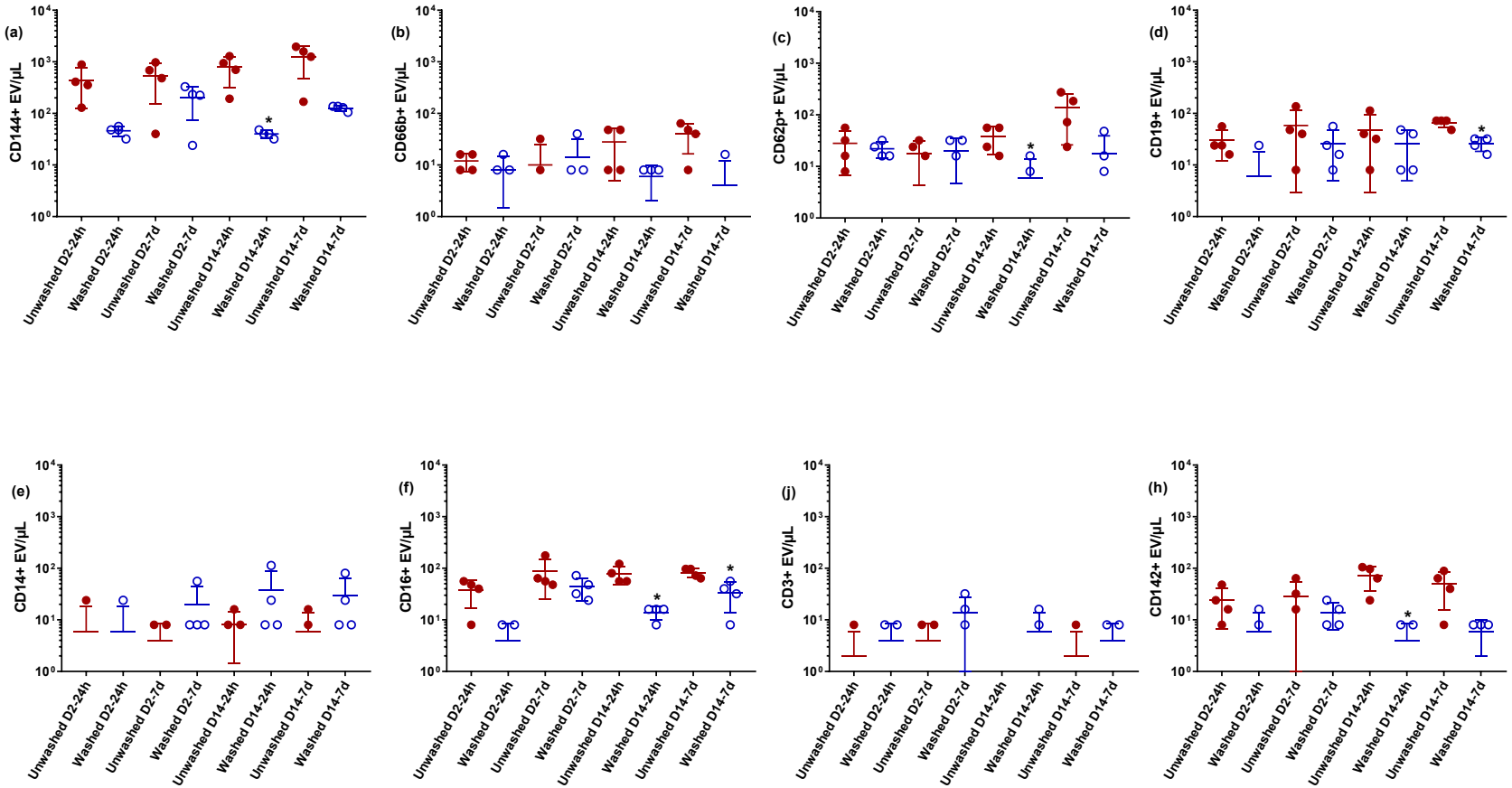

**Figure 5.7:** Monocyte cytokine production for **(1) washing study**; (A) IL-8, (B) MCP-1 following exposure of RCC supernatant from unwashed (filed/Red) and washed (unfiled/blue) units, and **(2) spiking study**; (C) IL-8, and (D) MCP-1 following exposure platelet-EVs (filed, black) only or the exposure of RCC supernatant from washed/spiked with platelet-EVs (filed/Red), or washed/spiked with PBS (unfiled/blue). *Significant level in comparison to control (*P<0.05; **P<0.01; ***P<0.001, ***P<0.0001).

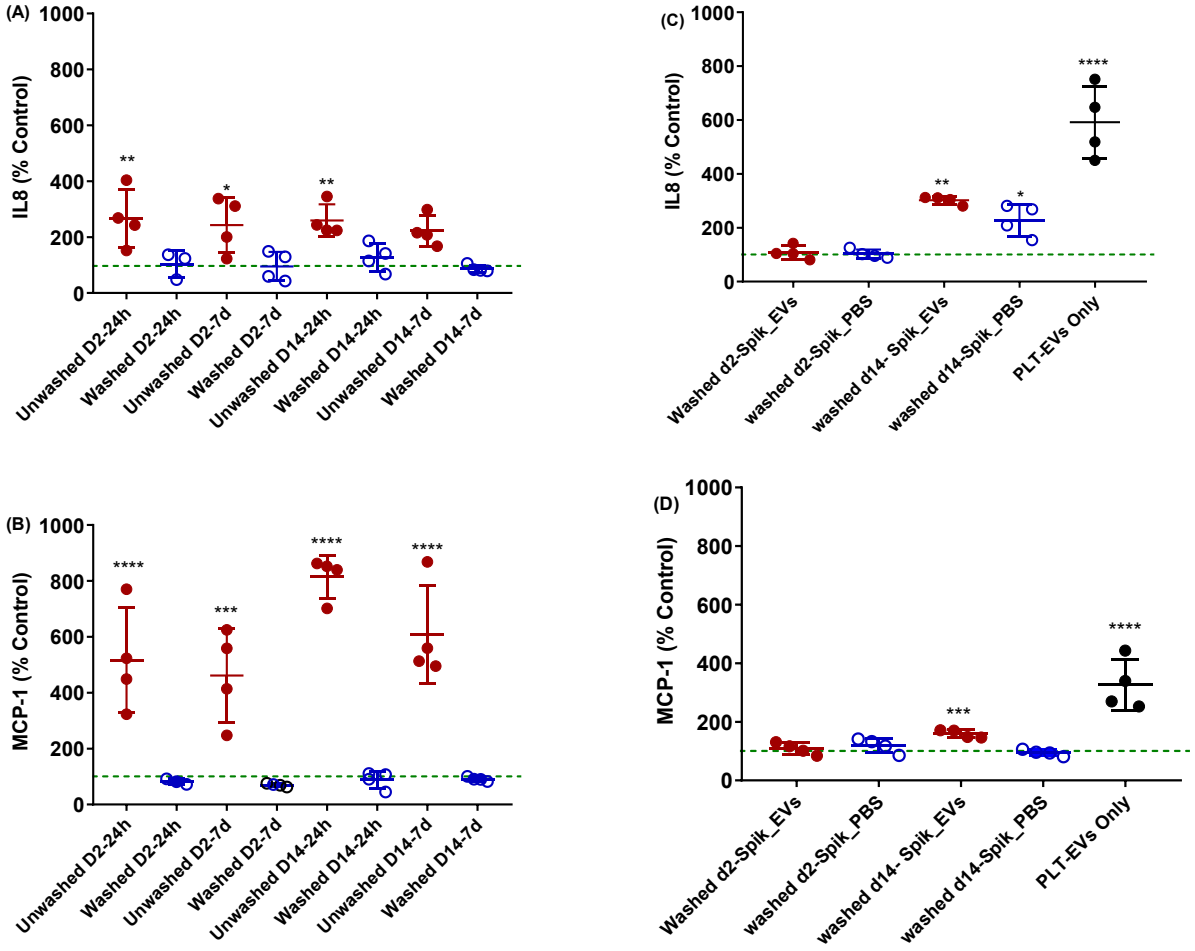

**Figure 5.8:** Expression of adhesion molecules on HUVEC for **(1) washing study**; (A) IL-8, (B) MCP-1 following exposure of RCC supernatant from unwashed (filed/Red) and washed (unfiled/blue) units, and **(2) spiking study**; (C) IL-8, and (D) MCP-1 following exposure platelet-EVs (filed, black) only or the exposure of RCC supernatant from washed/spiked with platelet-EVs (filed/Red), or washed/spiked with PBS (unfiled/blue). *Significant level in comparison to control (*P<0.05; **P<0.01; ***P<0.001, ***P<0.0001).

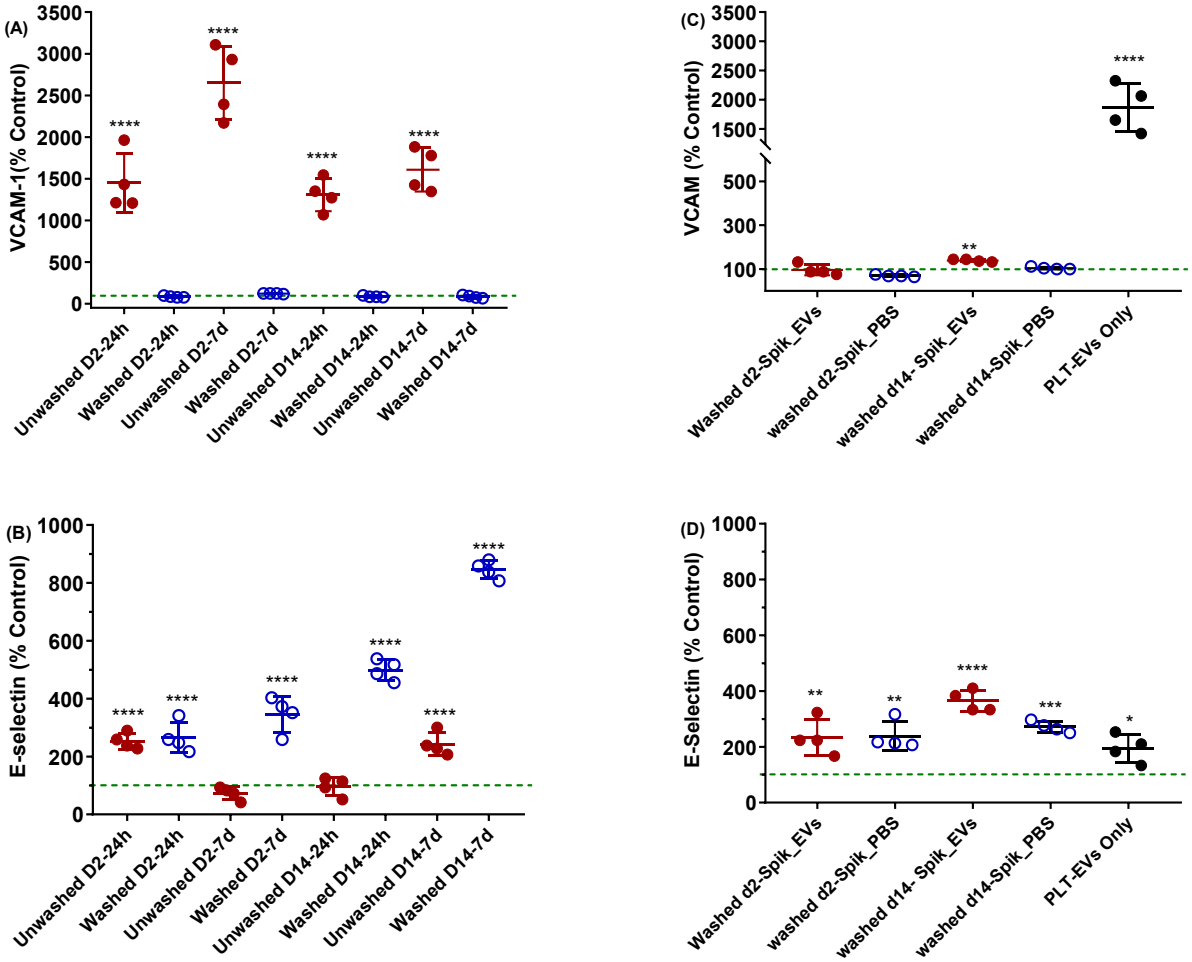

**Figure 5.9:** Overall correlations between EVs based on their cell of origin and monocyte cytokines production for all RCC units [washed RCCs (green), unwashed RCCs (red)] using Pearson correlation.

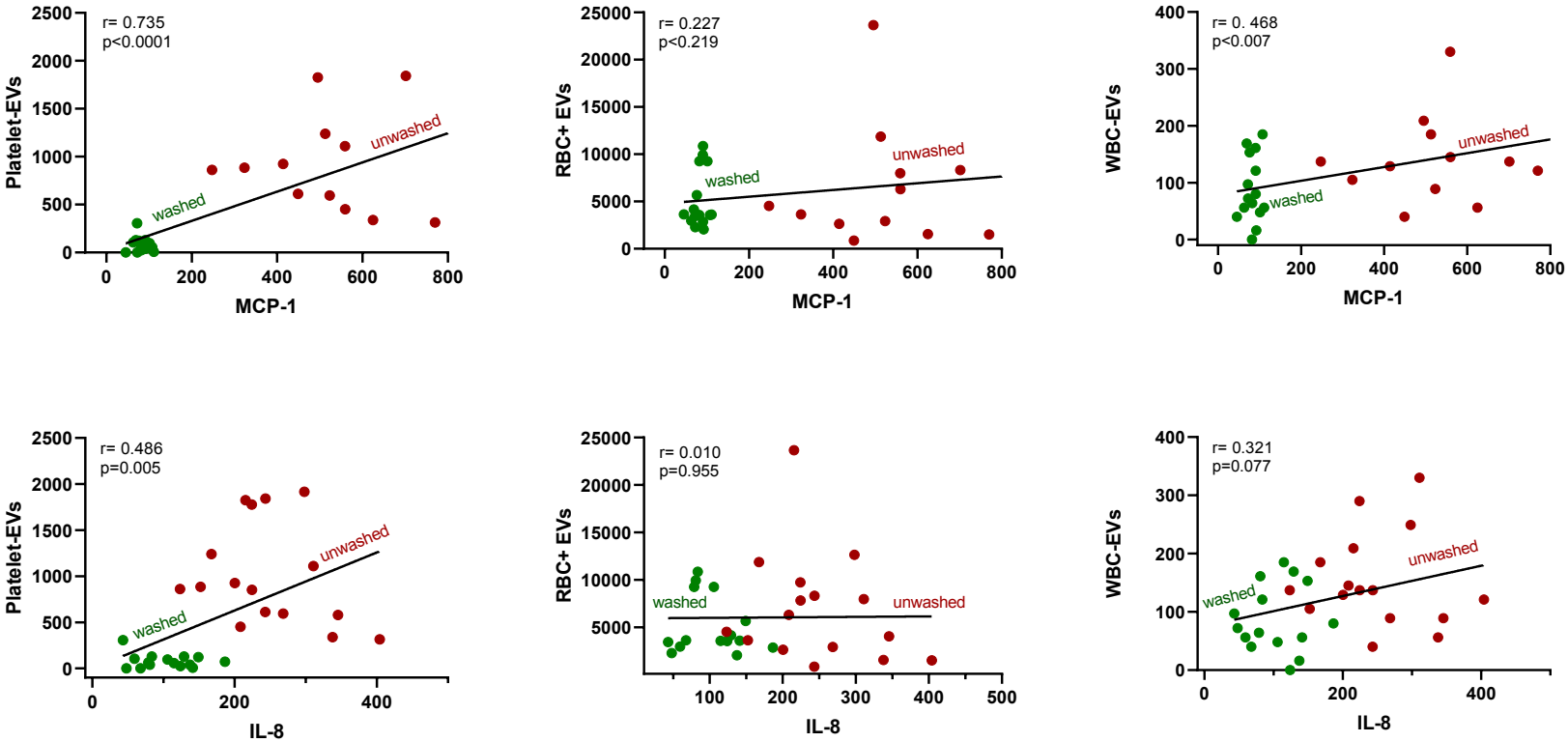

**Figure 5.10:** Overall correlations between EVs based on their cell of origin and HUVEC adhesion molecules expression for all RCC units [washed RCCs (green), unwashed RCCs (red)] using Pearson correlation.

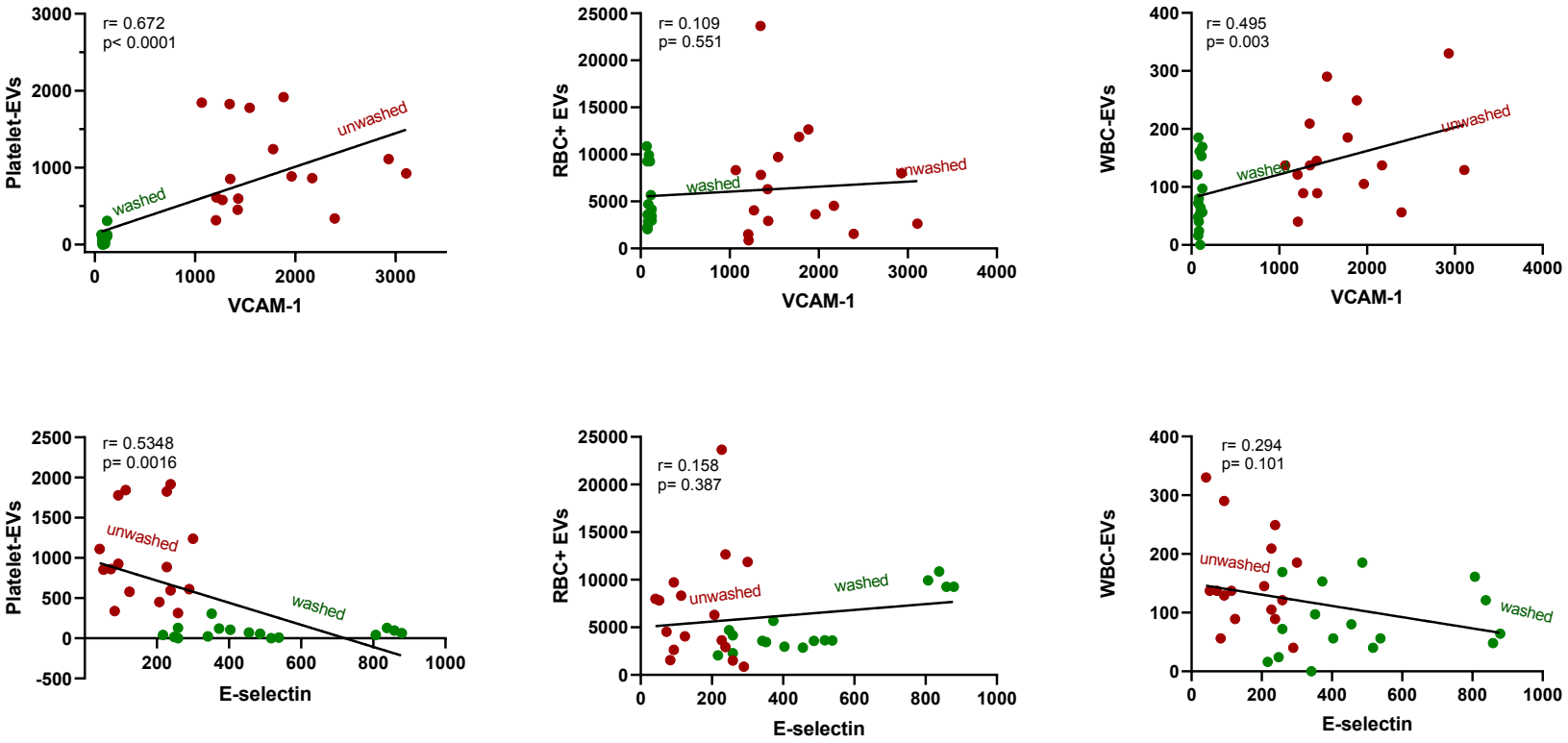

**Table 5.1:** Table represent the RBC hematological indices values measured by hematological analyzer. Data reported as mean ± standard deviation.

| | 24 h Post Wash (mean ± SD) | | | | 7 d Post Wash (mean ± SD) | | | |
|---|---|---|---|---|---|---|---|---|
| ***Parameters*** | **Washed d 2** | **Unwashed d 2** | **Washed d 14** | **Unwashed d 14** | **Washed d 2** | **Unwashed d 2** | **Washed d 14** | **Unwashed d 14** |
| Hct (%) | 52 ± 0*a | 58 ± 1 | 51 ± 1*a | 61 ± 0*c | 53 ± 0*a,b | 60 ± 0*b | 61 ± 1*a,b | 61 ± 0*b,c |
| THb (g/unit) | 54 ± 7 | 57 ± 7 | 55 ± 8.14*a | 56 ± 9 | 59 ± 6 | 51 ± 8 | 54 ± 11 | 55 ± 3 |
| MCV (fL) | 89 ± 2 | 88 ± 2 | 92 ± 3 | 91 ± 3 | 90 ± 2*b | 90 ± 2*b | 91 ± 3 | 92 ± 2*b |
| MCH(pg) | 28 ± 2 | 28 ± 3 | 29 ± 2*a | 28 ± 3 | 32 ± 3 | 27 ± 6 | 29 ± 6 | 29 ± 3 |
| MCHC(g/L) | 319 ± 31 | 347 ± 19 | 317 ± 38*a | 306 ± 38*c | 359 ± 32 | 300 ± 58 | 324 ± 63 | 318 ± 28 |

Data reported as mean ± standard deviation.
*a ($P < 0.05$); significant results compared with **unwashed** sample of the correspondent group
*b ($P < 0.05$); significant difference in comparison with **24 h** values of the correspondent group
*c ($P < 0.05$); significant difference between washed/unwashed **day 2** vs washed/unwashed **day 14** of the correspondent group

**Table 5.2A:** Monocyte cytokine production (% control) for **(A) washing study** following exposure of RCC supernatant from unwashed and washed units. *Significant level in comparison to negative controls ($P < 0.05$). OOR; out (below) of detection range.

| Groups /Cytokines | GM-CSF | IFN-y | IL-10 | IL-1B | IL-8 | MCP-1 | IL-4 | IL-6 | TNF-a | IL-12(p70) |
|---|---|---|---|---|---|---|---|---|---|---|
| Negative Control | 100 | 100 | 100 | 100 | 100 | 100 | OOR | 100 | 100 | OOR |
| Positive Control (LPS) | 1050 ± 129 | 261 ±61 | 486 ± 175 | 33274 ± 16883 | 1175 ± 361 | 99 ± 34 | OOR | 89941 ± 15139 | 40987 ± 8092 | OOR |
| Unwashed - 24h Post wash d2 | OOR | OOR | 239 ± 20* | OOR | 267 ± 104* | 516 ± 188* | OOR | OOR | 283 ± 102* | OOR |
| Washed - 24h Post wash d2 | OOR | OOR | OOR | OOR | 103 ± 48 | 82 ± 10 | OOR | OOR | ± | OOR |
| Unwashed - 7d Post wash d2 | OOR | OOR | OOR | OOR | 243 ± 100* | 461 ± 168* | OOR | OOR | 233 ± 97* | OOR |
| Washed - 7d Post wash d2 | OOR | OOR | OOR | OOR | 95 ± 52 | 70 ± 6 | OOR | OOR | OOR | OOR |
| Unwashed - 24h Post wash d14 | OOR | OOR | 232 ± 15* | OOR | 259 ± 58* | 814 ± 76* | OOR | OOR | 174 ± 17 | OOR |
| Washed - 24h Post wash d14 | OOR | OOR | OOR | OOR | 128 ± 50 | 88 ± 30 | OOR | OOR | OOR | OOR |
| Unwashed- 7d Post wash d14 | OOR | OOR | OOR | OOR | 222 ± 55 | 609 ± 175* | OOR | OOR | 294 ± 145* | OOR |
| Washed - 7d Post wash d14 | OOR | OOR | OOR | OOR | 87 ± 13 | 91 ± 8 | OOR | OOR | OOR | OOR |

**Table 5.2B:** Monocyte cytokine production (% control) for **spiking study** following exposure of platelet-EVs only, supernatant from washed RCC and spiked with platelet-EVs, or supernatant from washed RCC and spiked with PBS. *Significant level in comparison to controls ($P < 0.05$). OOR; out (below) of detection range

| Groups /Cytokines | GM-CSF | IFN-y | IL-10 | IL-1β | IL-8 | MCP-1 | IL-4 | IL-6 | TNF-a | IL-12(p70) |
|---|---|---|---|---|---|---|---|---|---|---|
| Negative Control | 100 | 100 | 100 | 100 | 100 | 100 | OOR | 100 | 100 | OOR |
| Positive Control (LPS) | 1269 ± 375 | 165 ± 36 | 284 ±112 | 21803 ± 6049 | 1509 ± 368 | 117 ± 48 | OOR | 36320 ± 11689 | 24335 ± 8913 | OOR |
| Washed-EVs Spike-Post wash d2 | OOR | OOR | OOR | OOR | 108 ± 25 | 108 ± 20 | OOR | OOR | OOR | OOR |
| Washed-PBS Spike-Post wash d2 | OOR | OOR | OOR | OOR | 103 ± 16 | 119 ± 24 | OOR | OOR | OOR | OOR |
| Washed-EVs Spike-Post wash d14 | OOR | OOR | OOR | OOR | 302 ± 15* | 159 ± 13* | OOR | OOR | OOR | OOR |
| Washed-PBS Spike-Post wash d14 | OOR | OOR | OOR | OOR | 228 ± 59* | 94 ± 10 | OOR | OOR | OOR | OOR |
| PLT-EVs only | OOR | OOR | 283 ± 22* | OOR | 591 ± 134* | 326 ± 86* | OOR | OOR | 229 ± 62* | OOR |

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# Chapter 6

## General Discussion and Conclusion

## 6.1. REVIEW OF THESIS OBJECTIVES AND SUMMARY OF RESULTS

Transfusion of red blood cell concentrates (RCCs) is a necessary, lifesaving medical intervention[1]. However, like any medical therapy, transfusion of RCCs comes with risks. A number of studies have revealed that transfusion of RCC products is still associated with increased risk of serious clinical outcomes that include immune and nonimmune adverse reactions[2-8]. Transfusion-related immunomodulation (TRIM) has emerged as a potential explanation for the increased immunosuppression or proinflammatory effects associated with infection, multi-organ dysfunction and mortality in some patients who receive blood transfusions[1,7,9]. However, the exact mechanisms responsible for TRIM are still undetermined. While efforts to understand the biological mechanisms are under way, the immunomodulatory potential of extracellular vesicles (EVs) in blood products have been shown to be important in transfusion medicine[10-12]. In addition, despite the fact that there are several ongoing prospective research and studies to understand the adverse clinical outcomes associated with transfusion, variations between RCCs produced by different manufacturing methods and issued for transfusion are not well appreciated. Our previous studies have revealed that RCC produced by different manufacturing methods are not all equal and characteristics of EVs, which may contribute in TRIM, are different across the RCC products[13-15]. Moreover, a linked database has shown an association between the production methods and transfusion outcomes[16]; however, the impact of RCC manufacturing process on transfusion-related immune responses has not been explored. Understanding the potential immunomodulatory effects of EVs and blood manufacturing methods can be used to improve blood production and lead to safer transfusions. Therefore, investigations in this research were to identify "what is in the bags" of differently

manufactured RCCs, highlight the presence of non-red blood cell (RBC) fractions in the RCC products including residual cells and cell-derived EVs. The second step of this work was conducted to understand the role that cell-derived EVs and blood manufacturing methods may play in the quality and immunomodulatory activity of RCC products with an attempt to relate this knowledge to the development of better manufacture processing, preservation and transfusion. Thus, the last aim of this research was to identify strategies that may eliminate the parameters responsible for poorer clinical outcomes.

The results of the experimental studies have been presented to test the following hypotheses: 1) produced and stored RCCs contain a mixed population of EVs and not all EVs in RCC are solely from the constituent RBCs; 2) the size, concentration, and population of EVs in RCCs are influenced by method of detection, blood component manufacturing and storage duration; 3) blood manufacturing methods affect RCC products characteristics and are associated with immunomodulatory activity of RCCs; 4) non-RBC generated vesicles in RCC are potent mediators of RCC immunomodulatory activity *in vitro;* and 5) washing RCC products using an automated cell processer can reduced immunomodulatory effects of RCC.

Microvesiculation is a controlled process by which EVs, small membranous vesicles, are formed and released *in vivo* and *in vitro* by many cells in response to a variety of conditions and stimuli including hypoxia, oxidative and shear stress[17-20]. An increase in microvesiculation during storage has been identified as a significant indicator of the red blood cell storage lesion[21-24]. However, the biological complexity of EVs including the variation in morphology, size, composition, cellular source and the biogenesis, create excessive challenges and difficulties in detecting, and characterizing these EVs[25,26]. Although there are a wide variety of methods and techniques being used to purify, identify, quantify and characterize these vesicles, there are

several limitations associated with these techniques[25,27-29]. In addition, most studies do not take into account the heterogeneity of EVs in the RCC products in terms of concentration, content, size, and phenotype (cell of origin). Therefore, the objective of the first experimental study in this thesis was to identity, characterize, and quantify EVs in RCC units using different techniques [tunable resistive pulse sensing (TRPS/qNano), flow cytometer (FC), and dynamic light scattering (DLS; Zetasizer)], and to assess the impact of different approaches to characterize EVs in stored RCC products **(Chapter 2)**. Ultimately, the goal was to evaluate the advantage and the disadvantages of each approach and select the optimum method(s) to be used consistently for the next studies. This study showed that the characterization of EV present in stored RCC products is significantly influenced by the method of detection used. The quantitative analysis in this study showed that an increase in the concentration of EVs ≥ 200 nm (large EVs/microparticles) during hypothermic storage of RCCs when FC and TRPS device were used. Notably, the TRPS method revealed a significant increase in the concentration of EVs < 200 nm (small EVs/exosomes) throughout storage. This change in exosome concentration was not detectable with FC or DLS due to limitations in their ability to resolve particles < 200 nm and/or accurately determine EV concentration. This study highlighted the important of understanding the advantages and the limitations of each technique before selecting specific method to characterize EVs. From this work, it is recommended that the use of different method of detection would be better in order to characterize the size, concentration and phenotype of EVs in stored RCCs.

After selecting and establishing the techniques required to identify and characterize the subpopulations of EVs in RCC, the next study (**Chapter 3**) aimed to assess the impact of different blood manufacturing methods and duration of hypothermic storage on these

subpopulations of EVs in relation to other commonly evaluated *in vitro* quality parameters of RCC products. Different manufacturing methods are used to produce blood components by separating red blood cells (RBCs), platelet concentrates, and plasma from the whole blood (WB) of the donor. In Canada, the blood processing methods include red cell-filtered (RCF, top-and-bottom) and whole blood-filtered (WBF, top-and-top) RCCs[16]. As the RCCs issued for transfusion are not created or treated equally, variability exists within the blood products themselves[30,31], which may contribute to the storage lesions and adverse clinical outcomes. In **Chapter 3**, we investigated the impact of RCF and WBF blood manufacturing methods and hypothermic storage duration on the characteristics of different subpopulations of EVs and *in vitro* quality parameters. This study revealed that the dynamic shift in the size and concentration of the EVs subpopulation is dependent on the blood manufacturing method and storage duration. The outcome of this study also showed a positive correlation between EVs and hemolysis, and a negative correlation between EVs and the level of ATP as well as deformability parameters. These correlations with the measured *in vitro* quality parameters suggest that EVs could be implemented as a quality indicator. Results of this study also revealed that heterogeneous submicron-sized vesicles are present in RCC products and that the frequency of the diverse populations of EVs is dependent on the blood manufacturing method. Furthermore, our novel data captured by the TRPS showed that the concentration of small EVs / exosomes (< 200 nm) was greater in WBF units in comparison to RCF units. As red blood cells are able to produce microvesicles but lack the capacity to release exosomes[32,33], data in this study suggested that the EVs < 200 nm are non-RBC derived EVs (platelet-derived EVs and WBC-derived EVs). Although great attention has been directed to the role of the 'contaminating' residual cells in RCCs, comparatively, very few studies focus on the role of the EVs derived from these residual

cells. Therefore, this study recommended further studies to investigate the cell of origin of these EVs, particularly of the small EVs, and their potential influence on the quality of the products as well as their immunomodulatory effects after transfusion.

The mechanisms of adverse effects related to red cell transfusion remain uncertain, though RCCs contain a host of biologically active mediators, in both soluble and cell-associated forms, which may contribute to organ dysfunction via alterations in recipient inflammation and immune cell function[7,34-36]. While many previous studies have focused on accumulation of potentially harmful immunomodulatory mediators during RCC storage[1,2,4], recent randomized clinical trials have failed to demonstrate benefit with fresh RCC transfusion in critically ill or hospitalized patients[37], thus calling into question the clinical relevance of storage-related TRIM effects. Noteworthy, it has been suggested that RCC manufacturing methods, which are rarely accounted for in interventional trials, may have confounded these results[31,38]. In view of this and the previous chapters, experiment in **Chapter 4** aimed to investigate the effect of different manufacturing methods on hemolysis, residual cells, cell-derived EVs, and immunomodulatory activity. In this study, thirty-two RCC units produced using whole blood filtration (WBF), red cell filtration (RCF), apheresis derived (AD), and whole blood derived (WBD) methods were examined (n=8 per method). This study showed that blood manufacturing methods significantly influence the immunomodulatory effects of RCC supernatant on monocytes *in vitro* and significantly affected RBC and non-RBC EVs characteristics throughout storage. Noteworthy, while an immunosuppressive effect was observed with the supernatants form AD and WBD RCCs as shown by the significant reduction in the release of the inflammatory cytokine (TNF-α) by monocytes in response to LPS-stimulation, supernatants from fresh WBF units resulted in significantly higher inflammatory cytokine (IL-8) production from the unstimulated

monocyte model in comparison to controls. Our exploratory analyses relating immunomodulatory activity to cell-derived EVs in this study suggested that platelet-derived EVs correlate with immunosuppressive activity. Interestingly, our study failed to identify significant correlations between RBC-EVs and monocyte cytokine production across manufacture methods for either fresh RCC or RCC at expiry. This study supports our hypothesis that non-RBC vesicles/fractions in RCC, which vary across manufacturing methods, are potent mediators of immunomodulatory activity *in vitro*. However, the effects of residual cells and cell-derived EVs on immunomodulatory activity and patient clinical outcomes worth additional examination. In addition, it is worth noting that the correlation analysis in this study was exploratory correlation only. Therefore, future studies are still warranted better explain the mixed immunomodulatory effects observed with different blood manufacturing methods and to identify the factor(s) or agent(s) that contribute to these effects.

In the previous chapters, we showed that EVs from leukoreduced and stored RCCs are heterogonous in quantity, size and cell of origin. In addition, we observed that EVs, depending on their phenotype, are associated with RCC immunomodulatory activity. Since studies suggest that several soluble contaminants and harmful substances could be removed by washing RCC products, which may improve the quality of RCC and may reduce or prevent some adverse outcomes post transfusion[39-41], the final study (**Chapter 5**) was aimed at investigating the effect of washing, using an automated cell processor (ACP-215), on RCC quality parameters, residual cells, extracellular vesicles, and on the immunomodulatory activity of WBF RCCs. Once the effects of washing were determined, the result of washed and unwashed RCC were compared to determine the potent factor(s) associated with the RCC immunomodulatory activity. We also investigated whether platelet-EVs, which were shown to be associated with immunomodulatory

activity in **Chapter 4**, induce pro- or anti-inflammatory response in monocytes and human umbilical vein endothelial cell (HUVEC). Investigation in this study showed that washing RCC with the ACP 215 was not sufficient to remove residual cells from the stored RCC products; however, all tested RCC units of all experimental groups exceeded the quality assurance criteria for hemolysis, hematocrit, and hemoglobin. Furthermore, the TRPS showed that washing significantly reduced the concentration of small/exosomes (< 200 nm) and large EVs /microparticles (≥ 200 nm) compared to unwashed RCCs. Noteworthy, results from monocyte and HUVECs models used in **Chapter 5** further emphasize that RCCs produced by WBF method are inflammatory as evidenced by significantly elevated production of the inflammatory cytokines and chemokines; TNF-α, MPC-1 and IL-8, and increased expression of VCAM-1 compared to controls. Washing these RCCs with the ACP 215 decreased the inflammatory phenotype observed. Additionally, the exploratory correlational analyses of **Chapter 5** showed that the non-RBC EVs, particularly platelet-EVs, were associated with the inflammatory phenotype observed with WBF RCCs and reducing these vesicles by washing, can attenuate the immunomodulatory activity of the examined RCCs. Taken together the results from **Chapter 4** and **Chapter 5** of this thesis, the data further suggest that the same type of EVs may bias toward inflammatory or immunosuppressive activity depending on the co-stimulatory factors, which in our study were blood manufacturing methods and storage duration. Therefore, the immune heterogeneity of EVs in RCCs needs to be more carefully considered when evaluating the immunomodulatory activity of blood products.

## 6.2. CONTRIBUTION TO SCIENCE AND FUTURE DIRECTION

This thesis has made a number of valuable contributions to the fields of the transfusion medicine and biopreservation, as demonstrated by five peer-reviewed published manuscripts in addition to a submitted paper in top journals in the field of transfusion medicine, blood research and biopreservation.

1) The work presented in this thesis has highlighted the limitation and the challenges related to monitoring extracellular vesicles in blood research and have addressed this with the development and recommendation of using different techniques and methods to detect, quantify and characterize EVs in stored RCC products. This knowledge is important to promote our understanding of EVs, report more accurate data on "what is in the RCC bags", and allow better comparison between the studies. Taking in consideration the findings of this work and the recommendations, this work will help in the development of a reference method (standardization) in how extracellular vesicles should be measured to support data sharing across research groups.
2) This work has also contributed to the investigation and current understanding of the impact of blood manufacturing methods and hypothermic storage duration on the characteristics of different subpopulations of EVs and *in vitro* quality parameters of RCCs produced by different manufacturing methods. The results highlighted in this study give emphasis to the important of understanding the differences and variability in the final manufactured products when new products, such as blood bag or storage solution, or even new manufacturing process are implemented. This will help providing better blood products for clinical care. Further studies are warranted to understand the individual and collective impact of other variables/factors on the quality of RCC

products, such as donor factors, pre-storage leukoreduction, and storage solutions, and to better understand the clinical impact.

3) This piece of work provided an explanation for the potential immunomodulatory activity associated with stored and differently manufactured RCC products as my work suggests that storage duration and blood manufacturing method used to produce the blood components could affect patient clinical outcomes. Therefore, throughout my thesis work, I strongly recommended additional studies and clinical investigation to further understand what could influence "what is in the bag", particularly for differentially manufactured RCC products, and what clinical consequences may result. This will help clarify the difference in the reported results of stored RCCs and provide a better understanding of the issues that exist with current blood products. Future work in this area should focus on elaborating the influence of these differences observed on transfusion reactions and patient clinical outcomes posttransfusion.
4) The work in this thesis is among the first to document a potential functional consequence related to the differences in RCC quality measures and EV characteristics that result from different manufacturing methods. Although further studies to clarify the mechanisms in which EVs and other untested soluble mediators in RCC result in immunomodulatory activity still required, the data of this work is a novel finding could provide a biological mechanism for the data recently published regarding the increased inflammatory responses associated with "fresh" but not "expired" RCC products. The data of this work provided solid evidences that RCC products vary in their content of the immunomodulatory mediators that are capable of altering the immune cell function. It is worth noting that this work showed that depending on the manufacturing method used,

some blood product can be associated with more harmful immunomodulatory activity, such as apheresis derived RCCs, compared to other RCC products (red cell filtered RCCs). The data of this thesis clearly emphases that not all RCC products are equivalent in terms of their contribution to TRIM because they vary in their content and concentration of the immunomodulatory agents/ factors, donor characteristics and manufacturing process.

5) This research identify and suggest new strategies that may eliminate the parameters responsible for poorer clinical outcomes for better transfusion. The findings of this work indicate that washing of RCC can reduce immunomodulatory mediators, such as non RBC-EVs, and attenuate bioactivity of the stored RCC, which may be an effective additional procedure to reduce the immunomodulatory effect of blood transfusion. Although further studies are still required to identify the other potential immunomodulatory factors in RCCs and to understand the mechanism associated with immunomodulation, the role of the non RBC-EVs/fractions in the augmented immunomodulatory effects observe in our study is worth further investigations to translate these in vitro findings to the bedside. Overall, the knowledge raised by this thesis suggest that the EVs can be a novel biological indicator for quality of RCC products, which may reflect the efficacy and safety of the blood product. Additionally, this thesis will provide novel and strong scientific foundation about role of EVs in immunomodulation. Optimistically, the knowledge of this thesis will help in the development of better manufacture processing, preservation and transfusion.

In conclusion, this thesis provides a better understanding not only regarding the issues that exist with current blood products, but also about the complex relationship between the

individual blood products (specific donor, collection, processing and handling) and their potential contribution to transfusion-related immunomodulation. The mixed immunomodulatory (immunosuppressive/inflammatory) effects we observed in this study, which has the potential to affect the quality of RCC product and the safety of our patients, may account for conflicting results reported with the studies examining the clinical effects of RCCs as a function of storage time. I believe that this thesis is promising study that will strongly contribute to explain the confounded results associated with the recent randomized clinical trials. In addition, this work provides solid scientific knowledge about EVs in RCC products. Using the tools, methods and knowledge of this research can be the beginning to recognize EVs as a novel biological indicator for the quality and the safety of blood products. Further examination of their potential immunomodulatory effects and clinical consequences is required to bring the development of safer blood transfusion.

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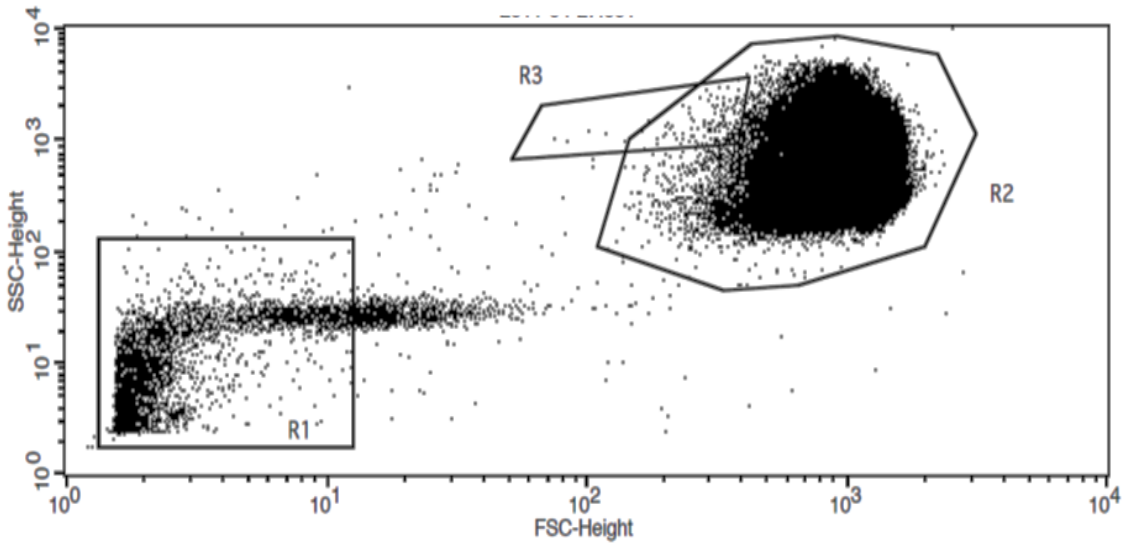
## Appendices

**Appendix A:** Flow Cytometer Gating Settings and Verification of Crosshairs for Red Blood Cell and Microparticles.

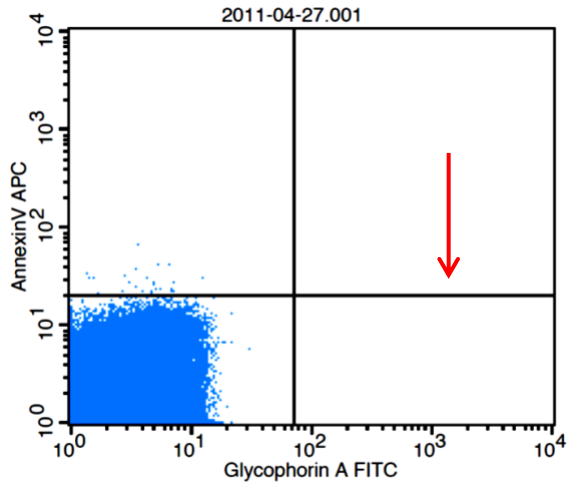
Below you will find a reference guide to verify crosshairs and gating settings. Scatterplots are presented for the untreated RBC sample, isotype RBC sample, stained RBC sample, size beads and TruCOUNT beads

## Untreated/Unstained RBC Sample

| Reference Guide for Untreated RBC Sample Scatterplots | |
|---|---|
|  | |
| Gating Key: | R1 = Gate containing microparticles<br>R2 = Gate containing RBCs<br>R3 = Gate Containing TruCOUNT beads (no TruCOUNT beads should be present) |

## RBC Unstained

| Crosshair Verification | % Gated Verification |
|---|---|



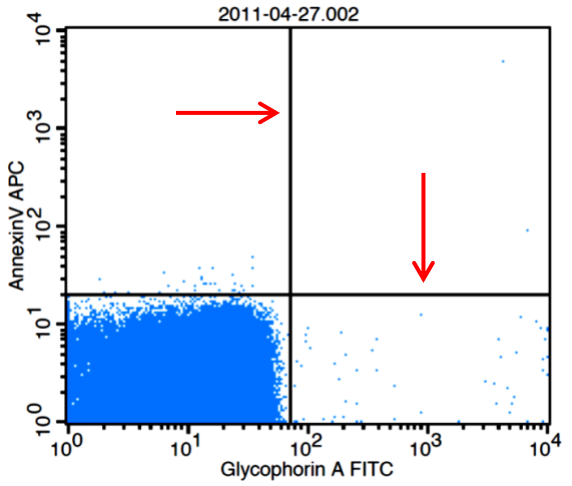

File: 2011-04-27.001
Sample ID: sample 1 unstained
Gate: G2
Total Events: 103200
Log Data Units: Linear \
Acquisition Date: 27-Ap
Gated Events: 98306
Quad Location: 70, 19

| Quad | Events | % Gated | % Total | X Geo Mean | Y Geo Mean |
|---|---|---|---|---|---|
| UL | 35 | 0.04 | 0.03 | 3.96 | 25.02 |
| UR | 0 | 0.00 | 0.00 | *** | *** |
| LL | 98271 | 99.96 | 95.22 | 3.46 | 2.18 |
| LR | 0 | 0.00 | 0.00 | *** | *** |

Ensure that the % gated population in the LL Quadrant is > 98%. If the % gated population is not > 98%, the crosshair denoted by the arrow needs to be adjusted.
*NOTE: If you adjust the Annexin V APC crosshair on this histogram, ensure that all other occurances of the Annexin V APC crosshair in G2 are placed in the same location on the histograms for this specific sample.*
*NOTE: All fluorochromes should appear negative in the unstained sample.*

## Isotype (APC and FITC)

| Crosshair Verification | % Gated Verification |
|---|---|



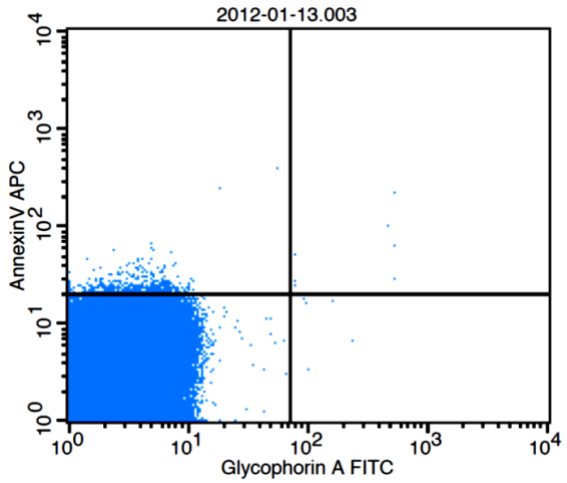

File: 2011-04-27.002
Sample ID: sample 1 isotype
Gate: G2
Total Events: 112155
Log Data Units: Linear Val
Acquisition Date: 27-Apr-1
Gated Events: 107955
Quad Location: 70, 19

| Quad | Events | % Gated | % Total | X Geo Mean | Y Geo Mean |
|---|---|---|---|---|---|
| UL | 44 | 0.04 | 0.04 | 12.54 | 23.92 |
| UR | 3 | 0.00 | 0.00 | 6024.87 | 204.12 |
| LL | 107850 | 99.90 | 96.16 | 11.49 | 2.22 |
| LR | 58 | 0.05 | 0.05 | 760.91 | 2.74 |

Ensure that the % gated population in the LL Quadrant is > 98%. If the % gated population is not > 98%, the crosshair denoted by the arrow needs to be adjusted.
*NOTE: If you adjust the Glycophorin A FITC crosshair on this dot plot, ensure that all other occurances of the Glycophorin A FITC crosshair in G2 are placed in the same location on the dot plots for this specific sample.*

## RBC APC

| Crosshair Verification | % Gated Verification |
|---|---|



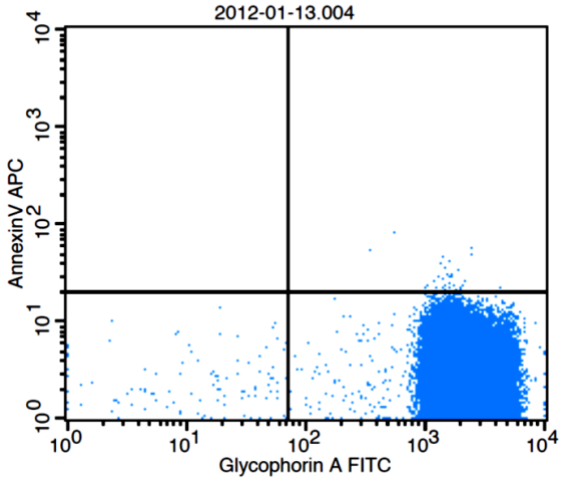

File: 2012-01-13.003 Log Data Un
Sample ID: RBC sample APC -Unstained S Acquisition D
Gate: G2 Gated Event
Total Events: 145350 Quad Locati

| Quad | Events | % Gated | % Total | X Geo Mean | Y Geo Mean |
|---|---|---|---|---|---|
| UL | 654 | 0.57 | 0.45 | 3.53 | 23.01 |
| UR | 7 | 0.01 | 0.00 | 226.42 | 53.28 |
| LL | 113730 | 99.42 | 78.25 | 3.25 | 4.53 |
| LR | 6 | 0.01 | 0.00 | 122.98 | 11.19 |

Ensure that the % gated population is in the UL and LL quadrants is >98% (FITC negative).
*NOTE: Fresh RBCs will not exhibit high levels of PS exposure and therefore you may not have a large population of APC positive events. See NEM PE for positive expression of PS on RBCs.*

## RBC FITC

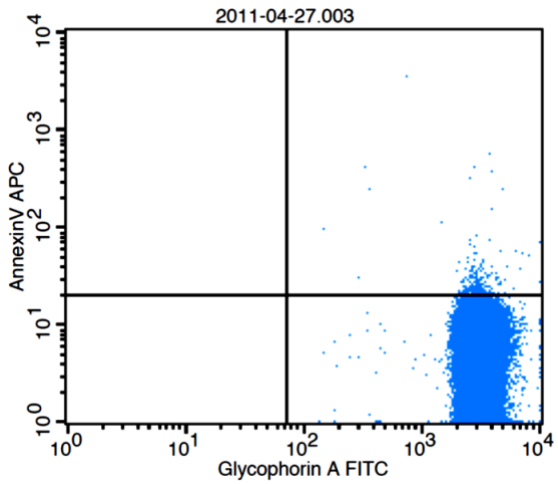
| Crosshair Verification | % Gated Verification |
|---|---|




File: 2012-01-13.004 Log Data Uni
Sample ID: RBC sample FITC Unstained S Acquisition D
Gate: G2 Gated Events
Total Events: 131520 Quad Locatic

| Quad | Events | % Gated | % Total | X Geo Mean | Y Geo Mean |
|---|---|---|---|---|---|
| UL | 0 | 0.00 | 0.00 | *** | *** |
| UR | 40 | 0.04 | 0.03 | 1513.83 | 26.84 |
| LL | 100 | 0.09 | 0.08 | 11.75 | 2.34 |
| LR | 109660 | 99.87 | 83.38 | 1716.33 | 2.48 |

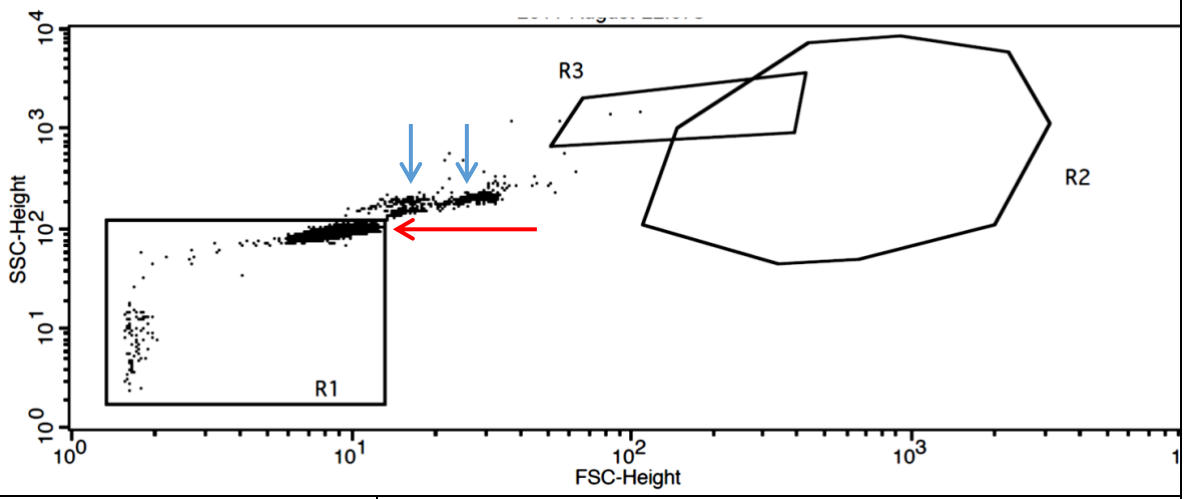
Ensure that the % gated population in the LL and LR quadrants is >98% (ACP negative).

| RBC Cocktail (APC and FITC) | |
|---|---|
| **Crosshair Verification** | **% Gated Verification** |
| Confirm that the population is visible on the dot plot. Do not adjust crosshairs. | |
|  | File: 2011-04-27.003<br>Sample ID: sample 1 cocktail<br>Gate: G2<br>Total Events: 115440<br>Log Data Units: Linear V<br>Acquisition Date: 27-Apr<br>Gated Events: 112650<br>Quad Location: 70, 19 |

| Quad | Events | % Gated | % Total | X Geo Mean | Y Geo Mea |
|---|---|---|---|---|---|
| UL | 0 | 0.00 | 0.00 | *** | ** |
| UR | 371 | 0.33 | 0.32 | 2942.87 | 25.8 |
| LL | 0 | 0.00 | 0.00 | *** | ** |
| LR | 112279 | 99.67 | 97.26 | 2998.66 | 4.0 |

| |
|---|
| Confirm that the population is visible on the dot plot. Do not adjust crosshairs. |

## 1.01 µm Size Beads

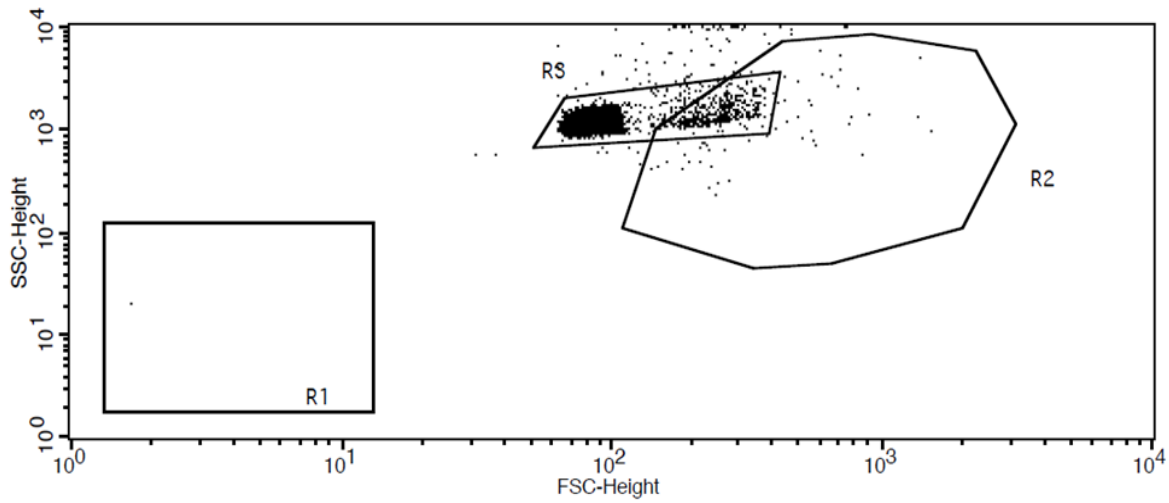
| Reference Guide for Size Bead Scatterplots | |
|---|---|
|  | |
| Gating Key: | R1 = Gate containing size beads<br>R2 = Gate containing RBCs (no RBCs should be present)<br>R3 = Gate Containing TruCOUNT beads (no TruCOUNT beads should be present) |

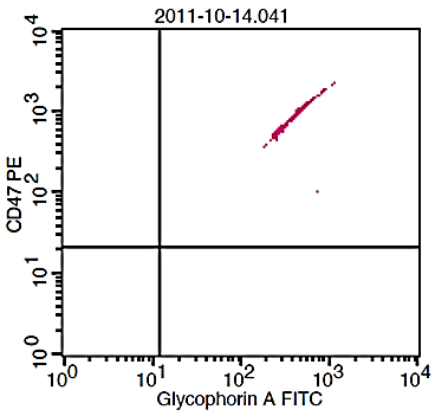
*NOTE: The 1.01 µm Size Beads are noted by the red arrow. The gate R1 contains events that are less than 1.01 µm and less in size. The bottom and left sides of the gate were determined to eliminate background noise and events that are below the limit of detection.*

*NOTE: The blue arrows denote the populations of aggregated beads that should not be included in the microparticle gate.*

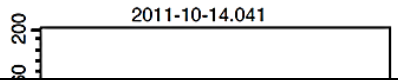
*NOTE: During analysis, ensure that the entire smallest population of beads is surrounded by gate R1. It is only critical to line up the right side of the gate (FSC-Height) with this population because this discriminates microparticles that are less than 1.0 µm. The position of the top of the gate (SSC) is not as critical, but should be as close to the top of the bead population as possible.*

## TruCOUNT Beads

| Reference Guide for TruCOUNT Bead Scatterplots | |
|---|---|
|  | |
| Gating Key: | R1 = Gate containing microparticles (no microparticles should be present)<br>R2 = Gate containing RBCs (no RBCs should be present)<br>R3 = Gate Containing TruCOUNT beads |

| TruCOUNT Beads | |
|---|---|
| **Crosshair Verification** | **% Gated Verification** |
|  | File: 2011-10-14.041<br>Acquisition Date: 14-Oct-11<br>Gated Events: 8087<br>Sample ID: Trucount beads 1<br>Gate: G3<br>Total Events: 8220 |

| Quad | Events | % Gated | % Total | X Mean | X Geo Mean | Y Mean | Y Geo Mean |
|---|---|---|---|---|---|---|---|
| UL | 0 | 0.00 | 0.00 | *** | *** | *** | *** |
| UR | 8087 | 100.00 | 98.38 | 291.74 | 288.40 | 629.30 | 620.98 |
| LL | 0 | 0.00 | 0.00 | *** | *** | *** | *** |
| LR | 0 | 0.00 | 0.00 | *** | *** | *** | *** |




Confirm that 100% of events are in the UR quadrant. Adjust the crosshairs if required to ensure only FITC and PE positive events are captured in the UR quadrant.