Exploring the Interplay between Donor Sex, CD71^{+} RBCs, Erythrophagocytosis and Hospital-Onset Sepsis

by

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Abstract

Transfusion of red cell concentrates (RCCs) is common for treating anemia in Intensive care unit (ICU) patients, but it may result in adverse outcomes like sepsis, potentially due to donor sex-related immune responses. $CD71^+$ RBCs, which are immature, may influence erythrophagocytosis, where the monocyte system removes damaged or aged RBCs—a process that could lead to complications if overwhelmed.

The hypothesis posits that the number of $CD71^+$ RBCs in blood products was affected by donor sex and the donor factors can predict post-transfusion hemoglobin levels and the rate of hospital-onset sepsis. Flow cytometry quantified $CD71^+$ RBCs, revealing that levels are generally higher in male donors, are affected by processing methods, and decrease with storage time. A positive correlation between $CD71^+$ RBCs and hemoglobin levels was noted.

In vitro experiments demonstrated that higher proportions of $CD71^+$ RBCs were associated with increases in the phagocytosis index (PI) of RBCs, elevated supernatant Hb, and loss of $CD14^+$ monocytes. The increase in PI and decrease in $CD14^+$ monocytes could be reversed after $CD71^+$ RBCs were treated with a reactive oxygen species (ROS) inhibitor. This suggests that $CD71^+$ RBCs with high ROS levels may exhibit an immunomodulatory role during *in vitro* erythrophagocytosis. Dose-dependent relationships were also observed between the PI of $CD71^+$ RBCs and the proportion of $CD71^+$ RBCs, as well as between supernatant Hb and the proportion of $CD71^+$ RBCs.

Retrospective analysis showed no significant link between donor sex and hospital-onset sepsis. However, blood from male donors resulted in higher hemoglobin increments, notably in female recipients. Factors like recipient age and the number of transfused RCC units were significant for sepsis, while the donor's hemoglobin level influenced the hemoglobin increment in the recipients.

This study underscores the need for further in vivo research to clarify the impact of donor sex on CD71+ RBCs and transfusion outcomes. It provides an immune perspective on donor sex-related mechanisms in transfusion responses. The findings suggest that both donor and recipient factors should be considered to optimize transfusion practices, as the data points to the potential of including $CD71^{+}$ RBCs as a factor in managing transfusion-related immunomodulation in ICU settings.

Preface

This thesis represents the original work of Wenhui Li as part of a research project titled "Evaluating the role of donor characteristics and blood component manufacturing on the quality of red cell concentrates" The research project obtained ethical approval from the Canadian Blood Services Ethics Board (CBSREB #2020.005 and #2020.049) and the University of Alberta (Pro00082512). Furthermore, for the third aim of the project, which focused on "The impact of donor sex on the Hb increment and sepsis" received additional ethical approval from MacMaster Center for Transfusion Research (HIREB-#14539-C), Canadian Blood Services (CBSREB #2022.015), and the University of Alberta Institutional Review Board (Pro00119217). These approvals ensured that the research adhered to ethical guidelines and protocols. The author of this thesis conducted the research within the approved framework and followed the necessary ethical considerations to protect research participants and ensure the integrity of documented studies.

In Chapter 2, the blood sample collection was carried out by qualified CBS technicians. This involved adhering to established protocols and procedures to ensure the collection of high-quality samples for subsequent analysis. The data collection for the hemolysis analysis was conducted with the assistance of doctoral student Nishaka William and technician Carly Olafson. The protocol for the validation inter-variation (Appendix 1) was executed with the help of doctoral student Nishaka William. The author of the thesis was responsible for collecting the remaining data for Appendix 1 and Chapter 2.

In Chapter 3, the data collection for the supernatant Hb was carried out with the assistance of MSc student Rafay Osmani and Dr. Anika T. Rahman. The blood collection process was performed with the assistance of CBS technicians Tanya Atallah, Michelle Aube, Allahna Elahie, and phlebotomist Sanaz Hemmatibardehshahi.

The data presented in Chapter 4 is part of a research collaboration led by me. The data retrieval process involved the cooperation of the CBS team Jason Liu, Dr. Bill Ferguson and CBS legal representative Rachel Ward and Dr. Chantale Pambrun, Dr. William Sheffield and McMaster university team Dr. Jonathan Bramson, Prof Nancy Heddle, and Dr. Yang Liu. Data analysis was performed with the assistance of Dr. Yang Liu from McMaster University. The data interpretation was conducted by the author with the assistance of Dr. Jason Acker and Prof. Nancy Heddle.

The experimental design in Chapter 2 was developed with the guidance and assistance of Dr. Jason Acker. The experimental design in Chapter 3 was developed solely by the author. The experimental design in Chapter 4 was developed with the assistance of Prof. Nancy Heddle and Dr. Jason Acker. The experimental design in the Appendix 1 was developed with the assistance of Dr. Jason Acker. The experimental design was developed by me in the Appendix 2.

The data analysis presented in Chapters 2, 3, 4, and the concluding Chapter 5 represents the original work of the author. The literature review in Chapter 1 was also authored by the author of the thesis. Dr. Qilong Yi, a statistician from Canadian Blood Service, aided with statistical analysis for Chapter 3, while Yang Liu from McMaster University assisted with statistical analysis for Chapter 4.

Chapter 1 of this thesis has been published as 2 review paper. The first paper was: Li, W. and Acker, J.P. $CD71^+$ RBCs: A potential immune modulator in the transfusion. *Transfusion and Apheresis Science* 2023; **62** (3): 103721. Dr. Acker was the supervisory author and was involved with concept formation and manuscript editing. Portion of this chapter was also published in: Abdulrahman, A., Li, W., Juffermans, N.P., Seghatchian, J. and Acker, J.P. Biological mechanisms implicated in adverse outcomes of sex-mismatched transfusions. *Transfusion and Apheresis Science* 2019; **58** (3):351-356.

Chapter 2 has been published as 2 papers: Li, W. William, N. and Acker, J. P. Blood donor sex, pre-donation Hb and manufacturing methods affect the quantity of $CD71^+$ erythroid

cells in RBC products. *Transfusion* 2023; **63** (3): 601-60I. Dr. Acker was the supervisory author and was involved with concept formation, data interpretation and manuscript editing. The protocol (Appendix 1) was published as Li, W. and Acker, J.P. Development and validation of a sensitive flow cytometric method for determining CEC in RBC products. *Clinica Chimica Acta* 2022; **530**: 119-125.

Chapter 3 includes one published abstract, one abstract that has been accepted for presentation, and one invited presentation at scientific conferences. The first published abstract, titled " Li, W. Hemmatibardehshahi, S. Osmani R and Acker, J. P. $CD71^+$ Red Blood Cells Mediate Increased Erythrophagocytosis and Reduced Monocytes in Human Suspension Assay. Vox Sanguinis, 2023, 118(S1), PA21-L03 . It was also accepted for an oral presentation at the International Society for Blood Transfusion (ISBT) 2023 Congress. I played a pivotal role in this research, handling data collection, analysis, and the subsequent presentation. Dr. Acker, as the supervisory author, provided guidance in concept formation, data analysis. The second abstract, entitled " Li, W. and Acker, J.P. ROS inhibitor reduces phagocytose of $CD71^+$ RBCs and protect CD14-monocytes in human monocyte suspension assay," was presented as a poster at the Canadian Society Transfusion Medicine (CSTM) 2023 Annual Conference. This work has also been invited for presentation at the 13th Annual Blood Matters 2023, Nov 3rd. I was responsible for the data collection, analysis, and presentation of this research.

Chapter 4 includes one published abstract, one accepted abstract : Li W, Liu Y, Lucie J K, Heddle M N. Acker P J. Effect of donor and recipient characteristics on hemoglobin increments in intensive care unit patients receiving red blood cell transfusion. Transfusion, 2023, 63(S5), P-TS-29; Li W, Liu Y, Lucie J K, Heddle M N. Acker P J. Effect of donor and recipient characteristics on sepsis in intensive care unit patients receiving red blood cell transfusion, Chin J Blood Transfusion 2023 (Accept). This work has been invited for presentation as “the effect of donor sex on Hb increment and sepsis among ICU patients” in Sichuan Blood Safety Symposium (2023) &

Inaugural Meeting on Adverse Transfusion Outcomes Committee of Sichuan Blood Transfusion Association Chengdu, China. **2023.12.26-2023.12.27.** The collaborative efforts of Wenhui Li, Yang Liu, Kayla J. Lucier, Prof. Nancy Heddle, and Dr. Jason P. Acker have been instrumental in the preparation of these two abstracts. Yang Liu played a vital role in data analysis, while Kayla J. Lucier contributed to coordination and editing. I, assumed multiple responsibilities, including obtaining data collection, analysis, and interpretation. Prof. Nancy Heddle and Dr. Jason P. Acker provided invaluable guidance as the supervisory authors, assisting in concept formation, data analysis, and editing.

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List of Abbreviations

ACD	acid citrate dextrose
APC	allophycocyanin
APCs	antigen presenting cells
ATP	adenosine triphosphate
BSA	Bovine serum albumin
BV711	brilliant violet 711
CBU	cord blood unit
CECs	$CD71^+$ erythroid cells
$CD71^+$ RBCs	$CD71^+$ red blood cells
CFSE	carboxyfluorescein diacetate succinimidyl ester
CI	confidence Interval
CST	cytometer setup and tracking
CV	coefficient of variance
DAD	discharge abstract database
DMSO	dimethyl sulfoxide
DPBS	dulbecco's phosphate buffer solution
2,3 - DPG	2,3-diphosphoglycerate
EDTA	ethylenediaminetetraacetic acid
EV	extracellular vehicles
FBS	fetal bovine serum
FC	flow cytometry
FMO	fluorescence minus one
FSC	forward scatter
GvHD	graft-versus-host disease
ICU	intensive care unit

Hct	hematocrit
Hb	hemoglobin
Hb_S	supernatant hemoglobin
Hb_T	total hemoglobin
Hb_v	venous blood hemoglobin
ICU	intensive care unit
IFC	imaging flow cytometry
IFN-γ	Interferon - gamma
IgG	immunoglobulin G
IL	interleukin
IQR	interquartile range
LIS	laboratory information system
LoB	limit of blank
LoD	limit of detection
LPS	lipopolysaccharides
MCHC	mean corpuscular hemoglobin concentration
MFI	median fluorescence intensity
MRD	minima residual disease
MSAR	methicillin-resistant staphylococcus aureus
NO	nitric oxide
NTBI	non-transferrin bound iron
OpRBC	Opsonized RBC
OR	odds ratio
PBS	phosphate buffer solution
PBMCs	peripheral blood mononuclear cells
PD - L1/2	programmed death-ligand 1/2
PE-Cy7	phycoerythrin-cyanine 7

Pre-donation Hb level	pre-donation fingerstick capillary hemoglobin level
PS	phosphatidylserine
RBCs	red blood cells
RCCs	red cell concentrates
RCF	red cell filtration
REB	research ethics board
RMS	root mean square
ROS	Reactive oxygen species
RPMs	red pulp macrophages
SAGM	sodium chloride, adenine, dextrose, mannitol
SCANDAT	Scandinavian donations and transfusions
SD	standard deviation
SI	separation index
SIRS	systemic inflammatory response syndrome
SSC	side scatter
TACO	transfusion associated circulatory overload
TM	trademark
TGF - β	tumor growth factor - beta
TNF - α	tumor necrosis factor- alpha
TRICC	Transfusion Requirements in Critical Care
TRIM	transfusion related immunomodulation
TRALI	transfusion related acute lung injury
Tregs	regulatory T cells
TRUST	Transfusion Research Utilization, Surveillance and Tracking
WB	whole blood
WBC	white blood cells
WBF	whole blood filtration

WCC	white cell count

Chapter 1 * Introduction

* A version of this chapter has been published as 2 review papers: Li, W. and Acker, J.P. $CD71^+$ RBCs: a potential immune modulator in the transfusion. Transfusion and Apheresis Science 2023 Jun;62(3):103721. Alshalani, A., Li, W., Juffermans, N.P., Seghatchian, J. and Acker, J.P. Biological mechanisms implicated in adverse outcomes of sex-mismatched transfusions. Transfusion and Apheresis Science, 2019, 58(3):351-356.

1.1 THE RISK ASSOCIATED WITH BLOOD TRANSFUSION

Transfusion of red cell concentrates (RCCs) is to improve oxygen delivery in patients who are in anemia or have lost lots of blood, which is a common medical practice nationally and globally. Annually In Canada, the number of RCC units administrated is approximately 1.2 million [1], world widely, the number of RCC units is estimated to be over 118.5 million [2]. Unfortunately, as with any medical intervention, transfusions of RCCs can lead to complications and even death [3, 4].

The risk of transmitting infectious diseases with blood transfusion has been minimized after substantial progress. After implementations of inventions, such as blood donor screening, laboratory testing, and continuous surveillance and feedback, the incidence of transfusion-related transmissible infections is now considerably very low [5-8].

However, other transfusion-associated risks present new challenges. Adverse events associated with transfusions, such as the susceptibility to infections [9-11], acute and delayed hemolytic reactions, transfusion-related acute lung injury (TRALI), and transfusion associated circulatory overload (TACO) [12], continue to contribute to transfusion-associated morbidity and mortality among blood product recipients.

Seeking explanations for the emerging new risks associated with RCC transfusions is necessary to ensure continued safety for recipients. Even though RCCs are kept in the blood bank refrigerator, they remain metabolically active, leading to ongoing age-related changes during storage [13]. This phenomenon, known as "hypothermic storage lesion", involves oxidation of the cell structure and vesiculation of the membrane, which causes a loss of membrane flexibility [14]. Additionally, there is a reduction in vital metabolites like adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG; a Hemoglobin [Hb] regulator,) [15]. Current insights into the red cell storage lesion suggest that older stored RCCs may increase patient morbidity and mortality [16]. Beyond this factor, research is still ongoing to investigate the effects of other factors, such as manufacturing methods and donor factors on the transfusion outcomes.

Manufacturing methods employed to separate RBCs, platelets, and plasma from donated whole blood play a critical role in the composition of transfused RCCs. The selection of blood component manufacturing is influenced by the desired end products. Different blood component manufacturing methods have been observed to lead to notable variations in product volume and RBC content, as well as extracellular vesicles present which can be derived from residual platelets, leukocytes, or RBCs [17, 18]. Some methods might be more efficient in separating and concentrating RBCs, leading to a higher volume of packed RBCs, while others might result in less RBC yield [19, 20]. It has been reported that manufacturing methods affect the efficacy of RCC transfusion [21, 22]. While the primary goal of blood component separation is to provide safe and effective products for transfusion, the chosen manufacturing method can significantly influence the quality and efficacy of the final products. Thus, continuous evaluation, research and optimization of RCC manufacturing are essential to ensure the optimal outcomes for patients receiving transfusions.

1.1.1 The Effect of Blood Donor Factors on the Quality of RCCs

In addition to the impact of hypothermic storage duration and manufacturing methods, donor factors may also contribute to the RCC quality and related transfusion outcomes. Both biological and non-biological donor factors have been reported to be associated with the quality of RCCs. Biological factors including donor sex, age and pre-donation Hb levels, not only affect the quantity of total Hb in the RCCs, but also the inherent quality of RBCs in RCCs. The mean Hb level in premenopausal females is approximately 12% lower than that of men [23] and the total Hb value could range from 56 g/ unit to 74 g/ unit among donors [24]. Moreover, RCCs derived from male donors tend to exhibit higher rates of storage hemolysis compared to those from female donors [25, 26].

Non-biological factors such as the inter-donation interval (frequency of donation) and smoking may also affect the overall quality of RCCs. Blood centers have established a minimum blood donation interval for donors to prevent iron deficiency and other potential adverse impacts

on their health [27]. Frequent blood donors are more likely to be iron-deficient, and their RBCs are characterised by being small (microcytic) and deficient in Hb (hypochromic) [28], reduced levels of endogenous antioxidants, and being more sensitive to oxidative stress [29, 30]. These differences can influence the variability of RCCs and may affect transfusion outcomes.

1.1.2 The Effect of Blood Donor Factors on the Mortality in Transfusion

The importance of factors related to blood donors has become increasingly evident in recent years, shaping our understanding of transfusion medicine's intricacies. Since 2014, studies have revealed that transfusions of RBCs from younger mice can positively affect older mice in various age-related functions, including reducing the decline in muscle regeneration [31], cognitive abilities [32] and reversal of cardiac hypertrophy [33]. This growing interest have spurred studies on the donor characteristics, particularly the effects of blood donor sex and age, on the mortality of recipients.

Currently, there are conflicting evidence regarding the association between the sex and age of RCC blood donors and the mortality observed among transfusion recipients. Some studies suggest that donor sex is associated with mortality [34-36]. For example, in a clinical trial involving 11,211 participants in the Netherlands, it was observed that recipients of single-sex blood transfusions from donors of the opposite sex had higher mortality rates [37]. Specifically, increased mortality was noted at 90 days, 180 days, and 1 year follow-ups [37]. A 2017 retrospective study of 31,118 participants found a correlation between the source of RCC transfusions and all-cause mortality [38]. Notably, male recipients who received RBCs from female donors with a history of pregnancy had higher mortality compared to those who received from male donors [38]. Similarly, a 2016 Canadian retrospective study of 30,503 RCC transfusion recipients utilized Cox proportional hazards regression models for analysis and the finding indicated that a significantly higher risk of mortality for recipients of transfusions from young female donors [39]. Further research conducted in 2019 also showed that donors aged 45 years or younger were associated with higher in-hospital death rates compared to recipients exclusively

exposed to RCCs from donors older than 45 years [40]. In light of these findings, the relationship between donor demographics and transfusion recipient outcomes emerges as a critical area for further investigation to guide safer transfusion practices.

However, other studies have yielded different results. A study using Scandinavian Donations and Transfusions (SCANDAT) database found that neither donor age nor sex was related to mortality of recipients after adjustment for the number of RCC units transfused [41]. These conflicting results may be attributed to the variations in study design and methodology. Furthermore, factors such as the severity of recipients' conditions and RCC manufacturing methods, which could influence the observed associations, were not accounted for in the analyses. In conclusion, the evidence regarding the impact of blood donor factors on recipient mortality remains inconclusive, underscoring the importance of considering a range of contributing factors and methodological differences across studies.

1.1.3 The Effect of Donor Sex on Critically Ill Patients' Susceptibility to Sepsis

Sepsis is life-threating characterized by immune dysregulation that can arise from bacterial, fungal, viral, and parasitic pathogens, as well as from non-immune disruptions as part of the host's dysregulated responses. Pathogens activate the immune system by expressing pathogen-associated molecular patterns (PAMPs) that interact with pattern recognition receptors (PRRs), triggering protective inflammatory responses [42]. An example of this mechanism is the activation of Toll-like receptors on monocytes by lipopolysaccharides (LPS) found on Gram-negative bacteria, leading to cytokine production [43]. However, an uncontrolled reaction can result in a cytokine storm, with pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) contributing to tissue damage and organ failure, or a systemic inflammatory response syndrome (SIRS) [44, 45]. These complex immunopathological processes may getting more complex in critically ill patients receiving RCC transfusions.

Regarding allogenic transfusion in critically ill patients in transfusion, unexpected Hb increment and alloimmunization may be associated with the development of sepsis. Transfusing

RCCs introduces a substantial amount of Hb into the patient, ideally increasing the Hb concentration by approximately 10 g/L per unit, as per transfusion efficacy criteria [46, 47]. However, poor Hb increment has been reported to be associated with heightened infection rates among transfused hospitalized patients [48]. Furthermore, patients receiving allogenic RBC transfusions can experience immune suppression [49, 50]. Immune suppression is a notable characteristic of sepsis progression, which initiated by intense inflammation period [51]. While the exact mechanisms by which poor Hb increments contribute to sepsis remain unclear, investigating Hb increments in critically ill patients following RCC transfusions is helpful to understanding the potential effects of transfusions on sepsis outcomes.

However, the diagnosis of sepsis in clinical settings is complicated by its variable symptoms, making it difficult to determine if a RCC transfusion preceded or followed the onset of sepsis. Sepsis diagnosis typically requires the presence of at least two SIRS characteristics. These characteristics include fever or hypothermia, abnormally fast heart rate (tachycardia), abnormally rapid breathing rate (tachypnea), and a white cell count (WCC) indicating more than 10% immature granulocytes (bands, myelocytes, and metamyelocytes), accompanied by a documented or suspected infection [52]. These overlapping clinical presentations further complicate the exploration of the potential influence of donor sex on sepsis outcomes.

While the influence of the blood donor's sex on sepsis in recipients remains under-researched, the role of donor sex in post-transfusion infections has attracted growing attention. In 2023, a randomized clinical trial with involving 8,719 patients study suggested that donor sex might influence the risk of methicillin-resistant staphylococcus aureu (MSRA) infection, a type of bacterial infection that is resistant to several antibiotics, including methicillin, oxacillin, penicillin and amoxicillin [53]. However, there is a possibility that this finding could be coincidental. The significance of the donor’s sex on the recipient’s outcome was only identified through subgroup analysis and conducting a multiplicity of analyses also increased the potential risk associated with the findings [53]. Although the impact of donor sex on recipients’ infection rates may seem

marginal, nearly one-third of critically ill patients receiving RCC transfusions [54-57] and infections being a primary trigger for sepsis, it is imperative to delve deeper into how donor sex affects sepsis in critically ill patients.

1.2 BIOLOGICAL MECHANISM OF TRANSFUSION-ASSOCIATED ADVERSE EVENT INTERMS of BLOOD DONOR SEX DIMORPHISM

Various biological mechanisms have been suggested to explain sex-related differences in transfusion outcomes. These include impact related to oxygen delivery, coagulation, and immunomodulation [58]. Blood donor sex leads to variations in the RBC distribution [59] and deformability [60, 61], which can impact oxygen delivery, particularly in the cardiovascular patients [62]. However, there is a current lack of experimental studies focused on the influence of donor variability on immune mediators and transfusion-related immunomodulation (TRIM) outcomes.

1.2.1 Donor Sex and TRIM

The immunologic effects of TRIM may largely contribute to the risk of transfusion. TRIM denotes the immunosuppressive effects following an allogeneic blood transfusion. Historically, in 1978, it was observed that kidney transplant recipients who had received transfusions displayed enhanced graft survival, suggesting a protective role of TRIM in graft acceptance [49]. However, by the 1990s, studies began to highlight potential risks; kidney transplant recipients with prior transfusions were found to have an increased risk of post-operative infections [63]. Furthermore, a higher rate of cancer recurrence was observed in transfused surgical patients, particularly those with colorectal cancer [64]. Illustrating the potential implications of transfusion in infections, the pivotal Transfusion Requirements in Critical Care (TRICC) study led by Hebert et al., which assessed transfusion strategies in 838 anemic critically ill patients were assessed for transfusion strategies. The study compared outcomes using a Hb threshold of 70 g/L (restrictive strategy) to 100 g/L (liberal strategy) and found that the latter group experienced more nosocomial infections [65]. This correlation raises pressing questions about the underlying mechanisms behind transfusion-related infections.

While the exact mechanisms of TRIM are unclear, several constituents of donated blood have been identified as potential contributors. These included donated white blood cells (WBCs), soluble WBC-derived mediators, bioactive lipids, accumulated cytokines, extracellular vehicles (EVs) and components released from red blood cells (RBCs) [66, 67]. WBCs are the body's primary defence against infections. When transfused, donated WBCs can interact with recipient's immune system, potentially leading to modulatory effects [68]. These effects might range from mild immune suppression to more significant immune reactions, such as graft-versus-host disease (GvHD). GvHD occurs when the donor's immune cells (the "graft") recognize the recipient's tissues (the "host") as foreign and attack them, potentially leading to life-threatening conditions if untreated, including severe infections, organ damage, or other complications [69]. Another factor of interest is extracellular vesicles, small vesicles that cells release and that can transport various bioactive molecules such as proteins, lipids, and nucleic acid [70]. These vesicles can trigger TNF-α production by monocytes and/or stimulate T cell proliferation [71, 72]. All these factors play vital roles in TRIM outcomes.

While the direct correlation between donor sex and TRIM outcomes remains to be definitively established, emerging evidence suggests a potential link. Studies have indicated an intrinsic difference in RBC hemolysis based on the donor's sex, which might contribute to TRIM. Hemolysis refers to the rupture of RBCs, causing the release of cell-free Hb into blood products. This phenomenon can also manifest as intravascular hemolysis, where cell-free Hb enters the bloodstream directly. This Hb can interact with nitric oxide (NO), a molecule essential for vasodilation — the expansion of blood vessels [73]. The binding of cell-free Hb to NO diminishes NO's availability, leading to endothelial dysfunction, elevated blood pressure in small blood vessels (microvascular hypertension) [74]. Additionally, the breakdown of Hb results in the release of its heme, which is further metabolized to release iron [75]. This iron can intensify inflammatory injury through the activation of the innate immune response [76, 77]. This chain of events underscores the importance of understanding the nuanced impact of donor sex on hemolysis and

the ensuing effects on the immune system. Moreover, exploring the role of other cell types, such as immature RBCs, $CD71^+$ RBCs, may deepen our understanding of the intricate biological implications for donor sex-related TRIM.

1.2.2 $CD71^+$ RBCs in the Context of Transfusion

1.2.2.1 The definition of $CD71^+$ RBCs

In this study, $CD71^+$ red blood cells （$CD71^+$ RBCs) are identified on their surface marker expression. They are characterized by the presence of the transferrin receptor protein (CD71) and erythroid lineage markers, glycophorin A (CD235a) in humans (**Figure 1-1**) and glycophorin A - associated protein (Ter119) in mice. CD71, a marker of immature RBCs, is a protein located on the surface of erythroid cells [78, 79]. It is important to highlight that while $CD71^+$ RBCs are included in the broader category of $CD71^+$ erythroid cells (CEC, $CD235a^+$ $CD71^+$), they are distinct from erythroblasts that carry the CD45 leukocyte marker [80, 81]. Specifically, $CD71^+$ RBCs do not express CD45. Additionally, CECs are naturally predominant in neonates and cord blood and may accumulate in extramedullary locations in adults under certain pathological conditions in adults [81]. In this study, the term $CD71^+$ RBCs refers to cells from whole blood samples obtained from healthy adult donors.

Typically, $CD71^+$ RBCs would undergo standard physiological processes within the donor's circulation, but donated $CD71^+$ RBCs instead diverted to RCC products. This diversion means that these cells, which are in a transitional stage of maturation, are preserved outside their natural environment. In the bone marrow, $CD71^+$ expression on hematopoietic stem cells marks a crucial phase of development, starting from the proerythroblast to the erythroblast stages [82]. Upon nuclear extrusion, erythroblasts transition into reticulocytes in blood circulation. Reticulocytes could be further divided into four stages namely CD71 high, CD71 medium, CD71 low and CD71 negative based on CD71 staining of cord blood-derived reticulocytes [83]. These reticulocytes fully mature happens within 24 to 48 hours [84]. When these cells are collected and placed into

RCC products, it raises questions about the potential impact on their natural development and function, as well as any possible implications for the recipients of transfusions.

1.2.2.2 The Rationale for the Potential of $CD71^+$ RBCs in Transfusion

Donated $CD71^+$ RBCs follow a unique maturation pathway compared to the standard physiological process, which suggest a potential influence on transfusion outcomes. In the context of transfusion, these cells are removed from their usual milieu and introduced into a recipient's body. This change could potentially disrupt their typical maturation process, leading to unintended effects. For instance, if these cells do not mature correctly, they might interact differently with the recipient's immune system. In blood products, donated $CD71^+$ RBCs can persist for the whole shelf life, up to 35 days [85, 86]. Once transfused to recipients, allogeneic immature donor reticulocytes can remain in circulation for up to four days [87]. Given the lifespan of donated $CD71^+$ RBCs in both blood products and after transfusion, it's important to consider the potential implications for recipients. Notably, various transfusion-related adverse events, such as febrile non-hemolytic transfusion reaction and TRALI, manifest within a 24-hour window [88]. This timeframe provides a sufficient opportunity for $CD71^+$ RBCs to enact their potential immune functions and potentially affect these reactions.

While the proportion of donated $CD71^+$ RBCs in RCCs is lower compared to that of mature RBCs, their presence remains significant and should not be overlooked, even considering the donor's sex. Given sex variation in reticulocytes [89], the sex-based differential in $CD71^+$ RBC concentration is estimated to be 10^9 / L. This concentration is three orders of magnitude higher than leukocyte concentration in leukoreduced RCCs (5×10^6 WBCs / L) [90]. Considering the known association between residual leukocytes and unfavorable immunomodulatory outcomes [91], it is plausible that $CD71^+$ RBCs, accounting for sex variations, are present in sufficient quantities to potentially elicit an immune response in recipients.

1.2.2.3 Factors Affecting $CD71^+$ RBCs in Blood Products

1.2.2.3.1 Blood Donor Factors

The generation of $CD71^+$ RBCs, also recognized as reticulocytes, is influenced by a wide range of blood donor factors, such as donor sex, donor age and donation times over the last 12 month. Reticulocytes are usually used as a parameter to assess erythropoiesis or blood production. The production could be stimulated by blood donation [92]. Approximately 100 days after blood donation, an increase in reticulocytes could be observed in donors with ferritin levels of 12 ng / mL or higher [93]. Ferritin is the major intracellular iron storage protein and used as indictive of the body's iron levels [94]. The body's iron status is a fundamental prerequisite for RBC production [95, 96]. A prospective cohort study involving 550 successful donors discovered that female blood donors, especially those who donate frequently, exhibit a greater propensity towards iron deficiency [97]. Therefore, donor sex and donation times over 12 months may collectively affect $CD71^+$ RBCs in blood products. Additionally, RBC production is also influenced by sex hormone [98]. In the treatment of some anemic patients, the administration of androgens have been found to stimulate several stages of the hematopoietic system [99, 100], while treatment of estrogen could significantly decrease erythropoietin (EPO) synthesis and gene expression during hypoxia in the animal model [101]. Furthermore, the capacity for erythropoiesis, encompassing hematopoietic stem cells and the local environment, changes with age. Experimental research has shown a decline in circulating peripheral blood $CD34^+$ cells associated with aging [102]. Studies on autologous stem cell transplantation indicates that rather than a diminished function of progenitor cells, it's the reserve capability of the bone marrow that might be affected [103, 104]. Additionally, research has shown an age-related elevation in serum EPO concentration and a notable inverse correlation between EPO and Hb levels in healthy elderly individuals without anemia, a pattern not observed in younger participants [105]. The culmination of these factors may profoundly impact the generation of $CD71^+$ RBCs in blood donors.

1.2.2.3.2 Manufacturing Methods

Blood manufacturing methods have been reported to affect the components of RCCs [106]. While many different blood manufacturing methods exist among international blood services, red cell filtration (RCF, top / bottom) and whole blood filtration (WBF, top / top) methods are the two most commonly used to produce whole blood-derived, leukoreduced RCCs in Canada [17]. The objective of blood manufacturing, which prepares packed red cells for transfusion, is to remove WBCs and other components like platelets and plasma, while retaining the majority of RBCs. The RBC filtration process includes removing "buffy coat", a layer containing WBCs and platelets that forms between the RBCs and plasma after centrifugation. Reticulocytes, which are immature RBCs, have been reported to exhibit a lower mean corpuscular Hb concentration (MCHC) [107] and a lower density compared to mature RBCs, which may make them more likely to be removed during the RCF process. This removal could be an unintentional consequence to separate blood components effectively. In whole blood filtration, where the goal is to remove plasma from blood without separating buffy coat, potentially allowing more reticulocytes to remain in the blood product. Given that RCF and WBF are the predominant methods in North America, they may differentially affect the reticulocyte concentration in the final RCC products.

Furthermore, the volume of RCC units varies depending on the manufacturing technique. RCCs produced through the RCF method typically have a reduced volume compared to those made via the WBF method, primarily because of the buffy coat removal [60]. This variation could influence the total number of $CD71^+$ RBCs in the RCC products.

1.2.2.3.3 Hypothermic Storage

After manufacturing process, RCC units are usually stored in hypothermic storage, which may extend the maturation and presence of $CD71^+$ RBCs. In blood banking, RCC units are stored at a low temperature range of 1 to 6 °C to deliberately slow down cellular metabolism. This is in contrast to the body's optimal physiological temperature of 37 °C, where enzymes facilitate

metabolism at their peak efficiency. This low-temperature storage helps minimize deterioration and extend RCC units' shelf life [108]. It is crucial to recognize that under these hypothermic conditions, reticulocytes or $CD71^+$ RBCs present in the RCC units undergo a marked reduction in their metabolic activity. This impedes the standard 24 to 48-hour maturation process of the $CD71^+$ RBCs [85]. Consequently, compared to their typical survival duration in the body's circulation, $CD71^+$ RBCs have an extended lifespan in storage bags.

Several studies have explored the impact of hypothermic storage on reticulocytes in stored blood. For instance, flow cytometric analysis with thiazole orange has shown that the persistence of reticulocytes in units of stored CPDA-1 blood remained largely consistent over 35 days when stored at 4℃, in contrast to those stored at 37 °C [85]. In a more recent investigation, the impact of extended storage on CD71 expression in leukocyte depleted RCCs containing SAGM additive solution, with a trace amount of CPD, was studied using samples from 20 male and 12 female blood donors over an 8-week storage period [109]. Utilizing flow cytometry, researchers discerned only minor variations in CD71 expression throughout this duration [109]. This suggests that RBCs with CD71 expression can endure for the entire 8-week storage period. While storage duration can vary based on additive solutions and regulatory standards across different countries [110], the *in vitro* kinetics of $CD71^+$ RBCs might also differ. Nevertheless, the fact that these $CD71^+$ RBCs can last in storage beyond their typical lifespan in human circulation emphasizes the need to comprehend its consequences on transfusion outcomes.

1.2.2.4 The Immunosuppressive Potential of $CD71^+$ RBCs

The multifaceted immunomodulatory functions of CECs and their subtypes have intrigued researchers with new insights suggesting potential roles in transfusion medicine. The immunomodulatory role of CECs has been associated with erythropoiesis in genetic low responder mice models, although distinctions between erythroblasts and reticulocytes were not made earlier studies from 1978 [111]. Reticulocytes, serving as markers for erythropoiesis [83],

may have an unclear immune role. Recent research has identified two specific subtypes of CECs based on their CD45 expression levels, which appear to affect their immunosuppressive capacities [112]. Specifically, $CD45^+$ CECs from the blood of tumor-bearing mice are more potently inhibit $CD8^+$ T cell proliferation *in vitro* compared to their $CD45^-$ counterparts [112]. Also, when splenic $CD45^-$ CECs sourced from tumor-induced bearing mice were introduced to mouse models, there was a marked increase in tumor growth and rapid disease progression, hinting their immunomodulatory role [113]. It is particularly interesting to note the potential immunomodulatory properties of $CD45^-$ CECs, given that $CD71^+$ RBCs exhibit similar $CD45^-$ and $CD71^+$ marker expressions. However, the extent of this immunomodulation in CECs seems to be influenced by both the host and location [114]. In the context of this research, the $CD71^+$ RBCs are derived from human blood donors, and their precise role remains to be elucidated. To further understand the significance of donated $CD71^+$ RBCs in the realm of transfusion medicine, the following sections aim to explore their potential functions and impacts.

$CD71^+$ RBCs may influence erythrophagocytosis. Erythrophagocytosis is a process where macrophages scrutinize passing RBCs and remove the aged / less deformable RBCs, as well as recycle the iron-containing Hb [115]. The known mechanism for aged RBC phagocytosis under physiological conditions includes phosphatidylserine (PS) exposure [116], band 3 (anion exchanger [AE1]) alteration, the loss of CD47 (thrombospondin-1 receptor, “do not eat me” signal) [117, 118]. Under pathological conditions that increase red cell clearance, the mechanisms involve decreased deformability [119, 120], and antibody-mediated destruction [121], along with oxidant injury [122] **(Figure 1-2).** In individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency, the diminished activity of the G6PD enzyme leads to a depletion of NADPH (nicotinamide adenine dinucleotide phosphate), a crucial cofactor required for managing oxidative stress in cells [123]. It has been reported that G6PD deficient RCC units are associated with decreased post-transfusion red cell recovery [124, 125]. While donated $CD71^+$ RBCs have not

demonstrated increased PS exposure or a loss of CD47 compared to mature RBCs, they do exhibit reduced deformability [126]. Furthermore, $CD71^+$ RBCs are more prone to mechanical fragility [127], and have a heightened expression of reactive oxygen species [112]. These characteristics may collectively contribute to the role of $CD71^+$ RBCs' influence in the process of erythrophagocytosis.

Macrophages, which engulf RBCs, also experience altered functions due to this interaction. Macrophages can secret TNF-α, a proinflammatory cytokine [128]. In a mouse model of T cell-induced colitis, a reduction of TNF-α was observed after red pulp macrophages (RPMs) phagocytosing CECs compared to those that didn't engulf CECs [129]. *In vitro*, CECs directly reduce TNF-α production from splenic RPM in a phagocytosis-dependent manner [129]. This suggests that the phagocytosis of CECs would be anti-inflammatory. This is different from findings associated with the phagocytosis of mature RBCs. For instance, Hod *et al.* demonstrated that in a murine model, transfusion of stored RBC products induced macrophages to produce proinflammatory cytokines, while transfusion of fresh RBC product did not induce any inflammatory effects [130]. Such interactions highlight the nuanced roles RBCs play in influencing macrophage behavior and inflammatory responses.

Additionally, CECs can also modulate adaptive immune response to infection. Adaptive immune response can be triggered by antigen-presenting cells that produce cytokines to eliminate infection [131]. However, following heat killed *Listeria monocytogenes* stimulation *in vitro,* the production of TNF-α by $CD11b^+$ cells (neutrophils) was suppressed with the incubation of CECs [132]. When CECs interact with splenocytes, they reduce the production of TNF-α, and this reduction varies based on the amount of CECs present [132]. CECs could also inhibit the proliferation of T cells through releasing immune mediators [133]. This is because they produce greater amounts of tumor growth factor-beta (TGF-β), potentially inducing the differentiation of T cells into regulatory T (Treg) cells [134]. Interestingly, in contrast, some studies found that both

early and late reticulocytes promote T cell proliferation under certain conditions [135]. These multifaceted interactions between CECs and immune cells highlight the complex and sometimes paradoxical roles that these cells play in shaping the body's immune response.

1.2.3 The Putative Roles of $CD71^+$ RBCs in Sepsis

Considering the influential role of CECs in mediating immune responses, $CD71^+$ RBCs, being a subset of CECs, could potentially contribute to the onset or progression of sepsis in critically ill patients receiving RCC transfusions.

Donated $CD71^+$ RBCs may facilitate RBC clearance, and this may in turn, overwhelm the mononuclear phagocytic system in critically ill patients (**Figure 1-3**). In an autotransfusion rat model, it was observed that RCC products with a higher proportion of reticulocytes (52%) had a shorter half lifespan after transfusion, lasting only about 6 days, compared to the half-life of 42 days reported for the RCC with a lower proportion of reticulocytes (12%) [136]. Excess RBC clearance may impose a burden of heme, free Hb, iron and Hb-containing RBC membrane microparticles [137, 138]. The free Hb or iron could serve as a proliferative substrate for bacteria and may modulate endothelial interactions [73, 130]. Furthermore, free Hb/ iron may interact with immune cells such as macrophages and neutrophils, prompting the release of pro-inflammatory cytokines like IL-6, IL-1β, and TNF-α [73, 130].. These cytokines, normally involved in the regulation of immune responses, can become dysregulated, contributing to systemic inflammation and endothelial dysfunction [139]. Furthermore, the capacity of mononuclear phagocytic system in female patients has been reported to be about 12% lower than in male patients [140]. It is possible that the mononuclear phagocytic system in female recipients may be more easily overwhelmed by the increased erythrophagocytosis of RBCs from male RCCs. This overloading of the erythrophagocytosis may be accompanied by progressively increasing free Hb in circulation, leading to a poor Hb recovery and an increased susceptibility to develop sepsis.

Donated $CD71^+$ RBCs may be involved in the dysregulation of T cells, which are essential for mounting specific responses against pathogens (**Figure 1-3**). During the course of sepsis, T cell depletion and anergy have been observed [141]. Ardie *et al.* reported that patients admitted to intensive care units for non-septic conditions who had a low lymphocyte count for more than 3 days were at greater risk of developing nosocomial infection [142]. The depletion of L-arginine, a pathway that can be caused by the presence of CECs, may lead to lymphopenia and suppression of T proliferation [143]. Pirns *et al.* suggested that the release of arginase 2 by RBCs may link transfusion with downstream immunosuppression [144]. It is possible that $CD71^+$ RBCs in the RCCs express arginase 2 and suppress T cell proliferation by depleting arginine.

Growing evidence demonstrates an immunomodulatory role for CECs both *in vitro* and *in vivo*. The quantity and immune activity of $CD71^+$ RBCs in blood products warrants further investigation. The role of $CD71^+$ RBCs in erythrophagocytosis needs to be examined to explore their impact on sepsis in critically ill patients receiving RCC transfusions.

1.3 HYPOTHESIS

Donated $CD71^+$ RBCs are potent immune mediators of erythrophagocytosis, and the quantity of $CD71^+$ RBCs is influenced by blood donor factors that is a predictor of change in post-transfusion Hb and hospital-onset sepsis in critically ill patients receiving RCCs (**Figure 1-4**).

1.4 THESIS OBJECTIVES AND APPROACH

Specific Research Aim #1: To investigate the effect of donor sex on the quantity of $CD71^+$ RBCs (Chapter 2).

Considering that immature RBCs are rare and fragile compared to mature RBCs, accurately determining $CD71^+$ RBCs in WB samples and leukoreduced red cells concentrates has been challenging. To establish a robust method for quantifying $CD71^+$ RBCs in blood products, the current flow cytometric methods using PBMCs from WB are optimized and validated by

assessing the limit of detection, linearity, inter-run imprecision, and tech-to-tech variability (**Appendix A**).

The presence of $CD71^+$ RBC in blood products may be affected by donor characteristics and manufacturing methods. To achieve the first objective, healthy donors who meet the blood collection service criteria are recruited for $CD71^+$ RBC and hematology analysis. Limited sample size and restricted access to donor information may limit the applicability of the finding of the effects of donor factors on $CD71^+$ RBCs to the general population.

Specific Research Aim #2: To assess the effect of $CD71^+$ RBCs on erythrophagocytosis of macrophages and supernatant Hb *in vitro* (Chapter 3).

Transfused RBCs are usually recycled within the first hours by recipient monocyte/macrophage functions. To mimic the impact of donated $CD71^+$ RBCs within recipients, an *in vitro* erythrophagocytosis- monocyte suspension assay are employed. This assay measures the phagocytosis index and supernatant Hb levels to understand the effects of $CD71^+$ RBCs on erythrophagocytosis *in vitro*. ROS antioxidants are applied to investigate the mechanism by how $CD71^+$ RBCs may affect erythrophagocytosis. Due to the *in vitro* nature of the assay, the generalizability of these results *in vivo* may be restricted.

Specific Research Aim #3: To retrospectively explore the association between donor sex and hospital-onset sepsis rate and Hb increment in critically ill patient (Chapter 4).

No observational studies to date have been reported on the impact of blood donor sex on sepsis rates in critically ill patients. Elevated erythrophagocytosis in recipients of transfusion s may lead to a state of free Hb overload, potentially heightening the risk of sepsis. For this retrospective analysis, information for eligible patients admitted to acute care hospitals in Hamilton, Canada, from 2010 to 2020, are sourced from the Transfusion Research Utilization, Surveillance and Tracking (TRUST) database, with corresponding donor data obtained from Canadian Blood Services. Univariate analysis is used to examine the effect of donor sex on sepsis rates and Hb increments, and regression analysis explores the relationships between donor

factors, sepsis rates, and Hb increments. The diagnosis of sepsis is determined using validated ICD -10 codes. The analysis also consider sample size, validation of sepsis codes, and potential confounding factors that may affect the validity and generalizability of the findings.

1.5 FIGURES

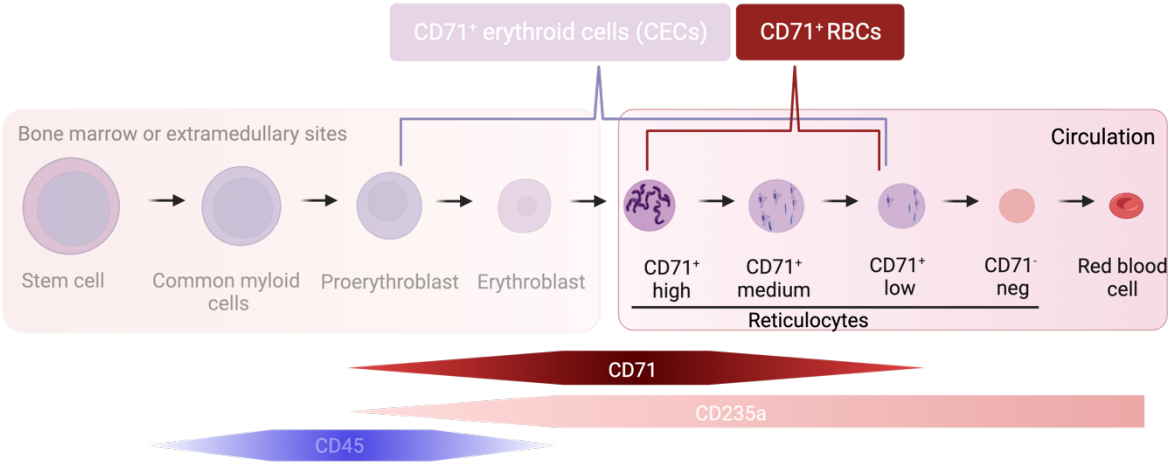

Figure 1-1 CD71$^+$ erythroid cells, reticulocytes and CD71$^+$ RBCs during erythropoiesis

CD71$^+$ RBCs originate from stem cells within the bone marrow or extramedullary sites. Initially, These progenitors express surface markers like CD45, CD235a, and CD71 early in development. As they mature, they transition through the erythroblast stages, shed their nucleus, and enter the bloodstream as young red cells, known as reticulocytes. As these cells continue their journey towards full maturity, they decrease their expression of CD71. This research distinguishes between the broader category of CD71$^+$ erythroid cells and the more specific CD71$^+$ RBCs. In this thesis, this focus is on CD235a$^+$CD71$^+$CD45$^-$ cells – young red cells akin to reticulocytes, identified by specific surface markers (Adapted from Grzywa et al. 2021).

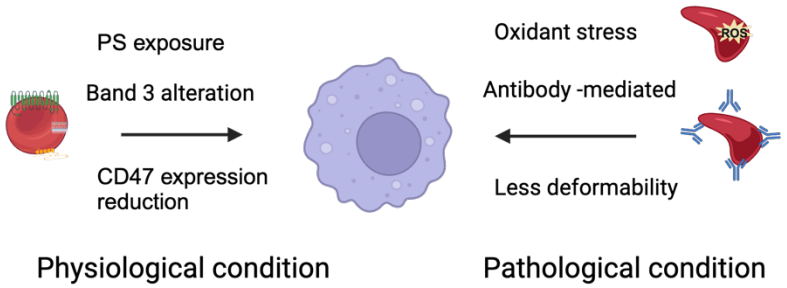

Figure 1-2 The mechanism for erythrophagocytosis under physiological and pathological conditions.

Phagocytosis of aged RBCs is regulated through mechanisms such as the exposure of phosphatidylserine (PS), alterations in band 3 (anion exchanger [AE1]), and the loss of CD47, which serves as a 'do not eat me' signal under normal physiological conditions. In contrast, pathological clearance of red cells is driven by factors including oxidative damage, reduced deformability, and immune-mediated destruction.

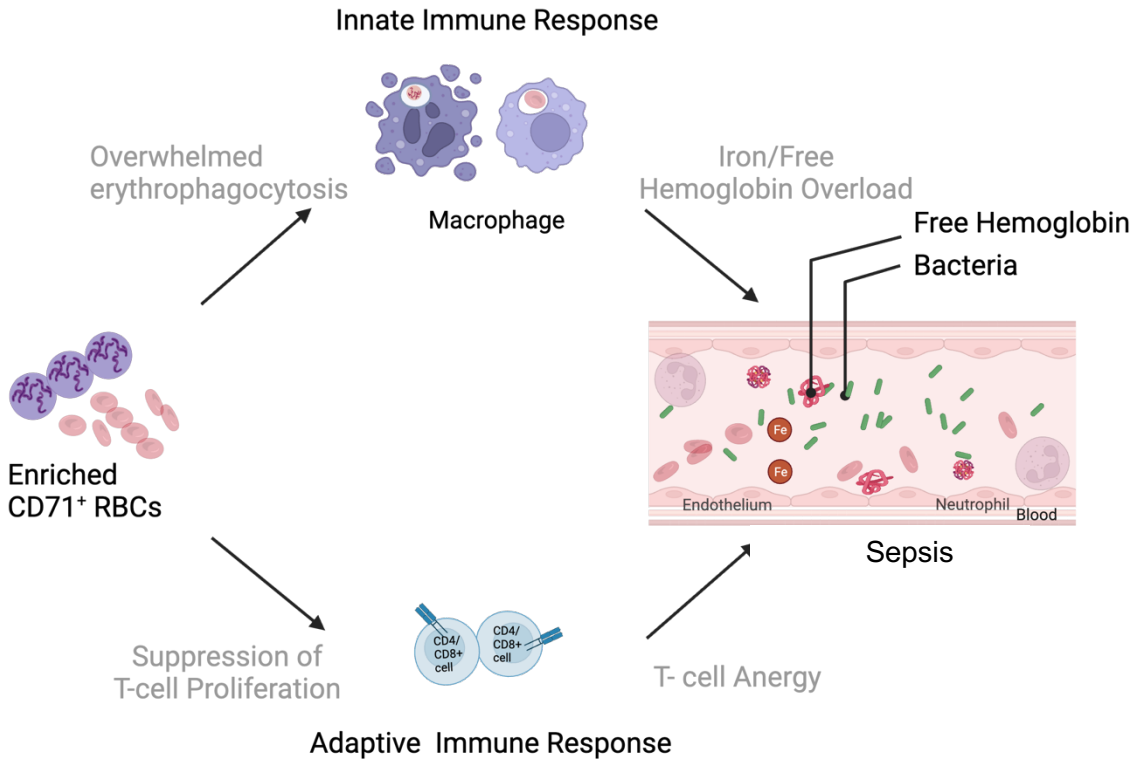

Figure 1-3 Diagrammatic representation of the proposed function of CD71$^+$ RBCs in modulating septic responses within the innate and adaptive immune systems.

Transfusion of CD71$^+$ RBCs is postulated to accelerate clearance by the mononuclear phagocytic system, potentially leading to overwhelmed erythrophagocytosis. This process may result in an excessive release of free Hb or iron which could serve as a proliferative substrate for bacteria and may modulate endothelial interactions, including effects on the glycocalyx—a key component in vascular integrity and immune cell trafficking. Furthermore, free Hb/ iron may interact with immune cells such as macrophages and neutrophils, prompting the release of pro-inflammatory cytokines like IL-6, IL-1β, and TNF-α, NO which are crucial mediators in the pathogenesis of sepsis. These cytokines, normally involved in the regulation of immune responses, can become dysregulated, contributing to systemic inflammation and endothelial dysfunction. Meanwhile, CD71$^+$ RBCs, might suppress monocyte function, by reducing their production of the inflammatory signal TNF-α after being consumed by these cells. Additionally, CD71$^+$ RBCs may influence T-cell function,

where their presence is correlated with the suppression of T-cell proliferation and the induction of T-cell anergy—processes potentially mediated by the altered cytokine environment. This complex cascade of events underscores the potential for $CD71^+$ RBCs to exacerbate immune dysregulation, thereby increasing the risk of sepsis.

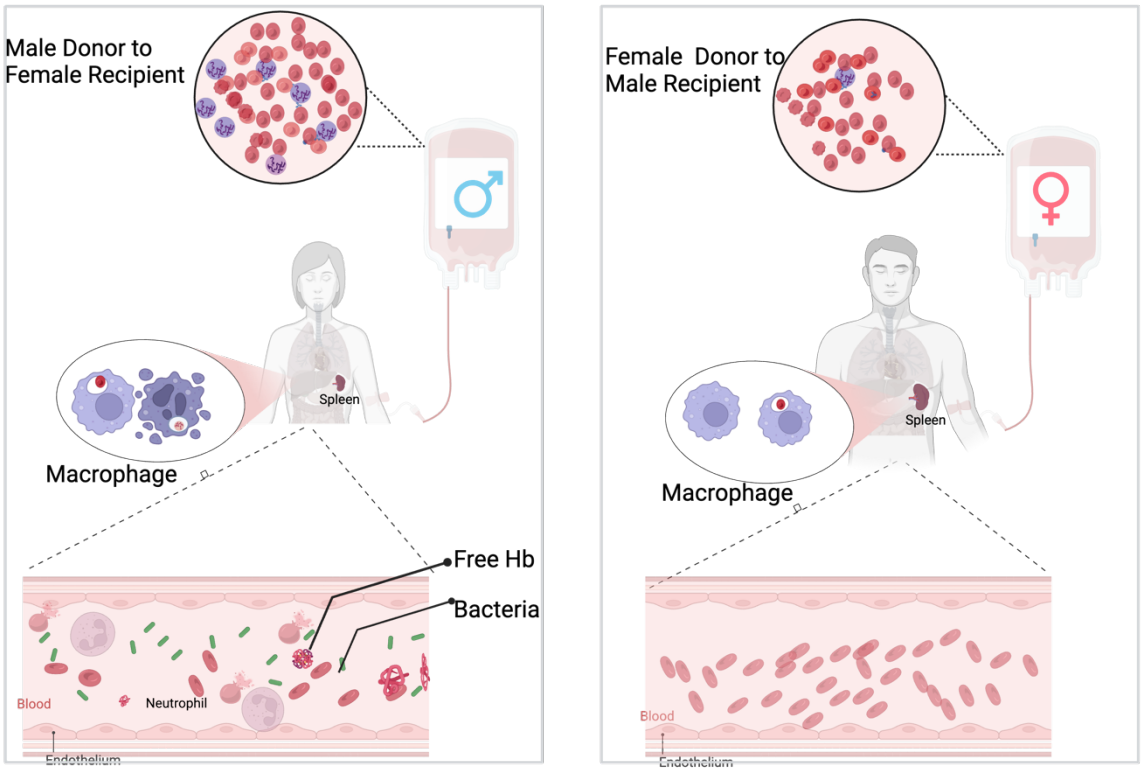

Figure 1-4 Proposed mechanism of the effect of donor sex on the transfusion-associated with sepsis.

When compared to female donor, male donor may have elevated $CD71^{+}$RBCs, potentially enhancing erythrophagocytosis. This process, which allows the mononuclear monocyte system to clear out old RBCs, can become overloaded in transfusion recipients. When patients receiving RCCs, their system can be overloaded, especially in female recipients who might have a lesser capacity than males. This overloaded may lead to a lower Hb increment and posed risk for sepsis development. Therefore, this project propose that $CD71^{+}$ RBCs in donated blood products are potent immune mediator of *in vitro* erythrophagocytosis and their quantity is influenced by blood factors that is a predictor of change in post-transfusion Hb and hospital-onset sepsis in critically ill patients receiving RCCs. Hb, hemoglobin

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Chapter 2 † Donor sex, Pre-donation Hb and Manufacturing Methods Affect CD71^{+}RBCs in Blood Products

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2.1 INTRODUCTION

Transfusion of allogeneic red cells is a common medical intervention to improve oxygen delivery in anemic patients, but it is in some cases associated with increased morbidity and mortality [1-3]. Research has shown that factors like donor characteristics, manufacturing methods, and hypothermic storage duration impact the quality of RCCs [4, 5] and transfusion outcomes [6-8]. Recent study report that male donors were associated with an increased risk of mortality in female recipients [9].This can be in large part attributed to deleterious immunologic effects that remain ill understood. Several factors, such as residual WBCs, soluble WBC-derived mediators, decompartmentalized RBC contents, bioactive lipids, and extracellular vehicles (EVs) contribute to transfusion-related immunomodulation (TRIM) [10-14]. $CD71^+$ erythroid cells ($CD71^+$, immature RBC marker) which include immature reticulocytes ($CD71^+$ RBCs) have recently been recognized as potent immunoregulatory cells in certain conditions [15-17]. Therefore, transfused donor $CD71^+$ RBCs may serve as an additional biological mechanism underlying TRIM in donor-recipient sex-mismatched transfusions.

However, the quantity of $CD71^+$ RBCs in RCCs from different donors is not well-determined. In healthy individuals, $CD71^+$ RBCs originate in bone marrow and are released into the peripheral blood where they mature. Iron is crucial for the development and maturation of $CD71^+$ RBCs, and its production can be impacted by blood loss [18]. Each whole blood (WB) donation, particularly from frequent donors and menstruating females, leads to a loss of 200 mg - 250 mg of iron, potentially causing iron deficiency even if pre-donation Hb levels and donation intervals meet Canadian standards for both female (125 g / L, 84 days) and male donors (130 g / L, 56 days) [19]. Factors such as donor age and erythropoiesis stimulation may also be related to iron status [20]. The influence of donor sex, age, Hb levels, and donation behaviors on the total number of $CD71^+$ RBCs in blood products remains unclear.

The number of $CD71^+$ RBCs in stored RCCs can also be influenced by various manufacturing methods and hypothermic storage time. While there are differences on manufacturing methods among international blood services, red cell filtration (RCF, top / bottom) and whole blood filtration (WBF, top / top) methods are the most used to produce whole blood-derived, leukoreduced RCCs. These processing methods differ in terms of leukoreduction and buffy coat removal timing [21], which may individually or collectively impact the quantity of $CD71^+$ RBCs in stored RCCs. Additionally, the metabolic slowing of RBC maturation during cold storage may influence the stability and *in vitro* maturation of $CD71^+$ RBCs, prolonging the process beyond the usual 24-48 hours typically observed in circulation.

To investigate the effect of donor factors, manufacturing methods and hypothermic storage on the number of $CD71^+$ RBCs, WB samples from healthy blood donors and RCCs will be utilized to examine RBC indices, $CD71^+$ RBC concentration and the total number of $CD71^+$ RBCs in RCC samples.

2.2 MATERIALS AND METHODS

2.2.1 Sample Collection and Preparation

This project was reviewed by both Canadian Blood Services' (Protocol 2020.005 and 2020.049) and the University of Alberta's (Protocol Pro00082512) Research Ethics Boards. Data on donor sex, donor age, pre-donation fingerstick capillary Hb level (pre-donation Hb level), and the number of WB donations over the previous 12 month were either self-reported or provided by the Canadian Blood Services. The prevalence of transgender donors is extremely low in the Canadian blood donor population [22], so donor sex was inferred. WB specimens (2 - 5 mL; EDTA; n = 80) were collected by the Canadian Blood Services' Blood for Research program. An aliquot of 500 µL was taken from each sample for hematology and flow cytometry analyses. In addition, WB samples (30 - 50 mL; acid citrate dextrose [ACD]; n = 6) were obtained from local healthy donors to perform small scale manufacturing as described below.

2.2.2 Assessment of Hematological Parameters

The pre-donation Hb level was determined using a fingerstick sample (Compo Lab, Fresenius Kabi LLC, Germany). RBC concentration, hematocrit (Hct) and pre-donation venous Hb level (Hb_v) of the samples were determined using a hematology analyzer (DxH520, Beckman Coulter, USA), with low, medium, high-level controls (B36872, Beckman Coulter, Ireland) performed daily.

2.2.3 Assessment of Hemolysis

To examine whether WB hemolysis could be a confounding factor influencing the effect of donor sex on $CD71^+$ RBC concentration or percentage, percent hemolysis was determined from male and female WB (**Figure 2-1**) using Drabkin's method. The Drabkin's method involves consists of following steps: Drabkin's reagent preparation (pH, 7.2 ± 0.2), a solution containing potassium ferricyanide (Cat # 244023, Sigma-Aldrich, USA; 0.61 mmol / L), potassium cyanide(Cat # P5379, Sigma-Aldrich, USA; 0.77 mmol / L), potassium dihydrogen phosphate (1.03 mmol / L) and Triton X-100 （Cat # X100, Sigma-Aldrich, USA; 4.38 mmol / L) prepared with distilled H_2O to create Drabkin's reagent, which reacts with Hb to form cyanmethemoglobin, a stable-colored compound; blood sample dilution, a small volume of each blood sample diluted with Drabkin's reagent at a ratio of 1:200 and allowed to stand for at least 5 min to ensure complete conversion of Hb to cyanmethemoglobin; Absorbance measurement, the absorbance of the resulting cyanmethemoglobin solution measured at a wavelength of 540 nm using a spectrophotometer (Spectrum Plus 384, Molecular Devices, USA); Hb concentration calculation, the SoftMax pro software (Molecular Devices, San Jose California, USA) programmed to automatically complete the calculation using the Lambert-Beer's law equation as follow [23]:

$$c = \frac{A_{540} \times M \times F}{\varepsilon_{540} \times l \times 1000} \qquad \text{Eq 2-1}$$

- Where c : Concentration of Hb (g / L)

- A_{540} : Absorbance of the solution at 540 nm
- M: Molecular mass of Hb monomer = 16114.5 mg / mmol
- F: Dilution factor (TotHb: F=201 and SuHb: F=26)
- ε_{540}: Millimolar absorptivity of HiCN at 540 nm = 11.0 cm-1· mM-1
- l : Light path (cm).

After determining the Hb concentration in the supernatant hemoglobin(Hb_S) and total hemoglobin (Hb_T) using the Drabkin's method, the hematocrit (Hct) of the blood sample was also measured using microhematocrit capillary tubes (Cat #: 22-362574, Fisher Scientific, USA). This sample was centrifuged at 12,500 x g for 5 min at room temperature using microhematocrit centrifuge (210, Hettich, Germany). The Hct was then measured by reading the results using with a Leica Biosystems laminated Critocaps Tube Reader card.

With these measurements, percent hemolysis can be calculated using the following equation [24] :

$$Percent\ hemolysis = \frac{\mathrm{Hbs}}{Hb_T} \times \frac{1}{(1 - hematocrit)} \times 100 \qquad \text{Eq 2-2}$$

Where:

- Supernatant Hb (Hb_S) is the concentration of free hemoglobin in the plasma or supernatant after centrifuging the blood sample(g / L).
- Total Hemoglobin (Hb_T) is the overall Hb concentration in the blood sample, which includes both free Hb and Hb within RBCs (g / L).
- Hematocrit (Hct) is the ratio of the volume of RBCs to the total volume of blood, expressed as a fraction or percentage.

2.2.4 Small-Scale RCF and WBF Method

WB samples (n = 6) were divided into two aliquots (10 mL each) for small-scale RCC using the RCF and WBF methods (**Figure 2-2**), which were adapted from the 500 mL WB processing

methods employed by Canadian Blood Services [24]. For RCF production: WB samples were centrifuged at 2000 × g for 11 min at 22 °C, and plasma was removed by vacuum aspiration using a glass sterile pasteur pipette. The buffy coat was extracted until the white layer was no longer visible above the packed red cells and SAGM (sodium chloride 150 mmol / L, adenine 1.25 mmol / L, dextrose 45 mmol / L, mannitol 30 mmol/L, pH=5.7) was added to resuspend the packed red cell. To obtain leukoreduced RCC with a Hct of 45% - 65%, the resuspend packed red cell were passed through an SAGM-primed WBC syringe filter (AP - 4952, Pall Corporation, USA) attached to a 10 mL syringe cylinder.

For WBF production: WB samples underwent leukoreduction using an SAGM-primed filter for leukoreduction. The leukoreduced WB was then centrifuged at 2200 × g for min at 22 °C, and the plasma in the upper layer was removed. SAGM was added to the packed RBCs to obtain a Hct of 45% - 65% (leukoreduced WBF RCC).

Duplicate 1 mL aliquots of the RCCs were tested for hemolysis. RCCs (0.5 mL) were aliquoted into sterile FACS flow tubes and stored at 2 - 6 °C to determine the RBC indices and total $CD71^+$ RBC numbers at day 1, 7 and 28. Hemolysis, Hct, and final RCC volume were also measured to evaluate whether the small-scale manufacturing method could accurately mimic the large-scale blood bank manufacturing process (**Figure 2-3**).

2.2.5 Determination of $CD71^+$ RBC Proportion, Concentration, and Total Number

A published flow cytometric method was employed to directly determine $CD71^+$ RBC proportion in whole blood (**Appendix A**) [25]. Briefly, 85 µL of diluted WB (volume ratio of WB sample to Dulbecco's buffered solution [DPBS] = 1:114) was incubated with anti-human PE-Cy7 CD235a (Cat # 563666, BD, USA), BV711 CD71(Cat # 745418, BD, USA) and APC CD45 (Cat # 555485, BD, USA) in triplicate. Fluorescence minus one (FMO) control were also prepared. The samples were incubated in the dark for 30 min at 20 - 24 °C. Then 400 µL of DPBS was added to

resuspend the cells at a concentration of 1 - 10 × 10^6 / mL. The samples were then analyzed using a flow cytometer (LSRFortessa X-20, BD, USA).

Data analysis was performed with FlowJo software (v10 10.7.1, FlowJo, USA). The proportion of CD71+ RBC was applied using the gating strategy shown in **Figure 2-4**. The $CD71^+$ RBC concentration was calculated by multiplying the proportion of $CD71^+$ RBC with the RBC concentration in the specimen. The total number of $CD71^+$ RBCs in the RCC sample was determined based on the $CD71^+$ RBC concentration and the volume of RCC samples.

2.3 STATISTICAL ANALYSIS

Univariable analysis between $CD71^+$ RBC concentration, donor sex, Hb_v level, and the number of WB donations over the previous 12 months was performed using a Mann-Whitney test for two group comparisons or a Kruskal-Wallis test for multiple group analyses due to the skewness of the $CD71^+$ RBC proportion in the RCCs (skewness > 0.5). A two-tailed t test was employed to examine differences in RBC concentration and Hb level between male and female. The association between pre-donation Hb level, $CD71^+$ RBC concentration and Hb_v Level was determined using Pearson's correlation coefficient. The Wilcoxon-matched pairs signed rank test was utilized to examine differences between the RCF and WBF methods given that same individual was used to investigate the effect of the manufacturing methods. All calculations were performed with Prism 9 for Mac, version 9.3.1 (GraphPad Software, USA). A p value < 0.05 was considered statistically significant difference.

2.4 RESULTS

2.4.1 Donor Demographics

There were 38 females (49.5 ± 21.0 yrs) and 42 male (48.9 ± 19.4 yrs) donors who participated in the study **(Table 2-1).** The minimum pre-donation Hb levels were 126 g / L for females and 141 g / L for males, surpassing the threshold Hb level of 125 g / L for females and 130 g / L for males, respectively. Only one of the 38 female (2.6%) donors had a low venous Hb

of 115.3 g / L. Significant differences were observed in the pre-donation Hb level ($p < 0.001$), Hb_v level ($p < 0.001$) and RBC concentration ($p < 0.01$) between female and male donors. The frequent donors (> 2 WB donations over the previous12 months), regular donors (1-2 WB donations over the previous 12 months) and first-time WB donors accounted for 39%, 52%, 8% of females and 33%, 60%, 7% of male donors, respectively. No statistically significance difference was found in the frequency of donation between the male and female donors.

2.4.2 Donor Sex, Age, Donation Frequency, and $CD71^+$ RBC Concentration

The median $CD71^+$ RBC (%) in male donors was of 0.20% (IQR 0.12%), which was higher than the 0.16% (IQR 0.14%), observed in female donors (**Figure 2-5A**; $p < 0.001$). The median $CD71^+$ RBC concentration in male donors was 10.4×10^9 / L (IQR 8.1×10^9 / L), higher than the 6.7×10^9 / L (6.8×10^9 / L) in female donors (**Figure 2-5B**; $p < 0.001$). Specifically, younger male donors (17 - 50 yr) had a higher $CD71^+$ RBC concentration than both younger (adjusted $p < 0.01$) and older female donors (adjusted $p = 0.001$) (**Figure 2-5C**). Due to the small number of first-time donors recruited, male and female donors were divided into regular donors and frequent donors to investigate the effect of donation frequency. The male group with 1-2 WB donations in the previous 12 months ($n = 25$) had a higher $CD71^+$ RBC concentration than female donors with the same frequency of donation **Figure 2-5D**; $n = 20$, $p < 0.01$).

2.4.3 Hb Level and $CD71^+$ RBC Concentration

To further investigate the effect of donor Hb level on the donor-recipient sex-mismatched transfusion outcomes in a retrospective study, pre-donation Hb, obtainable from Canadian Blood Services, should be used for grouping. However, to ensure sufficient sample size, 18 local donors were included without access to pre-donation fingerstick capillary Hb level. Hb_v levels were used as a proxy for pre-donation Hb levels, justified by demonstrating a strong correlation between the two, as shown as shown in **Figure 2-6**. A higher proportion of $CD71^+$ RBC was observed in the Hb_v level group above 155 g / L ($n = 23$) compared to the group below 140 g / L ($n = 26$) (**Figure**

2-7A; $p < 0.001$), which mirrors the comparison of $CD71^+$ RBC concentration between the two groups (**Figure 2-7B;** $p < 0.001$). Specifically, only males in the Hb_v level group above 160 g / L had a higher $CD71^+$ RBC concentration than the two Hb_v level (<135g / L and >140g / L) female groups (**Figure 2-7C**). Multiply linear regression revealed that donor sex did not change the correlation between pre-donation Hb level and $CD71^+$ RBC concentration, suggesting that pre-donation Hb level was correlated for both males and females (Data not shown). Consequently, the 62 CBS provided pre-donation Hb values were utilized and a medium positive correlation between pre-donation Hb level and $CD71^+$ RBC concentration was identified (**Figure 2-7D**; Pearson $r = 0.41$, $p < 0.001$). When potential outliers were removed, specifically those with Hb values at 170 g/L and 181 g/L correlating with $CD71^+$ RBC counts of 31.3×10^9/L and 33.0×10^9/L respectively, the correlation coefficient between pre-donation Hb levels and $CD71^+$ RBC concentrations modestly decreased to 0.36. This adjustment indicates that, although the outliers have a a tangible impact on the correlation, a consistent moderate positive correlation between pre-donation Hb levels and $CD71^+$ RBC concentrations remains evident.

2.4.4 The Effect of Small-Scale RCF and WBF on $CD71^+$ RBC Numbers in RCC Samples

WBF-produced RCCs had a higher total number of $CD71^+$ RBCs [5.45×10^7 / sample (IQR 2.15×10^7 / sample)] than RCF-produced RCCs [4.40×10^7 / sample (IQR 2.57×10^7 / sample) ($p < 0.05$). These values were 69.6% and 53.7% of the $CD71^+$ RBCs found in WB sample [8.23×10^7 / sample, IQR 2.57×10^7 / sample] respectively (**Figure 2-8A**). A lower total number of $CD71^+$ RBCs was observed in stored RCF-RCCs and WBF-RCCs (day 28), compared to day 7 fresh RCF-RCCs ($p < 0.05$) and WBF RCCs ($p < 0.05$) respectively (**Figure 2-8B**). Interestingly, no significant difference was seen between stored RCF-RCCs and WBF-RCCs (day 28, $p > 0.05$).

2.5 DISCUSSION

The study investigated the effect of donor characteristics, small-scale manufacturing processes, and hypothermic storage duration on the quantity of $CD71^+$ RBCs in blood products.

The findings revealed that male donors had higher $CD71^+$ RBC quantity than female donors and those with higher Hb levels (> 155 g/L) had higher $CD71^+$ RBC quantity than those with lower Hb levels (< 140 g/L). Additionally, the small-scale RCF method was associated with a significant decrease in the total number of $CD71^+$ RBCs compared to the small-scale WBF method. Fresh RCCs exhibited a higher $CD71^+$ RBC number compared to stored $CD71^+$ RBCs. Consequently, it is important to note that not all RCCs have the same total number of $CD71^+$ RBCs, which maybe serve as an additional biological mechanism in donor-recipient sex-mismatched transfusions.

Compared to female WB donors (0.16%; IQR 0.14%), a higher $CD71^+$ RBC proportion was observed in the male WB donors (0.20%; IQR 0.12%). These values are in line with immature reticulocyte fraction reported for healthy male and female individuals [26]. However, previous research has reported a higher proportion of $CD235a^+$ $CD71^+$ cells in females than that in non-WB blood donation male [27]. The participants in this study were blood donors, whose Hb level are no less than thresholds for donation (130 g/L for males;125 g/L for females) [19], yet some may be on the cusp of iron deficiency without crossing into anemia. In this study, 39% of females and 33% of males were frequent donors and 52% of female and 60% of male donors were regular donors (**Table 2-1**).

Typically, $CD71^+$RBCs or reticulocytes are recognized as indicators of erythropoiesis after blood loss and are usually elevated during anemia. However, frequent WB donation may hamper $CD71^+$ RBC production. Particularly, premenopausal female donors, are more likely to be iron deficient [28]. Studies have shown that iron deficient can hamper $CD71^+$ RBC production [18]. Consistently, as depicted in **Figure 2-5D**, frequent female WB donors had the lowest $CD71^+$ RBC concentration compared to other groups. Iron supplementation has been reported to stimulate the production of $CD71^+$ cells in pregnant women [29]. Additionally, androgen stimulates several stages of the hematopoietic system and their bioactive levels decrease with aging [30], while estrogen depress these activities [31]. This is consistent with the finding that male donor aged 17-

50 yrs had the highest $CD71^+$ RBC concentration compared to other groups (**Figure 2-7C**). Lastly, instead of PBMC preparation [27], WB preparation was used for $CD71^+$ RBC determination in this study, and there was a good correlation between these two methods with four WB samples tested (R^2 =0.73, data not shown). Thus, it is likely, instead of the preparation methods, donor sex, donation history and donor age collectively contribute to the different $CD71^+$ RBC concentrations in male and female donors.

A medium positive correlation was seen between pre-donation Hb level and $CD71^+$ RBC concentration in donors (**Figure 2-7D**), which contrasts with the negative correlation between Hb level and $CD71^+$ RBC concentration reported in anemic individuals [32]. This discrepancy could be attributed to the differences in iron status and Hb level of the participants. In this study, only one donor was anemic based on WHO criteria. However, iron deficiency, which is common in the regular and frequent donor population, may affect $CD71^+$ RBC production, as it relies on the availability of iron. Non-anemic donors with higher Hb (>155 g / L) had higher $CD71^+$ RBC proportions and concentrations compared to those with lower Hb levels(<140 g / L) (**Figure 2-7A**). In contrast, anemic and / or cancer patients may exhibit extramedullary hematopoiesis, leading to increased $CD71^+$ RBC production. To estimate the quantitative relationship among Hb level, and $CD71^+$ RBC concentration, the $CD71^+$ RBC concentration in the group with a Hb_v greater than 140 g / L were compared to the group with a Hb_v greater than 160 g / L, suggesting that a lower Hb of 20 g / L was associated with a significantly lower $CD71^+$ RBC concentration in donors (**Figure 2-7C).** This finding indicates a “U” shaped relationship between $CD71^+$ RBC concentration and Hb level (**Figure 2-9Error! Reference source not found.**). Further studies could investigate whether lower $CD71^+$ RBC concentrations and lower Hb levels are risk factors for developing anemic status.

A higher reduction in the total $CD71^+$ RBC number was observed in RCF-RCCs, compared to WBF-RCCs on day 1. This could be due to the lower density of $CD71^+$ RBCs compared to

mature RBC and the removal of the buffy coat after centrifugation in the RCF method. Interestingly, there was no statistical significance between RCF-RCCs and WBF-RCCs at day 7 and day 28. This finding suggests that the metabolism rates in stored RCCs with different numbers of $CD71^+$ RBCs may vary. As a small-scale model was applied in this study, it is possible that a more substantial difference in absolute $CD71^+$ RBC numbers between fresh RCF-RCCs and WBF-RCCs could be observed when full-scale manufacturing methods are employed. An interesting observation was that the two subpopulations (CD235a high and CD235a low, **Figure 2-8B, C**) are frequently observed in RCC samples from small-scale manufacturing processes, but they're seldom seen in whole blood. This distinction might be attributed to the small-scale manufacturing method's use of a white blood cell syringe filter to produce RCC, aiming to reduce WBC. Back gating these two populations shows remarkable similarities between them. To gain deeper insight into these subpopulations, imaging flow cytometry could be used to analyze their image or other protein expression side by side.

Given the variance in the total number of $CD71^+$ RBC among RCCs, further research is needed to explore the potential role of $CD71^+$ RBCs in the donor-recipient sex-mismatched transfusion. There are a few aspects that could be investigated: firstly, determining the required number of $CD71^+$ RBCs to exert an immunomodulatory role. It has been reported that the immunomodulatory effect of $CD71^+$ erythroid cells *in vitro* exhibits a dose-dependent manner incubated with splenocytes [15]. Monocyte monolayer assay, which assess the phagocytosis of RBCs that can occur with incompatible transfusion to avoid transfusion-related hemolysis in recipients, could be a functional assay to evaluate the immunity of $CD71^+$ RBCs *in vitro*. Secondly, Investigating the potential differences in immunity between $CD71^+$ erythroid cells from male and female donors: It has been reported that female mice possess a stronger immunosuppressive function on T cells with a higher expression of $CD45^+$ $CD71^+$ erythroid cells compared to male mice [27]. Although $CD45^+$ $CD71^+$ erythroid cells were not reported in significant numbers in this

study, examining the immunity of $CD71^+$ RBCs from male and female donors could provide valuable insights.

As multiple variables affect the $CD71^+$ RBC concentration in the donors, it requires a large sample size to control all variables. Donor age and the number of WB donations over the previous 12 months between male and female group were able to be controlled when investigating the impact of donor sex on $CD71^+$ RBC concentration. Given the observed effect of donor sex and pre-donation Hb level on RBC, further investigation into the impact of donation frequency is warranted. Other factors, such as exercise and inflammation status [33, 34], may also affect $CD71^+$ RBC concentration. However, these factors are not routinely collected by the Canadian Blood Service. Future studies could consider increasing the sample size, conducting longitudinal study to assess the donation frequency impact, and investigating additional factors, to better understand the relationship between donor characteristics and $CD71^+$ RBC concentration.

2.6 CONCLUSION

In conclusion, this study has demonstrated small-scale RCF-derived RCCs from female donors with low pre-donation Hb are associated with a low total quantity of $CD71^+$ RBCs. These finding emphasize that both donor factors and manufacturing methods can influence the number of $CD71^+$ RBCs in RCC products. Therefore, understanding the immunological impact of $CD71^+$ RBCs in donor - recipient sex - mismatched RBC transfusions becomes increasingly important.

2.7 TABLES / FIGURES

Table 2-1 Blood donor demographic characteristics

Characteristics	**Female**	**Male**	**p-value**
Number of participants	38	42	
Age (yr, Mean ± SD)	49.5 ± 21.0	48.9 ± 19.4	ns
Number of WB donations in the previous 12 months (median, [min, max])	2.0 [0, 4]	2.0 [0, 6]	ns
first-time WB donation (%)	8% (3/38)	7% (3/42)	
1 WB donation (%)	18% (7/38)	40% (17/42)	
2 WB donations (%)	34% (13/38)	20% (8/42)	
>2 WB donations (%)	39% (15/38)	33% (14/42)	
Pre-donation Hb level (g / L, Mean ± SD) [1]	144.6 ± 12.8	164.1 ± 14.0	***
Minimum (g / L)	126.0	141.0	
Hb_v (g / L, Mean ± SD)	137.3 ± 9.0	155.3 ± 13.3	***
Minimum (g / L)	115.3	132.0	
RBC ($\times 10^{12}$ / L, Mean ± SD)	4.6 ± 0.6	5.0 ± 0.5	**

[1] Sample sizes for pre-donation Hb levels within the female group (n=27) and male group (n=35).

ns, not statistically difference; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. A two-tailed t test was used to examine differences on age, RBC concentration and Pre-donation Hb level, Hb_v level between male and female. Mann-Whitney test was used for time of WB donation over 12 months.

WB, Whole blood; Hb_v, Venous hemoglobin; RBC, Red blood cells.

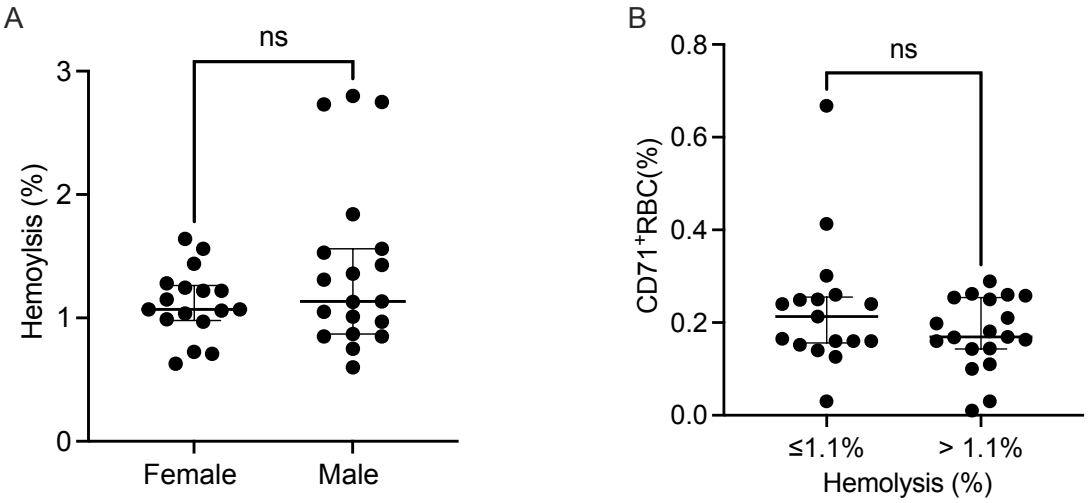

Figure 2-1 The WB hemolysis and the $CD71^+$ RBC proportion

(A) Hemolysis between female and male WB samples, median with interquartile range (error bar), Mann-Whitney test. (B) $CD71^+$ RBC (%) in the group with hemolysis above and below 1.1%, median with interquartile range (error bar), Mann-Whitney test. NS, not significant.

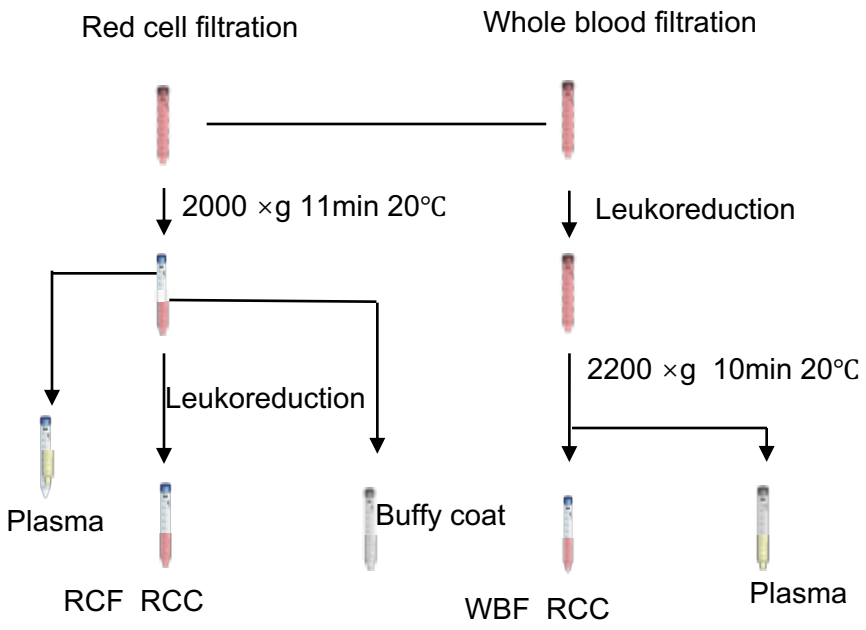

Figure 2-2 Schematic details of small-scale manufacturing methods for red cell filtration and whole blood filtration.

WB samples were divided into two aliquots for small-scale RCC samples using the RCF and WBF methods. For RCF production: WB samples were centrifuged at 2000 × g for 11 min at 22 °C, and plasma was removed by vacuum aspiration using a glass sterile pasteur pipette. The buffy coat was extracted until the white layer was no longer visible above the packed red cells and SAGM was added to resuspend the packed red cell. To obtain leukoreduced RCC the resuspend packed red cell were passed through an SAGM-primed filter attached to a 10 mL syringe cylinder. For WBF production: WB samples underwent leukoreduction using an SAGM-primed filter for leukoreduction. The leukoreduced WB was then centrifuged at 2200 × g for min at 22 °C, and the plasma in the upper layer was removed. SAGM was added to the packed RBCs (leukoreduced WBF RCC).WBF, whole blood filtration; RCF, red cell filtration.

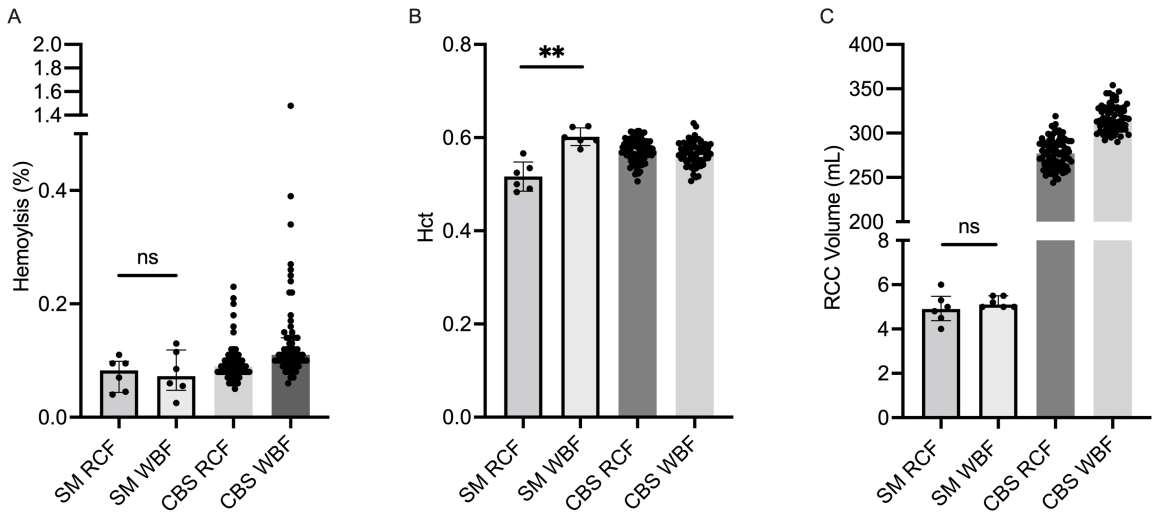

Figure 2-3 The profile of small-scale manufacturing method produced RCC sample and Canadian Blood Services RCC unit

The analysis of small-scale processed RCC samples (SM RCF, SM WBF; n=6) and Canadian Blood Service processed RCC unit (CBS RCF sample n=88, CBS WBF sample n=64). (A) Hemolysis in WBF RCC samples and RCF RCC samples, median with interquartile range (error bar), Wilcoxon-matched pairs test. (B) Hematocrit in WBF RCC samples, RCF RCC samples on day 1, day 7 and day 28; mean with standard deviation (error bar); paired t-test. (C) RCC volume in the SM RCF, WBF RCC and CBS; median with interquartile range (error bar), Wilcoxon-matched pairs test. **, $p < 0.01$; NS, not significant. CBS, Canadian Blood Service; RCF, red cell filtration; SM, small scale; WBF, whole blood filtration.

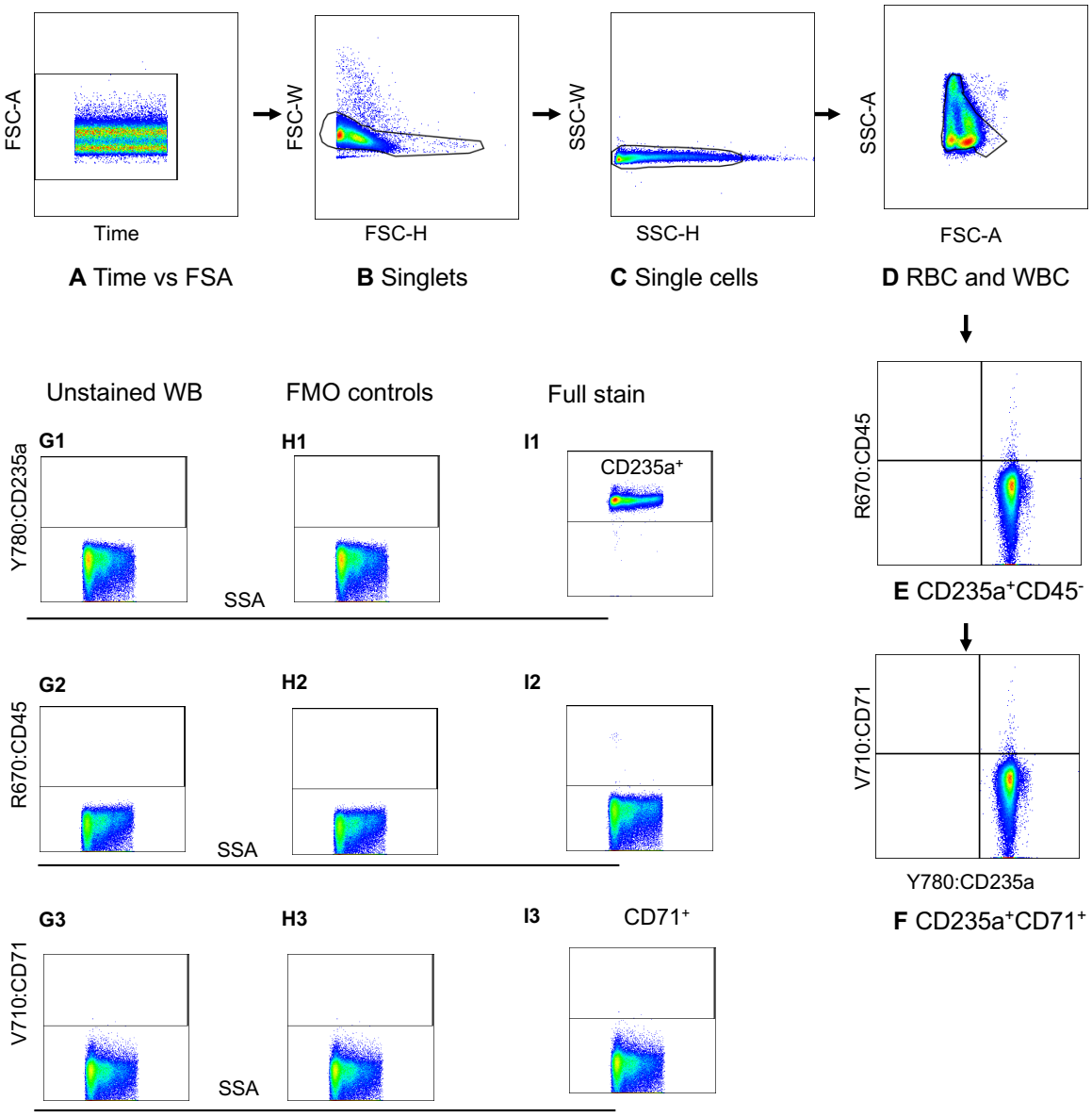

Figure 2-4 Representative nested gating strategy for CD71$^+$ RBCs in WB.

(A) Time vs FSA by adjusting the rectangular gate to include as many events as possible but getting rid of the interrupted events when the fluidics interrupted. (B) To create a polygon matching the distribution of the events in the FSH vs FSW dimension for singlets. (C) Polygon matching the distribution of the events in the SSH vs SSW for single cells. (D) FSA vs SSA (logarithm). (E) Gating for CD45$^-$ CD235a$^+$. (F) Gating for CD71$^+$CD235a$^+$. G1-3) Unstained WB. (H1) PE-Cy7

FMO control for gating CD235a^{+}. (H2) APC FMO control for gating CD45^{+}. (H3) FMO control for gating CD71^{+}. (I1-I3) Full stained sample. (J1-J2) Blank control. (K1-K2) Buffer cocktail control.

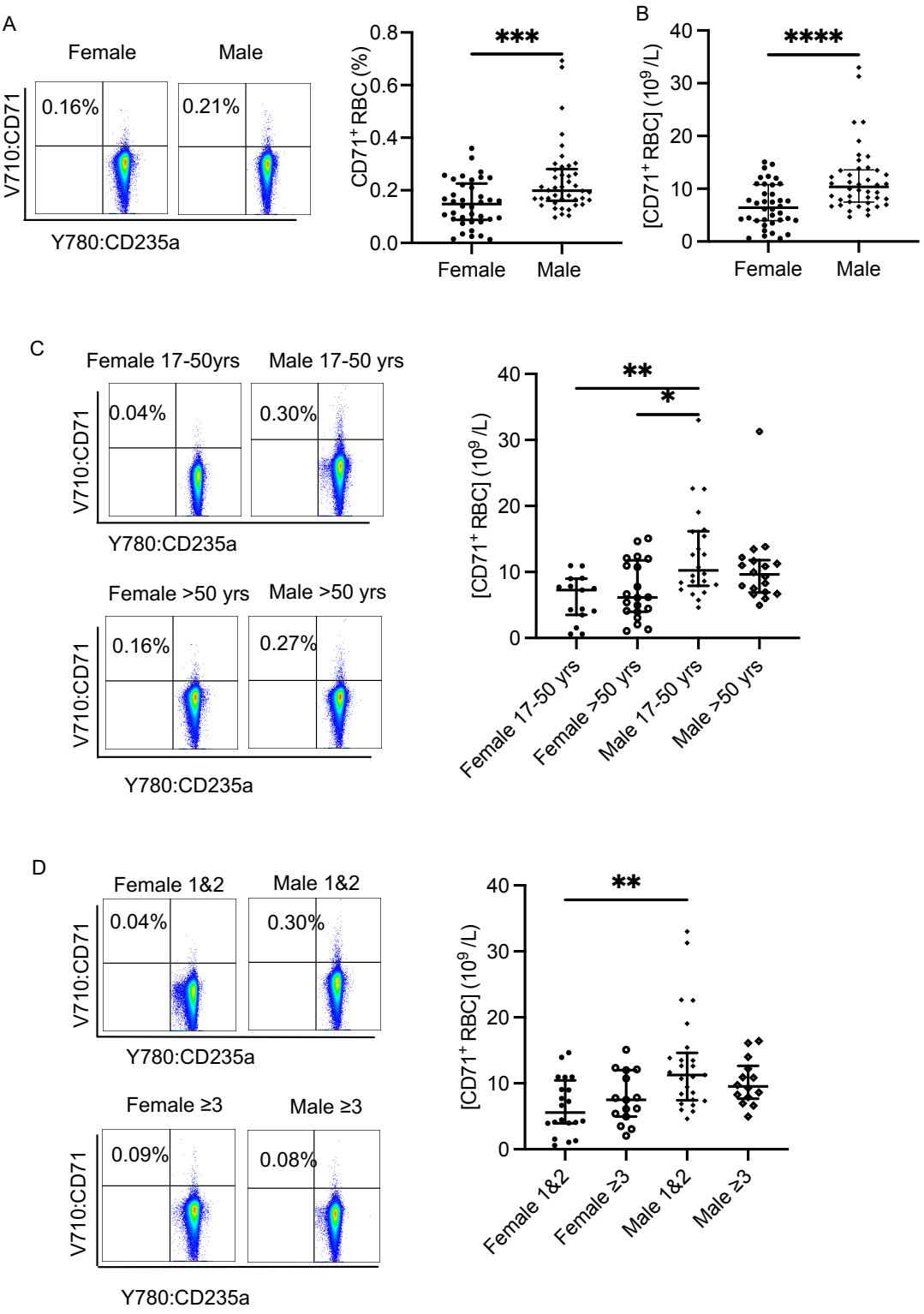
A
Female
Male
0.16%
0.21%
V710:CD71
Y780:CD235a
CD71+ RBC (%)
***
Female
Male
B
[CD71+ RBC] (10^9 /L)
****
Female
Male
C
Female 17-50yrs
Male 17-50 yrs
0.04%
0.30%
Female >50 yrs
Male >50 yrs
0.16%
0.27%
**
*
Female 17-50 yrs
Female >50 yrs
Male 17-50 yrs
Male >50 yrs
D
Female 1&2
Male 1&2
0.04%
0.30%
Female ≥3
Male ≥3
0.09%
0.08%
**
Female 1&2
Female ≥3
Male 1&2
Male ≥3

Figure 2-5 The $CD71^+$ RBC (%) and $CD71^+$ RBC concentration in male and female donors

(A) Representative images for $CD71^+$ RBCs and $CD71^+$ RBC (%) in female and male donors; median with interquartile range (error bar), Mann-Whitney test. (B) $CD71^+$ RBC concentration in female and male donors, median with IQR, Mann-Whitney test. (C) Representative images for $CD71^+$ RBCs and $CD71^+$ RBC concentration in female and male donors with two age groups, median with IQR, Kruskal - Wallis test with Dunn's multiple comparison test. (D) Representative images for $CD71^+$ RBCs and $CD71^+$ RBC concentration in female and male donors with different number of WB donations over the previous 12 months, median with interquartile range, Kruskal - Wallis test with Dunn's multiple comparison test. *, $p < 0.05$; ** , $p< 0.01$; ***, $p< 0.001$; ns, not statistically significant difference.

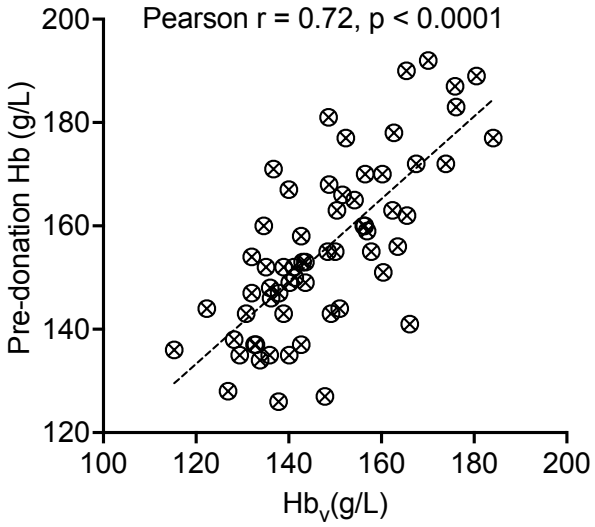

Figure 2-6 The relationship between pre-donation Hb and Hb_v.

Hb, hemoglobin; pre-donation Hb: pre-donation fingerstick capillary Hb level; Hb_v: pre-donation venous Hb level.

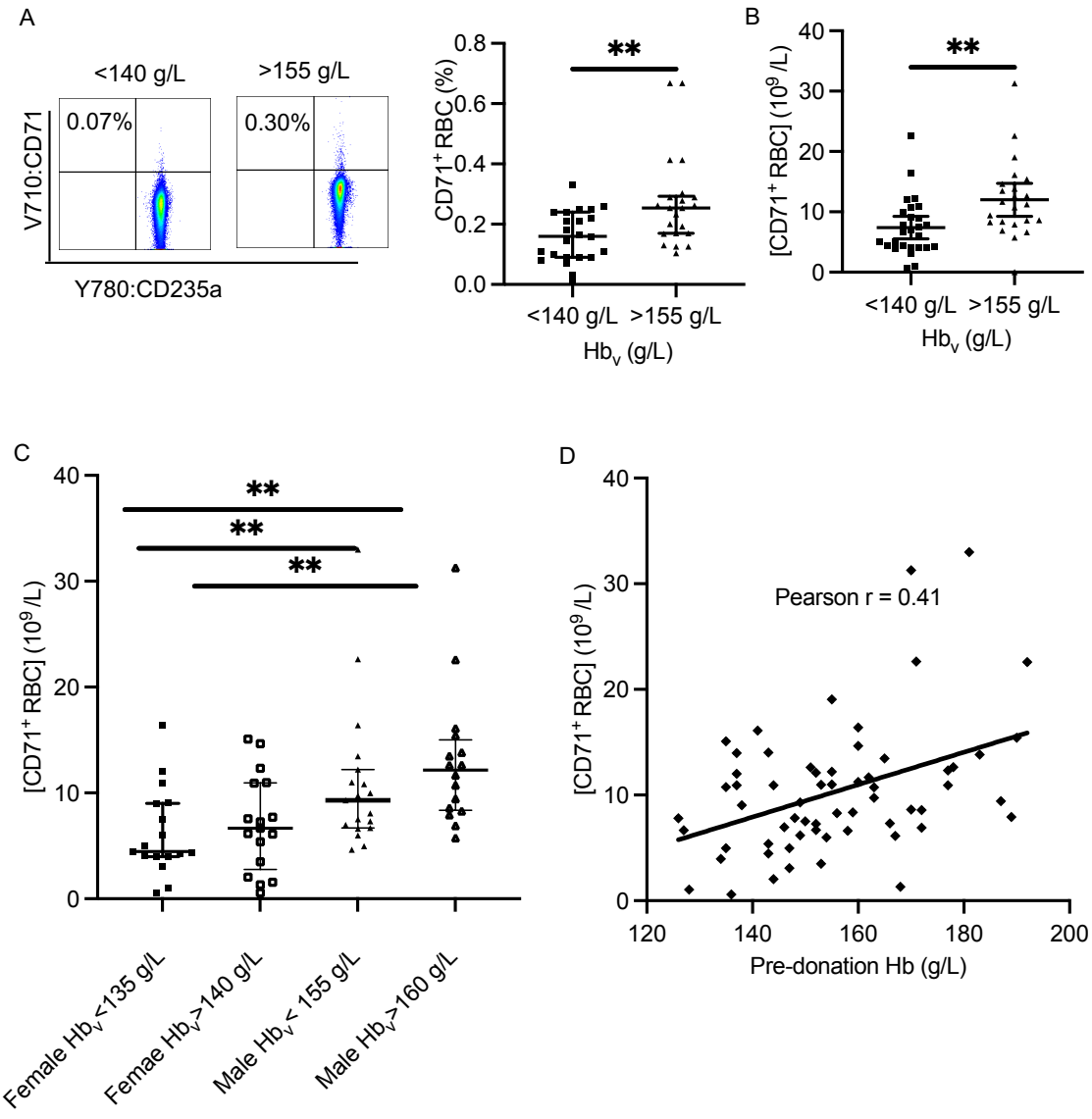
A
<140 g/L
>155 g/L
0.07%
0.30%
V710:CD71
Y780:CD235a
CD71+ RBC (%)
**
<140 g/L >155 g/L
Hbv (g/L)
B
[CD71+ RBC] (10^9 /L)
**
<140 g/L >155 g/L
Hbv (g/L)
C
[CD71+ RBC] (10^9 /L)
**
**
**
Female Hbv<135 g/L
Femae Hbv>140 g/L
Male Hbv< 155 g/L
Male Hbv>160 g/L
D
[CD71+ RBC] (10^9 /L)
Pearson r = 0.41
Pre-donation Hb (g/L)

Figure 2-7 The association between $CD71^+$ RBC concentration and Hb level in healthy blood donors

(A) Representative image for $CD71^+$ RBC in the two Hb_v groups (> 155 g / L and <140 g / L), median with interquartile range (error bar), Mann-Whitney test. (B) $CD71^+$ RBC (%) in the two Hb_v groups (> 155 g / L and <140 g / L), median with interquartile range (error bar), Mann-Whitney tests. (C) $CD71^+$ RBC concentration in the two Hb_v groups (> 155 g / L and <140 g / L) stratified by donor sex, median with interquartile range, Kruskal-Wallis test with Dunn's multiple comparison. (D) $CD71^+$ RBC concentration in the two Hb_v groups (> 155 g / L and <140 g / L) stratified by donor sex, median with interquartile range, Kruskal-Wallis test with Dunn's multiple comparison. (E) The correlation between $CD71^+$ RBC concentration and pre-donation Hb level. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not statistically significant difference. Hb_v: pre-donation venous Hb level; Hb, hemoglobin.

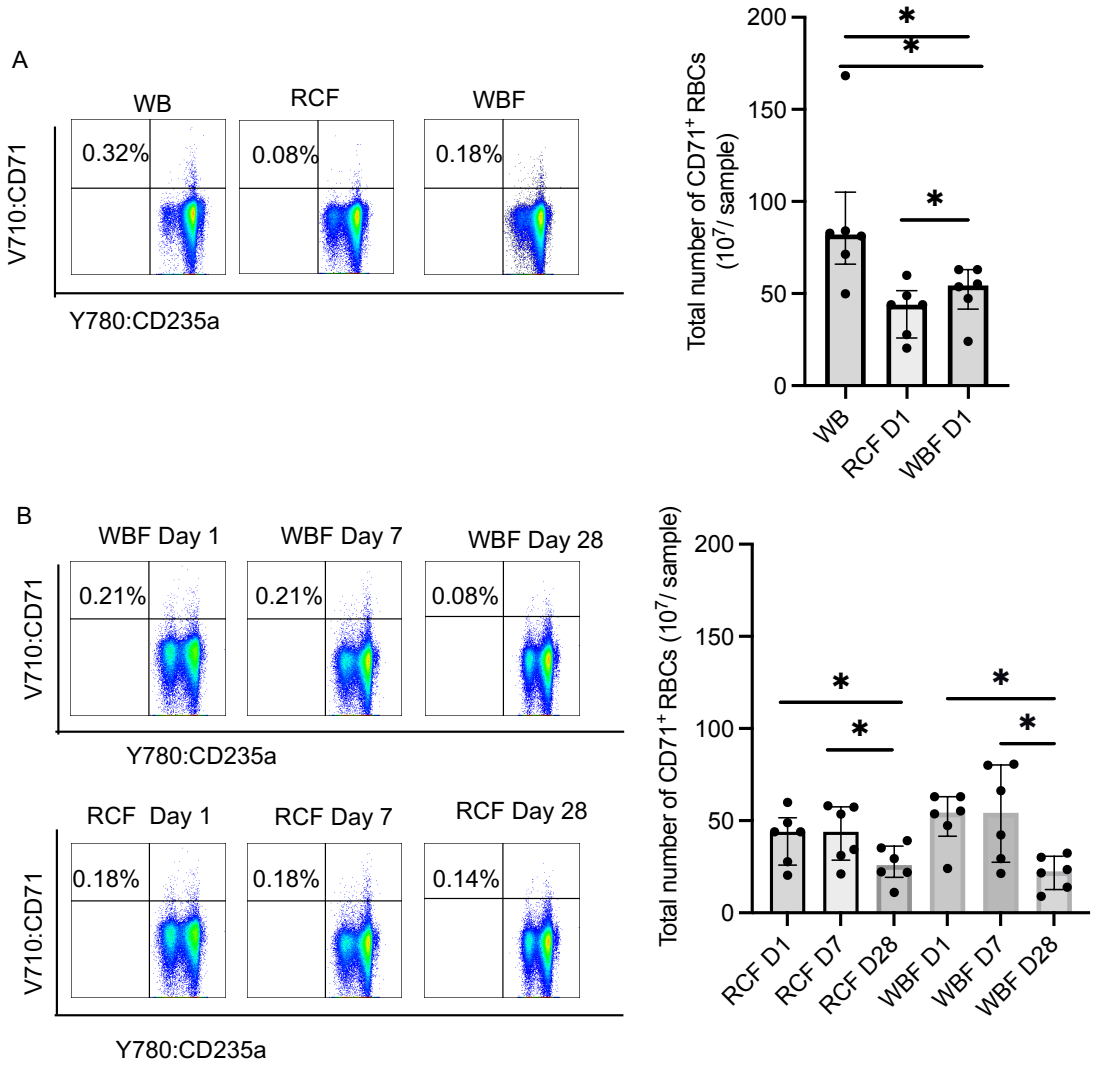

Figure 2-8 Total number of CD71$^+$ RBCs in RCF RCC samples and WBF RCC samples

(A) Representative image of CD71$^+$ RBCs and total number of CD71$^+$ RBCs in WBF RCC, RCF RCC and WB sample, median with IQR, Wilcoxon-matched pairs test. (B) Representative image of CD71$^+$ RBCs and total number of CD71$^+$ RBCs in WBF RCC and RCF RCC samples on day 1, day 7 and day 28, median with IQR, Friedman test with Dunn's multiple comparison test. *, P<0.05. RCC, red cell concentrate; RCF, red cell filtration; WBF, whole blood filtration.

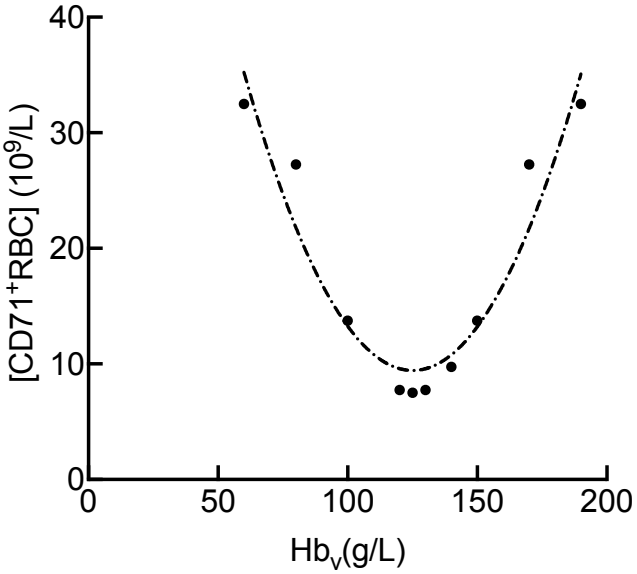

Figure 2-9 A proposed “U” shape relationship between Hb level and $CD71^+$ RBC concentration. Hb, hemoglobin.

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Chapter 3 CD71^{+} RBCs Mediate Increased Erythrophagocytosis and Reduced Monocytes in a Monocyte Suspension Assay

3.1 INTRODUCTION

Transfusion of allogeneic red blood cells (RBCs) is a critical medical intervention to improve oxygen delivery to tissues in critically ill patients. However, it can also be associated with mortality and infection in some cases [1-3], potentially sepsis, a life-threatening immune dysregulation to infection. These outcomes are believed to be linked not only to the duration of hypothermic storage and blood component manufacturing methods but also to blood donor characteristics, such as sex [4-6]. Recent studies reported that male donors were associated with an increased risk of mortality in female recipients [7-9], although there are conflicting evidences from other studies [10-12]. The sex-related difference on oxygen delivery, and coagulation has been proposed for the adverse outcomes [13]. Further research is still required to explore how blood donor sex differences might affect transfusion-related immunomodulation.

The risk of sepsis may be elevated after an RCC transfusion, as recipients may find it challenging to cope with the influx of heterogenous RBCs, particularly sourced from male donors. RCC units are derived from whole blood collections from healthy adult donors, encompassing both mature RBCs and their younger counterparts, reticulocytes. Interestingly, a higher proportion of reticulocytes was observed in male blood compared to female blood [14]. Under normal circumstances, as RBCs age or become damaged, they are cleared by macrophages, and their iron content is subsequently recycled in a process known as erythrophagocytosis [15, 16]. In mouse models, an increased RBC clearance was observed after receiving RCCs rich in reticulocytes [17]. If the rate of RBC clearance in a RCC transfused recipient exceed the iron recycling pace, there's potential for excessive iron and free Hb accumulation in the bloodstream [18, 19], which can set the stage for worsening infections or sepsis [20]. Reticulocytes predominantly consist of $CD71^+$ RBCs, with a smaller portion being $CD71^-$ RBCs [21]. As male and female exhibit varying levels of $CD71^+$ RBCs (discussed in **Chapter 2**), this donor sex difference may influence erythrophagocytosis rates, contributing to the risk of sepsis.

However, the effect of $CD71^+$ RBCs on monocyte phagocytosis and its implications in erythrophagocytosis have yet to be clearly understood. Notably, monocytes have been observed to phagocytose $CD71^+$ erythroid cells, a category that includes $CD71^+$ RBCs [22]. Among various assays, the monocyte monolayer assay (MMA) and the monocyte suspension assay stand out as effective tools for forecasting hemolysis risks linked with incompatible transfusions [23, 24]. In these assays, monocytes serve as the phagocytic cells and are exposed to opsonized RBCs. The resulting phagocytosis can be visualized and quantified using either flow cytometry or traditional microscopy. While flow cytometry excels in high-throughput analysis and data quantification, traditional microscopy provides an intricate visual of phagocytic events. However, a limitation of microscopy is its inability to discern between $CD71^+$ and $CD71^-$ RBCs during phagocytosis – a distinction made possible with fluorescence techniques. Considering the increasing use of imaging flow cytometry (IFC) for analyzing immune cell phagocytosis [25, 26], it shows promise in its potential to distinguish between $CD71^+$ and $CD71^-$RBCs during monocyte-mediated phagocytosis.

Moreover, the monocyte suspension assay presents a unique advantage; the supernatant from this assay can be analyzed to monitor Hb levels, offering further insights into the dynamics of erythrophagocytosis. In vitro experiments using the J774A.1 macrophage cell line, which was exposed to oxidized RBCs, demonstrated a pronounced erythrophagocytosis resulting in macrophage apoptosis [27]. As these macrophages undergo apoptosis, they could release Hb into the surrounding environment. Thus, a combined assessment using the phagocytic index, alongside the Hb levels in the supernatant, emerges as a potent toolset. Together, these metrics can be invaluable in delving deeper into the role and implications of $CD71^+$ RBCs in erythrophagocytosis processes.

In the context of *in vitro* erythrophagocytosis, the impact of $CD71^+$ RBCs on monocytes is intriguing. $CD71^+$ RBCs are a subset of CECs [22], which have been documented to significantly

influence the immune response, potentially due to elevated intracellular levels of reactive oxygen species (ROS) [28]. ROS are highly reactive molecules rich in oxygen, such as superoxide anion, hydrogen peroxide [29]. Furthermore, ROS can influence the immunosuppressive phenotype of macrophages, which in turn can affect their phagocytic capabilities [30, 31]. Moreover, the ROS inhibitor, apocynin, which inhibits the activity of the enzyme NADPH oxidase, counteracts the inhibitory effects of CECs observed on T cells [32]. Based on these observations, it is proposed that the ROS-rich profile of $CD71^+$ RBCs could influence how monocytes undertake erythrophagocytosis.

This work will investigate the effects of $CD71^+$ RBCs on the phagocytosis index and supernatant Hb, while also delving into the potential mechanism by which $CD71^+$ RBCs influence erythrophagocytosis through the use of ROS antioxidants. A comprehensive understanding of the influence of $CD71^+$ RBCs on erythrophagocytosis may provide insights into their potential as a contributing factor in adverse transfusion outcomes, especially in cases of donor-recipient sex mismatches.

3.2 METHODS AND MATERIALS

3.2.1 Enrichment of $CD71^+$ RBCs and $CD71^-$ RBCs

Human whole blood was collected under a protocol reviewed by both the Canadian Blood Services (CBS) Research Ethics Board (2020.005) and the University of Alberta Research Ethics Board (Pro00082512). Whole blood (WB) samples (EDTA, 50 mL) were centrifugated at 240 × g for 10 min at 20 °C to remove platelets and followed by another centrifugation to remove plasma (17 min at 20 °C). Soft spins were applied to avoid unintended activation of the platelets. The samples were then resuspended in 1 × PBS (phosphate buffered saline [pH 7.4, without Calcium or Magnesium; Cat # 1000100023, Gibco™, ThermoFisher, USA) to obtain RBC samples with a hematocrit (Hct) between 45% and 50%.

To isolate enriched $CD71^+$ RBCs and $CD71^-$ RBCs for monocyte suspension assay, the RBC samples were subjected to a Percoll density separation with a density of 1.084 g / L or 1.085 g / L (Cat # GE17-0891-01, Sigma-Aldrich, USA) (**Figure 3-1**). Briefly, a Percoll solution (3 mL) prepared from 1.5 mol / L NaCl, and deionized water was placed in separate 5-mL tubes (Cat # C353054, Falcon™, BD Biosciences, USA). RBC samples (1 mL) were layered on the Percoll solution and centrifuged at 3200 × g for 15 min at 20 °C (Eppendorf 5810R, Germany) with acceleration (Ace) and deceleration (Dce) setting of 4 and 1, respectively.

Resulting fractions were washed three times in 1 × PBS (1500 × g, 10 min, 20 °C) to remove residual Percoll solution. The less dense, younger population (containing $CD71^+$ RBCs) was further enriched by depletion of $CD45^+$ cells using CD45 microbeads (Cat #130-045-801, Miltenyi Biotec, Germany) following the manufacturer's instructions. Briefly, a young RBC population was prepared using a pre-separation filter (Cat # 130-041-407, Miltenyi Biotec, Germany) primed with MACS buffer containing PBS with 2 mmol / L EDTA (Cat # 130-091-222, AutoMACS™ rinsing solution, Miltenyi Biotec, Germany) and 0.5% bovine serum albumin (BSA, Cat # 130-091-276, Miltenyi Biotec, Germany). CD45 microbeads were added to the samples and incubated for 15 min at 2 - 8 °C. After being washed with MACS buffer and centrifuged at 300 × g for 10 min at 2 - 8 °C, the pellet was resuspended in MACS buffer at a concentration of 10^5 / mL. The cell suspension was applied to an LS separation column (Cat # 130-042-401, Miltenyi Biotec, Germany), which was attached to MACS multistrand (Cat # 130-042-303, Miltenyi Biotec, Germany). To enhance the removal of $CD45^+$ cells, a second magnetic activated cell filtration was performed using a new LS separation column. RBC concentration was adjusted to 2.3×10^7 / mL.

The older, dense population (containing $CD71^-$ RBCs) underwent leukoreduction through a filter primed with SAGM, and SAGM was added to the packed RBCs to obtain a Hct of 45% - 65% ($CD71^-$ RBCs). Both fractions were analyzed for the presence of $CD71^+$ RBCs and $CD45^+$

population using a validated flow cytometry method (**Table 3-1**). The purity of $CD71^+$ RBCs in the enriched $CD71^+$ RBC population was 32.4% ± 2.2%

3.2.2 Monocyte Suspension Assay

The human monocyte suspension assay is comprised of several steps as summarized in **Figure 3-2** and outlined in detail below.

3.2.2.1 Preparation of Monocytes

Cryopreserved PBMCs were isolated from residual buffy coats from volunteer blood donors and stored by Canadian Blood Services Diagnostic Testing program for use in their in-house erythrophagocytosis assay. The cryopreserved PBMCs were thawed in the 37 °C water bath for 2 - 3 min or until a sliver of ice remained in the tube. The thawed specimens were carefully transferred into a fresh conical tube, to which 1 mL of pre-warmed culture media (RPMI1640, [Roswell Park Memorial Institute 1640], Cat # R8755, Gibco™, Sigma-Aldrich, USA] with 10% [V / V] fetal bovine serum [FBS]) was added dropwise with gentle mixing and incubated for 30 s at room temperature. Then an additional of 9 mL of pre-warmed culture media was slowly added with gentle mixing and centrifuged 550 × g for 7 min at 20 °C (ace 9, dce 4). The supernatant was removed, and cells twice washed by adding 10 mL of pre-warmed culture media with gentle mixing to the cell pellet. The cell suspension, with a concentration of 1.5 - 2.5 × 10^6 cells / mL, was then plated in well plates (Cat # 145380, Nunc™, ThermoFisher, USA) and incubated for 12 hours at 37 °C and 5% CO_2 in a CO_2 incubator (Serial # 304806-2484, Mode 370, Steri-Cycle™ Thermo Scientific™, USA). After culture, the unbound cells were removed, and the monocytes were detached using ice-cold PBS for 3 - 5 min. The cell suspension was then centrifuged at 550 × g for 7 min at 20 °C (Ace 9, Dec 4). Ultimately, the pelleted monocytes were re-suspended to a concentration of 2.5 × 10^6 cells / mL using pre-warmed culture medium. The concentration was determined using the AOPI staining solution (Cat # CS2-0106, Viastain™, PE, USA) with an

automated cell counter (Cellaca MX, Nexcelom, USA). Viability was also assessed using this method and confirmed to be greater than 90%.

3.2.2.2 RBC Opsonisation

Opsonization of RBCs involves their coating via the attachment of antibodies and complement proteins in monocyte suspension assay. To opsonize $CD71^-$ RBCs, an opsonization mixture composed of anti-Rh D antibodies and serum was prepared in house. IgM + IgG monoclonal anti-Rh D antibodies (Cat # 5350, Novaclone, USA) were diluted with 0.2% BSA / PBS at a volume ratio of 1:11.5, with detailed titration available in Appendix B Session B.2.1 Serum, providing complement for opsonization, was separated from another donor's whole blood (clot activator, BD Vacuette®, BD, USA) by centrifugation at 1600 × g for 10 min at 20 °C. The opsonisation mixture was added to $CD71^-$ RBCs (0.35×10^9 / mL) at a volume ratio for 1 : 4 : 1.5 ($CD71^-$ RBC: diluted anti-D: serum) at 37 °C for 30 min. The ratio was referenced from Dr.Denomme Lab monocyte suspension assay (MSA) [23]. The opsonized $CD71^-$ RBCs were then washed twice (1200 × g ,10 min, 20 °C) with 0.2% BSA / PBS and resuspended in culture media to a cell concentration of 7×10^7 / mL (with 10% FBS [Cat # F4135, Gibco™, Sigma-Aldrich, USA]).

3.2.2.3 Monocyte Suspension Incubation

In an effort to mimic the cellular dynamics following a RCC transfusion, monocytes were cultured alongside RBCs at a ratio of 33 RBCs per monocyte. The culture conditions were maintained for 4 hours at 37 °C in a 5% CO_2 atmosphere, using 5-mL tubes. The RBC population consisted of non-opsonized $CD71^+$ RBCs, non-opsonized $CD71^-$ RBCs, with or without opsonized $CD71^-$ RBCs. The purpose of including opsonized $CD71^-$ RBCs was to investigate the influence of $CD71^+$ RBCs on the phagocytic activity toward $CD71^-$ RBCs, which are generally non-phagocytic unless opsonized *in vitro*. In experiments that involved opsonized $CD71^-$ RBCs, their

quantity was balanced with the combined total of non-opsonized $CD71^+$ and $CD71^-$ RBCs. Following incubation, samples were centrifuged at 1200 × g for 5 min to obtain the cell pellet for further surface and intracellular staining, and / or the supernatant for Hb measurement as needed. Control samples included negative controls (non-opsonized $CD71^-$ RBCs with monocytes, NC), positive controls (opsonized $CD71^-$ RBCs with monocyte, PC) and blank (Blk, only monocytes), set up as required.

3.2.2.4 Surface and Intracellular Staining

The cell pellet obtained after incubation was washed with PBS twice (550 × g, 5 min, 20 °C). The resuspended cells were incubated with CD14-PE-efluor 610 (Cat # 61-0149-42, BD pharmingen™, USA) at a 1:320 dilution (0.156 µg / mL) for 30 min at room temperature in the dark. Subsequently, the cells were washed twice with PBS, with each wash followed by centrifugation at 550 × g for 5 min. To fix the cells, 100 µL of fixation buffer (Cat # 88-8824-00, ThermoFisher™, USA) was added, followed by incubation on ice for 30 min. Once fixed, labelled samples were washed with PBS and centrifuged at 550 × g for 5 min to remove the supernatant. The pellet was resuspended in 200 µL of 1 × permeabilization buffer (Cat # 00-8333-56, eBioscience™, ThermoFisher™, USA) and incubated for 10 min at room temperature. Afterwards, the cells were washed with PBS and centrifuged at 550 × g for 5 min. The pellet was then resuspended in permeabilization buffer, followed by addition of antibody VioBlue-CD71 (Cat # 130-129-050, Miltenyi Biotec, Germany) at a 1:200 dilution (0.275 µg / mL) and FITC - CD235a (Cat # 559943, BD pharmingen™, USA) at a 1:320 dilution (1.56 µg / mL). Then the cell suspension were incubated in the dark at room temperature. Permeabilization buffer was added to the sample for two washes (550 × g for 5 min, 20 °C) and to resuspend the monocytes with a volume of 20 - 50 µL.

3.2.2.5 IFC Analysis

The analysis was performed using an imaging flow cytometer (Image Stream® Mark II, Amins, USA) in standard configuration, which include 405 nm, 488 nm and 640 nm lasers for excitation and a 785 nm laser for scatter signal detection, along with conventional filter set. Sample acquisition was performed using INSPIRE® software (AMINS™, USA). The instrument was calibrated daily that covered camera synchronization, spatial offset, side scatter calibration for different objectives, dark current, core stage position, retro calibration and horizonal laser calibrations. The ability to detect multiple light spectra concurrently is based on fluorescence principles, as well as the separation of emitted light spectra into specific wavelength ranges detected by dedicated channels. For example, brightfield is detected in channels 01 and 09, VioBlue in channel 07 with a 430-505 nm range, FITC in channel 02 and PE-efluor 610 in channel 04 with a 480-560 nm range, and side scatter in channel 06. Three compensation tubes, each containing a single stain (FITC, PE-eflour, VioBlue), were prepared as well as an unstained tube, all of which were prepared simultaneously with the test samples. During data collection, cell gating was based on the area and aspect ratio (size vs. circularity). A minimum of 5,000 single cells per sample were acquired for each sample.

3.2.3 Calculation of the Phagocytosis Index (PI)

Compensation matrices were generated by processing the data from single-stained cells using IDEAs software version 6.2 (AMINIS, Seattle, USA). The details for the gating strategy are summarized in **Figure 3-3**. The PI for $CD71^+$ RBC (%) and $CD71^-$ RBCs (%) were calculated as follow:

$$CD71^+RBC\ PI\ (\%) = \frac{monocytes\ that\ phagocytose\ \mathrm{CD71^+RBCs}}{total\ monocyte\ number} \times 100 \qquad \text{Eq 3-1}$$

$$RBC\ PI\ (\%) = \frac{monocytes\ that\ phagocytose\ \mathrm{CD71^+RBCs\ and\ CD71^-RBCs}}{total\ monocyte\ number} \times 100 \qquad \text{Eq 3-2}$$

3.2.4 Enumeration of Monocytes

To assess the effect of $CD71^+$ RBCs on the monocytes during erythrophagocytosis *in vitro*, the monocyte number was examined in different groups. This was done utilizing a surface staining protocol, outlined in 3.2.2.4, which involved the use of CD14, a known monocyte marker. This allowed for the identification and quantification via IFC. Considering the variety of monocytes among different runs of experiments and the potential attachment of monocytes to the tubes, the negative control, comprising of monocytes not exposed to $CD71^+$ RBCs, served as a reference point, representing a 0% reduction in erythrophagocytosis (the baseline). By comparing this baseline to the treatment group, the reduction of monocytes could be quantified in each group as follow.

$$Monocyte\ reduction\ (\%) = (1 - \frac{number\ of\ monocytes\ in\ each\ group}{number\ of\ monocytes\ in\ negative\ control}) \times 100 \quad \text{Eq 3-3}$$

3.2.5 Supernatant Hb Determination

Following the centrifugation of the incubation mixtures as described in section 3.2.2.3, the supernatant was carefully collected without disturbing the pellet. This supernatant underwent spectrophotometric analysis using the Drabkin's method for Hb determination. Given the low volume of supernatant and reduced Hb concentration, the assay was modified using a dilution factor of 1.35, allowing for a linear range from 2.2 mg /L - 100 mg / L with a detection limit of 1.0 mg / L. The SoftMax Pro Software (Molecular Devices, San Jose California, US) was programmed to automatically complete the calculation using the Lambert-Beer's law equation as follow [33]:

$$c = \frac{A_{540} \times M \times F}{\varepsilon_{540} \times l \times 1000} \quad \text{Eq 3-4}$$

Where :

- c : Concentration of Hb (g/L)
- A_{540} : Absorbance of the solution at 540 nm
- M: Molecular mass of Hb monomer = 16114.5 mg/mmol

- F: Dilution factor (1.35)
- ε_{540}: Millimolar absorptivity of HiCN at 540 nm = 11.0 cm-1·mM-1
- l : Light path (cm).

3.2.6 Determination of ROS Levels by IFC

Enriched $CD71^+$ RBCs were first incubated with PE-Cy7-CD235a (Clone GA-R2, Cat #563666, BD, USA), VioBlue - CD71, and APC -CD45 at room temperature for 30 min in the dark, followed by washing twice with PBS (550 × g, 5 min, 20 °C) and resuspended in PBS (2.3×10^7 cells / mL). Cells were then incubated at 37 °C with H_2DCF (2',7'-dichlorodihydrofluorescein diacetate; Cat # D399, Sigma-Aldrich, USA) for assessing ROS levels using an imaging flow cytometer. The analysis process followed that described in section 3.2.2.5, but using different fluorescence. To determine ROS, various channels were employed as follow: DCF was determined in channel 02 using a 488 nm laser, PE-Cy7 in channel 06 using a 488 nm laser, APC in channel 11 with a 642 nm laser, and VioBlue in channel 07 with a 405 nm laser.

3.2.7 ROS Inhibitor Treated-$CD71^+$ RBCs

ROS inhibitors, namely apocynin (ab120615, Abcam, UK; Cat # 472301, Sigma-Aldrich, USA) and Dimethyl Sulfoxide (DMSO; D8418, Sigma-Aldrich, USA) were incubated with at a Hct level of 3.3% for a 30 min at 37 °C. Apocynin was initially dissolved in DMSO and subsequently diluted in PBS to attain a final concentration of 100 µmol / L, and DMSO was further diluted in PBS to a volume ratio of 0.5%. PBS was utilized as a reference control in this setup. The chosen concentration of different ROS inhibitors was informed to maintain hemolysis below 0.8%, and it was guided by literature values [32]. Following the treatment, the cells underwent two washes with PBS (1200 × g for 5 min, 20 °C). They were then resuspended in culture media to achieve a concentration of 2.3×10^7 cells / mL, before being utilized in the monocyte suspension assay as detailed in section 3.2.2.

3.3 STATISTICAL ANALYSES

Statistical analysis was performed using GraphPad Prism software (version 9.5.1, GraphPad Software, Inc, USA). Due to small sample size, Shapiro-Wilk test was applied to assess normality. Data were presented as mean ± standard deviation or median ± interquartile range. For comparing two groups, a t test was performed if the data were normally distributed and a Mann-Whitney U test was applied for non-normally distributed data. For comparing three groups, one way ANOVA followed by Tukey's post-hoc test was utilized if normally distributed and homogeneity among groups; Kruskal-Wallis test followed by Dunn multiple comparisons test was utilized for non-normally distributed data. The level of significance was set as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$;****, $p < 0.0001$; ns, not significant.

3.4 RESULTS

3.4.1 Differentiating $CD71^+$ RBCs from $CD71^-$ RBCs Inside $CD14^+$ Monocytes

To examine whether non-opsonized $CD71^+$ RBCs were being phagocytosed, a gating strategy (**Figure 3-3**) was employed to identify $CD71^+$ RBC and $CD71^-$ RBCs inside monocytes. Initially, a dot plot was created based on area (size of cells) vs. aspect ratio (the ratio of the minor axis divided by the major axis) to identify cells of interest and exclude the doublets (**Figure 3-3A**). This was followed by a focus gate using gradient RMS (root mean square) in brightfield model to gate a clear visualization of cells (**Figure 3-3B)**. A histogram plot was then employed to identify $CD14^+$ positive cells (**Figure 3-3C**), 34.7% of focus population (**Figure 3-3B)**, 33.8% of singlets population (**Figure 3-3A**). Bivariate dot plots were then utilized to determine CD235a positive populations based on the maximum pixel intensity of CD235a and the fluorescence intensity for CD235a to exclude aggregated RBCs (**Figure 3-3D**). A histogram plot was utilized to capture internalized populations of interest (**Figure 3-3E**). Then a bivariate dot plot based on CD71 marker and CD235a marker was used to separately gate $CD71^+$ and $CD71^-$ RBCs within monocytes (**Figure 3-3F**) using negative control (**Figure 3-3G**) and positive control (**Figure 3-3H**) as

references. The representative images of the phagocytosis of $CD71^+$ RBC (**Figure 3-3I**) and $CD71^-$ RBCs are shown in **Figure 3-3J**.

3.4.2 The Effect of $CD71^+$ RBCs on Erythrophagocytosis

To examine the effect of $CD71^+$ RBCs on erythrophagocytosis, RBC PI and $CD71^+$ RBC PI were compared between $CD71^+$ RBC enriched group (non-opsonized enriched $CD71^+$ RBCs with the proportion of $CD71^+$ RBCs being 32.4% ± 2.2%) and $CD71^-$ RBC group (non-opsonized $CD71^-$ RBCs). The $CD71^+$ RBC group showed a significantly higher RBC PI (12.4% ± 2.7 %, mean ± SD) compared to the $CD71^-$ enriched RBC group (5.1% ± 1.9%; $p < 0.05$; **Figure 3-4A**). There was also a higher supernatant Hb in the $CD71^+$ RBC enriched group than the $CD71^-$ RBC group ($p < 0.05$, **Figure 3-4B).** This indicated that $CD71^+$ RBCs could boost erythrophagocytosis.

3.4.3 The Dose Effect of $CD71^+$ RBCs on the Phagocytosis Index

In order to ascertain at which level of $CD71^+$ RBCs could influence erythrophagocytosis, this study examined the effect of different proportions of $CD71^+$ RBCs on *in vitro* erythrophagocytosis. Different proportion of enriched $CD71^+$ RBCs were mixed with equal opsonized $CD71^-$ RBCs to obtain three different ratios (0.16:100, 1.6:100, 16:100), and this mixture was then incubated in the presence of monocytes. ($CD71^+$ RBCs / RBCs; 0.16:100, 1.6:100, 16:100; low, medium, high) and same number of monocytes. The lowest proportion was prepared at 0.16% to align with the proportion of $CD71^+$ RBCs in female blood **(Chapter 2)** . The RBC PI in the high group was 26.3% ± 13.8% (median ± IQR), significantly higher than the low group 13.4% ± 9.0% ($p < 0.05$; **Figure 3-5D**), but not different from the medium group (17.9% ± 5.9%, $p > 0.05$; **Figure 3-5B**, **Figure 3-5D**). The $CD71^+$ RBC PI in the high group was 6.4% ± 1.7% (mean ± SD), which was significantly higher than the low (1.5% ± 1.0%, $p < 0.0001$) and the medium group (2.4% ± 0.7%, $p < 0.0001$; **Figure 3-5E**). Additionally, there was a strong positive correlation between $CD71^+$ RBC PI and the proportion of $CD71^+$ RBCs ($R^2 = 0.77$, $p < 0.0001$; **Figure 3-5F**). This suggested a dose effect of $CD71^+$ RBC on $CD71^+$ RBC PI.

3.4.4 The Dose Effect of $CD71^+$ RBCs on Supernatant Hb

To deepen our understanding of how varying doses of $CD71^+$ RBCs affect erythrophagocytosis, supernatant Hb levels across the low, medium, and high $CD71^+$ RBC groups was measured. Supernatant Hb levels serve as an indirect indicator of erythrophagocytosis, with the source of the Hb being RBCs that have been consumed or broken down by monocytes or macrophages. We observed that supernatant Hb in the high $CD71^+$ RBC group (8.0 mg / L ± 1.8 mg / L, mean ± SD) was significantly higher than the low (3.2 mg / L ± 1.5 mg / L, $p < 0.001$), and the medium group (4.4 mg / L ± 2.1 mg / L, $p < 0.0001$; **Figure 3-6A**) respectively. There was a positive correlation between supernatant Hb ($R^2 = 0.56$, $p < 0.0001$) and $CD71^+$ RBC proportion (**Figure 3-6B**).

3.4.5 The effect of ROS Antioxidants on Monocytes and Erythrophagocytosis

To explore the potential mechanism of how $CD71^+$ RBCs affect erythrophagocytosis, both monocyte number and phagocytosis index was examined with the presence and absence of ROS antioxidants treated $CD71^+$ RBCs. The findings suggest that that ROS inhibitor treated $CD71^+$ RBCs can restore monocyte reduction and decrease $CD71^+$ RBC phagocytosis. Compared to the $CD71^-$ RBC group, there was a significant reduction (56.3 % ± 13.2 %, mean ± SD) in the $CD14^+$ monocyte number in the enriched $CD71^+$ RBC group ($p < 0.0001$; **Figure 3-7A).** A higher ROS expression was observed on the $CD71^+$ RBC population compared to the $CD71^-$ RBC population (**Figure 3-7B**) within the same sample. To further explore the effect of ROS on erythrophagocytosis, enriched $CD71^+$ RBC populations were treated with the ROS inhibitors apocynin, DMSO and PBS control. Compared to the control group (45.8 % ± 8.2 %, median ± IQR), the reduction of $CD14^+$ monocytes was partially rescued in the apocynin - treated group (37.3 % ± 4.2 %, $p < 0.05$), but not in the DMSO - treated group (42.2 % ± 17.8 %, $p > 0.05$; **Figure 3-7C**). Compared to the phagocytosis index of $CD71^+$ RBCs in control group (3.0 % ± 1.8 %, median ± IQR), a significant difference on $CD71^+$ RBC PI was observed on the apocynin-

treated group (1.4 % ± 1.0 %, $p < 0.05$; **Figure 3-7G**), but not the DMSO-treated group (1.8 % ± 1.4 %, $p > 0.05$). There was no significant difference observed in RBC PI among apocynin, DMSO and the control group ($p > 0.05$, **Figure 3-7H**).

3.5 DISCUSSION

Using a monocyte suspension assay combined with imaging flow cytometry, this study demonstrated that $CD71^+$ RBCs promoted *in vitro* erythrophagocytosis of $CD71^+$ RBCs in a dose-dependent manner. ROS inhibitors partially rescued the phagocytosis of $CD71^+$ RBCs and the $CD71^+$ RBCs - induced reduction in $CD14^+$ monocytes.

Immature RBCs, known as $CD71^+$ RBCs, display dual properties in that they can be subject to phagocytosis and also demonstrate immunomodulatory capabilities during the process of erythrophagocytosis. They can be phagocytosed without opsonization by monocytes (**Figure 3-4A**), which aligns with previous findings showing that CECs are engulfed by monocytes [22]. Additionally, both a higher RBC phagocytosis index and supernatant Hb levels in the non-opsonized $CD71^+$ RBC group was observed compared to non-opsonized $CD71^-$ RBC group (**Figure 3-4 A,B**). This may imply that monocytes prefer $CD71^+$ RBCs over $CD71^-$ RBCs. This is probably due to the high expression of ROS by $CD71^+$ RBCs (**Figure 3-7B**). When the ROS inhibitor apocynin - NADPH oxidase inhibitor – was administrated to $CD71^+$ RBCs, the phagocytosis index was found to be lower compared to control groups (**Figure 3-7G**). This suggests that specific types of ROS may play a critical role in erythrophagocytosis. However, the phagocytosis index of $CD71^+$ RBCs was relatively low in **Figure 3-7G,** and there was no significant difference on phagocytosis index of RBC between ROS inhibitor treated and control group (**Figure 3-7H).** Therefore, there may also be other factors that affect the preferential phagocytosis of $CD71^+$ RBCs, which may include their increased mechanical fragility [34] and elevated talin expression [35]. Talin, a cytoskeleton protein, has been shown to play a crucial role during $\alpha_M\beta_2$ - mediated phagocytosis [36]. $\alpha_M\beta_2$, an integrin protein found on the surface of

monocytes/macrophages, can facilitates cell-to-cell interactions [37]. Investigating the role of these factors in phagocytosis may provide further insights into the process by which $CD71^+$ RBCs are phagocytosed by monocytes.

Additionally, $CD71^+$ RBCs could induce a reduction in monocytes. The number of $CD14^+$ monocytes in the $CD71^+$ RBC group was significantly decreased compared to $CD71^-$ RBC group (**Figure 3-7A**). This reduction might be due to diminished or undetectable CD14 expression in monocytes, monocyte differentiation. This phenomenon has been found in the cases of RBCs from Gaucher patients [31]. The differentiation could be driven by higher ROS levels observed in $CD71^+$ RBCs compared to $CD71^-$ RBCs (**Figure 3-7B**). The decrease in monocyte numbers can also be caused by heme-induced programmed necrosis in monocytes [38, 39]. Since heme could be identified as ROS by using the DCF fluorescence method [40], both monocyte differentiation and monocyte programmed necrosis may explain monocyte reduction induced by $CD71^+$ RBCs with higher ROS. This aligns with the finding that using antioxidants, such as apocynin can partially restore the reduction (**Figure 3-7C).** ROS can mediate cytokine production by activating transcription factors such as nuclear factor-kappa B [41]. Therefore, it is possible that ROS produced after engulfing of $CD71^+$ RBCs are responsible for a reduction in monocytes, and that $CD71^+$ RBCs treated with certain ROS inhibitors can ameliorate this pro-oxidant effects. Therefore, while further research is reuqired to evaluate the impact of purified $CD71^+$ RBCs on monocytes by considering changes in monocytes' surface protein expression or its cytokine profiles, $CD71^+$ RBCs have an immunomodulatory function during erythrophagocytosis *in vitro* by reducing monocytes.

$CD71^+$ RBCs, in addition to higher levels of ROS level, are also marked by the presence of rRNA, which may influence the immune response in transfusion recipients. Reticulocytes are known to retain the unique ability to translate mRNA into proteins [44]. Leveraging this feature, mouse reticulocytes loaded with untranslated mRNA have been proposed as a vaccine strategy,

potentially inducing T cell responses and even anti-tumor immune reactions, highlighting the immune potential of reticulocytes [45]. In transfusion medicine, the maturation process of donated $CD71^+$ RBCs diverges from typical reticulocytes, which normally complete maturation within 24 to 48 hours post nucleus release from the bone marrow [46]. The presence of rRNA in these donated $CD71^+$ RBCs, especially when introduced to recipients, is an intriguing subject warranting further exploration.

With the immune potential of $CD71^+$ RBCs in transfusion medicine, our previous findings also revealed a differential presence of $CD71^+$ RBCs in blood products based on donor sex [42]. It's pivotal to determine the threshold variations of $CD71^+$ RBCs that might influence erythrophagocytosis *in vitro*. Our studies revealed a positive correlation between $CD71^+$ RBC phagocytic index, supernatant Hb and the proportion of $CD71^+$ RBCs (**Figure 3-5F**, **Figure 3-6B**). It has been reported higher than 10% of phagocytosis index could be considered hemolytic in certain patients [23]. This difference could also be observed between the high group with a percentage of 16% of $CD71^+$ RBCs (95% CI of median, 19.5 % - 34.4%; **Figure 3-5D**) and the low group 0.16% (10.2% - 19.6%). Despite the potential variation in phagocytosis calculation methods between the two studies, the observed difference between the groups in our study suggests that a proportionate discrepancy of 16% could have clinically significant implications for *in vivo* erythrophagocytosis. A higher supernatant Hb was also observed in high $CD71^+$ RBC, compared to low group (**Figure 3-6**A). As free Hb play a central role in the pathophysiology of sepsis, this difference may contribute to varied susceptibility to infection or sepsis. While the noteworthy difference of 16% in $CD71^+$RBCs in a real-life setting might seem unattainable, and additional *in vivo* studies to unravel the clinical implications of these $CD71^+$ RBC variations, this insight accentuates the potential role of donor sex plays in modulating infection and sepsis risk among critically ill patients.

IFC combined with monocyte suspension assay offers several advantages over the traditional monocyte monolayer assay. The MMA is a laborious assay requiring use of a microscope to examine the extent of phagocytosis and predict the potential for a patients' alloantibody to cause a hemolytic transfusion reaction. The use of IFC increases efficiency of the visualization of cell phagocytosis, and the automated analysis of a large cell population provides more robust and reliable results. However, one potential limitation to this study is that some $CD71^+$ RBCs and $CD71^-$ RBCs may be externally attached to the front or back of monocytes, leading to random false positive internalization events. Nevertheless, study have shown that the frequency of these events is consistent across all data files as it depends on cells orientation during imaging [43]. Moreover, IFC measurements of internalization rely on automated analysis of images of hundreds or thousands of cells. Conclusions were drawn from statistically significant differences between replicates of large population, rendering this method advantageous and considerably more robust despite the potential for random false events.

Another notable limitation of this study is the potential impact of donor variability on our results. Only 2 male donors met the required proportion of $CD71^+$ RBCs needed to perform these experiments. Functional differences on the $CD71^+$ RBCs among donors that are affected by donor factors, such as sex, can contribute to differences in cellular responses [44-46]. To mitigate the influence of donor variability, future studies will increase the sample size and utilize samples from a more diverse group of donors to make the study's findings more generalized.

3.6 CONCLUSION

In conclusion, this study delves into the intriguing realm of $CD71^+$ RBCs' immune potential in *in vitro* erythrophagocytosis, providing valuable insights into immature RBC interaction within immune cells and contributing to our understanding of $CD71^+$ RBCs' role in post-transfusion immunobiology. While *in vivo* studies are essential to explore the clinical implications of erythrophagocytosis differences due to donor sex on $CD71^+$ RBCs, this *in vitro study* offers a

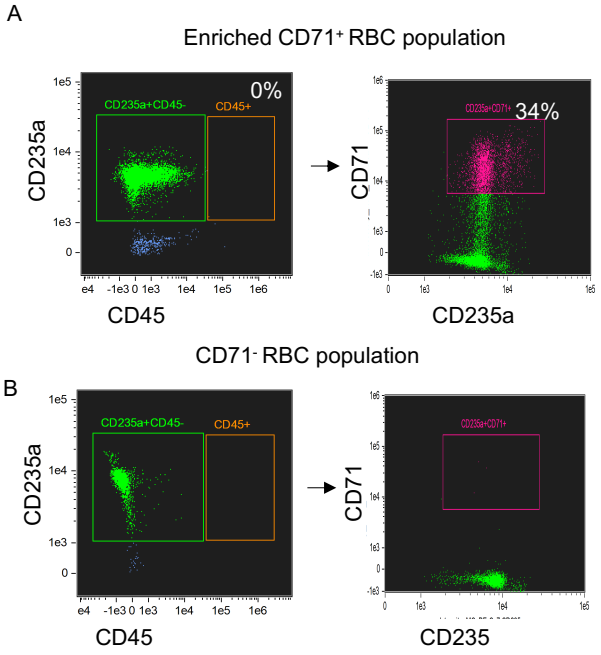
potential immunological perspective in understanding the impact of donor sex on transfusion outcomes.

3.7 TABLES/FIGURES

Table 3-1 The yield of $CD71^+$ RBCs in enriched $CD71^+$ RBC and $CD71^-$ RBC population*

	Percentage of $CD235a^+$ $CD45^-$ $CD71^+$ (n=3, mean ± SD)	**Percentage of $CD235a^-$ $CD45^+$ (n=3, mean ± SD)**
$CD71^+$ RBC population	32.4% ± 2.2%	0.02 ± 0.03%
$CD71^-$ RBC population	0.09% ± 0.06%	0.00%

* Representative image for $CD235a^+CD45^-CD71^+$ and $CD235a^-CD45^+$ in $CD71^+$RBC population (A) and $CD71^-$ RBC population (B)



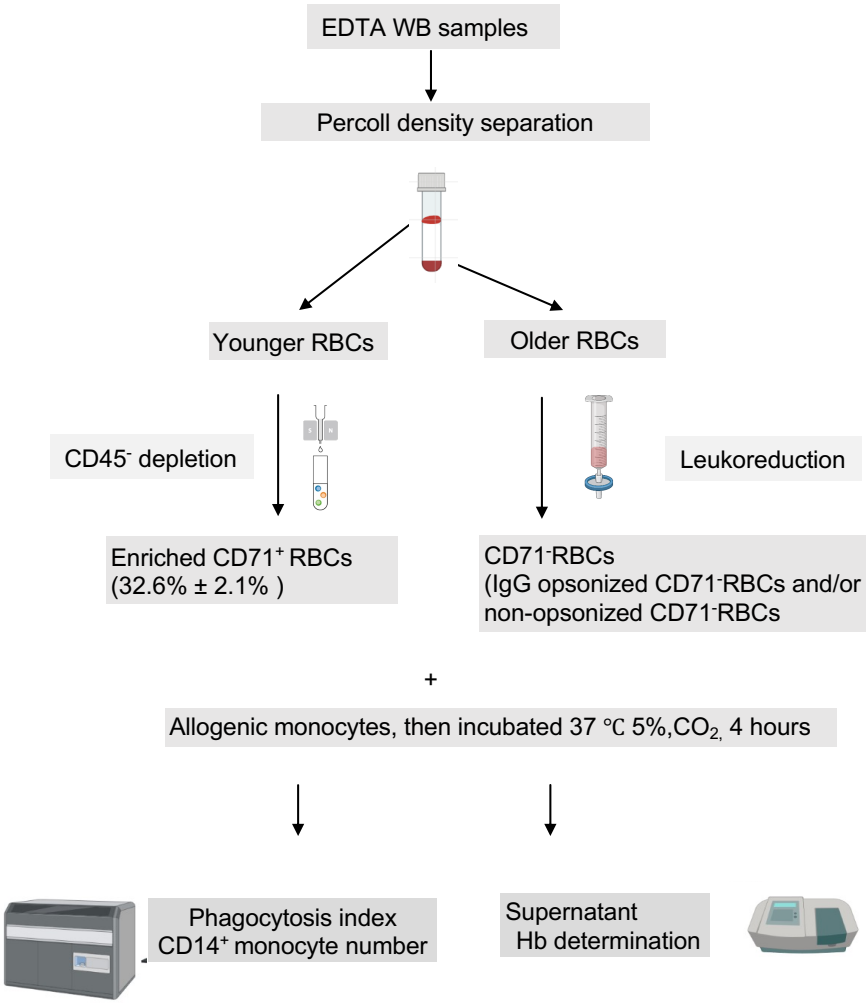


Figure 3-1 Schematic diagram of the preparation of monocyte suspension assay

EDTA: Ethylenediaminetetraacetic acid; WB whole blood.

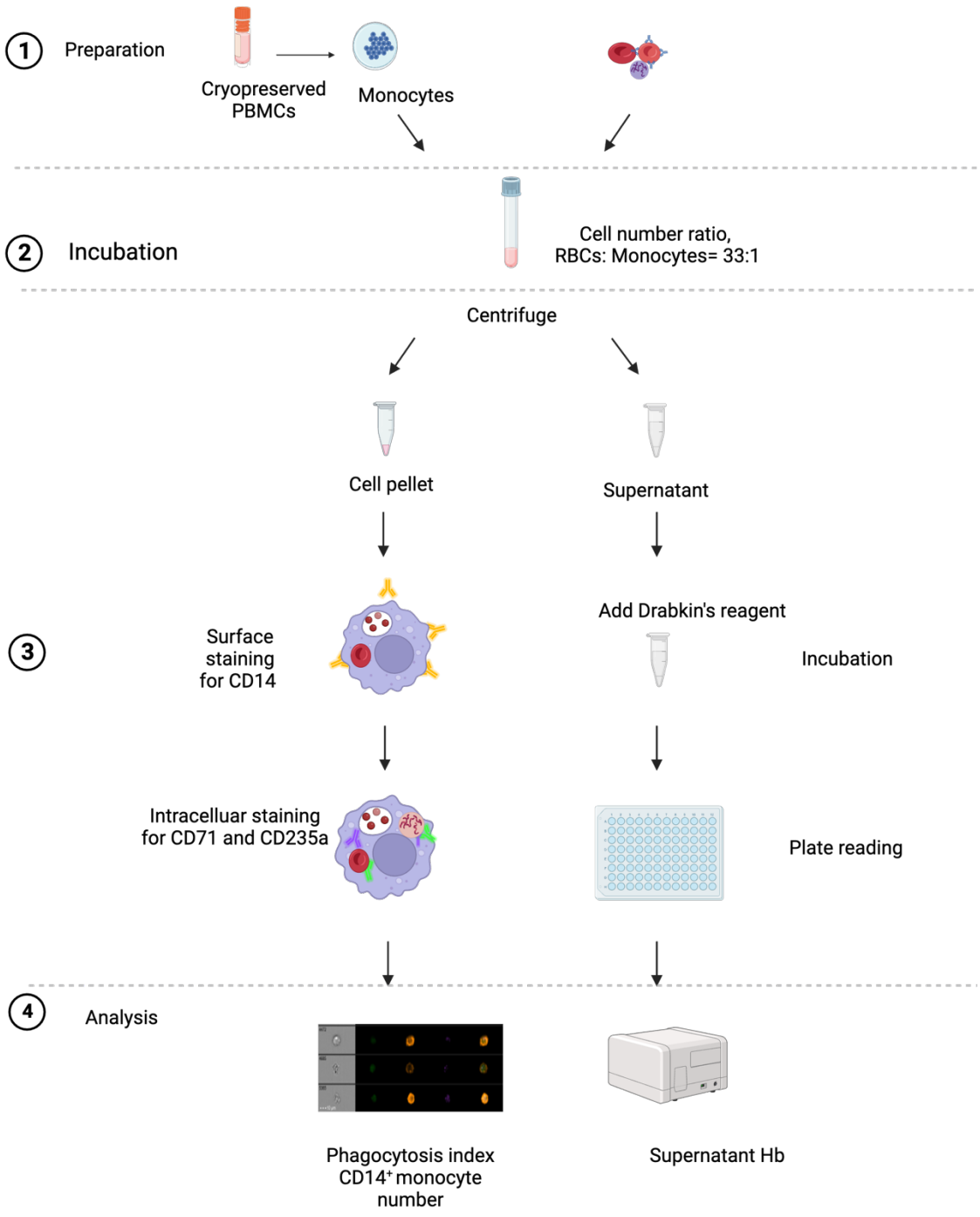


Figure 3-2 Schematic diagram of the details of monocyte suspension assay

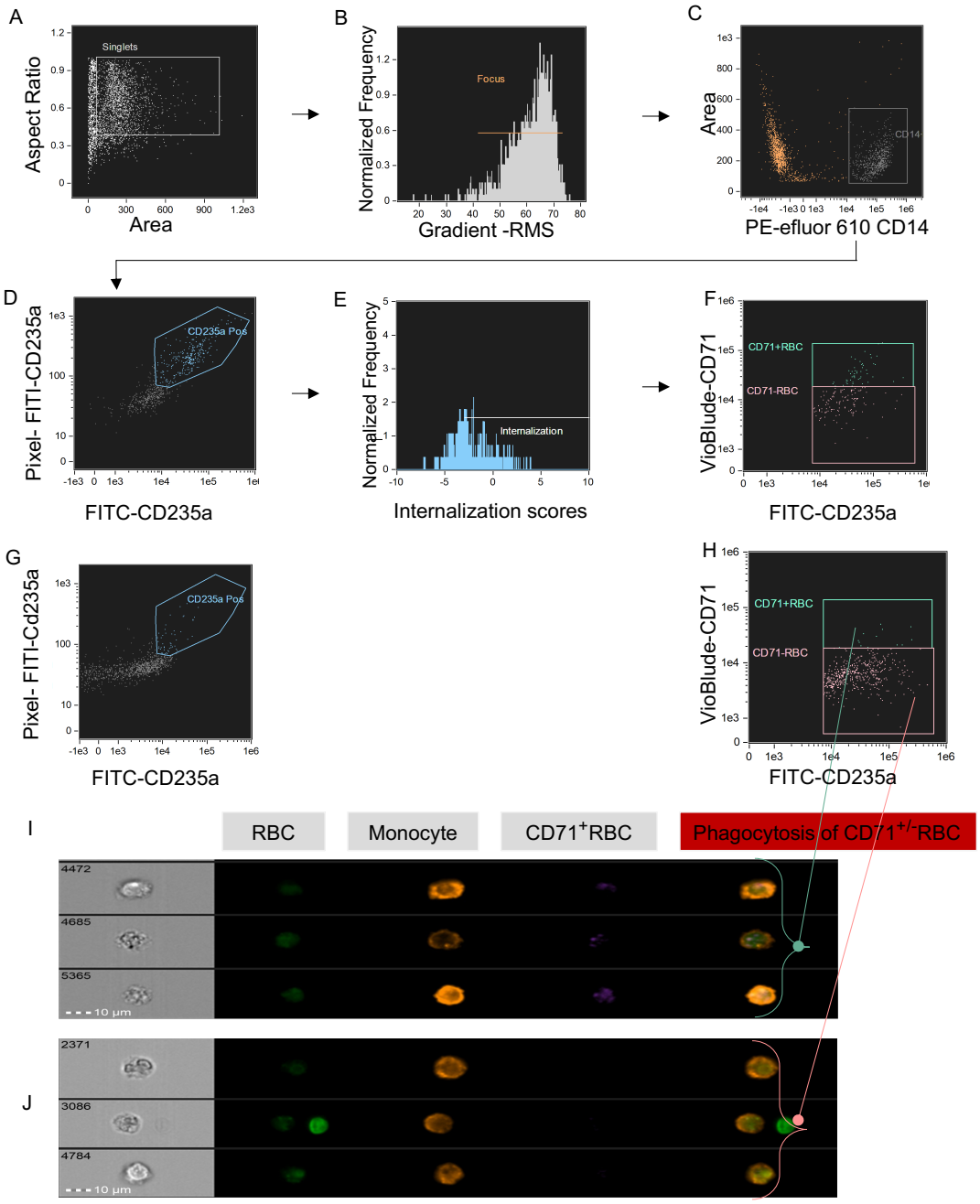


Figure 3-3 Gating strategy of phagocytosed CD71^{+} RBCs and CD71^{-} RBCs by monocytes

(A) A dot plot base on area vs. aspect ratio to gate single cells. (B) A histogram plot of the brightfield gradient root mean square (RMS) to gate focused cells. (C) A histogram plot for CD14^{+} fluorescence intensity to gate CD14 fluorescence positive population. (D) A scatter plot to gate CD235a^{+} positive populations based on the fluorescence intensity for CD235a and maximum pixel

of CD235a. (E) A histogram plot of internalization scores $CD14^+$ monocytes to capture internalized populations of interest. (F) A bivariate dot plot for CD71 and CD235a fluorescence intensity to gate $CD71^+$ RBC and $CD71^-$ RBCs within monocytes. (G) A negative control for $CD235a^+$ gating. (H) A negative control for $CD71^+$ gating, also served as positive control for CD235a gating. (I) Representative images of $CD71^+$ RBCs within monocytes. (J) Representative images of $CD71^-$ RBCs within monocytes. Negative control: non-opsonized $CD71^-$ RBCs and monocytes; positive controls opsonized $CD71^-$ RBCs and monocytes.

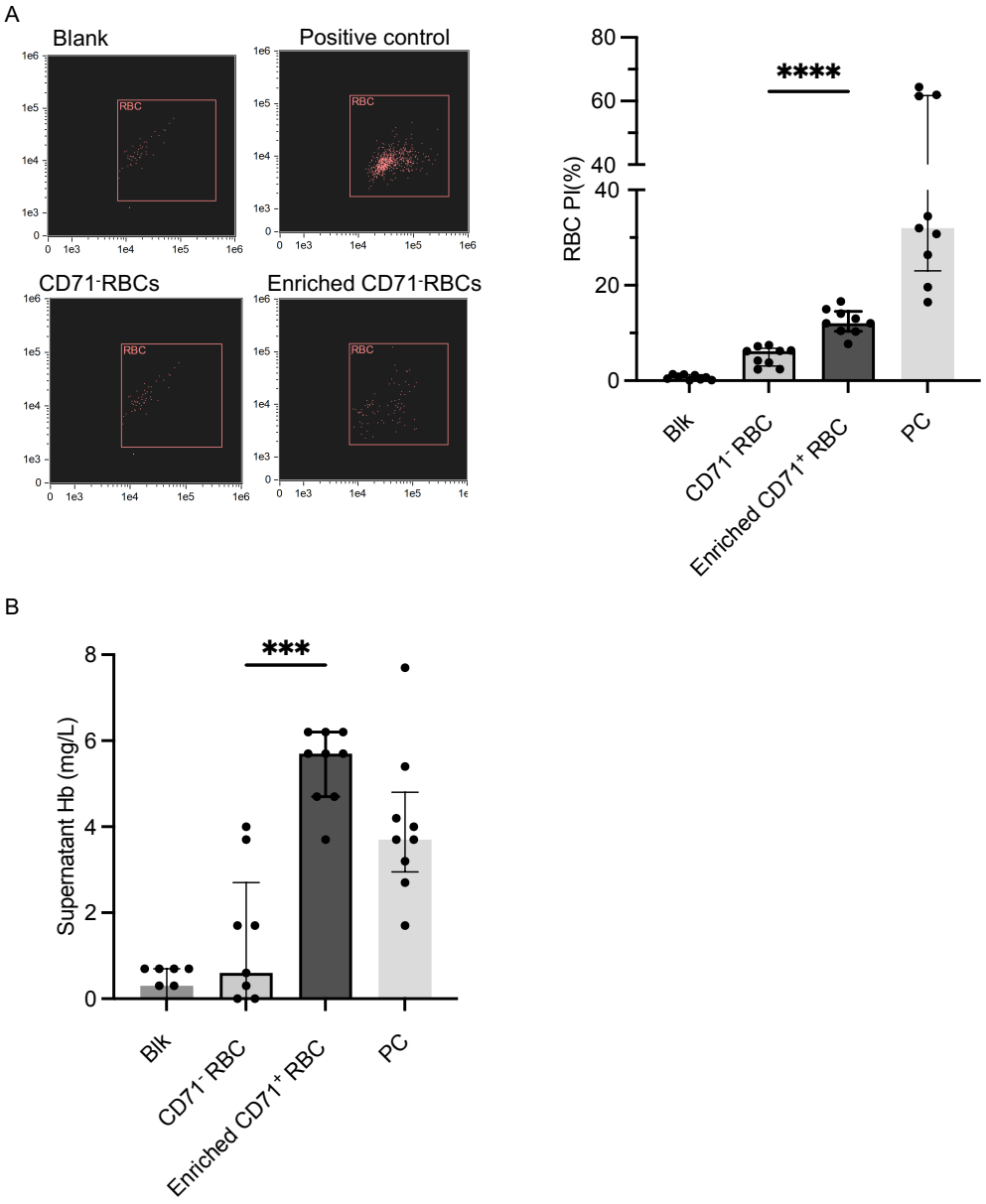


Figure 3-4 The effect of CD71$^+$ RBCs on erythrophagocytosis

Non-opsonized CD71$^-$ RBCs and enriched CD71$^+$ RBCs were incubated with allogenic monocytes, respectively. Positive controls (PC) with an equal number of opsonized CD71$^-$ RBCs and blank (Blk) containing only monocytes were prepared. (A) Representative images for CD71$^+$ RBC phagocytosis index and CD71$^-$ RBC PI in different groups, mean ± SD, unpaired t test after its normality confirmed with Shapiro-Wilk test. (B) Supernatant Hb in different groups, mean ± SD,

unpaired t test after its normality confirmed with Shapiro-Wilk test. Triplicates for each group across three experiments. ***, $p < 0.001$; ****, $p < 0.0001$.

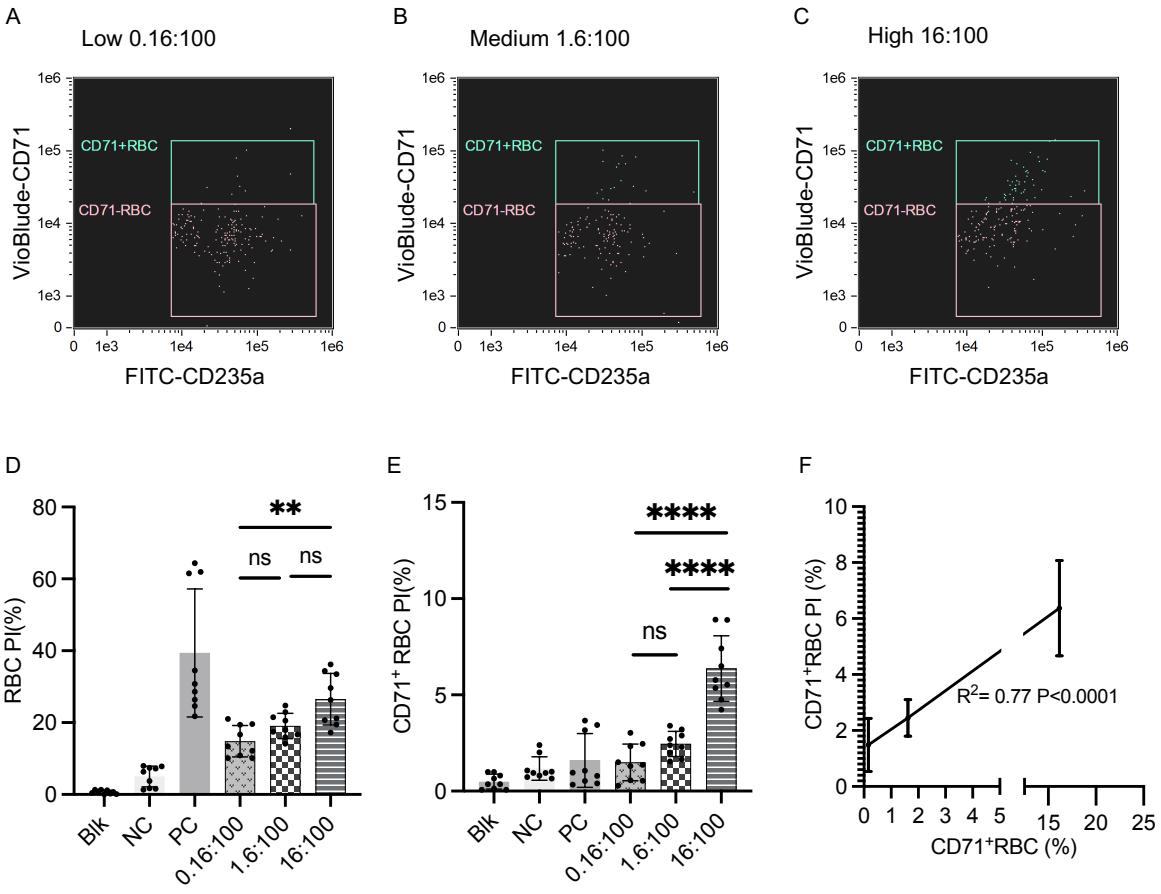


Figure 3-5 The dose effect of CD71$^+$ RBCs on the phagocytosis index

Different proportion of enriched CD71$^+$ RBCs were mixed with opsonized CD71$^-$ RBCs to obtain three different ratios (0.16:100, 1.6:100, 16:100), and this mixture was then incubated in the presence of monocytes. Negative controls (NC) with equal number of non-opsonized CD71$^-$ RBCs and monocytes, and positive controls with an equal number of opsonized CD71$^-$ RBCs (PC) and blank (Blk) containing only monocytes were prepared. (A) CD71$^+$ and CD71$^-$ gating in the low level of CD71$^+$ RBCs groups. (B) CD71$^+$ and CD71$^-$ gating in the medium level of CD71$^+$ RBCs groups. (C) CD71$^+$ and CD71$^-$ gating in the high level of CD71$^+$ RBCs groups. (D) RBC PI (%), median ± interquartile range, Kruskal-Wallis test used after its normality confirmed with Shapiro-

Wilk test and non-heterogeneity among groups confirmed with Levene's median test. (E) $CD71^+$ RBC PI (%), mean ± SD; one way ANOVA was used after its normality confirmed with Shapiro-Wilk test and equal variability of difference confirmed with Levene's median test. (F) Correlation between $CD71^+$ RBC PI (%) and $CD71^+$ RBC (%). Triplicates were performed across three experiments; **, $p < 0.01$; ****, $p < 0.0001$; ns, not significant.

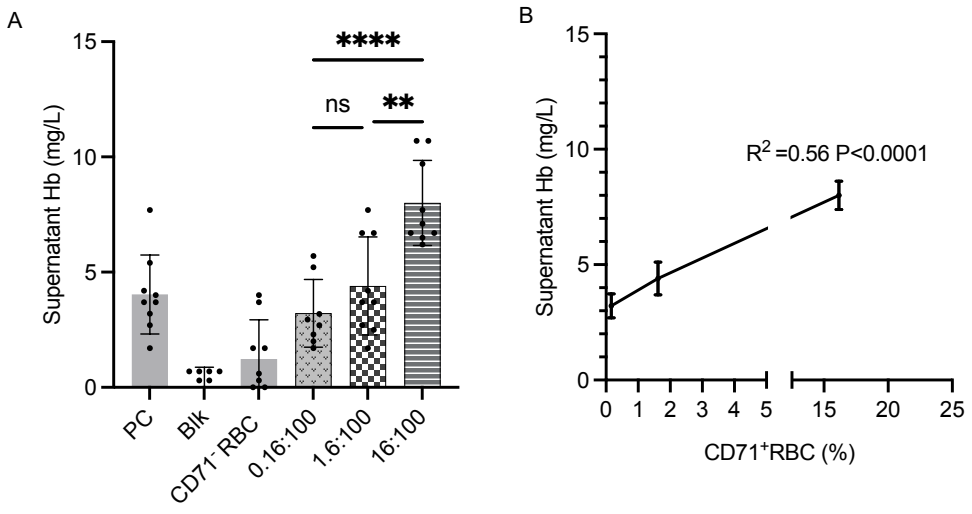
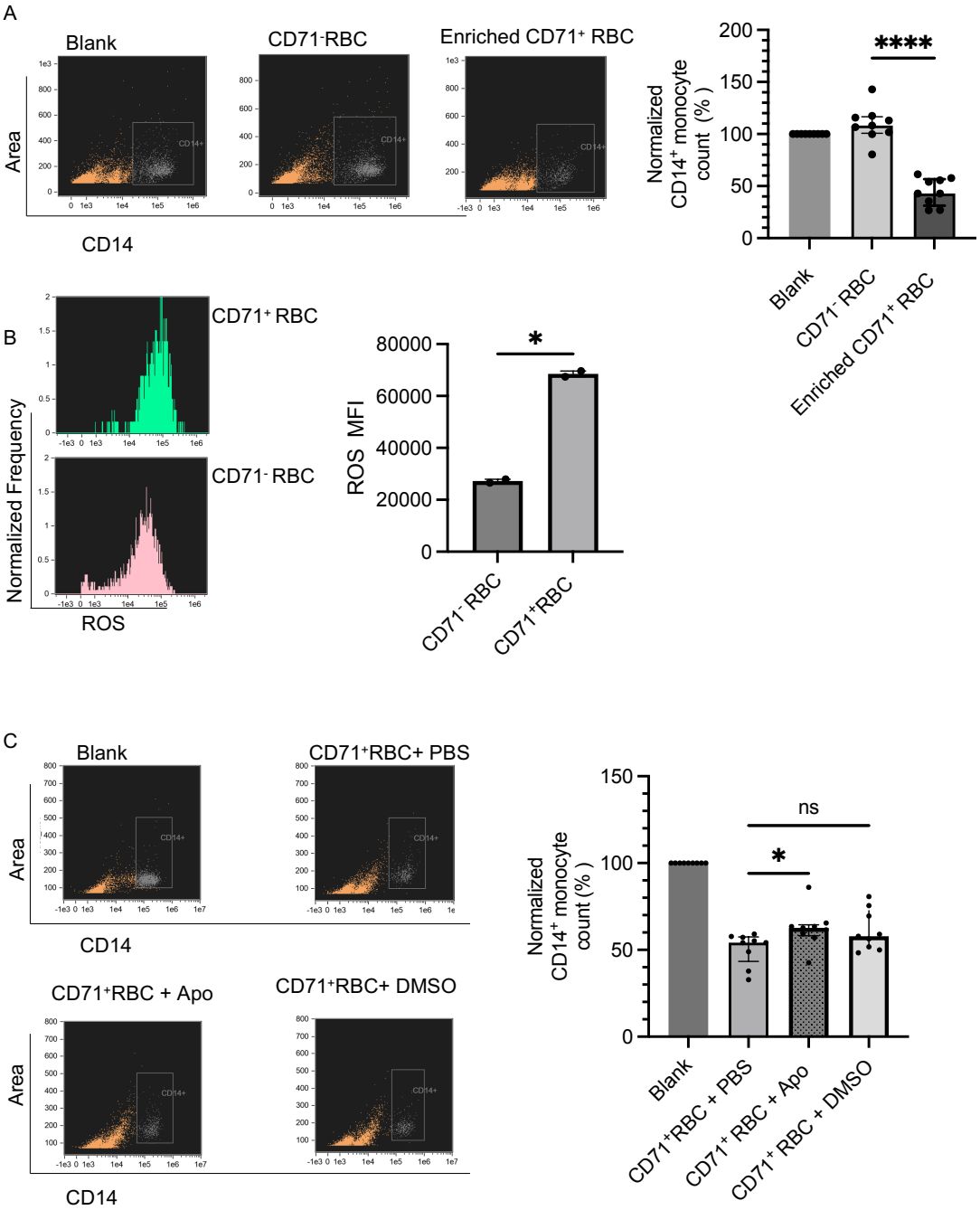


Figure 3-6 The dose effect of $CD71^+$ RBCs on the supernatant Hb

Different proportion of $CD71^+$ RBCs were mixed with opsonized $CD71^-$ RBCs in three different ratios (0.16:100, 1.6:100, 16:100), and this mixture was then incubated in the presence of monocytes. Negative control (NC) with equal number of non-opsonized $CD71^-$ RBCs and monocytes, positive control with an equal number of opsonized $CD71^-$ RBCs (PC) and blank (Blk) containing only monocytes were prepared. (A) Supernatant Hb, mean ± SD, one-way ANOVA utilized after its normality confirmed with Shapiro-Wilk test and equal variability of difference confirmed with Levene's median test. (B) Correlation between supernatant Hb and $CD71^+$ RBC (%). All groups were tested in triplicate with three experiments; **, $p < 0.01$; ****, $p < 0.0001$; ns, not significant.


A
Blank
CD71-RBC
Enriched CD71+ RBC
Area
CD14
Normalized CD14+ monocyte count (%)
****
Blank
CD71- RBC
Enriched CD71+ RBC
B
CD71+ RBC
CD71- RBC
Normalized Frequency
ROS
ROS MFI
*
CD71- RBC
CD71+RBC
C
Blank
CD71+RBC+ PBS
CD71+RBC + Apo
CD71+RBC+ DMSO
Area
CD14
Normalized CD14+ monocyte count (%)
ns
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CD71+RBC + PBS
CD71+ RBC + Apo
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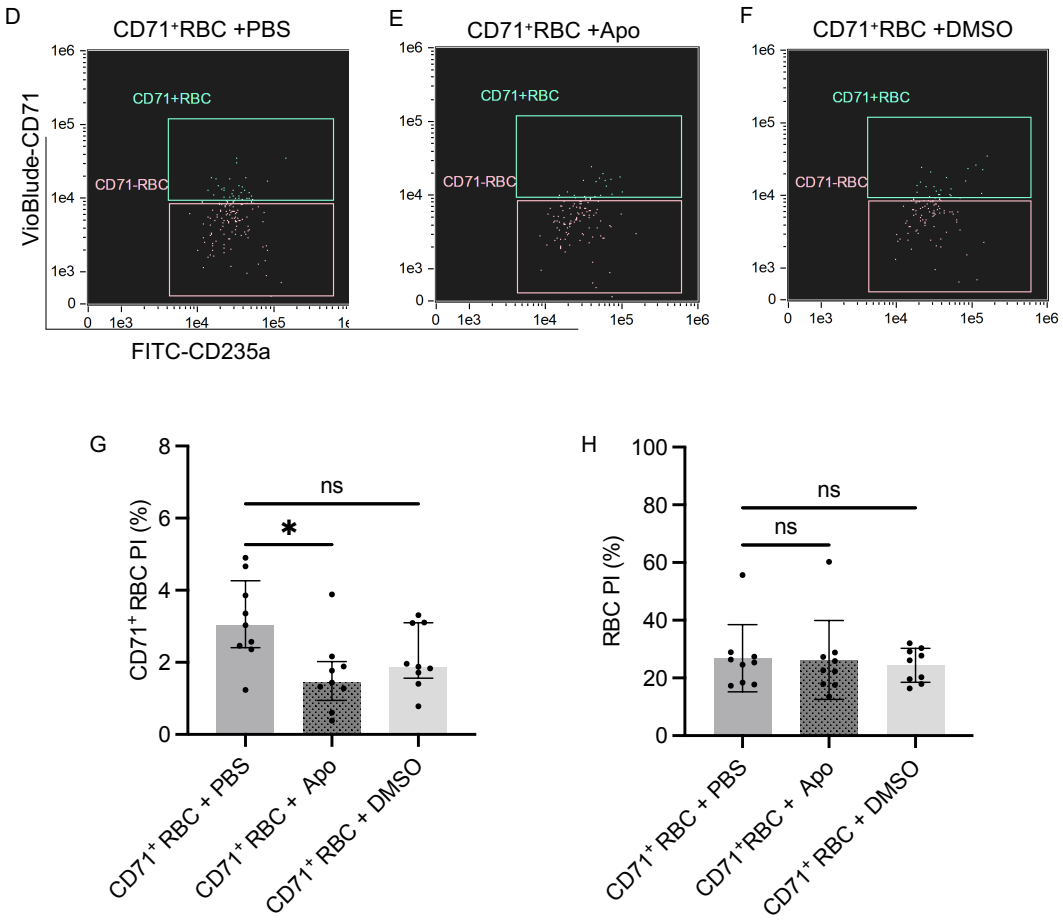

Figure 3-7 The effect of ROS inhibitors treated CD71$^+$ RBCs on the monocyte reduction and phagocytosis index

CD71$^+$ RBCs were treated with apocynin (1 mM), and 0.5% DMSO / PBS and then incubated with monocytes separately. (A) Representative image for CD14$^+$ population and CD14$^+$ monocyte number between CD71$^+$ RBC and CD71$^-$ RBC group, triplicates across three experiments, mean ± SD, unpaired t test. (B) Representative image for ROS expression and ROS median fluorescence intensity (MFI) in CD71$^-$ RBCs and CD71$^+$ RBCs, technical duplicates, mean ± SD, unpaired t test. (C) Representative image for CD14$^+$ population and CD14$^+$ monocyte number between CD71$^+$ RBC and ROS-inhibitors treated groups, triplicates across three experiments, median ± IQR, Kruskal - Wilks test followed by Dunn's multiple comparison test. (D) CD71$^+$ and

$CD71^-$ gating in the PBS-treated $CD71^+$ RBC group. (E) $CD71^+$ and $CD71^-$ gating in the apocynin-treated $CD71^+$ RBC group. (F) $CD71^+$ and $CD71^-$ gating in the DMSO-treated $CD71^+$ RBC group. (G) $CD71^+$ RBC PI (%), triplicates across three experiments; median ± IQR, Kruskal - Wilks test followed by Dunn's multiple comparison test. (H) RBC PI (%), triplicates across three experiments, median ± IQR, Kruskal - Wilks test followed by Dunn's multiple comparison test. *, $p<0.05$; ns, not significant.

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Chapter 4 The Effect of Donor and Recipient Sex on Sepsis Rates and Hb Increment Among Critically Ill Patients Receiving Red Blood Cell Transfusions

4.1 INTRODUCTION

Transfusion of RCCs is a common therapeutic approach for critically ill patients to improve anemia by evaluation of Hb level [1]. However, its potential association with an increased risk of infection and sepsis, adds a complex dimension to patient care [2-4]. Sepsis is a life-threatening condition that arises when the body's response to an infection injuries its own tissues and organs [5]. An observational study has identified blood donor sex as a contributing factor to mortality in critically ill patients [6] . Sepsis, a life-threatening condition, has been reported to significantly contribute to mortality in critically ill patients receiving RCC transfusion [6]. However, few studies have examined the effect of donor sex on hospital sepsis in critically ill patients.

In the specialized setting of intensive care units (ICU), poor Hb recovery is concerning which may increase the susceptibility to sepsis in critically ill patients receiving RCC transfusion. Critically ill patients often experience an unanticipated Hb increment, which frequently falls short of the expected 10 g / L increment following an RCC transfusion [7]. Among transfused hospitalized patients, a poor Hb increment has been reported to be associated with heightened infection rates [8]. Both suspected infection and documented infection could be the cause the of sepsis [9], and heightened infection rates suggests a higher susceptibility to sepsis. Additionally, study indicated that RCC transfusions sourced from female donors can result in a smaller Hb increase in male recipients when contrasted with those from male blood donors [10]. While in a separate study spanning from 2012 to 2019, which analyzed 32,691 single units of RCCs, the multivariate analysis indicated that the donor's sex did not significantly influence the hemoglobin increment in adult patients [11]. Therefore, the possible connection between blood donor sex, Hb increments, and its potential impact on sepsis in critically ill patients remains to be explored.

The development of comprehensive databases, such as the TRUST at the McMaster Centre for Transfusion Research (MCTR), has greatly enhanced the scope of exploratory research, paving the way for hypothesis testing. Spanning over two decades, TRUST integrates

various data sources, including electronic health records, hospitals' Laboratory Information System (LIS), administrative claims data, pharmacy records, and clinical trial data. These diverse sources amplify the depth and breadth of data available for research and analysis [12]. Among the various conditions monitored, sepsis has been meticulously tracked in TRUST since 2008, offering an unparalleled view into its prevalence and associated factors.

This comprehensive data provides invaluable insights into the intricacies of blood transfusions, especially in the context of critical care. It acts as a guiding tool, helping researchers understand the delicate balance between the urgent need for transfusion and the potential associated risks in critically ill patients. Leveraging this wealth of information, we conducted a retrospective study on adult ICU patients to understand the impact of donor sex on transfusion outcomes. We specifically examined changes in Hb levels and the onset of hospital-acquired sepsis following RCC transfusions sourced exclusively from either male or female blood donors."

4.2 METHOD AND MATERIALS

4.2.1 Study Design

This retrospective cohort study was conducted using electronic health records from TRUST and donation records from Canadian Blood Services. The Hamilton Integrated Research Ethics Board (HIREB-#14539-C), Canadian Blood Services Research Ethics Board (CBSREB # 2022.015) and University of Alberta Institutional Review Board (Pro00119217) have approved this study.

4.2.2 Recipient Population

All adults aged 18 years or older (at the time of hospital admission) admitted to the ICU at acute care hospitals within the Hamilton Health Sciences (HHS; Hamilton, Canada) and received their first single allogeneic RCC transfusion in the ICU between January 1, 2010, and December 31, 2020, were included. Patients were excluded if they received: autologous or directed donations; RCCs from a donor of unknown sex or sex recorded other than male or female (ex.

intersex); or RCCs from a blood supplier other than CBS. For the analysis focused on hospital-onset sepsis, specific exclusion criteria were employed: patients who met the criteria for community-onset sepsis, determined using sepsis International Classification of Diseases and Related Health Problems 10th Revision (ICD-10) codes (**Table 4-1**) in conjunction with a microbiology test conducted from 48 hours before admission to 48 hours post-admission. A microbiology test, commonly used to identify an infection-causing organism, was employed as a reference point, indicating that sepsis occurred in the hospital. Additionally, we did not include patients who received RCCs from both female and male donors or patients whose donor information missing. To study changes in Hb levels, we did not include certain patients in the research. These were patients who received more than one unit of RCC in the first transfusion episodes or with hema-oncology (purpura, with the exception of allergic purpura, and other hemorrhagic conditions; ICD-10 codes D691-D699); trauma (minimal residual disease [MRD] ICD-10 codes S00-T98); or hemolytic anemia (MRD ICD-10 codes D55-D59). We also left out any patients who did not have complete information about their blood donor information or their Hb levels. Patients' information was collected from TRUST, a comprehensive source of demographic, clinical transfusion, and laboratory test information on all transfusion patients within HHS. These data are updated monthly and sourced from the hospitals' Laboratory Information System (LIS) and Discharge Abstract Database (DAD). To ensure data reliability, validation studies have been conducted to confirm the accuracy of the information, data encryption, and merging process [13-15]

4.2.3 Donor Information

Information on blood donors was obtained from database of ePROGESA (MAK System International Group, Paris, France) database and provided by Canadian Blood Service. Data was collected blood at multiple CBS blood centers which supply blood components in all Canadian provinces except Québec province. All blood products were leukoreduced-depleted by pre-

storage filtration and all donors were at least 17 years old at time of donation. All RCCs transfused to eligible study patients were identified with their unique CBS unit number, ABO group and date of collection. Data collected include donor demographic characteristics (sex, age, pre-donation Hb), and blood component characteristics (ABO / Rh) for each unit from Canadian Blood Services.

4.2.4 Outcomes

The primary outcome focused on the rate of sepsis in the hospital setting (non-community) among four distinct groups (females who only received RCC units from female blood donor, males who only received RCC units from male blood donor, females who only received RCC units from male blood donor, and males who only received RCC units from female blood donor). The identification of sepsis diagnoses was based on the ICD-10 codes (**Table 4-1**), which include codes for severe sepsis and has been validated [9]. To better elucidate the relationship between the transfusion of RCCs and sepsis, we excluded cases of sepsis that had their onset in the community. Community onset sepsis was defined as a microbiology test performed between 48 hours prior to admission and 48 hours following admission in addition to sepsis determined by ICD-10 codes. Non-community onset sepsis, in contrast, is hospital-onset sepsis. The secondary outcome was Hb increment determined by Hb pre transfusion and Hb post transfusion. Hb pre transfusion was defined as the latest Hb result within 24 hours pre issue of RCC unit. Hb post transfusion was defined as the earliest Hb result within 4 -24 hours post issue of RCC unit without any other RCC unit(s) issued between issue of the RCC unit and time point of the Hb test.

4.2.5 Statistical Analysis

Statistical analyses were performed using statistical software SAS (Version 9.4, USA). Continuous variables will be reported as means with standard deviations and median with interquartile range (IQR). Categorical data were reported as proportions. Difference between study groups were assessed by Chi-squared test or Analysis of Variance (ANOVA). Further logistic regression model was conducted for hospital sepsis onset with a four-level exposure

variable (female to female, male to male, male to female, and female to male) and adjusting for additional covariates, including recipient demographics (age, most responsible diagnosis, ABO group), number of RCC unit transfused, and donor characteristics (donor age, pre-donation Hb). Odds ratio (OR) =1 exposure does not affect odds of hospital-onset sepsis, OR > 1 exposure associated with higher odds of hospital-onset sepsis, OR < 1 exposure associated with lower odds of hospital-onset sepsis. Meanwhile, a linear regression model was conducted for Hb change in the recipients with the four-level exposure (female to female, male to male, male to female, and female to male) and adjusting for additional covariates, including recipient demographics (age, most responsible diagnosis, ABO group, pre-transfusion Hb), time interval between post-transfusion Hb and transfusion, and donor characteristics (donor age, pre-donation Hb). A p value < 0.05 was considered statistically significant difference.

4.3 RESULTS

4.3.1 Study Population for Hospital-Onset Sepsis and Hb Increment Analysis

This research study encompassed a comprehensive review of 90,165 adult ICU patients from three HHS hospitals (**Figure 4-1**), out of which 25,585 patients received RCC transfusions. The 10,307 patients that received their first RCC transfusion in the ICU setting were included. Various exclusion criteria were enforced, such as patients who received RCC transfusions from both sexes (mixed donor cases, n=3,668), those with missing donor information (n=65), patients who developed community-onset sepsis (n=3,154), and patients receiving autologous (n=30) or non-CBS RCC transfusions (n=7). After the implementation of these exclusions, the study focused on a cohort of 3,410 patients who received RCC transfusions from a single-sex donor group. In this cohort, 581 females received transfusions from female donors and 906 females from male donors, while 737 males received transfusions from female donors and 1,189 males from male donors. Patient characteristics age, ABO groups and transfusion-related parameters (number of RCC unit transfused) showed no significant differences among the groups as

summarized in **Table 4-2.** There were significant difference in the most responsible diagnosis, and other transfusion (platelets, plasma, cryopreserved products), mean donor pre-donation Hb among the four groups. These factors were used for logistic analysis.

The Hb increment analysis started with a pool of 5,157 patients who received an initial transfusion of one unit of RCC with two other conditions including only one unit issued at the first transfusion of admission and no transfusion within 4 hours post the first transfusion (**Figure 4-1**). Certain patients were excluded due to various factors. This included one patient who received deglycerolized RCCs, one patient with non-CBS RCCs, 194 hema-oncology patients, 873 trauma patients, and 2 patients with hemolytic anemia. Due to missing donor information and Hb increment data, we further excluded 29 and 570 patients respectively. As a result, the final analysis consisted of 3487 patients. Patient characteristics age, recipient pre-transfusion Hb showed no significant differences among the groups as summarized in **Table 4-3**. There were significant difference in the most responsible diagnosis, and other transfusion (platelets, plasma, cryopreserved products), donor age, duration from time of Hb pre-transfusion to transfusion among the four groups. These factors were used for linear regression analysis.

4.3.2 The Impact of Single-Donor Sex Transfusion on Hospital-Onset Sepsis

Table 4-4 showed the rates of hospital-onset sepsis across the four distinct groups. Notably, there were no statistically significant differences observed in the incidence of hospital-onset sepsis among these single-sex transfusion groups. In the bivariate analysis (**Figure 4-2**), female donor was not associated with increased risk to sepsis compared to male donor, with an odds ratio (OR) of 0.92 [95% confidence interval (CI), 0.63 – 1.35; $p > 0.05$]. Interestingly, it was discernible that female recipients exhibited a higher susceptibility to sepsis compared to their male counterparts, with an OR of 1.52 (95% CI, 1.04 - 2.21;$p < 0.05$; **Figure 4-2**)

4.3.3 The Logistic Regression Model Estimating Hospital-Onset Sepsis in Critically Ill Patients Receiving RCC transfusions

The multivariate analysis revealed that the number of RCC unit transfused and the age of patients they receive are both significant predictors of hospital-onset sepsis (**Table 4-5**). In contrast, donor sex, recipient sex and whether the sex-matched or not did not significantly influence sepsis rates (**Table 4-5**). In the analysis where the number of RCC units transfused were treated as categorical data, it was found that there's a significant difference when comparing the effects of receiving just 1 RCC unit to receiving more than 5 units. Specifically, the odds of the latter have an OR of 5.5 (95% CI, 1.74-16.77; $p < 0.01$; **Table 4-5**), indicating a markedly increased effect. The effects of 3 units and 4 units are slightly less definitive, with each showing an OR of 2.19 (95% CI, 0.98 - 4.91; $p = 0.06$) and 2.58 (95% CI, 0.98 - 4.91; $p = 0.06$), respectively. Additionally, when considering it as continuous data assuming the potential equivalence among RCC units, the number of RCC units transfused has a notable influence on the likelihood of hospital-onset sepsis, demonstrated by an OR of 1.37 (95% CI, 1.14 -1.67; $p < 0.01$) (**Table 4-6).** Additionally, for every incremental year in patient age, there is a slight uptick in the risk for hospital-onset sepsis, evidenced by an OR of 1.02 (95% CI, 1.00 - 1.03; $p < 0.05$; **Table 4-5**). Furthermore, having a trauma diagnosis also heightens the likelihood of hospital-onset sepsis development, marked by an OR of 2.28 (95% CI, 1.00 - 5.19; $p < 0.05$; **Table 4-5**).

4.3.4 The Impact of Single-Sex Transfusion on Hb Increment in Critically Ill Patients Receiving RCC Transfusions

Female recipients exhibited lower average Hb increments from female donors (9.0 g / L ± 10.2 g / L, n = 654) than from male donors (10.9 g / L ± 10.3 g / L, n = 849; $p < 0.01$; **Figure 4-3A**). The median Hb increment in female recipients also mirrored this trend, showing a lower median increment from female donors than from male donors, as detailed in **Table 4-7**). In contrast, male recipients showed no significant difference in Hb increments when receiving blood from female

(6.2 g / L ± 9.7 g / L, n = 852) or male donors (6.8 g / L ± 10.0 g / L, n = 1132; p > 0.05; **Figure 4-3**A). Supporting these preliminary findings, the linear regression analysis further elucidated, in the model with main effect, recipient sex emerged as a decisive factor in Hb increments (p < 0.0001,**Table 4-8**). Concurrently, the donor's sex, although less definitive, presented a potential influence on the Hb increments (p = 0.06,**Table 4-9**). In Initial bivariate analysis, recipient receiving from male blood donor experience a Hb that was on average 1.2 g/L higher than those receiving from female blood donors(p < 0.001,**Table 4-8)**. When stratified by recipient using an interaction model analysis, male recipients' Hb increments remained unaffected by donor sex (p = 0.75, **Table 4-9**). Conversely, female recipients still experienced significant Hb increment variations contingent on the donor's sex (p = 0.007,**Table 4-9**).

4.3.5 The Linear Regression Model Estimating Hb Increment in Critically Ill Patients Receiving RCC transfusions

The multivariate analysis revealed donor pre-donation Hb, duration from pre-transfusion Hb to transfusion (the time interval between the measurement of pre-transfusion Hb levels and the execution of the actual RCC transfusion procedure), and other transfusion before RCC transfusion, recipient sex, age, and pre-transfusion Hb were significant predictors of Hb increments in the recipient (p < 0.05; **Table 4-9).** Each 10 g / L increase of blood donor pre-donation Hb resulted in a 0.3 g /L Hb increment in the recipient post-transfusion. Each 10 g / L increase of pre-transfusion Hb in the recipient resulted in a Hb decrease of 4.4 g / L in the recipient post-transfusion. Compared to female blood, male blood would result in 1.4 g/L increment in the female recipient.

4.3.6 Hb Increment in the Hospital-Onset Sepsis and Non-Sepsis Patient

To further explore the relationship between Hb increment and hospital-onset-sepsis in critical ill patients post transfusion, we analyzed the Hb increment in both hospital-onset sepsis and non-sepsis group, with further stratification by donor sex and recipient sex (**Table 4-10**). Out

of an initial 3487 patients who received a single unit of RCC (**Figure 1**), 1,097 were excluded due to patients with community onset sepsis, leaving a cohort of 2390 patients for analysis. There was not statistically difference in Hb increment between hospital-onset sepsis and non-sepsis critically ill patients (**Table 4-10**). A similar trend that Hb increment in male recipient was lower than female recipient regardless of hospital-onset sepsis status and donor sex ($p > 0.05$; **Figure 4-3B**).

4.4 DISCUSSION

In this retrospective study, the effect of donor and recipient sex on hospital-onset sepsis and Hb increment among critically ill patients were assessed and adjusted for covariates. We found there was no difference in hospital-onset sepsis in critically ill patients either receiving from male or female blood donors (**Table 4-4**) and compared with male donor, female donor are associated with a lower Hb increment in female critically ill patients, not in male patients (**Table 4-7**, **Table 4-8**). Recipient age and the number of RCC unit transfused appeared to be significant contributor to hospital-onset sepsis (**Table 4-5**). Factors influencing the rise in Hb were multi-faceted, including the donor's pre-donation Hb levels, recipient's sex and age, the primary diagnosis, and pre-transfusion Hb levels and duration from pre-transfusion Hb to transfusion, and other transfusion before RCC transfusion(**Table 4-9**). This underlines the importance of considering these factors for the optimal management of critically ill patients receiving RCC transfusions.

Our findings indicated that donor sex was not associated with hospital-onset sepsis. This is similar to a clinical trial demonstrated that observed effect of donor sex on infection might have occurred by chance [16]. Another study reported that donor sex does not significantly influence the incidence of nosocomial infection in recipient [6]. Infection or nosocomial infection was used as a subsite of sepsis since in these two studies since infection are usually the cause of sepsis [5].

More importantly, our data indicate that recipient's characteristics, particularly the number of RCC unit transfused, recipient age and responsible diagnosis, are closely correlated with the development of hospital-onset sepsis (**Table 4-5**,**Table 4-6**). In our study, critically ill patients showed an increased vulnerability to hospital-onset sepsis with each added unit of RCC transfusion, highlighting the effect of their compromised immune functions, including a potentially diminished capacity to recycle old RBCs [17, 18]. Notably, the efficiency of the phagocytic system, responsible for this recycling, reportedly declines with age [19], which could further elevate the risk for older recipients. Trauma's role as a significant predictor to hospital-onset sepsis further compounds this risk. While our data does not robustly link recipient sex to sepsis onset (**Table 4-5**), an intriguing trend emerged: female recipients had higher odds ratios than males in bivariate analysis **(Figure 4-2**). This raises questions about the interplay of RCC transfusion quantity, recipient age, sex, and other medical conditions. Further investigations are warranted to consider these relationships to examine the impact of the number of RCC units transfused on the hospital-onset sepsis in critically ill patients.

Our findings indicated that donor sex significantly influenced Hb change in female critically ill recipient but not in their male counterparts. This differs from earlier research where donor sex was believed to impact all hospitalized patients, irrespective of recipient sex [20]. Beyond different patient population, one potential explanation could be that the influence of donor sex observed in female recipients might also manifest in male recipients when both sexes are analyzed collectively. Upon further stratification by recipient sex, it was evident that the donor's sex did not significantly influence the Hb increment in male recipients. However, for female recipients, the donor's sex remained a significant determinant of Hb increment **(Table 4-9)**. The pre-donation Hb levels emerged as a significant determinant of Hb increment for all recipients in our stud (**Table 4-9**). This emphasizes the relevance of the transfused Hb volume, and by extension, the total RCC units. This perspective aligns with prior findings highlighting the critical role of the number RCC

units transfusion in the onset of hospital sepsis. Additionally, the disparity between male and female recipients may be due to their body weight, which has been identified as a contributing factor. Males represent 60% of the patient population within higher body weight ranges (80 kg-139.9 kg), and an increase in body weight has been associated with a decreased hemoglobin response to RBC transfusions [21]. Specifically, for every 20 kg increase in patient weight, there is an estimated 6.5% reduction in hemoglobin increment per RBC unit transfused, as determined by multivariate linear regression analysis [21]. These findings underscore the complex interplay between donor factors, recipient factors in determining the efficacy of blood transfusions.

In exploring the intricate dynamics between donor sex, Hb increment and hospital-onset speiss in critically ill patients, our results unveiled some interesting observations. It revealed that the average Hb increment in male recipients, either receiving from male blood donor (6.8 g/L) or female blood donor (6.2 g/L) (**Figure 4-3**A), was lower than 10.4 g/ L that reported by Roubinina et. al [20]. Furthermore, among those who received a single RCC unit, the Hb increment trend in septic male recipients mirrored those without sepsis, irrespective of the donor's sex (**Table 4-9**). And there was no different on Hb increment between sepsis and non-sepsis male recipient either from male blood donor (5.4 g / L) or female blood donor (6.2 g / L, **Figure 3B**). This suggests that hospital-onset sepsis are not associated with Hb increment in critically ill patients receiving a single RCC, which is different from finding that a poor Hb increment is associated with infection.

This study has several strengths. Firstly, it delves into the connection between the transfusion of RCCs and sepsis by focusing on hospital-onset sepsis rather than community-onset sepsis. This approach provides a clearer perspective on the onset of sepsis in relation to transfusion by leveraging microbiology test timings. As both suspected and confirmed infections can serve as potent markers for sepsis diagnosis [5] and it broadens the scope of the population under analysis. Secondly, RCC units were randomly allocated among recipients regardless of donor characteristics, such as blood donor sex and age, as is current transfusion practice,

rendering it a double-blinded study. Therefore, the chance of having systematic confounders among the study groups is minimal.

As an observational study relying on an administrative database, it carries certain constraints. Firstly, the identification of sepsis cases through ICD-10-CA codes depends on the precision of documentation and coding. The codes selected for sepsis identification in this research have been referenced in prior studies that utilized administrative data [13-15]. Martin and his team found in a validation study that these codes offer a reasonably reliable measure when used to define sepsis from administrative databases [22]. Secondly, while the timing of microbiology tests help confirm the presence of hospital-acquired sepsis, it cannot definitively determine if RCC transfusions occurred before or after the onset of sepsis. Given more comprehensive access to clinical data, we could more precisely delineate the relationship between the effect of blood donor sex and the onset of sepsis in critically ill patients.

4.5 CONCLUSION

In summary, blood donor sex was not associated with hospital-onset sepsis in critically ill patients receiving RCC transfusion. Male donor to female recipient transfusions are found to be associated with a higher Hb increment in critically ill patients. In addition, the pre-donation Hb level, rather than the donor sex, significantly affected Hb increment in critically ill patients. This factor should be considered when determining the number of RCC units to be transfused to critically ill patients, given its substantial influence on hospital-onset sepsis risk. Additionally, the age and condition of recipients play pivotal roles in influencing hospital-onset sepsis. These insights emphasize the importance of considering both donor and recipient factors in making decisions about RCC transfusion for critically ill patients to enhance the quality of care.

4.6 TABLES/ FIGURE

Table 4-1 ICD-10-CA codes used to define sepsis

ICD-10-CA	
Sepsis	Severe [&]
A039, A021, A207, A217, A227, A239, A241, A267, A280, A282, A327, A392, A393, A394, A40, A400, A401, A402, A403, A408, A409, A41, A410, A411, A412, A413, A415, A4150*, A4151*, A4152*, A4158*, A418, A4180*, A4188*, A419, A427, B007, B377, P360, P361, P362, P363, P364, P365, P368, P369, P352, P372, P375 A047 B9548 B956 B962 J189 J440 N390	R57.2 septic shock OR Sepsis codes with any of the following codes from CIHI definition Respiratory J96.0, J96.9, J80, R09.2 Cardiovascular R57.0, R57.1, R57.2, R57.8, R57.9, I95.1, I95.9 Renal N17.0, N17.1, N17.2, N17.8, N17.9 Neurological K72.0, K72.9, K76.3, F05.0, F05.9, G93.1, G93.4, G93.80 Haematological D69.5, D69.6, D65 Procedure codes 1GZ31CAND, 1GZ31CRND 1GZ31GPND

[&] In this study, instances of severe sepsis and septic shock were both categorized as sepsis.

Table 4-2 Description of participant demographics and transfusion data for hospital-onset sepsis analysis

Characteristic	Female Recipient		Male Recipient		
	Female Donor n=581	Male Donor n=903	Female Donor n=737	Male Donor n=1189	P value
Age (years), mean ± SD; median (IQR)	66.4 ± 16.1; 69(58-78)	66.3 ± 16.4; 69(58-78)	67.8 ± 14.1; 70(61-77)	66.6 ± 14.6; 68(59-77)	0.21
ABO blood group, number (%)					0.14
	1(0.2)*	0(0.0)	0(0.0)	0(0.0)	
A	246(42.3)	360(39.9)	299(40.6)	457(38.4)	
AB	20(3.4)	42(4.7)	22(3.0)	64(5.4)	
B	65(11.2)	124(13.7)	98(13.3)	135(11.4)	
O	249(42.9)	377(41.7)	318(43.1)	533(44.8)	
Most responsible diagnosis,					<0.001
Circulatory diseases	259(44.6)	392(43.4)	471(63.9)	745(62.7)	
Trauma	116(20.0)	176(19.5)	111(15.1)	193(16.2)	
Neoplasms	56(9.6)	64(7.1)	26(3.5)	55(4.6)	
Digestive diseases	40(6.9)	65(7.2)	38(5.2)	43(3.6)	
Musculoskeletal disorder	28(4.8)	47(5.2)	15(2.0)	26(2.2)	
Respiratory diseases	17(2.9)	35(3.9)	23(3.1)	40(3.4)	
Endocrine disorders	12(2.1)	20(2.2)	11(1.5)	20(1.7)	
Pregnancy and childbirth	16(2.8)	31(3.4)	0(0.0)	0(0.0)	
Unclassified signs	3(0.5)	13(1.4)	11(1.5)	19(1.6)	
Nervous system disorder	6(1.0)	19(2.1)	6(0.8)	11(0.9)	
Genitourinary disorder	9(1.5)	17(1.9)	3(0.4)	11(0.9)	
Infectious diseases	8(1.4)	1(0.1)	7(0.9)	4(0.3)	
Hematologic diseases	4(0.7)	5(0.6)	5(0.7)	5(0.4)	
Health accessibility	2(0.3)	9(1.0)	2(0.3)	3(0.3)	
Mental disorders	1(0.2)	2(0.2)	2(0.3)	8(0.7)	
Skin disorders	2(0.3)	5(0.6)	2(0.3)	4(0.3)	
Congenital disorder	2(0.3)	1(0.1)	2(0.3)	2(0.2)	
Special purposes	0(0.0)	0(0.0)	2(0.3)	0(0.0)	
Ear disorders	0(0.0)	1(0.1)	0(0.0)	0(0.0)	
Other transfusion					
Platelets	44(7.6)	98(10.9)	219(29.7)	406(34.1)	<0.0001
Plasma	50(8.6)	113(12.5)	150(20.4)	295(24.8)	<0.0001
Cryo	2(0.3)	11(1.2)	17(2.3)	49(4.1)	<0.0001
Other transfusion before RCC transfusion					
Platelets	29(5.0)	72(8.0)	205(27.8)	347(29.2)	
Plasma	37(6.4)	74(8.2)	121(16.4)	221(18.6)	
Cryo	1(0.2)	6(0.7)	9(1.2)	21(1.8)	
Number of RCC units transfused	1.5 ± 0.8; 1(1-2)	1.7 ± 0.9; 1(1-2)	1.6 ± 0.8; 1(1-2)	1.7 ± 1.0; 1(1-2)	>0.05
Exposed to donors age >50	285(49.1)	501(55.5)	359(48.7)	629(52.9)	0.02
Number of units from donors age >50					
1 unit	239(83.9)	387(77.2)	302(84.1)	472(75.0)	
2 units	44(15.4)	95(19.0)	53(14.8)	123(19.6)	
3 + units	2(0.7)	19(3.8)	4(1.1)	34(5.4)	
Exposed to donors age 18-50	418(71.9)	635(70.3)	535(72.6)	859(72.2)	0.72
Number of units from donors age 18-50					
1 unit	311(74.4)	436(68.7)	383(71.6)	565(65.8)	
2 units	87(20.8)	161(25.4)	119(22.2)	237(27.6)	

3 + units	20(4.8)	38(6.0)	33(6.2)	57(6.6)	
Donor Hb pre-donation, mean ± SD; median(IQR)	138.6 ± 8.2; 138(133-144)	152.0 ± 10.2; 152(145-158)	138.5 ± 8.5; 137(133-144)	151.9 ± 10.9; 152(145-159)	<0.0001
Recipient nadir Hb pre transfusion Hb , mean ± SD ;median(IQR)	77.3 ± 13.0; 75(70-82)	77.0 ± 13.2; 74(69-81)	77.7 ± 12.2; 75(70-82)	79.0 ± 13.9; 76(70-84)	0.0031

* Unless explicitly illustrated, the remaining data is represented as continuous data (proportions).

Cryo: cryopreserved products

Table 4-3 Description of participant demographics and transfusion data for Hb increment among critically ill patients *

	Female recipient		Male recipient		
	Female Donor n=654	**Male Donor n=849**	**Female Donor n=852**	**Male Donor n=1132**	**P value**
Age (years), mean ± SD; median(IQR)	65.9 ± 15.1; 68(58-77)	66.4 ± 15.5; 69(58-78)	68.1 ± 12.8; 70(61-77)	68.1 ± 12.5; 70(61-77)	0.04
ABO blood group					0.56
A	263(40.2)	328(38.6)	351(41.2)	474(41.9)	
AB	27(4.1)	36(4.2)	29(3.4)	55(4.9)	
B	76(11.6)	111(13.1)	116(13.6)	126(11.1)	
O	288(44.0)	374(44.1)	356(41.8)	477(42.1)	
Most responsible diagnosis					<0.0001
Circulatory diseases	294(45.0)	398(46.9)	548(64.3)	702(62.0)	
Respiratory diseases	46(7.0)	98(11.5)	63(7.4)	85(7.5)	
Digestive diseases	70(10.7)	79(9.3)	61(7.2)	73(6.4)	
Infectious diseases	66(10.1)	62(7.3)	56(6.6)	69(6.1)	
Neoplasms	57(8.7)	66(7.8)	38(4.5)	73(6.4)	
Musculoskeletal disorder	35(5.4)	38(4.5)	19(2.2)	26(2.3)	
Endocrine disorders	20(3.1)	20(2.4)	11(1.3)	28(2.5)	
Genitourinary disorder	18(2.8)	24(2.8)	15(1.8)	12(1.1)	
Nervous system disorder	10(1.5)	19(2.2)	14(1.6)	20(1.8)	
Unclassified signs factors	7(1.1)	14(1.6)	11(1.3)	17(1.5)	
Pregnancy and childbirth	12(1.8)	15(1.8)	0(0.0)	0(0.0)	
Skin disorders	5(0.8)	6(0.7)	5(0.6)	7(0.6)	
Mental disorders	2(0.3)	2(0.2)	5(0.6)	6(0.5)	
Health accessibility	5(0.8)	3(0.4)	2(0.2)	4(0.4)	
Hematologic diseases	5(0.8)	2(0.2)	1(0.1)	5(0.4)	
Congenital disorder	2(0.3)	1(0.1)	1(0.1)	3(0.3)	
Special purposes	0(0.0)	1(0.1)	2(0.2)	2(0.2)	
Ear disorders	0(0.0)	1(0.1)	0(0.0)	0(0.0)	
Other transfusion					
Platelets	77(11.8)	127(15.0)	266(31.2)	379(33.5)	<0.0001
Plasma	75(11.5)	111(13.1)	186(21.8)	291(25.7)	<0.0001
Cryo	9(1.4)	19(2.2)	29(3.4)	47(4.2)	0.0058
Other transfusion before RCC transfusion					
Platelets	40(6.1)	71(8.4)	210(24.6)	291(25.7)	<0.0001
Plasma	47(7.2)	70(8.2)	136(16.0)	206(18.2)	<0.0001
Cryo	1(0.2)	10(1.2)	16(1.9)	23(2.0)	0.04
Donor age (years), mean ± SD; median(IQR)	42.8 ± 15.2; 45(29-55)	45.6 ± 15.2; 48(32-58)	43.1 ± 15.5; 46(28-56)	44.8 ± 15.1; 47(31-57)	0.0004
Donor Hb pre-donation, mean ± SD; median(IQR)	138.6 ± 9.0; 138(131-144)	152.5 ± 12.1; 151(144-160)	138.1 ± 9.5; 136(131-144)	152.4 ± 12.0; 152(144-160)	<.0001
Recipient Hb pre- transfusion, mean ± SD; median(IQR)	77.6 ± 11.6; 75(70-82)	78.0 ± 12.6; 75(70-82)	78.7 ± 11.7; 76(71-83)	78.9 ± 12.2; 76(71-84)	0.11
Duration from Hb pre- transfusion to transfusion (hours), mean ± SD; median(IQR)	4.90 ± 3.70; 4.0(1.9-7.2)	5.00 ± 3.90; 4.2(2.0-7.2)	4.00 ± 3.60; 2.8(1.3-5.9)	3.90 ± 3.40; 2.8(1.2-5.8)	<0.0001
Hb post transfusion, mean ± SD; median(IQR)	86.6 ± 10.7; 85(80-93)	88.9 ± 11.4; 88(81-95)	84.8 ± 10.8; 84(78-91)	85.7 ± 10.7; 84(79-91)	<0.0001
Duration from transfusion to Hb post- transfusion (hours), mean ± SD; median(IQR)	11.20 ± 5.50; 10.1(6.2-15.7)	10.60 ± 5.10; 9.2(6.1-14.9)	10.60 ± 5.20; 9.0(6.2-14.3)	10.40 ± 5.20; 8.7(6.2-14.4)	0.0186

*Unless explicitly illustrated, the remaining data is represented as numerical values (proportions).

ǂ pre transfusion Hb was defined as the latest Hb result within 24 hours pre issue of RCC unit.

ǂǂ Hb post transfusion was defined as the earliest Hb result within 4 -24 hours post issue of RCC unit without any other RCC unit(s) issued between issue of the RCC unit and time of the Hb test.

Table 4-4 Association of single-sex transfusion and hospital-onset sepsis among critically ill patients

	Female Recipient		Male Recipient		P value
Variable	**Female Donor n=581**	**Male Donor n=903**	**Female Donor n=737**	**Male Donor n=1189**	
Hospital-onset sepsis, Yes # (%)	25 (4.3)	38 (4.2)	19 (2.6)	37 (3.1)	>0.05

Table 4-5 The logistic regression analysis for hospital-onset sepsis among critically ill patients*

	Model with interaction				Model with main effect			
	P value	OR	95%	CI	P value	OR	95%	CI
Recipient Age (one year older)	0.05	1.02	1.00	1.03	0.05	1.02	1.00	1.03
ABO group	0.72				0.72			
A vs. O	0.32	1.30	0.78	2.17	0.32	1.30	0.78	2.17
AB vs. O	0.88	1.09	0.37	3.22	0.90	1.07	0.36	3.16
B vs. O	0.37	1.39	0.68	2.84	0.37	1.39	0.68	2.85
Most Responsible Diagnosis	0.08				0.08			
Circulatory diseases vs. Other	0.96	0.98	0.43	2.22	0.97	0.98	0.43	2.22
Digestive diseases vs. Other	0.61	1.35	0.43	4.26	0.60	1.36	0.43	4.28
Musculoskeletal disorders vs. Other	0.54	1.54	0.39	6.12	0.55	1.53	0.39	6.05
Neoplasms vs. Other	0.25	1.86	0.65	5.33	0.25	1.86	0.65	5.31
Trauma vs. Other	0.05	2.28	1.00	5.19	0.05	2.29	1.00	5.21
Number of RCC unit transfused	0.01				0.08			
2 units vs. 1 unit	0.54	1.18	0.69	2.01	0.56	1.17	0.69	1.99
3 units vs. 1 unit	0.06	2.19	0.98	4.91	0.06	2.17	0.97	4.86
4 units vs. 1 unit	0.06	2.58	0.96	6.96	0.06	2.55	0.95	6.85
5 or more units vs. 1 unit	0.004	5.40	1.74	16.77	0.00	5.37	1.73	16.69
Other transfusion before RCC transfusion								
Platelets (yes vs no)	0.31	0.64	0.27	1.51	0.31	0.64	0.27	1.51
Plasma (yes vs no)	0.85	0.92	0.38	2.21	0.85	0.92	0.38	2.21
Mean donor pre donation Hb	0.72				0.82			
136 – 145 vs. 120-135	0.52	1.29	0.59	2.82	0.59	1.24	0.57	2.67
146-155 vs. 120-135	0.28	1.59	0.69	3.68	0.37	1.41	0.66	3.00
>155 vs. 120-135	0.31	1.61	0.65	4.01	0.42	1.38	0.63	3.04
Recipient pre transfusion nadir Hb	0.06				0.06			
0-70 vs. >85	0.47	0.75	0.34	1.64	0.47	0.75	0.34	1.64
71-75 vs. >85	0.23	1.57	0.75	3.26	0.23	1.57	0.75	3.26
76-85 vs. >85	0.16	1.67	0.82	3.39	0.15	1.68	0.83	3.40
Recipient sex and Donor sex↿								
Female: Female blood vs male blood	0.99	1.01	0.47	2.14				
Male: Female blood vs male blood	0.32	1.47	0.69	3.13				
Female recipient vs male recipient					0.53	1.17	0.72	1.93
Sex mismatch vs Sex match					0.45	1.21	0.75	1.96

* Hb level and the number of RCC unit transfused were analyzed as categorical data.

↿The interaction between donor sex; the interaction between female recipient and blood donor sex; the interaction between male recipient and blood donor sex were considered.

The model with interaction was applied to explore the potential interaction between donor sex and recipient.

Table 4-6 The logistic regression analysis for hospital-onset sepsis among critically ill patients*

	Model with interaction				Model with main effect			
	P value	**OR**	**95%**	**CI**	**P value**	**OR**	**95%**	**CI**
Recipient Age (one year older)	0.03	1.02	1.00	1.03	0.03	1.02	1.00	1.03
ABO group	0.78				0.78			
A vs. O	0.35	1.28	0.77	2.13	0.34	1.28	0.77	2.14
AB vs. O	0.82	1.13	0.39	3.32	0.85	1.11	0.38	3.25
B vs. O	0.46	1.31	0.64	2.68	0.45	1.31	0.64	2.68
Most Responsible Diagnosis	0.08				0.08			
Circulatory diseases vs. Other	0.99	1.00	0.44	2.25	0.99	1.00	0.44	2.25
Digestive diseases vs. Other	0.56	1.40	0.45	4.41	0.56	1.41	0.45	4.42
Musculoskeletal disorders vs. Other	0.47	1.66	0.42	6.51	0.49	1.62	0.41	6.37
Neoplasms vs. Other	0.21	1.95	0.69	5.55	0.21	1.94	0.68	5.53
Trauma vs. Other	0.05	2.28	1.01	5.18	0.05	2.28	1.01	5.18
Number of RCC unit transfused (one more RCC)	0.001	1.37	1.13	1.65	0.001	1.36	1.13	1.64
Other transfusion before RCC transfusion								
Platelets (yes vs no)	0.30	0.63	0.27	1.49	0.30	0.63	0.27	1.49
Plasma (yes vs no)	0.91	0.95	0.40	2.30	0.91	0.95	0.39	2.30
Mean donor pre donation Hb (per 10 g / L increase)	0.19	1.18	0.92	1.51	0.28	1.12	0.91	1.37
Recipient pre transfusion nadir Hb (per 10 g / L increase)	0.63	1.04	0.89	1.22	0.62	1.04	0.89	1.22
Recipient sex and Donor sex†	0.63							
Female: Female blood vs male blood	0.34	1.46	0.67	3.18				
Male: Female blood vs male blood	0.29	1.51	0.71	3.23				
Female recipient vs male recipient					0.57	1.15	0.70	1.89
Sex mismatch vs Sex match					0.47	1.19	0.74	1.94

* Hb level and the number of RCC unit transfused were analyzed as numerical data

†The interaction between donor sex; the interaction between female recipient and blood donor sex; the interaction between male recipient and blood donor sex were considered.

The model with interaction was applied to explore the potential interaction between donor sex and recipient.

Table 4-7 The effect of single-sex transfusion on Hb increment among critically ill patients

	Female Recipient		Male Recipient		P value
Variable	**Female Donor n=654**	**Male Donor n=849**	**Female Donor n=852**	**Male Donor n=1132**	
Hb Increment mean ± SD; median (IQR)	9.0 ± 10.2; 9 (5-15)	10.9 ± 10.3; 11 (6-17)	6.2 ± 9.7; 7 (2-12)	6.8 ± 10.0; 8 (2-13)	<0.0001

Table 4-8 Univariate analysis for Hb increment in recipient after receiving one RCC unit

	Mean (95% CI)	**Mean difference (95% CI)**	**p-value**
Univariate model			
Female received Female RCC	9.02 (8.3, 9.8)		
Female received Male RCC	10.9(10.3, 11.6)		
Male received Female RCC	6.2 (5.5, 6.8)		
Male received Male RCC	6.8 (6.2, 7.4)		
Female: male blood vs female blood		1.9 (0.9, 2.9)	0.003
Male: male blood vs female blood		0.6 (-0.6, 1.5)	0.17
Bivariate model			
Recipient: Male vs. Female		-3.6(-4.3, -2.9)	<0.0001
Donor: Male vs. Female		1.2 (0.5, 1.9),	<0.001

Table 4-9 The linear regression analysis for Hb increment among critically ill patients

	Model with interaction				Model with main effect			
	P-value	Mean Diff.	95% CI		P-value	Mean Dif.	95% CI	
Recipient Age (one year older)	<.0001	0.05	0.03	0.07	<.0001	0.05	0.031	0.075
ABO group	0.37							
A vs. O	0.11	-0.54	-1.21	0.12	0.10	-0.55	-1.22	0.12
AB vs. O	0.86	0.13	-1.38	1.64	0.88	0.11	-1.40	1.63
B vs. O	0.98	0.01	-0.99	1.02	0.95	0.03	-0.97	1.04
Most Responsible Diagnosis	0.02				0.02			
Circulatory diseases vs. Other	0.63	0.25	-0.78	1.29	0.61	0.27	-0.76	1.30
Digestive diseases vs. Other	0.19	0.93	-0.46	2.34	0.18	0.95	-0.45	2.35
Infectious diseases vs. Other	0.25	-0.84	-2.29	0.60	0.24	-0.87	-2.31	0.57
Musculoskeletal disorders vs. Other	0.60	0.56	-1.57	2.70	0.61	0.56	-1.57	2.70
Neoplasms vs. Other	0.01	1.94	0.46	3.44	0.01	1.91	0.42	3.40
Respiratory diseases vs. Other	0.13	1.08	-0.31	2.49	0.11	1.15	-0.25	2.55
Donor Age (one year older)	0.49	-0.01	-0.03	0.01	0.51	-0.01	-0.03	0.01
Donor pre-donation Hb (per 10 g / L increase)	0.03	0.31	0.03	0.59	0.03	0.31	0.03	0.59
Recipient pre transfusion Hb (per 10 g / L increase)	<.0001	-4.43	-4.69	-4.17	<.0001	-4.43	-4.69	-4.17
Duration from pre- transfusion Hb to transfusion (one more hour)	0.003	0.13	0.047	0.22	0.002	0.14	0.05	0.23
Duration from transfusion to post Hb (one more hour)	0.51	-0.02	-0.08	0.04	0.48	-0.021	-0.08	0.04
Other transfusion before RCC transfusion								
Platelets (yes vs no)	<.0001	-2.69	-3.63	-1.75	<.0001	-2.68	-3.62	-1.74
Plasma (yes vs no)	0.0004	-1.82	-2.83	-0.81	0.0004	-1.82	-2.83	-0.81
Cryo (yes vs no)	0.20	-1.68	-4.24	0.89	0.21	-1.66	-4.23	0.91
Recipient sex and Donor sex	<.0001							
Female: female blood vs male blood	0.007	-1.39	-2.40	-0.38				
Male: female blood vs male blood	0.75	-0.15	-1.07	0.77				
Female recipient vs male recipient					<.0001	2.35	1.71	2.99
Female donor vs. male donor					0.06	-0.70	-1.43	0.04

Mean Diff: Mean difference.
Duration from pre- transfusion Hb to transfusion : the time interval between the measurement of pre-transfusion Hb levels and the execution of the actual RCC transfusion procedure.
Duration from transfusion to post Hb: the time interval between the execution of the actual RCC transfusion procedure and the measurement of post-transfusion Hb levels.

Table 4-10 Hb increment in hospital-onset sepsis and non-sepsis patients stratified by donor and recipient sex

	Female Recipient		Male Recipient****	
Variable	**Female Donor**	**Male Donor**	**Female Donor**	**Male Donor**
Hospital-onset Sepsis# mean ± SD, number	8.9 ± 8.8, n=15	9.7 ± 7.6, n=26	5.8 ± 7.8, n=17	5.3 ± 11, n=31
Non-sepsis mean ± SD, number	8.9 ± 10.5 n=419	10 ± 11 n=497	5.4 ± 9.8 n=603	6.2 ± 10.6 n=782

**** $p < 0.0001$ between male and female recipient either from male or female blood donors

No significant difference among the hospital-onset sepsis and non-sepsis group

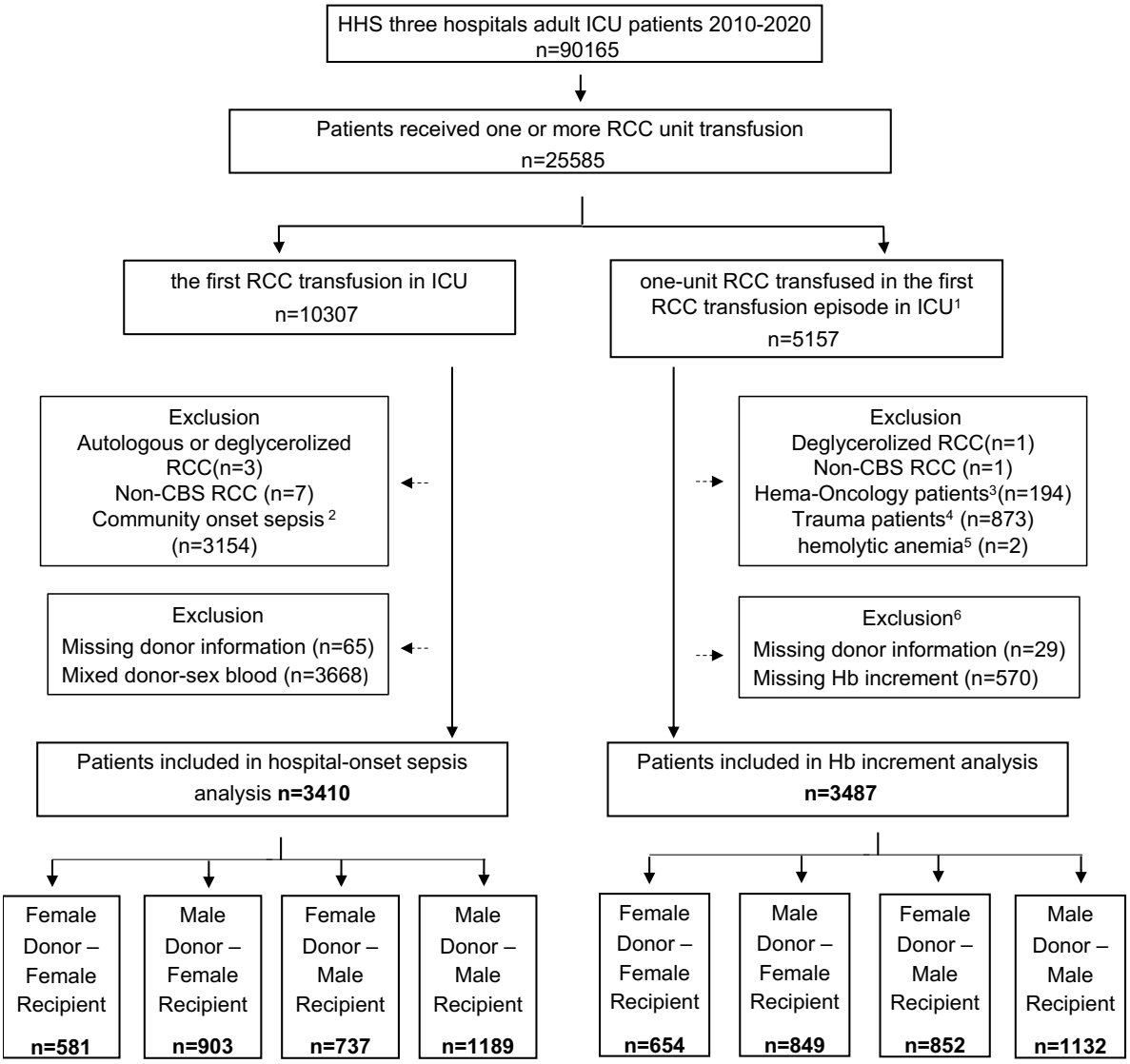

Figure 4-1 Study flow diagram illustrating the population selection process for the hospital-onset sepsis analysis and Hb increment analysis

1. Three conditions: (a) only one unit issued at the first transfusion of admission; and (b) first transfusion occurred during ICU admission; and (3) no transfusion within 4 hours post the first transfusion.
2. Community onset sepsis is defined as sepsis ICD 10 codes and had a microbiology test from 48 hours before admission to 48 hours post admission. Non-community onset sepsis (sepsis diagnosis ICD 10 codes) is hospital onset sepsis.
3. Any ICD 10 code D691- D699: Purpura (except for allergic purpura) and other hemorrhagic conditions

4. MRD ICD 10 code S00-T98: Injury, poisoning, and certain other consequences of external causes
5. MRD ICD 10 code D55-D59: Haemolytic anemia.
6. Hb pre transfusion was defined as the latest Hb result within 24 hours pre issue of RCC unit. Hb post transfusion was defined as the earliest Hb result within 4 -24 hours post issue of RCC unit without any other RCC unit(s) issued between issue of the RCC unit and time point of the Hb test.

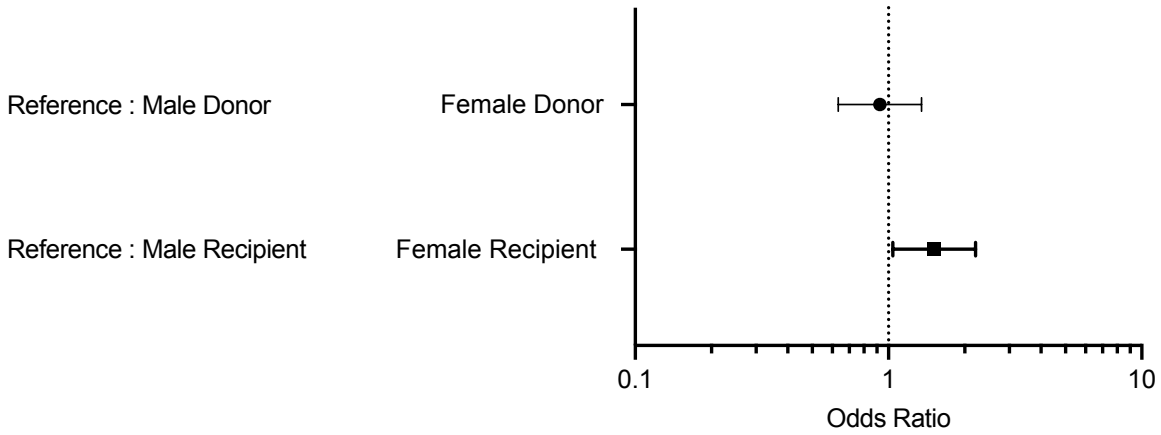

Figure 4-2 Forest plot depicting the odds ratios for the association between donor sex and hospital-onset sepsis.

Female donor was not associated with increased risk to sepsis compared to male donor, with an OR of 0.92 (95% CI, 0.63 – 1.35; $p > 0.05$). Female recipients exhibited a higher susceptibility to sepsis compared to their male counterparts, with an OR of 1.52 (95% CI, 1.04 - 2.21; $p < 0.05$). OR: odds ratios.

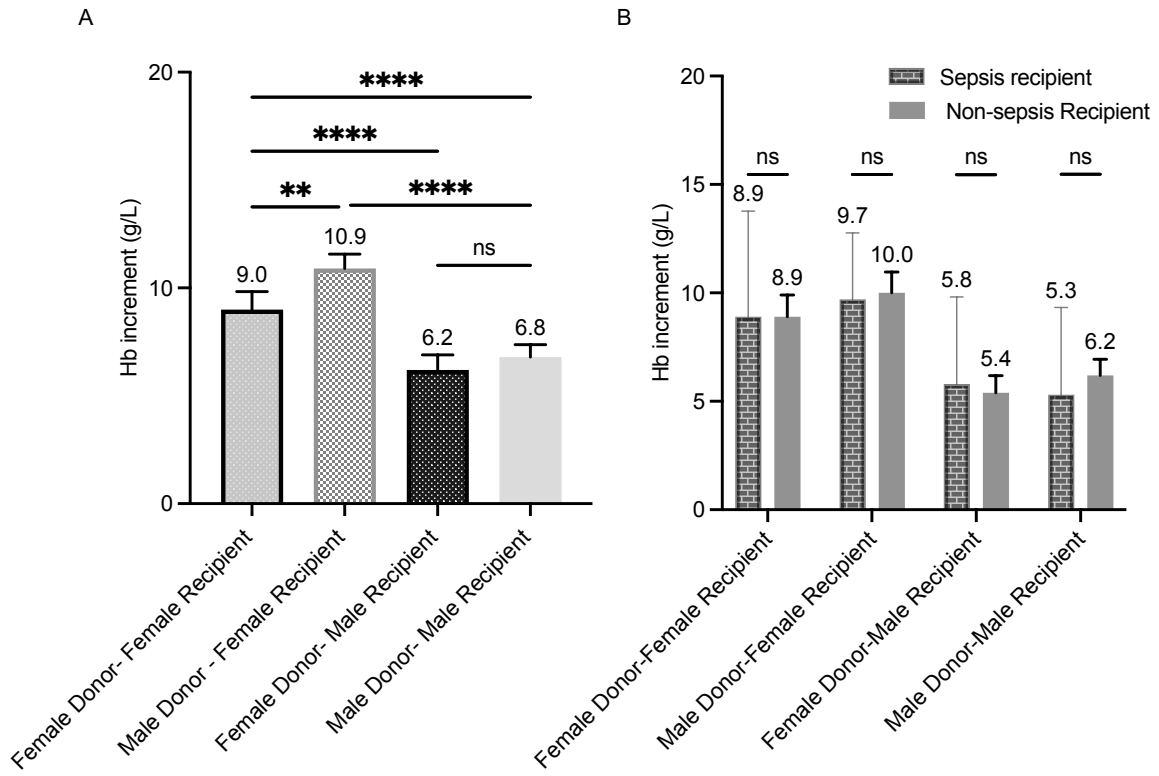

Figure 4-3 The impact of single-donor sex transfusion on Hb increment in different groups

(A) The effect of single-donor sex transfusion on Hb increment among the four groups (female-male, male-female recipient, female-male recipient, male-male recipient); mean with 95% CI (error bar), the mean value presented, one way ANOVA performed. (B) Hb increment in hospital-onset sepsis and non-sepsis patients stratified by donor and recipient sex; mean with 95% CI(error bar), the mean value presented, one way ANOVA performed. **, $p < 0.01$; ****, $p < 0.0001$, ns, not significant.

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Chapter 5 General Discussion and Conclusions

5.1 REVIEW OF THESIS OBJECTIVES AND SUMMARY OF RESULTS

The impact of donor sex on transfusion-associated infections and mortality has conflicting results. Some studies have reported the associations between donor sex and these outcomes [1, 2], while not all agree with the finding [3, 4]. An instance of this is the connection between blood donor sex and infections following RCC transfusions, which might merely be coincidental [5]. Given that infections can lead to sepsis [6], a life-threatening immune dysregulation condition and considering the vast number of RCCs transfused globally each year – over 100 millions [7], it becomes crucial to delve deeper into the potential connection between donor sex and the onset of sepsis in transfused patients in critical condition. Furthermore, exploring the potential biological mechanism behind sex differences, particularly in the context of transfusion-induced immunomodulatory outcomes, is pivotal to make sense of the varied effects of donor sex reported in literature.

Donor sex difference on RBC heterogeneity may influence erythrophagocytosis. Typically, RBCs have a lifespan of around 120 days in circulation [8], and as they age or sustain damage, they are recycled by macrophages in the liver and spleen, a process called erythrophagocytosis [9]. In a mouse transfusion models, faster RBC clearance has shown been associated with a higher proportion of donated reticulocytes [10]. In humans, studies have indicated that males generally have a higher concentration of reticulocytes than females [11]. Reticulocytes predominantly consist of $CD71^+$ RBCs, with a smaller portion being $CD71^-$ RBCs [12]. The implications of transfusing donor derived $CD71^+$ RBCs in erythrophagocytosis remain uncertain. Moreover, macrophages serve a dual function: they phagocytose RBCs and are also influenced by these cells in terms of their own functionality. For instance, in a mouse model of T-cell induced colitis, the phagocytosis of CECs by red pulp macrophages was shown to suppress the production of the inflammatory cytokine, TNF-a [13]. This points to the immunomodulatory role of CECs on macrophages. As part of CECs, $CD71^+$ RBCs might also modulate the function of monocytes and

macrophages. However, the effect of donated $CD71^+$ RBCs on transfusion outcomes, particularly from an immunological perspective, are still underexplored. As $CD71^+$ RBCs are differently present from male and female blood donors (discussed in **Chapter 1**), this donor sex difference on $CD71^+$ RBCs may influence erythrophagocytosis rates, increasing the risk of sepsis.

Understanding the role of $CD71^+$ RBCs on RBC clearance may provide an immunological mechanism for understanding the effect of donor sex on hospital-onset sepsis. Investigations in the first aim were to identify the quantity of $CD71^+$ RBCs in blood from male and female donors. The second aim of this work was to understand the role of $CD71^+$ RBCs on *in vitro* erythrophagocytosis with an attempt to analyze their dose - dependent effect on erythrophagocytosis. The last aim sought to effect of donor factors on the Hb increment and hospital-onset sepsis in critically ill patients who receive RCC transfusions. The results of the experimental and observational studies have been presented to explore the following hypothesis: **$CD71^+$ RBCs in blood products are potent immune mediators of *in vitro* erythrophagocytosis and the quantity of $CD71^+$ RBCs is influenced by donor factors which could serve as a predictor of change in post-transfusion Hb increment and hospital-onset sepsis in critically ill patients receiving red cell transfusion.**

The first aim was to examine the effect of donor factors on the quantity of $CD71^+$RBCs, which may be collectively affected by the blood component manufacturing methods and hypothermic storage duration. Using a simple and sensitive flow cytometric method (**Appendix A**), we found that male donors had a higher proportion of $CD71^+$ RBCs than female donors (**Chapter 2**). Additionally, a positive linear relationship was observed between pre-donation Hb and $CD71^+$ RBCs. Furthermore, WBF produced RCC samples had a higher total number of $CD71^+$ RBCs than RCF produced RCC samples at day 1. Fresh RCCs exhibited a higher $CD71^+$ RBC number compared to stored RCCs. Therefore, donor factors, manufacturing methods as well as

hypothermic storage duration all collectively influence the observed differences in $CD71^+$ RBCs between male and female blood derived products.

After identifying the sex-dependent differences in $CD71^+$ RBC concentration in donor products, the next aim (**Chapter 3**) assessed whether these differences could affect erythrophagocytosis and examined the threshold at which variations in the proportion of $CD71^+$ RBC impact erythrophagocytosis. A novel imaging flow cytometry-based monocyte suspension assay was developed to evaluate phagocytosis index and monocyte numbers (**Appendix B**). Furthermore, the measurement of supernatant Hb level *in vitro* as a way to determine the potential risk of hemolysis that may exist during transfusion. It was found that enriched $CD71^+$ RBCs increased the phagocytosis index and supernatant Hb level compared to control group **(Chapter 3).** The enriched $CD71^+$ RBC group exhibited a significant reduction in monocyte number compared to the $CD71^-$ RBC group. Compared to a non-treated group, the reduction of monocyte number was partially reversed in an ROS inhibitor apocynin-treated group. This suggests that $CD71^+$ RBCs may play an immunomodulatory role during *in vitro* erythrophagocytosis. To further understand how the variations of $CD71^+$ RBCs affect erythrophagocytosis, different proportions of $CD71^+$ RBCs were exposed to monocytes. Although significant differences in the phagocytosis index was only observed between the high $CD71^+$ RBC group and the low group, we found that there was a positive correlation between the proportion of $CD71^+$ RBCs and $CD71^+$ RBC PI, RBC PI and supernatant Hb. This demonstrated that $CD71^+$ RBCs promoted erythrophagocytosis in a dose-dependent manner *in vitro*. Given that the *in vitro* erythrophagocytosis setup mimics a transfusion scenario, our experimental findings hint at a potential impact of $CD71^+$ RBC proportions on transfusion outcomes in a real-world setting.

Given the findings from **Chapter 2**, which indicated that males have higher levels of $CD71^+$ RBCs compared to female donors, and their association with an increased phagocytosis index and supernatant Hb as shown in **Chapter 3**, it is plausible to speculate that blood products from

male donors may be associated with higher free Hb levels in critically ill patients. It is worth noting that a higher free Hb indicates a larger quantity of RBCs are being rapidly removed from an overwhelmed reticuloendothelial system, potentially contributing to poor Hb recovery and sepsis following transfusion. Therefore, the third aim (**Chapter 4**) was to investigate the effect of blood donor sex on Hb increment and hospital-onset sepsis in critically ill patients who receive RCC transfusion in a retrospective study. It was found that only female recipients exhibited lower Hb increments from female donors than from male donors. However, in multivariate analysis, pre-donation Hb level, instead of blood donor sex, was identified as the most significant contributor to Hb increment in both male and female recipient. Additionally, recipient sex, recipient age and pre-transfusion Hb, duration from pre-transfusion Hb to transfusion (the time interval between the measurement of pre-transfusion Hb and the execution of the actual RCC transfusion), and other transfusion before RCC transfusion, were also significant contributors to Hb increment. Similarly, blood donor sex was not associated with hospital-onset sepsis. The number of RCC units transfused, recipient age and major diagnosis (trauma) were significant contributors in the development of hospital-onset sepsis. This retrospective study demonstrated that instead of blood donor sex, donor pre-donation Hb, transfusion processing, recipient status are more significant contributors to the outcomes of the patients.

In summary, the number of RCC units to be transfused, which contributed to the risk of hospital-onset sepsis, was calculated based on the desired Hb increment for recipients, which was influenced by the blood donor pre-donation Hb level (**Chapter 4**). There exists a positive correlation between the blood pre-donation Hb level with the quantity of $CD71^+$ RBCs (**Chapter 2**), which in turn impact *in vitro* erythrophagocytosis (**Chapter 3**). Therefore, donated $CD71^+$ RBCs may be potent immune mediators in transfusion medicine and the quantity of $CD71^+$ RBCs influenced by donor factors could still serve as a predictor of transfusion outcomes in critically ill patients.

5.2 CONTRIBUTION TO SCIENCE AND FUTURE DIRECTIONS

The thesis has made a number of valuable and unique contributions to the fields of the transfusion medicine and biopreservation as summarized in at least four peer-reviewed published manuscripts. This work has made the following scientific contributions to the field:

1) This work has highlighted limitations and the challenges in quantifying $CD71^+$ RBCs in RCCs. To address this, we introduced a sensitive and precise flow cytometry method tailored for the direct measurement of $CD71^+$ RBCs in leukoreduced blood products. By bypassing the traditionally cumbersome step of manipulating WB for PBMC isolation, this approach offers a faster, more straightforward method for obtaining vital information about blood products. For medical professionals and diagnostic labs, this means potential reductions in testing times, resource usage, and the margin for procedural errors.
2) This work has also contributed to our current understanding of the impact of blood donor factors, blood component manufacturing methods and hypothermic storage duration on $CD71^+$ RBCs in blood products. We showed that there was a significant difference in the quantity of $CD71^+$ RBCs between male and female blood donors. This knowledge is important because it demonstrates that a sex difference in $CD71^+$ RBC concentration. The observation underscores the importance of considering donor sex as a factor that might influence the quality or composition of blood products. This may provide a biological mechanism to help understand the role of donor sex differences on transfusion outcomes. Furthermore, a correlation was established between pre-donation Hb level and $CD71^+$ RBCs, which is important information to help estimation of $CD71^+$ RBCs based on pre-donation Hb level. If the blood banks or hospitals need to know the concentration of $CD71^+$ RBCs for specific transfusion purposes, they might not need to perform additional tests to determine this. Instead, they can use the pre-donation Hb level, which is commonly measured, to estimate the $CD71^+$ RBCs.

3) This work provided a novel method using IFC to discern between $CD71^+$ RBC and $CD71^-$ RBC within monocytes, thereby enhancing the capabilities of the monocyte suspension assay. By leveraging the strengths of vivid visualization, high throughput, and precise cellular marker quantification, this method stands as a pivotal tool. It can illuminate the phagocytic system's intricate role in managing distinct RBC populations post-transfusion, such as the transfused $CD71^+$ RBCs versus aged RBCs. With this, it can guide researchers and clinicians in understanding the post-transfusion dynamics.
4) This work has influenced our existing theoretical understanding of $CD71^+$ RBC function and their immune potential in transfusion medicine. The monocyte reduction induced by $CD71^+$ RBCs demonstrates an immunomodulatory role for $CD71^+$ RBCs during *in vitro* erythrophagocytosis. This could revolutionize our approach to transfusions, emphasizing the importance of RBC biological age and its potential impact on recipient immunity. Additionally, $CD71^+$ RBCs promoted changes in phagocytosis index and supernatant Hb in a dose-dependent manner *in vitro* erythrophagocytosis. These findings not only deepen our insight into the intricate interactions of $CD71^+$ RBCs within the immune milieu and potentially guide optimized transfusion strategies.
5) This research offers transformative insights that challenge the traditional notions surrounding blood donor sex in the context of transfusion outcomes. By highlighting that donor pre-donation Hb is more consequential than donor sex in influencing recipient Hb increment, it underscores the need for a shift in transfusion practice paradigms. For researchers, this means that emphasis should be placed on evaluating the pre-donation Hb levels of donors rather than merely focusing on the donor's sex. It also accentuates the need for individualized, patient-centric transfusion strategies, considering the interconnected nature of various factors like the desired Hb increment, sepsis onset, and the number of transfused RCC units. Such a comprehensive approach could enhance

the safety and effectiveness of RCC transfusions, potentially improving outcomes for critically ill patients.

Given the insights elucidated in this thesis, other vital aspect that warrants exploration to have a further understanding of immune potential of $CD71^+$ RBCs in transfusion. The observed variances in $CD71^+$ RBCs, contingent upon donor sex, present themselves as potential intrinsic factors influencing erythrophagocytosis *in vitro (***Chapter 3***)*. As a logical next step, there's a compelling need to extend this research into diverse models and, where appropriate, in vivo studies, particularly when addressing the link to transfusion-associated sepsis. Sepsis is a multifaceted condition that begins with an inflammatory response but often evolves into a phase of immune suppression [14]. The immune suppression might be exacerbated by erythrophagocytosis. A study with a mouse model of transfusion showed that robust erythrophagocytosis could lead to splenic red pulp macrophages depletion [15]. Therefore, it's crucial to navigate through various research approaches to deeply understand the intricate mechanistic of erythrophagocytosis and sepsis, potentially mediated by $CD71^+$ RBCs.

First, to achieve a deeper understanding of erythrophagocytosis, there's a need for a refined monocyte suspension assay that can discriminate between different levels of erythrophagocytosis. Terms like "increased erythrophagocytosis", "stressed erythrophagocytosis" and "robust erythrophagocytosis" have been employed to describe elevated phagocytosis; however, these lack standardized definitions. Different levels of erythrophagocytosis have distinct impacts. Increased erythrophagocytosis could induce ferroptosis, an iron-dependent form of cell death, in red pulp macrophages in a mouse model [15]. Stressed erythrophagocytosis led to an immunosuppressive phenotype of macrophage after intrapulmonary *Klebsiella pneumoniae* in mice [16]. Robust erythrophagocytosis leads to macrophage apoptosis after ingestion of oxidized RBCs in J774A.1 cell line [17]. Therefore, one possible perspective is based on the monocyte status or monocyte / macrophage phenotype. Given that monocytes can be categorized into

classical, intermediate, and non-classical types, each expressing different types of cytokines and cell surface marker [18]. When polarized, monocytes may exhibit M1 or M2 characteristics, corresponding to pro-inflammatory cytokine and anti-inflammatory cytokines, respectively [19]. Therefore, for a more nuanced understanding of *in vitro* erythrophagocytosis, future research should set specific benchmarks for these different levels of erythrophagocytosis. These benchmarks should encompass diverse factors, from the phagocytosis index to supernatant Hb, monocyte number and monocyte phenotype, cytokine production. Such comprehensive parameters will likely enhance our grasp of *in vitro* erythrophagocytosis and might lead to more accurate estimations of *in vivo* erythrophagocytosis.

Gaining insight into the functionality of the phagocytic system in critically ill patients is paramount when exploring the relationship between RCC transfusion and the onset of hospital sepsis. Our research indicates that the risk escalates by approximately 2.19 times when patients receive 3 units as opposed to a single unit (**Chapter 4**). This statistic underscores the need for thorough patient assessments, especially given that instances of stressed erythrophagocytosis can lead to an immunosuppressive response, as seen with intrapulmonary *Klebsiella pneumoniae* exposure in mice[16]. Hence, gauging a critically ill patient's ability to manage a surge of transfused RCCs is crucial. A predictive model, taking into account factors like the patient's age, sex, Hb level, and primary diagnosis, might provide valuable insights into their phagocytic system's capacity. Such an understanding could empower medical professionals to reduce or even avoid certain transfusions, potentially mitigating the risk of sepsis in these vulnerable individuals.

Thirdly, the influence of $CD71^+$ RBCs extends beyond just their impact on the mononuclear phagocytic system. $CD71^+$ RBCs have been demonstrated to interact with a variety of cell types, such as lymphocytes and endothelial cells. For instance, a study revealed that, in comparison to controls using only a medium, cultured reticulocytes can enhance T cell proliferation [20]. Delving

into the specifics, the provenance of $CD71^+$ RBCs seems to have bearings on their functionality [21]. This necessitates an experiment where purified donor derived $CD71^+$ RBCs are incubated with T cells, initially to observe T cell proliferation and subsequently to deeply analyze the resultant T cell phenotype. The nuances of T cell subtypes merit particular attention here. While Th1 cells mediate the inflammatory response against intracellular against intracellular pathogens, Th2 cells combat extracellular organisms and play roles in allergic responses are the vanguard against extracellular threats and are pivotal in allergic responses[22]. Not to be overlooked, Tregs also play a pivotal role in maintaining immune system homeostasis by suppressing overactive immune responses, preventing autoimmune diseases, and ensuring self-tolerance [22]. Given these distinct functions, determining what type of T cells that $CD71^+$ RBCs might induce is vital, which represents the immune role of $CD71^+$ RBCs. Furthermore, A subsequent avenue of inquiry is the comparative analysis of $CD71^+$ RBCs with their $CD71^-$ counterparts regarding T cell interaction. Setting $CD71^-$ RBCs as the control and examining the resulting and T cell proliferation and T cell phenotype can offer invaluable insights into the impact of RBC subtypes on immune responses. The ultimate goal of this comprehensive exploration is to deepen our understanding of the immune potential inherent in $CD71^+$ RBCs.

With this immune potential of $CD71^+$ RBCs addressed above; an in vivo transfusion model would be a pivotal approach to assess their functional impact on transfusion outcomes. By labeling donated $CD71^+$ RBCs, one can effectively gauge RBC turnover and analyze the dynamics of phagocytosis after transfusion. Factors such as phagocytosis indices in the spleen, the production of various cytokines in the blood, susceptibility to infections, and subsequent mortality rates would provide invaluable insights. Such comprehensive evaluations would deepen our understanding of the role $CD71^+$ RBCs play in transfusion outcomes.

Additionally, to further explore the biological mechanism of donor sex -related on adverse transfusion outcomes, an intriguing direction for future research in transfusion outcomes revolves

around the inherent physiological and genetic differences between male and female blood donors. Beyond the mere quantity of $CD71^+$ RBCs (**Chapter 2**), the genetic landscape set by the differing sex chromosomes—XX for females and XY for males—may play a significant role. In females, X-inactivation largely silences one of the two X chromosomes, preventing gene overexpression [23]. Yet, some genes on the X chromosome escape this inactivation, potentially resulting in varied expression between sexes [24]. Intriguingly, although $CD71^+$ RBCs have lost their nucleus during maturation, the extent to which residual genetic or epigenetic factors might still influence the cell's function remains a mystery. Additionally, the role of sex hormones in modulating the characteristics of $CD71^+$ RBCs cannot be overlooked. As we delve deeper into these nuances, understanding the residual influence, if any, of the erstwhile nucleus on $CD71^+$ RBC function could be invaluable for advancing the field of transfusion medicine

In examining the immune potential of $CD71^+$ RBCs in transfusion recipients, it is crucial to consider both the transfused and endogenous populations of these cells. Many recipients, including those with anemia, sepsis, and neonates themselves, also exhibit elevated levels of $CD71^+$RBCs and / or CECs, which may influence their susceptibility to infections. Clinical observations have suggested a link between these cells and an increased susceptibility to infections. For instance, a study involving 112 critically ill adult septic patients, identified an elevated count of $CD235a^+CD71^+$ and $CD235a^+CD71^+CD45^+$ cells had been associated with a higher incidence of nosocomial infections [25]. The elevation was found to be associated with increased inflammatory cytokine levels of IL-6 and IFN-γ and decreased anti-inflammatory level of IL-10 [25]. Although the interaction between the cytokine and CECs in septic patients still need further study, it suggests that CECs could modulate their immune response. With the immune potential of transfused $CD71^+$ RBCs, investigating how transfused $CD71^+$ RBCs and recipient-derived CECs interact with immune system could enhance our understanding of transfusion-related immunological responses in vulnerable patient populations.

In conclusion, this thesis work has significantly contributed to our scientific understanding of $CD71^+$ RBCs in blood products, providing valuable insights into understanding the immune potential mechanisms by which blood donor factors affect transfusion-associated sepsis. While the clinical implication of the observed differences caused by blood donor factors needs further study, this work clearly highlight that both donor and recipient factors still need to be considered in efforts to optimize the transfusion practice of critically ill patients. The methods and knowledge presented in this study serve as a solid foundation for recognizing and comprehending the continuing significance of $CD71^+$ RBCs in transfusion medicine.

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Appendix A‡ Development and Validation of a Sensitive Flow Cytometric Method for Determining $CD71^+$ RBCs in Blood Products

‡ A version of this appendix has been published as Li, W. and Acker, J.P. Development, and validation of a sensitive flow cytometric method for determining CEC in RBC products. Clinica Chimica Acta. 2022; 530: 119-125, 2022

A.1 INTRODUCTION

$CD71^+$ RBCs, a subgroup of $CD71^+$ erythroid cells with immunomodulatory functions[1-3], are present in blood products and can circulate in the transfusion recipients, potentially influencing transfusion outcomes by exerting immune-suppressive properties. However, $CD71^+$ RBCs are rare and fragile compared to mature RBCs, which makes determination of the $CD71^+$ RBCs in whole blood (WB) samples and leukoreduced red cells concentrates (RCCs) difficult. Therefore, a robust method is needed to quantify $CD71^+$ RBCs in RCCs to investigate their potential immunomodulatory role in transfusion outcomes.

Current flow cytometric methods need to be optimized for quantifying $CD71^+$ RBCs in RCCs. Density gradient centrifugation to obtain enriched $CD71^+$ RBCs in PBMCs from WB is used in currently described methods [4, 5]. However, in leukoreduced RCCs the buffy coat and plasma are removed and there are limited PBMCs available for $CD71^+$ RBC determination. Additionally, these methods typically use lymphocyte separation media which has not been optimized for $CD71^+$ RBC separation. Manipulation of WB may reduce the accuracy for determination of $CD71^+$ RBCs due to their rarity and fragility. Alternatively, non-manipulated WB could be assessed directly, which has been used to determine rare cell populations at a frequency lower than 1 / 1,000 [6]. The application of counting beads (ie. BD Trucount™) to determine cell concentration can be expensive, particularly when large numbers of samples are being analyzed. Therefore, we aimed to develop a simple, sensitive, and cost-effective method for determining the proportion of $CD71^+$ RBCs in WB.

We selected cellular surface markers, CD71, CD235 and CD45 to define $CD71^+$ RBCs using flow cytometry. The expression of CD71 (transferrin receptor) increases before the proerythroblast development stage, decreases as the nucleus disappear, and is ultimately absent in mature RBCs [7, 8]. CD235a (glycophorin A) is abundantly expressed in erythroblast and mature RBC membranes [2], whereas CD45 is not present on the $CD71^+$ RBCs since this is

expressed principally on nucleated hematopoietic cells [9]. The processes and analysis used to determine the proportion of $CD71^+$ RBCs in WB will be discussed in this study. The analytical performance of the whole blood, flow cytometric method will be evaluated by examining its limit of detection, linearity, and inter- and intra-assay precision.

A.2 MATERIALS AND METHOD

A.2.1 Reagents and Equipment

BV711 anti-human CD71 (Clone L01.1, Cat #745418, Becton Dickinson, USA), PE-Cy7 anti-human CD235a (Clone GA-R2, Cat #563666, BD,USA), APC anti-human CD45 (Clone HI30, Cat #560973, BD, USA) were chosen based on prior studies [3] and the BD Horizon™ Guided Panel Solution tool. Anti-Mouse Ig, K / Negative control compensation particle set (Cat #552843, BD, USA) was used to optimize the three fluorescence compensation settings. Phosphate buffered saline (PBS) with 2 mM EDTA and 0.5% bovine serum albumin (BSA), at a pH of 7.2 (Cat #130-091-276, Miltenyi Biotec, Germany) was used to stabilize the $CD71^+$ RBCs. Flow cytometer (LSRFortessa X-20, BD, USA) was calibrated weekly by flow core facility staff using BD™ Cytometer Setup and Tracking (CS&T) beads with BD FACSDiva™ software 9.0. The LSRFortessa was configured with 3 lasers (Red 633 nm, Violet 405 nm, Yellow 561 nm) and the following filters were used: 670 / 30 nm, 710 / 50 nm, 780 / 60 nm respectively to measure the fluorescence of APC, BV711, PE-Cy7. High, medium, low-level controls were performed daily on a hematology analyzer (DxH520, Beckman Coulter, USA) to determine RBC and WBC)concentrations.

A.2.2 Sample Collection

Access to human blood was reviewed by both Canadian Blood Services (CBS) Research Ethics Board (2020.005 and 2020.049) and the University of Alberta Research Ethics Board (Pro00082512). Cord blood units (CBU, anticoagulant citrate-phosphate-dextrose) were obtained from the CBS national cord blood bank. All CBUs were stored at 1 to 6 °C. Whole blood

(anticoagulant EDTA) was collected from healthy volunteers using standard phlebotomy procedures. Residual buffy coats from processing of whole blood were obtained from CBS. While WB was used to develop the method, CBU was used during the validation as it allowed for the acquisition of a greater number of $CD71^+$ RBCs than from WB. To enrich $CD71^+$ RBCs , the peripheral blood mononuclear cells (PMBCs) in CBU were isolated using Ficoll-Paque^{TM} PLUS (Cat #17-1440-03, Cytiva, USA). We adapted the isolation steps from the manufacture's standard protocol (Cytiva) as follows:1) cord blood was diluted 1:4 with PBS and then layered over the Ficoll-Paque^{TM} PLUS followed by centrifugation at 400 × g for 35 min without break to obtain a "PBMC layer"; 2) the "PBMC layer" was mixed with PBS and centrifuged at 400 × g for 10 min to get a PBMC pellet; and 3) the PBMC pellet was resuspended in PBS with 2mM EDTA and 0.5% BSA (PBS PLUS) to a WBC concentration range of 5×10^6 - 10×10^6 cells /mL

A.2.3 Sample Preparation for Method Validation

A.2.3.1 $CD71^+$ RBC Labelling for Linearity and Limit of Detection

The proportion of $CD71^+$ RBCs in a sample was calculated as the number of $CD71^+$ RBC events detected on the flow cytometer divided by the number of intact RBCs and WBC gated events in a sample and expressed as a percentage. As an internal calibration bead was not used, the concentration of $CD71^+$ RBCs in an individual sample could be calculated using the percentage of $CD71^+$ RBCs and the concentration of WBC and / or RBC determined using the hematology analyzer, assuming that all the WBC and RBC are captured by flow cytometry. To prepare a sample with a high percentage of labelled $CD71^+$ RBCs (1.26%), CBU PBMC were spiked into unlabelled WB at a volume ratio of 153:1. The ratio was calculated based on the number of $CD71^+$ RBCs, WBC, and RBC in the PBMC and the number of RBC in WB. In the CBU PBMC, both RBC and WBC count were considered when determining the concentration of $CD71^+$ RBCs as their concentrations were similar (around 10^6 / mL). In contrast, in WB samples, only the RBC count was used as the RBC concentration was 3 logs higher than the WBC concentration.

The $CD71^+$ RBCs in PBMC were labelled by incubating with PE-Cy7 anti-human CD235a, APC anti -human CD45, BV711 anti-human CD71 for 30 min in the dark at 18 – 25 °C. The labelled CBU PBMC were centrifuged for 3 min at 400 × g to remove the supernatant and were re-suspended with PBS PLUS to obtain a CBU PBMC suspension ([WBC] 5 $\times 10^6$ to 1 × 10^7 / mL). This sample spiked with 1.26% labelled $CD71^+$ RBC was 2-fold serially diluted with 10 µL unlabeled WB to obtain 9 labelled $CD71^+$ RBC samples for the linearity evaluation. Triplicates were prepared for the 9 samples which had a final target proportion of $CD71^+$ RBCs of 1.26% to 0.005%. This approach was deliberately chosen to mimic the real biological context, where both $CD71^+$ and $CD71^-$RBCs co-exist. Six buffer cocktail controls were prepared with PBS PLUS and antibodies to calculate the limit of blank (LOB).

A.2.3.2 Inter- and Intra-Assay Precision

For the intra-assay precision (repeatability) assessment, freshly isolated CBU PBMC (day 4 of storage) were directly spiked into WB at the following ratios: 4.25:1, 8.5:1, 42.5:1 to obtain a low (0.06%), medium (0.12%) and high (0.6%) $CD71^+$ RBC sample. To determine the proportion of $CD71^+$ RBCs in WB, three FMO (Fluorescence Minus One) controls containing 85 µL of diluted WB, two of the target antibodies and 5 µL of PBS Plus were prepared. WB (5 µL) was added to 570 µL buffer to obtain unstained controls. These samples were kept in the dark for 30 min at 18 - 25 °C. Then 400 µL of PBS PLUS was added to resuspend the cells to a final concentration of 1× 10^6 to 1 × 10^7 cells / mL prior to analysis.

A.3 Flow Cytometry Setup and Antibody Titration

The forward scatter (FSC) and side scatter (SSC) voltage for WB were adjusted to get a clear separation among debris and the platelets, RBCs, and other cells. To get appropriate separation between the negative (fluorescence intensity around 10^2) and positive population (fluorescence intensity from 10^4 to 10^5), the voltage was adjusted for each of the three antibodies using WB or buffy coat (PE-Cy7-CD235a, BV711-CD71 antibodies). The three antibodies were

titrated with PBS to produce 5 serial dilutions of antibodies (1,1/2,1/4,1/8,1/16,1/32) (**Table A1)** using WB or compensation particles (BV711-CD71 antibody). Each sample was analyzed with a threshold of 1000 - 4000 events per second and a total of 100,000 events were collected. To determine the fluorescence compensation settings, anti-Mouse Ig, k / negative compensation particles were stained with each of the three antibodies separately.

A.3.1 Data Analysis and Analytical Performance

To calculate the separation index for each antibody titration and voltage titration, data was initially presented as median fluorescence intensity (MFI) for both the positive cell-populations and unlabelled populations (negative). The separation index was calculated as follows [10]:

$$SI = \frac{(\text{median positive - median negative})}{0.995(84\% \text{ percentile negative - median negative})} \quad \text{Eq A-1}$$

For quantification of $CD71^+$ RBCs , a spill over spreading matrix was calculated using FlowJo v10 10.7.1 for compensation. As calibration beads were not used, the proportion of $CD71^+$ RBCs was denoted as follow:

$$CD71^+ \text{ RBC (\%)} = \frac{\#\ CD71^+ \text{ RBC events}}{\#\ \text{intact RBCs and WBCs}} \times 100 \quad \text{Eq A-2}$$

where the number of intact RBC and WBC cells refer to “RBC and WBC” events (“RBC and WBC” gating in **Figure A 2**). The analytical performance of the WB flow cytometric method for determining the proportion of $CD71^+$ RBCs was evaluated by linearity, limit of detection, and inter- and intra-assay precision.

A.3.1.1 Linearity and Limit of Detection

The proportion of $CD71^+$ RBCs versus the reciprocal of the dilution factor was plotted to determine a reportable range. A trendline and R squared value was calculated for the linearity. The limit of blank (LOB) and limit of determination (LOD were calculated based on the following formulas [11]:

$$LOB = \text{mean}_{\text{buffer cocktail}} + 1.645 \times SD_{\text{buffer cocktail}} \quad \text{Eq A-3}$$

$$LOD = LOB + 1.645 \times SD_{low\ sample} \quad \text{Eq A-4}$$

where $SD_{low\ sample}$ is the standard deviation of three replicates of a sample containing the lowest proportion of $CD71^+$ RBCs where a change is observed; $mean_{buffer\ cocktail}$ is the mean of six replicates of the buffer cocktail, and $SD_{buffer\ cocktail}$ is the standard deviation of six replicates of the buffer cocktail.

A.3.1.2 Intra- and Inter-Assay Precision

To evaluate intra-assay precision, SD and CV (coefficient of variance) of 10 replicates of each of the three levels (low, intermediate, and high) were calculated. The inter-assay precision of 20 replicates for each level samples were calculated from 10 replicates by two operators.

A.4 RESULTS

A.4.1 Development of the Flow Cytometric Method to Determine $CD71^+$ RBCs

A.4.1.1 Voltage and Antibody Titrations

A FSC voltage of 320 V and SSC voltage of 192 V provided a clear separation of three target populations ("debris and platelets", RBCs and other cells) using a logarithmic amplification scale (**Figure A-1-A**). The optimal SI for the voltages of three antibodies were as follows: 160 at 600 V for PE-Cy7-CD235a, 76 at 572 V for APC-CD45, and 30.2 at 540 V for BV711-CD71(**Figure A-1B-D)**. The optimal SI for each antibody were as follows: 114 at 1.25 µg / mL for BV711-CD71, 61.4 at 0.038 µg / mL for PE-Cy7-CD235a, 69.0 at 0.31 µg / mL for APC-CD45 (**Table A1**)**.**

A.4.1.2 Gating Strategy for Determining $CD71^+$ RBCs in Whole Blood

To identify $CD235a^+CD45^-CD71^+$ cells in WB, a nested gating strategy which included two steps of singlet gating to reduce the interference of sample debris and platelets was applied. The time vs FSA gate was applied to check the stability of the instrument fluidics. A gating of FSC-H vs. FSC-W and SSC-H vs. SSC-W (15) was applied to remove doublet events (**Figure A 2-A**). Blank and buffer cocktail controls were prepared to examine event backgrounds. We applied the gate for CD71 positive population above 10^3 since the fluorescence intensity of the CD71 negative

population extended to 10^3 on the plot of the BV711-CD71 FMO control (**Figure A 2**). Whereas the gate for CD235a positive population was higher than 10^4 and the gate for CD45 negative population was lower than 10^3, respectively (**Figure A 2**).

A.4.2 Analytical Performance of the Flow Cytometry Method

A.4.2.1 $CD71^+$ RBCs in CBU PBMC

For isolated CBU PBMC used in the linearity and repeatability assays, we determined the proportion of CEC, and used WBC and RBC concentrations to calculate the sample $CD71^+$ RBC concentrations. The proportion of $CD71^+$ RBC used were 2.73 ± 0.05% (mean ± SD) and 2.70 ± 0.06%, respectively, using the gating strategy (**Figure A 3**). The concentration of WBC, RBC and $CD71^+$ RBCs in the CBU PBMC used in the linearity assay were 5.6×10^6 / mL, 1.0×10^7 / mL, $0.43 \pm 0.02 \times 10^6$ / mL, whereas intra- and inter-assay were 6.7×10^6 / mL, 2.0×10^7 / mL, $0.72 \pm 0.04 \times 10^6$ / mL respectively.

A.4.2.2 Linearity

A linear relationship between the proportion of labelled $CD71^+$ RBCs in a WB sample versus the reciprocal of dilution factors was found over the testing range of 0.01% to 1.32% with an R^2 of 0.98(**Figure A 4-A)**. The CV was lower than 20% when the test range was from 0.06% to 1.32%. The CV increased to 30%, 38%, 42%, when the test range was from 0.01% to 0.04%. Spiking labelled $CD71^+$ RBCs into a WB ensures our measurements are representative of actual biological conditions.

A.4.2.3 Limit of Detection

The $CD71^+$ RBC events in buffer cocktail controls was 0 ± 0 (n=6) and proportion of $CD71^+$ RBC in the limit of blank samples was 0%. The lowest percentage of spiked samples (256 times dilution) was 0.01% ± 0.003% (n=3). The limit of detection was 0.005% based on Eq A-3

A.4.2.4 Intra- and Inter-Run Precision

The proportion of $CD71^+$ RBCs in 10 replicates at the low, medium and high levels were 0.17 ± 0.02 %, 0.30 ± 0.02 %,1.40 ± 0.07%, respectively. The values are higher than the expected value (low: 0.06%, medium: 0.12%, high: 0.6%), probably due to the existence of WB $CD71^+$ RBCs before spiking PBMC-derived $CD71^+$ RBCs in the sample. However, the ratio of the three levels is approximately 1:2:10, which is similar with the volume ratio of spiked PBMC and WB (4.25:8.5:42.5) prepared for these three levels. The overall CV of all the three levels is 6.9% with individual CVs for the low, medium, and high being 8.6%, 7.1%, 5.1%, respectively (**Figure A-4-B**). The three level of $CD71^+$ RBCs in 20 replicates were 0.18 ± 0.02%, 0.33 ± 0.04%,1.45 ± 0.08% respectively (**Figure A-4-B**). The CVs of 20 replicates of the proportion of $CD71^+$ RBCs were 11.6%, 11.8%, 5.8% at three levels(**Figure A-4-C**).

A.5 DISCUSSION

In this study, a sensitive and efficient flow cytometry method was established to determine the quantity of $CD71^+$ RBCs in WB. Through standardizing sample handling, staining protocols, instrument setup and gating strategy, this procedure offers multiple advantages over those previously developed method [12, 13]. Using WB directly to determine $CD71^+$ RBCs not only saves time and reduces the blood volume required, but also provided an alternative method to determine $CD71^+$ RBCs when PBMC cannot be obtained, like in RCCs. Removing the density centrifugation step which requires use of no break, saved 1.5 h in processing one sample. Additionally, compared to the 3 mL of WB needed for separation, only 5 µL of WB is required for this reported method. A smaller volume of blood sample makes the assessment of large numbers of samples significantly easier and quicker. In addition to supporting our ongoing efforts examining the impact of blood donor factors on the quantity of $CD71^+$ RBCs in donated whole blood and RCCs, this method will be of value to studies examining $CD71^+$ RBCs in infants, pregnant women, and cancer where immunomodulation may be a concern.

As calibration beads to determine absolute cell numbers were not used, the concentration of $CD71^+$ RBCs could not be directly quantified in the WB samples. However, the proportion of $CD71^+$ RBCs relative to RBCs in a sample can be used to calculate the concentration of $CD71^+$ RBCs in the source sample. Although this may bring variation for $CD71^+$ RBC concentration when RBC concentration in PBMC is close to the lowest linear threshold of haematology analyzer ([RBC] 0.2×10^9 / mL), RBC indices of WB and RCCs are usually in its linear range ([RBC] 0.2 - 8×10^9 / mL) [14, 15]. Therefore, this method is relatively cost-effective, especially for studies where large sample sizes are required. For example, since we expect there to be a small difference in the proportion of $CD71^+$ RBCs in WB from male and female blood donors based on other published works [16], even with a low CV (< 15%) for the assay precision provided by this method, more than 600 samples would be required to investigate the impact of various blood donor factors (donor sex, age, pre-donation Hb level and donation frequency) on the $CD71^+$ RBCs in WB and RCCs.

This study presents several strengths through its detailed methodology aimed at mitigating false positives in the measurement of $CD71^+$ RBCs, which are critical for accurate clinical interpretations and avoiding the overestimation of their prevalence. The initial step involves careful antibody titration to prevent non-specific binding due to improper antibody concentrations, ensuring the determination of optimal levels for CD71, CD235a, and CD45 antibodies as illustrated in Figure A-1. Secondly, the establishment of baseline readings for $CD71^+$ RBCs utilizes both positive (labeled $CD71^+$ RBCs shown in Figure A-2- I1, I2, and I3) and negative controls (unstained control, seen in Figure A-2- G1, G2, and G3), a critical step in distinguishing true positives from mere background noise. Thirdly, the strategy of gating is implemented with precision to exclude non-target elements such as debris, dead cells, and aggregates, thereby preventing false identification of $CD71^+$ RBCs, as depicted in Figure A-2- B, C, and D. Additionally, the application of Fluorescence Minus One (FMO) control is integral for identifying spillover and

accurately setting gates, referenced in Figure A-2- H1, H2, and H3. Moreover, when utilizing multiple fluorophores such as APC, PE-Cy7, and BV711, compensation using specific beads is essential to avoid false positives. Performing the assay in triplicates contributes to the reproducibility, enhancing the consistency and dependability of the positive results. The application of meticulous validation, and calibration are paramount for refining the assay's quantitative capabilities for diagnostic purposes.

A limitation in this study was that we used WB spiked with PBMC-derived $CD71^+$ RBCs for serial dilution and repeatability instead of WB. The volume and cellular complexity of $CD71^+$ RBCs from PBMCs may be different than those found in WB (**Figure A 2-D and Figure A-1-A).** We did observe a small, separated population with a larger cell size (higher FSC intensity) in the "RBC" gate **Figure A 2-D,** compared to the cell distribution in **Figure A-1-A**. To determine the $CD71^+$ RBCs in the CBU PBMC, higher voltages for FSC (465 V) and SSC (275 V) were applied to these samples compared with what was used for the WB $CD71^+$ RBCs (320 V and 192 V). The observed proportion of $CD71^+$ RBCs was 1.32%, which was slightly higher than the 1.26% of $CD71^+$ RBCs spiked into the WB. However, the serial dilution was not affected by this variation and the linear relationship between the observed percentage of $CD71^+$ RBCs and reciprocal of dilution factors was good with an R^2 of 0.98 (**Figure A 3-A).** In sum, although using CBU $CD71^+$ RBCs may introduce variation, we found it to be acceptable for validation purposes.

A.6 CONCLUSIONS

We have developed a simple, sensitive, and cost-effective method for determining the portion of $CD71^+$ RBCs in non-manipulated WB. This method could be used in other RBC products without PBMC components, which could help understand the impact of RBC products on recipient transfusion outcomes.

A.7 TABLES /FIGURES

Table A 1 – Selection of antibody concentration based on separation index

Antibody	Clone	Label	Start [AB]† (µg / mL)	SI – 1	SI – 2	SI – 3	SI – 4	SI – 5‡	Final [AB]§ (µg / mL)
CD235a	GA-R2	PE-Cy7	0.075	3.16	6.42	22.6	61.4	39.7	0.038
CD45	HI30	APC	1.25	29.3	40.5	69.0	66.5	58.7	0.31
CD71	L01.1	BV711	5	71.2	102	114	85.0	40.0	1.25

† The starting highest antibody concentration (SI-5) for antibody titration.

‡SI-5: Separation index under the highest antibody concentration. 85 µL of diluted WB, 5 µL diluted antibody and 10 µL PBS in a 100 µL total staining volume were used for antibody titration under 5 serial dilutions of antibodies (1,1/2,1/4,1/8,1/16,1/32) at room temperature (20 ºC – 24 ºC) for 30 min.

§ Optimal concentration of the antibody in the staining volume.

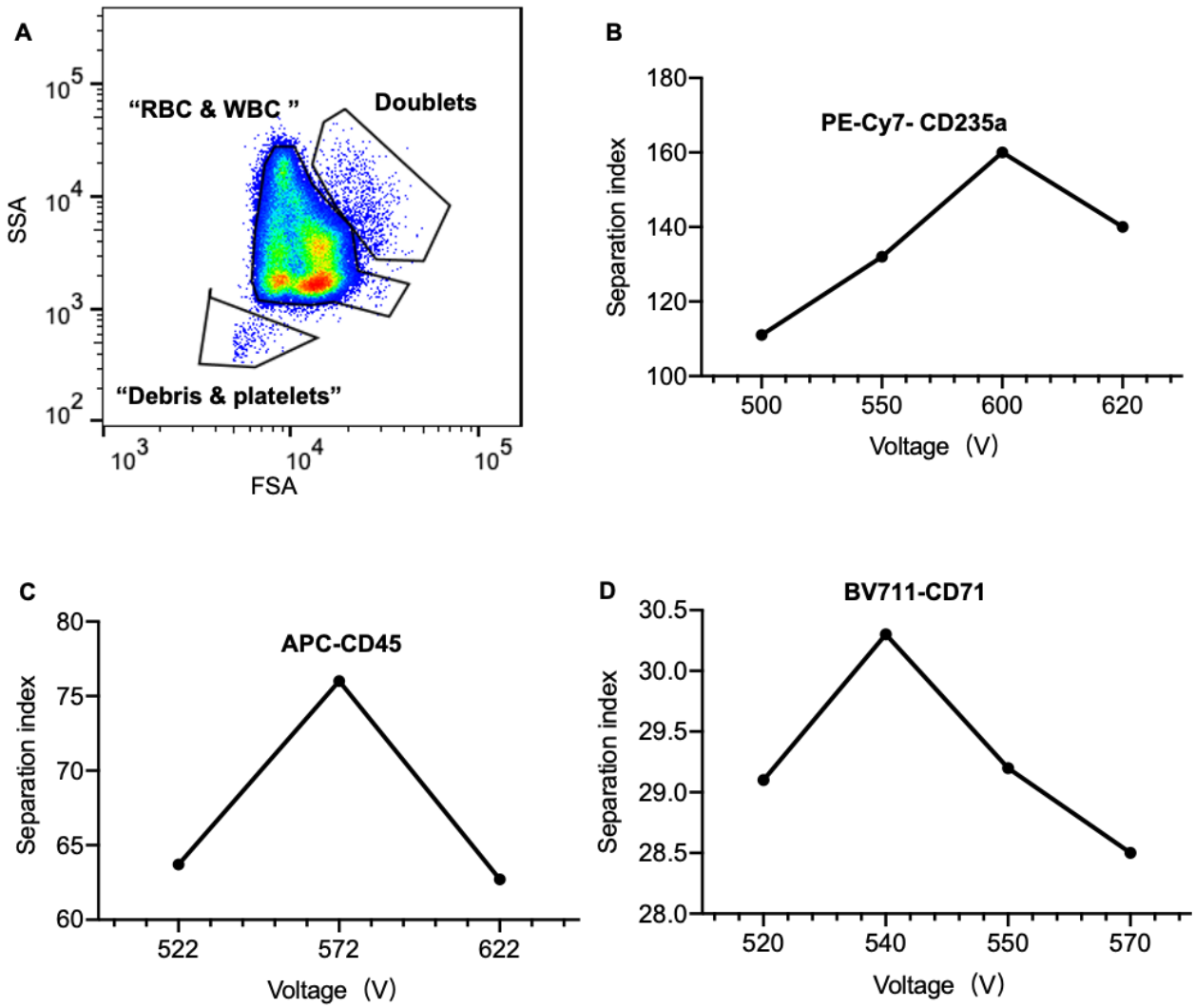


Figure A-1 Flow cytometer setup

(A) Forward light scatter vs side light scatter dot plot (logarithmic scale) of cells under a FSC voltage of 320 V and SSC voltage of 192 V. (B-D) Separation index for PE-Cy7-CD235a, APC-CD45, BV711-CD71under 5, 3, 4 levels of voltages. The final voltage used for PE-Cy7 CD235a, APC-CD45, BV711-CD71 were 600 V, 572V, 540V respectively.

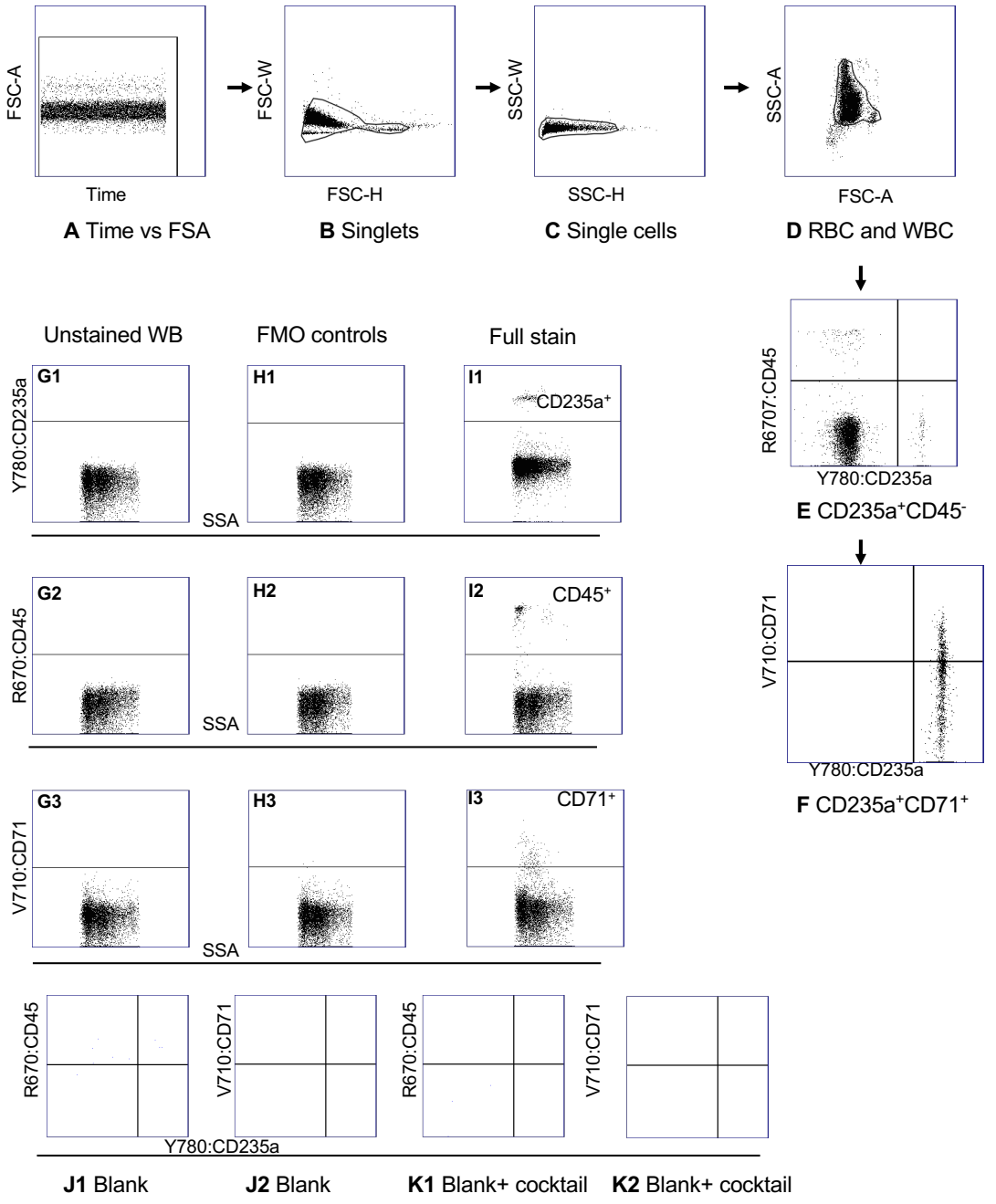


Figure A 2 A Representative nested gating strategy for labelled CD71$^+$ RBCs in unstained WB. A - F represents the addition of CD71$^+$ RBCs labelled with CD235a, CD71, CD45 antibodies into **unstained WB**. (A) Time vs FSA by adjusting the rectangular gate to include as many events as

possible but getting rid of the interrupted events when the fluidics interrupted. (B) To create a polygon matching the distribution of the events in the FSH vs FSW dimension for singlets. (C) Polygon matching the distribution of the events in the SSH vs SSW for single cells. (D) FSA vs SSA (logarithm); (E) Gating for $CD45^{-}$ $CD235a^{+}$. (F) Gating for $CD71^{+}CD235a^{+}$. G1-3) Unstained WB. (H1) PE-Cy7 FMO control for gating $CD235a^{+}$; (H2) APC FMO control for gating $CD45^{+}$. (H3) FMO control for gating $CD71^{+}$. (I1-I3) Full stained sample. (J1-J2) Blank control. (K1-K2) Buffer cocktail control.

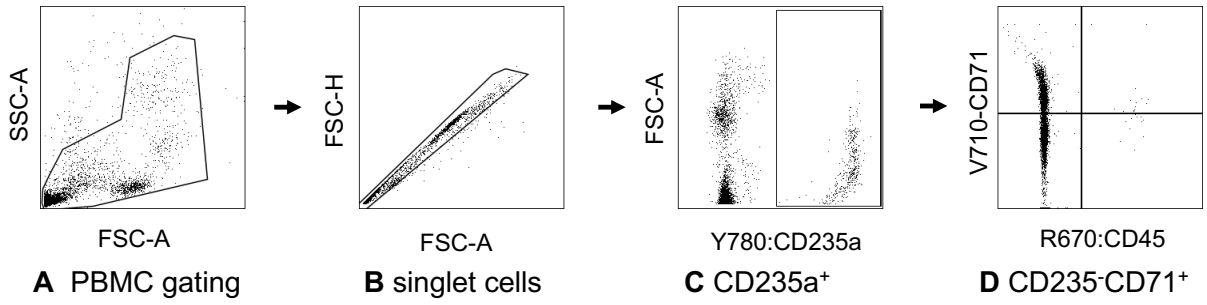
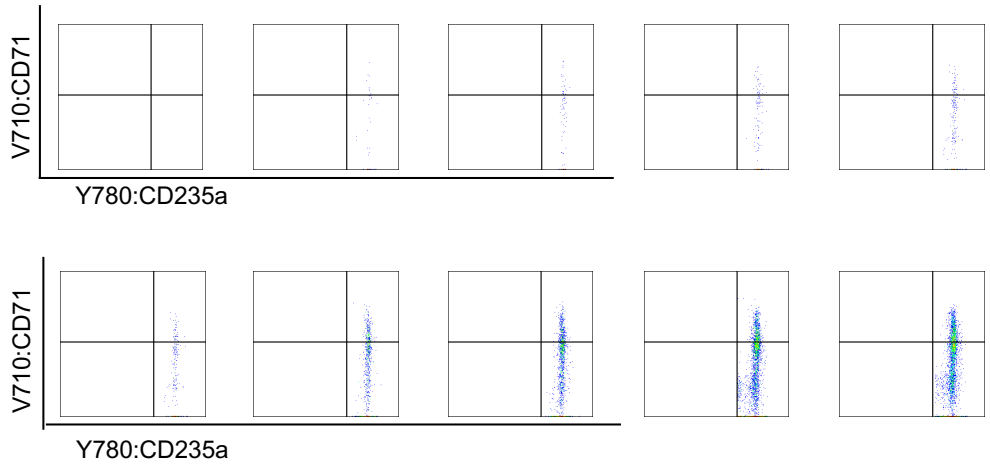
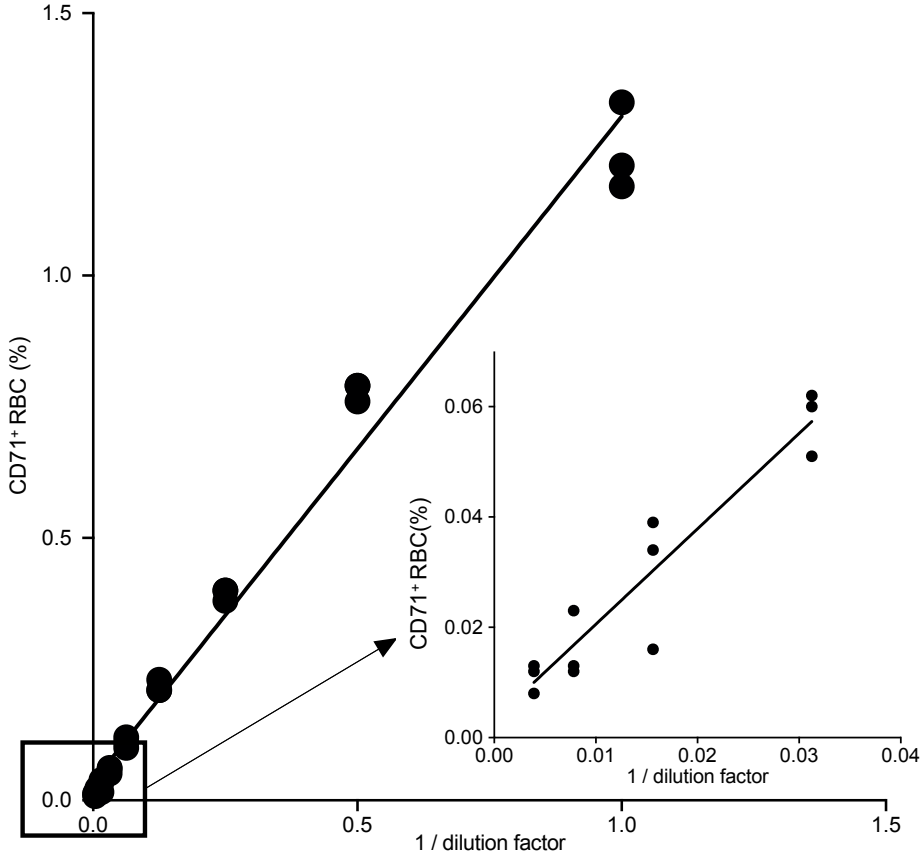


Figure A 3 Gating for $CD71^+$ RBCs in isolated CBU PBMC.

(A) Gating for PBMC. (B) Gating for single cells. (C) Gating $CD235a^+$ cells. (D) Gating for $CD235a^+CD45^-CD71^+$ cells.

A


V710:CD71
Y780:CD235a
V710:CD71
Y780:CD235a


CD71+ RBC (%)
1 / dilution factor
CD71+ RBC(%)
1 / dilution factor

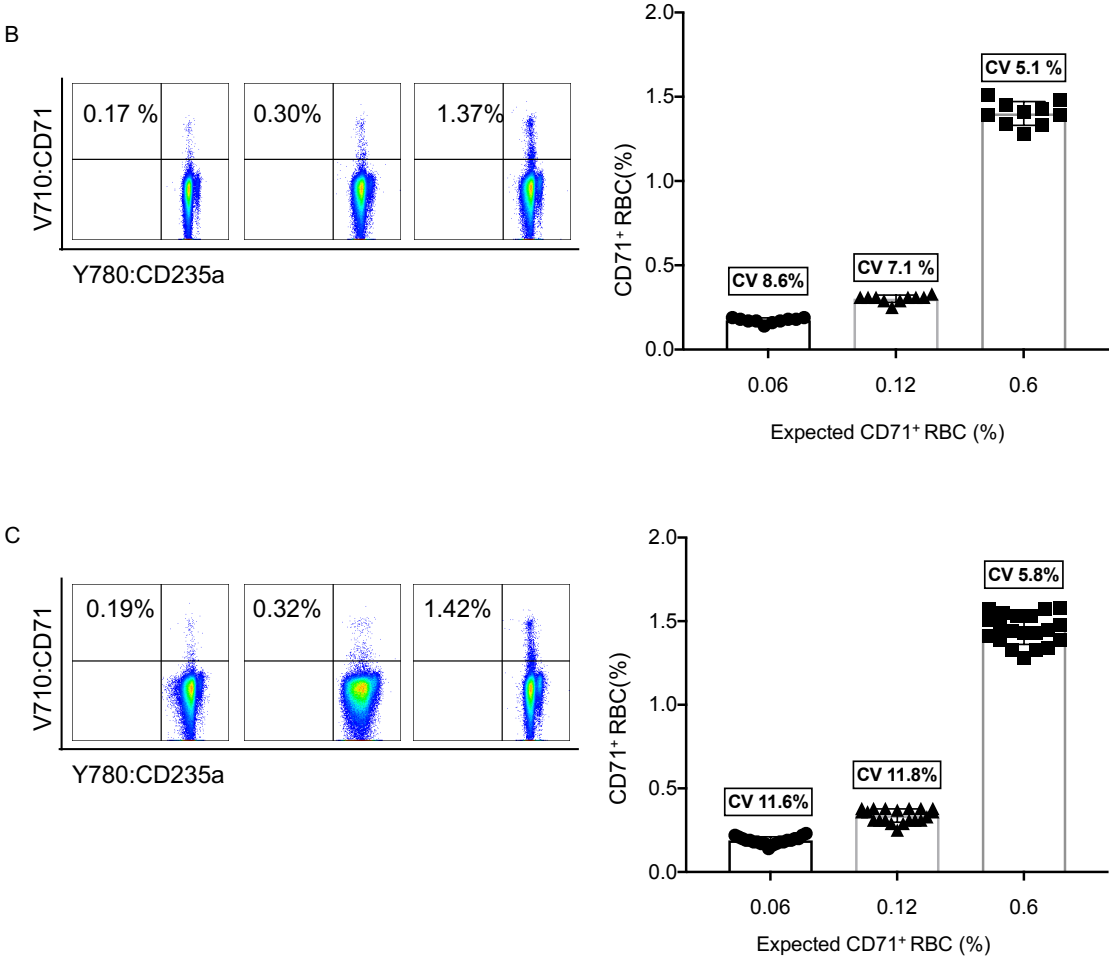


Figure A-4 Linearity, intra- and inter-run precision

(A) The linearity of observed percentage of labelled CD71$^+$ RBCs in WB vs the reciprocal of dilution factors from 1 to 1/256 and its representative images. The reciprocal of dilution factor from 1/64 to 1 in the drilled down graph. (B) CV of 10 replicates of CD71$^+$ RBCs% for low, medium, high proportions in WB samples spiked with CBU CECs and its representative images. (C) CV of 20 replicates of low, medium, high CD71$^+$ RBCs in WB samples spiked with CBU CD71$^+$ RBCs by two operators and its representative images.

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Appendix B

Development and Validation of an Imaging Flow Cytometric Method for Determining Phagocytosis Index

B.1 INTRODUCTION

The monocyte suspension assay (MSA) is a laboratory technique employed to evaluate the phagocytic activity of monocytes using conventional flow cytometry (FC), which holds particular significance in cases of incompatible complicated transfusion [1]. It aids in assessing the risk of transfusion-related hemolysis in recipients. This method was adapted from the traditional monocyte monolayer assay (MMA) using microscopy [2, 3], which has been widely employed for similar purposes.

The emergence of imaging flow cytometry (IFC) has introduced significant advancements in phagocytosis analysis. By combining the strengths of flow cytometry and imaging, IFC has become a valuable tool in the field of phagocytosis analysis. It enables researchers to observe and analyze phagocytic events with improved spatial information and visualization, enhancing the understanding of cellular interactions.

During erythrophagocytosis, monocytes engulf and degrade RBCs, which may result in the release of Hb into the surrounding environment. By quantifying the amount of Hb released, researchers can infer the degree of phagocytic activity exhibited by monocytes. Indeed, analyzing the release of Hb during MSA provides valuable insights into the phagocytic activity of monocytes. The assessment of Hb release aids in understanding the extent of RBC engulfment and subsequent hemolysis within monocytes.

In order to determine phagocytosis and supernatant Hb, various procedures were attempted based on the steps required (**Figure B 1**) as follows: titration of IgG for RBC sensitization, the incubation duration and determination of the cell count number of opsonized RBCs (OpRBCs) and monocytes. Furthermore, validation was carried out to compare the PI of RBCs between conventional FC and IFC. Furthermore, the MMA was also used to capture PI of RBCs to compare with MSA combined with IFC. The development and validation were briefly

outlined in the subsequent sections. A comprehensive outline of the materials and methodology can be referred to **Chapter 03**.

B.2 Development of Monocyte Suspension Assay

B.2.1 Titration of IgG for RBC sensitization

Immunoglobulin G (IgG) -mediated phagocytosis is a commonly observed mechanism by which cells are engulfed and ingested by phagocytes including monocytes. IgG antibodies, a type of immunoglobulin, which can bind to specific antigens present on the surface of target cells, such as RBCs. Sensitized RBCs or OpRBCs are commonly used to induce phagocytosis. Hemagglutinin reactions are used to grade the strength of the agglutination. It is recommended to use a grade higher than "2" for the strength of the agglutination in the assay, based on the protocol from Dr. Denomme's team [1]. In this study, the grade “2” will be used to determine whether the concentration of IgG is acceptable for further analysis.

The titration involved diluting IgM + IgG monoclonal Anti-Rh D antibodies (Cat # 5350, Novaclone, USA) with 0.2% BSA / PBS started from a concentration of 1:2 and subsequently at ratios of 1:4, 1:8, 1:16, and 1:32. These dilutions were likely performed to establish a range of antibody concentrations for agglutination. RBCs were incubated with diluted opsonization mixture at 37 °C for 30 min (details refer to 3.2.2.2). In addition, further concentrations were applied at ratios of 1:11.5 and 1:23 for the convenience of calculation. These additional dilutions might have been employed to simplify the subsequent calculations or to ensure that the concentrations fall within a specific range for experimental purposes. Based on observation, a titration of 1:16 and 1:11.5 can be graded “2 ” as shown in **Figure B 2.**

B.2.2 The Incubation Duration

To determine the concentration of Hb in the supernatant, different incubation durations were considered to allow sufficient time for Hb release during erythrophagocytosis. OpRBCs and non-OpRBCs were incubated with monocytes for 4 hrs and 24 hrs respectively. For the

preparation of non-OpRBCs and OpRBCs and monocytes and Hb determination refer to 3.2.2 and 3.2.5 respectively

The levels of supernatant Hb in the OpRBC group were successfully quantified at both 4-hour and 24- hour time points (**Table B 1**). Notably, after a 24-hour incubation period, monocyte presence could no longer be detected. To expedite the process, the monocyte suspension assay subsequently utilized a shortened incubation period of 4 hours.

B.2.3 The Cell Number Ratio of RBCs to Monocytes

The ratio of RBCs to monocyte was adopted from the protocols established by Kipkeu et al [4], which are used in both Dr. Acker and Dr.Denomme lab. Different RBC and monocyte counts were examined to identify the concentration of Hb in the supernatant. For preparation of monocyte suspension assay and supernatant Hb determination, refer to 3.2.2 and 3.2.3 respectively. All of the levels of supernatant Hb were within detectable range **(Table B2).** Considering the limited number of $CD71^+$ RBCs, the cell count ratio of RBC to monocyte was fixed at 33:1 ($8.4 \times 10^6 : 2.5 \times 10^5$)

B.3 Validation of IFC Method

B.3.1 Comparison Between IFC And Conventional FC Method

To assess the capability of the developed IFC method to capture phagocytosed RBCs, a comparative study was carried out against the conventional FC within the framework of a monocyte suspension assay.

In brief, RBCs were initially labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Cat # C34554, Invitrogen™, USA), subsequently opsonized, and then exposed to monocytes for a period of 4 hours to facilitate phagocytosis. The preparation procedures for opsonized RBCs for the monocyte suspension assay, and the phagocytosis index are detailed in sections 3.2.2 and 3.2.3. One deviation from this is the use of PE-Cy7-CD235a for intracellular staining for CD235a. Following this, the samples were evaluated using both the conventional flow

cytometer and the imaging flow cytometer. The efficacy of the assay was determined by computing the standard deviation and coefficient of variation values. Furthermore, the correlation between the two methodologies was identified and quantified to gauge their level of concordance.

The gating approach utilized for IFC was illustrated in **Figure B 3**. This demonstrates that the IFC technique can effectively identify RBCs within monocytes. Interestingly, a CFSE-positive population, which does not include monocytes, was not gated following visual inspection (**Figure B 3D**). A similar population was noticed in the upper right population of the traditional FC gating, as shown in **Figure B 4C**. This population was not gated out due to the inability to determine whether it had undergone RBC phagocytosis. The data showed a phagocytosis index for RBCs of 32.6% ± 4.2% (**Figure B 5**), differing from the phagocytosis index of 90.0% ± 2.6% obtained using conventional flow cytometry (**Figure B 5**). This discrepancy may be due to the high fluorescence of CFSE, which can cause false positive event (**Figure B 3D**). The IFC method's ability to discern the phagocytosed cells based on visualization illustrates its superiority. This demonstrated the advantage of IFC method in determining whether phagocytosed cells were inside or outside of monocytes. This feature underscores the value of IFC in such circumstances.

B.3.2 The MMA and IFC method

Given the discrepancy in the phagocytosis index of OpRBCs between the IFC method and the conventional FC method, an MMA procedure was implemented to to further validate the accuracy of the developed IFC method in capturing phagocytosed RBCs. Using similar preparation methods of OpRBCs, this assay was combined with traditional microscopy. The MMA protocol was established by Kipkeu et al. in the Acker lab [4].

Briefly, the method for preparing OpRBCs was outlined in section 3.2.2. Unlike the monocyte suspension assay, the MMA involves carefully seeding monocytes onto glass slides. After a 4-hour incubation period, the monocyte monolayer was gently washed to eliminate any unbound RBCs. Subsequently, the monocytes or macrophages and RBCs are then visualized

using a Wright-Giemsa stain (PROTOCOL Hema 3 Manual Staining System, 22-122911, Fisher Healthcare, Fisher Scientific, USA). For the analysis, conventional microscopy techniques were employed to assess the phagocytosis index, a quantitative measure of RBC uptake, in the prepared samples. The PI of RBCs was 30.2% ± 2.5% (**Table B 3**), a result that aligns with the PI obtained using the IFC method. This validates the precision of the IFC within a framework of monocyte suspension assay.

B.4 CONCLUSION

The study concluded that the monocyte suspension assay, combined with IFC method, proved to be effective in accurately identifying the presence of RBCs inside monocytes. The method involved several steps, including Anti-D IgG and serum as an opsonization mixture, followed by a 4-hour incubation period with a cell count ratio of 33 RBCs to 1 monocyte. Using specialized software analysis, the researchers were able to carefully examine the RBCs within monocytes, allowing for a comprehensive assessment of the phagocytosis index. This suggests that the method employed in the study provides a reliable means of determining the extent of RBC uptake by monocytes.

B.5 TABLES / FIGURES

Table B 1 The effect of incubation duration on supernatant Hb

	Incubation duration			
	4 hours		**24 hours**	
	Non-OpRBC	**OpRBC**	**Non-OpRBC**	**OpRBC**
Supernatant Hb (mean ± SD mg / L)	Not detectable	3.5 ± 2.0	Not detectable	3.0 ± 1.0

OpRBC : Opsonized red blood cells

Table B 2 The effect of the number of opsonized RBCs and monocytes on the supernatant Hb

	Ratios of RBC number to monocyte number (volume 540 µL)		
	$4.2 \times 10^6 : 1.25 \times 10^5$	**$8.4 \times 10^6 : 2.5 \times 10^5$**	**$12.8 \times 10^6 : 5.0 \times 10^5$**
Supernatant Hb (mg/L)(duplicates, mean ± SD)	2.7± 0.00	4.3 ± 0.14	7.9 ± 0.07

Hb: hemoglobin

Table B 3 The performance of phagocytosis index of the three methods for the opsonized RBC group*

	IFC	Conventional FC	MMA
RBC PI (%), mean ± SD	32.6% ± 4.2%	92.6% ± 4.2%	30.2 ± 2.5%
CV (%)	13.0	2.4	4.1

* The same samples were analyzed using both the IFC technique and the traditional flow cytometry method within a monocyte suspension assay framework. Comparable RBC opsonization preparations were set up for the monocyte suspension assay with the IFC technique and the monocyte monolayer assay integrated with microscopy. FC, flow cytometry; IFC, imaging flow cytometry; PI, phagocytosis index; RBC, red blood cell; MMA, monocyte monolayer assay.

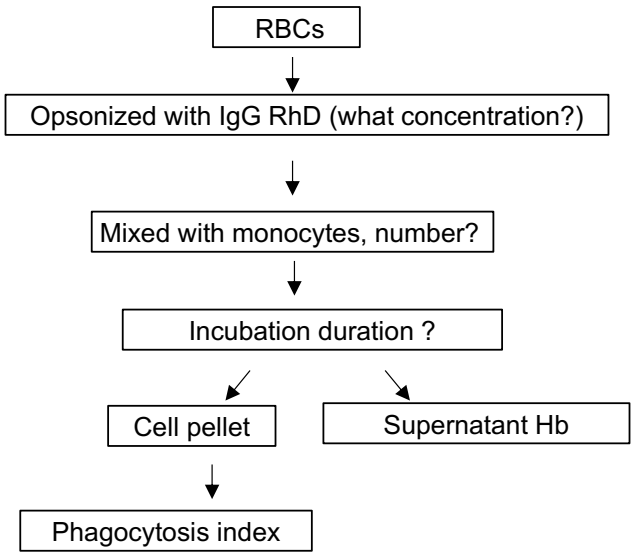

Figure B 1 Schematic diagram for monocyte suspension assay

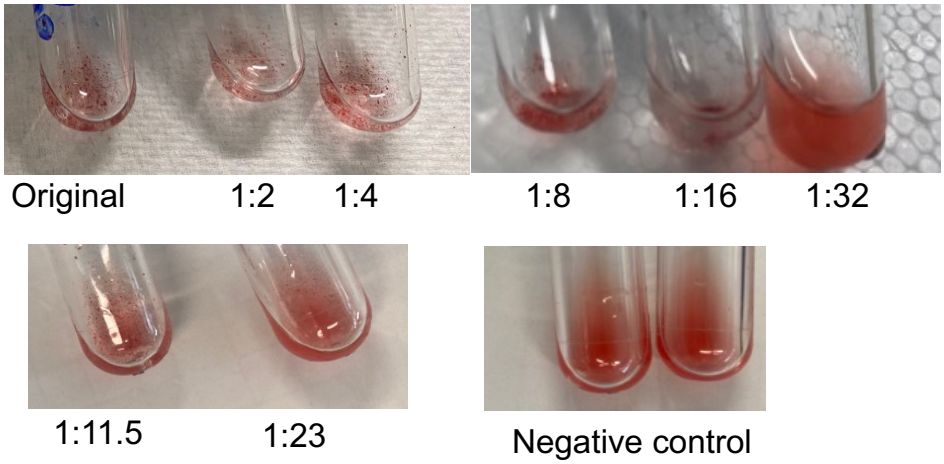

Figure B 2 The representative image of agglutination of RBCs for IgG titration.

Agglutination of RBCs for IgG titration refers to the process where RBCs clump together in the presence of IgG antibodies. In a laboratory setting, this process can be utilized to measure the concentration, or titer, of specific IgG antibodies in a blood sample. When Anti-D antigen present on the surface of RBCs, is exposed to a sample containing its corresponding IgG antibodies, the antibodies will bind to these antigens and cause the RBCs to clump or agglutinate. By observing this agglutination and comparing it to known standards, one can determine the titer of IgG antibodies required for sensitization or opsonization. IgG : immunoglobulin G

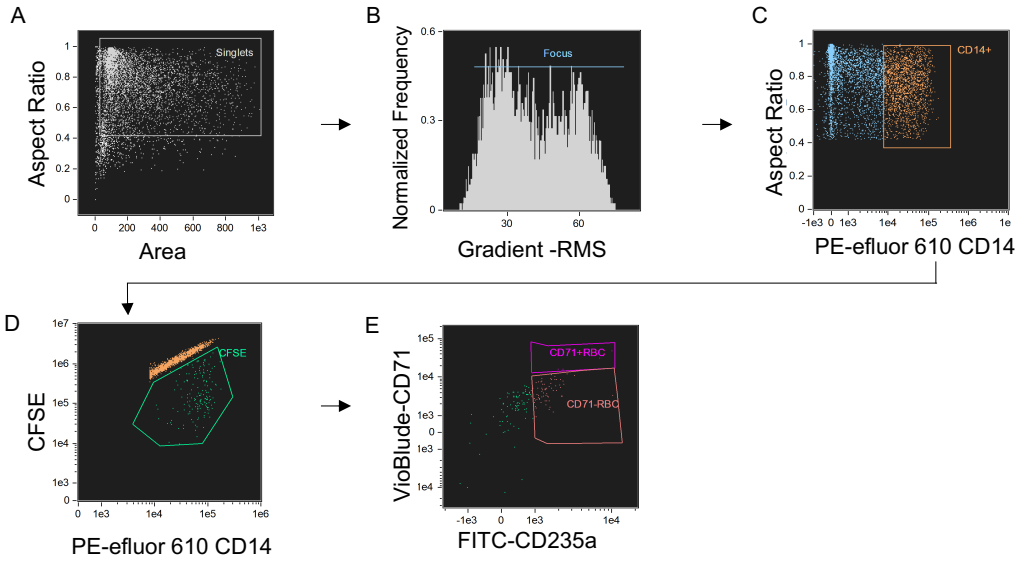

Figure B 3 Gating strategy of RBCs phagocytosed by monocytes with imaging flow cytometry (A) Dot plot base on area vs. aspect ratio to gate single cells. (B) Histogram plot of the brightfield gradient root mean square (RMS) to gate focused cells. (C) Histogram plot for $CD14^{+}$ fluorescence intensity to gate CD14 fluorescence positive population. (D) Bivariate dot plot for CFSE and CD14 fluorescence intensity to gate true CD14 positive and CFSE positive combined with visualization of images. (E) Bivariate dot plot for CD71 and CD235a fluorescence intensity to gate $CD71^{+}$ RBC and $CD71^{-}$ RBCs within monocytes. CFSE: carboxyfluorescein diacetate succinimidyl ester, a fluorescence dye.

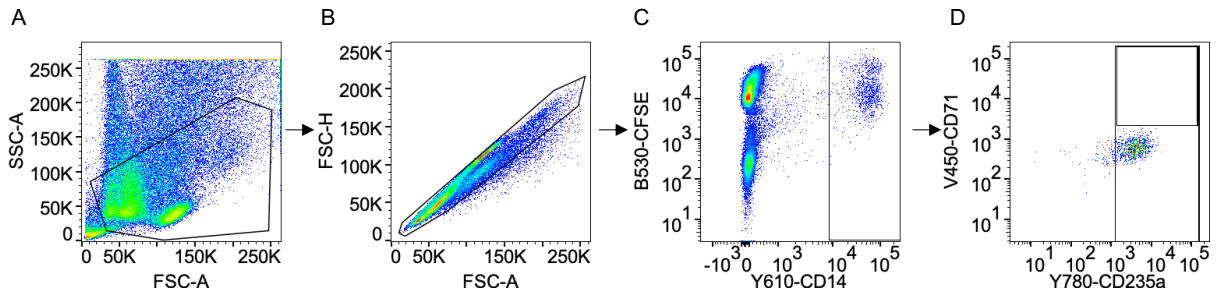

Figure B 4 Gating strategy of RBCs phagocytosed by monocytes with conventional flow cytometry. (A) A dot plot based on FSC vs. SSC to gate mononuclear cell population. (B) Dot plot base on FSC-A vs. FSC-H to gate singe cells. (C) Bivariate dot plot for CFSE and CD14 fluorescence intensity to gate CD14 positive and CFSE positive. (E) Bivariate dot plot for CD71 and CD235a fluorescence intensity to gate $CD71^+$ RBC and $CD71^-$ RBCs. FSC: forward scatter; SSC: side scatter; CSFE: carboxyfluorescein diacetate succinimidyl ester, a fluorescence dye.

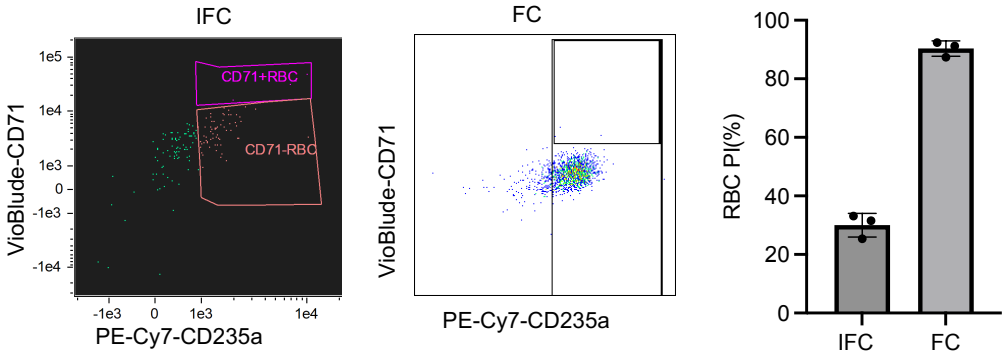

Figure B 5 Representative images of phagocytosis and the phagocytosis index of RBCs using IFC method and FC within a framework of monocyte suspension assay. Same samples were analyzed in imaging flow cytometer and conventional flow cytometer. Triplicates in each group, mean ± SD. FC, flow cytometry; IFC, imaging flow cytometry.

B.6 REFERENCES

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