Characterisation of dendritic cell immune profile in models of transfusion

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Abstract

Blood transfusion modulates recipients’ immune responses sometimes resulting in poor clinical outcomes. Further, transfusion with blood products at date-of-expiry has been associated with exacerbation of this concerning problem. In-vitro studies have demonstrated exposure to packed red blood cell (PRBC) and platelet products modulate the responses of leukocytes, however, there are limited studies on the impact of transfusion on dendritic cells (DC). Despite DC being central to the immune response, their role in transfusion-related immune modulation remains largely undefined. I hypothesised that exposure to blood components changes DC phenotype and function, which could be one mechanism underpinning transfusion-related immune modulation. The potential for blood components (PRBC, buffy-coat-derived platelet concentrates (PC) and cryopreserved platelets (cryo-PLT)) to modulate responses of myeloid dendritic cells (mDC) and the specialised subset blood DC antigen 3 (BDCA3+) DC, was investigated.

Amongst blood DC, BDCA3+ DC are the sole subset equipped with C-type lectin domain family 9 member A (Clec9A) receptor. Of interest to transfusion, Clec9A has been reported to bind filamentous actin (F-actin) when exposed, in artificially aged or modified red blood cells (RBC) and platelets. I further hypothesised Clec9A expressed on BDCA3+ DC has a role in transfusion-related immune modulation. Importantly, BDCA3+ DC are capable of cross-presentation of endogenous antigen via major histocompatibility complex (MHC) class I and have pathogen recognition receptors skewed towards recognition of viruses, unlike most mDC.

I investigated the suitability of the fluorescein conjugated function-spacer-lipid (FSL-FLRO4) construct for labelling PRBC. I demonstrated that FSL-FLRO4 is a suitable tool for labelling PRBC at different storage duration and can be retained until date-of-expiry, aiding in-vitro visualisation and tracking of PRBC during routine storage. This was utilised in an assessment of erythrophagocytosis in my model of PRBC transfusion (see below).

In addition, I investigated the unexpected loss of Clec9A surface expression on BDCA3+ DC observed during optimisation of the in-vitro transfusion model. Interestingly, I found loss of Clec9A was an effect of ethylenediaminetetraacetic acid (EDTA), as well as incubation temperature and duration. Therefore, recombinant human (rh)Clec9A was used to investigate a role for Clec9A in the context of transfusion, specifically whether this receptor bound fresh and/or stored PRBC, PC and cryo-PLT. Concurrently, I investigated
whether blood components exposing F-actin for Clec9A ligation were present within the blood product. rhClec9A did not bind fresh or stored PRBC, and this outcome was supported by the lack of detection of F-actin. Although, no binding of rhClec9A to PC or cryo-PLT was demonstrated, F-actin was detected on both types of platelet products. As binding of rhClec9A to PRBC, PC or cryo-PLT was not detected, EDTA blood collection tubes were used for the remainder of the study under the premise that lack of expression of this receptor would not impact on outcomes in my transfusion models.

I assessed the impact of fresh and/or stored blood products on mDC and BDCA3+ DC surface antigen and inflammatory profile using a human in-vitro whole blood model of transfusion. In parallel, to model the processes associated with viral or bacterial infection, polyinosinic:polycytidylic acid (polyI:C) or lipopolysaccharide (LPS) was added respectively. Exposure to PRBC and PC predominately suppressed surface antigen expression (CD40, CD80, CD83 and CD86) and inflammatory mediator production (interleukin (IL)-6, IL-8, IL-12, tumour necrosis factor-α and interferon-gamma inducible protein-10 or IL-10) on both DC subsets. These changes were often more evident in the presence of polyI:C and LPS, and when DC were exposed to stored PRBC. Similar modulation was evident for BDCA3+ DC when exposed to cryo-PLT alone and cryo-PLT in the presence of polyI:C and LPS. The impact of blood transfusion on the overall inflammatory response by leukocytes was also examined. The immunomodulatory effect of transfusion in-vitro was more pronounced in the presence of polyI:C and LPS. For PRBC, an additional erythrophagocytosis assay was conducted where the uptake of stored PRBC by mDC and BDCA3+ DC was significantly increased in comparison to fresher PRBC.

This study provided the first evidence that exposure of PRBC, PC and cryo-PLT modulates mDC and/or BDCA3+ DC maturation and activation. The changes were more pronounced when modelling processes associated with concurrent viral or bacterial infection. Experimental evidence suggested that modulation of BDCA3+ DC was independent of Clec9A-F-actin interaction. My study highlights the importance of considering mechanisms associated with transfusion-related immune modulation in specific cell subsets – with changes in cell responses in rare but functionally important cell subsets overlooked when only assessing the overall leukocyte inflammatory response, which largely represents the response of the more abundant leukocyte populations. My results add to our knowledge of the changes in immune profiles that could be predicted in transfusion patients, in particularly those with infectious complications. My PhD provided detailed evidence of
changes in DC phenotype and function in a model of blood transfusion, and I propose these changes are one mechanism underpinning transfusion-related immune modulation.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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List of abbreviations

α  Alpha
β  Beta
λ  Lambda
%  Percentage
μg/mL  Micrograms per millilitre
μL  Microlitre
pg/mL  Picograms per millilitre
18s  18s ribosome RNA
2°  Secondary
2,3 DGP  2,3 diphosphoglycerate
AA  Arachidonic acid
Ab  Antibody
ABLE  Age of blood evaluation
ABO  Blood group antigen
ADP  Adenosine di-phosphate
APC  Allophycocyanin
ARDS  Acute respiratory distress syndrome
ANZSBT  Australian and New Zealand society of blood transfusion
aPC  Activated protein C
ALI  Acute lung injury
ATP  Adenosine triphosphate
BAPTA  1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid
BATF3  Basic leucine zipper transcription factor, ATF-Like 3
BCL10  B-Cell CLL/Lymphoma 10
BD  Becton Dickinson
BDCA  Blood dendritic cell antigen
Blood Service  Australian Red Cross Blood Service
BV421  Brilliant violet 421
BV510  Brilliant violet 510
C51  Chromium 51
C3b  Complement protein 3b
Ca²⁺  Calcium
CARD9  Caspase recruitment domain family member 9
CAMD1 (Nect2)  Cell adhesion molecule 1 (nectins and nectin-like molecules)
CBA  Cytometric bead array
CCL  Chemokine (C-C motif) ligand
cDNA  Complementary DNA
c.f  Compared to
CHO  Chinese hamster ovary
Clec9A (DNGR-1)  C-Type lectin family 9 member A
                      (DC natural killer lectin group receptor 1)
CPD  Citrate phosphate dextrose
Cryo-PLT  Cryopreserved platelets
CTLD  C-type lectin domain
CXCL10 (IP-10)  Chemokine (C-X-C motif) ligand 10
                      (Interferon-inducible protein 10)
CXCR  Chemokine receptor
D  Day
DAMP  Damage associated molecular pattern
DC  Dendritic cell(s)
DFP  Deep frozen plasma
DMSO  Dimethyl sulfoxide
E  Echinocytes
EDTA  Ethylenediaminetetraacetic acid
EGF  Epidermal growth factor-like
EGTA  Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA  Enzyme-linked immunosorbent assay
ERAP1  Endoplasmic reticulum aminopeptidase 1
ERK (MAPK1)  Extracellular signal-regulated kinases
                      (mitogen-activated protein kinase 8)
F-actin  Filamentous actin
FAS  Tumour necrosis receptor
FBS  Foetal bovine serum
FDA  Food and Drug Administration
FFP  Fresh frozen plasma
FITC  Fluorescein isothiocyanate
FLT3  FMS-related tyrosine kinase
F/T  Freeze/thaw
FSL-FLRO4  Fluorescein conjugated function-spacer-lipid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>GCSAM</td>
<td>Germinal center-associated signalling and motility</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GIP</td>
<td>Gamma-tubulin complex protein</td>
</tr>
<tr>
<td>h</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>HAA</td>
<td>Haematology Society of Australia and New Zealand, Australian &amp; New Zealand Society of Blood Transfusion and the Australasian Society of Thrombosis and Haemostasis</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Human leukocyte antigen DR-1</td>
</tr>
<tr>
<td>HMBG1</td>
<td>High-mobility box group 1</td>
</tr>
<tr>
<td>HMVEC</td>
<td>Human pulmonary microvascular endothelial cell</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRF3</td>
<td>Interferon regulatory factor 3</td>
</tr>
<tr>
<td>JNK (MAPK8)</td>
<td>c-Jun N-terminal kinases (mitogen-activated protein kinase 1)</td>
</tr>
<tr>
<td>K</td>
<td>Potassium</td>
</tr>
<tr>
<td>LOS</td>
<td>Length of stay</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>Ly75</td>
<td>Lymphocyte antigen 75</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemoattractant protein</td>
</tr>
<tr>
<td>mDC</td>
<td>Myeloid dendritic cell(s)</td>
</tr>
<tr>
<td>MFI</td>
<td>Median fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>Min</td>
<td>Minutes</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MoAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MoDC</td>
<td>Monocyte-derived dendritic cell</td>
</tr>
<tr>
<td>MOD</td>
<td>Multiple organ dysfunction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>ms</td>
<td>Millisecond(s)</td>
</tr>
<tr>
<td>Na</td>
<td>Sodium</td>
</tr>
<tr>
<td>ng</td>
<td>Nanograms</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometers</td>
</tr>
<tr>
<td>No incu.</td>
<td>No incubation</td>
</tr>
<tr>
<td>No tx</td>
<td>No transfusion</td>
</tr>
<tr>
<td>NSW</td>
<td>New South Wales</td>
</tr>
<tr>
<td>oxLDL</td>
<td>Oxidised low-density lipoprotein</td>
</tr>
<tr>
<td>P</td>
<td>Population</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Platelet concentrate(s)</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell(s)</td>
</tr>
<tr>
<td>PDIA</td>
<td>Protein disulfide isomerise family A member 3</td>
</tr>
<tr>
<td>PF4</td>
<td>Platelet factor 4</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit Alpha</td>
</tr>
<tr>
<td>PolyI:C</td>
<td>Polyinosinic:polycytidylic acid</td>
</tr>
<tr>
<td>PP</td>
<td>Polypropylene</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PST</td>
<td>Polystyrene</td>
</tr>
<tr>
<td>PRBC</td>
<td>Packed red blood cell(s)</td>
</tr>
<tr>
<td>PSMD7</td>
<td>Proteasome (prosome, macropain) 26S Subunit, Non-ATPase, 7</td>
</tr>
<tr>
<td>RAC1</td>
<td>Ras-related C3 botulinum toxin substrate 1</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptors for advanced glycation end products</td>
</tr>
<tr>
<td>RANTES (CCL5)</td>
<td>Regulated on activation normal T-cell expressed and secreted (Chemokine (C-C motif) ligand 5)</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell(s)</td>
</tr>
<tr>
<td>RECESS</td>
<td>Red-cell storage duration study</td>
</tr>
<tr>
<td>REL</td>
<td>v-rel reticuloendotheliosis viral oncogene homolog</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>rhClec9A</td>
<td>Recombinant human Clec9A</td>
</tr>
<tr>
<td>RPL32</td>
<td>Ribosomal protein L32</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute Medium</td>
</tr>
<tr>
<td>s</td>
<td>Second(s)</td>
</tr>
<tr>
<td>SAGM</td>
<td>Saline, adenine, glucose and mannitol solution</td>
</tr>
<tr>
<td>sCD40L</td>
<td>Soluble CD40 ligand</td>
</tr>
<tr>
<td>scu-PA</td>
<td>Single chain urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SlanDC</td>
<td>6-Sulfo LacNAc-expressing dendritic cell(s)</td>
</tr>
<tr>
<td>SS</td>
<td>Sphero-echinocyte(s)</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>SL</td>
<td>Sphero-echinocyte(s)</td>
</tr>
<tr>
<td>sTM</td>
<td>Soluble thrombomodulin</td>
</tr>
<tr>
<td>SYK</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>TAFI</td>
<td>Thrombin activatable fibrinolysis inhibitor</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter antigen presenting molecule</td>
</tr>
<tr>
<td>TGA</td>
<td>Therapeutic Goods Administration</td>
</tr>
<tr>
<td>TGF</td>
<td>Tissue growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper</td>
</tr>
<tr>
<td>THBD</td>
<td>Thrombomodulin intronless gene</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TM</td>
<td>Thrombomodulin</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TRAP</td>
<td>Thrombin receptor-agonist peptide</td>
</tr>
<tr>
<td>TRALI</td>
<td>Transfusion-related acute lung injury</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>v450</td>
<td>Violet 450</td>
</tr>
<tr>
<td>vs</td>
<td>Versus</td>
</tr>
<tr>
<td>XCR1</td>
<td>X-C motif chemokine receptor 1</td>
</tr>
</tbody>
</table>
Chapter 1. Introduction

Synopsis: This introductory chapter provides a review of the literature around blood transfusion and immune modulation associated with transfusion. It presents an overview of clinical and laboratory studies of transfusion-related immune modulation. Specifically, this chapter discusses the potential mechanisms underpinning transfusion-mediated immune modulation, with particular emphasis on the potential role of dendritic cells (DC) and a specialised receptor on their surface - C-type lectin receptor family member 9 (Clec9A). The final section of this chapter provides a summary of current deficiencies in our understanding of transfusion biology, outlining the significance and motivation behind this Doctor of Philosophy thesis.
1.1. Introduction

The advent of modern transfusion science dates back to the 1900’s, with Karl Landsteiner’s discovery of the ABO blood group antigens providing a scientific basis for blood transfusion. Since then, various transfusion-associated infectious complications have emerged, including hepatitis C virus (HCV) and human immunodeficiency virus (HIV) [1]. Although the risk of acquiring transfusion-transmitted infection has been dramatically reduced in developed countries with advances in viral testing technologies, other transfusion-associated hazards remain including transfusion-related immune modulation [2]. The first observation of a form of transfusion-related immune modulation was in 1973 where Opelz et al. (1973) reported improved renal-graft survival with increased number of blood transfusions in humans [3]. More recent studies suggest that blood transfusions mediate immune modulation that contribute to poor patient outcomes, which can result in increased rates of morbidity, mortality, infectious complications and prolonged length of hospital stays (LOS). A further consideration in this matter is the highly controversial topic that prolonged ex-vivo storage of blood components prior to transfusion exacerbates transfusion-related immune modulation and in patient outcomes. A number of in-vitro and in-vivo studies have demonstrated that exposure to blood components alters responses of immune cells. Despite these laboratory based studies, the receptors and mechanisms underpinning transfusion-related immune modulation in patients remain largely undefined.

Therefore, to address knowledge deficiencies in this area, my PhD study focuses on identifying and understanding mechanisms underpinning transfusion-related immune modulation. I investigated the impact of the two most frequently used blood products, packed red blood cells (PRBC) and buffy-coat-derived platelet concentrates (PC; Figure 1.1), as well as an emerging product cryopreserved platelets (cryo-PLT; Figure 1.1) [4], on key immune regulatory cells and their surface receptors. Specifically, I studied responses in myeloid dendritic cells (mDC) and the specialised subset of mDC expressing blood dendritic cell antigen 3 (BDCA3+) and the C-type lectin receptor family member 9 (Clec9A). A more comprehensive review of these elements of my research is provided in the following sections of this dissertation.
Figure 1.1. **Blood component production scheme.** Reproduced with permission [5] with modifications.
1.2. PRBC transfusion
Transfusion of PRBC is widely used to treat decreased oxygen carrying capacity in patients caused by disease, severe traumatic or surgical blood loss. Although PRBC transfusion is life-saving, it is not without risk. An increased rate of poor patient outcomes following PRBC transfusion has been reported. Although storage-related PRBC modifications are well documented, whether these changes contribute to worse patient outcomes remains highly debated and controversial. Despite evidence of immune modulation, the exact receptors and mechanisms underpinning this phenomenon and driving poor patient outcomes remains largely undefined.

1.2.1. Clinical outcomes of PRBC transfusion
Clinical studies have been associated PRBC transfusion with adverse outcomes in several patient cohorts including trauma [6-10], intensive care (ICU) [6, 11, 12], surgery (general, cardiac, colorectal and orthopaedic) [6, 13-18] and oncology [19-21]. Outcomes of these studies were based on endpoints including morbidity, mortality, infectious complications, LOS, cancer recurrence and other adverse outcomes including multiple organ dysfunction (MOD) and acute respiratory distress syndrome (ARDS). A further consideration in modulation of patient outcomes following transfusion is the ex-vivo storage duration of PRBC used for transfusion. Studies demonstrated increased rates of mortality [22-28], morbidity [29], MOD [22, 27, 30] and infection [22, 31, 32] in trauma, cardiac surgery, cardiovascular and ICU patients receiving blood stored for two weeks or more. On the other hand, several other studies were unable to demonstrate that the storage duration of blood components utilised have contributed to adverse patient outcomes [24, 33-39]. Despite the numerous studies available, significant variation in patient cohorts, study designs and patient outcomes, have limited the conclusions that could be drawn. A number of systemic reviews have been published on the topic.

A systematic review of 45 observational studies, published between 1966 and 2007 has reported outcomes (mortality, infections, MOD and ARDS) in critically ill patients associated with PRBC transfusion, published by Marik and colleagues (2008) [6]. PRBC transfusion was identified as an independent risk factor increasing the rate of mortality, infectious complications and other adverse effects in patients. In a systemic review of 32 randomised observational studies (between 2006 and 2010), Hopewell et al. (2013) has reported that PRBC transfusion was associated with mortality in ICU, trauma, surgery patients and cancer patients [40]. A recent Cochrane Systematic Review lead by Brunskill
et al. (2015), analysed 16 randomised controlled clinical trials that compared mortality, MOD, infection or LOS following “fresh” versus (vs.) “old” PRBC transfusion, and fresher PRBC transfusion vs. standard care practice for all patient conditions [41]. The authors stated no definitive conclusion could be reached due to varying definitions of “fresh” and “old” PRBC (storage duration) and in study designs [41]. In a similar systemic review, 12 randomised clinical trials published between 2014 and 2015 were investigated [42]. Alexander and colleagues (2016) reported no correlation between prolonged PRBC storage duration and increased mortality in patients with moderate certainty and overall adverse outcomes with low certainty [42].

Three prospective multi-centre studies have been recently published (with one more in progress), with the aim to provide evidence of the impact of PRBC storage duration prior to transfusion on patient outcomes. The Red-Cell Storage Duration Study (RECESS), a randomised controlled clinical trial led by Steiner et al. (2015) investigated whether transfusion with “fresh” vs. “old” PRBC increased the risk of MOD and mortality in 1098 patients undergoing cardiac surgery [12]. Neither mortality rate (7 days and 24 post transfusion) nor MOD score differed between patients transfused with PRBC stored for ≤10 days (“fresh”; median of 7 days) or ≥ 22 days (“old”; median of 28 days). Outcomes from the Age of Blood Evaluation (ABLE) trial also report no association between mortality of critically ill adults transfused with “fresh” (mean of 6.1 ± 4.9 days) and standard issue or “old” (mean of 22.0± 8.4 days) PRBC [17]. In addition, the authors reported no differences in any of the secondary outcomes investigated, including major illnesses, LOS and transfusion reactions. Similarly, a randomised controlled study by Heddle et al. (2016) report that mortality rate between a total of 25594 patients (undergoing cardiovascular surgery or in ICU or with cancer) transfused with “fresh” (mean of 13 days) or “old” (mean of 23.6 days) PRBC did not vary significantly [43]. Furthermore, a current randomised double blinded phase III trial comparing mortality rate and MOD score between ICU patients receiving standard issue and freshest available PRBC, the STandaRd Issue TrANsfusion versus Fresher red blood cell Use in intenSive carE (TRANSFUSE) trial, is under way [44].

Although these recent prospective studies together failed to detect a correlation between PRBC storage duration and adverse clinical outcomes for the parameters measured, this topic remains controversial and further studies are required. In the majority of the above mentioned studies, the mean storage duration of around 22 days is considered “older”
PRBC, and due to current standard practice and ethical considerations, the capacity to randomise patients to receive PRBC at date-of-expiry (D42) is limited. It is without doubt that red blood cells (RBC) undergo significant biochemical and biomechanical changes during routine ex-vivo storage (section 1.2.2 and 1.2.3). Furthermore there is a substantial decline in PRBC quality after 21 days of ex-vivo storage. Therefore, conclusions that can be drawn in relation to the influence of PRBC storage duration and poor patient outcomes are precluded by restrictions in clinical trials to date. Whether transfusion of stored PRBC near date-of-expiry is associated with poor patient outcomes remains a highly controversial issue in transfusion medicine.

1.2.2. PRBC processing and storage
In Australia, PRBC are leukodepleted via filtration and stored in saline-adenine-glucose-mannitol (SAGM) at 4°C [45]. Fresh PRBC intended for clinical transfusion currently have an expiry of 42 days post-collection. This date-of-expiry is based upon the requirements of in-vivo survival of the transfused PRBC. In the 1980’s, the United States Food and Drug Administration (FDA) stipulated 24 hour (h) in-vivo recovery of $\geq 75\%$ for transfused PRBC [46].

1.2.3. “PRBC storage lesion”: Biochemical and biomechanical modifications
During ex-vivo storage, RBC products undergo significant modifications that can alter cellular function and viability [47]. This biological process is commonly referred to as the “PRBC storage lesion”. This phenomenon was originally described by Gabrio and colleagues’ [48] who demonstrated that refrigerated RBC age more rapidly than those in-vivo. Stored RBC have since been extensively studied and numerous biochemical and biomechanical modifications have been reported (Table 1.1), notably i) depletion of 2,3 diphosphoglycrate (2,3 DPG), ii) reduction of adenosine triphosphate (ATP), iii) destabilisation of RBC membrane, iv) reduction in nitric oxide (NO), and v) RBC microparticles (microvesiculation) [33, 49, 50].
Table 1.1. Overview of “PRBC storage lesion”: Parameters modified during ex-vivo storage. Table was reproduced with permission [51] and with partial modifications.

<table>
<thead>
<tr>
<th>Effects of “PRBC storage lesion”</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>↓ pH, ATP</td>
<td>↑ Osmotic fragility</td>
</tr>
<tr>
<td>↓ 2,3 DPG</td>
<td>↑ Mechanical fragility</td>
</tr>
<tr>
<td>↓ Glutathione</td>
<td>↑ Vascular adhesion</td>
</tr>
<tr>
<td>↑ Free heme</td>
<td>RBC microvesiculation</td>
</tr>
<tr>
<td>↑ Extracellular K⁺</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>Na⁺/K⁺ pump paralysis</td>
<td>↑ RBC aggregability</td>
</tr>
<tr>
<td>Echinocytosis</td>
<td>Alter NO metabolism</td>
</tr>
</tbody>
</table>

1.2.3.1. 2,3 DPG and ATP

2,3 DPG is an essential biochemical, which contributes to oxygen release in tissues by forming a low oxygen affinity complex when binding to deoxyhaemoglobin. At two weeks of routine PRBC storage, 2,3 DPG has been shown to be significantly depleted resulting in increased oxygen affinity and potentially impairing oxygen delivery to tissues post-transfusion. In addition to depletion of 2,3 DPG, the storage of PRBC results in loss of RBC ATP [52]. ATP is central to many biochemical pathways, therefore, depletion of this component in RBC from PRBC during ex-vivo storage has been reported to modulate a number of cellular processes. This consists of processes involved in maintenance of the potassium and sodium gradient (K⁺ Na⁺), stabilisation of the cell membrane and vasodilation under hypoxic environments [49]. With loss of ATP, the K⁺ Na⁺ gradient pump is inactivated, equilibrium is lost and a substantial amount of K⁺ ions are leaked into the PRBC storage medium.

1.2.3.2. De-stabilisation of RBC membrane

In addition to biochemical changes, during routine PRBC storage the stability of the cellular membrane is altered, resulting in phospholipid translocation and impaired deformability. Phospholipids, such as phosphatidylserine (PS), are translocated from the inner leaflet to the outer cell surface via a “flip-flop” and scrambling movement of the plasma membrane, induced by deregulation of three essential enzymes: flipase, floppase and scramblase [53-56]. Phospholipid translocation to the RBC surface initiates removal of cells by the host immune system, predominantly via macrophage mediated phagocytosis [57-60]. During storage, PRBC deformability is progressively reduced. Cells change from a
flexible discoid shape, which aids movement and oxygen delivery in circulation, to “stiff” stages such as i) echinocytes (E), ii) spher-echochinoctyes (spicules) and iii) spheroechinoctyes (SL; smooth; Figure 1.2) [47]. The impaired deformability results in reduced oxygen delivery to tissues and can lead to haemolysis [61].

![Microscopic images of fresh RBC and stored PRBC.](image)

**Figure 1.2. Microscopic images of fresh RBC and stored PRBC.** A) Fresh RBC and B) day (D)42 PRBC. (E: echinocytes, SS: spher-echochinoctyes (spicules), SL: spheroechinoctyes (smooth)). Reproduced with permission from Mitrofan-Oprea et al. Novel criteria for assessing red blood cell viability in blood banks. *Transfusion Clinique et Biologique* 2007; 14(4):p.393-401. Copyright © 2015 Elsevier Masson SAS. All rights reserved. [47]

### 1.2.3.3. NO

NO is also affected by routine PRBC storage. During storage, the release of NO scavengers (such as microparticles and free heme) results in NO reduction [62]. As NO contributes to vasodilatation, the reduction in NO is predicated to reduce in erythrocyte passage through blood vessels, contributing to a hypoxic environment [63]. Despite the ongoing characterisation of the biochemical and biomechanical changes observed as a result of PRBC storage, the precise mechanisms driving these cellular changes and how they impact on transfusion outcomes are still not well understood. The clinical relevance and significance of these modified components remain active areas in transfusion research. To further understand and elucidate the underlying mechanisms driving transfusion-related immunomodulation and that may compromise patient prognosis, *in-vitro* and *in-vivo* models have been developed to examine the immunological changes upon interaction of stored PRBC with “host” immune cells.

23
1.2.3.4. Microparticles
During routine storage, RBC release submicron fragments known as microparticles, and the number of microparticles increases with prolonged duration of storage [64-67]. These submicron particles have been shown to induce changes in NO pathway of vascular contractility due to the increased NO scavenging activity by haemoglobin carried by microparticles [68]. In addition, the microparticle surface has high levels of PS which is associated with pro-coagulant reactions [64, 69]. Exposure to microparticles also induce pro-inflammatory responses [70, 71] and augment PBMC survival as well as mitogen-induced T lymphocyte proliferation [70].

1.2.4. Emerging labelling technology for RBC in context of transfusion: Function-spacer-lipid (FSL)
The current standard techniques to label RBC for the assessment of cell morphology and survival in transfusion models involves the use of radioisotope chromium 51 ($^{51}$Cr) [72] or biotinylation [73, 74]. Both are reliable RBC labelling techniques, however, they also share some disadvantages. The use of radiation poses risks to individuals involved in preparing the labelled cells and for the transfusion recipients [72]. While biotinylation is safer, it is more laborious and requires a secondary reagent for detection, which may affect the consistency of the outcomes. In the last decade, an alternative method to label cells has been developed that involves the use of KODE technology, FSL constructs [75]. The constructs are comprised of three components: functional head (F), which can represent a variety of biological functional groups, a spacer (S) selected to provide spacing between F and the membrane and, a lipid (L) tail for spontaneous incorporation into cell membrane anchoring into the lipid bi-layer [75].

FSL constructs are not only useful in providing visualisation and tracking cell survival, but can be used to modify cells to generate a desired phenotype (e.g. blood group antigens) without affecting the host cell function [76]. Using FSL-fluorescein (FSL-FLRO4) or Atto488 fluorophores (FSL-FLRO4-II) in a zebrafish transplantation model, Lan et al. (2012) report efficient labeling and visualisation of transplanted kidney marrow cells from adult zebrafish to a recipient fish without affecting host cell function [77]. Moreover, using a murine transfusion model, Oliver and colleagues (2011) demonstrate the capacity of FSL-biotin labeled RBC to be recovered post-transfusion, reporting approximately 10% loss of labeled cells per day [78]. This can be a valuable technology to adopt for assessing PRBC survival for transfusion as well as transfusion-related research.
However, no previous studies have reported the stability of FSL labelling clinical grade PRBC at different *ex-vivo* storage duration.

### 1.2.5. *In-vitro* and *in-vivo* models of PRBC transfusion (related to immune modulation)

The potential for PRBC to modulate recipients’ immune response and impact patient outcomes has been studied using *in-vitro* [79-89] and *in-vivo* [90] models. An *in-vitro* study using a whole blood model reported increased interleukin (IL)-10 production and reduced tumour necrosis factor (TNF)-α production by the overall leukocytes in the culture supernatant following 24 h exposure to either autologous or allogeneic PRBC [81]. Similar studies consistently report suppression of TNF-α and augmentation of IL-10 responses following exposure to PRBC [89] or PRBC supernatants [85]. Schneider et al. (2009) have also noted a PRBC dose-dependent modulation when co-stimulated with lipopolysaccharide (LPS), with increased suppression of TNF-α at 50% and 75% blood replacement compared to 25% [89]. Karam et al. (2009) report that co-culture with PRBC supernatants also augmented IL-6 production [85]. Modulation of monocyte inflammatory responses have also been reported in two other independent studies, with significantly reduced LPS-induced TNF-α production following exposure to PRBC [86]. The authors also compared the impact of different *ex-vivo* storage durations (D7, D14 and D21) and additives (citrate-phosphate-dextrose solution (CPD), AS-1 and AS-3). A significant suppression of monocyte responses following exposure to D21 CPD-stored PRBC was observed [86]. PRBC supernatant-mediated neutrophil priming [82, 87], and superoxide production [83] was also reported.

In addition, the potential for blood transfusions to modulate T lymphocyte responses has also been demonstrated in a number of studies, although the outcomes of these studies were not concordant. In one study, T lymphocyte activation (CD25⁺) was not significantly affected following exposure to PRBC supernatants [85]. However, in another study PRBC supernatants increased the number of regulatory T lymphocytes (CD25⁺ Treg) and reduced the number of T responder lymphocytes (CD25⁻) [79]. A more recent study indicates B and T lymphocyte proliferation was suppressed following exposure to leukodepleted PRBC stored for two to three weeks, but not freshly collected leukodepleted RBC [88]. Suppression of T lymphocyte proliferation has also been reported in studies utilising PRBC in a whole blood model [84], and in a cell culture model utilising PRBC supernatants and Jurkat T lymphocytes [80].
To date, studies assessing the role of DC in transfusion outcomes are limited. Research at the Australian Red Cross Blood Service (Blood Service) report suppression of DC cytokine, chemokine and co-stimulatory molecule expression [84], and modulation of antigen presentation and signal transduction gene expression following exposure to PRBC in-vitro [84]. In addition, a murine transfusion model using non-leukodepleted PRBC demonstrated CD200⁺ (tolerising) DC may play a role in modulating transfusion-associated tumour growth [90]. Erythrophagocytosis by murine plasmacytoid (p)DC particularly in co-stimulation with TLR3 agonist, polyinosinic-polycytidylic acid (polyI:C)-driven inflammation has been reported [91]. Murine studies have also demonstrated that splenic DC are important for outcomes in terms of alloimmunisation [92, 93].

These in-vitro and in-vivo models suggest that transfusion modulates the function of key immune cells. The differences in models (isolated cells vs. whole blood models), choice of component (autologous vs. allogeneic; PRBC vs. PRBC supernatant only), dosage modelled (25%, 50% or 75% replacement volume) and additive storage solution used (SAGM vs. CPD) limit the broad applicability of the conclusions that can be drawn. However, the specific cells, receptors and pathways that mediate PRBC transfusion-related immune modulation remain largely undefined.
1.3. PC transfusion

Platelet transfusion is the required standard of care for treating patients with thrombocytopaenia and bleeding associated with severe trauma. Platelets are also transfused prophylactically in patients with bone marrow suppression or failure from ablative chemotherapy or neoplasia to maintain haemostasis and limit bleeding, especially when undergoing invasive bedside procedures or surgery. Although platelet transfusions are widely used, they are also associated with adverse patient outcomes. To date, mechanisms driving PC transfusion-related immune modulation in patients remain largely undefined.

Two main sources of platelets are used in clinical transfusion: buffy-coat-derived PC (pooled from multiple ABO compatible donors); and, apheresis platelets (single donor). Buffy-coat-derived PC are more frequently used in transfusion in Australia compared to apheresis platelets. In my dissertation, platelets derived from buffy coats stored at room temperature (liquid form), are the focus of my research.

1.3.1. Clinical outcomes of PC transfusion

While it is clear that PC transfusions are an effective therapy, studies suggest that PC transfusion is associated with higher rates of adverse patient outcomes than PRBC transfusion [94-96]. PC transfusion-related reactions are frequently reported, characterised by febrile non-haemolytic reactions (chills and rigors) and allergic (rash and urticaria) reactions [97-103]. A few mechanisms have been proposed, including the presence of donor leukocytes and the release of soluble human leukocyte antigens (HLA) during storage, as a trigger of these poor outcomes post-PC transfusion [104, 105]. Reactions mediated by HLA antibodies were responsible for a large proportion of reported febrile non-haemolytic reactions, which were significantly reduced following the implementation of leukodepletion [106, 107].

In addition, transfusion of PC has the potential to modulate the immune system, contributing to transfusion-related immune modulation with outcomes including increased mortality [108-111], organ injury (transfusion-related acute lung injury (TRALI)) [112], infectious complications and prolonged LOS [10, 108]. However, studies reporting the incidence of transfusion-related immune modulation are limited.
Spiess et al. (2004) analysed six randomised controlled trials in cardiac surgery patients [108]. The author reported frequently occurring stroke and mortality in 284 of 1720 patients following non-leukodepleted pooled PC and apheresis PC transfusion [108]. However, a retrospective study by McGrath et al. (2008) reported no association of PC transfusion with increased risk for postoperative mortality, infectious complications, neurologic events, and other adverse outcomes in perioperative patients [113]. Similarly, Karkouti and colleagues (2006) also report no association between PC transfusion and increased rate of mortality or morbidity in a study of 11459 cardiac surgery patients [114].

PC are routinely stored for up to five days before transfusion [115]. Currently, whether stored PC are associated with increased rates of poor patient outcomes are still controversial. A number of studies conclude that transfusion with stored PC results in increased febrile non-haemolytic reactions [97, 116-118], whilst Welsby et al. (2010) did not observe significant differences in postoperative infection, LOS or mortality among cardiac patients at different storage durations (D2, D3, D4, or D5) of the single leukodepleted apheresis platelet unit transfused [119]. Heddle et al. (1993) and Kauffman et al. (2014) observed an association between dosage of PC used for transfusion and adverse patient outcomes [97, 120]. Collectively these studies demonstrate PC storage, as well as potentially dosage, may mediate transfusion-related immune reactions and modulations.

1.3.2. PC processing and storage

In Australia, buffy-coat-derived PC are pooled from four units of ABO compatible buffy coats, followed by leukodepletion via filtration. PC are stored in ~72% SSP+/~28% plasma for up to five days at 20-24°C with continuous agitation [45]. The shelf life of PC has been extended to up to 7 days in some European countries [121, 122] and in the United States [123]. In the FDA guideline established in 1999, a minimum standard for in-vivo survival and recovery has not been implemented [124]. In 2002, the blood advisory committee of the American Society of Blood Transfusion recommended to set the criteria for in-vivo recovery and survival of platelet post-transfusions as 66% and 50% of fresh platelets [125]. Clinical studies used Cr\textsuperscript{51} and Indium\textsuperscript{111} as radioactive labels for platelets and assessed in-vivo recovery and survival of PC infused into healthy volunteers at various time points [125]. FDA later established ratios for both in-vivo recovery and survival of PC to be 66% of fresh platelets [125].
1.3.3. “PC storage lesion”: Biochemical and biomechanical modification

Concerns have risen in relation to platelet therapeutic efficacy and safety over the routine ex-vivo storage period. During ex-vivo PC storage, platelets undergo progressive biochemical and biomechanical modifications, referred to as “platelet storage lesion”. These include platelets becoming more activated with increased expression of CD62P (P-selectin) [126-129], alteration of matrix adhesion (gamma-tubulin complex protein (GIP)) properties [130], increased loss of membrane asymmetry [126-128, 131] and lactate generation [126]. Further, reduction in aggregation with adenosine di-phosphate (ADP) [126, 132], and hypotonic shock response [126] have also been reported. These changes have been associated with reduced in-vivo survival and recovery of stored platelets [126-128, 132-134].

In addition, increased release of microparticles bearing apoptotic molecules and inflammatory mediators such as soluble CD40 ligand (sCD40L) [102, 135, 136], histamine [137], RANTES (C-C motif chemokine 5 (CCL5), platelet factor-4 (PF4), transforming growth factor-β (TGF-β) and IL-8 [138] have also been identified in PC following ex-vivo routine storage.

1.3.3.1. sCD40L

sCD40L is one of the most abundant platelet-derived substances in PC. Accumulation of sCD40L in stored PC supernatants has been reported to have potential deleterious effect on the function of immune cells through inflammation, which impacts downstream immune responses such as release of cytokines and chemokines, and expression of adhesion molecules [102, 139-144]. In-vitro studies have demonstrated the capacity of sCD40L to mediate suppression of monocyte IL-12 production [139], and induce MoDC maturation and co-stimulation [102, 140-144]. sCD40L has also been reported to prime neutrophil oxidase mediating cytotoxicity in a human pulmonary microvascular endothelial cell (HMVEC) model [145].

1.3.3.2. Histamine

Histamine is essential in regulating allergic reactions and inflammation. Secretion of cytokines and chemokines from LPS stimulated monocyte-derived DC (MoDC) was reported in the presence of histamine [146, 147]. MoDC production of IL-12, IL-6, IP-10, MIP-31, IL-18 and RANTES was down-regulated, and production of IL-8 and IL-10 was
increased [146, 147]. In addition, histamine suppressed monocyte secretion of IL-12 and TNF-α, while inducing IL-10 secretion, acting as an anti-inflammatory mediator [148, 149].

1.3.3.3. PF4
During routine *ex-vivo* storage, PF4 is released by activated platelet alpha-granules promoting coagulation. *In-vitro* studies have demonstrated enhanced monocyte survival and mediation of monocyte differentiation into macrophages [150]. PF4 has also up-regulated MoDC IL-12 and TNF-α production in the presence of LPS but lowered expression of maturation molecule CD83 [151].

1.3.3.4. Microparticles
Platelet-derived microparticles have been reported to promote expression of adhesion molecules on monocytes and endothelial cells, enhancing their adherence [152]. Additionally, elevated release of microparticles bearing apoptotic molecules has been reported to increase macrophage phagocytosis [150]. MoDC differentiation was also modulated in the presence of microparticles resulting in reduced expression of HLA-DR [153]. Co-culture of microparticles with MoDC down-regulated LPS-stimulated expression of HLA-DR and CD80, and production of IL-1β, IL-6, IL-10, IL-12, TNF-α and interferon (IFN)-γ [153].

1.3.4. *In-vitro* and *in-vivo* models of PC transfusion (related to immune modulation)
A few mechanisms have been proposed, including the presence of donor leukocytes and the release of soluble HLA during storage, as a trigger of poor outcomes post-PC transfusion [105, 154]. Reactions mediated by HLA antibodies were responsible for a large proportion of reported febrile non-haemolytic reactions, which were significantly reduced following the implementation of leukodepletion [106, 107], however, the incidence of transfusion-related immune modulation remained largely unchanged.

In addition to the critical role platelets have in coagulation and haemostasis, there is emerging evidence of a fundamental role for platelets in regulating immune cells including neutrophils [155], monocytes [156, 157], B lymphocytes [158, 159], T lymphocytes [159, 160] and DC [142, 161, 162]. This provides a basis for potential mechanisms to investigate in the context of transfusion-related immune modulation.
*In-vitro* and *in-vivo* models have been used to study the potential mechanisms for the reported immune aberrations. *In-vivo* models have reported that a number of platelet-derived substances, such as sCD40L, RANTES and bioactive lipids, may have potential adverse effects in recipients. This includes induction of neutrophil priming, lung inflammation and coagulopathy, potentially serving as mechanisms of TRALI [145, 163, 164]. In addition, the expression of MHC class I surface molecules on platelets has been reported to mediate increased production of immunoglobulin G (IgG) underpinning skin graft adverse outcomes [165].

*In-vitro* studies have also reported activation of monocytes, B lymphocytes and T lymphocytes following exposure to PC stored for less than three days [166]. In addition, monocyte production of IL-12 was suppressed following exposure to PC with LPS [167]. Schneider et al. (2009) reported increased production of TNF-α and IL-10 by overall leukocytes after exposure to PC alone, with TNF-α reduced in the model of PC and LPS [89]. Recently PC supernatant has been shown to modulate mDC responses and the overall leukocyte inflammatory response [168]. In this model, PC supernatants predominantly suppressed mDC responses, particularly when modelling processes involved in bacterial infection with LPS. This study also reported that the modulation of the immune profile was dose-dependent rather than dependant on *ex-vivo* storage duration. In addition to blood DC, MoDC have also been used to assess immune modulation associated with PC transfusion, however, Hamzeh-Cognasse et al. (2008) found no changes in MoDC surface antigen expression or inflammatory profile when co-cultured with PC alone [162]. To date, the impact of PC transfusion on blood DC immune responses remains largely unexplored.
1.4. Cryo-PLT transfusion

Cryo-PLT transfusion is in routine use by the military to treat bleeding patients, and its therapeutic efficacy has been demonstrated. However, the capacity of cryo-PLT to mediate transfusion-related immune modulation in patients has not yet been investigated. As outlined above for conventional PC transfusion (section 1.3.1), there are two major sources of platelets used for clinical transfusion. In line with the study of PC transfusion, this dissertation focuses on cryo-PLT from buffy-coat-derived PC, stored at -80°C (solid form).

1.4.1. Clinical outcomes of Cryo-PLT

The use of cryo-PLT for transfusion has predominately been in military combat areas to treat bleeding patients, and therapeutic efficacy has been well documented. With the availability of cryo-PLT, the Netherlands military has reported abrogation of the backup plan, the “walking donor panel” since 2010 [169]. In combat areas, a “walking donor panel” is traditionally in place for when conventional PC are not available. This is a panel of pre-screened civilians where fresh whole blood donations can be collected and used without testing or leukodepletion prior to transfusion, carrying the potential risk of transmitting infection and transfusion-related acute reactions. In 1996, a case of transfusion-transmitted hepatitis B was reported with such a panel [169]. To date, the use of cryo-PLT and other cryopreserved blood components has reduced mortality rates in the military from 56% to 16% with no adverse transfusion outcomes reported [169].

Cryo-PLT products are not available in civilian clinical settings but in an effort to provide evidence to demonstrate their suitability, clinical trials have been conducted and one is underway. Clinical studies have demonstrated suitability of cryo-PLT for transfusion in thrombocytopaenic [170], leukaemic [171], cardiac [172] and trauma [173] patient groups. Schiffer et al. (1976) reported the capacity for cryo-PLT to control haemorrhage in 23 thrombocytopaenic patients [174]. In addition, low and high concentrations (2.7 and 9 x10^{12}/L) of cryo-PLT transfused were assessed and no significant differences in patient outcomes were reported [174]. In a separate study, the author also reported 225 transfusions of autologous cryo-PLT administered to 45 leukaemia patients with no adverse outcomes [175]. Between 1977-1982, post-transfusion platelet increments, in-vivo recovery, and the shortening of bleed time have been consistent [175].
Khuri et al. (1999) have reported a significant reduction in blood loss and total volume of blood products transfused for patients who received cryo-PLT in comparison to those receiving conventional PC [172]. A total of 73 cardiopulmonary bypass patients were involved, 24 patients received cryo-PLT and 29 patients received PC after heparin reversal with protamine was administered. However, in demonstrating the effectiveness of cryo-PLT in treating bleeding in a clinical setting, the study was under powered to satisfy clinician and regulatory organisation requirements preventing the adoption of cryo-PLT for use in the civilian setting. Subsequently, a large scale single-centre clinical trial was conducted where a total of 1082 units were transfused into 333 patients with no transfusion reactions reported [173]. The use of cryo-PLT in trauma patients when no tested conventional PC are available was approved by the Netherlands military and the German Civilian Paul Ehrlich Institute in 2012 [173].

The results from the aforementioned two studies led to the design of a randomised controlled multicentre clinical trial, which is currently underway [176]. This study aims to compare the bleeding time, mortality, LOS and infectious complications in 90 cardiac patients receiving cryopreserved vs. conventional apheresis platelets for surgical bleeding (CLIP trial, ACTRN12612001261808). It is hope that this trial will provide strong evidence in relation to cyro-PLT use for civilians.

1.4.2. Cryo-PLT processing and storage

Cryopreservation of platelets is an effective technique for prolonging ex-vivo shelf life up to two years at -80°C [170, 177]. The cryopreservation process for preserving the biological structure and/or function involves cooling cells at a very low temperature (typically at -80°C) [178]. To date, two approved platelet cryopreservation methods are available, first established by the Naval Blood Research Laboratory (freeze-thaw-wash) and later modified by the Netherlands Military Blood Blank (wash-freeze-thaw) [179]. Originally, apheresis-derived PC were stored at -80°C with 6% dimethyl sulfoxide (DMSO) cryoprotectant until required for transfusion. When required, cells were thawed at 37°C, followed by washing and centrifugation to remove DMSO prior to reconstitution in FFP [179]. While in the newly emerged method, the Netherlands Military Blood Blank removed supernatant containing DMSO by centrifugation prior to cryopreserving the PC [179]. In doing so, the post-thaw wash step can be eliminated, which then reduces the preparation time of cryo-PLT in times of emergency.
Cryopreservation of platelets can circumvent a number of issues associated with conventional PC stored at room temperature with a short shelf life, including logistics associated with supply while minimising wastage and the potential risk of bacterial contamination at prolonged storage [173, 178]. Therefore, supply of cryo-PLT is particularly appealing in military combat settings and for civilian use in remote areas. For transfusion, the FDA criteria require cryo-PLT for transfusion to have an *in-vivo* recovery of 66% and lifespan of 50% of fresh conventional PC [169].

1.4.3. “Cryo-PLT storage lesion”: Biochemical and biomechanical modification

The process of platelet cryopreservation for transfusion has been reported to modify cellular function [172, 180-184]. Valeri and colleagues (2005) compared the *in-vitro* quality of cryo-PLT processed using the original (freeze/with DMSO-thaw-wash) and modified (wash-freeze/without DMSO-thaw) method mentioned above in section 1.4.2 [185]. Freezing with DMSO resulted in an *in-vitro* recovery of 74-76%, while those with the DMSO removed prior to freezing had recovery of 69-78%. In addition, platelet-derived microparticles also increased by 10% in comparison to the original method. However, while using the modified procedure, Lelkens et al. (2006) and Johnson et al. (2011) reported similar recovery to those observed with the original method [179, 186]. In addition, Johnson et al. (2011) demonstrated that buffy-coat-derived cryo-PLT had an acceptable *in-vitro* function, with recovery similar to those made from apheresis product [186].

However, in comparison to conventional PC, *in-vitro* studies have reported that cryo-PLT are different in a number of parameters. Cryo-PLT (apheresis- or buffy-coat-derived) have a 77% average recovery of conventional PC [170, 172, 177, 179], with reduced capacity to aggregate arachidonic acid (AA) and ADP [172, 180, 181, 187], and respond to hypotonic stress [182]. Three independent studies have shown that cryo-PLT are more active compared to fresh PC (follow 24 h storage) [171, 186, 188], however, the proportion of platelets expressing activation markers CD62P and CD63 was similar between cryo-PLT and conventional PC at date-of-expiry [180]. In addition, Johnson et al. (2015) have also reported reduced expression of the key glycoprotein receptors GPIbα and GPIIb on cryo-PLT, indicating reduced adhesion capacity [180].

Cryo-PLT are also more haemostatically active with greater pro-coagulant activity, compared with conventional PC; increased levels of pro-coagulants such as factor V have
been demonstrated as well as thrombin and thromboxane following stimulation with AA or ATP [172, 180, 183, 187, 189]. For example, Khuri et al. (1999) observed significant differences in cryo-PLT in-vitro function (pH, hypotonic stress, aggregation and thromboxane generation) compared to “fresh” conventional PC and “stored” PC (after 24, 28 and 72 h ex-vivo storage) [172]. Notably, a significant increase in thromboxane level was reported in comparison to conventional PC following storage. This suggests thromboxane may be one of the factors contributing to cryo-PLT superior haemostatic capacity, as it plays an important role in this process, serving as constrictor of blood vessels and facilitator at the site of vessel injury to mediate platelet aggregation [190].

These modifications have been associated with a significantly higher number of non-discoid platelets [181], as well as microparticles expressing apoptosis mediators, such as PS, and other cytoskeletal membrane proteins within the unit itself [180, 183, 184]. Studies have reported that the cryo-PLT unit contains significantly higher numbers of PS- and tissue factor-positive microparticles than conventional PC [180, 183]. Increased levels of microparticles have been associated with reduced clotting time and increased thrombin generation [180, 183].

These changes may be due to excessive cellular dehydration, osmotic injury and extracellular ice crystal formation from the process of cryopreservation resulting in cryoinjury [191] (Figure 1.3). Although changes in cryo-PLT in-vitro are well documented, whether these changes in cryo-PLT mediate immune function modulation in transfusion recipients is currently unknown.

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**Figure 1.3. Diagrammatic representation of cryopreservation injury.** Reproduced from *Anaesthesia and Intensive Care* with the kind permission of the Australian Society of Anaesthetists [178].

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1.4.4. *In-vitro* and *in-vivo* models of cryo-PLT transfusion (related to immune modulation)

To date, no *in-vitro* studies have assessed the capacity of cryo-PLT to mediate transfusion-related immune modulation. However, with strong evidence to suggest that PC transfusion mediates immune aberrations in recipients, the potential for cryo-PLT to modulate immune responses should be considered.
1.5. DC and C-type lectin receptor: Potential mechanisms in transfusion-related immune modulation

To date, there are limited studies reporting the impact of blood transfusion on DC. Therefore, it is of interest to this PhD study to further explore the role of DC in the context of transfusion, given their importance in initiating and regulating the immune system.

1.5.1. DC

DC are bone marrow derived antigen presenting cells with a central function in the immune system to protect the host from tumours and invading pathogens, such as bacteria, fungi or virus [192, 193]. These specialised and unique cells initiate innate and adaptive immunity [194], stimulate and regulate primary B and T lymphocytes, antigen-specific T lymphocytes, and natural killer (NK) cells [193, 195]. DC also play a unique role in regulating self-tolerance [196] and sensitising and interacting with naïve T cells [197, 198]. These key DC properties are acquired following DC maturation.

DC maturation is a critical developmental pathway for initiation of immunity (Figure 1.4). Immature DC sample antigens from their local surroundings, facilitated by cytoplasmic projections expressing pattern recognition receptors which bind pathogen associated molecular patterns (PAMP) and/or damaged associated molecular patterns (DAMP). Upon ligation of DAMP or PAMP, antigens are then processed into MHC peptide complexes and presented on MHC class I or II receptors [199]. This process mediates migration of immature DC from the peripheral environment to the draining lymph node, where maturation is aided by cytokines, inflammatory or microbial stimulations [193, 200]. Mature stellate DC have increased expression of HLA-DR (MHC class II). DC maturation and expression of co-stimulatory molecules (CD40, CD80, CD83, CD86) are required for i) antigen presentation, ii) T lymphocyte stimulation and iii) secretion of cytokines and chemokines [195, 201]. Perturbation of DC maturation and activation has the potential to significantly affect host immune responses [193, 202, 203].
Within human peripheral blood, DC are broadly categorised into two major groups based on their lineage: myeloid (CD11c+) and plasmacytoid (CD123+) [193]. In addition, a number of DC subpopulations have been described based on phenotype, including myeloid BDCA1+ (CD1c+), myeloid BDCA3+ (CD141+), plasmacytoid BDCA2+ (CD303+) and plasmacytoid BDCA4+ (CD304+) DC [204, 205]. These cells have specialised functions shaped by the differential expression of numerous pattern recognition receptors including the relatively well-known and well-characterised toll-like receptors (TLR), and the less characterised C-type lectin receptors.

1.5.2. C-type lectin receptors

C-type lectin receptors are characterised by the presence of a highly evolutionarily conserved C-type lectin-like domain (CTLD) that commonly consists of six cysteine residues [206]. Based on evolutionary similarities, amino acid homology, phylogeny, and molecular architecture, Drickamer et al. (1999) originally proposed seven C-type lectin receptor subgroups [206]. Since then, numerous additional novel receptors have been described, expanding the C-type lectin receptor family into 17 subgroups [207, 208]. C-type lectin receptors encompass a broad group of receptors involved in recognition of both self and non-self, carbohydrate and non-carbohydrate ligands in either a calcium (Ca$^{2+}$)-dependent (“classic”) or calcium-independent (“non-classic”) manner [208].
Although the roles of C-type lectin receptors in transfusion medicine are not well understood, and reports are limited, a role for lectin-mediated recognition of changes in RBC and platelet properties as a result of storage has been demonstrated. Sorensen et al. (2012) [209] and Hoffmeister et al. (2011) [210] collated evidence from both in-vitro and in-vivo studies demonstrating that loss of terminal sialic acid from aging RBC [211, 212] and platelets [213, 214] exposes penultimate galactose, which mediates removal of cells by lectin receptors [215-218]. In addition, Sparrow et al. (2007) report stored PRBC had reduced lectin binding and suggest these changes were associated with altered carbohydrate moieties on the RBC membrane [219]. These studies highlight the potential importance of C-type lectin receptors that recognise DAMP in transfused cells and subsequent immune modulation in the transfusion setting.

1.5.2.1. C-type lectin receptor family member 9 (Clec9A)

Clec9A (also known as DC natural killer lectin group receptor 1 (DNGR-1)) is a more recently described Group V C-type lectin receptor expressed by a small subset of DC, monocytes and B lymphocytes, as well as an uncharacterised CD14+CD16+CD64+ cell population [220-222]. Of the four blood DC subsets, Clec9A is strictly expressed on BDCA3+ myeloid blood DC [220-222]. BDCA3+ DC are a rare cell population constituting only ~0.03-0.08% of human peripheral blood mononuclear cells (PBMC) [223, 224]. While the full extent of their contribution to the immune response and immune regulation is still to be elucidated, the high expression of CD62L (L-selectin) on BDCA3+ DC suggests efficiency in migration from the peripheral blood to lymph nodes [205]. In addition, studies reported superiority of BDCA3+ Clec9A+ DC in cross-presentation of dead cell-associated antigens to CD8+ cytotoxic T lymphocytes (CTL) [223-225], as well as cross-priming of CTL in respond to viral infection [226, 227].

Clec9A was first characterised as a DAMP receptor by Sancho and colleagues [221, 228]. The authors demonstrated cross-presentation of dead cell-associated antigens to T lymphocytes through coupling of necrotic cells with Clec9A expressed by murine CD8α+ DC [221, 228]. Based on phylogenetic homology, expression of Clec9A and several other surface markers (e.g. X-C motif chemokine receptor 1 (XCR1) [229]), human myeloid BDCA3+ DC have been proposed to be equivalent to murine CD8α+ DC subset [220-222, 230]. Both human and murine Clec9A recognise necrotic cells from nucleated and non-nucleated (including modified RBC and platelets) cells [231]. The ligand for Clec9A was
proposed to be filamentous actin (F-actin) [231-233], a key structural protein of eukaryotic cells, serving as a cell damage or death signal [234, 235].

1.5.2.1.1. Clec9A antigen presentation and signalling transduction pathways
Clec9A is an endocytic receptor with reported involvement in dead cell-associated antigen cross-presentation. Clec9A is reported to contribute to the regulation of dead-cell antigen associated CD8⁺ T cell response in a SYK-dependant manner via major MHC class I [220, 228, 236]. In mice, the intracellular pathways involved in cross-presentation are divided into “cytosolic” and “vacuolar”, but subdivisions of this pathway are not well characterised in human DC. It has been suggested that BDCA3⁺ DC cross-present antigen via cytosolic pathway [237]. This pathway transforms internalised proteins into peptides and delivers them to endocytic compartments for loading onto MHC class I complex for antigen presentation by transporter associated with antigen processing (TAP)1 and TAP2 [237, 238]. Moreover, it has been shown that transcription factor interferon regulatory factor 8 (IRF8) is essential for Clec9A cross-presentation [239], and amino-terminal peptidases, such as ER-associated aminopeptidase 1 (ERAP1), are required for trimming of polypeptides generated by proteases for presentation on MHC class I [240]. Clec9A DAMP antigen presentation to MHC class I subsequently activates signalling cascades mediated by serine-tyrosine kinase (SYK) pathway.

Clec9A contains a classical C-type lectin “hemi immunoreceptor tyrosine based activation motif” (hemiITAM) in its cytoplasmic tail (YxxL), which facilitates intracellular signalling via recruitment of SYK [220, 228, 241, 242]. It is postulated that the intracellular signalling pathway of Clec9A is mediated by SYK bridging two Clec9A molecules in an interaction similar to that which has been proposed for another C-type lectin receptor, Dectin-1 [220, 243]. The signalling pathway has been postulated as follows: coupling of DAMP to Clec9A triggers hemITAM interaction with tandem SH2 domains and, subsequently dual phosphorlyation of ITAM tyrosine residues activates SYK recruitment and subsequent downstream signalling [242]. Cellular responses are induced following downstream signalling, such as direct binding partners of SYK (e.g. p85a subunit of phosphoinositide 3-kinase (PI3K)) and signalling intermediates such as extracellular signal-regulated kinases (ERK), c-Jun NH(2)-terminal kinase (JNK), and nuclear factor of kappa B (NFκB) [242]. It has been demonstrated that Clec9A SYK-dependent signalling promotes activation of the key transcription factor NFκB in response to ligation of F-actin [227, 244].
Although it has been shown that Clec9A ligation results in activation of SYK signalling pathways and subsequent downstream signalling, investigation of changes in human BDCA3+ DC surface markers and cytokine production indicate Clec9A does not function as an immune activating receptor when anti-Clec9A monoclonal antibodies (mAb) are used for receptor ligation [223]. Co-stimulation of BDCA3+ Clec9A+ DC with anti-Clec9A mAb, polyinosinic-polycytidylic acid (poly:IC, TLR3 antagonist), and/or R848 (TLR7/8 antagonist), resulted in the expected up-regulation of maturation marker CD83 and co-stimulatory molecules (CD40, CD80 and CD86), as well as increased secretion of IL-1, IL-6, IL-8, interferon-gamma-inducible (IP)-10, (IFN-α and IFN-λ [223, 224, 245]. However, loss or blocked Clec9A receptor has been shown to reduce murine CD8α+ DC cross-presentation capacity but has no effect on BDCA3+ DC capacity to recognise and bind to damaged cells [228].

1.5.2.2. BDCA3/thrombomodulin (TM)

As mentioned, BDCA3 is a marker for distinguishing a rare subset of mDC. BDCA3+ DC have a different and unique profile to BDCA1+ DC. Compared to BDCA1+ DC, this DC subset is highly efficient in anti-viral responses, which are facilitated by the abundant expression TLR3 receptor (Figure 1.5) [224]. Additionally, BDCA3+ DC are superior in cross-presentation of antigens [223, 224, 228]. This so called “cross-presentation” has been reported to be important in mediating cytotoxic T lymphocyte anti-tumour immunity [224, 225, 246].

![Image](https://example.com/image.png)

**Figure 1.5. Differential profile of BDCA3+ and BDCA1+ DC.** Image reproduced with permission [247] with partial modifications.

Not only is BDCA3 a marker for distinguishing this rare specialised mDC subset, but it is also a membrane bound glycoprotein that was originally identified on endothelial cells as a
cofactor for generation of activated protein C (aPC), known as TM [248]. Subsequent to cloning and sequencing of the TM intronless gene (*THBD*), the putative structural organisation of human TM has been elucidated [249-251]. It consists of six major domains, beginning with i) N-terminus CTLD, followed by ii) six copies of epidermal growth factor-like (EGF) repeats, iii) O-linked domain, iv) hydrophobic transmembrane, and v) C-terminus cytoplasmic domain (Figure 1.6) [252]. Each domain corresponds to different localised activities, providing TM a multifaceted role and the capacity to bind many different molecules. Notably, the role of TM in coagulation, inflammation and immunity is largely associated with ligand interaction with CTLD and EGF domains [252].

Figure 1.6. Structure of TM and specific domains responsible for its function.
Schematic reproduced with permission [253].

TM-thrombin binding takes place at the EGF repeats 5-6 region [248], thereby triggering a cascade of changes, reducing coagulation and inflammation [253]. This process then accelerates aPC generation [248], activates thrombin activatable fibrinolysis inhibitor (TAFI) [254], inactivates complement protein (C)3b [255] and single chain urokinase-type plasminogen activator (scu-PA) [256] at the EGF region.
Further, the CTLD of TM binds to two known ligands – high-mobility box group 1 (HMGB1) protein and the carbohydrate Lewis Y. Binding of the CTLD with either ligand results in an anti-inflammatory profile [257, 258]. This occurs when TM binds chromosomal HMGB1 protein secreted by stressed cells, which subsequently prevents HMGB1 from triggering receptors for advanced glycation end products (RAGE) and activation of pro-inflammatory immunity [259]. For Lewis Y, a blood group antigen that can also be found on some bacteria, binding with TM induces agglutination and inhibits the LPS-induced infection in affected cells [258]. In-vitro and in-vivo studies have also shown that uptake of soluble TM (sTM) reduces BDCA3+ DC co-stimulatory surface antigen expression (CD80 and CD86), pro-inflammatory response (IL-6, TNF-α and IL-12) and co-stimulatory activity with T lymphocytes [260, 261], as well as modulation of gene expression of key immune pathways [262]. A similar BDCA3+ DC maturation and inflammatory profile following sTM treatment was reported in an allergy model, resulting in reduced allergic bronchial asthma [260]. In this study, Takagi et al. (2011) also associated BDCA3+ DC enhanced tolerogenicity with up-regulation of surface expression of TM following sTM [260]. In addition to sTM, expression can also be modulated by numerous other factors, such as heat shock, histamine, thrombin, soluble inflammatory mediators and more (Table 1.2) [253].

<table>
<thead>
<tr>
<th>TM up-regulation</th>
<th>TM down-regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat shock</td>
<td>TNF, IL-1β</td>
</tr>
<tr>
<td>Statins</td>
<td>Endotoxin</td>
</tr>
<tr>
<td>Retinoic acid, cAMP</td>
<td>Transforming growth factor (TGF)-β</td>
</tr>
<tr>
<td>Histamine</td>
<td>Hypoxia</td>
</tr>
<tr>
<td>VEGF</td>
<td>Oxidised low-density lipoprotein (oxLDL)</td>
</tr>
<tr>
<td>Thrombin</td>
<td>Shear stress</td>
</tr>
<tr>
<td>1,3 Dihydroxyvitamin D3</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>Adenosine</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>Prostaglandin E1</td>
<td>IL-4</td>
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</tbody>
</table>
Given the multifaceted role of DC in pattern recognition, phagocytosis, activation, signalling the adaptive immune response and coagulation, in particularly BDCA3^Clec9A^ DC, this specialised cell subset may be vulnerable to modulation by transfusion of blood products.
1.6. Rationale for study

Blood transfusion is reported to modify transfusion recipients’ immune responses, resulting in worse outcomes. In addition, *ex-vivo* storage of blood components has been proposed to further modulate recipients’ immune responses, however, the notion remains controversial. *In-vitro* and *in-vivo* studies have provided evidence that exposure to PRBC and PC modulates responses of both innate and adaptive immune cells. However, the precise receptors and mechanisms underpinning transfusion-related immune modulation in patients remain largely undefined. Furthermore, the immunomodulatory effect of cryo-PLT transfusion remains unexplored.

Therefore, this novel study investigated the role of DC (mDC and BDCA3⁺ DC) and their specialised pattern recognition receptor (Clec9A receptor) to help address these gaps in our current understanding of transfusion biology. DC have been selected as the basis of this study as they are central to the immune response, occupying a specific niche, functioning as innate antigen presenting cells and signalling the adaptive immune response. Yet, there are limited studies exploring the impact of transfusion on DC to date.

In addressing these deficiencies, my study generates important evidence regarding the potential mechanisms contributing to patients’ failure to mount an immediate immune response resulting in undesirable clinical outcomes. In addition it will provide additional support to whether storage duration prior to transfusion plays a role. Together, the newly assimilated knowledge can assist in further elucidating and understanding the nature of transfusion-related immune modulation.

1.6.1. Hypotheses and aims

1.6.1.1. Hypotheses

Given DC have a multifaceted role in pattern recognition, phagocytosis, activation and signalling the adaptive immune response, they may be particularly vulnerable to modulation by transfusion of blood products. I hypothesise that exposure to transfused blood components changes DC phenotype and function. I further hypothesise that Clec9A receptor expressed on DC have a role in transfusion-related immune modulation as it has been reported Clec9A binds the DAMP, F-actin on RBC ghosts and platelets. DC and Clec9A could be one mechanism that could potentially underpin transfusion-related immune modulation.
1.6.1.2. Aims

To address my research hypothesis, two main aims were established:

Aim 1.
To determine:

a) whether extracellular F-actin is present on PRBC, PC and cryo-PLT.

b) whether Clec9A receptors recognise and bind PRBC, PC and cryo-PLT.

Aim 2.
To characterise the immunomodulatory effects of PRBC, PC and cryo-PLT exposure on:

a) mDC and BDCA3+ DC surface antigen expression.

b) mDC and BDCA3+ DC cytokine and chemokine production.

c) overall leukocyte inflammatory response.

Chapter 3

Chapter 2, 4, 5 & 6
Chapter 2. Incorporation of fluorescein conjugated function-spacer-lipid constructs into the red blood cell membrane facilitates detection of labelled cells for the duration of ex-vivo storage

This chapter has been published as:


Additional data and discussion omitted in publication has been included in this chapter.

**Synopsis:** This chapter demonstrates the feasibility and capacity of Kode™ technology as a tool in labelling PRBC to aid *in-vitro* visualisation and tracking of PRBC during routine ex-vivo storage. To date, changes in stored PRBC physiology and morphology is well documented but the potential for PRBC storage duration to mediate transfusion-related immune modulation remains controversial. Therefore, a PRBC labelling method developed in this chapter was utilised for assessment of mDC and BDCA3⁺ DC erythrophagocytosis in Chapter 4, addressing thesis Aim 2.
2.1. Introduction

Transfusion-related immune modulation is a term used to broadly describe a variety of complications that have been associated with transfusion including increased rate of infection, LOS, mortality and morbidity in some patients [6]. The potential for PRBC transfusion to modulate recipient immune responses and result in poor patient outcomes is currently a central area of research in transfusion medicine. PRBC are routinely stored for up to 42 days (date-of-expiry) prior to transfusion and changes in both cell morphology and physiology occur during storage [33]. Length of ex-vivo PRBC storage prior to transfusion has been proposed to further mediate poor patient outcomes [33]. The capacity to evaluate and monitor PRBC recovery, survival and morphological changes is an important component of transfusion research.

To date, the use of radioactive $^{51}$Cr labelling has remained the benchmark for the measurement of RBC survival in human and animal models of transfusion [72]. Although this standard method is reliable, the use of radiation poses risks to individuals involved in preparing labelled cells, and the recipient [72]. Therefore, techniques that avoid use of radioactive compounds, such as covalently biotinylating RBC, have become more desirable and they have been used in both human and animal models successfully [73, 263]. However, RBC biotinylation has not been routinely adopted due to the potential risks associated with untargeted biotin reacting with surrounding residues (e.g. epsilon amino) [73]. Furthermore, in comparison to the standard $^{51}$Cr labelling, biotinylation is a more specialised and laborious technique and this approach can result in reduced RBC survival [264].

An alternative method to label cells involves the use of FSL. These constructs comprise of three components: functional head (F), which can represent a variety of biological functional groups; a spacer (S) selected to provide spacing between F and the membrane; and a lipid (L) tail for spontaneous incorporation into cell membrane via anchoring into the lipid bi-layer [75]. FSL constructs are not only useful in providing visualisation and tracking cell survival, but can be used to modify cells to generate a desired phenotype (e.g. blood group antigens) without altering the host cell function [75, 76]. This is a valuable technology that has been utilised to model transfusion reactions mediated by blood antigen incompatibility and predict in-vivo RBC survival in animals [78], and has the potential to be applied in human clinical studies, though this avenue has not yet been explored.
This chapter aimed to examine the suitability of FSL-FLRO4 constructs to label clinical grade PRBC, and to determine if the label could be retained during ex-vivo storage. The capacity to track and visualise PRBC at different points of routine storage has the potential to facilitate the development of tracking immunoassays, in particular, in-vitro erythrophagocytosis (Chapter 4). First, the binding and uptake of FSL-FLRO4 on PRBC at different storage duration was tracked using a time course approach to confirm feasibility. PRBC in circulation are frequently renewed due to eryptosis. The differences of FSL-FLRO4 in labelling recently enucleated (light-young) and older PRBC (dense-old, up to 120 days) within a PRBC unit during storage was investigated.
2.2. Materials and Methods

2.2.1. Ethics
Ethical approval was obtained from the Blood Service Human Research Ethics Committee (#100112, 2012) and The University of Queensland School of Medicine Low Risk Ethical Review Committee (2014-SOMILRE-009, 2014).

2.2.2. Reagents
FSL-FLRO4 constructs were obtained from Kode Biotech Materials Limited (Auckland, New Zealand). The construct is comprised of FLRO4 linked to an activated adipate derivative of dioleoylphosphatidylethanolamine (Figure 2.1) [75]. Phosphate buffered saline (PBS; Invitrogen, Carlsbad, California, USA) was used to reconstitute lyophilised FSL-FLRO4 (2 mg/mL stock concentration), prepare FSL-FLRO4 working concentration (50 µg/mL) and as a wash buffer following RBC labelling.

![Chemical structure of the FSL-FLRO4 construct.](image)

Figure 2.1. Chemical structure of the FSL-FLRO4 construct. Reproduced with permission [265].

2.2.3. Blood components
Leukodepleted PRBC units were obtained from the processing department of the Blood Service (Kelvin Grove, Queensland, Australia). PRBC units were obtained the day after standard processing and filtration procedures were completed (D2). Cells from a leukodepleted PRBC unit were labelled (see below) and assessed using a time course approach (n=6 independent experiments). To further assess the differences in the capacity of FSL-FLRO4 labelling, the PRBC unit was centrifuged to separate “young” and “old” cells (n=4 independent experiments). Based on the differential separation of young and old PRBC reported by Sparrow et al. (2007) the light-young- (top 10% of RBC layer) and dense-old-PRBC (bottom 10% of RBC layer) were obtained via density distribution centrifugation (3220 g, 30 min at 4°C) in Falcon tubes (50mL; Becton Dickinson (BD) Biosciences, San Jose, USA) [219].
2.2.4. FSL-FLRO4 labelling of RBC

100 µL of FSL-FLRO4 (50 µg/mL in PBS) was added to an equal volume of PRBC and mixed by vortexing [75]. Cells were incubated at 37°C for one hour, followed by three washes with PBS (1mL) and centrifugation (515 g, 5 min at 22°C). In parallel, a matched PRBC control was prepared with the omission of FSL-FLRO4 in 100 µL of PBS. Both FSL-FLRO4 and control PRBC were stored at 4 ± 2°C before flow cytometric assessment at the defined storage duration (D2, D7, D14, D21, D28, D35, D42). In addition, to assess whether different storage duration resulted in different uptake of FSL-FLRO4, an alternate labelling approach was used. Cells from the same PRBC unit were taken at weekly intervals and labelled with FSL-FLRO4 at each defined storage duration prior to flow cytometry.

2.2.5. Flow cytometry

At each time point, 3 µL of FSL-FLRO4 labelled PRBC or unlabelled PRBC control were assessed using FACSCanto II flow cytometer (BD, Franklin Lakes, New Jersey, USA). PRBC were gated based on FSC and SSC. FLRO4 emission of gated PRBC population was collected with 530/30 band pass filter following excitation with 488 nm laser. Unlabelled PRBC controls were used at each time point to establish the quadrants for determining FSL-FLRO4⁺ RBC.

2.2.6. Fluorescence microscopy

Fluorescence microscopy was carried out on PRBC at D2 and D42, to warrant visual confirmation of FSL-FLRO4 labelling of RBC at the beginning and end of the time course. An Olympus IX 73 fluorescence microscope (Shinjuku, Tokyo, Japan) with 60x objective was used. A volume of 5 µL of labelled PRBC or unlabelled PRBC control was diluted in PBS (2 mL). One drop of diluted cells were transferred onto a glass slide, and to enable a three dimensional observation of PRBC morphology, a cover slip was not applied. Fluorescence was set up to excite at 458 nm and detect the emissions at 518 nm from the FSL-FLRO4 labelled RBC. Images were first millisecond (ms) exposure time followed by the use of fluorescein filter for visualisation of FSL-FLRO4 labelled RBC using 2 second (s) exposure time.

2.2.7. Analyses and statistics

FCS Express V3 (De Novo Software, Glendale, California, USA) was used for analysis and representation of flow cytometric data. One way analysis of variance (ANOVA) with
Tukey’s post-test (D2 as the comparator) was used to assess changes in FSL-FLRO4 percentage (%) positive cells and median fluorescent intensity (MFI). For each time point, MFI was compared against D2 MFI (designated 100%). Unpaired t-test was used to compare means of FSL-FLRO4 MFI between light-young- and dense-old-PRBC at each time point. GraphPad Prism (GraphPad Software Inc., La Jolla, California, USA) was used for all analyses and graphic representation. $p<0.05$ was considered statistically significant.
2.3. Results

2.3.1. FSL-FLRO4 label was retained by PRBC for the duration of routine storage

Substantial biochemical and biomechanical changes occur during routine storage of PRBC [33]. To facilitate tracking and interaction of stored PRBC in both in-vitro and in-vivo transfusion models, a reliable method of PRBC labelling is required. The capacity of FSL-FLRO4 to be retained by PRBC during routine storage was assessed. 100% of labelled RBC remained positive for FSL-FLRO4 during routine PRBC storage (Figure 2.2A). Over the time course, a significant decrease ($p<0.0001$) in the intensity of FLRO4 was observed (Figure 2.2B). In comparison to the FSL-FLRO4 MFI at D2, an average reduction of 66% was observed by the date of PRBC expiry, D42 ($p<0.001$) (Figure 2.2C). This reduction was particularly apparent from PRBC at D35 ($p<0.0001$) onwards (Figure 2.2B, C). Despite this reduction over time, all cells were clearly identified as FSL-FLRO4+ following storage for 42 days. Together these data demonstrate FSL-FLRO4 is a suitable reagent for labelling RBC during storage of PRBC for visualisation and tracking.

![Figure 2.2. Determination of FSL-FLRO4 constructs suitability in labelling PRBC following ex-vivo storage for 42 days.](image)

(A) Histograms demonstrating binding of FSL-FLRO4 to PRBC at D2 and assessed weekly. D2 (red), D7 (purple), D14 (green), D21 (blue), D28 (maroon), D35 (yellow), D42 (black), unstained control (D2, solid grey). B) MFI of FSL-FLRO4 labelled PRBC. (C) Change in FSL-FLRO4 MFI compared to D2 (indicated as %). Bars indicate mean ($\pm$ standard error of the mean (SEM)). ANOVA indicated by $p$ values, Tukey's post-test analyses indicated as **$p<0.01$, ***$p<0.001$. Data derived from six independent time course assessments (n=6).
2.3.2. Age of RBC did not impact uptake of FSL-FLRO4 into the cell membrane

Mature enucleated RBC are constantly renewed from nucleated precursors in the bone marrow. Human RBC have an average life span of 120 days in circulation and a RBC donation for transfusion contains a heterogeneous population of both recently enucleated (light-young) and older PRBC (up to 120 days, dense-old). Given the decrease in FSL-FLRO4 intensity on labelled PRBC over time (Figure 2.2B, C), we considered that FSL-FLRO4 may remain more stably inserted in the membrane of younger PRBC, and the decrease in intensity of FSL-FLRO4 observed during storage may be due to reduced labelling of older PRBC. FSL-FLRO4 inserted into both light-young- and dense-old-PRBC, and both subsets remained FSL-FLRO4+ for the duration of the time course (Figure 2.3A, B). Assessment of mean MFI indicated that there was no difference in the capacity of light-young- and dense-old-PRBC to take up the label, and a similar loss of intensity was observed during storage (Table 2.1).

Figure 2.3. Assessment of whether both light-young- and dense-old-PRBC retain FSL-FLRO4 during routine storage. Histograms demonstrating binding of FSL-FLRO4 to (A) Light-young- and (B) dense-old-PRBC separated by density centrifugation at D2 and assessed weekly. D2 (red), D7 (purple), D14 (green), D21 (blue), D28 (maroon), D35 (yellow), D42 (black), unstained control (D2, solid grey).
Table 2.1. The impact of age of PRBC at collection on insertion or retention of FSL-FLRO4.

<table>
<thead>
<tr>
<th>Duration of PRBC storage</th>
<th>MFI of FSL-FLRO4 Mean (standard deviation)</th>
<th>(^1p) value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light-young-RBC</td>
<td>Dense-old-RBC</td>
</tr>
<tr>
<td>D2</td>
<td>57368 (6188)</td>
<td>58075 (8889)</td>
</tr>
<tr>
<td>D7</td>
<td>54310 (6254)</td>
<td>55237 (5185)</td>
</tr>
<tr>
<td>D14</td>
<td>50135 (6775)</td>
<td>52172 (4939)</td>
</tr>
<tr>
<td>D21</td>
<td>43780 (6516)</td>
<td>45598 (4539)</td>
</tr>
<tr>
<td>D28</td>
<td>40336 (5226)</td>
<td>41720 (4967)</td>
</tr>
<tr>
<td>D35</td>
<td>35371 (4018)</td>
<td>35455 (3454)</td>
</tr>
<tr>
<td>D42</td>
<td>31284 (7027)</td>
<td>30042 (3958)</td>
</tr>
</tbody>
</table>

\(^1p\) values obtained from unpaired \(t\)-test of FSL-FLRO4 labelled light-young- vs. dense-old-RBC. Data derived from five independent time course assessments (n=5).

2.3.3. Different storage duration of PRBC had minimal effect on FSL-FLRO4 uptake

Examination of whether different storage ages of PRBC differ in FSL-FLRO4 uptake was conducted using an alternate labelling approach. PRBC were labelled at weekly intervals at each time point prior to flow cytometric analysis. Over the time course, FSL-FLRO4 uptake was seen to be similar at different storage durations of PRBC with some reduction (\(p=0.041\)) in the intensity of FLRO4 (Figure 2.4A). An average reduction of 20% was observed by the date of PRBC expiry (Figure 2.4B). Despite the drop in MFI of FSL-FLRO4 with increased storage duration indicating reduced uptake, MFI >20000 was still achieved (Figure 2.4A). Collectively, these data suggest uptake FSL-FLRO4 were similar, although a reduction in MFI was evident, the MFI is more than suitable for detection by flow cytometry.
2.3.4. Fluorescence microscopy provided further evidence that storage duration of RBC did not affect FSL-FLRO4 uptake

FSL-FLRO4 labelled PRBC were visualised using fluorescence microscopy. RBC morphology during ex-vivo storage was altered; at D42, RBC no longer exhibited the healthy discoid shape as observed on D2 (Figure 2.5A, C, E, G). Despite changes in cell morphology, fluorescence microscopy confirmed uptake of FSL-FLRO4 by PRBC. Upon utilisation of fluorescence filter both labelled D2 (Figure 2.5D) and D42 (Figure 2.5H) PRBC emitted FLRO4+ signal, illustrated in green, unlike their matched unlabelled controls (Figure 2.5B, F). Together, fluorescence microscopy provided further evidence that storage duration of PRBC (D2 or D42), did not impact on FSL-FLRO4 uptake and demonstrated the capacity of FSL-FLRO4 to enable visualisation throughout storage. This suggests that FSL-FLRO4 constructs are appropriate for labelling clinical grade PRBC and detection by microscopy.
Figure 2.5. Fluorescence microscopy of FSL-FLRO4 labelling of D2 and D42 PRBC. PRBC were first identified using bright field (left panel; 60X objective), followed by usage of fluorescein filter to warrant visualisation of FSL-FLRO4 labelled RBC (green, right panel; 60X objective). (A, B) Unlabelled D2 and (E, F) D42 PRBC were set up in parallel as negative control for FSL-FRO4 labelled (C, D) D2 and (G, H) D42 PRBC.
2.4. Discussion
To study the interaction and survival of stored PRBC using in-vitro and in-vivo transfusion models, techniques providing visualisation and tracking are required. In this chapter, I report that the FSL-FLRO4 construct is a suitable reagent for labelling PRBC during routine storage. Recently developed FSL constructs provide a simple, direct and non-radioactive alternative to standard RBC labelling techniques ($^{51}$Cr, biotinylation). These KODE technology constructs spontaneously incorporate into RBC membranes providing visualisation and tracking cell survival without affecting the host cell function, even when modifying cells to generate a desired phenotype (e.g. blood group antigens) [76]. A range of FSL constructs have been introduced and used in in-vivo animal transfusion models [78]. However, to date the stability of insertion and longevity of the FSL-label for assessment of stored human PRBC has not been investigated. In this research chapter, FSL-FLRO4 constructs were utilised to assess whether this alternative method would be suitable for labelling and detecting PRBC over the duration of storage. Initially, D2 PRBC were labelled and both the percentage of positive cells and the FSL-FLRO4 MFI were tracked using a time course approach with flow cytometry. Despite a reduction of FSL-FLRO4 MFI over time, all cells were clearly distinguishable as FSL-FLRO4+ at the date of expiry. The reduction in FSL-FRO4 intensity may be attributed to morphological and biochemical changes in the PRBC that are known to occur during routine storage. This includes depletion of 2,3 DPG, reduction of ATP, NO, de-stabilisation of the RBC membrane and generation of microparticles [33]. It is of particular interest for future study to explore the significance of microparticle formation on the loss of FSL-FLRO4 on stored PRBC. Using FSL-Biotin in a murine transfusion model, Oliver and colleagues have reported a post-transfusion loss of FSL label approximating 10% loss per day post-transfusion [78]. However, this study was focused on post-transfusion survival of FSL labelled cells and only labelled RBC at a single time point before transfusion thus did not study stability of FSL labelling in different PRBC subsets and over the duration of storage.

Of note, these experiments were designed to determine whether FSL labelling was retained during PRBC storage to facilitate models focused on addressing differences associated with storage. In line with the goal of developing a method for reliable labelling of PRBC that would be retained during routine storage, further investigation demonstrated no differences in uptake and retention of the FSL-FLRO4 construct on light-young- and dense-old-PRBC. In addition, examination of whether different storage ages of PRBC differ in FSL-FLRO4 construct uptake was conducted using an alternate approach. PRBC
were labelled at weekly intervals at each time point and FSL-FLRO4 uptake was seen to be similar at different storage durations of PRBC. These data suggest biochemical and biophysical changes that occur naturally during RBC eryptosis *in-vivo*, or induced during *ex-vivo* storage, do not impact on uptake of FSL-FLRO4, and propose that this label is suitable for labelling and studying heterogeneous PRBC populations.
2.5. Conclusion

In conclusion, Kode™ technology FSL-FLRO4 is a reliable and suitable reagent for labelling both light-young- and dense-old-RBC during storage of PRBC. Labelling with FSL-FLRO4 enables visualisation and tracking of PRBC over the duration of ex-vivo storage. This method will be a valuable tool for studying stored PRBC in both in-vitro fluorescent immunoassays to investigate cellular interactions and in-vivo models of transfusion. In this thesis, the method of FSL-FLRO4 labelling of PRBC was developed and employed with the intention to provide further evidence to the heavily debated concept that pre-transfusion storage of PRBC may mediate transfusion-related immune modulation. This reliable tool was utilised for the first time to assess changes in mDC uptake of PRBC during storage, reported in Chapter 4.
Chapter 3. Elucidating the role of C-type lectin receptor family member 9 receptor in transfusion-related immune modulation

Synopsis: Chapter 3 focuses on investigating the role of Clec9A in transfusion-related immune modulation, given Clec9A is reported to bind modified RBC and platelets, addressing Aim 1 of this PhD project. The first part of this chapter discusses the unexpected reduction or loss of Clec9A expression on BDCA3⁺ DC surface while optimising the whole blood model of transfusion to be used in Chapters 4-6. In the second part of this chapter, the capacity of Clec9A to recognise and bind damaged or aged blood components was investigated and confirmed using a rhClec9A Fc chimeric protein. Understanding the mechanisms mediating the loss of Clec9A and knowledge of the interaction of Clec9A with different blood components may contribute to further elucidating the mechanisms underpinning transfusion-related immune modulation that potentially drive poor patient outcomes.
3.1. Introduction

Blood transfusion has been associated with poor clinical outcomes. Clinical studies, together with in-vitro and in-vivo models (all discussed in detail in Chapter 1) suggest that transfusion of blood components has the potential to modulate the recipients’ immune responses. DC are antigen presenting cells crucial for regulating both innate and adaptive immunity, and function as a bridge between these sides of the immune system [194]. DC protect the host from infections, exogenous and endogenous pathogens, as well as tumours, serving as guardians of the immune system [192, 193, 266].

In-vitro and in-vivo transfusion models have demonstrated that transfusion modifies DC function. Modulation of DC maturation and activation following exposure to either clinical PRBC [84, 90, 92, 93] or PC [162] or PC supernatants containing soluble factors [162, 168] has been reported. Despite these studies, receptors or mechanisms involved in the interaction between blood components and DC resulting in transfusion-mediated immune modulation of DC function were not explored.

As mentioned in the literature review (section 1.5), DC pattern recognition receptors are essential for the sustainability of immune responses against pathogens and altered self; induction of signalling pathways via recognition of PAMP and/or DAMP are required [51, 193, 267]. Clec9A is a more recently described DAMP receptor of the C-type lectin family, which is strictly expressed on a rare myeloid BDCA3+ DC subset amongst the DC population [220-222]. These cells constitute only ~0.03-0.08% of human PBMC [220-222]. Based on phylogenetic homology as well as the expression of Clec9A and several other surface markers (e.g. XCR1 [229]), human myeloid BDCA3+ DC are essentially equivalent to murine CD8α+ DC subset [220-222, 230]. It has been demonstrated that Clec9A expressed by murine CD8α+ DC facilitate cross-presentation of dead-cell-associated antigens to T lymphocytes through coupling of necrotic cells [221, 228]. In line with the above mentioned murine study, Schreibelt et al. (2012) report Clec9A on human BDCA3+ DC also mediate cross-presentation to antigen-specific T lymphocytes through the use of Clec9A antibody triggering [223]. Furthermore, both human and murine Clec9A complex with a conserved component within nucleated and non-nucleated cells, exposed when cells undergo necrosis. One such DAMP ligand is F-actin [231-233], a key structural protein of eukaryotic cells located in the inner leaflet of the plasma membrane, which serves as a cell damage or death signal upon extracellular exposure [235, 268]. Importantly, of interest to transfusion science research, Clec9A receptors have been
demonstrated to bind to modified or aged RBC (human and mouse) and platelets (mouse only) [231].

PRBC and PC have a limited shelf life; during ex-vivo storage, aging of these cells results in membrane integrity loss, micro-vesicle formation and increased levels of soluble products [33, 269]. Although the potential roles of C-type lectin receptors in transfusion medicine are not well understood and reports of their involvement are limited, two published reviews have provided support for the role of C-type lectin receptors in recognition and clearance of transfused stored RBC and platelets [209, 210]. Both authors suggest that aging RBC and platelets loose terminal sialic acid resulting in exposure of penultimate galactose, which mediates removal of cells by lectin receptors. Therefore in this chapter, I aimed to investigate the hypothesis that Clec9A is a receptor involved in the recognition and clearance of aged transfused RBC and platelets, mediating transfusion associated modulation of DC function.
3.2. Material and Methods

3.2.1. Ethics
Ethics approval statement as per Chapter 2 (section 2.2.1).

3.2.2. Confirmation of Clec9A surface antigen on BDCA3⁺ DC
Fresh whole blood samples were collected in ethylenediaminetetraacetic acid (EDTA) spray-coated blood collection tubes (BD Biosciences) from consented healthy volunteers, and used within one hour of collection. Whole blood samples (2 mL) from these volunteers were incubated for 4 h at 37°C (5% CO₂) in Falcon tubes (50 mL). In parallel, a no incubation control was also performed. Whole blood was centrifuged (515 g, 5 min, deceleration at 4) and plasma removed. Whole blood was then stained (15 min, 22°C) with the following DC panel of mouse anti-human monoclonal antibodies (mAb; all from BD Biosciences unless stated otherwise): lineage-fluorescein isothiocyanate (FITC; 7 µL; CD3, CD14, CD19, CD20, CD34, CD56), CD45-peridinin chlorophyll (PerCP; 7 µL), CD11c-brilliant violet (BV)421 (10 µL), BDCA3-phycoerythrin (PE; 40 µL) Clec9A-allophycocyanin (APC; 30 µL; Miltenyi Biotec, Bergisch Gladbach, North Rhine-Westphalia, Germany). Following staining, whole blood was washed with 3% foetal bovine serum in phosphate buffered saline (3% FBS/PBS; 20 mL; Invitrogen), centrifuged (1000 g, 2 min), supernatant was removed and cell pellet was resuspended in residual supernatant (~70 µL of 3% FBS/PBS). RBC were removed using FACS lyse (1x; 10 min, 22°C; BD Biosciences) and residual leukocytes were washed three times (50 mL, 3% FBS/PBS). Leukocyte pellets were resuspended (1 mL; 3% FBS/PBS) for flow cytometric analysis. BDCA3⁺ DC were gated as lineage⁻CD45⁺CD11c⁻BDCA3⁺Clec9A⁺⁻.

3.2.3. Investigation of the unexpected reduction in Clec9A surface antigen expression on BDCA3⁺ DC
During optimisation a significant reduction of Clec9A was observed without explanation, therefore, to understand and provide justification for this phenomenon various potential contributing factors were assessed. This was undertaken using a similar method as outlined above in section 3.2.2, with the following modifications: different incubation conditions (duration, temperature and presence/absence of 5% CO₂); type of culture media (Roswell Park Memorial Institute medium (RPMI) 1640 (Invitrogen) or PBS); culture vessel plastic type (polystyrene (PST; T25 flask; Sarstedt, Nümbrecht, Germany) or polypropylene (PP; 15 mL or 50 mL Falcon tube)); anti-human Clec9A antibody (clone, manufacturer); and, anticoagulant coating blood collection tubes (EDTA, sodium citrate or
lithium heparin (BD Biosciences) (Table 3.1). In addition, whether Clec9A reduction was associated with BDCA3⁺ DC activation and/or Clec9A internalisation was investigated (Table 3.1).

Table 3.1. Potential contributing factors assessed.

<table>
<thead>
<tr>
<th>Potential influencing factors</th>
<th>Parameters assessed &amp; additional procedures</th>
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<td>Culture media</td>
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<td>Anti-human Clec9A antibody</td>
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<td>• 8f9 (Miltenyi Biotec)</td>
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<td>• 30L2 (0.66 µL; Novus Biologicals LLC,</td>
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<td>Littleton, Colorado, USA)</td>
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<td>• 683409 (66 µL; R&amp;D Systems, Inc.,</td>
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<td></td>
<td>Minneapolis, Minnesota, USA)</td>
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<td>• 3A4 (3 µL; BD)</td>
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<td>Internalisation</td>
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<td>• Intracellular Clec9A expression</td>
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<td>Maturation</td>
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<td>Anti-coagulant</td>
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1 Anti-human Clec9A mAb were APC conjugated except clone 3A4, conjugated with PE. Thus, BDCA3-PE mAb was substituted with BDCA3-APC mAb (15.2 µL).
For surface staining, cells were stained as per DC panel outlined. For intracellular staining, cells were surface stained first, but Clec9A mAb was omitted, until they were permeablismed using FACS Perm (1x; 10 min, 22°C; BD Biosciences). Then Clec9A mAb was added (30 min, 22°C, dark), cells were washed (3% FBS/PBS) and resuspended in cell stabilising fixative (1x; 1 mL; BD Biosciences) for flow cytometric analysis.

Leukocytes were stained with DC panel outlined above and with additional CD80-BV510 (10 µL) or CD86-BV510 (10 µL). Matched BV510 (10 µL) conjugated isotype controls (IgG1) was utilised.

3.2.4. Blood components
Leukodepleted PRBC, buffy coat-derived PC and FFP units were obtained from the Blood Service (Kelvin Grove, Queensland, Australia) and were processed according to standard Blood Service protocols based on the Council of Europe Guidelines for the preparation and quality assurance of blood components [45]. Whole blood units (450 ± 45 mL) were collected into top-and-bottom bags with a built-in filter containing citrate phosphate dextrose (CPD; 66.5 mL; Macopharma, Mouvaux, Nord, France; product number: LQT6283LB; Figure 1.1), then stored at 20-24°C within 24 h of collection before processing. Whole blood units were spun at 3640 g for 10 min (at 22°C), followed by separation of RBC, buffy coat and plasma using a MacoPress Smart EVO with LAN cable and Auto Cannula Breaker component extractor (Macopharma; product number: 9MPSEVOLAN).

3.2.4.1. Preparation of PRBC
RBC were leukodepleted via filtration using a built-in filter within the collection bag and added into the bottom bag containing storage additive saline, adenine, glucose and mannitol solution (SAGM; 105 mL). PRBC units (≥185 mL) were stored at 2-6°C, and used “fresh” (D2) or “stored” (D42, date-of-expiry).

3.2.4.2. Preparation of PC
Each PC unit was created by pooling four buffy coats with additive solution (SSP⁺, Macopharma), resulting in ~28% plasma and 72% SSP⁺. The units were then centrifuged (500 g, 6 min) and leukodepleted via filtration (Terumo BCT, Lane Cove, new South Wales (NSW), Australia; product number: 1TFFP1AS9DB). PC units (platelet count: 284 x 10⁹ ± 40 x 10⁹; volume: 326 ± 14 mL) were stored at 20-24°C with continuous gentle agitation until use. PC were used “fresh” (D2) or “stored” (D5, date-of-expiry).
3.2.4.3. Preparation of FFP
FFP units derived from whole blood were frozen in a rapid plasma freezer (model Mark 4; Arrowsmith & Grant, Dandenong South, Victoria, Australia) to below -30°C within 18 h of collection and stored at -30°C. FFP were utilised to reconstitute cryo-PLT in section 3.2.4.4.

3.2.4.4. Preparation of cryo-PLT
For the production of cryo-PLT, cryoprotectant DMSO (Hybri-Max™, Sigma, Missouri, USA) solution was added to the PC unit at a final concentration of 5% following centrifugation at 1350 g for 10 min. DMSO was removed along with supernatant. The platelet unit was then frozen at -80°C with residual supernatant (~10-20 mL) for a minimum of seven days prior use [270]. Cryo-PLT were processed at the Sydney Blood Service Blood Processing Centre (Alexandria, NSW, Australia) and shipped in a cryo container with dry ice, which maintained the temperature at -80°C during shipment. For thawing, a single unit of cryo-PLT was reconstituted in one unit of whole blood-derived FFP (280-310 mL). Before reconstitution of cryo-PLT, both units were thawed in a 37°C water bath until a temperature of 28-30°C was reached. The temperature range was achieved within 20-35 min for FPP and 4 min for cryo-PLT [270]. Cryo-PLT were used with an hour following FFP reconstitution.

3.2.5. Assessment of recombinant human (rh)Clec9A binding to PRBC
To determine whether Clec9A receptors recognise and bind to fresh and/or stored PRBC, rhClec9A Fc chimera protein (R&D systems, Inc.) was used (n=5). An aliquot (100 µL) of D2 and D42 PRBC were washed twice (1 mL of PBS; 2 min, 1000 g) and resuspended in 1 mL of PBS. Washed D2 and D42 PRBC (50 µL each) were co-incubated with rhClec9A Fc chimera protein at various concentrations (0 ng (control), 20 ng, 200 ng and 2,000 ng) for 30 min at 22°C. At the end of incubation, cells were washed (1.5 mL of PBS), centrifuged (2 min, 1000 g) and resuspended in residual PBS (~50 µL). Mouse anti-human Fc secondary antibody conjugated with APC (2° ab-APC; 10 µL; 20 min, 22°C; R&D system, Inc.) was added for detection of PRBC-rhClec9A binding, followed by washing (1.5 mL of PBS; 2 min, 1000 g). Cell pellets were resuspended in 1.5 mL of PBS before flow cytometry. MFI for PRBC-rhClec9A binding was determined.
In parallel, matched positive controls were established in duplicate experiments where D2 PRBC were permeabilised prior to co-incubation with rhClec9A protein. Briefly, diluted PRBC were treated with 0.05% cold glutaraldehyde (1 mL; 10 min, 22°C; Sigma) and 0.1% Triton X-100 (500 µL; 10 min, 22°C; Sigma). PRBC were washed in PBS (3 mL; 200 g, 5 min) between incubations. Of note, centrifugation parameters for permeablisised RBC were altered to 200 g for 5 min due to fragility of cell membrane.

### 3.2.6. Detection of extracellular PRBC F-actin using phalloidin

To determine whether F-actin is exposed on the cell surface of fresh and/or stored PRBC, phalloidin-fluorescein (200 unit/mL; Invitrogen; n=5) was used. Phalloidin-fluorescein is a highly selective tool for F-actin detection [271]. In brief, washed D2 and D42 PRBC (50 µL) were made up to a final volume of 200 µL in PBS and co-incubated with phalloidin-fluorescein (5 µL) for 30 min at 37°C (in the dark). A matched no phalloidin control was carried out concurrently. Cells were washed (1.5 mL of PBS; 2 min, 1000 g) and resuspended in PBS (1.5 mL) before flow cytometric analysis. MFI for extracellular PRBC F-actin was determined. As per section 3.2.5, matched positive controls were established and stained with phalloidin-fluorescein.

### 3.2.7. Assessment of rhClec9A-PC or -cryo-PLT binding

Based on the procedure outlined above for PRBC (section 3.2.5), 1 x10^6 platelets from either D2 and D5 PC or cryo-PLT were co-incubated with rhClec9A Fc chimera protein (n=5). Platelets were then washed and resuspended in filtered 1x Tyrode’s buffer (1 mL; containing 12 mM sodium bicarbonate (VWR International Ltd., Dorset, England), 10 mM HEPES (Sigma), 137 MM sodium chloride (Ajax Finechem, NSW, Australia), 2.7 mM potassium chloride (VWR International Ltd.), 5 mM D-glucose (Sigma); pH 7.2-7.3) to prevent activation during the assay.

Matched positive controls were established in duplicate experiments where fresh PC, or cryo-PLT were permeabilised prior to co-incubation with rhClec9A protein. 1 x10^6 platelets from either PC or cryo-PLT were fixed (500 µL; 1x cell stabilising fixative, containing 2.81% formaldehyde and 4.01% disodium tetraborate decahydrate in a proprietary buffered solution; BD Biosciences), washed with Tyrode’s buffer (1 mL; 1000 g, 2 min) followed by permeabilisation of platelets (500 µL; 10 min, 22°C; 1x FACS Perm; BD Biosciences). Cells were then washed, centrifuged at 500 g for 5 min and resuspended before the
addition of rhClec9A or phalloidin. Of note, centrifugation parameters for permeabilised platelets were altered to 500 g for 5 min due to fragility of cell membrane.

3.2.8. Detection of extracellular PC or cryo-PLT F-actin using phalloidin
As per the procedure outlined above for PRBC (section 3.2.6), 1 x10^6 D2 and D5 PC or 1 x10^6 cryo-PLT were stained with phalloidin-fluorescein (n=5), and matched positive controls were carried out as per section 3.2.7.

3.2.9. Flow cytometry
Three laser FACSCanto II flow cytometer was used. For all multi-coloured antibody panels used in this thesis, compensation settings were applied to account for potential spectral overlap between fluorescent antibodies. Briefly, negative and positive compensation beads (BD Biosciences) were added into 3% FBS/PBS (100 µL), followed by addition of fluorochrome-conjugated antibodies of interest where each antibody was added into a separate tube. The beads-antibody mixture were then incubated for 15 min at 22°C (in dark), washed with 3% FBS/PBS and centrifuged for 10 min at 200 g. Supernatants were removed, beads resuspended in 3% FCS/PBS (500 µL) and run on the flow cytometer. Compensation was then calculated using FACS DIVA. Data were analysed using FCS express V3 and V5 and FCS express V5 was used for generation of dot plots and histograms for representation of data in figures.

3.2.10. Statistical analyses
GraphPad Prism was utilised to conduct all statistical analyses and representation of graphs, unless otherwise stated. ANOVA with Tukey’s post-test, and paired t-test were used to assess changes in the number and proportion of BDCA3^+ DC positive for Clec9A between different factors investigated. ANOVA with Tukey’s post-test was also used for the assessment of changes in rhClec9A binding, and phalloidin staining between D2 and D42 PRBC or D2 and D5 PC. Paired t-test was used to compare “no transfusion” control with cryo-PLT “transfusion”. p<0.05 was considered statistically significant.
3.3. Results

3.3.1. Unexpected reduction of Clec9A expression on BDCA3+ DC following in-vitro incubation

Expression of Clec9A on BDCA3+ DC in fresh whole blood was confirmed, with cells distinguished as lineage-CD45+BDCA3+Clec9A+ (Figure 3.1A). Surprisingly, Clec9A surface expression was drastically reduced following 4 h incubation at 37°C as BDCA3+ DC became Clec9A- (Figure 3.1B). This was observed for the first time during optimisation of my in-vitro whole blood model for investigating the effect of “blood transfusion” on mDC and BDCA3+ Clec9A+ DC. Herein, I sought to determine the potential contributing factors to provide further understanding of the mechanisms mediating this phenomenon. Of note, in the following sections, the results are presented as the proportion of BDCA3+Clec9A+ DC in all events gated (“Population 1” (P1)) and lineage- leukocyte population (“Population 2” (P2)) of each experiment.

Figure 3.1. Clec9A+ phenotype on BDCA3+ DC. Representative flow cytometric plots showing the presence of Clec9A on BDCA3+ DC. (A) Immediately after whole blood collection and (B) demonstrating the loss of Clec9A after 4 h incubation at 37°C (5% CO2). Cells were first gated based on forward scatter (FSC-A, size) and side scatter (SSC-A, complexity), all events were indicated by P1 and lineage- leukocytes were then gated indicated by P2. At the final gating, lineage-CD45+BDCA3+Clec9A+ DC were gated in P3 and lineage-CD45+BDCA3+Clec9A- DC were gated in P4.
3.3.2. Clec9A surface expression on BDCA3+ DC reduced with increased incubation duration

First, the rate at which Clec9A+ BDCA3+ DC became Clec9A− was assessed, where cells were incubated for different times at 37°C. BDCA3+Clec9A+ DC harvested from fresh whole blood without incubation (0 h) were used as a positive control. The number of BDCA3+Clec9A+ DC (positive control) ranged from 250-1500, which was 0.007-0.021% of P1 and 0.011-0.037% of P2 (Figure 3.2A-C). The number and proportion of BDCA3+Clec9A−DC were significantly reduced with increased incubation times (\(p<0.0001\)). Following 0.5 h incubation, the results were comparable to that of the positive control, but at 2 h >80% reduction in Cle9A was observed in comparison to the positive control. At the final time point (4 h), BDCA3+Clec9A+ DC were largely diminished (<130 cells, ≤0.001% of P1 and ≤0.028% of P2). In establishing the relationship between Clec9A expression and incubation time, I further investigated the impact of culture conditions including temperature and CO₂ status.

![Figure 3.2. Clec9A expression was reduced with increased incubation time.](image)

BDCA3+Clec9A+ DC (A) number, (B) proportion of P1, and (C) proportion of P2, in whole blood following various incubation times (0, 0.5, 2 and 4 h) at 37°C were assessed. Bars indicate mean ± SEM. Data were derived from seven independent experiments (n=7) and analysed using ANOVA (indicated by \(p\) value) with Tukey’s post-test (*\(p<0.05\), **\(p<0.01\), ***\(p<0.001\), ****\(p<0.001\)).

3.3.3. Reduction of Clec9A−BDCA3+ DC was associated with incubation temperature but independent of CO₂ status

Association between incubation temperature and CO₂ status with the reduction of Clec9A was examined. To closely represent a closed human system, maintaining pH and cell survival, 37°C incubation with 5% CO₂ is commonly employed for human cell culture. This standard culture condition was challenged to assist in understanding the mechanisms of
the reduction of Clec9A surface expression. Incubation with 5% CO₂ at 22°C (room temperature) for 4 h resulted in comparable numbers and proportions of BDCA3⁺Clec9A⁺DC to fresh whole blood (Figure 3.3A-C). While BDCA3⁺Clec9A⁺DC were significantly reduced following incubation at 37°C with 5% CO₂ for 4 h (Figure 3.3A-C). In addition to 22°C and 37°C, cells were incubated at 4°C without CO₂ (Figure 3.3D-F). At 4°C and 22°C, Clec9A⁺BDCA3⁺ DC expression was retained and remained similar to fresh whole blood without incubation (Figure 3.3D-F). In the absence of CO₂, significant reduction of Clec9A expression on BDCA3⁺ DC remained evident at 37°C (Figure 3.3D-F). These results were consistent to that observed in the previous experiments performed in this study and the reduction in Clec9A was clearly due to culture at 37°C, therefore an additional confirmatory experiment was carried out.

Figure 3.3. Reduction of Clec9A expression was associated with incubation temperature but independent of CO₂. BDCA3⁺Clec9A⁺ DC (A, D) number, (B, E) proportion of P1, and (C, F) proportion of P2 in whole blood following various incubation temperatures duration (4°C, 22°C or 37°C) with or without CO₂ for 4 h were assessed. Data were derived from one independent experiment (n=1).
3.3.4. Confirmation that incubation at 37°C mediated loss of Clec9A expression

In light of the established association of incubation temperature and duration with the loss of Clec9A, further experiments were conducted to determine whether the receptor would disappear following prolonged culture (24 or 48 h) at 4°C and 22°C or be retained after culture at 22°C followed by a short time at 37°C. Following incubation for 24 h at 22°C ± CO₂ and at 4°C without CO₂, the number and proportion of BDCA3⁺Clec9A⁺ DC were comparable to the no incubation control (Figure 3.4A-C). In addition to incubation at 22°C ± 5% CO₂ for 24 h, the cells were subjected to subsequent incubation at 37°C ± 5% CO₂ for 4 h, to determine whether this process modified the expression of Clec9A. Consistent with my previous observations when BDCA3⁺ DC were incubated at 37°C for 4 h, a clear reduction of Clec9A⁺ DC was still seen (Figure 3.4A-C). However, due to donor-to-donor variation in BDCA3⁺Clec9A⁺ DC numbers, statistical significance was not achieved.

I further extended my analyses using 48 h incubation under the same incubation conditions. However, the additional incubation at 37°C was not included, given loss was observed at 24 h, highlighting that 37°C incubation is one of the causes of loss of Clec9A. After 48 h, BDCA3⁺Clec9A⁺ DC numbers and proportion remained comparable to the control when incubated at 4°C (Figure 3.4D-F). While at 22°C some reduction was observed (Figure 3.4D-F). Collectively, these data demonstrate that reduction in Clec9A expression was associated with incubation at 37°C potentially the increased temperature mediated an acceleration of the phenomenon.
Figure 3.4. Loss of Clec9A expression was associated with incubation at 37°C. Assessment of BDCA3+Clec9A+ DC (A, D) number, (B, E) proportion of P1 and (C, F) proportion of P2 in whole blood with no incubation (no incu.) or following incubation temperatures duration at 4°C or 22°C ± continuation to 37°C with or without CO2 for 24 and 48 h. Bars indicate mean ± SEM. Data were derived from three independent experiments (n=3) and analysed using ANOVA (indicated by $p$ value) with Tukey’s post-test ($p<0.05$) or paired t-test.

3.3.5. Reduction of BDCA3+Clec9A+DC was independent of the plastic type of culture vessel used

I then looked into the potential effect of plastic material in which culture vessels were manufactured from. In addition to the already used 50 mL PP Falcon tubes, 15 mL PP Falcon tubes and PST T25 flasks were also investigated. The reduction in cell number following 4 h incubation at 37°C were comparable between the PP and PST culture vessels, reporting <100 BDCA3+Clec9A+ DC and <0.001% of both P1 and P2 (Figure 3.5A-C). In comaprison to fresh whole blood in 50 mL Flacon tubes, the no incubation control, reduced in Clec9A expression in both PST and PP culture vessel. This demostntrated the phenomenon observed for Clec9A is independent of type of culture vessel plastic.
Figure 3.5. Changes in Clec9A expression was not the result of plastic culture vessels. BDCA3⁺Clec9A⁺ DC (A) number, (B) proportion of P1, and (C) proportion of P2, in whole blood incubated in culture vessels manufactured with different plastic materials (PP and PST) at 37°C (with 5% CO₂) for 4 h were assessed. Data were derived from two independent experiments (n=2) and analysed using paired *t*-test.

3.3.6. Reduction of Clec9A⁺BDCA3⁺ DC was independent of culture media used

Although no culture media was employed in the preliminary investigation that demonstrated loss of Clec9A, it was of interest to determine if the use of RPMI 1640 culture media or PBS had the potential to facilitate the retention of Clec9A expression. This is because RPMI 1640 culture media is required in my transfusion model to maintain culture equilibrium, provide nutrients and maintain pH, which sustains cell survival. In addition, PBS is required for most wash steps and resuspending cells. The number and proportion of BDCA3⁺Clec9A⁺ DC were relatively similar between whole blood incubated alone, with RPMI 1640 media or PBS (Figure 3.6A-I). The presence of media or buffer did not prevent the reduction of Clec9A expression on BDCA3⁺ DC surface after 2 and 4 h incubation at standard temperature. These data demonstrate that the loss of Clec9A expression is independent of whether RPMI 1640 culture media or PBS is used.
Figure 3.6. Assessment of the effect of culture media on Clec9A expression on BDCA3⁺ DC. BDCA3⁺Clec9A⁺ DC (A, D, G) number, (B, E, H) proportion of P1, and (C, F, I) proportion of P2, in whole blood alone or whole blood co-incubated with RPMI 1640 media or PBS following various incubation duration (0, 0.5, 2 and 4 h) at 37°C (5% CO₂) were assessed. Bars indicate mean ± SEM. Data were analysed using non-parametric ANOVA (indicated by $p$ value) with Tukey’s post-test (*$p<0.05$, **$p<0.01$). Data were derived from replicates as follows for whole blood alone: n=5 at 0 h, n=4 at 0.5 h, n=4 at 2 h and n=3 at 4 h; for whole blood with RPMI 1640 media: n=6 at 0 h, n=4 at 0.5 h, n=5 at 2 h and n=4 at 4 h, and for whole blood with PBS: n=6 at 0 h, n=4 at 0.5 h, n=5 at 2 h and n=4 at 4 h.

3.3.7. Reduction of BDCA3⁺Clec9A⁺ DC was independent of Clec9A antibody clone or manufacturer

In addition to assessing factors involved within or during incubation, I began to assess factors or mechanisms external to the incubation process. Firstly, I investigated four different manufacturers supplying Clec9A mAb of different clones with similar immunogen (Table 3.2) to determine whether lack of binding of Clec9A was an issue.
Table 3.2. Details of mouse anti-human monoclonal Clec9A antibodies.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Clones</th>
<th>Immunogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clec9A-APC</td>
<td>Miltenyi MACS</td>
<td>8f9</td>
<td>Extracellular domain</td>
</tr>
<tr>
<td>Clec9A-APC</td>
<td>Novus Biological</td>
<td>30L2</td>
<td>Extracellular domain (Lys57-Val241)</td>
</tr>
<tr>
<td>Clec9A-APC</td>
<td>R&amp;D Systems</td>
<td>683409</td>
<td>Extracellular domain (Lys57-Val241)</td>
</tr>
<tr>
<td>Clec9A-PE</td>
<td>BD</td>
<td>3A4</td>
<td>Human Clec9A peptide</td>
</tr>
</tbody>
</table>

The matching antibody pairs were compared at 22°C and 37°C (Figure 3.7A-C). BDCA3⁺Clec9A⁺ DC reduction \((p<0.05)\) was evident for all antibody pairs studied when cells were incubated at 37°C in comparison to those incubated at 22°C (cell number: \(p=0.010\), proportion of P1: \(p=0.011\), proportion of P2: \(p=0.011\)). It is clear there were no differences observed between the four clones of Clec9A antibodies used, implying that the diminished Clec9A expression was not linked to use of antibody clones.

![Figure 3.7](image-url)  
**Figure 3.7. Assessment of the effect of antibody clone on Clec9A surface expression.** Different clones (8f9 (black); 30L2 (red); 683409 (green); 3A4 (blue)) and manufacturers of Clec9A mAb were investigated. BDCA3⁺Clec9A⁺ DC (A) number, (B) proportion of P1, and (C) proportion of P2 in whole blood following incubation at 22°C or 37°C for 4 h. Data were derived from one independent experiment for each antibody pair (n=1) and analysed using paired \(t\)-test \((*p<0.05)\).

3.3.8. Reduction of BDCA3⁺Clec9A⁺ DC may be associated with Clec9A internalisation

During the investigation of factors or mechanisms external to incubation, whether Clec9A were internalised was examined. It was evident that the reduction of BDCA3⁺Clec9A⁺ DC was associated with temperature, which had led to the postulation that temperature may have mediated internalisation of the receptor. To test this assumption, intracellular flow
cytometry was used. As established previously in this study, BDCA3+ DC incubated at 22°C after 4 h retained Clec9A surface expression while those incubated at 37°C did not (cell number: \( p = 0.021 \), proportion in P1: \( p = 0.016 \), proportion in P2: \( p = 0.019 \); Figure 3.8A-C). For intracellular staining, the number of BDCA3+Clec9A+DC at 37°C declined in comparison to incubation at 22°C (\( p = 0.040 \); Figure 3.8D). The proportion of intracellular stained BDCA3+ DC positive for Clec9A was similar for both incubation temperatures (Figure 3.8E, F), suggesting the occurrence of Clec9A internalisation and potentially a rationale for the reduction in Clec9A surface expression. Then I further hypothesised that the internalisation may be mediated by DC maturation.

Figure 3.8. Investigation of Clec9A internalisation. Surface and intracellular Clec9A for BDCA3+ DC (A, D) number, (B, E) proportion of P1, and (C, F) proportion of P2 were assessed in whole blood incubated at 37°C for 4 h. Bars indicate mean ± SEM. Data were derived from three independent experiments (n=3) and analysed using paired t-test (\(* p < 0.05\)).

3.3.9. Reduction of Clec9A expression was independent of BDCA3+ DC maturation

With an outcome suggesting internalisation, I was interested to address a potential underlying mechanism. Therefore, I investigated whether DC maturation was involved; the expression of DC surface molecules CD80 and CD86, which are associated with maturation and activation, were investigated. MFI for CD80 (\( p = 0.069 \)) and CD86 (\( p = 0.055 \))
were comparable between BDCA3⁺Clec9A⁺ DC obtained from no incubation control and those incubated at 22°C, and BDCA3⁺ Clec9A⁻ DC obtained from whole blood incubated at standard 37°C (Figure 3.9A, B). No maturation phenotype was observed, neither CD80 nor CD86 surface expression were up-regulated, which indicates that reduction or internalisation of Clec9A was not associated with maturation of BDCA3⁺ DC.

Figure 3.9. Assessment of DC maturation phenotype. Flow cytometric assessment of Clec9A⁺⁻BDCA3⁺ DC expression of DC (A) CD80 and (B) CD86 without incubation or incubation at 22°C and 37°C for 4 h were assessed. Bars indicate mean ± SEM. Data were derived from four independent experiments (n=4) and analysed using non-parametric ANOVA with Tukey’s post-test.

3.3.10. EDTA contributed to loss of Clec9A surface expression

The use of anti-coagulant coated in the blood collection tube, which is the first point of contact of whole blood drawn from volunteers, was also examined to determine whether it had an effect on Clec9A loss. BDCA3⁺Clec9A⁺ DC was drastically reduced in whole blood collected in EDTA with <50 cells (p<0.0001) where <0.0001% were positive for Clec9A in P1 (p=0.002) and P2 (p=0.01) after 2 and 4 h incubation at 37°C (Figure 3.10A-C). Interestingly, whole blood collected in both sodium citrate and lithium heparin retained the surface expression of Clec9A on BDCA3⁺ DC after 2 and 4 h at 37°C, which were comparable to the no incubation control (Figure 3.10D-I). In addition, use of sodium citrate or lithium heparin induced leukocyte clumping, which was not observed for EDTA (observation not shown). Together, these results indicate that EDTA as an anti-coagulant combined with 37°C incubation temperature for longer than 2 h, reduced Clec9A expression on BDCA3⁺ DC. Reduction in Clec9A was the combined result of anti-coagulants, temperatures and incubation durations used.
Figure 3.10. EDTA was associated with a rapid reduction in Clec9A. BDCA3^Clec9A^ DC (A, D, G) number, (B, E, H) proportion of P1, and (C, F, I) proportion of P2, in whole blood collected in EDTA (purple), sodium citrate (blue) and lithium heparin (green) blood collection tubes were examined following incubation at defined durations (0, 2 and 4 h) at 37°C (5% CO₂). Bars indicate mean ± SEM. Data were derived from replicates as follows: EDTA, n=3; sodium citrate, n=5; and, lithium heparin, n=5. Data were analysed using ANOVA (indicated by p value) with Tukey’s post-test (*p<0.05, **p<0.01, ****p<0.0001).
3.3.11. rhClec9A did not bind fresh or stored blood components

Collection of blood in EDTA coated tubes followed by incubation for 4 hours at 37°C resulted in the loss of Clec9A expression. This outcome was a concern for my transfusion experiments as I had hypothesised that Clec9A would be involved in the process of transfusion related immune modulation of DC function. A rhClec9A Fc chimera protein was therefore used to determine whether Clec9A could recognise and bind to fresh and/or stored PRBC, PC or cryo-PLT. This would enable me to determine whether Clec9A bound to the different blood products using a more direct approach assessing cell-receptor interaction. To demonstrate the functionality of the commercial rhClec9A protein, PRBC, PC or cryo-PLT were first permeabilised to expose F-actin followed by incubation with rhClec9A. Permeabilising the cells and exposing the F-actin was designed to serve as a positive control for the rhClec9A. rhClec9A did not bind to the permeabilisation PRBC at any concentration (Figure 3.11A, C). The higher background MFI observed for the PRBC positive control was an effect of glutaraldehyde used for permeabilisation. rhClec9A bound permeabilised PC and cryoPLT at 200 ng and 2000 ng (Figure 3.11B-C, E-F) indicating that the commercial recombinant Clec9A protein was functional. rhClec9A did not bind unmodified cells from D2 or D42 PRBC, D2 or D5 PC or cryo-PLT at any concentration studied (Figure 3.11A-C). This suggests that for PRBC, PC and cryo-PLT, F-actin is not exposed as a result of standard blood component preparation and storage. These data suggest rhClec9A may not be involved in recognition, clearance or uptake of stored blood components.
Figure 3.11. rhClec9A did not recognise and bind PRBC, PC and/or cryo-PLT. Representative histograms demonstrating binding of rhClec9a Fc chimera protein at 20 ng (light blue), 200 ng (medium blue), 2000 ng (dark blue), no rhClec9a control (solid grey) with cells from (A) PRBC, (B) PC and (C) cryo-PLT. Permeabilised blood components were used as positive control. Delta MFI of rhClec9A binding with cells from (D) PRBC (circle l), (E) PC (square) and (F) cryo-PLT (triangle) were also presented. MFI expressed by blood components (rhClec9A + 2° ab-APC) were compared to (c.f.) the matched 2° ab-APC only control (denoted as zero (dotted line)). Bars indicate mean ± SEM. Data derived from five independent experiments (n=5).
3.3.12. Extracellular F-actin detected on PC and cryo-PLT but not PRBC

My result of rhClec9A not binding to permeabilised PRBC was surprising. To further investigate the differences in Clec9A binding to permeabilised PRBC, PLT and cryo-PLT, phalloidin was used for detection of the Clec9A ligand F-actin. To demonstrate the functionality of the highly specific phalloidin-fluorescien stain, cells were permeabilised, to serve as a positive control. As expected, following permeabilisation F-actin was detected in all three cell types when phalloidin was used as a marker (Figure 3.12A-C). Exposure of F-actin was not evident on unmodified D2 and D42 PRBC (Figure 3.12A, D). A low level of F-actin was detected on D2 and D5 PC (Figure 3.12B, E). Greater phalloidin-fluorescien MFI was observed for cryo-PLT with ~50% positive for F-actin (MFI >800; Figure 3.12C, F). Together these results suggest that during routine preparation and storage of PC and cryo-PLT, F-actin may be exposed indicating activation and processes involved in cell death. Higher levels of F-actin were observed on cryo-PLT, probably due to the freeze-thaw involved in their preparation.

Figure 3.12. Investigation of whether F-actin is exposed on fresh and old PRBC and PC, and freeze-thawed cryo-PLT. Representative histograms illustrating the detection of F-actin on (A) PRBC, (B) PC and (C) cryo-PLT, using phalloidin-fluorescien stain. No phalloidin stain control (solid grey). Permeabilised blood components were used as positive control. Delta MFI of phalloidin-fluorescien expressed by (D) PRBC (circle), (E) PC (square) and (F) cryo-PLT (triangle) were also represented. Phalloidin-fluorescien MFI expressed by blood components c.f. matched no stain control (denoted as zero (dotted line)). Bars indicate mean ± SEM. Data derived from five independent experiments (n=5).
3.4. Discussion
To date, receptors and mechanisms involved in the interaction between blood components and DC resulting in transfusion-mediated immune modulation of DC function remains largely undefined. Clec9A is a receptor that binds the conserved cytoskeletal cell membrane protein F-actin. During ex-vivo storage or the freeze-thaw process PRBC, platelets and cryo-PLT undergo significant biochemical and mechanical modifications [33, 269]. Therefore, I investigated the hypothesis that Clec9A is the DAMP receptor involved in recognition and clearance of transfused RBC and platelets, mediating transfusion associated modulation of DC function. In the first part of this chapter, I investigated the unexpected and drastic reduction in the surface expression of Clec9A\(^+\) BDCA3\(^+\) DC following incubation, which was accelerated by increased temperature and prolonged incubation time, and demonstrated this loss of expression was associated with the type of anti-coagulant used to coat the blood collection tube. In the second part of this chapter, I observed no binding of rhClec9A with either “fresh” or “stored” clinical PRBC or PC, or freeze-thawed cryo-PLT. However, F-actin were detected on PC and cryo-PLT, but not on PRBC.

During the initial optimisation of my in-vitro transfusion model for studying the effect of “blood transfusion” on Clec9A\(^+\) BDCA3\(^+\) DC, loss in Clec9A surface expression on BDCA3\(^+\) DC after incubation at 37°C (4 h) was observed. Such a phenomenon had not been reported in the literature and I sought to determine the contributing factors involved as this may have significant impacts not only for my own work, but for other researchers studying Clec9A. I assessed different CO\(_2\) status, plastic type of culture vessel, culture medium during incubation, as well as different Clec9A antibody clones and manufacturers, and reported neither enhancement nor further reduction to the preliminary observation. However, when the standard culture temperature was challenged, where a closed human system was no longer represented, Clec9A expression was retained. BDCA3\(^+\) DC in whole blood incubated cold (4°C) or at room temperature (22°C) retained their Clec9A expression. In addition, this was only observed following ≥2 h of incubation and at the end of 4 h Clec9A expression was largely diminished at 37°C. The effect of temperature in generating differential protein expression has been demonstrated in Chinese hamster ovary (CHO) cells. Using lower incubation temperature has enhanced expression of recombinant therapeutic proteins of interest [272, 273].
Based on the association established with incubation temperature and duration, I then postulated that reduction in Clec9A expression was due to DC maturation and potentially internalisation mediated by temperature. I found BDCA3\(^+\)Clec9A\(^+\) DC shared a similar profile of antigen expression to Clec9A\(^-\) BDCA3\(^+\) DC where maturation phenotypes were not observed. Neither BDCA3\(^+\) DC populations expressed the anticipated up-regulation of key DC surface molecules CD40, CD80, CD83 and CD86 for DC [193]. Although DC maturation was not observed, internalisation of Clec9A was depicted where intracellular Clec9A expression was demonstrated in BDCA3\(^+\) DC following 4 h incubation at 37°C. This was not observed when BDCA3\(^+\) DC was surface stained. However, limited conclusions can be drawn from these data due to anti-Clec9A antibody not being specific for intracellular staining. Therefore, non-specific binding to both surface and intracellular Clec9A was likely to have occurred on BDCA3\(^+\) DC (positive control) incubated at room temperature (22°C), impeding the accuracy of the comparison. Additional experiments are required to confirm whether reduction in Clec9A expression was due to receptor internalisation. This could be achieved by either pre-saturating the surface Clec9A with a different fluorochrome conjugated Clec9A antibody prior to intracellular staining or using trypan blue to quench the fluorochrome signal of the surface bound antibody after permeabilisation and intracellular staining [274, 275].

Furthermore, the effect of the type of anti-coagulant used to coat the blood collection tubes was examined to further elucidate the mechanism of Clec9A reduction, as it is the first point of contact with these cells. In addition to EDTA, other anticoagulants including sodium citrate and lithium heparin were assessed. Interestingly, blood collected in sodium citrate and lithium heparin retained Clec9A expression on BDCA3\(^+\) DC even after 2 and 4 h of 37°C incubation. The results provided evidence that EDTA was a critical contributing factor in mediating the reduction of Clec9a surface protein expression. Anti-coagulants are often selected depending on the nature of the experiment that will be performed [276]. Heparin is a natural anticoagulant found in the body that prevents fibrinogen transforming into fibrin via inhibition of prothrombin transformation into active thrombin [277]. This agent does not prevent clotting, but rather delays the process (for 8-12 h). Both EDTA and sodium citrate share a similar mechanism of action in preserving and preventing blood from clotting through inhibition of thrombocyte agglutination by binding free Ca\(^{2+}\) ions [278]. However, in comparison, EDTA is often used for its more efficient blood preservation and prevention of clotting for prolonged duration without distorting RBC function and physiology. Therefore, EDTA was initially utilised in all the experiments.
discussed above. Although the mechanism of action is similar between EDTA and sodium citrate, it was speculated that the disparity in the results lies within the free Ca\(^{2+}\) ions in the blood; EDTA has a stronger chelating affinity depleting the majority of free Ca\(^{2+}\) ions [279], which may result in a series of reactions leading to Clec9A loss.

Zhang et al. (2012) report that stabilisation of Clec9a conformation is dependent on Ca\(^{2+}\) [231], therefore, I inferred that reduction of BDCA3\(^+\)Clec9A\(^+\) DC was due to destabilisation of the receptor mediated by the lack of Ca\(^{2+}\) ions available in whole blood. As a result, Clec9A antibodies may no longer recognise and bind to the extracellular domain of this receptor or may have provoked internalisation of this receptor. On the basis of my findings and the available literature, I further hypothesised that reduced detection of Clec9A surface expression via flow cytometry was due to the lack of Ca\(^{2+}\) ions present in whole blood from EDTA chelation resulting in destabilisation of Clec9A conformational and/or internalisation. Additional experiments are required to further elucidate and understand the precise mechanism of how EDTA mediated this phenomenon. This could include the assessment of Clec9A expression following Ca\(^{2+}\) spiking, exposure to different strength of Ca\(^{2+}\) chelators (such as 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) and ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA)) and determining if this was associated with depletion in intracellular or extracellular Ca\(^{2+}\). These experiments would be necessary to confirm whether Ca\(^{2+}\) is mediating the loss of Clec9A.

Given that leukocytes and their response following “transfusion” were the focus of my studies, EDTA is the most appropriate anticoagulant of choice. Leukocyte clumping was observed in both sodium citrate and lithium heparin, which could potentially interfere with the outcomes of my study. In addition, lithium heparin also has the capacity to interfere with leukocyte staining, inflammatory responses and signalling pathways [278, 280], therefore is not appropriate for my studies. To assist in the decision of whether the use of EDTA can be continued or should be substituted with sodium citrate blood collection tubes, I assessed the capacity of Clec9A to recognise and bind different blood components during ex-vivo storage.

Clec9A has been reported to recognise and bind extracellular F-actin on modified RBC and platelets [231-233], and mediate superior cross-presentation [223, 224]. In this study, use of permeabilised cells as positive controls provided indication of the commercial
rhClec9A protein functionality. Binding was observed when 200 ng and 2,000 ng rhClec9A protein was co-incubated with platelet positive controls. Surprisingly, the rhClec9A protein did not bind to PRBC positive control. This may be due to the permeabilisation method used for PRBC being too gentle preventing rhClec9A from complexing with F-actin in the comparison to method used for platelets. The use of western blot analysis may be useful for confirmation of rhClec9A functionality where cells are deconstructed fully exposing F-actin. Overall, no binding of the commercial rhClec9A with non-permeabilised fresh or old PRBC or PC, or freeze-thawed cryo-PLT was observed. Using phalloidin staining, I observed no detection of extracellular F-actin in D2 or D42 PRBC, suggesting that clinical PRBC stored at 4°C do not undergo necrosis. As translocation of the conserved F-actin from the inner leaflet to outer leaflet of the plasma membrane is a marker for cell death [281], it serves as cell damage or death signal, which mediate cell clearance in the immune system [235, 268]. However, in my study extracellular F-actin was evident on PLTs from PC. This was especially evident for cryo-PLT, which is not surprising given that cell death occurs due to the process of cryopreservation and thawing. Additionally, these experiments suggest that phalloidin and Clec9A bind to different parts of F-actin.

In contrast with the results from Zhang et al. (2012), I did not observe binding of Clec9A to permeabilised PRBC [231]. Of note, key differences that potentially influenced the outcomes in my studies were suspected to be associated with the source of Clec9A and whether the damaged cells were induced or naturally occurring. Zhang et al. (2012) examined different binding sites of Clec9A receptor generated in-house, one of which was the ectodomain and had been reported to successfully bind murine and human RBC, and murine platelets [231]. The region of ectodomain targeted between Zhang et al. (2012) and the rhClec9A protein used in this study (purchased from R&D systems Inc.), differed by 10 nucleotide base pairs. This may potentially contribute to the differential outcomes observed between our studies. In addition, Zhang et al. (2012) observed Clec9A binding of RBC and platelets that were treated with either permeabilising solution containing saponon or ABT-737 to induce apoptosis [231]. However, the focus of my study was the role of Clec9A role in transfusion, extracellular F-actin was exposed naturally rather than induced. Therefore, accessibility of F-actin may potentially be restricted. These factors discussed are suspected to have contributed to the different results observed and, further investigation will be required to provide a detailed explanation. The outcome of these experiments suggests that phalloidin and Clec9A bind to different parts of F-actin.
3.5. Conclusion

In summary, novel and important knowledge regarding Clec9A was acquired in this chapter. My study has shed more light into the nature of Clec9A and the unexplored relationship between Clec9A surface expression and EDTA. Additionally, lack of F-actin detected on PRBC in this model implies that PRBC do not express this cell death marker during \textit{ex-vivo} storage. Conversely for platelets with different storage conditions, in particularly cryo-PLT, extracellular F-actin was detected suggesting induction of cell death. In my study, I did not detect binding of rhClec9A to any of the blood components investigated, suggesting that Clec9A may not play a role in transfusion-related immune modulation of DC function. Based on my preliminary findings and the nature of this research, which focuses on leukocytes and their response in the context of transfusion, the use of EDTA blood collection tubes was used for my transfusion model in subsequent chapters.
Chapter 4. Mechanisms underpinning transfusion-related immune modulation: erythrophagocytosis and immune modulation in myeloid dendritic cells

Synopsis: In this chapter, the potential for PRBC to modulate responses of mDC and the specialised BDCA3⁺ subset was assessed using an *in-vitro* whole blood culture model of transfusion, addressing Aim 2 of this thesis. I proposed that DC erythrophagocytosis and modulation of DC activation and maturation are one mechanism underpinning transfusion-related immune modulation driving poorer patient outcomes. Knowledge of the *in-vitro* effect of PRBC transfusion and *ex-vivo* storage duration on the immune system will provide further evidence to our understanding of PRBC in transfusion-related immune modulation.
4.1. Introduction

Although transfusion of PRBC is a life-saving therapy, clinical studies have reported increased rates of morbidity, mortality, cancer reoccurrence and infectious complications in transfusion recipients [6, 282]. PRBC are routinely stored for up to 42 days prior to transfusion. While storage-related morphological and biochemical modifications are well documented [33], whether these changes contribute to worse patient outcomes remains controversial, with correlation of stored PRBC with poor patient outcomes in some studies, but not others [28, 42]. Whether PRBC transfusion negatively impacts patient outcomes remains a highly debated topic in the transfusion medicine arena.

The potential for PRBC to modulate recipients’ immune response and impact patient outcomes has been studied using in-vitro and in-vivo models. In-vitro studies demonstrate modulation of monocyte [86] and overall leukocyte [81, 85, 89] inflammatory responses following exposure to PRBC or PRBC components. These studies consistently report suppression of TNF-α and augmentation of IL-10 responses. Increased monocyte erythrophagocytosis of stored PRBC has also been reported [283]. Transfusion-related modulation of immune responses are not limited to innate cells, with exposure to PRBC reported to increase CD25+ regulatory T lymphocyte production, reduce the number of responder T lymphocytes [79], and suppress B and T lymphocyte proliferation [80, 88]. To date, studies assessing the role of DC in transfusion outcomes are limited. A murine model using non-leukodepleted PRBC reported CD200+ DC may play a role in modulating transfusion-associated tumour growth [90]. Erythrophagocytosis commonly occurs in the spleen via activated resident tissue macrophages in-vivo as opposed to circulating DC. However, erythrophagocytosis by murine pDC has been reported, particularly in combination with polyI:C-driven inflammation [91]. Murine studies have reported that splenic DC are important for outcomes in terms of alloimmunisation [92, 93]. Despite these murine studies, our understanding of whether DC play a role in poor patient outcomes following PRBC transfusion remains largely undefined.

DC play a crucial and central role in the immune system at the interface of innate and adaptive immune responses [194]. DC are classified into two major lineages, mDC and pDC [284], which are further divided into subsets based on their specialised functions and associated with differential expression of pattern recognition receptors. Pattern recognition receptors include receptors that recognise PAMP or DAMP, which enable DC to detect invading pathogens, or damaged cell components, respectively. mDC predominantly
express pattern recognition receptors for recognition of bacteria and subsequent antigen presentation via MHC class II. A rare subset of mDC, BDCA3⁺ DC are equipped with pattern recognition receptors skewed towards recognition of viruses, and these cells are specialised in dead cell-associated antigen cross-presentation (via MHC class I) [224, 225]. Following pattern recognition, DC become activated and undergo phenotypic changes essential for antigen presentation including expression of co-stimulatory molecules and secretion of cytokines and chemokines [195, 201]. Perturbation of these DC processes has the potential to significantly affect host immune responses [202, 203], which may contribute to poor outcomes in transfusion recipients.

While PRBC have been reported to modulate cells of the innate and adaptive immune response, the impact of transfusion on DC responses remains largely unexplored. As central cells in the immune response, we investigated whether mDC and the specialised subset BDCA3⁺ DC were modulated following exposure to PRBC in-vitro. This chapter aimed to investigate the hypothesis that PRBC transfusion modulates key DC processes, hindering the capacity of these cells to respond adequately to subsequent immune challenges.
4.2. Materials and Methods

4.2.1. Ethics
Ethics approval statement as per Chapter 2 (section 2.2.1).

4.2.2. Blood Components
Preparation and processing of PRBC were as described in Chapter 3 (section 3.2.4). Leukodepleted PRBC units were obtained from the Blood Service and stored at 2-6°C. PRBC were used “fresh” (D2) or “stored” (D42, date-of-expiry).

4.2.3. In-vitro whole blood model of transfusion
An in-vitro whole blood model was used to assess the potential of PRBC to mediate immune modulation in a “transfusion recipient” (Figure 4.1). This model was established to closely model the clinical scenario, after a series of optimisation experiments to maximise BDCA3+ DC number, and to minimise the amount of antibodies used. Fresh whole blood samples (6 mL) from different consented healthy volunteers (Blood Service staff) were collected in EDTA blood collection tubes and utilised as the “recipient (source of leukocytes including DC)”. A 25% blood replacement volume transfusion (representing two-three units) was modelled through culturing “recipient” whole blood (6 mL) with ABO compatible PRBC (D2 or D42 PRBC (from different units); 3 mL) and RPMI 1640 media (3 mL) for 5.5 h (37°C, 5% CO2). PolyI:C (50 μg/mL, high molecular weight; TLR3 agonist; Invitrogen) or LPS (1 μg/mL, Escherichia coli (055:B5); TLR4 agonist; Sigma) was added in duplicate tubes to model the processes activated by concurrent viral (polyI:C) or bacterial (LPS) infection. Matched “no transfusion” controls were included in parallel with PRBC volume replaced with RPMI 1640 (included polyI:C and LPS only controls). For experiments assessing intracellular mediators, protein transport inhibitor (1 µg/mL; BD Biosciences) was added for the last 4.5 h. Cells were harvested and supernatant collected from cultures without protein transport inhibitor (stored at -80°C for later analysis using cytometric bead array (CBA)). RBC (from 6 mL whole blood) were lysed with FACS lyse (40 mL) and residual leukocytes washed thrice in 50 mL of 3% FBS/PBS before staining for flow cytometry.
4.2.4. Assessment of DC surface antigen expression and intracellular inflammatory mediator production

Leukocytes from the whole blood transfusion model were stained (15 min, 4°C) with the following DC panel of mouse anti-human mAb: lineage markers (3.5 µL; CD3, CD14, CD19, CD20, CD34, CD56)-FITC, CD45-PerCP (3.5 µL), CD11c-BV421 (5 µL) and BDCA3-APC (5 µL). For assessment of surface antigen expression, leukocytes were also stained with CD40-PE (20 µL) and CD80-BV510 (5 µL) or CD83-PE (20 µL) and CD86-BV510 (5 µL). Cells were washed twice (2 mL of 3% FBS/PBS) and resuspended in 350-500 µL of 3% FBS/PBS for flow cytometric analysis.

For assessment of intracellular mediators, the selection of cytokines and chemokines assessed was altered between the two models using either polyI:C or LPS. IP-10 used in the polyI:C model was substituted by IL-10 in the LPS model (all other inflammatory mediators assessed remain the same in both models). IP-10 production is observed more in response to polyI:C/viruses, especially for BDCA3+ DC while LPS/bacteria induces higher level of IL-10 in comparison predominately in mDC. Following staining with the above DC panel, leukocytes were permeabilised (500 µL; 10 min, 22°C; FACS Perm) and labelled with intracellular PE-conjugated mAb (7.5 µL, 1 in 10 dilution in PBS) targeting IL-6, IL-8, IL-12, TNF-α and, IL-10 (LPS panel) or, IP-10 (polyI:C panel) for 30 min (22°C, dark). Cells were washed with 3% FBS/PBS (200 µL) and resuspended in 220-250 µL cell stabilising fixative for flow cytometry.

mDC were selected using the following gating strategy: SSC vs. CD45 was used to exclude granulocytes and lymphocytes, selected mononuclear cells were displayed as Lin vs. CD11c with Lin-CD11c+ cells selected as mDC (Lin-CD45+CD11c+). BDCA3+ cells were selected using the following gating strategy: SSC vs. CD45 was used to exclude granulocytes and lymphocytes, selected mononuclear cells were displayed as Lin vs.
CD11c with Lin^-CD11c^+ cells selected for gating of the BDCA3^+ DC subset (Lin^- CD45^-CD11c^-BDCA3^+). Compensation was applied as stated in Section 3.2.9. MFI for each surface antigen and inflammatory mediator expressed by mDC and BDCA3^+ DC was determined. Data presented were derived from a two-step approach. First, PRBC “transfusion” data were normalised to the matched “no transfusion” control (ratio of MFI). Results were then calculated as binary logarithm (log2) with no transfusion controls represented as zero. A twofold increase would be graphed +1 and a twofold suppression as -1. This approach establishes a baseline for each donor to account for donor-to-donor variation. Experiment replicates were as follows for assessment of surface antigen expression: n=16 PRBC only, n=8 PRBC + polyI:C and n=8 PRBC + LPS and intracellular mediators: n=11 PRBC only, n=6 PRBC + polyI:C and n=5 PRBC + LPS.

4.2.5. Assessment of overall leukocyte inflammatory response
Concentrations of inflammatory mediators (IL-1α, IL-1β, IL-8, IL-6, IL-10, TNF-α, IL-12p70, IP-10, IFN-α, IFN-γ, MCP-1, MIP-1α, MIP-1β) were assessed in culture supernatant from the transfusion model (n=16 PRBC only, n=8 PRBC + polyI:C or LPS) using CBA according to manufacturer’s directions (BD Biosciences). Briefly, culture supernatants (25 µL; 1:2 dilution with assay diluent) were incubated with capture beads (25 µL) for 1 h (22°C, dark) and, PE-labelled detection reagent was added and incubated for 2 h (22°C, dark). Unbound detection reagent was removed via addition of wash buffer (1 mL) followed by centrifugation (200 g, 5 min). Supernatants were removed and bead pellets were resuspended in 60 µL of wash buffer for analysis using flow cytometry. Concentrations were determined from standard curves (0, 10, 20, 40, 80, 156, 312.5, 625, 1250, 2500 pg/mL) run in parallel.

4.2.6. Assessment of erythrophagocytosis
D2 and D42 PRBC were labelled with FSL-FLRO4 as previously described [75, 285]. Briefly, an equal volume of PRBC and FSL-FLRO4 were vortexed and incubated (37°C, 1 h, dark), then washed and resuspended in PBS (500 µL). PBMC were isolated from fresh “recipient” whole blood (15 mL) using Ficoll-Paque reagent (GE Healthcare, Munich, Bavaria, Germany) density gradient centrifugation. Briefly, Ficoll-Paque reagent (15 mL) was layered under the diluted cell suspension (35 mL; whole blood in PBS) in a Falcon tube (50 mL), centrifuged (515 g, 20 min, without brake) and the PBMC layer was removed. PBMC were washed thrice with PBS (50 mL) and resuspended in residual PBS for staining with HLA-DR-PerCP, BDCA3-APC, CD11C-BV421, CD14-V500 (15 min, 4°C,
dark). FSL-FLRO4-PRBC (D2 or D42) were mixed with PBMC (10:1 ratio), and incubated (30 min, 37°C, 5% CO₂) before non-internalised PRBC were lysed with FACS lyse (1 mL). Phagocytosis of PRBC (expressed as percent positive and MFI) was determined by flow cytometry in gated monocyte (CD14⁺), mDC (CD14⁻HLA-DR⁺CD11c⁺) and BDCA3⁺ DC (CD14⁻HLA-DR⁺CD11c⁻BDCA3⁺) populations.

4.2.7. Flow cytometry
Three laser FACSCanto II flow cytometer was used and data analysed using FCS Express V5 or FCAP Array software (BD Biosciences).

4.2.8. Statistical analyses
GraphPad Prism was used for all statistical analyses and representation of graphs. ANOVA with Tukey’s post-test was used to assess the differences in surface antigen expression and inflammatory mediator production. Phagocytosis data were compared using paired t-test. p<0.05 was considered significant.
4.3. Results

4.3.1. Exposure to PRBC modulated mDC and BDCA3⁺ DC surface antigen expression

An in-vitro whole blood model of transfusion was used to assess the impact of PRBC on activation and maturation of mDC and BDCA3⁺ DC. PRBC alone down-regulated mDC expression of co-stimulatory molecules CD40 (p<0.0001) and CD80 (p=0.019), and up-regulated expression of CD83 (p<0.0001, Figure 4.1A-C). When polyI:C was co-cultured with PRBC to model the processes activated by viral infection, mDC expression of CD80 (p=0.006) was down-regulated and CD83 was up-regulated (p=0.006, Figure 4.1B, C). There was no change in expression of CD40 or CD86 compared to polyI:C alone (Figure 4.1A, D). When LPS was used to model the processes activated by bacterial infection, mDC expression of CD86 (p=0.030, Figure 4.1D) was down-regulated, and there was no change in CD40, CD80 or CD83 (Figure 4.1A-C).

For BDCA3⁺ DC, exposure to PRBC alone down-regulated expression of CD80 (p=0.006, Figure 4.1F) and up-regulated CD83 (p<0.0001, Figure 4.1G), with no changes in expression of CD40 or CD86 (Figure 4.1E, H). In the presence of polyI:C, BDCA3⁺ DC expression of CD80 (p=0.001) and CD86 (p=0.006) was suppressed (Figure 4.1F, H). In the presence of LPS, BDCA3⁺ DC expression of CD83 (p=0.005) was increased and CD86 (p=0.001) was suppressed in the presence of PRBC (Figure 4.1G, H). For both DC subsets, modulation of maturation and co-stimulatory molecules were particularly evident following exposure to stored (D42) PRBC (Tukey's post-test).
Figure 4.2. Assessment of mDC and BDCA3⁺ DC expression of maturation and costimulatory molecules following exposure to D2 and D42 PRBC. Data represent flow cytometric analysis of (A-F) mDC (purple) and (G-L) BDCA3⁺ DC (blue) surface antigen expression after being exposed to D2 or D42 PRBC with or without polyI:C or LPS (n=11 PRBC only, n=6 PRBC + polyI:C, n=5 PRBC + LPS. Data were normalised (log2) to the matched no transfusion control (dotted line at zero). Bars indicate mean ± SEM. Data analysed using ANOVA (*p<0.05, **p<0.01, ****p<0.0001) with Tukey’s post-test (a no transfusion vs. D2 or D42, b D2 vs. D42).
4.3.2. Exposure to PRBC down-regulated mDC and BDCA3+ DC production of inflammatory mediators

I further assessed the impact of PRBC on cytokine and chemokine production by mDC and BDCA3+ DC. Two panels of inflammatory mediators were used based on responses to polyI:C or LPS. Expression of inflammatory mediators was not significantly altered following exposure to PRBC alone for mDC (Figure 4.2A-F). When PRBC were co-cultured with polyI:C, mDC production of IL-6 \( (p=0.004) \), IL-8 \( (p=0.003) \), IL-12 \( (p=0.005) \) and TNF-\( \alpha \) \( (p=0.024) \) was reduced (Figure 4.2A-D). Similarly, co-culture of PRBC with LPS resulted in suppression of pro-inflammatory cytokines IL-6 \( (p=0.002) \) and TNF-\( \alpha \) \( (p=0.015) \) (Figure 4.2A, D).

For BDCA3+ DC however, a different response profile was found. Expression of IL-12 \( (p=0.028) \) and IP-10 \( (p=0.047) \) was suppressed following exposure to PRBC alone (Figure 4.2J, K). Co-culture of PRBC with LPS did not result in altered production of inflammatory cytokines, however, IL-6 \( (p=0.006) \), IL-8 \( (p=0.015) \), IL-12 \( (p=0.019) \), TNF-\( \alpha \) \( (p<0.0001) \) and IP-10 \( (p=0.001) \) production were significantly suppressed following co-culture of PRBC and polyI:C (Figure 4.2G-K). For both subsets, changes in production of inflammatory mediators were particularly evident following exposure to D42 PRBC (Tukey’s post-test).
Figure 4.3. Assessment of mDC and BDCA3⁺ DC cytokine and chemokine production following exposure to D2 and D42 PRBC. Data represent flow cytometric analysis of (A-F) mDC (purple) and (G-L) BDCA3⁺ DC (blue) expression of maturation and co-stimulatory molecules mediators after being exposed to D2 or D42 PRBC with or without polyI:C or LPS (n=11 PRBC only, n=6 PRBC + polyI:C, n=5 PRBC + LPS. Data were normalised (log2) to the matched no transfusion control (dotted line at zero). Bars indicate mean ± SEM. Data analysed using ANOVA (*p<0.05, **p<0.01, ****p<0.0001) with Tukey’s post-test (a no transfusion vs. D2 or D42, b D2 vs. D42).
4.3.3. Exposure to PRBC modulated the overall leukocyte inflammatory response

The impact of PRBC on recipients' overall cytokine and chemokine production was also examined. Exposure to PRBC alone did not result in modulation of the overall leukocyte inflammatory response (Figure 4.3A-M). In the presence of polyI:C, IL-8 production was augmented following exposure to D2 PRBC ($p=0.001$, Figure 4.3A). In the LPS, exposure to PRBC up-regulated IL-8 ($p=0.020$), IL-1β ($p=0.003$), IL-6 ($p=0.001$), MIP-1α ($p=0.020$), MIP-1β ($p=0.003$), and down-regulated IP-10 ($p=0.014$) production (Figure 4.3A-F). Changes in production of inflammatory mediators were particularly evident following exposure to D42 PRBC (Tukey's post-test).

Figure 4.4. Assessment of the overall leukocyte inflammatory response following exposure to D2 and D42 PRBC. (A-M) Concentration (pg/mL) of cytokines and chemokines secreted into culture supernatant after exposure to D2 or D42 PRBC with or without polyI:C or LPS (n=16 PRBC only; n=8 PRBC + polyI:C, n=8 PRBC + LPS). Presence and absence of PRBC, polyI:C or LPS were represented by plus (+) or minus symbols (-). Matched “No transfusion” controls were abbreviated as “No tx”. Bars indicate mean ± SEM. Data were analysed using ANOVA (*$p<0.05$, **$p<0.01$, ****$p<0.0001$) with Tukey’s post-test (a no transfusion vs. D2 or D5, b D2 vs. D5).
4.3.4. PRBC storage was associated with increased erythrophagocytosis

I assessed whether phagocytosis of PRBC may be a potential mechanism driving changes in mDC and BDCA3+ phenotype in the transfusion model. While increased uptake of stored PRBC by monocytes has been reported, whether mDC and BDCA3+ DC, contribute to PRBC clearance was unknown. I found that while monocytes were the primary cell associated with PRBC phagocytosis, mDC and BDCA3+ DC both phagocytosed D2 and D42 PRBC (Figure 4.4A). For all three myeloid subsets assessed, D42 PRBC were phagocytosed more than D2 PRBC (CD14+ monocytes, \( p=0.004 \); mDC, \( p=0.001 \); BDCA3+ DC, \( p=0.008 \); Figure 4.4A, B).

Figure 4.5. Assessment of erythrophagocytosis by mDC and BDCA3+ DC. (A) Percentage uptake and (B) MFI of phagocytosed FSL-FLRO4 labelled D2 and D42 PRBC by CD14+ monocytes (red), mDC (purple) and BDCA3+ DC (blue). Matched donors were used for assessment of update of D2 and D42 PRBC, data connected by a dotted line (n=5). Unpaired t-test *\( p<0.05 \), **\( p<0.01 \), ***\( p<0.001 \).
4.4. Discussion
Transfusion of PRBC has been reported to modulate recipients' immune responses. However, little is known about the mechanisms underpinning transfusion-related immune modulation. As DC play a central role in the initiation and regulation of both innate and adaptive immunity, I hypothesised that PRBC transfusion modulates key DC processes, impeding the capacity of these cells to adequately respond to subsequent immune challenges. Significant modulation of mDC and BDCA3+ DC phenotype following exposure to PRBC in my transfusion models was observed. Of particular importance for outcomes in transfusion recipients, I found changes to DC phenotype were predominantly found in our models mimicking the processes of infection, and stored PRBC had a more pronounced effect than fresh PRBC. This study provides the first evidence to suggest that patients with underlying infection may have worse outcomes following transfusion due to the impeded DC function.

DC maturation and co-stimulation are an essential process for downstream immune initiation and regulation [195]. I first assessed the effect of PRBC on mDC and BDCA3+ DC maturation and co-stimulatory surface antigen profile in my model of transfusion. I report augmentation of maturation molecule CD83 and suppression of co-stimulatory molecules (CD40, CD80, CD86) following exposure to PRBC. CD83 is up-regulated following exposure to pathogens [193] or TLR agonists including polyI:C (TLR3) and LPS (TLR4) [286]. Our finding that exposure to PRBC alone up-regulates CD83 suggests that either RBC themselves, or substances within the PRBC product have the capacity to stimulate DC maturation. Although CD83 increased, the co-stimulatory molecules were predominantly down-regulated following exposure to PRBC. This trend was similar for both mDC and BDCA3+ DC, and the suppression persisted even when the PRBC were co-stimulated with polyI:C or LPS. Given the complexity of the PRBC product and the changes that occur during storage, it is likely that multiple DC receptors are engaged resulting in differential expression of maturation and co-stimulatory molecules.

In addition to expression of co-stimulatory molecules, a balanced release of cytokines and chemokines by DC is also crucial for directing multiple immune pathways [93]. I found minimal modulation of cytokine responses following exposure to PRBC alone, however, both mDC and BDCA3+ DC responses to polyI:C and LPS were suppressed in the presence of PRBC. Together poor co-stimulation and lack of cytokine and chemokine production may be associated with inadequate T and B lymphocyte responses [79, 80, 88].
and my data suggest PRBC transfusion has the potential to impair downstream lymphocyte responses.

I hypothesised that changes in mDC and BDCA3⁺ DC phenotype may be in part due to phagocytosis of PRBC. Indeed I report that both mDC and BDCA3⁺ DC play a role in RBC uptake, and stored PRBC were phagocytosed more frequently and rapidly than fresh PRBC. Therefore, in addition to uptake by monocytes (human monocyctic cell line (THP-1)) as reported previously [283], mDC and BDCA3⁺ DC both preferentially recognise and remove stored PRBC. This is likely attributed to the significant changes in PRBC that occur during routine storage which result in expression of DAMP [287]. It has been reported that inflammation enhances erythrophagocytosis [91, 92] and the modulation of mDC and BDCA3⁺ DC phenotype observed in this study, particularly in the models associated with infection processes, may be the result of increased erythrophagocytosis.

In addition to studying mDC and BDCA3⁺ DC specific responses, the overall inflammatory response of the peripheral blood leukocytes in the culture was investigated. This effectively models the inflammatory profile that could be expected in plasma of transfused patients. I observed minimal impact on the overall leukocyte inflammatory response in the presence of PRBC alone, and PRBC co-cultured with polyI:C. When modelling processes associated with bacterial infection, PRBC augmented production of IL-1β, IL-8, MIP-1α and MIP-1β and suppressed the production of IP-10. I found it interesting that the overall leukocyte inflammatory response was largely augmented by exposure to PRBC whereas the specific cellular responses of mDC and BDCA3⁺ DC were attenuated. These results highlight the potential for mechanisms associated with specific cell subsets to be missed when only assessing overall leukocyte inflammatory response, which largely represent the response of the more numerous cell subsets such as neutrophils, lymphocytes and monocytes.

I studied not only mDC but also the rarer subset of mDC, BDCA3⁺ DC, as they have a specialised and unique role in the immune response. While mDC express pattern recognition receptors predominantly associated with recognition of bacteria, the pattern recognition receptor profile of BDCA3⁺ DC is skewed towards recognition of viruses. Following receptor ligation, mDC present antigen via the standard MHC class II pathway, but BDCA3⁺ are superior in cross-presentation of antigen from dead cells onto MHC class I. BDCA3⁺ DC have been shown to be important in CD8⁺ T cell responses [224, 225] and
have been identified as a key cell involved in anti-tumour immunity [246]. I report for the first time that PRBC significantly modulate BDCA3⁺ responses and propose these cells may play a role in increased rate of cancer recurrence in transfusion recipients.
4.5. Conclusion

This chapter provided evidence that exposure to PRBC modulated the cellular responses of both mDC and the specialised subset BDCA3⁺ DC. Importantly, I found modulation of DC function was particularly evident in models combining transfusion and TLR agonist that mimics the processes of concurrent infection. A more prominent affect was also observed following exposure to stored PRBC. These results suggest that patients who receive stored PRBC, particularly those with underlying infectious complications, may fail to mount an appropriate immune response precipitating worse patient outcomes. However patient studies will be necessary to confirm data derived from my *in-vitro* model of transfusion. I hypothesise DC are particularly vulnerable to modulation by transfusion due to their multifaceted role in pattern recognition, phagocytosis, activation and signalling the adaptive immune response and, propose changes in DC phenotype and function are potential mechanisms underpinning transfusion-related immune modulation.
Chapter 5. Platelet concentrates modulate myeloid dendritic cell immune responses

Synopsis: In this chapter, the potential for PC to modulate responses of mDC and the specialised BDCA3⁺ subset was assessed using an in-vitro whole blood culture model of transfusion, addressing Aim 2 of this dissertation. I proposed that modulation of DC activation and maturation may be one mechanism underpinning transfusion-related immune modulation driving poorer patient outcomes. Knowledge of the in-vitro effect of PC transfusion and ex-vivo storage duration on the immune system will provide further evidence to our understanding of PC in transfusion-related immune modulation.
5.1. Introduction

Transfusion of PC is the required standard of care for treating patients with thrombocytopaenia and bleeding associated with severe trauma. PC are also transfused prophylactically to maintain haemostasis and limit bleeding in patients with bone marrow suppression or failure from ablative chemotherapy or neoplasia, especially when undergoing invasive procedures or surgery. Although PC transfusions are widely used, they are also reported to be associated with adverse patient outcomes. Studies suggest that PC transfusion is associated with higher rates of adverse patient outcomes than PRBC transfusions [94-96].

PC transfusion-related reactions are frequently characterised by febrile non-haemolytic reactions (chills and rigors) and allergic (rash and urticaria) reactions [98]. In addition, transfusion-related immune modulation has been associated with PC transfusion with outcomes including increased mortality [108-110], TRALI [112, 288], infectious complications and prolonged hospital stays [10, 108, 119].

In Australia, PC are routinely stored up to 5 days at 20-24°C with continuous agitation [45]. Following the introduction of bacterial screening the shelf life of PC has been extended up to 7 days in some European countries [121, 122] and the United States [123], but remains at 5 days in Australia. During ex-vivo PC storage, platelets undergo progressive biochemical and biomechanical modifications. They become partially activated, lose membrane asymmetry associated with exposure of bioactive lipids/apoptosis molecules (e.g. PS), release microparticles and produce inflammatory mediators such as sCD40L [95, 269]. While the impact of ex-vivo storage on the basic biochemical and biophysical properties of PC have been comprehensively studied, there is limited understanding of how these changes impact on patient outcomes. Whether stored PC are associated with increased numbers of poor patient outcome remains controversial, with a number of studies concluding that transfusion with stored PC results in increased febrile non-haemolytic reactions [97, 116-118], whilst other studies are not concordant with these findings [119, 120]. These poorer clinical outcomes may be associated with modulation of recipients’ immune responses due to changes in the platelet itself, or due to the accumulation of platelet-derived substances during PC ex-vivo storage.

Prior to the introduction of leukodepletion, the presence of donor leukocytes and the release of soluble HLA during storage was reported as a trigger of poor outcomes post-PC
transfusion [105, 154]. Reactions mediated by HLA antibodies were responsible for a large proportion of reported febrile non-haemolytic reactions, which were significantly reduced following the implementation of leukodepletion [106, 107]. While febrile non-haemolytic reactions have been reduced, our understanding of other poor outcomes (mortality, morbidity, increased rates of infection) following PC transfusion remains limited. The mechanisms of transfusion-related immune modulation in patients reporting adverse outcomes remains largely undefined.

In addition to the critical role platelets have in coagulation and haemostasis of the circulatory system, there is emerging evidence of a fundamental role for platelets in regulating immune cells including neutrophils [155], monocytes [156, 157], B lymphocytes [158, 159], T lymphocytes [159, 160] and DC [142, 161, 162]. In-vitro and in-vivo models have been used to elucidate potential mechanisms underpinning PC transfusion-related immune modulation. In-vivo models have reported a number platelet-derived substances such as sCD40L, RANTES, bioactive lipids, and the expression of MHC class I surface molecules could be mediating poor patient outcomes [145, 163-165]. In-vitro studies have also reported that stored PC (≤D3) activate monocytes, B lymphocytes and T lymphocytes [166]. In addition monocyte production of IL-12 was suppressed following exposure to PC with LPS [167]. In another study, overall leukocyte production of pro-inflammatory TNF-α and anti-inflammatory IL-10 was increased following exposure to PC alone with TNF-α reduced in the model of PC and LPS [89]. Recently, Perros et al. (2015) reported that PC supernatant modulated mDC responses and the overall inflammatory response [168]. In this model, PC supernatants predominantly suppressed mDC responses, particularly when modelling bacterial infection with LPS. This study also reported that dose was more important than ex-vivo storage period in modulation of the immune profile. In addition to blood DC, MoDC have also been used to assess immune modulation associated with PC transfusion, however, Hamzeh-Cognasse et al. (2008) found no changes in MoDC surface antigen expression or inflammatory profile when co-cultured with PC alone [162]. To date, the impact of PC transfusion on blood DC immune responses remains largely unexplored.

DC provide a crucial linkage between the innate and adaptive immune systems and regulating the host immune response [193-198]. Within human peripheral blood, DC are broadly categorised into two major groups based on their lineage: myeloid and plasmacytoid. DC are further organised into four subsets: myeloid BDCA1⁺ and BDCA3⁺ DC, and, plasmacytoid BDCA2⁺ and BDCA4⁺ DC [204, 205]. These cells have specialised
functions shaped by the differential expression of pattern recognition receptors such as TLR or C-type lectin receptors that recognise PAMP or DAMP. As the predominant subset of mDC, BDCA1⁺ DC express high levels of TLR skewed towards recognition of bacteria and antigen presentation via MHC class II. The rare mDC subset, BDCA3⁺ DC is equipped with pattern recognition receptors that favour recognition of viruses, and are highly efficient in cross-presenting endogenous antigen via MHC class I [224, 225]. Upon recognition of PAMP or DAMP, DC become activated. Activation is accompanied by phenotypic changes for presenting antigen and initiating downstream signalling including expression of maturation and co-stimulatory molecules, and secretion of cytokines and chemokines [195, 201]. Interference of these DC processes can impinge on host immune responses significantly [202, 203], which may contribute to the undesirable consequences reported in PC transfusion recipients.

Although PC have been reported to modulate the responses of a number of immune cell subsets, very little is known about the impact of PC transfusion on blood mDC responses. In addition, the specialised BDCA3⁺ DC subset has not been studied in the context of PC transfusion. Therefore, we investigated the potential impact of PC on mDC and BDCA3⁺ DC responses in-vitro. This chapter aimed to investigate the hypothesis that transfusion of PC modulates key DC maturation and activation processes, impeding DC capacity to establish an adequate response to subsequent immune challenges.
5.2. Materials and Methods

5.2.1. Ethics
Ethics approval statement as per Chapter 2 (section 2.2.1).

5.2.2. PC
Preparation and processing of PC were as described in the Chapter 3 (section 3.2.4 and 3.2.4.2). Leukodepleted buffy-coat-derived PC were obtained from the Blood Service and stored at 20-24°C with continuous gentle agitation. PC were used “fresh” (D2) or “stored” (D5, date-of-expiry).

5.2.3. Blood collection for transfusion model
As outlined in Chapter 4 (section 4.2.3), fresh whole blood samples were collected from different consented healthy volunteers (Blood Service staff) and utilised as the “recipient” (source of leukocytes including DC) in the model of transfusion. Samples were collected in EDTA spray-coated blood collection tubes and used within one hour of collection.

5.2.4. In-vitro whole blood model of transfusion
A human in-vitro model was used to study the potential of PC to mediate immune modulation in a “transfusion recipient”. A whole blood model with a 25% blood replacement volume transfusion was used, as described in Chapter 4 (section 4.2.3).

5.2.5. Assessment of mDC and BDCA3+ DC surface antigen expression and intracellular inflammatory mediator production following PC exposure
Leukocytes from the whole blood transfusion model were stained with mouse anti-human mAb for assessment of surface antigen expression and intracellular mediators and compensations were applied as outlined in Chapter 4 (section 4.2.4). Experiment replicates were as follows for assessment of surface antigen expression and intracellular mediators: n=10 PC only, n=5 PC + polyI:C and n=5 PC + LPS.

5.2.6. Assessment of overall leukocyte inflammatory response following exposure to PC
Culture supernatants harvested from models without transporter protein inhibitor treatment were further processed via triple centrifugation (515 g for 5 min, 1600 g for 20 min, 12 000 g for 5 min). Supernatants were collected and stored at -80°C until CBA analysis. Concentrations of inflammatory mediators were assessed in culture supernatants from the
transfusion model (n=10 PC only, n=5 PC + polyI:C or LPS) using CBA, outlined in
Chapter 4 (section 4.2.4).

5.2.7. Flow cytometry

Three laser FACSCanto II flow cytometer was used and data were analysed using FCS
express V5 and FCAP Array software as per Chapter 4 (section 4.2.7).

5.2.8. Statistical analyses

All statistical analyses and representation of graphs were generated using GraphPad
Prism as per Chapter 4 (section 4.2.8). ANOVA with Tukey’s post-test was used to assess
the differences in surface antigen expression and inflammatory mediator production
following PC exposure. p<0.05 was considered statistically significant.
5.3. Results

5.3.1. Exposure to PC down-regulated mDC and BDCA3⁺ DC surface antigen expression

The capacity of mDC to initiate immunity is dependent upon their maturation stage [193], and is accompanied by phenotypic changes associated with antigen presentation [195]. The immunomodulatory effect of PC on mDC and BDCA3⁺ DC maturation and activation was assessed utilizing an *in-vitro* whole blood model of PC transfusion. Exposure to PC alone down-regulated expression of mDC co-stimulatory molecules CD40 (*p*=0.004) and CD80 (*p*=0.002; Figure 5.1A, B). Co-culture of PC with polyI:C, to model the processes activated by underlying viral infection, resulted in reduced expression of mDC CD40 (*p*=0.002) and CD86 (*p*=0.012; Figure 5.1A, C). Suppression of mDC activation and maturation molecules was not evident in the model of PC transfusion with LPS to model the processes activated by underlying bacterial infection (Figure 5.1A-D).

For BDCA3⁺ DC, we found a broader profile of immune modulation following exposure to PC. PC alone down-regulated BDCA3⁺ DC expression of CD40 (*p*=0.005) and CD80 (*p*=0.001; Figure 5.1E, F). In the presence of polyI:C, exposure to PC reduced BDCA3⁺ DC expression of CD40 (*p*=0.019), CD80 (*p*=0.001) and CD83 (*p*=0.004; Figure 5.1E, F, H). In the presence of LPS, exposure to PC decreased BDCA3⁺ DC expression of CD80 (*p*=0.013), CD86 (*p*=0.010) and CD83 (*p*=0.0002; Figure 5.1F-H).
Figure 5.1. mDC and BDCA3⁺ DC surface antigen expression profile following exposure to D2 and D5 PC. Flow cytometric assessment of (A-D) mDC (purple) and (E-H) BDCA3⁺ DC (blue) expression of maturation and co-stimulatory molecules after exposure to D2 or D5 PC with or without polyI:C or LPS for 6 h (n=10 for PC only, n=5 for PC + polyI:C, n=5 for PC + LPS). Data were normalised (log2) to the matched “no transfusion” control (dotted line at zero). Bars indicate mean ± SEM. Data analysed using ANOVA (*p<0.05, **p<0.01, ***p<0.001) with Tukey’s post-test (a no transfusion vs. D2 or D5).
5.3.2. Exposure to PC suppressed mDC and BDCA3⁺ DC production of inflammatory mediators

DC production of cytokines and chemokines are crucial for stimulating and regulating the immune system [204]. Therefore, I assessed a panel of inflammatory mediators produced by mDC and BDCA3⁺ DC to further investigate the potential impact of PC transfusion on recipient immune responses. In-vivo, DC activation is induced by mechanisms such as pathogens and inflammatory cytokines [193], therefore, to mimic the processes activated by such effects in-vitro, we used polyI:C or LPS. Based on the differential responses to polyI:C or LPS, two panels of inflammatory mediators were examined, with IP-10 used in the polyI:C model and IL-10 in the LPS model. mDC production of IL-8 (p=0.002), IL-12 (p=0.001) and TNF-α (p=0.003) was suppressed in the presence of PC alone (Figure 5.2A-C). The suppression was particularly evident for mDC IL-8 production following exposure to D5 PC. In the presence of polyI:C, mDC production of IL-12 (p=0.010) and IP-10 (p=0.008) was reduced in the presence of PC (Figure 5.2B, D). In addition, IP-10 production was reduced following exposure to D5 PC compared to D2 PC. In the presence of LPS, mDC production of IL-6 (p=0.009) and IL-10 (p=0.004) was reduced following exposure to PC (Figure 5.2E, F).

For BDCA3⁺ DC, exposure to PC alone suppressed production of IL-8 (p=0.002), IL-12 (p=0.002) and IL-6 (p=0.010; Figure 5.2G, H, K). In the presence of polyI:C, expression of IL-8 (p=0.008), IL-12 (p=0.001) and IP-10 (p=0.002) was suppressed following exposure to PC (Figure 5.2G, H, J), with further suppression of IL-8 in the presence of D5 PC. In the presence of LPS, PC suppressed TNF-α (p=0.001). IL-6 (p=0.001) and IL-10 (p=0.008) production by BDCA3⁺ DC (Figure 5.2I, K, L). For both DC subsets, exposure to D2 and D5 PC predominantly attenuated the production of inflammatory mediators.
Figure 5.2. mDC and BDCA3+ DC inflammatory profile following exposure to D2 and D5 PC. Intercellular flow cytometric assessment of (A-F) mDC (purple) and (G-L) BDCA3+ DC (blue) production of cytokines and chemokines after exposure to D2 or D5 PC with or without polyI:C or LPS for 6 h (n=10 for PC only, n=5 for PC + polyI:C, n=5 for PC + LPS). Data were normalised (log2) to the matched “no transfusion” control (dotted line at zero). Bars indicate mean ± SEM. Data were analysed using ANOVA (*p<0.05, **p<0.01, ***p<0.001) with Tukey’s post-test (a no transfusion vs. D2 or D5, b D2 vs. D5).
5.3.3. Exposure to PC modulated the overall leukocyte inflammatory response

The effect of PC on the “recipient” overall leukocyte inflammatory response was studied in culture supernatants from the transfusion model. This represents the expected changes in inflammatory profile in patient’s plasma following PC transfusion. Exposure to PC alone augmented IP-10 ($p=0.021$) and IFN-α ($p=0.037$) production (Figure 5.3A, B). When modelling processes associated with viral infection, exposure to PC suppressed the overall leukocyte production of IP-10 ($p=0.035$) and MCP-1 ($p=0.040$; Figure 5.3A, C). When modelling processes associated with bacterial infection, the level of IP-10 ($p=0.002$), IL-6 ($p=0.002$) and MIP-1β ($p=0.001$) was reduced, and IL-1β ($p=0.006$) and IL-8 ($p<0.0001$) was augmented, following exposure to PC (Figure 5.3A, D-G). IL-8 production further increased following exposure to D5 PC.

Figure 5.3. The overall leukocyte inflammatory profile following exposure to D2 and D5 PC. (A-M) Peripheral leukocytes in the whole blood model were exposed to D2 or D5 PC with or without polyI:C or LPS. Presence and absence of PC, polyI:C or LPS were represented by plus + or - symbols. Matched “No transfusion” controls were abbreviated as “No tx”. Levels (pg/mL) of cytokine and chemokine released into the culture supernatant were measured (n=10 for PC only, n=5 for PC + polyI:C, n=5 for PC + LPS). Bars indicate mean ± SEM. Data were analysed using ANOVA (*$p<0.05$, **$p<0.01$, ****$p<0.0001$) with Tukey’s post-test (a no transfusion vs. D2 or D5, b D2 vs. D5).
5.4. Discussion

PC transfusion has been proposed to modulate recipients’ immune responses. Clinical studies have reported increased rate of mortality, infectious complications and prolonged LPS in patients following PC transfusion [10, 108-110, 112, 119, 288]. However, the mechanisms driving these poor patient outcomes remain ambiguous. Given DC have a multifaceted role in both the innate and adaptive immune system, I hypothesised that PC transfusion alters DC maturation and activation processes, thereby hindering the establishment of an adequate response against immune challenges. Here, I report that exposure to PC significantly modulated maturation and activation of both mDC and specialised subset BDCA3+ DC. Interestingly, we found changes predominantly in the models mimicking the processes involved in infection. I have demonstrated for the first time that exposure to PC impedes both mDC and BDCA3+ DC function, and provided the basis for further assumption that these changes may be one mechanism underpinning worse outcomes in patients, in particularly those with underlying infection.

DC maturation and co-stimulation are essential for regulating and stimulating the immune system. Appropriate activation of DC and associated phenotypic changes are crucial for the initiation of an adequate and sustainable host immune response [195]. In this study I first assessed the impact of PC on mDC and BDCA3+ DC maturation and co-stimulatory surface antigen profile and found that exposure to PC alone down-regulated expression of CD40 and CD80 on both DC subsets. To date, no other studies have investigated modulation of mDC or BDCA3+ DC surface antigen expression in correlation with PC transfusion preventing a direct comparison of our results with previous publications. However, MoDC models have been used to assess immune modulation following exposure to PC or freshly isolated platelets. Hamzeh-Cognasse et al. (2008) report PC did not modulate MoDC phenotype in models that facilitated cell-cell interactions [162] and Kissel et al. (2006) reported that exposure to resting freshly isolated platelets did not affect MoDC expression of CD40, CD83 and CD86 [161]. These results are in contrast to my observations, however, different models were used. I have specifically chosen a whole blood culture based transfusion model, rather than a model using isolated cells as in the above mentioned studies [161, 162], to be more representative of changes that could be expected in transfusion recipients. My whole blood model facilitates cell-to-cell interactions of the blood component and the leukocyte subsets without the need for cell isolation procedures, involving density gradients or cell separation.
Not only did I assess the potential immune modulation associated with transfusion of PC only, we also incorporated models of the different types of concurrent infection. In the model of LPS-bacterial infection, expression of maturation and co-stimulatory molecules on mDC were not changed following exposure to PC. My mDC results are in line with Kissel et al. (2006) who reported no effect on the expression of MoDC maturation and co-stimulatory molecules in the presence of thrombin receptor-agonist peptide (TRAP)-activated platelets with LPS [161]. Although mDC phenotype was unchanged in our model of LPS-bacterial infection, we observed suppression of CD80, CD83 and CD86 on BDCA3+ DC following exposure to PC. This differential response suggests that BDCA3+ DC are more susceptible to immune modulation than mDC and I propose that this may be what happens in patients receiving PC transfusion who have underlying bacterial infections. In this study, the effect of PC transfusion was assessed for the first time in a model mimicking the processes activated by viral infection. I found co-culture of PC and polyI:C reduced expression of co-stimulatory and maturation markers on both DC subsets although a different profile of response was evident.

Together with maturation and co-stimulatory molecules, appropriate DC production of inflammatory mediators are also crucial to the downstream immune responses [204]. We examined the effect of PC on mDC and BDCA3+ DC production of cytokines and chemokines. I observed modulation of mDC and BDCA3+ DC pro-inflammatory cytokine and chemokine production with PC “transfusion” alone. Using a similar whole blood model, but with PC supernatant, Perros et al. (2015) reported little modulation of mDC inflammatory profile [168], suggesting that changes observed in our study may be via cell contact mediated process rather than the result of platelet-derived soluble factors alone.

The importance of cell-cell interactions in determining the outcome of the immune response to PC has also been reported by Hamzeh-Cognasse et al. (2008), who investigated the change in MoDC phenotype as a result of cell-to-cell contact or filter-separated models where soluble factors in the PC could exchange between compartments without direct cell contact [162]. These studies highlight the importance of cell-to-cell interactions in understanding mechanisms associated with transfusion-related immune modulation and better reflect the potential immune profile of transfusion in patients. Taken together, suppression of appropriate DC maturation and co-stimulatory molecules as well as an altered profile of cytokine production, may result in inadequate B and T and lymphocyte responses following transfusion of PC, especially in patients with underlying infection.
This study has been the first to investigate the impact of PC on BDCA3+ DC specific responses in a model of transfusion. BDCA3+ DC are a rare and specialised DC subset that have a unique role in the immune system. Traditionally, mDC are equipped with pattern recognition receptor favouring bacterial recognition and antigen presentation via standard MHC class II. BDCA3+ DC, however, favour viral recognition and these cells are capable of presentation of endogenous antigen on MHC class I [224]. This so called “cross-presentation” has been reported to be important in mediating cytotoxic T lymphocyte anti-tumour immunity [224, 225, 246]. In the context of PC transfusion for cancer patients, poorer clinical outcomes may be a consequence of suppressed BDCA3+ DC maturation and activation, however, additional patient studies will be required to confirm my findings. Moreover, BDCA3 is also known as TM, a membrane bound glycoprotein that was originally identified on endothelial cells as a cofactor for generation of aPC [248]. In addition to binding thrombin and accelerating generation of aPC, it has been reported TM has many binding partners and plays a role in linking coagulation, inflammation and immunity [252]. TM also binds TAFI and has a cofactor for inactivation of C3b. Moreover, TM has a C-type lectin domain that binds two known ligands – HMGB1 protein and the carbohydrate Lewis Y. Binding of the CTLD with either ligand results in an anti-inflammatory profile [257, 258]. In models of allergy, BDCA3+ DC are reported to be tolerogenic and it may be these cells play a similar role in preventing an inflammatory response to PC transfusion. The PC product is a complex mix of cells and cellular derived products that have the capacity to bind numerous receptors on DC resulting in modulation of DC responses. Additional studies to help elucidate the specific role of BDCA3/TM in modulation of the immune response post-PC transfusion are warranted.

In addition to specific changes in mDC and BDCA3+ DC, I investigated the overall inflammatory response of recipient peripheral leukocytes in my model of PC transfusion. This provides an in-vitro representation of the inflammatory mediators expected in patient’s plasma post-PC transfusion. We found minimal impact on the overall inflammatory response of leukocytes following exposure to PC alone, a similar result to that reported by Perros et al. (2015) using model with PC supernatant only [168]. In the present study, we also modelled the process associated with viral infection in the context of PC transfusion and the level of inflammatory mediators secreted remained largely unchanged following exposure to PC and polyI:C compared to polyI:C alone. However, when modelling the processes involved in bacterial infection the immunomodulatory capacity of PC was more apparent. Exposure to PC suppressed the LPS-driven production of IL-6, IP-10 and MIP-
1β, and an increased secretion of IL-1β and IL-8. These results are similar to a previous study which focused on responses to PC supernatants [168]. Given the importance of balanced secretion of inflammatory mediators in directing immune pathways, the up-regulated responses observed in this model may be associated with a number of feedback mechanisms resulting in an attempt to compensate for the suppression of other cytokines and chemokines. The array of inflammatory mediators modulated suggests that PC transfusion may perturb the production of pro-inflammatory cytokines and chemokines in patients’ especially those with underlying bacterial infection. This may hinder B and T lymphocyte responses as well as the recruitment of lymphocytes, neutrophils, monocytes, NK cells to the site of infection or injury [79, 80, 88, 289]. While changes in the overall leukocyte inflammatory response were evident, it is important to consider these results predominantly represent the response of the more abundant leukocyte subsets, such as neutrophils and lymphocytes. Specific, and sometimes critically important changes in small specialised immune cell subsets would not be evident using this approach, which is why cell specific mDC and BDCA3+ DC responses were also targeted.

The changes I observed in mDC and BDCA3+ DC specific responses, as well as the overall inflammatory response were predominately independent of ex-vivo storage duration of PC. The data provide evidence to suggest that transfusion of even freshly prepared PC may be immunomodulatory, and that changes that occur during routine storage do not necessarily result in further modulation of immune responses. This study is concordant with the work of Perros et al. (2015), who found dose was more important than ex-vivo storage duration when mDC response and the overall inflammatory response was assessed following exposure to PC supernatants [168]. This is an important consideration for administration of PC, indicating that dose, treatment regimens and appropriate product use may be the key to reducing potential immune modulation and possible poor patient outcomes post-PC transfusion.
5.5. Conclusion

In summary, my data demonstrated that in an in-vitro model, PC “transfusion” differentially suppressed mDC and BDCA3⁺ DC. Of particular importance, modulation of the cellular responses was more evident in both mDC and BDCA3⁺ DC in the models that mimic the processes involved in the concurrent infection. Taken together, these data presented the question that transfusion recipients, particularly those suffering from underlying infection, may have a higher rate of poorer patient outcomes due to failure to establish an adequate immune response. I further hypothesise that BDCA3⁺ DC may be important in regulating anti-inflammatory responses to PC transfusion, which could compromise the immediate response to immune challenge. For cancer patients with suppressed bone marrow, PC may further repress patients’ immunity and anti-tumour activity, resulting in increased cancer recurrence and infection complications. However, repressed DC response may potentially be beneficial for trauma patients where reducing excess inflammation could reduce organ injury and improve patient outcomes. Future studies in patients will be required to extend our understanding of BDCA3⁺ DC and their clinical significance in the context of transfusion. While the role of TM remains unclear as a mechanism associated with transfusion-associated immune modulation, the potential for BDCA3⁺ DC to serve as a biomarker to help assess or predict patients’ prognosis is worthy of further investigation.
Chapter 6. Cryopreserved platelets modulate BDCA3+ dendritic cell surface antigen expression and inflammatory response

Synopsis: In this chapter, whether cryo-PLT mediate modulation in mDC and BDCA3+ DC surface antigen expression, and cytokine and chemokine production was assessed using an in-vitro whole blood culture model of transfusion, addressing Aim 2 of this thesis. In this study, I proposed that exposure to cryo-PLT modulate DC immune profile. Knowledge of the in-vitro effect of cryo-PLT transfusion on the immune system will provide further evidence to our understanding of PC in transfusion-related immune modulation.
6.1. Introduction

Cryopreservation of platelets is an effective technique for prolonging ex-vivo shelf life for up to two years [177]. Cryopreservation can help overcome issues associated with short shelf life of this biological product and improve logistics associated with supply, while minimising wastage [173, 178]. The supply of cryo-PLT is particularly appealing in military combat settings and remote areas. The use of cryo-PLT for transfusion has predominately been in military combat areas to treat bleeding patients, and therapeutic efficacy has been well documented [169, 179, 290].

The use of cryo-PLT and other cryopreserved blood components has reduced mortality rates in the military from 56% to 16% with few adverse transfusion outcomes reported [169]. Other clinical studies report cryo-PLT transfusion reduced bleeding time [172, 174, 175] and required the use of fewer blood products [172]. The FDA criteria for cryo-PLT requires 66% recovery and 50% lifespan compared to fresh conventional PC (set at 100%) [169, 172, 174, 175, 179, 290]. However, cryo-PLT survival and platelet increment count post-transfusion are lower in comparison to conventional room temperature stored PC [169, 172, 174, 175, 179, 290, 291]. These platelet products are still very different from each other.

During the process of platelet cryopreservation (cooling to very low temperatures, typically -80°C) and preparation for transfusion (thawing at 37°C) platelet properties and function may be modified [172, 180-184]. In-vitro studies have reported significant reduction in cryo-PLT recovery [177], aggregation [172, 180, 181] and hypotonic stress response [182] in comparison to conventional PC. Cryo-PLT are reported to be more haemostatically active, with greater pro-coagulant activity, thrombin and thromboxane generation [172, 180, 183, 189]. These modifications are associated with a significantly higher proportion of non-discoid platelets [181] as well as microparticles expressing apoptotic mediators including PS, and other cytoskeletal membrane proteins (e.g. including actin, filamin, gelsolin, and tropomyosin) within the unit [180, 183, 184]. These changes may be due to the process of cryopreservation inducing cryoinjury due to excessive cellular dehydration, osmotic injury and extracellular ice crystal formation [191]. Whether these changes in cryo-PLT modulate immune function modulation in transfusion recipients is currently unknown.

With several reports demonstrating the capacity for conventional PC or PC supernatant “transfusion” to alter immune response of leukocytes [89, 166-168], MoDC [162] and blood
mDC [168] *in-vitro*, it is possible that cryo-PLT may exert a similar pattern of immune modulation. For example, as established in Chapter 5, exposure to PC predominately suppressed mDC and the specialised subset BDCA3⁺ DC responses, particularly in models of infection processes. In a clinical context, PC transfusion may be beneficial in preventing excessive inflammatory insult in trauma patients, but for cancer patients this may result in susceptibility to infection, driving poorer outcomes.

DC are professional antigen presenting cells that occupy a specific niche as a bridge between the innate and adaptive immune systems. They are specialised and unique cells with the capacity to initiate both the innate and adaptive immunity [194], stimulate and regulate primary B and T lymphocytes and NK cells [193, 195]. Myeloid and plasmacytoid are the two main lineages of DC, which are further divided into four subsets. Compared to pDC (BDCA2⁺ and BDCA4⁺) subsets, mDC (BDCA1⁺ and BDCA3⁺) subsets are more efficient in the uptake, processing and presentation of foreign antigens [195, 284]. Of interest, BDCA3⁺ DC are superior in cross antigen presentation and mediating immune responses against viruses [224, 225]. In addition, BDCA3⁺ DC are major producers of IFN-λ (IL-28/IL29) following ligation with viral components, thereby, effectively activating anti-viral immunity [245]. These specialised functions are shaped by the differential expression of pattern recognition receptors including those that recognise PAMP and DAMP. Upon PAMP or DAMP ligation, DC undergo activation and maturation, which DC are programmed to increase expression of maturation (CD83) and co-stimulatory (CD40, CD80 and CD86) molecules, and cytokines and chemokines for antigen presentation and initiating downstream signalling [195, 201]. As previously suggested, transfusion of blood components perturbs these DC processes in *in-vitro* model providing evidence of pathways for mediating significant modulation in host immune responses and, as a consequence, may lead to poorer clinical outcomes [168].

No evidence has been reported with respect to immunomodulatory capacity of cryo-PLT transfusion in recipients. Herein, this chapter aimed to investigate whether cryo-PLT mediate immune modulation of mDC and the specialised subset BDCA3⁺ DC using an *in-vitro* human whole blood model of transfusion. I hypothesised that cryo-PLT have the capacity to modulate maturation and activation of mDC and BDCA3⁺ DC in the model of transfusion, which can impair immune responses.
6.2. Material and Methods

6.2.1. Ethics
Ethics approval statement as per Chapter 2 (section 2.2.1).

6.2.2. Preparation and cryopreservation of PC (cryo-PLT)
Preparation and processing of cryo-PLT were as described in Chapter 3 (section 3.2.4). Leukodepleted buffy-coat-derived PC obtained from the Blood Service were stored at -80°C as cryo-PLT and, thawed at 37°C and reconstituted in FFP before used.

6.2.3. Blood collection for transfusion model
As outlined in Chapter 4 (section 4.2.3), fresh whole blood samples from different consented healthy volunteers (Blood Service staff) were collected in EDTA spray-coated blood collection tubes and utilised as the “recipient” (source of leukocytes including DC) in the model of transfusion.

6.2.4. In-vitro whole blood model of transfusion
A human in-vitro model was used to study the potential of cryo-PLT to mediate immune modulation in a transfusion recipient. A whole blood model with a 25% blood replacement volume transfusion was used, as described in Chapter 4 (section 4.2.3).

6.2.5. Assessment of mDC and BDCA3+ DC surface antigen expression and intracellular inflammatory mediator production following cryo-PLT exposure
As outlined in Chapter 4 (section 4.2.4), leukocytes from the whole blood transfusion model were stained with mouse anti-human mAb for assessment of surface antigen expression and intracellular inflammatory mediators, and compensations were applied. Experiment replicates were as follows for assessment of surface antigen expression and intracellular mediators: n=10 cryo-PLT only, n=5 cryo-PLT + polyI:C and n=5 cryo-PLT + LPS.

6.2.6. Assessment of overall leukocyte inflammatory response following exposure to cryo-PLT
As outlined in section 5.2.6, culture supernatants were harvested via triple centrifugation. Concentrations of inflammatory mediators were assessed in culture supernatants from the transfusion model (n=10 cryo-PLT only, n=5 cryo-PLT + polyI:C or LPS) using CBA, outlined in Chapter 4 (section 4.2.4). In addition to the panel of inflammatory mediators
outlined in section 4.2.4 for CBA, the level of IL-28a/IL-28b and IL-29/IL-28b (also known as IFN-λ) in the culture supernatants was quantified using enzyme-linked immunosorbent assay (ELISA; DuoSet ELISA kits; R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's specification. Absorbance at 450 nm was measured on a Synergy™ H1 monochromator-based microplate reader (BioTek Instruments Inc., Winooski, VT, USA) using the Gen5 version 2.06 software (BioTek). Wavelength correction was made against absorbance at 570 nm. Concentrations were extrapolated from standard curves performed in parallel.

6.2.7. Flow cytometry
Three laser FACSCanto II flow cytometer was used and data were analysed using FCS express V5 and FCAP Array software as per Chapter 4 (section 4.2.7).

6.2.8. Statistical analyses
GraphPad Prism was utilised to conduct all statistical analyses and representation of graphs. Differences in surface antigen expression and inflammatory mediator production between culture with cryo-PLT and the matched "no transfusion" control were assessed using paired t-test. \( p<0.05 \) was considered significant.
6.3. Results

6.3.1. Cryo-PLT significantly reduced the expression of BDCA3+ DC maturation and co-stimulatory surface antigen expression in response to polyI:C or LPS

The capacity of cryo-PLT to modulate mDC or BDCA3+ DC maturation and activation was examined using an *in-vitro* whole blood model of transfusion. Exposure to cryo-PLT alone up-regulated mDC expression of CD83 (p=0.012), and BDCA3+ DC expression of CD86 (p=0.006; Figure 6.1A, F). In the models of processes associated with infection, modulation of mDC surface antigen expression was not observed following exposure to cryo-PLT (Figure. 6.1A-D). For BDCA3+ DC, in the presence of both polyI:C and LPS, expression of CD83 (p=0.025 and p=0.012), CD40 (p=0.020 and p=0.015) and CD80 (p=0.017 and p=0.018) were reduced (Figure 6.1E, G, H). CD86 (p=0.002) was also down-regulated in the presence of cryo-PLT and LPS (Figure 6.1F). Collectively, I found exposure to cryo-PLT in combination with polyI:C or LPS had minimal impact on mDC but significantly modulated BDCA3+ DC surface antigen expression.
Figure 6.1. mDC and BDCA3⁺ DC maturation and co-stimulatory surface antigen expression after exposure to cryo-PLT. Data represent flow cytometric analysis of (A-D) mDC (purple) and (E-H) BDCA3⁺ DC (blue) surface antigen expression following exposure to cryo-PLT in the presence of absence of polyIC or LPS. Matched “No transfusion” controls were denoted as “No tx”. Data are representative of five independent experiments (n=5). Bars indicate mean ± SEM. Data were analysed using paired t-test (*p<0.05, **p<0.01).
6.3.2. Cryo-PLT suppressed BDCA3+ DC inflammatory mediator production

We further examined the effect of cryo-PLT on mDC and BDCA3+ DC release of inflammatory mediators. As indicated in Chapter 4 (section 4.2.4), the panel of inflammatory mediators assessed differed, with IP-10 employed when modelling processes associated with viral infection and IL-10 when modelling processes associated with bacterial infection. The remaining inflammatory mediators were kept the same. Exposure to cryo-PLT alone and cryo-PLT with either TLR or LPS did not modulate mDC production of cytokines and chemokines (Figure 6.2A-F). On the contrary, the BDCA3+ DC inflammatory profile was substantially modulated. BDCA3+ DC expression of IL-8 ($p=0.008$), TNF-$\alpha$ ($p=0.032$) and IP-10 ($p=0.010$) were suppressed following exposure to cryo-PLT alone (Figure 6.2G-I). In the presence of polyI:C, cryo-PLT suppressed IL-8 ($p=0.032$), TNF-$\alpha$ ($p=0.009$), IP-10 ($p=0.001$), IL-6 ($p=0.024$) and IL-12 ($p=0.006$) production (Figure 6.2G-K). In the presence of LPS, cryo-PLT suppressed IL-8 ($p=0.043$), TNF-$\alpha$ ($p=0.003$) and IL-12 ($p=0.015$) production (Figure 6.2G, H, K). Similar to our results for the surface maturation and co-stimulatory molecules, I found that cryo-PLT had no effect on mDC responses, but BDCA3+ DC inflammatory mediator production was significantly suppressed in the presence of cryo-PLT.
Figure 6.2. mDC and BDCA3⁺ DC cytokines and chemokines production after being exposed to cryo-PLT. Data represent flow cytometric analysis of (A-F) mDC (purple) and (G-L) BDCA3⁺ DC (blue) inflammatory mediator production following exposure to cryo-PLT in the presence of absence of polyI:C or LPS. Matched “No transfusion” controls were denoted as “No tx”. Data are representative of five independent experiments (n=5). Bars indicate mean ± SEM. Data were analysed using paired t-test (*p<0.05, **p<0.01).
6.3.3. Cryo-PLT mediated a modest reduction in the overall leukocyte inflammatory response

The effect of cryo-PLT on the overall leukocyte inflammatory response was also assessed, which represents changes that could be expected in patients’ plasma post transfusion. Exposure to cryo-PLT alone reduced levels of TNF-α \((p=0.015; \text{Figure 6.3A})\), with the other inflammatory mediators investigated unchanged (Figure. 6.3B-O). When modelling the process associated with viral infection, cryo-PLT reduced the production of IL-6 \((p=0.001)\), IP-10 \((p=0.013)\), IFN-α \((p=0.017)\), MIP-1α \((p=0.004)\), MIP-1β \((p=0.023)\) and MCP-1 \((p=0.041; \text{Figure 6.3B-G})\). Suppression of IL-6 \((p=0.023)\) and MIP-1α \((p=0.038)\) was also observed in the model of processes associated with bacterial infection (Figure 6.3B, E). In addition, IL-8 \((p=0.004)\) was augmented when co-cultured with cryo-PLT and LPS (Figure 6.3H). Overall, leukocyte inflammatory response was predominately suppressed after exposure to cryo-PLT, especially when modelling infection.
Figure 6.3. The overall inflammatory response of leukocytes after exposure to cryo-PLT. (A-O) Concentration (pg/mL) of cytokine and chemokine secreted into culture supernatant by the overall leukocytes in the whole blood model following exposure to cryo-PLT in presence of absence of polyI:C or LPS. Matched “No transfusion” controls were denoted as “No tx”. Data are representative of five independent experiments (n=5). Bars indicate mean ± SEM. Data were analysed using paired t-test (*p<0.05, **p<0.01).
6.4. Discussion

Transfusion of cryo-PLT has been reported to be more effective for treating bleeding in patients compared to PC stored at room temperature (22-24°C) [172, 174, 175]. Previous *in-vitro* studies suggested that this was due to significant changes in cryo-PLT properties and increased soluble factors within the PC resulting in altered platelet functionality such as haemostasis and anticoagulation [172, 177, 180-184]. Given the changes in the cryo-PLT product [172, 177, 180-184], and the capacity of PC to significantly alter recipients’ immune responses [89, 162, 166-168], the potential for cryo-PLT to mediate immune modulation in a transfusion recipient was considered. To allow for comparison of my PC findings (Chapter 5), I investigated the impact of buffy-coat-derived cryo-PLT on DC maturation and activation using an *in-vitro* whole blood model of transfusion. Use of this *in-vitro* whole blood transfusion model to investigate changes in immune response in recipients provides a reflection of *in-vivo* changes as the model facilitates cell-cell interactions, and in contrast to models using isolated cells, cells have not been subjected to a number of procedures prior to use. In this study, I have provided the first evidence that cryo-PLT had minimal impact on mDC responses, but significantly modulated the specialised subset BDCA3+ DC. Expression of BDCA3+ DC maturation and co-stimulatory molecules, and inflammatory mediators as well as the overall inflammatory response were predominantly suppressed after cryo-PLT “transfusion”. Of note, immune modulation following exposure to cryo-PLT was particularly prominent in the presence of TLR agonist (polyI:C or LPS) that model the processes associated with infection. The results from this study suggest that exposure to cryo-PLT modulates recipients’ immune responses and may interfere with BDCA3+ DC capacity to appropriately respond to invading pathogens.

The capacity of DC to initiate immunity is dependent upon their maturation stage, accompanied by phenotypic changes associated with antigen presentation [193, 195]. Here, we report that cryo-PLT had minimal impact on mDC surface antigen expression, with only an increase in expression of maturation molecule CD83 observed following exposure to cryo-PLT alone. It would be of interest to expand the current panel, further assessing whether cryo-PLT alone also augmented other mDC maturation and activator molecules such as MHC class I/II, CD205, CD209 and DC-specific intercellular adhesion molecule-3. Whilst in Chapter 5, exposure to buffy-coat-derived PC reduced mDC co-stimulatory molecules CD40 and CD80 expression, and in polyI:C-viral infection PC reduced CD40 and CD86. Comparing the results from both studies, cryo-PLT appeared to have lesser effect on mDC than PC. This may be associated with differential change in cell
properties and function as well as production of platelet-derived substances during ex-vivo storage [172, 180]. Herein, I also observed limited changes in BDCA3+ DC with cryo-PLT alone, but in the models mimicking the processes of infection, both maturation (CD83) and co-stimulatory (CD40, CD80 or CD86) surface antigens were reduced following exposure to cryo-PLT. A similar profile of modulation was observed in Chapter 5 for BDCA3+ DC after exposure to PC in combination with TLR agonist. Although differential profiles of modulation were reported for mDC between cryo-PLT and PC, both studies consistently demonstrate that platelet “transfusion” has a greater immunomodulatory effect on BDCA3+ DC than mDC. Collectively, my data suggest that BDCA3+ DC are more susceptible to immune modulation than mDC in platelet transfusion patients with concurrent infection.

I further investigated cryo-PLT immunomodulatory capacity on DC-specific release of cytokines and chemokines, an essential process required for DC to determine the nature of the downstream immune responses and signal other immune cells for adequate induction of immunity [193, 289]. Co-culture of cryo-PLT alone, and cryo-PLT with polyI:C or LPS did not affect mDC inflammatory profile. However, in Chapter 5 when mDC were exposed to PC alone or PC in the presence of polyI:C or LPS, the production of a number of inflammatory mediators were significantly down-regulated, including IL-6, IL-8, IL-12, TNF-α, IP-10 and/or IL-10. Using a similar model, Perros et al. (2015) investigated the effect of PC supernatant and reported a significant modulation of mDC inflammatory response in the LPS-bacterial infection model [168]. An implication of the present and previous studies is that the differences in modulation of mDC may be an effect of DC-platelet interaction and the presence of platelet-derived soluble factors generated during storage. However, BDCA3+ DC production of pro-inflammatory cytokines (IL-6, TNF-α and IL-12) and chemokines (IL-8 or IP-10) were significantly suppressed in both models of infection following cryo-PLT exposure, but the impact was minimal with cryo-PLT alone. A similar trend, of modulation, was reported for BDCA3+ DC inflammatory profile following PC exposure. Suppression of BDCA3+ DC inflammatory responses potentially impact on the recipient’s downstream immune responses as they are required for stimulation, proliferation or differentiation of B and T lymphocytes, as well as other leukocytes [289]. As a consequence, BDCA3+ DC capacity to induce adequate immune defence against invading pathogens are potentially impaired, which may result in latter transfusion-related immune modulation in patients.
I hypothesise that the increased susceptibility of BDCA3⁺ DC to cryo-PLT may be associated with their multifaceted and specialised role. BDCA3⁺ DC are a rare subset of mDC, comprising only 0.03-0.08% of the PBMC population. Unlike the prevailing subsets of mDC (BDCA1⁺) equipped with pattern recognition receptor (TLR4) that recognise bacteria and antigen present via MHC class II, BDCA3⁺ DC express high level of TLR3 that recognise viruses and are efficient in cross-presentation (MHC class I) of antigen to cytotoxic T lymphocytes [223, 224, 228]. This process is important for anti-tumour activity [223, 246]. If cryo-PLT were to be used for treating cancer patients in the future as are PC at present, a potential suppression of maturation and activation of DC may lead to enhanced tumour activity. In addition, BDCA3 is not only a marker for a mDC subset, but also a multifaceted receptor known as TM, with the capacity to complex with thrombin, accelerate generation of activate protein C and a marker associated with tolerogenicity [252, 260, 262]. The receptor also encompasses a lectin domain, which acts as pattern recognition receptor to HMGB1 protein and the carbohydrate Lewis Y [257, 258]. These processes have been associated with anti-inflammatory activities [252]. The plasma reconstituted cryo-PLT unit is multifarious mix of cells and soluble factors such as thrombin, TM, microparticles, tissue factors and membrane proteins [180, 183, 184]. These highly abundant factors may bind to BDCA3 or other receptors expressed on BDCA3⁺ DC, which may overwhelm the process of maturation, activation and signalling or trigger anti-inflammatory pathways mediating modulation of these cells. My data suggest for the first time that in addition to being excellent in controlling bleeding in patients, cryo-PLT may be capable of preventing severe insults to the immune system for those already suffering from inflammation. However, impairment of BDCA3⁺ DC responses could potentially compromise outcomes when challenged with infectious pathogens. Additional research is required to further elucidate and understand the mechanisms behind the specific modulation of cryo-PLT on BDCA3⁺ DC and how the receptor plays a role in transfusion-related immune modulation.

Not only did I assess mDC- and BDCA3⁺ DC-specific responses, using the whole model of transfusion also provided the opportunity to examine the impact of cryo-PLT on the overall inflammatory response of the recipients’ peripheral leukocytes in the culture. Importantly, quantification of the inflammatory mediator secreted into the culture supernatant enables a closer representation of the potential plasma profile expected of transfusion patients. I observed very little modulation of the overall leukocyte inflammatory response in the presence of cryo-PLT alone, or cryo-PLT with LPS. More profound changes were
demonstrated when modelling cryo-PLT “transfusion” and the processes activated by concurrent viral infection; a larger array of pro-inflammatory cytokines and chemokines were suppressed, including production of IL-6, IP-10, IFN-α, MIP-1α, MIP-1β and MCP-1. The observation for cryo-PLT alone was in accordance with the results from my previous chapter using PC (Chapter 5) or previous studies using PC supernatant [168] investigating a similar panel. In line with previous studies, I have also reported a more pronounced immunomodulatory effect when modelling the processes activated by infections. While most of the overall leukocyte responses were suppressed following exposure to cryo-PLT in the models of infection processes, IL-8 levels were up-regulated in the transfusion model of LPS-bacterial infection. It is pertinent to recognise that the outcomes were predominately representing the response of the more abundant leukocyte populations such as neutrophils and monocytes present in the culture. Up-regulation of IL-8 maybe a feedback mechanism of the immune system to counterbalance suppressed responses by heightening recruitment of leukocytes to site of injury or inflammation. Exposure to PC and PC supernatants have been reported to modulate the overall leukocyte inflammatory response in the LPS-bacterial infection model [89, 168], however, in the present study I observed limited changes. Nonetheless, modulation of these inflammatory mediators were evident in my study when modelling the processes involved in viral infection, which denote potential inability of the overall leukocyte population to activate immune responses, induce inflammation and recruitment of lymphocytes to the site of inflammation [289]. In the context of transfusion, this may be beneficial for recipients with underlying inflammation such as trauma or cardiac patients as the suppressed inflammatory response may reduce the immune insults from a mass production of cytokine and chemokines. Of note, differences between DC and the overall leukocyte inflammatory profile demonstrate the importance of assessing cell-specific responses, especially when the cell population is rare, as the potential response can be masked by other abundantly expressed immune cells.
6.5. Conclusion

This study demonstrated for the first time that cryo-PLT suppressed BDCA3\(^+\) DC maturation and co-simulation, and, cytokine and chemokine production in both models of concurrent infection. In the same models, cryo-PLT also largely suppressed the inflammatory profile of the overall peripheral recipient leukocytes. With initial evidence now demonstrating the capacity of cryo-PLT to modulate recipients’ immune responses in my \textit{in-vitro} transfusion model, it is of interest to determine if similar effects are observed with apheresis-derived cryo-PLT, which are of high demand and expense, utilised in clinical trials [172, 174-176] and military settings [169, 179, 290]. In addition, it would be pertinent to use isolated BDCA3\(^+\) DC to study their direct response, further defining the underlying mechanisms associated with the immune modulation mediated by cryo-PLT. Studies in cryo-PLT transfusion patients will be required to confirm the applicability of data generated from my \textit{in-vitro} model. Although further studies are required to determine the clinical significance of these outcomes, my data provided the basis for prediction of what could be expected in cryo-PLT transfusion patients, specifically for BDCA3\(^+\) DC.
Chapter 7. Overall discussion and conclusion

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**Synopsis:** This chapter is the final segment of my dissertation, summarising and discussing the major findings derived from my five research chapters. It discusses how my work aligns with the current literature in transfusion medicine and transfusion associated immune modulation, as well as ideas for future research avenues.
7.1. **Overall discussion**

Blood transfusion may be known as “the gift of life”, but it has been reported to modulate recipients’ immune responses potentially driving poor patient outcomes. As highlighted throughout this thesis, the receptors and mechanisms responsible for transfusion-related immune modulation are yet to be elucidated. Given DC have a multifaceted role in the immune system, they may be particularly vulnerable to modulation by transfusion of blood products. However, there have been limited studies investigating modulation of DC in the context of transfusion. I hypothesised that exposure to transfused blood components changes DC phenotype and function. I also hypothesised that Clec9A expressed on BDCA3+ DC has a role in transfusion-related immune modulation. This is the first study to provide an understanding of the impact of blood products on the immune response using different models and approaches, looking specifically at mDC and the specialised subset BDCA3+ DC. In addition, whether Clec9A recognises and binds PRBC, PC or cryo-PLT was investigated.

7.1.1. Chapter 2: Incorporation of FSL-FLRO4 constructs into the RBC membrane facilitates detection of labelled cells for the duration of ex-vivo storage

Although a range of FSL constructs have been introduced and used in animal transfusion models, the stability of insertion and longevity of this technology for assessment of stored human PRBC had not been investigated. Therefore, this study assessed the suitability of FSL-FLRO4 construct for labelling routine stored PRBC at various storage durations. FSL-FLRO4 was detectable on PRBC at all time points investigated during routine storage, including at the date-of-expiry. No difference in FSL-FLRO4 labelling of heterogeneous RBC (light-young and dense-old) populations from the same PRBC unit was observed. These data demonstrated the suitability of FSL-FLRO4 as a tool for labelling PRBC, and that it can be used to aid *in-vitro* and *in-vivo* visualisation and tracking of PRBC during routine ex-vivo storage. More importantly, FSL-FLRO4 facilitated my study (Chapter 4) in assessing mDC and BDCA3+ DC erythrophagocytosis, further contributing to the current knowledge base of transfusion biology.

7.1.2. Chapter 3: Elucidating the role of Clec9A in transfusion-related immune modulation

Clec9A has been previously reported to bind RBC ghosts and permeabilised platelets [231]. However, whether Clec9A binds to cells from standard blood components had not been studied. As Clec9A has the potential to bind F-actin exposed on cells from blood
products following standard processing and preparation, I questioned whether Clec9A expressed on BDCA3+ DC play a role in modifying DC immune responses in an *in-vitro* model of blood transfusion.

Interestingly, during initial optimisation of the transfusion model for BDCA3+Clec9A+ DC (for Chapters 4-6), an unexpected reduction of Clec9A surface expression was observed after 4 h at 37°C incubation. To date, no previous publications have reported this phenomenon, and a series of investigations were performed to troubleshoot. I found reduction of Clec9A expression was associated with EDTA (Ca2+ chelator) in the blood collection tubes, and that the reduction was accelerated by increased temperature (37°C) and prolonged incubation duration (≥2 h). Of note, retention of Clec9A expression was observed with leukocyte clumping when using sodium citrate, which has a similar mechanism of action to EDTA. This suggests reduction of Clec9A expression was linked to the lack of free Ca2+ in whole blood, as EDTA is a much stronger Ca2+ chelator than sodium citrate. Moreover, it has been reported that depletion of Ca2+ impedes the stabilisation of Clec9A conformation [231].

For my experiments modelling transfusion *in-vitro*, the finding that Clec9A was lost in culture was of concern as I had hypothesised that Clec9A would be involved in the process of transfusion-related immune modulation of DC function. A commercial rhClec9A protein was used to conduct a more direct assessment of cell-receptor interaction. This approach enabled the investigation of whether Clec9A could recognise and complex with fresh and/or stored PRBC, PC or cryo-PLT. However, no binding of rhClec9A protein with cells was found from any of the three blood products. Although binding of rhClec9A was not reported, F-actin was detected on platelets from PC or cryo-PLT, indicating that phalloidin and Clec9A may bind a different region of this ligand. Together these findings suggest DC may be modulated by blood products independent of Clec9A. In addition, the outcome of this part of the study facilitated the decision to continue using EDTA blood collection tubes for the remaining experiments as sodium citrate induces leukocyte aggregation and also under the premise that that lack of Clec9A would not impact on outcomes of the transfusion models.
7.1.2.1. Future directions: Importance of elucidating mechanisms underpinning reduction in Clec9A expression

My study demonstrated that the impact of anti-coagulants used on cells should be considered when designing experiments. Clec9A is a focus of a trending area of research, targeted for cancer immunotherapy. Therefore, it is particularly important to raise awareness of the impact of EDTA collection has on DC receptors, specifically Clec9A. Additionally, care should also be taken for studies interested in the role of specific receptors to validate the behaviour of cells bearing similar receptors.

7.1.3. Chapter 4: Mechanisms underpinning transfusion-related immune modulation: erythrophagocytosis and immune modulation in myeloid dendritic cells

My PhD work provides the first evidence that exposure to PRBC predominately suppressed mDC and BDCA3+ DC maturation and activation, and up-regulated the overall leukocyte inflammatory response. Outcomes of my transfusion models demonstrate DC are refractory to TLR agonists in the presence of PRBC. In addition, evidence of prolonged PRBC storage was associated with further modulation of DC and overall leukocyte response. Of note, an increased mDC and BDCA3+ DC erythrophagocytosis was observed when exposed to PRBC at date-of-expiry (“old”) rather than D2 (“fresh”). Despite recent clinical studies reporting no differences in patient adverse outcomes between transfusion of “fresh” and “old” PRBC [12, 17, 43], the topic remains of interest and investigation into the mechanisms associated with the reported undesirable outcomes are still required. Collectively, my results provide a basis to suggest that in patients who receive “old” PRBC, particularly those with underlying infectious complications, the immediate immune response may be compromised, precipitating worse patient outcomes. This will further address knowledge gaps associated with mechanisms underpinning transfusion-related immune modulation in patients and help provide an extended understanding of transfusion biology.

7.1.3.1. Future directions: Further characterisation of mDC and BDCA3+ DC immune profile in the model of PRBC transfusion

To fully understand the immunomodulatory consequent of PRBC transfusion, it would be of interest to separately study the effect of the cellular component and supernatants that contain the soluble factors, on mDC and BDCA3+ DC responses. It is also of interest to investigate the influence of PRBC transfusion dose on both DC subsets to more closely model the clinical scenario, using different transfusion replacement volumes to represent
small, medium and massive transfusions. PRBC dose-dependent modulation of inflammatory responses has been previously reported by Schneider et al. (2009) but for leukocytes only [89]. In addition, it would be worthwhile to study mDC and BDCA3+ DC erythrophagocytosis in the presence of poly(I:C) or LPS, given I observed a more pronounced modulation of DC immune profile when modelling the processes of infection, and Richards et al. (2016) has also reported enhanced pDC erythrophagocytosis during inflammation [91].

The transfusion models utilised herein examined the interaction of mDC or BDCA3+ DC with PRBC. Using a similar transfusion model incorporating endothelial cells could more accurately allow transfusion-related immune modulation to be modelled. Such models would incorporate a cellular surface to allow interaction of DC with other cells as well as PRBC, mimicking what could be expected in transfusion recipients. These additional studies would provide a more thorough characterisation of the immune profile of both DC subsets in the context of transfusion.

### 7.1.4. Chapter 5: PC modulate mDC immune responses

Outcomes similar to PRBC (section 7.1.3) were observed for mDC and BDCA3+ DC following exposure to PC. However, the overall leukocyte inflammatory response was predominately suppressed and immune modulation of DC and leukocytes were independent of PC storage duration. This is consistent with findings from a recently published *in-vitro* study reporting dose dependent modulation on mDC when assessing the impact of PC supernatants [168]. To date, the association of PC *ex-vivo* storage and increased rate of transfusion-related immune modulation in patients remains unclear. Clinical studies predominately report the effect of PC storage in relation to transfusion-related acute reactions (allergic and febrile reactions), rather than immune modulation (mortality, morbidity, TRALI, LOS). In addition to the speculation in the PRBC study, together with my results (Chapter 5) and the literature, I propose that changes observed in DC maturation and activation may be associated with dosage.

#### 7.1.4.1. Future directions: Further elucidation of mDC and BDCA3+ DC as one mechanism mediating PC transfusion-related immune modulation

The directions for future *in-vitro* PC transfusion research would be similar to those outlined for PRBC (section 7.1.3.1). It is also important to investigate the impact of PC vs. PC supernatants, different “transfusion” dosage and use of different models on mDC and
BDCA3⁺ DC immune profile. Such additional studies would provide an even more comprehensive analysis to further our understanding of transfusion-related immune modulation. Further, as exposure to both PRBC and PC result in significant perturbation of DC responses, it would be of interest to study the impact of the immune response of these products together, as these two blood products are often transfused in combination. I propose that exposure to PRBC and PC concurrently further promotes phenotypic and functional modulation of mDC and BDCA3⁺ DC, as well as the overall leukocyte population.

7.1.5. Chapter 6: Cryo-PLT modulated BDCA3⁺ dendritic cell surface antigen expression and inflammatory response
Consistent with my findings in the studies with PRBC and PC transfusion (section 7.1.3 and 7.1.4), modulation of both DC subsets and the overall leukocytes were observed following exposure to cryo-PLT, especially when modelling processes activated by concurrent viral or bacterial infection. More importantly, the changes were predominately exhibited in the profile of BDCA3⁺ DC, which was more specific than following exposure to PRBC and PC. Given BDCA3 have multifaceted functions and numerous binding partners, it may be one mechanism underpinning modulation associated with blood transfusion. In the whole blood model of transfusion co-stimulated with TLR agonist, BDCA3 would be exposed to numerous ligands simultaneously such as *E.coli* (LPS) expressing Lewis Y, thrombin from platelets or plasma, HGBM from activated/stressed leukocytes. This may increase BDCA3⁺ DC vulnerability to modulation by transfusion.

In addition, BDCA3⁺ DC tolerogenicity was reported to associate with increased BDCA3 surface expression, which down-regulates their co-stimulatory surface antigen expression, pro-inflammatory response and co-stimulatory activity with T lymphocytes. BDCA3 expression can be regulated by factors such as thrombin, histamine and inflammatory mediators, which can also be found in blood products. It may be these factors that play a role in preventing an inflammatory response to blood transfusion and invading pathogens.

7.1.5.1. Future directions: Further elucidation of cryo-PLT impact on BDCA3⁺ DC in the context of transfusion
Future directions for this study would be similar to what has been described for both PRBC and PC (section 7.1.3.1 and 7.1.4.1). Since I have demonstrated for the first time that buffy-coat-derived cryo-PLT have the capacity to modulate recipients’ immune responses...
in an *in-vitro* transfusion model, it would be of interest to study the effect of apheresis-derived cryo-PLT. Comparing mDC and BDCA3⁺ DC phenotype and function using apheresis-derived cryo-PLT is required to more closely mimic clinical trials and the military setting. Additional studies in patients are also required to see if similar modulation occurs *in-vivo*.

In this study, my data highlight the potential importance of BDCA3⁺ DC in transfusion-related immune modulation. To further define their role in the context of cryo-PLT transfusion, it would be pertinent to use isolated BDCA3⁺ DC to study direct responses and expression of genes relevant to the inflammatory response, antigen presentation and signalling transduction (Table 7.1). Again, studies in patients will be required to confirm the applicability and clinical significance of these specific *in-vitro* outcomes generated from my models of transfusion.
### Table 7.1. Potential gene array panel for BDCA3+ DC.

<table>
<thead>
<tr>
<th>Chemokines, cytokines and receptors</th>
<th>Antigen presentation</th>
<th>Signalling transduction</th>
<th>Housekeeping genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNL1 (IL-28A)</td>
<td>ERAP1</td>
<td>ERK (MAPK1)</td>
<td>GAPDH</td>
</tr>
<tr>
<td>IFNL2 (IL-29)</td>
<td>Ly75</td>
<td>JNK (MAPK8)</td>
<td>18s RNA</td>
</tr>
<tr>
<td>IFNA</td>
<td>PSMD7</td>
<td>BCL10</td>
<td></td>
</tr>
<tr>
<td>IFNB</td>
<td>TAP2</td>
<td>PI3K</td>
<td></td>
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<tr>
<td>CXCL10 (IP-10)</td>
<td>TAP1</td>
<td>FAS</td>
<td></td>
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<tr>
<td>CCL-3 (MIP-1α)</td>
<td>CLEC9A</td>
<td>RAC1</td>
<td></td>
</tr>
<tr>
<td>CCL-4 (MIP-1β)</td>
<td>HLA-A</td>
<td>CLNK</td>
<td></td>
</tr>
<tr>
<td>TNF</td>
<td>HLA-DRA</td>
<td>PDIA</td>
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<tr>
<td>CCL5</td>
<td>CD80</td>
<td>CARD9</td>
<td></td>
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<tr>
<td>IL12A (p35)</td>
<td>CD83</td>
<td>SYK</td>
<td></td>
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<tr>
<td>IL-12B (p40)</td>
<td>CD86</td>
<td>RELB</td>
<td></td>
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<tr>
<td>IL-10</td>
<td>CD40</td>
<td>RELA</td>
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<td>IL-8</td>
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<td>NFκB2</td>
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<td>IL-6</td>
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<td>NFκB1</td>
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<tr>
<td>TLR3</td>
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<td>GCSAM</td>
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<td>CAMD1 (Nect2)</td>
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<td>BATF3</td>
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<td>CAMD1 (Nect2)</td>
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<td>FLT3</td>
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<td></td>
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<td>IDO2</td>
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</table>
7.2. Conclusion

In summary, my PhD research has generated original information that contributes to the understanding of transfusion biology and addresses a number of deficiencies in the field. The capacity of FSL-FLRO4 as a tool for labelling PRBC during ex-vivo storage was established, which can potentially be useful for studying in-vivo survival and recovery of PRBC in humans post-transfusion. The newly assimilated knowledge of the relationship between Clec9A surface expression and Ca$^{2+}$ chelating anti-coagulants from this study, provides additional emphasis of how frequently used anti-coagulants can affect study outcomes and could lead to misinterpretation of results. This also sheds light into a potential pathway of investigation for understanding the nature of Clec9A receptor. In addition, binding of rhClec9A protein to PRBC, PC or cryo-PLT was not detected, indicating that Clec9A receptor may not have a role in modulating DC function in transfusion, however, additional studies are required to conclude this.

More importantly, my research provided a comprehensive analysis of DC immune profile in the context of transfusion. Changes in mDC, especially the maturation and activation of specialised BDCA3$^+$ DC, following exposure to three commonly used blood products in transfusion was observed in an in-vitro model of transfusion. I propose that these changes are one mechanism underpinning transfusion-related immune modulation. In addition, the impact of these blood products on DC was particularly pronounced in models associated with the processes of concurrent infection. My results provide the basis for proposing that patients who receive blood transfusion, in particularly those with underlying infectious complications, may fail to mount an immediate immune response precipitating worse patient outcomes. Together, the results derived from this PhD study enhance our knowledge of the DC immune profile that could be expected in transfusion patients.
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define human CD11c⁺CD141⁺ cells as homologues of mouse CD8⁺ dendritic cells.


