UTS UNIVERSITY OF TECHNOLOGY SYDNEY

Examining the non-haemostatic function of alternatively stored platelets

by Ben Winskel-Wood

Thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy (Science)

under the supervision of Dr. Lacey Johnson and Associate Professor Matthew Padula

University of Technology Sydney
Faculty of Science

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Certificate of original authorship

I, Ben Winskel-Wood declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy (Science), in the School of Life Sciences at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

This research is supported by the Australian Government Research Training Program.

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Date: 01/05/2023

Statement

This is a thesis by compilation. Chapters one, two, three and four are peer-reviewed published manuscripts. The figure and table numbers in each publication have been reformatted to match the chapter numbering used in the thesis. Australian English formatting has been applied throughout the thesis. All references have been compiled into a single list at the end of the thesis.

Ethics disclosure

Ethics approval was obtained from the Australian Red Cross Lifeblood Ethics Committee (Johnson 10052019). The University of Technology Sydney (UTS) Human Research Ethics Committee ratified this ethics approval (ETH19-3955).

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Publications

Journal articles arising from this thesis

1. **Wood B**, Padula MP, Marks DC and Johnson L. The immune potential of *ex vivo* stored platelets: a review. Vox Sanguinis. 2020; 116;5: 477-88. https://doi.org/10.1111/vox.13058
2. **Wood B**, Padula MP, Marks DC and Johnson L. Cryopreservation alters the immune characteristics of platelets. Transfusion. 2021; 61;12: 3432-42. https://doi.org/10.1111/trf.16697
3. **Winskel-Wood B**, Padula MP, Marks DC and Johnson L. Cold storage alters the immune characteristics of platelets and potentiates bacterial-induced aggregation. Vox Sanguinis. 2022; 117:8: 1006-15 https://doi.org/10.1111/vox.13293
4. **Winskel-Wood B**, Padula MP, Marks DC and Johnson L. The phenotype of cryopreserved platelets influences the formation of platelet-leukocyte aggregates in an *in vitro* model. Platelets. 2023; 34:1:2206916 https://doi.org/10.1080/09537104.2023.2206916

Published conference abstracts

1. **Wood B**, Padula MP, Marks DC and Johnson L. Cold-storage of platelet components induces changes in receptor expression associated with innate immune function. Vox Sanguinis. 2020; 115:S1
   Presented as a poster at the 36th International Conference of the International Symposium for Blood Transfusion (ISBT).
2. **Wood B**, Padula MP, Marks DC and Johnson L. Platelet refrigeration differentially affects the release of immunomodulatory factors. Transfusion. 2021; 61, S3
   Presented as a poster at the International Conference of the Association for the Advancement of Blood and Biotherapies (AABB).

Published journal articles unrelated to this thesis

1. Johnson L, Waters L, Green S, **Wood B** and Marks D. Freezing expired platelets does not compromise in vitro quality: An opportunity to maximise inventory potential. Transfusion. 2019; 60;3: 454-9 https://doi.org/10.1111/trf.15616
2. de Korte D, Bontekoe IJ, Fitzpatrick A, Marks D, **Wood B**, Gravemann U, Bohonek M, Kutner JM, The Biomedical Excellence for Safer Transfusion Collective. Evaluation of platelet concentrates prepared from whole blood donations with collection times between 12 and 15 min: The BEST Collaborative study. Vox Sanguinis. 2022; 117;5: 671-7. https://doi.org/10.1111/vox.13245
3. Johnson L, Vekariya S, **Wood B**, Tan S, Roan C and Marks DC. Refrigeration of apheresis platelets in platelet additive solution (PAS-E) supports *in vitro* platelet quality to maximise the shelf-life. Transfusion. 2021; 61;S1: S58-67. https://doi.org/10.1111/trf.16489
4. Johnson L, Vekariya S, **Wood B**, Tan S, Costa M, Waters L, Green S and Marks DC. The *in vitro* quality of X-irradiated platelet components in PAS-E is equivalent to gamma-irradiated components. Transfusion. 2021; 61;11: 3075-80. https://doi.org/10.1111/trf.16647

Abbreviations

A23187	calcium ionophore A23187
α-granules	Alpha-granules
4C	Refrigerated platelets
ACD	Anti-coagulant citrate dextrose solution
ADAM	A disintegrin and metalloproteases
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ApoT	Apotracker-Green
ATCC	American Type Culture Collection
BEST	Biomedical Excellence for Safer Transfusion
BRMs	Biological response modifiers
CAPs	Carboxy (alkyl-pyrrole) protein adducts
Coa	Coagulase
CCD	Charged coupled device camera
DAMPs	Damage associated molecular patterns
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid

ELISA	Enzyme linked immuno-assay
EV	Extracellular vesicle
FBS	Foetal bovine serum
FDA	Federal Drug Administration
FMO	Fluorescence minus one control
FNHTRs	Febrile non-haemolytic transfusion reactions
HMGB1	High mobility group box 1
IL-1β	Interleukin-1 beta
IL-8	Interleukin-8
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-13	Interleukin-13
IL-27	Interleukin-27
LPS	Lipoprotein polysaccharide
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
mtDNA	Mitochondrial DNA
NET	Neutrophil extracellular trap

PAMPs	Pathogen associated molecular patterns
PAS-E	Platelet additive solution E
PF	Pre-freeze
PF4	Platelet factor 4
PLA	Platelet-leukocyte aggregate
PRP	Platelet rich plasma
PSL	Platelet storage lesion
PT	Post-thaw
PT0	0 hours post thaw
PT24	24 hours post-thaw
PVC	Polyvinylchloride
RS	Resuspension solution
RT	Room-temperature
TNF-α	Tumour necrosis factor alpha
SD	Standard deviation
TLRs	Toll-like receptors
TRALI	Transfusion associated acute lung inflammation
vWbp	von Willebrand Factor binding protein

Thesis abstract

Platelet transfusion is commonly prescribed for patients suffering from acute haemorrhage resulting from trauma or surgery. Prompt platelet transfusion promotes haemostasis and improves patient outcomes, reducing morbidity and mortality. Currently, platelet components are stored at room-temperature for up to 7 days due to the risk of bacterial proliferation and a gradual degradation in haemostatic effectiveness. The relatively short shelf-life makes managing platelet inventories and suppling to remote medical centres, impractical. As such, there has been renewed interest in the evaluation of platelet storage methodologies which have the potential to extend platelet shelf-life while better preserving platelet haemostatic function. Consequently, two methods are currently under clinical evaluation, platelet refrigeration and platelet cryopreservation.

The effect of refrigeration and cryopreservation on the haemostatic properties of platelets is relatively well studied. However, platelets are now recognised as being significant modulators of the immune system. Platelets facilitate pathogen recognition, pathogen clearance, leukocyte activation and inflammation *in vivo*. In general, platelet immune function is beneficial, however dysregulation is associated with a range of pro-inflammatory disorders and in transfusion medicine, adverse transfusion events. Research has highlighted that *ex vivo* platelet storage conditions, including current conventional storage methods, can significantly alter the immune characteristics of platelets, which has been directly linked to the induction of adverse transfusion events. Unfortunately, the effect of alternative storage methods on the immune characteristics of platelets is

relatively unstudied. The effect of *ex vivo* storage on platelet immune characteristics is key information and would contribute significantly to the body of evidence required for the clinical evaluation of both storage modes. As such, the focus of this thesis was to examine the effect of refrigeration and cryopreservation on the immune characteristics of platelets.

Platelet refrigeration significantly altered the immune characteristics of platelets. The abundance of the leukocyte adhesion receptors P-selectin and the activated conformation of GPIIb/IIIa were increased on the platelet surface membrane over 14 days of storage. Further, the abundance of leukocyte co-stimulatory receptors (CD86, CD44, ICAM-2 and CD40) and pathogen recognition receptors (TLR1, TLR3, TLR4, TLR3, TLR7 and TLR9) was significantly lower on the surface membrane of refrigerated platelets compared to conventionally stored components. As a significant decrease in pathogen recognition receptors was observed the responsiveness of refrigerated platelets to bacterial stimulation was examined. Notably, refrigerated platelets exhibited increased responsiveness to both *E. coli* and *S. aureus* induced aggregation, which was linked to the function of the fibrinogen receptor, GPIIb/IIIa.

Cryopreservation significantly altered the immune characteristics of platelets. Cryopreserved platelets exhibited an altered surface receptor phenotype and concentration of soluble factors compared to conventionally stored components. Immediately post-thaw (PT0), the concentration of IL-1β, OX40L, IL-13, IL-27 and CD40L was significantly reduced in the supernatant of thawed cryopreserved platelets. However, other soluble factors including RANTES, PF4 and C3a which have the potential to be immunomodulatory, increased. Following extended post-

thaw storage at room temperature for 24 hours (PT24), the concentration of RANTES, PF4 and C3a increased significantly compared to PT0 and pre-freeze platelets. In contrast, CD40L increased significantly but only compared to PT0 platelets. The abundance of the leukocyte interaction receptors P-selectin, phosphatidylserine, siglec-7 and the pathogen recognition receptors TLR3, TLR7 and TLR9 were increased on the surface membrane of cryopreserved platelets. The surface abundance of most receptors examined increased at PT24 compared to PT0 and pre-freeze platelets.

Based on the changes in the abundance of leukocyte adhesion receptors, whether cryopreservation altered the capacity of platelets to bind to a monocyte-like cell line (THP-1 cells) was investigated. Platelets were examined pre-freeze, post-thaw, and following stimulation with TRAP-6 or A23187 alone and following co-culture with THP-1 cells for 1 and 24 hours. Platelet subpopulations were analysed by multi-colour imaging flow cytometry and categorised based on the abundance of phosphatidylserine, P-selectin and GPIbα. Cryopreserved platelet components contained a heterogenous mix of activated, procoagulant and a novel platelet subpopulation. Further, cryopreserved platelets more readily formed aggregates with THP-1 cells during co-culture compared to pre-freeze, but not TRAP-6 or calcium ionophore (A23187) stimulated platelets, which was primarily attributable to an increase in the procoagulant subpopulation. Finally, P-selectin exposure on the surface membrane correlated with an increase in platelet-THP-1 cell aggregates.

In conclusion, the data presented in this thesis demonstrates that refrigeration and cryopreservation of platelets significantly alters their immune characteristics.

Additionally, our data highlights that the observed changes result in specific functional changes to the way platelets interact and respond to bacteria and leukocyte-like cells *in vitro*. As such, changes in the immune characteristics of platelets should continue to be a key consideration during the evaluation of novel platelet storage or manufacturing methodologies.

Overview of the Thesis

This section provides a background on alternative platelet storage methodologies, highlighting the importance of the project. Subsequently, the aims of the thesis are provided and the structure of the dissertation is outlined.

Background and project significance

Currently, platelet components are stored at room-temperature, which limits the shelf-life to 5-7 days due to the risk of bacterial proliferation and a gradual reduction in haemostatic function. Due to the short shelf-life, maintaining platelet supplies to remote medical centres and military settings is often impractical. Further, between 10-20% of platelet products are discarded due to reaching their expiry date. Consequently, there has been renewed interest in the evaluation of alternative storage methodologies including platelet refrigeration and platelet cryopreservation. Both techniques allow for the potential extension of shelf-life while better preserving the haemostatic function of platelets compared to room-temperature storage. Recent findings have identified that both refrigeration and cryopreservation alter the abundance of receptors on the surface membrane and soluble factors released into the supernatant. Additionally, storage induced changes have been linked to alteration in the immune phenotype of platelets and an increased risk of adverse events post-transfusion in room-temperature stored components. In general, very little is known about the effects of refrigeration and cryopreservation on the immune characteristics of platelets. As such, the overall aim of this thesis was to address these knowledge gaps and contribute to the body of evidence being assembled to clinically evaluate refrigerated and cryopreserved platelets.

The platelet surface membrane is host to a range of receptors which facilitate pathogen recognition (TLRs, FcγRIIa), leukocyte interaction (P-selectin, ICAM-2) and leukocyte activation (CD40L). Platelet activation can mobilise receptors from internal stores to the surface membrane. This can influence the ability of platelets

to adhere to pathogens and leukocytes. Platelet-pathogen recognition is linked to direct pathogen killing, clearance and also immune evasion. In contrast, platelet-leukocyte interaction can lead to the formation of platelet-leukocyte aggregates which mediate anti-pathogenic activity, platelet clearance, leukocyte differentiation and pro-inflammatory function. In this dissertation, the effect of refrigeration and cryopreservation on the abundance of immune receptors on the platelet surface membrane was examined. This information provides insight into how alternatively stored platelets may interact with pathogens and other immune cells.

Platelet activation induced by storage or immunological stimuli can cause degranulation, releasing a range of soluble factors into the surroundings, facilitating pathogen clearance (PF4), cellular proliferation (PF4), leukocyte migration (RANTES) and inflammation (IL-1β, OX40L, IL-13, IL-27 and CD40L). Notably, the accumulation of pro-inflammatory soluble factors in the supernatant of platelet components has been directly linked with an increased risk of adverse events post-transfusion. The research presented in this thesis characterises the concentration of pro-inflammatory mediators in the platelet supernatant which may provide insight into the likelihood of adverse transfusion events.

Consequently, this dissertation aims provide a greater understanding of how alternative platelet storage methodologies affect the immune characteristics and function of platelets *in vitro*.

Aims of the Thesis

Platelets have key roles as regulators of the immune system. Further, dysregulation of platelet immune function has been linked to adverse transfusion events. However, a review of the literature identified several knowledge gaps regarding how storage, particularly novel storage modes affect the immune characteristics of platelets. This knowledge would be beneficial to the field of transfusion science, contributing to the body of evidence being assembled to clinically evaluate alternate platelet storage methodologies. As such, this thesis aimed to examine the effect of conventional storage, refrigeration and cryopreservation on the immune characteristics of platelets and the potential functional impacts.

This thesis had three aims:

Aim 1: Characterise the expression of immune-related receptors on stored platelets.

Aim 2: Characterise the releasate from stored platelets, with a particular focus on immune-modulatory soluble proteins.

Aim 3: Determine the effect of storage on platelet-bacteria and platelet-leukocyte interactions.

Thesis structure

Chapter One: Literature review

This first chapter outlines the general background of the thesis and provides a general outline of platelet immune function *in vivo*. This is followed by a discussion of how conventional platelet storage alters the immune characteristics of platelets, increasing the risk of adverse events. The last section highlights what is currently known about the effects of conventional storage, refrigeration and cryopreservation on the immune characteristics and function of platelets. This is followed by an outline of the key knowledge gaps in the current literature.

Chapter Two: The immune characteristics and function of refrigerated platelets

This chapter focuses on examining the effect of refrigerated storage on the immune characteristics and function of platelets *in vitro*. Refrigerated storage was found to significantly alter the abundance of receptors associated with the immune function of platelets. Notably, this included a significant reduction in pathogen recognition receptors on the surface membrane. In contrast, refrigerated platelets exhibited increased bacterial-induced aggregation, which was linked to the function of the activated form of the fibrinogen receptor GPIIb/IIIa.

Chapter Three: Cryopreservation alters the immune characteristics of platelets

Cryopreservation causes significant activation post-thaw, dramatically altering the haemostatic phenotype of platelets. As such, it was hypothesised that cryopreservation may also cause significant changes in the immune characteristics of platelets. Consequently, this chapter aimed to characterise cryopreservation induced changes in the abundance of surface receptors and soluble factor release related to platelet immune function. Cryopreserved platelets exhibited an altered immune phenotype on the surface membrane and a reduction in the concentration of pro-inflammatory mediators in the supernatant. Further, the surface phenotype continued to change following extended post-thaw storage.

Chapter Four: The phenotype of cryopreserved platelets influences the formation of platelet-leukocyte aggregates in an *in vitro* model

Previous work suggests that cryopreservation induced activation results in the generation of phenotypically heterogeneous platelet subpopulations. Platelet subpopulations may exhibit an altered capacity to bind to leukocytes and form PLAs. PLA formation is immunomodulatory, which can facilitate both pro- and anti-inflammatory signalling and platelet clearance. As such, it was hypothesised that cryopreservation may alter the likelihood of PLA formation in a model *in vitro* system, using monocyte-like cells (THP-1 cells). It was observed that cryopreserved platelets were composed of heterogeneous subpopulations post-thaw. Further, the likelihood of PLA formation was linked to the formation of specific platelet subpopulations and the exposure of P-selectin.

Chapter Five: General Discussion:

This dissertation is composed of three published original research manuscripts which examine the impact that refrigerated storage and cryopreservation has on the immune characteristics and function of platelets. In this final chapter, the results are discussed in context with the current literature and future avenues of research are highlighted.

Appendices

This section contains the supplementary figures referred to in Chapter Four.

1. The immune function of platelets

1.1. Foreword

This first chapter outlines the general background of the thesis and provides a general outline of platelet immune function *in vivo*. This is followed by a discussion of how conventional platelet storage alters the immune characteristics of platelets, increasing the risk of adverse events. The last section highlights what is currently known about the effects of conventional storage, refrigeration and cryopreservation on the immune characteristics and function of platelets. This is followed by an outline of the key knowledge gaps in the current literature.

1.2. Statement of authorship

The following section has been adapted from a review article originally published in *Vox Sanguinis* in 2020. Minor edits have been made to the formatting and wording of the manuscript to make it consistent with the rest of the Thesis. Further, additional relevant references which were released after the publication of this review have been added.

Wood B, Padula MP, Marks DC and Johnson L. The immune potential of *ex vivo* stored platelets: a review. Vox Sanguinis. 2020; 116;5: 477-88. https://doi.org/10.1111/vox.13058

All authors contributed to development of the concepts and design of the review article and critically reviewed the manuscript. Ben Wood and Lacey Johnson wrote the manuscript. Ben Wood prepared the figure.

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A/Prof Denese C. Marks

1.3. Introduction

Haemostasis has long been considered the primary role of platelets. However, contemporary research has highlighted that platelet function is much more complex, as they also play a significant role in regulating the immune system [1]. Platelets, as immune cells, contribute significantly to bacteria recognition, inflammation, leukocyte recruitment and activation [2-8] (Figure 1.1). Under normal conditions, the immune function of platelets is biologically beneficial, however, hyperactivity is linked to a number of pro-inflammatory disorders [2]. In transfusion medicine, the immune function of platelets has become topical due to its association with adverse transfusion outcomes [9-12].

Rapid transfusion of platelet components decreases the mortality and morbidity of patients suffering from acute haemorrhage [13]. Unfortunately, platelet components may be unavailable in remote or rural medical settings due to their short shelf-life [14]. However, platelet manufacturing and storage methodologies are constantly evolving, and a renewed interest in refrigeration and cryopreservation could allow an extension of the shelf-life to weeks or years respectively [15-17]. The evaluation of new storage methods has primarily focused on characterising the haemostatic characteristics of platelets to ensure that this functional capacity is maintained. However, considering what is now understood about the immune function of platelets, a broader understanding of how alternative storage modes may affect the immune characteristics of platelets may provide clinically relevant information.

This review outlines the ability of platelets to modulate aspects of the immune system in order to facilitate pathogen recognition, bacterial-induced aggregation,

leukocyte activation and inflammation. This article also highlights the link between platelet immune characteristics and adverse events, examines the impact that novel *ex vivo* storage modes may have on this functionality and outlines why further experimental investigation is justified.

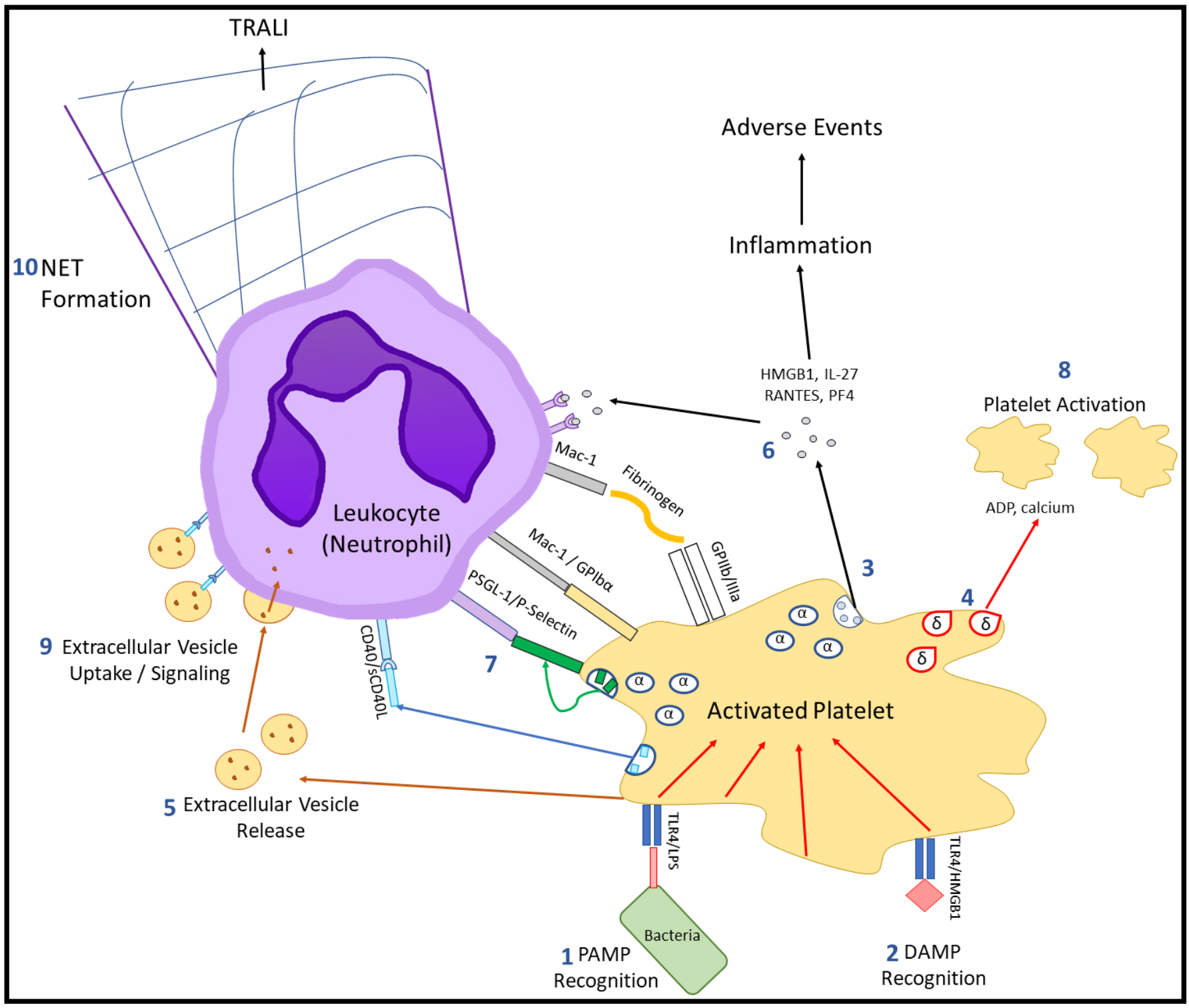

Figure 1-1. A simplified schematic depicting platelet-leukocyte interactions, which are mediated by the expression of surface receptors and release of soluble factors. Platelet-leukocyte interaction can be triggered by toll-like receptor (TLR) recognition of 1) pathogen or 2) damage associated molecular patterns (PAMPs; e.g. bacterial lipids or DAMPs; e.g. HMGB1). This triggers platelet activation and release of 3) α-granules (α), 4) dense granules (δ), and 5) EVs (brown lines). Degranulation of α-granules increases the surface expression of activation markers, including CD40L (blue rectangle) and P-selectin (green rectangle) and 6) the release of a range of biological response modifiers (BRMs; e.g. HMGB1, IL-27, RANTES, PF4; black lines) into the plasma which can influence surrounding leukocytes (purple receptors) and have been linked to inflammation and adverse events. 7) Activated platelets can attach to neutrophils through P-selectin, CD40L and GPIbα to PSGL-1, CD40 and Mac-1, respectively. Attachment can also occur through platelet GPIIb/IIIa and leukocyte Mac-1 through a fibrinogen intermediate. This interaction generally increases the activation of both platelets and leukocytes. 8) The activated platelet can then release internal agonist stores (ADP, calcium) which lead the activation of platelets in the surrounding area 9) EVs can activate leukocytes either through transport of their internal contents (e.g. mitochondria, mtDNA, microRNAs, proliferation factors and cytokines) or by receptor signalling. The activation of neutrophils can trigger 10) the generation neutrophil extracellular traps (NETs) which are associated with the pathogenesis of transfusion associated acute lung injury (TRALI).

1.4. Platelet immune function *in vivo*

1.4.1 The role of platelets in mediating leukocyte function

Similar to the initiation of haemostasis, the immune function of platelets *in vivo* is mediated through activation. Platelet immune activation can be induced through receptor recognition of a range of signalling factors, including pathogen associated molecular patterns (PAMPs), damage associated molecular patterns (DAMPs) and traditional platelet agonists (Figure 1.1) [2, 6, 7, 18-21]. The signals are then transduced across the cell membrane and propagated in the cytosol by downstream signalling, resulting in significant changes to internal cytoskeletal structure and leading to external morphological changes [2, 18]. Cytoskeletal rearrangement typically results in the release of α-granules and dense granules, dispersing a range of soluble factors into the circulation [22]. Extracellular vesicles (EVs) are also released, which act as mobile signalling mediators [20, 23-25]. *In vivo* these changes increase localised inflammation and encourage the detection of invading pathogens through the recruitment and activation of leukocytes, escalating the immune response [7, 20, 26, 27].

1.4.2 Immunoregulatory surface receptors

It has been established that platelets express a variety of surface molecules that enable them to moderate early immune responses to both infection and sterile tissue damage. Platelets express most of the known toll-like receptors (TLRs) on their surface membrane (TLR1, 2, 4, 5, 6), or internally within endosomes or platelet specific T-granules (TLR3, 7, 8, 9) [2, 28, 29]. Notably, platelets are also capable of expressing TLRs which are localised internally in nucleated cells (TLR3, 7, 9) or on the surface membrane [28, 29]. In general, platelet TLRs

recognise and bind highly conserved PAMPs, including lipids, lipoproteins, proteins, or nucleic acids derived from bacteria, viruses, fungi, or parasites, to initiate anti-microbial signalling [1, 2, 6, 19, 21, 28, 29]. TLRs can also bind a range of 'self'-derived DAMP molecules that can be secreted or released from damaged/apoptotic cells or accumulate in platelet components during storage, which induces pro-inflammatory signals (Figure 1.1; Table 1.1 & 1.2) [2, 12, 20, 24]. Platelet TLR-mediated responses are facilitated through their interactions with leukocytes [12, 19, 30]. The binding of PAMPs and DAMPs to TLRs initiates distinctive signal transduction responses, which induce platelet activation, the release of biological response modifiers (BRMs) and immunomodulatory EVs leading to leukocyte activation and recruitment [12, 19, 20, 31].

1.4.3 Release of biological response modifiers

Platelets possess a large internal store of soluble factors within α- and dense granules [22, 32], which are released following platelet activation (Figure 1.1). The platelet releasate contains a range of BRMs, including adhesion molecules, cytokines, chemokines and growth factors [22, 32, 33] (Table 1.1 & 1.2), which are able to modulate many aspects of the innate immune response following transfusion [11, 34, 35]. Specifically, granule release promotes leukocyte activation (sCD40L, RANTES, PF4, NAP2), proliferation (PF4, NAP2), adhesion (RANTES, IL-1β) and inflammation (IL-1β, RANTES) [30, 36, 37], as well as amplifying the activation of the original platelet and any surrounding platelets (ADP, calcium; Figure 1.1).

1.4.4 Release of extracellular vesicles

Platelets produce up to 90% of the EVs present in the circulation of healthy subjects [38, 39]. Initially, interest in platelet derived EVs was due to their haemostatic properties [40]. However, it is now apparent that platelet EVs are also key mediators of immune signalling [18, 20, 31, 38, 39, 41]. While low numbers of EVs are released from resting platelets, a significant increase is observed following activation [25, 31]. Platelet EVs express a range of surface receptors including P-selectin and CD40L, which facilitate attachment and activation of monocytes through Mac-1 and CD40 respectively [42, 43]. Procoagulant platelets also release EVs rich in surface exposed phosphatidylserine, which attach to monocytes and neutrophils *in vivo,* although the specific mechanics of this interaction and its immunomodulatory effects are still under evaluation [31, 41]. Platelet EVs can also transport and deliver a range of platelet derived immunomodulatory compounds to leukocytes including receptors (CD40L), mitochondria and mitochondrial DNA (mtDNA), microRNAs, cytokines (IL-1β, MIP-3), proliferation factors (PF4, CXCL-7) and chemokines (RANTES; Figure 1.1) [18, 24, 38, 41, 44, 45]. As a result, platelet EVs elicit wide-ranging effects and can influence the activation of most leukocyte populations, although interactions with neutrophils and monocytes are more common [18, 24, 31, 41, 44]. EV activated monocytes exhibit increased expression of several adhesion receptors (ICAM-1, LFA-1 and Mac-1), increasing their capacity to bind to endothelial cells [42, 43, 46, 47]. Additionally, EV-monocyte interaction causes the release of a range of monocyte derived pro-inflammatory cytokines (IL-8, IL-1β, TNF-α, MCP-1 and MMP-9) [42, 46, 47].

1.4.5 Platelet-pathogen interactions

Platelets are encountered at high density around sites of tissue damage, where pathogen entry is most probable. Thus, platelets are often the first cell to recognise and respond to microbial invaders [48, 49]. Platelet-pathogen interaction can occur directly, through TLRs, P-selectin, CLEC-2, DC-SIGN or indirectly through FcγRIIa. Platelet recognition of pathogens can lead to their immobilisation and in specific cases, destruction [5, 7, 20, 50, 51].

Aspects of platelets haemostatic function including the activation of GPIIb/IIIa receptor can facilitate platelet-bacteria interaction indirectly through fibrinogen [6, 21, 52, 53]. This can lead to bacterial-induced platelet aggregation and thrombus formation [6, 52]. Bacterial-induced platelet aggregation can be beneficial, inhibiting bacteria migration and proliferation [54]. However, it can also be utilised to facilitate immune evasion and the progression of infection [54].

Platelets can facilitate direct bacterial killing by interaction with the adaptive immune system. Circulating platelets can interact with IgG opsonised bacteria through FcγRIIa receptors and soluble PF4 [5, 7, 51, 55], which is released from the α-granules of activated platelets and binds to polyanionic lipopolysaccharides (LPS) on the surface membrane of Gram-negative bacteria [5, 55]. Upon binding to LPS, PF4 changes conformation, exposing neo-epitopes, leading to the subsequent formation of anti-PF4/polyanion antibodies. Platelets then encapsulate the PF4-coated bacteria via the FcγRIIa on the platelet membrane, exposing it to intracellular anti-microbial compounds including ROS and proteases (e.g. β-defensin) causing their destruction [5, 51].

1.4.6 Platelet-leukocyte interactions

The initial response of platelets to tissue damage is the initiation of haemostasis via adhesion to sub-endothelial structures and subsequent activation of the coagulation cascade; however they also play a key role in recruitment and activation of leukocytes in order to contain and eliminate any potential infiltrating pathogens [3]. Attachment to leukocytes requires platelet activation and degranulation, which can be induced by stimulatory signals, including haemostatic agonists (thrombin, collagen, calcium), PAMPs/DAMPs or *ex vivo* storage [4, 12, 19, 56, 57]. Specifically, platelet activation results in α-granule release, and the consequent localisation of P-selectin, CD40L and phosphatidylserine on the platelet surface, which facilitates leukocyte attachment (Figure 1.1 & Table 1.1) [3, 58]. P-selectin is often the first point of contact between platelets and leukocytes through binding to its ligand, PSGL-1 [3, 58]. While P-selectin/PSGL-1 binding alone is relatively weak, co-stimulation with CD40L induces downstream signalling in leukocytes [3, 37, 58]. This promotes Mac-1 expression on leukocytes cells, enabling more permanent attachment directly to platelet GPIbα (Figure 1.1) [3, 37] or indirectly through fibrinogen bound to platelet GPIIb/IIIa [59]. Activated platelets also externalise phosphatidylserine to the surface membrane, which has been shown to facilitate platelet attachment to macrophages and neutrophils through a range of potential surface receptors [56, 60]. In general, the formation of platelet-leukocyte aggregates results in the activation and degranulation of both cell types, increasing the local haemostatic and inflammatory effects [3].

While platelet-leukocyte aggregates can be beneficial to the immune response, an overabundance is associated with pro-inflammatory disorders and adverse

transfusion events such as transfusion associated acute lung injury (TRALI) [2, 3, 9, 61]. Activated platelets are able to attach to and circulate with all major leukocyte subgroups including neutrophils, monocytes, T-cells and B-cells [61]. However, association with monocytes and neutrophils is much more common and is associated with several pro-inflammatory disorders [37, 61]. Platelet-monocyte aggregates have been linked with a range of immune functions, contributing to platelet phagocytosis, coagulation and both pro- and anti-inflammatory signalling [62-64]. In contrast, polarised neutrophils, which display asymmetric receptor expression, can actively search for activated platelets in the bloodstream and attach through P-selectin/PSGL-1 [65]. The formation of platelet-neutrophil aggregates is pro-inflammatory and a key step towards the generation of platelet induced neutrophil extracellular traps (NETs) [4, 9, 65, 66]. However, platelet-neutrophil binding alone is usually insufficient to induce NET formation, which requires additional secondary signals such as pathogen recognition via platelet TLR4, signalling from traditional platelet agonists (thrombin), soluble factors (sCD40L, IL-1β, HMGB1) or EVs [4, 9, 12, 36, 39]. Following receipt of secondary signals, the neutrophil ejects its nucleus, releasing a web of DNA coated in a range of anti-microbial factors (Figure 1.1) [4]. The induction of NET formation is tightly controlled due to its inflammatory and procoagulant properties, which can cause significant collateral tissue damage, particularly to the sensitive alveoli of the lung [4, 9].

As platelet activation is key in mediating platelet immune function, it is important to note that a progressive degree of platelet activation occurs during *ex vivo* storage [57, 67, 68]. While the haemostatic impact of storage has been well

characterised, the storage-related effects on the immunological functions of platelets are still being unravelled.

1.5. The effect of *ex vivo* storage on platelet immune function

1.5.1 Conventional room-temperature stored platelets

While specific manufacturing methods vary between countries, platelet components are produced either by pooling and isolating platelets from the WB of multiple donors or by selectively isolating platelets from a single donor by apheresis [69]. Additionally, the solution in which the platelets are suspended can vary; consisting of 100% donor plasma or a combination of plasma and up to 70% platelet additive solution (PAS), which influences the initial composition and concentration of BRMs in the component [70].

Platelet concentrates are currently stored for up to 7 days at room-temperature (20 – 24 °C), due to the risk of bacterial proliferation and the effects of the platelet storage lesion (PSL) [57, 67, 71-73]. The PSL is a range of deleterious storage-related effects, which results from the storage of platelet components at room-temperature [57, 67, 72, 73]. Progression of the PSL impacts the platelet phenotype and haemostatic function, the composition of the supernatant of stored components (Table 1.1 and 1.2; conventional storage), and also affects the immunogenicity of the component, with older platelet components more likely to induce adverse transfusion events [10].

Room-temperature storage induces a gradual shift towards a more activated phenotype. This is observed by a loss of certain glycoproteins (GPIbα, GPIV, GPVI), increased surface expression of P-selectin, phosphatidylserine, CD40L and an increase in the activated conformation of GPIIb/IIIa (Table 1.1) [57, 67, 74]. These are all key mediators of platelet-leukocyte signalling (Figure 1.1) [3, 37]. There is also an accumulation of metabolic by-products, pro-inflammatory

cytokines, mtDNA and EVs in the supernatant (Table 1.2) [11, 70, 74-79]. The initial concentration of many soluble factors is reduced by supplementation with PAS. However, current data indicate that *ex vivo* storage can stimulate the release of BRMs from platelets to levels become several fold higher than baseline, regardless of the starting concentration in plasma or plasma/PAS (Table 1.2) [11, 33-35, 44, 77].

Conventional storage induces changes in platelet immune function over time, which is directly linked to the incidence of adverse events. A retrospective study by Losos *et al*. examined more than 50,000 leukoreduced platelet transfusions, and identified that the majority of transfusion reactions occurred after 3 days of storage, with the likelihood of adverse events increasing per day (OR, 1.30, 95% CI1.12 - 1.52) [10]. The induction of adverse events has been associated with platelet activation, high concentrations of HMGB1, soluble CD40L, OX40L, IL-27, IL-1β, mtDNA and mitochondria carrying EVs in platelet components (Figure 1.1 & Table 1.2) [11, 24, 34, 36, 76]. Further, DAMPs, such as HMGB1 and mtDNA, may accumulate in platelet components and have been implicated in the induction of adverse events, including febrile non-haemolytic transfusion reactions (FNHTRs) and TRALI [2, 9, 12, 24, 34, 76]. Although the mechanisms of TRALI are multi-factorial, transfusion of platelets is thought to trigger leukocyte activation and increase lung inflammation and NET formation, potentially via the action of activated platelets, TLR signalling, EVs and BRMs (Figure 1.1) [9, 12, 24, 36, 65]. Further, platelet depletion and inhibition by aspirin has been found to have a protective effect against acute lung injury in mice [65, 66].

1.5.2 Refrigerated platelets

Platelet components were commonly stored under refrigerated conditions (2-6 °C) until the practice was discontinued in the 1970s [16, 73, 80]. This was primarily due to the discovery that refrigerated storage caused platelets to be cleared from circulation significantly faster post-transfusion than platelets stored at room temperature [80]. Subsequent work has identified that refrigeration induced platelet activation causes the clustering of the GPIbα receptor on the surface membrane, which increases clearance by hepatocytes and macrophages in the liver [81]. However, there has been a resurgence of interest in platelet refrigeration as a storage methodology for use in the treatment of acute bleeding, which is less impacted by a faster rate of clearance *in vivo* [16]. Further refrigeration reduces anaerobic metabolism during storage, thus inhibiting bacterial growth and better preserving haemostatic function compared to room-temperature storage, allowing for a potential extension of the shelf-life to 21 days [16, 73, 80, 82].

Platelet refrigeration results in significant morphological change and more pronounced expression of activation markers in comparison to room-temperature stored components [83-86]. However, it is still unclear whether the refrigeration induced changes in platelets are comparable to agonist induced platelet activation [87-89]. Further, alterations to the immune characteristics of platelets induced by refrigeration are largely uncharacterised (Table 1.1 & 1.2). Changes in the abundance of adhesion receptors have been observed during refrigeration, including decreased GPIbα, increased CD40L and P-selectin and phosphatidylserine externalisation and activation of GPIIb/IIIa [84-86, 90] (Table 1.1), which could increase the likelihood of bacterial interactions and/or

platelet leukocyte aggregate (PLA) formation. Refrigeration appears to slow the rate of release of α-granule associated soluble factors, compared to conventionally stored platelets (Table 1.2) [33, 77]. A study by Johnson *et al*. demonstrated that refrigerated platelets stored for 14 days contained cytokine levels comparable to day 5 room-temperature stored platelets [33]. The release of phosphatidylserine-positive EVs is also significantly increased during refrigerated storage compared to conventional storage [75], and while they have been demonstrated to be haemostatic [33], the immunologic effects are as yet unknown.

Research examining the immune effect of refrigerated platelets *in vivo* is limited to a single study in a mouse model, whereby refrigerated platelets increased vascular leakage compared to conventionally stored components [91], which could facilitate increased leukocyte infiltration through endothelial cells. Contemporary clinical data on the safety of refrigerated platelets is also limited. While historical data supports that cold-stored platelets are safe, it may not be reflective of current platelet components, as significant changes in platelet manufacturing processes have occurred in the last 30 years [92, 93]. However, a number of studies are underway [16, 17, 94-96], with preliminary safety data reporting that cold-stored platelets are not associated with more adverse events than conventionally stored platelets in patients undergoing cardiac surgery [17].

1.5.3 Cryopreserved platelets

Platelet cryopreservation, pioneered by Valeri *et al.* [97], was used throughout the 1970s to supply autologous platelets to alloimmunised leukaemia patients presenting with thrombocytopenia [98]. However, more recent use of

cryopreserved platelets has been limited to the treatment of active bleeding, as a result of the reduced post-transfusion recovery [99]. In recent years, an emphasis has been placed on using cryopreserved platelets for the treatment of acute bleeding due to their increased haemostatic effectiveness [15, 100-102]. Platelet cryopreservation involves adding 5 – 6% DMSO as a cryoprotectant, before concentrating the platelets and removing excess DMSO prior to freezing at -80 °C [97, 103, 104]. This method allows for long term storage for potentially 12 years [105, 106]. When required, cryopreserved platelets can be thawed and reconstituted in saline, thawed plasma or additive solution [103, 107, 108].

The cryopreservation process alters the surface phenotype and increases degranulation and release of phosphatidylserine-positive EVs (Table 1.2) [33, 109]. In terms of membrane changes, 50-70% of cryopreserved platelets externalise phosphatidylserine (Table 1.1) [56, 110, 111]. There is some evidence that phosphatidylserine on cryopreserved platelets may be pro-inflammatory, mediating interactions with macrophages [56, 112]. Similarly, the abundance of GPIbα is reduced on a population of cryopreserved platelets (Table 1.1) [56, 104, 111, 113], which may affect their ability to bind to leukocytes. The concentration of soluble factors in the supernatant of cryopreserved components is significantly higher than conventionally stored platelet components (Table 1.2), including RANTES and TGF-β1, which have pro- and anti-inflammatory effects respectively [33, 113, 114]. Further, the number of EVs in cryopreserved platelet components is up to 15-fold higher than conventionally stored platelets, and 10-fold higher than refrigerated platelets at the end of storage, which contributes to their haemostatic function [33, 110]. Additionally, EVs from cryopreserved platelet have an altered surface receptor profile

compared to EVs from fresh platelet components, demonstrating increased expression of several adhesion receptors (CD36, GPIIb, GPIX and CD47) and exposure of phosphatidylserine [109].

The altered surface phenotype and the releasate of cryopreserved platelets has the potential to affect the way they interact with leukocytes following transfusion, although relatively few studies have been conducted to examine these phenomena (Table 1.1 & 1.2). In an *in vitro* human whole blood model of transfusion, cryopreserved platelets suppressed the responsiveness of $BDCA3^{+}$ myeloid derived dendritic cells to stimulation by LPS and poly(I:C), producing less IL-8, TNF-α and IP-10, suggesting a potentially immunosuppressive effect [115]. In contrast, studies using THP-1 cells and a murine model of controlled haemorrhage suggest that cryopreserved platelets may be pro-inflammatory, by increasing leukocyte release of TNF-α, IL-1β and IL-6 [56, 116]. While these contradictory findings are likely due to differences in experimental models, they highlight the need for further work in this area.

To date, very few adverse events have been reported following transfusion of cryopreserved platelets [15, 98, 100, 101, 108]. However, larger studies are underway [117-119], as the number of cryopreserved platelets transfused in a clinical trial setting is too low to provide confidence that they are not associated with adverse events of low incidence. Further, a greater focus on the clinical outcomes associated with the immunoregulatory functions of platelets in these studies would be valuable.

Table 1.1. Summary of the effect of storage methodologies on the surface expression of immune-related platelet proteins.

Platelet Surface			Expression/concentration change over storage			
Receptors	**Role in platelet mediated immunity**	**References**	**Conventional Storage γ**	**Refrigeration δ**	**Cryopreservation †**	**References**
TLR 1, 2, 4, 5 & 6	• recognises PAMPs (e.g. proteins, lipids) and DAMPs (e.g. HMGB1, Histone H4) • triggers platelet activation • can lead to pro-inflammatory effects • expressed on the surface membrane	[1, 2, 28, 29]	na	na	na	
TLR 3, 7 & 9	• recognises nucleic acids and HMGB1 (TLR9) • triggers platelet activation • expressed internally and on the surface membrane of activated platelets	[1, 2, 28, 29]	na	na	na	
Receptors/factors	**Role in leukocyte interaction**					
GPIIb/IIIa (Active conformation)	• facilitates indirect attachment to leukocytes through fibrinogen bound to Mac-1 • facilitates indirect attachment to bacteria	[1, 59]	↑	↑	↔ or ↓	[6, 52, 57, 68, 84-86, 94, 107, 111]
GPIbα	• facilitates attachment to leukocytes through Mac-1	[1]	↓	↓	↓	[56, 83-85, 104, 107, 111, 113, 114]
P-selectin	• facilitates attachment to leukocytes through PSGL-1 • facilitates attachment to bacteria	[1, 3, 50]	↑	↔ or ↑	↑	[56, 57, 68, 70, 83-85, 104, 107, 111, 113, 120]
Phosphatidylserine	• anti-inflammatory and immunosuppressive signal • mediates phagocytosis by macrophages	[56, 112]	↑	↔ or ↑	↑	[56, 57, 68, 83-85, 94, 104, 107, 110, 111, 113, 114]
CD40L (CD154)	• co-stimulatory molecule • pro-inflammatory	[3]	↑	↑	na	[83, 121]

Changes over storage are described as general trends and vary depending on the method of platelet manufacture, including collection method and plasma content.
γ conventionally stored platelets (20-24°C, with agitation) at day 5 – 7 compared to day 1

δ refrigerated platelets compared to conventionally stored platelets at same time point of storage

† thawed platelets compared to conventionally stored platelets at day 1
Abbreviations: PAMPs = pathogen associated molecular patterns, DAMPs = damage associated molecular patterns, TLR = toll-like receptor
Relative changes in receptor expression are expressed as the following: ↑ = increase; ↓ = decrease; ↔ = unchanged; na = no published data available

Table 1.2. Summary of the effect of storage methodologies on the release of immune related soluble factors.

Platelet Supernatant			Expression/concentration change over storage			
Biological Response Modifier	**Role in platelet mediated immunity**	**References**	**Conventional Storage γ**	**Refrigeration δ**	**Cryopreservation †**	**References**
PF4	• promotes haemostasis • attracts neutrophils and monocytes • anti-pathogenic function	[32, 49]	↑	↓	↔ or ↑	[5, 33, 70, 104, 113]
TGF-β1	• context sensitive signalling protein • pro/anti-inflammatory properties	[1, 26, 49]	↑	↔	↑	[70, 77, 78, 104, 107]
IL-1β	• key mediator or the inflammatory response	[1, 3, 18, 23, 26, 49]	↑	na	↓	[79, 104]
IL-8	• attracts neutrophils and granulocytes	[32]	↑	↓	na	[77]
IL-27	• context sensitive signalling protein • pro/anti-inflammatory properties	[1, 11]	↑	na	↔	[11, 114]
HMGB1	• pro-inflammatory signalling molecule • promotes cytokine release through TLRs	[1, 3, 34]	na	na	na	
RANTES	• pro-inflammatory • attracts leukocytes	[26, 122]	↑	↓	↔ or ↑	[33, 70, 74, 79, 104, 113, 114]
Soluble CD62P	• promotes leukocyte signalling and recruitment	[1, 58]	↑	↓	↑	[33, 70, 104, 107, 113, 114]
Soluble OX40L	• promotes endothelial inflammation and leukocyte activation	[11]	↑	na	na	[11, 76]
Soluble CD40L	• pro-inflammatory • platelet and leukocyte activator • facilitates leukocyte differentiation • co-stimulatory molecule	[1, 3, 11, 26]	↑	↑ or ↓	↓ or ↑	[33, 35, 44, 79, 83, 104, 107, 113]
EVs	• remote signalling factors • facilitate intracellular transfer of receptors, cytokines and miRNAs • activate leukocytes	[1, 3, 23, 25, 38, 67, 109]	↑	↔ or ↑	↑	[33, 75, 84, 94, 104, 109, 110, 114]
Mitochondrial DNA	• pro-inflammatory signal • recognised by TLRs	[76]	↑	na	na	[76]

Changes over storage are described as general trends and vary depending on the method of platelet manufacture, including collection method and plasma content.
γ conventionally stored platelets (20-24°C, with agitation) at day 5 – 7 compared to day 1

δ refrigerated platelets compared to conventionally stored platelets at same time point of storage
† thawed platelets compared to conventionally stored platelets at day 1
Abbreviations: TLR = toll-like receptors, EV = extracellular vesicles
Relative changes in factor expression/concentration are expressed as the following: ↑ = increase; ↓ = decrease; ↔ = unchanged; na = no published data available

1.6. Future areas of study

While the immune function of platelets is now acknowledged, and studies have begun to consider the effect of conventional and alternative storage methodologies, there are significant gaps in our current understanding. Specifically, the concentration of key BRMs which have been associated with adverse events (IL-1β, IL-8, IL-27, HMGB1 and sCD40L) and the expression of immune related receptors in platelets stored under novel conditions is largely uncharacterised, particularly those involved in pathogen recognition and leukocyte interaction (Table 1.1) [11, 76]. This information could potentially be used by blood collection centres to identify less immunogenic platelet components, and reserve these for the most at-risk of adverse events. Additionally, very few studies have examined how stored platelets interact with recipient leukocytes, which is believed to be a key factor in the incidence of adverse events [9, 65]. Continued research using *in vitro* and animal models of transfusion could be utilised to examine the likelihood of leukocyte activation by stored platelets. Ultimately, clinical trials, which are in progress for both cold-stored and cryopreserved platelets offer the perfect opportunity to assess the immunological changes occurring in the recipient post-transfusion and such outcomes should be incorporated into the study design of future trials. This knowledge would aid in characterising the pathogenesis of adverse events, support the development of preventative strategies and inform future changes to transfusion guidelines.

1.7. Concluding remarks

Platelets are no longer recognised as simply mediators of haemostasis, but also as having a tangible role as an immune cell. *Ex vivo* storage conditions have the capacity to significantly alter the characteristics of platelet components, which may affect their immune function and the likelihood of adverse events. Consequently, the impact of new manufacturing and storage methodologies on the immune characteristics of platelets should be considered alongside their haemostatic function prior to implementation.

2. Cold storage alters the immune characteristics of platelets and potentiates bacterial-induced aggregation

2.1. Foreword

This chapter focuses on examining the effect of refrigerated storage on the immune characteristics and function of platelets *in vitro*. Refrigerated storage was found to significantly alter the abundance of receptors associated with the immune function of platelets. Notably, this included a significant reduction in pathogen recognition receptors on the surface membrane. In contrast, refrigerated platelets exhibited increased bacterial-induced aggregation, which was linked to the function of the activated form of the fibrinogen receptor GPIIb/IIIa.

2.1.1 Contribution to thesis aims

This chapter addresses **Aim 1** and **Aim 2** and partially addresses **Aim 3**.

Aim 1: Characterise the expression of immune-related receptors on stored platelets.

Conclusion: Refrigeration was found to alter the abundance of immune-related receptors on the platelet surface membrane.

Aim 2: Characterise the releasate from stored platelets, with a particular focus on immune-modulatory soluble proteins.

Conclusion: Refrigeration did not significantly increase the concentration of soluble factors previously associated with adverse events in platelet components.

Aim 3: Determine the effect of storage on platelet-bacteria and platelet-leukocyte interactions.

Conclusion: Refrigerated platelets exhibited increased responsiveness to *E. coli* and *S. aureus* induced aggregation, which was linked to the function of the activated GPIIb/IIIa receptor on the platelet surface membrane.

2.2. Statement of authorship

This chapter is comprised of an original manuscript which was published in *Vox Sanguinis* in 2022. Minor edits have been made to the formatting and wording of the manuscript to make it consistent with the rest of the Thesis.

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Ben Wood, Lacey Johnson and Denese Marks conceived and designed the study. Ben Wood conducted the lab work and prepared the figures. Ben Wood and Lacey Johnson analysed the data and wrote the manuscript. Ben Wood, Matt Padula, Lacey Johnson and Denese Marks critically reviewed the manuscript.

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2.3. Abstract

Background and Objectives

Cold-stored platelets are currently under clinical evaluation and have been approved for limited clinical use in the United States. Most studies have focussed on the haemostatic functionality of cold-stored platelets; however, limited information is available examining changes to their immune function.

Materials and Methods

Two buffy coat derived platelet components were combined and split into two treatment arms: room-temperature (RT)-stored (20-24°C) or refrigerated (cold-stored, 2-6°C). The concentration of selected soluble factors was measured in the supernatant using commercial enzyme-linked immunosorbent assay (ELISA) kits. The abundance of surface receptors associated with immunological function was assessed by flow cytometry. Platelet aggregation was assessed in response to *E. coli* and *S. aureus*, in the presence and absence of RGDS to the block active conformation of GPIIb/IIIa.

Results

Cold-stored platelet components contained a lower supernatant concentration of C3a, RANTES and PF4. The abundance of surface bound P-selectin and GPIIb/IIIa, in the activated conformation, increased during cold storage. In comparison, the abundance of CD86, CD44, ICAM-2, CD40, TLR1, TLR2, TLR4, TLR3, TLR7, TLR9 was lower on the surface membrane of cold-stored platelets, compared to RT-stored components. Cold-stored platelets exhibited an increased responsiveness to *E. coli* and *S. aureus* induced aggregation

compared to RT-stored platelets. Inhibition of the active conformation of GPIIb/IIIa using RGDS reduced the potentiation of bacterial-induced aggregation in cold-stored platelets.

Conclusion

Our data highlights that cold storage changes the *in vitro* immune characteristics of platelets, including their sensitivity to bacterial-induced aggregation. Changes in these immune characteristics may have clinical implications post-transfusion.

Keywords: cold storage, platelets, immunology, bacteria

2.4. Introduction

Cold storage has re-emerged as an alternative platelet storage methodology. Several clinical trials are ongoing to determine the appropriate shelf-life and clinical indications, including 4CPLT (NCT02495506), CHIPS (NCT04834414) and CriSP-TBI (NCT04726410) trials. Additionally, cold-stored platelets have been approved for limited use by the FDA [16, 123], and delayed cold storage has been implemented in some blood centres to counter pandemic induced shortages [124]. Overall, cold storage better preserves the *in vitro* haemostatic function of platelets, while lowering the risk of bacterial proliferation [71, 73], compared to conventional room-temperature (RT)-storage [17, 33, 75, 84, 125, 126].

While traditionally considered haemostatic cells, platelets are also key mediators of the immune response [37, 127]. The platelet surface membrane houses a range of co-stimulatory (CD40, CD40L, HCAM, B7-2, MHC-I, siglec-7) and adhesion (P-selectin, ICAM-2, DC-SIGN, CLEC2, GPIIb/IIIa) receptors, which enable leukocyte activation and facilitate the formation of PLAs [59, 127-129]. Further, activation by haemostatic or immunological agonists can induce degranulation and release of biological response modifiers (BRMs) [33, 127, 130]. BRMs can have numerous effects, facilitating inflammation and leukocyte activation (CD40L, endocan, IL-1β, IL-13, IL-27, HMGB1, OX40L and RANTES), and anti-pathogenic activity (PF4, C3a, C4a and C5a) [11, 33, 34, 37, 79, 127, 131-133]. Notably, the accumulation of BRMs in platelet components has been associated with an increased incidence of adverse events following transfusion [11].

Platelet surface receptors also mediate the detection and elimination of infiltrating pathogens. Toll-like receptors (TLRs; TLR1, TLR2, TLR4 and TLR6) and adhesion molecules (P-selectin) are capable of recognising and binding pathogens *in vivo* [28, 29]. Additionally, platelets are unique in that TLR3, TLR7 and TLR9, which are generally expressed in intracellular compartments in other cells, are present on the surface membrane, where they are believed to assist in pathogen clearance from the circulation [28, 29]. Platelets can also adhere to pathogens indirectly through FcγRIIa and the activated form of GPIIb/IIIa and their bridging molecules IgG and fibrin/fibrinogen, respectively [5, 6, 134, 135]. Platelet-pathogen binding can be beneficial, facilitating pathogen immobilisation and destruction [5, 7, 8] or detrimental, allowing pathogens to evade aspects of the immune system [129].

Therefore, this study aimed to examine the effect of cold storage on the immune characteristics of platelets, with a particular focus on functions associated with pathogen interaction.

2.5. Methods

2.5.1 Experimental design

Ethics approval was obtained from the Australian Red Cross Lifeblood Ethics Committee (Johnson 10052019). Blood donations were obtained from voluntary blood donors. Pooled platelet components were prepared by combining buffy coats from 4 whole blood donations. Platelet components were resuspended in 30% plasma/70% PAS-E (SSP+; MacoPharma, Mouvaux, France) and leukoreduced by filtration (AutoStop BC filters; Haemonetics Corp. Boston, MA,

USA) before storage in 1300 mL PVC bags (ELX; Haemonetics Corp.). Each replicate consisted of two pooled platelet concentrates which were combined and split into two components of equal weight at day 1 post-collection. Components (n=8 in each group) were randomly assigned to be either RT-stored (20-24 °C with agitation) or cold-stored (2-6 °C with no agitation). The combined component was sampled on day 1 post-collection (10-15 mL) to establish baseline parameters, and components in each treatment arm were sampled on day 2, 5, 7, 9 and 14 post-collection. Platelet counts were obtained using an automated haematology analyser (CellDYN Emerald, Abbott Core Laboratory, Abbott Park, IL, USA).

2.5.2 Flow cytometry

The surface membrane abundance of platelet receptors was examined using flow cytometry (FACS Canto II, Becton Dickinson, Franklin Lakes, NJ, USA). Approximately 500, 000 platelets were individually stained with the following antibodies diluted in Tyrode's buffer for 30 minutes at 20-24 °C in the dark: CD40-PE (5C3), CD32-APC (FUN-2), CD154-APC (24-31), CD286-PE (TLR6.127), CD86-PE (IT2.2), CD44-APC (VI A034, BioLegend, San Diego, CA, USA), PAC1-FITC (PAC1), CD62P-PE (AC1.2, BD Biosciences, Franklin Lakes, NJ, USA), CD328-PE (194211), CD209-APC (120,507), MHC-I-PE (W6/32), CLEC2-APC (219133), TLR7-PE (533707, R&D Systems, Minneapolis, MN, USA), CD281-PE (GD2.F4), CD282-PE (TL2.1), CD283-PE (TLR-104), CD284-PE (HTA125), CD289-PE (eB72-1665), CD102-PE (V BP363, eBioscience, San Diego, CA, USA). Following incubation, each tube was diluted 1:20 with Tyrode's buffer and 10 000 gated platelet events were collected.

2.5.3 Soluble factor analysis

Platelet supernatant was collected at each timepoint by double centrifugation, as previously described [33]. Briefly, platelet concentrate was centrifuged at 1 600 x *g* for 20 minutes and again at 12 000 x *g* for 5 minutes and the supernatant was stored at - 80 °C for later analysis. The concentration of soluble factors in the supernatant was examined using commercially available ELISA kits: endocan (limit of detection: 10 pg/mL; Abcam, Cambridge, UK), HMGB1 (limit of detection: 19 pg/mL; Novus Biologicals, Littleton, CO, USA), C3a, C4a (limit of detection: 7 pg/mL and 6 pg/mL respectively; BD Bioscience), C5a, RANTES, PF4, IL-13, IL-27, IL-1β, OX40L and CD40L (limit of detection: 31 pg/mL, 16 pg/mL, 16 pg/mL, 94 pg/mL, 156 pg/mL, 4 pg/mL, 47 pg/mL and 16 pg/mL respectively; R&D Systems). Each sample was tested in duplicate or triplicate and compared to a standard curve, as per the manufacturer's instructions.

2.5.4 Platelet bacteria aggregometry

Platelet-bacteria aggregation was measured by light transmission aggregometry (Helena Laboratories, Beaumont, TX, USA). A common gram-positive (*S. aureus*; NCTC 10788) and a gram-negative (*E. coli*; NCTC 12923; Bioball, Biomerieux, Marcy-l'Etoile, France) bacterial strain were included in this study due to their association with post-surgical infection risk [136, 137]. Bacterial vials were rehydrated to a concentration of 1.1 x 10^9 in rehydration fluid for 30 minutes at 22 °C. Platelets were diluted to 300 x 10^6 cells/mL in fresh frozen plasma to obtain platelet-rich plasma (PRP). All reagents, including PRP, were pre-heated to 37 °C before use. Platelet aggregation was monitored for 40 minutes following stimulation with either 20 µM adenosine diphosphate (ADP; Sigma, MO, USA),

or bacteria at a ratio of 1:2 (bacteria: platelets, n=6). Further, at day 14 of storage, four of the replicates were randomly selected for incubation with RGDS peptide (400 μM, Cayman Chemical Company, MI, USA) prior to bacterial-induced activation. RGDS binds to the active site of GPIIb/IIIa, acting as a competitive inhibitor of fibrin/fibrinogen binding [138].

2.5.5 Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical analysis was conducted using GraphPad Prism 8.4.3 (GraphPad Software, Inc.). A two-way repeated-measures analysis of variance (ANOVA) was used to compare RT-stored and cold-stored platelets over the storage period. RGDS data was analysed by a one-way ANOVA comparing all treatment groups. Post-hoc Bonferroni multiple comparisons were performed to determine differences at each time-point or treatment. In all cases a p-value< 0.05 was considered to be statistically significant.

2.6. Results

The platelet components had a starting count of 380 ± 24 x 10^9 /unit which decreased by 13% over storage, but to the same degree in both arms. Platelets contain significant internal stores of soluble factors, which can be released upon activation, promoting inflammation and anti-pathogenic functions [5, 7, 127]. In general, cold storage did not significantly alter the release of most immunomodulatory factors examined. The concentration of CD40L, endocan, IL-1β, IL-13, IL-27, HMGB1 and OX40L did not vary significantly compared to RT-stored platelets at any time point (Figure 2.1A-G). However, the concentration

of RANTES and PF4 increased two and three-fold respectively in RT-stored platelets from day 9 onwards compared to cold-stored components (Figure 2H-I).

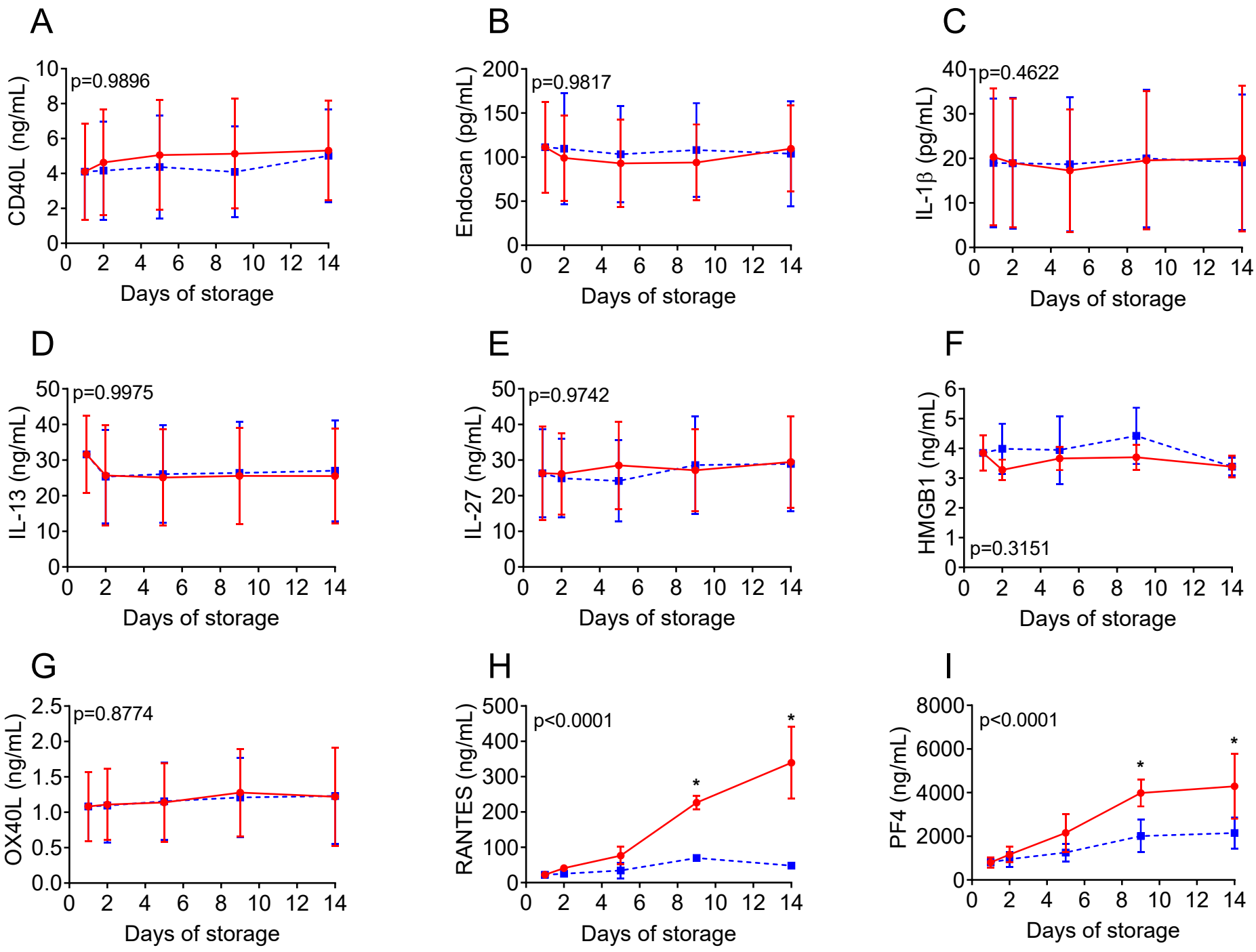

Figure 2-1. Refrigeration alters the concentration of cytokines in the supernatant. Platelet supernatants were collected from RT-stored (red lines) and cold-stored (broken blue lines) platelets stored at the indicated days post-collection. The concentration of (A) CD40L, (B) endocan, (C) IL-1β, (D) IL-13, (E) IL-27, (F) HMGB1, (G) OX40L, (H) RANTES and (I) PF4 were measured by ELISA. Data represents mean ± standard deviation (error bars, n=8 in each group). Significance was determined by two-way ANOVA comparing the effects of each treatment over the storage period. * indicates $p<0.05$ compared to RT-stored platelets.

Complement factors are stored in α-granules and can be released following platelet activation and degranulation [130]. The concentration of C3a gradually increased in RT-stored components, becoming two-fold higher than cold-stored platelets at days 9 and 14 (Figure 2.2A). In contrast, no significant difference was observed in the supernatant concentration of C4a or C5a between RT-stored and cold-stored platelets (Figure 2.2B-C).

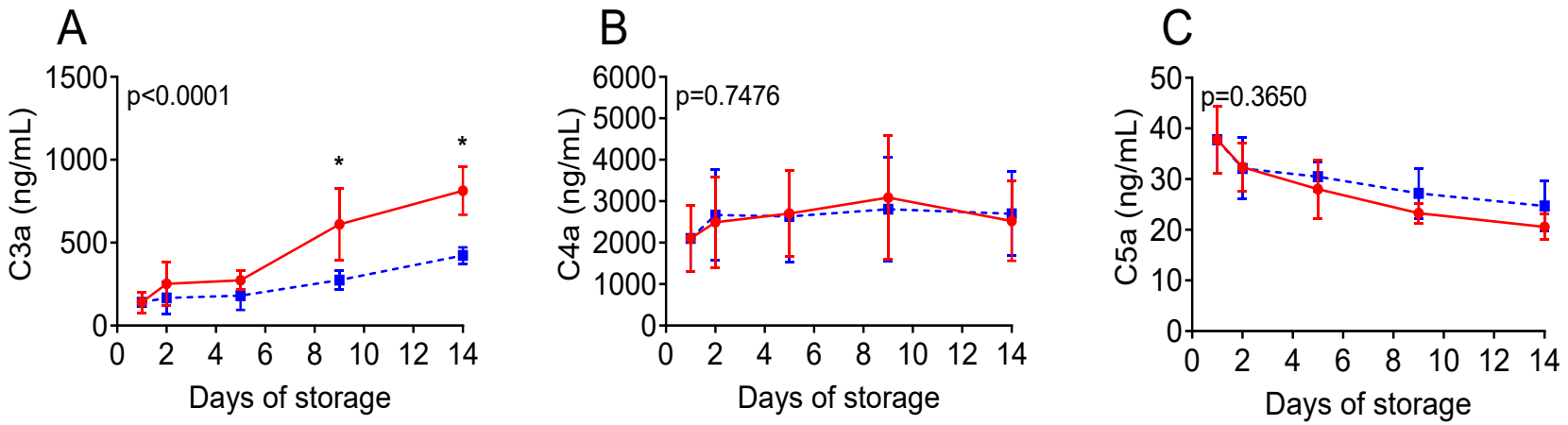

Figure 2-2. Refrigeration alters the concentration of complement factors in the supernatant. Platelet supernatants were collected from RT-stored (solid red lines) and cold-stored (broken blue lines) platelets stored at the indicated days post collection. The concentration of (a) C3a, (b) C4a and (c) C5a was measured by ELISA. Data represent mean ± standard deviation (error bars, n = 8 in each group). Significance was determined by two-way ANOVA comparing the effects of each treatment over the storage period. * indicates $p < 0.05$ compared to RT-stored platelets.

The platelet surface membrane contains a range of adhesion and co-stimulatory receptors, which allow binding and activation of leukocytes [127]. Cold storage significantly increased the abundance of P-selectin on the surface membrane from day 2, compared to RT-stored components (Figure 2.3A). In contrast, the abundance of B7-2, HCAM, ICAM-2 and CD40 were reduced by day 2 on the surface membrane of cold-stored platelets, compared to those stored at RT (Figure 2.3B-E). The abundance of DC-SIGN appeared lower on the surface membrane of cold-stored platelets; however, the reduction was not statistically significant (Figure 2.3F). In contrast, there was no difference in the membrane abundance of MHC-I, CD40L, siglec-7 or CLEC2 between treatment arms (Figure 2.3G-J). Overall, cold storage differentially altered the abundance of certain platelet receptors associated with immunological function.

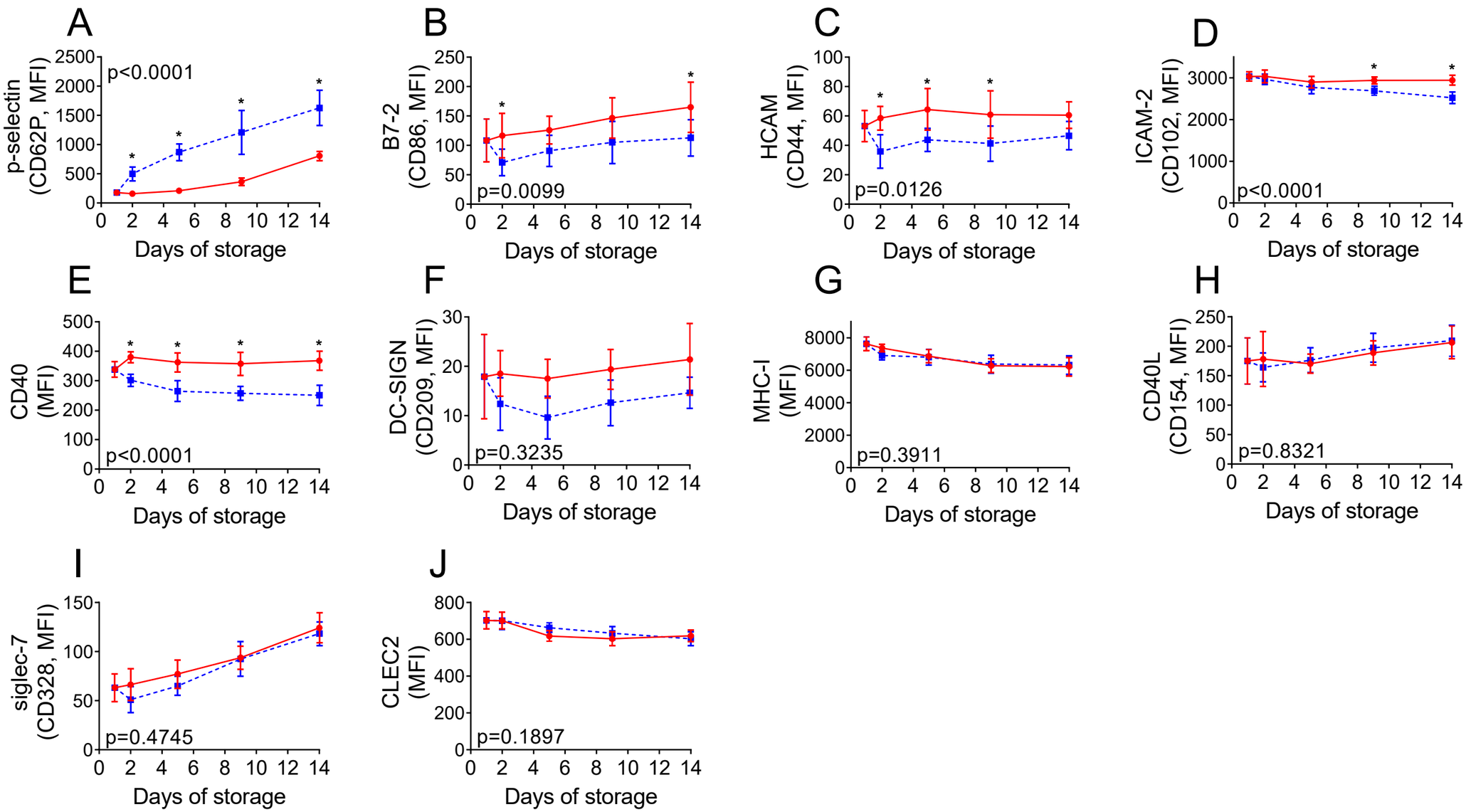

Figure 2-3. Refrigeration alters the abundance of leukocyte interaction receptors on the platelet surface membrane. The surface abundance of receptors was measured in RT-stored (solid red lines) and cold-stored (broken blue lines) platelets at the indicated days post-collection. Platelets were stained with (A) CD62P-PE, (B) CD86-PE, (C) CD44-APC, (D) CD102-APC, (E) CD40-PE, (F) CD209-PE, (G) MHC-I-PE, (H) CD154-APC, (I) CD328-APC and (J) CLEC2-APC. The median fluorescent intensity (MFI) was determined by flow cytometry. Data represents mean ± standard deviation (error bars, n=8 in each group). Significance was determined by two-way ANOVA comparing the effects of each treatment over the storage period. * indicates $p<0.05$ compared to RT-stored platelets.

Platelets can respond and adhere to pathogens directly through TLRs and indirectly through binding FcγRIIa or the activated conformation of GPIIb/IIIa [134, 135]. In general, RT-stored platelets exhibited a higher abundance of most TLRs and FcγRIIa on the surface membrane over storage (Figure 2.4A-H). The exception was TLR6 which was comparable between the treatment arms over storage (Figure 2.4D). Notably, only the abundance of the active conformation of GPIIb/IIIa was significantly increased by cold storage, compared to RT-stored components (Figure 2.4I).

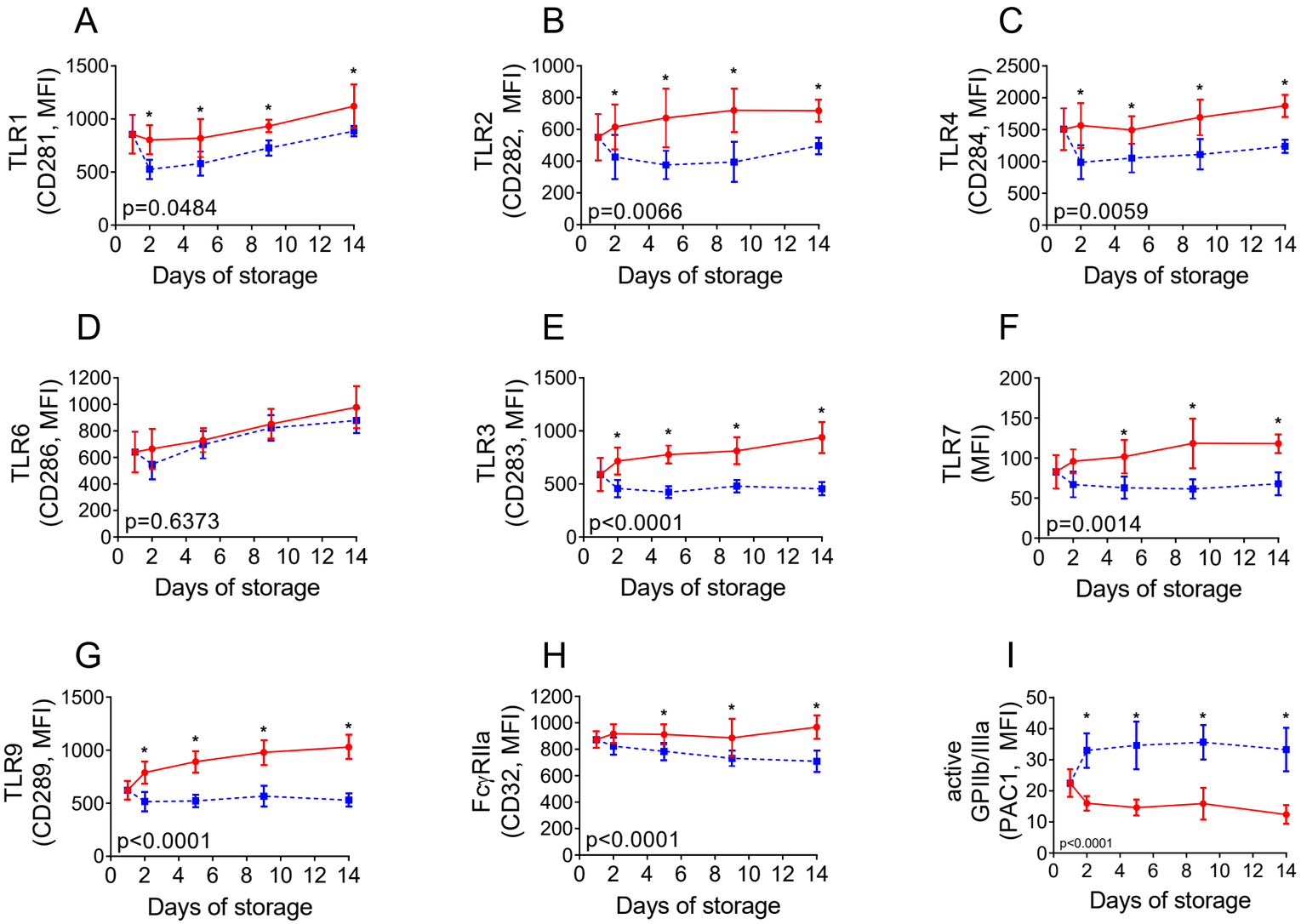

Figure 2-4. Refrigeration alters the abundance of pathogen recognition receptors on the platelet surface membrane. The surface abundance of receptors was measured in RT-stored (solid red lines) and cold-stored (broken blue lines) platelets at the indicated days post-collection. Platelets were stained with (A) CD281-PE, (B) CD282-PE, (C) CD284-PE, (D) CD286-PE, (E) CD283-PE, (F) TLR7-PE, (G) CD289-PE, (H) CD32-PE and (I) PAC1-FITC. The median fluorescent intensity (MFI) was determined by flow cytometry. Data represents mean ± standard deviation (error bars, n=8 in each group). Significance was determined by two-way ANOVA comparing the effects of each treatment over the storage period. * indicates $p<0.05$ compared to RT-stored platelets.

As cold storage reduced the abundance of pathogen receptors on the platelet surface membrane, aggregometry was used to determine if this impacted the ability of platelets to aggregate in the presence of common bacterial strains *(E. coli* and *S. aureus)*. Platelet aggregation in response to ADP decreased in both treatment arms but was better maintained in cold-stored platelets at days 7 and 14 (Figure 2.5A). *E. coli* induced a relatively low level of aggregation in RT-stored platelets, which remained stable over storage (Figure 2.5B). In contrast, a 2-fold higher aggregation response to *E. coli* was observed in cold-stored platelets at day 7 and day 14 (Figure 2.5B). Similarly, *S. aureus* induced aggregation in RT-stored platelets which increased slightly after 14 days of storage (Figure 2.5C). Notably, cold-stored platelets exhibited a significantly higher aggregation response to S. *aureus* at day 7, which further increased at day 14, compared to RT-stored components.

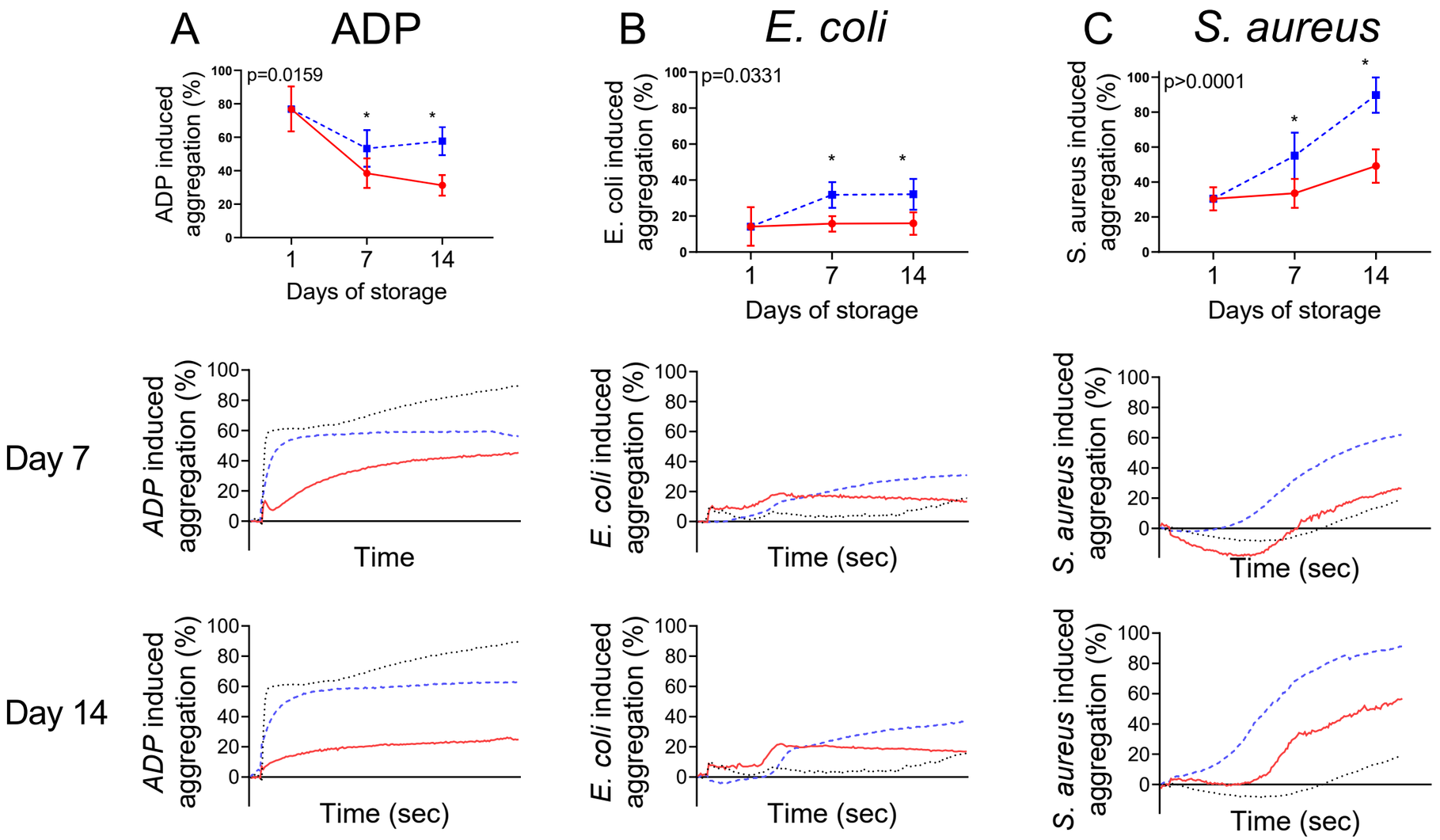

Figure 2-5. *E. coli* and *S. aureus* stimulation induces a higher aggregation response in refrigerated platelets. Light transmission aggregation was measured in RT-stored (solid red lines) and cold-stored (broken blue lines) platelets at the indicated days post-collection. Aggregation was induced by addition of (A) 20 µM ADP or bacteria (B) Escherichia coli (NTCC 12923) and (C) Staphylococcus aureus (NTCC 10788) at a ratio of 1:2 (bacteria: platelets). Data represents mean ± standard deviation (error bars, n=6 in each group). Significance was determined by two-way ANOVA comparing the effects of each treatment over the storage period. * indicates $p<0.05$ compared to RT-stored platelets. Representative aggregometry traces are presented below the corresponding graphs for day 1 (dotted black lines), 7 and 14 of storage.

The activated conformation of GPIIb/IIIa is a key mediator of *E. coli* and *S. aureus* induced aggregation [6, 52]. Inhibition of the active site of GPIIb/IIIa with RGDS did not significantly affect *E. coli* induced platelet aggregation in RT-stored platelets (Figure 2.6A). However, RGDS treatment significantly reduced *E. coli*-induced aggregation after cold storage, to levels comparable to RT-stored components (Figure 2.6A). Likewise, RGDS addition had no effect on *S. aureus*-induced aggregation of RT-stored platelets (Figure 2.6B) but inhibited the cold-stored potentiation of aggregation (Figure 2.6B).

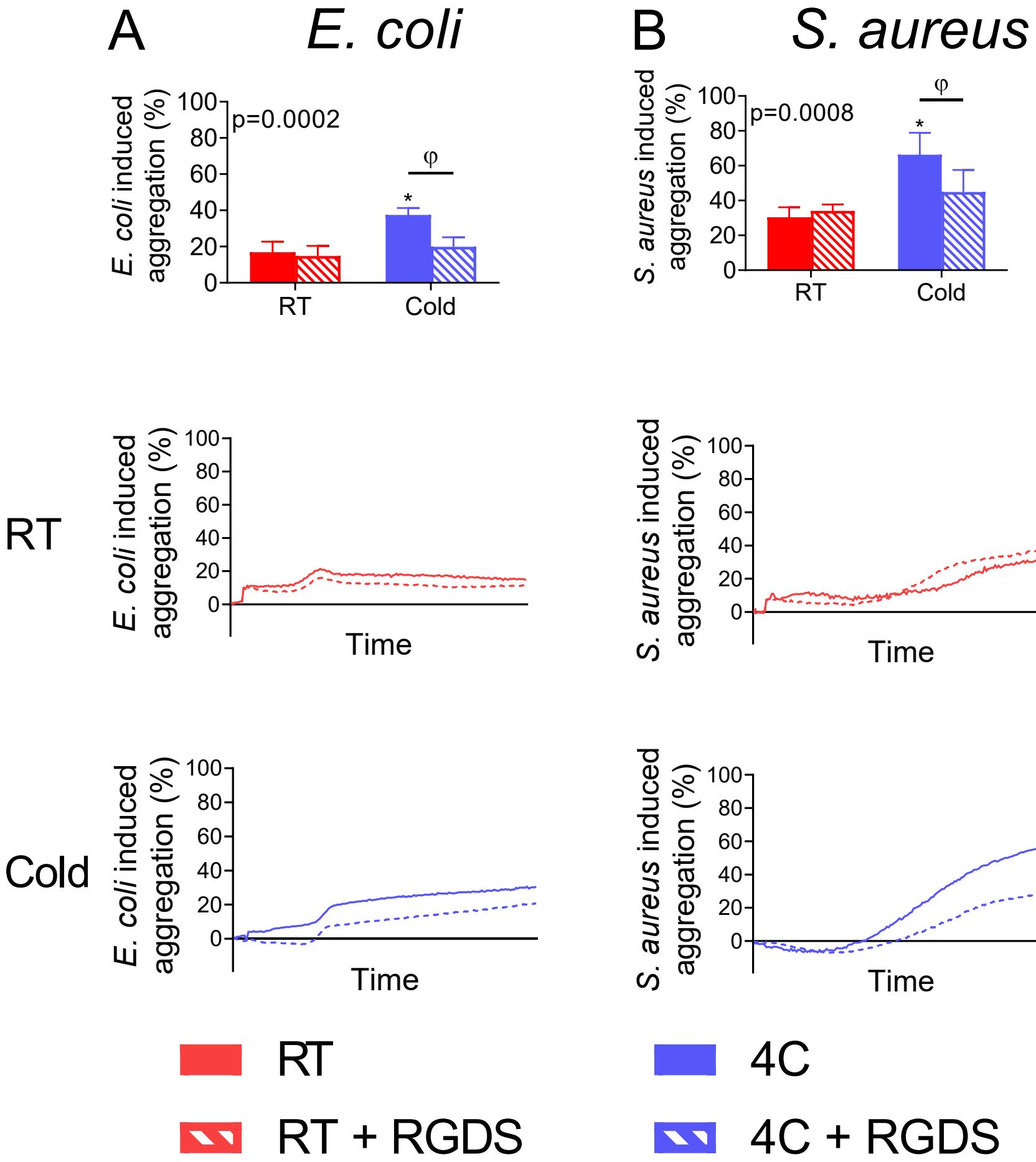

Figure 2-6. The activated conformation of GPIIb/IIIa mediates the cold-induced potentiation of *E. coli* and *S. aureus* aggregation response. Platelet aggregation was measured in the presence or absence of RGDS (400 μM) and (A) Escherichia coli (NTCC 12923) and (B) Staphylococcus aureus (NTCC 10788) at a ratio of 1:2 to RT-stored or cold-stored platelets at day 14 post-collection. Data represents mean ± standard deviation (error bars, n=4). Significance was determined by one-way ANOVA comparing means between treatments. * indicates $p<0.05$ compared to RT-stored platelets without RGDS. φ indicates $p<0.05$ compared to cold-stored platelets without RGDS. Representative aggregometry traces for untreated platelets (solid lines) and RGDS-treated platelets (dashed lines) are presented below the corresponding bar graph.

2.7. Discussion

Cold storage alters the haemostatic phenotype and function of platelets [75, 84, 126]. In this study, it was demonstrated that cold storage also induces changes in the immune characteristics of platelets. Specifically, cold storage reduced the release of a range BRMs into the supernatant and altered the surface abundance of receptors associated with immunological function. Cold storage also potentiated the aggregation responses induced by *E. coli* and *S. aureus,* in an activated GPIIb/IIIa dependent manner.

Platelet surface receptors facilitate haemostasis, pathogen recognition and leukocyte interaction [59, 127, 128]. Previous studies have demonstrated that cold storage results in reduced abundance of specific haemostatic receptors (GPIb, GPVI, GPIIb and GPIX), which is believed to be related to changes in the morphology of the platelet surface membrane [83-85]. This study reports a cold-associated decrease in the abundance of a range of TLRs and leukocyte associated receptors, which may occur through a similar mechanism. Additionally, RT storage results in a significant increase in surface abundance of TLRs during extended storage, which may be indicative of the mobilisation of internal stores.

Platelets are often the first cells to reach sites of tissue damage and thus have a high chance of encountering infiltrating pathogens [127]. Despite a decrease in the abundance of TLRs and FcγRIIa, which are typically associated with pathogen binding, an increase in bacterial-induced aggregation was observed in cold-stored platelets. While still under investigation, current research suggests

that cold storage better maintains metabolic parameters and potentially primes internal signal transduction pathways [75, 139, 140]. Previous work has identified that cold storage increases the phosphorylation of Src and Syk family of kinases [139, 140]. Notably, bacterial-induced platelet activation of TLRs, GPIIb/IIIa and FcγRIIa is reliant on signal transduction through Src and Syk [141, 142]. Consequently, cold-stored platelets may still be able to respond to activation by bacteria through TLRs and FcγRIIa, despite the observed reduction in pathogen adhesion receptors on the surface membrane.

Prior research has highlighted the importance of GPIIb/IIIa mediated fibrinogen binding in facilitating agonist mediated (ADP, collagen) and bacterial (*E. coli* and *S. aureus*) induced platelet aggregation [6, 52, 138, 143]. Notably, cold-stored platelets exhibited increased activation of the GPIIb/IIIa (higher PAC-1 binding), which was associated with increased aggregation in response to *E. coli* and *S. aureus.* Further, the addition of RGDS inhibited the cold-induced potentiation of bacterial-induced aggregation. As such, the activated conformation of GPIIb/IIIa may be responsible for the observed increase in bacterial-induced aggregation. Interestingly, RGDS inhibition reduced but did not eliminate *E. coli* and *S. aureus* induced aggregation. Additionally, platelets in both treatment arms exhibited increased aggregation in response to *S. aureus* at day 14 compared to day 7, despite no further change in GPIIb/IIIa activation. A potential explanation may be found in the increased abundance of P-selectin on the surface membrane of both RT and cold-stored platelets over storage. Surface P-selectin is known to mediate *S. aureus* induced platelet activation and adhesion [129], but is not the primary mechanism for *E. coli* induced aggregation [6].

Alterations in platelet immune function *ex vivo* can cause post-transfusion immunomodulation, contributing to adverse events [11, 76]. The likelihood of adverse events has been linked to changes in platelet activation state and release of immunomodulatory BRMs into the supernatant of components [11, 76]. The concentration of most BRMs was comparable between treatments. However, C3a, RANTES and PF4 concentrations were observed to increase in RT-stored components, which aligns with previous reports [33, 77, 131]. PF4 and C3a are involved in anti-pathogen responses [5, 144], while RANTES has been linked to a higher risk of adverse transfusion events [76]. Further, cold-stored platelets exhibited a lower abundance of several key leukocyte adhesion (HCAM, ICAM-2) and co-stimulatory (B7-2, DC-SIGN, CD40) receptors. Taken together these changes suggest a decreased capacity to bind and activate leukocytes. In contrast, cold storage results in an activated haemostatic phenotype, with phosphatidylserine and P-selectin exposure and activation of the GPIIb/IIIa. To date one study has reported a slight but not statistically significant increase in PLAs following the transfusion of refrigerated whole blood in coagulopathic rats [145]. While increased activation in RT-stored components is associated with a higher risk of adverse events [11, 76], the evidence is less clear for cold-stored platelets, warranting further investigation.

In this study, it was demonstrated that cold storage potentiates bacterial-induced aggregation using single strains of *E. coli* and *S. aureus*. However, bacterial-induced platelet aggregation varies significantly between species and strains [7, 135]. As such, further examination of a diverse range of bacteria, including other species associated with post-operative infection including

Pseudomonas, *Klebsiella and Enterobacter* would be informative [146]. Further, bacterial-induced platelet aggregation is associated with both bacterial destruction and immune evasion [5-8, 129]. Consequently, it is unclear what impact increased platelet-bacteria aggregation would have post-transfusion. Notably, most cold-stored components will be used for the treatment of acute bleeding resulting from trauma or surgery, which is associated with an elevated risk of bacterial infection post-surgery [136, 137], highlighting the need for further work in this area.

This study presents a general overview of the effects of cold storage on a range of characteristics associated with the immune function of platelets. The impact of these *in vitro* changes on clinical outcomes following transfusion of cold-stored platelets should be a major consideration as the implementation of cold-stored platelets progresses.

3. Cryopreservation alters the immune characteristics of platelets

3.1. Foreword

Cryopreservation causes significant activation post-thaw, dramatically altering the haemostatic phenotype of platelets. As such, it was hypothesised that cryopreservation may also cause significant changes in the immune characteristics of platelets. Consequently, this chapter aimed to characterise cryopreservation induced changes in the abundance of surface receptors and soluble factor release related to platelet immune function. Cryopreserved platelets exhibited an altered immune phenotype on the surface membrane and a reduction in the concentration of pro-inflammatory mediators in the supernatant. Further, the surface phenotype continued to change following extended post-thaw storage.

3.1.1 Contribution to thesis aims

This chapter addresses **Aim 1** and **Aim 2.**

Aim 1: Characterise the expression of immune-related receptors on stored platelets.

Conclusion: Cryopreservation significantly altered the abundance of pathogen recognition and leukocyte adhesion receptors on the platelet surface membrane.

Aim 2: Characterise the releasate from stored platelets, with a particular focus on immune-modulatory soluble proteins.

Conclusion: The profile of soluble factors was altered in cryopreserved platelets. A significant reduction in the concentration of pro-inflammatory factors previously associated with adverse events was observed post-thaw. Most soluble factors did not appear to be released from platelets following cryopreservation, and remained stable over storage, with the exception of PF4, RANTES and CD40L which increased.

3.2. Statement of authorship

The following section is an original manuscript which was published in *Transfusion* in 2021. Minor edits have been made to the formatting and wording of the manuscript to make it consistent with the rest of the Thesis.

Wood B, Padula MP, Marks DC and Johnson L. Cryopreservation alters the immune characteristics of platelets. Transfusion. 2021; 61;12: 3432-42. https://doi.org/10.1111/trf.16697

Ben Wood, Lacey Johnson and Denese Marks conceived and designed the study. Ben Wood conducted the lab work and prepared the figures. Ben Wood and Lacey Johnson analysed the data and wrote the manuscript. Ben Wood, Matt Padula, Lacey Johnson and Denese Marks critically reviewed the manuscript.

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Dr. Lacey Johnson

26/04/2023

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A/Prof Matthew P. Padula

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A/Prof Denese C. Marks

3.3. Abstract

Background

Cryopreserved platelets are under clinical evaluation as they offer improvements in shelf-life and potentially haemostatic effectiveness. However, the effect of cryopreservation on characteristics related to the immune function of platelets has not been examined.

Materials and Methods

Buffy coat derived platelets were cryopreserved at -80°C using 5–6% dimethylsulfoxide (DMSO, n=8). Paired testing was conducted pre-freeze (PF), post-thaw (PT0) and after 24 hours of post-thaw storage at room-temperature (PT24). The concentration of biological response modifiers (BRMs) in the supernatant was measured using commercial ELISAs and surface receptor abundance was assessed by flow cytometry.

Results

Cryopreservation resulted in increased RANTES, PF4 and C3a but decreased IL-1β, OX40L, IL-13, IL-27, CD40L and C5a concentrations in the supernatant, compared to PF samples. C4a, endocan and HMGB1 concentrations were similar between the PF and PT0 groups. The abundance of surface-expressed P-selectin, siglec-7, TLR3, TLR7 and TLR9 was increased PT0; while CD40, CLEC2, ICAM-2 and MHC-I were decreased, compared to PF. The surface abundance of CD40L, B7-2, DC-SIGN, HCAM, TLR1, TLR2, TLR4 and TLR6 was unchanged by cryopreservation. Following 24 hours of post-thaw storage, all

immune associated receptors and TLRs increased to levels higher than observed on PF and PT0 platelets.

Conclusion

Cryopreservation alters the immune phenotype of platelets. Understanding the clinical implications of the observed changes in BRM release and receptor abundance are essential, as they may influence the likelihood of adverse events.

Keywords: cryopreservation, storage, platelets, immunology, transfusion, adverse events

3.4. Introduction

Platelet cryopreservation presents an attractive option for long-term platelet storage [102, 147]. Cryopreservation can currently extend the platelet shelf-life to 2 years and alters aspects of haemostatic function compared to conventionally stored platelets [110, 147].

The evaluation of cryopreserved platelets has largely focused on changes to the surface phenotype and soluble factor release as they relate to haemostatic function; which changes significantly post-thaw [102, 110, 111]. However, platelets are also significant modulators of the immune system, with multiple roles encompassing pathogen recognition, leukocyte interaction, leukocyte activation and inflammation. A summary of the key roles of platelets in immune function, as well as the receptors and soluble proteins mediating these effects are summarised in Table 3.1 [30, 31, 59, 148-151].

Notably, platelets express most of the identified toll-like receptors (TLRs) including TLR1, 2, 3, 4, 5, 6, 7, 8 and 9 [4, 28, 29, 148, 151, 152]. TLRs enable the recognition of PAMPs and DAMPs, which can induce platelet activation and have pro-inflammatory effects (Table 3.1) [4, 12]. The platelet surface membrane is also host to a range of adhesion (phosphatidylserine, P-selectin, Siglec-7, DC-SIGN, ICAM-2, CLEC2, CD44) and co-stimulatory molecules (CD40, CD86, CD40L, B7-2, MHC-I) which enable platelet-leukocyte interaction (Table 3.1) [59, 128, 151, 152]. Platelet-induced leukocyte stimulation can lead to leukocyte activation, degranulation, the release of pro-inflammatory cytokines, the

generation of neutrophil extracellular traps (NETs) and further platelet activation [4, 31, 56, 59, 151].

Table 3.1. Factors associated with platelet immune function

Platelet Surface Membrane			
Pattern recognition receptors	**Clone**	**Role in platelet mediated immunity**	**References**
TLR1	GD2.F4	• recognize external and internal PAMPs and DAMPs • ligand recognition can lead to generation of a pro-inflammatory platelet phenotype • can recognize HMGB1, CAPs, mitochondrial DNA and histones in the plasma	[2, 12, 28, 29, 31, 149-152]
TLR2	TL2.1		
TLR3	TLR-104		
TLR4	HTA125		
TLR6	TLR6.127		
TLR7	533707		
TLR9	eB72-1665		
Receptors/factors associated with leukocyte interaction	**Clone**	**Role in platelet mediated immunity**	**References**
Phosphatidyl-serine	NA	• anti-inflammatory and immunosuppressive signal • mediates phagocytosis by macrophages	[56, 112]
P-selectin	AC1.2	• facilitates attachment to leukocytes through PSGL-1 • facilitates formation of platelet-leukocyte aggregates • involved in the initial stages of NET formation	[2, 59, 144, 148]
Siglec-7	194211	• mediates platelet apoptosis • may negatively regulate platelet induced inflammation	[2]
CLEC-2	219133	• co-stimulatory molecules • role in inflammation • promote platelet-leukocyte interaction • associated with antigen presentation, leukocyte activation and platelet activation	[2, 148, 149, 151]
ICAM-2	V BP363		
CD40L	24-31		
CD40	5C3		
B7-2	IT2.2		
DC-SIGN	120507		
CD44	VI A034		
Platelet Supernatant			
Cytokines		**Role in platelet mediated immunity**	**References**
PF4		• attracts neutrophils and monocytes • anti-bacterial function	[148, 149, 151]
RANTES		• linked to transfusion associated adverse events • pro-inflammatory • causes leukocyte activation • promote cytokine release from platelet, leukocytes and/or endothelial cells	[2, 11, 23, 149, 151, 153]
IL-1β			
IL-27			
HMGB1			
IL-13			
Endocan			
Complement Factors		**Role in platelet mediated immunity**	**References**
C3a		• pro/anti-inflammatory properties • anti-microbial function	[144]
C4a			
C5a			
Soluble Ligands		**Role in platelet mediated immunity**	**References**
sOX40L		• promotes endothelial inflammation and leukocyte activation	[11, 151]
sCD40L		• pro-inflammatory • platelet and leukocyte activator • facilitates leukocyte differentiation • linked to transfusion associated adverse events	[11, 148-151]

Abbreviations: PAMPs = pathogen associated molecular patterns, DAMPs = damage associated molecular patterns, CAPs = carboxy (alkyl-pyrrole) protein adducts, NA = not applicable, NET = neutrophil extracellular trap

Once activated, platelets release a range of biological response modifiers (BRMs) from internal granules or as a result of internal synthesis including proliferation factors (PF4), chemokines (RANTES), complement (C3a, C4a and C5a) and pro-inflammatory cytokines (IL-1β, OX40L, IL-13, IL-27 and CD40L; Table 3.1) [11, 23, 33, 151]. Importantly, BRMs have been shown to accumulate in platelet components during conventional storage, and these have been linked to an increased risk of adverse transfusion events [11].

This study examined the effects of cryopreservation on a broad range of characteristics related to the immune function of platelets. Specifically, changes in the concentration of soluble factors previously associated with adverse events and the abundance of surface receptors involved in platelet-leukocyte interaction and pro-inflammatory processes were examined.

3.5. Methods

3.5.1 Production of platelet concentrates

Ethics approval was obtained from the Australian Red Cross Lifeblood Ethics Committee prior to commencement of this study (Johnson 10052019). All blood donations were obtained from voluntary non-remunerated donors. Pooled platelet concentrates were generated by combining the buffy coats from four whole blood donations. The platelet components were suspended in 30% plasma/70% platelet additive solution (PAS-IIIM, SSP+; MacoPharma, Mouvaux, France) and leukoreduced using AutoStop BC filters (Haemonetics Corp. Boston, MS, USA) and stored in ELX 1300 mL PVC bags (Haemonetics Corp.) Platelet components were sampled on day 1 (10-15 mL), to establish baseline (pre-freeze) characteristics. Platelet components were stored at room-temperature (20 – 24 °C) on a platelet agitator (Helmer, Inc., Noblesville, IN, USA) until they were frozen.

Platelet components were cryopreserved on day 2 post-collection by addition of 27% (wt/vol) DMSO in 0.9% saline (Sypharma Pty. Ltd., Dandenong South, VIC, Australia) to achieve a final concentration of 5-6%. The platelet concentrate was then transferred to a PVC bag (MacoPharma) and centrifuged at 1350 x *g* for 10 minutes. Following centrifugation, the majority of the DMSO containing supernatant was removed and the pelleted platelets were gently re-suspended in the residual supernatant (~25 mL). The hyperconcentrated platelet component was frozen at -80 °C for an average of 72 ± 6 days (n=8).

Platelets were thawed in a 37 °C water bath for approximately 4 minutes. Once thawed, the components were transferred to a platelet agitator for 15 minutes then reconstituted in approximately 280 mL resuspension solution. A solution of 30% plasma/70% SPP+ was used to reconstitute the platelets, in order to minimise differences between the pre-freeze and post-thaw supernatant. Briefly, 10 whole blood derived plasma components were pooled and aseptically aliquoted in PVC bags and frozen at -30 °C until the day of thawing. Immediately prior to reconstitution, a plasma aliquot (85 mL) was thawed at 37 °C and aseptically combined with SSP+ (195mL, MacoPharma). Approximately 5 mL of the resuspension solution was retained and frozen at -80 °C for later analysis. Reconstituted platelet components were transferred to gas permeable platelet storage bags (ELX 1300 mL, Haemonetics Corp.) and stored for 24 hours on a platelet agitator at room-temperature (Helmer Inc.). Thawed components were sampled (10-15 mL) immediately after resuspension (PT0) and after 24 hours (PT24) of storage.

Platelet counts were obtained using a hematology analyser (CELL DYN Emerald, Abbott Core Laboratory, Abbott Park, IL, USA). Platelets were assessed for aggregates at each time point.

3.5.2 Flow cytometry

The abundance of platelet surface receptors was examined by flow cytometry (FACS Canto II, Becton Dickinson, Franklin Lakes, NJ, USA). Receptor abundance on day 1 was assessed in resting and activated platelets. Platelets were activated with TRAP-6 (10 μM; Sigma-Aldrich, St. Louis, MI, USA) for

10 minutes at 22 °C, as a positive control for degranulation. The abundance of platelet surface receptors was determined by staining 500 000 platelets diluted in Tyrode's buffer for 30 minutes at room-temperature in the dark with the following antibodies: CD40-PE, CD154-APC, CD286-PE, CD86-PE, CD44-APC (BioLegend, San Diego, CA, USA), CD62P-PE (BD Biosciences, Franklin Lakes, NJ, USA), CD328-PE, CD209-APC, MHC-I-PE, CLEC2-APC, TLR7-PE (R&D Systems, Minneapolis, MN, USA), CD281-PE, CD282-PE, CD283-PE, CD284-PE, CD289-PE, CD102-PE (eBioscience, San Diego, CA, USA) or annexin-V-APC diluted in annexin-V binding buffer (Biolegend). Clones for all monoclonal antibodies are listed in Table 3.1. After staining, all samples were diluted in Tyrode's or annexin-V binding buffer (Biolegend) and analysed immediately by flow cytometry. A total of 10, 000 gated platelet events were collected.

3.5.3 Soluble factor analysis

Platelet supernatant was prepared by double centrifugation as previously described [33] and then frozen at -80 °C for later analysis. Supernatant and retained resuspension solution were analysed for the concentration of soluble factors using the following commercially available ELISA kits: C3a, C4a (BD Bioscience), C5a, RANTES, PF4, IL-13, IL-27, IL-1β, OX40L, CD40L (R&D Systems), endocan (Abcam, Cambridge, UK) and HMGB1 (Novus Biologicals, Littleton, CO, USA). All samples were tested in duplicate or triplicate together with a standard curve, as per the manufacturer's instructions.

3.5.4 Statistical analysis

All results are presented as individual data points. Statistical analysis was conducted using GraphPad Prism 7 (ver. 9.0, GraphPad Software, Inc.). Repeated measures one-way analysis of variance (ANOVA) was used to compare differences between all groups, with post hoc Bonferroni's multiple comparisons test performed to identify specific differences. A p-value of less than 0.05 was considered to be significant.

3.6. Results

3.6.1 Platelet count and recovery

The mean platelet concentration at day 1 post-collection was 297 ± 35 x 10^9 cells/unit. Platelet cryopreservation and thawing reduced the platelet count to 248 ± 16 x 10^9 cells/unit, resulting in a mean recovery of 84.1 ± 9.1%. No persistent aggregates were observed in any platelet components following thawing.

3.6.2 Cryopreserved platelets exhibit lower supernatant concentrations of pro-inflammatory cytokines

Platelet components contain a range of BRMs, some of which have been linked to adverse transfusion events [11]. In general, the PT0 supernatant concentration of IL-1β, OX40L, IL-13, IL-27 and CD40L was two to four-fold lower than in PF platelets (Figure 3.1A-E). Additionally, endocan and HMGB1 concentrations were similar before and after freezing (PT0, Figure 3.1F-G). In contrast, RANTES and PF4 were three to four-fold higher in thawed platelets compared to PF platelets (Figure 3.1H-I). The supernatant concentration of IL-1β, OX40L, IL-13, IL-27, endocan and HMGB1 remained stable during 24 hours of post-thaw storage (PT24), while CD40L, RANTES and PF4 were increased compared to PT0 (Figure 3.1). Generally, the concentration of BRMs in the resuspension solution (RS) was very low (Figure 3.1). The exceptions were OX40L and HMGB1, which were similar in concentration in the RS and the PT0 and PT24 supernatants (Figure 3.1B&G), suggesting cryopreservation did not alter the supernatant concentration of these factors. Overall, cryopreservation differentially altered the composition of BRMs in the supernatant.

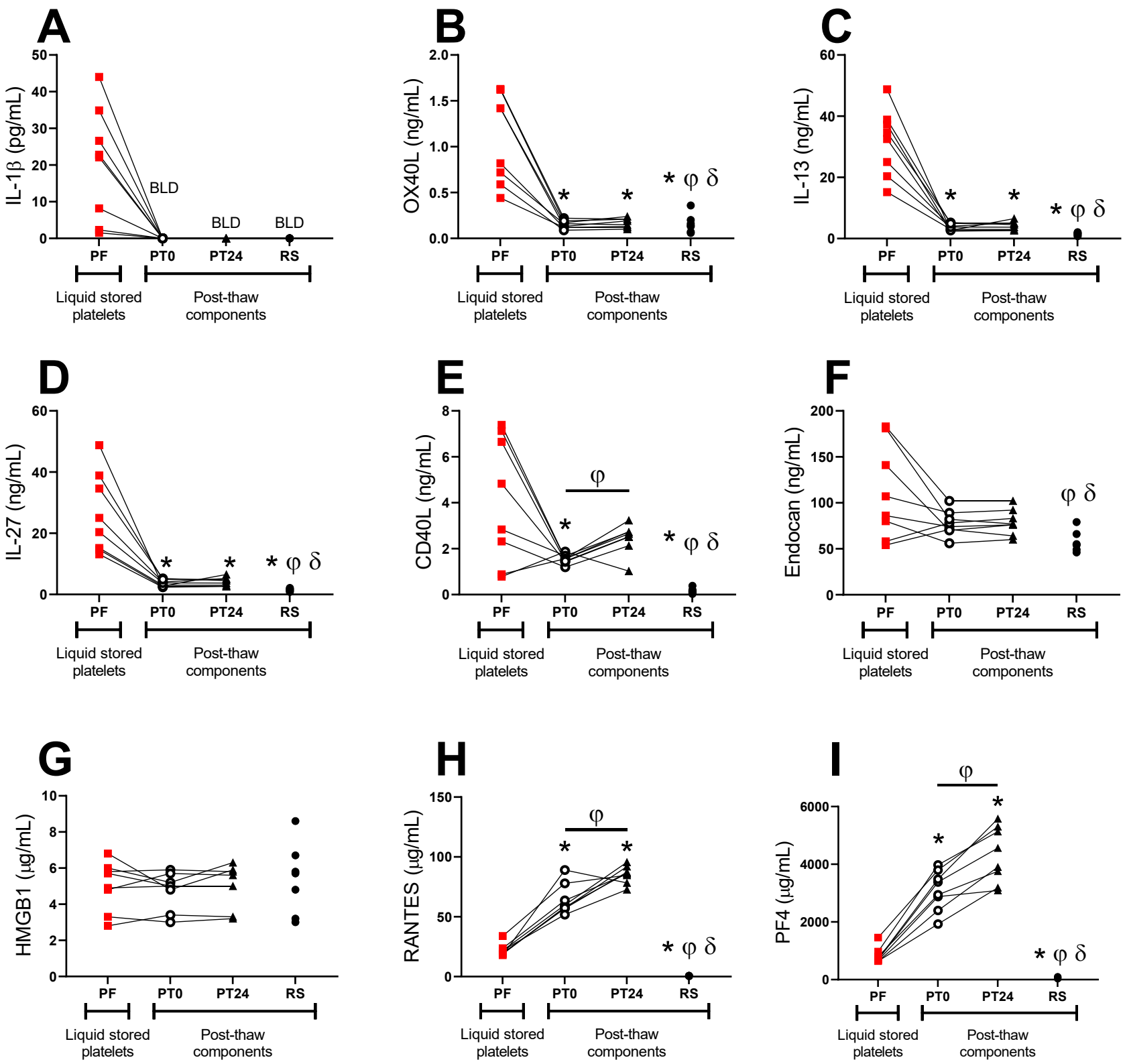

Figure 3-1. Cryopreservation alters the concentration of soluble factors in the supernatant of platelet components. Supernatant was collected pre-freeze (PF, □), immediately post-thaw and resuspension (PT0, ○), 24 hours post-thaw (PT24, Δ) and from the resuspension solution (RS, ●). The concentrations of (A) IL-1β, (B) OX40L, (C) IL-13, (D) IL-27, (E) CD40L, (F) Endocan, (G) HMGB1, (H) RANTES and (I) PF4 were measured by ELISA. Data are presented as individual data points (n=8). Significance was determined by one-way ANOVA with Bonferroni post hoc comparisons. * = $p<0.05$ compared to PF, ϕ = $p<0.05$ compared to PT0, δ = $p<0.05$ compared to PT24. BLD = values were below the limit of detection.

Platelets store complement factors internally in granules, which are released upon activation [130]. The supernatant of PT0 platelets contained higher concentrations of C3a, similar amounts of C4a and a lower concentration of C5a than pre-freeze components (Figure 3.2). However, the supernatant concentration of the complement proteins did not change significantly during post-thaw storage. The concentration of C3a and C4a in the resuspension solution was lower than the PT0 time points (Figure 3.2A-B). However, the concentration of C5a in the resuspension solution was higher than in PT0 and PT24 platelets (Figure 3.2C), indicating a loss of C5a from the supernatant as a result of platelet thawing and reconstitution. Overall, cryopreservation differentially affected the concentration of complement factors in the supernatant.

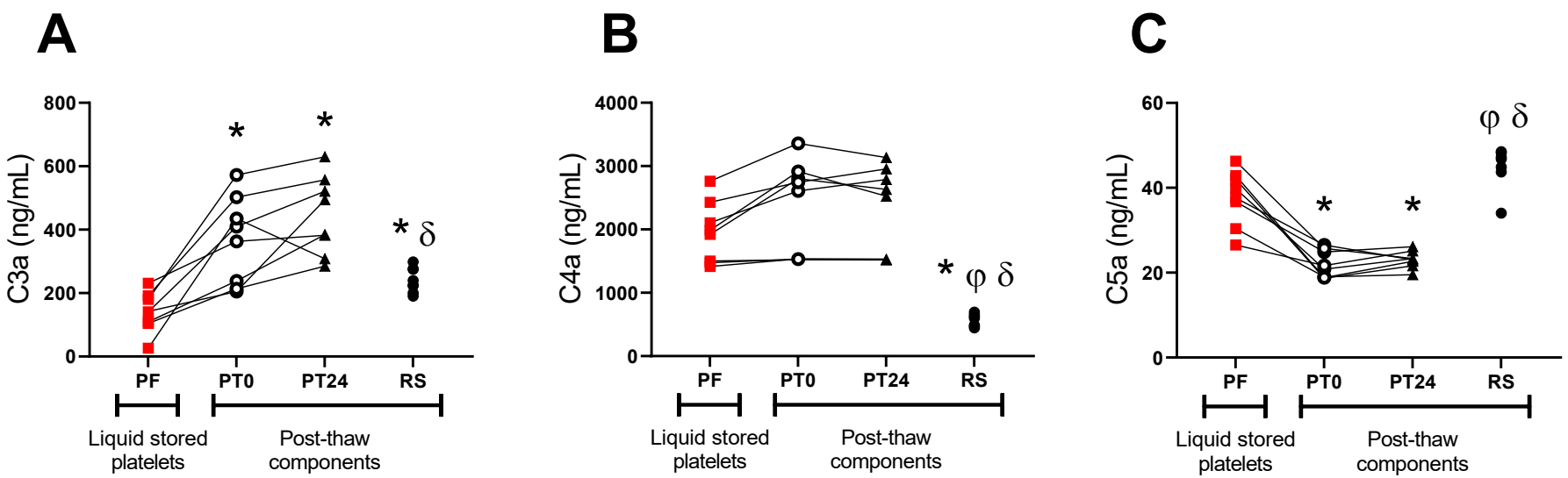

Figure 3-2. Cryopreservation alters the concentration of complement factors in the supernatant of platelet components. Supernatant was collected pre-freeze (PF, □), immediately post-thaw and resuspension (PT0, ○), 24 hours post-thaw (PT24, Δ) and from the resuspension solution (RS, ●). The concentrations of (A) C3a, (B) C4a and (C) C5a were measured by ELISA. Data are presented as individual data points (n=8). Significance was determined by one-way ANOVA with Bonferroni *post hoc* comparisons. * = $p<0.05$ compared to PF, ϕ = $p<0.05$ compared to PT0, δ = $p<0.05$ compared to PT24.

3.6.3 Platelet cryopreservation alters leukocyte associated receptor abundance on the surface membrane

The platelet surface membrane houses a range of receptors that facilitate platelet-leukocyte attachment and signalling [59]. Further, platelets are known to contain significant stores of haemostatic and immune associated receptors internally, which can be mobilised to the surface membrane following activation [28, 130, 152]. TRAP-6 was used as a positive control for receptor mobilisation from internal granules [154]. TRAP-6 stimulation resulted in a significant increase in the surface abundance of all receptors examined, compared to pre-freeze platelets (Figure 3.3), although the increase in DC-SIGN was not statistically significant (Figure 3.3J). While DC-SIGN was more modestly increased by TRAP-6 stimulation, the effect of cryopreservation on this marker was still of interest, due to its key role in platelet immune function [151]. Following thawing, phosphatidylserine exposure, P-selectin and siglec-7 were significantly increased compared to pre-freeze (Figure 3.3A-C). However, only phosphatidylserine exposure was significantly increased compared to TRAP-6-stimulated platelets. In contrast, the abundance of CD40, CLEC2, ICAM-2 and MHC-I was significantly lower at PT0 compared to both pre-freeze and TRAP-6 stimulated platelets, while CD40L, CD86, DC-SIGN and HCAM were similar to pre-freeze platelets (Figure 3.3D-K). Notably, at PT24 the abundance of all receptors increased compared to PT0 (Figure 3.3B-K), with the exception of phosphatidylserine, which decreased significantly (Figure 3.3A). In general, the abundance of immune receptors was variably affected by cryopreservation, but the abundance

of all receptors and activation markers examined, with the exception of phosphatidylserine was increased following post-thaw storage.

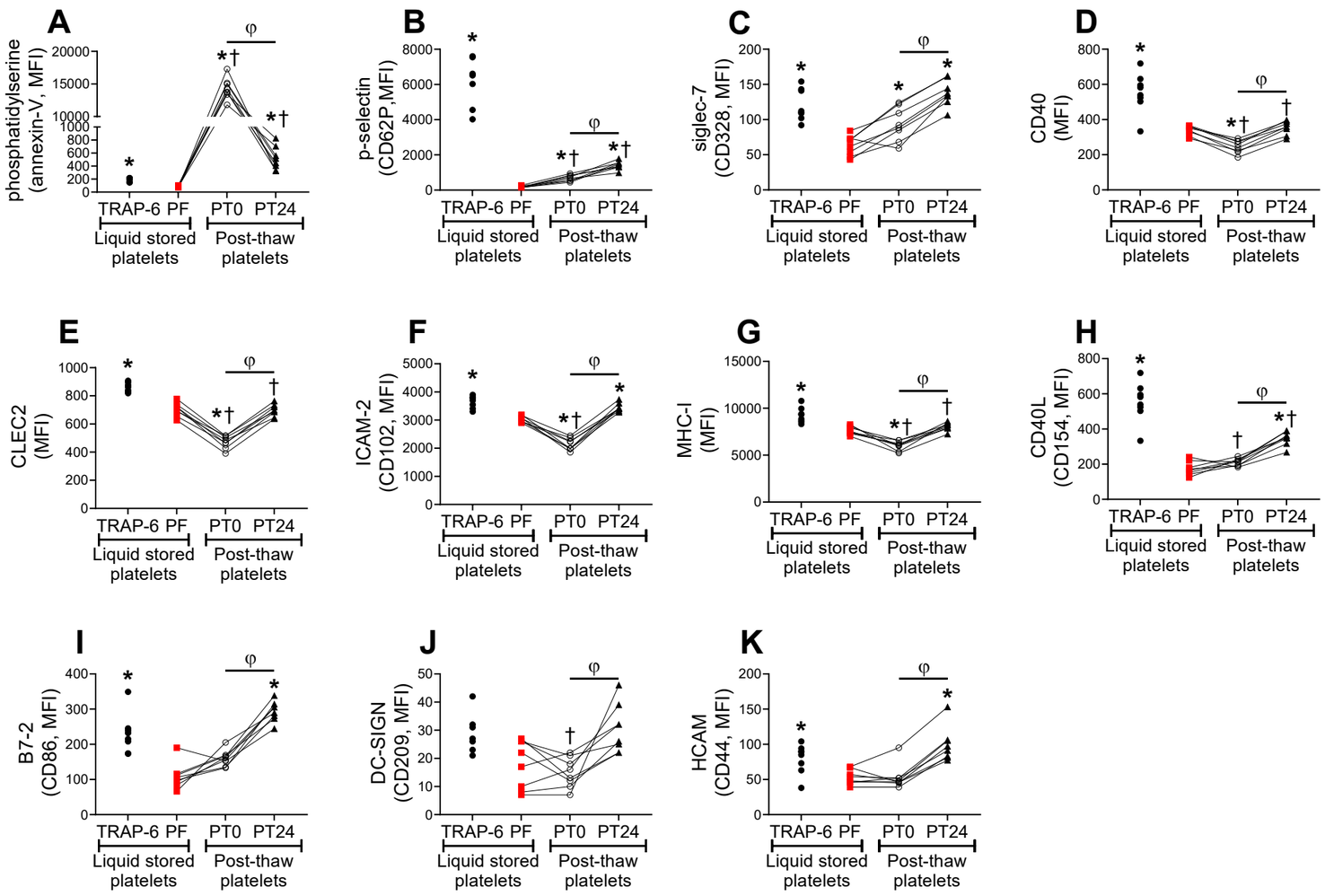

Figure 3-3. Cryopreservation alters the abundance of receptors associated with leukocyte attachment and signalling. Receptor expression was measured in platelets which were TRAP-6 stimulated (TRAP-6, ●), resting (PF, □), immediately post-thaw and resuspension (PT0, ○) and after 24 hours of storage (PT24, ∆). Platelets were stained with (A) annexin-V-APC, (B) CD62P-PE, (C) CD328-APC, (D) CD40-PE, (E) CLEC2-APC, (F) CD102-PE, (G) MHC-I-PE (H) CD154-APC, (I) CD86-PE, (J) CD209-APC and (K) CD44-APC. The median fluorescence intensity (MFI) was measured by flow cytometry following the collection of 10 000 platelet events. Data are presented as individual data points (n=8). Significance was determined by one-way ANOVA with Bonferroni post hoc comparisons. * = $p<0.05$ compared to PF; ϕ = $p<0.05$ compared to PT0, † = $p<0.05$ compared to TRAP-6.

3.6.4 Platelet cryopreservation alters the abundance of Toll-like receptors on the surface membrane

Platelets express most identified TLRs on their surface membrane, including TLR3, TLR7 and TLR9, which are typically expressed internally in nucleated cells [4, 28, 29]. Prior to freezing, platelets exhibited measurable surface expression of all TLRs (Figure 3.4). TRAP-6 stimulation significantly increased the surface abundance of TLR1, TLR2, TLR4, TLR6 and TLR3 by two-fold compared to pre-freeze (Figure 3.4A-E), whereas surface TLR7 and TLR9 abundance was unchanged (Figure 3.4F-G). The abundance of TLR1, TLR2, TLR4 and TLR6 at PT0 was similar to pre-freeze (Figure 3.4A-D). In comparison, cryopreservation increased the abundance of TLR3, TLR7 and TLR9 on the surface membrane compared to pre-freeze platelets (Figure 3.4E-G). At PT24, the membrane abundance of all TLRs was significantly higher than PT0, at levels comparable to or exceeding TRAP-6-stimulated platelets (Figure 3.4). Overall, only internally expressed TLRs (TLR3, TLR7, and TLR9) increased in abundance immediately post-thaw; whereas the abundance of all TLRs increased during post-thaw storage

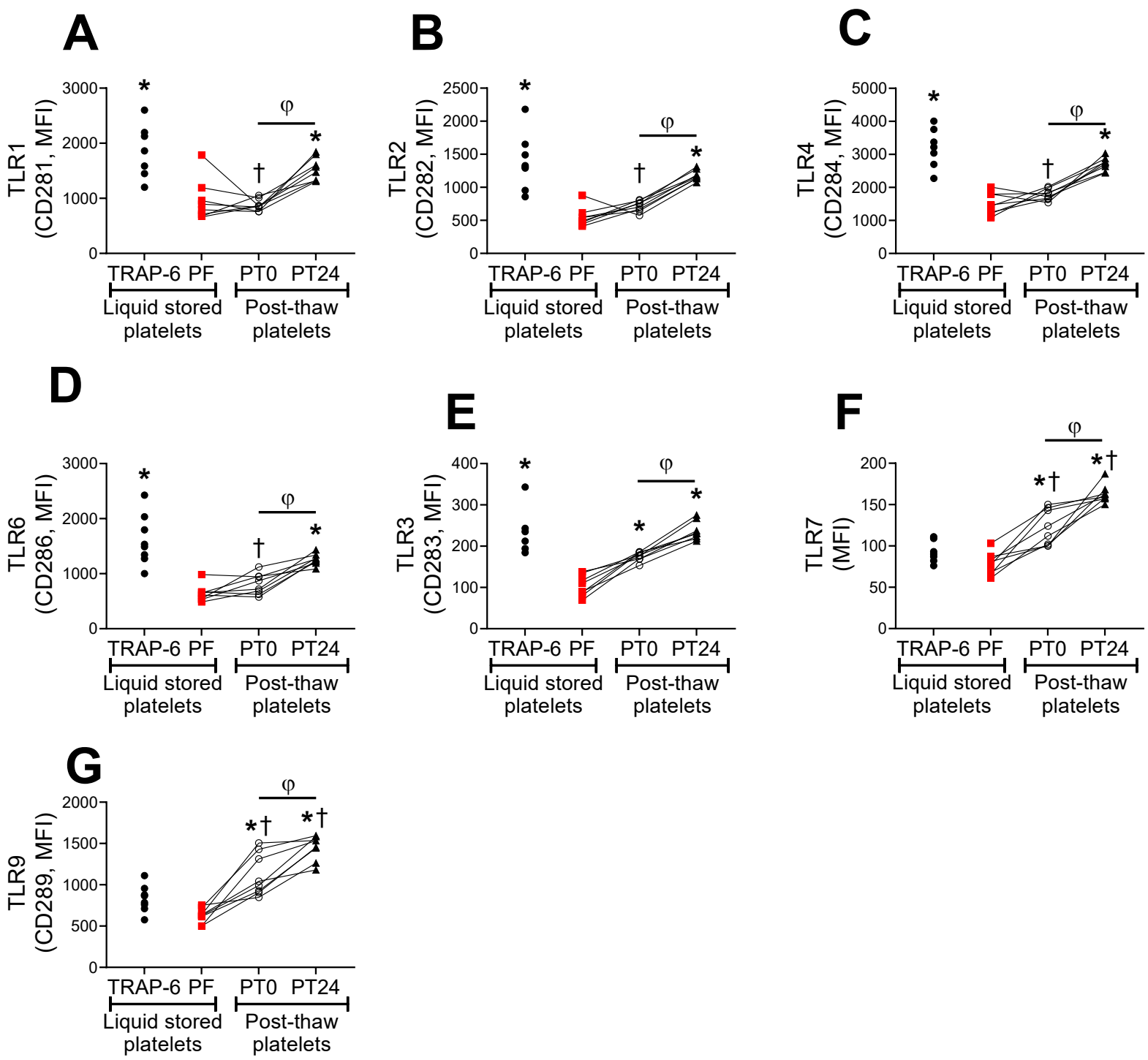

Figure 3-4. Cryopreservation alters the abundance of toll-like receptors (TLRs). Receptor expression was measured in platelets which were TRAP-6 stimulated (TRAP-6, ●), resting (PF, □), immediately post-thaw and resuspension (PT0, ○) and after 24 hours of storage (PT24, Δ). Platelets were stained with (A) CD281-PE, (B) CD282-PE, (C) CD284-PE, (D) CD286-PE, (E) CD283-PE, (F) TLR7-PE or (G) CD289-PE. The median fluorescence intensity (MFI) was measured by flow cytometry following the collection of 10 000 platelet events. Data are presented as the individual data points (n=8). Significance was determined by one-way ANOVA with Bonferroni post hoc comparisons. * = $p<0.05$ compared to PF; ϕ = $p<0.05$ compared to PT0, † = $p<0.05$ compared to TRAP-6.

3.7. Discussion

Cryopreservation dramatically changes the haemostatic capacity of platelets [110, 111]; however, little is known regarding the impact on the immune phenotype. This information is crucial to the clinical evaluation of cryopreserved platelets, as storage-induced alterations in platelet immune function have been linked to the incidence of adverse events [11]. In this study, it was demonstrated that cryopreservation significantly changes the immune phenotype of platelets. Specifically, cryopreserved platelets exhibit alterations in both the concentration of BRMs in the supernatant and the abundance of surface receptors involved with leukocyte interaction and PAMP/DAMP recognition.

Previous studies have demonstrated that cryopreserved platelets exhibit increased abundance of activation markers (phosphatidylserine and P-selectin), accompanied by degranulation and an elevated concentration of specific BRMs in the supernatant [33, 104, 113, 155]. Our study supports these previous findings and expands the repertoire of BRMs to include pro-inflammatory proteins. Additionally, our data suggests that different mechanisms mediate the changes in concentration of BRMs in the supernatant of cryopreserved platelet components.

Platelets store a range of soluble factors, TLRs and immune associated receptors internally in α-granules [33, 130, 154]. In this study, cryopreserved platelets exhibited a significant release of key α-granule BRMs (RANTES, PF4, C3a) and increased abundance of the receptors P-selectin and siglec-7 on the surface membrane. In contrast, the abundance of most immune associated receptors,

TLRs and the concentration of other α-granule associated BRMs (OX40L, IL-13, IL-27, CD40L and HMGB1) was initially either unchanged or decreased. Our data suggests that cryopreservation induces the release of specific BRMs and receptors, but not others, as a result of platelet degranulation or potentially, thaw-induced damage or lysis of the cellular membrane. Additionally, it has been suggested that α-granules may be packaged heterogeneously and/or granule cargo may be released differentially [156, 157]. Further, differential release of complement factors has been observed previously in conventionally stored platelet components [158].

BRMs released into the supernatant are capable of binding to their cognate receptors on the surface of platelets and extracellular vesicles [144]. This may account for the observed decrease in C5a concentration in the post-thaw supernatant, relative to the resuspension solution. Complement proteins are known to bind to the surface of activated platelets through complement receptors, P-selectin and the surface of platelet-derived extracellular vesicles, which are all increased following cryopreservation [110, 144, 159]. Additionally, HMGB1 was equivalent in post-thaw platelets and the resuspension solution. HMGB1 is reportedly stored in α-granules [160] and when released can bind to a range of surface receptors (TLR2, TLR4, TLR9 and RAGE) [160], and anionic lipids (such as phosphatidylserine) [161]. While these cognate receptors are significantly increased on the platelet membrane post-thaw, it is currently unclear whether HMGB1 is being selectively retained within the platelets following cryopreservation, or whether it is released, but then subsequently binds to the surface membrane.

Despite increased degranulation, the concentration of many of the BRMs examined post-thaw was significantly lower than in conventionally stored platelet components. This can largely be attributed to the process of manufacturing cryopreserved platelets, rather than dilution. In preparation for freezing, the supernatant of the platelet is removed and then replaced with fresh plasma or another solution at the time of thawing [103, 104], effectively washing the component. This results in the removal of most metabolic by-products and soluble factors that may be released from platelets during collection, processing and pre-freeze storage [11, 162, 163]. Given that the resuspension solution was the same composition as the pre-freeze samples, the data suggests that cryopreservation results in minimal release of several of the inflammatory BRMs examined. Notably, IL-1β, OX40L, IL-13, IL-27 and CD40L have previously been strongly associated with adverse reactions [11]. As such, a lower concentration of these particular mediators in the supernatant may be advantageous, particularly to minimise the risk of TRALI in critically injured patients undergoing massive transfusion [164]. However, other BRMs, including RANTES, are present in higher concentrations following thawing, which have also been linked to adverse events [11]. Consequently, it remains difficult to speculate on the overall immunogenicity of the cryopreserved platelet component without the collection of further data from appropriately designed clinical studies.

Cryopreservation is known to induce significant changes in the profile of key haemostatic surface receptors (GPIbα and GPVI) [107, 111, 165], and this study suggests a similar effect on the abundance of immune associated receptors. Cryopreservation results in a decreased abundance of several leukocyte

associated co-stimulatory (CD40 and MHC-I) and adhesion receptors (CLEC2 and ICAM-2). In contrast, large increases in P-selectin, siglec-7, and phosphatidylserine were observed, which also mediate platelet-leukocyte adhesion, DAMP recognition and platelet immune function [31, 56, 59, 152]. The abundance of P-selectin on the surface membrane is required for platelet attachment to neutrophils, monocytes and lymphocytes [128]. However, the formation of platelet-leukocyte aggregates is a multistep process requiring initial binding through P-selectin, followed by stabilisation by GPIbα, which is decreased on approximately 50% of platelets following thawing [56, 107, 111, 128]. Consequently, normal leukocyte binding may only be possible in a limited proportion of cryopreserved platelets. Signalling through siglec-7 in platelets can lead to apoptosis [153]; thus the higher surface abundance on cryopreserved platelets could increase their immunomodulatory potential and possibly the incidence of adverse events. Further, platelets with exposed phosphatidylserine on the surface membrane can interact with leukocytes, particularly macrophages, which facilitates clearance from the circulation [56, 166]. To date few studies have examined the effects of post-thaw platelet interaction with leukocytes and both immunosuppressive and pro-inflammatory effects have been reported [56, 115], illustrating the need for further work in this area.

Platelets are unique in that they are capable of expressing internal TLRs (TLR3, TLR7 and TLR9) on the surface membrane, which are typically used to detect internal pathogens in nucleated cells [29, 152]. TLR3 and TLR7 are thought to be stored in either α-granules or endosomes [152, 167, 168]. In contrast, while TLR9 is typically located in the endosomes of nucleated cells [169], it has been

proposed that TLR9 may be contained within platelet specific T-granules, which reside close to the surface membrane [28]. Notably, surface TLR7 and TLR9 were increased following cryopreservation, but not on TRAP-6 stimulated platelets, suggesting the release of endosomes or potentially T-granules may be due to cryopreservation-specific platelet activation. TLR3, TLR7 and TLR9 are capable of recognising a range of DAMPs released from damaged tissue including mRNA, CpG DNA, HMGB1 and carboxy (alkyl-pyrrole) protein adducts (CAPs) [152, 170, 171], which may generate a pro-inflammatory response in platelets [152, 171]. A higher abundance of TLRs on the surface membrane may increase DAMP recognition in cryopreserved platelets, but it is currently unclear if this interaction would be advantageous or detrimental. DAMP recognition by platelets can lead to leukocyte recruitment, which has been associated with inflammation and wound healing [31]. It is important to understand the implications of this and whether this interaction would be beneficial or detrimental, given that cryopreserved platelets would largely be used for the treatment of acute bleeding in trauma and during surgery [15, 100, 102], which have independently been associated with greater DAMP release [31, 172, 173]. Further, it would be useful to examine if TLRs present on cryopreserved platelets are capable of ligand binding and signal transduction, as this has been linked to platelet activation and the formation of platelet-leukocyte aggregates and NETs [4].

This study has presented a broad screen of largely unexamined *in vitro* immune characteristics of cryopreserved platelets and highlights the need for further work in this area. Research focusing on the interaction between leukocytes and both

the platelets and the accompanying releasate present in cryopreserved components is required to determine if the observed changes influence their immune function and potential to induce adverse transfusion events. This information is crucial as several BRMs including C3a and EVs are known to increase following cryopreservation and have the potential to be immunomodulatory [110, 144, 174]. Notably, extracellular vesicles, have been shown to induce TRALI in murine models through ceramide delivery [174]. Additionally, it remains to be determined whether cryopreserved platelets can induce the generation of pro-inflammatory platelet-leukocyte aggregates and if so, which receptors mediate this interaction (i.e. phosphatidylserine or P-selectin). Finally, cryopreserved platelets will likely be used for the treatment of bleeding associated with trauma [100, 102], which carries a significant risk of post-operative infection [175]. Therefore, investigations as to whether cryopreserved platelets are immunosuppressive or possess the anti-pathogenic capacity of conventional platelets are required.

The main advantage of cryopreservation is the extension of platelet shelf-life when frozen [147], ensuring components are available for transfusion in remote medical centres while minimising wastage. However, further utility may be gained if the post-thaw shelf-life could be extended beyond the current 4-6 hours [176-178]. In general, extended storage of post-thaw platelets only slightly reduces haemostatic function *in vitro* [104, 176]. However, our data suggests a much more significant change in the immune characteristics of post-thaw platelets, which has the potential to substantially alter the immunogenicity of the component in a currently unknown way. Consequently, prior to any extension of post-thaw shelf-

life, more data regarding the immune function of cryopreserved platelets is required. Additionally, mitigation strategies that minimise changes in post-thaw platelet phenotype, such as refrigeration [176], may be beneficial.

The data presented in this study highlights that changes in the immune characteristics of platelets should be a key consideration when evaluating novel *ex vivo* storage methods. At this stage, post-hoc analysis of the adverse event and transfusion reaction data generated from clinical trials of cryopreserved platelets that are currently underway, such as CLIP-II (NCT03991481) and CRYPTICS (NCT04709705), may provide insight into potential differences in immune function of cryopreserved platelets.

4. The phenotype of cryopreserved platelets influences the formation of platelet-leukocyte aggregates in an *in vitro* model

4.1. Foreword

Previous work suggests that cryopreservation induced activation results in the generation of phenotypically heterogenous platelet subpopulations. Platelet subpopulations may exhibit an altered capacity to bind to leukocytes and form PLAs, which can be immunomodulatory, facilitating both pro- and anti-inflammatory signalling and platelet clearance. As such, it was hypothesised that cryopreservation may alter the likelihood of PLA formation in a model *in vitro* system, using monocyte-like cells (THP-1 cells). Cryopreserved platelet components were composed of heterogenous subpopulations, post-thaw. Further, the likelihood of PLA formation was linked to the formation of specific platelet subpopulations and the exposure of P-selectin.

4.1.1 Contribution to thesis aims

This chapter addresses **Aim 3**.

Aim 3: Determine the effect of storage on platelet-leukocyte and platelet-bacteria interactions.

Conclusion: Cryopreserved platelets more readily form aggregates with a monocyte-like cell line (THP-1 cells) than conventionally stored platelets. P-selectin exposure on the surface membrane is linked to the formation of cryopreserved platelet-THP-1 cell aggregates.

4.2. Statement of authorship

The following section is an original manuscript which was published in *Platelets* in 2023. Minor edits have been made to the formatting and wording of the manuscript to make it consistent with the rest of the Thesis. The supplementary Figures 1-3S can be found in the Appendices.

Winskel-Wood B, Padula MP, Marks DC and Johnson L. The phenotype of cryopreserved platelets influences the formation of platelet-leukocyte aggregates in an *in vitro* model. Platelets. 2023; In press.
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Ben Wood, Lacey Johnson and Denese Marks conceived and designed the study. Ben Wood conducted the lab work and prepared the figures. Ben Wood and Lacey Johnson analysed the data and wrote the manuscript. Ben Wood, Matt Padula, Lacey Johnson and Denese Marks critically reviewed the manuscript.

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26/04/2023
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A/Prof Denese C. Marks

4.3. Abstract

Cryopreservation significantly alters the phenotype of platelets; generating distinct subpopulations, which may influence the formation of platelet leukocyte aggregates (PLA). PLAs are immunomodulatory and have been associated with transfusion-associated adverse events. As such, the aim of this study was to examine the effect of cryopreservation on the ability of platelets to form PLAs, using a monocyte-like cell line (THP-1). Platelets were tested pre-freeze, post-thaw and following stimulation with TRAP-6 or A23187, both alone and following co-culture with THP-1 cells for 1 and 24 hours (n=6). Platelet subpopulations and platelet-THP-1 cell aggregates were analysed using multi-color imaging flow cytometry using Apotracker Green (ApoT), CD42b, CD62P, CD61, and CD45. Cryopreservation resulted in the generation of activated (ApoT-/CD42b+/CD62P+), procoagulant (ApoT+/CD42b+/CD62P+) and a novel (ApoT+/CD42b+/CD62P-) platelet subpopulation. Co-incubation of cryopreserved platelets with THP-1 cells increased PLA formation compared to pre-freeze but not TRAP-6 or A23187 stimulated platelets. P-selectin on the surface membrane was correlated with increased PLA formation. Our findings demonstrate that cryopreservation increased the interaction between platelets and THP-1 cells, largely due to an increase in procoagulant platelets. Further investigation is required to determine the immunological consequences of this interaction.

Keywords: platelets, cryopreservation, THP-1, immunology, leukocytes

4.4. Introduction

Platelet transfusion is commonly used to promote haemostasis in patients presenting with acute bleeding. Currently, platelet components are stored at room-temperature (20-24 °C), limiting the shelf-life to 5-7 days due to the risk of bacterial proliferation and a gradual reduction in haemostatic effectiveness [67, 72]. Consequently, maintaining inventories in remote medical centres is difficult. This has prompted the evaluation of alternative storage methodologies, including platelet cryopreservation [15, 102, 179]. Cryopreservation extends component shelf-life to at least two years and enhances haemostatic parameters *in vitro*, addressing the primary limitations of room-temperature storage [110, 180]. The effect of cryopreservation on the haemostatic characteristics of platelets is well documented, and includes the increased exposure of activation makers (phosphatidylserine, P-selectin), decreased surface abundance of selected glycoproteins (GPVI, GPIbα) and an enhanced capacity to generate thrombin [15, 102, 108, 110, 179, 180]. However, less information is available regarding the impact of cryopreservation on the immune characteristics of platelets [33, 56, 181].

Platelets can interact with the immune system directly through receptors on the surface membrane and the release of soluble factors [11, 182]. Typically, platelet immune function is beneficial, however, dysregulation can contribute to a range of pro-inflammatory disorders and transfusion-associated adverse reactions [11, 183]. The cause of adverse transfusion events is multifactorial [183]. However, a strong link has been established between severe adverse events such as

Transfusion Related Acute Lung Injury (TRALI), storage-induced platelet activation, and the formation of platelet-leukocyte aggregates (PLAs) [11, 182].

The formation of PLAs is a multistep process, first requiring platelet activation and degranulation, exposing P-selectin on the surface membrane [3, 184]. P-selectin can then bind to leukocyte PSGL-1, which triggers leukocyte activation and the expression of $\alpha_M\beta_2$ (Mac-1) receptor on the surface membrane of leukocytes [184, 185]. Activated Mac-1 can bind platelet GPIbα stabilising the PLAs [184]. Further, the expression of platelet surface receptors such as CD40L, GPVI, GPIIb/IIIa, or phosphatidylserine can enhance adhesion or provide co-stimulatory signals to adherent leukocytes [184, 185]. PLA formation can modulate downstream immune function, promoting pro- or anti-inflammatory signalling, and platelet clearance by phagocytosis [64, 184, 186]. Notably, the likelihood of PLA formation and functional consequences can be influenced by the phenotype of the adherent platelet [187].

Once thought to be largely homogenous, distinct subpopulations of platelets have been described based on morphology, phenotype, and functional characteristics [31, 187, 188]. The current literature describes resting, aggregatory, procoagulant and apoptotic platelet subpopulations [187-191]. Platelet subpopulations were originally investigated to understand their distinct roles in haemostasis and thrombus formation [192]. However, recent work has established links between platelet subpopulations and the formation of PLAs associated with inflammatory disorders [186]. Recent examinations have highlighted that cryopreservation-induced platelet activation does not occur uniformly, and that the majority of cryopreserved platelets are phenotypically aligned with a

procoagulant subpopulation [188]. The selective formation of this subpopulation may impact the ability of platelets to bind to leukocytes and form PLAs.

The examination of PLAs has been made more accessible through imaging flow cytometry, which combines the high throughput benefits of multi-color flow cytometry with the imaging capabilities of microscopy [31]. THP-1 cells are commonly used to model platelet-leukocyte interactions *in vitro*, including previous work which has examined co-culture with cryopreserved platelets and the effect on phagocytosis and pro-inflammatory signalling [56]. In this study, imaging flow cytometry was used to examine the interaction of cryopreserved platelets with monocyte-like cells (THP-1 cell line) *in vitro*.

4.5. Methods

4.5.1 Platelet collection and processing

Ethics approval was obtained from the Australian Red Cross Lifeblood Ethics Committee prior to commencement of this study (Johnson 10052019). All blood donations were obtained from voluntary non-remunerated donors. Pooled platelet concentrates were manufactured by combining the buffy coats from four whole blood donations. Following centrifugation, the platelets and remaining plasma were diluted with platelet additive solution (PAS-E, SSP+; Macopharma, Tourcoing, France) to achieve a final supernatant composition of 30% plasma / 70% PAS-E and leukoreduced using AutoStop BC filters and stored in ELX 1300 mL PVC bags (Haemonetics, Boston, USA). Platelet components were stored at room temperature (20 – 24 °C) on a platelet agitator (Helmer, Noblesville, USA) until they were frozen. Samples were removed on day 2 (10-15 mL), to establish baseline (pre-freeze, PF) characteristics.

4.5.2 Platelet cryopreservation and thawing

Platelet components were cryopreserved within 24 hours of sampling at day 2-3 post-collection by addition of 27% (wt/vol) DMSO in 0.9% saline (Sypharma, Dandenong, Australia) to achieve a final concentration of 5-6%. The platelet concentrate was then transferred to a PVC bag (Macopharma) and centrifuged at 1350 *g* for 10 minutes. Following centrifugation, the majority of the DMSO containing supernatant was removed and the pelleted platelets gently resuspended in the residual supernatant (~25 mL) before being frozen at -80 °C.

Platelets were thawed in a 37 °C water bath for approximately 4 minutes. Once thawed, the components were transferred to a platelet agitator for 20 minutes then reconstituted in approximately 280 mL resuspension solution. A solution of 30% plasma / 70% SPP+ was used to reconstitute the platelets to minimize differences between the PF and post-thaw (PT) supernatant, as detailed previously [181]. Thawed components were sampled (10-15 mL) immediately after resuspension.

4.5.3 Cell culture

THP-1 cells (TIB-202, passage 6, American Type Culture Collection; ATCC) were cultured in Complete Media consisting of RPMI 1640 (Thermo Fisher Scientific, Waltham, USA) without phenol red, containing 10% foetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin (both from Thermo Fisher Scientific). A new vial of THP-1 cells (frozen at passage 9) was thawed for each replicate and cultured at a concentration between 2-8 x 10^5 cells/mL in an incubator (37 °C, 5% CO_2, Thermo Fisher Scientific). The media was refreshed, and THP-1 cell viability was assessed by cell counter (Countess 2, Thermo Fisher Scientific) every 2-3 days until expanded sufficiently for testing. THP-1 cells were then concentrated by centrifugation at 125 *g* for 5 minutes before resuspension at a concentration of 1 x 10^6 cells / mL in fresh Complete Media containing acid citrate dextrose solution (ACD, 10 mM trisodium citrate, 11 mM glucose, 8 mM citric acid). THP-1 cells were tested prior to co-incubation with platelet concentrates to ensure a consistent phenotype (Figure 1S).

4.5.4 Platelet co-incubation with THP-1 cells

Resting PF platelets were activated by 10 μM TRAP-6 or 5 μM calcium ionophore A23187 (both from Sigma-Aldrich, St. Louis, USA) for 20 minutes at 37 °C [181, 193]. Platelet stimulation using TRAP-6 or A23187 has previously been used to generate subpopulations of aggregatory and procoagulant platelets respectively [181, 187, 188, 193, 194]. In this study, TRAP-6 and A23187 stimulated platelets were used to assist the classification of cryopreserved platelets into subpopulations. THP-1 cells were analysed as a platelet free control or co-incubated with 5% (v/v) of PF, PT, TRAP-6 stimulated or A23187 stimulated platelet concentrates in 12 well culture plates (Corning, New York, USA) in an incubator (37 °C / 5% CO_2). Separate wells were co-incubated for either 1 or 24 hours. Following co-incubation, the supernatant was transferred by pipette to 5 mL tubes. Any adherent THP-1 cells were removed by incubation with 1 x TrypLE Express (no phenol red, Thermo Fisher Scientific) for 7 minutes in an incubator before it was combined with the corresponding sample tube containing non-adherent THP-1 cells.

4.5.5 Staining of platelet concentrates and platelet-THP-1 cell co-culture for analysis by imaging flow cytometry

To assess the platelet phenotype, independent of co-culture, approximately 2 x 10^6 platelets were diluted in 0.1μm filtered Tyrode's buffer and stained with Apotracker (ApoT)-Green (Biolegend, San Diego, USA), CD42b-PE-Dazzle 594 (HIP-1; Biolegend), CD62P-PE (AC1.2; BD Bioscience, Franklin Lakes, USA), CD61-APC (Y2/51; Dako, Glostrup, Denmark) for 15 minutes. ApoT is a calcium independent stain similar to Annexin-V, which is used to detect

phosphatidylserine exposure [195]. The presence of the calcium chelator ACD in the co-culture made staining with Annexin-V impractical.

To examine the phenotype of platelets adhered to THP-1 cells, the co-culture was stained with ApoT-Green, CD42b-PE-Dazzle 594 (HIP-1), CD62P-PE (AC1.2), CD45-APC-H7 (2D1, BD Bioscience) and CD61-APC (Y2/51, Dako). THP-1 cell phenotype were assessed by staining with CD11b-FITC (ICRF44), CD11a/CD18-APC (m24), CD45-APC-H7 (2D1, BD Bioscience), CD162-PE (KPL-1) and CD61-PE-Dazzle 594 (VI-PL2, Biolegend). Samples were incubated for 20 minutes in the dark prior to fixation with 1% paraformaldehyde for 20 minutes at 4 °C. Samples were concentrated by centrifugation at 125 *g* for 5 minutes after which the supernatant was removed and the pellet resuspended in 50 µL sterile filtered PBS (Sigma-Aldrich).

Platelets and THP-1 cell / platelet co-cultures were analysed using an Amnis Imagestream^X Mark II multi-spectral imaging flow cytometer (IS^x, EMD Millipore, Seattle, USA) equipped with one charge coupled device (CCD) camera and two excitation (488nm: 100 mW, 642nm: 150mW) lasers and a side scatter (785 nm: 2mW) laser. Laser voltages were set to maximize fluorescent signal while avoiding oversaturation. Data were acquired using INSPIRE acquisition software based on area, aspect ratio and CD61 or CD45 positivity to identify platelets and THP-1 cells, respectively. Data collection gates were set by using the relevant unstained and fluorescence minus one (FMO) controls. Single stained controls were employed to generate compensation matrices. Data were collected at a low flow rate with the camera set to 60x magnification with 10 000 platelet or THP-1 cell events collected.

All imaging flow cytometry data was analysed in IDEAS v6.2 (Luminex Corporation, Austin, USA). Fluorescence minus one (FMO) controls were employed to refine gates and exclude false positive events. The thresholds for intensity masks were set with reference to unstained, positive and FMO controls. Individual platelets and THP-1 cells were identified based on area and aspect ratio in conjunction with the cell specific markers (Figure 4.1A-B). Masks and the feature finder were then used to identify platelet subpopulations and platelet-THP-1 cell aggregates. A custom platelet mask based on CD61-APC fluorescence data was combined with the Spot Count feature to enumerate the number of platelets bound to each THP-1 cell (Figure 4.1C-D). Coincidental events (platelet and THP-1 cells in the same field but not attached), platelets occluded from view by other cells and extracellular vesicle-THP-1 cell aggregates were excluded from the data analysis. Platelet subpopulations were classified using CD42b-PE-Dazzle 594, CD62P-PE and ApoT-Green fluorescence (Figure 4.1E). Intensity masks were sequentially applied in order to classify platelets based on positive or negative antibody / dye binding. Boolean logic was applied to classify each platelet into a subpopulation based on current literature [187-191].

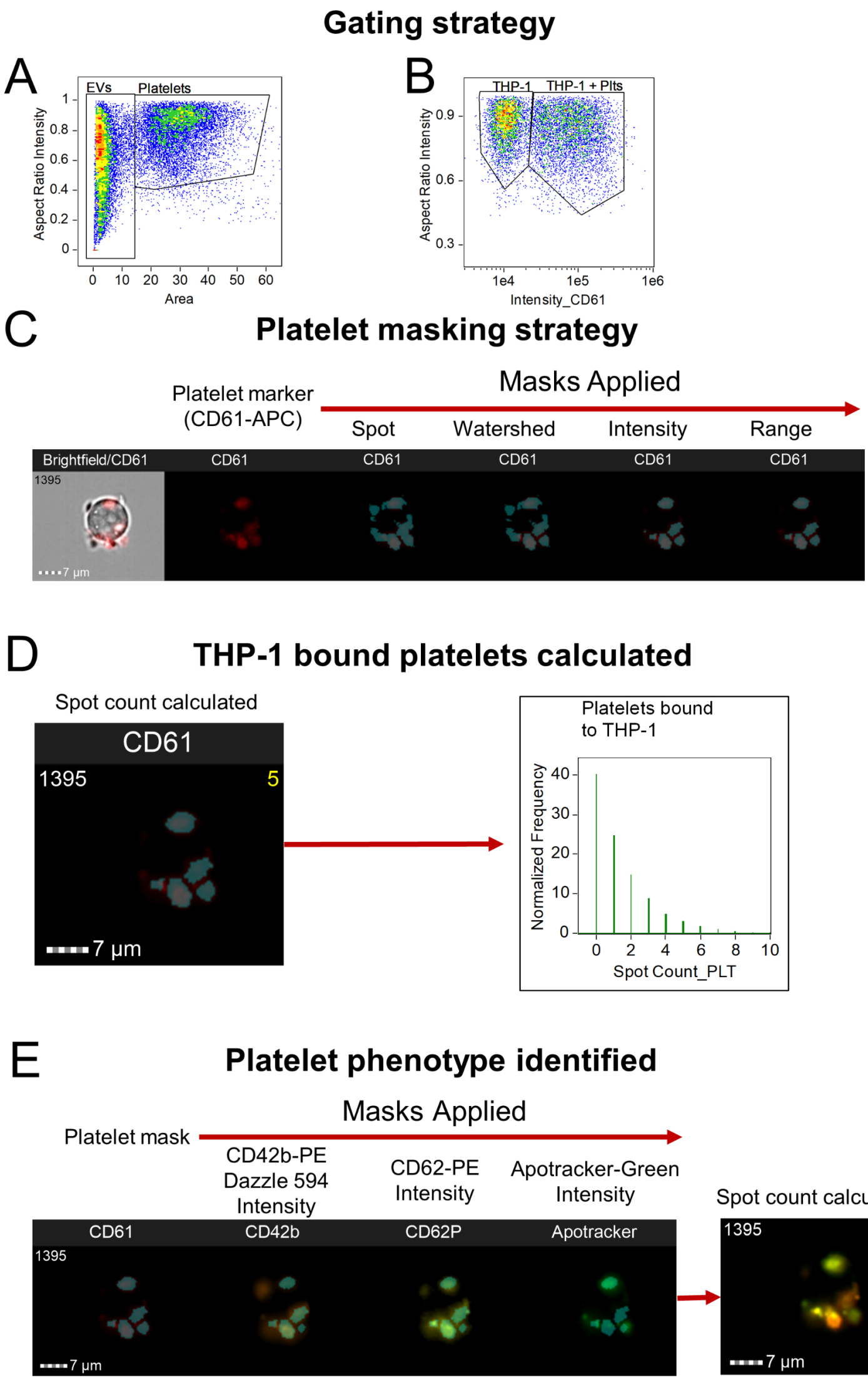


Figure 4-1. Gating and analysis strategy for enumerating and phenotyping platelet-THP-1 cell aggregates using imaging flow cytometry and IDEAS v6.2 analysis software. Gating and analysis strategy for enumerating and phenotyping platelet-THP-1 cell aggregates using imaging flow cytometry and IDEAS v6.2 analysis software. Gating strategies are shown for analysis of A) platelets and B) platelet-THP-1 cell co-culture. C) A fluorescence mask based on CD61-APC was created to enumerate the number of platelets bound to THP-1 cells. The platelet mask was created by sequentially applying Spot, Watershed, Intensity and Range masks (blue highlighted area) to reduce the fluorescence background and allow the analysis of individual platelets. D) The platelet mask was combined with the Spot Count feature to enumerate the number of platelets bound to each THP-1 cell. E) The phenotype of bound platelets was analysed based on the staining pattern of CD42b-PE Dazzle 594, CD62P-PE and Apotracker-Green fluorescence images with Intensity masks. The number of platelets counted in an image is indicated by the yellow number in the top right-hand corner of the image. EVs = extracellular vesicles.

4.5.6 Supernatant collection and analysis

Co-culture supernatant was isolated by three consecutive centrifugation steps at 125 *g* for 5 minutes*,* 1600 *g* for 20 minutes and 12000 *g* for 5 minutes to remove most THP-1 cells, platelets and EVs, respectively. The supernatant was frozen at -80 °C for later analysis. The concentration of soluble factors was measured using the following commercially available enzyme-linked immunosorbent assay (ELISA) kits: IL-1β, IL-6, IL-8, IL-10 and TNF-α (R&D Systems Minneapolis, MN, USA). All samples were tested in duplicate and measured against a standard curve, as per the manufacturer's instructions.

4.5.7 Statistical analysis

Statistical analysis was conducted using GraphPad Prism 9.4.1 (GraphPad Software, Inc.). Repeated measures one-way analysis of variance (ANOVA) was used to identify differences between groups. Post hoc Bonferroni's multiple comparisons tests were performed to determine differences between the specific groups. Pearson r-test was used to determine the correlation between data points. A p-value of less than 0.05 was considered to be statistically significant.

4.6. Results

Platelet activation and cryopreservation significantly altered the abundance of receptors involved in leukocyte adhesion on the surface membrane of platelets (Figure 4.2). The majority of PF and TRAP-6 platelets were positive for GPIbα (Figure 4.2A). In comparison, cryopreservation and stimulation with A23187 reduced the proportion of platelets positive for CD42b binding by approximately 10% and 15% respectively (Figure 4.2A). Only 10% of PF platelets exhibited CD62P binding, compared to approximately 50% of PT and 80-90% of TRAP-6 and A23187 stimulated platelets (Figure 4.2B). Further, PF and TRAP-6 platelets displayed negligible ApoT staining compared to PT and A23187, where 60-80% of the platelets had externalised phosphatidylserine (Figure 4.2C).

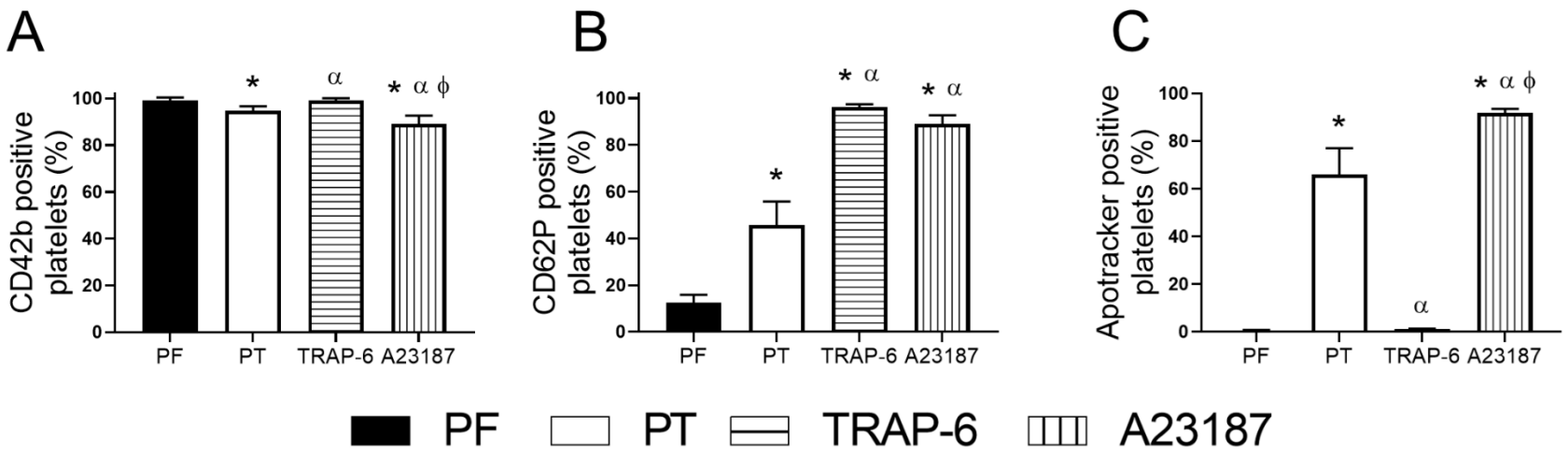


Figure 4-2. Cryopreservation and activation differentially alters the abundance of leukocyte adhesion receptors on the surface membrane of platelets. Platelets were stained with Apotracker-Green, CD62P-PE, CD42b-PE-Dazzle 594 and CD61-APC and analysed by imaging flow cytometry at 60x magnification. Platelet events were gated based on area, aspect ratio and CD61-APC fluorescence parameters. Pre-freeze (PF, ■), post-thaw (PT, □), TRAP-6 stimulated (▤) or A23187 stimulated (▥) platelets were identified using the CD61-APC mask, and the proportions of A) CD42b-PE-Dazzle 594, B) CD62P-PE and C) Apotracker-Green positive cells were determined. Data represent the mean + standard deviation (error bars, *n* = 6 in each group) of 10 000 gated platelet events. Significance was determined by one-way ANOVA. * = $p < 0.05$ compared to PF. α = $p < 0.05$ compared to PT. Φ = $p < 0.05$ compared to TRAP-6

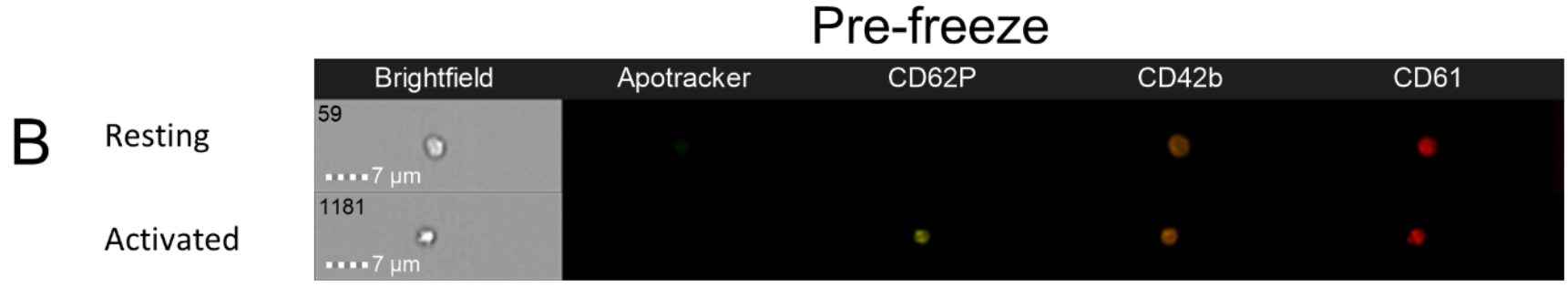
Platelet activation can occur through several pathways, generating phenotypically distinct subpopulations (Figure 4.3) [188, 192, 194]. In this study, platelet subpopulations were defined based on morphological and surface phenotype characteristics as described in previous literature [187, 188, 192, 194, 196]. Platelets were classified as resting (ApoT-/CD42b+/CD62P-), activated (ApoT-/CD42b+/CD62P+) or procoagulant (ApoT+/CD42b+/CD62P+). In line with previous publications, TRAP-6 and A23187 stimulation were used to generate reference populations of activated and procoagulant platelets, respectively [188, 192, 194, 196].

The majority of PF platelets exhibited a resting phenotype, with a small proportion (~10%) of activated platelets (Figure 4.3A&B). In comparison, cryopreservation resulted in the generation of three platelet subpopulations (Figure 4.3A&C). As expected, PT components contained activated and procoagulant platelets, as well as a population of platelets with a phenotype that did not align with the major subpopulations (ApoT+/CD42b+/CD62P-). Stimulation with either TRAP-6 or A23187 resulted in most platelets exhibiting an activated or procoagulant phenotype, respectively (Figure 4.3A, D-E).

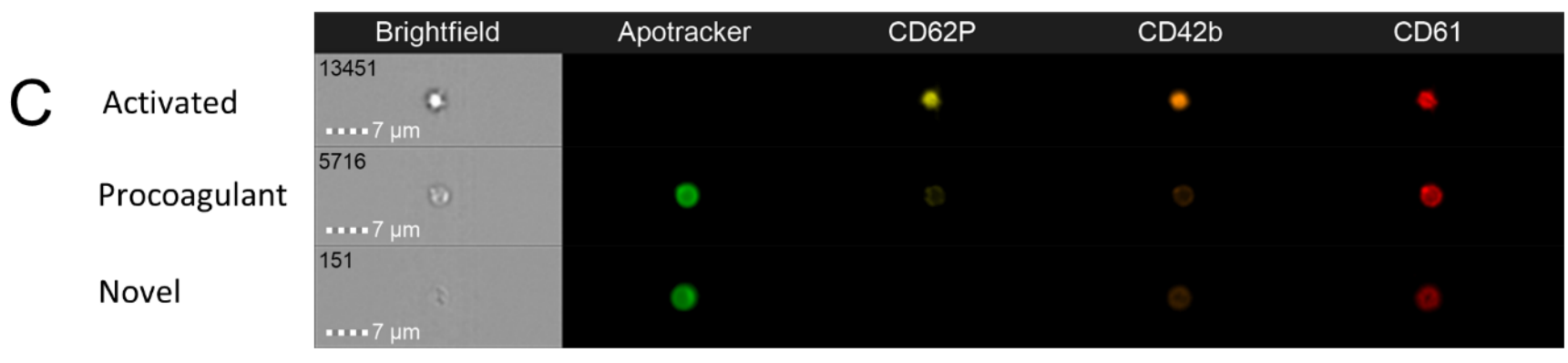
A

	Platelets classified by subpopulation							
	Dye / Fluorescent Antibody (%)							
	Apotracker-				Apotracker+			
Treatment	CD42b+ CD62P-	CD42b+ CD62P+	CD42b- CD62P-	CD42b- CD62P+	CD42b+ CD62P-	CD42b+ CD62P+	CD42b- CD62P-	CD42b- CD62P+
Pre-freeze	**84 ± 6**	11 ± 3	0 ± 1	0 ± 0	0 ± 0	1 ± 1	0 ± 0	0 ± 0
Post-thaw	7 ± 2*	**34 ± 16***	1 ± 1	0 ± 0	**44 ± 11***	**20 ± 2***	5 ± 4*	0 ± 1
TRAP-6	4 ± 1*α	**95 ± 2*α**	0 ± 0	0 ± 0	0 ± 0	1 ± 0	0 ± 0	0 ± 0
A23187	0 ± 0*α	3 ± 1*αΦ	2 ± 0	1 ± 1	7 ± 3*αΦ	**79 ± 6*αΦ**	3 ± 2	5 ± 2*αΦ
Platelet Subpopulation	**Resting**	**Activated**	**NC**	**NC**	**Novel**	**Procoagulant**	**NC**	**NC**

B



C



D

E

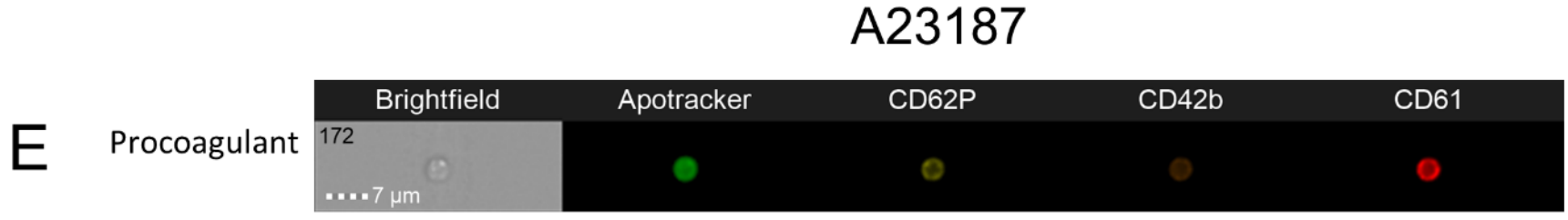


Figure 4-3. **Cryopreservation and activation promote the formation of different subpopulations.**Platelets were stained with Apotracker-Green, CD62P-PE, CD42b-PE-Dazzle 594 and CD61-APC and analysed by imaging flow cytometry at 60x magnification. A) The staining pattern of the three markers was used to classify platelets into the indicated subpopulations, where the proportions of each phenotype within that treatment group are displayed as a percentage of the total platelet population, with the greatest proportion presented in bold text. Representative brightfield and fluorescence images are shown for each of the major subpopulations in B) pre-freeze, C) post-thaw, D) TRAP-6 stimulated or E) A23187 stimulated platelets. Data represent the mean ± standard deviation (*n* = 6 in each group) of 10 000 gated platelet events. Significance was determined by one-way ANOVA comparing each treatment within the platelet subpopulations. * = $p < 0.05$ compared to PF. α = $p < 0.05$ compared to PT. Φ = $p < 0.05$ compared to TRAP-6. NC = no classification.

Platelet binding to THP-1 cells was defined by single colour staining, assessing the pattern of CD61, CD42b, CD62P and phosphatidylserine (ApoT) on THP-1 cells. As expected, THP-1 cells were negative for these markers in the absence of platelets (Figure 4.4A), and suspension of platelets alone in Complete Media did not affect the platelet phenotype (Figure 2S). Platelet-THP-1 cell aggregates were observed in all treatment groups following co-culture (Figure 4.4B-E). However, the proportion of platelets binding to THP-1 cells was significantly higher following thawing (PT), and stimulation by TRAP-6 and A23187, compared to PF (Figure 4.4B-E and Figure 4.5). Assessment of single markers demonstrated a similar pattern of staining for CD61, CD42b and CD62P, suggesting that the majority of the bound platelets expressed these three markers. Specifically, 40% of THP-1 cells stained positive for CD61, CD42b or CD62P following co-incubation with PF platelets compared to 60% in PT and 80% of TRAP-6 and A23187 stimulated samples (Figure 4.5A-C). The ApoT staining profile showed a different pattern between the treatment groups, with THP-1 cells demonstrating negligible staining following incubation with PF and TRAP-6 stimulated platelets. In contrast, ApoT+ platelet binding was significantly elevated in THP-1 cells incubated with PT (~40%) and A23187 (~90%) platelets (Figure 4.5D). In general, the staining pattern of the THP-1 cells mirrored the phenotype of the platelets prior to co-culture.

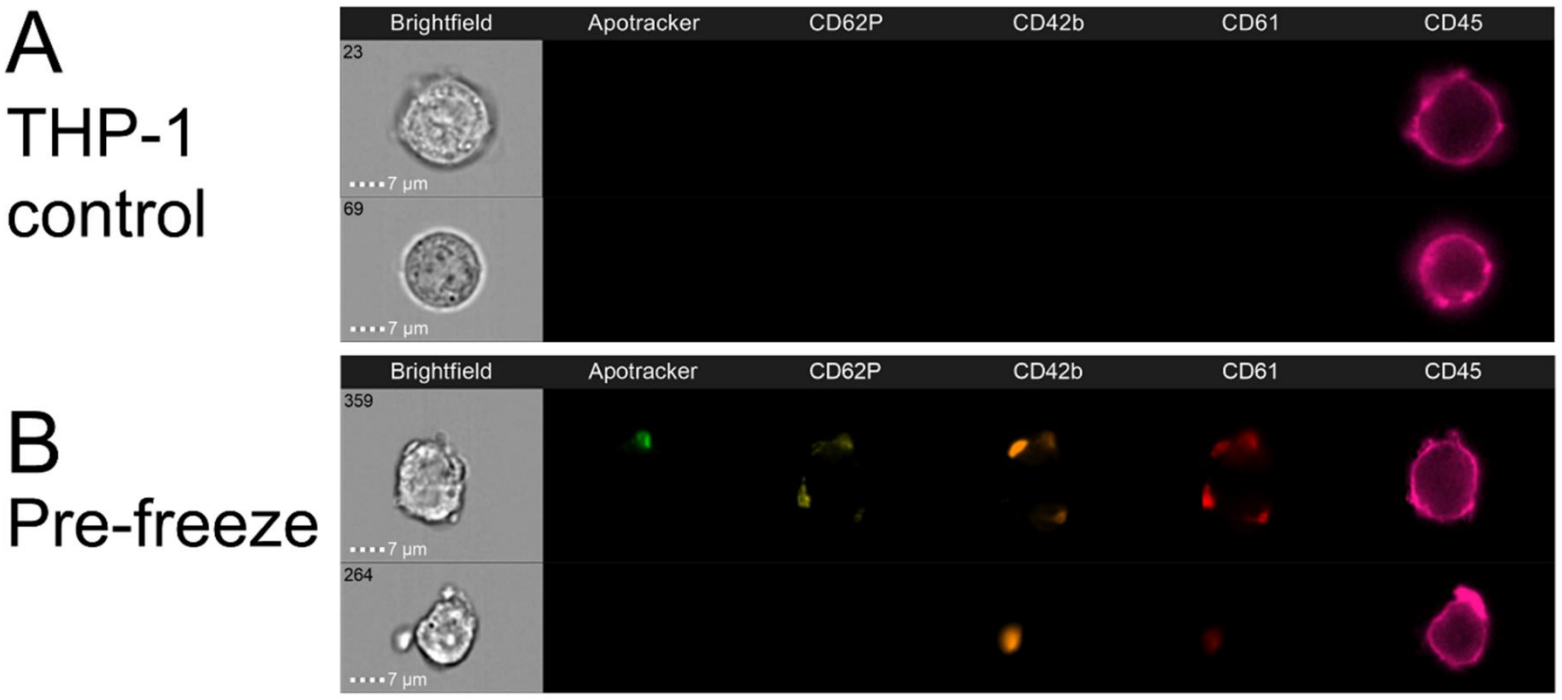


Figure 4-4. Visualisation and quantitation of platelet-THP-1 cell aggregates using imaging flow cytometry. THP-1 cells were examined A) alone or following co-incubation with B) pre-freeze, C) post-thaw, D) TRAP-6 stimulated or E) A23187 stimulated platelets for 1 hour. The platelet-THP-1 cell co-culture was stained with Apotracker-Green, CD62P-PE, CD42b-PE Dazzle 594, CD61-APC and CD45-APC-H7 and analysed by imaging flow cytometry at 60x magnification. Representative brightfield and fluorescence images are shown for each group.

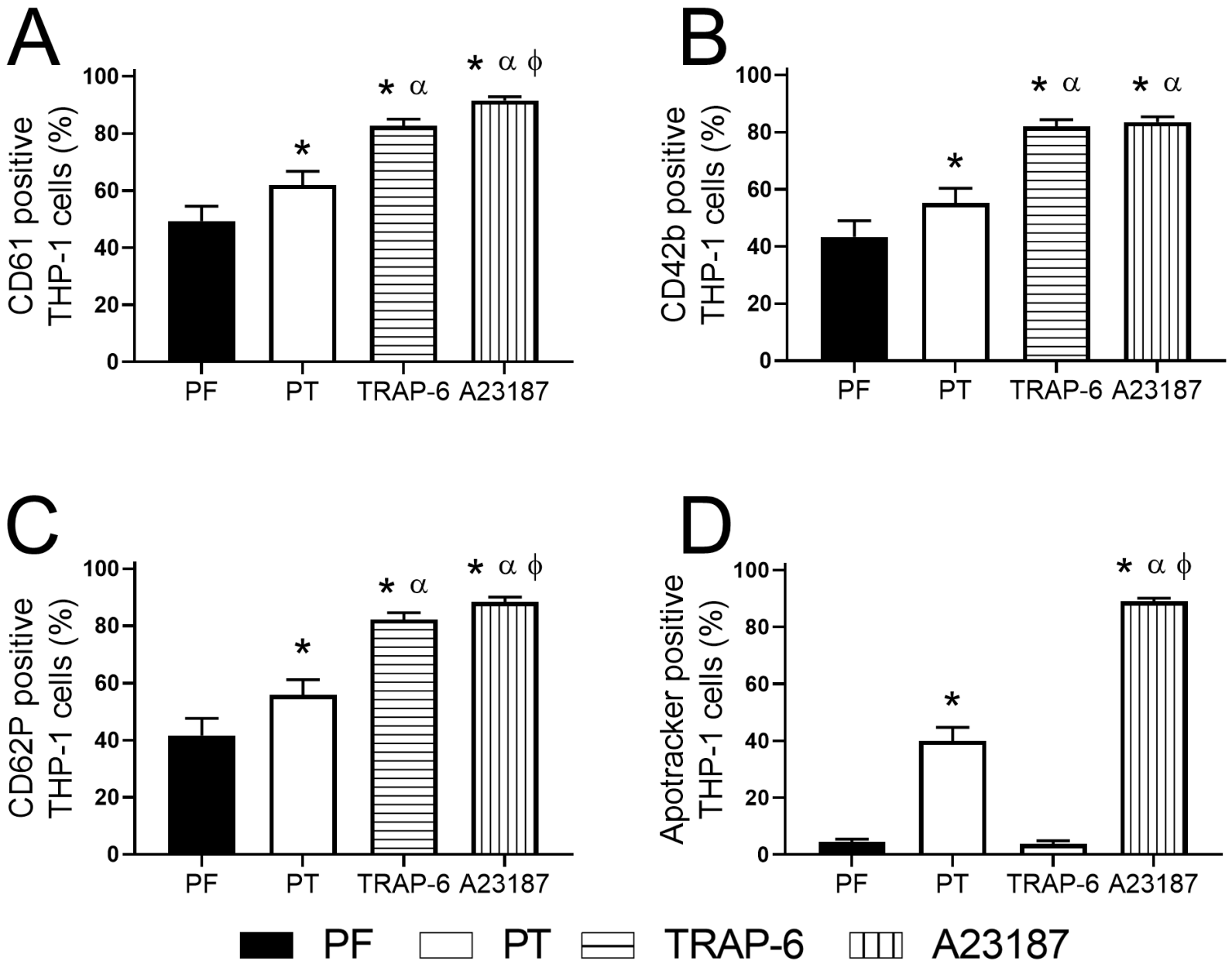


Figure 4-5. Platelet cryopreservation and activation increases the abundance of platelet-THP-1 aggregates. THP-1 cells were co-cultured with pre-freeze (PF, ■), post-thaw (PT, □), TRAP-6 stimulated (▤) and A23187 stimulated (▥) platelets for 1 hour. The platelet-THP-1 cell co-culture was stained with Apotracker-Green, CD62P-PE, CD42b-PE Dazzle 594, CD61-APC and CD45-APC-H7 and analysed by imaging flow cytometry at 60x magnification. The percentage of THP-1 cells positive for A) CD61-APC, B) CD42b-PE Dazzle 594, C) CD62P-PE and D) Apotracker-Green was measured for each treatment. Data represent the mean ± standard deviation (error bars, *n* = 6 in each group) of 10 000 gated THP-1 cells events per sample. Significance was determined by one-way ANOVA comparing each treatment. * = $p < 0.05$ compared to PF. α = $p < 0.05$ compared to PT. Φ = $p < 0.05$ compared to TRAP-6.

Characterisation of the phenotype of the platelets bound to the THP-1 cells was extended to assess the triple marker staining pattern to allow classification of the specific subpopulations bound (Figure 4.6). Most PF platelet-THP-1 cell aggregates exhibited an activated phenotype with a smaller proportion of resting platelets bound (Figure 4.6A-B). In contrast, most PT platelets adhered to THP-1 cells exhibited an activated or procoagulant phenotype (Figure 4.6A&C). In comparison, following TRAP-6 or A21287 stimulation, most adherent platelets exhibited an activated or procoagulant phenotype, respectively (Figure 4.6A, D-E).

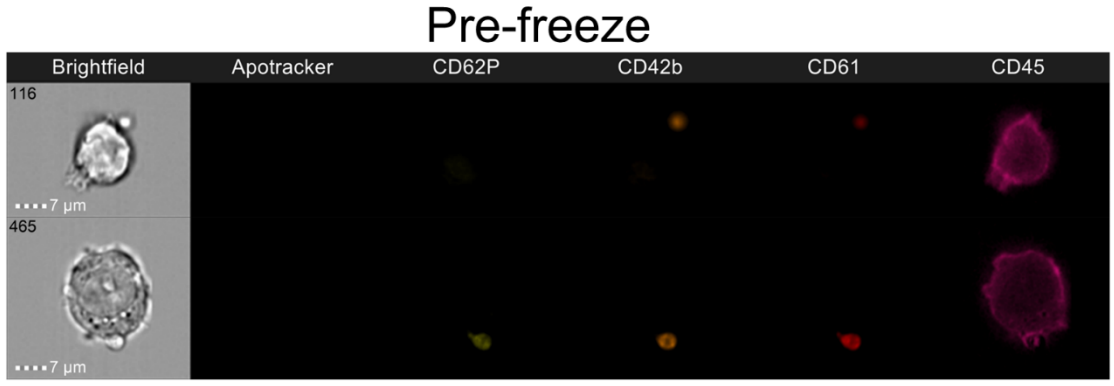
A

	%THP-1 cells bound by platelet subpopulations							
	Dye / Fluorescent Antibody (%)							
	Apotracker-				Apotracker+			
Treatment	CD42b+ CD62P-	CD42b+ CD62P+	CD42b- CD62P-	CD42b- CD62P+	CD42b+ CD62P-	CD42b+ CD62P+	CD42b- CD62P-	CD42b- CD62P+
Pre-freeze	**24 ± 5**	**43 ± 7**	2 ± 1	1 ± 1	0 ± 0	4 ± 1	0 ± 0	2 ± 1
Post-thaw	2 ± 2*	**44 ± 8***	1 ± 1	2 ± 1	2 ± 1	**42 ± 5***	3 ± 1	4 ± 3
TRAP-6	4 ± 1*	**83 ± 2*α**	0 ± 0	3 ± 0	0 ± 0	18 ± 6*α	0 ± 0	0 ± 0
A23187	0 ± 0*	1 ± 0*αΦ	0 ± 0	0 ± 0	0 ± 0	**89 ± 1***αΦ	0 ± 0	2 ± 1
Platelet Subpopulation	**Resting**	**Activated**	**NC**	**NC**	**Novel**	**Procoagulant**	**NC**	**NC**

B

Resting

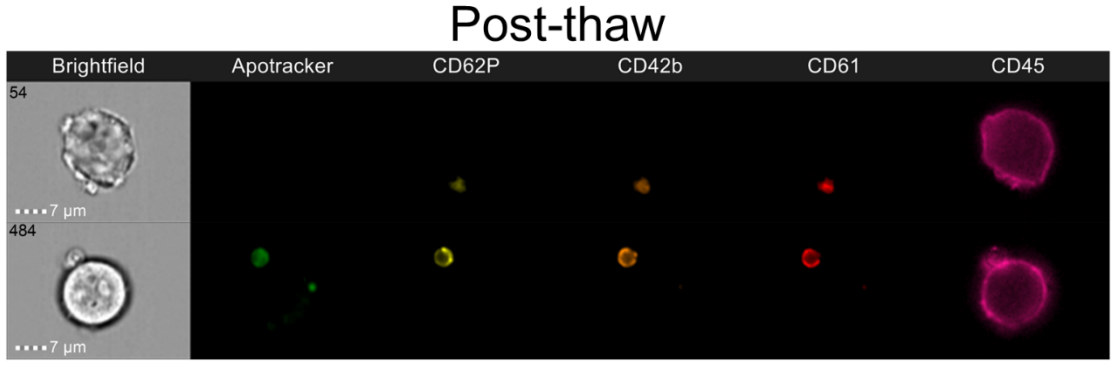
Activated



C

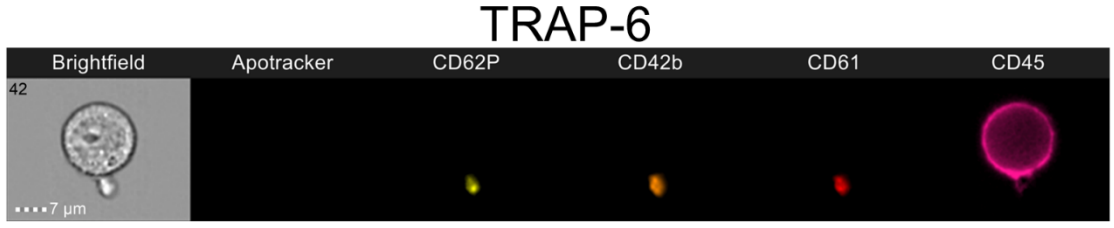
Activated

Procoagulant



D

Activated



E

Procoagulant

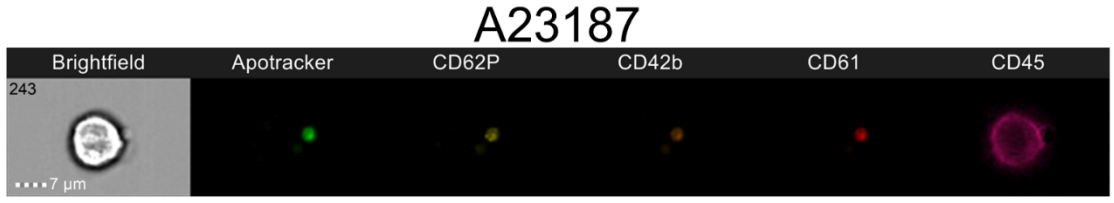


Figure 4-6. Cryopreservation increases the formation of procoagulant platelet-THP-1 cell aggregates. THP-1 cells were incubated with pre-freeze, post-thaw, TRAP-6 or A21387 platelets for 1 hour. The platelet-THP-1 cell co-culture was stained with Apotracker-Green, CD62P-PE, CD42b-PE Dazzle 594, CD61-APC and CD45-APC-H7 and analysed by imaging flow cytometry at 60x magnification. A) The staining pattern of the triple platelet markers was used to classify platelets into the indicated subpopulations. The proportion of THP-1 cells in each treatment group bound by platelets of a certain subpopulation are presented, with the major subpopulations highlighted in bold. The number of platelets bound to THP-1 cells was calculated post-collection using the mask and feature function. Representative brightfield and fluorescence images of B) pre-freeze, C) post-thaw, D) TRAP-6 stimulated or E) A21387 stimulated platelets bound to THP-1 cells are shown. Data represent the mean ± standard deviation (error bars, *n* = 6 in each group) of 10 000 gated THP-1 events per sample. Significance was determined by one-way ANOVA comparing the mean of each treatment within each platelet subpopulation. * = $p < 0.05$ compared to PF. α = $p < 0.05$ compared to PT. Φ = $p < 0.05$ compared to TRAP-6. NC = no classification.

The density of platelets bound to THP-1 cells was categorised for each treatment group. A classification of low, medium or high binding density was indicative of 1-2, 3-5 or 6-10 platelets bound to THP-1 cells, respectively (Figure 4.7A-B). The majority of THP-1 cells co-incubated with PF platelets displayed low platelet binding (40%, Figure 4.7A), with very few THP-1 cells having >2 platelets bound. A similar proportion of low density binding was observed in the PT platelets, although 15 and 5% of the THP-1 cells had medium and high density of platelets bound, respectively. In contrast, approximately 60% of THP-1 cells incubated with TRAP-6 or A23187 treated platelets displayed medium or high density adherence. The presence of CD62P on the platelet surface was associated with the density of platelets adhered to the THP-1 cells. Specifically, low CD62P positivity was correlated with low numbers of platelet adherence to THP-1 cells (Figure 4.7C; r= -0.86). In contrast, a higher proportion of CD62P positive platelets was positively associated with a medium (r=0.93) and high (r=0.88) density of platelets adhered per THP-1 cell (Figure 4.7D-E).

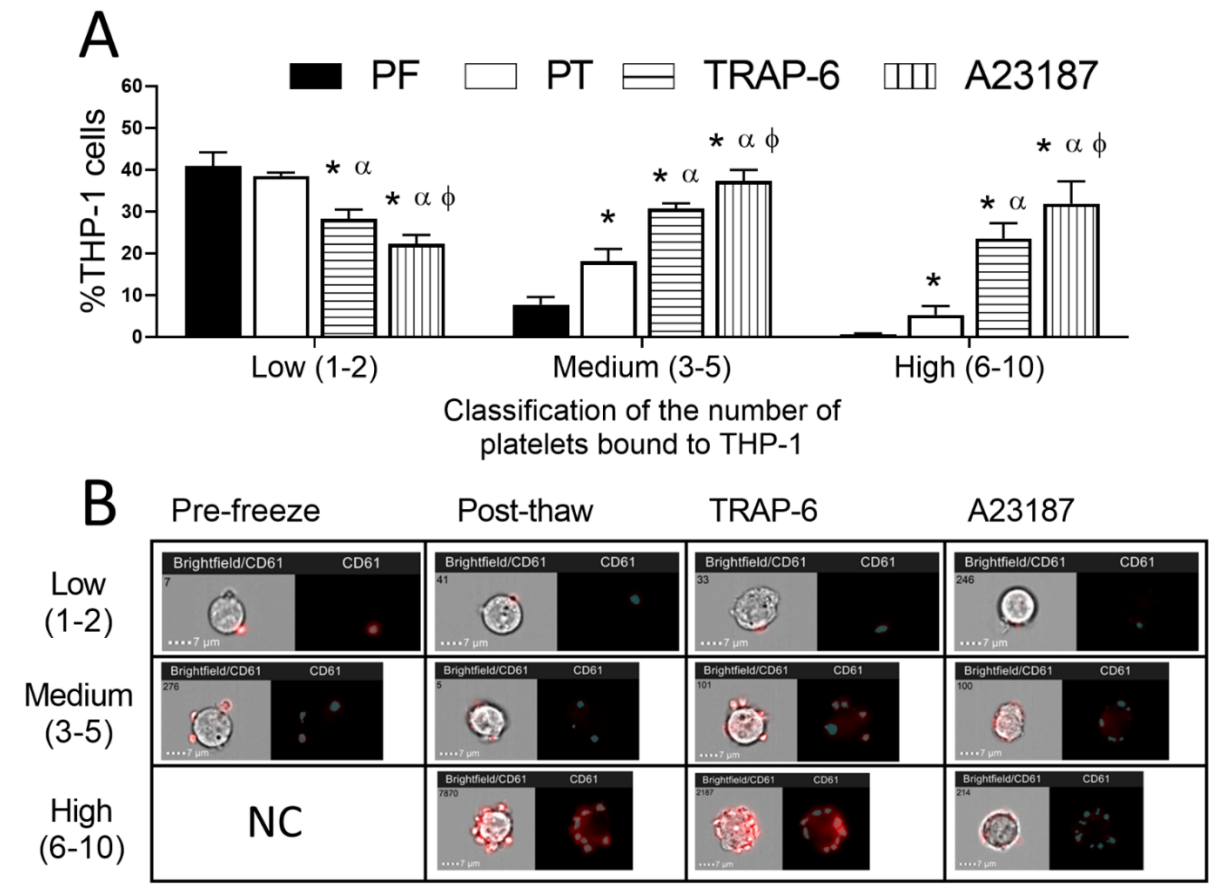


Figure 4-7. Platelet cryopreservation and activation increases the density of platelets present within the platelet-THP-1 cell aggregates. THP-1 cells were incubated with pre-freeze (PF, ■), post-thaw (PT, ▭), TRAP-6 (▤) or A23187 (▥) platelets for 1 hour. The platelet-THP-1 cell co-culture was stained with Apotracker-Green, CD62P-PE, CD42b-PE Dazzle 594, CD61-APC and CD45-APC-H7 and analysed by imaging flow cytometry at 60x magnification. A) THP-1 cells were classified into low (1-2), medium (3-5) and high (6-10) categories based on the number of bound platelets, shown in brackets, and the proportion of platelets in each category was calculated. B) Representative images of brightfield / CD61-APC composites next to CD61-APC fluorescence images with platelet masks applied (blue dots). The correlation between the percentage of platelets positive for CD62P-PE for PF (■), PT (□), TRAP-6 (x) or A23187 (◊) and the proportion of THP-1 cells exhibiting C) low, D) medium and E) high numbers of platelets bound was calculated. Data represent the mean ± standard deviation (error bars, $n = 6$ in each group) of 10 000 gated THP-1 events per sample or individual data points from each replicate. Significance was determined by one-way ANOVA comparing the mean of each treatment within each platelet subpopulation. * = $p < 0.05$ compared to PF. α = $p < 0.05$ compared to PT. Φ = $p < 0.05$ compared to TRAP-6. NC = no classification. Pearson r-test was used to determine correlation between data points. Linear regression was used to apply a line of best fit.

Upon PLA formation, platelets are capable of altering the soluble factor release and surface phenotype of leukocytes [56, 64]. Analysis of the supernatant from the platelet-THP-1 cell co-culture revealed that the concentration of IL-1β, IL-6, IL-8, IL-10 and TNF-α were all below the limit of detection of the assays following 1 or 24 hours of incubation (data not shown). Similarly, the percentage of THP-1 cells binding CD162, CD11a, LFA-1 and CD45 did not vary between treatment groups (Figure 3S), indicating their activation status did not change as a result of platelet binding.

4.7. Discussion

Platelet cryopreservation is currently under clinical evaluation as it offers potential improvements in shelf-life and haemostatic function compared to conventional storage [15, 179]. However, cryopreservation also alters the surface abundance of a range of receptors associated with immune function and leukocyte adhesion [56, 181], which may influence the risk of adverse events. The data in this study, demonstrates that cryopreservation-induced changes in receptor abundance increases the occurrence of platelet adhesion to monocyte-like cells (THP-1 cells) compared to PF components, *in vitro*. Further, it was demonstrated that cryopreserved platelets are phenotypically heterogenous, and only CD62P positive platelets have the capacity to bind to THP-1 cells.

Platelet subpopulations can be generated by various physiological and non-physiological stimuli [188, 194]. In line with previous publications, TRAP-6 or A23187 stimulation predominantly produced subpopulations of activated and procoagulant phenotype platelets, respectively [188, 194, 197]. Compared to agonist stimulation, cryopreservation-induced platelet activation resulted in a heterogenous mix of activated and procoagulant platelets along with a novel subpopulation. The mechanism by which cryopreservation induces platelet activation is still under investigation; however, preliminary work suggests that increased cytosolic calcium concentrations may be important [165]. The question remains as to why cryopreservation does not activate platelets uniformly. Notably, recent work in washed platelets has suggested that individual platelet characteristics, such as the number of mitochondria, may influence whether a platelet progresses from an activated to a procoagulant phenotype [189].

Previous work has highlighted that platelet activation, which leads to P-selectin exposure, increases the likelihood of PLA formation [3, 31, 64, 186], a finding reflected in this study. Cryopreservation caused an increase in CD62P positive platelets and it is unsurprising that an increase in platelet-THP-1 cell aggregates was evident compared to PF samples. Interestingly, the number of THP-1 cells bound by the 'activated' subpopulation was comparable between PF and PT samples. As such, the increase in platelet-THP-1 cell aggregates post-thaw was primarily attributable to an increase in procoagulant platelets. However, PT platelets formed platelet-THP-1 cell aggregates at a significantly lower rate than TRAP-6 or A23187 samples. A potential explanation of this finding is that approximately half of PT platelets were CD62P negative.

The ApoT+/CD42b+/CD62P- phenotype does not match current literature classifications. In this study, the novel subpopulation was observed to exhibit phenotypical similarities to procoagulant platelets, including a balloon-like morphology and ApoT binding on the surface membrane. However, this population was CD62P- and did not form platelet-THP-1 cell aggregates. The clinical significance of this subpopulation is currently unclear. But it would be of interest to examine if this subpopulation is present in circulation under physiological or pathological conditions, as well as being generated in platelet components stored under different blood bank conditions, including during cold storage or after pathogen inactivation.

It also remains to be determined how this novel phenotype arises. Preliminary work from our lab suggests a potential link between procoagulant platelets, the novel subpopulation and extracellular vesicle release, which are all increased

post-thaw [109, 188]. The novel subpopulation has been shown to exhibit decreased internal complexity, suggesting that degranulation has occurred, exposing P-selectin, which is subsequently lost from the surface membrane [188]. Notably, the concentration of P-selectin in the supernatant is significantly increased post-thaw, which supports this hypothesis [104, 198]. The mechanism by which P-selectin is removed from the surface membrane is immunologically relevant, as evidence suggests only the dimeric membrane-bound isoform is pro-inflammatory [199]. The dimeric form of P-selectin is found on the surface of extracellular vesicles, whereas proteolytic cleavage or release of soluble P-selectin generally occurs as a monomer [200]. Although the concentration of extracellular vesicles increases significantly post-thaw [110], they are not CD62P positive [109]. While further investigation is required, the available information suggests that cleavage of P-selectin from the surface of procoagulant platelets may result in the CD62P negative subpopulation.

The formation of PLAs occurs primarily through P-selectin and PSGL-1 [3, 182, 184, 201], although platelets are capable of leukocyte adherence through a range of other receptors, including ICAM-2 and GPVI [184, 185]. Notably, ICAM-2 and GPVI are reduced following cryopreservation [181, 188, 198]. This may help to explain how a proportion of resting platelets (CD62P negative) were observed to generate platelet-THP-1 cell aggregates in the PF group, while the CD62P negative PT platelets did not bind to the THP-1 cells. Alternatively, P-selectin can be shed from the platelet surface membrane shortly after binding to leukocyte PSGL-1 [202], which may account for the observed reduction in CD62P binding.

The post-transfusion recovery of cryopreserved platelets is approximately half that of freshly collected components [98]. Interestingly, the survival time (~7 days) of the cryopreserved platelets that remain in the circulation is lower than freshly isolated platelets, but better than conventionally stored components [98, 108]. Platelet clearance from the circulation is mediated through phagocytosis by leukocytes or ingestion by hepatocytes, depending on the surface phenotype. Non-antibody-mediated platelet clearance is typically facilitated by hepatocytes/macrophages in the liver and leukocytes in the circulation [64, 81, 184, 203, 204]. Platelet ingestion/phagocytosis in the liver occurs when GPIbα is clustered and becomes deglycosylated exposing β-N-acetylglucosamine (β-GlcNac) [81, 203, 204]. Recent work has suggested that cryopreservation does not increase GPIbα clustering [178] and β-GlcNac exposure is decreased PT compared to fresh platelet components [56]. Alternatively, platelets that are dual positive for P-selectin and phosphatidylserine can be phagocytosed by circulating leukocytes [56, 64, 181, 184, 205]. P-selectin facilitates platelet-leukocyte adhesion while phosphatidylserine provides a secondary signal, triggering internalisation and phagocytosis [56, 64, 184, 205]. In this study, it was observed that both activated and procoagulant platelets form aggregates with THP-1 cells. However, only procoagulant platelets exhibited phosphatidylserine exposure, which is required for phagocytosis [64, 184, 205]. Further, the blockade of phosphatidylserine, using annexin-V has been shown to reduce phagocytosis of cryopreserved platelets by macrophage-like THP-1 cells, *in vitro* [56]. Therefore, subpopulations may influence cryopreserved platelet survival post-transfusion.

The classification of platelets into their subpopulations is complicated by similarities in morphology and phenotype [187, 188]. Due to technical limitations, a marker for the activated conformation of GPIIb/IIIa (PAC-1) could not be included in the imaging flow cytometry panel. Consequently, aggregatory platelets could not be definitively identified. Instead, platelets with a comparable morphology and phenotype to the aggregatory subpopulation (ApoT-/CD42b+/CD62P+) were classified as “activated platelets”. Likewise, a marker for platelet apoptosis was not included, as recent work suggests that this subpopulation is not present in cryopreserved platelet components [188].

While many novel findings are presented, further work is required to fully understand the potential clinical consequences. In this study, the thawed platelets were resuspended in 30% plasma / 70% PAS-E to match the pre-freeze composition. However, it is likely that the thawed platelets will be reconstituted in 100% plasma in the clinical setting [15]. While the ratio of plasma can influence the concentration of soluble factors, our prior work has reported a negligible impact on the activation status (CD62P, phosphatidylserine externalisation) of post-thaw platelets [107], which is what appears to be mediating PLA formation. In addition, this study utilised THP-1 cells, which are commonly used to model platelet-leukocyte interaction *in vitro* [56, 201, 206], however, these results may not be representative of platelet-leukocyte interactions following co-culture with primary monocytes or neutrophils or leukocytes *in vivo*. Further investigation of platelet-neutrophil interactions would be informative due to their dual roles in platelet clearance and adverse events including TRALI [184].

Despite the differences in the number of platelet-THP-1 aggregates between samples, no measurable difference in THP-1 cell surface phenotype or soluble factor release was observed. Consequently, it is difficult to determine the potential immunomodulatory effects of the observed increases in cryopreserved platelet-THP-1 cell aggregates. However, our findings align with the current literature, which propose that platelets alone are insufficient to induce the more severe adverse transfusion events such as TRALI [182, 207]. Research suggests that TRALI requires the pre-priming of patient leukocytes, caused by an underlying co-morbidity or traumatic injury, which are then further activated by the transfusion of platelet components containing immunomodulatory soluble factors [182, 207]. However, cryopreserved platelet components contain lower concentrations of pro-inflammatory cytokines, but more extracellular vesicles than fresh components [109, 110, 181]. It is important to note that transfusion of cryopreserved platelets is reported to be safe and effective in the context of controlled trials and clinical use [15, 100, 102, 179].

Cryopreserved platelets offer advantages over conventionally stored platelets, enhancing haemostatic function *in vitro* and extending the shelf-life, enabling supply to remote medical settings [110, 180]. While further work is required, our findings contribute to the body of pre-clinical data being assembled to aid in the interpretation of clinical data as more becomes available.

5. General Discussion

This dissertation is composed of three published original research manuscripts which examine the impact that refrigerated storage and cryopreservation has on the immune characteristics and function of platelets. In this final chapter, the results are discussed in context with the current literature and future avenues of research are highlighted.

5.1. Introduction

The implementation of refrigeration and cryopreservation address certain limitations of conventional platelet storage. Both techniques allow for a potential extension of platelet shelf-life and improved haemostatic function compared to storage at room-temperature. Consequently, both techniques are under clinical evaluation or have recently been implemented to support inventory management during COVID-19. It is well established that both refrigeration and cryopreservation alter the haemostatic characteristics of platelets. However, prior to embarking on this study, comparatively little was known about the impact of alternative storage modes on the immune characteristics of platelets. This area of study is important as alterations in platelet immune characteristics have been directly linked with adverse events. As such, the aim of this dissertation, was to explore how refrigeration and cryopreservation affect the immune characteristics and function of platelet components. The data presented in this document contributes to our understanding of platelet immunobiology and the body of pre-clinical evidence being assembled to support the implementation of refrigerated and cryopreserved platelets.

5.2. Refrigeration alters the immune characteristics of platelets

Refrigeration is a logistically simple alternative to conventional platelet storage. Post-collection, platelets are placed under refrigerated conditions (2 - 6 °C) without agitation. Notably, refrigerated platelets exhibit an activated phenotype [83, 84, 89]. The exact mechanism of refrigeration-induced activation is still under investigation but may be caused by calcium leakage from storage organelles, dysregulation of membrane bound ion pumps or temperature induced activation of signalling pathways [87-89]. This results in the platelets transitioning from a resting, discoid shape to a spherocytic morphology and the extrusion of pseudopodia [87, 208, 209]. These changes lead to significant alterations in the abundance of certain surface receptors and the release of soluble factors and may be responsible for the more rapid clearance *in vivo* [33, 80, 123, 126, 210-212]. In this dissertation, refrigeration-induced activation was shown to significantly alter the immune characteristics and function of platelets *in vitro*.

5.2.1 Refrigerated storage reduces the abundance of receptors associated with immune function on the platelet surface membrane

Refrigeration was observed to significantly decrease the abundance of a range of receptors associated with the immune function of platelets. Specifically, the abundance of CD40, ICAM-2, FcγRIIa decreased gradually over storage. Previous work has established that refrigeration causes a gradual reduction in the abundance of haemostatic receptors (GPIbα, GPVI, GPIX, GPIIb, GPIV) on the platelet surface membrane [75, 83-85, 213, 214]. It has been suggested that

proteolytic cleavage by the calcium dependent A Disintegrin And Metalloprotease (ADAM) family of proteases may be causative [140, 192]. Consequently, proteolytic cleavage may also remove CD40, ICAM-2 and FcγRIIa from the surface membrane. Alternatively, as refrigerated platelets gradually release EVs over storage [33, 215, 216], the loss of surface proteins may be due to reallocation of membrane proteins to the EVs.

In contrast, the surface abundance of TLR1, TLR2, TLR3, TLR4, TLR7, TLR9, CD86 and CD44 decreased significantly during the first 24 hours of refrigerated storage but was comparatively stable thereafter. The exact mechanism behind this change remains to be identified, however, refrigeration causes significant alterations to the organisation of the cellular membrane and the composition of lipid rafts [139, 203]. Lipid rafts are sections of the surface membrane which exhibit increased concentrations of sphingolipids and cholesterol [139, 203]. These structures act as platforms which enhance receptor signalling by increasing the physical concentration of receptors and downstream signalling proteins [217, 218]. Notably, refrigeration has been shown to cause GPIbα to localise to lipid rafts, significantly altering its structure, causing deglycosylation, which leads to receptor clustering [139, 203]. Changes in the conformation of receptors can reduce antibody binding [219]. While yet to be examined in platelets, in other cells, activation signals cause the migration of immune receptors, including TLRs, to lipid rafts [217, 220]. This facilitates changes in receptor structure, the formation of receptor complexes and the priming of signalling pathways [139, 220]. Therefore, the initial reduction in receptor

abundance may be due to refrigeration-induced changes to the structure of the surface membrane.

Platelet bound TLRs mediate the recognition of PAMPs and DAMPs, facilitating the initiation of anti-pathogenic and pro-inflammatory signalling [28, 29, 167]. Platelet recognition of PAMPs and DAMPs is associated with anti-pathogenic effects, tissue repair, inflammation and adverse reactions [12, 76, 221]. As such a reduction in TLR abundance could reduce the capacity of platelets to recognise PAMPs and DAMPs. However, the responsiveness of platelets to stimuli is also reliant on the function of signalling pathways and metabolic function, which are better preserved during refrigerated storage [139, 140]. Signal transduction through TLRs requires phosphorylation of the Src and Syk family of kinases, which is increased following platelet refrigeration [139, 140]. Further, refrigeration of platelets has been demonstrated to alter the preferential partitioning of Src kinases and phospholipase-Cγ2 to lipid rafts [139], which may enhance signal transduction across the surface membrane. Additionally, storage at low temperature reduces enzyme kinetics, which has been shown to better preserve the metabolic parameters of platelets compared to room-temperature storage [75, 210]. A recent study by Zhao *et al.* examined the metabolome of stored platelets and reported that refrigeration leads to reduced protein lysis, glucose usage, lactate generation, damage caused by reactive oxygen species and mitochondrial activity compared to conventionally stored components [210]. Metabolic health was correlated with increased responsiveness to collagen induced stimulation, despite a reduction of GPVI on the surface membrane of refrigerated platelets [85, 210]. Consequently, refrigerated platelets may still be

able to respond to PAMP and DAMP stimulation despite a decrease in TLR abundance on the surface membrane.

5.2.2 Refrigeration alters the release of soluble factors mediators associated with immune function

The recognition of haemostatic and immunologic agonists by receptors on the platelet surface membrane triggers platelet activation and the release of soluble factors [48]. Platelet α-granules contain a wide range of soluble factors associated with haemostatic function, tissue repair and immune signalling [11, 28, 33, 127, 222]. Typically, α-granule release is measured indirectly by P-selectin exposure on the surface membrane and increased concentrations of soluble factors in the supernatant. Previous work has highlighted that platelet activation by shear stress, haemostatic (collagen, thrombin, ADP, TRAP-6) and immunological (LPS) agonists can result in different levels of P-selectin exposure and soluble factor release [48, 197, 223-226]. In this dissertation it was observed that both conventionally stored and refrigerated platelets exhibited an increase in P-selectin exposure. However, the concentration of most cytokines (CD40L, Endocan, IL-1β, IL-13, IL-27, HMGB1, OX40L) examined did not increase over storage. In contrast, the concentration of C3a, RANTES and PF4 did increase progressively during storage at room-temperature, but not refrigeration. Platelet activation is believed to occur through different mechanisms in response to refrigerated or room-temperature storage [227]. Refrigeration-induced activation occurs shortly after storage and is linked to calcium release [88, 89]. In contrast, room-temperature stored platelets become activated progressively throughout storage due to alterations in metabolic function, oxidative damage and

mitochondrial dysfunction [67, 75, 210]. Notably, platelets are capable of selectively releasing soluble factors. The concentration and type of soluble factor release is largely dependent on the mechanism of platelet activation [48, 225, 226]. Further, current evidence suggests that refrigeration induced activation may not be comparable to platelet activation through conventional pathways [87, 209]. This may explain the different supernatant compositions of conventionally stored and refrigerated platelets and why only specific soluble factors were released during storage.

In contrast to our findings, previous work has reported that conventional storage of platelets results in the release of IL-27, IL-1β, IL-8, HMGB1, OX40L and sCD40L [11, 77, 114, 227]. A potential explanation for the variation between our data and previous work may be the differences in the manufacture of platelet components [11, 77, 114, 227]. Notably, the components used in these studies were collected by apheresis, were suspended in 100% plasma or were conducted prior to the widespread implementation of leukoreduction. These factors can significantly affect the initial composition of the platelet supernatant and soluble factor release over storage [228-230], potentially explaining this discrepancy.

5.2.3 The impact of refrigeration on platelet-bacteria interaction

Platelets can interact with bacteria through surface receptors [5, 48]. Despite being anucleate, platelets possess TLRs on the surface membrane, which can recognise and bind to bacterial PAMPs [28, 29]. Further, aspects of platelet haemostatic function, including activated GPIIb/IIIa receptor, can facilitate indirect bacteria adherence through fibrinogen [6, 21, 52, 53]. Depending on the

bacteria this can aid in clearance of the infection or facilitate immune evasion [7, 52].

One of the main features driving the (re)implementation of refrigerated storage is the reduced risk of bacterial proliferation, allowing for an extension of the platelet shelf-life [71, 231]. As such, previous studies have largely focussed on examining the effect of refrigeration on bacterial proliferation in platelet concentrates [71, 231]. This work is the first to examine how refrigeration-induced changes in the immune characteristics of platelets affect bacteria-platelet interaction. Refrigerated platelets exhibited increased aggregation following exposure to *E. coli* and *S. aureus*. Previous work has demonstrated that refrigeration increases the abundance of activated GPIIb/IIIa on the surface membrane, which contributes to improvements in fibrinogen binding and haemostatic parameters *in vitro* [84, 85, 126]. The data presented in this dissertation highlights that GPIIb/IIIa also facilitates bacterial-induced aggregation with stored platelets *in vitro*.

Bacterial-induced aggregation was largely dependent on the function of activated GPIIb/IIIa on the surface membrane. Inhibition of the active site of GPIIb/IIIa with RGDS peptide reduced but did not eliminate bacterial-induced aggregation. Further, *S. aureus* induced platelet aggregation increased significantly over storage despite no further increase in the abundance of activated GPIIb/IIIa, in conventionally stored and refrigerated platelets. Depending on the species, bacteria can interact with platelets through multiple surface receptors. Notably, P-selectin, which is increased on the surface membrane of conventionally stored and refrigerated platelets can mediate *S. aureus*-platelet interactions [232]. However, *S. aureus* can also cause platelet aggregation through the release of

coagulase (coa) and von Willebrand factor binding protein (vWbp). Both proteins can activate prothrombin allowing for the conversion of fibrinogen to fibrin and clot formation [53]. Previous work has demonstrated that over storage phosphatidylserine is exposed on the surface of both room-temperature and refrigerated platelets and EVs [75, 85, 216]. Phosphatidylserine exposure on refrigerated platelets acts as a binding surface for coagulation factors (FV, FXIIIa), catalysing thrombin generation and enhancing procoagulant function [75, 214, 216]. As such, the procoagulant function of phosphatidylserine positive platelets may synergise with *S. aureus* induced coagulation, potentially contributing to the increase in bacterial-induced aggregation over storage.

The potential impact of the enhanced bacterial-induced aggregation induced by refrigeration post-transfusion is currently unclear. Currently, refrigerated platelets are primarily used for the treatment of acute bleeding, caused by surgery or traumatic injury. Notably, traumatic injury is associated with an increased risk of bacterial infection [136, 175]. As such, it is currently unclear if refrigerated platelets would be capable of interacting with bacteria at the site of injury and the potential clinical consequences. Additionally, an examination of the interaction of refrigerated platelets with other common bacteria would be informative, especially as platelet-bacteria interactions can be species specific, promoting both clearance and immune evasion [48, 53, 233-235]. As such, a further examination of refrigerated platelet-bacteria interactions with these strains *ex vivo* would likely provide useful information.

5.3. Cryopreservation alters the immune characteristics of platelets

Currently, platelet cryopreservation involves freezing platelets at -80°C with the addition of 5-6% DMSO [97]. The cryopreservation process causes significant platelet activation. The exact mechanism of activation is still under investigation; however, current evidence suggests that elevated cytosolic calcium, increased membrane permeability and mitochondrial membrane depolarisation may be causative [15, 100, 179]. Cryopreservation induced activation causes a loss of internal cytoskeletal structure, dissolution of the microtubular ring and peripheral organisation of granules, with most platelets displaying a balloon like morphology [188, 236]. The data presented in this dissertation has demonstrated that cryopreservation induced activation alters the immune characteristics and function of platelets.

5.3.1 Cryopreservation alters the abundance of receptors associated with immune function on the platelet surface membrane

Platelets can facilitate immune signalling through receptors on the surface membrane [31, 48, 167]. The studies included in this dissertation report that cryopreservation alters the abundance of a range of receptors associated with immune function. The abundance of TLR3, TLR7 and TLR9 increased immediately post-thaw. Additionally, the abundance of all TLRs examined increased significantly following extended post-thaw storage. Platelet TLRs facilitate the recognition of PAMPs and DAMPs, which can promote pathogen clearance, inflammatory signalling and tissue repair [2, 4, 31]. Notably,

cryopreserved platelets will primarily be used for the treatment of acute bleeding as a result of traumatic injury or surgery [15, 100]. Both conditions are associated with elevated DAMP release into the circulation [31, 172]. As such, understanding whether cryopreserved platelets can recognise and respond to DAMPs has clinical relevance.

Post-thaw, platelets exhibit a high level of activation and limited responsiveness to haemostatic agonists [237]. As such, it is unclear if cryopreserved platelets can respond to immunological signals. Previous work has highlighted that cryopreserved platelets exhibit reduced responsiveness to haemostatic agonists (ADP, TRAP-6 and collagen) *in vitro,* as measured by aggregation, activation marker exposure and soluble factor release [237]. This finding could be attributed to a significant reduction in the abundance of haemostatic receptors (GPVI, GPIbα, GPIIb) on the surface membrane [111, 114, 198]. However, recent work has demonstrated that the recovery of GPVI post-thaw, following extended post-thaw storage did not restore the loss of collagen-induced aggregation associated with cryopreservation [176]. The recognition of haemostatic and immunological agonists is facilitated by the phosphorylation of signalling molecules [18, 142]. However, cryopreserved platelets exhibit lower baseline phosphorylation of Lyn, Src, Akt, Erk and p38 MAPK. Additionally, agonist stimulation does not further increase phosphorylation of these signalling molecules [237]. As such, current findings suggest that PAMP or DAMP stimulation of cryopreserved platelets would not further activate cryopreserved platelets.

5.3.2 Cryopreservation alters the release of granular contents from platelets

Cryopreservation causes platelets to externalise P-selectin and release soluble factors into the supernatant [33, 111], and whether this is due to degranulation or platelet fragmentation post-thaw is still unknown. To examine the level of α-granule release, cryopreserved platelets were compared to platelets stimulated with TRAP-6. TRAP-6 is a non-physiological agonist which causes platelet activation through the PAR-1 receptor [238]. Sufficient stimulation of PAR-1 causes platelet activation and close to complete release of α-granule contents [48]. Interestingly, the surface abundance of receptors known to be contained within α-granules (TLR1, TLR2, TLR4, P-selectin, Siglec-7, MHC-I, CD40L) [28, 29, 224, 239] were significantly lower in cryopreserved compared to TRAP-6 stimulated platelets. This finding suggests that cryopreserved platelets are unable to completely release of α-granule contents to the surface membrane. Alternatively, cryopreserved platelets may exhibit reduced surface receptor abundance due to receptor shedding into the supernatant or transfer to EVs, both of which are significantly increased post-thaw [109, 110]. Notably, the abundance of TLR7 and TLR9 increased only after cryopreservation and not after TRAP-6 stimulation. While still a matter of debate, TLR7 and TLR9 are believed to be stored in endosomes or potentially platelet specific T-granules [188, 236]. Previous work has shown that TLR9 can be mobilised to the platelet surface membrane by thrombin stimulation [28]. Thrombin stimulation causes platelet activation through both PAR1 and PAR4 on the platelet surface membrane [240,

241]. Further, PAR4 but not PAR1 signalling is associated with prolonged calcium release, phosphatidylserine exposure and the formation of procoagulant platelets, characteristics shared by cryopreserved platelets [240, 241]. A deeper understanding of the dynamics of granule release in cryopreserved platelets would be useful and provide insight into the mechanics of activation post-thaw.

The accumulation of pro-inflammatory soluble factors is directly linked to the risk of transfusion-related adverse events [24, 34, 221]. This dissertation is the first to examine the release of pro-inflammatory cytokines previously associated with adverse events from cryopreserved platelets post-thaw. The concentration of IL-1β, OX40L, IL-13, IL-27 and CD40L was significantly lower in cryopreserved platelets. This was likely due the manufacturing process of cryopreserved platelets, which requires the removal of the supernatant prior to freezing and its replacement post-thaw, which has an effect similar to “washing” the component. In contrast to our current and previous findings [104], a recent publication by Tyngaard *et al.* reported that post-thaw platelets exhibited increased supernatant concentrations of IL-1β, CD40L, IL-7, IFN-γ, VEGF and TNF-α post-thaw [242]. This discrepancy may arise from differences in the manufacturing processes used, which can significantly affect the baseline concentration and release of soluble factors from platelets [104]. Notably, the cryopreserved platelets used by Tyngaard *et al.* were reconstituted in 100% plasma compared to 30% plasma / 70% SSP+ used in this study and in previous work, which can significantly affect cytokine release [104, 107]. The addition of PAS significantly reduces the risk of adverse events [228, 229], highlighting its potential utility for the resuspension of cryopreserved platelets post-thaw.

Cryopreserved platelet components contained a heterogenous mix of activated and procoagulant platelets, as well as those with a novel phenotype. Approximately 60-70% of post-thaw platelets exhibited a procoagulant or novel phenotype characterised by a balloon like morphology. Previous work has highlighted that balloon platelets generated by thrombin / collagen stimulation or following cryopreservation are largely degranulated [188, 236]. In line with this observation, post-thaw platelets exhibited an increased release of α-granule contents (RANTES, PF4, C3a). Interestingly, the concentration of these soluble factors was observed to increase following extended post-thaw storage. Notably, the activated platelet subpopulation exhibits similar internal complexity to resting platelets [188], suggesting the granular contents are retained post-thaw, which may allow for further release during extended storage.

Despite significant release of RANTES and PF4, the concentration of IL-13, IL-27, OX40L and HMGB1 did not increase post-thaw. As balloon platelets were largely degranulated, some release into the supernatant would be expected. Activated platelets expose phosphatidylserine on the surface membrane, which is capable of binding a range of soluble factors including HMGB1 [160, 161], which may account for decreased detection in the supernatant. Alternatively, platelets can package BRMs into EVs, which are significantly elevated post-thaw [24, 109]. Platelet derived EVs can act as delivery vectors for soluble factors and have been linked to inflammation and adverse events [24]. As such, further examination of the EV content post-thaw would be informative.

5.3.3 The impact of cryopreservation on platelet immune function

Platelet activation alters the surface phenotype of platelets which can influence the likelihood of PLA formation. Interestingly, the formation of platelet-THP-1 aggregates was increased post-thaw but still comparable to fresh components. PLA formation is primarily mediated by P-selectin exposure on the surface membrane, which binds to PSGL-1 on leukocytes [184]. Notably, only activated and procoagulant subpopulations, which exhibited P-selectin on the surface membrane, formed platelet-THP-1 aggregates during co-culture. In contrast, approximately 50% of cryopreserved platelets exhibited a novel phenotype post-thaw with negligible binding to THP-1 cells. Our findings highlight the importance of P-selectin in the formation of platelet-THP-1 cell aggregates.

This study was the first to demonstrate that cryopreservation induced changes in platelet phenotype increase the formation of PLAs during co-culture with a monocyte-like cell line (THP-1 cells) compared to fresh platelets. The formation of PLAs has been associated with a range of pro-inflammatory disorders and adverse events [182, 186]. However, PLA formation also facilitates a range of functions including the regulation of haemostasis, tissue repair and platelet clearance [62, 184, 243]. As such, further work is required to determine the immunological effect of cryopreserved PLAs. Additionally, in this study platelet-THP-1 cell interactions were examined immediately after thawing. However, cryopreserved platelets are currently approved for between up to 4-6 hours post-thaw [15, 106], with the potential to further extend this shelf-life using refrigerated storage [176]. Data presented in this dissertation, and previous work [33, 104, 244], highlights that post-thaw storage of platelets further alters the

surface abundance of leukocyte adhesion receptors. These changes may impact PLA formation. Importantly, future studies utilising primary cells, or a whole blood model of transfusion [115] are required to further examine functional impact of PLA formation by cryopreserved platelets.

5.4. Comparing the impact of refrigeration and cryopreservation on the immune characteristics of platelets

The data presented in this dissertation has demonstrated that refrigeration and cryopreservation significantly alter the immune characteristics and function of platelets compared to conventional storage. Key differences were observed between the immune characteristics of refrigerated and cryopreserved platelets, and functional attributes were investigated based on the specific changes observed. Accordingly bacterial versus leukocyte interactions were investigated with cold versus cryopreserved, respectively. As such, this final section hypothesises the potential impact that the opposite storage mode may have on the other functions, with reference to current literature and the findings reported in this dissertation.

5.4.1 The transfusion of refrigerated or cryopreserved platelets

The majority of current literature, including the studies included in this dissertation, compare refrigerated or cryopreserved platelets to conventionally stored components. In contrast, there are comparatively few studies directly comparing the effect of refrigeration or cryopreservation of platelets. Both methods differentially affect platelets haemostatic and immune characteristics [33, 75], which may make them more suited to the treatment of specific patient cohorts. Both refrigerated and cryopreserved platelets exhibit enhanced haemostatic function compared to conventionally stored components [188, 245]. Further, in this dissertation both refrigerated and cryopreserved platelet

components exhibited a significantly lower concentration of pro-inflammatory cytokines in the supernatant, potentially minimising the risk of adverse events. As such, refrigerated and cryopreserved platelets are likely suitable for the treatment trauma and surgical patients who often present with acute bleeding and are at greater risk of severe adverse events, including TRALI [100, 102, 246]. In contrast, conventionally stored platelets remain more suitable for prophylaxis as they exhibit lower clearance rates post-transfusion [98, 211, 212]. Both alternative platelet storage methodologies exhibit significant advantages over conventional storage. However, further work is required to determine how best to utilise refrigerated and cryopreserved platelet components.

5.4.2 The potential for cryopreserved platelets to aggregate with bacteria

During an examination of the immune characteristics of refrigerated platelets, a significant reduction in the abundance of pathogen recognition receptors (FcγRIIa, TLRs) was identified on the surface membrane. As such, the capacity of refrigerated platelets to aggregate with two common bacteria, *E. coli* and *S. aureus* was investigated. Unexpectedly, bacterial-induced aggregation was increased following platelet refrigeration, compared to components stored at room-temperature. The increase in bacterial-induced aggregation was linked the function of activated GPIIb/IIIa, which facilitates platelet-bacteria interaction indirectly through fibrinogen [6, 21, 52]. Platelet refrigeration has been shown to increase the baseline activation of GPIIb/IIIa and better preserve platelets capacity to activate it in response to agonist stimulation [126, 247]. Interestingly, previous work suggests that a small but significant proportion (10-30%) of

cryopreserved platelets exhibit activated GPIIb/IIIa on the surface membrane [178, 188, 237, 248, 249], which could potentially facilitate bacterial-induced aggregation. However, cryopreserved platelets display a negligible capacity to further activate GPIIb/IIIa following stimulation with haemostatic agonists (ADP, TRAP-6 and collagen) [237, 248]. This may potentially be due to the majority of platelets progressing to a procoagulant phenotype, which results in the inactivation of GPIIb/IIIa [250]. Whether this would limit the capacity of cryopreserved platelets to aggregate with bacterial strains where active GPIIb/IIIa is the main mediator of attachment would require experimental confirmation.

5.4.3 The potential of refrigerated platelets to form platelet-leukocyte aggregates

The formation of PLAs is primarily facilitated by the exposure of P-selectin on the platelet surface membrane, which binds to leukocyte bound PSGL-1 [184]. Approximately 50% of cryopreserved platelets expose P-selectin, which resulted in a higher number of platelet-THP-1 cell aggregates compared to fresh components. In comparison, between 20-30% of platelets exhibit P-selectin exposure after 24 hours of storage under refrigerated conditions [83-85, 90]. This increases to 40-60% at the end of 14 days of extended storage, which is comparable to post-thaw platelets [83-85, 90]. This finding suggests that refrigerated platelets would be more likely to form PLAs than fresh components, increasing further as storage progresses. Further, while refrigerated storage causes the gradual exposure of phosphatidylserine on the platelet surface membrane, it is reportedly 4-fold lower than cryopreserved platelets [85]. As phosphatidylserine is required to trigger leukocyte mediated phagocytosis [184],

it is likely that refrigerated platelets would be less likely to be cleared by this mechanism.

5.4.4 The potential impact of refrigeration on the responsiveness of platelets to activation by bacteria and leukocytes

In vivo, platelet interaction with bacteria and leukocytes can activate platelets leading to the release of soluble factors into the surrounding intercellular space, facilitating anti-bacterial function and leukocyte activation [5, 37, 48]. Consequently, the depletion of granular content during storage may reduce the ability of platelets to respond to immunological stimulation once transfused. Our findings align with current literature, demonstrating that refrigerated platelets release less soluble factors during storage compared to conventionally stored and cryopreserved platelets [33, 77, 227], suggesting that the granular contents are better retained. Refrigeration also better preserves the responsiveness of platelets to agonist stimulation [83, 84, 237], although whether this also facilitates degranulation is yet to be determined. As such, the retention of granular contents during refrigerated storage may allow for their targeted release post-transfusion, thereby promoting improved haemostatic and immune function.

5.4.5 The effect of alternate platelet storage methods and the risk of adverse events

At present, clinical trials are underway to examine the safety and efficacy of refrigerated (CHIPs, NCT04834414) and cryopreserved (CLIP-II, CT03991481) platelets [96, 117]. Currently available literature reports that refrigerated and cryopreserved platelet components appear to be safe for clinical transfusion.

However, more information, potentially from post-implementation haemovigilance programs will be required to determine the risk of low incidence adverse events. Nevertheless, adverse events have been linked to specific aspects of platelet immune function [11, 24, 34], which can provide insight into the potential risk factors associated with the transfusion of refrigerated and cryopreserved platelets.

The pathophysiology of adverse events is commonly explained by a two-hit model [251]. The first hit is caused by a pre-existing condition in the patient causing a pro-inflammatory environment such as cardiac disease, active infection, traumatic injury or recent surgery [246]. The second hit is delivered by the transfusion of a platelet component containing immunogenic antibodies or BRMs triggering pro-inflammatory signalling, leukocyte migration and PLA formation [12, 24, 182, 251]. Platelet activation during conventional storage is associated with an increased risk of adverse events post-transfusion [10, 11, 34]. However, this association is largely due to conventional storage causing both platelet activation and the release of pro-inflammatory cytokines (RANTES, HMGB1, sCD40L, OX40L, IL-27, IL-1β, mtDNA) into the supernatant [11, 24, 34, 76]. In contrast, while both refrigerated and cryopreserved platelets exhibit an activated phenotype, the concentration of pro-inflammatory cytokines was either equivalent or lower than conventionally stored platelets. As such, both refrigerated and cryopreserved platelets may carry a lower risk of adverse events than platelets stored at room temperature.

The formation of PLAs has also been associated with adverse events, particularly TRALI [9]. However, the role of PLAs in disease and adverse events is still a

matter of debate. It is currently unclear if PLA formation is symptomatic or causative [182, 252]. Further, it has yet to be determined if transfused platelets contribute to PLA formation. As activated platelets expose P-selectin, which increases the likelihood of PLA formation [184], both refrigerated and cryopreserved platelets may have a higher propensity to form PLAs, post-transfusion compared to conventionally stored platelets. Further, it is well established that both refrigerated and cryopreserved platelets exhibit faster clearance post-transfusion compared to conventionally stored components, which is partially facilitated by leukocytes in the circulation or the liver [81, 99]. Whether the removal of refrigerated or cryopreserved platelets from the circulation limits the potential for any immunogenic effects is currently unknown. As such, it remains unclear if an increase in the formation of PLAs due to refrigeration or cryopreservation induced platelet activation would influence the risk of adverse events.

5.5. Concluding remarks

This dissertation examined the effect of refrigeration and cryopreservation on the immune characteristics and function of platelets. A general screen of surface receptor abundance and soluble factor release was conducted in refrigerated and cryopreserved platelets. The data presented in this dissertation demonstrates that refrigeration and cryopreservation significantly alter the immune characteristics of platelets, albeit in different ways. Given the observed changes, the effect of storage conditions on platelet immune function was assessed. Refrigerated platelets exhibited increased responsiveness to bacterial-induced stimulation compared to conventionally stored platelets, despite a lower surface abundance of pathogen recognition receptors. Our findings linked this to the cold-induced activation of the GPIIb/IIIa receptor. Cryopreservation significantly increased the abundance of certain leukocyte adhesion receptors on the platelet surface membrane and led to the generation of phenotypically distinct subpopulations. Notably, the increase in the proportion of procoagulant platelets post-thaw correlated with an increase in PLA formation with a monocyte-like cell line (THP-1 cells). These results demonstrate that storage induced alterations in platelet characteristics can lead to changes in immune function *in vitro*. As these findings have the potential to influence the immune responses to platelet transfusion, platelet immune characteristics and function should be a consideration during the evaluation of new manufacturing and storage methodologies. These findings facilitate a greater understanding of platelet immune function and contribute to the ongoing pre-clinical evaluation of refrigerated and cryopreserved platelets.

5.6. Recommendations for future research

The data presented in this dissertation demonstrates that alternative platelet storage methods alter the immune characteristics and function of platelets. However, further work is required to build on these findings. Specifically, the following areas should be considered in future research.

- Refrigerated platelets exhibit better retention of their granular content compared to conventionally stored components. Further investigations should identify if refrigerated platelets can release soluble factors following stimulation by haemostatic or immunological agonists.
- Refrigerated platelets exhibited enhanced aggregation with bacteria. Bacterial-induced platelet aggregation can facilitate both bacterial clearance and immune evasion. As such, future studies should further examine the mechanics of refrigerated platelet-bacteria interaction and the potential clinical impact.
- Cryopreservation causes platelets to release significant amounts of EVs into the supernatant, which may be immunomodulatory. Future work should aim to examine the contents of cryopreserved platelet EVs.
- Platelet-bacteria and -leukocyte interaction was examined *in vitro*. However, examination of the effects of refrigerated and cryopreserved platelet interactions *in vivo* are required. Specifically, mouse models of TRALI and wound infection may provide clinically translatable information.
- Both refrigerated and cryopreserved platelets are being evaluated clinically. Further work should identify which patient cohorts would most benefit from the transfusion of alternatively stored platelet components.

- Our data suggests that refrigerated and cryopreserved platelets exhibit increased affinity for binding to bacteria and a leukocyte-like cell line, respectively. It may be advantageous to investigate whether this interaction could be of harnessed for other therapeutic benefits, such as the delivery of pharmacological agents or biotherapies.
- Refrigerated and cryopreserved platelets will primarily be used to treat acute bleeding, which is often associated with traumatic injury. Trauma is linked to platelet activation and the release of immunomodulatory soluble factors. As such, an examination of the interaction between donor and patient platelets may be informative.

6. Appendices

The appendices included in this dissertation contain supplementary information relevant to Chapter Four. Figures 1-3S were submitted as supplementary data in the following publication.

Winskel-Wood B, Padula MP, Marks DC and Johnson L. The phenotype of cryopreserved platelets influences the formation of platelet-leukocyte aggregates in an *in vitro* model. Platelets. 2023; Platelets. 2023; 34:1:2206916

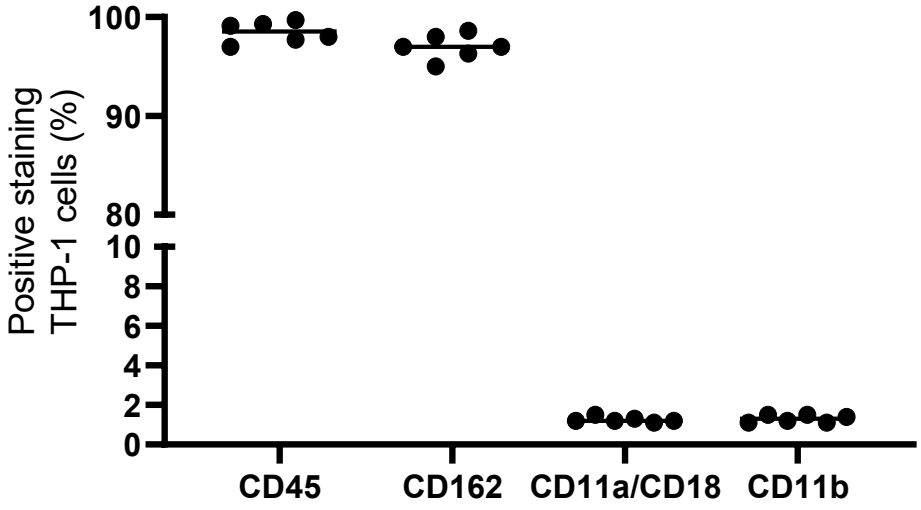


Figure 1S. The THP-1 cell phenotype was similar in all replicates tested prior to co-culture. THP-1 cells were stained with CD45-APC-H7, CD162-PE, CD11a/CD18-APC and CD11b-FITC and analysed by imaging flow cytometry at 60x magnification. The percentage of THP-1 cells positive for CD45-APC-H7, CD162-PE, CD11a/CD18-APC and CD11b-FITC was measured for each treatment. Each dot point represents the individual data from each replicate (*n* = 6) from a total of 5000 gated THP-1 cells events.

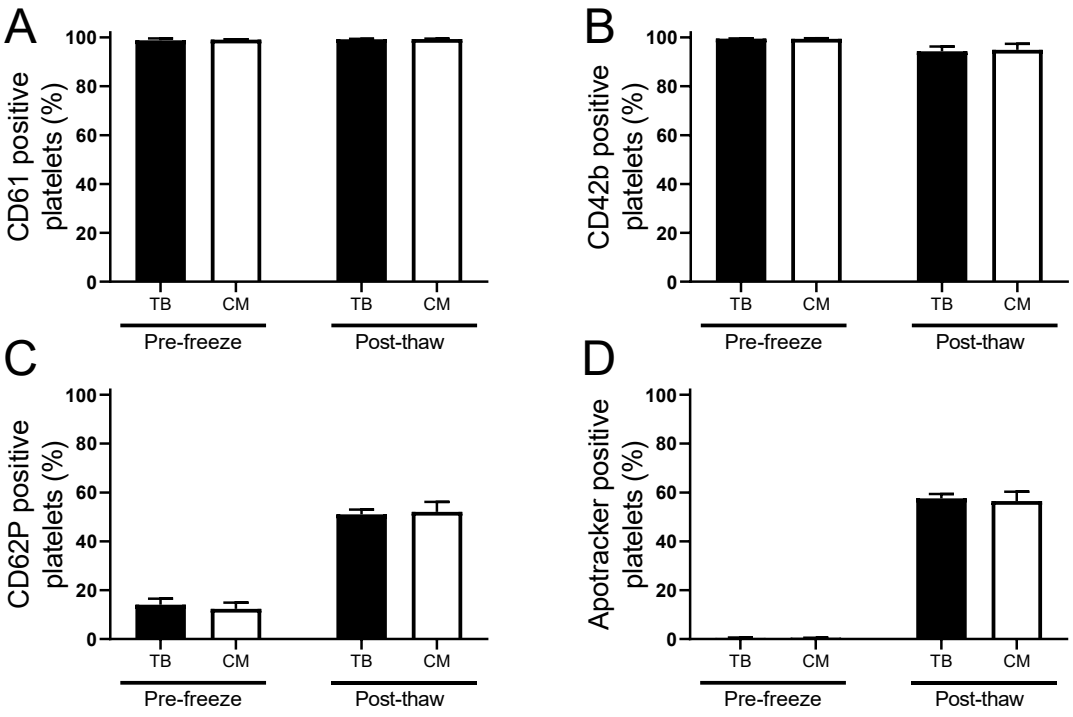


Figure 2S. The phenotype of platelets was not influenced by incubation in Complete Media. Pre-freeze and post-thaw platelets were diluted in Tyrode's Buffer (TB, ■) or Complete Media (CM, □). Platelets in TB were analysed immediately after dilution, whereas those in CM were incubated in a tissue culture incubator (37 °C, 5% CO_2) for one hour prior to staining to mimic co-culture conditions. Platelets were stained with Apotracker-Green, CD62P-PE, CD42b-PE-Dazzle 594 and CD61-APC and analysed by imaging flow cytometry at 60x magnification. Platelet events were gated based on area, aspect ratio and CD61-APC fluorescence parameters. Pre-freeze and post-thaw platelets were identified using the CD61-APC mask, and the proportions of A) CD61-APC, B) CD42b-PE-Dazzle 594, C) CD62P-PE and D) Apotracker-Green positive cells were determined. Data represent the mean + standard deviation (error bars, n = 3 in each group) of 10 000 gated platelet events. Significance was determined by one-way ANOVA. $p > 0.05$ for all treatment groups

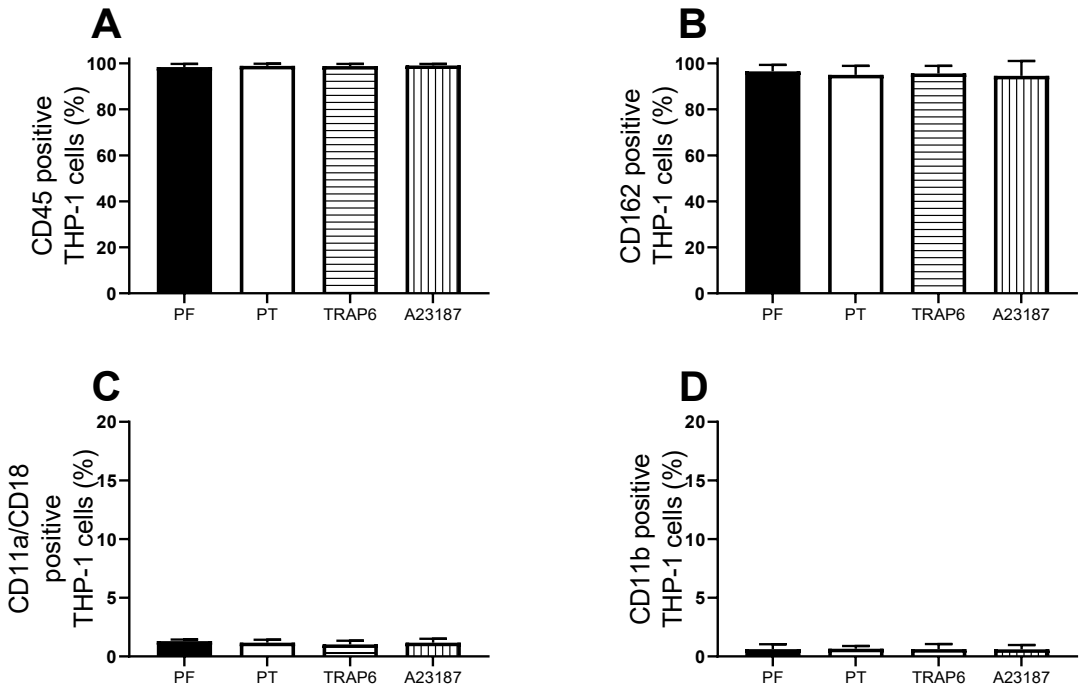


Figure 3S. The activation status of THP-1 cells is not affected by platelet co-culture for 24 hours. THP-1 cells were co-cultured with pre-freeze (PF, ■), post-thaw (PT, □), TRAP-6 stimulated (▤) or A23187 stimulated (▥) platelets for 24 hours. THP-1-platelet co-culture was stained with CD45-APC-H7, CD162-PE, CD11a/CD18-APC, CD11b-FITC and CD61-PE Dazzle 594 and analysed by imaging flow cytometry at 60x magnification. The percentage of THP-1 cells positive for A) CD45-APC-H7, B) CD162-PE, C) CD11a/CD18-APC and D) CD11b-FITC was measured for each treatment. Data represent the mean ± standard deviation (error bars, n = 4 in each group) of 10 000 gated THP-1 cells events per sample. Significance was determined by one-way ANOVA comparing each treatment. $p > 0.05$ between all treatments.

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