Détermination des prédicteurs de sévérité des effets indésirables receveurs au cours des transfusions de concentrés plaquettaires

Caroline Sut

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Sciences, Ingénierie, Santé

Spécialité de doctorat : Biologie moléculaire et cellulaire

Soutenue publiquement le 19/12/2017, par :

Caroline SUT

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Résumé

La transfusion sanguine est une thérapeutique ou un support thérapeutique indispensable pour laquelle il n'existe pas actuellement de substitut. La transfusion de produits sanguins labiles (PSL) est dans la grande majorité des cas très bien tolérée mais elle peut être à l'origine d'effets indésirables chez les receveurs (EIR) notamment de type inflammatoire dans un petit % de cas. Ceci dépend de plusieurs facteurs liés aux produits eux-mêmes et/ou liés aux receveurs de par leur prédisposition génétique éventuelle et de leur état clinique. Les concentrés plaquettaires (CP) transfusés sont la principale source de manifestations inflammatoires et/ou allergiques par rapport aux autres PSL (CGR et Plasma thérapeutique). Malgré la déleucocytation systématique de tous les PSL (en fait une leucoréduction à $\leq 10^6$ leucocytes résiduels par produit), ces réactions, bien que bien moindres que sans cette opération, subsistent. Ceci est notamment dû, en partie, à la capacité des plaquettes à sécréter une multitude de molécules ayant une activité pro- (davantage qu'anti-) inflammatoire. De plus, les processus de collecte, de préparation et de conservation induisent un stress vis-à-vis des cellules, qui peut activer les plaquettes et donc induire la production de produits de sécrétion inflammatoires dans les CP. Ces « lésions de stockage » provoquent notamment une sécrétion abondante de CD40 ligand soluble (sCD40L). Identifier l'ensemble des molécules impliquées dans ces réactions inflammatoires et préciser les mécanismes qui influencent sur leur production nous est apparu primordial pour améliorer les pratiques transfusionnelles et mettre en place des actions de prévention efficaces, notamment pour limiter les EIR.

Le but de ce travail de thèse a été dans un premier temps d'identifier les molécules les plus impliquées dans les manifestations inflammatoires. Ces études ont en particulier identifié le sCD40L comme étant largement impliqué dans les EIR après transfusion de plaquettes. Cependant, des concentrations élevées de cette molécule dans les CP transfusés n'induisent pas systématiquement des réactions chez les patients transfusés, et inversement des CP pauvres en sCD40L peuvent induire des EIR. Aussi, la composante inflammatoire de ces réactions est multifactorielle. Dans la deuxième partie de thèse, nous avons évalué le potentiel inflammatoire des CP sur l'endothélium vasculaire. Des différences d'activation des cellules endothéliales, dans un modèle *in vitro*, ont été observées lorsqu'elles sont en présence de surnageants de concentrés plaquettaires ayant induits un EIR.

Ce travail de thèse poursuit l'effort entrepris depuis plus d'une dizaine d'années de notre équipe de recherche, en vue de prédire la survenue d'EIR (et de la modéliser) et de

préciser les mécanismes qui influencent la physiopathologie plaquettaire transfusionnelle ; un corollaire de ces travaux est ainsi d'optimiser les processus de production et de conditionnement des CP transfusés afin de réduire ces réactions inflammatoires.

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Ac	Anticorps
ADP	Adénosine diphosphate
ADN	Acide désoxyribonucléique
ADNmt	ADN mitochondrial
Ag	Antigène
AMPc	Adénosine monophosphate cyclique
ANSM	Agence Nationale de Sécurité Médicale
ARN	Acide ribonucléique
ARNm	ARN messager
ATP	Adénosine triphosphate
BRM	Biological Response Modifier
CARD	Caspase Activation and Recruitement Domains
CCL	C-C Chemokine Ligand
CD	Cluster de différentiation
CP	Concentré Plaquettaire
CPA	Concentrés de Plaquettes d'Aphérèse
DAMP	Damage-Associated Molecular Patterns
DTS	Dense Tubular System
EIR	Effets indésirables des receveurs
ELISA	Enzyme Linked Immuno-Sorbent Assay
FcR	Récepteurs du fragment Fc
GP	Glycoprotéines
GTP	Guanosine triphosphate
HMEC	Human Microvascular Endothelial Cell
HMGB1	High Mobility Group Box 1
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM	Intercellular Adhesion Molecule
Ig	Immunoglobuline
IL	Interleukine
ITAM	Immuno-Tyrosine Based Activation Motifs
JAM	Junction Adhesion Molecule

LAMP Lysosomal Associated Membrane Protein

LPS Lipopolysaccharide

MAPK Mitogen-Activated Protein Kinases

MCP Mélange de Concentrés Plaquettaires

MCP1 Monocyte Chemoattractant Protein 1

MIP1α Macrophage Inflammatory Protein 1 α

MMP Matrix metallopeptidase

MP Microparticule

MPP Microparticule plaquettaire

NET Neutrophile Extracellular Traps

NFκB Nuclear Factor kappa B

NK Natural Killers

NLR Nucleotide-binding oligomerization domain-like receptors

NLRP Nucleotide-binding domain Leucine Rich repeat containing Protein

NO Oxyde nitrique

NOD Nucleotide-binding Oligomerization Domain

OCS Open Canalicular System

PAMP Pathogen-Associated Molecular Patterns

PAR Protease-Activated Receptor

PECAM Platelet Endothelial Cell Adhesion Molecule

PFC Plasma frais congelé

PF4 Platelet factor 4

PI3k Phosphoionositide 3 Kinase

PIP2 Phosphatidylinositol 4,5-bisphosphate

PIP3 Phosphatidylinositol 3,4,5-trisphosphate

PAS Platelet Additive Solution

PDGF Platelet-Derived Growth Factor

PLC Phospholipase C

PRR Pattern Recognition Receptors

PS Phosphatidylsérine

PSGL1 P Selectin Glycoprotein Ligand 1

PSL	Produit Sanguin Labile
RAGE	Receptor for Advanced Glycation Endproducts
RANTES	Regulated on Activation, Normal T cell Expressed and Secrete
RCPG	Récepteurs couplés aux protéines G
RFNH	Réactions Fébriles Non-Hémolytiques
ROS	Reactive Oxygen Species
RTK	Récepteurs à activité tyrosine kinase
SAMP	Self-Associated Molecular Patterns
SDF1	Stromal cell-Derived Factor 1
Siglec	Sialic acid binding Immunoglobulin-like lectins
SNARE	Soluble N-ethylmaleimide sensitive fusion Attachment protein Receptor
SSL	Staphylococcal Superantigen Like
TGFβ	Transforming Growth Factor β
TIMP	Tissue Inhibitor of Metalloproteinases
TLR	Toll-Like Receptor
TNFα	Tumor necrosis factor α
TPO	Thrombopoïétine
TRALI	Transfusion Related Acute Lung Injury
TXA2	Thromboxane A2
UTP	Uridine triphosphate
VAMP	Vesicle-Associated Membrane Proteine
VCAM	Vascular Cell Adhesion Molecule
vWF	von Willebrand Factor

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Introduction générale

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TRANSFUSION CLINIQUE ET BIOLOGIQUE

Transfusion Clinique et Biologique 24 (2017) 87–91

Research perspective

Determination of predictors of severity for recipient adverse reactions during platelet product transfusions

Détermination des prédicteurs de sévérité des effets indésirables receveurs au cours des transfusions de produits plaquettaires

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Abstract

The introduction of allogeneic cells is not a natural process, even if the transfusion is therapeutic and — when no alternative exists, as is often the case — essential. Transfusion of cellular products creates some level of danger sensed by recipients. Danger may manifest itself clinically or biologically, in which case we are dealing with recipient adverse reactions. Platelet concentrate transfusion in particular may be responsible for notable adverse reactions. Some appear to be inevitable, while others are tied to recipient factors: either health or genetic characteristics. The authors' research is specifically focused on platelet storage lesion and stress factors, and the means of controlling them to ensure greater recipient tolerance.
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Keywords: Transfusion; Platelets; Inflammation; Adverse reactions; Biological response modifier; Endothelial cells

Résumé

L'apport de cellules homologues (allogéniques) n'est pas physiologique quand bien même la transfusion est thérapeutique et essentielle en ce sens qu'il n'y a fréquemment pas d'alternative. La transfusion de produits cellulaires créée une situation de danger chez le receveur. Ces situations de danger peuvent occasionnellement s'exprimer cliniquement ou biologiquement : on parle alors d'effet indésirable receveur (EIR). La transfusion de concentrés de plaquettes en particulier peut être responsable d'effets indésirables notables, dont une partie semble évitable et une partie est liée à des facteurs du receveur, soit de par son état clinique, soit de par ses propres caractéristiques génétiques. Ce travail se focalise plus particulièrement sur les facteurs de stress ou lésions de stockage des produits plaquettaires et les moyens de les contrôler pour une meilleure tolérance par les receveurs.
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Mots clés : Transfusion ; Plaquettes ; Inflammation ; Effets indésirables receveurs ; Modificateurs du comportement biologique ; Cellules endothéliales

1. Introduction

In France, near 3,200,000 labile blood product (LBPs) transfusions take place each year. There are three kinds of LBPs: red cell concentrates (RCCs), platelet concentrates (PCs), and plasma for various therapeutic purposes. Most transfused LBPs are RCCs (79%), plasma comes in second (11%), and PCs follow

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close behind (10%) [1]. All LBPs are derived from voluntary, ethically obtained, unpaid whole blood or apheresis donations made by healthy donors. To ensure safe, optimal transfusion, strict rules apply to the collection, preparation, storage, and prescription of blood products.

During or following a blood transfusion — especially a platelet transfusion — the recipient may experience an adverse reaction (AR). The severity of ARs varies: they are most often benign, sometimes serious, and rarely lethal. Every LBP transfusion is affected by donor phenotypic characteristics, which exert effects during the process of preparing and storing the products; by recipient health characteristics; or by both. These parameters are the three variables of the transfusion triangle: donor–product–recipient. During preparation and storage of the PCs, accumulation of cytokines and chemokines may trigger an inflammatory reaction in the recipient [2].

Among the main cytokines/chemokines called Biological Response Modifiers (BRMs) are immunomodulatory molecules, many of which are pro-inflammatory and may be involved in several tissue pathologies [3]. These BRMs act as ligands for receptors on endothelial cells and most kinds of leucocytes that are in circulation or that adhere to the endothelium.

2. Platelet concentrates

Platelets play a specialized role in the haemostatic response that stops bleeding after damage to a blood vessel [4]. They also participate in immune responses by acting as modulators and mediators of the inflammatory response [5]. Platelets express receptors that bind ligands involved in various stages of innate or adaptive immune response [6], and they also produce soluble inflammatory factors [7]. PCs may be derived from whole blood or plateletpheresis donations. Each type is used to prepare a distinct product. Pooled whole-blood-derived platelet concentrates (PPCs) are obtained from the buffy coat layers of several whole-blood donations (presently limited to five in France). Apheresis platelet concentrates (APCs) are obtained using an apheresis machine that isolates the platelets from the blood of a single donor. In France, PPCs and APCs are stored in a mixture of 35% donor plasma and 65% additive solution. PCs are stored for five days or less at $22 \pm 2\,^{\circ}C$. They are constantly gently agitated to prevent platelet aggregation. All LBPs must undergo leucocyte reduction: after this process, there must be < 106 leucocytes per unit of product. LBPs are screened in compliance with EU and French standards, and they are labelled per regulations according to blood group.

3. Recipient adverse reactions

In spite of the strict precautions taken for LBP preparation and storage, there may be complications — i.e. ARs — following a blood transfusion. The principal ARs reported are alloimmunization, inflammation, excess blood volume, and infection. Though viral and parasitic infections are now rare, professionals are particularly apprehensive about bacterial infections — especially those associated with PCs. Inflammatory incidents include febrile non-haemolytic transfusion reactions (FNHTRs), allergic reactions, cardiovascular reactions (e.g. hypotension), and transfusion-related acute lung injury (TRALI) [8]. Though PCs account for only 10% of all LBP transfusions, they are responsible for 40% of all ARs [9]. Interactions between platelets, immune cells, and endothelial cells play a major role in the occurrence of an AR.

4. Platelets and inflammation

PCs are therapeutic products subject to regulatory and safety requirements. However, they contain immunomodulatory BRMs that may trigger ARs.

4.1. BRMs associated with platelets

Platelets contain soluble factors responsible for inflammatory or allergic reactions. It has been shown that during platelet transfusion, BRMs can induce immune responses [10] or post-transfusion reactions [11], affect haemostasis [12], or cause inflammation in recipients [13]. These substances — categorized as cytokines/chemokines — have immunomodulatory effects, and their secretions can vary depending on how platelets are activated. Which contents platelets release is a function of the kind of stimulus [14]. Although platelets are anucleate, they do contain α-granules, δ-granules, T granules, and lysosomes, which enclose molecules of many types involved in inflammatory and allergic reactions [15]. Some cytokines/chemokines and related molecules produced by platelets may play a part in the advent of an AR.

4.2. Identification of factors promoting BRM secretion

Concentration of BRMs in PCs is partly determined by the preparation and storage processes applied and by the age of the platelets. Platelets are subject to stress damage during collection, processing, and storage [16]. The kinds of stress to which platelets are subjected vary by PC preparation technique, and studies have shown that these techniques differ in their effects on pro-inflammatory reactions [17]. Here, we are interested in how preparation and storage affect platelet inflammatory properties, in order to optimize PC quality. Our team demonstrated that immunomodulatory factors are found in PC supernatants secreted during preparation and storage [2]. BRMs present during preparation of non-leucoreduced PCs are of three origins: from leucocytes, plasma, or platelets. PC leucocyte reduction considerably lowered the concentration of these factors, including TNF-, IL-6, and IL-8 [15], and consequently the incidence of ARs involving leucocyte cytokines. However, some cytokines are released by the platelets themselves during PC storage. These include sCD40L, PDGF-AA, RANTES, IL-6, and TGF-β [2]. In general, BRM production increases with length of PC storage. This may be linked to AR incidence, which has been observed to increase as storage time lengthens. To lessen this incidence, PCs should be transfused as soon as possible. Our team showed that the concentration of PC BRMs — sCD40L in particular — increases significantly starting on the third day of PC storage. These observations suggest that storage lesions have a major

effect on PC-induced inflammation. Furthermore, how PCs are processed during preparation and storage may affect platelet activation. Leitner et al. showed that the extent of initial platelet activation — as reflected in CD62P expression — was significantly greater when platelets were stored in Intersol™ than when other additive solutions (Composol™ et SSP + TM) were used [18]. Nevertheless, platelet storage in an additive solution has some benefits, including a reduction in the incidence of serious ARs [19]. Though PPCs and APCs are comparable in quality, there is disagreement as to which is safer. Daurat et al. showed ARs to be associated with PPCs less frequently than with APCs [20]. These findings suggest that APCs, currently in widespread use, should be limited to specific indications. For any given medical indication, a risk-benefit evaluation considering each kind of PC should allow prescription of the best product.

4.3. Identification of BRMs triggering ARs

PC transfusions transfer BRMs as well as platelets to recipients. Among these BRMs are soluble CD40 ligand (sCD40L), which has been described as partly responsible for FNHTRs following platelet transfusions [21]. We have shown that other soluble factors, such as IL-27 and sOx40L, are involved in FNHTRs [11]. Mathematical models using machine learning have shown ARs to be very reliably predicted by other soluble factors, including sCD40L, IL-13, and MIP-1α [22]. Indeed, this study shows that the concentration of sCD40L and IL-13 is correlated with the advent of ARs. In addition, the concentration of MIP-1α in supernatants associated with ARs appears to determine the kind of AR that occurs: NFHR or allergic reaction. PCs also contain mitochondrial DNA (mtDNA), which has been linked to ARs [23]. The correlation between BRMs and mtDNA can identify the AR [24]. The goals of our current research are to define the mechanisms by which platelet BRMs lead to ARs and to identify the main molecules behind these reactions. This would allow detection of high-risk product-patient combinations and construction of bioclinical decision trees for prescribing a specific PC to a given patient in light of his or her clinical history.

5. Interaction between platelets and endothelium

Under normal physiological conditions, circulating platelets do not adhere to intact endothelium. The adhesion of platelets to the endothelium is an important response to vessel damage, infection, or inflammation [25]. Platelets thus play an essential role in vessel repair and maintenance of haemostasis — especially primary haemostasis. Platelet membrane integrins can interact with elements of the damaged endothelium, causing the platelets to adhere. This leads to their activation and aggregation, resulting in the formation of a thrombus, which consists of platelets linked by fibrinogen (Fg). The thrombus, in association with other blood cells, seals the vessel breach [26]. When inflammation occurs, endothelial cells produce more thrombin, which triggers the release of storage granules known as Weibel-Palade bodies that contain von Willebrand factor (vWF) and P-selectin [27]. Any disruption of the endothelium can cause it to interact with platelets by very rapidly modulating the expression of adhesion molecules (i.e. E-selectin, ICAM-1, and VCAM-1). The most widely known function of these adhesion molecules is to promote leucocyte 'rolling', which brings the leucocytes to a stop at the site of the activated endothelial cells [28]. Frenette et al. showed that, like leucocytes, platelets can also roll along endothelium activated by calcium ionophore or an inflammatory cytokine like TNF-α [29]. Calcium ionophore induces release of Weibel-Palade body contents and the expression of P-selectin at the endothelial surface [5]. P-selectin that is expressed on the surface of activated platelets can also contribute to adhesion by allowing platelet interaction with the endothelium via the endothelial ligand induced by TNF-α [30]. After platelets have been activated by thrombin, they may adhere to the endothelium via GpIbα, which binds to its ligands (fibrinogen, fibronectin, and vWF) [31]. These ligands can in turn be bound to their endothelial receptors (i.e. ICAM-1 and αvβ3) [32]. When vWF is released and bound to its platelet ligand, GpIbα, platelet adhesion to infected endothelial cells — versus healthy cells — is greatly enhanced [31]. GpIbα has been identified as a platelet ligand of P-selectin, as well as of vWF [33]. GpIbα can promote platelet adhesion to the cells of activated endothelium and, when endothelial cells have been removed by a vessel rupture, to the subendothelium. Under inflammatory conditions, newly synthesized E-selectin is also found on the surface of endothelial cells. P-selectin and E-selectin appear to jointly promote platelet rolling [34]. Among the various selectin ligands that have been identified, PSGL-1 has been best described. It is expressed on the surface of platelets [35].

Activated platelets can promote the expression of various inflammatory mediators on endothelial cells. Activation of platelets significantly increases the secretion of IL-1β, MCP-1, MIP-1α, and ICAM-1 [36]. Moreover, the interaction of platelet CD40 ligand (CD40L) with CD40 expressed on endothelial cells causes these cells to release IL-8, tissue factor, and MCP-1. Binding with CD40 on endothelial cells also leads to greater expression of several kinds of adhesion molecules, including E-selectin, VCAM-1, and ICAM-1. The binding of platelet CD40L to endothelial CD40 ramps up production of inflammatory cytokines, adhesion molecules, and matrix metalloproteinases (MMPs) [37]. MMPs break down various proteins of the extracellular matrix (ECM) and promote inflammation and destruction of the inflamed tissue. Thus, sCD40L derived from platelets induces activation of MT-MMP-1 (membrane-type MMP-1), MMP-1, MMP-2, and MMP-9, which leads to breakdown of endothelial cell ECM. Furthermore, the MMP inhibitor TIMP-2 interacts with MT-MMP-1 and MMP-2 to form a complex after stimulation of platelets. It has been shown that platelets express this complex and that MMP inhibitors can modulate aggregation [38].

5.1. Effects of activated platelets on endothelium

Platelets secrete many BRMs, which act as ligands for receptors on endothelial cells and most of the leucocytes that are in circulation or that adhere to the endothelium. When there is an infection or inflammation, platelets adhere to the endothelial cells. Platelet adhesion to the endothelium leads to activation

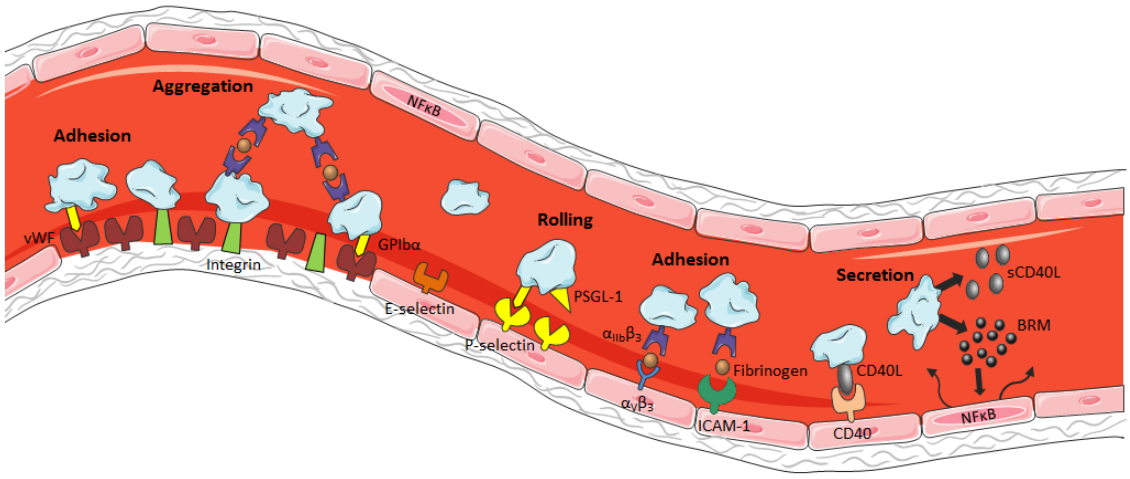

Fig. 1. Interaction of platelets and endothelial cells in event of vessel rupture or infection. At sites of vessel damage, platelets adhere to the subendothelial matrix. Platelet GPIbα binds to subendothelial vWF. Platelets may also interact directly with collagen via integrins such as GPIa/IIa, GPIV, and GPVI. Platelets aggregate through the action of fibrinogen, which binds to the integrin αIIbβ3, found on activated platelets. Activated endothelial cells express P-selectin, which enhances platelet rolling via GPIbα, and possibly PSGL-1. After changes to their conformation, αIIbβ3 integrins cause platelets to firmly adhere by binding to fibrinogen or vitronectin, both of which interact with the endothelial adhesion molecules $\alpha v\beta 3$ and ICAM-1. This causes exposure of P-selectin at the surface of platelets and the release of sCD40L and BRMs, including IL-1β, which stimulate endothelial cells to establish inflammatory conditions.

of endothelial cells and release of soluble factors. During PC transfusion, the extent of platelet activation is a function of the different variables described above. Transfused platelet BRMs can interact with the recipient's endothelial cells, leading to an inflammatory reaction in the recipient. It is useful to evaluate the degree of endothelial cell activation by different platelet supernatants — whose composition varies according to the PC preparation and storage processes applied. Broadly, interaction of platelets with the endothelium triggers a cascade of intracellular signalling events that lead to the secretion and release of products from both platelets and endothelial cells, which in turn prolongs inflammation (Fig. 1).

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Etat de la littérature

Chapitre 1 - Transfusion et inflammation

La transfusion de produits cellulaires est une thérapeutique ou un support thérapeutique fréquent, globalement très sûr. Cependant, une transfusion de PSL (CGR, CP ou PFC) peut parfois être perçue comme une source de danger par l'organisme receveur. Ces situations de danger peuvent occasionnellement s'exprimer cliniquement ou biologiquement, et être à l'origine d'incidents, ce sont en particulier des manifestations de type inflammatoire [1]. Ces effets inflammatoires, bien que rares, se rencontrent notamment lors de transfusion de concentrés plaquettaires (CP), et également de concentrés de globules rouges (CGR). Les plaquettes sont capables de secréter elles-mêmes une grande quantité de produits inflammatoires. La collecte des produits sanguins, leur préparation ainsi que leurs conditions de stockage sont des éléments pouvant induire un stress pour les cellules, pouvant entraîner leur activation ainsi que la libération de diverses molécules et donc promouvoir des réponses inflammatoires [2]. L'amélioration constante de ces processus liés à la préparation, notamment la leucoréduction systématique des PSL, a permis de réduire considérablement ces effets inflammatoires [3, 4].

Identifier et prévenir ces manifestations inflammatoires chez des patients fragilisés est indispensable afin d'améliorer la médecine transfusionnelle. En effet, l'apport de cellules homologues (allogéniques) n'est pas physiologique ni anodine sur le plan clinique, quand bien même la transfusion est thérapeutique et essentielle en ce sens qu'il n'y a fréquemment pas d'alternative.

La revue suivante vise à présenter une mise à jour en hématologie et en immunologie qui aide à comprendre les manifestations inflammatoires lors de transfusion, et de préciser les caractéristiques des globules rouges, et plus particulièrement des plaquettes qui contribuent à l'inflammation.

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REVIEW
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Transfusion as an Inflammation Hit: Knowns and Unknowns

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Transfusion of blood cell components is frequent in the therapeutic arsenal; it is globally safe or even very safe. At present, residual clinical manifestations are principally inflammatory in nature. If some rare clinical hazards manifest as acute inflammation symptoms of various origin, most of them linked with conflicting and undesirable biological material accompanying the therapeutic component (infectious pathogen, pathogenic antibody, unwanted antigen, or allergen), the general feature is subtler and less visible, and essentially consists of alloimmunization or febrile non-hemolytic transfusion reaction. The present essay aims to present updates in hematology and immunology that help understand how, when, and why subclinical inflammation underlies alloimmunization and circumstances characteristic of red blood cells and – even more frequently – platelets that contribute inflammatory mediators. Modern transfusion medicine makes sustained efforts to limit such inflammatory hazards; efforts can be successful only if one has a clear view of each element's role.

Keywords: inflammation, transfusion, allergy, blood components, leukocytes, platelets, erythrocytes, alloimmunization

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INTRODUCTION

Historically, inflammation was viewed as the compendium of all four stigmas: "*rubor*, *calor*, *dolor*, and *tumor*"; this concept fits well with the theory of humors; bloodletting – and surrogates (e.g., leeches and suction cups) – have long been applied to treat, if not cure, inflammation symptoms. As a matter of fact, iron depletion caused by bloodletting happened to alter bacterial growth and ameliorate certain disease conditions, as already observed by Tissot in 1761 (1). The Hippocratic theory of humors was probably the first to introduce the relationship between blood and inflammation, though using wrong descriptors. In its earliest days, transfusion was clearly associated with acute inflammation, though the connection was not acknowledged as such: indeed, the very first reported serious adverse events (SAEs) of "modern" transfusion in the early twentieth century were dual in nature: first, immune-hematological [i.e., antigen–antibody (ABO)] conflicts, and second, blood-borne and blood-transmitted infections, such as syphilis and malaria (2). Both conditions – presenting as very severe – were later on acknowledged as being dominated by cytokine storms and standing for acute inflammatory reactions (often lethal) (3, 4).

The concept of inflammation has been largely revisited by modern internal medicine; series of autoimmune and auto-inflammatory diseases have thus been acknowledged. No organ-specific disorder is actually beyond the scope of the large clinical inflammation spectrum, since a number of

neurological disorders (5) – as well as many cardiovascular lesions especially the atheroma plaque deposit (6) – are inflammation stigmas. The causality of inflammation in organ-specific lesions is being questioned, but combinations of genetic predisposition, lifelong hygienic habits, other environmental factors, and infectious triggers are commonly evoked. For decades now, clinical inflammation has not been restricted to acute Hippocratic symptoms and is acknowledged to present as more subtle symptoms of varying degrees.

We believe two major achievements have helped reconsider clinical inflammation as it may apply to transfusion medicine and cell, tissue, and organ transplantation. Neither was intended to apply to this discipline; however, the first is the (re)discovery of the danger signal theory, as proposed by P. Matzinger at the NIAID, NIH, in the 1990s, after its seminal conceptualization by E. Metchnikoff at the Pasteur Institute 100 years earlier. This discovery is basic to immunology and helps reframe the reading of immunology (7). The second is the conceptualization of the microbiota's role in immunity – initially presented as governing what Ph Sansonetti (at the Pasteur Institute in Paris) called "war and peace" at the mucosal surfaces. This concept helped show that inflammation spans the whole spectrum, from physiology to pathology (8–10). It has since been suggested that healing (e.g., of tissue attrition or organ lesions) is the ultimate step of inflammation (11, 12).

TRANSFUSION AND INFLAMMATION: FROM BEDSIDE TO BENCH

From the bedside, one can consider two periods in relation to transfusion-related hazards, especially inflammation. The initial period concerns acute symptoms of SAEs: inflammation is observed among other symptoms such as shock. Those accidents were principally reported with reference to their major cause(s): the ABO conflict, transfusion-transmitted bacterial, viral, or parasitic infections, and allergy. In 1983, a novel cause of transfusion-transmitted SAE was described: *transfusion-related acute lung injury* (TRALI) (13–15). Interestingly, this SAE is ascribed to a dual cause: an Ag/Ab conflict – within the human leukocyte antigen (HLA) or, more rarely but more severely, the human neutrophil antigen (HNA) systems – and an inflammatory layer: sepsis, stress, etc. Besides conflicting Abs (when identified, i.e., in two out of every three cases on average), the principal actors are leukocytes recruited or residing in lung capillaries. The TRALI concept prompted a reinvestigation of SAEs in transfusion and acknowledgment of serious inflammatory cases. This is also true for allergy: though one cannot exclude the possibility of pathogenic IgE transfer, it is rather felt that such an occurrence cannot account for one-third of adverse events (AEs) varying in severity (16, 17). Transfusion allergy is, in general, considered to present like allergy, though it is not believed to have a link with atopy or involve allergens or Abs specific to allergens. It is recognized as one of the most frequent inflammatory consequences of transfusion (18).

In summary, despite this is over-simplistic, one may acknowledge that inflammation symptoms manifested by a transfused patient and in relation with the transfusion process has two principal causes: it is either due to the transfer of pathogenic material collected from the donor or it is due to a conflict between high affinity receptors found on the recipients' cells or plasma molecules and ligands brought by the transfused component.

The majority of AEs in patients receiving blood (recipients) manifest either allergy or febrile non-hemolytic transfusion reactions (FNHTRs), both being clearly inflammatory conditions (19). Leukocytes transferred with blood were ascribed to as the principal causes of TT inflammation. Systematic "leucoreduction" – often inappropriately, but nevertheless officially, termed "leucodepletion" – was proposed at the start of the millenium by many countries or blood transfusion systems. However, leucoreduction has neither been become recommended nor a mandatory practice for mitigating inflammatory responses but is instead used to limit transfusion-transmitted viral risks as many "serious transfusion-associated viruses" are intracellular. Leucoreduction was principally aimed to reduce the risk of transmitting the Creutzfeldt–Jakob prion (20). Veterans of transfusion medicine very well recall the time when every single transfused patient was "shaking and heating," manifesting common symptoms that were subsequent to the therapy and introduced as such to patients (when patients happened to receive information). Pre-storage leucoreduction was then acknowledged to have largely improved comfort and safety in patients, suggesting a deleterious role for leukocytes (21, 22). When leucoreduction is performed post-storage (e.g., at the bedside, prior to the infusion of the blood component), inflammatory manifestation is intermediate, largely suggesting that not only leukocytes but also their secreted content play a role in the transfusion inflammation pathophysiology (23).

However, as the transfused patient profile changed, more and more recipients benefited from platelet components (PCs). This major change took place more or less at the same time as the implementation of systematic hemovigilance, and it soon became obvious that PCs – though representing no more than 10% of issued blood components – provide between one-quarter to one-half of reported AEs (24). This means that leukocytes were not the only cells associated with transfusion-associated inflammation.

Another population of patients benefiting from frequent transfusion episodes, sickle-cell disease patients, led to an important discovery: first, they manifest complex hemolytic reactions that involve activated complement and present as essentially inflammatory (25) and second, they are subjected to the most frequent rate of alloimmunization among tracked cohorts of transfused patients (26). This prompted specialists to also examine the inflammatory potential of stored erythrocytes.

THE MAJOR IDENTIFIED CAUSES OF TRANSFUSION-ASSOCIATED INFLAMMATION

There is good evidence in favor of an undesirable role for residual leukocytes in transfused patients; blood banks can get rid of such residual leukocytes with a high degree of efficacy by using filtration methods. Leucoreduction is highly recommended by the European Community and the American Association of Blood

Banks with a target of no more than 10^6 residual leukocytes per blood component after filtration (27, 28); most pre-storage methods allow scores of leucoreduction ranging between 2 and 5×10^5 residual leukocytes per component (29). However, clinical observations suggest that other constituents of pre-stored and leucoreduced cellular blood components still lead to some inflammatory manifestations in patients.

To simplify and summarize, pro-inflammatory factors in labile blood components fall into one of the following four categories: (1) infectious pathogens transmitted by blood that cause bacterial sepsis, acute or chronic viral infection, or acute parasitic infection (and often hemolysis); (2) pathogenic, undesirable, Abs (causing hemolysis when encountering target Ags, especially when capable of binding complement; causing TRALI, depending on circumstances or predisposition; causing Reagin-mediated allergy; and causing a number of non-hemolytic situations, now ascribed to FNHTRs); (3) leukocytes and their content, and especially their high loads of pro-inflammatory cytokines, chemokines, and the like, collectively termed *biological response modifiers* (BRMs); and (4) (pro)inflammatory material linked to platelet and erythrocyte pathophysiology, especially when cellular blood components [PCs and packed red blood cell components (pRBCCs)] are stored over time and undergo so-called storage lesions, which consist of extracellular vesicle emission and the freeing of membrane-bound molecules and intracellular content, either iron (erythrocytes) or BRMs (platelets). It is not unusual that extracellular vesicles are called microparticles.

The first two categories are chiefly inflammatory, involving two principal mechanisms: first, the triggering of a cytokine storm, with broad consequences for all systems, exposing the patient to multivisceral failure and severe central neurologic disorders. Second, if erythrocytes are ultimate targets of the Ab or infectious pathogens, there is acute hemolysis with obvious consequences. This essay will not further discuss such cases. Neither will it discuss the specific case of bacterial contamination of PCs, largely related to the storage temperature of 22 ± 2°C; 18.5 severe cases per million PCs delivered are recorded annually according to the latest French hemovigilance records; and one such case happens to be lethal, on average (30).

The latter two categories reveal that inflammation is not only the result of substantial levels of BRMs secreted by leukocytes, platelets, or lysed erythrocytes but also of products secreted as a consequence of cell–cell encounters after the blood component has been transfused. Cell–cell interactions occur mainly between (i) donor transfused cells and recipient circulating cells and (ii) donor transfused cells and recipient vascular endothelium cells. Transfusion is a dynamic process, but it is often regarded as the passive infusion of therapeutic components (31). This view is misleading and a source of errors for the interpretation of transfusion-associated inflammation.

MODELS OF TRANSFUSION-ASSOCIATED INFLAMMATION HIT

This section will address three main issues: (1) how transfusion can act as a stress for the recipient, subsequently triggering an immune defense; (2) how blood components can present with varying degrees of stress signals, accompanied by pathogenic storage lesions; and (3) how the stage is set for a recipient's adaptive immune response to donor cell Ags. These three points parallel three major aspects of the transfusion process or chain: donor-linked characteristics, additional pathogenic steps during blood component production, and recipient-linked characteristics.

Transfusion as a Stress and Donor-Linked Characteristics Account for Recipients' Inflammatory Symptoms

Transfusion is an unnatural process in the sense that the exchange of body parts between individuals – other than mothers and their embryos or fetuses – is not part of the human evolutionary program. Each individual's blood has potentially unique biological characteristics. Thus, when foreign cellular material, is tentatively grafted into a recipient, the latter identifies it as foreign and potentially dangerous, even when it has a therapeutic purpose. Indeed, many studies have shown that platelets express a large variety of pathogen sensors, promptly engaged by several kinds of the so-called pathogen-associated molecular pattern (molecules) or PAMPs (if stresses are infectious in nature) or damage-associated molecular pattern (molecules) or DAMPs (if stresses are internal, such as Abs). This has been principally found relative to platelets (32, 33), and similar findings have been reported for erythrocytes (34). Furthermore, donor platelets express HLA class I Ags that differ in general from those of recipients. Donor cells are thus likely to be sensed as foreign by recipients' circulating and vessel-lining leukocytes, which are prone to signaling this through a pro-inflammatory response, or by vessel endothelial cells. Experimental data suggest that endothelial cells can also signal the detection of foreign material by mounting a pro-inflammatory response (35–38). In general, though it is still difficult to link with certainty a host's innate inflammatory response with unmanipulated donor cells, the danger theory of innate immunity would largely predict it in transfusion.

Recent data offer newer evidence supporting the hypothesis. First, a large Canadian clinical trial recently reported that age and sex of donors influenced the outcome of transfusion in recipients, more than any other factor (e.g., age of blood or pathology) (39, 40). Although there is now good evidence that there are differences between males and females in pathology and in particular in immune responses to infection or vaccines and inflammation processes, the gender issue has not been specifically addressed satisfactorily in transfusion medicine (41): this is perhaps a path for further investigation.

Our own investigations have shown that donors present great variation in the genes coding for CD40L; CD40L was investigated because platelets are the major purveyors of sCD40L in the body (42), and this BRM influences both innate and adaptive immunity (43). CD40L gene polymorphism was found to influence the presentation of secreted CD40L (44). It has been hypothesized that this genetic characteristic of donors may affect pro-inflammatory secretion of donated platelets in BCs (45, 46). This type of result is plausible as well for other BRMs.

Blood Component Manufacturing and Storage Lesions with Pro-inflammatory Consequences in Recipients

A large body of reviews has documented this topic. We may consider two sets of data for illustrative purposes: one explores the secretory capacity of stored platelets over time or of platelets undergoing stress lesions upon collection, processing, and storage (47–50); the other explores the age of blood – and more precisely, the age of pRBCCs – at delivery. Both data sets incorporate diverse readouts: BRMs, oxidants, free iron, and extracellular vesicles (51–54). Despite such extracellular vesicles are reported to carry pro-inflammatory factors (55), some anti-inflammatory properties of extracellular vesicles have been reported (56), suggesting a fine-tune balance of inflammatory responses in relation of extracellular vesicles, likely depending on their sizes (57), origin, and abundance.

To summarize, it is generally reported that longer PC storage is accompanied by greater production of pro-inflammatory cytokines, which make up the majority of anti-inflammatory products (58–62) (**Table 1**). If leukocytes are still present in the PCs, leukocyte- and platelet-originating cytokines and other BRMs potentiate each other over time (63). Our own group has reported that there is a direct relationship between (i) the secretion of sCD40L [proved to exert a pathogenic effect in certain recipients, together with companion BRMs Ox40L and IL-27 (64, 65)] and the component shelf life; and (ii) between the net amount of sCD40L (alongside potentiating molecules IL-13 or MIP-1α) and the manifestation of an inflammatory AE in the recipient (62). Similar findings exist for mitochondrial DNA (66–68). In addition, it has also been proposed that the techniques used to obtain PCs influence pro-inflammatory reactions, as these techniques do not expose platelets to the same stress (62, 69). Together, these observations strongly suggest the platelet storage lesions have a role in PC induced inflammation and its balance in transfused patients.

The situation for erythrocytes is even more complex. Substantial experimental evidence suggests that the age of erythrocytes, and the subsequent freeing of iron, is directly responsible for inflammation in experimental models, both *in vivo* and *ex vivo/in vitro* (70–74). Thus far, however, clinical trials have consistently failed to support this hypothesis (75). It should nevertheless be noted

TABLE 1 | Blood product storage and biological response modifier release.

	Packed red blood cell concentrates	Platelet concentrates	Plasma for direct therapeutic use
Usual storage time	42 days	5 days	1 year
Main product transformation	Leucoreduction Irradiation Pediatric preparation Deplasmatization/washing Volume reduction Cryopreservation Reconstituted blood	Automated cell separation Centrifugation Leucoreduction Platelet additive solutions Occasionally pathogen Reduction or inactivation technology Irradiation Deplasmatisation/washing Cryopreservation Volume reduction	Leucoreduction Freezing/thawing Solvent-detergent Chemical and light pathogen inactivation Lyophilization
Lesion storage	Shape changes from a normal biconcave disk to echinocytes and spheroechinocytes ↑ Ammonium ↑ Free Hb in plasma ↑ K+ from ↓ ATP ↓ 2,3 DPG to <10% of original levels – replenished ↓ Labile proteins, e.g., complement, fibronectin, and coagulation factors ↓ to negligible ↓ Na+ ↓ pH ↓ NADH ↑ Bioactive substances (free Hb, hemin, microvesicles, iron, cytokines, lipids, and enzymes) ↓ S-nitrosohemoglobin (SNO-Hb) bioactivity	Shape changes from discoid to spheroid ↑ Activation (↑ release of granular contents) ↑ Proteolysis Altered platelet surface receptor expression ↑ Platelet aggregates Decreased mean platelet volume (MPV) ↑ Volume and density heterogeneity ↑ Procoagulant activity ↑ Platelet apoptosis ↓ pH, pO_2, and glucose ↑ pCO_2 ↑ Lactate production ↑ Glucose consumption ↓ Calcium ion flux ATP/ADP ratio change ↓ Mitochondrial oxidative respiration ↓ Fibrinogen binding	↑ Proteases ↑ Oxidation of Pro, Arg, Lys, Thr, Glu, or Asp side chains ↑ Cleavage of protein backbone ↑ Incorporation of lipid peroxidation products into Cys, His, or Lys residues ↑ Formation of advanced glycation end products ↑ Lipid peroxidation
Released/ increased factor	MPs, IL-8, TNF-α, RANTES, NAP-2, Gro-α, MIP-1α, SDF-1, ENA-78, TGF-β, … Microvesicles	EGF, ENA-78, Gro-α, IL-1β, IL-6, IL-7, IL-8, IL-27, Lyso-PCs, sOX40L, PAI-1, PDGF-AA, PF4, RANTES, sCD40L, TGF-β, TNF-α, VEGF, β-TG, … Microvesicles Mitochondrial DNA	MPO, ECP, and histamine increase after thawing IL-1β, IL-4, and IL-10 increase with freeze/thaw cycles MMP-7 increases with the number of freeze/thaw cycles IL-4, IL-12, and TNF-α increase with the number of freeze/thaw cycles …

that this is extremely difficult to investigate and that further trials are necessary to resolve the matter (76).

Consequences of Inflammation in Recipients: Manifestations of Adaptive Immunity

There are two main consequences of inflammation in blood component recipients. One – alloimmunization to foreign Ags – is rather clear. The other is transfusion-related immune modulation or TRIM. TRIM is a complex occurrence that involves a number of adaptive immune tools, of which suppressive CD8+ T cells, regulatory T (and probably B) cells, anti-idiotypic T cell clones, along with soluble HLA molecules and other supposed mediators (77–79). It must be made clear that, if the main visible consequence of immunization to foreign Ags is alloimmunization, there is a likely strong T cell immunity; however, it seems difficult to catch it up, and the majority of published works focus on Ab production. One may hypothesize that T cell immunity and TRIM rather explain cases where immunization is not productive in terms of Ab formation. Another consequence of TRIM is perhaps the likely depression of immune surveillance with the report of suspected increase of posttransfusion infections (that are quite well documented) and perhaps malignancies or organ dysfunctions (that are to be ascertained) (80–83).

Alloimmunization remains the most frequently reported AE of transfusion. It is often reported in pathologies where extended matching of red cells is difficult to achieve, for people needing repeated transfusions, such as sickle-cell disease or β-thalassemic patients. Indeed, as there are near 350 Ags on erythrocytes, a perfect match is unlikely. Recipient characteristics such as how well one presents HLA are also considered to have a major impact (84). In addition, residual leukocytes are highly potent immunizers (far more commonly in HLA groups than in HNA groups); platelets are also good immunizers in both HLA class I and HPA groups (85). However, it has been demonstrated that residual leukocytes can influence the global immunization score: as shown in experimental models, stringent but incomplete leucoreduction minimizes alloimmunization, while strict leucoreduction reinforces it, supposedly by erasing the TRIM effect (84).

Yet in spite of several attempts to decipher innate immune mechanisms acting as layers of inflammation that fuel Ag presentation, the details of alloimmunization largely remain a mystery (86).

Last, another consequence of inflammation is the reported enhanced erythrocyte phagocytosis by spleen cells. Inflammation created by excess iron and nitric oxide (NO) freeing – perhaps linked with aged erythrocytes – would aggravate anemia instead of correcting it by bringing Hb/O_2 (87). While there is good experimental evidence in favor of this pathophysiology, clinical relevance is not yet ascertained.

FROM BENCH TO BEDSIDE: PATHS TO IMPROVE PATIENTS' SAFETY

Pathophysiological studies of platelets reveal that any exposure to stress may have consequences. Depending on the nature of this stress, platelets can mobilize predefined patterns of BRMs (88, 89). Indeed, contrary to what we might expect, given that they are anucleate, platelets do not indiscriminately release granule content through an all-or-nothing mechanism but rather exhibit stress-dictated processes (32, 90–93). This may explain why certain patients having received PCs manifest allergic reactions (where δ-granule BRMs predominate) or FNHTR/inflammation (where α-granule BRMs predominate) (**Figure 1**; **Table 2**). A readout in the PC leftover is given by the predominance of either IL-13 or MIP-1α in addition to sCD40L (62). Platelets are extremely reactive cells, and it is almost impossible to not pre-activate them while processing PCs for transfusion purposes. However, it has been made clear that the collection process, i.e., apheresis vs. recovered platelets from whole blood; platelets recovered from platelet-rich plasma vs. from buffy coats; PAS vs. plasma; pathogen reduction/inactivation vs. no additional safety measure; and in the case of apheresis, type of cell separator – and the length of storage are important parameters to control activation (59, 62, 65, 94–97). Despite conflicting data (98), it cannot be ruled out that ABO compatibility vs. identity also affects the outcome of platelet transfusion (99, 100), but probably not by triggering pre-activation. Furthermore, PCs are almost always HLA incompatible, at least for the majority of expressed class I Ags. This has not appeared to be deleterious in terms of clinical outcome – in the PLADO trial (98), for example, but consistent recommendations suggest that refractoriness to platelet transfusion is better addressed with HLA-compatible PCs (97). The HPA case is barely addressed, unless a specific and pathogenic Ab is identified, or in the case of fetal/neonatal maternal incompatibility (101). Serious allergies, allergic reactions, or severe FNHTRs can be addressed – when further PC transfusions are needed – by washing the components. This process is nevertheless tedious as it may itself pre-activate the cells. Alternatively, some teams may absorb pathogenic BRMs on columns when available (102, 103). At present, there are parameters that cannot be controlled (recipients' genetic characteristics and – to a large extent – donors' characteristics) and those that can be partly controlled, i.e., manufacturing, ABO matching, and aging of the PCs (**Table 3**). Most efforts appear to focus on the last three issues to limit inflammation in recipients. Further investigations are needed to evaluate the actual impact of safety measures in PC recipients and determine whether efforts can be made to propose matching procedures that can calculate the most important factors to limit inflammatory responses in patients.

For pRBCCs, the appropriate strategy may be very simple or very complex. In theory, it should be simple if one considers that the enemy is alloimmunization, which concerns inflammation. To limit the risk of immunization, an improvement of blood group Ag matching would be ideal; however, considering the volume of blood components to be issued to millions of recipients, this is simply not achievable on a routine basis. Efforts are being made to facilitate matching for at-risk recipients, such as those routinely receiving transfusions, though success varies according to specific needs of ethnic groups transfused outside their native region, where erythrocyte Ag group distribution differs from their own. Further, if inflammation fuels alloimmunization, some genetic control of responders vs. non-responders – or more

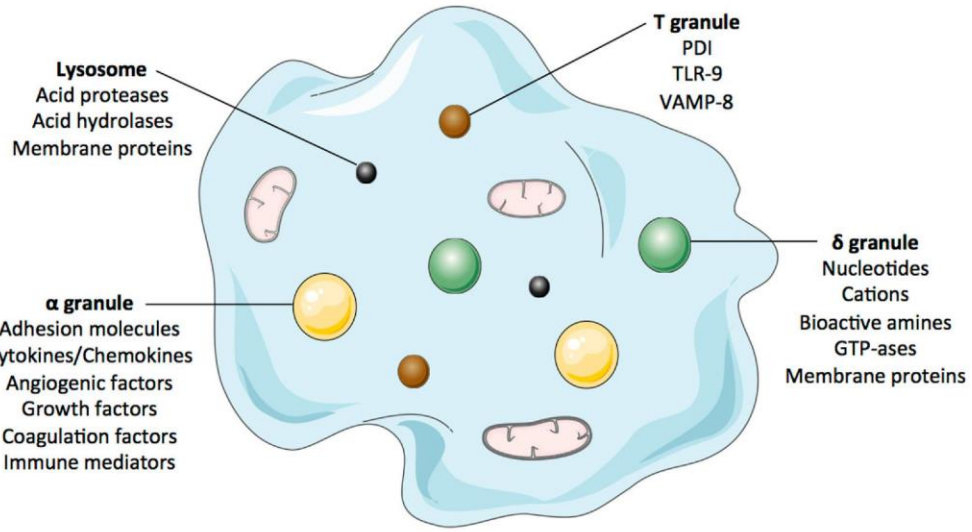

FIGURE 1 | The figure cartoons the platelet' main granules and their secretory content. Most products that can be released by platelets are listed in complementary **Table 2**.

TABLE 2 | Platelet granule main contents.

α granules	δ granules	T granules	Lysosomes
Adhesion molecules αIIbβ3, αVβ3, CD9, fibronectin, GPIbα, multimerin, osteonectin, PECAM, P-selectin, vitronectin, vWF Cytokines/chemokines β-thromboglobulin, CCL4, CCL17, ENA-78, Gro-α, IL-1, IL-7, IL-8, MCP-1, MCP-3, MIP-1α, NAP-2, PF4, RANTES, sCD40L, SDF-1 Angiogenesis/growth factors ADAM10, ADAMTS13, angiostatin, angiopoietin-1, BDNF, bFGF, BMP-2, BMP-4, BMP-6, CTAP-III, CTGF, EGF, endostatin, HGF, HGR, IGF-1, kininogen, MMP-1, MMP-2, MMP-9, PDGF, TGF-β, thrombospondin, TIMP-1, TIMP-4, VEGF Coagulation factors α_2-antiplasmin, α_2-antitrypsin, α2-macroglobulin, antithrombin, factor V–VIII–XI–XIII, fibrinogen, PAI-1, plasmin, plasminogen, protease nexin-2, protein S, prothrombin, TFPI Immune mediators β1H globulin, C1 inhibitor, complement factors, factor D, IgA, IgG, IgM, platelet factor H, thymosin-β4 Others Albumine, PDCI	Nucleotides ADP, ATP Cations Calcium, magnesium Bioactive amines Serotonin, histamine GTP-ases rab27a, rab27b Membrane proteins αIIbβ3, CD63, GPIb, LAMP-1, LAMP-2, P-selectin Others Polyphosphate, pyrophosphate	PDI TLR-9 VAMP-8	Acid proteases Acid phosphatase, arylsulphatase Carboxypeptidase A–B, cathepsin D–E, collagenase, elastase, proline carboxypeptidase Acid hydrolases α-arabinofuranosidase, α-fucosidase, β-fucosidase, α-galactosidase, β-galactosidase, β-glucoronidase, α-mannosidase, α-glucosidase, β-glucosidase, β-*N*-acetyl-hexosaminidase Membrane proteins CD63, LAMP-1, LAMP-2

ADP, adenosine diphosphate; ATP, adenosine triphosphate; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; BMP, bone morphogenetic protein; C, complement; CTAP-III, connective tissue-activating peptide III; CTGF, connective tissue growth factor; EGF, epidermal growth factor; HGF, hepatocyte growth factor; HGR, histidine-rich glycoprotein; Ig, immunoglobulin; IGF, insulin-like growth factor; IL, interleukin; LAMP, lysosomal-associated membrane protein; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; NAP, neutrophil-activating protein; PAI, plasminogen activator inhibitor; PDCI, platelet-derived collagenase inhibitor; PDGF, platelet-derived growth factor; PDI, protein disulfide isomerase; PECAM, platelet endothelial cell adhesion molecule; PF, platelet factor; RANTES, regulated on activation normal T cell expressed and secreted; SDF, stromal cell-derived factor; TFPI, tissue factor pathway inhibitor; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinases; TLR, toll-like receptor; VEGF, vascular endothelial growth factor; VAMP, vesicle-associated membrane protein; vWF, von Willebrand factor.

precisely, good vs. bad HLA presenters – seems to prevail: this has been observed for certain blood group Ags and is very likely true for all others (77, 104). The situation is more complex than for platelets because the triggers of inflammation are less clearly identified. The age of blood is a likely but unproven factor, and the effects of storage lesions and erythrocyte Ag alloimmunization (105). Various teams have provided indirect evidence after examining whether RBC collection can stress RBCs and subsequently stress endothelial cells exposed to such RBCs (at least in *ex vivo/in vitro* models) (106, 107). Here again, until more

TABLE 3 | Examples of preventable and not yet preventable causes of inflammation in transfusion medicine.

	Parameters that can be addressed	Parameters that cannot yet be addressed
Donor-related parameters	– So-called irregular antibodies to red blood cells or HLA – Autoantibodies – Potentially: allergens and IgE antibodies to allergens – Infectious pathogens and infectious pathogen-derived material (toxins, residues, superantigens) – …	Genetic parameters predisposing to inflammation
Processed component-related parameters	– Typically: leukocytes – Microvesicles/microparticles – All types of storage lesions – Age of blood[a] – …	
Recipient-related parameters	– Certain therapies (drugs)	– Genetic parameters that predispose to inflammation – Clinical state (causal disease or treatment being the cause of the transfusion need) – Most therapies, otherwise needed – Preexisting alloimmune Abs, autoimmune Abs – …
Standard of operation parameters (SOP)	– Main blood group matching – Blood component freshness[a]	– Fine-tuned blood group matching

[a]*Age of blood appears to fall into either category as it affects the release of biological response modifiers (storage lesions) and likely sustains TRIM, and it affects the release of, e.g., oxygen (SOP) and the recirculation or cells (and propensity to apoptosis or to be prone to phagocytosis).*

direct evidence becomes available, one may heed protocols that minimize stress to donors' RBCs and subsequently to recipients' vascular endothelium. Accordingly, some authors recommend not overexposing cellular blood components to radiation unless absolutely required as this may increase storage lesions (54, 108, 109). Closer examination is needed to determine the extent to which irradiation of BCs favors alloimmunization. Similar caution has been suggested for pathogen inactivation/reduction technologies, but there are conflicting claims in favor of reduction or alloimmunization based on impairment of indirect Ag presentation (84). This too calls for further investigation.

CONCLUSION

Transfusion is an old therapy, though it is not obsolete. In fact, it is quite modern if seen as cell therapy or biotherapy (110). It is very commonly used and is nowadays associated with few nosocomial AEs. Moreover, not all AEs are truly nosocomial as some are in fact linked to characteristics of recipients that can neither be dampened nor counteracted by matching blood components. When transfusion is associated with AEs, most can be related to an inflammatory state, which is either obvious (allergy, FNHTR, hypotension) or ascribed to such a state by current knowledge (alloimmunization). Indeed, transfusion-transmitted infection has become a rarity, and novel means are regularly applied to further minimize their occurrence. Means of decreasing the occurrence of transfusion-associated inflammation have received less attention and care, though they should be our new focus, to help patients, secure resources, and limit indirect costs. Platelet pathophysiology owes a lot to transfusion medicine: many of the major discoveries in this field were made by researchers who, questioning the role of PC transfusion in AEs (and occasionally SAEs), attempted to solve questions about platelet activation and secretion. This review has not considered the other side of the coin with respect to platelets and their role in the inflammation process. Namely, in addition to being the source of many pro-inflammatory BRMs, platelets also produce healing factors (the terminus of physiological inflammation) that may also be used as therapeutic tools (111).

AUTHOR CONTRIBUTIONS

OG drafted the manuscript; all other contributors contributed illustrations, discussion, and critical review, along with the production of original data supporting the synthesis as a review article.

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Chapitre 2 - Effets indésirables receveurs

Malgré toutes les précautions strictes de préparation et de stockage, des complications peuvent survenir après transfusion sanguine, appelées effets indésirables receveurs (EIR). En France, les principaux EIR déclarés sont de types immunologiques (allo-immunisation) [5], inflammatoires, de surcharge, ou infectieux [6]. Suite à une transfusion de PSL contaminés par des agents infectieux, les contaminations virales et parasitaires sont devenues exceptionnelles, seulement 0,1% des EIR. Les infections bactériennes, surtout liées aux CP, sont particulièrement redoutées, elles représentent 0,45% des EIR dont 74% survenus après transfusion de plaquettes (2,9 déclarés pour 100 000 CP cédés). Les incidents considérés comme inflammatoires sont principalement des réactions fébriles non-hémolytiques (RFNH) [7], allergiques/pseudo-allergiques [8-10], cardiovasculaires (hypotension), sans conflit antigène/anticorps (Ag/Ac) identifié [11]. Cependant, les EIR peuvent être incriminés dans des réactions gravissimes comme le syndrome de détresse respiratoire aigu post-transfusionnel, ou « Transfusion Related Acute Lung Injury » (TRALI) [12]. Les réactions peuvent, très rarement, être fatales, environ une transfusion sur 200 000-420 000 unités associées à un décès [13] ; ceci ne signifie pas que le produit transfusé a été létal mais que le décès est survenu dans le contexte d'une transfusion, décès dont la cause peut n'être qu'indirectement liée à cette transfusion. Bien que les CP ne représentent que 10% des PSL transfusés, ils sont responsables près de 40% des EIR [14].

La survenue d'EIR après transfusion est influencée par la triade transfusionnelle : « donneurs-produits-receveurs ». En effet, une transfusion de PSL fait intervenir des paramètres liés aux donneurs comme le sexe, phénotype, génotype ; aux produits notamment le process et la conservation ; et aux receveurs, en particulier son état clinique, phénotype, génotype [1].

Chapitre 3 - Plaquettes et inflammation

Les plaquettes sanguines sont des cellules spécialisées dans la réponse hémostatique et sont maintenant également reconnues pour intervenir au cours des processus immunitaires, en agissant comme modulateurs et médiateurs de la réponse inflammatoire [15-17]. Ce sont des cellules inflammatoires aux multiples facettes, ayant des fonctions dans les réponses immunitaires innées et adaptatives [18]. D'une part, les plaquettes expriment des récepteurs permettant la fixation des ligands impliqués dans différentes étapes de l'immunité innée ou adaptative [19, 20]. D'autre part, elles produisent des cytokines, chimiokines, regroupées sous le nom de modificateurs du comportement biologique ou « Biological Response Modifier » (BRM), ayant un rôle pro-inflammatoire excédant largement une plus faible composante anti-inflammatoire [21]. De nouvelles caractéristiques et fonction sont donc décrites pour définir les plaquettes comme des cellules de l'immunité et de l'inflammation [22]. Les plaquettes sont maintenant connues pour avoir un rôle dans l'inflammation, notamment en interagissant avec de nombreuses cellules immunitaires et en détectant –grâce à leurs « Pattern Recognition Receptor » (PRR) de membrane et cytoplasmiques– des pathogènes au sens le plus large du terme, c'est-à-dire des agents infectieux via leurs « Pathogen-Associated Molecular Patterns » (PAMP) membranaires mais aussi des éléments pathogènes cellulaires non infectieux via leurs « Damage-Associated Molecular Patterns » (DAMP) et « Self-Associated Molecular Patterns » (SAMP) membranaires [23]. Cependant, des études ont montré qu'elles peuvent également intervenir dans des pathologies inflammatoires, tels que l'athérosclérose, la polyarthrite rhumatoïde ou encore le lupus érythémateux [24, 25], ou encore la dissémination métastatique des cancers [26].

La revue suivante se focalise sur le potentiel inflammatoire des plaquettes transfusées ainsi que leur implication dans les EIR (revue « in review » dans *Frontiers*, Décembre 2017).

The Non-Haemostatic Aspects of Transfused Platelets

Running Title: Platelet Inflammation and Transfusion

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Abstract

Blood transfusion is a safe process, but during or after the process the recipient may experience an adverse reaction and occasionally a serious adverse reaction (SAR). In this review, we focus on the inflammatory potential of platelet components and their involvement in SARs. The involvement of platelet components in SARs could be related, at least in part, to the inflammatory functions of platelets. Blood platelets are involved in inflammation and various other aspects of innate immunity through the release of a plethora of immunomodulatory cytokines, chemokines and associated molecules, collectively termed biological response modifiers (BRMs) that behave like ligands for endothelial and leukocyte receptors and for platelets themselves. It has been observed that particularly platelets collected, processed and stored for transfusion purposes, secrete and release into the storage medium large quantities of BRMs that are proportional to the storage duration. This is linked to the platelets' propensity to be activated even in the absence of deliberate stimuli and to the occurrence of time-dependent storage lesions. This means that when a platelet component is transfused to a recipient, a huge amount of cytokines and chemokines is also transfused. One of the key molecules of platelet secretion is CD40 ligand (CD40L) which is a key molecule in that it bridges innate and adaptive immunity. The latter observation may be responsible for many non-hemostatic functions of transfused platelets.

Key words: Platelets, Transfusion, CD40L, Serious Adverse Reaction, Inflammation, Innate Immunity

1. Introduction

Blood platelets are small anucleate cells essentially originating from megakaryocyte (MK) fragmentation. These cells have a dense cytoskeleton that maintains their discoid shape in normal state and changes the platelets to a spherical form after their activation (1). Platelets play a key role in vascular repair and maintenance of homeostasis, particularly in primary hemostasis. The platelet membrane glycoproteins can interact with the elements of the injured endothelium, mediating their adhesion, followed by activation and finally aggregation, resulting in the formation of a thrombus formed by aggregation of interconnected platelets by fibrinogen to close the vascular gap (1, 2). Platelets also play an important role in innate and adaptive immunity by interacting directly or indirectly with other immune cells to trigger or maintain the inflammatory response (1, 2, 3). Several factors are involved in the platelet inflammatory process, in particular by membrane expression of several immune receptors, such as cytokines (CKs), chemokines (CHs), and a large number of soluble factors contained in their granules (in α-granules this includes CKs/CHs, immunomodulatory factors and growth factors, etc.) (4, 5) (Figure 1). Moreover, platelets also release other factors: i) growth factors promoting angiogenesis, which are also required to repair damage to inflammatory sites (6-8), ii) clotting factors required for platelet hemostatic functions (9, 10), iii) antibacterial peptides (1, 11), iv) adhesion factors (12), and v) inflammatory mediators, such as serotonin and histamine (13-15).

Among those factors, soluble CD40L (sCD40L), of which platelets are major providers, is a key molecule, characterized as one of the conductors of the immune system (16-19). Platelet secretion profiles seem dependent on the platelet receptor at stake; these differential profiles –a completely new concept with regard to an anucleate cell– also appear to be strictly regulated by intraplatelet signalling pathways, depending on the stimuli (20-23). This review summarizes current information concerning the inflammatory role of platelets in a transfusion context.

2. A Brief Overview of Platelet Functions

Blood platelets are important reservoirs of soluble, preformed mediators (CKs/CHs, hemostatic factors and immunomodulators) that are present in secretory granules, in particular α-granules, δ-granules, and lysosomes, which are released upon their activation (1,

15, 24-28). Platelets contain a large variety of CKs/CHs, which are mainly synthesized in the MK and stored in α-granules in most cases (Figure 1). CKs/CHs may directly interact with cells of the innate and adaptive immune system or indirectly through immune or non-immune relay cells, such as endothelial cells. CK/CH platelets help regulate the surrounding cells, including their proliferation, differentiation and activation (1). Interestingly, platelets also express receptors for several CKs/CHs that they secrete, showing their potential to establish autocrine and paracrine bidirectional loops. Platelet immunomodulatory factors include growth factors and CKs/CHs, but also molecules sharing the main characteristics of CHs and CKs, such as sCD40L/CD40L, soluble P-selectin/CD62P, platelet-derived growth factor AB (PDGF-AB), transforming growth factor β (TGF-β), interleukine 1β (IL-1β), regulated on activation normal T cell expressed and secreted (RANTES) and platelet factor 4 (PF4 ou CXCL4).

Although, platelets have generally not been considered central to innate immunity and inflammation, this paper proposes that they actually are. Recent data reports evidence that platelets can also recycle a number of CKs/CHs and regulatory products called BRMs for which they also express the pairing ligand, which is the case for sCD40L (platelets are the major purveyors of this molecule in the circulation) (17, 29). Platelets also express membrane CD40 and, unlike CD40L, CD40 is detectable on the surface of resting as well as activated platelets (1).

CKs and other platelet products can already be detected at the onset of acute inflammation (8). In this regard transfusion is an excellent model of the pathological process as here the mediators of inflammation are transfused with consecutively rare but then severe SARs. It is currently widely admitted that sCD40L is the master platelet-associated CKs (17, 30). When it was first described in 2001 in association with platelets, this was in the context of platelet component (PC) transfusion hazards. Subsequently, platelet-sCD40L and the CD40/CD40L pair have been described in many pathologies with the conclusion that e.g. Febrile non Hemolytic Transfusion Reaction, where sCD40L appears to be chiefly responsible for pathological symptoms, was indeed inflammatory, similar to their role in diabetes, cardiovascular disease, atheromatous plaques and inflammatory bowel disease, where CD40/CD40L have now been acknowledged as being influential (1, 31).

3. Platelet Interactions with Other Blood Cell Elements

Interactions of sCD40L with its CD40 receptor (expressed on immune cells or other cells, such as endothelial cells) can modulate the responses of each of the different cell partners (5, 32). Indeed, platelet sCD40L, interacting with CD40 on endothelial cells, induces inflammatory responses characterized by the expression of adhesion receptors (E-selectin, P-selectin, intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) for the release of proinflammatory CKs/CHs (CCL2, IL-6, IL-8) and the recruitment of leukocytes to the inflammatory sites (16). The *in vitro* engagement of neutrophil CD40 by sCD40L induces the generation of reactive oxygen species (ROS) and the destruction of lung endothelial cells suggesting this factor's role in transfusion-related acute lung injury (TRALI) (33). Moreover, platelet sCD40L creates a link between innate and adaptive immunity in promoting maturation (19, 34), activation (35), secretion (36) and presentation of dendritic cells (DCs) which are cells capable of activating naive T cells to induce an adaptive immune response (5). Elzey *et al.* have demonstrated that platelet sCD40L can, both *in vitro* and *in vivo*, amplify the activity of pathogen-specific CD8+ T lymphocytes (Lyt), which results in the production and function of IFNγ, and in the enhancement of their lytic function (37). Iannacone *et al.* have further shown that the number of cytotoxic Lyt during infection with lymphocytic choriomeningitis virus (LCMV) was dramatically reduced in the absence of platelets, involving CD40/CD40L: thrombocytopenia is estimated to result almost exclusively from the antiplatelet antibodies (38). CD40/CD40L also plays a major role in the interaction of CD4+ T lymphocytes (and CD8+) and B lymphocytes, which supports proliferation, differentiation and production of immunoglobulin by plasma cells. Platelet or megakaryocyte derived sCD40L, which is continuously released into the circulation in large quantities (37, 39, 40), is a key molecule regulating the immune system and increased release of sCD40L plays a major role in the pathogenesis of the immune mediated disease.

While for the role in primary hemostasis, platelets primarily interact with endothelial cells, they also interact directly or indirectly via their released CK/CH with many of cell types, hereby strongly influence their function. Platelets can, indeed, activate (and be mutually activated by) almost all types of leukocytes (monocytes, T-lymphocytes, B-lymphocytes and neutrophils) and DCs (1, 30, 41). When allogeneic (donor) platelets are transfused to patients, the recipients' circulating cells make foreign encounters (e.g. by human leucocyte antigen

(HLA) class I molecule expressed on platelets) and can potentially be activated by those encounters, and vice versa. This led to a recent re-examination of the concept of pathogens defense mechanisms, extending it to non-infectious “dangers” such as foreign (transfused) cells (15, 26, 27, 42, 43). Platelet components are stored for a maximum of five days (most countries) before being issued to a patient in need; prior to that, during their shelf life, platelets “spontaneously”, i.e. with no acknowledged exogeneous stimulus, release a number of CKs, particularly sCD40L (17, 30) in high enough quantities to exert functional activities on target cells possessing the ad hoc receptors. sCD40L was found to be consistently and significantly elevated in PCs that had led to SARs comprising various syndromes, including (antibody independent) TRALI (although this is disputed in such particular case) (30, 33).

4. A Brief Overview of Platelet Component Transfusion Benefits and Complications

Platelet component transfusions have two main indications, aimed at being either curative or prophylactic (44). Curative transfusions are given to patients presenting with active bleeding and low to very low platelet counts (in exceptional circumstances, the platelet count can be normal, but platelets are non-functional), or massive blood loss. Curative transfusions are not under debate, unlike the protocols and timing of other blood component transfusions (red blood cell concentrates and fresh plasma) and/or blood derivatives, such as prothrombin complex concentrate (PCC) or fibrinogen. There is no consensus on prophylactic transfusions, however, although many practitioners still recommend not exposing at-risk patients to bleed. Thresholds for transfusion and quantities of transfused platelets vary consistently in different countries and with different systems. In short, PC transfusion provides a benefit to patients and prevents bleeding and deterioration of otherwise serious clinical conditions. PC transfusion is supportive in many chemotherapy protocols and stem cell transplantation. On rare occasions (grade 1-3: 0.24%) PC transfusion can lead to immediate to short-delayed inflammatory adverse reactions that can occasionally be life threatening (grade 3: 0.006%) (45). The rationale for the relatively high number of SARs with PC transfusion (from 1/4 to 1/2 of all reported SARs, while PCs represent only about 10% of transfused blood components (BCs)) may be deduced from their propensity to secrete copious amounts of pro-inflammatory BRMs as outlined in the previous section). In addition, PC transfusion can be associated with volume overload, as PCs frequently come into large volumes and elevated levels of proteins and lipids

exerting a surfactant effect (46). The latter can be prevented by close patient monitoring, and by replacing 2/3 of plasma with platelet additive solutions (47). The case of TRALI and the responsibility of platelets have been presented elsewhere (43). Lastly, PC transfusion carries a greater risk of bacterial contamination which can be life threatening especially in severely immuno-compromised patients (48). The introduction of Pathogen Reduction Technologies has completely eradicated this hazard, albeit with a moderate worsening of storage lesions (49, 50). Pre-storage leukoreduction proved to significantly reduce inflammatory reactions as well as viral infections (51). In brief, PC transfusions can induce unwanted effects e.g. volume, plasma, inflammatory reactions, pathogen transmission etc. in addition to their therapeutically intended effect i.e. improving hemostasis. However, since PC-transfused patients are particularly fragile patients, close monitoring and careful dosing can prevent many complications such as volume overload.

5. Platelet Storage and Outcomes of Critically Ill Patients

Over the platelet storage period, certain biochemical and functional changes occur in the platelets and their storage medium. These changes, called storage lesions (Figure 2), include acidification of the storage medium secondary to anaerobic platelet metabolism, platelet activation (52), and an increase in CKs and lipids level in PCs (53, 54). These storage lesions may compromise the platelets' viability and functionality, and therefore the transfusion's efficacy (55). They may also lead to adverse reactions in the recipients.

Critically ill patients are the second largest patient group to receive platelet products after oncology-hematology patients. Around 15% of critically ill patients require a platelet transfusion during their intensive care unit (ICU) stay for treatment or prophylaxis of bleeding (56). Critically ill patients are characterized by a coexisting inflammatory state, making them theoretically more susceptible to blood product adverse reactions. A "two-hit" hypothesis has largely been used to explain the pathophysiology of transfusion adverse events including TRALI, the first hit being a pro-inflammatory condition and the second hit being the administration of antibodies or BRMs through blood component transfusion (57). Results of *in vitro* and animal studies suggest platelets storage lesions have a key effect on the occurrence of non-antibody mediated TRALI (33, 57). Khan *et al.* have observed an increase in sCD40L level over the platelet component storage period, and higher levels of sCD40L in platelet products

implicated in TRALI, suggesting that the accumulation of sCD40L during platelet storage induces TRALI (33). Consistent with these findings, Vlaar *et al.* have found that stored platelet supernatant compared with fresh platelet supernatant led to an increase in systemic and pulmonary coagulopathy in lipopolysaccharide pre-treated rats (57).

Prolonged platelet storage has been associated with a decrease in post-transfusion platelet increment and a shorter time to next platelet transfusion in oncology-hematology patients (58-60), but the clinical consequences of the platelets storage lesions remain uncertain (60-62). To our knowledge, no study has investigated the association between transfusion efficacy and platelet storage duration in critically ill patients. Five observational studies have investigated the association between PC storage duration and critically ill patient outcomes; one included post-cardiac surgery patients only, two studies included trauma patients only, and two all critically ill patients (63-67). There was no association between mortality and storage duration in the three studies evaluating this outcome (63, 64, 67). In a study of 381 trauma patients, those receiving platelets stored for five days developed more complications, including sepsis, than patients transfused with platelets stored for less than five days (5.5% sepsis in patients receiving platelets stored for three days or less, versus 16.7% in patients receiving platelets stored for five days, p=0.03) (64). After adjustment for confounders, patients receiving PCs stored for five days had a 2.4-fold higher risk of developing complications, including acute renal failure, acute respiratory distress syndrome and sepsis, than patients transfused with fresher platelets (64). All these studies are retrospective and have numerous limitations in their methods making it impossible to draw any definitive conclusion on the impact of platelet storage duration on clinical-centred outcomes. Prospective research is warranted to determine whether prolonged platelet storage has an impact on the prognosis of critically ill patients. In the meantime, better understanding of platelet transfusion-related immunomodulation may help us to understand the reported association between platelet transfusion and an increased risk of hospital-acquired infections (68, 69).

6. Transfusion-Associated Immunomodulation

Transfusion related immunomodulation or TRIM is a complex event with dual effects that are potentially beneficial, but in general mostly considered harmful (70). The long-term

effect of transfusions is suspected to modulate (dampen) immune responses and consequently favour the emergence of secondary malignancies and infections. It is, however, extremely difficult to decipher the respective roles of causal pathologies in severely sick intensive care patients or patients receiving chemotherapy and immunosuppressants, monoclonals and biosimilars, and blood components. TRIM induced by PCs would be best understood in patients having received PCs only, but it is almost impossible to delineate the immunosuppressive role of platelets relative to red blood cells as only very few patients receive PCs and no red blood cell concentrates (RBCCs). Furthermore, in case of plasma-rich BCs, plasma polyreactive immunoglobulins (Igs) may counterbalance certain immunosuppressive effects. In short, whether PC transfusions may be immunomodulatory remains elusive and difficult to assess, though it would be of interest to investigate this in order to provide patients with optimized care.

7. Platelets, Polyanions and Bacterial Defense

While the above-described mechanisms clearly indicate that platelets interfere with the immune system, only a few studies clearly show a causal link between platelet activation and immune-mediated disorders. One well-investigated example of the role of platelets in mediating immune reactions is the interaction of platelets with heparin.

The adverse drug effect of heparin-induced thrombocytopenia (HIT) will therefore be used to exemplify the interaction of platelets and the immune system. HIT is a prothrombotic adverse drug reaction caused by the transient production of IgG-class platelet-activating antibodies that recognize multimolecular complexes of the positively-charged PF4 and the polyanion drug heparin. These antibodies activate platelets and also monocytes via their FcγRIIa receptors. This causes transformation of an immune reaction into a prothrombotic reaction, resulting in massive thrombin generation and paradox thrombotic complications. If unrecognized, the risk for new thrombosis in affected patients is 5% per day and the risk of mortality is 25-30% (71). There is no doubt that with HIT, platelets mediate an extremely powerful reaction which results from concomitant activation of the immune system and the coagulation system.

The reason for this massive response is that HIT is likely a misdirected bacterial host defense (72). PF4 binds charge-related to gram-negative and gram-positive bacteria. On gram-

negative bacteria, lipid-A is the binding site for PF4 (73). The binding site of PF4 on gram-positive bacteria has not yet been identified. The question raised is how and why PF4 induces such a potentially dangerous immune response.

The following section summarizes our recently proposed working model (74, 75). All bacteria expose strong negative charges on their surface. This negative charge is likely a mechanism by which bacteria are kept apart from each other, and by which bacteria are protected from phagocytosis. The zeta potential-mediated repulsive forces generated by the negative charges push bacteria apart from each other and away from their “predators” (the reader is invited to watch the following YouTube video demonstrating this principle https://www.youtube.com/watch?v=Kb-m1uDoWfU). Eukaryotic cells, however, must not have this strong negative charge as the repulsive forces would be incompatible with a complex multi-cellular organism. In view of this consideration, we propose that a strong negative charge is a fundamental feature of prokaryotes. In line with this concept, basic mechanisms of the innate immune system, like the alternative and classic complement pathway, the intrinsic clotting system with factor XII and factor XI, as well as the kininogen-bradykinin pathway are strongly activated by negative charges (76). However, the adaptive immune system (T cell receptors, B cell receptors, antibodies) do not recognize charge, they recognize structures. The platelet-derived chemokine PF4 has the role of translating charge into structure. After binding to negative charges, PF4 undergoes complex structural changes (for review) (77, 78). These structural changes expose a neoepitope, which is recognized by anti-PF4/polyanion antibodies, the same antibodies that induce HIT. After binding of anti-PF4/P antibodies to PF4-labelled bacteria, these opsonized bacteria mediate very efficient phagocytosis by granulocytes (72). The evolutionary advantage of using such a mechanism is that it enables an early IgG response towards bacteria the organism has not seen before. The newly encountered bacteria also bind PF4; PF4 undergoes its conformational change due to the negative charge on the bacteria surface and is then recognized by the preformed anti-PF4/P antibodies. In line with this concept, natural anti-PF4/P antibodies are found in the general population where their presence is highly correlated with the presence of chronic infections like chronic periodontal disease (79). On the basis of this concept, these antibodies must be very common. Indeed, the adverse drug reaction HIT has helped to prove this. In HIT, anti-PF4/P IgG is formed in high titre between day five and day ten (80). As B cells cannot produce IgG antibodies during a primary

immune response within 5-10 days, HIT is always a secondary immune reaction, even in patients who have never received heparin before. As 65% of patients develop these antibodies after cardiac surgery, a plausible explanation for such frequent primary immunization is the above-outlined concept of bacterial infection-related priming of the immune system.

As the negative charge is a danger signal for the human defense system, bacteria have naturally developed counteracting methods to hide this danger signal. One of which is long lipopolysaccharide (LPS) chains covering and "hiding" the negative charges or the Fc-part of the anti-PF4/P antibodies bound to conformational-changed PF4 on the bacteria surface. Lipid A is the basis of LPS. PF4 has a diameter of 5 nm; when an IgG molecule (which is about 10 nm long) binds to conformationally-changed PF4 bound to lipid A, the entire complex has a height of about 15 to 18 nm. The LPS chain, however, can reach lengths of up to 25 nm. This covers the Fc part of the antibody and thereby recognition of opsonized bacteria by the immune system's Fc receptors. However, platelets, in addition to PF4, secrete polyphosphates from their δ-granules. Polyphosphates are also negatively charged and bind to the PF4 molecule on the bacteria surface, attracting other PF4 molecules and finally forming large multimolecular PF4/polyphosphate complexes which extend well out of the bacteria's LPS shield (81). This has two effects: conformationally-changed PF4 is now exposed for antibody recognition, and consequently several anti-PF4/P antibodies can bind to these complexes on the bacterial surface, forming immune complexes which are then readily recognized by the Fc receptors of human defense cells.

Platelets also have a direct antibacterial effect. When platelets are incubated with *E. coli* in the presence of PF4 and anti-PF4/P antibodies, platelets kill up to 75% of *E. coli* by direct platelet bacteria interaction. Upon investigating this mechanism in more detail, we found a new way in which platelets defend bacteria. It is well established that platelets can internalize IgG-coated targets (82-84), however, it is debated whether phagocytosis of bacteria (i.e. *Staphylococcus aureus*) (85) is really a major mechanism for bacterial host defense (86, 87). Although platelets store bactericidal substances in their α-granules (12), α-granules are designed to be released and it is not that phagocytosed bacteria are transported within the platelet into the α-granules. Such a mechanism would be incompatible with platelet shape change during activation where platelets are spread thinly over a large area with the α-granules concentrated within the immediate granulomere zone (88). We propose an

alternative mechanism, where platelets cover bacteria by widely extending their membranes and then actively contracting them, thereby centralizing bacteria until they are very close to the granulomere of the platelets, where the substances with antibacterial potency are stored (Palankar, JTH, 2017, in press). When a threshold concentration of platelet-activating signals is reached due to platelet interaction with the opsonized bacteria, the activated platelets release their α-granules preferentially at the site of the bacteria, thereby locally reaching high concentrations of antibacterial substances. This phenomenon is similar to the pore-forming perforin released from the granules at the immunological synapse potentiated by cytotoxic T lymphocytes (89).

The above-outlined mechanisms are not the only ways in which platelets interfere with bacteria and other pathogens (90-93). Through complex mechanisms involving the platelet Fc-receptor FcγRIIA (94-96), glycoprotein (GP) αIIbβ3, GPIbα, complement receptors (e.g. gC1q-R) and Toll-like receptors (e.g. TLR-2 and TLR-4), platelets interact with bacteria and become activated by bacteria (27, 97). Upon activation, platelets release antimicrobial substances such as ROS, antimicrobial peptides, defensins, kinocidins and proteases (11, 98-100).

However, the above-outlined concept of the role of PF4 and platelets as mediators between innate and specific immunity places platelets in a very special position, bridging two major parts of our immune defense system. Platelets secrete or expose many molecules with a specific role in immunity. Little information exists on how platelet storage modifies the structure of these molecules or their spatial presentation within platelet compartments or on the platelet surface. As exemplified by the structural changes of chemokine PF4 induced by polyanions like heparin, conformational changes in these proteins may transmit a danger signal to the transfusion recipient's immune system, which erroneously triggers potent pathogen defense mechanisms, resulting in adverse transfusion reactions. Although this has not been shown yet, it is conceivable that other platelet derived mediators such as sCD40L intensify and probably orchestrate the interaction of platelets and other immune cells with pathogens. If misdirected, this can cause SARs. The adverse drug reaction of HIT provides one of the most prominent examples of the potentially deleterious consequences for patients.

The risk that our immune system develops autoimmune-like reactions towards platelet proteins when they are modified during storage is probably quite low, although such autoimmune reactions may occur. Again, this has been demonstrated for the immune reaction

towards conformationally-changed PF4. In the past decade, it has become recognized that certain patients present with clinical symptoms and laboratory features of HIT despite not having previously received heparin either in the recent past or at all. Sera from these patients contain antibodies that strongly activate platelets even in the absence of heparin. To date, ≈ 20 cases of spontaneous HIT syndrome have been reported (101-110). In the plasma of these patients, antibodies are found which bind to PF4 with such high avidity that they cluster two PF4 molecules, thereby inducing the same conformational change as polyanions. These clusters of conformationally-changed PF4 attach to platelets and endothelial cells, giving the immune system a false signal of the presence of strong negative charges, which prompts the above-described bacterial defense mechanism.

Taken together, there is ample evidence that platelets play an important role in the defense of pathogens. Recognition of pathogens by platelets is at least partly mediated by conformationally-changed endogenous, platelet derived proteins. The challenge for transfusion medicine and immunohematology is to identify whether platelet proteins with an important role in danger signaling are also conformationally changed during platelet processing and storage, thereby presenting a danger signal with an increased risk of triggering misdirected host defense mechanisms.

8. Platelets, Polymorphisms and Alloimmunization

Platelet component transfusions are extremely difficult to match for surface antigens between donors and recipients, apart from the ABO groups (A and/or B antigens can be variably expressed on platelets) (111). Moreover, platelets exhibit numerous copies of highly polymorphic HLA class I antigens. The functions associates with HLA class I molecules on platelets are currently under debate, as platelets are not consensually considered capable of presenting antigens. HLA transfer to other cells has recently been evidenced experimentally in mice, opening up novel avenues on the subject. HLA immunization of patients is not uncommon, but pre-storage leukoreduction has proved to be tremendously efficacious in limiting it, since leukocytes –ten-times more loaded with HLA moieties than platelets– seem to potentiate immunization against platelet antigens, HLA and human platelet antigens (HPA) (112). HPA are actually polymorphic variants of platelet glycoproteins, presenting “platelet specific blood groups”. Almost twenty such molecules are recognized as being immunogenic,

with less than five being implicated in the most frequent immunization, while the others stand for rare antigens. Those HPA antigens usually come into antithetical moieties termed ‘a’ and ‘b’, ‘a’ being the frequent allele and ‘b’ the rarest. In certain circumstances, HLA or HPA testing and matching is the only option available to efficiently transfuse refractory patients. It should be noted that as residual red blood cells exist even in very small numbers in PCs, patients transfused with PCs can be immunized against red blood cell antigens, especially when these are highly immunogenic such as Rhesus-D. It is therefore strongly advised not to transfuse a Rhesus D negative recipient with platelets obtained from a Rhesus D positive donor unless prophylaxis is available if needed. Lastly, it has recently been hypothesized that ABO mismatched platelets favour alloimmunization, although this hypothesis has yet to be ascertained with respect to its clinical impact.

9. Conclusion

In conclusion, transfusion of platelets is generally safe and largely beneficial to patients. On rare occasions, SARs (which cannot be prevented by current measures), occur with clinical presentation of acute inflammation. In all cases investigated to date, either based on clinical observations or tested experimentally, BRMs (comprising chiefly of CKs and CHs and related molecules such as sCD40L) are found to be in close association. Potentially these SARs are misdirected physiological defense mechanisms. This we have exemplified by the complex pathogenesis of HIT, which, however, involves just one of the many immunomodulatory CHs released by platelets. Additional safety measures to prevent those SARs would be beneficial to patients; however, it is likely they would be extremely difficult to establish and would not be cost effective. Again, transfusion-linked inflammation is likely the result of a combination of factors related to the donor, the BC and the recipient. The only factor that can be targeted at present is the BC, and measures to improve BC quality are being implemented when identified within the industry, in partnership with blood establishments. The identification of parameters that may be related to patients (recipients) would be desirable to identify at-risk patients and apply measures to prevent the severity of the hazards. If parameters are linked to donors the situation becomes much more difficult, because further medical investigations in donors would scarcely be acceptable, and would have the potential to jeopardize BC stocks. How can one explain to a generous blood donor that he or she is perfectly safe and healthy, but “at risk” of

inflicting harm on "certain" recipients? This problem is medically, ethically and psychologically difficult to address. Alternatively, transfusion medicine may become one of the first medical specialties where personalized medicine comes into effect: "How can a given patient be given the BC most suited to his or her condition"?

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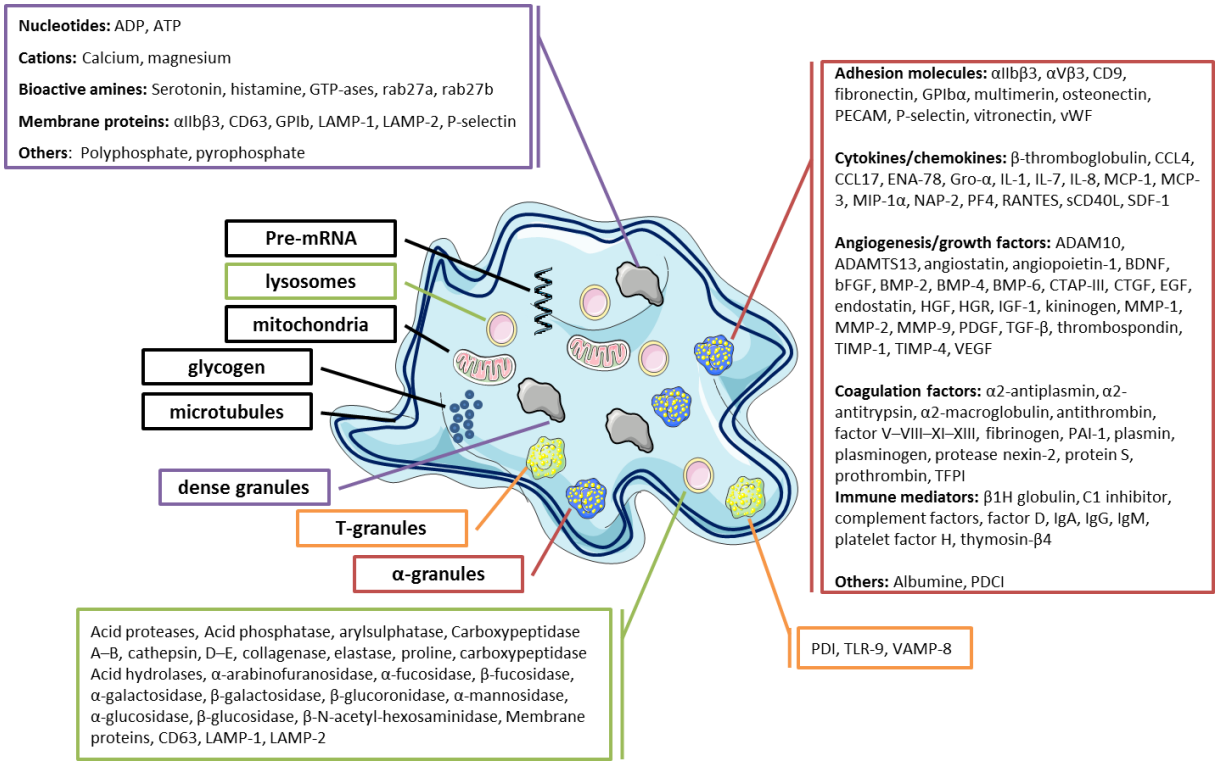

Figure 1: Diagram of the platelets' main granules and their secretory content. Most products that can be released by platelets are listed.

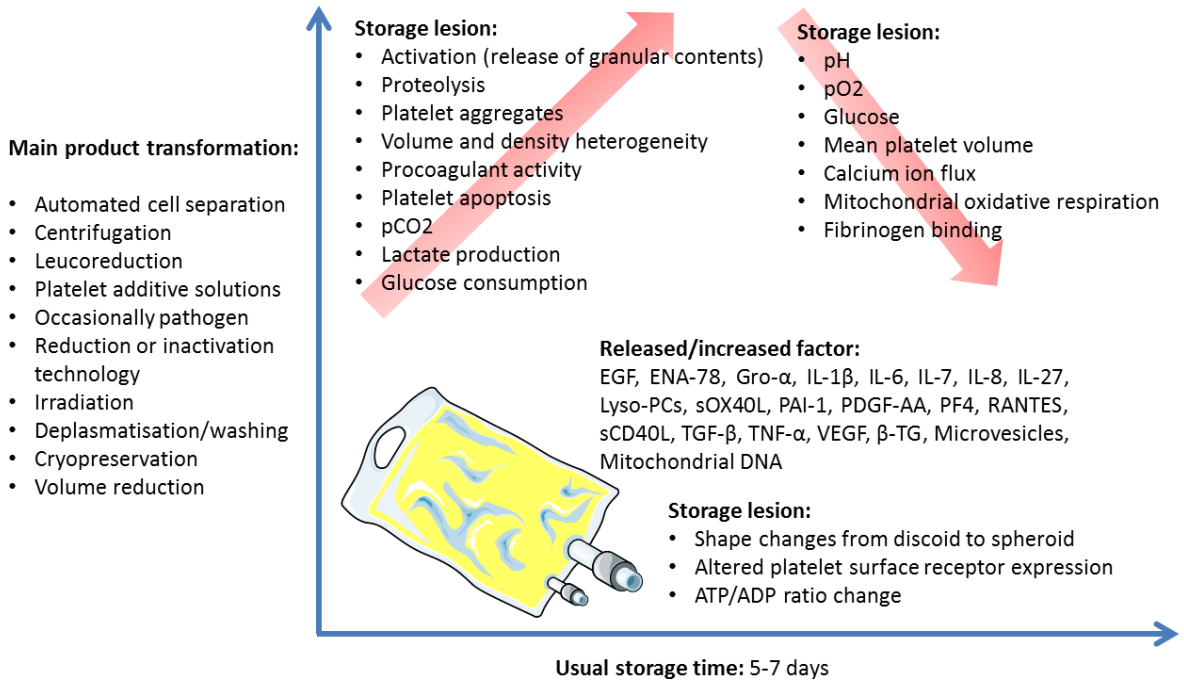

Figure 2: Platelet concentrate storage and biological response modifier release.

I - Les plaquettes sanguines

Les plaquettes sanguines sont des cellules anucléées issues des mégacaryocytes de la moelle osseuse, présentes dans la circulation sanguine [27, 28]. Une étude récente a également identifié les poumons comme site de production plaquettaire [29]. La production quotidienne des plaquettes est d'environ $2{,}5.10^{11}$ cellules avec une durée de vie moyenne de 7 à 9 jours [30]. Elles sont caractérisées par une forme discoïde d'environ 2 à 4 micromètres de diamètre. Lorsque les plaquettes sont activées, leur morphologie change, elles deviennent sphériques et émettent des pseudopodes.

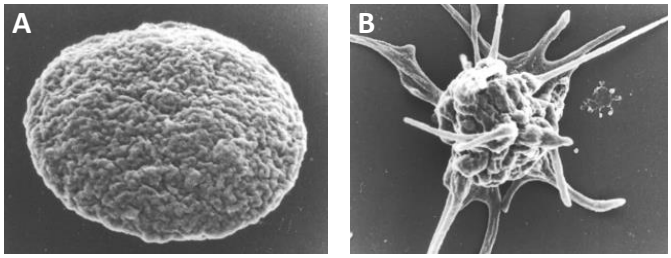

Figure 1. Morphologie des plaquettes en microscopie électronique à balayage

Observation d'une plaquette au repos (A) et d'une plaquette activée (B) [29].

1) La structure plaquettaire

Des plaquettes, observées en microscopie électronique, on distingue 3 parties : la membrane plasmique, le cytosquelette et les organites intracellulaires [31, 32].

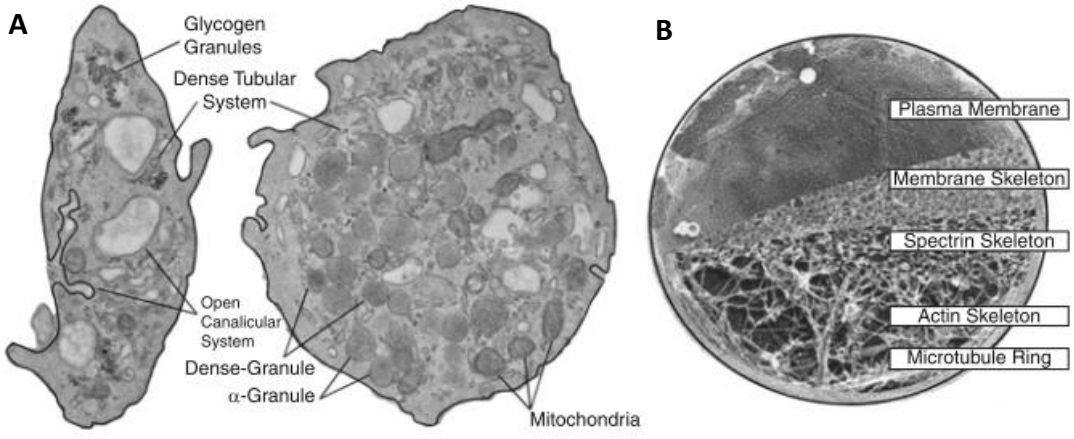

Figure 2. Structure des plaquettes en microscopie électronique à transmission

Observation des caractéristiques ultrastructurales d'une plaquette au repos : les différents organites (A), la membrane et le cytosquelette (B) [30].

a. La membrane plasmique

La membrane plaquettaire présente une structure trilaminaire composée d'une bicouche lipidique, qui maintient une couche riche en glycoprotéines [33]. La membrane est constituée de protéines et de lipides. Les protéines membranaires sont nombreuses, ce sont principalement des glycoprotéines et des intégrines. Les lipides membranaires sont essentiellement des phospholipides. La phosphatidylcholine et la sphingomyéline sont majoritairement situées dans le feuillet externe, tandis que la phosphatidyléthanolamine, la phosphatidylsérine (PS) et le phosphatidylinositol sont majoritaires sur le feuillet interne.

Les plaquettes possèdent un système canaliculaire ouvert ou « Open Canalicular System » (OCS) connecté à la surface, qui correspond à des invaginations de la membrane externe au travers du cytoplasme. Ce système permet aux plaquettes permet l'entrée des éléments externes dans les plaquettes. Les plaquettes possèdent également un système tubulaire dense ou « Dense Tubular System » (DTS), non connecté à la surface, qui correspond au réticulum endoplasmique lisse résiduel du mégacaryocyte. Ce système contient des enzymes du métabolisme lipidique, essentiellement des prostaglandines, mais aussi du calcium. Les deux systèmes membranaires OCS et DTS sont étroitement liés pour favoriser la libération du contenu plaquettaire, notamment celui des granules lors de l'activation plaquettaire [30, 34].

b. Le cytosquelette

La forme discoïde des plaquettes au repos est maintenue par un cytosquelette bien défini et hautement spécialisé [35]. Ce système complexe d'entretoises moléculaires et de poutres préserve la forme et l'intégrité des plaquettes qui sont exposées à des forces de cisaillement élevées dans la circulation sanguine. Le cytosquelette est composé de microtubules, de microfilaments d'actine et de filaments intermédiaires de vimentine [36].

La périphérie interne des plaquettes est constituée d'un faisceau de 8 à 24 microtubules, eux-mêmes composés de tubuline, permettant de maintenir la structure discoïde de la plaquette au repos.

Les microfilaments d'actine composent essentiellement le cytosquelette plaquettaire, responsable du maintien de l'intégrité plaquettaire. Le réseau de microfilaments d'actine situé

dans le cytoplasme, est relié à la membrane externe par de petits filaments d'actine. La profiline empêche la polymérisation des monomères d'actine G et les sites d'élongation sont bloqués par les « capping proteins » [37].

Le changement morphologique des plaquettes lors de leur activation est le résultat du désassemblage et d'assemblage des microtubules et d'actine du cytosquelette [32, 38].

c. Les organites

Le cytoplasme plaquettaire est riche en organites : des mitochondries, des composants de l'appareil de Golgi, du réticulum endoplasmique, des ribosomes et des peroxysomes [39]. De plus, elles contiennent quatre types de granules morphologiquement différentes : les granules α, les granules denses ou δ, les granules T et les lysosomes [15]. Au sein de ces granules, les plaquettes stockent différents médiateurs immunitaires, notamment des facteurs de croissance, des cytokines, des chimiokines, des molécules d'adhésion et des facteurs de coagulation [3, 40, 41]. Elles contiennent une très grande quantité de molécules solubles, plus de 300 [42]. Lorsque les plaquettes deviennent activées, elles ont la capacité de sécréter le contenu de ces granules par exocytose [43] via le système canaliculaire [30]. Les plaquettes peuvent sécréter divers facteurs pro-inflammatoires et cela de façon différentes selon le signal d'activation [40, 44].

Les granules α

Ce sont les granules les plus abondants dans les plaquettes, environ 80 par plaquettes, mesurant entre 200 et 500 nm [45]. Elles contiennent des facteurs de coagulation, des facteurs de croissance, des facteurs angiogéniques, des médiateurs de l'immunité mais également des molécules d'adhésion ainsi que des cytokines/chimiokines [3]. La plupart des molécules de la membrane plasmique plaquettaire sont présentes également sur la membrane des granules α, tels que les intégrines et glycoprotéines (GP) [46, 47].

Des études ont montré que les plaquettes contiennent des sous-populations distinctes de granules α, qui subissent une libération différentielle lors de l'activation plaquettaire [48, 49]. La sécrétion des granules α se fait rapidement pour améliorer la réponse hémostatique ou inflammatoire [15]. Ces molécules sont destinées à être livrées de manière précise sur des sites de lésions vasculaires et fonctionnent pour recruter d'autres cellules [30].

Tableau 1. Contenu des granules α (liste non exhaustive) [15, 30, 45, 50, 51]

Type	Exemples
Molécules d'adhésion	αIIbβ3, αVβ3, GPIbα-IX-V, GPVI, CD40L, PECAM, P sélectine, CD9, TLT1, Vitronectine, Fibronectine, vWF, Multimérine, Ostéonectine
Cytokines/chimiokines	β-thromboglobuline, CCL4, CCL17, ENA78, Groα, IL1, IL7, IL8, MCP1, MCP3, MIP1α, NAP2, PF4, RANTES, sCD40L, sCD62P, SDF1
Facteurs de croissance, angiogéniques	ADAM10, ADAMTS13, Angiostatine, Angiopoiétine-1, BDNF, bFGF, BMP2, BMP4, BMP6, CTAP-III, CTGF, EGF, Endostatine, HGF, HGR, IGF1, Kininogène, MMP1-2-9, PDGF, TGFβ, Thrombospondine, TIMP1-4, VEGF
Facteurs de coagulation	Factor V-VIII-XI-XIII, Fibrinogène, Plasminogène, Plasmine, Antithrombine, Prothrombine, α_2-antiplasmine, α_2-antitrypsine, α2-macroglobuline, PAI-1, Protéase nexin-2, Protéine S, TFPI
Médiateurs immuns	Précurseur du complément C3, précurseur du complément C4, Globuline β1H, facteur D, facteur H, inhibiteur C1, IgA, IgG, IgM
Peptides antibactériens	Thrombocidine 1-2, Thymosine-β4
Autres	Albumine, PDCI

Les granules denses

Ces granules sont beaucoup moins nombreux que les granules α, environ 3 à 8 par plaquettes, mesurant de 100 à 200 nm. Elles contiennent principalement du calcium, de la sérotonine, de l'adénosine diphosphate (ADP), de l'adénosine triphosphate (ATP) et des cathécolamines. La sécrétion des granules denses est observée lors de l'activation plaquettaire après stimulation par le collagène ou l'ADP [52]. Leur fonction principale est de recruter les plaquettes au site de dommage vasculaire. L'ADP, faible agoniste des plaquettes, déclenche le changement de forme plaquettaire, la libération des granules et l'agrégation [30].

Tableau 2. Contenu des granules denses (liste non exhaustive) [15, 30, 50, 52]

Type	Exemples
Protéines membranaires	αIIbβ3, CD63, GPIb, LAMP1, LAMP2, P sélectin
Nucléotides	ADP, ATP, UTP, GTP
Cations	Calcium, Magnésium, Potassium
Amines bioactives	Sérotonine, Histamine
GTP-ases	rab27a, rab27b
Phosphates	Polyphosphate, Pyrophosphate

Les lysosomes

Ce sont de petites organelles riches en enzymes de dégradation, notamment la cathepsine, la β-galactosidase, l'arylsulfatase, la β-glucuronidase, et les acides phosphatases [53]. La fonction principale des lysosomes est de participer à la dégradation du matériel ingéré par phagocytose ou pinocytose [30].

Tableau 3. Contenu des lysosomes (liste non exhaustive) [15, 30, 50]

Type	Exemples
Enzymes dégradant les protéines	Cathepsines, Elastase, Carboxypeptidase, Collagenase, Proline carboxypeptidase
Enzymes dégradant les glucides	Glucosidase, Arabinofuranosidase, Fucosidase, Galactosidase, Glucuronidase, Mannosidase, Hexosaminidase
Phosphates	Polyphosphate acide

Les autres organites

Les plaquettes possèdent d'autres organites, notamment des mitochondries, des granules T et des peroxysomes [30, 54]. Les plaquettes contiennent un nombre relativement faible de mitochondries, contenant de l'acide désoxyribonucléique (ADN) plaquettaire et contribuant au métabolisme énergétique de la cellule. Récemment identifiés, les granules T contiennent des molécules de l'immunité innée de type « Toll-Like Receptor » (TLR), en

particulier le TLR9 [55]. Les peroxysomes sont également présents dans le cytoplasme des plaquettes. Ce sont de petits organites qui contiennent l'enzyme catalase.

2) L'activation plaquettaire

L'activation plaquettaire est effectuée en plusieurs phases, à savoir l'adhérence initiale, l'activation au contact de la paroi lésée, la sécrétion, l'amplification de l'activation et enfin, l'agrégation des plaquettes entre elles. Deux grandes voies d'activation plaquettaires se distinguent, celles qui sont impliquées dans les phénomènes d'adhérence et d'activation au contact de la paroi vasculaire lésée, et celles qui contribuent à l'amplification des réponses et au recrutement de plaquettes circulantes par des médiateurs solubles libérés lors de l'activation. L'activation plaquettaire est caractérisée par un changement morphologique et phénotypique ainsi que la libération du contenu des granules plaquettaires.

a. Adhésion à la paroi vasculaire lésée

Les plaquettes sont capturées au niveau de la paroi du vaisseau blessé par l'interaction spécifique du complexe plaquettaire GPIb-V-IX avec le facteur de von Willebrand (vWF), lié au collagène exposé sur le sous-endothélium [56]. Une adhésion stable des plaquettes se produit par la liaison de la GPVI et des intégrines β1 plaquettaires avec le collagène et la fibronectine [57]. Les plaquettes subissent ensuite une série de changements biochimiques qui induisent l'activation de l'intégrine αIIbβ3 et de sa voie de signalisation, conduisant à une interaction d'affinité élevée avec des protéines d'adhérence comprenant le vWF, le fibrinogène et la fibronectine [58]. Ces interactions sont indispensables pour que les plaquettes forment des agrégats stables avec d'autres plaquettes activées et ainsi favoriser la croissance du thrombus [59].

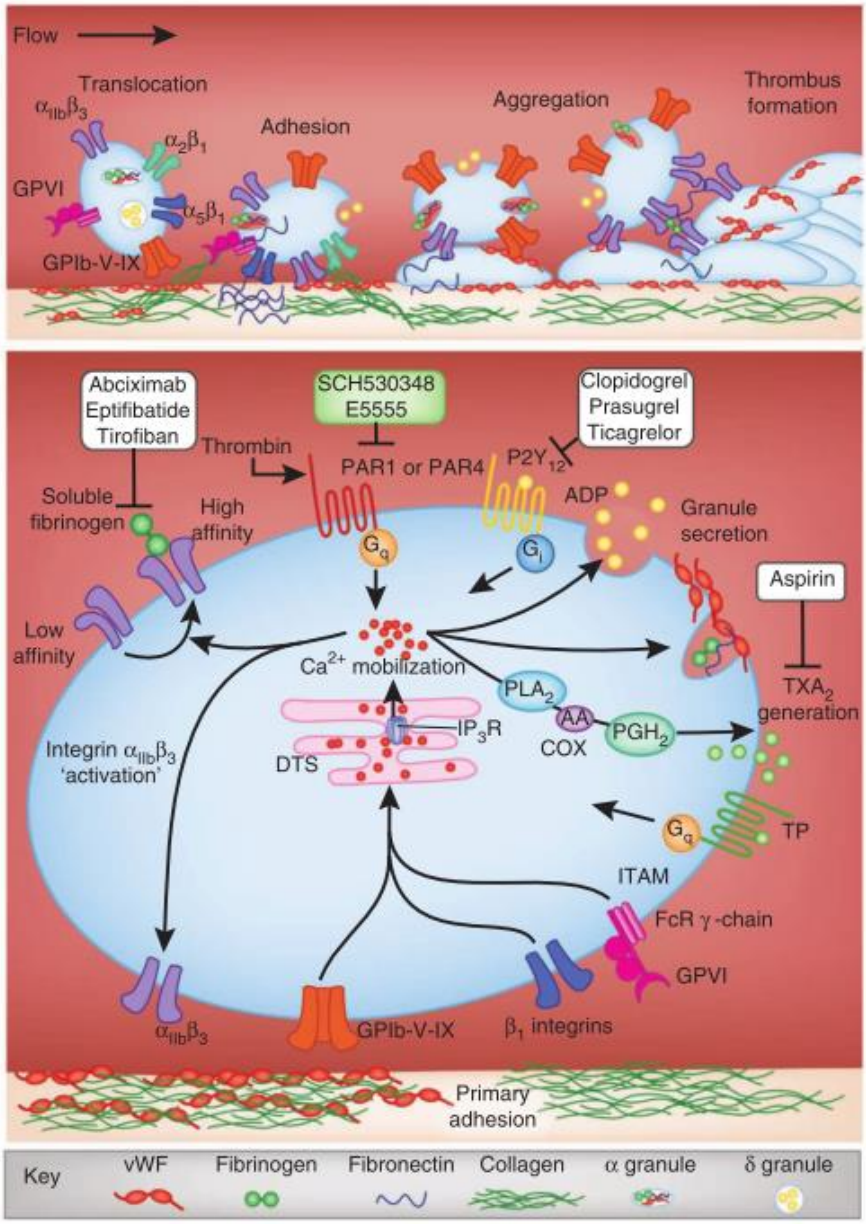

Figure 3. Mécanismes d'adhésion et d'activation plaquettaire

Principaux ligands et récepteurs, médiateurs de l'adhésion et de l'activation des plaquettes sur les sites de lésions vasculaires [59].

b. Activation par les agonistes solubles

Les plaquettes sanguines sont susceptibles d'être activées par de nombreux agonistes solubles, en particulier l'ADP, la thrombine et le thromboxane A2 (TXA2), mais également le collagène, la sérotonine et l'épinephrine [60]. Ces médiateurs aux effets autocrines et paracrines, agissent par l'intermédiaire de récepteurs couplés aux protéines G hétérotrimériques (RCPG), stimulant la cascade de signalisation intracellulaire et induisant une

mobilisation du calcium cytosolique [61]. Le calcium a un rôle clé dans la promotion de l'activation de l'intégrine αIIbβ3, la génération de TXA2, la sécrétion des granules et de la fonction procoagulante des plaquettes [59].

L'ADP

L'ADP est l'un des principaux composants libérés par les plaquettes activées, ayant un rôle essentiel dans le processus d'activation et d'agrégation plaquettaire. Il agit comme agoniste à deux récepteurs de type RCPG purinergiques, le $P2Y_1$ couplé à la protéine Gq et le récepteur $P2Y_{12}$ couplé à la protéine Gi_2 [50]. L'activation de $P2Y_1$ déclenche l'agrégation plaquettaire induite par ADP, et est responsable du changement de forme plaquettaire [62]. L'activation de $P2Y_{12}$ entraîne une amplification et une stabilisation de la réponse d'agrégation. Il existe une interaction complexe entre $P2Y_1$ et $P2Y_{12}$, et la co-activité des deux récepteurs est nécessaire pour l'agrégation complète des plaquettes [63]. En raison de son rôle prépondérant dans l'agrégation plaquettaire, le récepteur $P2Y_{12}$ est devenu une cible majeure du traitement antiplaquettaire.

La thrombine

La thrombine est l'agoniste plaquettaire le plus puissant. Il active les plaquettes après engagement des récepteurs activés par la protéase (PAR) et GPIb-IX-V [60]. Ce sont les récepteurs PAR1 et PAR4, couplés aux protéines Gq, G12/13 et Gi, qui interviennent principalement dans la réponse plaquettaire induite par la thrombine [50]. Bien que PAR1 soit sensible aux faibles taux de thrombine, le PAR4 déclenche l'activation et l'agrégation des plaquettes uniquement à des concentrations élevées de thrombine [64]. Des travaux antérieurs de notre équipe ont montré qu'une grande majorité des protéines membranaires et granulaires réagissent de manière similaire à l'engagement de PAR1 et PAR4. Cependant, des différences ont été observées au niveau de l'expression membranaire du CD40L, ainsi que la sécrétion par les granules de GROα et MDC [65]. Ceci soutient l'hypothèse que la stimulation plaquettaire par PAR1 et PAR4 déclenchent une mobilisation différentielle des protéines.

Le thromboxane A2

Le TXA2 est un métabolite instable de l'acide arachidonique. Il est à la fois un activateur des plaquettes et un puissant vasoconstricteur. Le TXA2 se lie avec aux récepteurs TP, qui

appartiennent à la classe des récepteurs des prostanoïdes, responsables de la mobilisation du calcium intracellulaire et de l'activation de la guanosine triphosphate (GTP)ase RhoA provoquant notamment des phénomènes contractiles des plaquettes [60].

c. Le réarrangement du cytosquelette

Les plaquettes au repos sont capables de se transformer rapidement lors de l'activation. Leur morphologie discoïde devient alors sphérique [32]. Ces modifications sont dues aux réarrangements du cytosquelette qui dépendent de la réorganisation des filaments d'actine [66]. Dans un premier temps, le réseau d'actine se désassemble et se contracte. Les filaments d'actine sont raccourcis sous l'action de la gelsoline. Dans un second temps, les filaments d'actine se réassemblent pour former des filopodes et des lamellipodes. Le réseau lamellipodial s'organise et est stabilisé par la filamine, les filaments courts sont le siège d'une élongation qui produit les filaments longs des filopodes. L'interaction entre l'actine et la myosine permet la contraction des filaments [37].

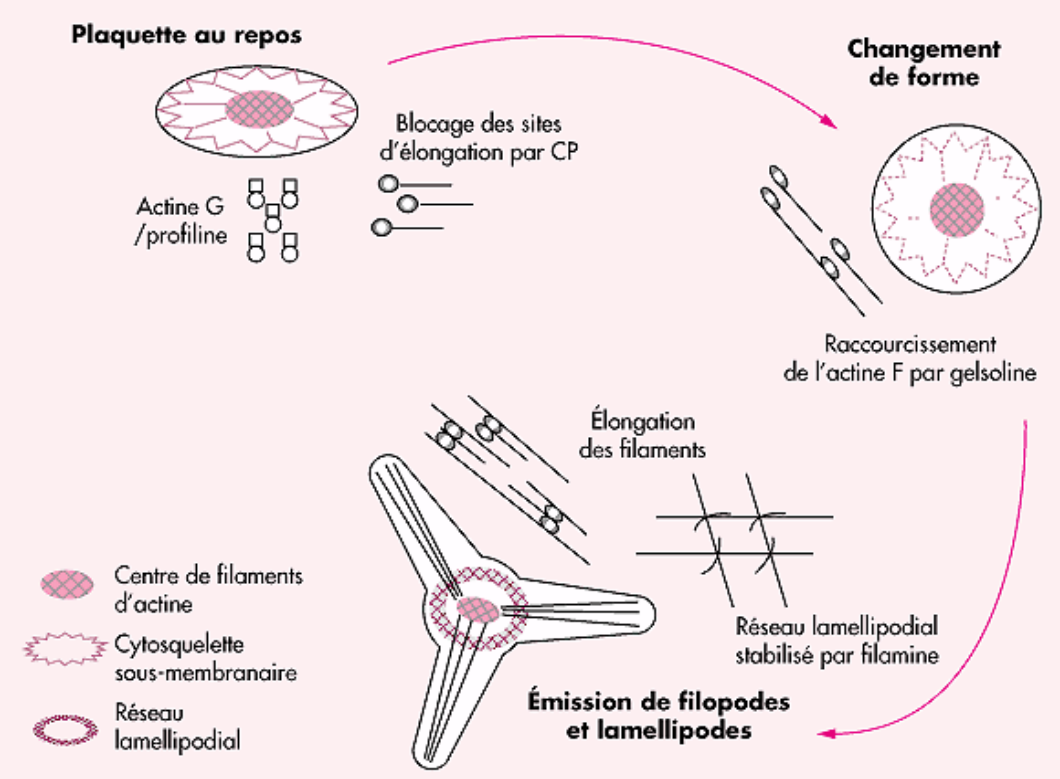

Figure 4. Réarrangement du cytosquelette

Réorganisation de l'actine lors de l'activation plaquettaire [37].

Ces changements morphologiques sont également associés à une fusion de la membrane des granules avec la membrane plasmique ou avec l'OCS conduisant à la sécrétion du contenu des granules plaquettaires.

d. La sécrétion des granules

Lors de l'activation, les plaquettes peuvent sécréter plus de 300 substances actives contenues dans leurs granules intracellulaires. L'exocytose des granules plaquettaires est réalisé soit par fusion des granules avec l'OCS, soit par fusion directe des granules avec la membrane plasmique [43]. Lors de la fusion des granules avec l'OCS, leur contenu est libéré dans l'OCS et se diffuse dans l'environnement extracellulaire [45]. Lors de la fusion de la membrane des granules avec la membrane plasmique, les protéines « Soluble N-ethyl-maleimide-sensitive factor attachment protein receptors » (SNARE) sont impliquées [67]. Cela implique le transport de la vésicule sur la membrane cible, les granules deviennent attachés aux régions de la membrane plasmique, enrichie en phosphatidylinositol 4,5-bisphosphate (PIP2). La GTPase rab27a sous forme active, s'associe aux facteurs de liaison Munc13-4 et à la protéine SLP4, qui peuvent tous deux s'associer à PIP2 de la membrane plasmatique opposée. Ensuite, un changement de conformation entraîne la fusion membranaire, puis finalement les protéines contenues dans les granules vont être libérés dans le milieu extérieur. Deux types de protéines SNARE sont retrouvés, des vésicules (vSNARE) et des SNARE appelés cible (tSNARE). Des vésicules vSNARE, tel que « Vesicle-associated membrane proteine » (VAMP)-8 jouent un rôle important lors de ce processus [68]. De plus, l'augmentation du calcium intracellulaire ainsi que les protéines kinases C, en particulier les isoformes α et β, supportent la libération des granules [50].

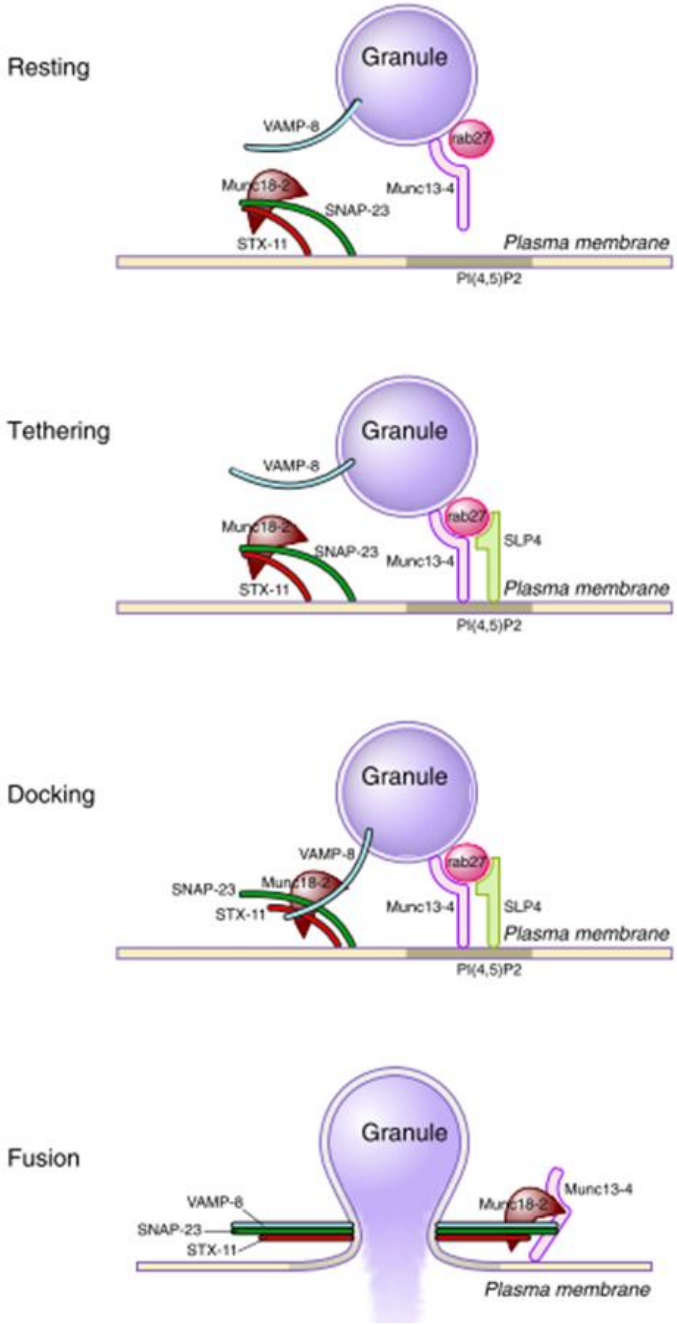

Figure 5. Libération du contenu des granules plaquettaires

Schéma illustrant les différentes étapes et les protéines impliquées dans la libération des granules plaquettaires lors de leurs activations [69].

En outre, les granules α montrent une certaine hétérogénéité lors de leur fusion à la membrane, cela peut-être notamment lié à l'existence de différents sous-types de granules [70]. Ces nombreuses études permettent d'identifier les mécanismes de sécrétion plaquettaires. Cependant des questions subsistent quant à la sécrétion différentielle des granules en fonction du stimulus.

II - Les récepteurs plaquettaires

Les plaquettes expriment de nombreux récepteurs à leur surface, ayant un rôle dans l'adhésion et l'activation plaquettaire et dans l'immunité innée. Ces différents récepteurs plaquettaires leurs permettent d'interagir avec d'autres cellules, en particulier les leucocytes et l'endothélium, notamment les neutrophiles via le CD62P, les lymphocytes B via des PRR, les lymphocytes T via le complexe CD40/CD40L, les cellules endothéliales et les érythrocytes via des intégrines [23]. La liaison de ces récepteurs à leurs ligands induit une activation plaquettaire, illustrant clairement la capacité des plaquettes à détecter et à répondre aux agents pathogènes [15].

1) Les récepteurs impliqués dans l'activation plaquettaires

Les plaquettes expriment également des récepteurs pour la thrombine, l'ADP et le thromboxane, qui favorisent l'agrégation et la sécrétion de BRM lorsqu'ils sont liés à leurs ligands [68]. Les voies d'activation plaquettaires diffèrent selon les agonistes, donc selon les récepteurs. Deux types de récepteurs se distinguent, les RCPG qui conduisent à la voie de la phospholipase C, et ceux qui induisent la voie d'activation impliquant les tyrosines kinases (RTK).

a. Les RCPG

Les médiateurs solubles activent principalement des récepteurs de type RCPG, qui sont des commutateurs moléculaires de la transduction des signaux. Ces récepteurs ont une structure commune à sept domaines transmembranaires. Concernant la physiologie des plaquettes, trois voies principales agissent de façon synergique [71] :

- la voie initiée par la protéine Gq qui active la phospholipase C β qui mène à l'activation de protéines kinases C et à la mobilisation des stocks intracellulaires de calcium

- la voie G12/13 qui mène à la phosphorylation de la chaîne légère de la myosine

- la voie Gi qui inhibe la formation d'adénosine monophosphate cyclique (AMPc), second messager inhibiteur des fonctions plaquettaires, et active la phosphatidylinositide 3-kinase (PI3K).

b. Les RTK

Les plaquettes contiennent une très forte activité tyrosine kinase, impliquée dans une voie de transduction lors de l'activation plaquettaire. Lors de l'adhésion des plaquettes, la GPVI, récepteur majeur du collagène, s'associe avec des immunorécepteurs à motifs « Immuno-Tyrosine Based Activation Motifs » (ITAM), conduisant aux voies de signalisation liées à des Src kinases, recrutant la tyrosine kinase Syk. Cette cascade déclenche des réactions de phosphorylation, conduisant à l'activation de la phospholipase Cγ2 (PLCγ2) et donc initialisant l'activation plaquettaire [72, 73].

2) Les molécules d'adhésion

Les plaquettes expriment également de nombreuses molécules d'adhésion, jouant un rôle primordial lors de l'adhésion, l'activation et l'agrégation des plaquettes entre elles.

a. Les intégrines

Les molécules d'adhésion les plus importantes exprimées par les plaquettes, au niveau membranaires sont les intégrines (β1 et β3), protéines hétérodimériques transmembranaires qui interviennent en réponse à une blessure vasculaire [74]. Les intégrines interviennent dans l'adhésion des plaquettes aux protéines de la matrice extracellulaire, telles que la fibronectine, la laminine, le collagène et le vWF [15]. Les plaquettes expriment des complexes glycoprotéiques et notamment la GP1b-IX-V, qui permet l'interaction des plaquettes avec le sous-endothélium [75], en se liant au vWF [76]. Les intégrines jouent également un rôle important dans la signalisation cellulaire. L'activation plaquettaire via les récepteurs RCPG et RTK, conduit à l'activation de l'intégrine αIIbβ3. Ce processus d'activation est communément appelé « Inside-out signaling». Lorsque l'intégrine ainsi modifiée est occupée par ses ligands, d'importants phénomènes de signalisation de l'extérieur vers l'intérieur de la plaquette ont lieu, appelés «Outside-in signaling» [77].

b. Les sélectines

Les plaquettes expriment une grande quantité de P sélectine (CD62P) qui, lors de l'activation, est libérée des granules α et rapidement mobilisée à la surface, où elle peut

participer à l'adhésion aux cellules immunitaires exprimant « P selectin glycoprotein ligand 1 » (PSGL1), principalement des neutrophiles, des monocytes et d'autres leucocytes, mais également les cellules endothéliales. La P sélectine plaquettaire a un rôle crucial dans le recrutement des cellules immunitaires, avec une double action en tant que molécule d'adhésion et de signalisation [78].

c. Les autres molécules d'adhésion

En plus des sélectines et des intégrines, les plaquettes expriment d'autres molécules qui peuvent faciliter leur interaction avec les leucocytes et les cellules endothéliales, telles que « Intercellular Adhesion Molecule 2 » (ICAM2), « Junctional Adhesion Molecules » (JAMA, JAMC) et « Platelet Endothelial Cell Adhesion Molecule 1 » (PECAM1) [79]. Ces molécules interagissent avec d'autres cellules par le biais d'un complexe homophile (PECAM1, JAMA) et hétérophile (ICAM2, JAMA, JAMC), servant de ligands pour les intégrines [15].

Le complexe CD40/CD40 Ligand (CD40L), fortement exprimé par les plaquettes, a un rôle central pour promouvoir l'interaction entre les lymphocytes et les cellules présentatrices d'antigènes [25]. En effet, les plaquettes contiennent une quantité significative de CD40L qui est exprimé et libéré lors de l'activation des plaquettes [19]. Une fois exprimée, le CD40L plaquettaire peut interagir avec le CD40 lié à la membrane sur les cellules endothéliales, déclenchant plusieurs réactions inflammatoires conduisant à la libération locale de molécules d'adhésion, incluant ICAM1, « Vascular Cell Adhesion Molecule 1 » (VCAM1) et « C-C Chemokine Ligand 2 » (CCL2) [80]. CD40 est exprimé par de nombreuses cellules immunitaires, y compris les monocytes, les cellules dendritiques et les lymphocytes B. Il contribue au développement de la réponse immunitaire acquise, en fonction de son activation et de la transduction du signal par CD40L [81]. Cela se produit en induisant le clivage de CD40L, qui aboutit à une forme soluble [82].

3) Les PRR

Les plaquettes expriment des récepteurs immunitaires fonctionnels, tels que les PRR [83], qui incluent les récepteurs du complément, les TLR [84], les « Nucleotide-binding oligomerization domain Like Receptors » (NLR) et les récepteurs Fc (FcR) reconnaissant les

immunoglobulines (Ig) [25]. Les PRR sont capables de reconnaître les signaux de dangers et de déclencher une réponse immunitaire innée [85]. Les signaux de danger peuvent être des éléments du « non-soi biologique », c'est-à-dire des pathogènes ou des fragments de pathogènes appelés PAMP, ça peut être également des signaux de danger du « soi », qui sont libérées par des cellules endommagées ou nécrosées appelés DAMP ou encore les SAMP qui sont des molécules endogènes, présentes sur la membrane plasmatique des cellules et les glycosaminoglycanes [86].

a. Les TLR

Les TLR sont une famille de récepteurs de reconnaissance des motifs conservés exprimés par des agents pathogènes PAMP [87]. Les TLR sont divisés en sous-familles selon le type de PAMP qu'ils reconnaissent [88]. Les TLR ne sont pas seulement exprimés par des cellules phagocytaires, mais sont également exprimés par les plaquettes, les TLR1, 2, 3, 4, 6, 7, 8 et 9 membranaires et les TLR3, 4, 7, 8 et 9 intracellulaires [89]. Les TLR 2, 4 et 9 ont un rôle fonctionnel, ce différemment selon leur état d'activation des plaquettes [90, 91]. Ainsi, les TLR permettent aux plaquettes de participer à la détection du danger, qui est généralement décrit pour les cellules du système immunitaire inné [92]. La stimulation des TLR induit l'activation de la voie « Nuclear Factor Kappa B » (NFκB) et « Mitogen-Activated Protein Kinases » (MAPK), porté par la production de cytokines pro-inflammatoires [89].

Le TLR4, largement étudié, reconnaît le lipopolysaccharide bactérien (LPS) [93]. Le LPS provoque la libération du CD40L soluble (sCD40L) à travers le TLR4 plaquettaire [94]. De plus, les plaquettes peuvent moduler leurs réponses avec une sécrétion de cytokines différentielle en fonction du ligand de TLR4 [95]. Des études ont étudié l'activité fonctionnelle du TLR4 plaquettaire, montrant un rôle *in vivo* des plaquettes stimulées par le LPS dans le déclenchement de la sécrétion de « Tumor Necrosis Factor α » (TNFα) et confirmant une thrombocytopénie dépendante du TLR4 [96]. En outre, la reconnaissance du LPS par le TLR4 plaquettaire induit une liaison plaquettaire-neutrophile et une activation des neutrophiles entraînant la formation de « Neutrophil Extracellular Traps » (NET) [97-99]. Des études sur des modèles murins ont validé la fonctionnalité des TLR4 plaquettaires, montrant que les plaquettes sont activées par du LPS, comme en témoigne l'augmentation de l'expression du récepteur GPIIb/IIIa et du CD40L, ainsi que l'augmentation de la fixation au fibrinogène [100].

Enfin, notre équipe a mis en évidence que la sécrétion de cytokines/chimiokines plaquettaires est sensible à des signaux différentiels en fonction des liaisons des TLR par différents ligands [101].

L'expression du TLR2 fonctionnel a également été démontrée dans les plaquettes. La stimulation du TLR2 par des agents pathogènes via la voie de signalisation de la PI3K est capable de directement activer les réponses plaquettaires prothrombotiques et pro-inflammatoires [102].

Récemment, il a été montré que l'activation des TLR9 plaquettaires est un facteur important reliant l'activation des plaquettes à la thrombose, au stress oxydatif et à l'immunité innée [103]. Le TLR9 plaquettaire est distribué de manière unique dans les granules T [55], et est associé à VAMP7 ou VAMP8. Les plaquettes expriment le TLR9 à leur surface lors de l'activation, ce qui facilite ensuite l'activation secondaire à travers les PAMP exprimés sur les agents pathogènes.

Les TLR ont un rôle essentiel dans la reconnaissance des acides nucléiques viraux. TLR3 reconnaît l'acide ribonucléique (ARN) bicaténaire résultant du matériel génétique ou produit pendant le cycle de vie de certains virus. TLR7 et TLR8 reconnaissent l'ARN monocaténaire et TLR9 reconnaît l'ADN viral contenant une séquence CpG non méthylée [104].

b. Les NLR

Parmi les PRR, les NLR sont des acteurs clés de l'immunité innée. Contrairement aux TLR qui se trouvent sur la membrane plasmique, les NLR sont des récepteurs cytoplasmiques. « Nucleotide-binding Oligomerization Domain 2 » (NOD2) est un NLR important, exprimée dans les plaquettes et ayant un rôle dans d'activation plaquettaire et de thrombose artérielle, éventuellement pendant l'infection [105].

Le « Nucleotide-binding domain Leucine Rich repeat containing Protein » (NLRP3) exprimé par les plaquettes peut s'activer en cas d'infection par le virus de la Dengue, et entraîner la sécrétion d'interleukine 1β (IL1β) et augmenter la perméabilité vasculaire [106]. Le NLRP3 coopère avec des protéines « Caspase Activation and Recruitement Domains » (CARD), la caspase 1 et la tyrosine kinase de Bruton pour former un complexe nommé inflammasome. L'inflammasome NLRP3 contribue à l'activation et l'agrégation plaquettaire [107].

4) Les récepteurs Siglec

Les « Sialic acid-binding immunoglobulin-like lectins » (Siglec) sont des récepteurs de type inhibiteur ayant un rôle indispensable dans le contrôle des processus physiologiques cellulaires et dans la régulation négative du système immunitaire inné et adaptatif vis-à-vis des signaux de danger [108]. Notre équipe a mis en évidence une expression plaquettaire importante du Siglec-7. L'engagement de ce récepteur plaquettaire est capable d'induire l'apoptose des plaquettes et cette fonction est strictement régulée par les voies de signalisation intraplaquettaire. Cette fonction du Siglec-7 sur les plaquettes est probablement un mécanisme de la régulation négative d'une réponse plaquettaire inflammatoire excessive [109].

III - Les BRM plaquettaires

Les plaquettes possèdent dans leurs granules un très grand nombre de cytokines, chimiokines et molécules apparentés, ayant un rôle immunomodulateurs, qui sont susceptibles d'être sécrétés différemment selon l'activation des plaquettes [110, 111]. En effet, les plaquettes peuvent libérer leurs contenus en fonction de différents stimuli [101]. Ces facteurs solubles peuvent activer et recruter différentes cellules, notamment les leucocytes, afin de moduler la réponse inflammatoire ou immunitaire.

1) Cytokines, chimiokines et molécules apparentées

La plupart des cytokines, chimiokines et molécules apparentées se trouvent dans les différentes granules plaquettaires. Lorsqu'elles sont libérées lors de l'activation plaquettaire, ces molécules peuvent servir de médiateurs à un large éventail d'interactions cellulaires. Par exemple, les granules α contiennent de nombreuses chimiokines tels que le « Platelet Factor 4 » (PF4), la β-thromboglobuline et CXCL5, qui peuvent moduler la chimiotaxie des neutrophiles [112] ; ou encore « Macrophage Inflammatory Protein 1α » (MIP1α), « Monocyte Chemotactic Protein 3 » (MCP3) et « Regulated on Activation, Normal T Cell Expressed and Secreted » (RANTES), qui ont sont connus pour recruter et activer les leucocytes [16, 113, 114]. Le PF4 peut rendre les monocytes résistants à l'apoptose et induire leur différenciation en

macrophages [115]. En outre, le PF4 peut améliorer l'adhésion des neutrophiles à l'endothélium non stimulé et à la libération des granules [116]. MIP1α peut stimuler la libération d'histamine à partir des basophiles et est chimiotactique pour les lymphocytes T [19].

L'IL1β produite et libérée par les plaquettes peut favoriser des réponses inflammatoires par les leucocytes et les cellules endothéliales [117, 118].

Les plaquettes libèrent également des facteurs de croissance ayant des activités immunomodulatrices, tels que le « Platelet-Derived Growth Factor » (PDGF) et « Transforming Growth Factor β » (TGFβ), un puissant facteur immunosuppresseur [20].

Des molécules exposées à la surface plaquettaire après activation peuvent également être clivées et donc devenir sous forme solubles, jouant un rôle actif dans l'immunomodulation, notamment le CD62P et le CD40L solubles (sCD62P, sCD40L). En effet, les plaquettes ont été identifiées comme étant la plus grande source de sCD40L, et les taux de sCD40L semblent être un indicateur du degré d'activation plaquettaire [82]. C'est une molécule qui a été décrite comme capable d'induire la production de « Reactive Oxygen Species » (ROS) et l'augmentation de l'expression des molécules d'adhérence par les neutrophiles, d'activer les macrophages, et d'induire une activation optimale des cellules T cytotoxiques *in vitro* et *in vivo* [119].

Les plaquettes sont la principale source de « High Mobility Group Box 1 » (HMGB1), considéré comme un DAMP, impliqué dans le recrutement de cellules immunitaires au niveau du site d'infection [120-122]. HMGB1 interagit avec plusieurs récepteurs plaquettaires, notamment les TLR2-4-9 et le « Receptor for Advanced Glycation Endproducts » (RAGE), induisant l'agrégation plaquettaire et la formation des NET [123, 124].

2) Microparticules plaquettaires

Il a été démontré que les plaquettes libèrent deux types de vésicules extracellulaires lorsqu'elles sont activées, des exosomes dérivés de l'exocytose des corps multivésiculaires et des granules α [125], et les microparticules (MP) produits par l'excrétion de surface [126].

Les MP sont des vésicules extracellulaires de taille comprise entre 0,1 et 1 µm, exprimant la phosphatidylsérine (PS) à leur surface et des récepteurs caractéristiques de leur

cellule d'origine, contenant une partie des éléments de leurs cellules parentales [127]. Les MP proviennent de corps multivésiculaires induits par exocytose, différents sous-groupes de MP peuvent exister [128]. Dans des conditions physiologiques, les MP circulent dans le sang [129]. Cependant, le nombre de MPP peut augmenter dans plusieurs situations pathologiques, tels que le syndrome coronarien aigu, ou encore des maladies inflammatoires vasculaires comme l'athérosclérose [130-133]. Les microparticules plaquettaires (MPP) sont les plus abondantes dans la circulation sanguine [134].

Elles possèdent des propriétés cytoprotectrices en participant au maintien de l'homéostasie vasculaire et en préservant les fonctions de l'endothélium mais peuvent aussi être initiatrice d'un processus délétère si elles sont libérées en excès ou si elles expriment des constituants pathogènes tels que des antigènes tumoraux. En effet, les MPP exposent une surface procoagulante [135] et agissent comme transporteur de molécules bioactives, notamment des facteurs de croissance, des molécules de signalisation et du matériel génétique, y compris des ARN messager (ARNm) [136], des microARN [137] et de l'ADN mitochondriale (ADNmt) [138]. Les MPP peuvent transférer leur contenu à d'autres cellules du système circulatoire, notamment les cellules endothéliales, et réguler l'expression et la fonction des gènes des cellules réceptrices [139, 140].

Au cours de l'inflammation, les MPP peuvent activer les cellules présentatrices d'antigène, moduler l'activation des cellules dendritiques, augmenter les réponses des lymphocytes T, induire la production d'immunoglobuline G (IgG) par les lymphocytes B et améliorer la formation du centre germinal en coopération avec les lymphocytes T [141].

3) L'ADN mitochondrial

Outre les BRM classiques, de l'ADNmt est également retrouvé dans le cytoplasme plaquettaire. L'ADNmt est classé comme signal de danger endogène (DAMP). Des études ont montré que l'ADNmt peut être sécrété lors de l'activation, et activer avec les neutrophiles afin de promouvoir l'inflammation [142]. En effet, les plaquettes activées libèrent des mitochondries fonctionnelles, soient contenues dans des MPP soient sous forme d'organelle libre. De plus, la mitochondrie est un substrat endogène de la phospholipase A2 IIA (sPLA2-IIA), spécifique des bactéries. L'hydrolyse de la membrane mitochondriale par le sPLA2-IIA produit

des médiateurs inflammatoires (lysophospholipides, acides gras et ADNmt) qui favorisent l'interaction avec les neutrophiles, déclenchant leur adhésion à la paroi endothéliale [143]. Les mitochondries produites par les plaquettes conduisent à des réponses inflammatoires.

4) Le CD40 Ligand

Parmi ces BRM immunomodulateurs plaquettaires, le principal est le sCD40L, qui joue un rôle charnière entre l'immunité innée et adaptative. Cette dernière molécule pro-inflammatoire est impliquée dans plusieurs processus pathologiques tissulaires. Le CD40L est exprimé à la fois sous forme membranaire et à la fois sous forme soluble après activation plaquettaire. Il semble intervenir dans de nombreux processus immunitaires notamment en interagissant avec d'autres cellules immunes ou non comme les cellules endothéliales.

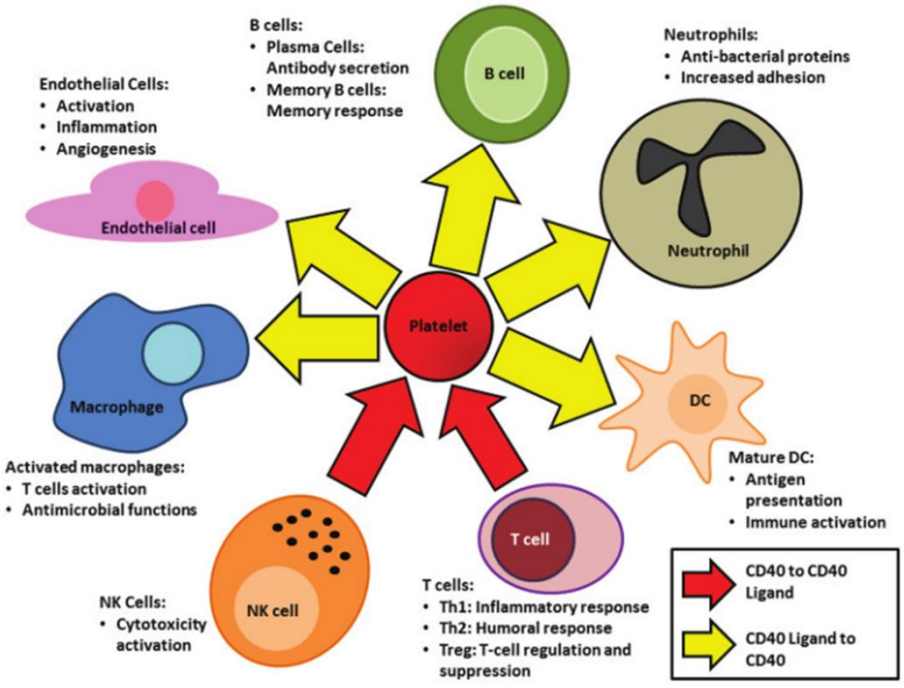

Figure 6. Interaction des plaquettes avec d'autres cellules via le couple CD40/CD40L

Les plaquettes peuvent interagir avec de nombreuses cellules immunitaires telles que les lymphocytes B et T, les neutrophiles, les macrophages, les cellules endothéliales, les cellules NK et les cellules dendritiques [110].

La revue suivante décrit le rôle immunomodulateur du CD40L plaquettaire dans des conditions physiologiques et pathologiques, en se concentrant sur les principales voies de signalisation activées après liaison du CD40L avec ses différents récepteurs.

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Review

The Signaling Role of CD40 Ligand in Platelet Biology and in Platelet Component Transfusion

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Abstract: The CD40 ligand (CD40L) is a transmembrane molecule of crucial interest in cell signaling in innate and adaptive immunity. It is expressed by a variety of cells, but mainly by activated T-lymphocytes and platelets. CD40L may be cleaved into a soluble form (sCD40L) that has a cytokine-like activity. Both forms bind to several receptors, including CD40. This interaction is necessary for the antigen specific immune response. Furthermore, CD40L and sCD40L are involved in inflammation and a panoply of immune related and vascular pathologies. Soluble CD40L is primarily produced by platelets after activation, degranulation and cleavage, which may present a problem for transfusion. Soluble CD40L is involved in adverse transfusion events including transfusion related acute lung injury (TRALI). Although platelet storage

designed for transfusion occurs in sterile conditions, platelets are activated and release sCD40L without known agonists. Recently, proteomic studies identified signaling pathways activated in platelet concentrates. Soluble CD40L is a good candidate for platelet activation in an auto-amplification loop. In this review, we describe the immunomodulatory role of CD40L in physiological and pathological conditions. We will focus on the main signaling pathways activated by CD40L after binding to its different receptors.

Keywords: CD40 ligand; CD40; inflammation; signaling pathways; p38 mitogen-activated protein kinases (MAPK); nuclear factor-KappaB (NF-κB)

1. Introduction

CD40 ligand (CD40L)—otherwise known as CD154—is of particular interest for several reasons. It is easily detectable in plasma; it is essential to immunity at large and central to adaptive immunity, being among the seminal molecules that tether antigen (Ag)-specific T and B-lymphocytes in the synapse; and it is indispensable for the formation of germinal centers (GCs) in lymph nodes [1–4]. CD40L is thus crucial for cell signaling in both adaptive and innate immunity, as it is expressed by a large variety of cells that take a role in immune responses [1,4]. Further, CD40L has genetic and molecular polymorphisms, with pathogenic and pathologic consequences [5]. Intriguingly, its soluble form is principally generated by platelets, and it is responsible for transfusion associated hazards [6–8]. Together, those properties require the attention of pathologists and clinicians, as CD40L is more important in medicine than initially thought.

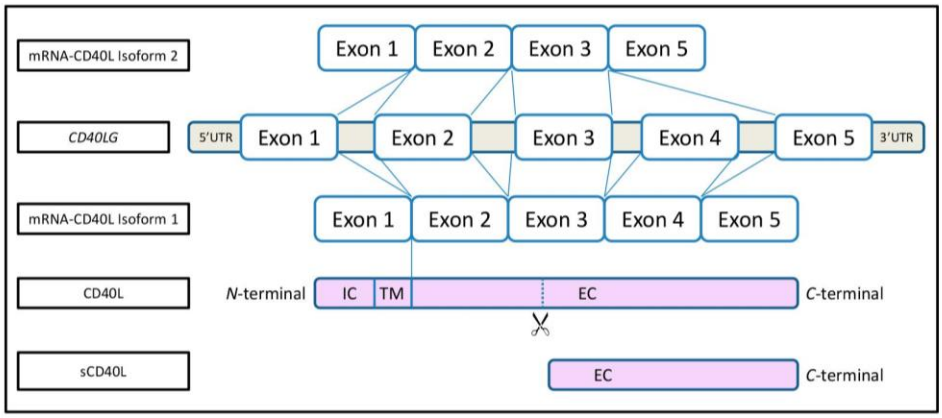
In this review, we will discuss the role of CD40L and its soluble form (sCD40L) in transfusion hazards. It is associated with high levels of inflammatory molecules such as chemokines, cytokines and biological response modifiers (BRMs) released by platelets during storage. sCD40L is a master pro-inflammatory BRM in transfusion [6–12]. Platelet sCD40L has been largely studied in inflammation and autoimmune disease [3,13–15], but the mechanism for its regulation is just beginning to be unraveled.

2. What Is CD40L?

CD40L is a 33 kDa type II transmembrane protein belonging to the Tumor Necrosis Factor (TNF) superfamily. The CD40L gene (*CD40LG*) encodes a 261 amino acid (AA) protein with a 22 AA cytoplasmic domain, a 24 AA transmembrane (TM) domain, and a 215 AA extracellular domain (Figure 1) [3]. CD40L is constitutively highly expressed by a panoply of hematopoietic and non-hematopoietic cells [1,3,4,16]. CD40L can be further expressed or overexpressed by activated cells, the most characteristic and best studied of which are activated and/or differentiated T cells [4]. Like other members of the TNF family, active CD40L at the cell surface or in its soluble form is composed of homotrimers [17]. This multimeric conformation of CD40L is of crucial importance for effective interaction with CD40 and the subsequent intracellular signaling [18]. Moreover, the

soluble forms of CD40L retain their ability to form trimers, which bind CD40 and deliver biological signals [18]. Membrane bound CD40L can be cleaved at methionine 113 of the extracellular domain and shed as a soluble form [19–21]. The principal isoform (isoform 1) is encoded by 5 exons. The second CD40L isoform (isoform 2) is poorly described (Figure 1). It is a truncated 240 AA protein lacking exon 4 in the *CD40LG* (extracellular domain), and the functional consequence of this is unknown [22]. Of note, membrane bound CD40L is expressed on B cells and dendritic cells (DCs). It is not expressed on non-activated T cells and platelets, but is weakly expressed on non-activated macrophages, neutrophils and endothelial cells [23]. It is highly expressed on activated T cells and platelets from which it can be cleaved as a soluble form, but it is not cleaved from B cells, DCs and macrophages. There is no up-regulation in neutrophils and endothelial cells, regardless of whether they are activated [1,3,4,23].

Figure 1. Sheme of the CD40 ligand gene structure and its different isoforms. Intracellular domain (IC), transmembrane domain (TM), extracellular domain (EC).



The main receptor for CD40L is CD40, which is constitutively expressed by antigen presenting cells (APCs) such as B cells, macrophages, and DCs [3,4,24]. CD40 is also expressed by platelets [25,26], neutrophils, endothelial cells [23] and T-cells [27–30]. Five distinct isoforms of CD40 are expressed with two isoforms predominating in human and mice [31–33]. Isoform 1 predominates and is membranous, but may be cleaved into a soluble form by a metalloproteinase, ADAM-17 [34]. In contrast, isoform 2 is produced as a soluble form resulting from alternative splicing [35]. It is hypothesized that the soluble forms act as competitive inhibitors for the membranous form, though this remains unclear [36]. Although CD40 is a type I TM protein that can form monomers, dimers and trimers, only the latter form fully activates cells [37–39].

CD40L can also bind to three integrins: the platelet glycoprotein αIIbβ3 (GPIIb/IIIa), otherwise known as receptor for fibrinogen and von Willebrand Factor [40,41]; α5β1 (CD49e/CD29), an integrin that binds to matrix macromolecules and proteinases and thereby stimulates angiogenesis [42–44]; and Mac-1, an integrin (otherwise known as CR3 (Complement Receptor 3), CD11b/CD18, or $\alpha_M\beta2$), mainly expressed by neutrophils, natural killer cells and macrophages to trigger a transduction signal and mediate inflammation [45]. The functional interaction of CD40L with α5β1 is independent of its binding to αIIbβ3 and CD40 [43,44]. Interactions between CD40L and α5β1 are not relevant in platelet physiology/physiopathology [45].

3. What Is the Function of CD40L?

The interaction between CD40 and CD40L is essential in the innate and adaptive immune systems, both in physiology and in physiopathology.

3.1. CD40/CD40L in Physiology

First characterized as a major marker on carcinoma cells, CD40 was next shown to be a key molecule shared by endothelial cells and most APCs, including B-cells, monocytes and DCs [46]. Interactions with CD40L are mandatory for the B-cell response to T-dependent Ags [2]. In particular, studies on patients with primary Ab immunodeficiencies targeting CD40 or CD40L have definitively established the requirement of these interactions for GC formation and the generation of memory B-cells and long-lived plasma cells [47]. More recent data on GC reactions and follicular helper T-cells (T_{FH}) show that the polarization of CD4 T-cells into T_{FH} is initiated by contact with DCs at the border of B-cell follicles and maintained by GC B-cells [48]. The expression of BCL6, the master regulator of T_{FH}, is dependent on CD40-CD40L and ICOS-ICOSL interactions outside follicles and within GCs [49]. CD40L-induced CD40 signaling in B-cells is crucial for inducing the expression of BCL6 and Ki67 in GC B-cells, allowing the proliferation of GC B-cells in the dark zone and expression of activation-induced deaminase (AID), a transcription factor required for somatic hypermutation (dark zone) and Ig class switching (light zone). CD40-CD40L interactions are further required for the selection of B-cell clones expressing high affinity BCR that takes place within the GC light zone. In physiological conditions, only selected B-cell clones differentiate into effector B-cells (memory and plasma cells). CD40 is also constitutively expressed by DCs and macrophages, and its triggering induces the expression of other co-stimulatory molecules and the release of cytokines that modulate T- and B-cell responses [24]. CD40 activation on macrophages also induces the release of nitric oxide and reactive oxygen species, contributing to the destruction of intracellular pathogens. Strikingly, CD40-induced CD40L signaling in CD8 T-cells rescues them from the exhaustion observed during chronic viral infections and is important to maintain their poly-functionality [50]. With CD40 being expressed on various B-cell lymphomas and carcinomas (nasopharynx, bladder, cervix, kidney and ovary), there is a renewed interest in CD40/CD40L in the control of tumor growth, leading to the development of new therapeutic strategies [51].

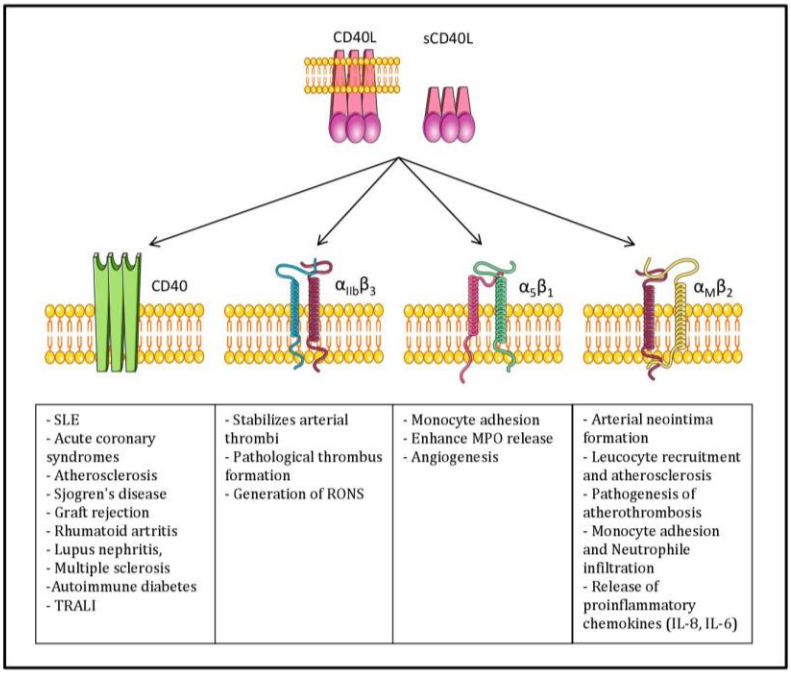
3.2. CD40L and Its Receptors in Inflammatory Pathologies

As already presented, in addition to the classical receptor CD40, CD40L also binds the $\alpha IIb\beta 3$, $\alpha 5\beta 1$, and Mac-1 ($\alpha M\beta 2$) integrins and induces different biological responses. Figure 2 illustrates the pathological role of each dyad interaction.

The CD40-CD40L system is associated with both pro-thrombotic and pro-inflammatory effects. Soluble CD40L contributes to the pathophysiology of atherosclerosis and atherothrombosis [52]. Because of its autocrine, paracrine, and endocrine activities, sCD40L enhances platelet activation, aggregation, and platelet-leukocyte conjugation that may lead to atherothrombosis [13,53,54]. CD40L binding may result in the activation of CD40 expressing cells with interleukin production [23,55].

The interaction of CD40L with CD40 on endothelial and other vascular cells upregulates adhesion molecules such as E-selectin, VCAM-1, ICAM-1 and proinflammatory cytokines such as regulated on activation normal T cell expressed and secreted (RANTES), interleukin (IL)-6, and IL-8 as well as matrix metalloproteinase (MMP)-1, -2, -3, and -9 [56]. Soluble CD40L also stimulates the expression of tissue factor (TF) on monocytes and on endothelial cells [57,58]. After CD40L and CD40 interact on the endothelial surface, thrombomodulin expression is decreased, facilitating thrombin generation [59]. CD40L-CD40 interactions activate endothelial cells via either sCD40L *in vivo* or by a specific antibody to CD40. Membrane-bound CD40L, but not sCD40L, induces the upregulation of pro-inflammatory cytokines and cell adhesion factors in endothelial cells. However, both forms of CD40L activate both classical and alternative NF-κB pathways [60]. In addition, sCD40L induces endothelial dysfunction with decreased NO synthesis and augmented oxidative stress [61]. These events may further contribute to endothelium injury and accompanying atherogenesis. sCD40L may play a pathogenic role in triggering acute coronary syndromes [54,62]. The involvement of CD40-CD154 interactions in autoimmunity and allo-immunity is also well documented. In fact, many tissue injuries and immune mediated pathologies such as graft allo-rejections involve this signaling pathway [63]. CD40-CD40L interactions play a significant role in the production of auto-antibodies in systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and other autoimmune diseases. An increased serum level of soluble CD154 was reported in SLE, RA, and Sjogren's disease, in correlation with the relevant auto-antibodies and with the clinical disease activity [14,64].

Figure 2. CD40L and its receptors: the binding of CD40L to CD40, αIIbβ3, α5β1, or Mac-1 (αMβ2) induces different inflammatory pathologies. Systemic lupus erythematosus (SLE), transfusion-related acute lung injury (TRALI), reactive oxygen and nitrogen species (RONS), Myeloperoxidase (MPO).



αIIbβ3 integrin was first identified as a receptor for CD40L by André *et al.* [65]. They showed that sCD40L can bind to αIIbβ3 integrin on activated platelets, thereby inducing platelet spreading and promoting platelet aggregation under high shear rates, as well as allowing stability of arterial thrombi [66]. The same group further reported that CD40L is a primary platelet agonist capable of inducing platelet activation, induction of fibrinogen binding and the formation of platelet microparticles by binding to its αIIbβ3 receptor and triggering outside-in signaling [67]. In addition, the engagement of αIIbβ3 by CD40L or other ligands that induce platelet adhesion upregulates CD40L surface exposure on platelets [68], enhancing the interaction of platelets with CD40+ cells, including ECs. Incubation of platelets with recombinant sCD40L led to enhanced P-selectin expression, aggregation, and platelet-leukocyte conjugation. The inhibition of either sCD40L or αIIbβ3 attenuated the generation of reactive oxygen and nitrogen species (RONS) by platelets [69].

Mac-1 is an important mediator of neutrophil and monocyte adhesion to the activated endothelium during inflammation. CD40L ligation to Mac-1 is involved in mediating CD40L/Mac-1-dependent monocyte and neutrophil adhesion and transmigration at the atherosclerotic lesion site, as well as neointimal formation during atherogenesis [42,70]. In transfusion, the sCD40L concentration increases in stored platelets compared to fresh platelets [9,10]. The neutrophil priming ability of stored platelets is significantly higher compared to fresh platelets [71]. Soluble CD40L and CD40-activated-neutrophils are essential to permit the adhesion and migration of neutrophils by Mac-1 secretion. This signal is the main system to recruit neutrophils into pulmonary tissue [72]. CD40+ neutrophils primed by CD40L+ activated platelets and sCD40L are recruited and over-stimulated by IL-6, IL-8 and IL-1β originating from alveolar macrophages and fibroblasts. In alveolar space, these neutrophils secrete ROS, proteases, PAF and elastase-α1-antitrypsin complexes that insult the pulmonary parenchyma [73]. In another study using the two-event TRALI mouse model, Hidalgo and colleagues demonstrated an increase in platelet interactions with adherent neutrophils in the systemic circulation [74]. These interactions were dependent on E-selectin expression on the endothelium interacting with E-selectin ligand on neutrophils, which ultimately led to the polarization of Mac-1 on the leading edge of the neutrophils. Circulating platelets interacted with the clustered Mac-1, although the platelet ligand mediating this interaction is not known [74]; could it be CD40L?

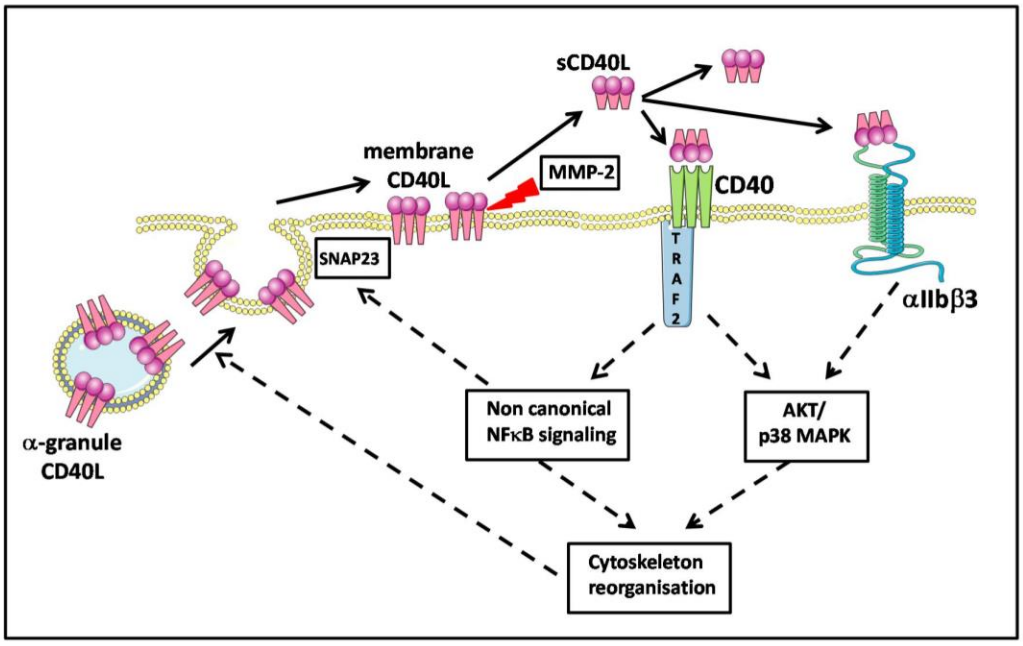
The α5β1 integrin is expressed by endothelial cells, smooth muscle cells, monocytes/macrophages and platelets. It is implicated in cell adhesion, migration, and proliferation as well as survival of many cell types. The binding of CD40L to a monocytic cell line expressing α5β1 integrin leads to the phosphorylation of the extracellular signal regulated kinases 1/2 (ERK-1/2) and expression of IL-8 mRNA in these cells [14]. However, unlike fibrinogen and vitronectin which are the natural ligands of α5β1, CD40L binds to the inactive rather than the active form of α5β1. Interestingly, CD40L/α5β1 interactions do not interfere with the binding of CD40L to CD40, indicating that CD40L can bind simultaneously to both receptors [43].

The role of α5β1 as a receptor for CD40L in α5β1-expressing-cells has not yet been investigated. Hassan *et al.* hypothesized the involvement of the CD40L/α5β1 dyad in angiogenesis and pathological conditions of the vascular system after the tethering of cells in inflamed tissues such as atherosclerotic lesion sites [75].

4. Platelet CD40L

The discovery in 1998 that platelets preferentially express many copies of CD40L on their surfaces upon activation was surprising because CD40L was thought to characterize immune reactive cells, and platelets were not yet acknowledged to display any immune function [25]. CD40L was then found in platelet cytoplasm [25,65,76,77], and years later more precisely identified as being docked in the platelet α-granules [78] (Figure 3). The discovery that, despite being non-nucleated cells devoid of DNA apart from mitochondrial DNA [79], platelets can retrotranscribe RNA using a spliceosome [80–83] and lead to detectable RNA messages for cytokines, questioned the possibility that CD40L is also produced *de novo* by activated platelets. Recently, some RNA-seq studies did not find CD40L mRNA in platelets [84–87]. This result suggests that a preformed protein is synthesized by megakaryocytes and stored in α-granules before platelet fragmentation [88–90].

Figure 3. Schematic overview of the regulation of platelet CD40L and the role of sCD40L in signaling after binding to platelet CD40 and αIIbβ3 inducing an auto-amplification loop. Synaptosomal-associated protein 23 (SNAP23), mitogen-activated protein kinase (MAPK), nuclear factor kappa B (NF-κB), protein kinase B (AKT) matrix metalloproteinase-2 (MMP-2), TNF receptor associated factor 2 (TRAF2).



After stimulation by different agonists, platelets undergo a degranulation process via a well characterized mechanism [91], and either export the α-granule molecules to the membrane in a fixed form or secrete them as a soluble form. Granules fuse with the platelet membrane and display their fixed CD40L on the surface. This process occurs within seconds to minutes after stimulation [25]. CD40L is thus expressed on the platelet surface only after activation, and this molecule is identical in terms of structure and physiological function to membrane bound CD40L

expressed in activated T-lymphocytes and other cells. It can notably generate signals for the recruitment and extravasation of leukocytes. It induces, through the engagement of CD40, the secretion of chemokines and the expression of adhesion receptors in endothelial cells [25]. It provides a powerful link between platelets and the immune system: CD40L expressed on activated platelets induces dendritic cell maturation, B-cell isotype switching, and augments CD8+ T-cell responses in both *in vitro* and *in vivo* models [92–95].

Platelets do not maintain CD40L on their surface for long. It is cleaved and released in a soluble form and may also be carried on the surface of microparticle-derived platelets [96]. Platelets are the major source of sCD40L in the circulation [65,97,98]. The normal range of sCD40L in the serum of a healthy adult is estimated at 0.79 to 4.7 ng/mL, by means of immunoassay techniques [99–101].

Of note, platelets constitutively express CD40 on their surfaces, both when resting and upon activation (Figure 3) [25,26,92]. This is surprising, as CD40 has long been considered to characterize APCs. Some sCD40L is reabsorbed on the platelet surface and principally binds CD40, a mechanism of recycling that must not be ignored when discussing platelet physiology and pathology.

5. Platelets, CD40L, and Molecular Signaling

5.1. Platelet Activation in Platelet Components and Molecular Signaling

Platelet activation and the signaling pathways involved in hemostatic conditions are well documented [102–104]. However, there is little information regarding the platelet components (PCs) prepared and processed for transfusion.

Several proteomic studies have investigated platelet changes after either resting (*ex vivo*) conditions or stimulation (*in vitro*) [105,106]. Most have tested activation markers such as shape change, glycolysis, supernatant pH levels, platelet CD62P and CD40L surface expression, reactivity to repeated activation by agonists, secretion of platelet granule products, cytoskeletal reorganization and expression of apoptotic markers [9,107,108]. Most of those studies, as well as the subsequent ones, were carried out with the purpose of improving the platelet physiology in the *ex vivo* conditions that lead to the possibility of storing platelets for a limited number of days and transfusing homologous donor platelets to a recipient patient. The signaling pathways involved in the "spontaneous" activation of platelets in PCs were investigated [105,109,110].

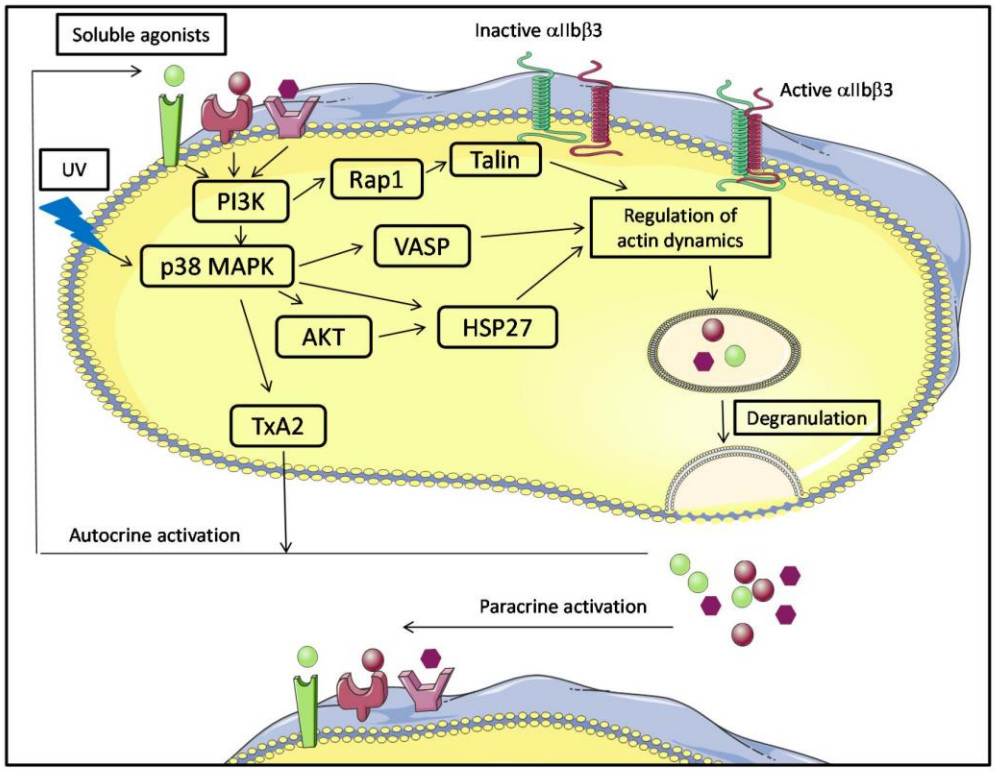
Schubert *et al.* [109] found evidence for a signaling pathway mediating PC storage lesions in which PI3-kinase-dependent Rap1 activation leads to integrin αIIbβ3 activation and platelet degranulation. This pathway involves two principal actors: Rap1, a small GTPase that modulates αIIbβ3 affinity, most likely through effects on the actin cytoskeleton [111], and Talin, an adaptor protein that links αIIbβ3 to the actin cytoskeleton. In hemostasis, this pathway is activated by soluble molecules after binding to different receptors, leading to the activation of the integrin αIIbβ3 [112].

Moreover, several studies identified the activation of the p38 MAPK signaling pathway during the aging of platelets not subjected to added stimulus [105,110], and/or after treatment of platelet concentrates with UV light with the intent of eradicating infectious pathogens. p38 MAPK is more

highly activated after UV exposure, a PI3-kinase-dependent mechanism that involves AKT, VASP and HSP27. AKT thus acts as a substrate for p38 MAPK. HSP27 is a substrate for AKT, and it regulates actin dynamics and degranulation. This confirms the earlier finding that MAPK activation stimulates platelet degranulation and TxA2 synthesis, which may in turn activate platelets via the TP receptor [113]. After degranulation, soluble factors (ADP, ATP, TxA2, Ca^{2+} and thrombin) are released and may act quickly to amplify autocrine activation of platelets as well as the activation of surrounding platelets (Figure 4) [88].

Platelets possess a variety of pathogen recognition receptors (PRRs) to sense bacterial and viral moieties and other receptors that could be involved in platelet activation in PCs [114–116]. Activated platelets can, consequently, secrete inflammatory cytokines and chemokines and other biological response modifiers (BRMs), including sCD40L, which could be a good candidate for such autocrine activation loops in platelets (Figures 3 and 4).

Figure 4. Principal signaling pathways inducing platelet activation in platelet components. Phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), protein kinase B (AKT), Thromboxane A2 (TxA2), Vasodilator-stimulated phosphoprotein (VASP), Ras-proximate-1 (Rap1), Heat shock protein 27 (HSP27).



5.2. Platelet Membrane CD40L Regulation and Shedding

As already stated, CD40L was initially thought to be almost absent from the surface of non-activated platelets [117]. It has been recently reported, however, that resting platelets express very low levels of CD40L on their surface but can translocate massive amounts of CD40L to the

surface within minutes of activation. This CD40L can subsequently be cleaved and released as a soluble molecule into the circulation [92,118,119]. Such CD40L would thus be mobilized from the α-granules (Figure 3).

Knowledge regarding CD40L cleavage, either from T-cells or from platelets, remains incomplete. Cleavage from T-cells has been attributed to two types of MMPs. Two other MMPs are also responsible for the cleavage of platelet membrane bound CD40L.

MMPs constitute a large family of more than 25 functionally related endopeptidases mediating the proteolytic cleavage of most matrix proteins, as well as several non-matrix proteins including cytokines, chemokines, adhesion molecules and surface receptors [120]. ADAM10 has been proposed as a candidate MMP for CD40L cleavage and shedding from T-cells [121]. ADAM17 has been shown to be another candidate in an *in vitro* model of Jurkat E6.1 T-cells, where the inhibition of both ADAM10 and ADAM17 nearly completely inhibited CD40L shedding from the cells, suggesting that no other MMP besides ADAM10 and ADAM17 is involved [122]. The mechanisms that cleave activated platelet CD40L appear to be quite different. Not only MMPs but also the integrin αIIbβ3 are mandatory. MMP2 is the best candidate, based on different experimental approaches [123–126]. A novel enzyme has revealed itself as a potential candidate, at least in pathological situations, as demonstrated in a mouse model of sepsis. Here, MMP9 was involved in the shedding of CD40L after platelet-neutrophil interaction. Again, different experimental approaches confirmed a role for MMP9 [127–130].

Interestingly, the enzymatic regulation of CD40L cleavage from CD40L-positive cells appears cell-dependent. Platelets and T-cells use different proteases to cleave sCD40L from their cell surfaces (MM2/MMP9, and ADAM10/ADAM17, respectively), despite both cell types containing all four identified enzymes. Among the possible explanations are the existence and particularities of the cytoplasmic or granule reservoirs. Those distinct mechanisms have functional consequences on signaling pathways triggered upon CD40L/CD40 activation between these two cell types.

In platelet CD40L cleavage, the need for functional and complete αIIbβ3 remains intriguing [123,131]. For example, Glanzmann thrombocytopenia patients fail to properly release sCD40L upon platelet activation [123].

5.3. Platelet and CD40L Signaling

CD40L production by platelets is an interesting intersection between hemostasis and inflammation. Hemostatic activation of platelets (by ADP, thrombin, collagen, *etc.*) induces inside-out signaling and, consequently, activation of αIIbβ3. This leads to outside-in signaling and degranulation, followed by CD40L expression on the membrane surface. After activation, CD40L is shed and released in an active form that can activate different cell types, including platelets.

Soluble CD40L may activate platelets via two independent receptors, CD40 and αIIbβ3. After sCD40L binding, both receptors activate AKT and enhance platelet p38 MAP kinase phosphorylation. One study showed that this signaling pathway initiates the generation of inflammatory molecules such as reactive oxygen and nitrogen species [69].

Soluble CD40L binding to platelet αIIbβ3 (through its KGD sequence) enhances thrombus formation and induces platelet spreading via outside-in integrin signaling in an auto-amplification

loop [65,67]. This phenomenon also induces the generation of microparticles, especially through phosphorylation of tyrosine-759 in the cytoplasmic domain of the β3 chain [67].

Soluble CD40L may also activate platelets via the CD40 receptor, which is present on platelet membranes [25,26]. In this case, the mechanism is outside-in independent. The CD40L/CD40 activation in platelets involves a CD40-dependent TRAF2/Rac1/p38 MAPK signaling pathway and triggers phosphorylation of IkBα [132,133]. Thus, the sCD40L/CD40 interaction also triggers NF-κB pathway activation in platelets. In this case, NF-κB acts as a signaling molecule and not a transcription factor. IκB phosphorylates SNAP23, a key protein for the fusion of alpha granules and the plasma membrane [134]. IKKb blockade inhibits SNAP 23 phosphorylation and prevents SNARE complex formation (SNARE complex formation reviewed in [90,135,136]) and platelet degranulation [134]. These mechanisms are outlined in Figure 3.

6. CD40L and Platelet Component Transfusion

The sCD40L association with platelets has been popularized because of the description of transfusion hazards [6–12]. Before that, although well published, this association received little consideration. For more than a decade, sCD40L-linked associated hazards also received modest consideration, probably because the attention of transfusiologists focused on preventable hazards, and residual leukocytes were considered to be responsible for all symptoms of inflammation [137]. Transfusion-linked inflammation was not yet acknowledged, but classed as discomfort. Accidents were attributed to other causes, which were sometimes reported as unidentified. Soluble CD40L gained attention when progress was made in the field of hemostasis and thrombosis, which outlined the role of platelets and leukocytes in the formation of atheroma plaque deposition and led to the proposal that cardiovascular disease is inflammatory [16,99,138–143].

Platelets in an inventory are generally stored no longer than 5 days (ranging from 3 to 7 days depending on country regulations). During storage, and without the addition of any stimulus intended to activate them, those so-called "resting" platelets are exposed to a number of stresses, including the process of constituting a PC, exposure to plastics, preservatives and gases, rotation, and changes in temperature [107,144,145]. Platelets are extremely reactive to external signals and are designed to sense external danger. They are equipped with many types of receptors and danger sensors, and they respond to multiple signals [83,116]. Anticoagulant factors and bacterial residues can modify the status of platelets that are believed to be "resting", but which in fact are lightly stimulated just above physiological steady state [83,116]. As platelets secrete more pro-inflammatory than anti-inflammatory BRMs, they begin to produce or secrete BRMs that are fairly detectable in the PC supernatant by day 3 [9,146]. Soluble CD40L is the most visible cytokine-like BRM which is thus made, and it is produced in amounts that are sufficient to activate CD40+ cells *in vitro*, including B-cells, dendritic cells, and macrophages [146,147]. It is therefore fully bioactive. The longer the PC is stored, the more BRMs are found, apart from some molecules with extremely short half-life [11,12,146]. CD40L has a short half-life outside the α-granule, but its secretion over day 3, for 2 to 4 days, still allows biological function [146]. In general, PC transfusion is safe and accomplishes what it is expected to do: prevent or stop bleeding in the allocated patient/recipient. In approximately 10% of cases, moderate intolerance symptoms are reported, which are referred to

as either febrile non-hemolytic transfusion reactions (FNHTRs) or allergic reactions (in fact, allergic-type reactions) [148]. In 2% of cases, the symptomatology is more severe, and presents more clearly as inflammatory [148]; such cases have been investigated by several groups, and there is a consensus on the responsibility of sCD40L that is found in excess in the PC or in the recipient's plasma [6,7,10–12]. Soluble CD40L does not carry the full responsibility, but it is chiefly to blame [7,11,12]. It is also responsible in part for the physiopathology of a severe transfusion hazard called TRALI (Transfusion-Related Acute Lung Injury) [10], despite one recent publication that disputed this [149]. An open question is why some PCs seem loaded with sCD40L. If platelets in PCs can be over-stimulated by some unexpected event in the process, it probably does not occur in all cases [7,150].

7. Concluding Remarks: Towards Molecular Medicine Based upon CD40L and CD40 Polymorphisms

As the CD40/CD40L molecular tandem is essential in many pathways of physiological but also pathological immune and inflammatory responses, its control is valuable in patient care. We and other groups have worked extensively during the past few years on the involvement of sCD40L in transfusion associated hazards, and we have recently obtained evidence that there are a number of *CD40LG* polymorphisms that may affect the behavior of platelets in a PC processed for the purpose of transfusion [151]. Combined with polymorphisms of *CD40*, this may affect the preferential decrease of inhibitory isoforms of the molecules and the increase of high affinity isoforms. Certain platelet donors may express high levels of sCD40L that are promptly cleaved [11,12,126], and/or certain recipients express high affinity CD40 receptors on both circulating cells and endothelial cells, favoring excess CD40/CD40L reactions and adverse events. Cell signaling through these interactions may prompt those cells to either synthesize or release copious amounts of bioactive BRMS with inflammatory potential. If proven, donor selection and/or patient investigation would allow better matching to prevent such adverse events. Serious adverse events would also benefit from the recent development of biologicals that target either CD40 or CD40L. In fact, Tanaka *et al.* [152,153] have succeeded to remove 80% to 90% of sCD40L in PCs using a column of adsorptive cellulose beads. However, there was a significant decrease in the recovery of platelets after adsorption. In other diseases, blockade of CD40/CD40L was performed using anti-CD40L Abs, but unfortunately these drugs have exhibited potentially adverse interactions with platelets in patients [154].

Molecular or personalized medicine is thus underway for patients presenting with high risk of potentially lethal acute inflammatory responses. If not yet implementable at a large scale, this may be forecast for the very near future.

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Author Contributions

Fabrice Cognasse, Sandrine Laradi and Olivier Garraud designed and supervised this manuscript. Chaker Aloui, Olivier Garraud and Yolande Richard wrote this manuscript. Antoine Prigent, Caroline Sut and Sofiane Tariket participated in the discussion and in the conception of the figures. Hind Hamzeh-Cognasse and Bruno Pozzetto reviewed the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Depuis la publication de cette revue, de nombreuses études se sont intéressées à l'implication des plaquettes lors d'une réponse inflammatoire, notamment par l'interaction du CD40L.

Nous avons vu précédemment que les pathogènes pouvaient induire une activation plaquettaire et entrainer la sécrétion de BRM. Une étude récente a montré qu'une protéine staphylococcique de type superantigène 5 (SSL5) pouvait induire des réponses pro-inflammatoires des monocytes par l'intermédiaire des MPP [144]. Comme attendu, SSL5 active les plaquettes et provoque le relargage de BRM, en particulier de MPP. Ces MPP se lient aux monocytes, entraînant la formation d'agrégats et la libération de médiateurs inflammatoires, comprenant IL1β, « Tumor necrosis factor α » (TNFα), MCP1 et « Matrix metallopeptidase » (MMP9). Le blocage des interactions CD40/CD40L réduit significativement la libération de médiateurs inflammatoires par les monocytes et la migration induite par les MPP. Ceci semble attribuable à l'activation de la voie de signalisation NFκB via le couple CD40/CD40L.

De plus, les plaquettes peuvent contribuer au maintien d'un milieu inflammatoire. En effet, une étude a montré dans un modèle de coculture *in vitro* que les plaquettes activées étaient capables d'induire significativement la libération de MCP1 par les cellules endothéliales, et ce de manière dépendante du CD40L [145]. Les taux élevés de sCD40L sont corrélés avec une augmentation de MCP1, reflétant un état inflammatoire.

Des études ont évalué la contribution des plaquettes dans l'inflammation cérébrale. Il a été démontré que l'hypertension est associée à une activation plaquettaire et une activation gliale dans le cerveau. Bhat *et al*., ont constaté qu'une hypertension augmente l'expression des molécules d'adhésion comme JAM1, ICAM1 et VCAM1 sur l'endothélium cérébral et entraîne le dépôt plaquettaire, qui est associé à l'augmentation du CD40 et CD40L et à l'activation des astrocytes et de la microglie dans le cerveau [146]. Ainsi, le CD40L plaquettaire est identifié comme molécule inflammatoire clé dans l'induction de l'activation des astrocytes et de la microglie, entraînant une neuroinflammation et la mort cellulaire neuronale dans des conditions *in vitro* et *in vivo*. D'autres travaux ont également montré qu'il y avait une corrélation entre la concentration du sCD40L et la gravité du déficit neurologique chez des patients souffrant d'hémorragie sous-arachnoïdienne [147]. Ce qui suggère que le sCD40L, mais pas uniquement, permet de prédire l'état neurologique chez ces patients.

Kuijpers *et al.* se sont intéressés au rôle du CD40L dans des conditions athérogènes. Les auteurs ont montré que le CD40L plaquettaire module la formation du thrombus. En effet, le CD40L stimule les interactions plaquettes-plaquettes induites par le collagène, en favorisant l'activation de l'intégrine αIIbβ3, la sécrétion et la croissance du thrombus via la voie de signalisation PI3K, et non celle du NFκB [148]. Le CD40 plaquettaire joue un rôle crucial dans l'inflammation en stimulant l'activation des leucocytes, et le recrutement et l'activation des cellules endothéliales, favorisant ainsi l'athérosclérose [149].

Le sCD40L est essentiellement produit par les plaquettes après leur activation. Sa concentration est augmentée au cours du stockage des CP. Des études ont rapporté que le taux de sCD40L peut être lié avec certains marqueurs génétiques. En effet, notre équipe a notamment identifié un polymorphisme du gène ITGA2 (région codante du récepteur plaquettaire au collagène), associé à une modification significative de la sécrétion de sCD40L [150].

Le sCD40L mais également le volume moyen plaquettaire, sont des biomarqueurs de l'activation plaquettaire et considérés comme un facteur de risque de maladie cardiovasculaire. Une étude a montré que la concentration en sCD40L est significativement plus élevée chez les patients atteints d'hypertension [151].

Des données ont impliqué le sCD40L dans le dysfonctionnement endothélial et l'angiogenèse. Napoleao *et al.* ont montré que la concentration du sCD40L augmentait au cours du temps après le début d'un infarctus du myocarde, associé à un polymorphisme de « Endothelial NOS » (eNOS) et à la concentration de « Vascular Endothelial Growth Factor » (VEGF) [152]. Ces résultats indiquent que le sCD40L ne doit pas être considéré comme impliqué seulement dans l'hémostase et l'inflammation, mais également comme ayant un rôle important dans la dysfonction vasculaire et endothéliale.

Des études se sont intéressées aux rôles inflammatoires des MPP qui sont libérées lors de l'activation plaquettaire. Notamment, Mobarrez *et al.* ont montré que l'administration de LPS chez des volontaires sains induisait une augmentation de MPP. De plus, ces MPP expriment du CD40L à leur surface mais contiennent également du sCD40L [153].

Ces différentes études renforcent le rôle des plaquettes comme étant des acteurs forts dans la modulation de l'inflammation vasculaire, mais également dans l'activation de l'endothélium.

Grâce à leurs contenus cytoplasmiques, leurs récepteurs ainsi que leurs nombreux BRMs pouvant être sécrétés lors de l'activation, les plaquettes ont un rôle clé dans la modulation des processus inflammatoires [154].

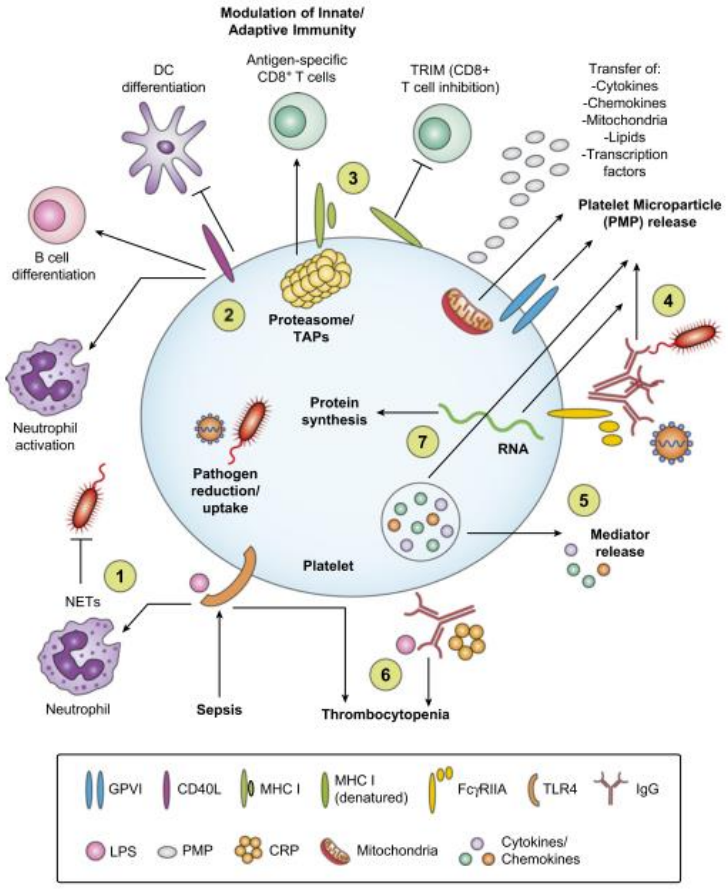

Figure 7. Le rôle clé des plaquettes dans la modulation des processus inflammatoires

Tout d'abord, les plaquettes peuvent détecter la présence de pathogènes et les absorbent. En effet, l'expression de TLR, en particulier le TLR4, leur permet d'activer les neutrophiles, et d'induire la sécrétion de NET (1). L'expression du CD40L leur permet d'interagir avec différentes cellules du système immunitaire, notamment d'activer les neutrophiles ainsi que la différenciation des lymphocytes B et/ou d'inhiber la différenciation des cellules dendritiques (2). De plus, des molécules CMH de classe I intracellulaires, sont exprimées lors de l'activation, et peuvent activer les lymphocytes TCD8+ spécifiques de l'antigène (par exemple, le paludisme). En revanche, les molécules CMH de classe I membranaires des plaquettes au repos, sont dénaturées et conduisent à l'inhibition des lymphocytes TCD8+ (3). Les plaquettes libèrent des MPP dans des conditions de stress, qui peuvent transporter différentes molécules (cytokines/chimiokines, mitochondries, lipides, facteurs de transcription) vers d'autres cellules

et sites d'inflammation (4). Les plaquettes contiennent de nombreux BRM ayant des effets pro-inflammatoires et anti-inflammatoires et, après activation, peuvent les libérer dans l'espace extracellulaire (5). Les interactions immunitaires avec les plaquettes peuvent mener à des états thrombocytopéniques sévères, comme dans le cas de sepsis, où les infections peuvent se lier aux plaquettes et provoquer leur séquestration et/ou leur destruction (6). Les plaquettes contiennent plusieurs espèces d'ARN, et celles-ci peuvent être exportées via des MPP ou des ARNm peuvent être traduits en synthèse protéique (7). L'ensemble de ces événements fait de la plaquette un formidable hôte immunomodulateur [19].

IV - Les lésions de préparation et stockage des CP favorisant la sécrétion de BRM

Les PSL sont des produits cellulaires vivants, ayant une durée de vie limitée et se dégradant selon des mécanismes physiologiques qui peuvent être accélérés ou aggravés par les conditions mécaniques de production et de stockage [155]. Les plaquettes préparées à des fins transfusionnelles subissent des lésions de stress lors du prélèvement, de la préparation et du stockage [156]. Confrontées à ces stress, les plaquettes vont subir des modifications morphologiques/métaboliques pouvant entraîner l'activation plaquettaire et une augmentation de la concentration des BRM sécrétés [157]. Le traitement *ex vivo* des plaquettes peut influer sur la sécrétion de BRM [158]. Ces paramètres favorisant la sécrétion de BRM entrainent des changements délétères conduisant à la détérioration progressive de la viabilité, de la structure et de la fonction plaquettaire.

1) Processus de préparation

En France notamment, les concentrés plaquettaires (CP) peuvent provenir de dons de sang total ou de dons de plaquettes d'aphérèse. Ces deux types de prélèvements permettent la préparation de deux types de produits distincts [159]. Les mélanges de concentrés plaquettaires (MCP) sont obtenus à partir de la couche leuco-plaquettaire (Buffy-Coat) de plusieurs dons de sang total (5 en France). Les concentrés de plaquettes d'aphérèse (CPA) sont obtenus à l'aide d'une machine d'aphérèse qui sépare les plaquettes du sang d'un donneur

unique [160, 161]. Tous les PSL sont déleucocytés (les leucocytes résiduels doivent être $<10^6$ par produit). De plus, les PSL sont qualifiés biologiquement selon les normes européennes et nationales, et étiquetés réglementairement selon leurs groupes sanguins [162].

Des études ont montré que les plaquettes pouvaient changer morphologiquement en fonction des méthodes utilisées pour préparer les CP [163]. Ces différentes techniques de préparation influencent de façon différentielle sur la physiologie plaquettaire, car ces techniques n'exposent pas les plaquettes aux mêmes contraintes [164, 165]. Lors de la préparation des CP, les plaquettes subissent de nombreux types de stress, notamment liés aux contraintes de cisaillement infligés par la centrifugation, ainsi qu'aux traitements des poches tels que la leucoréduction (par filtration), l'utilisation de différentes solutions additives de conservation, l'irradiation et/ou l'utilisation des traitements inactivateur/reducteur de pathogènes.

De plus, il a été montré que la dégranulation et la vésiculation plaquettaires sont influencées par les techniques de préparation [166, 167]. En effet, d'autres études ont montré que la sécrétion de MPP varie en fonction des différents processus de préparation [168]. Nousri *et al.* montrent que le type d'aphérèse active différemment les plaquettes, en particulier avec des aphérèse de types Amicus, où un pourcentage d'activation plaquettaire ainsi que de MPP est significativement plus élevé que dans les autres CP. Notre équipe avait également montré que les aphérèses de type Amicus semblent activer davantage les plaquettes comparés aux autres, notamment Trima [14]. Une autre étude a montré que la teneur en MPP dans les CP est prédite par les MPP présentes chez les donneurs [169].

Le traitement des CP a également un impact direct sur la production de BRM plaquettaire. Une étude a montré que la réduction des pathogènes par lumière UVC augmente l'accumulation de cytokines [170].

Au cours des processus de préparation des CP non déleucocytés, les BRM peuvent être de trois origines : leucocytaires, plasmatiques et/ou plaquettaires. La leucoréduction des CP a permis de diminuer considérablement la concentration de ces facteurs notamment leucocytaire, comme le TNFα, l'IL6 et l'IL8 [3] et donc réduire significativement l'incidence d'EIR impliquant des cytokines leucocytaires [171].

2) Lésions de stockage

Lors du stockage des CP, les plaquettes peuvent subir des changements qui sont liés principalement aux solutions de conservation, ainsi qu'aux conditions de conservation (agitation des plaquettes, la température et la durée de stockage) [172]. En France, les CP sont stockées au maximum 5 jours à 22°C ± 2°C, sous agitation constante et douce afin d'éviter toute agrégation des plaquettes entre elles. De plus, les MCP et les CPA sont conservées en suspension dans 35% de plasma du donneur ainsi que 65% de solution additive de conservation « Platelet Additive Solution » (PAS). Le stockage des plaquettes dans une solution additive a montré une meilleure préservation de la fonction plaquettaire par rapport au stockage dans un plasma autologue [173]. Les PAS sont généralement utilisés comme substitut du plasma pour (1) réduire la quantité de plasma transfusé; (2) éviter la transfusion de grands volumes de plasma pour réduire l'incidence des réactions indésirables et de surcharge circulatoire; (3) rendre possible un traitement photochimique pour l'inactivation des pathogènes et (4) améliorer les conditions de stockage [174].

Les lésions de stockage des plaquettes comprennent l'apparition de marqueurs d'activation plaquettaire, les changements morphologiques, le dysfonctionnement mitochondrial, la perte de l'expression de GPIbα et la sécrétion des granules α [175]. Le stockage des concentrés plaquettaires peut entraîner la sécrétion de plusieurs BRM, comme le sCD40L, PDGFAA, RANTES, IL1β, IL6, IL7, IL8, PF4, l'IL13, l'OX40L, l'IL27 et TGFβ [156, 176]. Généralement, le stockage prolongé des CP s'accompagne d'une plus grande production de BRM, qui peut être lié à une augmentation du pourcentage des EIR observée en fonction de la durée de stockage des CP. Afin de limiter les EIR, il serait préférable de transfuser les CP le plus tôt possible. Il convient cependant de mettre en regard cette conclusion à la contrainte de production et délivrance des CP par les EFS, et en fonction de la demande des produits des établissements de soins. Notre équipe a notamment montré qu'à partir du 3ème jour de stockage des CP, on peut observer une augmentation significative de la concentration de ces BRM, notamment le sCD40L [156]. Ces observations suggèrent que les lésions de stockage ont un rôle dans l'inflammation induite par les CP. En effet, sCD40L induit la production de « Reactive Oxygen Species »(ROS) pendant le stockage des CP, provoquant une augmentation de la production et libération de molécules pro-inflammatoires [177].

Au cours du stockage des plaquettes, des marqueurs apoptotiques sont augmentés [178-180]. De plus, il y a également une activation de la caspase 3 et le clivage conséquent de la gelsoline, indépendamment de l'activation plaquettaire [181, 182].

De plus, le type de traitement des CP utilisé lors du processus de préparation et stockage peut avoir un effet sur l'état d'activation plaquettaire. Leitner *et al.* ont montré que les plaquettes stockées dans une solution d'Intersol™ présentaient une activation initiale significativement plus élevée, d'après l'expression de CD62P, que d'autres solutions additives, Composol® et SSP+® [183]. Néanmoins, le stockage des plaquettes dans une solution additive a montré un certain nombre de bénéfices, notamment une réduction des réactions indésirables graves [184]. Bien que les différents types de CP aient une qualité comparable, il existe une controverse quant à leur sécurité. Daurat *et al.* ont montré que les EIR étaient moins fréquemment associés aux MCP qu'aux CPA [185]. Ces résultats remettent en question l'utilisation étendue des CPA et suggèrent leur prescription pour des indications spécifiques. L'évaluation du rapport risque/bénéfice associé à la transfusion des différents types de CP, permettrait de prescrire le produit optimal, en fonction de l'indication médicale.

V - Les BRM impliqués dans les EIR

Comme montré précédemment, les plaquettes sont pourvoyeuses de BRM. Lors d'une transfusion plaquettaire, les BRMs contenus dans le CP sont également transfusés. il a été démontré que des BRM peuvent induire des réponses immunitaires [186], des réactions post-transfusionnelles [187], affecter l'hémostase [188] et l'inflammation chez le receveur [189]. Les lésions de stockage déclenchées par des facteurs extrinsèques (méthodes de préparation) ou intrinsèques (facteurs plasmatiques et plaquettaires, leucocytes résiduels), pourraient être responsables en grande partie d'une diminution de l'efficacité thérapeutique de la transfusion des CP, mais également de l'induction d'EIR [190-192].

Parmi ces molécules, le sCD40L est décrit comme responsable, en partie, de RFNH après transfusions plaquettaires [193, 194]. Outre son rôle dans l'inflammation, le CD40L semble intervenir lors d'EIR. En effet, le sCD40L est présent dans les CP et sa concentration est augmentée au cours du stockage [156]. De nombreuses études ont montré que le sCD40L est impliqué lors de réactions après transfusion de CP [189, 195]. Nous avons également montré

que d'autres facteurs solubles tels que l'IL27 et sOX40L, étaient impliqués dans les RFNH [187]. Plusieurs facteurs solubles ayant une valeur prédictive élevée pour la survenue des EIR, ont été identifiés notamment par des modèles mathématiques de « Machine learning », comme le sCD40L, IL13, MIP1α [196]. Effectivement, cette étude a montré une corrélation entre la concentration en sCD40L et IL13 et la survenue d'EIR. De plus, MIP1α présent dans les surnageants induisant un EIR, semble capable selon sa concentration de distinguer le type d'EIR, entre RFNH ou allergies.

Les CP contiennent également de l'ADNmt qui est associé aux effets indésirables [197-199]. En effet, Boudreau *et al.* ont montré que les plaquettes activées libèrent des mitochondries, à la fois dans des microparticules encapsulées et comme organelles libres. Les mitochondries extracellulaires retrouvées dans les CP transfusés, sont présentes à des taux plus élevés chez ceux ayant provoqué des réactions aiguës (RFNH, manifestations cutanées et cardiovasculaires) chez les patients transfusés [143].

Il est bien admis en effet, que l'accumulation de cytokines et chimiokines dans les concentrés plaquettaires au cours de leur conservation, en l'absence de stimulus exogène décelable, peut participer au déclenchement d'EIR [156, 200].

Outre les cytokines/chimiokines, les MPP, médiateurs importants de l'inflammation et de la régulation des réponses immunitaires, semblent également être impliqués dans l'apparition d'EIR [134, 201]. Les MPP contenants des microARN peuvent également être impliqués dans une réponse physiopathologique à la suite d'une transfusion de CP. En outre, des études ont montré que les technologies de réduction des pathogènes, qui visent à réduire le risque potentiel d'infections transmises par transfusion, induisent une activation plaquettaire et une diminution des taux d'ARNm [202] et de microARN [203]. Ces changements de niveaux d'ARN sont corrélés avec une augmentation de la concentration de MPP. Par conséquent, il semble probable que les technologies de réduction des agents pathogènes peuvent augmenter la formation de MPP dans les CP [203]. Compte tenu des propriétés pro-inflammatoires des MPP, il est raisonnable de supposer que celles-ci peuvent exacerber les affections inflammatoires aiguës et chroniques des vaisseaux sanguins, telles que celles associées respectivement à la transfusion de plaquettes et à l'athérosclérose [24, 133].

Chapitre 4 - Plaquettes et endothélium

I - Les cellules endothéliales

L'endothélium est un système cellulaire hautement spécialisé composé de 1 à 6.10^{13} cellules endothéliales (EC) recouvrant une surface totale de 4000 à 7 000 m^2, qui joue un rôle clé dans les processus physiologiques tels que l'approvisionnement en sang, la distribution des nutriments, l'homéostasie métabolique et le trafic de cellules immunitaires, ainsi que des processus pathologiques tels que l'inflammation [204-206].

Les cellules endothéliales tapissent les parois internes de tous les vaisseaux sanguins et lymphatiques ainsi que l'intérieur des cavités cardiaques. Elles constituent la barrière entre le sang qui circule dans les vaisseaux et leurs parois. Elles assurent une surface glissante et contribuent à empêcher la coagulation sanguine, outre la participation de protéines plasmatiques anticoagulantes. Elles ont une forme extrêmement aplatie et polygonale. Elles reposent en général sur une membrane basale et elles sont liées entre elles par des desmosomes et des jonctions serrées [207]. Les cellules endothéliales contiennent des granules de stockage, dans lesquels se trouvent les corpuscules de Weibel-Palade, qui libèrent leur contenu en réponse à des agents comme la thrombine, l'histamine, les ionophores de calcium [207]. Les protéines stockées dans les cellules endothéliales (facteur vonWillebrand, P-sélectine) jouent un rôle important dans l'hémostase, dans la régulation du tonus vasculaire ou dans l'adhésion et la migration des leucocytes.

Lors d'une infection et/ou d'une inflammation, on observe une réaction caractérisée par l'augmentation de l'expression de protéines pro-inflammatoires comme les cytokines/chimiokines (TNFα, IL1β, IL6, IL8), les molécules d'adhésion (E sélectine ou CD162E, ICAM1, VCAM1). L'expression de la plupart de ces protéines pro-inflammatoires est contrôlée par NFkB, un activateur transcriptionnel activant les régions promotrices des gènes codant pour ces protéines. L'activation quasi immédiate du NFkB, lors d'une réaction inflammatoire, nécessite un système de signalisation intracellulaire [208].

Les cellules endothéliales expriment d'autres récepteurs, notamment PAR1 qui est un récepteur exprimé par les cellules endothéliales ainsi que par les plaquettes [209]. PAR1 est activé par la thrombine.

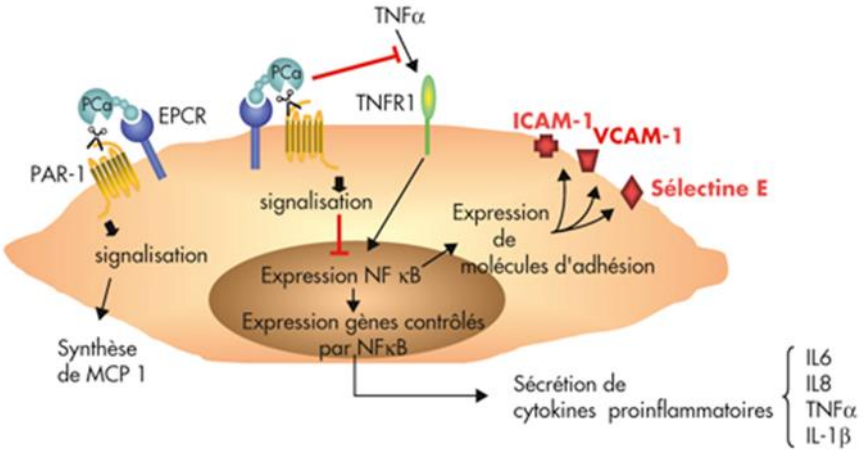

Figure 8. Cellule endothéliale lors de l'inflammation

Lors d'une réaction inflammatoire, les cellules endothéliales expriment des molécules d'adhésion telles que ICAM1, VCAM1 et la E sélectine. Elles sécrètent des cytokines pro-inflammatoires telles que l'IL6, IL8, TNFα et IL1β. La réponse des cellules endothéliales est contrôlée par NFκB [210].

L'endothélium intact, fortement antithrombogène, est remarquablement réfractaire à toute adhérence plaquettaire et de façon à préserver cet équilibre, les cellules endothéliales sécrètent des substances telles que la prostacycline, l'oxyde nitrique (NO) et l'ecto-ADPase qui empêchent l'activation plaquettaire et ses conséquences thrombotiques [211].

La thrombomoduline (CD141), un inhibiteur de la thrombine présent à la surface des cellules endothéliales, contribue aussi aux propriétés antithrombogènes de l'endothélium. Dans les conditions physiologiques, les cellules endothéliales jouent ainsi un rôle important dans la prévention de l'activation plaquettaire afin de maintenir un état non thrombogénique [212].

Différents signaux inflammatoires libérés dans les zones d'infection et/ou d'inflammation provoquent l'activation de l'endothélium. Parmi ces signaux, des cytokines et chimiokines sont principalement retrouvées (TNFα, IL1β) [213, 214].

L'activation des cellules endothéliales par les cytokines inflammatoires modifie la composition des corpuscules de Weibel-Palade et induit un phénotype procoagulant et pro-inflammatoire [215, 216], ayant pour conséquence d'entretenir la réaction inflammatoire et la mise en place des processus immunitaires. Ainsi, les cellules endothéliales constituent un des premiers remparts immunitaires.

II - Interaction des plaquettes sur l'endothélium

Dans un état physiologique normal, les plaquettes circulantes n'adhérent pas à l'endothélium intact. L'adhésion cellulaire des plaquettes à l'endothélium est une réponse pathologique importante due à la lésion du vaisseau, une infection et/ou à l'inflammation. Différents mécanismes d'adhérences ont été identifiés [217].

Lors d'une infection, les cellules endothéliales produisent d'avantage de thrombine, ce qui induit la sécrétion des granules de stockage, les corps de Weibel-Palade, qui contiennent le vWF et la P sélectine [218]. La libération du vWF et la liaison avec son ligand, la GpIbα plaquettaire entraînent une adhérence plaquettaire beaucoup plus importante sur les cellules endothéliales infectées que sur les cellules saines [219-221].

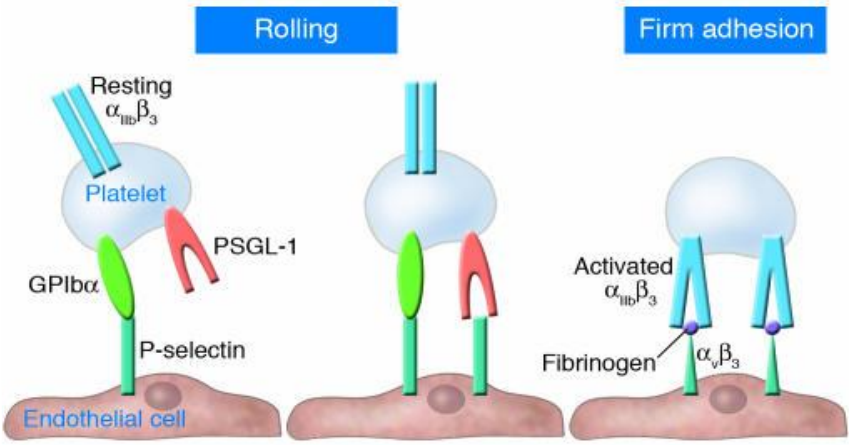

Figure 9. Adhérence des plaquettes sur l'endothélium

La surface de l'endothélium activé exprime la P sélectine. Les récepteurs plaquettaires GPIbα et PSGL1 interagissent avec la P sélectine endothéliale et initient le roulement des plaquettes. L'adhésion ferme subséquente est médiée par les intégrines β3 [222].

Toute perturbation de l'endothélium est susceptible d'induire l'interaction des plaquettes à l'endothélium grâce à une modulation très rapide de l'expression des molécules d'adhésion (E sélectine, ICAM1, VCAM1). La fonction la plus connue de ces molécules d'adhésion est de promouvoir le « rolling », c'est-à-dire le roulement conduisant à l'arrêt des leucocytes sur les cellules endothéliales activés [223]. Il a été montré que d'une façon similaire aux leucocytes, les plaquettes peuvent rouler sur l'endothélium activé par l'ionophore du calcium ou par une cytokine inflammatoire, comme le TNFα [224]. L'ionophore de calcium induit le relargage du contenu des corps de Weibel-Palade et l'expression de la P sélectine à la surface de l'endothélium [39]. La P sélectine exprimée aussi à la surface des plaquettes activées peut également participer à ce phénomène d'adhérence puisqu'elle permet l'interaction des plaquettes avec l'endothélium par l'intermédiaire du ligand endothélial induit par le TNFα [225].

De plus, le récepteur GpIbα a été identifiée comme ligand plaquettaire de la P sélectine, en plus du vWF [226]. Le récepteur GpIbα peut promouvoir d'une part, l'adhérence des plaquettes aux cellules endothéliales lorsque l'endothélium est activé, et d'autre part, l'adhérence des plaquettes aux collagènes du sous-endothélium suite à l'altération des cellules endothéliales lors d'une brèche vasculaire [227]. L'interaction du récepteur GpIbα et le collagène est régulé par PECAM1 pour éviter une activation inutile des plaquettes lors d'un cisaillement élevé [227]. Les plaquettes sont en effet capable de moduler la réponse des cellules endothéliales en cas de stress à travers l'expression de PECAM1 [228].

Dans des conditions inflammatoires, la E sélectine nouvellement synthétisée est également présente à la surface des cellules endothéliales. Les deux sélectines P et E semblent coopérer pour promouvoir le roulement des plaquettes [215]. Parmi les différents ligands des sélectines identifiés, le PSGL1 est le plus courant et est exprimé à la surface plaquettaire [229].

Après l'activation des plaquettes notamment par la thrombine, les plaquettes sont capables d'adhérer fermement à l'endothélium par l'intermédiaire de la GpIIb/IIIa (αIIbβ3) qui se lie à ses ligands (fibrinogène, fibronectine ou vWF) [220]. Ces ligands sont capables de se lier à leurs récepteurs endothéliaux (ICAM1, αvβ3 ou GpIbα) [230].

Cependant, même si l'endothélium vasculaire est intact, les plaquettes activées sont aussi capables d'interagir avec les cellules endothéliales mettant en jeu le couple PSGL1/P sélectine [229].

III - Effets des plaquettes activées sur l'endothélium

Les plaquettes activées peuvent contribuer à l'expression de divers médiateurs inflammatoires sur les cellules endothéliales. D'un côté, les plaquettes libèrent de l'IL1β, du TGFβ, du PDGF et du VEGF, chacun pouvant déclencher la transduction du signal des voies dans l'endothélium. De l'autre, les cellules endothéliales expriment des récepteurs à leurs surfaces ou des médiateurs solubles qui inhibent la fonction plaquettaire ou favorisent l'activation des plaquettes [231].

En présence de plaquettes activées, la sécrétion de l'IL1β, MCP1, MIP1α et ICAM1 est significativement augmentée par rapport à des plaquettes non activées [213]. Parmi eux, l'IL1β a été identifié comme étant un facteur majoritaire dans l'activation des cellules endothéliales, et ce de façon dépendante des plaquettes [232]. En effet, l'interaction de plaquettes activées sur des cellules endothéliales en culture induit une surexpression de molécules d'adhésion telles que ICAM1 [118]. L'IL1β plaquettaire induit également la sécrétion de facteurs chimioattractants, tels que l'IL6, IL8 et MCP1 [233, 234]. De plus, l'IL1β augmente l'expression de l'intégrine αvβ3 qui est capable de se lier ensuite à la fibronectine [222], et active la voie NFκB [79].

De plus, l'interaction du CD40L plaquettaire et du CD40 exprimé par les cellules endothéliales induit un phénotype inflammatoire des cellules endothéliales, avec une augmentation de la sécrétion de l'IL8 et de MCP1, principaux chimioattractants des neutrophiles et des monocytes [235]. L'activation de CD40 sur les cellules endothéliales augmente également l'expression de plusieurs molécules d'adhésion telles que E sélectine, VCAM1 et ICAM1, permettant la fixation des neutrophiles, des monocytes et des lymphocytes à l'endothélium [235]. La liaison du récepteur endothélial CD40 et de CD40L plaquettaire se traduit par une augmentation de la production de cytokines inflammatoires, des molécules d'adhésion et des protéases dégradant la matrice (MMP) [236]. Les MMP dégradent diverses protéines de la matrice extracellulaire et favorisent l'inflammation et la destruction du tissu

enflammé. Ainsi, le sCD40L dérivé des plaquettes induit l'activation des MT-MMP1 (MMP14), MMP1, MMP2 et MMP9 qui dégradent la matrice extracellulaire des cellules endothéliales. De plus, l'inhibiteur de MMP, appelé TIMP2, interagit avec MT1-MMP et MMP2 pour former un complexe en réponse à la stimulation des plaquettes. Il a été montré que les plaquettes exprimaient ce complexe (MT1-MMP1/TIMP2/MMP2), et que les inhibiteurs de MMP pouvaient moduler l'agrégation [237].

Lors de l'exposition à des cellules vasculaires exprimant CD40, le sCD40L dérivé de plaquettes peut induire également l'expression de molécules d'adhésion, telles que la E sélectine et la P sélectine, et initier la libération du facteur tissulaire. Ainsi, il devient de plus en plus clair que le complexe plaquettaire CD40/CD40L peut jouer un rôle central entre l'endothélium, la coagulation et l'inflammation [222].

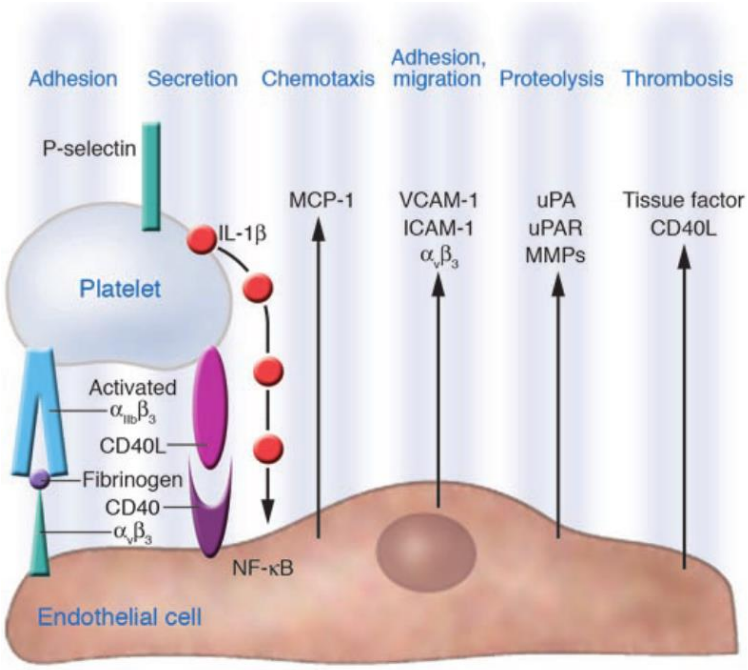

Figure 10. Les plaquettes adhérentes enflamment les cellules endothéliales

L'adhérence plaquettaire ferme impliquant αIIbβ3 induit une exposition à la plaquette de la P sélectine (CD62P) et la libération de CD40L et d'IL1β, ce qui stimule les cellules endothéliales à fournir un milieu inflammatoire qui supporte les altérations prothérapeutiques de l'endothélium [222].

Les plaquettes sont également capables d'assurer le transfert de matériel génétique vers l'endothélium. Les plaquettes contiennent des microARN, petits fragments d'ARN non

codants, qui jouent un rôle fondamental dans la régulation post-transcriptionnelle des gènes en ciblant l'ARNm, induisant sa dégradation ou la répression de la traduction [238]. La plupart des microARN caractérisés ont une préférence à se lier aux régions 3' non traduites de leurs ARNm cibles régulant ainsi près de 60% des ARNm codants pour des protéines [239]. Des études ont montré que les MPP contiennent également des microARN qui peuvent jouer un rôle régulateur sur les cellules endothéliales [139, 240, 241].

De plus, le RANTES libéré par les plaquettes activées peut être immobilisé et présenté sur l'endothélium activé, où il améliore le recrutement des monocytes [242]. L'activation de RANTES sur l'endothélium implique la P sélectine [243]. Nous avons vu précédemment que l'activation plaquettaire entrainait de multiples réponses cellulaires, y compris la formation de MP. En présence de RANTES, les MPP semblent intervenir avec l'endothélium en induisant l'adhésion et/ou la signalisation des récepteurs de RANTES [78], déclenchant l'arrêt des monocytes sur l'endothélium inflammatoire [244]. Ceci démontre une nouvelle fois que le recrutement des monocytes est dépendant des plaquettes lors de l'inflammation.

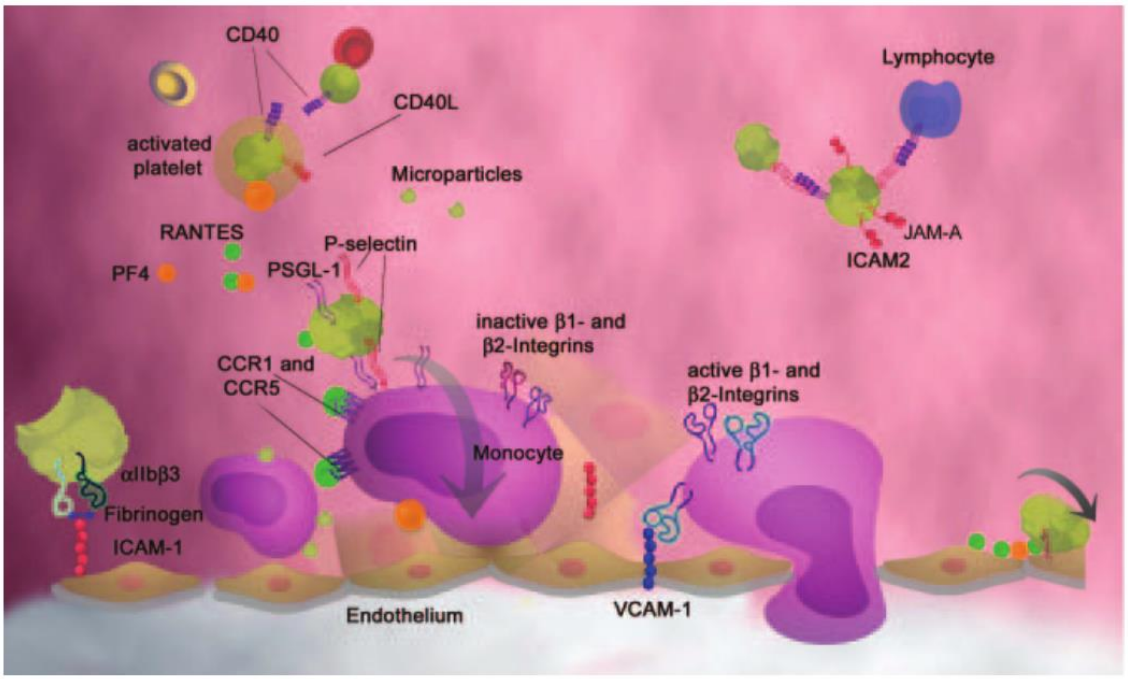

Figure 11. Interaction des plaquettes et des leucocytes régulent l'inflammation vasculaire

Une multitude de mécanismes moléculaires et de composants dérivés des plaquettes interviennent et régulent l'infiltration des leucocytes induite par les plaquettes, y compris le dépôt de BRM et l'activation des cellules endothéliales [78].

Objectifs de thèse

La transfusion de produits cellulaires peut créer une situation de danger chez le receveur. Au cours ou décours d'une transfusion sanguine, le receveur peut manifester un EIR, dont la gravité est variable, de bénigne à grave, voire létale. La transfusion de CP en particulier, est responsable dans quelques pourcentages des cas d'EIR notables, dont une partie semble évitable en partie et une partie est liée à des facteurs liés au receveur, soit de par son état clinique, soit de par ses propres caractéristiques génétiques. Il est bien admis en effet que l'accumulation de BRM dans le PSL au cours de sa conservation, en l'absence de stimulus exogène décelable, suffit à déclencher une réaction inflammatoire chez le receveur. Les principaux BRM plaquettaires sont des molécules immunomodulatrices, dont le principal est le CD40L soluble. Ces BRM se comportent comme des ligands pour des récepteurs des cellules endothéliales et la plupart des types leucocytaires circulants ou adhérents aux endothéliums.

Le but est de mon travail de thèse a été de déterminer, dans un premier temps, l'importance des facteurs de stress créés au cours du prélèvement, de la préparation et du stockage des CP, notamment en fonction des différents types de CP et les caractéristiques du don. On a aussi cherché d'identifier les BRM susceptibles d'induire un EIR, et de préciser les mécanismes par lesquels des BRM plaquettaires conduisent à un EIR.

Dans un second temps, nous nous sommes intéressés à l'effet des BRM des CP (via leurs surnageants) sur d'autres cellules, en particulier les cellules endothéliales. Il semblait en effet essentiel d'étudier comment les BRM plaquettaires affectent les différentes cellules du receveur.

Notre étude a permis de mettre en place une méthodologie d'étude de l'activation des cellules endothéliales vasculaires après activation par des CP, afin de mimer un paramètre « receveur » au cours d'une transfusion, en évaluant *in vitro* la capacité des surnageants des CP à activer des cellules endothéliales au travers l'étude de leurs proliférations, du signalosome, de l'expression de marqueurs d'activation et de la production de facteurs inflammatoires solubles.

Ce travail se focalise plus particulièrement sur les facteurs de stress liés au processus de préparation des CP et les moyens de les amoindrir pour accroître la tolérance de la transfusion par les receveurs. Ce projet, qui permet de préciser les mécanismes par lesquels des BRM plaquettaires conduisent à un EIR et d'identifier les molécules responsables, vise à détecter les

situations à risque (produits, patients) de sorte à proposer des arbres de décision biocliniques sur l'utilisation de tel ou tel CP chez un patient donné.

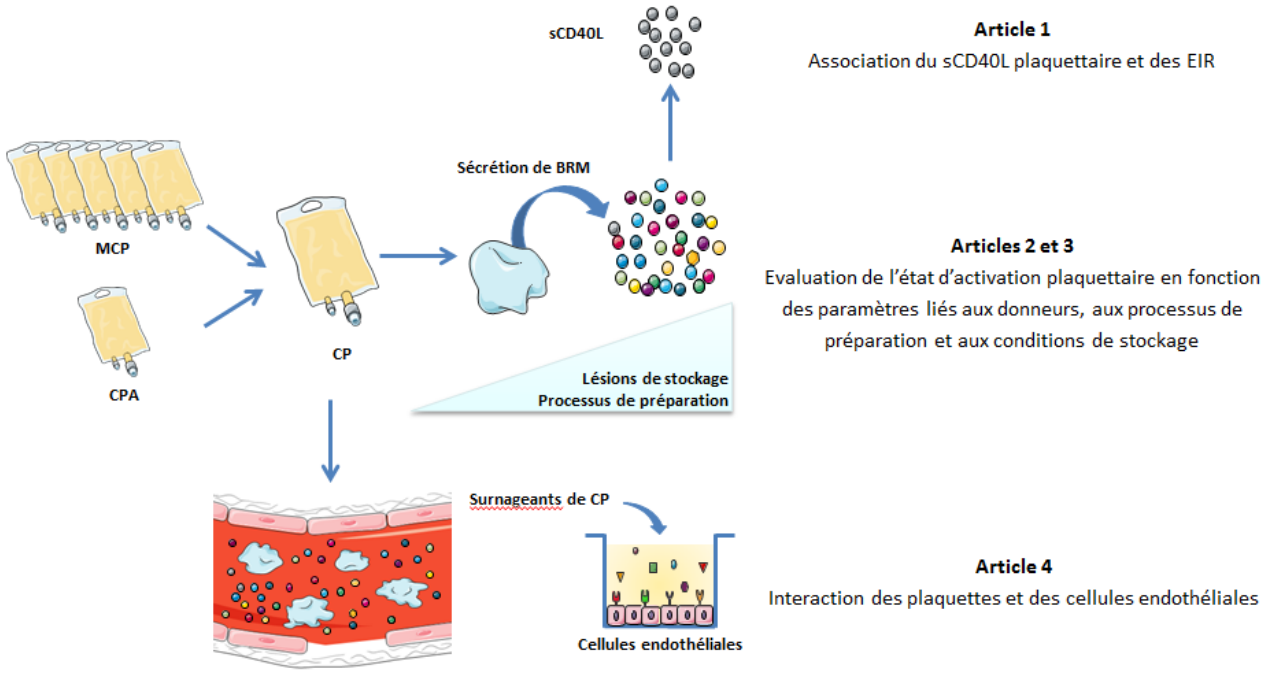

Figure 12. Objectifs et démarche expérimentale

Résultats

Article 1

Platelet soluble CD40-Ligand level is associated with transfusion adverse reactions in a mixed threshold and hit model

Article publié dans *Blood*, Juillet 2017

Le CD40-ligand soluble dérivé des plaquettes (sCD40L) est souvent associé à des réactions indésirables chez le receveur (EIR) après transfusion de concentrés plaquettaires (CP). Cependant, il n'est pas clair que les niveaux élevés de sCD40L soient toujours associés à ces réactions chez le receveur.

Dans cette étude, 9 206 échantillons de CP ont été recueillis au moment de la préparation, dont 2 850 pour lesquels l'échantillonnage était possible le jour de la délivrance du CP. La concentration de sCD40L a été évaluée pour tous ces échantillons. En lien avec les services d'hémovigilance, nous avons recueilli 140 CP ayant induit des EIR. Un modèle mathématique a identifié un seuil au-dessus duquel il existait une association significative entre les niveaux de sCD40L et les EIR, en permettant l'élimination de l'entropie d'un grand ensemble de données.

La plupart des surnageants n'ayant pas induit d'EIR sont inférieures au seuil pathogène calculé, soit 82% des concentrés plaquettaires d'aphérèse (CPA) et 54% des mélanges de concentrés plaquettaires (MCP) obtenus à partir de Buffy-Coat. A l'inverse, 60% des CPA et 82% des MCP ayant conduit à un EIR déclarées sont égales ou supérieures à ce seuil.

Ces résultats indiquent que sCD40L contenu dans les CP n'est pas toujours responsable de la pathogénicité chez les patients (EIR). Les seuils sont plus discriminants pour les MCP que pour les CPA.

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Platelet soluble CD40-ligand level is associated with transfusion adverse reactions in a mixed threshold-and-hit model

Fabrice Cognasse, Caroline Sut, Elisa Fromont, Sandrine Laradi, Hind Hamzeh-Cognasse and Olivier Garraud

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Hoffmann-La Roche (December 2015). The remaining authors declare no competing financial interests.

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To the editor:

Platelet soluble CD40-ligand level is associated with transfusion adverse reactions in a mixed threshold-and-hit model

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Platelets are the principal source of soluble CD40-ligand (sCD40L) found in blood.[1] This biological response modifier has been reported to be a candidate mediator for acute reactions after platelet transfusions.[2] Serious adverse reactions (SARs) associated with excessive levels of sCD40L are febrile nonhemolytic transfusion reactions, transfusion-related acute lung injury (TRALI), and allergic reactions,[2-4] although there is not a consensus on the role of sCD40L in TRALI.[5,6] An elevated level of sCD40L in SAR-causing transfusions was found to be 1 element of a broader spectrum of biological response modifiers.[7-9] sCD40L was modeled for likeliness to be predictive of SARs compared with other mediators:[10] in the presence of elevated levels of sCD40L, elevated levels of MIP1α were associated with febrile nonhemolytic transfusion reactions, whereas low levels were associated with allergic type reactions.[7] In SARs, exacerbated sCD40L was attributed to platelet secretion in bags.[9] Although there is extensive polymorphism in genes that control both sCD40L isotype and production in donors,[11,12] no such polymorphisms proved statistically associated with SARs.[12] We evaluated whether elevated levels of infused sCD40L associate with SARs by following a large series of platelet concentrate (PC) transfusions.[7,8,10] Most patients who developed SARs received a high amount of sCD40L, but many patients who also received high amounts of sCD40L did not develop a SAR. Thus, only a subgroup of recipients manifested inflammation.

Single-donor apheresis (SDA) PCs and pooled platelet concentrates (PPCs) were produced in 1 regional setting of the French Blood Establishment). All PCs were leukoreduced to $<10^6$/bag and suspended in 35% native plasma/65% nutritive solution.[7,8,10] PCs from female donors were tested for anti-HLA. Platelets became outdated 5 days (d5) after collection. The study included 9206 successive PCs (issued to patients in the 2 university hospitals), of which 2850 were sampled at the time of delivery to the patient, allowing case control for the day of processing. A total of 140 SARs (grades 2-3; accountability 2-3)[9] was reported, for which samples were shipped back to the Blood Bank. The processing procedure and the incident/accident declaration strictly conformed to the national protocols.[8] Platelets incriminated in a SAR were immediately shipped back the Blood Bank for investigation. All samples obtained from PCs not associated with SAR (referred to as "no.AR") were used as controls. All samples were prepared as reported,[7,8,10] stored at −80°C, assayed for sCD40L by Luminex (HCYTOMAG-60K-06, Merck Millipore, Molsheim, France), and analyzed using Bioplex Software (Biorad, Marnes-la-Coquette, France). Statistical analyses consisted in paired *t* and nonparametric Mann-Whitney *U* tests. Analysis of variance, followed by the Kruskal-Wallis test with Dunn posttest, were performed to compare sCD40L concentrations during storage (GraphPad, La Jolla, CA). "Pathogenic" thresholds were calculated by

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Table 1. Storage time of platelet components and adverse reactions

	No adverse reaction (n = 2710)*		Serious adverse reaction (n = 140)†	
Days	SDA PCs‡	Pooled PCs§	SDA PCs‡	Pooled PCs§
1-2‖	162	269	6	13
2-3	402	395	15	12
3-4	491	279	25	20
4-5	377	335	29	20
Total (n = 2850)	1432	1278	75	65

*No adverse reaction reported.
†Serious adverse reaction reported.
‡Single donor, apheresis platelet component.
§Whole blood buffy coat–derived blood component, pools of 5 ABO-RH:1 matched units, constituting 1 pooled platelet component.
‖Indicates that more than 24 hours have elapsed since the computer integrated timeframe of blood donation and less than 48 hours (maximum, 47 hours).

a "decision stump," which is a binary test focusing on an "attribute" (ie, sCD40L) and allowed minimizing the "entropy/uncertainty" (group impurity [ie, the risk of having negative data mixed with positive data in the same group, such as the SAR and no.AR groups]).[13]

Of 9206 consecutive PCs enrolled in this survey, 2850 were successfully processed, making this study highly powered (not all PCs could be sampled because of the constraints of normal practice). From these 2850 PCs (1507 SDA PCs and 1343 PPCs), for which biological material was available both at the time of labeling (d0-d1) and issuance (d1-d5), 2710 were not associated with a SAR (no.AR; 1432 SDA PCs and 1278 PPCs) and 140 were associated with a SAR (75 SDA PCs and 65 PPCs) (Table 1). Figure 1 shows sCD40L levels in SDA PCs (Figure 1A,C,E) and PPCs (Figure 1B,D,F) and the calculated thresholds (6424.01 and 6182.68 pg/mL, respectively) for each delivery day (d0-d5) that led to no.AR or to a SAR. Most no.AR values fell below the "pathogenic" sCD40L threshold calculated from a decision stump: 82% and 18% were $<$ and $\geq$6424.005 pg/mL sCD40L, respectively, for SDA PCs, and 54% and 46% were $<$ and $\geq$6182.68 pg/mL sCD40L, respectively, for PPCs (Figure 1C-D). Conversely, most (60% SDA PCs and 82% PPCs) SAR values were $\geq$6424.005 or $\geq$6182.28 pg/mL sCD40L, respectively (Figure 1E-F). The percentage of SARs in the SDA and PPC patient groups was similar (0.4977% vs 0.484%). However, the frequency of SARs increased with time in storage, particularly in the SDA PC group, in accordance with previous findings.[14]

Only patients who received only 1 platelet transfusion with the exclusion of other components were enrolled in the present survey to ensure that the considered SARs were indeed linked to the platelet transfusion; this does not preclude that other blood components were not transfused before the platelet component having caused the SAR. When that was the case, however, the last transfused blood component was given at least 16 hours before (16 hours being considered the timeframe to rely a SAR to a given blood component, according to the majority of published consensuses). A limit of this study is that it strictly depends on reporting. Many patients receiving PC transfusions received immunosuppressive drugs, such as corticosteroids, which may have masked symptoms[15] and caused underreporting. The aim of the methods used in this study to minimize entropy/uncertainty was to restore possibly altered data.[16] The influence of irradiation on sCD40L secretion[17] could not be assessed. Although most PCs were irradiated, samples were generally taken immediately before irradiation for logistical reasons. However, γ-irradiation does not augment platelet secretion of biological response modifiers, contrary to ultraviolet C.[18]

The present study did not aim to confirm previous data, but to provide new information on the link between sCD40L and SARs, namely to explore whether high levels of sCD40L are consistently associated with SARs, or not, in transfused patients. It has also been shown that the storage duration of PCs is associated with the secretion of sCD40L[19-21] and the occurrence of sCD40L-associated SAR.[12] Thus, we sought to further examine the relationship of sCD40L values and SARs, considering platelet concentrate storage time. We plotted the sCD40L levels in supernatants of the PCs associated with the 140 reported SARs against controls after various times of storage. The results are particularly suggestive of an "all or nothing" threshold phenomenon already proposed in case studies,[10] suggestive of the requirement for a second trigger in addition to higher sCD40L levels. Such a trigger is yet to be identified, but would fit well both the "2-hit" and "threshold" models of TRALI[22] and various clinical conditions.

We cannot rule out genetic causality, but in a previous study, we were unable to show an association between occurrences of SARs and genetic variants of *CD40LG*. We also cannot rule out the possibility that other genes are implicated, such as those regulating CD40 and CD40L pathophysiology.[11,12] Further genetic analyses of susceptibility may identify patients who are the most exposed to such a risk. This study clearly showed that sCD40L levels are not fully predictive of SARs, but leaves open the possibility of the comorbidities of the recipient, genetic susceptibility (high-affinity binding of sCD40L by off-target receptors), a causal disease condition, or all 3. Current means to reduce the risk of introducing excess sCD40L to transfusion recipients is either to use fresh ($\leq$3 day old) PCs (but susceptible patients may nevertheless develop SARs even with this safety measure) or to eventually wash platelet components.[23-25]

Considering that the storage lesion side product sCD40L is pathogenic, at least in a non-negligible of susceptible recipients, several options are opened. The platelet-washing procedure is not only time-consuming but also does not prevent platelets from continuing to secrete biological response modifiers, prone to accumulate during storage. In a number of diseases, recently developed biologicals to either CD40L or CD40L were successfully used; however, these drugs may interfere with patients' own platelets. Some studies have succeeded in removing 80% to 90% of sCD40L in PCs using a column of adsorptive cellulose beads: these processes usually decrease the recovery of platelets after adsorption but do not alter platelet function and may represent the best option to reduce severe nonhemolytic transfusion reaction if needed. It should be proposed that a batch system be engineered to absorb not only sCD40L but also inflammatory platelet biological response modifiers while preserving the functionality and quality of PCs, optimally being washable/reusable to make the process affordable and cost-effective.

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Contribution: O.G. undertook the study hypothesis and wrote the manuscript; F.C. designed the protocol, trained personnel in blood banks, and contributed to the writing of the manuscript; C.S. collected samples, did the experiments and statistical analyses, and cowrote the manuscript; E.F. performed all stump decision and big data analyses; and S.L. and H.H.-C. participated in all steps of the process and reviewed the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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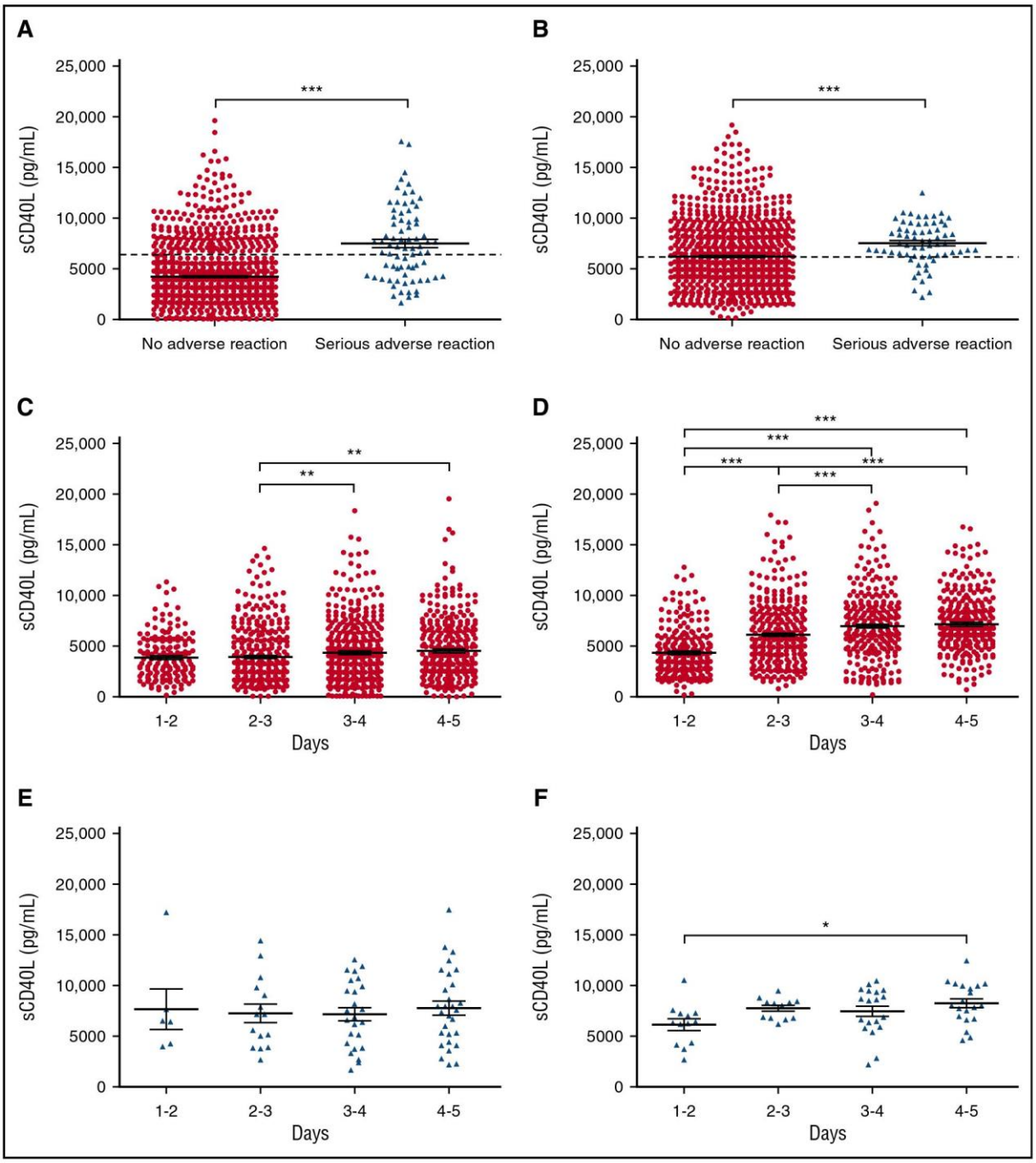

Figure 1. Increase of sCD40L in supernatants. Increase during the storage of SDA platelet components (A,C,E) and pooled platelet component (B,D,F). Day 5 represents the last day of storage for which SDA platelet components and pooled platelet component can be used for transfusion in our study. The production of sCD40L was quantified by Luminex technology. Values shown are deducted from background levels. Data (scatter plot and mean; n as indicated in Table 1) are expressed in picograms per milliliter. Group comparisons were performed by 1-way analysis of the variance followed by the Kruskal-Wallis test with Dunn posttest. The 2-tailed *t* test and Mann-Whitney test were used to compare 2 groups (*$P < .05$; **$P < .01$; ***$P < .001$).

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To the editor:

Hematologic relapse in AL amyloidosis after high-dose melphalan and stem cell transplantation

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Light-chain (AL) amyloidosis is a rare disease in which an underlying clonal plasma cell population generates aberrant immunoglobulin light chains that misfold and form amyloid fibrils, which are deposited in extracellular tissues and organs, resulting in impairment of vital organ function.[1,2] High-dose melphalan and autologous stem cell transplantation (HDM/SCT) can produce both hematologic and clinical remissions and extend survival in selected patients with AL amyloidosis.[3-5] However, there are limited data documenting outcomes for those who experience hematologic relapse after an initial hematologic complete response (CR) to HDM/SCT. We report on patients with AL amyloidosis treated with HDM/SCT to review long-term relapse and survival data and to help guide the intricate management and follow-up of this unique patient population.

Our group previously reported on 629 patients with AL amyloidosis who underwent HDM/SCT between 1994 and 2014. These patients were enrolled in several successive institutional review board–approved protocols with inclusion criteria as previously reported.[3] Hematologic response was assessed using international consensus criteria,[6,7] and 40.3% achieved a CR at 6 to 12 months following HDM/SCT. The CR rate was 34.8% by intention-to-treat. At the time of that report, hematologic relapse had occurred in 40 patients (18.2%), having previously achieved CR with a median time to relapse of 3.97 years (range, 1.89-12.45). The median overall survival (OS) for patients following hematologic relapse was 4.3 years. A retrospective review of 410 patients who underwent HDM/SCT for AL amyloidosis from 1996 to 2009 at Mayo Clinic revealed hematologic relapse or progression in 146 patients (36%) with a median time to relapse/progression of 1.97 years.[8] The median OS in this patient group after relapse or progression was 4.31 years, and the most common treatment regimen pursued after progression included lenalidomide or thalidomide. We note however that only 38 of the patients (26%) investigated with relapse/progression had achieved a hematologic CR post-SCT. Others have reported an event-free survival of ~4 years in patients undergoing SCT for AL amyloidosis independent of hematologic response and superior event-free survival in those individuals achieving CR at 1 year posttransplant.[5] Therefore, there remains a scarcity of information available regarding outcomes for relapsed patients after HDM/SCT in AL amyloidosis.

Article 2

Soluble CD40L and CD62P levels differ in single donor apheresis platelet concentrates and Buffy-coat-derived pooled platelet concentrates

Article accepté avec des révisions majeures dans *Transfusion*, Novembre 2017

Les lésions de stockage plaquettaires sont décrites par des changements structurels et biochimiques des plaquettes. Ces lésions de stockage dépendent des méthodes de production, des solutions additives, de la méthode d'inactivation des agents pathogènes, la durée de stockage.

Cette étude s'intéresse à la concentration de sCD40L et sCD62P contenue dans les concentrés de plaquettes d'aphérèse (CPA) et les mélanges de concentrés plaquettaires obtenus à partir de Buffy-Coats de plusieurs dons de sang total (MCP). Les CPA et MCP sont conservés en suspension dans du plasma ainsi que dans une solution additive de conservation. Deux solutions de conservation sont comparées, Intersol™ et SSP+®. Près de 9 000 échantillons ont été étudiés, selon la préparation et la durée de stockage. Les facteurs solubles ont été quantifiés dans les surnageants plaquettaires, en utilisant la technique Luminex.

Les CPA semblent plus activés que les MCP à la fin de l'étape de préparation, c'est-à-dire avant le stockage. Cependant, les facteurs solubles pro-inflammatoires contenus dans les MCP sont plus élevés au cours du stockage que pour les CPA. Pour les CPA, la concentration de sCD62P augmente légèrement avec Intersol plutôt que SSP+, alors que la concentration de sCD40L est nettement supérieure en présence d'Intersol comparée à SSP+.

Ces données soulignent l'importance du traitement et du stockage des CP.

Soluble CD40L and CD62P levels differ in single donor apheresis platelet concentrates and Buffy-coat-derived pooled platelet concentrates

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Running head: Inflammatory markers in platelets

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Abstract

Background: Platelet storage lesions are structural and biochemical changes in platelet components, and depend on variables in collection and processing, as well as secondary procedures and storage conditions; such lesions can be mitigated by the use of platelet additive solutions (PAS).

Study design and methods: This study investigated release of the inflammatory markers, sCD40L and sCD62P, by single donor apheresis platelet concentrates (SDA-PCs) and Buffy-coat-derived pooled platelet concentrates (PPCs) before and after storage. SDA-PC and PPC samples (n = 9089) processed by various methods and stored for different durations were obtained following production in one regional setting, the National Blood Service EFS. Soluble factors were quantified in PC supernatants immediately after processing and at the time of delivery, using Luminex technology.

Results: SDA-PCs appeared more activated than PPCs at the end of the production step (i.e., prior to storage); however, pro-inflammatory soluble factors exhibited greater increases in PPCs than in SDA-PCs during storage. In SDA-PCs, the PAS 65% Intersol led to reduced secretion of sCD62P but increased that of sCD40L, compared with the alternative PAS, SSP+.

Conclusion: These data stress the importance of the production (processing) steps of PC manufacture, and of storage. To what extent they affect patient outcomes awaits further investigation in clinical studies.

Keywords: Platelets; Transfusion; Inflammation; Preparation and Storage of Platelet Components; CD40L; CD62P

Introduction

Platelets are non-nucleated cellular elements with roles in both hemostasis and inflammation[1]. Platelet components (PCs) processed for transfusion contain numerous secretory products that have expected influence on hemostasis, but also effects on innate immunity[2]. Biological response modifiers (BRMs) increase during storage in supernatants of transfusion grade PCs[3-5], and can even affect transfusion profiles[6]. BRM secretion profiles can affect the pathogenic condition of recipients[4,5]. Some BRMs are reported to be instrumental in triggering inflammatory responses in recipients when their gross levels exceed a threshold (this finding was determined in model animals[4], and its clinical relevance is unknown). This study compared single donor apheresis (SDA-PCs) and whole blood, buffy-coat-derived, pooled PCs (PPCs), and investigated BRM secretions that reflect storage lesions[7], focusing on the soluble inflammatory platelet markers, sCD40L and sCD62P, as these BRMs are considered indicative of platelet metabolism and activation. We also compared two platelet additive solutions (PAS), Intersol and SSP+ for SDA-PCs preparation; PPCs were processed in Intersol only. Storage length was also examined with respect to BRM secretion. The method of preparation of SDA-PCs and PPCs differ in terms of centrifugation and filtration protocols, and have been well described[8,9].

This study was a well-powered ex vivo investigation of PCs processed for transfusion, including almost 9100 samples (SDA-PCs n = 3876 *vs.* PPCs n = 5213).

Materials and methods

Platelet component processing

SDA-PCs or PPCs were collected from anonymous blood donors who volunteered to provide blood for research purposes and signed a consent form after receiving specific information4. Methods for collecting SDA-PCs were reported previously[4]; briefly, blood was collected on ACD-A with Trima (Gambro BCT, Lakewood, CO, USA) or MCS (Haemonetics, Braintree, MA, USA) cell separators. All PCs were automatically resuspended in 35% autologous donor plasma and 65% platelet additive solution (PAS; InterSol, Fenwal, la Châtre, France; or SSP+, MacoPharma, Mouveaux, France). PPCs were prepared as pools of five ABO and RH:1-identical buffy coats, using an Optipress device with top and bottom separation (Baxter Healthcare Corporation, La Châtre, France); they were resuspended in 35% InterSol. PPCs were

leukoreduced by filtration immediately after pooling (pre-storage)[10]. PCs were stored at 22°C ± 2°C under gentle rotation and shaking (60 rpm) for a maximum of 5 days before being issued for transfusion. PC supernatant samples were collected after centrifugation (402 g; 10 min), as part of a research protocol detailed in Cognasse *et al.*[11], and aliquots frozen at −80°C. For this study, 9089 samples were collected, corresponding to different steps of processing and storage (Table 1).

Quantification of soluble proteins

Soluble CD40L (sCD40L) and CD62P (sCD62P) were quantified in PC supernatants using Luminex technology (Millipore, Molsheim, France), according to the manufacturer's instructions, using a Bioplex 200 system (BioplexManager software; Biorad, Marnes-la-Coquette, France)[4,5].

Statistical analyses

Group comparisons were performed by one-way analysis of variance followed by Kruskal-Wallis with Dunn's post-hoc tests. For comparisons between two groups, two-tailed t-tests and Mann-Whitney tests were performed. For all tests, p-values < 0.05 were considered significant. Results are presented as scatter plots and means. Statistical analyses were performed using GraphPad Prism (GraphPad, La Jolla, CA, USA).

Results

From March 2013 to April 2016, 9089 samples were collected, comprising 3876 (43%) SDA-PCs and 5213 (57%) PPCs. Samples were recovered on the day of processing and on the day of delivery (detailed in Table 1). In PPCs, the mean platelet number was 1259 x 10^3/µl (min–max, 772–1787 x 10^3/µl) and residual leukocyte concentration was 0.033 x 10^6 (min–max, 0.014–0.121 x 10^6). In SDA-PCs collected using a Trima-Gambro BCT instrument, mean platelet number was 1351 x 10^3/µl (min–max, 1004–1702 x 10^3/µl), with a residual leukocyte concentration of 0.022 x 10^6 (min–max, 0.016–0.098 x 10^6). Using the MCS/Haemonetics device the mean platelet number of SDA-PCs was 1250 x 10^3/µl (min–max, 1025–1609 x 10^3/µl) and residual leukocytes was 0.021 x 10^6 (0.015–0.073 x 10^6). These data are from the Quality Control Laboratory.

sCD62P and sCD40L were examined to evaluate the level of platelet activation after each PC processing method. As shown in Fig. 1A, pre-storage sCD62P levels was 167.6 ± 2.09 and 206.3 ± 2.7 ng/mL in PPCs and SDA-PCs, respectively, indicating that platelet processing for PPC production activates significantly fewer platelets than that for SDA-PCs ($p < 0.0001$). Similarly, pre-storage sCD40L concentrations were 2887 ± 25.43 and 4283 ± 53.47 pg/mL in PPCs and SDA-PCs, respectively ($p < 0.0001$) (Fig. 1B). Next, sCD62P (Fig. 1C) and sCD40L (Fig. 1D) were measured in PCs during storage. The concentration of sCD62P and sCD40L varied significantly over time in both SDA-PCs and PPCs ($p < 0.001$). Interestingly sCD62P and sCD40L increased more rapidly in PPCs than SDA-PCs at all-time points.

We next evaluated the influence of PAS (65%) on platelet activation during shelf-life; 836 SDA-PC samples were stored in Intersol and 1583 in SSP+; sCD62P (Fig. 2A) and sCD40L (Fig. 2B) were measured for each group during storage. Concentrations of sCD62P and sCD40L varied significantly over time (both $p < 0.0001$). Interestingly, in contrast to sCD62P, the concentration of sCD40L in SDA-PCs in Intersol was clearly superior to that of SDA-PCs in SSP+; on Day 5, the sCD62P concentrations were 140 and 120.4 ng/mL, respectively, while sCD40L concentrations were 7137 and 3447 pg/mL, respectively.

Discussion

Here, we report that platelet activation is lower in PPCs than SDA-PCs, based on concentrations of sCD62P and sCD40L secreted in PCs processed and stored for transfusion. The concentration of sCD40L and sCD62P varied significantly over time in PCs; however, their increases were more substantial in PPCs than SDA-PCs. Moreover, the level of sCD40L was greater in SDA-PCs stored in Intersol than those in SDA-PCs stored in SSP+, consistent with previous reports[12-15]. Storage of platelets in PAS has a number of benefits, including a reduction of serious adverse reactions (SARs)[16]. As low plasma content in platelet concentrates is a requirement for some pathogen inactivation processes, optimization of PAS has become a focus of interest[17]. Although differences can be demonstrated between PPCs and SDA-PCs, both processes conform to transfusionnel standards. Several reports indicate that platelets from apheresis and whole blood (Buffy-coats) are comparable both in terms of *in vitro* quality and clinically, although the advantages of one over the other are debated[18,19]; however, a French Haemovigilance survey clearly indicated the substantial superiority of PPCs over SDA-

PCs in terms of tolerance[20], data which was substantiated by the national hemovigilance report[21]. Platelet concentrates can be obtained from different sources as SDA-PCs and PPCs, and can be used for clinical applications, within the international directives and regulations set out in transfusion guidelines. Platelets from whole blood or apheresis are equivalent in quality; however, additional factors such as SARs, donor accessibility, ethical considerations, cost, infectious risks, and donor comfort may affect the final choice of which method of platelet collection is used for transfusion. Little attention, if any, has been given to the impact of procedural changes on the anti- and pro-inflammatory lesions potentially inflicted by stored platelets. The extent to which they affect patient outcome requires further investigation in clinical studies. Moreover, the extent to which anti- and pro-inflammatory lesions correlate with clinical outcomes is not fully understood. Further research will reveal the relationship between our *in vitro* data and clinical outcomes.

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Contributions

All authors were involved in data acquisition and approved the final version of the manuscript. FC and OG designed the study. CS, ST, CA, CAA, MAE, JF, PC, HHC, and SL performed experiments and acquired data. CS, OG, and FC analyzed the data and drafted the manuscript.

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Table 1. Study protocol, samples, and tests.

Platelet Additive Solution (PAS)		Samples tested prior to storage	Samples tested at the time of delivery			
			days 1–2	days 2–3	days 3–4	days 4–5
Single Donor Apheresis (SDA-PCs)	SSP+	1583	103	310	355	261
	Intersol	836	60	109	145	114
	Total	2419	163	419	500	375
Whole-Blood Buffy-Coat Pools of 5 (PPCs)	Intersol	3889	276	407	289	352
Total Platelet Concentrates (PCs)		6308	439	826	789	727

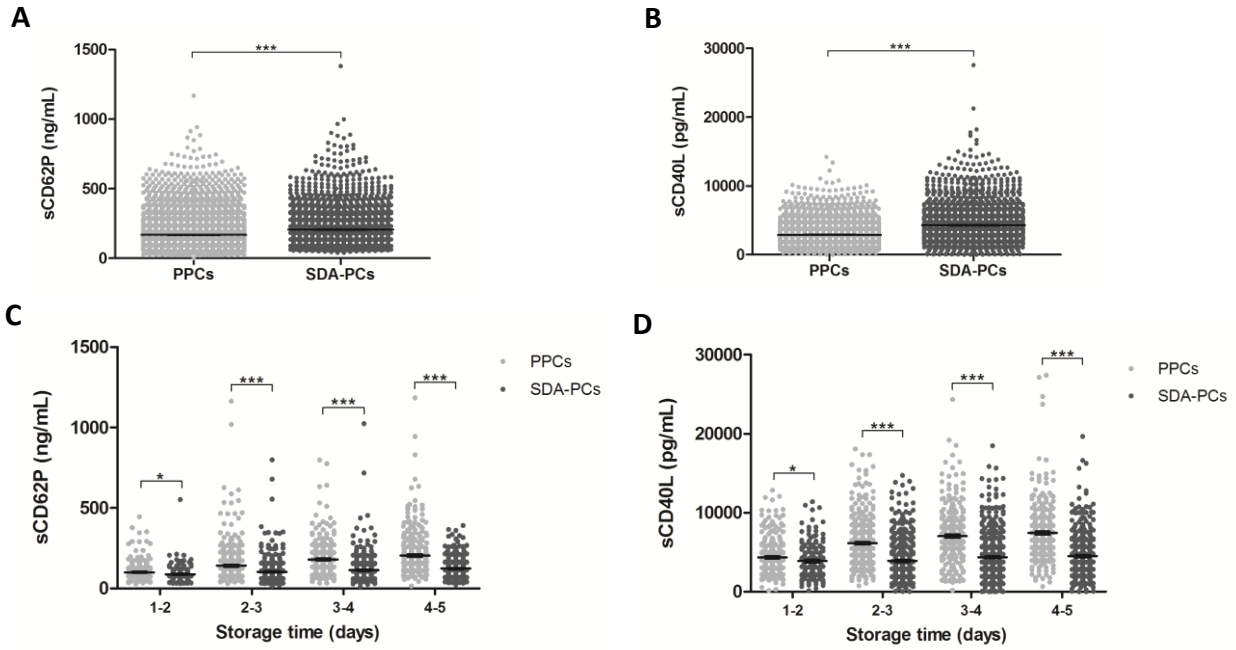

Figure 1. Elevation of soluble CD62P (A, C) and CD40L (B, D) in supernatants before storage (A, B) of PPCs and SDA-PCs and during storage (C, D) (Days 1–2, 2–3, 3–4, and 4–5).

Day 5 was the last day of storage for which PPCs and SDA-PCs could be used for transfusion. Production of sCD62P and sCD40L was quantified by Luminex technology. Values shown are with background levels deducted. Data (scatter plots and means; numbers of samples in each group are described in Table 1) are expressed in ng/mL (sCD62P) and pg/mL (sCD40L). Comparisons of groups were performed by one-way analysis of variance followed by Kruskal-Wallis with Dunn's post-hoc tests. For comparisons between two groups, two-tailed t-tests and Mann-Whitney tests were applied. *P < 0.05; ***P < 0.001.

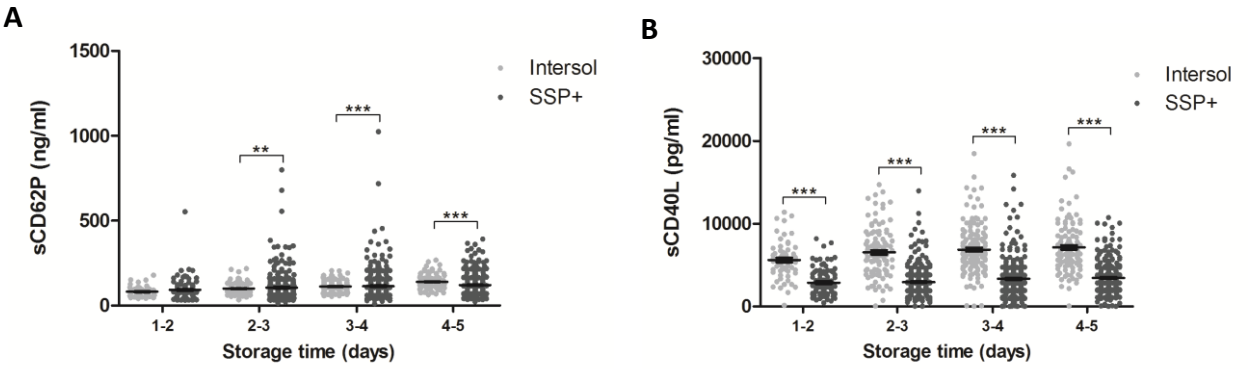

Figure 2. Elevation of soluble CD62P (A) and CD40L (B) in supernatants of SDA-PCs stored in Intersol and SSP+ (Days 1–2, 2–3, 3–4, and 4–5).

Day 5 was the last day of storage for which SDA-PCs could be used for transfusion. Production of sCD62P and sCD40L was quantified by Luminex technology. Values shown are with background levels deducted. Data (Scatter plot and mean; numbers of samples in each group are described in Table 1) are expressed in ng/mL (sCD62P) and pg/mL (sCD40L). Comparisons of groups were performed by one-way analysis of variance followed by Kruskal-Wallis with Dunn's post-hoc tests. Comparisons between two groups were performed using two-tailed t-tests and Mann-Whitney tests. **P < 0.01; ***P < 0.001).

Article 3

Assessment of soluble platelet CD40L and CD62P during the preparation process and the storage of apheresis platelet concentrates: absence of factors related to donors and donations

Article « *in review* » dans *Transfusion Clinique et Biologique*, Septembre 2017

Une transfusion de plaquettes peut être associée à des effets indésirables chez le receveur, potentiellement dû à la présence de modificateurs biologiques du comportement contenus dans les concentrés plaquettaires.

Cette étude a pour but d'identifier les paramètres reflétant une activation plaquettaire au cours du processus de préparation ainsi que du stockage des concentrés plaquettaires. Près de 4000 échantillons de concentrés plaquettaires d'aphérèse ont été étudiés au regard de paramètres liés au donneur en sus du processus de préparation et de leur stockage. Le CD40L et CD62P solubles ont été quantifiés dans les surnageants de concentrés plaquettaires à l'issue de leur préparation et au cours du stockage, en utilisant la technologie Luminex.

Nous avons observé une augmentation des facteurs solubles au cours du temps. Cependant, les différents paramètres étudiés en lien soit avec les donneurs soit avec les dons, comme : i) le sexe du donneur, ii) le groupe sanguin du donneur, iii) l'heure du prélèvement et iv) le type de séparateur d'aphérèse, semblent ne pas avoir d'influence significative sur l'état d'activation plaquettaire et la libération de CD40L et CD62P solubles.

Assessment of soluble platelet CD40L and CD62P during the preparation process and the storage of apheresis platelet concentrates: absence of factors related to donors and donations

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Conflict-of-interest disclosure: All other authors declare no competing financial interests.

Summary

Platelet transfusions may be associated with certain adverse effects in recipients, potentially caused by the presence of biological response modifiers contained in the platelet concentrates. The aim of this study is to identify the parameters that reflect platelet activation during both the preparation process and the storage of platelet concentrates. A total of 3,949 apheresis platelet concentrate samples were studied with regard to parameters related to the donor as well as to the preparation process and their storage. Key glycoproteins characteristic of platelet activation, i.e. soluble CD40L and CD62P, were quantified in platelet concentrate supernatants on completion of their processing and during storage, using Luminex technology. We observed an increase in soluble factors over time. However, the different parameters studied in connection either with the donors or with the donations, such as i) donor gender, ii) donor blood group, iii) time of collection and iv) type of apheresis separator, do not seem to have any effect on platelet activation or the release of soluble CD40L and CD62P.

Keywords: Transfusion, apheresis platelet concentrates, inflammation, preparation and storage of platelet concentrates

Introduction

Blood platelets are anucleate cells which play an essential role in haemostasis; they also play an important role in innate immunity (1). They are known to be involved in inflammation, namely by releasing molecules capable of modulating the inflammatory response (2). It has been shown that these immunomodulatory molecules may be present in Platelet Components (PCs) and that their concentration increases during storage of the products (3).

A transfusion of labile blood products (LBPs) and, more specifically, PCs entails variable parameters related to the donors, the preparation process and the recipients. The donor-related factors are primarily their genetic characteristics. During the collection and the preparation process of apheresis platelet concentrates (APCs) –45% of regional/national production– several parameters may differ, namely the following in our study: type of separator (Trima Accel® or MCS®+) and type of storage solution (Intersol™ or SSP+®). The time of day when the donation is made is seldom assessed as a parameter, which we also wanted to examine here. APCs are stored in one-third donor plasma and two-thirds platelet additive solution (PAS). The storage conditions and the storage time of APCs are significant factors. Indeed, platelets are capable of releasing an increasing number of biological response modifiers (BRMs) the longer they are stored (4-6).

Many strict rules in view of international recommendations are applied during the LBP preparation, qualification and storage process, but adverse reactions (ARs) in recipient are still sometimes seen (7). LBP leucocyte reduction has considerably reduced such reactions, especially when applied within hours of blood collection (referred to as “prestorage leukoreduction”) (8). However, despite all these precautions, the platelets still suffer damage related to preparation and storage (9). This is due in particular to the fact that platelets can be activated and therefore release BRMs. The use of PAS has also substantially reduced the occurrence of allergic/allergy-like and inflammatory adverse effects (10-13). Some of these BRMs can cause an inflammatory reaction in the recipient and thus be associated with ARs (14, 15). Platelet transfusions may therefore be associated with inflammatory effects in a non-negligible percentage of transfusion occurrences. This may be due in part to immune mediators either present in residual plasma or expressed by activated platelets, or released into the platelet medium during storage (16). Soluble CD40 ligand (sCD40L) and soluble CD62P (sCD62P) were selected to reflect platelet activation (17).

This study assesses various donor-related parameters, such as gender and blood group. Parameters related to collection were also studied, namely the time of donation and the type of apheresis machine.

Materials and methods

Platelet concentrates

The PCs were collected from anonymous regular blood donors who volunteered, after receiving specific information, to provide blood for research purposes and signed a consent form approved by the regulatory authorities. The PCs were obtained from a single donor by apheresis (APCs). The platelets were collected on cell separators using citrate dextrose-A with Trima Accel® (Terumo BCT, Lakewood, CO, USA) or MCS®+ (Haemonetics®, Braintree, MA, USA). The PCs were automatically resuspended in 35% autologous donor plasma and 65% PAS (InterSol™, Fenwal, Lake Zurich, IL, USA, or SSP+®, MacoPharma, Mouveaux, France). The PCs were stored at 22 ± 2°C with gentle rotation/agitation (60 rpm) for a maximum of five days before being delivered for transfusion. PC samples for this research survey were collected on the day of preparation at the end of the process or on the day of delivery (Table 1). The samples collected on the day of preparation were used as controls. The PC samples were transferred to a polypropylene tube and centrifuged at 1 500 rpm for 10 minutes. The supernatants were stored at -80°C before soluble factor testing.

Assays of soluble factors contained in the platelet concentrates

The sCD40L and sCD62P were quantified using the Luminex technique (HCYTOMAG-60K-06, Merck Millipore, Molsheim, France). Results were obtained using a Bioplex 200 system (BioplexManager software, Biorad, Marnes-la-Coquette, France).

Statistical analysis

The results are presented as mean values ± SEM. A value is considered significant if $p<0.05$. Group comparisons were made using a one-way analysis of variance (ANOVA) followed by a Kruskal-Wallis test and a Dunn's post-test. The Two-tailed t-test and Mann-Whitney test were used to compare two groups (GraphPad Software, La Jolla, CA, USA).

Results

Between March 2013 and April 2016, approximately 3,949 APCs were collected: 2,468 samples collected on the day of PC processing, as well as 1,481 samples obtained on the day of delivery (Table 1). The platelet count was 1,351x103 plts/µL (1,004–1,702) and the residual leucocyte concentration was 0.022x106 (0.016–0.098) in the APCs (Trima Accel®); 1,250x103 plts/µL (1,025–1,609) and 0.021x106 (0.015–0.073) in the APCs (MCS®+). We assessed sCD62P and sCD40L in order to determine the level of platelet activation based on various parameters related to the donor, the preparation process and the storage of the APCs. In general, the concentration of sCD40L and sCD62P varied over time (Figure 1). We found that sCD40L increased significantly from Day 2 of storage, while sCD62P increased from Day 1 of storage. However, the concentration of sCD62P was significantly higher on the day of preparation than during storage. More accurate data regarding the effect of the process and storage on sCD62P and sCD40L parameters has also been presented (Sut *et al.*, submitted for publication).

We then assessed the influence of various parameters related to donors and to collection. The concentration of sCD40L and sCD62P did not vary based on the gender of the donor, except between Day 2 and 3 where there are a slight difference for sCD40L (Figure 2A). As for the donor's ABO blood group, we observed no differences for any of the groups A, B or O (Figure 2B), despite variable expression of platelet surface A and B antigens. We also examined whether or not the time of donation might affect platelet activation: again, we observed no differences based on the time of collection (Figure 2C).

We subsequently focused on the type of apheresis separator (Trima Accel® or MCS®+). The concentration of sCD40L did not vary based on the type of separator, except from Day 5 of storage, when we saw a higher concentration for the platelets prepared using the MCP®+ procedure compared to Trima Accel® (Figure 2D). Furthermore, the concentration of sCD62P was 94.85 ± 3.3 ng/mL for MCS®+ and 186 ± 29.66 ng/mL for Trima Accel® between the second and third day of storage. Production of sCD62P released by the platelets was therefore significantly higher with MCS®+ separators from the second day of storage of the APCs. On the day of preparation, the concentration of sCD40L and sCD62P increased further with Trima Accel® separators, conversely during storage. The MCS®+ separator therefore seems to activate more platelets than the Trima Accel® separators ($p<0.0001$) (Figure 2D), with a visible effect at the end of platelet storage in particular.

Discussion

We confirm an increase in the concentration of sCD40L and sCD62P over time during platelet storage (3, 5). Many studies have in fact shown that platelets were activated over time through damage during storage and that it is therefore preferable to transfuse PCs before the shortest possible storage time, namely based on sCD40L, as this cytokine-like molecule is associated with the occurrence of ARs (18, 19). PCs have high levels of sCD40L, with the maximum levels observed 72 hours after platelet collection (20). However, this conclusion should be adapted based on future research into the impact of damage during storage depending on the recipient's disease.

Furthermore, unexpectedly, we noted that the concentration of sCD62P was higher on the day the APCs were prepared and lower during the initial storage time of the platelets. During APCs preparation, the platelets were subjected to stress related to the preparation process, namely centrifugation, which may therefore activate the platelets. From Day 1 of storage, the release of sCD62P was much lower, which can be explained by an internalization of sCD62P by the platelets. Generally speaking, platelet BRMs help regulate the neighbouring cells and, in particular, their proliferation, differentiation and activation. Interestingly, platelets also express the receptors of several of the BRMs that they secrete, thus illustrating their potential ability to form bidirectional autocrine and paracrine loops. Lastly, one of the study's limitations is the much higher number of samples on the day of preparation than on the day of storage, which may influence the results.

Based on the released levels of sCD40L and sCD62P in the APCs, we noted that the different parameters studied in the donors (gender and blood group) had no effect on the concentration of sCD40L and sCD62P, which indicates platelet activation. It should be noted that some authors have shown that there could be a difference in platelet storage between donors, according to metabolic differences such as pH in particular (21), but we did not find this to be the case (3). We also saw that the time of collection had no effect on platelet activation. On the other hand, we would like to point out a difference in sCD62P secretion based on the type of apheresis separator, indicating greater platelet activation for the APCs prepared with MCS®+. Noulsri *et al.* showed that there was high variability in BRM secretion – platelet microparticles in particular– and in platelet activation based on the different preparation processes (22). While differences can be proven, namely in the inflammatory

characteristics of the platelet products between the Trima Accel® and MCS®+ separators, both processes allow clinical-grade platelet preparation in accordance with international standards and recommendations.

However, according to available haemovigilance data, ARs occur more frequently with apheresis platelet concentrates (APCs) than with pooled whole-blood-derived platelet concentrates (PPCs) (23). It therefore seems more important to identify which parameters promote the occurrence of ARs based on the different types of PCs and their preparation (24). In conclusion, further research is required to establish the underlying causes and the implications of donors and PC preparation processes in order to understand the inflammatory role of blood platelets and their level of involvement in triggering ARs.

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Table 1. Information on donors, APC preparation and storage time.

Storage time		d0	d1-d2	d2-d3	d3-d4	d4-d5
Gender	**Male**	1572	90	279	323	270
	Female	814	64	123	156	103
Blood group	**A**	1135	76	207	231	195
	B	135	11	26	32	24
	O	1117	67	169	203	146
Time of donation	**8am-11am**	709	13	122	188	137
	11am-2pm	878	77	166	165	115
	2pm-6pm	798	64	114	113	113
Device	**Trima Accel®**	2084	135	348	404	318
	MCS®+	147	8	26	32	22
Total		**2468**	**164**	**423**	**505**	**389**

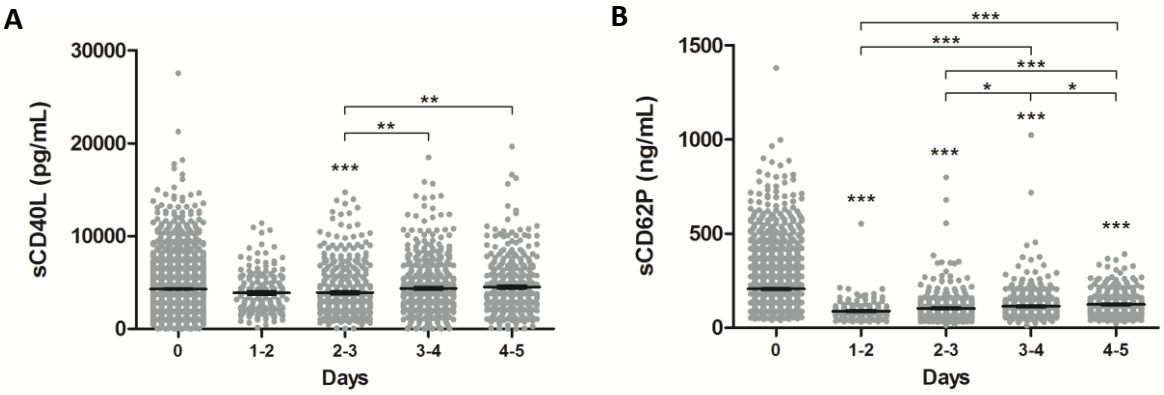

Figure 1. Concentrations of sCD40L and sCD62P over time.

The production of sCD62P and sCD40L was quantified using Luminex technology. The mean values ± SEM are represented. The data is expressed in ng/mL (sCD62P) and pg/mL (sCD40L). Group comparisons were made using a one-way analysis of variance (ANOVA) followed by a Kruskal-Wallis test and a Dunn's post-test (*<0.05, **<0.01, ***<0.001). Day 0 represents the day of preparation, and Day 5 represents the last day of storage for which the APCs could be used for transfusion.

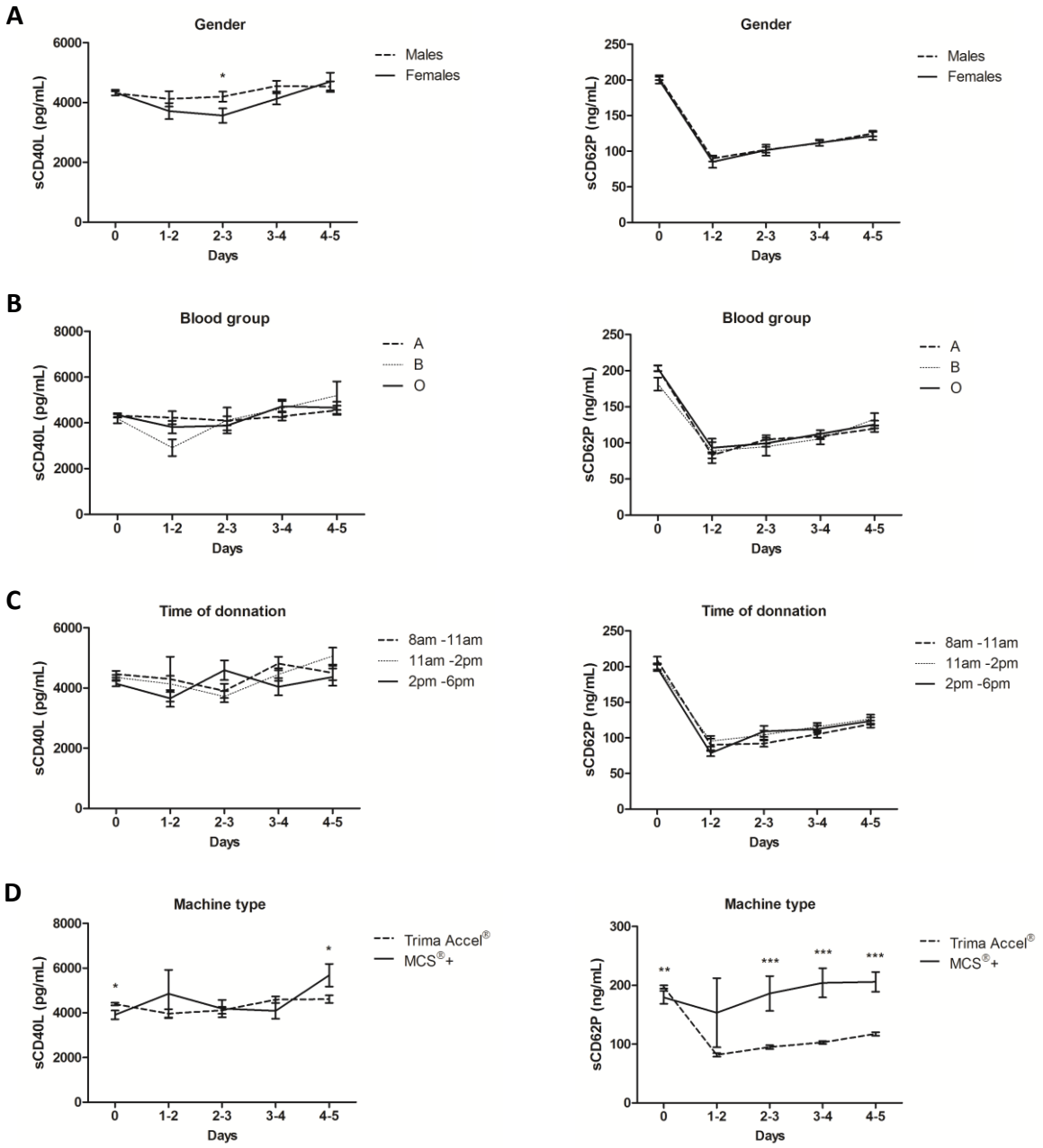

Figure 2. Concentrations of sCD40L and sCD62P over time according to gender, blood group, time of donation and type of separator.

The production of sCD62P and sCD40L was quantified using Luminex technology. The mean values ± SEM are represented. The data is expressed in ng/mL (sCD62P) and pg/mL (sCD40L). Group comparisons were made using a one-way analysis of variance (ANOVA) followed by a Kruskal-Wallis test and a Dunn's post-test. The Two-tailed t-test and Mann-Whitney test were used to compare two groups (*<0.05, **<0.01, ***<0.001). Day 0 represents the day of preparation, and Day 5 represents the last day of storage for which the APCs could be used for transfusion.

Article 4

Platelet biological response modifiers associated with transfusion adverse reactions on *in vitro* endothelial function

Article en cours de rédaction

Les plaquettes dans les CP sont la principale source de facteurs responsables de manifestations inflammatoires et/ou allergiques. Les plaquettes, durant leur conservation, secrètent de nombreux BRM, qui sont des ligands pour des récepteurs des cellules endothéliales et pour la plupart des types leucocytaires circulants ou adhérents aux endothéliums.

Notre projet permet de préciser les mécanismes par lesquels des surnageants plaquettaires conduisent à activer les cellules endothéliales, en fonction de la durée de stockage des CP et s'ils ont déclenché un EIR. Pour cela, des CP au jour de préparation (considérés comme témoins), des CP au jour de délivrance ainsi que des CP ayant entrainé un EIR, ont été collectés. Des cellules endothéliales EA.hy926 cultivées, *in vitro*, ont ensuite été mises en présence des différents surnageants plaquettaires. Par la suite, l'étude du signalosome des cellules endothéliales a été réalisée par des techniques de biologie moléculaire.

Nos résultats indiquent que certains gènes impliqués dans l'inflammation des cellules endothéliales sont plus ou moins exprimés en fonction des stimulations par différents surnageants plaquettaires. Ce criblage a permis d'identifier des gènes d'intérêt afin d'étudier les expressions protéiques membranaires et/ou solubles possiblement consécutives. L'analyse de l'expression de marqueurs d'activation, d'apoptose ainsi que la production de facteurs inflammatoires solubles des cellules endothéliales a également mis en évidence des différences d'expression en fonction des surnageants plaquettaires.

De plus, cette étude est complétée par un « Woud healing test ». Une brèche est réalisée sur les cellules endothéliales en culture, stimulées par les différents surnageants plaquettaires. La migration cellulaire est observée au cours du temps par microscopie.

Platelet Biological Response Modifiers that Usually Associate with Transfusion Adverse Reactions Alter Endothelial Functions in an *In Vitro* Model

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Running head: Platelet Inflammation and Transfusion

Abstract: 307 words, Main text: 3 724 words, References: 28, Figures: 4, Supplemental Data: 2

Abstract

Background: Platelet transfusions are safe, but can nevertheless cause adverse reactions (ARs). This study aimed to investigate the effects of platelet biological response modifiers (BRMs) that accumulate during storage and are commonly associated with transfusion ARs. To this end, endothelial cell lines were exposed to supernatants of platelet components (PCs) having caused and not caused ARs.

Study design and methods: Endothelial cells (ECs), i.e. EA.hy926 were exposed *in vitro* to supernatants of PCs that had both been involved and not been involved in transfusion serious adverse reactions (SARs). The EC Biology RT^2 Profiler PCR Array was used to simultaneously study 84 genes related to EC functions, and subsequently screen sets of molecules of interest. Surface expression of EC markers was determined by flow cytometry. Soluble cytokines in culture supernatants of ECs exposed to PC supernatants were measured by Luminex technology or specific ELISA. Apoptosis and scratch wound assays were performed using the IncuCyte technology.

Results: *In vitro* exposure of EA.hy926 monolayers with Day 0, Day 1-3 and Day 3-5 stored PC supernatants resulted in decreases in surface expression of EC markers on EA.hy926 cells, such as ICAM-1, ITGB3, NPPB and NPR1. Furthermore, there was differential production of soluble BRMs in the tested cell line. When EA.hy926 monolayers were exposed to PC supernatants that had been involved in SARs, there was little significant difference relative to the surface expression of EC marker expression. In contrast, concerning the secretion of soluble BRMs, EA.hy926 cells responded differently when cells were in the presence of platelet supernatants with different storage times or if they were involved in SARs.

Conclusions: Irrespective of the transfusion outcome, PC supernatants collected at Day 1-3 activate fewer EC cell lines compared with supernatants collected at Day 3-5. Moreover, PC supernatants involved in SARs appear to alter EC activation compared with the control and during storage length.

Key words: Transfusion, Endothelial Cells, Platelets, Biological Response Modifiers, Serious Adverse Reaction, Storage

Introduction

The haemostatic function of platelets has been known since the late 1800s[1]. More recently it has been recognized that platelets contribute to regulating immune functions and impact on vascular integrity[2]. The benefit of platelet transfusion has been acknowledged since the beginning of the 20th Century[3]. Later, platelet components (PCs) have been made available, although the processing of PCs leads to so-called storage lesions[4]. Platelet storage lesions are characterized by damaging changes in the structure and function of the platelets in PCs during collection, processing, preservation and storage before delivery for transfusion. Platelet storage leads to an accumulation in the PC of a wide variety of biological response modifiers (BRMs)[5]. In addition to soluble CD40 ligand (sCD40L) implicated in serious adverse reactions (SARs), other important BRMs accumulate in PCs during storage[6]. BRMs which platelets store inside dense and alpha granules are able to bind to receptors on the surface of endothelial cells (ECs) alongside receptors of circulating blood cells. Certain SARs can manifest as inflammation of the vascular system and/or vessels in particular sensitive organs such as the lungs; transfusion-related acute lung injury (TRALI) may be an example of such a pathology.

This work aims to model the inflammatory response at the EC level when exposed to platelet-originating BRMs, especially in conditions assumed to raise excessive levels of some of them. To this end, endothelial cell lines were exposed to various types of PC supernatants obtained at different timeframes between Day 1 and Day 5 of storage that were both associated and not associated with a SAR in the transfused patient. The induced activation of the modelled vascular endothelium resulted in the release of cytokines/chemokines and the modulation of the surface expression of adhesion molecules, specifically intercellular adhesion molecule 1 (ICAM-1)[7]. Supernatants from certain stored PCs both involved and not involved in SARs, create pro-inflammatory conditions as has been consistently shown in a large number of investigations by both ourselves and others[5,6,8-13]. However, no in-depth investigation had been performed to determine how pathogenic PC supernatants (BRMs) can alter EC functions; this investigation thus undertook to examine the way in which pathogenic platelet-originating BRMs alter EC gene regulation, protein membrane expression and cytokine/chemokine secretion, in order to assess whether or not there is a synergy between the pro-inflammatory signals delivered and platelets and the inflamed endothelium. This investigation was carried

out using an experimental model under real-condition PC supernatants and an EC line, namely EA.hy926.

Materials and Methods

Platelet Concentrate Processing

PCs were collected from anonymous regular blood donors who volunteered, after receiving specific information, to provide blood for research purposes and signed a consent form approved by the regulatory authorities[6]. PCs were obtained from single donors by apheresis (APCs). Platelets were collected on cell separators using citrate dextrose-A with Trima AccelTM (Terumo BCT Europe NV, Zaventem, Belgium) or MCS+TM (Haemonetics, Limonest, France). The PCs were automatically resuspended in 35% autologous donor plasma and 65% PAS (InterSol™, Fenwal, Lacs, France or SSP+TM, MacoPharma, Mouveaux, France). The PCs were stored at 22 ± 2°C with gentle rotation/agitation at 60 rpm for a maximum of five days before being delivered for transfusion. PC samples for this research survey were collected on the day of processing (at the end of the process) or on the day of delivery. PC supernatant was recovered from leftover PCs having led to a SAR, thanks to cooperation with clinical staff, or from control PCs to match the different storage timeframes. For each group (SARs and controls), 50 PC supernatants were analysed. All PC supernatant samples were transferred to a polypropylene tube and centrifuged at 402 g for 10 minutes. The supernatants were stored at -80°C prior to soluble factor testing.

Endothelial Cell Culture

EA.hy926 is a permanent cell line derived by fusing human umbilical vein endothelial cells (HUVEC) with cell line A549[14]. The cell line EA.hy926 was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). EA.hy926 was cultured in Dulbecco's Modified Eagle's Medium (DMEM), high glucose (PAA) supplemented with 10% foetal calf serum (FCS), 1% penicillin/streptomycin, 1% non-essential amino acid, 0.1% ciprofloxacin and maintained at 37°C under 5% CO2. Cells were cultured in 6-well and 96-well plates in order to obtain approximately 10^6 cells/ml. The cell number was quantitated with a TC10™ Automated Cell Counter (Bio-Rad, Marnes-la-Coquette, France). Cells were grown until confluent then passaged with 0.25% trypsin (Sigma-Aldrich, Saint-Quentin-Fallavier, France).

Stimulation of EA.hy926

ECs were cultured in 6-well plates (1.10^6 cells/well) or 96-well plates (5.10^4cells/well) and grown for 48 hours to reach a confluent state prior to the experiment. Confluent endothelial monolayers were incubated for 24 hours with the following treatment: control wells without any stimulation, platelet supernatants diluted at 1/5 in DMEM, and positive control wells stimulated by recombinant human TNF-α (PeproTech, Neuilly-sur-Seine, France) at a final concentration of 100 pg/ml. After 24 hours of incubation, supernatants were recovered and frozen at -80°C. After washing in phosphate buffered saline (PBS), the cells were centrifuged for 5 minutes at 1 200 rpm. The EC pellets were resuspended in 1% paraformaldehyde in PBS for 30 minutes and then washed. The fixed cells were resuspended in PBS.

RT^2 Profiler PCR Array

Total RNA was extracted from EC stimulated by PC supernatants using the RNeasy® Mini Kit (Qiagen, Courtaboeuf, France) following the manufacturer's instructions. The RNase-Free DNase Set was used to completely eliminate genomic DNA contamination. RNA purity was quantified using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Illkirch-Graffenstaden, France). Reverse transcription reaction was performed with the RT^2 First Strand Kit (Qiagen, Courtaboeuf, France). The same amount of total RNA was used for each sample: 500 ng. Real-time PCR was performed using the RT^2 SYBR® Green qPCR Mastermix according to the manufacturer's protocol. The Endothelial Cell Biology RT^2 Profiler PCR Array (PAHS-015ZA, Qiagen, Courtaboeuf, France) was used to simultaneously study 84 genes related to EC functions and five housekeeping genes, a genomic DNA control, three replicate reverse transcription controls, and three replicate positive PCR controls in 96-well plates. The amplification assays were performed using a CFX96 Touch qPCR System (Bio-Rad, Marnes-la-Coquette, France) following cycling conditions: 10 minutes at 95°C, 15 seconds at 95°C, and 1 minute at 60°C for 40 cycles.

Flow Cytometry Analyses

Surface expression of ECs was determined by flow cytometry. Cells were fixed beforehand. Direct labelling was performed for the following markers: endoglin/CD105 (BD

Biosciences, Le Pont de Claix, France), ICAM-1/CD54 (BD Biosciences, Le Pont de Claix, France), ITGB3/CD61 (BD Biosciences, Le Pont de Claix, France), and PDGFRA/CD140a (BD Biosciences, Le Pont de Claix, France). The cells were incubated for 30 minutes with antibodies. Double-labelling was performed for ApoE, NPPB and NPR1. The cells were incubated for 30 minutes with the following primary antibodies: anti-ApoE (Abcam, Cambridge, UK), anti-NPPB (Abcam, Cambridge, UK) and anti-NPR1 (Abcam, Cambridge, UK), simultaneously with the secondary antibody: FITC-conjugated anti-mouse antibody (Abcam, Cambridge, UK) or FITC-conjugated anti-rabbit antibody (Abcam, Cambridge, UK). In all the experiments, background labelling was assessed using the relevant fluorochrome-conjugated mouse IgG isotype control (BD Biosciences, Le Pont de Claix, France). Cells were centrifuged at 1 200 rpm for 5 minutes then washed in PBS. Data acquisition was performed using a Guava easyCyte™ HT Flow Cytometer (Merck Millipore, Molsheim, France) and the data were analysed using the Incyte program. At least 10 000 events were collected for each sample. Results were obtained as the mean fluorescence intensity (MFI).

Soluble Factors Quantification

The production of soluble cytokines in culture supernatants of endothelial cells both stimulated and not stimulated by PC supernatants was measured using the Luminex technology or specific enzyme-linked immunosorbent assays (ELISA). The Luminex technology was used to test MCP-1, fractalkine or CX3CL1, FGF-2, IL-1β, IL-6 and RANTES or CCL2 (panel HCYTOMAG-60K-06, Merck Millipore, Molsheim, France), according to the manufacturer's instructions. Results were obtained using a Bioplex 200 system (Bioplex Manager software, Bio-Rad, Marnes-la-Coquette, France). The levels of the following soluble cytokines: ICAM-1, IL-11, PTGS2, Cox-2 (DuoSet) and ApoE (Quantikine) were measured by commercially-obtained ELISA (R&D Systems, Lille, France) according to the manufacturer's instructions. NPPB production was assayed using pre-quote plates (Sigma-Aldrich, Saint-Quentin-Fallavier, France), according to the manufacturer's instructions. Absorbance at 450 nm was determined with an ELISA reader (Magellan Sunrise software, Tecan Group Ltd., Lyon, France).

Apoptosis Assays

The activity of caspase 3/7 was measured using the IncuCyte® Caspase 3/7 apoptosis assay reagent (Essen BioScience, Ann Arbor, Michigan, USA). This reagent was added directly to tissue culture wells stimulated by PC supernatants. Kinetic activation of caspase 3/7 was measured using live cell imaging after 35 hours of stimulation, and quantified using the IncuCyte™ ZOOM basic analyser.

Scratch Wound Assay

Cell migration was observed with the IncuCyte® Scratch Wound Assay (Essen BioScience, Ann Arbor, Michigan, USA). Cells were seeded (4.10^4 cells/ml in DMEM), and a wounding procedure was performed with WoundMaker™ to create precise and reproducible wounds in each well of a 96-well plate. After washing, some of the ECs were stimulated with PC supernatants. The IncuCyte ZOOM® 96-Well Scratch Wound Cell Migration data processing software module produces automated collection time-lapse images, measuring the spatial cell density in the wound area relative to the spatial cell density outside the wound area every three hours.

Statistical Analysis

RT² Profiler PCR data analyses were performed using web-based analysis software (https:// www.qiagen.com/fr/products/genes and pathways/data-analysis-center-overview-page/). Results were calculated using the $2^{-\Delta\Delta Ct}$ method and are expressed as a fold change. The results are presented as mean values ± SEM. A value is considered significant if $p<0.05$. Group comparisons were made using a one-way analysis of variance (ANOVA) followed by a Kruskal-Wallis test and a Dunn's post-test. The two-tailed t-test and Mann-Whitney test were used to compare two groups (GraphPad Software, La Jolla, California, USA).

Results

mRNA Expression Results of EA.hy926 Cells Stimulated by Platelet Component Supernatants

In this study, we used the Human Endothelial Cell Biology RT² Profiler™ PCR Array to examine the expression of 84 genes which related to the biology of ECs and the other 12 to

controls (Supplemental Table 1). The studied material consisted of stimulated EA.hy926 cells exposed to stimuli which were PC supernatants involved or not involved in SARs, collected at different timeframes during their storage. This PCR Array includes representative genes from various biological pathways: angiogenesis, vasoconstriction and vasodilation, inflammatory response, apoptosis, cell adhesion, and coagulation. EA.hy926 was stimulated with PC supernatants collected at Day [1-3[and Day [3-5[; supernatants collected on Day 0 of the PC processing were used as comparators. The significant differences observed in the EA.hy926 gene expression profiles are presented in Supplemental Figure 1. There was a 2-fold decrease in the expression for genes *ITGB3* and *IL-11* and a >2-fold upregulation for genes *PDGFRA*, *NPR1*, *IL-6* and *ICAM-1* when EA.hy926 cells were exposed to Day [4-5[vs Day 0 PC supernatants. When EA.hy926 cells were stimulated with PC supernatants involved in SARs, the expression of many genes (*ApoE*, *PDGFRA*, *NPPB*, *CX3CL1*, *IL-1β*, *NPR1*, *CCL5*, *IL-6*, *ICAM-1*, *CCL2*, *PTGS2* and *FGF-2*) was upregulated more than 2-fold compared to cell lines exposed to PC supernatants at Day 0.

Bioactivity of Control Platelet Component Supernatants Collected at Different Storage Times and Assessed on EA.hy926 Cells

PCs are usually stored for a maximum of five days under gentle rotation at 22°C (this is particularly the case in France). To evaluate the role of PC supernatants on endothelial barrier function during storage, PC supernatants with varying storage times were added to EA.hy926 cell monolayers; membrane expression markers of the assayed ECs were then measured. Three storage timeframes for the PC supernatants were evaluated: Day 0, Day [1-3[, and Day [3-5[; there were 50 different donor PCs per group. Membrane marker MFI significantly decreased on EA.hy926 cells treated with Day [1-3[and Day [3-5[PC supernatants compared with Day 0 supernatants for ICAM-1 (Fig. 1A), NPPB (Fig. 1B), ITGB3 (Fig. 1C) and NPR1 (Fig. 1H). Parallel findings were observed concerning the EA.hy926 cell soluble factor released after activation with PC supernatants of different storage timeframes; indeed, there was a decrease in sICAM-1 (Fig. 1E), ApoE (Fig. 1G), FGF-2 (Fig. 1K), IL-1β (Fig. 1L), IL-6 (Fig. 1M) and RANTES (Fig. 1P). In contrast, there was a significant increase in NPPB (Fig. 1F), IL-11 (Fig. 1N) and MCP-1 (Fig. 1O). There was no significant modulation of Cox-2 (Fig. 1I) and CX3CL1 (Fig. 1J) released by EA.hy926 cells after activation with PC supernatants collected at different storage timeframes.

Collectively, these data indicate that the longer PCs not involved in SARs are stored, the less they promote protein expression of EC lines *in vitro*.

Bioactivity of Platelet Component Supernatants Collected at Different Storage Times Having Led to SARs and Assessed on EA.hy926 Cells

PC supernatants collected at different storage timeframes and involved in SARs were added to the EA.hy926 cell monolayers and their effects on EC membrane marker expression were measured. PC supernatants with three different storage timeframes and involved in SARs were evaluated; there were 50 different donor PCs per group. MFIs for the markers ICAM-1 (Fig. 2A) and NPPB (Fig. 2B) were unaltered on ECs following exposure to PC supernatants involved in a SAR. There was a significant increase in PDGRFA MFI on ECs exposed to PC supernatants involved in SARs compared with the controls, however, PDGFRA MFI decreased as the storage duration increased when ECs were exposed to PC surpernatants both involved and not involved in SARs. (Fig. 2D). In contrast, there was a significant decrease in ITGB3 MFI (Fig. 2C) on ECs exposed to PC supernatants involved in SARs as the storage duration increased compared to the controls, but only with PC supernatants involved in SARs at Day [1-3[. Moreover, there was a significant decrease in NPR1 MFI (Fig. 2H) on ECs exposed to such PC supernatants involved in SARs compared to the controls. Those observations were made irrespective of the storage duration of the PCs having led to a SAR. The production of BRMs by EA.hy926 cells exposed to PC supernatants having led to a SAR was measured in parallel. There was an increase in sICAM-1 (Fig. 2E), FGF-2 (Fig. 1K) and IL-1β (Fig. 2L), and a decrease in NPPB (Fig. 2F), ApoE (Fig. 2G), Cox-2 (Fig. 2I), CX3CL1 (Fig. 2J), IL-6 (Fig. 2M), MCP-1 (Fig. 2O) and RANTES (Fig. 2P). Those observations were made irrespective of the storage duration of the PCs having led to a SAR, except for sICAM-1 and IL-6 where there was a different, but insignificant, secretion profile when ECs were exposed to PC supernatants involved in SARs at Day [1-3[and at Day [3-5[. Furthermore, there was an increase in IL-11 secreted by EA.hy926 cells exposed to supernatants involved in SARs and collected at Day [1-3[but a decrease at Day [3-5[(Fig. 2N). Besides the decrease in protein expression of EC lines *in vitro* with increased PC storage length, these data indicate that PCs involved in SARs alter protein expression, which may be increased or decreased depending on the marker.

Assessment of Apoptosis in EA.hy926 Cells Exposed to Platelet Component Supernatants

To further analyse the biological effect of stored PC supernatants involved in SARs, the caspase 3/7 (apoptosis) activity of EA.hy926 cells exposed to PC supernatants was measured. PC supernatants, irrespective of their storage length and whether or not involved in a SAR did not increase the caspase 3/7 activity of EA.hy926 cells *in vitro* (Fig. 3). Moreover, there was a decrease in caspase 3/7 activity with PC supernatants as storage duration increased and with PC supernatants involved in SARs. These data suggest that PC supernatants did not induce apoptosis.

Assessment of Platelet Component Supernatants on Endothelial Migration of EA.hy926 Cells

The effect of PC supernatants on EA.hy926 cell migration was then evaluated by means of an endothelial wound scratch assay. When PC supernatants were added to the culture medium, there was a significant decrease in platelet-dependent endothelial migration compared with the control (medium only) condition (Fig. 4). These data were irrespective of the PC storage duration and transfusion outcome (SAR or not). This indicates that the PC supernatants themselves inhibit the migration of endothelial cells and, potentially, re-endothelialization.

Discussion

This study provides experimental evidence that PC supernatants can alter the expression of EC genes (at least in EC-line conditions). Indeed, when ECs were stimulated with PC supernatants collected at different timeframes, i.e. storage length, four EC genes were upregulated and two other genes were downregulated when ECs were exposed to PC supernatants collected at Day [3-5[compared with Day 0. Differences were even more pronounced when PC supernatants were obtained from PCs having led to SARs in transfused patients, where twelve EC genes were upregulated, while the others remained unaltered. These PC supernatants further altered the expression of membrane proteins expressed by ECs and their secretion of soluble factors. The general trend was a decrease in EC activity paralleling longer PC storage for control PCs. When PCs led to SARs, there was both a decrease

and an increase in protein expression induced on ECs. ECs thus proved to be differentially activated depending on the PC supernatant's age and status, i.e. associated with a SAR or not. PC supernatants induced EC activation, but did not induce EC apoptosis.

Under homeostasis, ECs secrete antiplatelet factors, such as NO or PGI2, which prevent excessive adhesion and aggregation of platelets[15]. When the vascular endothelial wall is injured, platelets adhere to the injury site by interacting with subendothelial proteins and through their upregulated (platelet) integrins. However, even when the vascular endothelium is intact, activated platelets are capable of interacting with ECs by engaging the PSGL-1 (P-selectin glycoprotein ligand-1)/ P-selectin pair[16]. This mechanism is important to understanding the pathophysiology of inflammation, since platelets firmly adhered to the endothelium can increase inflammation locally. Indeed, it has been shown *in vivo* in mice that infusion of activated platelets induces the release of the contents of Weibel-Palade bodies in the mesenteric veins, resulting in the expression of CD62P by the endothelial cells. The expression of this integrin then promotes leukocyte rolling via the PSGL-1 receptor[17]. Moreover, platelet CD40L has been identified as a factor capable of modulating the endothelial phenotype. CD40L expressed on the surface of activated platelets has the ability to bind CD40 on endothelial cells. This would result in the release of pro-inflammatory cytokines/chemokines such as IL-6, IL-8, and the recruitment and extravasation of leukocytes to the inflammatory sites, i.e. the inflamed endothelium[17,18]. Similarly, the involvement of endothelial CD40 by platelet CD40L induces the expression of adhesion molecules, including E-selectin, VCAM-1 and ICAM-1 for attachment of neutrophils, monocytes, and lymphocytes to the endothelium[19]. In the same way, sCD40L secreted by platelets, in interacting with CD40 expressed on ECs, may induce an inflammatory phenotype on the ECs. Expression of ICAM-1 on cultured ECs after contact with activated platelets was confirmed by Gawaz *et al.*[18]. However, our study has shown that ICAM-1 expression was decreased when ECs were exposed to PC supernatants with longer storage times. In addition, ICAM-1 expression of ECs was increased in the presence of PC supernatants involved in SARs, particularly at Day 3-5. Unlike storage times, SARs seem to induce the expression of adhesion molecules.

Several platelet BRMs, like sCD40L, have been described as amplifying the inflammatory signal in the endothelium. Among them, IL-1β has been identified as the controlling factor in the activation of ECs depending on the platelet. Indeed, platelet IL-1β may initiate and regulate

the early phases of the endothelial inflammatory response[20], especially by releasing chemoattractant factors, such as IL-6, IL-8, and MCP-1[21]. Our results showed higher IL-1β concentrations when ECs were stimulated with PC supernatants involved in SARs at Day 3-5. However, this does not induce the release of IL-6 and MCP-1 by ECs. Indeed, a decrease in these markers was observed when ECs were treated with PC supernatants involved in SARs compared with the control. Platelets produce many BRMs during storage, which may be involved in SARs after PC transfusion. Such BRMs contained in PCs are capable of affecting EC functions and in particular of activating ECs. PCs are able to induce an inflammatory response in endothelial cells, especially when cells are already pre-activated[22], which can be the case in transfused patients.

Beyond its role in inflammation, platelet supernatants were observed to inhibit endothelial migration. Thus, platelet-derived BRMs seem to modulate endothelial migration and have an effect on vascular repair. It has been shown that platelet-derived CXCL14 prevents endothelial migration, which is consistent with our findings[23]. In contrast, platelets also secrete CXCL12 which increases neo-endothelization[24]. Witte *et al.* have shown that it is necessary to have higher concentrations of agonists to achieve the release of CXCL14 from platelets compared with CXCL12[23]. Those observations suggest that the secretion of platelet BRMs is regulated differentially and depends on stimuli. While platelets seem to modulate cell migration of endothelium, PC storage time and the association with SARs did not influence it. Nevertheless, it has been shown that platelets with different storage timeframes do not regulate vascular endothelial permeability in the same way. Indeed, fresh platelets have a protective effect on vascular endothelial permeability which is reduced as the platelet storage time lengthens[25]. These observations indicate that the regulation of vascular endothelial integrity and permeability decreases with platelet storage time, corresponding to storage lesions.

Communication between platelets and the endothelium can be done through platelet microparticles (PMPs), inducing a proinflammatory function[26]. PMPs comprise of many BRMs, such as IL-1β and sCD40L released under activation and capable of activating endothelial cells and promoting SARs[27]. Platelets are also capable of ensuring the transfer of genetic material to the endothelium through PMPs which contain microRNAs[28]. The inflammatory role of microparticles therefore includes their ability to participate in the interconnection with

endothelial cells. It is therefore highly possible that even unrecognized platelet function may impact on the inflammatory function of the endothelium.

To conclude, BRMs released from platelets and contained in PCs can promote the inflammatory status. Furthermore, platelet BRMs are able to regulate endothelial activation and migration at the site of vascular lesions. Platelets have the potential to modulate vascular permeability and integrity. Transfusion reactions depend on the blood products, but also on the patient and their pathology. Although several studies have sought to identify the factors that cause inflammatory reactions in transfused patients, the key compound responsible has not yet been discovered. Further investigations are needed to understand the extent to which PCs affect patients and their inflammatory status.

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Author contributions

FC, CS and OG made the study hypotheses and co-wrote the paper. FC and CS designed the protocol and trained personnel in the blood banks. CHK, CD, TB, CS, MAE and CAA collected samples, carried out the experiments and statistical analyses and reviewed the paper. SL and HHC participated in all steps within the process and reviewed the paper.

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Legends

Figure 1. Endothelial cell protein expression during PC storage.

EA.hy926 cells were stimulated with PC supernatants during storage at Day 0 (n=50), Day [1-3[(n=50), and Day [3-5[(n=50). Bioactivity in EA.hy926 endothelial cells of soluble factors and membrane receptor were measured during PC storage (Day 1-5). Day 5 represents the last day of storage for which PCs can be used for transfusion in our study. Membrane expression of ICAM-1 (A), NPPB (B), ITGB3 (C), PDGFRA (D) and NPR1 (H) was analysed after stimulation with PC supernatants. The release of ICAM-1 (E), NPPB (F), ApoE (G), Cox-2 (I), CX3CL1 (J), FGF-2 (K), IL-1β (L), IL-6 (M), IL-11 (N), MCP-1 (O) and RANTES (P) by endothelial cells was analysed after stimulation with PC supernatants. Data is expressed as a mean ± SEM. The difference was considered significant if *p<0.05; **p<0.01; ***p<0.001.

Figure 2. Endothelial cell protein expression according to the SAR.

EA.hy926 cells were stimulated with PC supernatants involved in SARs, Day [1-3[(n=50), Day [3-5[(n=50) and involved in SARs (n=26). Membrane expression of ICAM-1 (A), NPPB (B), ITGB3 (C), PDGFRA (D) and NPR1 (H) were analysed after stimulation with PC supernatants. The release of ICAM-1 (E), NPPB (F), ApoE (G), Cox-2 (I), CX3CL1 (J), FGF-2 (K), IL-1β (L), IL-6 (M), IL-11 (N), MCP-1 (O) and RANTES (P) by endothelial cells was analysed after stimulation with PC supernatants. Data is expressed as a mean ± SEM. The difference was considered significant if *p<0.05; **p<0.01; ***p<0.001.

Figure 3. Caspase 3/7 analyses of apoptosis induced by platelet supernatants in EA.hy926 cells.

EA.hy926 cells were treated with PC supernatants with different storage timeframes and involved or not involved in SARs. Caspase 3/7 activity was measured in EA.hy926 cells after 50 hours of stimulation with PC supernatants at Day 0 (n=50), Day [1-3[(n=50), Day [3-5[(n=50) and involved in SARs (n=26). Data is expressed as a mean ± SEM.

Figure 4. Cell migration of EA.hy926 cells stimulated with platelet supernatants.

Scratch wound healing was applied on EA.hy926 monolayers. Cells were treated with PC supernatants with different storage timeframes, Day 0 (n=50), Day [1-3[(n=50), Day [3-5[(n=50) and involved in SARs (n=26). Cell migration was measured with relative wound density (%). Data is expressed as a mean ± SEM. The difference between the cells stimulated with PC supernatants and unstimulated cells was considered significant if *p<0.05; **p<0.01; ***p<0.001.

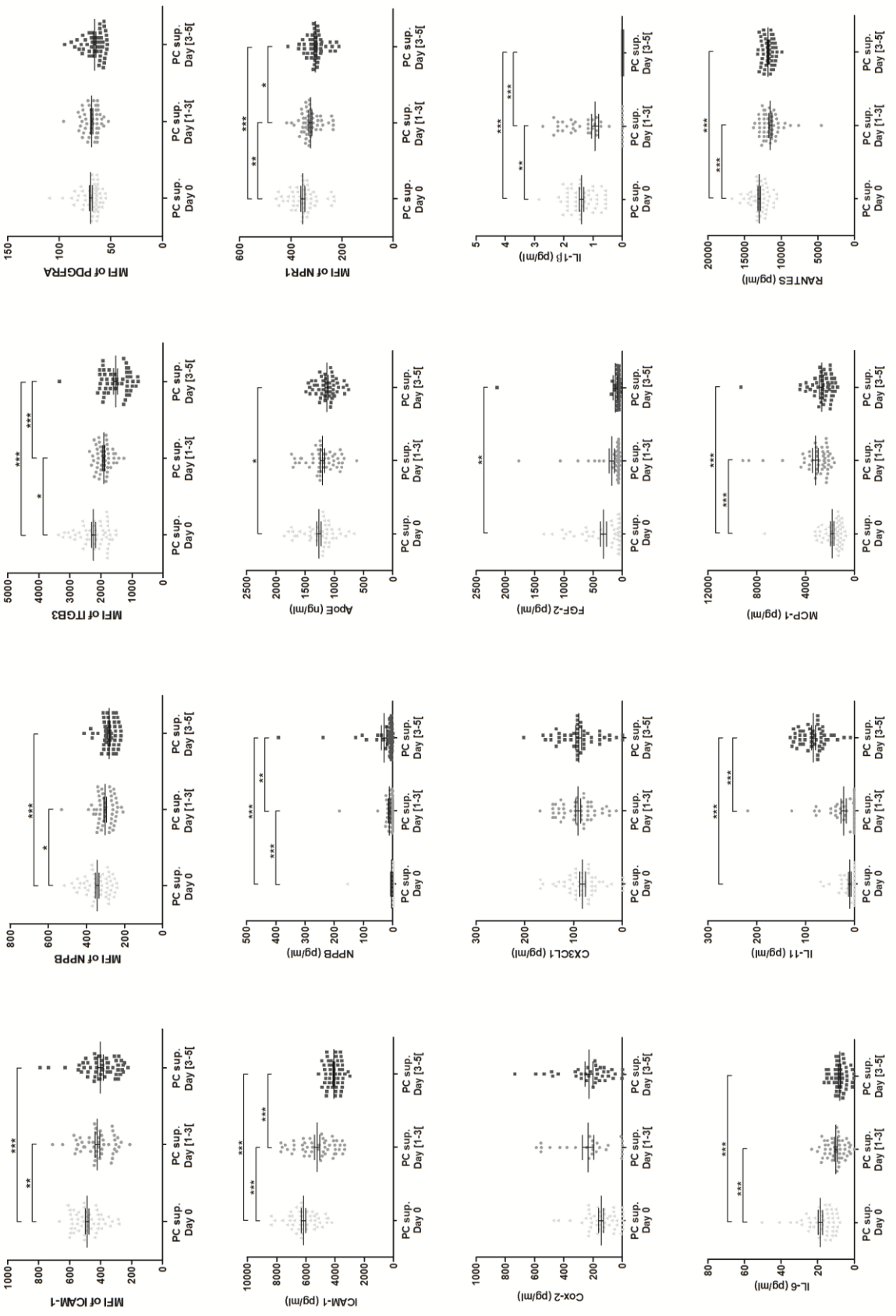

Figure 1. Endothelial cell protein expression during PC storage.

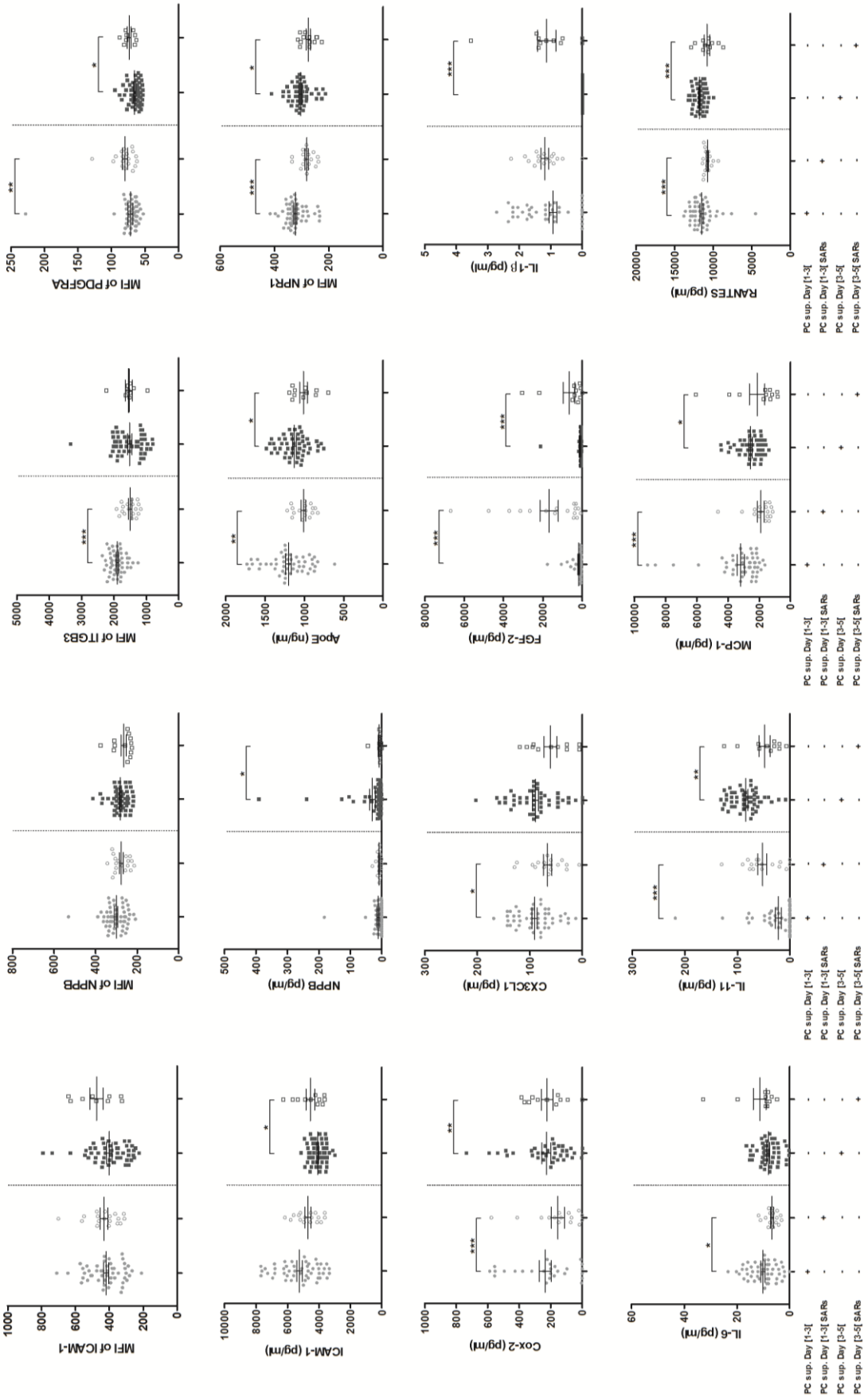

Figure 2. Endothelial cell protein expression according to the SAR.

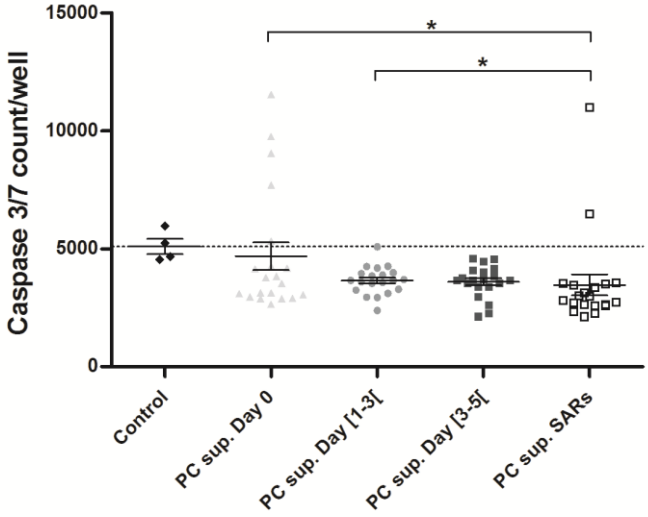

Figure 3. Caspase 3/7 analyses of apoptosis induced by platelet supernatants in EA.hy926 cells.

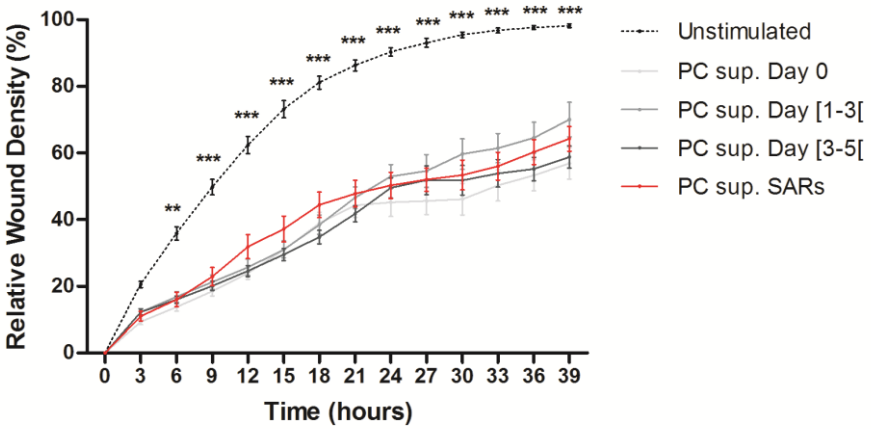

Figure 4. Cell migration of EA.hy926 cells stimulated with platelet supernatants.

Supplemental data

Gene table: RT² Profiler PCR Array Human Endothelial Cell Biology

Symbol	Description
ACE	Angiotensin I-converting enzyme (peptidyl-dipeptidase A) 1
ADAM17	ADAM metallopeptidase domain 17
AGT	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
AGTR1	Angiotensin II receptor, type 1
ALOX5	Arachidonate 5-lipoxygenase
ANGPT1	Angiopoietin 1
ANXA5	Annexin A5
APOE	Apolipoprotein E
BAX	BCL2-associated X protein
BCL2	B-cell CLL/lymphoma 2
BCL2L1	BCL2-like 1
CALCA	Calcitonin-related polypeptide alpha
CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta convertase)
CASP3	Caspase 3, apoptosis-related cysteine peptidase
CAV1	Caveolin 1, caveolae protein, 22kDa
CCL2	Chemokine (C-C motif) ligand 2
CCL5	Chemokine (C-C motif) ligand 5
CDH5	Cadherin 5, type 2 (vascular endothelium)
CFLAR	CASP8 and FADD-like apoptosis regulator
COL18A1	Collagen, type XVIII, alpha 1
CX3CL1	Chemokine (C-X3-C motif) ligand 1
EDN1	Endothelin 1
EDN2	Endothelin 2
EDNRA	Endothelin receptor type A
ENG	Endoglin
F2R	Coagulation factor II (thrombin) receptor
F3	Coagulation factor III (thromboplastin, tissue factor)
FAS	Fas (TNF receptor superfamily, member 6)
FASLG	Fas ligand (TNF superfamily, member 6)
FGF1	Fibroblast growth factor 1 (acidic)
FGF2	Fibroblast growth factor 2 (basic)
FLT1	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)
FN1	Fibronectin 1
HIF1A	Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix

	transcription factor)
HMOX1	Heme oxygenase (decycling) 1
ICAM1	Intercellular adhesion molecule 1
IL11	Interleukin 11
IL1B	Interleukin 1, beta
IL3	Interleukin 3 (colony-stimulating factor, multiple)
IL6	Interleukin 6 (interferon, beta 2)
IL7	Interleukin 7
ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)
ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)
ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
KDR	Kinase insert domain receptor (a type III receptor tyrosine kinase)
KIT	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homologue
KLK3	Kallikrein-related peptidase 3
MMP1	Matrix metallopeptidase 1 (interstitial collagenase)
MMP2	Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)
MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
NOS3	Nitric oxide synthase 3 (endothelial cell)
NPPB	Natriuretic peptide B
NPR1	Natriuretic peptide receptor A/guanylate cyclase A (atrial natriuretic peptide receptor A)
OCLN	Occludin
PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide
PECAM1	Platelet/endothelial cell adhesion molecule
PF4	Platelet factor 4
PGF	Placental growth factor
PLAT	Plasminogen activator, tissue
PLAU	Plasminogen activator, urokinase
PLG	Plasminogen
PROCR	Protein C receptor, endothelial
PTGIS	Prostaglandin I2 (prostacyclin) synthase
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
PTK2	PTK2 protein tyrosine kinase 2
SELE	Selectin E
SELL	Selectin L

SELPLG	Selectin P ligand
SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
SOD1	Superoxide dismutase 1, soluble
SPHK1	Sphingosine kinase 1
TEK	TEK tyrosine kinase, endothelial
TFPI	Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)
TGFB1	Transforming growth factor, beta 1
THBD	Thrombomodulin
THBS1	Thrombospondin 1
TIMP1	TIMP metallopeptidase inhibitor 1
TNF	Tumour necrosis factor
TNFSF10	Tumour necrosis factor (ligand) superfamily, member 10
TYMP	Thymidine phosphorylase
VCAM1	Vascular cell adhesion molecule 1
VEGFA	Vascular endothelial growth factor A
VWF	Von Willebrand factor
ACTB	Actin, beta
B2M	Beta-2 microglobulin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HPRT1	Hypoxanthine phosphoribosyltransferase 1
RPLP0	Ribosomal protein, large, P0
HGDC	Human Genomic DNA Contamination
RTC	Reverse Transcription Control
RTC	Reverse Transcription Control
RTC	Reverse Transcription Control
PPC	Positive PCR Control
PPC	Positive PCR Control
PPC	Positive PCR Control

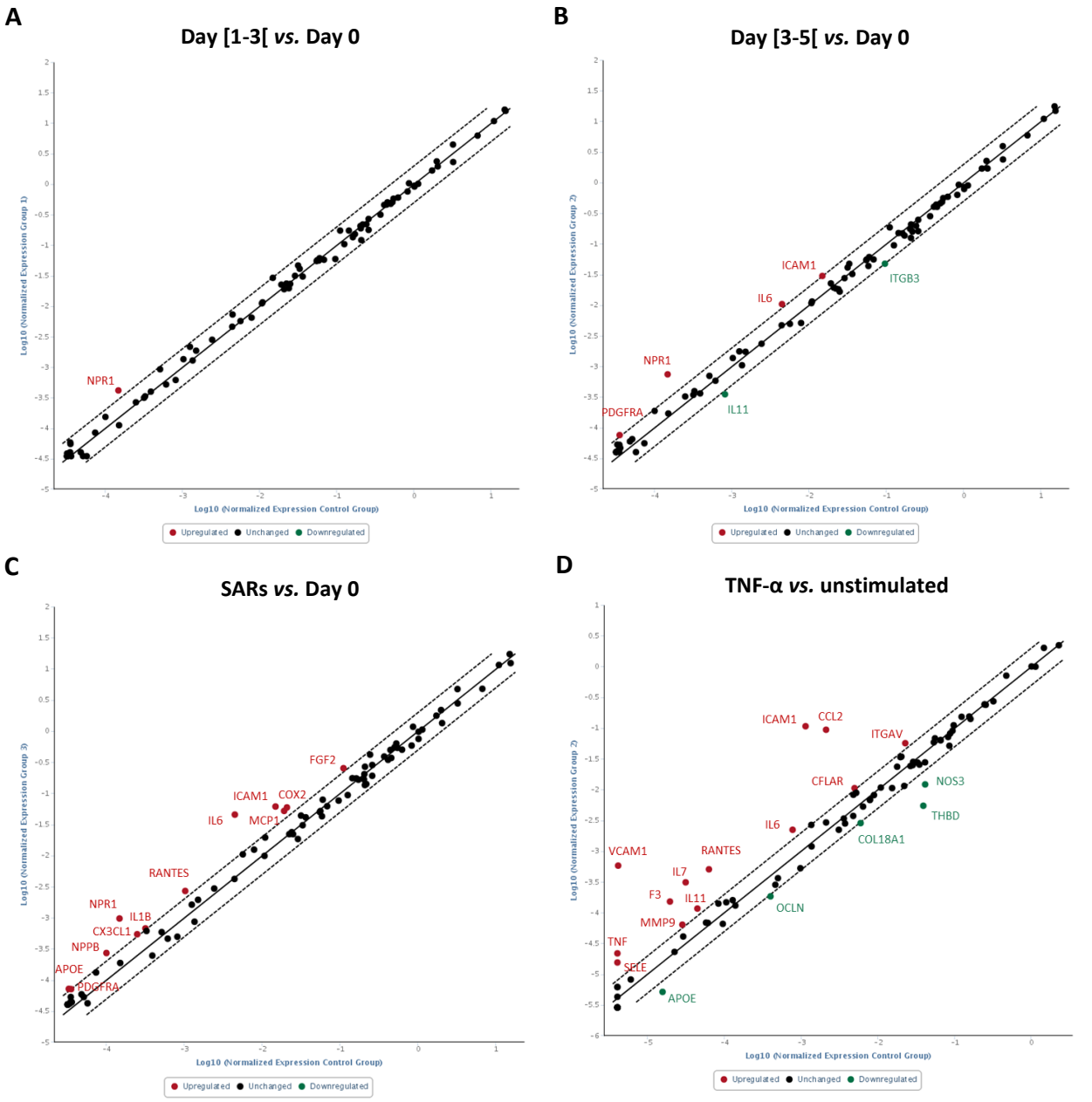

Figure 1. Expression comparison for 84 endothelial cell-related genes stimulated with platelet supernatants with varying storage times or involved in SARs.

The scatter plots compare the normalized expression of each gene using the Endothelial Cell Biology RT² Profiler PCR Array. EA.hy926 cells were stimulated with PC supernatants during storage: Day 0 (n=5), Day [1-3[(n=4), Day [3-5[(n=4), involved in SARS (n=5). Expression of endothelial cell genes stimulated with PC supernatants at delivery Day [1-3[(A), Day [3-5[(B) or involved in SARs (C) compared with PC supernatants on the processing day (D0). Expression of endothelial cell genes stimulated with TNF-α (D) compared with unstimulated cells. The analysis revealed significantly up-regulated genes (in red) and significantly down-regulated genes (in green) with a fold-change threshold of 2. Each gene is represented by a single point.

Discussion et perspectives

Les plaquettes sanguines ont un rôle clé dans le maintien de l'hémostase et interviennent également lors de lésions vasculaires [245]. Ces dernières années de nombreuses études ont montré que les plaquettes pouvaient être considérées comme des cellules immunitaires, ayant notamment un rôle inflammatoire important [21]. Nous avons vu précédemment que les plaquettes avaient la capacité de libérer un très grand nombre de médiateurs inflammatoires ayant un rôle immunomodulateur, appelés BRM. Outre la sécrétion des facteurs solubles, le rôle inflammatoire des plaquettes est également assuré par l'expression de nombreux récepteurs immunitaires, notamment des TLR [90], qui peuvent distinguer diverses pathogènes, activer des voies de signalisation spécifiques et la sécrétion différentielle du contenu plaquettaire [101]. Cela caractérise les plaquettes comme une cellule immunitaire complexe, étant capable de reconnaître les signaux de dangers et de participer à l'activation ainsi qu'à la régulation de la réponse inflammatoire.

Lors d'une transfusion sanguine, plusieurs types de complications peuvent survenir chez le receveur, appelées EIR [246-248]. Les réactions inflammatoires plus particulièrement retrouvées après transfusion plaquettaire, peuvent être de type RFNH, allergies, hypotensions ou TRALI [7, 249]. Il a été démontré que ces réactions inflammatoires peuvent être, en partie, provoquées par des niveaux élevés de BRM sécrétés par les plaquettes.

Les différentes données de la littérature consolident l'hypothèse que l'activation des plaquettes entretient une réponse inflammatoire. Ce travail de thèse vise tout d'abord à identifier les BRM qui sont susceptibles d'induire des EIR. Ensuite, nous avons évalué les caractéristiques des donneurs, les méthodes de préparation et les conditions de conservation des CP, afin de déterminer si ces différents éléments peuvent activer plus ou moins les plaquettes et donc entrainer une augmentation de la sécrétion de BRM. Puis, nous nous sommes intéressés aux potentiels inflammatoires de ces CP plaquettaires ayant induit un EIR ou non, sur l'endothélium vasculaire.

Les CP peuvent être impliqués dans la survenue de réactions post-transfusionnelles, en particulier de type inflammatoire. Ces réactions semblent incriminer la quantité importante de facteurs solubles qui sont sécrétés par les plaquettes, notamment lors du stockage des CP. Ces BRM contenus dans les CP transfusés, peuvent directement interagir avec les cellules du receveur. Plusieurs études ont montré que la survenue d'EIR est associée à la présence de taux

élevés de BRM dans ces CP [176, 200]. Toutefois, des taux élevés de BRM ne sont pas systématiquement corrélés à la survenue d'un EIR. Il est donc important de déterminer les BRM qui peuvent induire un EIR. Notre équipe a démontré les BRM tels que sOX40L, IL27 et sCD40L, s'accumulaient dans les surnageants de CP ayant induit des EIR inflammatoires [187]. De plus, une autre étude a permis de distinguer le type de réaction inflammatoire en fonction de la concentration de certains BRM, comprenant sCD40L, IL13 et MIP1α [196]. En effet, une augmentation significative des concentrations de sCD40L et IL13 est retrouvée dans les CP ayant entraîné un EIR en comparaison à des CP contrôles. De façon intéressante, le MIP1α semble capable de distinguer le type d'EIR. Aussi, la concentration de ce facteur permet de différencier un EIR de type RFNH d'un EIR allergique.

Nous avons souhaité poursuivre ce travail et en nous appuyant sur un nombre d'échantillons beaucoup plus important. Pour cela, nous avons collecté près de 9000 échantillons de CP. Cette puissance d'échantillonnage semble être de nature à permettre d'investiguer si les produits comprenant des concentrations théoriques en BRM se révèlent cliniquement pathogènes ou non, et vice-versa si des manifestations cliniques sont systématiquement en lien avec l'infusion de produits pathogènes. L'hypothèse sous-tendue par ce travail est celle d'un possible effet individuel (« receveur ») dans la pathogénicité, combiné avec un effet « produit ». Les surnageants de CP ont été recueillis le jour de la préparation de la poche ainsi que le jour de la délivrance, c'est-à-dire juste avant la transfusion. Au cours de cette même période, 140 EIR ont été reportés et collectés. Plusieurs études ont démontré l'implication du sCD40L lors de réactions post-transfusionnelles [187, 194, 196, 250]. Dans un premier temps, nous avons évalué le lien entre sCD40L et les EIR, afin de déterminer si des niveaux élevés de sCD40L sont associés de façon constante aux EIR. Ceci fait l'objet de la première étude [251]. L'objectif était de déterminer un seuil au-dessus duquel la concentration de sCD40L pouvait être associée à un EIR. Pour cela, un modèle mathématique a été utilisé permettant de minimiser l'entropie/l'incertitude afin de restaurer des données potentiellement altérées [252].

Tout d'abord, cette étude a confirmé que la durée de conservation des CP est associée à la sécrétion de sCD40L [150, 156, 188]. Ensuite, nous avons examiné plus en détail la relation entre la concentration de sCD40L dans les surnageants des CP associés aux 140 EIR signalés, par rapport aux témoins après divers temps de stockage. Les résultats sont particulièrement

suggestifs d'un phénomène de seuil «tout ou rien» déjà proposé dans l'étude précédente [187]. Ceci suggèrait l'existence d'un deuxième déclencheur, en plus des niveaux élevés de sCD40L. La causalité génétique n'est pas exclue et cette hypothèse de travail est investiguée par une autre équipe dans notre groupe de recherche. Cependant, dans une étude précédente, nous n'avons pu montrer une association entre les occurrences d'EIR et les variantes génétiques du gène CD40L [253]. En outre, la possibilité que d'autres gènes soient impliqués, tel que ceux qui régulent la physiopathologie du CD40 et du CD40L peuvent être envisagés [253, 254].

Cette étude a clairement montré que les niveaux de sCD40L ne sont pas pleinement prédictifs des EIR, mais laisse entrevoir la possibilité de comorbidité du receveur, la susceptibilité génétique ou une maladie causale. Une limite de cette étude concerne la déclaration des EIR, ces réactions peuvent parfois ne pas être perçues. De nombreux patients recevant des transfusions de CP sont traités avec des immunosuppresseurs ou parfois « prémédiqués » (paracétamol, antihistaminiques, glucocorticoïdes), qui peuvent masquer les symptômes [195].

Les moyens actuels de réduire le risque d'introduction du sCD40L en concentration élevée chez les patients transfusés consistent à utiliser des CP frais, c'est-à-dire inférieurs à 3 jours de stockage. Cependant, les patients sensibles peuvent néanmoins développer des EIR, même avec cette mesure. Afin de minimiser le risque d'EIR, il faudrait envisager de réduire la concentration du sCD40L et des BRM identifiés comme étant impliqués dans ces réactions, notamment accumulés lors des lésions de stockage. L'utilisation de plaquettes lavées, avec élimination totale du plasma, pourrait être envisagée, cependant cette procédure prend du temps supplémentaire, avec un surcoût et n'empêche pas les plaquettes de continuer à sécréter des BRM. De plus, c'est un traitement supplémentaire qui pourrait également entraîner un stress et une activation des plaquettes, ou encore altérer leur fonction (super-lésions de stockage). Un système de colonnes plus raisonnable sur le plan conceptuel pourrait être conçu, pouvant absorber non seulement le sCD40L mais aussi les BRM plaquettaires inflammatoires, tout en préservant la fonctionnalité et la qualité des PC (mais les coûts seront probablement très élevés). On note que lavages par colonnes ont été proposées par quelques cliniques transfusionnelles qui ont rapporté leur expérience [255, 256].

Les CP contiennent des BRM inflammatoires qui sont susceptibles d'entrainer des EIR. Outre l'identification des BRM induisant ces réactions, il semble important de déterminer les éléments qui peuvent entrainer la production et l'accumulation de ces produits inflammatoires dans les CP transfusés. Nous avons vu précédemment que le processus de collecte des PSL, les différentes méthodes de préparation de CP et également les conditions de stockage peuvent influencer la sécrétion de ces BRM. En effet, ceci soumet les plaquettes à de multiples stimulations ou stress, tels que les processus de préparation variables. Ces phénomènes peuvent conduire aux différentes propriétés inflammatoires des produits, en termes d'expression de récepteur et de sécrétion de BRM plaquettaires. L'évaluation des différentes méthodes de préparation de CP a fait l'objet de la deuxième étude.

Les CP peuvent être de deux types, soient des CPA obtenus par aphérèse à partir d'un donneur, soient des MCP obtenus à partir d'un mélange de Buffy-Coats de 5 dons de sang total. Dans cette étude, nous avons analysé l'état d'activation des plaquettes des deux méthodes de préparation au cours du stockage, au regard de deux marqueurs d'activation plaquettaire, le CD40L et le CD62P solubles. Après le processus de préparation, nous observons des concentrations significativement plus importantes dans les CPA comparés aux MCP. Ceci suggère une activation plaquettaire plus importante lors de la préparation des CPA. Les concentrations de sCD40L et de sCD62P augmentent significativement au cours du temps, ce qui confirme une nouvelle fois que les lésions de stockage induisent la sécrétion de BRM [156]. Cependant, pendant le stockage, l'augmentation est plus importante dans les MCP que dans les CPA, contrairement au jour de préparation. Bien que des différences puissent être démontrées entre les MPC et les CPA, les deux processus sont conformes aux normes transfusionnelles. Plusieurs études indiquent que les plaquettes provenant d'aphérèse et de sang total (Buffy-Coat) sont comparables en termes de qualité *in vitro* et cliniquement. Cependant, les avantages de l'un par rapport à l'autre sont parfois discutés [257, 258]. En effet, une étude française basée sur les données du rapport d'hémovigilance [6], a clairement montré la supériorité en termes de tolérance des MPC par rapport aux CPA [185].

Outre le type de préparation de CP, différentes solutions de conservation sont également utilisées. Dans notre cohorte, les CPA sont conservés soient dans une solution Intersol soit SSP+. La concentration de sCD40L est plus élevée dans les CPA stockés avec Intersol que les CPA stockés avec SSP+. Ces résultats montrent que les différentes solutions de

conservation peuvent influencer la sécrétion de BRM au cours du stockage, ce qui est cohérent avec les rapports précédents [183, 259-261]. Le stockage des plaquettes dans le PAS présente un certain nombre d'avantages, notamment une réduction des EIR graves [184]. Ceci est notamment dû à une concentration en plasma moins importante dans les CP. Étant donné qu'une faible teneur plasmatique en CP est requise pour certains processus d'inactivation des agents pathogènes, l'optimisation du PAS est devenue un sujet d'intérêt [174].

Bien que des différentes subsistent entre les plaquettes de sang total ou d'aphérèse, ou encore les solutions de conservation, tous les CP sont de qualités équivalentes et respectent les normes transfusionnelles. Cependant, ces résultats suggèrent une réponse plaquettaire différentielle en fonction des processus de préparation et des PAS. L'évaluation de l'impact des facteurs pouvant stimuler les plaquettes permettrait de réduire la propriété pro-inflammatoire de ces produits.

Une transfusion de PSL fait intervenir des éléments liés au donneur, au produit et au receveur. Les paramètres liés aux donneurs sont en particulier des variables phénotypiques polymorphes. Outre les caractéristiques génétiques, nous nous sommes intéressés si ces paramètres pouvant influencer l'activation plaquettaire, et donc entrainer la sécrétion de produits pro ou anti-inflammatoire.

Dans cette troisième étude, nous avons évalué des paramètres du donneur, comprenant le sexe et le groupe sanguin. Pour cela, seuls les CPA ont été analysés, les MCP étant obtenus à partir de 5 donneurs n'étant pas forcément du même sexe. Au regard des marqueurs d'activation sCD40L et sCD62P, aucune différence n'est observée. Le groupe sanguin et le sexe du donneur ne semblent donc pas influencer l'état d'activation des plaquettes. Cependant, les caractéristiques des donneurs peuvent influencer des lésions de stockage, en particulier des différences au regard du pH. Bontekoe *et al.* ont montré que les plaquettes des CP ayant un pH inférieur à 6,7, expriment plus de CD62P et de phosphatidylsérine, ainsi qu'un potentiel membranaire mitochondrial plus faible [262].

Outre les paramètres liés aux donneurs, nous avons également analysé les éléments liés aux produits, plus particulièrement à la collecte, l'heure du don ainsi que le type de machine d'aphérèse. Là encore, l'heure du don ne semble pas influencer une activation plaquettaire. Concernant le type d'aphérèse, plusieurs machines sont utilisées. Nous avons pu constater une

augmentation significative du sCD62P avec les aphérèses de type MCS comparées aux aphérèses de types Trima Accel. L'aphérèse est une méthode de préparation qui induit un stress aux plaquettes, qui peut être différent selon le type de machine. Noulsri *et al.* ont en effet montré une différence de sécrétion de BRM en fonction du processus de préparation, en particulier concernant les MP [168]. Malgré ces différences, tous respectent les normes de sécurité transfusionnelle.

L'évolution constante des techniques de collecte, de préparation, de conservation a permis de réduire considérablement les EIR après transfusion de plaquettes. Les principales réactions sont notamment dues aux propriétés inflammatoires qu'ont les plaquettes, en particulier de leur capacité à sécréter une pléthore de molécules ayant un rôle immunomodulateur. La déleucocytation systématique de tous les PSL en particulier, a réduit les EIR. Cela permet de diminuer nettement la concentration de la plupart des BRM induits par les leucocytes, tels que TNFα, IL6 et IL8 dans les CP [171], et donc réduire significativement l'incidence d'EIR lié à la composante inflammatoire des CP. Cependant les plaquettes sont aussi capables de sécréter des BRM et cette sécrétion augmente au cours du stockage des CP [156]. Ceci explique l'augmentation significative de la concentration de ces BRM, en particulier le sCD40L, notamment après 3 jours de stockage, ce qui entraîne une augmentation du pourcentage des EIR au cours du stockage. Les résultats que nous avons obtenus sont comparables à ceux d'autres études de différents pays [263]. Les différents traitements que subissent les plaquettes influencent également leur sécrétion. Effectivement, les traitements qui permettent l'inactivation des pathogènes agissent différemment. L'irradiation γ n'augmente pas la sécrétion plaquettaire des BRM contrairement à l'ultraviolet C [257].

De façon intéressante, les différentes études montrent que les plaquettes peuvent sécréter des BRM, mais elles ne libèrent pas la totalité de leur contenu lorsqu'elles sont activées. En effet, cette sécrétion est programmée et varie en fonction du stimulus et de la situation inflammatoire. La plupart de ces facteurs d'origine plaquettaire sont contenus dans les granules, notamment les granules α. La sécrétion plaquettaire est régulée notamment par des signaux de transduction intraplaquettaire. Au cours de la conservation des CP, la sécrétion semble dépendante des stimuli endogènes, comme les DAMP [85] ainsi que les lésions de stockage [175]. Effectivement, les plaquettes reconnaissent ces signaux par leurs différents récepteurs, tels que les TLR, ce qui déclenche la libération de BRM de façon différentielle en

fonction de l'engagement de ces récepteurs [101]. De plus, les produits sécrétés, sont aussi des stimuli pour les plaquettes en favorisant eux aussi la sécrétion plaquettaire, c'est le cas notamment du sCD40L. Il en est de même, concernant l'activation plaquettaire, elles répondent différemment en fonction des différents stresse qu'elles subissent, force de cisaillement [264], stress oxydatif [265], pathogènes [101], contacts avec les agonistes [266]. Nous supposons que l'engagement de chaque voie de signalisation est lié à un profil de sécrétion plaquettaire respectif. La composante inflammatoire des plaquettes est dépendante des signaux qu'elles reçoivent depuis l'environnement.

De plus, ces différentes études montrent que la composante inflammatoire des plaquettes dans les EIR est multifactorielle. Effectivement, nous avons pu observer une corrélation entre la concentration élevée de BRM dans les surnageants des CP ayant induit des EIR. Cependant, une concentration importante de ces BRM n'induit pas systématiquement des réactions chez le receveur. La survenue d'un EIR est due à une accumulation de plusieurs BRM et non à un seul. La composante inflammatoire d'un CP peut être également liée à des paramètres du donneur, mais également du receveur. La limite de ces études *in vitro* est due à l'évaluation individuelle du rôle de chaque facteur dans la réponse inflammatoire, alors qu'il faudrait évaluer l'ensemble des rôles de ces BRM.

En perspective de ces travaux, il faudrait poursuivre l'étude des différents processus de préparation et de stockage afin d'identifier les signaux de dangers susceptibles d'intervenir, capables de modifier la fonction plaquettaire et leur rôle inflammatoire. Ce travail permettrait de trouver les points clés afin d'optimiser les processus de préparation et de stockage des CP, en vue d'une transfusion.

De plus, identifier les BRM impliqués dans les EIR, et mettre en place un modèle mathématique permettant de définir des concentrations seuils de ces BRM, au-delà desquelles elles induisent une réaction, améliorait considérablement la sécurité transfusionnelle. Pour cela, le dosage des BRM de prédiction, reflétant la qualité des CP, serait réalisé avant transfusion afin d'éliminer les produits à fort risque d'induction d'EIR.

Il serait également important d'étudier dans quelle mesure, ces BRM affectent directement les patients transfusés. Ceci nécessite une étude plus approfondie d'une corrélation entre les BRM plaquettaires et les manifestations cliniques chez les receveurs.

L'idéal serait de pouvoir établir une médecine personnalisée, permettant de définir un PSL à transfuser en fonction de sa composition, et également en fonction de la pathologie du receveur.

Dans la seconde partie de ce travail de thèse, nous nous sommes intéressés aux rôles des BRM plaquettaires sur les cellules du receveur, et plus particulièrement à l'interaction des plaquettes et de leurs produits de sécrétion sur l'endothélium vasculaire. En effet, les plaquettes sécrètent de nombreux BRM capable d'interagir directement avec d'autres cellules, telles que les leucocytes et cellules endothéliales. A l'homéostasie, les cellules endothéliales sécrètent des facteurs antiplaquettaires, tels que NO ou PGI2, qui empêchent l'agrégation et l'adhésion plaquettaire [267]. Lorsque la paroi endothéliale vasculaire est lésée, les plaquettes adhèrent au site de la lésion (sur des protéines sous-endothéliales exposées) par leurs intégrines [268]. Cependant, les plaquettes activées sont également capables d'interagir avec les cellules endothéliales, même si l'endothélium vasculaire est intact, en impliquant le couple PSGL1/P sélectine, le PSGL1 étant exprimé par les plaquettes et la P sélectine exprimée par les cellules endothéliales [229]. Ensuite, les BRM sécrétés par les plaquettes, tels que sCD40L, interagissent avec le CD40 exprimé sur les cellules endothéliales, provoquant un phénotype inflammatoire, caractérisé par l'expression des récepteurs d'adhésion : E sélectine, P sélectine, ICAM1, VCAM1, et la libération de cytokines/chimiokines pro-inflammatoires, telles que CCL2, IL6, IL8 et le recrutement et l'extravasation de leucocytes au niveau de l'endothélium enflammé [222].

Le but de cette quatrième étude, est d'identifier les effets de ces BRM lorsqu'ils sont transfusés, et plus particulièrement comment ils affectent l'endothélium vasculaire. Pour cela, un modèle *in vitro* a été mis en place, qui étudie l'activation des cellules endothéliales et modélise la réponse de celles-là dans un contexte transfusionnel, en l'occurrence de transfusion de CP mais adaptable à d'autres produits sanguins labiles. La lignée endothéliale EA.hy926 a été cultivée et stimulée par les différents surnageants plaquettaires, ayant ou non induit un EIR.

Cette étude a permis d'identifier des modifications différentielles d'expression génique des cellules endothéliales de lignée exposées à différents types de surnageants plaquettaires. Ces derniers ont été étudiés par rapport à leur durée de stockage (de J0 à J5) et à leur

association (ou non) à un EIR. L'étude du signalosome, en utilisant la méthode RT² Profiler PCR Array a permis de mettre en évidence des changements d'expression en fonction du temps de stockage des CP. Plusieurs gènes tels que ICAM1, IL6, NPR1, NPPB et PDGFR3 sont surexprimés au fur et à mesure du temps de stockages des CP. En effet, la durée de stockage des CP peut induire une modification de l'expression de certains gènes impliqués dans l'inflammation des cellules endothéliales. Ces résultats sont en phase avec plusieurs autres études montrant, elles, des modifications protéiques. Nous mettons aussi en évidence la sécrétion accrue de facteurs immunomodulateurs en lien avec la durée de stockage des CP, c'est notamment le cas pour l'IL6 qui augmente entre J4 et J5 [156]. De nombreuses modifications ont également été observées pour les CP ayant entraîné un EIR. Plusieurs gènes tels que IL6, ICAM1, IL1β, RANTES sont surexprimés lorsque les CP ont été associés un EIR. IL6 et IL1β sont des cytokines fortement sécrétées lors de réactions inflammatoires [269]. RANTES est une chimiokine abondamment produite par les plaquettes, qui est décrite comme associée à des pathologies inflammatoires [20]. RANTES pourrait donc être impliqué dans les EIR.

Afin de compléter ces résultats, nous avons ensuite étudié l'expression membranaire et soluble de ces gènes plus ou moins exprimés en fonction des différents surnageants plaquettaires, c'est-à-dire au cours du stockage des CP et s'ils ont induit ou non un EIR. Au regard du temps de stockage des CP, nous avons observé une réponse différentielle des cellules EA.hy926, en particulier pour les facteurs solubles libérés. Cependant, nos données suggèrent une perte progressive de la capacité des plaquettes à réguler l'activation endothéliale. Lorsque les cellules endothéliales sont stimulées par les surnageant ayant induit un EIR, les plaquettes semblent influencer l'activation endothéliale. Les BRM contenus dans les surnageants plaquettaires impliqués dans les EIR semblent jouer un rôle important dans l'activation *in vitro* des cellules EA.hy926.

Ce projet doit être poursuivi afin de confirmer quels BRM plaquettaires conduisent à une potentielle activation des cellules endothéliales et de préciser les mécanismes par lesquels ils contribuent à une réaction inflammatoire. Plusieurs facteurs plaquettaires ont été décrits qui amplifient le signal inflammatoire dans l'endothélium. Parmi eux, IL1β a été identifié comme le facteur déterminant dans l'activation des cellules endothéliales dépendante des plaquettes. Le contact *in vitro* des plaquettes stimulées par la thrombine et des cellules endothéliales induit –dans la cellule endothéliale– la surexpression de molécules d'adhésion,

telles que ICAM1, la libération de facteurs chimioattractants, tels que IL6, IL8, MIPα et MCP1 [213]. Une pré-incubation des plaquettes avec un anticorps anti-IL1β inhibe cette réponse endothéliale, suggérant que cette molécule plaquettaire peut initier et réguler les phases précoces de la réponse inflammatoire endothéliale [232]. De plus, cette étude semble en phase avec une autre étude ayant montré qu'IL1β relargué par les plaquettes activées induit l'activation des cellules endothéliales dans des pathologies telles que le lupus érythémateux disséminé [232].

En plus de l'IL1β, le CD40L plaquettaire a été identifié comme un facteur capable de moduler le phénotype endothélial [270]. Le CD40L exprimé sur la surface des plaquettes activées à la capacité de lier le CD40 dans les cellules endothéliales. Cela se traduit par la libération d'IL8 et de MCP1 [271], deux agents chimioattractifs primaires pour les neutrophiles et les monocytes. De même, l'implication du CD40 endothélial par le CD40L plaquettaire induit l'expression de molécules d'adhésion, notamment E sélectine, VCAM1, et ICAM1, permettant également la fixation des neutrophiles, des monocytes et des lymphocytes aux endothéliums [235]. Slupsky *et al.* ont également rapporté que cette même liaison induit dans les cellules « Human Umbilical Vein Endothelial Cells » (HUVEC), l'expression du facteur tissulaire et une diminution de l'expression de la thrombomoduline montrant un rôle supplémentaire des plaquettes dans la réponse thrombo-inflammatoire [272]. La fixation des plaquettes activées aux cellules endothéliales initie également l'expression et la libération d'un autre type de molécule, les enzymes MMP, qui sont des protéines dégradant la matrice extracellulaire. Leur activité contribue grandement à la destruction et au remodelage des tissus affectés par les processus inflammatoires [237].

La communication entre les plaquettes et l'endothélium n'est pas nécessairement directe ou juxtacrine. Au-delà de leur activité pro-coagulante, les MPP ont une fonction pro-inflammatoire [273]. Les MPP contiennent également différents BRM, tel que IL1β, libérés lors de l'activation plaquettaire, capable d'activer les cellules endothéliales. Xie *et al.* ont montré que les MPP expriment une concentration importante de sCD40L, qui favorise les dommages d'un endothélium microvasculaire « Human Microvascular Endothelial Cell » (HMEC) induits par les neutrophiles, et peuvent être mis en cause dans le TRALI [274].

Outre les BRM, les plaquettes sont également capables d'assurer le transfert du matériel génétique à l'endothélium. En effet, il a été démontré que les plaquettes contiennent

une gamme abondante et diversifiée de microARN [275, 276]. De plus, ce matériel génétique peut être contenu dans les MMP [277]. Laffont *et al.* ont démontré que ces MPP peuvent être internalisés par les cellules endothéliales de la veine ombilicale humaine (HUVEC), conduisant à l'accumulation de microARN fonctionnels dérivés des plaquettes [139]. Dans cette même étude, il est également montré que les MPP agissent comme des transporteurs intercellulaires de complexes fonctionnels, qui peuvent exercer une régulation hétérotypique de l'expression des gènes dans les cellules endothéliales, et éventuellement d'autres cellules receveuses du système circulatoire.

Le transfert de matériel génétique par l'intermédiaire des microparticules est quelque chose de relativement nouveau. Le rôle inflammatoire des MPP inclut donc leur capacité à participer à l'interconnexion avec les cellules endothéliales. Il devient maintenant clair que les plaquettes peuvent avoir un impact sur la fonction inflammatoire de l'endothélium. A présent, il serait important de déterminer l'effet des MPP contenu dans les CP sur l'endothélium.

Peu d'études se sont intéressées au rôle inflammatoire des plaquettes sur l'endothélium dans un contexte transfusionnel. Urner *et al.* comparent pour la première fois la réponse inflammatoire dans les cellules endothéliales lors d'une exposition à des PSL [278]. Ils ont notamment montré que les CP semblent être plus puissants que les autres PSL en tant que déclencheurs de réaction inflammatoire dans le compartiment vasculaire. De plus, les teneurs élevées en lipides dans les PSL sont corrélées avec une réaction inflammatoire accentuée des cellules endothéliales. En effet, une autre étude, basée sur les globules rouges, montre que les lipides contenus dans les surnageants s'accumulent pendant le stockage, entrainant l'activation de l'endothélium ainsi qu'une prédisposition au TRALI [279].

Une équipe s'est plus particulièrement focalisée sur le rôle des CP et la perméabilité vasculaire. Une combinaison de tests *in vitro* et *in vivo* a montré que les CP à 1 jour de stockage ont un potentiel protecteur plus important que les CP conservés 5 jours concernant la perméabilité endothéliale vasculaire [280]. Ceci montre une variabilité entre les donneurs marqués au cours du stockage, suggérant que les plaquettes régulent la stabilité endothéliale vasculaire, en fonction de leur durée de conservation. Cette même équipe a montré *in vitro*, que les plaquettes stockées à 4°C présentent une plus grande capacité à inhiber la perméabilité de l'endothélium vasculaire que les plaquettes stockées à 22°C [281]. Cependant, ils ont constaté *in vivo*, que les avantages endothéliaux des plaquettes stockées à 4°C sont

diminués par rapport à celles stockées à 22°C, ce qui est probablement dû à l'élimination rapide des plaquettes à 4°C dans la circulation.

Outre la perméabilité vasculaire, les plaquettes via leurs BRM sécrétés ont un rôle important dans la réparation/régénération au niveau des sites de lésions vasculaires. Les plaquettes sont pourvoyeuses de molécules pro-inflammatoires, telles que CXCL14. Witte *et al*. ont montré que le CXCL14 dérivé des plaquettes inhibe la migration des cellules endothéliales, contrairement au CXCL12, et module donc l'angiogenèse en tant que chimiokine angiostatique [282]. Par conséquent, sur le site des lésions vasculaires, le CXCL14 dérivée des plaquettes activées pourrait favoriser l'inflammation vasculaire et empêcher la régénération endothéliale.

Une équipe s'est intéressée au rôle des CP sur l'endothélium, et plus particulièrement à la réponse des cellules endothéliales selon le processus de préparation. En effet, nous avons vu précédemment que les différentes procédures de préparation des CP pouvaient influencer la sécrétion de BRM et donc induire un état inflammatoire. Chen *et al.* ont montré que CP provenant d'aphérèse pouvait contenir des concentrations plus importantes de P sélectine [283]. De plus, l'augmentation de ce marqueur d'activation plaquettaire semble être due aux aphérèses répétées chez les donneurs. La procédure d'aphérèse connue pour pouvoir engendrer un stress aux plaquettes, influence la sécrétion de P sélectine dans les CP. Il reste à déterminer si cela à une conséquence et dans quelle mesure active l'endothélium.

La transfusion plaquettaire améliore l'hémostase. Cependant les différents composés contenus dans les CP peuvent être responsable de réactions inflammatoires chez les receveurs. Pendant la préparation et le stockage, la physiologie des plaquettes elles-mêmes peut être modifiée, ce qui peut entraîner une altération des fonctions *in vivo*, dans la production et la libération de médiateurs pro-inflammatoires ; ceux-ci peuvent aussi avoir des effets néfastes lorsque les plaquettes sont transfusées. Bien que de nombreuses études menées au cours des dernières années aient fait évoluer les techniques de préparation et de conservation permettant une diminution de la production de BRM, ces réactions subsistent, la fréquence étant tout de même moindre. Plusieurs études ont permis d'identifier certains facteurs pouvant être responsables de maladies inflammatoires induites par les produits sanguins chez les patients. Cependant, ces réactions sont multifactorielles et ne dépendent pas uniquement d'un facteur. Il est donc important de poursuivre ces travaux en identifiant les mécanismes

provoquant un état inflammatoire chez les patients, notamment via l'interaction des plaquettes avec les cellules du receveur, telles que celles de l'endothélium vasculaire.

D'une manière générale, l'ensemble des travaux présentés dans cette thèse montre que les plaquettes sont capables de recouvrir la majorité des étapes de l'inflammation et confirme ainsi leur implication, à part entière, dans cet état physiopathologique.

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Annexes

I - Travaux annexes

Au cours de cette thèse, nous nous sommes également intéressés aux globules rouges, et notamment l'impact de la durée de stockage sur la production de marqueurs inflammatoires. Ceci est présenté dans la revue suivante publiée dans *Blood Transfusion* [284].

Une étude portant sur les propriétés des composants de globules rouges provenant de patients atteints d'hémochromatose héréditaire a fait l'objet d'une publication dans le journal *Transfusion* [285]. Les globules rouges contiennent de grandes quantités de fer. La phlébotomie thérapeutique périodique est donc le traitement principal de l'hémochromatose héréditaire. Cependant, le don de produits issus du prélèvement (saignée) thérapeutique de ces patients asymptomatiques à des fins de transfusion reste controversé. Dans cette étude, nous avons comparé la qualité des concentrés de globules rouges (CGR) obtenus chez ces patients avec ceux de donneurs sains, dans la période de stockage autorisée de 42 jours. Le potentiel inflammatoire de ces CGR sur l'endothélium a été évalué à partir du modèle *in vitro* mis en place. Cette étude suggère que les globules rouges de patients hémochromatose héréditaire, tout en présentant des différences subtiles, conviennent à la transfusion selon les normes actuelles transfusionnelles, mais qui restent néanmoins à réévaluer pour un certain nombre de transfuseurs et de cliniciens.

REVIEW

Duration of red blood cell storage and inflammatory marker generation

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Abstract

Red blood cell (RBC) transfusion is a life-saving treatment for several pathologies. RBCs for transfusion are stored refrigerated in a preservative solution, which extends their shelf-life for up to 42 days. During storage, the RBCs endure abundant physicochemical changes, named RBC storage lesions, which affect the overall quality standard, the functional integrity and *in vivo* survival of the transfused RBCs. Some of the changes occurring in the early stages of the storage period (for approximately two weeks) are reversible but become irreversible later on as the storage is extended. In this review, we aim to decipher the duration of RBC storage and inflammatory marker generation. This phenomenon is included as one of the causes of transfusion-related immunomodulation (TRIM), an emerging concept developed to potentially elucidate numerous clinical observations that suggest that RBC transfusion is associated with increased inflammatory events or effects with clinical consequence.

Keywords: red blood cell, inflammation, storage.

Introduction

An emerging transfusion community interest concerns the ability for blood transfusion to modulate the immune system of recipients[1-5]. Transfusion-related immunomodulation (TRIM) has been implicated in adverse clinical outcomes[6,7]. The present "gold standard" for maximum shelf-life of red blood cells (RBCs) is six weeks (42 days)[8]. Even if storage of blood at 4 °C is proposed to slow down RBC metabolism and the accumulation of soluble factors, amongst other main pointers of quality and safety of stored blood, it does not stop the overall process often referred to as storage lesions. Storage lesions have been extensively researched[9-11]. Classically, storage lesions in RBCs are categorised as either biochemical or rheology changes. However, inflammatory markers are poorly evaluated in the literature. Even if the rise in inflammatory markers observed in transfusion-related immunomodulation[12] may be improved through leucocyte reduction, several immunomodulatory factors stored in RBCs participate in inflammation[13-16]. The potential of other modes of processing for creating storage lesions, e.g. through degradation of nucleic acids to enhance pathogen safety of red cell concentrates, deserves to be fully established. Therefore, a goal of the current review is to summarise the biochemical or rheology changes occurring in relation to the duration and processing of RBC storage, focusing on the generation of inflammatory markers.

Biomechanical changes in stored RBCs

During the typical storage conditions of blood, abundant biochemical alterations take place[17-21]. Such changes primarily refer to the generation of aggregates and biochemical debris that accumulate in the supernatant during prolonged storage of RBCs (Figure 1). Biomechanical storage lesions occur in the cytoskeleton and cellular membranes, defined as membrane and cytoskeleton protein oxidation, membrane phospholipid loss, abnormal rearrangement of membrane phospholipids, and morphological changes[22,23]. As an example, increased storage induces an increased level of extracellular potassium, lactate, and a decrease of sodium and glucose which leads to acidosis, particularly obvious by the end of the second week of storage (approximately after day [d]14). Extended RBC storage is also identified, resulting in reduced levels of ATP and 2,3-diphosphoglycerate (2,3-DPG) (Figure 1). Taken together, the above events are useful markers that could be indicative of a storage lesion in a given stored unit of blood[17,18,24-28]. Moreover, stored RBCs reveal functional changes to RBCs during storage, and particularly reduced deformability and increased rigidity, which may affect the flow of transfused RBCs through micro-capillaries, cell-to-cell aggregation, and adhesion to endothelial cells. The decreased levels of 2,3-DPG

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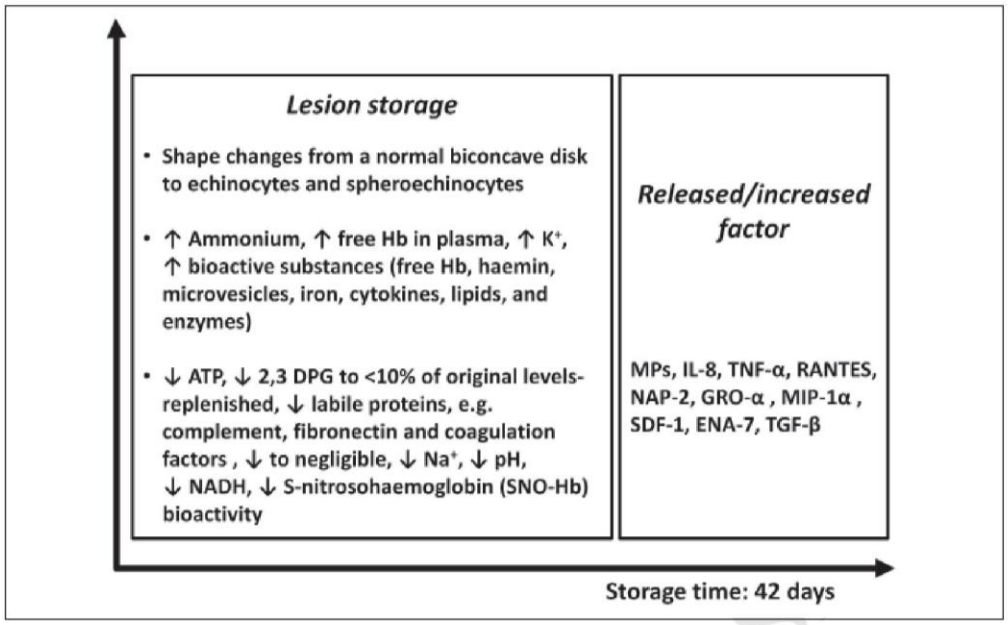

Figure 1 - Red blood cell product storage: lesion storage and biological response modifier release. Hb: haemoglobin; ATP: adenosine triphosphate; 2,3-DPG: 2,3-diphosphoglycerate; NADH: nicotinamide adenine dinucleotide; MPs: microparticles; IL-8: interleukine 8; TNF: tumour necrosis factor; RANTES: regulated on activation, normal T cell expressed and secreted; NAP: neutrophil-activating peptide; GRO: growth-related oncogene; MIP: macrophage inflammatory proteins; SDF: stromal cell-derived factor; ENA-7: epithelial neutrophil-activating protein 7; TGF: transforming growth factor.

increase the affinity of haemoglobin to oxygen, which results in reduced oxygen delivery[26,29-32]. D'Alessandro *et al.* performed comprehensive metabolomics and quantitative tracing metabolic experiments that revealed that mature RBCs can metabolise substrates other than glucose, such as citrate. This observation was highly relevant to Transfusion Medicine, influencing particularly the process of RBC preparation and the formulation of novel additives[33,34].

Red blood cell-derived lipids during storage

During RBC storage, the implications of RBC membrane breakdown and release of potentially harmful bioactive lipids could be quantified, and contributed to the quality assessment of RBC[18,22,26,28,35,36]. The damaging oxidative storage effects on the RBC lipid membrane have numerous functional implications. As an example, increasing oxidative stress on stored RBC is a determining primer to increase phosphatidylserine translocation to the RBC surface membrane[13,37,38]. This phenomenon could mediate adhesion of transfused RBC to endothelial cells and induce the shedding and accumulation of bioactive microvesicles (Mvs)[26,35,39,40]. These bioactive lipids have been implicated in transfusion-related acute lung injury (TRALI) pathogenesis due, mainly, to their[26] polymorphonuclear neutrophil (PMN) priming abilities. In 2011, Silliman *et al.* explored the effect of infusing d1 or d42 lipids that were isolated from healthy human donor into lipopolysaccharide (LPS) or saline-treated male rats[41]. The study evaluated the PMN-priming capacity of the lipids as well as the effect of their infusion on acute lung injury as part of the "two-hit" TRALI model[41-44].

Red blood cell-derived microvesicles during storage

One implication of RBC membrane failure could be the release of potentially injurious bioactive microvesicles[26,35,39,40]. Phospholipids of the membrane are released during the microvesiculation process, which was first defined by Rumsby *et al.* in 1977[45]. Microvesiculation is a cellular process that leads to intracellular communication and cell apoptosis. Membrane lipid oxidation and cytoskeletal protein oxidation can dislocate the plasma membrane and cytoskeleton[10,22,46-48]. This disturbance could be a key phenomenon contributing to the increased release, during the RBC storage, and accumulation of bioactive microvesicles. Recently, several studies investigating the composition of the RBC membrane, particularly during microvesiculation, revealed a significant increase of RBC-derived microparticles as storage exceeded day 42[22,40,49-53]. Moreover, these RBC membrane-derived microvesicles present a significant

physiological and inflammatory pathophysiological process, principally involving vascular dysfunction. However, there is little clinical and *in vivo* evidence linking the effects of microvesicles during transfusion. As defined and summarised elegantly by Michel Prudent *et al.*[54], analysis of *in vitro* data highlights the presence of reversible and irreversible storage lesions demonstrating that RBCs exhibit two limits during storage: one around two weeks and another one around four weeks of storage. Microvesiculations could be considered irreversible storage lesions as degradation/oxidation of proteins, protein aggregations, protein activation, such as the proteasome 20S, shape change and deformability[54].

Reisz *et al.*[55] hypothesised that routine storage of erythrocyte concentrates promotes metabolic modulation of stored RBCs by targeting functional thiol residues of GAPDH and identified ex vivo functionally relevant reversible and irreversible (sulfinic acid; Cys to dehydroalanine) oxidations of GAPDH without exogenous supplementation of excess pro-oxidant compounds in clinically relevant blood products. Palia *et al.* propose that 8 compounds (lactic acid, nicotinamide, 5-oxoproline, xanthine, hypoxanthine, glucose, malic acid, and adenine) strongly correlate with the metabolic age of packed RBCs, and can be prospectively validated as biomarkers of the RBC metabolic lesion[56]. In the same way, Wither *et al.* show that several of the oxidised residues identified play well-established roles in haeme iron co-ordination, 2,3-diphosphoglycerate binding, and nitric oxide homeostasis[57].

Recently, Straat *et al.* hypothesised that extracellular vesicles in RBC products during storage contribute to a pro-inflammatory host response in recipients, which is related both to their amount as well as to the storage duration[58]. The authors clearly demonstrate that incubation of whole blood with both fresh and stored supernatant containing extracellular vesicles induced a strong host response with production of tumour necrosis factor (TNF), interleukin(IL)-6 and IL-8. Moreover, once supernatant was depleted from extracellular vesicles, this host response was completely abolished.

Immunomodulatoty factors in stored red blood cell concentrates

The accumulation of immunomodulatoty factors in stored RBC concentrates has been implicated as a potential cause of transfusion reactions associated with the use of such products[8,9,25,26,29,32,59-61]. Data suggest high concentrations of TNF, IL-1 and IL-6 in random donor RBC concentrates[62-64], and an association between the period of storage of blood components and the risk of developing acute transfusion reactions to platelet concentrates (PCs) and RBCs[3,22,53,65,66].

Cytokine could orchestrate a systemic inflammatory response. Kristiansson *et al.* report that plasma-soluble immunomodulatory factor concentration increases on the first post-operative day after major surgical trauma, the Author observing a relationship between the amount of RBC concentrates transfused perioperatively and post-operative systemic plasma IL-6 concentration[67]. The interaction between these cytokines is complex, each being able to up-regulate and down-regulate their own expression as well as that of the other cytokines. Nevertheless, the cytokine content may reflect the presence of leucocytes, in other words, an association with the initial amounts of leucocytes in RBC concentrates. Leucoreduction may significantly decrease febrile non-haemolytic transfusion reactions and may decrease cardiopulmonary transfusion reactions (TRALI and transfusion-associated circulatory overload)[3,8,41,68,69]. Presumably, this ensues through reduced levels of bioactive lipids and soluble CD40L in leucoreduced RBCs, which would have been produced by leucocytes, had they not been removed from the blood product. Donor leucocytes release cytokines and lipid factors able to activate neutrophils in a time-dependent course during RBC storage[70]. Pre-storage leucoreduction decreases the release of metabolites and cellular components into the RBC product. TNF-α, a multipotent cytokine, perfoms several immunological functions and is involved in maintaining the homeostasis of the immune system. It is known, that TNF-α, like IL-1 and IL-6, suppresses erythropoiesis by direct inhibitory effects on bone marrow RBC production[71,72]. Moreover, TNF-α, being an endogenous pyrogen, is able to induce fever, apoptotic cell death, cachexia, inflammation and to inhibit tumourigenesis and viral replication, and respond to sepsis *via* IL-1 and IL-6 producing cells. IL-6 is an important mediator of fever and of the acute phase response. It is capable of crossing the blood-brain barrier and initiating synthesis of prostaglandin E2 (PGE2) in the hypothalamus, thereby changing the body's temperature set point. In muscle and fatty tissue, IL-6 stimulates energy mobilisation that leads to increased body temperature[73,74].

Therefore, Muylle *et al.* demonstrated a relationship between TNF-α and IL-6 levels and febrile transfusion reactions[75]. IL-8, also known as neutrophil chemotactic factor, has two primary functions. It induces chemotaxis in target cells, primarily neutrophils, but also other granulocytes, causing them to migrate toward the site of infection. IL-8 is also known to be a potent promoter of angiogenesis[76,77]. Cases of TRALI have been consistently associated with high levels of cytokines/chemokines, specifically IL-8[78], which has been shown to promote assembly of cholesterol-enriched microdomains or so-called lipid rafts on human neutrophils[79]. Moreover,

McKenzie *et al.*, proposed that antibodies bind monocytes (instead of neutrophils), leading to increased IL-8, which results in neutrophilic pulmonary infiltrate with subsequent TRALI[80]. IL-1 is intensely produced by tissue macrophages, monocytes, fibroblasts, and dendritic cells, but is also expressed by B lymphocytes, natural killer (NK) cells and epithelial cells[81]. They form an important part of the inflammatory response of the body against infection. These cytokines increase the expression of adhesion factors on endothelial cells to enable transmigration (also called diapedesis) of immunocompetent cells, such as phagocytes, lymphocytes and others, to sites of infection[82,83]. They also affect the activity of the hypothalamus, the thermoregulatory centre, which leads to fever. IL-1, appears to be associated with the occurrence of febrile non-haemolytic transfusion reaction (FNHTR) and other transfusion reactions, such as urticaria, hypotension, anaphylaxis, or TRALI[62,84,85]. The main critical factors in determining the accumulation of cytokines are considered to be the WBC content and the age of the blood component; moreover, accumulation is heterogeneous and there is a large inter-individual variation related to donors' hereditary and social habits[86]. Interestingly, the cytokines/chemokines in RBCs might be caused by haemolysis of the cells. This could be a comparable phenomenon to that detected in haemolytic transfusion reactions *in vitro* where there are high concentrations of cytokines/chemokines[87,88].

In the majority of the cases, antibodies against HLAs and/or human neutrophil antigen (HNA) present in the transfused product are thought to be responsible for initiating TRALI. TRALI is assumed to result from two hits, the first hit being caused by the underlying clinical condition of the patient, whereas the second occurs when the antibodies or factors are transferred to the recipient during transfusion[89]. Peters *et al.* investigated 18 healthy male volunteers (aged 18-35 years) infused with LPS to induce systemic inflammatory response syndrome. Two hours later, each participant received either one unit of 2-day stored autologous RBCs, 35-day stored autologous RBCs, or an equal volume of saline. Every 2 hours up to 8 hours after LPS infusion, haemoglobin, haemolysis parameters, and iron parameters, including non-transferrin bound iron (NTBI), were measured. The author concluded that 35-day stored autologous RBCs do not cause haemolysis or increased levels of NTBI during human endotoxemia[90,91]. Production of cytokines/chemokines could originate from an activation of RBC contact with the storage bag system during the storage period, indicating that these storage lesions should also be considered for future evaluations. Foreign material may stimulate cytokine synthesis and release, though this may be less likely during storage at a temperature of 4 °C.

Recently[13], our group focused on the characterisation of stored RBC with regard to cytokine/chemokine content, and investigated the possible influence of storage time (Figure 1). Individual RBC concentrate (RBCC) supernatants were processed by double centrifugation at 2,600 *g* for ten minutes. Samples were kept frozen at −80 °C and shipped on dry ice to the sample-processing laboratory. Levels of soluble cytokines growth-related oncogene (GRO)-α, IL-16, epithelial-derived neutrophil-activating protein 78 (ENA-78), macrophage inflammatory protein 1α (MIP-1α), monocyte chemoattractant protein-1 (MCP-1), stromal cell-derived factor 1 (SDF-1) and transforming growth factor (TGF) β1, 2, and 3 were measured in triplicate from aliquots using Luminex technology[92], and amounts were expressed in ng/RBC unit. Supernatants from RBCCs were collected over time and tested for the presence of a variety of soluble chemokines and cytokines. GRO-α, IL-16, ENA-78, MIP-1α, MCP-1, SDF-1 and TGF β1, 2, and 3 were selected on the basis of previous reports[25,59,93]. There were no differences in ENA, GROα, MIP1α, MCP1, SDF1, IL-16 or TGF β3, either between the groups or over time[13]. However, TGF β1 and TGF β2 decreased over time in both RBCC groups, with a significant difference at d0 *vs* d42[13]. The biological activities of TGF-β are not species-specific. TGF-β isotypes share many biological activities and their actions on cells tend to be qualitatively similar, though there are a few examples of distinct activities. In some systems, TGF-β3 appears to be more active than the other isotypes. TGF-β2 is the only variant that does not inhibit the growth of endothelial cells. TGF-β2 and TGF-β3, but not TGF-β1, inhibit the survival of cultured embryonic chick ciliary ganglionic neurons. TGF-β is the most potent known growth inhibitor of normal and transformed epithelial cells, endothelial cells, fibroblasts, neuronal cells, lymphoid cells and other haematopoietic cell types, hepatocytes, and keratinocytes. Although TGF-β inhibits endothelial cell growth, it promotes angiogenesis in several bioassays, though TGF-β may also inhibit angiogenesis under certain circumstances[94,95]. At higher concentrations, TGF-β stimulates the growth of these cells. TGF-β has mainly suppressive effects on the immune system by inhibiting the IL-2 dependent proliferation of T cells and B lymphocytes. TGF-β inhibits the proliferation of B lymphocytes and thymocytes induced by IL-2 and IL-1, respectively, and inhibits the maturation of B cells[96]. It also suppresses interferon-induced cytotoxic activity of NK cells, cytotoxic T-lymphocyte activity, and the proliferation of lymphokine-activated killer cell precursors. TGF-β also inhibits the synthesis of immunoglobulin (Ig)G and IgM by B lymphocytes and

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stimulates the synthesis of IgA. TGF-β1 is the most potent known chemoattractant for neutrophils[97,98].

In this same report[13], we performed a functional assay of RBCC supernatant on EA.hy926 endothelial cells. The human endothelial hybrid cell line EA.hy926 was obtained by fusion of primary umbilical vein endothelial cells with the human lung carcinoma cell line A459/8 (ATCC #CRL-2922). EA.hy926 cells were cultured in Dulbecco's modified MEM medium supplemented with 10% foetal calf serum and 1% penicillin-streptomycin and then incubated at 37 °C in a humidified atmosphere in 5% CO_2 until the cell monolayer reached confluence. The cells were then exposed to stored RBCC supernatants. IL-6, sCD141 and sCD62E levels were measured by enzyme-linked immunosorbent assay. In this *in vitro* model, we investigated the bioactivity of soluble immunomodulatory factors in endothelial cells *in vitro*. We tested the potential bioactivities of the soluble immunomodulatory factors from RBCs over time using EA.hy926. There was a difference in the expression of marker molecules generally associated with EA.hy926 cell activation (CD141) during storage (d1-d42) and similar results were observed for the expression of soluble markers generally associated with EA.hy926 cell activation (sCD141, sCD62E and IL-6). This result, revealing the bioactivities of soluble immunomodulatory factors in the supernatant of RBCCs on endothelial cells *in vitro*, suggests a potential generation of inflammatory markers during RBC storage. Further investigation could be carried out to determine the nature of these inflammatory markers.

Conclusions and perspectives

The increase of inflammatory soluble markers observed in transfusion-related immunomodulation[12,99] is reduced by leucocyte reduction[60,63,100-103]. However, stored RBCs deliver large quantities of iron to the monocyte/macrophage system and could thus induce inflammation, and transfusion of older, stored RBCs, therefore, produces a proinflammatory response associated with increased iron levels in the liver, spleen, and kidney, and increased circulating levels of non-transferrin bound iron[3,104].

However, it is currently unclear whether the storage lesions simply reflect an accelerated ageing of the RBCs, or something else, and the consequences *in vivo* (after transfusion) remain largely unknown. In addition, RBC preparation and storage processes (cryopreserved for extended periods of time, cryoprotectant, plastic bag, etc.) could be investigated to quantify the RBC inflammatory soluble markers observed in transfusion-related immunomodulation. In this context, we note that several current concepts of intervention (reduction of biological response modifiers) focus on methods to attenuate the cytokine response[105-108]. Another characteristic could be considered concerning the transfusion-related immunomodulation, as for platelet component[2,109-112]. The variability in cytokine/chemokine concentration in RBCs could reveal a biological variation in donors with regard to their capacity to synthesise and release mediators. Moreover, differences in measured cytokine/chemokine concentrations associated with various commercial immunoassay kits should be considered and standardised in the future.

The clinical implications of transfusing RBCs containing cytokines/chemokines to critically-ill patients have not been clarified[113]. It might be that the cytokine content of transfused RBCs may fuel the systemic inflammatory reaction in conditions of trauma and infection, and simulate non-haemolytic transfusion reactions. Investigations have confirmed that stored RBC transfusions seem to up-regulate proinflammatory gene expression in the leucocytes of the transfusion recipient[114]. Moreover, McFaul *et al.*[115] observed an *in vivo* inflammatory effect of transfusion with an increasingly proinflammatory RBC function of storage.

Numerous studies have evaluated a wide variety of photosensitisers and alkylating agents as candidates for a pathogen inactivation process of RBC suspensions, but few with a focus on the inflammatory role of RBC. Consequently, future questions could probably investigate:

i) how this blood component differs from classical RBC components in use;
ii) what are the benefits to the patients of possible pathogen inactivation processes to be used for RBC suspensions;
iii) whether there a reduction in acute transfusion reactions in patients receiving future pathogen-reduced RBC (febrile non-haemolytic and/or allergic transfusion reactions, TRALI).

Future animal and clinical studies could probably increase knowledge of the effect of RBC storage on post-transfusion outcomes and TRALI, with a specific focus on the inflammatory soluble markers observed in TRIM. Moreover, knowledge of TRIM could help answer questions concerning a possible difference between fresh and old blood, and, more interestingly, the medical effects of transfusing stored RBCs. As elegantly defined in animal models by James C. Zimring[16], the question now is to understand the "induction of cytokine storm" on RBCs during storage, and the potential promotion of acute transfusion reactions.

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Properties of donated red blood cell components from patients with hereditary hemochromatosis

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BACKGROUND: Red blood cells (RBCs) contain large amounts of iron, and periodic therapeutic phlebotomy is thus the main treatment for hereditary hemochromatosis (HH). However, the donation of therapeutic phlebotomy products from asymptomatic patients for transfusion purposes remains controversial. In this study, we compared the quality of RBCs obtained from HH patients with those of non-HH RBCs, within the allowed 42-day storage period.

STUDY DESIGN AND METHODS: RBCs were obtained from HH patient donors and random regular blood donors by whole blood collection. RBCs were stored for up to 42 days, according to national regulations and standard blood bank conditions in France. The following variables were assessed: hematologic and biochemical results, RBC membrane and soluble inflammatory markers, and the proinflammatory potential of HH RBC supernatant toward endothelial cells in an in vitro model.

RESULTS: There were no major differences between the two groups in terms of biophysical, biochemical, or soluble immunomodulatory factors. However, we observed small but significant differences in changes in RBC membrane proteins during storage, including increased phosphatidylserine expression and decreased hemolysis in HH compared with normal RBCs. However, there were no differences in terms of bioactivity of soluble immunomodulatory factors in the RBC supernatant during storage between HH and control donors, as determined by their effects on endothelial cells in vitro.

CONCLUSIONS: These in vitro studies suggest that RBCs from HH patients appear, while exhibiting subtle differences, to be suitable for transfusion purposes according to currently accepted criteria.

Hereditary hemochromatosis (HH) is a genetic disorder of iron metabolism characterized by increased iron absorption and storage, resulting in progressive and multisystemic oxidative organ damage.[1,2] The human hemochromatosis gene *HFE*

ABBREVIATIONS: ENA-78 = epithelial-derived neutrophil-activating protein 78; HH = hereditary hemochromatosis; MCP-1 = monocyte chemoattractant protein-1; MIP-1α = macrophage inflammatory protein 1α; SDF-1 = stromal cell–derived factor 1; TfR = transferrin receptor; WB = whole blood.

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has been identified on Chromosome 6,[3] and the missense mutation C282Y has been characterized as the main mutation responsible for hemochromatosis.[3] Homozygosity for the C282Y mutation has been identified in approximately 1 in 200 to 250 people of northern European and Celtic descent, but the phenotypic expression of the mutation is highly variable, as are the clinical presentation and extent of the disease.[4,5] C282Y homozygotes account for more than 80% of patients with HH.[6] A second mutation, H63D, has also been described in HH patients.[7] Although this mutation does not affect cell surface protein expression, its ability to interfere with the HFE–transferrin receptor (TfR) interaction may explain its relation to iron overload.[8] HH is caused by a mutation in a gene that controls the amount of iron your body absorbs from the food you eat. Nowadays, four main genes are implicated in the pathophysiology of clinical syndromes classified as non-HFE hemochromatosis: hemojuvelin (HJV, Type 2A juvenile HH), hepcidin (HAMP, Type 2B juvenile HH), TfR2 (Type 3 HH), and ferroportin (SLC40A1, Type 4 HH).[9]

Red blood cells (RBCs) contain large amounts of iron, and periodic therapeutic phlebotomy is thus the main treatment for HH.[10,11] In light of the occasional shortage of blood donation products and the possibility of switching from whole blood (WB) donations to apheresis donations, especially plasma apheresis, it has been suggested that therapeutic phlebotomy products from patients with mild or moderate disorders and no visceral damage may be donated, under specified conditions and the physician's advice, and with the donor's agreement.[12-14] The ethical aspects of this issue have been debated with the various stakeholders, and the practice has now largely been accepted in many countries where HH is prevalent within populations. Eligibility criteria have been established by the French regulatory authorities (Arrêté du 12 janvier 2009 fixant les critères de sélection des donneurs de sang—NOR: SJSP0901086A) and RBCs from eligible, voluntary, HH patients are now commonly accepted as part of the nonremunerated blood donation system. However, there are no specific procedures for the labeling, processing, handling, storage, and distribution or delivery of HH RBCs, which thus enter the regular RBC pool. The only differences between HH and regular blood donations in France relate to the likely higher frequency of HH donations, and donation initiated in a day care hospital and followed at a fixed site, with no donations from HH donors at mobile collection sites.

However, recent studies have suggested that iron, especially free iron, is toxic, and largely responsible for RBC transfusion–mediated inflammation.[15] This has led to reconsideration of the safety of HH blood donations for transfusion with regard to possible inflammatory outcomes, despite their compliance with quality control (QC) requisites and current regulations.[14]

The aim of the current study was to investigate the quality of RBCs obtained from HH patients. We compared target variables in HH and regular RBCs, stored for up to 42 days. The study variables were the results of extensive hematologic and biochemical analyses performed until the last day of storage (Day 42), RBC membrane and soluble inflammatory markers, and the proinflammatory potential of HH RBC supernatant in relation to endothelial cells in an in vitro model. The global objective of this study was to confirm the suitability of HH RBCs for general donation.

MATERIALS AND METHODS

Patient samples

WB donations were accepted from eligible donors according to national regulations. Donors were divided into two groups: regular blood donors without HH (six men, four women; mean ± SD age, 45.60 ± 4.59 years) and genetic HH donors (eight men, four women; mean ± SD age, 54.17 ± 4.05 years). All donors agreed to give blood for nonmedical purposes and gave their signed consent. No other specific ethical committee agreement was required as French legislation permits such activities (Arrêté du 12 janvier 2009 fixant les critères de sélection des donneurs de sang—NOR: SJSP0901086A). The absence of organ failure was confirmed by medical examination, and laboratory tests were performed to measure iron and ferritin stores before determining eligibility for the WB donation program. Details of the blood donors are given in Table 1. Because the therapeutic phlebotomy products from the HH patients meet the criteria for donation, these individuals are referred to as HH donors, rather than patients, in this article.

RBC preparation

Approximately 45-mL WB units were collected from regular and eligible HH donors using the same equipment (R8481, Fenwal, Inc.) with CPD. WB units were processed by leukofiltration at room temperature, centrifugation at 5000 × *g* for 10 minutes, and manual separation into plasma and RBC units containing 100 mL of SAGM. RBCs were stored upright at 4°C for 42 days in bis(2-ethylhexyl)phthalate–plasticized PVC storage containers (PL146 for WB, PL1813 for Alyx, Fenwal, Inc.), with weekly mixing and sampling through to Day 42. RBCs were mixed thoroughly on each sampling day and sampled using a sterile syringe. All samples were analyzed on the day of sampling, except for supernatants and RBC extracts, which were kept frozen at −80°C until further analysis.

Cell count, hematocrit, mean cell volume, hemoglobin, pH, and O_2/CO_2 partial pressure

Automated cell counts and mean cell volume (MCV) were measured using a differential cell counter (KX-21N,

TABLE 1. Characteristics of regular blood donors and hemochromatosis patients with HFE gene mutation

	Sex	Age (years)	ABO group	Rhesus	Kell	HIV	HTLV	SYPH	PALU	HBC	AgHBS	HCV	CMV	MCHC	WBCs	RBCs	Hb	Hct %	PLTs	MCH	MCV	Serum iron (μmol/L)	Transferrin (g/L)	Ferritin (μg/L)	Transferrin saturation	Therapeutic phlebotomy
Characteristics of blood donors																										
	F	52	B	+	–	–	–	–	–	–	–	–	–	33.40	7.59	5.04	14.60	43.70	248.00	29	86.70					
	M	70	O	+	–	–	–	–	–	–	–	–	–	32.50	4.71	5.17	13.80	42.50	216.00	26.70	82.20					
	F	51	A	–	–	–	–	–	–	–	–	–	–	34.00	5.12	4.31	13.30	39.10	239.00	30.90	90.70					
	M	44	O	+	–	–	–	–	–	–	–	–	–	34.00	5.72	4.89	15.00	44.10	221.00	30.70	90.20					
	F	42	O	+	–	–	–	–	–	–	–	–	–	33.70	7.21	4.66	13.40	39.80	241.00	28.80	85.40					
	F	28	A	+	–	–	–	–	–	–	–	–	–	34.10	4.39	4.24	13.30	39.00	237.00	31.40	92.00					
	M	21	O	+	–	–	–	–	–	–	–	–	–	33.80	6.38	5.20	16.40	48.50	290.00	31.50	93.30					
	M	46	O	+	–	–	–	–	–	–	–	–	–	34.10	6.98	5.19	15.40	45.20	233.00	29.70	87.10					
	M	40	B	+	–	–	–	–	–	–	–	–	–	34.00	6.89	5.26	15.50	45.60	211.00	29.50	86.70					
	M	62	B	+	+	–	–	–	–	–	–	–	–	34.00	5.49	5.10	15.10	44.40	220.00	29.60	87.10					
Mean ± SEM	F/M: 4/6	45.60 ± 4.59												33.76 ± 0.16	6.05 ± 0.35	4.91 ± 0.12	14.58 ± 0.34	43.19 ± 0.98	235.60 ± 7.16	29.78 ± 0.46	88.14 ± 1.06					
Characteristics of hemochromatosis patients																										
Homozygous for C282Y	F	56	A	–	–	–	–	–	–	–	–	–	–	34.5	5.08	4.1	12.7	36.8	165	31	89.8	21.1	2.03	98	41	32
Homozygous for C281Y	F	41	A	+	–	–	–	–	–	–	–	–	–	33.7	7.66	4.65	13.6	40.3	255	29.2	86.7	ND	ND	294	ND	10
Homozygous for C282Y	M	61	O	+	–	–	–	–	–	–	–	–	–	35	8.01	4.85	15.7	44.8	288	32.4	92.4	ND	ND	132	ND	47
Dysmetabolic hyper-ferritinemia + homozygous for H63D	M	57	O	+	–	–	–	–	–	–	–	–	+	35.2	6.73	4.67	15.4	43.7	187	33	93.6	25.9	2.48	1206	42	15
Dysmetabolic hyper-ferritinemia + homozygous for C282Y	M	66	O	–	–	–	–	–	–	–	–	–	–	35.1	6.85	4.81	16.2	46.1	128	33.7	95.8	14.5	2.91	123	19.9	20
Homozygous for C281Y	F	49	A	–	–	–	–	–	–	–	–	–	+	35	8.35	4.49	14.6	41.7	247	32.5	92.9	15.8	2.29	53.9	ND	8
Homozygous for C281Y	M	33	A	+	–	–	–	–	–	–	–	–	–	36.3	5.75	5.37	17.2	47.4	208	32	88.3	41	1.85	259.1	93	20
Homozygous for C282Y	M	52	A	+	–	–	–	–	–	–	–	–	ND	33.4	5.01	4.38	14.2	42.5	177	32.4	97	ND	ND	94.1	ND	87
Heterozygous for C282Y/H63D	F	78	A	+	–	–	–	–	–	–	–	–	ND	32.2	5.98	4.25	13.7	42.5	247	32.2	100	ND	ND	137	ND	12
Homozygous for C282Y	M	31	O	+	–	–	–	–	–	–	–	–	ND	34.3	6.94	4.5	14	40.8	207	31.1	90.7	27.9	1.97	312.5	56.6	16
Heterozygous for C282Y/H63D	M	58	O	+	–	–	–	–	–	–	–	–	ND	34.4	6.27	4.88	14.6	42.5	316	29.9	87.1	ND	ND	77	ND	8
Homozygous for C282Y	M	68	O	+	–	–	–	–	–	–	–	–	ND	33.1	5.85	5.84	16.7	50.5	194	28.6	86.5	ND	ND	97.6	ND	26
Mean ± SEM	F/M: 4/8	54.17 ± 4.05												34.35 ± 0.32	6.54 ± 0.31	4.73 ± 0.14	14.88 ± 0.39	43.30 ± 1.03	218.30 ± 15.55	31.50 ± 0.45	91.73 ± 1.26	24.37 ± 3.97	2.25 ± 0.16	240.4 ± 91.31	50.5 ± 13.6	25.08 ± 6.50

Sysmex America, Inc.). Spun hematocrits (Hcts) were tested using a microcentrifuge (IEC Micro-MB Centrifuge, Thermo Scientific). Residual white blood cell (WBC) counts were performed by flow cytometry (LeukoCOUNT kit, FACScan flow cytometer, Becton Dickinson). Hemoglobin (Hb) as a surrogate measure of hemolysis was measured using a hematology instrument (ABX Pentra XL80, HORIBA ABX SAS). pH was measured at 37°C using an automated gas analyzer (Nova Bio Profile pHOx, Nova Biomedical Corp.). O_2/CO_2 partial pressure in WB samples was measured using a blood gas analyzer (ABL Radiometer).

Glucose, phosphorus, adenosine triphosphate, 2,3-diphosphoglycerate, iron, and ferritin

RBC supernatants from random regular donors and eligible HH donors were processed by double centrifugation at 2600 × *g* for 10 minutes (Sorvall RT Legend, Thermo Fisher Scientific). Glucose and phosphorus were measured using a standard chemical analyzer (Olympus AU400e, Olympus America). RBC extracts were prepared by combining 1 mL of RBCs with 3 mL of 8% trichloroacetic acid followed by centrifugation and filtration (Surety Column, Evergreen). RBC extracts were neutralized with 3 mol/L K_2CO_3 and analyzed for adenosine triphosphate (ATP) using the Beutler NADP+ reduction method. 2,3-Diphosphoglycerate (2,3-DPG) was measured using a commercially available kit (Roche Diagnostics). ATP and 2,3-DPG were both finally measured using a manual spectrophotometer (Lambda 40, Perkin-Elmer). Iron and ferritin concentrations were measured using a clinical chemistry analyzer (Cobas 6000, Roche Diagnostics).

RBC flow cytometry assays

RBCs from both groups (non-HH, 10; HH, 12) were processed individually for flow cytometry analysis. Labeling was performed with fluorochrome-conjugated monoclonal antibodies (MoAbs) against CD44 (Clone G44-26), CD71 (Clone M-A712), CD36 (Clone CB38), CD58 (Clone 1C3), CD47 (Clone B6H12), CD147 (Clone HIM6), CD108 (Clone KS-2), and annexin V and phosphatidylserine expression (BD Biosciences). All clusters of differentiation are expressed on cells involved in the inflammatory process.[16-18] Background labeling was assessed in all experiments using a fluorochrome-conjugated mouse immunoglobulin (Ig)G isotype-negative control (BD Biosciences). Positive events were recorded by flow cytometry and analyzed using its accompanying software (FACSCalibur and CellQuest Pro, respectively, BD Biosciences). Analyzed variables were expressed as percentage of cells expressing the markers and mean fluorescence intensity (MFI) of RBC membrane proteins throughout storage.

Cytokine assays

Individual RBC supernatants from each group were processed by double centrifugation at 2600 × *g* for 10 minutes (Sorvall RT Legend, Thermo Fisher Scientific). Samples were kept frozen at −80°C and shipped on dry ice to the sample-processing laboratory (GIMAP-EA3064, Université de Lyon). Levels of soluble cytokines GRO-α; interleukin (IL)-16; epithelial-derived neutrophil-activating protein 78 (ENA-78); macrophage inflammatory protein 1α (MIP-1α); monocyte chemoattractant protein-1 (MCP-1); stromal cell–derived factor 1 (SDF-1); and transforming growth factor (TGF)-β1, -2, and -3 were measured in triplicate from aliquots using Luminex technology.[19] Amounts are expressed in ng/RBC unit. All cytokines and soluble factors are involved in the inflammatory process.[20-22] The levels of TfR and hepcidin were measured by enzyme-linked immunosorbent assay (ELISA) obtained commercially (R&D Systems Europe Ltd.) according to the manufacturer's instructions. The content of transferrin was assayed using prequote plates (Enzo), according to the instructions of the manufacturer. Absorbance at 450 nm was determined with an ELISA reader (Magellan Software Sunrise, Tecan Group Ltd.).

In vitro functional testing of RBC supernatant in EA.hy926 endothelial cells

Immortalized endothelial cell lines are very often used as a model of endothelium for studies of various processes connected with its functions. The human endothelial hybrid cell line EA.hy926 was obtained by fusion of primary umbilical vein endothelial cells with the human lung carcinoma cell line A459/8 (ATCC #CRL-2922); it is presently the best characterized macrovascular endothelial cell line, indeed, as Baranska and colleagues[23] showed significant similarity of endothelial cell lines with primary HUVECs, although they also pointed out marked phenotype differences. ICAM-1, VCAM-1 and E-selectin, basically exhibit similar expression pattern in both EA.hy926 cells and HUVECs.[24] EA.hy926 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin and then incubated at 37°C in a humidified atmosphere in 5% CO_2, until the cell monolayer reached confluence. The cells were then exposed to RBC supernatants from each group, or to tumor necrosis factor (TNF) α (100 pg/mL as a positive control or neutral medium as a negative control).[25]

EA.hy926 cells were analyzed by flow cytometry for surface marker expression under various simulation conditions. Labeling was performed with fluorochrome-conjugated MoAbs against CD54 (Clone HA58), CD31 (Clone WM59), and CD141 (Clone 1A4; BD Biosciences). Background labeling in all experiments was assessed using a fluorochrome-conjugated mouse IgG isotype-negative control (BD Biosciences). Positive events were

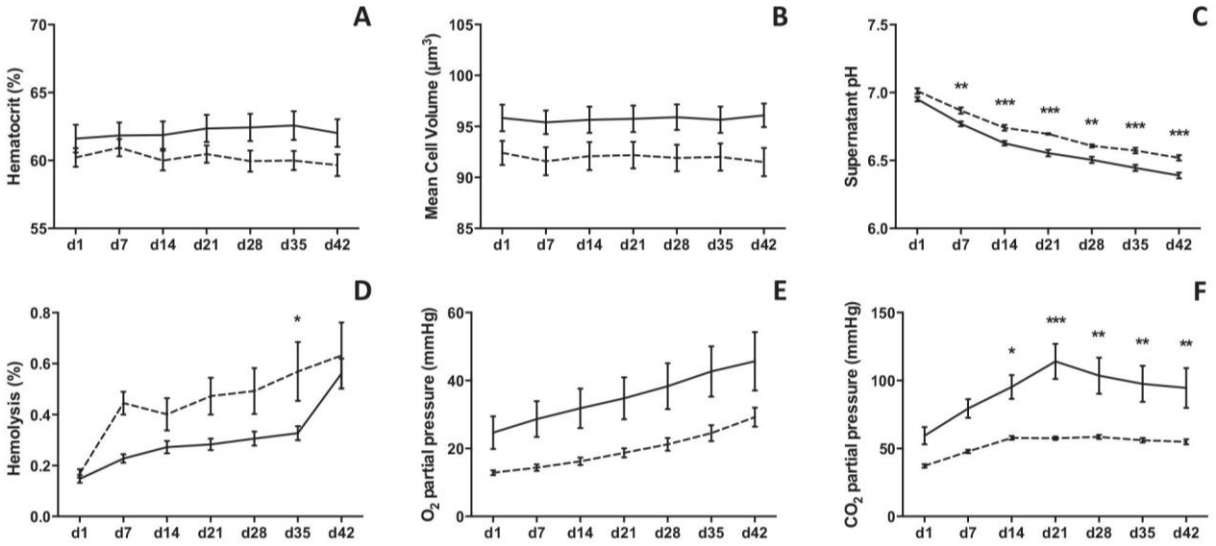

Fig. 1. Hct (A), MCV (B), pH (C), hemolysis (D), pO_2 (E), and pCO_2 (F) in regular (control) donors (- - -, n = 10) and eligible patients with HH (—, n = 12) during RBC storage (Days 1-42). Data are expressed as mean ± SEM. The difference was considered significant for $p < 0.05$: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

recorded with a flow cytometer and analyzed using the accompanying software (FACSCalibur and CellQ Pro, respectively, BD Biosciences). IL-6, sCD141, and sCD62E levels were measured by ELISA according to the manufacturer's instructions (R&D Systems Europe Ltd).

Statistical analysis

Determination of means, standard error of the mean (SEM), and all statistical analyses were carried out using computer software (GraphPad Prism 5, GraphPad). Two-way analysis of variance with Bonferroni posttest was used to evaluate any significant effect of the processing method on RBC variables on each day of storage. The difference was considered significant for p values of less than 0.05 (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

RESULTS

Biophysical and biochemical variables of RBCs

There was no significant difference in Hct and MCV between the two groups at any time up to Day 42 (Figs. 1A and 1B). Although the pH on Day 1 was similar in regular and HH RBCs (7.01 ± 0.02 vs. 6.95 ± 0.02, respectively; Fig. 1C), it decreased gradually over time to approximately 6.52 ± 0.02 and 6.4 ± 0.02, respectively, after 42 days of storage (Fig. 1C). There was a slightly significant difference between the two groups starting on Day 7, with HH RBCs having a lower pH than regular RBCs, although the pH in both groups was within the acceptable range of QC.[26] Hemolysis was significantly less pronounced in HH RBCs compared with regular RBCs on Day 35 (Fig. 1D). This was always within the limits of the national QC program, which mandates that there should be no more than 0.80% hemolysis on Day 42, in 80% of the products (randomly checked for normal RBCs and 100% checked for HH RBCs), according to the Council of Europe guidelines. There was no difference between the two groups in terms of pO_2 levels, which increased during storage (Fig. 1E), but pCO_2 levels were consistently higher in HH RBCs compared with regular RBCs (Fig. 1F).

2,3-DPG (Fig. 2A) and ATP (Fig. 2B) levels were similar in both groups at all time points. Glucose concentrations decreased consistently in both groups, but were significantly lower in HH RBCs from 28 days, compared with regular RBCs (Fig. 2C). Phosphorus (Fig. 2D) and potassium (Fig. 2E) increased, while sodium decreased (Fig. 2F) over time in both groups, with no significant difference between them, except for potassium on Day 7. Increased iron absorption throughout life in patients with HH may lead to progressive iron deposition and injury to multiple tissues and organs, with liver parenchyma, endocrine organs, and the heart and joints being particularly susceptible to damage.[27,28] In accordance with the causality of the disease, free iron levels were higher in HH RBCs than in regular RBCs at all times and differ in manner significantly as of Day 35; before this time, iron increased in parallel in both groups over time (Fig. 2G). As expected, free ferritin levels were 14-fold higher in HH RBCs compared with regular RBCs, with little change over time in regular RBCs, but an inconsistent increase in free ferritin from HH RBCs over time (Fig. 2H).

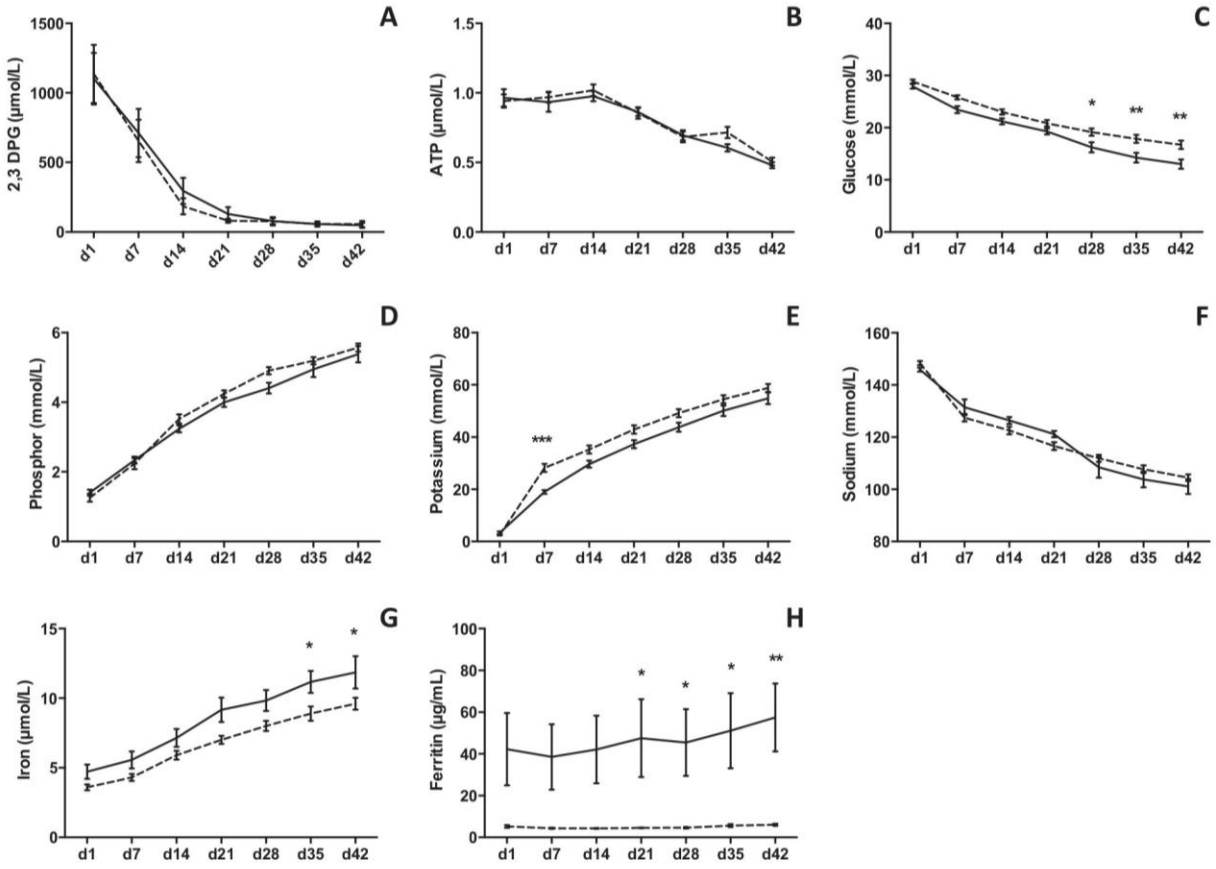

Fig. 2. 2,3-DPG (A), ATP (B), glucose (C), phosphor (D), potassium (E), sodium (F), iron concentrations (G), and ferritin concentration (H) in regular (control) donors (- - -, n = 10) and eligible patients with HH (—, n = 12) during RBC storage (Days 1-42). Data are expressed as mean ± SEM. The difference was considered significant for $p < 0.05$: *$p < 0.05$; **$p < 0.01$.

RBC membrane protein modulation during RBC storage

The RBC surface was examined in both RBC groups by flow cytometry to evaluate changes in the cell membranes over time. We compared the percentage of cells expressing (acquiring or loosing) defined markers (Figs. 3A, 3C, 3E, 3G, 3I, and 3K), and the MFI of marker expression on RBCs at each sampling time in each group (Figs. 3B, 3D, 3F, 3H, 3J, and 3L). RBC CD44 expression was similar in both groups and at all time points, except on Day 21 (Fig. 3A). However, MFI was significantly higher in HH RBCs compared with regular RBCs (Fig. 3B). Baseline expression levels of CD71 and CD36 were similar in both groups and levels remained unchanged or little changed for the first 28 days; however, expression of these markers increased in some among the regular RBCs unlike the HH RBCs, from Day 35 onward (Figs. 3C-3E). In contrast, the MFI of CD71 was significantly higher in HH RBCs compared with regular RBCs (Fig. 3D), although there was no significant difference in the MFI of CD36 (Fig. 3F). RBC CD58 expression was almost constant in both groups over time; apart for the extremes (Day 0 and Day 42), it was below that of regular RBCs; the percentage was slightly decreased in HH RBCs on Day 1 (Fig. 3G) and MFI was increased in HH RBCs on Day 42 (Fig. 3H). CD108 expression was increased in HH RBCs 2 weeks before regular RBCs (percentage only, as MFI remained unchanged).

Regarding phosphatidylserine expression, no RBCs expressed this apoptosis marker on Day 1, but its expression increased sooner (Day 7) in the HH compared with the regular RBC group (35 days), with a significantly higher percentage expression in the HH group at all times (Fig. 3I). Similarly, phosphatidylserine MFI was higher in HH RBCs compared with normal RBCs at all times and this was significant on Day 42 (Fig. 3J). There were no significant changes in the percentages of CD47 and CD147 expression and MFI during storage in either group (data not shown).

Release of soluble immunomodulatory factors by RBCs during storage

Supernatants from RBCs were collected over time and tested for the presence of a variety of soluble chemokines

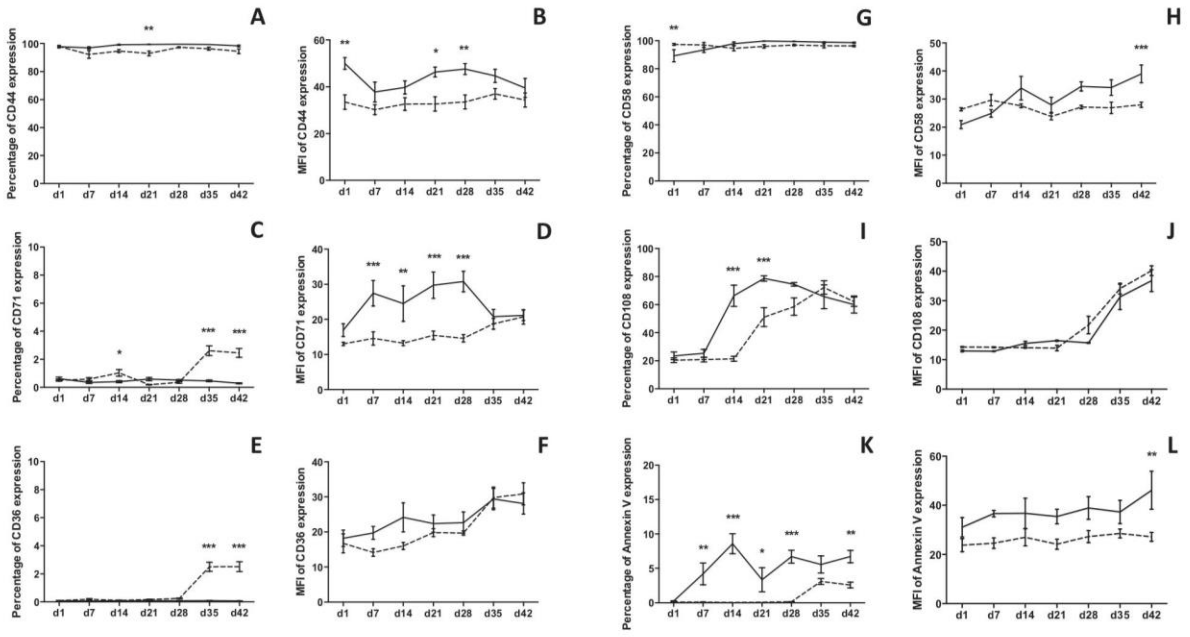

Fig. 3. Flow cytometric analysis of percentage expression and MFI of RBC membrane proteins during storage. CD44 (A, B), CD71 (C, D), CD36 (E, F), CD58 (G, H), CD108 (I, J), and annexin V and phosphatidylserine expression (K, L) in regular (control) donors (- - -, n = 10) and eligible patients with HH (—, n = 7) during RBC storage (Days 1-42). Data are expressed as mean ± SEM. The difference was considered significant for $p < 0.05$: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

and cytokines. GRO-α; IL-16; ENA-78; MIP-1α; MCP-1; SDF-1; and TGF-β1, -2, and -3 were selected on the basis of previous reports.[29-32] There was neither a difference in ENA-78, GRO-α, MIP-1α, MCP-1, SDF-1, IL-16, hepcidin, or TGF-β3 between both groups nor a difference over time (Figs. 4A-4G). However, TGF-β1 (Fig. 4I) and TGF-β2 (Fig. 4J) decreased over time in both RBC groups, with no significant difference between the groups.

TfR was higher in HH RBCs compared with regular RBCs at all times, and this became significant on Day 21 (Fig. 4K). Transferrin was decreased significantly in the HH group compared to the regular RBC groups, as seen as of Day 1 until Day 42 (Fig. 4L).

Bioactivity of soluble immunomodulatory factors in endothelial cells in vitro

We tested the potential bioactivities of the soluble immunomodulatory factors from both RBC groups over time using the human endothelial hybrid cell line EA.hy926. There was no difference in the expression of marker molecules generally associated with EA.hy926 cell activation (CD54, CD31, and CD141)[33,34] at any sampling point (Days 1-42), using TNF-α as a control (Figs. S1A-S1C, available as supporting information in the online version of this paper).[25,35] Similar results were observed regarding the expression of soluble markers generally associated with EA.hy926 cell activation (sCD141, sCD62E, and IL-6; Figs. S1D-S1F). Our results indicate that TNF-α induced an expression on the CD54 cell surface on human endothelial hybrid cell line EA.hy926, but not CD31 and CD141. These results are consistent with those of another report. Romer and coworkers[35] showed that the surface expression of most endothelial cell adhesion molecules involved in WBC interfaces with the vascular endothelium during wound is regulated (with a decrease in expression) by soluble immunomodulatory factors such as IL-1, TNF-α, and IFN-γ. The supernatant of RBCs is a complex medium and we can exclude that RBCs contain sCD141 or sCD162 and/or human endothelial hybrid cell line EA.hy926 is more sensitive to RBC soluble immunomodulatory factors compared to TNF-α used as a control.

DISCUSSION

This study investigated the suitability of HH RBCs for transfusion. The variations in biophysical and biochemical variables and soluble immunomodulatory factors in both HH and normal RBCs were within the acceptable range of QC defined by the European Directorate for the Quality of Medicines & HealthCare.[26] We demonstrated small but significant differences in RBC membrane protein modulation between HH and regular RBCs during storage, but no differences in other variables, such as CD47 expression, which usually indicate pathology and

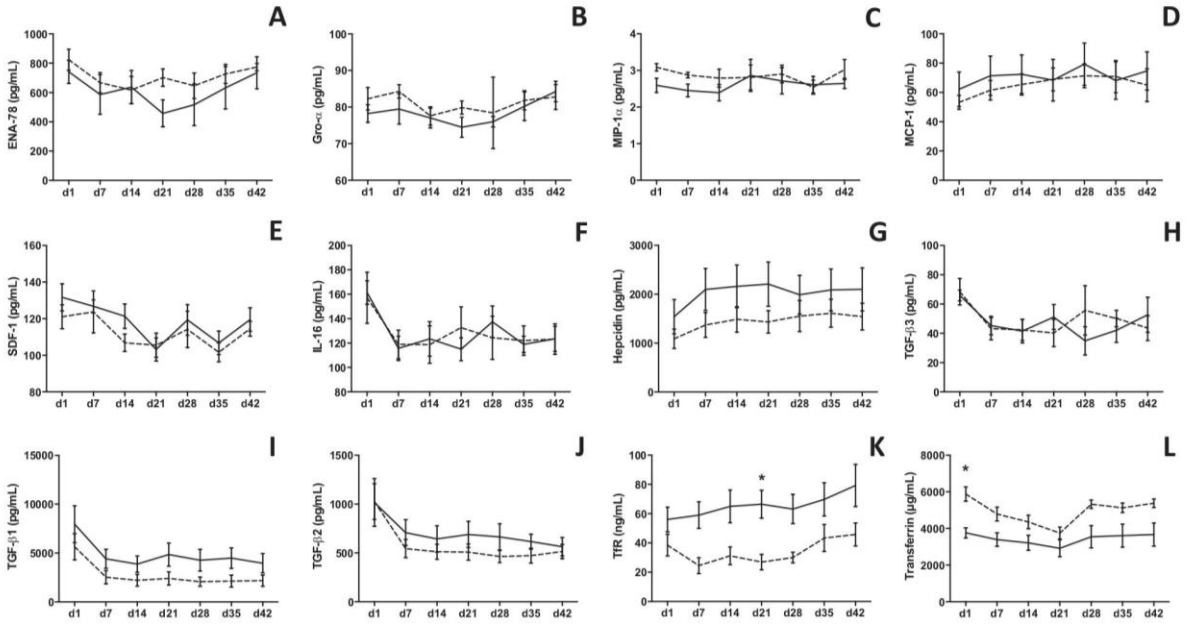

Fig. 4. Release of soluble immunomodulatory factors ENA-78 (A), Gro-α (B), MIP-1α (C), MCP-1 (D), SDF-1 (E), IL-16 (F), hepcidin (G), TGF-β3 (H), TGF-β1 (I), TGF-β2 (J), TfR (K), and transferrin (L) from regular (control) donors (- - -, n = 10) and eligible patients with HH (—, n = 7) during RBC storage (Days 1-42). Data are expressed as mean ± SEM. The difference was considered significant for $p < 0.05$.

stimulate phagocytosis. Interestingly, there were also no differences between the groups regarding the bioactivities of soluble immunomodulatory factors in the supernatant of RBCs in terms of their effects on endothelial cells in vitro.

Although it is possible that blood donors with undiagnosed HH may be enrolled unknowingly, this is unlikely because of systematic Hct measurement (despite the lack of ferritin testing). However, the possible impact of using HH patients as blood donors remains unclear. Despite extensive advances in the understanding of the pathogenesis of HH since the identification of the *HFE* gene,[36] therapy for HH has changed little since regular therapeutic phlebotomy was shown to reduce iron stores safely in most patients and thus prevent the progression of organ damage.[37,38] Blood from HH patients is used for transfusion purposes in several countries, with no reported side effects in the recipients.[5,13] However, hemovigilance alone may not be adequate to detect possible deleterious effects such as moderate inflammation, which may be confounded by other symptoms, and delayed pathology (such as iron overload). De Buck and colleagues[39] used three databases (The Cochrane Library, MEDLINE, and Embase [until January 2012]) and showed evidence of effectiveness and safety of blood for transfusion when derived from hemochromatosis patients who do not suffer from complications or organ damage. The authors insist on the need for harmonization of the blood donor selection policy among nations allowing hemochromatosis patients who do not suffer from problems of iron overload to donate blood, once iron levels are controlled. Moreover, future research should: 1) focus on patients who received blood from hemochromatosis patients; 2) examine closer infections of iron-overloaded patients; and 3) test for other products such as platelets (PLTs) and plasma.

Iron overload is known to increase the risk of infections by not only bacteria, which may utilize iron, but also by viruses and fungi. However, no such adverse events have been recorded as a result of hemovigilance in countries where HH donation is currently practiced, although such adverse effects may not have been closely investigated.[40] Moreover, Prestia and coworkers[41] described, in a mouse models, that transfusion of older RBCs increases infection with both Gram-negative pathogens. The author proposed that iron transfer to macrophages could be an underestimated phenomenon mediating at least some of the adverse effects of RBC transfusions.

To the best of our knowledge, only one previous report has evaluated the quality of RBC products from HH patients.[42] This study stated that RBCs from HH patients complied with the in vitro quality requirements for transfusion. Our present study confirmed the general compliance of HH RBCs to the QC guidelines, while highlighting several small but significant differences, notably in

phosphatidylserine expression, supernatant pH, pCO_2, and hemolysis. The goal of this study concerned the comparison of HH and the regular RBC groups concerning the potential bioactivities of the soluble immunomodulatory factors from both RBC groups. This is interesting to show that, although there is no statistical difference for the markers tested in this system between RBCs derived from healthy donors or RBCs from HH patients, sCD141 concentration and CD141 MFI increase after stimulation with the supernatants, while the positive control, TNF, shows a small decrease. Transfusion-related immunomodulation has developed as a concept to clarify clinical observations that suggest that RBC transfusion is associated with increased proinflammatory or immunosuppressive effects. Our data do not support the hypothesis that RBC storage is more injurious than that of fresh RBCs in healthy people. Further investigation is needed to explore the proinflammatory or immunosuppressive effects, as the transfusion-related immunomodulation concept. The goal of this study concerned the comparison of HH and the regular RBC groups concerning the potential bioactivities of the soluble immunomodulatory factors from both RBC groups. We observed no difference in the expression of marker molecules generally associated with EA.hy926 cell activation (CD54, CD31, and CD141) at any sampling point (Days 1-42).

Iron overload–mediated oxidative stress is involved in damage to cellular macromolecules, including polysaccharide depolymerization and DNA oxidation. HH patients experience severe oxidative stress, but markers of nucleic acid oxidation are normalized after therapeutic phlebotomy. A recent Finnish survey indicated that HH donors, irrespective of their C282Y and H63D genotype, suffered from less ferritin and iron deprivation in women and men, respectively, compared with normal donors.[43] However, no previous study has addressed the effects of HH blood products in the recipients. We observe no strict correlation between the number of therapeutic phlebotomy and ferritin levels (sample size, 12; correlation coefficient (r), −0.20; data not shown). Barton and colleagues[44] reported that voluntary blood donation before diagnosis of hemochromatosis is not significantly correlated with the number of therapeutic phlebotomy units needed to induce iron depletion. These findings have implications for the understanding of the severity of iron overload and its complications in hemochromatosis, for advising persons with hemochromatosis about treatment, and for considering persons with hemochromatosis as possible blood donors.

Pretorius and coworkers[45] proposed a study to assess whether RBCs had an altered morphology in individuals with HH, as well as some others who displayed hyperferritinemia. It would be valuable to test for RBC morphologic changes in HH individuals and outcome of any inflammatory symptom.

The elevation of inflammatory markers seen in transfusion-related immunomodulation may be ameliorated by WBC reduction. However, stored RBCs deliver large amounts of iron to the monocyte-macrophage system thus inducing inflammation, and transfusion of older, stored RBCs therefore produces a proinflammatory response associated with increased iron levels in the liver, spleen, and kidney and increased circulating levels of non–transferrin-bound iron.[46,47] It has also been reported that freed iron, such as in old RBCs, may increase the risk of distress and acute lung injury in patients presenting with severe conditions, although these reports did not specifically address the case of HH RBCs. In this observation, HH RBCs were transfused similarly to other RBCs, that is, between 5 and 15 days after collection, depending on the ABO and RH1 blood groups, with rare units being allowed longer storage. This highlights the problems of considering the deleterious effects of "old blood"; although the impact of "old blood" versus "young blood" transfusions has been the subject of lively debates, the disadvantages of old blood might be restricted to certain pathologies or high-risk conditions, such as extracorporeal circulation or sepsis.[46-50]

Increased levels of annexin V in the supernatant of stored RBCs have been previously reported.[51] Our data are in agreement with those of other reports.[16,52,53]

Human RBCs discard their nucleus during maturation and are thought not to be able to synthesize proteins. Nevertheless, several proteomic approaches described the supernatant of stored RBCs. The proteomic approach produced evidence about the proteins that accumulate under standard blood banking storage conditions and the effect of process on the nature and range of protein species. Plasma proteins contributed to the specific profile of the protein maps of RBC supernatants during the storage period.[54-56] Interestingly, it was recently reported that RBCs have various populations of plentiful microRNAs.[57] At this time there is only limited knowledge concerning microRNAs and their function in mature RBCs, but according to recent data, there is strong evidence that anucleate PLTs contain a functional spliceosome.[58-61]

We observed a small but significant increase in phosphatidylserine expression in HH, compared to normal, RBCs. Phosphatidylserine avidly binds annexin V, which is thus employed to identify eryptotic cells.[62] Chadebech and coworkers[63] show that delayed hemolytic transfusion reaction can occur in the absence of detectable antibody in a sickle cell disease model; however, eryptosis was proposed. It would thus be interesting to investigate whether RBC phosphatidylserine expression during storage can reflect posttransfusion eryptosis.

In conclusion, normal RBCs and HH RBCs are equally suitable for transfusion purposes, according to currently defined in vitro criteria. However, the results of this study showed that HH RBCs are not identical to regular RBCs

with regard to apoptosis and hemolysis during storage. These differences could have clinical consequences that are undetectable by current hemovigilance assessments. Even if the clinical relevance of young or fresh blood (the definition of which needs to be improved) is unclear, evidence suggests that transfusion-associated iron release may be toxic, especially in recipients with existing inflammation.

Given that a dedicated, prospective study of the possible toxicity of iron-overloaded RBCs is unlikely to be feasible, we suggest that recipients commonly considered as being "at risk" (such as patients undergoing extracorporeal circulation, etc.) should be identified and the use of HH RBCs avoided in these individuals. This recommendation would be extrapolated to neonates and infants under the principle of precaution and awaiting further document study, although—in general—this population is transfused with no more than 5-day-old RBCs to ensure optimal RBC function and limit detrimental potassium overload. This would require RBCs to be tagged in some way, either physically or electronically, to allow their source to be identified within the inventory.

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AUTHOR CONTRIBUTIONS

CS, VB, CA, SA, MV, NB, CAA, and MAE performed the experiments and analyzed the data; FC, HHC, SL, BP, CF, PT, and OG conceived and designed the experiments, analyzed the data, and wrote the paper; and all authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1. Bioactivity in EA.hy926 endothelial cells of soluble immunomodulatory factors from RBC supernatants from control donors (n = 10) and eligible patients with HH (n = 7) during storage (Days 1, 21 and 42). TNFα (100 pg/mL) was used as a positive control and unstimulated cells as a negative control. CD54 **(A)**, CD31 **(B)** and CD141 expression **(C)**; sCD141 **(D)**, sCD62E **(E)**, and IL-6 **(F)** release in regular (control) donors (n = 10) and eligible patients with HH (n = 7) during RBC storage (Days 1, 21 and 42). Data expressed as mean ± SEM. The difference was considered significant for $p < 0.05$: *< 0.05; **< 0.01; ***< 0.001.

II - Publications

1. Cognasse F, **Sut C**, Fromont E, Laradi S, Hamzeh-Cognasse H, Garraud O. Platelet soluble CD40-Ligand level is associated with transfusion adverse reactions in a mixed threshold and hit model. Blood. 2017.

2. **C. Sut**, S. Tariket, F. Cognasse, O. Garraud. Determination of predictors of severity for recipient adverse reactions duringplatelet product transfusions. Transfusion Clinique et Biologique. 2017.

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4. Garraud O, Tariket S, **Sut C**, Haddad A, Aloui C, Chakroun T, et al. Transfusion as an Inflammation Hit: Knowns and Unknowns. Frontiers in immunology. 2016;7:534.

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III - Communications scientifiques

1) Communications affichées

1. Properties of donated red blood cell components from patients with hereditary haemochromatosis

Caroline Sut, Hind Hamzeh-Cognasse, Sandrine Laradi, Vincent Bost, Christine Aubrège, Sophie Acquart, Martine Vignal, Nadia Boutahar, Charles Antoine Arthaud, Marie Ange Eyraud, Bruno Pozzetto, Pierre Tiberghien, Olivier Garraud, Fabrice Cognasse

- 25th Regional Congress of the ISBT, Londres, Juin 2015
- XXVII^{e} Congrès de la SFTS, Montpellier, Septembre 2015
- 34th International Congress of the ISBT, Dubaï, Septembre 2016

2. Interaction des plaquettes et des cellules endothéliales lors de l'inflammation

Caroline Sut, Marie Ange Eyraud, Charles Antoine Arthaud, Jocelyne Fagan, Fabrice Cognasse, Olivier Garraud

- Journée de la Recherche IFRESIS, Saint-Etienne, Juin 2016

3. Release of soluble CD40L and serum amyloid a during platelet storage: gender-specific difference

Caroline Sut, Hind Hamzeh-Cognasse, Charles-Antoine Arthaud, Marie-Ange Eyraud, Olivier Garraud, Fabrice Cognasse

- 34th International Congress of the ISBT, Dubaï, Septembre 2016

4. Apheresis platelet concentrates versus buffy-coat derived pooled platelet concentrates: focus on sCD40L and sCD62P

Caroline Sut, Sofiane Tariket, Chaker Aloui, Charles-Antoine Arthaud, Marie-Ange Eyraud, Jocelyne Fagan, Patricia Chavarin, Hind Hamzeh-Cognasse, Sandrine Laradi, Olivier Garraud, Fabrice Cognasse

- 27th Regional Congress of the ISBT, Copenhague, Juin 2017
- XXVIII^{e} Congrès de la SFTS, Bordeaux, Septembre 2017

5. Soluble CD40-Ligand in platelet components associates with transfusion adverse reactions in certain but not all recipients in a mixed threshold and hit model

Fabrice Cognasse, Caroline Sut, Elisa Fromont, Sandrine Laradi, Hind Hamzeh-Cognasse, Olivier Garraud

- 27th Regional Congress of the ISBT, Copenhague, Juin 2017

2) Communications orales

1. Etude des molécules inflammatoires des plasmas issus d'aphérèses plasmatiques simples et mixtes

- XXVII^{e} Congrès de la SFTS, Montpellier, Septembre 2015

2. La concentration de sCD40L dans les concentrés plaquettaires est associée à des effets indésirables receveurs après transfusion dans un sous-groupe des receveurs

- XXVIII^{e} Congrès de la SFTS, Bordeaux, Septembre 2017

Détermination des prédicteurs de sévérité des effets indésirables receveurs au cours des transfusions plaquettaires

Résumé : La transfusion sanguine est une thérapeutique indispensable pour laquelle il n'existe pas actuellement de substitut. La transfusion de produits sanguins labiles est dans la grande majorité des cas très bien tolérée mais elle peut être à l'origine d'effets indésirables chez les receveurs (EIR) notamment de type inflammatoire. Ceci dépend de facteurs liés aux produits eux-mêmes et/ou aux receveurs de par leur prédisposition génétique et de leur état clinique. Les concentrés plaquettaires (CP) sont la principale source de manifestations inflammatoires et/ou allergiques. Ceci est notamment dû, en partie, à la capacité des plaquettes à sécréter une multitude de molécules ayant une activité inflammatoire. De plus, les processus de collecte, de préparation et de conservation induisent un stress vis-à-vis des cellules, qui peut activer les plaquettes et donc induire la production de produits inflammatoires dans les CP. Le but de ce travail de thèse a été dans un premier temps d'identifier les molécules les plus impliquées dans les manifestations inflammatoires. Le sCD40L en particulier est identifié comme étant largement impliqué dans les EIR après transfusion de CP, mais pas systématiquement. Aussi, la composante inflammatoire de ces réactions est multifactorielle. De plus, nous avons évalué le potentiel inflammatoire des CP sur l'endothélium vasculaire. Des différences d'activation des cellules endothéliales, dans un modèle *in vitro*, ont été observées lorsqu'elles sont en présence de surnageants de CP ayant induits un EIR. Ce travail de thèse poursuit l'effort entrepris par notre équipe de recherche, en vue de prédire la survenue d'EIR et de préciser les mécanismes qui influencent la physiopathologie plaquettaire transfusionnelle ; un corollaire de ces travaux est ainsi d'optimiser les processus de production et de conditionnement des CP transfusés afin de réduire ces réactions inflammatoires.

Mots clés : Transfusion, EIR, Plaquettes, Inflammation, BRM, Cellules endothéliales

Determination of severity predictors of adverse reactions during platelet transfusions

Abstract: Blood transfusion is an indispensable therapy for which there is currently no substitute. Transfusion of blood products is in the great majority of cases very well tolerated but it can be at the origin of serious adverse reactions (SARs), notably of inflammatory reactions. This depends on the factors related to the products themselves and/or to the recipients, their genetic predisposition and clinical condition. Platelet concentrates (PCs) are the main source of inflammatory and/or allergic manifestations. This is due, in part, to the ability of platelets to secrete a multitude of molecules with inflammatory activity. In addition, the collection, processing and storage conditions induce stress on cells, which can activate platelets and thus induce the production of inflammatory products in PCs. The purpose of this work is to identify the molecules involved in inflammatory manifestations. sCD40L was identified as being involved in SARs after PCs transfusion, but not systematically. Also, the inflammatory component of these reactions is multifactorial. In addition, we evaluated the inflammatory potential of PCs on the vascular endothelium. Differences in endothelial cell activation, in an *in vitro* model, were observed when they were in the presence of PC supernatants involved in SARs. This thesis work continues the effort undertaken by our research team to predict the occurrence of SARs and to clarify the mechanisms that influence transfusional platelet physiopathology; a corollary of this work is to optimize the production and conditioning process of PCs transfused in order to reduce these inflammatory reactions.

Keywords: Transfusion, SAR, Platelets, Inflammation, BRM, Endothelial cells