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Liste des abréviations

AABB	*American Association of Blood Banks*
ADP	Adénosine diphosphate
AHTR	(*Acute Hemolytic Transfusion Reaction*) Réaction hémolytique aiguë
ARN	Acide ribonucléique
ATP	Adénosine triphosphate
ATR	Réaction transfusionnelle adverse
BC	(Buffy-Coat) Couche leucoplaquettaire
β-TG	β-thromboglobuline
CD40L	CD40 Ligand
CD62P	P-sélectine
CjD	Maladie de Creutzfeldt-Jacob
CO_2	Dioxyde de carbone
COX-2	Cyclooxygenase-2
DAMPs	*Damage-Associated Molecular Patterns*
DHTR	(*Delayed Hemolytic Transfusion Reaction*) Réaction hémolytique retardée
DMSO	Diméthylsulfoxyde
FACS	(*Fluorescence-activated cell sorting*) Cytométrie en flux
FADH	Flavine adénine dinucléotide
FcεR	Récepteur Fc pour les IgE
FcγR	Récepteur Fc pour les IgG
FDA	*Food and Drug Administration*
FNHTR	(*Febrile Non-Hemolytic Transfusion Reaction*) Réaction fébrile non-hémolytique
HLA	(*Human Leukocyte Antigen*) Antigènes leucocytaires humains
HPA	(*Human Platelete Antigen)* Antigènes plaquettaires humains
HSCs	(*Hematopoietic Stem Cells*) Cellules souches hématopoïétiques
Ig	Immunoglobuline
IL	Interleukine
LDH	Lactate deshydrogénase
Lyso-PCs	Lysophosphatidylcholine
MIP	*Macrophage inflammatory protein*
MPs	Microparticules
mRNA	ARN messager
miRNA	micro ARN
mtDNA	ADN mitochondrial
NADH	Nicotinamide adénine dinucléotide
O_2	Oxygène
OCS	(*Open Canalicular System*) Système canaliculaire ouvert
PAF	*Platelet activating factor*
PAMPS	*Pathogen-associated molecular patterns*
PCs	(Platelet Concentrates) Concentrés plaquettaires
PDGF	*Platelet derived growth factor*

PF4	*Platelet factor* 4
PGE_2	Prostaglandine E_2
pH	Potentiel hydrogène
PRP	Plasma riche en plaquettes
PS	Phosphatydylsérine
PSL	(*Platelet Storage Lesion*) Lésions d'entreposage des plaquettes
PTP	(*Post-Transfusion Purpura*) Purpura post-transfusion
RANTES	*Regulated on Activation, Normal T Cell Expressed and Secreted*
Rh	Rhésus
RNA	Acide ribonucléique
SPADE	*Spanning-tree Progression Analysis of Density-normalized Events*
sCD40L	CD40 Ligand soluble
SLE	(*Systemic Lupus Erythematosus*) Lupus érythémateux disséminé
$sPLA_2$-IIA	Phospholipase A_2 sécrétée de type IIA
TRALI	(*Transfusion-Related Acute Lung Injury*) Syndrome respiratoire aigu post-transfusionnel
TACO	(*Transfusion Associated Circulatory Overload*) Surcharge circulatoire liée à la transfusion
TA-GVHD	(*Transfusion-Associated Graft-Versus-Host Disease*) Réaction du greffon contre l'hôte
TEM	(*Transmission Electron Microscopy*) Microscopie électronique à transmission
TF	(*Tissue Factor*) Facteur tissulaire
TGF	*Transforming growth factor*
TH	Thrombaphérèse
TLR	*Toll-like receptors*
TNF	*Tumor necrosis factor*
TOM	(*Translocase of the Outer Membrane*) Translocase de la membrane externe
TIM	(*Translocase of the Inner Membrane*) Translocase de la membrane interne
UV	Ultra-violets
VIH	Virus de l'immunodéficience humaine
VHA	Virus de l'hépatite A
VHB	Virus de l'hépatite B
VHC	Virus de l'hépatite C
VHG	Virus de l'hépatite G
vWf	Facteur von Willebrand

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L'article qui constitue le chapitre 2, intitulé **Platelets release mitochondria serving as substrate for bactericidal group IIA-secreted phospholipase A2 to promote inflammation** a été publié dans le journal *Blood* :
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Chapitre 1 : Introduction

1.1 La transfusion

1.1.1 But

La transfusion de produits sanguins est l'une des procédures médicales les plus communes.[1] Elle a pour but de substituer les éléments du sang manquants à la suite d'une hémorragie,[2] d'une maladie ou d'un traitement.[3] Ces produits sont rigoureusement contrôlés et leur usage est limité.[1] Aujourd'hui, la transfusion est indiquée uniquement lorsque les bénéfices sont supérieurs aux risques résiduels de la transfusion.[4]

1.1.2 Produits labiles impliqués

La transfusion du sang total n'est plus utilisée étant donné sa courte durée de vie,[5] et parce que chaque composant du sang est utilisé pour une indication différente. Ainsi, la séparation du sang en différents composants permet de maximiser l'utilité d'un don.[6] Les produits sanguins peuvent être obtenus par centrifugation du sang total ou par aphérèse et comprennent les globules rouges, le plasma congelé, les plaquettes, les globules blancs et le cryoprécipité.

La transfusion de globules rouges a pour but de normaliser le transport d'oxygène vers les tissus.[7] Elle est nécessaire en cas d'anémie.[8]

La transfusion de granulocytes est indiquée pour le traitement d'infections bactériennes ou fongiques qui ne répondent pas aux agents microbiens usuels en présence de neutropénie où d'un défaut qualitatif de granulocytes.[7]

La transfusion de plaquettes, sous forme de concentrés plaquettaires (PCs), est utilisée pour traiter ou prévenir les saignements en cas de thrombocytopénie ou de plaquettes défectueuses.[7] Bien que les autres composants sanguins soient

davantage transfusés, les transfusions de plaquettes sont apparemment associées à un risque plus élevé de réactions.[9]

Le plasma frais congelé est utilisé pour remplacer des facteurs de coagulation déficients, dans le but de prévenir une hémorragie ou de l'arrêter. Il peut être aussi traité pour fabriquer un cryoprécipité, qui contient une concentration supérieure en fibrinogène et en facteur XIII et VIII.[10]

1.1.3 Les groupes sanguins

Notre sang présente une combinaison d'éléments classifiés selon divers systèmes de groupage sanguin pour les différentes cellules du sang, soit les érythrocytes, les leucocytes et les plaquettes.

1.1.3.1 Groupes sanguins érythrocytaires

Comme ce fut le premier groupe étudié, le groupe sanguin érythrocytaire est généralement utilisé comme point de référence pour le groupe sanguin. Environ 328 antigènes érythrocytaires ont été décrits à ce jour.[11] Les trois systèmes antigéniques les plus importants pour la transfusion sont les systèmes ABO, Rhésus et Kell. Le système ABO a été découvert au début du 20^{e} siècle par Karl Landsteiner et a permis le développement de la transfusion et les bases pour l'étude des autres systèmes.[12] Il est d'une grande importance, car A et B, des sucres présents à la surface des globules rouges, sont fortement antigéniques. Le gène ABO, qui comprend 7 exons, code pour une glycosyltransférase, soit N-acétyl-galactosamine-transférase pour l'antigène A ou une galactose-transférase pour l'antigène B.[13] L'allèle O, quant à lui, résulte d'une mutation dans la séquence codante, causant un transcrit incomplet et donc une enzyme inactive.[13] À l'état homozygote, il conduit à l'absence d'antigène A et B sur les hématies, correspondant au phénotype O. La combinaison de ces allèles donne quatre groupes différents, soit A (AA ou AO), B (BB ou BO), AB ou O (OO). Des anticorps anti-A et anti-B apparaissent naturellement dans le plasma des personnes qui n'ont pas l'antigène. Ceux-ci appartiennent aux classes d'immunoglobulines (Ig) M et G et peuvent induire une réaction d'hémolyse

importante lors d'une transfusion avec des hématies non-compatibles.[14]

Le groupe Rhésus (Rh) est le second groupe le plus important pour la médecine transfusionnelle.[13] Il est hautement polymorphique et code pour plus de 45 antigènes.[15] Contrairement aux anti-A et anti-B, les anticorps dans le système Rhésus résultent d'une réponse immunitaire induite par une grossesse ou une transfusion sanguine incompatible (rencontre avec l'antigène). Le gène RhD est le plus important pour le système Rh, car il est fortement immunogénique et fréquemment impliqué dans les réactions transfusionnelles.[16] Il code pour l'antigène D qui est une protéine exprimée à la surface de la membrane des globules rouges. Cette protéine peut être traduite (RhD^+) ou non-traduite (RhD^-).[16] Cet antigène est impliqué dans l'incompatibilité foeto-maternelle.[15] Dans le cas où la mère est RhD^- et le foetus est RhD^+, la mère peut en effet développer des allo-anticorps lors de l'accouchement. Ces allo-anticorps dirigés contre l'antigène D peuvent nuire sévèrement lors de grossesses subséquentes en causant la destruction des globules rouges du fœtus.[15] Une transfusion sanguine incompatible pour le groupe Rh va, elle aussi, entrainer la lyse des globules rouges.

Le troisième groupe est le système Kell qui inclut 35 antigènes.[17] Comme pour le groupe Rh, les anticorps résultent d'une réponse immunitaire induite par une grossesse ou une transfusion sanguine incompatible.[18] L'antigène Kell est une glycoprotéine membranaire de type II, associée à d'autres protéines de la membrane cellulaire des globules rouges.[18] Différents phénotypes de Kell sont obtenus à la suite d'une seule mutation.

1.1.3.2 Groupes sanguins leucocytaires

Le système d'antigènes leucocytaires humain (HLA) comprend les antigènes d'histocompatibilités présents sur toutes les cellules de l'organisme. Il code pour des molécules de surface qui présentent les peptides antigéniques, qui sont reconnus, directement ou indirectement, par les cellules du système

immunitaire.[19] Un facteur qui contribue à l'immunogénicité du système HLA est son fort polymorphisme, qui augmente la probabilité d'incompatibilité, donc de complications lors de transfusion.[20] Basé sur des caractéristiques moléculaires et fonctionnelles, 2 classes de HLA ont été décrites, soit les HLA classe I et classe II. Les HLA classe I sont exprimés sur la majorité des cellules sanguines et des tissus et peuvent former un complexe avec les peptides endogènes qui est reconnu par les cellules T $CD8^+$.[21] Dans le sang, environ 70% des antigènes HLA sont exprimés sur les plaquettes, alors que 15 à 20% sont sous forme soluble.[21] Les HLA classe II sont exprimées sur les cellules présentatrices d'antigènes comme les lymphocytes, les monocytes et les cellules dendritiques ainsi que sur les lymphocytes B et les cellules T activées.[21] Ces cellules présentent les peptides exogènes aux cellules T CD4+.[19] Plusieurs réactions transfusionnelles peuvent être médiées par le HLA, comme la réaction fébrile non-hémolytique (FNHTR), la réaction allergique, les réactions hémolytiques, le syndrome respiratoire aigu post-transfusionnel (TRALI), ou la réaction du greffon contre l'hôte (TA-GVHD).[21] La leucoréduction des produits sanguins s'avère un moyen efficace pour prévenir les réactions transfusionnelles reliées aux HLA.

1.1.3.3 Groupes sanguins plaquettaires

Pour les groupes sanguins plaquettaires, on utilise le système HPA (antigènes plaquettaires humains). Les plaquettes ont à leur surface les glycoprotéines GPIb/XI, GPIa/IIa, et GPIIa/IIIa.[22] Le polymorphisme dans la séquence codante de ces protéines permet l'obtention de différent phénotype HPA. Le cas le plus commun d'allo-immunisation contre le système HPA est l'incompatibilité fœto-maternelle plaquettaire et implique dans 75-80% des cas le système HPA-1.[22] Les anticorps anti-HPA sont impliqués dans certaines réactions immunitaires, telles que le purpura post-transfusion (PTP), le FNHTR et la thrombocytopénie allo-immune.[23-25] De plus, les receveurs peuvent développer un état réfractaire à la suite de multiples transfusions de plaquettes.[24]

1.2 Les réactions transfusionnelles

1.2.1 Les formes de réactions transfusionnelles

La transfusion de sang est une procédure courante et bien réglementée de nos jours. Malheureusement, il est important de considérer l'équilibre des risques et bénéfices, car cette procédure médicale présente certains risques pour le receveur qui peuvent mener à des réactions transfusionnelles graves ou à la transmission de maladies infectieuses. Ces risques peuvent donc être classés en deux catégories, soit les risques infectieux et non-infectieux.

1.2.1.1 Risques infectieux

Le risque de contracter une infection demeure aujourd'hui une préoccupation transfusionnelle. D'ailleurs, la réaction transfusionnelle la plus fréquente est le sepsis associé à la transfusion d'un produit sanguin contaminé.[26] Au moins trois sources de contamination des produits sanguins sont connues : le sang du donneur est contaminé, car il est lui-même infecté, un élément de la flore bactérienne est introduit dans le composant sanguin lors du prélèvement ou une contamination survient lors de la préparation.[27]

Dans les années 1980, la principale préoccupation concernait la transmission d'infections virales lors de transfusion de sang contaminé.[28] Les principaux virus visés étaient le virus de l'immunodéficience humaine (VIH), de l'hépatite B et C (VHB et VHC). Pour pallier ce problème, des critères stricts et stigmatisants d'exclusion ont été appliqués lors de la sélection des donneurs et les poches de sang sont aujourd'hui testées pour prévenir la transmission de maladies. Le questionnaire pour la détermination d'éligibilité au don est une composante importante pour la sécurité du sang. Parmi les critères d'exclusion, on y retrouve, entres autres, les pratiques sexuelles à risques, telles que « relation non protégée ou avec de multiples partenaires ». Ce critère, apparu dans les années 1980, a permis une diminution dramatique de la transmission du VIH par le sang et de 30% pour l'hépatite.[29, 30] Un autre critère implique les voyages à l'étranger, limitant les risques de contracter la malaria ou la maladie de Creutzfeldt-Jacob (CJD) et sa

variante. Une sélection restrictive des donneurs de sang permet d'éviter la transmission de certains pathogènes pour lesquels il n'existe pas de tests ou pour lesquels les tests ne sont pas effectués systématiquement.[31] Une autre façon de réduire les risques infectieux est l'utilisation de donneurs non-rémunérés.[32, 33]

Voici une liste non-exhaustive des risques infectieux non bactériens visés par les critères de sélection ou les tests de dépistages.[34] Parmi les parasites, on compte *Plasmodium falciparum* causant la malaria*, Babesia sp.* causant la babesia, *Leishmania* sp. causant la leishmania ainsi que *Toxoplasma gondii* et *Trypanosoma cruzii.* Les virus comprennent le virus de l'immunodéficience humaine (VIH), le virus T-lymphotrophe humain (HTLV I/II), le cytomégalovirus (CMV), le parvovirus B19, le virus d'Epstein-Barr (EBV), le virus de l'hépatite A (VHA), le virus de l'hépatite B (VHB), le virus de l'hépatite C (VHC), le virus de l'hépatite G (VHG), le virus de SEN, le virus herpes humain 8 et le virus du Nil occidental (VNO). Enfin, pour les prions sont visés le CJD sa variante. Cependant, le risque de contracter l'une de ces maladies est très faible dans les pays développés.[35]

Dans les années 1990, les efforts se sont davantage concentrés sur la réduction de l'incidence de la contamination bactérienne des produits sanguins, principalement les PCs.[28] Les PCs sont habituellement conservés avec agitation entre 20 et 24°C pour 5 à 7 jours jours selon les directives des services transfusionnels.[36] Ces conditions sont idéales pour une croissance bactérienne, pouvant amener un simple contaminant microbien à atteindre une concentration dangereuse pour le receveur. Le risque de recevoir un PC contaminé par une bactérie est alors 50-250 fois plus élevé que de contracter une maladie virale à la suite d'une transfusion.[26] Les pathogènes les plus fréquemment identifiés sont *Staphylococcus epidermidis* et *Staphylococcus aureus,* généralement présents sur la peau.[37] Des procédures simples, comme la désinfection efficace de la peau au site de prélèvement, permettent une réduction des bactéries de la flore normale jusqu'à 99%.[38] Plusieurs méthodes sont présentement utilisées. Certaines

comprennent une ou deux étapes désinfection qui durent généralement 30 secondes.[39] Les principaux désinfectants, utilisés seuls ou en combinaisons, sont l'alcool isopropylique 70%, la chlorexidine 0,5%, le peroxyde d'hydrogène 0,125% et la povidone iodine.[38]

Bien que la désinfection de la peau soit importante, elle ne limite pas la contamination causée par la carotte de peau pouvant se retrouver dans l'aiguille lors de la phlébotomie et entrainer dans le dispositif de prélèvement avec le sang. En effet, ceci se produit dans à peu près 65% des ponctions veineuses.[40] Afin d'éviter ce problème, la méthode de diversion des premiers millilitres de sang a été instaurée et elle a déjà prouvé son efficacité pour réduire la contamination des produits sanguins.[41, 42] Elle consiste à récupérer ces premiers millilitres de sang, qui seront utilisés pour les tests de dépistage, dans une poche annexe pour prévenir la contamination par la flore du donneur.[43] La Croix-Rouge américaine a rapporté une diminution de contamination de 27,2 à 14,7 pour 100 000 PCs avec l'ajout de la dérivation et d'une bonne technique de désinfection.[44]

Avant 2004, la fréquence de contamination bactérienne des PCs se situait à 1 pour 1000 à 3000 unités transfusées.[45] Pour réduire ce taux, *l'American Association of Blood Banks* (AABB) a exigé en 2004 une implantation de méthodes visant à détecter la contamination bactérienne.[45-47] Parmi ces méthodes, le BacT/ALERT (bioMerieux, Canada) est un système de culture commercial approuvé par la *Food and Drug Administration* (FDA) et utilisé par les fournisseurs de produits sanguins nord-américains et européens pour tester les PCs.[48] Un aliquot du PC est inoculé 24 heures après le prélèvement dans une bouteille aérobique et anaérobique. Ces bouteilles sont mises en culture et la production de CO_2 par les bactéries présentes dans le produit sanguin est détectée par l'appareil. Deux autres systèmes du même type existent, soit le Bactec (Becton Dickson, USA) et le VersaTREK (Trek Diagnostics, USA).[49, 50] Un autre système approuvé par la FDA est le eBDS (Haemonetics, USA) qui utilise la réduction de la concentration d'oxygène pour détecter la présence de croissance bactérienne.

Bien que ces méthodes soient efficaces,[51] elles présentent le désavantage d'être peu rapide et donnent parfois de faux-négatifs ou de faux-positifs,[52, 53] pouvant entrainer la transfusion d'un produit contaminé ou un délai dans le traitement du produit respectivement.

Dans le but constant d'améliorer la sécurité des produits sanguins et plus précisément des PCs, des méthodes visant à inactiver les pathogènes sont apparues. Elles comprennent, entre autres, l'ajout d'un agent chimique photosensibilisant, soit une combinaison d'amotosalen et d'hydrochloride[54, 55] ou de la riboflavine[56-58] qui, couplé à une exposition aux ultra-violets (UV) A, ciblent l'acide nucléique des bactéries empêchant ainsi leur croissance.[31] Une exposition unique aux UVC[59] a aussi été évaluée ainsi que l'ajout de solvant-détergent[60] ou de bleu de méthylène,[61] afin d'inactiver les pathogènes. Malheureusement, ces méthodes affectent le métabolisme et l'activation des plaquettes *in vivo*,[59, 62] et ont une forte toxicité.[63-66] Elles doivent dont être utilisées avec précaution. Une autre méthode est l'irradiation qui permet d'inactiver les bactéries à fortes doses (100-150 grays). Par contre, la qualité des plaquettes est aussi compromise.[67]

1.2.1.2 Risques non-infectieux

De nombreuses mesures ont été implantées pour diminuer les risques infectieux, permettant ainsi aux risques non-infectieux de devenir une cause plus importante de réactions transfusionnelles.[68] Parmi les risques non-infectieux sont compris la réaction hémolytique aiguë (AHTR) ou retardée (DHTR), la FNHTR, la réaction allergique mineure ou majeure, le TRALI, la surcharge circulatoire liée à la transfusion (TACO), le TA-GVHD et le purpura post-transfusion (PTP).

Une réaction hémolytique est une destruction accélérée des globules rouges résultant d'une incompatibilité entre les anticorps du donneur et les globules rouges du receveur. Ceci peut mener à de l'hypotension et une incapacité rénale aiguë pouvant avoir des conséquences fatales.[69] Elle peut aussi être de nature non-immunologique, lorsque des cellules endommagées sont transfusées. Elle est

considérée AHTR lorsque les symptômes surviennent dans les 24 heures suivant la transfusion et DHTR s'ils surviennent entre 24 heures et trois semaines post-transfusion.[70]

Une FNHTR est caractérisée par une élévation de température égale ou supérieure à 1°C près de la fin de la transfusion qui ne peut être reliée à la condition du patient ou à un autre type de réaction transfusionnelle.[70] Bien qu'une FNHTR puisse être causée par plusieurs facteurs, il a été démontré que les antigènes HLA et HPA peuvent jouer un rôle comme déclencheur immunologique.[71] Des anticorps réagissant contre les leucocytes sont retrouvés dans plus de 70% des patients atteints d'une FNHTR.[72] Ce mécanisme explique bien les FNHTR causés par une transfusion d'érythrocytes, mais les FNHTR pour les transfusions de plaquettes sont majoritairement causées par une autre voie immunologique impliquant les cytokines.[73] L'âge du composant sanguin a ici une importance, car il existe une corrélation linéaire entre les niveaux de cytokines, la quantité de leucocytes et la durée d'entreposage, avec une accumulation plus importante de cytokines pour les PCs entreposés à 22°C que les globules rouges entreposés à 4°C.[73, 74]

Une réaction cutanée à la suite d'une transfusion est considérée comme une réaction allergique mineure. Si cette réaction est accompagnée d'hypotension, de dyspnée, d'une respiration sifflante, de douleurs à la poitrine, ou d'une tachycardie elle est considérée comme anaphylactique (majeure).[70] Le mécanisme classique mis en cause dans les réactions allergiques est une hypersensibilité de type I due à la transfusion d'IgE présentes dans le plasma.[75]

Le TRALI est caractérisé par une détresse respiratoire aiguë, non cardiogénique, d'œdème pulmonaire bilatérale et d'une hypoxémie qui se produisent généralement dans les 2 heures qui suivent la transfusion d'un produit sanguin contenant du plasma.[19, 70] Des études à l'aide de modèles animaux ont permis de démontrer que cette réaction est initiée par l'activation des granulocytes, qui induisent alors la libération d'anaphylatoxines, de cytokines et de chimiokines qui

promeuvent l'agrégation et la chimiotaxie des neutrophiles dans les poumons,[19] causant ainsi des dommages à l'endothélium et un oedème pulmonaire.[76] La majorité des TRALI impliquent la transfusion de produits sanguins contenant du plasma, comme le sang total, les PCs et le plasma congelé.[77] Deux hypothèses impliquant l'activation des granulocytes et un processus inflammatoire ont été postulées pour expliquer les mécanismes du TRALI. Cette réaction serait médiée soit par des anticorps et/ou un médiateur soluble. Dans les cas de TRALI où aucun anticorps n'est détecté, l'activation des granulocytes semblerait médiée par une substance lipidique soluble qui s'accumule lors de l'entreposage du produit sanguin.[78] Des études rétrospectives ont mis en évidence que des facteurs propres au receveur pouvaient influencer le développement du TRALI.[79, 80] Ainsi est apparue l'hypothèse du « *Two-hit* », où deux évènements, l'implication d'un anticorps ou d'un médiateur soluble combiné à la condition clinique du receveur, sont nécessaires pour le développement du TRALI. [81, 82]

Le TACO est causé par une incapacité du coeur à pomper adéquatement le sang dont le volume est augmenté à la suite de transfusion. Il en résulte une insuffisance cardiaque et un oedème pulmonaire aigu avec dyspnée et cyanose dans les 6 heures suivant une transfusion.[83] Il est possible d'identifier certains facteurs de risques pour le TACO, soit l'âge du patient, une balance fluidique positive avant la transfusion ou des problèmes cardiaques et rénaux.[83]

Le TA-GVHD est une condition majoritairement fatale (90%) qui résulte d'une destruction des cellules du receveur par les lymphocytes du donneur.[70] Pour que le TA-GVHD se développe, il doit y avoir présence d'une différence HLA entre le donneur et le receveur, la présence de cellules immunocompétentes dans le produit transfusé et une incapacité du receveur à les rejeter.[19]

Le PTP est caractérisé par une thrombocytopénie survenant de 5 à 10 jours après la transfusion.[70] Il est généralement accepté que les plaquettes du donneur et du receveur soient détruites lors de la réaction. Dans 85% des cas, les receveurs

n'ont pas l'HPA 1a alors qu'il était présent dans le produit transfusé,[84] bien que d'autres antigènes plaquettaires soient impliqués. Plusieurs hypothèses ont été suggérées pour expliquer le PTP, soit l'adsorption du complexe antigène-anticorps ou la réactivité croisée des anticorps ou auto-anticorps.[84] Toutefois, le mécanisme exact de destruction reste inconnu.

1.2.2 Facteurs connus à ce jour

1.2.2.1 Alloimmunisation

La majorité des cas de TRALI sont reliés à des allo-anticorps anti-leucocytaires dirigés contre les HLA de classe I et II.[85] Ces anticorps sont fréquemment retrouvés chez les femmes ayant un antécédent de grossesse ou chez les donneurs ayant déjà été transfusés.[86, 87] L'utilisation de donneurs masculins, féminins sans antécédents de grossesse ou sans historique de transfusion permet de réduire les risques de réactions transfusionnelles.[88-90]

1.2.2.2 Allergènes

Les réactions allergiques induites par une transfusion sont causées par des protéines plasmatiques comme l'IgA, l'IgE et l'haptoglobine.[75, 91, 92] Certains allergènes chimiques ou alimentaires présents dans le produit sanguin peuvent aussi induire des réactions.[9]

1.2.2.3 CD40 ligand

Le CD40 ligand (CD40L), aussi nommé CD154, est un médiateur pro-inflammatoire qui se retrouve sous forme soluble (sCD40L) ou associé aux cellules.[93] Le CD40L est entreposé dans les granules-α des plaquettes jusqu'à leur activation.[94] Le sCD40L s'accumule dans les produits sanguins lors de l'entreposage.[95] Il est reconnu pour activer les macrophages, induisant la libération de diverses cytokines incluant l'interleukine (IL)-1, l'IL-6 et CXCL8/IL-8, et induisant l'expression de la cyclooxygenase-2 (COX-2) et la production d'une grande quantité de prostaglandine E_2 (PGE_2).[96] Il est associé aux réactions transfusionnelles impliquant des PCs.[97]

1.2.2.4 Cytokines

Plusieurs cytokines semblent impliquées dans les réactions transfusionnelles. Parmi celles-ci, il y a l'IL-1α, l'IL-1β, l'IL-2, l'IL-6, CXCL8/IL-8, le *Tumor Necrosis Factor* (TNF), l'interféron-α, l'interféron-γ, le CXCL4/*Platelet Factor* 4 (PF4), la β-thromboglobuline (β-TG), CCL5/RANTES (*Regulated on Activation, Normal T Cell Expressed and Secreted*), le CCL3/*Macrophage Inflammatory Protein* (MIP)-1α, le *Transforming Growth Factor* (TGF)-β, le *Platelet Derived Growth Factor* (PDGF) AB. [73, 74, 98-107]

1.2.2.5 Lipides

Différents lipides bioactifs semblent être impliqués dans les réactions transfusionnelles. Parmi ceux-ci, certains lysophosphatidylcholines (lyso-PCs), dont les lyso-PAF (*Platelet Activating Factor),* sont relargués par les macrophages, les basophiles et les plaquettes. Ces lipides augmentent lors de l'entreposage des PCs[108] et peuvent activer les neutrophiles. [109, 110]

1.3 Les concentrés plaquettaires

1.3.1 Les plaquettes

1.3.1.1 Description générale

En 1862, un nouvel élément du sang, ayant un rôle important pour l'hémorragie et la thrombose, a été décrit par Giulio Bizzozero.[111] Connus maintenant sous le nom de plaquettes, ces éléments qui parcourent les vaisseaux sanguins sont petits (environ 2 µm de diamètre), et leur rôle physiologique primaire est de détecter les vaisseaux endommagés et de s'y accumuler pour initier la coagulation.[112] Une plaquette mature ne possède pas de noyau, mais quelques fragments de Golgi et une traduction protéique minimale y sont toujours présents.[113] Les plaquettes sont toutefois très spécialisées, car elles possèdent des récepteurs de membrane capable de reconnaitre la plupart des protéines de la matrice extracellulaire, un cytosquelette capable de se modifier rapidement lors de l'activation et des granules sécrétoires contenant des protéines impliquées dans l'hémostase, des facteurs de croissance et des chimiokines.

La membrane plasmique de la plaquette est constituée d'une bicouche de phospholipides, généralement lisse, à l'exception d'invaginations qui déterminent les entrées du système canaliculaire ouvert (OCS).[112] Ce système permet la perméabilisation du cytoplasme pour l'importation de protéines et de molécules et leur libération lors de l'activation[112] et constitue aussi une source importante de membrane mobilisable lors du changement de conformation des plaquettes.[114] L'organisation asymétrique des phospholipides régule la coagulation.[115] Enfin, la membrane plasmique possède un glycocalyx qui est recouvert de glycolipides et de glycoprotéines qui permettent l'adhésion, l'activation, l'agrégation et l'interaction des plaquettes avec les autres cellules.[116]

La membrane plasmique est supportée par un cytosquelette très développé, constitué de trois composantes majeures, soit la spectrine, l'actine et les microtubules. Lorsque la plaquette s'active, subit un changement de conformation

important, passant de sa forme discoïde à sphérique.[117] Ce processus est initié par un influx de calcium[112] et implique le cytosquelette d'actine.[117]

Le cytoplasme de plaquettes contient de nombreux organites, dont la mitochondrie, et différents types de granules sécrétoires. Parmi les différents types de granules, les granules α sont les plus abondantes.[118] Elles renferment des chimiokines, des facteurs de coagulation, la β-TG, des protéines d'adhésion, des facteurs de croissance et de la P-sélectine (CD62P), ce qui leur confère un rôle dans la coagulation, l'inflammation, la défense contre les pathogènes et la mitogénèse.[119] Les granules denses contiennent de petites molécules comme l'ADP et l'ATP, le calcium et la sérotonine qui confèrent une apparence opaque en microscopie.[113] Des lysosomes et des peroxysomes sont aussi présents dans le cytoplasme des plaquettes.[120]

Environ un trillion de plaquettes circulent dans le sang pour une période d'environ 8 à 10 jours.[112, 121] Pour maintenir un compte plaquettaire normal (150-400 x 10^9/litre de sang), 100 milliards de plaquettes doivent être générées quotidiennement.[122] Cette production peut être multipliée par 10 en cas de baisse subite du compte plaquettaire, suggérant une homéostasie réactive.[123] Ce processus, nommé thrombocytopoiëse, requière de nombreuses étapes durant lesquelles un seul mégacaryocyte peut libérer des milliers de plaquettes.[124, 125]

1.3.1.2 Mégacaryocytogénèse et thrombopoiëse

Les mégacaryocytes sont des cellules présentes dans la moelle osseuse dont la fonction est de produire et de libérer des plaquettes dans la circulation.[126] Tout comme les autres cellules sanguines,[127, 128] ils proviennent des cellules souches hématopoïétiques (HSCs). Les HSC peuvent soit s'auto-renouveler ou bien se diriger vers une lignée cellulaire spécifique, voie pour laquelle la cellule perd sa multipotence et restreint son potentiel développemental au fur et à mesure qu'elle se spécialise.[128] Ainsi, avant de devenir un mégacaryocyte, la cellule passe par différents stades, soit le progéniteur commun myéloïde, puis le

progéniteur mégacaryocyte/érythrocyte pour devenir progéniteur mégacaryocytaire. Cette régulation se fait via l'action de différentes cytokines, majoritairement la thrombopoïétine.[129]

Les précurseurs mégacaryocytaires vont subir deux mécanismes fondamentaux caractéristiques de la mégacaryocytopoïèse, soit la polyploïdisation par le processus d'endomitose et la maturation cytoplasmique. L'endomitose est une mitose incomplète,[130] menant à la formation de cellules polyploïdes, en moyenne 16 N, entrainant de ce fait la modification de l'expression génique et l'augmentation de la synthèse protéique.[131, 132] La maturation comprend la formation d'un réseau de membranes de démarcation, l'augmentation du volume du cytoplasme, la production de granules sécrétoires en particulier de type α et l'expression des protéines plaquettaires.[133] Ces évènements permettront aux précurseurs mégacaryocytaires d'effectuer la formation des proplaquettes.

La formation des proplaquettes implique les éléments du cytosquelette, notamment le système de microtubules,[134, 135] ainsi qu'un réarrangement du cytoplasme de façon à former une succession de renflements reliés par de fins ponts cytoplasmiques. Les mégacaryocytes vont ainsi produire des extensions ressemblant à un collier de perles jusqu'à l'utilisation complète du cytoplasme et des membranes. Lors de ce processus, le contenu des mégacaryocytes, incluant l'ARN messager (mRNA), les microARNs (miRNA) et les organelles sont transférés aux proplaquettes,[136-138] essentiellement grâce au réseau de microtubules et aux protéines associées. Les plaquettes, ayant un chimiotactisme faible, doivent être libérées directement dans le courant sanguin.[139] Les extensions vont alors s'insérer dans les jonctions de la paroi des vaisseaux sanguins, où des forces de cisaillement dues au débit sanguin vont poursuivre la fragmentation pour la production de plaquettes individuelles.[140]

1.3.1.3 Rôles dans la coagulation

Les plaquettes et les facteurs de coagulations ont un rôle majeur dans l'hémostase, en patrouillent les vaisseaux sanguins à la recherche de dommages

vasculaires, où ils s'activent.[141] La coagulation comporte une cascade de réactions enzymatiques impliquant les plaquettes et les facteurs de la coagulation **(Figure 1)**. Plus spécifiquement, les plaquettes utilisent leurs récepteurs de surface pour le collagène sous-endothélial et le facteur von Willebrand (vWf) lié au collagène,[141] ce qui induit leur adhésion, leur activation et leur agrégation pour permettre la formation du caillot.[142] La plaquette activée expose alors la phosphatydylsérine (PS) à sa surface, pour fournir un échafaud aux complexes enzymatiques de coagulation.[143] De façon concomitante, le facteur VII cible les sites d'exposition du facteur tissulaire (TF) pour démarrer la cascade de coagulation impliquant les facteurs de coagulation (I à XIII). Cette cascade induit la conversion de la prothrombine en thrombine, ce qui permet la transformation du fibrinogène soluble en filaments de fibrine.

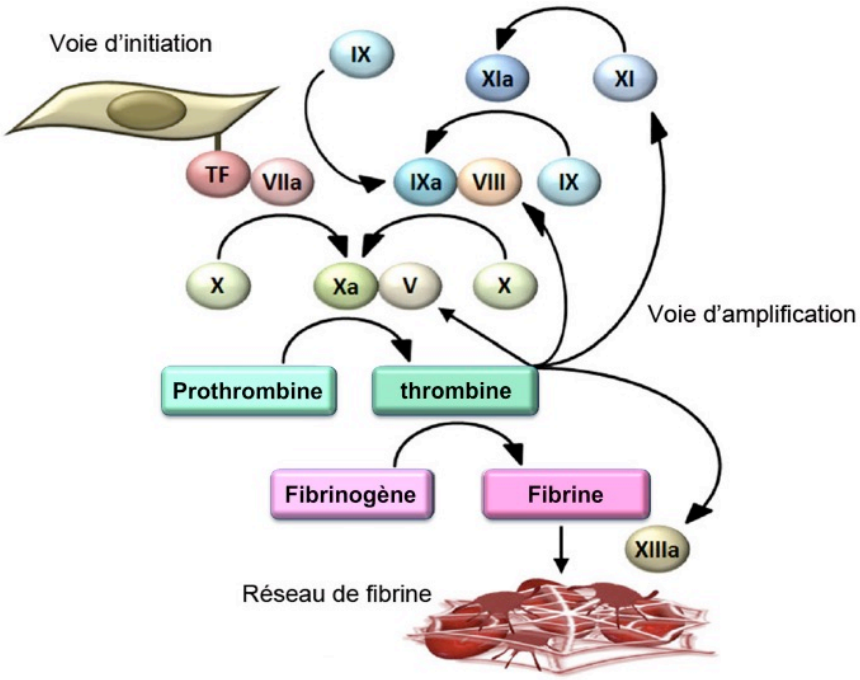

Figure 1: **Cascade de coagulation.** Le facteur de coagulation VII cible les sites de lésion vasculaire où le facteur TF est exposé. La liaison du facteur de coagulation VIIa au TF résulte en une cascade de réactions de coagulation du sang, conduisant à la génération de thrombine (voie d'initiation). Une fois que de petites quantités de thrombine sont générées par cette voie, la thrombine joue un rôle

crucial dans les phases d'amplification et de propagation de la coagulation par l'activation des facteurs de coagulation V, VIII et XI (voie d'amplification). Cela conduit à une augmentation de la génération de thrombine, ce qui est essentiel pour la formation de fibrine. Le facteur XIII réticule ensuite les fibres de fibrine, un processus fondamental pour la stabilisation des caillots. Traduite de Ito, T.[141]

1.3.1.4 Rôles dans l'immunité et l'inflammation

En plus de maintenir l'homéostasie, les plaquettes ont aussi un rôle important dans la réponse inflammatoire.[112] Elles sécrètent ou exposent à leur surface de nombreux médiateurs inflammatoires, immuno-modulateurs ou antimicrobiens. Parmi ceux-ci sont compris l'histamine, la sérotonine, le thromboxane A_2, le PAF, le PDGF et le TGF-β, différentes chimiokines (CXCL4/PF4, CCL5/RANTES, β-TG) et cytokines (CD40L, IL-1β).[144]

Des études récentes ont démontré que ces propriétés inflammatoires peuvent induire des dommages aux poumons.[145, 146] Lorsque les plaquettes sont inhibées, lors d'une thérapie anti-plaquettaire, elles atténuent les dommages médiés par leur association aux neutrophiles.[147]

Les plaquettes expriment de nombreux membres de la famille des TLR (*toll-like receptors*) incluant le TLR1, le TLR2, le TLR4, le TLR6, le TLR8, et le TLR9.[148] Les TLRs jouent un rôle critique dans l'immunité innée par la reconnaissance de PAMPs (*pathogen-associated molecular patterns*), des motifs moléculaires associés aux pathogènes indiquant la présence des micro-organismes envahisseurs. Des études antérieures indiquent également que les plaquettes se localisent aux sites de l'invasion bactérienne, s'accumulent dans les exsudats inflammatoires et ciblent les tissus qui sont sujets aux dommages médiés par une réaction antigène-anticorps.[146] Ces TLRs semblent avoir un rôle lors d'inflammation due à une infection et dans les maladies vasculaires athérosclérotiques.[148]

En plus d'exprimer différents TLRs, les plaquettes expriment un récepteur Fc pour les IgG (FcγR) soit le FcγRIIA.[149] Ce récepteur, en plus d'être important pour l'activation des plaquettes par le vWf,[150] contribue aussi à l'élimination des complexes contenant des IgG de la circulation.[151] Une altération des FcγRs contribue à la pathogenèse des certaines maladies auto-immunes comme le lupus érythémateux disséminé (SLE).[152] En plus de leurs rôles bien définis dans le déclenchement de l'activation des cellules effectrices innées, les FcyRs participent à la présentation antigénique et à la maturation médiée par le complexe immun des cellules dendritiques en plus de réguler l'activation des cellules B et la survie des plasmocytes.[153] Les plaquettes expriment aussi le récepteur FcεRI, un récepteur pour les IgE (FcεR).[154] La stimulation des plaquettes via le FcεRI induit la libération de sérotonine et de CCL5/RANTES, appuyant le rôle des plaquettes dans l'inflammation allergique.[154]

1.3.2 Procédés de préparation des PCs

Avant le début des années 1970, la seule source de plaquettes viables provenait de sang total frais.[155] Avec l'avancement de différentes technologies, il est maintenant possible de préparer des PCs de bonne qualité, facilitant ainsi la thérapie transfusionnelle.[156] De plus, l'isolation des autres composants permet de conserver les plaquettes dans des conditions optimales.[6] Les PCs peuvent être préparés soit par centrifugation du sang total ou par aphérèse en utilisant un séparateur de cellules automatisé.

1.3.2.1 Par centrifugation du sang total

Deux méthodes principales, distinctes principalement par les vitesses de centrifugation, sont utilisées pour préparer des PCs à partir de sang total, soit celle du Plasma Riche en Plaquettes (PRP) ou celle de la couche leucoplaquettaire (BC-*Buffy Coat*). La technique du PRP est utilisée majoritairement aux États-Unis et celle du BC en Europe et maintenant au Canada.[157] Dans les deux cas, environ 450 ml de sang sont prélevés puis mélangés avec 63 ml d'un

anticoagulant. Dans le cadre de ce projet de recherche, le citrate-phosphate-double-dextrose (CP2D) a été utilisé pour la technique PRP et le citrate-phosphate-dextrose (CPD) pour la technique du BC. Le sang est ensuite laissé au repos pour diminuer l'activation des plaquettes causée par le prélèvement.

Pour la technique du PRP, une première centrifugation avec une force g faible permet la séparation des érythrocytes du plasma riche en plaquettes (**Figure 2)**.[156] Cette dernière fraction est transférée dans une poche satellite puis soumise à une centrifugation avec une force g supérieur permettant l'obtention d'un culot de plaquettes. La majorité du plasma est transféré dans une autre poche satellite et le culot plaquettaire est conservé dans 40 à 60 ml de plasma.[121] Le concentré est gardé au repos de une à deux heures qui suivent la centrifugation,[158, 159] puis mis en agitation.

Les PCs préparés par la méthode du BC sont, quant à eux, centrifugés à une force g élevée,[160] ce qui permet d'obtenir trois phases, soit une phase d'érythrocytes, une phase qui comprend les globules blancs et les plaquettes (la couche leucoplaquettaire) et une phase qui contient le plasma **(Figure 2)**. Comme les plaquettes reposent sur une couche de globules rouges, cette méthode induit une activation moindre comparativement à la méthode PRP où les plaquettes sont en culot au fond de la poche.[161] La couche leucoplaquettaire est ensuite récupérée puis mise au repos pour 1 à 2 heures. Enfin, de 4 à 6 couches leucoplaquettaires compatibles sont regroupées avec une unité de plasma et centrifugées à basse vitesse et leucoréduits à l'aide d'un filtre pour éliminer les leucocytes et les érythrocytes résiduels.

Les concentrés de type PRP causent environ 3 fois plus de réactions transfusionnelles que les autres méthodes.[162] De plus, l'activation des plaquettes, démontrée par l'expression de CD40L et de CD62P,[163] est plus élevée pour les PRP que celles préparées par BC.[161, 164]

La fabrication de PCs à partir de sang total présente quelques avantages. Les plaquettes sont obtenues en combinaison avec des globules rouges et du plasma, ce qui amortit la perte si jamais le PC ne peut être utilisé.[165] De plus, les risques pour le donneur de sang total sont moindres que pour les donneurs d'aphérèse.[166] Toutefois, pour obtenir une dose thérapeutique identique aux TH,[121] il est nécessaire de grouper plusieurs PRP ou BC, ce qui augmente les risques de contaminations et de réactions transfusionnels adverses.[167]

1.3.2.2 Par aphérèse

Dans le cas d'un PC préparé par aphérèse, le sang du donneur est prélevé à l'aide d'un cathéter puis acheminé dans l'appareil d'aphérèse afin que les plaquettes soient séparées des autres cellules sanguines qui retournent au donneur **(Figure 2)**. Les procédures de sédimentations diffèrent entre les appareils d'aphérèse pour la séparation des plaquettes,[168, 169] mais sont généralement basés sur la taille des cellules, ce qui génère un PC avec des plaquettes de taille inférieure à celles d'un BC ou d'un PRP.[170] Le processus est dit automatisé, car l'appareil prend en compte le poids du donneur, son compte plaquettaire et son hématocrite. De 4 à 5 litres de sang vont circuler dans l'appareil lors de la procédure qui dure jusqu'à 90 minutes.

L'obtention de PC par aphérèse présente aussi ses avantages. Il y a, par exemple, un risque plus faible d'allo-immunisation et de transmission de maladies au patient étant donné une réduction du nombre de donneurs.[171] Toutefois, les coûts associés à ce type de prélèvement sont beaucoup plus élevés et le donneur doit se déplacer au centre de prélèvement plutôt que de se présenter à une collecte mobile.[172]

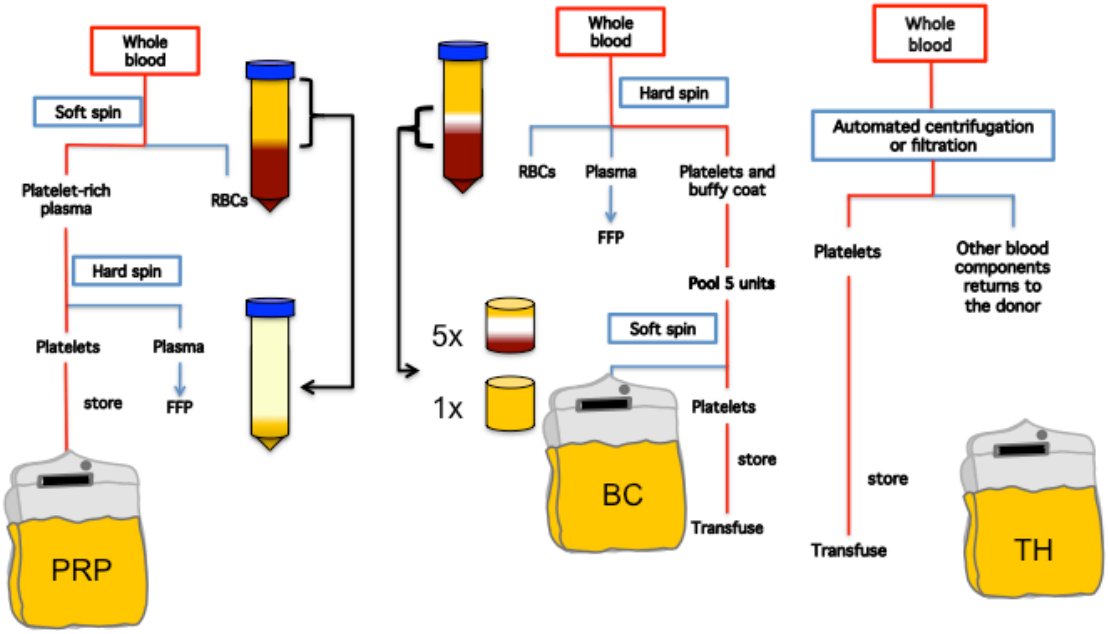

Figure 2: **Préparation des différents concentrés plaquettaires.** Le PRP est obtenu à partir du sang total à l'aide d'une centrifugation lente (2005g x 3 min 50 sec), où l'on récupère le plasma et les plaquettes, suivie d'une centrifugation rapide (4395g x 5 min) qui permet de concentrer les plaquettes. Le BC est obtenu par une centrifugation rapide qui permet d'isoler la fraction qui contient les plaquettes et les leucocytes. Cinq de ces fractions sont combinées à une fraction de plasma puis centrifugées à basse vitesse pour obtenir le BC. Le PC préparé par aphérèse (TH) est obtenu à partir d'un appareil automatisé.

1.3.3 Paramètres affectant la qualité des PCs

De nombreux facteurs affectent la qualité des plaquettes durant l'entreposage. En plus du procédé de fabrication abordé précédemment, il est important de prendre en considération le temps de repos, la température d'entreposage ainsi que la durée, la variation de pH, la poche contenant le PC, ce qui inclut aussi sa perméabilité aux gaz, le type d'agitation et l'utilisation ou non d'une solution d'entreposage.

1.3.3.1 Période de repos

Une période de repos d'une heure est recommandée avant de resuspendre les PCs.[173] Cette période permet de diminuer le niveau d'activation des plaquettes causé par la sédimentation[161, 163, 164] ainsi que d'améliorer leur morphologie.[174]

1.3.3.2 Température

Les PCs étaient initialement conservés à 4°C comme les globules rouges, mais leur fonction et leur viabilité étaient fortement compromises.[156] Les plaquettes sont actuellement entreposées entre 20 et 24°C, ce qui a fortement augmenté leur viabilité post-transfusion.[175] Des plaquettes cryopréservées dans le DMSO à -80°C sont aussi utilisées par l'armée pour prévenir les saignements.[176] Ces PCs contiennent plus de microparticules (MPs) et ont une expression plus faible de glycoprotéines plaquettaires importantes.[177]

1.3.3.3 Durée d'entreposage

Les PCs sont généralement entreposés pour 5 jours afin de maintenir leurs fonctions et limiter la prolifération bactérienne.[36, 175, 178] Dans les pays qui appliquent cette règle, il est estimé que près de 30% des PCs sont jetés avant d'être transfusés.[179] Leur durée de vie courte crée un problème pour leur disponibilité.

Une performance *in vivo* acceptable pour des plaquettes conservées 7 jours a été récemment rapportée.[180-182] De plus, les récents développements pour la préparation, combinés à l'introduction de nouvelles technologies pour détecter, réduire ou prévenir la contamination bactérienne rendent maintenant possible une prolongation de la période d'entreposage qui est maintenant appliqué dans plusieurs pays.[59, 183, 184]

1.3.3.4 La variation du pH

Il est bien connu que les plaquettes subissent des dommages et une perte de viabilité en association avec une variation du pH lors de l'entreposage.[185] Une forte concentration de plaquettes, combinée à une perméabilité à l'oxygène insuffisante de la poche de conservation, peut être responsable d'un pH faible.[186] En effet, une accumulation de lactate, produit de la glycolyse anaérobique, induit une baisse de pH qui provoque une détérioration et une perte de fonction des plaquettes.[187]

Une baisse de pH est aussi associée à des changements morphologiques marqués de la plaquette.[188-191] Une diminution en dessous de 6.7-6.8 entraine un passage de la forme discoïde à sphérique et un phénomène de gonflement.[189, 191-193] Ces changements sont, pour la majeure partie, réversible quand les plaquettes sont resuspendues dans du plasma frais à 37°C et à pH physiologique.[189] Toutefois, si le pH descend en dessous de 6.1, ces changements sont irréversibles.[185] Dans un pH alcalin (>7,3), des dommages tels que la lyse ou un gonflement des plaquettes peuvent être observés.[186] Bien que le pH soit encore utilisé pour évaluer la qualité des PCs, la majeure partie des facteurs qui affectent le pH sont bien controlés.[194]

1.3.3.5 Type de poche

Pour maintenir le pH lors de l'entreposage des plaquettes à 20-24 °C, il est nécessaire d'avoir des contenants avec une perméabilité suffisante à l'oxygène (O_2) et au dioxyde de carbone (CO_2).[187, 188] Les poches de première génération avaient une perméabilité pauvre à l'oxygène, limitant l'entreposage à 3 jours.[195] Les méthodes de traitement et d'entreposage des PCs ayant considérablement évolué, plus de vingt différentes poches pour la conservation des PCs sont maintenant disponibles sur le marché et sont fabriquées à partir de différentes matières plastiques avec des perméabilités aux gaz, des géométries et d'épaisseurs variables.[196] Les poches pouvant contenir un grand volume et une

pellicule plastique moins épaisse permettent un meilleur échange gazeux grâce à leur superficie accrue et donc un entreposage prolongé.[196]

1.3.3.6 Agitation

Une agitation lors de l'entreposage est considérée nécessaire pour une diffusion adéquate des gaz et pour la maintenance d'un pH adéquat.[185, 197] Les PCs entreposés sans agitation ont une morphologie et une recouvrance osmotique inférieure, ce qui indique une perte de fonction.[198] Cette procédure s'effectue donc de routine pour la conservation des PCs.[156, 185, 190]

Le type d'agitation affecte la qualité des plaquettes. Une forme douce d'agitation, comme un mouvement circulaire ou à plat (horizontal) donne les meilleurs résultats.[199] Une agitation de type elliptique, au contraire, entraine une libération plus grande de lactate deshydrogénase (LDH) et de β-TG.[198] De plus, comme cette agitation est plus vigoureuse, la morphologie de la plaquette s'en trouve affectée.[198] Une agitation constante peut aussi avoir des effets néfastes sur les plaquettes, causant leur activation et des dommages lorsque ces dernières entrent en contact entres-elles et avec les parois de la poche.[197]

Une interruption brève de l'agitation survient fréquemment lors du transport des PCs vers les hôpitaux. Il a été démontré qu'une interruption n'excédant pas 24 heures n'entraine pas ou peu d'effet sur les plaquettes.[200-203]

1.3.3.7 Solution d'entreposage

Les PCs sont généralement conservés dans du plasma. Toutefois, plusieurs solutions d'entreposage ont été développées et maintiennent aussi la structure et les fonctions des plaquettes.[204]

L'utilisation d'une solution d'entreposage présente plusieurs avantages : réduction des lésions plaquettaires, le retrait de certains composants du plasma pouvant

causé des réactions transfusionnelles tels que les MPS, certaines enzymes relâchées par la plaquette et les HLA solubles,[205] ainsi que l'augmentation de la quantité de plasma disponible pour les produits de fractionnement comme le facteur de coagulation VIII.[206] Toutefois, le nombre de plaquettes en circulation post-transfusion peut être diminuée.[207] À ce jour, les solutions d'entreposage ne remplacent pas parfaitement le plasma.

1.3.4 Lésion d'entreposage des plaquettes

Même sous des conditions optimales d'entreposage, il se produit des changements morphologiques, structuraux et fonctionnels des plaquettes entre le moment de leur collecte et le moment de leur transfusion à un patient. Ces altérations qui se produisent lors de l'entreposage des PCs sont nommées lésions d'entreposage des plaquettes (PSL) et résultent en une diminution progressive des fonctions hémostatiques et une survie *in vivo* réduite des plaquettes.[208] Les PSL comprennent le changement des protéines de surface des plaquettes,[209-211] un réarrangement du cytosquelette,[212] la perte de la forme discoïde ainsi que l'accumulation de cytokines.[100] L'activation des plaquettes et leur dégranulation se produit lors de l'entreposage, marqué par l'accumulation de *β*-TG, de PF4 et la présence à leur surface de CD62P et de PS,[213] Toutefois, les plaquettes perdent rapidement leur CD62P et continuent de circuler *in vivo*.[214] Enfin, une autre caractéristique des PSL due à l'activation des plaquettes est la production de MPs lors de l'entreposage.

1.4 Les vésicules extracellulaires

1.4.1 Description générale

Les vésicules extracellulaires sont formées d'une membrane sphérique de petite taille et sont dérivées de cellules à la suite de leur activation ou de leur apoptose. Elles sont divisées en 3 groupes selon leur taille **(Figure 3)**, leur composition et leur processus de formation. Ces différentes caractéristiques affectent leurs fonctions.[215]

Les exosomes ont une taille comprise entre 50 et 100 nm de diamètre, taille s'approchant de celle des virus et sont formés par l'exocytose des corps multi vésiculaire.[216] Ils sont libérés constitutivement ou par induction selon le type cellulaire.[215]

Les corps apoptotiques ont une taille est comprise entre 1 et 5 µm, chevauchant celle des plaquettes.[216] Ils sont issus de la fragmentation de cellules en apoptose.

Les MPs, également appelées microvésicules, sont des structures délimitées par une membrane et de taille comprise entre 100 nm et 1 µm.[216] Leur taille chevauche celle des bactéries et des complexes immuns **(Figure 3)**.[217] Les MPs sont le type de vésicules extracellulaires qui a été ciblé au cours des travaux de recherche et qui sera défini plus en détail dans la section suivante.

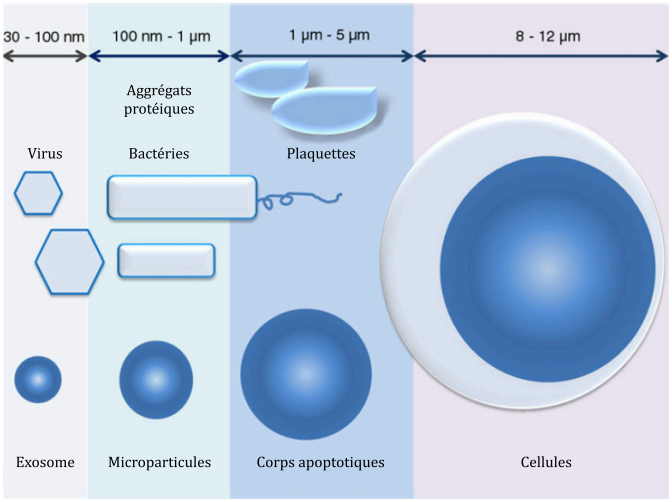

Figure 3: **Échelle de taille des vésicules extracellulaire.** Tirée de György, B. et al.[216] Taille respective des cellules et des différentes vésicules extracellulaires en comparaison avec d'autres éléments connus.

1.4.2 Les microparticules

1.4.2.1 Caractéristiques

Les MPs circulent dans le sang et peuvent provenir de différentes cellules vasculaires (plaquettes, monocytes, cellules endothéliales, globules rouges et granulocytes). Elles sont formées lorsque les lipides de la membrane plasmique perdent leur distribution asymétrique,[218] avec l'externalisation de la PS comme indicateur.[219] Leur formation implique aussi une augmentation du calcium intracellulaire ainsi qu'un réarrangement du cytosquelette qui contribue à l'asymétrie de la membrane.[219]

En plus de la PS, les MPs expriment des antigènes de surface et transportent des mRNA, des miRNA et des protéines qui proviennent de la cellule mère, suggérant que les différentes MPs pourraient avoir une fonction distincte selon la nature de la cellule de laquelle elles proviennent.[220]

1.4.2.2 Isolation et détection des microparticules

Différentes méthodes peuvent être utilisées pour isoler et détecter les MPs. En ce qui concerne leur isolation, elle présente différents obstacles. Le milieu extracellulaire duquel elles sont isolées est complexe, étant donné que plusieurs fluides corporels contiennent de l'ARN extracellulaire,[221] des agrégats protéiques ou des lipoprotéines qui ont des tailles similaires aux MPs,[222] et qui peuvent contaminer la préparation. Différentes méthodes peuvent être utilisées pour l'isolation, comme la centrifugation, l'ultracentrifugation par gradient de densité, des kits commerciaux,[221] ou la chromatographie d'exclusion de taille.[222]

Les différentes approches analytiques incluent l'analyse du suivi individuel de particules,[223] la microscopie électronique,[223] la microscopie à force atomique, les immunobavardages de type western, la cytométrie en flux (FACS) ou des analyses protéomiques globales utilisant la spectrométrie de masse. [221] Le FACS est toutefois l'approche la plus largement utilisée pour la détection des MPs.[220] Un cytomètre conventionnel offre une faible résolution pour des MPs de taille inférieures à 0,5 µm.[224] Heureusement des cytomètres de haute sensibilité ont été développés et offrent une résolution suffisante pour différentier les sous-types de MPs, incluant ceux qui contiennent des organelles.[225, 226]

1.4.2.3 Fonctions physiologiques et pathologiques des microparticules

Les MPs ont de nombreuses fonctions. Entre autres, elles ont une activité procoagulante.[227, 228] En effet, elles adhèrent au collagène de type I, au fibrinogène, au vWf et à la surface des plaquettes,[229]et elles accélèrent la génération de thrombine.[230] Elles exposent de la PS qui est importante pour l'hémostase, tel que montré par le syndrome de Scott et le défaut de Castaman, deux troubles de la coagulation qui sont caractérisés par une incapacité à former des MPs qui exposent la PS.[231, 232] Elles expriment aussi du TF actif, soit la protéine transmembranaire qui, avec le facteur VII, permet l'initiation de la cascade de coagulation *in vivo*. [233]

Les MPs peuvent aussi jouer un rôle dans la communication intracellulaire grâce aux différents éléments qu'elles transportent (facteurs de croissance, protéines, organelles dont des mitochondries, etc).[216, 234] Par exemple, les MPs peuvent réguler les cellules à des niveaux post-transcriptionnels par l'échange d'acide nucléique (mRNA, miRNA et autres types d'ARN).[235-237]

Les MPs ont aussi un rôle dans l'inflammation en libérant de nombreux médiateurs pro-inflammatoires. En effet, les MPs de diverses origines cellulaires induisent la production de différentes cytokines. Par exemple, des MPs de leucocytes peuvent induire la production d'IL-6 par les cellules endothéliales,[238] mais aussi de CXCL8/IL-8 et de MCP-1 (*Monocyte Chemoattractant Protein*) en plus d'exprimer des molécule d'adhésion comme la E-sélectine, ICAM-1 (*InterCellular Adhesion Molecule*) et VCAM-1 (*Vascular Cell Adhesion Molecule*).[239] Les MPs peuvent aussi transporter certains de ces médiateurs comme l'IL-1β ou CCL5/RANTES.[240, 241]

Les MPs de différentes origines cellulaires sont détectées en quantité supérieure dans les fluides de patients atteints de maladies auto-immunes. Par exemple, les MPs de plaquettes et de cellules endothéliales sont en concentration supérieures chez les patients atteints du syndrome anti-phospholipides, une maladie immunitaire qui entraine des complications thrombotiques et obstétriques.[242] Les MPs de plaquettes jouent aussi un rôle dans l'arthrite,[243] où elles sont très abondantes dans le liquide synovial des patients. Les MPs de plaquettes sont aussi plus nombreuses chez les patients atteints de lupus érythémateux systémique.[244]

1.4.3 Les microparticules de plaquettes

Les MPs de plaquettes, anciennement appelées poussière de plaquettes,[245] représentent environ 80% des MPs circulantes.[246] Elles sont formées lors de l'activation des plaquettes par divers agonistes comme la thrombine, le collagène et le complément,[247, 248] mais aussi en absence d'agoniste lors de

l’entreposage des plaquettes. [177, 206, 210, 225, 228, 249-254] Lors de leur genèse, elles peuvent apporter avec elles des mitochondries, abondantes dans les plaquettes et situées à proximité de la membrane plasmique.[225] En effet, les PCs contiennent une population hétérogène de MPs qui peut être divisée en trois catégories,[225] soit les MPs qui ne contiennent pas de mitochondries (nommées MPs), les MPs qui contiennent une ou des mitochondries (mitoMPs) et finalement les mitochondries extracellulaires, qui ne sont pas entourées d’une membrane de plaquette(freeMitos). Bien que les freeMitos ne répondent pas à tous les critères d’une MP (principalement concernant le mécanisme de genèse) elles seront considérées ici comme une catégorie de MP.

1.5 La mitochondrie

1.5.1 Structure

La mitochondrie possède une membrane interne, site de la respiration cellulaire, qui est repliée de façon à former de nombreuses invaginations. Elle possède aussi une membrane externe qui est formée de nombreuses porines pour permettre le passage de différentes molécules. Bien que la majorité des protéines mitochondriales soient encodées par les gènes nucléaires, certaines restent encodées par la mitochondrie.[255, 256] Pour que les protéines nucléaires puissent être importées dans la mitochondrie, elles doivent passer par deux translocases différentes. La première est la translocase de la membrane externe (TOM) et la seconde celle de la membrane interne (TIM).[257] Le complexe TOM permet de faire le lien entre le cytosol et l'espace intermembranaire.[257] Il est formé de différents composants (**Figure 4**), dont le TOM22, un récepteur de surface utilisé pour isoler les mitochondries extracellulaires dans les articles de ce mémoire. La mitochondrie possède son propre génome qui s'apparente a celui de *Rickettsia prowazekii*.[256] L'ADN mitochondrial (mtDNA) est une molécule circulaire, double-brin, de 16 569 paires de bases qui codent pour 2 ARN ribosomaux, 22 ARN de transfert et 13 protéines,[258] et qui possède des îlots CpG non-méthylés.[259]

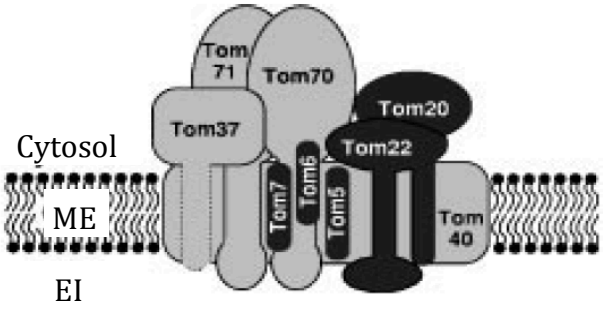

Figure 4 : Représentation schématique du complexe TOM. Schémtisation des différents éléments formant le complexe TOM et leur positionnement. Le récepteur TOM22 est situé à la surface de la mitochondrie. Abbréviation : ME, membrane externe; EI, espace intermembranaire. Traduite de Neupert, W.[257]

1.5.2 Fonction des mitochondries

1.5.2.1 La production d'énergie

La mitochondrie possède le système enzymatique qui permet la conversion des sucres, des graisses et des protéines en une forme utilisable d'énergie qui est l'ATP.[260] Ces trois substrats entrent dans le cycle de l'acide citrique **(Figure 5)**, aussi connu sous le nom de cycle de Krebs, sous forme d'acetyl-CoA, où ils seront convertis en nicotinamide adénine dinucléotide (NADH) et flavine adénine dinucléotide (FADH). Les électrons récupérés lors des réactions d'oxydoréduction permettent à la pompe à protons de générer une force protomotrice pour alimenter la synthèse d'ATP.[255]

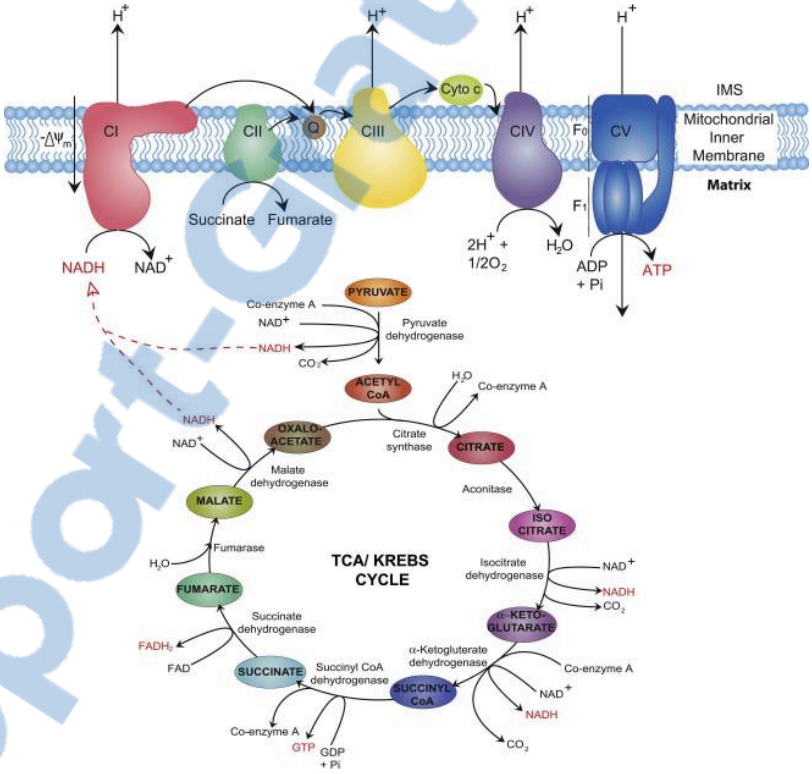

Figure 5 : Bioénergétique du cycle de Krebs et de la chaine de transport d'électrons. Le pyruvate est converti en molécules fortes en énergie comme le NADH, le GTP et le $FADH_2$ (en rouge) par les enzymes du cycle de Krebs. Le NADH généré est transféré au complexe I (CI) et est converti en NAD+. Puis le transfert d'électrons maintient le potentiel membranaire en pompant les protons

dans l'espace intermembranaire (IMS), pour former l'ATP par phosphorylation de l'ADP par le complexe V (CV : ATP synthase). Tirée de Osellame[255]

1.5.2.2 La biosynthèse des hormones stéroïdes

Le cytochrome P450, situé dans la membrane interne de la mitochondrie, permet la conversion du cholestérol en prégnénolone, un précurseur commun à toutes les hormones stéroïdes.[261]

1.5.2.3 L'homéostasie du calcium

Les mitochondries sont en communication constante avec le cytosol afin de coordonner l'équilibre entre la demande d'énergie de la cellule et la production d'énergie par la phosphorylation oxydative. Cette communication s'effectue principalement à l'aide du calcium, qui permet un état d'activation des cellules et gouverne plusieurs processus qui requièrent une demande importante d'énergie telle que la motilité.[255] La membrane interne de la mitochondrie possède un canal sélectif, le MCU (*mitochondrial calcium uniporter*), qui permet d'emmagasiner le calcium.[262]

1.5.2.4 L'apoptose

Les mitochondries jouent également un rôle majeur dans la mort cellulaire en participant activement à l'apoptose. Elles régulent la mort cellulaire en intégrant des signaux pro-apoptotiques via la famille de protéines BCL-2 (*B-cell lymphoma 2*).[263] Ces signaux contrôlent l'intégrité de la membrane externe de la mitochondrie,[264] pouvant causer la libération de facteurs apoptotiques comme le cytochrome c (Cyt c).

1.5.3 DAMPS

La mort cellulaire ou certains dommages aux cellules peuvent entrainer une libération ou une exposition de molécules intracellulaires appelées DAMPs qui sont reconnues par le système immunitaire. Récemment, la mitochondrie s'est révélée être elle aussi une source importante de DAMPs (**Figure 6**), puisqu'elle possède plusieurs caractéristiques communes avec les procaryotes.

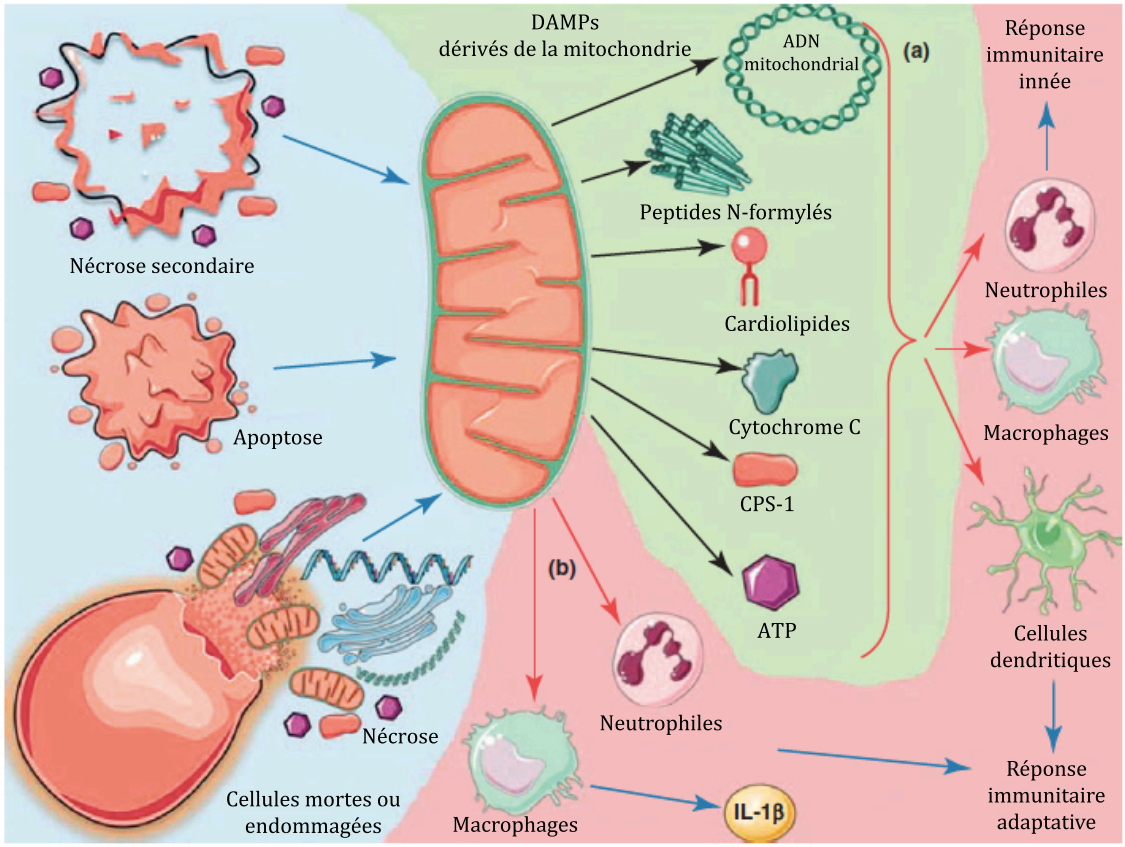

Figure 6. DAMPs dérivés de la mitochondrie. La mitochondrie est une source de DAMPS qui peuvent être libérés lors de différents processus de mort cellulaire (apoptose, nécrose secondaire ou nécrose) ou une lésion tissulaire (section en bleu). Ces DAMPs comprennent la mtDNA (mitDNA), des peptides formylés, des cardiolipides, du cytochrome c, du carbamoyl-phosphate synthétase (CPS-I) et de l'ATP (section en vert). Une fois que les DAMPs sont libérés dans l'espace extracellulaire, ils peuvent stimuler la réponse innée ou adaptative (section en rose). Adaptée de Krysko et al.[265]

1.5.3.1 La mtDNA

La présence de mtDNA est associée à de nombreuses conditions physio-pathologiques comme le sepsis,[266, 267], le cancer du sein,[268] des dommages aux organes[269, 270] et l'arthrite.[271] Elle peut aussi être liée à l'évolution clinique chez les patients gravement blessés.[272-275] Il y a aussi présence de

mtDNA dans les produits sanguins, incluant les PCs.[225, 276] Via le TLR9, la mtDNA peut induire une réponse immunitaire et être un médiateur de ces différentes physio-pathologies, mais aussi être impliquée dans le développement de certaines réactions transfusionnelles comme le TRALI.[276]

1.5.3.2 Les peptides formylés

Les peptides formylés sont aussi une caractéristique procaryotique et activent les récepteurs de peptides formylés pour induire une réponse pro-inflammatoire.[277] Ces peptides s'accumulent aux sites de dommages tissulaires et jouent un rôle dans l'accumulation de cellules inflammatoires comme les neutrophiles.[278]

1.5.3.3 Les cardiolipides

Les cardiolipides (CL) sont des phospholipides négativement chargés situés dans la membrane interne des mitochondries,[279] et qui permettent l'ancrage du cytochrome c.[280] Les CL peuvent s'exposer à la surface des cellules apoptotiques et sont reconnus par des anticorps antiphospholipides purifiés de patients atteints du syndrome des antiphospholipides.[281]

1.5.3.4 Le cytochrome c

Le Cyt c est une protéine de la chaine de transport d'électrons, donc unique aux mitochondries, qui est située dans l'espace intermembranaire et qui est transférée dans le cytoplasme des cellules tôt lors de l'apoptose.[282] C'est aussi un autoantigène ciblé par les anticorps de certains patients ayant une autoimmunité systémique,[283] comme le SLE ou l'arthrite.[284, 285]

1.6 Objectifs

Les plaquettes favorisent l'hémostase en jouant un rôle important dans la coagulation, mais elles ont aussi un rôle dans l'immunité. Lors de leur activation, elles produisent des microparticules qui peuvent induire la production de différents médiateurs inflammatoires, mais aussi agir comme transporteur de différentes protéines et ARN. Dépourvues de noyau, elles contiennent des mitochondries fonctionnelles. Comme l'ADN mitochondrial (mtDNA) est un déclencheur inflammatoire très puissant, nous avons émis l'hypothèse que les plaquettes activées pourraient libérer leurs mitochondries.

Le but de cette étude vise d'une part à approfondir les connaissances fondamentales sur les microparticules de plaquettes et leurs rôles dans l'inflammation. Plus précisément, nous nous sommes intéressés aux différents types de MPs, c'est-à-dire les MPs, les mitoMPs et les freeMitos, afin de mieux comprendre comment les mitochondries extracellulaires peuvent conduire à des réponses inflammatoires. Pour ce faire, nous avons vérifié si les mitochondries pouvaient sortir des plaquettes et rester fonctionnelles dans le milieu extracellulaire. Nous avons aussi vérifié leur présence dans des liquides synoviaux de patients arthritiques et dans des concentrés plaquettaires pour transfusion où elles ont été détectées en concentration supérieure dans les concentrés plaquettaires ayant causé des réactions transfusionnelles.

En Amérique du Nord, les concentrés plaquettaires sont actuellement conservés 5 jours afin d'assurer le maintien des fonctions plaquettaires et de prévenir la contamination bactérienne. Pour valider ce délai, les MPs ont été étudiées pour des PCs de type PRP, BC et TH. Les PCs ont été suivis sur une période de 7 jours pour les paramètres biochimiques, les marqueurs d'activation, les MPs (incluant les mitochondries extracellulaires) et l'ADN mitochondrial extracellulaire.

Chapitre 2: Les plaquettes libèrent des mitochondries servant de substrat pour la phospholipase bactéricide A2 sécrétée du groupe IIA afin de promouvoir l'inflammation

2.1 Résumé

Les plaquettes sont abondantes dans le sang où elles favorisent l'hémostase. Dépourvues de noyau, elles contiennent des mitochondries fonctionnelles et produisent des microparticules lors de leur activation. Comme l'ADN mitochondrial (mtDNA) est un déclencheur inflammatoire très puissant, nous avons émis l'hypothèse que les plaquettes pourraient libérer leurs mitochondries lors de l'activation. En effet, nous avons montré qu'elles libèrent différents sous-types de microparticules, incluant des mitochondries extracellulaires. Celles-ci se trouvent dans les concentrés plaquettaires utilisés pour la transfusion et sont présentes à des niveaux supérieurs dans ceux qui provoquent des réactions transfusionnelles. La membrane mitochondriale peut être hydrolysée par la phospholipase A2 sécrétée IIA, ce qui libère des médiateurs inflammatoires favorisant l'activation des leucocytes. Lors d'une transfusion chez l'animal, les mitochondries extracellulaires interagissent avec les neutrophiles *in vivo* et déclenchent leur adhésion à la paroi endothéliale. Nos résultats ont permis d'identifier les mitochondries extracellulaires au centre d'un mécanisme puissant conduisant à des réponses inflammatoires.

Platelets release mitochondria serving as substrate for bactericidal group IIA secreted phospholipase A2 to promote inflammation

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2.2 Abstract

Mitochondrial DNA (mtDNA) is a highly potent inflammatory trigger and is reportedly found outside the cells in blood in various pathologies. Platelets are abundant in blood where they promote hemostasis. While lacking a nucleus, platelets contain functional mitochondria. Upon activation, platelets produce extracellular vesicles known as microparticles. We hypothesized that activated platelets could also release their mitochondria. We show that activated platelets release respiratory-competent mitochondria, both within membrane-encapsulated microparticles and as free organelles. Extracellular mitochondria are found in platelet concentrates used for transfusion and are present at higher levels in those that induced acute reactions (febrile non-hemolytic reactions, skin manifestations and cardiovascular events) in transfused patients. We establish that the mitochondrion is an endogenous substrate of secreted phospholipase A_2 IIA (sPLA$_2$-IIA), a phospholipase otherwise specific for bacteria, likely reflecting the ancestral proteobacteria origin of mitochondria. The hydrolysis of the mitochondrial membrane by sPLA$_2$-IIA yields inflammatory mediators (i.e. lysophospholipids, fatty acids and mtDNA) that promote leukocyte activation. Two-photon microscopy in live transfused-animals revealed that extracellular mitochondria interact with neutrophils *in vivo*, triggering neutrophil adhesion to the endothelial wall. Our findings identify extracellular mitochondria, produced by platelets, at the midpoint of a potent mechanism leading to inflammatory responses.

Key points:

1) When activated and in platelet storage bags, platelets release respiratory-competent mitochondria, a recognized damage-associated molecular pattern.
2) Mitochondria, descendant of *Rickettsia prowazekii*, serve as substrate for bactericidal sPLA$_2$-IIA to promote inflammation.

2.3 Introduction

Platelets are small (2-4 μm) discoid anucleate cell fragments released by megakaryocytes present in the bone marrow.[1] During this process, megakaryocytes transfer components to daughter platelets, including messenger RNA[2] and microRNA[3] as well as cytoplasmic organelles, such as granules and mitochondria.[4,5]

Platelets are highly abundant in blood where they promote hemostasis.[6] However, platelets are also activated in multiple inflammatory responses in which they participate via the liberation of their broad arsenal of mediators.[7,8] Upon platelet activation, platelet cytoplasmic granules (alpha, dense and lysosomes) fuse with the plasma membrane leading to the release of granule contents into the extracellular milieu.[9] Activated platelets also shed thrombotic and pro-inflammatory membrane vesicles termed microparticles (MPs).[7]

Mitochondria are thought to be descendant of the Alphaproteobacterium Rickettsia prowazekii[10], and as a result they can generate highly potent pro-inflammatory signals when present extracellularly.[11-15] In blood circulation, platelets appear as a major reservoir of mitochondria. Although platelet mitochondria have been implicated in platelet activation and thereby thrombosis,[16,17] whether platelets can release their mitochondria is unknown. We made the surprising observation that stimulated platelets relocate mitochondria toward the cell membrane and then release respiratory-competent mitochondria into the extracellular milieu, both as free organelles and encapsulated within microparticles. Extracellular mitochondria are internalized by bystander leukocytes and, importantly, become a substrate for bactericidal secreted phospholipase A_2, leading to the liberation of pro-inflammatory lipid mediators and mitochondrial DNA (mtDNA). Taken together our work identifies a novel mechanism by which platelets mediate inflammation, with considerable relevance to blood transfusion, and provides a potential explanation

for the increased levels of extracellular mtDNA reported in blood in multiple pathologies.[13,18,19]

2.4 Methods

More details are presented in **supplementary materials and methods section**.

2.4.1 Mice

All studies were approved by the institutional review board protocol (CRCHUQ, Université Laval). Guidelines of the Canadian Council on Animal Care were followed in a protocol approved by the Animal Welfare Committee at Laval University. For our studies, we used 6-10 week old male mice (C57BL/6N and BALB/c, Charles River). For *in vivo* experiments in which $sPLA_2$-IIA contribution is evaluated, we used C57BL/6J (Jackson laboratories) and $sPLA_2$-IIA sufficient mice as previously reported.[20]

2.4.2 Cells and human fluid preparation

Blood was obtained from healthy human volunteers (citrate as anticoagulant) under an approved institutional review board protocol (CRCHUQ, Université Laval) and in accordance with the Declaration of Helsinki. Platelets, platelets microparticles (96% of them expressing CD41), and human polymorphonuclear leukocytes were prepared as previously described.[21] Platelet-free plasma was obtained from platelet storage bag preparations as previously reported.[22] Briefly, leukoreduced platelet concentrates were prepared from 6 healthy blood donors and incubated for 5 days at 20-24°C with agitation. Platelet-free plasma samples were obtained on day 0, 1 and 5 and were monitored immediately after collection (without freezing). Increase in platelet P-selectin expression was less than 2% during total storage time (day 1 vs day 5). Synovial fluids of rheumatoid arthritis and osteoarthritis patients were obtained from volunteers under the approval of the institutional review board protocol (Brigham and Women's Hospital) and were used to assess mitoMPs. The freshly obtained synovial fluid of rheumatoid arthritis patients (**Supplementary Table 1**) was cleared of leukocytes by centrifugation at 1,900g for 30 min at 4°C.

2.4.3 Isolation of mouse liver mitochondria

Mitochondria were isolated from the liver of C57BL/6N mice with the Qproteome mitochondria isolation kit (QIAgen) according to the manufacturer's protocol. The mitochondria pellet was resuspended in Tyrode Buffer pH7.4 (134 mM NaCl, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 12 mM $NaHCO_3$, 20 mM HEPES, 1 mM $MgCl_2$, 5 mM glucose and 0.5 mg/ml BSA), labeled with MitoTracker® Deep Red (100 nM, Invitrogen) and counted by flow cytometry on a BD FACS Canto II SORP small particle option.

2.4.4 High sensitivity flow cytometry (hs-FCM) configuration

A forward scatter (FSC) coupled to a photomultiplier tube (PMT) "small particles option" (FSC-PMT) (rather than the usual diode) with a 488 nm solid state, 100 mW output blue laser (rather than the conventional 20 mW), a 633 nm HeNe, 20 mW output red laser and a 405 nm solid state diode, 50 mW output violet laser were mounted on the BD FACS Canto II Special Order Research Product used for all our studies (BD Biosciences). The chosen parameters were optimal to detect particles from 100 to 3,500 nm simultaneously on the FSC-PMT.

2.4.5 Platelet stimulation

Platelets were labeled with MitoTracker® Deep Red and PKH67 Green Fluorescent Cell Linker as described above. Platelets were washed, and then were left non-activated or activated using heat-aggregated IgG (HA-IgG) 1 mg/ml; thrombin 0.5 U/ml; collagen 5 µg/ml; CRP-XL 1 µg/ml or PMA 100 nM for 4 h at RT. Platelets (20 µl) were then diluted into 500 µl of PBS and analyzed by flow cytometry. For extracellular mitochondria release experiments, platelets were treated with cytochalasin B (20 µM, Sigma-Aldrich), cytochalasin D (1 µM, Sigma-Aldrich), cytochalasin E (4 µM, Cayman Chemical), latrunculin A (10 µM, Cayman Chemical) or nocodazole (5 µM, Sigma- Aldrich). To process the data quantitatively, 100,000 polystyrene microsphere (15 µm diameter) (Polysciences) were added to each tube, and 1,000 microspheres were acquired. The included

MitoTracker+ and/or PKH67+ events were portrayed as FSC-PMT versus side scatter (SSC) graph, and the relative dimensions were displayed according to the acquisition of Sky Blue microspheres of mean diameter of 90, 220, 450, 840 and 3,200 nm (Spherotech).

2.4.6 Mitochondrial activity

Oxygen consumption was measured with mitochondrial preparations (final concentration of ~0.15 mg protein/ml) using a temperature-controlled polarographic O_2 monitoring system with 1 ml chambers (Rank Brothers Ltd). Temperature was maintained at 37°C by a circulating refrigerated water bath (Haake G8, Polyscience). The oxygen probes were calibrated with air-saturated reaction buffer and corrected for temperature and atmospheric pressure. All components were dissolved in reaction buffer (140 mM KCl, 20 mM HEPES and 5 mM K_2HPO_4, pH 7.3 with 0.5% fatty acid free bovine serum albumin), except rotenone and antimycin A that were dissolved in 95% ethanol.

Oxygen consumption due to flux through complexes I-IV was estimated from rates of pyruvate + malate + succinate oxidation (3.45 mM, 0.37 mM and 6 mM) in the presence of 5 mM ADP and corrected for residual rates after inhibition of complex I by rotenone (1 µg/ml final concentration) and complex III by antimycin A (5 µg/ml final concentration). Preliminary experiments showed that 5 mM adenosine diphosphate was sufficient to maintain state 3 rates for the time required for the complete series of measurements. Preliminary experiments also established optimal substrate and inhibitor concentrations for these measurements from platelet mitochondria.

Mitochondria membrane potential was performed as described in the JC-1 Mitochondrial Membrane Potential Detection Kit protocol (Cayman Chemical). Platelet supernatant was incubated with JC-1 (1/10) as recommended by the manufacturer and with 5 µl of anti- CD41a-APC (BD Biosciences) for 30 min at

37°C. Samples were diluted and analyzed using hs-FCM.

2.4.7 Air pouch model

Air pouch model was performed on C57BL/6N mice as previously reported.[23] Briefly, sterile air was subcutaneous injected on day 0 and 3. TNF-α (50 ng) was injected into pouch on day 6. On day 7, mitochondrial membrane hydrolysis products (100 µl of 5 x 10^8 mitochondria treated with $sPLA_2$-IIA), diluent (PBS), $sPLA_2$-IIA or mitochondria alone were injected in the air pouch. After 4 h, air pouch was washed with 1.2 ml of PBS. Cytokines were quantified using BD Cytometric Bead Array System (CBA) (BD Biosciences) by flow cytometry on a BD FACS Canto II and analyzed using FCAP Array Software v3.0.

2.5 Results

2.5.1 Distribution of mitochondria in platelets

Using fluorescence and TEM, we found that unactivated platelets contain an average of ~4 mitochondria, frequently located in the vicinity of the plasma membrane (**Figure 1AC, Supplementary video 1** and **Supplementary Figure 1A**). Remarkably, a fraction of these mitochondria promptly localize in pseudopodia upon activation by thrombin, a serine protease that participates in blood coagulation (**Supplementary video 2** and **Supplementary Figure 1B**).

2.5.2 Activated platelets release functional mitochondria

In addition to promoting release of granule contents,[24] platelet activation triggers cytoplasmic membrane budding and the shedding of submicron vesicles called MPs.[7,25] Taking into account the localization of mitochondria in the vicinity of the cytoplasmic membrane, we hypothesized that mitochondria may be packaged within MPs and form mitochondria-containing microparticles (mitoMPs).

Mitochondria are recognized as the powerhouse of the cell, producing the energy (ATP) required for most metabolic reactions, mostly via oxidative phosphorylation.[26] To determine whether platelets release mitochondria, we first evaluated specific mitochondrial O_2 consumption, mediated by flux through complexes I-IV, using specific inhibitors of complexes I and III (roteonone and antimycin A respectively). Unless mitochondria have been isolated and free in the milieu, permeabilization of the cytoplasmic membrane is necessary to allow the access of exogenous substrates added exogenously to stimulate mitochondrial respiration.[27,28] To evaluate respiration by putative mitoMPs in platelet-free supernatants, we thus used an established permeabilization method for our assays.[28] We found that the supernatant from activated platelets harvested by centrifugation (cell-free, **Supplementary Figure 2**) consumes O_2 (**Figure 2A**). In contrast, the supernatant from isolated resting platelets exhibited no detectable O_2 consumption (**Figure 2A**). Quite unexpectedly, significant O_2 consumption was

detected even in the absence of detergent (**Figure 2A**). These observations suggest that, in addition to active mitoMPs, platelets may also release respiration-competent free mitochondria (freeMito) into the extracellular milieu (**Figure 2B**).

We next examined the presence of freeMitos and mitoMPs, using a series of quantitative and qualitative approaches. Using a monoclonal antibody directed against a specific mitochondrial outer membrane receptor (TOM22, **Supplementary Figure 3**), we found intact freeMitos in the supernatants from thrombin-activated platelets, quantified by a PCR approach targeting mtDNA sequences (**Figure 2C**). TEM and confocal fluorescence microscopic analyses using fluorescent dyes to discriminate the plasma membrane and mitochondria further establish the production of freeMitos and mitoMPs by activated platelets (**Figure 2D,E**).

While the transfer of organelles from megakaryocytes to platelets is mediated by cytoskeleton components[4], we assessed whether the cytoskeleton is also involved in the extrusion of mitochondria from platelets. Using actin and tubulin polymerization inhibitors along with hs-FCM[29] to resolve the submicron particle populations (i.e. MPs, mitoMPs and freeMitos, **Figure 2B**) produced by platelets, we observed that the release of mitochondria (freeMitos and mitoMPs) implicates actin and occurs independently of microtubules (**Figure 2F,G**). Thus, via cytoskeletal contribution, activated platelets are a source of mitoMPs and respiration-competent freeMitos.

Thrombin is a highly potent agonist of platelet activation, and we next aimed at determining whether thrombin as well as other well-recognized platelet stimuli (**Supplementary Figure 4A,B**) promote the release of extracellular mitochondria. We found that all the platelet stimuli tested lead to the production of both mitoMPs and freeMitos (**Figure 2H**). Interestingly, freeMitos were found to fulfill the current structural definitions of conventional MPs. In fact, freeMitos are smaller than intact platelets, have submicron dimensions and a membrane moiety (**Figure 2F**),

providing an explanation for the recognized heterogeneity found among platelet-derived MPs.[30]

2.5.3 Relevance of extracellular mitochondria in health

The broad diversity of stimuli capable of promoting concomitant release of mitochondria and MPs points to the biological relevance of this phenomenon. We thus sought to determine whether mitochondria are released *in vivo* in sterile inflammatory pathologies where platelet MPs are known to be produced. Using hs-FCM, anti-TOM22 coprecipitation of mtDNA, as well as TEM, we could detect significant levels of extracellular mitochondria in synovial fluid from patients with rheumatoid arthritis (RA) (**Figure 3A-C**), consistent with the accumulation of platelet MPs[29,31,32] and mtDNA[33] reported in RA synovial fluid. For comparison, lower concentrations of extracellular mitochondria of platelet origin ($CD41^+$ mitoMPs) were measured in the synovial fluid of osteoarthritis patients (**Figure 3A**), a joint disease in which platelet MPs are also less abundant.[31,32] Similar observations were made in bronchoalveolar lavage fluids from the experimental transfusion-related acute lung injury (TRALI) mouse model (**Supplementary Figure 5A,B**). While the identified CD41+ mitoMPs are very likely produced by platelets, damaged cells as well as activated mast cells are potential sources of freeMitos.[11,12] We thus endeavored to confirm the platelet origin of extracellular mitochondria in a relevant biological context where platelets have been reported to release MPs *ex vivo*. Given their pivotal functions in hemostasis,[34] platelet transfusion is frequently used to restore optimal platelet levels in thrombocytopenic patients. In contrast to red blood cell (RBC) concentrates, which are stored at ~4°C for up to 42 days, platelet concentrates used for transfusion are stored at 20-24°C. Adverse reactions [febrile nonhemolytic reactions (fever or chills), anaphylaxis, transfusion-related sepsis and TRALI] are more frequently observed with platelet than RBC transfusion.[35,36] It is generally thought that this difference may be due to the presence of bacteria in platelet concentrates grown at permissive temperatures. From a phylogenetic view, mitochondria are thought to have originated from the endosymbiosis of alphaproteobacteria (Rickettsiales) during the

early evolution of eukaryotic cells.[37] We hypothesized that extracellular mitochondria (organelles that are lacking in RBC) present in platelet concentrates might trigger adverse reactions similar to those observed with infectious agents.

We thus evaluated the presence of extracellular mitochondria in platelet concentrates used for human transfusion in the course of their conservation. As measured using the O_2 consumption assay, as well as by TOM22 co-precipitation of mtDNA, hs-FCM and TEM, we demonstrate significant levels of freeMitos and mitoMPs in platelet concentrates (**Figure 3D-G**). In keeping with the coupling of O_2 utilization with energy production, mitochondria present in MPs and free mitochondria in platelet concentrates display JC-1 dye aggregates, a cationic dye that accumulates in energized cell mitochondria (**Figure 3H**). Most importantly, we established that platelet concentrates that had been associated with adverse transfusion reactions in human recipients contain higher concentrations of extracellular mitochondria (**Figure 3I**). Thus, extracellular mitochondria, which have the alphaproteobacterium *Rickettsia prowazekii*[10] as an ancestor, are present in platelet concentrates used for transfusion, particularly in those that triggered transfusion-related incidents, and exhibit a significant degree of functionality.

2.5.4 The mitochondrion is an endogenous substrate for the bactericidal secreted phospholipase A2-IIA

What are the implications of the release of free mitochondria by platelets? Extracellular mitochondria are already well recognized as highly potent damage-associated molecular patterns (DAMPs), capable of mediating inflammation locally[12,14] as well as systematically[13,15] through their bacteria-like components (i.e. N-formylated peptides and mtDNA). In this study, we aimed to identify unprecedented roles for extracellular mitochondria in inflammation. The secreted phospholipase A_2 group IIA ($sPLA_2$-IIA), initially identified in platelets[38] and present abundantly in this cellular lineage (**Figure 4A**), hydrolyzes the sn-2 acyl bond of glycerophospholipids, resulting in the release of free fatty acids and lysophospholipids. $sPLA_2$-IIA is found in plasma and is induced in chronic and

acute inflammatory conditions.[39] Although the promotion of host defense via bacterial membrane hydrolysis is an established function for this enzyme[39], $sPLA_2$-IIA is only poorly active towards the plasma membrane of eukaryotic cells, including platelets[40], and its endogenous substrate in sterile inflammation has thus far remained unclear.

The ancestral similarities between bacteria and mitochondria[10,37] prompted us to examine whether the mitochondrial membrane is susceptible to hydrolysis by $sPLA_2$-IIA. We observed that $sPLA_2$-IIA binds mitochondria (**Figure 4B,C**) leading to the release of various lysophospholipids and free fatty acids (**Figure 4D,E**), and in so doing, severely affects mitochondrial structural integrity (**Figure 4F**). Similarly to bacteria, the mitochondrial genome is rich in unmethylated CpG motifs, a recognized DAMP[13] and which is found outside cells in multiple disorders.[13,18,19] To determine whether mitochondrial digestion by $sPLA_2$-IIA might lead to mtDNA release, we used confocal microscopy and an assay specifically designed to quantify soluble DNA. With this combination of approaches, we identified $sPLA_2$-IIA as an enzyme capable of promoting the liberation of mtDNA (**Figure 4G,H**), Thus, the mitochondrion is an endogenous substrate of $sPLA_2$-IIA and its hydrolysis leads to the generation of recognized proinflammatory signals (arachidonic acid[41], lysophospholipids[42] and mtDNA[13]). Further, this result raises the possibility that a previously unrecognized function of $sPLA_2$-IIA is to assist in the degradation of freeMitos released by platelets and potentially other cells.

2.5.5 Extracellular mitochondria interact with neutrophils

We next endeavored to identify a relevant cellular lineage that may be regulated by extracellular mitochondria. We found that fluorescent mitochondria intravenously injected in mice associate with neutrophils, a polymorphonuclear leukocyte cell lineage that plays key roles in inflammation[43] (**Figure 5A**). Intravital investigations in mice using two-photon microscopy demonstrated that extracellular mitochondria present in the bloodstream prompt neutrophil interactions with the vascular wall and rolling (**Figure 5B** and **Supplementary video 3**). Using qualitative scanning

electron microscopy, we found that extracellular mitochondria consistently lead human neutrophils to display striking ultrastructural features, similar to pseudopodia (**Figure 5C**). Together, these observations suggest that extracellular mitochondria can interact with neutrophils, thereby modulating the activities of the latter.

2.5.6 Inflammation is amplified by the combined action of extracellular mitochondria and sPLA2-IIA.

To address the possibility that the combination of $sPLA_2$-IIA and mitochondria might trigger activities other than those induced by either mitochondria or $sPLA_2$-IIA alone, we assessed whether $sPLA_2$-IIA and extracellular mitochondria could associate together with neutrophils. Interestingly, fluorescent $sPLA_2$-IIA and exogenously labeled mitochondria rapidly associate with human neutrophils and colocalize intracellularly (**Figure 6A,B**) through dynamin, clathrin, and caveolin-dependant endocytosis (**Figure 6C** and **Supplementary Figure 6**). Arachidonic acid (20:4, n-6) liberated by $sPLA_2$-IIA may contribute to the biosynthesis of inflammatory eicosanoids such as leukotrienes by neighboring leukocytes.[41] To determine whether the association of mitochondria, $sPLA_2$- IIA and neutrophils might promote cell activation, we measured the release of leukotriene B_4 (LTB_4) by neutrophils. We found that neutrophils produce copious amounts of LTB_4 when both extracellular mitochondria and $sPLA_2$-IIA are present (**Figure 6D**). Importantly, such LTB_4 production is strictly dependent on $sPLA_2$-IIA catalytic activity since it takes place even in presence of the $cPLA_2\alpha$ inhibitor pyrrophenone and it is not observed when a catalytically inactive $sPLA_2$-IIA mutant form is used (**Figure 6D** and **Supplementary Figure 7**).

Platelets are implicated in the activation of neutrophils and participate in the formation of neutrophil extracellular traps (NETs),[44] a recognized feature found in RA and transfusion adverse reactions like TRALI.[45,46] Interestingly, freeMitos are also NET inducers, a phenomenon that is amplified in the presence of $sPLA_2$-IIA (**Figure 6E**). Consistent with their recognized proinflammatory potency, the

different hydrolytic products derived from sPLA2-IIA activity (arachidonic acid, lysophospholipids and mtDNA) are all highly potent at inducing NETosis (**Figure 6E**). Thus, the sPLA2-IIA/mitochondria complex associates with neutrophils and promotes cellular activation that is dependent on sPLA$_2$-IIA activity.

We next examined the significance of this pathway in the inflammatory response *in vivo*. In this set of experiments, we used C57BL/6J mice (which naturally lack sPLA$_2$-IIA) to evaluate whether mitochondrial hydrolytic products are active. Consistent with our *in vitro* observations, we found that the products derived from freeMitos via human recombinant sPLA$_2$-IIA activity promote a rapid, significant decrease of body temperature (**Figure 7A**) and induce proinflammatory cytokine release when injected in sPLA$_2$-IIA-deficient mice (**Figure 7B**). Conversely, intact freeMitos injected in these mice elicited only a modest response, confirming that freeMitos can trigger an inflammatory response that is dependent on hydrolysis by sPLA$_2$-IIA. To determine whether endogenous sPLA$_2$-IIA can produce mediators from freeMitos *in vivo*, we examined the effect of intact freeMitos injected in transgenic C57BL/6J mice expressing sPLA$_2$-IIA. Interestingly, we found that the latter treatment induced a delayed but albeit significant lowering of body temperature (**Figure 7A**), suggesting that the endogenous sPLA$_2$-IIA expression is sufficient to promote inflammatory reactions. To determine whether freeMitos can modulate physiological processes in discrete organs, we evaluated their localization upon injection via tail vein. We observed that the bulk of injected freeMitos accumulate in the liver, kidney, lungs and lymph nodes (**Supplementary Figure 8A**). Consistent with the concomitant localization of sPLA$_2$-IIA (**Supplementary Figure 8B**) and neutrophils[47] in liver, freeMito accumulation in this organ triggers the expression of a broad variety of proinflammatory genes recognized as relevant to neutrophil functions (**Figure 7C**). Our observations demonstrate that the combined activity of extracellular mitochondria and sPLA$_2$-IIA generate inflammatory signals *in vivo*

2.6 Discussion

Mitochondrial components secreted from cells might act as autopathogens, a term first coined by Zhang and colleagues.[11] Owing to their numerous similarities to bacteria, extracellular mitochondria can stimulate the immune system and thereby trigger inflammation. Indeed, cell-free mtDNA levels are increased in blood in several pathologies and can be used as potent biomarker. Our study establishes that platelets can release functional mitochondria (free or shuttled via MPs), which can be transferred to other cells such as neutrophils.

The concentration of $sPLA_2$-IIA is increased in inflammatory exudates, such as in the plasma of septic shock patients and RA synovial fluid.[48,49] Consistently, the $sPLA_2$-IIA expressing transgenic mice used in our study[20] display higher levels of plasma $sPLA_2$-IIA and develop accelerated blood vessel inflammation and autoimmune arthritis.[49,50] While $sPLA_2$-IIA is a bactericidal enzyme that has been studied for decades, its endogenous substrates in sterile inflammation were thought to be limited to dying cells[51] and MPs.[52] Our study identifies mitochondria as an overlooked source of biologically active mediators that can be liberated via $sPLA_2$-IIA. In addition to arachidonic acid conversion to eicosanoids[41], other lipid mediators may also promote inflammation (**Figure 7D**). Lysophospholipids are released through $sPLA_2$-IIA activity towards the mitochondrial membrane and can trigger inflammation.[39,42] In the context of platelet transfusion, lysophospholipid levels accumulate in platelet concentrates during storage[53] and promote adverse effects.[54] It is thus highly plausible that these lysophospholipids are in fact derived from $sPLA_2$-IIA activity, which is also abundant in platelet concentrates (Supplementary Figure 9A), towards extracellular mitochondria. Beyond the release of lipid mediators, $sPLA_2$-IIA also participates in the extrusion of mtDNA (**Figure 4F-H**). This process may occur in an inflammatory microenvironment where $sPLA_2$-IIA and freeMitos (originating from platelets, mitoMPs or other activated/damaged cell lineages) are both present, such as in RA[49], as well as in the context of transfusion. Indeed, soluble mtDNA concentrations in platelet

storage bags increase concomitantly with $sPLA_2$-IIA levels (**Supplementary Figure 9A,B**) and are higher in concentrates associated with adverse transfusion reactions (**Figure 3I**). Since intravenous injection of mtDNA triggers lung inflammation[13], this may provide insight into how platelet transfusions induce lung injury.

Mitochondrial components that are not dependent on $sPLA_2$-IIA activity might also contribute to the promotion of inflammation. Respiratory competence displayed by platelet-derived mitochondria implies an increased production of ATP, a recognized DAMP[55], as well as an upsurge in extracellular reactive oxygen species, which are danger signals also.[56] Similar to bacteria, mitochondria express N-formylated peptides that can recruit leukocytes to the site(s) of inflammation.[12,57] The presence of these different inflammatory components, whose production does not require $sPLA_2$-IIA, may explain how intravenous injection of mitochondria induce neutrophil rolling along the vascular wall (**Figure 5B** and **Supplementary video 3**), which is typically reminiscent of neutrophil priming, and how intact mitochondria may induce significant NETosis (**Figure 6E**).

While we have focused our study on innate and acute inflammatory responses triggered by extracellular mitochondria, the latter may also be involved in adaptive immune responses and chronic inflammation. Indeed, the generation of antibodies directed against mitochondrial components may occur in chronic rheumatic diseases in which mitochondria are constantly liberated by activated platelets and in patients repeatedly transfused with extracellular mitochondria. Interestingly, cardiolipin, a phospholipid uniquely expressed by mitochondria (and bacteria), may be also highly antigenic, providing an explanation for the prevalence of anti-cardiolipin in rheumatic diseases implicating platelets like systemic lupus erythematosus and anti-phospholipid syndrome.[58] We predict that future investigations will determine to what extent mitochondria contribute in these processes.

Platelet activation under flow condition induces the formation of long tubes, called flowinduced protrusions, and the plasma of healthy subjects contains tubular extracellular vesicles. We thus foresee that these tubular structures might also express mitochondria.[59,60] Keeping in mind that platelets rapidly respond to vascular injuries to prevent bleeding, and that mitochondria might also be released in this context, we further speculate that extracellular mitochondria could contribute to the hemostatic functions of platelets. Like MPs, mitochondria might serve to tissue factor deposition and to the initiation of the coagulation cascade, which is by itself a well-controlled inflammatory reaction.[61] Subsequent studies will undoubtedly uncover additional physiological role(s) played by extracellular mitochondria.

Platelets are classically considered first and foremost as key players in hemostasis. However, mounting evidence suggests that these cells actively participate in inflammation.[8] The identification of mitochondria, with their bacterial ancestry, and of a bactericidal phospholipase A_2, $sPLA_2$-IIA, as entities that are released from platelets and that work together in many inflammatory disorders, suggests that they may both be key mediators in sterile inflammatory conditions.

2.7 References

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2.8 Figures and legends

Figure 1

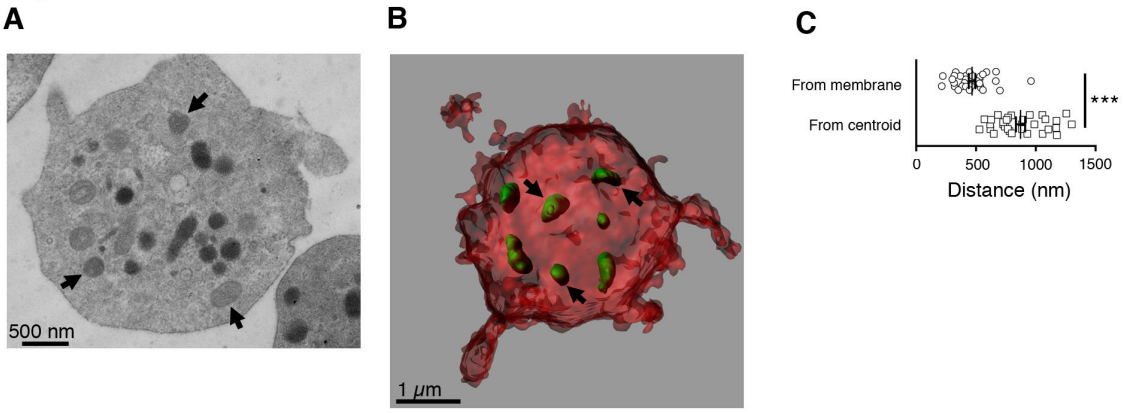

Figure 1. Mitochondrial distribution within resting platelets.

Mitochondria (black arrows) in resting platelets examined by (A) transmission electron microscopy (TEM) and (B) confocal scanning laser microscopy (CSLM). (C) Mitochondria are located proximally to the platelet plasma membrane (n=31; data represent the mean ± SEM, ***P<0.0001, *t*-test).

Figure 2

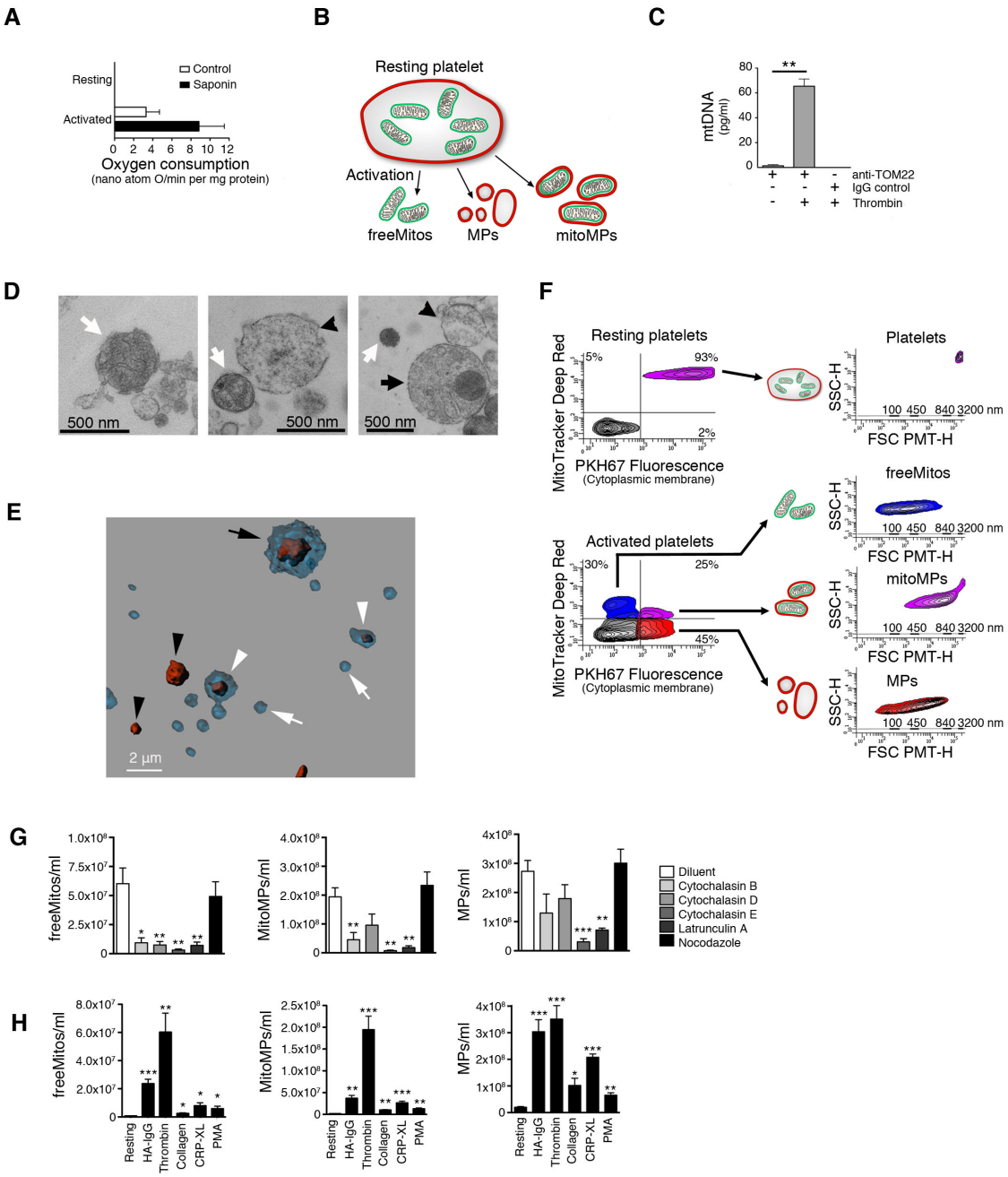

Figure 2. Activated platelets release extracellular mitochondria.

(A) Platelet-free supernatants resulting from the isolation of thrombin-activated platelets consume O_2 via the electron transport chain following cell permeabilization with saponin detergent (50 µg/ml). No O_2 consumption is detected in supernatants obtained from resting platelets (n=4; data are mean ± SEM). (B)

Three predicted types of extracellular microparticles (MPs) produced upon platelet activation: mitochondria (freeMitos), mitochondriacontaining MPs (mitoMPs) and MPs lacking mitochondria (MPs). (C) Isolation of freeMitos using anti-TOM22 microbeads (or IgG control) in thrombin-stimulated platelets and mitochondrial DNA (mtDNA) quantification (n=4; data are mean ± SEM, **P<0.005, *t*-test). (D) TEM visualization of freeMitos (white arrows), mitoMPs (black arrows) and MPs (black arrowheads) released from thrombin-activated platelets. (E) 3DCSLM reconstruction of the supernatant of thrombin-activated platelets. Populations represented in image are platelets (black arrow), MPs (white arrows), mitoMPs (white arrowheads) and freeMitos (black arrowheads). (F) High-sensitivity flow cytometry (hs- FCM) analysis of resting platelets (*upper* panel, top right quadrant) and thrombin activated platelets, which show 3 additional, distinct populations of particles, i.e. freeMitos (bottom panel, top left quadrant, blue), mitoMPs (bottom panel, top right quadrant, pink) and mitochondria-free MPs (bottom panel, bottom right quadrant, red). Bottom left quadrant of both *upper* and *lower panel* represent background noise (gray). FSC-PMT and SSC dot plots of platelets (first right panel) and 3 populations of microparticles, freeMitos (second right panel), mitoMPs (third right panel) and MPs (fourth right panel). The relative diameters are presented according to size-defined microsphere calibrations. (G) Release of freeMitos (left panel), mitoMPs (middle panel) and MPs (right panel) from thrombin-activated platelets require intact actin microfilament dynamics. Mitochondrial release is significantly reduced upon addition of actin inhibitors (cytochalasin B, D, E and latrunculin A), but not tubulin polymerization inhibitor (nocodazole) (n=4; data are mean ± SEM, *P<0.05, **P<0.005 and ***P<0.001, *t*-test). (H) Heat-aggregated IgG (HA-IgG), thrombin, collagen, crosslinked collagen related peptide (CRP-XL) and phorbol 12-myristate 13-acetate (PMA) trigger the release of extracellular freeMitos (left panel), mitoMPs (middle panel) and MPs (right panel) quantified by hs-FCM (n=4; data are mean ± SEM. *P<0.05, **P<0.005 and ***P<0.001 vs. supernatant from resting platelets, *t*-test).

Figure 3

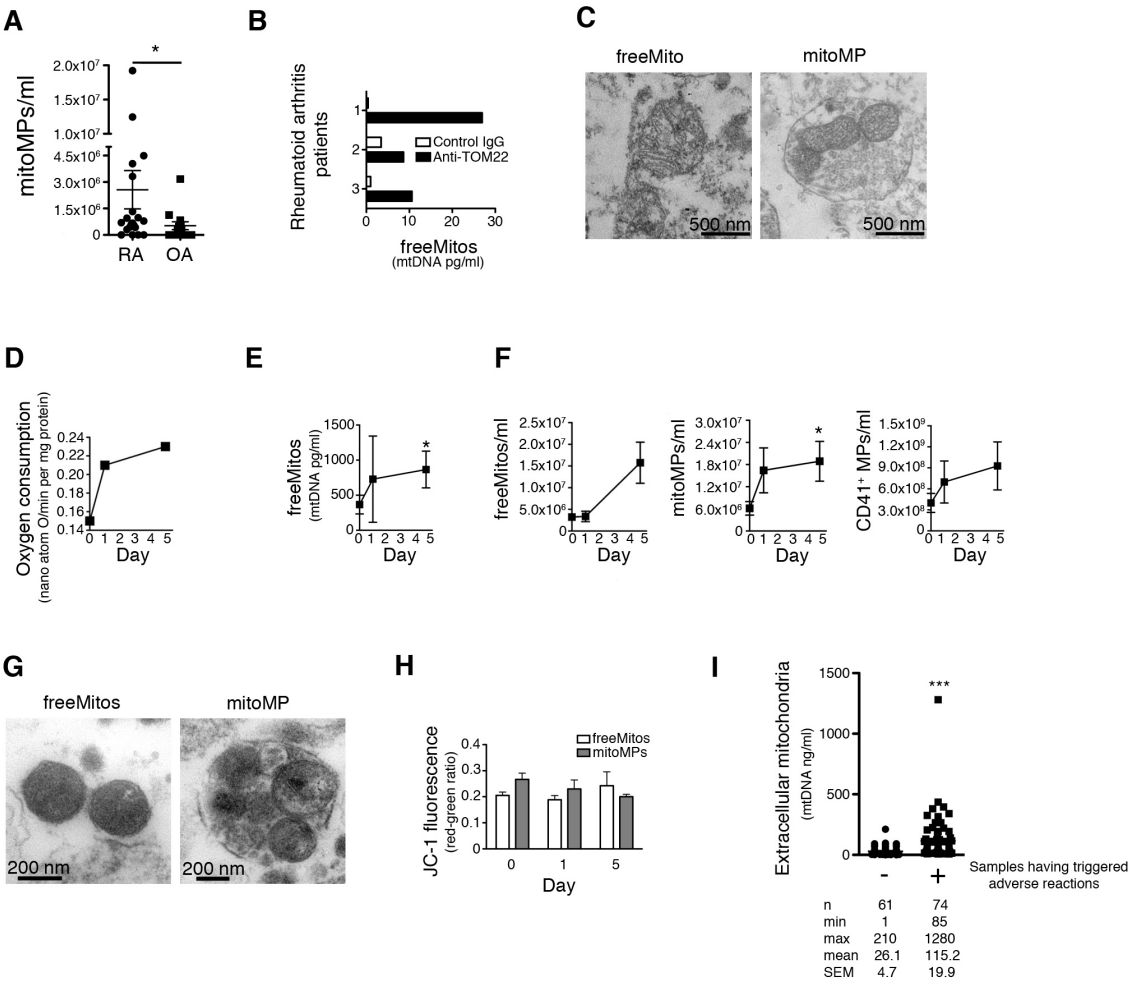

Figure 3. Extracellular mitochondria are present in various situations where platelets are known to be activated.

(A) Platelet mitoMPs (CD41+MitoTracker+) are found in higher concentrations in the synovial fluid of rheumatoid arthritis patients (RA, n=20) than in the synovial fluid of osteoarthritis patients (OA, n=14; data are mean ± SEM, *P<0.05, Mann Whitney test). (B) FreeMitos are detected in fresh SF of RA patients. Isolation of freeMitos in RA SF (from 3 different patients) with anti-TOM22 microbeads (or control IgG, Supplementary Figure 3) and mtDNA quantification. (C) TEM imaging of a freeMito (left panel) and a mitoMP (right panel) from fresh RA SF. (D) O_2 consumption is observed in platelet-free plasma (PFP) obtained at the indicated time intervals from platelet storage bags. (E) Isolation of freeMitos (Supplementary Figure 3) in PFP along with mtDNA quantification reveals an abundance of freeMito at day 5 (n=6;

data are mean ± SEM, *P<0.05 vs. day 0, paired *t*-test). (F) High-sensitivity flow cytometry (hs-FCM) analysis of resting platelets (*upper* panel, top right quadrant) and thrombin-activated platelets, which show 3 additional, distinct populations of particles, i.e. freeMitos (bottom panel, top left quadrant, blue), mitoMPs (bottom panel, top right quadrant, pink) and mitochondria-free MPs (bottom panel, bottom right quadrant, red). Bottom left quadrant of both *upper* and *lower panel* represent background noise (gray). FSC-PMT and SSC dot plots of platelets (first right panel) and 3 populations of microparticles, freeMitos (second right panel), mitoMPs (third right panel) and MPs (fourth right panel). The relative diameters are presented according to size-defined microsphere calibrations. (G) TEM imaging of PFP collected on day 5 confirming the presence of freeMitos (left panel) and mitoMPs (right panel). (H) Mitochondrial membrane potential is detected in freeMitos and mitoMPs collected from PFP, as measured by a JC-1 assay using hs-FCM (red to green ratio) (n=5; data are mean ± SEM). (I) Extracellular mitochondria (as detected by mtDNA quantification) are found at higher concentration in PFP of platelet storage bags that have cause adverse transfusion reaction to the recipient (no adverse reaction group (n=61) vs adverse reaction group (n=74) matched in term of storage duration; data are mean ± SEM, ***P<0.001, t test). Adverse reaction measured include mainly febrile non-hemolytic reactions, skin manifestations such as itching or skin rash and cardiovascular events such as hypotension or tachycardia.

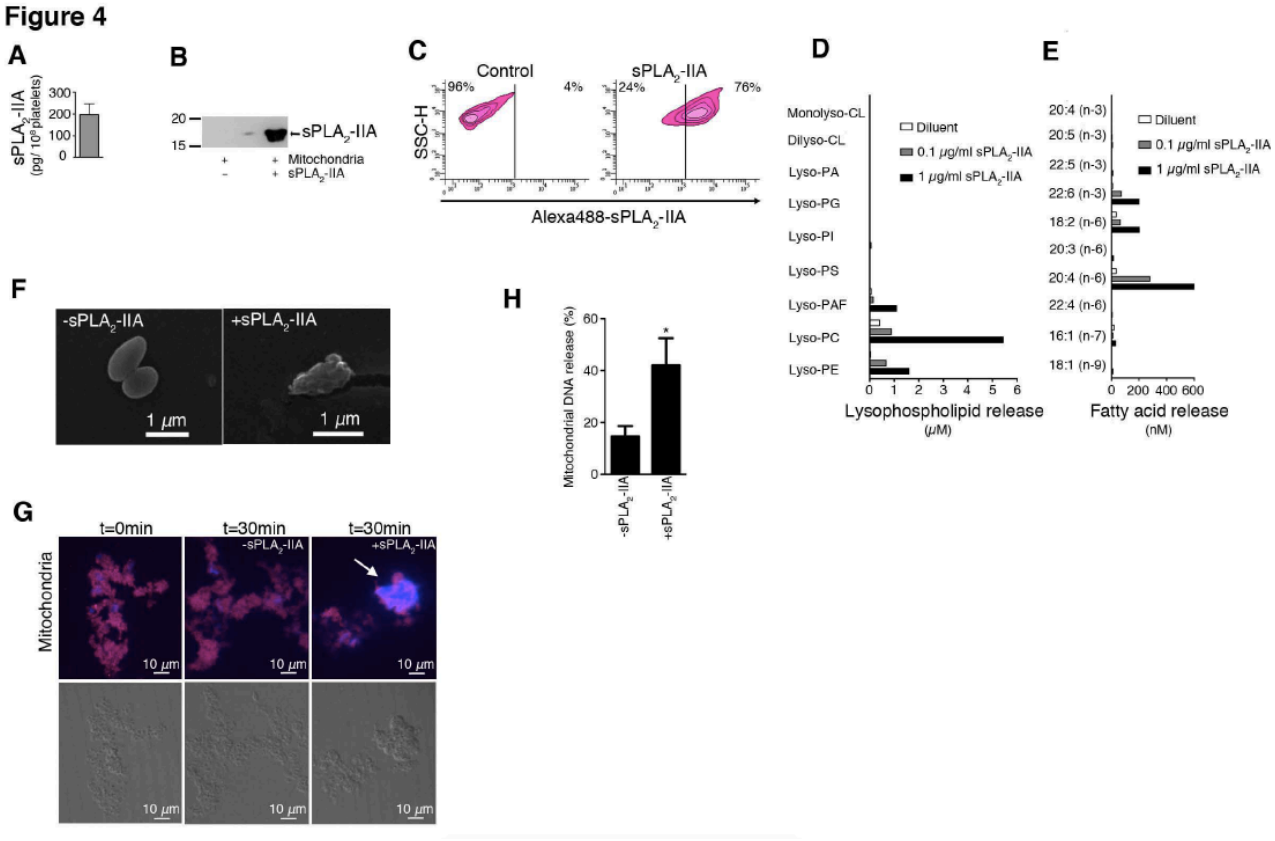

Figure 4. The mitochondrion is a substrate for the bactericidal secreted phospholipase A2-IIA (sPLA2-IIA).

(A) Quantification of sPLA2-IIA in human platelets by time-resolved immunofluorescence (n=3; data are mean ± SEM). (B) sPLA2-IIA immunoblotting of mitochondria isolated with anti-TOM22 microbeads reveals binding of human recombinant sPLA2-IIA to mitochondria (Supplementary Figure 3). (C) Mitochondria were incubated in the absence (left panel) or presence (right panel) of Alexa488-conjugated sPLA2-IIA and analyzed by hs-FCM. The significant shift in the fluorescent population size (right panel) indicates that sPLA2-IIA binds mitochondria. (D, E) Catalytic activity of human recombinant sPLA2-IIA (or PBS as vehicle) towards mitochondria. Mitochondrial membrane phospholipid hydrolysis by sPLA2-IIA yields lysophospholipids (D) and fatty acids (E) as quantified by mass spectrometry. (F) sPLA2- IIA affects mitochondrial structural integrity. Scanning electronic micrographs of mitochondria incubated in the absence (left panel) or

presence (right panel) of human recombinant sPLA$_2$-IIA. (G) Mitochondria (magenta) release mtDNA (blue) upon incubation with recombinant sPLA$_2$-IIA (*upper panels*). Extracellular mtDNA accumulation (arrow) is apparent in the presence of sPLA$_2$-IIA. Differential interference contrast images are shown for reference (*lower panels*). (H) mtDNA extrusion is amplified in presence of human recombinant sPLA$_2$-IIA (0.2 µg/ml, 30 min at 37°C), as quantified by Sytox® Green nucleic acid stain assay (n=6; data are mean ± SEM, *P<0.05, *t*-test).

Figure 5

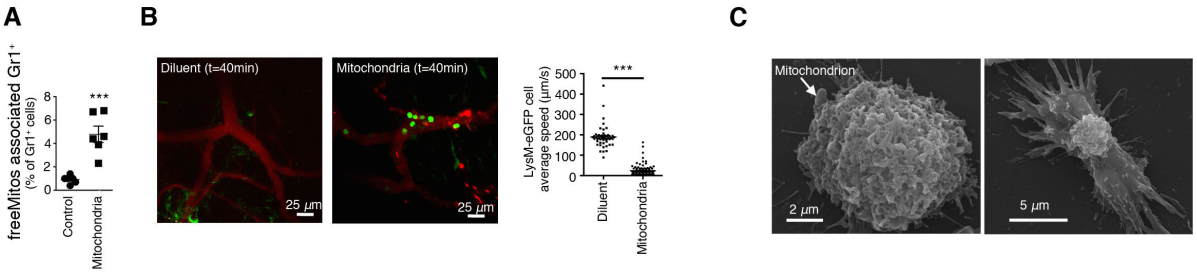

Figure 5. Extracellular mitochondria interact with neutrophils

(A) Intravenously injected fluorescence-labeled mitochondria (MitoTracker® Deep Red) associate with mouse neutrophils (Gr1+ cells) *in vivo* as measured by flow cytometry (n=6; data are mean ± SEM, ***P<0.001, *t*-test). (B) Intravenous injection of mitochondria induces neutrophil rolling in LysM-eGFP mice. Neutrophil (green) velocity is significantly reduced (middle and right panel (n=89), Supplementary video 3) in blood (red) following intravenous injection of mitochondria when compared to Tyrode Buffer as vehicle (left panel (n=51), data are mean ± SEM, ***P<0.001, *t*-test) (C) Scanning electronic micrographs of mitochondria in association with freshly isolated human neutrophil (left panel) and ensuing neutrophil structural change (29.2 ± 2.11%, n=3) after 30 min incubation in presence of human recombinant sPLA$_2$-IIA (right panel).

Figure 6

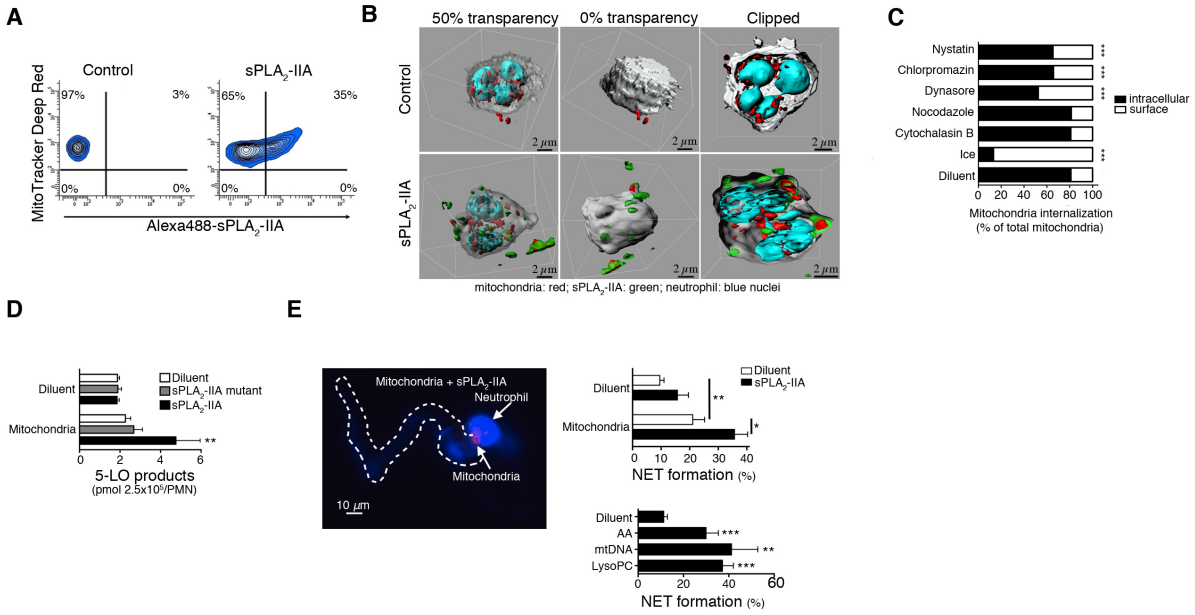

Figure 6. The interaction of human neutrophils with the mitochondria/sPLA2-IIA complex promotes the release of proinflammatory mediators.

(A) Human neutrophils associate with the mitochondria/sPLA2-IIA complex *in vitro* as measured by flow cytometry analysis of human neutrophils incubated with fluorescently-labeled mitochondria (MitoTracker® Deep Red) in the absence (left panel) or presence (right panel) of Alexa488-conjugated sPLA2-IIA. (B) 3D-CSLM reconstruction of mitochondria (red) and sPLA2-IIA (green) colocalizing within neutrophils (blue nuclei, Hoechst stain and gray cytoplasm, CMPTX). (C) Mitochondria are internalized in human neutrophils via an endocytosis-dependent pathway. Graph bars representation of the relative localization (surface vs intracellular) of the mitochondria inside neutrophils following pre-treatment with indicated inhibitors (nystatin for inhibition of caveolinmediated endocytosis; chlorpromazine for inhibition of clathrin-mediated endocytosis; dynasore for inhibition of dynamin-mediated endocytosis; nocodazole for inhibition of polymerization of microtubule (endocytosis and phagocytosis); cytochalasin B for inhibition of polymerization of actin (endocytosis and phagocytosis)). Data were obtained from 100 neutrophils per condition repeated 3 times (n=3, *P<0.01, **P <0.001, ***P <0.0001, Mann Whitney test compare to diluent). (D) Mitochondrial

hydrolytic products derived from the action of the mitochondria/sPLA$_2$-IIA complex (Figure 4D,E) induce proinflammatory responses in human neutrophils. The total 5-lipoxygenase products (5-LO products) were quantified by high-performance liquid chromatography (n=4; data are mean ± SEM, **P<0.005, vs. control, *t*-test). (E) The freeMito fraction induces NET formation *in vitro* and is enhanced by sPLA$_2$-IIA. NET formation (left panel, DNA, blue, white dotted line) was confirmed by confocal imaging after treatment of mitochondria (red, right panel) with sPLA$_2$-IIA. sPLA$_2$-IIA significantly enhances NET formation by mitochondria (*upper* right panel, n≥7; data are mean ± SEM, *P<0.05 and **P<0.005, *t*-test). Hydrolysis products from mitochondria/sPLA2-IIA complex activity also induce significant NET formation (*lower right panel*, n≥3; data are mean ± SEM, **P<0.005 and ***P<0.001, *t*-test).

Figure 7

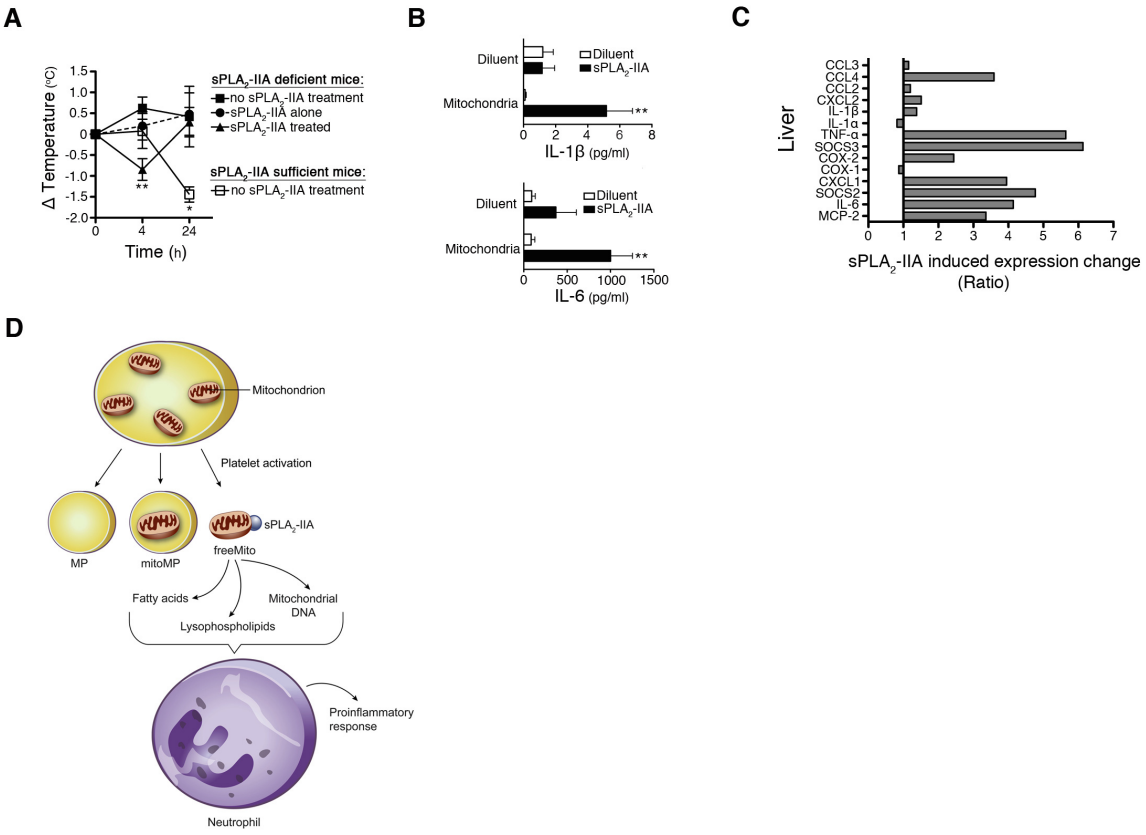

Figure 7. Extracellular mitochondria and sPLA2-IIA amplify inflammation *in vivo*.

(A) Intravenous injection of mitochondrial hydrolytic products (sPLA2-IIA-treated mitochondria, black triangle) in sPLA2-IIA-deficient mice significant lowers body temperature (Δ temperature, vs. PBS-injected mice of respective background) after 4 h (n=6/group; data are mean ± SEM, **P<0.005 compared to sPLA2-IIA-untreated mitochondria (■) or sPLA2-IIA alone (●)). Intravenous injection of mitochondria (sPLA2-IIA-untreated, □) in sPLA2-IIA-sufficient mice results significantly lowers body temperature after 24 h. Only modest temperature decrease was observed in sPLA2 IIAuntreated mitochondria (■) in sPLA2-IIA deficient mice (n≥3/group; data are mean ±
SEM, **P<0.005). (B) sPLA2-IIA-generated mitochondrial products trigger inflammation *in vivo*. Mitochondria incubated in the presence of recombinant

sPLA2-IIA and injected into the air pouch of C57BL/6N mice induce the production of IL-1β (left panel) and IL-6 (right panel). Diluent (PBS), sPLA2-IIA alone or untreated mitochondria induce modest cytokine production when injected separately (n=7; data are mean ± SEM, **P<0.005, compared to mitochondria incubated in the absence of sPLA2-IIA). (C) Mitochondria accumulation in the liver induces numerous proinflammatory genes that are amplified in the presence of endogenous sPLA2-IIA. Messenger RNA expression of inflammatory genes relevant to neutrophil function was quantified in the liver of sPLA2- IIA-sufficient and -deficient mice intravenously injected with mitochondria (n=3 per group; data expressed as the ratio of specific mRNA expression ratio (sPLA2 IIAsufficient/-deficient mice). (D) Schematic representation of the mechanism of action of extracellular mitochondria and sPLA2-IIA in sterile inflammatory conditions. Upon activation, platelets release MPs, mitoMPs and freeMitos. Mitochondrial membrane phospholipids may be hydrolyzed by sPLA2-IIA, generating bioactive mediators (fatty acids, lysophospholipids and mtDNA) and promoting neutrophil proinflammatory responses.

2.8 Supplementary materials and methods

2.8.1 Isolation and quantification of free mitochondria

Free mitochondria were isolated from diverse samples with anti-TOM22 microbeads kit (Miltenyi Biotec) with a minor modification. The initial step consisting of cell lysis was omitted since only soluble free mitochondria were targeted for isolation. Free mitochondria were subsequently isolated via magnetic field. Mitochondrial DNA was extracted from eluted anti-TOM22 processed samples with the QIAamp DNA Micro extraction kit (QIAgen) according to the manufacturer's protocol and quantified by real- time quantitative PCR (Rotor Gene 3000, QIAgen) with the Rotor-Gene Probe PCR kit (QIAgen). Primers and probes (Integrated DNA Technologies) were used for specific amplification of human (forward 5'-ACGCCTGAGCCCTATCTATTA-3', reverse 5' GTTGACCTGTTAGGGTGAGAAG-3' and probe 5'-/56 FAM/TGACAAGCG/ZEN/CCTATAGCACTCGAA/3IABkFQ/-3') and mouse (forward 5'-GGAACAACCCTAGTCGAATGAA-3', reverse 5'-GCTAGGGCCGCGA-TAATAAA-3' 5' /56-FAM/ACAAAGCCA/ZEN/CCTTGACCCGATTCT/3IABkFQ/-3' mitochondrial DNA. The qPCR cycling condition consisted of an initial step of 95°C for 3 min followed by a two-step amplification of 95°C for 3 s and 60°C for 10 s (40 cycles). Mitochondrial DNA extracted from platelet was used for generation of standard curve.

2.8.2 Mitochondrial DNA quantification in platelet concentrates associated with acute transfusion reactions

Of the 10,600 apheresis platelet concentrate transfusions performed over 2 consecutive years, 30 platelet concentrates were associated to Acute Transfusion Reactions (ATRs). Only severe cases were considered in our study, Grade 3 of the International Society Blood Transfusion (ISBT) scale. Symptoms were mainly febrile non-hemolytic reactions (42% of ATRs), skin manifestations such as itching or skin rash (38% of ATRs) and cardiovascular events such as hypotension or tachycardia (20% of ATRs). Recipients were women in 53% of cases and mean

age was 47.3 ± 10.5 years. All platelet concentrate collections were subjected to quality control to ensure their consistency and their conformity with French and European standards (EDQM, 16th Edition, 2010). Several parameters were assessed including volume (mean volume = 419 ± 30 ml), mean platelet count (5.6 ± 0.6 × 10^{11} platelets/bag), mean residual leukocyte count (0.095 ± 0.087 × 10^{6} leukocytes/bag) and mean pH (7.3 ± 0.1).[1] MtDNA quantification in the platelet concentrates (ATR vs. Matched controls storage duration) was performed by qPCR as described above.

2.8.3 Transmission electron microscopy and determination of relative position of mitochondria within platelets

Platelets and platelets MPs (freshly obtained and never frozen) were fixed in 2.5% glutaraldehyde for 30 min at room temperature (RT) then stored at 4°C until paraffin inclusion. Samples were stained and analyzed on a FEI Tecnai G2 Spirit BioTWIN transmission electron microscope at 80kV. Relative positioning was calculated using a custom made macro for NIH ImageJ. Each platelet contour was drawn with the free hand tool to calculate their centroid, which was subsequently marked on the picture as a landmark. Minimum and maximum radii of the platelet were then measured using the line tool. Centroids were also determined for each mitochondrion and marked on the microphotograph. The distance between the platelet centroid and the mitochondria centroids was measured as well as the shortest distance between the mitochondria centroids and the plasma membrane.

2.8.4 Scanning electronic microscopy imaging

Samples were fixed with 2.5% glutaraldehyde for at least 24 h then processed for standard dehydration. Briefly, samples were first washed (3 x 10 min) with sodium cacodylate buffer (0.1 M, pH 7.3) then fixed with osmium tetroxide (1 % in sodium cacodylate buffer) for 90 min. Samples were washed again (3 x 10 min) in sodium cacodylate buffer and subsequently processed for alcohol dehydration steps (50, 70, 95 and 100% EtOH, 10 min each steps). Samples were then dipped in 100% EtOH for 40 and 10 min respectively, and air-dried overnight. Samples were then

coated with palladium and observed with a JEOL 6360LV scanning electron microscope (Tokyo, Japan).

2.8.5 Live cell imaging of platelet activation and confocal immunofluorescence microscopy

Mitochondrion staining was performed on isolated platelets (10^8 cells/ml) in the presence of MitoTracker® Green FM or Deep Red FM (100 nM, Invitrogen) incubated for 45 min at 37°C. Cell membrane staining was achieved with the addition of wheat germ agglutinin (WGA) Alexa Fluor 594-conjugate (5 µg/ml, Invitrogen) or PKH67 Green Fluorescent Cell Linker (0.75 µM, Sigma-Aldrich) that was added 15 min before the end of incubation. Cells for live cell imaging were incubated in 8 well-chamber slides and maintained at 22°C within a top-stage incubator (Tokai Hit ZILC-F1) during the entire acquisition. Single plane of platelets were acquired every 20 s for 105 min.

For confocal immunofluorescence microscopy, resting labeled cells were immediately fixed with 2% paraformaldehyde (PFA) for 5 min at RT and smeared on a Superfrost Plus glass slide (Fisher Scientific). Fluoromount (Sigma-Aldrich) was added as mounting agent. For activated platelets, platelets were stimulated with 0.5 U/ml of thrombin for 2 h at RT. The supernatant of activated platelets was labeled with anti-CD41-V450 and MitoTracker® Deep Red FM (100 nM) for 30 min at RT in the dark. The reaction was stopped with PFA 2 % fixation and sample was smeared on a Superfrost Plus glass slide. Fluoromount was added and samples were then analyzed by confocal laser scanning microscopy. Confocal laser scanning microscopy was performed with an IX81-ZDC microscope equipped with a FV1000 scanning head and an Olympus 60X OSC NA 1.4 objective lens. Confocal images were acquired by sequential scanning with the 488 nm, 546 nm and 633 nm laser lines, and the variable bandwidth filters were set optimally according to the spectral properties for MitoTracker® Green FM and WGA Alexa Fluor 594-conjugate. The Fluoview imaging software ASW3.1a (Olympus America Inc) was used to acquire and export the z-stacks. Maximum intensity projections

and volume rendering were calculated using the Surpass module in Bitplane Imaris 7.5.1 (Zurich, Switzerland). Colocalization analysis was performed with the Bitplane Imaris 7.5.1 colocalization module using the Costes' estimation for automatic threshold, which compares the Pearson's coefficient for non-randomized vs. randomized images and calculates the significance.[2] Colocalization channel of mitochondria with sPLA2-IIA was generated for visual representation, and Pearson's coefficients were calculated.

2.8.6 Internalization of mitochondria by human neutrophils.

Neutrophils ($5x10^6$cell/ml) were labeled with CMPTX (1 µM, Invitrogen) for 15 min at 37°C in HBSS1X. Neutrophils were pre-treated with 1 µM final concentration of cytochalasin B (Sigma), 10 µM of nocodazol (Sigma), 50 µM of dynasore (Sigma), 10 µg/ml of nystatin (EMD milipore), or 40 µM of chlorpromazine (LKT Laboratories) for 10 min at 37°C. Cells were then incubated for 30 min at 37°C in presence of $5x10^5$ mitochondria/µl (labeled with 100 nM final of MitoTracker® Deep Red, Invitrogen) and recombinant human sPLA2-IIA (0.2 µg/ml). Cells were finally labeled with Hoestch (1 µg/ml) fixed in 2% PFA and cytospined at 500 RPM for 3 min. Mitochondrial internalization in human neutrophils was then evaluated by confocal microscopy.

2.8.7 Multiphoton microscopy and leukocyte speed quantification

Heterozygous LysM-eGFP *knock-in* mice[3] were anesthetized with 2-3% isoflurane in O2, hairs from their right ear were removed using depilatory cream (Nair®) and the ear was held in place with physiological glue (MSI-EpiDermGlu). Vasculature was visualized by injecting 1 % Qdot 705 (Life Technologies) diluted in sterile Tyrode Buffer pH 7.4 in the tail vein. Blood vessels between 14-20 µm in diameter were localized with epifluorescence and used for leukocyte speed quantification. A volume of 100 µL of mitochondria ($5x10^8$) or Tyrode Buffer (diluent) was next injected, i.v., at which point continuous acquisition started for 40 minutes. The average leukocyte speed was measured as the distance travelled (in µm) in a given number of images acquired at 0,859 frames per second. Body temperature

was maintained at 37°C during all procedures with a temperature controlling device (RWD Life Science Co). All images were acquired on an Olympus FV1000 MPE 2-photon microscope as previously described.[4] Images recorded for the 40 minutes quantification period and for stacks had a resolution of 256 X 256 and 320 X 320 pixels, respectively.

2.8.8 Generation of recombinant sPLA2-IIA Alexa Fluor 488-conjugated

Recombinant sPLA2-IIA labeled with an Alexa Fluor 488 fluorescent dye was prepared as follows. The S36C mutation was created using the QuickChange kit (Agilent Technologies) and confirmed by DNA sequencing of the full coding region of the protein expression plasmid.5 The inclusion body protein from bacterial expression was refolded to give the protein containing an extra cysteine residue disulfide linked to cysteine-36.[5] The disulfide was cleaved by mild dithiothreitol treatment and labeled with Alexa Fluor 488 C5-maleimide (Life Technologies). The labeling method and purification of the labeled protein free of excess dye reagent was carried out as described previously for site selective spin labeling of sPLA2-IIA on surface cysteine residues.[5] The catalytically inactive H48Q mutant of human sPLA2-IIA was produced as previously described.[6]

2.8.9 sPLA2-IIA binding to mitochondria

Mitochondria (10^6) from mouse liver were labeled with 100 nM of MitoTracker® Deep Red and incubated with 10 ng of sPLA2-IIA Alexa Fluor 488 (final volume 10 µl) in HBSS with 5 mM of CaCl2 for 30 min on ice. Samples were diluted and the presence of sPLA2-IIA Alexa Fluor 488 on fluorescent mitochondria was analyzed by flow cytometry.

Interaction between mitochondria and sPLA2-IIA was also assessed by immunolabeling and co-elution. Unstained mouse liver mitochondria (10^7) in HBSS with 5 mM CaCl2 were incubated with 250 ng of sPLA2-IIA (final volume 100 µl) for 30 min on ice. Anti- TOM22 microbeads labeling was then performed as described above and processed on a magnetic for mitochondria isolation. Mitochondria were

then pelleted and lysed in 1X lysis buffer. Samples were electrophoresed, transferred onto membranes and incubated in 0.2% Milk/TBS-Tween solution containing rabbit anti-sPLA2-IIA antibody (1/1000, Cayman Chemical) for 48 h at 4°C. The membrane was washed, treated with Peroxidase- AffiniPure anti-rabbit-IgG (Jackson ImmunoResearch) and reactive proteins were visualized by chemiluminescence (Perkin Elmer).

For immunofluorescence visualization, neutrophils (5 x 10^6/ml) were labeled with CMPTX Cell Tracker (5 µM, Invitrogen) for 15 min at RT and incubated with 5 x 10^5 mitochondria (pre-incubated with sPLA2-IIA) during 30 min at 37°C. Hoechst (1 µg/ml) was added 10 min before the end of incubation time and reaction was stopped with addition of PFA 4%. Cells were analyzed by flow cytometry and were also prepared for microscopy using a cytospin protocol (500 rpm for 5 min at 4°C) and analyzed by confocal laser scanning microscopy as described below.

2.8.10 Mass spectrometry analysis of lysophospholipids and fatty acid released from mitochondrion membranes by human recombinant sPLA2-IIA

Mitochondria were incubated in presence of 0.1 µg/ml and 1 µg/ml of human recombinant sPLA2-IIA at 37°C for 0.5 and 6 h. Mitochondria were also incubated in absence of sPLA2 to determine the content of basal free fatty acid. Following incubation, the reaction was stopped with the addition of 20 mM of EGTA. Lysophospholipid analysis by mass spectrometry was carried out as described.7 Samples of sPLA2-IIA- treated mitochondria (200 µl) was mixed with 800 µl of chloroform/methanol (2/1) followed by addition of 15 µl of internal standard mixture.8 Samples were extracted as described and analyzed by combined liquid chromatography/tandem mass spectrometry.[7] Fatty acids were analyzed by conversion to their AMPP amide derivatives8 and then analyzed by combined liquid chromatography/tandem mass spectrometry.[8]

2.8.11 Stimulation of neutrophils for leukotriene generation

Human recombinant wild-type sPLA2-IIA[9], its catalytically inactive homologous form H48Q6 or vehicle diluent were incubated 18 h at 5 µg/ml in presence of mouse liver mitochondria (5 x 10^5 mitochondria/µl in Tyrode Buffer pH 7.4 supplemented with 5 mM CaCl2) at 37°C. Human neutrophils were primed and stimulated for leukotriene biosynthesis as previously described.[10,11] To evaluate sPLA2-IIA mediated release of arachidonic acid, cPLA2α inhibitor pyrrophenone (100 nM) was added 5 min before stimulation. Stimulation was initiated by addition of 5 µl of pre-treated mitochondria, or control, to prime neutrophils. The reaction was stopped by addition of 500 µl of cold MeOH:CH3CN (1:1) containing 12.5 ng of prostaglandin B2 as internal standard. Samples were then processed and analyzed by reversed-phase high performance liquid chromatography using on-line extraction as previously described.[12]

2.8.12 NET quantification

Activated human neutrophils (5 x 10^6 cells/ml) were incubated in presence of labeled mitochondria (5 x 10^5 mitochondria/ml, MitoTracker® Deep Red, 100 nm) and sPLA2- IIA (0.1 µg/ml) or diluent (PBS) for 2 h at 37°C. Cells were then fixed with PFA 2% and DNA staining was performed with Hoestch 33342 (1 µg/ml, Invitrogen). Cells were cytospined on a slide at 500 RPM for 3 min. NET formation (%) was determined by the following equation: (NETs / PMN counted) * 100. Preliminary experiments confirmed that mtDNA is readily distinguished from neutrophils NETs.

2.8.13 Quantification of mitochondrial DNA release following incubation with human recombinant sPLA2-IIA

Mitochondria were seeded at 5 x 10^8 mitochondria/ml (Tyrode Buffer pH 7.4 + 5 mM CaCl2) in a flat bottom well plate (Costar, Corning). Human recombinant sPLA2-IIA (5 µg/ml) or diluent (PBS) was added and samples were then incubated

for 30 min at 37°C. Nucleic acid stain Sytox® Green (2.5 µM, Invitrogen) was added to the mix and incubated for 10 min at room temperature. Fluorescence was obtained with a Tecan apparatus. To calculate the percentage of mitochondrial DNA released in the milieu, Triton X-100 (0.1% PBS) lysis of an equivalent amount of mitochondria (5 x 10^8 mitochondria/ml) was performed, determining the total amount of mitochondrial DNA present per well.

2.8.14 mRNA quantification of inflammatory genes

Mitochondria (or Tyrode Buffer as diluent) were intravenously injected in sPLA2-IIA sufficient or deficient mice. After 1 h, mice were sacrificed and organs (heart, thymus, spleen, liver, kidneys, lymph nodes and lungs) were recovered and immediately processed for total RNA extraction. Total RNA was isolated using Trizol (Life Technologies Inc) according to the manufacturer's protocol. RNA was quantified using a Qubit® Fluorometer (Life Technologies Inc). Reverse transcription was performed using 1 µg of total RNA with Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) following the manufacturer's instructions. Real-time PCR was performed as described previously.13 Briefly, cDNA amplification was carried out in a Rotor-Gene Q operated with the Q series software version 2.0.2 (Qiagen) using 35 cycles of 95oC for 17 s, 58oC for 25 s and 72oC for 25 s. Each sample consisted of 40 ng of cDNA, 2 µl of 10X buffer (100 mM Tris, 500 mM KCl, 30 mM MgCl2, 1.5 % Triton X-100), 100 µM dNTP, 500 nM of primers, 0.1 unit of Taq DNA polymerase (Roche Applied Science) and SYBR® Green I dye (Life Technologies) in a reaction volume of 20 µL. For each gene of interest, specific primers were designed as described previously.[13]

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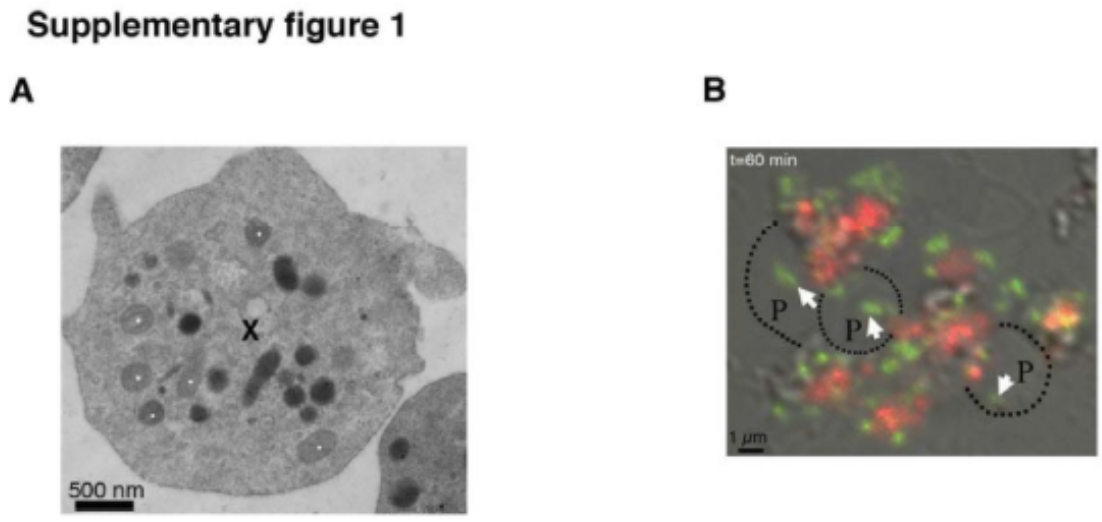
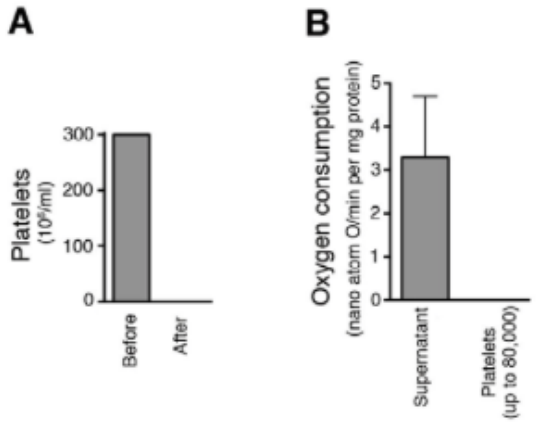
2.10 Supplementary figures and legends



Supplementary Figure 1. Relative positioning of mitochondria within the platelet.

(A) Platelet centroid (indicated by X), as well as mitochondrial centroids (indicated by white dots), were calculated using a custom-made macro for NIH ImageJ and marked on the microphotograph. The distance between the platelet centroid and the mitochondrial centroids was measured along with the shortest distance between the mitochondrial centroids and the plasma membrane. (B) Time-lapse imaging of mitochondria movement in thrombin-activated platelets. A proportion of mitochondria (green, white arrow) are found within pseudopodia (P, dotted lines) of thrombin-activated platelets (t=60 min).

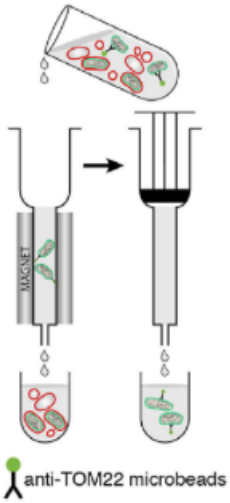
Supplementary figure 2



Supplementary Figure 2. Characterization of platelet supernatants.

(A) Platelets were counted in platelet preparations (before) and following centrifugation (after) by microscopy to detect residual platelet contamination in the supernatant. No detectable platelet remains in supernatants with the centrifugation protocol used. (B) No respiratory activity was detected in Tyrode's buffer following the addition of intact platelets (≤80,000/mL) indicating the detection limit of the approach. For comparison, the supernatant obtained after isolating activated platelets, which contains extracellular mitochondria, displays significant respiration.

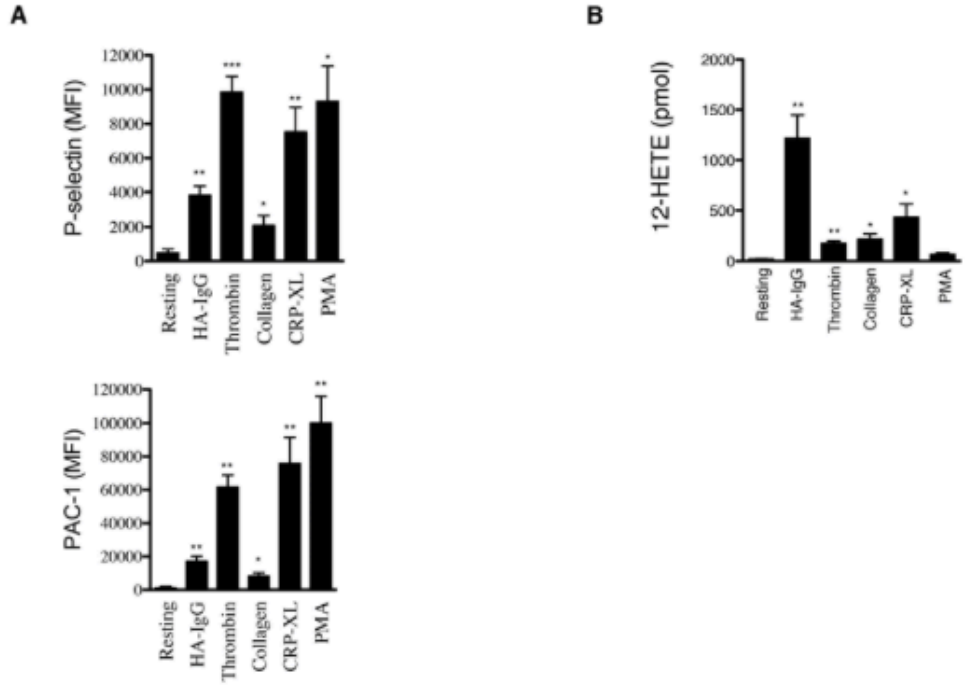
Supplementary figure 3



Supplementary Figure 3. Magnetic purification of freeMitos.

The platelet supernatant, which contains freeMitos, mitoMPs and MPs, is incubated with anti-TOM22 microbeads, and freeMitos are then isolated using a magnetic field. Magnetic field removal allows the elution of freeMitos from the column, which are then used for various purposes.

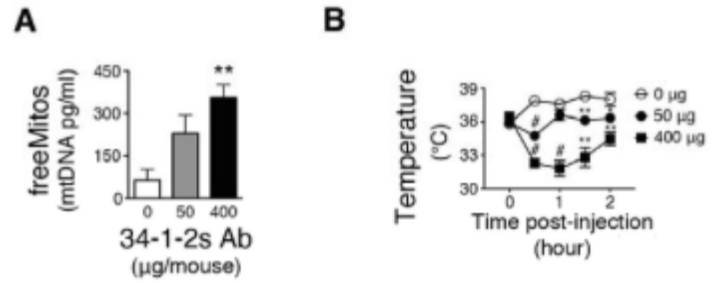
Supplementary figure 4



Supplementary Figure 4. Platelet are activated under various stimuli.

(A and B) Platelets were activated using heat-aggregated IgG (HA-IgG), thrombin, collagen, cross- linked collagen related peptide (CRP-XL) and phorbol 12-myristate 13-acetate (PMA) for 4 hours at room temperature. (A) P-Selectin (*upper panel*) and activated glycoprotein IIb/IIIa expressions (PAC-1 antibody, *lower panel*) in activated platelets by FCM. Values represent the mean fluorescence intensity (MFI) (n=3; data are mean ± SEM. *P<0.05, **P<0.005 and ***P<0.001 vs. resting platelets, *t*-test). (B) 12-Hydroxyeicosatetraenoic acid (12-HETE) quantification by high-performance liquid chromatography of activated platelets.

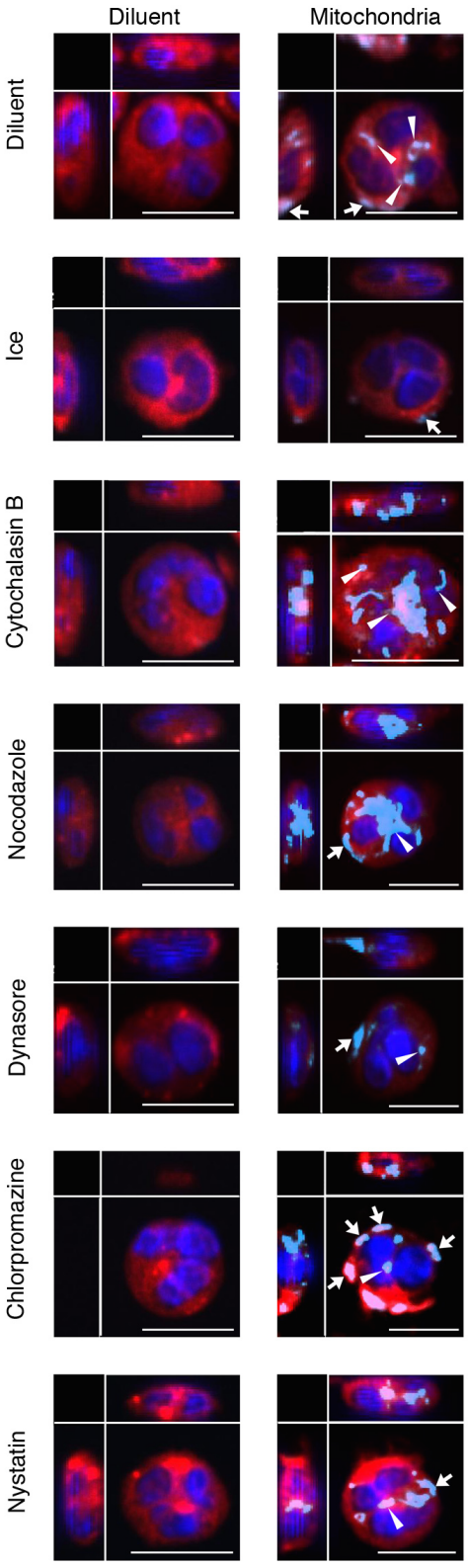
Supplementary figure 5



Supplementary Figure 5. Extracellular mitochondria are present in various sterile inflammatory pathologies.

(A and B) A transfusion-related acute lung injury (TRALI) animal model was obtained by intravenous injection of the indicated concentration of 34- 1-2s antibody to BALB/c mice. A significant temperature drop is observed in mice 1 h after antibody injection, that correlates with an upsurge of freeMitos as measured by TOM22-mediated mtDNA isolation in bronchoalveolar lavages (n=3; data are mean ± SEM, *P<0.05, **P<0.01 and #P<0.001 vs. control at 0 mg, *t*-test).

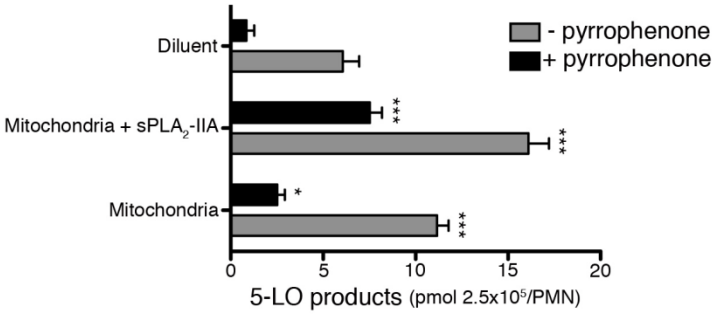
Supplementary figure 6



Supplementary Figure 6. Exogenous mitochondria are internalized via an endocytosis-dependent pathway by human neutrophils.

Representative confocal microscopy analyses of neutrophils cytoplasm and nuclei (shown in red and blue respectively) incubated with exogenous mitochondria (cyan) for 30 min at 37°C. To assess passive internalization (ice condition), exogenous mitochondria and neutrophils were incubated on ice for 30 min. Neutrophils were pre-treated with either cytochalasin B, nocodazol, dynasore, nystatin, or chlorpromazine for 10 min at 37°C. Cells were then incubated for 30 min at 37°C in presence of mitochondria/µl (labeled with 100 nM final of MitoTracker® Deep Red, Invitrogen) and recombinant human sPLA2-IIA (0.2 µg/ml). Scale bars represent 10 µm. Data are representative of three independent experiments.

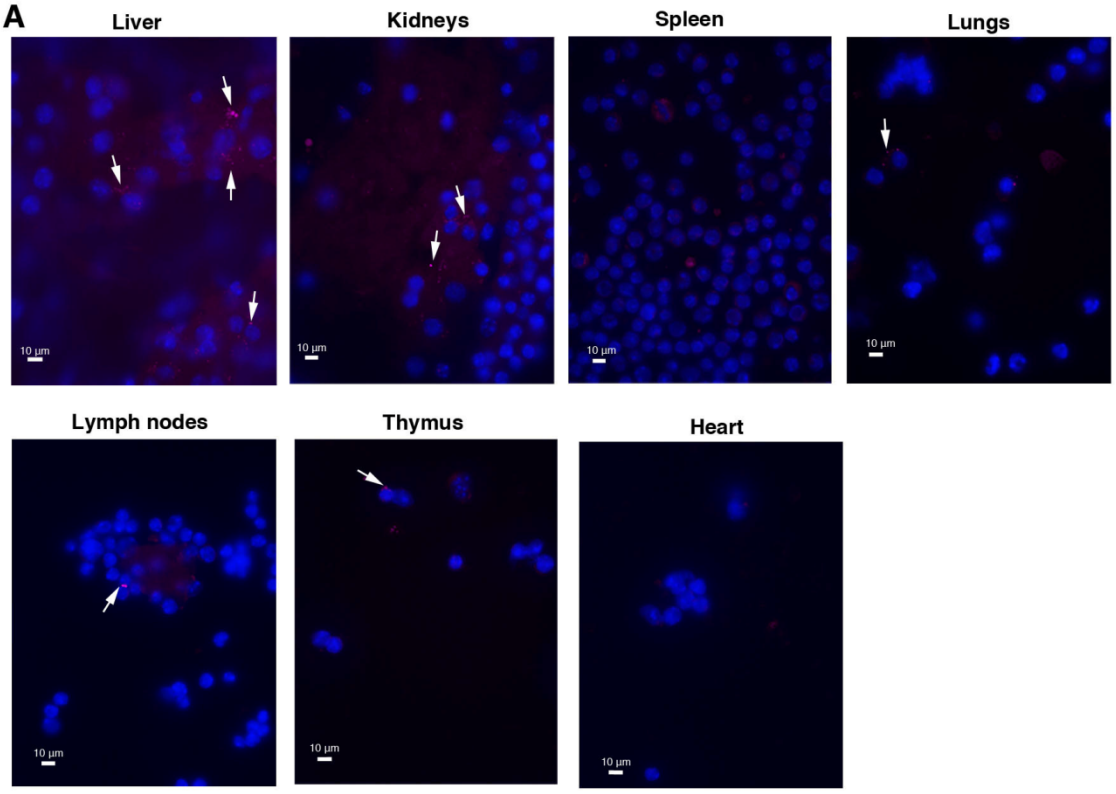
Supplementary figure 7

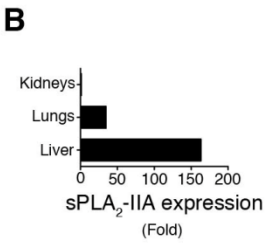


Supplementary Figure 7. sPLA2-IIA and cLA2-α work in concert to promote generation of 5-lipoxygenase products in human neutrophils.

Human neutrophils pre-treated with the cPLA- α inhibitor pyrrophenone (or diluent) were incubated in presence of exogenous mitochondria and mitochondria/sPLAIIA complex. Proinflammatory lipid mediators release was then evaluated. The total 5-lipoxygenase products (5-LO products) were quantified by high-performance liquid chromatography (n=6; data are mean ± SEM, *P<0.05 and ***P<0.001 vs. control, *t*-test).

Supplementary figure 8

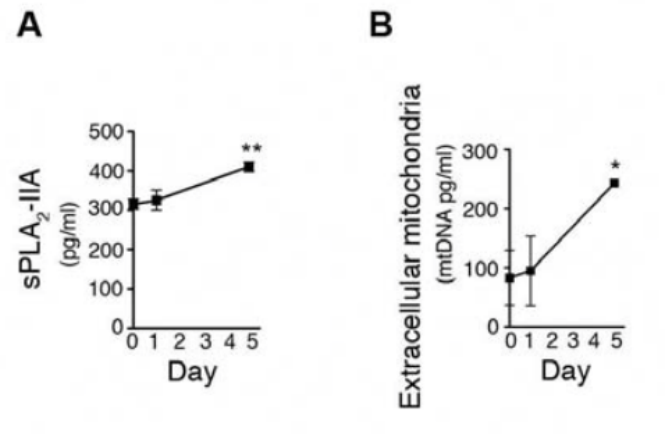




Supplementary Figure 8. Localization of extracellular mitochondria following their intravenous injection.

(A) Mitochondria (magenta, white arrow) are found primarily in liver, kidneys, lymph nods and lungs (blue nuclei, Hoechst stain) of C57BL/6N mice. (B) IIA sufficient mice. sPLA -IIA sufficient mice. IIA expression was obtained when coIIA expression in lungs and liver compared to the level found in kidneys. sPLA-IIA is predominantly expressed in the liver of sPLA-IIA sufficient mice (n=3 for each phenotype).

Supplementary figure 9



Supplementary Figure 9. sPLA2-IIA and mtDNA levels increase in platelet concentrates during storage.

Platelet storage bags (n=6) were incubated for the indicated time at 22°C with constant agitation. PFP samples were obtained on days 0, 1 and 5 for the following analyses: (A) quantification of sPLA2-IIA by time-resolved immunofluorescence (n=3; data are mean ± SEM, *t*-test); (B) extracellular mtDNA abundance by quantitative PCR (n=3; data are mean ± SEM, *P<0.05, **P<0.01, *t*-test).

2.11 Supplementary table

Supplementary Table 1: Description of synovial fluid specimen from rheumatoid arthritis patients.

Specimen	Gender	Age	Specimen description	RF	CCP
1	Male	48	Knee SF	N/A	N/A
2	Male	35	Knee SF	<30	3
3	Male	72	Knee SF	134	37

SF: Synovial fluid; RF: Rheumatoid Factor; CCP: Cyclic citrullinated peptide; CRP: C-reactive protein; ESR: Erythrocytes sedimentation rate

2.12 Supplementary videos

Supplementary video 1. Mitochondria localization in resting platelets.

Non-activated platelets were labeled with the plasma membrane WGA Alexa Fluor® 594 (red) and mitochondrial MitoTracker® Green FM (green) dyes, and visualized by CSLM.

Supplementary video 2. Mitochondria relocalize in the pseudopodia of activated platelets.

Platelet plasma membrane and mitochondria were labeled with WGA Alexa Fluor® 594 (red) and MitoTracker® Green FM (green) dyes, respectively. Platelets were seeded in 8-well chamber slides maintained at 22°C (Tokai Hit ZILC-F1 stage-top incubator), and activated by addition of thrombin (0.5 U/ml) and CaCl2 (5 mM). Single planes of platelets were acquired every 20 s for 105 min.

Supplementary video 3. Intravenous injection of mitochondria induces neutrophil rolling in LysM-eGFP mice.

Neutrophil (green) velocity is significantly reduced in blood (red) following intravenous injection of mitochondria (A, t=40min) as opposed to Tyrode Buffer (B, vehicle).

Chapitre 3: Propriétés biochimiques des plaquettes et libération de microparticules durant l'entreposage prolongé des concentrés plaquettaires.

3.1 Résumé

Les concentrés plaquettaires sont généralement conservés 5 jours afin d'assurer le maintien des fonctions plaquettaires et de prévenir la contamination bactérienne. Cette durée de vie limitée est problématique considérant la production coûteuse et la difficulté liée au recrutement. Avec les avancées, une extension de la conservation des concentrés plaquettaires est envisageable. La possibilité d'extension sans une détérioration de la qualité a été étudiée. Les concentrés plaquettaires de type plasma riche en plaquette, couche leucoplaquettaire et d'aphérèse ont été suivis sur une période de 7 jours pour les paramètres biochimiques, les marqueurs d'activation, les microparticules et l'ADN mitochondrial extracellulaire. Aucune différence significative n'a été observée lors de l'entreposage. Ces résultats indiquent que les MPs dans les concentrés plaquettaires varient en fonction de la méthode de préparation plutôt que lors de l'entreposage et que les Concentrés âgés de 7 jours respectent les standards de qualité.

Platelet biochemical properties and microparticle release during extended storage of platelet concentrates

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The authors have disclosed no conflicts of interest.

Running title: Platelet storage and microparticles

Keywords: Platelets, platelet concentrates, storage, transfusion, mitochondrial DNA, platelet-derived microparticles

3.2 Abstract

Background: Platelet concentrates (PC) are generally stored for up to 5 days in order to maintain platelet function and to prevent bacterial contamination. The limited lifespan of PCs adds pressure on blood banks due to their high production cost and challenges in the recruitment of blood donors. Given the recent improvements in preparation and storage of PCs and the strategies to limit bacterial contaminations, extending the storage duration of PCs is envisioned. The possibility to store the platelet concentrates for up to 7 days without a decrease in product quality was evaluated.

Study design and methods: Platelet-rich plasma, buffy coat and apheresis PCs were tested on day 1, 3, 5 and 7 for biochemical parameters, platelet activation markers, platelet-derived microparticle concentrations and extracellular mitochondrial DNA.

Results: No significant changes in the different parameters evaluated were observed in PCs at day 7 of storage compared to PCs examined on day 1, 3 and 5.

Conclusions: These results show that PCs comply with the quality standards for transfusion at day 7 of storage, suggesting that extending the storage period by two additional days might be considered.

3.3 Introduction

Platelet transfusion is a common medical procedure essential in raising platelet count in thrombocytopenic patients. In the United States alone, about thirteen million platelet concentrates (PCs) are transfused yearly.[286] Three different separation methods are commonly used by blood banks to generate PCs from human blood: 1) the platelet-rich plasma (PRP), 2) the buffy coat (BC) and 3) the apheresis (AP) methods. PCs are generally kept until 5 days between 20-24°C to maintain platelet function and to prevent bacterial contamination.[36, 175, 178] However, keeping in mind the high production cost and the difficulty to recruit and keep blood donors (especially those who are HLA-typed), the short half-life of PCs is a serious concern for blood banks. Taking in account the recent development of preparation and storage techniques, several studies are seeking to know whether extending the shelf life of PCs is now possible.[183, 184]

Although the production of blood components is highly standardized, adverse transfusion reactions (ATRs) occur in approximately 7.5% of PC transfusions.[287] Non-infectious reactions include acute or delayed hemolytic transfusion reactions, febrile non-hemolytic transfusion reactions (FNHTR) allergic (minor or major), transfusion-related acute lung injury (TRALI), volume overload (TACO), transfusion-associated graft-versus-host-diseases (TA-GVHD) and post-transfusion purpura (PTP).[68, 70] Some of these adverse reactions, such as FNHTR, appear less frequent with PCs that were 3-day old or less,[288] suggesting that mediator(s) implicated in sterile adverse reaction might be generated during storage.

Platelet storage lesion (PSL) such as platelet activation,[163, 164, 289]modification of biochemical parameters, and accumulation of platelet-derived microparticles (MPs),[177, 206, 210, 225, 228, 249-254, 276] could participate in ATRs. Mitochondrial DNA (mtDNA) has also been detected in the extracellular milieu in all

blood components, including PCs.[225, 276] From the evolution standpoint, mitochondria might originate from the endosymbiosis of an α-Proteobacteria[290] during the development of the eukaryotic cells.[291, 292] Mitochondria share significant levels of similarities with bacteria. Both the bacteria and the mitochondria have a double membrane that comprises cardiolipins, have a circular genome with hypomethylated CpG DNA motifs, and present N-formyl-peptides.[277, 293] Notably, the genome of mitochondria has been linked to the genome of the *Rickettsia prowazekii*.[256] These common features are such that the mitochondrion is recognized as an important source of damage-associated molecular patterns (DAMPs), and could thus play a key role in the promotion of innate immune responses.[294, 295] As extracellular mtDNA is present at higher levels in PCs associated with ATRs,[225] this suggests that mtDNA could be a novel marker of PSL.

In this study, we aimed to determine whether PCs can be stored for 2 more days (7 days rather than 5 days). Herein, we evaluated biochemical parameters, activation level of platelets, MP and mtDNA content in PRP, BC and AP at day 1, 3, 5 and 7 of storage.

3.4 Methods

3.4.1 Blood collection, PC preparation and storage

Whole-blood units (WB) were collected from healthy human volunteers after informed consent according to the standards as stated by Héma-Québec research ethics committee. For platelet-rich plasma (PRP), six WB (450 ml) were collected with 63 ml of citrate–phosphate–double dextrose (CP2D) in Leukotrap WB systems (Leukotrap RC-PL, Pall Medical Corporation, East Hills, NY). For buffy coats (BC), WB (450 ml) was collected with 63 ml of citrate–phosphate–dextrose (CPD) in Atreus blood collection systems (Terumo BCT, Zaventem, Belgium). After separation, six pools made of five ABO-compatible buffy coats were prepared with the OrbiSac (Terumo BCT). For apheresis (AP), six PCs were prepared using the Trima Accell cell separators (Terumo BCT) and platelets were resuspended in 100 percent plasma containing acid phosphate dextrose (ACD-A). All steps were carried out at 20 to 24°C. All blood collection systems were used according to manufacturer's instructions. After a rest period of 60 to 120 minutes, platelets were stored under continuous horizontal agitation in a platelet incubator (Helmer Laboratories, Fort Wayne, IN).

All blood components met AABB and Health Canada's quality criteria for PCs.[296, 297] Aliquots were taken on day 1 and 7 of storage and every PCs were tested for aerobic and anaerobic bacterial growth in 7-day cultures, with the BacT/ALERT (bioMérieux, St-Laurent, QC, Canada). Samples used for our assays were drawn aseptically during storage on days 1, 3, 5, and 7 by using sampling site couplers inserted in a spike entry port of the component storage bags. To obtain a platelet-free supernatant, platelets from PCs were centrifuged at 1430 g x 25 min and then twice at 3200 g x 5 min. Supernatant was kept frozen until testing and platelet discarded after each step.

3.4.2 Platelet activation measurements

CD62P expression (basal and stimulated) levels were measured as described previously.[298] The amino acid sequence Glycine-Proline-Arginine-Proline (GPRP peptide) and human α-thrombin were from Sigma Chemical Co (St. Louis, MO, USA). PE anti-CD62P was from Beckman Coulter (Mississauga, ON, Canada) and *Streptomyces chartreusensis* A23187 was from Calbiochem (Etobicoke, ON, Canada). For phosphatidylserine surface expression, PCs ($200x10^6$/ml) were labeled with AlexaFluor 488-conjugated Annexin-V (Life Technologies, Burlington, ON, Canada), APC-conjugated anti-human CD41a (BD Bioscience, Mississauga, ON, Canada) in Annexin-V buffer (Life Technologies) (1X) for 15 min. Then 900 µl of Annexin-V buffer (1X) was added and samples were analyzed by flow cytometry with the BD Accuri C6 (BD Biosciences) and FCS Express software (De Novo Software,Glendale, CA, USA)

3.4.3 Transmission electronic microscopy

Platelets from a PRP (day 7) were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde for 30 min at room temperature (RT) then stored at 4˚C until paraffin inclusion. Samples were stained with uranyl acetate 3% for 5 minutes and platelets, MPs and mitochondria were observed with the JOEL JEM-1230 transmission electron microscope at 80kV.

3.4.4 Platelet microparticle detection

PCs were labeled with 100 nm MitoTracker Deep Red (Life Technologies), which accumulates in active mitochondria, and V450 anti-human CD41a (BD Bioscience), which targets the platelet glycoprotein IIb, immediately after sampling. After a 30 min incubation period at room temperature, labeled PCs were diluted with PBS pre-filtered on a 0.22 µm pore size membrane (Fisher Scientific, ON, Canada) and analyzed by flow cytometry with a BD FACSCanto II Special Order Research Product (BD Biosciences), as described previously.[220, 226] Briefly, a forward scatter (FSC) coupled to a photomultiplier tube (PMT) ‘small particles option’ (FSC-PMT) was mounted on the FACS to allow the detection of smaller particles. Flow

cytometer performance tracking was performed daily before all analyzes. For FSC-PMT, the assigned voltage was 363 Volts and the threshold was 200. For SSC, the assigned voltage was 407 Volts and the threshold was 200. MP acquisition was done at low speed (~10 µl/min) and, to be quantitative, a known quantity of fluorescent beads (15 µm diameter: Polysciences, PA, USA) were added in each tube and a constant number of beads was acquired for each sample. Polystyrene microspheres (Spherotech Inc. Lake Forest, IL) of known dimensions (1µm and 3 µm in diameter) were used to estimate the dimensions of the detected cells and microparticles. As polystyrene microspheres cannot perfectly permit the assessment of particle sizes, we defined microparticles as being smaller of 1µm microspheres and platelets.

3.4.5 Flow cytometry data analyses

A Spanning-tree Progression Analysis of Density-normalized Events (SPADE),[299] (www.cytobank.org) containing 50 nodes was performed to objectively identify the different MP subpopulations and their relationships,[300, 301] on the basis of CD41 and MitoTracker expression, size (FSC-PMT-H) and granularity (SSC-H). The platelet population was identified at first, and events smaller in size were considered to be microparticle subtypes: ie. Microparticle expressing CD41 and mitotracker at lower intensity levels than full size platelets (mitoMP), and microparticles expressing CD41, and not expressing mitotracker (MPs). Finally, the nodes were regrouped in 5 distinct populations; 1) other cells, 2) platelets, 3) mitoMPs, 4) MPs and 5) debris/*freemitos*.

3.4.6 Isolation and quantification of extracellular mitochondria

In the extracellular milieu, mitochondria might be present encapsulated in MPs (mitoMP) or as free organelles (freeMito).[225]

Total mtDNA: In these conditions, mtDNA (potentially soluble in the extracellular milieu), mitoMPs and freeMitos were quantified through measurements of total mtDNA. For each sample, mitochondrial DNA was extracted from 100 µl of platelet

supernatant with the QIAamp DNA Micro extraction kit and quantified by real-time quantitative PCR as described below.

FreeMitos: For each samples, 1.5 mL of supernatant was incubated with 50 µL α-TOM22 (an antigen on surface of mitochondria) microbeads kit (Miltenyi Biotec, San Diego, CA, USA) or 10 µL IgG control (α-FITC). Cell lysis was omitted to target only free mitochondria that were subsequently isolated via magnetic field. DNA was extracted with the QIAamp DNA Micro extraction kit (QIAGEN, Toronto, ON, Canada) and quantified by real-time quantitative PCR (Rotor Gene-3000, QIAGEN) with the Rotor-Gene Probe PCR kit (QIAGEN). Human mitochondrial DNA (forward 5'-ACGCCTGAGCCCT-ATCTATTA-3', reverse 5'-GTTGACCTGTTAGGGTGAGAAG-3' and probe 5'-/56-FAM/TGACAAGCG/ZEN/CCTATAGCACTCGAA/3IABkFQ/-3'), specific primers and probes (Integrated DNA Technologies, Coralville, IA, USA) were used for amplification. The standard curve was made by serial dilution of human platelet extracted mitochondrial DNA.[225]

3.4.7 Statistical analyses

The results are presented as mean ± SEM. The differences between days 1 and 7 were compared using a Wilcoxon matched-pairs signed rank test and were considered significant (marked by an asterisk*) with a p value of less than 0.05. These statistical calculations were performed with Prism 6 (GraphPad Software, CA, USA).

3.5 Results

3.5.1 Platelet Concentrate characteristics

To confirm the quality of PCs and whether they can be used for transfusion, several *in vitro* parameters are routinely measured in blood banks. Bacterial growth[45, 46] and the presence of residual leukocytes are monitored to avoid sepsis and TA-GVHD.[70] Moreover, pH at 22°C ($pH_{22°C}$), pressure in CO_2 (pCO_2) and O_2, (pO_2), the concentrations of glucose and lactate as well as platelet counts and sterility were also measured to assess the quality of PCs.

A total of eighteen PCs (6 PRPs, 6 BCs and 6 APs) were collected and stored for 7 days. All PCs were negative for aerobic and anaerobic bacterial growth. Residual leukocytes were under 8.3 x 10^5/unit for PRP (1.9 ± 2.8 x 10^5/unit) and under 5 x 10^6/unit for BC and AP (0.30 ± 0.08 x 10^6/PCs). At day 7 of storage, $pH_{22°C}$ was maintained above 6.4 and below 7.8 (acceptable values for transfusion) for PRP-PCs, BC-PCs and AP-PCs **(Table 1)**. All PCs showed a decrease in pCO_2 and an increase in pO_2 over storage **(Table 1)**. These two parameters reflect efficient permeability of the container, which prevents acidification of the milieu.[195] Furthermore, over the 7 days of storage, all PCs showed a decrease in glucose and an increase in lactate **(Table 1)**, consistent with the production of ATP through anaerobic glycolysis. Finally, platelet concentration for each PCs remained stable over storage and complied with AABB and Health Canada's standards[296, 297] **(Table 1)**. Hence, our results demonstrate that every PCs fulfill the criteria for transfusion at 7 days of storage.

3.5.2 Determination of platelet activation level in PCs during storage

Having demonstrated that all PC characteristics fulfilled the Canadian standards for transfusion,[296] we wished to study the impact of extended period of storage on platelet activation. As readouts, we examined the surface expression of phosphatidylserine, which is only expressed on activated or apoptotic platelets,[213] by binding of annexin-V. We also verified the expression of P-selectin (CD62P) (basal and stimulated), present on activated platelets, by binding

of monoclonal antibody directed against this receptor.[302]

We observed a slight but significant increase of annexin-V binding on the platelet surface in every PCs during storage **(Figure 1A)**. CD62P expression, on the other hand, remained stable through storage, with the exception of AP platelets, which displayed significant increased levels of CD62P at day 7 compared to levels seen at day 1 ($p<0.05$). Furthermore, we observed that the expression levels of phosphatidylserine and of CD62P in PRP were significantly higher than those seen in BC and AP ($p<0.05$) **(Figure 1B)**. These observations, which are consistent with previous studies,[298, 303-305] suggest that the storage of platelets up to 7 days does not significantly impact platelet activation.

Next, we wished to verify the platelet reserve activity to thrombin. While PRP platelets displayed constant, but lower, reserve activity during storage, we found a significant decrease of thrombin response for BC and AP platelets at day 7 of storage **(Figure 1C)**. The observed reduction of reserve activity seen at day 7 in BC and AP platelets was very modest (representing less than 9 %, significantly better than PRP at day 1), suggesting that the activation of platelets in the different PCs remains acceptable through 7 days of storage.

3.5.3 Detection and quantification of platelet-derived microparticles in PCs

As another evidence of platelet activation, we measured the production of submicron extracellular vesicles shed from the cytoplasmic membrane, called microparticles (also known as microvesicles). Microparticles are recognized playing a role in the clotting process and an excessive concentration of microparticles in PCs might trigger thrombi formation in transfused patients.[228] In order to verify whether PCs can be transfused after an extended period of storage, we thus assessed microparticles in PCs.

Using transmission electron microscopy (TEM) to visualize platelets and microparticles in PRP at day 7 of storage, we observed platelets that had lost their discoid shape and presenting a dilated open canalicular system (OCS) or

vacuoles, an indication of slight activation.[306] The dilated OCS (or vacuoles) sometime contained mitochondria, (**Figure 2A, panel I**), further pointing to active mitophagy that might be occurring in these platelets.[307] We also observed different types of platelet-derived microparticles, such as microparticles not containing any apparent organelles (MP), microparticles containing organelles (potentially dense granules or mitochondria (mitoMPs)), and free mitochondria (freeMitos) in the PRP **(Figure 2A)**.

We next used a quantitative approach to assess platelet-derived microparticles present in PCs through storage and to further characterize them. SPADE analyses were performed to distinguish subpopulations present within PCs, which were portrayed on the basis of granularity and size (SSC vs FSC-PMT, respectively). We could distinguish 5 main cellular/microparticle populations present in PCs **(Figure 2B-D)**; namely 1) the larger (approximately 10 µm in diameter) $CD41^-$ $mitochondria^+$ cells (other cells, possibly the contaminating leukocytes), 2) $CD41^+$ and $mitochondria^+$ cells with a diameter of approximately 3 µm (platelets), 3) $CD41^+$ $mitochondria^+$ particles smaller than platelets and 1 µm diameter microspheres (mitoMPs), 4) $CD41^+$ $mitochondria^-$ particles smaller than platelets and 1 µm diameter microspheres (MPs) and 5) $CD41^-$ $mitochondria^{-\ or\ dim}$ particles smaller than platelets and 1 µm diameter microspheres. Note that although the latter population ($mitochondria^{dim}$) also appeared positive for TOM22, an antigen expressed on mitochondrial surface (data not shown), we judged preferable to conservatively consider this population as debris/freeMitos. The different cell and microparticle populations were also portrayed on the basis of fluorescence, size and granularity (**Figure 2E,F**) further confirming that they are distinct from each other.

Having verified our flow cytofluorometric approach, we quantified the different microparticle subpopulations in PRP, BC and AP in function of duration of storage. No significant changes in MP and mitoMP levels were seen through storage in the 3 types of PCs, although PRP contained significantly more MPs and mitoMPs than other PCs (**Figure 2G,H**). Thus, based on the determination of microparticle

subtypes, PCs appear stable during storage for up to 7 days.

3.5.4 Quantification of extracellular mitochondria

Mitochondria represent an important source of DAMPs, capable of promoting innate immune responses if present extracellularly.[265, 275] PCs contain mtDNA in the extracellular milieu,[225, 276] which concentrations correlate with the occurrence of ATRs in transfused patients.[225] To determine if PCs might be safely transfused until day 7 of storage, the levels of mtDNA was determined by a quantitative PCR analysis.

Although significant levels of mtDNA were detected in all types of PCs, it remained stable between day 1 and day 7 of storage. (**Figure 2I**). Moreover, the concentrations of freeMitos tended to increase over time in BC (P=0.03) and AP (P=0.22) products **(Figure 2J)**. Importantly, the concentration of mtDNA detected in each PC, notably at day 7, remained far below the lower concentration detected in PCs associated with ATR (represented by the dotted line in **Figure 2I)**. Thus, our data suggest that PCs might be stored 7 days without impact on mitochondrial release.

3.6 Discussion

Blood banks in some countries are already storing PCs up to 7 days. In other countries, such as Canada, this feasibility is still questioned. As the new sampling method for bacterial growth (BacT/alert) allows extended lifespan for PCs, a 7-day storage could be considerably beneficial for blood banks and patients. Furthermore, the recent implementation of pathogen reduction systems might permit to extend PC storage. This study thus verified whether PCs might be stored up to 7 days and still could fulfill blood bank standards, based on known measured of platelet quality and on additional parameters that include the evaluation of microparticles and mitochondrial release.

There exist different methods for the preparation of PCs. As each of them may induce distinct changes to platelet biochemistry, we wished to verify the impact of the mode of preparation on extended storage. The metabolic parameters evaluated in this study varied significantly between each type of PC. The slight acidification of PRP-PCs may be explained by the plastic composition of the bags.[196] The higher concentration of glucose on day 1 and of lactate on day 7 in PRP-PCs is due to the type of anticoagulant Citrate Phosphate Double Dextrose (CP2D) used during blood collection. CP2D contain twice the amount of glucose when compared to Citrate Phosphate Dextrose (CPD) (used for BC-PCs) and Acid-Citrate Dextrose (ACD) (used for AP-PCs), leaving more glucose available for glycolysis. Considering those metabolic parameters with a confirmed negative bacterial growth, all PCs, independently of its method of preparation, respected quality standards for transfusion over the 7-days storage.

Platelet activation is influenced by the method of processing[161, 164] and increases during storage.[161] Although phosphatidylserine and CD62P expressions increased during storage, platelets remained responsive to thrombin, even when used on day 7, pointing to sufficient activation reserve in these platelets. With a higher stress level inflicted upon sedimentation of platelets with the PRP technique, it was expected to find higher levels of activation and reduced

thrombin response in PRP-PCs compared to platelets in BC and AP.[161, 164] As transfused degranulated platelets rapidly lose surface P-selectin and continue to circulate and to function,[214] it suggests that platelets, even when stored up to 7 days, might be able to accomplish their pro-hemostatic role in the transfused patient. On the other hand, phosphatidylserine expression engages platelet clearance, suggesting that less platelets can actually contribute to hemostasis if prepared as PRP.[183] Future studies are thus needed to examine *in vivo* function and half-life of platelets stored for an extended period of time.

Microparticles are released from platelets during storage as a result of platelet activation.[251] Consistently, we observed higher concentrations of microparticles in PRP-PCs than in BC-PCs and AP-PCs, which correlates with the observed higher activation levels of platelets in PRP-PCs. To our surprise, we did not observe any significant increase in microparticle levels during storage in any PCs (between day 1 and 7). Although other studies showed that levels of microparticles increased during storage,[177, 225, 228, 249-252, 254] microparticles were monitored using annexin-V probes and in some case from day 0 (immediately after platelet isolation). As not all microparticles express surface phosphatidylserine,[308] this may suggest that only phosphatidylserine positive microparticles are released during storage. These studies also suggest that most of the microparticle production might occur early after platelet isolation (within the first 24h), possibly during blood processing, and exclude constant production of microparticles inside PC, in agreement with the observations made by others.[309] Several types of microparticles circulate in the blood, exposing surface proteins and membrane lipids originating from their parent cells. Most are derived from platelets and range in size from 100 nm to 1 µm.[216] Platelet microparticles can support blood coagulation[228] and may also play a role in cell-cell communication through transportation of its cargo (growth factors, protein, organelles, messenger RNAs, and microRNAs).[216, 234] The fact that different subtypes of microparticles are present in storage bags suggest that each of them may impact cells in the transfused patients differently. Future studies will undoubtedly delineate the

consequences of the transfusion of microparticle subtypes.

Extracellular mitochondria are recognized DAMPs, triggering potent innate immune responses mainly through Toll-like receptor 9 (TLR9),[275] the receptor ligand for mtDNA, and formyl peptide receptors, which are activated by mitochondrial formyl peptides.[278, 310, 311] The presence of extracellular mtDNA is associated with many physio-pathological conditions,[266-275] and is found at higher levels in PCs associated with ATRs (115.2 ± 19.9 ng/ml).[225] Even at day 7 of storage, none of the 18 studied PCs reached 85 ng/ml of mtDNA, the minimum amount of mtDNA detected in PCs implicated in ATRs, and were at least 3 fold under this concentration. As extracellular mtDNA might be considered as a novel marker of quality, these data suggest that PCs stored up to 7 days do not contain overly high concentrations of DAMPs and that it might be transfused. As duration of storage nor the method of production of PCs do not seem to be the explanation for mtDNA release, other studies are needed to identify the actual trigger of mitochondrial release in the subset of PCs that cause ATRs. Furthermore, additional work is required to determine the impact of pathogen reduction systems, such as INTERCEPT, and of platelet additive solution, on extension of PC storage.

Based on a set of measurements, this study suggests that PC storage for up to 7 days is not detrimental to platelet quality. Upon confirmation of the maintenance of platelets' functions in transfused recipients, we conclude that the extension of the storage of PCs is a promising avenue to improve blood bank productiveness.

3.7 References

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3.8 Figures and legends

Figure 1

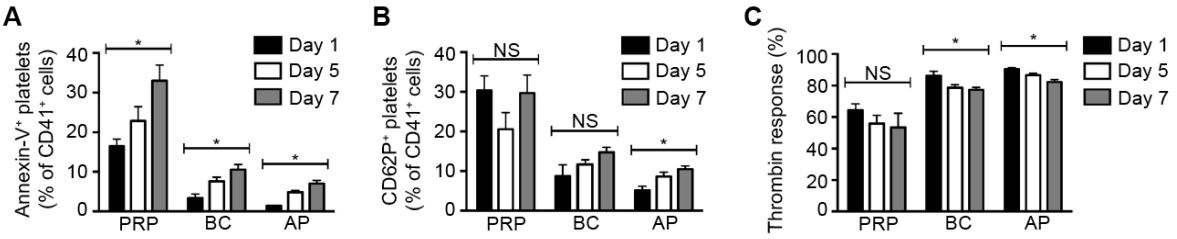

Figure 1. Platelet activation during storage.

Percentage of Annexin-V$^+$ **(A)** and CD62P$^+$ **(B)** platelets (CD41$^+$ cells) and thrombin response (i.e. the percentage of stimulated platelets expressing CD62P$^+$ minus the percentage of basal CD62P$^+$ platelet) **(C)** during 7 days of platelet storage. The data are expressed as the mean ± SEM. Differences between day 1 and 7 were verified using t-test, *$p<0.05$.

Figure 2

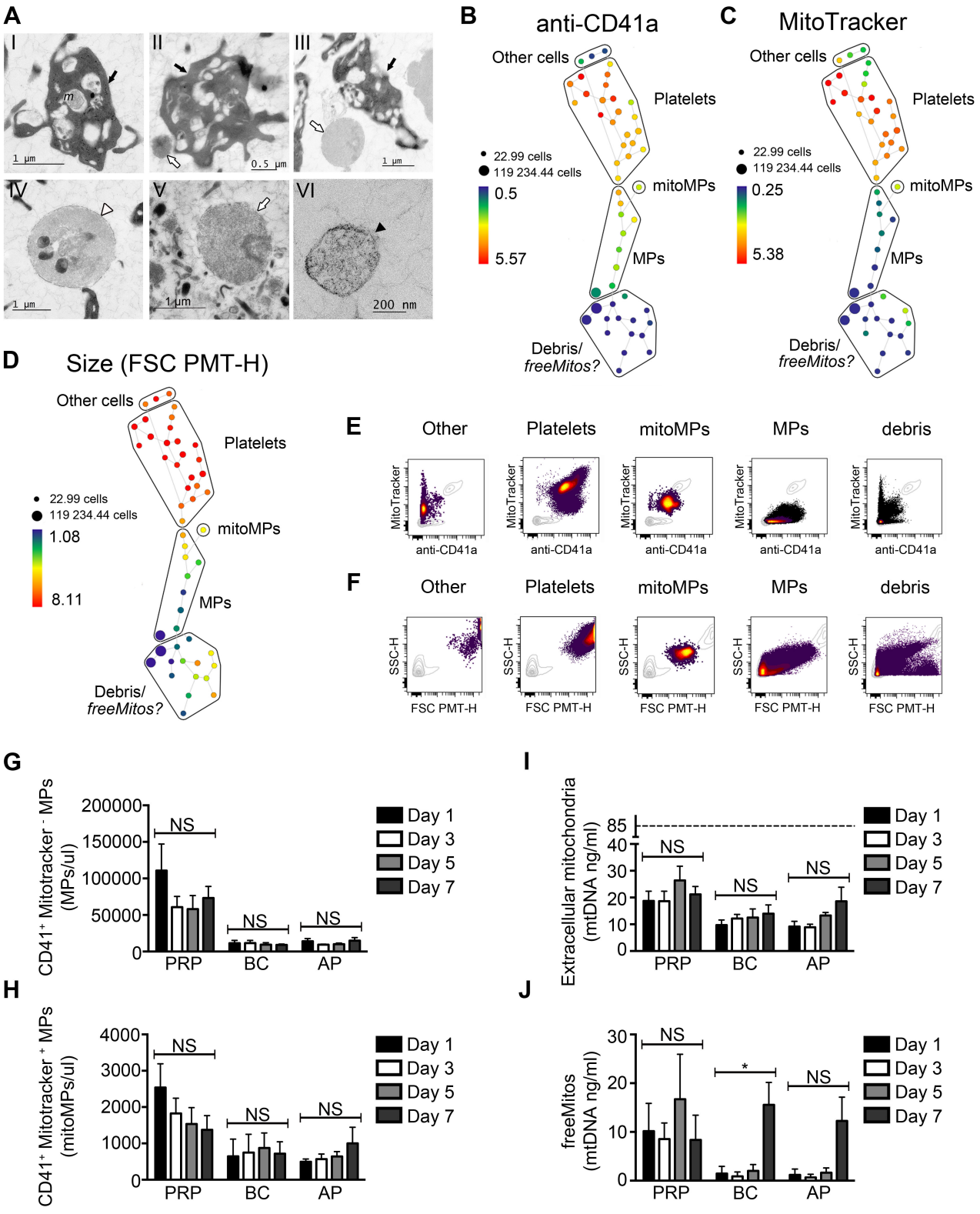
A
I
II
III
IV
V
VI
m
1 µm
0.5 µm
200 nm
B
anti-CD41a
Other cells
Platelets
mitoMPs
MPs
Debris/
freeMitos?
22.99 cells
119 234.44 cells
0.5
5.57
C
MitoTracker
0.25
5.38
D
Size (FSC PMT-H)
1.08
8.11
E
Other
Platelets
mitoMPs
MPs
debris
MitoTracker
anti-CD41a
F
SSC-H
FSC PMT-H
G
CD41⁺ Mitotracker⁻ MPs (MPs/ul)
200000
150000
100000
50000
0
NS
PRP
BC
AP
Day 1
Day 3
Day 5
Day 7
H
CD41⁺ Mitotracker⁺ MPs (mitoMPs/ul)
4000
3000
2000
1000
I
Extracellular mitochondria (mtDNA ng/ml)
85
40
30
20
10
J
freeMitos (mtDNA ng/ml)
*

Figure 2. Analysis of platelets and microparticles.

(A)TEM visualization of platelets and microparticles present in PC at day 7 of storage. Populations represented in image are platelets (black arrow) with multiple vacuoles (I, II and III), some containing mitochondrion (*m*) (I), MPs (white arrows) (II, III and V), microparticle containing organelles (mitoMP) (white arrowhead) (IV) and freeMitos (black arrowhead) (VI). Scale bars are presented under each panel. SPADE tree used to distinguish clusters of particles expressing different levels of anti-CD41a **(B)**, MitoTracker **(C)** and size (FSC-PMT-H) **(D)**. Five different populations obtained with SPADE analysis according to fluorescence for MitoTracker and anti-CD41a **(E)** and according to size **(F)**. Quantification of MPs **(G)** and mitoMPs **(H)** during 7 days of platelet storage. The data are expressed as the mean ± SEM. Quantifications were compared to day 1 using t-test. No significant difference was found over time (NS). Quantifications of mtDNA from extracellular mitochondria **(I)** and from freeMitos **(J)** during 7 days of platelet storage. The dotted line in (I) indicates the minimum level of mtDNA detected in ATRs-involved PCs[225]. The data are expressed as the mean ± SEM. Concentrations in the same group were compared to day 1 using t-test, *$p<0.05$.

3.9 Table

TABLE 1. Characteristics of platelet concentrates over the storage period*

	Day				Acceptation criteria[34]
	1	3	5	7	
PRP-PCs (n=6)					
$pH_{22°C}$	7.47±0.11 (7.30-7.63)	7.42±0.25 (6.99-7.63)	7.31±0.14 (7.11-7.46)	7.02±0.34 (6.51-7.34)	6.4-7.8†
pCO_2 (mmHg)	37±3 (33-41)	27±3 (23-29)	31±13 (20-49)	21±9 (10-31)	-
pO_2 (mmHg)	119±18 (94-144)	132±11 (115-142)	140±18 (120-168)	151±14 (137-168)	-
Glucose (mM)	33.7±2.1 (30.0-35.2)	29.2±1.8 (26.2-31.0)	28.0±0.9 (27.2-29.4)	24.9±3.5 (18.0-26.9)	-
Lactate (mM)	4.2±0.7 (3.5-5.5)	10.3±2.8 (7.8-13.8)	14.5±1.2 (12.8-16.2)	21.1±3.7 (17.4-27.4)	-
Platelets (10^{10}/unit)	6.4±1.1 (5.4-8.5)	NT	6.3±1.0 (5.4-8.2)	6.2±1.1 (5.4-8.4)	≥ 5.5 x 10^{10}/unit‡
BC-PCs (n=6)					
$pH_{22°C}$	7.31±0.12 (7.11-7.42)	7.48±0.04 (7.41-7.53)	7.42±0.11 (7.23-7.57)	7.38±0.05 (7.32-7.44)	6.4-7.8¶
pCO_2 (mmHg)	64±5 (59-70)	31±2 (29-33)	26±2 (24-30)	24±2 (21-26)	-
pO_2 (mmHg)	81±8 (73-95)	97±20 (69-121)	105±16 (77-123)	115±15 (98-136)	-
Glucose (mM)	16.1±1.0 (14.4-17.3)	14.9±0.9 (13.5-15.8)	13.8±0.8 (12.3-14.7)	12.4±0.8 (11.0-13.3)	-
Lactate (mM)	7.5±1.4 (5.9-9.7)	9.4±1.2 (7.8-10.8)	12.2-1.1 (10.2-13.2)	13.7±0.7 (12.8-14.6)	-
Platelets (10^{11}/unit)	3.6±0.6 (3.1-4.4)	NT	3.6±0.6 (3.0-4.4)	3.5±0.6 (3.0-4.4)	≥ 2.4 x 10^{11}/unit‡
AP-PCs (n=6)					
$pH_{22°C}$	7.47±0.05 (7.37-7.52)	7.51±0.07 (7.42-7.61)	7.37±0.11 (7.23-7.50)	7.28±0.11 (7.12-7.45)	6.4-7.8¶
pCO_2 (mmHg)	41±4 (34-45)	29±2 (26-31)	25±2 (22-27)	22±2 (20-25)	-
pO_2 (mmHg)	92±13 (79-115)	89±16 (75-116)	105±14 (89-123)	107±17 (91-130)	-
Glucose (mM)	17.9±1.3 (16.5-19.9)	16.2±1.5 (14.0-18.0)	14.7±14 (12.6-16.8)	12.8±1.7 (10.7-15.3)	-
Lactate (mM)	3.0±1.1 (2.1-4.8)	6.0±0.5 (5.5-6.5)	9.6±0.1 (8.4-10.9)	12.8±1.1 (11.3-14.0)	-
Platelets (10^{11}/unit)	3.5±0.1 (3.4-3.6)	NT	3.5±0.0 (3.4-3.6)	3.5±0.1 (3.4-3.5)	≥ 3.0 x 10^{11}/unit‡

NT Not tested
* Data are presented as mean ± SD (range)
† In 95% of tested PCs
‡ In 75% of tested PCs

Chapitre 4 Discussion

Bien que plaquettes soient classiquement considérées comme des acteurs clés dans l'hémostase, des preuves s'additionnent pour suggérer que ces cellules participent activement à inflammation.[112] Leur capacité à libérer lors de leur activation différents types de MPs, incluant des mitochondries extracellulaires, suggère encore un rôle lors d'un processus inflammatoire.

La mitochondrie possède différents composants reconnus via le TLR9 et le récepteur des peptides formylés qui peuvent agir comme DAMPs et entrainer le recrutement des leucocytes aux sites d'inflammation.[275, 278, 310-312] Grâce à ses caractéristiques bactériennes, la mitochondrie peut stimuler le système immunitaire et déclencher l'inflammation.

Via l'action de la $sPLA_2$-IIA, une enzyme dont la concentration est augmentée dans les exsudats inflammatoires,[313, 314] la membrane mitochondriale devient une source importante de médiateurs bioactifs. La prévalence d'anticorps anti-cardiolipine dans les maladies rhumatismales impliquant les plaquettes, telles que le SLE et le syndrome anti-phospholipide,[315] peut être expliquée par la présence de cardiolipine, un élément de la membrane mitochondriale. En plus de convertir l'acide arachidonique en différents écosanoïdes pouvant médier l'inflammation,[316] elle libère aussi des lysophospholipides inflammatoires,[317, 318] et de l'mtDNA.

En effet, la concentration de mtDNA libre augmente de façon concomitante avec les niveaux de $sPLA_2$-IIA dans les poches de PCs. De plus, la présence de mtDNA extracellulaire, qui est associée à plusieurs conditions physio-pathologiques,[266-275] a été retrouvée en concentration supérieure dans les PCs ayant causé des réactions et pourrait être mis en cause dans les réactions transfusionnelles telles que le TRALI puisque l'injection de mtDNA induit une inflammation pulmonaire.[311]

Maintenant que nous avons identifié les mitochondries extracellulaires comme médiateurs de l'inflammation et que nous avons observé un lien entre l'mtDNA et les réactions transfusionnelles, nous avons étudié la libération de microparticules et de mitochondries extracellulaires lors de l'entreposage de trois types de PCs. Bien que chaque type de PCs soient thérapeutiquement semblables, leur procédé de préparation diffère et peut induire des changements biochimiques aux plaquettes.

Tout comme l'activation des plaquettes,[161, 164] la libération des microparticules semble influencée par le procédé de fabrication. Avec un niveau de stress plus élevé infligé lors de la sédimentation des plaquettes avec la technique PRP, il était attendu d'observer des niveaux plus élevés d'activation et une réserve fonctionnelle réduite pour les PRP-PCs en comparaison avec les concentrés de type BC-PCs ou AP-PCs.[161, 164] De façon consistante avec l'activation des plaquettes,[251] nous avons observé des concentrations plus élevées de microparticules dans les PRP-PC.

Bien que d'autres études aient montré que les niveaux de MPs augmentent durant l'entreposage,[177, 225, 228, 249-252, 254, 319, 320], nous n'avons observé aucune augmentation significative entre les jours 1 et 7. Plusieurs éléments peuvent expliquer cette discordance. Dans cette étude, nous nous sommes intéressés aux MPs de plaquettes. Bien qu'elles soient moins abondantes,[246] d'autres types de MPs circulent dans le sang, tels que les MPs de globules rouges, de leucocytes ou de cellules endothéliales, peuvent être présents dans les PCs et avoir un impact sur les niveaux de MPs totaux. Aussi, puisque les MPs n'expriment pas toutes de la PS en surface,[308] l'expérience au chapitre 3 a été conçue de façon a inclure aussi les MPs PS négative. À cause de contraintes expérimentales, les MPs ont été mesurées à partir du jour 1. Nieuwland et al. [309] ont observé que la majorité de la production des MPs se produit tôt après l'isolation des plaquettes (dans les premières 24 heures), probablement durant le procédé de préparation des PCs. Ceci exclut la production constante de microparticules dans le PC, et

concorde avec nos observations et celles faites par d'autres,[309]. De plus, comme les MPs de plaquettes peuvent soutenir la coagulation,[228] les irrégularités dans le plastique des dispositifs contenant les PCs sont un endroit propice à l'adhésion des MPs, ce qui peut expliquer qu'elles ne soient pas détectées dans le surnageant des PCs.

Comme la durée d'entreposage, la méthode de production de PCs ne semble pas être l'explication pour la libération de mtDNA. D'autres études sont nécessaires pour identifier le facteur réel qui déclenche la libération mitochondriale dans les PCs. En outre, des travaux supplémentaires sont nécessaires pour déterminer l'impact des systèmes de réduction des agents pathogènes et des solutions additives plaquettaires.

Les microparticules présentes dans les PCs forment une population hétérogène et chacune d'elles peut avoir un impact différent sur les cellules des patients transfusés comment elles jouent un rôle dans la communication intracellulaire. Il est évident que les mitochondries extracellulaires sont reconnues comme DAMPs. Toutefois, nous en savons très peu sur le rôle physiologique et l'impact spécifique des mitoMPs puisque ce type de mitochondrie extracellulaire est encapsulé dans une membrane de plaquette. Celle-ci peut potentiellement camoufler la mitochondrie et empêcher les DAMPs d'être reconnus par le système immunitaire. De plus, il est inconnu à ce jour si les freeMitos proviennent directement des plaquettes ou si elles proviennent de la dégradation d'une mitoMPs. Des études futures sauront sans doute définir les conséquences de ces sous-types de microparticules sur les cellules du receveur lors d'une transfusion, mais aussi dans d'autres contextes inflammatoires aigus ou chroniques.

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