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Le sort des femmes, c'est rarement fade. Et c'est rarement simple. »

Nathalie Loiseau, livre « Choisissez TOUT », 2015

RÉSUMÉ

Le sepsis est défini depuis 2016 comme la présence d'une infection associée à la survenue de dommages tissulaires à distance du foyer infecté. Le choc septique en est une des complications les plus graves. Le sepsis est une pathologie inflammatoire médiée par l'activation du système immun inné avec la reconnaissance par l'hôte de produits microbiens et de signaux de danger par des récepteurs cellulaires spécifiques et l'activation de multiples voies de signalisation cellulaires. Un dysfonctionnement de la barrière endothéliale survient précocement compromettant l'intégrité de l'endothélium maintenue par le cytosquelette cellulaire, les molécules d'adhérence intercellulaire et les protéines de soutien. Combinée à l'augmentation de l'expression des sélectines et des intégrines, la liaison des leucocytes à la surface endothéliale entraîne une fuite du liquide vasculaire et la migration des leucocytes par extravasation à travers la barrière endothéliale altérée. Bien que cette réponse permette aux plaquettes et aux cellules immunitaires d'atteindre les sites tissulaires sièges d'une infection, le sepsis induit des réponses systémiques excessives et prolongées qui peuvent engendrer des lésions tissulaires majeures. Le sepsis entraîne un état d'hypercoagulabilité caractérisé par l'apparition de thrombi dans la microcirculation, de dépôts de fibrine, la formation de NETs (Neutrophil Extracellular Traps) et de lésions endothéliales. La formation de thrombi particulièrement dans la microcirculation entraîne un défaut de perfusion des organes. L'inhibition de l'activation plaquettaire réduit les interactions plaquettes-cellules inflammatoires-cellules endothéliales, ce qui théoriquement pourrait bloquer la réaction en cascade entre l'inflammation et la coagulation. Le potentiel des médicaments antiplaquettaires pour réduire les défaillances d'organes et améliorer le pronostic du sepsis est discuté.

Cependant, l'activation plaquettaire et le détail de la cinétique d'activation plaquettaire dans le sepsis restent peu décrits, particulièrement chez l'Homme. Parallèlement, la crise COVID-19 a soulevé de nombreuses interrogations quant à la pathogénicité du virus et son impact sur la coagulation des patients de réanimation, présentant plus d'évènements thrombo-emboliques que de façon habituelle. Le rôle des plaquettes dans la physiopathologie de l'infection grave à SARS-CoV-2 reste encore à préciser.

Ainsi le but de ce travail de thèse était double, travailler sur les fonctions plaquettaires au cours du sepsis puis, dans un second temps, mieux comprendre le rôle des plaquettes dans la pathogenèse de l'infection à SARS-CoV-2. Dans un premier temps, nous avons tenté de

déterminer le modèle animal précis d'étude du sepsis nous permettant une analyse de la cinétique d'activation plaquettaire. Nous avons ensuite utilisé ce modèle pour caractériser la cinétique d'activation des plaquettes au cours du choc septique. Nous avons dans un troisième temps réalisé la comparaison de l'activation plaquettaire chez des patients admis en réanimation polyvalente pour choc septique avec des sujets sains. Nous avons parallèlement étudié si le volume moyen plaquettaire (VMP) pouvait constituer un biomarqueur d'intérêt pour prédire la mortalité du choc septique chez l'Homme. Ensuite, nous avons travaillé sur les plaquettes des patients hospitalisés pour COVID-19 en réanimation.

Dans ce document, une revue de la littérature est présentée en première partie. Elle expose la physiopathologie du sepsis/choc septique, les grands principes de sa prise en charge thérapeutique. Elle y décrit également le rôle des plaquettes dans le sepsis notamment leur implication dans la réaction inflammatoire, les interactions plaquettes-cellules, plaquettes-pathogènes, et les conséquences cliniques et biologiques de leur mise en jeu. La seconde partie est consacrée aux travaux de recherche fruits de ce travail.

ABSTRACT

Sepsis has been defined since 2016 as the presence of an infection associated with the occurrence of tissue damage remote from the infected site. Septic shock is one of its most serious complications. Sepsis is an inflammatory pathology mediated by activation of the innate immune system with host recognition of microbial products and danger signals by specific cellular receptors and activation of multiple cellular signaling pathways. Endothelial barrier dysfunction occurs early compromising the integrity of the endothelium maintained by the cellular cytoskeleton, intercellular adhesion molecules and supporting proteins. Combined with increased expression of selectins and integrins, leukocyte binding to the endothelial surface leads to leakage of vascular fluid and migration of leukocytes by extravasation across the altered endothelial barrier. Although this response allows platelets and immune cells to reach tissue sites of infection, sepsis induces excessive and prolonged systemic responses that can result in major tissue damage. Sepsis leads to a state of hypercoagulability characterized by the appearance of thrombi in the microcirculation, fibrin deposits, the formation of NETs (Neutrophil Extracellular Traps) and endothelial damage. Thrombi formation, particularly in the microcirculation, leads to a defect in organ perfusion. Inhibition of platelet activation reduces platelet-inflammatory cell-endothelial cell interactions, which could theoretically block the cascade reaction between inflammation and coagulation. The potential of antiplatelet drugs to reduce organ failure and improve the prognosis of sepsis is discussed.

However, platelet activation and the details of platelet activation kinetics in sepsis remain poorly described, particularly in humans. At the same time, the COVID-19 crisis has raised many questions about the pathogenicity of the virus and its impact on coagulation in resuscitation patients, who present more thrombo-embolic events than usual. The role of platelets in the pathophysiology of severe SARS-CoV-2 infection remains to be clarified.

Thus, the aim of this thesis was twofold: to work on platelet functions during sepsis and, in a second step, to better understand the role of platelets in the pathogenesis of SARS-CoV-2 infection. First, we tried to determine the precise animal model for the study of sepsis allowing us to analyze the kinetics of platelet activation. We then used this model to characterize the activation kinetics of platelets during septic shock. In a third step, we compared platelet activation in patients admitted to a polyvalent intensive care unit for septic shock with healthy subjects. In parallel, we studied whether the mean platelet volume (MPV)

could be a biomarker of interest for predicting mortality in human septic shock. Then, we worked on the platelets of patients hospitalized for COVID-19 in the ICU.

In this thesis, a review of the literature is presented in the first part. It explains the physiopathology of sepsis/septic shock and the main principles of its therapeutic management. It also describes the role of platelets in sepsis, in particular their involvement in the inflammatory reaction, platelet-cell and platelet-pathogen interactions, and the clinical and biological consequences of their involvement. The second part is devoted to the research work resulting from this work.

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INDEX DES ABRÉVIATIONS

ABP : Actin Binding Protein
ACE2 : Enzyme de Conversion de l'Angiotensine 2
ADN : Acide Désoxyribonucléique
ALI : Acute Lung Injury
APC : Activated Protein C
ARN : Acide Ribonucléique
ATP : Adénosine TriPhosphate
BTK : Bruton Tyrosine Kinase
CAM : Cell Adhesion Molecules
CCL : Chemokine Ligand
CIVD : Coagulation Intravasculaire Disséminée
CLP : Cecal Ligation and Puncture
COVID-19 : Coronavirus Disease 19
COX : Cyclo-oxygenase
DAMPS : Damage-associated Molecular Patterns
DC-SIGN : Dendritic-Cell-Specific ICAM3-Grabbing Non Integrin
DENV : Dengue Virus
EMA : European Medicines Agency
FDA : Food and Drug Administration
FT : Facteur tissulaire
FvW : Facteur von Willebrand
GAG : Glycosaminoglycanes
HCV : Virus de l'hépatite C
HIV : Virus de l'Immunodéficience Humaine
HMGB1 : High Mobility Group Box 1
ICAM : Intercellular Adhesion Molecule
IFN : Interferon
IgG : Immunoglobuline G
IL : interleukine
LAMP :
LPS : Lipopolysaccharide
MAPkinase : Mitogen-activated protein kinase
MMP9 : Matrix Metallo Protease 9
NETs : Neutrophil Extracellular T
NLRP3 : NOD-like receptor family pyrin domain containing 3

NO : Oxyde Nitrique

NOD : Nucleotide-like receptors

PAI-1 : Plasminogen activator inhibitor-1

PAMPs : Pathogen associated molecular patterns

PAR : Protease-Activated Receptor-2

PDF : Produits de Dégradation de la Fibrine

PDGF : Platelet-derived growth factor

PGI2 : Prostacycline

PICS : Persistant inflammation immunosuppression and catabolism syndrome

PKC : Protein Kinase C

PMP : Platelet Microparticles

PRR : Pattern Recognition Receptors

PSGL-1 : P-Selectin Glycoprotein Ligand

RANTES : Regulated upon Activation Normal T Cells Expressed and Presumably Secreted

RBC : Red Blood Cells

SARS-CoV-2 : Severe Acure Respiratory Syndrome Coronavirus 2

SCO : Système Canaliculaire Ouvert

SIC : Sepsis Induced Coagulopathy

STD : Système Tubulaire Dense

TAFI : Thrombin activable fibrinolysis factor

TFPI : Tissue factor pathway inhibitor

TLR : Toll-Like Receptor

TNF : Tumor Necrosis Factor

TXA2 : Thromboxane A2

VCAM : Vascular cell adhesion protein

VMP : Volume Moyen Plaquettaire

LISTE DES FIGURES ET TABLEAUX

1. SEPSIS ET CHOC SEPTIQUE

1.1. Définitions

Le sepsis est un des plus anciens syndromes décrits en médecine. Avicenne le définissait comme un processus par lequel le sang et les tissus se putréfiaient dans un contexte de fièvre (Majno 1991). Plus tard décrits par Boerhaave, Von Liebig, Semmelweis, Pasteur, Lister, Lennhartz et plus récemment par Bone (Bone et al. 1992), le sepsis et sa prise en charge ont intéressé les praticiens depuis près de 3000 ans.

Depuis 1991, les définitions ont évolué dans la littérature jusqu'à la plus récente de Singer and coll. en 2016 qui décrit désormais le sepsis comme la présence d'une infection associée à la survenue de dommages tissulaires à distance du foyer infecté (Singer et al. 2016). Le choc septique devient une entité à part entière dans laquelle le risque de mortalité augmente de façon substantielle, caractérisé par une hypotension qui persiste malgré un remplissage vasculaire, et requiert *in fine* l'usage d'agents vasopresseurs.

L'étude des traitements du sepsis reflète les progrès de notre compréhension de la physiopathologie humaine et des interactions entre l'hôte et les micro-organismes. Les premières recherches se sont penchées sur les micro-organismes et leur pathogénicité. A partir des années 80, grâce aux nouvelles technologies et particulièrement au clonage moléculaire et au séquençage des gènes humains liés à l'inflammation, les investigations se sont davantage tournées vers la réponse de l'hôte pour inactiver les pathogènes que vers la pathogénicité même de ces agents infectieux (Le and Vilcek 1989; Dinarello 1984; Beutler and Cerami 1989). La découverte de comment l'hôte reconnaît le soi et le non-soi a contribué à améliorer notre connaissance du sepsis et de sa pathogenèse. En effet, la reconnaissance du signal « danger » supporte le fait que le système de l'immunité innée reconnaît des patterns microbiens et des produits cellulaires comme des signaux de danger ou des « alarmines » de l'invasion microbienne ou des lésions tissulaires. Cependant, des travaux ont montré également que la physiopathologie de la progression du sepsis était bien plus complexe que la simple mise en jeu de l'inflammation ou d'une reconnaissance d'un pattern microbien ou de l'hôte. Le sepsis concerne aussi le tissu endothélial et la microcirculation, les tissus lymphoïdes, la coagulation et les tissus parenchymateux (poumon, cerveau…) (Deutschman and Tracey 2014; Levi, Schultz, and van der Poll 2013; L. E. White et al. 2013).

Parallèlement, en dépit d'une nette amélioration de notre compréhension du sepsis, de ses origines, sa progression et sa résolution (guérison ou décès), notre capacité à intervenir et modifier le cours de la pathologie n'est que partiellement atteinte (Kaukonen et al. 2014). Autrement dit, notre compréhension physiopathologique a souvent échoué à améliorer le devenir des patients. Ainsi, bien que la mortalité intra-hospitalière liée au sepsis ait diminué durant la dernière décennie, cette amélioration est principalement attribuable au fait que l'on reconnaît les sepsis plus précocement et

que l'on suit de façon plus complète les recommandations dans leur prise en charge (Ferrer et al. 2008; Levy et al. 2015).

1.2. Épidémiologie & mortalité

En dépit d'un taux de mortalité élevé, les données épidémiologiques au niveau mondial sont manquantes. Une tentative d'extrapolation à partir de données de pays dits « développés » suggère que près de 31,5 millions de cas de sepsis surviennent chaque année dans le monde, causant près de 5,3 millions de décès par an (Fleischmann et al. 2016). Ces chiffres sont simplement des estimations car l'incidence et la mortalité par sepsis dans les pays plus pauvres restent mal connues en raison du manque de données (Fleischmann et al. 2016; Jawad, Lukšić, and Rafnsson 2012; Becker et al. 2009).

L'augmentation de l'incidence du sepsis dans des systèmes de soins pourrait être attribuable à une amélioration de sa reconnaissance et/ou à des incitations financières pour améliorer le remboursement des services codant le diagnostic « sepsis ». En tant que telles, les données épidémiologiques récentes des pays à revenu élevé ne permettent pas de saisir le poids réel du sepsis, mais il est certain qu'il reste l'objet d'un défi considérable dans le monde entier.

Dans certains pays, la mortalité par choc septique avoisine encore les 50%, alors que dans d'autres, elle serait de 20-30% (Murray and Lopez 2013; Vincent 2006). Cela tient aussi au recrutement de patients assez différents en termes de sévérité à la phase initiale. La diminution globale de la mortalité par sepsis et choc septique à l'hôpital est encourageante. Cependant, étant donné que l'incidence globale du sepsis semble augmenter à un rythme plus rapide, la mortalité globale ne s'améliore pas de manière significative, ce qui démontre l'ampleur persistante du défi que représente la prise en charge de ces patients.

1.3. Physiopathologie du sepsis

1.3.1. Initiation de l'inflammation

Le sepsis est une pathologie inflammatoire médiée par l'activation du système immun inné. Deux points clefs caractérisent la réponse immunitaire innée du sepsis. Le premier est le fait que le sepsis est généralement initié par la reconnaissance simultanée de multiples produits microbiens et de signaux endogènes de danger par le système du complément et par des interactions avec des récepteurs cellulaires spécifiques de surface (Takeuchi and Akira 2010). Les cellules innées incluent des populations immunes, épithéliales et endothéliales constamment exposées à leur environnement local. La liaison des *pathogen-associated molecular patterns* (PAMPs) ou *damage-associated molecular patterns* (DAMPS) au système du complément, des récepteurs *Toll-like* (TLR), des *nucleotides-binding oligomerization domain* (NOD)-*like receptors*, des *retinoic acid-inducible gene* (RIG)-*like*

receptors, *mannose-binding lectin* et aux récepteurs scavengers, induit des voies de signalisation intracellulaire complexes avec des réponses redondantes et complémentaires (Figure 1) (Tang et al. 2012).

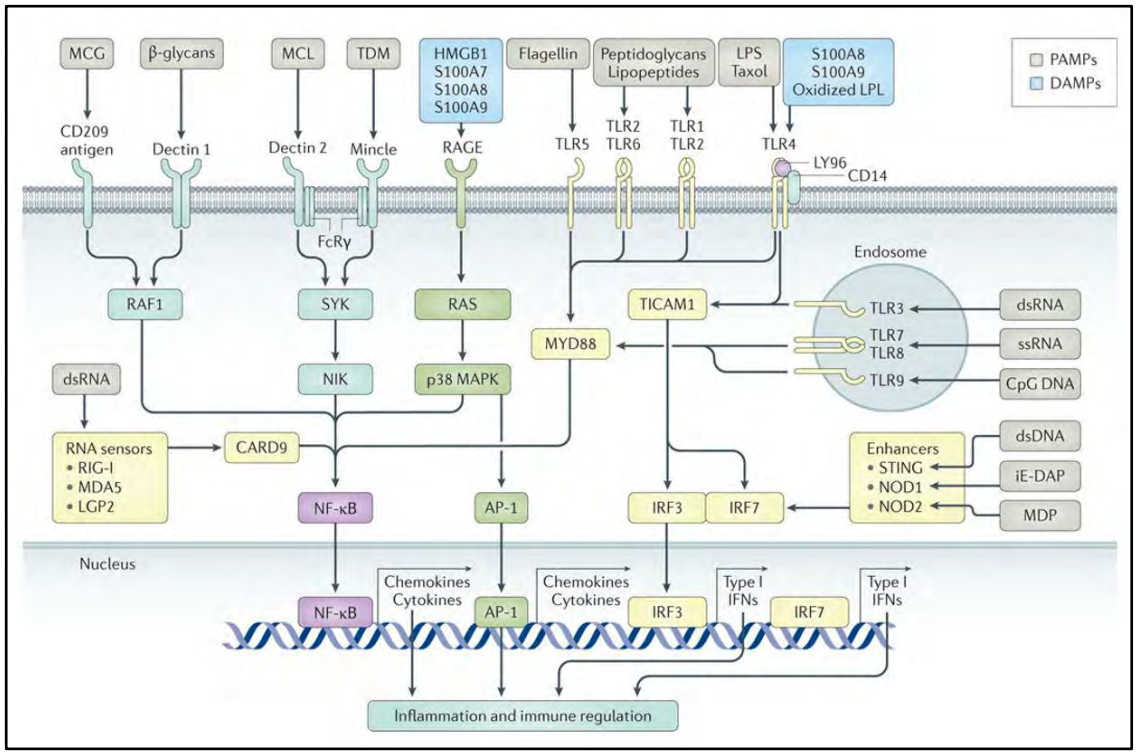


Figure 1 : Récepteurs de surface et intra-cellulaires responsables de la reconnaissance des produits microbiens et des signaux de danger (alarmines)

Initiation du sepsis par la reconnaissance de PAMPs et activation de voies de signalisation inflammatoires. Exemples de PAMPs et DAMPs reconnus par des PRR (pattern-recognition receptors) tels que les TLR (Toll-Like Receptors), dectine 1, dectine 2. Une fois activée, la voie de signalisation en aval des TLR converge généralement vers la signalisation de l'IRF (Interferon Regulatory Factor) et la voie NFκB. IRF est responsable de la production d'interferon de type 1 (IFN). La signalisation de NFκB et d'AP-1 (Activator Protein 1) sont principalement responsables de l'activation précoce de gènes pro-inflammatoires comme le TNF (Tumor Necrosis Factor), Interleukine 1 (IL1) et ceux codant pour des molécules de surface des cellules endothéliales (D'après Hotchkiss, *Nat Rev*, 2016)

Le deuxième point clef correspond à l'activation de multiples voies de signalisation qui mènent *in fine* à l'expression de plusieurs classes de gènes communes engagées dans l'inflammation, l'immunité adaptative et le métabolisme cellulaire.

Associées, ces deux caractéristiques de l'immunité innée assurent un schéma de réponse commun, dont l'intensité et la direction peuvent être finement régulées par le niveau et la variation du répertoire des PAMPs et DAMPs et des voies de signalisation activées.

Concernant l'activation précoce de gènes, la translocation nucléaire de NF-κB et l'activation de son promoteur en particulier, induit l'expression de multiples gènes d'activation précoce, incluant des cytokines associées à la réponse inflammatoire (Tumor Necrosis Factor (TNF), IL-1, IL-12, IL-18 et les interférons de type 1 (IFNs)). Ces cytokines initient une cascade d'autres cytokines inflammatoires et chemokines (incluant IL-6, IL-8, IFNγ, CC-chemokine ligand 2 (CCL2), CCL3 et CXC-chemokine ligand 10 (CXCL 10)), aussi bien que la polarisation et la suppression de composants de l'immunité

adaptative. L'activation de ces réseaux inflammatoires débute dans les minutes qui suivent la reconnaissance de PAMPs et DAMPs. Simultanément, l'activation de ces récepteurs sentinelles de l'immunité innée, l'activation du complément et/ou la production de cytokines inflammatoires ont un effet majeur sur la coagulation et l'endothélium vasculaire et lymphatique, résultant en l'augmentation de l'expression de sélectines et de molécules d'adhésion (Bierhaus and Nawroth 2003). Parallèlement, des protéases pro-inflammatoires induisent l'internalisation de la VE-cadhérine entraînant la perte des jonctions serrées et augmentant la perméabilité vasculaire (Parikh 2013). Les modalités de réponse de l'hôte au sepsis sont résumées dans la figure 2.

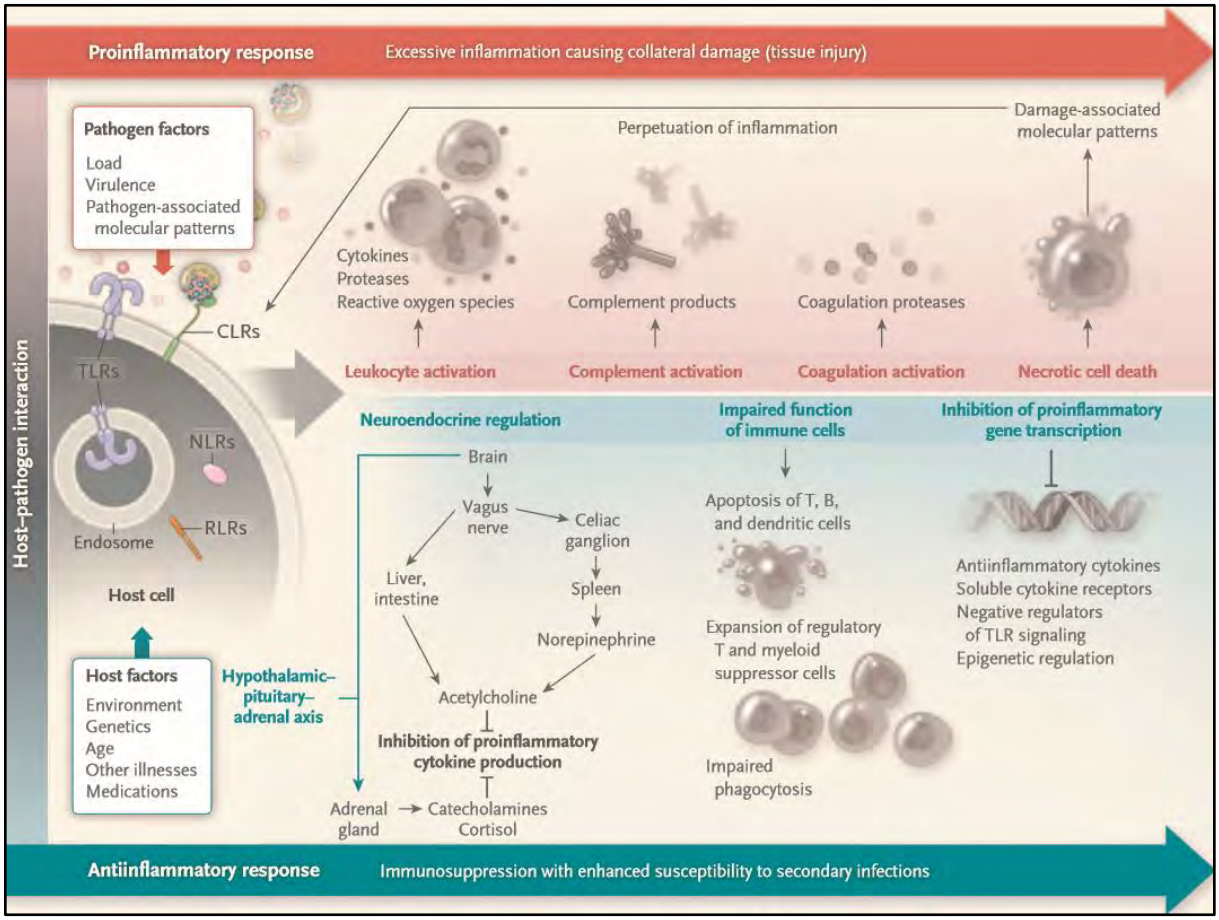


Figure 2 : Réponse de l'hôte au sepsis

La réponse de l'hôte est caractérisée à la fois par une réponse pro-inflammatoire (partie supérieure en rouge) et par une réponse immunosuppressive anti-inflammatoire (partie inférieure en vert). La durée, la direction et l'importance de ces réactions sont déterminées par des facteurs liés à l'hôte et des facteurs liés au pathogène. Les réponses inflammatoires sont initiées par les interactions entre les PAMPs exprimés par les pathogènes et les PRR exprimés par les cellules de l'hôte (à la surface, dans les endosomes ou dans le cytoplasme). Les conséquences d'une inflammation exagérée sont des lésions tissulaires collatérales et la mort cellulaire par nécrose, qui résultent en la libération de DAMPs, ou « molécules de danger », qui perpétuent l'inflammation en agissant à leur tour sur les mêmes PRR précédemment cités (D'après Angus DC, *NEJM* 2013)

1.3.2. Mise en jeu de l'axe complément C5a

L'activation du complément est considérée comme l'un des signes distinctifs du sepsis et est initiée immédiatement après exposition aux PAMPs et DAMPs (Figure 3). Elle contribue au contrôle de l'infection et est clairement bénéfique aux premiers stades du sepsis. Cette activation entraîne la

génération de peptides du complément (nommés C3a et C5a). Le C5a est l'un des peptides inflammatoires les plus actifs produits durant le sepsis et est un des plus puissants chimio-attractants pour les neutrophiles, monocytes et macrophages. Au contact des neutrophiles, le C5a entraîne un burst oxydatif générant des espèces réactives de l'oxygène et le relargage d'enzymes contenues dans les granules, impliquées dans les dommages tissulaires inflammatoires (Guo and Ward 2005). En outre, le fragment C5a entraîne la synthèse et le relargage de cytokines pro-inflammatoires et de chemokines, amplifiant ainsi les réponses inflammatoires. Ces mécanismes contribuent à la vasodilatation, aux dommages tissulaires, et aux défaillances viscérales en cas d'inflammation aiguë.

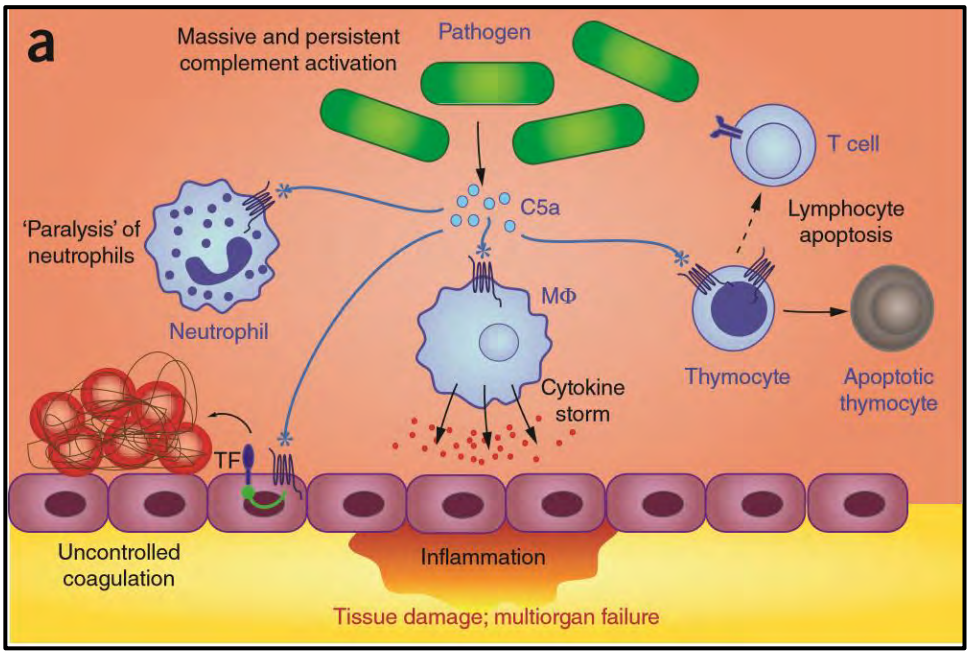


Figure 3 : Rôle du complément dans le sepsis

Durant le sepsis, la présence d'agents infectieux dans le sang et les tissus cause l'activation excessive du complément avec la libération de la fraction C5a qui participe aux effets dévastateurs allant de la suppression immune à l'inflammation majeure et la coagulation intravasculaire disséminée via le facteur tissulaire (D'après Daniel Ricklin, *Nat Immunol* 2010)

1.3.3. Développement d'une suppression Immune

Lorsque la réponse inflammatoire systémique précoce spécifique du sepsis est passée, apparaît une phase dite d'immunosuppression chez l'hôte. Les patients qui survivent à un sepsis sont souvent hospitalisés de façon prolongée et présentent à la fois les caractéristiques d'une suppression immunitaire chronique et d'une inflammation. Ce syndrome a récemment été qualifié de « PICS » pour *Persistent Inflammation/immunosuppression and Catabolism Syndrome* (Gentile et al. 2012; Hu et al. 2014; Vanzant et al. 2014) (Figure 4).

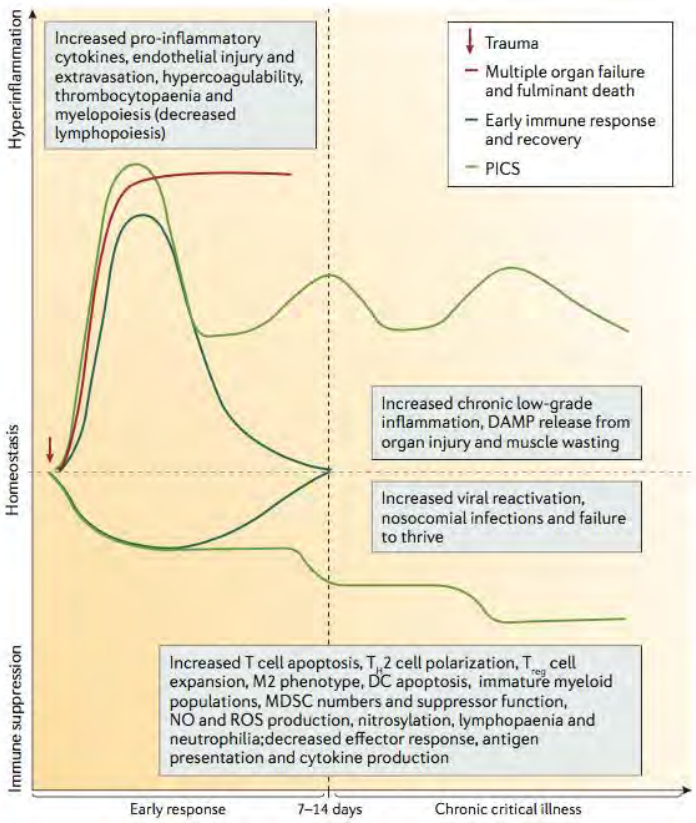


Figure 4 : modèle conceptuel de l'évolution possible d'un sepsis d'après Hotchkiss

Le succès de la réanimation lors du sepsis peut survenir après une phase hyper-inflammatoire (ligne bleue) permettant une sortie de réanimation. Quelques patients présentent une réponse inflammatoire précoce prononcée en réponse aux signaux de danger, menant aux défaillances d'organe et parfois au décès (ligne rouge). D'autres patients survivent à la phase précoce très inflammatoire mais présentent une chronicisation de leur état en réanimation (lignes vertes) qui est caractérisé par une inflammation persistante, une immunosuppression et un syndrome catabolique (PICS), associé à la réactivation d'infections virales latentes, la survenue d'infections nosocomiales et d'un déclin cognitif à plus long terme (D'après Hotchkiss, *Nat Rev* 2016).

Le PICS est probablement alimenté par des DAMPs et des alarmines produites par des organes et des tissus lésés, tels que l'ADN mitochondrial et les nucléosides, les histones, HMGB1 (*High Mobility Group Protein B*-1), la protéine S100A ou l'ATP (Rubartelli and Lotze 2007). Comparativement à des sujets témoins sans sepsis, les patients septiques présentent des taux de réactivation plus élevés des virus latents, l'ADN viral étant détecté dans le sang de 42% des patients atteints de sepsis contre 5% des patients non septiques (Walton et al. 2014).

Les changements dans l'immunité adaptative en réponse au sepsis sont profonds. Une lymphopénie, la production de neutrophiles immatures (Delano et al. 2007; Taneja et al. 2008), la diminution des cytokines inflammatoires monocytaires (Munoz et al. 1991) ainsi qu'un nombre accru de cellules suppressives dérivées de la lignée myéloïde libérées dans la circulation sanguine (Cuenca et al. 2011) sont des caractéristiques courantes lors d'un sepsis.

1.3.4. Dysfonction endothéliale

En plus de profonds changements dans l'immunité protectrice de l'hôte, la fonction de barrière endothéliale fait partie intégrante de la réponse au sepsis. La phase liquide du compartiment vasculaire est séparée des tissus par une barrière endothéliale continue qui recouvre le système vasculaire. Dans des conditions physiologiques, l'endothélium sert de surface anticoagulante et régule les flux de gaz, d'eau, de solutés, d'hormones, de lipides, de protéines, et d'une multitude d'autres macromolécules de la microcirculation. Le sepsis entraîne une dysrégulation notamment des communications cellule-cellule qui participent à l'homéostasie dans des conditions physiologiques (Deutschman and Tracey 2014) avec un dysfonctionnement de la barrière endothéliale qui survient précocement. La barrière endothéliale, en situation normale ou pathologique, est hautement dynamique grâce à la cellule endothéliale, pivot de la régulation de cette barrière. L'endothélium sain a pour rôle de garantir la couverture de la membrane capillaire et de l'adventice, afin d'éviter l'exposition des fibres de collagène et du facteur von Willebrand (FvW) qui interagit avec. Lorsqu'elle est exposée au sang, la matrice sous-endothéliale riche en collagène fournit une surface adhésive capable de recruter et d'activer les plaquettes. Dans le même temps, l'endothélium activé peut exprimer le facteur tissulaire qui va lier le facteur VII circulant et entraîner ainsi l'initiation de la cascade de la coagulation par la voie du facteur tissulaire (ou voie extrinsèque) (Angus and van der Poll 2013).

L'intégrité de l'endothélium est maintenue par le cytosquelette cellulaire (actine), les molécules d'adhérence intercellulaire (jonctions serrées) et un ensemble de protéines de soutien. Durant le sepsis, ces structures sont perturbées principalement en réponse à l'adhésion plaquettaire et des neutrophiles et à la libération des médiateurs inflammatoires et d'intermédiaires toxiques oxydants et nitrés. Combinée à l'augmentation de l'expression des sélectines et des intégrines, la liaison des leucocytes à la surface endothéliale entraîne une fuite du liquide vasculaire et la migration des leucocytes par extravasation à travers la barrière endothéliale altérée. Bien que cette réponse permette aux plaquettes et aux cellules immunitaires d'atteindre les sites tissulaires sièges d'une infection, le sepsis induit des réponses systémiques excessives et prolongées qui peuvent engendrer des lésions tissulaires majeures. Parallèlement, le glycocalyx est aussi altéré. Il est composé d'une couche de glycoprotéines-polysaccharides qui recouvre l'endothélium et maintient l'état anticoagulant et les jonctions serrées. Le sepsis altère la continuité du glycocalyx ce qui concourt à une augmentation de la perméabilité endothéliale (Chelazzi et al. 2015).

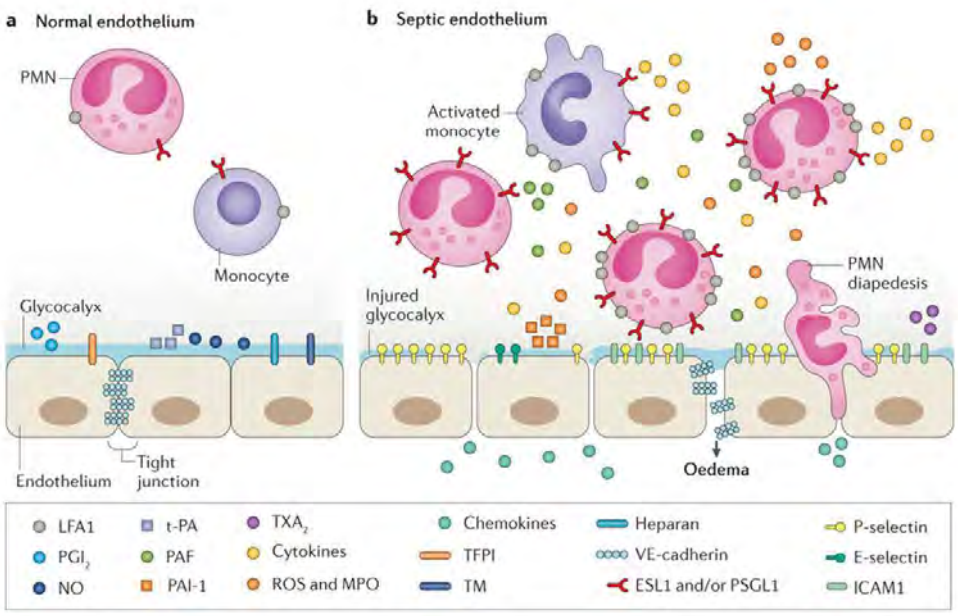


Figure 5 : Changements de l'endothélium vasculaire en réponse aux stimuli inflammatoires du sepsis

L'endothélium vasculaire au repos présente un phénotype anticoagulant (a). Le sepsis induit de profonds changements qui entraînent un état anticoagulant (b). L'altération de la barrière endothéliale se traduit par une fuite capillaire au travers de la perte des jonctions serrées et par le recrutement, l'attachement et l'extravasation de cellules inflammatoires au travers de l'endothélium. L'activation de la cascade de la coagulation potentialise l'inflammation et participe au cercle vicieux dans lequel l'inflammation induit et exacerbe les lésions endothéliales et la coagulopathie (D'après Hotchkiss, *Nat Rev* 2016)

L'hyperperméabilité capillaire crée une fuite massive de protéines intravasculaires et de fluides plasmatiques vers l'espace extravasculaire (Figure 5). La vasodilatation étendue tout le long de la microcirculation modifie le flux sanguin capillaire contribuant à une mauvaise perfusion tissulaire et, finalement, à un état de choc septique. Dans le choc septique, la dysfonction endothéliale entraîne un choc distributif au cours duquel le maintien de la pression sanguine n'est pas obtenu lors de l'administration de fluides intraveineux et nécessite un agent vasopresseur. L'apport de solutés intravasculaires pour maintenir la pression sanguine artérielle contribue secondairement à l'apparition des œdèmes du fait de la fuite capillaire.

1.3.5. Thromboinflammation et coagulopathie septique

Inflammation et coagulation sont étroitement liées aux mécanismes de défense lors d'un sepsis et auto-amplifiées par stimulation réciproque (Aird 2003).

1.3.5.1. Thrombose et inflammation

L'activation coordonnée des réponses hémostatique et inflammatoire suite à une infection est un mécanisme de défense phylogénétiquement conservé que l'on peut retrouver en remontant aux organismes invertébrés (Herwald and Theopold 2011; Loof et al. 2011). Dans ces organismes

primitifs, une cellule unique (l'hémocyte) peut supporter des fonctions basiques inflammatoires, immunes et hémostatiques. Cependant, l'évolution des mammifères vertébrés s'est faite vers un système complexe multicellulaire comprenant les plaquettes et de multiples populations de leucocytes. En dépit de cette évolution, la thrombose et l'inflammation ont traditionnellement été décrites comme deux processus complémentaires distincts. La thrombose peut être définie par une réponse hémostatique exagérée menant à la formation d'un thrombus occlusif dans la circulation sanguine. En comparaison, l'inflammation est le terme employé pour décrire la réponse immune complexe protectrice en réponse à un stimulus dangereux comme la présence d'un pathogène. En réalité, l'inflammation stimule la thrombose comme la thrombose promeut l'inflammation, témoins d'une interdépendance fonctionnelle (Jackson, Darbousset, and Schoenwaelder 2019) (Figure 6).

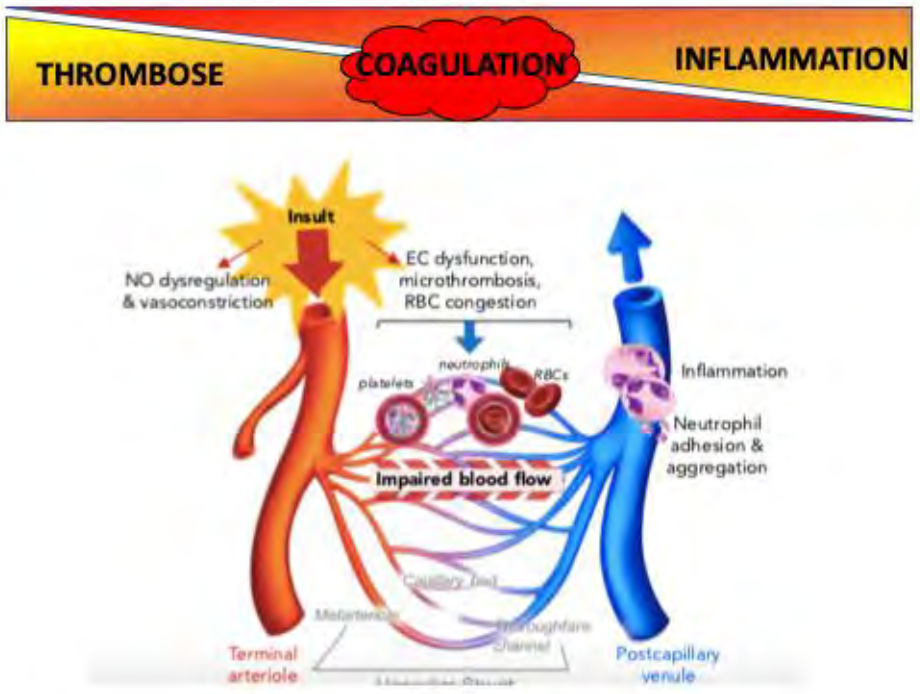


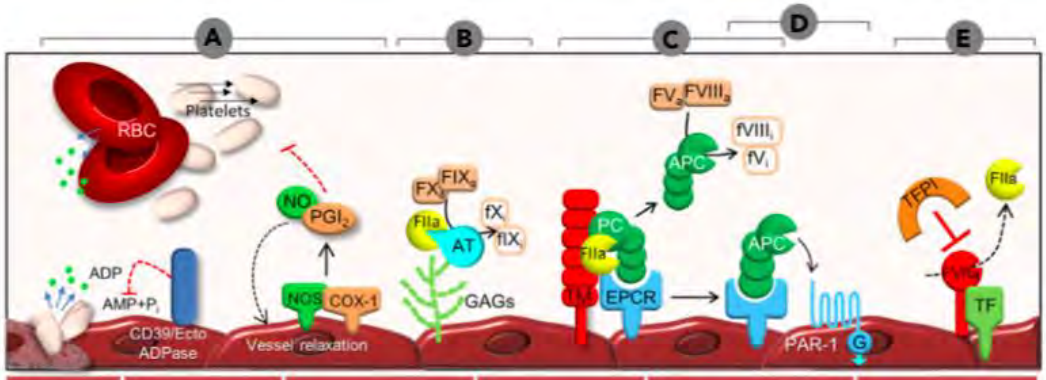
Figure 6: La thromboinflammation au carrefour de la thrombose et de l'inflammation

Les lésions des tissus ou des organes par divers mécanismes pathogènes sont généralement associées à des réponses thrombo-inflammatoires microvasculaires. L'obstruction microvasculaire peut être médiée par tous les éléments cellulaires du sang, y compris les plaquettes, les neutrophiles et les globules rouges (RBCs), l'occlusion stable étant généralement liée à l'activation de la coagulation et de la génération de fibrine. L'issue finale de ces troubles est fortement influencée par l'ampleur des réponses microvasculaires thrombotiques et inflammatoires. NO, oxyde nitrique, EC, cellule endothéliale. (D'après Watson SP, *Blood* 2019)

1.3.5.2. L'endothélium, régulateur critique de la thrombo-inflammation

Comme évoqué plus tôt, à l'état de repos, l'endothélium possède des propriétés anti-agrégantes, anticoagulantes et anti-inflammatoires. Les cellules endothéliales préviennent l'adhésion et l'activation plaquettaires en produisant de puissants antagonistes (l'oxyde nitrique ou NO, la

prostacycline ou PGI2, l'ectoadénosine diphosphatase). Ces molécules participent au maintien de l'homéostasie de l'endothélium. Le NO diminue le recrutement des leucocytes en diminuant l'expression de P-sélectine à la surface des cellules endothéliales, en diminuant l'expression des chemokines et en réduisant la transcription de molécules d'adhésion telles que E-sélectine, VCAM-1 et ICAM-1. La prostacycline (PGI2) diminue l'inflammation en réduisant l'adhésion, l'activation et l'extravasation leucocytaires. En outre, l'endothélium supporte un vaste répertoire de voies anticoagulantes et anti-fibrinolytiques naturelles, impliquant les glycosaminoglycanes (GAG), la thrombomoduline (TM), la voie de la protéine C activée (APC) et l'inhibiteur de la voie du facteur tissulaire (TFPI) (Figure 7). En revanche, lors du sepsis, les composants de la paroi cellulaire bactérienne activent les récepteurs de type PRR à la surface endothéliale, ce qui entraîne la production de cytokines. Les endotoxines bactériennes stimulent également fortement l'expression du facteur tissulaire (FT) et augmentent le taux de l'inhibiteur de l'activateur du plasminogène 1 (PAI-1), bloquant la fibrinolyse et favorisant le phénotype procoagulant de l'endothélium.



Molecule or Pathway	CD39 – Ecto ADPase (A)	NO and PGI$_2$ (A)	GAGs and ATIII (B)	TM, Protein C & EPCR (C and D)	TFPI (E)
ACTION	Degradation of ADP	Production of vasoactive hormones nitric oxide (NO) & prostacyclin (PGI2)	Inhibition of Thrombin (FIIa), FXa and FIXa, [GAGs include heparan- sulfate]	C: FVa & FVIIIa inactivation; D: Activation of PAR-1	Blocks TF pathway
OUTCOME	Anti-platelet	Vessel relaxation Anti-platelet; [indirect anti-coagulant via platelet inhibition]	Anti-coagulant [anti-inflammatory; anti-platelet via thrombin reduction]	A: Anti-coagulant [anti-inflammatory; anti-platelet via thrombin reduction]; B: Cytoprotection; barrier protection	Anti-coagulant [anti-inflammatory; anti-platelet via thrombin reduction]

Figure 7: Fonctions anti-thrombotiques des cellules endothéliales

(A) L'endothélium présente un phénotype antiadhésif garanti par 3 voies intrinsèques : la voie CD39/ecto-adénosine diphosphatase (ecto-ADPase), et les voies PGI2 et NO, qui inhibent l'activation plaquettaire. L'endothélium utilise plusieurs mécanismes pour neutraliser l'α-thrombine. (B) Premièrement, l'antithrombine (AT) se lie aux GAG à la surface des cellules endothéliales et inactive l'α-thrombine (IIa), le FXa et plusieurs autres protéases de coagulation. (C-D) Deuxièmement, la thrombine peut être convertie en protéase anticoagulante dans la microcirculation en liant la thrombomoduline (TM) et le récepteur de la protéine C endothéliale (EPCR). Une fois liée à ces récepteurs, la thrombine clive et active la protéine C (PC) pour générer l'APC. (C) L'APC fonctionne comme un anticoagulant en inactivant protéolytiquement le FVa et le FVIIIa. (D) Si l'APC reste liée à l'EPCR, elle induit une signalisation par l'intermédiaire de PAR1 afin d'obtenir une cytoprotection et une augmentation de la fonction de barrière endothéliale. (E) Un endothélium sain exprime également des niveaux élevés de TFPI qui inhibent la formation du complexe facteur tissulaire (TF)/FVIIa, empêchant ainsi le déclenchement de la coagulation et la génération ultérieure de thrombine. AMP, ecto-adénosine monophosphate ; PAR1, récepteur 1 activé par la protéase ; RBC, globule rouge. (D'après Jackson SP, *Blood* 2019)

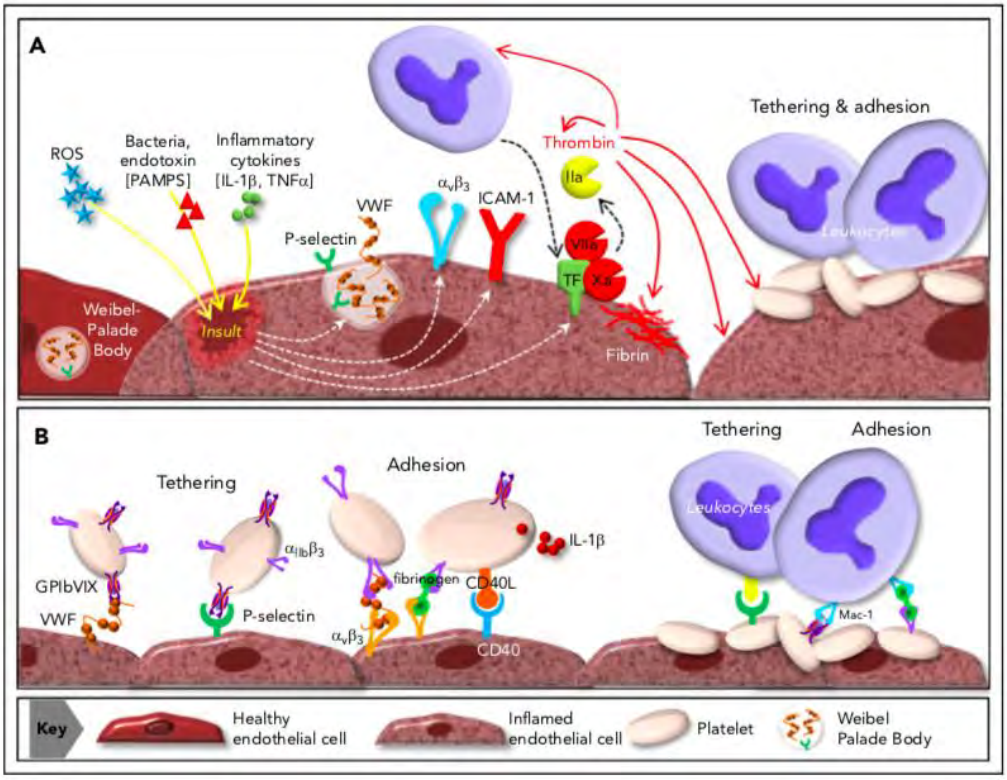


Figure 8: Fonctions pro-inflammatoires et pro-thrombotiques des cellules endothéliales activées par l'inflammation

A) l' « agression » de l'endothélium lors d'un sepsis entraîne une augmentation de l'expression des molécules d'adhésion, telles que le facteur von Willebrand (VWF) et les P-sélectines, $\alpha_v\beta3$ et ICAM-1, ce qui conduit au recrutement de plaquettes et de leucocytes. Les cellules endothéliales activées expriment également le facteur tissulaire (TF), ce qui entraîne l'activation du FVII, du FXa et, finalement, la génération de thrombine. La thrombine clive le fibrinogène pour générer de la fibrine, ainsi que de multiples récepteurs activés par la protéase à la surface des cellules endothéliales, des plaquettes et des leucocytes, propageant ainsi le processus thromboinflammatoire. (B) Recrutement des plaquettes et des leucocytes sur les sites d'endothélium lésé : l'expression du VWF et de la P-sélectine à la surface des cellules endothéliales endommagées favorise la fixation et le roulement des plaquettes. Par la suite, une adhésion stable des plaquettes se produit par le biais de complexes fibrinogène-intégrine $\alpha_{IIb}\beta_3$ se liant à αvβ3 ou à ICAM-1 sur les cellules endothéliales. Les plaquettes adhérentes sécrètent de nombreuses substances bioactives qui modifient les propriétés chimiotactiques et adhésives des cellules endothéliales. L'interleukine-1 (IL-1) dérivée des plaquettes induit une expression active du FT sur l'endothélium. L'expression de la P-sélectine sur les plaquettes activées adhérentes induit la fixation des leucocytes suivie de leur adhésion, principalement via Mac-1 lié au complexe GPIb-GPV-GPIX et fibrinogène-$\alpha_{IIb}\beta_3$. PAMPs, Pathogen Associated Molecular Patterns ; TNFα, Tumor Necrosis Factor. (D'après Jackson SP, *Blood* 2019)

1.3.5.3. α-thrombine : médiatrice centrale de la thrombo-inflammation

L'expression du FT dans le système vasculaire est considérée comme une étape essentielle pour initier et maintenir la coagulation dans un large éventail de maladies thrombo-inflammatoires (Erlich et al. 2000). Le FT est un puissant activateur de la coagulation grâce à sa liaison de haute affinité avec le facteur VII activé (FVIIa). Bien qu'il soit principalement exprimé par les cellules entourant la paroi du vaisseau, y compris les péricytes et les fibroblastes, le FT peut également être produit en intravasculaire par les cellules endothéliales, les monocytes et les microparticules circulantes (Levi and van der Poll 2017). Les monocytes peuvent synthétiser et exprimer le FT et sont considérés comme une source majeure de FT sanguin.

Le FT est l'initiateur le plus important de la génération de thrombine durant le sepsis (Levi and van der Poll 2010).

Au cours du sepsis, les PAMPs exprimés par les bactéries envahissantes, sont reconnus par les PRR présents à la surface des cellules endothéliales, des plaquettes et des leucocytes. Les PRR transduisent des signaux conduisant à la libération de cytokines et de chemokines inflammatoires et à une expression accrue des molécules d'adhésion des leucocytes. Ils limitent également le système anticoagulant et fibrinolytique naturel des cellules endothéliales et augmentent la production de FT par les monocytes et les cellules endothéliales (Opal and Esmon 2003; Pawlinski and Mackman 2010).

Une autre voie favorisant la génération d'α-thrombine est la voie dite contact de la coagulation (voie intrinsèque). La thrombine est également un puissant activateur des plaquettes, facilitant leur fonction pro-coagulante et la production de fibrine par l'expression de surface de la phosphatidylsérine (Jackson and Schoenwaelder 2010).

1.3.5.4. Les multiples facettes de la thrombine

Étant donnée l'importance centrale de la thrombine dans la propagation de la thrombose microvasculaire et de l'inflammation, la définition des mécanismes de la génération dérégulée de thrombine et une meilleure compréhension des mécanismes par lesquels cette sérine-protéase favorise la thrombo-inflammation durant le sepsis sont importants pour le développement futur de thérapies anticoagulantes et anti-inflammatoires efficaces.

La thrombine peut augmenter sa propre génération notamment via une boucle d'activation rétroactive du complexe thrombine-Facteur XI (Matafonov et al. 2011). De nombreuses fonctions de la thrombine sont médiées par des récepteurs PAR (protease-activated receptors) couplés aux protéines G. Quatre PAR ont été identifiés et peuvent être activés par un large éventail de protéases.

La thrombine a de nombreux effets dans le système vasculaire. Une partie de ses effets pro-inflammatoires est médiée par la stimulation des cellules endothéliales. L'α-thrombine active les cellules endothéliales principalement par la protéolyse de PAR1, induisant l'expression du FT et la mobilisation des corps de Weibel-Palade, ce qui entraîne une augmentation de l'expression de la P-sélectine et la libération du FvW (Tull et al. 2012). L'α-thrombine induit également la libération de chimiokines par l'endothélium, de cytokines et de facteurs de croissance, et elle régule l'expression des molécules d'adhésion, notamment VCAM-1, ICAM-1 et E-sélectine (Okada et al. 2006).

L'α-thrombine stimule l'activité pro-coagulante des plaquettes par l'intermédiaire des PAR1 et PAR4 (PAR3 et PAR4 chez la souris). Le clivage des PAR plaquettaires déclenche la libération du contenu des granules, libérant ainsi l'ADP et la sérotonine, entraînant l'expression de la P-sélectine et du CD40 ligand, la génération de thromboxane A2, et la libération d'un large éventail de molécules

pro-inflammatoires, y compris des chimiokines et des facteurs de croissance (Shankar et al. 2006; Coppinger et al. 2004). Enfin, l'α-thrombine est un puissant activateur de l'intégrine $\alpha_{IIb}\beta_3$, induisant l'agrégation plaquettaire (Figure 9).

1.3.5.5. Coagulopathie septique

La formation du thrombus et l'activation de l'immunité innée constituent un avantage pour la survie en contenant localement l'infection. Cependant, en cas d'activation généralisée de la coagulation et de l'inflammation, comme c'est le cas dans la coagulation intravasculaire disséminée (CIVD), les conséquences peuvent être dévastatrices et mener au choc septique potentiellement mortel. La survenue de saignements semble être inconstante compte-tenu du phénotype procoagulant du sepsis avec initialement un état d'hypercoagulabilité et des dépôts de fibrine, mais ce processus hémorragique apparaît souvent dans un second temps, comme étant le résultat de la thrombocytopénie et de la consommation de facteurs de coagulation. La transition d'un état d'hypercoagulabilité vers une CIVD est caractérisée par une fibrinolyse qui entraîne la circulation de produits de dégradation de la fibrine (PDF), une thrombopénie, l'épuisement des réserves hépatiques de prothrombine, fibrinogène, facteur X et V.

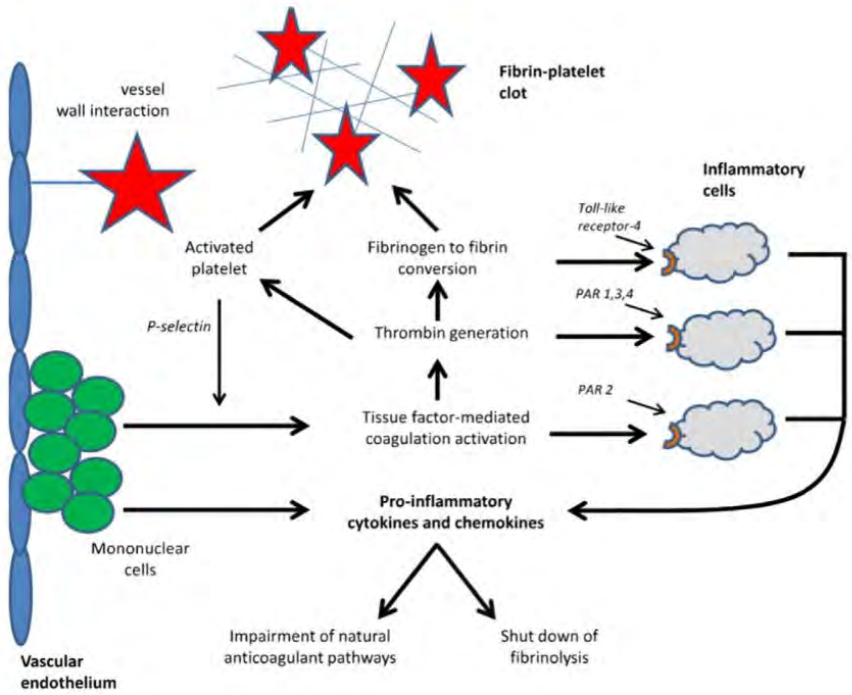


Figure 9: Interaction entre inflammation et coagulation durant le sepsis

L'expression de facteur tissulaire par les monocytes entraîne la génération de thrombine suivie par le clivage du fibrinogène qui polymérise alors en fibrine. De façon concomitante, les interactions entre les plaquettes et l'endothélium et l'activation des plaquettes contribuent à la formation de microthrombi vasculaires. L'augmentation de l'expression de la P-selectine à la surface des plaquettes activées favorise leur interaction avec les monocytes ce qui augmente l'expression du facteur tissulaire sur les monocytes. La liaison du facteur tissulaire, de la thrombine et d'autres protéases de coagulation aux récepteurs activés par les protéases (PAR) et la liaison de la fibrine au récepteur Toll-like

4 (TLR-4) sur les cellules inflammatoires majore l'état d'inflammation par la libération consécutive de cytokines et chimiokines pro-inflammatoires, qui modulent à leur tour la coagulation et la fibrinolyse. (D'après Lévi M, Thromb Res 2017)

1.3.6. Effets sur les organes

Le sepsis est un désordre systémique qui peut affecter tous les organes, probablement en raison du panel de cytokines et autres médiateurs inflammatoires libérés dans la circulation générale dès le début de l'infection. Les signes cliniques présentés ainsi que les symptômes sont variables et dépendent des organes affectés. On peut schématiquement distinguer 6 types de dysfonctions d'organes prédominantes dans le sepsis : neurologique, pulmonaire, cardiovasculaire, rénale, hématologique et hépatique. En détail,

Atteinte neurologique : typiquement, les patients présentent une altération neurologique, qui peut se manifester par une léthargie, une confusion ou un délirium. Occasionnellement, l'état de conscience est si altéré qu'il est nécessaire de sécuriser les voies aériennes du patient (procéder à une intubation orotrachéale). L'examen neurologique est typiquement sans atteinte focale. Durant l'évaluation clinique, les autres causes de troubles neurologiques (par exemple l'hypoxémie, l'hypoglycémie, la toxicité médicamenteuse ou une infection du système nerveux central) doivent être éliminées ou confirmées.

Atteinte pulmonaire : une des manifestations les plus communes du sepsis est l'augmentation de la fréquence respiratoire. La polypnée peut être associée à des gaz du sang artériel anormaux, typiquement une alcalose respiratoire à la phase initiale. Une hypoxémie et/ou hypercapnie peuvent également apparaître, souvent en lien avec une fatigue des muscles respiratoires. Ainsi, l'hypoxémie ou l'hypercapnie qui en découlent peuvent nécessiter le recours à l'intubation orotrachéale. L'étiologie de la défaillance respiratoire dans le sepsis est en partie due au dommage alvéolaire diffus des membranes capillaires des alvéoles du fait de l'inflammation. Ces lésions pulmonaires médiées par les cytokines se manifestent par un œdème alvéolaire non cardiogénique qui cause une diminution de la compliance pulmonaire et altère les échanges en oxygène et l'élimination du dioxyde de carbone. La diminution de la compliance pulmonaire et l'activation des récepteurs juxta-capillaires entraînent une augmentation de la ventilation minute et sont en partie responsables de la tachypnée. Le cliché radiologique des poumons montre en général des infiltrats pulmonaires bilatéraux. Même si les patients peuvent présenter une hypoxémie profonde mettant en jeu leur pronostic vital, la plupart des patients ne décède pas d'hypoxémie mais plutôt de défaillance multiple d'organes.

Atteinte cardio-vasculaire : la défaillance myocardique peut s'associer ou non à un état de vasoplégie majeure, caractérisée par une hypotension artérielle nécessitant l'emploi d'un traitement vasopresseur. Une augmentation modérée des troponines cardiaques est fréquente au cours du sepsis et en lien avec la sévérité du sepsis. La dépression myocardique affecte en général les deux ventricules ce

qui différencie cette étiologie d'une lésion artérioscléreuse coronarienne. La dysfonction myocardique liée au sepsis peut être majeure avec une diminution des fractions d'éjection des ventricules droit et gauche, nécessitant le recours à des agents inotropes (Sato and Nasu 2015).

Atteinte rénale : la dysfonction rénale du sepsis qui peut déboucher sur une insuffisance rénale aiguë sévère est une cause majeure de décès (L. E. White et al. 2013). Bien que le mécanisme de l'insuffisance rénale aiguë du sepsis soit mal connu, les cliniciens peuvent réduire l'incidence de l'atteinte rénale sévère par une réanimation agressive et optimisée avec remplissage vasculaire pour essayer de limiter l'impact du sepsis. Du fait de la diminution du volume intravasculaire par la fuite capillaire et la vasodilatation, les patients requièrent habituellement un volume de remplissage majeur pour compenser l'hypovolémie. Les cliniciens doivent limiter l'emploi de traitements néphrotoxiques autant que possible chez les patients septiques. L'absence de récupération d'une fonction rénale complète est associée à un mauvais devenir des patients. De ce fait, la prise en charge de cette atteinte rénale durant le sepsis est primordiale. Il faut noter que même une augmentation mineure des concentrations plasmatiques de créatinine est associée à une augmentation de la mortalité (Ricci et al. 2011).

Atteinte hématologique : la CIVD est une des manifestations les plus bruyantes du sepsis. Elle peut se manifester par 2 entités cliniques différentes : avec un saignement diffus ou au contraire avec des thromboses des petits et moyens vaisseaux. La raison de ces différences frappantes de présentation clinique vient du fait que les systèmes de coagulation représentent sans cesse une balance entre la coagulation et la fibrinolyse. Pour un même sepsis, chaque système peut prédominer. Si la fibrinolyse est dominante, le patient pourra présenter un profil plutôt hémorragique. A l'inverse si l'activation du système procoagulant prédomine, le patient pourra présenter des extrémités décolorées, cyanosées voire nécrotiques avec une gangrène des extrémités possible.

Atteinte hépatique : la dysfonction hépatique est fréquente dans le sepsis bien que l'insuffisance hépatique elle-même induite par le sepsis reste rare, survenant chez moins de 2% des patients (Angus et al. 2001). L'atteinte hépatique du sepsis se manifeste par une augmentation des concentrations plasmatiques des enzymes hépatiques et des taux élevés de bilirubine. L'étiologie exacte de l'atteinte hépatique reste méconnue. De façon indubitable, une part importante de la dysfonction hépatique est à mettre en lien avec la nécrose centro-lobulaire du foie secondaire à la baisse de la perfusion hépatique. Les hépatocytes subissent plusieurs mécanismes de mort cellulaire au cours du sepsis. Des séries autopsiques ont montré des zones d'hépatocytes nécrotiques dans les régions entourant les veines centrales (Boomer et al. 2011; Hotchkiss et al. 1999). En plus des

phénomènes de nécrose, de nombreuses cellules subissent une apoptose. De façon intéressante, la microscopie électronique a montré qu'il y avait une augmentation de vacuoles d'autophagie au sein des hépatocytes chez les patients septiques. Dans de rares cas, les vacuoles d'autophagie étaient si extensives que l'on constatait une mort cellulaire induite par autophagie (Watanabe et al. 2009).

1.4. Diagnostic du choc septique, biomarqueurs

L'étude des biomarqueurs du sepsis comme éléments permettant d'identifier formellement un sepsis ainsi que sa gravité a majoritairement été décevante. Cependant, de nombreux biomarqueurs basés sur l'ampleur de la réponse inflammatoire, tels que l'IL-6, IL-10, CCL2, CXCL10 et HMGB1, ont montré une bonne corrélation avec la gravité du sepsis mais souffrent d'un manque de spécificité lors de la réponse inflammatoire précoce. Notre capacité à distinguer le sepsis d'une autre maladie grave non infectieuse ainsi que la capacité à pronostiquer l'issue de cette maladie est très limitée.

La seule exception concerne l'utilisation de la procalcitonine pour distinguer le sepsis d'une maladie grave non infectieuse et pour guider l'utilisation d'un traitement antibiotique (Rowland, Hilliard, and Barlow 2015). La procalcitonine est un peptide précurseur de l'hormone calcitonine produite par les cellules parafolliculaires de la thyroïde et par les cellules neuroendocrines du poumon et de l'intestin. Chez les individus en bonne santé, les taux de procalcitonine sont presque indétectables. Initialement, il y avait un enthousiasme considérable pour que les concentrations de procalcitonine puissent distinguer le sepsis d'une pathologie grave non septique et pour prédire les résultats cliniques mieux que les cytokines inflammatoires ou les critères cliniques. Cependant, à ce jour, son utilisation reste controversée (Rowland, Hilliard, and Barlow 2015; Casserly et al. 2015).

1.5. Prise en charge du choc septique

Une fois le sepsis/choc septique identifié, une prise en charge appropriée précoce et agressive doit être mise en place sans délai. Le traitement repose sur trois composantes : le contrôle du foyer infectieux, la stabilisation hémodynamique et la modulation de la réponse septique.

1.5.1. Contrôle de l'infection

Un traitement anti-infectieux adapté, généralement antibiotique, doit être initié le plus tôt possible de façon probabiliste puis secondairement adapté après obtention des données de culture. L'importance du choix initial du traitement est primordiale, car elle réduit considérablement le risque de mortalité (Kumar et al. 2009; Zahar et al. 2011). Le choix des molécules administrées repose sur le spectre anti-infectieux large, de la source probable d'infection, de la flore microbiologique locale et des profils de résistance, des thérapeutiques anti-infectieuses précédemment administrées et de

l'écologie des établissements de santé. Le traitement anti-infectieux en association est préférable au traitement par un agent unique, en particulier dans les cas les plus graves (Kumar et al. 2010; Micek et al. 2010). Une fois que les résultats de la culture sont disponibles, le choix des thérapeutiques doit être réévalué et la réduction du spectre sur un spectre plus étroit doit être effectuée dans la mesure du possible. Cette approche optimisera l'efficacité du traitement, en limitera la toxicité, aidera à prévenir le développement d'une pharmaco-résistance et réduira les coûts. Néanmoins, dans certains cas, plusieurs organismes sont incriminés et, chez 30% des patients, les résultats de la culture demeurent négatifs (Vincent et al. 2009; Sprung et al. 2006) de sorte qu'une désescalade n'est pas toujours possible (Heenen, Jacobs, and Vincent 2012).

Parallèlement, le contrôle du foyer infectieux par une intervention chirurgicale, un drainage ou le retrait un dispositif médical infecté peut s'avérer nécessaire au contrôle du sepsis.

1.5.2. Stabilisation hémodynamique

La prise en charge hémodynamique des patients en choc septique peut être envisagée en plusieurs phases, de l'optimisation à la désescalade en passant par la stabilisation (P. J.-L. V. MD et al. 2014). L'objectif général est de fournir un soutien hémodynamique immédiat pour prévenir les lésions et défaillances multiples d'organes (P. J.-L. V. MD et al. 2014) en assurant une pression artérielle de perfusion suffisante (en général, pression artérielle moyenne supérieure à 65 mmHg). A la phase initiale, l'administration de fluides doit être généreuse (Dellinger et al. 2013). Dans la phase d'optimisation, une approche individualisée est nécessaire. Les critères de précharge dépendance ou réponse au remplissage vasculaire, doivent être très régulièrement réévalués afin de titrer le volume d'expansion volémique nécessaire. Après la période de stabilisation, une phase de désescalade doit être menée, dans laquelle le bilan hydrique devrait devenir négatif.

Sur la base d'essais contrôlés randomisés de grande envergure, les recommandations actuelles recommandent l'utilisation de cristalloïdes pour la réanimation liquidienne et suggèrent l'utilisation d'albumine humaine en cas de choc septique lorsque les patients ne peuvent pas être stabilisés avec des cristalloïdes (Dellinger et al. 2013). L'utilisation d'hydroxyéthylamidon chez les patients atteints de sepsis a été interdite par la FDA (Food Drug Administration) et l'Agence européenne du médicaments (EMA), en raison de l'augmentation de la mortalité; l'utilisation d'autres colloïdes synthétiques est également déconseillée (Reinhart et al. 2012). Les quantités excessives de solutions salines doivent également être évitées, car l'hyperchlorémie peut avoir des effets indésirables, en particulier au niveau rénal (Myburgh 2015).

L'usage d'agents vasoactifs est également souvent nécessaire parallèlement à l'administration de liquides dans le but d'éviter une hypotension prolongée pouvant nuire à la perfusion tissulaire. La noradrénaline est recommandée par rapport à la dopamine en raison de la réduction des effets

indésirables et de la mortalité (De Backer et al. 2010; Marshall 2014). La dobutamine s'emploie en tant qu'agent inotrope afin d'augmenter le débit cardiaque et la délivrance d'oxygène aux tissus. Le suivi de l'évolution du taux de lactate sanguin artériel peut aider à évaluer l'efficacité de la réanimation.

Enfin, une assistance respiratoire appropriée est souvent nécessaire en raison de la prépondérance élevée des lésions pulmonaires aiguës et de l'hypoxémie. Cela passe par la réalisation d'une intubation endotrachéale pour ventilation mécanique contrôlée associée à une sédation-analgésie. L'objectif primordial est le maintien d'une oxygénation adéquate tout en minimisant la fraction d'oxygène et des volumes inhalés, dans le but d'obtenir un sevrage du ventilateur le plus rapidement possible.

1.5.3. Modulation de la réponse inflammatoire

La réponse inflammatoire précoce exagérée demeure une cible d'intérêt dans la recherche fondamentale et clinique autour du choc septique. À l'heure actuelle, au moins 150 essais cliniques ont été menés sur PRR, les PAMPs eux-mêmes ou les cytokines et médiateurs inflammatoires précoces du sepsis. Aucun n'a démontré à ce jour son efficacité, même si plusieurs sont encore en cours d'essai. L'analyse rétrospective de sous-groupes a souvent montré un bénéfice significatif dans les sous-groupes plus petits avec antagoniste du récepteur de l'IL-1 et inhibiteurs du TNF, mais ceux-ci n'ont pas été validés de manière prospective. Il a été suggéré que les raisons de l'échec de ces essais cliniques incluaient le moment choisi pour l'administration du médicament et l'impossibilité d'identifier de manière prospective les patients présentant un sepsis qui pourraient bénéficier de ces traitements (Marshall 2014). Chez certaines populations de patients, le traitement avec des agents anti-inflammatoires a en réalité augmenté la mortalité (Qiu et al. 2011), ce qui suggère que, dans certains cas, la production endogène de ces médiateurs pourrait être essentielle pour l'immunité protectrice. À l'heure actuelle, de nombreux agents anti-inflammatoires et immunostimulants font l'objet d'essais cliniques dans cette indication.

2. RÔLE DES PLAQUETTES DANS LE SEPSIS

2.1. Les plaquettes sanguines : structure et fonctions hémostatiques

2.1.1. Structure plaquettaire

Les plaquettes sont les plus petits éléments figurés du sang (2 à 3 µm). Ce sont des fragments cellulaires provenant des mégacaryocytes. Ces énormes cellules (50 à 120 µm de diamètre) proviennent des mêmes cellules totipotentes qui donnent naissance aux érythrocytes et aux leucocytes. Chaque mégacaryocyte produit environ 2000 plaquettes. Ce processus a principalement lieu au niveau de la moelle osseuse mais un site de biogénèse tel que le poumon a été décrit récemment (Lefrançais et al. 2017). La durée de vie des plaquettes est de 8 à 10 jours chez l'humain. Elles sont éliminées par les macrophages de la rate et du foie (Hartwig and Italiano 2006; Hartwig 2006). Dans le sang normal humain, le compte plaquettaire est compris entre 150 000 à 400 000/µL. A l'état normal, les deux tiers de la masse plaquettaire circulent dans le sang et un tiers est séquestré dans la rate. Les plaquettes présentent à l'état de repos une forme discoïde avec un volume d'environ 6 à 8 μm^3. Les progrès de la microscopie électronique ont permis de mieux connaître l'ultrastructure plaquettaire. La plaquette est dépourvue d'ADN puisqu'elle ne contient pas de noyau mais elle contient quelques traces d'ARN. La plaquette renferme un grand nombre de granules et d'organelles subcellulaires. On trouve ainsi des mitochondries, des peroxysomes et des particules de glycogène. Trois sortes principales de granules de sécrétion peuvent être observées : les granules denses, les granules alpha et les lysosomes. Les éléments contenus dans ces granules apportent une importante contribution dans la cascade de réactions conduisant à l'agrégation plaquettaire discutée plus loin. La membrane plaquettaire est un système complexe constitué de la membrane plasmique, du système canaliculaire ouvert (SCO) connecté avec le milieu extracellulaire par de profondes invaginations et un système tubulaire dense (STD) uniquement intracellulaire (Figure 10). **La membrane plasmique** présente une structure comprenant 2 feuillets lipidiques externe et interne maintenant une couche riche de glycoprotéines (GP).

Les glycoprotéines sont enchâssées dans la membrane lipidique et la traversent une ou plusieurs fois grâce des leurs domaines transmembranaires hydrophobes. Elles possèdent une partie intra-cytoplasmique et une partie extracellulaire. L'interaction avec le cytoplasme se fait soit directement avec l'actine, soit grâce à des protéines qui lient l'actine (*Actin Binding proteins* ABP) (Fox 1985). Des glycoprotéines spécifiques permettent l'adhésion des plaquettes à la matrice extracellulaire. Leur rôle est primordial. En cas de perte d'expression ou de défaut fonctionnel d'un complexe glycoprotéique majeur, on peut assister à des syndromes hémorragiques plus ou moins sévères. D'autres glycoprotéines telles les CAM (*Cell Adhesion Molecules*) régissent les interactions inter-plaquettaires ou entre les plaquettes et les complexes immuns.

Le système canaliculaire ouvert constitue un réservoir de membrane qui s'invagine dans la plaquette au travers du cytoplasme. Ce SCO joue un rôle à la fois dans l'exocytose lors de la sécrétion des granules plaquettaires, et dans l'endocytose de molécules d'origine plasmatique.

Le système tubulaire dense constitue un lieu important de stockage et de régulation du calcium ionisé rapidement mobilisable lors de l'activation plaquettaire.

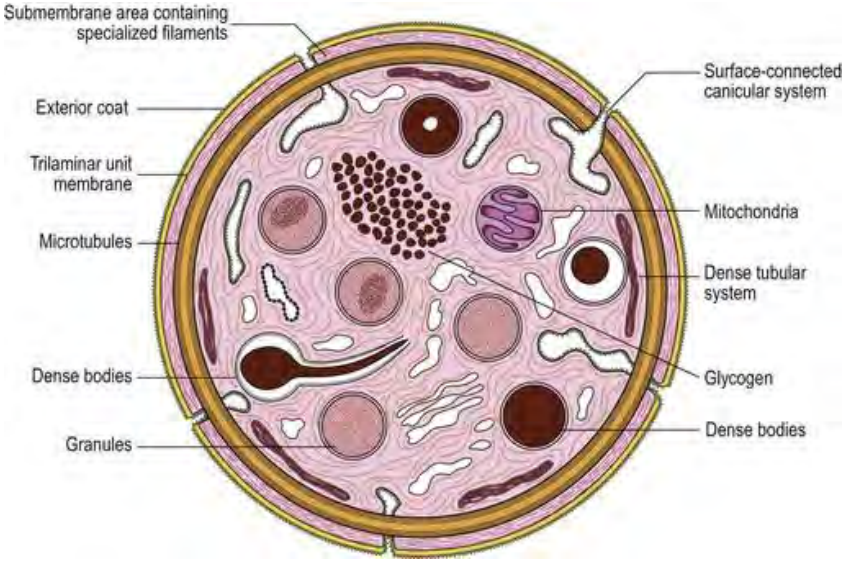


Figure 10 : Schématisation de l'ultrastructure plaquettaire dans le plan équatorial d'après JG White, disorders affecting megakaryocytes and platelets, Chap 32.

Les 3 types de granules (alpha, denses, lysosomes) participent à l'activation plaquettaire par la sécrétion de leur contenu.

Les granules α sont les plus nombreux (5 à 10 fois plus nombreux que les granules denses) et occupent 10% du volume plaquettaire. Ils contiennent de nombreuses protéines adhésives (le fibrinogène, le facteur von Willebrand, la vitronectine, la fibronectine et la thrombospondine), des protéines de la coagulation (facteurs V, VIII, IX, XIII, kininogène de haut poids moléculaire, plasminogène, protéine S) de nombreux facteurs de croissance (PDGF (*platelet derived growth factor*), EGF (*epidemal cell growth factor*), VEGF (*vascular epidermal growth factor*), IGF1 (*Insulin Growth Factor 1*), TGFβ (*transforming growth factor β*). On y retrouve également des inhibiteurs de la fibrinolyse (PAI-1 (plasminogen activator inhibitor-1) et TAFI (*thrombin activatable fibrinolysis inhibitor*)), des immunoglobulines G, des protéines plaquettaires telles que le PF4 (Platelet Factor 4) et la β-thromboglobuline. C'est dans ces granules que l'on trouve également des glycoprotéines de surface.

Les granules denses (δ) sont estimés au nombre de 6 à 8 par plaquette humaine et se présentent sous l'aspect de vésicules sphériques ou parfois allongées, de 150 à 300 nm de diamètre. Ces organelles présentent une structure dense aux électrons qui contraste fortement avec le reste de leur contenu (J. G. White 2008). Les granules δ contiennent des composés non protéiques dont du calcium, de l'adénosine diphosphate (ADP), de l'adénosine triphosphate (ATP), des pyrophosphates, de la sérotonine et de

l'histamine (McNicol and Israels 1999). La concentration importante en calcium et la présence de polyphosphates et de sérotonine sont responsables de l'aspect caractéristique de ces organelles en microscopie électronique.

Les plaquettes contiennent des **lysosomes** primaires et secondaires qui renferment essentiellement des enzymes telles que les cathepsines D et E, des carboxypeptidases et présentent une importante hétérogénéité (McNicol and Israels 1999). Ces organelles sont des vésicules sphériques de 175 à 250 nm (Bentfeld-Barker and Bainton 1982), qui présentent souvent un aspect similaire aux granules α. Le nombre de lysosomes présents dans les plaquettes est faible, mais aucune donnée quantitative précise n'est disponible dans la littérature. Les lysosomes intègrent des molécules par la voie endocytique en interagissant avec des vésicules recouvertes de clathrine. Ils présentent à leur membrane des protéines lysosomales ubiquitaires de la famille LAMP (King and Reed 2002). Les lysosomes sont sécrétés lors de fortes activations plaquettaires.

2.1.2. Fonctions plaquettaires dans l'hémostase

La paroi vasculaire recouverte par une surface endothéliale est cruciale pour le maintien de l'homéostasie du système vasculaire. L'endothélium vasculaire contient 3 principaux thromborégulateurs – l'oxyde nitrique, la prostacycline et l'ectonucléotidase CD39 – qui lui confèrent un phénotype anticoagulant au repos. En cas de brèche vasculaire, le collagène et le facteur tissulaire des couches profondes de la paroi sont exposés au courant sanguin, initiant ainsi la formation du thrombus. L'exposition du collagène entraîne l'accumulation et l'activation des plaquettes alors que l'exposition du facteur tissulaire initie la formation de thrombine qui non seulement convertit le fibrinogène en fibrine, mais active également les plaquettes (B. Furie and Furie 2008) (Figure 11).

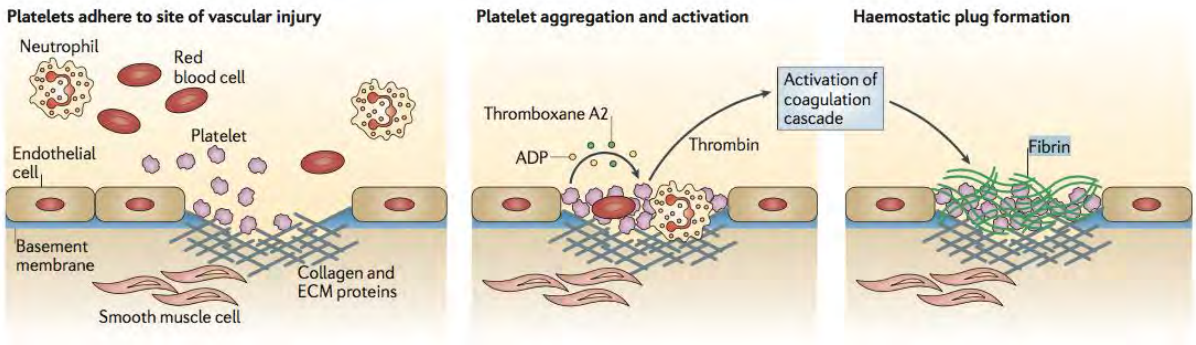


Figure 11 : fonctions plaquettaires dans l'hémostase

Les plaquettes ont une fonction physiologique primaire qui est l'hémostase lors d'une lésion de l'endothélium vasculaire qui expose le collagène et les protéines de la matrice permettant aux plaquettes d'adhérer. Les plaquettes vont se lier ainsi au sous-endothélium et s'agréger entre elles, libérant des médiateurs comme l'ADP et le thromboxane A2 activant à leur tour d'autres plaquettes. Suite à l'activation, les plaquettes par leur activité procoagulante conduisent à la production de la thrombine qui catalyse la génération de fibrine. Il en résulte la formation d'un clou plaquettaire stable permettant le comblement de la brèche vasculaire (D'après Semple JW, *Nat Rev Immunol* 2011).

Sont classiquement décrites 2 voies indépendantes d'activation plaquettaire. La première est en lien avec la mise à nu du sous-endothélium avec exposition de collagène qui initie l'activation plaquettaire, la seconde est initiée par le facteur tissulaire présent dans les fibroblastes des couches profondes de la paroi vasculaire, entraînant lors d'une blessure la formation de thrombine.

Le paradigme des plaquettes en tant que simples médiatrices de l'hémostase a été progressivement remplacé par le fait que les plaquettes jouent un double rôle : hémostastique et inflammatoire. Elles sont des acteurs essentiels dans la réponse immunitaire innée et adaptative, capables d'interagir avec presque toutes les cellules immunes connues. Ces interactions plaquettes-cellules immunitaires représentent une caractéristique de la réaction immunitaire car elles peuvent puissamment améliorer les fonctions des cellules immunitaires et dans certains cas même constituer un préalable aux mécanismes de défense de l'hôte comme la NETose (Figure 12). Nous allons les détailler dans le prochain chapitre.

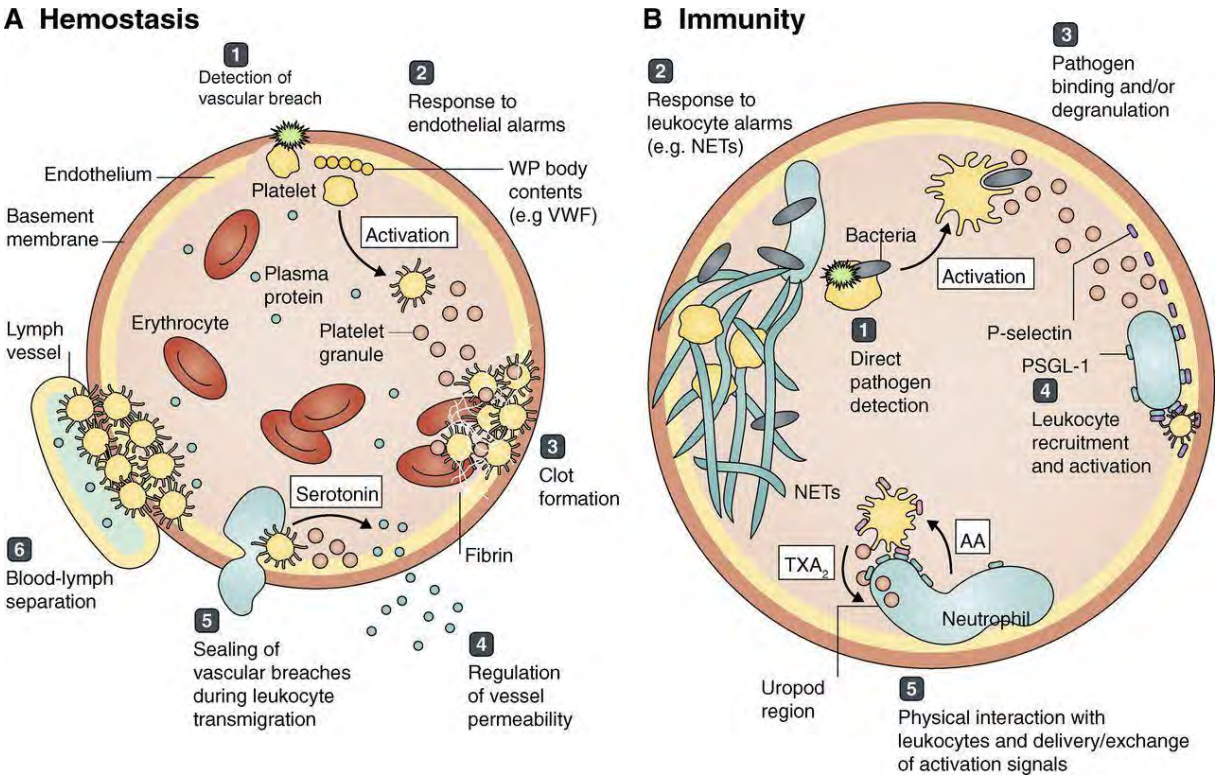


Figure 12: Résumé des principaux rôles des plaquettes dans l'hémostase et l'immunité

Les plaquettes circulent dans le sang, surveillant le système vasculaire pour contrer (A) les menaces hémostatiques et (B) les menaces immunitaires.
(A) Les plaquettes détectent les brèches vasculaires à l'aide de divers récepteurs, tels que ceux qui lient le collagène exposé (1). Elles réagissent aux signaux de danger tels que l'ADP ou le contenu des corps de Weibel-Palade (WP), libérés par les cellules endothéliales endommagées ou activées (2). Lors de leur activation, les plaquettes peuvent former un thrombus (3) tout en régulant la perméabilité des vaisseaux (4). Elles agissent également comme des gardiennes, empêchant la perte d'érythrocytes lors de la transmigration des leucocytes (5), ainsi qu'à la jonction lympho-veineuse à l'état d'équilibre ou pendant la lymphangiogénèse (6).
(B) Les plaquettes peuvent reconnaître les menaces immunitaires directement à l'aide de récepteurs de type PRR (patter-recognition receptors) par l'évolution (1) ou indirectement par le biais de signaux leucocytaires, tels que les pièges extracellulaires de neutrophiles (TNE) ou les cytokines (2). Les plaquettes peuvent se lier et s'enrouler autour des agents pathogènes, déclenchant la dégranulation pour effectuer la mise à mort (3) et le recrutement direct/indirect des leucocytes (4). En outre, les plaquettes interagissent souvent physiquement avec les

leucocytes pour délivrer ou échanger des signaux pro-inflammatoires, par exemple en absorbant l'acide arachidonique (AA) des neutrophiles pour synthétiser le thromboxane A2 (TXA2 ; 5). (d'après Li JL, JEM 2017)

2.2. Fonctions immunes des plaquettes

Depuis quelques années, une littérature robuste alimente le fait que les plaquettes ont un rôle majeur comme sentinelles et cellules effectrices, particulièrement au cours de pathologies infectieuses, puisqu'elles exercent un rôle crucial dans le continuum hémostatique, inflammatoire et immun. Après irruption d'un pathogène dans l'organisme, elles peuvent directement le reconnaître via des récepteurs spécifiques ou des intégrines. Patrouillant constamment dans le système circulatoire, elles scannent l'endothélium à l'affût du moindre signal d'alarme. Elles agissent également indirectement à travers leurs interactions avec les leucocytes et l'endothélium. A travers différents mécanismes, les plaquettes activées par les pathogènes peuvent directement les séquestrer ou les tuer, ou bien faciliter leur élimination en activant les neutrophiles, macrophages et en promouvant la formation de NETs, en formant des agrégats et des microthrombi.

2.2.1. Patrouilleuses autonomes de la surveillance immune

Détection rapide des sites de l'infection au contact de l'endothélium lésé

Dans le courant sanguin, les plaquettes circulent préférentiellement proches du mur vasculaire, on parle de « margination plaquettaire » (Chang et al. 2018). Cela les met en position de surveillance constante de l'intégrité de l'endothélium vasculaire par des liaisons transitoires ou du « rolling ». Ces interactions répétées avec l'endothélium vasculaire permettent aux plaquettes de répondre rapidement à des signes locaux d'infection.

Les cellules endothéliales sont recouvertes, à l'état physiologique, par une couche riche en protéoglycanes, glycosaminoglycanes, glycoprotéines et glycolipides avec une charge négative, située sur la face endoluminale et nommée glycocalyx. Le glycocalyx participe à la régulation de la microcirculation en prévenant l'adhésion plaquettaire et le recrutement leucocytaire, jouant ainsi un rôle central dans le maintien de l'intégrité de l'endothélium vasculaire. Lors de situations inflammatoires comme le sepsis, le glycocalyx est dégradé par des mécanismes encore mal connus, entraînant la mise à nu des cellules endothéliales pouvant être activées directement par des pathogènes ou indirectement via des médiateurs inflammatoires. Chez l'Homme, la dégradation du glycocalyx durant les 24 premières heures de la prise en charge en réanimation pour sepsis est associée à une augmentation de la mortalité (Beurskens et al. 2020).

L'activation des cellules endothéliales suite à l'infection entraîne la libération des corps de Weibel-Palade contenant de la P-sélectine et du facteur von Willebrand, l'expression de molécules d'adhésion comme les ICAM 1 (InterCellular Adhesion Molecule 1) et des intégrines renforçant le rolling des plaquettes et intensifiant les interactions endothélium-plaquettes. L'activation des cellules endothéliales se traduit également par l'expression du facteur tissulaire entraînant la cascade de la coagulation et la formation d'un réseau de fibrine participant au recrutement d'autres plaquettes. Durant la phase d'adhésion plaquettaire, les plaquettes activées exposent à leur surface ou libèrent des molécules qui à leur tour majorent l'activation des cellules endothéliales. Par exemple, les plaquettes activées expriment le CD154 (ou CD40L) qui s'associe au CD40 exprimé par les cellules endothéliales pour up-réguler l'expression d'E-sélectine, des VCAM 1 (Vascular Cell Adhesion Molecule 1) et ICAM 1 aussi bien que pour augmenter la sécrétion de médiateurs endothéliaux pro-inflammatoires (CCL2 et IL-8). Les plaquettes en parallèle, bien que dépourvues de noyau, ont la capacité de synthétiser *de novo* des protéines à partir d'ARN. Ainsi, la synthèse d'IL-1β augmente l'activation endothéliale et la perméabilité vasculaire soutenant ainsi le recrutement des leucocytes.
De fait, ces mécanismes réciproques d'activation endothéliale et plaquettaire amplifient la réponse inflammatoire au site de l'infection.

Modalités d'interactions des plaquettes avec les pathogènes

En plus des interactions avec l'endothélium vasculaire lésé lors du sepsis, les plaquettes sont capables de reconnaître la présence de pathogènes via les nombreux récepteurs de surface qu'elles possèdent incluant les récepteurs au complément, le FCγRIIa, les *toll-like receptors* mais aussi les intégrines classiquement engagées dans la réponse hémostatique. Les interactions sont possibles avec les bactéries, mais aussi les virus, les champignons, les protozoaires.

Les bactéries et les virus sont les principaux pathogènes rencontrés lors des infections chez l'Homme. Les mécanismes d'interactions actuellement décrits entre les plaquettes et les bactéries sont au nombre de 3. Il existe des interactions indirectes des bactéries via des protéines plasmatiques avec les plaquettes, des liaisons directes aux récepteurs plaquettaires et des liaisons des plaquettes aux produits bactériens sécrétés, particulièrement des toxines. La figure 13 illustre ces interactions multiples que nous détaillons ci-après.

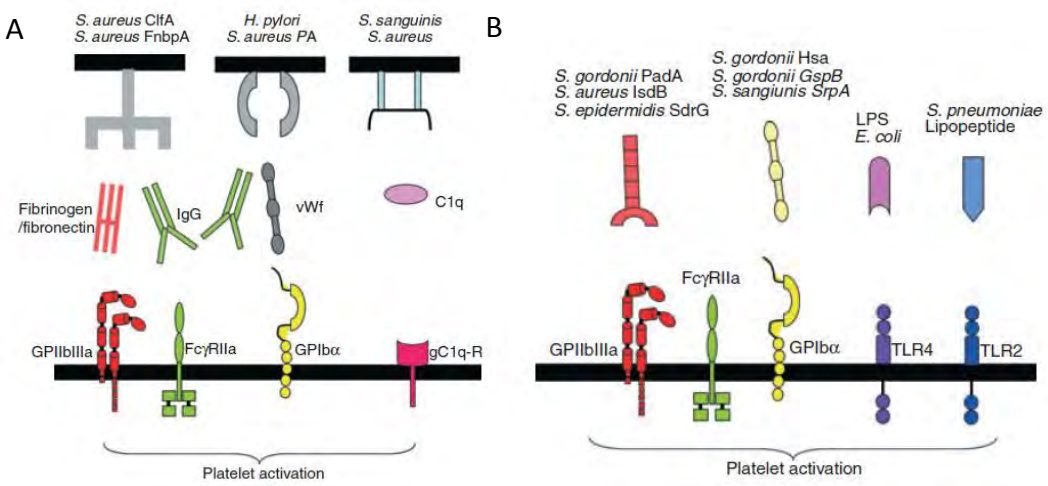


Figure 13: Illustration des interactions directes et indirectes des plaquettes avec les bactéries ou leurs produits

Résumé des interactions indirectes (A) et directes (B) entre les bactéries et les plaquettes. ClfA, clumping factor A, C1q, complement 1q, FnbpA, fibronectine-binding protein A; GP, glycoprotéine; PA, protéine A ; vWF, von Willebrand Factor ; GspB, glycosylated strepococcal protein B, Hsa, Hemaglutinin salivary antigen, PadA, platelet adhesion protein, LPS, lipopolysaccharide, SdrG, serine-aspartate repeat G, Srp, serine-rich protein A, TLR, toll like receptor. (D'après Cox, *JTH* 2011)

a) Interactions avec les bactéries et leurs produits

Plusieurs récepteurs plaquettaires peuvent être engagés dans la reconnaissance des bactéries lors du sepsis.

Rôle de la glycoprotéine IIb-IIIa

Elle est le récepteur du fibrinogène et son activation se solde par l'adhésion et l'agrégation plaquettaires. Les *staphylococci* expriment des récepteurs de surface spécifiques au fibrinogène et à la fibronectine. Ils sont caractérisés par des régions riches en motifs sérine-aspartate appartenant à la famille des composants microbiens de surface reconnaissant les molécules de la matrice adhésive (MSCRAMM) (Josefsson et al. 1998). Ces molécules permettent aux bactéries d'adhérer aux tissus, étape critique de la progression de l'infection. Le staphylocoque doré (*s. aureus*) en exprime plusieurs dont ClfA, ClfB, fibronectin-binding protein (Fnbp) (McDevitt et al. 1994). Toutes ces molécules se lient de façon indirecte à GPIIb-IIIa via le fibrinogène. D'autres bactéries telles que le *Streptococcus Pyogenes* et le *S. Mitis* peuvent également lier le fibrinogène et interagir ainsi de façon indirecte avec les plaquettes (Cox, Kerrigan, and Watson 2011b).

Plus récemment il a été démontré que les bactéries étaient capables d'interagir directement via des protéines de surface avec GPIIb-IIIa indépendamment du fibrinogène. C'est le cas par exemple du SdrG de *S. Epidermidis* (Brennan et al. 2009) (Brennan et al. 2009), de la protéine Isd (Iron-regulated surface determinant) de *S. Aureus* (Miajlovic et al. 2010) ou de PadA (platelet adherence protein) de *S. Gordonii* (Petersen et al. 2010).

Rôle de la glycoprotéine Ibα

GPIb est une glycoprotéine membranaire appartenant à la famille des protéines à motifs leucine répétés. Elle lie divers ligands et est essentielle à l'hémostase primaire grâce à sa haute affinité avec le vWF. Plusieurs espèces de *Streptococci* sont capables de s'y lier directement. Ces interactions mettent en jeu une famille de protéines bactériennes riches en sérines, hautement glycosylées. On retrouve SrpA (serine-rich protein A) du *S. Sanguinis* (Plummer et al. 2005), GspB (glycosylated streptococcal protein B et Hsa (hemagglutinin salivary antigen) du S. *gordonii* (Bensing, López, and Sullam 2004). Quelques protéines bactériennes peuvent aussi lier GPIb indirectement via le vWF telles que la protéine A du *S. Aureus*.

Récepteurs du complément

La littérature décrit la capacité des plaquettes à se lier au système du complément au cours du sepsis. *S. Sanguinis* peut induire l'agrégation plaquettaire via le complément. ClfA et ClfB du *S. Aureus* induisent également une agrégation dépendante du complément (Loughman et al. 2005). Les plaquettes expriment le récepteur gC1q-R, récepteur du fragment C1q du complément et peuvent ainsi servir de récepteur aux bactéries recouvertes de ce facteur du complément (Peerschke, Murphy, and Ghebrehiwet 2003).

Récepteur FcγRIIa

L'expression de ce récepteur immun qui reconnaît le domaine Fc des immunoglobulines (IgG) est décrit chez les phagocytes comme les neutrophiles et les monocytes. FcγRIIa est capable de lier et d'internaliser des complexes immuns engageant des IgG, qu'ils soient solubles ou cellulaires. Les plaquettes sanguines expriment le FcγRIIa. Les IgG liées aux bactéries sont capables d'interagir avec ce récepteur présent sur les plaquettes, entraînant l'internalisation du complexe lié à l'IgG (Worth et al. 2006). Les plaquettes expriment environ 5000 copies du FcγRIIa (Cox, Kerrigan, and Watson 2011a), faisant ainsi, du fait du nombre important de plaquettes circulantes, une cellule majeure dans la réponse anti-bactérienne.

Interactions avec les toxines bactériennes

Les bactéries sécrètent des toxines capables d'activer les plaquettes. *Porphyromonas gingivalis* sécrète des gingipaïnes. Ces toxines sont reconnues par le protease-activated receptor (PAR) 1 et le clivent de façon similaire à la thrombine, le rendant ainsi fonctionnel (Fitzpatrick, Wijeyewickrema, and Pike 2009). L'alpha-toxine du *S. Aureus* se lie à la bicouche lipidique plaquettaire pour la rendre poreuse (Arvand et al. 1990) tout comme peuvent le faire la streptolysine

du *S. Pyogenes* (Bryant et al. 2005) et la pneumolysine du *S. Pneumoniae* (Johnson, Boese-Marrazzo, and Pierce 1981).

b) Interactions avec les virus

Les plaquettes possèdent différents récepteurs à leur surface capables d'interagir avec des virus. Les plaquettes et les mégacaryocytes expriment des ARN messagers (mRNA) et/ou des protéines pour les TLR (TLR1, TLR2, TLR3, TLR4, TLR6, TLR7, TLR8 et TLR9) qui détectent et lient des composants viraux présents à leur surface et les acides nucléiques viraux (Cognasse 2015). Les TLR font partie des PRR et sont des récepteurs non catalytiques qui reconnaissent des molécules structurellement conservées dont celles dérivées des virus. Les TLR fonctionnent comme des dimères mais peuvent dépendre d'autres co-récepteurs pour une sensibilité totale au ligand comme dans le cas de la reconnaissance du LPS par le TLR4 qui nécessite MD-2. Une fois activés, les TLR recrutent des molécules adaptatrices nécessaires à la propagation du signal pour conduire à l'induction ou la suppression de gènes qui orchestrent la réponse inflammatoire.

Les plaquettes expriment également plusieurs récepteurs du complément qui agissent comme récepteurs aux virus qui entraînent de multiples fonctions de défense antimicrobienne incluant la lyse, l'opsonisation et la chimiotaxie. Le récepteur DC-SIGN (Dendritic-Cell-Specific ICAM3-Grabbing Non Integrin) contenu dans les granules est capable de lier le virus de la Dengue (DENV) une fois exprimé à la surface plaquettaire. L'intégrine α2β1 et la glycoprotéine GPVI (récepteur principal du collagène) sont capables de lier respectivement les rotavirus et le virus de l'hépatite C (HCV) (Flaujac, Boukour, and Cramer-Bordé 2010). Les plaquettes expriment également un récepteur aux virus coxsackies, le Coxsackie-Adeno Receptor (CAR) negrotto (Negrotto et al. 2015).

La capacité des plaquettes à internaliser des particules du virus de l'immunodéficience humaine (HIV) a été initialement décrite par Zucker-Franklin et *al* (Zucker-Franklin, Seremetis, and Zheng 1990). L'incubation de plaquettes avec un surnageant de lymphocytes pendant 30 minutes montre que le VIH est internalisé dans les plaquettes (Flaujac, Boukour, and Cramer-Bordé 2010). Récemment, Banerjee et al. ont démontré que les plaquettes endocytent des virions de VIH et s'activent via des cascades de signalisation impliquant les TLR (Banerjee et al. 2020). La figure 14 illustre les interactions possibles entre les virus et leurs récepteurs à la surface de la plaquette.

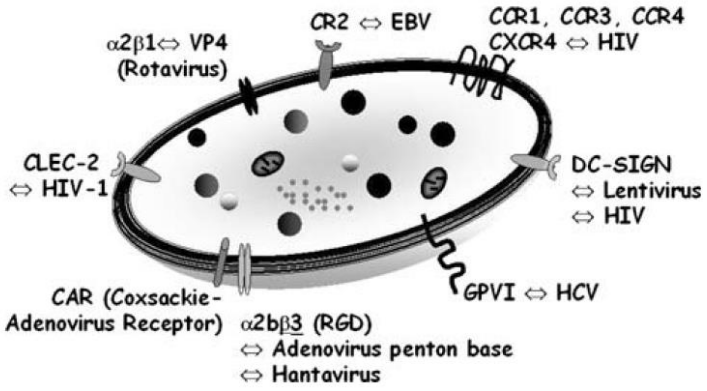


Figure 14: illustration des récepteurs plaquettaires impliqués dans les interactions avec les virus

(D'après Flaujac, Cell Mol Life Sci 2010)

Récemment, en lien avec la pandémie à SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2), plusieurs hypothèses quant à la capacité des plaquettes à internaliser le SARS-CoV-2 ont été émises. Koupenova fait l'hypothèse que, du fait de leur abondance, les plaquettes pourraient être les premières cellules sanguines à internaliser des particules virales et à induire une réponse au pathogène (Koupenova 2020). Par exemple, dans le cas de l'infection grippale au virus Influenza, il a été démontré que les plaquettes étaient capables d'internaliser activement de nombreuses particules du virus (Koupenova et al. 2019) (Figure 15).

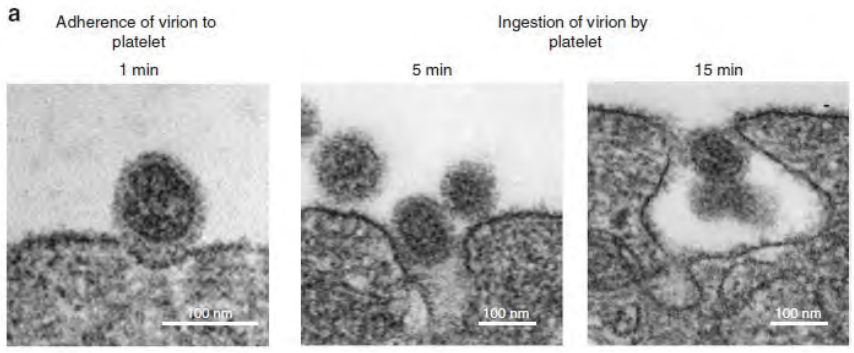


Figure 15: Internalisation du virus influenza par les plaquettes in vitro en microscopie électronique à transmission

Images représentatives de différents stades de phagocytose dans des structures « phagosomes-like » des plaquettes capturant le virus WSN/33 influenza incubé avec des plaquettes de donneur sain. (D'après Koupenova, *Nat Comm*un 2019)

Le virus de la grippe, est comme le SARS-CoV-2, un virus à RNA simple brin qui peut infecter les cellules épithéliales. Chez l'Homme, la réponse plaquettaire au contact de virus RNA simple brin est médiée de façon prédominante par le TLR7 (Koupenova et al. 2014; Koupenova et al. 2019). Le TLR7 est situé dans les endolysosomes plaquettaires et requiert l'internalisation des particules virales et le pH acide des endolysosomes pour sa propre activation et sa signalisation. Les

coronavirus SARS-CoV et SARS-CoV-2 utilisent le récepteur de l'enzyme de conversion de l'angiotensine 2 (ACE2) pour infecter les cellules (Hoffmann et al. 2020; Zhou et al. 2020). D'autres protéases incluant TMPRSS2 (transmembrane protease serine 2), la basigine (aussi appelée CD147) et potentiellement la cathepsine B et L sont requises (Zhou et al. 2020; Hoffmann et al. 2020). La présence du récepteur de l'ACE2 dans les plaquettes est discutée. Manne BK and *coll*. n'ont pas détecté d'ARNm de l'ACE2 ni de protéine dans les plaquettes (Manne et al. 2020). Cependant, de l'ARNm du SARS-CoV-2 a été détecté chez 2 patients des 25 patients séquencés suggérant la capacité des plaquettes à internaliser le SARS-CoV-2 indépendamment de l'ACE2. En revanche, l'équipe de Zhang S a publié des résultats en faveur de l'expression de l'ACE2 dans les plaquettes humaines et a détecté par RT-PCR de l'ARN et des protéines d'ACE2 (Zhang et al. 2020). L'ARNm du récepteur à l'enzyme de conversion de l'angiotensine n'a pas été détecté lors du séquençage des plaquettes (Clancy et al. 2017; Rowley et al. 2011). Plus récemment encore, Koupenova et al. ont réussi à amplifier l'ARNm de SARS-CoV-2 dans les plaquettes de 17 patients COVID-19 en utilisant plusieurs amplicons à partir du génome viral fragmenté (Koupenova et al. 2021). Par ailleurs, après incubation de virions de SARS-CoV-2 avec des plaquettes, l'équipe a démontré que les plaquettes pouvaient internaliser le virus, suggérant 3 modes d'entrée : via des endosomes, des vacuoles de phagocytose ou par attachement à des microparticules.

Il est décrit que les virus à ARN simple brin, comme le virus grippal, peuvent être internalisés par d'autres mécanismes indépendants d'une reconnaissance liée à un récepteur. La micropinocytose de vésicules d'endocytose (vésicules « cargo » de grande taille) ou la phagocytose de fragments apoptotiques de cellules infectées par l'influenza virus ont été décrites comme mécanismes non spécifiques d'entrée virale dans d'autres types cellulaires (Edinger, Pohl, and Stertz 2014; Hashimoto et al. 2007). Il est convenu que les plaquettes sont capables d'acquérir activement de l'ARN à partir des cellules endothéliales et d'internaliser des vésicules circulantes, des débris, des mitochondries, des particules de pollen etc. (Koupenova et al. 2018).

2.2.2 Modulatrices versatiles de l'inflammation : caractéristiques de la réponse plaquettaire à l'infection

De l'activation à l'agrégation plaquettaire

L'interaction des bactéries avec les plaquettes est capable d'entraîner une activation plaquettaire pouvant dans certaines situations, conduire jusqu'à une agrégation plaquettaire (Cox, Kerrigan, and Watson 2011a). Quand ce phénomène se produit de façon localisée, cela peut conduire à la formation d'un thrombus. Les différentes étapes nécessitent l'adhésion du pathogène à la bactérie,

l'activation et la transduction d'un signal plaquettaire qui peut, *in fine*, aboutir à une agrégation plaquettaire (Figure 16).

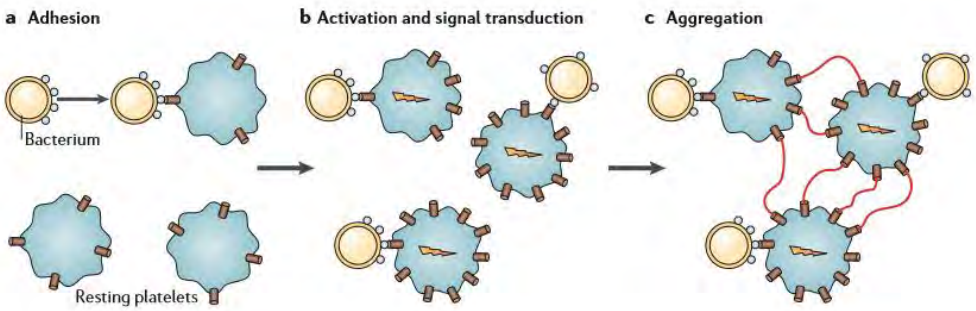


Figure 16 : Schématisation de l'activation et l'agrégation plaquettaires en réponse aux bactéries

Les bactéries se lient au récepteur plaquettaire, directement ou via un ligand. Une cascade de transduction du signal est initiée qui mène à l'activation de l'intégrine plaquettaire GPIIb/IIIa et à l'expression de protéines de surface plaquettaires. GPIIb/IIIa activée lie le fibrinogène plasmatique entraînant l'agrégation . (D'après Fitzgerald JR, *Nat Rev Microbiol* 2006)

Après recrutement rapide au site infecté, les plaquettes ciblent activement les pathogènes et s'accumulent localement aux sites riches en protéines bactériennes, en protéines du complément C3a et C5a et en protéines de la phase aiguë de l'inflammation. Les interactions pathogènes-plaquettes entraînent une réorganisation du cytosquelette et une mobilisation du calcium responsables de l'activation plaquettaire et de la dégranulation. Les plaquettes expriment des récepteurs pour de multiples chemokines et kinocidines générées au site de l'infection. Les signaux de chimiotaxie en plus des fractions du complément détectés par les plaquettes incluent 4 classes de chemokines qui engagent 4 classes de récepteurs. Ainsi, la réponse immédiate plaquettaire à l'infection initie une défense rapide et ciblée de l'hôte. Les plaquettes peuvent ainsi être activées et agréger. La libération du contenu granulaire (ADP, sérotonine…) participe au recrutement d'autres plaquettes et à l'amplification de la réponse plaquettaire (Figure 17) menant *in fine* à la formation de thrombi.

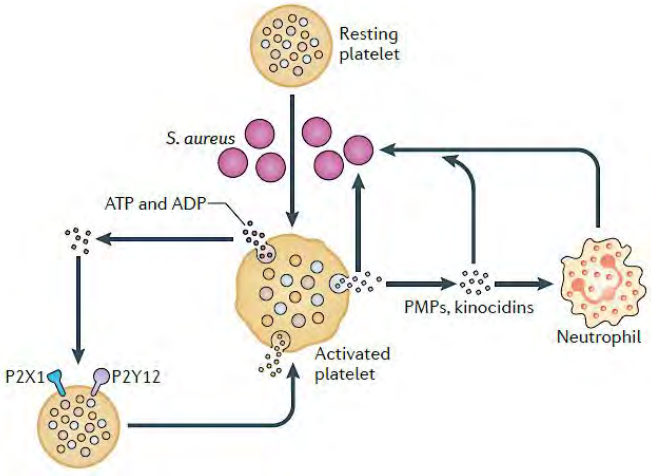


Figure 17: activation et amplification des mécanismes plaquettaires antimicrobiens

Les plaquettes sont activées par les pathogènes. L'activation plaquettaire est suivie par une seconde vague d'amplification de la réponse antimicrobienne. Les plaquettes libèrent le contenu de leurs granules dont de nombreux agonistes. L'ADP et l'ATP stimulent les récepteurs tels que P2Y12 ou P2X1 des plaquettes adjacentes. En retour, le cytosquelette se réorganise pour entraîner de nouvelles dégranulations plaquettaires, augmentant encore la libération d'agonistes plaquettaires aussi bien que des PMPs (platelet microbicidal proteins) et des kinocidines. Les PMPs libérées et les kinocidines ciblent les pathogènes comme le S aureus, résultant en une action antimicrobienne directe et la potentialisation des fonctions immunes des cellules de l'immunité innée telles que les neutrophiles. (D'après Yeaman MR, *Nature* 2014).

Les interactions bactéries-plaquettes qu'elles soient directes ou indirectes sont insuffisantes pour une forte activation plaquettaire. Elles requièrent un anticorps circulant spécifique dirigé contre des protéines bactériennes pour engager le récepteur FcγRIIa dont la signalisation conduit à une agrégation plaquettaire et à la formation d'un thrombus.

Watson et *al*. ont étudié les mécanismes moléculaires de l'activation plaquettaire en réponse à *Escherichia Coli* (C. N. Watson et al. 2016). Le rôle critique du FcγRIIa, d'$\alpha_{IIb}\beta_3$ a été mis en lumière, ainsi que des tyrosines kinases Src et Syk pour permettre l'activation des plaquettes en réponse à *E. Coli* (Moriarty et al. 2016). La présence d'IgG et l'activation d'$\alpha_{IIb}\beta_3$ sont nécessaires pour l'activation du FcγRIIa. De plus, l'ADP et le TxA2 libérés sont essentiels pour entraîner une agrégation plaquettaire (Arman et al. 2014). L'utilisation d'aspirine lors de l'adhésion du *streptococcus sanguinis* aux plaquettes inhibe totalement l'agrégation suggérant un rôle de la cyclo-oxygénase (COX) et de la production de TxA2 dans l'agrégation (Cox, Kerrigan, and Watson 2011a).
L'agrégation induite par le contact des plaquettes avec les bactéries est différente de l'agrégation classique en réponse aux agonistes ADP, ATP, thrombine. Le délai d'initiation (« lag time ») de l'agrégation est généralement plus long en réponse aux bactéries par comparaison à l'activation par les agonistes classiques. Alors que quelques secondes sont nécessaires en réponse à la stimulation par des agonistes, quelques bactéries ont un lag time court de 90 à 120 secondes alors que d'autres peuvent nécessiter plus de 20 minutes (Kerrigan and Cox 2010). Plusieurs hypothèses ont été formulées pour expliquer la variation de lag time selon les bactéries : (1) le temps nécessaire à la liaison bactérie-plaquettes particulièrement si la liaison est indirecte ; (2) l'activation des récepteurs qui pourrait ne pas être aussi forte.

L'agrégation plaquettaire induite par les bactéries reste cependant controversée, et plusieurs études ont montré que la stimulation par des bactéries ne résultait pas en une agrégation mais plutôt en une réponse inflammatoire ciblée (libération de chemokines, activation leucocytaire, formation de NETs).

Recrutement des cellules immunes, action antimicrobienne et anti-inflammatoire

Les plaquettes sont impliquées dans la détection précoce des agents pathogènes microbiens, ainsi que dans l'activation et le recrutement de défenses de l'hôte complémentaires. De plus, elles ont des fonctions antimicrobiennes directes qui sont médiées par la sécrétion de molécules effectrices antimicrobiennes, y compris les PMP (platelet microbicidal proteins) et les kinocidines.

Comme les neutrophiles et autres granulocytes professionnels, les granules de plaquettes contiennent une gamme de protéines et de peptides à action antimicrobienne directe. Au moins quatre familles de protéines microbicides sont contenues et libérées par les plaquettes (kinocidines, défensines, thrombocidines et peptides dérivés antimicrobiens). Les autres classes sont résumées sous le nom de PMP (Yeaman 2010b; Yeaman 2014; Yeaman 1997; Yeaman 2010a). Le nom « kinocidines » reflète les doubles fonctions de ces protéines, à savoir un rôle de chimiokine et un effet microbicide (Yeaman 2010b; Yeaman 2014; Yeaman 1997; Yeaman 2010a). Les plaquettes contiennent de nombreuses kinocidines qui exercent une activité antimicrobienne directe. Les kinocidines sont stockées de manière constitutive dans les granules de plaquettes et sont rapidement libérées après l'activation des plaquettes, par dégranulation en réponse aux PAMPs notamment. Cependant, certaines kinocidines, telles que CXCL4, CXCL7 et CXCL8, sont également exprimées par des lignées cellulaires monocytaires et macrophages humains (Schaffner et al. 2005). La plus abondante au sein des plaquettes est CXCL4. Les kinocidines plaquettaires sont libérées dans le courant sanguin en réponse à des agents microbiens ou des lésions tissulaires à la différence de peptides antimicrobiens classiques souvent présents chez des phagocytes professionnels ou sécrétés localement. Les kinocidines ont des fonctions immunomodulatrices dont le chimiotactisme et l'activation du complément des cellules de l'hôte. Les PMPs matures et les kinocidines subissent des clivages protéolytiques et leurs dérivés peuvent maintenir une activité antimicrobienne.

L'interleukine 1β (IL-1β) est un médiateur clef de la réponse inflammatoire. Elle est essentielle dans la réponse de l'hôte aux pathogènes (Dinarello 1996). Elle est produite et sécrétée par de nombreuses cellules comme les monocytes et macrophages. Elle est produite via un précurseur inactif, la pro-IL-1β. Les données concernant la possibilité qu'elle soit sécrétée par les plaquettes sont contradictoires.
La synthèse d'IL-1β provient de l'assemblage d'inflammasomes, des complexes de protéines intracellulaires, comme NLRP3 (Nod-like receptor nucleotide-binding domain leucine-rich repeat containing protein 3) qui contrôle l'activation de la caspase-1 qui clive le précurseur en IL-1β.

Cornelius et *al*. a publié 2 récents articles concernant l'inflammasome plaquettaire. Un premier article traite de l'activation de l'inflammasome dans un modèle septique murin de ligature perforation caecale (Cornelius et al. 2019). Les auteurs mettent en évidence dans les plaquettes une augmentation de l'IL-1β dans le sepsis et une activation de l'inflammasome NLRP3. Dans un article plus récent, l'équipe propose le fait que l'inactivation pharmacologique de l'inflammasome NLRP3 dans le même modèle animal diminue la synthèse de cytokines pro-inflammatoires, avec une diminution de IL-1β (Cornelius et al. 2020). L'inhibition de l'inflammasome semble diminuer les lésions d'organe et la dysfonction endothéliale. Parallèlement, Vogel et *al*. proposent que l'inflammasome NLRP3 plaquettaire est activé dans la drépanocytose et que l'inactivation pharmacologique de la Bruton tyrosine kinase (BTK) qui semble contrôler l'activité de la caspase 1 dans ce modèle diminue cette-dernière (Vogel et al. 2018).

Cependant, une récente publication remet en cause l'existence d'un inflammasome plaquettaire qui pourrait fournir une production plaquettaire d'IL-1β. Rolfes et *al*. remettent en cause l'existence de ces structures au sein des plaquettes (Rolfes et al. 2020). L'équipe démontre au contraire que les plaquettes activent fortement la production d'IL-1β par l'inflammasome des cellules immunes. En revanche, après préparation de plaquettes dont la purification a été attestée en cytométrie de flux excluant la présence de leucocytes, on ne retrouvait aucune présence des composants de l'inflammasome (pas de NLRP3, pas d'ASC ni de caspase-1 et d'IL-1β) au sein des plaquettes. Ainsi, le fait que les plaquettes soient une source d'IL-β est fortement remis en cause.

Partenariat avec les cellules de l'immunité innée dans l'immunité intra-vasculaire dans le sepsis

Les plaquettes sont des effecteurs versatiles de l'immunité anti-bactérienne contribuant à la fois aux propriétés bactéricides et bactériostatiques en synergie avec les autres cellules de l'immunité innée.

a) Illustration de la collaboration dans la réponse à l'infection

Les propriétés antibactériennes des plaquettes sont largement attribuées au fait qu'elles produisent des peptides antimicrobiens comme les β-défensines et les PMP (Yeaman 2014). Cependant, en plus de ces actions chimiques microbicices, il a été observé qu'elles étaient capables d'engloutir de façon rudimentaire les bactéries (Youssefian et al. 2002). Cependant, l'impact fonctionnel de ces propriétés autonomes anti-bactériennes dans le contexte de défense de l'hôte *in vivo* reste peu établi. En revanche, les plaquettes ont des fonctions antibactériennes *in vivo* bien établies qui résultent de partenariats synergiques avec d'autres cellules immunitaires (Figure 18).

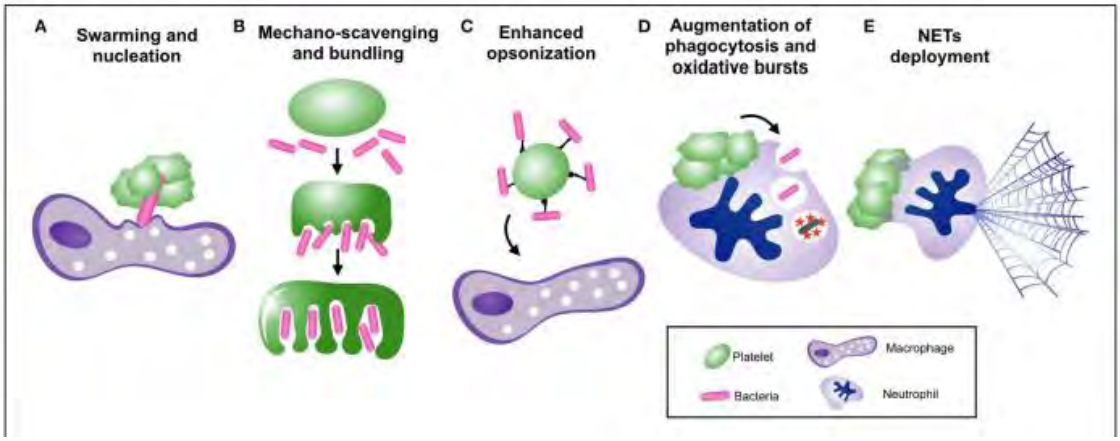


Figure 18: Collaboration plaquettes-cellules immunitaires en réponse à l'infection bactérienne

Les plaquettes collaborent avec les neutrophiles et autres cellules du système immunitaire pour faciliter l'élimination des bactéries du courant sanguin. Les mécanismes d'élimination incluent (A) une surveillance vasculaire et la nucléation des bactéries préparant la phagocytose des

macrophages, (B) le regroupement des pathogènes dans la microcirculation, (C) l'opsonisation des bactéries circulantes, (D) la phagocytose médiée par les neutrophiles et (E) l'induction de la formation de NETs. (D'après McDonald Front Immunol 2019)

Par exemple, les plaquettes sont cruciales pour la survie de souris contaminées lors d'une bactériémie à *Bacillus cereus* et *Staphyloccus aureus* en raison de leur capacité à collaborer avec les macrophages hépatiques pour éliminer les bactéries circulantes (Wong et al. 2013). Dans ce modèle, on observe que les plaquettes s'agrègent sur les macrophages hépatiques chargés de bactéries, ce qui constitue un signal essentiel pour les macrophages de la circulation menant à l'élimination des bactéries. De plus, les plaquettes circulantes peuvent capturer les bactéries du sang et améliorer leur acheminement vers les phagocytes (Verschoor et al. 2011).

b) Interactions plaquettes-neutrophiles

Un des mécanismes les plus étudiés et probablement un des plus puissants de la réponse plaquettaire à l'infection réside dans la collaboration plaquettes-neutrophiles. Les interactions plaquettes-neutrophiles induisent et/ou augmentent de nombreuses fonctions antibactériennes des neutrophiles afin d'améliorer l'élimination des pathogènes de l'organisme. Les interactions plaquettes-neutrophiles sont orchestrées par les protéines de surface et les molécules sécrétées. Les plaquettes activées expriment le CD62-P à leur surface qui se lie au P-selectine protein ligand (PSGL-1) à la surface des neutrophiles. Sinon, GPIb ou l'intégrine $\alpha_{IIb}\beta_3$ des plaquettes interagissent avec $\alpha M\beta_2$ à la surface des leucocytes soit directement soit indirectement via le fibrinogène comme molécule « pont » (figure 19). En revanche, des molécules sécrétées comme la cathepsine G produite par les neutrophiles activés peuvent compromettre ces interactions en clivant GPIb ou PSGL-1.

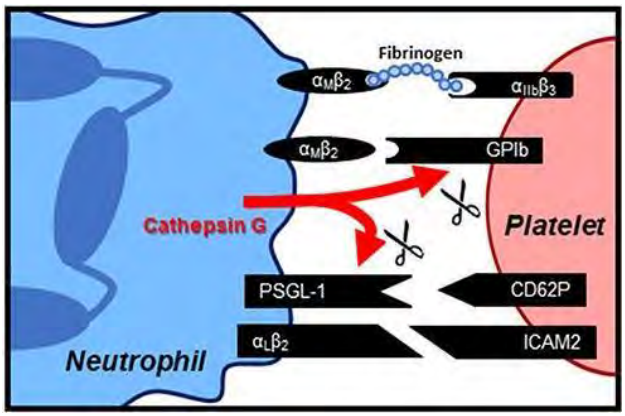


Figure 19: molécules d'adhésion clés des interactions neutrophiles-plaquettes

Les interactions neutrophiles-plaquettes permettent non seulement une liaison mais aussi entraînent une cascade de signalisation intracellulaire responsable d'une activation cellulaire, résultant en une régulation positive des molécules d'adhésion et effectrices. (D'après Zucoloto AZ, Front Cardiovasc Med 2019)

Récemment, il a été démontré que les produits dérivants des plaquettes pouvaient moduler le recrutement des neutrophiles, leur activation et fonctions. Par exemple, le CD40L sécrété par les plaquettes régule de façon positive l'expression des intégrines des neutrophiles (Rahman et al. 2013).

La sérotonine et le CXCL4 sont impliqués dans le recrutement des neutrophiles par les plaquettes dans des modèles animaux d'inflammation et de pancréatite aiguë (Wetterholm et al. 2016). Certains médiateurs libérés après activation plaquettaire améliorent les capacités de phagocytose des neutrophiles. En effet, Assinger et al. ont démontré que les plaquettes, activées par des pathogènes de parodontites, augmentaient la clairance des bactéries de plus de 20% via des interactions directes avec les bactéries, en lien avec le TRL2 plaquettaire (Assinger et al. 2012). D'autre part, Gaertner et al. ont démontré pour la première fois en 2017 que les plaquettes sont capables de regrouper des bactéries et de favoriser leur phagocytose par les neutrophiles (Gaertner et al. 2017).

La coopération entre les plaquettes et les neutrophiles ne s'arrête pas là. Les plaquettes participent activement à la capacité de transmigration des neutrophiles lors du sepsis. En effet, les fonctions des neutrophiles dans la réponse immune contre les pathogènes dépendent étroitement de leur capacité à migrer au sein et au travers des vaisseaux. La migration des neutrophiles est initiée par leur attachement et leur rolling sur les veinules inflammées, un processus médié par les sélectines endothéliales. La capacité des neutrophiles à migrer au travers de la barrière endothéliale dépend de leur capacité à adopter une morphologie polarisée avec une distribution asymétrique de leurs récepteurs. Sreeramkumar and *coll*. ont démontré en 2014 que les neutrophiles recrutés dans les veinules inflammées présentent un domaine dans la lumière vasculaire où les clusters de PSGL-1 (P-selectine glycoprotein ligand 1) scannent les plaquettes activées dans la circulation (Sreeramkumar et al. 2014). Lorsque les interactions plaquettes neutrophiles sont efficaces, la transduction du signal de l'extérieur vers l'intérieur conduit à une redistribution des récepteurs de surface Mac-1 (αMβ2 integrin) et CXCL2, générant des microdomaines récepteurs polarisés essentiels pour une migration efficace vers le site infecté. Cette découverte a révélé que les neutrophiles adhérents recherchent les plaquettes activées dans le système vasculaire afin de permettre des interactions physiques qui orientent la migration des neutrophiles vers des cibles appropriées.

c) Implication des plaquettes dans la NETose

Un des mécanismes effecteurs antimicrobiens les plus puissants déclenchés en réponse aux interactions entre les plaquettes et les neutrophiles est la formation de NETs. Les NETs correspondent à des filets de chromatine décondensée chargée d'enzymes protéolytiques et d'autres molécules antibactériennes qui sont expulsées des neutrophiles activés (Brinkmann et al. 2004) (figure 20).

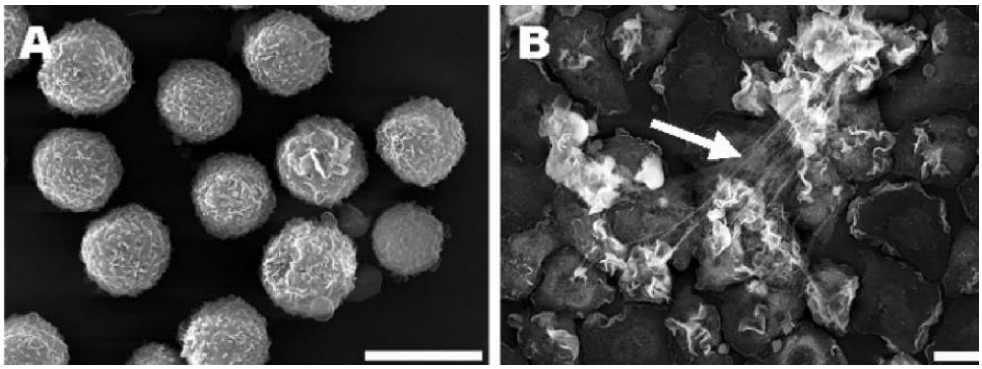


Figure 20: Visualisation de NETs en microscopie électronique

Analyse en microscpie électronique de neutrophils au repos et actives. (A) neutrophiles au repos (B) neutrophils après stimulation avec apparition de protrusions et formation de NETs (flèche). (Brinkmann, *Science* 2004)

La NETose est initiée par l'activation de la peptidylarginine desaminase 4 (PAD4) qui induit la citrullination des histones 3 et 4, entraînant le démantèlement du nucléosome (Wang et al. 2009; Leshner et al. 2012). De plus, l'activation de plusieurs voies de signalisation (PKC, NFκB,…) entraîne la phosphorylation de plusieurs kinases résultant en l'assemblage d'un complexe NADPH oxydase (nicotinamide adenine dinucleotide phosphate) pour la génération d'espèces réactives de l'oxygène. L'élastase neutrophile libérée entraîne la décondensation de la chromatine et le clivage des histones. Après désintégration de l'enveloppe nucléaire et la rupture de la membrane cytoplasmique, les NETs sont libérés (Papayannopoulos et al. 2010; Metzler et al. 2014). Cette première voie s'apparentant à un « suicide cellulaire » par NETose est différente de la voie non lytique, apparentée à de la NETose « vitale » (Pilsczek et al. 2010; Yipp et al. 2012). Par exemple, *staphylococcus aureus* entraîne la libération de NETs en quelques minutes via la sécrétion du contenu granulaire neutrophile et de la chromatine, en l'absence de mort cellulaire. Ce phénomène génère à la fois des NETs mais aussi des cytoplastes anucléés qui phagocytent les bactéries ((Pilsczek et al. 2010; Yipp et al. 2012) (Figure 21).

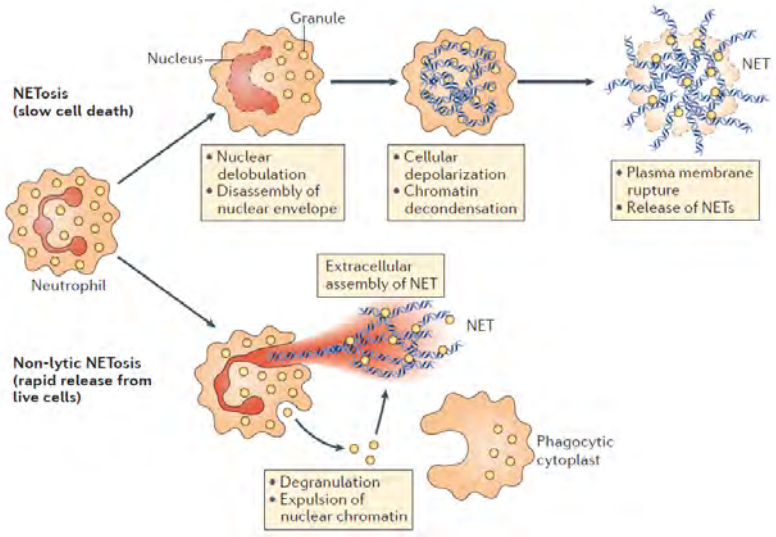


Figure 21: Voies de formation des NETs

Les NETs peuvent se forment selon 2 processus différents. Le premier correspond à une voie avec mort cellulaire nommée NETose qui débute par une délobulation nucléaire et le désassemblage de l'enveloppe nucléaire, continue par la perte de la polarisation de la cellule, la décondensation de la chromatine et la rupture de la membrane plasmique. Le second correspond à une forme non lytique de NETose qui peut survenir indépendamment et fait intervenir l'expulsion de la chromatine nucléaire accompagnée par la libération des protéines granulaires via dégranulation. Ces composant se regroupent en dehors de la cellule et laissent derrière eux des cytoplastes anucléés actifs qui continuent à ingérer des microorganismes. (D'après Papayannopoulos V, *Nat Rev Immunol* 2018)

Les NETs sont capables de capturer et de tuer des pathogènes extra-cellulaires incluant les bactéries, les champignons, les parasites, et ont des propriétés antivirales (Papayannopoulos 2018).
La co-incubation de plaquettes et neutrophiles issus de donneurs sains avec du plasma de patients septiques favorise l'adhésion des plaquettes aux neutrophiles d'une manière dépendant du TLR4 comme on peut le voir en les co-incubant avec du LPS (Clark et al. 2007; Carestia et al. 2016). En outre, il a été démontré que la NETose intravasculaire induite par le LPS et la capacité à piéger de l'*E. Coli* par les NETs étaient augmentées en présence de plaquettes (Clark et al. 2007). La NETose médiée par les plaquettes est observée en présence des agonistes plaquettaires classiques (thrombine, ADP, acide arachidonique, collagène…) de même qu'en présence de ligands classiques des TLR ; cependant dans ces modèles, une activation plaquettaire est nécessaire (Clark et al. 2007; Carestia et al. 2016). La NETose réalisée *in vitro* nécessite des plaquettes activées. Le CD62P est aussi nécessaire pour la NETose induite par les plaquettes puisque des plaquettes $CD62P^{-/-}$ ne peuvent induire la libération de NETs alors que la surexpression de CD62P augmente la NETose (Clark et al. 2007). Bien qu'il soit admis que l'adhésion plaquette-neutrophile joue un rôle central dans la formation de NETs, il semble que les interactions directes entre les plaquettes et les neutrophiles ne soient pas indispensables puisque les plaquettes activées voient leur CD62P être clivée. En effet, les neutrophiles en présence de *streptococcus mutans* et de CD62P soluble (sCD62P) sont capables de produire des NETs, tout du moins *in vitro* (Jung et al. 2015). En plus de la signalisation CD62P/PSGL-1, d'autres médiateurs sont capables d'entraîner la formation de NETs. Par exemple, le blocage par anticorps de la protéine HMGB-1 (High mobility group box 1) inhibe la formation de NETs in vitro (Maugeri et al. 2014). De plus, il a été démontré que HMGB-1 est nécessaire à l'activation des voies d'autophagie, nécessaires à la NETose de manière dépendante de RAGE (Receptor for advanced glycation endoproducts), récepteur clé d'HMGB-1 (Pluskota et al. 2008). Le rôle du TxA2 a également été démontré dans la libération des NETs par les plaquettes. Dans une étude de Caudrillier et al. , il a été démontré que des plaquettes activées induisent la formation de NETs in vitro. Ce processus dépend de la signalisation médiée par le récepteur TXA2, qui active la voie des MAP kinases (MAPK) (Caudrillier et al. 2012). Les chimiokines RANTES (CCL5) et PF4 (CXCL4) sont aussi de forts inducteurs de NETs (Carestia et al. 2016).

Ainsi, la NETose peut être médiée directement par les interactions plaquettes-neutrophiles ou via des médiateurs plaquettaires de façon indirecte (figure 22).

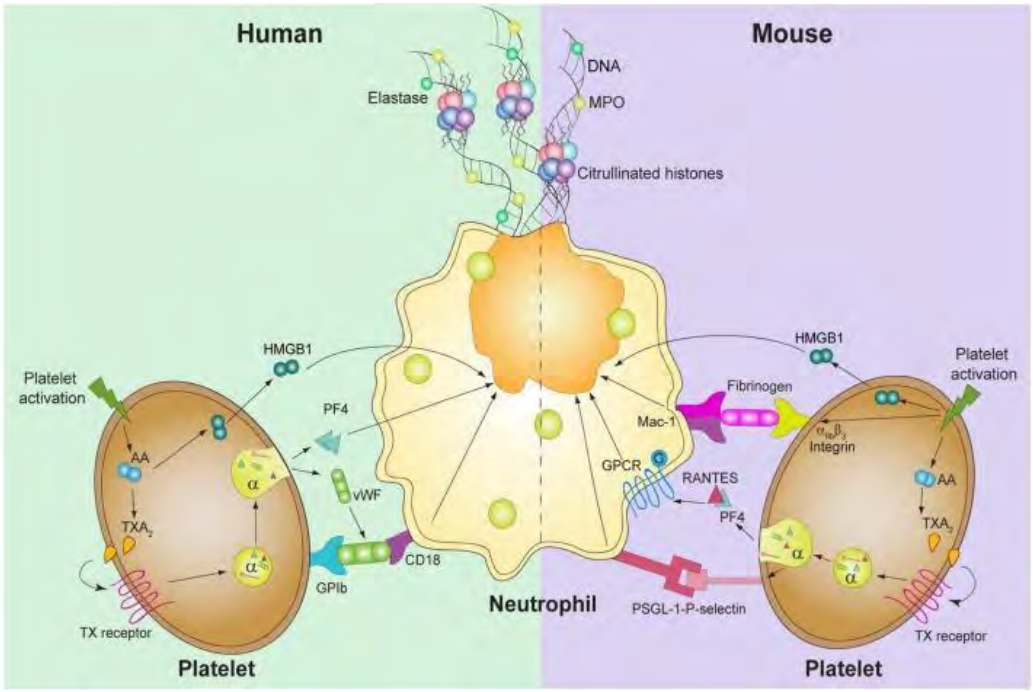


Figure 22: Molécules connues impliquées dans la formation de NETs médiée par les plaquettes

Chez l'Homme (gauche), l'activation plaquettaire induit la formation de thromboxane A2 (TxA2) qui entraîne la libération d'HMGB-1 (High Mobility Group Box 1), de von Willebrand fator (vWF) et de PF4 (platelet factor 4). Le vWF se lie à son récepteur, la glycoprotéine GPIb, qui se lie au CD18 des neutrophiles. PF4 et HMGB-1 agissent directement sur les neutrophils et induisent la libération d'ADN. Chez la souris (droite), le TXA2, HMGB-1 mais aussi PF4 et RANTES induisent la formation de NETs médiée par les plaquettes. De même, les interacions P-selectine-PSGL1 (P selectine ligand 1) et αIIbβ3-Mac-1 sont nécessaire à la libération de NETs (D'après Carestia A, *Front immunol* 2016).

2.2.3 Rôle des plaquettes dans l'immunothrombose

Le terme d'immunothrombose a été introduit pour la première fois par Engelmann (Engelmann and Massberg 2012). Il n'est pas sans rappeler le terme de thrombo-inflammation dont il pourrait être une des composantes essentielles de la lutte contre les pathogènes. Ce processus physiologique décrit la formation de microthrombi à l'intérieur des microvaisseaux. Ces microthrombi agissent comme des matrices anti-microbiennes qui assurent la protection de l'hôte contre les agents pathogènes. L'immunothrombose est déclenchée et maintenue par l'accumulation locale de cellules immunitaires innées (en particulier les monocytes et les neutrophiles), un processus qui est susceptible d'impliquer l'adoption d'un phénotype pro-adhésif par les cellules endothéliales microvasculaires qui sont exposées aux agents pathogènes. Dans le sepsis, une fois activés, les neutrophiles libèrent des NETs et de nombreux composants antibactériens dont la myélopéroxydase, l'élastase neutrophile, la lactoferrine, la matrix metalloproteinase 9 (MMP9). Cependant les NETs n'ont pas seulement une fonction antibactérienne, mais ils sont capables d'induire une forte réponse procoagulante (Figure 23). Les NETs peuvent lier et activer les plaquettes dans le cadre des thromboses veineuses profondes par exemple (Fuchs et al. 2010). De plus, les nucléosomes extracellulaires au sein des NETs forment une plateforme catalytique qui stimule l'activité protéolytique de l'élastase neutrophile qui, à son tour, entraîne la coagulation (Massberg et al. 2010).

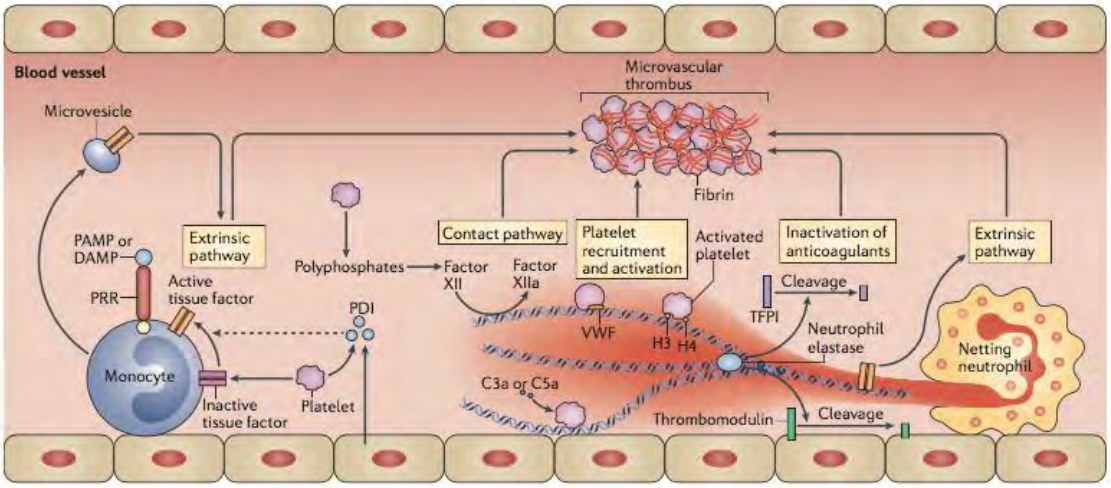


Figure 23 : Principes de bases de l'immunothrombose

En réponse à des PAMPs ou des DAMPs, les monocytes et leurs microvésicules expriment et délivrent du facteur tissulaire intravasculaire au site infecté, initiant la voie extrinsèque de la coagulation. Les NETs (Neutrophil Extracellular Traps) sont constitués de brins d'ADN et d'histones et entraînent l'immunothrombose de plusieurs façons. D'une part, les NETs peuvent directement activer le facteur XII (phase contact). D'autre part, les NETs se lient au vWF et recrutent des plaquettes. Mais aussi les histones H3 et H4 présentes dans les NETs peuvent activer les plaquettes. Les NETs concentrent des enzymes localement comme l'élastase neutrophile et les myélopéroxydases qui clivent et inactivent les protéines anticoagulantes dont le TFPI (tissue factor pathway inhibitor) et la thrombomoduline. Les plaquettes jouent un rôle important dans l'immunothrombose. Le système du complément (C3a, C5a) joue un rôle également en activant les plaquettes (D'après Engelmann, *Nat Rev Immunol* 2013).

Un composant majeur de l'immunothrombose correspond à la formation des NETs qui agissent comme des surfaces catalytiques qui favorisent et compartimentent le système de coagulation. L'action pro-coagulante des NETs implique l'activation du facteur XII (Brühl et al. 2012) et la dégradation des molécules anticoagulantes telles que le TFPI et probablement la thrombomoduline. La perfusion d'anticorps spécifiques anti-histones H2A et H2B et de l'ADN, qui démantèlent les NETs, atténue la coagulation dans la microcirculation hépatique des souris exposées à une infection systémique à *Escherichia coli* (Massberg et al. 2010).

L'immunothrombose favorise la défense contre les pathogènes. Engelmann et Massberg suggèrent que le processus d'immunothrombose est un élément majeur du système immunitaire inné intravasculaire et qu'il remplit au moins quatre fonctions physiologiques différentes (Engelmann and Massberg 2012). Premièrement, il aide à capturer et à piéger les agents pathogènes circulant dans le sang et limite ainsi la dissémination des agents pathogènes en retenant les micro-organismes dans le réseau de fibrine. Deuxièmement, il empêche l'invasion des tissus par les agents pathogènes grâce à la formation de microthrombi dans les microvaisseaux. Troisièmement, les thrombi intravasculaires génèrent un compartiment distinct qui concentre les stratégies antimicrobiennes et leurs cibles pathogènes et favorise ainsi la destruction des agents pathogènes. Cette mise à mort implique des stratégies antimicrobiennes, qui sont fournies par les cellules immunitaires innées, et des peptides antimicrobiens qui sont générés lors de l'activation de la coagulation sanguine et/ou libérés par les plaquettes activées

aux sites d'immobilisation des agents pathogènes (Yeaman 2010b). Quatrièmement, l'accumulation et le dépôt microvasculaires de fibrinogène ou de fibrine favorisent le recrutement de cellules immunitaires supplémentaires sur le site de l'infection et/ou de la lésion tissulaire, ce qui renforce la reconnaissance des agents pathogènes et coordonne la réponse immunitaire (Szaba and Smiley 2002) (Figure 24).

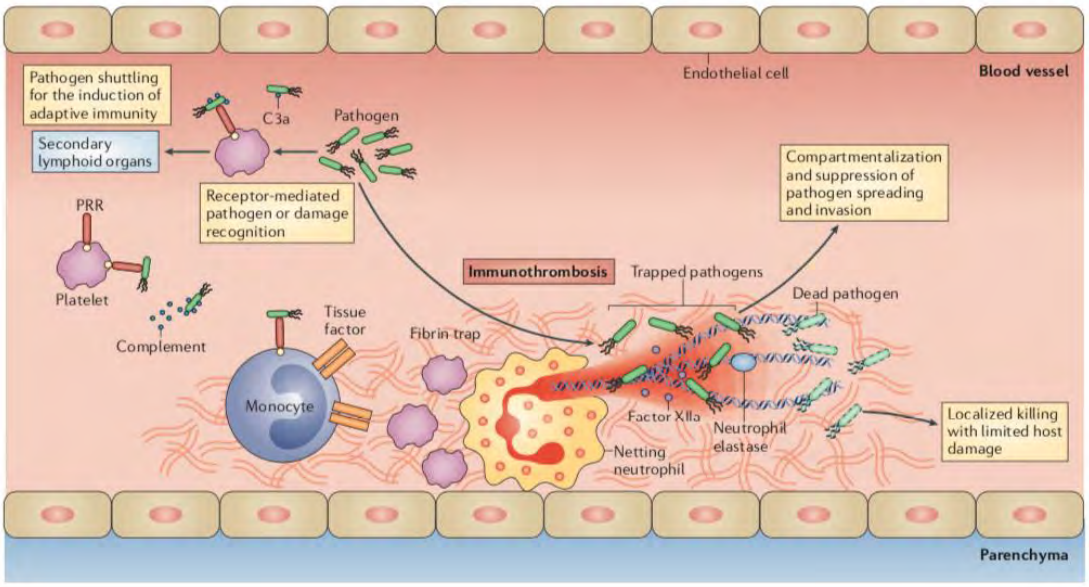


Figure 24: illustration de la rétention des pathogènes par l'immunothrombose

Engelmann propose l'existence d'une forme de thrombose physiologique qui met en jeu les défenses de l'immunité innée pour lutter contre les pathogènes. L'immunothrombose permet une stratégie cruciale impliquant la reconnaissance de pathogènes, leur compartimentalisation et leur capture, la prévention de leur dispersion, et leur destruction. L'immunothrombose est cantonnée au compartiment intravasculaire et pourrait n'entraîner que des lésions minimes des organes. (PRR, pattern-recognition receptor) (D'après Engelmann, *Nature* 2012)

Durant la COVID-19, le phénomène d'immunothrombose est décrit (Bonaventura et al. 2021).
Les NETs semblent impliqués dans l'immunothrombose liée à l'infection au SARS-CoV-2 et leur taux est d'ailleurs corrélé à la sévérité de l'infection (Middleton et al. 2020; Zuo et al. 2021). Des thrombi riches en neutrophiles sont décrits dans les microvaisseaux pulmonaires (Middleton et al. 2020) mais aussi au niveau du cœur et des reins des patients COVID-19 (Nicolai et al. 2020; Bonaventura et al. 2021) et plusieurs auteurs font l'hypothèse qu'une immunothrombose dérégulée participe aux manifestations cliniques de la COVID-19 en lien avec la survenue de thrombi dans les microvaisseaux, en particulier pulmonaires.

2.2.4 Rôle dans la résolution de l'inflammation

Une fois la phase inflammatoire installée, il est nécessaire qu'elle soit contrôlée dans le temps et dans l'espace. Une inflammation non freinée peut mener à des dommages d'organes sévères ou une chronicisation de certaines lésions. Le contrôle de l'inflammation et la phase de résolution de l'inflammation sont nécessaires pour restaurer la physiologie des tissus et éliminer les cellules inflammatoires apoptotiques ainsi que les débris cellulaires, impliquant une communication entre macrophages, plaquettes, lymphocytes, composants de la matrice extracellulaire, et cellules progénitrices (Rossaint, Margraf, and Zarbock 2018; Zarbock, Polanowska-Grabowska, and Ley 2007).

De façon intéressante, les plaquettes possèdent des propriétés anti-inflammatoires. On sait que les plaquettes interagissent avec les cellules T régulatrices et améliorent leurs réponses, ce qui entraîne une augmentation des niveaux d'IL-10 (Bergmann et al. 2016; Zhu et al. 2014). De plus, les plaquettes activées sont connues pour moduler la polarisation des macrophages vers un phénotype anti-inflammatoire avec une libération accrue d'IL-10 et une sécrétion réduite de TNF-α (Linke et al. 2017).

CLEC-2 semble jouer un rôle dans la résolution de l'inflammation dans un modèle murin de sepsis avec délétion de CLEC-2 associée à une aggravation des lésions d'organes. Comme mentionné précédemment, les plaquettes induisent également la formation de NETs (Rayes et al. 2017). Même si ce processus est principalement considéré comme pro-inflammatoire, les NETs sont également connus pour limiter l'inflammation par dégradation des cytokines et des chimiokines, telles que l'IL-10, l'IL-6, la MCP-1, la MIP-1α etβ, l'IL-1β et le TNF (148), même si ces résultats sont débattus (Reber et al. 2016).

Les plaquettes sécrètent aussi des médiateurs nommées résolvines. Ces médiateurs de type lipoxines ont un rôle protecteur pour les organes ou influencent la restauration des organes dans différents modèles (Motwani et al. 2018; Sansbury and Spite 2016). Les principales résolvines sont par exemple les résolvines D1 et D3, la résolvine E1 et la marésine 1. Les plaquettes expriment des récepteurs pour les SPMs (Specialized proresolving mediators) nommé ChemR32 (récepteur à la résolvine E1), GPR32 (récepteur de la résolvine D1) et ALX (récepteur de la lipoxine A4). La stimulation des plaquettes par la marésine 1 entraîne un changement de phénotype dans lequel la sécrétion des médiateurs pro-inflammatoires des plaquettes est inhibée (Lannan et al. 2017).

Cependant, la versatilité des plaquettes dans l'apparition et la résolution de l'inflammation est loin d'être totalement comprise. Les mécanismes et les actions précises qui conduisent à passer d'un recrutement pro-inflammatoire des neutrophiles à un arrêt anti-inflammatoire de l'afflux de neutrophiles, ainsi qu'au recrutement et à l'amorçage de la résolution des lymphocytes T et des macrophages régulateurs avec libération de médiateurs pro-résolution, restent flous. On ne sait pas encore s'il existe un programme de résolution de l'inflammation intrinsèque aux plaquettes, qui peut

être activé au cours de l'inflammation, ou s'il s'agit d'une réaction purement passive basée sur des changements de médiateurs extérieurs.

2.3 Conséquences clinico-biologiques du sepsis sur les plaquettes en réanimation

Outre les aspects physiopathologiques précédemment décrits, les plaquettes jouent un rôle non négligeable dans l'impact clinique du sepsis, particulièrement en réanimation au cours du choc septique. Elles participent à l'état de défaillance multiviscérale qui peut mettre en jeu la vie des patients, à la dysfonction endothéliale, et leur compte diminue à la phase précoce du choc septique, témoin de la sévérité de l'atteinte des patients.

Un des marqueurs les plus pertinents et visibles est la survenue d'une thrombocytopénie au cours du sepsis. Les étiologies sont multiples et souvent intriquées. A l'examen clinique, la survenue de pétéchies, d'hématomes, de saignements, voire d'un purpura extensif doivent alerter le clinicien. Des anomalies de la coagulation et un risque hémorragique surajouté peuvent survenir chez le patient septique.

2.3.1 Thrombopénie septique : étiologies et implications

L'incidence de thrombocytopénie en réanimation est variable, principalement selon les pathologies rencontrées (médicale et/ou chirurgicale) et du seuil utilisé pour définir la thrombocytopénie dans les études (100 ou 150 G/L). Une thrombocytopénie peut survenir avant l'arrivée du patient ou durant son séjour en réanimation. Le délai de survenue de la thrombopénie par rapport à la maladie causale est variable selon les circonstances. D'ailleurs, la cinétique de survenue de la thrombopénie est utile à intégrer dans l'anamnèse puisque elle peut aider à suggérer un diagnostic ou un autre. La survenue d'une thrombopénie est corrélée à la morbi-mortalité des patients septiques (Moreau et al. 2007; Akca et al. 2002).

Les mécanismes de survenue d'une thrombocytopénie en réanimation sont généralement multiples. Une des causes principales est la consommation de plaquettes en réponse à leur activation médiée par la thrombine. Dans certains cas, il existe d'autres critères cliniques et biologiques de consommation de facteurs de la coagulation conduisant au diagnostic de CIVD (Kelton et al. 1979). Outre la consommation accrue, d'autres mécanismes de thrombocytopénie sont à évoquer en réanimation. Il peut s'agir d'une destruction des plaquettes dans la circulation sanguine, de l'effet d'un remplissage vasculaire important générant une hémodilution, de la séquestration des plaquettes dans la rate ou de la diminution de la production plaquettaire médullaire (Greinacher and Selleng 2016).

Dans le choc septique, les mécanismes de la thrombopénie sont multiples et probablement intriqués. L'agrégation plaquettaire et l'adhésion à des leucocytes et des cellules endothéliales est un mécanisme

courant de thrombopénie. L'activation des cellules endothéliales est caractéristique du sepsis est associée à la libération de grandes quantités de multimères du facteur de Willebrand et à la diminution réciproque des quantités de la protéase ADAMTS13. L'activation majeure des cellules endothéliales permet la fixation d'un grand nombre de plaquettes à leur surface. De même, la formation d'agrégats plaquettes-neutrophiles et de complexes plaquettes-monocytes participe à la consommation de plaquettes.

L'activation de la voie du complément peut entraîner une activation plaquettaire qui dans des cas extrêmes d'inflammation majeure comme le choc septique peut conduire à une consommation accrue de plaquettes (Peerschke et al. 2010). Le syndrome hémolytique et urémique atypique (SHUa) est un modèle pathologique classique où l'incapacité à contrôler l'activation du complément peut entraîner une thrombocytopénie et une anémie hémolytique avec micro-angiopathie.

La libération de NETs et d'histones promeut la thrombose et participe à la consommation plaquettaire dans le sepsis. L'infusion d'histones dans le courant sanguin provoque artificiellement une thrombocytopénie rapide et profonde par agrégation plaquettaire (Fuchs et al. 2010). Alhamdi et al ont récemment montré que des taux plasmatiques élevés d'histones pendant un séjour en réanimation prédisaient fortement l'apparition d'une thrombopénie modérée à grave (Alhamdi and Toh 2016).

Enfin, la thrombopénie peut être mise en lien avec un syndrome d'activation macrophagique, un mécanisme auto-immun ou du fait de l'introduction d'un traitement avec toxicité médullaire.

En résumé, les mécanismes de survenue d'une thrombopénie chez les patients septiques sont multiples et souvent intriqués, d'origine centrale et périphérique à la fois, même si les mécanismes périphériques prédominent souvent.

2.3.2 Coagulopathie induite par le sepsis

Les patients septiques présentent très fréquemment une coagulopathie associée au sepsis (Kinasewitz et al. 2004). Dans une récente étude observationnelle menée au Japon, parmi près de 2000 patients hospitalisés en réanimation pour sepsis, 29% présentaient les critères d'une coagulopathie induite par le sepsis ou « sepsis-induced coagulopathy, SIC » (Saito et al. 2019). Chez les patients septiques, la dysrégulation de la balance entre la génération de thrombine (coagulation) et la lyse du caillot (fibrinolyse) est une réponse pivot qui survient durant le sepsis qui peut être responsable de l'apparition de dysfonctions d'organes (Gando et al. 2012).

Les voies principales menant à la SIC et la CIVD font intervenir l'activation de la coagulation, les plaquettes et d'autres cellules inflammatoires (neutrophiles, lymphocytes...) en plus de lésions de l'endothélium vasculaire activé. Dans le sepsis, les neutrophiles sont activés, libérant les NETs, les histones et d'autres protéines granulaires des neutrophiles, aux propriétés hautement prothrombotiques. De plus, la libération de DAMPs à partir des cellules lésées augmente l'activité

prothrombotique. L'ADN libre circulant et les protéines du noyau résultant des dommages cellulaires possèdent également une forte activité prothrombotique. Pour contrebalancer ces effets prothrombotiques, les protéines plasmatiques circulantes telles que l'antithrombine, les protéines C et S possèdent d'importants effets anticoagulants. La contribution antithrombotique de l'endothélium vasculaire est importante dans des conditions physiologiques pour prévenir la formation de thrombi, maintenir l'intégrité vasculaire et réguler le tonus vasculaire. Cependant, en conditions septiques, l'expression du facteur tissulaire et la libération de facteur von Willebrand augmentent le côté prothrombotique.

Au final, la dysfonction endothéliale et le phénotype prothrombotique sont les caractéristiques de la coagulopathie induite par le sepsis.

2.4 Ciblage plaquettaire dans le sepsis

Il est suggéré depuis plusieurs années que les plaquettes pourraient représenter des cibles thérapeutiques d'intérêt dans le sepsis. Des études précliniques et observationnelles plaident pour cette hypothèse (Tsai et al. 2015).

Aspirine : il y a plus de 50 ans, il était démontré que l'aspirine améliorait la survie dans un modèle de sepsis canin à *E. Coli* (Hinshaw et al. 1967), résultat également démontré quelques années plus tard dans un modèle murin de choc septique à *Salmonella enteritidis* (halushka pv, j pharmacol exp ther 1981). Chez l'Homme, même si l'aspirine semble avoir un effet sur des sous-groupes de patients très sélectionnés en réanimation ou lors d'une pneumonie (Blasco-Colmenares et al. 2009; Otto et al. 2012; Bonaca et al. 2016), elle ne paraît pas avoir d'impact sur la sévérité du sepsis et n'améliore pas la survie (Gross et al. 2013). Publié en 2020, l'essai randomisé contrôlé multicentrique ANTISEPSIS (aspirine contre placebo) incluant des patients de plus de 70 ans hospitalisés pour un sepsis n'a pas montré, sur 16703 participants, une diminution du taux de mortalité liée au sepsis chez les patients recevant 100mg d'aspirine en prévention primaire (Eisen et al. 2021).

Antagonistes des récepteurs P2Y12 : ces antagonistes (clopidogrel, prasugrel, ticagrelor) sont largement utilisés en médecine cardio-vasculaire. Ils ont démontré leur capacité à réduire les interactions leuco-plaquettaires et les marqueurs inflammatoires, et ont montré qu'ils amélioraient la fonction pulmonaire dans des modèles murins de pneumonie (Akinosoglou and Alexopoulos 2014; Liverani et al. 2016). Dans un modèle animal de ligature perforation caecale, Rahman a démontré que l'usage du ticagrelor réduisait l'infiltration pulmonaire des leucocytes, l'œdème pulmonaire et les lésions pulmonaires (Rahman et al. 2013). De la même manière, le ticagrelor inhibait la formation d'agrégats leuco-plaquettaires et réduisait la thrombopénie septique. De façon similaire, ces inhibiteurs ont récemment montré une amélioration de la fonction respiratoire chez les patients atteints de

pneumopathie, bien que ces études aient des limites dans leur design et nécessitent une confirmation sur de plus larges effectifs (Gross et al. 2013) . Récemment, dans le contexte de crise épidémique actuelle, Omarjee L et al. posent la question de l'utilisation du ticagrelor pour réduire les agrégats leuco-plaquettaires, la libération de NETs et la fuite capillaire et ainsi prévenir la coagulopathie secondaire au sepsis liée au SARS-CoV-2 (Omarjee et al. 2020).

Récepteurs couplés au motif ITAM : les progrès dans la définition des voies engagées dans la thromboinflammation suggèrent que les récepteurs plaquettaires couplés au motif ITAM (immunoreceptor tyrosine-based activation motif) pourraient constituer une cible thérapeutique potentielle. Les plaquettes contiennent le récepteur C-type lectin-like-2 (CLEC-2) associé au motif ITAM, qui lie la podoplanine, et le récepteur du collagène GPVI. Ces récepteurs sont des cibles thérapeutiques d'intérêt parce que leur déficience n'entraîne pas de désordre hémorragique majeur. L'administration d'anticorps anti-GPVI chez la souris a montré un effet bénéfique dans un modèle de sepsis par pneumopathie sans augmenter le risque de saignement (Claushuis et al. 2016). La délétion spécifique de CLEC-2 (mais pas GP VI) réduit les thromboses hépatiques dans un modèle d'infection par *salmonella typhirium* (Hitchcock et al. 2015). Cependant, la perte de CLEC-2 semble entraîner une inflammation systémique et augmenter les lésions d'organe dans des modèles murins de sepsis (Rayes et al. 2017).

Cibler les interactions neutro-plaquettaires : plaquettes et neutrophiles font partie des cellules dont l'impact est majeur en terme de réaction thrombo-inflammatoire durant le sepsis. De plus, la formation des agrégats neutro-plaquettaires contribue à l'obstruction microvasculaire et à la réaction inflammatoire, notamment dans l' « Acute Lung Injury » (ALI), les syndromes coronariens, l'accident vasculaire cérébral ischémique (Zarbock, Polanowska-Grabowska, and Ley 2007; Zarbock, Singbartl, and Ley 2006; Otto et al. 2012). A l'heure actuelle, seules des études précliniques ont confirmé que cibler les molécules d'adhésion des plaquettes (P-sélectine, GPIb, $\alpha_{IIb}\beta_3$) et les neutrophiles (PSGL-1, Mac-1) inhibait les agrégats neutro-plaquettaires et améliorait la dysfonction microvasculaire et l'inflammation. Un essai de phase 1 a confirmé la sécurité et le dosage de l'inclacumab, un anticorps monoclonal dirigé contre la P-sélectine, et a établi l'absence de majoration du temps de saignement, ou l'impact sur l'agrégation plaquettaire (Schmitt et al. 2015).

RÉSULTATS EXPÉRIMENTAUX ET CLINIQUES

PARTIE 1

CHOC SEPTIQUE & RÉACTIVITÉ PLAQUETTAIRE

PARTIE 2

SEPSIS VIRAL : L'EXEMPLE DE LA COVID-19

PARTIE 1

- CHOC SEPTIQUE ET RÉACTIVITÉ PLAQUETTAIRE -

Contexte scientifique n°1

Chez les patients septiques, l'histoire de la progression de la maladie avant l'hospitalisation, et particulièrement le début du sepsis, restent souvent inconnus. Les modèles animaux aident à comprendre ces processus précoces et la disponibilité de souches de souris génétiquement modifiées a contribué à l'identification de voies de signalisation ou de gènes distincts en tant que biomarqueurs ou cibles de médicaments potentiels. Ainsi, les modèles de rongeurs, et en particulier de souris, se sont révélés être des modèles expérimentaux d'intérêt pour étudier les mécanismes cellulaires et moléculaires du sepsis dans des contextes définis. Les sepsis étant de multiples origines, des modèles d'infection des voies aériennes ou d'infection intra-abdominale ont été développés.

La ligature-ponction caecale (CLP ou cecal ligation and puncture) représente l'un des modèles de sepsis les plus couramment utilisés car elle reproduit le sepsis chez l'Homme en termes de réponses biochimiques, hémodynamiques et immunitaires, notamment l'hypotension artérielle, la leucopénie et la thrombocytopénie avec un phénotype concomitant pro-thrombotique et pro-coagulant, des niveaux élevés de cytokines pro-inflammatoires ainsi que de marqueurs de dysfonction d'organes (Dejager et al. 2011; Li et al. 2018). La perforation du caecum imite une brèche de la barrière intestinale avec la dissémination secondaire de la flore microbienne intestinale dans le péritoine. Dans le modèle CLP, cette péritonite est associée à une nécrose du tissu ligaturé et à une éventuelle dissémination à la périphérie, entraînant une septicémie et des lésions d'organes distants (Dejager et al. 2011). Cependant, ce modèle est sujet à des variations dues à l'opérateur et les réponses immunitaires locales peuvent parfois contenir les bactéries dans un abcès, empêchant ainsi la progression du choc septique (Buras, Holzmann, and Sitkovsky 2005). Si le nombre de ponctions et la taille de l'aiguille sont bien décrites dans les études animales, la hauteur de la ligature caecale est souvent ignorée, rendant l'extrapolation des résultats discutables entre les études. Le premier objectif du travail était de mieux définir notre modèle expérimental murin de CLP, et plus particulièrement d'analyser les conséquences de 3 hauteurs de ligatures différentes sur la mortalité, et la sévérité clinique et biologique du sepsis créé.

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RESEARCH Open Access

Sepsis modeling in mice: ligation length is a major severity factor in cecal ligation and puncture

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Abstract

Background: The cecal ligation and puncture (CLP) model, a gold standard in sepsis research, is associated with an important variability in mortality. While the number of punctures and needle size is well described in CLP animal studies, the length of cecal ligation is often not. The relationship between cecal ligation and survival in mice is briefly reported in the literature; therefore, we devised an investigation in mice of the consequences of three standardized cecal ligation lengths on mortality and the severity of the ensued sepsis.

Methods: Male C57BL/6J mice underwent standardized CLP. The cecum was ligated at 5, 20, or 100 % of its total length and further perforated by a single 20-G puncture. Mortality was analyzed. We assessed blood lactate, serum creatinine levels, and serum cytokines (TNF-α, IL-1β, IL-6, and IL-10) after procedure in a control group and in ligated mice.

Results: Mortality was directly related to ligation length: median survival was 24 h for the "100 %" group and 44 h for the "20 %" group. Blood lactate increased proportionally with the ligation length. At 6 h post-procedure, pro-inflammatory cytokines significantly increased in the ligated group with significantly higher serum levels of IL-6 in the 100 % group compared to the other ligated groups. The 20 % group exhibited the characteristics of septic shock with hypotension below 65 mmHg, pro-inflammatory balance, organ dysfunction, and hyperlactatemia.

Conclusions: Cecal ligation length appears to be a major limiting factor in the mouse CLP model. Thus, this experimental model should be performed with high consistency in future protocol designs.

Keywords: Cecal ligation and puncture, Mice, Cytokine, Sepsis

Background

Severe sepsis results from a complex and dynamic pathophysiology; therefore, a better understanding of the inflammatory process leading to sepsis is essential [1]. Although they do not reflect entirely the clinical complexity, animal models remain a valuable approach to developing new therapeutic strategies. Various animal models of sepsis have already been developed such as intravascular infusion of endotoxin (lipopolysaccharide (LPS)), live bacteria or viruses, bacterial peritonitis, cecal ligation and puncture (CLP), soft tissue infection, pneumonia model, and meningitidis model [2–6]. However,

since 1998 Deitch pointed out that an important number of failures in new therapeutic approaches may be due to the use of inappropriate experimental models [7]. An endotoxic model (LPS injection) mimics poisoning more than infection. In the LPS endotoxic model, the cytokines peak early and transiently, whereas in the CLP model, the pro-inflammatory response is delayed and persists over time [8]. LPS model mortality occurs early, most likely due to the effects of the intense inflammatory response on the cardiovascular system, whereas in the CLP model, mortality is delayed with multiple organ failure complicating induced peritonitis. In humans, endotoxic shocks are rare and sepsis origin is often localized. The CLP model is the most widely used model for experimental sepsis and is currently considered as a gold standard in research since it mimics the nature and evolution of severe sepsis in humans [5, 9]. Ensuing a simple procedure, the model induces sepsis secondary to a stercoral peritonitis, followed by a polymicrobian translocation in the blood circulation with an early inflammatory phase, after which an anti-inflammatory response develops [2]. However, significant variability on mortality from one experimental protocol to another can lead to differing interpretations of the results. That being said, survival rates can vary from 20 to 50 %. The main determinants of mortality are the size of the needle used for cecal puncture; the number of punctures, generally between 1 and 4; and the use of antibiotics and/or fluid resuscitation [10, 11]. While the number of punctures and needle size is standardized, the length of cecal ligation is often not described in CLP animal studies.

To the best of our knowledge, only few brief descriptions exist regarding cecal ligation and survival in the mouse CLP model [10, 12]. We investigated in non-resuscitated C57BL/6J male mice the consequences of several standardized distances of CLP on mortality and sepsis severity. To do so, we used organ failure markers such as serum creatinine levels (as an early sign of acute kidney injury), serum lactate, and the kinetics of the inflammatory state reflected by cytokine synthesis including TNF-α, IL-1β, IL-6, and IL-10.

Methods

Animals

C57BL/6J wild-type mice were obtained from Harlan (Harlan France, Gannat, France). We used male animals aged 20 weeks, weighing 25–30 g. Animal experimentation was performed according to national and institutional animal care and ethical guidelines and was approved by the local board. Mice were housed in a temperature-controlled room on a 12-h night-dark cycle. Four animals were placed in a cage and had access to water and food ad libitum. The mice were not fasted prior to CLP procedure. The animals were shocked or control-operated and euthanized at different times after surgery.

Protocol design

Sepsis was induced following a modification of a previously published method of CLP [10]. Briefly, animals were anesthetized with intraperitoneal injection of ketamine and xylazine (250 and 10 mg/kg, respectively). After adequate anesthesia, the lower quadrants of the abdomen were shaved and the surgical area was disinfected. A longitudinal midline incision was made using a scalpel, and scissors were used

to extend the incision into the peritoneal cavity. After intramuscular, fascial, and peritoneal incision, the cecum was located and exteriorized. In our experiments, the cecum was ligated at different lengths below the ileocecal valve to avoid bowel obstruction. Total cecal length was measured from the tip of the ascending cecum to the tip of the descending cecum. The cecum was then ligated at 5, 20, and 100 % of its total length. For the "100 %" group, the cecum was ligated to the longest possible without bowel occlusion (Fig. 1).

The cecum was then perforated by a single puncture midway between the ligation and the tip of the cecum with a 20-G needle. We chose this needle diameter to obtain mid-grade lethal sepsis [5, 13, 14]. After removing the needle, a small amount of feces was extruded. The cecum was relocated, after which the fascia, abdominal musculature, and peritoneum were closed via simple running sutures; the skin was also sutured. The control mice were anesthetized and underwent laparotomy without puncture or cecal ligation and served as the control. The animals were shocked or control-operated and euthanized at different times depending on the set of experiments.

Immediately post-procedure, 1 ml of saline was administered subcutaneously for fluid resuscitation (circa 0.045 ml/g) [8, 14]. Pain control for CLP and sham mice was achieved with 0.05 mg/kg buprenorphine every 12 h.

Spontaneous mortality determination

The first set of experiments consisted of observation of spontaneous mortality for each ligation length. The mice were then redistributed into subgroups of three to four in order to repeat the observation of mortality. The mortality was followed for 4 days after the CLP protocol.

Renal function study and lactate dosage

In the second set of experiments, control and CLP animals were euthanized at 6 h.

We used serum lactates as a severity marker of septic state and serum creatinine concentration as a marker of renal dysfunction. Samples were collected 6 h after surgery by intracardiac puncture under general anesthesia (intraperitoneal injection of

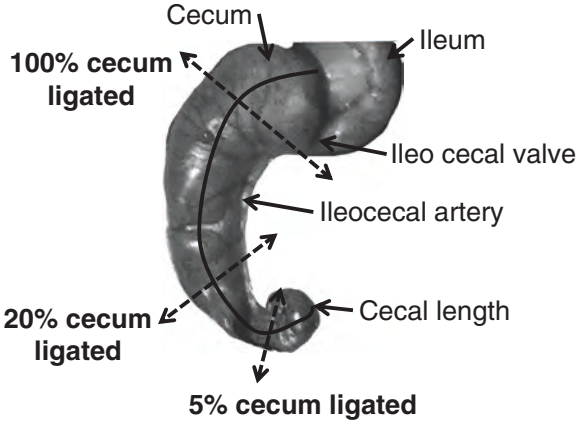

Fig. 1 Description of cecal length ligation methods. The total length of the cecum is represented by the *full line*. *Dotted arrows* are placed at each level of cecal ligation: 5, 20, and 100 % of the total of cecum length

250 mg/kg ketamine and 10 mg/kg xylazine). Serum obtained after centrifugation was immediately frozen and stored at −80 °C before being analyzed at the phenotyping platform (GenoToul Anexplo, Toulouse, France).

Serum cytokines, bacterial blood culture, and leukocyte count

In the third set of experiments, the mice were put down at 6 and 24 h for the control, "5 %," "20 %," and "100 %," ligated groups. For the control and 20 % ligated animals, we performed supplementary analysis at 48 h.

We measured serum cytokines TNF-α, IL-1β, and IL-6. IL-10 concentrations were determined at 24 and 48 h for the control and 20 % ligated groups. The samples were collected by intracardiac puncture under general anesthesia (intraperitoneal injection of 250 mg/kg ketamine and 10 mg/kg xylazine). The samples were then immediately frozen at −80 °C and analyzed by Luminex technique (Bio-Rad Y60-00000YU Pro Mouse Cytokines Group 4-plex 1 x 96, Bio-Rad, Hercules, CA, USA) on the phenotyping platform (GenoToul Anexplo, Toulouse, France).

Leukocyte count was performed at the Phenotyping platform (GenoToul Anexplo, Toulouse, France) on the MICROS-60 hematology analyzer (Horiba ABX-Diagnostics, MA, USA). Datum is expressed in leukocytes per milliliter. Analysis was performed on 20-μl samples of heparinized blood in the first hour following cardiac blood puncture.

Bacterial blood cultures were extracted for the 20 % group 24 h after CLP. The samples were collected by cardiac puncture. Blood was serially diluted and cultured on a tryptic soy blood agar plate at 37 °C for 48 h ($n = 10$ mice).

Histological analysis

A macroscopic examination was performed to look for abscess and pus collections in the peritoneal cavity at 24 h after surgery. Liver samples, preserved in 10 % buffered formalin, were dehydrated and embedded in paraffin. Four-micrometer sections were stained with hematoxylin-eosin. The sections were then evaluated for signs of hypoperfusion and ischemic hepatitis or "shock liver."

Mean arterial pressure measurement

Mean arterial pressure (MAP) was measured under anesthesia in the "20 %" ligated and control groups before surgery and at 24 h post-procedure. The femoral artery was catheterized. After surgery, a 5-min stabilization period was observed and femoral arterial blood pressure was monitored using a blood pressure analyzer (via a Statham P10 EZ transducer coupled to a TA 4000; Gould, Eichstetten, Germany) for 10 min. The published results are the mean of MAP values measured every 30 s.

Statistics

Values are not normally distributed and are expressed as median and range or interquartile range (IQR). To assess whether the measurements changed over time, Friedman's test was used. When Friedman's test was significant ($p < 0.05$), pair comparisons were performed using Wilcoxon's signed-rank test. Time comparison between groups was made using non-parametric Kruskal–Wallis test. When the Kruskal–Wallis test was significant ($p < 0.05$), then comparisons were made using

the Dunn's post hoc test. Survival was analyzed by log-rank test. Analysis was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, La Jolla, CA USA, www.graphpad.com. Results with $p < 0.05$ were considered statistically significant.

Results

Mortality and organ dysfunctions are correlated with ligation length

Median total cecal length was 29 mm (25–30 mm). For the ligated groups, measurements of ligated ceca were the following: 2 mm (1–2 mm) for the 5 % ligated group, 6 mm (5–7 mm) for the 20 % ligated group, and 21 mm (19–25 mm) for the 100 % ligated group. Mortality was evaluated at different ligation lengths of standardized CLP by simple puncture with a 20-G needle. Our results indicate that the ligation length influences mortality (Fig. 2). At the end of the 96-h follow-up period, we observed 100 % mortality in the 100 % ligated group, 88 % mortality in the 20 % ligated group, and 20 % mortality in the 5 % ligated group ($p < 0.001$). The median survival time was 24 h for the 100 % ligated group and 44 h for the 20 % group. The median survival time of the 5 % ligated group could not be determined because of the low number of deaths at the end of the observation period (80 % of survival). Because of death rapidity in the 100 % group, we macroscopically analyzed ceca 24 h post-procedure when the animals were put down for serum collection. The mice with cecum ligated at 100 % presented with ischemia of the ligated component in contrast to other groups (Fig. 3c). Animals in the 20 % group developed macroscopic cecal abscesses (Fig. 3a, b).

Serum creatinine and blood lactate also varied with ligation distance. Blood lactate increased proportionally with the length of ligated cecum (Fig. 4a). For the 5 % ligated group, it did not differ from the control group. For the 20 % and 100 % ligated groups, blood lactate increased up to 2.6 mmol/l (1.2–4.2) for the 20 % group and up to 3.2 mmol/l (1.2–5.7) for the 100 % group. At 6 h, serum creatinine increased by 1.5-

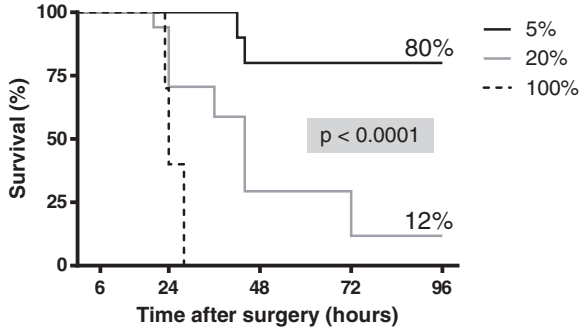

Fig. 2 Survival following ligation of 5 % ($n = 10$), 20 % ($n = 17$), and 100 % ($n = 10$) of total cecal length within 96 h after CLP procedure. At 24 h, survival was 100 % for the 5 % group, 70 % for 20 % group, and 40 % for 100 % group (log-rank test, $p < 0.05$). At 96 h, we observed 100 % mortality in the 100 % ligated group, 88 % mortality in the 20 % ligated group, and 20 % mortality in the 5 % ligated group (log-rank test, $p < 0.0001$). The median survival time was 24 h for the 100 % ligated group and 44 h for the 20 % group. The median survival of the 5 % ligated group could not be determined because of the low number of deaths at the end of the observation period (80 % of survival)

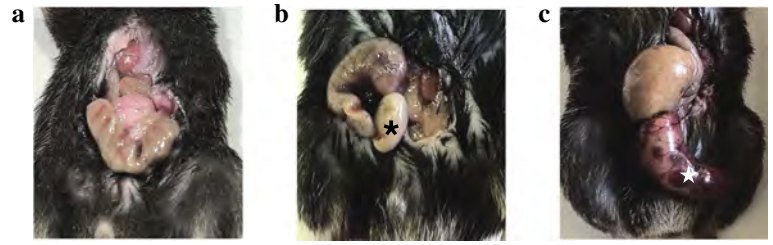

Fig. 3 Macroscopic aspects of ceca 24 h after CLP procedure. **a** Sham-operated mouse. **b** Mouse with 20 % of cecum ligated; we observed cecal abscess (✲). **c** Mouse with cecum ligated at 100 % presented an ischemia of the ligated part (★)

fold in the 100 % group compared to the control mice: 26.2 μmol/l (17.4–73.8) vs 15.2 μmol/l (6.6–23.9). In the 20 % group, serum creatinine was higher at 16.9 μmol/l (12.5–39) when compared to the control group (Fig. 4b).

Inflammatory status differs in accordance with ligation length

Inflammatory response to CLP length was evaluated by serum cytokines. TNF-α, IL-1β, and IL-6 concentrations were measured at 6 and 24 h for all the groups. We calculated an IL-6/IL-10 ratio at 24 h to determine the balance between pro- and anti-inflammatory responses. At 24 h post-procedure, only four animals in the 100 % group were still alive.

At 6 h after the procedure, pro-inflammatory cytokines were significantly increased in the ligated groups compared to the control group. TNF-α was increased in the 5 % group (212.4 pg/ml (56.7–313.7)), 20 % (187.6 pg/ml (105.6–317)), and 100 % group (190.9 pg/ml (112.1–317.1)) compared to the control group (136.7 pg/ml (50.3–181); $p < 0.05$). We did not observe any difference in amongst the ligated groups (Fig. 5a). At 24 h, TNF-α quickly decreased or animals subsequently died. There was no difference between the 100 % groups because of the few number of survivors at 24 h (less than five) (Fig. 5a).

IL-1β significantly increased at 6 h for the groups 5 % (218.8 pg/ml (26–320.8)) and 20 % (213.4 pg/ml (119–388.6)) compared to the control group (93.5 pg/ml (67.5–

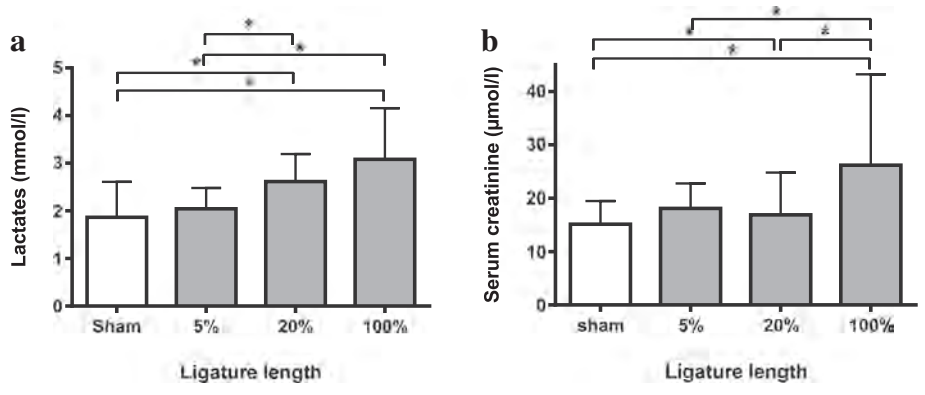

Fig. 4 Serum creatinine and blood lactate variations according to ligation distance at 6 h after procedure as markers of organ dysfunction. **a** Blood lactate increased proportionally with the length of the ligated cecum. **b** At 6 h, serum creatinine augmented significantly 1.5 times in the 100 % group compared to the sham mice. Results are reported as median ± IQR. *$p < 0.05$; $n = 14$ to 20 per group

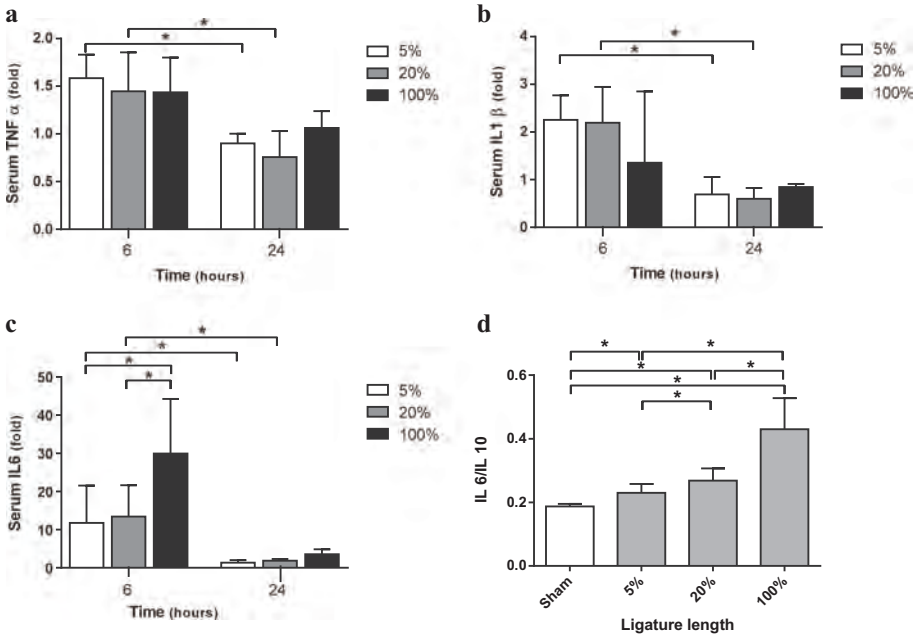

Fig. 5 Inflammatory response to CLP length evaluated by serum cytokines. TNF-α, IL-1β, and IL-6 concentrations were measured for all the groups at 6 h ($n = 10$ for sham-operated and each length of the ligated cecum) and 24 h ($n = 10$ for the sham-operated, 5 %, and 20 %; $n = 4$ for the 100 % ligated group). At 24 h after procedure, only 40 % of the animals in the 100 % group were alive. We expressed serum TNF-α, IL-1β, and IL-6 concentrations as fold increases relative to time-matched sham laparotomy (**a**–**c**). **a** Serum TNF-α concentrations. We did not observe any difference between the ligated groups at H6 or H24, but a decrease in time. **b** Serum IL-1β concentrations. We observed a decrease in time, but no difference between the ligated groups at the same time. **c** Serum IL-6 concentrations. Besides decrease in time, we observed significant higher levels of serum IL-6 at H6 in the 100 % compared to the 5 % and 20 % ligated groups. **d** Serum IL-6/IL-10 ratio. The longer the ligated cecum was, the more the imbalance was significantly in favor of pro-inflammatory response. Results are reported as median ± IQR; *$p < 0.05$

150.1); $p < 0.05$). On the other hand, IL-1β serum concentrations of the 100 % ligated group were not different from the control group (128.4 pg/ml (60.8–354.9)). IL-1β concentrations did not increase in accordance with the length of ligation at 6 h but decreased at 24 h nevertheless (Fig. 5b).

At 6 h, IL-6 serum concentrations of ligated groups were at least 15 times higher than in the control group (Fig. 5c; $p < 0.05$). Serum IL-6 concentrations reached 1916 pg/ml (961–4141) for the 20 % ligated group and were evidently increased for the 100 % group, with a median value of 4262 pg/ml (2070–7723). Like other pro-inflammatory cytokines, IL-6 concentrations decreased at 24 h (Fig. 5c).

At 24 h, when observing the pro- and anti-inflammatory balance (IL-6/IL-10), the longer the ligated cecum, the more significant the pro-inflammatory status was (Fig. 5d).

The "20 %" ligated group presented all characteristics of septic shock

With these results, we more closely monitored the 20 % ligated group. At 24 h after surgery, without any resuscitation the animals presented with a decreased MAP below 65 mmHg compared to the control mice (Fig. 6a).

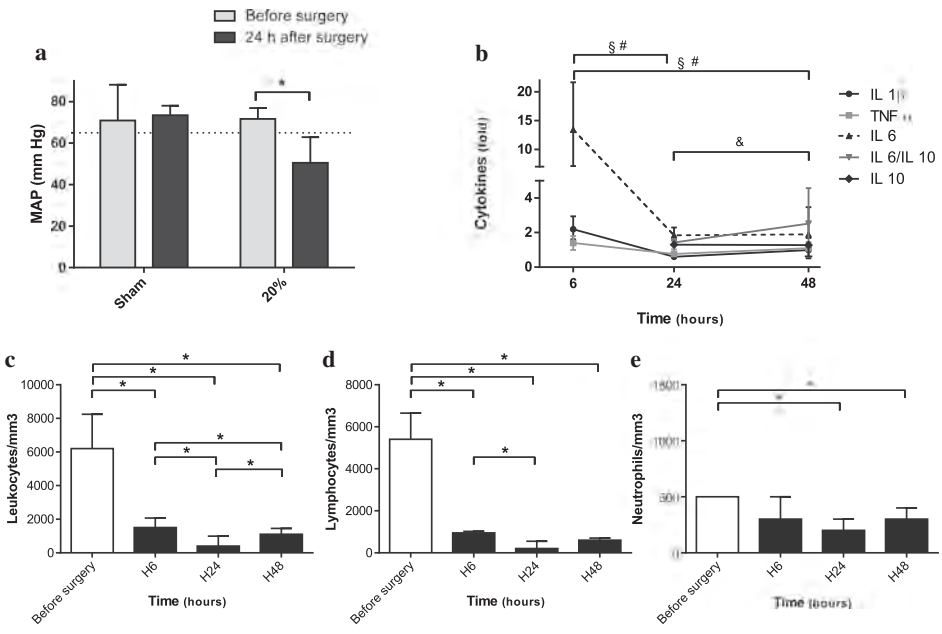

Fig. 6 Clinical and biological characteristics of the 20 % ligated group. Results are reported as median ± IQR. **a** Invasive mean arterial pressures (MAP) were measured in the 20 % ligated and sham groups before surgery and at 24 h post-procedure. At 24 h after surgery, the animals presented a decreased MAP below 65 mmHg compared to sham mice. *$p < 0.05$. **b** TNF-α, IL-1β, IL-6, IL-10, and IL-6/IL-10 ratio serum concentrations were measured at 6 h (H6), 24 h (H24), and 48 h (H48) after surgery. We expressed serum cytokine concentrations as fold increases relative to time-matched sham laparotomy ($n = 10$ for the sham-operated and 20 % group at H6, H24, and H48). We observed a significant imbalance in favor of pro-inflammatory status. $^{\S}p < 0.05$ for IL-1β between H6 and H24, and H6 and H48 after CLP. $^{\#}p < 0.05$ for TNF-α between H6 and H24 and H6 and H48 after CLP. &$p < 0.05$ for IL-6/IL-10 between H24 and H48. **c, d** Leukocyte (**c**), lymphocyte (**d**), and neutrophil (**e**) counts in the 20 % ligated group before surgery, at H6, H24, and H48. We observed a drop after surgery, more pronounced at H24, with an increase at 48 h. *$p < 0.05$

This hypotension was associated with sepsis in the 20 % ligated group. We observed cecal abscesses when compared to control mice (Fig. 7a, b), and blood cultures at 24-h post-procedure were positive with enteric bacteria such *Citrobacter braakii* and *Enterococcus faecalis* (40 %).

This was associated with a drop in leukocyte count due to leucopenia, which peaked at 24 h (Fig. 6c, d, e). At 48 h, leukocyte count increased but was diminished compared to time before surgery. The cytokine profile was pro-inflammatory, including status at 48 h. IL-6 levels remained high in survivors at 2.9 compared to those in the control and were not counterbalanced by IL-10 levels. At 48 h, IL-6/IL-10 ratio increased compared to 24 h after CLP, what highlights the persistence of inflammation (Fig. 6c).

When observing macroscopic liver morphology, we found a patchy appearance corresponding to pale ischemic areas in contrast to the control liver (Fig. 7c, d). These areas displayed centrilobular necrosis of hepatocytes (Fig. 7f).

Discussion

While the CLP model is the most widely used model for experimental sepsis, the length of cecal ligation is often inaccurately described. Our results underline that, in mice, length of cecal ligation is a major determinant of mortality and sepsis severity. Organ

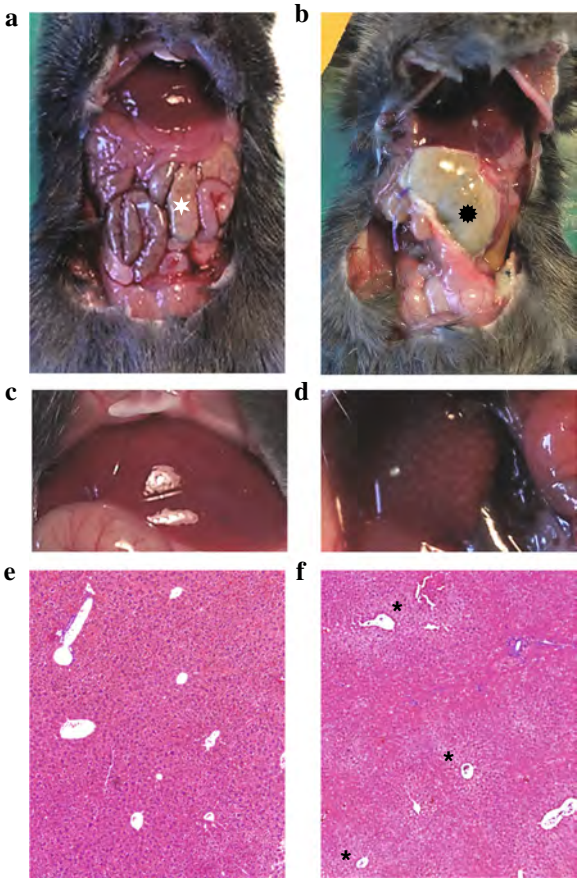

Fig. 7 Aspect of peritoneal cavity of the sham-operated mice (**a, c, e**) and the 20 % ligated mice (**b**, **d**, **f**) 24 h after surgery. **a** The cecum is identified by a *white star*. **b** The 20 % ligated animal with cecal abscess (✹). When observing liver macroscopic morphology, we found a patchy appearance corresponding to pale ischemic areas (**d**) in contrast to the sham liver (**c**). **f** These areas were centrilobular necrosis of hepatocytes (*asterisk*) (**e** and **f** hematoxylin-eosin coloration ×50)

dysfunction markers and pro-inflammatory status increased with ligation length. The "20 %" ligated group presented all characteristics of septic shock with a delayed mortality compared to the "100 %" group, allowing further studies as to the effect of different treatments or physiopathology. In the "20 %" group, we confirmed the presence of sepsis associated with hypotension below 65 mmHg, pro-inflammatory balance with high IL-6 levels and augmented IL-6/IL-10 ratio, organ dysfunction, hyperlactatemia, elevated serum creatinine, and hepatocyte centrilobular necrosis.

Length of ligated cecum influenced mortality in our mice model with high lethality in the 20 % and 100 % groups (at 4 days, 88 and 100 % mortality, respectively). As we found in our results, the percentage of cecum ligation is more accurate than a standard length (as length can vary from 25 to 30 mm). Rittirsch briefly described this influence in his mouse CLP procedure but did not characterize the model except for survival rate and for pro-inflammatory cytokines in his mid-grade sepsis group [10, 12]. Singleton showed the same influence more extensively but in a rat CLP model [15]. In his model, at 96 h a ligation length of 20 % allowed a 60 % survival rate whereas "25 %" of a ligated cecum caused higher mortality with only a 24 % survival rate. Our data confirm the importance of this variable in mouse CLP model. Singleton's results could not be

transposed a priori because of differences in anatomy and vascularization of these rodents' ceca. In fact, the same proportion of ligated cecum between these two breeds of animals does not exactly have same consequence in terms of lethality. Our study was not designed to determine which mechanisms were involved in this phenomenon. However, we suppose that mortality in the 100 % ligated group is related to intestinal ischemia rather than sepsis. We observed macroscopic cecal necrosis early after procedure. As discussed in Singleton's study concerning rats, the response amplitude to cecum ligated length may be due to the amount of feces stored in the ligated portion and thus create bacterial inoculum that may translocate or be locally pathogenic (e.g., by forming abscesses like in the 20 % group).

Inflammatory response mediated by cytokines plays a major role in sepsis evolution [16, 17]. Pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6 are responsible for severe manifestations in sepsis and septic shock [17, 18]. In our model, apart from IL-6 measurements, differences between groups for other cytokines are not clinically relevant when considering the same measurement time. Serum TNF-α levels peak at 120 min in mouse CLP models [19]. It is the first cytokine produced in response to aggression, and it promotes the activation of immune cells and the release of immunoregulatory mediators [20]. In our model, TNF-α was significantly increased at 6 h in ligated groups compared to control mice. However, serum TNF-α concentrations were not proportional to ligation length as described previously in rats [15]. We detected serum TNF-α in the control mice as well and did not find any differences between the ligated groups; this finding was opposite to Singleton et al.'s in rats [15]. These findings are in accordance with other studies observing severity in mice CLP models. Serum TNF-α levels did not differ between the more severe groups which were defined by the puncture size [14]. One explanation may be the difference in pro-inflammatory response influenced by genetic background. Serum TNF-α profiles over time are different after CLP between A/J and C57BL/6J mice; therefore, we infer it could be dissimilar between rats and mice [21]. Moreover, TNF-α neutralization does not improve survival in mouse CLP model [22–24]. This underlines that serum TNF-α levels are not necessarily linked to severity of insult in this model.

Serum IL-1β levels were higher in the "5 %" and "20 %" groups at 6 h compared to the levels at 24 h. We did not observe any difference between groups at the same time or between 6 and 24 h for the "100 %" ligated group. There is little data in the literature concerning IL-1β in mouse CLP modeling. Initial descriptions of the inflammatory profile in this mouse model did not detect serum IL-1β [8]. In the mouse CLP model, this cytokine seemed to be significantly elevated in the sera of animals that died before the fifth day post-procedure [25]. In a study evaluating needle size in CLP, only the group with the largest puncture presented with a significant elevation in serum Il-1β at 24 h [13]. In our case, the lack of difference between groups may be explained by the regulation of IL-1β secretion and the small number of surviving animals in the 100 % group at 24 h [26].

Concerning serum IL-6, our model is in accordance with previous results indicating that serum IL-6 levels increase proportionally with mortality at 6 h after mouse CLP [27, 28]. As described in the literature, the 20 % and 100 % groups, which presented with the highest mortality rates, had serum IL-6 concentrations near or superior to

2000 pg/ml. This breakpoint predicts mortality within 3 days with a specificity of 97 % and sensitivity of 58 % [27]. When observing the pro- and anti-inflammatory balances, the IL-6/IL-10 ratio was higher in groups with larger lengths of ligated cecum. This ratio has been shown to be predictive of the outcome of patients with systemic inflammatory response syndrome [29–31].

We use in our model low dose of buprenorphine (0.05 mg/kg every 12 h) to achieve analgesia as stated by the Office of Laboratory Animal Welfare [32]. Morphine is known to increase pro-inflammatory mediators; however, low dose of buprenorphine seems to have no effect on mortality and inflammatory response [33, 34]. Moreover, we gave this analgesic drug both to sham and CLP mice; hence, we believe that buprenorphine is not a cofounding factor in our model.

In our study, we focused on the 20 % ligatured group. This was done since we chose this group for further experimentation and wanted to ensure that the results were consistent. Furthermore, this group corresponds to a septic shock group. The animals of this group presented both clinical and biological elements of septic shock. They were hypotensive with a MAP below 65 mmHg. The cytokine profile was in favor of a pro-inflammatory imbalance with markers predictive of mortality, such as IL-6 and the IL-6/IL-10 ratio [27, 30]. At 48 h, IL-6/IL-10 ratio increased compared to that at 24 h after CLP, which shows the persistence of inflammation. Moreover, this group presented with a drop in leucocyte count secondary to lymphopenia, which decreased even further at 24 h. Other studies described this change in a complete blood count, with the same kinetics [8, 13, 35, 36]. The lymphopenia was secondary to sepsis-induced apoptosis and is correlated to the severity of an immunosuppressive phase and its late complications [37, 38].

We acknowledge that our study has potential limitations. First, we did not perform the same analysis in the "100 %" group as we did in the "20 %" group because of the high lethality rate. We chose animals with the same genetic background, age, and gender to limit experimental variability secondary to differences in inflammatory response and maturity of the immune system [11, 39]. Because of the mice's age, we did not have enough animals to compensate for the mortality of the 100 % ligated group. Furthermore, our study lacked evaluation of anti-inflammatory balance within the first hours post-procedure. As previous studies on mouse CLP modeling described late IL-10 serum elevation, we chose to measure levels at 24 and 48 h [13, 14]. We were not able to determine if the 100 % group had very early pro- and anti-inflammatory imbalance, which has been shown to be predictive of mortality [25]. Third, we are aware that we have not analyzed exhaustively the cytokine response to cecal ligation. For example, other cytokines such as IL-12 or interferon-γ play a central role in septic inflammatory response [40].

Conclusions

Our study suggests that the length of cecal ligation is a major severity factor in the mouse CLP model when needle size and the number of punctures are controlled. Furthermore, it underlines differences in the inflammatory response between rats and mice. Therefore, this experimental model should be performed with high consistency in future protocol designs. In order to accurately compare studies, ligature length used in protocols should be described.

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Authors' contributions
SR conceived and designed the experiments. SR, FV-B, VM-D, and MB performed the experiments. SR, VM-D, and JMC analyzed the data. SR, FV-B, VM, IT, and OF wrote the paper. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Ethics approval and consent to participate
Animal experimentation was performed according to national and institutional animal care and ethical guidelines and was approved by the local board.

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Contexte scientifique n°2

Après avoir mieux caractérisé notre modèle animal d'étude, nous avons souhaité étudier la cinétique de l'activation des plaquettes dans le sepsis après réalisation d'une ligature perforation caecale. L'objectif secondaire était d'étudier les propriétés thrombotiques des plaquettes à haut shear afin d'évaluer l'impact de l'inhibition de la PI3Kβ.

SCIENTIFIC REPORTS

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Platelet activation and prothrombotic properties in a mouse model of peritoneal sepsis

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Sepsis is associated with thrombocytopenia and microvascular thrombosis. Studies have described platelets implication in this pathology but their kinetics of activation and behavior remain poorly known. We show in a mouse model of peritonitis, the appearance of platelet-rich thrombi in organ microvessels and organ damage. Complementary methods are necessary to characterize platelet activation during sepsis as circulating soluble markers and platelet-monocyte aggregates revealed early platelet activation, while surface activation markers were detected at later stage. A microfluidic based *ex-vivo* thrombosis assay demonstrated that platelets from septic mice have a prothrombotic behavior at shear rate encountered in microvessels. Interestingly, we found that even though phosphoinositide-3-kinase β—deficient platelet mice formed less thrombi in liver microcirculation, peritoneal sepsis activates a platelet alternative pathway to compensate the otherwise mandatory role of this lipid-kinase to form stable thrombi at high shear rate. Platelets are rapidly activated during sepsis. Thrombocytopenia can be attributed in part to platelet-rich thrombi formation in capillaries and platelet-leukocytes interactions. Platelets from septic mice have a prothrombotic phenotype at a shear rate encountered in arterioles. Further studies are necessary to unravel molecular mechanisms leading to this prothrombotic state of platelets in order to guide the development of future treatments of polymicrobial sepsis.

Sepsis is a major cause of mortality and critical illness in the world[1,2] and is considered as a major public health concern whose incidence is increasing[3]. Severe sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection[4].

It is now well documented that the role of platelets includes an immune response function during the host response to infections[5,6]. Platelets are thought to play a major role in sepsis with thrombocytopenia being recognized as an independent risk factor for mortality of patients admitted to the intensive care units with severe sepsis or septic shock[7]. Platelets are anucleated circulating cells playing an essential role in hemostasis and thrombosis. They are highly reactive to extracellular stimuli through activation of a variety of specific membrane receptors for soluble agonists or adhesive proteins allowing platelet adhesion, activation, secretion and aggregation to form a plug, which, together with activation of the coagulation system, safeguards vessel integrity and prevent hemorrhage[8]. Under conditions of high shear, the initial recruitment of platelets to an injured vessel wall is mediated by the reversible interaction with von Willebrand factor (VWF) that binds to collagen fibers of the subendothelial matrix and the platelet receptor glycoprotein GPIb. This interaction allows platelets to establish contacts with collagen leading to their activation and the release of secondary mediators such as adenosine diphosphate (ADP) and thromboxane A_2 (TxA_2). These mediators, together with thrombin generated by the coagulation cascade, further activate platelets leading to their aggregation and the stabilization of the platelet-rich thrombus[9]. Sepsis is a multistep disease in which platelets are implicated through several mechanisms including recruitment of immune cells. This recruitment contributes to a hyper-inflammatory state[10] with subsequent development of

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microvascular occlusive syndromes and thus worsening multiple organ failure[11,12]. The procoagulant state in sepsis and formation of neutrophils extracellular DNA traps (NETs) and microthrombi as a defense strategy increase the risk of vaso-occlusive complications[13]. It is important to note that at certain stages of sepsis platelets have a protective role through tempering macrophage-dependent inflammation[14] and limiting clinical severity through the podoplanin-CLEC2 axis[15].

Several reports suggest that platelets may be a relevant therapeutic target in sepsis. *In vitro* studies have described bacterial-induced platelet activation processes and identified different mechanisms of interactions between platelets and bacteria[16–19]. Evidence is accumulating that inhibition of platelet function can modulate inflammatory markers[20]. Drugs inhibiting platelet activation, such as acetylsalicylic acid (ASA) or P2Y12 inhibitors, may have a benefit in reducing thrombo-inflammation, arterial microthrombi and in turn multiple organ failure in critically ill septic patients. Furthermore, new targets for antithrombotic therapy have been proposed such as Class IA phosphoinositide 3-kinase (PI3K) β isoform that participates in the regulation of a range of functional platelet responses, including sustained activation of $\alpha_{IIb}\beta_3$ integrin. It has been shown that *in vivo*, isoform-selective PI3Kβ inhibitors prevent occlusive thrombus formation but do not prolong the bleeding time[21,22]. Such inhibitor could be of interest in the treatment of septic patients. However, it is essential to get further insights on the time course of platelet activation during sepsis, on the consequences of sepsis on platelet prothrombotic properties at arterial shear rate and to evaluate the impact of platelet PI3Kβ inhibition. Only a few studies have investigated platelet activation during sepsis in humans[13,23–26] or in experimental animal models[27,28]. Recently, a study of platelet activation dynamics during the progression of a *streptococcus pyogenes* infection has proposed that monitoring platelet activation may provide prognostic information in this type of sepsis[29].

The aim of the present work was to characterize platelet activation during the development of a cecal ligation and puncture (CLP) model of polymicrobial peritoneal sepsis in mice. Furthermore, we studied platelet prothrombotic properties at high shear rate encountered in microvessels and evaluated the impact of platelet PI3Kβ inhibition.

Results

Characterization of sepsis and platelet parameters over time in the CLP mice model. To analyze sepsis development and progression in our mouse model of peritonitis a series of biological parameters were measured at different time points after CLP (Fig. 1). There was significant weight loss in the CLP group (sham −4.1 [−6.7–3.0] % versus CLP −15.9 [−17.2–−11.9] %) accompanied by an overall mortality of 47% three days after surgery (Fig. 1a,b). The plasma levels of IL-1β, TNFα and IL-6 were significantly increased in the CLP group after 6 hours (Suppl Fig. 1) indicating a pro-inflammatory stage rapidly generated following CLP. A marked elevation of liver transaminases AST and ALT was measured in the CLP group (sham AST 30.0 [25.8–33.8] IU/L versus CLP AST 58.0 [48.5–107.5] IU/L, n = 6, $p = 0.005$ and sham ALT 15.5 [10–20] IU/L versus CLP ALT 27.5 [20.3–34.8] IU/L, n = 6, $p = 0.019$) indicating liver cytolysis (Fig. 1c,d). Lactate dehydrogenase (LDH) was also significantly increased in the CLP group (Fig. 1e). There was no difference in serum creatinine level measured between the 2 groups (Suppl Fig. 1d). Analysis of these selected biochemical parameters indicated organ cytolysis 48 hours after CLP.

There was a significant leukocyte count decrease in the CLP group after 8 hours that persisted until 48 hours of sepsis (5.8 [4.7–6.2] 10^9/L in sham group versus 1.05 [0.8–1.23] 10^9/L in CLP group, (n = 30), $p < 0.0001$) (Fig. 1f). Lymphocyte and monocyte counts were particularly low 48 hours after the surgery while neutrophil count declined significantly 4 hours after CLP (Suppl Fig. 2a–c).

Histological assessment of lung sections revealed significant inflammatory infiltrates as demonstrated by interalveolar thickening, interstitial edema and a significantly elevated mean acute lung injury score 48 hours after CLP (Fig. 1g,h). Neutrophil infiltration in both lung and liver was increased in the CLP group (Suppl Fig. 3a).

As shown in Fig. 2A, we observed a progressive platelet count decrease in the CLP group that became significant after 24 hours (sham 780 [657–913] 10^9/L versus CLP 431 [380–515] 10^9/L, n = 15, $p < 0.0001$) and persisted two days post CLP (sham 864 [785–1016] 10^9/L versus CLP 519 [393–622] 10^9/L, n = 30, $p < 0.0001$). A significant increase in mean platelet volume was also observed at 48 hours suggesting occurrence of platelet renewal (sham 5.5 [5.0–7.0] fl versus CLP 6.9 [6.2–7.3] fl, n = 30, $p = 0.001$) (Fig. 2b). Interestingly, the circulating platelet count decrease in the CLP group correlated with the appearance of platelet-rich thrombi in tissue microvessels as shown by immunohistopathology (Fig. 2c). Thrombi could be observed in the heart (upper panels), in periportal capillaries (middle panels), and in pulmonary microvessels (lower panels) of the CLP group of mice while no thrombus formation could be seen in the sham group (Fig. 2c).

Of note, identification of bacteria from blood cultures obtained 48 hours after CLP indicated the presence of *enterococcus faecalis*, and of *citrobacter braakii* (Suppl Fig. 3b).

Kinetics of platelet activation during sepsis. To investigate the level and kinetics of platelet activation during sepsis we analyzed platelet intrinsic markers including membrane exposure of CD62P and fluorescently labeled fibrinogen binding to activated GpIIbIIIa ($\alpha_{IIb}\beta 3$), formation of heterotypic aggregates between platelets and leukocytes and soluble markers in plasma such as soluble CD40L (sCD40L) and eicosanoids.

P-selectin (CD62P) exposure at the platelet surface assessed by flow cytometry at intervals following CLP indicated a significant platelet activation 24 hours after CLP that persisted at 48 hours (Fig. 3a). Consistent with these results, fibrinogen fixation on activated GpIIbIIIa was significantly enhanced 24 hours after CLP and further increased at 48 hours (Fig. 3b). However, activated platelets may be rapidly removed from the circulation and/or conjugated to leukocytes[30]. As shown in Fig. 3c, as soon as 4 hours monocyte-platelet interactions significantly increased with a maximal increase 24 hours after surgery. In spite of an important decline of circulating monocytes (Suppl Fig. 2b), platelet-monocyte aggregates were still significantly elevated 48 hours after CPL. Figure 3d is a representative confocal image showing that several platelets interacted with monocytes 24 hours after CLP.

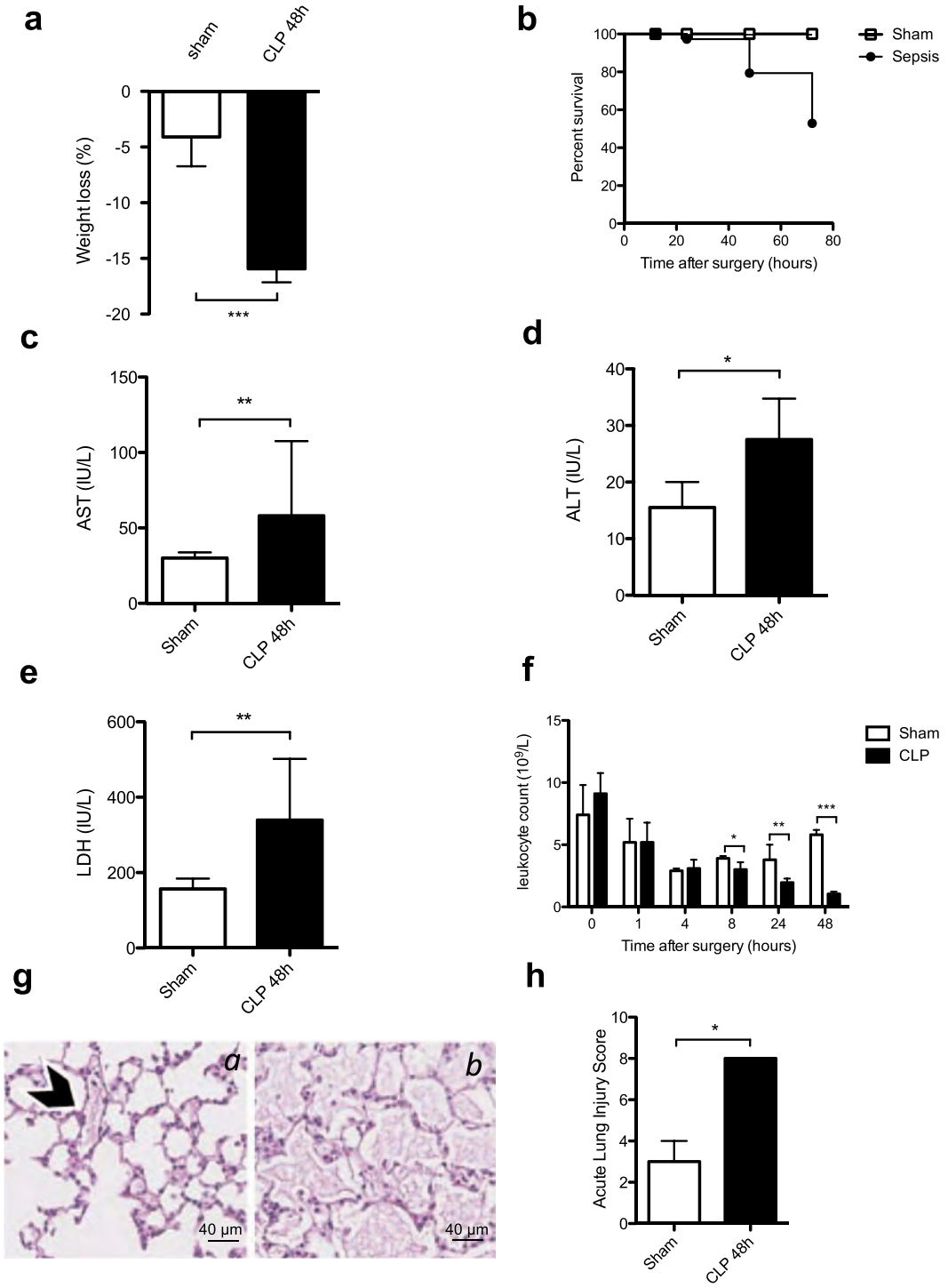

Figure 1. Characterization of sepsis after cecal ligation and puncture. (**a**) Weight loss was increased 48 hours post procedure in the CLP group of mice (black bar) compared to the sham group (white bar). Results are expressed as percentage of weight loss and are median [25–75th percentiles] (n = 14, ***$p < 0.001$). (**b**) Survival was quantified at 72 hours post CLP. At 72 h, the overall mortality was 47% in CLP group. Results are expressed as percentage of survival (n = 36, $p < 0.05$). Biochemical analysis were performed with a PENTRA 400 ABXc analyzer for aspartate aminotransferase (AST) (**c**), alanine aminotransferase (ALT) (**d**) and lactate dehydrogenase (LDH) (**e**). Results are presented as median [25–75th percentiles] (n = 6, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **(f)** Leukocyte count was measured 48 h post surgery and compared in sham versus CLP group. Results are expressed as median [25–75th percentiles] (n = 6 to 30 *$p < 0.05$,**$p > 0.001$, ***$p < 0.0001$). **(g)** Representative images of lung sections stained with hematoxylin and eosin 48 h post surgery. The arrowhead shows a blood vessel section which integrity is conserved in a sham-operated mouse (**a**). In the CLP group of mice (*b*) important alveolar injuries are observed as quantified by the Acute Lung Injury (ALI) Score 48 h post CLP induction (**h**). Results are median ± IQR of 7 independent experiments (*$p < 0.05$) and representative images are shown (**g**).

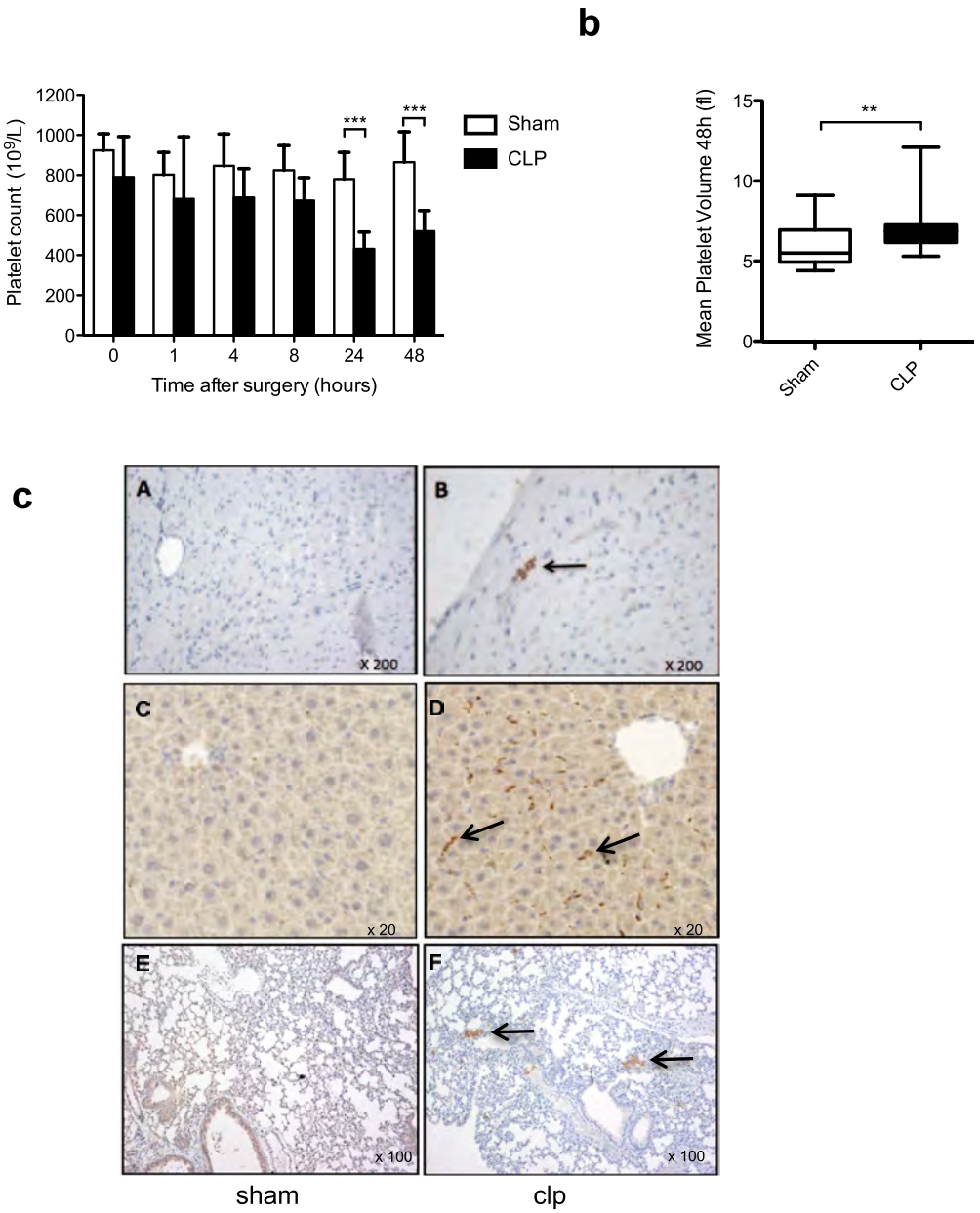

Figure 2. Sepsis promotes thrombocytopenia and thrombus formation in lung capillaries after cecal ligation and puncture. (**a**) Whole blood platelet count kinetics at 48 h post CLP surgery. Results are expressed as platelets x 10^9/L and are median ± IQR of 30 independent experiments (**$p < 0.01$, ***$p < 0.001$). **(b)** Comparison of Mean Platelet Volume (MPV) 48 h post CLP surgery. Whisker boxes are constructed as follow: min, max, median, 25–75th percentiles (n = 30, **$p < 0.01$). (**c**) Representative histological sections of heart (A,B), liver (C,D) and lung (E,F) tissues 48 h post surgery. Sections from CLP (B,D,F) or sham-operated animals (A,C,E) as controls were stained with Masson's trichrome and platelets were specifically labeled with an anti-αIIb antibody. Arrows highlight platelet-rich thrombi in microvessels. Images (x20, x100 and x200 magnification) shown are representative of 3 independent experiments.

Interestingly, at that time point, the density of platelets per monocyte, estimated by median fluorescence intensity of the platelet marker CD41[31], was strongly increased following sepsis compared to Sham operated mice (Fig. 3d). Of note, the formation kinetics of neutrophil-platelet aggregates formation were different. Indeed, these heterotypic aggregates were observed later in the sepsis and were significantly increased 2 days after surgery with an increase in the density of platelets per neutrophil compared to Sham mice (Fig. 3e, right panel).

Analysis of soluble markers of platelet activation, such as plasmatic sCD40L and eicosanoids, provides the opportunity to detect low grade circulating platelet activation or platelet activation even if activated platelets are no longer circulating (i.e. bound to the endothelium) or in complex with leukocytes[32]. CLP significantly increased plasma levels of sCD40L as soon as 4 hours (1.75 [1.48–1.99] fold increase $n = 3$, $p = 0.04$) and 8 hours (2.53 [1.66–3.27] fold increase, $n = 4$, $p = 0.006$) after surgery (Fig. 4a). This increase was no longer significant 2 days after CLP.

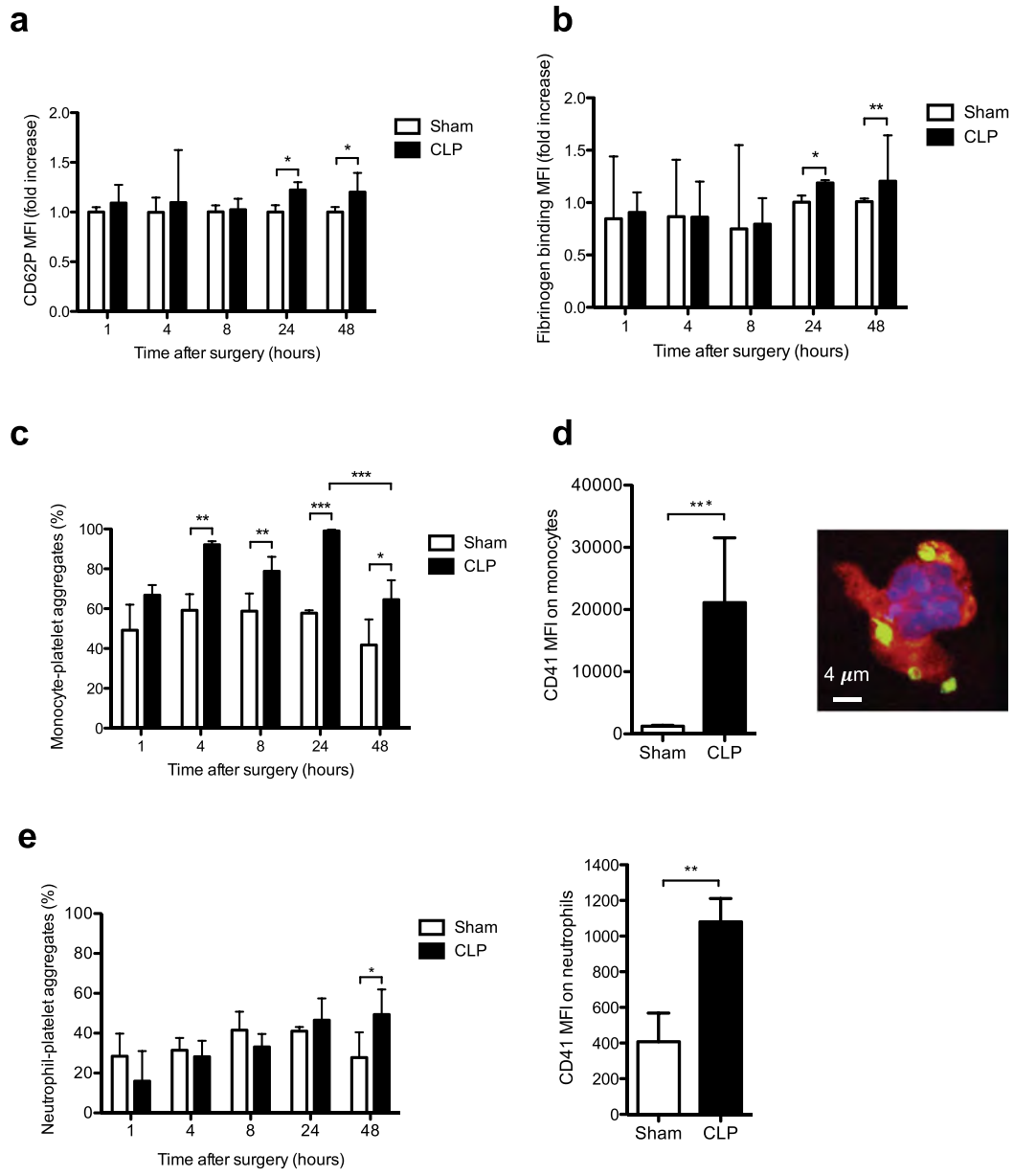

Figure 3. Expression of surface platelet activation markers and elevation of leukocyte-platelets interactions during sepsis. (**a**) Expression of the surface platelet activation marker CD62P analyzed by flow cytometry during sepsis. (**b**) Activation of $\alpha_{IIb}\beta_3$ (GpIIbIIIa) integrin at the platelet surface assessed by oregon green fibrinogen binding and flow cytometry analysis. Results are expressed as median fluorescence intensity and are median fold increase ± IQR of 6 to 8 independent experiments (*$p < 0.05$, **$p < 0.01$). (**c**) Whole blood monocyte-platelet aggregates quantified at different times post surgery in sham and CLP-operated mice. Results are expressed as percentage of monocyte-platelet aggregates and are median ± IQR of 4 to 6 independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (**d**) Density of platelets per monocytes. The MFI values of the platelet marker (CD41) on monocytes was measured 24 h after CLP by flow cytometry to evaluate the platelet density per monocyte (left panel). After sorting by flow cytometry the platelet-monocyte aggregates were spin down onto poly-lysine coated slides and observed by confocal microscopy (right panel). A representative confocal image is show to illustrate the interaction of platelets (CD41, green) and monocyte (CD115, red) 24 h post CLP. The monocyte nucleus was labeled with DAPI (blue). (**e**) Whole blood neutrophil-platelet aggregates quantified at different times after surgery in sham and CLP-operated mice. Results are expressed as percentage of neutrophil-platelet aggregates and are median ± IQR of 3 to 7 independent experiments (*$p < 0.05$) (left panel). The MFI values of the platelet marker (CD41) on neutrophils was measured 48 h after CLP to evaluate the platelet density per neutrophil (right panel).

Eicosanoids are locally acting bioactive signalling lipids derived from arachidonic acid and related polyunsaturated fatty acids that regulate a diverse set of homeostatic and inflammatory processes[33]. After activation, platelets produce eicosanoids via cyclooxygenase and lipooxygenase pathways, particularly thromboxane A2

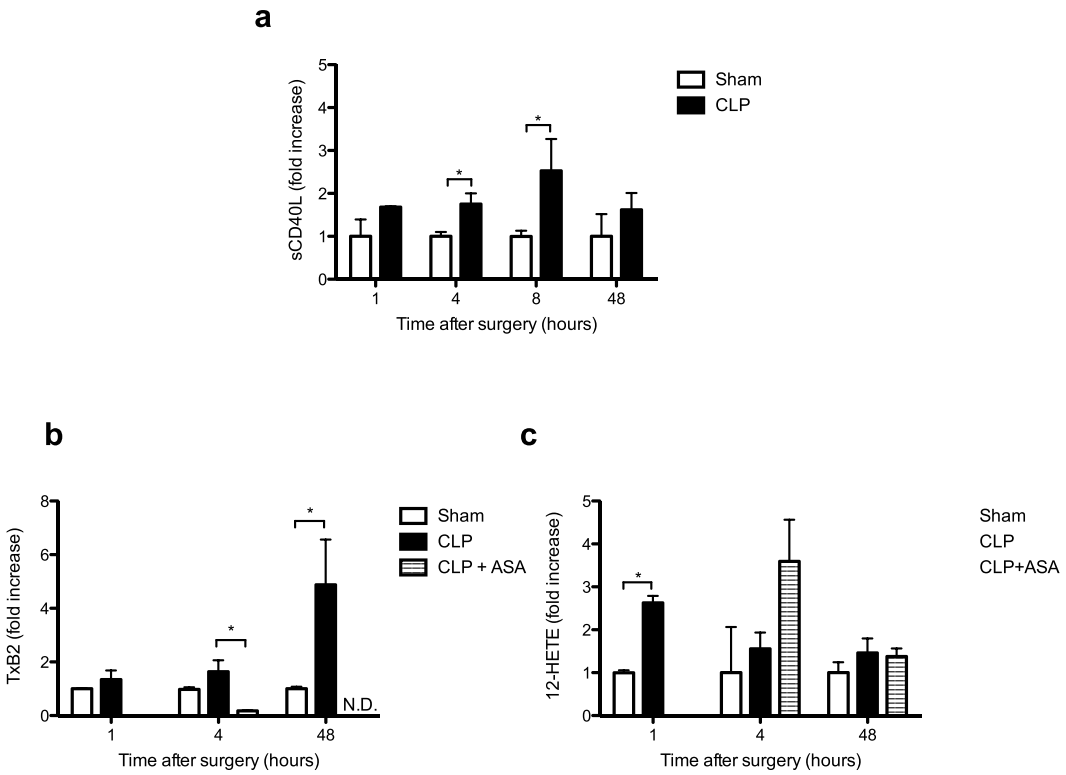

Figure 4. Early elevation of soluble markers of platelet activation during sepsis. (**a**) Levels of plasma soluble CD40L (sCD40L) and eicosanoids at different times in sham and CLP mice. Results are expressed as fold increase and are median (25–75th percentile) of 4 to 7 independent experiments (*$p < 0.05$). (**b**) Kinetics of TxB2, the stable metabolite of TxA2, and (**c**) 12-HETE production in plasma of sham, CLP-operated mice and CLP-operated mice treated with aspirin. The quantification was performed by a lipidomics LC-MS/MS technique. Results are expressed as fold increase and are median (25–75th percentile) of 3 to 6 independent experiments (*$p < 0.05$, **$p < 0.01$). N.D., not detectable.

(TxA2) and 12-hydroeicosatetraeinoic acid (12-HETE). Thromboxane B2 (TxB2), the stable metabolite of TxA2, increased 4 hours after CLP to become significantly more abundant after 48 hours in the CLP group (Fig. 4b). As a control, inhibition of cyclooxygenase by aspirin treatment of mice fully inhibited TxB2 production following CLP. The lipoxygenase product 12-HETE was also rapidly produced, measured 1 hour after CLP its plasma concentration was significantly elevated compared to the sham group of mice (Fig. 4c). In this case, as expected, inhibition of cyclooxygenase by aspirin did not affect the production of this lipoxygenase product (Fig. 4c).

Overall, these data show that there is early platelet activation in sepsis which can be detected by quantification of monocyte-platelets aggregates in whole blood and soluble markers in plasma (sCD40L and eicosanoids).

Sepsis induces a prothrombotic platelet state under flow and brings out an alternative mechanism enabling platelets to form stable thrombus at high shear rate in the absence of PI3Kβ. To further investigate the effect of sepsis on platelets we studied thrombus formation under flow conditions encountered in microcirculation. Interestingly, despite a decreased platelet count at 48 hours, the CLP group exhibited a significantly faster adhesion and formation of platelet thrombus compared to the sham group of mice. However, after forty seconds of flow, the surface coverage was not significantly different in the two groups (Fig. 5a). Sepsis thus induced a pre-activation stage of platelets allowing them to very rapidly interact with the collagen surface at a shear rate of 1500 s^{-1} to form a growing thrombus.

Class I phosphoinositide 3-kinase β (PI3Kβ) is known to play an important role in platelet activation and has been proposed as a potential antithrombotic target[21,22,34]. To evaluate its role during sepsis we took advantage of our mouse model presenting an invalidation of the catalytic subunit of PI3Kβ (p110β) specifically in platelets[35]. As shown in Fig. 5b, following CLP, the number of thrombi in periportal zone of the liver was significantly decreased in the absence of PI3Kβ in platelets. However, this decrease had no effect on weight loss or the mean acute lung injury score 48 hours after CLP (Suppl Fig. 4). Deficiency of PI3Kβ in platelets is known to induce an instability in arterial thrombus at high shear rate[35]. Therefore, we performed CLP in wild type and p110β-deficient platelet mice and analyzed their capacity to form stable thrombi at high shear rate *ex-vivo*. Blood from both groups of mice was first perfused on collagen at 1500 s^{-1} for 150 seconds to form comparable thrombi and then an acceleration of the blood flow was generated to reach a high shear rate of 4000 s^{-1}. In the wild type sham or CLP groups the thrombi formed at 1500 s^{-1} were stable and continued to grow at 4000 s^{-1} (Fig. 5c–e). As expected, in the absence of p110β, platelet thrombi rapidly destabilized at high shear rate, leaving a single platelet layer on the collagen surface with some small aggregates

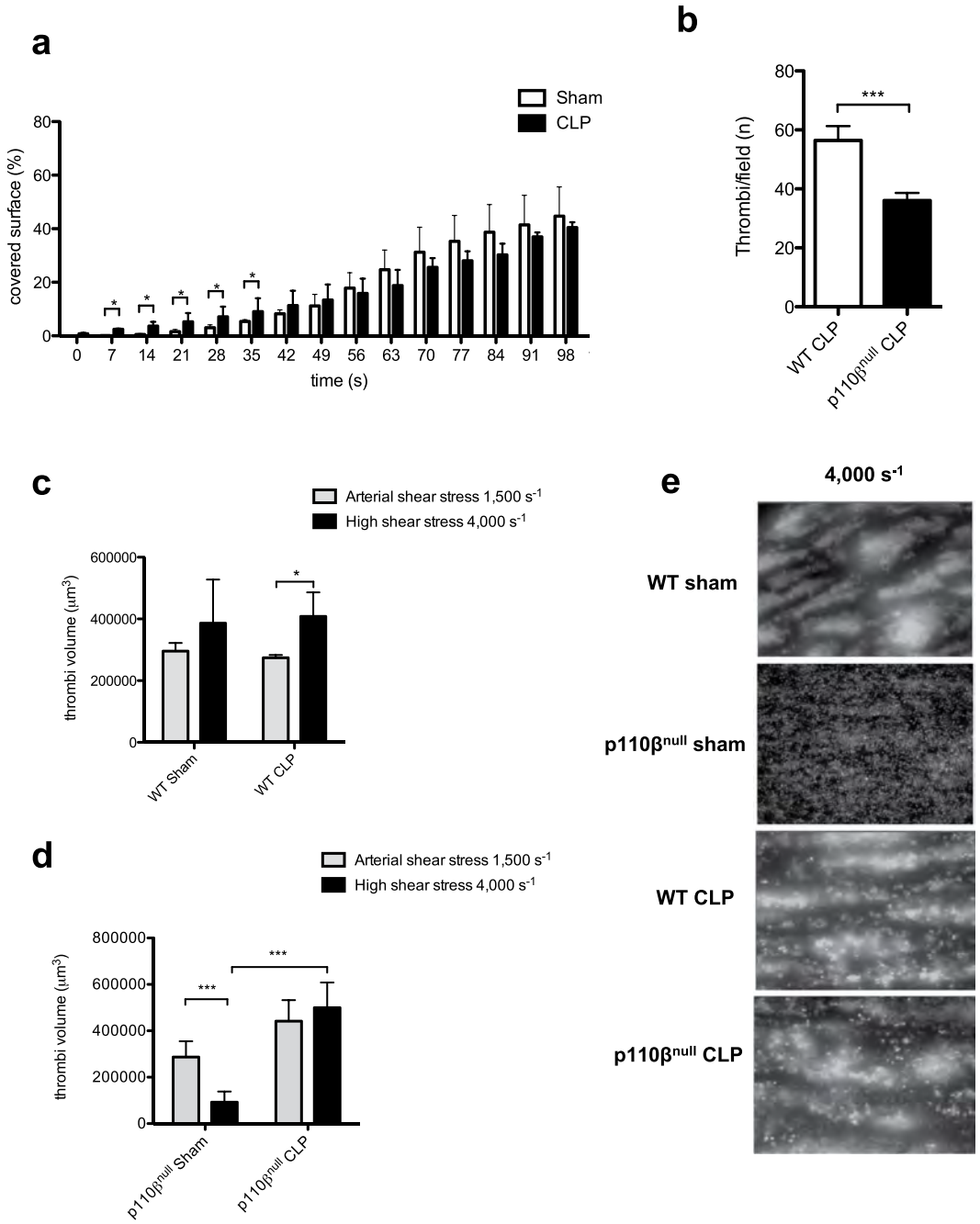

Figure 5. Platelet pro-thrombotic properties at arterial flow and bypass of PI3Kβ for thrombus stability at high shear rate during sepsis. (**a**) DIOC6-labeled platelets in whole blood from the CLP (black bar) or sham (white bar) mice 48 h post intervention were perfused through a collagen-coated microcapillary at a physiological arterial rate of 1500 s^{-1}. Surface coverage (%) by fluorescent platelets was analyzed using ImageJ software. Results shown are median ± IQR of 4 independent experiments (*$p < 0.05$). (**b**) Platelet-rich thrombi formed in the liver 48 h post CLP were detected as in Fig. 2 (C-D) and quantified. 5 mice from each group and 5 to 10 field per mice were analyzed. Results are expressed as median ± IQR (***$p < 0.001$). (**c**–**e**) DIOC6-labeled platelets in whole blood from platelet PI3Kβ-deficient mice (p110β^{null}) or wild type mice (WT) were perfused through a collagen-coated microcapillary at a physiological arterial shear rate of 1500 s^{-1}, followed by a high shear rate of 4000 s^{-1}. Thrombi volumes (μm^3) were analyzed using ImageJ software. Results are expressed as median ± IQR of 4 to 6 independent experiments (***$p < 0.001$). (**e**) Representative images showing the platelet thrombi remaining after 1 min of high shear rate (4000 s^{-1}).

(Fig. 5d,e). Strikingly, septic conditions reversed the thrombus instability at high shear rate in p110β-deficient platelet mice. Indeed, following CLP, platelet thrombi were stable at 4000 s^{-1} indicating that sepsis allowed platelets to overcome the defect of thrombus stability induced by PI3Kβ invalidation at high shear rate (Fig. 5d,e).

Discussion

Sepsis is a complex disease which can rapidly evolve to septic shock and subsequent organ failure in the absence of required early and aggressive management generally needed to stop the progression to septic shock and subsequent organ failure. Platelets are probably among the first responding cells during sepsis development and their roles and influences are likely different during sepsis progression. They can have protective roles by tempering macrophage-dependent inflammation, regulatory functions by maintaining inflammation or negative roles by precipitating microvessels dysfunction and in turn multiple organ failure. Here we analyzed the kinetics and characteristics of platelet activation and their behavior during sepsis in a mouse model of peritoneal sepsis[36]. CLP in rodents has become the most widely used model for experimental sepsis and considered a standard in sepsis research[37]. The survival rates found in our study, the severity of sepsis and multiple organ failure were comparable to those reported in the literature[36–38]. Lung histology revealed inflammatory acute lung injury as also reported in experimental polymicrobial peritonitis[36]. Thrombocytopenia was present significantly one day after CLP and persisted at 48 hours. Thrombocytopenia is common in Intensive Care Unit (ICU) patients and a decline in platelet count, even without thrombocytopenia, adds significant prognostic information to the current parameters used in the current ICU scoring system[39,40]. Several studies have reported that failure of the platelet count recovery to normal level during critical illness is associated with a higher mortality[41,42].

The causes of thrombocytopenia in sepsis are still unclear but platelet consumption following activation and aggregation or adhesion to the endothelium and/or leukocytes likely contribute. Indeed, 48 hours after CLP we observed platelet-rich thrombi in the microcirculation of different organs including liver, kidney and lung. This appearance of platelet-rich thrombi correlated with organ failure and likely contributed to ischemic complications.

To study the kinetics of platelet activation during sepsis we analyzed different parameters including intrinsic and soluble platelet markers as well as formation of heterotypic platelet leukocytes aggregates. CD62P (P-selectin) was significantly increased at 24 hours post onset of sepsis. Consistent with this a significant increase in $\alpha_{IIb}\beta_3$ activation assessed by fibrinogen binding on the platelet surface was observed 24 hours after CLP and persisted at 48 hours. In septic patients, Gawaz *et al.*[24] also observed increased $\alpha_{IIb}\beta_3$ activation on the platelet surface compared to controls. However, since activated platelets may rapidly be eliminated from circulation, measurement of intrinsic circulating platelet activation markers may not be sensitive enough to detect platelet activation, particularly in the first hours of sepsis[24]. Indeed, in patients with septic shock it has been suggested that the most active cells with high adhesion potential might be sequestered from circulation and thus escape detection[30,43]. In this context, we quantified soluble platelet markers and analyzed circulating heterotypic aggregates between platelets and leukocytes as valuable indicators of platelet activation during sepsis.

The formation of platelet-leukocyte aggregates is now recognized as a sensitive marker of *in vivo* platelet activation and is a feature of inflammatory conditions[30,44,45]. These aggregates are mediated via multiple ligands and receptors including P-selectin which is translocated to the surface of activated platelets[44]. We found an increase in monocyte-platelet aggregates formation, as early as 4 hours after CLP that persisted until 48 hours despite a drop in circulating monocyte count. The density of platelet per monocyte was also strongly increased. In contrast, the level of circulating neutrophil-platelet aggregates showed no significant differences until the first 24 hours but significantly increased 48 hours after CLP. An increase of platelet-leukocyte adhesion has been reported in patients with sepsis, and these heterotypic interactions were shown to decrease when patients developed multiple organ failure[24]. The exact mechanisms, regulations and clinical significance of such platelet-leukocyte interaction in sepsis are still poorly understood[46]. In our sepsis model the rapid increase in platelet-monocyte aggregates suggests early activation of a set of platelets in circulation. Consistent with this, the plasma level of sCD40L significantly increased 4 hours after CLP. These levels remained significantly high 8 hours after CLP but decreased 48 hours later. CD40L shedding from platelets following CLP has been shown to involve the matrix metalloprotease-9[47]. sCD40L contributes to the regulation of neutrophil recruitment and lung damage in sepsis. Higher sCD40L levels were found in 49 patients with meningococcal sepsis and 15 patients with African tick bite fever compared to controls[48,49]. In septic patients, a multicenter prospective study has shown that circulating sCD40L levels were significantly higher in septic patients than in controls, and in non-survivors compared to survivors[50].

Activated platelets are known to generate several bioactive lipids including eicosanoids acting as regulators of a diverse set of homeostatic and inflammatory processes[33]. We found that circulating levels of the cyclo-oxygenase product TxB2 (the stable metabolite of TxA2) were significantly elevated in the early phase of sepsis and remained high 48 hours after CLP. As expected, treatment of mice with the cyclo-oxygenase inhibitor aspirin abolished this production. The lipo-oxygenase product 12-HETE was also significantly elevated 1 hour after CLP and was not affected by aspirin treatment. Overall, these data show that CLP-mediated experimental peritonitis in mice promotes a rapid activation of platelets detectable after 1 to 4 hours by measuring circulating soluble markers of platelet activation and monocyte-platelet aggregates. Activation markers at the surface of circulating platelets such as P-selectin expression and $\alpha_{IIb}\beta_3$ activation are only significantly detectable 24 to 48 hours after CLP, when thrombocytopenia is already well established. Therefore, these results show that it is important to investigate both soluble and intrinsic platelet markers of activation to determinate the onset of platelet activation during sepsis.

Besides markers of platelet activation, another important question concerns the prothrombotic feature of platelets during sepsis, particularly at a shear rate encountered in microvessels. The dynamics of platelet thrombus formation and stabilization under flow is yet poorly studied in septic conditions. Using videomicroscopy analysis we observed that septic conditions significantly accelerated platelet adhesion and thrombus growth at a shear rate found in microvessels. Of note, this was observed with blood from mice 48 hours after CLP, a stage where a significant thrombocytopenia is present. This data reveals a prothrombotic behavior of platelets at arterial flow conditions during sepsis. Importantly, the thrombus formed were stable, even at very high shear rates. We and others have previously shown that PI3Kβ is mandatory for platelet activation and thrombus stability in both human and mouse models at high shear rate. PI3Kβ inhibitors have thus been proposed as potential antithrombotic drugs[21]. Therefore, we checked whether such inhibitors could be relevant to treat septic patients to prevent ischemic events

arising from thrombosis in microcirculation, where the shear rate is elevated. Using p110β-null platelet mice, we found that absence of PI3Kβ significantly decreased the number of thrombi formed in the liver following CLP but was not sufficient to impair weight loss and lung injury. This decreased number of thrombi suggests that PI3Kβ contributed to the processes of platelet activation during sepsis. However, once formed these thrombi appeared stable at high shear rate even in the absence of PI3Kβ. In fact, sepsis restored the ability of platelets to form a stable thrombus at high shear rate in the absence of platelet PI3Kβ, indicating that septic conditions allow platelets to compensate for PI3Kβ deficiency. Thus, despite a decrease in the number of thrombi formed in the periportal zone of the liver, our results suggest that PI3Kβ inhibitors may not be sufficient to efficiently treat septic patients. How sepsis can modify platelets to allow them to form a stable thrombus at high shear rate in the absence of PI3Kβ remains to be established. It is noteworthy that the GpIb-VWF axis is exacerbated and important in sepsis[51–53] and may contribute to compensate the lack of PI3Kβ in thrombus stabilization at high shear rates.

Conclusions

Our results indicate that platelets are rapidly activated in the CLP model of peritonitis and that soluble and surface expression markers as well as monocyte-platelet aggregates should be quantified to determine platelet activation during sepsis. Thrombocytopenia can be attributed in part to platelet-rich thrombi formation in capillaries and platelet-leukocytes interactions. Platelets from septic mice have a prothrombotic phenotype at a shear rate encountered in arterioles. We propose that sepsis activates an alternative mechanism enabling platelets to bypass the normally mandatory role of PI3Kβ to form stable thrombus at high shear rates. Further studies are now necessary to unravel the molecular mechanisms leading to this prothrombotic state of platelets at high shear rate as this may unravel new risk markers and guide the development of future treatments of polymicrobial sepsis.

Methods

Animals. All animal procedures were in accordance with the guidelines of the Midi-Pyrénées Ethics Committee on Animal Experimentation (Comité National de Réflexion Ethique sur l'Expérimentation Animale – Midi-Pyrénées) and with the French Ministry of Agriculture license. This study was approved by the Midi-Pyrénées Ethics Committee on Animal Experimentation (N°MP/02/39/05/12, date 2012/10/02).

Male C57BL/6 J mice were obtained from Janvier Labs (Saint-Berthevin, Mayenne, France). PF4-cre/p110β$^{flox/flox}$ mice were generated by crossing a mouse line in which exons 21 and 22 of the kinase domain of p110β are flanked by loxP sites (p110β flox/flox) with transgenic animals expressing the Cre recombinase specifically in megacaryocytes under the control of the PF4 promoter (PF4-Cre/p110wt/wt). PF4-Cre/p110β flox/wt mice were then crossed with p110β flox/flox mice to produce platelet-specific p110β-null mice PF4-Cre/110β flox/flox. They were obtained in a mendelian ratio, and were healthy, with no growth abnormalities. These animals exhibited normal size and platelet count[35,54]. We used 20-week-old animals, weighing 25–30 g. Mice had access to food and water *ad libitum* and were not fasted prior to CLP.

Experimental protocol design of sepsis. Polymicrobial sepsis was induced by a CLP procedure as previously described[37,38]. Briefly, a laparotomy was performed under general anaesthesia, with the cecum ligated at 20% of its total length, below the ileocecal valve, and was punctured once with a 20-gauge needle. The cecum was then returned into the peritoneal cavity. Sham mice underwent the laparotomy without ligation and puncture. When indicated, mice were treated with aspirin (10 µg/g, intraperitoneal injection). Spontaneous mortality was followed four days after the surgery.

Systemic platelet and leukocyte counts. Blood samples were collected by puncture of the lower vena cava utilizing heparinized syringe (0.1 ml heparin (100 IU/ml) for 0.9 ml of blood). Blood cells counts were performed on MICROS-60 analyzer (ABX-Diagnostics, Baden, Switzerland).

Bacteriology. One milliliter of blood from sham and CLP group was cultured in Petri dishes with sheep blood and incubated for 48 hours at 37 °C in an aerobic atmosphere. Bacterial colonies identification was conducted using VITEK® automated system (BioMerieux, USA).

Biochemical diagnostic assays. Serum was obtained after centrifugation (2800 *g*, 10 min) and immediately frozen and stored at −80 °C. Dosages were performed with PENTRA 400 ABX© (Horiba Medical ®) biochemical analyzer for serum creatinine, lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

Flow cytometry. In order to analyze surface molecules expression (CD62P and $\alpha_{IIb}\beta_3$) on circulating platelets, blood was collected (1:10 acid citrate dextrose (ACD: 3% trisodium citrate 5.5-hydrate, 1.4% citric acid, 2% anhydrous glucose)) at different time after surgery. Platelet Rich Plasma (PRP) was incubated (10 min, RT) with an anti-CD62P FITC-conjugated monoclonal antibody (BD Pharmigen™) to analyze CD62P expression. PRP was also incubated with fluorescent fibrinogen (Fibrinogen Oregon green® 488, Invitrogen™) permitting analysis of the changes in affinity of αIIbβ3 for fibrinogen. Platelet-leukocyte interactions were studied using blood samples (collected in 1:10 ACD), fixed prior to analysis with CELL-FIX© for 20 min and washed in 2 ml PBS. After centrifugation (300 *g*, 4 min), the pellet was resuspended and incubated with anti-CD41 FITC-conjugated monoclonal antibody (BD Pharmingen™), PE-conjugated anti-Ly-6G Gr-1 monoclonal antibody, PE-conjugated anti-CD115 monoclonal antibody (eBioscience) or with the corresponding isotypes. ACK buffer (0.14 M ammonium chloride, 0.017 M Tris HCl, pH 7.4) was used for red blood cells lysis.

Samples were analyzed by flow cytometry using FACS-Verse™ (BD Biosciences) and the FACS Suite™ software. The platelet density per monocyte or neutrophil was evaluated by measuring the median fluorescence intensity (MFI) of the platelet marker (CD41) on monocytes or neutrophils as previously reported by Rutten *et al.*[31].

ELISA. Platelet poor plasma (PPP) was frozen in liquid nitrogen and stored at −80 °C before analysis. Soluble CD40 ligand (sCD40L) was measured in plasma with mouse sCD40L platinium ELISA kit (Affymetric eBioscience™).

Histology. Lungs were excised and formalin-fixed for 24 hours. Then, they were immersed in 70% ethanol solution. Four randomly selected sites from each inflation-fixed lung were embedded in paraffin, and sections were cut for hematoxylin and eosin staining. Lung injury severity was quantified in a blinded manner by adoption of a semi-quantitative scoring system, using the Acute Lung Injury (ALI) score taking into account 4 variables (leukocyte infiltration, fibrin/alveolar edema, alveolar wall thickness, intra-alveolar haemorrhage). The severity of each variable was quantified from 0 to 4 (absence, minimal: 1 to 3 alveoli, light > 3 alveoli, moderate: 2/3 of the surface area, severe: all the alveoli)[55]. Maximum score was 16. Leukocyte infiltration was studied by immunohistochemistry using the Ly6B.2 antibody (AbD Serotec™ Bio-Rad Compagny).

Plasma eicosanoids measurement by high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). PPP samples were stored at −80 °C until lipid extraction. LC-MS/MS was performed as detailed elsewhere[56] using HPLC grade methanol, methyl formate and acetonitrile (Sigma-Aldrich). Briefly, lipid preparation from all samples was carried out through solid-phase extraction using hydrophobic polystyrene-divinylbenzene resin in dedicated 96-well plates (Chromabond multi96 HR-X 50 mg; Macherez-Nagel). After complete loading, columns were washed twice with H_2O/MeOH (90/10, v/v) and dried under aspiration for 15 min. Samples were dried using nitrogen, dissolved again in methanol (10 μL) and transferred to liquid chromatography before LC-MS/MS analysis.

Flow assays on collagen matrix. Biochips microcapillaries (Vena8Fluro +, Cellix) were coated with a collagen fibril suspension (50 μg/ml) and incubated at 37 °C for one hour prior to being saturated with a solution of 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) without Ca^{2+}/Mg^{2+}. Mouse blood was drawn into heparin (10 IU/mL), and DIOC6 (2 μM) was used to label platelets. Using a syringe pump (Legato 200, KDScientifics) to apply a negative pressure, labeled blood was then perfused through a microcapillary for indicated time at a wall shear rate of 1500 $seconds^{-1}$, and, when indicated, formed thrombi were then exposed to a high shear rate of 4000 $seconds^{-1}$ as described[35]. Platelet adhesion and thrombus formation was visualized with a x40 oil immersion objective for both fluorescent and transmitted light microscopy; light source was provided by Colibri LED System (Zeiss) and was recorded (high resolution CCD cooled camera, Orca-R2, Hamamatsu) in real time (1 frame every 5 seconds). Image sequences of the time-lapse recording and analysis of surface coverage were performed offline on a single frame by quantification of pixel surface after manual thresholding using ImageJ. Thrombi volumes are calculated by thresholding of surface covered by thrombi on slice of Z-stack images and addition of voxel (automatically converted into μm^3 by ZenZeiss software).

Statistical analysis. Values are not normally distributed and are expressed as median and interquartile range (IQR). To compare differences between groups, the Mann-Whitney test was used. Analysis was performed using GraphPad Prism (version 5.0a for Mac). P value < 0.05 was considered significant and n represents the number of animals pooled together from each experiment.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. All data generated or analyzed during this study are included in this published article and its supplementary information file.

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Author Contributions

F.V.B., A.J., V.M., M.-P.G., M.M., C.G., J.S. designed and performed most experiments and analyzed data; H.H.-C., P.S., V.M. and B.P. designed experiments and analyzed data, F.V.B. and B.P. wrote the article.

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Contexte scientifique n°3

Le sepsis est caractérisé par une physiopathologie complexe faisant intervenir les plaquettes comme acteurs de la réponse inflammatoire et de la formation de microthromboses. Leur activation est précoce et se traduit par l'expression de marqueurs spécifiques, à la fois membranaires, mais aussi circulants. Leur activation est aussi médiée par leur capacité à interagir avec d'autres cellules telles que les cellules endothéliales mais aussi les leucocytes au cours du sepsis.

Si des études sur l'activation plaquettaire dans des modèles animaux de sepsis existent, les études chez l'Homme, et particulièrement en choc septique, sont rares depuis la définition de 2016. Russwurm S et *al*. a montré qu'il existait une corrélation positive entre l'expression de P-sélectine (CD62P) à la surface des plaquettes et la sévérité des patients septiques (Russwurm et al. 2002), mais cette étude est ancienne (année 2002). La P-sélectine soluble (sCD62P) a été proposée comme biomarqueur dans le syndrome de réponse inflammatoire systémique (SIRS), dont le sepsis, mais le SIRS n'est pas spécifique au sepsis (Schrijver et al. 2017). La présence d'agrégats monocytes-plaquettes était associée à une augmentation de la mortalité des patients âgés de plus de 65 ans dans l'étude de Rondina et al. mais l'étude n'a pas analysé d'autres marqueurs d'activation plaquettaire (Rondina et al. 2015). Il a également été montré que les plaquettes de patients septiques présentaient une diminution de leur capacité d'agrégation en réponse à différents agonistes en comparaison à celles de patients non septiques (Adamzik et al. 2012) sans que des marqueurs spécifiques d'activation plaquettaire ne soient décrits dans cette étude.

Ainsi, après avoir caractérisé dans un modèle murin l'activation des plaquettes et leurs propriétés pro-thrombotiques, nous avons souhaité décrire l'activation plaquettaire dans les premières 72h d'un choc septique chez les patients hospitalisés en réanimation. C'est l'objet de l'étude PASS (Platelet Activation in Septic Shock) qui a reçu un soutien institutionnel au CHU de Toulouse au travers de l'attribution d'une AOL.

EVOLUTION OF PLATELET ACTIVATION PARAMETERS DURING SEPTIC SHOCK IN INTENSIVE CARE UNIT

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Running head: Kinetics of platelet activation in septic shock

ABSTRACT

During severe sepsis, platelet activation may induce disseminate microvascular thrombosis, which play a key role in critical organ failure. Crucially, most of the studies in this field have explored platelet-leukocyte interactions in animal models, or explored platelets under the spectrum of thrombocytopenia or disseminated intravascular coagulation and have not taken into account the complex interplay that might exist between platelets and leukocytes during human septic shock nor the kinetics of platelet activation.

Here, we assessed platelet activation parameters at the admission of patients with sepsis to the intensive care unit (ICU) and 48 hours later. Twenty-two patients were enrolled in the study, thirteen (59.1%) of whom were thrombocytopenic. The control group was composed of twelve infection-free patients admitted during the study period.

The activation parameters studied included platelet-leukocyte interactions, assessed by flow cytometry in whole blood, as well as membrane surface and soluble platelet activation markers measured by flow cytometry and dedicated ELISA kits. We also investigated platelet aggregation and secretion responses of patients with sepsis following stimulation, compared to controls. At admission, the level of circulating monocyte-platelet and neutrophil-platelet heterotypic aggregates was significantly higher in sepsis patients compared to controls and returned to a level comparable to controls or even below 48 hours later. Basal levels of CD62P and CD63 platelet membrane exposure at admission and 48 hours later were low and similar to controls. In contrast, plasma level of soluble GPVI and soluble CD40 ligand was significantly increased in septic patients, at the two times of analysis, reflecting previous platelet activation. Platelet aggregation and secretion responses induced by specific agonists were significantly decreased in septic conditions, particularly 48 hours after admission.

Hence, we have observed for the first time that critically ill septic patients compared to controls have both an early and durable platelet activation while their circulating platelets are less responsive to different agonists.

Key words: platelets – septic shock – platelet activation – platelet-leukocyte aggregates

INTRODUCTION

Sepsis has been defined as "life-threatening organ dysfunction caused by a dysregulated host response to infection"[1]. Sepsis is a major contributor to the global burden of diseases, further aggravated by the general development of antimicrobial resistance. Septic shock constitutes a subset of sepsis in which both circulatory and cellular metabolism abnormalities occur. Sepsis-related morbi-mortality is linked to multiple organ failure (MOF) development that is partly due to microvascular thrombosis and endothelial dysfunction. Thrombosis develops in micro-vessels such as arterioles, capillaries (sinusoids) and venules, impairs oxygen delivery to cells[2], causing organs dysoxia, ischemia and eventually MOF. Converging evidence support the idea that platelets play an important role in innate immune responses, including immunothrombosis[3,4]. For example, thrombocytopenia is the most common hemostatic disorder in the intensive care unit (ICU) with a prevalence of around 50%[5]. Thrombocytopenia is well recognized as a poor prognostic marker[6-10]. It is worth noting that mechanisms underpinning sepsis-related thrombocytopenia are multifactorial[11] and still under debate. The recruitment of platelets to neutrophil extracellular traps (NETs) and their interaction with leukocytes and endothelial cells contribute to thrombocytopenia[12]. Platelets play a complex role in sepsis; they are able to modulate their own function but also that of cells around them including innate immune cells[13]. Neutrophils and monocytes are the first line of innate immune defense against infection. Platelet-leukocyte interaction engages P-selectin receptor and P-selectin Glycoprotein Ligand-1 (PSGL-1) on neutrophil and monocyte[14]. Platelet-leukocyte interaction via P-selectin is a crucial step in the activation and recruitment of leukocytes to the lung in acute lung injury (ALI) [15]. Importantly, by interacting with neutrophils, platelets enhance the formation of NETs contributing to the antimicrobial reaction[13]. Moreover, platelet activation contributes to microvascular thrombosis and organ failure in systemic inflammation, particularly in septic conditions. It is well known that during

severe sepsis, platelet activation may induce disseminate microvascular thrombosis, which play a key role in critical organ failure[16,17]. However, the mechanisms that support platelet activation in this setting are poorly understood[18,19]. Crucially, most of the studies in this field have explored platelet-leukocyte interaction in animal models[20,21], or explored platelets under the spectrum of thrombocytopenia or DIC[22] and have not taken into account the complex interplay that might exist between platelets and leukocytes during human septic shock nor the kinetics of platelet activation.

Aiming to pave the way for preclinical and clinical studies, it seems important to further characterize platelet activation at different times of septic shock in patients. In this context, our study aimed to investigate a series of platelet activation markers and parameters in septic shock in adult patients at ICU admission and 48 hours later. This should improve our understanding of the role of platelets in the pathophysiology of septic shock and could help to establish new therapeutic strategies to reduce MOF.

METHODS

Study patients

This was a single-center, prospective, 5-month study (February-June 2016) comparing patients suffering from septic shock with control subjects. Inclusion criteria were adult patients (≥18-year-old) with septic shock according to the Third International Consensus Definitions for Sepsis and Septic shock (Sepsis-3)[1], admitted in a tertiary ICU for septic shock, less than 3 days at the time of inclusion, with SOFA (Sequential Organ Failure Assessment) score > 8. Inclusion criteria for control subjects were adult patients (≥18-year-old) free from infection included preoperatively before cholecystectomy or hip/knee replacement, or patients admitted in continuing care units for anything other than an infection. Exclusion criteria were a malignant blood disease, constitutional hemostatic disorder (thrombocytopenia, thrombopathia), in case of pregnancy or if they were adults under tutorship or guardianship. The study protocol complies with the Declaration of Helsinki and was approved by the Toulouse University Hospital Human Research and Ethics Committee (n° IDRCB 2015-A01680-49, 04/02/2016). Written consent was obtained from all the patients in this study.

Characteristics at ICU admission and data collection

Upon admission to ICU, the following baseline characteristics were recorded: gender, age, body mass index, previous treatment by statins, antiplatelet agent or anticoagulant. The use of antibiotics, vasopressors, support technique and site of infection were collected. SOFA score and SAPS II (Simplified Acute Physiology Score) were computed. Biological variables were collected from each enrolled patient at admission. Mortality was defined as death occurring

within 28 days after admission. We also collected patient's ICU and hospital mortality rates. In the control group, venous blood tests were collected only at the inclusion.

Sample collection

Venous blood was collected at the inclusion for controls and both at the inclusion (H0) and 48 hours later (H48) for septic patients. For hematological data, whole blood was drawn on EDTA Vacutainer tubes (Becton Dickinson), inverted to ensure adequate mixing and transported at room temperature to the laboratory within 30 minutes. Hemoglobin, platelet count, mean platelet volume (MPV) and leukocytes count were assessed. For biochemical data, blood from septic group was drawn in Vacutainer heparin tubes and several parameters were analyzed including lactic acid, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), serum creatinine, troponin and total bilirubin, at 2 different time points (inclusion (H0) and 48 hours after inclusion (H48)) (Department of biochemistry, Rangueil Academic Hospital, Toulouse, France).

Platelet activation tests

Flow cytometry analysis

Whole blood was carefully drawn on citrate sodium (0.109 M) Vacutainer tubes (Becton Dickinson), inverted to ensure adequate mixing and transported at room temperature to the laboratory within 30 minutes. Flow cytometry was performed with FACSVERSE Analyzer and FACSUITE software for analysis (BD Biosciences). The flow cytometer was calibrated daily and cleaned carefully before each sample acquisition. All antibodies were obtained from BD Biosciences. Staining was performed immediately after blood sampling.

For platelet-leukocyte interaction measurement, 100 µL of citrated whole blood were immediately fixed with 500 µL of CELL-FIX© 1X solution (CELLFIX Biosciences ref 340181). After 15 minutes of incubation at room temperature (RT), fixed whole blood was diluted with 2 mL of phosphate buffer saline (PBS) and centrifuged at 190 g for 10 minutes at RT. The pellet was resuspended in 300 µL of PBS and incubated with anti-CD61 (platelets) PE-conjugated antibodies (CD61 PE BD Biosciences ref 555754), anti-CD14 (monocytes) alexafluor 488-conjugated antibodies (CD14 AF488 BD Biosciences ref 562689) and anti-CD66b (neutrophils) PerCP-CY5.5-conjugated antibodies (CD66b PercpCY5.5 BD Biosciences ref 562254) or with the corresponding isotypes (Isotype antibody AF488 BD Biosciences ref 558716, isotype antibody PE BD Biosciences ref 400114, isotype antibody PercpCY5.5 BD Biosciences ref 558304 and isotype antibody FITC BD Biosciences ref 555748) for 30 minutes in the dark at room temperature. After a washing step, erythrocytes were lysed by addition of a 3 ml ACK lysing buffer (0.15 M NH_4Cl; 1mM $KHCO_3$; 0.1mM Na_2EDTA pH 7.3). After 30 min at RT, samples were centrifuged (190 g, 10 minutes) and the pellet suspended in 500 µl PBS for FACS analysis.

For platelet α- and δ-granule secretion, citrated whole blood was centrifuged (190 g, 10 min, RT) to obtain platelet-rich plasma (PRP). Ninety µL of PRP were stimulated with agonists (10 µg/mL CRP, 25 µM TRAP, 5 µM U46619) (10 min, 37°C) and 25 µl were stained with conjugated primary antibodies (anti-CD62P FITC-conjugated antibody, BD Biosciences ref 555523, anti-CD63 FITC-conjugated antibody BD Biosciences ref 557288; 5 µg/ml) for 30 minutes at RT. Samples were then diluted with 1 ml of PBS and kept in the dark until analysis by flow cytometry. Results are expressed as median fluorescence intensity (MFI).

Measurement of sGPVI and sCD40L in plasma samples

Poor platelet plasma (PPP) from control and septic groups were isolated from citrated blood by 2 steps of centrifugation (2800 *g* for 10 minutes). Collected PPP was conserved at -80°C until analysis. For the analysis, samples were thawed and sGPVI levels were measured by sandwich enzyme-linked immunosorbent assay (ELISA kit, MyBioSource, San Diego, CA, USA) and sCD40L was determined by human sCD40L Platinum ELISA kit (eBioscience™, Fisher Scientific, Strasbourg, France), according to the manufacturer's instructions.

Platelet aggregation

Blood sample collected in citrated tubes was centrifuged (190 g for 10 minutes, RT) to obtain platelet-rich plasma (PRP). Platelet aggregation responses in PRP to adenosine diphosphate (ADP; ref A2754 Sigma Aldrich), collagen-related peptide (CRP; 10 µg/ml, from Pr Richard Farndale laboratory, Cambridge, UK), stable analogue of thromboxane A2 (U46619; 5 µM; ref D8174 Sigma Aldrich) or thrombin-receptor-activating peptide (TRAP; 25 µM; ref S7152 Sigma Aldrich) were assessed by turbidimetry (1000 rev/min, 37°C, TA-V8 aggregometer, SD Medical) during 10 minutes and results are expressed as percentage of maximal platelet aggregation.

Statistical analysis

Variables distribution was verified with Shapiro–Wilk test and after the first step of descriptive analysis, the population was separated in two groups (controls and sepsis). Characteristics of the patients were compared using non-parametric tests as appropriate (*t*test or Mann–Whitney for continuous variables and χ^2 or Fisher's exact test for categorical

variables). Then, data are presented as medians and extremes values and categorical data are expressed as numbers and percentages.

$P<0.05$ was considered statistically significant.

SPSS Statistics 20.0 software (Chicago, Il, USA) was used for the analysis and GraphPad Prism (La Jolla, CA, USA) was used to realize the figures.

RESULTS

Clinical characteristics

Patients admitted to the ICU for septic shock during the study period were analyzed for inclusion (Figure 1) and twenty-two patients met inclusion criteria. Table 1 summarizes the main clinical and physiological characteristics of the two groups at study entry. Clinical and biological characteristics of septic patients at inclusion (H0) and 48 hours after (H48) are summarized in Table 2.

At inclusion, 15 (68.2%) patients were mechanically ventilated and 2 patients were placed on extra renal therapy the same day (one on continuous extra-renal therapy and one on intermittent hemodialysis). Median duration of mechanical ventilation and CRRT were 9 [0-30] and 0 [1-10] days, respectively. Patients received a median vascular filling volume of 1900 [1250-4500] ml during the first 24 hours and a median dose of 0.58 [0.18-1.30] µg/kg/min of norepinephrine during their initial management. The median duration of norepinephrine use was 3.5 [0-17] days and median lengths of stay in ICU and in hospital were 15.5 [1- 60] and 23.5 [1-74] days, respectively. Patients in the control group were comparable in age and BMI but were more likely to be female (Table 1). The control patients were included before planned cholecystectomy (n=5), before hip/knee replacement (n=5) or after admission in continuing care units for mild trauma without hemorrhage. Long-term treatments were comparable between the two groups. Septic patients had more anemia and hyperleukocytosis compared to patients in the control group.

Impact of sepsis on platelet count and activation

Thirteen patients in 22 (59.1%) were thrombocytopenic (platelet count < 150G/L) of whom 11 (84.6%) already upon admission and only one patient (8.3%) in the control group. Relative platelet reductions were calculated from the initial platelet counts on day 0 (at admission) to

the minimum value on day 1 and on day 2. On day one, the relative platelet decrease was of -17.4% [IQ -29.4 to 5.9] (n=21 platelet count) and on day two, the relative platelet decrease was of -28.4 [IQ -29.4 to 5.9] (n=19 platelet count). During the first 4 days, the percentage of platelet count decline is negative for 75% of the values (the 75° percentiles are below the red line (i.e. 0% reduction) except for day 1) (Supplemental Figure 1) "

We compared platelet activation in the control group and in the sepsis group at H0 and H48 by analyzing platelet-leukocyte interactions as well as platelet membrane and soluble activation markers.

Platelet leukocyte interactions

As shown Figure 2, we observed a significant increase in circulating platelet-leukocyte aggregates, particularly in monocyte-platelet aggregates in septic patients 67.4% [45.4-87.6] at H0 *versus* 45.3% [29.6-54.5] in controls (p=0.0049) (Figure 2A) while the number of circulating monocytes was comparable in the two groups (Table 1). The increase in neutrophil-platelet aggregates was more modest, reaching 24.83% [19.0-42.2] at H0 in septic patients *versus* 18.1% [14.6-22.9] in controls (p=0.003) (figure 2C). Interestingly, after 48 hours these interactions returned to a level comparable to controls, 29.7% [24.8-52.7] for monocyte-platelet aggregates (Figure 2A) and 10.8% [6.7-22.7] for neutrophil-platelet aggregates (Figure 2C). Of note, the reference range of the laboratory for circulating platelet-leukocyte aggregates obtained from healthy blood donors were clearly below the values measured in the sepsis and the control groups (Figure 2A and C). The density of platelets per monocyte, assessed by median fluorescence intensity of the platelet marker CD61, was significantly increased in sepsis patients at H0 compared to control and decreased after 48 hours (Figure 2B). This was different for neutrophils since the density of platelets per neutrophil did not increase in the sepsis group and was even significantly lower than in the

control group at H0 and H48 (Figure 2D).

Membrane and soluble platelet activation markers:

The basal level of platelet membrane exposure of CD62P (P-selectin), a marker of α-granule secretion, and CD63, a marker of dense granule secretion was low, both in the control group and in the sepsis group (H0 or H48) (Figure 3A and 3B). Soluble markers of platelet activation are surrogate of a platelet activation that already happened in patients. As shown Figure 4A, the plasma level of a reliable soluble biomarker of platelet activation, soluble GPVI (sGPVI) shed from the platelet membrane, significantly increased in the sepsis group (H0: 256.5 [155.3-324.4] ng/ml and H48: 253.7 [90.2-336] ng/ml) compared to the control group (48.9 [36.6-71.6] ng/ml, $p<0.0001$ and $p=0.0001$, respectively). The shedding of this collagen receptor was detected at admission and the plasma level of sGPVI was still significantly elevated 48 hours later. Consistent with these results, another plasmatic biomarker of platelet activation, the soluble CD40 ligand (sCD40L), was also increased in the septic group compared to the control group (3.05 [2.1-5.5] ng/ml for sepsis H0, 3.3 [1.9-6.1] ng/ml for sepsis H48 and 1.6 [1.3-2.2] ng/ml for control, $p=0.0027$ and $p=0.0127$, respectively) (Figure 4B).

In vitro platelet aggregation and secretion responses:

We then investigated platelet aggregation response in platelet rich plasma following stimulation by adenosine diphosphate (ADP), collagen related peptide (CRP), the stable analogue of thromboxane A2 (U46619) and the thrombin receptor agonist peptide (TRAP) (Figure 5A-D). The aggregation responses were in the normal range in the control group but significantly reduced in the sepsis group, particularly 48 hours after admission, except for CRP-induced platelet aggregation (Figure 5). This decrease in platelet aggregation in response to the high concentrations of agonists used in these experiments suggests a desensitization

mechanism.

We also assessed platelet secretion response by measuring membrane exposure of CD62P (α granules) and CD63 (dense granules) following activation by CRP and U46619 (Figures 3A and B). Following stimulation by U46619, both α and dense granules were significantly secreted in the control and the sepsis groups. However, as shown in Figure 3A, α granule secretion decreased significantly in platelets from sepsis at H48 compared to controls after U46619 stimulation. Following stimulation by CRP, the secretion of α granules tended to decrease (Figure 3A). After stimulation by U46619, CD62P median fluorescent intensity in the control group was 214 (±28.7) a.u. *versus* 89.5 (±15.1) in the sepsis group at H48 (p=0.0011) (Figure 3A). In the same way, dense granule secretion was significantly decreased following stimulation by CRP or U46619 in the sepsis group (both at H0 and H48) compared to control (Figure 3B).

DISCUSSION

We observed that septic shock induced an early and durable elevation of platelet activation markers as shown by the significant increase in soluble markers such as sGPVI and sCD40L at admission and 24 hours later. This increase in soluble markers of platelet activation is probably due to the shedding of platelet surface receptors and externalized proteins during platelet activation, leading to the release of soluble ectodomain fragments detectable in plasma[16]. Platelet activation was also identified by an increase in heterotypic monocyte-platelet aggregates and, to a lesser extent, neutrophil-platelet aggregates in the sepsis group at admission. Of note, the percentage of leukocyte-platelet interactions and the density of platelets interacting with monocytes significantly decreased after 48 hours in the ICU while the level of soluble platelet activation markers remained high. The decrease in platelet-monocyte heterotypic interactions may have different explanations including the developed thrombocytopenia, platelet desensitization, P-selectin cleavage and/or the potential impact of patient care decreasing the pro-inflammatory state. In contrast, activation markers expressed at the surface of circulating platelets, such as P-selectin (CD62P) and CD63, were not significantly increased in the sepsis group. These data suggest that activated platelets, with high adhesion potential, were likely sequestered from the circulation through interactions with the endothelium, formation of microthrombi and association with leukocytes and NETs. This is consistent with the frequent report of thrombocytopenia in septic shock patients. However, we cannot exclude the possibility that circulating platelets in the sepsis group did not exhibit the intrinsic surface activation markers P-selectin and CD63 because these proteins were rapidly proteolyzed. It has been shown that loss of platelet surface P-selectin fully accounted for the increase in the plasma concentration of soluble P-selectin[17]. Thus, as we previously observed in a mouse model of peritoneal sepsis both intrinsic and soluble markers of platelet activation should be investigated in this pathological situation[18].

GPVI is a major platelet signaling receptor for collagen and fibrin and is expressed only on megakaryocytes and platelets[19]. In physiology, GPVI levels are stable on circulating platelets but the receptor undergoes rapid metalloproteolyctic cleavage on activation[20-22]. Two recent studies have observed elevated sGPVI levels in sepsis[16,23]. In a cohort of ICU patients, elevated sGPVI significantly associated with 28-day and 90-day mortality[16]. In this study, the production of sGPVI was triggered by interaction of platelets to fibrin polymers which generation was increased in these patients. Thus, the elevated level of sGPVI in our septic patient group at inclusion and after 48 hours may suggest a formation of fibrin. Interestingly, Weiss et al. [23] found that besides reduction of surface GPVI expression, sepsis platelets were hyporesponsive to multiple agonists and failed to transduce GPVI-mediated signaling.

The increase in sCD40L is also a reflection of platelet activation. Resting platelets are known to express minute levels of CD40L on their surface, but can translocate massive amounts of this molecule to their surface within minutes of activation where it can subsequently be cleaved and released as a soluble molecule into the circulation[24-27]. In a mouse model of abdominal sepsis, Rahman et *al*. have shown that cecal ligation and puncture induced a decrease of platelet surface CD40L and a concomitant increase in sCD40L levels[28]. Our results are also in line with the study of Lorente et *al*.[29] showing in a multicenter prospective study that serum levels of circulating sCD40L were significantly higher in septic patients than in controls, and in non-survivors compared to survivors.

Leukocyte-platelet aggregates were also elevated at admission in our sepsis patient group, particularly platelet-monocytes heterotypic aggregates. The number of platelets interacting with monocyte was also elevated. Formation of monocyte-platelet aggregates is known to involve interactions between P-selectin expressed at the surface of activated platelets and PSGL-1 (P selectin glycoprotein ligand 1) at the surface of monocytes. Of note, a correlation between the rate of monocyte-platelets aggregates and mortality has been

observed in septic patients[30].

Indeed, all patients in the sepsis group presented a drop in their platelet count and some of them were thrombocytopenic (<150G/L). Importantly, rather than the absolute value of platelet count, the percentage drop has been shown as a decisive prognosis factor[10,31]. Indeed, in a large cohort of patients, Moreau and *al*. showed that a 30% decline in platelet count independently predicted death (odds ratio, 1.54; 95% CI 1.12 to 2.14)[32]. In our patient group, we observed a 47.5% mean decline in platelet count underlying the severity of the illness, in correlation with high SAPS II and SOFA scores (51[26-87] and 11 [8-18] respectively).

Our study presents limitations inherent in such a study. It is a monocentric study that can lead to the observation of a "center effect" even if the nature of the septic shocks included is varied. However, due to the fragility of blood platelets and the technical level of the specialized laboratory, it was not possible to carry out a multicenter study with this study design. We did not fit the patients with their controls, but this was a pilot study and the number of patients needed to be included did not allow it.

In summary, to evaluate platelet reactivity in patients with septic shock we assessed platelet aggregation and secretion following agonists stimulation. We observed a moderated but significant decrease in platelet reactivity to specific agonists in the sepsis group suggesting a reduced efficiency of the committed receptors (TPα, P2Y1/P2Y12, PAR1, GPVI). Indeed, surface P-selectin and CD63 expression as well as maximal platelet aggregation were both decreased in the sepsis group. A potential desensitization and/or shedding of the surface receptors after prolonged exposition to platelet agonists, in a pro-inflammatory context, may explain a reduced platelet reactivity in the sepsis group. Thus, the question arises whether drugs inhibiting platelet activation may have a benefit in critically ill patients, particularly in

septic shock to limit microvascular occlusion and multiple organ failure. Preclinical evidence for a beneficial impact of aspirin and adenosine diphosphate (ADP) receptor P2Y12 inhibitors in experimental models of sepsis have been reported[33,34]. Some clinical studies on the use of antiplatelet agents in septic critically ill patients have also reported encouraging results[35,36]. However, given inherent limitations from observational studies, only randomized controlled trials may answer the question of the pertinence and the effectiveness of antiplatelet agents in septic shock to reduce organ failure and morbi-mortality. Recently, a first placebo-controlled, randomized trial did not provide support for a potential benefit of low dose aspirin for the primary prevention of sepsis[37].

CONCLUSION

In critically ill patients with septic shock, platelets are activated early and durably, which may explain the thrombocytopenia observed, the formation of leukocyte-platelet interactions and the occurrence of microvascular thrombosis. However, the functional characteristics of the remaining circulating platelets are different from those activated in the initial phase of septic shock. They are less reactive to stimulation and appear partly desensitized. A better understanding of the pathophysiology of hemostasis alterations, particularly of platelet functions, may lead to the identification of diagnostic/prognostic markers of sepsis but also of the potential introduction of anti-platelet therapies at the very initial phase and during the evolution of septic shock in order to counteract the dramatic organ consequences related to septic shock.

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DECLARATION OF INTEREST STATEMENT

None

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DATA AVAILABILITY STATEMENT: the data supporting the results can be send on a reasonable demand.

TABLES AND FIGURES

FIGURES LEGENDS

Figure 1: **Flow chart of the inclusions**

Figure 2: **Leukocytes-platelets aggregates in control and sepsis groups**. The circulating heterologous aggregates between platelets and monocytes (A) or neutrophils (C) were assessed by flow cytometry in the control group (n=12) and the sepsis group at admission (H0, n=21) and after 48 hours (H48, n=13). Results are expressed as percentage of leukocyte-platelet aggregates (median, 25^{th} and 75^{th} percentiles). The amount of platelets on monocytes (B) or neutrophils (D) was assessed by measuring the mean fluorescence intensity (MFI) of the platelet marker CD61 on monocytes or neutrophils with the anti-CD61 antibody coupled to PE. Whisker box plots with median [25^{th}-75^{th} percentile] (upper extern and lower extern values) are shown. Statistical analysis was performed using nonparametric test U Mann-Whitney. *p<0.05, **p<0.01 and *** p<0.001. The reference range of the laboratory for circulating platelet-leukocyte aggregates obtained from a series of 9 healthy blood donors are indicated by the gray line.

Figure 3: Surface expression of platelet activation markers in resting and activated platelets. Secretion of platelet alpha granules (A) and dense granules (B) was assessed by flow cytometry using selective anti-CD62P or anti-CD63 antibodies, respectively. Platelets from control group (n=12) and sepsis group at admission (n=21) and 48 hours later (n=13) were analyzed either in resting conditions (Basal) or following 10 minutes stimulation by CRP (Collagen Related Peptide) (10 µg/ml) or U46619 (5 µM). Results are expressed as MFI and are mean ± SEM. Statistical analysis was performed using nonparametric test U Mann-Whitney or Wilcoxon test (#) for compare H0 and H48 * p<0.05, ** p<0.01 and *** p<0.001.

Figure 4: **Soluble platelet activation markers in resting and sepsis platelets.** Levels of plasma soluble GPVI (sGPVI) (A) and soluble CD40 ligand (sCD40L) (B) were measured in the control group (n=12) and in the sepsis group (sepsis H0 n=18 and sepsis H48 n=17). Results are expressed as whisker box plots with median [25^{th}-75^{th} percentile] (upper extern and lower extern values). Statistical analysis was performed using a non-parametric test U Mann-Whitney. *p<0.05, **p<0.01 and *** p<0.001.

Figure 5: Maximal platelet aggregation response in the control group versus the sepsis group. Platelets from control were compared with sepsis at H0 and H48 after addition in platelet-rich plasma (PRP) of adenosine diphosphate (ADP, 10 µM) (A), collagen-related peptide (CRP, 10 µg/ml) (B), thromboxane A2 analog (U46619, 5 µM) (C) and thrombin-receptor activation peptide (TRAP, 25 µM) (D). Platelet aggregation was assessed by light transmission aggregometry under stirring at 1000 rpm during 10 minutes at 37°C. Results are expressed as % maximal platelet aggregation and are mean ± standard deviation (n=11 for control, n=11 for sepsis H0 and n=11 for sepsis H48). Statistical analysis was performed using a non-parametric test U Mann-Whitney. *p<0.05, **p<0.01, *** p<0.001, **** p<0.0001.

Supplemental Figure 1: Relative platelet decrease during the first days after ICU admission for sepsis. Kinetics of platelet counts during 4 days are represented as median and 25-75 percentiles. The red line represents the median platelet count at admission as our reference.

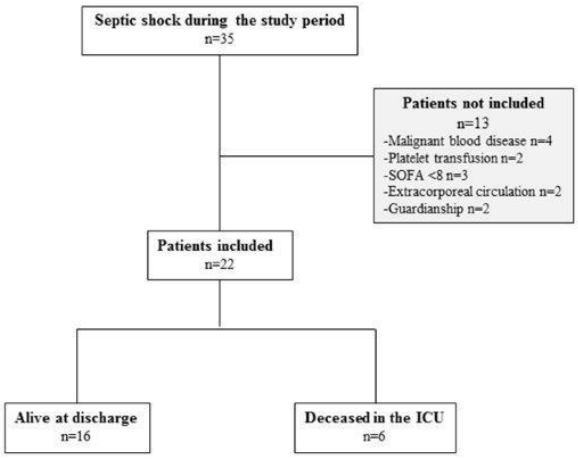

Septic shock during the study period
n=35
Patients not included
n=13
-Malignant blood disease n=4
-Platelet transfusion n=2
-SOFA <8 n=3
-Extracorporeal circulation n=2
-Guardianship n=2
Patients included
n=22
Alive at discharge
n=16
Deceased in the ICU
n=6

Figure 1

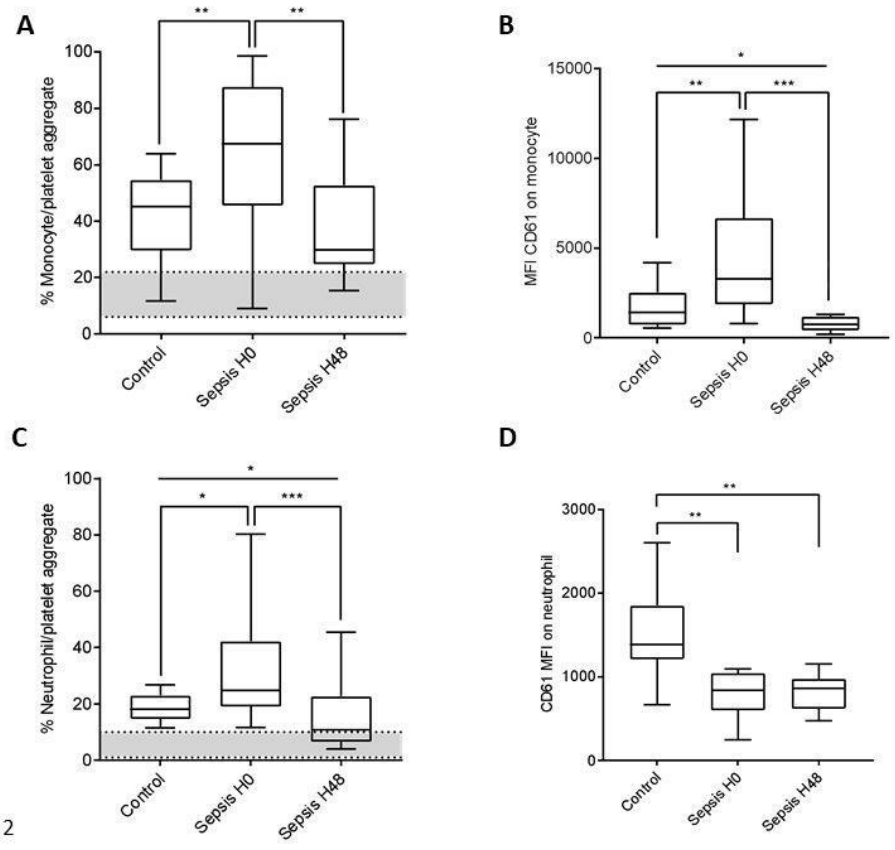

A
% Monocyte/platelet aggregate
100
80
60
40
20
0
**
**
Control
Sepsis H0
Sepsis H48
B
MFI CD61 on monocyte
15000
10000
5000
0
*
**
***
Control
Sepsis H0
Sepsis H48
C
% Neutrophil/platelet aggregate
100
80
60
40
20
0
*
*
***
Control
Sepsis H0
Sepsis H48
D
CD61 MFI on neutrophil
3000
2000
1000
0
**
**
Control
Sepsis H0
Sepsis H48

Figure 2

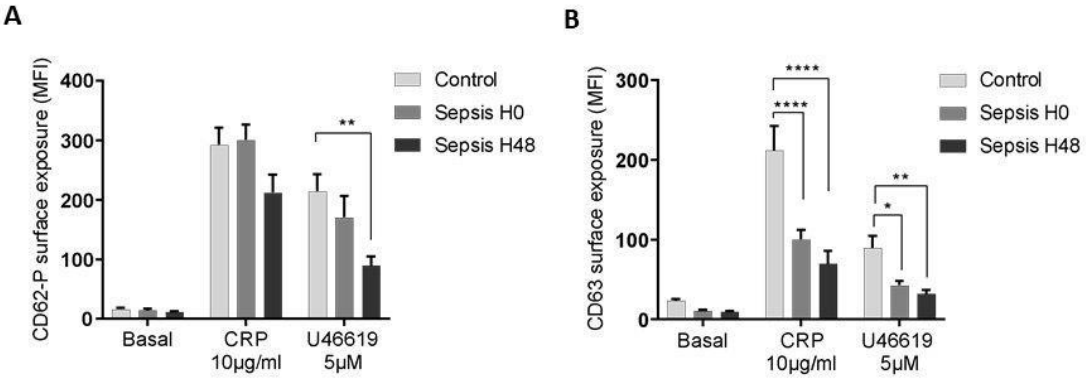

A
CD62-P surface exposure (MFI)
400
300
200
100
0
**
Control
Sepsis H0
Sepsis H48
Basal
CRP
10µg/ml
U46619
5µM
B
CD63 surface exposure (MFI)
300
200
100
0
****
****
**
*
Control
Sepsis H0
Sepsis H48
Basal
CRP
10µg/ml
U46619
5µM

Figure 3

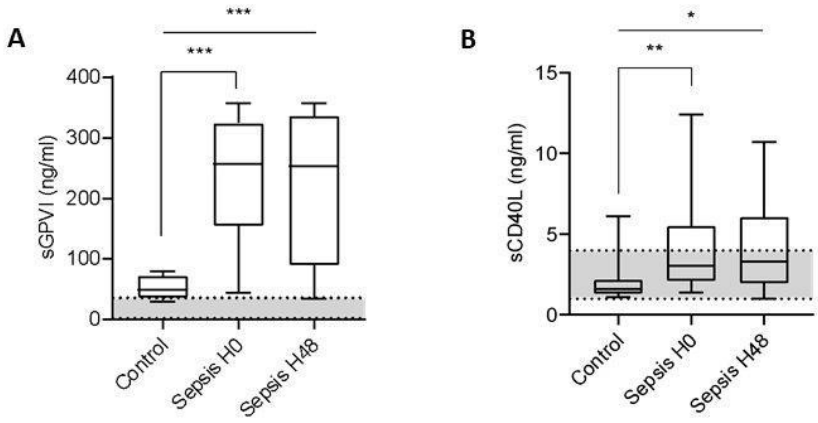

A
***
***
400
300
200
100
0
sGPVI (ng/ml)
Control
Sepsis H0
Sepsis H48
B
*
**
15
10
5
0
sCD40L (ng/ml)
Control
Sepsis H0
Sepsis H48

Figure 4

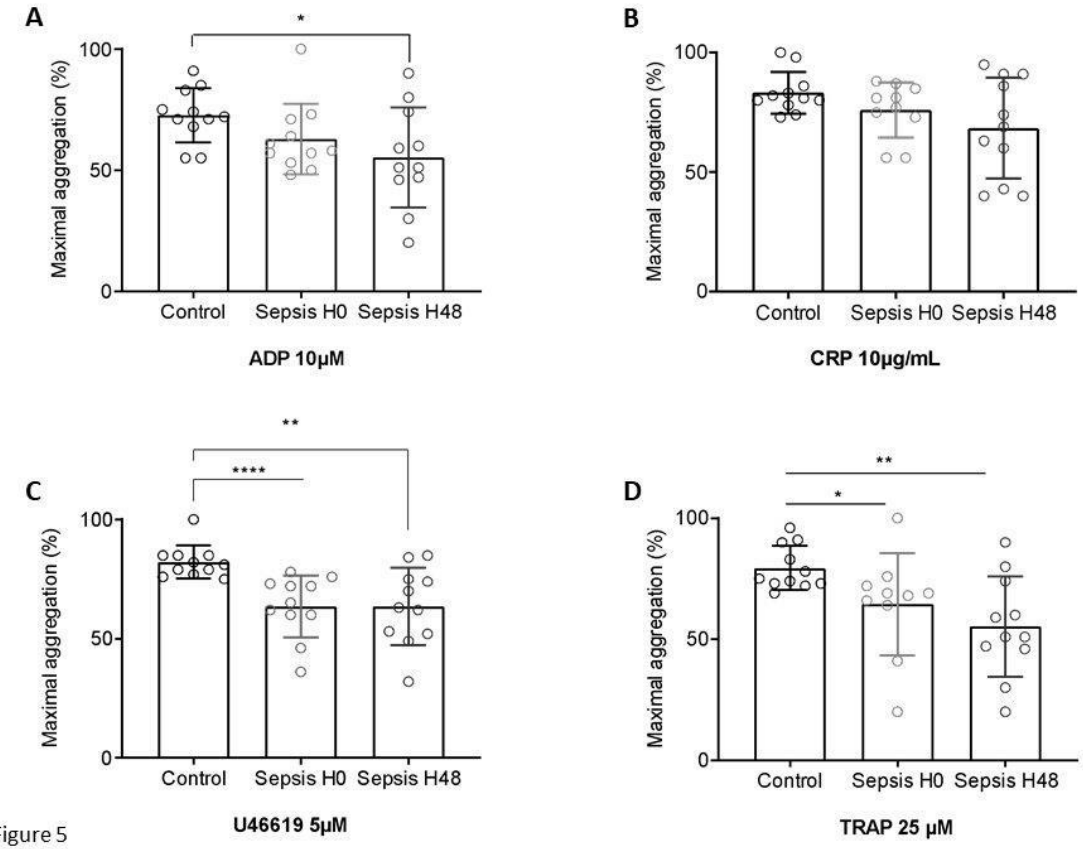
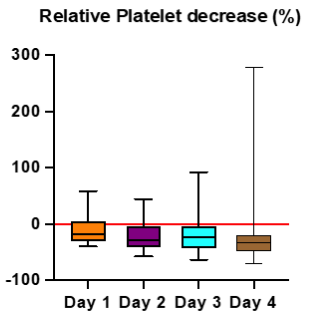

A
Maximal aggregation (%)
100
50
0
*
Control
Sepsis H0
Sepsis H48
ADP 10µM
B
Maximal aggregation (%)
100
50
0
Control
Sepsis H0
Sepsis H48
CRP 10µg/mL
C
Maximal aggregation (%)
100
50
0
**
****
Control
Sepsis H0
Sepsis H48
U46619 5µM
D
Maximal aggregation (%)
100
50
0
**
*
Control
Sepsis H0
Sepsis H48
TRAP 25 µM

Figure 5



Supplemental figure 1

Table 1: Main clinical and physiological characteristics of control and sepsis patients at the time of inclusion

Variables	Total population (n=34)	Control (n=12)	Septic shock (n=22)	*P* value
Clinical data				
Age (years)	63 [19-88]	65.5 [37-86]	63 [19-88]	0.97
Sex ratio F/M	9 (26.5%) /25 (73.5%)	6 (50%) /6 (50%)	3 (13.6%) / 19(86.4%)	**0.04***
BMI (kg/m²)	26.1 [15.9-41.1]	26.7 [18.9-35.6]	25.5 [15.9-41.1]	0.94
SAPS II	-	-	51 [26-87]	
SOFA	-	-	11 [8-18]	
Treatments				
Statins	9 (26.5%)	3 (25%)	6 (27.3%)	0.99
Aspirin	5 (14.7%)	1 (8.3%)	4 (18.2%)	0.63
P2Y12 inhibitors	2 (5.9%)	1 (8.3%)	1 (4.5%)	0.99
Beta-blockers	9 (26.5%)	3 (25.0%)	6 (27.3%)	0.99
Antihypertensive drug	10 (29.4%)	1 (8.3%)	9 (40.9%)	0.06
Hematological data				
Hemoglobin (g/dL)	11.1 [7.9-19.4]	13.1 [10.7-19.4]	10.3 [7.9-16.7]	**0.0026***
Platelets (G/L)	179 [62-549]	218 [101-448]	129 [62-549]	0.06
VMP (fL)	10.9 [9.2-13.8]	10.75 [9.3-11.6]	11.3 [9.2-13.8]	0.16
Leukocytes (G/L)	9.51 [2.76-40.9]	6.57 [2.7-12.5]	11.49 [2.77-40-9]	**0.0174***
Neutrophils (G/L)	6.72 [0.45-38.76]	4.19 [0.45-10.25]	11.56 [2.36-38.76]	**0.0009***
Monocytes (G/L)	0.65 [0-3.62]	0.60 [0.27-3.62]	0.69 [0-1.77]	0.72

BMI: Body Mass Index; SAPS II: Simplified Acute Physiologic Score; MPV: Mean Platelet Volume; SOFA (Sepsis-Related Organ Failure Assessment). Date are expressed as median and extreme values, except for sex ratio and treatment data (n, %). * $p<0.05$.

Table 2: Clinical and biological characteristics of patients in septic shock

	At inclusion (H0)	48h after inclusion (H48)	*P* value
Clinical data			
HR (bpm)	101 [63-160]	86 [50-113]	**0.0019***
MAP (mmHg)	66 [63.9-74]	66 [60-96]	0.11
Temperature (°C)	38 [34.3-40]	37.6 [35.5-40.9]	0.16
Biochemical data			
pH	7.33 [6.80-7.49]	7.43 [7.27-7.51]	**0.0003***
Excess base (mmol/L)	-5.9 [-20- +9]	-1.2 [-12.9- +16]	**0.0094***
PaO2 (mmHg)	75 [49-210]	75.5 [51.9-136]	0.51
PaCO2 (mmHg)	36 [23-95]	36 [25-51]	0.22
Lactic acid (mmol/L)	2.5 [0.6-12]	1.4 [0.8-2.7]	**0.0031***
Serum creatinine (mol/L)	100 [40-406]	77.5 [34-486]	0.64
GFR (ml/min/1.73m²sc)	71.5 [13-164]	94 [11-185]	**0.0068***
Serum bilirubin (mg/L)	16 [3-174]	14 [3.4-163]	0.11
SGOT(UI/L)	72 [11-370]	53.5 [18-1864]	0.59
SGPT (UI/L)	45 [13-447]	40 [18-737]	0.63
Troponin (ng/L)	43 [14-265]	-	-

HR: heart rate, MAP: mean arterial pressure, GFR: glomerular filtration rate, SGOT: Serum Glutamo-Oxaloacetate Transferase; SGPT: Serum Glutamo-Pyruvate Transferase. Results are expressed as median and extreme values. * $p<0.05$.

Contexte scientifique n°4

L'étude des mécanismes à l'origine des dysfonctions d'organes dans le sepsis a mis en évidence que certains médiateurs, tels que les cytokines par exemple, avaient une valeur diagnostique et pronostique (Calandra et al. 1991; Linder et al. 2009; Song et al. 2019). Cependant, leur dosage n'est pas réalisé en routine. Au lit du patient, la valeur pronostique individuelle des scores de gravité utilisés dans le sepsis (Indice Gravité Simplifiée (IGS 2), Sequential Organ Failure Assessment (SOFA)) et de la plupart des biomarqueurs est limitée. Une stratification des patients en fonction du pronostic peut guider le clinicien vers une optimisation ou au contraire une limitation de certaines approches thérapeutiques. Certains auteurs ont proposé d'utiliser des paramètres plaquettaires comme marqueurs prédictifs de la mortalité dans le sepsis (Kim et al. 2015; Zampieri et al. 2014; Tajarernmuang et al. 2016; Oh et al. 2017). Parmi les paramètres plaquettaires facilement disponibles, le volume moyen plaquettaire (VMP) est rendu par le laboratoire en routine. La variation de VMP entre l'admission et 24h (Zampieri et al. 2014) ou entre l'admission et 72h (Kim et al. 2015) est associée à la mortalité hospitalière et à J28 dans le sepsis, respectivement. Par ailleurs, le rapport VMP/compte plaquettaire a une valeur pronostique de mortalité à J28 chez les patients en choc septique (Oh et al. 2017). Cependant, ces paramètres ont été étudiés sur des durées relativement courtes lors du choc septique en réanimation.

Le but de notre étude chez les patients de réanimation était d'évaluer si la cinétique du compte plaquettaire et du VMP avaient une valeur pronostique chez les patients en choc septique.

PLOS ONE

RESEARCH ARTICLE

Kinetics of mean platelet volume predicts mortality in patients with septic shock

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Abstract

Introduction

Thrombocytopenia is well recognized as a poor prognosis sign associated with increased mortality and prolonged Intensive Care Unit (ICU) stay, particularly in septic patients. Mean platelet volume (MPV) could represent a relevant predictive marker of mortality. Here we investigated whether MPV kinetics during the first 15 days after hospital admission has a potential prognostic value for clinical outcome in septic shock.

Methods

We performed a retrospectively analysis of a cohort of 301 septic patients admitted in ICU. Three-month mortality was the primary endpoint. The prognostic value of the covariates of interest was ascertained by multidimensional analysis. We proposed a classification and regression trees analysis to predict survival probability.

Results

MPV kinetics was significantly different between 90-day survivors and non-survivors when followed during 15 days (except on day 3). 10-day MPV >11.6fL was an independent predictive factor of 90-day mortality (Hazard Ratio (HR) 3.796, 95% Confidence Interval (CI) [1.96–7.35], p = 0.0001) in multivariate analysis. Base excess on day 4 <1.9mmol/L was also a predictive factor of mortality (HR 2.972, 95%CI [1.38–6.40], p = 0.0054.

Conclusion

MPV increase during the first 15 days after ICU admission in non-survivors was observed during septic shock and 10-day MPV >11.6fL was an independent predictive factor of 90-day mortality. This could be explained by the emergent response to acute platelet loss during septic shock, leading to megakaryocyte rupture to produce new but potentially immature platelets in the circulation. Therefore, continuous monitoring of MPV may be a useful parameter to stratify mortality risk in septic shock.

Introduction

Sepsis has recently been defined as a "life-threatening organ dysfunction caused by a dysregulated host response to infection" [1]. Septic shock constitutes a "subset of sepsis in which both circulatory and cellular metabolism abnormalities occur". Sepsis-related mortality is linked to multiple organ failure (MOF) development. MOF is partly due to microvascular thrombosis and endothelial dysfunction, involving thrombocytes. Thrombocytopenia is the most common hematologic disorder in the Intensive Care Unit (ICU) with a prevalence of around 50% [2]. Thrombocytopenia is well recognized as a poor prognosis sign and is associated with increased mortality and with a prolonged ICU stay [3–6]. Mean platelet volume (MPV) describes the average size of platelets in a blood sample. Many physicians have recently shown interest in MPV in several human studies in term of prognosis on short periods (one or two days) [7–10]. A mechanism that could explain the changes in MPV values is an adapted response to acute platelet loss during an inflammatory condition. Indeed, in physiological conditions, platelet count and thrombopoiesis from bone marrow megakaryocytes (MK) are tightly inter-regulated processes. In the presence of thrombopoietin (TPO), MK exhibit microtubule-dependent extensions of elongated pseudopodal structures called proplatelets allowing the release of newly generated platelets in the blood stream [9,11,12]. It is well known that younger platelets have a higher MPV. However, using intravital microscopy, Nishimura et *al*. have suggested that this process may not be sufficient to support a rapid platelet turnover, especially when the platelet need is acute [13]. Their team highlighted a mechanism for the rapid production of platelets and their release into the bloodstream thanks to a MK rupture process which leads to the rapid fragmentation of cytoplasmic prolongations. This leads to the release of a large number of thrombocytes into circulation. These platelets exhibit an increase in MPV and their morphology is somewhat different. This mode of release of young platelets from bone marrow megakaryocytes would restore a pool of circulating platelets in acute consumption situations such as sepsis. However, these platelets may have important functional differences due to a lesser organization of their microtubules and could therefore contribute to a poor clinical impact in septic patients. Little is known about the potential influence of MPV changes on mortality in a homogenous group of septic patients. Therefore, we focused our study on the MPV kinetics during the first 15 days after hospital admission to check if this parameter has a prognostic value for clinical outcome in septic shock.

Materials and methods

Patients and study design

This retrospective cohort study included patients with microbiologically proven septic shock who were admitted to the Intensive Care Unit (ICU) of a tertiary-care teaching hospital between January 2012 and January 2016. Patients received early gold-directed therapy (EGDT) as recommended.

Patients were excluded if they met these criteria: age <16, hemorrhagic shock, hemorrhagic surgery, patient under extracorporeal life support (ECLS), cardiac arrest, platelet transfusion and pre-existing thrombocytopenia or thrombocytopenia induced by chemotherapy.

This study was reviewed and approved by the institutional review board of Toulouse University Teaching Hospital, France (n°09–0916). All data were fully anonymized before we accessed them. No consent was necessary for this retrospective study.

The primary end-point was 90-day mortality.

Data collection

We collected baseline characteristics including demographic information, neurologic and hemodynamic factors, laboratory data, duration of mechanical ventilation, renal replacement

therapy, stay in ICU and clinical outcome 28 days after ICU admission, at 3 months (e.g. 90 days) and intrahospital mortality. For disease severity assessment, both new Simplified Acute Physiology Score (SAPS II) and Sequential Organ Failure Assessment (SOFA) were determined according to the worst values within the initial 24 hours of ICU admission.

Platelet count, serum mean platelet volume, hemoglobin (Hb), white blood cell (WBC) count, lactic acid, pH and base excess were measured during the 15 days after admission. Venous blood samples for laboratory counts were collected from all patients in tubes containing ethylenediamine tetra-acetic acid (EDTA) and analyzed with an SYSMEX XN 1000 hematology analyzer (Canada) within 30 minutes of sample collection. The normal reference range for MPV in our laboratory hospital is 8.5 to 12.2fL. Thrombocytopenia was defined by platelet count <150 G/L.). Day -1 corresponded to the day before the onset of sepsis for the patients already in hospital for another reason such as elective surgery.

Statistical analysis

Continuous variables are expressed as median, 95% confidence interval (95% CI) and extreme values and categorical variables as numbers with percentages. Patients who died within 90 days after ICU admission were defined as "non-survivors". Baseline characteristics are presented according to the occurrence of the primary outcome (survivors *vs*. non-survivors) and were compared between the 2 groups. We compared continuous variables using Mann-Whitney test and categorical variables using χ^2 tests or Fisher exact tests. Correlations between the quantitative variables were realized by the Spearman Rank method.

Considering time-to-event, we constructed time-dependent receiver operating characteristics (ROC) curves to assess threshold and predictive values of the covariates of interest. Kaplan-Meier survival curves were produced using 90-day mortality based on MPV threshold value.

Covariate selection for the multivariate analysis was based on *P* value <0.2 in univariate analysis. The prognostic value of the covariates of interest was ascertained by Cox proportional hazards model, using covariates whose AUC contained 0.8 in the 95% confidence interval and the results are presented as hazard ratios (HR) with 95% CI. To highlight patients with the best survival chances, a partitioning of the population was represented using a Classification and Regression Trees (CART) analysis. The advantage of this approach is to describe the means of distribution of the population in homogeneous groups according to 90-day survival and the covariates selected from the multidimensional analysis[14].

Statistical analyses were conducted using SPSS® for Window version 23.0 (IBM Corporation, Chicago, IL). A *P* value <0.05 was considered statistically significant.

Results

Baseline characteristics

As shown in **Fig 1** illustrating the flow chart of the enrollment of patients used in our study, of the 316 consecutive patients admitted to the ICU, 15 were excluded. Among the 301 patients included, 102 (33.9%) were deceased at 3 months. The baseline demographic, clinical, and biological data of each group stratified by 90-day all-cause mortality are presented in **Table 1**. The main infection sites were lung (45.5%), and intra-abdominal cavity (29.2%), followed by urinary tract (20.9%). As shown in Table 1, 90-day survivors were younger than 90-day non-survivors. As expected, SOFA and SAPS II scores were higher in non-survivors. At admission, platelet count was not different between the 2 groups. However, MPV value, serum creatinine, total serum bilirubin and lactic acid were higher in non-survivors. The proportions of patients

who received renal replacement therapy (RRT) and who required mechanical ventilation were significantly higher in non-survivors.

Kinetics of MPV during the first 15 days in ICU

Monitoring the MPV during the 15 days following admission in ICU indicated a significant difference between 90-day survivors and non-survivors since their admission for sepsis (10.4fL 95%CI [10.2–10.6] *vs* 10.8fL 95% CI [10.5–11.1] (p = 0.035) (Fig 2**).** Strikingly, the non-survivors exhibited a higher MPV all along the kinetics. Except for day 3, this difference was significant for all time points and was particularly clear after day 7. In the survivors group, MPV values were stabilizing as early as day 2 then declining after 7 days in ICU. MPV value was inversely significantly correlated with the value of the platelet count at each time (S1 Table). MPV was also significantly correlated with SOFA score at each time (S2 Table).

Kinetics of platelet count during the first 15 days in ICU

Platelet count decreased significantly during the first days of ICU admission to reach a nadir on day 3 in the survivors group and on day 4 in the non-survivors group (Fig 3). The difference between the two groups was significant from day 6 and was particularly exacerbated on day 10 with 326 [16–993] G/L, 95% CI [276–361] in the 90-day survivors group *vs* 198 [11–681] G/L, 95% CI [131–224] in the 90-day non-survivors group (p = 0.0001). In the survivors group, the platelet count returned to the admission value at the end of the first week and continued to rise to become significantly greater than that at admission. In the non-survivors group, the platelet count did not return to the admission values, even after 15 days.

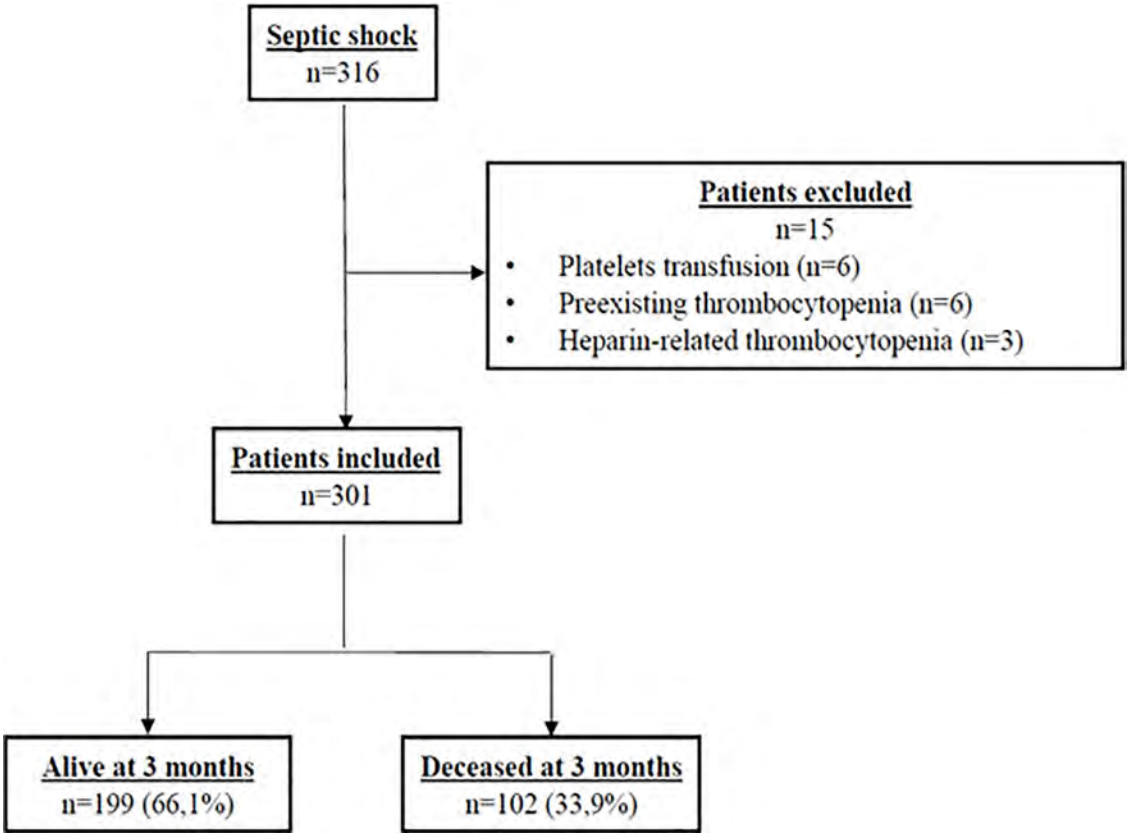

Fig 1. Flowchart of the enrolment of the patients in the study.

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Table 1. Characteristics of the patients.

	Total Population (n = 301)	90-day survivors (n = 199)	90-day non survivors (n = 102)	*P*
Demographic Data				
Age (years)	66 (64–69)	64 (62–68)	71.5 (66–74)	0.0002***
Male sex. n (%)	197 (65.4)	130 (65.3)	67 (65.7)	
SOFA	11 (11–12)	10 (9–11)	14 (13–15)	< 0.0001***
SAPS II score	57 (52–60)	52 (48–56)	69 (61–76)	< 0.0001***
Antiplatelet therapy: yes (%)	34.6	32.8	38	ns
Time between onset of sepsis and ICU admission(D)	2 (2–2)	2 (2–2)	2 (2–2)	ns
Lenght of stay in ICU (D)	11 (10–13)	11 (10–14)	9 (4–12.4)	0.0044**
Biochemical data at admission				
Platelet count (G/L)	195 (187–218)	195 (181–219)	193 (178–239)	ns
MPV (fL)	10.5 (10.4–10.7)	10.4 (10.2–10.6)	10.8 (10.5–11.1)	0.0035**
Leukocyte count (G/L)	12.68 (11.26–14.069)	12.7 (10.9–14)	12.6 (11–15)	ns
Serum creatinine (μmol/L)	127 (119–140)	115 (99.6–127)	148 (129.6–198)	< 0.0001***
Total bilirubin (mmol/L)	11 (10–12.9)	10 (9.3–12)	13.5 (11–19)	0.0183*
Lactic acid (mmol/L)	2.6 (2.26–3.04)	2.3 (2–2.78)	3.3 (2.44–3.99)	0.0009***
pH	7.33 (7.31–7.35)	7.35 (7.32–7.37)	7.28 (7.25–7.33)	0.0022**
Base Excess (mmol/L)	-5.7 (-6.5; -4.90)	-5.1 (-6.13; -4.16)	-6.85 (-9.54; -5.56)	0.011*
Day of platelet count nadir (D)	3 (3–3)	3 (3–4)	3 (2–4)	ns
Number of days of thrombocytopenia (D)	2 (1–3)	1 (1–3)	2 (1–3)	ns
Infection site. n (%)				
Lung	137 (45.5)	88 (44.2)	49 (48)	0.0018**
Intra-abdominal site	88 (29.2)	48 (24.1)	40 (39.2)	
Urinary tract	63 (20.9)	52 (26.1)	11 (10.8)	
Bacteremia	13 (4.3)	11 (5.5)	2 (2)	
Digestive tract	88 (29.2)	48 (24.1)	40 (39.2)	0.00754**
Mechanical Ventilation n (%)	245 (81.4)	150 (75.4)	95 (93.1)	< 0.0001***
Acute kidney injury n (%)	209 (69.3)	120 (60.1)	89 (87.3)	< 0.0001***
Renal Replacement Therapy n (%)	84 (28)	37 (18.7)	47 (46.1)	< 0.0001***
Vasopressor use n (%)	276 (91.7%)	177 (88.9%)	99 (97.1%)	0.015*

Data are median (CI 95%) or n (%). SOFA: Sequential Organ Failure Assessment; SAPS II: Simplified Acute Physiology Score Index 2; MPV: Mean Platelet Volume

*p<0.05

**p<0.01

***p<0.001

ns: non-significant.

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Kinetics of blood pH and lactic acid

The study of metabolic parameters kinetics revealed that blood pH and serum lactic acid were different between survivors and non-survivors, from the day of admission in ICU to day 4 for lactic acid and to day 6 for blood pH (S1 and S2 Figs). The main differences were observed at day 1 and day 2.

ROC curves analysis

Considering time-to-event, we constructed time-dependent receiver operating characteristics (ROC) curves to assess on which day after the onset of the sepsis MPV provided the better prognostic value for 90-day mortality (**Fig 4**). Areas under the curves (AUC) of the main continuous clinical and biological variates discriminating survivors and non-survivors are shown

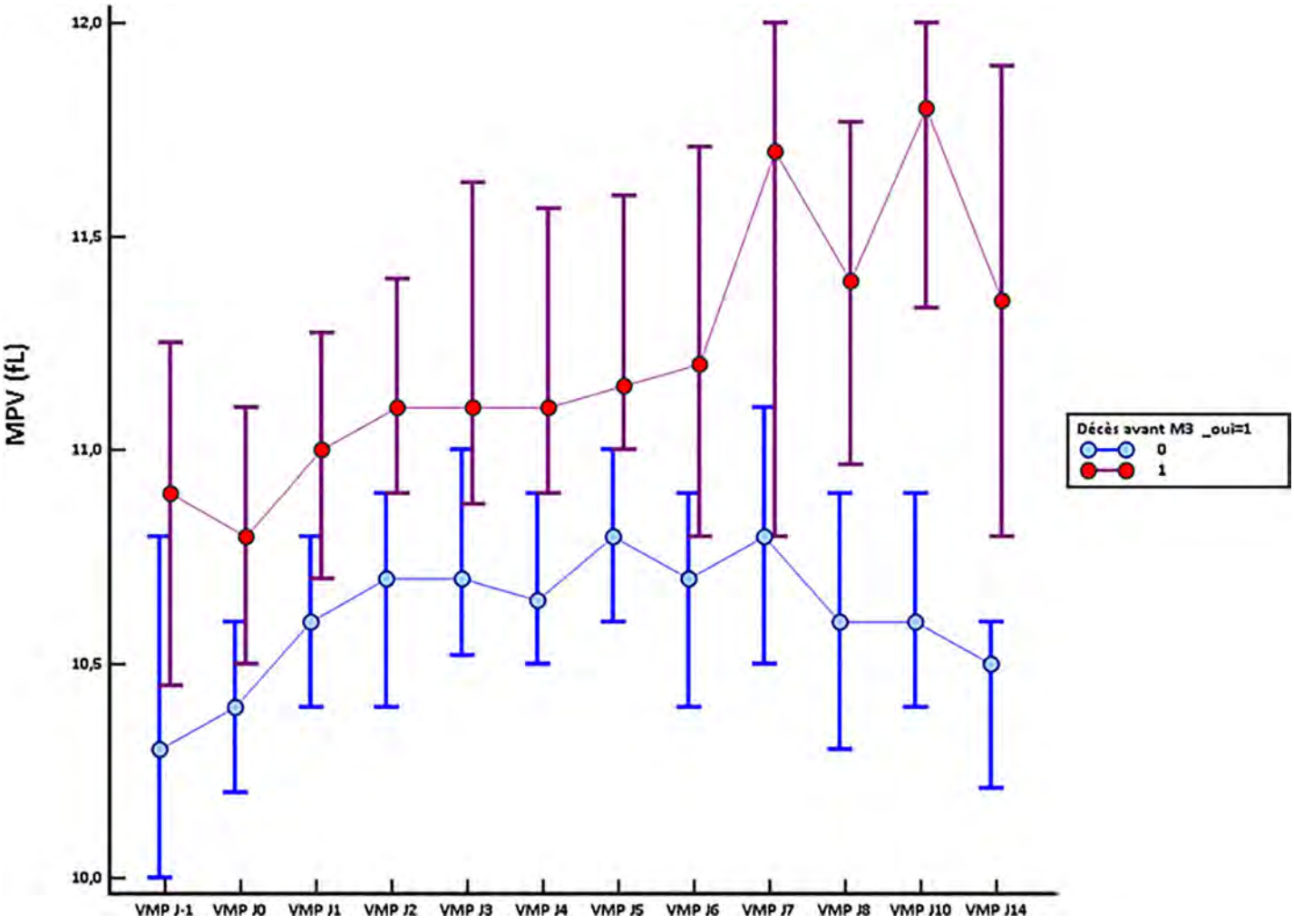

Fig 2. Comparison of MPV kinetics during 15 days according to 90-day survival.

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in Table 2. Best MPV value was the 10-day value with a Youden index >11.6fL. Other parameters did not provide acceptable prognostic value in view of AUC or sensitivity/specificity.

Kaplan-Meier curve

Kaplan-Meier survival curves were produced using 90-day mortality based on 10-day MPV value >11.6 or ≤ 11.6fL. As shown in Fig 5, survival probability was better in patients with 10-day MPV≤11.6fL ($p<0.0001$) with a survival probability of 86.3%.

Multivariate analysis

Among the 301 patients included in the study, 102 (33.9%) died within 90 days following admission in ICU. We used multivariate Cox proportional analysis to assess the effect of several covariates on 90-day mortality by adjusting for other significant variables. Covariates included in the multivariate analysis were SOFA score >11, serum lactic acid on admission >5.2 mmol/L, day 4 base excess ≤1.9 mmol/L, 10-day MPV >11.6fL and 10-day/platelet >4.14 (Table 2). Cox Model exhibited that 10-day MPV >11.6fL was an independent predictive factor of 90-day mortality (Hazard Ratio (HR) 3.796, 95% CI [1.96–7.35], p = 0.0001) and

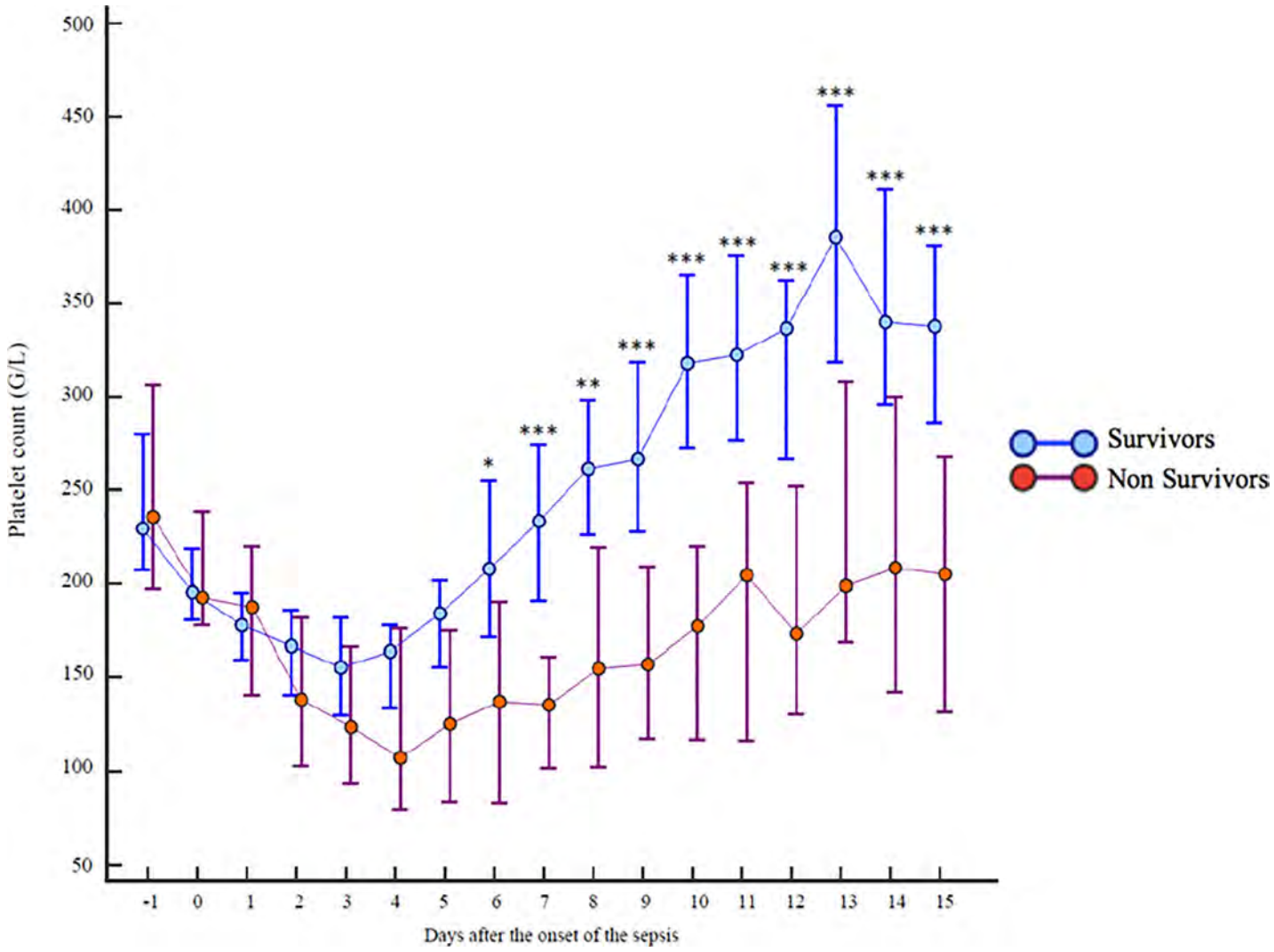

Fig 3. Comparison of platelet counts during 15 days according to 90-day survival.

https://doi.org/10.1371/journal.pone.0223553.g003

base excess on day 4 <1.9mmol/L was also a predictive factor of mortality (HR 2.972, 95%CI [1.38–6.40], p = 0.0054 (Table 3).

Results of Classification and Regression Trees (CART) are shown in Fig 6. CART have been used extensively as an alternative to the classical linear and additive prediction models. Results are presented in tree form of a decision rule with a hierarchical sequential structure that can be easily understood and applied in clinical practice. The percentage of estimation of this CART analysis was greater than 80%. For example, patients with 10-day MPV ≤10.5fL and MPV/platelet ratio <4.14 had a 90-day predicted survival of 98.5%. This corresponded to 66 patients from the study (21.9%). In contrast, patients with 10-day MPV >11.6fL and with SOFA score >11 at the day of admission presented a 90-day risk of mortality of 64.6%.

Discussion

Septic shock is a major cause of mortality in ICU; therefore, it is crucial to detect patients at high risk of death in order to improve their management. In this context, the main findings of

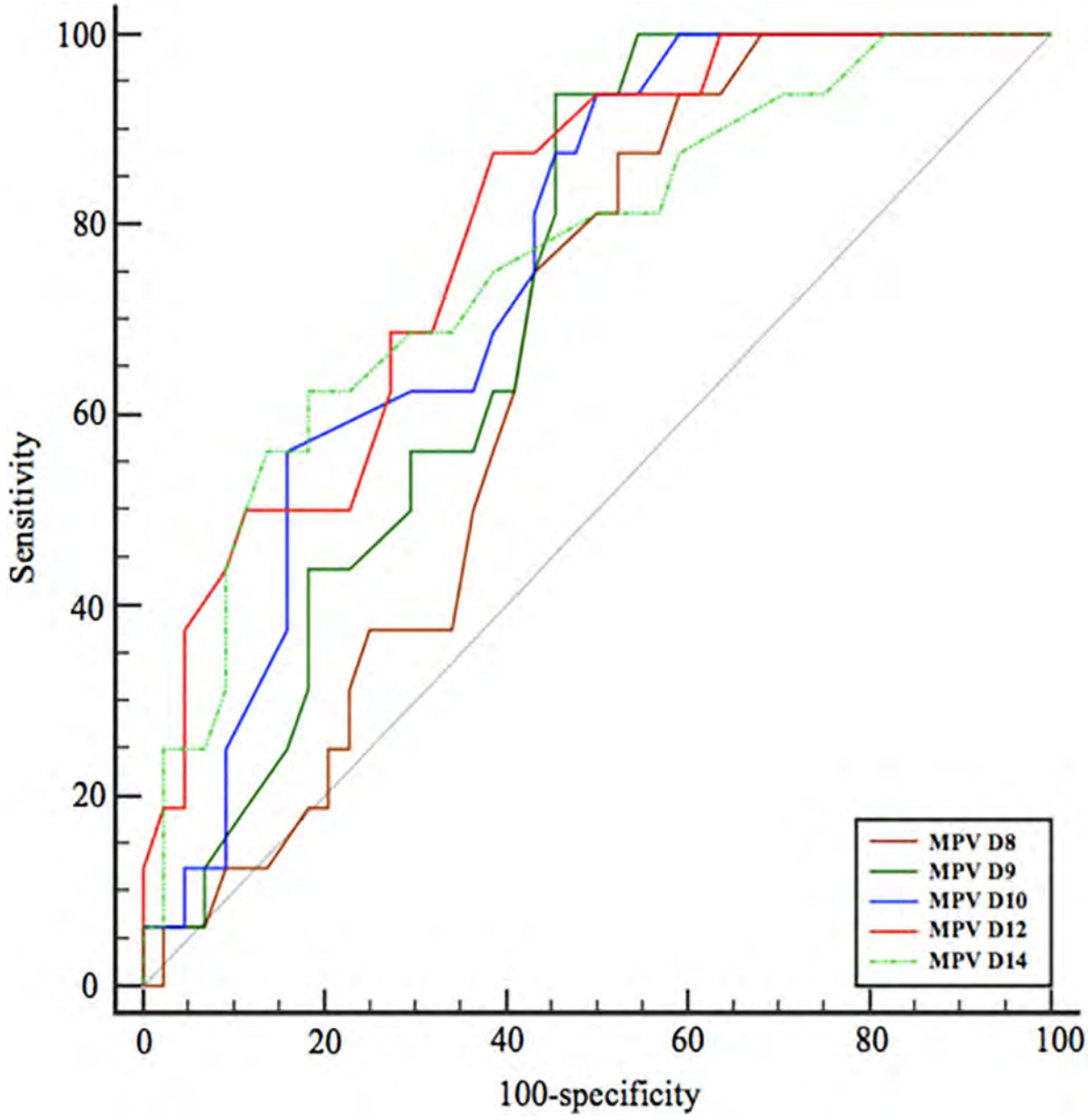

Fig 4. Comparison of areas under ROC curves according to Mean Platelet Volume.

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this study bring new insight that may be useful for the monitoring of septic patients. First, we found that 10-day MPV >11.6 fL was an independent predictive factor of 90-day mortality. Second, we verified the significant increase of MPV during the first 15 days after ICU admission in non-survivors compared to survivors. Third, we confirmed that platelet count in the survivors group, after an early drop, returned to the admission value during the first week whereas it did not in the non-survivors group. The AUC values and multidimensional analysis (COX and CART) showed that MPV was an important factor linked to mortality not sufficiently discriminating if used alone. Other covariates (usually used for mortality prediction in sepsis shock) should be associated, including base excess and lactic acid, as shown by the thresholds of these covariates for the partitioning of patients using CART analysis. This is also

Table 2. Comparison of area under the curves of continuous clinical and biological variates discriminating survivors and nonsurvivors.

	AUC	95% CI	Threshold	Sensitivity	Specificity	PPV	NPV
MPV (fL) (Day 10)	0.76	0.63–0.86	> 11.6	57.45	82.28	49.1	86.7
SOFA Score	0.74	0.69–0.79	> 11	73.53	62.81	50.3	82.2
Ratio MPV/platelet (Day 10) (%)	0.73	0.59–0.84	> 4.14	72.73	64.08	38.6	88.3
Base excess (mmol/L) (Day 4)	0.653	0.58–0.72	≤ 1.9	84.48	41.89	36.3	87.3
Lactic acid (mmol/L) (Day 0)	0.614	0.42–0.78	> 5.2	37.11	80.98	50.7	71.0

MPV: Mean Platelet Volume; SOFA: Sequential Organ Failure Assessment; PLT: Platelet; AUC: Area under the curve; CI: Confidence Interval; PPV: positive predictive value, NPV: Negative predictive value

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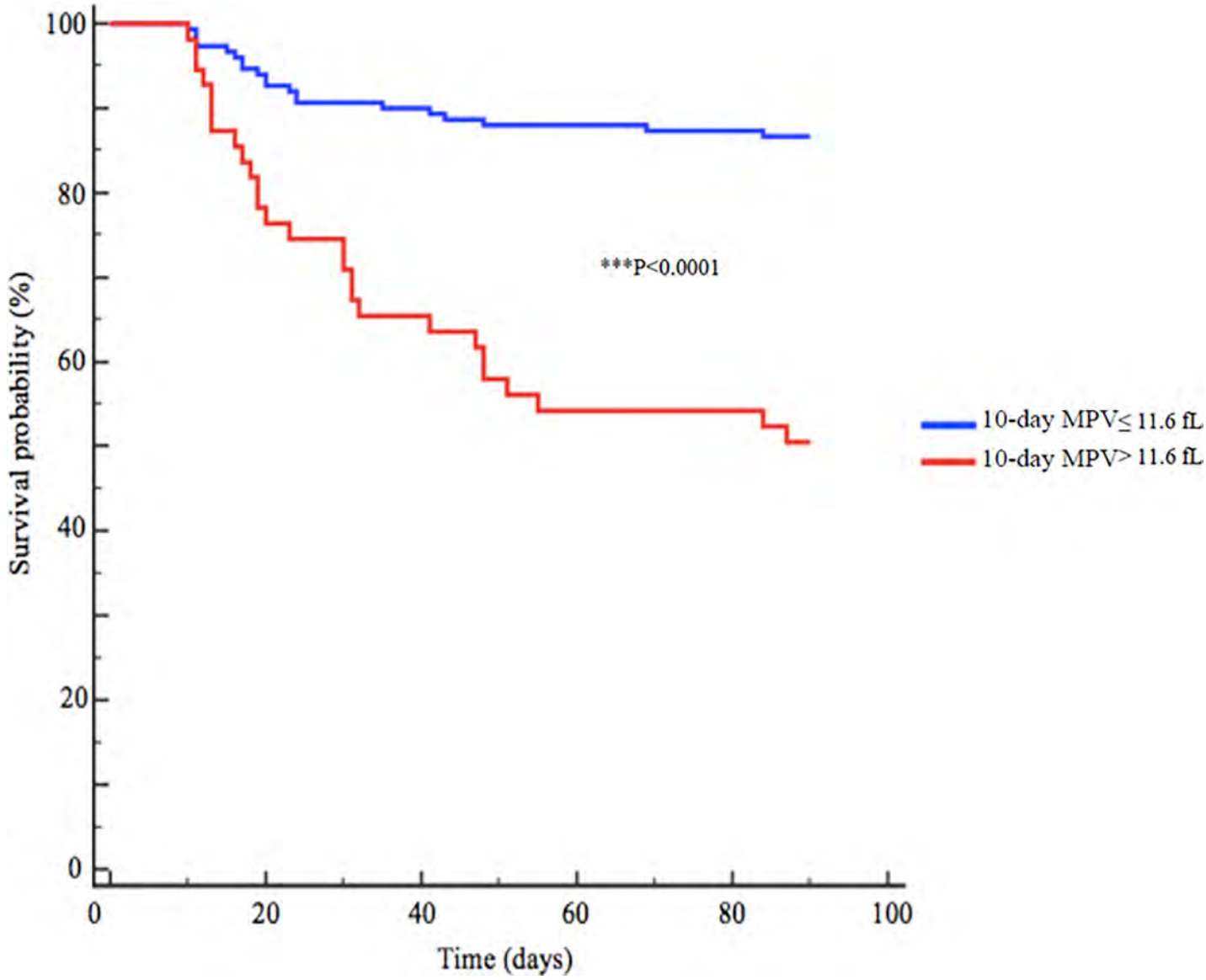

Fig 5. Survival probability Kaplan Meier curve depending on 10-Day MPV.

https://doi.org/10.1371/journal.pone.0223553.g005

Table 3. Multivariate analysis (Cox Model).

Significant Covariates	Hazard-Ratio	95% CI	*P value*
10-day MPV >11.6 fL	3.796	1.96–7.35	0.0001
4-day Base Excess <1.9 mmol/L	2.972	1.38–6.40	0.0054
Variables not included			
Renal Replacement Therapy	1.596	0.77–3.29	ns
SOFA > 11	0.957	0.47–1.96	ns

MPV: Mean Platelet Volume; SOFA: Sequential Organ Failure Assessment; CI: Confidence Interval; ns: not significant. Overall model significance with P—0.0001—AUC 0.76 [0.69 to 0.82]

https://doi.org/10.1371/journal.pone.0223553.t003

the first time that a multidimensional analysis has been completed by a population partitioning in a study on MPV, thus refining the predictability of survival in septic shock with relevant covariates.

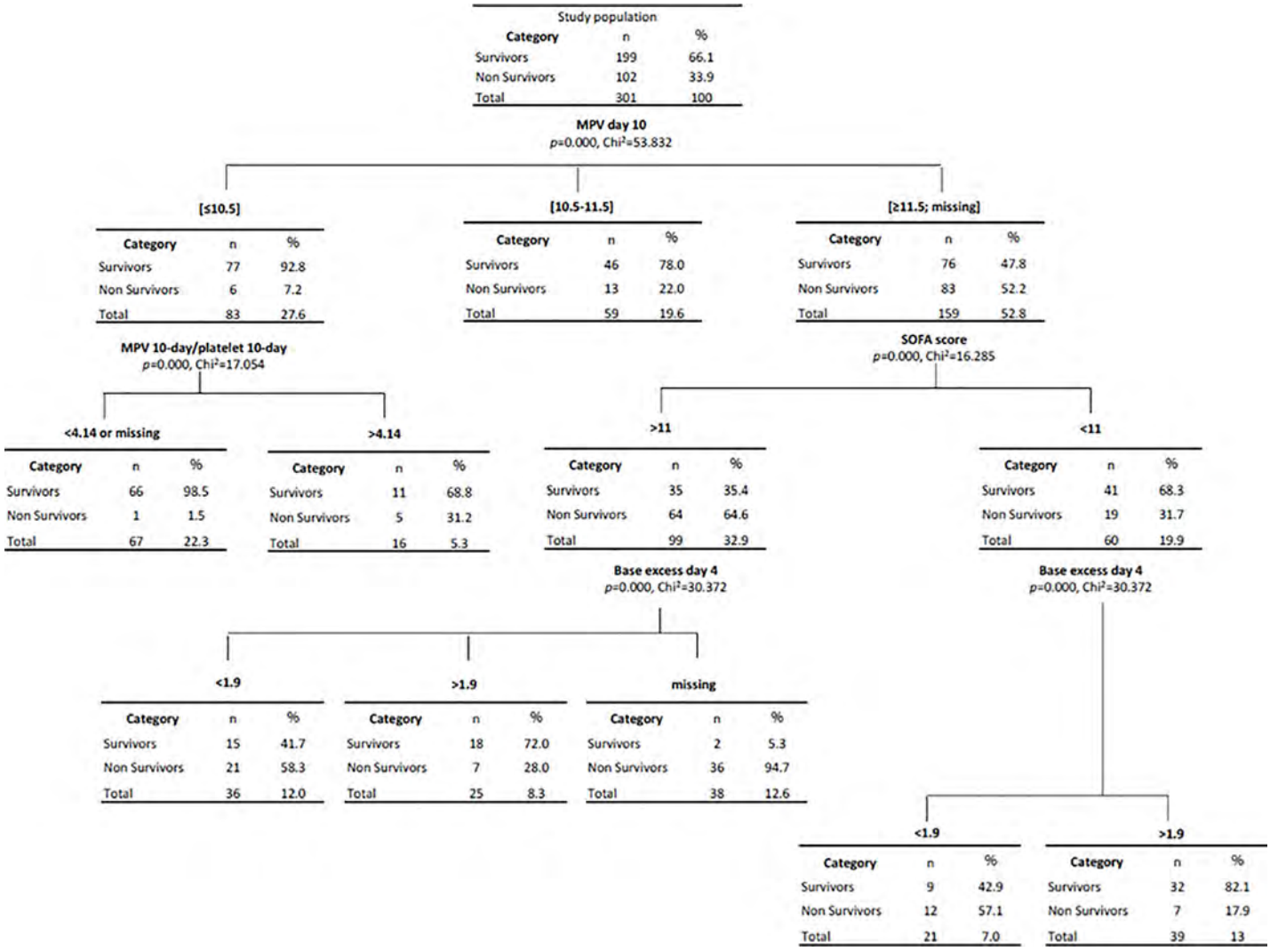

Fig 6. Classification and regression tree (CART) according to 10-day MPV, 10-day MPV/platelet ratio, SOFA score and 4-day base excess.

https://doi.org/10.1371/journal.pone.0223553.g006

Concerning the MPV values, we observed a significant difference from the first day of admission in ICU for septic shock between survivors and non-survivors. Moreover, in the deceased patients group, there was a gradual increase in the MPV values during the first 15 days following the onset of septic shock, whereas, in the survivors group, MPV increased during two days, remained stable until day 7 and then decreased. An increase in MPV value could be associated with uncontrolled infection, as well as linked to illness severity and patient outcome [15,16]. Some studies have highlighted an increase in MPV in infectious endocarditis associated with embolic complications and death while the MPV value decreased in completely healed patients [15,16]. The dosage of MPV and its monitoring during sepsis is relatively simple and would track the evolution of the disease. Several studies have previously demonstrated that the increase in MPV was statistically significant in the first 3 days of gram-positive sepsis [7], could predict 28-day mortality in septic shock [8], and was a risk factor for poor clinical outcome.

Daily monitoring of MPV value would stratify the risk of death in these patients. Special attention should be paid to the evolution of MPV during the first week after the onset of sepsis because the lack of return to its starting value is correlated to an unfavorable outcome. Indeed, septic patients with increased MPV value without a return to the base value are most likely to die and should be tracked in order to improve their management. Some authors propose to use MPV/platelet ratio on admission and at 24 hours to predict mortality at 28 days [10] and that is why this ratio was included in our CART analysis.

The increase in MPV is an important platelet production index that has been shown to correlate with increased platelet reactivity. Beside the physiological platelet production process, previous study has shown that in response to a sharp fall in the platelet count, a rapid production of platelets was possible after megakaryocyte rupture with cytoplasmic fragmentation [13]. Thanks to this alternative mechanism, a large number of platelets is released rapidly into the blood stream with a high proportion of thrombocytes with a large VMP and hence a risk of procoagulant phenotype[16]. As a result, these large platelets are more activated than smaller platelets. An explanation can be that larger platelets, indicating an increased MPV, have more intracellular thromboxane A2 and increased levels of procoagulant surface proteins, such as P-selectin and glycoprotein IIIa, thus presenting a greater prothrombotic potential [17].

MPV is a strong predictive factor of mortality, particularly after day 10. However, rather than the absolute value, kinetics of MPV values appeared to be of major significance, with an absence of decrease in the non-survivors group. Our findings are consistent with a study showing that an increase in MPV after admission to an ICU was independently associated with higher hospital mortality [18]. These findings suggest that progressive MPV increase during sepsis without returning to the initial value is linked to a more severe illness. The trends in changes in MPV and platelets counts are more reliable markers of poor prognosis than the corresponding absolute values.

Platelet count was not different during the first 4 days of the disease between the two groups, and became significantly different at day 6 until the end of the observation (day 15). Platelet count kinetics generally corresponds to a biphasic course that has previously been reported in patients after surgery [19,20] and acute myocardial infarction [21] and could be a physiologic response to stress. In critically ill patients, a similar biphasic pattern has been reported in a study of 18 surgical patients with severe sepsis [22]. Indeed, non-survivors had persistent thrombocytopenia, whereas survival was related to the degree of thrombocytosis within 2 weeks. In critically ill patients, the evolutionary profile of the platelet count is different depending on whether the patient will survive or not. It has even been shown that late thrombocytopenia was more predictive of death than its early onset [22]. In our cohort, the platelet count decreased significantly in the first days after admission to reach a nadir on day 3 in

survivors or day 4 in non-survivors. In the survivor group, the platelet count returned to the admission value by the end of the first week and continued to rise to become significantly greater than the admission value by day 8 whereas, in the non-survivors group, platelet count never returned to the admission value during follow-up.

Aydemir et al. have evaluated kinetics of platelet counts and MPV in adult with sepsis to determine whether the responses were specific to the type of infection [7]. They found a day of nadir different between Gram-positive septic patients, gram-negative septic patients and fungal septic patients. They concluded that fungal sepsis had a stronger association with thrombocytopenia and increased MPV. We did not find a difference in platelet counts according to the pathogen agent. This may be explained by the fact that the authors excluded all patients deceased before day 10 or with negative blood cultures contrary to our study. Previous studies have already reported such conflicting results [23,24]; therefore, it is difficult to predict the type of pathogen agent depending on the kinetics of MPV or platelet count.

Mean platelet volume therefore represents a prognostic marker of interest in septic shock and its value is higher in patients who will die. Similar results have previously been shown in other diseases than sepsis. MPV appeared to be as a useful marker for early mortality and neurologic outcomes in patients who achieved return of spontaneous circulation after out-of-hospital cardiac arrest [25]. An elevated MPV was independently associated with increased 30-day mortality, with the highest discriminative value being obtained upon admission after cardiac arrest. An elevated MPV on admission was also associated with poor neurologic outcomes [26]. In another study, MPV was an independent predictor of the risk of stroke among individuals with a history of stroke or transient ischemic attack. Concerning inflammatory diseases like rheumatoid arthritis, MPV has been correlated with inflammatory markers and with the measures of disease activity [26,27].

Other indices of platelets have been described, including platelet volume distribution width (PDW), plateletcrit (PCT), and platelet large cell ratio (PLCR). All these indices can be measured by an inexpensive and readily available routine blood count; however their use and application in septic shock remains unknown [7] and these parameters are not routinely done in our lab, and have not been reported for this study.

The novelty of the study is the use of classification and regression tree (CART) methodology that is a recursive partitioning method for predicting continuous dependent variables (regression) and categorical predictor variables (classification). It is a simple, accurate prediction model for outcome in patients with septic shock easily usable for clinicians [14]. Models are easy to read and interpreted using a flow chart diagram.

Our study presents limitations. The retrospective and monocentric nature of the study can limit the external validity of the results. The variation of standard MPV value between different laboratories is a weak point for the realization of a larger scale study. However, our population was similar to that reported in the literature in terms of age, severity and site of infection [10,28]. 90-day mortality in our study was 33.9% which was comparable with most studies [8,10] and in a recent publication reporting the "third International Consensus definitions for sepsis and septic shock"[1]. We did not have plateletcrit values, at the time of the study that could have been markers of interest.

Conclusion

Mortality related to sepsis and septic shock remains high. Our work confirms the existence of early biological anomalies such as thrombocytopenia and biological markers of tissue hypoperfusion (pH, base excess, lactic acid). Our results suggest that an increase in MPV value is correlated with mortality. 10-day MPV and 4-day base excess values were the main covariates

predicting 90-day survival. Based on these data, we propose a segmentation analysis with 80% predictability of 90-day mortality. This study shows that particular attention must be paid to platelet counts and MPV value variations in septic shock.

Supporting information

S1 Table. Spearman rank correlation between platelet count and MPV.
(DOCX)

S2 Table. Correlation coefficients between SOFA score at admission and MPV.
(DOCX)

S1 Fig. Comparison of serum lactic acid kinetics according to 90-day survival.
(TIF)

S2 Fig. Comparison of blood pH kinetics according to 90-day survival.
(TIF)

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Les 2 articles suivants sont des revues de la littérature publiées en parallèle des travaux de recherche. Elles abordent dans un premier temps le rôle fondamental joué par les plaquettes dans le sepsis (Vardon-Bounes F, Int J Mol Sci 2019). Nous y reprenons les éléments évoqués dans la revue de la littérature rédigée en début de manuscrit. Le second article passe en revue le rôle des lipides bioactifs produits par les plaquettes et leur participation aux phénomènes d'hémostase et de thrombose, mais aussi dans le maintien de l'intégrité du mur vasculaire, dans l'inflammation, le remodelage tissulaire et la cicatrisation des plaies (Vardon-Bounes F, Advances in Biological Regulation, 2018).

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Review

Platelets Are Critical Key Players in Sepsis

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Abstract: Host defense against infection is based on two crucial mechanisms: the inflammatory response and the activation of coagulation. Platelets are involved in both hemostasis and immune response. These mechanisms work together in a complex and synchronous manner making the contribution of platelets of major importance in sepsis. This is a summary of the pathophysiology of sepsis-induced thrombocytopenia, microvascular consequences, platelet-endothelial cells and platelet–pathogens interactions. The critical role of platelets during sepsis and the therapeutic implications are also reviewed.

Keywords: platelets; sepsis; endothelium; immunothrombosis

1. Introduction

The definition of sepsis has recently been modified. According to the Third International Consensus Definitions for Sepsis and Septic Shock, it is defined as a "life-threatening organ dysfunction caused by a dysregulated host response to infection" [1]. It is commonly admitted that sepsis management requires monitoring and intervention, including admission to the emergency department of the intensive care unit, if necessary. When circulatory and cellular metabolism anomalies occur, sepsis is called "septic shock". These anomalies significantly increase morbimortality. The number of hospitalizations for sepsis continues to grow, which highlights the importance of having a clearer understanding of the pathogenesis to aid in future improvements [2].

The development of multiple organ failure (MOF) increases sepsis-related mortality. MOF is partly due to endothelial dysfunction with hyperpermeability and microvascular thrombosis. Extensive microvascular thrombosis impairs oxygen delivery to cells [3]. This phenomenon results in tissue ischemia and cellular hypoxia leading to partial or complete inhibition of organ function. For example, hepatic arterioles occlusion leads to a decrease in hepatic function and hepatic impairment. Platelets and coagulation are both involved in thrombosis which is generally considered to be a pathological deviation of hemostasis [4]. However, recent findings suggest that intravascular thrombosis also involves processes that are distinct from hemostasis and which occur mainly in pathological situations such as sepsis. The participation of neutrophils and monocytes, as well as dendritic cells leads to a "thrombosis-related signature" which initiates and propagates fibrin formation and triggers platelet activation during the development of thrombosis. Recent works mention a phenomenon named "immunothrombosis". This suggests that under certain circumstances, thrombosis is a physiological process which constitutes an effective mechanism of innate immunity in which platelets play an important part [4].

2. Thrombocytopenia is Common in Sepsis and is Correlated to Mortality

Thrombocytopenia is generally defined as platelet count <150 G L^{-1} and it is classified as severe if platelet count is <50 G L^{-1} [5–7]. It is the most common hemostatic disorder in the intensive care unit (ICU) with a prevalence of approximately 50% [8]. Interestingly, platelet count is a part of the SOFA score (Sepsis-related Organ Failure Assessment) [9] which aims to assess the severity of organ dysfunction in critically ill patients.

Between 5% and 20% of patients develop severe thrombocytopenia that can be associated with bleeding and even a moderate-degree thrombocytopenia is associated with organ failure and an unfavorable prognosis. Thrombocytopenia in patients admitted to the ICU is well recognized as a poor prognostic sign [9–11] and is associated with a prolonged ICU stay [10,12,13]. Akca et al. reported that there was a biphasic temporal pattern in the way platelet counts changed in a large population of medical and surgical ICU patients [14]. They found an initial acute decrease followed by an increase in the platelet count. The authors showed that thrombocytopenia was associated with an increase in the rate of mortality. Moreover, mortality was associated with prolonged thrombocytopenia and the absence of a relative increase in platelet count. Platelet count reached nadir on day four. Thrombocytopenia was more predictive of death on day 14 than earlier in the ICU (66% mortality versus 16% for patients without thrombocytopenia, $p < 0.05$). At the same time in a large database of critically ill medical and surgical patients, Moreau et al. demonstrated that a 30% decline in platelet count on day 4 strongly predicted hospital mortality which is a better predictive value than the absolute number [15]. Vanderschueren et al. reported that the SAPS II (Simplified Acute Physiology score) and occurrence of thrombocytopenia remained significantly related to ICU mortality (odds ratio (OR) = 4.2, 95% confidence interval (CI) 1.8–10.2) [13].

Sepsis accounts for approximately 50% of all thrombocytopenia in the severely ill [16]. In septic patients, thrombocytopenia is frequently associated with a dysregulated host response [17,18]. The SAPS II is an inexpensive and easy-to-perform test that could serve as an early alert for clinicians.

Thrombocytopenia is a risk marker, rather than a cause, of mortality in the ICU. Clinicians confronted with this hemostatic disorder or a significant decrease in platelet count should actively identify and try to correct the underlying cause(s). A detailed history and careful physical examination are keys to achieving the right diagnosis. This should be supported by some laboratory results along with an interpretation of the data within the clinical context.

3. Mechanisms that Contribute to Thrombocytopenia in Sepsis

Thrombocytopenia is a common and multifactorial phenomenon occurring during sepsis. The main causes are decreased platelet production, hemodilution, platelets consumption, increased sequestration of platelets in microvessels, and immune-mediated destruction of platelets. The combination of a decrease in the production associated to an increase of platelets consumption and destruction coexists.

The first step for the clinician consists of eliminating pseudo-thrombocytopenia due to an artefact of laboratory parameters cause by in vitro platelet agglutination in EDTA-anticoagulated blood. Measuring normal platelet count in citrated blood is usually sufficient to confirm the diagnosis.

During sepsis, peripheral mechanisms of thrombocytopenia are preponderant, and a myelogram is therefore unnecessary, except in specific cases. Classically, "platelets consumption" via thrombin-mediated platelet activation is the most common mechanism. In severe forms of sepsis, disseminated intravascular coagulation (DIC) can occur and is characterized by the widespread activation of coagulation, which results in the intravascular formation of fibrin and ultimately thrombotic occlusion of small and midsized vessels [19]. It is an acquired disorder that occurs in a wide variety of clinical conditions, and particularly in septicemia. The activation of diffuse coagulation is triggered by cell-specific membrane components of the microorganism, such as endotoxin, lipopolysaccharide or bacterial exotoxins. The use and subsequent depletion of platelets and coagulation factors at the same time, resulting from the ongoing coagulation, may induce severe bleeding.

Another diagnosis that should not be overlooked in sepsis thrombocytopenia is acquired hemophagocytic lymphohistiocytosis (HLH) [20]. This rare pathology is characterized by clinical and biological abnormalities resulting from the dysregulated activation and proliferation of lymphocytes, leading to an overproduction of cytokines. The main clinical and biological data consists of fever, spleen and liver enlargement, cytopenias (thrombocytopenia, anemia and leukopenia), liver dysfunction, high serum levels of triglycerides and ferritin, and histological evidence of hemophagocytosis. Thrombocytopenia is often a warning signal when associated with other criteria suggested by the Histiocyte Society [21]. Bone marrow evaluation may reveal macrophage activation with hemophagocytosis. In severe HLH cases, multi-organ dysfunction develops and eventually leads to death, demonstrating the importance of a prompt diagnosis for early initiation of treatment.

Activated platelets have also been shown to promote neutrophil recruitment to the site of injury [22] and the formation of neutrophil extracellular traps (NETs) which trap and help kill pathogens [23]. Recently, extracellular histones were described as a cause of thrombocytopenia in critically ill patients [24]. Fuchs et al. demonstrated that NETs, which are extracellular DNA fibers comprising histones and neutrophil antimicrobial proteins, were formed inside the vasculature in infectious and noninfectious diseases [25]. They reported that NETs provided a heretofore unrecognized scaffold and stimulus for thrombus formation. NETs perfused with blood cause platelet adhesion, activation and aggregation, whereas NETs formation in animal models caused rapid and profound thrombocytopenia.

Platelet aggregation/adhesion to leukocytes and endothelial cells is a common mechanism for a type of thrombocytopenia called “immune thrombocytopenia”. Platelet-associated IgG (PAIgG) are found in 30%–40% of septic patients [12,26] and, in the ICU, 30% of thrombocytopenic patients are positive for PAIgG. This population is described as being more subject to sepsis and to have a medical history of cardiopulmonary bypass [27].

In summary, the mechanisms for thrombocytopenia in sepsis are multiple. But in most cases, sepsis resolution allows the slow resolution of this hemostatic disorder. Table 1 summarizes the main etiologies.

Table 1. Etiologies of thrombocytopenia during sepsis.

Main Etiologies of Thrombocytopenia during Sepsis
Pseudothrombocytopenia
Laboratory artefact (*in vitro* agglutination in EDTA-anticoagulated blood)
Decreased platelet production
Viral infection (EBV, CMV, HCV, HIV)
Bone marrow suppression due to medication (antibiotics, proton pump inhibitor)
Hemodilution
Massive vascular infusion of fluids
Increased platelet consumption/sequestration
Thrombin-mediated platelet activation
Disseminated Intravascular Coagulation
Acquired hemophagocytic lymphohistiocytosis (HLH)
Platelet aggregation/adhesion to leukocytes and endothelial cells
Thrombus formation in extracellular DNA fibers from neutrophil extracellular traps (NETs)
Immune-mediated destruction
IgG antibodies associated to platelets (PAIgG)
Autoantibodies directed against platelets glycoproteins
Heparin-induced thrombocytopenia

4. The Role of Platelets in Sepsis

Thrombocytes play a complex role in sepsis as they are able to modulate not only their own function but also that surrounding of cells. During sepsis, coagulation cascades and inflammatory response, together with endothelial tissue damage, constantly cause the activation of platelets which

can be further stimulated by direct interactions with pathogens (Figure 1) [28]. During hemostasis, platelets adhere and aggregate at sites of endothelial injury to form a plug which warrants vascular integrity and prevents hemorrhage. In fact, when the vascular wall is damaged, platelets immediately adhere to the subendothelium, newly exposed via the vWF (von Willebrand factor), various collagens, fibronectin, fibrinogen and other adhesive molecules such as laminin and thrombospondin. The adhesion of platelets to the injured subendothelium is ensured by three types of receptors: the Glycoprotein GPIb-V-IX glycoprotein complex (vWF receptor), Glycoprotein VI (GPVI) and $\alpha_2\beta_1$ integrin (collagen receptors), and $\alpha_{IIb}\beta_3$ integrin. At first, the circulating platelets interact with the vWF linked to collagen fibers via the GPIb-V-IX complex and subsequently with collagen via the $\alpha_2\beta_1$ integrin and the GPVI glycoprotein.

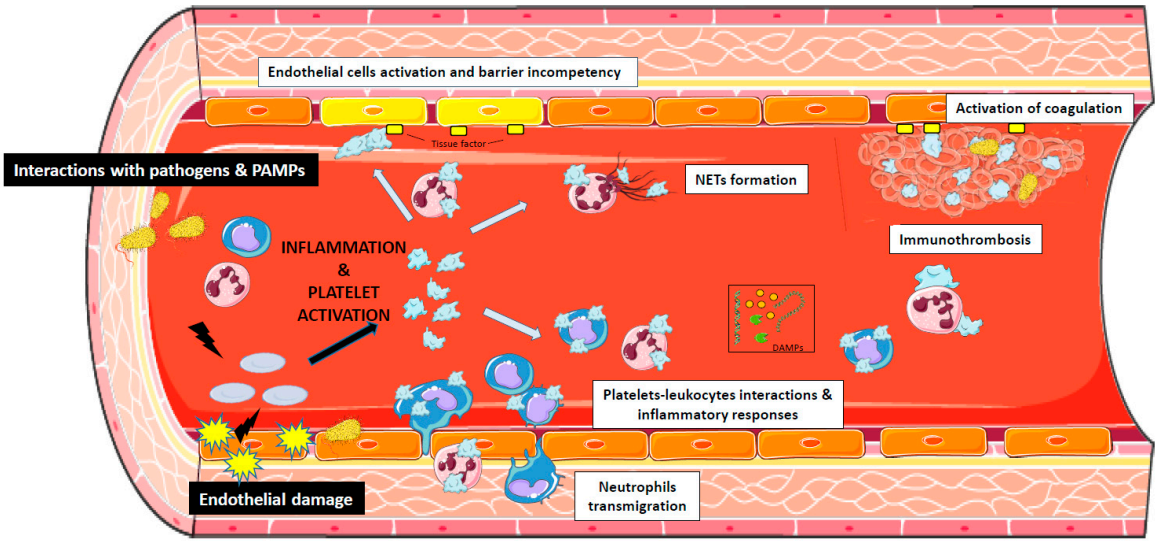

Figure 1. Selected examples of pro-inflammatory role of platelets during sepsis: endothelial damage and interactions with pathogens such as pathogens-associated molecular patterns (PAMPs) activate platelets that interact with endothelium, with monocytes and neutrophils, promoting neutrophils extra-cellular Trap (NET)osis, neutrophils transmigration, activation of coagulation through tissue factor release and immunothrombosis. DAMPs: damage associated molecular patterns (microvesicles, free DNA, protease).

These interactions allow transient adhesion of platelets to the surface of the exposed subendothelium. Platelets can then either detach from the subendothelium and return to the bloodstream (i.e., undergo translocation as long as the disc shape is maintained), or undergo rotation or "rolling" with a change in the shape of the pads that then become spherical. Platelets activate and secrete the contents of their granules. This activation also leads to a conformational change in the $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ integrins which can then only bind respectively to collagen and fibrinogen. These two integrins allow stable and firm adhesion of platelets to the subendothelium, which is then able to spread out and form a platelet monolayer.

During the various processes described above, platelet activation is amplified by soluble agonists secreted or generated by the platelet, such as ADP (Adenosine diphosphate) or TXA_2 (thromboxane A_2), or from the coagulation cascade, such as thrombin. Thrombin is the most effective activator of platelets capable of inducing a change in their shape, secretion, and aggregation. It is also the main effector in coagulation, allowing the transformation of fibrinogen to fibrin for thrombus consolidation [29]. TXA2 is a prostanoid produced from arachidonic acid by the action of COX-1 (Cyclooxygenase-1) and thromboxane synthase. As a result of its short half-life, the action of TXA2 is highly localized. Dense granules contain a high concentration of ADP which is salted out during platelet activation. Although considered a weak platelet agonist, it has now been recognized that it plays a role, in vivo,

in multiple stages of thrombosis. On activation, ADP is secreted at the site of vascular injury or it amplifies the platelet response and contributes to the stabilization of the thrombus. The interaction of soluble agonists (ADP, thrombin and TXA2) with their seven heterotrimeric G-protein-coupled transmembrane domain receptors generates "inside-out" signaling, leading to the activation of αbβ3 integrin. This mechanism enables the recruitment of circulating platelets in the thrombus which also affects its growth. Furthermore, it is becoming clear that platelets are involved in other processes such as immunity.

To begin with, platelets are able to induce the acute phase response to infection [30,31]. This acute phase response corresponds to the production of proteins such as complement proteins, fibrinogen, and C-reactive protein. These proteins destroy or inhibit the growth of microorganisms and exert procoagulant effects that may limit infection by trapping pathogens within local blood clots. During activation of the acute phase response, platelets are known to be a major source of interleukin-1β (IL-1β) which is not granule stored but is produced upon platelet stimulation (after splicing of pre-messenger RNA (mRNA)). Platelet-derived IL-1β plays a major role in inducing this acute phase response to infection.

In addition, platelets promote innate immune cells responses. Neutrophils and monocytes are the first line of innate immune defense against infection. Activated platelets drive responses in target leukocytes that modulate host response to infection. After platelet activation, they express P-selectin on their surface. Platelet–leukocyte interactions engage the P-selectin receptor P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils and monocytes [32]. Zarbock et al. showed that platelet–leukocyte interaction via P-selectin was a crucial step in the activation and recruitment of leukocytes to the lung in acute lung injury (ALI) [33].

Interactions between platelets and neutrophils are critical for cell trafficking and to deliver molecular signals. In fact, the formation of neutrophil extracellular traps (NETs) is a prime example. Clark et al. demonstrated that platelet toll-like receptor 4 (TLR4) detected TLR4 ligands in blood and induced platelet binding to adherent neutrophils [34]. These interactions lead to robust neutrophil activation and the formation of NETs. NETs ensnare bacteria within the vasculature, primarily in pulmonary capillaries and liver sinusoids. These NETs have a proteolytic activity that can trap and kill microbes in tissues. Data from Clark et al. suggest that this event only happens under extreme conditions such as severe sepsis. It should also be noted that NETs formation has recently been reported in various diseases such as non-autoimmune and auto-immune disorders [35–37]. In fact, in addition to their role in the host defense, recent data suggest that the formation of NETs contributes to the pathophysiology of many diseases such as diabetes, auto immune and renal diseases and heparin-induced thrombocytopenia [38–42]. Sreeramkumar et al. reported that neutrophil recruited to injured vessels in an animal model of inflammation, extended a domain into the lumen, where PSGL-1 clusters scanned for the presence of activated platelets [43]. Their findings revealed that the dynamic reorganization of neutrophil domains and receptors allow simultaneous interactions with both the vascular wall and activated platelets in circulation, to provide a rapid and efficient regulatory mechanism in the early inflammation process. These observations underscore the crucial role of platelet–leukocyte interactions which participate in the defense against infections during inflammation and sepsis.

Toll-like receptors are known to promote NETs formation. They are a highly preserved family of pattern recognition receptors (PRR) that bind pathogen-associated molecular patterns (PAMPs) molecules that are broadly expressed by many infectious organisms. One of the most studied PAMPs is lipopolysaccharide (LPS), which is a part of the gram-negative bacteria membrane, and it is a major TLR ligand. Platelets express numerous TLR family members. Platelet TLR4 signaling leads to platelet activation, the shedding of IL-1β-rich microparticles, and platelet interactions with others cells [44]. TLR2 platelet stimulation increases P-selectin surface expression, the activation of the integrin αIIbβ3, the generation of reactive oxygen species, and in human whole blood, the formation of

platelet–neutrophil heterotypic aggregates [45]. Other TLRs may also have platelet-related functions but their role in inflammation and sepsis remains to be studied.

Platelet adhesion and activation, vascular endothelial cell activation, innate immune cell recruitment, NET formation and fibrin deposition, all contribute to an increased propensity of thrombosis in septic conditions [46,47]. Thrombosis and microthrombosis play a major role in innate immunity and "immunothrombosis", which is the term that describes this process [4]. Immunothrombosis constitutes a line of host defense that is supported by several specific molecular mechanisms that fight against pathogen dissemination and survival. In fact, innate immune cells (especially monocytes and neutrophils) trigger immunothrombosis by their local accumulation in microvessels. They generate a procoagulant surface on microvascular endothelial cells with local delivery of tissue factor, degradation of endogenous anticoagulants, and the provision of a procoagulant matrix consisting of extracellular nucleosomes. Consequently the recruitment of platelets leads to clot growth and NETs formation by neutrophils, resulting in bacterial entrapment in microvasculature [48]. The procoagulant action of NETs involves the activation of factor XII [49] and the clearance of anticoagulants such as tissue factor pathway inhibitor (TFPI) and probably thrombomodulin [50]. In the immunothrombosis phenomenon, fibrin plays a crucial role since it has direct antimicrobial activity and may limit the spread of pathogens [51]. Indeed, fibrin network helps by retaining microorganisms circulating in the blood. Fibrin formation can be initiated by extracellular nucleosomes from NETs resulting in the direct activation of the coagulation contact pathway. Factor XII is thus activated in factor XIIa. At the same time, histones released into the NETs can result in platelet activation via toll-like 2 (TLR2) and TLR4 receptors [47,52,53]. Fuchs et al. were the first to demonstrate that NETs were able to induce platelet-adhesion molecule deposition and thrombin-dependent fibrinogen conversion into fibrin [25].

On the other hand, platelets also influence acquired immune responses including T-cell functions. They may activate platelets through an interaction involving T-cell CD40L (CD40 ligand) and platelet CD40 causing them to release RANTES (Regulated upon Activation, Normal T cell Expressed, and Secreted) which binds to endothelial cells and mediates T-cell recruitment [54]. Platelet CD40L is able to interact with many other cells besides lymphocytes and dendritic cells, granulocytes, fibroblasts, macrophages and monocytes, including other platelets [55–57].

5. Platelets and Endothelial Cells Interactions During Sepsis

Endothelial cells play a central role in a host's response to sepsis, including inflammation, coagulation, and vascular permeability. Endothelial cells are not classically considered to be immune cells but they express innate immune receptors such as TLRs, which can be activated by pathogen components or a myriad of host-derived factors, including complement, cytokines, leucocytes, fibrin, and activated platelets and leukocytes [58].

The surface of the vascular endothelium is covered by a structure called a "glycocalyx", a gel-like layer, which regulates thrombus formation, vascular permeability, and inflammation. The glycocalyx is comprised of a membrane-binding domain containing core proteins (such as proteoglycans and glycosaminoglycans) and plasma proteins (such as albumin and antithrombin). Under inflammatory conditions, the glycocalyx is disrupted by glucuronidases, reactive oxygen species (ROS), and other proteases. The shedding of this structure results in the synthesis and exposure of adhesion molecules, such as P-selectin, E-selectin and Intercellular Adhesion Molecule 1 (ICAM-1), and subsequently in the recruitment of leukocytes and platelets [59,60]. On the other hand, the activated endothelial TLRs induce a shift to an endothelial procoagulant phenotype, with a decrease in the synthesis of tissue factor pathway inhibitor (TFPI), tissue plasminogen activator (tPA) and heparan, and an increased expression of tissue factor (TF) and plasminogen activator inhibitor 1 (PAI-1) [58]. TF exposure to blood leads to thrombin formation which in turn activates platelets and converts fibrinogen into fibrin. The activated platelets may in turn also accelerate fibrin production [61].

This complex interaction between circulating platelets, endothelial cells, and subendothelial structures is mediated by cellular receptors on the surface of platelets and endothelial cells, such as integrins and selectins, as described above, and by adhesive proteins, such as fibrinogen and von Willebrand factor (vWF). vWF allows initial platelet adhesion to the injured vessel wall by binding the platelet receptor GPIb-IX-V. Large and ultra-large vWF multimers, the most active forms in the promotion of platelet aggregation are cleaved by A Disintegrin and Metalloproteinase with Thrombospondin type 1 motif-13 (ADAMTS-13). The release of significant ultra-large vWF multimers following endothelial damage, associated with a decrease in its cleavage by a consumption of ADAMTS-13, could explain the increase of the platelet-vessel wall interaction during sepsis [62].

6. The Interaction of Bacterial Pathogens with Platelets

Several research teams have evaluated the ability of bacteria to bind to platelets. Interactions are broadly represented by three types of mechanisms: 1) secretion of bacterial products (i.e., toxins that interact with platelets), 2) direct bacterial binding to a platelet receptor, and 3) binding of a plasma protein, which is a ligand for a platelet receptor, to bacteria [63].

Bacteria can induce platelet aggregation with a characteristic lag-time that varies from seconds to several minutes. This aggregation is an "all-or-nothing" phenomenon.

Bacteria secrete toxins that can activate platelets. For example, *Staphyloccocus aureus* secretes a major cytolysin called α-toxin. Arvand et al. showed that α-toxin promotes blood coagulation via the exocytotic release of factor V from α-granules and the enhanced capacity of platelets to bind external factor V leading to the assembly of prothrombinase complexes. In addition, α-toxin binds to the platelet lipid bilayer creating a transmembrane pore that leads to an influx of calcium-triggering platelet activation [64]. Lourbakos et al. showed that cysteine proteases produced by *Porphyromonas gingivalis* (called gingipains) induce an increase in intracellular calcium in human platelets and cause platelet aggregation with an efficiency comparable to thrombin [65]. Gingipains appeared to activate the protease-activated receptors (PAR-1 and PAR-4) expressed on the surface of the platelets.

Platelets can also interact with bacteria via direct interactions. Different species of bacteria contain mimetic ligand motifs that act as agonists on platelet receptors. Lipopolysaccharide from *Escherichia coli* can bind to TLR4 and *S. pneumoniae* lipopeptide to TLR2. Recently, reports have demonstrated that *S. epidermidis*, *S. aureus,* and *S. gordonii* expressed proteins could directly bind to GPIIb-IIIa in the absence of a bridging molecule [66–68]. Other platelet surface proteins can bind to bacteria. Bacterial components are able to bind indirectly to platelet receptors via different proteins such as fibrinogen, fibronectin, and von Willebrand factor [69]. In fact, several strains of bacteria can bind to plasma proteins that bridge with their specific platelet receptors, resulting in their activation [69]. For example, *Staphylococcus aureus* can bind vWf that interacts with GPIbα platelet receptor thus triggering its activation [70]. *S. aureus* can also bind via clumping factor A (ClfA) several plasma proteins such as fibrinogen or fibronectin that can in turn bind to GPIIb-IIIa [71,72].

7. Therapeutic Implications

Platelet activation contributes to microvascular thrombosis and organ failure in systemic inflammation, particularly in septic conditions. Based on this observation, the question of whether drugs that inhibit platelet activation may have a benefit in critically ill patients arises, particularly in septic shock to limit microvascular occlusion and multiple organ failure.

Preclinical evidence for aspirin and $P2Y_{12}$ inhibitors in sepsis in animal models is already published. In fact, Winning et al. pre-treated mice with clopidogrel for four days prior to intraperitoneal administration of endotoxin (LPS) from *E. coli*. Clopidogrel eliminated the LPS-induced drop in platelet count and reduced fibrin deposition in lung tissue [73].

In a model of abdominal sepsis, Rahman et al. showed that pulmonary infiltration of neutrophils was reduced by 50% in ticagrelor-treated animals [74]. Moreover, ticagrelor abolished CLP-provoked lung edema and decreased lung damage score by 41%. Another study showed that mice injected with

Salmonella enteritidis endotoxin and pre-treated with aspirin 30 minutes prior to infection exhibit a significant 24-hour survival rate benefit with different dosages of aspirin [75]. However, the results of the animal studies are sometimes contradictory. In a recent article, in a mouse model of septic shock by cecal ligation and puncture, the authors did not find any protective role of the $P2Y_{12}$ purinergic receptor using mice deficient for the $P2Y_1$ receptor and with clopidogrel.

The $P2Y_{12}$ receptor has also been studied for his modulation in inflammation. In $P2Y_{12}$ null mice, clopidogrel is shown to have pleiotropic effects, especially on neutrophils, with an effect on their number, even in the absence of a platelet receptor [76].

Class I phosphoinositide 3-kinase β (PI3Kβ) could be an interesting target for antithrombotic therapy in sepsis. In fact, this PI 3-kinase isoform is shown to play an important role in thrombus formation and stability [77–81]. In vivo, isoform-selective PI3Kβ inhibitors eliminate occlusive thrombus formation but do not prolong bleeding time. However, such treatments have not yet been tested in this indication in phase II trials. Interestingly, even though invalidation of the catalytic subunit of PI3K β (p110β) specifically in platelets is known to induce instability of the thrombus at high shear rate [80], it was shown in a mouse model of sepsis that septic conditions reversed the thrombus instability at a high shear rate, bringing out an alternative mechanism enabling platelets to form stable thrombus [82].

Clinical data on the use of antiplatelet agents in critically ill septic patients also reported encouraging results [83]. In a retrospective study, Eisen et al. showed a strong association between acetyl salicylic acid (ASA) and survival in the ICU [84]. The ASA group had a 10.9% in-hospital mortality compared with 17.2% in the nonusers after propensity matching.

However, there is a lack of prospective randomized controlled studies. Valerio-Rojas et al. performed a retrospective cohort study of severe sepsis and septic shock in adult patients. They showed that antiplatelet therapy was associated with a decreased incidence of acute lung injury and acute respiratory distress syndrome.

Otto et al. analyzed the medical records of 886 septic patients who were admitted to the surgical ICU of a university hospital [85]. Logistic regression analysis indicated that patients who were treated with ASA (100mg/d) had a significantly lower mortality.

Given the inherent limitations of observational studies, only randomized controlled trials could answer the question concerning the interest and effectiveness of antiplatelet agents to reduce organ failure and morbimortality. Currently, there are two ongoing relevant clinical trials. "Aspirin for the treatment of sepsis" (NCT01784159) involves the beneficial effect of seven days of aspirin treatment on organ dysfunction and the duration of ventilation in severe septic patients. The other trial is "Aspirin to inhibit sepsis (ANTISEPSIS, ACTRN12613000349741) which examines the effect of daily aspirin administration on mortality and admission to the ICU for sepsis. The results of these trials will help to elucidate the role of aspirin in treating sepsis.

8. Conclusions

Platelets play key roles against infection and are involved in various mechanisms to promote the immune response and the activation of coagulation. Thrombocytopenia is common in the ICU during sepsis, causes are multiple, and low platelet count is correlated with poor outcome. A better understanding of platelet activation mechanisms and crosstalk between endothelial cells, immune cells, and pathogens would provide the perspective to target several deleterious pathways in sepsis, particularly in platelet activation.

Conflicts of Interest: The authors declare no conflict of interest.

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The importance of blood platelet lipid signaling in thrombosis and in sepsis

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ABSTRACT

Blood platelets are the first line of defense against hemorrhages and are also strongly involved in the processes of arterial thrombosis, a leading cause of death worldwide. Besides their well-established roles in hemostasis, vascular wall repair and thrombosis, platelets are now recognized as important players in other processes such as inflammation, healing, lymphangiogenesis, neoangiogenesis or cancer. Evidence is accumulating they are key effector cells in immune and inflammatory responses to host infection. To perform their different functions platelets express a wide variety of membrane receptors triggering specific intracellular signaling pathways and largely use lipid signaling systems. Lipid metabolism is highly active in stimulated platelets including the phosphoinositide metabolism with the phospholipase C (PLC) and the phosphoinositide 3-kinase (PI3K) pathways but also other enzymatic systems producing phosphatidic acid, lysophosphatidic acid, platelet activating factor, sphingosine 1-phosphate and a number of eicosanoids. While several of these bioactive lipids regulate intracellular platelet signaling mechanisms others are released by activated platelets acting as autocrine and/or paracrine factors modulating neighboring cells such as endothelial and immune cells. These bioactive lipids have been shown to play important roles in hemostasis and thrombosis but also in vessel integrity and dynamics, inflammation, tissue remodeling and wound healing. In this review, we will discuss some important aspects of platelet lipid signaling in thrombosis and during sepsis that is an important cause of death in intensive care unit. We will particularly focus on the implication of the different isoforms of PI3Ks and on the generation of eicosanoids released by activated platelets.

1. Introduction

Blood platelets are anucleated circulating cells playing an essential role in hemostasis and thrombosis. Following vessel injury, they rapidly form a plug to prevent bleeding. Platelets are highly reactive to extracellular stimuli through the activation of a variety of specific membrane receptors for soluble agonists or adhesive proteins allowing platelet adhesion, activation and aggregation to form a plug at the site of vascular injury (Furie and Furie, 2008). Defective platelet activation is associated with hemorrhagic disorders (Wei et al., 2009). Conversely, platelet hyperactivity in various diseases can provoke adverse effects associated to arterial thrombosis,

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a major cause of death and disability worldwide (Jackson et al., 2009). This is especially the case in coronary artery disease where activated platelets can obstruct blood circulation during atherothrombosis leading to ischemic complications and myocardial infarction (Badimon and Vilahur, 2014; Davi and Patrono, 2007). The formation of a platelet-rich thrombus is a highly regulated and structured process involving platelet adhesion and aggregation through the coordination of complex intracellular signaling mechanisms (Furie and Furie, 2008; Jackson et al., 2009; Welsh et al., 2014). Following vascular injury von Willebrand factor (vWF) binds to collagen fibers of the subendothelial matrix allowing platelets to tether to vWF via the GpIb-IX-V complex. Platelets then bind to collagen via GPVI and α2β1 leading to integrin-dependent stable adhesion. Activated platelets secrete soluble agonists such as adenosine 5′-diphosphate (ADP) and thromboxane A_2 (TXA_2) which, together with thrombin generated by the coagulation cascade, further activate platelets leading to their aggregation and the stabilization of the platelet-rich thrombus. Following rupture of an unstable atherosclerotic plaque, comparable processes occur but platelets are also strongly activated by components of the plaque leading to the formation of a massive thrombus which can be partly or totally occlusive thus preventing oxygen supply.

Besides their well characterized functions in hemostasis and thrombosis, it is becoming clear that platelets are also involved in the onset of immune and inflammatory responses and are thus at the crossroad of hemostatic and immune surveillance (Li et al., 2017).

Platelet activation is associated with profound changes in lipid metabolism and several newly generated lipids are strongly involved in platelet activation processes, thrombus formation and immune cells modulation (Slatter et al., 2016; O'Donnell et al., 2014). Some of these lipids and their metabolizing enzymes will be discussed below.

2. Lipid signaling in platelet activation and thrombosis

Phosphoinositides (PIs) are glycerophospholipids accounting for about 10–15% of membrane phospholipids. They are crucial molecules in the regulation of platelet production by megakaryocytes and platelet signaling (Min and Abrams, 2013). The different PIs specifically interact with proteins to organize the spatiotemporal formation of multi-protein complexes involved in intracellular signaling, vesicular trafficking or cytoskeleton dynamics (for reviews see Di Paolo and De Camilli, 2006; Viaud et al., 2016). A set of specific kinases and phosphatases ensure production, degradation and inter-conversion of PIs. Several of these enzymes are directly involved in pathologies such as cancer and genetic or infectious diseases and specific PIs kinases are now targeted by selective inhibitors in clinic (Viaud et al., 2016).

The phospholipases C (PLC) pathway uses phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P_2) to produce the second messengers inositol trisphosphate (IP3) and diacylglycerol (DAG). This pathway, using either PLCβ downstream of G-protein coupled receptors (GPCR) or PLCγ downstream of immunoreceptor tyrosine-based activation motif (ITAM)-coupled receptors, is crucial for platelet activation (Harper and Poole, 2010; Varga-Szabo et al., 2009). Phosphoinositide 3-kinases (PI3Ks) are also key players in different aspects of platelet production and activation. Class I, II and III PI3Ks are expressed in platelets. Class I PI3Ks (α, β, γ, δ) generate the second messenger phosphatidylinositol 3,4,5 trisphosphate (PtdIns(3,4,5)P_3) at the plasma membrane following stimulation by various agonists. This lipid interacts with several PH-domain containing signaling proteins such as Akt (PKB) (Payrastre and Cocco, 2015) or RASA3 (Schurmans et al., 2015) to organize, in a spatio-temporal manner, functional signaling complexes. Using biochemical, pharmacologic and genetic approaches, it has been shown that class I PI3Kβ is a key isoform in platelet signaling (for reviews see Laurent et al., 2014; Guidetti et al., 2015). This isoform is activated by GPCR, ITAM-coupled receptors and integrins and its stimulation is strongly increased by the Gi pathway, likely through β/γ subunits binding to p110β catalytic subunit. Interestingly, inhibition of class I PI3Kβ prevents occlusive thrombus formation in mouse, rat and dog with a limited increase in bleeding risk and acceptable safety in humans (Jackson et al., 2005; Nylander et al., 2012, 2015). Using genetic and pharmacological approaches we have recently shown the strong implication of class I PI3Kβ in thrombus stability at high shear rate (Laurent et al., 2015). In these conditions, PI3Kβ inhibition causes thrombus fragmentation in the blood stream with a potential risk of distal embolization in downstream microvessels. Association of class I PI3Kβ inhibitor and aspirin has been shown to be of potential interest as antithrombotic treatment (Nylander et al., 2015). However, whether this association can prevent arterial thrombosis without increasing the risk of bleeding and of distal embolization remains to be clearly demonstrated. Concerning the other class I PI3Ks isoforms; PI3Kδ is weakly implicated in platelet activation (Senis et al., 2005), PI3Kγ is mainly activated downstream of the ADP receptor and contributes to platelet activation (Hirsch et al., 2001) and PI3Kα is involved in platelet priming through IGF1 (Blair et al., 2014; Hers, 2007; Kim et al., 2007) and activation by low doses of collagen (Gilio et al., 2009; Laurent et al. in preparation).

Class II and III PI3Ks do not produce the second messenger PtdIns(3,4,5)P_3 but are thought to generate PtdIns3P and, likely for class II, PtdIns(3,4)P_2. PtdIns3P binds to protein domains such as FYVE and PX domains and is implicated in the regulation of vesicular trafficking, particularly in the endosomal pathway and in autophagy (Schink et al., 2013). Class II PI3Kα and β as well as class III PI3K (Vps34) are expressed in megakaryocytes and platelets and their role in theses cells is just emerging (Valet et al., 2016). Class II PI3Kα (PI3KC2α) has been shown in two different studies, using different mouse models, to control the membrane structure of megakaryocytes and platelets (Mountford et al., 2015; Valet et al., 2015). This platelet membrane phenotype was associated with a significant enrichment of the so-called barbell-shaped proplatelets in the bloodstream (Valet et al., 2015). We found that PI3KC2α is not responsible for the agonist-inducible pools of PtdIns3P and PtdIns(3,4)P_2 but is necessary for the production of a housekeeping pool of PtdIns3P in platelets. This particular pool of PtdIns3P may regulate membrane skeleton integrity. Indeed, deficiency in PI3KC2α activity leads to a reduced recruitment of several membrane skeleton proteins including spectrin and myosin associated with changes in the biophysical properties of platelet membrane such as reduced elasticity (Valet et al., 2015). These two pioneering studies on PI3KC2α in megakaryocytes and platelets have revealed a role for this lipid kinase in the control of membrane structure and dynamics. It will be important to further characterize the role of class II PI3Ks in this system as these kinases may become novel drug targets (Falasca et al., 2017).

Recently, it was shown that invalidation of the other class II PI3K expressed in platelet, PI3KC2β, has no obvious impact on platelet count, size, morphology and functions (Petitjean et al., 2016). Thus, the role of class II PI3Ks in megakaryocytes and platelets is just emerging and further investigations are needed to fully understand their mechanisms of regulation and their roles.

Class III PI3K (Vps34), the most ancient form of PI3Ks, is thought to produce PtdIns3P through PtdIns phosphorylation. Loss of Vps34 expression in mouse causes early embryonic lethality and this lipid kinase has been implicated in the regulation several important mechanisms including endocytosis, vesicular trafficking and autophagy (for a recent review see Backer, 2016).

Using 3-methyladenine, a poorly specific Vps34 inhibitor, it has been suggested an implication of this lipid kinase in platelet autophagy and in collagen and thrombin-induced platelet aggregation (Feng et al., 2014). Using the cre-lox system we have recently generated a mouse line deficient in Vps34 in megakaryocytes and platelets. These mice have a moderated microthrombopenia associated with platelet granule defects (Valet et al., 2017). In megakaryocytes lack of Vps34 is associated with a significant decrease in the basal level of PtdIns3P leading to a defect in receptor endocytosis and recycling and a loss of migration directionality leading to a release of platelets into the bone marrow. In platelets, the basal level of PtdIns3P is only weakly affected by the absence or inhibition (with the new selective inhibitors) of Vps34 but the synthesis of the stimulation-dependent pool of PtdIns3P is significantly decreased. Consistent with this we observed an increase in the specific lipid kinase activity of Vps34 following acute platelet stimulation. Interestingly, Vps34 deficiency or inhibition is associated with a defect of platelet granule secretion (i.e. exacerbated secretion) and a reduction of thrombus growth under flow conditions both in *ex-vivo* assays and *in vivo*. This study uncovers a dual role for Vps34 as a regulator of platelet production by megakaryocytes and as a modulator of platelet secretion and thrombus growth under flow conditions (Valet et al., 2017). Further work will be necessary to better understand the complex regulation and function of Vps34 in megakaryocytes and platelets.

Besides activation of the phosphoinositide metabolism, platelets also generate sphingosine 1-phosphate (S1P), a sphingolipid metabolite which synthesis requires the intervention of a sphingosine kinase catalyzing the phosphorylation of sphingosine to S1P (Spiegel and Milstien, 2003). Platelets express high amounts of sphingosine kinase, lack S1P lyase activity and abundantly produce and store S1P (Tani et al., 2005; Yatomi et al., 1995; Ulrych et al., 2011; Hla et al., 2012). Following activation, platelet release S1P that acts extracellularly through specific GPCR on platelets and neighboring cells to regulate many normal and pathophysiological processes (for reviews see Vito et al., 2016; Maceyka et al., 2012). S1P can induce platelet activation through a surface receptor (Yatomi et al., 1997) and has been proposed as a master regulator of efficient thrombopoiesis in mouse (Zhang et al., 2012). It is guiding the elongation of megakaryocyte proplatelets from the interstitium into bone marrow sinusoids to deliver platelets into the blood stream. However, among the pleiotropic effects of S1P its immune-modulating properties are particularly important (for a recent review on sphingolipids in inflammation see Espaillat et al., 2017). Platelet-derived S1P contributes to inflammation, maintenance of the endothelial barrier integrity and to leukocyte activation and recruitment at sites of injury (Vito et al., 2016).

Another important lipid produced and released by activated platelets is platelet activating factor (PAF) (Chignard et al., 1979; Chap et al., 1981; Alam and Silver, 1986). PAF is an inflammatory phospholipid that regulates activation of platelets, neutrophils, and other cells of the innate immune system (Prescott et al., 2000). It has been shown that activated platelets produce PAF through acetylation of 1-alkyl-glyceryl-3-phosphorylcholine (Chap et al., 1981; Alam and Silver, 1986). PAF acts through a widely expressed GPCR receptor which invalidation suppresses anaphylactic responses in mice (Ishii et al., 1998). Platelet-generated PAF is likely an important mediator of platelets as a circulating source of pro-inflammatory molecules.

3. Eicosanoid production during platelet activation

Eicosanoids are oxidative metabolites of C20 polyunsaturated fatty acids (e.g. arachidonic acid) abundantly generated and released by activated platelets (Marcus, 1978; O'Donnell et al., 2014; Lagarde et al., 2010). These lipid mediators are produced through activation of cytosolic phospholipase A2 (PLA2), a major early response of platelets to stimulation, producing arachidonic acid (AA) from platelet membrane phospholipids (Mouchlis and Dennis, 2016). AA is then rapidly metabolized, mainly by platelet cyclooxygenase-1 (COX-1) and 12-lipoxygenase (12-LOX), into eicosanoids. These molecules are released and act as autocrine and/or paracrine effectors on platelets and other vascular cells. TXA2 is generated in large amounts by the action of COX-1 and thromboxane synthase and released by platelets. It binds to the TXA2 receptors (TPs) which are RCPG present on platelets and other vascular cells, inducing prothrombotic and vasoconstrictive actions. Aspirin, an irreversibly inhibitor of COX-1, is largely used at small doses to protect against heart attacks and strokes (for a review see Majerus, 2014).

Fig. 1 shows the effect of thrombin (0.5 UI/ml) and collagen (5 μg/ml) stimulation on the production of a series of eicosanoids by washed human platelets. Eicosanoids were quantified by a LC/MS/MS method as previously described (Le Faourder et al., 2014). TXA2 is unstable and rapidly transformed into TXB2, which was quantified. The impact of class I PI3Kβ and PLA2 on eicosanoids production was analyzed by using two pharmacological inhibitors, TGX-221 (a selective inhibitor of PI3Kβ) and methyl arachidonyl fluorophosphonate (MAFP, an inhibitor of PLA2). As expected, thrombin, a potent platelet agonist acting through 2 GPCRs, PAR1 and PAR4, induced a robust production of TXB2, 12-hydroxyeicosatetraenoic acid (12-HETE), produced by the 12-LOX, but also to a lesser extent of 15-HETE and 8-HETE through the 15-LOX and 8-LOX activities, respectively. Collagen, acting through two specific membrane receptors, GPVI and α2β1, and activating a tyrosine kinase pathway, also induces the generation of COX-1 and LOX products but to a lesser extent compared to thrombin. The PLA2 inhibitor MAFP strongly inhibited the synthesis of the different eicosanoids following thrombin and collagen stimulations. The calcium sensitive cPLA2α is thought to be the main responsible for AA production in platelets (Adler et al., 2008). Interestingly, the class I PI3Kβ inhibitor had no effect on the production of eicosanoids induced by thrombin but significantly reduced their production following collagen stimulation. This striking difference is likely due to the fact that class I PI3Kβ and its product PtdIns(3,4,5)P_3 are required for full PLCγ2 activation downstream of GPVI while PLCβ

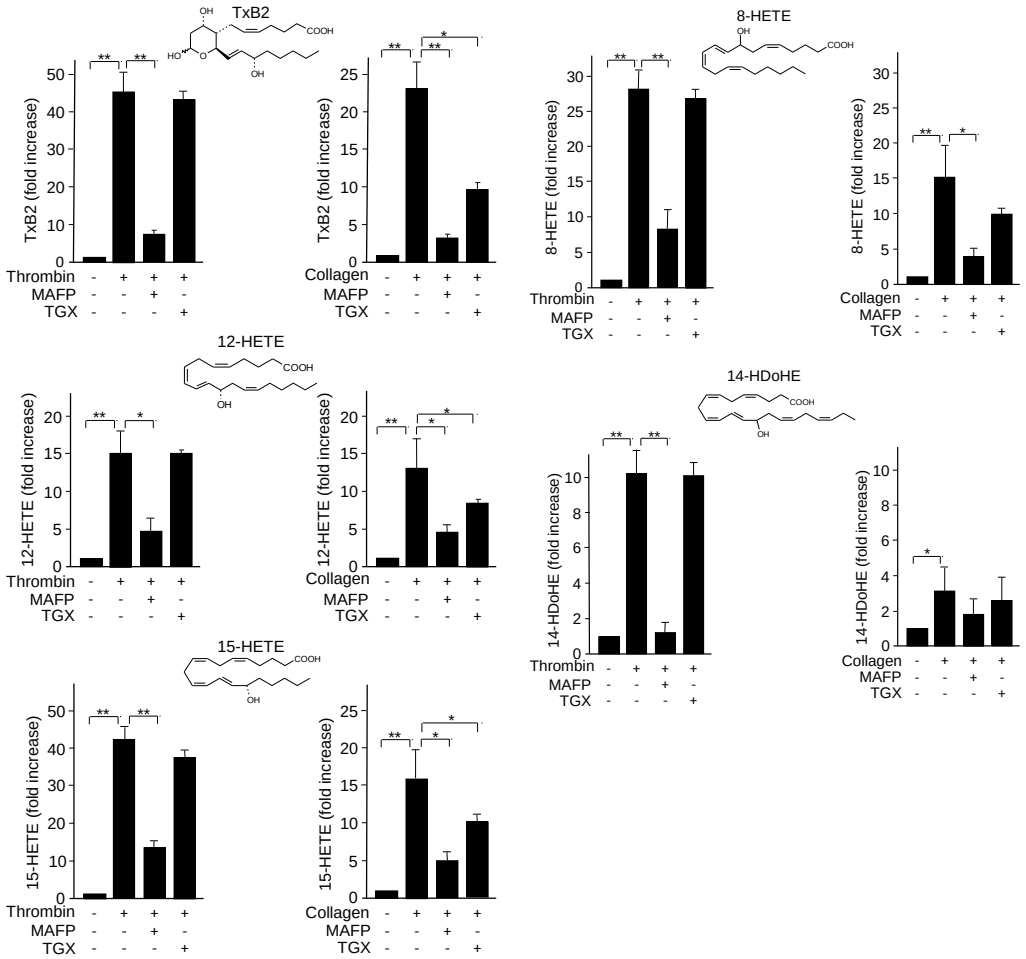

Fig. 1. Production of eicosanoids by human platelets stimulated by thrombin or collagen, implication of PLA2 and class I PI3Kβ.
Human washed platelets were prepared from healthy donors who had not ingested aspirin or any anti-platelet and anti-inflammatory drugs in the previous 10 day as described (Levade et al., 2014). Platelets were stimulated during 5 min with thrombin (0.5 U/ml) or collagen (5 μg/ml) and the lipids were then immediately extracted and analyzed by a LC/MS/MS method as described (Le Faourder et al., 2014). To test the implication of PLA2 or class I PI3Kβ, washed platelets were pretreated during 10 min with 1 μM of TGX-221 (a selective inhibitor of PI3Kβ) or 20 μM of methyl arachidonyl fluorophosphonate (MAFP), an inhibitor of cPLA2 and iPLA2 before stimulation. The amount of TXB2 (the stable metabolite of TXA2) produced by 7.5×10^7 platelets was 61.5 ± 20 ng following thrombin stimulation and 13 ± 6.5 ng following collagen stimulation. Thrombin-stimulated platelets (7.5×10^7) also produced 75 ± 25 ng of 12-HETE, 0.7 ± 0.2 ng of 8-HETE, 0.62 ± 0.1 ng of 15-HETE and 0.7 ± 0.2 ng of 14-HDoHE while collagen-stimulated platelets (7.5×10^7) produced 12 ± 5 ng of 12-HETE, 0.13 ± 0.06 ng of 8-HETE, 0.15 ± 0.9 ng of 15-HETE and 0.18 ± 0.05 ng of 14-HDoHE. Results are mean ± SEM of 3–5 independent experiments and are expressed as fold increase of the resting value. Student *t*-test $^{**}p < 0.001$, $^{*}p < 0.05$.

activation downstream of PAR1 is insensitive to class I PI3Kβ and PtdIns(3,4,5)P_3 (Gratacap et al., 1998, 2011). As cytosolic calcium increase is mandatory for cPLA2 activation the reduction of calcium mobilization following inhibition of class I PI3Kβ in collagen stimulated platelets strongly impacts AA production and in turn eicosanoids generation. Fig. 1 also shows that platelets stimulated by thrombin produce 14-hydroxy-docosahexaenoic acid (14-HDoHE) in a PLA2-dependent manner and independently of class I PI3Kβ. Free 14-HDoHE is formed from docosahexaenoic acid (DHA), which is hydrolyzed from the *sn*-2 position of platelet membrane phospholipids by calcium-dependent $cPLA_2$ and then submitted to lipoxygenation (likely via 12-LOX). Compared to thrombin, we found that collagen is a weak inducer of 14-HDoHE production by human platelets.

12-HETE is known to bind a GPCR, GPR31, but its roles remain unclear as controversial reports suggest anti- or pro-thrombotic activity of this lipid. 15-HETE has been involved in angiogenesis and inflammation, the role of 8-HETE is still poorly known and 14-HDoHE has been shown to reduce platelet activation (Lagarde et al., 2010). These results highlight the power of activated platelets in producing a range of eicosanoids that likely play important roles in physiopathology.

4. Sepsis and platelets

Sepsis has recently been defined in the third international consensus definitions for sepsis and septic shock as “life-threatening organ dysfunction caused by a dysregulated host response to infection” (Singer et al., 2016). Septic shock constitutes a subset of sepsis

in which both circulatory and cellular metabolism abnormalities occur. The incidence of sepsis is increasing with a consequent rise in hospitalizations. Sepsis-related morbi-mortality is linked to multiple organ failure development partly due to microvascular thrombosis and endothelial dysfunction with hyperpermeability. Thrombosis develops in microvessels (arterioles, venules, capillaries and sinusoids) impairing oxygen delivery to tissues (Pfeiler et al., 2014). Capillaries occlusion by microthrombi cause ischemia resulting in a partial or complete inhibition of organ function. Thrombosis in sepsis involves coagulation and platelet activation but also immune cells and may be considered as “immunothrombosis” (Engelmann and Massberg, 2013).

Thrombocytopenia (platelet count below 150 G/L) is a common feature of sepsis and is frequently associated with a dysregulated host response (Tsirigotis et al., 2013; Claushuis et al., 2016). A platelet count below 100 G/L during the first day after onset of septic shock is associated with a significantly increased risk of death (Thiery-Antier et al., 2016). Early thrombocytopenia is a major prognostic factor in septic shock and should represent an early alert for the clinician. During sepsis, thrombocytopenia is a multifactorial phenomenon involving decreased platelet production, splenic sequestration and increased platelet consumption. Platelet aggregation/adhesion to leukocytes and endothelial cells may be a common mechanism for this type of thrombocytopenia. Recently it was shown that during sepsis, neutrophil extracellular traps (NETs) that are extracellular DNA fibers comprising histones and neutrophil antimicrobial proteins which can trap and kill microbes, formed inside the vasculature, provide a scaffold and stimulus for thrombus formation (Fuchs et al., 2010; Alhambdi and Toh, 2016; Semeraro et al., 2011). Indeed, NETs are able to induce platelet adhesion, activation and aggregation contributing to thrombocytopenia. In most cases, the resolution of sepsis allows a slow correction of thrombocytopenia.

Platelets contribute to the antibacterial response of the host and play complex roles along sepsis as they control thrombosis and hemostasis and also modulate the inflammatory process. A report suggests that at certain stages of sepsis, platelets can have a protective role by reducing the plasma level of pro-inflammatory cytokines such as TNF-α and IL-6 and inhibiting macrophage-dependent inflammation via the COX1/PGE_2/EP4-dependent pathway (Xiang et al., 2013). Several mechanisms lead to platelet activation during sepsis including the inflammatory and coagulation cascades, the endothelial damage and the direct interaction with pathogens (Jenne and Kubes, 2015). Platelet adhesion and activation, vascular endothelial cell activation, innate immune cell recruitment, NET formation and fibrin deposition, all contribute to an increased propensity of thrombosis in septic conditions (Yaguchi et al., 2014; Semeraro et al., 2011). Several reports underline the crucial role of platelet-leukocyte interactions during inflammation and sepsis to participate in the defense against infections through intervention of a variety of specific receptors (for reviews see Jenne and Kubes, 2015; Li et al., 2017).

Bacteria can also directly interact or modulate platelets. These interactions are broadly represented by three types of mechanisms: secretion of bacterial products (i.e. toxins that interact with platelets), direct bacterial binding to a platelet receptor and binding to bacteria of a plasma protein that is a ligand for a platelet receptor (Cox et al., 2011). In vitro, incubation of pathogenic bacteria with human platelets can lead to platelet aggregation with a characteristic lag-time that varies from seconds to several minutes (Arman et al., 2014).

Based on these different observations, the question arises whether drugs that inhibit platelet activation may have a benefit in septic shock to limit microvascular occlusion and multiple organ failure (Akinosoglou and Alexopoulos, 2014). Preclinical and clinical data on the use of antiplatelets agents in septic critically ill patients have reported some encouraging results (Chabert et al., 2017; Otto et al., 2013). A retrospective cohort study has shown a strong association between acetyl salicylic acid and survival of patients with sepsis in intensive care units (Eisen et al., 2012). Randomized controlled trials are however required to answer this question. Two ongoing clinical trials called “Aspirin for the treatment of sepsis” (NCT01784159) and “Aspirin to inhibit sepsis” (ANTISEPSIS, ACTRN12613000349741) should help to elucidate the role of aspirin in the treatment of sepsis.

5. Platelet lipid signaling during sepsis

Interaction of platelets with different pathogenic bacteria species has been shown to cause platelet activation through mechanisms involving immunoglobulin G (IgG)/FcγRIIa, GpIIbIIIa (αIIbβ3 integrin), TXA2 and ADP (Arman et al., 2014). The low affinity Fc receptor FcγRIIA is expressed in human platelets and appears important for platelet activation by a variety of bacterial species (*S. sanguinis, S. gordonii, S. oralis*) (for a review see McNicol, 2015). IgG-bound bacteria stimulate FcγRIIA which ITAM sequence becomes tyrosine phosphorylated to trigger activation of the tyrosine kinase Syk and the organization of a signaling complex involving two key enzymes of the phosphoinositide metabolism, PLCγ2 and class I PI3Kβ. These enzymes and their products (i.e. PtdIns(3,4,5)P_3, DAG and inositol trisphosphate) are critical in the process of platelet activation via FcγRIIA (Gratacap et al., 1998). It was also shown that the combination of FcγRIIA activation upon recognition of IgG-coated bacteria plus integrin αIIbβ3 engagement (possibly through binding to bacteria or as a result of inside-out platelet signaling) induce a signal sufficient to release ADP and TXA_2 which are essential for platelet aggregation induced by bacteria (Watson et al., 2016). As observed for GPVI, we have shown that inhibition of class I PI3Kβ impairs PLCγ2 activation and calcium mobilization following FcγRIIA triggering (Gratacap et al., 1998). Thus, it is likely that calcium-dependent cPLA2 activation and the subsequent eicosanoid production will be impaired upon inhibition of class I PI3Kβ in human platelets stimulated via FcγRIIA.

The role of platelets along sepsis is complex and, as mentioned above, despite their pejorative role in microvessel occlusion, they may have some protective roles by tempering macrophage-dependent inflammation (Xiang et al., 2013). This beneficial effect of platelets is likely to be due to platelet COX1-dependent generation of prostaglandin E2 that acts on macrophage EP4 receptors to modulate their function and the production of TNF-α and IL-6. Of note the phosphoinositide metabolism is also playing important roles in the regulation of macrophage functions (Kudo et al., 2016).

The impact of the different eicosanoids produced by platelets during sepsis is still poorly characterized and likely quite complex as

these highly active molecules are now recognized as key mediators determining progression and resolution of inflammation (Dennis and Norris, 2015).

One important pro-inflammatory lipid generated by activated platelets is PAF. Interestingly, it has been shown that blood and urinary concentrations of PAF correlated with some of the clinical and laboratory parameters related to the severity of sepsis (Mariano et al., 1999). Despite a negative correlation with the number of circulating platelets, the origin of PAF during sepsis remains unclear. However, it has been shown that the serum levels of PAF acetylhydrolase, which inactivates PAF through conversion into lyso-PAF, are decreased in severe sepsis. Unfortunately, administration of recombinant PAF acetylhydrolase to septic patients did not give the expected promising results during clinical trials (Rabinovici, 2003).

In contrast to PAF, the serum levels of S1P are reduced in septic patients and are inversely associated with disease severity (Coldewey et al., 2016) however the reason for this change in S1P levels is unclear.

Thus, although these relatively scattered data suggest an important contribution of the platelet lipid signaling to sepsis, further work is needed to better understand the kinetics of activation of the different platelet lipid signaling pathways along sepsis and their implication in the development of this complex disease.

6. Concluding remarks

As highlighted in this review, platelet activation is associated with significant changes to membrane lipids with the production of diverse bioactive lipids playing essential roles not only in platelet signaling and activation itself but also in influencing immune cells and endothelial cells to regulate vascular integrity and inflammation. These lipids also play a key role in pathologies, including arterial thrombosis and infection. In sepsis, platelets can have protective roles by tempering macrophage-dependent inflammation, regulatory functions by maintaining inflammation or pejorative roles by precipitating microvessel dysfunction and in turn multiple organ failure. How the bioactive lipids produced by platelet regulate these different effects during the development of sepsis is an important question. Clearly, exciting challenges remains to further characterize the role of these platelet-derived bioactive lipids in pathologies, to analyze their changes in patient populations by lipidomics approaches, and to test if therapeutic manipulation of products of phospholipases, or kinases may help to modulate platelet activation in order to keep their positive effects and dampen their pejorative actions in thrombosis and sepsis.

Conflict of interest

The authors declare no competing financial interests.

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PARTIE 2

SEPSIS VIRAL : L'EXEMPLE DE LA COVID-19

Contexte scientifique

La maladie liée au nouveau coronavirus (SARS-CoV-2) ou COVID-19 (Coronavirus 19 Disease) a émergé en France en début d'année 2020. Dans les formes graves, elle entraîne une pneumopathie hypoxémiante pouvant évoluer jusqu'au Syndrome de Détresse Respiratoire Aiguë (SDRA) en réanimation. Les patients de réanimation sont à haut risque de développer des évènements thrombo-emboliques. Plusieurs études suggèrent une augmentation de l'incidence des thromboses et une coagulopathie chez les patients admis pour SDRA COVID-19, dans un contexte de syndrome inflammatoire systémique majeur. L'augmentation des D-Dimères et un faible compte plaquettaire sont corrélés à une plus grande mortalité chez ces patients. Une thrombocytopénie modérée, des changements dans l'expression génomique des plaquettes, des modifications de fonctionnalités plaquettaires ainsi que la présence de thrombi riches en plaquettes au sein des poumons ont d'ores et déjà été rapportés. Cependant, la contribution propre des plaquettes à la physiopathologie de l'infection à SARS-CoV-2, aux processus thrombo-inflammatoires et leurs interactions avec le virus, restent peu décrites.

L'objectif de notre travail était d'étudier les paramètres plaquettaires d'activation, les changements morphologiques au cours de l'infection grave à SARS-CoV-2, dans une cohorte prospective de patients admis en réanimation. L'objectif secondaire du travail était d'analyser la capacité des plaquettes à internaliser des particules virales.

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Platelet activation and partial desensitisation is associated with viral xenophagy in severe COVID-19 patients

Running title: Platelet selective autophagy in severe COVID-19

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Key points**:**

- During severe COVID-19, platelets get activated and become partly desensitized through mechanisms involving glycoprotein shedding.
- Platelets from severe COVID-19 patients internalize SARS-CoV-2 and develop viral xenophagy.

Abstract

Mild thrombocytopenia, changes in platelet gene expression, enhanced platelet functionality and presence of platelet-rich thrombi in the lung have been associated to the thromboinflammatory complications of COVID-19 patients. However, whether SARS-CoV-2 gets internalized by platelets and directly alters their behaviour and function in infected patients remain elusive. Here, we investigated platelet parameters and the presence of viral material in platelets from a prospective cohort of twenty-six severe COVID-19 patients admitted consecutively in intensive care unit. A combination of specific assays, tandem mass spectrometry and flow cytometry indicated high levels of protein and lipid platelet activation markers in the plasma from severe COVID-19 patients associated with an increase of pro-inflammatory cytokines and leukocyte-platelets interactions. Platelets were partly desensitized as shown by a significant reduction of αIIbβ3 activation and granule secretion in response to stimulation and a decrease of surface GPVI, whereas plasma from severe COVID-19 patients potentiated washed healthy platelet aggregation response. Transmission electron microscopy indicated the presence of SARS-CoV-2 particles in a significant fraction of platelets as confirmed by immunogold labelling and immunofluorescence imaging of Spike and nucleocapsid proteins. Compared to platelets from healthy donors or patients with bacterial sepsis, severe COVID-19 platelets exhibited enlarged intracellular vesicles and autophagolysosomes. They had large LC3-positive structures and increased levels of LC3II with a co-localisation of LC3 and Spike suggesting that platelets can digest SARS-CoV-2 material by xenophagy in critically ill patients. Altogether, these data show that during severe COVID-19, platelets get activated, become partly desensitized and develop a selective autophagy response.

Introduction

The acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes coronavirus disease 2019 (COVID-19) displaying variable clinical severity. To date, the pandemic has caused more than 4.6 million deaths worldwide (https://coronavirus.jhu.edu/map.html). During COVID-19, blood platelets have been shown to be activated and recruited at the site of infection, particularly in the lungs, and participate in the activation of the body's inflammatory response as well as in the appearance of coagulopathy related complications. [1-3] Lung is an important target of the SARS-CoV-2 virus and patients with severe COVID-19 have respiratory failure with major systemic inflammatory syndrome. A high proportion of pulmonary embolism is found in severe COVID-19 patients, up to 1 patient out of 5 in some intensive care units (ICU). [4,5] Electron microscopy and histochemistry analyses have shown platelet-rich thrombi in the lung of patients who have succumbed to COVID-19. [6-8] Together with other mechanisms of pulmonary circulatory failure, such as thrombotic microangiopathy, these complications might explain the relatively poor efficacy of standard prophylaxis with anticoagulants and, in the most severe cases, the failure of standard assisted ventilation techniques. [9-12]

Severe COVID-19 patients are characterized by an exacerbated host immune/inflammatory response to SARS-CoV-2 leading to acute respiratory distress syndrome (ARDS) and multi-organ failure. [9] In general, the pathophysiology of ARDS in its early phase combines local inflammation, accumulation and activation of leukocytes and platelets, uncontrolled activation of coagulation and alteration of endothelial and epithelial permeability. [13] In COVID-19, the thromboembolic events are multifactorial but endotheliopathy and thrombocytopathy are thought to take a central stage. [3]

In addition to their well-known function in haemostasis, as first responders of vascular injury in concert with plasma coagulation, blood platelets have an important role in inflammation and in host defense against pathogens. [14-17] They have also been shown to protect the epithelium of the pulmonary alveoli. [18] Several recent reports indicate platelet dysfunction in COVID-19 patients including a moderate thrombocytopenia, that has been proposed as a marker of severity of the pathology [19-21] but also platelet hyperactivation and interaction with monocytes leading to monocyte-derived tissue factor expression. [22-24] A current hypothesis concerning the underlying mechanisms is that, during COVID-19 disease, blood platelets are activated and recruited at the site of infection, particularly damaged endothelium of the pulmonary vasculature, and participate in the activation of the body's inflammatory response as well as in the appearance of complications related to coagulopathy. Several studies have shown that changes in platelet activation is associated with disease severity and mortality. [21,23-25] However, further studies are needed to characterize platelet activation in COVID-19 and to understand their role in the establishment of the most severe forms of the disease. An important question is to know whether platelets are activated through endothelial cell stimulation and dysfunction, via the exacerbated inflammatory conditions, directly by SARS-CoV-2 or by a combination of these different triggers. Several viruses, including dengue and influenza, can infect megakaryocyte and have been found in platelets. [26,27] SARS-CoV-2 RNA has been detected in platelets from COVID-19 patients suggesting the presence of viral particles. [22] However, the presence of the receptor for SARS-CoV-2 binding, the angiotensin-converting enzyme 2 (ACE2), on the platelet surface is a subject of controversy. [22,23,28] Recently, Koupenova *et al*. detected viral RNA from SARS-CoV-2 in the platelets from COVID-19 patients and demonstrated that, following incubation with SARS-CoV-2 virions, platelets can actively internalize the virus by different pathways including through endosomes, in phagocytic vacuoles and by attachment to microparticles. [29]

Here we have investigated a series of platelet parameters to assess their activation and responsiveness in a cohort of severe COVID-19 patients hospitalized in ICU. We sought to analyse the ultrastructure of the patient's platelets and the presence of SARS-CoV-2 particles within platelets and its consequences. Our data shed new light on the behaviour of platelets during severe COVID-19 including their activation, partial desensitization and the presence of viral particles and/or proteins leading to selective autophagy/xenophagy.

METHODS

Study design

Patients were enrolled in a single-centre, prospective, observational study conducted in the University Hospital of Toulouse from April 07-2020 to April 12-2021. Patients were included in the "COVID group" after admission in the ICU for severe acute respiratory failure due to SARS-CoV-2 infection as confirmed by RT-PCR. Patients from the "sepsis group" were included if they were admitted for a non-COVID-19 sepsis in the ICU according to the international definition of sepsis. [30] All patients from the "COVID group" and the "sepsis group" were enrolled under a study protocol approved by the Comité de protections des personnes du sud-ouest et outre-mer (CPP2020-04-042a / 2020-A00972-37 /20.04.08.64705). Healthy donors were recruited under a protocol approved by the Toulouse Hospital Bio-Resources biobank, declared to the Ministry of Higher Education and Research (DC2016-2804). They were asymptomatic and had negative serology within 3 months of inclusion. Patients and healthy donors were excluded from the study if they suffered from malignant blood disease, hemostatic disorder (thrombocytopenia, thrombopathy), disease associated with an activation of inflammation (cryopyrinopathy, Crohn's disease, rheumatoid arthritis,

sickle cell disease), in case of pregnancy, if they were majors under tutorship or guardianship. Functional tests in patients and healthy donors were not performed if aspirin or steroids was taken within the previous 10 days. All were recruited after informed consent and study procedures were conducted in accordance with the declaration of Helsinki. Upon admission to ICU, baseline characteristics, biological variables and outcome were recorded and detailed in Table 1.

Statistical analysis

Characteristics of the patients were compared using nonparametric tests (Mann-Whitney). $P<0.05$ was statistically significant. Fischer's test was used for morphological analysis by electronic microscopy. GraphPad Prism (La Jolla, CA, USA) was used to realize the figures.

Details regarding the sources of materials and additional methods are provided in the supplemental Materials and Methods.

RESULTS

Characteristics of the COVID-19 patients at admission and outcome

A total of twenty-six patients admitted in the ICU for severe COVID-19 were included in the study (median length of 4 days after admission). They were tested positive for SARS-CoV-2 infection on RT-PCR and their clinical and biological characteristics at the admission are detailed in Table 1. Patients admitted for a non-COVID-19 sepsis in the ICU (sepsis group, n=4) were also included in the study (Supplemental Table 1). Eighty percent of the patients were admitted for bacterial pneumonia and 20% for urinary sepsis.

Increased soluble platelet activation markers and decreased platelet reactivity in severe COVID-19 patients

Specific soluble platelet activation markers including soluble GPVI (sGPVI), soluble P-selectin (sCD62P) and soluble CD40 ligand (sCD40L) were significantly increased in the plasma from COVID-19 patients compared to healthy controls (Figure 1A-C). Consistent with the increased plasma concentration of sGPVI, the number of copies of this collagen receptor was significantly decreased at the surface of platelets from severe COVID-19 patients (Figure 1B). This result complies with a shedding of GPVI known to occur during platelet activation. The plasma concentration of eicosanoids derived from the metabolism of arachidonic acid during platelet activation through the cyclooxygenases (COX) and the lipoxygenases (LOX) pathways was then analysed. The plasma level of thromboxane B2 (TxB2), the stable metabolite of thromboxane A2 (TXA2), and of the LOX product 12-hydroxyeicosatetraenoic acid (12-HETE) were very high in severe COVID-19 patients and low in controls (Figure 1D). Other LOX products such as 15-hydroxyeicosatetraenoic acid (15-HETE), 5-hydroxyeicosatetraenoic acid (5-HETE) and 14-hydroxydocosahexaenoic acid

(14-HDoHE) were also significantly elevated in patients compared to healthy controls (Figure 1D).

Platelet activation was also highlighted by a significant increase in circulating heterotypic monocyte-platelet (Figure 1E) and neutrophil-platelet (Figure 1F) aggregates in the COVID-19 patient group compared to the control group. As expected, this was associated with an increase in the blood concentration of pro-inflammatory cytokines such as interleukin 1β (IL1β), interleukin 6 (IL-6) and interleukin 8 (IL-8) (Figure 1G).

We then investigated the level of activation of the platelet-specific αIIbβ3 integrin (GpIIbIIIa) by binding of the PAC1 antibody on the platelet surface, as well as the membrane expression of the α-granule secretion marker P-selectin (CD62P) and the dense granule secretion marker CD63 at rest or following stimulation. Non-stimulated platelets from severe COVID-19 patients and controls showed comparable levels of activated αIIbβ3 as well as CD62P and CD63 membrane expression (Figure 2A-D). The basal surface expression of αIIbβ3 per platelet was significantly decreased in COVID-19 patients suggesting the occurrence of a shedding process (Figure 2B). We next assessed platelet reactivity following stimulation by either collagen-related peptide (CRP), thrombin receptor agonist peptide (TRAP) or the stable analogue of TXA2 (U46619). The intensity of activation of the fibrinogen receptor αIIbβ3 was significantly reduced in severe COVID-19 patients compared to healthy controls in response to all agonists tested (Figure 2A). Similarly, while the number of α-granule and dense granules was comparable in the two groups (supplemental Figure 1), their secretion was significantly reduced in the severe COVID-19 group (Figure 2C-D). These results suggest that platelets from severe COVID-19 patients have a significant reduction of reactivity to physiological agonists. The maximal platelet aggregation response

measured by light transmittance aggregometry after 6 minutes of stimulation tended to decrease in response to U46619 and TRAP and was significantly reduced following collagen stimulation in the severe COVID-19 patient group (Figure 2E). Overall, these data suggest that platelets get activated and partly desensitized during severe COVID-19.

To check whether the plasma from severe COVID-19 patients would modify the aggregation response of platelets from healthy donors, we performed cross-aggregation tests. Washed platelets from healthy donors were resuspended in plasma from either healthy donors or COVID-19 patients and stimulated by low doses of CRP (0.075 µg/ml) or TRAP (25 µM). As shown Figure 3A-D, the plasma from severe COVID-19 patients significantly potentiated platelet aggregation induced by CRP (Figure 3A-B) or TRAP (Figure 3C-D). This potentiating effect, which may contribute to platelet desensitisation in severe COVID-19 patients, was not due to platelet FcγRIIA triggering since specific blockade of this Fc receptor with a neutralizing antibody did not impair the effect of the COVID-19 plasma (Figure 3E). As expected, this neutralizing antibody totally inhibited platelet aggregation induced by plasma from heparin-induced thrombocytopenia (HIT) patients (Figure 3E).

Platelets from severe COVID-19 patients contain viral material

Ultrastructure analysis of platelets from ten severe COVID-19 patients by TEM indicated the presence of characteristic crown-like structures of the expected size of SARS-CoV2 (80-120 nm of diameter) in 22.7 ± 6.3% of their platelets (Figure 4A-B). Such characteristic viral-like particles identified and quantified on the basis of specific criteria were observed in platelets from all ten patients tested but not in platelets from bacterial sepsis patients or from healthy donors (Figure 4B). The presence of SARS-CoV2 proteins in platelets from severe COVID-19 patients was confirmed by immunofluorescence and super-resolution confocal microscopy

using a specific Spike S1 antibody (Figure 4C). Moreover, immunogold labelling of Spike S1 and nucleocapsid applied to TEM showed the presence of these viral proteins in platelets from severe COVID-19 patients (Figure 4D) while no labelling was found in control platelets (supplemental Figure 2). Of note, the Spike S1 labelling was often found in large vesicle-like structures while nucleocapsid labelling was mainly in smaller vesicles suggesting different processing of these proteins.

Specific platelet ultrastructural changes in severe COVID-19 patients

Analysis of platelet ultrastructure by TEM revealed that a number of platelets from severe COVID-19 patients exhibited enlarged surface areas of low electron density reminiscent of vesicular structures or open canalicular system (OCS) (Figure 5A). Interestingly, these vesicle-like structures were rare in platelets from patients admitted to the ICU for bacterial sepsis (Figure 5A). Analysis of electron micrographs of platelet complete cross-sections indicated that 63.1 ± 12.5% of platelets from severe COVID-19 patients exhibited enlarged vesicle-like structures (Figure 5B). To check whether those structures would belong to the surface-connected and dilated OCS we used a specific labelling with the electron-dense extracellular tracer tannic acid. This labelling revealed that the vesicle-like structures observed in platelets from COVID-19 patients were not in continuity with the plasma membrane and the OCS as they show no tannic acid labelling (Figure 5C). These enlarged vesicle-like structures suggest a modification of the intracellular trafficking machinery in platelet from severe COVID-19 that could be linked to viral particles up-take as Spike S1 was found in such vesicles (Figure 4D). The number and area of these vesicle-like structures were quantified using electron micrographs of platelet complete cross-sections from healthy donors (n=186 micrographs), bacterial sepsis patients (n=160 micrographs) and severe COVID-19 patients (n=240 micrographs). The platelet cross-sectional area and the number of

vesicle-like structures per platelet were comparable between the 3 groups (Figure 5D-E). In contrast, the vesicle-like area/platelet area ratio and the mean area of vesicle-like structures were significantly increased in the severe COVID-19 group compared to control and bacterial sepsis groups (Figure 5F-G). The electron micrographs analysis also allowed to quantify the number of microtubules and pseudopodia emitted as signs of platelet activation. Microtubules are intracytoplasmic structures that can be easily visualized by TEM in resting platelets. Following platelet activation, microtubules rapidly reorganize and become much less visible by TEM. The number of visible platelet microtubules was significantly decreased both in the bacterial sepsis and in the severe COVID-19 groups compared to controls, suggesting platelet activation (Figure 5H). Of note, 74% of platelets exhibiting viral-like particles had invisible microtubules. Consistent with the decrease in microtubules, the number of pseudopodia significantly increased in the bacterial sepsis and the severe COVID-19 groups (Figure 5I). These data point to platelet activation in the two groups of patients while only platelets from severe COVID-19 patients exhibited enlarged vesicle-like structures.

Large LC3-positive structures characterize platelets from severe COVID-19 patients, evidence for xenophagy

The autophagy pathway can act as an intrinsic antiviral defense mechanism to degrade viral material through a process called xenophagy (foreign-eating) or virophagy. [31] Selective autophagy allows to deliver trapped viral cargo to the lysosome for degradation through autophagosome formation. [31,32] Immunofluorescence and super-resolution confocal microscopy analysis clearly indicated the presence of large LC3-positive puncta in platelets from severe COVID-19 patients (Figure 6A). A 3-dimensional representation of LC3-positive structures highlights their important size and tubular nature compared to healthy control platelets (Figure 6B). Consistent with this observation, LC3BII, a standard marker for

autophagosomes and autolysosomes generated by conjugation of cytosolic LC3BI to phosphatidylethanolamine, was hardly detectable by Western blotting in healthy control platelets and strongly present in severe COVID-19 platelets (Figure 6C). The LC3BII/actin ratio, and, to a lesser extent, the LC3BI/actin ratio, were significantly increased in platelets from severe COVID-19 patients compared to control platelets (Figure 6C, right panel). Consistent with these data, immunogold labelling of LC3B indicated an increase of gold particles in platelets from COVID-19 patients (Figure 6D) and the presence of LC3B around phagosome like structures (Figure 6E). Autolysosomes can derive from fusion of autophagosomes with lysosomes or from fusion with late endosomes structures which may give rise to LAPosomes that subsequently fuse with lysosomes. In order to investigate the origin of these LC3-positive vesicles, we searched for autophagy patterns. TEM analysis clearly demonstrated the presence of different characteristic autophagic structures including elongation membranes (phagophores), autophagosomes and autophagolysosomes in a substantial fraction of platelets from the ten COVID-19 patients analysed (Figure 6F and supplemental Figure 3). These structures were not visible or very rare in control platelets. Immunofluorescence and super-resolution confocal microscopy analysis demonstrated a significant degree of colocalization of Spike S1 and LC3B in platelets from severe COVID-19 patients as shown by a Pearson's correlation coefficient of 0.667 ± 0.142 and a Manders coefficient of 0.521 ± 0.209 (Figure 7A and 7D). There was much less colocalization of Spike S1 with the early endosome marker EE1 but a partial colocalization with the late endosome marker Rab7 (Figure 7B-D) suggesting the presence of structures derived from the endosomal pathway that may be related to LAPosomes or amphisomes. Finally, co-labelling experiments with fibrinogen, a marker of α-granules, and CD63, a marker of dense granules excluded the presence of Spike S1 in platelet granules (supplemental Figure 4).

Discussion

The high incidence of thromboembolic events in severe COVID-19 patients is now well established and is associated with increased morbidity and mortality. [4,21,25,33-35] The mechanisms underlying these thrombotic complications are multifactorial with however a central role of thrombocytopathy and endotheliopathy. [3,25,36] Platelets are emerging as important players in the coordinated action of the uncontrolled inflammatory and thrombotic response observed in SARS-CoV-2-mediated severe disease. [22-24] Here, we show that severe COVID-19 patients with ARDS hospitalized in the ICU have high levels of plasma platelet activation markers including sCD40L, sGPVI and sP-selectin. Our study confirms the significant increase in circulating heterotypic monocyte-platelet and neutrophil-platelets aggregates highlighting *in vivo* platelet activation. [37] These aggregates are observed in thrombo-inflammatory diseases such as sepsis, acute lung injury and cardiovascular diseases [38] and are associated with increased mortality in older septic patients. [39] Eicosanoids produced by activated platelets such as TXB2, the stable metabolite of TXA2, and 12-HETE were present at a very high level in patient's plasma. The concentration of other members of this family of bioactive lipids produced by the LOX pathway either by activated platelets and/or by immune cells such as 5-HETE and 15-HETE were also very high in the plasma from severe COVID-19 patients. Consistent with previous studies [22,40-42], our cohort of severe COVID-19 patients exhibited high levels of circulating pro-inflammatory cytokines including interleukin 1β (IL1β), interleukin 6 (IL-6) and interleukin 8 (IL-8). This first set of data confirms platelet activation in severe COVID-19 patients in complement to previous studies. [22-24,43] Importantly, when platelet reactivity was tested ex-vivo, we observed a partial but significant desensitisation to physiological agonists. The significant decrease in the activation of the platelet-specific integrin αIIbβ3 assessed by PAC-1 labelling after stimulation

correlated with a decrease in the number of αIIbβ3 copies on the surface of platelets from severe COVID-19 patients. Moreover, the secretion response of α and dense granules was also significantly reduced as well as platelet aggregation following collagen activation. A drop in the number of the collagen receptor GPVI copies on the surface of platelets was associated with an increase of sGPVI indicating a shedding of this platelet-specific receptor after proteolytic cleavage known to occur following contact of the receptor with its ligand or binding antibodies, or as a result of elevated shear stress in the microcirculation during sepsis. Acquired platelet GPVI receptor dysfunction in critically ill patients with non-COVID-19 sepsis, particularly in the early phase of sepsis, has recently been described. [44] The disintegrin and metalloprotease ADAM10 is known to cleave the extracellular domain of GPVI. [45] Factor Xa can also mediate coagulation-dependent shedding of GPVI. [46] The proteasic activity of ADAM10, which increases following platelet activation, may explain the acquired platelet GPVI dysfunction we observed during severe COVID-19 as a potential programmed downregulation function of the receptor. [47,48]

Manne et al. [23] have also reported a reduced activation of αIIbβ3 in COVID-19 patients. Reduction of platelet procoagulant response and platelet desensitization to TRAP have also been described. [49] Of note, hypoxia may be involved in the reduction of platelet responses. [50] However, several reports have shown a hyperactivity of platelets from COVID-19 patients. [22-24] The partial desensitization observed in our cohort of patients may have taken place secondary to the thromboinflammatory events as the median for blood sampling was four days after ICU admission. Consistent with this, activation of normal platelets by CRP or TRAP in the platelet-poor plasma from severe COVID-19 patients induced a significant potentiation of the aggregation response highlighting the prothrombotic nature of patient's plasma. Moreover, analysis of the ultrastructure of platelets from severe COVID-19 patients

by TEM indicated a decrease in the number of microtubules and an increase in pseudopodia emitted which are signs of platelet activation. Overall, these data indicate that platelets from severe COVID-19 patients undergo in vivo activation and then become partly desensitized.

Interestingly, using TEM, immunogold labelling and immunofluorescence approaches we found the presence of viral particle and viral proteins in a significant proportion of platelets from severe COVID-19 patients. Viral-like particles were found in 22.7% of platelets from the ten severe COVID-19 patients analysed in this study. How SARS-COV-2 enters platelets remains elusive. While Manne et al. [23] did not find ACE2 mRNA or protein in patient platelets, Zaid et al. [22] could detect ACE2 mRNA in platelets and Zhang et al. [28] demonstrated that they express ACE2 as well as its partner, the transmembrane protease serine 2 (TMPRSS2) known to proteolytically cleave and activate the viral Spike protein to facilitate virus entry. Koupenova et al. [29] found low level of ACE2 and TMPRSS2 proteins in platelets and detected SARS-CoV-2 RNA in the platelets from COVID-19 patients. They also show that following in vitro incubation with SARS-CoV-2 virions platelets internalize the virus through endosomes, in phagocytic vacuoles and by attachment to microparticles. Moreover, an ACE2-independent mechanism allowing interaction of SARS-CoV-2 with megakaryocyte and platelet has been suggested. [51] Finally, CD147, which is expressed in platelet [52] has been proposed as a potential receptor or co-receptor for SARS-CoV-2 that can promote virus entry into host cells. [53] Thus, evidence is accumulating that platelets can uptake SARS-CoV-2 by different mechanisms.

Analysis of Spike S1 protein by immunogold labelling in TEM indicated its presence in large vesicles. Compared to control platelets or platelets from patients with bacterial sepsis, platelets from severe COVID-19 patients exhibited a higher proportion of large vesicles that were not in continuity with the plasma membrane and the OCS. These vesicles are

reminiscent to the endocytic vesicle described by TEM in platelets infected by HIV. [54,55] Recently, it was shown that platelets engulf HIV-1 virion and traffic them from the endocytic pathway to an LC3-decorated compartment which may correspond to LC3-associated phagosomes or LAPosomes. [56] Here we found that platelets from the ten severe COVID-19 patients analysed exhibited characteristic autophagic structures including elongation membranes, autophagosomes and autophagolysosomes. Crosstalk between host autophagy and viruses has been frequently reported [31,57], including for SARS-COV-2. [58] The strong presence of LC3B-decorated large tubular structures in platelets from severe COVID-19 patients associated with a significant increase in LC3B-II levels and a colocalisation of LC3B and Spike S1 protein strongly suggest that selective autophagy recognizes intracellular viral components to degrade them via autophagosomes and autophagolysosomes. This process also termed xenophagy or, in the case of viruses, virophagy is known to play an important role in the resistance to infections. [31] Besides entry of the virus itself, platelets may uptake circulating viral proteins including Spike S1. [59]

The intraplatelet processing of soluble viral protein and of the whole SARS-COV-2 virus may require different pathways. Moreover, modulation of these autophagy-related pathways by the virus to escape degradation may also occur. [31] Mechanisms governing xenophagy in response to viral infection are of particular current interest in the context of the COVID-19 pandemic. Our data showing the partial colocalization of Spike S1 with Rab7 suggests the formation of structures derived from the endocytic pathway that may be related to LAPosomes. The endolysosomal and the autophagic pathways may co-exist and interconnect in platelets from severe COVID-19 patients. This is consistent with the different modes of entry of the virus recently described in platelets. [29] Thus, platelet have conserved a specialized form of selective autophagy to target viruses or components thereof to

autophagosomes and autophagolysosomes for elimination. Of note, platelets have the ability to process exogenous antigens to antigenic peptides through a vacuolar (phagosome-to-cytosol) pathway and present antigen via histocompatibility complex class I. [60,61] An attractive hypothesis is that platelets could contribute to T cell triggering in order to promote SARS-COV-2 protective immune responses.

In conclusion, platelets from patients admitted in ICU for COVID-19 ARDS are activated and get partly desensitized through mechanisms including the shedding of surface glycoproteins such as GPVI. Ultrastructural analysis of patient's platelets demonstrates for the first time in vivo that a significant proportion of platelets internalize SARS-CoV-2 and viral material and develop xenophagy through the endolysosomal and autophagic pathways contributing to viral material clearance.

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Authorship

Contribution: conception-design of the work: BP, CG, FVB, VM; patient inclusion, clinical management duties and ethical approvals: FVB, JAD, MP; experimental design and execution of the majority of the experiments: CG, JAD, MP, FVB, BP; data compilation, analysis and interpretation: CG, JAD, AR, PS, SV, VM, FVB, BP; manuscript writing: CG, BP, FVB; manuscript review and critical editing: all authors.

Conflict-of-interest statements

The authors declare no competing financial interests.

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Table 1: Characteristics of the severe COVID-19 patients

	Severe COVID-19 (n=26)
Demographic characteristics	
Age – median (range)	62 (55-73)
Male sex – no. (%)	21 (78)
BMI – median (range)	28.6 (26.7-33.7)
SAPS II – median (range)	49.5 (32.8-56.5)
Baseline SOFA – median (range)	5 (4-6)
Co-existing conditions – no. (%)	16 (59.3)
Hypertension	
Diabetes mellitus	10 (37)
Dyslipidemia	5 (18.5)
Previous myocardial infarction	2 (7.4)
Chronic respiratory disease	3 (11.1)
Obesity	11 (40.7)
Chronic cardiac disease	5 (18.5)
Chronic liver disease	1 (3.7)
Chronic kidney disease	3 (11.1)
Thrombo-embolic disease	4 (14.8)
Prior antiplatelet agent	4 (14.8)
Anticoagulation	2 (7.4)
Biology (at admission) – median (range)	
CRP (mg/mL)	143.8 (59.9-179)
Leukocytes (G/L)	7.7 (5.4-10.8)
Lymphocytes (G/L)	1.0 (0.6-1.1)
Neutrophils (G/L)	7.2 (4.1-8.3)
Platelet count (G/L)	197 (177-251)
D-dimers (ng/mL)	850 (715-1340)
Fibrinogen (g/L)	7.1 (6.9-7.4)
aPTT ratio	1.03 (0.99-1.11)
PT ratio	1.09 (1.04-1.17)
Outcomes – median (range) or no.(%)	
Duration of mechanical ventilation (days)	18.5 (8.25-26)
ICU LOS (days)	18 (13.5-29)
Hospital LOS (days)	31 (18-36)
Death – no. (%)	8 (30.7)

aPTT: Activated Partial Thromboplastin clotting Time; BMI: Body Mass Index, SAPS II: Simplified Acute Physiology Score, SOFA: Sepsis-related Organ Failure Assessment, COPD: Chronic obstructive pulmonary disease, CRP: C-Reactive Protein, ICU: Intensive Care Unit, LOS: Length of Stay; PT ratio: Prothrombin time.

Figures legends

Figure 1: Platelet activation markers in severe COVID-19 patients compared to controls

Soluble markers of platelet activation including soluble P-selectin (A), soluble GPVI (B, left panel) and soluble CD40L (C) were quantified in the plasma from severe COVID-19 patients and healthy donors (A: n=12 controls and n= 6 patients, B: n=20 controls and n=22 patients, C: n=11 controls and n=15 patients). The number of copies of platelet surface GPVI was also quantified (B, right panel, n=16 controls and n=15 patients). Eicosanoids (D) known to be produced by activated platelets, including TXB2, 12-HETE, 14-HDoHE, 15-HETE, 5-HETE were quantified in plasma from 7 severe COVID-19 patients and 6 controls using a mass spectrometry-based targeted lipidomic approach. The presence of heterotypic monocyte-platelet (E) and neutrophil-platelet (F) aggregates were quantified by flow cytometry in 13 severe COVID-19 patients and in 11 healthy donors. The concentration of interleukin-1beta, interleukin-6 and interleukin-8 were also quantified using appropriate ELISA kits (G) in the plasma of 6 to 10 severe COVID-19 patients and in 4 to 8 healthy donors. Results are mean ± SEM and each circle represents an individual. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$ according to the non-parametric Mann-Whitney test.

Figure 2: Platelet reactivity following stimulation in severe COVID-19 patients compared to healthy donors

PRP from 15 healthy donors and 16 severe COVID-19 patients were stimulated or not with TRAP (50 µM), U46619 (5 µM) and CRP (0.9 µg/ml) during 10 minutes in non-stirring conditions at 37°C and activation of αIIbβ3 (GpIIb-IIIa) was assessed by flow cytometry using PAC-1 antibody (A). The number of copies of surface GpIIb-IIIa in resting platelets

was also quantified (B, n=8 controls and n=9 patients). The surface expression of P-selectin (CD62P), a marker of α-granules secretion (C), and of CD63, a marker of dense granules secretion (D) was quantified in resting and stimulated platelets. The platelet aggregation response (% of maximal platelet aggregation) from healthy donors and severe COVID-19 patients was also assessed by light transmission aggregometry in response to TRAP (50 µM), U46619 (1 µM) and collagen (0.75 µg/ml) in stirring conditions during 10 minutes (E). Results are mean ± SEM, each circle represents an individual. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$ according to the non-parametric Mann-Whitney test.

Figure 3: Effect of plasma from severe COVID-19 patients on washed healthy platelet aggregation response

Washed platelets from 6 to 7 healthy donors were resuspended in plasma from 11 to 15 severe COVID-19 patients and 9 healthy donors. The platelet aggregation response (% of maximal platelet aggregation) was then assessed by light transmission aggregometry in response to low doses of CRP (0.075 µg/ml) (A-B) and TRAP (25 µM) (C-D) during 10 minutes. Representative aggregation traces are shown (A,C). The potential impact of FcγRIIA receptor on the potentiation of severe COVID-19 patient's plasma on CRP-induced platelet aggregation was assessed by using the IV.3 neutralizing antibody (E). As a positive control of the IV.3 antibody efficiency, three sera from heparin-induced thrombocytopenia (HIT) patients were used in the presence of heparin (0.5 IU/ml) to induce healthy platelet aggregation via FcγRIIA. The specificity of the reaction was assessed by addition of a large excess of heparin to impair platelet aggregation induced by HIT sera (F). Results are mean ±

SEM, each circle represents an individual. $^{**}p<0.01$ according to the non-parametric Mann-Whitney test.

Figure 4: Detection of SARS-CoV-2 in platelets from severe COVID-19 patients

Analysis of transmission electron micrographs of platelets from severe COVID-19 patients revealed the presence of characteristic crown-like structures reminiscent to SARS-CoV-2 particles (A). Representative images from 3 different patients are shown for illustration. Viral-like particles were identified on the basis of specific criteria including the size of the viral particle (100 nm ± 20%), the presence of a white halo around the particle, the size of the endosomal structure containing the viral particle (between 100 and 150 nm) and the crown-like spikes on the surface of the particle. Quantitative analysis was performed on transmission electron micrographs (186 from n=6 healthy donors (31 for each), 160 from n=4 non-COVID-19 septic patients (40 for each) and 240 from n=10 severe COVID-19 patients (24 for each). The percentage of platelets from healthy controls, patients with bacterial sepsis and patients with severe COVID-19 containing viral-like particles was quantified (B). Results are mean ± SEM, each circle represents an individual. **p<0.01, ***p<0.001 according to the non-parametric Mann-Whitney test. The presence of SARS-CoV-2 Spike S1 protein within platelets was analysed by immunofluorescence and super-resolution confocal microscopy with the Airyscan module (C). F-actin staining using phalloidin-AF488 allowed platelet visualisation. Representative images are shown (scale bar = 5 μm). Immunogold labelling of SARS-CoV-2 Spike S1 (D) and nucleocapsid (E) proteins analysed by transmission electron microscopy confirmed the presence of these viral proteins in platelets

from severe COVID-19 patients. Representative images from 3 different patients are shown for illustration.

Figure 5: Specific ultrastructural modifications in platelets from severe COVID-19 patients

PRP from healthy donors (n=6), non-COVID-19 septic patients ("Bacterial Sepsis", n=4) and severe COVID-19 patients (n=10) were fixed and analysed by transmission electron microscopy (A). None of the healthy donors or patients were treated with hydroxychloroquine. Representative electron micrographs at different magnification are shown. Different parameters and cellular elements were quantified using electron micrographs of platelet complete cross-sections (186 from n=6 healthy donors (31 for each), 160 from n=4 non-COVID-19 septic patients (40 for each) and 240 from n=10 severe COVID-19 patients (24 for each)). The percentage of platelets exhibiting enlarged vesicles (vesicle surface/platelet cross section surface ratio >6.1 which is the upper quartile for control platelets) was quantified (B). **$p<0.01$, ***$p<0.001$ according to the non-parametric Mann-Whitney test. To check whether the vesicle-like structures were part of the open canalicular system (OCS), PRP from 4 healthy donors and 6 severe COVID-19 patients were fixed and stained with tannic acid before analysis by transmission electron microscopy (C). Representative transmission electron-micrographs are shown. Magnifications of selected areas (x10000) show that the vesicles were not labelled with tannic acid excluding there belonging to the OCS. The cross-sectional surface (D), the number of vesicle-like structures per cross-section (E), the surface of vesicle-like structures per cross-section (F) and the vesicle-like surface/platelet cross-section surface ratio (G) were quantified. ****$p<0.0001$

according to the non-parametric Mann-Whitney test. The presence or absence of microtubules (H) and of pseudopodia (I) per platelet cross-section was also quantified as parameters of platelet activation. Fischer's test was used for statistical analysis.

Figure 6: Presence of LC3B-positive vesicles in platelets from severe COVID-19 patients

Washed platelets from healthy donors (n=3) and severe COVID-19 patients (n=3) were fixed and labelled with a specific anti-LC3B antibody and analysed by super-resolution confocal microscopy with the Airyscan module (A). F-actin staining using phalloidin-AF488 allowed platelet visualisation. Representative images are shown (scale bar = 1 μm). Images reconstructed in three dimensions (B) show LC3B spatial distribution in healthy control and severe COVID-19 patients platelets. Western blotting analysis of LC3B-I and LC3B-II levels in platelets from heathy donors (n=4, H1 to H4) and from severe COVID-19 patients (n=4, P1 to P4) (C, left panel). The quantification of the LC3B/actin ratio for LC3B-I and LC3B-II is shown in the right panel. Results are mean ± SEM. $^{***}p<0.001$ according to the non-parametric Mann-Whitney test. Immunogold labelling of LC3B protein analysed by transmission electron microscopy show the number of gold particles by cross section (30 from n=3 healthy donors (10 for each), 30 from n=3 severe COVID-19 patients (10 for each)) (D). Results are mean ± SEM. ***p<0.001, according to the non-parametric Mann-Whitney test. The presence of LC3B associated to a large vacuole reminiscent to autophagosome in a platelet from severe COVID-19 patient (E). Transmission electron microscopy sections of platelets from severe COVID-19 patients showing structures typical of elongation membrane (EM), autophagosome (AP) and autophagolysosome-like (AL) (F).

Figure 7: Localization of Spike S1 protein in platelets from severe COVID-19 patients

The intraplatelet localization of Spike S1 protein was investigated by immunofluorescence and super-resolution confocal microscopy with the Airyscan module using a specific anti-Spike S1 antibody. Its colocalization with LC3B, a marker of autophagosomes (A), EEA1, a marker of early endosomes (B) and Rab7, a marker of late endosomes (C) was analysed and quantified by Pearson's correlation coefficient and Manders' coefficient calculation (D). Quantification was performed from the analysis of 30-40 platelets from severe COVID-19 patients (n=3). Representative images (from 2 different severe COVID-19 patients out of 3) are shown for each colocalisation study (scale bar = 1 μm). Results are mean ± SEM. *p<0.05, ***p<0.001, ****p<0.0001 according to the non-parametric Mann-Whitney test.

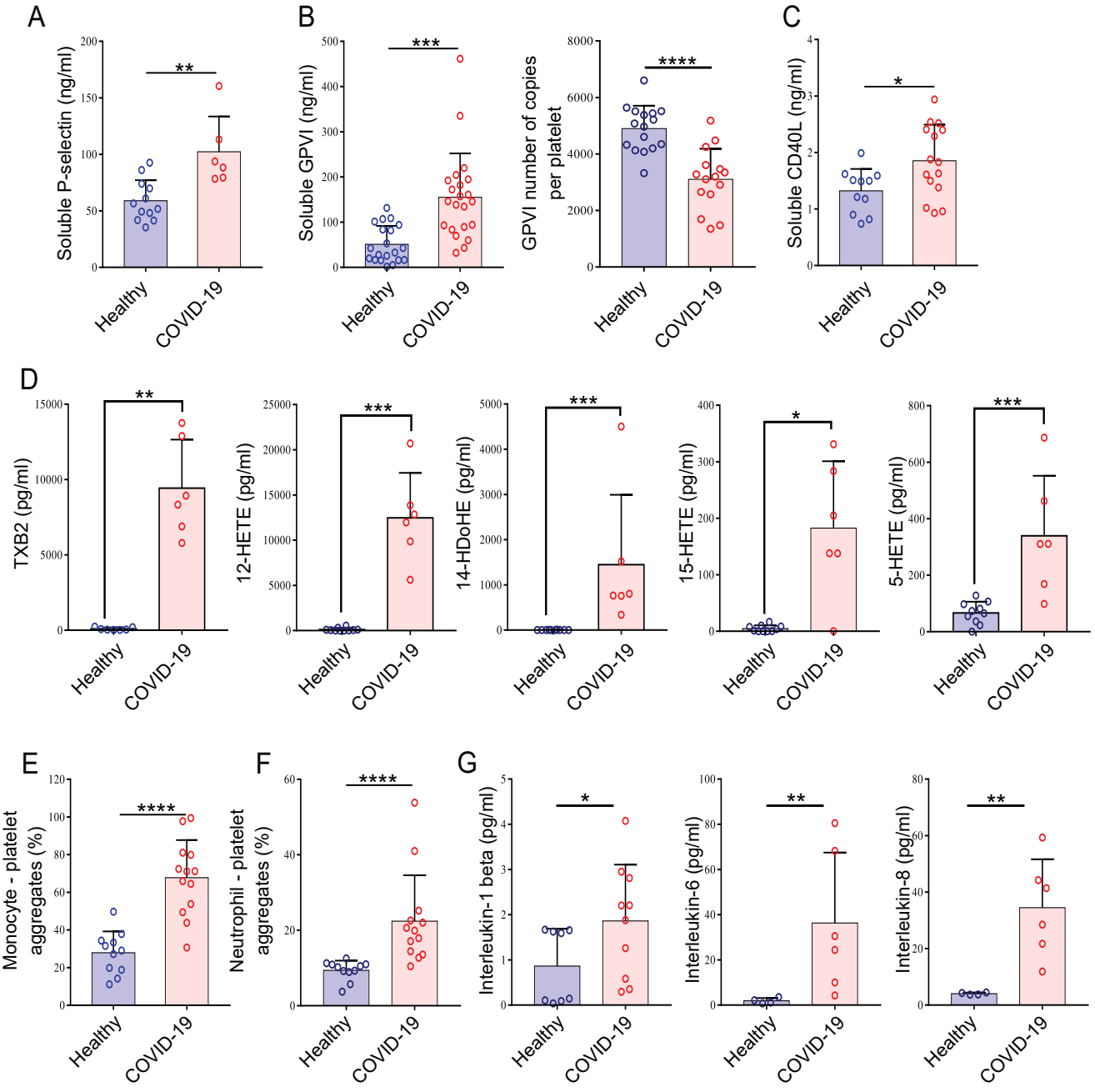

A
Soluble P-selectin (ng/ml)
**
Healthy
COVID-19
B
Soluble GPVI (ng/ml)
***
GPVI number of copies per platelet
****
C
Soluble CD40L (ng/ml)
*
D
TXB2 (pg/ml)
12-HETE (pg/ml)
14-HDoHE (pg/ml)
15-HETE (pg/ml)
5-HETE (pg/ml)
E
Monocyte - platelet aggregates (%)
F
Neutrophil - platelet aggregates (%)
G
Interleukin-1 beta (pg/ml)
Interleukin-6 (pg/ml)
Interleukin-8 (pg/ml)

Figure 1

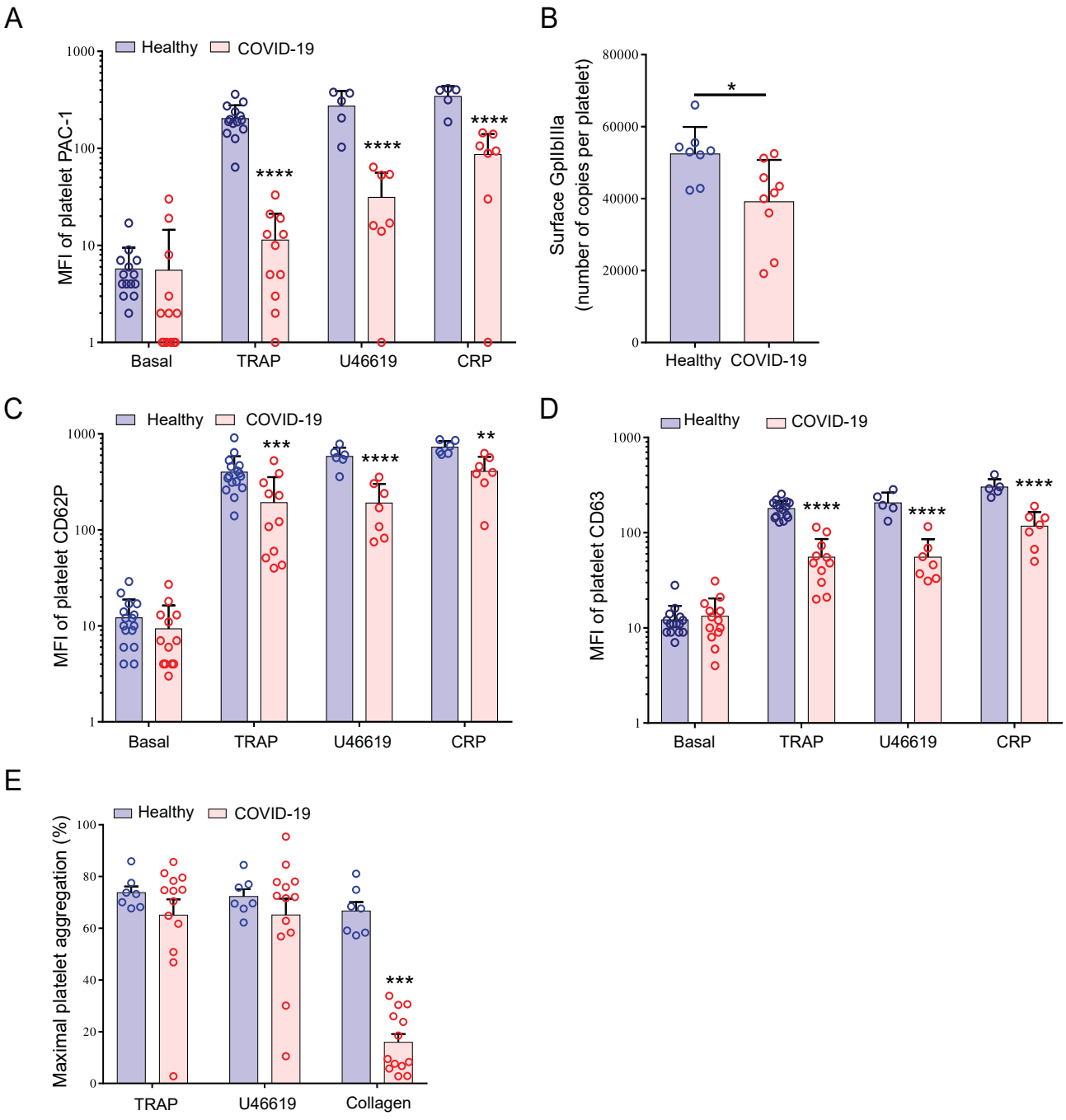

A
Healthy
COVID-19
MFI of platelet PAC-1
1000
100
10
1
****
****
****
Basal
TRAP
U46619
CRP
B
Surface GpIIbIIIa
(number of copies per platelet)
80000
60000
40000
20000
0
*
Healthy
COVID-19
C
Healthy
COVID-19
MFI of platelet CD62P
1000
100
10
1
***
****
**
Basal
TRAP
U46619
CRP
D
Healthy
COVID-19
MFI of platelet CD63
1000
100
10
1
****
****
****
Basal
TRAP
U46619
CRP
E
Healthy
COVID-19
Maximal platelet aggregation (%)
100
80
60
40
20
0
***
TRAP
U46619
Collagen

Figure 2

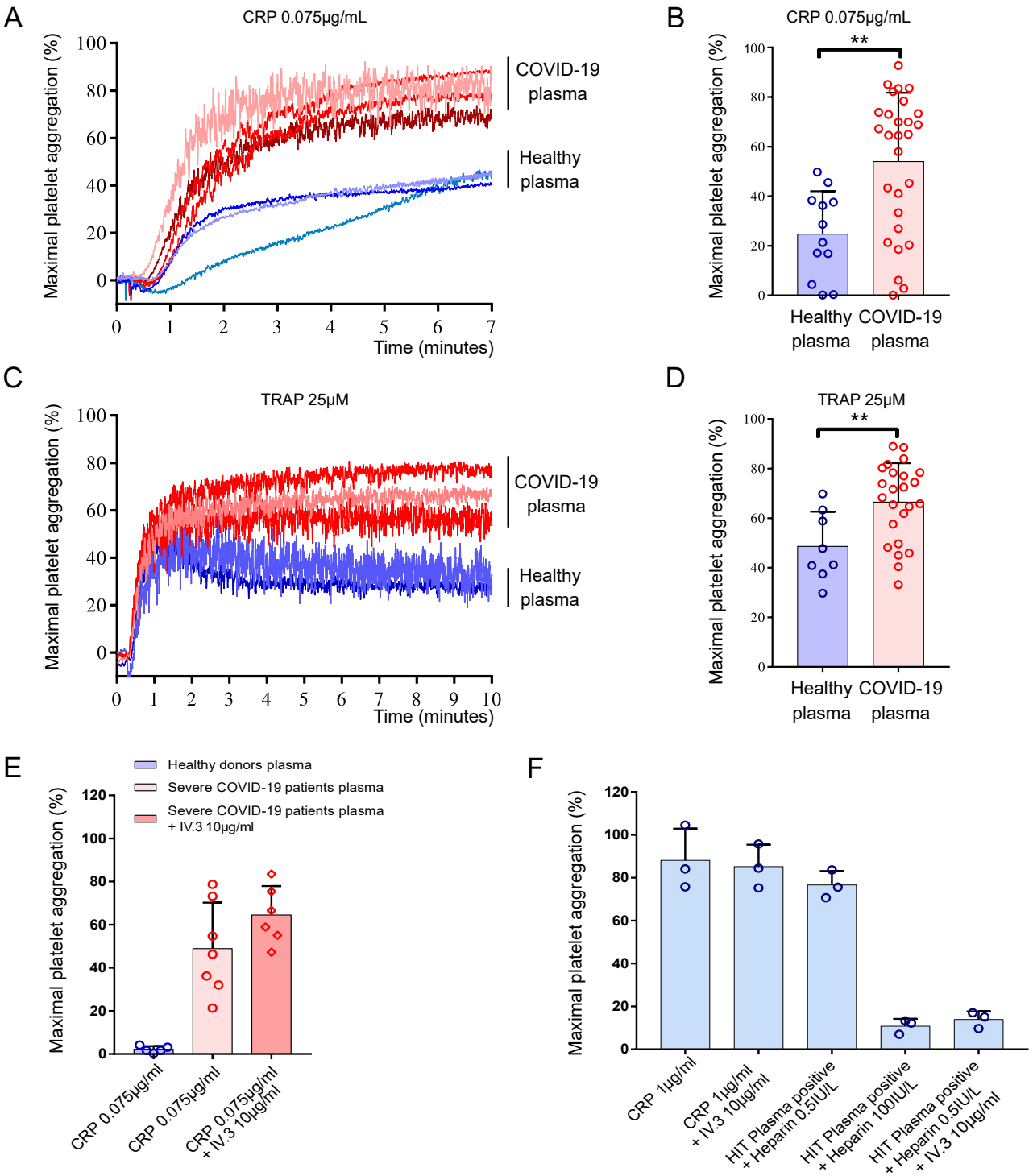

A
CRP 0.075µg/mL
Maximal platelet aggregation (%)
Time (minutes)
COVID-19 plasma
Healthy plasma
B
CRP 0.075µg/mL
**
Maximal platelet aggregation (%)
Healthy plasma
COVID-19 plasma
C
TRAP 25µM
Maximal platelet aggregation (%)
Time (minutes)
COVID-19 plasma
Healthy plasma
D
TRAP 25µM
**
Maximal platelet aggregation (%)
Healthy plasma
COVID-19 plasma
E
Healthy donors plasma
Severe COVID-19 patients plasma
Severe COVID-19 patients plasma + IV.3 10µg/ml
Maximal platelet aggregation (%)
CRP 0.075µg/ml
CRP 0.075µg/ml
CRP 0.075µg/ml + IV.3 10µg/ml
F
Maximal platelet aggregation (%)
CRP 1µg/ml
CRP 1µg/ml + IV.3 10µg/ml
HIT Plasma positive + Heparin 0.5IU/L
HIT Plasma positive + Heparin 100IU/L
HIT Plasma positive + Heparin 0.5IU/L + IV.3 10µg/ml

Figure 3

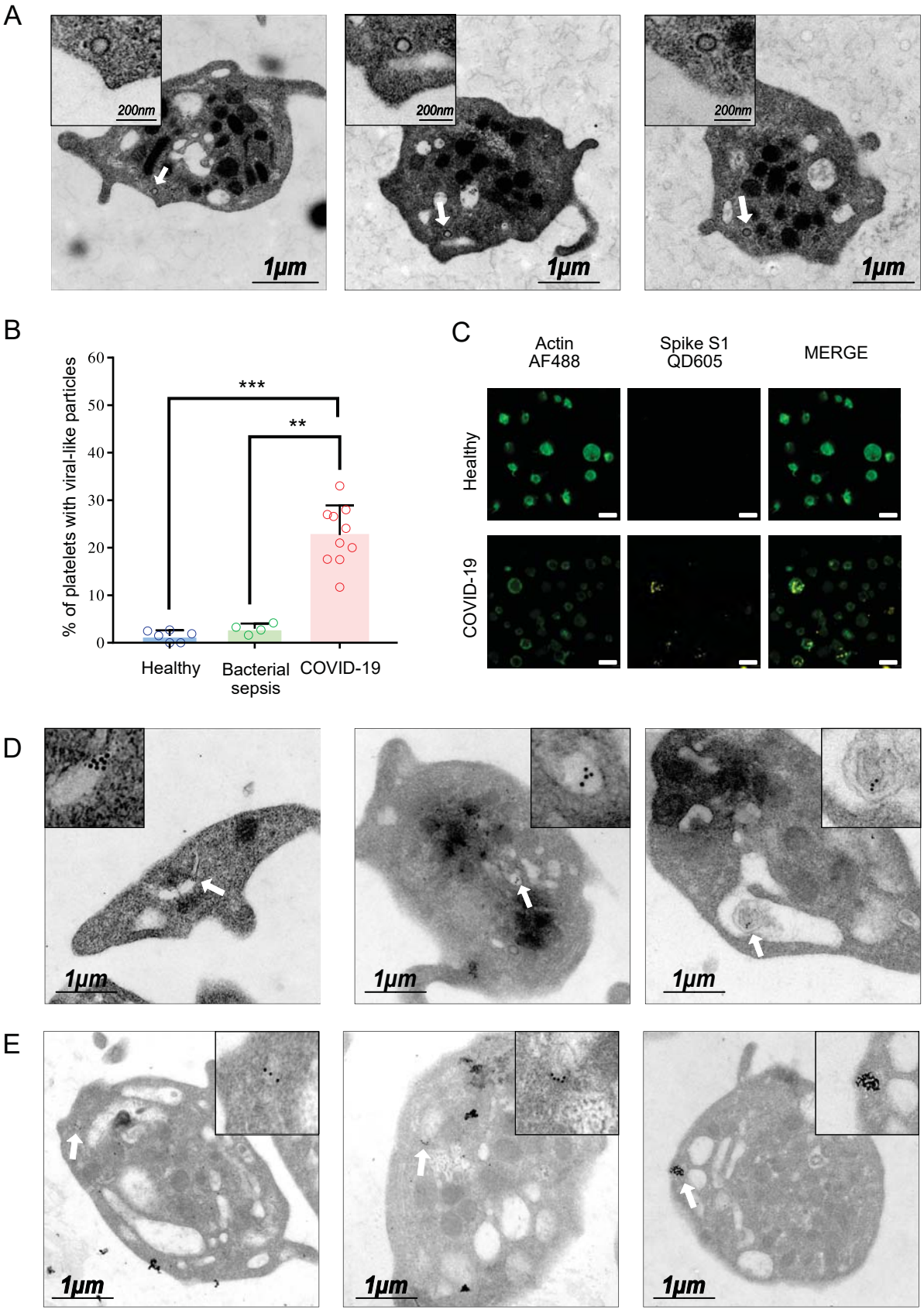

A
200nm
1µm
B
% of platelets with viral-like particles
60
50
40
30
20
10
0
***
**
Healthy
Bacterial sepsis
COVID-19
C
Actin AF488
Spike S1 QD605
MERGE
Healthy
COVID-19
D
1µm
E
1µm

Figure 4

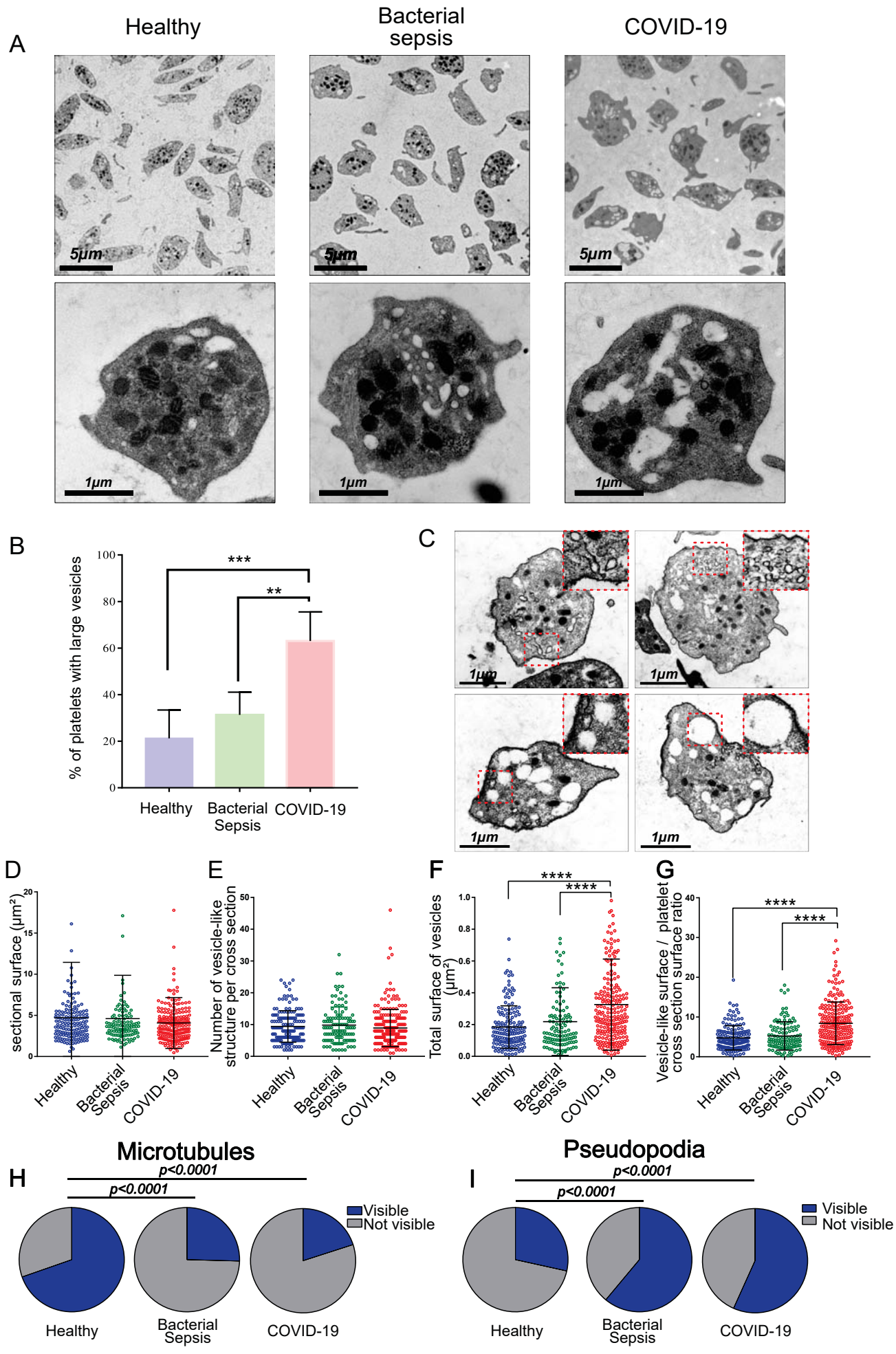

A
Healthy
Bacterial sepsis
COVID-19
5µm
1µm
B
% of platelets with large vesicles
***
**
Healthy
Bacterial Sepsis
COVID-19
C
1µm
D
Platelet cross sectional surface (µm²)
E
Number of vesicle-like structure per cross section
F
Total surface of vesicles (µm²)
****
G
Vesicle-like surface / platelet cross section surface ratio
H
Microtubules
p<0.0001
Visible
Not visible
I
Pseudopodia

Figure 5

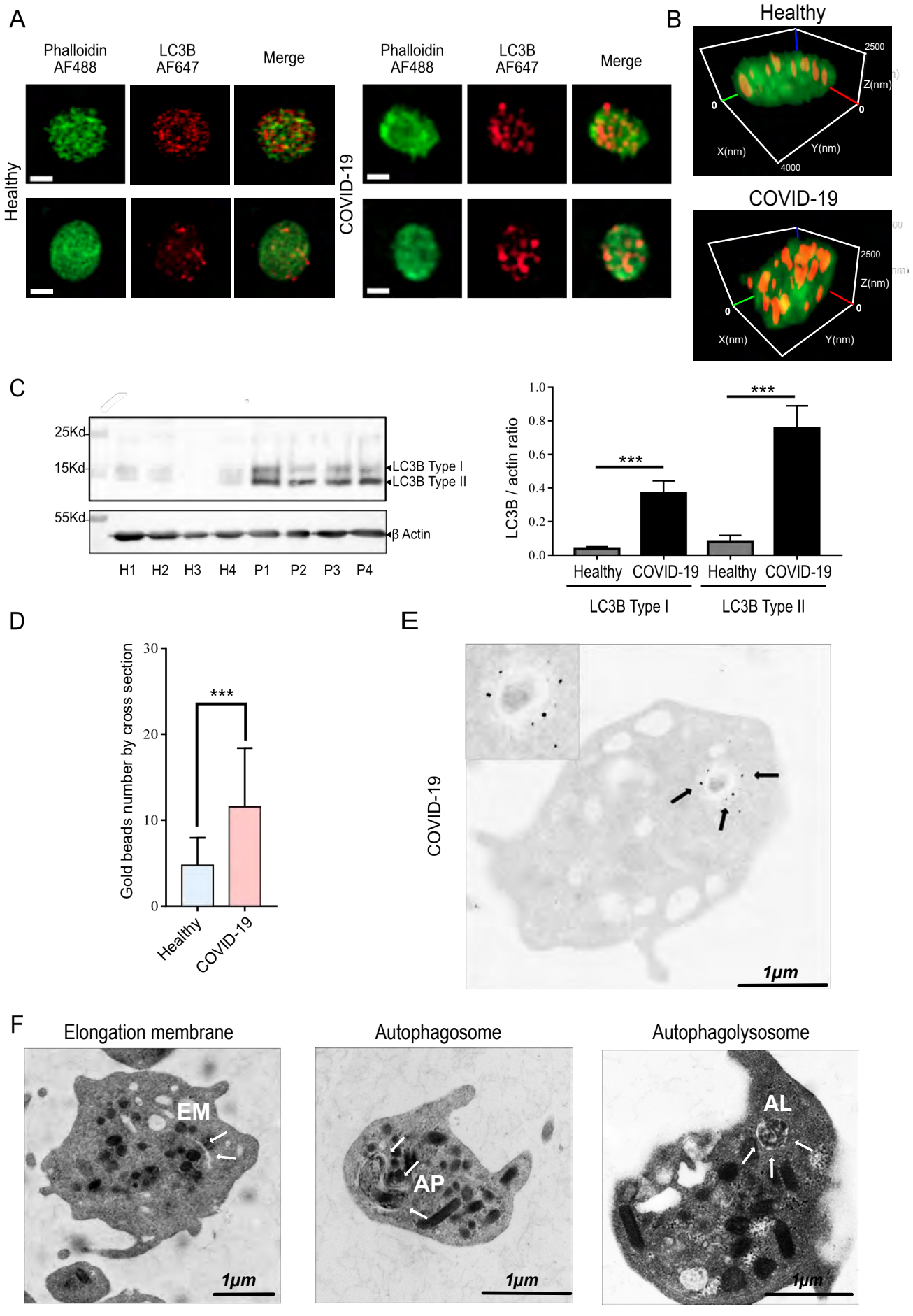

A
Phalloidin AF488
LC3B AF647
Merge
Healthy
COVID-19
B
Healthy
COVID-19
2500
Z(nm)
0
X(nm)
Y(nm)
4000
C
25Kd
15Kd
55Kd
LC3B Type I
LC3B Type II
β Actin
H1 H2 H3 H4 P1 P2 P3 P4
LC3B / actin ratio
***
Healthy COVID-19 Healthy COVID-19
LC3B Type I
LC3B Type II
D
Gold beads number by cross section
Healthy
COVID-19
E
COVID-19
1μm
F
Elongation membrane
Autophagosome
Autophagolysosome
EM
AP
AL
1μm

Figure 6

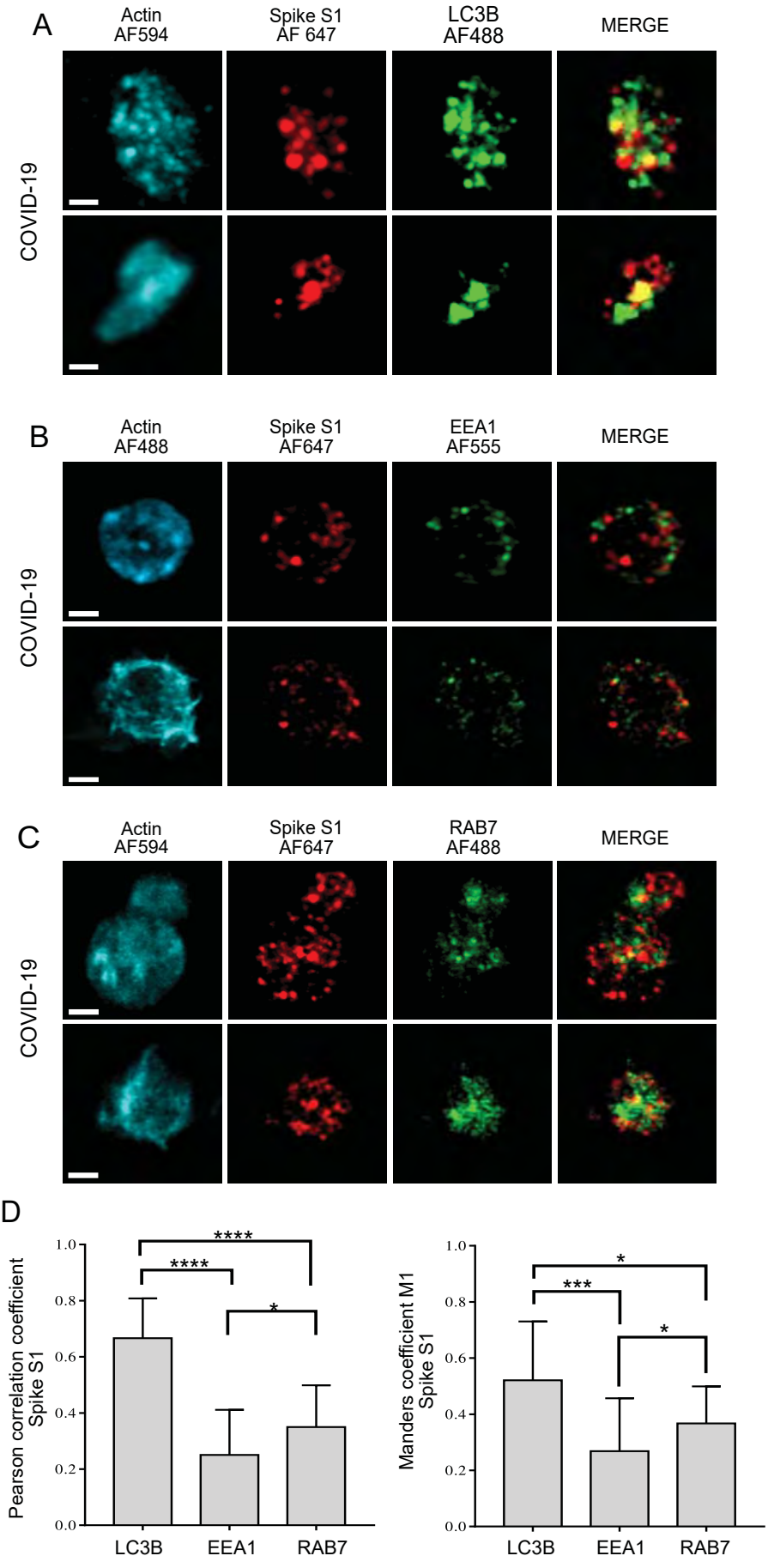

A
Actin AF594
Spike S1 AF 647
LC3B AF488
MERGE
COVID-19
B
Actin AF488
Spike S1 AF647
EEA1 AF555
MERGE
COVID-19
C
Actin AF594
Spike S1 AF647
RAB7 AF488
MERGE
COVID-19
D
Pearson correlation coefficient Spike S1
1.0
0.8
0.6
0.4
0.2
0.0
****
****
*
LC3B
EEA1
RAB7
Manders coefficient M1 Spike S1
1.0
0.8
0.6
0.4
0.2
0.0
*
***
*
LC3B
EEA1
RAB7

Figure 7

Platelet activation and partial desensitisation is associated with viral xenophagy in severe COVID-19 patients

Supplemental Methods

Materials

CRP was from Pr. R. Farndale laboratory (Cambridge, UK), collagen reagent Horm® (equine) suspension was purchased from Takeda (St. Peter Strasse, Austria), anti-CD62P conjugated FITC, anti-CD63 conjugated FITC, anti-PAC conjugated FITC, anti-CD61 conjugated Phycoerythrin, anti-CD14 Alexa fluor 488 and anti CD66B conjugated PerCP-Cy5.5 antibodies were from BD biosciences and the anti-GPVI from Biocytex (Marseille, France). Anti-CD63 uncoupled antibody was from Santa Cruz biotechnology (Dallas, USA). The anti-FcγRIIA monoclonal antibody IV.3 was from Medarex (Annandale, USA) and the anti-mouse IgG F(ab′)2 to cross-link FcγRIIA-IV.3 complexes was from Jackson Immunoresearch Laboratories (Baltimore Pike, USA). All other reagents were purchased from Sigma Aldrich (Saint-Louis, USA).

Antibodies references

Antibody	Isotype	Provider	Reference
SARS Coronavirus Spike S1	Rabbit	Abcam	ab272504
SARS/SARS-CoV-2 Coronavirus Nucleocapsid	Mouse	Thermofisher	MA1-7404
LC3B (E5Q2K) Mouse mAb (Alexa Fluor® 488 Conjugate)	Mouse	Cell Signaling Technology	#83506
LC3B (E7X4S) XP® Rabbit mAb	Rabbit	Cell Signaling Technology	#43566
Alexa Fluor™ 488 Phalloidin		Thermofisher	A12379
Alexa Fluor™ 594 Phalloidin		Thermofisher	A12381
EEA1 (E9Q6G)	Mouse	Cell Signaling Technology	#48453
Rab7 (E9O7E)	Mouse	Cell Signaling Technology	#9367
CD63 MX-49.129.5	Mouse	Santa Cruz Biotechnology	sc 5275
Fibrinogen alpha chain	Sheep	Abcam	ab118533
Goat anti-Mouse IgG (H+L), Superclonal™ Recombinant Secondary Antibody, Alexa Fluor 555		Thermofisher	# A28180
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488		Thermofisher	# A-11008
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647		Thermofisher	# A-21244
Donkey anti-Rabbit IgG (H+L) Secondary Antibody, Qdot® 605 conjugate		Thermofisher	# Q22083
Donkey anti-Sheep IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488		Thermofisher	A-11015
F(ab')2 goat anti rabbit gold bead 10nm		Aurion	
F(ab')2 goat anti mouse IgG gold bead 10nm		Aurion	

Haemostasis variables

Several haemostasis parameters were analyzed including the prothrombin time, activated partial thromboplastin time, fibrinogen, D-dimers and platelet count. All were performed at the Laboratory of haematology, Rangueil Academic Hospital (Toulouse, France).

Blood sampling and plasma preparation

Whole blood samples were collected in 3.2% citrate (Vacutainer, Becton Dickinson). Platelet-rich plasma (PRP), platelet-poor plasma (PPP) and washed platelets were obtained by differential centrifugation as previously described.[1] PPP for measurement of soluble markers were stored at -80°C.

Light transmission platelet aggregation tests

Platelet aggregation was monitored with a turbidimetric method (SD Medical, STAGO, France) as previously.[1] PRP was stimulated by different agonists (collagen-related peptide [CRP] 0.9 µg/mL, thrombin receptor agonist peptide [TRAP] 50 µM, collagen 0.75 µg/mL, thromboxane A2 analog U46619 1 µM) for 10 minutes under continuous stirring conditions (1000 revolution min^{-1}) at 37°C in siliconized glass cuvettes. Maximal aggregation percentage was analyzed with thrombosoft 1.6 (SD Medical). Washed platelets were resuspended in modified HEPES-Tyrode's buffer containing 2 mM CaCl2 (pH 7.4) at a density of $2x10^8$ platelets/mL for confocal microscopy and western-blot analysis. For plasma cross-testing, washed platelets at a density of 4×10^8 platelets/mL were resuspended in one volume of plasma from healthy donors or severe COVID-19 patients. After 5 minutes of incubation at 37°C, agonists (CRP 0.075 µg/mL and TRAP 25 µM) were added.

Flow cytometry analysis

Flow cytometry was performed using a FACSVERSE or a LSR II Fortessa (BD Biosciences) and FASCsuites software for analysis (BD biosciences). Platelet granule secretion was assessed by measuring surface expression of CD62P and CD63, markers of α and δ granules, respectively. [2] Following platelet stimulation by different agonists (TRAP 50 µM, U46619 5 µM and CRP 0.9 µg/ml) during 10 minutes in non-shaking conditions, PRP was incubated for 15 minutes at room temperature with appropriate antibodies. Phosphate buffer saline (PBS) was then added before cytometer analysis. Results were expressed as median fluorescence intensity (MFI). The copy number of platelets GpIa, GpIb, GpIIIa and GpVI was quantified using a specific commercial kit (Platelet GP Screen, Biocytex) according to the manufacturer's instructions.

Platelet-leukocyte aggregates were measured as previously described. [2] Briefly, 100 µL of whole blood were fixed with 500 µL-Cellfix 1X solution (BD Biosciences). After 15 minutes of incubation, 2 mL of PBS was added and the suspension was centrifuged at 190*g* for 10 minutes. Cell pellet was resuspended in 300 µL PBS and incubated with anti-CD61 (BD biosciences) conjugated phycoerythrin (PE) (platelets), anti-CD14 conjugated Alexa fluor 488 (monocytes) and anti-CD66b conjugated PerCP-Cy5.5 (neutrophils) antibodies for 30 minutes at room temperature. After 2 steps of erythrocyte lysis (Ammonium Chloride solution lysis) cells were washed with PBS before FACS analysis.

ELISA assay

The PPP was immediately frozen and stored at -80°C before determination of the plasma level of sGPVI using a quantitative ELISA kit (MyBioSource, San Diego, CA, USA) likewise for sCD40L and sCD62P (eBioscience™, Fisher Scientific, Strasbourg, France). [2] The pro-inflammatory cytokine interleukin 1-β, was measured in human plasma by ELISA (Life Technologies SAS, Thermofischer, Courtaboeuf, France) following the manufacturer's

indications. The other interleukins were determined by microELISA using ELLA technology (Proteinsimple).

Plasma eicosanoids measurement by high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

PPP samples were stored at -80°C until lipid extraction and LC-MS/MS analysis as previously described.[3] Briefly, internal standards containing eicosanoid species deutrium tagged (Cayman Chemicals, Ann Arbor, MI, USA) was added before lipid extraction. TXB2, 5, 12 and 15-hydroxyeicosatetraenoic acids (HETE), 14-hydroxydocosahexaenoic acid (HDoHE) were separated on a ZorBAX SB-C18 column using Agilent 1290 Infinity HPLC system coupled to an ESI-triple quadruple G6460 mass spectrometer (Agilent Technologies, Santa Clara, California, USA). Data were acquired in Multiple Reaction Monitoring mode with optimized conditions (ion optics and collision energy). Peak detection, integration, and quantitative analysis were done using Mass Hunter Quantitative analysis software (Agilent Technologies) based on calibration lines built with internal standards.

Western blotting

Washed platelets from patients or control donors were lysed and analysed by immunoblotting with specific antibodies following standard procedures.[1]

Transmission electron microscopy (TEM) and ultrastructural immunocytochemistry

Platelets were fixed in glutaraldehyde, prepared as previously described [4] and examined on a HT 7700 Hitachi electron microscope. Electron microscopy image analysis was performed using Zen (Zeiss) and Qupath software for the measurement of platelet vesicle surface. Immunogold labelling was performed as follows: sections were incubated with ammonium chloride (50 mM, pH 7.8) for 10 minutes at RT, blocked with blocking solution (1% bovin

serum albumin (BSA)-5% normal-goat-serum-0.2% Tween 20 in PBS, pH 7.4) for 15 minutes at RT prior incubation with the different antibodies (anti-spike antibody, anti-nucleocapsid antibody and anti-LC3B antibody (dilution 1/50 for all)) in blocking solution for 3 hours at RT. They were then washed at RT in 0.1% BSA-PBS three times for 10 minutes each and then with 0.1% fish skin gelatin in PBS for 5 minutes prior incubation for 1 hour at RT with 10 nm gold conjugated goat anti-rabbit or anti-mouse antibodies (dilution 1/50 in PBS containing 0.1% fish skin gelatin). Grids were then washed in PBS 1X, fixed for 5 minutes in 1% glutaraldehyde, rinsed with distilled water, and post-stained with 3% uranyl acetate in 50% ethanol before being examined on a HT7700 Hitachi electron microscope at an accelerating voltage of 80 KV.

Confocal microscopy immunocytochemistry

Platelets (2.10^8/mL) were fixed with paraformaldehyde 1.5% during 30 minutes at room temperature, applied to 0.01% poly-L-lysine-coated coverslips, and permeabilized with 0.1% Triton X100. After blocking with 1% BSA-PBS for 1 hour, cells were incubated overnight at 4°C with primary antibodies (dilution 1/250 in Tris-buffer-saline containing 3% goat serum). After washing, platelets were incubated 2 hours with secondary antibodies (dilution 1/1000) and washed twice in PBS. Confocal images were acquired using 63X objective and a LSM900 confocal laser microscope (Carl Zeiss). Super resolution imaging was performed using the Airyscan module. For the quantification of colocalization, the FIDJI software with the JACOP plug-in was used to calculate the Pearson's correlation coefficient and the Manders' M1 coefficient.

References.

1. Series J, Garcia C, Levade M, et al. Differences and similarities in the effects of ibrutinib and acalabrutinib on platelet functions. *Haematologica*. 2019;104(11):2292–2299.

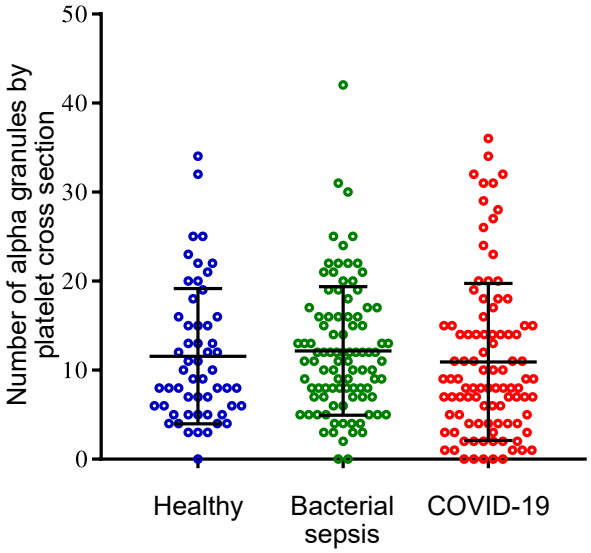
2. Dumonteil N, Levade M, Garcia C, et al. Platelet Activation Is Limited during Transcatheter Aortic Valve Implantation in Patients on Aspirin Monotherapy and without per Procedural Clinical Complications. *TH Open*. 2019;3(2):e146-e152.
3. Le Faouder P, Baillif V, Spreadbury I, et al. LC–MS/MS method for rapid and concomitant quantification of pro-inflammatory and pro-resolving polyunsaturated fatty acid metabolites. *Journal of Chromatography B*. 2013;932:123–133.
4. Valet C, Batut A, Vauclard A, et al. Adipocyte Fatty Acid Transfer Supports Megakaryocyte Maturation. *Cell Rep*. 2020;32(1):107875.

Supplemental Table 1: Characteristics of the sepsis group patients

	Sepsis (n=4)
Demographic characteristics	
Age – median (min-max)	59 (44-68)
Male sex – no. (%)	4 (100)
BMI – median (range)	30.5 (29.5-36)
SAPS II – median (min-max)	62 (43-83)
Baseline SOFA – median (range)	12 (11-16)
Co-existing conditions – no. (%)	1 (25)
Hypertension	
Diabetes mellitus	1 (25)
Dyslipidemia	1 (25)
Previous myocardial infarction	1 (25)
Chronic respiratory disease	2 (50)
Obesity	4 (100)
Chronic cardiac disease	2 (50)
Thrombo-embolic disease	1 (25)
Prior antiplatelet agent	2 (50)
Anticoagulation	1 (25)
Biology (at inclusion) – median (min-max)	
Leukocytes (G/L)	12.5 (9.4-15.1)
Lymphocytes (G/L)	1.0 (0.4-1.1)
Platelet count (G/L)	175 (146-318)
Fibrinogen (g/L)	5.2 (2.7.-7.4)
Outcomes, median (min-max) or no.(%)	
Duration of mechanical ventilation (days)	17 (7-53)
ICU LOS (days)	43 (12-115)
Hospital LOS (days)	59 (12-115)
Death – no. (%)	0 (0)

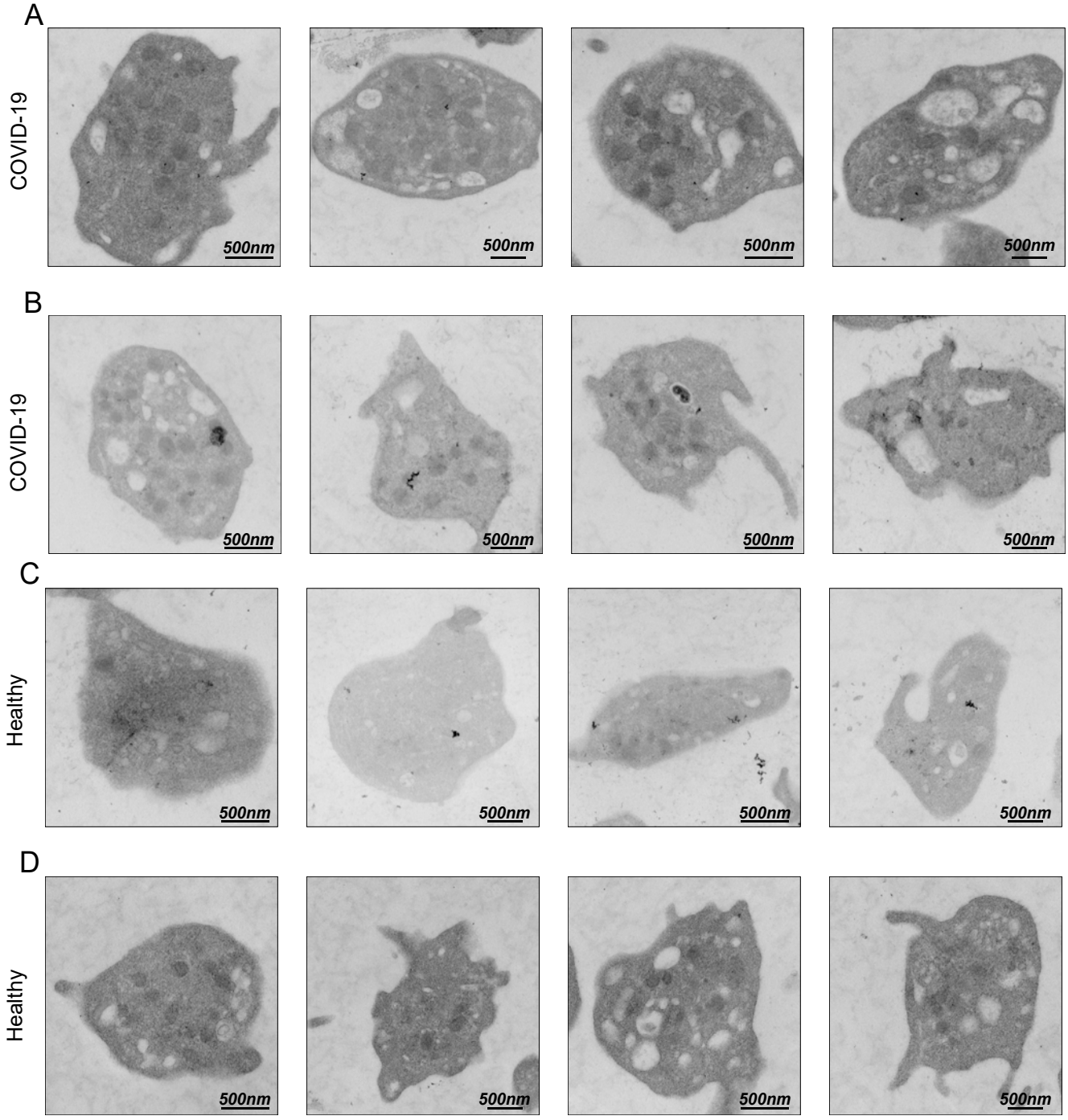
BMI: Body Mass Index, SAPS II: Simplified Acute Physiology Score, SOFA: Sepsis-related Organ Failure Assessment, COPD: Chronic obstructive pulmonary disease, CRP: C-Reactive Protein, ICU: Intensive Care Unit, LOS: Length of Stay.

Figure S1. Comparable number of platelet α-granules in normal and severe COVID-19 patients



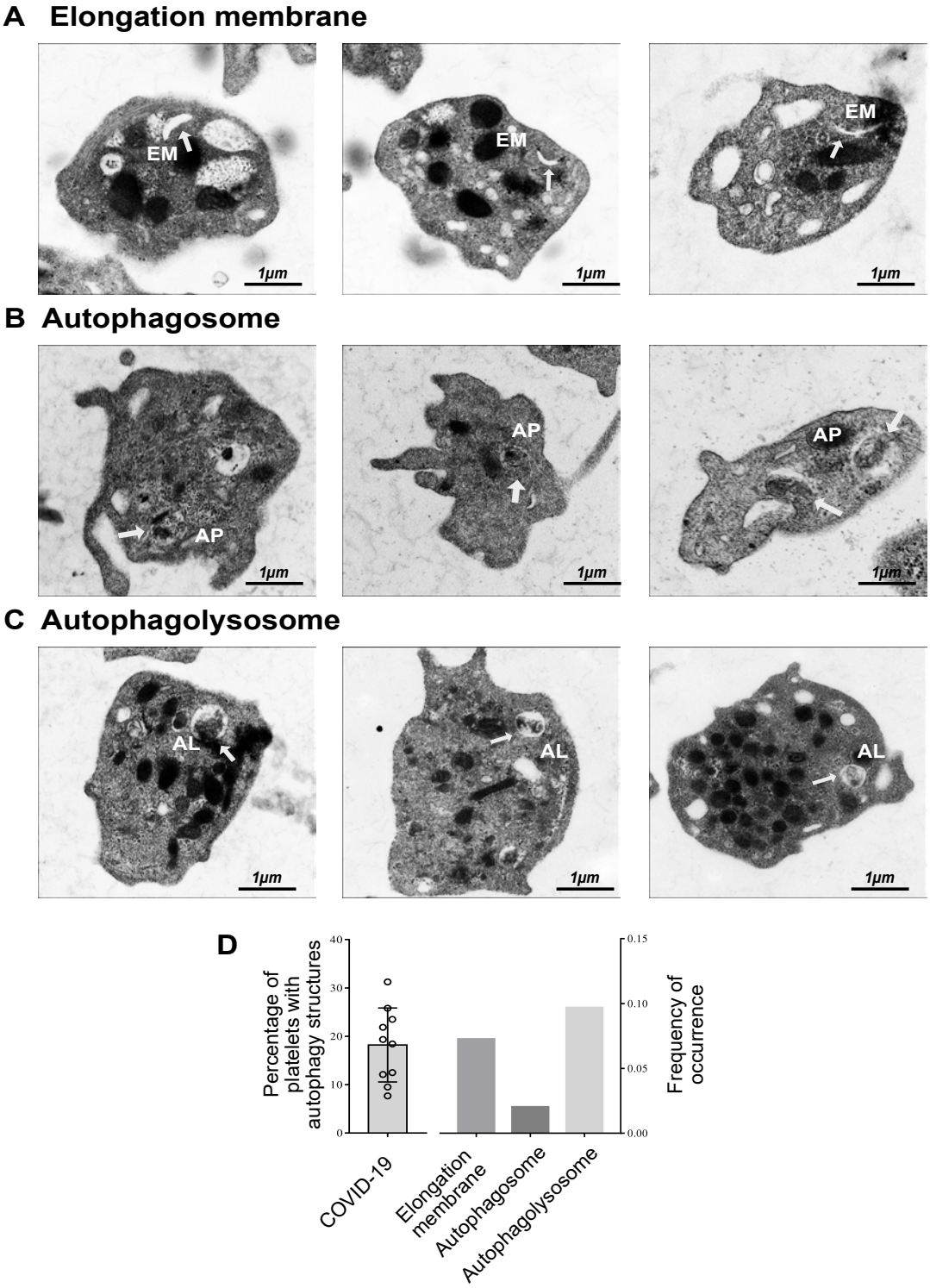
The α-granules were quanti ed using electron micrographs of platelet complete cross-sections (186 from n=6 healthy donors (31 for each), 160 from n=4 non-COVID-19 septic patients (40 for each) and 240 from n=10 severe COVID-19 patients (24 for each)).

Figure S2. Illustration of experimental quality controls



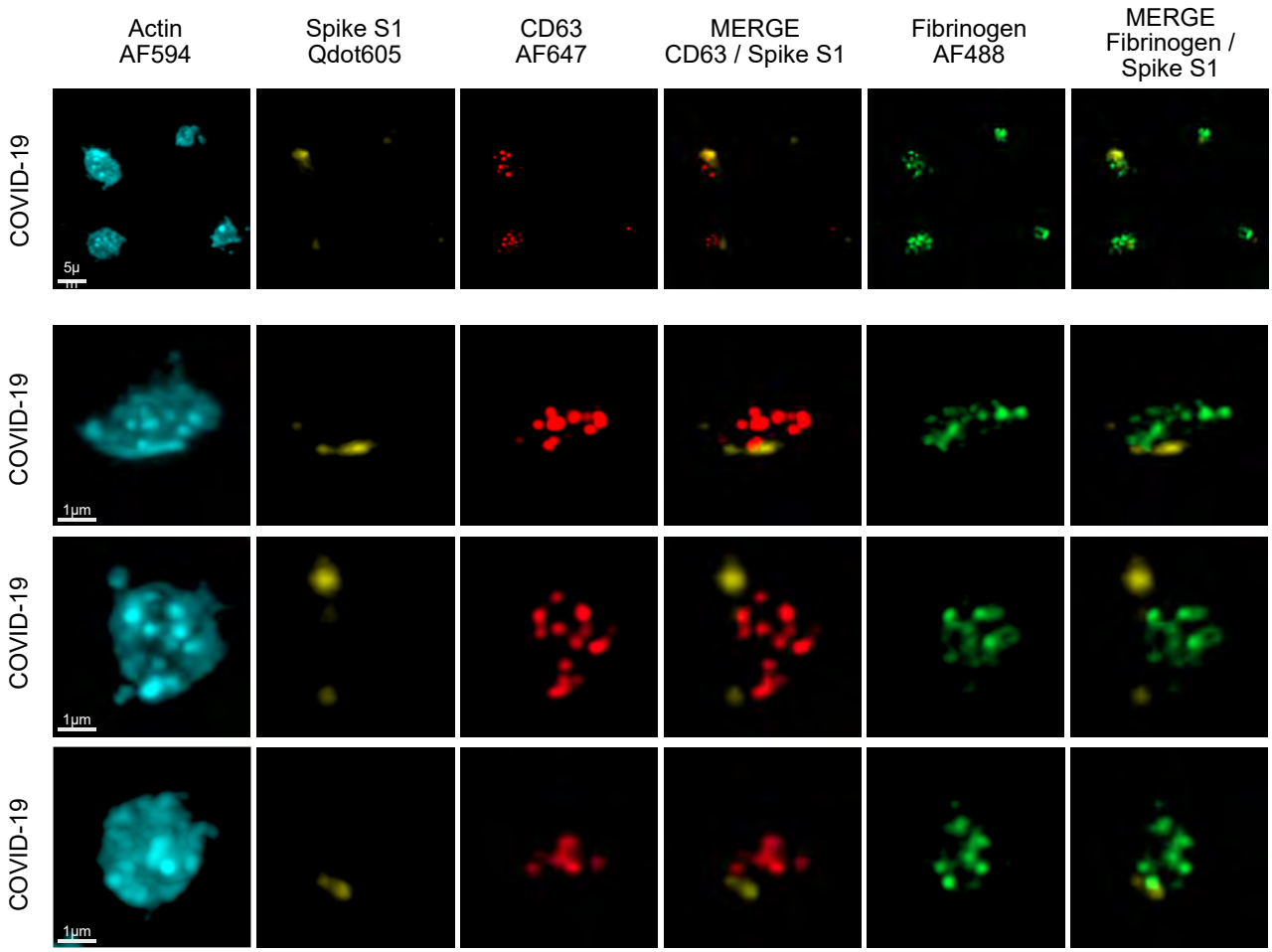
The quality controls of immunogold labelling of Spike S1 protein (A,C) and nucleocapsid (B,D) with the secondary antibody alone were performed in platelet from severe COVID-19 patients (A,B) and from healthy controls (C,D). The gallery of representative image show the absence of gold labbelling.

Figure S3. A gallery of macroautophagy patterns in platelets from severe COVID-19 patients



Macroautophagy is characterized by typical morphological features visualized by transmission electron microscopy in platelets from severe COVID-19 patients. This gallery shows platelets exhibiting elongation membranes (A), autophagosomes (B) and autophagolysosomes-like structures (C). Representative transmission electron micrographs from 8 severe COVID-19 patients are shown. The percentage of platelets with autophagy structures and the frequency of occurrence of the di erent types of structures in platelet from the 10 severe COVID-19 patients were quanti ed (D).

Figure S4. Spike S1 protein does not co-localize with α granules or dense granules in platelets from severe COVID-19 patients



The intraplatelet localization of Spike S1 protein was investigated by imm uorescence and super-resolution confocal microscopy with the Airyscan module using a speci c anti-Spike S1 antibody. Its colocalization with CD63, a marker of denses granules and brinogen, a marker of alpha granules was analysed. Representative images from 3 di erent severe COVID-19 patients are shown for each colocalization study.

Les 2 articles suivants sont d'une part une revue de la littérature rédigée avec l'équipe de recherche dans le cadre de nos travaux portant sur la COVID-19 (Ribes A, Vardon-Bounes F et al, Ad Biol Reg 2020). Nous réalisons dans cette revue de la littérature un état des lieux des connaissances sur les manifestations thrombo-emboliques, la perturbation des paramètres hémostatiques et la physiopathologie de la thrombo-inflammation associées à la COVID-19. Nous discutons en parallèle les modalités de traitement anticoagulant et d'autres potentielles options thérapeutiques pour limiter le risque thrombo-embolique.

Le dernier article proposé présente une cohorte de patients admis au CHU de Toulouse avec un diagnostic d'infection à SARS-CoV-2, dans laquelle nous avons inclus des patients admis en réanimation polyvalente. Nous mettons en évidence un lien entre la survenue d'une thrombocytopénie et un devenir défavorable dans cette pathologie (Maquet J et al. (Vardon-Bounes F collaborator), BJH 2020).

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Thromboembolic events and Covid-19

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ABSTRACT

The novel Corona virus infection (Covid-19) first identified in China in December 2019 has rapidly progressed in pandemic leading to significant mortality and unprecedented challenge for healthcare systems. Although the clinical spectrum of Covid-19 is variable, acute respiratory failure and systemic coagulopathy are common in severe Covid-19 patients. Lung is an important target of the SARS-CoV-2 virus causing eventually acute respiratory distress syndrome associated to a thromboinflammatory state. The cytokinic storm, thromboinflammation and pulmonary tropism are the bedrock of tissue lesions responsible for acute respiratory failure and for prolonged infection that may lead to multiple organ failure and death. The thrombogenicity of this infectious disease is illustrated by the high frequency of thromboembolic events observed even in Covid-19 patients treated with anticoagulation. Increased D-Dimers, a biomarker reflecting activation of hemostasis and fibrinolysis, and low platelet count (thrombocytopenia) are associated with higher mortality in Covid-19 patients. In this review, we will summarize our current knowledge on the thromboembolic manifestations, the disturbed hemostatic parameters, and the thromboinflammatory conditions associated to Covid-19 and we will discuss the modalities of anticoagulant treatment or other potential antithrombotic options.

1. Introduction

Compared with severe acute respiratory syndrome coronavirus (SARS-CoV-1) and Middle East respiratory syndrome (MERS-CoV), the novel corona virus infection (Covid-19), which first case was described in China in December 2019, has spread more rapidly and the epidemic has unevenly affected nearly all continents. This new disease has made more than 370000 confirmed victims worldwide at the end of May 2020, probably underestimated due to weaknesses in the census or the lack of publications. Around 5–10% of Covid-19 patients are severely affected and admitted to intensive care unit (ICU) for mechanical ventilation because of pneumonia. The pathophysiology of SARS-CoV-2 infection goes far beyond the only pulmonary attack and is still under characterization. This review will focus on the thromboembolic events frequently observed in severe Covid-19 patients. It is mainly based on publications of isolated clinical cases or short series, with a retrospective collection of data, still rare histopathological data, and as far as our subject is concerned, results of fairly standard routine tests, and antithrombotic treatment practices by analogies with other pathologies,

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which have been adapted over the course of experience.

2. Clinical manifestation of thromboembolic complications in Covid-19

From the first cases of the disease, symptoms of severe acute respiratory infection accompanied by acute respiratory distress syndrome (ARDS) revealed the lung tropism of this new virus. Autopsy studies of Covid-19 patients (Fox et al., 2020; Wichmann et al., 2020) confirm that lung is a major target organ for SARS-CoV-2, although other organ failure (heart, kidney) and neurological disorders may be observed (Guan et al., 2020; Huang et al., 2020). The SARS-CoV-2 virus uses ACE2 (angiotensin-converting enzyme 2) receptor expressed by pneumocytes in the epithelial alveolar lining to infect the host's lung. Around 5–10% of Covid-19 patients need hospitalization in ICU and require mechanical ventilation. Associated with the severe lung disease, cases of Covid-specific coagulopathy were first reported in China and then all over the world (Wang et al., 2020a; Tang et al., 2020a; Connors and Levy, 2020). The patients presenting with more severe disease symptoms had more pronounced coagulopathy associated with a pejorative prognosis. Severe Covid-19 patients are prone to develop thrombotic events including pulmonary embolism (PE), deep vein thrombosis (DVT), arterial thrombosis and intracatheter thrombosis (Klok et al., 2020a; Helms et al., 2020; Lodigiani et al., 2020; Bikdeli et al., 2020; Joly et al., 2020; Ranucci et al., 2020). Cases of disseminated intravascular coagulopathy (DIC) are also described. This increased Covid-19 patient's predisposition to thrombotic diseases has been highlighted in recent reviews (Connors and Levy, 2020; Bikdeli et al., 2020). Of note, the thrombotic risk is influenced by race and ethnicity and is significantly lower in Chinese compare to Caucasian individuals (Fogarty et al., 2020).

Patients with infection by pathogens admitted to ICU for sepsis are known to frequently develop thromboembolic events that contribute to multi-organ failure. The International Society on Thrombosis and Haemostasis (ISTH) has individualized this medical entity under the acronym sepsis-induced coagulopathy (SIC) and developed a SIC score along with a DIC score. This ISTH definition has been used in reports concerning the Covid-19 patients presenting coagulopathy. Some specificities of SARS-CoV-2 infection induced coagulopathy have however stimulated the emergence of other acronyms like pulmonary intravascular coagulopathy (PIC) (Fogarty et al., 2020) which, given the tropism and viral involvement, would be appropriate, or Covid-19 associated coagulopathy (CAC) which is now frequently used.

The first experiences in the Wuhan province in China, and then in other parts of the world, reported in the form of retrospective and then prospective studies, allowed to propose an incidence of thromboembolic complications ranging from around 15% to 85%, depending on the diagnostic mode (systematic screening or clinical manifestation) and on the severity of the patient population studied (ICU or traditional hospitalization). In these first studies, the size of the patient cohorts for which the incidence of thrombotic events has been quantified was rather small with a probable overestimation of the actual rates of thrombosis all causes included. The nature of thromboembolic episodes was not always precisely specified, and the terms “any cause” or “cumulative incidence” have been used in several reports (Middeldorp et al., 2020). Nevertheless, the multiplicity of these studies, combined with post-mortem analyses have led to a better understanding of the pathogenesis and strongly supports the association between Covid-19 and

Table 1
Incidence of venous thromboembolic events among the different studies.

References	Patient number and setting	Thromboembolic events	Study relevance
Cui et al. (2020)	81, ICU	Incidence of VTE is 25%	Retrospective and single-center study
Demelo-Rodriguez et al. (2020)	156, non ICU	Incidence of asymptomatic DVT is 15%	Prospective and single-center study
Helms et al. (2020)	150, ICU	43% of VTE of which 17% is PE	Prospective and multicentric study
Klok et al. (2020a)	184	Cumulative incidence of VTE of 31% (CTPA confirmed in 27%) of which 81% is PE	Prospective and multicentric study
Llitjos et al. (2020)	26, ICU	Cumulative incidence of peripheral VTE of 69% of which 23% is PE	Retrospective and single-center study
Lodigiani et al. (2020)	388	Cumulative rate of VTE is 21% (28% in ICU and 7% in the general ward)	Retrospective and single-center study
Marone and Rinaldi (2020)	30, non ICU	53% with positive signs or symptoms suggestive of DVT	Retrospective and single-center study
Middeldorp et al. (2020)	198	Cumulative incidence of VTE is 26% at D7, 47% at D14, 59% at D21 in ICU	Retrospective and single-center study
Poissy et al. (2020)	107, ICU	Cumulative incidence of PE is 21%	Retrospective and single-center study
Ren et al. (2020)	48, ICU	86% of lower extremity DVT of which 75% are distal and 10% proximal	Retrospective and multicentric study
Spiezia et al. (2020)	22, ICU	23% of DVT	Retrospective and single-center study
Zhang L et al. (2020a)	143	47% developed lower extremity DVT (35% proximal and 65% distal)	Retrospective and single-center study

ICU, intensive care unit; VTE, venous thromboembolism; DVT, deep vein thrombosis; CTPA, computed tomography pulmonary angiogram; PE, pulmonary embolism; D, day.

coagulopathy (Table 1).

Klok et al. reported a high percentage of PE (Klok et al., 2020b) but other studies report that PE does not seem to be a primary mechanism in contrast to DVT (Helms et al., 2020; Lodigiani et al., 2020).

DIC may complicate SARS-CoV-2 infection (Lillicrap, 2020). Tang and colleagues (Tang et al., 2020a) revealed that 71.4% of the non-survivors from Covid-19 matched the grade of overt-DIC in comparison with survivors (0.6%) according to ISTH criteria (Levi et al., 2009). It has been suggested that DIC might not be only a concomitant finding of Covid-19, but is part of the process leading to organ failure with microthrombi and tissue damage (Seitz and Schramm, 2020). It is still unclear whether DIC results from the progression of the early coagulation changes in Covid-19. Recently, it was proposed that thrombosis occurs within the pulmonary circulation, in the absence of apparent embolism, due to *in situ* macro and microthrombi formation (Deshpande, 2020). Whether the presence of these thrombi correlates with the presence of DIC during SARS-CoV-2 infection is unknown.

Autopsy reports provided important information and demonstrated thrombotic microangiopathy (Wichmann et al., 2020; Konopka et al., 2020; Menter et al., 2020). A German prospective series including 12 Covid-19 patients found a high incidence of DVT (58%) and PE in 4 patients as a direct cause of death (Wichmann et al., 2020). Histopathological study of the lungs revealed diffuse alveolar damage, as classically described in ARDS (Thompson et al., 2017), associated with the presence of microthrombi in small caliber pulmonary vessels. A Swiss autopsy report of 21 patients also revealed diffuse alveolar damage, significant pulmonary capillary congestion, the presence of fibrin deposits and microthrombi despite well-conducted anticoagulation in these patients (Menter et al., 2020). A French series based on 5 autopsies discusses the histological pattern of acute fibrinous and organizing pneumonia in Covid-19 patients with accumulation of intra-alveolar fibrin deposits (Copin et al., 2020). Overall, it appears that DVT and thrombotic microangiopathy of the pulmonary capillaries are more frequently observed in severe SARS-CoV-2 infection than PE. As discussed below, different mechanisms may occur according to vascular territories, the caliber of the vessels and their sensitivity to the highly inflammatory environment.

Arterial territories of all calibers seem less prone to embolic episodes since infarction, lower limb ischemia, mesenteric ischemia and stroke are under-represented (Helms et al., 2020; Lodigiani et al., 2020; Kashi et al., 2020).

Of note, “chilblains like” skin lesions have also been reported whose physiopathology is still debated (De Masson et al., 2020) but the hypothesis of an acro-ischemic lesion has been suggested following observation of microthrombi in rare skin biopsies (Piccolo and Bassi, 2020; Fernandez-Nieto et al., 2020), again pointing to the potential tropism of the virus for small vessels.

3. Disturbed hemostasis parameters in Covid-19

Covid-19 patients with coagulopathy being at increased risk of mortality (Tang et al., 2020a; Zhou et al., 2020), it is important to monitor biological and hemostatic changes during the course of the disease. The most frequently reported changes are an increase in D-dimer levels (a degradation products of fibrin acting as a biomarker reflecting fibrin formation and fibrinolysis), a moderate thrombocytopenia, and a slight prolonged prothrombin time. Early in the epidemic, Huang et al. described that Covid-19 patients hospitalized in the ICU have significantly higher levels of D-dimer compared to Covid-19 patients not admitted in ICU (Huang et al., 2020). Moreover, in a cohort of 191 patients, it was shown that D-dimer levels > 1 mg/L were significantly associated with an increase in hospital mortality (Zhou et al., 2020). In a chinese cohort of 1099 patients, 46.4% of the patients had D-dimer levels > 0.5 mg/L (Guan et al., 2020).

Prothrombin time was significantly longer in IUC compared to non-IUC Covid-19 patients, but the absolute difference was small (Huang et al., 2020). Accordingly, prothrombin time was significantly longer in non-survivors compared to survivors (Tay et al., 2020).

Unlike patients conventionally admitted to ICU for septic shock, where thrombocytopenia is often deep, long-lasting and correlates with morbidity and mortality (Akca et al., 2002), it appears to be moderate in SARS-CoV-2 infection. Indeed, Huang et al. report 5% of patients with a platelet count < 100 G/L and 8% in patients admitted to ICU (Huang et al., 2020). The reasons for this relative preservation of platelet counts are unknown. Very low platelet counts (< 20 G/L) or sudden fall in platelet count suggest additional causes of thrombocytopenia (immune or drug-induced). Guan et al. found a 36.2% incidence of thrombocytopenia < 150 G/L, rising 57.7% in patients admitted to ICU (Guan et al., 2020). A meta-analysis of 9 studies with 1779 Covid-19 patients indicates that the most severe patients had lower platelet counts (Lippi et al., 2020). In a subgroup analysis of 4 studies, thrombocytopenia was associated with a 5-fold increased risk of developing severe Covid-19 (Guan et al., 2020; Huang et al., 2020; Zhou et al., 2020; Liu et al., 2020). Several mechanisms of thrombocytopenia in Covid-19 have been proposed (Xu et al., 2020a). Briefly, SARS-CoV-2 may reduce platelet production by infection of bone marrow cells, hemophagocytosis, or alteration of the pool of megakaryocytes present in the lungs. Other coronaviruses have been shown to infect the bone marrow resulting in hematopoiesis impairment (Yang et al., 2005a). SARS-CoV-2 virus may thus reduce platelet production by affecting megakaryopoiesis. Destruction of haematopoietic cells by activated macrophages may also occur leading to cytopenias including thrombocytopenia. The “cytokine storm” which has been widely discussed in the context of the Covid-19 epidemic appears to be associated in some patients with hemophagocytosis that may contribute to thrombocytopenia (Mehta et al., 2020). The SARS-CoV-2 virus may also increase platelet destruction with specific auto-destruction through autoantibodies and immunes complexes similarly to other infections (Zulfiqar et al., 2020). Finally, the virus may increase platelet consumption via the formation of thrombi in the microcirculation as strongly suggested by autopsy reports showing the presence of platelet microthrombi in small caliber pulmonary vessels (Menter et al., 2020; Fox et al., 2020).

Fibrinogen levels during the course of Covid-19 are elevated (Tay et al., 2020; Panigada et al., 2020; Maier et al., 2020; Spiezia et al., 2020), particularly in ARDS (Ranucci et al., 2020), as expected in any strong inflammatory syndrome, but in contrast to

fibrinogen degradation products (D-Dimers), it is not a marker of poor prognosis (Tang et al., 2020a). Elevated fibrinogen may contribute to the plasma hyper-viscosity observed in Covid-19 patients (Maier et al., 2020), a factor known to increase endothelium damage and the risk of thrombosis.

In a series of 216 patients positive for SARS-CoV-2, 35 patients had prolonged activated partial thromboplastin time (APTT), uncorrected by mixture with normal plasma, and a lupus anticoagulant (LA) was found using clotting tests (dilute Russel Viper Venom Time and/or LA-sensitive APTT) (Bowles et al., 2020). Unfortunately, immunological testing (anticardiolipin, anti-β2 glycoprotein-1) was not performed and LA could not be confirmed in the follow-up. The significance of this disorder is therefore difficult to assess, but these cases indicate that a prolonged APTT warrants complete exploration and is not *per se* an obstacle to anticoagulant treatment. Of note in a small series of 3 patients, ischemic stroke was associated to the presence of anticardiolipin and anti-β2 glycoprotein-1 (Zhang et al., 2020b).

A moderated decrease in factor XII, a contact phase protein, was observed in 91% of patients who had a prolongation of the APTT. Contact pathway activation supports inflammation and thrombin generation and could play a role in immunothrombosis (Bowles et al., 2020), thus representing a potential therapeutic target in Covid-19 patients (Shatzel et al., 2020).

Severe Covid-19 patients have an endothelialopathy (endotheliitis) characterized by the accumulation of inflammatory cells and viral inclusions in endothelial cells (Becker, 2020). Laboratory parameters associated with endothelium activation, cell recruitment and activation may be lymphopenia, thrombocytopenia and elevated levels of Von Willebrand Factor (VWF) and factor VIII (FVIII). In a small series of severe Covid-19 patients, a massive elevation of VWF and FVIII levels was observed while ADAMTS13 activity (a disintegrin and metalloprotease known to regulate the size of VWF) was normal (Escher et al., 2020). It is interesting to note that a high VWF/ADAMTS13 ratio has already been associated with poor clinical outcome in acute ischemic brain injury (Taylor et al., 2020).

Another approach to demonstrate a hypercoagulability state of SARS-CoV-2 infection consists in the use of thromboelastography (TEG), a global method to assess coagulation status (Spiezia et al., 2020). In a recent report, it was shown that TEG parameters were consistent with a state of hypercoagulopathy in a series of 24 blood samples from ICU patients (Panigada et al., 2020). Of note, this hypercoagulability was not associated to a significant decrease in coagulation inhibitors such as antithrombin, protein C or protein S.

4. Thromboinflammation in Covid-19

Although one cannot exclude that the SARS-CoV-2 virus may have some direct procoagulant properties, the hemostasis abnormalities described above are thought to be largely related to the high-grade systemic inflammatory state characterizing severe Covid-19 patients (Connors and Levy, 2020). The concept of “cytokine storm” is associated with a marked increase in pro-inflammatory cytokines such as IL-1 and IL-6 and chemokines together with a Th1 response (England et al., 2020; Tay et al., 2020). The interplay between inflammation and thrombosis, also called thromboinflammation (or immunothrombosis), has been clinically recognized in different human pathologies including sepsis (for a recent review see Jackson et al., 2019). These intricate inflammatory and hemostatic reactions are part of an evolutionary conserved defense process against pathogens involving highly sophisticated molecular and cellular mechanisms in mammals. In this pathophysiological situation, several components of the coagulation system, the complement and the fibrinolytic system are involved in concert with endothelial cells, leukocytes and platelets activation (Jackson et al., 2019; Ekdahl et al., 2015).

A major cause of mortality in patients with Covid-19 is linked to acute lung injury related to ARDS. Several reports and observations suggest that the so-called “cytokine storm”, exacerbated by the lack of prior acquired immunity, is a major input for the occurrence of ARDS in Covid-19 patients (Coperchini et al., 2020). As mentioned above, PE and other mechanisms of pulmonary circulatory failure, such as thrombotic microangiopathy, may explain the relatively modest efficacy of standard prophylaxis with anticoagulants in severe Covid-19 patients (Llitjos et al., 2020) and, in the most severe cases, the failure of standard assisted ventilation techniques.

The pathophysiology of ARDS in its early phase is known to combine local inflammation, the accumulation and activation of leukocytes and blood platelets, uncontrolled activation of coagulation and alteration of endothelial and epithelial permeability (Matthay et al., 2012; Washington et al., 2020). A pathology autopsy report on a small number of Covid-19 patients indicates the presence of pulmonary emboli and thrombotic microangiopathy restricted to the lung (Xu et al., 2020b). This study also reports the presence of platelet-rich clot formation and inflammatory cells (particularly neutrophils) aggregated with fibrin and platelets in small vessels. These features are consistent with a thromboinflammatory response associated with acute lung injury in Covid-19.

Blood platelets are anucleate cells released from megakaryocytes playing a key role in hemostasis in concert with plasma coagulation. Furthermore, it is now well recognized that platelets have important roles in the defense function and in the maintenance of vascular integrity (Koupenova et al., 2018; Rayes et al., 2019). A recent study also highlights their contribution to the protection of the epithelium of the pulmonary alveoli (Washington et al., 2020). Thrombocytopenia has been reported in ARDS (Yang et al., 2005). In Covid-19, thrombocytopenia, generally moderate, is a marker of severity of the disease (Lippi et al., 2020; Yang et al., 2020). A relevant hypothesis to explain thrombocytopenia and poor prognosis is consumption of platelets to form pulmonary thrombi (Thachil, 2020a; Xu et al., 2020a).

To contribute to our body's defense against infectious agents (viral, bacterial, or fungal) platelets are capable of interacting with leukocytes thus helping neutrophils extravasation on inflammatory sites, they also secrete cytokines (IL-1ß, TGFß, RANTES, sCD40L) and produce bioactive lipids (eicosanoids) modulating the inflammation (Koupenova et al., 2018; Rayes et al., 2019). In addition, during infection with the dengue virus it has been shown that the platelet inflammasome, still poorly characterized, is activated leading to the release of pro-inflammatory cytokines, in particular IL-1β (Hottz et al., 2013). In a rat model of bacterial sepsis,

activation of the NLRP3 inflammasome in platelets has been associated with inflammation, increased vascular permeability and multi-visceral failure (Cornelius et al., 2019).

The interaction between platelets and neutrophils leads to their reciprocal activation and contributes to the release of chromatin and histone nets also called neutrophil extracellular traps (NETs) known to bind and immobilize pathogens. The secretion of histones amplifies the alteration of the alveolar endothelium and the NETs support the formation of thrombi (by activating platelets and the contact pathway of coagulation), thus increasing the risk of pulmonary microcirculation occlusion (Caudrillier et al., 2012).

In this context, it is important to note that the lung is, after the bone marrow, the richest organ in megakaryocytes and that in mice, the *in situ* platelets production in lungs is important (Lefrançais et al., 2017). Little is known about the physiological impact of this production in humans. Nevertheless, this recent discovery suggests that platelets are abundant in the alveolar tissue and that they can play a role in the pathophysiology of Covid-19, both in terms of protection by potential phagocytosis of the virus, and aggravation by amplifying local inflammatory processes and increasing the risk of occlusion of the pulmonary capillaries. A pathology report (Fox et al., 2020) shows megakaryocytes producing platelets within alveolar capillaries of Covid-19 patients. Whether this local production of platelets plays an important role following viral lung infection or during ARDS remains to be established. Overall, one can hypothesize that, during Covid-19, blood platelets are activated and recruited at the site of infection, particularly in the lungs, and participate in the activation of the inflammatory response as well as in the appearance of complications related to coagulopathy. Platelet studies in Covid-19 are currently lacking and would represent an important puzzle piece in our understanding.

Another important player in thromboinflammation is the endothelium. While the healthy endothelium is largely antithrombotic, under pathological conditions endothelium activation can lead to immune cells and platelets recruitment and activation (Yau et al., 2015). Endothelium activation and dysfunction has been described in severe Covid-19 patients (Escher et al., 2020). The ACE2 receptor is expressed on endothelial cells allowing direct infection by the SARS-CoV-2 virus leading to endothelial inflammation (endotheliitis) in several organs (Varga et al., 2020).

Following activation, endothelial cells are known to upregulate expression of adhesion molecules including VCAM-1, ICAM-1, and E-selectin that are important for leukocyte adhesion, activation and extravasation. Inflamed endothelial cells also express P-selectin and secrete VWF on their surface allowing platelet recruitment. Tissue factor (TF), a potent activator of coagulation, and plasminogen activation inhibitor 1 (PAI-1), blocking fibrinolysis, can be expressed by activated endothelium. TF is also known be produced by activated monocytes leading to activation of the extrinsic coagulation cascade and α-thrombin generation. α-Thrombin is a key enzyme in fibrin generation but is also a potent platelet activator and has numerous effects on the vasculature (Jackson et al., 2019). The effects of α-thrombin are amplified by the reduction of endothelial antithrombotic actions of molecules such as thrombomodulin, of the activated protein C pathway and of tissue factor pathway inhibitor (TFPI). Activated platelets further interact with leukocytes, secrete proinflammatory molecules and polyphosphates that promote the contact phase of the coagulation pathway. Platelets aggregate via integrin GpIIbIIIa activation and their surface become procoagulant through phosphatidylserine expression. α-Thrombin being able to enhance its own generation via different mechanisms and the endothelium having downregulated its natural antithrombotic properties, microvascular thrombosis and inflammation will further propagate.

Activation of the complement system (observation of deposit of the complement components C5b-9 and C4d in the microvasculature) may also contribute to microvascular injury and thrombosis in the pathogenesis of severe Covid-19 as suggested in a short series of patients (Magro et al., 2020).

Thus, endothelial cells, platelets and leukocytes in concert with the blood coagulation cascades are critical players of the thromboinflammatory reaction leading to thromboembolic events in severe Covid-19 patients (Fig. 1). These mechanisms are even further amplified by hypoxia and decreased blood flow.

5. Current treatment, bleeding risk and new perspectives

Following observations of a high incidence of venous thrombosis linked to mortality in severe Covid-19 patients admitted to the hospital, the thromboprophylaxis has been defined as standard care (Thachil et al., 2020b,c; Klok et al., 2020a,b; Tang et al., 2020b; Ti et al., 2020; Llitjos et al., 2020; Helms et al., 2020; Wichmann et al., 2020; Bikdeli et al., 2020). The heparin prophylactic treatment is associated with a better outcome in critically ill patients with high sepsis induced coagulopathy (SIC) score ≥ 4, and with D-Dimers > 0.3 μg/mL (Tang et al., 2020b). This study however shows some potential biases (observational study without propensity score, no mortality difference in entire cohort but in subgroup analysis) making definitive conclusions difficult.

Interestingly, in addition to the antithrombotic effect, the anti-inflammatory function of heparin may be relevant in this setting. Heparan sulfate proteoglycans bind to SARS-CoV2 spike proteins and may decrease binding to host protein, and reduce the proinflammatory activities of damage associated molecular patterns (DAMPs), chemokines, neutrophil chemotaxis and leukocyte migration (Thachil, 2020b; Liu et al., 2020; Lindahl and Li, 2020). Using surface plasmon resonance, it was shown that SARS-CoV-2 Spike S1 protein binds to heparin inducing a structural change (Mycroft-West et al., 2020).

In a meta-analysis concerning the effectiveness and safety of heparins for the prevention of venous thromboembolism in acutely ill patients (Alikhan et al., 2014), heparins, among 16 reported studies, reduced the incidence of thrombosis with a slightly stronger decrease in the low molecular weight heparin (LMWH) group compared to the unfractionated heparin (UFH) one. However, no significant difference between UFH and LMWH was observed in the incidence of PE. The authors found no clear difference in all-cause mortality but they specified that the studies were not powered to show a difference in mortality.

Several studies report high incidence of venous thrombosis arising in Covid-19 patients hospitalized in ICU units, or with higher risks factors of venous thrombosis such as obesity (BMI > 30 kg/m^2), despite using standard venous thromboembolism thromboprophylaxis according to the ESC or ASH guidelines (Kostantidines et al., 2019; Schünemann et al., 2018; Poissy et al., 2020;

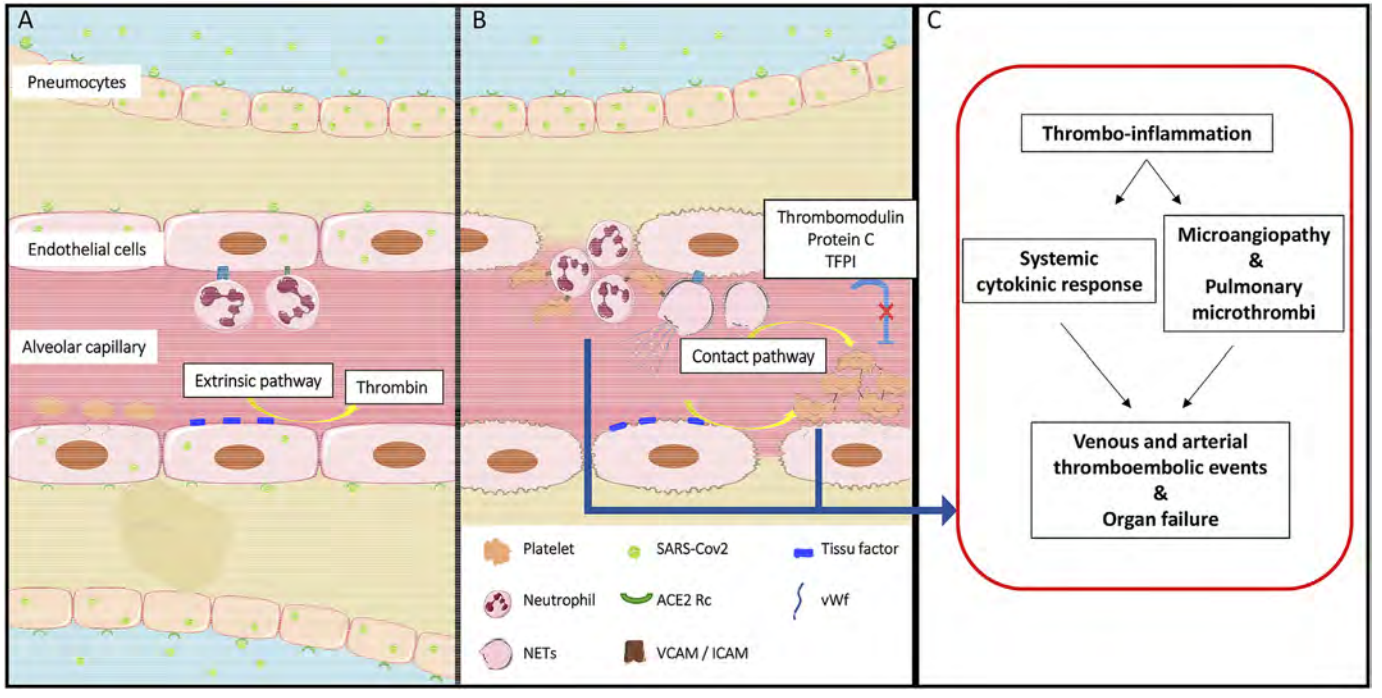

Fig. 1. Immunothrombosis mechanisms in pulmonary circulation during SARS-CoV-2 infection. A. Recruitment phase. SARS-CoV2 infects the alveolar epithelium and endothelial cells through binding to the ACE2 receptor (ACE Rc) exposed at the surface of these cells. Activation of endothelial cells by SARS-CoV-2 upregulates the externalization of Von Willebrand factor (vWF) and leukocytes' adhesion molecules (i.e. ICAM-1 (Intercellular Adhesion Molecule 1), VCAM-1 (Vascular cell adhesion protein-1), E-selectin) allowing adhesion of platelets and neutrophils. Activation of endothelial cells also triggers expression of tissue factor (TF) activating the extrinsic coagulation pathway and in turn α-thrombin generation leading to fibrin generation and platelets activation. B. Activation phase. Activated platelets interact with neutrophils leading to mutual amplification of neutrophil and platelet activation and eventually formation of Neutrophhils Extracelullar Traps (NETs) by DNA decondensation and externalization, allowing activation of the contact pathway. Thrombin generation through Tissue Factor (extrinsic) and contact pathways lead to clot formation and microvascular occlusion. Furthermore, clots are stabilized by the downregulation of endothelial antithrombotic properties. Meanwhile, cytokine release is triggered by platelets and leukocytes. C. Schematic representation of the systemic consequences of microvascular thromboinflammation.*This figure was created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License;* https://smart.servier.com.

Middledorp et al., 2020). The use of LMWH is proposed for all patients except those with severe renal impairment. For those patients, the prophylactic anticoagulant treatment is proposed with 5000 or 7500 unfractioned heparin (UFH) twice a day (Thachil et al., 2020b,c; Cohoon et al., 2020). Several authors suggest the use of a thromboprophylactic strategy with LMWH at low or intermediate dose (i.e. enoxaparine 4000 IU once, or twice daily if BMI > 30 kg/m^2) (Fogarty et al., 2020; Cohoon et al., 2020; Spyropoulos et al., 2020). The optimal thromboprophylaxis in Covid-19 patients is unknown (Hunt et al., 2020) and in absence of published results of randomized studies, many prophylactic strategies based on international, national or institutional expert consensus (Marietta et al., 2020; Susen et al., 2020; Cohoon et al., 2020; Khider et al., 2020) have been suggested in order to manage the risk of venous thrombosis. All these strategies take into account the personal risk factors such as obesity (BMI > 30 or > 40 kg/m^2), active cancer, previous venous thrombosis, age > 60 years, to consider the increase in treatment intensity in the higher risk patients. Even in outpatients with at least one risk factor of venous thrombosis, a thromboprophylaxis is proposed with prophylactic or intermediate doses. According to the VTE risk, the heparin treatment may be considered for up-to 45 days after hospital discharge (Paranjpe et al., 2020). Several authors specify that their guidance documents may be frequently updated. Many clinical trials are ongoing in the Covid-19 in the field of thromboprophylaxis.

It is noteworthy that some cases of thrombotic Heparin Induced Thrombocytopenia (HIT) were reported in Covid-19 patients (3 cases in a small series of patients with ARDS but only one confirmed by serotonin release assay) (Riker et al., 2020). The diagnosis of this rare immune complication of heparin treatment may be difficult in Covid-19 patients, particularly in ICU patients who have frequent thrombotic events and a mild thrombocytopenia.

When they are hospitalized, patients on long-term anticoagulation with oral anticoagulants, vitamin K antagonists (VKA) or direct oral anticoagulants (DOAC) should be switched to full-dose parenteral anticoagulation (mainly LMWH). Besides logistical challenges, such as drug intake difficulties or frequent INR monitoring for VKA, pharmacokinetics interactions of oral anticoagulants with multiple treatments, notably the antiviral therapies (lopinavir, ritonavir, darunavir) substrates of the P-glycoprotein and/or CYP3A4 make uncertain both their efficacy and safety (Testa et al., 2020; Cohoon et al., 2020; Khider et al., 2020; Susen et al., 2020).

Drug-drug interaction (DDI) may affect the levels of active metabolites of P2Y12 antagonists and protease inhibitors in patients requiring a double antiplatelet therapy. When an investigational antiviral treatment is introduced, the choice and the dose of P2Y12 antagonist agents should be carefully balanced in terms of benefit-risk at the lumen of pharmacokinetics of each drug. For example, ritonavir decreases the levels of active metabolites of the prodrug thienopyridines (clopidogrel, prasugrel) and thereby reduces their efficacy. Conversely, ritonavir increases the levels of ticagrelor, which is directly active but metabolized by CYPA4, and thereby increases the bleeding risk (Itkonen et al., 2019; Ancrenaz et al., 2013; Marsousi et al., 2016). Remdesivir is an inducer of CYP3A4 but dose adjustments for oral antiplatelet agents is currently not recommended. Of note, there are no major DDI between investigational Covid-19 therapies and parenteral antiplatelet agents such as cangrelor and integrin GpIIb/IIIa inhibitors (Bikdeli et al., 2020).

A few anecdotal reports on fibrinolytic off-label intravenous administration (tPA) in Covid-19 ICU patients have shown transient pulmonary perfusion improvement (Wang et al., 2020b). The diagnosis of DIC is probably overestimated in Covid-19 (Escher et al., 2020). In case of DIC, the management follows the basics principles.

Clinically-overt bleeding is uncommon in the setting of Covid-19. In summary, the mainstay of blood product transfusion is as follows: i) platelet concentrate to maintain platelet count > 50 G/L in DIC patients with active bleeding or > 20 G/L in those with a high risk of bleeding or requiring invasive procedures, ii) fresh frozen plasma (FFP) (15–25 mL/kg) in patients with active bleeding with either prolonged PT and/or APTT ratios (> 1.5 times normal) or decreased fibrinogen (< 1.5 g/L) and iii) fibrinogen concentrate to patients with persisting severe hypofibrinogenemia (< 1.5 g/L). With the existing data, tranexamic acid, which suppresses the conversion of plasminogen to plasmin, should not be used routinely in Covid-19-associated DIC. However, it should be noted that some clinical trials (NCT04390217; NCT04338074; NCT04338126) evaluate tranexamic acid use in order to reduce the virulence of SARS-CoV-2 because plasmin has been proposed to cleave a viral protein resulting in increased infectivity.

6. Conclusion

During the past six months, on the base of early pre-published experience, our knowledge on the pathophysiology of severe forms of the disease has rapidly evolved towards a systemic inflammatory storm with vascular changes in multiple organs particularly in the lungs. Covid-19 is challenging many clinicians and researchers worldwide to better understand the pathophysiology of the disease, particularly of the ARDS and the thromboinflammatory state. The cytokinic storm mainly described in severe patients may contribute to thrombogenicity and to multiple organ failure leading to death. Many publications report an unusual high incidence of thromboembolic events increasing mortality. Disturbed hemostasis parameters are observed, and coagulopathy is associated with poor outcome, emphasizing the importance to monitor biological and hemostatic changes during the course of the disease. Thromboprophylaxis is a standard of care even if adjustments seem necessary in patients with previous risk factors of thromboembolic disease. Whereas the pandemic Covid-19 is declining, at least in Asia and Europe, more than 1300 clinical trials, one-fourth in the setting of ICU, are registered in the World Health Organization's International Clinical Trials Registry Platform. No doubt that new data will be published in coming months/years, with possible changes in our perception of the disease, its prevention and treatments. This is clearly necessary to improve Covid-19 patient's prognosis.

Declaration of competing interest

The authors declare no competing financial interests.

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Letter to the Editor

Title: Thrombocytopenia is independently associated with poor outcome in patients hospitalized for COVID-19

Running title: Thrombocytopenia in COVID-19

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Thrombocytopenia (defined by platelet count <150 x 10^9/L) has been observed in up to 36% of patients with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus responsible for Coronavirus disease 2019 (COVID-19; Guan *et al*, 2020). In this setting, thrombocytopenia is usually mild, caused by platelet activation and consumption (Thachil, 2020; Pavord *et al*, 2020). In a recent paper published in the British Journal of Haematology, Jiang *et al.* conducted a meta-analysis of 31 studies involving 7613 participants and found a significant association between thrombocytopenia and severe COVID-19 hospitalized patients or poor outcome in this setting (Jiang *et al*, 2020). However, other clinical, biological and radiological factors strongly impact COVID-19 outcome. Whether thrombocytopenia is independently associated to poor outcome in this population is unknown. This study was aimed at addressing this question.

We conducted a study within the Covid-Clinic-Toul cohort that is the cohort of all patients hospitalized for SARS-CoV-2 at Toulouse University hospital, South of France (2800 beds, unique tertiary hospital covering an area of about 3 million inhabitants), with a SARS-CoV-2 infection proven by reverse transcriptase polymerase chain reaction (RT-PCR). First patients (from March 11 to April 1) were retrospectively included and data from patients hospitalized after April 1 were prospectively collected. The unique exclusion criterion was the opposition to data collection. All patients, or their representatives for those not able to understand the purpose of the study, were informed by a letter given at admission to hospital and/or sent to their place of residency. This cohort has been approved by institutional review board (n°RnIPH 2020-31), in accordance with the French data protection authority (MR004, *Commission Nationale de l'Informatique et des Libertés*, CNIL). In the present study, we selected the patients included in the Covid-Clinic-Toul cohort up to April 20, 2020.

The platelet count within the first 24 hours upon admission was considered. Thrombocytopenia was defined by platelet count <150 x 10^9/L. The primary outcome was composite, including admission to ICU, need of mechanical ventilation and death occurring during the 14 days after admission to the hospital. Secondary outcome was death occurring during the 14 days after admission to the hospital. Covariables, assessed upon admission at the time of platelet count measurement, were the age (≥65 years vs. <65 years), sex, presence of comorbidities (≥1 vs. 0, including arterial hypertension, cardiovascular disease, cerebrovascular disease, chronic pulmonary disease, chronic liver disease, chronic kidney disease, diabetes, cancer, obesity and

immunosuppression), oxygen saturation ≤92% or need of oxygen therapy, lymphopenia (<1.5 x 10^9/L), C-reactive protein (≥5 mg/dl vs. <5 mg/dl), and severe extension of lesions on chest computed tomography scans (≥50% of lung parenchyma, vs. moderate or mild involvement, defined by extension <50% of lung parenchyma). In adjusted models, missing values were handled by multiple imputation. We conducted logistic regression models providing odds ratios (ORs) with their 95% confidence intervals (CIs). Statistical analyses were performed using SAS V9.4™ (SAS institute, Cary, NC, USA).

A total of 263 patients were included in this study. The median age was 64 years (IQR: 53-76), and 116 patients (57.7%) were men. The median duration of symptoms at the time of admission to hospital was 7 days (interquartile range: 4-10). Platelet count within admission was assessed in 253 patients. The median platelet count was 186 x 10^9/L (range: 63-795). Thrombocytopenia was observed in 63 (24.9%) patients. Patient's characteristics are described in Table 1. Overall, 122 (46.4%) patients met the primary outcome criteria, and 19 (7.2%) died. The prevalence of thrombocytopenia upon admission was 35.8% and 52.6% in these two groups, respectively, while it was 15.0% in patients who did not achieve the composite outcome at day 14 after admission. Results regarding the association of thrombocytopenia upon admission and outcomes are presented in Table 2. In adjusted models, thrombocytopenia was associated to primary outcome occurrence with an OR of 2.48 (95% CI: 1.17 – 5.23). Thrombocytopenia was associated to mortality with an OR of 2.70 (95% CI: 0.91 – 8.01).

The population of this study was older and more severe than the initial cohorts from China (Guan *et al*, 2020; Yang *et al*, 2020; Jiang *et al*, 2020). However, the prevalence of thrombocytopenia was close to the prevalence previously observed (Terpos *et al*, 2020). We confirmed that thrombocytopenia is often mild in the setting of hospitalized patients for COVID-19 (Thachil, 2020; Pavord *et al*, 2020), in all cases in our cohort. The adjusted results of this model highlight the importance of thrombocytopenia upon admission as associated to poor outcome and mortality in patients hospitalized for SARS-CoV-2 pneumonia, like it has been described in other settings of hospitalized patients, notably community-acquired pneumonia (Brogly *et al*, 2007; Aliberti *et al*, 2008; Mirsaeidi *et al*, 2010; Moulis *et al*, 2020). However, this study suffers from limitations. First, acquisition of data was retrospective for the first patients included in the cohort. Second, some data were missing. However, they were very few (see Table 1 and Table 2) and corrected by multiple imputation. Lastly, the size of the cohort resulted in large

95% CI for the assessment of the link between thrombocytopenia and death in the multivariate model. However, the value of the OR (2.70) is so high that we can reasonably suppose that there exists a major association. Similarly, the size of the cohort prevented from assessing the association between various levels of thrombocytopenia and outcome occurrence. Of note, the aim of this study was to assess the impact of thrombocytopenia upon admission on poor outcomes whatever the mechanism. It should be noted that some mechanisms of thrombocytopenia may be particularly associated with worsening, such as disseminated intravascular coagulation in patients admitted ICU, and need to be evaluated in specific studies.(Al-Samkari *et al*, 2020)

This study confirms that thrombocytopenia upon admission is a strongly associated to poor outcome and mortality in patients hospitalized for SARS-CoV-2 pneumonia, independently from other markers of severity.

Aknowlegments

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Authors contributions

J.M., M.L., A.S. and G.M. designed the study. J.M. and M.L. carried out the data management and wrote the manuscript. J.M. and G.M. conducted the statistical analyses. The collaborators included in the "Covid-clinic-Toul investigators group" included the patients and participated to data collection. All authors participated in the interpretation of the results, critically reviewed the manuscript and gave final approval for submission. All authors also had full access to all of the data (including statistical reports) in the study and can take responsibility for the integrity of the data and the accuracy of the data analysis.

Conflicts of interest

The authors declare that they have no competing interest related to this study. The Covid-clinic-Toul cohort is funded by Toulouse University hospital.

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Table 1. Characteristics of patients admitted to Toulouse University hospital for COVID-19 pneumonia (n=263).

Variables	Total (n=263)	Thrombocytopenia* upon admission	
		Yes (n=63)	No (n=190)
Age ≥65 years, n (%)	132 (50.2%)	40 (63.5%)	88 (46.3%)
Women, n (%)	108 (41.1%)	17 (27.0%)	86 (45.3%)
Presence of comorbidities, n (%)	227 (86.3%)	58 (92.1%)	161 (84.7%)
Oxygen saturation ≤92% or need of oxygen therapy*, n (%)	117 (44.8%)	37 (58.7%)	78 (41.5%)
Lymphopenia (<1.5 x 10^9/L)*, n (%)	189 (82.9%)	54 (93.1%)	132 (80.0%)
C-reactive protein ≥5 mg/dl*, n (%)	131 (51.6%)	33 (53.2%)	95 (50.8%)
Severe extension of lesions on chest CT*, n (%)	70 (27.7%)	20 (31.7%)	48 (25.8%)
Death, n (%)	19 (7.2%)	10 (15.9%)	9 (4.7%)
Admission to ICU, mechanical ventilation or death, n (%)	122 (48.2%)	43 (68.3%)	77 (40.5%)

*Missing values: platelet count, n=10; oxygen saturation, n=2; lymphocyte count, n=35; C-reactive protein, n=9; extension of lesions on chest computed tomography scans, n=10.

Abbreviations: CT: computed tomography; ICU: intensive care unit.

Table 2. Association of thrombocytopenia upon admission and outcomes at Day 14 after admission to hospital for COVID-19 pneumonia (n=263).

	Admission to ICU, mechanical ventilation or death			Death		
	N	**Crude OR (95% CI)**	**Adjusted* OR (95% CI)**	**N**	**Crude OR (95% CI)**	**Adjusted* OR (95% CI)**
Thrombocytopenia	43 (35.8%)	3.16 (1.74 – 5.87)	2.48 (1.17 – 5.23)	10 (52.6%)	3.79 (1.46 – 10.03)	2.70 (0.91 – 8.01)

*Adjusted for the age (≥65 years vs. <65 years), sex, presence of comorbidities (≥1 vs. 0, including hypertension, cardiovascular disease, cerebrovascular disease, chronic pulmonary diseases, chronic liver disease, chronic kidney disease, diabetes, cancer, obesity and immunosuppression), oxygen saturation ≤92% or need of oxygen therapy, lymphopenia (<1.5 x 10^9/L), C-reactive protein ≥5 mg/dl vs. <5 mg/dl, and severe extension of lesions on chest computed tomography scans (≥50% of lung parenchyma, vs. moderate or mild involvement defined by extension <50% of lung parenchyma). Missing values: oxygen saturation, n=2; lymphocyte count, n=35; platelet count, n=10; C-reactive protein, n=9, extension of lesions on chest computed tomography scans, n=10.

Abbreviations: CI, confidence interval; ICU, intensive care unit; OR, odds ratio.

DISCUSSION ET PERSPECTIVES

Ces travaux, menés à la fois chez l'animal et chez l'Homme, nous permettent de confirmer que les plaquettes sont des acteurs essentiels de l'inflammation au cours du sepsis. Divers marqueurs, notamment solubles, témoignent de leur activation et de leur implication dans la physiopathologie du sepsis. La thrombopénie qui résulte du sepsis est à mettre en lien avec l'activation des plaquettes, les interactions leuco-plaquettaires, la formation de thrombi dans la microcirculation et avec leur engagement direct dans la lutte contre les agents pathogènes.

Les modèles animaux permettant d'étudier le sepsis sont variés et leur définition/caractérisation est primordiale pour l'interprétation et la reproductibilité des données collectées. Notre caractérisation du modèle de ligature perforation caecale a permis de mieux comprendre la réaction inflammatoire produite par la péritonite perforée chez la souris et de trouver un compromis entre la sévérité de l'atteinte septique induite, la hauteur de la ligature et la mortalité. Ce travail a servi de base à nos travaux ultérieurs.

Ensuite, grâce à ce modèle, nous avons pu mieux décrire la cinétique d'activation plaquettaire au cours des premières heures du choc septique. Nous avons également montré que les caractéristiques prothrombotiques du sepsis étaient conservées malgré l'absence de PI3Kβ (p110 β) plaquettaire. Cette isoforme de PI3K est connue pour son rôle clé dans la stabilisation du thrombus en condition de flux élevé. Nos résultats indiquent qu'il existe des voies de signalisation alternatives dans le cas du sepsis, capables de stabiliser le thrombus en condition de hautes forces de cisaillement, et ce, malgré la thrombopénie. Ce modèle de sepsis et nos souris déficientes en p110β spécifiquement dans les mégacaryocytes/plaquettes pourrait permettre d'identifier la ou les voies de signalisations mises en

jeux pour activer durablement l'intégrine aIIbβ3 et stabiliser le thrombus en condition de sepsis, indépendamment de la PI3Kβ.

Chez l'Homme, même si le début réel du choc septique est souvent inconnu, les résultats sont comparables, avec la mise en évidence de marqueurs d'activation précoces (dès l'inclusion) et persistant à 48h avec des signes de désensibilisation des plaquettes. Au cours d'un choc septique, le praticien est amené à s'interroger sur la fonctionnalité plaquettaire, particulièrement lors de thrombopénies sévères. En effet, l'incidence de la thrombopénie apparaissant en réanimation varie de 14 à 44% selon le seuil choisi dans les études (H. P. MD et al. 2011; Williamson et al. 2013) et il est bien démontré que sa survenue est associée à une augmentation de la mortalité en réanimation (Williamson et al. 2013; Thiery-Antier et al. 2016; Tsirigotis et al. 2016). Les mécanismes menant à la survenue d'une thrombopénie sont pluriels, allant de la diminution de la production, à la séquestration, aux interactions leuco-plaquettaires, mais également à une augmentation de la destruction et à la consommation plaquettaire (Zarychanski and Houston 2017; Greinacher and Selleng 2016). Le diagnostic étiologique repose sur un interrogatoire précis et un examen minutieux des thérapeutiques prescrites et des événements survenus au cours de l'hospitalisation (sepsis, saignement, usage d'une circulation extra-corporelle…). Si le diagnostic de thrombopénie est facile à porter, il est plus difficile d'évaluer la fonctionnalité plaquettaire au cours du sepsis au lit du patient. Nos travaux montrent qu'au cours du sepsis, à l'état basal, les plaquettes circulantes ne présentent pas de marqueurs de surface d'activation, mais le taux élevé des marqueurs circulants (GPVI soluble, CD40 ligand soluble, eicosanoïdes) est un stigmate d'activation. De plus, les plaquettes paraissent en partie désensibilisées lors d'une stimulation comme en témoignent l'analyse en cytométrie en flux des marqueurs de sécrétion (CD63, P-selectine) et les tests d'agrégation plaquettaire. Cependant, au cours du sepsis en réanimation, chez les patients thrombopéniques, la survenue de symptômes hémorragiques semble plus pertinente que le compte plaquettaire lui-même pour décider la prescription d'une transfusion de plaquettes. Du fait du manque d'essais randomisés, des incertitudes majeures subsistent quant à

l'utilité et l'innocuité de transfusion plaquettaire prophylactique chez les patients de réanimation thrombopéniques. Un essai contrôlé randomisé de 2019 publié dans le NEJM (Curley, Stanworth, and New 2019) a montré de façon étonnante une surmortalité chez les nouveau-nés préterme qui avaient reçu une transfusion plaquettaire en-dessous de seuil de 25G/L versus 50G/L. Les auteurs discutaient du potentiel effet inflammatoire de la transfusion plaquettaire chez les nouveau-nés, avec la possibilité que la transfusion soit accompagnée par la libération d'espèces réactives de l'oxygène et par des facteurs pro-angiogéniques qui pouvaient aggraver une dysplasie bronchopulmonaire (Curley, Stanworth, and New 2019). Chez les patients adultes de réanimation, 5 études observationnelles post-transfusion montrent que le compte plaquettaire ne remonte pas au-dessus de 100 G/L et que l'augmentation escomptée est en général de 15 G/L après une transfusion plaquettaire (Lieberman et al. 2014). Au final, les effets cliniques - bénéfiques ou non - de la transfusion plaquettaire chez les patients thrombopéniques de réanimation restent inconnus à ce jour, particulièrement au cours du sepsis. L'intérêt de documenter les fonctions plaquettaires chez les patients thrombopéniques lorsque se pose la question d'une transfusion est peu décrit. Au lit du patient ou sur la paillasse, peu de tests de fonctionnalité plaquettaire sont réalisables dans un délai court qu'est celui demandé en général par la prise en charge réanimatoire d'un patient. Au cours du sepsis, certains auteurs proposent l'utilisation de la thromboélastométrie pour documenter les paramètres de coagulation des patients (Sivula et al. 2009; Daudel et al. 2009; Andersen et al. 2014). Elle semble pouvoir fournir des résultats intéressants mais reste peu disponible en pratique clinique courante et réservée à certains centres. Son coût et la technicité qu'elle nécessite sont des freins à son utilisation en pratique courante, pour un bénéfice, au final, discutable.

Durant nos travaux, nous avons également mis en évidence que l'évolution du volume moyen plaquettaire (VMP) au cours des premiers jours du choc septique était différent selon que les patients survivaient ou non à 3 mois de leur admission en réanimation. Notre étude ne permettait pas d'expliquer l'augmentation du VMP en lien avec la mortalité, en particulier d'en préciser le

mécanisme physiopathologique. Plusieurs hypothèses sont envisageables pour la genèse de ces plaquettes dont la taille est augmentée. Nishimura S et *al.* ont montré qu'il existait une biogenèse alternative à la biogenèse physiologique des plaquettes, avec identification d'une fragmentation cytoplasmique rapide des mégacaryocytes avec libération d'un nombre important de plaquettes dans la circulation, en réponse à un stress (Nishimura et al. 2015). Les plaquettes immatures sont caractérisées par une augmentation de leur contenu en ARN par rapport aux plaquettes dites "matures", et sont aussi appelées plaquettes réticulées. Elles correspondent à des plaquettes au volume moyen augmenté, qui entraînent une augmentation de l'IPF (Immature Platelet Fraction). Des études ont précédemment démontré que les plaquettes immatures étaient hémostatiquement plus actives que des plaquettes matures, et de ce fait pouvaient potentiellement contribuer à l'augmentation du risque de maladie thrombo-embolique (Choi et al. 2017; Kovács et al. 2019). Les plaquettes immatures produiraient plus de TXA2, agrègeraient plus rapidement et contiendraient davantage de granules que les plaquettes matures (Bath and Butterworth 1996). Dans une revue récente, Thorup CV et *al* suggèrent qu'une augmentation du nombre de plaquettes immatures est associée à avec une augmentation de la sévérité des patients septiques (Thorup, Christensen, and Hvas 2020).

Enfin, dans la dernière partie de ma thèse, nous montrons pour la première fois que dans le cas d'un sepsis viral, les plaquettes sont en capacité d'internaliser des particules virales de SARS-CoV-2 et présentent des stigmates d'autophagie spécifique chez des patients admis en réanimation pour SDRA COVID-19. Il s'agit de la première étude à proposer une description aussi détaillée de l'ultrastructure plaquettaire au cours de la pneumonie virale sévère à SARS-CoV-2. Nos travaux montrent que les patients COVID-19 de réanimation ont des marqueurs d'activation plaquettaire protéiques et lipidiques solubles très élevés en comparaison à des contrôles sains. Les interactions leuco-plaquettaires sont aussi largement augmentées, en lien avec la situation inflammatoire de ces patients. Ces données valident notre population eu égard aux cohortes précédemment publiées et viennent les compléter. Nous mettons en évidence par ailleurs une moindre réponse au collagène en agrégométrie.

Ce résultat est cohérent avec la mise en évidence d'un « shedding » accru de la GPVI à la surface des plaquettes, et d'une augmentation du fragment soluble (sGPVI) qui pourrait s'expliquer par une augmentation de l'activité protéasique de la métalloprotéase ADAM10 au cours de l'activation plaquettaire, possiblement pour limiter les conséquences d'une hyperactivation.

L'analyse par microscopie électronique de l'ultrastructure plaquettaire des patients COVID-19 sévères a montré des signes en faveur d'une activation avec une diminution du nombre de microtubules et une augmentation du nombre de pseudopodes émis. De façon intéressante, nous avons mis en évidence des structures coronales dans une proportion significative de plaquettes des malades COVID. Nous avons confirmé la nature virale (SARS-CoV-2) de ces formations par un immunomarquage avec des billes d'or et en microscopie confocale avec un marquage anti-Spike S1 ou nucléocapside. Le mode d'entrée du virus dans les plaquettes reste débattu. Récemment, l'incubation de virions SARS-CoV-2 avec des plaquettes a confirmé la possibilité d'une internalisation très active et rapide (en quelques minutes) (Koupenova et al. 2021) via des endosomes, des vacuoles de phagocytose et possiblement par l'attachement à des microparticules. Cependant, des études supplémentaires sont nécessaires pour confirmer ces modes d'entrée. Un mécanisme indépendant du récepteur ACE2 est suggéré. Le récepteur CD147 exprimé par les plaquettes pourrait être un récepteur potentiel et permettre l'entrée du coronavirus dans les plaquettes mais ces hypothèses restent à confirmer.

Enfin, nous avons montré que la protéine Spike S1 était présente au sein de vésicules élargies dans les plaquettes des patients COVID-19 sévères. Des structures d'autophagie ont été mises en évidence avec des membranes d'élongation, des autophagosomes et des autophagolysosomes, confirmées par un marquage spécifique de LC3B qui co-localise avec Spike S1. Ce mécanisme d'autophagie spécifique est apparenté à la xénophagie, ou encore virophagie, connu pour jouer un rôle majeur dans la clairance des pathogènes. Les plaquettes semblent avoir conservé ce mécanisme mais de nombreuses interrogations subsistent : comment le processus de xénophagie est-il initié dans ce cas précis ? quels mécanismes sont mis en route ? via quelles protéines ? comment s'effectuent le ciblage spécifique,

l'endocytose et la destruction ciblée du SARS-CoV-2 ? quels sont les liens avec l'activation plaquettaire ? Nous tenterons d'aborder ces questions dans la poursuite de nos travaux concernant l'impact des plaquettes chez les patients atteints de la forme sévère de la COVID-19. Nous nous attacherons à poursuivre la caractérisation des modifications ultrastructurales, en particulier des structures d'autophagie spécifiques, mises en place par les plaquettes au cours de la COVID-19. Nous analyserons également les mégacaryocytes afin de rechercher la présence éventuelle de structures virales en leur sein. Nous analyserons la morphologie plaquettaire à distance de l'infection à SARS-CoV-2 afin de documenter le niveau de persistance des structures vacuolaires plaquettaires, notamment dans les formes de COVID-19 long. La mise en place d'un modèle expérimental de COVID-19 chez la souris serait très utile mais reste difficile. Outre la description détaillée des plaquettes de patients, un certain nombre d'expériences pourront être conduites in vitro, en mettant les plaquettes humaines ou de souris (sauvages ou KO pour un certain nombre de récepteurs de surface et de protéines signalisation) en présence de protéine Spike S1 recombinante. Nous avons en effet observé des taux circulants de Spike S1 élevés chez nos patients COVID-19 sévères. Nous testerons l'hypothèse selon laquelle les plaquettes pourraient capter cette protéine virale exogène pour la dégrader par un processus apparenté à la xénophagie.

De façon plus large, nous nous emploierons à analyser si les plaquettes présentent des caractéristiques spécifiques au cours du sepsis bactérien ou viral qui émaneraient d'une reprogrammation des mégacaryocytes durant le sepsis en lien avec leurs fonctions immunes.

Les travaux de recherche issus de cette thèse nous apportent une meilleure définition d'un modèle animal d'étude du choc septique, soulignent le rôle majeur des plaquettes dans la physiopathologie du sepsis et les positionnent comme cellules clés dans le déroulement de la réponse inflammatoire et des processus thrombo-inflammatoires. Nos travaux de recherche menés durant la pandémie à SARS-CoV-2 apportent des éléments cruciaux tant sur les modifications de l'ultrastructure

plaquettaire que sur les capacités d'internalisation de particules virales durant le SDRA COVID-19, suggérant un rôle majeur de l'autophagie dans les processus d'élimination virale.

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