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# Rôle inflammatoire des plaquettes sanguines lors du sepsis

Adrien Chabert

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Adrien Chabert. Rôle inflammatoire des plaquettes sanguines lors du sepsis. Médecine humaine et pathologie. Université de Lyon, 2017. Français. NNT : 2017LYSES024 . tel-02079232

**HAL Id: tel-02079232**

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**UNIVERSITÉ  
JEAN MONNET**  
SAINT-ÉTIENNE

**Université de Lyon**  
**Université Jean Monnet**  
*Ecole doctorale Science, Ingénierie, Santé*

Thèse de doctorat en Sciences, Ingénierie, Santé

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**Adrien CHABERT**

Présentée et soutenue publiquement le 19 Juillet 2017

Pr. F. ADER	Rapporteur	Université de Lyon
Dr. B. HO-TIN-NOE	Rapporteur	Université Paris 7
Dr. M-P. GRATACAP	Examineur	Université de Toulouse
Pr. B. POZZETTO	Examineur	Université Jean Monnet
Dr. H. HAMZEH-COGNASSE	Co-encadrante	Université Jean Monnet
Pr. O. GARRAUD	Directeur de thèse	Université Jean Monnet





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*« La vérité scientifique sera toujours plus belle que les créations de notre imagination et que les illusions de notre ignorance. »*

*Claude Bernard*

# Remerciements

*Je souhaiterais remercier sincèrement l'ensemble des membres de mon jury, le professeur Florence Ader, les docteurs Marie-Pierre Gratacap et Benoît Ho-tin-noe, d'avoir accepté et pris de leurs précieux temps, pour évaluer ce travail.*

*Je voudrais également remercier chaleureusement, le Professeur Bruno Pozzetto, directeur du laboratoire GIMAP durant les deux premières années de ma thèse, d'avoir accepté de présider et examiner ce travail doctoral ainsi que de m'avoir accueilli au sein de son laboratoire. Je voudrais, par ailleurs, le remercier dans son implication pour mon obtention pour le poste d'attaché temporaire à l'enseignement et à la recherche.*

*Un grand merci au Professeur Thomas Bourlet, actuel directeur du GIMAP, de m'avoir soutenu jusqu'à la fin de ma présence dans ce laboratoire et d'avoir contribué à mes enseignements au sein de la faculté de médecine.*

*Je ne pourrais jamais remercier assez le Professeur Olivier Garraud, pour sa direction lors de ces quatre années. Je le remercie pour la lumière qu'il m'a apportée dans ces différents projets, ses relectures si précieuses et son expertise dans le domaine de la publication.*

*Bien évidemment, un immense merci à ma co-encadrante de thèse, le Docteur Hind Hamzeh-Cognasse, de m'avoir fait confiance. Merci pour ta disponibilité, tes conseils techniques et théoriques. Je suis bien conscient de certains de mes défauts et j'honore ta patience dans certains moments. Cette histoire, je la dois, en grande partie à toi et j'espère que je repartirai en laissant une trace, autant scientifique que personnelle. Dans tous les cas, je repars le cœur stéphanois.*

*Je remercie également le Docteur Fabrice Cognasse, pour son encadrement scientifique qui a permis à ce projet d'avancer davantage. Merci pour les financements qui nous ont permis de surpasser les défauts de la comptabilité de la faculté. Tu auras contribué en grande partie à ma formation de jeune chercheur, et pour cela, je t'en remercie.*

*Je souhaiterais remercier tous nos collaborateurs dans ce projet ANR qui auront permis, par leurs aides, l'avancé de ce travail doctoral. Merci tout particulièrement, aux Professeurs Gérard Lina et Bernard Payraastre. Merci également au Docteur Laurence Panicot-Dubois qui lors d'un congrès m'a gentiment proposé de venir doser les microparticules au sein du*

*laboratoire du Professeur Françoise Dignat-George. Ces trois jours intenses avec le Docteur Stéphane Robert ont été intéressants et formateurs.*

*Je remercie la région Rhône-Alpes et l'école doctorale Science Ingénierie Santé 488 pour l'attribution de sa bourse Régionale, l'établissement Français du sang pour les produits sanguins labiles qui m'ont permis de travailler, les donneurs de sang de l'EFS sans qui ce travail n'aurait pu exister. Merci à l'université Jean Monnet l'attribution du poste d'attaché temporaire à l'enseignement et à la recherche. Je voudrais remercier le doyen de la faculté de médecine, le professeur Fabrice Zeni pour son implication dans l'obtention de ce poste et de sa confiance dans mes enseignements. Merci à tous ceux avec qui j'ai pu travailler lors de ces enseignements, particulièrement Pierre Flori, Florence Grattard, Paul Verhoeven, Sylvie Pillet, Stéphane Paul, Nathalie Perek.*

*Merci aux personnes avec qui j'ai pu travailler outre GIMAP : merci Zhigo pour tes conseils en microscopie et le résultat de ces photos si difficiles à obtenir, merci à Jérôme Morel pour ces échanges si éclairants grâce à votre point de vue clinique. Merci aux personnels du PLEXAN et tout particulièrement à Priscilla et Ghislaine.*

*Je voulais dire un immense merci à toutes les personnes du GIMAP ! Merci pour votre accueil que je ne retrouverais sûrement jamais ailleurs. Dès mon premier jour, le ventre serré, j'ai découvert un environnement de travail agréable et tous les matins durant ces quatre années, cela a contribué à la motivation nécessaire pour l'accomplissement de cette thèse. Directeurs, chercheurs, ingénieurs, techniciens et étudiants, vous avez tous, d'une manière ou d'une autre, participé à ma maturation scientifique et personnelle. J'en oublierai sûrement mais merci à Fabienne, Blandine, Agathe, Olivier, Séverine, Thibault, Rémi, Eva, Kiki, l'interne, l'autre (Alex), Anne, Youssouf... Merci à toi Benjamin pour ta gestion des commandes avec tant de professionnalisme ! Bientôt la retraite, tiens bon !*

*Un remerciement sans limite à l'« EFS Team » ! Merci à Paupau, Kim Ahn et Chaker pour vos conseils d'« anciens ». Marie-Ange, merci à toi pour tous ces moments de partage. Ce fût un réel plaisir de travailler avec toi, de « galérer » avec toi sur certaines manip' mais également de s'évader en parlant voyage... Charles-Antoine, merci pour ta joie de vivre, pour tes blagues, pour tes pics. Autant dire que j'aurais été ta plus belle cible durant ces quatre années, j'espère donc que tu en trouveras une autre après mon départ. L'« arnaque du siècle »*



*aura pris beaucoup de plaisir à travailler avec toi. Je garderai un souvenir marqué par votre familiarité et je ferai en sorte de recroiser vos routes dès que possible ! Merci à mes coloc' de bureau, Soso et Caro... tant de temps passé à vos côtés ! Nous avons muri côte à côte et j'ai apprécié tous ces discussions/débats scientifiques, ces discussions extra-professionnelles, ou encore nos sessions « photo of » !*

*Je souhaiterai remercier toutes mes rencontres stéphanoises. Que ce soit avec les amis du labo, Amélie, Nico et Alex avec qui j'ai passé tant de merveilleux moments...les sessions wakeboard ou les soirées d'œnologie. Que ce soit les autres rencontres comme Jet, Karine, Axelle, Rod et j'en passe. Merci à mes colocataires du château qui ont partagé mon quotidien durant ces années sur Saint-Etienne.*

*Une immense pensée me vient vers mes amis avignonnais, Machim, Valoche, Tibo, Océ, la jeje, Camille, Nico et Julie, Benoïte et Coco, Manue, Boris, Pascalou sans qui ma vie ne serait pas la même. Merci d'être encore là pour moi après tant d'années. Suite à cette aventure de thèse, j'espère écrire encore de nombreuses pages avec vous.*

*Merci à ma famille pour son éternel soutien. Merci à mes grands-parents pour leur sagesse. Merci à mes parents pour qui j'essaie de les rendre fier. Merci à mes frères et sœurs (de quelques nationalités et génotype qui soit) pour votre amour et votre bienveillance. Merci Cyril, qui sous son œil de MCU, m'a guidé au cours de ce voyage.*

*Enfin, merci à toi ma petite Lola. Je suis conscient de tous les sacrifices que tu as faits mais sache que je te serai éternellement reconnaissant pour tous les choix justes que tu as fait pour moi depuis trois ans. Merci pour ton soutien quotidien, pour ta patience et ton amour. Maintenant arrive l'époque tant attendue d'une vie commune afin qu'à présent je puisse me consacrer à toi.*

## **Serment éthique des Chercheurs en Sciences de la Vie**

« Je jure d'être fidèle à l'éthique du respect des personnes et des vies humaines et de contribuer au développement de la connaissance et à la plus large diffusion du savoir.

Je respecterai toutes les espèces dans leur biodiversité : ce respect inspirera mes actes et mes projets, notamment au cours de mes expérimentations sur les animaux ou les tissus humains.

Je m'efforcerai de soulager les souffrances de tous les êtres vivants.

Admis à avoir accès à l'intimité tissulaire ou génétique des personnes, je tairai leur identité et m'astreindrai au secret médical.

Même sous la contrainte, je ne ferai pas usage de mes connaissances contre les lois de l'humanité.

Je préserverai l'indépendance nécessaire à l'accomplissement de ma mission. Je m'informerai et réfléchirai au sens de mes expérimentations et à leurs connaissances.

Je veillerai à ce que mes travaux et recherches ne soient pas utilisés à des fins de destruction ou de manipulation.

Je respecterai les savoirs des ethnies et des sociétés traditionnelles.

Je n'aurai garde d'oublier mes responsabilités à l'égard des générations présentes et futures.

Je n'accepterai pas que des considérations de nationalités, de culture, de politiques ou d'avantages matériels me détournent de mes devoirs.

J'interviendrai pour défendre, s'il m'en est donné l'occasion, l'ensemble de ces règles.

Que les hommes et mes confrères m'accordent leur estime si je suis fidèle à mes promesses.

Que je sois déshonoré et méprisé si j'y manque. »

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# Résumé

TITRE : ROLE INFLAMMATOIRE DES PLAQUETTES SANGUINES LORS DU SEPSIS

RESUME : Depuis quelques années, les plaquettes sanguines sont reconnues comme véritable élément de l'immunité. En effet, de nombreux rôles clés lors du processus inflammatoire sont attribués aux plaquettes, tels que la détection d'un signal, la libération de nombreux immunomodulateurs ou une forte interaction avec les autres cellules immunitaires. Le constat clinique d'une corrélation entre le taux plaquettaire et la mortalité du sepsis permet de relever l'importance de cette cellule dans la physiopathologie du sepsis. Ainsi ce travail doctoral a eu pour objectif de mettre en relief la part inflammatoire des plaquettes lors des interactions entre les facteurs de virulence de *Staphylococcus aureus* (*S. aureus*) que sont différentes exotoxines ou de souches de *S. aureus* issues de bactériémies de patients septiques. Un modèle expérimental de sepsis murin nous a permis de comprendre la composante inflammatoire jouée par les plaquettes ainsi que leurs implications dans la dysfonction pulmonaire issue lors du sepsis. Enfin, nous avons évalué la modulation de molécules antiplaquettaires, et particulièrement de l'acide acétylsalicylique, sur les nombreux rôles pathogéniques des plaquettes durant cette pathologie.

MOTS CLES : Plaquettes – Sepsis – Inflammation – *Staphylococcus aureus*

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TITRE : ROLE INFLAMMATOIRE DES PLAQUETTES SANGUINES LORS DU SEPSIS

ABSTRACT : During some years, platelets are recognized as an key element of immunity. In fact, platelets play several roles, as signal detection, immunomodulator release and interaction with other immunity cells. The clinical significance of a correlation between platelet rate and sepsis mortality reveals the importance of this cell in the pathophysiology of sepsis. Thus, the purpose of this doctoral work was to highlight the inflammatory role of platelets in the interactions between the virulence factors of *Staphylococcus aureus* (*S. aureus*) that are different exotoxins or strains of *S. aureus* from bacteremia of septic patients. An experimental model of murine sepsis allowed us to understand the inflammatory component played by platelets as well as their implications in pulmonary dysfunction resulting from sepsis. Finally, we evaluated the modulation of antiplatelet molecules, particularly acetylsalicylic acid, on the numerous pathogenic roles of platelets during this pathology.

KEYWORDS : Platelets – Sepsis – Inflammation – *Staphylococcus aureus*

# Index des abréviations



**A:**

**ASA** : Acetylsalicylic acid (Acide acétylsalicylique)

**ADAM** : A disintegrin And metalloproteinase domain-containing protein

**ALI** : Acute Lung Injury

**C:**

**CD40L** : CD40 ligand

**CLP** : Cecal Ligation Puncture

**CRP** : C reactive protein (Protéine c réactive)

**CIVD** : Coagulation Intravascularaire Disséminée

**E:**

***E. coli*** : Escherichia coli

**I:**

**IL** : Interleukine

**L:**

**LPS** : Lipopolysaccharide

**M:**

**MP** : Microparticule

**MCP** : Monocyte chemoattractant protein

**N:**

**NETs** : Neutrophil Extracellular Traps

**P:**

**PCT** : Procalcitonine

**PF4** : Platelet Factor 4 (Facteur plaquettaire 4)

**PS** : Phosphatidylsérine

**P-Sélectine** : Platelet-Selectin (CD62P)

**R:**

**RANTES** : Regulated on activation, normal T cell expressed and secreted

**S:**

**SOFA** : Sequential Organ Failure Assessment

***S. aureus*** : Staphylococcus aureus

*S. condimenti* : *Staphylococcus condimenti*

**SDRA** : Syndrome de Détresse Respiratoire Aigue

**T** :

**TxA<sub>2</sub>** : Thromboxane A<sub>2</sub>

**TLR** : Toll Like Récepteurs

**TRALI** : Transfusion Related Acute Lung Injury

**TRAP** : Thrombin Receptor Activating Peptide

**TREM-1** : Trigerring Receptor Expressed on Myeloid cell-1

**TGF-β** : Tumor Growth Factor-Beta

**TNF-α** : Tumor Necrosis Factor-alpha

**W** :

**vWF** : von Willebrand Factor (facteur de von Willebrand)

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# Etat de la littérature

# **Chapitre 1 – Le sepsis**

Le sepsis représente un sérieux problème de santé publique puisque cette maladie est l'une des premières causes de mortalité dans le domaine hospitalier avec un taux de mortalité de 40% dans les unités de soins intensifs<sup>1,2</sup>. Au niveau mondial, l'incidence du sepsis est estimée à 31 millions de cas résultant de plus de 8 millions de décès<sup>3</sup>. De plus, une augmentation de l'incidence a été observée et devrait continuer à croître à cause du vieillissement de la population, de l'accroissement de l'utilisation de thérapies immunosuppressives et de chimiothérapies ou de procédures invasives<sup>4,5</sup>. Malgré cette augmentation, la mortalité associée au sepsis diminue grâce à un diagnostic plus rapide et une meilleure prise en charge des patients septiques<sup>6</sup>.

## 1. Définition

La nouvelle définition du sepsis, définie lors du troisième consensus international Sepsis-3, est la dysfonction d'organe avec un pronostic vital engagé et causée par une dérégulation de la réponse de l'hôte suite à une infection<sup>7</sup>. Elle préconise l'utilisation de marqueurs d'infection afin de diagnostiquer un sepsis et de définir si les dysfonctions observées chez un patient sont dues à l'infection et donc au sepsis<sup>8</sup>. Les dysfonctions d'organes (reins, poumons, cœur...) peuvent être mises en évidence par le score clinique « Sequential Organ Failure Assessment » (SOFA) supérieur ou égal à 2<sup>9</sup>. Ce score, basé sur différents paramètres cliniques des différents systèmes (respiratoire, cardiovasculaire, rénal, système nerveux central...), permet une identification rapide du sepsis via la dysfonction d'organe dans un contexte inflammatoire tandis que l'infection est plus difficile à mettre en évidence<sup>10</sup>. On parle de choc septique lorsque des dysfonctions circulatoires, cellulaires et métaboliques sont observées<sup>7</sup>. Le choc septique est une sous classe du sepsis avec un niveau de gravité plus important et taux de mortalité corrélé. Cette redéfinition est importante puisque de nouveaux mécanismes physiopathologiques ont été décrits depuis la dernière définition datant de 2001 et l'apparition de nombreux scores cliniques ont induit une grande variabilité dans les essais cliniques et épidémiologiques<sup>11</sup>.



## 2. Germes impliqués dans le sepsis

Malgré une augmentation de l'incidence du sepsis d'origine fongique, les bactéries sont les pathogènes responsables de la majorité des infections <sup>12</sup>. Sur la fin du 20<sup>ème</sup> siècle, l'épidémiologie a évolué puisque les bactéries gram positives étaient plus fréquemment impliquées dans le sepsis que les bactéries gram négatives (**Figure 1**) <sup>5,12</sup>. Cependant, une récente étude prospective multicentrique (1265 unités de soins intensifs dans 75 pays) a montré que les bactéries gram négatives (62.2%) étaient plus communément retrouvées que les bactéries à gram positives (46.8%) <sup>13</sup>.

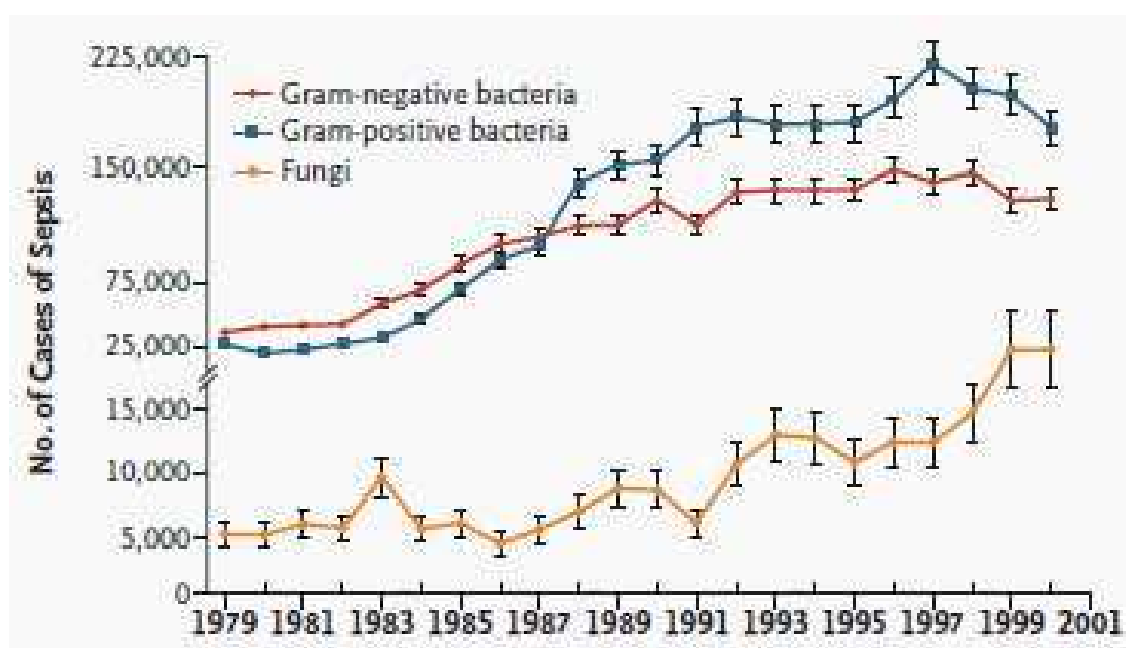


Figure 1 : Nombre de cas de sepsis aux Etats-Unis selon les germes en cause de 1979 à 2000 (d'après Martin et al. <sup>12</sup>)

Dans cette étude, il s'avère que *Staphylococcus aureus* (*S. aureus*, 20,5%), l'espèce *Pseudomonas* (19,9%) et *Escherichia coli* (*E. coli*, 16,0%) sont les germes qui induisent la grande majorité des cas de sepsis (l'ensemble des germes est retrouvé dans le **tableau 1**) <sup>5</sup>. Ces données se confirment avec une étude européenne (incluant 24 pays) qui a montré ces trois mêmes germes comme responsable du sepsis <sup>14</sup>. Le type de pathogène à l'origine d'un sepsis est important dans le devenir du patient. En effet, chaque espèce bactérienne ne possède pas les mêmes facteurs de virulence. Selon le site infectieux et les facteurs de risques du patient, les germes n'induisent pas la même pathogénicité. Ainsi, ceux les plus communément retrouvés ne

sont pas forcément associés à un taux de mortalité important. Une méta-analyse incluant plus de 55000 infections indique que les bactéries gram négative responsables d'infections systémiques sont associées à un taux de mortalité plus important que les bactéries gram positive<sup>15</sup>. Cependant, *S. aureus*, *Pseudomonas aeruginosa* et *E. coli* qui sont les germes les plus communément retrouvés induisent un taux de mortalité relativement faible (25%, 31% et 19% respectivement) comparativement à l'espèce *Acinetobacter* qui étant plus rare induit une mortalité de 40%<sup>15</sup>.

	Frequency (%)	OR (95% CI)
<b>Gram-positive</b>	46.8	
<i>Staphylococcus aureus</i>	20.5	0.8 (0.6–1.1)
MRSA	10.2	1.3 (0.9–1.8)
<i>Enterococcus</i>	10.9	1.6 (1.1–2.3)
<i>S. epidermidis</i>	10.8	0.9 (0.7–1.1)
<i>S. pneumoniae</i>	4.1	0.8 (0.5–1.4)
Other	6.4	0.9 (0.7–1.2)
<b>Gram-negative</b>	62.2	
<i>Pseudomonas species</i>	19.9	1.4 (1.2–1.6)
<i>Escherichia coli</i>	16.0	0.9 (0.7–1.1)
<i>Klebsiella species</i>	12.7	1.0 (0.8–1.2)
<i>Acinetobacter species</i>	8.8	1.5 (1.2–2.0)
<i>Enterobacter</i>	7.0	1.2 (0.9–1.6)
Other	17.0	0.9 (0.7–1.3)
<b>Anaerobes</b>	4.5	0.9 (0.7–1.3)
<b>Other bacteria</b>	1.5	1.1 (0.6–2.0)
<b>Fungi</b>		
<i>Candida</i>	17.0	1.1 (0.9–1.3)
<i>Aspergillus</i>	1.4	1.7 (1.0–3.1)
Other	1.0	1.9 (1.0–3.8)
<b>Parasites</b>	0.7	1.3 (0.5–3.3)
<b>Other organisms</b>	3.9	0.9 (0.6–1.3)

OR, odds ratio; CI, confidence interval; MRSA, methicillin-resistant *S. aureus*

**Tableau 1: Types d'organismes retrouvés chez les patients infectés et le risque de mortalité associé (d'après Mayr et al. 5)**

En plus d'être associé à une mortalité importante, le sepsis entraîne un séjour prolongé des patients et de fréquente réadmission post-sepsis entraînant un coût important pour le système hospitalier<sup>16</sup>. Le sepsis représente un budget de près de 17 milliards de dollars aux Etats-Unis<sup>1</sup>. Le traitement des patients septiques par une antibiothérapie à large spectre résulte d'une

croissance de résistance aux antibiotiques expliquant ainsi l'augmentation de l'incidence de cette pathologie.

### 3. Etiologie et facteurs de risque du sepsis

Etant dépendant de la sensibilité du système immunitaire de l'hôte, de nombreux facteurs de risque sont associés au sepsis. Le facteur de risque majeur apparaît comme le degré de dysfonction immunitaire préexistant. En effet, une personne immunodéprimée ou atteinte d'une maladie inflammatoire sera plus sujette à développer un sepsis. Beaucoup de maladies chroniques, comme le cancer<sup>17</sup> ou la cirrhose<sup>18</sup> induisent une augmentation du risque de déclarer un sepsis. De même, les patients infectés par HIV peuvent présenter une coïnfection entraînant un sepsis, cependant le développement des thérapies antirétrovirales a permis de réduire l'incidence du sepsis chez ces patients immunodéprimés<sup>19</sup>. Les maladies métaboliques sont fortement corrélées à la survenue de sepsis. Ainsi, 17% des personnes diabétiques sont susceptibles de déclencher un sepsis caractérisé par plus de lésions rénales que les patients septiques non diabétiques<sup>20</sup>. Concernant l'obésité, les études restent encore controversées et il n'y a pas de corrélation notable entre le sepsis et ce désordre métabolique cependant l'inflammation due à l'obésité induit une augmentation des dysfonctions d'organes lors du sepsis<sup>21</sup>. En effet, il y a de nombreuses interactions entre les adipocytes et les cellules immunitaires, ainsi on peut observer une différenciation des macrophages affectée lors de l'obésité, ou encore des taux de cytokines pro-inflammatoires élevés (CD40 ligand, Tumor Necrosis Factor  $\alpha$ , Interferon  $\gamma$ , Interleukines 5, 10, 12, 13...) <sup>22</sup>. Si l'impact du tabagisme sur le risque de développer une bactériémie reste encore controversé, la consommation d'alcool augmentent le risque de développer un sepsis<sup>23,24</sup> alors qu'une activité physique module les réponses inflammatoires et permet de limiter la mortalité due au sepsis en baissant les taux de cytokines inflammatoires (IL-6, IL-1 $\beta$ ) et l'expression de marqueurs d'apoptose dans plusieurs organes<sup>25,26</sup>.

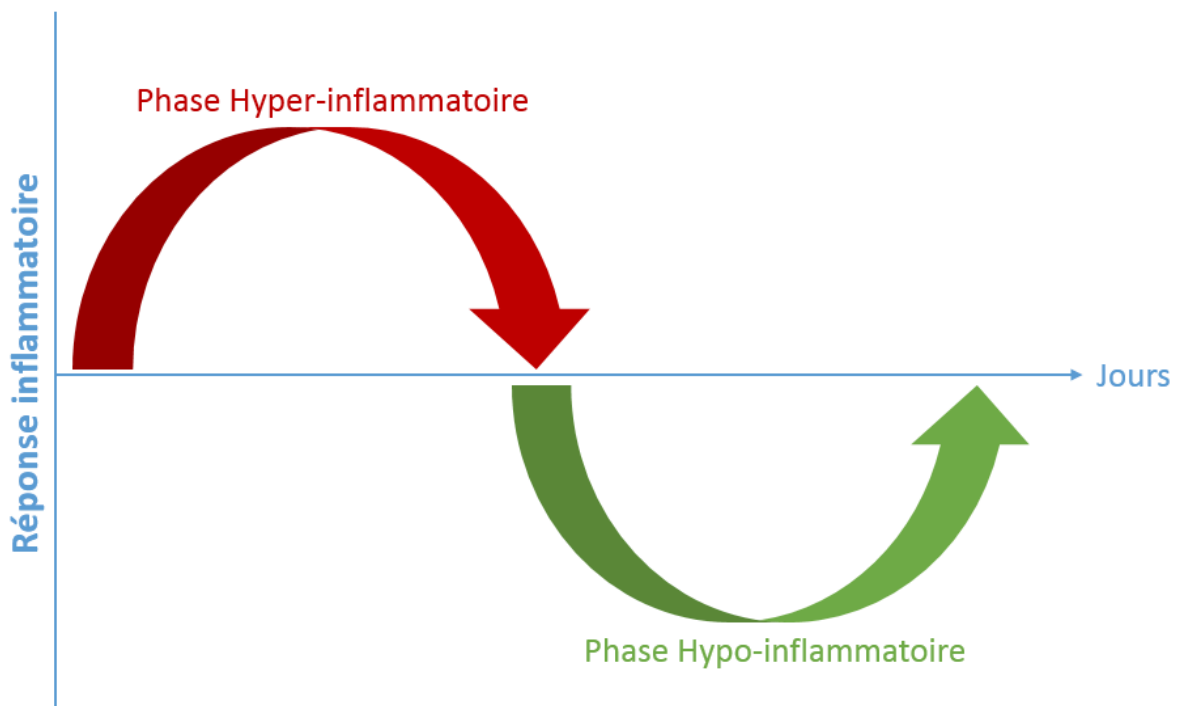
L'âge est un facteur de risque évident du sepsis puisqu'il est généralement corrélé à une immunosénescence. Ainsi, plus d'un tiers des patients septiques sont âgés de plus de 65 ans<sup>5</sup>. Le sexe montre également des différences vis-à-vis de l'incidence du sepsis. En effet, les hommes ont un risque plus élevé de déclencher un sepsis que les femmes<sup>12</sup>. Cela peut s'expliquer par les différences hormonales entre les hommes et les femmes, qui influencent le

système immunitaire. Les hormones sexuelles masculines, comme la molécule androgène 5 $\alpha$ -dihydrotestotérone, tendent à réprimer la réponse immunitaire cellulaire alors que les hormones sexuelles féminines (estrogènes et ses précurseurs) limitent la sécrétion de cytokines pro-inflammatoires et montrent des effets protecteurs vis-à-vis du sepsis <sup>27,28</sup>. En plus d'une incidence plus élevée, les hommes survivent moins au sepsis (31%) comparés aux femmes (74%) <sup>27,29</sup>. Ces différences de mortalité sont multivariées et peuvent s'expliquer par les variabilités hormonales et immunologiques ainsi que de styles de vie apparentés à plus de facteurs de risque (alcoolisme, tabagisme, activité physique) associées à une susceptibilité au sepsis plus importante chez les hommes.

La population noire américaine (standardisée sur l'âge et le sexe) a une prévalence et un taux de mortalité lors du sepsis nettement plus important que la communauté blanche <sup>30</sup>. Les mécanismes de cette disparité ethnique dans le sepsis sont encore incompris. De nombreux facteurs, comme l'accès aux systèmes de soins, le niveau de vie, ou la qualité de l'alimentation, peuvent expliquer cette différence ; cependant, cette étude met également en avant l'importance de facteurs génétiques comme facteurs de risques dans la survenue du sepsis <sup>30</sup>. Sans catégoriser le sepsis comme maladie mendélienne, de multiples gènes permettent aux cellules de l'organisme d'interagir avec les pathogènes et peuvent influencer la susceptibilité de l'hôte au sepsis <sup>31</sup>. Une étude sur l'impact de déterminants génétiques sur la prédisposition à une bactériémie a montré que la combinaison des gènes NOD2/TLR4 est associée à l'induction d'une bactériémie <sup>32</sup>. De plus, l'étude des polymorphismes de patients de réanimation indique que les patients portant un variant du TLR4 et de NOD2 ont une mortalité plus élevée (58,3%) que les patients ne portant qu'un seul des deux variants (38,4%) <sup>32</sup>. L'avancée technologique des études de génomes à grande vitesse va permettre l'étude des variations génétiques à l'origine d'une prédisposition à déclarer un sepsis afin, dans un futur proche, d'avoir une approche personnalisée du diagnostic biologique et/ou du traitement <sup>33</sup>.

#### 4. Physiopathologie du sepsis

Le sepsis se caractérise par une réponse inflammatoire systémique et incontrôlée lors de l'infection par un pathogène. Une réponse immunitaire exacerbée est à l'origine de la dysfonction de nombreux systèmes tels que la voie du complément, la fonction endothéliale, la coagulation qui vont entraîner la dysfonction d'organes. Les pathogènes retrouvés dans la circulation sanguine vont, via les « pathogen-associated molecular pattern » (PAMPs) qu'ils exposent, capables de stimuler les récepteurs de l'immunité qui vont répondre par une sécrétion de cytokines inflammatoires telles que le Tumor Necrosis Factor (TNF)- $\alpha$ , l'Interleukine-1 $\beta$  (IL-1 $\beta$ ) ou encore l'Interleukine 6 (IL-6). On observe généralement chez les patients atteints de sepsis, une première phase hyper-inflammatoire en réponse à l'action de nombreuses cellules de l'immunité suivie d'une phase hypo-inflammatoire (**Figure 2**).



**Figure 2 : Cinétique inflammatoire du sepsis** (inspirée d'après Hotchkiss et al. <sup>34</sup>)

Cette phase de dépression immunitaire est due au « syndrome de réponse anti-inflammatoire compensatoire » (CARS) qui est accompagné de nombreuses dysfonctions d'organe et d'un mauvais pronostic des patients <sup>35</sup>. En effet, une paralysie inflammatoire

entraîne des infections secondaires par des pathogènes nosocomiaux. Ce schéma de la cinétique inflammatoire lors du sepsis n'est pas forcément applicable à chaque patient ; cette variabilité reste encore floue mais peut s'expliquer par l'état général (âge, pathologies associées, facteurs génétiques) du patient qui entraîne une susceptibilité différente de réponse inflammatoire à des pathogènes. Cependant, la compréhension du profil de la cinétique inflammatoire est important pour prédire la mortalité <sup>36</sup>. La dérégulation de l'inflammation va initier la dérégulation de nombreux processus à l'origine de la défaillance d'organes. Les cytokines pro-inflammatoires libérées par les cellules de l'immunité entraînent un processus d'autostimulation induisant une exacerbation de libération de facteurs immuno-modulateurs tels que les interleukines IL-1/IL-2/ IL-6/ IL-8/ IL-10, le facteur d'activation plaquettaire ou encore l'oxyde nitrique. Cette boucle d'activation des processus inflammatoires crée alors une dissonance immunologique néfaste. Il y a une dérégulation circulatoire due aux médiateurs vasoactifs comme l'oxyde nitrique. En effet, il y a une vasodilatation réactionnelle et une augmentation de la perméabilité microvasculaire. Cette dérégulation vasculaire va avoir de nombreuses conséquences. En effet, on observe dans ce cas, des changements des performances cardiaques à l'origine d'hypoperfusion. L'oxygène n'étant plus distribué convenablement jusqu'aux organes, un stress oxydant va entraîner la défaillance de multiples organes. Le TNF- $\alpha$  va altérer la chaîne respiratoire et induire une cytotoxicité sur différents types cellulaires. Les cellules dysfonctionnelles sont éliminées par un phénomène apoptotique ce qui participe également aux dysfonctions d'organes comme le foie. Enfin, le désordre immunitaire favorise les événements thrombotiques qui peuvent entraîner une ischémie de certains microvaisseaux. A terme, les facteurs de la coagulation sont consommés et des saignements incontrôlés peuvent apparaître. Au niveau pulmonaire, l'augmentation de la perméabilité endothéliale permet le passage des neutrophiles. Il en résulte un œdème interstitiel à l'origine du syndrome de détresse respiratoire aigu (SDRA).

Plusieurs études ont mis en évidence l'interconnexion entre l'inflammation et la coagulation, ainsi de nombreuses cytokines pro-inflammatoires vont fortement favoriser un état pro-coagulant, alors que divers facteurs de la coagulation influencent l'inflammation <sup>37</sup>. Le déclenchement incontrôlé de la coagulation induit la consommation des facteurs de l'hémostase secondaire et des plaquettes, qui couplé à l'inhibition de la fibrinolyse, favorise le dépôt de fibrine au niveau des vaisseaux. La coagulation intravasculaire disséminée (CIVD) est clairement un facteur de mortalité lors du sepsis. Lors de la dégradation de la fibrine, les

produits de dégradation comme les D-dimères reflètent l'activité fibrinolytique, associée entre autres à la CIVD. Ainsi, l'évaluation des CIVD à travers les dosages des D-dimères pourrait contribuer à prédire la dysfonction d'organes et le niveau de sévérité des patients septiques <sup>38</sup>. Concernant la dérégulation hémostatique, les microparticules (MP) jouent un rôle primordial durant la pathogénèse du sepsis puisque il a récemment été montré que les MP d'origine plaquettaire, leucocytaire, érythrocytaire et endothéliale augmentent chez les patients atteints de sepsis et participent, via l'exposition de phosphatidylsérine (PS), à l'activité pro-coagulante <sup>39</sup>. Depuis quelques années, les MP cellulaires ont été largement investiguées dans de nombreuses pathologies inflammatoires <sup>40-42</sup>. Ces éléments microvésiculaires sont formés à partir de la membrane de leur cellule d'origine par retournement de la couche phospholipidique lors de l'activation ou de l'apoptose des cellules. Ils possèdent donc de nombreuses caractéristiques de leurs cellules d'origines (protéines, récepteurs, ARNs...) dont la PS et le facteur tissulaire. De nombreuses études ont montrés l'effet régulateur des MP au niveau de l'inflammation ainsi que de la coagulation <sup>43</sup>. Les MP ont divers rôles physiologiques (inflammatoires, angiogéniques...) mais, dans un contexte infectieux, une libération excessive de ces microvésicules entraine des conséquences pathologiques. Lors du sepsis, les MP ont des propriétés pro-inflammatoires et pro-coagulantes. Elles jouent un rôle critique dans l'initiation et la pathogénèse du sepsis <sup>44</sup>. En effet, les MP participent à la dysfonction endothéliale, puisqu'elles favorisent l'hyperréactivité vasculaire en activant l'endothélium et sont donc à l'origine des complications comme le SDRA ou les lésions rénales aiguës <sup>45,46</sup>. Les MP d'origine plaquettaire ont plusieurs actions inflammatoires <sup>47</sup>. Une belle étude a observé la formation de complexes immuns à la surface dans les MP, qui suite à la reconnaissance d'immunoglobulines, favorisent l'activation des neutrophiles <sup>48</sup>. La formation de MP par les plaquettes participe à la communication cellulaire, puisque les MP contiennent de nombreuses cytokines et chimiokines telles que IL-1 ou la protéine « regulated on activation, normal T cell expressed and secreted » (RANTES) ainsi que des enzymes ou médiateurs comme le thromboxane A<sub>2</sub> ou encore même des acides nucléiques <sup>44</sup>.

## 5. Marqueurs du sepsis

Actuellement, aucun traitement spécifique n'existe concernant le sepsis. Cependant, un diagnostic précoce et une bonne prise en charge des patients permettent de limiter la mortalité<sup>49</sup>. La rapidité du diagnostic se fait grâce à la mesure de paramètres cliniques tels que la pression sanguine, les constantes hémodynamiques, la tension artérielle... cellulaires et biochimiques comme la formule sanguine et le taux de cytokines inflammatoires. En tout, plus de 170 paramètres ont été dénombrés pour aider les cliniciens à distinguer une réponse inflammatoire d'un véritable sepsis. Ces marqueurs reflètent d'une modulation anormale des divers événements composés de la réponse immunitaire (innée et adaptative) mais également du système du complément, de la cascade de la coagulation ou de la perméabilité endothéliale. Des variabilités sont observées selon les patients puisque de nombreux facteurs tels que l'âge, l'état nutritionnel ou des facteurs génétiques fluctuent la réponse immunitaire et donc les biomarqueurs du sepsis.

Les différents paramètres biologiques mesurés au cours du sepsis peuvent refléter soit l'état inflammatoire du patient, soit la dysfonction d'un organe. Il est évident que l'inflammation étant la source de la défaillance des organes, ce sont les molécules inflammatoires qui sont les plus précocement retrouvées. Le TNF- $\alpha$ , l'IL-1 $\beta$  et l'IL-6 sont les cytokines pro-inflammatoires majeures qui vont moduler l'inflammation lors d'une infection systémique. L'IL-1 $\beta$  reste encore très controversée dans la physiopathologie du sepsis et l'utilisation de thérapies ciblant le TNF- $\alpha$  n'affecte pas le sepsis. C'est pour ces raisons que ces deux cytokines ne sont pas des marqueurs de choix pour mettre en évidence le sepsis. A contrario, il s'avère que le taux d'IL-6 est fortement élevé chez les patients septiques, significativement associé à la mortalité et en corrélation avec les patients ayant un risque important de développer un sepsis<sup>50,51</sup>. Ces cytokines ne sont pas spécifiques du sepsis donc l'utilisation des taux de TNF- $\alpha$ , d'IL-1 $\beta$  et d'IL-6 comme biomarqueur ne permet qu'un pronostic et non pas de véritable diagnostic. Les chimiokines sécrétées lors du sepsis révèlent être de meilleurs marqueurs que l'IL-6. Par exemple, les taux d'IL-8 et de « monocyte-chemoattractant protein » (MCP-1) sont prédictifs de la mortalité du sepsis<sup>52,53</sup>.

Au cours de l'inflammation, on observe une sécrétion accrue de protéine C réactive (CRP) au niveau hépatique. La CRP est un biomarqueur précoce, sensible et spécifique de la réaction inflammatoire augmentant proportionnellement à son intensité. Par ailleurs, sous l'effet des protéines inflammatoires telles que le TNF- $\alpha$  et l'IL-1 $\beta$ , de nombreux types cellulaires vont



sécréter la procalcitonine (PCT). Cette pro-hormone, stockée dans les granules des cellules, va être un marqueur biologique précoce des infections bactériennes <sup>54</sup> pouvant parfois être utilisé pour distinguer une infection à gram positive d'une infection à gram négative <sup>55</sup>. La PCT semble être plus sensible et relativement spécifique que la CRP pour diagnostiquer une infection bactérienne <sup>56</sup>. Les taux circulants de CRP, de PCT et d'IL-6 permettent d'évaluer la sévérité du sepsis <sup>57</sup>. Depuis quelques années, de plus en plus de marqueurs de la phase d'immunodépression sont étudiés pour mettre en avant de nouveaux biomarqueurs pouvant être utilisés à un moment spécifique de la cinétique de cette pathologie.

En plus des protéines inflammatoires, il est possible d'évaluer l'activité de certaines cellules pour diagnostiquer un patient en état de sepsis. En effet, l'IL-6 entraîne la production de polynucléaires dans la moelle osseuse. Ainsi, au cours du sepsis, on peut observer une augmentation des taux circulants de granulocytes circulants ou immatures. Plusieurs protéines de surface telles que le CD11b, le CD64 et TREM-1 (triggering receptor expressed on myeloid cell-1) pourraient constituer des marqueurs d'aide au diagnostic du sepsis <sup>58-60</sup>. La forme soluble du CD14 peut aussi être utilisée pour la prise en charge des patients septiques puisque cette molécule est corrélée au degré de sévérité <sup>61</sup>.

La barrière endothéliale est fortement impactée lors du sepsis. L'inflammation va causer une dysfonction endothéliale associée à un sepsis sévère. L'augmentation des taux circulants de marqueurs de l'activation endothéliale, tels que les molécules d'adhésion (Vascular Cell Adhesion Molecule-1, Intracellular Adhesion Molecule 1), l'endocane ou encore l'angiopoïétine sont corrélés à la pathogénèse du sepsis <sup>62-64</sup>. D'ailleurs, les marqueurs de l'activation endothéliales sont corrélés au devenir des patients septiques <sup>65</sup>. La fonction endothéliale est clairement en lien avec le système de coagulation. Or, le système de coagulation met en jeu les plaquettes sanguines qui, dans un état inflammatoire, vont participer à la coagulation et à l'inflammation dans la mesure où ces cellules prennent une part active à ces deux mécanismes de manière physiologique ou dans des états pathologiques (comme décrit plus amplement dans les Chapitre 2 et 3). Les plaquettes se placent ainsi comme véritable acteurs de la pathogénèse du sepsis. D'ailleurs, la thrombopénie observée chez la majorité des patients septiques est corrélée au taux de mortalité <sup>66,67</sup>.

Nous avons vu précédemment que les microparticules étaient des éléments importants de la physiopathologie du sepsis. De ce fait, on observe des taux largement plus élevés de MP

d'origine plaquettaire, en marge de celles d'origine leucocytaire, et endothéliale, chez les patients septiques <sup>68,69</sup>. Les MP leucocytaires circulantes pourraient ainsi servir de biomarqueurs pour évaluer le devenir des patients septiques déclenchant un SDRA <sup>70</sup>.

Outre des marqueurs inflammatoires ou des niveaux cellulaires, la dysfonction de certains organes est prise en considération. L'élaboration de scores cliniques comme le score APACHE (Acute physiology and chronic health elevation) ou encore le score SOFA ont permis de standardiser et d'évaluer le degrés d'atteintes des organes <sup>71</sup>.

Dans la mesure où l'expression clinique du sepsis est extrêmement variable d'un patient à l'autre, il n'existe pas de biomarqueur qui soit spécifique de la pathologie mais il est envisageable qu'une combinaison de marqueurs sous forme de score puisse constituer un biomarqueur qui permette un diagnostic plus rapide et d'établir une prise en charge plus adaptée du patient. Plus le nombre de marqueurs inflammatoires est important, meilleure sera l'identification des cas de sepsis <sup>72,73</sup>. De nombreuses études tentent de trouver le panel de marqueurs permettant de faire émerger les caractéristiques pro-inflammatoires et anti-inflammatoires ainsi que les défaillances d'organes <sup>74</sup>.

## 6. Les traitements du sepsis

L'incidence du sepsis étant en constante augmentation, il est primordial de mettre en place de nouveaux traitements ainsi que de nouvelles stratégies visant à adapter le traitement au patient. Nous avons vu précédemment que les conséquences physiopathologiques du sepsis sont dues aux dérégulations immunitaires et hémostatiques causées par un pathogène infectieux (bactérien). Depuis la récente redéfinition du sepsis, une nouvelle campagne indiquant les lignes directrices quant aux prises en charge des patients atteints de sepsis a été réalisée <sup>49</sup>. Le sepsis est une urgence médicale nécessitant un traitement et une réanimation le plus tôt possible. Actuellement, les premiers traitements mis en place pour les patients septiques sont une antibiothérapie afin de cibler le pathogène responsable de l'infection ainsi que des mesures permettant d'améliorer la perfusion et l'oxygénation tissulaire. Des corticostéroïdes sont recommandés pour les patients en choc septique, ne répondant pas aux traitements <sup>49</sup>. Plusieurs stratégies sont utilisées afin de contrôler l'état des patients septiques. Premièrement, la neutralisation des facteurs de virulence des pathogènes mis en jeu dans les cas de sepsis s'avère intéressante. Ainsi, l'inhibition du récepteur CD28 avec les toxines staphylococciques limite la

mortalité dans un modèle de souris septiques et semble améliorer l'état des patients atteints de sepsis <sup>75,76</sup>. Il est établi que le système du complément, constituant clé de l'élimination des pathogènes via l'activation de la cascade de nombreux facteurs et facilitant la destruction des pathogènes, stimule l'inflammation et participe à la pathogénèse du sepsis <sup>77</sup>. L'inhibition de ce système permettrait donc une réduction importante de la coagulation ainsi qu'une amélioration de l'état général des organes <sup>78</sup>.

Selon un autre point de vue, la stratégie de moduler la coagulation pour contrôler l'inflammation semble être judicieuse pour limiter le sepsis. Plusieurs molécules s'avèrent intéressantes : la protéine C activée (Drotrecogin alfa) a montré des résultats assez contradictoires puisqu'une première étude a révélé une efficacité significative de cette molécule vis-à-vis de la mortalité due au sepsis mais d'autres études n'ont, à l'inverse, observé aucun effet bénéfique <sup>79-81</sup>. D'autres agents pharmacologiques tels que la thrombomoduline, l'anti-thrombine ou l'inhibiteur de la voie du facteur tissulaire (TFPI) permettent la réduction de la CIVD mais n'ont, en contrepartie de leurs effets indésirables, qu'un effet bénéfique modéré sur la mortalité associée au sepsis <sup>82</sup>.

Plusieurs traitements ciblent la dérégulation de l'inflammation qui semble être la base des conséquences pathogéniques du sepsis. Il paraît donc primordial d'arriver à moduler la réponse immunitaire, en baissant la phase hyperinflammatoire ou au contraire en stimulant l'immunité lors de la paralysie immunitaire. Des agents immunostimulants tels que le granulocyte-macrophage colony-stimulating factor (GM-CSF), ou l'IFN $\gamma$  montrent des résultats prometteurs <sup>83-85</sup>.

La diversité du rôle des plaquettes dans la physiopathologie du sepsis ouvre plusieurs possibilités quant à l'élaboration d'anti-plaquettaires qui pourrait moduler une réponse inflammatoire exacerbée.

## 7. La place centrale des plaquettes dans le sepsis

Les plaquettes occupent une place particulière dans le sepsis. Du fait de leur nombre important dans la circulation sanguine et de leur hyper-sensibilité à tout changement dans leur environnement, les plaquettes se positionnent comme de véritables cellules sentinelles. Via leur

arsenal de récepteurs, les plaquettes apparaissent comme parmi les premières cellules pouvant détecter un élément pathogène au niveau systémique.

L'observation clinique d'une chute du compte plaquettaire chez les patients atteints de sepsis a véritablement révélé la position centrale de cette cellule dans la pathogénèse de cette maladie. En effet, selon de nombreuses études, environ 20 à 50% des patients septiques présentent une thrombopénie <sup>66,67,86</sup>. Plusieurs hypothèses ont été proposées pour expliquer la cause de cette thrombopénie. L'étiologie de cette conséquence hématologique est multifactorielle. Ainsi, l'adhésion des plaquettes au facteur de von Willebrand (vWF) de l'endothélium ou aux leucocytes circulants limiterait la détection des plaquettes circulantes <sup>87-90</sup>. En outre, dans un contexte inflammatoire, l'activation plaquettaire par la thrombine serait l'une des causes majeures de « consommation » des plaquettes. L'endothélium et les plaquettes libèrent du facteur tissulaire à l'origine de la génération de thrombine. Ainsi, malgré que la thrombine participe naturellement à la coagulation physiologique non pathologique, elle pourrait servir d'indicateur pour le développement de dysfonctions d'organes <sup>87,91</sup>. L'adhésion des plaquettes au vWF de l'endothélium ou aux leucocytes circulants favorisent la diminution de la détection des plaquettes circulantes <sup>87,88</sup>. Les patients septiques ont un taux de complexes leuco-plaquettaire plus important <sup>89,90</sup>. Le système du complément est essentiel l'immunité innée. Lors du sepsis, les facteurs C5a, C5b9 et C3a activent les leucocytes et potentialisent la libération de cytokines inflammatoires, ayant pour conséquence directe une dérégulation de la coagulation <sup>92</sup>. La génération de thrombine induite via les facteurs du complément, activerait les plaquettes sanguines favorisant la CIVD et la sur-consommation plaquettaire <sup>92</sup>. La P-sélectine (CD62P) plaquettaire exprimée par les plaquettes activées se lie au C3b et stimule la voie alternative du complément <sup>93</sup>. De plus, les plaquettes activées libèrent du sulfate de chondroïtine entraînant l'augmentation des facteurs C3a et sC5b-9 du complément <sup>94</sup>. Les plaquettes expriment plusieurs récepteurs aux protéines du complément (gC1qR, complement receptor 1, complement receptor 2) et contiennent certains facteurs du complément (C3/C4) dans leurs granules indiquant leurs capacités à orchestrer la voie du complément <sup>95-98</sup>. Ainsi, la destruction des plaquettes mises en jeu lors de l'activation du complément, pourrait contribuer à la

thrombopénie. Ainsi lors d'une réaction inflammatoire systémique due à une infection, la balance hémostatique est complètement dérégulée et favoriserait un état pro-coagulant <sup>37</sup>.

Un autre mécanisme pouvant expliquer la thrombopénie observée durant le sepsis, serait la formation de « Neutrophil Extracellular Traps » (NETs) via le récepteur TLR4 et la glycoprotéine GPIb $\alpha$  des plaquettes <sup>99,100</sup>. Ce réseau chromatinien extrudé par les neutrophiles suite à leur activation dans le but de piéger et de tuer les bactéries aurait cependant un effet pro-inflammatoire délétère lors du sepsis. Ce processus contribue à la réduction du taux plaquettaire circulant. Récemment, un nouveau mécanisme physiopathologique observé lors du sepsis a permis d'éclairer la diminution du compte plaquettaire. Les nombreuses cellules de l'immunité répondant à l'orage cytokinique s'activent, meurent et libèrent des histones au niveau plasmatique, contribuant à l'activité anti-microbienne durant une infection. Cependant, la présence d'histones favoriserait la coagulation et participerait à la thrombopénie observée lors du sepsis <sup>101</sup>. D'ailleurs, il a très récemment été montré que le niveau plasmatique d'histone peut être corrélé à la thrombopénie chez les patients atteints de sepsis <sup>102</sup>. Enfin, les plaquettes étant des cellules anucléées mais pourvues d'une machinerie apoptotique, leur rencontre avec un pathogène entraînerait la mort plaquettaire <sup>103,104</sup>.

Outre la consommation plaquettaire, on observe par ailleurs une diminution de la thrombopoïèse concourant à la thrombopénie chez certains patients septiques. Alors que les plaquettes libèrent des quantités importantes de thrombopoïétine chez les patients infectés, qui devrait favoriser la mégacaryocytopoïèse, il est observé une hémophagocytose des précurseurs plaquettaires via les macrophages recrutés par le macrophage-colony-stimulating factor (M-CSF) <sup>105-107</sup>.

L'ensemble de ces processus favorisant la thrombopénie montre que les plaquettes sanguines jouent un rôle dans de nombreux processus physiopathologiques et sont un élément cellulaire à ne pas négliger vis-à-vis du sepsis qui présente une dérégulation hémostatique et inflammatoire. Chez les patients septiques thrombopéniques, une remontée du taux plaquettaire est généralement observée chez les patients survivants alors qu'une chute de 30% du compte en plaquettes est prédicteur de léthalité <sup>108-110</sup>.

## 8. Contribution des plaquettes à la physiopathologie du sepsis

Bien que les plaquettes contribuent grandement à la mise en place de la réponse de l'hôte à l'agent infectieux au cours du sepsis, elles participent par différentes voies à sa physiopathologie. Ces petites cellules ont différentes propriétés les plaçant au cœur de la dérégulation de la balance hémostatique et inflammatoire du fait de leur place centrale dans ces deux les phénomènes.

Lors d'un état inflammatoire, le niveau d'activation des plaquettes apparaît corrélé au degré de gravité du sepsis dans la mesure où les patients septiques ont un niveau d'expression de CD62P plaquettaire plus important que des sujets sains <sup>111</sup>. La libération du contenu granulaire des plaquettes est également synonyme d'activation. Ainsi les taux plasmatiques CD62P soluble (sCD62P), de « platelet factor » 4 (PF4), de « Triggering receptor expressed on myeloid cells-like transcrit-1 (sTLT-1) <sup>112-114</sup>. Cependant, la concentration circulante de CD40-Ligand soluble (sCD40L), majoritairement libéré par les plaquettes, ne semble pas être corrélée au degré de thrombopénie et à la sévérité du sepsis <sup>115</sup>. Les complications cliniques du sepsis, en particulier, la CIVD, sont corrélées aux concentrations plasmatiques de sTLT-1, ce qui ferait de ce récepteur un bon biomarqueur de CIVD <sup>113</sup>. La réactivité plaquettaire étant plus importante chez les patients septiques, les plaquettes forment plus de complexes avec les leucocytes circulants et ont une réponse inflammatoire plus importante (PF4, Thrombomoduline, sCD62P) lorsque le germe responsable est gram positif <sup>116</sup>.

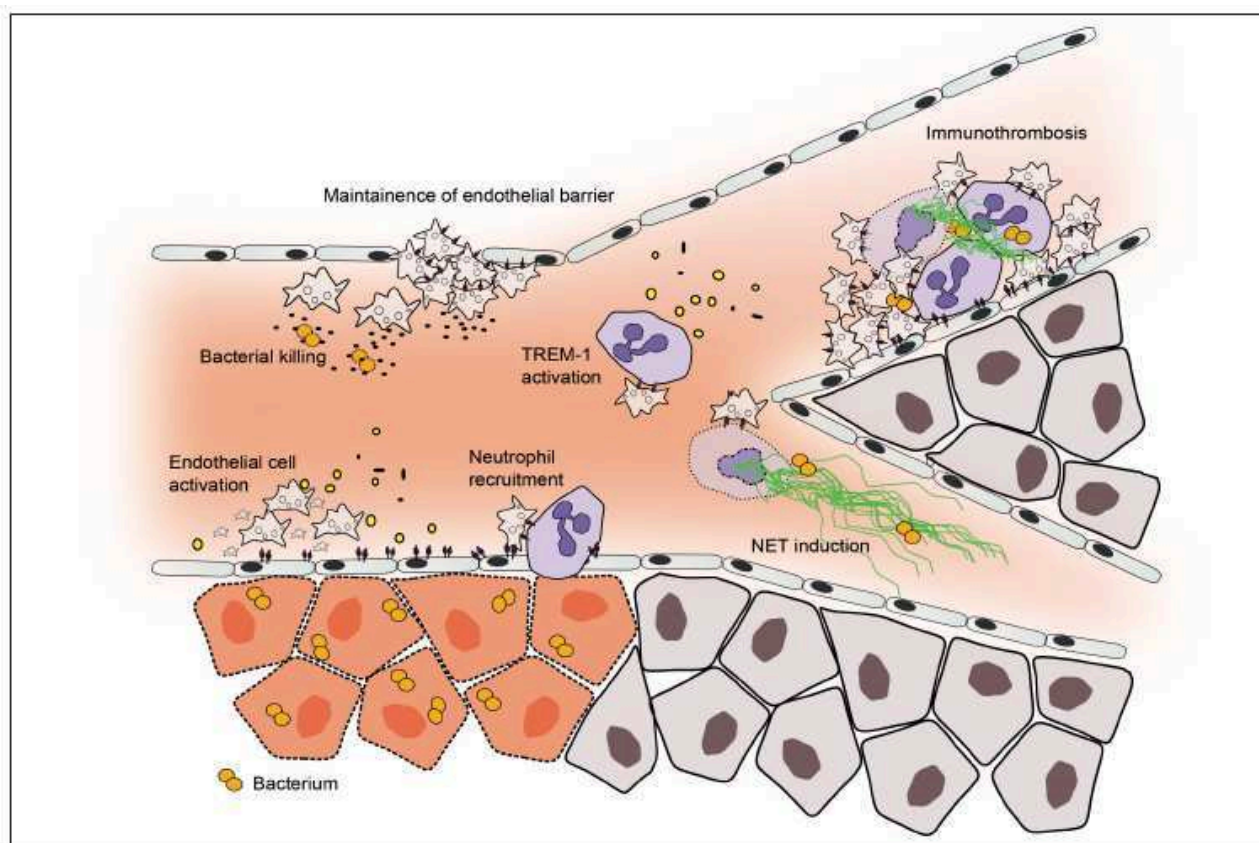
Suite à leur activation, les plaquettes entrent progressivement en mort cellulaire, et la mesure de la dépolarisation de la membrane mitochondriale (signe d'apoptose) serait un marqueur précoce de la sévérité de la pathologie chez un patient septique <sup>117,118</sup>. La mort des plaquettes est également associée à la libération de MP portant les marqueurs de membrane plaquettaires, exposant des PS et présentant capacité pro-coagulante importante <sup>39</sup>. Chez les patients en sepsis, il a été observé que le niveau de MP plaquettaires circulantes augmentait et serait associé à l'inflammation systémique et/ou la CIVD <sup>119,120</sup>. Ainsi les microparticules plaquettaires ont une activité apoptotique via la NADPH-oxydase et la génération de peroxy-nitrite sur les cellules endothéliales favorisant la dysfonction vasculaire et l'atteinte de nombreux organes, en particulier cardiaque <sup>121-123</sup>. Les MP d'origine plaquettaire présentent aussi une activité pro-coagulante via leur surface riche en PS, permettant ainsi aux facteurs de la coagulation et du complément de réagir <sup>39,124,125</sup>.

Plusieurs études ont révélé l'aspect pathogénique des plaquettes lors de la dysfonction d'organe observée durant le sepsis. L'accumulation des plaquettes dans divers organes (principalement les poumons, les reins et la rate) induit plusieurs mécanismes physiopathologiques : l'exacerbation de l'inflammation par libération de cytokines pro-inflammatoires ou le recrutement de cellules de l'immunité ; la dérégulation du système de coagulation ; un effet cytotoxique des plaquettes et le relargage de microparticules ayant un effet inflammatoire et pro-coagulant <sup>78,82,126,127</sup>. Au niveau de la rate et des poumons, les plaquettes s'accumulent via la glycoprotéine GPIIb/IIIa et induisent une apoptose des cellules tissulaires suite à la libération de granzyme B <sup>128</sup>. La libération plaquettaire de  $\beta$ -defensine humaine (hBD-1) favorisent la formation de NETs et ainsi de thrombose <sup>128,129</sup>. Les plaquettes activées et adhérentes à l'endothélium, le recrutement des cellules de l'immunité, la fibrinofomation vont contribuer au phénomène d' « immunothrombose » <sup>126,130</sup> (**Figure 3**). En effet, ce processus physiologique initié par les cellules de l'immunité est mis en jeu pour contrôler une infection, cependant l'exacerbation de l'immunothrombose induit des pathologies thrombotiques <sup>131</sup>. Ces microthrombi sont à l'origine de problèmes ischémiques ou thromboemboliques (arrêt vasculaire cérébral, embolies pulmonaires...). Paradoxalement, la consommation excessive des plaquettes et des facteurs de coagulation induit un risque hémorragique élevé <sup>132</sup>.

D'autre part, il est connu que les plaquettes interviennent dans de nombreux mécanismes de régulation de l'activité leucocytaire et de réparation vasculaire <sup>133</sup> (**Figure 3**). Ainsi, lors du sepsis, les plaquettes ont un rôle modulateur important dans le contact entre l'endothélium et les leucocytes circulants <sup>134</sup> (**Figure 3**). De plus, la formation de complexes leuco-plaquettaires importantes chez les patients septiques contribuent aux dommages rénaux et pulmonaires <sup>135,136</sup>. Un étude ancienne a également montré qu'au niveau pulmonaire, le niveau de protéines granulaires d'origine plaquettaire (PF4, Thrombomoduline) dans le liquide broncho-alvéolaire et la détresse respiratoire étaient corrélés <sup>137</sup>. Plus récemment, d'autres études ont mis en évidence l'implication de cytokines proinflammatoires associés aux plaquettes, telles que le RANTES, le PF4, le Thromboxane A2 (TxA2) et le sCD40L dans l'induction de dysfonction pulmonaire lors du sepsis <sup>138-140</sup>.

Les plaquettes favorisent en outre la formation de NETs lors du sepsis (**Figure 3**). La reconnaissance des pathogènes via le récepteur Toll-Like Receptor (TLR)2 ou TLR4 permet la

libération de PF4, vWF et TxA2 pour ainsi lier la glycoprotéine GPIb $\alpha$  au récepteur CD18 des neutrophiles afin d'induire la formation de NETs <sup>99,100</sup>. La formation incontrôlée de NETs entraîne des dommages tissulaires et a pour incidence un état hyperinflammatoire et d'hypercoagulabilité du patient <sup>141,142</sup>.



**Figure 3: Les actions immunitaires des plaquettes lors du sepsis. (D'après de Stoppelaar et al. <sup>126</sup>)**



## 9. Utilisation de molécules antiplaquettaires dans le sepsis

L'investigation des molécules antiplaquettaires dans l'optique de limiter la mortalité associée au sepsis a montré, expérimentalement et cliniquement, des résultats intéressants. Lors d'études expérimentales effectuées sur l'animal (souris et lapins), le blocage de la GPIIb/IIIa plaquettaire permet de réduire la létalité en limitant l'apoptose et le développement de dysfonctions rénales<sup>128,143</sup>. Les thiéno-pyridines, molécules ciblant les récepteurs P2Y<sub>12</sub> plaquettaires, ont révélé quant à elles des résultats contradictoires. Certaines études montrent des effets bénéfiques du clopidogrel sur la survie sur des modèles murins ou humains, en diminuant la formation de complexes leuco-plaquettaires et la migration des neutrophiles dans le parenchyme pulmonaire alors qu'une étude d'endotoxémie chez le porc n'a décrit aucun effet bénéfique du clopidogrel<sup>144-147</sup>. Une étude chez le cheval indique un faible effet du clopidogrel par son activité anti-agrégante lors de l'endotoxémie mais ce modèle semble peu pertinent quant à la faible activité plaquettaire<sup>148</sup>. D'un point de vue clinique, un modèle expérimental d'injection de lipopolysaccharide (LPS) chez l'homme a montré que ce récepteur ne semble pas être impliqué dans l'inflammation et les conséquences observées lors d'une endotoxémie<sup>149</sup>. L'étude PLATO (PLATelet inhibition and patient Outcomes) indique que les patients atteints de syndrome coronaire aigu traités au ticagrelor ont une mortalité, due au sepsis ou une complication pulmonaire comme événement indésirable, moins importante lorsqu'ils sont traités au clopidogrel<sup>150</sup>. Il semblerait que le ticagrelor ait une meilleure action sur les plaquettes que le clopidogrel permettant ainsi une réduction de l'action délétère des plaquettes lors du sepsis. Cela pourrait s'expliquer par un effet sur un spectre cellulaire plus large du ticagrelor comparé au clopidogrel, comme par exemple, l'inhibition de la recapture de l'adénosine par les érythrocytes, impliquée dans de nombreux processus inflammatoire<sup>150</sup>.

En ce qui concerne l'acide acétylsalicylique (ASA), plus connu sous le nom d'aspirine, qui permet d'inhiber la cyclo-oxygénase 1 (COX-1) et donc la libération de TxA<sub>2</sub>, son utilisation est associée à une diminution de marqueurs inflammatoires, une réduction des détresses respiratoires et améliore l'état de patient atteints de sepsis<sup>151,152</sup>. Plusieurs études prospectives ou rétrospectives ont corrélé la prise d'ASA à la réduction de la mortalité de patients septiques<sup>151,153,154</sup>. Etant donné que le thromboxane est l'un des médiateurs utilisés par les plaquettes pour la génération de NETs, l'ASA montrerait donc des effets protecteurs quant à ce mécanisme anti-infectieux qui favorise cependant la coagulation<sup>100</sup>. De plus, le

thromboxane semble être impliqué dans les dysfonctions rénales lors d'une endotoxémie chez la souris <sup>155</sup>. La réduction de la libération plaquettaire de thromboxane par l'ASA améliore le taux de filtration glomérulaire chez des souris soumises à une injection de LPS <sup>156</sup>.

Etant donné que l'acide acétylsalicylique est un traitement fréquemment utilisé dans bon nombre de pathologies inflammatoires chroniques, certaines études se sont intéressées à l'impact de cette molécule sur le sepsis et montrent des résultats contradictoires à ceux indiqués précédemment. En effet, une étude effectuée sur près de 1000 patients de réanimation, chroniquement traités à l'ASA, révèlent que ce traitement n'est en aucun cas associé à la sévérité du sepsis <sup>157</sup>. Cette étude, basée sur un score de propension, utilise un échantillonnage trop faible pour faire ressortir un véritable effet de l'acide acétylsalicylique sur le sepsis. Cependant, l'utilisation d'ASA dans les 24h après diagnostique, a montré une baisse de la mortalité (27.4% par rapport à 42.2% des patients non traités) <sup>151</sup>. Lors d'un modèle d'endotoxémie chez l'homme (2 ng/ml), l'ASA montre une diminution de la réactivité plaquettaire, malgré aucune modification de la libération de sCD62P et vWF, légèrement augmenté (de 50 à 65 ng/ml pour le sCD62P et de 100 à 350 ng/ml pour le vWF) 4h après l'injection de LPS <sup>158</sup>. L'effet anti-inflammatoire apporté par l'ASA et protecteur vis-à-vis du sepsis reste encore contesté puisque certaines études supposent plutôt un effet pro-inflammatoire de cette molécule responsable de cet effet bénéfique <sup>159</sup>. Ceci pourrait s'expliquer par le blocage de la libération de prostaglandines qui ont un effet anti-inflammatoire. Malgré les divergences quant aux mécanismes d'action, l'effet protecteur de l'ASA reste encore controversé par les nombreux résultats contradictoires qui peuvent s'expliquer sur les critères d'inclusions utilisés dans les études cliniques. Cette molécule possède des effets ubiquitaires sur plusieurs types cellulaires; ainsi son effet lors du sepsis ne passe pas uniquement par les plaquettes. D'un point de vue général, l'utilisation de thérapies antiplaquettaires semble avoir un véritable effet bénéfique sur le sepsis, révélant une fois de plus, la place importante occupée par les plaquettes, que ce soit autant au niveau hémostatique qu'inflammatoire, dans cette pathologie.

**Chapitre 2 – Plaquettes sanguines et immunité :  
de la physiologie à la pathologie**

**(Revue publiée dans  
Transfusion Clinique et Biologique en Mai  
2017)**



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Transfusion Clinique et Biologique 24 (2017) 83–86

Review article

# Platelets and immunity: From physiology to pathology

## *Plaquettes sanguines et immunité : de la physiologie à la pathologie*

O. Garraud<sup>a,b,\*</sup>, A. Chabert<sup>a</sup>, H. Hamzeh-Cognasse<sup>b</sup>, S. Laradi<sup>b,c</sup>, F. Cognasse<sup>b,c</sup>

<sup>a</sup> EA 3064, Saint-Étienne Faculty of Medicine, université de Lyon, 42023 Saint-Étienne, France

<sup>b</sup> National Institute of Blood Transfusion, 6, rue Alexandre-Cabanel, 75015 Paris, France

<sup>c</sup> French Blood Establishment (EFS), Rhône-Alpes-Auvergne, 42023 Saint-Étienne, France

Available online 4 May 2017

### Abstract

Blood platelets are cells acting during primary haemostasis. The thrombocytopenia observed in many different types of infectious processes begs the question of the relationship between cells and infectious pathogens and the role of platelets in the detection of biological hazards. This in turn brings us back to the role of platelets – via their molecular, membrane and secretory arsenal – in the detection and repair of vascular hazards. The common denominator between a breakdown of haemostasis and the risk of infection has been shown to be a cutoff point in the inflammatory continuum between physiology and physiopathology. The trophic role of platelets – as topical factor and as platelet transfusions – and their inflammatory complexities appear to correlate this proposed model, as reported in this short review.

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**Keywords:** Blood platelets; Haemostasis; Innate immunity; Inflammation; Infectious pathogens; Blood transfusion

### Résumé

Les plaquettes sanguines sont les cellules de l'hémostase primaire. L'observation de thrombopénies observées lors de plusieurs types de processus infectieux a permis de questionner la relation entre ces cellules et les pathogènes infectieux et le rôle des plaquettes dans la détection des dangers biologiques. Cela a à son tour permis de revisiter le rôle des plaquettes – via leur arsenal moléculaire membranaire et sécrétoire – dans la détection et la réparation des dangers vasculaires. Le point commun entre la rupture d'hémostase et le risque infectieux a été démontré comme un point de césure dans le continuum inflammatoire, entre physiologie et physiopathologie. L'observation du rôle trophique des plaquettes – comme topiques tissulaires et des transfusions plaquettaires – et leurs complications inflammatoires semblent corrélées cette proposition de modèle, ainsi qu'il l'est rapportée dans cette courte revue.

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**Mots clés :** Plaquettes sanguines ; Hémostase ; Immunité innée ; Inflammation ; Pathogènes infectieux ; Transfusion sanguine

Blood platelets are anucleated elements that are formed by the fragmentation of medullary megakaryocytes. Platelets are small cells (2–5  $\mu\text{m}$ ), with a short life-span (about ten days) that are mainly found in the intravascular space in physiological conditions.

Platelets are clearly associated to primary haemostasis: in case of a vascular breach they bind together and form a platelet clot that clogs the breach [1]. A collapse in the number of circulating platelets, whatever the reason, and/or a peripheral hyperconsumption of these same platelets exposes the subject to a risk of bleeding. Some patients may also suffer from bleeding although their circulating platelet count appears normal: this may be the case if they are either taking medication that directly or indirectly acts on the platelets' receptors, such as aspirin and many others or if they carry a genetic deficit that ren-

\* Corresponding author. National Institute of Blood Transfusion, 6, rue Alexandre-Cabanel, 75015 Paris, France.

E-mail address: [ogarraud@ints.fr](mailto:ogarraud@ints.fr) (O. Garraud).

ders platelets partially or totally nonfunctional (thrombopathic states). Thrombocytopenia and thrombopathic states thus represent two major risk factors for patients. Platelet transfusions may alleviate these deficits and restore primary haemostasis and/or prevent bleeding for a limited time. However, platelet transfusions to counteract an overdose of platelet inhibitors are generally advised against, with the exception of in life-threatening emergencies.

Thrombocytopenia may be due to one of two major causes: central causes due to a failure in production and peripheral causes due to excessive consumption or accelerated destruction of platelets. Among the main causes for thrombocytopenia with an increased risk of bleeding are infectious causes: viral, bacterial and parasitic causes, which, to some extent, combine central and peripheral factors [2,3]. This begs the question of whether there is a correlation between platelets and infectious agents. Investigations of this relationship – which is generally very complex – have also shown that the relationship between platelets and infectious agents is more often ternary than binary, with a notable interaction of leukocytes [4]. The relationship between platelets, bacteria and leukocytes, such as can be observed in sepsis, also puts primary haemostasis at risk along with soluble and membrane coagulation factors (platelet, leukocyte and erythrocyte counts) [5]. Recent studies of the coagulation sequence, that demonstrate that platelets intervene first before molecular factors engage, have also shown that these factors are not exclusively soluble but also occur on a membrane level, linked to platelets, vascular endothelial cells, leukocytes and erythrocytes. Experimental models have shown the transfer of platelets of the circulatory compartment, which was thought to be geared exclusively towards extravascular tissue, not free but rather linked to either inflammatory leukocytes or in the form of microparticles or microvesicles [6,7].

All of the facts set out above, call into question the unicist model of the blood platelet acting only in primary haemostasis. Other arguments drawn from experimental and clinical findings highlight the trophic role of platelets or platelet extracts in cell or tissue cultures as well as in the healing of epithelial, mucous (cornea, retina, skin, mucous membranes), sinewy or bony tissue [8,9]. Thus, platelets are much more complex than they appear at first glance: it is thus tempting to review their mode of operation not by starting with the pathology to explain the physiology but by beginning with the physiology to explain the pathology.

Blood platelets create platelet plugs to clog injured blood vessels. A reductionist view would be to confine their activity to the pathology and, a fortiori, to an exacerbated lesion such as a wound. Blood vessels and especially the capillaries are exposed to lesions due to the friction caused by blood cells passing “under pressure” through blood vessels with a smaller vascular diameter than that of the cells passing through them on a daily basis. Platelets play a role in repairing blood vessels (a role that has already been studied in prophylactic transfusions and in the topical application of platelets to damaged tissue): to exercise this function, which, as we already know, is linked to the granular content of the platelets that are rich in factors concerning healing (for instance, factors necessary for cell and tissue growth and differentiation), the platelets need to detect

the lesions beforehand. To do so, platelets are equipped with an arsenal allowing them to detect signals from endothelial vascular lesions whose exacerbation puts into motion the platelet sequence “activation – aggregation – adhesion” that is already well-known from primary haemostasis. This primary haemostasis also engages leukocytes and erythrocytes in addition to soluble plasma factors: platelets are the principal providers of factor V, a potent agonist in the formation of thrombin in the actual coagulation phase [1,10]. By reviewing their function in primary haemostasis, as an assistant to coagulation, platelets may be described as cells that detect (vascular) danger signals and repair lesions. Platelets – which comprise hundreds of distinct molecules on their membrane surface and more than thousand different molecules for secretion – thus discern which of these to activate during this process [11]. We have described platelets as “cells”. Using the term cell instead of cellular debris to describe platelets is not consensual, however, we (within our research group) have described in detail why platelets are not just cells but intelligent cells at that, capable of choosing an activation programme and differentiation options out of several possible options in their metabolome and, in particular, in their transcriptome [12].

This leads us to take a fresh look at the other findings made in connection with platelets in pathological conditions and, in particular, at thrombocytopenia induced by different types of infectious agents. Earlier works revealed that different infectious agents can be seen inside platelets, such as intracellular bacteria or HIV [13]. We now know that, if this is the case, it is that the host cell – here, the platelet – displays a sufficient amount of the necessary receptors to bind the infectious agent and actively penetrate it on its membrane surface. These receptors are not selective to target specific types of infections but are in most cases accidental or shared, as is the case for the expression of members of the “toll-like receptor” (TLR) family on human platelets. In 2005, we were able to demonstrate the expression of TLR on human platelets for the first time followed by their functional and selective role towards bacterial lipopolysaccharides [14,15]. The mechanisms that account for thrombocytopenia following a *Staphylococcus* infection resulting in sepsis, according to the accepted descriptors, are complex and include a central component (thrombopoiesis reduction) and a peripheral destruction which in itself is multicomposite with, in particular, platelet-induced apoptosis [16,17] (Chabert et al., manuscript under review). Here as elsewhere, likely in the case of viral infections with haemorrhagic viruses such as dengue or in the case of *Plasmodium falciparum* malaria, platelets seem to be the victims because they express receptors for different parts of each of these infectious agents [18,19]. In the case of malaria, this is even more complex, as platelets, infectious agents and leukocytes (bacteria) and/or endothelial cells (plasmodia) engage in haemostatic relations and an alternation between hyper and hypercoagulable pathological states can be observed [20].

All of the situations described above have one point in common: inflammation. Inflammation has long been considered only in its pathological expression. We now know that it is a continuum in which the visible component represents

the pathology of the disease but the underlying component is physiological. Philippe Sansonetti at the Institut Pasteur has even managed to conceptualise physiological inflammation in the case of mucous membranes confronted with saprophytic bacteria and viruses (now called microbiota) [21]. This physiological phase of inflammation currently includes two distinct parts: first, a component strictly confined to innate or natural immunity which is based on a dialogue with modified exterior or interior elements and the capacity to discern whether they are dangerous and, if they are, their level of danger and on the other hand, to their capacity to repair or heal wounds; secondly, an element transitioning between innate and adaptive immunity, essentially by aiding the presentation of antigens from captured and degraded pathogens. Platelets, although expressing major histocompatibility complex molecules, do not seem capable of effective presentation of antigens, however, in some instances; their secretion profiles promote an environment favourable to antigen presentation.

Taking into account all of these findings, the following pattern may be proposed: blood platelets are cells that are naturally employed in the detection of hazardous situations and the degree of danger they pose, the first level of danger – the most natural for cells – being vascular attrition. Equipped, as they are, with a rich arsenal of risk detectors, platelets are capable of binding infectious agents and probably also noninfectious pathogens such as immune complexes, especially with auto- and alloantibodies, although this last potential remains to be formally demonstrated (platelets express receptors for specific Fc fragments of IgG and complementary fractions) [22,23]. Some teams, including ours, regard platelets as not just participating in immunity but as immune cells in their own right, mainly due to their inflammatory component [24].

This, in turn, has a knock-on-effect on transfusion management for patients who require a platelet transfusion. Platelet transfusions may be administered for their haemostatic effectiveness, in case of profuse bleeding or as prophylaxis; also being immune and inflammatory cells, the transfused platelets physiologically use the inflammatory aspect to heal the injured endothelium, however, they are also conducive to creating a pathological inflammatory response [11]. As a matter of fact, transfusions of platelet concentrate cause most adverse reactions in recipients, mainly inflammatory in nature, even once the other causes for inflammation have been reduced as happens with prestorage leukodepletion or when decreasing the plasma volume of platelet concentrates [11]. This is mainly attributed to the platelets' content of dense and alpha-granules and platelet mitochondrial DNA [25,26]. Soluble CD40 ligand is among the most inflammatory molecules released by the platelets; but it is not the only one. Our team has identified several non-stochastic associations of soluble and membrane-bound inflammatory mediators in platelet concentrates that are generally released during storage and evolve along with it [27,28]. We also examined whether there are platelet donors who are more at risk and recipients who are (genetically) more vulnerable but we have not yet been able to formally proof our hypothesis of pathogenic transfusion by matching donor/recipient parameters [29]. Nevertheless, we suggest – in order to limit the patho-

logical inflammatory effect and retain only the physiological inflammatory effect – to refrain from hyperactivating platelet in concentrates intended for transfusion, possibly by assessing the methods of separation and concentration and solutions for conservation and storage [30,31].

All in all, platelets appear to be very complex cells that intervene in a number of physiological and immunophysiological areas, acting as tools for the detection of biological hazards and the repair of damaged tissue. By using several molecular and secretory biological tools to operate, platelets are involved in a great number of infectious and probably auto-inflammatory pathophysiological processes that use the same tools to circumvent them. The healing and trophic properties of topic platelets are a great example of their physiological importance along with the transfusion of nonactivated platelets. The occurrence of a posttransfusion inflammatory event in a transfused patient highlights the delicacy of the border between physiology and physiopathology, which is so remarkable for this cell.

### Disclosure of interest

The authors declare that they have no competing interest.

### Acknowledgements

Authors wish to acknowledge past and present PhD studies having helped contributing data discussed in the present manuscript, namely Julien Berthet, Sandrine Lafarge, Kin Ahn Nguyen, Chaker Aloui, Sofiane Tariket and Caroline Sut, as well as the former and present heads of the research group, Professor Bruno Pozzetto and Professor Thomas Bourlet for support. Studies cited in this article have been made possible through grants from Établissement français du sang, Agence nationale de la santé et du médicament, Région Rhône-Alpes, université Jean-Monnet de Saint-Étienne, Association les amis de Rémi and Association recherche transfusion.

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**Chapitre 3 – Impact des infections bactériennes  
sur la coagulation et l’hémostase**

**(Revue publiée dans  
Sang, Thrombose, Vaisseaux en Avril 2017)**



# Plaquettes et coagulation lors d'une infection bactérienne

Adrien Chabert<sup>1</sup>, Hind Hamzeh-Cognasse<sup>1</sup>, Fabrice Cognasse<sup>1,2</sup>, Olivier Garraud<sup>1,3</sup>

<sup>1</sup> EA3064-GIMAP, Université de Lyon, 42023 Saint-Étienne, France

<sup>2</sup> EFS Auvergne-Loire, 25, boulevard Pasteur, 42023 Saint-Étienne, France

<sup>3</sup> Institut national de la transfusion sanguine, 6, rue Alexandre Cabanel, 75015 Paris, France  
<ogarraud@ints.fr>

**Résumé.** Le sepsis est une entité évolutive, caractérisée par une condition clinique menaçant le pronostic vital, en lien à une réponse inappropriée ou dérégulée à une infection bactérienne de l'hôte infecté. On observe fréquemment une dérégulation de la coagulation ainsi qu'une thrombopénie. Les plaquettes, cellules clés de l'hémostase, exerçant en parallèle un rôle pro-inflammatoire, interviennent de façon exacerbée à ce double titre. De nombreuses études utilisant des agents antiplaquetaires montrent un effet bénéfique sur la mortalité dans des modèles expérimentaux de sepsis, mais également chez les patients en état septique grave. Une stratégie thérapeutique, ciblant les plaquettes, pourrait ainsi être envisagée dans le sepsis afin de contrôler tant la coagulation que la boucle inflammatoire. Cette revue a pour objectif de faire un point d'actualité sur la relation plaquettes/bactéries/inflammation, d'une part, et plaquettes/coagulation/sepsis, d'autre part, et d'aborder l'utilisation de molécules antiplaquetaires dans les essais cliniques.

**Mots clés :** plaquette, sepsis, coagulation, inflammation

## Abstract

### Platelets and coagulation during bacterial infections

Sepsis is an evolutive pathology that affects the prognosis and is characterized by an inappropriate or dysregulated inflammatory and immune response to a bacterial infection. The alteration of coagulation processes and the occurrence of thrombocytopenia are also frequently observed during the course of sepsis. Thus, platelets, which are both major coagulation cells and active players of the inflammatory response, play a dual role in the pathophysiology of sepsis. Many studies show that antiplatelet molecules improve survival in experimental sepsis models but is also beneficial for sepsis patient outcome. Therefore, a therapeutic strategy targeting platelets could be considered in sepsis in order to reduce coagulation disorders as well as the platelet-related exacerbation of the inflammatory loop. The aim of this review is to look over the knowledge about the relationships between platelets, bacteria and inflammation, about the contribution of platelets to the dysfunction of coagulation during sepsis, but also about the use of antiplatelet molecules in clinical trials.

**Key words:** platelet, sepsis, coagulation, inflammation

Les plaquettes sanguines sont des éléments cellulaires issus de la fragmentation des mégacaryocytes ; ce sont les cellules clés de l'hémostase. De nombreuses études ont mis

en évidence leur rôle pro-inflammatoire tant physiologique que physiopathologique [1]. En effet, les plaquettes expriment un large panel de récepteurs membranaires et intracellulaires permettant de détecter ou de reconnaître différents types de pathogènes (en particulier de nature infectieuse), déclenchant alors une activation plaquettaire

## Tirés à part :

O. Garraud

avec une double conséquence, hémostatique et inflammatoire [2]. Plaquettes et bactéries ont des relations bilatérales puisque les plaquettes peuvent aussi exercer des effets antibactériens de différentes natures. Ces effets sont, d'une part, directs grâce à la sécrétion de molécules bactériostatiques ou bactéricides, et d'autre part, indirects grâce à l'émission de signaux d'activation de cellules bactériostatiques et phagocytaires, elles-mêmes agissant à plusieurs niveaux, par la phagocytose, la toxicité moléculaire, la neutralisation ou l'émission de *neutrophil extracellular traps* (NET), entre autres mécanismes. Plaquettes et bactéries ont ainsi des relations extrêmement complexes, encore incomplètement comprises ou décrites, dont l'importance a probablement été sous-estimée tant sur le plan de la compréhension de la physiopathologie que des perspectives thérapeutiques [3]. Cette revue met en avant les mécanismes de dérégulation des réponses inflammatoires joués par les plaquettes lors d'une infection. Elle est centrée sur la compréhension entre les plaquettes et les outils de la coagulation et de ceux de l'inflammation lorsque les signaux qui les mobilisent sont des bactéries. Seront envisagés les processus pouvant expliquer la thrombopénie induite lors du sepsis ainsi que ces répercussions cliniques, puis les hypothèses thérapeutiques ciblant les plaquettes et visant à corriger les problèmes de coagulation et/ou d'inflammation en vue d'améliorer la survie d'un patient atteint de sepsis.

## Le sepsis

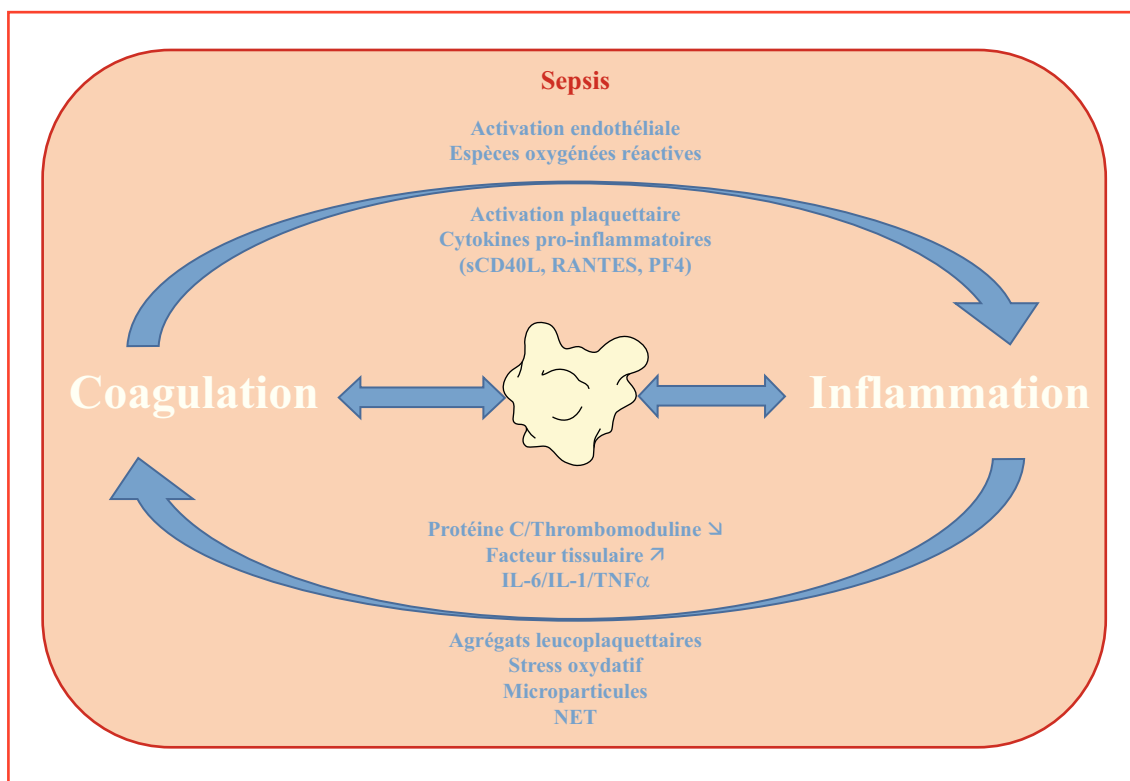
Le sepsis a récemment été redéfini comme une dysfonction d'un ou de plusieurs organes causée par une dérégulation de la réponse inflammatoire de l'hôte suite à une infection [4] majoritairement d'origine bactérienne, principalement *Staphylococcus aureus* (20,5 %), l'espèce *Pseudomonas* (19,9 %) et *Enterobacteriaceae* (*Escherichia coli*, 16,0 %) [5]. Ce consensus international permet alors, grâce à des scores cliniques tels que le *Sequential [Sepsis-related] Organ Failure Assessment* (SOFA), de standardiser la définition du sepsis afin de diminuer les différences dans les études épidémiologiques et cliniques concernant cette pathologie. Elle a aussi pour avantage de réduire les catégories dans l'ensemble « sepsis » afin de faciliter précocement le diagnostic et ainsi d'améliorer le management. On observe, chez ces patients, une inflammation induite sous la dépendance d'une signalisation cellulaire intégrante de l'immunité innée exagérée, induisant alors divers processus physiopathologiques (augmentation de la perméabilité cellulaire, coagulation incontrôlée, etc.) entraînant la défaillance d'organes (reins, foie, poumons, etc.). Le type et la virulence du ou des pathogènes entraînant le sepsis, ainsi que l'état clinique du patient induisent alors une diversité

de complications cliniques. La majorité des patients présentant un sepsis développe une coagulation intravasculaire disséminée (CIVD), ainsi que la formation de complexes leucoplaquettaires contribuant à la chute du compte plaquettaire. Il a été rapporté une relation significative entre le degré de la thrombopénie et la mortalité des patients [6, 7]. Il existe une association entre la baisse du taux de plaquettes et le statut inflammatoire (augmentation du relargage de cytokines inflammatoires et de l'activation des cellules endothéliales) responsable de la pathogenèse du sepsis [6]. Cette corrélation entre CIVD, taux plaquettaire et mortalité souligne l'importance centrale des plaquettes dans le sepsis et leur place entre coagulation et inflammation.

## Effets inflammatoires des plaquettes lors d'une infection bactérienne

Les plaquettes sont des cellules sentinelles qui contribuent de manière non négligeable à l'immunité anti-infectieuse. Elles vont interagir avec les bactéries *via* plusieurs mécanismes :

- une liaison directe de la bactérie avec un récepteur plaquettaire distinct selon le type bactérien ;
  - une fixation indirecte entre la bactérie et une protéine plasmatique reconnue elle-même par un récepteur plaquettaire ;
  - ou encore la reconnaissance par un récepteur plaquettaire de produits sécrétés par la bactérie, tels que des toxines [3].
- Les plaquettes expriment, à leur surface et dans leur cytoplasme, plusieurs récepteurs de la famille des *Toll-like receptor* (TLR) (TLR1/TLR2/TLR3/TLR4/TLR6/TLR7/TLR9) mais également de type *Sialic acid-binding immunoglobulin-type lectins* (SIGLEC), permettant de réguler la réponse immunitaire [2]. Elles expriment le récepteur FcγRIIA reconnaissant les complexes immuns ou les cellules ayant opsonisé un anticorps. Ce récepteur joue un rôle majeur dans la thrombose induite lors du sepsis [8]. Suite à la reconnaissance des pathogènes, les plaquettes vont s'activer, exprimer ou surexprimer un grand nombre de protéines à leur surface (P-sélectine, GPIIb/IIIa, GPIbα, etc.) mais également relarguer le contenu de leurs granules. Les plaquettes contiennent plus de 300 molécules solubles et membranaires telles que des peptides antimicrobiens, des facteurs de croissance, des protéines de la coagulation ainsi que de nombreux immunomodulateurs [9]. Le relargage granulaire peut induire un orage cytokinique pro-inflammatoire (*platelet factor 4*, RANTES, sCD40 ligand) sous certaines conditions (cliniques en particulier) ou initier l'interaction avec différents types leucocytaires (neutrophiles, monocytes, lymphocytes B) en hyper-régulant l'expression d'intégrines comme le



**Figure 1.** Place centrale de la plaquette entre la coagulation et l'inflammation induite lors du sepsis. La coagulation va être à l'origine de l'inflammation via l'activation endothéliale et plaquettaire, ce qui induit une libération d'espèces oxygénées réactives ou de cytokines pro-inflammatoires tels que sCD40L, RANTES ou PF4. À l'inverse, de nombreux processus inflammatoires (diminution de la protéine C, augmentation du facteur tissulaire, agrégats leucoplaquettaires, stress oxydatif, microparticules et formation de *neutrophil extracellular traps* [NET]) vont contribuer à la coagulation.

*P-selectin glycoprotein ligand 1* (PSGL-1), TREM-1 ou  $\alpha_M\beta_2$  (Mac-1) ; les chimiokines inflammatoires stimulent aussi le recrutement leucocytaire au niveau du site d'infection [10]. Les plaquettes facilitent la phagocytose du pathogène par les neutrophiles via le récepteur Fc $\gamma$ RIIA ou le TLR2 [11] ou par les macrophages via la GPIb $\alpha$  [12]. L'activation plaquettaire et l'expression du TLR2 et TLR4 permettent de potentialiser la formation de NET par les neutrophiles circulants [13]. Après reconnaissance du lipopolysaccharide (LPS) par les récepteurs TLR2, les plaquettes sécrètent des espèces oxygénées réactives (ion superoxyde et peroxyde d'hydrogène) exerçant un effet microbicide supplémentaire [14].

### La plaquette à l'interface entre coagulation et inflammation

Il existe une réelle inter-connexion entre la coagulation et l'inflammation participant à la physiopathologie du sepsis. La dérégulation de l'inflammation contribue à l'activité

pro-coagulante observée lors du sepsis et en retour une activation de l'hémostase est clairement à l'origine de problèmes inflammatoires (figure 1). Cette interface entre la coagulation et l'inflammation peut s'expliquer par différents mécanismes. Dans le contexte inflammatoire du sepsis bactérien, il y a une inhibition de la sécrétion de la protéine C par les cellules endothéliales et la « down régulation » de la thrombomoduline, activatrice de la protéine C. La déplétion de ces agents fibrinolytiques physiologiques provoque un état d'hypercoagulabilité à l'origine de la CIVD. Par ailleurs, les cellules endothéliales et les leucocytes activés sous l'effet de cytokines pro-inflammatoires (IL-1 et TNF $\alpha$ ) sécrètent du facteur tissulaire (FT) à l'origine de la génération de thrombine, procoagulante. Le facteur d'activation plaquettaire (PAF) sécrété par les mastocytes et polynucléaires lors d'une réaction inflammatoire active les plaquettes via le récepteur dédié, entraînant ainsi la dégranulation des plaquettes et l'initiation de la cascade de coagulation [15]. Les cellules endothéliales et plaquettes activées vont relarguer l'inhibiteur de l'activateur du plasminogène 1 (PAI-1), lui-même inhibiteur des enzymes

(tPA et uPA) responsables de la plasminogénèse, à l'origine de la fibrinolyse [16]. Les histones sécrétées par les neutrophiles, ainsi que le PF4 relargué par les plaquettes vont moduler le système thrombomoduline/protéine C et favoriser les événements prothrombotiques [17]. L'inflammation créée par les cellules de l'immunité (neutrophiles, monocytes) active les plaquettes qui se lient au CD40 de l'endothélium *via* leur CD40 ligand et induisent l'apoptose des cellules endothéliales sensibles à l'action des caspases 8/9 sécrétées et la production d'espèces oxygénées réactives. Cela entraîne l'exposition de la matrice extracellulaire et l'adhésion des plaquettes *via* les couples facteur Willebrand/GPIIb et fibrinogène/GPIIb/IIIa [18]. Cette hémostase pathologique va activer les plaquettes qui vont alors amplifier l'inflammation systémique due à l'infection en orchestrant les nombreuses actions inflammatoires précédemment décrites. L'activation de ces plaquettes entraîne par la suite une agrégation ainsi que la formation de complexes leucoplaquettaires [19, 20]. Ces agrégats constituent des plateformes circulantes pouvant former des thrombi obstruant les microvaisseaux et à l'origine d'ischémies et de dommages tissulaires. *Via* leur glycoprotéine GPIIb, les plaquettes se lient au récepteur CD18 (Intégrine  $\beta 2$ ) des neutrophiles favorisant alors la formation de NET, un réseau de filaments chromatiques (ADN) et d'enzymes pouvant capter les pathogènes [13]. Ce mécanisme de défense, mis en place par les neutrophiles, est utile pour tuer les pathogènes mais il est en contrepartie cytotoxique pour les cellules endothéliales. De plus, des études ont montré que les NET amplifient l'hypercoagulabilité chez des patients septiques [21]. En effet, la libération de NET par les neutrophiles, en présence de plaquettes, est associée au relarguage de protéines de la coagulation telles que le facteur tissulaire, initiant la voie extrinsèque, et le facteur XII, qui lui agit sur la voie intrinsèque de la cascade de coagulation [22, 23]. La NETose est donc favorable à la thrombose intravasculaire à l'origine de nombreuses complications lors d'une infection systémique [24, 25]. Suite à leurs actions avec les bactéries et les leucocytes, les plaquettes vont être induites à l'apoptose ; elles expriment différents marqueurs apoptotiques (Bcl-2, Bax, Caspase 3) et elles relarguent des microparticules plaquettaires (MPP), un phénomène reconnu comme un bon biomarqueur inflammatoire [26]. Ces MPP vont libérer des espèces oxygénées réactives, amplifiant l'inflammation au niveau endothélial mais également avoir une forte activité pro-coagulante [27].

## Sepsis et thrombopénie

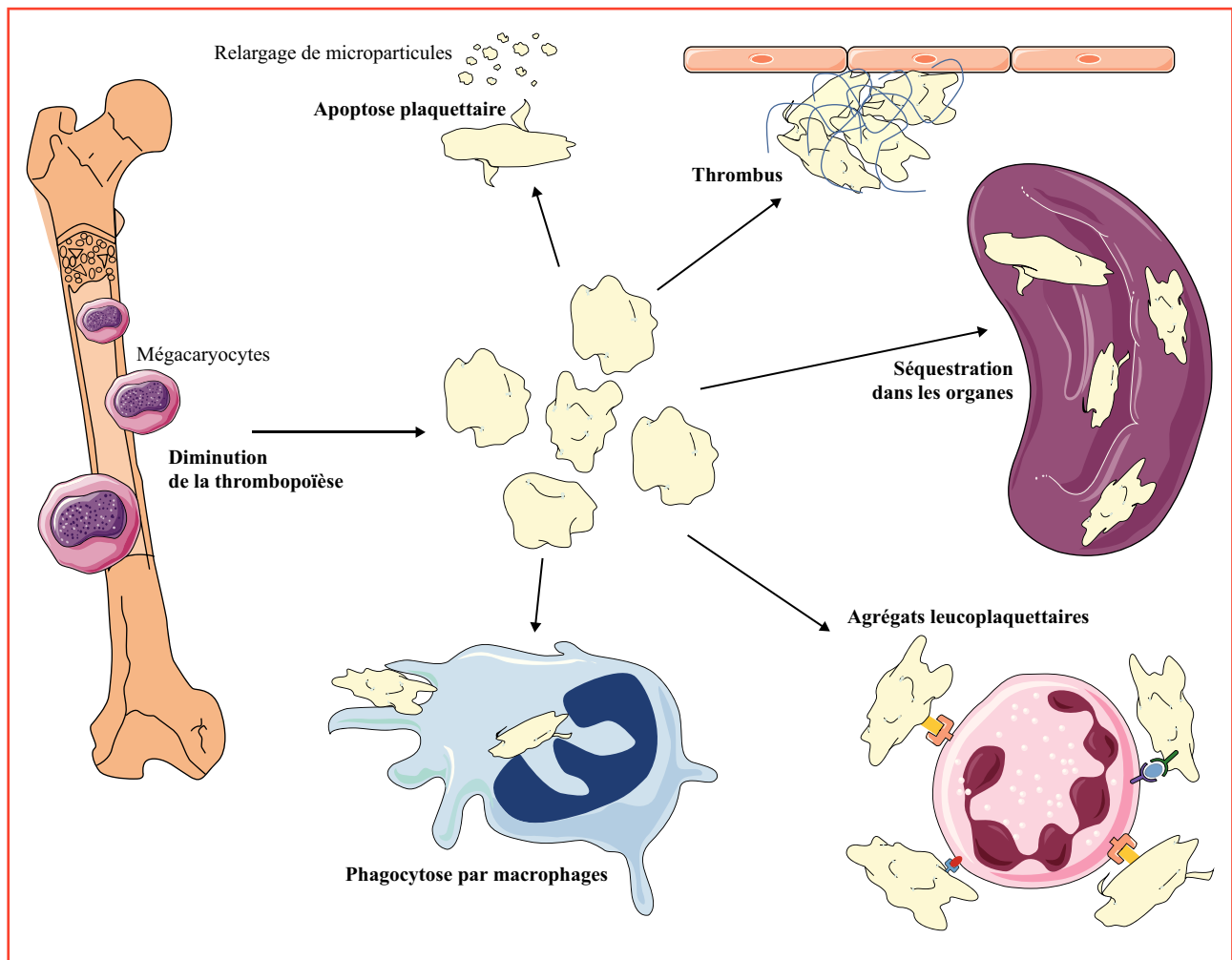
La constatation d'une thrombopénie ( $< 150 \times 10^9/L$ ) est fréquente (de 22 à 58 % selon les études) chez les patients

présentant un sepsis [7]. La thrombopénie a différentes origines (*figure 2*) :

- une perturbation de la thrombopoïèse : malgré une augmentation des taux de TNF $\alpha$  et d'IL-6 stimulant la thrombopoïèse chez les patients présentant un sepsis, une dérégulation de la production de thrombopoïétine (TPO) ainsi qu'une hémophagocytose des mégacaryocytes diminuent la thrombopoïèse. Chez les patients thrombopéniques septiques, un biomarqueur est la fraction de plaquettes immatures (proportion de plaquettes réticulées circulantes) qui se trouve corrélée à la diminution de la thrombopoïèse [28] ;
- une agrégation des plaquettes au niveau de l'endothélium lésé ou la formation de complexes leucoplaquettaires : après contact entre plaquettes et bactéries, l'activation plaquettaire permet l'expression de glycoprotéines (P-sélectine, CD40L, GPIIb/IIIa). Les plaquettes vont réagir en s'agrégeant entre elles grâce aux interactions CD40/CD40L ou par l'intermédiaire du fibrinogène se fixant à la GPIIb/IIIa. Elles se lient également avec des cellules de l'environnement telles que les neutrophiles (en créant des agrégats neutroplaquettaires, dont l'avenir est de migrer et s'accumuler dans des organes distaux comme les poumons et le foie). Les plaquettes adhèrent aux cellules endothéliales *via* le CD40/CD40 ligand et vont être séquestrées dans la microvasculature ;
- une apoptose plaquettaire induite directement par les bactéries ;
- une phagocytose des plaquettes adhérentes *via* la GPIIb aux cellules de Küpffer ou des macrophages circulants dans le but d'éliminer les bactéries [12]. Les macrophages participent à la thrombopénie induite lors du sepsis puisqu'ils phagocytent les plaquettes ayant opsonisé un complexe immun *via* le récepteur au fragment Fc des immunoglobulines (Fc $\gamma$ RIIA).

## Les traitements antiplaquettaires utilisés lors du sepsis bactérien

Lors d'une infection bactérienne, on observe une dérégulation vasculaire due à une diminution de la perméabilité membranaire ainsi qu'un déséquilibre des facteurs de la coagulation. L'induction de cette coagulation physiopathologique va avoir pour incidence de limiter la dissémination de l'infection. En effet, les pathogènes vont être piégés dans les thrombi ainsi formés, avant d'être dégradés par les cellules macrophagiques. La CIVD participe à la physiopathologie du sepsis. Une activation du système de coagulation (facteur tissulaire, thrombine, etc.), la formation de fibrine (relarguage plaquettaire d'inhibiteur de l'activateur du plasminogène 1) et la génération d'un thrombus peuvent déclencher des pathologies thrombotiques comme



**Figure 2.** Mécanismes pouvant induire une thrombopénie dans un contexte de sepsis. Lors d'une infection, la thrombopoïèse est diminuée en raison d'une réduction de la production de thrombopoïétine (TPO), les macrophages peuvent phagocyter les plaquettes *via* l'induction d'auto-anticorps dirigés contre les plaquettes. L'activation des plaquettes par les pathogènes entraîne une interaction avec les leucocytes et forme alors des agrégats leucoplaquettaires. Une activation plaquettaire induit également une apoptose et donc la formation de microparticules. Enfin, les plaquettes peuvent migrer et être séquestrées *in situ* (au niveau de la rate, du foie ou des poumons), diminuant alors le compte plaquettaire.

l'embolie pulmonaire ou encore des microangiopathies vasculaires à l'origine de complications d'organes (foie, poumons, reins). *A contrario*, une activité hémostatique exacerbée va entraîner une consommation des facteurs de la coagulation ainsi que des plaquettes, induisant par la suite des problèmes hémorragiques. Les plaquettes étant à l'interface entre les mécanismes inflammatoires et de coagulation semblent être une cible thérapeutique intéressante pour diminuer la mortalité du sepsis.

Différentes approches thérapeutiques afin de contrôler la coagulation (inhibiteur de la voie du facteur tissulaire (TFPI), thrombomoduline, anti-thrombine, protéine C activée, activateur tissulaire du plasminogène, etc.) ont montré un effet modéré sur la mortalité de patients septiques

puisque toutes ces approches induisent en contrepartie des effets indésirables [29]. Le fait de limiter l'action des plaquettes, autant d'un point hémostatique qu'inflammatoire, semble intéressant pour limiter la mortalité causée par le sepsis [30]. L'utilisation d'antiplaquettaires protège d'événements thrombotiques et limite également l'inflammation systémique, avec la diminution de cytokines pro-inflammatoires et de complexes leucoplaquettaires, comme l'a démontré l'historique de leur utilisation.

### Aspirine

Selon les critères d'inclusions, certaines études cliniques montrent un effet protecteur de l'aspirine sur la

mortalité des patients septiques alors que d'autres n'observent aucune association entre l'utilisation d'aspirine et la survie de ces patients [30]. Cette molécule a un effet pléiotrope et peut agir sur d'autres types cellulaires exposant les deux isotypes de cyclo-oxygénase (cellules endothéliales, macrophages, etc.). L'aspirine permet également de synthétiser le 15-epi-lipoxin A4 (ATL) qui augmente la production de monoxyde d'azote. Ce composé chimique permet d'inhiber l'activité plaquettaire, la libération de cytokines pro-inflammatoires ainsi que la formation de complexes entre les cellules endothéliales et les leucocytes, limitant alors leur recrutement. L'aspirine permet de réguler l'agrégation des plaquettes avec les neutrophiles ainsi que la séquestration des plaquettes au niveau de la muqueuse pulmonaire participant au syndrome de détresse respiratoire aigu (SDRA) [31]. De manière contradictoire, il a été montré que les plaquettes protégeraient du sepsis par leur capacité inhibitrice de l'inflammation causée par les macrophages ; ainsi, un traitement avec l'aspirine, sur un modèle murin d'endotoxémie, a un effet délétère sur la survie [32].

### Inhibiteur de GPIIb/IIIa

Le récepteur GPIIb/IIIa est primordial à l'étape finale de l'agrégation plaquettaire. Le blocage de cette glycoprotéine, dans un modèle murin de sepsis, montre une amélioration du sepsis due à la diminution de sécrétion de granzyme B ce qui réduit l'apoptose [33]. Une étude, utilisant un modèle d'endotoxémie chez le lapin, montre un effet bénéfique sur la mortalité, expliqué par une diminution de la sécrétion de facteur tissulaire par les monocytes, régulant ainsi la coagulation, mais également une amélioration des dysfonctions endothéliales [34]. Une étude sur les babouins montre quant à elle une protection dans le développement de microangiopathie hémolytiques après infusion de *E. coli* [35]. Cependant, un modèle clinique de faible endotoxémie (non comparable au sepsis) ne révèle aucun effet sur l'activation de la coagulation induite par le facteur tissulaire [36]. Il n'y a, pour l'instant, aucune étude clinique mettant en avant une diminution de la mortalité chez les patients septiques [30].

### Thiénopyridine

Cette catégorie de molécules ciblant les récepteurs P2Y<sub>2</sub>1 (récepteur à l'ADP) permet d'inhiber l'activation plaquettaire, conduisant à l'agrégation. L'utilisation de thiénopyridines lors du sepsis, limite l'activation des plaquettes, et des interactions avec leucocytes. Cela

permet de diminuer l'inflammation systémique (diminution de TNF $\alpha$ ), la formation d'agrégats leucoplaquettaires, à l'origine de thrombi mais également de limiter la migration des neutrophiles vers le parenchyme pulmonaire [37]. L'étude clinique PLATO, incluant plus de 18 000 patients, montre un effet bénéfique du ticagrelor et du clopidogrel sur la mortalité du sepsis et de ses répercussions [38]. Des études rétrospectives ont montré que l'usage préexistant d'antiplaquettaires (aspirine, thiénopyridines, etc.) a un effet bénéfique sur la mortalité des patients septiques et un effet protecteur quant à la défaillance d'organes [39, 40]. ■

### Conclusion

La plaquette est un « baromètre » pouvant évaluer le niveau d'infection et alors adapter la réponse immunitaire en recrutant les leucocytes (macrophages/neutrophiles) ou encore en mettant en jeu la formation de NET considérés « comme la dernière chance de défense ». Il a bien été établi, par de nombreuses études, que les plaquettes contribuent au sepsis, par leur potentiel hémostatique et inflammatoire. En effet, les plaquettes contribuent au déséquilibre de la balance hémostatique et favorisent les complications observées lors du sepsis (CIVD, microangiopathie thrombotique, saignements, etc.). Selon la cinétique inflammatoire qu'on peut observer dans la physiopathologie du sepsis, il semble intéressant de considérer la plaquette comme cible thérapeutique, afin de pouvoir moduler l'inflammation. Dans une optique d'approche curative innovante vis-à-vis du sepsis, davantage d'études prospectives, cliniques et expérimentales sont essentielles afin de pouvoir, le cas échéant, utiliser les antiplaquettaires lors de cette pathologie.

**Remerciements.** Les auteurs remercient la communauté de recherche académique (ARCI) de la région Rhône-Alpes pour son attribution de bourse régionale à Adrien Chabert, l'Agence nationale de la recherche (ANR-12-JSV1-0012-01), l'EFS Rhône-Alpes-Auvergne ainsi que l'association « Les amis de Rémi ».

**Liens d'intérêts :** les auteurs déclarent n'avoir aucun lien d'intérêt en rapport avec l'article.

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**Chapitre 4 – Impact des plaquettes sur  
l’inflammation lors d’infections bactériennes**

**(Revue publiée dans  
Frontiers in Immunology en Février 2015)**





# Platelets and infections – complex interactions with bacteria

Hind Hamzeh-Cognasse<sup>1</sup>, Pauline Damien<sup>1</sup>, Adrien Chabert<sup>1</sup>, Bruno Pozzetto<sup>1</sup>, Fabrice Cognasse<sup>1,2</sup> and Olivier Garraud<sup>1,3\*</sup>

<sup>1</sup> GIMAP-EA3064, Université de Lyon, Saint-Etienne, France

<sup>2</sup> Etablissement Français du Sang Auvergne-Loire, Saint-Etienne, France

<sup>3</sup> Institut National de la Transfusion Sanguine, Paris, France

## Edited by:

Claudia Monaco, Catholic University of Rome, Italy; Imperial College London, UK

## Reviewed by:

Kottarappat N. Dileepan, The University of Kansas Medical Center, USA

Kai Fang, University of California Los Angeles, USA

## \*Correspondence:

Olivier Garraud, Faculty of Medicine, GIMAP-EA3064, 15 rue Ambroise Pare, Saint-Etienne Cedex 2 42023, France  
e-mail: olivier.garraud@univ-st-etienne.fr

Platelets can be considered sentinels of vascular system due to their high number in the circulation and to the range of functional immunoreceptors they express. Platelets express a wide range of potential bacterial receptors, including complement receptors, FcγRII, Toll-like receptors but also integrins conventionally described in the hemostatic response, such as GPIIb–IIIa or GPIb. Bacteria bind these receptors either directly, or indirectly via fibrinogen, fibronectin, the first complement C1q, the von Willebrand Factor, etc. The fate of platelet-bound bacteria is questioned. Several studies reported the ability of activated platelets to internalize bacteria such as *Staphylococcus aureus* or *Porphyromonas gingivalis*, though there is no clue on what happens thereafter. Are they sheltered from the immune system in the cytoplasm of platelets or are they lysed? Indeed, while the presence of phagolysosome has not been demonstrated in platelets, they contain antimicrobial peptides that were shown to be efficient on *S. aureus*. Besides, the fact that bacteria can bind to platelets via receptors involved in hemostasis suggests that they may induce aggregation; this has indeed been described for *Streptococcus sanguinis*, *S. epidermidis*, or *C. pneumoniae*. On the other hand, platelets are able to display an inflammatory response to an infectious triggering. We, and others, have shown that platelet release soluble immunomodulatory factors upon stimulation by bacterial components. Moreover, interactions between bacteria and platelets are not limited to only these two partners. Indeed, platelets are also essential for the formation of neutrophil extracellular traps by neutrophils, resulting in bacterial clearance by trapping bacteria and concentrating antibacterial factors but in enhancing thrombosis. In conclusion, the platelet–bacteria interplay is a complex game; its fine analysis is complicated by the fact that the inflammatory component adds to the aggregation response.

**Keywords:** platelet, inflammation, adhesion, infection, cytokine, chemokine, sepsis

## INTRODUCTION

The molecular make-up of platelets, which are specialized in repair and in innate immunity (1–4), makes these “cells” unique blood elements. The recognition of platelets as cells is still controversial, primarily due to their lack of a nucleus. However, the multitude of functions that have recently been attributed to them and that are presented in this manuscript support our decision to consider them as such throughout these studies. The platelet response,

which was once believed to only be involved in hemostasis, is in fact extremely complex and probably adapts when required. In this review, we will address the inflammatory potential of platelets when confronted with pathogenic invasion, and more specifically when it involves bacteria or viruses. We will focus on their ability to directly trigger an immune response, ranging from recognition of the pathogen to the orchestration of its elimination.

**Abbreviations:** Bcl, B-cell lymphoma; C1q, first complement component; CD40L, CD40Ligand; Clf, clumping factor; CTAP3, connective tissue activating protein-3; DIC, disseminated intravascular coagulation; *E. coli*, *Escherichia coli*; Fas, apoptosis stimulating fragment; Fnbp, fibronectin-binding protein; gC1q-R, gC1q receptor; Gsp, glycosylated streptococcal protein; hBD-1, human beta-defensin-1; IE, infectious endocarditis; Ig, immunoglobulin; IL, interleukin; Isd, iron-regulated surface determinant; ITAM, immunoreceptor tyrosine-based activation motif; LPS, lipopolysaccharide; Mac-1, macrophage-1 antigen; mRNA, messenger RNA; MSCRAMM, microbial surface components recognizing adhesive matrix molecules; NET, neutrophil extracellular trap; NO, nitric oxide; NOS, nitric oxide synthase; OCS, open canalicular system; *P. gingivalis*,

*Porphyromonas gingivalis*; PAF, platelet-activating factor; PAFR, platelet-activating factor receptor; PAR, protease-activated receptor; PF4, platelet factor-4; PMP, platelet microparticles; PmP, platelet microbicidal proteins; PS, phosphatidylserines; RANTES, regulated on activation, normal T cell expressed and secreted; ROS, reactive oxygen species; *S. aureus*, *Staphylococcus aureus*; *S. epidermidis*, *Staphylococcus epidermidis*; *S. gordonii*, *Streptococcus gordonii*; *S. pyogenes*, *Streptococcus pyogenes*; *S. sanguinis*, *Streptococcus sanguinis*; sCD40L, soluble CD40L; Sdr, serine–aspartate dipeptide repeat; SEB, staphylococcal enterotoxin B; SrpA, serine-rich protein A; SSL, staphylococcal superantigen-like; TLR, Toll-like receptor; TNF, tumor necrosis factor; Tx, thromboxane; vWF, von Willebrand factor; ΔΨ<sub>m</sub>, mitochondrial membrane potential.

## PLATELETS AND BACTERIAL INFECTIONS

### PLATELETS AT THE INTERFACE BETWEEN BACTERIAL INFECTION AND THROMBOSIS

#### Example of infectious endocarditis

Cardiovascular diseases, although varied, may have infectious origins, as was described by Beynon et al. concerning infectious endocarditis (IE) (5). The main bacterial agents involved in IE are *Staphylococcus aureus*, *Streptococcus sanguinis*, and *Streptococcus gordonii* (*S. gordonii*). According to epidemiological studies, bacteremia that leads to the development of this disease may be the consequence of a local intervention, but there may be a more distant origin such as the recurrent administration of a drug or a surgical dental procedure.

By creating an inflammatory environment, bacteria adhere to the valvular endothelium and increase its permeability, thus leading to the exposure of subendothelial tissue factors. The circulating platelets then adhere to the subendothelium, and their hemostatic activation causes the formation of a thrombus, which can then lead to arterial ischemia, and even pulmonary embolism (6). IE is therefore a disease that links inflammation and hemostasis, though it was long accepted that platelet activation occurred indirectly.

In IE, analysis of a newly formed thrombus in the myocardium showed the presence of bacteria inside the platelet clot (7). The first hypotheses suggested that this bacterial presence stabilized the platelet clot due to the activity of bacterial enzymes specialized in coagulation [coagulase for *S. aureus* (8) or the “clumping factor” (Clf) for the other staphylococci (9)], although without direct participation in platelet activation.

In sepsis, microthrombi form in the blood capillaries (10). As with IE, this phenomenon was attributed to the inflammatory environment that promoted platelet aggregation. The study by Osterud et al. also supports this hypothesis, since the authors show that in severe sepsis, the circulating monocytes show an increased expression of tissue factors, which thus support platelet aggregation (11).

Beginning in 2005, publications describing the role of platelets in immunity put prior observations related to the direct interaction of platelets and bacteria back in the spotlight (12). They then suggested that the binding of bacteria to platelets should even be considered a factor in the immune response. Today, the growing number of studies based on the inflammatory potential of platelets show that these cells express a variety of receptors, soluble molecules, and signaling factors (both hemostatic and inflammatory), enabling them to secure their position as direct effectors of antibacterial defense. This function is presented in the following sections.

#### Inflammatory and thrombotic role of platelet microparticles

Platelets also form the link between thrombosis and inflammation through the production of microparticles. Platelet microparticles (PMP) are phospholipid vesicles (100–1000 nm) that are released after budding from the platelet plasma membrane. As a result, PMP express the same antigens as their parent cells, i.e., GPIIb–IIIa, GPIb, CD31, CD61, and CD62P. This distinguishes them from microparticles derived from other cell types (red blood cells, leukocytes, monocytes, endothelial cells). PMP thus make up between 70 and 90% of the circulating vesicles. PMP differ from exosomes

by their size, but also due to the fact that they are not derived from exocytosis of multivesicular bodies (13).

Platelet microparticles are released by the activated platelets in apoptosis or senescence. A central factor in the induction of this event is the detachment of the actin cytoskeleton from the plasma membrane, which occurs primarily through the increase in intracellular calcium concentration. The calcium then interacts directly with the proteins involved in proteolysis of the cytoskeleton, such as calpain (14, 15). The formation of PMP may also occur independent of calcium, in which case it involves the C5B–9 complement factor and activation of protein kinases, such as calmodulin (16).

Bacterial infection also appears to be a source of PMP formation. This has already been well described during the involvement of platelet Toll-like receptor (TLR) 4 (17–19) but also in response to the Shiga toxin (20).

Platelet microparticle formation results in an asymmetrical distribution of the membrane phospholipids. The circulating PMP thus express phosphatidylserines (PS), which are highly procoagulant phospholipids, on their surface (21). PMP also express tissue factor, the major initiator of the coagulation cascade (22–24). PMP have also been described as a surface that enables the *in vitro* generation of plasmin; this ability was not found in microparticles isolated from endothelial cells (25).

Platelet microparticle are also capable of issuing immunomodulatory factors, such as regulated on activation, normal T cell expressed and secreted (RANTES) (26), interleukin (IL)-1 $\beta$  (18), and CD40Ligand (CD40L) (27), and can also modulate the activation of inflammatory cells such as neutrophils (28, 29). PMP even seem to be able to exert their pro-inflammatory activity outside of the blood compartment (30). The pro-inflammatory function of PMP is referred to in greater detail throughout this manuscript.

Finally, the proportion of PMP in the circulation is increased in some illnesses, such as cardiovascular disease (22), sepsis (31), or HIV infection (32), which suggests they may be involved in the pathophysiology of these diseases.

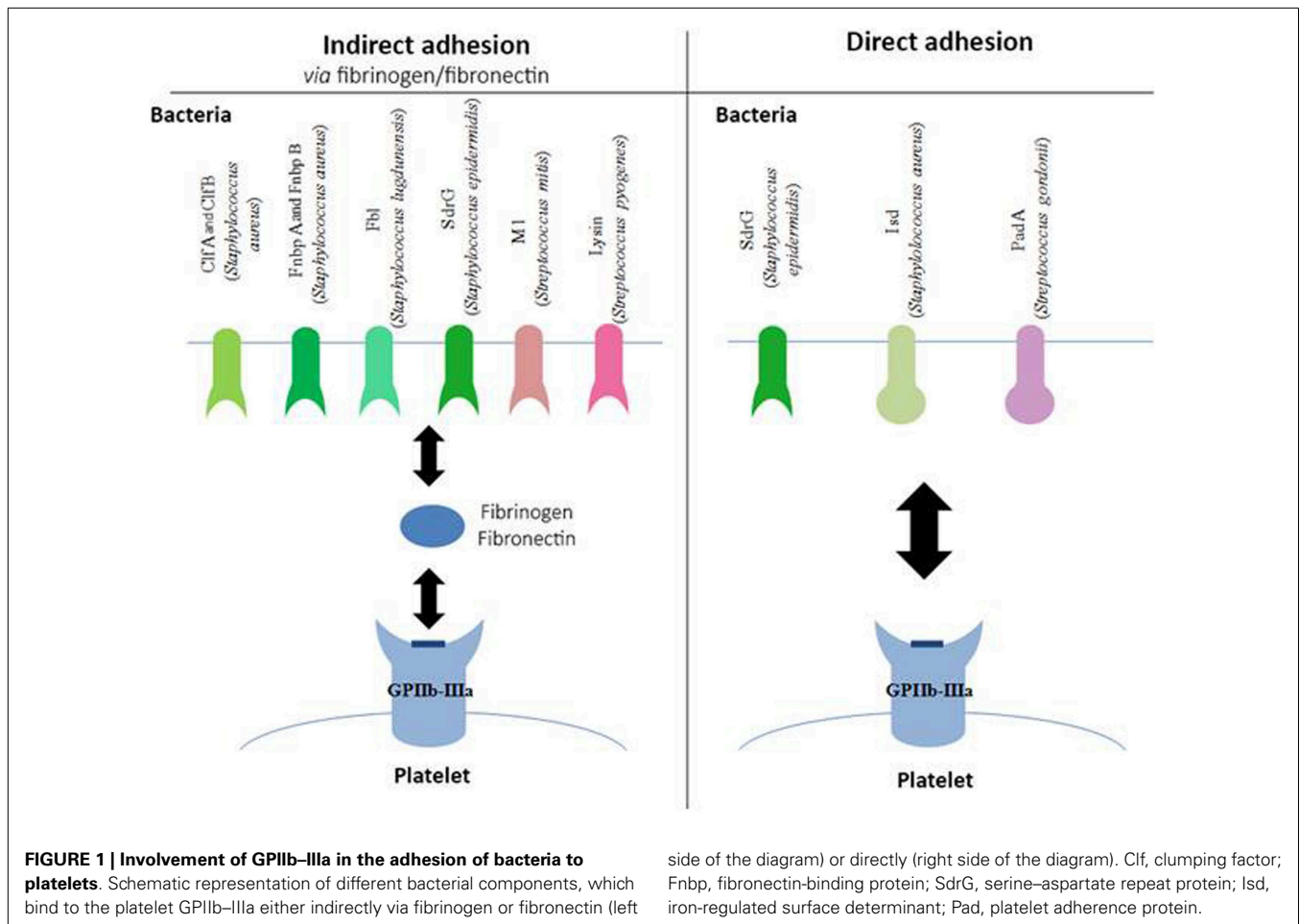
### MECHANISMS OF INTERACTION BETWEEN PLATELETS AND BACTERIA

Three mechanisms of interactions between bacteria and platelets have been described to date: (1) the indirect binding of bacteria to a plasma protein, which itself is a ligand of a platelet receptor; (2) the direct binding of bacteria to platelet receptors; and (3) the binding of secreted bacterial products, particularly toxins, to platelets. The mechanisms of interaction are made more complex by the diversity of platelet receptors involved in bacterial recognition.

#### Role of glycoprotein IIb–IIIa

In addition to ensuring their usual function in hemostasis, the platelet glycoproteins play a role in the adhesion to bacteria. The first platelet receptor identified as such was GPIIb–IIIa. This integrin, specifically from the megakaryocyte cell line, is the receptor for fibrinogen. Its involvement results in adhesion and platelet aggregation (33, 34).

*Staphylococci* express surface receptors that are specific for fibrinogen and fibronectin (Figure 1). These are surface proteins characterized by regions rich with serine–aspartate repeats,



belonging to the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) family (35). These molecules enable bacteria to adhere to tissues, a critical step in the establishment of infection. Some of the most common examples of MSCRAMM are found in *S. aureus*: ClfA (36), ClfB (37), fibronectin-binding protein (Fnbp) A, and FnbpB (38). *Staphylococcus lugdunensis* binds to fibrinogen via its Fbl protein, which is 58% identical to ClfA (39).

The binding of *Staphylococcus epidermidis* was long unknown, even though it had already been shown that trypsin treatment in a culture of *S. epidermidis* prevented its adhesion to platelets (40), suggesting the involvement of a membrane factor. In 2009, Brennan et al. showed that serine-aspartate dipeptide repeat (Sdr) G proteins expressed on the surface of *S. epidermidis* are necessary for the adhesion of bacteria to platelets via fibrinogen (41).

Even though these various MSCRAMM are highly similar proteins, they bind to fibrinogen via different binding sites. ClfA and Fbl, as well as FnbpA and B, bind to the C-terminal region of the fibrinogen  $\gamma$ -chain. ClfB has its binding site on the C-terminal region of the fibrinogen  $\alpha$ -chain, and SdrG on the  $\beta$ -chain. Other types of bacteria can also bind to fibrinogen, particularly *Streptococcus pyogenes* (*S. pyogenes*) via the M1 protein, and *Streptococcus mitis* via the enzyme, lysine (42).

More recently, bacteria have been described that also express surface proteins, enabling them to bind directly to GPIIb-IIIa, independent of fibrinogen (Figure 1). Such is the case for SdrG from *S. epidermidis*, which in addition to binding fibrinogen, can also directly target the platelet glycoprotein (41).

*In vivo*, *S. aureus* must find a source of iron that will enable it to grow and ensure its pathogenicity. To do so, it expresses iron-regulated surface determinant (Isd) proteins that are capable of binding the heme from hemoglobin and internalizing it. Yet, it has been shown that IsdB in particular can bind to GPIIb-IIIa in the absence of plasma protein. This adhesion is inhibited in the presence of platelets that have been pre-incubated with anti-GPIIb-IIIa antibodies, and in bacterial strains mutated for IsdB, confirming the specificity of the binding (43). *S. gordonii* also expresses a platelet adherence factor that has been recently described, platelet adherence protein A (PadA), and for which no other known function has been found to date (44).

The binding site(s) involved with GPIIb-IIIa have still not been mapped; however the use of peptides mimicking the arginine-glycine-aspartic acid chain, the ligand usually described for the involvement of the glycoprotein in hemostatic conditions, prevents the direct attachment of bacteria on platelets (42). This

observation suggests that the bond may be the same type as with fibrinogen.

### Role of glycoprotein Iba

GPIb $\alpha$  is a membrane glycoprotein and is also only found in the megakaryocyte cell line. It belongs to the family of leucine-rich repeat proteins. It is capable of binding several ligands and is essential in primary hemostasis through its high affinity with von Willebrand factor (vWF). It is important to remember that GPIb $\alpha$  is found as a complex with GPIb  $\beta$ , GPIIX, and GPV at a ratio of 2:2:2:1 (34).

It has been shown that several species of *Streptococcus* are able to bind directly to GPIb $\alpha$  (Figure 2). This interaction involves a family of highly glycosylated, serine-rich bacterial proteins. This family includes serine-rich protein A (SrpA) from *S. sanguinis* (45), as well as glycosylated streptococcal protein B (GspB) and hemagglutinin salivary antigen (Hsa) from *S. gordonii* (46). These bacterial proteins, which are highly similar, bind to the sialic acids of the host's receptors. The staphylococcal accessory regulator (Sar) P protein expressed by *S. aureus* also allows adhesion to platelets (47). The fact that SrpA and GspB are molecularly very close has led to the hypothesis that the SrpA–platelet bond could involve GPIb $\alpha$ .

Bacterial proteins are capable of binding to vWF, although they are fewer than those binding to fibrinogen (Figure 2). It has been

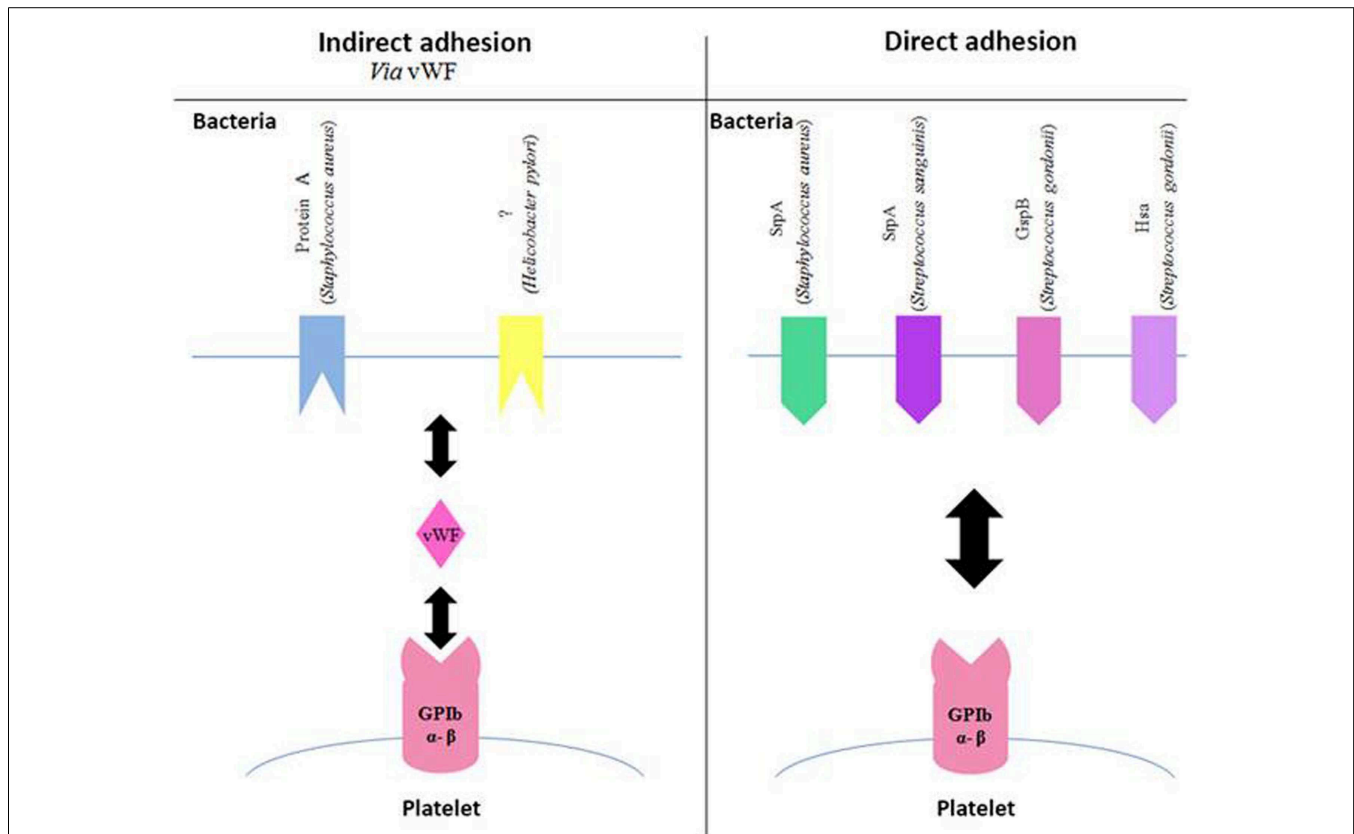
shown that protein A from *S. aureus* is capable of binding to vWF (48–50), which in turn interacts with GPIb  $\beta$ . The same applies to a surface protein of *Helicobacter pylori*, though it has still not been completely characterized. This study on the platelet–bacteria interaction involving vWF shows that if vWF is already bound to bacteria, it does not require shear forces to adhere to GPIb  $\beta$  (51).

### Complement receptors

The literature describes platelets' ability to interact with the complement system. This is mainly observed in activated platelets, thereby allowing their clearance, but also in platelets in a pathological environment (Figure 3). For example, C5b–9 is found at detectable levels on the surface of platelets in 14% of patients with coronary artery disease. From a molecular perspective, complement is capable of activating platelets by inducing the expression of pro-coagulant factors, such as prothrombinase complex, on the surface of the cells (52).

Complement proteins also interact with bacteria both through the conventional pathway and the alternative pathway (53, 54). *S. sanguinis* for example induces platelet aggregation involving complement (55). ClfA and ClfB from *S. aureus* also induce aggregation that is dependent on complement (56).

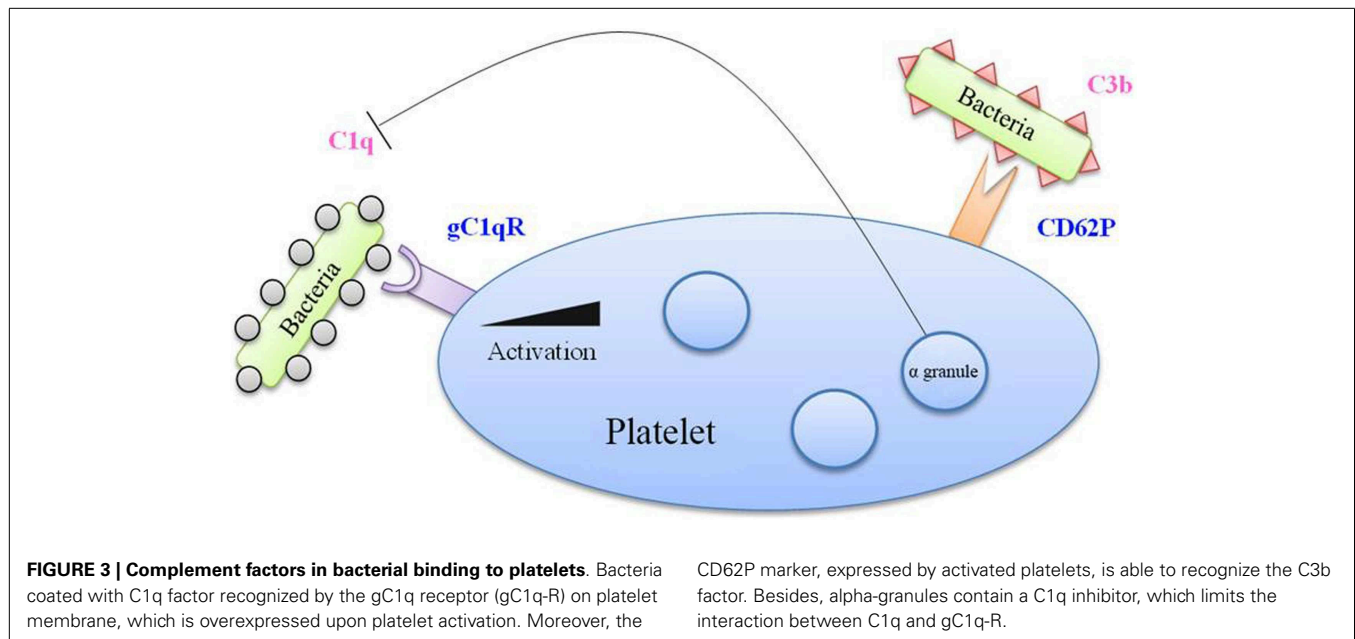
Platelets express gC1q-R, the receptor of C1q, and could thus serve as a receptor for bacteria coated with these complement factors. Following platelet activation, the expression of gC1q-R on the



**FIGURE 2 | Involvement of GPIb in the adhesion of bacteria to platelets.**

Schematic representation of different bacterial components, which bind to the platelet GPIb either indirectly via the von Willebrand Factor (vWF, left side

of the diagram) or directly (right side of the diagram). Gsp, glycosylated streptococcal protein; Srp, serine-rich protein; Hsa, sialic acid-binding hemagglutinin.



platelet surface is significantly increased (57). Platelet activation also leads to the increase of CD62P at the membrane, which has been reported to bind C3b, another complement protein (58).

This interaction of platelets with complement can be bivalent. On the one hand, platelets can assist in the destruction of bacteria by increasing the activity of complement, but since they bind complement proteins, they themselves can become the target of complement's lytic activity. This is notably what occurs in the case of thrombocytopenic purpura (52). Platelets however possess a C1 inhibitor in their  $\alpha$ -granule, which, during platelet stimulation, would enable complement activation to be modulated (59).

This concept of platelet–bacteria binding by complement molecules involves a mechanism that is more immunologic than hemostatic, which therefore highlights the dual function of platelets.

#### **Fc $\gamma$ RIIIa receptor**

The expression of this immune receptor, which recognizes the Fc domain of immunoglobulin (Ig)G, is typically described for phagocytes, such as neutrophils and monocytes. Fc $\gamma$ RIIIa enables the binding and internalization of immune complexes involving IgG, whether soluble or cellular. This mechanism is regulated by the fact that complexed IgG have a strong affinity for the receptor, while it is very weak for monomeric IgG (60).

Blood platelets also express Fc $\gamma$ RIIIa (61), and it is the only type of Fc $\gamma$  receptor that has been described on platelets to date. One of the first functions associated with platelet Fc $\gamma$ RIIIa involves its role in the pathophysiology of autoimmune disorders. In cases of heparin-induced thrombocytopenia, Reilly et al. described autoantibodies that recognized the platelet factor-4 (PF4)–heparin complexes binding to the platelet Fc $\gamma$ RIIIa. The involvement of the receptor then results in strong hemostatic activation, followed by clearance of the activated platelets (62).

Immunoglobulin G bound to bacteria are also capable of being taken by this platelet receptor. As with leukocytes, the immune

complexes that bind Fc $\gamma$ RIIIa can even be internalized by the platelets (63).

The stimulation of other platelet receptors by bacteria very often requires the simultaneous involvement of Fc $\gamma$ RIIIa in order to obtain an effective platelet response. This suggests a link between the involvement of Fc $\gamma$ RIIIa and the mechanisms of aggregation.

On average, platelets express approximately 5,000 copies of Fc $\gamma$ RIIIa (42). Considering the large number of circulating platelets, these are thus the richest reservoir of Fc $\gamma$ RIIIa, and indeed, they are therefore a significant cell in the antibacterial platelet response.

#### **BACTERIAL TOXINS**

Bacteria may also secrete toxins that are capable of activating platelets. *Porphyromonas gingivalis* secretes a family of cysteine proteases called gingipains. These toxins are capable of recognizing the platelet protease-activated receptor (PAR) 1 and cleave it in a manner similar to that of thrombin, thereby making it functional (64).

Alpha-toxin, expressed by strains of *S. aureus*, binds to the lipid bilayer membrane of platelets to form a pore, followed by a flow of calcium, similar to that induced by calcium ionophore (65). Other toxins capable of forming pores on the platelet surface have also been described. These include streptolysin O from *S. pyogenes* (66) and pneumolysin from *Streptococcus pneumoniae* (67).

*Staphylococcus aureus* and *S. pyogenes* produce a superfamily of toxins called staphylococcal superantigen-like (SSL) toxins, which have a known superantigenic effect. Of them, SSL5 interacts directly with GPIIb $\alpha$  via the sialyl lactosamine residues that terminate its glycan chain. This toxin also has a direct affinity for GPIV (42).

#### **EFFECTS OF BACTERIA ON PLATELET FUNCTION**

Most studies focusing on the adhesion of bacteria to platelets show that it is a result of aggregation. The scope of this manuscript does

not include coagulation; only the characteristics of aggregation and inflammation after bacterial contact are addressed.

### INTERNALIZATION OF BACTERIA

During systemic bacterial infection, pathogens are generally captured by phagocytes. When Clawson studied the interaction between platelets and bacteria in the 1970s, he occasionally observed the internalization of *S. aureus* in some platelets (12, 68, 69). Youssefian et al. (70) confirmed these observations, which concerned the internalization of *S. aureus* in particular. Electron microscopy photographs show the internalization of *S. aureus* in platelets, within vacuoles that are independent of the open canalicular system (OCS), suggesting active internalization.

Platelets have a better capacity for internalization when they are activated by a conventional agonist [adenosine diphosphate (ADP) or thrombin], which underscores a common mechanism between activation and internalization. Immunohistochemical labeling of vacuoles containing *S. aureus* shows the presence of CD62P and GPIIb–IIIa, but not GPIb, the phenotype that corresponds to that of an activated platelet membrane. The vacuole may therefore be formed through invagination of the plasma membrane (endocytosis) after activation.

A Japanese team confirmed the internalization of *S. aureus* in platelets but only after activation of the latter by ADP. The same study shows that *P. gingivalis* can also be internalized in platelets. There appears however to be a different mechanism of internalization at work in both bacteria. Indeed, *P. gingivalis* is capable of inducing it alone, without the addition of another platelet agonist, as the platelet aggregates are adequate for internalization of the bacteria (71).

*Staphylococcus aureus* and *P. gingivalis* share a common element with regard to internalization, as both types of bacteria are internalized in the vacuoles independent of the OCS (71). Although the final result is the same, it is thus possible that Gram-positive and Gram-negative bacteria may have different internalization mechanisms, suggesting that one of them has an additional molecule promoting its internalization. It must be emphasized that on the pictures from the study by Li et al., the presence of some *P. gingivalis* cells at the OCS may be explained by passive trapping of bacteria during platelet aggregation (71).

Platelet FcγRII may also initiate the internalization of IgG–pathogen complexes (63). One study indeed shows that platelets are capable, after involvement of FcγRII, of internalizing polystyrene beads (0.5–1.5 μm diameter) covered with IgG. This internalization is inhibited by cytochalasin D, suggesting the need to remodel platelet actin for bacteria to be internalized (72).

There remains a question as to the outcome of the internalized bacteria. White et al. reviewed arguments favoring the inability of platelets to degrade/kill bacteria. Their main argument is the absence of phagolysosomes in platelets (73). The internalization of bacteria in platelets would enable them to escape the immune system. Platelets could however use another pathway for destroying bacteria. Indeed, the endosome containing the pathogens has the ability to merge with the alpha-granules containing many bactericidal molecules (70). Finally, it is possible for *Escherichia coli* (*E. coli*) to be destroyed by platelets via internalization by FcγRII, provided that the bacteria have first been opsonized by IgG (72).

The fate of the bacteria internalized in the platelets thus remains a subject of discussion, being either a means of defending the host or an escape mechanism for the bacteria. Without progressing to internalization however, adhesion of the bacteria or bacterial products on the platelet surface is sufficient for inducing a defense response from the platelets. The main reactions are described in the following section.

### PLATELET ACTIVATION BY BACTERIA

#### Effect on aggregation

Since bacterial binding to platelets includes receptors that are also involved in hemostasis, some data show aggregation to be dependent on bacteria. Bacteria that indirectly use GPIIb–IIIa (via fibrinogen or fibronectin) bring about aggregation similar to that observed with other fibrinogen-coated surfaces (42). However, when there is direct adhesion between the bacteria and GPIIb–IIIa, a different mechanism is used, and aggregation induction is often controversial in the literature. *S. gordonii*, for example, which binds directly to GPIIb–IIIa through its PadA protein, does not induce aggregation (44), potentially due to a weaker affinity for the receptor.

Bacteria that bind to GPIb via vWF can (contrary to soluble or immobilized vWF) cause aggregation in the absence of shear force. This is the case for *S. sanguinis* and *S. gordonii*, which bind to platelets through their SrpA and GspB proteins, respectively. Deletion of these two proteins completely eliminates this aggregation (42). These bacterial components are capable of substituting for the shear forces, particularly by themselves ensuring platelet rolling.

For other bacteria, *S. pyogenes* and *S. aureus* in particular, shear forces are not necessary to induce the thrombus formation, but the observed aggregation may involve other platelet proteins. The hypothesis issued by Cox et al. is that the binding of bacteria to GPIb might bring them close to functional platelet receptors such as FcγRIIa or GPIIb–IIIa (42).

After stimulation by *S. sanguinis*, which involves GPIb, the platelets release the contents of their dense granules that contain vasoactive substances, including the adenosine nucleotides, adenosine triphosphate (ATP) and ADP. Once released, ATP is taken by the ecto-ATPases present on the surface of *S. sanguinis* and hydrolyzed to ADP. The newly formed ADP, as well as that released previously, will bind to their platelet receptor. The P2Y pathway is therefore involved (42).

The use of aspirin during the adhesion of *S. sanguinis* to platelets totally inhibits aggregation, also suggesting the role of cyclooxygenase and the production of thromboxane (Tx)A<sub>2</sub>. The platelets exposed to *S. sanguinis* produce TxA<sub>2</sub>, and the TPα receptor then amplifies platelet activation by binding the newly released TxA<sub>2</sub>.

Platelet activation induced by *S. sanguinis* may also involve the MAP kinase pathways. McNicol et al. recently showed that MAP kinases Erk2 and p38 underwent the triphasic stages of phosphorylation/dephosphorylation observed in other phosphoproteins. Aspirin has no effect on phosphorylation and dephosphorylation of Erk2 but is able to inhibit its rephosphorylation stage (74).

There is little data available concerning platelet signaling related to the adhesion of *S. aureus*. Cox et al. showed that during the

interaction of *S. aureus* with platelets, the induced aggregation is dependent on the cyclooxygenase and Tx pathways (75). This study focused mainly on the mechanisms of interaction, and therefore further details on the intracellular mechanisms were not provided.

Several studies on platelet aggregation after bacterial adhesion have also highlighted the need for Fc $\gamma$ RIIa involvement if there is to be an effective response (42). However, Fc $\gamma$ RIIa functioning could differ from that which is usually described, since, although the observed aggregation requires Fc $\gamma$ RIIa, IgG does not seem to be essential (76). The colocalization of Fc $\gamma$ RIIa with GPIIb $\alpha$  during bacterial stimulation might be the first step in signal transduction (76, 77).

One of the most accepted hypotheses concerning the alternative role of Fc $\gamma$ RIIa is based on platelet remodeling, since the GPIIb sequence that binds to Fc $\gamma$ RIIa (R542G543R544) is the same that binds to actin during platelet activation (77).

A similar type of study was conducted on Fc $\gamma$ RIIa and GPIIb–IIIa. Newman et al. showed that Src residue from GPIIb–IIIa, capable of ensuring a role of tyrosine kinase, phosphorylates the immunoreceptor tyrosine-based activation motif (ITAM) residue of Fc $\gamma$ RIIa and thus amplifies the platelet activation signals (78). Phosphorylation of the ITAM motif may take place within 30 s following contact, which shows that secondary signaling pathways can be established very quickly.

Aggregation induced by bacteria is different from that observed with the conventional ADP, ATP, and thrombin platelet agonists. It is “binary” aggregation, meaning that aggregation is not observed below a certain bacterial density, and aggregation is already at a maximum when above that density (79).

The lag time required before the appearance of aggregation is another parameter that differs according to whether the platelet stimulation is bacterial or not. This lag time is generally longer with bacterial stimulation than with hemostatic activation. Although about 10 s are needed with a hemostatic agonist, some bacteria can have a very quick lag time of 90–120 s, while others may need more than 20 min before inducing platelet aggregation. Increased bacterial density can decrease this time to a limited extent (79). Several hypotheses have been proposed to explain the lag time variations according to the bacteria: (1) the time required for platelets to bind bacteria, particularly if it done indirectly; and (2) activation of the receptor, which may not be as strong.

Bacteria-induced platelet aggregation nevertheless remains controversial, and several studies show that bacterial stimulation may not result in aggregation but a more targeted inflammatory response [chemokines release, leukocyte activation, neutrophil extracellular trap (NET) formation]. Based on current knowledge, particularly on platelet TLR, other platelet responses to bacteria, in addition to aggregation, may include the release of adapted molecules.

### **Effect on the release of immunomodulatory factors**

Platelets possess many bioactive molecules in their alpha-granules, including cytokines/chemokines, which are released during their activation and enable them to act during the immune response (80, 81). The Canadian team that partners with our laboratory and studies intraplatelet signaling after *S. sanguinis* stimulation also performed a study on the secretion of platelet cytokines. Four

strains of *S. sanguinis* and one of *S. gordonii* were used for the study.

The observation of aggregation and phosphorylation of signaling molecules such as PCg2 and Erk confirms the pro-thrombotic role of streptococci and also shows the release of RANTES, PF4, soluble (s)CD40L, and platelet-derived growth factor (PDGF-AB). CD62P is only released in the presence of one strain of *S. sanguinis* (82). Another study had also shown that platelets were capable of releasing soluble CD40L (sCD40L) and RANTES after stimulation with IgG-bead complexes, with the absence of aggregation and a very weak expression of CD62P (72).

Epinephrine, known to stimulate fibrinogen binding and aggregation (83), causes the opposite effect on the release of platelet cytokines. The mechanism of inhibition used is unknown but could be linked to the activation of type 3 nitric oxide synthase (NOS) after involvement of the  $\beta_2$  adrenoreceptors; the resultant generation of nitric oxide (NO) and cGMP has already been described in platelet activation inhibition (82). This clearly demonstrates that platelet aggregation and exocytosis of immunomodulating molecules are two independent functions.

One other platelet cytokine that is important in the antibacterial response is PF4. In heparin-induced thrombopenia syndrome (HIT syndrome), PF4 links to heparin through its positive charge, and together they form a neoantigen that is recognized by IgG. Likewise, soluble PF4 can bind to bacteria and thus form a new recognition site for IgG and the effector immune cells (84). This is particularly seen in Gram-negative bacteria, since PF4 presents an affinity for bisphosphorylated lipid A of lipopolysaccharide (LPS) bacteria. The newly formed complex is taken up the phagocytic cells. Platelet PF4 might thus facilitate the clearance of certain bacteria.

Finally, CD40L is the lead immunoregulatory molecule of platelets. It has been found that over 95% of plasma sCD40L originate from platelets (85). By comprising the main source of this molecule, platelets become an indisputable immunoregulatory factor; they participate both in the activation of the effector cells of innate immunity and that of adaptive immunity, since CD40L is involved in immunological synapses, as well as in class switching of B lymphocytes.

Following platelet activation, CD40L is first exposed to the membrane in trimeric form (the biologically most active form) and is then cleaved by proteolytic activity. Matrix metalloprotease (MMP)-9 is currently thought to be the most likely candidate. It should also be pointed out that the soluble form of platelet CD40L may also have an autocrine effect due to the presence of CD40 on the platelet surface (4, 86).

The involvement of CD40L in the full range of platelet functions is substantiated throughout this manuscript. This molecule is particularly implicated in the platelet response to bacteria, since the involvement of platelet TLR-2 (87–91) and TLR-4 (87, 88, 90–99) by means of their bacterial ligands, can specifically modulate the release of sCD40L. As with leukocytes, the following triad is thus observed: bacterial stimulation–platelet–detected release of immunoregulatory molecules. Hence, the role of platelet sCD40L in the pathophysiology of sepsis is an expanding field of study.

The platelet immunoregulatory molecules presented above are of great interest in the context of the immune system activation,

leading to bacterial elimination. The growing number of studies on the interaction of bacteria and platelets shows that platelets can also directly influence the elimination of microorganisms through the release of bactericidal molecules.

### Release of antibacterial platelet molecules

Kraemer et al. recently showed that platelets incubated with *S. aureus* limit the growth of this microorganism (100). The smallest platelet concentration for obtaining a bacteriostatic effect may even be possible to determine, though this would vary depending on the bacterial species. A 2013 study conducted on 17 volunteers was able to determine a critical platelet concentration for pathogens from the human oral cavity: *E. faecalis* (resistant or non-resistant to vancomycin), *C. albicans*, *S. agalactiae*, *S. oralis*, and *P. aeruginosa* (101). It should be noted that the growth of *P. aeruginosa* was not inhibited by platelet-rich plasma (PRP).

Despite these very recent studies, the first demonstration of the antibacterial role of platelets occurred very long ago. As Yeaman recounts in his literature review on the subject, Fodor already reported in 1887, the bactericidal effect of heated sera (102). The thermostable molecule involved was then identified and named  $\beta$ -lysine. Its platelet origin is based on the fact that it is released in coagulated plasma and is not found in the other blood cells.

Yeaman et al. were particularly interested in this platelet function and established terminology that classified these antimicrobial platelet molecules as platelet microbicidal proteins (PmP) (102), also known as thrombocidins. PmP, of which there are two (PmP1 and PmP2), are released under the induction of thrombin or bacteria, and differ from classically described defensins by their molecular mass, their sequence, and the chaining of lysine and arginine residues, which gives them a cationic charge. To become functional, these molecules must be cleaved by thrombin; the two sub-units then act in an autonomous but complementary manner by alternating the permeability of the bacterial wall (102).

The platelet signaling pathways that lead to the release of PmP depend primarily on the ATP/ADP pair and the P2 receptors. The signal is amplified through the release of ADP, and autocrine activation of the platelets is produced, which can even extend to neighboring platelets (103).

The PMP family was enlarged through the integration of kinocidins, which includes the platelet cytokines that have a direct bactericidal effect (104). They are divided into two subgroups according to the nomenclature of the cytokines. Les  $\alpha$ -kinocidins include the CXC-cytokines [PF4, platelet basic protein (PBP), connective tissue activating peptide (CTAP3), and neutrophil activating peptide (NAP2)], while the  $\beta$ -kinocidins are the CC-type (RANTES). These molecules even have a synergistic effect among themselves. For example, CTAP3 does not have an effect on the viability of *E. coli*, but the presence of PF4 potentiates its activity and thereby reduces the bacterial density by 2 logs. This result is not obtained for PF4 alone (105). Structural biochemical analyses identified the 60–74 structural domain in PF4 as being responsible for the bactericidal activity (106).

Kinocidins are integrated in the mechanisms of innate immunity, to the degree that they conserve their primary role, which is the chemoattraction of leukocytes, enabling cooperation between platelet and leukocyte factors in bacterial clearance (107, 108).

In addition, Kraemer et al. showed the presence of human  $\beta$ -defensins 1 (hBD-1) in megakaryocytes and platelets at the level messenger RNA (mRNA) and peptides (100). Platelet hBD-1 is thus released in response to the alpha-toxin of *S. aureus*; it is not released however in response to thrombin, thrombin receptor activating peptide, or platelet-activating factor (PAF), suggesting that hBD-1 release is independent of degranulation. hBD-1 may therefore not be found in the alpha-granules, especially since the authors did not observe colocalization of the markers of these granules and of hBD-1 (100).

While Kraemer et al. were unable to observe the expression of mRNA coding for hBD-2 and -3 in the platelets, other studies have demonstrated their presence through ELISA, Western Blot, and immunohistochemistry, as well as their microbicidal activity (109, 110). As a result, it appears that platelets are involved in the infectious immune response, both directly through the release of antimicrobial factors, and indirectly through the release of cytokines, enabling them to modulate the cell-mediated immune response.

### PLATELETS–BACTERIA: FOCUS ON STAPHYLOCOCCUS AUREUS INFECTION

There are many studies on the interaction of platelets with *Streptococcus*, particularly oral streptococci, while comparatively few concern *S. aureus*. In 2005, a prospective study was conducted in 39 medical centers throughout 16 countries that included 1779 patients with IE. The final analysis showed that *S. aureus* was the most common pathogen implicated, with 31.6% of cases versus 18% of *Streptococcus viridans* (111). Invasive infections by methicillin-resistant *S. aureus* generally tend to spread in health care centers and result in a high level of mortality (112). It is therefore of interest to consider the role of *S. aureus* on platelets.

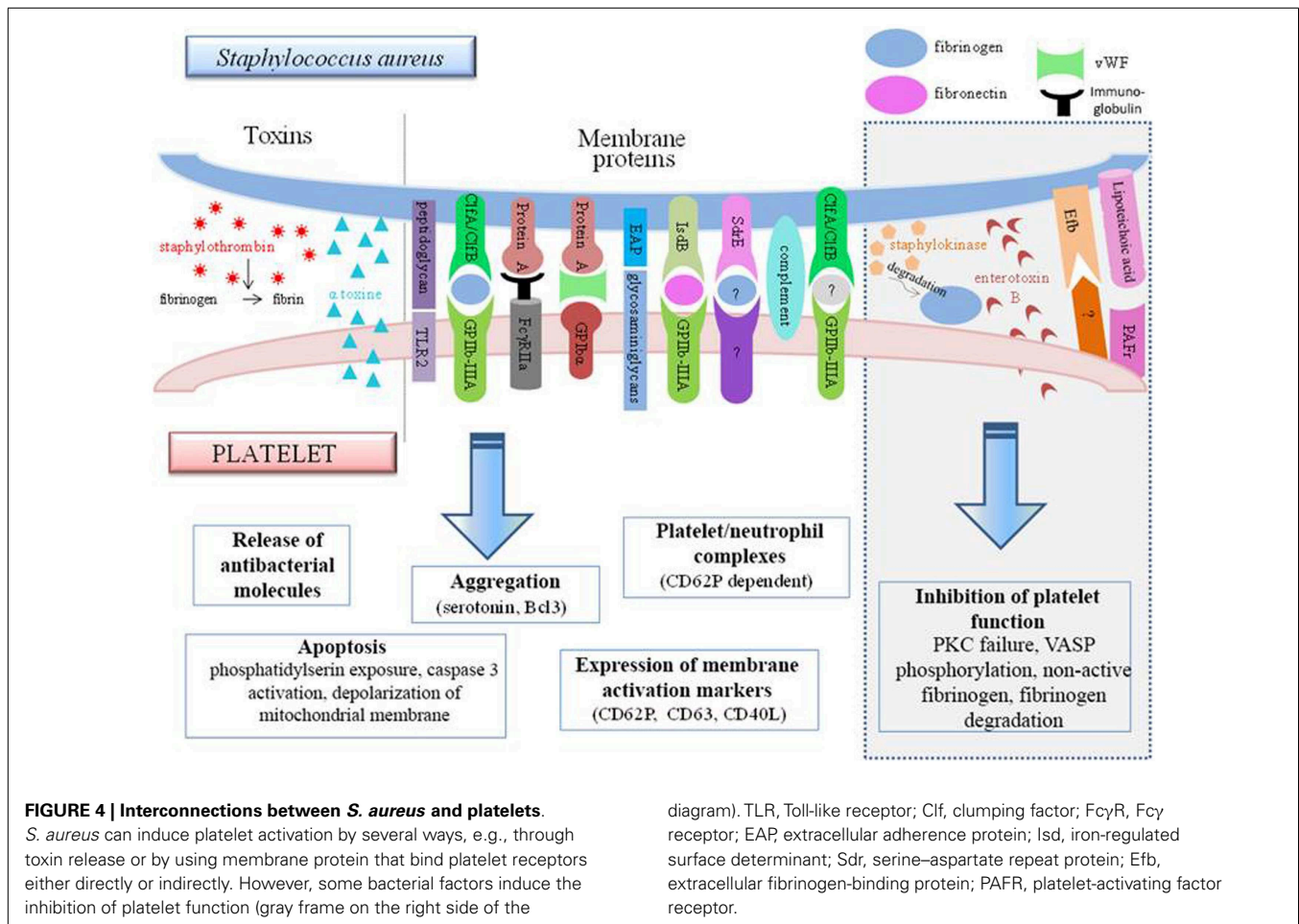
Most of the studies focus on molecules involved on both sides during the adhesion of *S. aureus* to platelets (Figure 4). We first looked at the interactions between the molecules present in the membrane of *S. aureus* and platelets.

Protein A, which is a surface protein of *S. aureus*, can be identified by anti-*S. aureus* antibodies. The immune complexes formed can attach to the Fc $\gamma$ RII of the platelets, resulting in serotonin release and platelet aggregation. This reaction is dependent on the stimulation time and the quantity of immune complexes formed. The activation was found to be optimal at 5 min, and from two bacteria per platelet (75). In a more recent study, protein A was found to be incapable of inducing aggregation by itself, but it was able to maintain it (113).

Protein A from *S. aureus* can also attach to vWF, which binds to GPIIb $\alpha$ . The use of an antibody to block vWF partially inhibits the platelet activation by *S. aureus* (79) providing evidence of the involvement of several adhesion pathways.

Clumping factor A from *S. aureus* is also involved in its attachment to platelets via fibrinogen (114). An alternative receptor exists however, since *S. aureus* adheres to platelets through the intermediary of fibrinogen/fibronectin, which does not necessarily involve GPIIb–IIIa (115). This could be shown by the persistence of aggregation, even if the two fibrinogen-binding sites on GPIIb–IIIa are blocked beforehand. It is not impossible for bacteria to transform fibrinogen so that it can be recognized by another receptor.





This might thus be a method for the bacteria to increase their pathogenicity.

Fc $\gamma$ RII and GPIIb–IIIa both have a functional role in the adhesion of *S. aureus*. The interconnection between these two receptors explains why the aggregation induced by *S. aureus* is dependent on Fc $\gamma$ RII. This hypothesis also reflects the fact that the involvement of Fc $\gamma$ RII alone is not sufficient for inducing aggregation, since its presence is meant to optimize the functionality of GPIIb–IIIa (79).

The identification of genetic mutations of *S. aureus* and the expression of candidate proteins in *L. lactis* (non-aggregative bacteria) showed that ClfB and the SdrE protein are also involved in platelet aggregation. The results also confirm the involvement of ClfA, which is moreover the first factor, before ClfB, that leads to aggregation, since it is the protein inducing the shortest lag time. Aggregation induced by both these factors can be inhibited by GPIIb–IIIa antagonists, aspirin, or prostaglandin E<sub>1</sub> (75). Aggregation is also seen with filtered platelets, suggesting a direct link between *S. aureus* and platelets, independent of fibrinogen. Different results have been observed for the SdrE protein. In order to attach to platelets, the bacterial protein requires the presence of a plasma protein other than fibrinogen, which has yet to be identified.

An important element to take into consideration is that *S. aureus* does not express the same factors depending on its stage

of growth, which could be a bias in *in vitro* studies. ClfA is the dominant pro-aggregant protein in the stationary phase of growth, whereas in the exponential phase, FnBP expression dominates (79). As a result of these indications, it becomes difficult, for example, to evaluate the results of Fitzgerald, which confirm his modeling of the interaction between FnBP and platelets on bacteria in the stationary phase (116).

The complement system is important in platelet aggregation induced by *S. aureus*. It is capable of substituting for ClfA. In this instance, the lag time will be longer (between 8 and 20 min), but simultaneous involvement of the Fc $\gamma$  receptor remains necessary (56).

The *Staphylococcus* protein, IsdB, could promote the adhesion and internalization of bacteria within platelets in the presence of fibronectin (117). In addition, this protein, unlike the IsdA and IsdH proteins, might induce platelet aggregation (117).

Studies on the interaction between platelets and *S. aureus* began in the 1970s and still continue today. The extracellular adherence protein, EAP, in the form of an oligomer, can bind directly to the glycosaminoglycans of platelets (118). This leads to the stimulation of thiol isomerase in the platelet and resultant platelet activation, ranging from stabilization of the fibrinogen binding, to the membrane expression of platelet activation molecules, such as CD62P, CD63, and CD40L.

In addition to having to integrate the alternative role of hemostasis receptors when confronted with *S. aureus*, there are also newly described platelet receptors that increase the range of functions. This particularly applies to TLR-2, which, by responding to the peptidoglycan of *S. aureus*, results in platelet activation after 30 min in association with a process of apoptosis characterized by depolarization of the mitochondrial membrane, exposure of PS to the plasma membrane, and caspase 3 activation.

In addition to the membrane components and the bacterial wall, *S. aureus* toxins can also modulate the platelet response (Figure 4). It has been shown *in vitro* that the alpha-toxin can interact with the platelet membrane and induce the production of microbicidal proteins and lysis of bacteria (102). This toxin might also be capable of generating dose-dependent platelet aggregation (119). It can also lead to the *de novo* synthesis in the platelet of B-cell lymphoma-3 (Bcl<sub>3</sub>) (119), a protein involved in the withdrawal of the platelet plug. This exotoxin is also the source of the formation of many platelet–neutrophil complexes via CD62P, an activation marker expressed on the platelet surface. Formation of the complexes increases the activation of neutrophils, which can be measured through the increase of CD11b. These aggregates could thus participate in the destruction of alveolar capillaries and be the cause of *S. aureus* hemorrhagic pneumonia (120).

*Staphylococcus aureus* releases staphylocoagulase and vWF-binding protein. Both of these molecules bind to prothrombin and form the enzymatic complex known as staphylothrombin (121). Staphylothrombin has no direct action on platelet activation, but by transforming fibrinogen to fibrin, it plays a role in stabilization of the aggregation, as well as in the initiation of secondary activation.

It has been noted, however, that several *S. aureus* molecules seem to actually inhibit the hemostatic function of platelets. In this respect, staphylococcal enterotoxin B (SEB) has been observed to cause platelet overactivation of protein kinase C (PKC). This enzyme, essential for platelet response, is therefore no longer found in physiological conditions and therefore cannot ensure its function. This explains why platelets incubated with SEB are incapable of ensuring correct aggregation in response to thrombin (122).

Lipoteichoic acid uses the PAF receptor to increase the level of cAMP within platelets. This latter then increases its phosphorylation activity on vasodilator-stimulated phosphoprotein (VASP) and inhibits aggregation and thrombus formation (123). The anti-thrombotic role of *S. aureus* can also be attributed to the extracellular fibrinogen-binding protein (Efb). *In vitro*, this protein has been described as adhering to platelets (on a non-characterized receptor or on fibrinogen). Once attached, it recruits fibrinogen but in a non-conventional form that is rather inclined to inhibit platelet activation. The inhibitory action of this molecule was confirmed *in vivo*, in which it is able to prevent thrombosis following treatment with platelet agonists (124).

Finally, staphylokinase also exerts inhibitory activity on the platelets although indirectly. This enzyme degrades plasmin and fibrinogen, thereby preventing aggregation (125). Several groups are also studying the interaction of *S. aureus* and platelets under conditions similar to those seen *in vivo*, particularly with regard to preservation of the necessary shear forces. Mice infected by *S. aureus* develop thrombi through a ClfA-dependent mechanism

(126). The use of a molecule occupying the binding site of ClfA on fibrinogen completely prevents aggregation, which demonstrates the predominance of ClfA, despite the multitude of other factors present in the microenvironment.

Dogs with *S. aureus* infection quickly develop sepsis, accompanied by platelet dysfunction (reduction in the capacity of growth in response to a PAR-4 agonist). This latter result suggests that beyond their role in IE due to *S. aureus*, platelets participate in the pathophysiology of *S. aureus*-induced sepsis (127).

In addition, a duality can be seen in the effect of *S. aureus* bacteria on platelets, indicating the complexity of the interaction. All of the studies are based on whether the bacteria have pro-aggregant or non-aggregant abilities. There is no data however on the release of platelet cytokines, which is nevertheless a very important component of bacterial infection.

## ROLE OF PLATELETS IN THE PATHOPHYSIOLOGY OF SEPSIS AND COAGULOPATHY

Data from the early 2000s show that between 30 and 50% of patients with severe sepsis have disseminated intravascular coagulation (DIC), resulting in organ hypoxia (128). During the inflammatory response, the neutrophils release tissue factors that trigger the coagulation cascade, leading to platelet activation. IL-1 and IL-6 are also strong inducers of coagulation (129, 130). This phenomenon is amplified by deregulation of the anticoagulant balance. Patients with sepsis have a strong release of PAI-1, a natural plasmin inhibitor. There is also a reduction in protein C, the active form of which is an inhibitor of coagulation factors Va and VIIIa (10, 129–133). In addition, these natural anticoagulants have their role in thrombin generation, with anti-inflammatory properties influencing nuclear factor  $\kappa$ B (NF $\kappa$ B) (134).

Reactive oxygen species (ROS) released in massive quantities during the acute phase of sepsis are also responsible for coagulopathies. In mice with induced sepsis that are knocked-out for NOS (the enzyme that produces ROS), vasoconstriction is reduced compared to wild mice. By favoring vasoconstriction, ROS participates in circulatory alteration in the blood capillaries. ROS also have a direct effect on the hemostatic activation of platelets (132).

The fourth element favoring excessive coagulation is the increase of adhesion factors. In endotoxemia models, the expression of adhesion molecules is increased in both the platelet and endothelial membranes. The adhesion of platelets to the endothelium promotes mutual activation and an accumulation of platelets, resulting in vessel occlusion (118, 123). Furthermore, in endotoxemia induced in a mouse model, it was seen that overexpression of the endothelial PAI-1 molecule in the lungs limits the *in situ* recruitment of regulatory T lymphocytes but promotes that of neutrophils (135).

Sepsis-related coagulation disorders have highlighted the role of platelets in the pathophysiology of sepsis, particularly through their hemostatic function. However, it is now clear that platelets also possess an inflammatory function that may enable them to directly participate in the amplification of inflammation associated with the early phases of sepsis. In addition to their sensitivity to thrombin, adhesion molecules, and cytokines/chemokines, the hypothesis of their direct involvement in sepsis is also supported by the TLR expression on their surface and the broad range of

inflammatory molecules that they can release during bacterial stimulation.

### SEPSIS AND INFLAMMATORY PLATELET MOLECULES

The first elements supporting the participation of platelets in sepsis-associated inflammation come from studies showing that the level of circulating sCD40L is greater in patients with sepsis than in individual controls (age- and gender-matched) but independent of the severity of the sepsis (136–141). This sCD40L released during sepsis comes from the platelets, since mice that have undergone platelet depletion do not present this increased plasma level (142). One recent study suggests that matrix metalloproteinase-9, which is also increased during sepsis, might be the source of platelet CD40L cleavage (143). This molecule has important inflammatory properties affecting many cells, both immune and non-immune, and may significantly amplify inflammation (144, 145).

In sepsis, sCD40L participates in the recruitment of neutrophils. CD40L gene-deficient C57BL/6 mice that had sepsis induced through cecal ligation puncture do not show neutrophil activation, edema formation, or neutrophil infiltration in the lungs, and they maintain their alveolar microarchitecture (142, 146).

Soluble CD40L may enable the expression of the macrophage-1 antigen (Mac-1) adhesion protein by neutrophils, promoting their recruitment at the mucosa. The mechanism of action of sCD40L may involve macrophage inflammatory protein 2 (MIP-2) and its receptor, CXCR2. This is supported by the fact that *in vitro* recombinant sCD40L does not increase the expression of Mac-1 on the neutrophils in culture (142).

Platelet-activating factor is a molecule synthesized by various cells and that possesses a cytokine function. Its receptor, platelet-activating factor receptor (PAFR), is attached to G proteins and is expressed by the cells that participate in immune defense and coagulation, including platelets. Platelet PAFR activation results in the release of inflammatory factors, degranulation, and the initiation of coagulation cascades. In physiological conditions, signaling associated with PAFR is finely regulated in order to avoid an excessive thrombo-inflammatory response. During sepsis however, this regulatory balance is disrupted, and PAF is then involved in the activation of neutrophils, monocytes, platelets, and in the formation of leukocyte–endothelium, leukocyte–platelet, and platelet–endothelium complexes (147).

A recent study suggested an association between the duration of storage of apheresis platelets before transfusion and the occurrence of complications in patients from a trauma center. This study was conducted on 381 patients who had been admitted to a trauma center and received apheresis platelet concentrates that had been stored for 3 days or less, 4 days, or 5 days. The results show that the transfusion of platelets stored for over 3 days may increase the risk of complications for the patient, sepsis in particular (148). It was shown that during the storage of platelet concentrates, the platelets are activated, and the platelet inflammatory factors, including sCD40L, are released and then accumulate (149–152). This suggests that the inflammatory molecules that accumulate during the storage of platelet concentrates could promote the onset of sepsis.

It has also recently been shown that IL-27 could be a predictive molecule of sepsis in children (153), and that the activated platelets could be a significant source of this cytokine (154). Thrombin formed during sepsis could lead to this release of platelet IL-27.

In addition, an increase in the level of circulating microparticles has been reported in septic patients. These vesicles (granulocytes, monocytes, endothelial cells, and platelets) may arise from several cell types (22). In sepsis, the release of PMP is accompanied by an increase of CD62P at the platelet membrane and an increase of platelet–monocyte aggregates (31). The role of microparticles in sepsis requires further exploration. It has been described however that they possess strong pro-coagulant pathogenicity through the expression of tissue factors. Some studies conducted in other contexts have shown that PMP are rich in CD40L (155) and IL-1 $\beta$  (18, 30), which are two pro-inflammatory molecules that are strongly associated with the pathophysiology of sepsis. It therefore becomes important to consider PMP in the development of inflammation during sepsis.

The role of platelets in the inflammatory phase of sepsis may not be limited to the production of inflammatory molecules. Indeed, in patients with uncomplicated sepsis, the level of circulating platelet–leukocyte complexes is higher than in controls. However, in sepsis complicated by organ failure, the number of platelet–leukocyte complexes is decreased. This can be explained by sequestration of the complexes in the damaged organs, for example the lungs (156). The same type of observation occurred *in vitro*, in which strains of *S. aureus* isolated from bacteremia result in aggregation, the formation of platelet–neutrophil complexes, and activation of these neutrophils (157).

Moreover, since platelets are capable of binding to bacteria, and even keeping them alive intracellularly, they could promote their dissemination within the body. This mechanism was proposed in a mouse model infected by *S. pyogenes* via the intraperitoneal route. Platelet-depleted animals are unable to ensure the transport of bacteria in the blood, lungs, and spleen, which is characterized by a reduction in the bacterial load (through a CFU count) in these organs after sacrifice of the animals (158).

### PLATELET APOPTOSIS IN THE MICROENVIRONMENT OF SEPSIS

The involvement of platelets in sepsis is also characterized by persistent thrombocytopenia in patients (159, 160). There have been various studies done that consider thrombocytopenia a predictive factor of the mortality rate of patients admitted to intensive care (160, 161). Several hypotheses have been made with regard to the decrease in circulating platelets and are presented below.

During sepsis, platelets express activation factors that promote their sequestration in the spleen and then their destruction (162). Platelet depletion can be accelerated if the platelet–bacteria contact involves the complement system (52) or Fc- $\gamma$  receptor (163). Furthermore, sepsis is a pathological state that might promote hemophagocytosis of platelets by macrophages which is partly dependent on macrophage colony-stimulating factor (164).

The failure of thrombopoiesis is an unlikely hypothesis to the degree that the plasma levels of IL-6, tumor necrosis factor (TNF)- $\alpha$ , and thrombopoietin are increased in sepsis (129, 130, 165). On the contrary, the involvement of megakaryocyte TLRs might rather promote an overproduction of platelets.

Another hypothesis points at sepsis-induced coagulopathy leading to DIC, in which disseminated thrombi may immobilize the platelets (166). The results of Tylml et al. confirmed this in a mouse model of sepsis (132).

More recently, the scientific community has been interested in platelet apoptosis. This mechanism of cellular death involves an important step of nuclear transformation involving chromatin condensation, followed by DNA fragmentation (167–169).

Apoptosis, which is known as “programed cell death,” can normally be triggered by two types of stimuli. These are referred to as the intrinsic pathway and the extrinsic pathway.

The extrinsic pathway involves cell death receptors, such as the apoptosis stimulating fragment (Fas) receptor, the ligands of which are the proteins from the TNF family. The involvement of Fas causes trimerization of the receptor, which then becomes active, enabling it to recruit an adaptor molecule, Fas-associated protein with death domain (FADD), which also contains a binding domain for procaspase-8. The formation of this complex leads to cleaving of caspase-8, which is then produced in its active dimeric form. Caspase-8 will then either activate the sequential cascade of the different caspases, or the previously described mitochondrial pathway.

The intrinsic pathway is triggered following cellular stress, such as oxidative stress. Under physiological conditions, Bcl<sub>2</sub> protein and other similar proteins maintain the integrity of the mitochondrial membrane. Under stress conditions, these proteins are degraded. Transition pores then form on the mitochondrial surface, causing them to swell and then burst. Cytochrome C is then released in the cytoplasm and can activate the caspase cascade (170).

In both cases, the pathways converge to activate caspase-3, which has many substrates. The effects of caspase-3 are seen both on the nuclear proteins involved in DNA repair and on cytoplasm proteins such as gelsolin, which is a cytoskeletal regulator. Cleaving of the molecules involved in cellular structure and repair causes DNA and cytoskeletal fragmentation, but the plasma membrane remains intact. During the formation of apoptotic bodies, a change in the distribution of phospholipids is produced, termed “membrane flip flop,” which exposes the PS on the membrane surface. The PS constitute an “eat me” signal for the phagocytic cells, which enables the elimination of the apoptotic bodies (170).

Platelets have also been found to possess apoptotic machinery. The platelets express caspases 1, 3, 4, and 9. Caspases 2, 6, and 8A have a more reduced expression, and no expression for caspases 5, 7, and 10 has been demonstrated. Fas are not expressed in the platelets, but they have other cell death receptors, such as DR3, DR5, TNF-receptor p55, and RIP. Platelets also express proteins from the Bcl-2 family: Bcl-X, Bfl1, Bad, Bak, Bax, and Mcl1 (171).

Activation of these apoptotic molecules has been demonstrated during storage of platelet concentrates. After 5 days of storage in standard blood bank conditions, platelet viability decreases. This platelet death is accompanied by an increase in PS at the cell surface and an increase in caspase-3 activity. Caspase activation occurs in a specific manner, since the use of an inhibitor (z-VAD-fmt) stops this process. Platelet death is thus well associated with a specific apoptosis (171).

This first study showed the independence of apoptosis and platelet activation, since PS exposure was independent of the increase in the expression of the CD63 activation marker, and thrombin activation had no effect on caspase-3 activity. The scientific community however is not in unanimous agreement. It has even been shown that thrombin stimulation induced depolarization of the mitochondrial membrane, the expression of pro-apoptotic molecules (Bax, Bak), activation of caspase-3, and exposure of PS to the membrane (172). The action of thrombin may not be direct and may require the release of platelet factors that can induce apoptosis. During storage, platelets release many soluble factors, including TNF- $\alpha$ , which is a strong inducer of apoptosis through the induction of caspases.

The life span of platelets could therefore be controlled through apoptosis. Caspase-9, for example, is required for platelet and megakaryocyte death but is not involved in their production or their functionality (159).

The Bcl-X family is also at the center of platelet survival. These molecules may even determine their life span. Two missense mutations of Bcl-X are capable of accelerating platelet death resulting in severe thrombocytopenia (173).

It has also been shown that a strain of isolated *E. coli* in a patient with sepsis was able to induce apoptotic manifestations of platelets, such as actin condensation, a decrease of the mitochondrial potential, and degradation of Bcl-X. The use of mutant and non-pathogenic strains showed that only pathogenic strains that release toxins forming pores have the ability to degrade Bcl-X. This applies to the alpha-toxin of *E. coli* and *S. aureus*. These toxins probably act on calpain, since this protein is the source of Bcl-X degradation. However, proteasome inhibition is ineffective in preventing degradation of the pro-apoptotic protein (174). This study is the first demonstration that pathogenic bacteria can influence the intrinsic initiation of platelet apoptosis. The degradation of Bcl-X suggests a new mechanism by which bacteria may be able to cause the thrombocytopenia observed in patients with bacteremia. This mechanism could then explain why depolarization of the mitochondrial membrane potential ( $\Delta\Psi_m$ ) is increased in the platelets of patients with SIRS (175). A decrease in  $\Delta\Psi_m$  could even be associated with SRIS progression.

#### INVOLVEMENT OF PLATELETS IN SEPSIS-RELATED NETose

Another phenomenon, the formation of NETs involving platelets, neutrophils, and bacteria, has been demonstrated and could play an important role in sepsis. It was first described in 2004. Neutrophils that have been activated, particularly by IL-8, release their granular (peptides and enzymes) and nuclear (chromatin and histones) components in the microvessels where they combine to form a network called NET (176). The use of high-resolution electron microscopy has confirmed the structure of NET, which is characterized by extracellular chromatin stretches that are associated with globular proteins. It has also been shown that a bacterial environment, or that mimicked through the injection of LPS, results in the *in vivo* release of NET that are able to trap bacteria and thus reduce their spread during sepsis (177).

It was noted that bacterial trapping at NET is greater under flow conditions, as in the blood circulation (94). NET can reach a

diameter of 25 nm, and once combined, they form a structure that can be over 100 nm both in diameter as well as length (94).

Aligned with DNA, NET contain the histones H1, H2A, H2B, and H4, as well as granular proteins such as elastase, myeloperoxidase, and bactericidal permeability increasing protein, which enable bacterial degradation (176). NET are able to stop both Gram-positive and Gram-negative bacteria. The histones and BPI have a proteolytic action on the alpha-toxin of *S. aureus*, as well as on the IpaB of *S. flexneri* (177). *C. albicans*, although not a bacteria, is also sensitive to NET, but its destruction may only depend on granular proteins and not histones (177).

Neutrophil extracellular trap release occurs within 5–10 min after stimulation of the neutrophils. This time period is too short for the implementation of an apoptosis or necrosis mechanism. It is therefore an active mechanism and not the consequence of disintegration of the neutrophilic plasma membrane (176).

From a molecular viewpoint, the formation of NET involves (178, 179):

- peptidylarginine deiminase type 4, for chromatin decondensation;
- ROS formation, which is NADPH oxidase-dependent for disintegration of the nuclear membrane;
- actin cytoskeleton and microtubules for NET release.

Neutrophil extracellular trap can be released according to three mechanisms (180): (1) a rapid mechanism (30–60 min) involving vesicles; in this case, the neutrophils remain viable; (2) a slower mechanism (3–4 h), resulting in rupture of the neutrophilic plasma membrane; or (3) directly from the mitochondria. At present, the third mechanism and the existence of NET composed from mitochondrial DNA remains controversial, although one study shows that NET might be majorly composed of mitochondrial DNA rather than nuclear DNA (181).

Lipopolysaccharide, which has traditionally been described as a neutrophil activator, is surprisingly incapable of inducing *in vitro* NET release by neutrophils. In contrast, in 2007, Clark et al. showed in a mouse model that the intravenous injection of LPS leads to NET formation within the first 5 min (94). A more detailed investigation showed that LPS-induced NET formation is not direct and requires platelet participation. Indeed, platelet stimulation by LPS in the presence of neutrophils may not cause a standard platelet response but might promote their adhesion; the neutrophils would then be activated and form NET (94).

Kraemer et al. then showed that type 1 beta-defensins released by platelets after bacterial stimulation were responsible for NET formation (100).

Kubes presents platelets as a barometer that can detect a substantial level of bacteria. The platelets are activated by an LPS concentration 100 times greater than that inducing neutrophil activation. The platelets may therefore come to the assistance of the neutrophils by enabling them to form NET when the bacterial load is too high and their normal functions are insufficient for correctly eliminating the bacteria (177). NET could be the innate “last chance” defense.

Neutrophil extracellular traps have been shown to have a positive impact on the destruction of pathogens during bacteremia.

Conversely, they may alter the microvascular circulation by promoting the formation of microthrombi, thereby also preventing the immune cells from reaching the bacteria. In addition, their components may have a toxic effect on the host cells. This was confirmed *in vitro* on human umbilical vein endothelial cell (HUVEC) (94). Hepatotoxicity has been observed *in vivo* following the release of NET, which was measured by the release of alanine aminotransferase and an occlusion of the liver sinusoids (94).

The histones released in the extracellular medium possess prothrombotic activity and are capable of activating the platelets via their TLR-2 and -4 (90). This phenomenon may be extrapolated to the NET histones. In this case, NET would be responsible for platelet overactivation, which may lead to thrombi formation. The potential detrimental effect of NET is another illustration of the alteration of platelet function during sepsis.

## CONCLUSION

The implication of platelets in the inflammatory response marks a veritable turning point in the understanding of platelet physiology and opens new fields of investigation that have been up to now somewhat neglected. The identification of platelets in the various inflammatory mechanisms places them at the center of innate immunity, whether in the recognition of pathogens, signal transduction, or the release of cytokines/chemokines. This functional similarity with leukocytes shows that both of these cell types are not so different.

Anucleated platelets are only found in mammals. In the lower vertebrates, such as birds, reptiles, amphibians, or fish, hemostatic function is ensured by nucleated thrombocytes. With regard to invertebrate species, they do not possess platelets *per se*, but the hemolymph contains a type of nucleated cell called a hemocyte that expresses TLR, which is capable of phagocytizing the foreign body or secreting antimicrobial proteins. It is this same cell however that regulates coagulation and healing. By compiling all of these characteristics, it is possible to discuss a possible common evolution between platelets and leukocytes, followed by a dissociation, proportional to the evolution of the species (1, 3, 4, 182), despite this is debated.

All of the studies presented in this thesis generally show that platelets are able to cover the majority of the steps of inflammation and thus confirm their total involvement in the orchestration of this pathophysiological state. By modulating both the acute effector phase of inflammation and the maintenance of this process, platelets may become a therapeutic target.

## AUTHOR CONTRIBUTIONS

All authors contributed substantially to the conception of this review. HC, PD, and AC drafted it while BP, FC, and OG revised it critically for important intellectual content. All authors approved the final version and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

## ACKNOWLEDGMENTS

Authors would like to thank Mr. Charles Antoine Arthaud, Mrs. Marie Ange Eyraud, and Jocelyne Fagand for their contribution to the team's work cited in the review. They also acknowledge

the staff of Etablissement Français du Sang Auvergne-Loire, Saint-Etienne, France and the healthy volunteer blood donors for their participation. The studies of the team that are cited were supported by grants from the French National Blood Service – EFS (Grant APR), France; the Association for Research in Transfusion (ART), Paris, France; the Agence Nationale de la Sécurité et du Médicament et des produits de santé (ANSM – AAP-2012-011, Reference 2012S055); the “Agence Nationale de la Recherche” (ANR), reference ANR-12-JSV1-0012-01; and the Association “Les Amis de Rémi,” Savignieux, France. The authors declare they have no conflict of interest.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 November 2014; accepted: 11 February 2015; published online: 26 February 2015.

Citation: Hamzeh-Cognasse H, Damien P, Chabert A, Pozzetto B, Cognasse F and Garraud O (2015) Platelets and infections – complex interactions with bacteria. *Front. Immunol.* 6:82. doi: 10.3389/fimmu.2015.00082

This article was submitted to *Inflammation*, a section of the journal *Frontiers in Immunology*.

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# Contextualisation de ma thèse dans notre équipe de recherche

La place des plaquettes en tant que cellule de l'inflammation a toujours été une question centrale de notre équipe de recherche. Cette hypothèse a fait naître deux thématiques : d'une part la capacité inflammatoire des plaquettes dans les produits sanguins labiles et son lien avec les effets indésirables receveurs (EIR); et d'autre part la relation étroite entre les pathogènes et les plaquettes ainsi que les mécanismes inflammatoires qui en découlent. C'est dans ce deuxième axe que ma thèse s'inscrit.

Nous avons ainsi décrit la capacité de certains virus à se lier et réagir aux plaquettes <sup>160</sup>. Les plaquettes peuvent reconnaître les protéines d'enveloppe GP120/GP41 du virus de l'immunodéficience humaine (HIV) <sup>161</sup>. D'ailleurs, dans les cas des patients séropositifs (HIV+), une trithérapie induit la modulation de la réponse inflammatoire des plaquettes <sup>162</sup>.

Même si de nombreux récepteurs hémostatiques permettent la reconnaissance de pathogènes, l'évidence des récepteurs de types TLRs au niveau plaquettaire a renforcé le caractère immunitaire de cette cellule. En effet, notre équipe a pu mettre en évidence l'expression des TLR2, 4 et 9 <sup>163</sup> et, dans cette continuité, la reconnaissance fonctionnelle du lipopolysaccharide (LPS) bactérien par le TLR4 plaquettaire, induisant la libération de nombreuses cytokines comme le Tumor Growth Factor (TGF- $\beta$ ), l'interleukine 8, le PF4, RANTES, le CD62P soluble ou encore le CD40L soluble <sup>164,165</sup>. Plus récemment, plusieurs membres de notre équipe se sont intéressés aux voies de signalisation utilisées par les plaquettes lors de la reconnaissance d'un stimulus d'origine bactérienne. Nous avons ainsi montré que les plaquettes ont la capacité de différencier l'isoforme du LPS en activant des voies signalétiques différentes <sup>166</sup>. De plus, il apparaît que le CD14 soluble est un véritable co-facteur de la reconnaissance du LPS au niveau du TLR4 plaquettaire <sup>167</sup>. Nous avons également observé que la stimulation des récepteurs TLR2 et PAR-1 plaquettaires entraîne l'activation de la voie NF- $\kappa$ B afin d'induire une sécrétion de molécules inflammatoires <sup>168</sup>. Enfin, le récepteur de type SIGLEC-7, naturellement présent à la surface des cellules « Natural Killer », est exprimé par les plaquettes et permet de réguler l'apoptose plaquettaire <sup>169</sup>. Ce récepteur a un rôle dans l'immunité anti-infectieuse jouée par les plaquettes et pourrait être à l'origine, via ce phénomène apoptotique, des thrombopénies fréquemment observées lors d'une infection. Par ailleurs, nous avons également observé qu'une bactérie à gram positif, *Streptococcus sanguinis*, présente dans la cavité orale et responsable d'endocardite infectieuse, est capable d'induire une réponse pro-inflammatoire plaquettaire avec la libération de l'enzyme matrix

metalloprotéinase 1 (MMP1) <sup>170,171</sup>. L'utilisation d'inhibiteur de la glycoprotéine GPIIbIIIa, de la GPIb $\alpha$  et des voies de la cyclo-oxygénase et de la phosphatidylinositol 3-kinase inhibe complètement cette réponse, ce qui met en relief la complexité de la signalisation intracellulaire en réponse à *Streptococcus sanguinis* <sup>170</sup>. Il est bien connu que le sCD40L est un facteur immuno-modulateur intervenant dans de nombreux processus inflammatoires et principalement libéré par les plaquettes <sup>172,173</sup> ; de plus il est responsable de nombreuses répercussions transfusionnelles <sup>174</sup>. Notre équipe s'est intéressée aux relations des plaquettes avec d'autres éléments circulants, principalement leucocytaire, acteurs majeurs de l'immunité. Le contact direct des plaquettes ou de leurs produits de sécrétion entraîne un effet direct de la maturation des cellules dendritiques ou permet la libération d'immunoglobuline par les lymphocytes B après activation <sup>175,176</sup>. Les cellules mononuclées du sang périphérique sécrètent de l'interleukine 8, lorsqu'elles sont exposées à des plaquettes préalablement stimulées par du LPS <sup>166</sup>.

Durant la pathogénèse du sepsis, les plaquettes font le lien entre l'immunité innée et adaptative. Notre équipe a mis en évidence la contribution des plaquettes dans la hausse des taux plasmatiques d'interleukine 27, cytokine pouvant servir de biomarqueur du diagnostic des patients atteints d'infections bactériennes <sup>177</sup>.

Durant ce travail de thèse, j'ai eu pour objectif d'approfondir nos connaissances sur les interactions plaquettes/bactéries et de mieux caractériser la composante inflammatoire consécutive à une infection à *S. aureus*, germe communément retrouvé dans les cas de sepsis.

# Problématique et objectifs de thèse

Actuellement, le sepsis est un sérieux problème de santé publique avec une prévalence croissante malgré l'amélioration de la rapidité du diagnostic et du management des patients. Cette pathologie est paradoxale puisqu'un mécanisme physiologique tel que le système immunitaire va entraîner, dans un contexte infectieux, une exacerbation de l'inflammation qui va être à l'origine de nombreuses complications et d'une mortalité importante. Les processus inflammatoires sont liés à de multiples mécanismes physiologiques et en particulier la coagulation. En effet, il est connu que l'inflammation et la coagulation sont interdépendantes et conduisent à la physiopathologie du sepsis. Depuis quelques années, les plaquettes se révèlent être un élément clé de l'inflammation. Elles jouent un rôle dans de divers mécanismes inflammatoires et sont à l'interface de la coagulation et de l'inflammation. A ce jour, malgré le nombre important et grandissant d'études témoignant du rôle inflammatoire des plaquettes, il apparaît important de mieux comprendre les relations entre les plaquettes et les bactéries afin de pouvoir mieux appréhender l'implication des plaquettes dans la pathogénèse du sepsis. Etant donné de la complexité des mécanismes inflammatoires mis en place par les plaquettes ainsi que de leurs nombreuses interactions avec d'autres types cellulaires, une meilleure compréhension permettra la mise en place de cibles thérapeutiques visant à limiter les problèmes inflammatoires et hémostatiques d'origine plaquettaire.

Au vu de l'implication des plaquettes dans divers mécanismes inflammatoires, il aurait été raisonnable de penser qu'une diminution du taux circulant plaquettaire réduirait l'inflammation et améliorerait l'état général du patient. Or, il convient de considérer que le rôle primaire des plaquettes est l'hémostase. En effet, une réduction du compte plaquettaire entraîne des problèmes hémostatiques et thrombotiques, très souvent fatals pour le patient. Plusieurs études montrent une corrélation entre thrombopénie et taux de mortalité confirmant que le maintien de la concentration circulantes en plaquettes est une constante physiologique primordiale pour l'état d'un patient. L'hypothèse de ce travail de thèse est, que dans un contexte d'infection bactérienne systémique, cibler les plaquettes d'un point de vue thérapeutique permettrait de limiter l'action des pathogènes sur divers paramètres plaquettaires et de réduire leur « pouvoir » inflammatoire, dans l'espoir, à terme, de diminuer la mortalité associée au sepsis.

Ainsi, ce projet a pour but d'évaluer le rôle inflammatoire des plaquettes lors du sepsis sévère mais également d'évaluer, si l'utilisation de molécules antiplaquettaires

permet de limiter l'inflammation induite par les plaquettes. Ce travail de thèse s'est effectué selon différentes approches *in vitro* et *in vivo* dans un modèle murin qui, par leur complémentarité, permettent de mieux comprendre les mécanismes d'action des plaquettes en réponse à un stimulus bactérien.

Dans un premier temps, il paraissait important d'estimer si les plaquettes avaient la capacité de détecter et de répondre à des facteurs de virulence des bactéries que sont les toxines. Ainsi, j'ai analysé la réponse inflammatoire *in vitro* des plaquettes à quatre exotoxines de *S. aureus*, l'un des germes les plus communément retrouvés dans les cas de sepsis, à savoir la toxine du syndrome de choc septique (TSST-1), le leucocidine de Panton-Valentine (PVL) et les hémolysines  $\alpha$  (Hl $\alpha$ ) et  $\gamma$  (Hl $\gamma$ ). Dans cette première étude, il était important de mettre en évidence les récepteurs potentiellement impliqués dans la réponse cytokinique inflammatoire plaquettaire suite à l'exposition à ces toxines staphylococciques.

Dans un deuxième temps, j'ai étudié les effets cytotoxiques, pro-agrégants et inflammatoires *in vitro* sur les plaquettes de différentes souches vivantes de *S. aureus*, de références ou cliniques issues de bactériémies. Par ailleurs, j'ai voulu observer si des molécules telles que l'acide acétylsalicylique (aspirine) ou encore la fluvastatine modulent les effets des souches de *S. aureus* sur les plaquettes.

Enfin, la troisième approche expérimentale, j'ai mis en place un modèle sepsis murin selon la procédure chirurgicale de CLP. Grâce à ce modèle, j'ai étudié la participation inflammatoire des plaquettes au sepsis. En effet, il était important de caractériser les mécanismes inflammatoires en lien avec les plaquettes, tels que leur participation dans la transmigration des neutrophiles au niveau pulmonaire et dans l'induction de NETs. La compréhension de ces processus inflammatoires *in vivo* est primordiale pour déterminer si l'action de molécules antiplaquettaire telle que l'acide acétylsalicylique permet de réduire l'inflammation systémique impliquée dans la pathogénèse du sepsis.

Dans la perspective de cibler la réponse inflammatoire plaquettaire dans un contexte infectieux, ces trois études combinées devraient permettre d'approfondir nos connaissances quant à la participation des plaquettes à l'inflammation associée à l'infection bactérienne et d'évaluer si une molécule anti-plaquettaire peut la moduler (Figure 5).



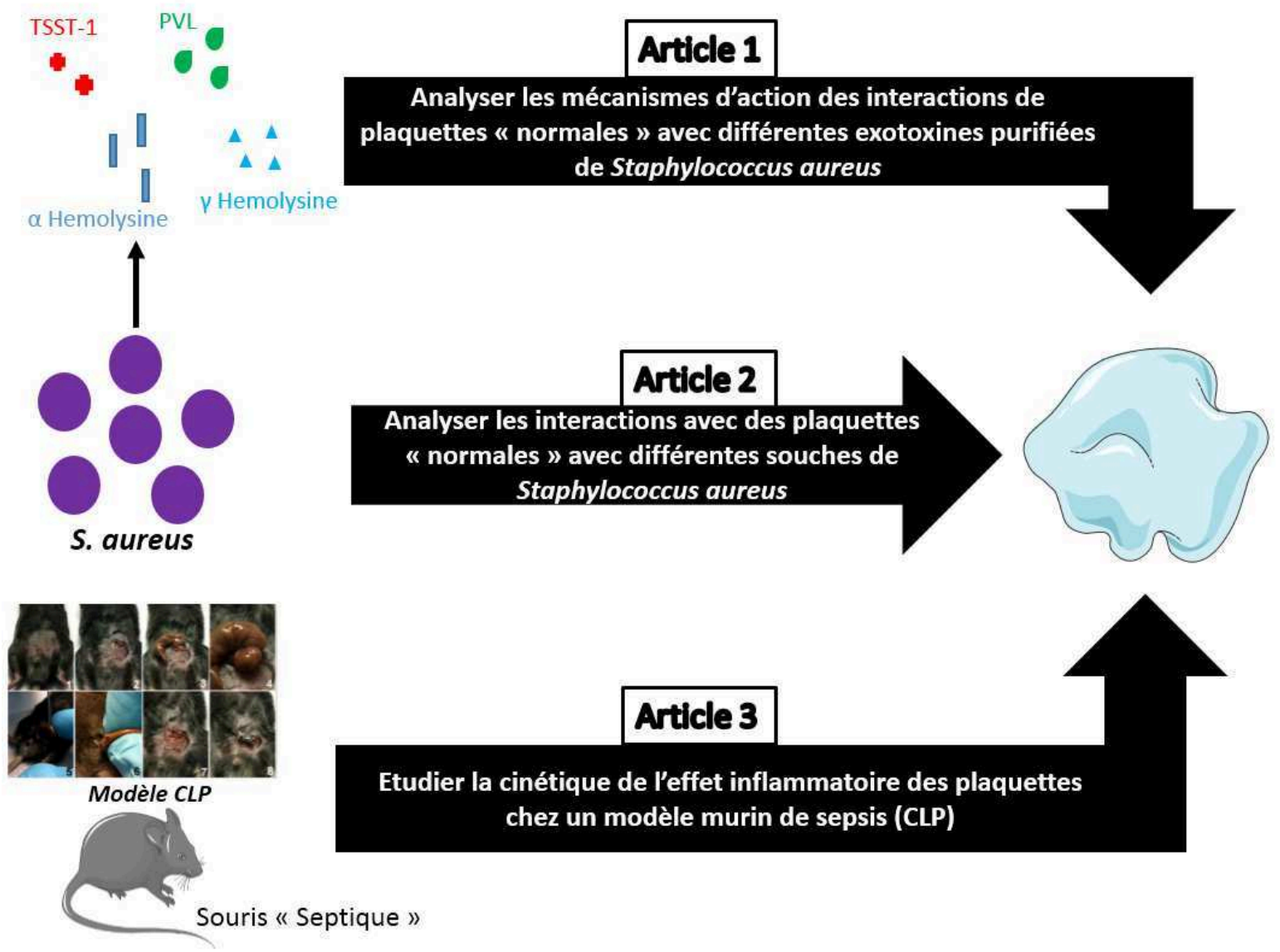


Figure 4 : Représentation des objectifs de thèse

# Résultats

## **Article I**

**Human platelets differentially sense various  
*Staphylococcus aureus* exotoxins and adapt their  
subsequent immune-modulatory molecule  
secretion profiles**

**(Article in progress)**

## Introduction de l'article

*S. aureus* est une souche bactérienne commensale à l'origine d'environ 30% des cas de sepsis au niveau mondial (dont 10% de *S. aureus* résistant à la méticilline - SARM) <sup>5,13</sup>. *S. aureus* est un germe majoritairement retrouvé dans le domaine hospitalier, puisque c'est l'une des principales bactéries responsables de maladies nosocomiales ou d'intoxications alimentaires entraînant une durée prolongée des patients ou encore l'utilisation d'une antibiothérapie. Sa pathogénicité est principalement due à sa grande diversité de facteurs de virulences. En effet, *S. aureus* possède un arsenal de facteurs de virulences permettant l'adhésion aux tissus, l'évasion du système immunitaire et la cytotoxicité vis à vis des cellules de l'hôte. Lors de bactériémies à *S. aureus*, ces facteurs sécrétés par la bactérie vont entraîner la dérégulation de processus physiologiques (coagulation, système immunitaire, intégrité vasculaire) et ainsi contribuer à la pathogénèse du sepsis.

En effet, *S. aureus* contient de multiples facteurs d'adhérences associés à sa paroi permettant son attachement aux protéines de la matrice cellulaire, aux protéines filamenteuses comme la fibrine ou encore aux plaquettes <sup>178</sup>. Les « clumping factors A et B » (Clf A/Clf B) et les « Fibrinonectin-binding protein A et B » (FnBPA/FnBPB) peuvent se lier au fibrinogène et à la fibronectine, facilitant ainsi la colonisation des vaisseaux <sup>179</sup>. *S. aureus* utilise également des éléments de sa paroi tels que l'acide teichoïque pour se lier au facteur de Willebrand (vWF) afin d'adhérer à l'endothélium <sup>180</sup>. Les lipoprotéines de la paroi du *S. aureus* sont capables de se fixer au TLR 2 et d'induire une réponse TNF- $\alpha$  et IL-6 par les monocytes/macrophages. Des composants tels que le peptidoglycane ou l'acide lipoteichoïque induisent la libération de cytokines pro-inflammatoires telles que l'interleukine 1 (IL-1) ou l'interferon- $\gamma$  (IFN- $\gamma$ ) <sup>181</sup>. *S. aureus* utilise des facteurs de virulence pour limiter la réponse immunitaire de l'hôte. Les protéines « Chemotaxis Inhibitory Protein of Staphylococci » (CHIPS) et « Staphylococcal Complement INhibitory » (SCIN) diminuent l'activation du complément et la chimioattraction des leucocytes. Etant synthétisés durant la phase de croissance de *S. aureus*, ces deux facteurs favorisent l'évasion du système immunitaire <sup>182</sup>. Les facteurs de virulence de *S. aureus* sont aussi à l'origine de la dérégulation hémostatique. Des enzymes telles que la staphycoagulase, la « von Willebrand factor-binding protein » (vWbp) se lient à la prothrombine et permettent la formation de fibrine <sup>183</sup>. D'autres enzymes comme les nucléases, protéases, lipases ou

collagénase, en détruisant les cellules de l'hôte, produisent une source de nutriments essentielle à la prolifération bactérienne.

Enfin, les exotoxines excrétées par *S. aureus* participent également à la virulence de la bactérie. Il est très clairement connu que les souches de *S. aureus* les plus virulentes sont les souches ayant une production accrue d'exotoxines<sup>184</sup>. De plus, il a été constaté que le taux d'anticorps dirigés contre les exotoxines est inversement corrélé au risque de développer un sepsis<sup>184,185</sup>. Cela montre toute l'importance des toxines comme facteurs de virulence sur la pathogénèse que peuvent induire certaines souches de *S. aureus*.

Plusieurs types d'exotoxines sont synthétisés par *S. aureus*<sup>186</sup>:

- « Pyrogenic toxin superantigen » (PTSAgs) : cette classe d'exotoxine regroupe plusieurs toxines produites par *S. aureus* comme la « Toxic shock syndrome toxin-1 » (TSST-1) ou encore les entérotoxines staphylococciques. Ces toxines sont regroupées sous la même classe pour leur effet pyrogénique et superantigénique. TSST-1 a la capacité unique de pouvoir traverser les muqueuses. Les superantigènes synthétisés de manière intracellulaire et sécrétés par *S. aureus* ont une action cytotoxique, déclenchent une réaction inflammatoire et jouent un rôle majeur dans de nombreuses pathologies infectieuses, telles que le sepsis<sup>187</sup>. Les PTSAgs activent les lymphocytes T en se liant aux molécules du complexe majeur d'histocompatibilité de classe II et utilisent sur les cellules endothéliales ces mêmes molécules pour exercer leur effet cytotoxique. Les métalloprotéases « A Disintegrin and metalloproteinase domain-containing protein » (ADAM) 10, ADAM17 et le récepteur de l'Epidermal Growth Factor reconnaissent TSST-1 et induisent une sécrétion pro-inflammatoire (IL-8 et syndécane-1) par les cellules épithéliales<sup>188</sup>.
- Les hémolysines : il existe quatre types d'hémolysines ( $\alpha$ ,  $\beta$ ,  $\delta$ ,  $\gamma$ ) :
  - L'hémolysine  $\alpha$  est la toxine la plus étudiée par son large spectre d'action et sa grande virulence. De plus, cette exotoxine est fréquemment retrouvée chez *S. aureus* (59 à 67%)<sup>189</sup>. L'hémolysine  $\alpha$  se lie à la métalloprotéase ADAM10 pour induire la formation de pores entraînant la lyse de nombreuses cellules telles que les cellules épithéliales et endothéliales, les lymphocytes T, les

monocytes/macrophages et neutrophiles <sup>190-193</sup>. En plus de son action cytotoxique, cette hémolysine altère la signalisation intercellulaire, la sécrétion cytokinique et va donc participer à la réponse inflammatoire. Elle joue ainsi un rôle non négligeable à la pathogénèse des infections à *S. aureus*.

- L'hémolysine  $\beta$ , produite par de nombreuses souches de *S. aureus* (57%) lors de la phase exponentielle de croissance bactérienne, a une activité phosphorylase C dérégulant ainsi la signalisation lipidique des cellules <sup>189</sup>. Elle semble avoir une action principalement sur les érythrocytes. Il a été montré également que l'hémolysine  $\beta$  stimule le syndecan-1 qui favorise le développement de lésions pulmonaires lors d'une bactériémie à *S. aureus* <sup>194</sup>.
- L'hémolysine  $\delta$  est sécrétée par 97% des souches de *S. aureus* <sup>186</sup>. Il a été montré que cette toxine, qui se fixe à la membrane pour lyser les cellules cibles, active les mastocytes, contrairement à l'hémolysine  $\alpha$  <sup>195</sup>. Cependant, l'ensemble des mécanismes d'action, des cellules cibles et des effets cytotoxiques de cette toxine restent encore à approfondir.
- L'hémolysine  $\gamma$  affecte les érythrocytes ainsi que les leucocytes tels que les neutrophiles et macrophages. Cette toxine est constituée de plusieurs protéines sécrétées de manière non associée ; Hlg A, Hlg B et Hlg C qui associés par deux permettront l'effet cytotoxique. L'Hlg A et C sont des composants « S » (pour Slow selon leur purification) à la différence de l'Hlg B qui est un composant « F » (Fast). L'hémolysine  $\gamma$  AB utilise les récepteurs CXCR1, CXCR2, CCR2 alors que l'hémolysine  $\gamma$  CB les récepteurs au complément 5a (CD88) et C5L2 pour cibler les cellules phagocytaires <sup>196</sup>. Les récepteurs CXCR1, CXCR2 étant majoritairement retrouvé sur les leucocytes, l'hémolysine  $\gamma$  AB possède un tropisme cellulaire plus large que l'hémolysine  $\gamma$  CB. Les trois exoprotéines de l'hémolysine  $\gamma$  peuvent s'associer au composants « S » ou « F » de la PVL entraînant différentes réactions physiopathologiques <sup>197</sup>.
- La leucocidine de Pantone-Valentine (PVL) est une toxine sécrétée en deux composants ; LukS-PV et LukF-PV. Elle est retrouvée uniquement dans 2 à 3% des souches de *S. aureus*. La PVL induit la sécrétion granulaire des leucocytes polynucléaires entraînant une importante réaction inflammatoire <sup>198</sup>.

D'un point de vue général, les différentes exotoxines sécrétées par *S. aureus* possèdent un large spectre de cellules cibles expliquant l'importante virulence de cette bactérie. De nombreuses études ont permis de montrer que *S. aureus* possède une relation complexe avec les plaquettes<sup>199</sup>. Par ailleurs, les bactéries sécrètent des toxines qui vont permettre l'action du germe à plus grande distance et agir de manière plus spécifique à un type cellulaire. Ces facteurs de virulence modulent l'action des plaquettes, contribuant ainsi aux dysfonctions hémostatique et inflammatoire observées lors des maladies infectieuses.

Une étude datant d'une cinquantaine d'années avait déjà montré l'effet pro-agrégant de l'hémolysine  $\alpha$ , par le biais de l'activation plaquettaire (changement de morphologie et du volume plaquettaire) induisant alors la libération des facteurs pro-coagulants contenus dans les plaquettes<sup>200</sup>. Outre cette action hémostatique induit par les toxines, ces facteurs de virulence peuvent générer une réponse inflammatoire altérant l'adhésion plaquettaire, en formant des complexes neutro-plaquettaire, des NETs ou en induisant l'apoptose des plaquettes<sup>103,201-203</sup>. L'ensemble de ces effets pourraient être observés au cours d'un sepsis à *S. aureus*<sup>203</sup>.

Actuellement, peu d'études se sont intéressées à l'effet des exotoxines de *S. aureus* sur les plaquettes sanguines d'un point de vue inflammatoire. Dans cette étude, nous avons étudié l'effet de différentes exotoxines purifiées de *S. aureus* (Hémolysine  $\alpha$  et  $\gamma$ , TSST-1 et PVL) utilisées seules ou en combinaison afin d'être plus proche de la physiopathologie. Nous avons mesuré, outre le potentiel pro-agrégant, la capacité de ces toxines à induire une réponse sécrétoire pro-inflammatoire plaquettaire suite à l'exposition avec ces exotoxines. Nous avons mis en évidence les récepteurs utilisés par les toxines pour agir sur les plaquettes.

1 **Human platelets differentially sense various *Staphylococcus aureus* exotoxins and adapt**  
2 **their subsequent immuno-modulatory molecule secretion profiles**

3

4 Adrien Chabert<sup>\*1</sup>, Pauline Damien<sup>\*1</sup>, Fabrice Cognasse<sup>\*,†</sup>, Cédric Badiou<sup>‡</sup>, Fabrice Zeni<sup>\*,§</sup>,  
5 Marie Ange Eyraud<sup>†</sup>, Charles Antoine Arthaud<sup>†</sup>, Bruno Pozzetto<sup>\*,¶</sup>, Olivier Dauwalder<sup>‡,||</sup>,  
6 Francois Vandenesch<sup>‡,||</sup>, Olivier Garraud<sup>\*,#</sup>, Gérard Lina<sup>‡,||</sup>, Hind Hamzeh-Cognasse<sup>\*</sup>

7

8 <sup>\*</sup>Université de Lyon, GIMAP-EA3064, F42023, Saint Etienne, France

9 <sup>†</sup>EFS Auvergne-Loire, F42000, Saint-Etienne, France

10 <sup>‡</sup>CIRI, International Center for Infectiology Research, LabEx Ecofect, Université Lyon1,  
11 Inserm U1111, Ecole Normale Supérieure de Lyon, CNRS UMR5308, 69008 Lyon,  
12 France

13 <sup>§</sup>Service de Réanimation Médicale, CHU de Saint-Etienne, 42270 Saint-Etienne, France

14 <sup>¶</sup>Laboratoire de Bactériologie-Virologie- Hygiène, Centre Hospitalo-Universitaire de Saint-  
15 Étienne, 42270 Saint-Etienne, France

16 <sup>||</sup>Centre National de Référence des Staphylocoques, Hospices Civils de Lyon, 69500 Bron,  
17 France

18 <sup>#</sup>Institut National de la Transfusion Sanguine, 75739 Paris, France

19 <sup>1</sup> A.C. and P.D. contributed equally to this work

20

21 Corresponding author:

22 Dr. Hind HAMZEH-COGNASSE, Université de Lyon, GIMAP-EA3064, Campus Santé  
23 Innovations, Faculté de Médecine, 10 rue de la Marandière, 42270 Saint-Priest-en-Jarez,  
24 France. Tel.: +33 4 77 42 14 67. Fax: +33 4 77 42 14 86.

25 E-mail: hind.hamzeh@univ-st-etienne.fr



26

27 **Running title:** Platelet response to *S. aureus* exotoxins

28

29

30

31 **Abstract**

32 Sepsis is accompanied by several features including acute systemic inflammation. Platelets  
33 are involved in the pathophysiology of sepsis, as shown by the frequent occurrence of  
34 thrombocytopenia although the mechanisms of platelet depletion are not completely  
35 understood. Platelets react to bacterial components and secrete amounts of proinflammatory  
36 molecules. *Staphylococcus aureus*, one of the most frequent bacterial causes of sepsis in  
37 Europe, and its components can bind to, and activate, platelets. To investigate the effects of *S.*  
38 *aureus* exotoxins on the capacity of human platelets to release inflammatory molecules,  
39 human platelets were exposed to staphylococcal  $\alpha$ - and  $\gamma$ -hemolysin, Panton-Valentine  
40 leukocidin and toxic shock syndrome toxin-1. Platelet activation, aggregation and the release  
41 of soluble (s)CD62P; soluble CD40-ligand (sCD40L); RANTES and stromal cell-derived  
42 factor-1 $\alpha$  (SDF-1 $\alpha$ ) were assessed. Statistical significance of data was analyzed using a two-  
43 way ANOVA with repeated measures and a Bonferroni post-hoc test. No phenotypic platelet  
44 activation or aggregation was observed upon toxin stimulation regardless of the toxin. Profiles  
45 of secreted immunomodulators appeared to be dependent on the staphylococcal exotoxin that  
46 was used for stimulation, with differential release of sCD62P, sCD40L and RANTES by  
47 platelets. Platelet stimulation with combinations of exotoxins used at suboptimal  
48 concentrations resulted in variable release of sCD40L and RANTES. In addition, some  
49 exotoxin combinations inhibited the release of RANTES by platelets upon prolonged  
50 exposure. These results suggest that platelets differentiate between staphylococcal toxins and  
51 adapt their inflammatory response to perceived specific “danger” signals. This is of particular  
52 importance in the context the inflammatory phase of sepsis.

53

54 **Keywords:** cytokines, exotoxins, inflammation, platelet, *Staphylococcus aureus*

55 **Introduction**

56 *Staphylococcus aureus* is a main cause of mucosal and skin infections, and it is also a  
57 prominent contributor to bloodstream infections and to respiratory and other invasive diseases  
58 (1, 2). Along with *Pseudomonas* species, *S. aureus* is the most frequent agent causing sepsis  
59 in European intensive care units (3). *Staphylococcus aureus* strains express a variety of  
60 exotoxins, including cytotoxins such as Panton–Valentine leukocidin (PVL) and  $\alpha$ -toxin,  
61 which induce leukocyte activation (4, 5) and cell death (1, 6), and superantigens such as toxic  
62 shock syndrome toxin-1 (TSST-1), which induces T cell polyclonal proliferation and a burst  
63 of cytokine release (7). This is of particular importance in sepsis because recent studies  
64 suggest that antibodies for *S. aureus* exotoxins might protect against *S. aureus*-related sepsis  
65 (8).

66 *Staphylococcus aureus* interacts with platelets and alters their hemostatic function.  
67 The interactions between platelets and bacteria have been extensively reviewed (9-11).  
68 *Staphylococcus aureus* activates platelets indirectly by mechanisms that depend on IgG and  
69 its associated platelet receptor Fc $\gamma$ RIIA, fibrinogen and its receptor  $\alpha$ IIB $\beta$ 3, von Willebrand  
70 factor and its receptor GPIb/IX/V and complement and its receptors (12). Thus, an efficient  
71 interaction between bacteria and platelets may require plasma components, as seen for platelet  
72 inflammatory response to LPS, which relies on plasma soluble CD14 (13). Similarly, Arman  
73 *et al.* showed that platelet activation by several strains of *Streptococcus sp.* and *S. aureus*  
74 required the presence of plasma IgG and involved Fc $\gamma$ RIIA and  $\alpha$ IIB $\beta$ 3 integrin on platelets  
75 (14). Staphylococcal superantigen-like 5 causes platelet activation and aggregation in a  
76 GPIb $\alpha$ -dependent manner, which has been hypothesized to be the mechanism by which  
77 *S. aureus* is able to colonize the vascular bed and evade the immune system (10). Mutants  
78 defective in iron-responsive surface determinant (Isd) B, but in not IsdA or IsdH, were found  
79 to be unable to adhere to or aggregate platelets (15). Other *S. aureus* components, such as

80 fibrinogen binding proteins, clumping factor A, staphylothrombin, protein A, serine-aspartate  
81 repeat protein SdrE and extracellular adherence protein, have also been reported to activate  
82 platelets either directly or indirectly and to promote aggregation (16-20). Recent studies on  
83 the effects of *S. aureus*  $\alpha$ -hemolysin on platelets have reported that this toxin induces platelet  
84 aggregation and Bcl3 synthesis (21), alters platelet viability (22) and also activates platelets  
85 and prompts them to aggregate with neutrophils (23).

86         Some *S. aureus* components inhibit the hemostatic functions of platelets, for example,  
87 lipoteichoic acid (24), extracellular fibrinogen binding protein (25), staphylokinase (26),  
88 enterotoxin B (27) and  $\alpha$ -toxin (28).

89         Increasing evidence suggests that, besides having a key role in primary hemostasis,  
90 platelets mediate inflammation during infection by various mechanisms that are not yet  
91 completely understood (29). During HIV infection, platelets have been reported to release  
92 inflammatory molecules, including neutrophil activating peptide 2, soluble CD62P (sCD62P),  
93 regulated on activation, normal T cell expression and secretion (RANTES) and soluble CD40-  
94 ligand (sCD40L) (30, 31). Similarly, bacterial stimulation of platelets results in the release of  
95 many immuno-modulatory molecules, particularly sCD40L (32), in associations that depend  
96 on the stimulus and receptors that are involved (33-36).

97         Most studies that have investigated the effects of toxins on platelets have focused on  
98 their hemostatic function and not the immunoregulatory role of platelets, particularly the  
99 potential of the secreted inflammatory molecules (9, 37). However, it is essential to consider  
100 this other side of the platelet response. In the current study, we examined the *in vitro* response  
101 of platelets to several *S. aureus* exotoxins to investigate the capacity of platelets to release  
102 inflammatory molecules in response to different danger signals given off by these exotoxins.

103

104

105 **Materials and Methods**

106 ***Exotoxin production and purification***

107 PVL,  $\alpha$ -hemolysin,  $\gamma$ -hemolysin and TSST-1 were produced in *Escherichia coli* M15 as His-  
108 tagged recombinant toxins and purified by affinity chromatography on a nickel affinity  
109 column, according to the manufacturer's instructions (New England Biolabs, Ipswich, MA,  
110 USA), as previously described (38, 39). Protein purity was verified using SDS-PAGE. LPS  
111 was removed from toxin solutions using affinity chromatography (Detoxi-GEL endotoxin  
112 Gel®, Pierce, Rockford, IL, USA). The QCL-1000 *Limulus* amebocyte lysate assay®  
113 (Cambrex-BioWhittaker, Walkersville, MD, USA) showed that the endotoxin content of the  
114 recombinant exotoxin solutions was less than 0.005 units/mL.

115

116 ***Platelet preparation and stimulation***

117 Platelet-rich plasma (PRP) was prepared as previously described (40 ). According to the L.  
118 1223-3 Article of the French Public Health Code, donated blood can be used for non-  
119 therapeutic purposes when the volunteer blood donor, after being informed, signs the donation  
120 form indicating that he does not preclude the use of his donation for non-therapeutic purposes.  
121 Thus, peripheral blood was collected from healthy donors in endotoxin-free tubes with 3.2%  
122 sodium citrate (Becton Dickinson, San Jose, CA, USA) and centrifuged at  $150 \times g$  for 12 min  
123 at 22°C. There was no detectable contamination of platelets by residual mononuclear cells as  
124 assessed by flow cytometry (CD3-T cells, CD15-neutrophils, CD19-B cells or CD14-  
125 monocytes; Supplemental Figure 1) (33). An aliquot of PRP was centrifuged at  $300 \times g$  for 5  
126 min at 22°C and washed twice in PBS to obtain washed platelets (WP). Platelets from PRP or  
127 WP ( $3 \times 10^8$ ) were stimulated for 30 at room temperature with 50  $\mu\text{g/mL}$  thrombin receptor  
128 activator peptide (TRAP, Sigma-Aldrich, Saint Quentin-Fallavier, France) as a positive  
129 control, Tyrode's buffer as a negative control or with the following staphylococcal exotoxins:

130  $\alpha$ - and  $\gamma$ -hemolysin, PVL or TSST-1, either alone in a concentration ranging between 0.01  
131 and 100  $\mu\text{g}/\text{mL}$  or in a two-exotoxin combination. A wide range of exotoxin concentrations  
132 was used to stimulate platelets and mimic pathological conditions, and the highly  
133 inflammatory concentration of 100  $\mu\text{g}/\text{mL}$  was used in first steps to ensure the functionality of  
134 exotoxins on platelets.

135

### 136 **Assessment of platelet aggregation**

137 The thrombo-aggregometer 4V (SD Medical, Frouard, France) was calibrated using PRP (0%  
138 light transmission, 0% aggregation) and platelet poor plasma (PPP, 100% light transmission,  
139 100% aggregation). The PPP was obtained by centrifugation of PRP at  $3000 \times g$  for 20 min at  
140 room temperature. Samples were treated with TRAP as a positive control, or with  
141 staphylococcal exotoxins for up to 60 min, at  $37^\circ\text{C}$ , with constant stirring. Platelet  
142 aggregation was monitored using the thrombo-aggregometer 4V instrument TA4V and  
143 analyzed using Thrombosoft 1.6 software (SD Medical).

144

### 145 **Analysis of the platelet activation phenotype**

146 CD62P and CD63 expressions, which characterize activated platelets, were measured on  
147 platelets using flow cytometry after gating on CD41-positive events (CD41 is specifically  
148 expressed by platelets), as described previously (13). Platelets from PRP and WP were stained  
149 with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (Abs) against  
150 human CD62P (clone AK-4), phycoerythrin-conjugated mAbs against human CD63 (clone  
151 H5C6) and allophycocyanin-conjugated mAbs against human CD41 (clone HIP8). For the  
152 assessment of PRP purity, FITC-conjugated mAbs against human CD3 (clone HIT3a), CD14  
153 (clone M5E2), CD15 (clone HI 98) or CD19 (clone SJ25CI) were used. All antibodies were  
154 purchased from BD Biosciences (Le Pont de Claix, France). Positive events were recorded

155 using a CANTO II cytometer (BD Biosciences) and analyzed with BD FACSDiva™ Software  
156 (BD Biosciences).

157

158 ***Enzyme-linked immunosorbent and Luminex® assay for cytokine assessment in platelet***  
159 ***supernatant***

160 sCD62P, RANTES, SDF-1 $\alpha$ , sCD40L and serotonin levels were measured in duplicate in  
161 supernatants of platelets stimulated with Tyrode's buffer (negative control) or TRAP-  
162 stimulated (positive control) in supernatants of PRP and WP stimulated by staphylococcal  
163 exotoxins used alone or in combination. Soluble cytokine ELISA kits (sCD62P) and serotonin  
164 were obtained from R&D Systems Europe Ltd. (Lille, France) and from IBL International  
165 (Hamburg, Germany) respectively. Absorbance at 450 nm was measured on an ELISA reader  
166 (Magellan software, Sunrise™, Tecan Group Ltd., Lyon, France), as described previously  
167 (33, 41). sCD40L, and RANTES and SDF-1 $\alpha$  were quantified using a multiplexed detection  
168 technique (Merck Millipore, Guyancourt, France), according to the manufacturer's  
169 instructions. Results were read on a Bioplex 200® system (Bioplex Manager™ software,  
170 Biorad, Marnes-la-Coquette, France).

171

172 ***Measurement of endotoxin levels in platelet-rich plasma***

173 To ensure that no LPS contamination influenced any *in vitro* observation, PRP were examined  
174 for bacterial growth by inoculation of 1 mL of platelet-rich plasma in conventional blood  
175 culture medium and incubated in a BD Bactec™ automated system (Becton Dickinson, Le  
176 Pont de Claix, France). Absence of endotoxins was confirmed using the QCL-1000 *Limulus*  
177 amebocyte lysate assay® (Lonza/Ozyme, Montigny-Le-Bretonneux, France).

178

179 ***Statistical analysis***

180 A two-way ANOVA with repeated measures and a Bonferroni post-hoc test were used for  
181 inter-experimental comparisons of cytokine/chemokine concentrations under various culture  
182 conditions. All values are reported as the mean  $\pm$  standard ecart mean (SEM). A P value of  
183  $<0.05$  was considered statistically significant.



184 **Results**

185 ***Staphylococcus aureus* exotoxins influence the release of cytokines/chemokines from**  
186 **platelets**

187 Platelet stimulation by a thrombin agonist such as TRAP validated their ability to respond to a  
188 specific trigger by releasing large amounts of several cytokines including sCD62P, sCD40L  
189 and RANTES, and lower but significant amounts of SDF-1 $\alpha$  and serotonin (Figure 1A-D,  
190 supplemental Figure 2). Release of the former three, but of neither SDF-1 $\alpha$  nor serotonin, was  
191 observed upon stimulation with all tested exotoxins, although with differing profiles (Figure 1  
192 A-D and supplemental Figure 2). This suggests an adapted platelet response to the trigger,  
193 because the entire cytokine content was not released upon stimulation and some of the content  
194 of the  $\alpha$ -granule has been released while the content of the dense granules was not. Thus,  
195 platelet stimulation with TSST-1,  $\alpha$ -hemolysin or PVL did not cause a significant release of  
196 sCD62P compared with the control (Figures 1 A, B and D, respectively); however,  $\gamma$ -  
197 hemolysin induced significant sCD62P release ( $P < 0.05$ ), ranging from  $10807 \pm 3420$  pg/mL  
198 for the weakest concentration ( $0.01 \mu\text{g/mL}$ ) to  $18747 \pm 5088$  pg/mL for the highest  
199 concentration ( $100 \mu\text{g/mL}$ ; Figure 1C).

200 A 30-min stimulation of platelets with all *S. aureus* exotoxins caused significant releases of  
201 RANTES (Figures 1A-D) when used at  $10 \mu\text{g/mL}$  and higher concentrations; the only  
202 exception was PVL, which required a concentration of  $100 \mu\text{g/mL}$  to induce significant  
203 RANTES release (Figure 1D). Platelet stimulation with TSST-1,  $\alpha$ -hemolysin,  $\gamma$ -hemolysin  
204 and PVL resulted in the significant release of  $8076 \pm 2122$  pg/mL,  $20636 \pm 8354$  pg/mL,  
205  $23734 \pm 7393$  pg/mL and  $10461 \pm 3544$  pg/mL RANTES, respectively, compared with  
206 unstimulated platelets ( $4598 \pm 3882$  pg/mL;  $P < 0.05$ ).

207 The minimal concentrations of exotoxins that induce significant sCD40L release were  $100$   
208  $\mu\text{g/mL}$  for TSST-1,  $1 \mu\text{g/mL}$  for  $\alpha$ -hemolysin and  $10 \mu\text{g/mL}$  for  $\gamma$ -hemolysin (Figures 1A–C).

209 PVL failed to induce sCD40L release at all concentrations tested (Figure 1D). sCD40L levels  
210 rose significantly from  $40 \pm 27$  pg/mL (in the absence of stimulation) to  $90 \pm 28$  pg/mL with  
211 TSST-1 at  $100 \mu\text{g/mL}$  ( $P < 0.05$ ; Figure 1A), to  $147 \pm 75$  pg/mL with  $\alpha$ -hemolysin at  $1 \mu\text{g/mL}$   
212 ( $P < 0.05$ ; Figure 1B) and to  $169 \pm 88$  pg/mL with  $\gamma$ -hemolysin at  $10 \mu\text{g/mL}$  exposure ( $P < 0.05$ ;  
213 Figure 1C).

214 With the exception of  $\gamma$ -hemolysin used at its highest concentration ( $100 \mu\text{g/mL}$ ), no  
215 aggregation was observed upon stimulation of platelets either with the highest toxin  
216 concentrations that induced platelet cytokine release, or with suboptimal exotoxin  
217 concentrations, that were defined as the maximal exotoxin concentration that did not induce a  
218 significant release of cytokines/chemokines after 30 min of stimulation, i.e. 1, 0.1, 0.001 and  
219  $10 \mu\text{g/mL}$  for TSST-1,  $\alpha$ - and  $\gamma$ -hemolysin, and PVL, respectively (Figure 2 A).

220 Next, we observed that concentrations below  $1 \mu\text{g/mL}$  for  $\alpha$ -hemolysin (Figure 3B),  $\gamma$ -  
221 hemolysin (Figure 3C) and PVL (Figure 3D) did not induce a significant expression of the  
222 CD62P platelet activation marker, while TSST-1, in contrast did not induce at all the  
223 expression of CD62P. CD63 expression appeared to be not significant in all conditions  
224 (Supplemental Figure 3) except for  $1 \mu\text{g/mL}$  of  $\alpha$ -hemolysin that induced a slight but  
225 significant expression of this marker (Supplemental Figure 3B). In addition, it appeared that  
226 washed platelets exhibited the same activation profiles as platelets in PRP upon activation  
227 with each exotoxin (Figure 3A-D) with the exception of the  $1 \mu\text{g/mL}$  concentration of  $\alpha$ -  
228 hemolysin (Figure 3B, Supplemental Figure 3B) and of PVL (Figure 3D) that induced CD62P  
229 or CD63 expression on washed platelets but not on platelets in PRP. This suggests that plasma  
230 is not required to induce a significant platelet activation phenotype upon *S. aureus* exotoxin  
231 stimulation. This was also confirmed for RANTES and sCD40L and sCD62P release  
232 upon single toxin stimulation (Supplemental Figure 4 A-D).

233

234 **Platelet cytokine/chemokine release after combined staphylococcal exotoxin activation.**

235 The apparent low sensitivity of platelets to the *S. aureus* toxins tested prompted us to test for  
236 the effects of pairwise combinations of TSST-1,  $\alpha$ -hemolysin,  $\gamma$ -hemolysin and PVL at  
237 suboptimal concentrations, for which they showed inflammatory factor secretion when used  
238 alone (i.e. 1, 0.1, 0.001 and 10  $\mu$ g/mL for TSST-1,  $\alpha$ - and  $\gamma$ -hemolysin and PVL,  
239 respectively). These combinations may not be representative of all virulence factors that could  
240 be simultaneously present on/in a *S. aureus* strain but enable the assessment of the synergistic  
241 effects of several exotoxins. We observed that the combinations of exotoxins induced neither  
242 platelet aggregation while they retained their ability to aggregate upon TRAP stimulation  
243 (Figure 2B), nor activation of platelets, in PRP as well as in washed platelets (Figure 3E,  
244 Supplemental Figure 3E). We then assessed sCD62P, RANTES, sCD40L and SDF-1 $\alpha$   
245 release, and there were no significant changes in sCD62P and SDF-1 $\alpha$  release when platelets  
246 were stimulated with any combination of suboptimal *S. aureus* exotoxin concentrations  
247 (Figure 4). However, all combinations of sub-optimal toxin concentrations induced platelets  
248 to differentially release significant amounts of RANTES and sCD40L after stimulation.  
249 RANTES and sCD40L levels were lower but comparable to the levels observed with  
250 exotoxins used alone at higher concentrations. For example, RANTES levels ranged between  
251  $15926 \pm 2628$  and  $20973 \pm 4038$  pg/mL, and sCD40L levels ranged between  $115 \pm 28$  and  
252  $215 \pm 40$  pg/mL for combinations of exotoxins at suboptimal concentrations, while the  
253 maximum release upon individual exotoxin stimulation was  $31158 \pm 2222$  pg/mL for  
254 RANTES and  $330 \pm 110$  pg/mL for sCD40L (Figure 1). Thus, the release of platelet immuno-  
255 modulatory factor that was induced by combinations of exotoxins was independent of the  
256 activation marker expression or aggregation.

257 Finally we examined whether plasma soluble factors were required for an efficient platelet  
258 secretion of inflammatory factors upon exposition to exotoxin combination and we observed

259 that, as seen for single toxins, washed platelets responded to the same extent as platelets from  
260 PRP confirming that platelet inflammatory response to exotoxins could occur in the absence  
261 of plasma factors (Supplemental Figure 4E).

262

263

264 **Discussion**

265 In this study, we investigated whether exposure of normal platelets to various staphylococcal  
266 exotoxins, such as TSST-1,  $\alpha$ - and  $\gamma$ -hemolysin and PVL, could signal platelets to release  
267 soluble immuno-modulator factors. We were particularly interested in molecules that have  
268 been reported to play a role in the inflammatory phase of sepsis and those that platelets can  
269 potentially secrete, such as sCD62P, sCD40L, RANTES and SDF-1 $\alpha$  (42-48). We first  
270 confirmed that exotoxins induced neither platelet aggregation (except  $\gamma$ -hemolysin at its  
271 highest concentrations) nor a huge phenotypic activation, which is in agreement with other  
272 observations (28). We then observed varying sCD62P, sCD40L and RANTES levels, but not  
273 SDF-1 $\alpha$  levels, after 30 min of staphylococcal exotoxin stimulation. Thus, platelet  
274 inflammatory response is not binary but it is rather adapted to the stimulation, because some  
275 immuno-modulatory factors such as sCD62P, sCD40L and RANTES are released upon  
276 exotoxin stimulation, whereas other factors such as SDF-1 $\alpha$  or serotonin are not. We also  
277 observed that a 30 min-stimulation with a combination of exotoxins at suboptimal  
278 concentrations resulted in a significant release of sCD40L and RANTES, but not of SDF-1 $\alpha$ ,  
279 serotonin or sCD62P, the release of the latter remained insensitive to exotoxin combinations,  
280 compared with each exotoxin alone. For all parameters analyzed, we also observed that  
281 washed platelets responded to exotoxins in a similar extend as compared to platelets in PRP,  
282 which raises the issue about the contribution of plasma factors in the platelet response to  
283 bacteria, which has been demonstrated for the efficacy of TLR4-induced platelet response  
284 (13). Platelets might interact with whole *S. aureus* bacteria either directly or indirectly by  
285 involving several plasma proteins, as described previously (12). *S. aureus* might divert plasma  
286 IgG molecules to bind to platelets via their Fc $\gamma$ RIIA receptor, and with the concomitant action  
287 of fibrinogen and platelet factor 4, promote their activation and aggregation (14). However, in  
288 our conditions, platelets do not seem to require plasma factors to react to *S. aureus* exotoxins

289 by expressing activation markers or releasing soluble factors, although TRAP seemed to be  
290 more efficient on washed platelets, which could reflect some increased reactivity of platelets  
291 to stimulation due to the washing procedure. This suggests that these toxins may interact with  
292 platelets rather directly than indirectly. Thus, the platelet receptors involved in such  
293 interactions remain to be evidenced, although some studies demonstrated the involvement of  
294 ADAM10 for instance in the platelet response to  $\alpha$ -hemolysin (28).

295         Innate immune cells show shared characteristics in response to pathogens such as the  
296 release of soluble immuno-modulatory factors. Platelets secrete many cytokines, chemokines  
297 and biological response modifiers, and express TLRs and other pattern recognition receptors  
298 (34, 49). Recently, interest in platelets has increased because of their rapid accumulation,  
299 aggregation and activation in response to infectious pathogens (50). This raised the issue of  
300 whether they might function as sentinels and transfer information to peripheral immune cells  
301 in the early stages of infection. For example, platelet TLR4 was reported to be important in  
302 the induction of neutrophils that are in contact with bacteria to release a sticky “net” of DNA  
303 and histones, which traps and kills pathogens even after the neutrophil no longer exists (51).  
304 Because platelets may recognize danger-associated molecular patterns to further perpetuate  
305 the inflammatory response (29, 52-55), it has been proposed that a complex network of  
306 inflammatory cytokines/chemokines, some derived from platelets and others derived from  
307 platelet partners (such as leukocytes), play a major part in facilitating, augmenting and  
308 propagating inflammation (56-59).

309         *S. aureus* is a significant human pathogen that causes multiple illnesses (60), and its  
310 exotoxins stimulate massive cytokine production by several types of immune cells (1, 6, 61-  
311 65). However, few studies have examined the effects of staphylococcal components on  
312 platelet inflammatory responses. The majority of studies examining platelets and sepsis have  
313 investigated the molecular mechanisms by which *S. aureus* and platelets interact, either

314 directly or indirectly, and these studies have analyzed the influence of these interactions on  
315 platelet aggregation (15-17, 20, 21, 66-70). Few studies have focused on *S. aureus* exotoxins  
316 and even fewer have examined the inflammatory components of the platelet response.

317 In the current study, all exotoxins tested stimulated platelet cytokine/chemokine  
318 production in varying patterns rather than in an on-off manner. This reinforces the hypothesis  
319 that platelets discriminate among triggering signals and adapt their inflammatory response to  
320 the specific trigger, as previously reported for *S. sanguinis* strains (36) and LPS isoforms (33).  
321  $\alpha$ - and  $\gamma$ -Hemolysin seem to induce a higher and wider platelet inflammatory response than  
322 TSST-1 and PVL, suggesting that these toxins can further contribute to the inflammatory  
323 response associated with infection by stimulating platelets. In addition, none of these toxins  
324 were able to induce significant SDF-1 $\alpha$  and serotonin release, demonstrating that bacterial  
325 compound-induced triggering of platelets does not lead to the release of their entire content.  
326 This observation is of particular importance for sepsis, and especially for the early pro-  
327 inflammatory phase. The amounts of RANTES and sCD40L released by platelets stimulated  
328 by these toxins are comparable to those observed in the plasma of sepsis patients (43, 71) or  
329 to those in patients with systemic meningococcal disease (72) and both platelet-derived  
330 molecules were shown to be crucial for the development of acute lung injury related to sepsis  
331 (48). The physiological significance of the platelet release of the inflammatory could be  
332 further assessed on target cells such as neutrophils, which are important partners of platelets  
333 in inflammation related to infection.

334 This observation, in addition to studies showing that some *S. aureus* toxins activate  
335 platelets and promote their interaction with neutrophils, reinforces the potential participation  
336 of platelets in the inflammation that is associated with systemic bacterial infection. Therefore,  
337 the release of inflammatory factors by platelets could be targeted by new strategies aiming at  
338 reducing the systemic inflammation in bacteremia or sepsis.

339           Because some *S. aureus* strains display several exotoxins, it was important to assess  
340 whether the exotoxins that do not strongly activate platelets were more effective in  
341 combination, even at suboptimal concentrations. Because of the lack of available data in  
342 humans and animals concerning exotoxin levels in plasma, it is difficult to draw conclusions  
343 about the pathophysiological relevance of the calculated suboptimal exotoxin concentrations.  
344 However, it was shown, at least for TSST-1 and streptococcal lymphotoxin- $\alpha$ , that  
345 concentrations of these exotoxins were above 440 and 350 pg/ml respectively in plasma from  
346 sepsis patients (73, 74). In addition, when compared to clinical endotoxin levels assessed in  
347 septic shock patients (after conversion of Endotoxin Units into ng/ml) that ranged from  
348 200pg/ml to 210ng/ml (75), suboptimal concentrations of TSST-1 (1 $\mu$ g/ml) and PVL  
349 (10 $\mu$ g/ml) appear to be higher than the pathophysiological values of LPS, whereas those of  $\alpha$ -  
350 and  $\gamma$ -hemolysins (100ng/ml and 1ng/ml, respectively) were compatible. Thus, combined  
351 staphylococcal exotoxins at suboptimal concentrations induced significant platelet  
352 cytokine/chemokine production, close to that of exotoxins alone, and suggest a cumulative  
353 effect after 30 min of stimulation.. These observations are in agreement with, and complete  
354 those of, Powers *et al.*, who elegantly demonstrated how  $\alpha$ -hemolysin alters collagen-induced  
355 platelet aggregation by increasing ADAM10-induced glycoprotein VI proteolysis (28).  
356 Although this study shows a moderate expression of membrane CD62P on platelets upon  
357 stimulation with 10 and 1  $\mu$ g/mL PVL, of  $\alpha$ -hemolysin and  $\gamma$ - hemolysin, which accounts for  
358 neutrophil interaction and activation, we observed no upregulation of CD62P or CD63 for  
359 lower concentrations of these toxins or for any concentrations of TSST-1. In addition neither  
360 CD62P nor CD63 were expressed by platelets submitted to combined exotoxins at suboptimal  
361 concentrations. This suggests that, in the absence of any significant CD62P membrane  
362 expression, platelet-neutrophil interaction could operate yet, for instance via ICAM-2 on  
363 platelets and CD18/CD11a on neutrophils, as previously described (76). However, the



364 participation of platelets to the pathophysiology of sepsis could be more complex since a  
365 protective role of platelets has also been demonstrated in a murine model of sepsis (77),  
366 indicating that the platelet response might also change over time, thus differently modulate  
367 immune cells for instance during the immunosuppression that accompanies the late phase of  
368 sepsis.

369 In conclusion, the participation of platelet inflammatory responses to staphylococcal  
370 infection over time should not be underestimated, especially in sepsis where the inflammatory  
371 phase has to be controlled.

372

### 373 **Acknowledgements**

374 The authors thank the excellent scientific and technical participation of present and former  
375 collaborators: M. Lauhlé, A. Mondière and O. Decreuse. The authors are grateful to Dr. P.  
376 Chavarin and Dr. A. Lefebvre at the EFS Auvergne-Loire, and to the generous blood donors.

377

### 378 **Authors' contributions**

379 FC and HHC designed the experiments, analyzed the data and wrote the manuscript. AC, PD,  
380 CB, MAE, CAA and CB performed the experiments and analyzed the data. FZ, BP, FV, OD  
381 and GL designed the study. OG, GL, FC and HHC coordinated the study.

382

383

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609 **Financial support**

610 This work was supported by EFS Auvergne-Loire, the French “Agence Nationale de la  
611 Recherche” (ANR-12-JSV1-0012-01) and the Academic Research Community-1 of the  
612 Rhône-Alpes Region.

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615 **Abbreviation list**

616 Isd: iron-responsive surface determinant ; PRP: platelet-rich plasma; PVL: Panton-Valentine  
617 leukocidin; *S. aureus*: *Staphylococcus aureus*; sCD40L: soluble CD40-ligand; sCD62P:  
618 soluble CD62P; SDF-1: stromal cell-derived factor-1; TRAP: thrombin receptor activator  
619 peptide; TSST-1: toxic shock syndrome toxin-1, WP: washed platelets.

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622

623 **Figure legends**

624 **Figure 1. sCD62P, RANTES, sCD40L and SDF-1 $\alpha$  release by platelets upon *S. aureus***  
625 **exotoxin stimulation**

626 sCD62P, RANTES, sCD40L and SDF-1 $\alpha$  levels were quantified using ELISA or Luminex®  
627 assays after a 30 min-stimulation of platelets with TSST-1 (A),  $\alpha$ -hemolysin (B),  $\gamma$ -hemolysin  
628 (C) or PVL (D).. Platelets were stimulated with either Tyrode's buffer as a negative control  
629 (NS) or with TRAP as a positive control. Data (mean  $\pm$  SEM; n= 12 experiments) are  
630 expressed in pg/mL. \* P<0.05 (two-way ANOVA with repeated measures and Bonferroni  
631 post-hoc test; stimulated vs. negative control, NS).

632

633 **Figure 2. Platelet aggregation upon *S. aureus* exotoxin stimulation**

634 Aggregation of normal human platelets was assessed on a thrombo-aggregometer upon  
635 stimulation with either a range single *S. aureus* exotoxin concentrations (the highest and the  
636 suboptimal concentrations are shown, panel A) or a combination of two toxins used at a  
637 suboptimal concentration (panel B). Platelets were stimulated with TRAP as a positive control  
638 after up to 60 min of stimulation with exotoxins. (One representative experiment is presented;  
639 n = 5 experiments).

640

641 **Figure 3. Platelet CD62P expression upon *S. aureus* exotoxin stimulation**

642 CD41/CD62P positive cells on normal human platelets in PRP or washed platelets (WP) was  
643 assessed by flow cytometry upon a 30-min stimulation with individual *S. aureus* exotoxins ;  
644 TSST-1 (A);  $\alpha$  hemolysin (B);  $\gamma$  hemolysin (C); PVL (D) or combinations of two toxins used  
645 at a suboptimal concentration (E). Platelets were stimulated with Tyrode's buffer as a  
646 negative control (NS) or TRAP as a positive control. (Data are presented as mean  $\pm$  SEM; n =  
647 5 experiments). ### p<0.001, ##### p<0.0001 (for PRP conditions, two-way ANOVA with

648 repeated measures and Bonferroni post-hoc test; stimulated vs. negative control, NS).  $\square\square$   
649  $p < 0.01$ ,  $\square\square\square p < 0.001$ ,  $\square\square\square\square p < 0.0001$  (for WP conditions, two-way ANOVA with repeated  
650 measures and Bonferroni post-hoc test; stimulated vs. negative control, NS).

651

652

653 **Figure 4. Platelet inflammatory factor release after a 30-min exposure to a combination**  
654 **of *S. aureus* exotoxins**

655 Platelets from PRP were stimulated for 30 min with two exotoxins simultaneously at  
656 suboptimal concentrations that were defined in the previous single toxin stimulation assays  
657 (i.e. 1, 0.1, 0.001 and 10  $\mu\text{g}/\text{mL}$  for TSST-1,  $\alpha$ - and  $\gamma$ -hemolysin (Hla, Hlg) and PVL,  
658 respectively). sCD62P, RANTES, sCD40L and SDF-1 $\alpha$  levels were quantified using ELISA  
659 or Luminex® assays. Platelets were stimulated with Tyrode's buffer as a negative control  
660 (NS) or with TRAP as a positive control. Data (mean  $\pm$  SEM; n = 5 experiments) are  
661 expressed in pg/mL. \*  $P < 0.05$  (two-way ANOVA with repeated measures and Bonferroni  
662 post-hoc test; stimulated vs. unstimulated, NS).

663

664

665 **Supplemental Figure 1. Purity of platelet-rich plasma preparation**

666 Platelet purity was assessed using flow cytometry after staining cells with monoclonal  
667 antibodies directed against CD3 (specific marker for T cells), CD14 (specific marker for  
668 monocytes), CD15 (specific marker for neutrophils) CD19 (specific marker for B cells) or  
669 CD41 (specific marker for platelets). Data (mean  $\pm$  SEM; n = 5 experiments) are expressed as  
670 the percentage of positive cells.

671

672 **Supplemental Figure 2. Serotonin release by platelets upon *S. aureus* exotoxin**

673 **stimulation**

674 Serotonin levels were quantified using ELISA assays after a 30 min-stimulation of platelets  
675 with TSST-1 (A),  $\alpha$ -hemolysin (B),  $\gamma$ -hemolysin (C) PVL (D) or with two exotoxins  
676 simultaneously (E) at suboptimal concentrations that were defined in the previous single toxin  
677 stimulation assays (i.e. 1, 0.1, 0.001 and 10  $\mu$ g/mL for TSST-1,  $\alpha$ - and  $\gamma$ -hemolysin (Hla, Hlg)  
678 and PVL, respectively). Platelets were stimulated with either Tyrode's buffer as a negative  
679 control (NS) or with TRAP as a positive control. Data (mean  $\pm$  SEM; n= 12 experiments) are  
680 expressed in pg/mL. \* P<0.05 (two-way ANOVA with repeated measures and Bonferroni  
681 post-hoc test; stimulated vs. negative control, NS).

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684 **Supplemental Figure 3. Platelet CD63 expression upon *S. aureus* exotoxin stimulation**

685 CD41/CD63 positive cells on normal human platelets in PRP or washed platelets (WP) was  
686 assessed by flow cytometry upon a 30-min stimulation with individual *S. aureus* exotoxins ;  
687 TSST-1 (A);  $\alpha$  hemolysin (B);  $\gamma$  hemolysin (C); PVL (D) or combinations of two toxins used  
688 at a suboptimal concentration (E). Platelets were stimulated with Tyrode's buffer as a  
689 negative control (NS) or TRAP as a positive control. (Data are presented as mean  $\pm$  SEM; n =  
690 5 experiments). #### p<0.001, ##### p<0.0001 (for PRP conditions, two-way ANOVA with  
691 repeated measures and Bonferroni post-hoc test; stimulated vs. negative control, NS). □□  
692 p<0.01, □□□ p<0.001, □□□□ p<0.0001 (for WP conditions, two-way ANOVA with repeated  
693 measures and Bonferroni post-hoc test; stimulated vs. negative control, NS).

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697 **Supplemental Figure 4. Inflammatory factor release by platelets from PRP and washed**  
698 **platelets after exposure to a *S. aureus* exotoxins used as single toxins or in combination**

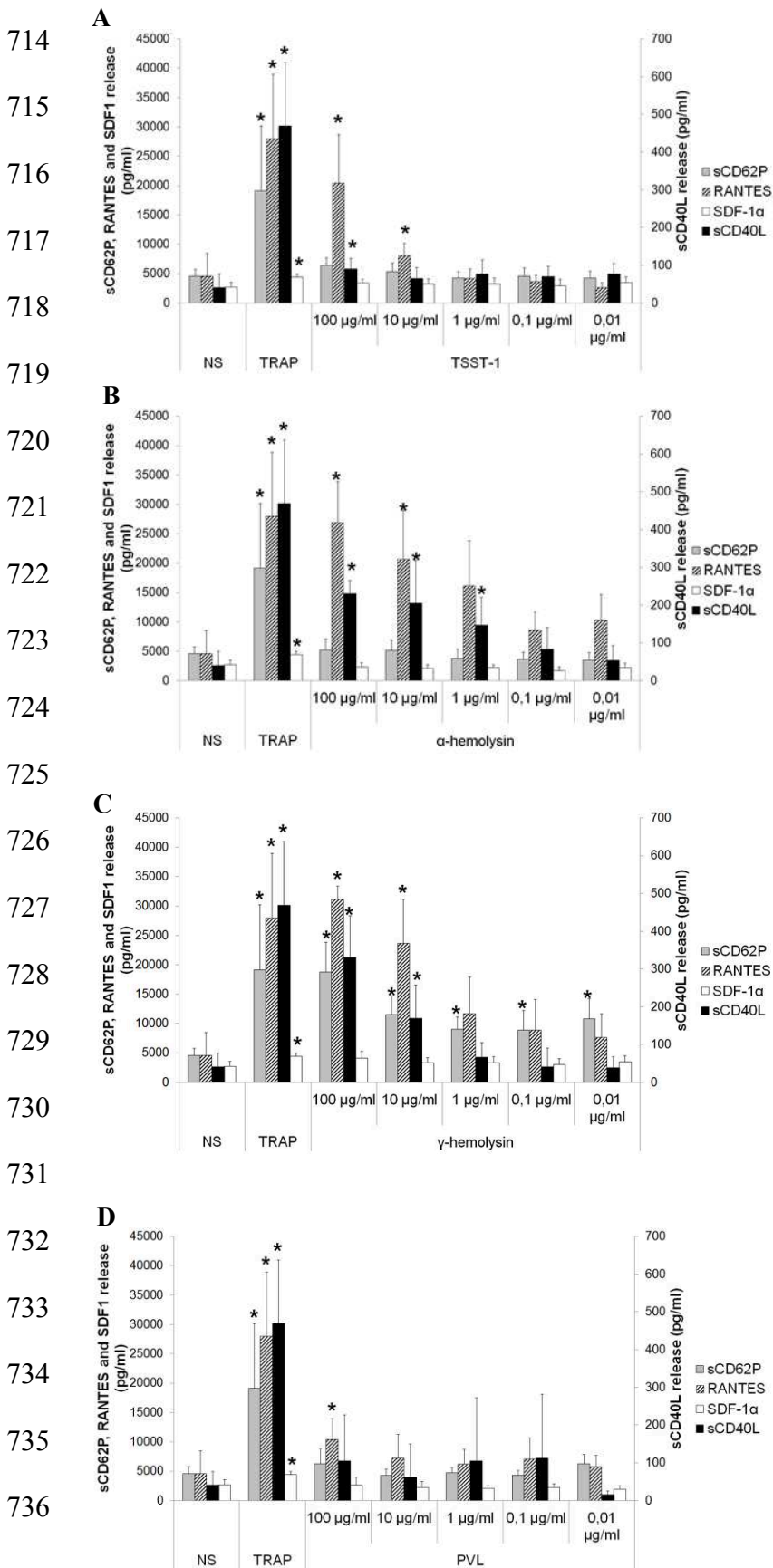
699 Platelets from PRP or washed platelets (WP) were stimulated for 30 min with TSST-1 (A),  $\alpha$ -  
700 hemolysin (B),  $\gamma$ -hemolysin (C) PVL (D) or with two exotoxins simultaneously (E) at  
701 suboptimal concentrations that were defined in the previous single toxin stimulation assays  
702 (i.e. 1, 0.1, 0.001 and 10  $\mu$ g/mL for TSST-1,  $\alpha$ - and  $\gamma$ -hemolysin (Hla, Hlg) and PVL,  
703 respectively). Platelets were stimulated with either Tyrode's buffer as a negative control (NS)  
704 or with TRAP as a positive control. sCD62P, RANTES, sCD40L and SDF-1 $\alpha$  levels were  
705 quantified using ELISA or Luminex<sup>®</sup> assays. Platelets were stimulated with Tyrode's buffer  
706 as a negative control (NS) or with TRAP as a positive control. Data are expressed as ratios  
707 between stimulated platelets and negative control (mean  $\pm$  SEM; n = 5 experiments). \* P<0.05  
708 (two-way ANOVA with repeated measures and Bonferroni post-hoc test; PRP vs. washed  
709 platelets WP).

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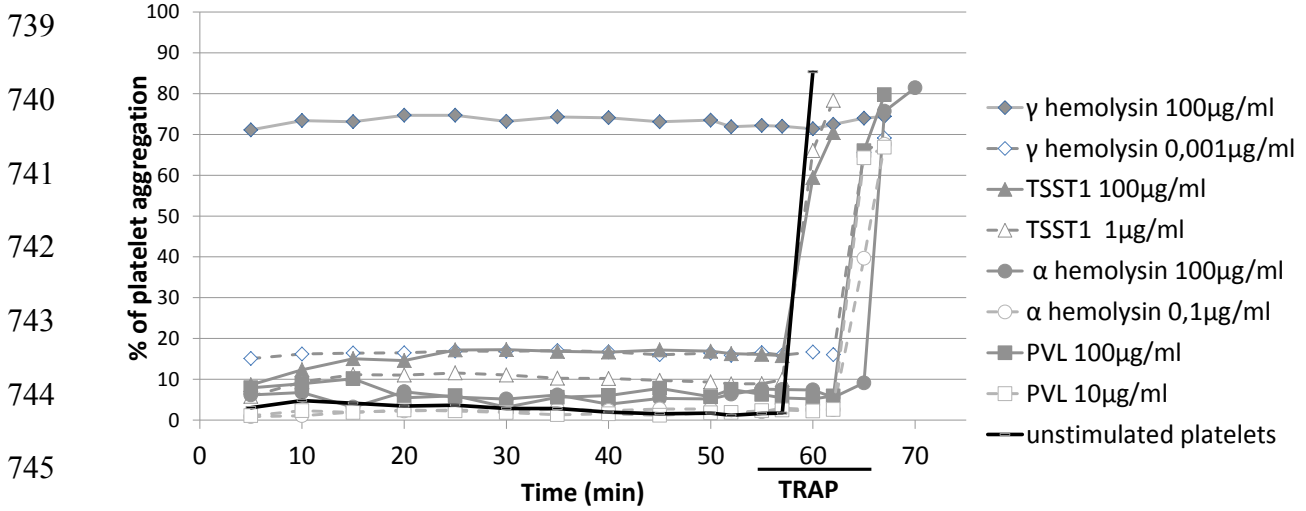
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713 **Figure 1**

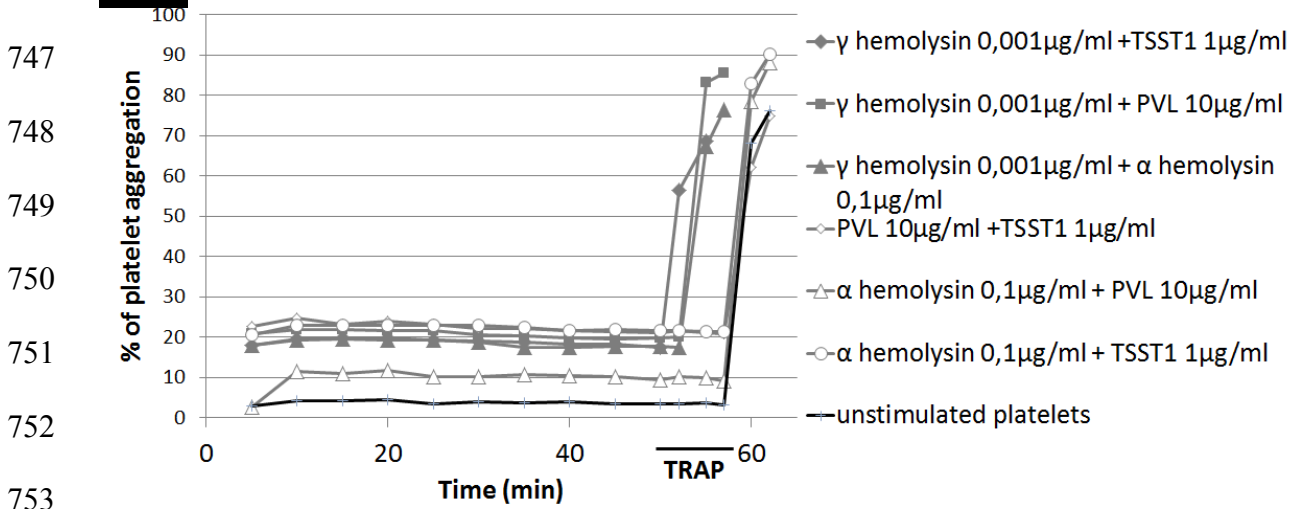


737 **Figure 2**

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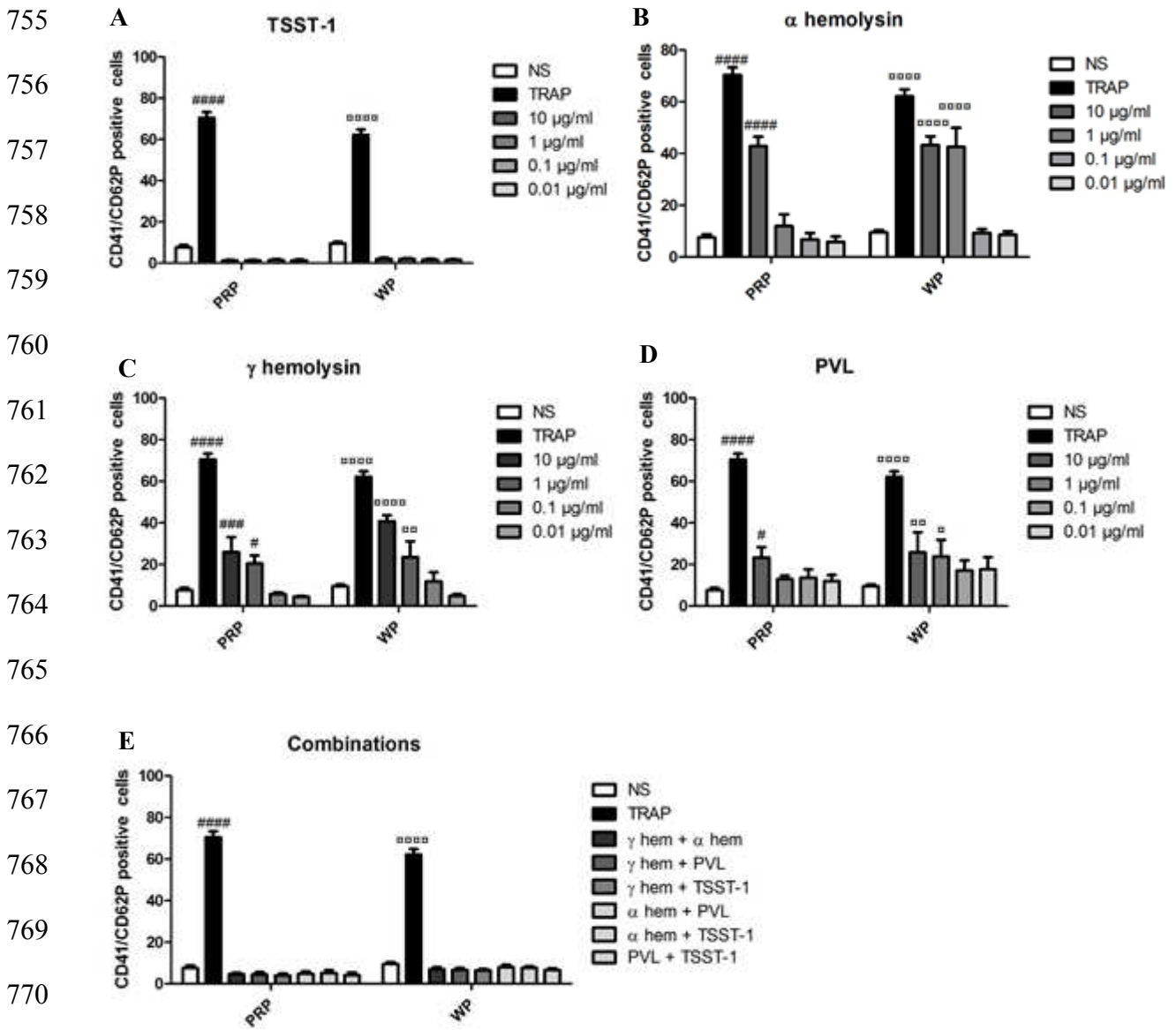


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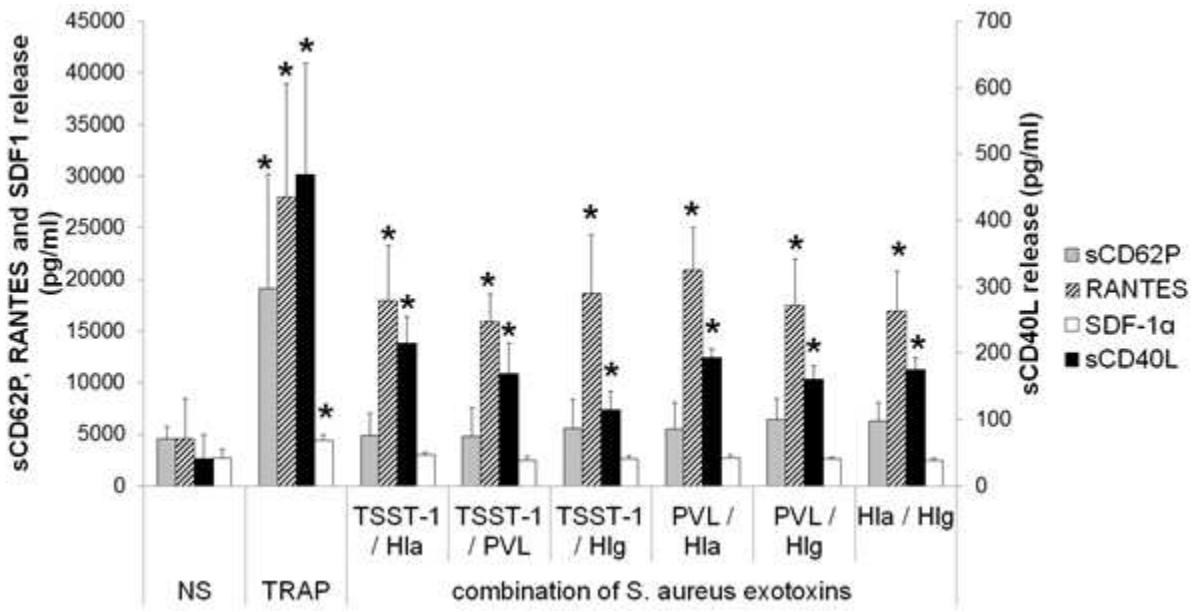


754 **Figure 3**



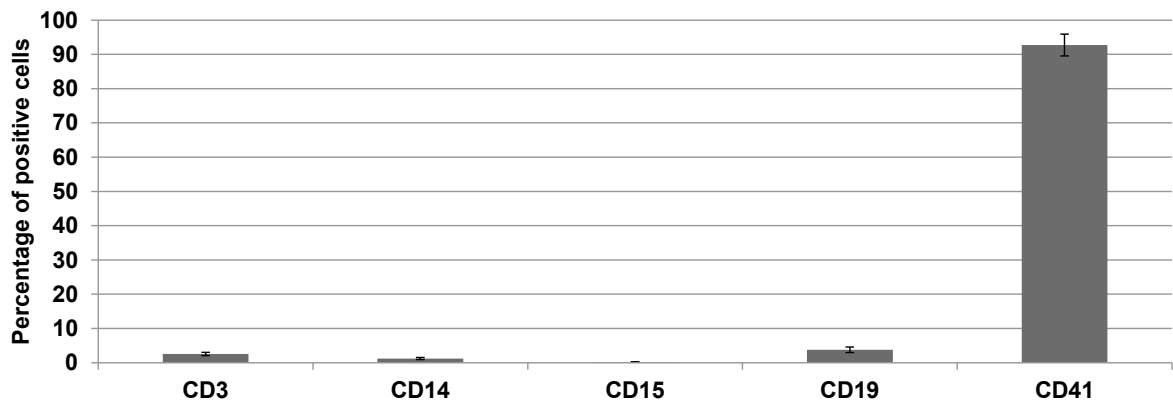
773 **Figure 4**

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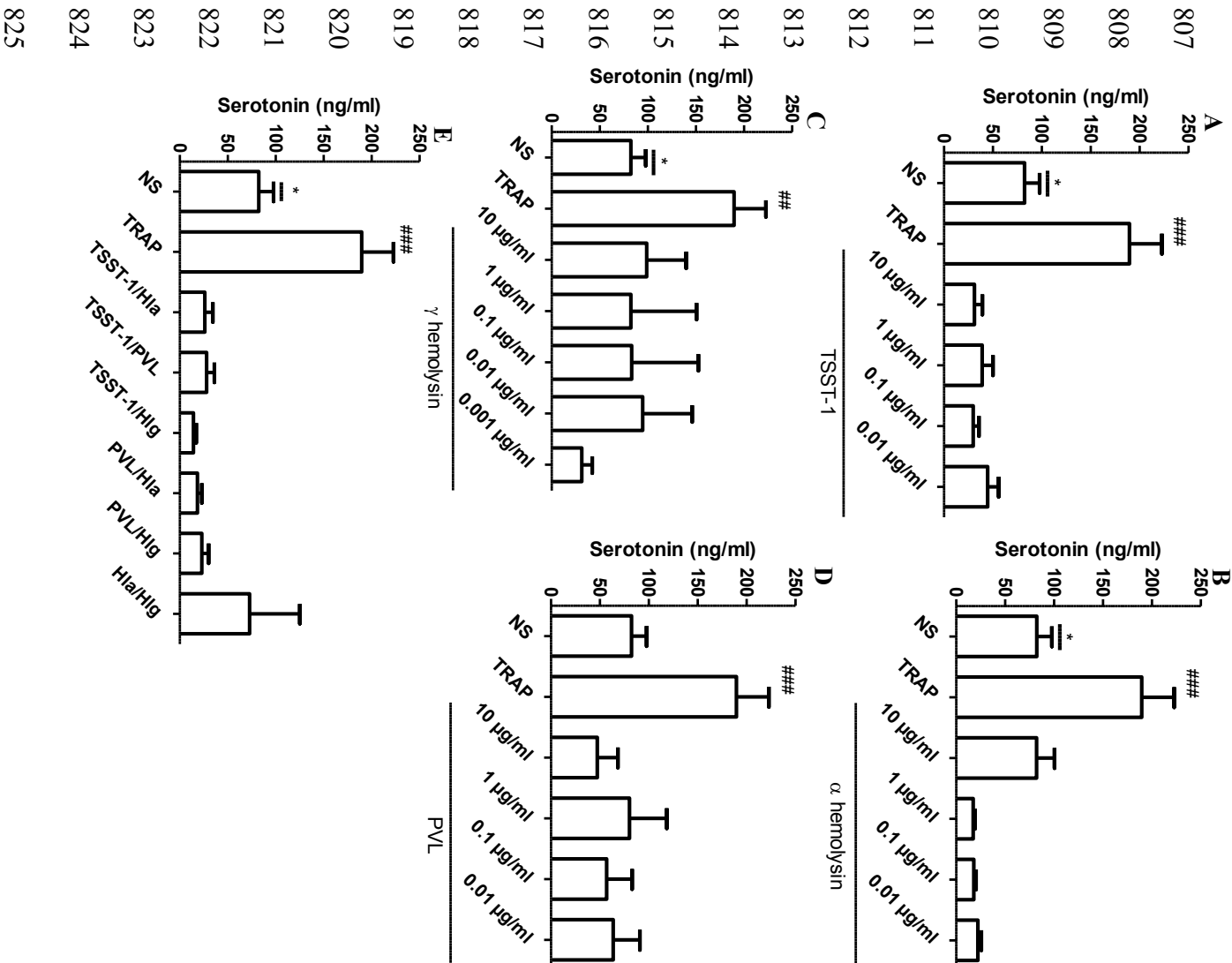


798 **Supplemental Figure 1**

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806 Supplemental Figure 2



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826 Supplemental Figure 3

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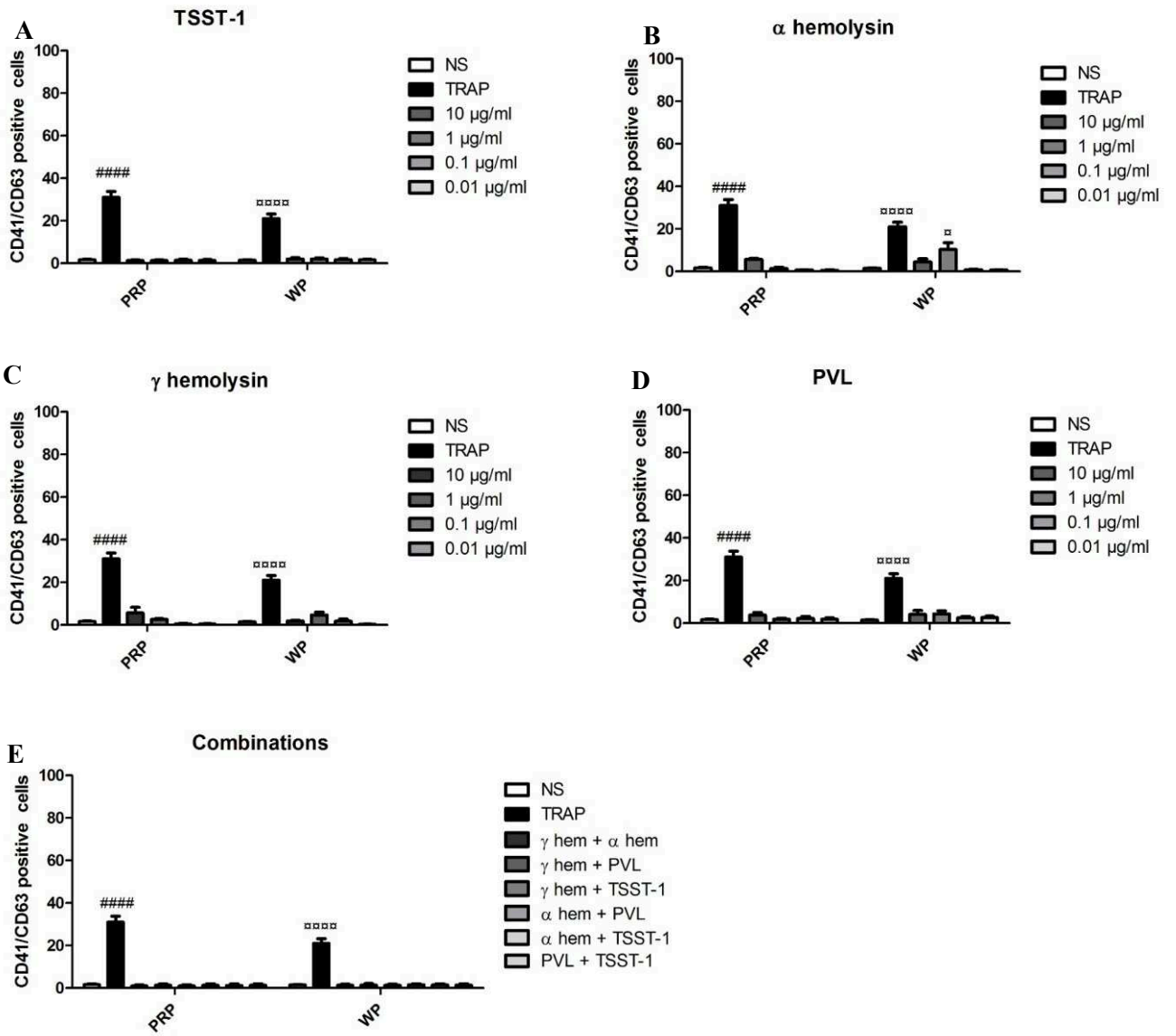
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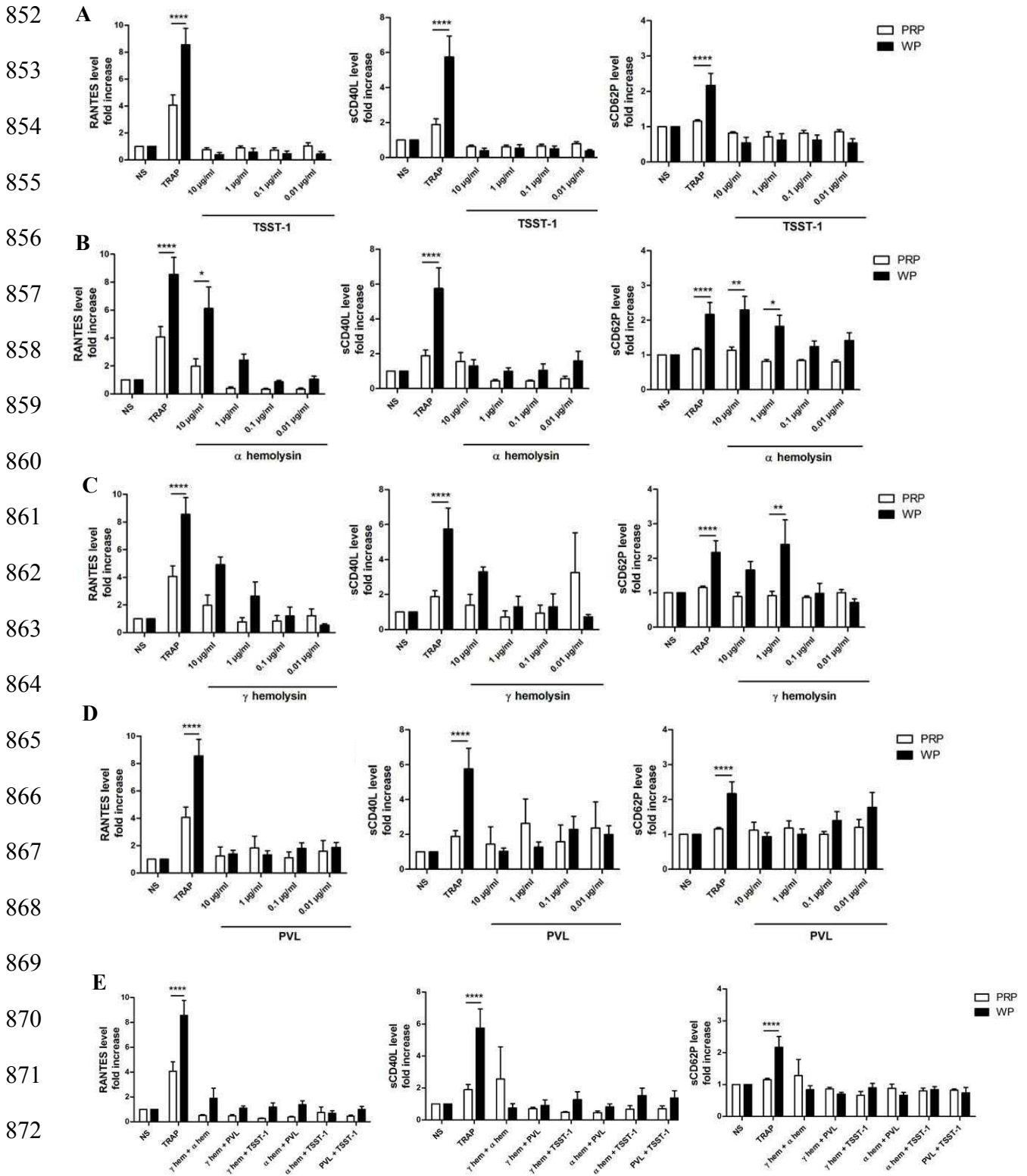
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851 **Supplemental Figure 4**



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## Points clés de l'étude

Cette étude a permis de mieux comprendre les interactions entre différentes exotoxines de *S. aureus* et les plaquettes. Nous avons pu montrer que seule l'hémolysine  $\gamma$ , à sa plus forte concentration (100 $\mu$ g/ml), induit une activation et une agrégation plaquettaire. D'un point de vue inflammatoire, il apparaît que chaque toxine entraîne un profil de sécrétion de facteurs inflammatoires, issus des granules  $\alpha$ , différent selon la toxine, et particulièrement visible pour les hémolysines ( $\gamma$  et  $\alpha$ ). Ceci est en accord avec de précédentes études effectuées par notre équipe et permet de renforcer la capacité des plaquettes à discriminer un signal de danger et d'en adapter leur réponse inflammatoire<sup>166,168</sup>.

Nous avons également évalué si la réponse aux exotoxines de *S. aureus*, mobilisait également les granules denses. Nous avons donc mesuré la libération de sérotonine, principalement retrouvée dans les granules denses. L'absence de libération de sérotonine par les plaquettes exposées aux quatre exotoxines, confirme une réponse inflammatoire orientée des plaquettes, impliquant principalement les granules  $\alpha$  et pas les granules denses.

Pour nous rapprocher des conditions plus physiologiques, nous avons stimulé les plaquettes par des combinaisons de deux exotoxines, utilisées à des concentrations suboptimales. Malgré aucun effet notable de ces combinaisons sur l'expression de marqueurs d'activation et sur l'agrégation des plaquettes, ces dernières réagissent à l'effet cumulatif de deux stimuli via le relargage d'immuno-modulateurs, tels que le sCD40L et RANTES.

La présence de nombreux facteurs plasmatiques pouvant moduler l'action cellulaire, nous avons cherché à déterminer si ces facteurs avaient une incidence sur la réponse plaquettaire aux exotoxines. Les résultats montrent, qu'en absence du plasma, les plaquettes restent activables notamment au travers de l'expression membranaire de CD62P suggérant ainsi que les facteurs plasmatiques n'interviennent pas dans notre modèle d'étude.



## **Article II**

**Acetylsalicylic acid differentially limits the  
activation and expression of cell platelet death  
markers in human platelets exposed to various  
*Staphylococcus aureus* strains**

**(Article published in Scientific Reports)**

## Introduction de l'étude

Après avoir évalué l'effet d'exotoxines de *S. aureus* sur les plaquettes, il était important de continuer ce travail *in vitro* avec des bactéries entières et vivantes afin de prendre en compte la virulence bactérienne dans sa globalité. En effet, nous avons vu que les plaquettes possèdent de nombreux récepteurs pouvant être mis en jeu pour interagir, directement ou indirectement, avec des pathogènes de type bactérien ou viral <sup>160,199</sup>. Aussi, pour prendre en compte les différentes possibilités d'interaction, il était important de passer à un modèle plus proche de la physiopathologie avec des bactéries entières, qui de plus sont issues de bactériémies.

Suite à leur reconnaissance par les plaquettes, les souches de *S. aureus* ont de nombreux effets sur les plaquettes. L'activation des plaquettes par les bactéries et l'induction de l'intégrine GPIIb/IIIa entraîne une forte activation des plaquettes, une adhérence endothéliale ainsi qu'une agrégation plaquettaire <sup>180,204,205</sup>. Cependant, hormis leurs aspects hémostatiques (activation, adhésion et agrégation plaquettaire, libération de facteur de la coagulation), peu d'études se sont intéressées aux aspects inflammatoires des plaquettes lors d'une exposition à une bactérie <sup>206,207</sup>. Il a récemment été montré que l'exoprotéine Staphylococcal superantigen-like 5 (SSL5) induit la formation de microparticules plaquettaires qui provoquent la libération de cytokines inflammatoires (IL-1 $\beta$ , TNF- $\alpha$ , MCP-1) par les monocytes circulants <sup>208</sup>. Actuellement, aucune étude n'a mis en évidence les facteurs immuno-modulateurs libérés par les plaquettes suite à leur activation par *S. aureus*.

Bien que les plaquettes soient des cellules anucléées, elles possèdent les molécules et mécanismes impliqués dans les voies apoptotiques. En effet, les voies intrinsèques et extrinsèques ont très bien été décrites au niveau plaquettaire <sup>104</sup>. L'apoptose plaquettaire permet la régulation physiologique de leur temps de vie. L'idée de la participation de phénomènes apoptotiques lors de la thrombopoïèse dans la formation de pro-plaquettes a été proposée mais elle a été abandonnée au profit de celle proposant que ce mécanisme était utilisé dans la différenciation des mégacaryocytes plutôt que dans celle des plaquettes <sup>209-212</sup>. Suite à une exposition des plaquettes au peptidoglycane de *S. aureus* ou de la bactérie *E. coli*, on observe une apoptose plaquettaire or actuellement, aucune étude n'a montré d'effet de la bactérie *S. aureus* direct sur l'apoptose plaquettaire <sup>103,213</sup>. Il est évident que l'apoptose plaquettaire est l'un des mécanismes participant à la thrombopénie fréquemment observée lors du sepsis et

corrélé au taux de mortalité<sup>66,67,214,215</sup>. De plus, les plaquettes jouent un rôle dans de nombreux mécanismes physiopathologiques du sepsis et notamment dans la dérégulation de l'inflammation ou de la fonction hémostatique<sup>126,216</sup>. Aussi, l'utilisation de molécules visant à inhiber l'activation plaquettaire et l'inflammation subséquente mais également l'apoptose des plaquettes pourraient constituer une nouvelle stratégie thérapeutique.

L'utilisation de molécules anti-inflammatoires comme l'acide acétylsalicylique montrent des effets bénéfiques sur la mortalité au cours du sepsis<sup>153</sup>. D'autres études ont montré que l'utilisation chronique d'ASA diminuait la durée des patients septiques et améliorait le pronostic<sup>217</sup>. Cet effet peut certainement être attribué à l'inhibition de l'activation plaquettaire par l'ASA, qui permet ainsi alors de limiter de la libération de facteurs immunomodulateurs, des interactions avec d'autres types cellulaires (leucocytes, cellules endothéliales...) ou encore le recrutement de neutrophiles et la formation de NETs, délétères lors du sepsis<sup>139</sup>.

Par ailleurs, en plus de leur effet hypocholestérolémiant, les statines possèdent de nombreux effets pléiotropes<sup>218</sup>. En effet, les statines inhibent la réductase de la 3-hydroxyl-3-méthylglutaryl coenzyme (HMG CoA) ce qui entraîne des fonctions anti-oxydantes, anti-apoptotiques et anti-inflammatoires. Une étude effectuée dans un modèle murin de sepsis, par ligature et ponction du caecum, révèle un effet protecteur de certaines statines (Simvastatine, pravastatine) en abolissant les complications inflammatoires associées au modèle<sup>219</sup>. Une étude prospective chez les patients atteints de sepsis a montré une légère réduction du développement cependant ces résultats restent encore controversés<sup>220,221</sup>. De plus, peu d'études ont démontré des effets directs des statines sur les plaquettes ; les seules études se sont focalisées sur le rôle anti-agrégant et n'ont pas investigué la réponse inflammatoire des plaquettes<sup>222,223</sup>.

Finalement, il semble que les patients traités avec de l'acide acétylsalicylique et des statines préalablement à leur hospitalisation sont ceux qui ont des taux de mortalité les plus faibles vis-à-vis du sepsis<sup>224</sup>. D'un point général quant aux données disponibles concernant l'utilisation de l'ASA et des statines sur la pathogénèse du sepsis, il semblerait qu'un effet protecteur sur la pathogénèse du sepsis soit observé mais il reste encore à définir si les plaquettes sont effectivement ciblées par ces traitements<sup>225</sup>.

Par conséquent nous avons cherché, dans notre étude, à déterminer les effets de différentes souches de référence ou issues de bactériémies de *S. aureus* sur la réponse inflammatoire et l'apoptose des plaquettes. Nous avons décidé de travailler avec du « plasma riche en plaquettes » (PRP) afin d'être les plus proches possibles des conditions physiopathologiques et parce que des facteurs plasmatiques pourraient être nécessaires aux interactions entre les plaquettes et *S. aureus* comme décrit dans la littérature et bien que nous ne l'ayons pas observé pour les exotoxines de *S. aureus*<sup>226</sup>. Nous avons également évalué si l'ASA qui a montré des effets bénéfiques lors du sepsis et la fluvastatine, modulaient l'effet des souches de *S. aureus* sur les plaquettes. Cette étude permettra d'évaluer dans quelle mesure les interactions entre plaquettes et *S. aureus* contribuent d'une part à la thrombopénie observée chez certains patients septiques, et d'autre part à la phase hyper-inflammatoire précoce au cours du sepsis. Enfin, nous pourrions investiguer la modulation potentielle de l'ASA et/ou de la fluvastatine, sur la réponse plaquettaire et la pertinence de les cibler lors du sepsis dans le cadre d'une stratégie thérapeutique spécifique.

1 **Acetylsalicylic acid differentially limits the activation and expression of cell**  
2 **death markers in human platelets exposed to Staphylococcus aureus strains**

3  
4 Adrien Chabert<sup>1</sup>, Pauline Damien<sup>1</sup>, Paul O. Verhoeven<sup>1,2</sup>, Florence Grattard<sup>1,2</sup>, Philippe  
5 Berthelot<sup>1,2</sup>, Fabrice Zeni<sup>1,3</sup>, Laurence Panicot-Dubois<sup>4</sup>, Stéphane Robert<sup>4</sup>, Françoise Dignat-  
6 George<sup>4</sup>, Marie-Ange Eyraud<sup>5</sup>, Bruno Pozzetto<sup>1,2</sup>, Bernard Payrastré<sup>6</sup>, Fabrice Cognasse<sup>1,5</sup>,  
7 Olivier Garraud<sup>1,7</sup> and Hind Hamzeh-Cognasse<sup>1\*</sup>

8 <sup>1</sup> EA3064—GIMAP, Université de Lyon, 42023 Saint-Etienne, France

9 <sup>2</sup> Laboratoire des *Agents Infectieux et d'Hygiène*, CHU de Saint-Etienne, 42055 Saint-  
10 Etienne, France

11 <sup>3</sup> Service Réanimation polyvalente, 42055 CHU de St Etienne, France

12 <sup>4</sup> Université Aix Marseille, INSERM UMR-S1076, VRCM, Marseille, France

13 <sup>5</sup> EFS Rhone-Alpes-Auvergne, 42023 Saint-Etienne, France

14 <sup>6</sup> Inserm/UPS UMR 1048 - I2MC, Toulouse, France

15 <sup>7</sup> Institut National de la Transfusion Sanguine, 75015, Paris, France

16  
17 **Correspondence to:** Hind Hamzeh-Cognasse, EA3064—GIMAP, Université de Lyon, 42023  
18 Saint-Etienne, France; Hind.Hamzeh@univ-st-etienne.fr

1 **Abstract**

2 Beyond their hemostatic functions, platelets alter their inflammatory response according to the  
3 bacterial stimulus. *Staphylococcus aureus* is associated with exacerbated inflammation and  
4 thrombocytopenia, which is associated with poor prognosis during sepsis. Acetylsalicylic acid  
5 and statins prevent platelet aggregation and decrease the mortality rate during sepsis. Therefore,  
6 we assessed whether these two molecules could reduce in vitro platelet activation and the  
7 inflammatory response to *S. aureus*. Platelets were exposed to clinical strains of *S. aureus* in the  
8 presence or absence of acetylsalicylic acid or fluvastatin. Platelet activation, aggregation, and  
9 release of soluble sCD62P, sCD40 Ligand, RANTES and GRO $\alpha$  were assessed. Platelet cell  
10 death was evaluated by analyzing the mitochondrial membrane potential, phosphatidylserine  
11 exposure, platelet microparticle release and caspase-3 activation. All *S. aureus* strains induced  
12 platelet activation but not aggregation and decreased the platelet count, the expression of cell  
13 death markers and the release of RANTES and GRO $\alpha$ . Acetylsalicylic acid but not fluvastatin  
14 limited platelet activation and inflammatory factor release and restored the platelet count by  
15 protecting platelets from *Staphylococcus*-induced expression of cell death markers. This study  
16 demonstrates that acetylsalicylic acid limits *S. aureus*-induced effects on platelets by reducing  
17 cell death, revealing new strategies to reduce the platelet contribution to bacteremia-associated  
18 inflammation.

19

## 1 **Introduction**

2 Platelets are chiefly known for their hemostatic role, but they have many other functions,  
3 particularly in innate immunity, host defense against infection and inflammatory processes <sup>1-3</sup>.  
4 Platelets act as sentinels of the vascular system due to the variety of receptors they express, such  
5 as  $\alpha$ IIb $\beta$ 3, GPIb and Toll-like Receptors (TLRs), which enable them to interact with pathogens  
6 as well as endothelial and circulating immune cells for various effector and regulatory functions  
7 <sup>2,4</sup>. Several studies have demonstrated that platelets can sense pathogen-associated molecular  
8 patterns (PAMPs) through pathogen recognition receptors (PRRs) <sup>1,5,6</sup>. Moreover, platelets  
9 produce numerous biological response modifiers (BRMs) such as CD40-ligand (CD40L), a  
10 major immunoregulatory molecule during immune—including inflammatory—responses <sup>7,8</sup>.  
11 *Staphylococcus aureus* (*S. aureus*) is a pathogen that frequently causes sepsis, which can be  
12 particularly severe <sup>9-11</sup>. Indeed, *S. aureus* bears and releases numerous virulence factors,  
13 including enzymes and toxins <sup>12</sup>, which induce either the formation <sup>13</sup> or the degradation of  
14 neutrophil extracellular traps (NETs) that can lead to neutrophil or macrophage apoptosis <sup>14,15</sup>.  
15 Exotoxins are particularly important in the interaction between *S. aureus* and platelets. Indeed,  
16  $\alpha$ -hemolysin accelerates platelet-neutrophil aggregate formation <sup>16</sup> or triggers B-cell lymphoma  
17 (Bcl-3) synthesis by platelets <sup>17</sup>. However, there are much conflicting data concerning platelet  
18 aggregation upon stimulation with *S. aureus*. Staphylothrombin, for instance, by converting  
19 fibrinogen into fibrin promotes the aggregation of platelets <sup>18</sup>. Similarly, Clumping Factor A,  
20 associated with specific antibodies, also induces platelet aggregation <sup>19</sup>. In contrast, lipotechoic  
21 acid has been shown to inhibit platelet aggregation by preventing the mobilization of  
22 intracellular calcium <sup>20</sup>. As very nicely described in the review by Cox et al. <sup>2</sup>, *S. aureus* can  
23 interact with platelets either directly or indirectly through plasma soluble factors, and this  
24 diversity of potentially involved mechanisms further complicates the elucidation of bacteria-  
25 induced platelet aggregation.

26 Sepsis is frequently accompanied by thrombocytopenia; a previous study showed that the latter  
27 follows the course of bloodstream infection and parallels adverse outcomes <sup>21</sup>. These findings  
28 collectively suggest that platelets have a crucial role in the pathophysiology of sepsis <sup>22,23</sup>.  
29 Several mechanisms may cause this reduction in platelet count. An infectious environment can  
30 induce platelet activation and promote disseminated intravascular coagulation, which retains  
31 platelets within thrombi formed around the pathogens and thus removes them from circulation  
32 <sup>24</sup>. Sepsis-associated thrombocytopenia can also result from increased platelet apoptosis. Indeed,  
33 platelets have recently been reported to display features associated with apoptosis pathways

1 such as mitochondrial membrane depolarization and caspase cascade activation<sup>25,26</sup>. Indeed,  
2 increased mitochondrial membrane depolarization in platelets has been described as a biomarker  
3 of severity in sepsis<sup>27,28</sup>. *S. aureus* has also been shown to induce (in vitro) the degradation of  
4 the survival protein Bcl-x<sub>L</sub>, indicating that this bacterium triggers platelet apoptosis<sup>29</sup>.  
5 Molecules such as acetylsalicylic acid (aspirin, ASA) and statins have been used to reduce  
6 complications and improve survival in patients with a bloodstream infection involving *S. aureus*  
7<sup>30-33</sup>, and the antiplatelet effects of aspirin reduce severity markers in experimental *S. aureus*  
8 endocarditis<sup>34</sup>. However, recent studies have also shown that aspirin induces platelet apoptosis  
9<sup>35</sup> and reduces the platelet life span<sup>36</sup>; thus, the effects of this drug may be complex in sepsis  
10 patients<sup>37</sup>. Overall, it appears particularly important to counteract the occurrence of  
11 thrombocytopenia during sepsis and to identify the effects of antiplatelet molecules<sup>38</sup>.  
12 Elucidating the mechanisms that are collectively responsible for platelet impairment in sepsis,  
13 using ASA as a tool, can help determine previously unknown causes and may be translated into  
14 clinical solutions.  
15  
16



## 1 **Results**

### 2 **S. aureus strains differentially alter the platelet count**

3 The effects of several live *S. aureus* clinical strains isolated from bacteremia on normal  
4 platelets were evaluated under ex vivo conditions to assess the residual post-exposure counts of  
5 viable normal platelets. These strains were compared to the effects of a non-clinical, food-  
6 derived strain, *Staphylococcus condimenti*, which is poorly associated with human pathologies  
7 <sup>39</sup>, and a reference methicillin-resistant strain of *S. aureus*, ATCC43866. We observed that *S.*  
8 *condimenti* did not affect the platelet counts compared with the controls (platelets not exposed to  
9 *S. aureus* strains but to a vehicle control, Tyrode's buffer) ( $146.3 \pm 15.6 \times 10^6$  Pl/ml vs.  
10  $163.2 \pm 5.3 \times 10^6$  Pl/ml, respectively,  $p > 0.05$ ). In contrast, all clinical *S. aureus* strains isolated  
11 from bacteremia (SaB) drastically and significantly reduced platelet counts ( $p < 0.001$ )  
12 comparably to the effects of Thrombin receptor activator peptide (TRAP)-stimulation (Figure 1).  
13 Indeed, from an initial count of  $163.2 \pm 5.3 \times 10^6$  Pl/ml, the platelet counts decreased to a range  
14 from  $14.2 \pm 4.9 \times 10^6$  Pl/ml to  $25.2 \pm 7.3 \times 10^6$  Pl/ml after SaB31 and SaB19 stimulation,  
15 respectively, and reached  $8.77 \pm 0.8 \times 10^6$  Pl/ml after stimulation with TRAP.

16 The reduction in platelet counts appeared to be related to an alteration in platelet  
17 morphology that prevented their automatic counting according to their size/structure by the  
18 hematological analyzer. Indeed, fluorescence microscopy experiments showed that 48% of  
19 unstimulated platelets maintained their usual size (less than 4  $\mu\text{m}$ ) and discoid shape  
20 (Supplemental Figure 1a), and 31% of TRAP-stimulated platelets retained their size; the  
21 remainder spread and aggregated (Supplemental Figure 1b), and only 21% of platelets exposed  
22 to the SaB24 bacteremia strain maintained a size less than 4  $\mu\text{m}$  (Supplemental Figure 1c).  
23 Moreover, the SaB24 strain promoted a dramatic change in morphology involving the spreading  
24 and emission of pseudopods.

### 25 **S. aureus strains promote platelet activation but do not lead to platelet** 26 **aggregation**

27 We evaluated the impact of *S. aureus* stimulation on platelets by assessing the expression  
28 of the platelet activation markers CD62P and CD63. TRAP, which was used as a positive  
29 control, induced strong and significant expression of CD62P ( $79.3 \pm 2.3\%$ ) (Figure 2a/  
30 supplemental Figure 2a) and CD63 ( $40.4 \pm 2.6\%$ ) (Figure 2b/ supplemental Figure 2b). The non-  
31 pathogenic staphylococcal strain *S. condimenti* promoted the modest expression of CD62P on

1 platelets ( $37.2\pm 5.4\%$  vs.  $15.6\pm 2.2\%$ ,  $p<0.001$ ) (Figure 2a/ supplemental Figure 2a) and no  
2 significant increase in CD63 ( $10.9\pm 2.6\%$  vs.  $3.4\pm 0.6\%$ ,  $p>0.05$ ) (Figure 2b/ supplemental Figure  
3 2b). Clinical and reference *S. aureus* strains induced significant expression of both CD62P and  
4 CD63 (Figure 2a and 2b/ supplemental Figures 2a and 2b, respectively).

5 *S. condimentii* failed to induce any significant release of RANTES, GRO $\alpha$ , sCD62P,  
6 thromboxane B2 or sCD40L by platelets ( $11816\pm 1276$  pg/ml,  $381\pm 62$  pg/ml,  $41\pm 5$  ng/ml,  
7  $19.8\pm 3.5$  ng/ml and  $690\pm 303$  pg/ml, respectively) compared with unexposed platelets  
8 ( $6753\pm 597$  pg/ml,  $836\pm 311$  pg/ml,  $44\pm 5$  ng/ml,  $36.7\pm 7.2$  ng/ml and  $632\pm 195$  pg/ml for  
9 RANTES, GRO- $\alpha$ , sCD62P, thromboxane B2 and sCD40L, respectively, Figure 3).

10 Every *S. aureus* strain exposed to platelets resulted in the release of significant amounts of  
11 RANTES, with levels ranging between  $16222\pm 3131$  pg/ml and  $21023\pm 2142$  pg/ml, for the  
12 SaB31 and ATCC43866 strains, respectively; this secretion is copious but was less than that  
13 induced by platelet exposure to TRAP ( $36688\pm 1022$  pg/ml) (Figure 3a). Platelets exposed to  
14 ATCC43866, SaB32 and SaB24 strains released significantly elevated amounts of GRO- $\alpha$   
15 ( $2580\pm 296$  pg/ml,  $3112\pm 323$  pg/ml and  $3477\pm 348$  pg/ml, respectively) (Figure 3b). However,  
16 these levels were less than those induced by TRAP ( $4642\pm 1470$  pg/ml vs.  $836\pm 311$  pg/ml in  
17 unexposed platelets,  $p<0.0001$ , Figure 3b). The SaB19 and SaB31 strains induced no significant  
18 release of GRO- $\alpha$  by platelets (Figure 3b). All *S. aureus* strains, excluding SaB19 and SaB31,  
19 the effects of which were not statistically significant, induced a significant increase in specific  
20 cytokines, in contrast to the non-pathogenic strain *S. condimentii*.

21 Only SaB24 stimulation resulted in an increased production of sCD40L, although it was non-  
22 significant ( $4642\pm 1470$  pg/ml, and  $2410\pm 842$  pg/ml, respectively, vs.  $632\pm 195$  pg/ml; (Figure  
23 3c). Neither the *S. aureus* strains nor the non-pathogenic strain *S. condimentii* altered the baseline  
24 production of sCD62p, in contrast to TRAP ( $103\pm 17$  ng/ml compared with the unstimulated  
25 value of  $44\pm 5$  ng/ml,  $p<0.001$ , Figure 3d). The same results were observed for thromboxane B2  
26 release ( $167.6\pm 15.9$  ng/ml for TRAP compared with the unstimulated value of  $36.7\pm 7.2$  ng/ml,  
27  $p<0.001$ , Figure 3e). Overall, similar to the non-pathogenic *S. condimentii* strain, all *S. aureus*  
28 strains failed to induce the release of thromboxane B2, sCD40L and sCD62P by platelets, except  
29 the SAB24 strain, which induced an increase in sCD40L release, although this increase was not  
30 significant. However, the strains succeeded in differentially mobilizing platelet stores, as  
31 demonstrated by the significant release in either RANTES or GRO- $\alpha$ . Thus, *S. aureus* strains

1 altered the secretion profile of exposed platelets with different patterns rather than a unique  
2 pattern.

3 We next assessed whether the aggregation capacity of platelets was altered in the  
4 presence of clinical and reference *S. aureus* strains (because platelets tend to clump around  
5 bacteria to seclude them and limit infection<sup>40</sup>). We did not observe any platelet aggregation  
6 during a 30-min stimulation, a time frame that allowed aggregation in response to TRAP or to  
7 the weaker agonist ADP, with any staphylococcal strains (Figure 4). Five of 35 platelet-rich  
8 plasma (PRP) samples resulted in partial aggregation (Supplemental Figure 3), suggesting a  
9 modest donor dependency, and they were not included in the whole aggregation dataset.

### 10 **S. aureus strains promote platelet cell death**

11 The apparent effect of clinical *S. aureus* strains on viable platelet counts, platelet  
12 activation, changes in morphology and the release of inflammatory factors suggested a robust  
13 upheaval in platelet physiology and led us to question whether exposure to bacteria in vitro  
14 could trigger a platelet cell death signal. To achieve this goal, we assessed membrane  
15 depolarization in platelet mitochondria using DIOC6(3) staining after exposure to various  
16 staphylococcal strains.

17 There was a significant decrease in DIOC6(3) mean fluorescence intensity (MFI) in platelets  
18 following stimulation with a TRAP agonist ( $4239 \pm 447$  arbitrary unit, AU) or the apoptosis  
19 inducer A23187 ( $2229 \pm 151$  AU,  $p < 0.0001$ ) compared with the control ( $12019 \pm 571$  AU,  
20  $p < 0.0001$ ). Exposure of the platelets to all *S. aureus* strains, except *S. condiment*, led to a  
21 significant decrease in the DIOC6(3) MFI ( $3704 \pm 488$  AU for SaB19,  $3153 \pm 601$  AU for SaB24,  
22  $5223 \pm 721$  AU for SaB31,  $4028 \pm 700$  AU for SaB32 and  $3163 \pm 688$  AU for ATCC 43866  
23  $p < 0.0001$ ;  $10614 \pm 1167$  AU, for *S. condiment*,  $p > 0.05$ ) (Figure 5a).

24 Next, we examined platelet phosphatidylserine (PS) exposure, which corresponds to a later stage  
25 of platelet cell death, using Annexin V binding. The TRAP agonist and apoptosis inducer  
26 A23187 promoted significant platelet expression of Annexin V ( $33.7 \pm 1.3\%$ ,  $p < 0.0001$ ;  
27  $31.1 \pm 2\%$ ,  $p < 0.0001$ , respectively) compared with unstimulated platelets ( $16.3 \pm 0.8\%$ ). Platelet  
28 Annexin V expression increased after exposure to all staphylococcal strains, except *S.*  
29 *condiment* ( $28.4 \pm 1.4\%$  for SaB19,  $p < 0.005$ ;  $27.3 \pm 0.7\%$  for SaB24,  $p < 0.01$ ;  $31.4 \pm 3.4\%$  for  
30 SaB31,  $p < 0.005$ ;  $36.5 \pm 2.3\%$  for SaB32,  $p < 0.0001$ ;  $29.8 \pm 2.7\%$  for ATCC 43866,  $p < 0.01$ ),

1 compared with the control ( $16.3\pm 0.8\%$ ), indicating increased PS exposure after *S. aureus*  
2 treatment (Figure 5b).

3 We also measured platelet microparticle (PMP) formation. Both TRAP and A23187 induced  
4 significant release of PMPs ( $3194\pm 595$  PMPs/ $\mu\text{l}$ ,  $p<0.01$ ;  $3075\pm 500$  PMPs/ $\mu\text{l}$ ,  $p<0.01$ )  
5 compared with unstimulated platelets ( $1576\pm 118$  PMPs/ $\mu\text{l}$ ). However, despite a non-significant  
6 increase in PMP formation in the presence of clinical strains, no difference was observed for *S.*  
7 *condimenti* ( $1405\pm 287$  PMPs/ $\mu\text{l}$ ) and ATCC 43866 ( $1518\pm 243$  PMPs/ $\mu\text{l}$ ), but every other strain  
8 exhibited increased PMP formation ( $2248\pm 283$  PMPs/ $\mu\text{l}$  for SaB19;  $2400\pm 303$  PMPs/ $\mu\text{l}$  for  
9 SaB24;  $2182\pm 326$  PMPs/ $\mu\text{l}$  for SaB31;  $2389\pm 243$  PMPs/ $\mu\text{l}$  for SaB32), in contrast to the  
10 unstimulated platelets ( $1576\pm 118$  PMPs/ $\mu\text{l}$ ) (Figure 5c). Similarly, the level of active caspase-3  
11 increased only upon TRAP stimulation ( $3.0\pm 0.7$ -fold increase vs unstimulated control, NS,  
12  $p<0.001$ , Figure 5d). Although not significant, the ATCC43866 *S. aureus* strain induced an  
13 increase in activated caspase-3 expression comparable to that triggered by the A23187 apoptosis  
14 inducer ( $1.7\pm 0.1$ -fold increase for ATCC43866 vs  $1.9\pm 0.3$ -fold increase for A23187  
15 stimulation).

### 16 **ASA, in contrast to fluvastatin, reduces the *S. aureus*-induced reduction in** 17 **platelet counts**

18 We next examined whether antiplatelet drugs can interfere with the effects of *S. aureus*  
19 strains on platelets. We selected two distinct antiplatelet drugs, ASA and fluvastatin, which  
20 target cyclooxygenase and 3-hydroxy-3-methylglutaryl coenzyme A reductase, respectively<sup>38,41</sup>.

21 First, we assessed the ability of antiplatelet molecules to prevent the platelet count decrease after  
22 exposure to *S. aureus* strains. Notably, when there was no reduction in platelet counts (such as  
23 in the presence of *S. condimenti*), and neither ASA nor fluvastatin had any detectable effect. In  
24 contrast, ASA and fluvastatin did not prevent the platelet count reduction induced by platelet  
25 exposure to TRAP. Furthermore, fluvastatin did not reverse the platelet count reduction  
26 observed after the platelets were exposed to any of the clinical or reference strains of *S. aureus*.  
27 In sharp contrast, ASA partially prevented the platelet count reduction caused by platelet  
28 exposure to the SaB31, SaB32 and ATCC 43866 strains compared with the control vehicle  
29 (water) ( $71\pm 14.7$  vs.  $14.2\pm 4.9\times 10^6$  Pl/ml,  $p<0.0001$ ;  $58.7\pm 13.6$  vs.  $16.2\pm 4.2\times 10^6$  Pl/ml,  $p<0.01$ ;  
30  $63.5\pm 9.6$  vs.  $22.2\pm 3.3\times 10^6$  Pl/ml,  $p<0.01$ , respectively) (Figure 6). ASA was less effective when

1 platelets were exposed to SaB19 or SaB24 ( $49.7 \pm 10.4$  vs.  $25.2 \pm 7.3 \times 10^6$  Pl/ml, and  $40.5 \pm 6.7$  vs.  
2  $17.3 \pm 4.7 \times 10^6$  Pl/ml,  $p > 0.05$ , respectively) (Figure 6).

### 3 **ASA limits S. aureus-induced platelet activation**

4 Next, we examined whether ASA affects the platelet activation phenotype following  
5 platelet exposure to bacteria. We observed a significant decrease in CD62P or CD63 expression  
6 by platelets following ASA treatment after exposure to any of the Staphylococcus strains—the  
7 clinical ones as well as *S. condimentii* (Figure 7a/b). Indeed, CD62P<sup>+</sup> platelet expression was  
8 reduced to  $22.2 \pm 3.8\%$ ,  $18.4 \pm 0.9\%$ ,  $18.6 \pm 2.0\%$ ,  $10.3 \pm 1.9\%$ ,  $23.8 \pm 3.3\%$  and  $27.6 \pm 3.7\%$   
9 following exposure to *S. condimentii*, SaB19, SaB24, SaB31, SaB32 and ATCC 43866,  
10 respectively, in the presence of ASA, compared with  $37.2 \pm 5.4\%$ ,  $54.3 \pm 4.2\%$ ,  $61.3 \pm 3.7\%$ ,  
11  $55.8 \pm 4.0\%$ ,  $70.8 \pm 3.4\%$  and  $65.0 \pm 5.0\%$  for the same strains, respectively, in the absence of ASA  
12 ( $p < 0.0001$ ).

13 A similar phenomenon was observed for CD63 expression, which was significantly reduced in  
14 the presence of ASA, regardless of the *S. aureus* strain used. For example, the maximal CD63  
15 expression by platelets upon *S. aureus* SaB32 exposure reached  $28.2 \pm 5.1\%$  but was reduced to  
16  $5.6 \pm 1.2\%$  in the presence of ASA ( $p < 0.0001$ ). Notably, fluvastatin did not reverse any of the *S.*  
17 *aureus*-induced activation signals assessed under these conditions (Figure 7a/b).

18 Thereafter, we assessed whether ASA and fluvastatin could reduce the release of  
19 immunomodulatory factor by platelets exposed to the various *S. aureus* strains. Again,  
20 fluvastatin had no noticeable effects and did not prevent platelet secretion following exposure to  
21 clinical *S. aureus* strains (Figure 7c-d). In contrast, ASA significantly reduced the secretion of  
22 RANTES or GRO $\alpha$ , although for a limited number of *S. aureus* strains (Figure 7c/d). ASA  
23 reduced the secretion of RANTES when platelets were exposed to SaB31 or ATCC43866  
24 ( $8038 \pm 1770$  pg/ml and  $14617 \pm 1315$  pg/ml, respectively, vs.  $16222 \pm 3101$  pg/ml and  
25  $21023 \pm 2142$  pg/ml;  $p < 0.05$ ). ASA limited the secretion of GRO $\alpha$  by platelets when they were  
26 exposed to SaB24 and SaB32 but not to others ( $1920 \pm 484$  pg/ml and  $1216 \pm 305$  pg/ml,  
27 respectively, vs.  $3477 \pm 348$  pg/ml;  $p < 0.01$  or  $3112 \pm 323$  pg/ml  $p < 0.001$ ) (Figure 7d). A moderate  
28 decrease in the secretion of GRO $\alpha$  by platelets exposed to SaB19, SaB31 and ATCC43866 was  
29 observed but remained modest and non-significant (Figure 7d).

30 We next investigated whether the modest aggregation observed for the very few PRP samples  
31 ( $n=5$ ) exposed to staphylococcal stimuli was altered by either ASA or fluvastatin. Indeed, we

1 observed that ASA but not fluvastatin efficiently protected platelets against *S. aureus*-induced  
2 aggregation (Supplemental Figure 3). These data are limited in that the results were not  
3 universal among our platelet samples, but they indicate the potential benefit of ASA at least for  
4 platelets that aggregate in response to *S. aureus*.

### 5 **ASA efficiently protects platelets against *S. aureus*-induced cell death**

6 We then investigated whether ASA affects the reduction in *S. aureus*-induced platelet  
7 counts, i.e., induced cell death. As expected, since fluvastatin had little effect on the reduction in  
8 platelet counts in the presence of bacteria, this drug did not prevent membrane depolarization in  
9 platelet mitochondria induced by *S. aureus* (Figure 8a). In contrast, ASA had a strong—almost  
10 complete—protective effect against *S. aureus*-induced platelet cell death. Indeed, the DIOC6(3)  
11 MFI was reduced to values between  $3153\pm 601$  and  $5253\pm 721$  AU upon bacterial exposure in the  
12 absence of treatment. However, the DIOC6(3) MFI was significantly restored to levels between  
13  $8466\pm 426$  and  $9390\pm 965$  AU in the presence of ASA ( $p<0.0001$  for SaB19, SaB24 and  
14 ATCC43866 strains;  $p<0.001$  for SaB32 strain and  $p<0.01$  for SaB31 strain), which were  
15 comparable to that of unstimulated platelets ( $12019\pm 571$  AU).

16 Concerning platelet PS exposure, which is usually observed during the final step of apoptosis  
17 and during necrosis<sup>42</sup>, ASA reduced platelet Annexin V expression induced by all *S. aureus*  
18 strains but showed no effect on platelets exposed to *S. condimentii* since this strain did not  
19 induce significant PS exposure. Indeed, the percentage of cells with CD41/Annexin V decreased  
20 from values between  $27.4\pm 0.7\%$  and  $36.6\pm 2.3\%$  after staphylococcal exposure alone to  
21  $16.7\pm 1.4\%$  and  $19.1\pm 0.8\%$  in the presence of ASA ( $p<0.0001$  for SaB32,  $p<0.05$  for SaB19,  
22 SaB24, SaB31 and ATCC43866 strains, Figure 8b). These levels were comparable to those of  
23 unstimulated platelets ( $16.57\pm 0.9\%$ ), indicating a strong protective effect of ASA on platelet cell  
24 death induced by *S. aureus* strains.

25 Finally, no difference was observed for each *Staphylococcus* strain in the presence of ASA with  
26 respect to the release of PMPs (Figure 8c) or the expression of active caspase-3 (Figure 8d). The  
27 presence of fluvastatin had no effect either on the formation of PMPs or on the expression of  
28 active caspase-3.

29

## 1 **Discussion**

2 We found a pathogenic effect of clinical and reference strains of *S. aureus* on platelets  
3 from healthy donors, while *S. condimentii*, a food-derived strain associated only once with  
4 bloodstream infection <sup>39</sup>, had a moderate effect. Indeed, *S. aureus* is predominantly reported  
5 during bacteremia and causes a variety of infections that frequently result in death <sup>9,10</sup>. We  
6 observed that freshly obtained platelets exposed to clinical and reference *S. aureus* strains for 30  
7 min showed an altered morphology and expressed activation markers, such as CD62P and  
8 CD63, with a strong effect on CD62P, regardless of the strains used. Genotyping of the strains  
9 revealed that while some genes associated with virulence were present in all strains, for instance  
10 ClfA/B, FnbA/B, LukS/F, Hla, others, such as Cna EntA or SasG, were expressed only by some  
11 strains (Supplemental Table 1). However, no strain appeared to have a more pronounced effect  
12 than another on the platelet response. Thus, the use of isogenic mutants to examine the  
13 participation of each virulence factor in the induction of the platelet response would be helpful.  
14 In addition, none of the tested strains induced platelet aggregation, except for 5 donors. Indeed,  
15 strain SAB24 induced aggregation in 2 donors, as did strain SAB32, whereas strain  
16 ATCC43866 induced the aggregation of only one donor. Therefore, the host specificity of the  
17 response should also be taken into account.

18 Concerning immunomodulatory factors, every strain of *S. aureus* but not *S. condimentii*  
19 induced significant release of RANTES and of GRO $\alpha$  for some strains. Thus, platelets exposed  
20 to *S. aureus* strains could actively contribute to the elevated plasma levels of RANTES and  
21 GRO $\alpha$  that have been described during the course of *S. aureus* bloodstream infection and sepsis  
22 <sup>43,44</sup>. GRO $\alpha$  levels have also been shown to correlate with several clinical scores (Pediatric Risk  
23 of Mortality, Sepsis-related Organ Failure Assessment and Disseminated Intravascular  
24 Coagulation scores) <sup>45</sup>. Regarding RANTES, murine models of lipopolysaccharide-induced  
25 acute lung injury <sup>46</sup> and of polymicrobial sepsis <sup>47</sup> revealed the involvement of platelet-derived  
26 RANTES in lung damage, underscoring the contribution of this chemokine and the potential  
27 implication of platelets in sepsis-associated complications. Interestingly, we observed in our  
28 study that in the presence of ASA, the expression of activation makers on platelets was restored  
29 to unstimulated levels, and the release of RANTES and GRO $\alpha$  was reduced.

30 Given that we used a multiplicity of infection (MOI) of 0.1 and that the bacterial  
31 suspension was washed to remove soluble bacterial products, it was likely that platelet  
32 activation was propagated by thromboxane B2 released by platelets since ASA could reduce this

1 activation. However, we did not observe any release of this factor in the presence of *S. aureus*  
2 strains. This finding suggests that under the present conditions, ASA either might reduce  
3 prostaglandin E2 rather than thromboxane B2, or exert Cox-independent effects, as observed for  
4 NF- $\kappa$ B, although described for high doses of ASA <sup>48</sup>.

5 In addition to their well described PS-related procoagulant activity <sup>49</sup>, platelet  
6 microparticles have been shown to be predominantly found in the circulation during sepsis <sup>50</sup>, to  
7 have an inflammatory role in rheumatoid arthritis <sup>51</sup> and also to have immunosuppressive  
8 properties <sup>52,53</sup>. Therefore, their contribution to the inflammatory/ immunosuppressive  
9 component of sepsis should not be underestimated. Although not significant under our  
10 conditions, probably because of a requirement for a longer contact duration, the increased  
11 release of platelet microparticles following *S. aureus* exposure could synergize with the release  
12 of RANTES and GRO $\alpha$  to promote an immunomodulatory environment, contributing to sepsis  
13 pathophysiology.

14 These data, on the one hand, confirm that platelet secretion triggered by exposure to  
15 infectious pathogens is not “all or none,” as diverse *S. aureus* strains elicited different secretion  
16 profiles, and on the other hand, show that platelet inhibitors such as ASA also have differential  
17 effects on these secretion patterns. These results support previous studies showing that human  
18 platelets have the capacity to differentially detect external signals and adapt their inflammatory  
19 responses <sup>54-56</sup>. In addition to their hemostatic role, platelets have an important inflammatory  
20 function. In fact, platelets are sentinels in the circulation due to the number of immunoreceptors  
21 expressed on their surface (Fc $\gamma$ RII, Toll-like receptors, complement receptor or  $\alpha$ IIb $\beta$ 3), which  
22 detect pathogens and participate in inflammation. Moreover, platelets interact with macrophages  
23 and neutrophils, leading to pathogen phagocytosis and NET formation <sup>4,57</sup>. Although many  
24 studies have investigated the effects of bacteria on platelet hemostatic responses <sup>58-60</sup>, few  
25 studies have focused on their immunoregulatory role <sup>61,62</sup>, although this could be a potential  
26 target to reduce bacterial sepsis <sup>38</sup>.

27 Previous studies have reported platelet apoptosis in response to *Escherichia coli* or  
28 peptidoglycan, a component of *S. aureus* cell walls <sup>29,63</sup>. In this study, we showed that in  
29 contrast to the food-derived *S. condimentii* strain, exposure to clinical or reference strains of *S.*  
30 *aureus* promoted PS exposure and mitochondrial membrane depolarization, which are features  
31 of both apoptosis and necrosis in platelets. However, the slight increase in both PMP release and  
32 active caspase-3 expression, although not significant, suggests the initiation of an apoptotic



1 process that might require a longer exposure to *S. aureus* strains but does not exclude the  
2 occurrence of a necrotic process. Indeed, it has previously been elegantly shown that  
3 mitochondrial membrane depolarization and PS exposure were induced either by an apoptosis  
4 inducer or by the thrombin/collagen combination that induces necrotic platelets <sup>42</sup>. Thus, the use  
5 of a cell death marker, such as GSAO, associated with the expression of CD62P would help  
6 discriminate between apoptosis and necrosis under our conditions.

7 Conversely, we did not observe platelet aggregation during staphylococcal strain exposure,  
8 indicating that platelets have the capacity to distinguish inflammatory from aggregation  
9 responses depending on the bacterial types <sup>59,64</sup> or concentration <sup>65</sup>. However, there remains  
10 some controversy over the induction of platelet aggregation by *S. aureus* or its compounds.  
11 Several very nice studies report that *S. aureus* promotes platelet aggregation through Clumping  
12 Factor A <sup>60</sup>, which binds fibrinogen and IgG and activates the platelet GPIIb/IIIa and FCγRIIa  
13 receptors through the thromboxane A<sub>2</sub> and ADP pathways <sup>66</sup>. However, platelets also harbor  
14 innate immunity receptors, such as TLR2 and TLR4, which, when triggered, may <sup>66,67</sup> or may  
15 not <sup>20,68</sup> lead to platelet aggregation. These phenomena raise questions regarding the platelet  
16 response according to the receptor engaged by bacteria and, more likely, the synthesis of the  
17 signalling pathways within platelets when several receptors are simultaneously engaged due to  
18 the multiplicity of potentially involved ligand/receptor pairs. Moreover, it is also important to  
19 consider the variability in virulence factor expression by bacterial strains because some, such as  
20 staphylothrombin, may promote aggregation <sup>18</sup>, while others, such as alpha-hemolysin, may  
21 inhibit it, as elegantly demonstrated by Powers et al. <sup>69</sup>. Finally, parameters of the host might  
22 also intervene, as observed in our study showing that under the same stimulation conditions,  
23 platelets from 5 of 35 donors aggregated in response to 3 of our strains.

24 We also evaluated the ability of molecules with marked antiplatelet activity, such as ASA and  
25 fluvastatin, to reduce platelet activation and secretion of inflammatory factors and potentially  
26 also limit platelet cell death <sup>30-33,70</sup> and PMP release <sup>71</sup>. Regarding statins, we observed that  
27 fluvastatin had no inhibitory effect on platelets after any in vitro *S. aureus* strain exposure,  
28 whereas recent studies have shown an inhibition of platelet hemostatic function with fluvastatin  
29 via downregulation of thromboxane A<sub>2</sub> platelet synthesis <sup>72-74</sup>. Statins such as fluvastatin, but  
30 particularly simvastatin, have shown a protective effect against abdominal sepsis predominantly  
31 by improving endothelial function and decreasing pulmonary inflammation <sup>75</sup>. Additional  
32 studies are needed to understand the effects of statins on platelet function during sepsis.

1 According to Merx et al., enhanced nitric oxide and cyclic GMP formation could play a role in  
2 the antiplatelet activity of simvastatin after sepsis induction <sup>70</sup>.

3 In contrast, in the present study, the administration of ASA (500  $\mu$ M) was shown to modify  
4 platelet functions with a protective effect on the decrease in platelet count, a significant decrease  
5 in the activation phenotype and complete protection against platelet-induced cell death after  
6 exposure to clinical or reference *S. aureus* strains. Platelet cell death, whose mechanism could  
7 be related to apoptosis or necrosis <sup>76</sup>, would thus be implicated in the decreased platelet counts  
8 that occur during *S. aureus* bacteremia. Conversely, it has been shown that ASA (used at 5 mM,  
9 10 times more concentrated than in our study) induced platelet apoptosis via the upregulation of  
10 pro-apoptotic proteins or mitochondrial pathways <sup>35,36</sup>.

11 Platelet cell death induced by *S. aureus* strains can promote deleterious responses during sepsis  
12 via different mechanisms: (1) thrombocytopenia, which represents an important complication of  
13 sepsis; (2) immunomodulatory factors present in platelets or in microparticles, which are  
14 secreted and participate in the inflammatory phase of sepsis; and (3) the reduction in ‘vital  
15 NETosis’, in which platelet-induced NETs help capture and clear bacteria <sup>77</sup>. However, the  
16 detrimental role of NETs should not be underestimated because NETs have been shown to  
17 enhance thrombosis <sup>78</sup>, although new evidence tends to distinguish the effects of intact NETs  
18 from those of DNA and histones in the induction of coagulation <sup>79</sup>.

19 Due to their dual hemostatic and inflammatory roles, platelets could be targeted by ASA to limit  
20 the harmful effects of *S. aureus* on platelets and improve clinical outcomes in *S. aureus*-induced  
21 bacteremia <sup>80,81</sup>. However, in the lipopolysaccharide-induced sepsis model in mice, ASA  
22 pretreatment reduced the survival rate by inhibiting the protective effect of platelets on  
23 macrophage-dependent inflammation <sup>82</sup>. Thus, more studies with more complex models are  
24 needed to better understand the inflammatory role of platelets during sepsis. Drugs such as ASA  
25 that have direct and indirect effects on platelets may be promising treatments to limit the  
26 severity of clinical sepsis; however, further studies are needed.

27

## 1 **Methods**

### 2 **Ethical considerations**

3 In accordance with the French Public Health Code (article L. 1223-3) and the  
4 procedures of the French Blood Bank ethical board (Etablissement Français du Sang – EFS),  
5 volunteer blood donors signed the donation form indicating that they did not preclude the use  
6 of their donation for non-therapeutic purposes. Thus, in accordance with the Declaration of  
7 Helsinki, informed and written consent has been obtained from all the healthy donors who  
8 participated in this study for the sampling of blood for scientific purposes. Analyses have  
9 been performed anonymously, in accordance with the relevant guidelines and regulations of  
10 the Lyon University (UJM, Faculty of Medicine, Saint-Etienne, France).

### 11 **Preparation of platelets and Staphylococcus strains**

12 PRP was prepared as previously described<sup>83</sup>. Briefly, peripheral blood was collected  
13 from healthy donors in endotoxin-free tubes with 3.2% sodium citrate (Becton Dickinson, San  
14 Jose, CA, USA) and centrifuged at  $150 \times g$  for 10 min at 22°C. There was no detectable  
15 contamination of platelets by residual mononuclear cells as assessed by flow cytometry (CD3-  
16 T cells, CD19-B cells, or CD14-monocytes) (Supplemental Figure 4). Platelets were counted  
17 in freshly prepared PRP with a MS4s Hematology analyzer (Melet Schloesing, Osny, France),  
18 and cell suspensions were adjusted to  $3 \times 10^8$  Pl/ml with Tyrode's buffer (Sigma-Aldrich,  
19 Saint Quentin-Fallavier, France) containing 0.04 U/ml of apyrase (Sigma-Aldrich, Saint  
20 Quentin-Fallavier, France) and 0.3  $\mu\text{g/ml}$  of PGI<sub>2</sub> (Sigma-Aldrich, Saint Quentin-Fallavier,  
21 France).

22 The tested staphylococcal strains were the *S. aureus* ATCC43866 reference strain, 4  
23 *S. aureus* clinical strains isolated from bacteremia patients (SaB) 19, 24, 31, 32 and the non-  
24 pathogenic food-derived *S. condimentii* (SC) strain. For each experiment, the bacterial strains  
25 were plated, from frozen cryobead stocks, on blood agar plates and incubated overnight at  
26 37°C. Then, after checking for the absence of contaminating colonies and variants, a Mueller-  
27 Hinton broth was seeded with a small number of colonies and cultured under agitation for 24  
28 h at 37°C. A Mueller-Hinton broth was then seeded with 2 ml of the 24 h-culture and  
29 incubated at 37°C for 4 h to reach the exponential phase. Bacterial cells were washed,  
30 resuspended in Tyrode's Buffer and adjusted to  $1.2 \times 10^8$  CFU/ml.

## 1 **Platelet exposure to Staphylococcus strains**

2 Staphylococcal strains (50  $\mu$ l, corresponding to  $6 \times 10^6$  CFU) or Tyrode's Buffer (50  $\mu$ l,  
3 as a non-stimulated (NS) control) were added to freshly prepared platelets (200  $\mu$ l,  
4 corresponding to  $6 \times 10^7$  platelets) in a 96-well uncoated polystyrene culture microplate to  
5 obtain a multiplicity of infection (MOI) of 0.1 (i.e., 1 bacteria for 10 platelets). Next, 50  $\mu$ l of  
6 either ASA (500  $\mu$ M final concentration, Sigma-Aldrich, Saint Quentin-Fallavier, France),  
7 fluvastatin (10  $\mu$ M final concentration, Sigma-Aldrich, Saint Quentin-Fallavier, France) or  
8 control vehicle (water, the resolubilization liquid for both ASA and fluvastatin) were added to  
9 the suspension. The concentrations of ASA and fluvastatin used in vitro to antagonize the  
10 effects of staphylococcal stimulation were determined by stimulating the platelets with the  
11 ATCC43866 reference strain in the presence of increasing concentrations of antiplatelet  
12 molecules, ranging from 0 to 5000  $\mu$ M for ASA and 100  $\mu$ M for fluvastatin, based on  
13 previous studies<sup>72,84</sup>. The lowest concentration of antiplatelet molecule that inhibited  
14 RANTES release by platelets was chosen for further experiments (i.e., 500  $\mu$ M final  
15 concentration, Supplemental Figure 5). However, 10  $\mu$ M fluvastatin failed to inhibit RANTES  
16 release by platelets and was therefore used as a control for further experiments. The culture  
17 plate containing the platelet and bacterial mix was then gently centrifuged for 5 min at  $300 \times$   
18 g to facilitate and standardize contact between platelets and bacteria. The plate was then  
19 incubated, without stirring, for 30 min at room temperature. As positive controls for either  
20 activation or platelet cell death, platelets were also stimulated for 30 min at room temperature  
21 with 50  $\mu$ g/ml thrombin receptor activator peptide (TRAP-SFLLRN, Sigma-Aldrich, Saint  
22 Quentin-Fallavier, France) or with the apoptosis inducer A23187 (Merck Millipore,  
23 Guyancourt, France), respectively.

24 After stimulation, platelets were resuspended by gentle pipetting, and an aliquot of the  
25 suspension was collected for viable normal platelet count and flow cytometry analysis. The  
26 plate was then re-centrifuged at  $300 \times$  g for 10 min to harvest the platelets, and the  
27 supernatants were collected and kept frozen at  $-80^\circ\text{C}$  until analysis.

## 28 ***Assessment of platelet mitochondrial membrane potential ( $\Delta\Psi_m$ )***

29 To determine whether platelet exposure to staphylococcal strains induced platelet cell  
30 death, the mitochondrial membrane potential ( $\Delta\Psi_m$ ) was assessed in platelets. After bacterial  
31 stimulation, 40 nM DiOC6(3), a cell-penetrable green fluorescent cationic dye (Invitrogen,

1 Saint Aubin, France), was added to the cell suspension for 15 min at room temperature. The  
2 samples were then diluted in phosphate-buffered saline (PBS) prior to flow cytometry  
3 analysis on a CANTO II flow cytometer with BD FACSDiva™ software (BD Biosciences, Le  
4 pont de Claix, France). Depolarization of the mitochondria was defined as a decrease in the  
5 mean fluorescence intensity of platelet-bound DIOC6(3), as observed in the FL1 channel.

## 6 **Assessment of platelet phosphatidylserine externalization**

7 Phosphatidylserine exposed on the membrane of apoptotic or necrotic cells was  
8 quantified by flow cytometry. In brief, after stimulation, platelets were resuspended in  
9 Annexin Binding Buffer (MabTag, Oldenburg, Germany) for immunostaining. PS exposure  
10 was determined after gating on CD41-positive cells (CD41 is a specific marker for platelets).  
11 Allophycocyanin (APC)-conjugated anti-human Annexin V (clone), fluorescein  
12 isothiocyanate (FITC) anti-human CD41 (clone HIP-8) and fluorochrome-conjugated mouse  
13 IgG isotypic controls were purchased from BD Biosciences (Le pont de Claix, France). All  
14 analyses were performed with a CANTO II flow cytometer and analyzed with BD  
15 FACSDiva™ software (BD Biosciences).

## 16 **Platelet microparticle preparation and flow cytometry analysis**

17 After stimulation, PRP was centrifuged at 1,100 x g for 15 min to obtain platelet-poor plasma  
18 (PPP) containing PMPs. Then, PPP was centrifuged at 7,000 x g for 3 min. Supernatants were  
19 collected and kept frozen at -80°C until flow cytometry analysis. PMPs were measured using  
20 Gallios flow cytometry as previously described by Robert et al.<sup>85</sup>. Briefly, PMPs were  
21 defined as CD41 and Annexin V positive, gating with Megamix beads (Biocytex, Marseille,  
22 France). PMPs were labeled following a 30-min incubation of PPP with APC-conjugated anti-  
23 human (MabTag, Oldenburg, Germany) and FITC anti-human CD41 (BD Biosciences) or  
24 fluorochrome-conjugated mouse IgG isotypic controls. During immunostaining, Annexin  
25 binding buffer was added to improve the binding of Annexin-V to PS. PMPs were fixed by  
26 addition of paraformaldehyde (4%); Cytocount beads (Dako/Agilent, Les Ulis, France) were  
27 used to determine the concentration. All analyses were performed using Kaluza® flow  
28 analysis software.

## 29 **Assessment of caspase-3 activation**

1 Stimulated platelets were resuspended in BD Cytotfix/Cytoperm™ solution (BD Biosciences,  
2 Le pont de Claix – France) and incubated for 20 min on ice according to the manufacturer’s  
3 instructions. Platelets were then centrifuged  $300 \times g$  for 5 min, and the pellet was washed  
4 twice with BD Perm/Wash™ buffer. Platelets were resuspended in Perm/Wash™ buffer,  
5 stained with 20  $\mu$ l of FITC rabbit anti-active Caspase-3 antibody (BD Biosciences) for 30 min  
6 at room temperature, washed once and analyzed with a CANTO II flow cytometer and BD  
7 FACSDiva™ software (BD Biosciences).

## 8 **Assessment of cytokine release by platelets**

9 The release of sCD62P, RANTES, GRO $\alpha$  and sCD40L by platelets was measured in  
10 duplicate in the supernatants of unstimulated (negative control) or TRAP-stimulated (positive  
11 control) platelets and in the supernatants of platelets exposed to staphylococcal strains in the  
12 presence or absence of ASA (500  $\mu$ M) or fluvastatin (10  $\mu$ M). RANTES and Thromboxane  
13 B2 were quantified by ELISA (R&D Systems Europe Ltd., Lille, France). The absorbance at  
14 450 nm was measured on an ELISA reader (Magellan software, Sunrise™, Tecan Group Ltd.,  
15 Lyon, France). Soluble CD40L, sCD62P and GRO $\alpha$  were quantified using a multiplexed  
16 Luminex® system (Merck Millipore, Guyancourt, France) according to the manufacturer’s  
17 instructions. The results were read using a Bioplex 200 system (Bioplex Manager™ software,  
18 Bio-Rad, Marnes-la-Coquette, France).

## 19 **Assessment of platelet aggregation**

20 Platelet aggregation was assessed throughout the platelet stimulation (30 min) with the  
21 staphylococcal strains described above using a Thrombo-aggregometer TA 4V with constant  
22 stirring and was analyzed with Thrombosoft 1.6 software (SD Innovation, Frouard, France).  
23 At the end of the platelet stimulation, TRAP (50  $\mu$ g/ml) or ADP (10  $\mu$ M) was added to the  
24 PRP suspension to confirm that the platelets were still responsive to stimulation. Calibration  
25 of the thrombo-aggregometer utilized PRP (0% light transmission, 0% aggregation) and PPP  
26 (100% light transmission, 100% aggregation), which was obtained by centrifuging the  
27 unstimulated PRP at  $3000 \times g$  for 20 min. The data for the PRP samples that revealed an  
28 aggregation, even partial, was not included in the whole aggregation dataset and were  
29 presented individually, as shown in Supplemental Figure 3.

## 30 **Analysis of platelet activation markers**

1 Platelet membrane expression of the CD62P and CD63 activation markers after  
2 stimulation was assessed using immunostaining of PRP after gating on CD41-positive cells  
3 (CD41 is a specific marker for platelets). APC-conjugated anti-human CD41 (clone HIP-8),  
4 FITC anti-human CD62P (clone AK-4), phycoerythrin (PE) CD63 (clone HSC-6) and  
5 fluorochrome-conjugated mouse IgG isotypic controls were purchased from BD Biosciences  
6 (Le pont de Claix, France). All analyses were performed with a CANTO II flow cytometer  
7 and analyzed with BD FACSDiva™ software (BD Biosciences).

8

### 9 **Platelets and *S. aureus* fluorescence microscopy**

10 The staphylococcal strain SAB24 expressing eGFP, obtained by transforming the SAB24  
11 strain with the pbSU101 plasmid (a kind gift from Prof. Spellerberg, University of Ulm,  
12 Germany) as previously described<sup>86,87</sup>, TRAP (as a positive control) or Tyrode's Buffer (as a  
13 non-stimulated (NS) control) were added to freshly prepared platelets as described in the  
14 "Platelet exposure to Staphylococcus strains" paragraph and placed in a µClear® polystyrene  
15 microplate with a bottom thickness of 190 µm +/- 20 µm (Greiner Bio-One, Courtaboeuf,  
16 France). The culture plate containing the platelet and bacterial mix was then gently  
17 centrifuged for 5 min at 300 × g to facilitate and standardize contact between platelets and  
18 bacteria and was incubated for 30 min at room temperature. The platelets were then fixed with  
19 4% paraformaldehyde, washed twice with PBS and stained with an anti-human CD41 Dylight  
20 550 antibody (Leinco Technologies®/ Interchim, Montluçon, France; 10 µg/ml). Finally, the  
21 platelets were washed once with PBS and observed with an IX81 fluorescence inverted  
22 microscope (Olympus, Germany) using Cell^P software (Olympus, Germany). The total  
23 number of platelets and the number of platelets with a normal size (less than 4 µm) was  
24 determined in each field, and the percentage of normal-sized platelets was calculated.

25

### 26 **Measurement of endotoxin levels in PRP**

27 The absence of any contaminating bacterial endotoxin in PRP was confirmed by  
28 inoculation of 1 ml of PRP into conventional blood culture medium, incubation in a BD  
29 Bactec™ automated system (Becton Dickinson) and analysis using the QCL-1000 Limulus  
30 amoebocyte lysate assay® (Lonza/Ozyme, Montigny-Le-Bretonneux, France).

31

## 1 **Statistical analysis**

2           One or two-way ANOVA with repeated measures and a Bonferroni post hoc test were  
3 used for inter-experimental comparisons. All values are reported as the mean  $\pm$  standard error  
4 of the mean (SEM). A p-value of  $< 0.05$  was considered statistically significant. Dixon tests  
5 were used to identify abnormal values. Statistical analyses and graphs were performed with  
6 GraphPad Prism 5 (La Jolla, USA) and FlowJo V10 (Ashland, USA) software.

7



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30

## Figure Legends

**Figure 1. Platelet counts following exposure to Staphylococcus strains.** After a 30-min exposure to the staphylococcal strains, TRAP (as a positive control) or Tyrode's buffer (as a negative control, NS), platelet counts were assessed with a MS4s Hematology analyzer [Tyrode (n=42), TRAP (n=40) or Staphylococcus strains: clinical strains (SaB), *S. condimentii* (Sc) or *S. aureus* ATCC 43866 (ATCC43866)] (n=12). The data (mean  $\pm$  SEM) are expressed as the platelet count  $\times 10^6$  /ml \*\*\*\*p<0.0001 (one-way ANOVA with repeated measures and the Bonferroni post hoc test; stimulated vs. unstimulated, NS).

**Figure 2. Platelet activation level after Staphylococcus aureus stimulation.** Expression levels of the CD62P (a) and CD63 (b) activation markers on platelets were analyzed after stimulation for 30 min with staphylococcal strains [clinical strains (SaB), *S. condimentii* (Sc) or *S. aureus* ATCC 43866 (ATCC43866)], TRAP (as a positive control) or Tyrode's buffer (as a negative control, NS). Membrane expression of CD62P and CD63 after gating on CD41 was assessed by flow cytometry, and data (mean  $\pm$  SEM; n = 10 experiments) are expressed as the percentage of positive cells. \*p<0.05; \*\*p<0.01\*\*\*; p<0.001; \*\*\*\*p<0.0001 (one-way ANOVA with repeated measures and the Bonferroni post hoc test; stimulated vs. unstimulated, NS).

**Figure 3. Platelet cytokine release in response to S. aureus stimulation.** RANTES (a) GRO $\alpha$  (b) sCD40L (c) sCD62P (d) and thromboxane B2 (e) release by platelets were analyzed after stimulation for 30 min with staphylococcal strains [clinical strains (SaB), *S. condimentii* (Sc) or *S. aureus* ATCC 43866 (ATCC43866)], TRAP (as a positive control) or Tyrode's buffer (as a negative control, NS). Immunomodulatory factor levels were assessed by ELISA or Luminex® assays, and data (mean  $\pm$  SEM; n = 10 experiments) are expressed as pg/ml or ng/ml (sCD62P and thromboxane B2). \*p<0.05; \*\*p<0.01\*\*\*; p<0.001; \*\*\*\*p<0.0001 (one-way ANOVA with repeated measures and the Bonferroni post hoc test; stimulated vs. unstimulated, NS). The dotted line represents the unstimulated value.

**Figure 4. Platelet aggregation after S. aureus exposure.** Aggregation was assessed using a thrombo-aggregometer after exposure (a/c) to Tyrode's buffer (NS), *S. condimentii* (Sc) and *S.*

aureus ATCC43866 (ATCC 43866) or **(b/d)** to clinical strains (SaB). TRAP **(a/b)** or ADP **(c/d)** was added as a positive control at the end of the stimulation (one representative experiment is presented; n = 5-10 experiments). The aggregation baseline was approximately 20% due to the added volume.

**Figure 5. S. aureus-induced platelet cell death.** **(a)** MFI of platelet-bound DIOC6(3) **(b)** Annexin V binding **(c)** PMP concentration or **(d)** fold increase in active caspase-3 expression were analyzed by flow cytometry after 30 min of exposure to Tyrode's buffer (NS), TRAP, staphylococcal strains [clinical strains (SaB), *S. condimenti* (Sc) or *S. aureus* ATCC 43866 (ATCC43866)] or the A23187 apoptosis inducer as a positive control. The data (mean  $\pm$  SEM; n = 8 experiments) are expressed as the AU (a), % expression (b) or fold increase compared with NS (d). The PMP concentration (mean  $\pm$  SEM; n = 6-27 experiments) (c) is expressed as microparticles/ $\mu$ l. \*p<0.05, \*\*p<0.01, \*\*\*\*p<0.0001 (one-way ANOVA with repeated measures and the Bonferroni post hoc test; stimulated vs. unstimulated, NS).

**Figure 6. Effect of ASA and fluvastatin on the S. aureus-induced reduction in platelet counts.** Platelet counts after a 30-min exposure to staphylococcal strains [clinical strains (SaB), *S. condimenti* (Sc) or *S. aureus* ATCC 43866 (ATCC43866)], TRAP (as a positive control) or Tyrode's buffer (as a negative control, NS), ASA, fluvastatin or water (as a control vehicle) [control vehicle (n=42), TRAP (n=40) or *Staphylococcus* strains (n=10)]. The data (mean  $\pm$  SEM) are expressed as the platelet count  $\times 10^6$ /ml. \*p<0.05, \*\*p<0.01; \*\*\*\*p<0.0001 (two-way ANOVA with repeated measures and the Bonferroni post hoc test; ASA or fluvastatin vs. control vehicle).

**Figure 7. Effect of ASA and fluvastatin on S. aureus-induced platelet activation.** Expression levels of the CD62P **(a)** and CD63 **(b)** activation markers and immunomodulatory factor release of RANTES **(c)** and GRO $\alpha$  **(d)** by platelets were analyzed after stimulation for 30 min with staphylococcal strains [clinical strains (SaB), *S. condimenti* (Sc) or *S. aureus* ATCC 43866 (ATCC43866)] with TRAP (as a positive control) or Tyrode's buffer (as a negative control, NS), ASA, fluvastatin or water (as a control vehicle). Membrane expression of CD62P and CD63 after gating on CD41 was assessed by flow cytometry, and data (mean  $\pm$

SEM; n = 10 experiments) are expressed as the percentage of positive cells. Immunomodulatory factor levels were assessed by ELISA or Luminex® assays, and data (mean ± SEM; n = 14-30 experiments) are expressed as pg/ml or ng/ml (sCD62P). \*p<0.05; \*\*p<0.01; \*\*\*; p<0.001, \*\*\*\*p<0.0001 (two-way ANOVA with repeated measures and the Bonferroni post hoc test; ASA or fluvastatin vs. control vehicle).

**Figure 8. Effect of ASA and fluvastatin on S. aureus-induced platelet cell death.** (a) The MFI of platelet-bound DIOC6(3) (b) Annexin V binding (c) PMP concentration or (d) fold increase in active caspase-3 expression were analyzed by flow cytometry after 30 min of exposure to Tyrode's buffer (as a negative control, NS), TRAP, staphylococcal strains [clinical strains (SaB), *S. condimentii* (Sc) or *S. aureus* ATCC 43866 (ATCC43866)] or the A23187 apoptosis inducer (as a positive control), with ASA, fluvastatin or water (as a control vehicle). (a) The data (mean ± SEM; n = 8) are expressed as AU. (b) The data (mean ± SEM; n = 8) are expressed as the % expression (c) The data (mean ± SEM; n = 9-27 experiments) are expressed as microparticles/μl. The data (mean ± SEM; n = 6-15 experiments) are expressed as the fold increase in active caspase-3 expression. \*\*p<0.01; \*\*\*; p<0.001, \*\*\*\*p<0.0001 (two-way ANOVA with repeated measures and the Bonferroni post hoc test; ASA or fluvastatin vs. control vehicle).

### Acknowledgements

This work was supported by grants from the Academic Research Community-1 of the Rhône-Alpes Region, which provided Adrien Chabert's PhD fellowship and financial support, the French "Agence Nationale de la Recherche" (ANR-12-JSV1-0012-01), EFS Rhone-Alpes-Auvergne and the "Les Amis de Rémi" association. The authors are grateful to Dr. P. Chavarin and Dr. A. Lefebvre at EFS Rhone-Alpes-Auvergne, to the generous blood donors and to Dr. S. Charaoui-Boukerzaza for providing the eGFP SAB24 *S. aureus* strain. The authors very warmly thank Dr. Zhiguo He for his invaluable assistance with the visualization of platelet-eGFP SAB24 interactions.

### Author contributions



AC, PD, FC and HHC designed and performed the experiments, analyzed the data and wrote the manuscript. AC, PD, MAE, PV and SR performed the experiments. FG, PV, FZ, PB, BPo, BPa, LPD and FDG designed the study. OG, FC and HHC coordinated the study.

### **Competing Financial Interest Statement**

The authors declare that they have no competing financial interests.

Figure 1

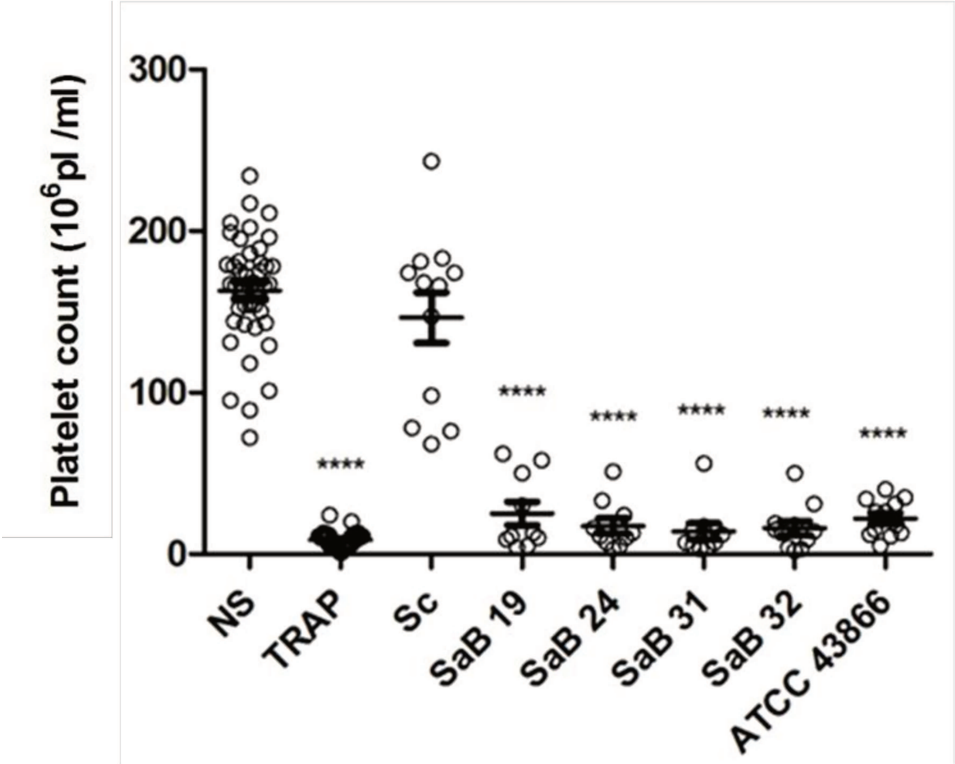


Figure 2

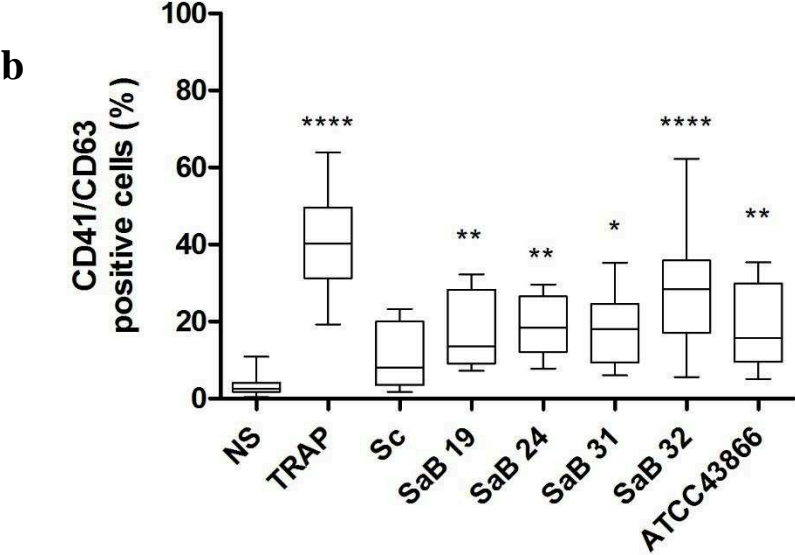
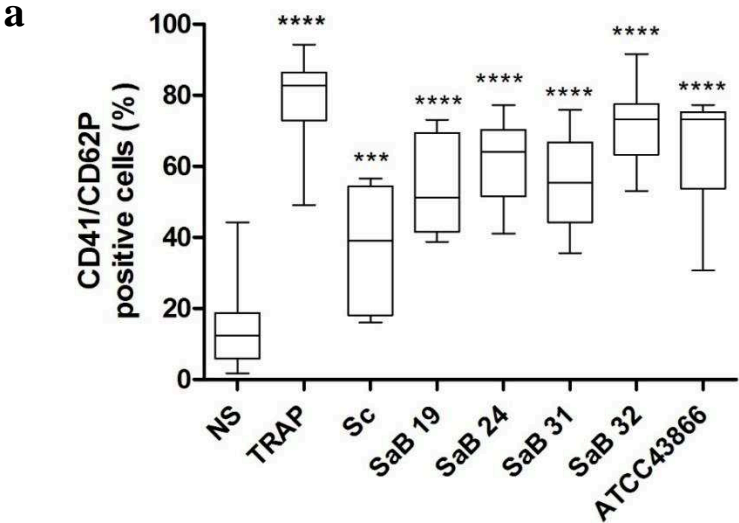


Figure 3

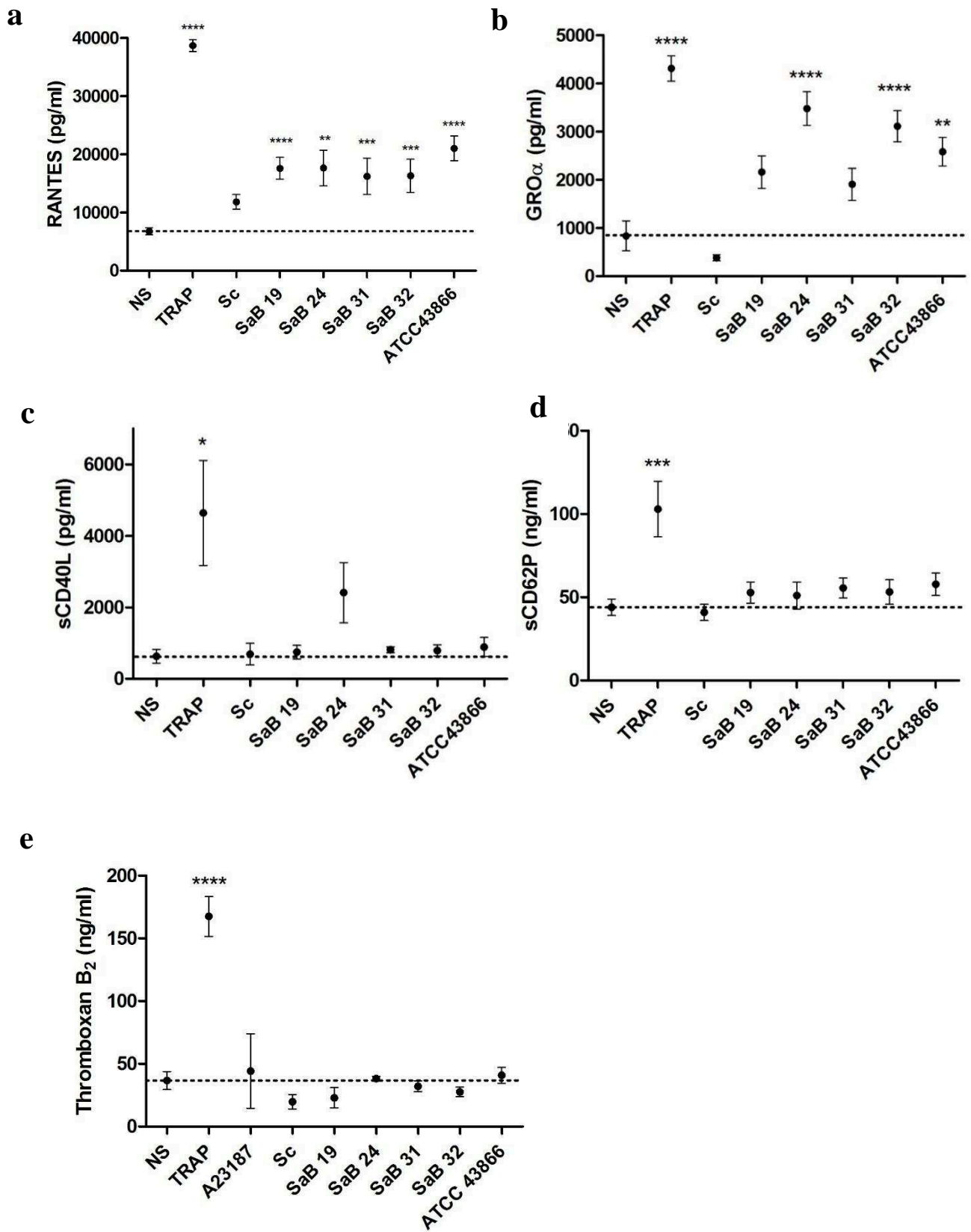
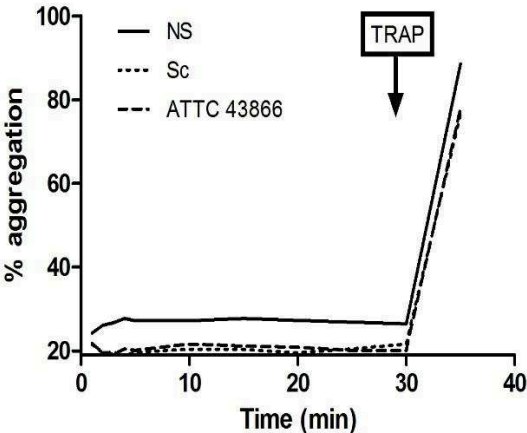
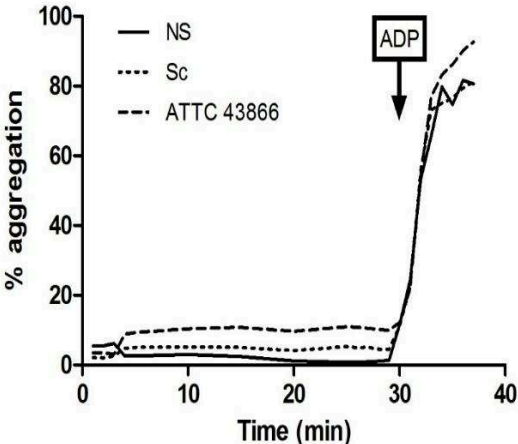


Figure 4

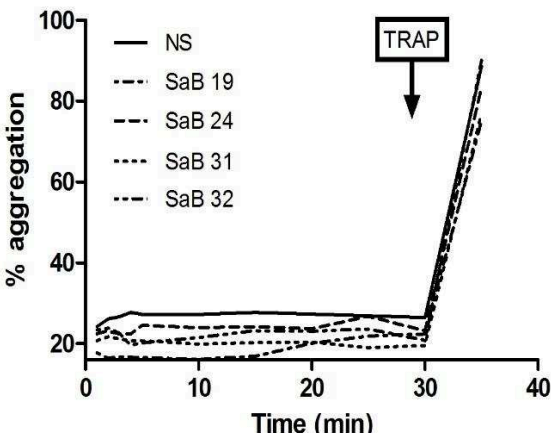
a



c



b



d

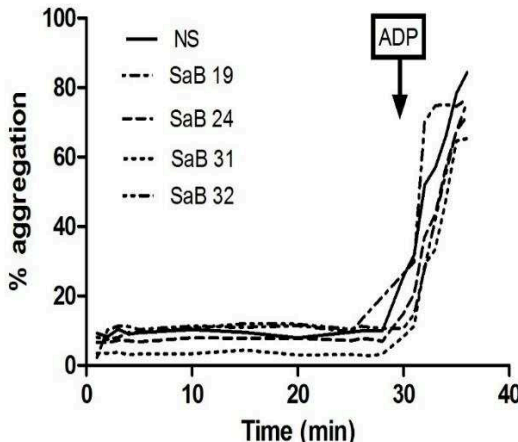


Figure 5

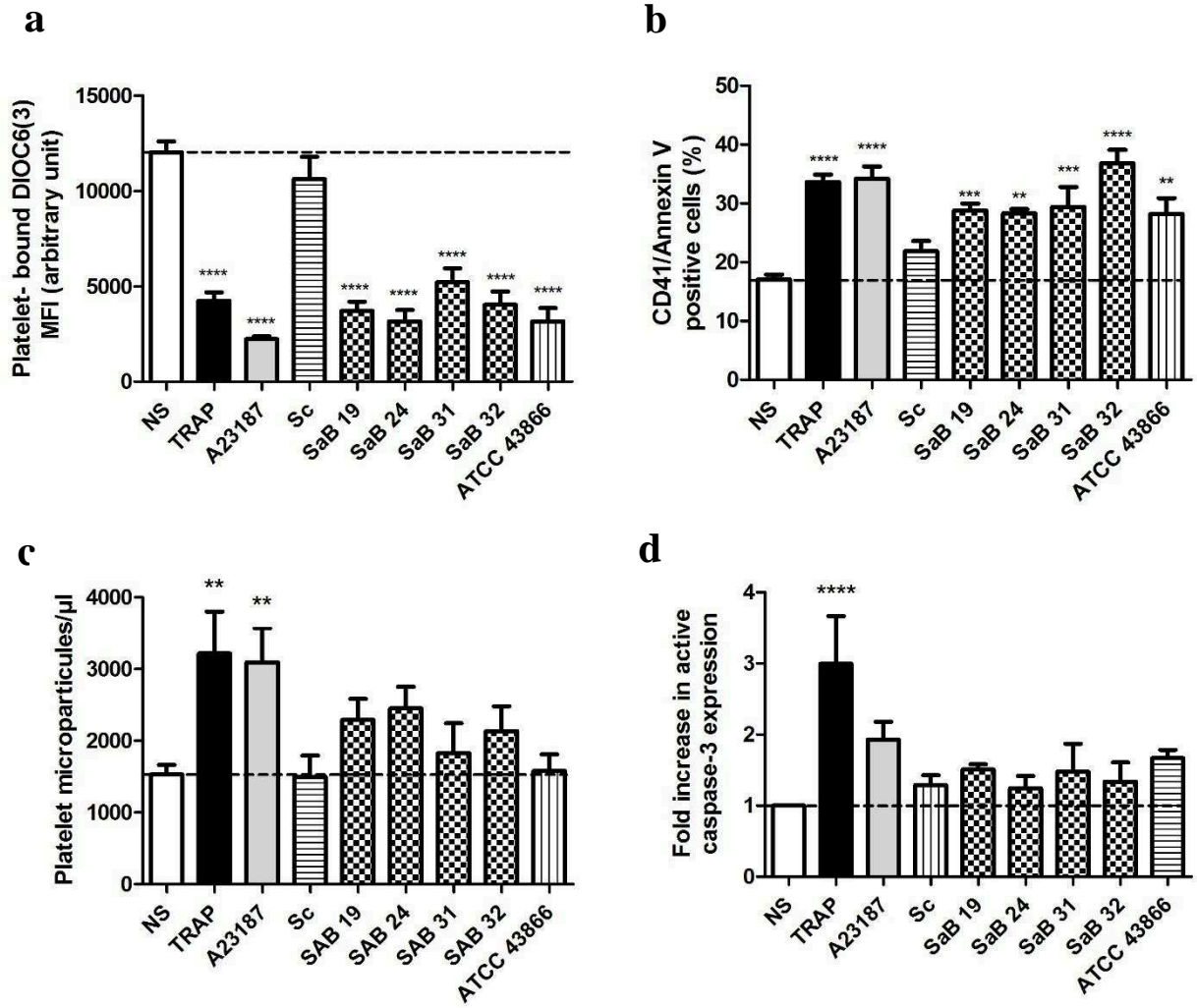
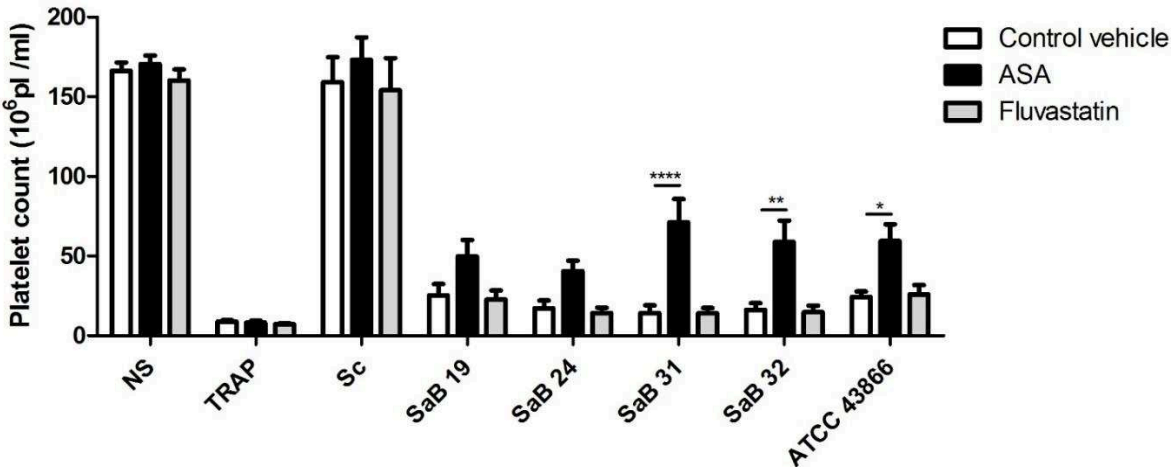
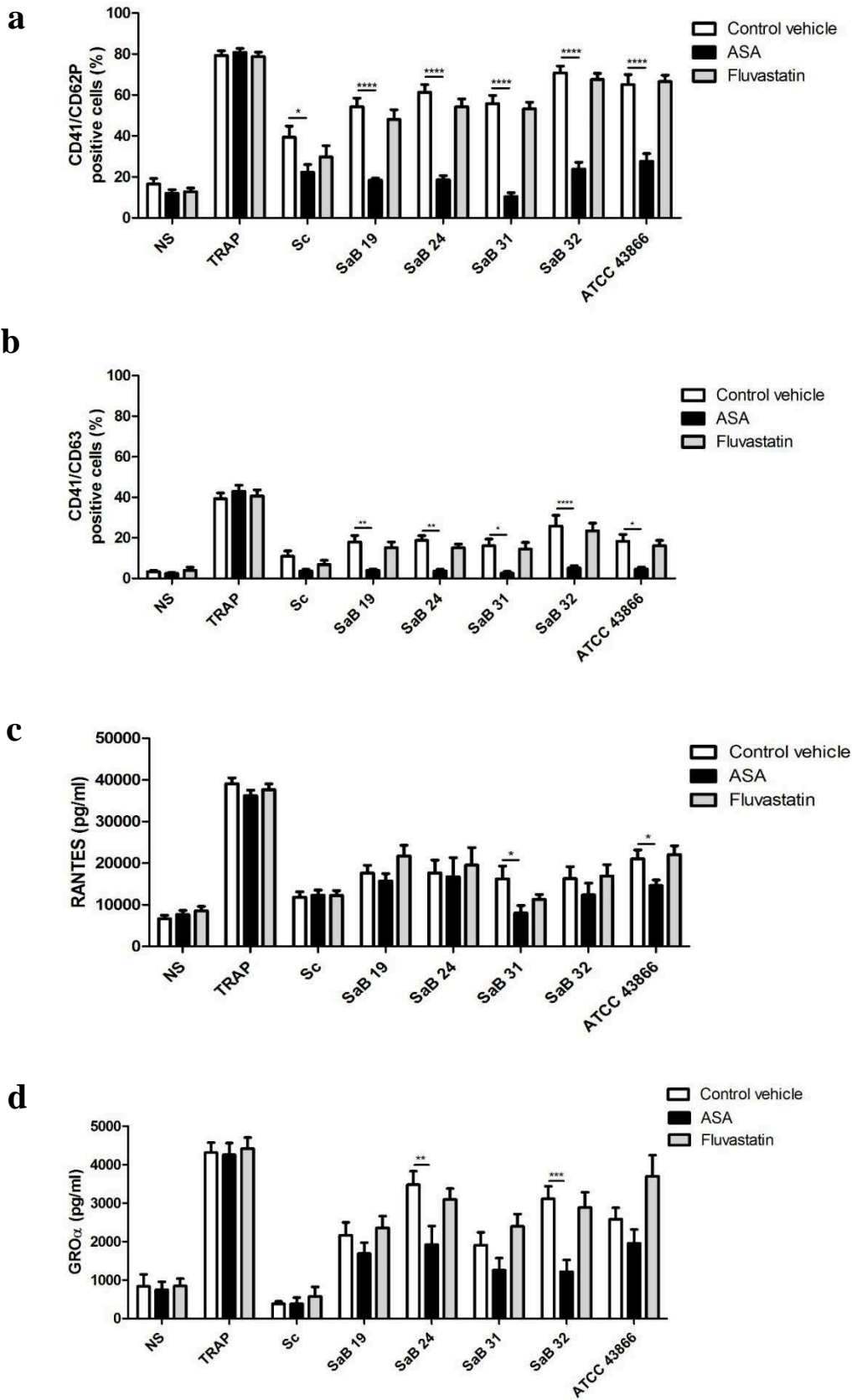


Figure 6



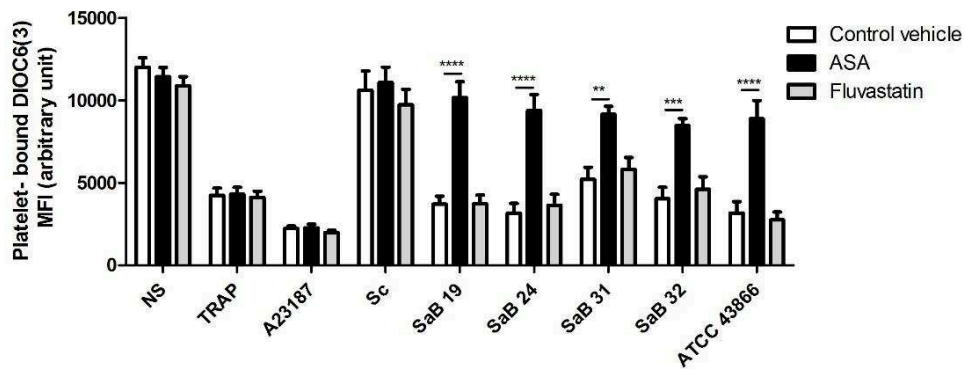
**Figure 7**



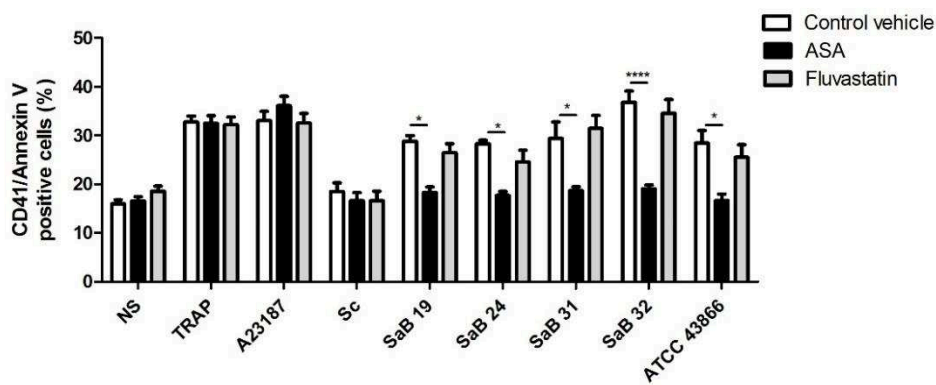


**Figure 8**

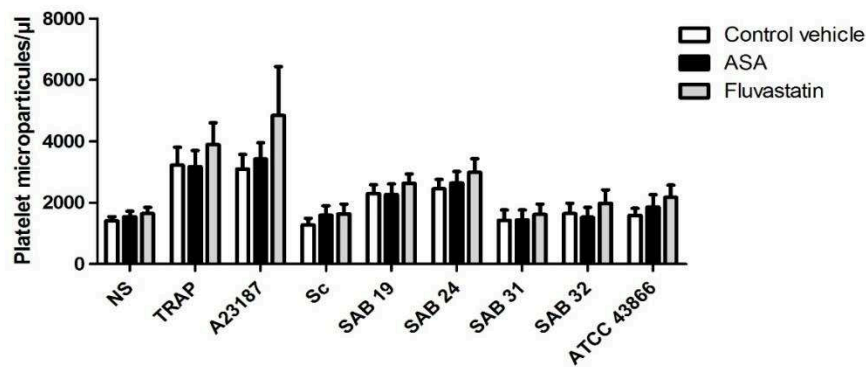
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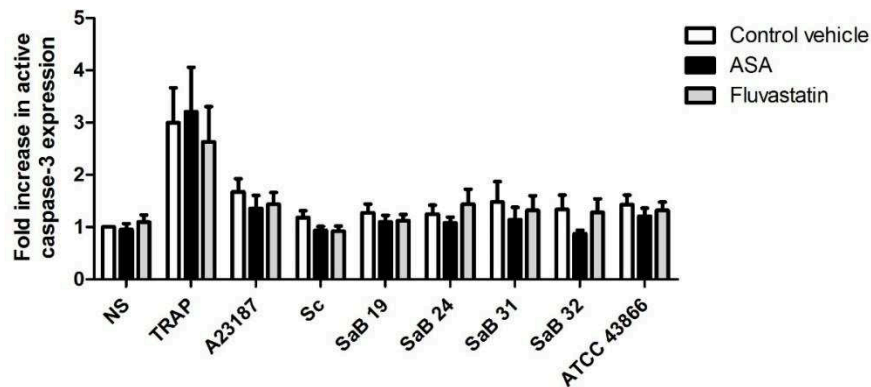
**b**



**c**



**d**



## **Acetylsalicylic acid differentially limits the activation and expression of cell death markers in human platelets exposed to *Staphylococcus aureus* strains**

Adrien Chabert<sup>1</sup>, Pauline Damien<sup>1</sup>, Paul O. Verhoeven<sup>1,2</sup>, Florence Grattard<sup>1,2</sup>, Philippe Berthelot<sup>1,2</sup>, Fabrice Zeni<sup>1,3</sup>, Laurence Panicot-Dubois<sup>4</sup>, Stéphane Robert<sup>4</sup>, Françoise Dignat-George<sup>4</sup>, Marie-Ange Eyraud<sup>5</sup>, Bruno Pozzetto<sup>1,2</sup>, Bernard Payrastré<sup>6</sup>, Fabrice Cognasse<sup>1,5</sup>, Olivier Garraud<sup>1,7</sup> and Hind Hamzeh-Cognasse<sup>1\*</sup>

<sup>1</sup> EA3064—GIMAP, Université de Lyon, 42023 Saint-Etienne, France

<sup>2</sup> Laboratoire des *Agents Infectieux et d'Hygiène*, CHU de Saint-Etienne, 42055 Saint-Etienne, France

<sup>3</sup> Service Réanimation polyvalente, 42055 CHU de St Etienne, France

<sup>4</sup> Université Aix Marseille, INSERM UMR-S1076, VRCM, Marseille, France

<sup>5</sup> EFS Rhone-Alpes-Auvergne, 42023 Saint-Etienne, France

<sup>6</sup> Inserm/UPS UMR 1048 - I2MC, Toulouse, France

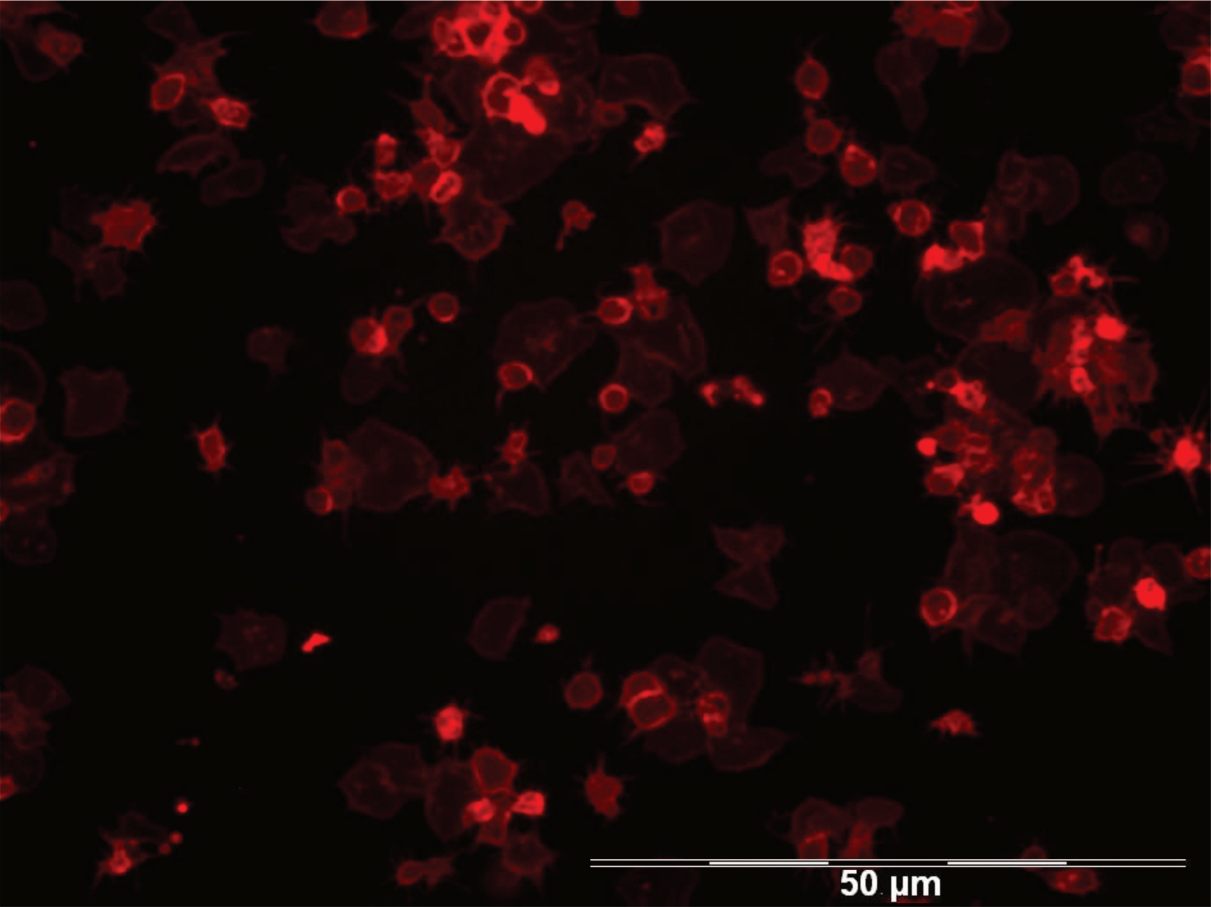
<sup>7</sup> Institut National de la Transfusion Sanguine, 75015, Paris, France

**Correspondence to:** Hind Hamzeh-Cognasse, EA3064—GIMAP, Université de Lyon, 42023 Saint-Etienne, France; Hind.Hamzeh@univ-st-etienne.fr

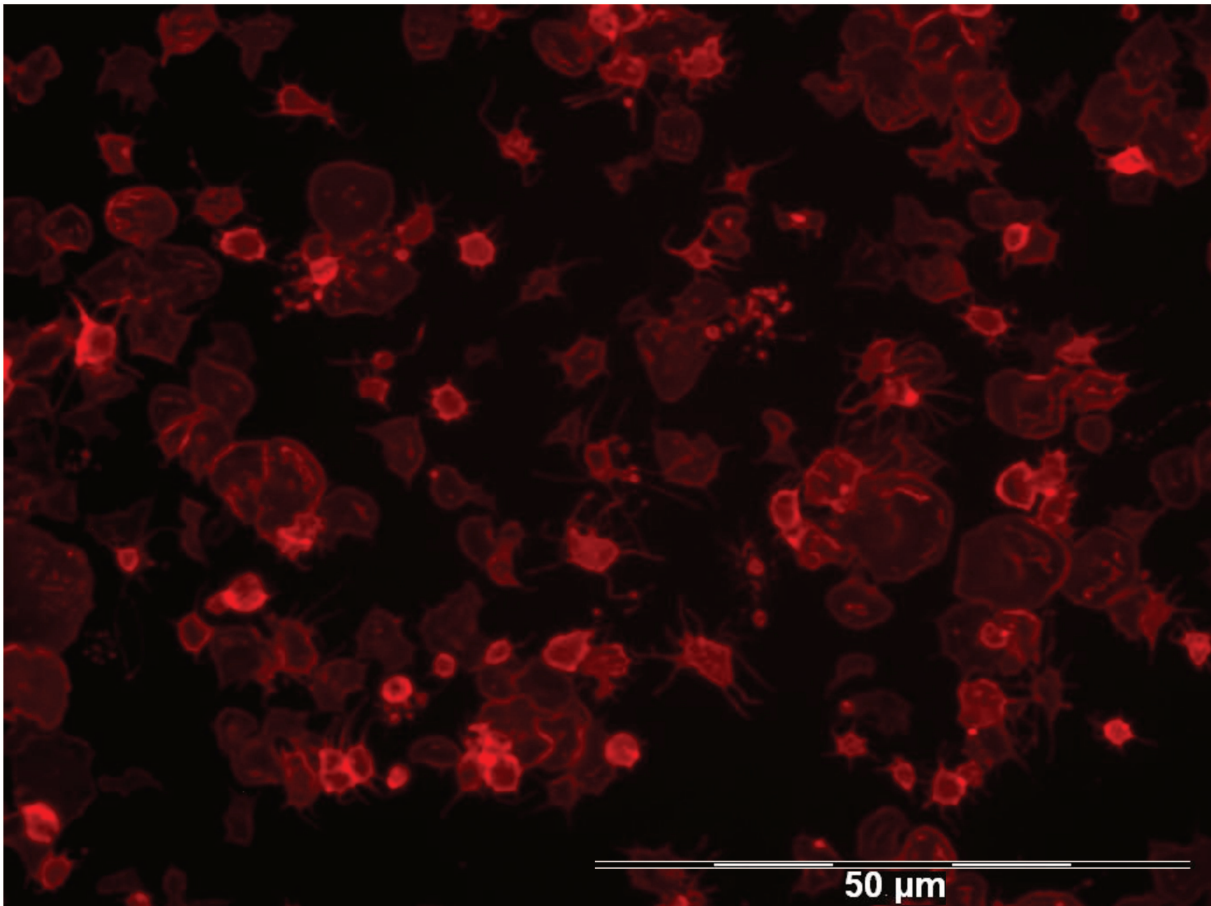
Supplemental data

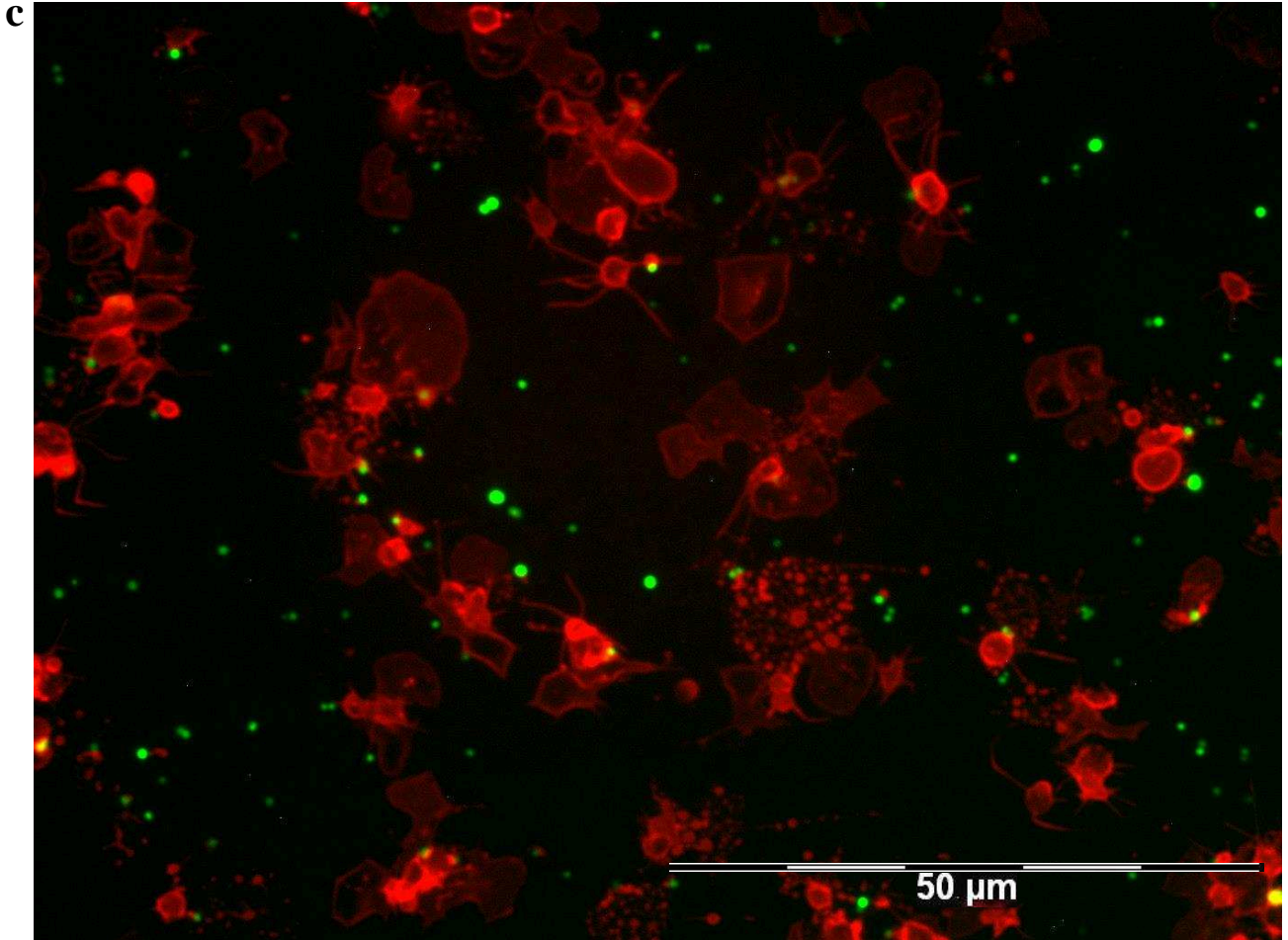
Supplemental Figure 1

a



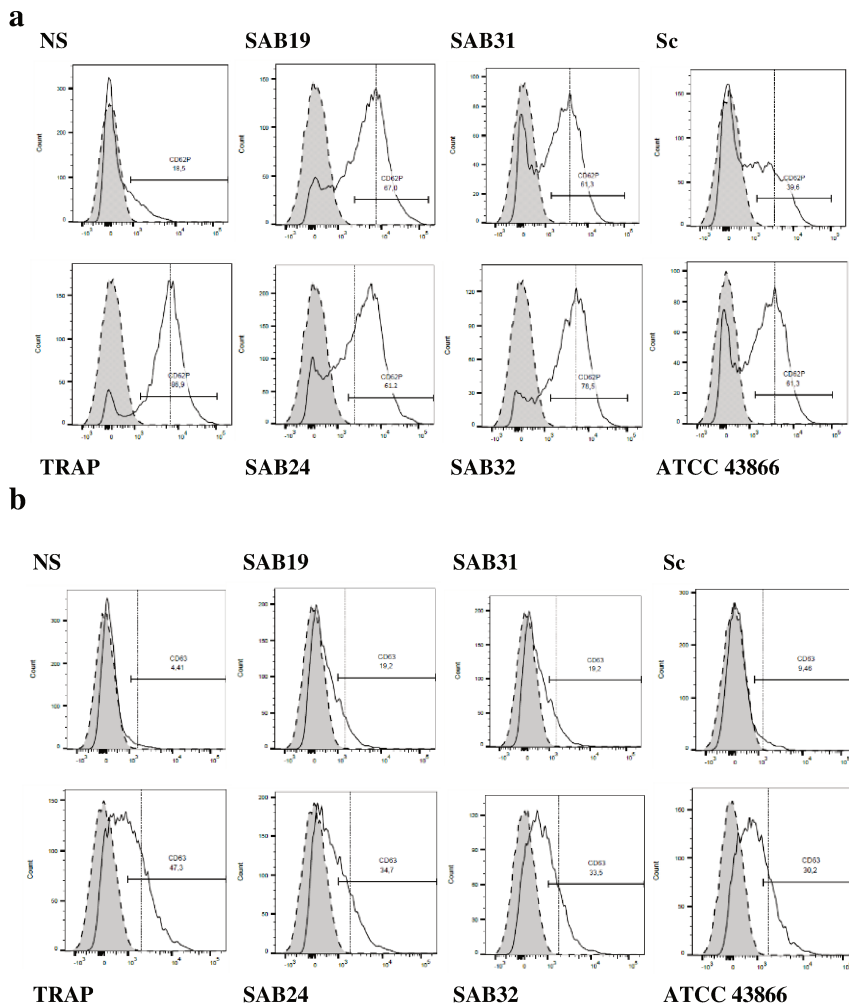
b





**Supplemental Figure 1. *S. aureus* SaB24 alters platelet morphology.** Platelets from healthy blood donors were stained with a Dylight 550 anti-CD41 antibody and analyzed by immunofluorescence microscopy after a 30-min exposure to (a) Tyrode's buffer (negative control), (b) TRAP (positive control), or (c) eGFP *S. aureus* clinical strain SaB24. Images are representative of 5 independent experiments.

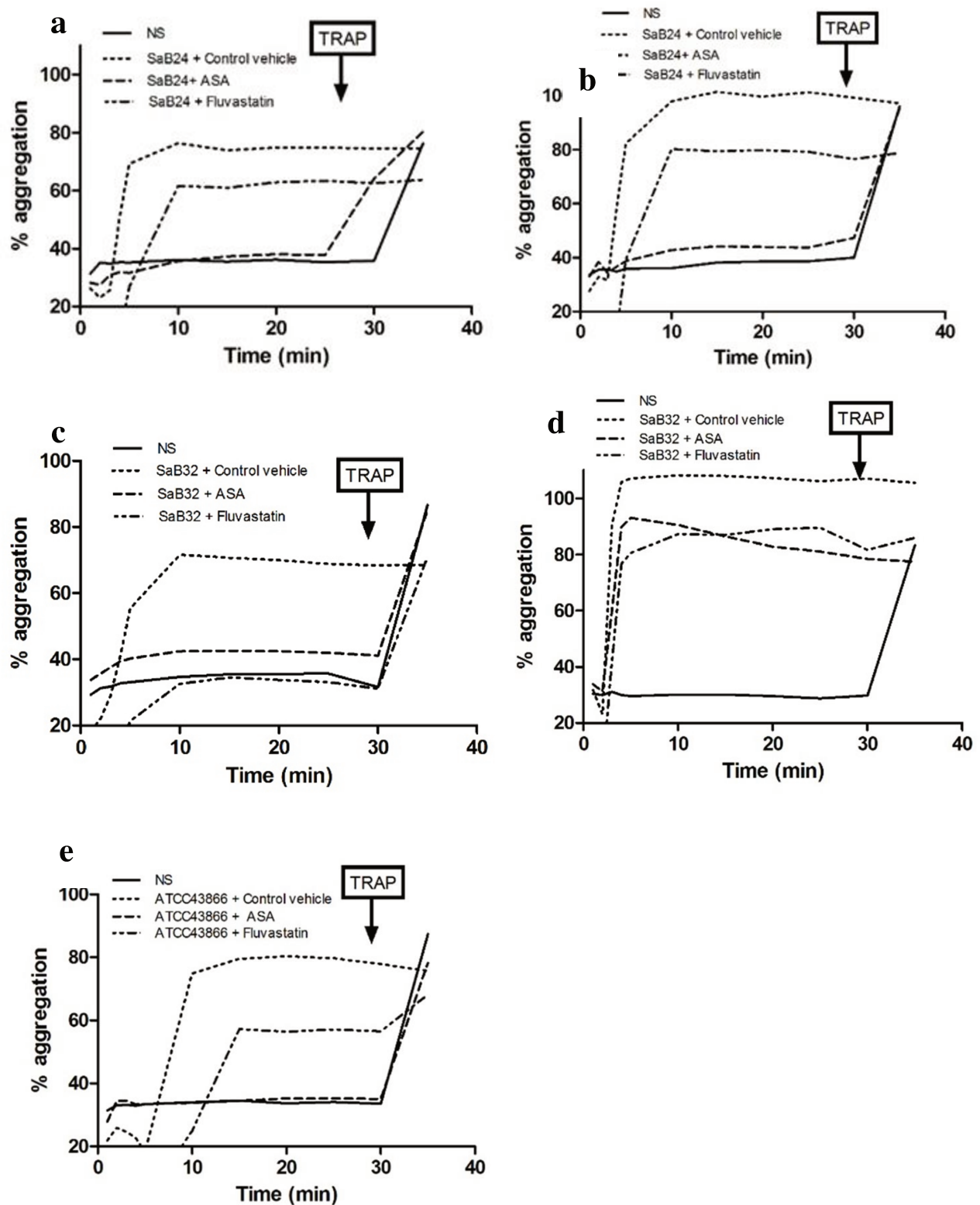
## Supplemental Figure 2



### Supplemental Figure 2. Level of platelet activation after *Staphylococcus aureus* stimulation.

Expression levels of the CD62P (a) and CD63 (b) activation markers on platelets were analyzed after stimulation for 30 min with staphylococcal strains [clinical strains (SaB), *S. condimentii* (Sc) or *S. aureus* ATCC 43866 (ATCC43866)], TRAP (as a positive control) or Tyrode's buffer (as a negative control, NS). Membrane expression of CD62P and CD63 after gating on CD41 was assessed by flow cytometry. One representative cytogram is presented for each condition with the isotype control (gray fill and dashed lines) and the marker CD62P or CD63 (white fill and solid lines).

Supplemental Figure 3

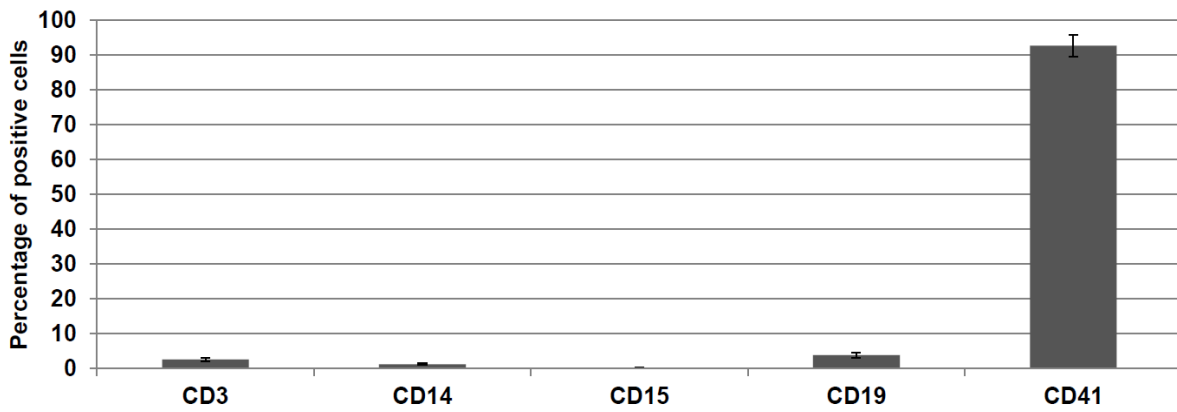


Supplemental Figure 3. Effect of ASA on *S. aureus*-induced platelet aggregation observed for 5/35 platelet samples.

Platelet aggregation was assessed using a thrombo-aggregometer following stimulation for 30 min with staphylococcal strains (a, SaB24; b, SaB24; c, SaB32; d, SaB32; e, ATCC43866), ASA,

fluvastatin or control vehicle (water). TRAP was added as a positive control at the end of the stimulation. The data from the 5/35 platelet samples that presented aggregation upon bacterial stimulation are shown. The aggregation baseline was approximately 20% due to the added volume.

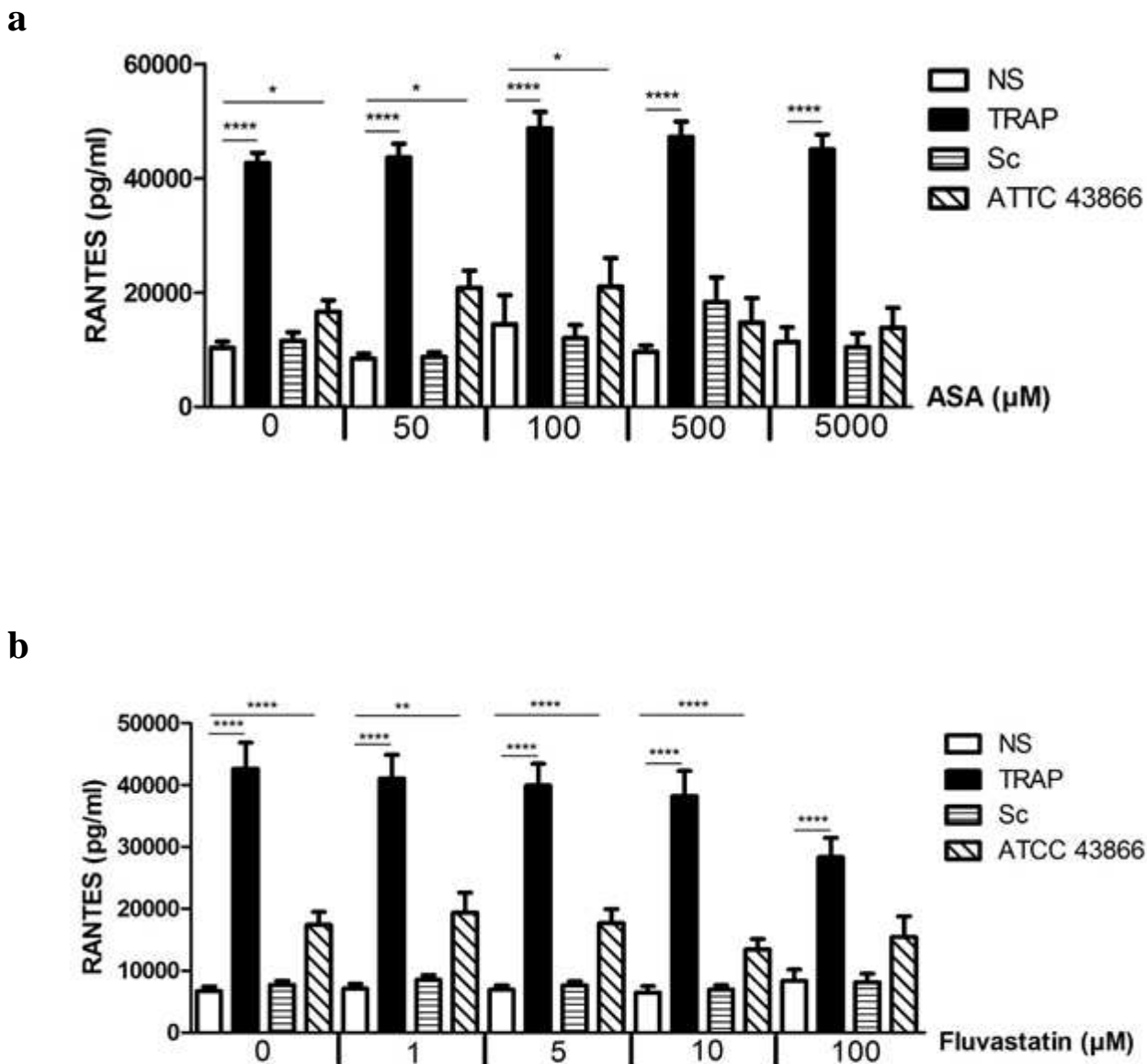
### Supplemental Figure 4



**Supplemental Figure 4. Purity of the PRP preparation.** Platelet purity was assessed using flow cytometry after staining cells with monoclonal antibodies against CD3 (specific marker for T cells), CD14 (specific marker for monocytes), CD15 (specific marker for neutrophils), CD19 (specific marker for B cells) or CD41 (specific marker for platelets). The data (mean  $\pm$  SD; n = 5 experiments) are expressed as the percentage of positive cells.



Supplemental Figure 5



**Supplemental Figure 5. Determination of the optimal concentrations of ASA and fluvastatin.**

RANTES release by platelets following a 30-min exposure to *S. condimentii* or *S. aureus* ATCC43866 strains (MOI of 0.1) in the presence of increasing concentrations of ASA (**a**, ranging from 0 to 5000 μM) or fluvastatin (**b**, ranging from 0 to 100 μM). \*p<0.05; \*\*p<0.01; \*\*\*\*p<0.0001 (two-way ANOVA with repeated measures and the Bonferroni post hoc test; stimulated vs. unstimulated, NS).

**Supplemental Table 1**

	SAB19	SAB24	SAB31	SAB32	ATCC43866
cap 1	-	-	-	-	-
cap 5	-	+	-	-	+
cap 8	+	-	+	+	-
icaA	+	+	+	+	+
icaC	+	+	+	+	+
icaD	+	+	+	+	+
bap	-	-	-	-	-
bbp	+	+	+	+	+
clfA	+	+	+	+	+
clfB (cons)	+	+	+	+	+
cna	+	+	-	-	-
ebh (cons)	+	+	+	+	+
ebpS	+	+	+	+	+
eno	+	+	+	+	+
FIBTOT	+	+	+	+	+
fnbA	+	+	+	+	+
fnbB	+	+	+	+	+
map	+	+	+	+	+
sasG	-	-	+	-	+
sdrC	+	+	+	+	+
sdrD	+	+	+	+	+
vwb	+	+	+	+	+
SSL-1total	+	+	+	+	+
SSL-2total	+	+	+	+	+
SSL-3total	+	-	+	+	+
SSL-4total	+	-	+	+	+
SSL-5total	+	+	+	+	+
SSL-6total	+	-	+	-	+
SSL-7total	+	+	+	+	+
SSL-8total	+	-	+	+	+
SSL-9total	+	+	+	+	+
SSL10total	+	+	+	+	+
SSL11total	+	-	+	-	+
SETB3total	+	+	+	+	+
SETB2tot	+	+	-	+	+
setB1	+	+	+	+	+
setC	+	+	+	+	+
Tst1 (consens)	-	-	-	-	-

	SAB19	SAB24	SAB31	SAB32	ATCC43866
entA	-	-	-	-	+
entA (N3+5) / entP	-	-	-	-	-
entB	-	-	-	-	-
entC	-	-	-	-	-
entD	-	-	-	-	-
entE	-	-	-	-	-
entG	-	-	-	-	-
entH	-	-	-	-	-
entI	-	-	-	-	nd
entJ	-	-	-	-	-
entK	-	-	-	-	-
entL	-	-	-	-	-
entM	-	-	-	-	-
entNTOT	-	-	-	-	-
entN (cons)	-	-	-	-	-
entN (other than RF122)	-	-	-	-	-
entO	-	-	-	-	-
entQ	-	-	-	-	-
entR	-	-	-	-	-
entU	-	-	-	-	-
entCM14 probe1	-	-	-	-	-
entCM+4 probe2	-	-	-	-	-
lukF	+	+	+	+	+
lukSTOT	+	+	+	+	+
lukS	+	+	+	+	+
lukS (ST22+ST45)	nd	+	+	+	-
hlgA	+	+	+	+	+
lukFndPV	-	-	-	-	-
lukSndPV	-	-	-	-	-
lukFndPV (P83)	-	-	-	-	-
lukM	-	-	-	-	-
lukD	+	-	+	+	+
lukE	+	-	nd	+	+
lukX	+	+	+	+	+
lukYTOT	+	-	+	+	+
lukY	+	nd	+	+	+
lukY (ST3-+ST45)	-	-	-	-	-
hl	+	+	+	+	+

	SAB19	SAB24	SAB31	SAB32	ATCC43866
hla	+	+	+	+	+
hIIIITOT	+	+	+	+	+
hIII (cons)	+	+	+	+	+
hIII (other than RF+22)	+	+	+	+	+
hIbTOT	+	+	-	+	+
hIbndprobe 1	+	nd	-	+	+
hIbndprobe 2	+	+	-	+	+
hIbndprobe 3	+	-	-	+	nd
unndtruncated hIb	-	-	-	-	-
sak	+	-	-	+	+
chp	-	+	+	-	-
scn	+	+	+	+	+
etA	-	-	-	-	-
etB	-	-	-	-	-
etD	-	-	-	-	-
edinA	-	-	-	-	-
edinB	-	-	-	-	-
edinC	-	-	-	-	-
arcAndSCC	-	-	-	-	-
arcBndSCC	-	-	-	-	-
arcCndSCC	-	-	-	-	-
arcDndSCC	-	-	-	-	-
aurTOT	+	+	+	+	+
aur (cons)	+	+	+	+	+
aur (Other than MRSA252)	+	-	+	+	+
aur (MRSA252)	-	+	-	-	-
splA	+	-	+	+	+
splB	+	-	+	+	+
splE	+	-	+	+	+
sspA	+	+	+	+	+
sspB	+	+	+	+	+
sspPTOT	+	+	+	+	+
sspP (cons)	+	+	+	+	+
sspP (other than ST93)	+	+	+	+	+
AGR	+	+	+	+	+

**Supplemental Table 1. Genotyping of clinical and reference *S. aureus* strains.**

The *S. aureus* reference strain ATCC43866 strain and 4 *S. aureus* clinical strains isolated from bacteremia patients (SaB) 19, 24, 31, 32 were genotyped with the *S. aureus* Genotyping Kit 2.0 (Alere Technologies GmbH, Jena, Germany). Briefly, strains were grown on Colombia blood agar, and the DNA was extracted, amplified and assessed as recommended by the manufacturer.

## Points clés de l'étude :

Dans cette étude, plusieurs éléments mécanistiques des interactions entre les plaquettes et les souches de *S. aureus* sont ressortis. Dans un premier temps, nous avons mis en évidence que certaines souches de *S. aureus* altéraient la morphologie plaquettaire au point d'affecter le compte plaquettaire et induisaient l'expression de marqueurs de mort cellulaire, tels que la dépolarisation de la membrane mitochondriale, l'exposition des phosphatidylsérines et la libération de microparticules plaquettaires ces dernières étant également un marqueur de réponse inflammatoire. Cette observation pourrait rendre compte de l'un des processus inducteurs de thrombopénie chez les patients atteints de sepsis. Conjointement à l'expression des marqueurs de mort cellulaire, nous avons observé que les plaquettes s'activent, libèrent certaines molécules immuno-modulatrices (GRO $\alpha$  et RANTES) sans toutefois s'agréger. GRO $\alpha$  est un marqueur soluble corrélé à l'état des patients septiques suggérant que les plaquettes participent à l'inflammation délétère du sepsis<sup>227</sup>. Par ailleurs, RANTES est une cytokine connue pour refléter l'orage cytokinique lors du sepsis et pour favoriser la migration des neutrophiles dans le parenchyme pulmonaire, induisant alors une défaillance des poumons<sup>138,228</sup>. Il faut noter la dichotomie dans la réponse pro-agrégante et la réponse pro-inflammatoire. Dans notre étude, nous n'avons pas observé d'agrégation plaquettaire suite à l'exposition par les différentes souches de *S. aureus* mais une libération de cytokines pro-inflammatoires. La concentration et le type de bactérie est un élément primordial dans la réponse plaquettaire puisque selon les récepteurs et ligands engagés par la bactérie, les plaquettes utilisent différentes voies signalétiques et adaptent leur réponse.

Pour limiter les effets des souches de *S. aureus* sur les plaquettes, nous avons évalué l'efficacité de l'ASA et la fluvastatine. Nos résultats montrent un effet protecteur de l'ASA concernant la chute du compte plaquettaire. En adéquation avec ces résultats, l'utilisation d'ASA limite l'activation plaquettaire, la libération de cytokines pro-inflammatoires, telles que GRO $\alpha$  et RANTES et protège de la mort plaquettaire en prévenant totalement la dépolarisation mitochondriale et l'exposition des PS membranaires. La fluvastatine en revanche n'a montré aucune efficacité pour prévenir les effets des souches de *S. aureus* sur les plaquettes.

D'un point de vue général, cette étude apporte de nouveaux éléments quant à l'effet cytotoxique exercé par *S. aureus* au niveau des plaquettes ainsi que la finesse dans la réponse

plaquettaire suite à l'exposition par cette bactérie. De plus, cela confirme l'intérêt d'utiliser l'ASA dans le but de limiter l'inflammation exercée par les plaquettes ainsi que de limiter la mort plaquettaire pour limiter la mortalité due au sepsis.

## **Article III**

**Acetylsalicylic acid limits neutrophil and platelet recruitment responsible for lung damage during experimental sepsis**

**(Article in progress)**

## Introduction de l'étude

Après avoir étudié le rôle inflammatoire de plaquettes sanguines issues de donneurs sains et exposées à différents stimuli d'origine bactérienne, il était important de s'intéresser au sepsis dans un système plus complet. De plus, nous voulions évaluer si l'effet inhibiteur de l'ASA observé *in vitro* sur l'expression de marqueurs de mort cellulaire par les plaquettes suivant leur exposition à des souches de *S. aureus* était également observé *in vivo* au cours du sepsis. Pour cela, nous avons décidé d'utiliser un modèle de sepsis murin expérimental. Il existe différents modèles pour induire un sepsis chez l'animal <sup>229</sup>. Le choix du modèle est d'une importance capitale dans la mesure où il conditionne la pertinence clinique des observations puisque les réponses immunitaires enclenchées diffèrent selon le modèle <sup>230</sup>. Le modèle d'endotoxémie consistant en l'injection de l'endotoxine bactérienne (LPS), est pertinent d'une intoxication endotoxémique. Ce modèle permet de mieux comprendre les effets d'une toxine mais ne prend pas en compte l'ensemble des facteurs de virulence appartenant à une bactérie et la dynamique de prolifération bactérienne. L'injection de LPS est un modèle simple et très reproductible mais ne permettant uniquement d'investiguer que certaines voies d'activation utilisant le TLR-4 ou certaines caspases <sup>231</sup>. Il induit une réponse rapide et aigüe qui diffère du sepsis chez l'homme. Un autre modèle, s'apparentant plus du choc septique, consiste en l'inoculation de bactéries. Même si la densité bactérienne de la suspension étant contrôlée, ce modèle reste pourtant assez variable, selon les études, à cause de la charge bactérienne ou encore de la voie d'administration utilisée. Enfin, le modèle le mieux décrit et le plus robuste est la technique intitulée « Cecal and Ligation Puncture » consistant en une ligature et ponction du cecum afin de libérer une partie du microbiote intestinal. Cette technique permet d'induire un sepsis polymicrobien ce qui recrée la progression physiopathologique et un profil cytokinique, représentatif de la pathologie chez l'homme <sup>232</sup>. Ce modèle se caractérise, selon le type de chirurgie (longueur de la ligature, nombre et taille de la ponction), d'une mortalité retardée (2 à 10 jours) ce qui permet l'induction de complications de multiples organes <sup>233</sup>. Ainsi, il s'apparente bien plus à la pathologie humaine que les autres modèles. Cependant, ce modèle murin entraîne une bactériémie impliquant majoritairement des bactéries gram négatif ce qui ne rend pas compte des sepsis humains à bactéries à gram-positif, bien que *Pseudomonas* et *E. coli* soient fréquemment impliqués (*S. aureus* (20,5%), *Pseudomonas* (19,9%) et *E. coli* (16%)). De plus, il est clair que l'expérimentation animale permet une standardisation (fond génétique, âge, sexe, même procédure d'induction du sepsis) favorisant une reproductibilité des

observations. Il s'avère donc intéressant d'utiliser ce modèle pour comprendre les mécanismes immunitaires joués par les plaquettes ainsi que la capacité de certaines molécules à contrôler les dérégulations inflammatoires observées lors du sepsis.

L'équipe du Pr. Thorlacius a beaucoup étudié le rôle inflammatoire des plaquettes lors du sepsis et a montré que la sécrétion de facteur plaquettaire 4 (PF4), de CD40L soluble (sCD40L) ou de chimiokines telle que RANTES par les plaquettes permet le recrutement de neutrophiles au niveau du parenchyme pulmonaire<sup>140,234-236</sup>. La séquestration de ces cellules dans le compartiment broncho-alvéolaire va induire la défaillance du poumon qui va causer un SDRA. De plus, les plaquettes favorisent la formation de NETs par les neutrophiles<sup>99,100</sup>. Ce processus pro-inflammatoire permettant de piéger et tuer les pathogènes peut toutefois exercer un rôle néfaste lors du sepsis puisqu'il active les macrophages alvéolaires et favorise l'œdème pulmonaire ou contribue à la coagulation<sup>142,237</sup>. Par ailleurs, le récepteur P<sub>2</sub>Y<sub>12</sub>, principalement retrouvé au niveau de la surface plaquettaire, semble moduler l'inflammation lors du sepsis puisque l'utilisation de souris génétiquement inactivée pour ce récepteur dans un modèle d'endotoxémie, ou l'utilisation de clopidogrel montre une réponse cellulaire et inflammatoire moins prononcée que les souris non modifiées indiquant le rôle de ce récepteur et des plaquettes dans la modulation de l'inflammation lors du sepsis<sup>238</sup>. Le blocage de la thrombopoïétine, facteur de la thrombopoïèse, limite plusieurs dysfonctions d'organes, suggérant un rôle de ce facteur, et des plaquettes, dans la physiopathologie du sepsis<sup>239</sup>.

Diverses stratégies thérapeutiques visant à limiter l'action plaquettaire lors du sepsis ont révélé des résultats prometteurs. L'utilisation de thiéno-pyridines, molécules ciblant le récepteur P<sub>2</sub>Y<sub>12</sub>, permet de limiter l'activation plaquettaire, leur interaction avec les leucocytes dans les complexes leuco-plaquettaires et la défaillance des poumons chez des souris septiques<sup>147,240</sup>. L'utilisation de l'ASA ou d'anticorps ciblant la glycoprotéine GPIIb/IIIa permet de réduire la formation des NETs et la défaillance pulmonaire induite expérimentalement<sup>241</sup>.

Cependant, certains résultats sont controversés puisque d'autres études (de sepsis polymicrobien ou par injection de *Klebsiella pneumoniae*) ne montrent aucune implication des plaquettes dans le recrutement des neutrophiles lors du SDRA ou encore un effet protecteur des plaquettes vis-à-vis de l'action inflammatoire des macrophages<sup>242,243</sup>. Il est donc important de considérer que d'autres études concernant le rôle inflammatoire des plaquettes lors d'un sepsis



expérimental murin sont à réaliser afin de mieux comprendre les mécanismes d'action joués par les plaquettes dans le sepsis ainsi que dans ses complications telles que le SDRA.

Dans cette étude, nous avons évalué la contribution inflammatoire des plaquettes dans un modèle expérimental de sepsis murin par CLP. Nous avons voulu investiguer les relations étroites entre les plaquettes et les neutrophiles dans la migration au niveau pulmonaire ou dans l'induction de NETs. De plus, nous avons évalué l'efficacité de l'ASA à améliorer l'état général des souris et à limiter la participation des plaquettes à l'inflammation associée au sepsis et ses complications

# **Acetylsalicylic acid limits neutrophil and platelet recruitment responsible for lung damage during experimental sepsis**

Adrien Chabert<sup>1</sup>, Charles-Antoine Arthaud<sup>2</sup>, Bernard Payraastre<sup>3</sup>, Fabrice Cognasse<sup>1,2</sup>, Olivier Garraud<sup>1,4</sup> and Hind Hamzeh-Cognasse<sup>1</sup>

<sup>1</sup> EA3064—GIMAP, Université de Lyon, 42023 Saint-Etienne, France

<sup>2</sup> EFS Auvergne-Rhône-Alpes, 42023 Saint-Etienne, France

<sup>3</sup> Inserm/UPS UMR 1048 - I2MC, Toulouse, France

<sup>4</sup> Institut National de la Transfusion Sanguine, 75015, Paris, France

## Abstract

**Introduction:** Platelets are implicated in numerous inflammatory responses and promote pulmonary failure through interactions with locally recruited neutrophils. The platelets' roles in inflammation during sepsis remain inconclusively understood. The aim of this study was thus to evaluate the innate immunity and inflammation associated functions played by blood platelets during polymicrobial sepsis using an experimental murine model and their actions during sepsis-induced acute lung injury (ALI). As antiplatelet molecules revealed potential in reversing bacterium induced-platelet death—at least experimentally—, we then evaluated the effect of acetylsalicylic acid in such experimental sepsis model, and particularly lung alteration.

**Methods:** Sepsis was induced with cecal ligation and puncture (CLP) on wild-type C57BL/6 mice previously treated—or not—with acetylsalicylic acid (ASA). Mice were sacrificed 24h and 48h after the sepsis induction. The survival rate during the following 72h was assessed. Aggregation, inflammatory cytokine secretion (Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ), Interleukine-6 (IL-6), RANTES, soluble CD40L (sCD40L), soluble CD62P (sCD62P)), bronchoalveolar and blood platelet, total leucocytes and neutrophil counts were

evaluated at 24h and 48h after surgery and sepsis induction.

**Results:** ASA reduced sepsis-induced mortality by 42%; this was accompanied by a significant decrease in recruitment of neutrophils and platelets in the lungs. ASA, at this protective concentration (25 mg/kg) had neither significant effect on immunomodulator secretion.

**Conclusion:** Platelets participate to the sepsis pathophysiology through the release of inflammatory cytokines and likely contribute to the lung failure through interactions with leucocytes, rendered prone to migrate.

**Keywords:** Platelets; Neutrophils; Sepsis; Acetylsalicylic acid

## Introduction

Platelets are central to hemostasis and thrombus formation but also play seminal roles during inflammation and infection [1, 2]. Numerous studies have further shown the generally underestimated role of platelets in different inflammatory pathologies such as atherosclerosis and rheumatoid arthritis, in cancer and metastasis, and also in viral, parasitological and bacterial infections and foremost in sepsis, suggesting that platelets as a key elements of the immune surveillance [3-7]. Platelets display numerous receptors, such

as toll like receptors, Fc $\gamma$ RII, complement receptors and integrins conventionally described for the hemostatic response (GPIIb-IIIa and GPIb) which can recognize infectious pathogens including numerous bacteria and viruses [2, 8, 9]. These interactions induce platelet activation and the release of multiple immune modulating factors from the platelet granules; such mediators alter the host defence. Via CD40L and P-selectin surface glycoproteins, platelets enhance leukocyte functions—as most leukocytes express CD40—and ease the T cell, B cell, monocyte and granulocyte crosstalk with platelets; of particular notice is the fast and potent interaction of CD40/CD40L associated interactions between platelets and neutrophils both in steady state and pathological conditions [10-12].

Sepsis is one of the most causes of mortality in critical care units despite improved patient management and care. During sepsis, platelets are activated and sequestered (in the circulation or in different organs as liver, kidney or lungs [13, 14]) resulting with frequent thrombocytopenia in septic patients, influencing adverse outcome and mortality [15]. Acute lung injury (ALI) secondary to sepsis causes important mortality [16, 17]; it is characterized by an insult to the alveolar-capillary barrier inducing leakage

and alveolar fluid infiltration, causing hypoxemia [18]. Neutrophils are central to the causative origin of ALI, as they accumulate in the lung where they are attracted by chemokines/cytokines, suffer stresses and in turn stress the lung epithelium, favoring leakage and leucocyte extravasation [19, 20]. Lung edema, endothelial and epithelial injury are augmented by a transmigration of neutrophils into the interstitium and bronchoalveolar compartment. Neutrophils are central to the development of ALI, but platelets amplify it [14, 21]. It has been shown that platelet-derived sCD40L, RANTES and Platelet Factor 4 (PF4) support neutrophil extravasation to the lung and participate to the induction of lung edema in ALI [14, 21-23]. Moreover, as platelets are required for maintenance of endothelial barrier integrity, a thrombocytopenia—such as induced by bacteria in blood—lead to increased permeability of systemic and pulmonary vessels.

Antiplatelet therapy, such as the acetylsalicylic acid (ASA), can reduce ALI in patients [24] and in experimental models as well [25, 26]. Several studies proved the involvement of platelets during such ALI [27]. The platelet receptor P2Y<sub>12</sub>, implicated in aggregation and granules release, can influence inflammation and

sepsis-induced ALI [28, 29]. The deletion of CD40L and the use of thienopyridin or statins has been shown to prevent neutrophil lung infiltration in abdominal sepsis murine model [14, 23, 28]. Platelets can also participate to the inflammation, particularly with TNF $\alpha$  release or by contributing to NET formation, during endotoxemia via TLRs [30]. ASA could also reduce NET formation by platelets, contributing to acute lung injury [26, 31].

Nevertheless, there are contradictory results, because studies have shown that platelets are not required for lung neutrophil infiltration in the lungs [32] and prevent macrophage cytokines release during sepsis [33]. Therefore, additional studies are needed to clarify platelet involvement in sepsis-related pulmonary complication.

In this study, we observed the close communication between platelets and neutrophils in acute lung injury during an experimental mouse sepsis. Finally, we focused on ASA effect during acute lung injury and platelet-induced inflammation.

## **Materials and methods**

### ***Mice***

C57Bl/6J male, 8 to 12 weeks age, were purchased from Charles River Laboratories (Wilmington, USA). All animals were

housed at the “Plateforme d’expérimentations et Analyses” (Plexan, Saint-Etienne, France). The protocol was approved by the Ethics Committee of CREEA (authorization CU15N03). Animals were anesthetized by intraperitoneal (i.p) administration of 10 mg xylazine (Bayer, Lyon, France) and 100 mg of ketamine (Virbac, Carros, France) per kg body weight.

### ***Experimental study design***

Before and after CLP surgery, weight and temperature were measured. ASA (25  $\mu$ g/g) or Phosphate Buffer Saline (PBS, Sigma Aldrich, Saint Quentin-Fallavier, France) (placebo) was injected by i.p 30 min prior to sepsis induction. Sepsis was induced by ligation and puncture of the ceacum as described previously [21]. Briefly, animals were anesthetized, the ceacum was externalized and ligatured with standard length (1cm). A double perforation was achieved with a 21-gauge needle and ceacum was reintroduced into the peritoneal cavity. Mice were stitched with a suture and rehydrated with by 1ml of 0.9% isotonic saline. Sham mice underwent the the same surgical procedure but the ceacum was neither ligated nor punctured. Then, 24h or 48h after surgery, mice were reanesthetized and blood was drawn by intracardiac puncture using a needle coated with 10% citrate dextrose solution (Sigma Aldrich ,

Saint Quentin-Fallavier, France). Bronchoalveolar lung fluid (BALF) were collected with 3ml of 0.9% isotonic saline, centrifugated at  $300 \times g$  5 minutes, pelleted cells were resuspended in 100 $\mu$ l of PBS for platelets, total leucocytes and neutrophil numeration and supernatants were stored at  $-80^{\circ}\text{C}$  for cytokine quantification. Lungs, heart, intestines and liver were recovered and fixed 24h in paraformaldehyde 4% for histology. 200  $\mu$ l of blood were used for numeration of total leucocytes, neutrophils and platelets, and the remaining blood sample was centrifuged at  $300 \times g$  for 10 minutes to obtain plasma. Plasma were collected and kept frozen at  $-80^{\circ}\text{C}$  until cytokine analysis.

### ***Cells count***

Whole blood or BALF cells (Total leucocyte, neutrophils and platelets) were quantified with a MS4s Hematology analyzer (Melet Schloesing – Osny – France).

### ***Clinical observation score***

Clinical signs were recorded and a score was assigned for each parameter as follows: general behavior (Normal = 1; excited and aggressive = 2; prostrate = 3; contraction and dorsal stretching = 4), look (normal pelling and rond eyes = 1; Rough coat and orbital tightening = 2; fur lost = 3),

physiology (normal breathing = 1, high cardiac frequency = 2, anormal physiological signs = 3), interactive behaviour (normal = 1 , limited = 2, none = 3), solitude (absent = 0 , present = 1); resulting in a maximum score of 14. Dead mice were scored with the highest clinical score.

### ***Tail bleeding time assay***

Tail bleeding times were performed after a 3 mm amputation of the mice tail tip from and immersion in PBS at  $37^{\circ}\text{C}$  during 10 minutes. Bleeding time was defined as a complete stop of bleeding of at least 20 sec.

### ***Histology and Immunohistology on mice lungs***

Fixed mice lungs were then embedded in OCT embedding matrix (CellPath, Newtown, UK). Sections (8 $\mu$ m) were performed with stained with Hematoxylin and Eosin or with antibodies against CD41 (clone MWReg30) or Ly6G (clone 1A8) purchased from BD Bioscience, (Le pont de Claix - France). The immunohistochemical staining procedure was performed as follow: endogen peroxidase activity was blocked by incubating sections with 0.3% hydroxide peroxide then unspecific binding sites were blocked with bovine serum albumin (10% or 1% for platelets and neutrophils respectively). Sections were

incubated 1 hour at room temperature with primary antibodies CD41 (25 µg/ml) or Ly6G (50µg/ml) then 30 minutes with a biotinylated antibody (clone G15-337, 5 µg/ml). The revelation was performed with streptavidin-HRP during 30 minutes then with with 3-3'-diciminobenzidine tetrahydrochloride {DAB}. Samples were examined with Nikon Eclipse Ti fluorescence inverted microscope (Nikon France S.A, Champaigny sur Marne, France).

### ***Assessment of enzyme activity***

Alamine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were measured in the plasma with activity assay kit (Sigma-Aldrich, Saint Quentin-Fallavier, France) according to the manufacturer's instructions.

### ***Assessment of cytokine release***

Release of mouse sCD62P, RANTES, IL-6, TNF $\alpha$  and sCD40L was measured in plasma. using a multiplexed Luminex® technology (Merck Millipore, Guyancourt, France) according to the manufacturer's instructions. Results were read on a Bioplex 200 system (Bioplex Manager™ software, Biorad, Marnes-la-Coquette, France).

### ***Statistical analyses***

A two-way ANOVA with repeated measures and a Bonferroni post-hoc were used for inter-experimental comparisons. All values are reported as the mean  $\pm$  standard error of the mean (SEM). A p-value of  $< 0.05$  was considered statistically significant. A Log-rank (Mantel-Cox) test was used to compare survival distributions. Dixon test was used to discriminate an abnormal value. Statistical analyses and graphs were performed with GraphPad Prism 5 (San diego, USA) software.

## Results

### Acetylsalicylic acid reduces mortality induced by sepsis without affecting inflammation plasma cytokines

Clinical scores and mortality were evaluated during 72h following sepsis induction with CLP surgery. Every Sham control mice survived (100%) whereas only 26.7% mice survived the CLP procedure (Mantel-Cox test;  $p < 0.01$ ). However, mice pre-injected with ASA (25 mg/kg) 30 minutes prior to the sepsis induction displayed a survival rate compared to mice that underwent the CLP procedure but received the placebo (68.7 % vs. 26.7% survival, respectively), (**Figure 1 A**,  $p < 0.01$ ). The ASA was used at a concentration which didn't modify bleeding time (**supplemental figure 1**).

The clinical score, as defined in the Material and Methods section increased in each mouse that declared sepsis and received a placebo and reached a value of 8,5 24h post surgery while it remained at a value of 4 in Sham control mice ( $p < 0.001$ ) (**Figure 1 B**). Septic mice pre-treated with ASA (4.6 at 24h) have lower clinical score compared to septic mice having received the placebo (8.5 at 24h;  $p < 0.001$ ) (**Figure 1B**). In particular, weight and body temperature were lower in the sepsis group than in the control Sham mouse group ( $p < 0.01$  for

temperature and  $p < 0.0001$  for weight, **Supplemental figure 2 A et B**).

Moreover, the activity of ALT, AST and LDH enzymes—usually released into the circulation after liver, hepatic and myocardial damage—, was quantified 24 and 48 hours in plasma after sepsis induction to evidence any organ failure associated to sepsis. In correlation with the elevated clinical scores, there was a significant increase of LDH activity at 24h ( $277.67 \pm 42.77$  mU/ml) (**Figure 1 C**) and AST activity at 48h (**Figure 1 D**) in sepsis mice compared to Sham control mice ( $90.38 \pm 23.02$  mU/ml,  $p < 0.001$ ). A non significant increase of ALT activity was observed in sepsis mice at 24h and 48h ( $1.97 \pm 0.32$  mU/ml and  $1.39 \pm 0.46$  mU/ml, respectively) (**Figure 1 E**). However, despite the reduction of the sepsis-induced mortality mediated by the ASA treatment, this molecule could not reduce the activity of all three enzymes neither 24h nor 48h after sepsis induction (**Figure 1 C/D/E**).

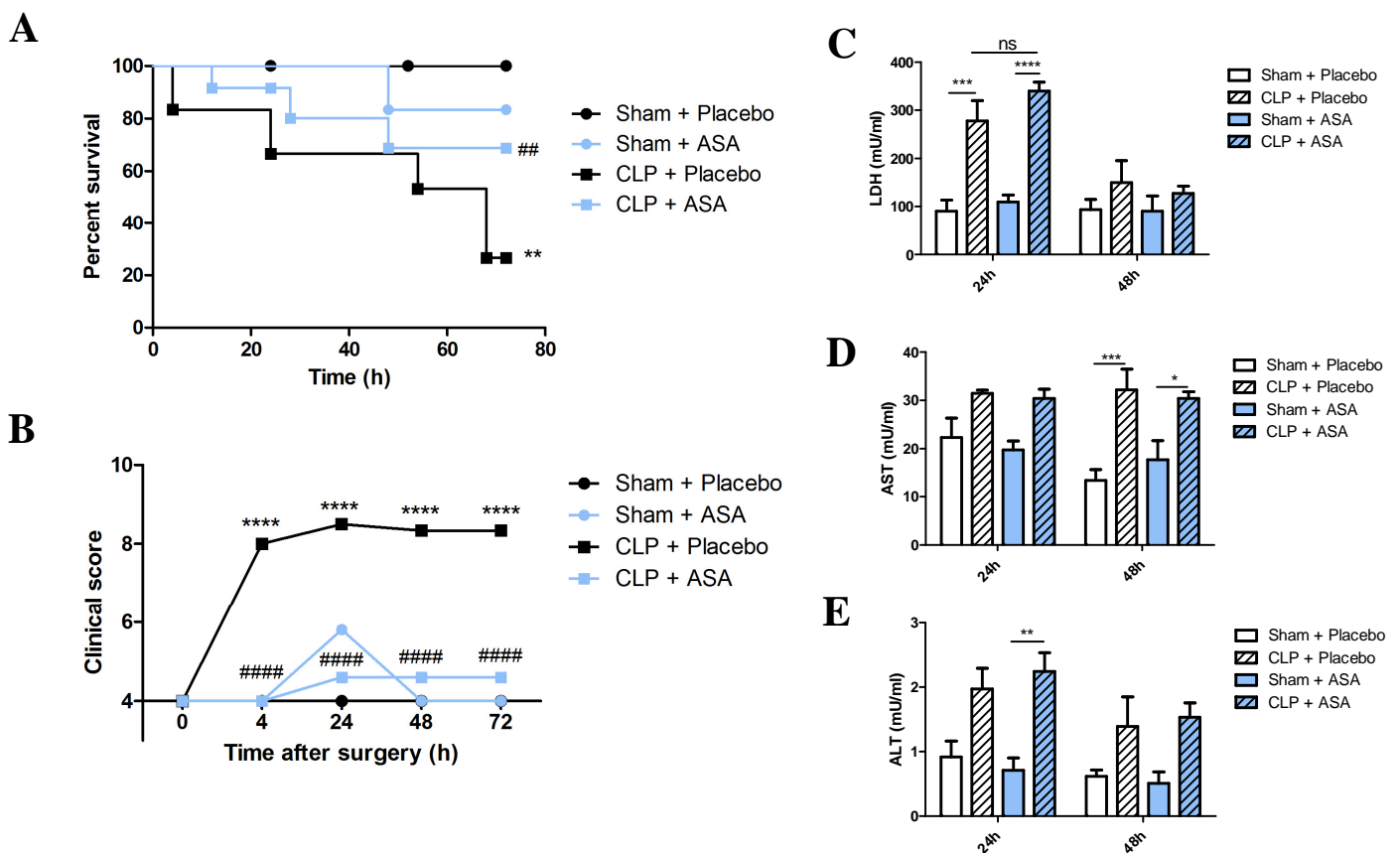
Since ALI is a frequent complication of sepsis [34], we next investigated lung alterations in our model. Thus, septic mice displayed macroscopically haemorrhagic



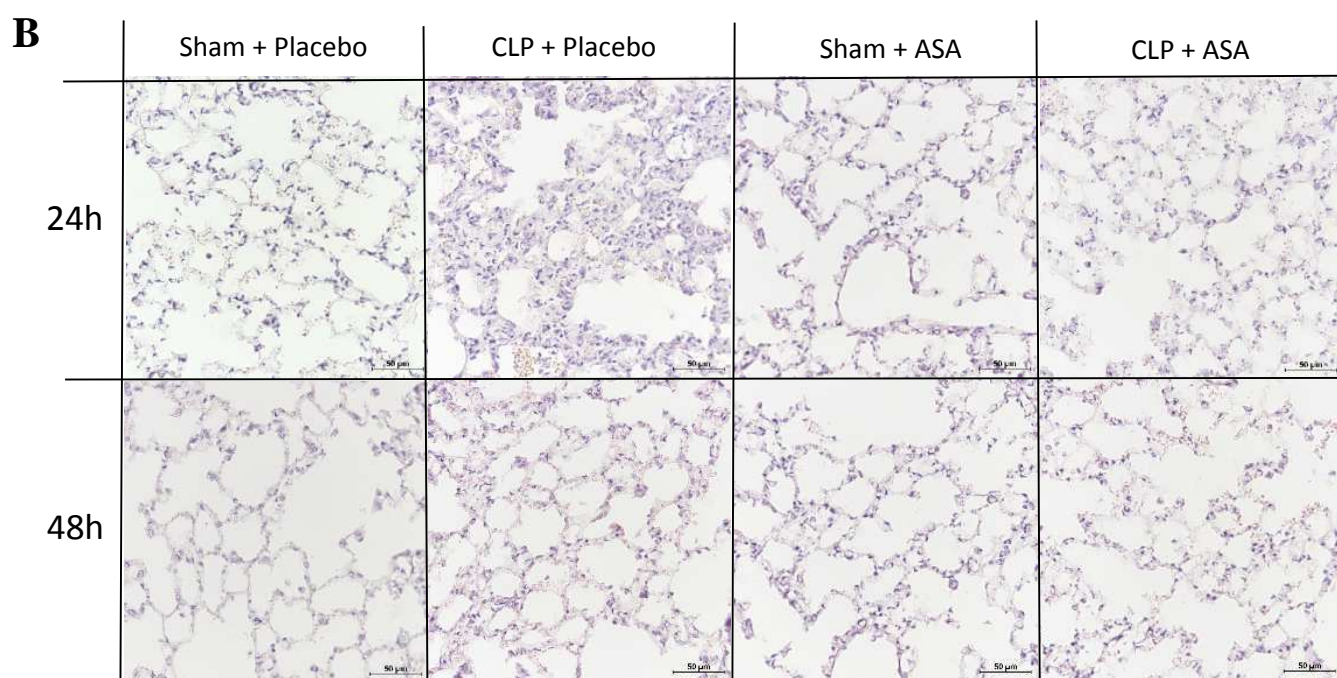
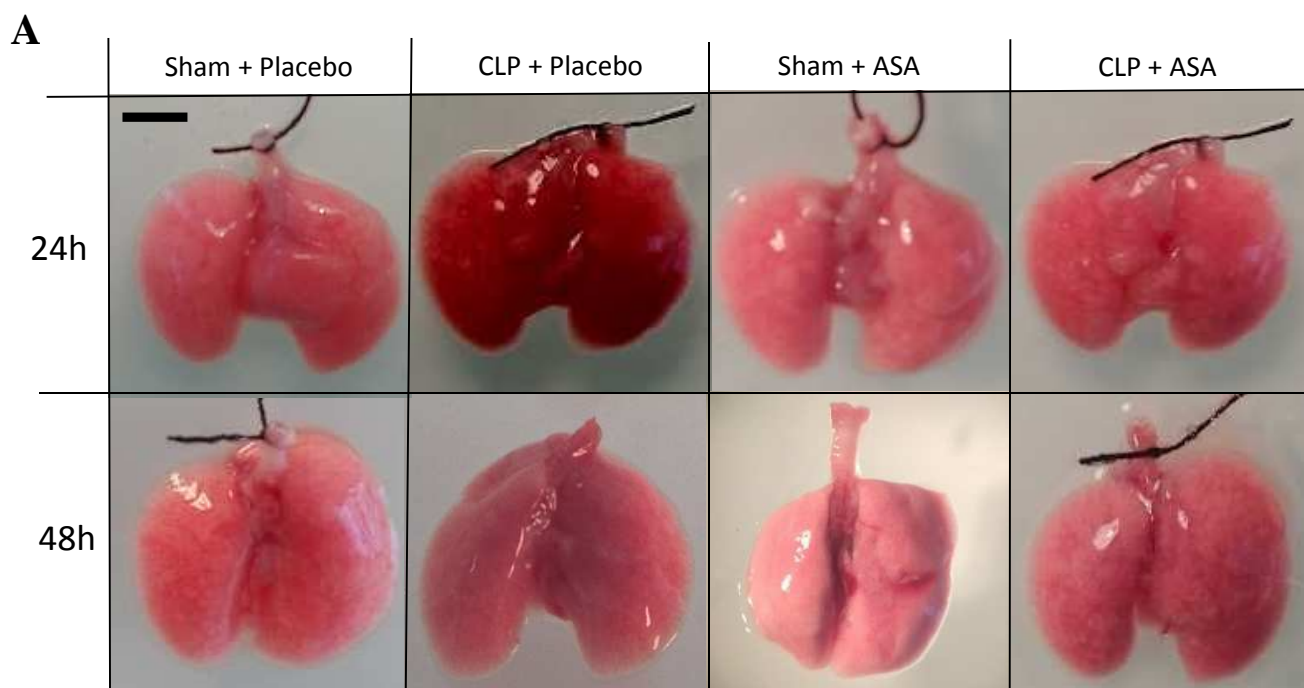
lungs compared to Sham control mice (**Figure 3 A**). Microscopic observation of lung hematoxylin and eosin (H/E)-stained lung tissue sections revealed alveolar septal thickening of the lungs at 24h and 48h post surgery in septic mice compared to Sham control mice (**Figure 2 B**). There was an accumulation of inflammatory cells and an influx of protein rich-fluid into alveolar spaces in septic mice (**Figure 2 B**) These

results evidence that experimental sepsis caused by CLP induces serious tissue injuries in the lungs, likely associated with organ failure (as suggested by plasma enzyme elevated levels) that could account for worsening mortality.

Treatment with ASA seemed to reduce lung injury, which could contribute to the limitation of mice mortality.



**Figure 1. Sepsis enhances mortality and worsens organ failure.** Survival rate (A) and clinical scores (B) were assessed during 72 hours post surgery on mice having CLP or Sham control surgery with treatment with ASA or Placebo (n=7-12). Activity of LDH (C), AST (D) and ALT (E) were measured 24 and 48h post surgery on CLP and control sham mice treated with placebo or ASA (n=5/group). p value was calculated using Mantel-Cox test or two way ANOVA with Bonferroni post-test. \* p<0.05, \*\* p<0.01, \*\*\* p<0.001, \*\*\*\* p<0.0001 comparison of sham+placebo vs. CLP+placebo. ## p<0.01 ##### p<0.0001 comparison of CLP+placebo vs. CLP+ASA.



**Figure 2. Acetylsalicylic acid reduces sepsis-induced lung damage.** (A) Representative photos of lungs of CLP and Sham control mice treated or not with ASA (25mg/kg) 30 minutes prior surgery. Scale bar = 0.5cm. (B) Sections of lungs were stained with haematoxylin and eosin and images were captured Original magnification, X400. Scale bar = 0.05mm. One experiment representative of 5 different experiments.

## **Neutrophils and platelets are involved together (or in concert) in lung damage**

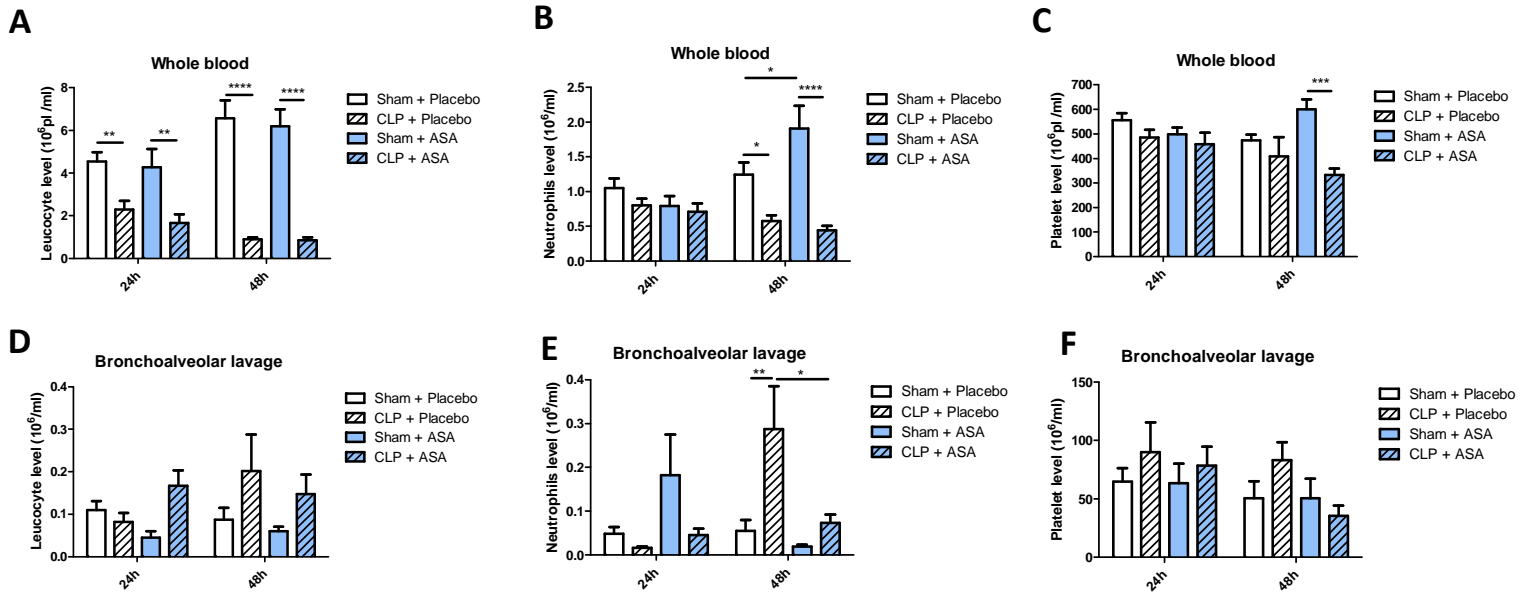
During sepsis-induced lung damage, neutrophils migrate to alveolar capillary barriers, causing an increased vascular permeability resulting in lung edema and ALI. We have thus assessed whether—in our experimental sepsis model—, blood neutrophils migrate to the lungs. Firstly, after the induction of sepsis, we observed that blood circulating leucocyte counts significantly decreased from  $4.54 \pm 0.44 \times 10^6/\text{ml}$  in control Sham mice to  $2.29 \pm 0.42 \times 10^6/\text{ml}$  in CLP mice and from  $6.57 \pm 0.84 \times 10^6/\text{ml}$  to  $0.90 \pm 0.08 \times 10^6/\text{ml}$  24h and 48h after sepsis induction, respectively ( **$p < 0.01$  at 24h and  $p < 0.0001$  at 48h, Figure 3A**). A neutrophil blood count drop was seen 48h, and not 24h, after the sepsis induction in septic mice when compared to as in Sham control mice ( **$p < 0.05$ , Figure 3B**). Secondly, there was no significant difference in platelet counts between Sham control and CLP mice (**Figure 3C**); the platelet count softly decreased in both Sham control and CLP mice at from 24h to 48h post surgery (**Figure 3A**). Reciprocal to the situation in blood, there was an increase of leucocytes in BALF from CLP mice 48h after sepsis induction, although not significant (**Figure 3D**). The number of

neutrophils in BALF was significantly more elevated in septic compared to as in Sham control mice at 48h ( $0.287 \pm 0.098 \times 10^6/\text{ml}$  vs  $0.055 \pm 0.025 \times 10^6/\text{ml}$ ;  **$p < 0.01$** ) (**Figure 3E**). There was also more platelets in BALF at 24h ( $90.11 \pm 25.29 \times 10^6/\text{ml}$ ) and 48h ( $83.14 \pm 15.47 \times 10^6/\text{ml}$ ) in septic compared to as in Sham control mice at 24h ( $64.78 \pm 11.55 \times 10^6/\text{ml}$ ) and 48h ( $50.37 \pm 14.75 \times 10^6/\text{ml}$ ) (**Figure 3F**). However this increase remained not significant.

Next, we observed that ASA treatment could slightly reduce the leucocyte count in BALF 48h after sepsis induction in septic mice (**Figure 3D**). We also observed that ASA treatment significantly reduced neutrophil lung sequestration 48h after sepsis induction (**Figure 3E**) despite it did not restore the leucopenia observed during sepsis (**Figure 3B**). There was also a decrease in platelet infiltrating lungs following the ASA procedure (**Figure 3F**) despite this molecule has no visible effect on circulating platelets in septic mice although it seemed to be associated with an increase of circulating blood platelet counts, at least in Sham control mice treated with ASA, pertaining the likeliness that ASA has

protected septic mice from extended platelet drop (**Figure 3C**). In total, platelets and neutrophils seem to migrate from blood

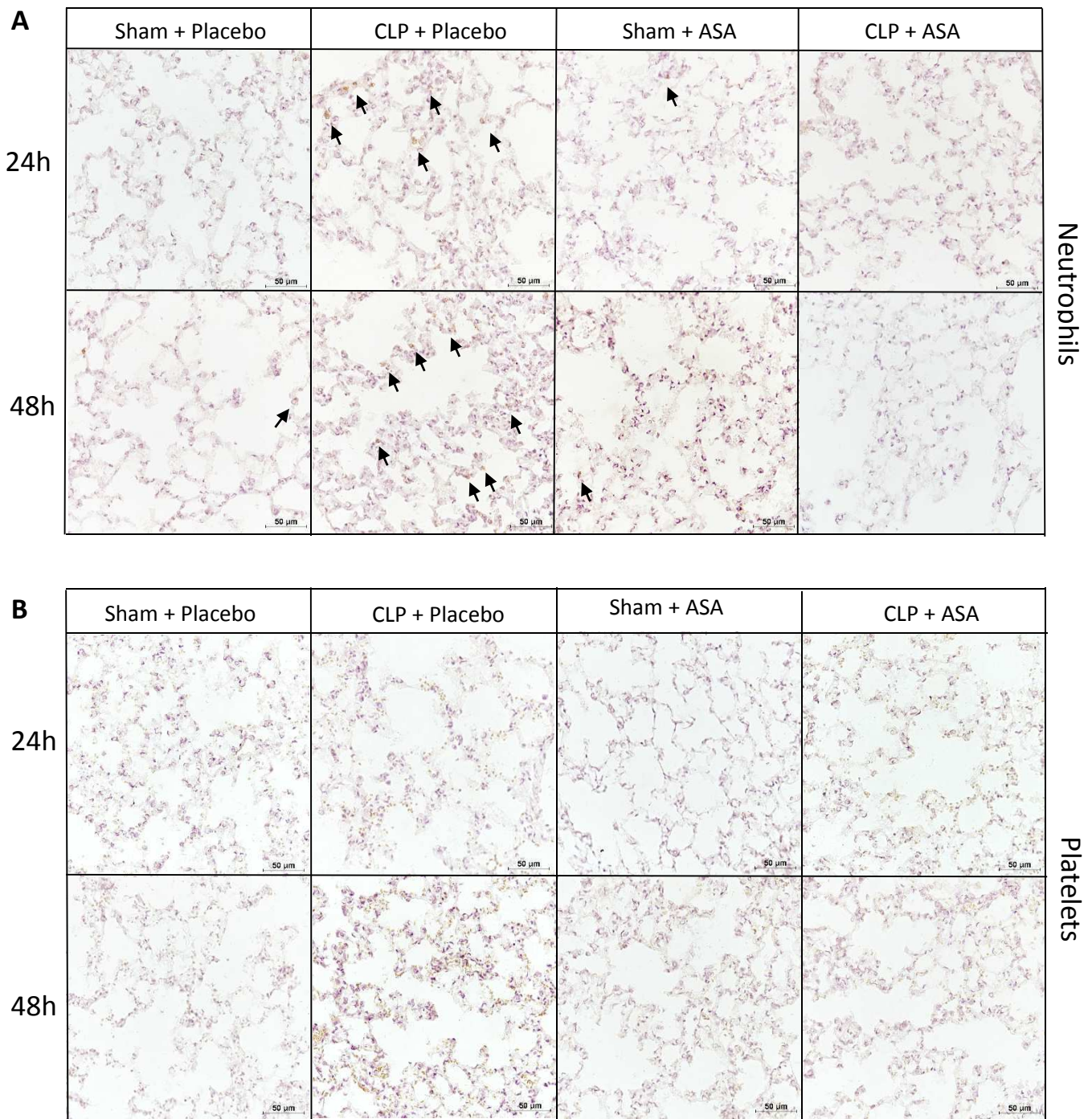
to the pulmonary septum mainly 48h after surgery.



**Figure 3. Neutrophils and platelets migrates from blood to lung during sepsis.** Quantification of circulating blood (n=7-13) (A-C) leucocyte counts (A) neutrophil counts (B) and platelet counts (C) or BALF (n=6-12) (D-F) leucocyte counts (D), neutrophil counts (E) and platelet counts (F) in Sham control or CLP mice treated with placebo or ASA before surgery. p value was calculated using two way ANOVA with Bonferroni post-test (\* p <0.05; \*\*, p <0.01; \*\*\*, p <0.001; \*\*\*\* p <0.0001).

These data were confirmed at the anatomical level by immuno-histology microscopy targeting neutrophils (Ly6G) and platelets (CD41) visualized directly in lung tissue. Parallel to findings in BALFs, there were more neutrophils in pulmonary septum in septic compared to as in Sham control mice at 24 and foremost 48h (**Figure 4A**). There were also significantly more platelets sequestered in the lung in the

CLP compared to the Sham control mice (**Figure 4B**). Septic mice treated with ASA exhibited less neutrophil and platelet lung recruitment as observed within pulmonary parenchyma. In all, this molecule can be hypothesized to mediate protection against some level of pathogenicity and potentially interactions between of platelets and neutrophils in septic mice displaying ALI.



**Figure 4. Neutrophils and platelets participate to lung failure during sepsis.** Representative histological images of neutrophils (LY6G) (A) and platelets (CD41) (B) were stained by immunohistochemistry on lung section from mice having CLP or Sham control surgery, treated by placebo or ASA (25mg/kg). Original magnification, X400. Scale bar = 0.05mm. One experiment representative of 5 different experiments.

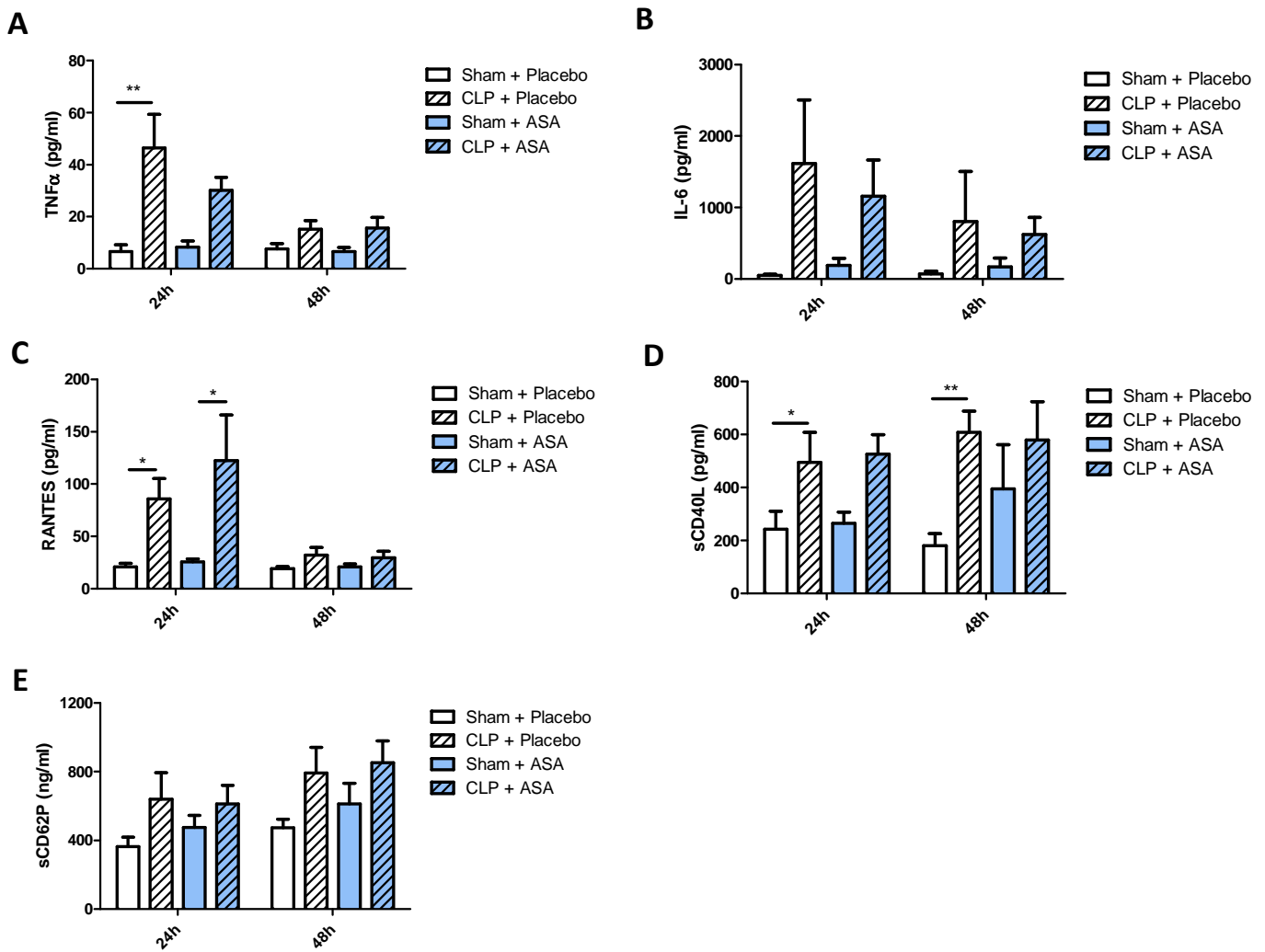
## **Acetylsalicylic acid has no effect on sepsis induced systemic inflammation**

Sepsis is characterized by an exacerbated systemic inflammation. This was indeed ascertained in the CLP group compared to as in the Sham mice control group, by measuring plasma concentrations of inflammatory cytokines (TNF $\alpha$ , IL-6 and RANTES) in plasma (compared to as in Sham control mice). There were indeed elevated levels of TNF $\alpha$  (46.4 $\pm$ 12.9 pg/ml vs 6.6 $\pm$ 2.6 pg/ml; p<0.01) (**Figure 5A**), IL-6 (845.1 $\pm$ 488.4 pg/ml vs 50.3 $\pm$ 19.2 pg/ml) (**Figure 5B**) and RANTES (85.9 $\pm$ 19.3 pg/ml vs 20.8 $\pm$ 3.4pg/ml; p<0.05) (**Figure 5C**) 24h after surgery. This returned to baseline levels at 48h. Platelets are prone to release many immunomodulator molecules and participate to inflammation during sepsis [35]. Indeed, sCD40L and sCD62P levels were significantly more elevated in the CLP group compared to as in the Sham

control group. sCD40L at 24h tittered 494.7 $\pm$ 113.2 pg/ml vs 242.0 $\pm$ 67.5 pg/ml, p<0.05 and 608.1 $\pm$ 80.2 pg/ml vs 179.4 $\pm$ 46.1 pg/ml, p<0.01 at 48h (**Figure 5D**). In contrast, sCD62P levels were significantly increased (at 24h and 48h), neither in septic nor in control mice (**Figure 5E**), although a tendency was observed.

No significant reduction of classical or platelet-associated inflammatory cytokines was observed in septic mice treated with ASA compared to Placebo (**Figure 5A/B/C/D/E/F**), apart from a weak decrease of TNF $\alpha$  levels in the CLP + ASA group compared to the CLP + placebo group (**Figure 5A**).

Every data (Mean $\pm$ SEM) were resumed in the **supplemental table 1**.



**Figure 5. Experimental sepsis induces inflammatory cytokines release.** TNF $\alpha$  (A), IL-6 (B), RANTES (C), sCD40L (D) and sCD62P (E) were measured in plasma, by an enzyme-linked immunosorbent assay or luminex technologies, from CLP and Sham control mice 24h and 48h post surgery, treated with ASA (25mg/kg) or Placebo. p value was calculated using two way ANOVA with Bonferroni post-test (\* p < 0.05; \*\* . p < 0.01)

## Discussion

In this study, an experimental murine sepsis model was performed by CLP surgery, that induced systemic inflammation, lung structure alteration, and resulted in a high mortality rate (73.3% 72h after surgery). Human sepsis typically associated with an increase their body temperature while mice exhibit a decrease in body temperature when sepsis is created by CLP [36]. In CLP septic mice TNF $\alpha$ , IL-6, RANTES plasma levels strongly increased 24h after sepsis induction but decreased and returned to baseline at 48h. This resembles the inflammatory storm followed by an immune depression observed in patients [37]. These data also correlate with clinical observations and experimental studies using the same model or using LPS injection to model of sepsis [22, 29, 38-40] in which platelets might contribute to TNF $\alpha$  levels after LPS ligation via their TLR4 [41]. However, increased levels of serum TNF $\alpha$  have also been reported in mice infected by *Escherichia coli* but depleted from platelets [33]. This indicates that, in addition to platelets, other cells contribute to the production of TNF $\alpha$ , such as TLR4-LBP expressing lymphocytes or monocytes/macrophages [33, 42, 43]. Additional platelet-related immunomodulators, such as sCD62P and sCD40L, were also found elevated in mice

after sepsis, significantly for the latter. This is in accordance with the studies by the Thorlacius group which demonstrated the role of platelet-originating sCD40L and PF4 in the pathogenesis of sepsis-induced ALI in CLP models by either depleting mice from platelets or using CD40L-deficient mice [14, 44-46]. Our work along with those of the Thorlacius group confirms the implication of platelets sCD40L in lung inflammation during sepsis. Besides our observation that sCD62P increased during sepsis, although not significantly, is interesting since it has been observed that sCD62P levels correlated to organ damage during infection by *Streptococcus pyogenes* in mice [13].

However, in our model, ASA, although greatly improving mice survival, failed to reduce the plasma levels of inflammatory cytokines. Indeed, there are conflicting data on ASA efficacy, depending on the mice models used: in a LPS injection model in mice, ASA worsened mortality, that was attributed to the regulatory role played by platelet via COX-1 on macrophage inflammation [33]. On the contrary, during aerosolized LPS induced ALI, ASA reduced the neutrophil influx into the lung, the release of platelet-derived RANTES and PF4, and protected from ARDS [31]. Besides, in our model, the kinetics of inflammatory factor secretion could also be



faster and the participation of platelets to the cytokine storm could be earlier than 24h. therefore platelet-associated inflammatory cytokines could be investigated at earlier time points, within the few hours after the induction of sepsis, for instance.

The uncontrolled inflammatory state in mice infected with a broth of bacteria such as observed after the CLP procedure, was thus likely to induce organ failure and be responsible to the increased plasmatic concentration of different enzymes released during injury or cell death. Indeed, we observed LDH, AST and ALT levels were substantially elevated in the plasma of CLP mice, in accordance to what was reported by other investigators using endotoxemia mouse models [33]: this is fairly evocative of organ distress and organ lesions. Again, ASA treatment failed to reduce these markers, indicating that, although aspirin significantly reduced the mortality of septic mice, it did not prevent some organ dysfunction. We evidenced further a pulmonary failure with disruption of tissue architecture and edema in CLP as opposed to Sham control mice. There was infiltration of neutrophils and platelets in the lung interstitium, which raises the importance of the communication of those two cell types in the pathophysiology of pulmonary complication of sepsis. Platelets support—via immunomodulatory factors release and

directly by glycoprotein expression such as CD62P—the recruitment of neutrophils to the lungs in CLP-induced sepsis [45, 47, 48].

We could observe in septic mice that neutrophils and platelets seem to have been recruited from the circulation to the lung, as 1) circulating neutrophils were substantially decreased while increased in BALF and in the pulmonary parenchyma, 2) platelets were sequestered in the lung, although their circulating numbers remained stable. This could be explained either by a sustained thrombopoiesis, compensating platelet migration thanks to high level of circulating thrombopoietin [49, 50] or by local thrombopoiesis within the lungs, as recently and elegantly demonstrated in mice [51]. ASA was able to significantly prevent lung damage in septic mice by preventing alveolar septal thickening as well as neutrophil and platelet infiltration. This effect of ASA could contribute to the improvement of septic mice survival. However, other mechanisms of ASA action can not be ruled out insofar as this molecule has numerous effects on cells, such as preventing endothelial cell activation, macrophage activation and transmigration, neutrophil chemotaxis and apoptosis [52] which may occur in a deregulated manner during the early pro-inflammatory acute phase of sepsis. For instance, ASA has been

shown to reduce neutrophil-platelet complexes and Neutrophil Extracellular Traps [25, 26, 31]. In all, there seem to be several layers of controls in the crosstalk of platelets and neutrophils that attract them together in sites such as lungs and render them responsive to ASA, according to mechanisms that have not been completely clarified. Several molecules with

antiplatelet activities, such as thienopyridines (Ticagrelor, Clopidogrel...) and ASA, showed beneficial effects during human or animal sepsis [13, 29, 53, 54]; however, further evidence is necessary to elucidate how these molecules, and ASA particularly, acts.

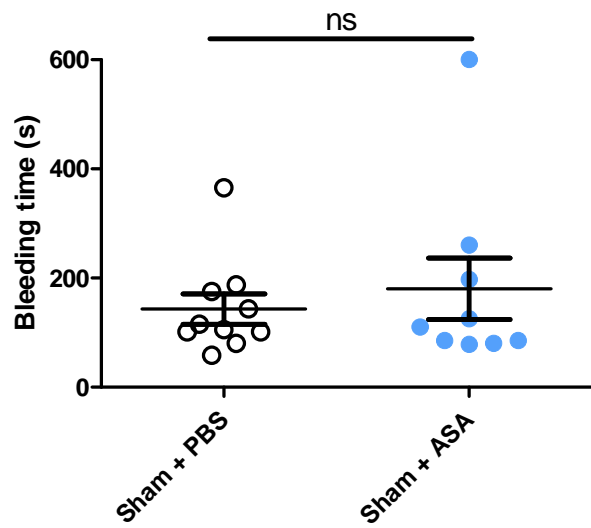
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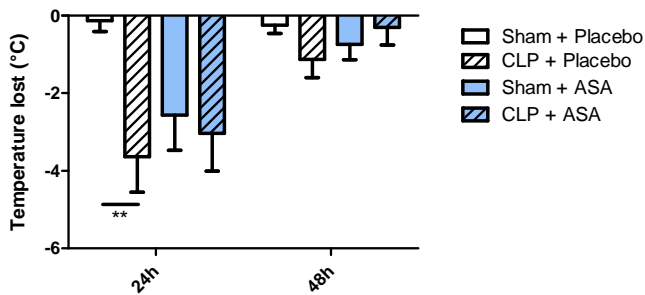
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## Supplemental data

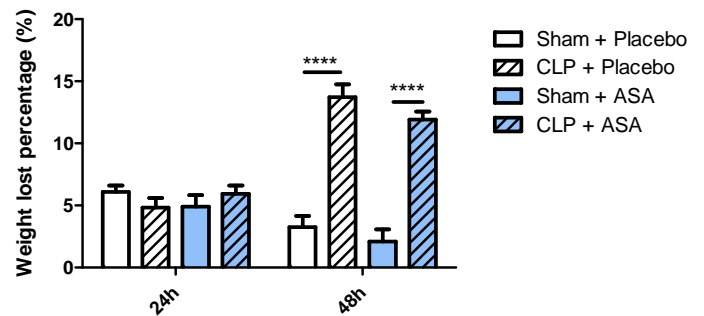


**Supplemental figure 1. ASA does not modify bleeding time.** Treatment with ASA has no effect concerning bleeding time in Sham control pre-treated with placebo (PBS) (n=10) compared to Sham control pre-treated with ASA (n=9). Observation of bleeding was performed during 10 minutes.

**A**



**B**



**Supplemental figure 2. Sepsis induced temperature and weight drop.** Temperature (A) and weight (B) were measured before and after surgery in CLP and Sham control mice 24h and 48h post surgery, treated with ASA (25mg/kg) or Placebo. p value was calculated using two way ANOVA with Bonferroni post-test (\*\* p < 0.01; \*\*\*\*. p < 0.001).

## Supplemental table

N	Time	Sham + Placebo	CLP + Placebo	Sham + ASA	CLP + ASA	Cytokines
5-13	24h	6,6±2,6	46,4±12,9	8,3±2,3	30,2±5,0	TNFα (pg/ml)
7-9	48h	7,5±2,1	15,2±3,2	6,6±1,5	15,7±4,1	
7-12	24h	50,3±19,2	845,1±488,4	191,1±100,0	1162,3±501,7	IL-6 (pg/ml)
7-9	48h	72,4±35,1	806,8±698,8	48,5±15,8	626,9±234,1	
8-12	24h	20,8±3,4	85,9±19,3	25,8±2,6	122,4±43,4	RANTES (pg/ml)
7-9	48h	19,2±1,8	32,1±7,4	20,7±3,1	29,6±6,3	
8-12	24h	242,0±67,5	494,7±113,2	264,6±41,9	525,8±73,8	sCD40L (pg/ml)
8-10	48h	179,4±46,1	608,1±80,2	394,9±116,8	578,9±145,1	
7-12	24h	364,2±54,3	640,6±152,9	475,0±70,4	612,7±106,4	sCD62P (ng/ml)
7-9	48h	473,1±50,6	791,2±149,2	612,8±118,2	851,1±128,3	
7-8	24h	215,9±75,5	50,2±18,4	258,0±78,5	32,9±13,2	PF4 (ng/ml)
6-8	48h	102,0±44,8	37,2±18,5	116,4±55,8	45,2±24,5	

**Supplemental table 1.** Data were the table indicating (Mean±SEM) and the number of animals studied per experimental group for each cytokines (TNFα, IL-6, RANTES, sCD40L and sCD62P) measured in plasma, by an enzyme-linked immunosorbent assay or luminex technologies, from CLP and Sham control mice 24h and 48h post surgery, treated with ASA (25mg/kg) or Placebo.

## Points clés et perspectives de l'étude

Plusieurs aspects semblent encore être incompris et de nombreuses perspectives de travail sont à réaliser afin de finaliser cette étude. La cinétique de cette pathologie apparaît un élément primordial à considérer puisque les plaquettes sanguines ont un niveau d'activation différentiel selon la progression d'une infection et ne jouent pas un rôle unilatéral dans la physiopathologie du sepsis<sup>244</sup>. Nous avons donc décidé d'évaluer la participation des plaquettes à la réponse inflammatoire aigüe du sepsis dans ce modèle de sepsis murin polymicrobien. Nous avons pu montrer une participation des plaquettes notamment au travers de la libération de sCD40L à 24h et 48h post-induction du sepsis, mais nous n'avons pas été en mesure de montrer un effet protecteur de l'ASA. Il apparaîtrait intéressant d'effectuer ces investigations à des temps plus précoces, dans les premières heures suivant l'induction du sepsis afin de pouvoir évaluer, dans l'éventualité où la réponse des plaquettes serait très précoce, l'effet de l'ASA.

La formation de microparticules plaquettaires est un processus physiopathologique car il favorise la génération de thrombine et ainsi induire des coagulations intravasculaires<sup>127,245</sup>. L'analyse des microparticules circulantes d'origine plaquettaire et l'effet de l'ASA sur ce phénomène pathogénique seront importants à étudier.

De plus, les interactions entre les plaquettes et les neutrophiles apparaissent centrales dans la pathogénèse du sepsis et particulièrement dans la dysfonction pulmonaire. Il semble essentiel de continuer à déchiffrer les actions jouées par les plaquettes et neutrophiles dans ce modèle murin. Pour confirmer la formation de complexes neutro-plaquettaires dans le parenchyme pulmonaire, des marquages d'immunofluorescence afin d'observer une co-localisation des deux types cellulaires devront être effectués. L'utilisation d'un modèle de souris rendues thrombopénique permettrait également de déterminer si les plaquettes favorisent de manière significative la migration des neutrophiles, et plus généralement, quelle est la part de leur contribution à l'orage cytokinique précoce au cours du sepsis. En effet, des études utilisant des souris thrombopéniques ont rapporté une protection contre une bactériémie à *S. aureus*<sup>246</sup> ou lors d'une endotoxémie<sup>243</sup> mais n'ont pas investigué le modèle de sepsis par CLP.

Il sera aussi essentiel d'évaluer la formation de complexes neutro-plaquettaires et NETs dans le sang et dans les poumons des souris contrôles et septiques ayant préalablement reçues



un traitement placebo ou de l'ASA dans la mesure où les NETs peuvent contribuer d'une part au contrôle de l'infection et d'autre part à la survenue de complications thrombotiques. En effet, l'ASA semble avoir la capacité de moduler la formation de NETs et de complexes neutro-plaquettaires durant des lésions des poumons induites lors de modèles murins par LPS aérosolisé ou de TRALI <sup>247,248</sup>.

De plus, les plaquettes favorisent la formation de NETs et ainsi le phénomène d'immunothrombose au cours du sepsis <sup>141,237</sup>. En effet, la coagulation intravasculaire disséminée est une complication fréquente du sepsis et les plaquettes participent à la thrombose <sup>82</sup>. L'évaluation de l'immunothrombose et la place des plaquettes dans notre modèle de sepsis murin polymicrobien permettra d'apporter de nouveaux arguments pour comprendre les effets délétères des plaquettes dans cette pathologie.

Le sepsis étant caractérisé par des défaillances pouvant affecter de multiples organes, il semble donc important de poursuivre les investigations en analysant comme nous l'avons fait pour le poumon, d'autres organes tels que le cœur, l'intestin et le foie pour distinguer le rôle des plaquettes dans les différentes dysfonctions d'organes <sup>249</sup>.

Ce travail constitue donc une base permettant de poursuivre les investigations pour mieux comprendre l'action inflammatoire menée *in vivo* par les plaquettes au cours du sepsis.

# Discussion

Depuis les premières observations de Schultze et Bizzozero à la fin du XIX<sup>ème</sup> siècle, les connaissances sur la plaquette, initialement décrite comme « le 3<sup>ème</sup> élément du sang » ont considérablement évolué<sup>250</sup>. En effet, les premiers travaux sur la plaquette ont rapidement mis en avant son importante capacité d'adhérence et d'agrégation dans le but de former une « plaque » ainsi que l'histologiste italien Bizzozero l'avait observé en qualifiant ces cellules de « Piastrine », signifiant « petite plaque ». De par leur rôle hémostatique, et inflammatoire, les plaquettes sont impliquées dans de nombreuses pathologies. En effet, nous avons vu que les plaquettes possèdent une variété de récepteurs pouvant reconnaître les pathogènes (bactéries, virus, parasites...) et interagir avec cellules de l'organisme et en particulier les cellules endothéliales et les cellules de l'immunité. Les plaquettes sont bien évidemment un élément clé de l'arbre vasculaire, mais de plus en plus d'études soulignent leurs implications dans le continuum inflammatoire. Lors de ce travail doctoral, nous avons étudié les mécanismes inflammatoires joués par les plaquettes en réponse à un stimulus bactérien, une souche entière de *S. aureus*, ou au sein d'un modèle expérimental de sepsis murin.

La pathogénicité d'une souche bactérienne est déterminée par les caractéristiques de virulence de cette bactérie. *S. aureus* détient un arsenal de facteurs de virulence afin de faciliter son adhésion aux tissus, une évasion immunitaire ou encore une attaque directe sur les cellules de l'hôte. Dans notre cas, nous avons décidé d'étudier les effets de différentes exotoxines staphylococciques au niveau plaquettaire. Lors d'infections bactériennes, les interactions entre les toxines sécrétées et les plaquettes circulantes sont tout à fait possibles. Cependant, peu d'études ont décrit les réponses inflammatoires des plaquettes dans ce contexte, la majorité s'étant focalisée sur le versant hémostatique de leur réponse. Dans la première étude présentée lors de ce travail doctoral, nous avons montré que dans les exotoxines de *S. aureus* utilisées expérimentalement (TSST-1, PVL, hémolysine  $\alpha$  et hémolysine  $\gamma$ ), seule l'hémolysine  $\gamma$  induisait une agrégation plaquettaire à sa plus forte concentration. La littérature présente une certaine hétérogénéité quant à l'induction de l'agrégation plaquettaire par cette toxine. Ainsi Bhakdi *et al.* et plus récemment, Schubert *et al.* avaient déjà montré dans une étude relativement ancienne, une action pro-agrégante de l'hémolysine  $\alpha$  à des concentrations identiques à celles que nous avons utilisées<sup>251,252</sup>. Cette différence peut s'expliquer du fait que, dans leur étude et contrairement à nos conditions, les plaquettes n'étant pas stimulées en présence de plasma, l'absence de facteurs plasmatiques et donc d'immunoglobulines dirigées contre ces toxines

pourrait empêcher l'inhibition de l'action pro-agrégante de l'hémolysine  $\alpha$  vis-à-vis des plaquettes <sup>253</sup>. Il apparaît que l'utilisation d'un inhibiteur de la glycoprotéine GPIIb/IIIa inhibe cette agrégation <sup>251</sup>.

De même Parimon *et al.* ont montré que l'hémolysine  $\alpha$  peut activer les plaquettes afin de former des complexes neutro-plaquettaires <sup>201</sup>. Or, les plaquettes utilisées dans cette étude proviennent de sang prélevé avec de l'héparine alors que notre équipe utilise du sang citraté. Dans la mesure où il a été montré que la libération de PF4 était variable selon l'anticoagulant utilisé, ceci suggérerait que l'anticoagulant aie également un effet sur la capacité des plaquettes à être activées <sup>252</sup>. En revanche, et en accord avec nos observations, Powers *et al.* ne constatent aucune agrégation plaquettaire en présence d'hémolysine  $\alpha$  et expliquent leur observation par le fait que la toxine forme un complexe avec la métalloprotéase ADAM10 qui inhibe l'agrégation et l'adhésion plaquettaire en clivant notamment la GPVI <sup>203</sup>. Enfin, nos résultats concordent avec ceux précédemment observés pour la TSST-1 indiquant que cette toxine n'a pas d'incidence sur l'agrégation plaquettaire. Concernant la leucocidine de Pantone-Valentine (PVL), nous n'avons observé qu'une très faible action sur la libération de RANTES ce qui est en accord avec une précédente étude indiquant aucune interaction entre cette toxine et les plaquettes <sup>201</sup>.

La reconnaissance plaquettaire des exotoxines de *S. aureus* induit une libération d'immunomodulateurs avec un profil différent selon l'exotoxine. En effet, il est connu que le passage du contenu granulaire se fait par le système caniculaire ouvert. Les canaux de ce système sont plus ou moins ouverts selon le niveau d'activation plaquettaire et permettent ainsi une libération différente <sup>254</sup>. De plus, notre étude révèle une synergie dans le mécanisme d'action des exotoxines. Les souches de *S. aureus* sécrétant généralement plusieurs types d'exotoxines, nous avons voulu nous placer dans des conditions plus physiopathologiques en combinant les toxines par deux, à leurs concentrations sub-optimales. Ainsi, lorsque TSST-1 et PVL sont utilisées individuellement, il n'y a que peu de sCD40L libéré pour les concentrations de toxines les plus importantes, voire aucune pour les concentrations sub-optimales. Cependant, une exposition de ces exotoxines combinées à des concentrations pourtant sub-optimales montre un relargage plaquettaire de sCD40L. Notre équipe a déjà décrit l'incidence de stimuli bactériens, comme le LPS, sur la libération de molécules immuno-modulatrices <sup>167</sup>. Ainsi comme les plaquettes ont la capacité de distinguer les isomorphes d'un élément pariétal d'une bactérie, ces cellules ont

aussi la faculté de différencier les exotoxines <sup>166</sup>. Il nous reste à présent à mettre en évidence que les cytokines libérées suite à la reconnaissance des exotoxines staphylococciques peuvent avoir un effet physiologique, or d'autres études ont montré que des molécules telles que RANTES ou sCD40L ont une incidence sur des cellules de l'immunité, *in vitro* <sup>166,175</sup>. De plus, ces cytokines pro-inflammatoires ont une part non négligeable dans la pathogénèse du sepsis et participent, entre autres, à la dysfonction de nombreux organes tels que les poumons <sup>228</sup>. Dans un modèle d'endotoxémie humaine, le taux de PF4 augmente rapidement <sup>255</sup>. Cette même étude montre également, *in vitro*, une activation des plaquettes par le LPS se traduisant par l'expression membranaire de CD62P et CD40L et la formation de complexes leuco-plaquettaires. Ceci suggère que la dégranulation plaquettaire suivant l'exposition des exotoxines que nous avons observée dans notre étude pourrait également initier l'interaction des plaquettes avec les leucocytes. D'un point de vue clinique, la libération plaquettaire de sCD40L et RANTES induite par les différentes exotoxines sécrétées par *S. aureus*, pourrait contribuer aux taux inflammatoires observés chez les patients septiques <sup>256</sup>.

De plus, il apparaît que l'inhibition des récepteurs P2X protège de l'action cytotoxique de l'hémolysine  $\alpha$  au niveau des monocytes <sup>257</sup>. Ces récepteurs étant également exprimés par les plaquettes, il est possible qu'ils soient également impliqués au niveau plaquettaire. On peut suggérer que cette exotoxine utilise le récepteur P2X1 afin de recruter le calcium intra-plaquettaire et induire la formation de pores au niveau de la membrane <sup>258</sup>. D'autres exotoxines synthétisées par l'espèce *Staphylococcus*, comme Staphylococcal superantigen-like protein 5, activent les plaquettes et favorisent la formation de microparticules plaquettaires, à fort capacité pro-inflammatoire et pro-coagulante, ce qui constituerait une manière supplémentaire pour les plaquettes de contribuer à la physiopathologie du sepsis <sup>208</sup>.

Cependant, il faut prendre en considération qu'au-delà de ces fonctions inflammatoires, les plaquettes ont aussi des capacités anti-microbiennes. En réponse à une sécrétion excessive d'hémolysine  $\alpha$  par *S. aureus*, les plaquettes libèrent une quantité de protéines microbicides plaquettaires <sup>259</sup>. Ce mécanisme permettrait une réduction de la virulence lors d'une endocardite infectieuse, suggérant la même action des plaquettes lors du sepsis. Toutefois, il est à prendre en considération que si la libération de protéines microbicides plaquettaires survient lors de la lyse des plaquettes, alors sont libérés également des nutriments issus du cytoplasme plaquettaire qui pourraient contrebalancer l'effet antimicrobien des protéines microbicides plaquettaires.

Après avoir étudié les relations étroites qui existent entre des facteurs de virulence libérées par *S. aureus*, tels que les exotoxines, nous avons voulu connaître l'effet global exercé par une bactérie entière issue de souches cliniques de *S. aureus*. Il est connu que les patients atteints de sepsis montrent une activation et agrégation plaquettaire à l'origine de thrombopénie en lien direct avec la mortalité <sup>97</sup>. Beaucoup de processus sont encore mal compris et reste à être élucidés dans le but de réduire cette chute du compte plaquettaire et de limiter l'hyper-inflammation précoce dans le sepsis.

Dans notre deuxième article, nous avons mis en évidence l'effet activateur de différentes souches de *S. aureus* issues de bactériémies sur les plaquettes. Ces résultats sont en accord avec la littérature démontrant l'ensemble des récepteurs (GPIIb/IIIa, GPIb $\alpha$ , Fc $\gamma$ RIIa...) impliqués dans la reconnaissance des bactéries par les plaquettes <sup>199,204,260,261</sup>. *S. aureus* est capable de synthétiser deux molécules, la protéine de liaison au facteur de von Willebrand et la staphylocoagulase, qui en liant à la prothrombine forment le complexe staphylothrombine <sup>183</sup>. Ce complexe protéique permet la fibrinoformation à l'origine de l'agrégation et activation plaquettaire. Par opposition, il s'avère que l'acide lipotéichoïque, constituant important de la paroi de *S. aureus*, altère la fonction plaquettaire, en inhibant l'activité de la phospholipase C, la libération de thromboxane A<sub>2</sub>, ou encore la mobilisation intracellulaire de Ca<sup>2+</sup> <sup>262</sup>. Cela montre qu'au sein d'une même bactérie, de nombreux éléments peuvent avoir des résultats antagonistes.

Sans évoquer la mécanistique mise en place avec d'autres espèces bactériennes, la capacité de *S. aureus* à induire une agrégation plaquettaire est reconnue depuis longtemps. Les équipes de Cox et Kerrigan ont beaucoup décrit les mécanismes d'interactions entre *S. aureus* et les bactéries <sup>204,260,261</sup>. Dans leurs études, Arman *et al.* et Miajlovic *et al.* indiquent que *S. aureus* induit l'agrégation des plaquettes. Or dans notre cas, nous n'avons observé aucune agrégation suite à l'exposition par les souches cliniques staphylococciques. Cette différence peut s'expliquer par des composants de *S. aureus*, inhibant les fonctions hémostatiques des plaquettes. En effet, l'acide lipoteichoïque de *S. aureus* inhibe l'agrégation des plaquettes ainsi que la formation de thrombus <sup>263</sup>. *S. aureus* sécrète la protéine d'adhésion au fibrinogène extracellulaire qui, en se fixant au fibrinogène plaquettaire, limite l'agrégation <sup>264</sup>. La staphylokinase, enzyme de virulence de *S. aureus* possède une activité fibrinolytique liée à une diminution de l'agrégation plaquettaire <sup>265</sup>. Selon le récepteur de l'immunité innée engagé par

les plaquettes lors de l'interaction avec les bactéries (TLR2/TLR4), il peut y avoir une agrégation plaquettaire ou pas <sup>261,263,266,267</sup>. Il en ressort que les bactéries, telles que *S. aureus*, possèdent des caractéristiques antagonistes permettant d'expliquer les différences de résultats quant à l'agrégation des plaquettes. Par ailleurs, il faut également considérer les paramètres propres à l'hôte qui de fait ne sont pas équivalents. En effet, nous avons observé l'agrégation plaquettaire de 5 donneurs sur les 35 utilisés, en réponse aux mêmes souches de *S. aureus*, qui dans des conditions expérimentales comparables, n'ont pas induit d'agrégation plaquettaire des 30 autres donneurs. Ainsi, cela complexifie l'analyse des interactions. Cela complexifie d'autant plus l'analyse des interactions entre les plaquettes et les bactéries.

En accord avec l'activation plaquettaire induite par les souches staphylococciques, une libération de différents immuno-modulateurs est observée. Nous avons montré l'augmentation du relargage des chimiokines RANTES et GRO $\alpha$  par les plaquettes alors que seulement une autre étude indiquait quant à elle, la sécrétion de PF4 <sup>261</sup>. En effet, beaucoup d'études se sont intéressées à la sécrétion des granules denses via la libération d'ADP ou de sérotonine, mettant ainsi en avant la boucle d'activation plaquettaire, mais peu ont investigué la part inflammatoire de la réponse plaquettaire en évaluant la sécrétion des granules  $\alpha$  <sup>261</sup>. Au regard du premier article, il serait également à suggérer que les plaquettes ont la capacité de distinguer des signaux de signaux (dans ce cas, des souches de *S. aureus* issues de bactériémies différentes) et n'ont pas le même comportement en dépit du pouvoir de virulence des souches de *S. aureus* vis-à-vis de la libération de molécules inflammatoires.

Nous avons également montré que *S. aureus* présente une action apoptotique, ou du moins induit l'expression de marqueurs de mort cellulaire, en diminuant le potentiel de membrane mitochondrial, en augmentant légèrement l'activité de la caspase 3, en exposant des PS à la surface plaquettaire et en libérant des MP. Leytin *et al.* ont décrit les mécanismes de signalisation mis en place lors de l'apoptose plaquettaire, malgré que ces cellules soient anucléés <sup>104</sup>. Ainsi nos observation convergent avec les quelques études qui se sont intéressées aux stimuli d'origine bactérienne dans l'induction de l'apoptose plaquettaire. Yeh *et al.* ont montré qu'*Helicobacter pylori* est responsable de l'apoptose des plaquettes et de leur agrégation, favorisant ainsi la chute du compte plaquettaire <sup>268</sup>. Kraemer *et al.* indiquent que les bactéries, comme *E. coli* ou *S. aureus*, agissent en dégradant la protéine de survie Bcl-XL, afin d'induire une apoptose cellulaire <sup>103</sup>. En délétant *E. coli* du gène de l'hémolysine  $\alpha$ , cet effet est

annihilé, démontrant l'implication directe de cette toxine dans l'effet cytotoxique observé. Cependant, l'utilisation d'agonistes comme le l'analogue de la thrombine TRAP, le Platelet Activating Factor, ou le LPS, n'induit pas la dégradation de la protéine de survie Bcl-x<sub>L</sub> ce qui met en avant que les voies de signalisation engagées menant à l'activation, les processus inflammatoires ou encore la mort plaquettaire sont complexes. En effet, ces voies sont indépendantes malgré qu'un stimulus puisse engager différentes voies d'activation simultanément <sup>168</sup>. Towhid *et al.* ont montré un phénomène apoptotique généré par du peptidoglycane de *S. aureus* simultanément avec l'activation plaquettaire, ce qui est en parfait accord avec nos résultats <sup>213</sup>. Cet auteur indique, dans une autre étude, que l'utilisation de la balhimycine, un antibiotique fréquemment utilisé lors des infections à *S. aureus*, stimule l'apoptose des plaquettes et participe à la thrombopénie <sup>269</sup>. Il faut donc considérer l'effet cytotoxique des thérapies utilisées afin de ne pas promouvoir la physiopathologie du sepsis. Nos résultats s'accordent parfaitement avec la corrélation clinique observée entre la diminution du potentiel de membrane des plaquettes et la sévérité des patients atteints de sepsis <sup>270</sup>. Concernant le mécanisme de mort cellulaire mis en jeu dans cette étude, il nous a été impossible de mettre en évidence une différence entre l'apoptose et la nécrose des plaquettes. En effet, il n'y a pas de différence quant à l'expression de la caspase 3 permettant de certifier du processus apoptotique induit par les souches de *S. aureus*. Cette absence d'augmentation peut s'expliquer par la durée limitée d'exposition (30 minutes), temps auquel Kraemer *et al.* n'ont pas observé de formation de la protéine Bax, autre protéine impliquée dans la signalisation de l'apoptose plaquettaire <sup>103</sup>. Il en ressort que la mort plaquettaire induit par les souches de *S. aureus* s'effectue suite à un changement morphologique, une perte de l'intégrité de la membrane cellulaire (dépoliarisation de la membrane mitochondriale et exposition de PS) leur conférant ainsi un pouvoir pro-coagulant et pro-inflammatoire <sup>104,271,272</sup>.

Puis, nous avons évalué si l'ASA et la fluvastatine pouvaient moduler l'expression des marqueurs de mort cellulaire des plaquettes exposées aux souches de *S. aureus*, ces deux molécules s'étant montrées efficaces dans des études chez l'homme, pour la première, et chez l'animal pour la seconde. Ainsi, alors qu'aucun effet n'est observable pour la fluvastatine sur les plaquettes, l'ASA permet de limiter l'activation, la diminution du potentiel de membrane mitochondriale, l'exposition de PS et la chute du compte plaquettaire. Ces résultats peuvent s'expliquer par l'implication des récepteurs P<sub>2</sub>Y<sub>12</sub> dans l'apoptose plaquette, qui par une stratégie ciblant ces récepteurs, permettrait de compléter les effets de l'ASA <sup>273</sup>. A contrario,



Zhao et ses collaborateurs révèlent que cette molécule entraîne l'apoptose des plaquettes <sup>274</sup>. Lors de cette étude, l'ASA n'induit l'exposition des PS qu'à partir d'une concentration dix fois plus concentrée que celle que nous avons utilisée suggérant peut-être un effet délétère de l'ASA à trop forte dose. Nayak *et al.* indiquent que les plaquettes traitées à l'ASA (à des concentrations 4 à 10 fois plus concentrées que celle que nous avons utilisées) sont sujettes à un phénomène d'apoptose facilitant leur phagocytose par les macrophages <sup>275</sup>. Les études de Zhao *et al.* et Nayak *et al.* utilisent des plaquettes sans aucun facteurs plasmatiques ce qui permet de penser de leurs implications dans la protection contre le phénomène de mort plaquettaire. Il en ressort également que selon la concentration d'ASA utilisée, on n'observe pas le même effet sur les plaquettes.

Outre les effets de l'ASA sur les plaquettes, Kupferwasser *et al.* ont mis en évidence que cette molécule permet de réduire la virulence de *S. aureus* en modérant la sécrétion de l'hémolysine  $\alpha$  et l'attachement de la bactérie à la fibronectine et au fibrinogène <sup>276</sup>. Ces résultats se corrélaient parfaitement à ceux de Kraemer indiquant le rôle de l'hémolysine  $\alpha$  dans l'induction de l'apoptose plaquettaire <sup>103</sup> ou encore le fait que l'interaction entre les plaquettes et *S. aureus* se fait en partie par l'intermédiaire de la fibronectine <sup>199</sup>. Nous n'avons pas vérifié la pathogénicité de nos souches de *S. aureus* préalablement traitées à l'ASA sur les plaquettes mais c'est une perspective de travail intéressante. Cet effet antiplaquettaire et antimicrobien pourrait contribuer à la protection contre l'endocardite infectieuse à *S. aureus* voire le sepsis <sup>277</sup>. L'inhibition de l'enzyme cyclo-oxygénase 1 par l'ASA ne semble pas avoir d'effet inhibiteur du relargage de protéines microbicides et kinocidines, soulignant encore davantage l'intérêt d'utiliser cette molécule dans le sepsis <sup>278</sup>.

Les interactions mises en jeu entre les plaquettes et les bactéries sont très complexes et dépendent de nombreux facteurs comme l'espèce bactérienne et ses caractéristiques pathogéniques (facteurs d'adhésions, de virulence...). En effet, divers pathogènes n'utilisent pas les mêmes mécanismes d'action pour cibler l'action pro-coagulante, pro-inflammatoire ou cytotoxique des plaquettes <sup>279,280</sup>. Il y a donc une multitude de phénomènes se chevauchant et complexifiant la compréhension du rôle des plaquettes durant le sepsis. Afin d'évaluer la part inflammatoire apportée par les plaquettes lors du sepsis, nous avons mis en place un modèle expérimental murin de septicémie polymicrobienne.

Dans le premier temps de cette 3<sup>ème</sup> étude, nous avons voulu connaître la contribution apportée par les plaquettes sanguines dans le processus inflammatoire constaté durant le sepsis, et comme dans l'étude précédente évaluer l'effet bénéfique de l'ASA sur l'état général des souris et des marqueurs inflammatoires. Le modèle de ligature et ponction du ceacum que nous avons utilisé pour induire un sepsis permet l'apparition d'un sepsis rapide et sévère. Ce modèle est assez variable, selon la souche de souris utilisée ou encore la taille ou le nombre de ponction effectuée <sup>281,282</sup>. Dans les 72h après l'opération, seulement 26.7% de survie est constaté chez les souris ayant subi une chirurgie CLP alors que d'autres études utilisant le même modèle observent une mortalité moins importante sur la même durée <sup>283</sup>. Nous observons que les souris préalablement traitées à l'acide acétylsalicylique ont un taux de survie de 68.7% indiquant un véritable effet protecteur de cette molécule lors du sepsis. Nous avons d'ailleurs vérifié au préalable que la concentration d'ASA que nous avons choisi, en accord avec la bibliographie, n'induit pas de problèmes hémorragiques <sup>243</sup>.

En corrélation avec la mortalité, les souris septiques libèrent plus de lactate déshydrogénase (LDH), d'alanine aminotransférase (ALT) et d'aspartate aminotransférase (AST). Ces enzymes sont le reflet de dysfonctions d'organes (foie, cœur, poumons, reins) et sont également retrouvées augmentées dans les modèles d'endotoxémie ou de sepsis polymicrobien par injection intrapéritonéale de fèces humaines <sup>145,243</sup>. Malgré une réduction de la mortalité par l'ASA, nous n'avons pas observés de diminution des taux plasmatique de ces trois enzymes reflétant la dysfonction d'organe. Il est possible que malgré une diminution des processus physiopathologiques par l'ASA, certains organes soient tout de même atteints et sécrètent ces enzymes. Dans un modèle de péritonite chez la souris (injection de fèces), le clopidogrel permet une réduction de la sécrétion de LDH <sup>145</sup>.

Notre modèle indique une inflammation systémique avec l'augmentation des taux de RANTES ou de TNF- $\alpha$  principalement 24h après avoir effectuée l'induction du sepsis. Cuccurullo *et al.* utilisant un modèle de sepsis murin par CLP, indique que 18h post-chirurgie, les taux de TNF- $\alpha$  et d'IL-6 sont fortement augmentés par rapport au groupe témoin <sup>239</sup>. Ces cytokines inflammatoires sont moins présentes à 48h, suggérant peut-être l'intervention d'autres molécules immuno-modulatrices comme le sCD40L ou un syndrome de réponse anti-inflammatoire compensatoire comme cela est observée dans l'étude de Osuchowski *et al.* <sup>36</sup>. L'augmentation des taux plasmatique de TNF- $\alpha$  est, en partie, due aux plaquettes grâce aux

récepteurs TLRs présents à leurs surfaces pour la reconnaissance des éléments bactériens <sup>284</sup>. Un modèle murin de détresse respiratoire par inhalation de LPS a révélé l'importance de RANTES dans l'orchestration de la coopération plaquettes/neutrophiles à l'origine de la dysfonction pulmonaire <sup>138</sup>.

Les animaux septiques présentent une augmentation des concentrations de sCD40L et sCD62P plasmatique à 24h et 48h. Il semblerait donc que les plaquettes soient activées et participent à l'inflammation systémique via le relargage de leurs granules  $\alpha$ . En effet, le sCD40L, principalement d'origine plaquettaire, est clivé par la métalloprotéinase matricielle 9, sous le contrôle de Rac-1, permettant ainsi l'activation des neutrophiles et leur migration au niveau pulmonaire <sup>140,285</sup>. Dans notre modèle d'étude, il semblerait qu'il y ait une diminution de la libération de facteur plaquettaire 4 (PF4) chez les souris septiques alors que Hwaiz *et al.* ont montré, sur le même modèle de sepsis expérimental murin que nous avons utilisé, une augmentation des taux de PF4 6h après l'induction du sepsis <sup>234</sup>. Il semblerait que la sécrétion plaquettaire de PF4 permettait de stimuler la sécrétion de GRO $\alpha$  et MIP-2 $\alpha$  par les macrophages alvéolaires, ce qui participait au recrutement des neutrophiles dans les poumons <sup>234</sup>. La dynamique d'activation plaquettaire est très variable durant le sepsis et dépendante du modèle utilisé. Après injection de *Streptococcus pyogenes* chez la souris, Hurley *et al.* observent une augmentation des taux de sCD62P corrélés à l'activité d'AST, synonyme d'une association entre l'activation plaquettaire et la dysfonction d'organes <sup>244</sup>. Nous n'observons, en revanche, aucun effet notable de l'ASA quant au relargage des cytokines inflammatoires. A l'inverse, l'ASA utilisant le récepteur plaquettaire COX-1, amplifierait l'inflammation causée par les macrophages en inhibant l'action modulatrice des plaquettes vis-à-vis de la production macrophagique de TNF $\alpha$  et IL-6 et favoriserait donc un état inflammatoire aigu <sup>243</sup>. De même, des souris rendues thrombopéniques et sujettes à une bactériémie montrent une augmentation de certains marqueurs inflammatoires solubles <sup>246</sup>. Cependant, notre modèle n'est pas comparable à ces deux dernières études qui utilisent une injection de bactéries (*E. coli* ou *S. aureus*), leurs modèles s'apparentant plus à un choc septique qu'un sepsis.

Le modèle utilisé dans ce troisième manuscrit montre une neutropénie induite par le sepsis à 48h qui peut s'expliquer par la migration de ces cellules dans les organes tels que les poumons ou le foie <sup>228,234,286</sup>. Cependant, notre modèle n'induit pas de thrombopénie chez les souris déclarant un sepsis comme cela a pu être observé dans l'étude de Rahman *et al.* <sup>240</sup>. Cette

différence peut, hypothétiquement, s'expliquer par la différence de prélèvement sanguin puisque Rahman *et al.* ont prélevé le sang à partir de la veine cave alors que nous prélevé par voie intracardiaque. De plus, les différences dans la procédure de numération cellulaire peuvent induire des variabilités de résultats. Par la suite, nous avons cherché à déterminer l'éventuelle contribution des plaquettes dans l'induction des défaillances pulmonaires durant le sepsis. Nous observons que dans ce contexte inflammatoire, plaquettes et les neutrophiles s'associent dans la circulation sous forme de complexes et migrent dans le parenchyme pulmonaire. Ces résultats sont en accord avec ceux de l'équipe de Thorlacius, qui a beaucoup travaillé sur l'interaction des plaquettes et des neutrophiles dans l'induction de défaillances pulmonaires associées au sepsis<sup>140,228,234,240,285</sup>. En effet, ils ont mis en évidence que le relargage plaquettaire de différents immuno-modulateurs (PF4, sCD40L, RANTES) permettait la communication avec les neutrophiles et intervenait dans l'induction des mécanismes de défaillances pulmonaires.

Dans notre étude, nous avons constaté que l'ASA semblait limiter la migration des plaquettes et neutrophiles au niveau pulmonaire. Shoemaker *et al.* ont montré que l'ASA réduisait l'activation plaquettaire provoquée par les bactéries et suivie d'une libération de thromboxane associée à une hypertension artérielle pulmonaire, en partie responsable des défaillances des poumons<sup>287</sup>. De plus, dans un modèle d'endotoxémie provoquant une insuffisance pulmonaire, Tilgner *et al.* indiquent que l'ASA limite la migration des neutrophiles<sup>247</sup>. Dans un modèle de détresse respiratoire induite chez l'homme par inhalation de LPS, une récente étude montre l'aspect bénéfique de l'ASA sur l'inflammation apportée par les neutrophiles au niveau pulmonaire<sup>288</sup>. Enfin, Ortiz-Munoz et ses collaborateurs indiquent, sur un modèle de détresse respiratoire par inhalation de LPS, un effet protecteur de l'ASA sur la formation de complexes neutro-plaquettaires<sup>289</sup>. Ainsi, nos données sont bien en accord avec la littérature. Depuis 2007, Clark *et al.* ont montré que les plaquettes participent à la formation de NETs durant la pathogénèse du sepsis<sup>99</sup>. Une autre étude a révélé l'implication de la glycoprotéine GPIIb/IIIa plaquettaire et du CD18 des neutrophiles ainsi que des facteurs vWF et PF4 dans la régulation des NETs par les plaquettes<sup>100</sup>. Récemment, Mc Donald *et al.* confirment, grâce à de images intravitales, l'induction de NETs par les neutrophiles via l'action des plaquettes démontrant une fois de plus la coopération entre ces deux types cellulaires dans un contexte inflammatoire<sup>237</sup>. Cependant, ce processus à visée antibactérienne favorise aussi une action pro-coagulante<sup>237</sup>. Dans notre étude, nous avons observé une augmentation de la formation de NETs au niveau circulant. Les souris septiques préalablement traitées à l'ASA retrouvent des niveaux de base

de NETs. Ceci est en accord avec les résultats de Caudrillier et al. qui chez des souris déclarant une détresse respiratoire transfusionnelle expérimentale, révèlent des effets protecteurs de l'ASA <sup>241</sup>.

Outre leurs rôles délétères dans l'inflammation et la physiopathologie de dysfonctions d'organes, l'action bénéfique des plaquettes ne devrait pas être négligée. Une thrombopénie induite lors d'un modèle de pneumosepsis à *Klebsiella pneumoniae* ou de bactériémie à *S. aureus* rehausse, de manière corrélée au taux plaquettaire, la présence bactérienne dans différents organes, en particulier dans le rein <sup>246</sup> et le poumon <sup>242</sup>. En effet, les plaquettes contiennent des protéines microbicides, des enzymes et des défensines capables d'agir directement pour les pathogènes, cependant le sang étant riche en nutriments, cette activité microbicide jouée par les plaquettes reste limitée. En vue du peu de données concernant cet effet antimicrobien joué par les plaquettes, il serait intéressant, dans la suite de notre étude, d'évaluer cette activité dans notre modèle de sepsis murin polymicrobien.

Une activation de l'endothélium et du système de coagulation, coïncidant avec une augmentation de cytokines pro-inflammatoires, est constatée durant la thrombopénie associée au sepsis. Il est donc important de considérer les différents rôles antagonistes que peuvent jouer les plaquettes durant cette pathologie. Ces cellules sont ambivalentes, d'un côté actrices de nombreux mécanismes inflammatoires et thrombotiques associés au sepsis et à ses complications et d'un autre côté, modulatrices de l'inflammation et de l'hémostase.

L'originalité de ce travail de thèse est l'analyse à différentes échelles de la réponse inflammatoire plaquettaire à un signal de danger bactérien, qu'il soit une toxine, une bactérie entière ou un sepsis polymicrobien. En comprenant les interactions possibles entre *S. aureus* ou ses exotoxines, il est plus facile, pour la communauté scientifique, d'appréhender les processus inflammatoires mis en place par les plaquettes lors du sepsis. L'utilisation d'un modèle murin de sepsis expérimental nous a permis de confirmer l'intérêt de poursuivre les investigations sur l'utilisation de l'ASA afin d'améliorer la prise en charge de cette pathologie et réduire les complications qui lui sont associées. La complémentarité expérimentale des trois études effectuées lors de ce travail doctoral a permis de nouvelles avancées quant à la place de la plaquette en tant que cellule de l'inflammation.

D'un point de vue général, les modèles utilisés dans ce travail de thèse sont pertinents par rapport à ceux retrouvés dans la littérature. L'évaluation de l'effet inflammatoire d'exotoxines de *S. aureus* sur les plaquettes nous a permis d'appréhender l'effet inflammatoire d'un facteur de virulence bactérien. Ce travail a permis de comparer différentes exotoxines, dont les conséquences au niveau plaquettaire avaient été peu, voire jamais, étudiées pour certaines toxines. Par ailleurs, le phénomène apoptotique induit lors de la reconnaissance d'une bactérie par les plaquettes est un sujet récent. L'utilisation de souches de *S. aureus* issues de bactériémies permet de rendre compte de la variabilité des caractéristiques phénotypiques bactériennes qui peut ainsi générer des réponses différentes chez les plaquettes. Par ce modèle d'étude, nous avons constaté un effet bénéfique de l'ASA sur les différents paramètres plaquettaires étudiés. Pour confirmer cet effet bénéfique de l'acide acétylsalicylique observé précédemment, nous avons utilisé le modèle le plus physiologique pour développer un sepsis chez la souris. Effectivement, la CLP est connue pour être le modèle de référence recréant la progression de l'orage cytokinique induit dans le contexte inflammatoire du sepsis <sup>232</sup>. Ce modèle s'avère être le plus cliniquement pertinent malgré qu'il soit complexe et entraîne de nombreuses variabilités selon les études. Quoiqu'il en soit, il demeure impossible de refléter parfaitement la réalité clinique du sepsis du fait de la variabilité des facteurs de l'hôte et de ceux du ou des pathogènes impliqués dans la pathologie.

Plusieurs perspectives se profilent quant à ce travail doctoral avec en particulier la troisième étude de ce manuscrit qui relève quelques interrogations méritant d'être éclaircies. L'implication des plaquettes dans la pathogénèse de la détresse respiratoire induite lors du sepsis ne sera mise en évidence qu'avec l'utilisation de souris rendues thrombopéniques. D'autre part, il serait intéressant d'étudier l'apoptose plaquettaire dans ce modèle de sepsis expérimental afin de confirmer que ce phénomène de mort cellulaire est, en partie, responsable de la thrombopénie associée au sepsis. Nous avons vu qu'une chute du compte plaquettaire était en général assimilée à une hausse de l'inflammation et de la mortalité. La stratégie d'utiliser des molécules antiplaquettaires, telles que l'ASA, afin de diminuer l'inflammation mais également l'apoptose, semble donc judicieuse pour limiter les conséquences du sepsis. De plus, de nombreux types cellulaires expriment les cyclooxygénases ce qui fait de l'ASA une molécule à forte capacité pléiotrope. Enfin, dans la continuité de ce projet, une étude clinique chez les patients en état septique grave est actuellement en cours au sein de notre laboratoire, en collaboration étroite avec le Centre d'Investigation Clinique - Épidémiologie Clinique CIC-

EC1408 et le CHU de Saint-Etienne. Cet essai a pour but d'évaluer si le niveau d'activation plaquettaire, l'association des plaquettes aux monocytes et aux neutrophiles dans les complexes circulants et la capacité des plaquettes à libérer du sCD40L sous l'action du TRAP. Ces données seront comparées à celles d'un groupe contrôle de patients présentant un syndrome inflammatoire sans infection associée et d'un groupe contrôle constitué par des donneurs de sang bénévoles de l'EFS Auvergne-Rhône-Alpes. Dans un deuxième temps, il sera investigué si le type de germe responsable du sepsis, influe sur la libération de sCD40L plaquettaire ou l'interaction des plaquettes avec les monocytes et les neutrophiles.

En conclusion, ce projet doctoral a permis de mettre en évidence l'incidence de différentes exotoxines staphylococciques, de souches de *S. aureus* sur la réponse inflammatoire plaquettaire, ainsi que l'effet de l'utilisation de molécules antiplaquettaires sur ces effets. Cela apporte d'autant plus d'éléments mettant en avant la plaquette au cœur de l'inflammation et de son rôle central dans de la pathogénèse du sepsis. L'ensemble de ces résultats pourrait, à terme, ouvrir de nouvelles perspectives dans la prise en charge des patients en état septique grave.

# Annexes



**Revue 1 : Human platelets and their capacity of  
binding viruses : meaning and challenges ?**

**Revue publiée dans  
BMC immunology en Avril 2015**

REVIEW

Open Access

# Human platelets and their capacity of binding viruses: meaning and challenges?

Adrien Chabert<sup>1</sup>, Hind Hamzeh-Cognasse<sup>1</sup>, Bruno Pozzetto<sup>1,2</sup>, Fabrice Cognasse<sup>1,3</sup>, Mirta Schattner<sup>4</sup>, Ricardo M Gomez<sup>5</sup> and Olivier Garraud<sup>1,6,7\*</sup>

## Abstract

Blood platelets are first aimed at ensuring primary hemostasis. Beyond this role, they have been acknowledged as having functions in the maintenance of the vascular arborescence and, more recently, as being also innate immune cells, devoted notably to the detection of danger signals, of which infectious ones. Platelets express pathogen recognition receptors that can sense bacterial and viral moieties. Besides, several molecules that bind epithelial or sub-endothelial molecules and, so forth, are involved in hemostasis, happen to be able to ligate viral determinants, making platelets capable of either binding viruses or even to be infected by some of them. Further, as platelets express both Fc-receptors for Ig and complement receptors, they also bind occasionally virus-Ig or virus-Ig-complement immune complexes. Interplays of viruses with platelets are very complex and viral infections often interfere with platelet number and functions. Through a few instances of viral infections, the present review aims at presenting some of the most important interactions from pathophysiological and clinical points of view, which are observed between human viruses and platelets.

**Keywords:** Platelets, Receptors, Viruses, Infection, Hemostasis

## Introduction

There is a large body of evidence that several types of viral infections led to sometimes severe and even life-threatening bleeding. In some of those infections, the platelet count drops dramatically; however, the precise mechanisms involved either in platelet destruction or impairment are still largely unclear, as well as the relative pathology counterpart of platelets and vascular endothelial cells. More largely, thrombocytopenia is common during or after viral infections and several mechanisms have been proposed to contribute to the drop of platelet count, including platelet destruction mediated by platelet-associated immunoglobulin G (IgG) or platelet-leukocyte aggregation, possibly leading to sequestration by macrophages, sequestration of platelets in the enlarged spleen, impaired production of thrombopoiesis, and direct effect of viruses on platelets.

Besides, albeit apparently infrequent though poorly examined in depth, platelets may bind and internalize

infectious agents, with little clue on the outcome of the agent and of the “infected” platelet. The presence of viruses in platelets and their capacity of entry within those cells has been acknowledged for more than half a century: as early as of 1959, influenza viruses were forced to enter platelets experimentally, offering beautiful electronic microscopic images [1]. Questions relative to this situation have not been addressed then, perhaps because platelets were not yet acknowledged as “cells” but as debris. Viruses indeed find panoply of receptors that are not specific but rather permissive (as cartooned in Figure 1). FcγR bound viruses can also enter platelets as those cells can ingest immune complexes [2]. However, it is not yet clear whether platelets, by taking up viruses, contribute to the transport and the dissemination of infection *in vivo* or if, on the contrary, they help the host organism to defend against infection. Platelet-virus interactions may have an ambivalent role, depending on the virus species and on the platelet and megakaryocyte (MK) environment [3].

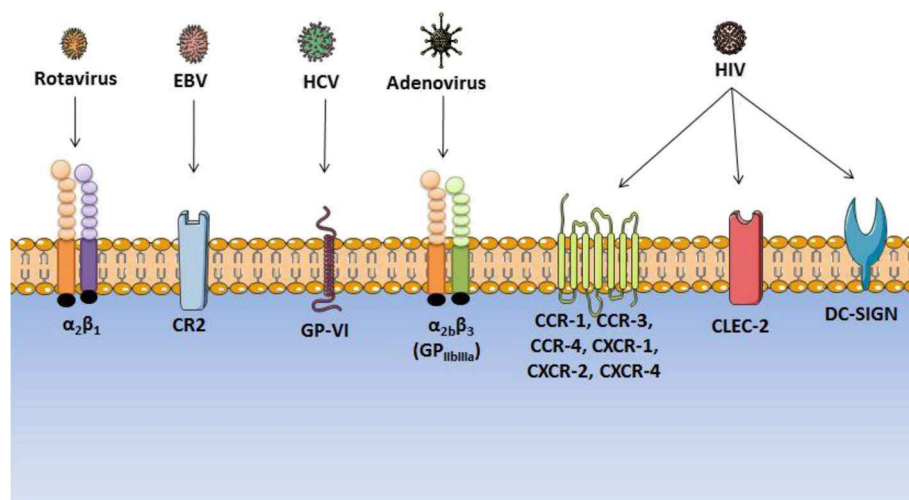
This mini-review aims at addressing the apparent complex issue of mutual relationships between certain viruses and platelets.

\* Correspondence: ogarraud@ints.fr

<sup>1</sup>EA3064—GIMAP, Université de Lyon, 42023 Saint-Etienne, France

<sup>6</sup>Institut National de la Transfusion Sanguine, 75015 Paris, France

Full list of author information is available at the end of the article



**Figure 1** Platelet receptors acknowledged to bind viruses, along with their primary molecular targets. CLEC-2: C-type lectin-like type II transmembrane receptor. CR-2: Complement Receptor type 2. CCR-1, CCR-3, CCR-4: C-C chemokine Receptor type 1, 3 and 4. CXCR-1, CXCR-2, CXCR-4: C-X-C chemokine receptor type 1, 2 and 4. DC-SIGN: Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin. GP-VI: Glycoprotein VI. PAR-1/PAR-4: Platelet Activating Receptor 1/4.

### Platelets and human immunodeficiency virus type 1 (HIV-1)

HIV-1 is a single positive-stranded RNA virus belonging to the *Retroviridae* family, genus *Lentivirus*. It was first visualized in 1990 in platelets and MKs of experimentally-infected mice [4]. In 2002, Youssefian et al. consistently identified HIV-1 in human MKs and platelets [5]. Several studies have thus clearly indicated that, during infection with HIV-1, there is a direct interaction between the virus, MKs and platelets; indeed, intact HIV-1 particles enclosed in endocytic vesicles were found to be sheltered from platelet secretory products while, when located in the surface connected canalicular system (SCCS), they were in contact with platelet secreted proteins [6]. These observations indicate that (1) platelets are able to endocytose HIV-1 virions; (2) HIV-1 can be enclosed within platelets, sheltered from host immune system aggression and transported by circulating platelets within the entire human body; and (3) viruses can come into contact with platelet-secreted alpha-granule contents leading to their destruction. In platelets incubated with HIV-1, characteristic endocytic vacuoles were identified close to the plasma membrane, tightly surrounding 1 or 2 viral particles. Examination of platelets from a patient with acquired immunodeficiency syndrome and high viremia suggested that HIV-1 endocytosis may also occur *in vivo* [5].

Regarding the receptors that favor HIV-1 entry in MKs and platelets, it has been found that MKs express the CD4 receptor for HIV-1 [7] as well as the co-receptors CXCR1, 2, 4, and CCR3 [8]. CXCR4+ MKs

have been shown to be able to transfer CXCR4 to CXCR4 negative cells, rendering them capable of being infected by HIV-1 [9]. Platelets do not, however, express CD4 but only bear the HIV-1 co-receptors CXCR1, 2, 4 and CCR 1,3 and 4 [10-13]. Dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN), a C-type lectin receptor present on both macrophages and dendritic cells, has also been identified on platelets [6], with the capacity of binding HIV-1 on platelets [6,14]. Platelets also express the C-type lectin-like receptor 2 (CLEC-2). Further, a combination of DC-SIGN and CLEC-2 inhibitors strongly reduced the association of HIV-1 with platelets, indicating that these lectins are required for efficient HIV-1 binding to platelets [14].

Besides its entry in platelets, HIV-1 can activate infected as well as non-infected—neighboring—platelets. Indeed, HIV-1 trans-activator of transcription protein (Tat) directly interacts with platelets and induces platelet activation resulting in platelet micro-particle (PMP) release. This activation by Tat requires the chemokine receptor CCR3 and  $\beta_3$ -integrin expression on platelets, as well as calcium flux. Tat-activated platelets secrete CD154 (CD40L) [15,16]. In general, enhanced platelet activation in HIV-1-infected patients seems a hallmark of the infection progression, as it strongly correlates with plasma viral load. Indeed, HIV-1 and platelet interactions engage the natural platelet partner that is vascular endothelium: levels of soluble endothelial activation markers (sVCAM-1, sICAM-1, and von Willebrand factor) are elevated in HIV-1 infected patients, and this elevation may be associated with a thrombotic state [7]. As stated, HIV-1 infection potentiates the pro-inflammatory

potential of platelets: CD40L and HIV-1 Tat have been reported to synergize and contribute to inflammation [17]. Soluble CD40L also promotes the formation of complexes between inflammatory monocytes and activated platelets, which are detected by flow cytometry as monocytes::platelet doublets, which are positive for both CD14 and CD61 [18]. On the contrary, platelets can also secrete CXCL4/PF4 that is supposed to inhibit HIV-1 infection of T cells [19,20]; this exemplifies the complex relationship and interactions of platelets and viruses (for instance HIV1). This (PF4) platelet-associated chemokine indeed blocks HIV-1 entry and neutralizes HIV-1 activity, that suggests a role for platelets in the defense against HIV-1 infection. However, platelet-derived PF4 facilitates HIV-1 replication in human macrophage [21], and potentiates HIV activity [22]; this demonstrates that platelets products may exert dual actions regarding HIV infection, an example of definitely complex platelet::virus interaction.

Further, the clearance of senescent platelet by macrophages and neutrophils operates via P-Selectin/PSGL-1 [23]; platelets with bound-HIV seem to use the same pattern, which indicates that HIV uses platelets for protect itself from the immune system; however, since the first descriptions of HIV interactions with platelets, there has been no substantiated indication of productive replication of virus within platelets and by extend of a so-called platelet infection with HIV [5]; there is neither any evidence that HIV induces platelets to enhanced apoptosis.

A considerable reduction in the incidence of severe thrombocytopenia after the introduction of highly active antiretroviral therapy (HAART) was found, probably due to its ability to limit bone marrow damage induced by uncontrolled HIV-1 replication and opportunistic infections [24]. However, protease inhibitors or non-nucleoside reverse-transcriptase inhibitors do not reduce levels of the soluble platelet activation markers P-selectin and CD40L [25]. HAART alters inflammation linked to platelet cytokines in HIV-1-infected patients [26].

In all, HIV-1 is capable of entering platelets but outcome of this entry is still largely unclear, as platelets are not thought to consistently disseminate infection, nor are their considered to be reservoirs. HIV-1 also activates infected as well as non-infected platelets and contributes to local vascular inflammation. However, antiretroviral therapy—if it ameliorates the platelet decrease in numbers—accelerates the pro-inflammatory potential of platelets on vascular endothelium.

#### **Platelets and Hepatitis C virus (HCV)**

HCV is a positive-sense single-stranded RNA virus of the *Flaviviridae* family, genus Hepacivirus. Chronic HCV infection is associated with the development of several

extra-hepatic manifestations including thrombocytopenia [27]. When HCV treatment consists in associating pegylated interferon and ribavirin, thrombocytopenia is also among the numerous side effects of this bitherapy, and the condition can potentially be induced or exacerbated during treatment [28]. It renders chronically infected patients either ineligible for such a treatment or eligible for an adapted course (reduced or discontinued doses), which can potentially decrease the probability of successful HCV treatment [27]. Some studies have shown a decreasing trend of platelet count with increasing fibrosis and cirrhosis among patients with chronic liver disease. Among these patients, the reduction in platelets is mainly caused by platelet destruction from splenic pooling, because of portal hypertension. Indirect evidence supports also the hypothesis of bone marrow suppression by HCV, partly mediated by a reduction of thrombopoietin (TPO) production due to liver cirrhosis and/or fibrosis [29-31]. However, in HCV infection, the virus itself or other mechanisms can also lead to low platelet counts [32-34]. Thrombocytopenia in HCV infection is complex and multiple mechanisms are involved. Besides the central decrease of megakaryocytosis due to TPO defects and possible direct viral suppression [35,36], increased peripheral immune-mediated destruction of platelets and hypersplenism may lead also to increased splenic platelet sequestration [29,37].

To explain the immune destruction of platelets, several hypotheses have been put forward: the HCV binding to platelet membrane (with consequent binding of anti-HCV antibody and phagocytosis of platelets), and an impairment of host immune system triggering the production of autoantibodies against platelet glycoproteins are the two most frequently postulated immune mechanisms explaining increased peripheral platelet destruction in HCV-infected cases [35,38-40]. Thrombocytopenia is now considered as a marker of HCV infection, which involves that all patients with low platelet count must be screened for the virus, especially in regions with high rates of infection [41,42].

In case of thrombocytopenia, the ultimate aim in HVC-positive patients is not to normalize the platelet counts [43], but to attain and maintain a safe hemostatic level that avoids hemorrhage on one hand and thrombosis on the other. Administration of Eltrombopag, a synthetic agonist of the TPO receptor that activates JAK2/STAT signaling pathways and induces increased proliferation and differentiation of human bone marrow progenitor cells into MKs and thus increased platelet production [44,45], has been proposed to meet this purpose [46,47].

#### **Platelets and dengue virus**

Dengue is a tropical infectious disease caused by the single positive-stranded RNA virus of the *Flaviviridae*

family, genus *Flavivirus*. It is now regarded as a possible threat in temperate climates where permissive mosquitoes are present [48,49]. It is always feared because of the possible severity of clinical symptoms (1% of cases) and because of the possibility that the disease evolves to a hemorrhagic form, with considerable drop in platelet count [50]. Even non-hemorrhagic dengue infections present with decreased numbers of circulating platelets [51]. In the recent past years, the role of platelets in regulating endothelial integrity and dengue-associated plasma leakage has gained increased interest [52-57]. Studies using platelet aggregometry showed reduced aggregation in dengue patients [58-60], but because the reliability of aggregometry is compromised in conditions with thrombocytopenia, these findings need to be interpreted with caution. There are, meanwhile, other platelet function alterations that are associated with plasma leakage [61]: dengue infection is generally associated with platelet activation with an increased expression of the activated fibrinogen receptor ( $\alpha$ IIb $\beta$ 3), the lysosomal marker CD63 and the alpha-granule marker CD62P (P-selectin). Indeed, Hottz et al. have reported a consistent increase of CD62P expression in isolated platelets from dengue patients [57]. The concentration of serotonin is increased in platelets of patients, perhaps as a consequence of platelet destruction or due to some upregulation of serotonin transporter (SERT), as it has been described to occur in association with activation of the  $\alpha$ IIb $\beta$ 3 integrin [62]. Upon maximal platelet activation by TRAP (*Thrombin* Receptor Activating Peptide), platelet function defects were observed with a significantly reduced maximal activated  $\alpha$ IIb $\beta$ 3 and CD63 expression and reduced platelet-monocyte and platelet-neutrophil complexes. Dengue infection stimulates platelets to produce pro-IL-1 $\beta$ , possibly *de novo*; mature IL-1 $\beta$  increases the production of PMPs, which themselves augment vascular permeability [56]. A molecular pattern for the binding of Dengue Virus on Platelets is DC-SIGN [63], the very same receptor that characterizes DV binding on the primary target i.e. dendritic cells [64]. This DV::platelet ligation enhances Phosphatidylserine expression by platelets—a signature of apoptosis—and a call for phagocytosis by macrophages [65].

Regarding platelets, dengue infection is not exempt of unexpected and somewhat paradoxical data: whereas Tsai et al. [66] and Onlamoon et al. [67] found an increase in platelet-leukocyte aggregates during dengue infection in humans and macaques, respectively, the circulating level of monocytes and polymorphonuclear cells was decreased. Further, at the febrile phase, interferon gamma-induced protein 10 (IP-10) is fairly detected in dengue patients with and without warning signs, and during defervescence as well; however, MIP-1 $\beta$  (Macrophage Inflammatory Protein-1  $\beta$ ) and MCP-1

(Monocyte chemotactic protein-1) are elevated only in patients with warning signs at this phase and G-CSF (Granulocyte colony-stimulating factor) is significant in patients without warning signs. There are further significant correlations between the levels of VEGF (vascular endothelial growth factor), RANTES (regulated on activation, normal T cell expressed and secreted), IL-7, IL-12, PDGF (Platelet-derived growth factor) and IL-5 with platelets; VEGF with lymphocytes and neutrophils; G-CSF and IP-10 with atypical lymphocytes and various other cytokines with the liver enzymes are reported [68]. In all, these data suggest complex interactions between virus, platelets and leukocytes. Because platelets and leukocytes are seminal to primary hemostasis and further to clotting, it may be envisioned, although experimental data is lacking, that those intertwined relationships are defective at a stage, allowing vessel leakage and hemorrhage.

The situation seems worsened because dengue virus infection is accompanied by a dampening of central megakaryogenesis. The down-regulation of haematopoiesis is probably a protective mechanism of the micro-environment that limits injury to the marrow stem/progenitor cell compartment during the subsequent process of elimination of infected cells [3].

Dengue fever is one of the rare infectious diseases that necessitates platelet component (PC) transfusion; PC transfusion is not systematic as infected individuals who do not bleed or present early signs of bleeding must not be transfused, even with a prophylactic perspective. PC transfusion is however indicated and must not be delayed in bleeding patients; this therapy has become very safe regarding the risk of transmission of other infectious agents at least in economically wealthy countries, and the immunological risk is kept minimal in occasional transfusion [69]. One must however be aware that dengue outbreaks pose threats to the PC inventory because the needs increase considerably while the number of healthy, non infected blood donor candidates is correlatively diminished, and because additional tests must be applied to detect viral markers (antigens and, recently, viral RNA). This also poses an economical burden to the public health systems.

#### **Platelets and arenaviruses**

Several members of the *Arenaviridae* family are etiologic agents of viral hemorrhagic fevers (VHFs) in humans, a syndrome characterized by fever and bleeding complications that may ultimately lead to shock and death [70]. Different experimental models shed light on the role of platelets in VHF [50]. It was reported that mice infected with an acute strain (Armstrong) of lymphochoriomeningitis virus (LCMV), a prototype representative of *Arenaviridae* that was shown able to be episodically

pathogen in the human species, developed a mild hemorrhagic anemia that became severe and eventually lethal in animals depleted of platelets or lacking integrin  $\beta 3$ . In addition to the life-threatening hemorrhagic anemia, platelet-depleted mice failed to mount an efficient cytotoxic T lymphocyte (CTL) response and could not clear LCMV. Remarkably, lethal hemorrhage was less frequently associated with thrombocytopenia and instead was more closely associated with platelet dysfunction mediated by high type I interferon levels [71]. These observations confirm that platelets are necessary to protect vascular integrity and are critical mediators of viral clearance; they also outline the generally underscored and underappreciated relationships between primary hemostasis, viral infection and immunosuppression.

It is noteworthy that there are important differences between the LCMV mouse model for thrombocytopenia in which IFN- $\alpha/\beta$  mediates the decrease in platelet numbers, platelet dysfunction and bleeding, and the LCMV primate model, in which the IFN- $\alpha/\beta$  expression is not correlated to the presence of bleeding signs [71,72]. However, the role of type I IFN in mediating thrombocytopenia and platelet dysfunction was further supported by the infection of human CD34<sup>+</sup> cells by Junin virus, an arenavirus that causes Argentinian Haemorrhagic Fever. In this disease, thrombopoiesis is selectively impaired by a decrease of pro-platelet formation, platelet release and P-selectin externalization [73]. This mechanism is supported by the recent observation that MKs express functional type I IFN receptors, suggesting that megakaryo/thrombopoiesis regulation by type I IFN is associated with a specific interaction with its receptor, and that these cells may play a role in the antiviral defense by being both type I IFN producers and responders [74].

Very recently, it was shown that mice profoundly depleted of platelets (<2.5%) and infected with the Armstrong LCMV strain developed systemic bleeding and hemorrhagic spots in several organs along with high viral titers, generalized splenic necrosis and increased mortality. Interestingly, the partial depletion (15%) of platelets was sufficient to prevent vascular damage but not viral replication, necrotic destruction of innate and adaptive immune splenocytes or CTL exhaustion [75]. The existence of different platelet requirements for controlling vascular integrity and immune response is an interesting novel concept, which suggests that these 2 events may be mediated by separated pathways at the platelet level. Moreover, these data give further support to the increasing evidence of the critical role of the physical interaction between platelets and immune cells to the host immune response during viral infection. However an important issue that requires further clarification is the molecular basis governing platelet interaction with immune cells in VHF.

The role of platelets in the immunosuppression occurring after infection by clone-13 of LCMV, a chronic, persistent strain, and treatment with TPO was also investigated [76]. Despite an increase in the number of platelets, no differences were seen in LCMV viral titers, indicating that other mechanisms mediate the deficient immune response seen in LCMV clone-13 infection. Interestingly the authors hypothesized that the selective inactivation of IFN- $\alpha/\beta$  signaling in MKs without altering their important antiviral activity in immune and stromal cells was found able to completely overcome the thrombocytopenia induced by infection with LCMV clone-13.

Among the different pathogenic mechanisms that account for the bleeding complications of VHF induced by arenaviruses, the presence of a platelet inhibitor has been reported in the serum of Lassa [77] and Junin virus infected-patients. This factor was not neutralized by plasma containing high titers of protective antibodies against Junin virus [78], suggesting that it is not a virus-derived molecule. Although this inhibitor has not yet been characterized, thrombomodulin could be a potential candidate. Indeed, thrombomodulin was shown to bind thrombin and block the ability of thrombin to activate platelets. Moreover, peripheral blood mononuclear cells (PBMC) exposed to a pathogenic Lassa virus increase expression of thrombomodulin mRNA. This pattern was observed also at the protein level in PBMC and dendritic cells for both soluble and membrane-bound thrombomodulin [50]. Thus, it is conceivable that the platelet aggregation inhibitor found in Lassa or Junin virus-infected patients is thrombomodulin, awaiting for laboratory confirmation.

Collectively, these data indicate that homeostatic abnormalities together with the antiviral immune response and high levels of viremia observed in arenavirus infection are partly regulated by platelet-virus interactions.

## Conclusion

In the light of the preceding considerations, the relationship between platelets and viruses appears to be very complex and heterogeneous. The commonest feature is probably the decrease of platelet count during viral infection, with sometimes threatening levels and bleeding. Two other common features are a profound disturbance of the coagulation process at large (primary hemostasis, clotting and fibrinolysis) and a sustained inflammation, some platelet factors intervening in both hemostasis and inflammation; a special link between these two events is very likely during viral infection but not yet fully understood. The functional properties of platelets are profoundly altered during viral infection and in particular their capacity to control the leukocyte containment within circulation or emigration to tissues; viral infections

where platelet disturbances are noticeable seem to be accompanied by an altered vascular integrity, allowing leakage. The role of platelets as exerting virostatic effects would also need to be addressed; although it seems rather limited or at least over-helmed by the global inflammatory environment, the fact that platelets are induced to massive death as consequences of direct attacks of viruses or virus-infected cells must be taken into consideration. Finally, a better understanding of the interplay between platelets and viruses would certainly help correcting the platelet defects that are commonly observed and that may be severe, with no treatment identified so far; platelet component transfusions may be needed but, even though, with imprecise and non consensual recommendations for prescriptions and use. In aggregate, roles of viral platelets in viral infection are dominated by pathologic inflammation with evident but not exclusive consequences in hemostasis. Further research in platelet mediated-immunity during viral infection would be helpful, both from a conceptual point of view due to the variety of current pathophysiological situations and the likely severity of certain clinical pictures, especially when hemorrhagic symptoms are present.

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

AC and HHC drafted the manuscript. BP, FC, MS, RC contributed original sections of the manuscript. OG reviewed the final work up of the manuscript. All authors read and approved the final manuscript.

#### Acknowledgements

Adrien Chabert's PhD fellowship is supported by the Region Rhône-Alpes, which is also greatly acknowledged for its invaluable support. The Platelet Team activity within the EA3064 group benefits from grants from EFS, ANSM, ANR, ART and "Les Amis de Rémi", to which/whom the authors express their gratitude.

#### Author details

<sup>1</sup>EA3064—GIMAP, Université de Lyon, 42023 Saint-Etienne, France. <sup>2</sup>Service des Agents infectieux et d'Hygiène, CHU de Saint-Etienne, 42055 Saint-Etienne, France. <sup>3</sup>EFS Auvergne-Loire, 42023 Saint-Etienne, France. <sup>4</sup>Laboratorio de Trombosis Experimental, Instituto de Medicina Experimental, ANM-CONICET, Buenos Aires, Argentina. <sup>5</sup>Laboratorio de Virus Animales, Instituto de Biotecnología y Biología Molecular, UNLP-CONICET, La Plata, Argentina. <sup>6</sup>Institut National de la Transfusion Sanguine, 75015 Paris, France. <sup>7</sup>INTS, 6 rue Alexandre-Cabanel, 75015 Paris, France.

Received: 13 January 2015 Accepted: 3 April 2015

Published online: 28 April 2015

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**Article 1 : Non-leukodepleted red blood  
transfusion in sepsis patients : beyond  
oxygenation, is there a risk of inflammation ?**

**Article publié dans  
Critical Care en Décembre 2014**

LETTER

# Non-leukodepleted red blood cell transfusion in sepsis patients: beyond oxygenation, is there a risk of inflammation?

Olivier Garraud<sup>1,2\*</sup>, Adrien Chabert<sup>1</sup>, Bruno Pozzetto<sup>1,3</sup>, Fabrice Zeni<sup>1,4</sup>, Fabrice Cognasse<sup>1,5</sup> and Hind Hamzeh-Cognasse<sup>1</sup>

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Donati and colleagues [1] evaluated the benefits of fresh, leukodepleted (LD) versus non-leukodepleted (nLD) erythrocyte transfusions on the microcirculation in sepsis patients. Oxygenation appeared equal in both groups. Differences between the two kinds of blood cells are presented in Table 1. In the reported study, there is a difference between the age of erythrocytes in the two groups (1.5 to 3 days for nLD versus 3.5 to 5 days for LD erythrocytes; a U test indicates  $P < 0.05$ ); this suffices to influence oxygen delivery mediators (Figure 1). The content of leukocytes prior to (and after) leukodepletion were not tested, nor was the freeing - which is usually very fast - of secreted or docked, soluble biological response modifiers. Considering the dynamics of secreted biological response modifiers in platelet components [2], there must be differences within the two groups, which possibly

influenced sepsis conditions. Residual plasma within erythrocytes given by female donors may include anti-human leukocyte antigen (HLA) antibodies which attack the recipient's lung alveolar epithelium neutrophils, and as sepsis is characterized by the pathology of neutrophils that release microparticles and neutrophil extracellular traps that target lung epithelium [3], it may be feared here that the nLD condition aggravates pulmonary lesions.

The re-evaluation of procedures is infrequent in transfusion despite the rapid evolution of techniques and materials; for example, differential stresses are inflicted on erythrocytes, depending on the collection process (apheresis versus conventional whole blood), with consequences for neutrophils and (vascular) endothelial cells upon transfusion [4]. Clinical investigations and registered trials such as Donati and colleagues are valuable.

## Authors' response

Abele Donati, Elisa Damiani, Erica Adrario, Rocco Romano, Paolo Pelaia and Can Ince

We thank Professor Garraud and colleagues for their interest in our study [1]. As underlined, the difference in the age of transfused red blood cells (RBCs) between the nLD group (4 (3.5 to 5) days) and the LD group (3 (1.5 to 3) days) may have influenced the RBC oxygen-delivery capacity. Stored RBCs lose their ability to release vasodilators (nitric oxide, ATP) during hypoxia [5,6]. We showed similar changes in microvascular reactivity (tissue oxygen saturation (StO<sub>2</sub>)-upslope) and oxygenation (StO<sub>2</sub>) after nLD or LD RBC transfusions [1]. This may indicate that oxygen-delivery mediators were not sufficiently affected to determine relevant variations in the response observed.

Alternatively, heterogeneity in the study population prevented detection of subtle differences. Variability in the response to treatments is common during sepsis. The patient heterogeneity was underlined as a limitation of our investigation [1].

LD RBCs showed a more favorable effect on microcirculatory convective flow [1]. This may depend on the lower adhesiveness of LD RBCs to the endothelium [7]. The transfusion of nLD RBCs decreased blood flow velocity and increased glycocalyx damage markers [1]. Similar effects may reasonably occur in the lungs. As highlighted, anti-HLA antibodies in nLD blood from female donors may contribute to aggravate pulmonary lesions. Nonetheless, the evaluation of respiratory function went beyond our goals.

\* Correspondence: [ogarraud@ints.fr](mailto:ogarraud@ints.fr)

<sup>1</sup>Université de Lyon, GIMAP-EA3064, Saint Etienne 42023, France

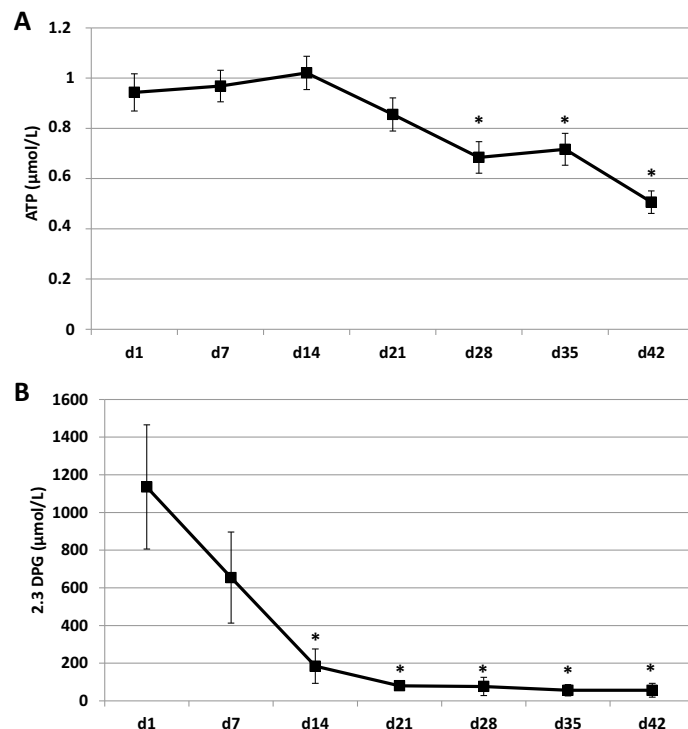
<sup>2</sup>Institut National de Transfusion Sanguine (INTS), Paris 75739, France

Full list of author information is available at the end of the article

**Table 1 Possible consequences of variation in transfusion conditions and the type of packed red blood cells administered**

Variable	Primary or immediate consequences (efficacy)	Secondary or delayed consequences (hazards)
<b>Total volume</b>	Possibly needs to be adjusted according to the patient's needs	Possibly needs to be adjusted to correct for anemia
<b>Hematocrit</b>	Possibly needs to be adjusted according to the patient's needs	Possibly needs to be adjusted to correct for anemia
<b>Residual plasma volume</b>	Possibly includes anti-HLA antibodies (from female donors) Possibly affects the amount of soluble, free biological response modifiers	Increases the risk of TRALI Increases the risk of inflammation and aggravates the risk of TRALI
<b>Leukocytes</b>	No pre-test (possibly affects donor eligibility) Pre-activation of leukocytes Release of biological response modifiers Release of microparticles and neutrophil extracellular traps HLA antibody targets Infectious risk (intracellular viruses)	Increases the risk of inflammation Aggravates sepsis Increases the risk of TRALI Increases the risk of viral infections
<b>Age of blood</b>	Decreases the benefit of oxygen transport Release of microparticles Expression of stress signals on red blood cells Free iron release NO and iNOS release Oxygenated lipid and lipid degradation	Increases the risk of inflammation Potentiates the risk of TRALI by stressing target neutrophils Possibly increases the risk of allo-immunization

HLA, human leukocyte antigen; iNOS, inducible nitric oxide synthase; NO, nitric oxide; TRALI, transfusion-related acute lung injury.



**Figure 1 ATP and 2,3-diphosphoglycerate (2,3-DPG) in packed red blood cells. (A)** ATP and **(B)** 2,3-DPG in packed red blood cells measured after 1, 7, 14, 21, 28, 35 and 42 days (d) of storage were re-evaluated by one-way analysis of variance. Inter-experiment differences in ATP and 2,3-DPG concentrations at different time points were analyzed by Wilcoxon paired test (XLSTAT® 2010 software, Addinsoft, Paris, France). *P*-values  $\leq 0.05$  were considered to be significant (\**n* = 10). PLT, platelet.

The efficacy of blood transfusion depends on multiple RBC- and patient-related factors. Understanding the response to transfusion during sepsis is a challenging task. Targeting predetermined hemoglobin levels and/or macrohemodynamics is clearly not sufficient. Monitoring the microcirculation may get us closer to the answer [8].

#### Abbreviations

HLA: Human leukocyte antigen; LD: Leukodepleted; nLD: Non-leukodepleted; RBC: Red blood cell; StO<sub>2</sub>: Tissue oxygen saturation.

#### Competing interests

The authors declare that they have no competing interests.

#### Author details

<sup>1</sup>Université de Lyon, GIMAP-EA3064, Saint Etienne 42023, France. <sup>2</sup>Institut National de Transfusion Sanguine (INTS), Paris 75739, France. <sup>3</sup>Département de Microbiologie Hôpital Nord, CHU de Saint-Etienne, Saint-Etienne 42055, France. <sup>4</sup>Service de Réanimation Médicale et Polyvalente Hôpital Nord, CHU de Saint-Etienne, Saint-Etienne 42055, France. <sup>5</sup>Etablissement Français du Sang Auvergne-Loire, Saint-Etienne 42100, France.

Published online: 08 December 2014

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doi:10.1186/s13054-014-0690-y

**Cite this article as:** Garraud *et al.*: Non-leukodepleted red blood cell transfusion in sepsis patients: beyond oxygenation, is there a risk of inflammation? *Critical Care* 2014 **18**:690.

**Article 2 : Streptococcus sanguinis-induced  
cytokine and matrix metalloproteinase-1 release  
from platelets.**

**Article publié dans  
BMC immunology en Décembre 2014**

RESEARCH ARTICLE

Open Access

# *Streptococcus sanguinis*-induced cytokine and matrix metalloproteinase-1 release from platelets

Fabrice Cognasse<sup>1,2\*</sup>, Hind Hamzeh-Cognasse<sup>2†</sup>, Adrien Chabert<sup>2</sup>, Elke Jackson<sup>3,4</sup>, Charles-Antoine Arthaud<sup>1</sup>, Olivier Garraud<sup>1,2</sup> and Archie McNicol<sup>3,4</sup>

## Abstract

**Background:** *Streptococcus sanguinis* (*S.sanguinis*), a predominant bacterium in the human oral cavity, has been widely associated with the development of infective endocarditis. Platelets play both a haemostatic function and can influence both innate and adaptive immune responses. Previous studies have shown that *S.sanguinis* can interact with, and activate, platelets.

**Results:** The aim of this study was to determine whether *S.sanguinis* stimulates the release of matrix metalloproteinases (MMPs) 1, 2 and 9 and the pro-inflammatory mediators SDF-1, VEGF and sCD40L, from platelets and to subsequently pharmacologically address the release mechanism (s). *S.sanguinis* stimulated the release of MMP-1, SDF-1, VEGF and sCD40L from platelets and inhibitors of cyclooxygenase and phosphatidylinositol 3-kinase, and antagonists of the  $\alpha\text{IIb}\beta\text{3}$  integrin and glycoprotein Ib, each inhibited the secretion of all factors.

**Conclusions:** Therefore the release of MMP-1, SDF-1, VEGF and sCD40L occurs late in the platelet response to *S.sanguinis* and highlights the complex intracellular signalling pathways stimulated in response to *S.sanguinis* which lead to haemostasis, MMP and pro-inflammatory mediator secretion.

**Keywords:** Platelets, Cytokines, Signalling, Oral cavity, Inflammation, *Streptococcus sanguinis*

## Background

The oral cavity is one of the most common gateways of entry of bacteria, including *Streptococcus sanguinis* (*S. sanguinis*) an organism which has been implicated in the development of infective endocarditis [1], into the blood [2]. A correlation exists between periodontitis, where there is an increased incidence of bacteremias [3], and the development of cardiovascular disease [4], although the causality is controversial [5]. Multiple studies have demonstrated that *S. sanguinis* can activate human platelets in a strain and donor dependent manner [6], and therefore it is possible that platelets play a central role in any relationship between periodontitis and cardiovascular pathologies [6].

Traditionally platelets have been considered solely as components of haemostasis and by extension the pathological process of thrombosis [7,8]. However, it is now clear that platelets are innate immune cells and are

associated with the early stages of atherosclerosis and other inflammatory conditions [9-11]. Platelets contain in, and secrete from, alpha granules a range of soluble immunomodulatory factors such as Stromal cell-derived factor (SDF)-1/CXCL12, a member of the CXC chemokine family and Vascular Endothelial Growth Factor (VEGF), a sub-family of growth factors, stimulate vasculogenesis and angiogenesis [12]. Furthermore, CD40 ligand (CD40L, CD154), of which approximately 95% of its soluble form (sCD40L) is generated from platelets, is a modulator of humoral and cellular immunity, has pro-inflammatory properties and provides a link between the immune system and atherothrombosis [13].

Several recent data, including our own, demonstrate that platelets have the capacity to sense external signals through a single type of pathogen recognition receptor and differentially adjust the innate immune response by the appropriate secretion of a number of cytokines/chemokines and some of their receptors [14,15]. Italiano and colleagues initially demonstrated that there are two discrete sub-populations of platelet alpha granules, one containing pro-angiogenic factors and one containing anti-angiogenic factors [16,17];

\* Correspondence: fabrice.cognasse@univ-st-etienne.fr

†Equal contributors

<sup>1</sup>Etablissement Français du Sang (EFS) Auvergne-Loire, Saint-Etienne, France

<sup>2</sup>GIMAP-EA3064, Université de Lyon, Saint-Étienne 42023, France

Full list of author information is available at the end of the article

indeed subsequent studies have suggested more heterogeneity among these granules [18]. This has led to the concept of differential alpha granule release, although the mechanisms remain unknown [17].

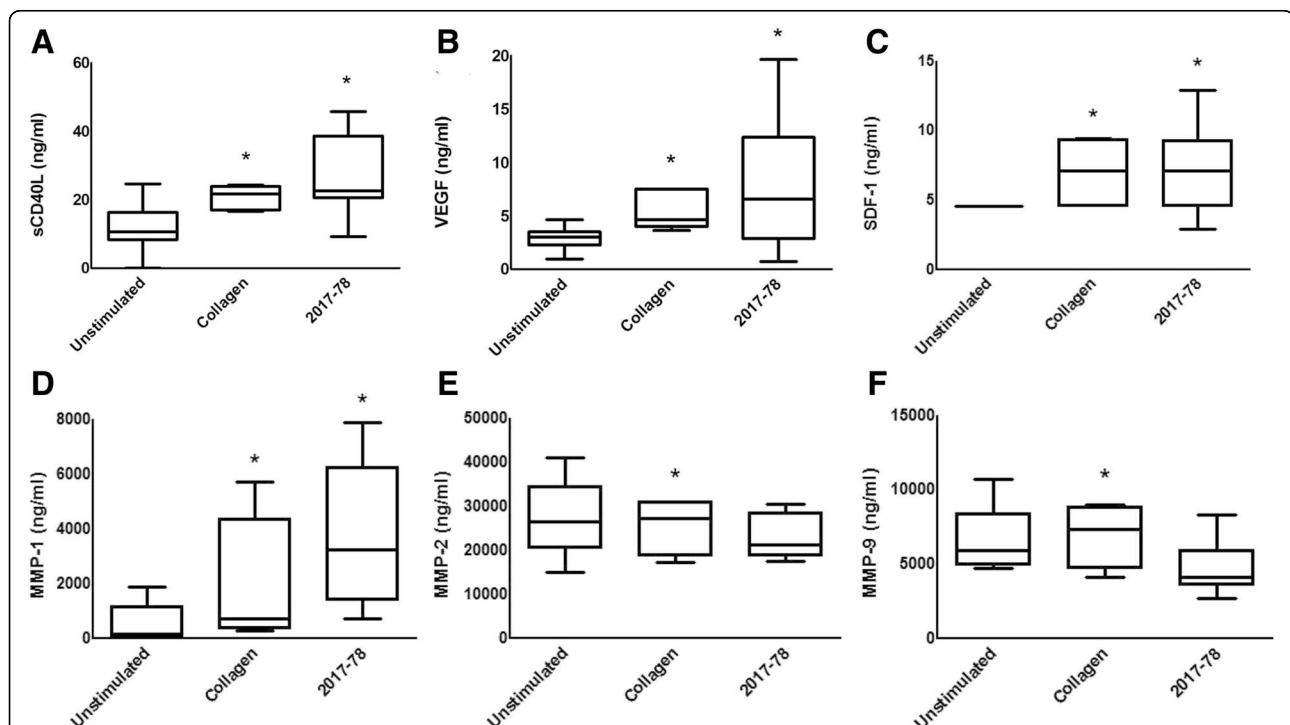
Matrix Metalloproteinases (MMPs) constitute a family of zinc- and calcium-dependent proteinases that are involved in the turnover of the extracellular matrix (ECM) of connective tissue. They degrade most components of the ECM and participate in a variety of pathological processes, including atherosclerosis, myocardial infarction and aortic aneurysms, as well as tumour growth and metastasis [19]. MMP-1 is expressed on the surface of resting platelets and, following platelet activation, its levels are upregulated and its activity engaged [20]. There is less MMP-2 than MMP-1 on the platelet surface, and the presence of both MMP-3 and MMP-9 is controversial [20]. In addition to its effects on the extracellular matrix, MMP-1 can regulate outside-in signalling in platelets resulting in the phosphotyrosine phosphorylation, and subsequent redistribution, of  $\beta 3$  integrins as a pre-requisite for platelet aggregation [20].

Interestingly, collagen can activate MMP-1, which in turn cleaves the platelet protease activating receptor, PAR-1, with the resultant engagement of the receptor and enhanced platelet activation [21]. To date there are no reports of the effects of *S. sanguinis* on MMPs in platelets.

The aim of this study was to determine whether *S. sanguinis* releases platelet MMPs (MMP-1, 2 and 9) and to pharmacologically address the mechanism by which the MMPs and pro-inflammatory mediators (SDF-1, VEGF and sCD40L) are released.

## Results and discussion

Kerrigan and colleagues have suggested that the interaction between *S. sanguinis* and GPIb on platelets is not only important for the pathogenesis of infective endocarditis but may also play a contributory role in some cases of myocardial infarction [22]. Studies have shown that plasma levels of sCD40L is a predictor of recurrent cardiovascular disorders (e.g. myocardial infarction and stroke) [23]. As previously observed [24], *S. sanguinis* 2017-78 stimulated the release of significant amounts of sCD40L from platelets (Figure 1A), consistent with Kerrigan and colleagues proposed role for platelets in *S. sanguinis*-induced myocardial infarction [22]. The secretion of sCD40L was accompanied by the secretion of VEGF (Figure 1B), SDF-1 (Figure 1C) and MMP-1 (Figure 1D). In each case, the level of secretion elicited by *S. sanguinis* 2017-78 was statistically similar to that elicited by the soluble positive control collagen (Figure 1A-D).



**Figure 1** *Streptococcus sanguinis*-induced soluble molecule secretion from platelets. Platelets were stirred in with either *S. sanguinis* strain 2017-78 (n = 15), collagen (2  $\mu$ g/ml; n = 5) or saline control (Unstimulated; n = 11). Release reactions were terminated and the levels of (A) sCD40L, (B) VEGF, (C) SDF-1, (D) MMP-1, (E) MMP-2 and (F) MMP-9 in the releasates determined by multi-plex luminex. Results are mean  $\pm$  SEM of several individual experiments using the platelets from different donors and subsequently analyzed using the Mann-Whitney U-test ( $^{\#}P < 0.05$  with respect to saline control;  $^{*}P < 0.05$  with respect to the saline control).



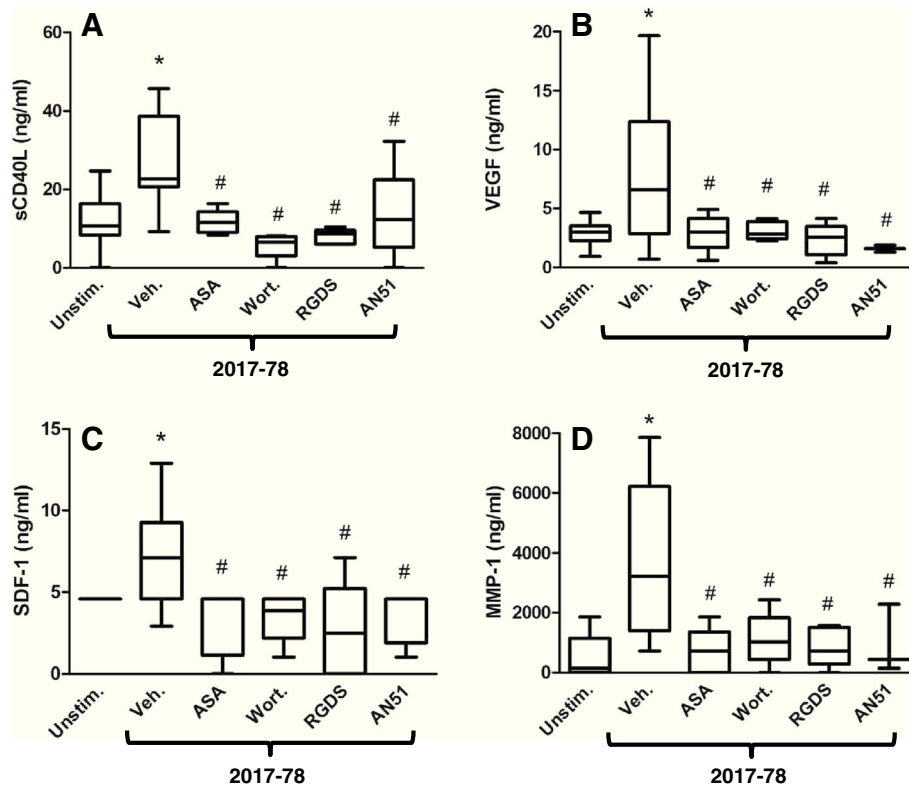
In contrast *S. sanguinis* 2017–78 did not elicit the release of MMP-2 (Figure 1E) or MMP-9 (Figure 1F). This constitutes the first demonstration that the oral micro-organism *S. sanguinis* can induce the secretion of a tissue-destructive molecule (MMP-1) from platelets to the local vasculature, thus potentially participating in the breakdown of the ECM at sites of vascular lesion. The functional significance of the secretion of MMP-1 is unclear. A novel autocrine role for MMP-1 in collagen-stimulated platelets has been proposed, whereby collagen converts inactive MMP-1 to active MMP-1 which in turn cleaves the thrombin receptor PAR-1 exposing the ligand and leading to full platelet activation [20]. A similar mechanism in *S. sanguinis*-induced platelet activation is attractive as *S. sanguinis* and collagen utilise many similar intracellular signalling pathways [25]. To date the effects of PAR-1 receptor antagonists, such as SCH 530398, on platelet activation induced by either collagen or *S. sanguinis* have not been examined. Interestingly, however, certain strains of *Porphyromonas gingivalis* have been shown to activate platelets via the PAR-1 and PAR-4 thrombin receptors on platelets [26]. Inhibition of this proteolytic action had no

effect on this activation, and therefore it is clear that a complex mechanism is involved [27] and a role for MMP-1 cannot be discounted.

The absence of MMP-2 secretion may reflect the subcellular localisation of MMP-2 within the platelet. Studies by Sawicki and colleagues suggest that MMP-2 is localised to the cytosol rather than to granules [28]. Although in the same studies MMP-2 was secreted in activated platelets [28], it is possible that the weak nature of platelet activation by *S. sanguinis* cannot stimulate this release.

The presence, secretion and release of MMP-9 from platelets is controversial. Some studies suggest that platelets do not secrete MMP-9 [29,30], whereas others report, not only that MMP-9 is secreted from platelets *in vivo*, but that it plays a crucial role in sCD40L release in pathological conditions such as Behçet's disease [31] and abdominal sepsis [32].

To determine the potential roles played by individual signalling pathways in the secretion of sCD40L, VEGF, SDF-1 and MMP-1 in response to *S. sanguinis*, we employed various inhibitors of biochemical pathways at concentrations previously reported to have maximal effects. Such concentrations



**Figure 2** Effects of inhibitors on *Streptococcus sanguinis* induced sCD40L (A), VEGF (B), SDF-1 (C) and MMP-1 (D) secretion from platelets. Platelets were pre-incubated with either aspirin (100  $\mu$ M; 20 mins; n = 7), wortmannin (100 nM; 2 mins; n = 5), RGDS (1 mM; 5 mins; n = 5), AN51 (0.165 mg/mL, 5 mins; n = 5) or the saline control then stirred with *S. sanguinis* strain 2017–78. Release reactions were terminated and the levels of sCD40L in the releasates determined by multi-plex luminex. Results are shown as box plots with whiskers for the data range representing median, upper and lower quartiles of several individual experiments using the platelets from different donors and subsequently analyzed using the Mann–Whitney U-test (\*P < 0.05 with respect to saline control; #P < 0.05 with respect to *S. sanguinis* strain 2017–78).

optimise the conditions to identify individual signalling pathways, although recognising the potential for non-selective actions.

We have previously shown roles for GPIb, COX, the  $\alpha$ IIb $\beta$ 3 integrin and PI3K in *S. sanguinis*-induced platelet aggregation [25,33,34]. Consistent with the aggregation results [24], pre-treatment of platelets with the GPIb antagonist AN51 (0.165 mg/mL, 5mins.), the COX inhibitor aspirin (100  $\mu$ M; 20 min), the  $\alpha$ IIb $\beta$ 3 integrin antagonist RGDS (1 mM; 5 mins.) or the PI3K inhibitor wortmannin (100 nM; 2 mins.) each significantly decreased *S. sanguinis* strain 2017-78-induced secretion of sCD40L (Figure 2A).

Pretreatment of platelets with the same inhibitors also decreased the secretion of VEGF (Figure 2B), SDF-1 (Figure 2C) and MMP-1 (Figure 2D) consistent with roles for GPIb, COX, the  $\alpha$ IIb $\beta$ 3 integrin and PI3K in the secretion of these proteins. The precise sublocalisation of SDF-1, VEGF and MMP-1 within the individual heterogenic population of granules is unknown. However, the current observations suggest, either that SDF-1, VEGF and MMP-1 are contained in the same granule population, or that the granule populations containing the proteins are regulated by the same signalling pathways.

## Conclusions

The concept of differing signalling pathways regulating the release of the individual populations of alpha granules was introduced by Italiano and Batinelli [17]. The pathway(s) involved in secretion has not been established. However as secretion was sensitive to inhibition by RGDS, it appears that it occurs distal to the engagement of the  $\alpha$ IIb $\beta$ 3 integrin. Speculatively this could lead to the incorporation, and activation, of ERK into the cytoskeleton [35], Rac activity, cytoskeletal rearrangement [36] and the subsequent alpha granule secretion [37]. Further description of their mechanisms of action will expand our understanding of platelet activation and oral/mucosal inflammatory process and produce new therapeutic strategies.

## Methods

### Materials

*S. sanguinis* strain 2017-78 was a gift from Dr M. Herzberg (University of Minnesota) [38]. Blood Agar Base No. 2 was obtained from Oxoid Inc. (Nepean, ON, Canada) and sheep's blood from Atlas Laboratories (Winnipeg, MB, Canada). Collagen, the cyclo-oxygenase (COX) inhibitor aspirin, the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin, the peptide  $\alpha$ IIb $\beta$ 3 integrin antagonist Arginine-Glycine-Aspartic Acid-Serine (RGDS), and the GP (glycoprotein) Ib receptor blocker AN51 were all purchased from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada).

### Blood collection

The study was approved by the Research Ethics Board of the University of Manitoba. Blood was collected after informed consent, from healthy human volunteers who had denied taking medication known to interfere with platelet function. Platelet-rich plasma (PRP) was prepared by centrifugation (200  $\times$  g; 20 min). *S. sanguinis* strain 2017-78 was prepared as previously reported [24]. PRP was stirred with *S. sanguinis* strain 2017-78 in the presence of the inhibitor or vehicle control, and was aggregation monitored continuously. At a time corresponding to that which *S. sanguinis* strain 2017-78 induced maximal aggregation, the release was terminated, the sample was centrifuged and the supernatants stored at -80  $^{\circ}$ C until assayed [24].

### Soluble factor detection

The levels of soluble factors MMPs (MMP-1, 2 and 9) and pro-inflammatory mediators (SDF-1, VEGF and sCD40L) were measured in duplicate from the releasates by multiplex luminex kits (Bio-Rad, Marnes-la-Coquette, France). Absorbance at 450 nm was determined using an ELISA reader (Miltiskan EX, Labsystem, Helsinki, Finland), as previously reported [39].

### Statistical analysis

Inter-experiment comparisons of *S. sanguinis* strain 2017-78-induced MMPs and cytokines release were analyzed by means of the paired *t*-test. The effects of inhibitors on *S. sanguinis* strain 2017-78-induced secretion were analyzed using the non-parametric Mann-Whitney *U*-test. Results are shown as box plots with whiskers for the data range representing median, upper and lower quartiles and a *P*-value < 0.05 was considered to be significant.

### Abbreviations

*S. sanguinis*: *Streptococcus sanguinis*; MMP: Matrix metalloproteinase; SDF1: Stromal cell-derived factor 1; VEGF: Vascular endothelial growth factor; sCD40L: Soluble CD40 ligand; COX: Cyclooxygenase; PI3K: Phosphatidylinositol 3-kinase; RGDS: Arginine-glycine-aspartic acid-serine peptide; GP: Glycoprotein; ECM: Extracellular matrix; PRP: Platelet-rich plasma.

### Competing interests

The authors declare no competing financial interests and no conflict of interest regarding this study.

### Authors' contributions

FC, HHC, AMcN, OG: Analysis and interpretation of results; FC, CAA, HHC, AC, EJ, AMcN, OG: Preparation of manuscript. All authors read and approved the final manuscript.

### Acknowledgements

The authors would like to thank Mrs. Marie-Ange Eyraud for her experimental expertise, and Dr. Sara Israels and Pr. Bruno Pozzetto for helpful discussions and critical reading of this manuscript. This study was funded by grants from the Heart and Stroke Foundation of Manitoba, L'Établissement Français du Sang, La Plaine - Saint-Denis, France, the French Agence Nationale de la Recherche (ANR) under reference ANR-12-JSV1-0012-01 and the Rhône-Alpes Region - ARC 2012-1200870001.

#### Author details

<sup>1</sup>Etablissement Français du Sang (EFS) Auvergne-Loire, Saint-Etienne, France. <sup>2</sup>GIMAP-EA3064, Université de Lyon, Saint-Étienne 42023, France. <sup>3</sup>Oral Biology and Pharmacology & Therapeutics, University of Manitoba, Winnipeg, MB, Canada. <sup>4</sup>International Centre for Oral Systemic Health, University of Manitoba, Winnipeg, MB, Canada.

Received: 30 October 2013 Accepted: 7 April 2014

Published: 22 April 2014

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doi:10.1186/1471-2172-15-15

**Cite this article as:** Cognasse et al.: *Streptococcus sanguinis*-induced cytokine and matrix metalloproteinase-1 release from platelets. *BMC Immunology* 2014 **15**:15.

# Communications scientifiques

## COMMUNICATIONS AFFICHEES

### « Platelet-associated inflammation in severe sepsis »

Adrien Chabert, Fabrice Cognasse, Gérard Lina, Marie-Ange Eyraud, Cyril Badiou, Florence Grattard, Paul Verhoeven, Fabrice Zeni, Bruno Pozzetto, Bernard Payrastre, Hind Hamzeh-Cognasse, Olivier Garraud

*Journée de l'ARC (Academic Research Community) 1 Santé Rhône-Alpes, Isle d'Abeau (FR),  
Septembre 2014*



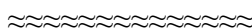
### « Acetylsalicylic acid differentially limits the activation and expression of cell death markers in human platelets exposed to various *Staphylococcus aureus* strains »

Adrien Chabert, Pauline Damien, Paul O. Verhoeven, Florence Grattard, Philippe Berthelot, Fabrice Zeni, Laurence Panicot-Dubois, Stéphane Robert, Françoise Dignat-George, Marie-Ange Eyraud, Bruno Pozzetto, Bernard Payrastre, Fabrice Cognasse, Olivier Garraud and Hind Hamzeh-Cognasse

*Journée annuelle de l'Institut Fédératif de Recherche en Sciences et Ingénierie de la Santé (IFRESIS), Saint-Etienne (FR), Mai 2016*



*Retraite du Centre International de Recherche en Infectiologie (CIRI), Chamonix (FR),  
Mai 2016*



*Journée de la recherche de l'école doctorale (EDSIS 488), Saint-Etienne (FR), Juin 2016*



*34th International Congress of the ISBT, Dubaï (EA), Septembre 2016*

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*International Society of Thrombosis and Haemostasis 2017 Congress, Berlin (ALL),*

*Juillet 2017*

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**« Acetylsalicylic acid limits neutrophil and platelet recruitment responsible  
for lung damage during experimental sepsis »**

Adrien Chabert, Charles-Antoine Arthaud, Thomas Bourlet, Bernard Payrastre, Fabrice  
Cognasse, Olivier Garraud and Hind Hamzeh-Cognasse

*Journée de l'ARC (Academic Research Community) 1 Santé Rhône-Alpes, Isle d'Abeau (FR),  
Septembre 2016*

## COMMUNICATIONS ORALES

### **« Inflammation associée aux plaquettes dans le sepsis sévère »**

Adrien Chabert

*Journée de l'ARC (Academic Research Community) 1 Santé Rhône-Alpes, Isle d'Abeau (FR),  
Septembre 2015*



### **« Les plaquettes sanguines adaptent leur sécrétion de facteurs immuno-modulateurs aux différentes exotoxines de *Staphylococcus aureus* »**

Adrien Chabert, Pauline Damien, Fabrice Cognasse, Cédric Badiou, Fabrice Zeni, Marie-Ange Eyraud, Charles-Antoine Arthaud, Bruno Pozzetto, Olivier Dauwalder, François Vandenesch, Gérard Lina, Olivier Garraud, Hind Hamzeh-Cognasse

*Congrès du Club français des plaquettes et mégacaryocytes (CFPM), Strasbourg (FR),  
Novembre 2015*



Présentée par Hind Hamzeh-Cognasse

*GREMI meeting, Vascular Inflammation : Blood vessels under attack, Paris (FR),  
Novembre 2015*



**«Acetylsalicylic acid differentially limits the activation and expression of cell death markers in human platelets exposed to various *Staphylococcus aureus* strains»**

Adrien Chabert, Pauline Damien, Paul O. Verhoeven, Florence Grattard, Philippe Berthelot, Fabrice Zeni, Laurence Panicot-Dubois, Stéphane Robert, Françoise Dignat-George, Marie-Ange Eyraud, Bruno Pozzetto, Bernard Payrastra, Fabrice Cognasse, Olivier Garraud and Hind Hamzeh-Cognasse

*CYTIMA 2016 – 17<sup>ème</sup> journées stéphanoises de cytométrie, imagerie cellulaire et tissulaire, Andrieux-Bouthéon (FR), Juin 2016*



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## Sponsors financiers



TITRE : ROLE INFLAMMATOIRE DES PLAQUETTES SANGUINES LORS DU SEPSIS

RESUME : Depuis quelques années, les plaquettes sanguines sont reconnues comme un véritable élément de l'immunité. En effet, de nombreux rôles clés lors du processus inflammatoire sont attribués aux plaquettes, tels que la détection d'un signal, la libération de nombreux immunomodulateurs ou une forte interaction avec les autres cellules immunitaires. Le constat clinique d'une corrélation entre le taux plaquettaire et la mortalité du sepsis permet de relever l'importance de cette cellule dans la physiopathologie du sepsis. Ainsi ce travail doctoral a eu pour objectif de mettre en relief la part inflammatoire des plaquettes lors des interactions entre les facteurs de virulence de *Staphylococcus aureus* (*S.aureus*) que sont différentes exotoxines ou de souches de *S. aureus* issues de bactériémies de patients septiques. Un modèle expérimental de sepsis murin nous a permis de comprendre la composante inflammatoire jouée par les plaquettes ainsi que leurs implications dans la dysfonction pulmonaire issue lors du sepsis. Enfin, nous avons évalué la modulation de molécules antiplaquettaires, et particulièrement de l'acide acétylsalicylique, sur les nombreux rôles pathogéniques des plaquettes durant cette pathologie.

MOTS CLES : Plaquettes – Sepsis – Inflammation – *Staphylococcus aureus*

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TITRE : ROLE INFLAMMATOIRE DES PLAQUETTES SANGUINES LORS DU SEPSIS

ABSTRACT : During some years, platelets are recognized as an key element of immunity. In fact, platelets play several roles, as signal detection, immunomodulator release and interaction with other immunity cells. The clinical significance of a correlation between platelet rate and sepsis mortality reveals the importance of this cell in the pathophysiology of sepsis. Thus, the purpose of this doctoral work was to highlight the inflammatory role of platelets in the interactions between the virulence factors of *Staphylococcus aureus* (*S. aureus*) that are different exotoxins or strains of *S. aureus* from bacteremia of septic patients. An experimental model of murine sepsis allowed us to understand the inflammatory component played by platelets as well as their implications in pulmonary dysfunction resulting from sepsis. Finally, we evaluated the modulation of antiplatelet molecules, particularly acetylsalicylic acid, on the numerous pathogenic roles of platelets during this pathology.

KEYWORDS : Platelets – Sepsis – Inflammation – *Staphylococcus aureus*