Plaquettes sanguines et insuffisance rénale aiguë : rôle du couple CD154/CD40 dans la constitution des lésions tubulaires

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PLAQUETTES SANGUINES ET INSUFFISANCE RENALE AIGUË : RÔLE DU COUPLE CD154/CD40 DANS LA CONSTITUTION DES LESIONS TUBULAIRES

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Titre : Plaquettes sanguines et insuffisance rénale aiguë : rôle du couple CD154/CD40 dans la constitution des lésions tubulaires

Résumé :

L'insuffisance rénale aiguë (IRA) est une pathologie fréquente en réanimation. Elle est associée à une mortalité et une morbidité importante. Le sepsis en est la cause la plus fréquente. La compréhension de la physiopathologie du sepsis et de ses complications a beaucoup progressé ces dernières années mais ne s'est pas encore traduite par des avancées thérapeutiques significatives en pratique clinique. Le paradigme d'une altération de la perfusion sanguine comme paramètre clé de la constitution des lésions rénales a ainsi été remis en question, plusieurs travaux révélant que le débit sanguin rénal n'est pas toujours altéré en cas de sepsis, et qu'une IRA peut se développer en cas de débit sanguin rénal préservé, voire augmenté. Le sepsis est caractérisé par de profondes perturbations de la réponse immunitaire et une réaction inflammatoire disproportionnée. A l'origine de l'atteinte rénale, l'inflammation et les altérations de la microcirculation sont maintenant considérés comme des mécanismes physiopathologiques fondamentaux. Au-delà de leur rôle dans l'hémostase, la contribution des plaquettes sanguines à la réponse inflammatoire, au maintien de l'intégrité tissulaire et à la défense contre les infections a considérablement élargi le spectre de leurs compétences et en a fait des acteurs physiopathologiques potentiels dans le sepsis. Les plaquettes sanguines exercent la plupart de ces fonctions grâce à l'expression de nombreux médiateurs membranaires ou solubles. Parmi eux, le CD154 tient une place particulière : les plaquettes sont une source essentielle de CD154 dans l'organisme et il joue un rôle central dans la réponse inflammatoire. Nous proposons dans ce travail un aperçu de ces avancées physiopathologiques récentes et nous discutons de la contribution des plaquettes et du CD154 dans les atteintes microcirculatoires et les défaillances multi-viscérales dans le sepsis. Nous nous sommes intéressés au rôle pro-inflammatoire du CD154 en conditions d'hypoxie au niveau de l'épithélium tubulaire rénal. Des données récentes soulignent en effet l'importance de l'hypoxie dans la réaction inflammatoire. Le contrôle de la production d'interleukine (IL)-6, une cytokine centrale de la réponse inflammatoire, par le CD154 a été étudié dans un modèle de culture de cellules épithéliales tubulaires (CET) rénales. Un modèle murin d'IRA par ischémie/reperfusion rénale a également été mis au point et appliqué à des souris déficientes en CD154 et CD40. Nos travaux révèlent que le CD154 induit fortement la sécrétion d'IL-6 par les CET en conditions d'hypoxie et que les souris déficientes en CD154 régénèrent plus rapidement leur épithélium tubulaire après ischémie/reperfusion rénale. Ces résultats pourraient ouvrir la voie à de potentielles pistes thérapeutiques pour la prise en charge des IRA d'origine septique.

Mots clés :

Insuffisance rénale aiguë ; plaquettes sanguines ; sepsis ; CD154

Title : Platelets and acute kidney injury : role of the CD154/CD40 dyad in the generation of tubular lesions

Abstract :

Acute kidney injury (AKI) is a common complication in critically ill patients and is associated with increased morbidity and mortality. Sepsis is the most common cause of AKI. The understanding of sepsis pathophysiology and its complications has progressed significantly in recent years but has not yet been translated into significant therapeutic advances in clinical practice. The traditional paradigm that sepsis-induced AKI is linked to renal hypoperfusion has been challenged by recent evidences showing that renal blood flow is not universally impaired during sepsis, and that AKI can develop in the presence of normal or even increased renal blood flow. Sepsis is characterized by profound alterations of the immune response and a disproportionate inflammatory response. Inflammation and microcirculatory dysfunction are now considered as fundamental pathophysiological mechanisms at the origin of renal injuries. Beyond haemostasis, the contribution of platelets in inflammation, tissue integrity and defence against infections has considerably widened the spectrum of their role and made them potential physiopathological actors in sepsis. Platelets fulfil most of these functions through the expression of membrane-bound or soluble mediators. Among them, CD154 holds a peculiar position, as platelets represent a major source of CD154 and as CD154 is a central regulator of inflammation. Here, we provide an overview of these recent pathophysiological advances and discuss the platelets and CD154 contribution to microcirculatory alterations in multi-organ dysfunction in sepsis. We investigated the pro-inflammatory role of CD154 under hypoxic conditions in the renal tubular epithelium as recent data highlight the importance of hypoxia in the inflammatory reaction. We studied the control of interleukin (IL)-6 production, a key cytokine involved in inflammation, by CD154 in oxygen deprivation conditions using a kidney tubular epithelial (TEC) cell line model. We also studied a murine model of kidney injury after ischemia/reperfusion, a model that was applied in CD154 and CD40 deficient mice. We found that CD154 is a potent inducer of IL-6 secretion by TEC in hypoxia and that CD154-deficient mice regenerate earlier the tubular epithelium after ischemia/reperfusion injury. These findings may provide potential avenues for septic AKI management and therapy.

Keywords : Acute Kidney Injury ; Sepsis ; Platelets ; CD154

Unité de recherche

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A Elise, à mes enfants, à toute ma famille,

A mes amis,

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ABBREVIATIONS

CET, cellules épithéliales tubulaires
DAMPs, Damage-Associated Molecular Patterns
DFG, débit de filtration glomérulaire
DSR, débit sanguin rénal
EER, épuration extra-rénale
HIF, hypoxia-inducible factor
HTA, hypertension artérielle
ICAM, Intercellular Adhesion Molécule
IRA, insuffisance rénale aiguë
IR, index de résistance vasculaire
IL, interleukine
I/R, ischémie/reperfusion
LPS, lipopolysaccharide
MPP, microparticules d'origine plaquettaires
NGAL, Neutrophil gelatinase–associated lipocalin
NTA, nécrose tubulaire aiguë
NO, oxyde nitrique
PAM, pression artérielle moyenne
PAMPs, Pathogen-Associated Molecular Patterns
RT-qPCR, real time quantitative polymerase chain reaction
TCD, tube contourné distal
TLR, récepteurs Toll-like
TNF, tumor necrosis factor
WT, Wild Type

Le travail de recherche présenté dans ce manuscrit débute par une description de la physiopathologie de l'insuffisance rénale aiguë (IRA) d'origine septique. L'implication des **phénomènes hémodynamiques** est tout d'abord discutée, accompagnée de deux publications de notre équipe. La première décrit les résistances vasculaires rénales mesurées par échographie doppler chez des patients admis en réanimation pour un sepsis. La seconde présente les résultats originaux d'une étude menée récemment ; elle analyse l'intérêt d'optimiser la pression de perfusion rénale pour améliorer la filtration glomérulaire en cas d'IRA dans ce contexte clinique.

Nous discutons ensuite la part respective des phénomènes d'ischémie/reperfusion (I/R) et de l'**inflammation** dans la physiopathologie de l'IRA, illustrés par une publication décrivant l'intérêt clinique des biomarqueurs des lésions tubulaires rénales. Les **plaquettes sanguines** pourraient être potentiellement impliquées dans la constitution de ces lésions d'IRA. En effet, au-delà de leur rôle dans la coagulation, les plaquettes sont maintenant reconnues comme des acteurs de la réaction inflammatoire. Une revue générale des données concernant le **rôle potentiel des plaquettes dans l'IRA associée au sepsis** est présentée.

Les mécanismes moléculaires en cause lors de la constitution de l'IRA d'origine septique restent cependant à élucider. Dans le schéma général du rôle des plaquettes sanguines dans l'inflammation, notre travail s'est focalisé sur le **CD154** et de son récepteur le **CD40**. Les plaquettes sanguines sont en effet une source importante de CD154. La mise en évidence de l'expression du CD154 (ou CD40-ligand) par les plaquettes et de son rôle clé dans la réaction inflammatoire, en particulier celle concernant l'endothélium vasculaire, nous ont conduit à nous intéresser à son implication dans la constitution des lésions d'IRA. Une revue générale des données concernant le **CD154 plaquettaire** est exposée, laquelle permet de positionner les principales fonctions du CD154 plaquettaire et son rôle en pathologie inflammatoire.

Notre hypothèse de travail est qu'un des mécanismes par lesquels le CD154 contribue à l'inflammation tubulaire, passe par l'induction de cytokines pro-inflammatoires. En effet, le CD154 est un inducteur important de la production de cytokines pro-inflammatoires par tout un ensemble de cellules, dont les cellules endothéliales et les cellules épithéliales des tubules rénaux. Notre travail expérimental est alors présenté. Il décrit le **rôle du CD154 sur la sécrétion d'interleukine-6 (IL-6) par les cellules tubulaires rénales**. Cette étude a été effectuée dans des conditions hypoxiques, en raison de l'importance grandissante qui est donné à l'association inflammation/hypoxie, comme rencontré dans les lésions tubulaires de l'IRA. Cette étude a été conduite *in vitro* mais aussi abordée *in vivo* grâce à un modèle murin d'I/R rénale que nous avons développé et appliqué à des souris déficientes en CD154 et en CD40. Nous discuterons nos résultats, ainsi que nos perspectives de recherche.

1. Introduction

L'IRA est une maladie fréquente. Elle survient en ville comme à l'hôpital et dans toutes les spécialités médicales et chirurgicales. Quelle que soit son étiologie, l'IRA est un facteur reconnu de mauvais pronostic à court et à long terme pouvant engendrer des coûts d'hospitalisation importants. L'incidence de l'IRA augmente régulièrement, elle atteint approximativement 2100 personnes par million d'habitant. Elle touche aux Etats-Unis jusqu'à 7 % des patients hospitalisés, soit plus d'1 million de patients sur un volume total de plus de 36 millions d'hospitalisations (1,2).

L'IRA est une maladie très fréquente en réanimation. Depuis l'homogénéisation des classifications diagnostiques AKIN, RIFLE puis KDIGO (annexe 1) (3), son incidence est estimée à environ 50% des patients (4). Dans une étude récemment publiée par notre équipe, une IRA touchait dans les 24h suivant leur admission en réanimation 31% des patients (5). **Le sepsis est la cause la plus fréquente d'IRA**, il représente plus de la moitié des cas (6,7). Dans une étude incluant 192980 patients atteints de sepsis sévère provenant de sept États américains, les auteurs soulignaient une incidence de 22%, associée à une mortalité de 38% (8). L'étude SOAP incluant 3147 patients admis dans 198 réanimation européennes mettait en évidence un taux de septicémie de 37% compliquée dans 51% des cas d'une IRA, avec une mortalité en réanimation de 41% (9). L'étude FINNAKI portait sur 2901 patients admis dans 17 réanimations finlandaises (10). Parmi les 918 patients atteints de sepsis sévère, 53% présentaient une IRA selon les critères KDIGO. Enfin dans l'étude VANISH, une IRA survenait chez environ 45% des patients en choc septique et une épuration extra-rénale (EER) était nécessaire dans 30% des cas (11).

L'impact pronostic de l'IRA a été aussi largement souligné au cours de la dernière décennie chez les patients de réanimation. Le paradigme d'une récupération complète de la fonction rénale après un épisode d'IRA a notamment été complètement bouleversé avec la mise en évidence d'un véritable continuum entre IRA et insuffisance rénale chronique (12,13). Un épisode d'IRA augmente non seulement le risque et la gravité d'une atteinte chronique mais peut être directement responsable d'une insuffisance rénale chronique terminale. L'impact sur la santé publique de l'IRA est ainsi une préoccupation croissante (14).

2. Implication de l'altération de la pression de perfusion rénale dans la constitution des insuffisances rénales aiguës

La physiopathologie de l'IRA se compose de phénomènes complexes et souvent intriqués. Les phénomènes d'I/R rénale ont été considérés pendant longtemps comme l'origine de l'atteinte rénale lors des états de choc persistants ou insuffisamment réanimés avec la constitution de lésions de nécrose tubulaire aiguë (NTA).

1.1. Vascularisation rénale

Le débit sanguin rénal (DSR) représente 25% du débit cardiaque soit 600 ml/mn/1,75m^2 pour chaque rein. La vascularisation rénale est complexe, comportant deux réseaux capillaires en série. Le premier, uniquement cortical artério-artériel, correspondant aux capillaires glomérulaires, représente 95% du DSR et joue un rôle prépondérant dans la filtration glomérulaire. Le deuxième, artério-veineux, est à l'origine des *vasa recta* qui peuvent naître directement des artères arquées irriguant la médullaire externe et à un moindre degré la médullaire interne. Se suivent ainsi les capillaires glomérulaires, où règne une pression hydrostatique élevée, contribuant à l'ultrafiltration pour aboutir à la formation de l'urine primitive, les capillaires péritubulaires où règne une pression oncotique très élevée contribuant à la réabsorption puis les capillaires des *vasa recta* où règne une pression osmotique qui peut être la plus élevée de l'organisme, jouant un rôle essentiel dans les mécanismes de concentration de l'urine.

Du fait de son organisation architecturale et de sa vascularisation médullaire, le rein est un organe très sensible aux chutes de la pression partielle en oxygène et aux phénomènes d'I/R. Le débit sanguin médullaire ne représente qu'un quart du DSR et ce bien que les cellules tubulaires aient des besoins énergétiques très importants, liés à la présence de la pompe NA-K-ATPase (15). La disposition des *vasa recta* explique la diffusion de l'oxygène de la branche descendante vers la branche ascendante, avec pour conséquence une diminution progressive du contenu en O2 et une baisse de la pression partielle en oxygène tissulaire au fur et à mesure que l'on s'enfonce dans la médullaire. Cette pression partielle en oxygène y est ainsi de 10 à 20 mmHg contre 50 mmHg au niveau du cortex (16). Les structures tubulaires à hautes contraintes métaboliques sont confrontées à des apports en oxygène limités et sont extrêmement sensibles aux variations de la perfusion rénale. Ceci est contrebalancé par le haut pouvoir d'extraction en oxygène de la partie médullaire du néphron (16,17).

1.2. Autorégulation du DSR

Afin de s'adapter aux variations du DSR, le rein développe des mécanismes de protection par autorégulation :

- Autorégulation intrinsèque, dépendant essentiellement des adaptations des résistances artériolaires post et surtout pré-glomérulaires :

    - La vasodilatation de l'artériole afférente au glomérule et la vasoconstriction de l'artériole efférente, lorsque la pression artérielle systolique reste comprise entre des valeurs variant de 80 à 200 mmHg (18), permet même en cas de baisse de la pression artérielle moyenne (PAM) de maintenir une pression hydrostatique constante au niveau du glomérule et ainsi un débit de filtration glomérulaire (DFG) stable. Cette autorégulation est locale et persiste même en cas de dénervation du rein (19). La vasodilatation est essentiellement secondaire aux prostaglandines et à la production locale de monoxyde d'azote. Les prostaglandines sont synthétisées dans le cortex et la médullaire à partir de l'acide arachidonique par la voie de la cyclo-oxygénase. Les prostaglandines I2, E2, D2 entraînent sur la micro circulation glomérulaire une vasodilatation entraînant une baisse des résistances artériolaires des artérioles afférentes et efférentes responsables d'une augmentation du débit sanguin glomérulaire et d'une augmentation du DFG. Les prostaglandines, en particulier E2, auraient un rôle tonique vasodilatateur sur la circulation des vasa recta médullaires. La vasoconstriction de l'artériole efférente est secondaire à l'activation du système juxta-glomérulaire et à l'action fortement vasoconstrictrice de l'angiotensine II (20,21). L'angiotensine II agit sur 3 niveaux dans le parenchyme rénal :
        - Préférentiellement au niveau de l'artériole efférente. Elle entraîne une vasoconstriction de l'artériole efférente provoquant une chute du débit sanguin glomérulaire avec une augmentation de la pression hydrostatique capillaire glomérulaire et une augmentation de la fraction de filtration.
        - Au niveau des cellules mésangiales glomérulaires qui comportent des récepteurs spécifiques. Ces cellules vont alors se contracter entraînant une baisse du coefficient de filtration glomérulaire
        - L'angiotensine II a également un rôle tonique vasoconstricteur sur la circulation médullaire grâce à la présence de nombreux récepteurs spécifiques de l'angiotensine dans la zone profonde de la médullaire externe.

      Ce rétrocontrôle négatif tubulo-glomérulaire repose sur l'appareil juxta glomérulaire et notamment sur la *macula densa*. Le signal au niveau de la *macula densa* serait une augmentation de la quantité de NaCl et en particulier de chlore arrivant dans le tube contourné distal. Ainsi, une augmentation de la pression de perfusion dans l'artère rénale augmente immédiatement le débit sanguin glomérulaire et de ce fait le DFG. L'augmentation de la réabsorption de NaCl entraîne une vasoconstriction de l'artériole afférente et de ce fait une diminution du débit sanguin glomérulaire et du DFG proche de sa valeur initiale. Un mécanisme inverse intervient en cas de diminution de la pression de perfusion rénale. Ces mécanismes expliquent la grande susceptibilité des patients traités par inhibiteur de l'enzyme de conversion ou par

anti-inflammatoires non stéroïdiens aux baisses de PAM, avec un risque de chute du DFG.

- Le reflexe myogène local est secondaire aux contraintes mécaniques d'étirement de la paroi artérielle lors du pic de pression artérielle systolique (22). Cette réaction met en jeu les cellules musculaires lisses de la paroi et permet rapidement de modifier le tonus vasculaire de l'artériole afférente au glomérule avec comme réponse à l'étirement une vasoconstriction réflexe (23,24). Ce mécanisme est lié à l'ouverture des canaux calciques et à l'entrée de calcium dans la cellule musculaire. Il est contrecarré par les inhibiteurs calciques.

- Autorégulation extrinsèque, qui associe des effets extra rénaux et des effets sur l'hémodynamique intra-rénale et participe à la régulation de la pression artérielle systémique :

    - Une régulation nerveuse existe également due à l'innervation sympathique exclusive des vaisseaux corticaux (principalement noradrénergique, et à un degré moindre dopaminergique). Les terminaisons nerveuses sympathiques se distribuent à la totalité des vaisseaux du cortex, à tous les éléments de l'appareil juxta glomérulaire et aux tubules. L'activation sympathique permet de modifier le tonus vasculaire et ainsi, en situation d'hypovolémie, d'induire une vasoconstriction des vaisseaux glomérulaires (via un effet α-adrénergique direct et par un effet β-adrénergique sur les cellules juxta-glomérulaires) permettant de maintenir une pression de perfusion glomérulaire satisfaisante et donc un DFG stable. Il est à noter que cette vasoconstriction touche à la fois les artérioles pré et post glomérulaires avec un effet plus important sur ces dernières.

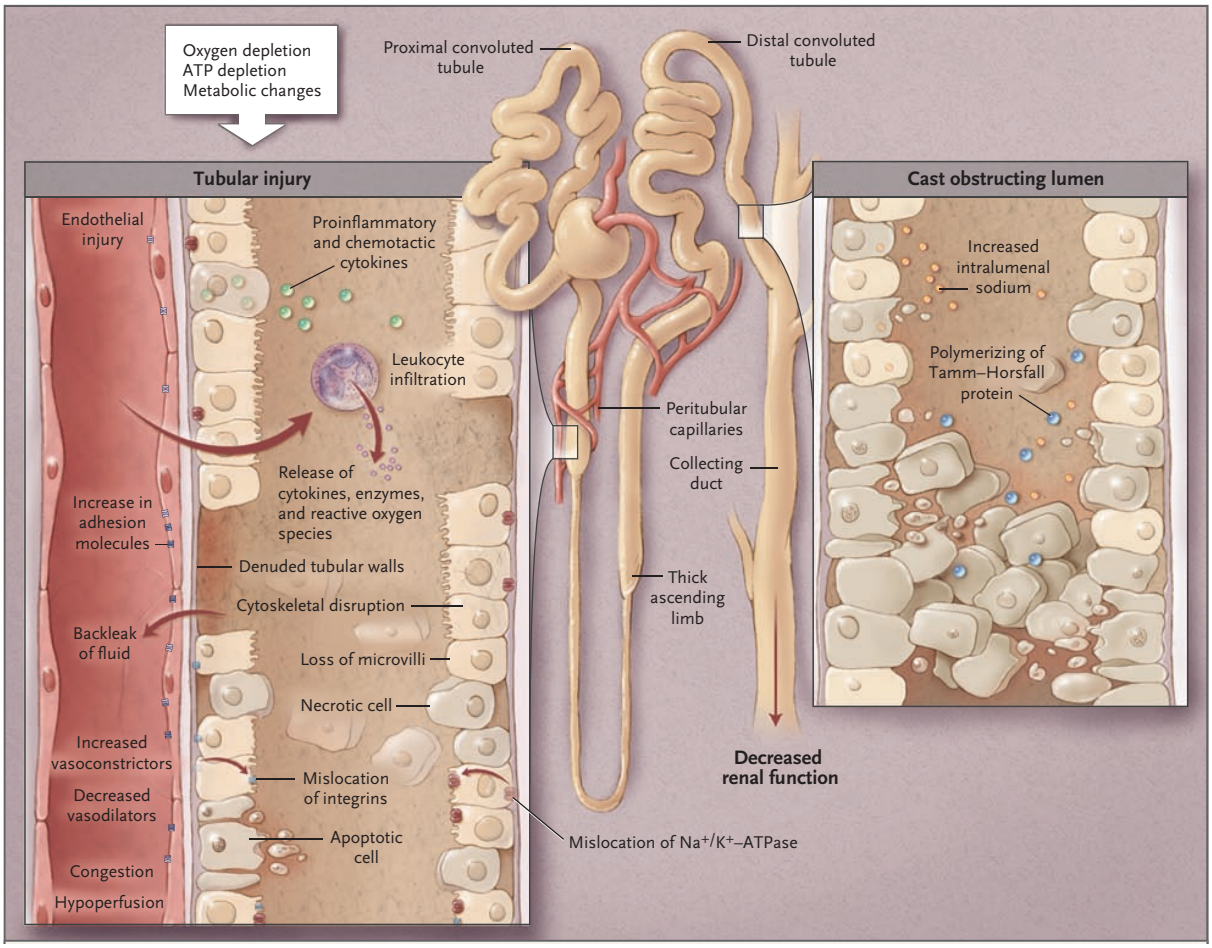
1.3. Physiopathologie « classique » de l'IRA

L'IRA est dite **pré-rénale ou fonctionnelle** dans les cas d'hypoperfusion lorsque les mécanismes cités plus haut sont dépassés. Cette hypoperfusion rénale apparait dans des contextes de troubles hémodynamiques localisés (syndrome cardio-rénal, syndrome hépatorénal) ou systémiques (choc hypovolémique, choc cardiogénique). Initialement, le rein garde son pouvoir de concentration des urines car les fonctions tubulaires ne sont pas altérées. A ce stade, l'atteinte est réversible en cas d'amélioration de la perfusion rénale.

Si l'hypoperfusion se prolonge, ou en cas de néphrotoxicité associée (produit de contraste iodé, médicaments néphrotoxiques), peuvent apparaître des signes de souffrance des cellules tubulaires. Ces phénomènes sont considérés comme responsables des lésions de NTA, majorés, lors de la reperfusion du rein, par la production de radicaux libres dérivés de l'oxygène (25). Les cellules tubulaires vont également perdre leur pouvoir de concentration des urines avec rétrodiffusion de l'urine primitive par perte des jonctions serrées aggravant les phénomènes inflammatoires. La nécrose de ces cellules serait la principale lésion anatomique puisque les vaisseaux, glomérules et interstitium restent intacts. Ces lésions s'accompagnent d'une accumulation intra-tubulaire de débris cellulaires nécrotiques

et de cellules desquamées responsable d'une obstruction de la lumière du tubule (figure 1). Le recrutement de médiateurs vasoactifs et pro-inflammatoires entretient les phénomènes de vasoconstriction intra-rénale pré et post-glomérulaire avec une diminution constante et durable du flux sanguin rénal. Une atteinte de la membrane basale tubulaire est également observée. Elle est responsable d'une rétrodiffusion tubulaire de l'ultrafiltrat, majorant l'œdème interstitiel et la réduction du calibre des capillaires et des tubules. L'arrêt de la filtration glomérulaire est le résultat d'une augmentation de la pression intra-tubulaire par obstruction des tubules rénaux et d'une baisse de la pression dans le capillaire glomérulaire secondaire à une altération de la microcirculation rénale. Du fait de la capacité de régénération des cellules tubulaires, ce phénomène est considéré comme réversible. Cependant, une réparation incomplète est possible, particulièrement en cas d'insuffisance rénale chronique, et constituerait le substrat d'une progression vers l'insuffisance rénale chronique (26). Dans un rein antérieurement sain, les cellules épithéliales tubulaires (CET) viables se divisent, se dédifférencient, migrent vers la membrane basale dénudée, prolifèrent puis se différencient pour rétablir l'intégrité et la fonction des tubules. Ce phénomène résulte en un retour à une fonction rénale normale sans lésion résiduelle. En cas de processus incomplet, il persiste une réaction inflammatoire tubulo-interstitielle et une prolifération inadaptée de fibroblastes responsable d'une fibrose locale par dépôt extracellulaire d'une matrice de collagène.

Figure 1 : Physiopathologie de l'IRA (27)



1.4. L'hémodynamique systémique est-elle le seul déterminant de la perfusion rénale ? Présentation d'un article clinique : « Intérêt de l'index de résistance vasculaire rénal comme reflet de la régulation du tonus vasculaire rénale dans l'IRA d'origine septique » (28)

L'index de résistance vasculaire rénale (IR) mesuré par échographie Doppler au niveau des artères interlobaires rénales (par application de la sonde en région lombaire) compare l'importance relative de la vitesse diastolique en comparaison à la vitesse maximale systolique. L'IR est un reflet des conditions hémodynamiques lié au niveau de résistance circulatoire dans le territoire d'aval. Il permettrait en pratique clinique d'estimer la pression de perfusion rénale. Une augmentation des résistances vasculaires rénales associée à une baisse du débit sanguin peut en effet contribuer à l'ischémie rénale (29,30). L'interprétation de ce paramètre reste cependant difficile. Chez des patients présentant un choc septique, l'augmentation de la PAM par l'administration de médicaments vasopresseurs (le plus souvent de la noradrénaline) peut être associée à une diminution des résistances vasculaires rénales (31). En revanche, certains auteurs ont rapporté que l'IR n'était pas influencé par les traitements vasoactifs (32,33). Nous présentons ici une étude clinique dont le but était d'évaluer la relation entre la PAM et les résistances vasculaires rénales étudiées par échographie doppler chez des patients de réanimation septiques souffrant ou non d'IRA.

96 patients étaient inclus dans cette étude observationnelle. Une IRA était définie selon les critères RIFLE (**annexe 1**) et son caractère transitoire ou persistant selon la récupération de la fonction rénale dans un délai de 3 jours. Les résultats de ce travail confirmaient les données de la littérature mettant en évidence des IR élevés chez tous les patients souffrant d'une IRA. Il est à noter que nous n'observions pas d'altération du débit cardiaque dans ce contexte d'IRA d'origine septique. Il n'y avait pas de différence d'IR selon le caractère transitoire ou persistant de l'IRA ainsi que chez les patients traités par vasoconstricteur (noradrénaline), sans corrélation avec la dose reçue. Il existait en revanche une faible corrélation entre l'IR et la PAM, la pression artérielle en oxygène et l'âge uniquement chez les patients ne souffrant pas d'IRA.

Ces résultats suggèrent que les mécanismes d'autorégulation du DSR sont préservés chez les patients septiques sans atteinte rénale, même en cas d'administration de noradrénaline. **En revanche en cas d'IRA septique, les déterminants de l'IR semblent multiples et complexes et ils ne sont pas uniquement la conséquence de variations de l'hémodynamique systémique, pouvant laissant supposer d'autres déterminants**. L'optimisation de l'hémodynamique en réanimation par l'administration de noradrénaline risque donc d'être insuffisante pour améliorer la fonction rénale lorsque les lésions tubulaires sont déjà installées, et doit être mis en balance avec les effets indésirables d'une vasoconstriction excessive.

Lorsque les patients constituent une IRA dans un contexte septique de réanimation, une atteinte dite fonctionnelle de la fonction rénale coexiste probablement avec des lésions tubulaires déjà installées au niveau des néphrons les plus sensible au stress hypoxique, qui ne récupèreront pas avec l'amélioration de la perfusion. Le niveau

d’atteinte rénale dépend ainsi du capital néphronique de chaque patient (13). Pour minimiser l’atteinte rénale, il est recommandé de maintenir des niveaux de PAM supérieurs à 60-65 mmHg en cas de choc vasoplégique (34). Mais ce niveau de PAM pourrait être insuffisant, et Derrudre et al avait suggéré que la mesure de l’IR pourrait aider à optimiser la pression de perfusion rénale individuellement (31). Notre travail a évidemment un certain nombre de limites, inhérentes à ce type d’étude observationnelle, dont les principales sont l’absence d’estimation du syndrome inflammatoire biologique, du DFG, ou de la microcirculation rénale. Mais cette étude souligne que l’estimation de la pression de perfusion rénale par la mesure du RI peut être hasardeuse chez des patients de réanimation en IRA en raison de la multiplicité de ses déterminants. Une recommandation formalisée d’experts a depuis confirmé qu’il ne faut probablement pas utiliser l'IR mesuré par Doppler rénal pour diagnostiquer ou traiter une IRA (35).

CRITICAL CARE

RESEARCH Open Access

Doppler resistive index to reflect regulation of renal vascular tone during sepsis and acute kidney injury

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Abstract

Introduction: Renal resistive index (RI), determined by Doppler ultrasonography, directly reveals and quantifies modifications in renal vascular resistance. The aim of this study was to evaluate if mean arterial pressure (MAP) is determinant of renal RI in septic, critically ill patients suffering or not from acute kidney injury (AKI).

Methods: This prospective observational study included 96 patients. AKI was defined according to RIFLE criteria and transient or persistent AKI according to renal recovery within 3 days.

Results: Median renal RIs were 0.72 (0.68-0.75) in patients without AKI and 0.76 (0.72-0.80) in patients with AKI (P=0.001). RIs were 0.75 (0.72-0.79) in transient AKI and 0.77 (0.70-0.80) in persistent AKI (P=0.84). RI did not differ in patients given norepinephrine infusion and was not correlated with norepinephrine dose. RI was correlated with MAP (ρ= -0.47; P=0.002), PaO_2/FiO_2 ratio (ρ= -0.33; P=0.04) and age (ρ=0.35; P=0.015) only in patients without AKI.

Conclusions: A poor correlation between renal RI and MAP, age, or PaO_2/FiO_2 ratio was found in septic and critically ill patients without AKI compared to patients with AKI. These findings suggest that determinants of RI are multiple. Renal circulatory response to sepsis estimated by Doppler ultrasonography cannot reliably be predicted simply from changes in systemic hemodynamics. As many factors influence its value, the interest in a single RI measurement at ICU admission to determine optimal MAP remains uncertain.

Introduction

In critically ill patients, sepsis and acute kidney injury (AKI) are very common diseases and are associated with increased hospitalization and elevated in-hospital mortality rates [1,2]. AKI is a dynamic process that evolves from an early reversible condition to an established disease and leads to sustained renal impairment, cell death, and delayed renal recovery [3,4]. Consequently, prompt resuscitation of the circulation and optimal perfusion pressure are the primary therapies for critically ill patients with AKI. These methods are based principally on the appropriate management of intravenous fluid replacement and vasopressor administration under strict hemodynamic monitoring [5].

To date, the pathogenesis of septic AKI is not completely known [6]. An increase in renal vascular resistance (RVR), associated with reduced renal blood flow (RBF) and, thus, potential renal ischemia has been reported [7,8]. Conversely, some experimental studies have demonstrated that septic AKI may be associated with renal vasodilatation and increased RBF [9,10]. Renal resistive index (RI), determined by Doppler ultrasonography, allows an estimation of RVR at the bedside. Although this parameter provides interesting information, its interpretation remains difficult in clinical practice. In patients with septic shock, the norepinephrine (NE)-induced increase in mean arterial pressure (MAP) from 65 to 75 mm Hg is associated with significant decreases in RVR and RI [11]. In contrast, some authors have reported that RI was not influenced by vasoactive drug therapy [12,13]. Furthermore, a recent publication reported that increased RI was associated with persistent AKI in

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critically ill patients who require mechanical ventilation [12]. We tested the hypothesis that the relationship between renal RI and MAP may be influenced by renal injury. The primary objective of this study was to evaluate whether MAP is a determinant of renal RI in septic and critically ill patients who do or do not have AKI.

Materials and methods

Setting and patients

Approval for this non-interventional study was obtained from our institutional review board (Comité de Protection des Personnes Sud-Ouest et Outre Mer III, agreement number DC2009/43, Bordeaux, France). Because data were collected while the care of patients conformed to standard procedures currently used in our institute, authorization to waive informed consent for the study was granted. From January 2010 to February 2011, patients admitted to our 22-bed mixed medical and surgical intensive care unit (ICU) from less than 24 hours for proven or suspected infection with severe sepsis or shock according to the American College of Chest Physicians/Society of Critical Care Medicine classification [14] were prospectively included. Non-inclusion criteria were pregnancy, age of less than 18 years, known renal artery stenosis, known end-stage renal disease or chronic kidney disease defined by a glomerular filtration rate (GFR) of less than 30 mL/minute per 1.73 m^2, angiotensin-converting enzyme inhibitor and non-steroidal anti-inflammatory treatment, cirrhosis with hepatorenal syndrome, and a major cardiogenic component in septic shock defined as a need for epinephrine or dobutamine or both. Patients presenting obstructive acute renal failures, assessed by echography, were also excluded.

Study protocol

Each patient was studied within the first 24 hours after ICU admission and after obtaining hemodynamic stabilization defined as an MAP of greater than 60 mm Hg for more than 1 hour without any change in the rate of catecholamine infusion or fluid vascular loading. Variation in pulse pressure by arterial catheterization (radial or femoral) was used as a trigger for fluid challenge. NE was the only vasopressor used in this study. Owing to the observational nature of the study, the optimum MAP was left to the discretion of the attending physicians. Fluid balance on inclusion day was calculated by hourly recording of all intravenous fluid intakes (including colloids, crystalloids, total parenteral nutrition, or blood products) and urinary or digestive output from each patient. The ventilator respiratory settings and sedative infusion rates were unchanged for at least 1 hour before Doppler sonography. Sedation was given according to individual needs as assessed by the Behavioral Pain Scale [15] and the Sedation-Agitation Scale [16]. Renal replacement therapy was initiated only in patients in the Failure stage of Risk, Injury, Failure, Loss, and End-stage kidney disease (RIFLE).

Transthoracic echocardiography and renal echocardiography were systematically performed by intensivists certified in echocardiography. A Vivid-i ultrasound machine with a 4-MHz curved-array multifrequency transducer was used (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The cardiac output by using the Doppler method at the level of the left ventricular outflow tract and renal perfusion indices were measured [17]. Intrarenal Doppler signals were obtained from two or three representative proximal interlobar arteries. The peak systolic velocity (V_{max}) and the minimal diastolic velocity (V_{min}) were determined by pulse wave Doppler, and the RI was calculated as $(V_{max} - V_{min})/V_{max}$ [13]. The results from three consecutive measurements on both kidneys were averaged. The reproducibility of RI measurements was assessed in 20 patients by two investigators (AD and BM).

Definitions

The Simplified Acute Physiology Score II (SAPS II) and the Sepsis-related Organ Failure Assessment score were assessed to evaluate the severity of disease of each patient at inclusion in the study. AKI was evaluated at inclusion and was defined by using the consensus definition from the Acute Dialysis Quality Initiative group, which proposes criteria for three grades of increasing severity (risk of acute renal failure, injury to the kidney, and failure of kidney function) and two outcome classes (loss of kidney function and end-stage kidney disease) (RIFLE classification). In the absence of known baseline serum creatinine (sCr) levels, the nadir of sCr after renal recovery was recorded to evaluate baseline renal function. In the absence of renal recovery, baseline sCr was estimated by using the Modification of Diet in Renal Disease (MDRD) formula. Transient AKI was defined as AKI that resolved within 3 days after inclusion by using conventional treatment in the ICU. Recovery from AKI was defined as a 50% decrease in sCr or normalization of urine output in the absence of diuretics or both. Persistent AKI was defined as persistent elevated sCr or oliguria (less than 0.5 mL/kg per hour) for at least 3 days [18-21].

Statistical analyses

Continuous variables were presented as mean (standard deviation) and median (interquartile range) for non-Gaussian variables and were compared by the Mann-Whitney *U* test. Categorical variables were presented as number (percentage) and were compared by the Pearson chi-squared test or Fisher exact test. Comparisons between more than two groups were made by using Kruskal-Wallis one-way analysis of variance with *post*

hoc analysis. Intra-observer reliability and inter-observer reliability of measurements were determined by the corresponding intraclass correlation coefficients and their 95% confidence intervals (CIs) [22]. Inter-observer measurements were compared by a Bland and Altman analysis. Correlation tests were performed by using the Spearman correlation coefficient. A logistic regression was performed to identify variables significantly associated with an RI of less than 0.75. All tests were two-sided, and a *P* value of less than 0.05 was considered statistically significant. Statistical analysis was carried out with JMP for Windows, version 6.0 (SAS Institute Inc., Cary, NC, USA).

Results

Ninety-six patients were prospectively screened during the study period. Poor-quality images from two patients made Doppler measurements for any lobar arteries impossible. Consequently, 94 patients were included in the statistical analyses (Table 1). Doppler measurements could be obtained from only one kidney in 15 patients. In this case, the right kidney has been preferentially observed (11 patients out of 15). For the 79 patients in whom bilateral Doppler could be performed, matched *t* test comparisons of right and left RI measurements showed no significant differences: 0.73 (95% CI 0.70 to 0.76) versus 0.73 (95% CI 0.70 to 0.75). Intra-observer variation was 3.4%, and the intraclass correlation coefficient was 0.94 (95% CI 0.92 to 0.96). Inter-observer variation was 6.2%, and the intraclass correlation coefficient was 0.92 (95% CI 0.81 to –0.97). The mean bias of inter-observer measurements was 0.00 (95% CI –0.10 to 0.09). RI was not correlated with cardiovascular risk factors and was correlated only with age in patients without AKI (ρ = 0.35; *P* = 0.015). RI was higher in patients presenting with an arterial partial pressure of oxygen/fraction of inspired oxygen (PaO_2/FiO_2) ratio of less than 200: 0.76 (0.72 to 0.80) versus 0.72 (0.68 to 0.78) (*P* = 0.02).

For patients with transient AKI, diagnosis was made at Risk, Injury, or Failure levels in 42%, 33%, and 25% of cases, respectively. For patients with persistent AKI, diagnosis was made at Risk, Injury, or Failure levels in 7%, 11%, and 82% of cases, respectively. Median renal RIs were 0.72 (0.68 to 0.75) in patients without AKI and 0.76 (0.72 to 0.80) in patients with AKI (*P* = 0.001) (Table 2). Median RIs were 0.75 (0.72 to 0.79) in cases of transient AKI and 0.77 (0.70 to 0.80) in cases of persistent AKI (*P* = 0.84).

RI did not differ between patients who received or did not receive NE - respectively, 0.73 (0.69 to 0.78) versus 0.75 (0.68 to 0.78) (*P* = 0.94) - and was not correlated with the NE dose (ρ = -0.06; *P* = 0.69). Among patients without AKI, RIs were 0.73 (0.68 to 0.76) in patients who received NE and 0.70 (0.68 to 0.75) in patients who did not receive NE (*P* = 0.30) and were also not correlated with NE dose (ρ = 0.20; *P* = 0.43).

Table 1 Characteristics of patients included in the study (n = 94)

Variables	
Male gender, mean (standard deviation)	59 (63)
Age in years, median (interquartile range)	62 (17)
Body mass index in kg/m^2, median (interquartile range)	25 (22-28)
SAPS II at inclusion, median (interquartile range)	43 (33-59)
SOFA score at inclusion, median (interquartile range)	7 (3-9)
Mechanical ventilation, number (percentage)	60 (64)
ICU length of stay in days, mean (standard deviation)	14 (13)
Patients receiving norepinephrine, number (percentage)	47 (50)
Norepinephrine dose in μg/kg per minute, mean (standard deviation)	0.37 (0.34)
Cardiovascular risk factors, number (percentage)	
Tobacco	35 (37)
Hypertension	47 (50)
Diabetes	12 (13)
Elevated cholesterol	21 (22)
Obesity and physical inactivity	9 (10)
Origin of sepsis, number (percentage)	
Abdominal	55 (59)
Pulmonary	26 (28)
Mediastinitis	6 (6)
Catheter	2 (2)
Other	5 (5)
Diuretic use, number (percentage)	20 (21)
Acute kidney injury, number (percentage)	52 (55)
Transient	24 (25)
Persistent	28 (30)
Renal replacement therapy, number (percentage)	20 (21)
28-day all-cause mortality, number (percentage)	18 (20)

ICU, intensive care unit; SAPS II, Simplified Acute Physiology Score II; SOFA, Sepsis-related Organ Failure Assessment.

Among patients without AKI, RI was not correlated with fluid balance on the day of inclusion (ρ = 0.21, *P* = 0.18) or with the cardiac index (ρ = -0.21; *P* = 0.37) but was significantly correlated with the MAP (ρ = -0.47; *P* = 0.002) (Figure 1) and the PaO_2/FiO_2 ratio (ρ = -0.33; *P* = 0.04). Among patients with AKI, RI was not correlated with fluid balance (ρ = -0.15, *P* = 0.29), the cardiac index (ρ = -0.21; *P* = 0.37), the MAP (ρ = 0.006; *P* = 0.97) (Figure 2), or the PaO_2/FiO_2 ratio (ρ = -0.23; *P* = 0.11). In cases of transient or persistent AKI, RI was not correlated with the MAP (ρ = 0.009, *P* = 0.99, and ρ = -0.0003, *P* = 0.99, respectively). When patients were divided into three groups according to MAP, RI was only inversely proportional to MAP in patients without AKI (*P* = 0.04) (Table 3). In contrast, in cases of AKI, RI remained elevated regardless of the MAP range. Logistic regression, using a univariate analysis, indicated

Table 2 Characteristics of patients according to acute kidney injury

	No AKI (n = 42)	Transient AKI (n = 24)	Persistent AKI (n = 28)	*P* value
Mean arterial pressure, mm Hg	78 (67-86)	70 (65-87)	70 (65-87)	0.4
Heart rate, beats per minute	92 (78-105)	90 (83-120)	96 (79-117)	0.5
Fluid balance on inclusion day, mL	1,585 (837-2,612)[a]	1,725 (1,205-3,002)	2,650 (1,556-3,487)[b]	0.01
Norepinephrine dose, μg/kg per minute	0.18 (0.06-0.37)[a]	0.29 (0.11-0.45)[a]	0.48 (0.20-0.78)[b,c]	0.01
Cardiac index, L/minute per m^2	3.1 (2.3-4.6)	3.3 (2.3-3.8)	3.1 (2.4-4.2)	1
Blood lactate, mEq/L	1.2 (0.9-2.0)[a]	1.5 (1.0-2.9)	2.4 (1.3-3.9)[b]	0.02
Hematocrit, percentage	30 (27-33)	31 (28-38)	29 (25-32)	0.3
PaO_2/FiO_2 ratio	240 (140-320)	240 (140-305)	180 (120-260)	0.1
Serum creatinine at inclusion, μmol/L	69 (45-81)[a,c]	160 (98-196)[a,b]	191 (134-243)[b,c]	<0.0001
FeNa, percentage	0.5 (0.2-1)[a,c]	1.5 (0.6-2.4)[b]	2.7 (0.6-6.7)[b]	0.008
FeU, percentage	28 (16-39)	36 (31-45)	31 (12-46)	0.2
Resistance index	0.72 (0.68-0.75)[a,c]	0.75 (0.72-0.79)[b]	0.77 (0.70-0.80)[b]	0.005

Results are expressed as median (interquartile range). FeNa, fractional excretion of sodium, was calculated as [(urine sodium/plasma sodium)/(urine creatinine/plasma creatinine)] × 100. FeU, fractional excretion of urea, was calculated as [(urine urea/plasma urea)/(urine creatinine/plasma creatinine)] × 100. *P* value refers to between-group comparisons (Kruskal-Wallis one-way analysis of variance with *post hoc* analysis). [a]*P* <0.05 compared with persistent acute kidney injury (AKI) group. [b]*P* <0.05 compared with no AKI group. [c]*P* <0.05 compared with transient AKI group. PaO_2/FiO_2, arterial partial pressure of oxygen/fraction of inspired oxygen.

that the factors that predicted an RI of greater than 0.75 in patients without AKI were the MAP ($P = 0.02$) and SAPS II ($P = 0.002$).

Discussion

In this study, renal RI was evaluated in critically ill patients with sepsis after hemodynamic stabilization. A poor correlation between RI and MAP, age, or PaO_2/FiO_2 ratio was found in septic and critically ill patients without AKI compared with patients with AKI. Our understanding of the pathogenesis of septic AKI is limited because currently we do not have a clinically validated method to measure RBF. In patients with renal hypoperfusion, compensatory mechanisms to maintain GFR include attenuation of afferent arteriolar vasoconstriction, afferent arteriolar dilation, efferent arteriolar constriction, and neuro-/hormonal effects to increase tubular reabsorption of fluid and maintenance of cardiac output [18]. An increase in RVR, representing renal vasoconstriction and a reduced RBF leading to renal ischemia, has repeatedly been proposed to be

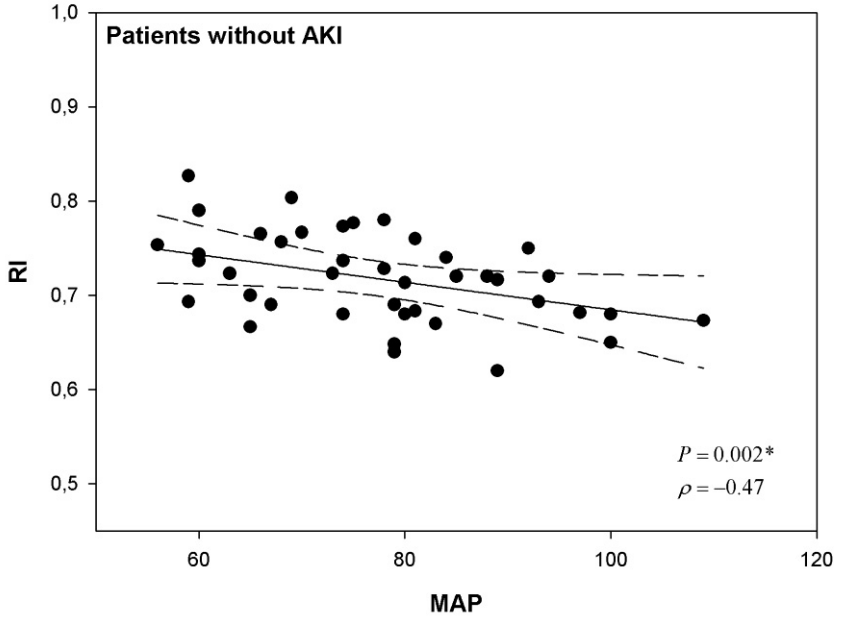

Figure 1 Relationship between resistive index (RI) and mean arterial pressure (MAP) in patients without acute kidney injury (AKI). Correlations were assessed by using Spearman correlation coefficient.

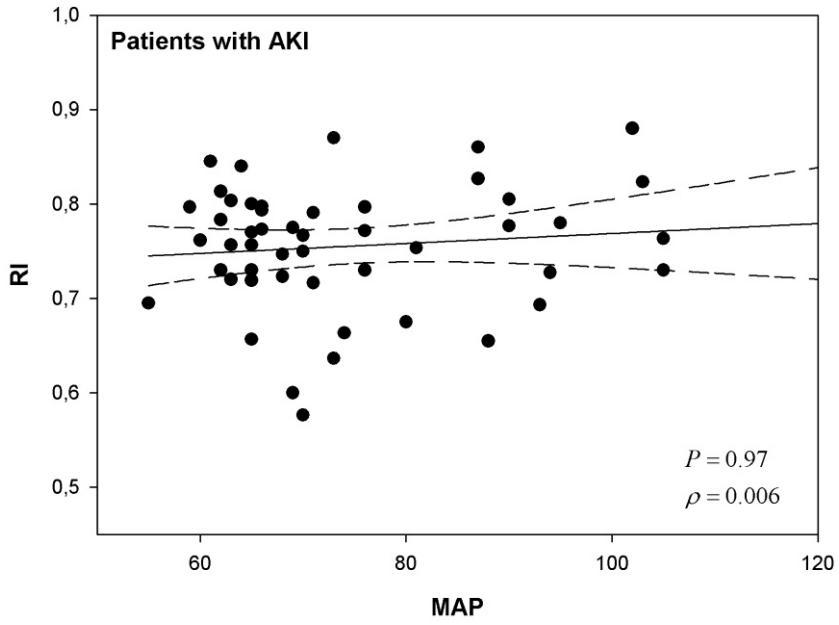

Figure 2 Relationship between resistive index (RI) and mean arterial pressure (MAP) in patients with acute kidney injury (AKI). Correlations were assessed by using Spearman correlation coefficient.

central to the pathogenesis of septic acute renal failure [7,8]. This paradigm has been derived from animal studies, using a variety of models, and from techniques for the measurement of RBF and for induction of sepsis without hemodynamic support [23]. However, many studies do not report decreased RBF in sepsis. Shortly after induced septic shock in ewes without significant fluid resuscitation or NE administration, Langenberg and colleagues [10] reported marked renal vasodilatation, which led to a transient increase in RBF. In parallel, sCr significantly increased after 24 hours whereas fractional excretion of sodium and fractional excretion of urea decreased in the

Table 3 Characteristics of patients according to mean arterial pressure

	<70 mm Hg	70 to 85 mm Hg	>85 mm Hg	*P* value
No acute kidney injury	(n = 13)	(n = 19)	(n = 10)	
Mean arterial pressure, mm Hg	63 (59-66)[a,b]	79 (74-81)[b,c]	93 (89-100)[a,c]	<0.001
Heart rate, beats per minute	88 (77-92)[a]	98 (90-115)[b,c]	90 (64-96)[a]	0.026
Fluid balance on inclusion day, mL	1,400 (865-2,735)	1,680 (990-2,560)	1,237 (699-2,376)	0.7
Norepinephrine infusion, number (percentage)	6 (46)	9 (47)	3 (30)	0.6
Norepinephrine dose, μg/kg per minute	0.20 (0.06-0.38)	0.19 (0.05-0.36)	0.17 (0.06-0.47)	0.9
Cardiac index, L/minute per m^2	2.6 (1.7-3.0)	4.1 (3.1-7.8)	2.7 (1.9-5.0)	0.1
PaO_2/FiO_2 ratio	280 (116-310)	240 (107-302)	229 (193-395)	0.4
Resistance index	0.74 (0.70-0.78)	0.72 (0.68-0.77)	0.69 (0.67-0.72)[c]	0.04
Acute kidney injury	(n = 25)	(n = 14)	(n = 13)	
Mean arterial pressure, mm Hg	65 (62-66)[a,b]	73 (70-76)[b,c]	94 (89-104)[a,c]	<0.001
Heart rate, beats per minute	95 (83-124)	90 (82-117)	89 (79-112)	0.7
Fluid balance on inclusion day, mL	2,605 (1,303-3,767)	2,650 (2,184-3,481)	1,480 (1,105-2,400)	0.1
Norepinephrine infusion, number (percentage)	16 (64)	10 (71)	3 (23)	0.02
Norepinephrine dose, μg/kg per minute	0.20 (0.06-0.38)	0.19 (0.05-0.36)	0.17 (0.06-0.47)	0.9
Cardiac index, L/minute per m^2	2.8 (2.4-3.6)	3.0 (1.8-5.2)	3.7 (3.1-5.9)	0.4
PaO_2/FiO_2 ratio	140 (110-270)	252 (180-310)	180 (134-270)	0.06
Resistance index	0.76 (0.73.80)	0.74 (0.66-0.78)	0.78 (0.73-0.82)	0.1

Results are expressed as median (interquartile range). *P* value refers to between-group comparisons (Kruskal-Wallis one-way analysis of variance with *post hoc* analysis). [a]*P* <0.05 compared with mean arterial pressure (MAP) 70-85 mm Hg group. [b]*P* <0.05 compared with MAP >85 mm Hg group. [c]*P* <0.05 compared with MAP <70 mm Hg group. PaO_2/FiO_2, arterial partial pressure of oxygen/fraction of inspired oxygen.

first 48 hours (as seen in pre-renal azotemia). In human sepsis, sustained systemic vasodilatation with a high cardiac output is the dominant clinical finding during the early phase of AKI [24,25].

Determination of increased RI on the first day of septic shock is known to have predictive value for acute renal failure in septic shock [13]. Darmon and colleagues [12] recently confirmed these results in patients who required mechanical ventilation. A Doppler renal RI value of more than 0.795 also predicted persistent AKI with good sensitivity and specificity. Our study has confirmed that RI is increased in cases of AKI, particularly in cases of persistent AKI. However, we were not able to precisely distinguish transient from persistent AKI in our selected patients. We assume that hypoperfusion (pre-renal component) and injury (renal component) often occurred at the same time in injured kidneys given that each tubular injury is a continuum from volume responsiveness to necrosis. The definition of pre-renal azotemia has conceptual or diagnostic limitations or both, and a precise renal RI cutoff point to distinguish clinical entities was difficult to determine in this study.

Alterations in renal hemodynamics may be induced by multiple factors in critically ill patients. Improving regional perfusion and inducing excessive vasoconstriction must be balanced when NE therapy is required. Indeed, intra-renal infusion of NE is used to induce AKI in animals, and NE has been shown to decrease RBF in healthy volunteers. In contrast, too low a dose of NE in patients with vasodilatory shock may result in an MAP that is below the limit of renal autoregulatory capacity. Guidelines for hemodynamic support of adult patients with sepsis advocate that vasopressors be titrated to the minimum level required to provide effective organ perfusion [26]. To prevent AKI, maintaining an MAP of at least 60 to 65 mm Hg during vasodilatory shock has also been recommended [27].

Deruddre and colleagues [11] suggested that measuring renal RI could help optimize renal perfusion in critically ill patients. In that study, a significant decrease in renal RI was observed in 11 patients with septic shock when MAP was increased from 65 to 75 mm Hg by NE titration. However, measurements of renal RI remain difficult to interpret in clinical practice. Our findings confirm previous results, showing that RI is dependent on factors apart from NE infusion [12,13]. In patients with acute lung injury, low oxygenation levels have been reported to increase renal RI [28]. In our study, RI was inversely correlated with the PaO_2/FiO_2 ratio in patients without AKI. Recently, Darmon and colleagues [12] also found a significant negative correlation between MAP and RI in critically ill patients receiving mechanical ventilation (see Online Supplementary Material). However, in that study, MAP was not statistically different between patients without AKI and patients with transient or persistent AKI, despite an increasing RI.

Our study has reported that RI is inversely correlated with MAP in septic critically ill patients without AKI but not in patients with AKI. This may have been because of unresponsive renal vasoconstriction or renal vascular obstruction caused by a sustained kidney injury, despite hemodynamic support, which led to increased RI. On the other hand, in septic critically ill patients without AKI, an inverse correlation might reflect increased renal perfusion pressure with associated reduced activation of RVR, reflected by RI, leading to pressure-dependent renal perfusion. Sepsis or sedation could explain this modified renal vasomotor response in our patients. However, this association seems to be relatively poor. Indeed, the correlation coefficient that we found between RI and MAP means that most of the changes in RI lie somewhere other than MAP. Consequently, renal circulatory response to sepsis estimated by Doppler ultrasonography cannot be reliably predicted simply from changes in systemic hemodynamics. This finding is consistent with a recent study showing that the development of septic AKI often seems to be accompanied by an uncoupling between systemic vascular resistance and RVR [29].

The following points need to be considered when assessing the clinical relevance of our study. First, this study was purely observational as it was difficult to perform a blinded study. Second, the present study included a single-center population of patients who had developed vasodilatory shock. Patients exhibiting an important cardiogenic component of septic shock, defined by the need for epinephrine or dobutamine or both, were deliberately excluded. Third, the pressure of the renal interstitium was impossible to measure in clinical practice, and renal Doppler measurements do not reflect microcirculation vessel tone. In addition, measurement of GFR and renal oxygenation by continuous renal vein thermodilution was not conducted, as we were unable to use invasive hemodynamic monitoring in this observational study. No patient included in this study presented clinical intra-abdominal hypertension, but this was not assessed by intravesical measurement. Other confounding factors not identified in this study also may have influenced our results. Finally, the subjective part of renal ultrasound and the precision of measurements, assessed by the inter-observer variation in comparison with the Doppler measurement differences, are the main limitations when determining renal RI.

Although future AKI studies need to determine the impact of early recognition of pressure-dependent renal perfusion on renal recovery, the timing of volume replacement to facilitate early restoration of kidney perfusion and to optimize vasopressor infusion dose can be an important determinant in preventing the progression of AKI and

facilitating recovery. Estimating renal circulatory response to sepsis by Doppler ultrasonography can be hazardous because many factors influence RI value. Increased RI may be associated with low renal perfusion pressure or low oxygenation levels in patients without AKI but may also be associated with sustained renal injury. Thus, its value in septic critically ill patients in the ICU should be interpreted carefully before concluding that there is renal injury or estimating optimal MAP.

Conclusions

A poor correlation between renal RI and MAP found only in septic and critically ill patients without AKI suggests that determinants of RI are numerous. Consequently, renal circulatory response to sepsis estimated by Doppler ultrasonography cannot reliably be predicted simply from changes in systemic hemodynamics. The value of a single RI measurement at ICU admission to determine optimal MAP remains uncertain.

Key messages

- RI, measured by Doppler ultrasonography, is correlated to MAP, age, and PaO_2/FiO_2 ratio only in septic and critically ill patients without AKI.
- RI was increased in cases of AKI, but theses correlations were abolished.
- RI did not differ between patients who received or did not receive NE and was not correlated with the NE dose.
- This correlation between RI and MAP in septic and critically ill patients without AKI is poor, suggesting that the determinants of RI are numerous.
- A single RI measurement at admission of septic critically ill patients to predict persistent AKI or to determine optimal MAP seems insufficient.

Abbreviations

AKI: acute kidney injury; CI: confidence interval; GFR: glomerular filtration rate; ICU: intensive care unit; MAP: mean arterial pressure; NE: norepinephrine; PaO_2/FiO_2: arterial partial pressure of oxygen/fraction of inspired oxygen; RBF: renal blood flow; RI: resistive index; RVR: renal vascular resistance; SAPS II: Simplified Acute Physiology Score II; sCr: serum creatinine.

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Authors' contributions

AD conceived, designed, and coordinated the study. BM and JC helped to collect the clinical data. AO helped to carry out the statistical analysis. OJ-B, CF, HR, JR, GJ, CC and AO helped to critically revise the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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1.5. L'optimisation de la PAM permet-elle d'améliorer le DFG ? Présentation d'un article clinique (en préparation)

Le traitement de la défaillance hémodynamique dans le choc septique repose sur l'association d'un remplissage vasculaire à la phase initiale de la prise en charge et l'administration d'agents vasoconstricteurs. L'objectif de ces mesures est de maintenir un niveau de PAM suffisant pour maintenir une pression de perfusion tissulaire permettant la bonne oxygénation des tissus. Comme abordé précédemment, la relation entre l'objectif de PAM et la survenue d'une IRA au cours du choc septique reste incertaine et le niveau optimal de PAM est toujours débattu. Certaines données suggèrent que des niveaux de PAM élevés (>65mmHg) pourraient diminuer la survenue des IRA (36-38). Une étude prospective multicentrique observationnelle finlandaise publiée en 2014 (étude FINNAKI) incluant 423 patients en sepsis sévère dont 153 présentaient une altération de la fonction rénale dans les premiers cinq jours d'hospitalisation (39) mettant en évidence des niveaux de PAM plus bas chez les patients développant une IRA. La valeur seuil de PAM pour prédire la survenue d'une IRA était ainsi de 73 mmHg. D'anciennes études rétrospectives avançaient déjà ces résultats. En 2005, Varpula *et al.* rapportaient une augmentation de la mortalité avec le temps passé en deçà de 65 mmHg dans une cohorte de 111 patients (40) tandis que Dunser *et al*. ne retrouvaient pas d'amélioration de la survie pour des valeurs de PAM supérieures 70 mmHg dans une étude incluant 290 patients (41). Une autre analyse rétrospective de 274 patients en choc septique rapportait des niveaux de PAM plus élevés (73 vs 66 mmHg) chez les survivants à la 24ème heure (37). Legrand et al. ont également montré qu'une pression artérielle diastolique basse dans les 24 premières heures d'une admission en réanimation était associée à la survenue d'une IRA (42). Quatre études prospectives ont comparé l'effet d'une titration de noradrénaline pour obtenir des niveaux de PAM jusqu'à 90 mmHg. Elles n'ont pas montré d'amélioration des paramètres métaboliques ou de la perfusion tissulaire à ces hauts niveaux de PAM (43-46). Il ne semble cependant pas que des niveaux de PAM aux alentours de 75-90 mmHg soient responsables d'effets indésirables (47-50).

En 2014, l'étude multicentrique et randomisée française SEPSISPAM (51) étudie la mortalité à J28 de près de 800 patients hospitalisés en réanimation pour un choc septique sous deux régimes de PAM différents : 65-70 mmHg vs 80-85 mmHg, obtenue dans les six heures suivant le diagnostic de choc septique. L'étude ne met pas en évidence de différence de mortalité (36,6% dans le groupe sous haut régime de PAM vs 34% dans le groupe à faible régime de PAM, P=0,57). Dans l'analyse en sous-groupe de patients aux antécédents d'hypertension artérielle (HTA), on relève cependant une réduction du nombre de patients doublant leur créatininémie dans le groupe à haut régime de PAM (52% vs 38,9%, P=0,02). Il en est de même pour le recours à l'EER (42,2 % vs 31,7 %, P=0,04).

Nous présentons les résultats originaux d'une étude clinique dont l'objectif était d'évaluer l'effet de hauts régimes de PAM (80-85) sur le DFG de patients en choc septique aux antécédents d'HTA. Nous avons inclus 26 patients, comparés en *cross-over* sur deux périodes consécutives de 6h à haut et à bas régime de PAM. Le DFG était calculé avec la formule de référence UV/P et une formule plus récente, le KeGFR. Les résultats originaux de ce travail sont la mise en évidence d'une amélioration du DGF mesuré par la formule UV/P à des régimes de pression plus

élevés, conséquence principalement d'une augmentation de la diurèse. Le DFG évalué par la formule du KeGFR ainsi que la créatinine urinaire n'étaient pas significativement améliorés à des régimes de PAM plus élevés. Ces résultats suggèrent ainsi qu'une optimisation de la PAM améliore principalement la filtration glomérulaire par le biais de la diurèse et de la natriurèse chez les patients aux antécédents d'HTA, avec un intérêt potentiel pour une meilleure maîtrise de la balance hydrosodée des patients septiques en IRA, mais que ces mesures ne permettent probablement pas de corriger l'atteinte tubulaire. En cas d'atteinte rénale plus sévère, attesté par un DFG inférieur à 30ml/min, le bénéfice de cette majoration de la PAM semble disparaître.

Cette étude présente un certain nombre de limites, là encore inhérentes à son « design » observationnel. La gravité des patients était relativement modérée en comparaison à l'étude SEPSISPAM, notamment en raison de l'exclusion de patients nécessitant d'emblée une EER. L'inclusion des patients après la phase initiale de réanimation et de remplissage vasculaire, indispensable avant de majorer les doses de noradrénaline et le tonus vasoconstricteur, pourrait également minimiser l'effet de cette optimisation de la pression de perfusion rénale. Les méthodes de mesure du DFG en réanimation sont aussi toujours sujettes à discussion. Enfin l'intérêt pronostique de cette optimisation du DFG par des mesures hémodynamiques n'était pas l'objectif de cette étude et ne peut pas être affirmé.

L'atteinte tubulaire en cas d'IRA d'origine septique ne semble ainsi pas améliorée par une simple optimisation de l'hémodynamique systémique, laissant entrevoir le rôle d'autres facteurs physiopathologiques, tels que l'inflammation, le stress oxydatif ou l'atteinte de la microcirculation.

Mean arterial pressure optimization to improve the glomerular filtration rate in septic critically ill patients with acute kidney injury: a case-crossover study

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Abstract:

Background: The optimal mean arterial pressure level (MAP) in case of septic shock is still debated. MAP between 75 and 85 mm Hg may reduce the risk for patients with chronic arterial hypertension to develop an acute kidney injury (AKI). The main objective of this study was to assess the value of high MAP level on the GFR in critically septic patients with prior hypertension.

Methods:

This case-crossover study included 26 consecutive patients in two intensive care units (ICU) in the first 24h of a septic shock and after hemodynamic stabilization and fluid loading. Data were collected during 2 distinct 6-hours periods of MAP regimen: a low MAP target (65-70mmHg) and a high MAP target (80-85mmHg). Glomerular filtration rate (GFR) was calculated with the UV/P and the KeGFR formulas and compared.

Results:

High MAP level resulted in a diuresis and natriuresis increase and a serum creatinine (sCr) decrease without significant uCr evolution and serious adverse effects. The GFR assessed by the UV/P formula increased from 35 [22-61] mL/mn to 49 [24-88] mL/mn (P=0.001). This increase depended on the level of AKI at inclusion. The concordance between the UV/P and the KeGFR formulas to estimate the GFR was low, without significant change in the KeGFR.

Conclusions:

Diuresis is a determinant parameter for the GFR improvement assessed by the UV/P formula. The tubular function may not been improved by higher MAP regimen. The prognostic benefit of higher MAP target in septic patients with prior hypertension to optimize the diuresis and natriuresis requires further investigations.

Keywords:
Acute Kidney Injury, Septic Shock, Intensive Care, Blood Pressure, Norepinephrine

Introduction

Acute kidney injury (AKI) is a common clinical problem in intensive care units (ICU) with over 50% of patients in the ICU developing stage 1 AKI. AKI remains associated with a poor outcome, a prolonged stay in ICU, a high cost of treatment, and long term deterioration of the quality of life with a risk for chronic kidney failure following hospitalization [1]. There have not been any specific measures targeted to the kidney that have been successful and management strategies continue to reflect supportive measures focusing on fluid delivery, diuretics, avoidance of nephrotoxic agents and RRT for approximately 10% of patients [2].

Sepsis is a well recognized cause of AKI in ICU [3]. One of the main treatments of AKI in this setting, apart from the etiological management, is the optimization of blood pressure to maintain the kidney perfusion pressure and therefore the glomerular filtration rate (GFR). This is made possible with vascular fillings and pressor amines such as norepinephrine. As prolonged hypotension increases mortality, The Surviving Sepsis Campaign guidelines [4] recommend that mean arterial pressure (MAP) should be targeted above 65 mmHg. However, the MAP target to optimize the GFR remains debated [5-9] and a MAP of around 75 to 85 mm Hg may reduce the risk for patients with chronic arterial hypertension to develop acute kidney injury [10].

The main objective of this study was to assess the value of high level of MAP on the GFR in critically septic patients with a history of chronic hypertension.

Patients and methods

Patients and Setting

Approval was obtained from our institutional review board (Comité de Protection des Personnes Sud-Ouest et Outre Mer III, France, number of agreement DC 2016/81). All patients agreed to participate. If patients were unable to receive this information, it was given to their family. During a 12-month study period (August 2016–July 2017), we included consecutive patients with a history of chronic hypertension diagnosed with AKI in the first 24 hours following the onset of a septic shock and after hemodynamic stabilization in two ICU's of the Bordeaux University Hospital: one medical and surgical ICU (thoracic or abdominal surgery) and one medical ICU. Non-inclusion criteria were pregnancy, patients aged ≤ 18 years, obstructive renal disease, known end-stage renal disease, or severe chronic kidney disease defined by a glomerular-filtration rate (GFR) of < 30 mL/min/1.73 m^2, RRT or anuria at the time of inclusion or a presumed life expectancy of < 24h.

The attending physician treated the selected cohort according to international recommendations and standards of care. Antibiotics were administered within the first hours after admission. Patients were included after the initial management including a fluid challenge to achieve a minimum of 30 mL/kg of crystalloids. Fluid administration was continued if there was hemodynamic improvement based on dynamic criteria (eg, change in pulse pressure, stroke volume variation). The hemodynamic was monitored in all patients using an arterial line and echocardiography. According to the surviving sepsis campaign recommendations [4], the administration of norepinephrine targeted a minimum MAP as low as 65 mmHg. As a MAP of 65 mmHg might be too low in this cohort of patients, and following the guidelines specifying that "*optimal MAP should be individualized as it may be higher*

in patients with atherosclerosis and/or previous hypertension than in young patients without cardiovascular comorbidity", the optimal MAP was titled by the attending physician. Patients were studied during two consecutive periods of MAP target, a high-target period with a MAP of either 80 to 85 mm Hg and a low-target period with a MAP of either 65 to 70 mm Hg *(figure 1).*

Definitions

Patients were defined as chronic hypertensive if there was at least one antihypertensive treatment (calcic inhibitor, angiotensin converting enzyme inhibitor (ACEI), beta-blockers or thiazide diuretic) in their usual medical treatment.

Sepsis was defined by a life-threatening organ dysfunction caused by the inappropriate host to an infection with an increase of SOFA (Sequential Organ Failure Assessment) of at least 2 points [11]. Septic shock was defined by a persisting hypotension requiring vasopressors to maintain MAP ≥65 mmHg and a serum lactate level >2 mmol/L (18 mg/dL) despite adequate volume resuscitation. Hemodynamic stabilization was defined by stable or decreasing dose of norepinephrine for a period of 3 hours

AKI was defined according to the KDIGO classification as urine volume <0.5 mL/kg/h for 6h, a serum creatinine (sCr) increase of ≥0.35mg/dL within 48h, or a sCr increase of 1.5 times the baseline value, which was known to have occurred in the previous 7 days [12]. The baseline sCr level was obtained by calling the referring doctor or by analyzing the patient's medical record.

Data collection

Data was collected for each patient during the two different periods of MAP target

proposed by the attending physician. All data was consecutively collected during 6 hours for each MAP target and indifferently during the escalation or the de-escalation of the MAP target. Thanks to our patient monitoring software (Metavision, iMDSoft), all variables were continuously recorded and averaged, including the MAP, fluids administration and norepinephrine dose. SCr and urine samples were collected at each change in MAP target. Urine samples were collected on one hour worth of urine. Adverse events of high dose of norepinephrine were also researched by analyzing supraventricular arrhythmias events.

GFR calculation

As Cockroft and Gault, MDRD, CKD-EPI equations to evaluate kidney function cannot be applied in the ICU setting, the estimated GFR (eGFR) was calculated as recommended from the sCr, the urinary creatinine (uCr) and the urine output using the formula UV/P [13], where U is the creatinine urine concentration, V the volume of urine, and P the sCr concentration. A new GFR formula proposed by Chen *et al*. [14] [15] estimating the kinetic eGFR (KeGFR) was also proposed and calculated as follow:

$$KeGFR = \frac{Baseline\ sCr \times baseline\ GFR}{Mean\ sCr}\ X\ \left(1 - \frac{24\ X\ \Delta sCr}{\Delta Time \times Max\Delta sCr/Day}\right)$$

Baseline GFR was calculated using the CKD-EPI equation in order to avoid overestimation in undernourished patients [16]. For the patients without reference value, an inverted formula was applied with an arbitrary GFR at 75 mL/mn/1.73m^2 [17]. Baseline sCr and GFR reflect the creatinine production rate of each patient. The

mean sCr corresponds to the average of sCr during a delta of time (Δtime) (which was 6h in our study). ΔsCr corresponds to the sCr variation during this period. The maximal increase in sCr per day that could occur if renal function was completely lost (MaxΔsCr /Day) was 1.5 mg/dl (0,425 mg/dl per 6 hours), as described by Chen [14]. Variations of GFR have been analyzed before and at each change in MAP target using both formulas.

Statistical Analysis

The sample size of the study was computed to achieve 90% power in showing a 10% eGFR variation during each MAP target, determining that a minimum, determining that a minimum of 15 patients was required. Quantitative parameters are reported as their medians and interquartile ranges, and qualitative parameters are expressed as numbers and percentages. Categorical variables were compared using the $\chi 2$ test or Fisher's exact test, when appropriate. Continuous variables were compared using the Mann–Whitney U test. Matched sets of variables were compared with the Wilcoxon's signed-rank test. A two-factor analysis of variance for repeated measurements (ANOVA) was performed to assess the GFR variation during the high and low MAP target according to the initial GFR at the time of inclusion assessed by the UV/P formula (below or above 30ml/min). Correlations between GFR calculations were analyzed using the Spearman's correlation coefficient (ρ). Concordance between GFR was evaluated using the Bland and Altman method. Bias was defined as the mean difference between measurements, whereas precision was defined as the mean absolute value of this difference. The limits of agreement were calculated as the bias ± 1.96 SD. All tests were two-sided, and a P-value of <0.05 was considered statistically significant. Statistical analysis was performed using SAS version 9.3

(SAS Institute, Cary, NC) and GraphPad Prism version 6.00 (GraphPad Software, La Jolla California USA).

Results

Study Population

We included 26 patients in the study. Main patients' characteristics are described in table 1. Data were consecutively collected for 12 patients during the escalation of the MAP target and for 14 patients during the de-escalation of the MAP target. According to our inclusion criteria, all patients presented an AKI at inclusion with a KDIGO 2 stage in half of the cases and all patients had chronic hypertension before hospitalization, treated by a calcic inhibitor (50%), an angiotensin converting enzyme inhibitor (39%), beta-blockers (42%) or thiazide diuretics (8%). The sCr baseline was 71 [53-90] µmol/L and the baseline GFR, calculated according to the CKD-EPI formula, was 92 [75-104] mL/mn/1,73m^2. The fluid balance was largely positive before the introduction of norepinephrine with an input-output balance of 46 [45-74] ml/Kg.

MAP was significantly different between the two distinct periods of study (P<0.001). During the six-hour period targeting a low MAP level, the MAP was 69 [67-75] mmHg. During the six-hour period targeting a high MAP level, the MAP was 85 [82-88] mmHg (*Figure 2*). To obtain a high MAP target, patients had a significantly greater need for norepinephrine (0.3 [0.13-0.5] µg/kg/mn vs 0.5 [0.33-0.6] µg/kg/mn; P<0.001). There was no difference in the fluid balance during the two MAP target period 5[3-10] ml vs 6 [4-13] ml/Kg in the low and high MAP target respectively, P=0.7).

GFR evolution according to the MAP target

The eGFR calculated with the UV/P formula increases with the rise of the MAP from 35 [22-61] mL/mn during the low-target period to 49 [24-88] mL/mn during the high-target period (P=0.001) (table 2). This increase was not retrieved when GFR was estimated using the KeGFR formula with a value ranging from 42 [26-62] mL/mn/1,73m^2 to 40 [16-78] mL/mn/1,73m^2 (P=0.93) during the low and the high-target period respectively. The MAP increase was associated with an improvement in the diuresis (0.7 [0.3-1] mL/kg/h to 0.9 [0.4-1.4] mL/kg/h; P=0.05), in natriuresis (30 [22-55] mmol/L to 48 [29-71] mmol/L; P=0.04) and a significant sCr decrease (128 [84-192] µmol/L to 126 [76-196] µmol/L; P=0.008) *(figure 3)*. However, the proteinuria was persistent without albuminuria (1 [0.7-1.5] g/L to 1.2 [0.6-1.5] g/L; P=0.43), and without change in urinary osmolarity (408 [350-420] mOsm/L to 410 [359-486] mOsm/L; P=0.75).

GFR comparison

Spearman's correlation coefficient (ρ) between eGFR and keGFR was 0.71 (P<0.001). The analysis of the concordance of the two formulas used for the estimation of GFR via Bland Altman method finds a bias at 1.3 mL/mn. The limits of agreement were very wide [-53 to 55 mL/mn] (*Figure 3*).

Interaction of the AKI level on the eGFR variation during MAP optimization

The eGFR at the time of inclusion estimated by the UV/P formula interacted on the eGFR variation during the two MAP target period. Patients with an eGFR below 30 mL/mn at inclusion presented a mean eGFR variation of 10 (+/-22) ml/min during the

high MAP target period vs 37 (+/-62) ml/mn for patients with an eGFR at inclusion above 30ml/m (P=0.04).

Adverse events of high norepinephrine infusion rate

The use of a higher dose of norepinephrine to obtain a higher level of MAP was not at the origin of a more important rate of adverse events. Seven patients (27%) presented with rhythm disorders in the type of atrial fibrillation and those regardless of MAP level (P=1). In our study, no patient suffered from acute coronary events or cutaneous necrosis (troponin =0.06 [0.005-0.145] ng/mL vs 0.03 [0-0.11] ng/mL for the two periods of MAP; P=0.42). Similarly, lactate levels and pH remained no different (1.4 [1.1-1,2] mmol/L vs 1.4 [1-1.9] mmol/L; P=0.7 and 7.40 [7.32-7.44] vs 7.39 [7.33-7.44] respectively; P=0.13).

Discussion

The main findings of our study is that a higher MAP target after fluid optimization may be beneficial on the renal filtration function, with a significant diuresis and natriuresis increase and a significant sCr decrease for septic critically ill patients with chronic hypertension. The improvement of GFR by higher MAP level, assessed by the UV/P formula, depends on the level of AKI at inclusion. The concordance between the UV/P and the KeGFR formulas to estimate the GFR was low, without significant change in the KeGFR. Diuresis is then a determinant parameter for this GFR improvement and the tubular function does not seem to improve in higher MAP regimen of MAP, as confirmed by the uCr stability.

Several studies investigated the effects of MAP level on AKI. In a retrospective cohort study of 274 septic patients, Dünser et al. [5] showed a linear association between the time below 60 mmHg during the first 24h after ICU admission and 28-day mortality while the need for RRT was highest when MAP was below 75 mmHg. More recently, Legrand et al. [8] showed that diastolic arterial pressure during the first 24h after ICU admission was significantly lower in patients developing an AKI. However in a post hoc analysis of 290 patients of a multicenter trial in which MAP was maintained above 70 mmHg during shock, Dünser et al. [7] showed that a MAP ≥ 70 mm Hg was not associated with increased mortality, but elevating MAP above 70 mmHg by increasing vasopressor infusion rates was associated to more disease-related events and increased 28-day mortality. Badin et al. [9] later suggested that a MAP between 72 and 82 mmHg could be necessary to avoid AKI in patients with septic shock and initial renal function impairment. The FINNAKY study including 423 septic patients also confirmed that patients who developed an AKI had lower MAP levels [18]. In this study, the MAP threshold value for predicting the occurrence of

AKI was 73 mmHg. In the SEPSISPAM trial [10], 778 patients with septic shock were stratified according to previous hypertension history and were treated with "*low*" (65–70 mmHg) versus "*high*" (80–85 mmHg) MAP target. In patients with previous hypertension treated with a higher MAP target, there was significantly less renal failure—as defined by the doubling of plasma creatinine (38.8 versus 52.0%, $p < 0.05$)—and less requirement for RRT between day 1 to day 7 (31.7 versus 42.2%, $p < 0.05$). Conversely, for patients without prior hypertension, there was no benefit to increase MAP target. However there was no difference for 28-day mortality, whatever the MAP group, and the occurrence of de novo atrial fibrillation was more frequent in the group treated with a higher MAP. Our study completes these results by demonstrating that a high MAP target after fluid optimization may be beneficial on glomerular filtration for patients with prior hypertension, leading to a significant diuresis increase and a significant sCr decrease. Another important parameter noticed in our study to predict an improvement in the glomerular filtration when MAP increases is the GFR at inclusion. Indeed, patients with a GFR above 30 mL/mn at inclusion better improved their GFR. These results may be the consequence of more severe renal lesions with tubular damage for patients with a GFR below 30 mL/mn at inclusion that cannot be improved by the optimization of renal perfusion.

Concerning the estimation of the GFR in critically ill patients, the recommended formula is UV/P [13]. This formula presents the advantage of involving diuresis on a period and thus can be used in absence of the stable state on the opposite of other equations (Cockroft ang Gault, MDRD, CKD-EPI) [19]. It is nevertheless subject to the same limitations than those equations, notably the unpredictable character of tubular secretion of creatinine, representing 10 to 40% of its urinary elimination. Many drugs used in ICU can inhibit tubular creatinine secretion, but only one patient

in our study received this type of treatment (cilastatine). As the relative increase in sCr depends on the patient's baseline sCr in case ok AKI [19], the KeGFR formula developed by Chen *et al*. includes this value and its corresponding clearance, with an endogenous production of creatinine a priori stable for a same patient whether with AKI or not [14]. To our knowledge, few studies have evaluated this formula, the KeGFR only better predicting the short-term regression of AKI in comparison with other biomarkers (NGAL and [TIMP-2]*[IGFBP7]) in 57 ICU patients [15]. However our study revealed a poor concordance between these two formulas. One of the explanations is certainly the absence of integration of diuresis in the KeGFR calculation, as in our study the sCr and the uCr increase during the high MAP target period. Furthermore this formula includes the MaxΔsCr/Day corresponding to the maximum sCr increase that can occur per day under consideration that renal function is totally lost. Current literature has shown that in the case of an AKI with anuria, the maximum increase in daily sCr was 1 to 1.5 mg/dL. By convention, Chen *et al.* recommends using a value of 1.5 mg/dL, but it may not be true for all patients. However the KeGFR may better reflect the tubular impairment in case of AKI as we only found in our study a slight and non-significant decreased in UCr for higher MAP regimen.

One of the strength of our study is the case-crossover design, which allows us to compare two distinct periods of evaluation in each patient with a limited number of inclusions. However this study has several limits. The first limit is the patient's severity, which was moderately low compared to the SEPSISPAM study. The patients were also included after the onset of the septic shock, as the fluid loading was first optimized during the early resuscitation period. The lactate level was then

already corrected at inclusion. The method of measurement of the sCr et uCr could also be subjected to criticism. In our laboratory, sCr is measured via the Jaffe method with a correction of -18 µmol/L corresponding to the non-creatine chromatogen calibrated by IDMS (Isotope Dilution Mass Spectrometry). Since all samples were taken at the Bordeaux Hospital, there was no inter-laboratory variability. Concerning the intra-laboratory variability, the coefficient of the automata variation is less than 2,5% and thus satisfy the SFBC (Société Française de Biologie Clinique) standards.

Despite a GFR improvement during a higher MAP target, its prognostic advantage could be questioned as in the SEPSISPAM study mortality was similar under the two different regimens of MAP [10]. The significant improvement of diuresis and urinary sodium demonstrated by this study could be theoretically an advantage to control the hydric balance of critically ill patients and reduce the number of patients requiring a RRT for patients with chronic hypertension. However the pathophysiological mechanisms of sepsis-induced acute renal failure are still a matter of debate. It is now well documented that AKI can occur during hyperdynamic sepsis despite increased total renal blood flow, suggesting that other mechanisms are involved [20, 21]. Several mechanisms are therefore likely to lead to sepsis-induced renal dysfunction, including hypoperfusion, venous congestion, microcirculation alterations, but also mechanisms independent of hemodynamic impairments, like inflammation and oxidative stress. Consequently, the effect of higher MAP target in septic critically ill patient with prior hypertension to reduce tubular lesions needs further investigations.

Conclusion

A higher MAP regimen significantly improved the GFR, assessed by the UV/P formula, the diuresis and the urinary sodium without causing serious adverse effects in septic critically ill patients with prior hypertension. This improvement depended on the level of AKI at inclusion. However the GFR assessed by the KeGFR formula did not change under the two different regimens of MAP, reflecting the importance of the diuresis, and suggesting that the tubular function did not change. The prognostic benefit of higher MAP target in septic hypertensive patients to optimize the diuresis and natriuresis requires further investigations.

Figure legends:

Figure 1: Study design

Figure 2: MAP according to the norepinephrine infusion rate during the high MAP target period (A) and the low MAP target period (B)

Figure 3: Serum creatinine (A), urine creatinine (B), diuresis and urinary sodium variations during the low and high MAP target periods

Figure 4: Concordance of UV/P and KeGFR formulas for estimation of GFR

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Table 1 : Patients 'characteristics

	n= 26
Characteristics at admission	
Age (year)	69 [61-75]
Male	21 (80%)
Size (cm)	170 [165-175]
Weight (kg)	80 [65-94]
Body Mass Index	27 [24-32]
Medical/Surgical patients	12 (46%)/14 (54%)
Comorbidity	
Hypertension	26 (100)
Coronaropathy	7 (27)
Valvular disease	3 (12)
Rhythmic disease	8 (31)
Heart Failure	4 (15)
Peripheral arterial disease	1 (4)
Ischemic stroke	1 (4)
Chronic kidney failure	4 (15)
Respiratory disease	8 (31)
Diabetes mellitus	10 (39)
Thromboembolic disease	1 (4)
Hepatic disease	1 (4)
Neoplastic disease	10 (39)
Smoke	7 (27)
Antihypertensive treatment	
Calcic inhibitor	13 (50)
Angiotensin converting enzyme inhibitor	10 (39)
Beta-blockers	11 (42)
Thiazide diuretic	2 (8)
SOFA	9 [7-13]
IGS2	52 [36-62]
Serum lactate level (mmol/L)	2.7 [2.1-3.1]
Prior kidney function	
Baseline sCr (µmol/L)	71 [53-90]
Baseline GFR* (mL/mn/1,73m^2)	92 [75-104]
Characteristics at inclusion	
KDIGO	
1	9 (35)
2	13 (50)
3	4 (15)
Fluid balance (mL/kg)	46 [45-74]
MAP (mm Hg)	71 [65-72]
Time between norepinephrine initiation and inclusion (hours)	19 [12-42]
Serum lactate levels (mmol/L)	1.5 [1.1-2.8]
Nephrotoxic treatment	24 (92)
Evolution	
Renal replacement therapy	6 (24)
ICU, length of stay (days)	15 [13-33]
Hospital, length of stay (days)	30 [25-48]
ICU Mortality	10 (43)
Hospital Mortality	11 (50)

Data are expressed as their medians [interquartile range] or number (%).
*calculated using the CKD-EPI formula

Table 2: Evolution of blood and urine parameters according MAP level

	Inclusion	Low MAP target period	High MAP target period	P
MAP (mmHg)	71 [65-72]	69 [67-75]	85 [82-88]	< 0.001
Norepinephrine infusion rate (µg/kg/mn)	0.3 [0.2-0.5]	0.3 [0.13-0.5]	0.5 [0.33-0.6]	< 0.001
sCr (µmol/L)	124 [75-192]	128 [84-192]	126 [76-196]	0.008
uCr (mmol/L)	5.5 [3.5-9.3]	5.9 [3.2-9.7]	5.7 [3.3-13.6]	0.06
Diuresis (mL/kg/h)	0.6 [0.3-1]	0.7 [0.3-1]	0.9 [0.4-1.4]	0.04
Proteinuria (g/L)	1.2 [0.5-1.6]	1 [0.7-1.5]	1.6 [0.6-1.5]	0.4
Albuminuria (g/L)	0.05 [0.01-0.3]	0.04 [0.02-0.09]	0.04 [0.01-0.2]	1
Urinary osmolarity (mOsm/L)	420 [353-466]	408 [350-420]	410 [359-486]	0.7
Urinary sodium (mmol/L)	37 [20-57]	30 [22-55]	48 [29-71]	0.04
KeGFR (mL/mn/1,73m^2)	NA	42 [26-62]	40 [16-78]	0.9
eGFR (mL/mn)	35 [22-61]	35 [22-61]	49 [24-88]	0.001

Data are expressed as their medians [interquartile ranges].

Figure 1:

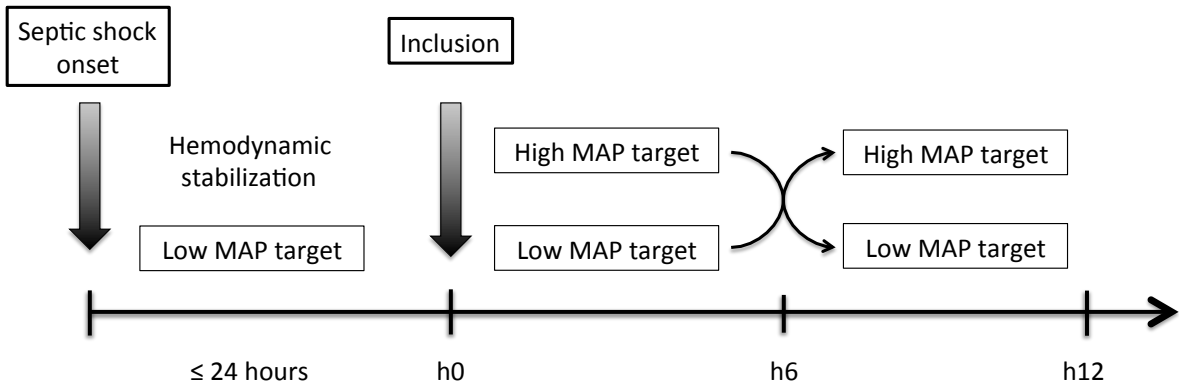


Figure 2:

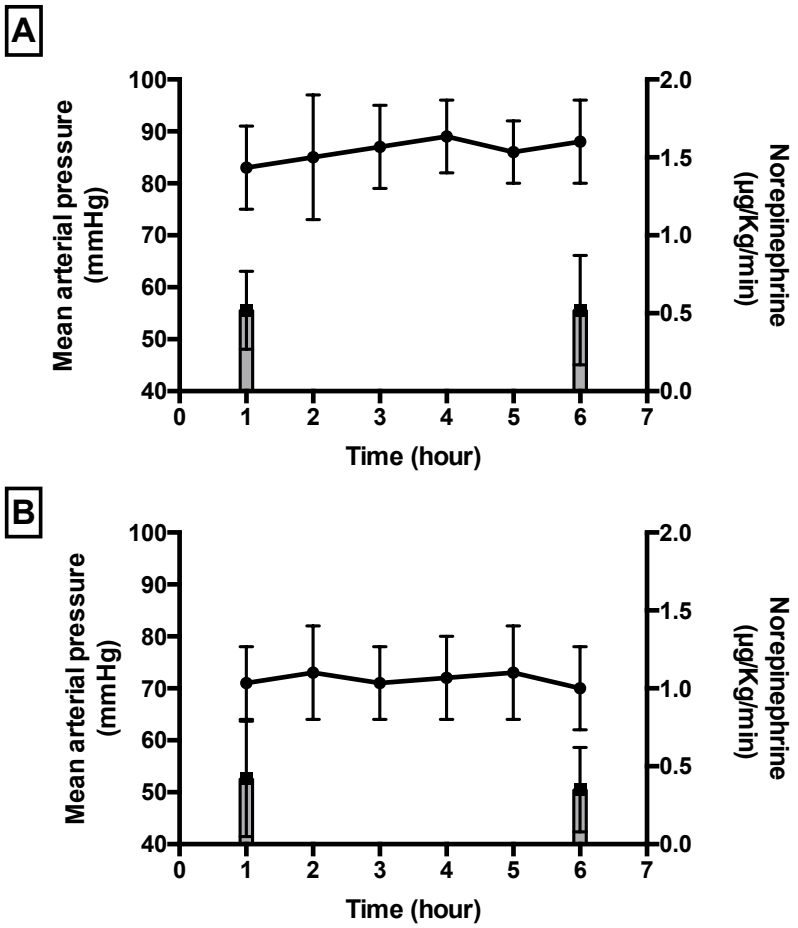
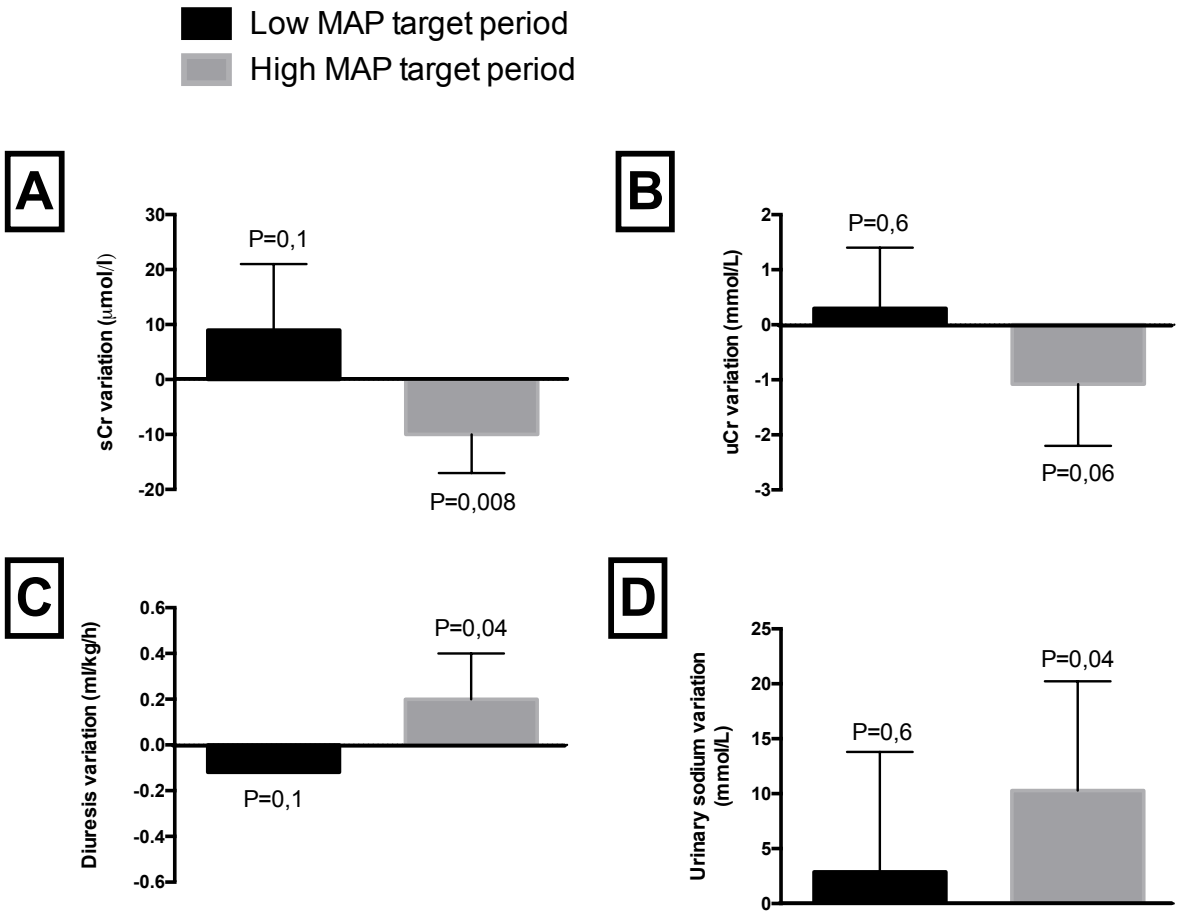


Figure 3:



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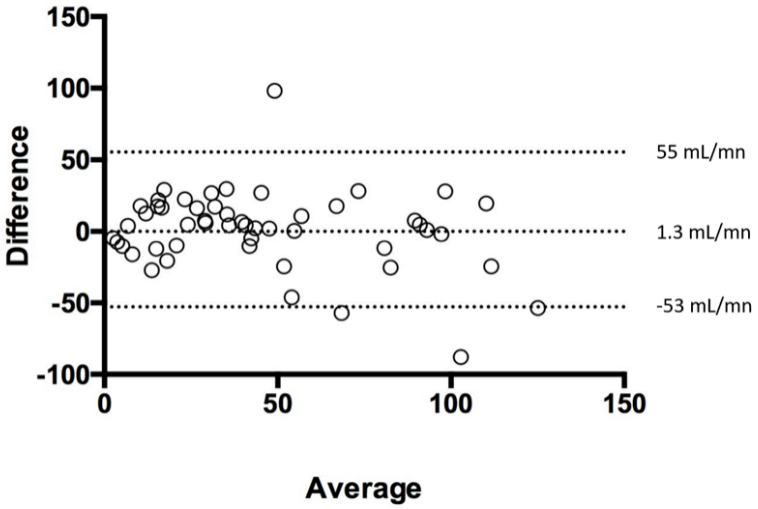

2. Nouvelles théories physiopathologiques de l'IRA associée au sepsis

La NTA a été considérée pendant longtemps comme la lésion à l'origine de la dysfonction rénale observée en pratique clinique. Plusieurs études cadavériques (52,53) et expérimentales (54) sont cependant venues contredire ce concept dans l'IRA d'origine septique en ne mettant que rarement en évidence des lésions de NTA. L'analyse anatomopathologique révèle en effet dans ce contexte une atteinte tubulaire discrète, localisée, avec infiltration leucocytaire ou signes d'apoptose, mais la plupart des néphrons restent normaux.

2.1. Rôle de la microcirculation rénale

Les modèles expérimentaux d'I/R par clampage de l'aorte ou de l'artère rénale permettent d'induire une IRA par chute de la pression de perfusion rénale accompagnée d'une augmentation des résistances vasculaires rénales (55). Ces situations d'I/R rénale « pures » sont cependant relativement peu fréquentes chez l'homme, se résumant à la transplantation rénale ou à la chirurgie vasculaire avec clampage aortique. En revanche ces phénomènes d'I/R se retrouvent dans de nombreuses situations cliniques, comme les états de choc, les détresses respiratoires avec hypoxémie, l'arrêt cardiaque réanimé, la chirurgie cardiaque...

La mise au point de modèles animaux d'IRA septique permettant des mesures sophistiquées et invasives du DSR et de la pression de perfusion corticale et médullaire a récemment révélé que le seul concept d'atteinte macrocirculatoire était insuffisant pour expliquer l'apparition d'une IRA. Les premières études expérimentales mettaient pourtant en évidence une chute progressive du DSR après administration d'endotoxines et constitution d'un état de choc (30). Mais des travaux plus récents proposant des modèles expérimentaux de sepsis hyperkinétique (avec augmentation du débit cardiaque en réponse à la chute des résistances vasculaires systémiques induite par le sepsis), plus proches de la clinique humaine, ont démontré qu'une IRA peut se développer en présence d'un DSR augmenté (56-58). Un modèle ovin d'IRA septique étudiant simultanément la fonction rénale et le DSR a ainsi permis d'obtenir des biopsies rénales séquentielles sur 48 h (59). La consommation en oxygène et le DSR restaient inchangées lors de la constitution de l'IRA et la seule anomalie histologique observée en microscopie électronique était une légère prolifération mésangiale. En dépit de cet hyperdébit sanguin rénal, une oligurie et une IRA se développent en quelques heures dans ces modèles, phénomène également observé chez l'homme (60).

Cette dissociation entre DFG et DSR pourrait s'expliquer en partie par une redistribution du débit sanguin intra-rénal. Une vasodilatation prédominante sur l'artère efférente peut en effet expliquer une chute du DFG par la baisse de la pression de filtration glomérulaire. Mais c'est surtout la microcirculation rénale qui serait un élément déterminant de la constitution des lésions puis de la récupération tubulaires, d'autant qu'elle se situe à l'interface des cellules endothéliales et immunitaires (61). Ainsi, malgré l'augmentation du DSR, des phénomènes ischémiques peuvent toujours intervenir (27). Plusieurs données expérimentales

objectivent une redistribution du flux sanguin de la médullaire rénale vers le cortex avec réduction de la pression partielle en oxygène médullaire (62-64). Ces modifications de la distribution sanguine rénale pourrait impliquer l'ouverture de shunts intra-rénaux (65).

2.2. Importance du stress oxydatif et de la dysfonction endothéliale dans les altérations de la microcirculation rénale

L'influence des radicaux libres d'azote semble également déterminante. L'oxyde nitrique (NO) joue un rôle primordial dans la genèse des anomalies microcirculatoires au cours du sepsis. L'augmentation de la concentration de NO induite par l'activation de la iNOS rénale (inductible NO synthase) est responsable d'une production accrue de radicaux libres d'azote impliqués dans la formation des lésions cellulaires au niveau des tubules rénaux. Son inhibition sélective atténue également la dysfonction rénale aigue d'origine septique dans des études animales (66). Kalakeche et al ont montré que la reconnaissance des lipopolysaccharides (LPS) par les récepteurs membranaires TLR-4 au niveau des cellules épithéliales situées au niveau du segment S1 du tubule proximal, entrainait l'internalisation du complexe par endocytose et déclenchait la production de radicaux libres dans les cellules épithéliales des segments tubulaires adjacents (S2 et S3) (67).

La dysfonction endothéliale est un autre facteur important de l'altération de la microcirculation. L'activation des différents éléments figurés du sang (en particulier les leucocytes et les plaquettes sanguines) et des cellules endothéliales lors du sepsis génère une diminution importante du flux sanguin capillaire péritubulaire et une augmentation de la perméabilité vasculaire (68). Elle résulte de l'adhésion des cellules circulantes, d'un déséquilibre de la coagulation en faveur d'une action procoagulante et antifibrinolytique, ainsi qu'un déséquilibre entre les déterminants du tonus vasculaire en faveur d'une vasoconstriction. La stagnation du flux sanguin dans ces zones augmenterait le temps de transit des leucocytes et favoriserait le recrutement des polynucléaires neutrophiles, par l'intermédiaire de molécules d'adhésion comme ICAM-1 (Intercellular Adhésion Molecule 1). L'augmentation du temps d'exposition de l'endothélium et des CET aux médiateurs de l'inflammation pourraient amplifier ce stress oxydatif. Ainsi, Wu et al (69) démontrent chez une souris dont l'expression d'ICAM-1 est absente une diminution de l'infiltration des polynucléaires neutrophiles et de la proportion de lésions tubulaires. De même, plusieurs études montrent que les cellules tubulaires où sont concentrés les radicaux libres d'azote et d'oxygène sont situés au contact des capillaires péritubulaires dans lesquels la perfusion est réduite (70,71). Ces témoins du stress oxydatif sont localisés au niveau des vacuoles apicales des CET (72), seule caractéristique histologique uniformément retrouvée dans l'IRA d'origine septique.

2.3. Activation de la réponse inflammation locale et systémique

L'inflammation est un dénominateur commun quelles que soient les causes d'IRA (58,73). La réponse inflammatoire est d'ailleurs importante dans les modèles d'I/R, elle est induite par l'ischémie et exacerbée par la reperfusion (74). La relation entre atteinte des tubules rénaux et processus inflammatoires a fait l'objet de nombreux

travaux récents. Le sang, chez les patients septiques, contient de nombreux médiateurs de l'inflammation de petits poids moléculaire (cytokines, chimiokines, fragments du complément) qui peuvent avoir un effet toxique sur les cellules tubulaires lorsqu'ils sont concentrés dans l'ultrafiltrat. L'IRA d'origine septique est ainsi associée aux marqueurs inflammatoires tels que les cytokines (75,76). Certains médiateurs de l'inflammation peuvent aussi provenir des agents pathogènes et des cellules lésées au cours de l'agression tissulaire. Ils constituent respectivement les PAMPs (Pathogen-Associated Molecular Patterns) et les DAMPs (Damage-Associated Molecular Patterns). Les PAMPs comprennent de nombreuses molécules, qui proviennent soit de la membrane bactérienne tels que le LPS ou le peptidoglycane (PG), soit de la dégradation des pathogènes comme des fragments d'ADN ou d'ARN. Les DAMPs regroupent des molécules aussi diverses que les histones ou des produits d'origine mitochondriale. Ils interagissent avec les cellules tubulaires au niveau de leur pole urinaire (77) mais aussi probablement par l'intermédiaire des capillaires péri-tubulaires (78). Ces motifs moléculaires peuvent notamment interagir avec les récepteurs Toll-like (TLR) sur les cellules tubulaires. L'administration d'antagonistes des TLR peut ainsi atténuer une IRA (78). De plus, les cellules endothéliales et tubulaires rénales expriment des récepteurs cytokiniques et libèrent des molécules pro-inflammatoires capables de recruter des lymphocytes T dans le rein et le sang de patients septiques, pouvant conduisant à une apoptose tubulaire in vitro (78). La plupart des effecteurs cellulaires et solubles de l'immunité innée sont impliqués dans l'IRA ischémique (79). Les CET ne sont pas seulement une cible des médiateurs de l'inflammation, ils sont aussi acteurs de la réponse inflammatoire. Ils expriment alors aussi un certain nombre de médiateurs contribuant à l'auto-amplification de la réponse inflammatoire (74).

2.4. Importance de la réponse adaptative des cellules tubulaires

Une des réponses de la cellule tubulaire rénale à ces situations de stress consiste en la diminution de sa consommation d'énergie. Plusieurs études sur le rongeur modélisant un sepsis par l'injection de LPS bactérien retrouvent une diminution des capacités d'endocytose mais aussi du transport actif transmembranaire des cellules tubulaires (80). La diminution de l'activité du cotransporteur Na/K/2Cl au niveau du tubule proximal pourrait contribuer à la baisse de la filtration glomérulaire, d'autant que l'augmentation de la charge en NaCl délivrée au niveau de la macula densa stimule le rétrocontrôle tubulo-glomérulaire responsable d'une vasoconstriction de l'artère afférente, avec comme conséquence une diminution de la pression hydrostatique dans le glomérule et donc du DFG (81). Les cellules touchées pourraient également déclencher par voie paracrine l'adaptation du métabolisme des cellules environnantes afin de limiter le processus lésionnel, expliquant la présence de cellules saines et lésées au niveau des tubules. Cette adaptation du métabolisme cellulaire offrirait aux tubules rénaux la capacité de recouvrir ensuite leur fonction.

Les cellules tubulaires rénales présentent d'importantes capacités d'autophagie et de mitophagie (82). Ces mécanismes permettent l'élimination et le remplacement des protéines et des organites non fonctionnels, favorisant la survie en condition de stress. Ce phénomène peut être induit, au niveau des cellulaires tubulaires, par différents facteurs comme l'inflammation (74) et le stress oxydatif (83). Il diminuerait la production de radicaux libres. En limitant l'apparition de signaux pro-apoptotiques,

ces mécanismes favoriseraient la survie cellulaire. Au cours du sepsis, une diminution de l'activité de mitophagie est associée à une anomalie du fonctionnement du tubule proximal. A l'inverse, l'induction de l'autophagie contribue à maintenir une fonction rénale normale (84,85).

Cette réponse est aussi orchestrée par la mitochondrie. Les mitochondries lésées sont retrouvées fréquemment chez les patients atteints d'IRA d'origine septique (53). Elles stopperaient les activités les plus consommatrices pour favoriser les activités essentielles à sa survie, comme le maintien du potentiel et de l'intégrité membranaires par l'intermédiaire du transport actif transmembranaire des ions (86). La mitochondrie est également impliquée dans la régulation des différentes étapes du cycle cellulaire avant d'aboutir à la mitose. La phase de transition G1-S, fortement consommatrice d'énergie, semble jouer un rôle important au cours du sepsis. Yang et al (87) montrent dans un modèle de sepsis induit par une ligature caecale perforée chez le rongeur, que l'arrêt du cycle lors de la phase G1-S est associé à une dysfonction rénale et que la phase de récupération rénale est associée à la reprise du cycle cellulaire 48h plus tard. Ces capacités remarquables d'adaptation métabolique des cellules tubulaires rénales pourraient expliquer la faible proportion de cellules nécrotiques ou apoptotiques visualisée lors d'analyse histologique de reins de patients souffrant d'IRA.

Ces découvertes ont aussi permis l'émergence de nouveaux biomarqueurs d'IRA, dit « lésionnels », en comparaison aux marqueurs de la filtration glomérulaire utilisés classiquement, comme la créatinine plasmatique. Un des biomarqueurs le plus prometteur à l'heure actuelle est la combinaison de deux médiateurs impliqués dans l'arrêt du cycle à la phase G1-S, TIMP-2 (tissue inhibitor of metalloproteinases 2) et IGFBP-7 (insuline-like growth factor-binding protein 7) (88).

2.5. Les nouveaux biomarqueurs de lésions tubulaires permettent-ils d'estimer le niveau d'atteinte rénale et de prédire la récupération d'une IRA ? Présentation d'un article clinique (5)

La recherche de moyen de détection précoce d'une agression rénale est un axe de recherche important (89,90). Dans cette optique, le développement de biomarqueurs rénaux permettant la détection précoce d'agressions rénales aiguës s'est accéléré ces 10 dernières années. Ces biomarqueurs sont essentiellement des protéines synthétisées en cas de lésions tubulaires rénales. La littérature évaluant la pertinence du dosage de biomarqueurs rénaux plasmatiques ou urinaires est ainsi extrêmement riche (91).

Nous présentons ici une étude clinique dont l'objectif était de comparer l'intérêt de certains de ces nouveaux biomarqueurs des lésions tubulaires rénales aux classiques marqueurs de la filtration tubulaire utilisés en pratique clinique. Nous avons ainsi testé la NGAL (Neutrophil gelatinase–associated lipocalin) et la combinaison de TIMP-2 (tissue inhibitor of metalloproteinase-2) et d'IGFBP7 (urine insulin-like growth factor–binding protein 7). Ces biomarqueurs ont pour caractéristique de prédire la survenue d'une IRA avant l'augmentation de la créatinine plasmatique (88,92,93). La plupart des patients admis en réanimation ont déjà été exposés à des événements agressant les tubules rénaux et notre hypothèse

était que ces biomarqueurs permettent de mieux évaluer le niveau d'atteinte rénale à l'admission en réanimation et ainsi de prédire une récupération rapide de la fonction rénale.

Nous avons inclus 57 patients présentant une IRA dans les 24 premières heures d'admission en réanimation. Nos résultats principaux sont qu'effectivement ces biomarqueurs sont capables de prédire une récupération rénale rapide ainsi que les complications associées à une IRA, incluant la nécessité d'une EER. La NGAL atteignait en revanche fréquemment la valeur maximale de dosage proposée par le kit commercial, limitant son intérêt. Ce biomarqueur a depuis été retiré du marché. La performance diagnostique de ces biomarqueurs était en revanche médiocre. L'analyse de la cinétique de décroissance de créatinine ainsi que le calcul du DFG avec la formule du KeGFR avait une performance diagnostique comparable, bien qu'imposant un certain délai.

Ce travail confirme les données de la littérature soulignant l'intérêt de ces biomarqueurs pour évaluer précocement les lésions rénales et leur sévérité, mais aussi la complexité de leur interprétation. Ces différents biomarqueurs traduisent en effet des mécanismes d'agression différents. Leur site de synthèse est différent, tout comme leur cinétique d'apparition après agression et lésion rénale (94). Et malgré l'importante littérature portant sur ces biomarqueurs rénaux, il n'existe pas de travaux mettant en évidence leur utilité clinique chez les patients à risque d'IRA. Les recommandations d'experts portant sur l'IRA en péri-opératoire et en réanimation spécifient d'ailleurs qu'il ne faut pas utiliser les biomarqueurs rénaux pour faire le diagnostic précoce d'IRA (grade faible) (35). Une meilleure compréhension des mécanismes physiopathologiques conduisant à l'IRA s'impose donc toujours avant de généraliser l'utilisation de biomarqueurs d'agression rénale.

3. Implication des plaquettes dans la physiopathologie de l'IRA associée au sepsis

3.1. Plaquettes sanguines et sepsis

Notre compréhension de la physiopathologie du sepsis a considérablement évolué ces dernières décennies, allant de pair avec les remarquables avancées sur l'identification et la compréhension du mode d'action des acteurs de la réponse immunitaire. Un rôle déterminant est donné aujourd'hui à la réaction inflammatoire dans la progression du sepsis et de ses complications. Un état hyper-inflammatoire, caractérisé par l'activation de multiples cascades inflammatoires, est en effet un événement clé du sepsis chez les patients et dans les différents modèles expérimentaux. La récente définition du sepsis (95) souligne la notion de dérégulation du système immunitaire et de la réaction immuno-inflammatoire à l'infection. De nombreux auteurs ont également insisté sur le rôle joué par le défaut des réponses immunitaires innées et adaptatives, conduisant à un état d'immunosuppression chez ces patients (96) (97).

La réaction inflammatoire en réponse à une infection ou une lésion tissulaire fait intervenir la réponse coordonnée de tout un ensemble de médiateurs solubles et cellulaires, interagissant sous la forme de réseaux complexes et encore mal compris, impliquant de nombreuses boucles d'amplification et de contrôle, dont le but ultime est l'élimination de l'agent infectieux et la réparation des tissus. Beaucoup de travaux ont été effectués sur le rôle des différents acteurs de l'inflammation dans le sepsis (cytokines, système du complément, rôle des leukocytes…), mais un effort de recherche plus récent est consacré au rôle potentiel des plaquettes sanguines. Ce d'autant plus qu'une thrombopénie est très souvent observée au cours de l'évolution du sepsis. La thrombopénie est en effet un facteur pronostique reconnu chez les patients septiques (98,99). Elle est observée chez plus d'un patient sur quatre à l'admission en réanimation (100,101) et jusqu'à la moitié des patients durant leur séjour (98), posant ainsi de nombreuses questions au réanimateur quand à ses mécanismes et à la conduite à tenir.

En effet, au-delà de l'hémostase, le rôle joué par les plaquettes dans la réponse immunitaire est de mieux en mieux décrit. La contribution des plaquettes à la réponse inflammatoire, au maintien de l'intégrité tissulaire et à la défense contre les infections a considérablement élargi le spectre de leurs compétences. De très nombreuses et importantes questions concernent ainsi non seulement les mécanismes de l'activation plaquettaire au cours du sepsis (rôle des agents infectieux, de la coagulopathie associée au sepsis, des médiateurs de l'inflammation, de l'atteinte de l'endothélium, de l'altération de la microcirculation) mais aussi la place des plaquettes dans la progression du sepsis. Quel est le rôle des plaquettes dans la physiopathologie des dysfonctionnements d'organe compliquant le sepsis ? La compréhension du rôle des plaquettes pourrait ainsi avoir d'importantes conséquences en terme de prise en charge des patients (79).

3.2. Rôle des plaquettes dans l'atteinte rénale

Dans le contexte des idées physiopathologiques récentes soulignant la place centrale de la réaction inflammatoire dans le sepsis, les plaquettes pourraient jouer un rôle important dans l'initiation et la progression des lésions microvasculaires rénales et dans l'atteinte des cellules tubulaires rénales (74). L'atteinte de l'endothélium entraîne une activation plaquettaire avec des conséquences pro-inflammatoires et pro-coagulantes potentiellement délétères. Dans un modèle de lésion endothéliale rénale spécifique, il existe des signes d'activation plaquettaire et la déplétion plaquettaire ou le blocage du récepteur P2Y12 ont alors un effet protecteur tandis que la P-sélectine plaquettaire intervient dans le recrutement des neutrophiles après ischémie (102,103). Les plaquettes pourraient aussi favoriser les défaillances multiviscérales, par exemple en contribuant à une souffrance rénale en cas de pneumopathie bactérienne (104). Le taux de microparticules d'origine plaquettaires (MPP) est inversement corrélé à la fonction rénale dans le sepsis (105).

3.3. Présentation d'une revue de la littérature : plaquettes sanguines et sepsis, une nouvelle cible thérapeutique chez les patients de réanimation ? (106)

Dans la revue de la littérature qui suit, nous décrivons les nouvelles fonctions plaquettaires et nous décrivons leur potentielle importance dans la constitution des lésions tissulaire accompagnant les défaillances multiviscérales compliquant le sepsis. Nous discutons la place que ces informations peuvent avoir dans la prise en charge des patients, en particulier à travers l'utilisation des traitements anti-plaquettaires et les problèmes posés par la thrombopénie du sepsis.

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Blood platelets and sepsis pathophysiology: A new therapeutic prospect in critical ill patients?

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Abstract

Beyond haemostasis, platelets have emerged as versatile effectors of the immune response. The contribution of platelets in inflammation, tissue integrity and defence against infections has considerably widened the spectrum of their role in health and disease. Here, we propose a narrative review that first describes these new platelet attributes. We then examine their relevance to microcirculatory alterations in multi-organ dysfunction, a major sepsis complication. Rapid progresses that are made on the knowledge of novel platelet functions should improve the understanding of thrombocytopenia, a common condition and a predictor of adverse outcome in sepsis, and may provide potential avenues for management and therapy.

Keywords: Platelets, Sepsis, Inflammation, Intensive care

Background

Sepsis is a syndrome based on a dysregulated immune response to infection also involving non-immunologic mechanisms, including neuroendocrine, cardiovascular and metabolic pathways [1–3]. Due to its prevalence and high mortality rate, sepsis is a major public health issue [4, 5]. The contribution of blood platelets to sepsis pathophysiology has been the subject of renewed attention. First, alterations of platelet count are commonly encountered in the intensive care unit (ICU). Using common platelet counts thresholds, thrombocytopenia accounts for 20–50% of patients for the whole part of intensive care settings [6–9]. Thrombocytopenia or the non-resolution of thrombocytopenia is associated with poor outcome [8, 10–15]. Second, platelets are well-known players in coagulation and likely to contribute to disseminated intravascular coagulation (DIC). Third, beyond the confines of haemostasis and thrombosis, platelets are now acknowledged as essential actors of the immune response, reacting to infection and disturbed tissue integrity and contributing to inflammation, pathogen killing and tissue repair [16–21]. These advances in platelet biology have opened perspectives on the knowledge of sepsis pathophysiology and on its management. The matter is a complex one as platelets are not only vectors of inflammation contributing to vascular and tissue injury in acute or chronic inflammation [18, 22, 23], but also play an important role in the resolution of inflammation, vascular protection and the repair of damaged tissues. The friend and foe dialogue between platelets and endothelium has been extensively studied and is thought to be relevant to sepsis complications. Here we examine this enlarged spectrum of platelet functions and their relevance to the pathophysiology of multi-organ dysfunction (MOD) and discuss some potential links between these advances and sepsis management.

Sepsis as a dysregulated host response to infection

Recent definition of sepsis [24] emphasizes the non-homoeostatic host response to infection that drives life-threatening organ dysfunctions. Activation of innate immune responses in sepsis realizes a systemic inflammatory condition. The inflammatory phase is

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characterized by the production of pro-inflammatory mediators and immune cell activation [25–29], and sepsis prognosis is linked to the magnitude and duration of this inflammatory response, high circulating cytokine levels being, for example, associated with poor outcome [30–32]. The triggering of innate immune responses by pathogens and pathogen-associated molecular patterns (PAMPs) has been identified as an early and primary mechanism [2, 31, 33–36]. Interestingly, mechanisms of non-septic systemic-associated inflammatory response syndrome (SIRS) as met in major surgery, severe trauma, extensive burns or pancreatitis may share common features with sepsis-associated SIRS, taking the form of a comparable early inflammatory storm that is triggered by alarmins released by damaged tissues [37]. However, the role played by this hyper-inflammatory phase in the progression of sepsis and its prognostic is to be understood in the context of an accompanying anti-inflammatory response and immunosuppression state, and much effort is made in elaborating a coherent vision of these opposite and complex events [38–43].

Platelets: multifunctional tiny cytoplasmic fragments

Platelets are small (2–4 μm), anucleate, discoid-shaped cytoplasmic fragments released in the bloodstream during the fragmentation of polyploid megakaryocytes in bone marrow sinusoidal blood vessels [44]. In humans, a regulated steady platelet supply and clearance maintains numbers of 150,000–400,000 platelets per microlitre of blood. Platelet production is critically dependent on thrombopoietin (TPO) that acts for an important part on megakaryocyte progenitor proliferation/differentiation and on megakaryocyte maturation [45]. Platelets have a short lifespan, of up to 10 days. They are cleared out from the circulation by mechanisms involving lectin–carbohydrate recognition by splenic and liver macrophages and hepatocytes [46, 47].

Platelets harbour a large variety of mediators stored in a pool of morphologically distinct granules [48]. Granule cargo loading is carried out in megakaryocytes. Platelets also transport mediators, such as serotonin, that they uptake from plasma and can deliver at sites of activation. The cataloguing of platelet-derived mediators reflects the remarkable versatility of platelets in haemostasis, thrombosis and immune responses [49, 50].

The secretion of granule content following platelet activation by agonists is central to platelet functions. Platelet activation induces the expression of membrane proteins and the release of mediators via several mechanisms. Many of these mediators are preformed and stored in granules such as cytokines/chemokines and coagulation factors, others can be synthesized by translational pathways, such as IL-1β, and others are released by yet incompletely defined mechanisms such as CD154. Activated platelets also release vesicles, which include platelet microparticles (PMPs) and exosomes [51]. Platelets represent a major source of circulating MPs [52].

In pathological conditions associated with platelet activation, multiple agonists are generated. In fact, apart from classical strong agonists such as thrombin or collagen, there is an expanding list of agonists that can contribute to platelet activation. These additional platelet agonists have allowed a re-appreciation of mechanisms and role of platelet activation in vascular inflammation and thrombotic events associated with a range of infectious and inflammatory conditions [53].

The archetypal function of platelets is haemostasis. Platelets encounter inhibitory signals that prevent their activation in the healthy vasculature, such as nitric oxide and prostacyclin, which are released by endothelial cells (ECs). Platelets circulate in close proximity to the vessel wall, and the disruption of EC lining overcomes inhibitory signals and drives platelet adherence, activation and aggregation, which temporarily plug the damaged vessel. In this process, platelets also activate and confine coagulation at site of damage, particularly via the exposure of an efficient catalytic phospholipidic surface [54].

Besides binding to damaged vessels and preventing bleeding, platelets support a large spectrum of more recently studied functions, as could be reflected by the diversity of platelet mediators [55–57]. Platelets are activated in conditions that disrupt tissue homoeostasis and exert, directly and indirectly, a complex control over the different stages of inflammation, contributing to pathogen clearance, wound repair and tissue regeneration (Figs. 1, 2). As such, platelets are now acknowledged as essential components of the innate immune response, monitoring and rapidly responding to noxious signals.

Platelets as key players in the inflammatory reaction; critical links with coagulation

Activated platelets secrete a profusion of pro-inflammatory material, cytokines/chemokines, vasoactive amines, eicosanoids, and components of proteolytic cascades that directly or indirectly, through the activation of bystander target cells, fuel inflammation [23, 58, 59]. ECs and leucocytes are prime targets for platelets. Endothelium is a non-adhesive, non-thrombogenic surface in normal conditions; when stimulated by inflammatory mediators, ECs undergo profound changes, collectively designed as "EC activation", which include the expression of cell adhesion molecules and tissue factor, production of von Willebrand factor, cytokines/chemokines, proteases and vasoactive substances such as nitric oxide. Platelets adhere to activated ECs, following a multi-step process in which

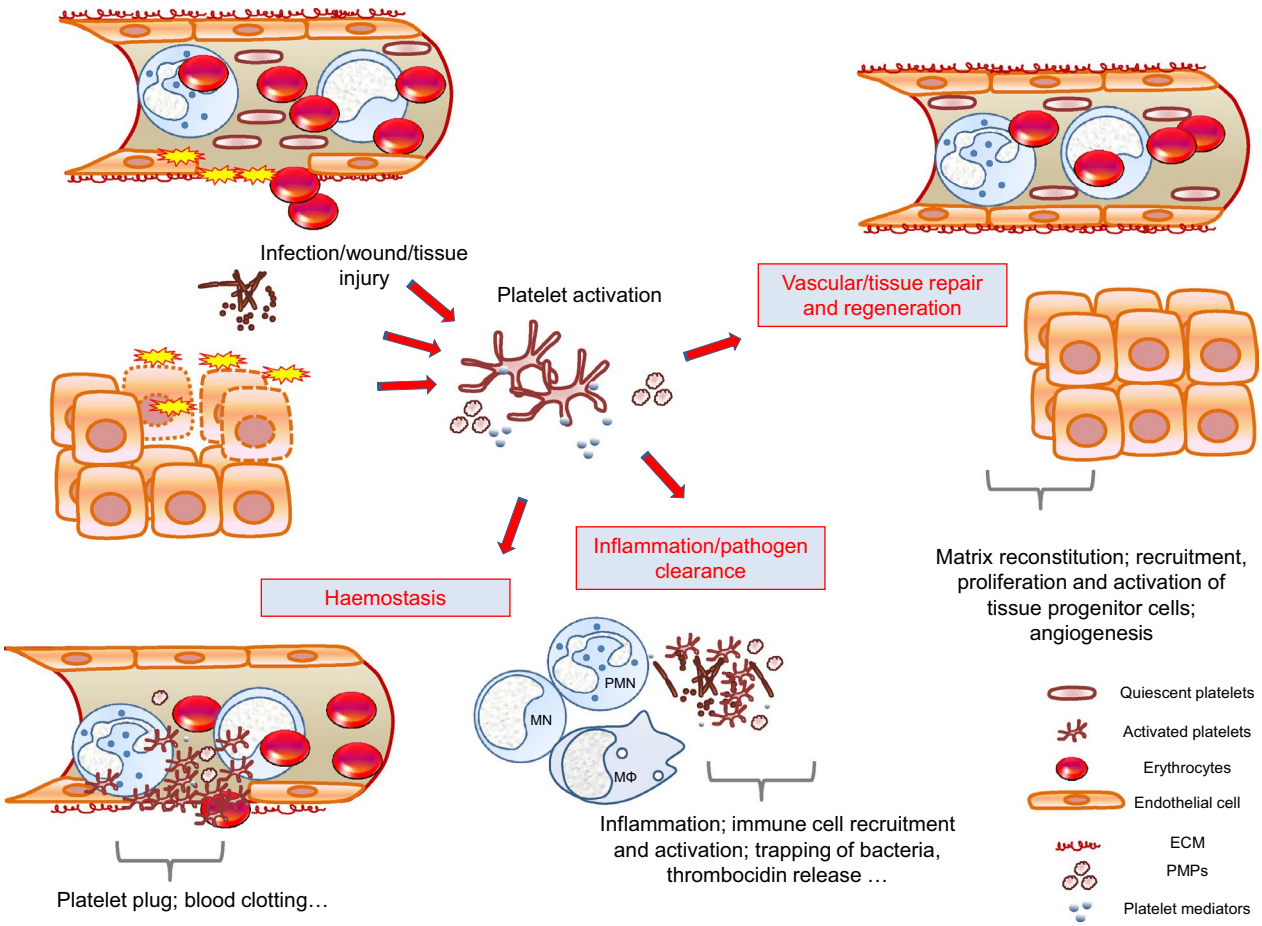

Fig. 1 Platelets are integral players in the immune response, linking haemostasis, thrombosis, inflammation, pathogen clearance and tissue repair: a schematic representation. A growing body of evidence highlights a role for platelets beyond the confines of haemostasis and thrombosis. Some of platelet interfaces in innate immune response are schematized. Platelets are activated at sites of infection/tissue injury. Platelets and platelet-derived mediators contribute to arrest bleeding, to clear pathogens directly or indirectly by acting on various steps of the immune response, and to drive vascular/tissue repair by providing matrix building blocks and a multiplicity of signals that remodel matrix, attracting tissue progenitor cells and reconstructing the vascular frame. In doing so, platelets provide a coherent biological response contributing to cure infection and re-establish tissue architecture and homoeostasis. Scales are arbitrary. Platelet-derived microparticles (PMPs) recapitulate several of activated platelet functions. *ECM* extracellular matrix, *MN* monocytes, *PMN* polymorphonuclear neutrophils, *MΦ* macrophages

glycans play a critical role [60–62]. Inflammation can also alter the protective EC glycocalyx barrier, favouring platelet adhesion [63, 64]. During the adhesion process, platelets can be activated and in turn activate ECs. Platelet activation in inflammation can alter the vascular tone and lead to deleterious effects on vasculature integrity, by increasing vascular barrier permeability and contributing to the generation of cytopathic signals, for example by mediating reactive oxygen species generation by neutrophils [65]; these effects have to be paralleled with the opposed protective role of platelets (below) [66–69]. Leucocytes are a second critical target for platelets, the platelet/leucocyte dialogue being essential in inflammation; here, we focus on neutrophils and monocytes. Platelet/leucocyte interactions are a critical step in leucocyte recruitment, activation and migration in inflammation [70]. Platelet/neutrophil or platelet/monocyte interactions can occur at the EC surface, in clot/thrombi and in circulating blood [18, 70, 71], and platelets direct neutrophil/monocyte migration to sites of tissue injury [72, 73]. Moreover, platelets activate neutrophils and monocytes upon interaction, via several mechanisms, including the triggering of TREM-1 on neutrophils, leading to various pro-inflammatory responses [65, 74–77]. The formation of platelet/leucocyte aggregates in blood depends on platelet activation and is an early phenomenon in sepsis progression. For example, platelet/neutrophils complexes are elevated at early phases, while being reduced in

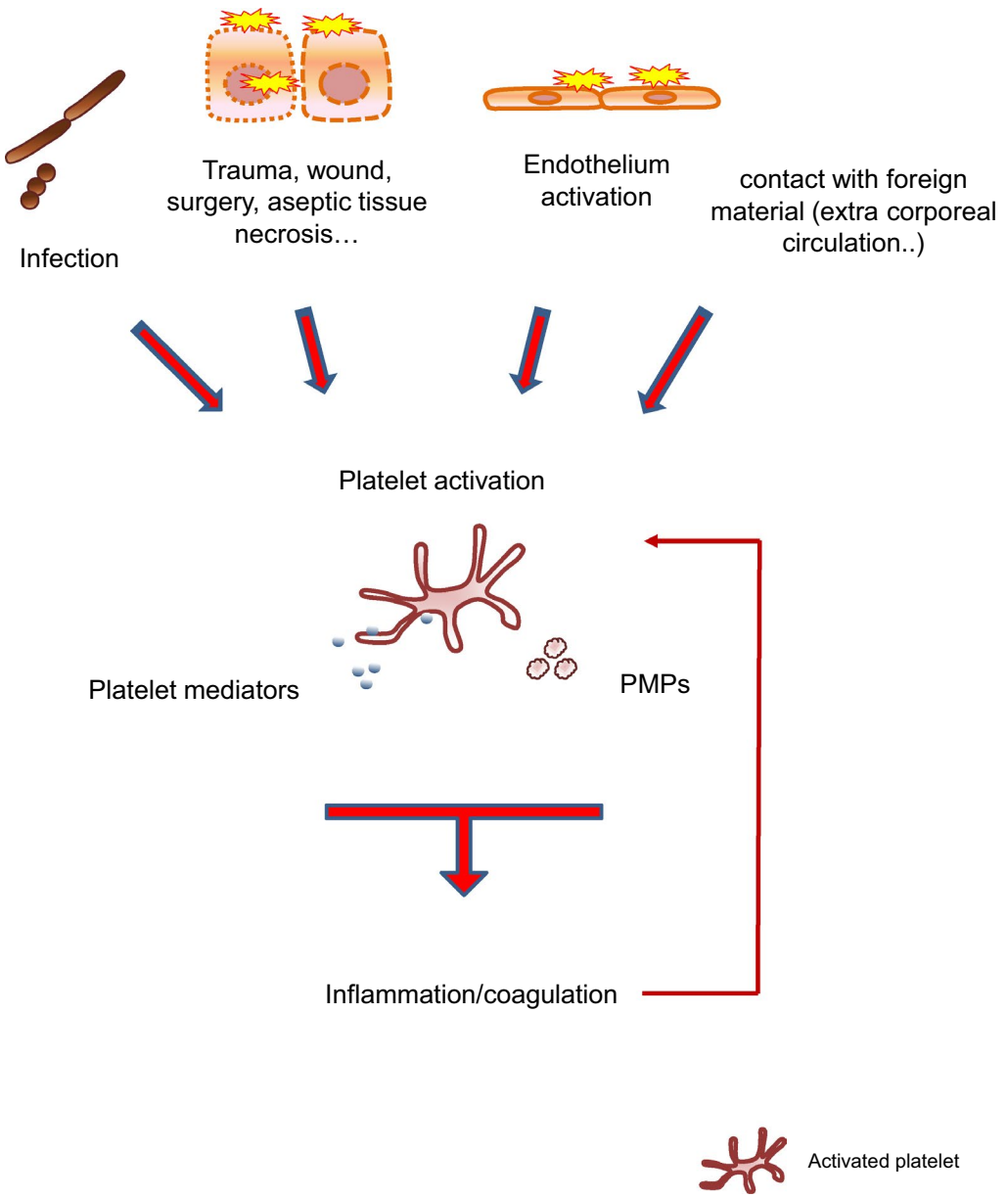

Fig. 2 Platelets monitor and are activated in response to noxious signals. Platelets sense and are activated by multiple signals generated in danger situations met by the organism. Interaction with pathogens, endothelial cell/tissue injury and interaction with foreign material activate platelets (see text for details). Platelet activation sparks off a broad range of responses, including the activation of various inflammation and coagulation pathways. Signals generated in inflammation and coagulation can in return activate platelets (thin arrow). *PMPs* platelet microparticles

complicated sepsis possibly reflecting peripheral sequestration or sepsis-associated thrombocytopenia [78, 79], and endotoxin administration in humans leads to an increased circulating platelet/neutrophil aggregates that follows a brief decrease [80]. Amplification of inflammation results from the reciprocal activation between platelets and their target cells [66], and circulating monocyte/and neutrophil/platelet aggregates may contribute to disseminate inflammatory signals [81]. Platelets also link several inflammatory cascades; for example, they propagate the activation of the complement system [82]. Commonly, cytokines have an induced expression that is regulated at the transcriptional/translational level. Most of platelet-derived inflammatory mediators are very rapidly released from activated platelets, making platelets instant providers of pro-inflammatory material. Cytokine

bioactivity at organs remote from their source is debated as cytokine bioactivity may be hampered in plasma. Platelet transport may protect inflammatory mediators from otherwise degradation. Therefore, platelets play a central role in the inflammatory reaction. Importantly, they also contribute to the control and resolution of inflammation via several mechanisms, including the release of anti-inflammatory cytokines and inflammation pro-resolving mediators [83].

The activation of coagulation and inflammation cascades are consequences of platelet activation, and inflammation and coagulation pathways crosstalk [84]. For example, some platelet mediators have both inflammatory and pro-coagulant properties, such as polyphosphates [85]. Pro-inflammatory cytokines released by platelets can also activate the coagulation cascade at various steps [86]. Conversely, the activation of coagulation by platelets generates a number of inflammatory effectors, such as thrombin. Further, inflammatory mediators can impair anticoagulant and fibrinolysis pathway mechanisms, which may contribute to coagulation dysregulation in sepsis [87–89]. Platelet inflammatory mediators may thus contribute to sepsis coagulopathy [88–91]. DIC is a frequent and major complication of sepsis [41], and various mechanisms concur to involve platelets in DIC; only some can be mentioned. First, platelets support the generation of thrombin. Second, platelet links inflammation and coagulation. Third, platelets are major inducers of the release of pro-thrombotic scaffolds neutrophil extracellular traps (NETs) [92–96].

Notwithstanding, the involvement of PMPs in vascular inflammation and inflammatory disorders, including sepsis, has been emphasized [97–102]. PMPs retain many pro-inflammatory and pro-coagulant features of parent platelets and are thought to disseminate inflammatory and coagulation signals. Although they represent potential pathophysiological players in inflammatory disorders [52, 99, 100, 103, 104], their role in sepsis remains ill-understood.

Platelets in vascular and tissue integrity

In normal wound healing, platelets establish regulatory crosstalks between soluble and cellular actors of tissue repair that concur to the various phases of inflammation and reestablishment of tissue homoeostasis [50, 83, 104, 105]. Platelets accumulate early, are activated at sites of tissue injury and intervene at each stage of tissue repair, the inflammatory, new tissue formation and remodelling stages. In fact, platelet-healing properties are already translated to the clinics [50, 55]. The best studied role of platelets in tissue homoeostasis is the preservation of resting and injured endothelium integrity, a critical point in MOD pathophysiology [71, 106–108]. The importance of platelets is exemplified by the disruption of the endothelium barrier associated with thrombocytopenia [109]. How platelets contribute is incompletely understood. Mechanisms include gap filling, production of EC mitogenic factors and factors enhancing the vascular barrier [71, 107]. On injured endothelium, platelets adhere to the vascular wall at sites of damage and immediate proximity, a first step in a sequence of events that lead to the initiation and the propagation of haemostasis, thrombosis and bleeding arrest [110, 111]. Platelets provide material for endothelium repair, including EC growth-promoting, antiapoptotic mediators, and attractants for progenitor cells endowed with vascular healing properties [104]. They help restoring the disrupted vascular network, providing positive and negative regulators of angiogenesis and stimulating angiogenic mediator production by target cells. Platelets are also important contributors to extracellular matrix (ECM) repair as they are a rich source of ECM components, ECM remodelling proteins, and fibrocompetent cell activators. Platelets have however been found to both promote and prevent vascular permeability in inflammation. The differential regulation of vascular permeability by platelets has been studied for a large part in acute lung injury (ALI) models and will be presented in the corresponding section. Importantly, platelets are highly efficient at preventing bleeding in an inflammatory context [76, 107, 112]. The platelet count threshold needed for vasculoprotection in humans in normal or inflammatory conditions is an important question that remains to be answered [107]. More generally, platelets may play a broader role in organ regeneration. Platelets not only prevent blood loss but also provide key signals for matrix architecture reconstruction and for the recruitment, proliferation, survival and differentiation of cells endowed with new tissue formation, such as fibroblasts, smooth muscle cells and tissue-specific progenitors cells [113]. This is remarkably illustrated by the requirement of platelets in liver regeneration [114]. PMPs are also thought to contribute to vascular repair [115]. Hence, platelet activation is both necessary to tissue integrity and undesirable as it generates tissue-damaging signals. The complex network of signals that organize this fine-tuned equilibrium is only recently being biochemically dissected [55, 104]. Many questions remain on this balanced platelet friend or foe contribution, although they are of key importance to the pathophysiology of microvascular dysfunctions, such as in sepsis [68].

Platelets contribute to the innate immune response against infection

The role of platelets in the defence against infection is increasingly stressed [83, 116]. Platelets are now acknowledged as *bona fide* pathogen sensors interacting directly

or indirectly with a number of bacterial, viral, fungal and protozoan pathogens and their products, contributing to their clearance. Platelet interaction with bacteria depends on the nature and concentration of bacteria, interaction time, and involves multiple mechanisms. Toll like receptors-dependent and independent mechanisms, such as those involving Fcγ receptors, complement receptors or glycoproteins GPIIb-IIIa and GPIbα, contribute to platelet–bacteria interactions. Indirect interactions are also involved, such as via the binding of plasma proteins, including fibrinogen, von Willebrand factor, complement proteins or IgG, that bridge pathogens and platelets or via interaction with bacterial toxins. Interaction with pathogens can lead to platelet adhesion or to their activation, aggregation and release of platelet mediators [83, 117–120]. Mechanisms of pathogen clearance by platelets may be direct, through the release of various antimicrobial peptides and indirect via the release of platelet-derived mediators that coordinate chemotaxis and activation of immune cells [83, 116–118, 120, 121]. Infection is commonly associated with tissue injury. Injured and dying cells generate mediators such as alarmins that fuel inflammation [122]. Mediators generated by cell damage such as complement activation products and histones can activate platelets [123, 124]. Notwithstanding, platelets also contribute to the adaptive immune response to infection [17, 18, 22, 125].

The aforementioned role of platelets in the defence against pathogens suggests that they can interfere with the progression of infection. How can these observations be translated to sepsis pathophysiology [120, 124, 126]? Models suggest a protective role of platelets, as, for example, in streptococcal endocarditis, malaria or gram-negative pneumonia [127–129], and thrombocytopenia could be a risk factor for bacterial or fungal infection. Alternatively, platelets could contribute to spreading infection, via the transport of pathogens [130].

Platelets in MOD pathophysiology

Endothelium in MOD: a common pathophysiological denominator

The pathophysiology of sepsis and its complications remains uncertain as much caution has to be applied in extrapolating to clinical sepsis results obtained in rodent models which have their own inherent complexities [131–134]. Within these extrapolation limits, experimental models have, however, yielded significant knowledge. Numerous studies have emphasized the orchestrating role of endothelium in sepsis, and endothelium injury could be one of the *primum movens* pathophysiological events in sepsis complications [102, 135–148]. Markers of endothelium injury are elevated in sepsis patients, although variably associated with sepsis severity [29, 32, 149]. Inflammation, thrombosis, capillary perfusion alterations are among key features of MOD microvascular alterations [102, 136, 150, 151]. Platelet activation can be detected in sepsis patients and sepsis models, and studies reported association with sepsis severity [79, 124, 152, 153]. Many signals can activate ECs and platelets in sepsis, including pathogens and mediators generated by inflammation and coagulation. Activated platelets may thus contribute to MOD via their role in inflammation and coagulation (Fig. 3) [23, 56, 83, 124, 141, 144, 152, 154].

Platelets in acute lung injury (ALI) in sepsis

There are arguments to involve platelets in ALI pathophysiology [155–158]. Dysregulated inflammation and coagulation are central pathophysiological events in ALI and lung vascular endothelium injury is a primary cause of the alteration of the alveolar-capillary barrier leading to pulmonary oedema [159] [160]. Platelets are sequestered early in lung microvascular beds in ALI models and may contribute to the initial insult of lung endothelium [155, 161–164]. EC activation/injury by inflammatory stimuli, PAMPs and alarmins can generate signals mediating platelet accumulation and activation. Entrapment and activation of platelets in pulmonary capillaries will consequently feed the deleterious cascade of pro-inflammatory and pro-coagulant events in the lung [71, 156, 158, 159, 165]. Platelets can also induce apoptosis in the lung in sepsis models [166]. Among these events, platelet/neutrophil interactions have received considerable attention. Neutrophils are of critical importance in MOD, neutrophil influx being a hallmark of ALI and their inappropriate activation leading to tissue damage signals [167]. Platelets play an important role in neutrophil recruitment and activation in the lung, and platelet-mediated neutrophil activation results in the release of cytokines, chemokines, reactive oxygen species and NET generation [71]. Indeed, experimental models highlight the deleterious role of platelet/neutrophil and also platelet/monocyte interactions in the alteration of the alveolar-capillary integrity [71, 108, 168]. Coagulation activation and alveolar fibrin deposition are common findings in ALI, and platelets are thought to be key contributors to the dysregulation of coagulation in ALI, through their role in coagulation and NET generation [169]. Therefore, several studies suggest a platelet involvement in ALI. In fact, platelet depletion, blocking of platelet/neutrophil interaction, NET dismantling or antiplatelet treatments are protective in experimental models [96, 162–164, 170]. Although in vitro experiments show pro-inflammatory and pro-coagulant effects of PMPs, there is little evidence for a specific deleterious role of PMPs in ALI, a study difficult to address due

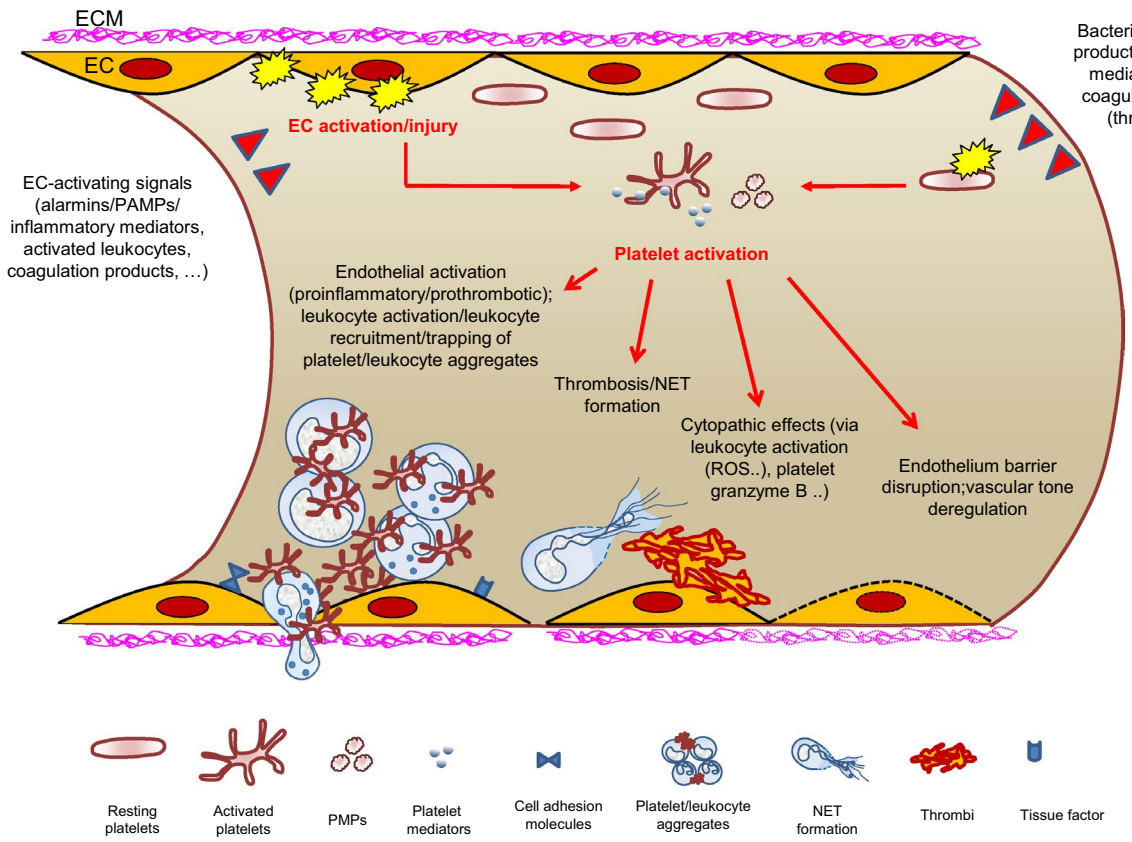

Fig. 3 Microvasculature is a critical target of platelet activation in sepsis. Platelets circulate more concentrated close to the vascular wall and sense endothelium disturbances. In sepsis, many cellular and soluble actors concur to activate the endothelium. Activated/injured endothelium is a key driver of platelet activation. Signals generated by infection, inflammation and coagulation can also activate platelets in sepsis. The relative importance of platelet activation by PAMPs in sepsis is not well established. Platelet activation contributes to fuel various pro-inflammatory and pro-coagulant pathways with potential deleterious consequences on endothelium homoeostasis and integrity. Unmitigated platelet activation in sepsis may take a significant part in the complex global scenario that leads to impairment of the endothelium barrier and microcirculatory failure, a leading cause of organ dysfunction in sepsis. Only some pathophysiological events are schematized (see text for details). Scales are arbitrary. *ROS* reactive oxygen species, *EC* endothelial cells, *NET* neutrophils extracellular traps, *PMPs* platelet microparticles, *ECM* extracellular matrix

to microparticle identification uncertainties and to the simultaneous presence of microparticles from various origins with heterogeneous functions [158, 171].

Increased vascular permeability is the basis for oedema in inflammation. The concept that platelets protect the basal barrier of alveolar capillaries is supported by experimental evidences, and thrombocytopenia put the lung capillary integrity at risk, particularly in inflammatory conditions. Indeed, severe thrombocytopenia results in increased alveolar-capillary permeability [71, 107, 108]. However, as mentioned above, platelet activation in inflammation can also disrupt endothelium barrier integrity, and platelet depletion is protective in several ALI models [69, 71, 162, 172]. How this dual endothelial barrier-stabilizing versus barrier-destabilizing property of platelets is organized and contribute to ALI progression, as well as the specific role of PMPs, is not understood. Such a differential effect of platelets in controlling endothelial barrier integrity is likely to be based on a complex balance between characteristics of inflammation in vascular beds, early or late phase, magnitude, role of other inflammatory players, i.e. leukocytes, and experimental models used. The changing relative importance during sepsis progression of platelet-activating signals, platelet count and proteome, interactions with leucocytes and ECs, underline the difficulty to dissect these mechanisms [69, 71, 76, 108, 173]. Moreover, platelets may play a positive role in the control and resolution of inflammation in lung injury, a mechanism that is only recently being understood [158]. The genetic background also plays a role in ALI-associated mortality and morbidity [174]. Platelet count is determined by genetic factors, and genetic studies point to an association between low platelet count and acute respiratory distress syndrome

(ARDS) risk. Genetic variants within the LRRC16A locus (6p22) are associated with a low platelet count. Interestingly, a low platelet count links a single nucleotide polymorphism within this locus to ARDS risk [175].

Platelets and acute kidney injury (AKI) in sepsis

Acute kidney injury (AKI), a major sepsis complication, is accompanied by hemodynamic disturbances such as decreased glomerular filtration rate and microcirculation alterations [146, 176–180]. The extent of apoptosis and necrosis in tubular lesions is debated. Subtle, heterogeneous, potentially reversible, cytopathic and adaptive cellular events (metabolic changes, mitochondrial dysfunction, autophagy, cell cycle arrest, etc.) may characterize tubular lesions in sepsis AKI [181–184]. Beyond the classic paradigm of renal hypoperfusion, the role of immune response pathways and particularly inflammation in AKI progression is increasingly stressed [1, 178, 185–195]. Alarmins, PAMPs, inflammatory mediators and leukocytes can activate ECs in the renal microcirculation bed, leading to inflammation/thrombosis, metabolic alterations, oxidative stress, concurring to microvascular dysfunction. Due to the close dependence between TECs and tubular microvascularization, compromise blood flow and inflammation in the microvascular beds can lead to tubular epithelial cells (TECs) injury, driving inflammation, mitochondrial/metabolic alterations and various adaptive responses, including cell cycle arrest. Alarmins, PAMPs and inflammatory mediators may also impact TECs after being filtered, and TECs are active participants in kidney inflammation [192, 196–198].

In the highly vascularized kidney, platelet/endothelium interactions can be postulated to be of specific importance. In an AKI model in which selective kidney endothelial injury is realized, there are evidences for platelet contribution [199]. Platelets will be arrested and activated on the kidney endothelium activated by circulating deleterious signals. Inflammation-mediated alteration of EC glycocalyx can also favour platelet adhesion [141, 146, 200–203]. Platelets can also be activated by ischaemic blood flow disturbances in the septic kidney. Therefore, and although much remains to be understood, platelets may be pathophysiological players in sepsis AKI. On the other hand, as mentioned above, platelets contribute to the resolution of inflammation and vasculature integrity. Important questions remain with reference to the identification of soluble and cellular effectors that contribute to the resolution of inflammation and tubular regeneration in the kidney [204]. Microparticles, and PMPs more specifically, are elevated in sepsis and sepsis complicated by AKI [101, 102, 193]. However, their specific role remains to be addressed.

Platelets and organ-to-organ crosstalk in sepsis

Despite the importance of the deleterious organ-to-organ communication in sepsis, underlying mechanisms are only beginning to be unravelled. Inflammatory signals are implicated in these communications [205]. Can platelets vectorize the exchange of pro-inflammatory and/or pro-coagulant signals that link injuries in distant organs? Interestingly, the activation of platelets at remote sites may mediate lung injury, as shown in mesenteric ischaemia/reperfusion models [206]. Platelets can mediate remote kidney damage induced by pneumonia [207]. Among platelet-derived mediators that could convey such a deleterious action, platelet factor 4 (CXCL4) and CD154 have been identified [208, 209]. When activated, platelets express CD154 and release a soluble form of CD154 [22, 210]; CD154 may bear a particular responsibility as, for example, the CD154/CD40 dyad plays a deleterious role in ALI, including pancreatitis-associated lung injury [211, 212], and as it could be brought to lung microcirculation via PMPs. Further, platelet CD154 mediates neutrophil recruitment in septic lung injury [213]. Although these results suggest a role for platelets, the extent and relative contribution of platelets, platelet-derived mediators, PMPs or circulating platelet/leucocyte aggregates in conveying deleterious signals at distance in patients with sepsis is unknown.

Platelet count in sepsis and the dilemma of platelet transfusion

Platelet count and dynamics of platelet count as determinants of clinical outcome in sepsis patients

Thrombocytopenia is common in sepsis and more generally in critically ill patients and has long been recognized as an independent risk factor for mortality in ICU patients and a sensitive marker for disease severity; the severity of sepsis is a risk factor for thrombocytopenia [6, 8–15, 214–221]. For these reasons, the platelet count is included in the ICU severity of illness scoring system. Platelet count kinetics is often biphasic in ICU patients, characterized by a moderate initial decrease in the first days followed by a rise [11, 216, 222]. Early thrombocytopenia and new-onset thrombocytopenia during ICU hospitalization are associated with a poor prognostic; the magnitude and duration of thrombocytopenia and the absence of relative increase in the platelet count have been linked to the poor outcome [6, 9, 11, 216, 221–227]. In a large recent study, which included 931 sepsis patients, a low platelet count at admission in the ICU was associated with an increased mortality risk [29]. Notably, patients with low platelet counts were more severely ill at ICU admission. Understanding pathophysiological links between platelet count alterations and clinical outcomes

is therefore an important issue for the intensive care physician.

The multiple causes of thrombocytopenia in sepsis patients

The association between thrombocytopenia and clinical outcome does not establish causality, and identifying the causes of thrombocytopenia is essential to patient management. Management of the underlying condition is a primary focus, and an important issue is platelet transfusion. Platelet transfusion may be ineffectual and deleterious in patients with, for example, intravascular platelet activation and have their own risks [228–230]. In a recent report, sepsis was identified as associated with ineffectual platelet transfusion, as evaluated by inadequate platelet count increase [231].

Several mechanisms, acting alone or in combination, can be responsible for a low platelet count in sepsis, and all steps of platelet life may be concerned. Decreased platelet production in the bone marrow can result from pre-existing conditions or from the inhibitory effect of pathogen toxins, drugs or inflammatory mediators on haematopoiesis. Peripheral mechanisms are essential causes of thrombocytopenia [15, 214, 218, 227, 232, 233]. The reduction in platelet half-life and their consumption/destruction may be linked to the many events of platelet activation occurring in sepsis, intravascular coagulopathy and immune mechanisms. Drug-induced thrombocytopenia, hemophagocytosis, bleeding, hemodilution are also major explanatory factors. Laboratory artefact of pseudothrombocytopenia can be encountered, and assessing the reality of thrombocytopenia is an important point [230].

Systematic investigations with routinely available tests can help to delineate mechanisms of thrombocytopenia [218, 232, 234]. An early rise of reticulated platelets follows endotoxin administration in humans, and the percentage of immature platelet fraction that evaluates thrombopoietic rate could be a useful tool to witness early bone marrow reaction predicting sepsis development [80, 235]. Apart from altering platelet count, sepsis and sepsis medications can also result in platelet function defect, adding another pathophysiological interface [236, 237]. A detailed description of these mechanisms and diagnostic/management guidance has been excellently reviewed and is beyond the scope of the present work [154, 222, 230, 233, 238–242]. A difficulty in approaching thrombocytopenia and its management is related to the paradox of platelets being potentially both deleterious and beneficial during sepsis course. In a first point of view, platelet count reduction is related to sepsis via consumption mechanisms including pathogen and pathogen product-mediated activation, induction of apoptosis, lysis and increased phagocytic clearance. Acute infections often lead to thrombocytopenia [58], and bloodstream infection is associated with lower platelet counts [221]. Coagulopathy, particularly DIC, platelet sequestration by leucocytes and by inflammatory vascular beds are also commonly stressed mechanisms of thrombocytopenia. Through these mechanisms, platelets can be perceived as bystanders whose destruction is related to the severity of infection and to the characteristics of the host response to the infectious challenge. In that case, the use of platelet transfusion may be perceived as being detrimental, via the fuelling of inflammation and coagulation. On the other hand, platelets are active players in pathogen clearance, leading to the possibility that a low platelet count and platelet function alteration may first favour infection. Further, platelets also protect vascular integrity; hence, maintaining an adequate threshold of platelet count seems a necessary target to prevent bleeding. In fact, platelet transfusions are mostly used to prevent or treat bleeding [228]. Conciliating such a paradox of platelets being both deleterious and beneficial is a challenging point for platelet-targeted therapeutic interventions in sepsis.

Can platelets represent therapeutic targets and diagnostic tools in sepsis?

The clinical management of sepsis remains a difficult challenge, and pathophysiological advances have not yet been translated into effective therapeutic protocols [2]. Notably, strategies to counteract the runaway pro-inflammatory state in sepsis, such as inhibition of specific inflammatory mediators, have given disappointing results [243]. However, current knowledge on sepsis pathophysiology, highlighting multiple humoral and cellular factors in the inappropriate inflammatory response to infection, suggests that therapies targeting a single mediator will not demonstrate effectiveness [41]. Additional complexity is linked to individual disease susceptibilities and medical comorbidities that would necessitate individual approaches. Accumulating evidence therefore speaks for an integrated approach of sepsis treatment based on a better knowledge of its natural history.

The recently described involvement of platelets at the crossroads of several immune response pathways has led to the assumption that platelets or platelet-derived effectors represent therapeutic targets in sepsis. Platelet activation can drive multiple inflammatory and coagulation pathways, and targeting platelets offer the theoretical perspective of targeting simultaneously several deleterious pathways. Although the clinical relevance of animal models has many drawbacks, it is of interest that platelet depletion, inhibition of platelet functions and anti-platelet drugs show protection in experimental ALI or

AKI [124]. P2Y12 inhibitors reduce inflammatory and pro-thrombotic mechanisms after endotoxin administration in humans [244]. Several observational and retrospective clinical studies have shown that antiplatelet agents such as acetylsalicylic acid, platelet P2Y12 inhibitor clopidogrel or GPIIb/IIIa antagonists reduce mortality or complications in critically ill patients [245–255]. However, some studies are conflicting [249, 252, 256] (Table 1). There is therefore a strong need for large randomized controlled clinical trials to investigate the effects of antiplatelet therapy in sepsis. The complexity of such studies relates in part to the heterogeneity of sepsis patients in terms of nature of the causal germ, site and severity of infection, multiple comorbidities, gender, age and genetic background. There is also individual variability in the concentration of antiplatelet agents that efficiently inhibits platelet function. A defective response to clopidogrel or aspirin treatment may concern up to 30 or 40% individuals, respectively [257–259]. Also, antiplatelet treatments have differential effects on platelet functions. Platelets treated with aspirin can still be activated by strong agonists, such as thrombin or ADP. Hence, in a full-blown pro-inflammatory/pro-coagulant condition as met in sepsis, it remains to be determined whether platelet activation is efficiently inhibited by antiplatelet treatments. Platelets are an important blood reservoir of pro-inflammatory molecules and may contribute to the "cytokine storm" that characterizes sepsis. However, many cellular players, including leucocytes and EC, also produce such mediators, and the relative contribution of platelets is not understood. In a recent study, antiplatelet therapy did not significantly reduce plasma pro-inflammatory cytokines levels in sepsis patients [260]. Antiplatelet agents have also been shown to have indirect off-platelet effects, a mechanism which importance is not yet established [261]. Finally, the impairment of platelet function may have undesirable consequences, such as bleeding or the blunting of platelet protective functions.

As mentioned above, elucidating mechanisms of thrombocytopenia in sepsis are essential with reference to transfusion. Platelet transfusion is mostly used to prevent/treat bleeding [228, 229]. The risk of bleeding increases with the severity of thrombocytopenia [222]. The threshold of platelet count ensuring protection may be higher in sepsis patients, reflecting the severity of the disturbance of the vascular beds. Commonly advocated threshold of platelet count is in the range of 10–50 $\times$ 10^9/L, depending on clinical situations, additional bleeding risks, evidence for central thrombocytopenia. The risk of bleeding is, however, not straightforwardly linked to the depth of thrombocytopenia, in the context of a sustained production of platelets, and additional parameters in the critically ill patient may interfere; indeed, the risk of bleeding is also increased for platelet counts between 50 and 100 $\times$ 10^9/L [8, 9, 222, 229]. In fact, there is a poor evidence-based clinical benefit of platelet transfusion in the non-bleeding ICU patient [154, 228–230, 242]. The lack of a clear understanding of thrombocytopenia causes makes the risk/benefit assessment difficult, as there is a theoretical risk to aggravate the underlying pathophysiology [229]. The main regulator of platelet production, TPO, is elevated in sepsis and related to the platelet count [262, 263], which may be linked to the reduction in platelet mass or stimulation of TPO production by inflammatory mediators. Experimental models show that TPO neutralization reduces the severity of organ damage [264]. However, in the clinics, the potential benefit of TPO administration in thrombocytopenic patients in sepsis has been recently suggested [265]. At this stage, results from randomized controlled trials remain necessary to evaluate TPO interest in sepsis.

If the interpretation of thrombocytopenia in sepsis patients is made difficult by the multiplicity of underlying mechanisms, the platelet count by itself may hold valuable information [242]. The platelet count may represent a surrogate marker of the severity of organ dysfunction. A low platelet count occurring even early in sepsis patients is indeed recognized as a sign of poor prognostic; however, a single platelet count at admission may have little pertinence [266], and the kinetics of platelet counts appears to have a deeper meaning. Two alterations of this kinetics have been shown to be of clinical interest in sepsis patients, suggesting that they must be given specific attention. Both the magnitude of the drop in platelet count rather that thrombocytopenia per se, and the non-resolution of thrombocytopenia are strong predictors of mortality in sepsis [9, 15, 267]. The onset and dynamics of thrombocytopenia have been stressed as potential diagnostic approaches in ICU patients [222].

Conclusion

Platelets play key roles in various aspects of the immune response, suggesting that they take a significant part in sepsis pathophysiology. Therapeutic control of platelet functions would offer the perspective of targeting simultaneously several deleterious pathways in sepsis. The difficult extrapolation of experimental models to clinical sepsis and the conflicting results of clinical studies do not allow us today to introduce an antiplatelet agent in clinical practice. However, septic critically ill patients treated with long-term antiplatelet agent may benefit from the continuation of their treatment in the absence of bleeding risk, avoiding a rebound of platelet reactivity. The multiple facets of platelet involvement in sepsis therefore represent substantial challenges to the clinician and call for a deeper understanding of the relative importance of

Table 1 Summary of cohort studies on antiplatelet agents and sepsis

Authors	Study year	Study type and setting	Patient number	Antiplatelet agent	Patients	Study conclusions	Potential limitations
Wang et al. [268]	2016	Meta analysis of cohort studies	14,612	ASA, clopidogrel, ticlopidine	ICU patients with ARDS predisposing conditions	Reduced mortality and lower incidence of ARDS	Non-sepsis patients included Treatment bias of antiplatelet agents
Kor et al. [269]	2012–2014	Multicenter, double-blind, placebo-controlled, randomized clinical trial	390	ASA	Patients with elevated risk for developing ARDS in the emergency department	ASA did not reduce the risk of ARDS and 28-day or 1-year survival	Non-sepsis patients included Low rate of ARDS development
Wiewel et al. [260]	2011–2014	Prospective observational study with propensity matching	972	Mostly ASA	Sepsis within 24 h after admission in 2 mixed medical/surgical ICU	Antiplatelet therapy was not associated with alterations in the presentation or outcome of sepsis or the host response	Treatment bias of ASA Inadequate patient number and power
Osthoff et al. [270]	2001–2013	Retrospective cohort study with propensity matching	689	ASA	Patients with *S. aureus* and *E. coli* bloodstream infection admitted in a single medical/surgical ICU	Low-dose ASA at the time of bloodstream infection was strongly associated with a reduced short-term mortality in patients with *S. aureus* bloodstream infection	Treatment bias of ASA at the time of enrolment Severity at presentation was not included in the analysis model Inadequate patient number and power
Tsai et al. [255]	2000–2010	A nation-wide population-based cohort and nested case–control study	683,421	ASA, clopidogrel, ticlopidine	Sepsis	Antiplatelet agents were associated with a survival benefit in sepsis patients	Claims database
Chen et al. [253]	2006–2012	Secondary analysis of prospective cohort with propensity matching	1149	ASA	Patients admitted in a mixed ICU for at least 2 days	Decreased risk of ARDS	Non-sepsis patients included Treatment bias of ASA
Boyle et al. [271]	2010–2012	Prospective observational study	202	ASA	ICU patients requiring invasive mechanical ventilation	Reduced risk of ICU mortality	Treatment bias of ASA Non-sepsis patients included
Valerio-Rojas et al. [249]	2007–2009	Retrospective cohort with propensity matching	651	ASA, clopidogrel	ICU patients with sepsis	No decrease in hospital mortality but decreased incidence of ARDS	Inadequate patient number and power Unmeasured bias and confounding
Otto et al. [251]	2013	Retrospective cohort	886	ASA, clopidogrel	Surgical ICU patients with sepsis and a minimum length of stay of 48 h and a history of atherosclerotic vascular diseases	ASA treatment reduced the ICU and hospital mortality. Combination of ASA with clopidogrel did not show any significant effect on mortality. Clopidogrel alone might have a similar benefit	Unmeasured bias and confounding

Table 1 continued

Authors	Study year	Study type and setting	Patient number	Antiplatelet agent	Patients	Study conclusions	Potential limitations
Sossdorf et al. [250]	2013	Retrospective cohort	979	ASA	Septic patients admitted to a surgical ICU	Decreased mortality with ASA or NSAIDs was associated with decreased hospital mortality. No benefit when ASA and NSAIDs are given together	Unmeasured bias and confounding
Eisen et al. [248]	2000–2009	Retrospective cohort study with propensity matching	7945	ASA	ICU patients with SIRS/sepsis on ASA at the time of SIRS/sepsis	ASA was associated with survival	Treatment bias of ASA at the time of enrolment and confounders
O'Neal et al. [272]	2006–2008	Cross-sectional analysis of a prospective cohort	575	ASA and Statin	Patients admitted in a mixed ICU for at least 2 days	ASA was not associated with the diagnosis of ALI/ARDS, sepsis or hospital mortality	Treatment bias of ASA Unmeasured bias and confounding Non-sepsis patients included
Erlich et al. [246]	2006	Retrospective cohort	161	ASA, clopidogrel, ticlopidine	Adult patients admitted in a medical ICU with a major risk factor for ALI	Reduced incidence of ALI/ARDS	Treatment bias of ASA Non-sepsis patients included
Kor et al. [256]	2009	Second analysis of prospective multicenter observational study	3855	ASA	Consecutive, adult, non-surgical patients with at least one major risk factor for ALI	ASA was not associated with ICU or hospital mortality and ICU or hospital lengths	Treatment bias of ASA Non-sepsis patients included Unmeasured bias and confounding
Storey et al. [273]	2006–2008	Post hoc analysis PLATO study	18,421	Ticagrelor vs clopidogrel	Patients with acute coronary syndrome	Reduced mortality following pulmonary infection and sepsis in acute coronary syndrome with ticagrelor	Unmeasured bias and confounding
Winning et al. [245]	2007–2009	Retrospective cohort	615	ASA, clopidogrel	Consecutive patients admitted in a mixed ICU	Reduction in organ failure and mortality in critically ill patients with pre-existing medication	Non-sepsis patients included Treatment bias of ASA
Winning et al. [274]	2002–2007	Retrospective cohort	224	ASA, clopidogrel ticlopidine	Patients admitted for CAP not receiving statins and using antiplatelet drugs for more than 6 months	Reduction in need of intensive care treatment and length of hospital stay	Unmeasured bias and confounding
Gross et al. [275]	2001–2005	Retrospective cohort	417,648	Clopidogrel	All adult (≥ 18 years) Medicaid beneficiaries in Kentucky	Increased CAP incidence and no significant reduction in severity	Claims database

ASA Acetylsalicylic acid, *ARDS* acute respiratory distress syndrome, *ALI* acute lung injury, *CAP* community-acquired pneumonia, *NSAID* non-steroidal anti-inflammatory drug

platelet contribution to determine their ultimate clinical significance.

Abbreviations
ALI: acute lung injury; ARDS: acute respiratory distress syndrome; PAMPs: pathogen-associated molecular patterns; DIC: disseminated intravascular coagulation; EC: endothelial cell; ICU: intensive care unit; MOD: multi-organ dysfunction; PMPs: platelet microparticles; SIRS: systemic inflammatory response syndrome; TEC: tubular epithelial cell; AKI: acute kidney injury; ECM: extracellular matrix; NET: neutrophil extracellular traps; TPO: thrombopoietin; TREM-1: triggering receptor expressed on myeloid cells 1.

Authors' contributions
AD and JR conceived, designed and coordinated this review. SL, JV, CR, CC and AO helped to critically revise the manuscript. All authors read and approved the final manuscript.

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4. Rôle du couple CD40/CD154 (107)

Des questions importantes subsistent en ce qui concerne l'identification des effecteurs solubles et cellulaires qui contribuent à la réponse inflammatoire et à la régénération des tubules rénaux (102). Parmi les médiateurs plaquettaires, le CD154, le ligand du CD40, pourrait jouer un rôle important.

4.1. Rôle du CD154 dans l'organisme

Le CD154 est une glycoprotéine membranaire présentant des homologies de séquence avec les molécules de la famille du tumor necrosis factor (TNF). Le gène codant pour le CD154 humain est localisé sur le chromosome X. Les voies de signalisation activées par l'engagement du récepteur du CD40 à la surface des cellules cibles font en particulier intervenir les protéines TRAF (TNF-receptor associated factor) (108). Le rôle immunologique du CD154 est bien décrit. Exprimé par les lymphocytes T activés, il conduit à un signal de costimulation dans l'activation T-dépendante des lymphocytes B. Il est indispensable aux différentes étapes du programme de différenciation des lymphocytes B. Chez l'homme, l'importance du couple CD40/CD154 est illustrée par le syndrome de déficit immunitaire lié à l'X (109). Des mutations ponctuelles ou des délétions du gène codant pour le CD154 sont à l'origine de ce désordre immunitaire, cliniquement caractérisé par des infections répétées, où il existe un déficit en IgG et en IgA, tandis que les concentrations sériques en IgM sont élevées.

De nombreux travaux plus récents ont considérablement élargi le spectre d'expression du CD40 et du CD154, ce qui a en particulier conduit à l'identification de nombreux rôles additionnels au couple CD154/CD40. De nombreuses cellules, leukocytes et non-leukocytes expriment le CD154, expression qui est essentiellement inductible, en particulier par des cytokines pro-inflammatoires. De manière intéressante, le CD40, dont la distribution est également ubiquitaire, voit son expression également augmentée par des stimuli pro-inflammatoires (110). Entre autres, les cellules endothéliales et les cellules épithéliales, y compris les cellules tubulaires proximales rénales, expriment le CD40. Le rôle de l'interaction CD154/ CD40 dans ces cellules n'est pas encore totalement compris ; un des concepts actuels met l'accent sur son importance dans la réaction inflammatoire.

4.2. Le CD154 plaquettaire

A la fin des années 1990, il a été montré que le CD154 est également exprimé par les plaquettes sanguines. Le CD154 est exprimé à leur surface au cours de leur activation. Les plaquettes secrètent également une forme soluble du CD154 au cours de leur activation, par des mécanismes qui restent encore mal compris. Les plaquettes activées sont la source principale de CD154 soluble dans le sang circulant (111-113). L'endothélium est une cible importante du CD154 plaquettaire. Le CD154 plaquettaire est un acteur direct ou indirect de la réponse inflammatoire, de la coagulation, du remodelage tissulaire et de la défense contre les infections, tous ces processus se croisant à plusieurs niveaux. Le CD154 plaquettaire induit l'expression de molécules pro-inflammatoires et chimiotactiques et l'expression de

molécules d'adhésion (CD62e, CD54, CD106, ...), conduisant au recrutement et à l'activation des leucocytes. Il induit l'expression du facteur tissulaire, contribuant à la génération de thrombine. L'induction de métalloprotéases contribue aussi à réguler l'activité protéolytique de l'endothélium (114). Une fois activées, les cellules cibles du CD154 recrutent et activent d'autres cellules à leur tour ; plusieurs boucles d'amplification sont ainsi générées dont l'activation plaquettaire par le CD154 soluble lui-même. Le CD154 plaquettaire active également des effecteurs cellulaires des réponses immunitaires innées et adaptatives : les cellules polynucléaires, les monocytes, les macrophages, les cellules dendritiques. Des études chez la souris ont montré que le CD154 plaquettaire agit également sur la réponse immunitaire adaptative en influençant directement les réponses des lymphocytes au cours de l'infection virale (111,115,116). La contribution du CD154 plaquettaire a pu être démontrée dans des états pathologiques inflammatoires tels que l'athérosclérose (117,118) ou le lupus érythémateux disséminé (119). Les microparticules plaquettaires exprimant le CD154 partagent ces fonctions.

4.3. Présentation d'une revue de la littérature sur le CD154 plaquettaire (107)

Nous présentons une revue de la littérature qui décrit ces données générales concernant le **CD154 plaquettaire**.

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REVIEW Open Access

New frontiers for platelet CD154

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Abstract

The role of platelets extends beyond hemostasis. The pivotal role of platelets in inflammation has shed new light on the natural history of conditions associated with acute or chronic inflammation. Beyond the preservation of vascular integrity, platelets are essential to tissue homeostasis and platelet-derived products are already used in the clinics. Unanticipated was the role of platelets in the adaptative immune response, allowing a renewed conceptual approach of auto-immune diseases. Platelets are also important players in cancer growth and dissemination. Platelets fulfill most of their functions through the expression of still incompletely characterized membrane-bound or soluble mediators. Among them, CD154 holds a peculiar position, as platelets represent a major source of CD154 and as CD154 contributes to most of these new platelet attributes. Here, we provide an overview of some of the new frontiers that the study of platelet CD154 is opening, in inflammation, tissue homeostasis, immune response, hematopoiesis and cancer.

Keywords: Platelets, CD154

Introduction

Platelets are cytoplasmic fragments released in the bloodstream during the fragmentation of polyploid megakaryocytes (MK), a phenomenon critically dependent on thrombopoietin [1-3]. The mammalian platelet is thought to result from a phylogenic trend to ensure hemostasis under high vascular shear forces; indeed, it can specifically form arterial thrombi sustaining high shear stress [4]. It is thought that the platelet coopted attributes of a nucleated cell ancestor endowed with a multifunctional role in coagulation, inflammation and defense against infections [5,6]. Platelets have a short lifespan, of around 7 days; mechanisms responsible for their clearance are ill-understood; lectin-carbohydrate recognition of aged and damaged platelets by splenic and liver macrophages and hepatocytes is emphasized [7]. The best-defined function of platelets is hemostasis. Disruption of the endothelial cell (EC) lining leads to platelet activation, platelet adherence and aggregation which temporarily plug the damaged vessel. In this process, platelets also drive and confine coagulation at sites of tissue damage. Indeed, deficiencies in platelet production or function are associated to bleeding disorders, while increases in platelet number or gain of function are associated to thrombosis. The role of platelets in health and disease extends beyond hemostasis; non-hemostatic platelet functions include inflammation, innate and adaptative immune responses and tissue homeostasis (Figure 1). Decisive advances in understanding platelet function have been made through the characterization of platelet receptors and their ligands and platelet-derived mediators [8]. Among platelet mediators, CD154, the ligand of CD40, has attracted specific attention as it orchestrates many of these new platelet attributes.

CD154

CD154, the CD40 ligand, a member of the Tumor Necrosis Factor (TNF) family, is central to the immune response [9,10]. CD154 was discovered as mediating humoral immunity and was originally considered to be restricted to activated helper T cells. The CD154/CD40 interaction drives B cell proliferation, antibody production and isotype switching and is involved in thymic selection. This interaction is required for B memory cell generation and germinal center formation. Accordingly, CD154 deficiency is associated with an impairment of the humoral immune response to T-cell dependent antigens, including defective immunoglobulin class switching; patients with the X-linked hyper-IgM syndrome caused by mutations

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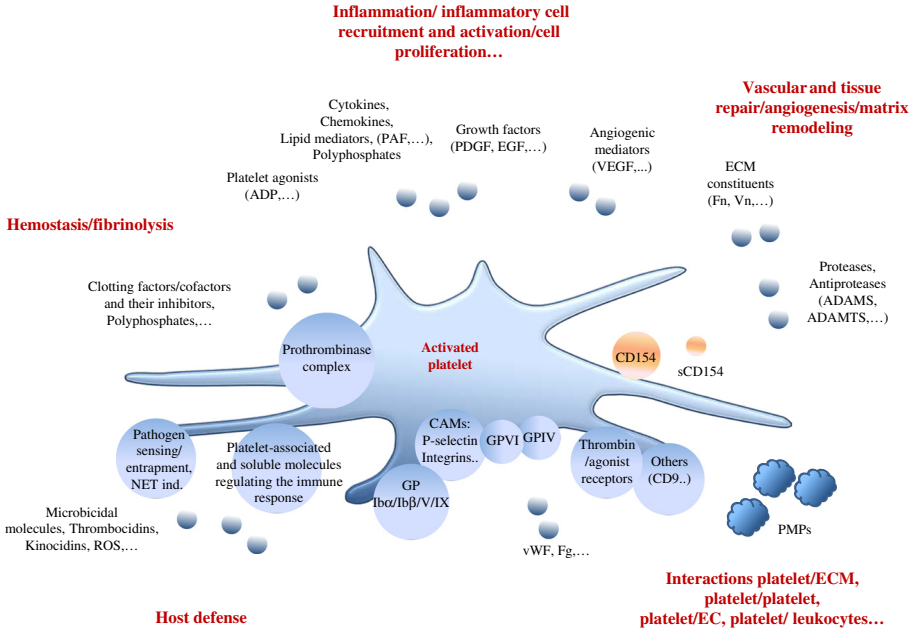

Figure 1 Platelets have a pleiotropic range of biological roles that extend beyond hemostasis. The breaching of tissue homeostasis leads to platelet activation, a common event in various causes of tissue injury, traumatic, infectious, ischemic, autoimmune… Platelet activation, apart from its essential role in bleeding arrest, is the source of a flow of information that fuels the inflammatory reaction. Platelets represent host defense machines against infection, via the clearing of pathogens and the expression of membrane-bound and soluble signals that regulate the innate and adaptative arms of the immune response. Pathways activated in inflammation, coagulation, vascular/tissue repair and host defense are connected via soluble and cell-mediated signals, providing a coherent biological response aiming at arresting bleeding, curing infection and reestablishing tissue homeostasis. CD154 interfaces with many of these pathways (see Figures 2 and 3); activated platelets express a membrane-bound form of CD154 and release a soluble form (sCD154). Platelet derived microparticles (PMPs) recapitulate several of activated platelet functions (see text for details). Only some relevant molecules have been depicted. Small circles symbolize secreted molecules, large circles membrane-associated molecules. Abbreviations: CAMs, cell adhesion molecules; Fg, fibrinogen; Fn, fibronectin; ECM, extracellular matrix; NET ind., neutrophil extracellular traps induction; PAF, platelet activating factor; ROS, reactive oxygen species; Vn, vitronectin; vWF, von Willebrand factor.

of the *CD154* gene, generally present low serum IgG and IgA, but normal or increased serum IgM, and are susceptible to opportunistic infections. Mice with a disrupted *Cd154* gene fail to undergo isotype switching to T-cell dependent antigens while normally responding to T-cell independent antigens. In line with its regulatory role on the adaptative immune response, the CD40/CD154 interaction contributes to autoimmune disorders in a number of animal models [11-15]. Manipulation of the CD154/CD40 interaction has been used in efforts to develop novel strategies in autoimmune diseases, results in animal models being encouraging [13]. Clinical trials have been launched with humanized anti-CD154 monoclonal antibodies. Clinical interest of this strategy remains mixed, and is strongly limited by thrombotic complications [12-14].

Apart from B cells, CD40 is expressed by various cells, including dendritic cells (DC), monocytes, T lymphocytes, EC, a variety of epithelial cells, smooth muscle cells, fibroblasts; its expression is low in basal conditions and is stimulated by inflammatory mediators [16-19]. CD40 expression is increased by CD154, however it is not known whether this induction is direct or indirect [20,21]. CD40 is not the sole receptor for CD154; alternative receptors have been described, such as integrins α5β1, αIIbβ3 and αMβ2; CD154 binding depends on their activation states [22-25]. These additional receptors are of significance in the pathophysiology of atherogenesis and are important to consider when comparing CD40- and CD154-deficient mouse phenotypes.

CD154 is a transmembrane protein and a proteolytic soluble form, sCD154, which keeps the CD40-binding domain, is released by a partially understood mechanism. The release of sCD154 was first documented in activated T-lymphocytes [26]. CD154 has a trimeric configuration, required for functional activity [27-30]. A complex signaling cascade is triggered by CD40 ligation, involving TNF receptor-associated factors (TRAF) as proximal transducing signal initiators [10,20]. Several signaling pathways, including nuclear factor-κB (NF-κB), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase pathways, are activated by CD40 ligation; however, there is a differential outcome depending upon which TRAF member binds preferentially, and which cell/conditions

are involved [31]; the binding of TRAF-6 is critical in vascular inflammation and metabolic complications associated with obesity [32,33].

CD154 expression is also observed in natural killer cells, DC, cells of the monocyte/macrophage lineage, endothelial, smooth muscle and epithelial cells [20]. Basal CD154 expression is very low, or undetectable, as in EC and epithelial cells for example [34], and is increased by a variety of stimuli, most notably inflammatory cytokines [20]. This suggests that CD154 expression may mostly have relevance when induced, as in inflammation. CD154 is also expressed by blood platelets, being cryptic in unstimulated platelets and rapidly exposed at the platelet surface following platelet activation [35].

CD154 expression by platelets

The distribution of CD154 in platelets is partly understood. CD154 was found in α-granules, as shown by immunoelectron microscopy or quantitative immunofluorescence approaches [36,37]. Accordingly, patients presenting a Gray-platelet syndrome, are characterized by platelets that lack α-granules, and do not release CD154 upon activation [37]. CD154 is highly coclustered with insulin growth factor in α-granules, the signification of which is unknown [36]. One question is whether CD154 is also cytosolic, as found in resting platelets [38].

Pre-mRNAs and mature mRNAs are present in platelets and a functional spliceosome and translational apparatus allow platelets to process them, in response to platelet-activating signals [39,40]. Detecting CD154 mRNA by RT-PCR in platelets is challenging because of purity issues. However, CD154 mRNA was evidenced in mouse platelets, introducing other potential regulatory layers of CD154 expression by platelets [34].

When activated, platelets express a membrane form and release a soluble form of CD154

Platelets are activated by immobilized or soluble agonists. The activation-driven secretion of granule content is a primary phenomenon [41-46]. Platelets also synthetize mediators, including interleukin-1β, tissue factor (TF), fibrinogen, thrombospondin, von Willebrand Factor, αIIbβ3, through a translational-dependent pathway triggered by platelet activation [47,48].

Soluble CD154 is released by an activation-driven proteolytic mechanism. Agonists, including thrombin, thrombin receptor-agonist peptide, ADP or collagen, stimulate CD154 expression at the platelet membrane and the release of sCD154; long-term platelet activation leads to complete conversion of CD154 to sCD154 [38,49-53]. A matrix metalloproteinase (MMP)-dependent proteolytic event is involved. The involvement of MMPs, MMP-2 and/or MMP-9, [51,54-57], differs from the release of sCD154 by activated T-cells, which involves ADAM10 and 17 [58]. A role for αIIb/β3 has been put forward, as αIIb/β3 antagonists inhibit sCD154 release and as Glanzmann platelets show reduced sCD154 release rate [53,54,59]. An interaction between αIIb/β3 and MMP-2 is involved [57]. The roles of NADPH activation and reactive oxygen species (ROS) generation as well as CD154 binding to platelet CD40 have been underlined [50,60]. The particularity of sCD154 release may explain its specific response to agonists and secretion kinetics [38,53]; however, how sCD154 is released remains be fully understood, as shown for example by the effects of inhibitors added after platelet activation, suggesting complex, intra-platelet mechanisms [53]. A debate remains about the parallel biological activities of platelet-derived soluble and membrane-associated CD154; recombinant soluble forms, particularly trimeric forms, are active [50,61-63]. Finally, sCD154 activates platelets by itself, suggesting feedback amplification of its secretion [64,65].

The megakaryocytic origin of platelet CD154

The assembly and loading of granules mainly occur in MK; granules are distributed in proplatelets via a microtubule-dependent mechanism [2,66,67]. The main origin of platelet CD154 is likely to be the MK that express CD154 mRNA, as shown in MK derived by differentiation of human and mouse hematopoietic progenitor cells and in MK of immune thrombocytopenic purpura (ITP) patients [68,69]. CD154 mRNA expression is increased upon MK differentiation [69]. CD154 protein is also found in MK cell lines and in MK from ITP patients [38,68,69]. As for T cells, the calcium-dependent activation of nuclear factor of activated T cells-c2 and the early growth response transcription factor EGR-1 contribute to *CD154* gene activation in MK [69,70].

Translation from endogenous mRNAs contributes to platelet content. Its significance in quiescent platelets is unclear. However, pre-mRNA processing and mRNA translation are driven by platelet activation [40,48,71]. The contribution of such mechanism in CD154 expression during platelet lifespan is unknown.

Platelets also carry mediators present in plasma and possibly concentrated and/or modified within platelets [72,73]. Fibrinogen, albumin, immunoglobulins, amino acids, inflammatory and angiogenic mediators including vascular endothelial growth factor (VEGF), histamine or serotonin, are among them. Soluble CD154 is not detected in platelets, making unlikely its uptake from plasma.

Platelets are a significant reservoir of CD154 in the organism

Platelets carry approximately 5 ng of CD154/mL of blood [52]. Correlation studies suggest a link between platelet count and plasma or serum sCD154 [37,52,74-78]. Such a correlation is also found in experimental ITP [78]. In ITP, albeit platelet CD154 is elevated [68], plasma sCD154 is

reduced [78], again suggesting relationship between the platelet count and circulating sCD154. However, there are contrasting studies, and a correlation between the platelet count and sCD154 is not always found [79,80].

Importantly, platelet activation is associated to elevated sCD154 and, indeed, platelet activation markers correlate with sCD154 in blood [81-83]. For this reason, serum seems inappropriate to evaluate circulating sCD154; in fact, sCD154 levels are higher in serum than in plasma, clotting resulting in increased sCD154 generation [52,79,80,84-88]. Hence the importance of a preanalytical standardization of blood samples processing, conditions such as temperature, length of storage, centrifugation, interfering with measurement [84,89]. Further, plasma/serum sCD154 may correspond to a pool of free soluble and microparticle-bound CD154 [84] and ELISA may not discriminate between sCD154 and platelet microparticles (PMP)-associated CD154 [90]. Circulating sCD154 is linked to platelet activation state; in patients with recent thrombotic events, plasma sCD154 correlates with platelet count, but this correlation is not found in patients with non-thrombotic, non-inflammatory conditions [84]. Finally, in patients with cardiovascular conditions, commonly used drugs such as statins, interfere with sCD154 releasing, a point that has also to be considered [91-93]. The baseline presence of sCD154 in the plasma of healthy subjects may be secondary to basal platelet activation, as in high shear stress flow areas [94]. PMP are released upon platelet activation [95]. A functional CD154 is expressed by PMP [63,96]. The importance of the contribution of PMP-bound CD154, in comparison with the "true" soluble CD154, to plasma sCD154 has been emphasized [90]. Questions also remain on the fate and half-life of sCD154 in blood and how the CD154 information can be delivered at distance from platelet activation sites.

Platelet CD154: a critical mediator of the inflammatory reaction

Platelets orchestrate a subtle balance between tissue injury and repair; they are a key source of material for reestablishing tissue homeostasis but they also contribute to tissue injury. CD154 mediates several platelet functions in tissue homeostasis (Figure 2).

Platelet CD154 and inflammation Regardless of its cause, the inflammatory milieu is rich in platelet-activating material, including chemokines [98]. The dialog between EC and platelets in inflammation has been widely studied as EC are primary platelet partners. Upon CD40 ligation, EC switch to an activated phenotype, expressing molecules that contribute to an inflammatory and thrombotic scenario, including cytokines/chemokines, adhesion molecules, and tissue factor [16,20,99]. Platelets/EC reciprocal activation is critical in atherosclerosis and cardiovascular conditions [100-103]. The pathogenic role of platelet CD154 is a major theme in atherosclerosis and cardiovascular diseases [25,62,74,100-109].

The role of platelet CD154 in inflammation extends beyond the dialog with EC, as activated platelets interact with various CD40 expressing-cells. Platelets are brought to inflammatory sites via vascular injury/permeability, attachment to activated leukocytes, and also chemotactic recruitment [110]. CD40 ligation on inflammatory cells at sites of tissue injury is a potent stimulus for the expression of a variety of proinflammatory mediators including cytokines, chemokines, eicosanoids, products of the proteolytic cascades, ROS generation, and of adhesion molecules [49,111], making platelet CD154 a versatile fuel for inflammation. The platelet contribution in many inflammation-associated disorders, including rheumatic, lung, gastrointestinal, neuro-inflammatory and metabolic diseases is actively studied [112-120] and the specific pathogenic role played by platelet CD154 in these disorders is a recently opened frontier. Soluble CD154 levels were found to correlate with disease activity as in systemic lupus erythematosus [121]; whether sCD154 could represent a potential useful marker in inflammation-associated disorders is an interesting question. PMP also contribute to inflammatory disorders [122-128]; the specific role of PMP-associated CD154 remains however to be fully understood.

Platelet CD154 and tissue repair The effectors of inflammation are orchestrated to cure infection and restore tissue integrity [129-131]. At various steps of tissue repair, platelets are a source of relevant material, including growth factors, pro- and anti-apoptotic mediators, matrix and matrix remodeling proteins [132-135] (Figure 1). Platelets contribute to maintain resting and injured endothelium integrity [136]. On injured endothelium, platelets provide EC growth-promoting and anti-apoptotic mediators, attractants for progenitor cells endowed with vascular healing properties [135]. They contribute to restoring the vascular network, by secreting regulators of angiogenesis [137-139]. Beyond endothelium, a remarkable role for platelets in organ regeneration has been substantiated. Platelets contribute to liver regeneration, serotonin being essential [140-142]. It is tempting to speculate that platelets will be found to have a broader role in organ regeneration by providing key mitogenic signals in various organs, such as for example fibroblast growth factor or platelet-derived growth factor that contribute to muscle or brain repair [143,144]. This is also in line with the known ability of platelet lysates to sustain the growth of primary cell cultures. PMP also contribute to vascular integrity [145-148] and promote tissue repair [128,149]. Platelet products have already found various applications in the clinics [150-154].

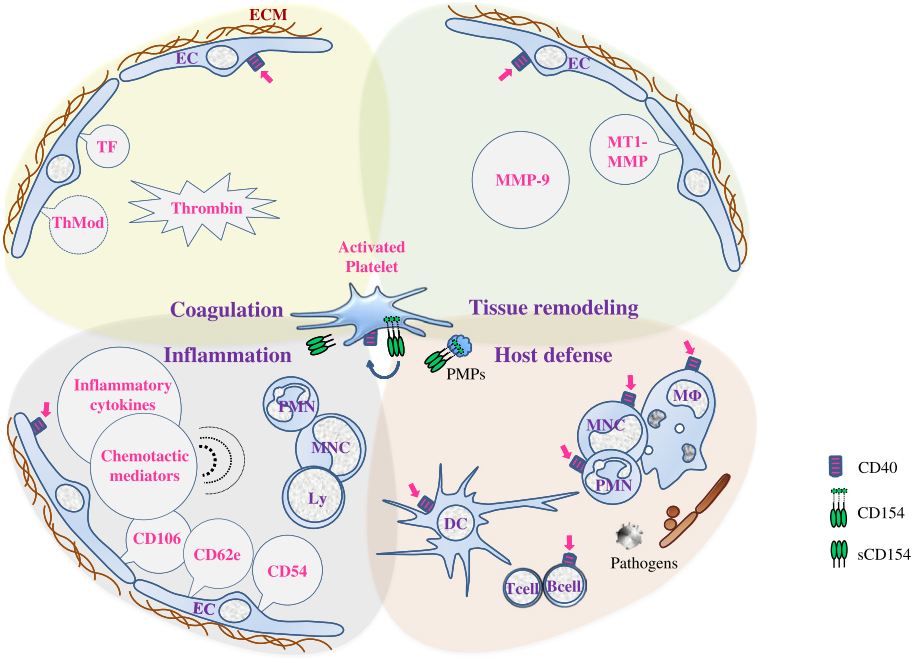

Figure 2 CD154 is a universal contributor to platelet functions. Activated platelets display CD154 at their membrane and release a soluble form (sCD154). Platelet CD154, directly or indirectly, is a molecular driver of inflammation, coagulation, tissue remodeling, and host defense, processes that intersect at multiple levels. Endothelium is a primary target. Platelet CD154 induces tissue factor (TF) expression and activity, thereby contributing to thrombin generation and upregulates urokinase plasminogen activator receptor which is at the interface between fibrinolysis/inflammation/tissue remodeling. MMP-9 and MT1-MMP induction contribute to regulate endothelium proteolytic activity [97]. Platelet CD154 also induces pro-inflammatory and chemotactic (dotted semicircles) molecule expression, and adhesion molecule expression (CD62e, CD54, CD106,…), leading to leukocyte recruitment and activation. Once activated, target cells recruit and activate other cells through multiple inputs; several amplification loops are thus generated including platelet activation by sCD154 itself (blue arrow). Platelet CD154 also activates cellular effectors of the innate and adaptative immune responses, polymorphonuclear cells (PMN), monocytes (MNC), macrophages (MΦ), dendritic cells (DC); how platelet CD154 contributes to host defense is schematized in Figure 3. CD154-expressing platelet microparticles (PMPs) share most of these functions. Depicted molecules do not comprehensively represent the range of platelet-derived mediators that are controlled by platelet CD154, and other interfaces, such as with endothelin-1, continue to be identified. Magenta arrows depict interaction with CD40. Dotted line for thrombomodulin (ThMod) represents inhibition; full line for others represents stimulation. Dotted semicircles symbolize chemotaxis. Abbreviations: Ly, lymphocytes; MMP, matrix metalloproteinases.

The specific role of CD154 has been mainly studied in EC. CD154 promotes EC survival, proliferation and migration, capillary-like tube formation *in vitro* and angiogenesis *in vivo*. Mechanisms include activation of the phosphatidylinositol-3 kinase/Akt pathway, induction of angiogenic mediators and matrix remodeling protein production [155-157]. CD40 signaling contributes to neointima repair, TRAF6 signaling intermediate being critical [32,158,159]. However, platelet CD154 was shown to inhibit the VEGF-induced EC migration via increased ROS generation, and sCD154 to inhibit VEGF-induced angiogenesis [160]. Soluble CD154 also promotes oxidative stress in endothelial outgrowth cells (EOC), reducing their viability and proliferation [161], while promoting endothelial repair via increased production of MMP-9 by EOC [162]. These findings may be context-dependent; they emphasize the importance of platelet CD154 in vascular homeostasis and the complexity of its biological interfaces. Other tissues for which platelet CD154 is likely to show importance for repair are skin and bone. CD40 ligation stimulates keratinocyte differentiation, suggesting contribution to skin wound repair [163]. Regulation of osteoclastogenesis by CD154 is suggested by the reduced bone mineral density together with elevated urine markers of osteoclast activity in patients with the X-linked hyper-IgM syndrome, and the reduced bone mineral density in CD154 deficient mice [164,165]. CD40 is expressed by osteoblastic cells and CD154 is anti-apoptotic in these cells [166]. Therefore, much remains to be found about the role of platelet CD154 in tissue repair. As CD40 is largely distributed, platelet CD154 could be conjectured to be generally involved, to one degree or another, in tissue repair.

Platelet CD154 as a mediator of tissue injury The model of platelets promoting tissue repair is to be compared to their deleterious role in acute and chronic tissue injury. Difficult points are raised by this friend or foe facet, implicating balanced therapeutic approaches [119].

Ischemia/reperfusion (I/R) underscores platelet deleterious role, and the importance to control platelet activation in this context. In I/R, platelet activation in the microcirculation vascular bed leads to tissue injury, as shown in lung, liver or kidney. Platelet depletion or antiplatelet treatments are protective in several experimental I/R models [167-169]; CD154 is contributing: mice deficient in CD154 are protected from I/R-mediated injury in brain, lung, liver or intestine; in lung I/R-mediated injury platelet CD154 is specifically contributing [170-172].

Platelet CD154 and the immune response: unanticipated new frontiers

Platelets participate to the control of infection via direct and indirect mechanisms [6,173-178]. The significance of platelet Toll-like receptors (TLR) has been emphasized; TLR ligation activates platelet secretion of mediators regulating the immune response, including sCD154 [6,179-184]. Platelets also regulate several steps of the adaptative immune response [6,182-194]. Moreover, platelets can present antigen [195]; they express MHC class I molecules and T cell costimulatory molecules, including CD86 and CD40 and harbor a functional proteasome [196-199]. Among platelet mediators, CD154 proved to be critical in linking platelet and immunity (Figure 3).

Although much remains to be understood, particularly with reference to the innate immune response, the specific role of platelet CD154 in immunity is strengthening. Several pathogen-clearing mechanisms are stimulated by CD154, including platelet aggregation [173], phagocytosis and production of defense proteins, such as complement proteins and interferon-α, by cells of the innate immune system [6,20,201]. CD40 contributes to the regulation of innate immune response, including induction of TLR expression, cooperation in TLR-mediated B cell activation, engagement in the crosstalk between intracellular MHC class II molecules and TLR signaling pathway [202-204]. The specific role of platelet CD154 in these mechanisms remains to be precised. However, it is now appreciated that platelet CD154 controls many facets of the interface between innate and adaptive immune responses [173,187,191,205]. Platelet CD154 induces DC

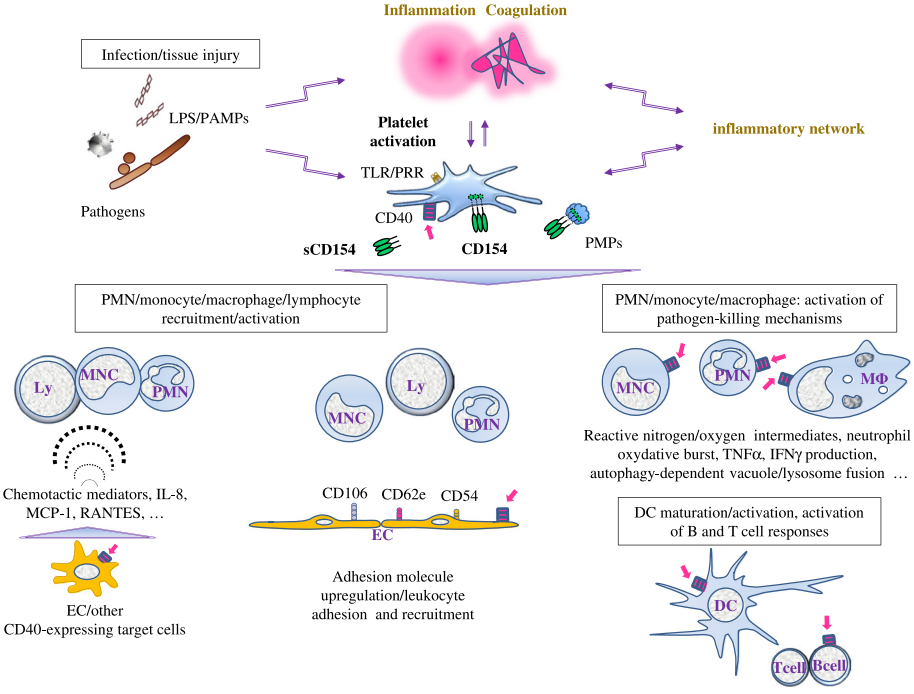

Figure 3 Platelet CD154 contributes to the host defense against infections. Infection triggers inflammation and coagulation. The interaction with pathogens, pathogen-derived molecules such as lipopolysaccharide (LPS), inflammation and coagulation concur to activate platelets, leading to CD154 display at the platelet membrane and the release of soluble CD154 (sCD154). Multiple inputs amplify the platelet activation scenario, including soluble and cellular effectors of the inflammatory network. Platelet CD154 targets several immune response effectors, including contribution to the chemotactic recruitment (dotted semicircles symbolize chemotaxis) of leukocytes to sites of infection, *e.g.* through the induction of adhesion molecules on EC (CD62e, CD54, CD106) and activation/upregulation of integrins such as αMβ2 on neutrophils [158,200]. CD40 triggering is a major inducer of pathogen-killing mechanisms by phagocytic cells. These responses are amplified by inflammatory mediators generated upon CD40 ligation; this schematic representation does not represent all interfaces that are directly or indirectly regulated by platelet CD154. Platelet CD154 influences the adaptative immune response, through several mechanisms, including the activation/maturation of antigen presenting cells (see text for details). Magenta arrows depict interaction with CD40. Abbreviations: PAMPs, pathogen-associated molecular patterns; PRR, pathogen recognition receptors; TLR, Toll-like receptors.

maturation, can activate B cells, antibody production and isotype switching, contributes to germinal center formation, and enhances $CD8^+$ T cell responses [188,206-213]. Platelet CD154 helps mounting a protective cytotoxic T cell immune response to viral or bacterial challenge [206,214]. Platelet CD154 may promote the immune response in the context of low antigen challenge by lowering the antigen threshold, and improve B cell response in regulatory T-cell limiting settings [210,215]. Further, sCD154 *per se* induces cardiac allograft rejection [212]. Many questions remain. How platelet CD154 enters the draining lymph nodes to regulate the adaptive immune response machinery is not known; PMP may convey this information, as CD154 associated to PMP is functional: it enhances DC activation, germinal center formation, B cell proliferation and IgG production [63,216]. Several questions are also raised with reference to platelet CD154 in autoimmunity; this "dark side" [14,217] feature of platelet CD154 is a recently opened frontier. Platelet CD154 is competent to increase production of antiplatelet antibodies in immune thrombocytopenic purpura [68] and, in systemic lupus erythematosus, platelet CD154 activates antigen presenting cells contributing to enhanced interferon-α production [218].

Platelet CD154: a new hematopoietic regulator?

Hematopoiesis can be adapted in response to inflammation/infection by signals generated at bone marrow distal sites [219-224]. Platelets are activated at sites of inflammation/infection and are a major source of circulating sCD154. Could platelets deliver a CD154 signal, through sCD154, platelet- or PMP-associated CD154 that regulates hematopoiesis? Platelet mediators enhance hematopoietic stem cell proliferation and platelet-derived signals may contribute to CD34+ cell mobilization [225,226]. Several studies have demonstrated CD154 involvement in hematopoiesis. CD154 regulation of early B cell lymphopoiesis is suggested by the sCD154-induced increased number of B cell progenitors (BCP) in mice after bone marrow transplantation (BMT) [227]. CD40 is expressed on BCP, and a positive effect of CD40 ligation on BCP proliferation can be observed on pre- and immature B cells in human and pro-B cells in the mouse [228,229]. In the mouse, there is clear experimental evidence for a positive role of CD154 in B cell hematopoiesis and, particularly in stress conditions, as after BMT [229]. However, normal numbers of circulating B cells in patients with X-linked hyper-IgM syndrome would rule out an absolute requirement for the CD154/CD40 signaling in early B cell development. CD154 may therefore mostly play a significant role in emergency B cell hematopoiesis [229]. More is known about CD154 regulation of the lymphoid system maturation, which has been fully reviewed [230]. A role for platelet CD154 on myelopoiesis is suggested by the sCD154-mediated increased granulocyte and platelet recovery after BMT in the mouse and by the neutropenia and thrombocytopenia observed in patients with X-linked hyper-IgM syndrome [227]. *In vitro*, sCD154 promotes the differentiation of CD34+ cells towards the granulocytic/monocytic and megakaryocytic lineages in CD34+/stromal cell cocultures. The mode of action of sCD154 appears to be essentially indirect, through the induction of hematopoietic cytokines by bone marrow stromal cells [231,232]. Platelet CD154 may therefore play a role in regulating emergency hematopoiesis. However, many questions remain unsolved, particularly which and how platelet CD154 signals could be delivered and interact with bone marrow stem/progenitor cells.

Platelet CD154 and cancer: a rapidly expanding frontier

There is strong evidence for the involvement of platelets in cancer progression; mechanisms are multiple [233-240]. Platelets are activated in the tumor environment and bind tumor cells. Mediators released upon platelet activation are key to tumor angiogenesis [241,242] and are likely to contribute to the tumor-supporting inflammatory environment [243,244]. Platelets play a positive role in metastasis [234,238,245-249]. However, this may not be true for all organs [250]. In hematogenous dissemination, platelet/cancer cell microthrombi provide protection, including shielding from shear flow, or immune evasion; during the arrest and extravasation phases, platelet mediators facilitate tumor cell arrest on EC, extravasation, survival and growth after seeding [251]. Platelet MPs are also contributing [124,252,253].

Many tumor cells express CD40. The outcomes of CD40 ligation on tumor cells are ambivalent depending on the models studied. In one hand, CD40 ligation promotes anti-tumor immune surveillance through a variety of mechanisms including antigen-presenting cell activation, restoration of malignant cell immune recognition, activation of tumoricidal-infiltrating macrophages, immunostimulatory cytokine production. CD40 ligation also induces tumor growth arrest and sensitization to apoptotic signals. On the other hand, CD40 ligation has positive consequences on tumor growth, survival and resistance to chemotherapy and metastatic potential. The interpretation of CD154 effects on cancer cells is made complex, first by the existence of several receptors for CD154, potentially explaining variable outcomes of CD154 treatment of tumor cells, and second, by the difficulty in assessing direct versus indirect effects. The contribution of the CD40 signaling in cancer, and prospects offered by targeting the CD40 signaling for cancer treatment have recently been underlined and reviewed [254-258]. However, the specific role played by platelet CD154 remains a new important frontier. If platelet activation is likely to result in expression of CD154 and generation of sCD154 in

the tumor cell environment, this study is made complex as there are extra platelet sources of CD154.

Conclusion

There have been recent and rapid advances in our current knowledge of the non-hemostatic functions of platelets, placing them in the middle of the spectrum of mechanisms that maintain homeostasis, and highlighting their role in a variety of inflammatory and immune disorders. However, platelets store and release such a wide diversity of biologically active mediators that major gaps remain in our understanding of which and how these mediators collectively fulfill these functions. Platelet CD154 has attracted considerable attention as it recapitulates several of non-hemostatic platelet attributes. Considering the large number of different cells expressing CD40, the complex signaling cascade and the wide range of effectors activated by the CD154/CD40 interaction, it can be anticipated that future investigations will further extend the contribution of platelet CD154 in health and disease. For example, recent publications on the CD154/CD40 dyad have pointed to its role in obesity and hepatic steatosis [259-263], and it is tempting to speculate that platelet CD154 contributes to metabolic homeostasis. In the same direction, the number of physiological or pathological conditions associated with platelet activation is enlarging. For example, platelet activation has been found associated to aging, to emotional or environmental stresses…; platelet CD154 might represent a significant link between these conditions and accompanying pathologies, such as cardiovascular events [264]. However, platelet CD154 is always acting in a multicytokine context, including inhibitors and activators released at the same time by platelets; understanding how this complexity is tuned and evidencing the specific role of platelet CD154 remains a difficult challenge.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors contributed to the writing of the manuscript. All authors read and approved the manuscript.

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5. Rôle potentiel du CD154 dans l'inflammation tubulaire dans l'IRA ; étude de la régulation de la production de l'IL-6 par le CD154.

5.1. Interaction du CD154 avec le CD40 au niveau rénal

L'activation des plaquettes sanguines pourrait ainsi jouer un rôle dans les phénomènes inflammatoires des tubules rénaux au cours de l'IRA. Le CD154 est un élément important de l'arsenal de médiateurs de l'inflammation exprimés par la plaquette. Son interaction avec le CD40 récapitule quelques-unes des nombreuses conséquences pro-inflammatoires de l'activation des plaquettes. Cette interaction peut s'exercer au niveau du CD40 de l'endothélium rénal, immédiatement accessible aux plaquettes, mais aussi au niveau des CET. Toute une série de travaux ont souligné l'importance potentielle dans les maladies rénales à composante inflammatoire de l'expression du CD40 par les CET et des conséquences pro-inflammatoires de l'interaction du CD40 de l'épithélium tubulaire avec le CD154 (120-122). Au cours de l'inflammation tubulo-interstitielle, les plaquettes et les lymphocytes T exprimant le CD154 sont étroitement associés à l'épithélium tubulaire exprimant le CD40 (121,123-125). La filtration potentielle du CD154 soluble par le rein et sa possible interaction avec la surface apicale des cellules de l'épithélium tubulaire est très mal comprise. Le CD154 soluble n'est en effet pas détecté dans les urines, phénomène pouvant être en rapport avec une réabsorption au niveau de l'épithélium tubulaire.

5.2. IL-6 et lésions inflammatoires en pathologie rénale

Les phénomènes reliant inflammation et interaction CD154/CD40 au niveau de l'épithélium tubulaire sont rendus complexes par la multiplicité des mécanismes impliqués. Nous avons choisi d'étudier le rôle de l'induction de la cytokine pro-inflammatoire IL-6. De nombreuses études en effet ont souligné le rôle de l'IL-6 dans la genèse des lésions inflammatoires en pathologie rénale (126-129). L'IL-6 a été découverte en 1986 en tant que médiateur capable de stimuler les lymphocytes B pour la production d'immunoglobulines (130). Son rôle clé dans l'initiation et le contrôle de la réponse inflammatoire a été depuis souligné et largement étudié (129,131-134). L'IL-6 joue un rôle essentiel dans le contrôle de la production des protéines de la phase aiguë de l'inflammation par les hépatocytes (135). Elle appartient a une famille de protéines, incluant l'oncostatine M, le leukemia inhibitory factor, le ciliary neurotrophic factor, la cardiotropin-1, la cardiotrophin-like cytokine, la neuropoietin, l'IL-11, l'IL-27, et l'IL-31 ayant en commun la glycoprotéine de membrane gp130 comme sous unité de transmission du signal dans leur récepteur (excepté pour l'IL-31). L'activation de la gp130 par l'IL-6 sur les cellules cibles conduit en particulier à l'activation de la voie de signalisation Jak/STAT et à l'activation de STAT3 (136). L'importance de la signalisation de l'IL-6 en pathologie inflammatoire est soulignée par les résultats cliniques des essais utilisant l'anticorps monoclonal humanisé anti-récepteur de l'IL-6, le tocilizumab (137,138).

Les cellules résidentes du rein, comprenant les cellules endothéliales, les cellules mésangiales, les podocytes et les CET sont des sources d'IL-6. La production d'IL-6 par ces cellules est en particulier stimulée par différents médiateurs de l'inflammation, comme l'IL-1 ou le TNFα. Différentes études ont souligné le rôle joué par l'IL-6 dans l'inflammation associée à l'IRA. Une élévation locale et systémique de l'IL-6 est observée dans des modèles d'IRA ischémique (139,140) et d'origine toxique. A travers la production d'IL-6, les CET ont ainsi démontré leur rôle clé dans l'inflammation associée à l'IRA (120).

5.3. Rôle du couple CD40/CD154 dans l'induction d'IL-6 en condition d'hypoxie

Le rôle délétère du couple CD154/CD40 a été souligné dans de nombreux modèles d'I/R, y compris rénal (141). Cependant les mécanismes en jeu restent mal compris. L'induction de la production de cytokines pro-inflammatoires est un des effets majeurs de l'interaction CD154/CD40. Les CET sont activées par le CD154 et répondent en particulier par une production accrue de médiateurs inflammatoires (120,142). Le CD154 pourrait jouer un rôle délétère dans l'inflammation tubulaire au cours de l'IRA via l'induction de cytokines pro-inflammatoires. Le CD154 est un inducteur de la sécrétion d'IL-6 dans différents types cellulaires, incluant les cellules endothéliales et épithéliales, dont les CET (120,143,144).

Une question importante reste l'étude de ce phénomène en conditions d'hypoxie. En effet hypoxie et inflammation sont en étroite interdépendance et leur association est de plus en plus soulignée dans de nombreuses pathologies à composante inflammatoire. Les foyers inflammatoires présentent des stigmates d'hypoxie avec l'expression de facteurs de transcription dépendant de l'hypoxie, comme l'« hypoxia-inducible factor » (HIF). D'un autre côté, les situations associées à une réduction d'oxygénation sont associées à une réponse inflammatoire, comme dans l'I/R. L'IRA est un exemple important de l'interdépendance entre hypoxie et inflammation. Des modèles expérimentaux ont souligné l'existence d'une hypoxie associée à l'ischémie rénale avec ou non reperfusion ; chez l'homme les données sont plus rares et contradictoires reflétant peut-être la limitation des méthodologies d'évaluation de l'hypoxie tissulaire. Comme mentionné plus haut, des contraintes énergétiques rendent certaines régions du rein comme la partie terminale du tube contourné proximal très sensible aux phénomènes d'ischémie (35). Inflammation et hypoxie sont ainsi étroitement associées dans les mécanismes lésionnels et la progression de l'IRA (145).

5.4. Présentation d'un article original : le CD154 induit la sécrétion d'IL-6 par les cellules tubulaires rénale en condition d'hypoxie (en préparation)

Notre travail porte donc sur le rôle proinflammatoire du CD154 sur les CET en conditions d'hypoxie en nous focalisant sur la synthèse d'IL-6.

CD154 induces interleukin-6 secretion by kidney tubular epithelial cells under hypoxic conditions.

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Abstract

The inflammatory milieu is commonly associated to hypoxia. Hypoxia pathways drive pro- and anti-inflammatory responses but are also regulated by inflammatory mediators. CD154, the ligand of CD40, is a central regulator of inflammation. Yet, how CD154 directs inflammation in hypoxic conditions remains unclear. Here, we studied the control of the pro-inflammatory cytokine interleukin (IL)-6 production by CD154 in O_2 deprivation conditions, using kidney tubular epithelial cell (TEC) line HK-2 as a model. HK-2 cells express CD40 and this expression was not altered in hypoxic conditions. Hypoxia moderately stimulated IL-6 secretion by HK-2 cells and the hypoxia-induced IL-6 production was markedly increased by CD154. Both intracellular IL-6 expression and IL-6 secretion were increased by CD154 and were associated to a strong up-regulation of IL-6 mRNA. These results indicate that CD154 is a potent inducer of IL-6 secretion by TECs in hypoxic conditions, results that may have pathophysiological significance in kidney diseases associated with tubular inflammation.

Introduction

Inflammation is a central manifestation of tissue response to stress and injury. Accumulating evidence underscore the universal association of hypoxia and inflammation and their interdependence. Decreased O_2 availability is a key feature of the inflammatory milieu and the role of hypoxia in inflammatory disorders is increasingly stressed [1-7]. Hypoxia is linked to the progression of inflammation via complex and yet ill-understood mechanisms. On one hand, hypoxia stimulates the expression of pro-inflammatory cytokine genes via several mechanisms, including those involving the hypoxia-inducible factor-1 (HIF-1) pathway. On the other hand, hypoxia also drives anti-inflammatory responses [6, 8-12]. Moreover, inflammation-associated metabolic disturbances, such as hypoxia and glucose depletion also induce a state of endoplasmic reticulum (ER) stress [3, 13-15]. ER stress pathways are also emerging as important pathophysiological players in the natural history of inflammation, as the secretion of inflammatory cytokines is an outcome of the ER stress response [14-20]. Therefore, much remains to be understood on how hypoxia stress pathways cooperatively control the different stages of inflammation [14, 21-24]. Importantly, these pathways not only drive pro- and anti-inflammatory responses, but are also regulated, for example by inflammatory mediators themselves, suggesting complex regulatory feedback mechanisms in the progression of inflammation.

Acute kidney injury (AKI) is an important example of the interdependence between inflammatory and hypoxic pathways. The central importance of inflammation in orchestrating the deleterious sequence of events that result in microcirculatory alterations and tubular cell injury in AKI is increasingly stressed [25-34]. In sepsis-associated AKI, current pathophysiological concepts give a particular importance to tubule-interstitial inflammatory events and underline the importance to identify inflammatory mediators involved [35, 36]. Among the plethora of inflammatory mediators, clinical and experimental studies have underscored the contribution of the pro-inflammatory cytokine interleukin (IL)-6 to renal injury in kidney inflammatory conditions, including AKI [32, 37-39]. IL-6 mediates acute inflammation in response to tissue damage [40], is involved in both promotion

and resolution of acute inflammation [39, 41, 42] and IL-6 signaling has deep relevance to inflammatory disorders as shown for example by the clinical efficiency of anti-IL-6 receptor antibody in a variety of inflammatory disorders [43, 44]. Via the production of inflammatory mediators such as IL-6, tubular epithelial cells (TECs) are key contributors to AKI-associated inflammation [45]. In fact, TECs stand at the interface of various inflammatory events in AKI. TECs are not only targets of inflammatory mediators but also active participants in kidney inflammation in response to inflammatory challenges, providing ground for self-amplifying inflammatory loops [46, 47]. Additionally, constitutive and induced IL-6 expression in TECs are not inhibited by glucocorticoids, emphasizing the need to understand how this cytokine is regulated for the management of kidney tubular inflammation [48]. Importantly, the AKI inflammatory milieu is associated with hypoxia and mechanisms of IL-6 production in such a context remain ill understood.

CD154, the ligand of CD40, controls key stages of innate and adaptive immune responses. The induction of pro-inflammatory cytokine secretion is a major outcome of CD154 binding to CD40 [49-53]. TECs are activated in response to CD154 and respond by the production of inflammatory mediators, including pro-inflammatory cytokines [45, 54]. CD154 is deleterious in kidney ischemia-reperfusion injury, as the inhibition of CD40 signalling is protective, but underlying mechanisms remain unclear [55]. CD154 is thought to be important in initiating and sustaining inflammation in kidney tubules particularly via the CD154-mediated induction of pro-inflammatory cytokines. Indeed, CD154 triggers IL-6 secretion in a variety of cells, including endothelial and epithelial cells [56-58] and more specifically in TECs [45]. However, it is not known how CD154 exerts its pro-inflammatory role in acute or chronic inflammation in the context of hypoxia.

Therefore, coincidental occurrence of inflammation and hypoxia and cross-regulations between hypoxic and inflammatory pathways underline the importance of studying how are pro-inflammatory cytokines expressed and regulated under hypoxic conditions. In this work, we used HK-2 cells as a TEC model to study the control of IL-6 secretion by CD154 in oxygen deprivation conditions.

Materials and Methods

Cells

The immortalized human proximal TEC cell line HK-2 [59] was from American Tissue Type Culture Collection (LGC standards, Molsheim, France). Cultures were free of mycoplasma as evaluated by PCR [60]. Cells were grown in complete medium consisting of Dulbecco's modified Eagle's medium (DMEM, glucose concentration 1g/L), containing 10% fetal calf serum (FCS), 100IU/mL penicillin and 100μg/mL streptomycin. Cultures were carried out in a humidified atmosphere under normoxic (21% O_2) conditions with a balance of 95% N_2, 5% CO_2. Cultures in hypoxic conditions (0.1% O_2) were performed in a hypoxia incubator chamber (Biospherix®, NY, USA). Cells were grown in 6-well plates for 1 to 24 h in 1mL culture medium. Culture medium was pre-equilibrated 1h in the hypoxic chamber before being added to cell cultures. For CD154 treatment of cells, recombinant soluble CD154 (MegaCD40LTM, Coger SAS, Paris, France) was added at the beginning of hypoxic cultures and used at a final concentration of 200 ng/mL. In actinomycin D experiments, actinomycin D (Sigma-Aldrich, Saint Quentin Fallaviers, France) was used at a concentration of 5 μg/mL and added to cells 30 minutes before starting the culture in hypoxia. In cycloheximide experiments, cycloheximide (Sigma-Aldrich) was used at a concentration of 50 μg/mL and added to cells 15 minutes before starting the culture in hypoxia. For tunicamycin treatment, cells were incubated in DMEM/10% FCS containing 2.5 μg/mL tunicamycin (Merck, Darmstadt, FRG).

Real-Time quantitative PCR (RT-qPCR)

Total RNA was extracted from HK-2 cells using a RNA extraction kit (Macherey-Nagel, Hoerdt, France) following manufacturer's instructions, and quantified spectrophotometrically (Thermo Spectronic, Cambridge, UK). RNA Integrity was assayed using the Agilent RNA Screen Tape Assay Bioanalyzer 2200 (Agilent Technologies, Les Ulis, France). Complementary DNA (cDNA) was synthesized using hexaprimers and oligo-dT from 1 μg of total RNA in a final volume of 20 μL, using the First Strand

cDNA Synthesis Kit for RT-PCR (Quantitect Reverse Transcription kit (Sigma-Aldrich)), according to manufacturer's instructions.

The qPCR was performed in triplicate on a CFX 384 (Bio-Rad, Marnes la Coquette, France) using SYBR® Premix Ex Taq ^{TM} bulk (Takara, Ozyme, Saint-Quentin en Yvelines, France). Cycling parameters for the qPCR reaction included a 3 min hot start followed by 40 cycles of denaturation at 90°C for 10 seconds, annealing at 60°C for 30 seconds and elongation at 72°C for 30 seconds. Post PCR Melt Curve analysis was performed (65°C to 95°C/ 0.5° each 5 seconds). Ribosomal phosphoprotein L0 (RPL0) and 18S rRNA were used as internal controls. Differential expression was performed using the ΔΔCq method. The Ct value of the housekeeping gene (18S rRNA) was removed from the Ct value of the target gene to gain the ΔCt. The normalized fold change in the target mRNA expression was shown as $2^{-\Delta\Delta Ct}$ [61]. IL-6 primers were from Sino Biologicals (Interchim, Montluçon, France). Primer sequences used are described in supplemental experimental procedures section. Measurement of the ratio spliced to unspliced *XBP1* mRNA (*XBP1*s/u) was performed as in [62].

Immunohistochemistry

Human kidney sections were deparaffinised in toluene, then rehydrated using decreasing concentrations of alcohol ending in tris-buffered saline (10 mM Tris HCl pH 7.5, 140 mM NaCl (TBS)). Sections were then subjected to steam heat antigen retrieval in citrate buffer (0.01 M, pH 6.0) for 30 minutes, and washed in TBS. Sections were then treated with 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature (RT) to block endogenous peroxidase activity. Sections were subsequently incubated for 1h at RT with the primary antibody (rabbit polyclonal anti-CD40, Biosource International, Camarillo, USA) at a dilution of 1:50. After washing, epitopes were detected with the Envision+ system horseradish peroxydase (HRP) detection and revealed with diaminobenzidine (Dako A/S, Glostrup, Denmark) according to the manufacturer's instructions. The sections were then counterstained with haematoxylin and mounted. Negative control with omission

of the primary antibody was also performed. Antibodies used in this study are described in the supplemental experimental procedure section.

Western blotting

Cells were lyzed in RIPA buffer (Sigma-Aldrich) supplemented with 10µL/mL protease inhibitor cocktail (Sigma-Aldrich). Cell lysates were centrifuged at 15000g for 10 min, at 4°C. Protein quantification was performed using the Pierce BCA Protein Assay kit (Thermoscientific, Pierce, Brebières, France). Samples were denatured in SDS-PAGE buffer containing 2% SDS and 5% β-mercaptoethanol. Samples, 10µg protein, were then separated on a 10 or 15% acrylamide bis acrylamide gel. After transfer on PVDF (Immobilon-P membrane, EMD Millipore, Darmstadt, FRG), membranes were blocked with TBST (TBS containing 0.1% Tween 20) containing 2.5% BSA, and then incubated overnight with the indicated antibody at 1µg/mL at 4°C. After several washes with TBST, the membrane was incubated for 1h at RT with the appropriate secondary antibody conjugated to HRP. The detection was performed using an enhanced chemiluminescent substrate, ECL plus (GE healthcare) and the Image Quant LAS 4000 mini camera. Densitometry was performed using the Image Quant software. Antibodies used are described in the supplemental experimental procedure section.

Immunofluorescence

Cells grown on coverslips were fixed in 4% paraformaldehyde (PFA) for 10 minutes. After several washes with TBS, cells were permeabilized for 10 minutes in 0.1% Triton X100, washed in TBS and blocked with TBS containing 1% BSA and 1% FCS. Cells were incubated with a mouse monoclonal anti-CD40 antibody, at a 1/100 dilution (Biosource) in blocking buffer, for 2h, at RT. After several washes, cells were incubated with Alexa Fluor® 488 goat anti-mouse antibody at a 1/200 dilution, for 1h at RT (Molecular Probes, Invitrogen, Saint-Aubin, France). After several washes, nuclei were labelled with 1 µg/mL of DAPI solution for 1 minute. Then, coverslips were mounted on slides using

Fluoromount-G (Southern Biotech, Birmingham, USA). For negative control, cells were incubated with and identical isotype immunoglobulin and secondary antibody. Samples were analysed with a confocal microscope (Leica Microsystems, Nanterre, France) and Leica software.

Flow cytometry

Cytometry was performed on an Epics XL2 (Beckman coulter) flow cytometer and the EXPO 32 ADC software (Beckman Coulter).

Flow cytometry studies on HK-2 cells: Cells were incubated 2h at RT with anti-CD40 monoclonal antibodies (Santa-Cruz Biotechnologies, H-10, Clinisciences, Nanterre, France) at 10µg/mL in phosphate buffered saline (PBS) containing 1% BSA. Live cells were washed once with PBS and further incubated 30 min at RT with a 1/400 dilution of Alexa Fluor 488 (Life Technologies, Courtaboeuf, France).

Flow cytometry studies on platelets: whole blood (anticoagulated with anticoagulant citrate dextrose solution, solution A) was obtained from blood donors (convention established with the Etablissement Français du sang Aquitaine-Limousin). To minimize platelet activation, temperature variations and agitation where avoided during transport of blood samples and platelet preparation was performed within 2 hours. Briefly, platelet-rich plasma (PRP) was prepared by low speed centrifugation at 150g for 10 minutes at RT followed by a second centrifugation at 250g for 10 minutes at RT. After addition of apyrase (Sigma-Aldrich), at 0.05 UI/mL, platelets were pelleted by centrifugation at 1000g, 10 minutes and the platelet pellet was resuspended and washed in Tyrode buffer (Sigma-Aldrich). After fixation of platelets for 20 minutes at RT with 1% PFA, P-selectin and CD154 expressions were analyzed by flow cytometry with a monoclonal anti-P-selectin antibody (Beckmann Coulter, Villepinte, France) and a polyclonal rabbit anti-CD154 antibody (Santa-Cruz Biotechnologies). Monoclonal goat anti-mouse and goat anti-rabbit phycoerythrin-conjugated antibodies were used as secondary antibody (Beckmann Coulter).

Enzyme-linked immunosorbent assay (ELISA)

IL-6 in cell culture supernatants or cell lysates were measured with commercial ELISA kits (R&D systems Lille, France and RayBiotech, Tebu-Bio Le Perray en Yvelines, France, respectively), according to manufacturer's recommendations. VEGF measurements, IL-1β and TNF-α in cell culture supernatants were measured with commercial ELISA kits from R&D.

Transepithelial Paracellular Permeability assay

Transepithelial Paracellular Permeability was assayed by measuring permeability cell monolayer to dextran. HK-2 cells were grown on polycarbonate-permeable inserts (8 μM pore size, 6,5 mm diameter (Costar Corp, Cambridge, MA)). Apical to basal permeability of a FITC-labeled dextran (70 kDA, 25 mg/mL (Molecular Probes, Leiden, Netherlands)) was assayed using a multiwall fluorescence plate reader.

Viability, apoptosis, toxicity and proliferation assays

Cell viability was assessed with the Alamar blue cell viability reagent (Life Technologies SAS, Courtaboeuf, France). Lactate deshydrogenase Pierce colorimetric kit assay (Life Technologies SAS, Courtaboeuf, France), was used to measure cellular cytotoxicity. Cell counts were performed on a Malassez cell. Apoptosis measurement was performed by flow cytometry with the Annexin V-FITC apoptosis staining/detection kit (Abcam, Paris, France).

Statistics

All Data are presented as mean ± SD. Differences in the mean values between 2 groups were assessed by 2-tailed Student t-test. $P<0.05$ was taken to imply statistical significance.

Results

HK-2 cells are tolerant to hypoxic challenge.

We studied the viability of HK-2 cells grown in O_2 deprivation conditions. As no significant HK-2 cell apoptosis or necrosis is detected at 1% O_2 for up to 48 hours of culture [63], HK-2 cell susceptibility to 0.1% O_2 conditions was studied. As reported for 1% O_2 culture conditions, HK-2 cells tolerated hypoxic conditions, as no significant increase of apoptosis or loss of viability following hypoxic challenge or also following hypoxia/reoxygenation (not shown) was detectable (Figure 1A and B). Hypoxic culture conditions inhibited cell growth (Figure 1C). We also studied another consequence of hypoxia, the induction of epithelial cell permeability. In HK-2 cells, 1% O_2 conditions rapidly increases dextran permeability and morphological alterations of intercellular spacing [63]. The paracellular permeability of a 70 kDa FITC-labeled dextran was indeed found to be significantly increased by 24 hours of culture at 0.1% O_2 (Figure 1D).

CD40 expression by HK-2 cells is not altered by hypoxic stress.

We analyzed whether CD40 expression was modified by hypoxic conditions. We first analyzed CD40 expression by HK-2 cells. As expected [64], HK-2 expressed CD40 messenger RNA (mRNA) and CD40 protein (Figure 2A,B,C,). CD40 expression is known to be increased by inflammatory mediators [50]. The expression of CD40 was stimulated by TNFα and IL-1α in HK-2 cells. IL-1α was a strong inducer both at mRNA and protein levels (Figure 2C and D). CD154 binding to CD40 on HK-2 cells was functional as shown by activation of ERK and JNK kinases (Figure 2E). We also reinvestigated CD40 expression in human kidney. On kidney tissue sections, CD40 was expressed on proximal TECs and, particularly, on the apical brush bordure and basal Ruschka's labyrinth (Figure 2F). Next, we studied CD40 expression on HK-2 cells grown under hypoxic conditions. CD40 mRNA and protein expression were found not to be modified in hypoxic culture conditions or following a reoxygenation step after hypoxic stress (Figure 3A, B, C).

Hypoxic stress induces rapid HIF-1α expression and late endoplasmic reticulum stress features in HK-2 cells.

Hypoxic stress induced a strong increase of HIF-1α expression in HK-2 cells; the induction of HIF-1α could be detected as early as 2h post hypoxic stress (Figure 4A). Treatment of HK-2 cells with CD154 did not significantly modify HIF-1α expression (Figure 4A). We also studied the expression of HIF-1α target gene vascular endothelial growth factor (VEGF). Hypoxic stress led to the accumulation of VEGF in cell culture supernatants (Figure 3B). ER stress is commonly associated to hypoxia. To monitor ER stress, we studied the ER chaperone BiP (GRP78) and unfolded protein response (UPR) markers, phosphorylated eukaryotic translation-initiation factor 2α (eIF2α) and the expression of the alternative spliced form of *XBP-1* mRNA [65]. ER stress induction was found to be dependent on the duration of hypoxia. Indeed, ER stress markers were not detectable for short hypoxic challenges such as 1 or 3 hours (not shown). Increased BiP expression and eIF2α phosphorylation were detected at 24h and time thereafter (Figure 4C). Increased splicing of *XBP-1* mRNA was also observed from 24h and depicted a bell-shaped curve kinetics, characterized by early increase and attenuation at later time-points (Figure 4D). CD154 increased the splicing of *XBP1* mRNA, as was apparent at the onset of *XBP1* mRNA splicing and subsequent time points. In anoxia, as realized by incubating cells for 1 h with 50 ng/ml antimycin A, a mitochondrial respiratory chain blocking agent, splicing of *XBP-1* mRNA was moderately induced but no effect of CD154 on XBP-1 mRNA was detectable. However, upon removal of antimycin A, mimicking reoxygenation, splicing of *XBP-1* mRNA was further increased and the stimulatory effect of CD154 on *XBP1* mRNA splicing was apparent (supplemental Figure 1A).

CD154-dependant increased splicing of *XBP1* mRNA was also confirmed in HK-2 treated with tunicamycin, a N-glycosylation inhibitor and a strong inducer of ER stress (supplemental Figure 1B). Altogether these results showed that HIF-1α induction is an early feature of hypoxic stress and that ER stress is essentially observed at late culture times, likely to be related to the addition of other ER stress-promoting conditions during the progression of culture, such as glucose consumption [66].

CD154 stimulates IL-6 secretion by HK-2 cells in hypoxic conditions.

We next investigated the regulatory role of CD154 on IL-6 secretion in hypoxic stress conditions. IL-6 secretion was moderately increased after a brief (1h) hypoxic stress, an effect that was not visible after 24h of hypoxic stress (supplemental Figure 2). Treatment with CD154 strongly stimulated the secretion of IL-6 following 3 and 24h hypoxic stress or after hypoxia-reoxygenation (Figure 5A, B and C). Neither IL-1β nor TNF-α was induced by a brief (3 hours) hypoxic challenge, whether CD154 was added or not; TNF-α secretion could only be detected after a 48h hypoxic stress (not shown). IL-6 did not modify CD40 expression on HK-2 cells (not shown). IL-6 measurements in cell lysates confirmed secretion studies, showing that under hypoxic conditions treatment with CD154 increased intra-cellular IL-6 content following 3 and 24h hypoxic stress (Figure 5D and E). The induction of IL-6 secretion by CD154 in hypoxia was not due to differences in cell death or proliferation (Figure 1A, B and C). Altogether, the rapid CD154-mediated induction of IL-6 in hypoxic conditions suggested a role for CD154 in the regulation of IL-6 production by HK-2 cells in conditions associated with hypoxia.

CD154 is a potent inducer of IL-6 mRNA expression in HK-2 cells in hypoxic conditions.

Hypoxia significantly but only moderately induced IL-6 mRNA. The addition of CD154 potently stimulated the expression of IL-6 mRNA in HK-2 cells in hypoxic conditions (Figure 6A). Therefore, the stimulation of IL-6 production by CD154 was associated to an increased expression of IL-6 mRNA. Actinomycin experiments did not evidence a stabilization of IL-6 mRNA by CD154 (Figure 6B). The addition of cycloheximide resulted in a rapid decrease of IL-6 in HK-2 cells, indicating degradation and unstability of IL-6 protein; no stabilization of IL-6 protein by CD154 was observed (Figure 6C). Altogether, these results indicated a rapid and strong induction of IL-6 expression by HK-2 cells in hypoxia. The absence of detectable protein or mRNA stabilization by CD154 in experiments where transcription was blocked by actinomycin D suggested regulatory action at the transcriptional level.

Platelet activation by hypoxic stress leads to CD154 expression and release of soluble CD154

The contribution of CD154 to the regulation of IL-6 production raises the question of its generation in acute inflammation. Platelets are a major reservoir of CD154 in the blood and are readily brought in the inflammatory milieu as a consequence of blood leakage and chemotactic recruitment. Platelet activation is an early step in inflammation and leads to the display at the platelet surface and secretion of inflammatory mediators, including CD154. We therefore studied whether hypoxic stress alone activated platelets. Platelet activation was evaluated by assaying P-selectin expression at the platelet membrane. The expression of CD154 was followed both by its expression at the platelet membrane and its release as sCD154 in activated platelet supernatants. Results indicated that challenging platelets with hypoxic stress results in their activation, expression of CD154 at their surface and the release of soluble CD154 (supplemental Figure 3).

Discussion

Hypoxia is a common feature of inflamed tissues and is linked for an important part to an unbalanced O_2 demand/supply [11]. Over the past several years, the role of hypoxia in mediating and controlling inflammatory events has been increasingly appreciated. In the various contexts of tissue injury, the regulation of immune cell function for example operates in the context of O_2 deprivation [67]. Pro-inflammatory cytokines are key players in the progression of inflammation, and much remains to be understood in mechanisms regulating the cytokine network in hypoxia. Results of our study indicate that CD154 strongly induce IL-6 secretion in HK-2 cells grown under O_2 deprivation conditions. Although significant, the induction of IL-6 secretion by hypoxic stress *per se* remains limited. Such a moderate induction of IL-6 by hypoxia was also observed in other cellular models, as in human EC, epithelial cell lines or macrophages [68] [69] or was only observed following a reoxygenation step [70, 71]. The amplitude or duration of IL-6 induction by hypoxia also shows variability, according to several studies [68, 71-74]. Hypoxic stress is generally associated to an increased expression of IL-6 mRNA, as shown in fibroblasts, vascular smooth cells, ECs, astrocytes or cardiac myocytes, and in human (our study) and mouse TECs, after a brief or a prolonged hypoxic challenge [71-73, 75, 76]. Mechanisms linking the signaling pathways activated by hypoxia to the increased expression of IL-6 mRNA remain to be fully understood; however, some clues may be brought by transcription studies that show increased transcription of the IL-6 gene upon hypoxic stress [71-73, 75]. Indeed, the IL-6 gene promoter contains hypoxia-responsive elements [77].

The strong stimulation of IL-6 secretion by CD154 suggests a regulatory role for CD40 signaling in IL-6 production under hypoxic conditions. As no stabilization of IL-6 protein or mRNA was induced by CD154 treatment, our study suggests that a likely mechanism underlying CD154 stimulatory effect is the stimulation of IL-6 transcription. CD40 signaling is an activator of IL-6 promoter and of IL-6 gene transcription in a variety of cells [78, 79] including TEC [64]. The regulation of IL-6 gene expression has been indeed mainly found to be transcriptional. IL-6 mRNA is very unstable as for several cytokines and growth factors, a phenomenon that is believed to be critical to the kinetics of the

inflammatory response [80-83], however there is little characterization of IL-6 posttranscriptional regulators [84-88]. IL-6 was found in intracellular stores in some cells, allowing another potential mechanism for rapid IL-6 mobilization [89-91].

A rapid activation of IL-6 secretion is likely to be necessary to mount an inflammatory response in the context of tissue injury. In the inflammatory environment there are multiple potential source for CD154 as various immune cells express CD154 and release a soluble form when stimulated by inflammatory signals [50]. Activated platelets are also present in inflamed tissues and represent a significant source of CD154 [92, 93]. The hypoxic/inflammatory environment is likely to contribute to platelet activation via multiple mechanisms, including via the liberation of alarmins [94]. *In vitro* experiments with washed platelets, as showed in our study, and *in vivo* higher platelet reactivity observed in low O_2 environment may suggest that hypoxia is an inducer of platelet activation [95]. However, whether hypoxia *per se* is an inducer of platelet activation remains debated [96]. Moreover, there are signs of platelet activation in rodent ischemic AKI models rodent models [97]. Platelet activation is likely to coincide with blood flow impairment, hypoxia and ongoing inflammation in the tubular microcirculation in AKI, leading to CD154 expression on platelets and release of sCD154 and CD154-expressing microparticles. Platelets are also readily present at sites of tissue injury. AKI tubular inflammatory milieu is therefore a likely source of CD154, the ligand for CD40 that is expressed on endothelial cells and TECs. The rapid kinetics of platelet activation and CD154 expression makes CD154 a potential front line mediator of inflammation in the tubular microenvironment. *In vivo*, the silencing of CD40 improves renal inflammatory status in rodent model of warm and cold ischemia, strongly suggesting contribution of the CD154/CD40 dyad in the progression of inflammation during AKI [55]. It is therefore tempting to speculate that the CD154/CD40 dyad exerts a proinflammatory role at very early stages of tubular injury and that one of the underlying mechanisms involves the induction of IL-6 production.

The amplification of *XBP-1* mRNA splicing by CD154 in hypoxia demonstrates intersection between the UPR and CD40 signaling. Alternative *XBP-1* mRNA splicing is induced by CD40 triggering [62, 98,

99]. Our results confirm that CD154 is an UPR regulator. In fact, many recent evidences point to the regulated nature of the UPR, the ER being envisioned as a platform integrating regulatory cues [14, 15, 100]. The IRE-1/XBP-1 pathway is strongly linked to inflammation and the activation of the IRE-1 pathway in kidney epithelial cells activates NF-kB pathway and secretion of inflammatory cytokines [66]. The regulation of XBP-1 by CD40 signaling may therefore represent a regulatory interface in inflammation. The consequences of the regulation of the XBP-1 pathway by CD154 in TECs are an important issue that will need further studies.

Various mechanisms coordinating the control of cytokine production are operating in distinct biological contexts [101, 102]. In the ischemic kidney, multiple inflammation modifiers concur to tubular epithelium injury, lesion and regeneration and have to be understood in the context of hypoxia that is associated to kidney inflammatory lesions [32]. This multiplicity first underlines the limitation of addressing the role of single mechanistic pathways and treating one target at a time [103]. Second, the coincidental occurrence of hypoxia and inflammation add further complexity, as the production of inflammatory mediators can be understood within a cross-regulation context between hypoxic and inflammatory pathways. The powerful induction of IL-6 in hypoxia is likely to contribute CD154 deleterious effects that have been evidenced in a variety of I/R models, including the kidney [104, 105]. Indeed, IL-6 is a key pathophysiological player in kidney I/R tubular injury [106, 107]. Moreover, inhibition of CD40 signalling has demonstrated encouraging protective effects in a kidney ischemia-reperfusion injury [55].

In conclusion, our results cast light on the regulatory role of CD154 on IL-6 expression in hypoxic conditions. A better understanding of the role of CD154 in hypoxia-associated inflammation could bring new insights in the pathogenesis of inflammatory diseases.

Figure legends

Figure 1

HK-2 cells are tolerant to hypoxic challenge.

HK-2 cells were subjected to a hypoxic stress for the indicated time and viability was assayed by (A) the percentage of annexin-positive cells (positive control, cells treated with Fas-ligand (n=3,*p<0.05) and (B) LDH release (positive control from LDH cytotoxicity assay kit (n=4, *p<0;05). (C) Hypoxia inhibits HK-2 cell growth. HK-2 cells were plated at various density and cultures performed under hypoxic or normoxic conditions and cells counted. (D) Hypoxia increases HK-2 para cellular permeability. The increased trans-epithelial flux of 70 kDa FITC-labeled dextran was evaluated in supernatants of HK-2 cells grown 24h in hypoxic conditions (n=3, *p<0.05).

Figure 2

CD40 expression by HK-2 cells. CD40 expression on HK-2 cells was analyzed by flow cytometry (A) and immunofluorescence microscopy (B). Nuclei are counterstained with DAPI. The insert depicts control isotype staining. Scale bar: 50 μm. (C and D) HK-2 cells were treated with IL-1α (200IU/mL), TNF-α (10ng/mL) or interferon-γ (200IU/mL) for 24 hours and CD40 mRNA expression was analyzed by RT-qPCR (n=4, *p<0.05), insert illustrates the increase of CD40 mRNA expression after IL-1α treatment (C), or by flow cytometry (n=3, * p<0.05) (D). (E) Treatment of HK-2 cells with CD154 activates ERK and JNK pathways. HK-2 cells were incubated with rsCD154 and at indicated time, cells lysed. For each time point, total cell lysate was analyzed by immunoblotting with the indicated antibodies. (F) CD40 is expressed on kidney tubular proximal epithelium. The expression of CD40 was studied by immunohistochemistry on tissue sections of cortical renal parenchyma (top and bottom panels, low and high magnifications, respectively). The epithelium of all proximal tubules expresses CD40; strong labelling is observed on both the brush border and the basal pole of tubular epithelial cells (arrows).

Figure 3

CD40 expression is not modified by hypoxic stress. HK-2 cells were incubated under hypoxic condition, and CD40 mRNA expression was analyzed by RT-qPCR (n=3) (A) and flow cytometry (n=3) (B). (C) HK-2 cells were subjected to variable hypoxia times followed by 24h culture in normoxic conditions and CD40 expression was analyzed by flow cytometry (n=3).

Figure 4

Inductions of HIF-1α and endoplasmic reticulum stress in HK-2 cells grown under hypoxic conditions. (A) HIF-1α is induced in response to hypoxic stress. Top panel: cells were subjected to hypoxia for depicted times in the presence or not of 200ng/mL rsCD154 and immunoblotting with anti-HIF-1α antibody was performed on cell lysates (CNX, calnexin, used as a loading control). Bottom panel: HK-2 cells were subjected to short hypoxia times and immunoblotting with anti-HIF-1α antibody was performed on cell lysates. (B) VEGF is increased in HK-2 cells supernatants in response to hypoxic stress: VEGF expression was quantified by ELISA in supernatants of HK-2 cells grown under hypoxic conditions during depicted times (UD, undetectable levels). (C) Hypoxia induces time dependent ER stress features in HK-2 cells. HK-2 cells were grown under hypoxic conditions for the indicated times and immunoblotting with antibodies against BiP, eIF2α and Phospho-eIF2α (P-eIF2α) was performed on cell lysates (CNX, calnexin, used as a loading control). (D) Fold induction of the spliced/unspliced ratio of XBP-1 (XBP1 s/u) mRNA in HK-2 cells subjected to hypoxic stress for the indicated times in the presence or absence of rsCD154.

Figure 5: CD154 stimulates IL-6 production in HK-2 cells under hypoxic conditions. Cells were subjected to hypoxia for various times in the presence or not of 200ng/mL rsCD154, and the expression of IL-6 measured by ELISA. IL-6 concentrations in HK-2 cell supernatants after 3h (A) or 24h (B) culture in hypoxia. (C) IL-6 concentrations in HK-2 cell supernatants after the indicated

hypoxia times followed by 24h culture in normoxic conditions. IL-6 concentrations in HK-2 cell lysates after 3h (D) or 24h (E) culture in hypoxia.

Figure 6: CD154-mediated stimulation of IL-6 secretion by HK-2 cells is associated to a strong IL-6 mRNA induction. (A) HK-2 cells were incubated 3h under hypoxic conditions in the presence or not of rsCD154 and IL-6 mRNA expression was analyzed by RT-qPCR (n=8, *p<0.05). (B) HK-2 cells were incubated 3h under hypoxic conditions in the presence or not of rsCD154 and actinomycin, and IL-6 mRNA expression was analyzed by RT-qPCR (n=8, *p<0.05). (C) HK-2 cells were incubated 3h under hypoxic conditions in the presence or not of rsCD154 and CHX, and cells lysed. For each condition, IL-6 expression was analyzed from cell lysate by ELISA (n=3, *p<0.05).

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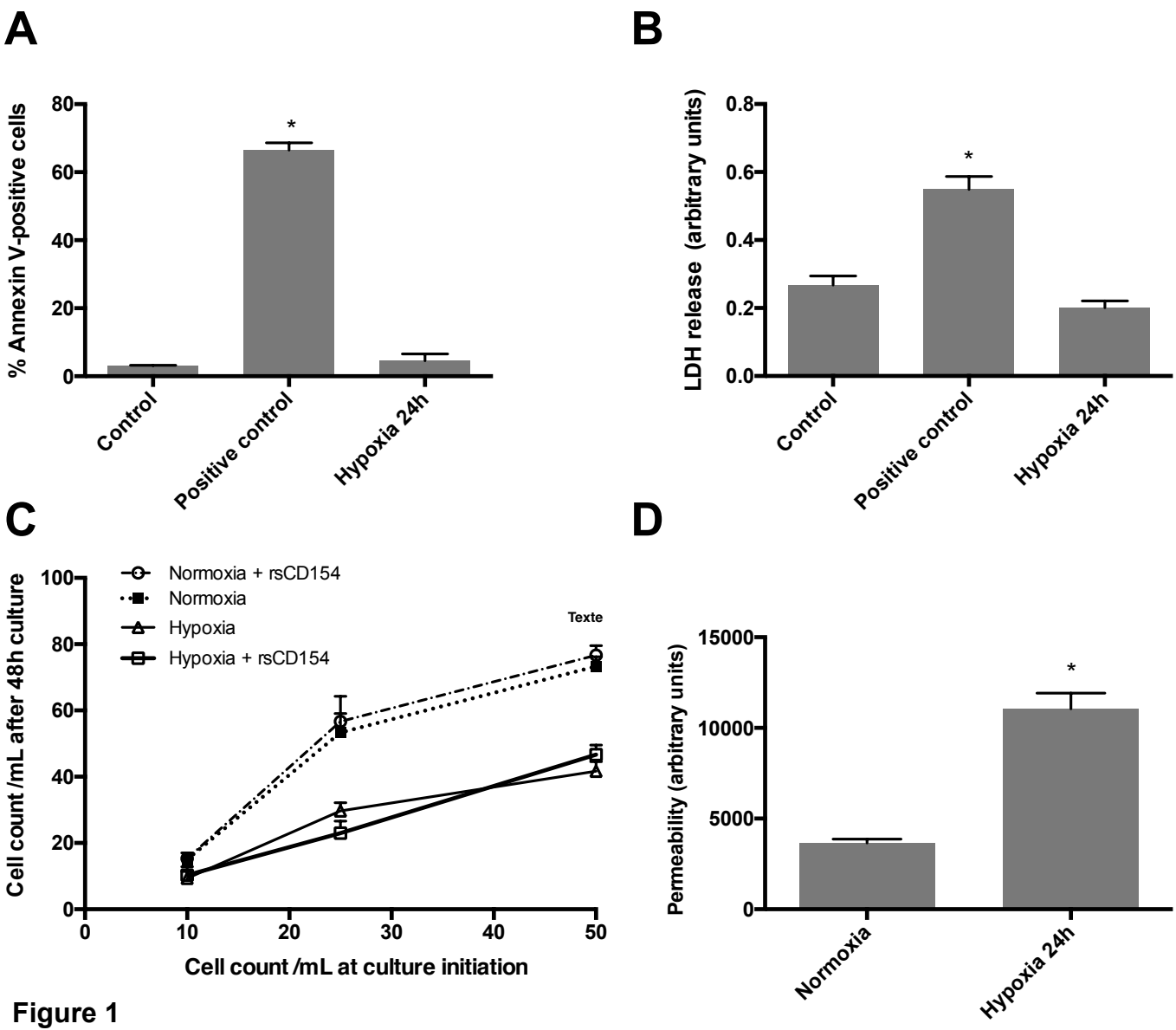
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LDH release (arbitrary units)
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0.4
0.2
0.0
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Control
Positive control
Hypoxia 24h
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Figure 1

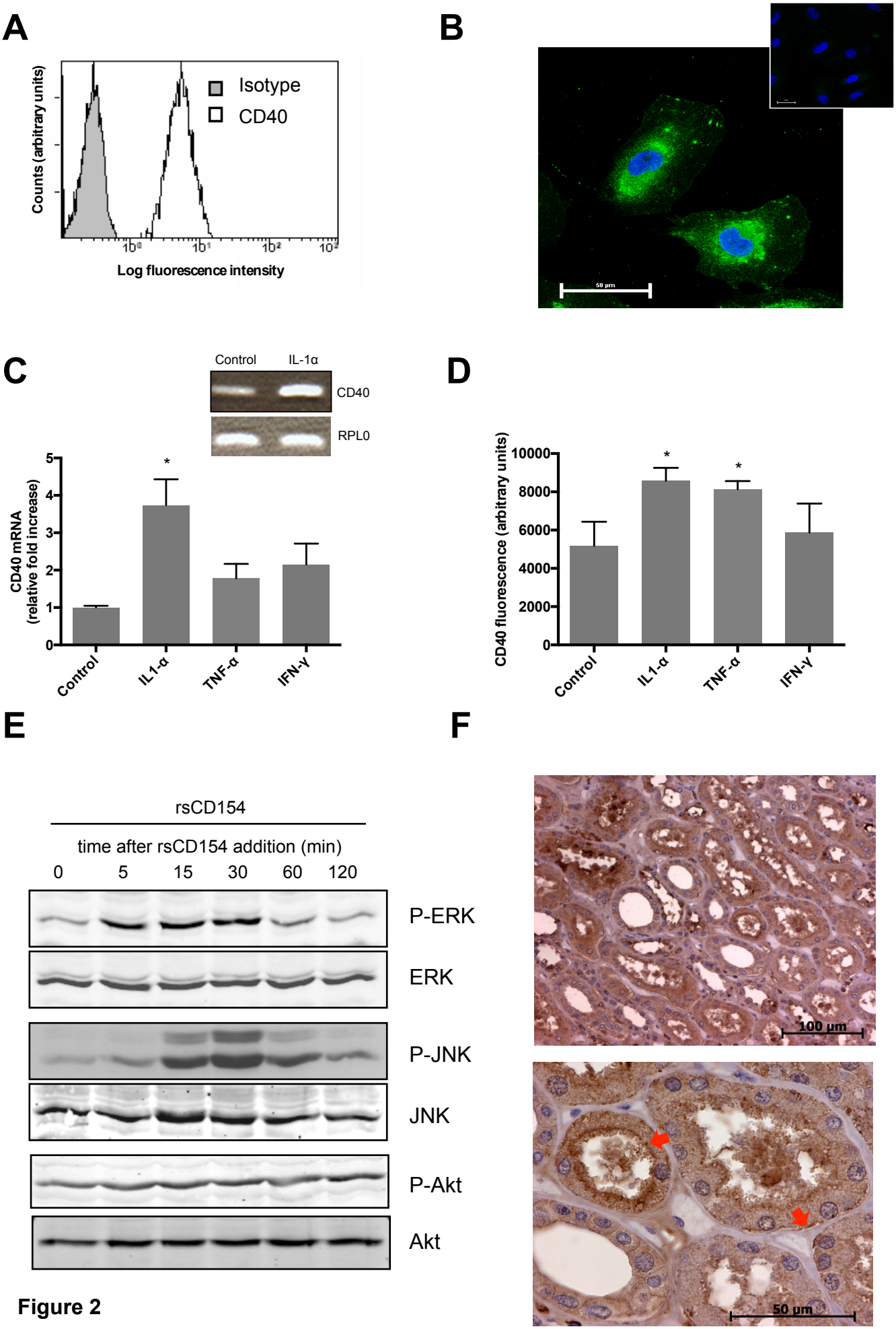
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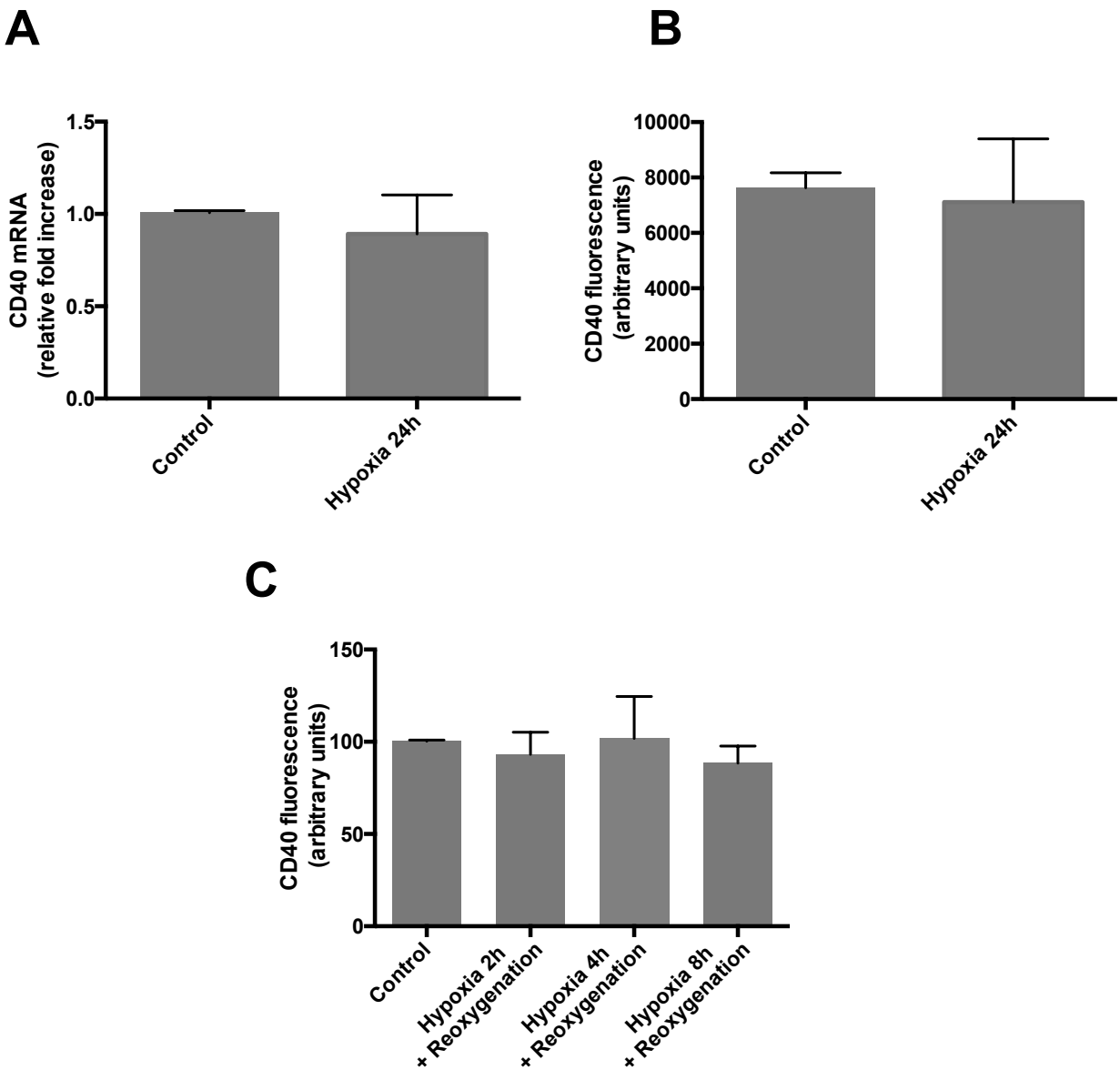
Figure 2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2000
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Hypoxia 24h
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(arbitrary units)
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Control
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+ Reoxygenation
Hypoxia 4h
+ Reoxygenation
Hypoxia 8h
+ Reoxygenation

Figure 3

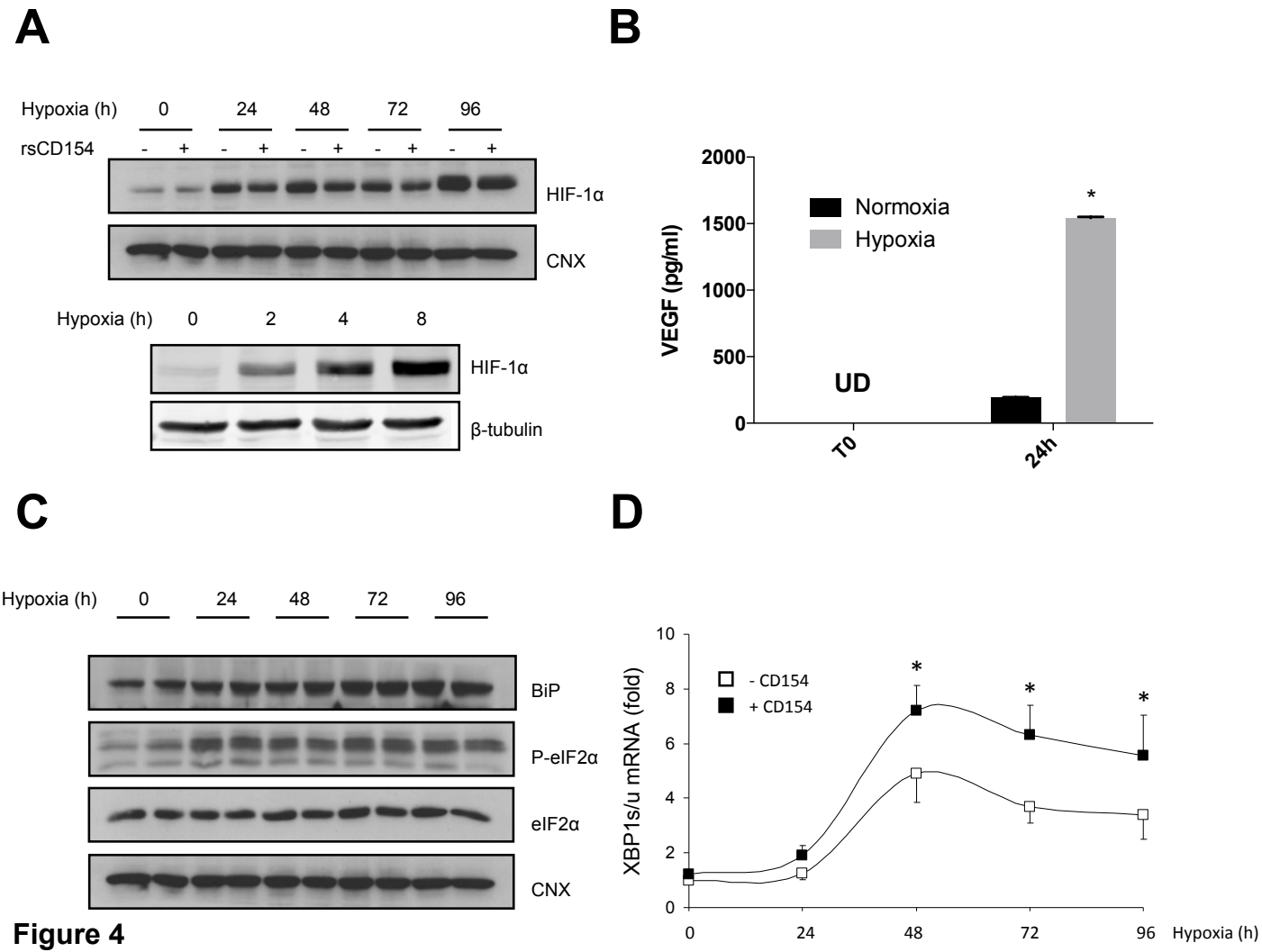

A
Hypoxia (h) 0 24 48 72 96
rsCD154 - + - + - + - + - +
HIF-1α
CNX
Hypoxia (h) 0 2 4 8
HIF-1α
β-tubulin
B
VEGF (pg/ml)
2000
1500
1000
500
0
Normoxia
Hypoxia
*
UD
T0
24h
C
Hypoxia (h) 0 24 48 72 96
BiP
P-eIF2α
eIF2α
CNX
D
XBP1s/u mRNA (fold)
10
8
6
4
2
0
- CD154
+ CD154
*
*
*
0 24 48 72 96 Hypoxia (h)

Figure 4

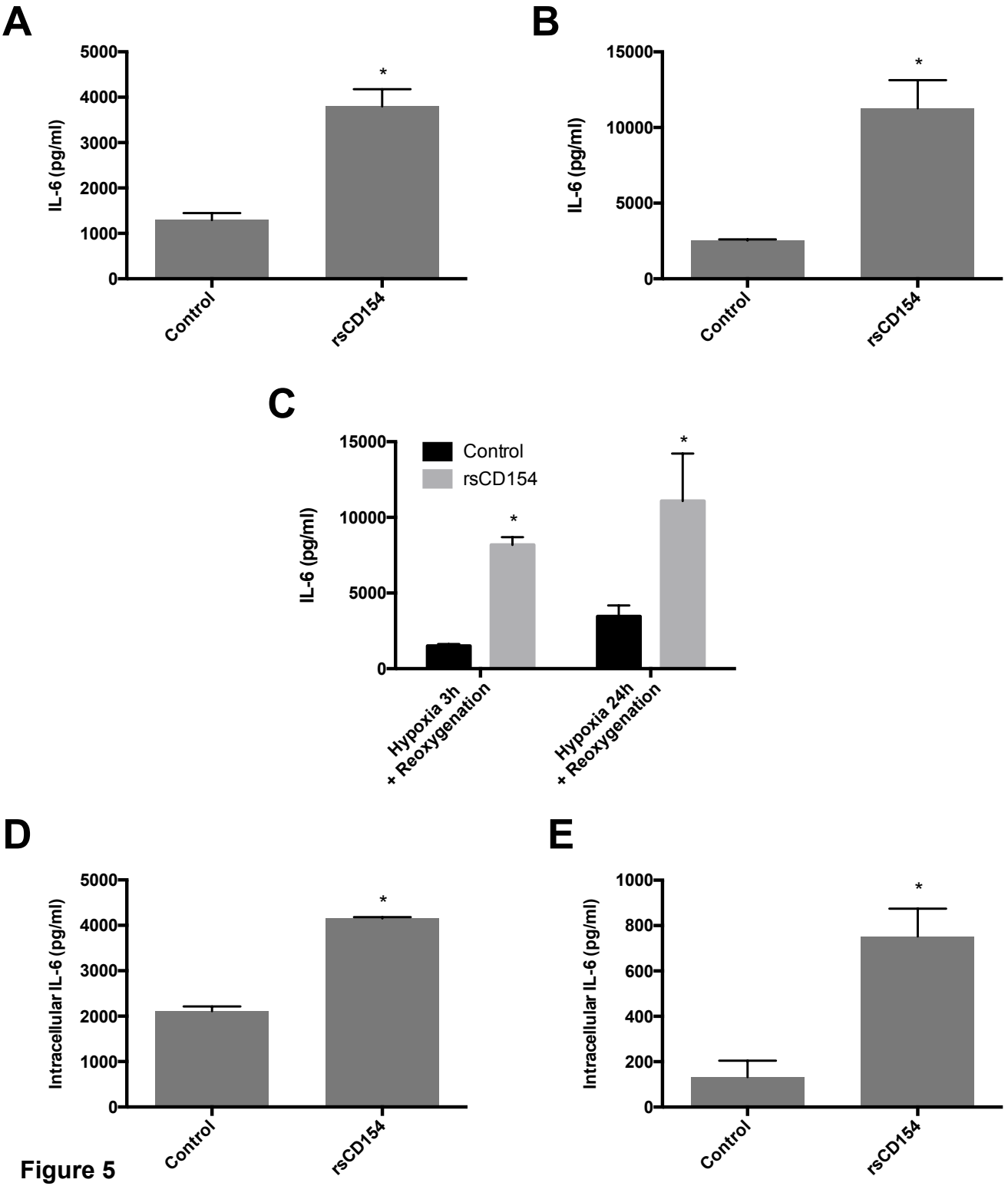
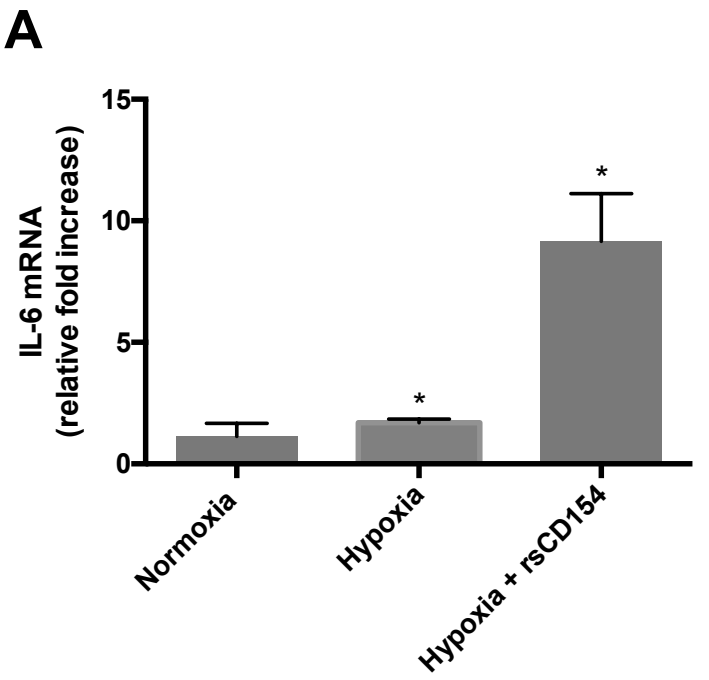
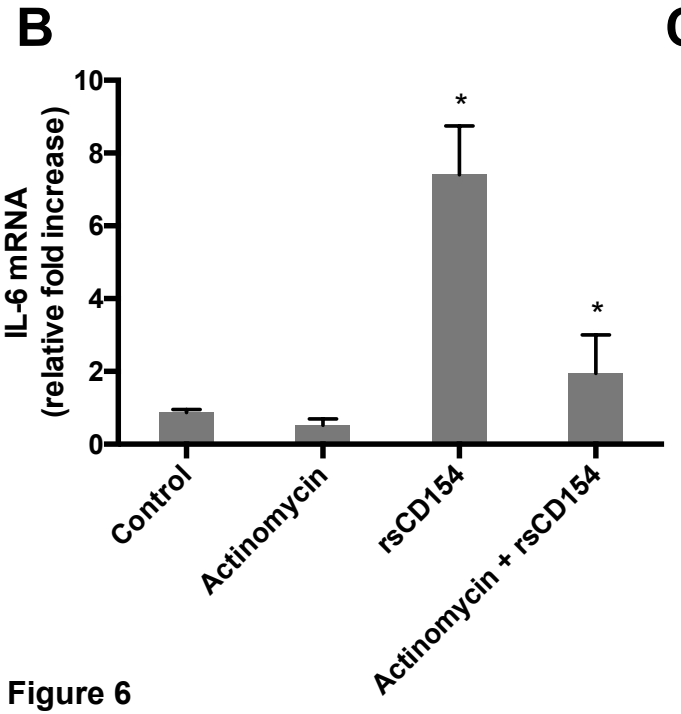

A
5000
4000
3000
2000
1000
0
IL-6 (pg/ml)
*
Control
rsCD154
B
15000
10000
5000
0
IL-6 (pg/ml)
*
Control
rsCD154
C
15000
10000
5000
0
IL-6 (pg/ml)
Control
rsCD154
*
*
Hypoxia 3h
+ Reoxygenation
Hypoxia 24h
+ Reoxygenation
D
5000
4000
3000
2000
1000
0
Intracellular IL-6 (pg/ml)
*
Control
rsCD154
E
1000
800
600
400
200
0
Intracellular IL-6 (pg/ml)
*
Control
rsCD154

Figure 5


A
IL-6 mRNA
(relative fold increase)
15
10
5
0
*
*
Normoxia
Hypoxia
Hypoxia + rsCD154


B
IL-6 mRNA
(relative fold increase)
10
8
6
4
2
0
*
*
Control
Actinomycin
rsCD154
Actinomycin + rsCD154

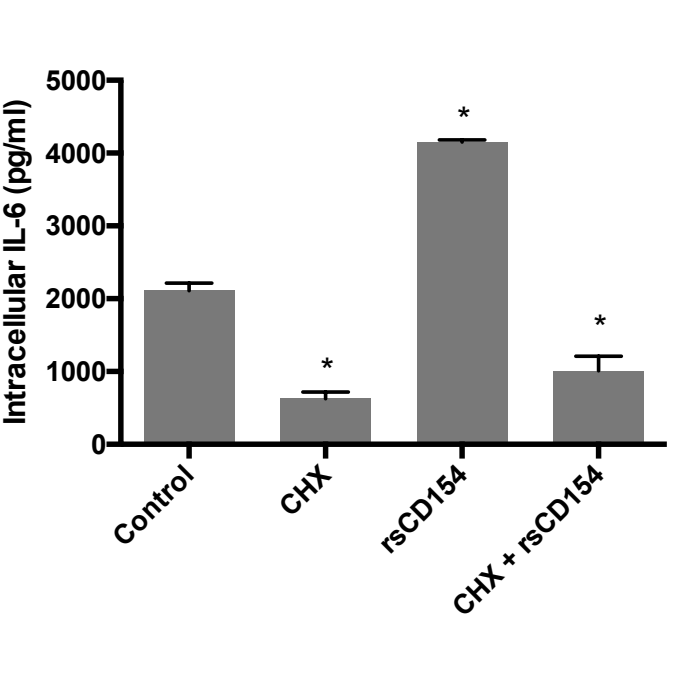
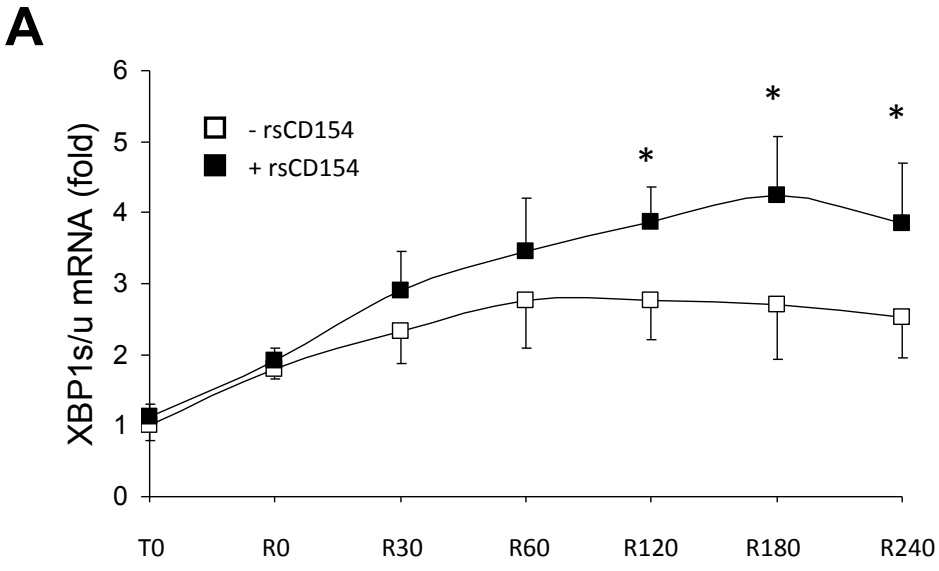
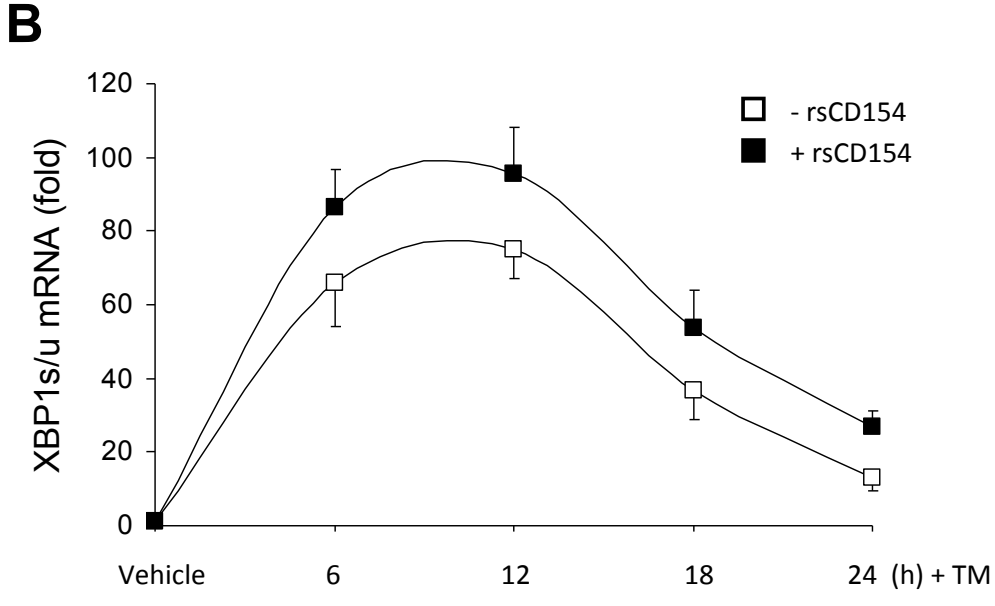
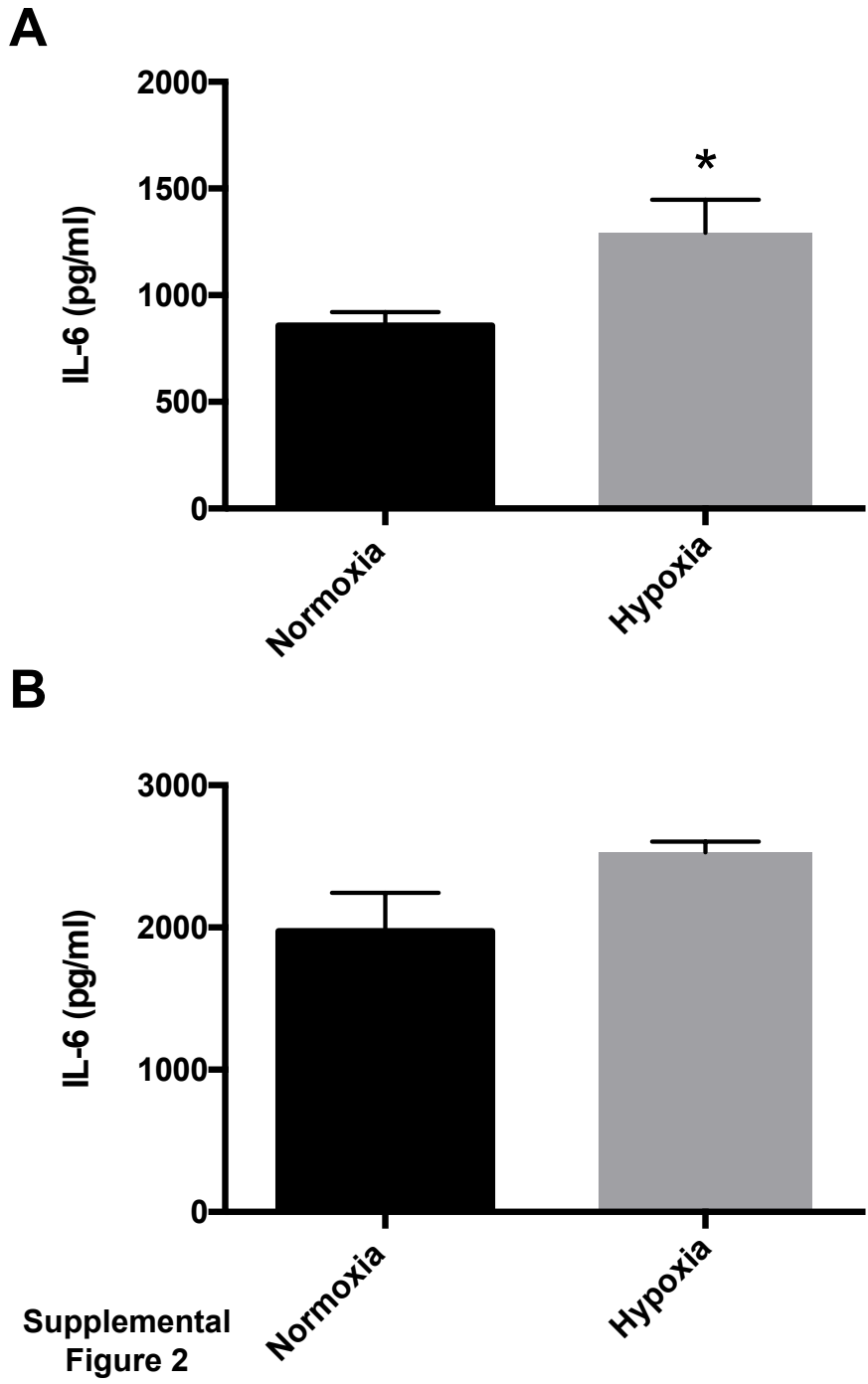

C
Intracellular IL-6 (pg/ml)
5000
4000
3000
2000
1000
0
*
*
*
Control
CHX
rsCD154
CHX + rsCD154

Figure 6

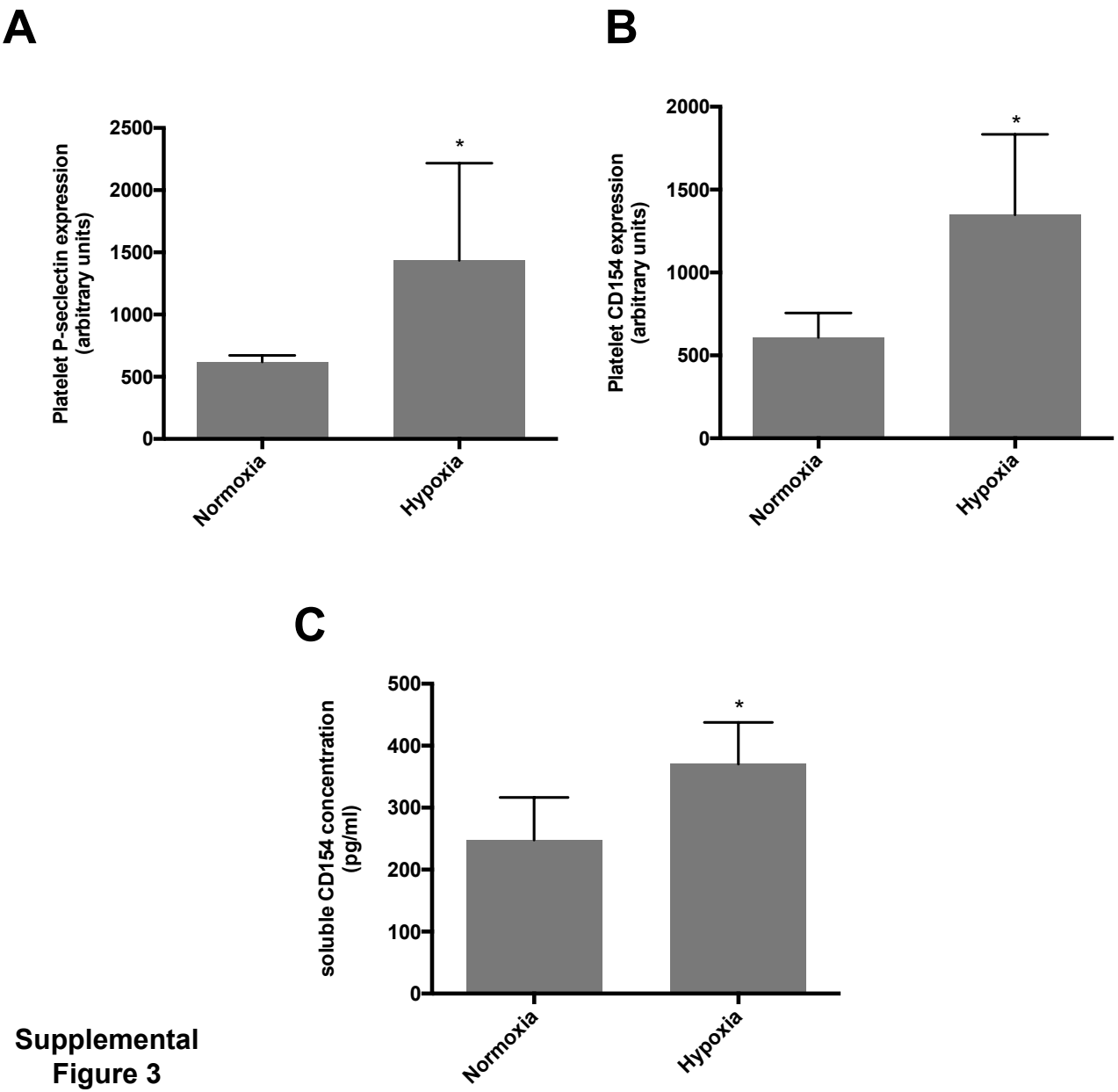




Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3

6. Rôle potentiel du CD154 dans la génèse des lésions tubulaires de l'IRA sur un modèle murin d'I/R rénale (travail en cours)

Dans une approche parallèle, nous avons étudié le rôle du couple CD154/CD40 dans la physiopathologie des lésions tubulaires dans l'IRA en nous appuyant sur l'utilisation de deux groupes de souris transgéniques, des souris déficientes pour le CD154 (CD154KO) ou pour le CD40 (CD40KO). L'importance de l'étude de souris déficientes pour le CD40 est notamment du au fait que des travaux récents ont identifié des récepteurs additionnels au CD154 (intégrines αIIbβ3, α5β1, and αMβ2). La réponse des souris CD154KO et CD40KO pourrait être ainsi différente, comme décrit dans d'autres modèles, par exemple celui de la réponse inflammatoire au stress métabolique (146).

Comme discuté précédemment, une des grandes difficultés de compréhension des mécanismes moléculaires intervenant dans la physiopathologie de l'IRA réside dans la difficile extrapolation aux patients des résultats obtenus à l'aide de modèles animaux. Ces modèles ont tous des limites et ne reproduisent que peu ou partiellement les événements entrant en jeu dans ce processus lésionnel particulièrement complexe. Un modèle animal idéal devrait reproduire la cinétique du sepsis, les événements hémodynamiques, immunologiques (inflammatoires/anti-inflammatoires) et les lésions histologiques dans les organes clés comme le rein.

Ces modèles ont cependant permis des progrès significatifs, notamment ceux par administration de LPS, création d'un foyer infectieux polymicrobien par ligature et brèche du caecum ou par infection avec une bactérie spécifique. Les modèles de clampage/déclampage du pédicule rénal ont permis également de faire des progrès significatifs dans la compréhension du rôle délétère joué par les phénomènes d'I/R et de comparer les caractéristiques des lésions rénales dans les deux modèles. Dans une étude récente comparant deux modèles lésionnels, I/R et ligature/perforation du caecum, les auteurs concluent sur la différence importante entre les lésions observées (nécrose tubulaire absente mais signes d'apoptose tubulaire, infiltration minime par les neutrophiles, dans le modèle septique...) et donc sur l'existence de processus physiopathologiques distincts (147). Mais ces phénomènes sont complexes et cette dichotomie entre I/R et inflammation semble discutable. Comme résumé dans la revue, différentes études font état soit d'une réduction du flux sanguin rénal avec augmentation des résistances vasculaires rénales, soit de l'absence d'une telle réduction dans l'IRA associée au sepsis (148). Il est probable que les deux types d'événements ont lieu de manière simultanée et d'une manière hétérogène quant à leur distribution locale et régionale dans le rein. En effet, ces processus inflammatoires pourraient contribuer à altérer la microcirculation sanguine, avec une réduction de la pression partielle en oxygène médullaire malgré l'augmentation du DSR (62-64). La distribution irrégulière des lésions de nécrose tubulaires est un reflet probable de cette hétérogénéité des altérations de perfusion. A l'inverse, l'atteinte microcirculatoire est un événement déclencheur de différentes cascades de la réaction inflammatoire (148,149).

Dans le but d'étudier le rôle des plaquettes sanguines et du couple CD154/CD40 dans l'IRA du sepsis, nous avons choisi de développer dans un premier temps un modèle d'I/R rénal. Trois modèles de souris transgéniques ont été développés dans cet objectif, des souris CD154KO, CD40KO et des souris thrombopéniques.

6.1. Animaux et méthodes

6.1.1. Animaux

Ce projet a été effectué dans le respect des règles d'éthique en expérimentation animale sous la responsabilité d'un personnel autorisé. Le protocole a été approuvé par les comités national et régional d'éthique pour l'expérimentation animale (avis 201705171524280-V2 APAFiS # 9960). Les animaux ont été hébergés dans l'animalerie transgénique de l'université de Bordeaux site Carreire) avec un accès libre à l'eau et à la nourriture.

Les souris utilisées pour le protocole expérimental sont des souris de lignée BALB/c mâles sauvages WT (Wild Type) et des souris dont les gènes codant pour le CD154 et le CD40 ont été invalidés (CD154KO et CD40KO). Toutes les expériences ont été effectuées sur des souris de même fond génétique BALB/c, de même sexe et de même stade de développement. Il existe en effet des susceptibilités différentes à l'I/R rénale selon le fond génétique et, dans le même fond, selon le sexe de l'animal. Par exemple les souris NIH Swiss sont résistantes (150) ; les fonds BALB/c et C57BL/6JRj sont régulièrement utilisés pour les protocoles d'I/R rénale. Dans le fond BALB/c, les mâles sont plus susceptibles au développement des lésions tubulaires après I/R rénale (151).

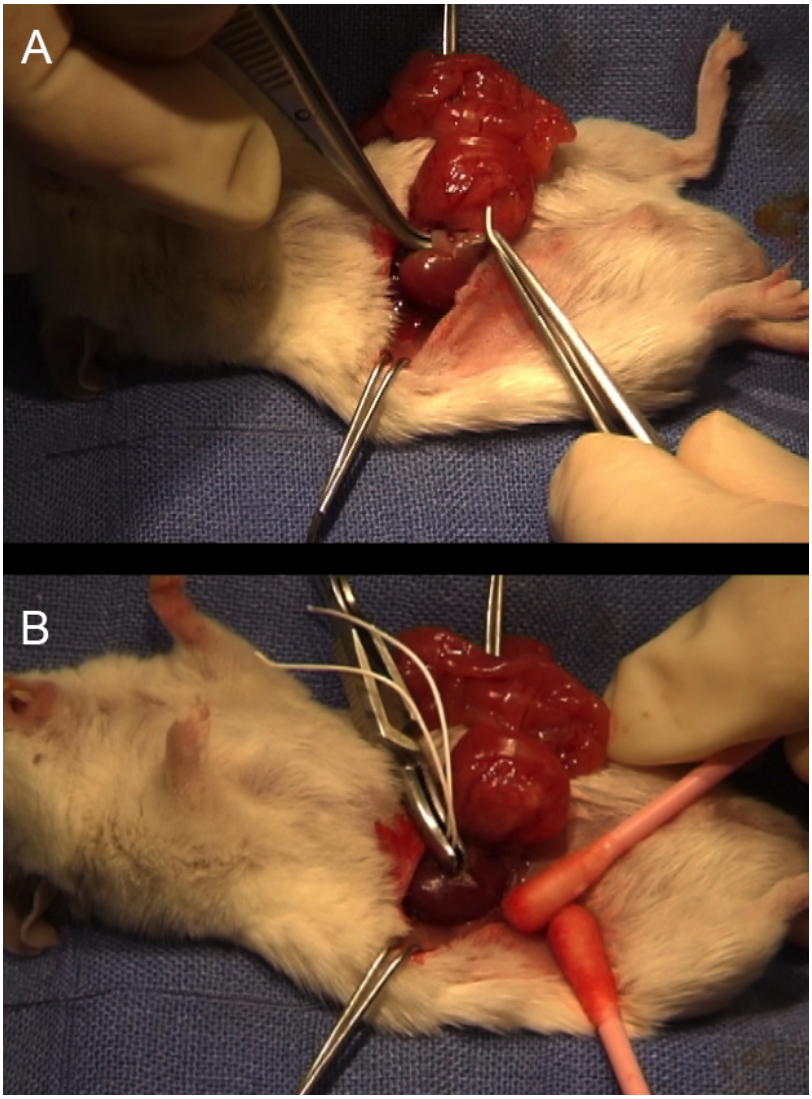
Les souris WT proviennent du laboratoire Charles River, Bois des Oncins France. Les souris CD154KO sur fond génétique C57BL/6JRj proviennent du Jackson Laboratory, Bar Harbor, USA (lignée 007074). Les souris CD40KO sur le fond génétique BALB/N, proviennent du Dr Claudia Chiodoni, Immunotherapy and Gene Therapy Unit, Department of Experimental Oncology, Milan. Les souris génétiquement thrombopéniques (cMplKO homozygotes, dont le taux de plaquettes est abaissé de 90% par rapport aux souris WT (152) proviennent du Dr Michelle Souyri, CNRS UMR7622 "Biologie du développement", Paris. Nous avons transmis les mutations des gènes codant pour le CD154 et le CD40KO sur le fond génétique BALB/c par croisements répétés (>10). Les expérimentations ont par la suite été réalisées après au minimum 10 générations, au-delà desquelles il est considéré que moins de 0.1% du patrimoine génétique de la lignée originelle persiste chez les descendants. Le suivi génétique des souris a été effectué par génotypage (Plateforme Génome Transcriptome de Bordeaux, Université de Bordeaux).

6.1.2. Protocole d'I/R du pédicule rénal

Des souris mâles âgées de 12 à 20 semaines ont été utilisées. Nous avons fait le choix de clamper le rein droit tout en gardant intact le rein gauche, ce qui permet de conserver la fonction rénale, tout en préservant la pertinence scientifique du modèle, contrairement à des protocoles de clampage bilatéral induisant une mortalité importante.

Après rasage de l'abdomen et désinfection cutanée, les souris sont anesthésiées par un mélange anesthésique et analgésique (kétamine 100mg/kg + xylazine 10mg/kg, administration intrapéritonéale) puis un complément est apporté en fin de procédure. Les animaux sont placés sur une table chauffante. Le pédicule rénal droit est abordé par une laparotomie médiane *(**Figure 1A**)*. Un clampage atraumatique du pédicule vasculaire rénal droit est alors mis en place pour une durée de 30 minutes *(**Figure 1B**)*. Du sérum physiologique à 37°C est instillé durant toute la durée de l'intervention. Après ischémie, le clamp est retiré. La bonne recoloration du rein est vérifiée puis l'abord chirurgical est suturé en deux plans. Le réveil des souris se fait en salle d'expérimentation en cage sur un tapis chauffant. L'analgésie postopératoire est assurée par du tramadol pendant 48h.

Figure 1 : protocole de clampage du pédicule rénal droit. (A) abord du pédicule et mise en place du clampage ; (B) clampage atraumatique du pédicule



Les souris sont ensuite observées quotidiennement. Toute souris présentant des signes de souffrance et/ou détresse dans les 48 premières heures (perte de poids > 20%, état prostré, poil ébouriffé, déshydratation, dyspnée), et en l'absence de

récupération rapide, a été euthanasiée par dislocation cervicale. Les souris sont ensuite sacrifiées par dislocation cervicale après une durée de reperfusion variable selon le protocole.

6.1.3. Données recueillies

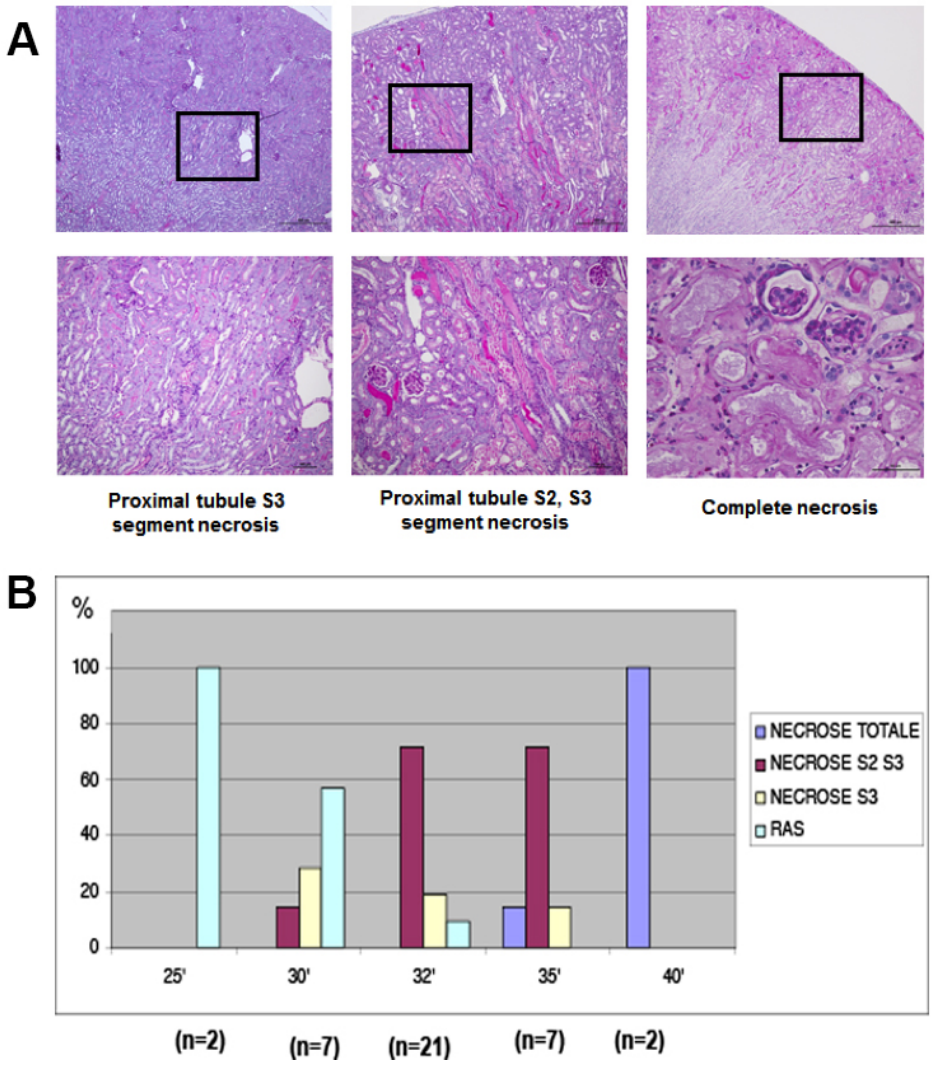
Les données ont été recueillies à J1, J2, J3, J4, J7, J10, J15, et J21 après reperfusion. Les temps tardifs 15 et 21 jours ont été choisis à partir des données de la littérature scientifique, il s'agit de temps auxquels la régénération des cellules tubulaires est visible histologiquement. A chaque temps, un prélèvement sanguin de 50 µL par prélèvement intracardiaque juste après sacrifice) pour dosage des médiateurs de l'inflammation a été effectué. Les reins ont également été prélevés pour analyse histologique, morphologique et immunohistochimique, et quantification d'ARN messagers par RT-qPCR (real time quantitative polymerase chain reaction).

6.2. Résultats

6.2.1. Mise au point de la durée d'ischémie

Un point important a été de définir la durée optimale d'ischémie. Une ischémie trop courte ne permet pas d'obtenir des lésions tubulaires. Une ischémie trop longue entraîne des lésions tubulaires qui ne permettent pas d'analyse histologique comparative et rendent impossible la régénération des cellules tubulaires et une reprise de fonction du rein. Dans le but de réduire au maximum le nombre des souris utilisées, des I/R pilotes ont été effectuées sur des souris WT. La première zone de souffrance correspondait aux parties terminales (S2, S3) du tube contourné proximal. La partie S2 est corticale et la partie S3 est à cheval sur la corticale et la médullaire rénale. Dans une première phase, nous avions déterminé un temps optimal d'ischémie permettant d'avoir histologiquement des lésions nécrotiques après un jour de reperfusion, touchant l'épithélium tubulaire des segments S3 et S2, sans avoir de nécrose extensive du parenchyme (**Figure 2A et B**). La durée optimale d'ischémie était de 32 minutes.

Figure 2 : Evolution de la nécrose tubulaire après 32 min d'ischémie et 24h de reperfusion. (A) Gradation des lésions : pas de lésion visible (RAS), nécrose du segment S3 du tube contourné distal (TCD), nécroses des segments S2 et S3 du TCD ou nécrose totale ; (B) Quantification des lésions (%).

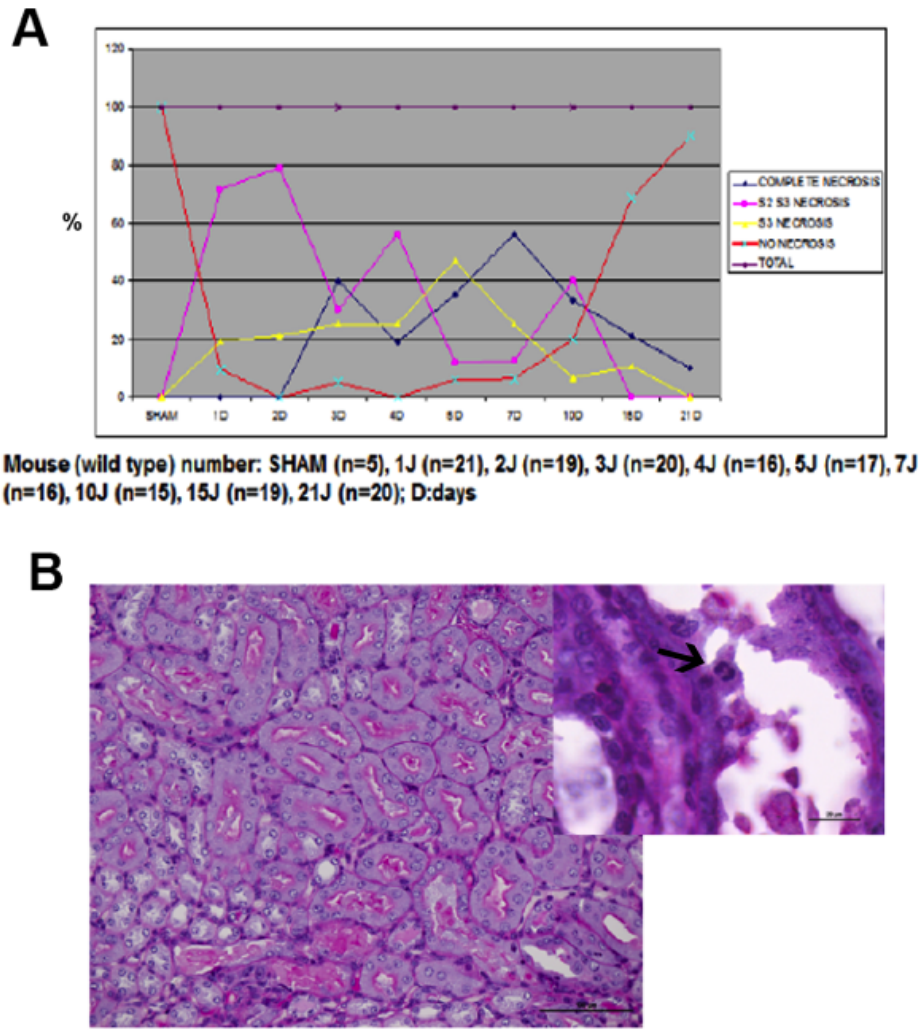


6.2.2. Cinétique d'évolution des lésions tubulaires

Nous présentons les résultats concernant l'analyse comparative entre souris WT et CD154KO (les coupes correspondant aux souris CD40KO sont en cours d'analyse). Une fois le temps d'ischémie déterminé, nous avons réalisé plusieurs temps de reperfusion pour évaluer l'évolution des lésions dans le temps (**Figure 3A**). Les résultats de l'analyse histologique montrent que les lésions sont établies dès le premier jour après la reperfusion et persistent jusqu'à 15 jours après celle-ci. Dans chaque cas le rein controlatéral non clampé était également analysé. Nous nous sommes efforcés de réaliser des groupes de souris pour chaque temps suffisamment importants (>10) pour pallier aux différences individuelles de réaction au stress rénal. Comme attendu, les lésions initiales concernent avant tout la région S2-S3. Histologiquement, nous avons remarqué que la régénération était précoce avec des images de mitose au niveau de l'épithélium tubulaire dès 48h après la reperfusion

(**Figure 3B**). Morphologiquement, l'amendement des lésions est bien visible à partir de 15 jours et complète à 21 jours. Une étude immunohistochimique est en cours, à l'aide d'un anticorps anti-Kidney Injury Molecule-1 (KIM-1), un marqueur lésionnel de plus en plus utilisé. KIM-1 est une molécule transmembranaire comportant un domaine immunoglobulin et mucine et dont l'expression est très augmentée sur l'épithélium proximal du rein ischémique (153). Nous évaluons également la prolifération cellulaire à chacune des étapes par marquage avec un anticorps anti Ki-67, antigène présent sur les cellules en phase de mitose.

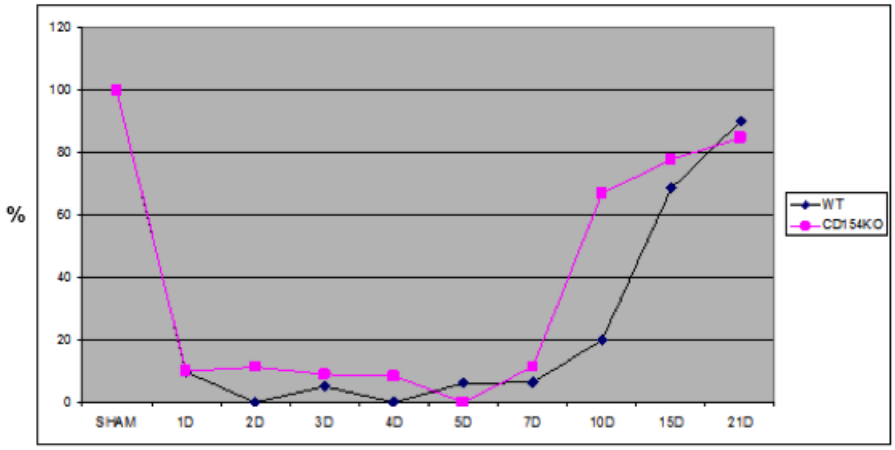
Figure 3 : cinétique de récupération des souris WT après I/R rénale. (A) analyse histologique des lésions rénale (%) ; (B) prolifération des cellules tubulaires après 48h de reperfusion avec apparition de images de mitose



6.2.3. Rôle du couple CD154/CD40 dans la cinétique lésion/ régénération de l'épithélium tubulaire

Ce protocole d'I/R a ensuite été appliqué pour l'étude comparative de l'évolution lésionnelle au cours du temps dans les différents groupes de souris, sauvages, CD154KO et CD40KO. Les résultats obtenus montrent une différence dans la cinétique de régénération des souris WT versus CD154KO, l'épithélium tubulaire des souris CK154KO régénérant plus précocement. A J10, 20% de souris sauvages ne présentent pas de lésions nécrotiques tubulaires contre 80% pour les souris CD154KO **(Figure 4)**.

Figure 4 : Cinétique de régénération de l'épithélium tubulaire (pourcentage de souris ne présentant pas de lésion)



CD154KO mouse number: SHAM (n=6), 1J (n=10), 2J (n=9), 3J (n=11), 4J (n=12), 5J (n=11), 7J (n=18), 10J (n=9), 15J (n=9), 21J (n=13)

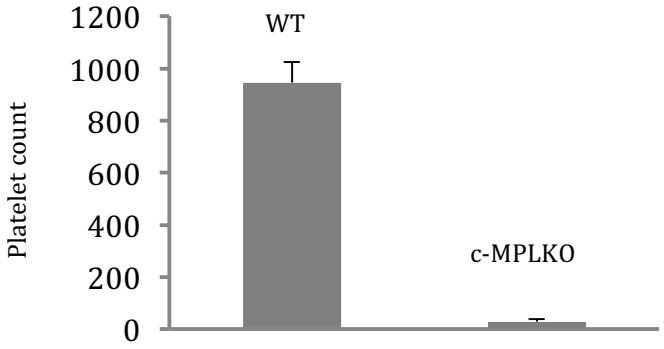
6.3. Discussion et perspectives

La cinétique de récupération plus rapide chez les souris CD154KO peut avoir deux explications. D'une part, la sévérité lésionnelle aux temps initiaux peut être réduite en l'absence de CD154. Ceci est très difficile à évaluer morphologiquement. L'analyse immunohistochimique (anticorps anti-KIM1) permettra de confirmer cette hypothèse. Une seconde hypothèse serait que l'absence de CD154 influence la régénération tubulaire en favorisant celle-ci (migration, prolifération des CET à l'origine de la régération). Nous privilégions à ce stade la première hypothèse, l'activation du couple CD154/CD40 ayant, comme discuté auparavant, des conséquences pro-inflammatoires, et donc potentiellement pro-lésionnelles. En fait, le rôle pro-lésionnel du CD154 a été mis en évidence dans d'autres modèles d'I/R (mésentère, cerveau, foie poumon…) (154-157). L'analyse par RT-qPCR et les dosages dans les prélèvements sanguins permettront également d'établir et de comparer l'induction de la synthèse d'IL-6 et sa concentration plasmatique.

La question suivante est donc de savoir si cet effet peut également être mis en évidence chez les souris CD40KO. Le travail d'analyse est en cours. Comme indiqué plus haut les phénotypes des souris CD154KO et CD40KO ne sont pas superposables en raison notamment de la présence de ligands additionnels pour le CD154 et l'engagement du CD154 avec ces ligands étant pro-inflammatoire (induction de cytokines pro-inflammatoires).

Le rôle des plaquettes a été seulement indirectement apprécié dans l'I/R rénale par l'utilisation d'anti-plaquettaires comme le clopidogrel (158,159). Pour évaluer directement le rôle des plaquettes nous calquerons le même schéma expérimental avec des souris thrombopéniques. Nous avons généré des souris génétiquement thrombopéniques qui sont prêtes à être utilisées (c-MPLKO, ***Figure 5***). Elles ne présentent pas de saignement spontané ni d'altération de la coagulation majeure, un allongement modéré du temps de saignement est simplement observé (160). Nous évaluerons comparativement aux souris WT, par analyse morphologique et immunohistochimique, le décalage la courbe de cinétique lésion/ réparation des lésions tubulaires. Nous apprécierons comparativement la régénération de l'épithélium tubulaire à travers l'indice de prolifération cellulaire (immunohistochimie avec l'anticorps KI67).

Figure 5 : les souris homozygotes c-MPLKO sont thrombopéniques



7. Conclusions et perspectives

Outre la finalisation de l'étude de l'I/R rénale avec les souris CD154KO et CD40KO et thrombopéniques, nous projetons de débuter une étude translationnelle combinant en parallèle une expérimentation animale et une étude clinique descriptive, prospective observationnelle multicentrique promue par le CHU de Bordeaux. Ce projet vise à mettre au point un **modèle de sepsis murin** pour le comparer aux résultats de notre modèle d'I/R et à mettre en place une **étude clinique** analysant le niveau d'activation plaquettaire chez des patients en sepsis**.** Nous avons obtenu un soutien de la fondation MSDavenir (www.msdavenir.fr) pour financer ces projets.

Le modèle murin de sepsis par ligature et perforation du caecum est un protocole d'utilisation courante, considéré comme un « gold standard » (12). Nous étudierons comparativement la sévérité de ce sepsis (survie des souris, atteinte histologique d'organes vitaux, notamment rénale et pulmonaire, et « pattern » cytokinique pro-inflammatoire dans le plasma (IL-6, TNF-α…), chez des souris sauvages et les souris transgéniques mentionnées plus haut. L'activation plaquettaire dans ce modèle sera également étudiée par analyse des marqueurs membranaires d'activation plaquettaire et quantification des médiateurs d'origine plaquettaire (Platelet Factor 4 et β-thromboglobuline).

La partie clinique de ce travail aura pour objectif principal d'analyser l'association entre des marqueurs d'activation plaquettaire (concentrations plasmatiques et urinaires de CD154 soluble, concentrations plasmatiques de Platelet Factor 4 (PF4), P-sélectine et de β-thromboglobuline) et la gravité des défaillances d'organe à la phase aiguë d'un sepsis. L'objectif secondaire sera d'évaluer l'aspect pronostique de ces marqueurs d'activation plaquettaire.

Les résultats attendus de ces travaux sont une meilleure compréhension du rôle des plaquettes et du couple CD154/CD40 dans les défaillances multi-viscérales associées au sepsis. Un meilleur contrôle des fonctions plaquettaires offrirait la perspective de cibler simultanément l'activation de plusieurs voies qui peuvent devenir délétères en cas de sepsis. Mais sans une meilleure compréhension de ces phénomènes physiopathologiques, il est difficile de proposer à l'heure actuelle des agents antiplaquettaires à tous les patients septiques. Nos travaux pourraient contribuer à mieux discerner en pratique clinique quand et comment les plaquettes deviennent des promoteurs d'une réaction inflammatoire incontrôlée, ouvrant alors de nouvelles perspectives pour les traitements antiplaquettaires dans le sepsis.

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Annexes

Annexe 1 : critères diagnostiques de l'IRA (89,90)

	Serum creatinine criteria			Urine output criteria
	RIFLE	AKIN	KDIGO	
Definition	Increase in serum creatinine of >50 percent developing over <7 days	Increase in serum creatinine of 0.3 mg/dL or >50 percent developing over <48 hours	Increase in serum creatinine of 0.3 mg/dL developing over 48 hours or >50 percent developing over 7 days	Urine output of <0.5 mL/kg/hr for >6 hours
Staging				
RIFLE-Risk AKIN/KDIGO stage 1	Increase in serum creatinine of >50 percent	Increase in serum creatinine of 0.3 mg/dL or >50 percent	Increase in serum creatinine of 0.3 mg/dL or >50 percent	Urine output of <0.5 mL/kg/hr for >6 hours
RIFLE-Injury AKIN/KDIGO stage 2	Increase in serum creatinine of >100 percent	Increase in serum creatinine of >100 percent	Increase in serum creatinine of >100 percent	Urine output of <0.5 mL/kg/hr for >12 hours
RIFLE-Failure AKIN/KDIGO stage 3	Increase in serum creatinine of >200 percent	Increase in serum creatinine of >200 percent	Increase in serum creatinine of >200 percent	Urine output of <0.3 mL/kg/hr for >12 hours or anuria for >12 hours
RIFLE-Loss	Need for renal replacement therapy for >4 weeks			
RIFLE-End-stage	Need for renal replacement therapy for >3 months			

AKIN: Acute Kidney Injury Network; KDIGO: Kidney Disease/Improving Global Outcomes.