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# Rôle de l'inflammasome NLRP3 dans l'immunodépression induite par le sepsis : étude chez les patients de réanimation

Rémy Coudereau

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# THESE de DOCTORAT DE L'UNIVERSITE CLAUDE BERNARD LYON 1

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Soutenue publiquement le 17/10/2023, par :  
**Rémy COUDEREAU**

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## **Rôle de l'inflammasome NLRP3 dans l'immunodépression induite par le sepsis : étude chez les patients de réanimation**

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*Je dédie cette thèse à Monique Cerf, dites Barbara,  
décédée d'un sepsis le 24 novembre 1997*

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*« La vie n'est facile pour aucun de nous. Mais quoi, il faut avoir de la persévérance, et surtout de la confiance en soi. Il faut croire que l'on est doué pour quelque chose, et que, cette chose, il faut l'atteindre coûte que coûte. » Marie Curie*

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## Liste des abréviations

AIM2 : absent in melanoma 2  
Arg1 : arginase 1  
ARNm : ARN messenger  
ASC : *apoptosis-associated speck-like protein containing CARD*  
Atg : *autophagy-related genes*  
ATP : adénosine triphosphate  
BCR : *B cell receptor*  
C/EBP $\beta$  : *CCAAT-enhancer binding protein- $\beta$*   
CAPS : *cryopyrin-associated periodic syndrome*  
CAR-T cells : *chimeric antigenic receptor-T cells*  
CARD : *caspase activation and recruitment domain*  
CFU-GM : *granulocyte-macrophage colony-forming units*  
CINCA : *chronic infantile neurologic cutaneous articular*  
CIVD : coagulation intravasculaire disséminée  
CLR : *C-type lectin receptors*  
COVID-19 : *coronavirus disease-2019*  
CRP : protéine C réactive  
CTLA-4 : *cytotoxic T lymphocyte antigen 4*  
DAMP : *Danger Associated Molecular Pattern*  
FCAS : *familial cold autoinflammatory syndrome*  
G-CSF : *granulocyte colony-stimulating factor*  
GM-CSF : *granulocyte-macrophage colony-stimulating factor*  
gp130 : glycoprotéine 130  
GSDMD : gasdermine-D  
HIF1 $\alpha$  : *hypoxia-inducible factor 1 $\alpha$*   
HMGB-1 : *High-Mobility Group Box 1*  
IKK : I $\kappa$ B kinase  
IFN- $\gamma$  : interféron  $\gamma$   
IL-1 : interleukine-1  
IL-1 $\beta$  : interleukine-1 $\beta$   
IL-1R : récepteurs de l'IL-1 $\beta$   
IL-1R1 : récepteur de type I de l'IL-1 $\beta$

IL-1R2 : récepteur de type II de l'IL-1 $\beta$   
IL-10 : interleukine-10  
IL-18 : interleukine-18  
IL-18BP : *IL-18 binding protein*  
IL-18R : récepteur de l'IL-18  
IL-18R $\alpha$  : chaîne  $\alpha$  du récepteur de l'IL-18  
IL-18R $\beta$  : chaîne  $\beta$  du récepteur de l'IL-18  
IL-6 : interleukine-6  
IL-6R : récepteur de l'IL-6  
iNOS : oxyde nitrique synthase inductible  
IRAK : *IL-1 receptor-associated kinase*  
IRAKM : *IL-1 receptor-associated kinase M*  
JAK : *Just Another Kinase*  
LAG3 : *lymphocyte activation gene 3 protein*  
LDG : *low density granulocytes*  
LDL : *low-density lipoprotein*  
LOX-1 : *lectin-type oxidized LDL receptor 1*  
LPS : lipopolysaccharide  
LRR : *leucin-rich repeat*  
MAPK : MAP kinases  
M-CSF : *macrophage colony-stimulating factor*  
mHLA-DR : *monocyte human leukocyte antigen-DR*  
MDSCs : *myeloid-derived suppressor cells*  
MLKL : *mixed lineage domain-like pseudokinase*  
m-MDSCs : *monocytic myeloid-derived suppressor cells*  
mTOR : *mechanistic target of rapamycin*  
MWS : syndrome de Muscle-Wells  
MyD88 : *myeloid differentiation primary response 88*  
NACHT : *nucleotide-binding and oligomerization domain*  
NETs : *neutrophil extracellular traps*  
NF- $\kappa$ B : *nuclear factor-kappa B*  
NFI-A : *nuclear factor I A*  
NK : (lymphocytes) *natural killer*  
NLR : *nod-like receptors*

NLRP1 : *NACHT, leucin-rich repeat and pyrin-containing receptor 1*  
NLRP3 : *NACHT, leucin-rich repeat and pyrin-containing receptor 3*  
NLRP3-AID : *NLRP3-associated autoinflammatory disease*  
NLRC4 : *NACHT leucine rich repeat and CARD domain containing 4*  
NOD : *nucleotide oligomerization domain*  
NOMID : *neonatal onset multisystem inflammatory disease*  
OMS : *Organisation mondiale de la Santé*  
PAF : *platelet activating factor*  
PAI-1 : *inhibiteur de l'activateur du plasminogène de type 1*  
PAMP : *Pathogen Associated Molecular Pattern*  
PBMC : *cellules mononuclées du sang périphériques*  
PD-1 : *programmed cell death protein 1*  
PD-L1 : *programmed cell death-ligand 1*  
PD-L2 : *programmed cell death-ligand 2*  
PMN : *polynucléaires neutrophiles*  
PMN-MDSCs : *Polymorphonuclear MDSCs*  
PRR : *Pattern Recognition Receptors*  
PYD : *pyrin domain*  
qSOFA : *quickSOFA*  
RIP1 : *kinase receptor-interacting protein 1*  
ROS : *reactive oxygen species*  
SARS-CoV-2 : *Severe acute respiratory syndrome coronavirus 2*  
SCF : *stem cell factor*  
SDRA : *syndrome de détresse respiratoire aigüe*  
SIRS : *syndrome de réponse inflammatoire systémique*  
SOFA : *Sequential Organ Failure Assessment*  
SR : *scavenger receptors*  
STAT3 : *signal transducer and activator of transcription 3*  
TAK1 : *transforming growth factor-activated kinase 1*  
TCR : *T cell receptor*  
TF : *facteur tissulaire*  
TH : *lymphocyte T helper*  
TH1 : *lymphocytes T helper 1*  
TH17 : *lymphocytes T helper 17*

TIM3 : *T cell immunoglobulin mucin receptor 3*  
TLR : *toll-like receptors*  
TLR-4 : *toll-like receptor 4*  
TNF- $\alpha$  : *Tumour Necrosis Factor- $\alpha$*   
TNFR1 : récepteur 1 du TNF- $\alpha$   
TNFR2 : récepteur 2 du TNF- $\alpha$   
TNFR : récepteurs du TNF  
TRAF6 : *TNF receptor-associated factor 6*  
TRAIL : *TNF-related apoptosis-inducing ligand*  
Tregs : lymphocytes T régulateurs  
TREM-1 : triggering receptor expressed on myeloid cells-1  
TRIF : *TIR domain-containing adapter inducing interferon- $\beta$*   
VEGF : *vascular endothelial growth factor*  
VIH : virus de l'immunodéficience humaine  
vWF : facteur de von Willebrand

## INTRODUCTION



## Introduction

Le sepsis est une pathologie dont l'incidence ne cesse d'augmenter ces dernières années, reconnu comme **enjeu de santé publique majeur par l'OMS**. Sa physiopathologie implique à la fois des réponses pro-inflammatoires, responsables des dysfonctions d'organes et de la mortalité précoce, et des réponses anti-inflammatoires, qui régulent le versant pro-inflammatoire. Chez certains patients, ces mécanismes régulateurs évoluent vers une profonde immunosuppression, conduisant à des infections nosocomiales et aux décès tardifs. Dans ce contexte, les thérapies immunostimulantes sont de plus en plus étudiées pour la prise en charge de ces patients, nécessitant une meilleure connaissance de la physiopathologie et de ces altérations afin d'identifier les traitements les plus pertinents et les patients pouvant en bénéficier.

L'inflammasome NLRP3 est un complexe protéique intracellulaire impliqué dans la réponse pro-inflammatoire en produisant les IL-1 $\beta$  et IL-18 au cours du sepsis. Dans d'autres contextes cliniques où l'inflammation chronique joue un rôle central dans la physiopathologie, NLRP3 a été associé à l'immunosuppression, notamment via l'induction de cellules myéloïdes aux capacités suppressives (MDSCs), décrites dans le sepsis. Cependant, aucune étude n'a encore examiné le rôle de l'inflammasome NLRP3 dans le développement des altérations immunitaires chez les patients septiques.

L'hypothèse de ce travail était que **l'activation aiguë ou persistante de l'inflammasome NLRP3 pourrait être impliquée dans les altérations immunitaires survenant au cours du sepsis, notamment dans l'induction de MDSCs**.

Dans le cadre de ce projet, je me suis focalisé sur le versant clinique, avec la volonté d'étudier l'association entre l'activation de l'inflammasome NLRP3 et les altérations immunitaires dans une étude clinique observationnelle chez les patients septiques. Plusieurs études ont été menées dans le cadre de ce travail. Nous avons tout d'abord recherché et caractérisé les PMN-MDSCs chez les patients présentant un sepsis bactérien ou viral (**Article 1, Journal of Leukocyte Biology, 2021**). Ensuite, nous avons exploré le lien entre NLRP3 et immunosuppression dans une cohorte prospective de patients septiques de réanimation, et ces résultats ont été confirmés dans une cohorte rétrospective (**Article 2, soumis en août 2023**). Enfin, nous avons développé une technique de monitoring permettant de suivre l'activation et les capacités de réactivation de l'inflammasome NLRP3 en sang total, utilisable en recherche clinique (**Article 3, Cells, 2022**).

En préambule à la présentation de ces résultats, une revue de la littérature est effectuée pour décrire l'état actuel des connaissances sur le sepsis, l'inflammasome NLRP3, les MDSCs, l'inflammation chronique et le lien entre ces différentes entités.

REVUE BIBLIOGRAPHIQUE

# Revue bibliographique

## I. Sepsis & choc septique

### 1. Historique et définitions

#### 1.1. Premières définitions et Sepsis-1

Le mot « sepsis » dérive du grec ancien σηψις et signifie « décomposition animale, végétale ou organique en présence de bactéries ». La première utilisation médicale du mot sepsis est retrouvée il y a près de 3000 ans dans les poèmes de l'aède grec Homère, où « sepo » (σηπω) signifie littéralement « je pourris »<sup>1</sup>. Il est aussi retrouvé dans les écrits d'Hippocrate et de Galien<sup>1</sup>, pères fondateurs de la médecine et de la pharmacie. Le sepsis n'est donc pas une pathologie nouvelle, mais la compréhension de son origine prendra plusieurs millénaires et connaîtra de spectaculaires avancées avec les travaux du 19<sup>e</sup> siècle, notamment ceux de Lister, Koch et Pasteur. La première définition moderne de sepsis est donnée par Hugo Schottmuller en 1914, il parle alors de la septicémie comme « un état d'invasion microbienne à partir d'une porte d'entrée sanguine qui provoque des signes de maladie »<sup>2</sup>. Après cette première définition, les termes de « sepsis », « septicémie », « bactériémie », « syndrome septique » et « choc septique » sont employés de manières interchangeable pour définir la réponse systémique face à une infection<sup>3</sup>, « sepsis » et « syndrome septique » étant parfois employés pour des états inflammatoires non infectieux<sup>4</sup>.

En 1991, une conférence de consensus est organisée par l'American College of Chest Physicians et la Society of Critical Care Medicine afin d'établir une définition claire du sepsis et d'homogénéiser les pratiques concernant le diagnostic, le suivi et la prise en charge des patients<sup>4</sup>. Le sepsis est alors défini ou suspecté comme l'association d'une infection documentée et d'un « syndrome de réponse inflammatoire systémique » (SIRS).

Le SIRS est caractérisé par la présence d'au moins deux des quatre critères suivants :

- Fièvre  $>38^{\circ}\text{C}$  ou hypothermie  $<36^{\circ}\text{C}$
- Tachycardie  $> 90$  bpm
- Polypnée  $> 20$  respirations par minute ou hyperventilation ( $\text{PaCO}_2 < 32\text{mmHg}$ )
- Hyperleucocytose ou leucopénie (globules blancs  $> 12\ 000/\text{mm}^3$  ou  $< 4\ 000/\text{mm}^3$ ) ou présence de polynucléaires immatures  $>10\%$

Dans le même temps, cette convention clarifie plusieurs termes :

- Septicémie et syndrome septique : termes ne devant plus être utilisés
- Bactériémie : présence de pathogènes dans le sang
- Sepsis sévère : sepsis compliqué d'une dysfonction d'organe, de signes d'hypoperfusion ou d'hypotension artérielle
- Choc septique : sepsis associé à une hypotension artérielle persistante malgré un remplissage vasculaire adéquat ou requérant l'administration de médicaments vasopresseurs

### 1.2.Sepsis-2

En 2001, des experts internationaux se réunissent afin d'améliorer cette définition<sup>5</sup>. La définition de sepsis est remise en cause, celle-ci étant peu précise, non spécifique et trop sensible. En effet, l'utilisation du SIRS et de ses critères cliniques ne permet pas de détecter avec précision l'origine du syndrome, celui-ci étant retrouvé dans de nombreuses situations cliniques (*e.g.* brûlures, pancréatite, traumatismes...). La plupart des patients hospitalisés en unité de soins intensifs satisfont donc à cette définition sans forcément présenter d'infection et donc de sepsis. Néanmoins, aucun marqueur n'est disponible pour permettre le diagnostic de certitude. Les experts gardent donc la précédente définition établie en 1992 et y apportent les modifications suivantes :

- Ajout de critères biologiques (protéine C réactive plasmatique, procalcitonine plasmatique...)
- Ajout de critères cliniques (présence d'œdèmes, iléus...)
- La documentation de l'infection n'est plus obligatoire : l'infection peut être seulement suspectée

### 1.3.Sepsis-3

Devant les avancées physiopathologiques, les résultats d'essais cliniques et l'accumulation de données épidémiologiques, un 3<sup>e</sup> consensus a été organisé en 2016 et donne la définition la plus récente du **sepsis** à ce jour : une **défaillance d'organe mettant en jeu le pronostic vital et liée à une dérégulation de la réponse de l'hôte face à une infection**<sup>6</sup>. Le choc septique est quant à lui décrit comme l'aggravation d'un sepsis, présentant des anomalies circulatoires, cellulaires et métaboliques suffisamment profondes pour augmenter considérablement la mortalité. Le **choc septique** est défini cliniquement par l'**utilisation de**

traitements vasopresseurs pour maintenir une pression artérielle moyenne  $\geq 65$  mmHg et présentant une lactatémie  $>2$  mmol/L en l'absence d'hypovolémie.

Avec cette révision, le SIRS disparaît de la définition du sepsis, les auteurs rapportant que celui-ci peut simplement refléter une réponse appropriée de l'hôte. De même, la notion de sepsis sévère est jugée redondante et ne doit plus être utilisée<sup>6</sup>. Le sepsis n'est donc plus le simple fait d'une infection associée à une réponse hyperinflammatoire, mais une entité à part entière avec une physiopathologie propre. Pour la première fois, la dérégulation des réponses de l'hôte est au cœur de la définition, pointant du doigt à la fois les réponses hyperinflammatoires et immunosuppressives observées au cours du sepsis.

#### 1.4. Diagnostic

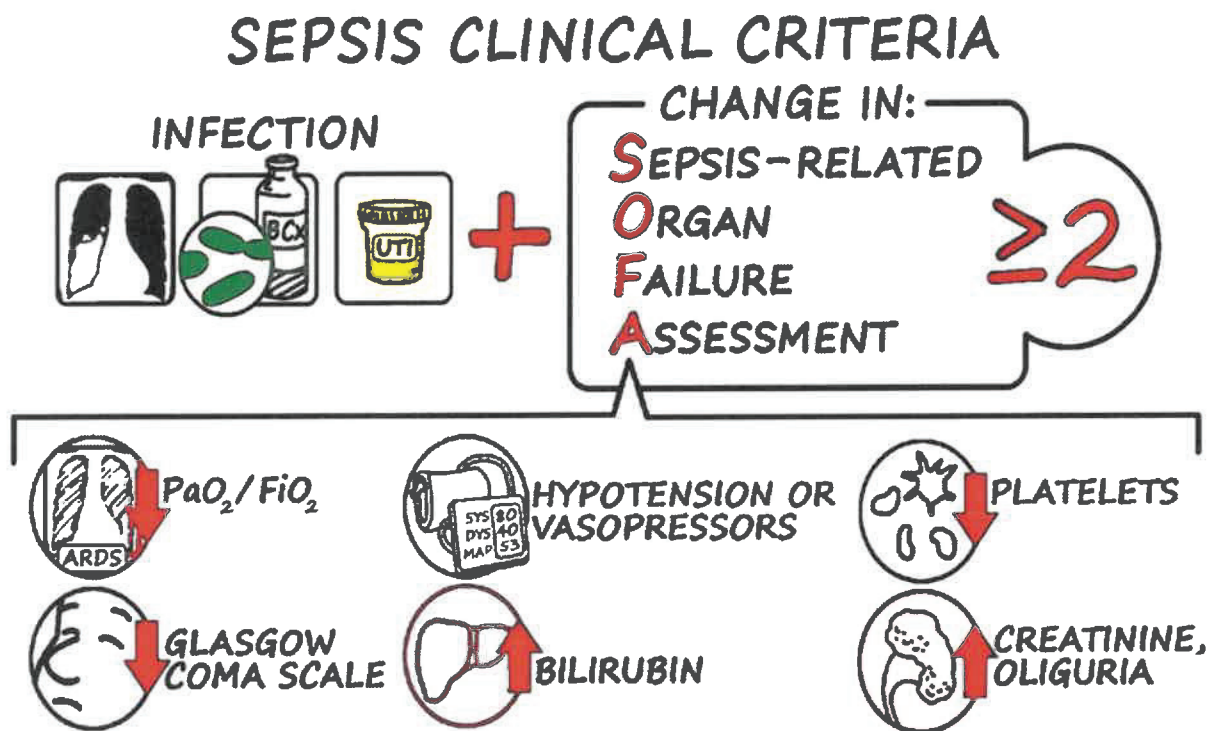


Figure 1. Critères diagnostics du sepsis selon la définition Sepsis-3  
Adapté de <http://jamasepsis.com>

Depuis la définition Sepsis-3, la recherche de défaillances d'organes est essentielle chez tout patient suspecté d'une infection et fait partie intégrante du diagnostic. L'appréciation de cette dysfonction est réalisée à l'aide du score SOFA (*Sequential Organ Failure Assessment*)<sup>7</sup> qui recherche les défaillances d'organes listées en **figure 1**, objectivées par des troubles clinico-biologiques associés. Le diagnostic clinique de sepsis est ainsi posé devant la documentation ou la suspicion d'une infection associée à une augmentation de 2 points du score SOFA. Le calcul du score SOFA nécessite néanmoins la réalisation d'analyses biologiques pour mesurer

divers paramètres (*e.g.* créatinine plasmatique, plaquettes...) et peut ainsi retarder le diagnostic dans les services autres que la réanimation. Il a donc été développé un qSOFA (*quickSOFA*) pour dépister plus rapidement les patients à risques de sepsis basé sur plusieurs paramètres : troubles de la conscience, fréquence respiratoire  $\geq 22/\text{min}$ , tension artérielle systolique  $\leq 100$  mmHg<sup>6</sup>.

Le consensus de 2016 rappelle que le score SOFA n'est pas destiné à être utilisé comme un outil de prise en charge du patient, mais comme un moyen de caractériser cliniquement un patient septique.

## 2. Épidémiologie et impact socio-économique

Le sepsis et le choc septique sont actuellement les **principales causes de décès dans les services de réanimation adultes**. Bien que sa prise en charge s'améliore continuellement, les études épidémiologiques montrent une augmentation de son incidence au cours des 20 dernières années<sup>8</sup>. En conséquence, le sepsis a été reconnu comme **enjeu de santé publique majeur par l'Organisation mondiale de la Santé (OMS) en 2017**<sup>9</sup>.

Cependant, les données épidémiologiques sont limitées et difficilement comparables. Tout d'abord, il n'existe pas de marqueur spécifique pour le sepsis et le choc septique, leur diagnostic repose sur un ensemble de critères clinico-biologiques. De plus, ces critères ont évolué au cours des 20 dernières années, ce qui a conduit à une importante hétérogénéité dans les définitions de sepsis utilisées, compliquant la comparaison des études. En outre, les standards de soins varient en fonction des pays (*e.g.* accessibilité à un hôpital de proximité, remboursement des soins de santé). Obtenir des données sur les pays en voie de développement peut également être difficile. Ces difficultés entraînent des différences dans les données concernant l'incidence et la mortalité entre les études et les pays, comme le montre la **figure 2**. Une récente étude a ainsi mis en évidence les disparités d'incidence et de mortalité du sepsis en fonction de la répartition géographique (**figure 3**), montrant que les pays en voie de développement sont les plus touchés<sup>10</sup>.

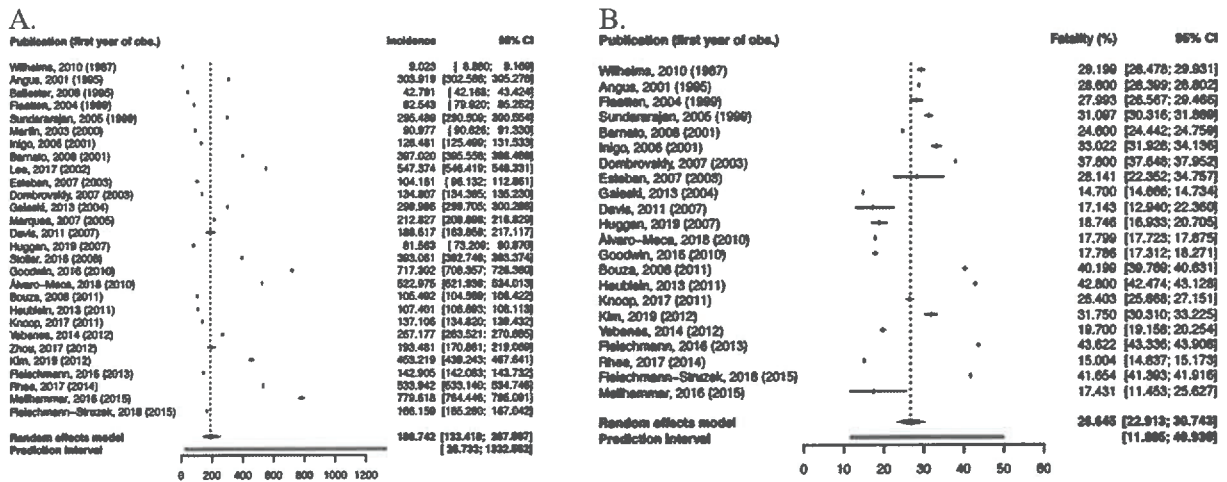


Figure 2. Incidence et mortalité du sepsis

Incidence (A) et mortalité (B) du sepsis sévère (définition Sepsis-1 et Sepsis-2) et du sepsis (définition Sepsis-3) rapportées par les études publiées entre 2001 et 2019. L'incidence est exprimée en nombre de cas pour 100 000 habitants, la mortalité en % pour 100 cas de sepsis. Adapté de Fleischmann-Struzek et al<sup>11</sup>.

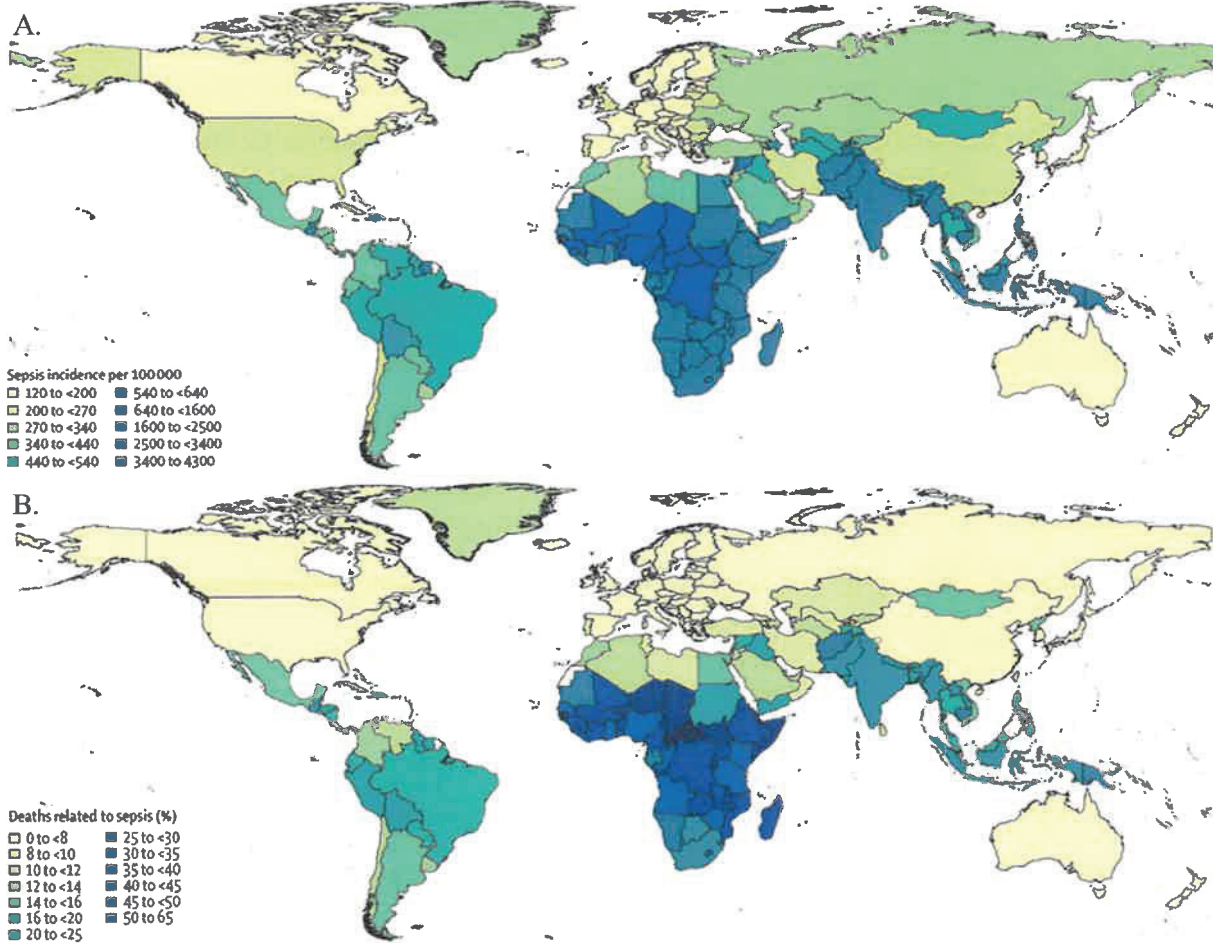


Figure 3. Répartition géographique du sepsis dans le monde en 2017

Incidence du sepsis exprimée en nombre de cas pour 100 000 habitants (A) et des décès liés au sepsis exprimés en % (B) dans le monde en 2017. Adapté de Rudd et al<sup>10</sup>.



## 2.1. Incidence

Les dernières données disponibles concernant l'incidence mondiale du sepsis montrent une diminution des patients hospitalisés entre 1990 et 2017, avec une réduction de 37% pour une incidence de 677 cas pour 100 000 habitants en 2017<sup>10</sup>. Il est à noter que cette année-là, **48,9 millions de cas de sepsis ont été rapportés de par le monde**. Néanmoins, cette diminution de l'incidence est en contradiction avec de nombreuses études mentionnant quant à elle une augmentation ou une stagnation des cas de sepsis<sup>11-13</sup>. Fleischmann-Struzek et collègues défendent l'augmentation de l'incidence (prouvée par leur méta-analyse de 51 études dans 22 pays de 4 régions du monde différentes) en argumentant la différence des données entre pays en voie de développement et pays industrialisés. Dans les pays développés, le vieillissement de la population et le nombre élevé de personnes âgées présentant des comorbidités, ainsi que l'augmentation d'utilisation du matériel invasif (*e.g.* cathéters), des traitements complexes (*e.g.* immunothérapies et chimiothérapies) et le taux élevé de résistance aux antimicrobiens sont des arguments objectifs à l'augmentation des taux d'incidence du sepsis. Au contraire, la diminution d'incidence constatée par Rudd *et al* pourrait être due à la baisse du nombre de décès dus aux maladies infectieuses dans les pays en voie de développement. Les méthodologies employées ainsi que les différentes définitions de sepsis utilisées sont aussi d'autres facteurs permettant d'expliquer ces différences. En France, une étude publiée en 2020 employant les critères ICD-10 (*International Classification of Diseases, Tenth Revision*) et des critères de dysfonctions d'organes (donc correspondant à la définition Sepsis-3 *in fine*) montrait une augmentation de l'incidence du sepsis et du choc septique, de 206 à 243 et de 135 à 171 cas pour 100 000 habitants, respectivement<sup>12</sup>.

Devant les discordances soulevées et la difficulté de comparer des données employant des définitions de sepsis différentes, il paraît important de privilégier les études épidémiologiques utilisant les définitions fournies par Sepsis-3 pour les diagnostics de sepsis et de choc septique.

## 2.2. Mortalité

En 2017, **11 millions de décès liés au sepsis** ont été estimés mondialement, représentant 19,7% de la mortalité globale et la **1<sup>re</sup> cause** devant le cancer et les maladies cardiovasculaires<sup>10</sup>. En 2019, une étude a montré que 13,7 millions de décès étaient reliés à des causes infectieuses (non définis comme sepsis dans cette étude)<sup>14</sup>. Globalement, la mortalité confondue du sepsis et du choc septique serait d'environ 25% (**figure 2B**).

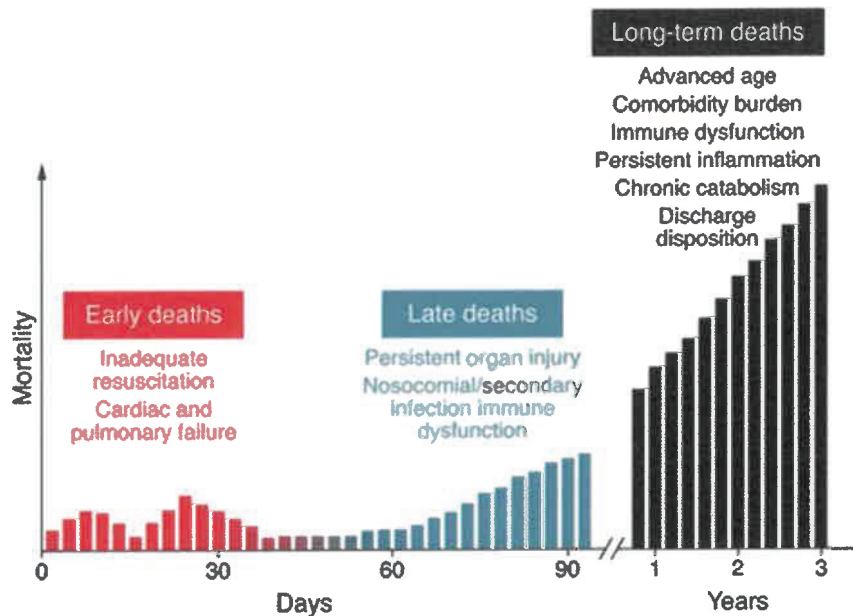


Figure 4. Distribution de la mortalité du sepsis au cours du temps  
Adapté de Delano & Ward<sup>15</sup>

Les décès observés à la suite d'un sepsis font partie de 2 grandes catégories : les **décès précoces**, attribuables aux dysfonctions d'organes observées durant les premiers, et les **décès tardifs** (comprenant les décès tardifs et à long terme), reposants sur la persistance des dysfonctions d'organes et le développement d'infections secondaires, notamment dues aux troubles immunitaires sous-jacents (**figure 4**). Depuis plusieurs décennies et la mise en place des premières recommandations pour la définition et la prise en charge du sepsis, **une diminution de la mortalité** est observée, que ça soit au niveau mondial<sup>10,16</sup> ou dans les pays industrialisés<sup>12,17,18</sup>. Entre 1990 et 2017, la mortalité mondiale du sepsis a ainsi chuté de 29,7%<sup>10</sup>. En France, ce constat est aussi rapporté avec une diminution des décès de 60,1% à 39,5% en réanimation entre 1993 et 2010<sup>19</sup>. Cette diminution des décès chez les patients septiques est expliquée par une meilleure compréhension de la physiopathologie de la maladie<sup>20</sup> permettant l'édition de recommandations pour permettre un diagnostic plus précoce<sup>6</sup>, l'amélioration de la prise en charge des patients et l'utilisation de la médecine personnalisée<sup>15</sup>. Néanmoins, une **stagnation de la mortalité a été rapportée depuis quelques années**. Une méta-analyse d'études provenant de pays industrialisés entre 2009 et 2019 a montré une diminution de la mortalité entre 2009 et 2011, mais cette différence n'était plus retrouvée ensuite<sup>17</sup>. Cette observation a aussi été faite par Zhao et ses collègues, qui ne retrouvent pas de différence de survie entre 2008 et 2015<sup>18</sup>. En France, les résultats sont similaires entre 2010 et 2015, où la mortalité du sepsis reste de 34% et celle du choc septique diminue discrètement de 46 à 44%<sup>12</sup>. Cette stagnation est expliquée par une amélioration de la prise en charge initiale,

mais qui entraîne une **surmortalité tardive**. En effet, les patients survivent mieux à la phase initiale du sepsis et développent secondairement des altérations immunitaires, dont les processus physiopathologiques moins bien compris sont encore à l'étude aujourd'hui<sup>20</sup>. Les patients entrant dans cette seconde phase sont plus sensibles aux infections secondaires et opportunistes (*e.g.* infections fongiques), celle-ci représentant aujourd'hui la plus grande part de la mortalité attribuable au sepsis<sup>18,21</sup>.

### 2.3. *Impact socio-économique*

En raison de sa gravité et de sa lourde prise en charge, le sepsis et le choc septique engendrent une charge économique conséquente. Aux États-Unis, une étude rétrospective de patients hospitalisés entre 2010 et 2016 (avant la définition Sepsis-3) a été réalisée. Cette étude a montré que la durée de séjour ainsi que le coût d'hospitalisation augmentaient avec la gravité, pour une durée de 7,7 jours et un coût moyen de 16 324\$ pour les sepsis sans dysfonctions organiques (ancienne définition), 10 jours et 24 638\$ pour les sepsis sévères et 12,6 jours et 38 298\$ pour les chocs septiques<sup>22</sup>. En 2017, le sepsis était **la première cause de dépense du système de santé aux États-Unis**, avec un coût de plus de 38 milliards de dollars, représentant 8,8% des dépenses totales ainsi que 2,86 millions de jours d'hospitalisation<sup>23</sup>. En France, la durée moyenne d'hospitalisation est de 17 jours pour les patients septiques (7 jours en service de réanimation) contre 19 pour les chocs septiques (8 jours en service de réanimation), engendrant un coût de 11 400€ et 16 439€ respectivement<sup>12</sup>.

Dans sa globalité, le coût de prise en charge d'un sepsis varie d'un pays à l'autre, pour un montant compris entre 1101€ et 91 951€, avec une moyenne de 36 161€<sup>24</sup>. Néanmoins, ces chiffres ne prennent pas en compte l'accompagnement des patients présentant des séquelles post-sepsis (ces séquelles seront décrites dans une autre partie). De plus, les patients septiques seraient aussi plus à risque de développer des cancers selon une récente étude, montrant une association avec 9 types de cancer (*e.g.* thyroïde, poumon, colorectal) suivant les 5 ans du diagnostic de sepsis<sup>25</sup>. Les coûts de prise en charge du sepsis, déjà importants, pourraient donc être largement sous-estimés.

## 3. Étiologies et facteurs de risque

### 3.1. *Étiologies*

Au niveau mondial, la **porte d'entrée digestive**, les **infections pulmonaires** et les **pathologies maternelles** sont les causes de sepsis les plus répandues entre 1990 et 2017, l'origine digestive restant la première cause<sup>10</sup>. Concernant la mortalité, les 3 origines les plus

mortelles ne changent pas entre 1990 et 2017, les **infections pulmonaires représentent la première cause de décès dans le sepsis**, suivi par la **porte d'entrée digestive** et les **sepsis du nourrisson**<sup>10</sup>. Une étude récente a étudié la mortalité causée par 33 espèces bactériennes dans 11 syndromes infectieux différents. Si le syndrome responsable de la majorité de la mortalité variait selon les pays, les trois principaux syndromes étaient systématiquement les infections des voies respiratoires inférieures, les bactériémies et les infections intra-abdominales et péritonéales<sup>14</sup>. Ces syndromes représentaient ainsi la 3<sup>e</sup>, la 7<sup>e</sup> et la 13<sup>e</sup> cause de décès dans le monde en 2019, devant le VIH ou le cancer colorectal par exemple. Si l'on s'intéresse aux germes prépondérants dans chacun des syndromes précédemment cités, *Streptococcus pneumoniae* est prédominant dans les infections des voies respiratoires inférieures, tandis que *Staphylococcus aureus* et *Escherichia coli* le sont dans les bactériémies et les infections intra-abdominales respectivement<sup>14</sup>. Toutes causes confondues, *Staphylococcus aureus* était la bactérie engendrant le plus de décès dans le monde avec plus d'un million de morts, suivie d'*Escherichia coli*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, et *Pseudomonas aeruginosa* avec plus de 500 000 morts chacune<sup>14</sup>. Les auteurs rappellent aussi que les causes non bactériennes d'infections pouvant conduire à un sepsis doivent être reconnues tels que les champignons (*e.g.* les mucorales), les virus (*e.g.* coronavirus) ou les parasites (*e.g.* *Plasmodium sp.*).

### 3.2. Facteurs de risque

De nombreux facteurs de risques du sepsis sont recensés dans la littérature et récapitulés dans le **tableau 1**. Concernant les facteurs propres à l'hôte, le plus décrit concerne l'**âge**, où un pic est observé dans la petite enfance suivi d'un second pic chez les adultes plus âgés<sup>10</sup>, où le sepsis est retrouvé chez les personnes de plus de 65 ans dans plus de 60% des cas (dans les pays industrialisés)<sup>26</sup>. La **présence de comorbidités** est aussi bien documentée, 50% des patients septiques en possédant au moins une<sup>26</sup>. La présence de susceptibilité génétique est sujette à débat, mais les polymorphismes touchant les *toll-like receptors* (TLR) ou le *Tumour Necrosis Factor- $\alpha$*  (TNF- $\alpha$ ) sont les plus documentés<sup>27</sup>.

Concernant les facteurs extérieurs et environnementaux, l'utilisation de **procédures invasives** comme la ventilation mécanique, la pose de cathéters ou la chirurgie d'urgence entraînent un risque accru de sepsis et d'infections secondaires<sup>26</sup>. La limitation de la durée d'utilisation de ces dispositifs ainsi que la réduction du temps d'hospitalisation apparaissent essentielles chez

les personnes hospitalisées. L'utilisation de traitements immunosuppresseurs, souvent vitale, va être un facteur supplémentaire au développement du sepsis.

Ces facteurs de risques concernent l'incidence du sepsis, mais nombreux sont aussi ceux qui font partie des facteurs influençant la mortalité comme les comorbidités, l'âge, *etc*<sup>28</sup>. D'autres ne sont pas liés à l'hôte, mais aux micro-organismes impliqués comme le facteur de virulence, la présence de co-infection, la résistance aux traitements anti-infectieux ou l'implication de pathogènes peu connus<sup>28</sup>. Il ne faut pas oublier que la plupart de ces facteurs de risques sont ceux observés dans les pays industrialisés où les études disponibles sont plus nombreuses. En 2017, **85% des cas de sepsis étaient observés dans les pays en voie de développement** où d'autres facteurs de risques sont observés<sup>10</sup>. Parmi ceux-ci, de **nombreux facteurs modifiables**, tels qu'un manque d'accès aux soins, une vaccination incomplète, de mauvaises conditions d'hygiène et la présence d'infections liées aux soins ont été associés à un risque accru de mortalité à l'hôpital<sup>28,29</sup>. Une étude comparant la mortalité du sepsis entre pays industrialisés et pays en voie de développement montrait ainsi une surmortalité significative dans les pays en voie de développement, et particulièrement chez les plus pauvres<sup>30</sup>. Dans ces derniers pays, l'accès aux soins reste un réel problème de santé publique : la mortalité rapportée dans les hôpitaux publics était plus importante que dans les cliniques<sup>31</sup>.

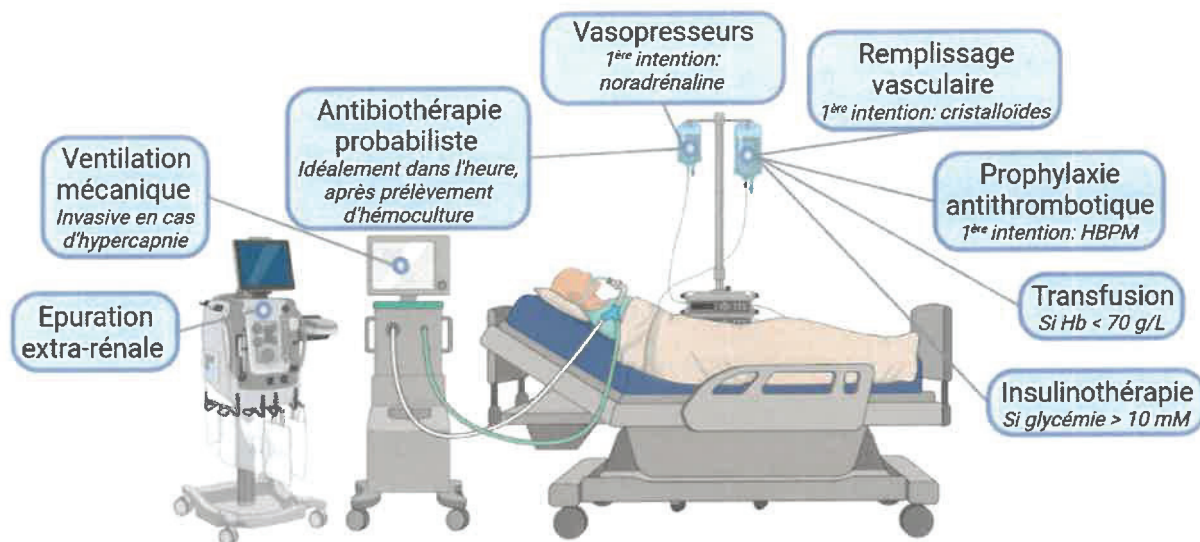
Un **antécédent de sepsis représente un facteur de risque significatif** chez les patients, à la fois en ce qui concerne l'incidence et la mortalité. Une étude française publiée en 2022 portant sur plus de 170 000 patients ayant survécu à un épisode de sepsis a révélé que 73,8% de ces patients sont réhospitalisés dans l'année suivant leur sepsis, dans différents services (médecine, chirurgie, etc.)<sup>32</sup>. Parmi ces réhospitalisations, 18,1% étaient liées à un **sepsis récurrent**. De plus, parmi l'ensemble des patients, 22,3% sont décédés dans l'année suivant leur sepsis. La mortalité à un an est significativement plus élevée chez les patients réhospitalisés pour un sepsis récurrent, avec un odds ratio ajusté de 2,9<sup>32</sup>.

<b>Âges extrêmes</b>	Patients < 1 an Patients > 65 ans
<b>Sexe</b>	Sexe masculin
<b>Prédisposition génétique</b>	Polymorphisme des TLR, du TNF- $\alpha$
<b>Comorbidités</b>	Pathologies chroniques : cancer, diabète, maladies pulmonaires, troubles hépatiques, insuffisance cardiaque, insuffisance rénale Infections chroniques : Virus de l'immunodéficience humaine (VIH), pneumopathies, infections urinaires, infections cutanées
<b>Désordres de l'immunité</b>	Thérapeutiques : Traitements immunosuppresseurs, chimiothérapie, radiothérapie, transplantation Pathologies : troubles de l'immunité
<b>Brèches des barrières naturelles</b>	Chirurgie, traumatisme, brûlure, intubation ou pose de cathéter, entérocolites.
<b>Autres</b>	Alcoolisme chronique, hospitalisation, malnutrition, nutrition parentérale, déficit en vitamine D

Tableau 1. Facteurs de risque de développer un sepsis<sup>26,27,33</sup>

#### 4. Prise en charge : recommandations de 2021

Bien que la prise en charge du sepsis soit une **urgence vitale** nécessitant une hospitalisation en services de réanimation, **il n'existe pas de traitement spécifique à ce jour**. De plus, les disparités entre les pays cités précédemment ont un effet sur la mortalité du sepsis (**figure 3B**). Dans le but d'homogénéiser et d'améliorer les pratiques, un groupe d'experts internationaux regroupés sous l'égide « *Surviving Sepsis Campaign* » ont créé des recommandations en 2004<sup>34</sup>, qu'ils ont fait évoluer au cours des années<sup>35-37</sup>, avec une dernière révision en 2021 (**figure 5**)<sup>38</sup>. Ces recommandations (93 au total) s'articulent autour de 4 grands axes.



**Figure 5. Prise en charge d'un sepsis en réanimation**

Représentation basée sur les recommandations de 2021 du Surviving Sepsis Campaign<sup>38</sup>. Les recommandations fortes sont représentées en bleu, les recommandations faibles en blanc.

#### *i. Détection des patients et diagnostic précoce*

La forte mortalité chez les patients atteints de sepsis doit impliquer une **suspicion précoce du diagnostic** (et sa confirmation ultérieure) et nécessite un dépistage méticuleux associé à une prise en charge appropriée. De nombreux outils de screening sont disponibles, mais aucun consensus n'est actuellement disponible. Par rapport aux recommandations de 2016, les experts déconseillent d'utiliser le qSOFA comme seul outil de screening, celui-ci ayant une trop faible sensibilité.

#### *ii. Contrôle de la source infectieuse*

Chez tout patient suspecté de sepsis ou de choc septique, des prélèvements microbiologiques (comprenant hémocultures et site(s) anatomique(s) suspecté(s) d'infection) doivent être réalisés avant l'initiation du traitement anti-infectieux, seulement si ceux-ci ne retardent pas l'instauration de la prise en charge médicamenteuse (*i.e.* < 45 min). La présence ou la forte suspicion d'un sepsis (ou la seule présence d'un choc) doit faire administrer une antibiothérapie probabiliste à large spectre **immédiatement et au plus tard dans l'heure de sa détection**. Si la présence d'un sepsis est possible, il est important de rechercher des causes non infectieuses. Si le doute n'est pas levé dans les 3 heures, il est recommandé d'administrer un traitement anti-infectieux.

### *iii. Traitement des dysfonctions d'organes*

Réanimation initiale et stabilisation hémodynamique : une réanimation appropriée, rapide et efficace est cruciale pour la stabilisation de l'hypoperfusion tissulaire induite par le sepsis et le choc septique. Celle-ci doit débiter dès la suspicion d'un sepsis ou d'un choc septique.

Ventilation mécanique : l'insuffisance respiratoire aiguë peut résulter d'un sepsis découlant d'une pneumopathie ou d'infections non pulmonaires entraînant un syndrome de détresse respiratoire aiguë (SDRA). Les patients présentant un SDRA peuvent être pris en charge à l'aide de ventilation non invasive ou de ventilation mécanique.

### *iv. Autres recommandations*

Transfusion : l'administration de concentrés érythrocytaires n'est recommandée que chez les patients présentant une anémie objectivée par une concentration en hémoglobine < 70 g/L et doit prendre en compte l'état clinique du patient.

Prophylaxie de la thrombose veineuse : sauf contre-indication, il est recommandé de mettre en place une thérapie prophylactique de la thrombose veineuse, en utilisant préférentiellement les héparines de bas poids moléculaires.

Épuration extrarénale : la prise en charge d'une défaillance rénale aiguë chez le patient septique doit faire intervenir une épuration extrarénale. Néanmoins, aucune recommandation n'est donnée concernant le choix de la thérapie à adopter (continue ou intermittente).

Contrôle de la glycémie : une hyperglycémie supérieure à 10 mmol/L doit ainsi entraîner l'administration d'insuline, la glycémie cible étant ensuite située à 8–10 mmol/L.

Prise en charge post-hospitalisation et objectifs de soins : la compréhension dans la physiopathologie du sepsis, notamment sa mortalité tardive, fait apparaître de nouvelles recommandations sur la prise en charge post-sepsis, tels que la conciliation médicamenteuse, le suivi des patients post-hospitalisation, la gestion des séquelles, etc.



## II. Réponse immuno-inflammatoire au cours du sepsis : hypothèses et évolution

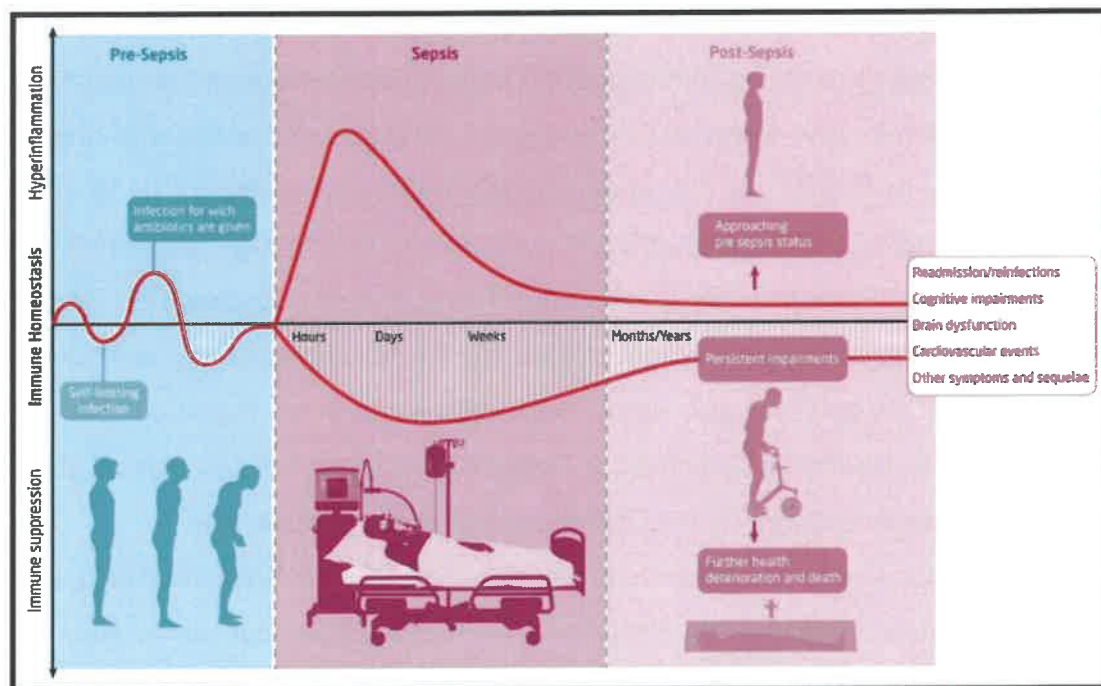
La compréhension de la *physiopathologie* du sepsis n'a cessé de croître depuis ses premières descriptions. Il y a plus de 100 ans, *sir* William Osler, un médecin anglais, décrivait l'importance de la réaction inflammatoire au cours du sepsis dans son ouvrage « *The Evolution of Modern Medicine* » en 1904, en écrivant « sauf en de rares occasions, le patient semble mourir de la réaction de l'organisme à l'infection plutôt que de celle-ci ». Cette hypothèse proposant le sepsis comme le résultat d'une réaction inflammatoire excessive est alors largement acceptée et étudiée pendant de nombreuses années. La symptomatologie des patients (signes cardinaux de l'inflammation, défaillances d'organes), les concentrations plasmatiques élevées de cytokines pro-inflammatoires (interleukine-1 (IL-1), IL-6, TNF- $\alpha$ ...) et les effets protecteurs des anti-inflammatoires sur différents modèles animaux de sepsis ont contribué à la mise en place d'essais cliniques anti-inflammatoires chez les patients. Après de nombreuses années et de nombreuses thérapies explorées dans différentes classes thérapeutiques, le **ciblage de l'inflammation s'est avéré être un échec en matière de survie des patients (absence d'efficacité voire effet délétère dans certains essais)**<sup>39-41</sup>.

Dans ce contexte d'échec thérapeutique, la réanalyse de données anciennes de la littérature a permis de faire évoluer le dogme purement inflammatoire du sepsis. Avant les années 2000, une moindre réponse aux tests cutanés d'hypersensibilité était décrite chez les patients septiques ou ayant subi une intervention chirurgicale<sup>42,43</sup>, ainsi qu'un excès d'apoptose<sup>44</sup> et une dérégulation de la production des cytokines *in vitro*<sup>45</sup>. Il faudra attendre les années 2000 pour que les premières hypothèses sur la place de l'immunosuppression dans la physiopathologie du sepsis émergent<sup>46,47</sup>. Ces premières hypothèses décrivent une prédominance initiale de la réponse pro-inflammatoire accompagnée d'une réponse anti-inflammatoire qui prend peu à peu le dessus, conduisant dans certains cas à une immunosuppression. Les décès survenant au décours de la réponse pro-inflammatoire sont dus à un excès de production des cytokines pro-inflammatoires, tandis que les décès survenant plus tard au cours de la phase anti-inflammatoire sont dus à l'incapacité à contrôler les agents pathogènes. Une étude post mortem a ainsi montré que chez 80% des patients septiques, des signes d'infection non résolue étaient retrouvés malgré une antibiothérapie adaptée, prouvant l'incapacité du système immunitaire à lutter efficacement contre l'infection<sup>48</sup>. La survenue d'infections nosocomiales et les réactivations virales observées chez cette population de patients est un autre argument en faveur de l'immunosuppression<sup>20,49</sup>.

Aujourd'hui, la **coexistence des réponses inflammatoires et immunosuppresseives** est établie (**figure 7**). L'ampleur de ces réponses et leur variation dans le temps définissent alors des **modèles de trajectoires immuno-inflammatoires** au sein des patients<sup>50,51</sup>. Ces trajectoires sont caractérisées par la survenue des évènements suivants (**figure 7**) :

- Une inflammation initiale excessive conduisant aux décès précoces
- Une réponse immunosuppresseive trop importante : développement d'infections opportunistes ou réactivation virale associée à une mortalité retardée
- La persistance d'une inflammation latente et/ou d'une immunosuppression au long cours provoquant des séquelles persistantes, des réinfections, *etc.*
- Une résolution adéquate des phases pro-inflammatoires et de compensation : retour à un état d'homéostasie semblable à celui avant le développement du sepsis

Bien que ces phénomènes soient simultanés chez le patient septique, par souci de clarté, la réponse inflammatoire et l'immunodépression induite par le sepsis seront traitées successivement dans les chapitres suivants.



**Figure 7. Trajectoires immunitaires du sepsis et ses conséquences cliniques<sup>52</sup>**

### III. La réponse inflammatoire au cours du sepsis

#### 1. Inflammation initiale

La réaction inflammatoire, plus généralement appelée inflammation, correspond à **l'activation des cellules immunitaires résidentes des tissus lésés ou agressés**, notamment les polynucléaires et les macrophages. Cliniquement, cette activation va se traduire par les 4 signes cardinaux de l'inflammation, décrits dès l'antiquité par Celsius : rougeur (*rubor*), chaleur (*calor*), douleur (*dolor*) et œdème (*tumor*). Les cellules majoritairement responsables de la réaction inflammatoire sont les **polynucléaires neutrophiles (PMN)** et les **monocytes/macrophages** qui vont alors sécréter des composés pro-inflammatoires (*i.e.* favorisant l'inflammation) pour lutter contre le danger détecté<sup>53</sup>.

##### *1.1. Cellules immunitaires impliquées dans l'inflammation*

Les PMN et les monocytes/macrophages sont les cellules pro-inflammatoires spécialisées du système immunitaire, jouant un **rôle crucial dans la réponse inflammatoire** en se déplaçant rapidement vers les sites infectieux ou inflammatoires.

Les PMN sont les cellules immunitaires **les plus abondantes** dans l'organisme et sont les premières à être recrutées en réponse à des signaux de danger, constituant ainsi la **première ligne de défense**. Ils libèrent des **cytokines pro-inflammatoires** (comme l'IL-1 $\beta$  et l'IL-6), des chimiokines (qui attirent d'autres cellules immunitaires vers le site de l'inflammation) ainsi que des **médiateurs lipidiques** tels que les leucotriènes et les prostaglandines. De plus, les PMN libèrent des **enzymes protéolytiques**, telles que les métalloprotéinases matricielles, qui contribuent à la dégradation des tissus endommagés et à la migration des cellules inflammatoires. Ils participent également à l'élimination des agents pathogènes grâce à la synthèse de **composés toxiques** (lors du burst oxydatif) et à la **phagocytose**<sup>54</sup>.

Les monocytes sont des cellules circulantes amenées à se différencier en macrophages après migration dans les tissus par diapédèse. Les macrophages sont des acteurs clefs de l'inflammation, produisant une variété de **médiateurs pro-inflammatoires** tels que l'IL-1 $\beta$ , l'IL-6 et le TNF- $\alpha$ . Ces cytokines jouent un rôle important dans l'amplification de la réponse inflammatoire en recrutant et activant d'autres cellules immunitaires. De plus, les macrophages synthétisent des **chimiokines**, des **prostaglandines** et des **facteurs de croissance** qui contribuent à la régulation de l'inflammation et à la cicatrisation des tissus endommagés. Les macrophages participent également à l'élimination des dangers par **phagocytose**<sup>54</sup>.

Les polynucléaires neutrophiles et les monocytes/macrophages sont donc des **acteurs clefs dans le processus inflammatoire** et représentent les **cellules de veilles** de l'immunité innée par excellence. Leur recrutement, leur activation et leur interaction avec d'autres cellules immunitaires sont essentiels pour orchestrer une réponse inflammatoire efficace et la lutte contre les infections.

### *1.2.Reconnaissance des signaux de danger*

Pour que la réponse immunitaire puisse se mettre en place, les cellules impliquées doivent détecter la présence de signaux de dangers. En 1989, les premiers travaux proposèrent que les cellules du système immunitaire inné reconnaissent ces dangers à l'aide de récepteurs spécifiques : il s'agit des *Pattern Recognition Receptors* (PRR) (**figure 8**)<sup>55</sup>. Les PRR sont exprimés par diverses cellules de l'immunité innée : les PMN, les monocytes/macrophages et les cellules dendritiques. De nombreux PRR sont retrouvés chez l'homme, tels que les TLR, les *C-type lectin receptors* (CLR), les *nod-like receptors* (NLR), les *scavenger receptors* (SR). Ces récepteurs sont intracytoplasmiques (NLR), retrouvés à la surface des cellules (SR) ou les 2 à la fois (TLR, CLR), permettant une diversité de réponse partiellement spécifique aux signaux de danger<sup>55</sup>.

Ces différents types de récepteurs peuvent reconnaître 2 grands types de signaux. Le 1<sup>er</sup> correspond aux **éléments clefs retrouvés uniquement chez les agents pathogènes**, on parle de *Pathogen Associated Molecular Pattern* (PAMP)<sup>55</sup>. Ceux-ci sont nécessaires à la survie du micro-organisme et sont hautement conservés : on peut citer le lipopolysaccharide (LPS) retrouvé à la surface de la membrane externe des bactéries à Gram négatif et reconnu par le *toll-like receptor 4* (TLR-4), ou le peptidoglycane, composant essentiel de la paroi bactérienne. Le second regroupe les **molécules intracellulaires de l'hôte et est normalement inaccessible aux cellules immunitaires**, on parle de *Danger Associated Molecular Pattern* (DAMP) ou **alarmines**<sup>56</sup>. Ces molécules sont libérées dans le milieu extracellulaire à la suite d'un stress ou de la mort cellulaire, comme les protéines S100A8 et S100A9, une molécule associée à la chromatine : *High-Mobility Group Box 1* (HMGB1), les acides nucléiques, l'adénosine triphosphate (ATP)... Lorsque les barrières de l'organisme sont rompues ou que celui-ci détecte un élément pathogène, ces signaux de dangers vont être captés par les PRR : le système immunitaire inné se met alors en place et provoque l'initiation de la réaction inflammatoire.

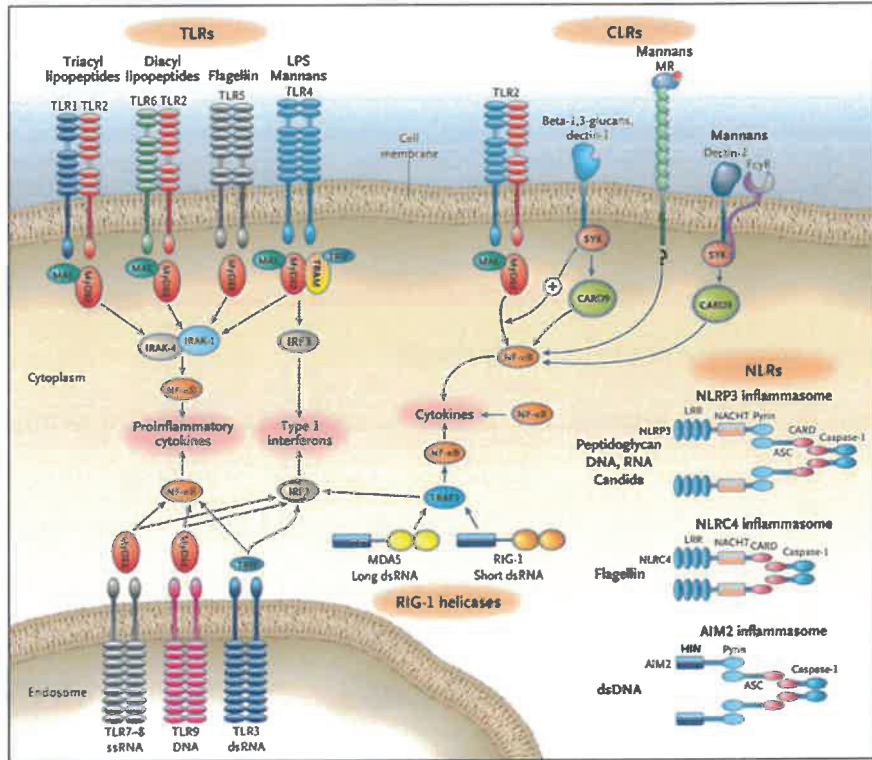


Figure 8. Différentes classes majeures de PRR et leurs principaux ligands<sup>57</sup>

### 1.3. Dysfonctions endothéliales et coagulation

Les cellules endothéliales du système vasculaire tapissent les vaisseaux sanguins et jouent un rôle essentiel dans l'homéostasie, la régulation de l'hémostase et la réponse immunitaire locale. Leur fonction de barrière est assurée par leurs cytosquelettes, la présence du glycocalyx (une membrane riche en glucides) et des jonctions serrées entre les cellules endothéliales. Cet endothélium régule les échanges de solutés et prévient les dysfonctionnements de l'hémostase en régulant finement les processus de coagulation et de fibrinolyse. Lorsqu'il y a une altération de cette barrière ou la détection d'un pathogène, les cellules endothéliales **augmentent la perméabilité vasculaire et provoquent une vasodilatation** pour accroître le débit sanguin local<sup>58</sup>. Cela entraîne la formation d'un **œdème** et **facilite le recrutement des cellules immunitaires** du sang vers le tissu concerné, ce qui se manifeste cliniquement par les 4 signes cardinaux de l'inflammation<sup>53</sup>.

Dans le cas du sepsis, la fonction endothéliale est altérée par de nombreux phénomènes tels que la présence excessive de médiateurs inflammatoires et oxydants, la destruction de la membrane plasmique, du glycocalyx, des jonctions serrées, *etc.* Cette altération de l'endothélium entraîne une augmentation du passage des solutés et des protéines vers les tissus, amplifiant ainsi les œdèmes et pouvant conduire à une hypovolémie, une hypotension artérielle et une défaillance cardiovasculaire<sup>58</sup>. Plus spécifiquement, la destruction du glycocalyx expose

des molécules d'adhésion telles que les sélectines et les intégrines, et active des composés impliqués dans l'hémostase primaire. L'hémostase est un système complexe impliquant l'endothélium, des facteurs solubles, les plaquettes et les leucocytes. Ces composés maintiennent l'équilibre entre les forces de coagulation et de fibrinolyse, tout en dirigeant la coagulation vers les zones appropriées. Les cellules endothéliales synthétisent et expriment des molécules essentielles à la régulation de l'hémostase, telles que le facteur de von Willebrand (vWF), le facteur tissulaire (TF) et l'inhibiteur de l'activateur du plasminogène de type 1 (PAI-1). Lorsque l'endothélium est endommagé, le facteur tissulaire s'active et le vWF se lie au collagène exposé, ce qui entraîne le recrutement des plaquettes et la synthèse de fibrine, amorçant ainsi le processus de coagulation<sup>58</sup>.

L'état hyperinflammatoire observé au cours du sepsis entretient un état prothrombotique et antifibrinolytique, ce qui peut entraîner des thromboses, une ischémie des tissus et de multiples dysfonctions d'organes. Cliniquement, ce phénomène peut se manifester par une coagulation intravasculaire disséminée (CIVD) ou un purpura thrombotique thrombocytopénique. La CIVD et la consommation des facteurs de coagulation peuvent conduire le patient vers un état où la fonction des plaquettes est gravement altérée, entraînant des hémorragies<sup>58</sup>.

#### *1.4. Activation du complément*

Le système du complément est ensemble de protéines circulantes représentant un mécanisme de défense parmi les plus anciens du système immunitaire inné. La reconnaissance des PAMP et des DAMP conduit à la synthèse des anaphylatoxines C3a et C5a et de l'opsonine C3b ainsi qu'à la formation du complexe d'attaque membranaire. La synthèse de ces composés permet de lutter contre le pathogène ou le danger détecté de plusieurs façons. C5a est un puissant chémoattracteur et va permettre de **recruter les PMN et les macrophages** qui vont pouvoir former des pièges extracellulaires afin d'améliorer la clairance bactérienne en plus de participer à la production de médiateurs pro-inflammatoires. L'opsonisation des agents pathogènes par C3b **facilite la phagocytose** de ces composés et leur destruction. Le complexe d'attaque membranaire est quant à lui formé de multiples protéines de la voie du complément, permettant la lyse osmotique de la cible et l'activation des cellules endothéliales<sup>59</sup>.

Bien que l'activation de ce système soit étroitement régulée par des systèmes inhibiteurs, son hyperactivation délétère a été décrite au cours du sepsis<sup>59,60</sup>. Les produits du complément sont impliqués dans les dysfonctions cellulaires et les défaillances d'organes

observés au cours du sepsis, mais aussi dans l'hyperinflammation et les troubles de la coagulation (formation de thrombi). Les fortes concentrations en anaphylatoxines sont ainsi associées à la sévérité du sepsis et à sa mortalité<sup>59,60</sup>.

## 2. Amplification de la réponse inflammatoire

### 2.1. Recrutement cellulaire

Après la détection des signaux de danger et l'initiation de la réponse inflammatoire, les cellules effectrices, telles que les PMN et les monocytes/macrophages, doivent être recrutées. Cette migration se réalise grâce à un gradient de concentration de molécules chimioattractantes émises par l'agent pathogène (*e.g.* protéines bactériennes), induites par celui-ci (*e.g.* les leucotriènes), par les anaphylatoxines issues du complément et par les chimiokines. Des récepteurs pour ces composés sont présents à la surface des PMN ou des monocytes/macrophages et induisent une migration orientée dans le sens du gradient : on parle de **chimiotactisme**. Ces cellules circulantes peuvent donc adhérer aux cellules endothéliales des vaisseaux, se glisser entre celles-ci par diapédèse et se diriger de façon orientée vers leur cible tissulaire. Cette migration dépend d'une part des molécules d'adhérence exprimées par les cellules circulantes, et d'autre part par les cellules endothéliales. Les premières cellules à migrer vers les foyers infectieux sont les PMN, puis dans un second temps les monocytes, qui se différencieront en macrophages dans les tissus. Le passage de l'endothélium et la migration tissulaire est réalisé en 4 étapes<sup>61</sup> (**figure 9**):

- *La capture* : adhérence réversible des cellules faisant intervenir des protéines de la famille des sélectines (*e.g.* CD62E, CD62P), exprimées à la fois par la cellule et par l'endothélium, permettant un ralentissement des cellules
- *Le roulement* : suite de la capture avec interaction des sélectines. La cellule s'active peu à peu jusqu'à s'arrêter complètement ou repartir dans la circulation générale
- *L'adhérence* : les intégrines (*e.g.* VLA4, LFA1) exprimées par les cellules immunitaires se lient à l'endothélium via des protéines appartenant à la superfamille des immunoglobulines (*e.g.* VCAM1, ICAM1). Cette fixation est irréversible et provoque l'arrêt de la cellule
- *La diapédèse* : il s'agit du passage de la cellule de la circulation sanguine vers le tissu lésé et fait intervenir différents processus (rampement et transmigration paracellulaire ou transcellulaire) et différents facteurs d'adhésions (*e.g.* PECAMs)

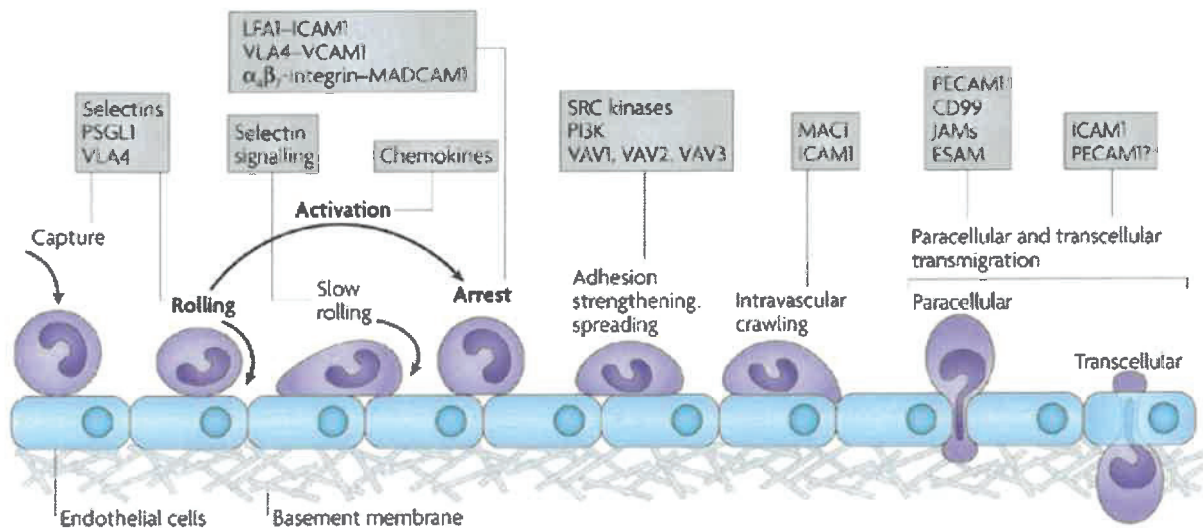


Figure 9. Processus de diapédèse<sup>61</sup>

## 2.2. Cytokine storm

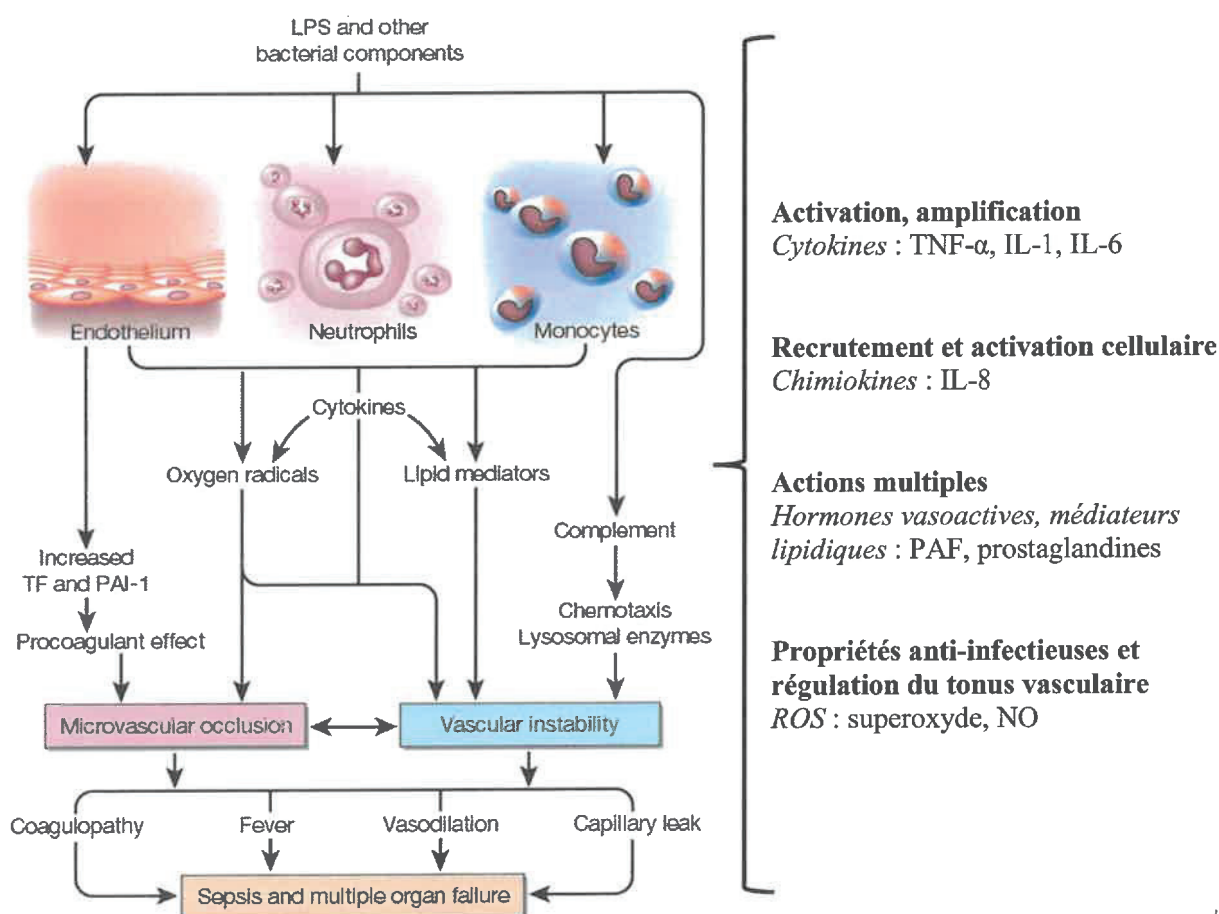
La tempête cytokinique ou *cytokine storm* est un **syndrome inflammatoire systémique potentiellement mortel** caractérisé par des niveaux élevés de cytokines plasmatiques et une hyperactivation des cellules immunitaires. Ce phénomène peut être déclenché par des thérapies qui ciblent le système immunitaire, divers agents pathogènes ou encore certaines pathologies (figure 10). Les cytokines sont des protéines clefs impliquées dans le développement et la régulation des réponses immunitaires, et plus particulièrement dans le processus inflammatoire. Elles sont produites par de nombreux types cellulaires afin d'éliminer le danger détecté. Néanmoins, cette synthèse doit être finement régulée pour repousser la menace tout en évitant une trop forte production pouvant provoquer des dommages tissulaires collatéraux. Les cytokines ont ainsi une demi-vie courte pour limiter leurs effets aux tissus concernés, bien que de forts taux de cytokines plasmatiques puissent être observés en cas de dissémination de l'infection, comme dans le sepsis. Ainsi, une réponse inflammatoire importante peut être appropriée pour contrôler une infection si un dysfonctionnement excessif des organes n'est pas observé. L'hyperactivation des cellules immunitaires observée dans la tempête cytokinique peut résulter de plusieurs mécanismes<sup>62</sup> (figure 10) :

- Activation en l'absence de signaux de dangers (*e.g.* troubles génétiques)
- Réponse excessive ou inappropriée (*e.g.* en réponse à un traitement par *chimeric antigenic receptor-T cells* (CAR-T cells))
- Une charge bactérienne très élevée (*e.g.* au cours d'un sepsis)



Lors de la tempête cytokinique, on observe une défaillance des mécanismes de rétrocontrôles négatifs censés prévenir la propagation de la réponse inflammatoire ainsi qu'une surproduction de cytokines et de médiateurs solubles pro-inflammatoires. Cette production excessive entraîne une hyperinflammation et une dissémination (anormale) de cette réponse en dehors du tissu concerné, pouvant aboutir à de graves conséquences sur les organes, et entraîner le décès<sup>62</sup> :

- Cœur : hypotension, insuffisance cardiaque...
- Système circulatoire : cytopénies, coagulopathies et hémorragies...
- Système nerveux central : confusion, convulsions...
- Rein : insuffisance rénale aiguë
- Poumons : œdème pulmonaire, SDRA...
- Foie : hypoalbuminémie, insuffisance hépatique aiguë...
- ...



**Figure 10. Physiopathologie de la tempête cytokinique**  
Adapté de Cohen<sup>63</sup>.

### 3. Médiateurs solubles de l'inflammation

En réponse à la détection des dangers par les PRR et l'activation de diverses voies de signalisation, de nombreux composés sont produits par les cellules immunitaires au cours de la réaction inflammatoire, dont des cytokines, des interleukines, des médiateurs solubles, *etc.* La description exhaustive de tous ces médiateurs n'étant pas l'objectif de ce travail, nous présenterons uniquement les composés étudiés dans notre travail personnel, à savoir l'IL-1 $\beta$ , l'interleukine 18 (IL-18), l'IL-6 et le TNF- $\alpha$ , qui sont tous des produits de l'activation de la voie de signalisation du facteur nucléaire kappa B (NF- $\kappa$ B pour *nuclear factor-kappa B*)<sup>64</sup>.

La voie NF- $\kappa$ B répond à de nombreux stimuli, notamment aux ligands de divers récepteurs de cytokines, aux PRR comme le TLR-4 (impliqué dans la synthèse des cytokines décrites ci-après), aux membres de la superfamille des récepteurs du TNF (TNFR). Lors de la fixation d'un ligand (*e.g.* LPS) ou d'une cytokine (*e.g.* TNF- $\alpha$ ) à son récepteur (*e.g.* TLR-4 ou TNFR), il y a recrutement de l'adaptateur MyD88 (*myeloid differentiation primary response 88*). Celui-ci va ensuite recruter les protéines à activité kinase de la famille IRAK (*IL-1 receptor-associated kinase*), qui stimulent à leur tour l'activité E3 ubiquitine ligase de TRAF6 (*TNF receptor-associated factor 6*), permettant à TRAF6 de réaliser une auto-ubiquitination, impliquée dans l'activation d'une kinase dépendante de l'ubiquitine, TAK1 (*transforming growth factor-activated kinase 1*). Après activation, TAK1 active la kinase IKK (I $\kappa$ B kinase), qui à son tour phosphoryle l'inhibiteur de NF- $\kappa$ B, I $\kappa$ B $\alpha$ , conduisant à sa dégradation et à l'activation de NF- $\kappa$ B<sup>64</sup>.

Le TLR4 a la particularité d'activer cette voie de signalisation via une autre protéine adaptatrice, TRIF (*TIR domain-containing adapter inducing interferon- $\beta$* ). TRIF mobilise ensuite la protéine RIP1 (*kinase receptor-interacting protein 1*) permettant le recrutement et l'activation de IKK pour conduire à l'activation finale de NF- $\kappa$ B comme précédemment. Cette activation permet la synthèse d'ARNm de nombreux composés pro-inflammatoires, dont l'IL-1 $\beta$ , l'IL-18, l'IL-6 et le TNF- $\alpha$ <sup>64</sup>.

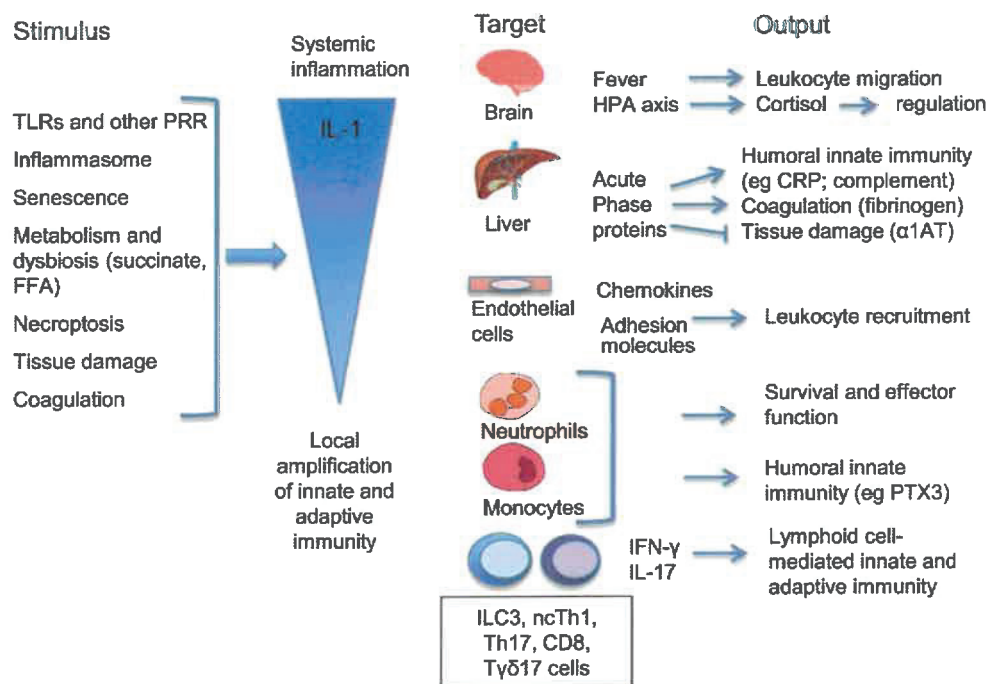
#### 3.1. L'interleukine 1 $\beta$

L'IL-1 $\beta$  est majoritairement synthétisée dans les cellules immunitaires telles que les PMN et les monocytes/macrophages après détection de signaux de dangers sous la forme d'un précurseur de 33 kDa (pro-IL-1 $\beta$ )<sup>65</sup>. Cette forme est néanmoins inactive et seule la forme clivée de 17 kDa est active. La maturation de l'IL-1 $\beta$  est réalisée par une enzyme protéolytique : la caspase-1. Il existe deux récepteurs de l'IL-1 $\beta$  (IL-1R) : le récepteur de type 1 (IL-1R1) qui

transmet un signal, et le récepteur de type 2 (IL-1R2) qui fixe l'IL-1 $\beta$  mais ne transmet pas de signal ; on parle de récepteur « leurre ». Les détails de la voie de production de l'IL-1 $\beta$  seront abordés dans la partie consacrée à NLRP3<sup>65</sup>.

Les effets de la fixation de l'IL-1 $\beta$  à son récepteur sont nombreux (**figure 11**) et dépendent de la région de l'organisme considérée<sup>66</sup> (non exhaustif) :

- Au niveau du système nerveux central : induction de fièvre et stimulation de l'axe corticotrope pour favoriser la production de cortisol
- Au niveau du foie : synthèse des protéines de la phase aiguë, comme la CRP (protéine C réactive), les protéines du complément, de la coagulation...
- Au niveau des cellules endothéliales : l'IL-1 $\beta$  favorise la production de chimiokines et de facteurs d'adhésions pour le recrutement des leucocytes
- Au niveau du système immunitaire : l'IL-1 $\beta$  favorise la survie et les fonctions effectrices des PMN et des macrophages, régule la différenciation et la fonction des cellules lymphoïdes innées et adaptatives en induisant par exemple la polarisation des lymphocytes T en lymphocytes T helper 17 (TH17)



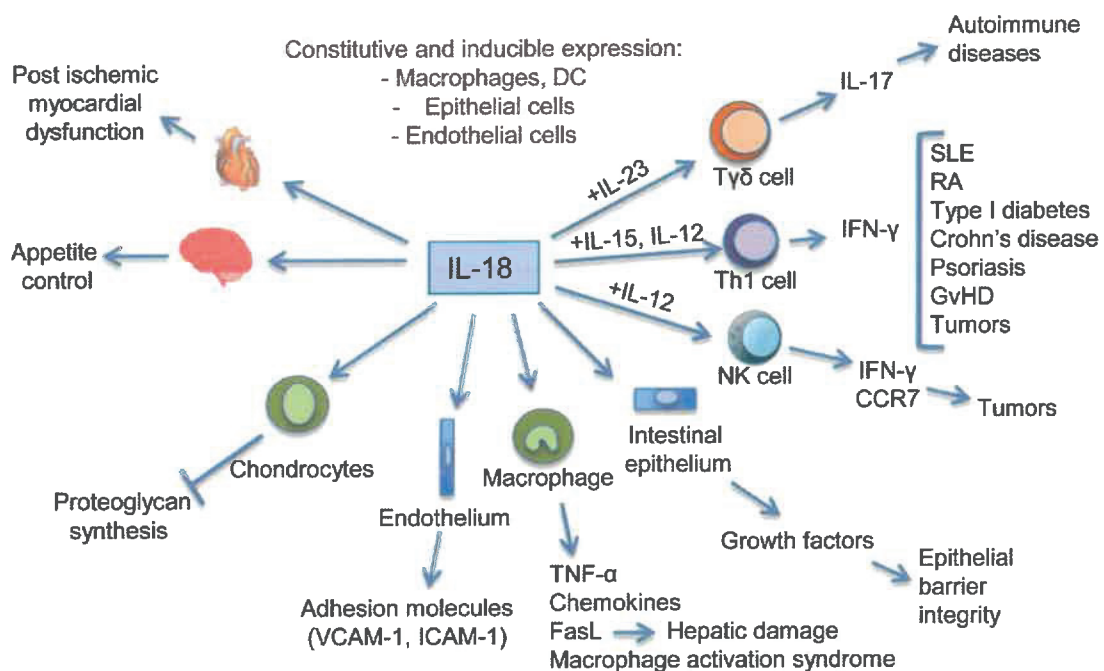
**Figure 11. Effet de l'IL-1 dans l'immunité**  
Adapté de Garlanda et al<sup>66</sup>

### 3.2. L'interleukine 18

L'IL-18 est étroitement apparentée à l'IL-1 $\beta$ . Comme celle-ci, elle est synthétisée sous la forme d'un précurseur inactif (pro-IL-18) nécessitant un clivage par la caspase-1 pour produire la cytokine sous sa forme active<sup>67</sup>. Contrairement à l'IL-1 $\beta$ , **l'IL-18 est synthétisée de manière constitutive** : l'ARNm de l'IL-18 ainsi que la pro-IL-18 sont retrouvés dans les cellules mononuclées du sang périphériques (PBMC) de volontaires sains alors qu'aucune expression d'IL-1 $\beta$  n'est retrouvée dans les mêmes échantillons<sup>67</sup>. Les détails de la voie de production de l'IL-18 seront abordés dans la partie consacrée à NLRP3.

Le récepteur induisant la signalisation de l'IL-18 (IL-18R) est un hétérodimère formé d'une chaîne  $\alpha$  de faible affinité (IL-18R $\alpha$ ) et d'une chaîne  $\beta$  de haute affinité (IL-18R $\beta$ ). Si le corécepteur  $\beta$  est présent lors de la fixation de l'IL-18 à l'IL-18R $\alpha$ , un complexe de haute affinité se forme alors, induisant le signal. De la même façon que l'IL-1 $\beta$ , l'IL-18 possède un récepteur leurre (soluble) ne permettant pas la transduction du signal, l'*IL-18 binding protein* (IL-18BP)<sup>66</sup>.

L'IL-18 est majoritairement impliquée dans la polarisation des lymphocytes T en TH1 (lymphocytes T helper 1), dans la production d'interféron  $\gamma$  (IFN- $\gamma$ ) par les TH1 et les lymphocytes *natural killer* (NK) (**figure 12**). Elle favorise aussi l'interaction entre les macrophages et les cellules NK, en plus d'activer les cellules endothéliales vasculaires pour que celles-ci produisent des facteurs d'adhésions<sup>66</sup> (**figure 12**).



**Figure 12. Impact de l'IL-18 sur la physiologie et dans la réaction inflammatoire**  
 Adapté de Garlanda et al<sup>66</sup>

### 3.3. L'interleukine 6

La majorité des cellules stromales et immunitaires produisent de l'IL-6, celle-ci étant présente à une très faible concentration au niveau basal. L'IL-1 $\beta$  et le TNF- $\alpha$  sont les principaux facteurs permettant la synthèse et la sécrétion d'IL-6, bien que d'autres voies comme les TLR, les prostaglandines et d'autres cytokines soient aussi impliquées<sup>68</sup>.

Le récepteur de l'IL-6 (IL-6R) se compose d'une sous-unité  $\alpha$  qui se lie à l'IL-6 sans induire d'effet, et d'une sous-unité, la gp130, qui ne se lie pas à l'IL-6 mais transmet le signal. Ainsi, le signal classique de l'IL-6 est émis lorsque les cellules expriment conjointement l'IL-6R et la gp130<sup>68</sup>.

L'IL-6 a d'abord été caractérisée par sa capacité à promouvoir l'expansion et l'activation des cellules T, la différenciation des cellules B et la régulation de la réponse à la phase aiguë. Ainsi, les enfants présentant des auto-anticorps dirigés contre l'IL-6 développent des infections récurrentes comme des cellulites staphylococciques et des abcès sous-cutanés<sup>69</sup>. On sait aujourd'hui que l'IL-6 est une cytokine pléiotropique avec de nombreux effets sur l'organisme, affectant le système cardiovasculaire, le métabolisme des lipides, la résistance à l'insuline, les activités mitochondriales, le système neuroendocrinien et le comportement neuropsychologique<sup>68</sup>.

### 3.4. Le Tumor Necrosis Factor $\alpha$

Le TNF- $\alpha$  est un puissant médiateur pro-inflammatoire principalement produit par les monocytes/macrophages, mais aussi par divers types de cellules telles que les cellules NK, les lymphocytes T et les polynucléaires éosinophiles en réponse à divers stimuli<sup>70</sup>. Initialement synthétisé sous la forme d'une protéine transmembranaire, le TNF- $\alpha$  est ensuite exprimé à la surface des cellules sous la forme d'un trimère. Son clivage par une enzyme de conversion permet sa libération sous forme soluble dans la circulation. Ses formes membranaire et soluble exercent toutes deux des fonctions physiologiques en se liant à deux récepteurs distincts sur les cellules cibles, le récepteur 1 du TNF- $\alpha$  (TNFR1) et le récepteur 2 du TNF- $\alpha$  (TNFR2)<sup>70</sup>.

TNFR1 est exprimé sur l'ensemble des cellules de l'organisme et est activé à la fois par les formes soluble et membranaire du TNF- $\alpha$ . Ce récepteur est impliqué dans l'initiation de l'apoptose via l'activation de la caspase 8 ainsi que dans l'initiation de la nécroptose via la mobilisation de la pseudokinase MLKL (*mixed lineage kinase domain-like pseudokinase*)<sup>70</sup>. TNFR1 possède aussi une voie de signalisation commune au TNFR2, qui lui est exprimé par certains types cellulaires tels que les lymphocytes CD4<sup>+</sup> et CD8<sup>+</sup> activés ou les cellules

endothéliales et est principalement activé par la forme membranaire du TNF- $\alpha$ . Cette voie de signalisation aboutit à l'activation de NF- $\kappa$ B et des MAP kinases (MAPK) afin de participer à la prolifération et la survie des cellules, la dégénérescence des tissus et à la défense de l'hôte tout en induisant la synthèse des médiateurs de l'inflammation et en activant la réponse inflammatoire à la phase aiguë (diapédèse, coagulation...) <sup>70</sup>.

#### 4. Les inflammasomes

##### 4.1. Généralités

Les **inflammasomes** sont des **plateformes macromoléculaires intracellulaires** composées de 3 protéines : un récepteur, un adaptateur et un effecteur, la caspase-1. NLRP1 (*NACHT (nucleotide-binding and oligomerization domain), leucin-rich repeat and pyrin-containing receptor 1*) a été le 1<sup>er</sup> inflammasome découvert en 2002 <sup>71</sup>. Depuis, de nombreux autres ont été décrits dans la littérature : NLRP3 (*NACHT, leucin-rich repeat and pyrin-containing receptor 3*), NLRC4 (*NACHT, leucine rich repeat and CARD domain containing 4*), AIM2 (*absent in melanoma 2*) et l'inflammasome pyrine <sup>72</sup>. De nombreux autres inflammasomes appartenant à la famille des NLR (*nod-like receptors*) ont été décrits : NLRP2, NLRP6, NLRP7, NLRP9 et NLRP12 <sup>72</sup>. Ceux-ci sont présentés en **tableau 2** en fonction de leurs récepteurs et des stimuli nécessaires à leur formation.

Récepteurs des inflammasomes	Stimuli activateurs
AIM2	<i>F.novicida</i> , ADN double brin intracytosolique
NLRC4	<i>S.typhimurium</i> , flagelline
NLRP1	Toxine létale de <i>B.anthraxis</i> , clivage autocatalytique
NLRP3	PAMP et DAMP, flux ioniques (efflux de K <sup>+</sup> ), cristaux, dommages mitochondriaux et ROS ( <i>Reactive Oxygen Species</i> ), déstabilisation lysosomale
Pyrine	C.difficile et inactivateurs de la Rho-GTPase

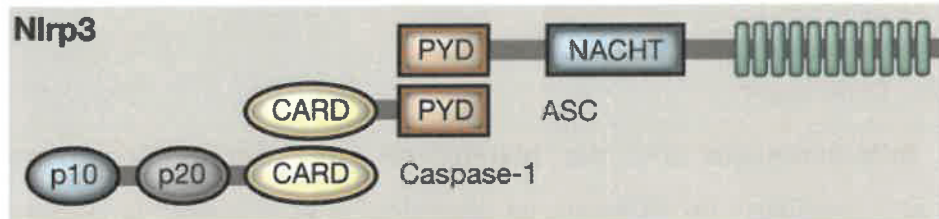
Tableau 2. Principaux récepteurs des inflammasomes associés à leurs stimuli activateurs <sup>72</sup>

Les inflammasomes dits « canoniques » (dont NLRP3 fait partie) convertissent la procaspase-1 en caspase-1 catalytiquement active et sont composés de PRR (e.g. NLRP3). Il existe également des inflammasomes dits « non canoniques » qui ne nécessitent pas de PRR pour effectuer leur activité catalytique : il s'agit de la caspase-11 chez la souris et des caspases-4 et 5 chez l'homme <sup>73</sup>. Dans la suite, nous nous focaliserons sur l'inflammasome le plus largement décrit : l'inflammasome NLRP3.

#### 4.2. Composition de NLRP3

L'inflammasome NLRP3 est constitué de 3 composants (**figure 13**) :

- Un récepteur : NLRP3
- Un adaptateur : ASC (*apoptosis-associated speck-like protein containing CARD*)
- Un effecteur : la caspase-1



**Figure 13. Composition de l'inflammasome NLRP3**

L'inflammasome NLRP3 est composé du récepteur cytosolique NLRP3, divisé en un domaine LRR, un domaine NACHT et un domaine PYD (pyrin domain), d'un adaptateur ASC divisé en un domaine PYD et un domaine CARD et de l'effecteur caspase-1 divisé en domaine CARD et des sous-unités p10 et p20. Adapté de Lamkanfi & Dixit<sup>74</sup>.

##### i. Le récepteur NLRP3

NLRP3 est un récepteur intracytoplasmique qui, en réponse à différents stimuli (voir ci-après), va déclencher l'activation de l'inflammasome NLRP3.

NLRP3 est une protéine de 115 kDa constituée<sup>75</sup> :

- D'un domaine LRR (*leucin-rich repeat*) C-terminal, dont la fonction régulatrice est mal comprise
- D'un domaine central d'oligomérisation NACHT aussi appelé NOD (*nucleotide-binding and oligomerization domain*) et présentant une activité ATPase
- D'un domaine PYD (*pyrin domain*) N-terminal capable de recruter l'adaptateur ASC

Après détection des signaux de danger, NLRP3 va recruter l'adaptateur ASC via des interactions homotypiques PYD-PYD (**figure 13**).

##### ii. L'adaptateur ASC

ASC (*apoptosis-associated speck-like protein containing CARD*) est une protéine assurant le lien entre la détection des signaux de danger et l'effecteur de l'inflammasome NLRP3. En effet, contrairement à NLRP3, ASC contient le domaine CARD nécessaire au recrutement de la caspase-1 (**figure 13**). Ces protéines, naturellement présentes dans de nombreuses cellules au repos, se relocalisent dans le cytosol après détection de signaux de dangers pour se **polymériser au niveau de la région périnucléaire sous la forme d'un complexe appelé speck**<sup>76</sup>. Ce n'est qu'après la formation de ce speck que la caspase-1 pourra être recrutée et activée via des interactions homotypiques CARD-CARD (**figure 13**).

### iii. L'effecteur caspase-1

La caspase-1 est une enzyme à activité protéase clivant ses substrats inactifs en protéines actives<sup>75</sup>. Elle est initialement synthétisée dans le cytosol sous la forme d'un précurseur inactif : la pro-caspase-1. Après formation de speck, les domaines CARD des pro-caspase-1 interagissent avec leurs homologues situés sur la protéine ASC (**figure 13**), induisant leur dimérisation puis leur clivage autoprotéolytique. Cela conduit à la production de la forme active : la caspase-1<sup>75</sup>. La structure ainsi formée de NLRP3, ASC et de la caspase-1 est appelée **inflammasome (figure 14)**.

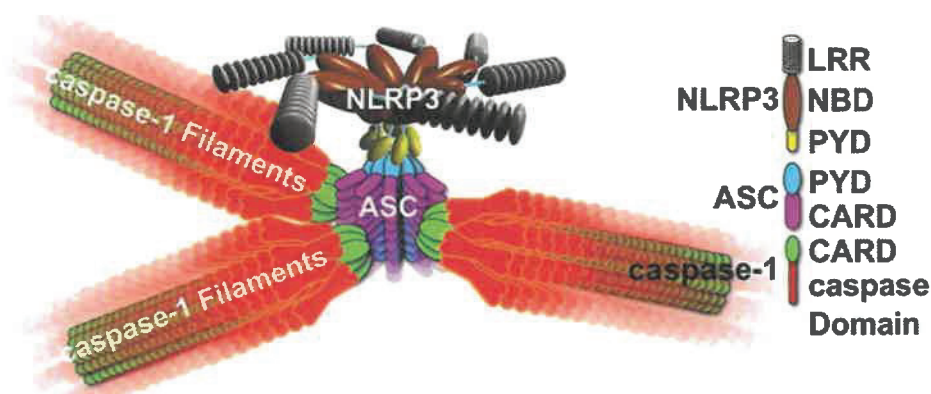


Figure 14. Représentation schématique de l'inflammasome NLRP3 activé<sup>77</sup>

### 4.3. Activation de NLRP3

L'inflammasome NLRP3 est activé en réponse à de nombreux agents pathogènes, des toxines formant des pores (*e.g.*, nigéricine), des cristaux, des agrégats (*e.g.*, plaque  $\beta$ -amyloïde) et des DAMP (*e.g.*, ATP)<sup>74</sup>. Deux signaux complémentaires sont nécessaires : le **priming** (signal 1) et le **signal d'activation** (signal 2).

#### i. Le signal de priming

Le **priming**, ou signal 1, est l'étape de sensibilisation permettant la synthèse de NLRP3 et de la pro-IL-1 $\beta$  (**figure 15**). Cette sensibilisation est le résultat de la détection d'un signal de danger (PAMP ou DAMP) qui va induire l'activation du facteur de transcription NF- $\kappa$ B permettant la synthèse des ARNm (ARN messagers) des protéines NLRP3 et pro-IL-1 $\beta$ <sup>78</sup>. L'ASC, la pro-caspase 1 et la pro-IL-18 sont par ailleurs **constitutivement produites**, indépendamment de l'étape de priming.



*ii. Le signal d'activation*

Le signal d'activation, ou signal 2, **permet la formation de l'inflammasome NLRP3.**

Trois grands types de signaux activateurs sont actuellement décrits.

*Les mouvements d'ions*

L'augmentation de la perméabilité membranaire (avec l'activation du récepteur P2X7 par la fixation d'ATP), ainsi que la formation de pore dans la membrane plasmique par action de la nigéricine (toxine bactérienne) ou du complexe d'attaque membranaire induisent un efflux de potassium dans la cellule<sup>79</sup>. La diminution de la concentration intracellulaire en potassium est suffisante pour activer l'inflammasome NLRP3<sup>79</sup> (**figure 15**).

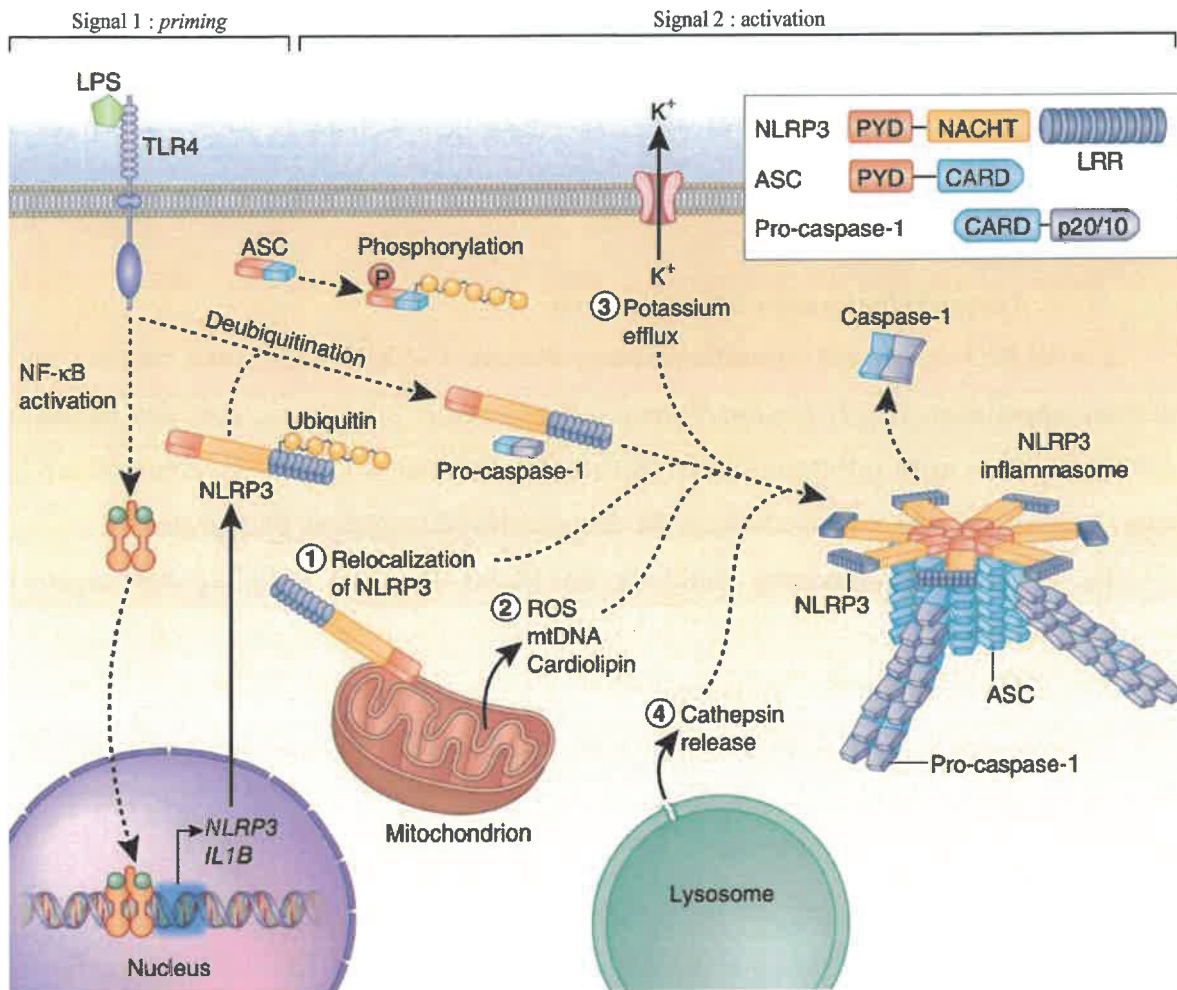
*Les dysfonctions de la mitochondrie*

Les dysfonctions mitochondriales représentent un autre type de signal activateur. Celles-ci produisent des ROS et de l'ADN mitochondrial identifiés comme signaux d'activation<sup>80,81</sup> (**figure 15**). Une autre possibilité est représentée par l'interaction directe de NLRP3 avec la mitochondrie au moyen des microtubules<sup>82</sup> ou des cardiolipides<sup>83</sup> qui la constituent (**figure 15**).

*Les troubles du lysosome*

La déstabilisation lysosomale est le dernier grand type de signal induisant l'activation de l'inflammasome NLRP3. On peut citer le relargage de cathepsine B (protéase lysosomale), la détection des dommages et de la rupture des phagolysosomes après endocytose de cristaux, comme la silice<sup>84</sup> (**figure 15**).

Les signaux de priming puis les signaux d'activation conduisent au recrutement de la procaspase-1 (**figure 15**) puis à son clivage autoprotéolytique conduisant à la production de la forme active : la caspase-1.



**Figure 15. Mécanismes d'activation de l'inflammasome NLRP3**

L'étape de priming correspond à la reconnaissance du danger (e.g., fixation du LPS au TLR4) et à l'activation des gènes codants pour NLRP3 et l'IL-1 $\beta$  par le facteur de transcription NF- $\kappa$ B. L'étape d'activation permet la formation de l'inflammasome NLRP3 après détection de divers signaux : dysfonctions mitochondriales (1 et 2), diminution intracellulaire du potassium (3) ou troubles du lysosome (4). Adapté de Guo et al<sup>85</sup>.

### iii. Conséquences de l'activation de NLRP3

L'inflammasome NLRP3 ainsi activé va permettre le clivage de 3 substrats inactifs en protéines actives : les pro-IL-1 $\beta$  et les pro-IL-18 en IL-1 $\beta$  et IL-18 respectivement (décrites précédemment), ainsi que la pro-gasdermine-D en **gasdermine-D** (GSDMD)<sup>86</sup>. Ces différents acteurs vont alors participer au processus inflammatoire.

La GSDMD est une protéine qui va aller s'insérer dans la membrane plasmique pour provoquer la mort de la cellule par pyroptose, une **mort inflammatoire dépendante des caspases**, à différencier de l'apoptose, une mort programmée non inflammatoire<sup>86</sup>. Celle-ci est caractérisée par la lyse de la cellule et le relargage de son contenu dans l'espace extracellulaire, dont un **relargage massif des IL-1 $\beta$  et IL-18** préalablement synthétisés<sup>87</sup>. La pyroptose n'est pas le seul mécanisme de sécrétion des IL-1 $\beta$  et IL-18 bien que ceux-ci restent encore discutés

dans la communauté scientifique. On peut néanmoins citer les travaux rapportant l'utilisation des exosomes<sup>88</sup>, de l'autophagie<sup>89</sup> ou de la relocalisation de l'IL-1 $\beta$  à la membrane plasmique<sup>90</sup> comme mécanismes de sécrétion de l'IL-1 $\beta$  indépendants de la GSDMD.

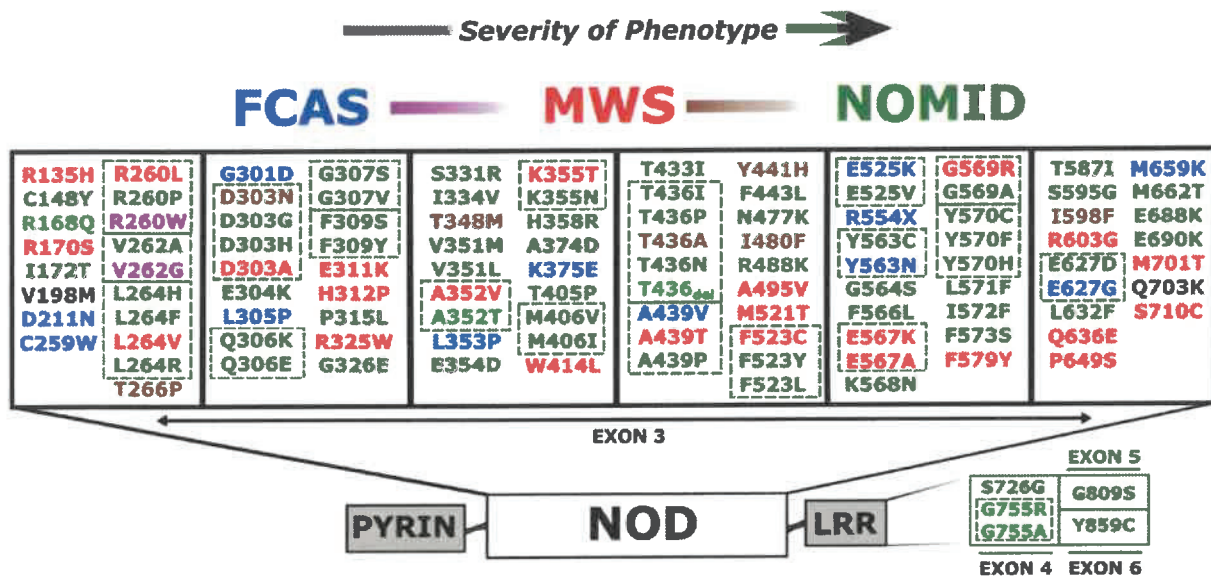
#### 4.4. *NLRP3 en pathologies*

##### *i. Les pathologies auto-inflammatoires*

Les ***NLRP3-associated autoinflammatory diseases*** (NLRP3-AID), aussi connues sous l'ancienne appellation CAPS (*cryopyrin-associated periodic syndrome*), sont des maladies **héréditaires rares auto-inflammatoires** décrites dès les années 1940<sup>91</sup>. Au cours du siècle dernier, 3 phénotypes de ces maladies ont été documentés (du moins au plus sévère)<sup>92</sup> :

- La fièvre méditerranéenne familiale ou FCAS (*familial cold autoinflammatory syndrome*)
- Le syndrome de Muscle-Wells (MWS)
- Le syndrome CINCA (*chronic infantile neurologic cutaneous articular*) et la NOMID (*neonatal onset multisystem inflammatory disease*)

Ces maladies sont caractérisées par des tableaux cliniques similaires, variables dans leurs intensités. On retrouve ainsi des atteintes cutanées (rougeur, urticaire), systémiques (fièvre, fatigue), musculosquelettiques (arthralgies, myalgies), oculaires (conjonctivite, kératite), auditives (perte d'audition), centrales (céphalées, méningites stériles) et la présence d'amyloïdose<sup>92</sup>. Il faudra attendre 2001 pour identifier la cause génétique de ces pathologies: des **mutations sur le gène CIAS1**<sup>93</sup> (l'un des anciens noms de NLRP3). Ces mutations gain de fonction associées au NLRP3-AID, à **transmission autosomale dominante**, conduisent à un **inflammasome dont l'activation n'est plus contrôlée** et entraîne alors une **libération accrue des cytokines pro-inflammatoires IL-1 $\beta$  et IL-18** qui elles-mêmes mènent à une inflammation systémique et tissulaire à l'origine des symptômes des pathologies précédemment décrites<sup>92</sup>. Les mutations recensées conduisant aux NLRP3-AID sont indiquées en **figure 16** et touchent majoritairement le domaine NACHT (appelé aussi domaine NOD).



**Figure 16. Mutations du gène codant la protéine NLRP3 et conduisant aux NLRP3-AID<sup>92</sup>**  
 Les mutations sont majoritairement localisées au niveau de l'exon 3. Les multiples mutations codant pour le même acide aminé sont indiquées par les encadrés en pointillés. Les codes couleurs font correspondre les mutations à leurs maladies : FCAS (bleu), FCAS/MWS (violet), MWS (rouge), MWS/NOMID (marron), NOMID (vert) et les mutations à faible pénétrance (noir).

### ii. Les pathologies métaboliques

Le syndrome métabolique se traduit par la présence de plusieurs désordres physiologiques et biochimiques regroupant des troubles de santé d'origine lipidique, glucidique ou vasculaire associés à un excès de poids. L'ensemble de ces désordres métaboliques augmente considérablement le risque de diabète de type 2 et de maladies cardiaques<sup>94</sup>. Ces troubles conduisent au relargage de signaux de dangers, comme le palmitate ou les lipides et favorisent l'activation de NLRP3<sup>95</sup>. La libération d'IL-1 $\beta$  qui en résulte provoque une résistance à l'insuline<sup>96</sup> et réduit l'absorption du glucose dans les tissus cibles de l'insuline tels que les muscles, le foie et les tissus adipeux, favorisant une progression vers le diabète et l'obésité<sup>95</sup>.

Des concentrations importantes des composants de l'inflammasome NLRP3 et de ces produits (*i.e.* IL-1 $\beta$  et IL-18) ont été retrouvées au niveau de plaques d'athéromes (dépôt des lipides oxydés conduisant à l'athérosclérose)<sup>97</sup>. Une corrélation entre l'expression de NLRP3 et la sévérité de la sténose des artères coronariennes chez l'homme a aussi été rapportée<sup>98</sup>. Ces informations sont à relier aux nombreuses études montrant le rôle de l'inflammasome NLRP3 dans l'infarctus aigu du myocarde et ses conséquences sur l'insuffisance cardiaque<sup>99</sup>.

Enfin, l'activation de la voie de NLRP3 est aussi observée dans les crises de goutte, où les cristaux d'urate de sodium alliés à la présence d'acides gras sont suffisants pour la synthèse d'IL-1 $\beta$  et d'IL-18, créant une réaction inflammatoire à l'origine de douleurs articulaires<sup>100</sup>.

### *iii. Les pathologies neurodégénératives*

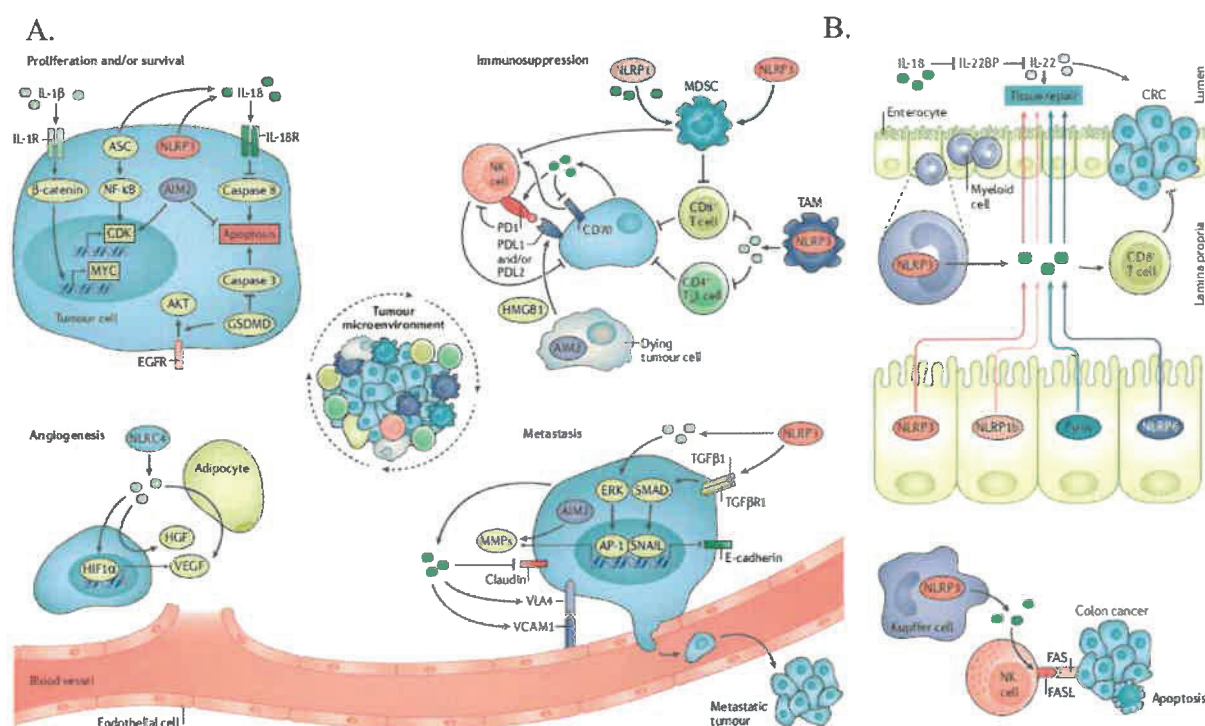
L'inflammation est une caractéristique du microenvironnement neurodégénératif et, malgré son impact essentiel dans l'homéostasie tissulaire, de plus en plus de preuves suggèrent un rôle de l'agression inflammatoire chronique à la neurodégénérescence progressive<sup>101</sup>. Il a ainsi été montré de nombreux liens entre physiopathologie et dérégulation de la voie de NLRP3. Les modèles animaux utilisés dans la maladie d'Alzheimer, la maladie de Parkinson ainsi que dans la sclérose en plaques ou la sclérose latérale amyotrophique montrent une hyperactivation de la voie de l'inflammasome NLRP3. L'utilisation d'inhibiteurs ou de souris KO pour les composants de l'inflammasome entraîne une diminution de la sévérité ainsi qu'une amélioration des fonctions cognitives<sup>101-104</sup>.

En clinique, des éléments en faveur de ce qui a été observé sur modèles animaux ont été observés. Chez les patients atteints de la maladie d'Alzheimer, les prélèvements post mortem de cortex et d'hippocampe montrent une augmentation de l'expression de la caspase-1<sup>105</sup> ainsi qu'une augmentation des ARNm des composants de l'inflammasome dans les monocytes et une production accrue d'IL-1 $\beta$  et d'IL-18 plasmatiques<sup>106</sup>. Dans la maladie de Parkinson, une augmentation des composants de l'inflammasome (ARN et protéines) est observée chez les patients ainsi qu'une production accrue d'IL-1 $\beta$ , dont cette dernière corrèle avec la dégradation des scores cliniques cognitifs et moteurs<sup>107,108</sup>. Chez les patients atteints de sclérose en plaques, le polymorphisme des gènes de l'inflammasome NLRP3 confère un risque accru de développer la maladie<sup>101</sup> et une étude du transcriptome a prouvé l'existence d'une signature inflammasome, reliant l'expression du gène de l'IL-1 $\beta$  à sa sévérité<sup>109</sup>. Enfin, sur des prélèvements post mortem de patients atteints de sclérose latérale amyotrophique, l'activité du gène codant NLRP3 est augmentée dans les neurones et les cellules gliales<sup>110</sup> et une élévation de la pyroptose est observée dans le cortex moteur, associée à une neurodégénérescence microgliale<sup>111</sup>.

### *iv. Les pathologies tumorales*

L'inflammation est l'une des principales caractéristiques du développement et de la progression tumorale<sup>95</sup>. Une étude récente a montré une différence d'expression des gènes liés à l'inflammasome NLRP3 dans 15 des 24 cancers humains étudiés. Ces différences d'expression génique, rapportant des augmentations ou des diminutions, étaient comparées entre les tissus sains et les tissus tumoraux chez tous les patients<sup>112</sup>. Ces résultats sont cohérents avec le rôle controversé de NLRP3 dans la tumorigenèse, pouvant favoriser la progression du processus tumoral ou en protéger<sup>113</sup>. De nombreuses revues récentes traitent de ce sujet<sup>95,113-115</sup>; nous n'aborderons donc pas en détail les différents processus. Nous pouvons mentionner

que le rôle de l'inflammasome NLRP3 dans la progression tumorale repose sur sa capacité à favoriser la prolifération des cellules tumorales, l'immunosuppression, l'angiogénèse et le processus métastatique (**figure 17**). Au contraire, ses capacités répressives de la tumorigénèse, majoritairement illustrées dans le cancer colorectal<sup>116</sup>, seraient attribuées à l'activation de la caspase-1 et à la sécrétion d'IL-18 (**figure 17**).



**Figure 17. Rôle dichotomique de l'inflammasome NLRP3 dans le processus tumoral**  
**A.** La production des interleukines IL-1β et IL-18 permet la progression tumorale en favorisant la survie et/ou la prolifération des cellules tumorales, la paralysie du système immunitaire, l'angiogénèse et le processus métastatique. **B.** L'IL-18 a une fonction répressive de la tumorigénèse dans le cancer colorectal (CRC) en régénérant les cellules épithéliales, en modulant la biodisponibilité de l'IL-22 et en favorisant l'action des lymphocytes T CD8+. L'IL-18 libérée par les cellules de Kupffer améliore les fonctions tumoricides des cellules NK, limitant les métastases hépatiques du cancer colorectal. Adapté de Karki & Kannan<sup>113</sup>.

#### v. Les pathologies infectieuses

Comme énoncé précédemment, l'inflammation permet le recrutement des cellules immunitaires pour combattre les infections. Il est donc tout à fait normal que l'activation de la voie de NLRP3 soit impliquée dans la physiopathologie des maladies infectieuses. De nombreux pathogènes (bactéries, virus et organismes fongiques) peuvent ainsi activer cet inflammasome (**tableau 3**), induisant les réactions immunitaires décrites plus haut.

<b>Pathogènes</b>	<b>Activateurs</b>
<b>Bactéries</b>	
<i>Streptococcus pneumoniae</i>	Pneumolysine
<i>Klebsiella pneumoniae</i>	Inconnu
<i>Bordetella pertussis</i>	Toxine adénylate cyclase A
<i>Clostridium difficile</i>	Toxine A et toxine B
<i>Yersinia species</i>	Protéine externe J
<i>Staphylococcus aureus</i>	Hémolysines
<i>Vibrio cholerae</i>	Hémolysine A, toxine MARTX <sub>Vc</sub> , toxine B
<i>Escherichia coli</i>	ARN, LPS, entérotoxine thermolabile
<i>Citrobacter rodentium</i>	Inconnu
<i>Streptococcus pyogenes</i>	Streptolysine O
<i>Chlamydia pneumoniae</i>	Inconnu
<i>Neisseria gonorrhoeae</i>	Inconnu
<i>Mycobacterium tuberculosis</i>	Inconnu
<i>Listeria monocytogenes</i>	Listériolysine O, flagelline
<i>Aeromonas hydrophila</i>	Aérolysine
<i>Salmonella typhimurium</i>	Flagelline, protéine PrgJ
<b>Organismes fongiques</b>	
<i>Candida albicans</i>	Inconnu
<i>Aspergillus fumigatus</i>	Inconnu
<b>Virus</b>	
Sendai virus	Inconnu
Influenza A virus	Protéine M2, ARN viral
Adenovirus	Inconnu
Varicella zoster virus	Inconnu
Herpes virus	Inconnu
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)	ARN ; protéines spike et 8b, N ; viroporines E et 3a

*Tableau 3. Activation de l'inflammasome NLRP3 par les pathogènes  
Activateurs bactériens, fongiques et viraux de l'inflammasome NLRP3. Adapté de Tartey &  
Kanneganti<sup>117</sup>.*

Dans les modèles murins de sepsis, il est ainsi observé une hyperactivation de la voie de l'inflammasome NLRP3, avec une augmentation des concentrations plasmatiques en IL-1 $\beta$  et en IL-18<sup>118,119</sup>. Les modifications apportées à NLRP3<sup>118</sup>, l'utilisation de souris KO pour NLRP3 ou d'inhibiteurs dirigés contre celui-ci<sup>120,121</sup> permettent d'améliorer la survie des animaux sur modèles murins de sepsis, montrant l'impact important de cette voie de signalisation dans la physiopathologie du sepsis. En clinique, des études ont aussi rapporté une augmentation de la production de ces cytokines chez des patients septiques<sup>122</sup> ou atteints de bactériémie à *Staphylococcus aureus*<sup>123</sup>, la surproduction d'IL-18 permettant de prédire la mortalité dans cette dernière étude.

Si les études précédemment citées sont toutes en relation avec des infections d'origine bactériennes, de pareilles observations ont été faites dans le cadre d'infections virales. Par exemple, dans un modèle murin d'infection par le virus *Influenza A*, l'utilisation d'un inhibiteur

spécifique de NLRP3 empêche l'emballage du système immunitaire et est associée à une diminution de l'inflammation pulmonaire, à une diminution de la perte de poids et à une augmentation de la survie des animaux<sup>124</sup>. Plus récemment chez l'homme, dans le cadre de la pandémie de la COVID-19, il a été montré une activation de la voie de l'inflammasome NLRP3 *ex vivo* dans les monocytes de patients, de même qu'*in situ* par analyse de coupes histologiques de poumons réalisées chez des patients décédés de la maladie<sup>125</sup>. Cette activation est reliée à la tempête cytokinique et aux dommages pulmonaires observés chez les patients atteints de forme grave de la COVID-19<sup>126</sup>. Enfin, comme dans les bactériémies à *Staphylococcus aureus*, les patients avec les concentrations plasmatiques les plus importantes en IL-18 présentaient un risque significativement augmenté de décès<sup>127</sup>.

#### *vi. Implication de l'inflammasome NLRP3 dans les pathologies : synthèse*

L'activation de l'inflammasome NLRP3 et la sécrétion des cytokines pro-inflammatoires reliées contribuent au développement d'une inflammation systémique pouvant entraîner un **état inflammatoire chronique dans l'organisme et moduler la physiopathologie des maladies où l'inflammation est une composante essentielle**. L'intérêt autour de la voie de NLRP3 ne cesse de croître, il est donc important de mieux comprendre et caractériser l'activation de cet inflammasome, aussi bien au niveau expérimental qu'au niveau clinique.

#### 5. L'inflammation comme cible thérapeutique dans le sepsis

La réponse inflammatoire joue **rôle crucial dans la physiopathologie du sepsis** et peut **conduire au décès** dans certains cas, notamment pendant la phase aiguë de la maladie. Face à cette constatation, de **nombreux essais cliniques ont tenté de réduire cette inflammation systémique**. Différentes approches anti-inflammatoires issues de différentes classes pharmacologiques ont été testées : des traitements non spécifiques des composés inflammatoires comme les AINS (anti-inflammatoires non stéroïdiens) ou les corticostéroïdes ainsi que des thérapeutiques ciblant les produits spécifiques de l'inflammation comme le TNF- $\alpha$ , l'IL-1 $\beta$ , leurs récepteurs, les signaux de dangers eux-mêmes, *etc.* Plus de 150 essais cliniques ont été conduits avec l'objectif de réduire la mortalité chez les patients septiques<sup>128</sup>. Les résultats de ces essais, présentés en **tableau 4**, convergent vers des conclusions similaires : **une absence ou une efficacité limitée pour réduire la mortalité chez les patients**. Dans certaines études, une surmortalité est même rapportée chez les patients traités par des thérapies ciblant la



réponse inflammatoire<sup>129-131</sup>. Les deux derniers essais majeurs visant à moduler la réponse inflammatoire chez tous les patients septiques se sont également soldés par des échecs<sup>40,41</sup>, malgré un nombre important de patients inclus (>1000).

Cibles	Classe médicamenteuse	Médicament	Résultats
<b>Anomalies endocriniennes</b>	Corticoïdes	Corticoïdes	Effet bénéfique ou pas d'effet
	Vasopresseurs	Vasopressine	Pas d'effet
<b>Endotoxines</b>	Anticorps monoclonaux	HA-1A, E5	Pas d'effet
	Émulsion lipidique	GR270773	Pas d'effet
	Bactericidal/permeability-increasing protein (BPI)	rBPI21	Peu d'effet
<b>TLR4</b>	Antagonistes du TLR4	TAK-242	Pas d'effet
		Eritoran	Pas d'effet
<b>CD14</b>	Anticorps monoclonaux	IC14	Non concluant
<b>TNF-<math>\alpha</math></b>	Anticorps monoclonaux	afelimomab	Peu d'effet
		BAY 1351	Pas d'effet
	Antagonistes du récepteur	Lenercept, Etanercept	Pas d'effet ou effet délétère
<b>IL-1</b>	Antagonistes des récepteurs	Anakinra	Pas d'effet
<b>PAF</b>	Antagonistes du PAF	TCV-309, lexipafant	Pas d'effet
<b>Eicosanoïdes</b>	AINS	Ibuprofène	Pas d'effet
	Inhibiteur de la phospholipase A2	Varespladib	Pas d'effet
<b>C5a</b>	Anticorps monoclonaux	CaCP29	En cours
<b>Monoxyde d'azote</b>	L-N-méthylarginine	546C88	Peu d'effet
	Agents réducteurs	Bleu de méthylène	Peu d'effet
<b>Système de coagulation</b>	Concentrés de protéine C activée	drotrécogine $\alpha$ (activée)	Effet bénéfique ou pas d'effet
	Inhibiteur de la voie du facteur tissulaire	Tifacogin	Pas d'effet
	Antithrombine	Antithrombine	Pas d'effet
	Anticorps anti-facteur tissulaire	ALT-836	Pas d'effet
	Héparine	Héparine	Peu d'effet
	Thrombomoduline	ART-123	Peu d'effet
<b>Autres</b>	Immunoglobulines (IV)	Immunoglobulines	Pas d'effet
	Antioxydant	Sélénium	Pas d'effet
	Antagonistes de la bradykinine	Deltibant	Pas d'effet
	N-acetylcystéine	N-acetylcystéine	Pas d'effet
	Statines (hypolipémiants)	Atorvastatine, simvastatine, rosuvastatine	Pas d'effet
	Hémoperfusion	Adsorbent EN500	Pas d'effet
	Inhibiteur de TREM-1	Nangibotide	Pas d'effet

Tableau 4. La réponse inflammatoire au cœur des essais cliniques dans le sepsis  
Adapté de Hotchkiss et al<sup>128</sup>.

Hormis l'utilisation des corticostéroïdes dans certaines conditions (hydrocortisone en association à la fludrocortisone)<sup>132</sup>, le dernier essai clinique randomisé étudiant l'effet de l'hydrocortisone ne montre pas d'amélioration de la survie (n=3658 patients)<sup>133</sup>. Actuellement, le faible niveau de preuve ne permet donc pas leur utilisation dans tous les cas de sepsis. En conséquence, **aucune thérapeutique anti-inflammatoire n'est recommandée au niveau international dans la prise en charge du sepsis**<sup>38</sup>.

3 principales raisons sont rapportées pour expliquer les échecs de ces essais cliniques. La première concerne la réaction inflammatoire elle-même. L'inflammation est un processus extrêmement complexe dont les effets délétères sont attribués à de multiples médiateurs interdépendants<sup>134</sup>. Ainsi, l'injection de faibles doses d'endotoxine à des volontaires sains a montré l'altération de plus de 3000 gènes dans les leucocytes circulants<sup>134,135</sup>. Il semble donc peu probable que le blocage d'un seul médiateur ou d'une seule voie de signalisation puisse, à lui seul, permettre une amélioration de la survie de tous les patients présentant un sepsis.

La seconde cause d'échec des essais cliniques précédents ciblant l'inflammation est probablement liée à l'utilisation de ces thérapies chez des patients qui ne présentent plus un état hyperinflammatoire, mais au contraire, un état d'immunodépression prédominant. La littérature scientifique comporte plusieurs cas démontrant qu'en présence d'une évolution défavorable du sepsis, qui menait à un pronostic fatal malgré plusieurs semaines de traitement conventionnel, l'introduction de thérapies immunostimulantes a permis la survie de ces patients<sup>136-138</sup>.

Cela conduit à la troisième cause principale de l'échec de ces essais cliniques : **l'absence de sélection des patients et la volonté de trouver un traitement universel** (« one-size-fits-all »).

Le sepsis est une pathologie dont l'origine peut être due à de nombreux pathogènes, affectant des populations très différentes, allant des nouveau-nés aux personnes âgées ou aux personnes jeunes présentant des comorbidités. Il est donc difficile d'imaginer que chaque patient réagisse de la même manière à une infection et encore moins que des infections différentes induisent la même intensité de réponse inflammatoire. À titre d'exemple, une étude clinique observationnelle a montré une forte variabilité dans les réponses pro- et anti-inflammatoires, avec des concentrations plasmatiques en IL-6 et IL-10 allant de 0,58 à 395 989 pg/mL et 1,01 à 10 902 pg/mL respectivement<sup>139</sup>. Il paraît impossible de penser que tous les patients visés par une thérapie anti IL-6 réagiraient de la même façon au vu de ses variations chez les patients. De nombreuses études ont fait ce constat et sont renforcées par d'autres objectivant les différentes trajectoires ou endotypes décrits chez les patients septiques<sup>50,52,139,140</sup>. Il apparaît donc important de **stratifier les patients en fonction de leur statut immunitaire**. Ainsi, une des rares études montrant un effet bénéfique limité utilisait l'afelimomab, un anticorps monoclonal dirigé contre le TNF- $\alpha$ , et stratifiait les patients en fonction de la concentration plasmatique en IL-6<sup>141</sup>.

De plus, des analyses post-hoc de différents essais cliniques ont montré des résultats positifs après stratification basée sur l'utilisation de biomarqueurs. Malgré le manque d'effets bénéfiques globaux observés dans les essais cliniques ciblant le récepteur de l'IL-1 $\beta$  à l'aide d'anakinra et ayant entraîné l'arrêt de ces essais<sup>142-144</sup>, une réanalyse des mêmes données en

stratifiant les patients en fonction de la présence de caractéristiques du syndrome d'activation macrophagique (CIVD + insuffisance hépatobiliaire dans cette étude) a démontré une diminution de la mortalité chez les patients traités<sup>145</sup>. De même, la stratification de patients septiques en fonction de la concentration plasmatique en IL-1Ra a permis d'augmenter la survie en traitant les patients présentant des concentrations élevées par anakinra<sup>146</sup>. Une observation similaire peut être faite concernant la réalisation d'endotypes. L'essai clinique HARP-2, qui étudiait l'utilisation de la simvastatine dans la prise en charge du SDRA, n'a pas démontré d'avantage global en ce qui concerne l'amélioration de la survie<sup>147</sup>. Cependant, une analyse post-hoc de cette étude a révélé qu'un **sous-phénotype hyperinflammatoire** traité avec de la simvastatine avait une amélioration de la survie à 28 jours et à 90 jours par rapport au placebo<sup>140</sup>. Dans le contexte de patients atteints de COVID-19 et présentant un SDRA, 2 groupes distincts ont été identifiés, dont un groupe hyperinflammatoire présentant une mortalité significativement plus élevée à 90 jours. Le traitement par corticostéroïdes a montré une diminution de la mortalité dans ce groupe particulier de patients, tandis que la survie est restée inchangée dans le groupe hypoinflammatoire<sup>148</sup>.

Cette approche thérapeutique basée sur l'utilisation de biomarqueurs est désormais préconisée. À titre illustratif, l'essai randomisé en double aveugle contre placebo ASTONISH (*Efficacy, Safety and Tolerability of Nangibotide in Patients With Septic Shock*, NCT04055909) a utilisé la concentration plasmatique de TREM-1 soluble (*triggering receptor expressed on myeloid cells-1*) pour stratifier les patients bénéficiant du traitement par nangibotide, un peptide inhibiteur de la voie de TREM-1<sup>149</sup>. Bien que cette étude n'ait pas montré d'amélioration du critère de jugement principal chez les patients traités (score SOFA), le nangibotide à forte dose a entraîné une amélioration du score SOFA par rapport au placebo chez les patients présentant des concentrations plus élevées de sTREM-1 (supérieures au seuil fixé initialement)<sup>149</sup>. De la même manière, un essai randomisé en double aveugle (NCT04990232) intitulé ImmunoSep (*Personalized Immunotherapy in Sepsis*) est en cours dans plusieurs pays d'Europe. Cet essai cible les patients manifestant une inflammation excessive et leur propose soit un traitement par anakinra, soit un placebo, chaque option étant complétée par une prise en charge standard.

## IV. L'immunodépression induite par le sepsis

Comme vu précédemment ([réponse immune au cours du sepsis](#)), l'état hyperinflammatoire observé au cours du sepsis a longtemps été au cœur de la recherche dans le but d'améliorer la survie des patients septiques. Presque 50 ans après les premières observations des troubles de la réponse immune, l'immunodépression qui y est associée est maintenant reconnue et est au cœur de la recherche chez les patients pour en approfondir les mécanismes physiopathologiques. Cette partie présentera dans un premier temps les mécanismes généraux d'apparition de l'immunosuppression associée au sepsis en se basant sur de récentes revues de la littérature<sup>20,52</sup>, avant d'aborder les altérations précises des différents types cellulaires du système immunitaire et leurs implications cliniques.

### 1. Mécanismes d'apparition des altérations immunitaires

#### 1.1. Mort cellulaire

L'**apoptose** est un mécanisme physiologique de mort cellulaire programmé, essentiel à la régulation du renouvellement cellulaire. Deux principales voies de signalisation sont décrites : la **voie intrinsèque ou mitochondriale** et la **voie extrinsèque ou voie des récepteurs de morts**. La première est activée par divers stimuli tels que les ROS et conduit au recrutement de la caspase 8 tandis que la seconde est activée par la fixation de différents ligands comme le TNF- $\alpha$  ou FAS ligand à leurs récepteurs et entraîne le recrutement de la caspase 9<sup>150</sup>. La caspase 9 active ensuite la caspase 3, conduisant à la formation de corps apoptotiques. Ces corps apoptotiques ne libèrent pas leur contenu cellulaire dans la circulation générale ou dans le milieu extérieur, **évitant ainsi l'induction d'une réaction inflammatoire**, et sont ensuite éliminés par les macrophages. Au cours du sepsis, on observe principalement une apoptose des lymphocytes T CD4+ et CD8+, des cellules B, des lymphocytes NK et des cellules dendritiques folliculaires, entraînant une **forte réduction de leur concentration dans la circulation générale**<sup>20,52</sup>.

L'**autophagie** est un processus protecteur qui permet l'élimination du contenu cellulaire redondant ou non fonctionnel et la mort de la cellule. Ce processus est médié par l'activation de gènes spécifiques appelés *Autophagy-related genes* (Atg), qui permettent la formation des autophagosomes et la dégradation du contenu cellulaire par le recrutement des lysosomes<sup>151</sup>. Des altérations de l'autophagie ont été observées sur modèles murins de sepsis, et l'utilisation de souris KO pour certains gènes Atg entraîne des **altérations immunitaires et une surmortalité**<sup>20,52</sup>.

La **pyroptose** est une **mort inflammatoire** principalement observée dans les monocytes, les macrophages et dans une moindre mesure les PMN, ces derniers y étant naturellement résistants. Elle est initiée par l'insertion de la GSDMD dans la membrane plasmique (voir [les inflammasomes](#)). Si la pyroptose, par le biais de la réaction inflammatoire qu'elle déclenche, joue un rôle protecteur au stade précoce de l'infection, une réaction excessive peut entraîner une réponse inflammatoire incontrôlée, des **dommages tissulaires et un mauvais pronostic au cours du sepsis**<sup>152</sup>.

### *1.2. Tolérance à l'endotoxine*

Le LPS, également appelé endotoxine, est un puissant inducteur de la réponse inflammatoire en stimulant le TLR4. Lors d'une exposition prolongée au LPS ou d'une injection sublétales chez l'animal, des mécanismes se mettent en place pour limiter une réponse inflammatoire excessive : on parle alors de **tolérance à l'endotoxine**. Cette tolérance ne se limite pas au LPS, car la stimulation par différents stimuli via divers récepteurs (*e.g.* TLR2, TLR4 et TLR5) peut entraîner une réduction de la capacité à produire des cytokines pro-inflammatoires. Chez les patients septiques, cela se traduit par des altérations caractérisées par une **diminution de la production de multiples cytokines pro-inflammatoires** telles que le TNF- $\alpha$ , l'IL-1 ou l'IL-6 lors d'une stimulation *ex-vivo*, tandis que la sécrétion des cytokines anti-inflammatoires comme l'IL-10 reste inchangée ou est augmentée. De plus, les capacités de chimiotactisme et de présentation antigénique sont réduites (diminution des molécules HLA de classe II), bien que les capacités de phagocytose soient conservées. Ces altérations seraient expliquées par des changements dans la transcription de gènes anti et pro-inflammatoires, mais aussi par des troubles de phosphorylation de NF- $\kappa$ B, dans le but de favoriser un état de tolérance<sup>20,52</sup>.

### *1.3. Épuisement immunitaire*

Le système immunitaire est finement régulé par des signaux activateurs et inhibiteurs, impliquant des protéines membranaires connues sous le nom de « **immune checkpoints** ». L'une des protéines inhibitrices les plus étudiées est **PD-1** (*programmed cell death protein 1*). Majoritairement décrite dans les lymphocytes T, l'activation de cette voie de signalisation est associée à la sécrétion de molécules aux capacités suppressives (*e.g.* IL-10), à une diminution des capacités de prolifération et à l'apoptose chez les patients septiques. Elle induit ce que l'on appelle **l'anergie lymphocytaire** des cellules T. On observe également l'expression de cette

protéine dans les monocytes et les PMN, où elle est associée à une diminution des capacités de phagocytose.

L'expression d'autres récepteurs inhibiteurs est aussi décrite au cours du sepsis comme CTLA-4 (*cytotoxic T lymphocyte antigen 4*), TIM3 (*T cell immunoglobulin mucin receptor 3*), LAG3 (*lymphocyte activation gene 3 protein*) ou TRAIL (*TNF-related apoptosis-inducing ligand*), contribuant également aux altérations des lymphocytes T<sup>20</sup>.

#### 1.4. Altérations de l'immunométabolisme

L'étude du métabolisme énergétique des cellules immunitaires, ainsi que son lien avec leurs fonctions, est un domaine vaste appelé « **immunométabolisme** ». Il explore les processus de production d'énergie des cellules immunitaires, tels que la phosphorylation oxydative et la glycolyse, et examine comment ces processus influencent les fonctions immunitaires.

La phosphorylation oxydative est un mécanisme métabolique par lequel les cellules utilisent efficacement le glucose en présence d'oxygène pour produire de l'ATP. En revanche, la glycolyse est un processus métabolique qui génère moins d'ATP en l'absence d'oxygène, mais de manière plus rapide. La **phosphorylation oxydative est la voie métabolique principale des cellules immunitaires en condition de repos**. Cependant, lorsqu'elles sont activées et ont besoin de plus d'énergie, elles basculent vers la glycolyse, un phénomène appelé effet **Warburg** (utilisation préférentielle de la glycolyse en présence d'oxygène)<sup>20</sup>.

Les patients atteints de sepsis présentent des dysfonctionnements de cet immunométabolisme, notamment une **diminution de l'effet Warburg** associée à des **altérations de la voie de signalisation mTOR** (*mechanistic target of rapamycin*) dans les monocytes. Ces anomalies d'activation de la voie mTOR sont également observées dans les lymphocytes T des patients septiques, associées à un **hypométabolisme** et entraînant un défaut de prolifération. De plus, une augmentation de la production de HIF1 $\alpha$  (*hypoxia-inducible factor 1 $\alpha$* ) est observée dans les monocytes des patients, ce qui entraîne une augmentation de l'expression de l'IRAKM (*IL-1 receptor-associated kinase M*), une diminution de la transcription des gènes pro-inflammatoires liant ainsi les **troubles métaboliques et le profil de tolérance des monocytes**<sup>20</sup>.

#### 1.5. Troubles épigénétiques et transcriptomiques

L'épigénétique englobe les **modifications de l'activité des gènes sans altérations de la séquence d'ADN**. Ces modifications impliquent des mécanismes tels que les modifications

post-transcriptionnelles des histones (*e.g.* acétylation, méthylation) ou les modifications chimiques de l'ADN, qui entraînent des changements d'accessibilité des gènes cibles aux facteurs de transcription. Au cours du sepsis, des modifications épigénétiques ont été rapportées et liées aux altérations immunitaires. Par exemple, la **méthylation** de certaines histones serait ainsi impliquée dans les phénomènes décrits précédemment (mort cellulaire, tolérance à l'endotoxine et altérations de l'immunométabolisme). La **persistance de l'immunodépression** peut aussi être reliée à l'épigénétique, ces **modifications pouvant être héritées par les cellules filles** lors de la division cellulaire. Des études sur modèles murins ont ainsi montré des modifications épigénétiques persistantes dans la moelle osseuse, associées à des altérations immunitaires, comme la diminution des fonctions des macrophages (*e.g.* la phagocytose). Ces modifications des histones sont également susceptibles d'être associées à une différenciation préférentielle des lymphocytes T vers une population spécifique de cellules régulatrices appelées lymphocytes T régulateurs (Tregs), en raison de l'augmentation de la transcription du gène Foxp3, un facteur de transcription caractéristique des Tregs<sup>20,52</sup>.

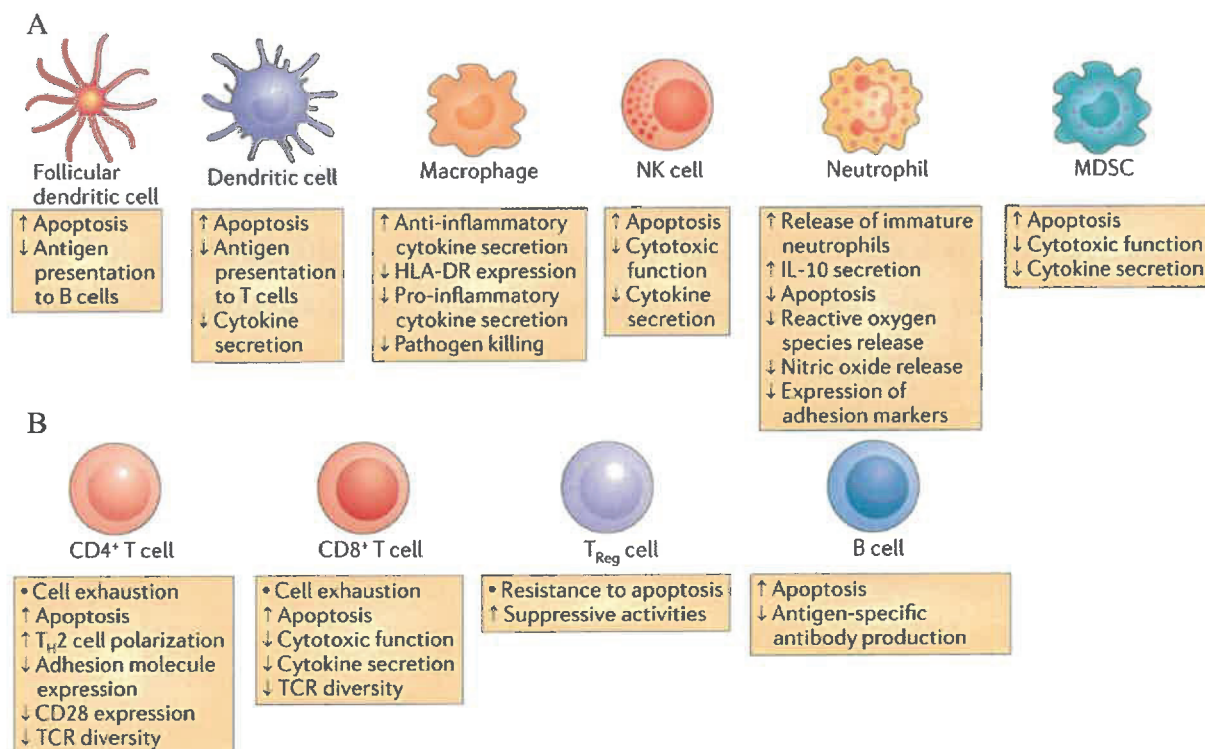
Le transcriptome est l'ensemble des ARN résultant de la transcription du génome, reflétant ainsi les altérations génomiques et les changements épigénétiques. Les analyses menées chez les patients septiques ont montré que plus de 70% du transcriptome leucocytaire subit une reprogrammation affectant leurs fonctions<sup>153</sup>. Des études ont également révélé l'expansion d'une population de monocytes avec des propriétés immunosuppressives associée à une diminution d'expression des protéines du CMH de classe 2 ainsi que de la production du TNF- $\alpha$  après stimulation *ex vivo*<sup>52</sup>. L'étude du transcriptome et l'établissement de scores permettent également de prédire la gravité des patients septiques<sup>51</sup>.

#### *1.6.Régulation centrale par le système nerveux central*

Le système nerveux central participe à la régulation de la réponse immunitaire, contribuant ainsi à une réponse efficace lors d'une infection. Il exerce son influence sur les fonctions immunitaires à travers l'action des **systèmes nerveux autonomes sympathiques et parasympathiques**, par l'intermédiaire de l'**axe hypothalamo-hypophyso-surrénalien** et la synthèse de neuromédiateurs impliqués dans la production de glucocorticoïdes. Cependant, la contribution spécifique de ces interactions neuro-immunitaires dans l'immunosuppression observée chez les patients septiques reste à établir<sup>20</sup>.

## 2. Conséquences sur le système immunitaire

Les mécanismes décrits précédemment peuvent affecter l'intégralité du système immunitaire, à la fois inné et adaptatif; contribuant ainsi à l'immunosuppression observée au cours du sepsis. Dans la suite, nous présenterons les différentes altérations présentées qui se manifestent au niveau des cellules du système immunitaire. Un résumé est proposé en **figure 18**.



**Figure 18. Altérations immunitaires observées au cours du sepsis**  
*Effet du sepsis sur le système immunitaire inné (A) et sur le système immunitaire adaptatif (B). Adapté de Venet et al<sup>20</sup>.*

### 2.1. Altérations du système immunitaire inné

#### i. PMN

Chez les patients septiques, les neutrophiles subissent une apoptose et présentent de multiples altérations de leurs capacités de défense. On observe une diminution des facteurs d'adhésions (*e.g.* CXCR1, CXCR2), entraînant une **réduction du recrutement de ces cellules dans les tissus infectés**. De plus, bien que les capacités de phagocytose de ces cellules soient conservées<sup>154,155</sup>, une réduction de la synthèse des produits participant à la destruction des pathogènes comme les ROS, la lactoferrine ou la myéloperoxydase<sup>155</sup> pourrait entraîner une **déficience dans l'élimination des bactéries**. Sur modèle murin de sepsis, il a été montré que les PMN pouvaient exercer des fonctions anti-inflammatoires, via la sécrétion d'IL-10<sup>156</sup>. Une



**formation excessive de pièges extracellulaires des neutrophiles** (NETs pour *neutrophil extracellular traps*) est aussi rapportée, corrélant avec les dysfonctions d'organes des patients présentant un sepsis<sup>157</sup>.

Parallèlement, de nouvelles sous-populations de PMN apparaissent chez ces patients. La myélopoïèse réactionnelle d'urgence entraîne la production de **PMN immatures**, caractérisés notamment par une **sous-expression des marqueurs CD10 et CD16**<sup>155</sup> (CD10<sup>low</sup>CD16<sup>low</sup>). D'autres phénotypes immatures de PMN sont décrits chez les patients (*e.g.* CD14<sup>neg</sup>/CD24<sup>pos</sup>CD16<sup>low</sup>, exprimant l'IL-1R2) et présentent des **capacités immunosuppressives**. Ces cellules sont capables de diminuer la prolifération ou l'activation des lymphocytes T *ex vivo*<sup>158</sup>, ainsi que d'induire leur mort<sup>159</sup>. Chez le volontaire sain, la réalisation d'une endotoxémie entraîne la production de PMN CD10<sup>high</sup>CD16<sup>high</sup>CD11b<sup>low</sup> capables de diminuer la prolifération des lymphocytes T<sup>160</sup>. Les mécanismes à l'origine de cette immunosuppression impliqueraient une surexpression de PD-L1 (*programmed cell death-ligand 1*)<sup>161</sup>. La persistance de ces PMN immatures chez les patients atteints de sepsis a été rapportée dans de nombreuses études<sup>139,155,161</sup> et a été **associée au développement d'infections nosocomiales**<sup>139</sup>, **ainsi qu'à une mortalité accrue**<sup>155,162</sup>. La présence de ces polynucléaires immatures est aussi retrouvée chez les patients atteints de COVID-19<sup>163</sup>. Ceux-ci sont présents en plus grande proportion chez les patients hospitalisés en réanimation et présentant un score de gravité plus élevé<sup>164,165</sup> (*e.g.* SOFA) ainsi que chez les patients non-survivants<sup>165</sup>.

#### *ii. Monocytes et macrophages*

Au cours du sepsis, on observe une diminution de la sécrétion de ces cytokines par les monocytes de patients après stimulation *ex vivo*, quel que soit le stimulus : il s'agit de la tolérance à l'endotoxine présentée plus haut. En revanche, les capacités de sécrétion des médiateurs anti-inflammatoires ne sont pas altérées, et l'on observe ainsi la sécrétion d'IL-10 ou d'IL-1Ra après stimulation *ex vivo*<sup>166</sup>. On assiste donc à une **reprogrammation des monocytes**, où ceux-ci présentent un **phénotype immunorégulateur**. Ce changement de phénotype se manifeste également par une diminution des capacités de présentation de l'antigène par les monocytes et les macrophages, remarqué par une **diminution d'expression du mHLA-DR**, un récepteur du complexe majeur d'histocompatibilité de classe II. Ainsi, une diminution d'expression de HLA-DR par les monocytes est un indicateur **d'anergie monocytaire**. Largement étudié dans le sepsis avec plus de 200 publications sur le sujet, le HLA-DR monocytaire représente **le biomarqueur le plus robuste pour caractériser l'immunosuppression induite par le sepsis**, ainsi que dans d'autres contextes où des

altérations immunitaires sont observées (*e.g.* patients brûlés, présentant des traumatismes)<sup>167</sup>. Plus récemment, il a été étudié lors de la pandémie de COVID-19, où une diminution de son expression a été observée, illustrant l'immunosuppression chez ces patients hospitalisés en réanimation<sup>168</sup>.

Du point de vue clinique, l'intensité et la persistance de la diminution de l'expression du mHLA-DR chez les patients présentant un sepsis sont associées à la survenue d'infections nosocomiales et à une augmentation de la mortalité. Cette diminution constitue un facteur prédictif, à la fois pour les infections secondaires et le décès, même après ajustement utilisant les facteurs cliniques confondants habituellement utilisés en analyses multivariées<sup>167</sup>. De plus, la valeur de mHLA-DR est également utilisée comme **marqueur de stratification** dans plusieurs études cliniques en cours dans le sepsis, afin de sélectionner les patients les plus immunodéprimés pouvant bénéficier d'une thérapie immunostimulante (*e.g.* IGNORANT, NCT05843786 et ImmunoSep, NCT04990232).

### *iii. Cellules dendritiques*

Les cellules dendritiques ont pour origine un précurseur médullaire et sont divisées en 2 populations principales : les cellules dendritiques myéloïdes et les cellules dendritiques plasmocytoïdes. Elles jouent le **rôle de sentinelles** dans l'organisme et sont impliquées dans **l'initiation de la réponse immunitaire adaptative**, ainsi que dans le processus de tolérance aux antigènes du soi. En effet, ces cellules sont capables de détecter divers types de lésions tissulaires et sont les **principales cellules présentatrices d'antigènes** spécialisées dans la capture, le transport, l'apprêtement et la présentation des antigènes aux lymphocytes T<sup>169</sup>.

Dans le contexte du sepsis, les cellules dendritiques sont **particulièrement sensibles à l'apoptose**, ce qui se manifeste par une diminution de leur nombre dans les prélèvements post mortem de rates de patients septiques<sup>170,171</sup>. Ces cellules présentent aussi une diminution des protéines de costimulation comme le CD86 ainsi que la molécule de présentation antigénique HLA-DR. Elles présentent aussi une surproduction d'IL-10 et surexpriment PD-L1, favorisant donc un **phénotype tolérogène des cellules dendritiques**<sup>171-174</sup>. Ces altérations se traduisent par une anergie des lymphocytes T associée à une diminution de leur prolifération et à une augmentation de l'induction de Tregs<sup>174</sup>. Sur le plan clinique, la persistance de la diminution des cellules dendritiques chez les patients pendant plusieurs semaines est associée à la survenue d'infections nosocomiales<sup>172</sup> et à la mortalité<sup>175</sup>.

#### *iv. Lymphocytes Natural Killer*

Les cellules NK sont un type de lymphocytes appartenant au système immunitaire inné. Elles jouent un **rôle de surveillance** en éliminant les cellules anormales, telles que les cellules cancéreuses ou infectées, tout en préservant l'intégrité des cellules saines de l'organisme grâce à un phénomène de tolérance. Une fois activées (détection du soi manquant ou phénomène de stress cellulaire), les cellules NK déploient leurs **capacités cytotoxiques**. Elles libèrent ainsi de la **perforine**, une protéine qui provoque la formation de pores dans la membrane plasmique de la cellule cible, ainsi que la **granzyme B**, une enzyme qui induit l'apoptose. Les cellules NK synthétisent également des cytokines, notamment l'**IFN- $\gamma$** .

Chez les patients atteints de sepsis, les lymphocytes NK présentent une **sensibilité accrue à l'apoptose**, des altérations de leurs capacités de sécrétion, notamment une **diminution de la production d'IFN- $\gamma$**  et une baisse de la cytotoxicité<sup>176-178</sup>. Des modèles murins de sepsis ont révélé une corrélation entre la diminution de la synthèse d'IFN- $\gamma$  et l'expression de TIM-3<sup>179</sup>, ainsi qu'une surexpression des protéines PD-1 et PD-L1<sup>180</sup>. Sur le plan clinique, on observe une diminution plus marquée de la production d'IFN- $\gamma$  chez les patients septiques présentant une réactivation du cytomégalovirus, et ce avant même la réactivation<sup>177</sup>. Les altérations des cellules NK pourraient être liées aux réactivations virales observées chez les patients.

#### *v. MDSCs*

Les MDSCs, pour *myeloid-derived suppressor cells*, représentent une population hétérogène de **cellules immatures d'origine myéloïde aux capacités suppressives** touchant à la fois le système immunitaire inné et adaptatif<sup>181</sup>. Initialement découvertes et majoritairement étudiées en cancérologie, les MDSCs sont de plus en plus étudiées dans le sepsis<sup>182,183</sup>. Ces cellules sont décrites de manière approfondie dans la [partie suivante](#), en prenant majoritairement appui sur les études réalisées en cancérologie puis en présentant les différentes études disponibles dans le sepsis.

### *2.2. Altérations du système immunitaire adaptatif*

#### *i. Lymphocytes T*

Les lymphocytes T jouent un rôle central dans toutes les réponses immunitaires adaptatives dirigées contre les antigènes. Ces cellules résident dans les organes et les tissus lymphoïdes secondaires, puis sont recrutées vers les sites où des signaux de danger sont détectés. Les cellules T expriment un récepteur spécifique de l'antigène, le **TCR** (*T cell*

*receptor*), qui leur permet de reconnaître les antigènes présentés par les cellules présentatrices d'antigènes via les protéines du complexe majeur d'histocompatibilité. L'interaction entre le TCR et l'antigène présenté entraîne **l'activation du lymphocyte T**. Selon l'environnement et les stimuli présents, les cellules T peuvent se polariser en différentes populations de cellules effectrices. Chaque sous-type de cellule T effectrice a des fonctions et des mécanismes d'action distincts, leur permettant de contribuer de manière spécifique à l'élimination des agents pathogènes et à la modulation de la réponse immunitaire.

### Lymphocytes T CD4

Les réponses immunitaires médiées par les lymphocytes T CD4<sup>+</sup> sont essentielles pour éliminer les infections virales, bactériennes, fongiques ou parasitaires grâce à la sécrétion de cytokines et à l'activation des autres cellules immunitaires. Les lymphocytes T CD4<sup>+</sup> exercent également une action sur les lymphocytes B afin de les aider à produire des anticorps. Sous l'effet des cytokines produites par les cellules présentatrices d'antigènes, ces cellules se différencient en **cellules T effectrices auxiliaires ou lymphocyte T helper (Th)**. Différentes populations sont décrites, présentant des caractéristiques distinctes concernant leur synthèse, leur capacité de sécrétion, leur fonction dans la réponse immune, *etc*<sup>184</sup>. Ces données sont résumées en **figure 19**. Par ailleurs, les Tregs jouent un rôle central dans le contrôle de l'auto-immunité, l'homéostasie immunitaire et les réponses immunitaires aux agents pathogènes.

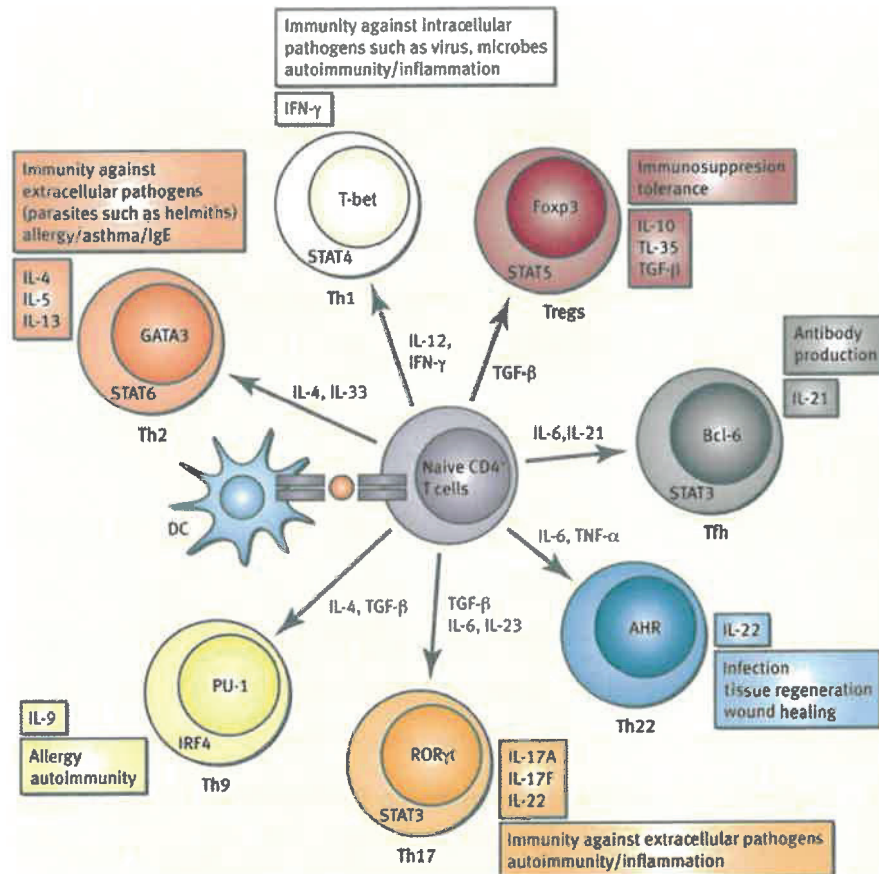


Figure 19. Caractérisation des sous-populations lymphocytaires T CD4<sup>+</sup><sup>184</sup>

Chaque sous-population de lymphocytes T CD4<sup>+</sup> est représentée par un cercle de couleur spécifique associé aux cytokines sécrétées (petits rectangles) ainsi qu'à ses fonctions dans l'immunité (grands rectangles) avec les mêmes couleurs. Les cytokines, sécrétées par la cellule présentatrice d'antigène (DC sur le schéma), qui permettent d'orienter la différenciation des lymphocytes T CD4<sup>+</sup> sont représentées le long de chaque flèche.

### Lymphocytes T CD8

Les lymphocytes T CD8<sup>+</sup> jouent un rôle important dans la réponse immunitaire cellulaire. Ces cellules sont principalement impliquées dans la **reconnaissance et l'élimination des cellules infectées** par des agents pathogènes tels que les virus. Ils sont capables d'éliminer directement les cellules cibles infectées grâce à la libération de molécules cytotoxiques, telles que la perforine et les granzymes. De plus, les lymphocytes T CD8<sup>+</sup> peuvent sécréter des cytokines comme l'IFN- $\gamma$  et le TNF- $\alpha$ . Leur activation nécessite la présentation de peptides antigéniques par les cellules présentatrices d'antigènes utilisant les molécules du complexe majeur d'histocompatibilité de classe I<sup>185</sup>.

Durant le sepsis, les lymphocytes T dans leur ensemble, qu'ils soient de sous-population CD4+ ou CD8+, subissent de profondes altérations<sup>20,184,185</sup>. Une grande partie de ces lymphocytes T subit une apoptose<sup>186</sup>, entraînant une lymphopénie et une diminution des lymphocytes T spécifiques capable de lutter contre les pathogènes. Cette lymphopénie et sa persistance sont associées à un risque accru d'infections secondaires et de mortalité chez les patients septiques<sup>187,188</sup>. De plus, les cellules T présentent une diminution de la synthèse de cytokines pro-inflammatoires, telles que l'IFN- $\gamma$  ou le TNF- $\alpha$ , ainsi qu'une capacité réduite de prolifération<sup>49,171</sup>. Ces altérations sont particulièrement marquées chez les patients non-survivants<sup>171</sup>. Chez les patients septiques, une augmentation des corécepteurs inhibiteurs comme PD-1, TIM-3 et CTLA-4 a été observée sur les lymphocytes T. Par exemple, une expression accrue de PD-1 a été retrouvée chez les patients présentant des infections nosocomiales et chez les patients non-survivants<sup>189</sup>. Cette altération de la synthèse des cytokines, la diminution des capacités de prolifération et l'expression de marqueurs tels que PD-1 caractérisent l'épuisement des lymphocytes T (ou *exhaustion* en anglais)<sup>20,185</sup>. Dans cet état d'épuisement, il a également été montré une diminution de la diversité du TCR<sup>190</sup> et de l'expression du CD127 (chaîne  $\gamma$  du récepteur à l'IL-7)<sup>189</sup> ainsi qu'une altération de la mémoire immunitaire chez les lymphocytes T<sup>185</sup>.

Néanmoins, une population de lymphocytes T n'est pas affectée par l'apoptose lors du sepsis, il s'agit des Tregs. En effet, cette population régulatrice est résistante à l'apoptose et est augmentée au cours du sepsis<sup>191</sup>. Les Tregs ont été associés à une diminution de la lymphoprolifération après stimulation *ex vivo*<sup>191</sup>, ainsi qu'à la diminution des fonctions des autres cellules du système immunitaire comme les monocytes, les PMN ou les lymphocytes T<sup>49</sup>. La persistance de ces cellules a été liée à la mortalité chez les patients septiques<sup>192</sup>.

Dans le contexte de la pandémie de COVID-19, les patients présentaient des caractéristiques similaires en ce qui concerne la réponse lymphocytaire T. Une lymphopénie associée à la gravité de la maladie et à sa mortalité a été largement rapportée<sup>193</sup>. L'épuisement des lymphocytes T a également été décrit, avec la surexpression des corécepteurs inhibiteurs tels que PD-1<sup>194</sup>, une diminution de la lymphoprolifération *ex vivo*<sup>194</sup>, ainsi qu'une altération profonde de la réponse IFN<sup>193</sup>. Cet épuisement des lymphocytes T a lui aussi été associé à la mortalité observée au cours de la COVID-19<sup>193</sup>.

#### ii. *Lymphocytes B*

Les lymphocytes B jouent un rôle crucial dans la réponse immunitaire en tant que **cellules clefs de la réponse humorale**. Ils sont responsables de la synthèse des

immunoglobulines et de la formation des lymphocytes B mémoires, qui contribuent à la **mémoire immunitaire**. En tant que cellules présentatrices d'antigènes via le BCR (*B cell receptor*), elles sont impliquées dans la détection des signaux de danger, la production de cytokines et l'activation des lymphocytes T.

Chez les patients septiques, les lymphocytes B sont aussi **sensibles à l'apoptose**<sup>20,186,195-197</sup>. Une diminution des cellules B circulantes a été observée dès l'admission des patients septiques, persistante persiste jusqu'à 28 jours après leur admission en réanimation<sup>196</sup>. Cette diminution a été associée à une **diminution de la survie**<sup>197</sup>. Des **altérations phénotypiques** ont également été observées, telles qu'une expression accrue du récepteur PD-1<sup>198</sup> ou de lymphocytes B coexprimant les protéines de costimulation CD80 et de mort cellulaire Fas, cette dernière population étant retrouvée en plus grande proportion chez les patients non-survivants<sup>196</sup>. Une diminution de l'expression de HLA-DR ainsi que de certains marqueurs comme le CD21 a été rapportée<sup>199</sup>. Des **altérations fonctionnelles** sont aussi présentes, notamment une diminution des immunoglobulines M circulantes qui est associée à la mortalité<sup>197</sup>, ainsi qu'une activation et une prolifération réduites des lymphocytes B *ex vivo*<sup>199</sup>. Pendant le sepsis, des **populations de la lignée B aux capacités régulatrices** apparaissent. Une étude a rapporté la présence de lymphocytes B avec un phénotype « *exhausted-like* », présentant une diminution d'expression de HLA-DR et une plus forte production d'IL-10 après stimulation *ex vivo*<sup>199</sup>. Cette étude signalait également une **plasmocytose** chez les patients. Une plasmocytose similaire a été observée sur modèles murins de sepsis ainsi que chez des patients atteints de choc septique ou de COVID-19 hospitalisés en réanimation. Ces cellules présentaient un pouvoir immunosuppresseur grâce à la production d'IL-10 et à une expression accrue de PD-L1 (*Gossez et al*, en cours de révision). Chez la souris comme chez l'homme, d'autres populations B régulatrices sont décrites, comme les **lymphocytes B régulateurs**<sup>200</sup> et les **plasmablastes**<sup>201</sup>, mais il n'existe pas encore de consensus sur leur classification.

Dans le contexte de la COVID-19, qui est une forme de sepsis d'origine virale, ces cellules ont également été identifiées. Une plasmocytose a été observée (*Gossez et al*, en cours de révision), ainsi qu'une augmentation de lymphocytes B extrafolliculaires et des plasmablastes, éléments associés à la sévérité des patients<sup>202-205</sup>.

### 3. MDSCs

#### 3.1. Définitions

Sur la base de caractéristiques phénotypiques et morphologiques, ces cellules sont subdivisées en 2 groupes principaux : les **m-MDSCs** (*monocytic myeloid-derived suppressor*

cells) et les **PMN-MDSCs** (Polymorphonuclear MDSCs). En raison de l'absence de marqueurs phénotypiques spécifiques pour ces cellules, il existe **différentes définitions et caractérisations** utilisées dans la littérature pour l'étude des MDSCs. Pour remédier à cela, un groupe d'experts a publié des recommandations en 2016 concernant leur nomenclature et leur caractérisation<sup>181</sup>. Les MDSCs sont définies par 2 caractéristiques principales : un **phénotype immature** et des **capacités immunosuppressives**. Parmi les **PBMC**, les **m-MDSCs** sont définies par l'expression des CD14 et CD11b, l'absence d'expression du CD15 et une sous-expression de HLA-DR (**CD11b<sup>+</sup>CD14<sup>+</sup>HLA-DR<sup>-low</sup>CD15<sup>-</sup>**). Les **PMN-MDSCs** sont quant à elles définies par l'expression du CD11b et du CD15 ou du CD66b associée à une absence d'expression du CD14 (**CD11b<sup>+</sup>CD14<sup>-</sup>CD15<sup>+</sup> ou CD11b<sup>+</sup>CD14<sup>-</sup>CD66b<sup>+</sup>**). Cependant, cette seule caractérisation phénotypique n'est pas suffisante pour qualifier les cellules de MDSCs, les capacités immunosuppressives doivent également être démontrées. Pour cela, **l'inhibition des lymphocytes T** est considérée comme le « *gold standard* » pour évaluer la fonction des MDSCs<sup>181</sup>. 2 tests sont décrits : **l'inhibition de la prolifération des cellules T** ou de la **production d'IFN-γ** induites par billes anti-CD3/CD28 (ou par la phytohémagglutinine) par **l'ajout de populations de MDSCs candidates**. Le retrait de la population suspectée d'être des MDSCs est parfois utilisé en co-culture pour démontrer l'amélioration de la prolifération ou des capacités de sécrétion des cellules T.

En 2016, l'équipe de Gabrilovich a introduit l'utilisation du **marqueur LOX-1** (*lectin-type oxidized LDL receptor 1*) pour identifier les PMN-MDSCs chez les patients atteints de cancer, en **sang total**<sup>206</sup>. Ces cellules répondaient aux critères de définition des MDSCs, présentant des **caractéristiques immatures** ainsi que la **capacité d'inhiber la prolifération des lymphocytes T** en co-culture. L'utilisation de ce marqueur pourrait **simplifier la recherche** autour de ces cellules dans les études cliniques, les PMN-MDSCs pouvant être directement détectées dans le sang total, évitant ainsi la séparation des PBMC par gradient de centrifugation, comme cela était requis auparavant.



### 3.2. Expansion et activation

L'accumulation des MDSCs n'est pas entièrement comprise, mais plusieurs équipes ont proposé un modèle pour l'expliquer, basé sur deux types de signaux : **l'expansion initiale suivie d'une activation** pathologique des MDSCs<sup>182,183,207</sup> (figure 19).

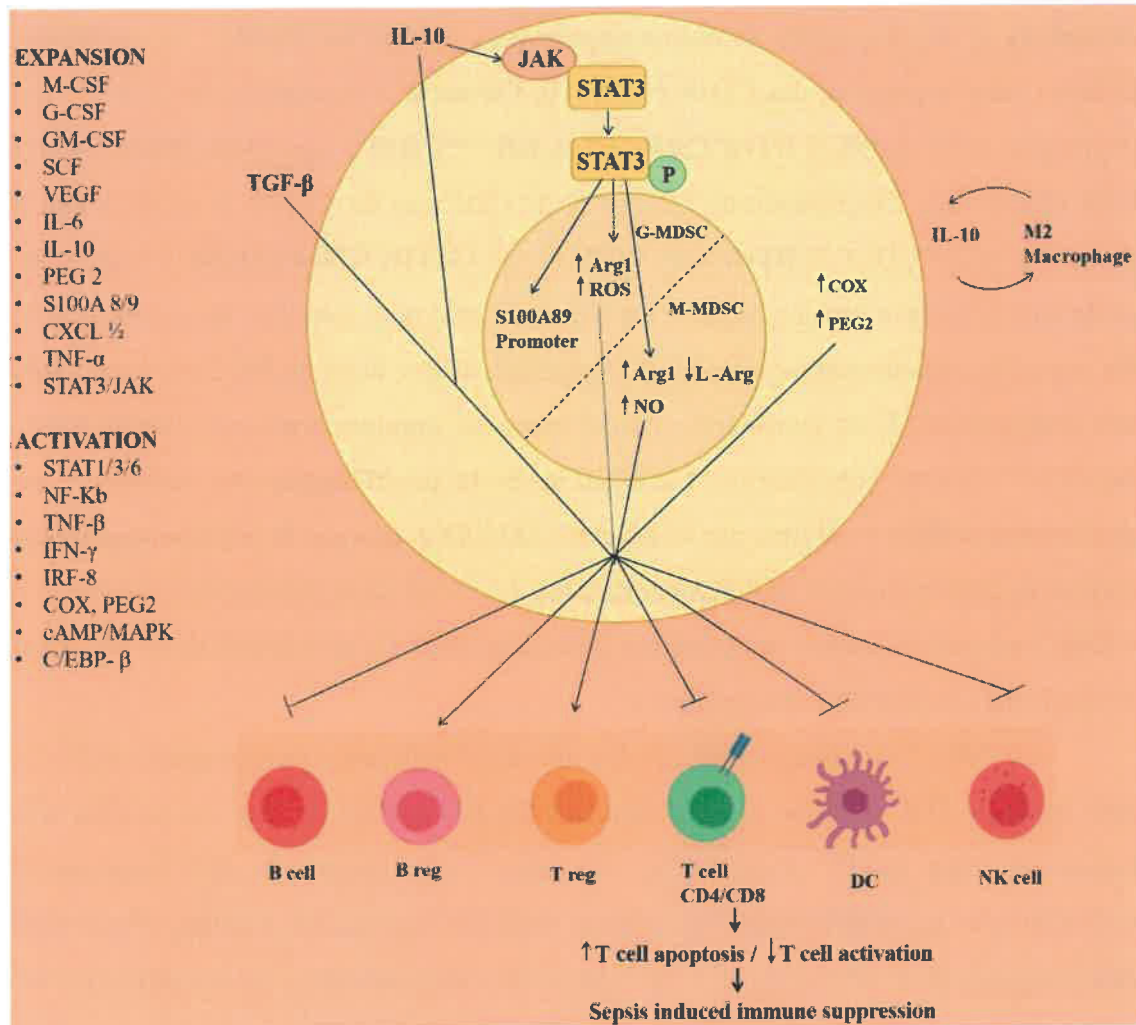


Figure 19. Développement des MDSCs et implication dans l'immunosuppression induite par le sepsis  
Adapté de Malavika et al<sup>183</sup>.

La **phase d'expansion** est initiée dans la moelle osseuse ou la rate, où les cellules souches hématopoïétiques, sous l'influence de mitoses et de multiples facteurs de croissance, génèrent des progéniteurs granulo-monocytaires (également appelés *granulocyte-macrophage colony-forming units* (CFU-GM)). En conditions normales, ces cellules immatures se différencient en cellules matures (ici en PMN et monocytes). Mais en conditions pathologiques (e.g. cancers ou sepsis), ces cellules **conservent leur état immature** et sont **incapables de se différencier**. De nombreux composés sont responsables de l'induction des MDSCs<sup>182,183,207</sup> (figure 19):

- Des facteurs de croissance : *macrophage colony-stimulating factor* (M-CSF), *granulocyte colony-stimulating factor* (G-CSF), *granulocyte-macrophage colony-stimulating factor* (GM-CSF), *stem cell factor* (SCF) ou le *vascular endothelial growth factor* (VEGF)
- Des interleukines et des DAMP : IL-6, IL-10, S100A8 et S100A9
- Des chimiokines : CXCL1, CXCL2

Parmi les voies de signalisation cellulaires décrites, les voies de **STAT3** (*signal transducer and activator of transcription 3*) et **JAK** (*Just Another Kinase*) jouent un rôle important dans l'expansion des MDSCs. STAT3 **bloque l'apoptose** de ces cellules et **favorise leur expansion** en activant des inhibiteurs de l'apoptose comme la cycline D, c-Myc, ou Bcl-XL. La fixation du TNF- $\alpha$  sur le TNFR2 joue aussi un rôle important dans l'expansion des MDSCs<sup>183</sup>.

La **phase d'activation** permet aux MDSCs **d'acquérir leurs capacités immunosuppressives**. Elle implique de nombreux composés produits par l'environnement tumoral ou l'organisme à la suite de la réponse inflammatoire, faisant intervenir de nombreuses voies de signalisations, notamment les voies de STAT1, STAT3, STAT6 et NF- $\kappa$ B<sup>182,183,207</sup> (**figure 19**). Parmi ces composés, nous pouvons citer :

- PAMP et DAMP : LPS, endotoxines, HMGB1
- Cytokines : IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-10, *etc.*
- Protéines de la phase aiguë :  $\alpha$ 2-macroglobulin, protéine sérique amyloïde A

Ainsi, la protéine MyD88, recrutée en réponse à l'activation du récepteur de l'IL-1 ou des TLR, permet l'activation de la voie de NF- $\kappa$ B, tandis que la glycoprotéine 130 (gp130) intervient dans les voies de signalisation de STAT3 et C/EBP $\beta$  (*CCAAT-enhancer binding protein- $\beta$* )<sup>208,209</sup>. Ces voies de signalisation jouent un rôle clef dans le **développement des MDSCs**. Les modèles murins de sepsis chez des souris KO pour la protéine gp130 au niveau hépatique ou pour MyD88 montrent une diminution de l'expansion de ces MDSCs<sup>208,209</sup>. Les **protéines S100A8 et S100A9** ont aussi un rôle important dans ce processus. Alors que S100A8 peut stimuler la voie de NF- $\kappa$ B, la translocation intranucléaire de S100A9 sous l'effet de l'IL-10 permet la synthèse de microARN (miRNA-21 et miRNA-181b) qui induisent le recrutement d'un facteur de transcription, NFI-A (*nuclear factor I A*)<sup>210,211</sup>. S100A9 est impliqué à la fois dans l'expansion et l'activation des MDSCs. L'utilisation de modèles murins de sepsis chez des souris KO pour NFI-A montre que les cellules isolées comme MDSCs ne possèdent pas de capacités immunosuppressives et se différencient normalement en macrophages et en cellules

dendritiques<sup>212</sup>. L'utilisation de ces mêmes modèles chez les souris KO pour S100A9 rapporte les mêmes résultats, avec une amélioration de la survie des animaux, surtout dans la phase tardive<sup>211,213</sup>. D'autres mécanismes sont également décrits, tels que l'impact de l'épigénétique ou la régulation des capacités immunosuppressives par d'autres microARN<sup>182,183</sup>.

### 3.3. Fonctions immunosuppressives

Comme illustré en **figure 19**, les MDSCs exercent leurs fonctions immunosuppressives sur de nombreuses cellules immunitaires, mais les **lymphocytes T sont les principales cibles de cette immunosuppression**. Les MDSCs peuvent inhiber les fonctions des cellules T par plusieurs mécanismes. Tout d'abord, le **catabolisme de la L-arginine** représente une cible importante : l'augmentation de l'activité de 2 enzymes, **l'oxyde nitrique synthase inductible (iNOS)** et **l'arginase 1 (Arg1)**, entraîne une **dégradation accrue** de cet acide aminé<sup>214</sup>. La réduction de la quantité d'arginine dans les cellules T entraîne une **diminution de l'expression de la chaîne  $\zeta$  du CD3 et entrave la réponse immune**. La **séquestration de la cystéine** est un autre mécanisme immunosuppresseur<sup>215</sup>. En présence de MDSCs, celles-ci captent la cystéine extracellulaire, ce qui la rend **indisponible pour les lymphocytes T** qui ne possèdent pas les enzymes nécessaires pour la synthétiser *de novo*. La cystéine est un acide aminé essentiel **nécessaire à la prolifération des cellules T**, mais aussi nécessaire à leur fonction de **présentation antigénique**. De plus, les MDSCs perturbent la fonction des lymphocytes T en générant un stress oxydatif conduisant à la production de **radicaux libres et de peroxy-nitrites**. Lorsque les cellules T entrent en contact avec ces composés, le TCR et le CD8 subissent une **nitration**, ce qui les rend **incapables de répondre de manière spécifique à une stimulation antigénique**<sup>216</sup>. En outre, grâce à la sécrétion d'IL-10, les MDSCs peuvent **favoriser la différenciation et l'expansion des Tregs**, en plus de rendre les lymphocytes T anergiques<sup>217</sup>.

### 3.4. Implications cliniques dans le sepsis

La recherche de l'impact clinique des MDSCs chez les patients septiques présente certaines difficultés. Ces difficultés sont liées à l'utilisation de multiples phénotypes pour décrire ces cellules ou au manque de caractérisation complète de leurs capacités immunosuppressives.

En ce qui concerne les m-MDSCs, **aucune étude n'a établi de lien clair entre la caractérisation complète de ces cellules (i.e. phénotype CD11b<sup>+</sup>CD14<sup>+</sup>HLA-DR<sup>-/low</sup>CD15<sup>-</sup> + capacités immunosuppressives) et les conséquences cliniques chez les patients présentant un**

sepsis. Certaines études décrivent parfois le caractère immunosuppresseur des monocytes HLA-DR<sup>-/low</sup> sans parvenir à faire un lien avec des conséquences cliniques<sup>218,219</sup>. Dans d'autres cas, un lien est effectué avec les conséquences cliniques, mais sans caractériser complètement le phénotype ou les capacités immunosuppressives des cellules étudiées<sup>220</sup>. Considérant que la diminution de l'expression de HLA-DR à la surface des monocytes est la principale caractéristique phénotypique des m-MDSCs, on peut supposer que **la diminution de mHLA-DR** (illustrée précédemment) décrite chez les patients depuis de nombreuses années **correspond à des m-MDSCs**. Une réévaluation des données sur l'expression de mHLA-DR (résultat en nombre de sites par monocytes) chez les patients atteints de sepsis, exprimées en pourcentage de m-MDSCs, a mis en évidence une **association entre la persistance de ces cellules et un risque accru d'infections nosocomiales, ainsi qu'une mortalité plus élevée**<sup>221</sup>. Cependant, la nature rétrospective de cette étude ne permettait pas de prouver les capacités immunosuppressives de ces cellules. Néanmoins, d'autres études ont démontré ces capacités chez les monocytes exprimant faiblement HLA-DR après un sepsis<sup>218-220</sup>.

En ce qui concerne les PMN-MDSCs, plusieurs phénotypes sont décrits dans la littérature, comme les **PMN immatures ou les LDG** (*low density granulocytes*), retrouvés dans la fraction des PBMC après séparation par gradient de centrifugation<sup>182</sup>. Il existe également des études qui utilisent la nomenclature publiée en 2016<sup>181</sup>. Sans apporter la preuve des capacités immunosuppressives de ces cellules, il est difficile de conclure sur leur classification en tant que MDSCs. Par conséquent, nous ne discuterons que des études fournissant à la fois les caractéristiques phénotypiques et immunosuppressives des PMN-MDSCs. Uhel *et al*, étudiant les LDG CD15<sup>+</sup> capables d'inhiber la prolifération des lymphocytes T, ont rapporté qu'un taux initial élevé de ces cellules était **prédictif de la survenue d'infections nosocomiales**<sup>219</sup>. Une autre étude réalisée par Mathias et collègues a analysé les cellules CD33<sup>+</sup> CD11b<sup>+</sup> HLA-DR<sup>-/low</sup> CD14<sup>-</sup> CD15<sup>+</sup>, capable d'inhiber la prolifération des cellules T et la production d'IFN- $\gamma$ . Les résultats ont montré que la présence globale de MDSCs (c'est-à-dire m-MDSCs et PMN-MDSCs) est **associée à un risque accru d'infections nosocomiales et à une augmentation de la mortalité**<sup>220</sup>. Enfin, une dernière étude portant sur les cellules CD33<sup>+</sup> CD11b<sup>+</sup> HLA-DR<sup>-/low</sup> CD14<sup>-</sup> CD15<sup>+</sup> a décrit **l'acquisition tardive des capacités immunosuppressives** (inhibition de la prolifération des lymphocytes T), ces **cellules suppressives étaient retrouvées jusqu'à 21 jours post-sepsis**<sup>222</sup>. Cette étude met en évidence **l'impact des MDSCs sur les altérations immunitaires persistantes** observées chez les patients ayant développé un sepsis. Concernant l'utilisation du marqueur LOX-1, si de nombreuses études lient la présence de ces cellules avec les conséquences cliniques en oncologie (*e.g.* diminution de la survie, rechute,

résistance aux chimiothérapies), les **seules études s'intéressant aux PMN-MDSCs LOX-1+ dans le sepsis bactérien sont celles publiées par notre laboratoire (Article 1 et 2)**.

Dans le contexte spécifique de la COVID-19, les m-MDSCs et les PMN-MDSCs ont été identifiées et ont montré des capacités immunosuppressives similaires à celles décrites précédemment<sup>223</sup>. Leur présence a été associée à la **gravité de la maladie** ainsi qu'à **un risque accru de mortalité**. Cependant, il est important de noter que les résultats des différentes études peuvent varier et que certaines ne trouvent pas d'association significative avec des conséquences cliniques<sup>223</sup>. Concernant le marqueur LOX-1, il a été montré que les neutrophiles immatures exprimant ce marqueur (dont les capacités immunosuppressives ne sont pas démontrées) sont présents en plus grande proportion chez les patients présentant une forme grave de la maladie<sup>224</sup>, développant des thromboses et des SDRA, ainsi que chez les patients non-survivants<sup>165,225</sup>.

#### 4. Synthèse

Globalement, au cours du sepsis, des altérations affectent l'ensemble du système immunitaire. À l'heure actuelle, même si des marqueurs sont proposés pour refléter l'état du système immunitaire (*e.g.* mHLA-DR), **il n'existe pas encore de marqueur largement utilisé pour diagnostiquer l'immunosuppression acquise dans le sepsis**, à l'instar de la numération des lymphocytes CD4+ chez les patients infectés par le VIH (virus de l'immunodéficience humaine). Par conséquent, la seule façon de démontrer la présence d'une **immunosuppression** en pratique clinique est **d'observer la présence d'altérations immunitaires associées à une incidence accrue d'infections secondaires et/ou opportunistes**. Étant donné que ces deux éléments ont été démontrés précédemment, il existe un **rationnel pour envisager des approches thérapeutiques immunostimulantes afin d'inverser l'immunoparalysie observée dans le sepsis**.

#### 5. Traitements immunostimulants dans le sepsis

À la lumière de l'immunosuppression observée chez ces patients, les thérapies immunostimulantes ont suscité un intérêt croissant dans la prise en charge de cette population. Néanmoins, cette approche a mis du temps à être évalué, en partie à cause du **risque théorique d'une réactivation excessive de la réponse pro-inflammatoire initiale**. À ce jour, plusieurs

immunothérapies ont ainsi été étudiées chez les patients septiques, dont une liste non exhaustive est présentée en **tableau 4**.

### IFN- $\gamma$

L'IFN- $\gamma$  est une cytokine majoritairement produite par les lymphocytes TH1, CD8+ et NK, jouant un rôle important pour **activer les monocytes et les macrophages**. Ses effets sur l'organisme comprennent l'amélioration des capacités de présentation des antigènes et des propriétés phagocytaires<sup>20</sup>. Cependant, chez les patients atteints de sepsis, la **synthèse de cette cytokine est altérée**.

La première étude rapportant l'utilisation de l'IFN- $\gamma$  chez l'homme remonte à 1997, où les patients septiques traités ont montré une amélioration des fonctions monocytaires, notamment une augmentation de l'expression de HLA-DR et une augmentation de la sécrétion de TNF- $\alpha$  après stimulation *ex vivo*. Depuis cette étude, d'autres recherches ont confirmé ces résultats, démontré la bonne tolérance de ce traitement et son efficacité potentielle (**tableau 5**). Quelques cas rapportés (en association ou non avec une thérapie anti-PD-1) montrent une **amélioration des troubles immunitaires associée à la survie des patients à la suite du traitement, alors que ceux-ci ne répondaient pas aux thérapies conventionnelles**<sup>136–138,226</sup>. L'utilisation de l'IFN- $\gamma$  comme thérapie pour traiter l'immunosuppression chez les personnes en réanimation est un sujet de recherche actif, mais il convient de noter que son utilisation clinique n'est pas encore généralisée et que des études sont en cours pour évaluer pleinement son efficacité (NCT05843786).

### GM-CSF

Le GM-CSF est un facteur de croissance hématopoïétique qui joue un rôle crucial dans la **synthèse**, la **stimulation** et la **survie des monocytes** ainsi que dans la **production des PMN**<sup>20</sup>. Depuis les premières utilisations du GM-CSF dans les années 2000, quelques études ont examiné son efficacité dans le traitement du sepsis. Ces études ont montré que son administration pouvait améliorer les fonctions des monocytes, notamment en augmentant leur capacité de présentation antigénique et leur capacité de sécrétion de cytokines. De plus, le GM-CSF a démontré sa capacité à **prévenir les infections secondaires chez les patients atteints de sepsis** et a été associé à une **réduction de la durée de ventilation mécanique et à une diminution de la durée de séjour en réanimation (tableau 5)**. Bien que quelques cas isolés affichent une efficacité sur la survie des patients, les essais cliniques randomisés contre placebo

montrent une absence de réduction de la survenue d'infections nosocomiales ou d'amélioration de la survie.

### IL-7

L'interleukine-7 est une cytokine principalement produite par le thymus et la moelle osseuse. Elle joue un rôle essentiel à la **prolifération, à la différenciation et aux fonctions des lymphocytes T**, tout en réduisant la population de Tregs circulants. Compte tenu de la forte lymphopénie observée au cours du sepsis, l'utilisation d'une forme recombinante de l'IL-7 a été étudiée chez les patients septiques. Son utilisation, par **voie intramusculaire**, montre une bonne tolérance chez les patients et une **amélioration de la lymphopénie** ainsi que des fonctions des lymphocytes T. Néanmoins, l'utilisation par voie intraveineuse semble présenter des risques, avec la survenue de SDRA (**tableau 5**).

### Anti-PD-1 & Anti-PD-L1

Le système de checkpoints immunitaires inhibiteurs, composé du récepteur PD-1 et de ses ligands PD-L1 et PD-L2 (*programmed cell death-ligand 1*), joue un rôle clef dans le rétrocontrôle des réponses immunes. La fixation des ligands PD-L1 ou PD-L2 sur le récepteur PD-1 induit notamment des altérations des lymphocytes T, comme l'apoptose ou l'anergie. Les traitements utilisant des anticorps monoclonaux ciblant PD-1, tels que le pembrolizumab ou le nivolumab, ont été initialement développés en oncologie, mais ont également été étudiés dans la prise en charge des patients présentant une immunosuppression au cours du sepsis. Les premières études récentes ont montré une bonne tolérance de ces traitements ainsi qu'une **amélioration de la lymphopénie et de l'expression du mHLA-DR (tableau 5)**. Une étude a aussi montré la bonne tolérance des anticorps anti-PD-L1 (**tableau 5**). Ces dernières années, des cas de sepsis ont été rapportés où l'utilisation d'une bithérapie combinant l'IFN- $\gamma$  et des anticorps anti-PD-1 a permis **d'améliorer les altérations immunitaires et d'obtenir des améliorations cliniques spectaculaires**, conduisant à la survie des patients qui ne répondaient pas aux thérapies conventionnelles telles que les agents anti-infectieux et la chirurgie<sup>136,138</sup>.

Thérapeutiques	Nombre de patients*	Principaux résultats	Références
IFN- $\gamma$	9	Amélioration du mHLA-DR et des fonctions sécrétrices des monocytes <i>ex vivo</i>	Döcke, Nat Med, 1997
	21	Amélioration du mHLA-DR et diminution des pneumopathies associées à la ventilation mécanique	Nakos, Crit Care Med, 2002
	18	Bonne tolérance du traitement, associée avec une amélioration des troubles immunitaires	Payen, BMC Infect Dis, 2019
	En cours	/	NCT05843786
GM-CSF	9	Amélioration du mHLA-DR, du nombre de leucocytes et des fonctions sécrétrices des monocytes <i>ex vivo</i>	Nierhaus, Intensive Care Med, 2003
	38	Amélioration des fonctions monocytaires. Diminution de la durée de ventilation mécanique et de séjour en réanimation	Meisel, Am J Respir Crit Care Med, 2009
	17	Amélioration des fonctions sécrétrices des monocytes <i>ex vivo</i> , prévention des infections secondaires	Hall, Intensive Care Med, 2011
	38	Amélioration du mHLA-DR	Pinder, Thorax, 2018
	66	Amélioration du mHLA-DR et des scores de gravités cliniques. Aucun effet sur la survenue d'infections secondaires ou sur la mortalité	Sun, Medicine, 2023
	98	Amélioration du mHLA-DR, mais aucun effet sur la survenue d'infections secondaires ou sur la mortalité	Vacheron, Journal of Critical Care, 2023
En cours	/	NCT03769844	
IL-7	27	L'injection intramusculaire est bien tolérée, n'induisant pas de cytokine storm. Permet d'inverser la lymphopénie et restaurer la prolifération/activation des cellules T	Francois, JCI Insight, 2018
	21	L'administration intraveineuse inverse la lymphopénie. Par rapport à l'administration intramusculaire, association à un SDRA sans séquelles à long terme. Arrêt de l'étude	Daix, Ann Intensive Care, 2023
Anti-PD-1	31	Pas d'effets indésirables majeurs	Hotchkiss, Crit Care Med, 2019
	13	Bonne tolérance du traitement, amélioration du mHLA-DR et de la lymphopénie	Watanabe, Shock, 2020
Anti PD-L1	24	Bonne tolérance du traitement, pas d'effets indésirables majeurs	Hotchkiss, Crit Care Med, 2019
Anakinra ou IFN- $\gamma$	36	Pas de différence de mortalité entre placebo et immunothérapie à 28 jours	Leventogiannis, Cell Rep Med, 2022
	En cours	/	NCT04990232
Thymosine $\alpha$ -1	1480	Méta-analyse de 12 essais cliniques. Diminution de la mortalité à 28 jours.	Li, Int J Infect Dis, 2015
Cellules souches	9	Bonne tolérance du traitement par cellules souches mésenchymateuses	McIntyre, Am J Respir Crit Care Med, 2018

Tableau 5. Essais cliniques immunostimulants dans le sepsis

Liste non exhaustive. \* Nombre de patients traités par placebo ou immunothérapies, adapté de Fabri et al<sup>227</sup> et Garduno et al<sup>228</sup>

Les études visant à stimuler le système immunitaire dans la prise en charge du sepsis connaissent une augmentation constante de leur incidence. Celles-ci ont généralement rapporté une **bonne tolérance** lors du traitement, ainsi qu'une **restauration des altérations immunitaires observées**. Certaines études ont également montré une **efficacité sur l'évolution clinique des patients septiques**. Cependant, il est important de souligner que ces études



présentent des limitations. Tout d'abord, de nombreux cas cliniques isolés sont rapportés et les essais cliniques incluent un faible nombre de patients, avec seulement 2 études prospectives incluant plus de 40 patients. **Ces dernières n'ont pas montré d'impact** sur les paramètres cliniques habituellement suivis, tels que la **réduction de la survenue d'infections secondaires ou l'amélioration de la survie**. Les méta-analyses ont adopté une approche prudente dans leurs conclusions, soulignant que les résultats doivent être interprétés avec précaution en raison de la moindre qualité de certaines études incluses<sup>229</sup>.

Si les résultats de ces études peuvent sembler convaincants, ils convergent tous vers une conclusion essentielle : **l'immunothérapie ne peut pas être considérée comme un traitement universel pour tous les patients atteints de sepsis**. Ces thérapies doivent être envisagées uniquement dans des contextes où une **immunosuppression est présente**. Ainsi, des **marqueurs de stratification** sont utilisés dans ces études, parmi lesquels la diminution d'expression du **mHLA-DR et la lymphopénie** sont les plus fréquemment rapportées. De la même manière que pour l'approche anti-inflammatoire, le manque de sélection des patients pourrait être un argument expliquant l'échec des essais d'immunostimulation<sup>134</sup>. Par exemple, une récente étude clinique de phase 2 évaluant l'effet de l'IFN- $\gamma$  contre placebo dans la prévention des pneumopathies secondaires chez les patients sous ventilation mécanique a été **interrompue prématurément** en raison de la survenue d'effets indésirables graves dans le groupe traité par l'IFN- $\gamma$ . Cet essai clinique n'avait pas cherché à stratifier les patients en fonction de marqueurs d'immunosuppression, mais les traitait tous de manière indifférenciée dès l'admission.

Les essais cliniques en cours dans la prise en charge du sepsis ont pris en considération les erreurs du passé et **appliquent désormais cette stratification des patients**. Une étude européenne multicentrique randomisée, en double aveugle, contre placebo (IMMUNOSEP, NCT04990232) se concentre sur l'utilisation de thérapies immunostimulantes, en se basant sur l'expression de mHLA-DR. Une étude antérieure avait montré une différence de survie chez les patients en fonction de la présence d'un syndrome hyperinflammatoire (ferritinémie > 4420 ng/mL) ou d'une immunosuppression (mHLA-DR < 5000 sites/monocytes)<sup>230</sup>. Les **patients présentant un syndrome hyperinflammatoire sont traités par anakinra, tandis que ceux présentant une immunosuppression reçoivent de l'IFN- $\gamma$** . Une étude française randomisée, en double aveugle, contre placebo (IGNORANT, NCT05843786) est en cours pour évaluer **l'effet de l'administration d'IFN- $\gamma$  sur la durée de la ventilation mécanique** après le premier épisode de pneumopathie acquise sous ventilation mécanique chez les patients ayant une immunosuppression objectivée par un mHLA-DR < 8000 AB/C.

Lors de la recherche de nouvelles thérapies pour améliorer la prise en charge des patients, il est crucial de tenir compte des enseignements tirés des erreurs passées liées aux traitements visant à lutter contre l'inflammation dans le sepsis. **Il est désormais bien établi qu'un seul traitement ne peut être appliqué de manière uniforme à tous les patients, car différents endotypes d'évolution de la maladie existent au cours du sepsis<sup>50,51,140</sup>.** Certains patients peuvent présenter une phase caractérisée par une hyperinflammation, tandis que d'autres présentent une immunosuppression. Par conséquent, il est essentiel **de mieux comprendre la physiopathologie du sepsis, en particulier le lien entre le phénomène inflammatoire et l'immunosuppression.** Cette caractérisation plus approfondie de la maladie permettra de développer des **stratégies thérapeutiques plus ciblées et personnalisées** pour répondre aux besoins spécifiques de chaque patient.

## V. Lien entre inflammation chronique et immunosuppression

### 1. PICS : Persistent Inflammation, Immunosuppression, and Catabolism Syndrome

Comme mentionné précédemment, la prise en charge précoce du sepsis a connu une amélioration significative au cours des dernières décennies, tout comme notre compréhension de sa physiopathologie. Les avancées ont considérablement **réduit la mortalité précoce**, principalement due à la tempête cytokinique et aux dysfonctions d'organes. Cette amélioration a entraîné une augmentation du nombre de patients en réanimation et dans les unités de réadaptation. Les patients survivants présentent des séquelles à long terme, telles qu'une diminution des capacités physique, une malnutrition et une perte de masse musculaire ainsi que des infections récurrentes, des dysfonctions d'organes. Ils sont également exposés à un risque accru d'évènements cardiovasculaires, à une détérioration neurocognitive à long terme, une forte limitation de l'autonomie et à un surrisque de développer des cancers<sup>25</sup>. Ces séquelles engendrent des coûts supplémentaires considérables imputables au sepsis.

Le terme « *Chronic Critical Illness* » (CCI) a été introduit pour décrire les patients ayant un **séjour prolongé et complexe en soins intensifs de plus de 14 jours et présentant les séquelles décrites ci-dessus**. En 2009, ces patients ont représenté un coût de plus de 20 milliards de dollars au système de santé aux États-Unis<sup>231</sup>. Plus de 60 % des patients étaient admis avec un diagnostic de sepsis, et seulement 20 % des patients atteints de CCI ont pu rentrer chez eux, tandis que près de 30 % sont décédés à l'hôpital<sup>231</sup>. La plupart de ces patients ne parviennent jamais à retrouver leur autonomie fonctionnelle et ont une **mauvaise espérance de vie à long terme**, avec une estimation de survie à un an inférieure à 50 %<sup>231</sup>.

En 2012, Gentile *et al* ont défini un sous-ensemble du CCI appelé « *Persistent Inflammation, Immunosuppression, and Catabolism Syndrome* » (PICS)<sup>232</sup>, terme repris depuis par de nombreux auteurs<sup>20,128,231,233</sup>. Comme son nom l'indique, ces patients présentent des **signes d'inflammation persistante**, une **immunosuppression**, un catabolisme des protéines, une perte de masse musculaire et des besoins nutritionnels non satisfaits. Dans une revue consacrée à ce syndrome, Mira et ses collègues définissent le PICS comme un **cercle vicieux** récurrent agissant sur la moelle osseuse où la réaction inflammatoire joue un **rôle déclencheur** (myélopoïèse d'urgence) à l'origine de cellules immatures (**Figure 20**), puis, dans un deuxième temps, elle joue un **rôle de perpétuation via la production de MDSCs** qui contribuent à un **état d'immunosuppression**. Cliniquement, ces patients souffrent d'infections nosocomiales récurrentes, de réactivations de virus latents, d'une mauvaise cicatrisation des plaies, et ne peuvent pas retourner chez eux, mais sont préférentiellement placés dans des centres de

rééducation de longue durée où ils connaissent des récurrences de sepsis nécessitant des réhospitalisations. Cela crée un cercle vicieux où les infections récurrentes amplifient l'inflammation, entraînant une myélopoïèse aberrante et une expansion continue des MDSCs, ce qui induit à son tour une immunosuppression et conduit souvent à une mort indolente.

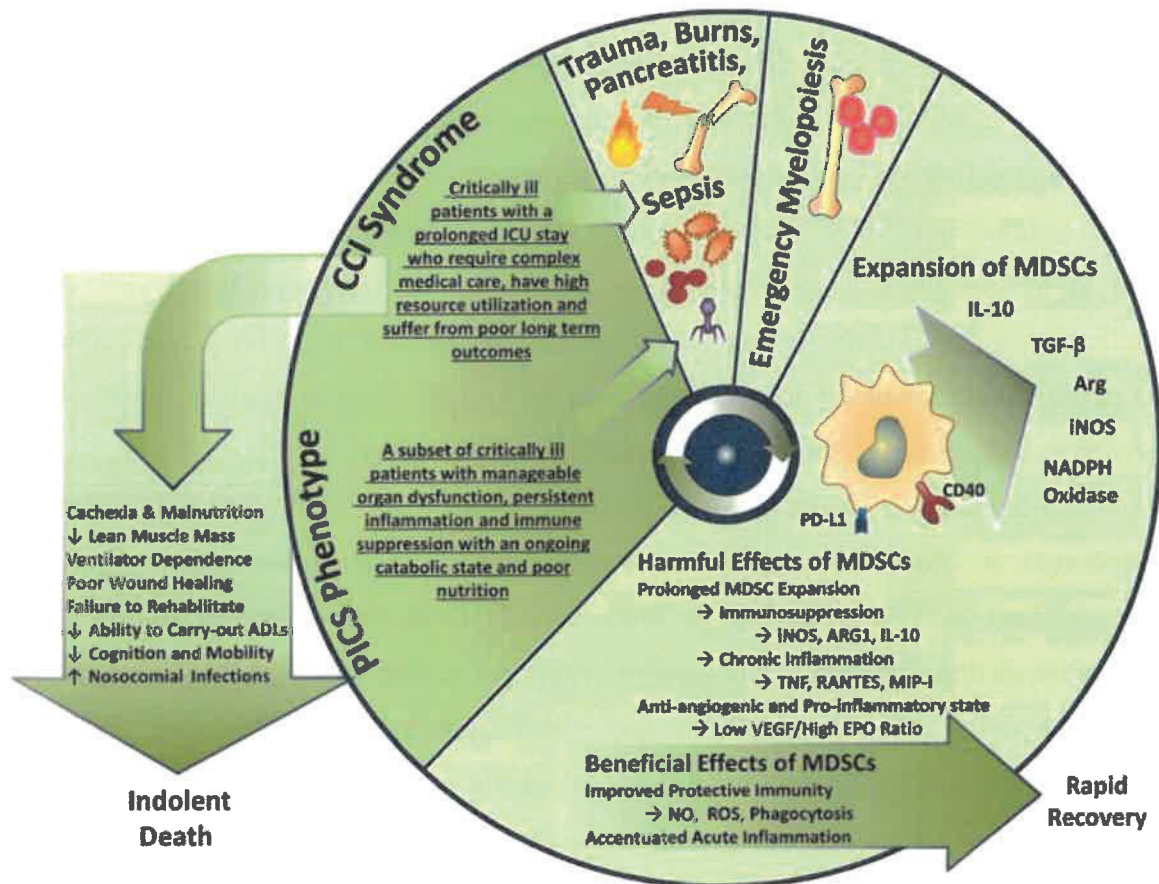


Figure 20. Développement du PICS<sup>233</sup>

De manière intéressante, ce processus partage des similitudes avec la cancérologie.

## 2. Immunosuppression et inflammation en cancérologie

L'inflammation est un processus nécessaire à l'éradication des tumeurs. Comme son nom l'indique, le *Tumour Necrosis Factor- $\alpha$*  est une cytokine impliquée dans la réponse immunitaire antitumorale en activant les cellules immunitaires, en favorisant la destruction des cellules cancéreuses et en régulant l'interaction entre les cellules tumorales et les cellules immunitaires. Néanmoins, d'une manière générale, **l'inflammation chronique est associée à la tumorigenèse**, y compris celle générée par le TNF- $\alpha$ .

Dès 1863, le docteur Rudolf Virchow émet l'hypothèse selon laquelle l'inflammation chronique pourrait être à l'origine du cancer<sup>234</sup>. Plus d'un siècle après cette hypothèse, la

relation de causalité entre l'inflammation, l'immunité et le cancer est largement acceptée. **Environ 15 à 20 % des cas de cancer sont précédés d'une infection, d'une inflammation chronique** ou d'une auto-immunité dans le tissu ou l'organe concerné<sup>235</sup>. Parmi les causes les plus connues, nous pouvons citer les maladies inflammatoires de l'intestin, l'hépatite chronique ou la gastrite induite par *Helicobacter pylori* augmentant respectivement le risque de cancer colorectal, de cancer du foie ou de cancer de l'estomac.

Avant la détection de la tumeur, les réponses inflammatoires abritent des mécanismes puissants qui entraînent l'accumulation de mutations et de divers changements épigénétiques dans les cellules épithéliales<sup>235</sup>. Une fois que le microenvironnement inflammatoire tumoral est établi, les facteurs inflammatoires utilisent la signalisation des récepteurs de cytokines dans les cellules mutées pour induire des voies de survie (médiées par NF- $\kappa$ B, STAT3, *etc.*) conduisant à la **prolifération cellulaire et prolongeant la survie cellulaire** en activant initialement les oncogènes, puis en inactivant les gènes suppresseurs de tumeurs<sup>235</sup>.

L'inflammation chronique joue un rôle crucial dans la progression tumorale en **contribuant à l'immunosuppression**, créant ainsi un microenvironnement propice au développement et à la formation de métastases<sup>236</sup>. Cette immunosuppression résulte du recrutement de diverses cellules aux capacités suppressives dans ce microenvironnement, telles que les macrophages de type M2, les lymphocytes T régulateurs, les MDSCs, *etc.* Ces cellules créent un microenvironnement favorable à l'angiogenèse et propice à la croissance tumorale, tout en entravant l'action du système immunitaire, notamment l'immunité antitumorale des lymphocytes T<sup>235</sup>, ce qui conduit à un **échappement immunitaire**. De nombreuses études se concentrent sur ces interactions entre l'inflammation et l'immunosuppression. Dans le tableau ci-dessous (**tableau 6**), nous nous restreignons à lier l'inflammasome NLRP3 et les MDSCs en résumant les principaux résultats des études établissant une telle association (et avec les cellules suppressives pour les études publiées avant la publication de la nomenclature des MDSCs).

Contexte		Principaux résultats	Références
Modèle murin de fibrosarcome	de	La sécrétion par la tumeur ou l'injection d'IL-1 $\beta$ est responsable d'altérations hématologiques et du développement de cellules immatures suppressives.	Song, J Immunol, 2005 <sup>237</sup>
Modèle murin de carcinome mammaire + d'IL-1 $\beta$	autosécrétion	Les souris avec les cellules sécrétant l'IL-1 $\beta$ ont une plus forte progression tumorale et plus de MDSCs qui inhibent la prolifération des lymphocytes T.	Bunt, J Immunol, 2006 <sup>238</sup>
Modèle murin de carcinome mammaire		Les souris IL-1R KO présentent une réduction de la progression tumorale et des métastases, ainsi que moins de MDSCs. Les souris IL-1Ra KO ont plus de MDSCs qui sont plus immunosuppresseurs.	Bunt, Cancer Res, 2007 <sup>239</sup>

Modèle d'autosécrétion d'IL-1 $\beta$	murin	L'expression de l'IL-1 $\beta$ chez les souris transgéniques conduit à une inflammation gastrique et à des carcinomes, corrélant avec le recrutement de MDSCs. Le traitement par IL-1Ra inhibe le recrutement de MDSCs et le développement de carcinome.	Tu, Cancer Cell, 2008 <sup>240</sup>
Prélèvements de patients et modèle murin de cancer de l'estomac		Expression de l'IL-18 et de son récepteur dans les tissus tumoraux et les lignées cellulaires. L'utilisation de siARN bloquant l'ARNm de l'IL-18 réduit la masse tumorale et le développement de métastases. L'IL-18 pourrait faciliter l'échappement immunitaire des cellules cancéreuses.	Kang, Carcinogenesis, 2009 <sup>241</sup>
Modèle murin de mélanomes		NLRP3 est exprimé par les MDSCs. Les souris NLRP3 KO présentent une forte réduction des MDSCs. La déplétion des MDSCs améliore la survie des souris WT, mais pas des souris NLRP3 KO. NLRP3 est critique pour l'accumulation des MDSCs.	Van Deventer, Cancer Res, 2010 <sup>242</sup>
Modèles de métastases	murins	L'administration de faible dose d'IL-18 favorise la dissémination des métastases. L'IL-18 est aussi sécrétée par la tumeur et augmente les lymphocytes NK aux capacités suppressives (PD-1+/PD-L1+/B7-H1+).	Terme, Cancer Res, 2011 & 2012 <sup>243,244</sup>
Modèle murin de cancer		La gemcitabine et le 5-fluorouracil (5FU) activent NLRP3 dans les MDSCs via la cathepsine B et induisent la production d'IL-1 $\beta$ associée à la résistance aux traitements et à la progression tumorale.	Bruchard, Nat Med, 2013 <sup>245</sup>
Modèle murin de mélanome		Le traitement <i>ex vivo</i> de BMDMs avec IL-18 +GM-CSF induit la synthèse de m-MDSCs. Les m-MDSCs ainsi produites étaient plus immunosuppressives que les m-MDSCs synthétisées sans IL-18.	Lim J Immunol, 2014 <sup>246</sup>
Modèle murin de cancer du sein métastatique		Les souris NLRP3 KO montrent une diminution de la croissance tumorale, moins de métastases, une amélioration de la survie, en lien avec la concentration d'IL-1 $\beta$ aux sites tumoraux et l'accumulation de MDSCs et TAM. Le traitement par IL-1Ra diminue cette accumulation, la croissance tumorale et le processus métastatique.	Guo, Sci Rep, 2016 <sup>247</sup>
Prélèvements de patients et modèle murin de carcinome pancréatique		Les TAM ( <i>tumor associated macrophages</i> ) aux capacités suppressives surexpriment NLRP3 chez l'homme et la souris. NLRP3 stimulent l'expansion des TAM, des Tregs, TH1. Les souris NLRP3 KO présentent une meilleure survie, moins de TAM et une amélioration de l'immunosuppression.	Daley, J Exp Med, 2017 <sup>248</sup>
Prélèvements de patients et modèle murin de carcinome de la tête et du cou		Augmentation de NLRP3 dans les tissus et plasma des patients. L'inhibition de NLRP3 réduit la progression tumorale, diminue les Tregs, les TAM, les MDSCs, et réduit l'épuisement des lymphocytes T en plus d'augmenter les cellules T CD4+ et CD8+.	Chen, Cell Mol Life Sci, 2018 <sup>249</sup>
Prélèvements de patients et modèle murin de myélome		Chez les patients, des niveaux élevés d'IL-18 dans la moelle osseuse sont associés à une diminution de la survie. Chez la souris, l'IL-18 favorise les capacités immunosuppressives des MDSCs. L'addition d'anti-IL-18 au bortezomib améliore la survie des souris.	Nakamura, Cancer Cell, 2018 <sup>250</sup>
Prélèvements de patients et modèle murin de mélanome		L'immunothérapie anti-PD1 induit le recrutement de PMN-MDSCs. L'utilisation de souris NLRP3 KO ou d'inhibiteurs de NLRP3 diminue le recrutement des PMN-MDSCs et améliore l'efficacité de l'immunothérapie.	Theivanthiran, J Clin Invest, 2020 <sup>251</sup>
Prélèvements de patients et modèle murin de cancer pancréatique		Plus d'expression d'IL-1 $\beta$ dans les tissus tumoraux de patients. Chez la souris, l'IL-1 $\beta$ favorise l'immunosuppression dans le microenvironnement tumoral (TAM, Breg, MDSC). L'utilisation de thérapie bloquant l'IL-1 $\beta$ améliore le traitement par anti-PD-1.	Das, Cancer Res, 2020 <sup>252</sup>

Modèle murin de cancer colorectal	L'utilisation d'une molécule inhibant NLRP3 dans les MDSCs, en combinaison avec le 5FU, améliore l'immunité antitumorale (infiltration CD4+ et CD8+...).	Xu, Toxicol Appl Pharmacol, 2021 <sup>253</sup>
Modèle murin de mélanome	L'IL-1 $\beta$ crée de l'inflammation dans le microenvironnement tumoral. L'inhibition de NLRP3 entraîne une réduction de la masse tumorale et une diminution de l'expression de gènes immunosuppresseurs des MDSCs.	Tengesdal, Front Immunol, 2021 <sup>254</sup>
Données génomiques et prélèvements de patients, modèle murin de mélanome	Plus forte activation des gènes de NLRP3 et de l'IL-1 $\beta$ chez les patients. L'utilisation de lignées cellulaires NLRP3 KO pour le modèle de mélanome diminue la progression tumorale. L'utilisation d'inhibiteur de NLRP3 réduit l'expansion des MDSCs et améliore l'infiltration des lymphocytes T CD8+ dans la tumeur.	Tengesdal, PNAS, 2021 <sup>255</sup>
Prélèvements de patients et modèle murin de lymphome	Chez les patients, l'expression du gène de l'IL-18 dans les tissus tumoraux est augmentée et corrèle avec l'expression de PD-1. L'utilisation d'inhibiteur de NLRP3 réduit la progression tumorale, l'expression de PD-L1 et le développement de cellules suppressives (TAM, Tregs, MDSCs).	Lu, Cancer Lett, 2021 <sup>256</sup>
Données génomiques de patients et modèle murin de mélanome	Les gènes de la voie de l'inflammasome NLRP3 sont associés à une diminution de la survie des patients atteints de mélanome et de cancer du poumon. Le KO de NLRP3 chez la souris réduit la progression tumorale, améliore l'immunité et diminue l'activité suppressive des MDSCs.	Papafragkos, Front Immunol, 2022 <sup>257</sup>
Prélèvements de patients et modèle murin de carcinome œsophagien	L'infection par <i>Fusobacterium nucleatum</i> chez les patients est associée à une hyperactivation de NLRP3 et au développement de MDSCs ainsi qu'à une diminution de la survie.	Liang, Ann Med 2022 <sup>258</sup>
Modèle murin de cancer du sein métastatique	Les souris NLRP3 KO montrent une moindre progression tumorale et une amélioration de la survie. L'inhibition de NLRP3 diminue l'infiltration de MDSCs et augmente celle des NK et lymphocytes CD8+. Amélioration de la survie par bithérapie avec anti PD-1 vs anti-PD-1 seul.	Tengesdal, Pharmaceuticals, 2022 <sup>259</sup>
Prélèvements de patients et modèle murin de cancer colorectal	Chez les patients, surexpression de NLRP3 dans les tissus tumoraux. Chez l'animal, <i>C. Tropicalis</i> induit l'activation des gènes de la voie de NLRP3 dans les MDSCs. Lors de l'inhibition de NLRP3, <i>C. Tropicalis</i> est moins tumorigène.	Zhang, Int Immunopharmacol 2022 <sup>260</sup>
Patients avec syndrome myélodysplasique	Augmentation d'expression des gènes de la voie de NLRP3 chez les patients, corrélant avec TIM-3. L'augmentation des gènes des composants ou des produits de l'inflammasome NLRP3 est associée à une diminution de la survie. En co-culture, TIM-3 active la voie de NLRP3 dans les MDSCs.	Yu, Immunology, 2023 <sup>261</sup>
Prélèvements de patients et modèle murin de carcinome de la tête et du cou	L'expression de NLRP3 est augmentée dans les TAM et le nombre de TAM expriment fortement NLRP3 est associée à une diminution de la survie chez les patients. Les souris NLRP3 KO ont une moindre progression tumorale, moins de TAM et de MDSCs.	Chen, Cancer Immunol Immunother 2023 <sup>262</sup>

*Tableau 6. Études associant activation de NLRP3 et cellules immunosuppresseurs en cancérologie. Listes non exhaustives des études chez l'homme ou sur modèles animaux montrant une association entre l'inflammasome NLRP3, l'immunosuppression et/ou les MDSCs en cancérologie.*

En résumé, quel que soit le cancer considéré, l'expression des gènes de l'inflammasome NLRP3 ou son activation est liée au développement de la tumeur, à sa progression et à la propagation des métastases. **L'activation de la voie de NLRP3 favorise la**

**création d'un microenvironnement immunosuppresseur** en recrutant de nombreuses cellules aux capacités immunosuppressives comme les **MDSCs**. Les thérapies qui ciblent et inhibent la voie de NLRP3 ou ses produits améliorent la survie chez les animaux, et quelques études chez les patients ont montré que la surexpression de la voie de l'inflammasome NLRP3 ou de ses produits est associée à une diminution de la survie.

### 3. Immunosuppression et inflammation dans le sepsis

Compte tenu des similarités observées entre le cancer et le sepsis (*e.g.* présence d'une inflammation, présence d'immunosuppression, voir **figure 21**), l'hypothèse du lien observé entre inflammation (aiguë ou chronique) et immunosuppression dans le cancer a émergé dans la recherche sur le sepsis.

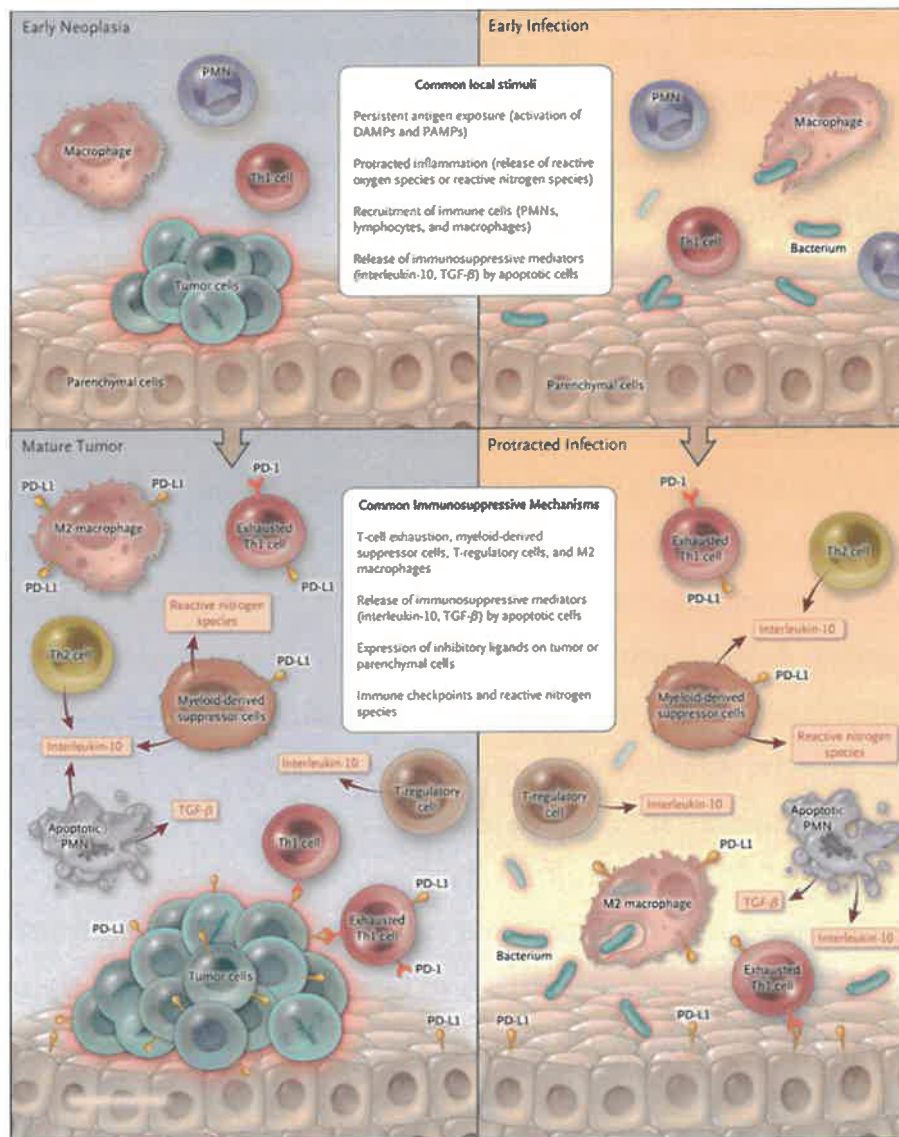


Figure 21. Similarités de la réponse immune dans le cancer et les infections graves<sup>263</sup>



Cependant, peu d'études cliniques ont pu établir ce lien de manière concrète. Une équipe dirigée par Tom Van der Poll a réalisé des recherches sur des patients hospitalisés pour pneumopathies communautaires, indépendamment de la présence d'un sepsis. Ils ont observé une diminution des capacités des cellules des patients à libérer des cytokines pro-inflammatoires telles que le TNF- $\alpha$ , l'IL-1 $\beta$  et l'IL-6 après stimulation *ex vivo*, associée à une plus forte réponse inflammatoire (e.g. sécrétion de résistine, sTREM-7)<sup>264</sup>. Dans une autre étude menée sur des patients graves en réanimation, dont certains atteints de sepsis, la même équipe a démontré que **les patients présentant la plus forte immunosuppression montraient également les signes les plus prononcés de réponse inflammatoire systémique**. En effet, les patients présentant la capacité la plus faible de production de TNF- $\alpha$  *ex vivo* présentaient les concentrations systémiques les plus élevées en CRP, IL-6 et IL-8<sup>265</sup>. Récemment, la même équipe a montré que les patients de réanimation qui développent une pneumopathie acquise sous respiration mécanique (comparés à ceux qui n'en développent pas) présentent des réponses pro-inflammatoires initiales plus importantes ainsi qu'une moindre diminution ou une augmentation des marqueurs inflammatoires (e.g. IL-6) entre l'inclusion et le diagnostic<sup>266</sup>. Néanmoins, aucune mesure directe des marqueurs cellulaires de l'immunosuppression, telle que la diminution d'expression du mHLA-DR ou la lymphopénie, n'a été utilisée dans ces études. L'étude la plus complète mesurant de nombreux marqueurs d'immunosuppression (lymphopénie, expression du mHLA-DR, apparition de PMN immatures...) et des paramètres inflammatoires (diverses cytokines, études transcriptomiques des voies de l'inflammation, des alarmines...) est REALISM (*REAnimation Low Immune Status Markers*, NCT02638779)<sup>139</sup>. L'analyse des données et les études de trajectoires montrant un **lien entre inflammation chronique et immunosuppression** au cours du temps sont en cours de publication (Bodinier *et al*, soumis). Selon Rincon et ses collègues, la **libération continue de médiateurs inflammatoires** pendant le sepsis (e.g. S100A, HMGB-1, IL-6...) peut **altérer la fonction de la moelle osseuse**, entraînant une activation persistante des cellules myéloïdes et le développement ultérieur d'un endotype immunologique avec une **immunosuppression persistante**.

Une récente étude publiée par Yao et ses collègues fournit des éléments supplémentaires pour soutenir le lien entre l'inflammation et l'immunosuppression<sup>218</sup>. Dans cette étude, les auteurs ont identifié différents phénotypes de monocytes chez les patients atteints de sepsis. Ils ont également observé l'existence de monocytes exprimant fortement S100A9 sur modèle murin de sepsis et ont démontré leur capacité à réduire la prolifération des lymphocytes T *ex vivo*. Les auteurs montrent donc que ces cellules sont, *in fine*, des m-MDSCs. De manière

intéressante, les souris S100A9 KO montraient une diminution de l'immunosuppression associée au sepsis. Le traitement par inhibiteur de liaison du S100A9 au TLR4 dans un modèle murin de sepsis montre une diminution de l'immunosuppression, une amélioration de la survie et donc la contribution de S100A9 à la formation de m-MDSCs après un épisode de sepsis. Bien que cette étude présente certaines limitations, elle montre de manière convaincante **l'impact de l'inflammation chronique induite au niveau de la moelle osseuse sur l'immunosuppression** observée chez les patients septiques. Ces résultats renforcent les études qui ont mis en évidence l'effet de S100A9 dans la régulation de l'immunosuppression médiée par les MDSCs sur modèle murin de sepsis<sup>210-213</sup>.

Néanmoins, ces résultats restent parcellaires et à ce jour, aucune étude n'a établi une association ou une relation de causalité entre l'immunosuppression observée dans le sepsis et l'inflammation chronique. Ceci est d'autant plus vrai quand on considère l'inflammasome NLRP3, alors que de nombreuses études montrent son activation au cours du sepsis, l'expression génique de ses composants ou les protéines elles-mêmes, et les conséquences cliniques observées, comme présenté en **tableau 7**. Il convient de noter que nous ne discuterons pas des études cliniques mentionnant la seule production d'IL-1 $\beta$  ou d'IL-18 chez les patients septiques, de nombreuses revues ayant déjà traité ce sujet<sup>66,267,268</sup>.

Contexte	Principaux résultats	Références
Patients avec sepsis bactérien + contrôles	La stimulation <i>ex vivo</i> de PBMC de patients septiques montre une diminution des capacités de sécrétion (dont l'IL-1 $\beta$ ) par rapport aux volontaires sains. Cette diminution est plus marquée chez les sepsis sévères et les patients en choc septique.	Giamarellos-Bourboulis, Crit Care 2011 <sup>269</sup>
Patients en réanimations dont sepsis (+/- SDRA)	Plus d'expression des gènes caspase 1, IL-1 $\beta$ et IL-18 (particulièrement chez les patients présentant un SDRA). Persistance de l'IL18 entre J1 et J7 chez les patients. Corrélation de l'IL18 avec sévérité et mortalité chez tous les patients.	Dolinay, Am J Respir Crit Care Med 2012 <sup>270</sup>
Patients avec sepsis bactérien + contrôles	L'IL-18 plasmatique dans les 24h post-admission est un facteur prédictif de la mortalité, indépendamment de tous les autres médiateurs inflammatoires.	Eidt, Clin Chim Acta, 2016 <sup>271</sup>
Patients avec sepsis bactérien + contrôles	L'analyse des gènes de NLRP3, IL-1 $\beta$ et IL-18 montrent une expression augmentée chez les non-survivants. Persistance d'une forte IL-18 plasmatique jusqu'à J7 chez les patients.	Esquerdo, Clin Exp Immunol, 2017 <sup>272</sup>
Patients avec sepsis + modèle murin de sepsis	Diminution de production d'IL-1 $\beta$ et de specks d'ASC <i>ex vivo</i> qui permet la définition d'un phénotype « <i>NLRP3 immunocompromised</i> » associé à HMGB1, IL-6, et TNF- $\alpha$ et la détection de 80% des patients non-survivants.	Martinez-Garcia, Nat Commun, 2019 <sup>273</sup>
Bactériémie à <i>S.aureus</i> + chocs septiques	Activation de la caspase 1 dans les monocytes et les PMN pendant la 1 <sup>re</sup> semaine. La persistance de forte concentration d'IL-18 plasmatique à J5 et J7 est associée à une diminution de la survie.	Rasmussen, Microbiol Immunol, 2019 <sup>123</sup>

Patients avec sepsis	La pyroptose des PBMC et la concentration en IL-18 dans les 24h post admission est plus important chez les non-survivants à J28.	Wang, Biomed Res Int, 2020 <sup>274</sup>
Patients en réanimation dont sepsis + contrôles	L'IL-1 $\beta$ plasmatique et l'expression du gène de NLRP3 étaient plus élevées chez les patients septiques par rapport aux autres patients de réanimation. L'expression de NLRP3 à J7 est plus élevée chez les survivants.	Garnacho-Montero, Shock, 2020 <sup>275</sup>
Patients avec COVID-19 (toutes sévérités)	Plus d'activation de l'inflammasome NLRP3 associée à la sévérité de la maladie. L'IL-18 plasmatique est plus élevée chez les non-survivants.	Rodrigues, J Exp Med, 2021 <sup>127</sup>
Patients avec sepsis bactérien + contrôles	Une mutation gain de fonction entraînant la diminution d'activation de NLRP3 est décrite dans la population normale et chez les patients. Cette mutation était associée à une augmentation de la survie à 28 jours.	Lu, Sci Rep, 2021 <sup>276</sup>
Patients avec sepsis ou choc septique	Hyperproduction d'IL-1 $\beta$ à J1, d'IL-18 à J1 et persistance à J7. À J6 et J7, les patients formant peu de specks d'ASC au niveau basal ont une survie diminuée.	Cui, Front Immunol, 2021 <sup>122</sup>
Patients avec sepsis bactérien + contrôles	Les patients présentent un fort taux de NLRP3 plasmatique, particulièrement les patients en choc. La concentration de NLRP3 était corrélée aux scores de gravité clinique et les patients avec de forts taux présentaient une diminution du taux de survie	Huang, Cytokine, 2022 <sup>277</sup>
Patients avec COVID-19 sévère	Activation de la voie de NLRP3 (ASC, caspase 1 et NLRP3) chez les patients et activation spontanée <i>ex vivo</i> de l'inflammasome NLRP3.	Bertoni, J Allergy Clin Immunol, 2022 <sup>278</sup>
Patients avec sepsis	L'expression des gènes de l'inflammasome NLRP3 (NLRP3, ASC, caspase 1) et ses produits (IL-1 $\beta$ et IL-18) restent élevés jusqu'à 1 an post-sepsis.	Gritte, Crit Care Explor, 2022 <sup>279</sup>
Patients avec COVID-19 hospitalisés	Les fortes concentrations plasmatiques en IL-18 libre 15j après l'apparition des symptômes sont associées à la gravité et à la mortalité de la COVID-19	Nasser, Intensive Care Med Exp, 2023 <sup>280</sup>

*Tableau 7. Études cliniques s'intéressant à l'inflammasome NLRP3 dans le sepsis  
Listes non exhaustives des études chez l'homme montrant une association entre  
l'inflammasome NLRP3 et les conséquences cliniques.*

Ces études montrent que les **produits de l'inflammasome NLRP3** (*i.e.* l'IL-1 $\beta$  et l'IL-18), ses **composants circulants ou cellulaires** (*e.g.* NLRP3 plasmatique ou ASC specks) ainsi que **l'expression des gènes associés sont reliés à la mortalité observée dans le sepsis**. Certaines de ces études ont révélé une persistance des composants de l'inflammasome NLRP3 ou de ses produits à long terme, sans toutefois établir de lien avec l'immunosuppression observée au cours du sepsis. Une seule étude, sur modèle murin de sepsis, semble indiquer un lien entre activation de l'inflammasome NLRP3 et développement de MDSCs. Les auteurs ont rapporté que l'utilisation de souris KO pour l'IL-1R1 ou le traitement par anakinra a réduit l'infiltration de MDSCs dans le foie des souris septiques<sup>281</sup>. Cependant, aucune diminution des MDSCs dans la moelle osseuse n'a été observée, et plus important, aucune démonstration du pouvoir immunosuppresseur des cellules identifiées comme MDSCs n'a été apportée, les auteurs se basant uniquement sur leur caractérisation phénotypique.

Dans un autre contexte, un lien direct a été effectué entre l'activation de l'inflammasome NLRP3 et le développement de MDSCs. En effet, dans les NLRP3-AID, où une mutation gain de fonction de l'inflammasome NLRP3 est présente, **une étude a rapporté la présence de MDSCs**<sup>282</sup>. Ces cellules présentaient toutes les caractéristiques des MDSCs : elles étaient immatures et avaient des capacités suppressives, pouvant réduire la prolifération des lymphocytes T en co-culture et la sécrétion d'IFN- $\gamma$ . Néanmoins, l'apparition d'une immunosuppression chez ces patients n'a jamais été rapportée.

En conclusion, de nombreuses données montrent l'impact de l'inflammation chronique sur l'immunosuppression dans le cancer, avec une implication directe des MDSCs et de l'activation de l'inflammasome NLRP3. Dans le sepsis, l'activation de la voie de NLRP3 ainsi que l'occurrence de MDSCs aux capacités immunosuppressives ont été démontrées autant sur modèle murin que lors d'études cliniques. Néanmoins, **l'exploration conjointe de NLRP3, de l'inflammation chronique et de l'immunosuppression n'a jamais été réalisée en recherche clinique**. C'est ce qui a constitué l'objectif principal de mon travail personnel.

## OBJECTIF ET PRÉSENTATION DU TRAVAIL DE THÈSE

## Objectif et présentation du travail de thèse

Le présent travail avait pour but général de **mieux décrire l'activation de l'inflammasome NLRP3 au cours du sepsis** et plus particulièrement son lien avec **l'immunodépression observée chez les patients**. Sur la base des connaissances acquises en cancérologie et des mécanismes immunosuppresseurs impliqués dans le sepsis, **nous avons émis l'hypothèse que l'activation de l'inflammasome NLRP3, de manière aiguë ou persistante, pourrait être impliquée dans l'immunodépression induite par le sepsis, en particulier via l'induction de MDSCs.**

Ce travail s'inscrit dans un projet de recherche translationnelle, associant en parallèle recherche fondamentale (*i.e.* approche expérimentale sur modèle murin de sepsis) et recherche clinique. Tandis que l'aspect fondamental était développé par une autre étudiante en thèse de doctorat, mon travail s'est concentré sur le versant clinique. Pour cela, j'ai pu mener des études observationnelles (prospective et rétrospective) sur différentes cohortes de patients septiques hospitalisés en réanimation au sein des Hospices Civils de Lyon. La première cohorte est constituée de patients en choc septique (cohorte IMMUNOSEPSIS, NCT02803346). La deuxième cohorte, initiée pendant la réalisation de ma thèse du fait de la pandémie de COVID-19, est constituée de patients de réanimation infectés par SARS-CoV-2, *i.e.*, présentant une forme sévère de COVID-19 (cohorte RICO, NCT04392401). Enfin, j'ai pu avoir accès à une base de données clinico-biologiques issue de l'étude REALISM, constituée de patients présentant un sepsis ou un choc septique (cohorte REALISM, NCT02638779).

Le présent travail a permis la rédaction est de 3 articles en premier auteur qui seront présentés successivement dans les pages suivantes.

Mon travail de thèse est constitué de 3 articles :

- Article n° 1 : Emergence of immunosuppressive LOX-1+ PMN-MDSC in septic shock and severe COVID-19 patients with acute respiratory distress syndrome (*J Leukoc Biol.* 2022 Feb ; 111(2) : 489-496)
- Article n° 2 : Persistent NLRP3 inflammasome activation is associated with delayed MDSCs in septic patients (article soumis pour publication)
- Article n° 3 : Monitoring NLRP3 Inflammasome Activation and Exhaustion in Clinical Samples: A Refined Flow Cytometry Protocol for ASC Speck Formation Measurement Directly in Whole Blood after Ex Vivo Stimulation (*Cells.* 2022 Oct 20 ; 11(20) : 3306)

Par ailleurs, ce travail s'inscrit dans la thématique de l'équipe de recherche de l'EA 7426 (Université Lyon I) visant à mieux comprendre les mécanismes induisant une immunodépression après un épisode inflammatoire aigu. Ainsi, ayant participé à la réalisation du suivi immunitaire dans d'autres protocoles de l'équipe, j'ai également contribué comme co-auteur à la publication de plusieurs articles sur le sujet. N'étant pas en lien direct avec NLRP3 et MDSC, mais s'intégrant néanmoins au contexte de l'immunodépression induite par le sepsis, ces articles sont présentés en annexe par ordre chronologique :

- **Annexe 1 : Immune monitoring of interleukin-7 compassionate use in a critically ill COVID-19 patient**, Monneret G, de Marignan D, Coudereau R, Bernet C, Ader F, Frobert E, Gossez M, Viel S, Venet F, Wallet F, *Cell Mol Immunol.* 2020 Sep ; 17(9) : 1001-1003
- **Annexe 2 : Monocyte HLA-DR Measurement by Flow Cytometry in COVID-19 Patients: An Interim Review**, Benlyamani I, Venet F, Coudereau R, Gossez M, Monneret G, *Cytometry A.* 2020 Dec;97(12):1217-1221
- **Annexe 3 : Characterization of Circulating IL-10-Producing Cells in Septic Shock Patients: A Proof of Concept Study**, Fabri A, Kandara K, Coudereau R, Gossez M, Abraham P, Monard C, Cour M, Rimmelé T, Argaud L, Monneret G, Venet F, *Front Immunol.* 2021 Feb 4;11:615009
- **Annexe 4 : Monocyte CD169 expression in COVID-19 patients upon intensive care unit admission**, Ortillon M, Coudereau R, Cour M, Rimmelé T, Godignon M, Gossez M, Yonis H, Argaud L, Lukaszewicz AC, Venet F, Monneret G, *Cytometry A.* 2021 May ; 99(5) : 466-471
- **Annexe 5 : Longitudinal assessment of IFN-I activity and immune profile in critically ill COVID-19 patients with acute respiratory distress syndrome**, Venet F, Cour M, Rimmelé T, Viel S, Yonis H, Coudereau R, Amaz C, Abraham P, Monard C, Casalegno JS, Brengel-Pesce K, Lukaszewicz AC, Argaud L, Monneret G; RICO study group, *Crit Care.* 2021 Apr 12;25(1):140.
- **Annexe 6 : Recombinant human interleukin-7 reverses T cell exhaustion ex vivo in critically ill COVID-19 patients**, Bidar F, Hamada S, Gossez M, Coudereau R, Lopez J, Cazalis MA, Tardiveau C, Brengel-Pesce K, Mommert M, Buisson M, Conti F, Rimmelé T, Lukaszewicz AC, Argaud L, Cour M, Monneret G, Venet F ; RICO Study Group, *Ann Intensive Care.* 2022 Mar 5;12(1):21
- **Annexe 7 : T cell response against SARS-CoV-2 persists after one year in patients surviving severe COVID-19**, Venet F, Gossez M, Bidar F, Bodinier M, Coudereau R, Lukaszewicz AC, Tardiveau V, Brengel-Pesce K, Cheynet V, Cazalis MA, Pescarmona R, Garnier L, Ortillon M, Buisson M, Bouscambert-Duchamp M, Morfin-Sherpa F, Casalegno JS, Conti F, Rimmelé T, Argaud L, Cour M, Saadatian-Elahi M, Henaff L, Vanhems P, Monneret G; RICO study group, *EBioMedicine.* 2022 Apr;78:103967
- **Annexe 8 : L'inflammasome NLRP3 dans la physiopathologie des infections virales Un focus sur la COVID-19**, Chemarin M, Dufies O, Mazet A, Mellan E, Coudereau R, F Py B, Boyer L, Venet F, *Med Sci (Paris).* 2022 Jun-Jul ; 38(6-7) : 545-552

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## RÉSULTATS

## Résultats

Article 1 : Emergence of immunosuppressive LOX-1+ PMN-MDSC in septic shock and severe COVID-19 patients with acute respiratory distress syndrome (J Leukoc Biol. 2022 Feb ; 111(2) : 489-496)

### Contexte et objectif

Les MDSCs (*myeloid-derived suppressor cells*) représentent une population hétérogène de cellules myéloïdes immatures aux propriétés immunosuppressives, difficilement caractérisable en sang total. Leur exploration imposait une étape pré-analytique de séparation (Ficoll) peu compatible avec des études de recherche clinique. En cancérologie, LOX-1 (récepteur de surface) a été identifié comme marqueur spécifique des MDSCs identifiable en sang total.

L'objectif de cette étude était d'objectiver la présence, en sang total, de cellules MDSCs LOX-1 positive dans les sepsis d'origine bactérienne et virale (COVID-19), d'évaluer leurs propriétés immunosuppressives et d'en faire le monitoring pendant l'hospitalisation en réanimation.

### Principaux résultats

Les cellules LOX-1+ ont été caractérisées sur le plan phénotypique, morphologique et fonctionnel dans le sang total de 39 patients atteints de COVID-19 et 48 patients en choc septique bactérien hospitalisés en réanimation. Des analyses ont été réalisées sur une période de 3 semaines dans les unités de soins intensifs et comparés à 22 volontaires sains inclus

Les polynucléaires neutrophiles LOX-1<sup>+</sup> présentaient un phénotype immature (diminution d'expression des CD10 et CD16, morphologie caractérisée par un noyau réniforme peu segmenté). En co-culture avec des lymphocytes T, ces cellules étaient capables de diminuer la production d'interféron  $\gamma$ . Ainsi, les polynucléaires neutrophiles LOX-1<sup>+</sup> présentaient les principales caractéristiques de MDSCs granulocytaires.

Chez les patients, nous avons observé une émergence significative de MDSCs LOX-1+. Le pic de MDSCs LOX-1<sup>+</sup> était retardé d'une semaine par rapport à l'admission en réanimation. Chez les patients atteints de COVID-19, leur élévation était plus marquée chez ceux présentant un syndrome de détresse respiratoire aiguë (SDRA).

### Conclusion

L'émergence progressive au cours de la première semaine d'hospitalisation en réanimation de polynucléaires neutrophiles LOX-1<sup>+</sup> présentant un phénotype, une morphologie et une fonction MDSCs a été détectée pour la première fois chez des patients présentant un sepsis, quelle que soit son étiologie (i.e., virale ou bactérienne). Chez les patients atteints de COVID-19, leur augmentation était plus prononcée chez les patients présentant un SDRA. De plus, nous avons démontré la faisabilité de la mesure des MDSCs LOX-1<sup>+</sup> en sang total par cytométrie en flux, ce qui devrait faciliter les études en recherche clinique à plus grande échelle.



# Emergence of immunosuppressive LOX-1+ PMN-MDSC in septic shock and severe COVID-19 patients with acute respiratory distress syndrome

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## Abstract

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of immature myeloid cells with immunosuppressive properties. In cancer patients, the expression of lectin-type oxidized LDL receptor 1 (LOX-1) on granulocytic MDSC identifies a subset of MDSC that retains the most potent immunosuppressive properties. The main objective of the present work was to explore the presence of LOX-1+ MDSC in bacterial and viral sepsis. To this end, whole blood LOX-1+ cells were phenotypically, morphologically, and functionally characterized. They were monitored in 39 coronavirus disease-19 (COVID-19, viral sepsis) and 48 septic shock (bacterial sepsis) patients longitudinally sampled five times over a 3 wk period in intensive care units (ICUs). The phenotype, morphology, and immunosuppressive functions of LOX-1+ cells demonstrated that they were polymorphonuclear MDSC. In patients, we observed the significant emergence of LOX-1+ MDSC in both groups. The peak of LOX-1+ MDSC was 1 wk delayed with respect to ICU admission. In COVID-19, their elevation was more pronounced in patients with acute respiratory distress syndrome. The persistence of these cells may contribute to long lasting immunosuppression leaving the patient unable to efficiently resolve infections.

## KEYWORDS

flow cytometry, HLA-DR, immunosuppression, sepsis

**Abbreviations:** ARDS, acute respiratory distress syndrome; COVID-19, coronavirus disease-19; HV, healthy volunteers; LOX-1, Lectin-type oxidized LDL receptor 1; MDSC, Myeloid-derived suppressor cells; mHLA-DR, monocyte HLA-DR; M-MDSC, Monocyte myeloid-derived suppressor; PMN-MDSC, Polymorphonuclear myeloid-derived suppressor cells; SAPS II, Simplified acute physiology score; SOFA, Sepsis-related organ failure assessment score

## 1 | INTRODUCTION

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of immature myeloid cells with immunosuppressive properties as they are potent repressors of T-cell responses.<sup>1</sup> Two main subtypes of MDSC are currently described.<sup>2</sup> Polymorphonuclear MDSC (PMN-MDSC), containing granulocytic progenitors, are phenotypically and morphologically similar to neutrophils. Monocytic MDSC (M-MDSC), containing monocytic progenitors, are phenotypically and morphologically similar to monocytes. Early MDSC are also described, only in mice. They are mostly immature and do not express any lineage markers.<sup>3</sup> MDSC have been first recognized for their role in attenuating immune surveillance and antitumor immune response in various cancer conditions.

More recently, MDSC have been reported to be involved in other clinical contexts including acute and chronic inflammation.<sup>4</sup> The salient feature of MDSC in these conditions is their ability to inhibit T cell function and thus to contribute to the occurrence of subsequent immunosuppression. As an illustrative example, sepsis, defined as a life-threatening organ dysfunction caused by a dysregulated inflammatory host response to infection,<sup>5</sup> is characterized by a delayed step of immunosuppression.<sup>6</sup> Recent studies have demonstrated the association between elevated circulating MDSC (both PMN- and M-MDSC) and increased and/or higher risk of nosocomial infections during sepsis.<sup>7-9</sup> Interestingly, the most severe cases of coronavirus disease-19 (COVID-19, caused by SARS-CoV-2) are viral sepsis by virtue of international definition (i.e., infection and one organ failure that is acute respiratory failure). Once COVID-19 patients develop acute respiratory distress syndrome (ARDS), they similarly present with features of immunosuppression: altered type-I IFN signaling,<sup>10</sup> decreased monocyte HLA-DR (mHLA-DR),<sup>11</sup> lymphopenia,<sup>12</sup> and altered lymphocyte functions.<sup>13</sup> A couple of recent articles reported on the early emergence of PMN-MDSC especially in the most severe COVID-19 patients.<sup>14,15</sup>

Although there is a growing interest in monitoring MDSC in clinical practice, the lack of a specific phenotypic marker usable in whole blood protocol renders their assessment difficult on a routine basis due to the necessity to perform cell purification before staining. This is especially true for PMN-MDSC, which are investigated among PBMCs after isolation by density gradient centrifugation.<sup>3</sup> In 2016, Condamine et al. reported that the expression of lectin-type oxidized LDL receptor 1 (LOX-1) identified a subset of MDSC that retained the most potent immunosuppressive properties.<sup>16</sup> Importantly, LOX-1 staining can be performed in whole blood.<sup>16</sup> Since this pioneering description, LOX-1 expression in MDSC have been confirmed in few human cancer studies.<sup>17,18</sup>

In this context, the question of the emergence of LOX-1+ PMN-MDSC in bacterial and viral sepsis remained unsolved. The main objective of the present work was thus to highlight the presence of these cells in two etiologies of sepsis, that is, bacterial and viral (COVID-19)

and to verify that these cells identified in whole blood possessed all the characteristics of MDSC.

## 2 | MATERIALS AND METHODS

### 2.1 | Study population

This clinical study was conducted on patients admitted to the intensive care unit (ICU) of Edouard Herriot Hospital (Hospices Civils de Lyon, Lyon, France). This project is part of two prospective observational studies: IMMUNOSEPSIS (on septic shock patients), and RICO (REA-IMMUNO-COVID on COVID-19 ICU patients). Extended details are provided in supplemental material.

### 2.2 | Whole blood phenotyping

Fresh peripheral blood was collected using EDTA anticoagulant tubes. A total of 100  $\mu$ l of whole blood was labelled with antibodies: CD45-KrO/CD10-PC7/CD16-PB (Beckman Coulter, Brea, CA, USA), CRTH2-APC (Miltenyi, Bergisch Gladbach, Germany), CD15-AF700, and LOX-1-PE or mouse IgG2a isotype control (Biolegend, San Diego, CA, USA) and incubated for 15 min at room temperature in the dark. Cells were then incubated with 1 m of lysing solution (Versalyse, Beckman Coulter) for 10 min at room temperature in the dark and washed with PBS before being analyzed by flow cytometry. Samples were run on a Navios flow cytometer (Beckman Coulter). Calibration beads (Flow Check and Flow Set, Beckman Coulter) were run daily to check for routine alignment, day-to-day stability, and long-term performance validation. Gating strategy involved a first step of PMN selection on a biparametric CD45/side scatter histogram. Then, eosinophils were excluded from gated PMN according to CRTH2 expression. LOX-1 expression was finally evaluated on the whole neutrophil population (positivity threshold was based on isotype values set up at 0.5%). Results were expressed as percentage of LOX-1 positive cells among neutrophils. Maturity of PMN or PMN-MDSC was also assessed based on the expression of CD10 and CD16.

### 2.3 | Cell isolation

Fresh PBMCs were isolated from whole blood by Ficoll density gradient centrifugation. For coculture and microscopy (May-Grünwald-Giemsa staining performed by an SP10 automatic system, Sysmex), MDSC were sorted by flow cytometry on a FACSAria II flow cytometer (BD Bioscience, San Jose, CA, USA). The gating strategy for cell sorting experiments was to select CD45dim LOX1+ cells for PMN-MDSC and CD45+ LOX1- cells for regular neutrophils among whole CD15+ neutrophil population (>90% purity). Viability of sorted cells was assessed using propidium iodide staining (>90% viability). For coculture, T cells

were isolated from healthy volunteers (HV) samples by antibody-based negative selection and Ficoll density gradient centrifugation using a human T-cell enrichment cocktail (Rosette Sep, StemCell Technologies, Grenoble, France), as described in the manufacturer's instructions. T cell purity was assessed by flow cytometry (cells were labelled with anti-CD3-APC [Beckman Coulter]).

## 2.4 | Suppression assay

Freshly purified T cells were stimulated with anti-CD2/3/28 coated-beads (T cell Activation/Expansion kit, 2 beads per T cell, Biolegend, Bergisch Gladbach, Germany) and cultured alone or cocultured with heterologous CD45<sup>dim</sup> LOX1<sup>+</sup> or CD45<sup>+</sup> LOX1<sup>-</sup> PMN at a 1:1 ratio for 72 h at 37°C and 5% CO<sub>2</sub>. T cell functionality was assessed by measuring IFN- $\gamma$  production in culture supernatant (ELLA, Proteinsimple, San Jose, CA, USA), according to the manufacturer's instructions.

## 2.5 | Statistical analysis

Results regarding expression of CD45<sup>dim</sup>LOX1<sup>+</sup> cells in patients are presented as individual values and Tukey boxplots. For clinical parameters, continuous data and biologic parameters are presented as medians and interquartile ranges (IQR) whereas categoric data are presented as numbers of cases and percentages among total population (median and IQR). Results regarding IFN- $\gamma$  production of T cells in coculture are presented as means  $\pm$  SD. Nonparametric Mann-Whitney, Fisher, and  $\chi^2$  tests were used to assess differences between HV and patients or differences between viral and bacterial sepsis, and the nonparametric Wilcoxon paired test was used to assess variations between patients themselves at different time points. *P*-values lower than 0.05 were considered statistically significant.

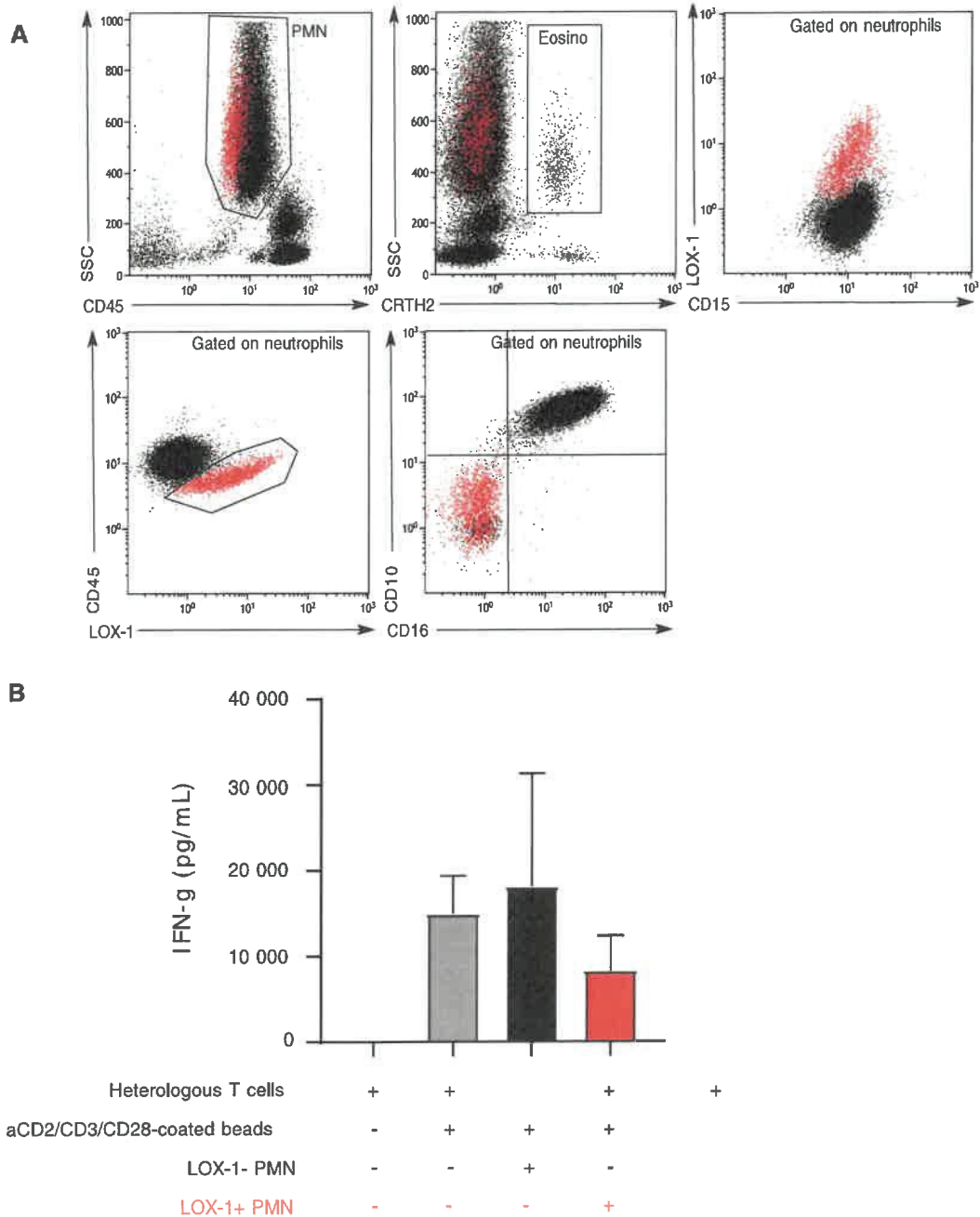
## 3 | RESULTS AND DISCUSSION

As a preliminary step, we first developed a whole blood protocol to assess LOX-1 expression on circulating neutrophils. As regularly performed in clinical flow cytometry, we used CD45 expression to draw a leukogate in order to exclude debris. Then, neutrophils were identified based on CD15 positivity and lack of CRTH2 (in order to exclude eosinophils). As depicted in Figure 1, we were able to identify a subset of neutrophils expressing LOX-1. These cells presented with diminished CD45 expression (Fig. 1A) and an immature phenotype as they did not express CD16 nor CD10 (Fig. 1A). We next performed LOX-1 staining after Ficoll purification on cells from both Ficoll ring and pellet. As expected, LOX-1<sup>+</sup> CD15<sup>+</sup> neutrophils were found in the Ficoll ring with PBMCs and were absent from the pellet (Supporting Information Fig. S1). We confirmed that these LOX-1<sup>+</sup> cells presented with low CD10 and CD16 expressions. In addition, after staining with May-Grünwald Giemsa, sorted CD45<sup>dim</sup> CD15<sup>+</sup> LOX-1<sup>+</sup> population appeared as neutrophil precursors presenting with

immature morphology (i.e., poorly segmented reniform nucleus). In contrast, CD45<sup>+</sup> LOX-1<sup>-</sup> cells (found in the pellet) appeared as mature neutrophils exhibiting a well-segmented nucleus (Supporting Information Fig. S1). In order to further qualify LOX-1<sup>+</sup> neutrophils as MDSC, we next investigated their suppressive functions.<sup>2</sup> Due to the limited biologic resources (i.e., small amount of available blood from severely ill ICU patients), we assessed inhibition of IFN- $\gamma$  production by T cells that require fewer cells than proliferation assay. To this end, CD15<sup>+</sup> CD45<sup>dim</sup> LOX-1<sup>+</sup> and CD15<sup>+</sup> CD45<sup>+</sup> LOX1<sup>-</sup> cells were sorted and cocultured with heterologous T cells from healthy donors. After 72 h of culture, IFN- $\gamma$  release was measured in culture supernatant. Although addition of regular neutrophils (CD15<sup>+</sup> CD45<sup>+</sup> LOX-1<sup>-</sup>) had no effect on IFN- $\gamma$  release by stimulated T-cells, coculture with CD15<sup>+</sup> CD45<sup>dim</sup> LOX-1<sup>+</sup> cells induced a fall in IFN- $\gamma$  production (Fig. 1B). Overall, our results identified a subpopulation of CD15<sup>+</sup> CRTH2<sup>-</sup> CD10<sup>low</sup> CD16<sup>low</sup> CD45<sup>dim</sup> LOX-1<sup>+</sup> neutrophils, which presented with immature morphology, low-density characteristics, and possessed immunosuppressive properties. This agreed with first report on LOX-1 cells by Gabrilovich's group<sup>16</sup> and identified these cells as PMN-MDSC. This allowed use of this phenotype in clinical studies for the whole blood phenotyping of MDSC. Thereafter, those cells will be named LOX-1 PMN-MDSC.

Forty-eight septic shock patients, 39 ICU COVID-19 patients, and 22 HV were included. Clinical and biologic characteristics are listed in Table 1. Overall, patients mostly presented with low mHLA-DR values and lymphopenia reflecting an immunosuppression state as usually described in sepsis.<sup>6</sup> Upon admission, patients with bacterial septic shock did not exhibit increased percentage LOX-1 PMN-MDSC in comparison with HV (median = 0.15% [0.04–0.38] vs. 0.165% [0.063–0.25], respectively) (Fig. 2A). Afterward, percentage LOX-1 PMN-MDSC significantly increased at D3 (0.26% [0.13–0.87]; *P* = 0.021) with a peak at D6 (3.10% [1.02–6.58] *P* = 0.003). D6 values were significantly higher than those observed at D1 (*P* = 0.003) and D3 (*P* = 0.005). On patient's discharge from ICU, percentage LOX-1 PMN-MDSC decreased (0.43% [0.15–0.73]) without returning to completely normal values. Indeed, discharge values were still significantly higher than admission values (*P* = 0.004). Emergence of LOX-1 PMN-MDSC was also observed in COVID-19 patients (Fig. 2B). At each time point, values were significantly higher than HV, even at patients' ICU admission (0.73% [0.47–1.94]; *P* = 0.007). Values of CD45<sup>dim</sup>LOX1<sup>+</sup> MDSC then rose up at D3 (2.83% [0.65–5.14]; *P* = 0.003) and reached a plateau at D6 (2.71% [1.52–5.47]; *P* = 0.009). The last day of their follow-up, patients still had significantly higher LOX-1 PMN-MDSC values than HV (1.47% [0.745–3.450]; *P* = 0.007). Last, COVID-19 patients were analyzed in regard with the development or not of ARDS within the first 3 d after ICU admission. COVID-19 patients who developed ARDS showed a significant elevation of LOX-1 PMN-MDSC values between the first two samples (*P* = 0.002), whereas LOX-1 PMN-MDSC did not rise in patients without ARDS (Fig. 2C).

Sepsis, which by international definition is defined as an organ dysfunction due to a dysregulated host response to an infection,<sup>19</sup> is characterized by the occurrence of delayed immunosuppression that associates with poor outcome and nosocomial infections.<sup>6,20</sup>



**FIGURE 1** Lectin-type oxidized LDL receptor 1 (LOX-1) neutrophils characteristics. (A) Representative example of CD45<sup>dim</sup> LOX-1 phenotypic characterization in septic shock patient. Polymorphonuclear cells (PMN) were first gated out from other cells on a biparametric CD45/side scatter histogram. Then, eosinophils were excluded from gated PMN on the expression of CRTH2. LOX-1 expression was finally evaluated on whole neutrophil population (positivity threshold was defined based on isotype values set up at 0.5%). LOX-1 expression is either represented among CD15<sup>+</sup> cells or CD45<sup>+</sup> cells. Red cells represent CD45<sup>dim</sup> LOX-1<sup>+</sup> cells gated on the biparametric CD45/LOX1 dotplot. CD10 and CD16 expressions gated on the CD45<sup>dim</sup> LOX-1 cells is also represented to illustrate the immature nature of these cells. (B) LOX-1<sup>+</sup> MDSC (myeloid-derived suppressor cells) immunosuppressive function on T cell IFN $\gamma$  release. IFN- $\gamma$  concentrations in the supernatants of T cells purified from healthy volunteers (HV) were measured ( $n = 3$  independent experiments). T cells were either incubated alone, with stimulation beads, or cocultured at 1:1 ratio with CD45<sup>+</sup> LOX-1<sup>-</sup> or CD45<sup>dim</sup> LOX-1<sup>+</sup> MDSC purified from 3 septic shock patients. Data are presented as means  $\pm$  SD

**TABLE 1** Demographic, clinical, and immunologic data for coronavirus disease-19 (COVID-19) and septic shock patients

Parameters	COVID-19 (n = 39)	Septic shock (n = 48)	P-value
Age (years)	65 [59–71]	72 [55–78]	0.3
Gender—male, n (%)	27 (69)	25 (52)	0.5
McCabe score			0.07
0, n (%)	36 (92)	34 (71)	
1, n (%)	3 (8)	13 (27)	
2, n (%)	0 (0)	1 (2)	
Delay between first symptoms (days)	8 [5–9]	NA	
SAPS II score	33 [28–40]	54 [47–70]	<0.001
SOFA score	4 [3–5]	9 [7–12]	<0.001
Respiratory dysfunction (COVID-19)			
Mechanical ventilation, n (%)	34 (87%)	NA	
PaO <sub>2</sub> /FiO <sub>2</sub> ratio on admission (mmHg)	108 [90–158]	NA	
Site of infection, n (%)			<0.001
Pulmonary	39 (100)	9 (19)	
Abdominal	0 (0)	16 (33)	
Other	0 (0)	23 (48)	
Follow-up			
28 d nonsurvivors, n (%)	3 (8)	7 (15)	0.03
Secondary nosocomial infections, n (%)	18 (46)	13 (27)	0.003
Immunologic parameters			
Monocyte HLA-DR (mHLA-DR; AB/C)	8344 [5986–8458]	5440 [3622–8169]	0.046
Absolute CD4+ T cell count (cell/ $\mu$ l)	319 [169–398]	367 [274–570]	0.05

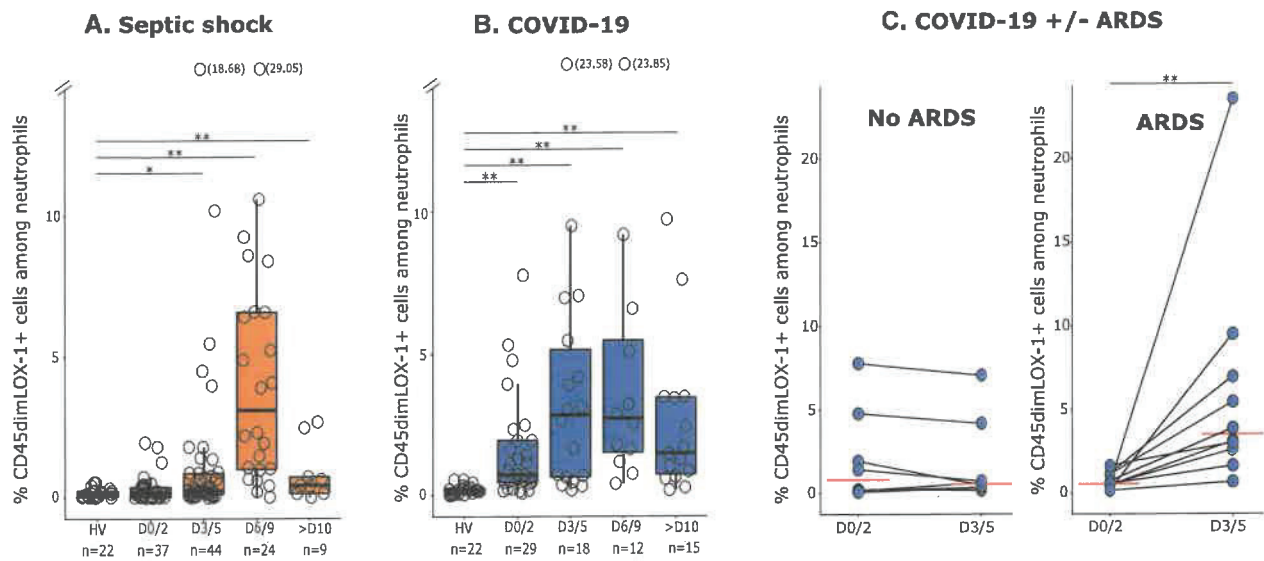
Continuous data and biologic parameters are presented as medians and interquartile ranges [Q1–Q3]. For clinical parameters, categorical data are presented as numbers of cases and percentages among the total population in brackets. SAPS II (simplified acute physiology score II) and McCabe scores were calculated at admission. SOFA (sequential organ failure assessment) score was measured during the first 24 h after ICU admission. Absolute CD4+ T cell count was calculated on day 3 as well as mHLA-DR, expressed as numbers of anti-HLA-DR antibodies bound per monocyte (AB/C). Reference values from our lab: mHLA-DR: > 15 000 ABC, CD4+: 336–1126 cells/ $\mu$ l. P-values: nonparametric Mann-Whitney, Fisher, and  $\chi^2$  tests when appropriate.

In this condition, MDSC emergence has been described and is reported to contribute to mechanisms sustaining this state of immunosuppression.<sup>7,21</sup> Although mainly due to bacterial and fungal etiologies, viral origin is also a cause for sepsis. COVID-19 is an illustrative example of viral sepsis because patients infected by SARS-CoV-2 may develop (in worst cases) ARDS (that is a major pulmonary dysfunction). Interestingly, COVID-19 patients also present with marked immunosuppression characterized by severe lymphopenia, decreased mHLA-DR expression, or increased IL-10 concentrations.<sup>11,22–24</sup>

In addition to lymphopenia, T cell altered functionality, and decreased mHLA-DR, the current results illustrate that LOX-1+ MDSC occurrence constitutes another similarity between sepsis and cancer.<sup>25</sup> Considering their potent immunosuppressive properties (T cell inhibition, arginine depletion, reactive oxygen species synthesis, production of PGE<sub>2</sub>, and anti-inflammatory cytokines), it is reasonable to speculate on their participation in septic patients worsening due to immunosuppression. In line, their elevation is a marker of severity associated with occurrence of ARDS.

Another striking result of the present study is the similar rise of these immunosuppressive cells in bacterial and viral sepsis and the delayed maximal appearance of LOX-1+ MDSC because they peaked approximately 1 wk after ICU admission in both etiologies of sepsis. This agrees with previous results showing that decreased mHLA-DR, observed in septic shock and COVID-19 patients, is associated with unfavorable outcomes in a delayed manner, that is, from day 4 after septic shock.<sup>6,20</sup> Over days persistence of low mHLA-DR predicted nosocomial infections occurrence as well as mortality,<sup>8,26,27</sup> whereas inaugural values did not. Here, at ICU discharge, patients still presented elevated LOX-1+ MDSC values. Importantly, despite constant improvements in the management of patients, the long-term mortality in sepsis has remained dramatically high.<sup>28</sup> Increased mortality may last few years after sepsis.<sup>29</sup> The first cause of rehospitalization and death after sepsis are infectious diseases evoking a role for a long-term persistence of immunosuppression. This phenomenon is hypothesized to be due to persistent inflammation, immunosuppression, and catabolism syndrome, and/or chronic critical illness, defined as





**FIGURE 2** Monitoring of lectin-type oxidized LDL receptor 1 polymorphonuclear myeloid-derived suppressor cells (LOX-1+ PMN-MDSC) overtime in septic shock and coronavirus disease-19 (COVID-19) patients. Percentages of LOX-1+ PMN MDSC among total neutrophils were measured in peripheral blood from 22 healthy volunteers (HV) and (A) 48 septic shock patients (day 0/2,  $n = 37$ ; day 3/5,  $n = 44$ ; day 6/9,  $n = 24$ ; above day 10,  $n = 9$ ), or (B) 39 COVID-19 ICU patients (day 0/2,  $n = 29$ ; day 3/5,  $n = 18$ ; day 6/9,  $n = 12$ ; above day 10,  $n = 15$ ). Missing values corresponded to patients who died or left ICU before the end of the study and to missing samples during the weekends for which staining was not possible since lab was not operating 24/7. Data are presented as individual values and Tukey boxplots. (C) Acute respiratory distress syndrome (ARDS) in COVID-19. For COVID-19 ICU patients with paired samples at day 0/2 and day 3/5, kinetics of CD45dim LOX-1+ percentage in patients with ( $n = 10$ ) or without ( $n = 8$ ) ARDS was evaluated. Nonparametric Mann-Whitney test was used to assess differences between HV and patients. The nonparametric Wilcoxon paired test was used to assess variations between patients themselves at day 0/2 and day 3/5; \* $P < 0.05$ , \*\* $P < 0.01$

more than 14 d spent in ICU associated with organ dysfunction and immunosuppression. In both cases, chronic low-grade inflammation perpetuates long-term immunosuppression that in turn favors additional infectious events. In that respect, proinflammatory compounds such as alarmins (S100A9/S100A8, HMGB-1) are believed to induce MDSC proliferation/differentiation during late bacterial sepsis by promoting immune repressor functions in bone marrow.<sup>9,30–33</sup> Most importantly, S100A8 (also known as calgranulin) has been demonstrated to be an important early maker of severity in COVID-19 patients.<sup>34</sup> We can thus speculate that this nonresolving inflammation observed in septic shock and COVID-19 patients, which is also a feature of various cancers may represent a common mechanism leading to the induction of MDSC known to lower immune surveillance.<sup>25</sup>

Nevertheless, our study has limitations. Immunosuppressive abilities of LOX-1+ MDSC may be confirmed with additional functional testing such as lymphocyte proliferation. The relatively small sample size of the cohort (less than 50 patients in each cohort) did not allow us to deeply study the relationships with clinical outcomes. However, considering the previously described roles of these cells in cancer and their kinetics in sepsis (i.e., peak that synchronously occurs during secondary immunosuppressive step of the disease), we may hypothesize that LOX-1+ MDSC participate in a deleterious immunosuppressive process.<sup>7,21,35–37</sup> Further larger studies are needed to confirm our preliminary results.

To conclude, emergence of LOX-1+ neutrophils with proven MDSC phenotype, morphology, and function was detected for the first time

in septic patients, whatever sepsis origin. In COVID-19, their elevation was more pronounced in patients with ARDS. The peak of LOX-1+ MDSC was delayed with respect to ICU admission. In addition, we demonstrated the feasibility of LOX-1+ MDSC measurement in whole blood by flow cytometry, which should facilitate larger clinical research studies. Upon demonstration of a causative and deleterious role of MDSC in sepsis-induced immunosuppression leaving the patient unable to effectively resolve infections, the repositioning of various drugs proved to be efficient as adjunctive anticancer agents by tackling MDSC could appear as a realistic therapeutic option.<sup>38</sup> This is all the more true because, in contrast to other putative immunoregulatory targets (regulatory T cells, inhibitory checkpoint molecules), MDSC are not present (or at very low concentration) in steady-state conditions providing thus a unique opportunity to act on immune response while minimizing side effects on immune homeostasis.<sup>38</sup>

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#### AUTHORSHIP

R.C., L.W., R.P., L.J. and A.F. performed the experiments. R.C., F.V., M.G. and G.M. wrote the report and designed the experiments. M.C., T.R., H.Y., A-C.L. and L.A. were involved with selection of the patients. All authors discussed the data, drafted or critically revised the manuscript for important intellectual content, have accepted responsibility for the entire content of this submitted manuscript, and have approved its submission.

#### DISCLOSURES

The authors declare no conflicts of interest.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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**Emergence of immunosuppressive LOX-1+ PMN-MDSC in septic shock  
and severe COVID-19 patients with acute respiratory distress syndrome**

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**Material and Methods**

*Study population*

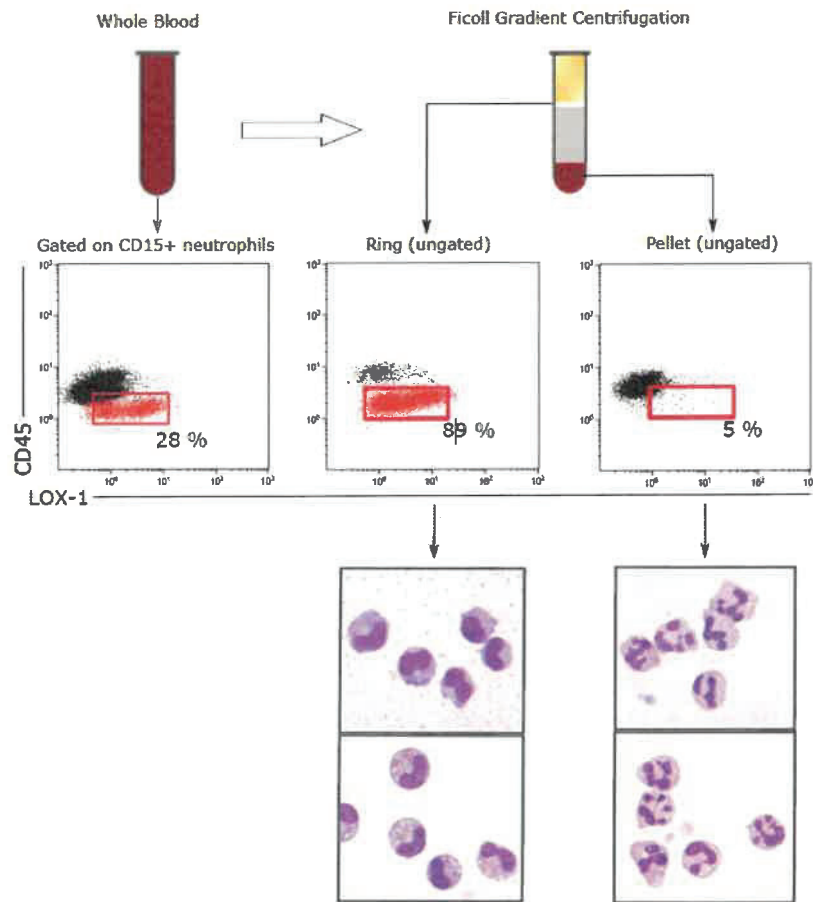
This clinical study was conducted on patients admitted to the intensive care unit (ICU) of Edouard Herriot Hospital (Hospices Civils de Lyon, Lyon, France). This project is part of 2 prospective observational studies: IMMUNOSEPSIS (on septic shock patients), and RICO (REA-IMMUNO-COVID on COVID-19 ICU patients). Regarding IMMUNOSEPSIS cohort, septic shock patients were identified according to the diagnostic criteria of the Third International Consensus Definitions for Sepsis and Septic Shock. Briefly, septic shock diagnosis was based on the combination of an identifiable site of infection, evidence of a systemic inflammatory response, vasopressor therapy needed to elevate mean arterial pressure  $\geq 65$  mmHg despite adequate fluid resuscitation and lactate  $>2$  mmol/L (18 mg/dL). Patients were excluded if under 18 years of age or presented with aplasia or pre-existent immunosuppression. The Sepsis-related Organ Failure Assessment score (SOFA) (1) and the Simplified Acute Physiology Score (SAPS II) (2) were used to evaluate the presence of organ dysfunctions and initial severity respectively. Clinical and biological parameters were collected, including demographic characteristics, date and cause of ICU admission, status at day 28 after inclusion, type of infection. This project was approved by Institutional Review Board for ethics ("Comité de

Protection des Personnes Ouest II”, n° RCB: 2019-A00210-57). This study is registered at the French Ministry of Research and Teaching (#DC-2008-509), at the Commission Nationale de l’Informatique et des Libertés, and on clinicaltrials.gov (NCT02803346). Regarding RICO cohort, COVID-19 ICU patients over 18 years with a confirmed diagnosis of SARS-CoV2 infection by RT-PCR testing in one respiratory sample were enrolled in the study. This project was approved by Institutional Review Board for ethics (“Comité de Protection des Personnes Ile de France 1”, n° RCB: 2020-A01079-30). These studies are registered at the French Ministry of Research and Teaching (#DC-2008-509), at the Commission Nationale de l’Informatique et des Libertés, and on clinicaltrials.gov (NCT04392401). Oral information and non-opposition to inclusion in the study were mandatory and recorded in patients’ clinical files for both studies. Peripheral blood from healthy volunteers (HV) was provided by the “Etablissement Français du Sang (EFS)” from Lyon. According to EFS standardized procedures for blood donation and to provisions of the article R.1243–49 and following ones of the French public health code, a written non-opposition to the use of donated blood for research purposes was obtained from HV. The blood donors’ personal data were anonymized before transfer to our research laboratory.

### **Supplemental references**

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## Legend to supplemental figures



**Supplemental Figure 1. Phenotypic and morphologic characterization of CD15+CD45dim LOX-1+ cells after density gradient centrifugation in septic shock patients.** LOX-1 stainings were simultaneously performed in whole blood and after Ficoll density gradient centrifugation in cells included in the ring and pellet obtained from the same sample. One representative example in a sample from septic shock patients is shown. In addition, CD15+CD45+ LOX1- and CD15+CD45dim LOX-1+ cells were sorted on a FACS Aria II flow cytometer, stained with May-Grunwald-Giemsa staining and analysed under the microscope.



Article 2 : Persistent NLRP3 inflammasome activation is associated with delayed MDSCs in septic patients  
(Article soumis pour publication)

Contexte et objectif

Après avoir développé un protocole permettant l'étude prospective des MDSCs en clinique, l'objectif de cette étude était d'évaluer au cours du temps l'association entre l'activation de l'inflammasome NLRP3 et les altérations immunitaires, en particulier l'émergence de cellules suppressives dérivées de la moelle osseuse (MDSCs), chez les patients septiques.

Dans une cohorte prospective de patients septiques présentant différentes étiologies (n=17 choc septique, n=15 COVID-19), nous avons évalué les médiateurs solubles de l'activation du NLRP3 inflammasome (IL-1 $\beta$ ; IL-18 et récepteurs solubles) ainsi que les marqueurs cellulaires de l'immunosuppression (LOX-1 PMN-MDSCs, neutrophiles immatures et mHLA-DR). Nous avons également réalisé une analyse transcriptomique des ARNm liés à l'inflammasome NLRP3 combinée à des cytokines et au phénotype cellulaire sur une cohorte plus large de 107 patients septiques pour confirmer nos résultats.

Principaux résultats

Dans la cohorte prospective, nous avons observé une activation marquée et prolongée de l'inflammasome NLRP3 ainsi que l'établissement d'un état d'immunosuppression (mHLA-DR diminué, lymphopénie T), ainsi qu'une augmentation progressive des MDSC LOX-1<sup>+</sup>. Les marqueurs de l'activation de la voie de NLRP3 (concentrations plasmatiques d'IL-18 et d'IL-1R2) ont été corrélés avec les marqueurs d'immunosuppression (LOX-1 PMN-MDSC, IL-10, faible mHLA-DR). L'analyse transcriptomique de la cohorte rétrospective a confirmé l'association entre la voie NLRP3 dérégulée et les altérations immunitaires retardées. Plus précisément, deux trajectoires distinctes ont été définies parmi les patients, avec un groupe exprimant une réponse inflammatoire initiale excessivement élevée qui a persisté au-delà de la période de suivi de 28 jours, associée à une immunosuppression marquée et persistante ainsi qu'à une mortalité significativement plus élevée à 28 jours.

Conclusion

À notre connaissance, la présente étude clinique objective pour la première fois au cours du sepsis, une association entre une activation persistante de l'inflammasome NLRP3 et une immunosuppression. Cette association était associée à une augmentation de la mortalité à 28 jours. L'identification d'endotypes présentant des trajectoires immuno-inflammatoires distinctes pourrait avoir des implications cliniques majeures en termes de thérapeutique individualisée.





Persistent NLRP3 inflammasome activation is associated with delayed LOX-1 myeloid derived suppressor cells in septic patients

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Running title: Acute and persistent NLRP3 inflammasome activation is associated with immunosuppression in septic patients

Keywords: Sepsis, COVID-19, inflammation, NLRP3 inflammasome, MDSCs, immunosuppression

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## **Abstract**

**Background:** Sepsis represents a significant health challenge, often leading to life-threatening complications. It triggers a complex response marked by the simultaneous presence of pro-inflammatory and anti-inflammatory/immunosuppressive elements, disrupting the mechanisms intended to maintain homeostasis. While the NLRP3 inflammasome has been implicated as a major contributor to the inflammatory aspect of this process, its connection with delayed sepsis-induced immunosuppression remains unclear.

**Methods:** We conducted a prospective study involving septic shock patients (n=17) and COVID-19 ICU patients (n=15) to investigate the association between NLRP3 inflammasome activation-related inflammation and induced immunosuppression. We measured various inflammatory markers and assessed LOX-1 MDSC and other cellular parameters using flow cytometry. To validate our findings, we conducted a transcriptomic study on an additional cohort of 107 patients.

**Results:** Septic patients exhibited both a hyper-inflammatory response and immune suppression. NLRP3 inflammasome activation markers correlated with delayed immunosuppression (as measured on day 7). The transcriptomic analysis highlighted elevated mRNA levels of NLRP3-associated genes and revealed two distinct endotypes. One cluster displayed inflammation rapidly returning to normal values, while the other exhibited a higher inflammatory response persisting beyond the 28-day follow-up period. This latter cluster was characterized by persistent marked immunosuppression and higher 28-day mortality.

**Conclusions:** Our study demonstrates that the activation of the NLRP3 inflammasome and its persistence were associated with marked immunosuppression and increased mortality rates. Identifying endotypes with different pro-/anti-inflammatory trajectories could hold important clinical implications for the management of sepsis.



## Introduction

Based on the third international consensus definitions for sepsis and septic shock, sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection [1]. Despite decades of research and multicenter trials, no efficacious therapy against sepsis is yet available, and the overall mortality remains high: 24.4% to 26.7% for sepsis [2] and 34.7% to 44.5% for septic shock [2,3]. The disease course is complex and heterogeneous. It is now recognized that activation of both pro- and anti-inflammatory pathways occurs simultaneously in septic patients [4,5].

The initial acute inflammatory response is characterized by the massive release of various pro-inflammatory mediators that may lead to organ failure and death. Experimental studies have highlighted the crucial role of the NACHT, leucine-rich repeat and pyrin-containing receptor 3 (NLRP3) inflammasome in sepsis pathophysiology [6], responsible for IL-1 $\beta$  and IL-18 secretions, both potent inflammatory cytokines.

In parallel, regulatory mechanisms are initiated to reduce the excessive pro-inflammatory response. In some patients, this leads to the development of an immunosuppression associated with increased nosocomial infections and viral reactivations [7], responsible for delayed mortality. Mechanisms sustaining this sepsis-induced immunosuppression include cellular anergy, lymphopenia, the release of immunosuppressive mediators or the over-expression of inhibitory ligands [4,8]. Of them, the occurrence of myeloid-derived suppressor cells (MDSCs) has emerged as a potential mechanism hypothesized to be associated with increased mortality and/or nosocomial infections [9–13].

In other contexts, a link has been established between NLRP3 inflammasome activation and immunosuppression, particularly the development of MDSCs. In cryopyrin-associated periodic syndromes (CAPS), caused by gain-of-function mutations in the NLRP3 gene leading to the overproduction of IL-1 $\beta$  and other NLRP3 inflammasome products, patients showed significantly elevated MDSCs [14]. In cancer patients, few studies have underscored the

association between NLRP3 inflammasome and the development MDSCs, which in turn has been associated with decreased overall survival [15,16]. Of interest, cancer and infectious diseases share similarities regarding the occurrence of induced immunosuppression [17]. In septic patients, while data linking NLRP3 inflammasome components (NLRP3, ASC, caspase-1) or its products (IL-1 $\beta$ , IL-18) to mortality are available, no comprehensive study established a direct link between NLRP3 inflammasome activation and sepsis-induced immunosuppression [18–24]. Therefore, we hypothesize that acute or chronic inflammation due to NLRP3 inflammasome activation may contribute to the immune alterations observed in sepsis, specifically the emergence of MDSCs.

The objective of this study was to examine concomitantly the initial and persistent inflammation triggered by NLRP3 inflammasome activation and the profound delayed immunosuppression observed in septic patients, with a focus on MDSCs appearance. To comprehensively investigate the kinetics of MDSCs, we enrolled septic patients with both bacterial and viral infections in a prospective manner. In order to validate the observed associations, we subsequently conducted a retrospective analysis using a large cohort of septic patients (REALISM study), in which we established immune trajectories and identified clusters that associated increased NLRP3 markers with indicators of immunosuppression.

## **Methods**

### **Prospective cohorts**

Patients admitted to intensive care unit (ICU) of Edouard Herriot Hospital (Hospices Civils de Lyon, Lyon, France) were included in two prospective observational studies: RICO (REA-IMMUNO-COVID) [25] and IMMUNOSEPSIS4. Regarding RICO cohort, COVID-19 ICU patients over 18 years with a confirmed diagnosis of SARS-CoV2 infection were enrolled. This project was approved by Institutional Review Board for ethics ("Comité de Protection des Personnes Ile de France 1", n° RCB: 2020-A01079-30). This study is registered at the French Ministry of Research and Teaching (#DC-2008-509), at the Commission Nationale de l'Informatique et des Libertés, and on clinicaltrials.gov (NCT04392401). Regarding IMMUNOSEPSIS4 cohort, patients over 18 years were enrolled for septic shock based on Sepsis-3 criteria [1]. This project was approved by Institutional Review Board for ethics ("Comité de Protection des Personnes Ouest II", n° RCB: 2019-A00210-57). This study is registered at the French Ministry of Research and Teaching (#DC-2008-509), at the Commission Nationale de l'Informatique et des Libertés, and on clinicaltrials.gov (NCT02803346). For both cohort, patients' clinical samples and data were collected three times: within 48 h after admission (day 0: D0), between D3 and D5 (D3) and between D7 and D9 (D7).

### **Retrospective cohort**

Septic or septic shock patients over 18 years were enrolled in the REALISM study [26,27]. This project was approved by Institutional Review Board (Comité de Protection des Personnes Sud-Est II, Bron, France) and the French National Security agency for drugs and health-related products (Approval code: 69HCL15\_0379, 30 November 2015). This study was registered on clinicaltrials.gov (NCT02638779). Patients' clinical samples and data were collected three times during the first week after admission: within the first 48 h after admission (D0), on D3 or

D4 (D3), and between D5 or D7 (D7), as well as at two additional time points on day 14 (D14) and day 28 (D28). Details of sampling schedules for each cohort are available in additional file 1 (Supplementary Figure 1).

### **Healthy volunteers**

Peripheral blood from healthy volunteers (controls) was provided by the “Etablissement Français du Sang (EFS)” from Lyon. According to EFS standardized procedures for blood donation and to provisions of the article R.1243–49 and following ones of the French public health code, a written non-opposition to the use of donated blood for research purposes was obtained from controls. The blood donors' personal data were anonymized before transfer to our research laboratory

### **Patient characteristics**

Patients' demographics, comorbidities, diagnosis, severity and clinical outcome were prospectively recorded. A longitudinal follow-up was conducted for 28 days. The following data were collected: age, gender, disease severity, and presence of organ dysfunction measured by the Simplified Acute Physiological Score II (SAPSII) [28] and the Sequential Organ Failure Assessment (SOFA) [29] score at ICU admission, respectively. Chronic health status was defined using the Charlson comorbidity index [30]. Additionally, the use of mechanical ventilation, the ratio of the arterial partial pressure of oxygen to the fractional inspired oxygen ( $\text{PaO}_2/\text{FiO}_2$  ratio) and the site and type of infections were documented upon admission. Survival were recorded after 28 days as well as the occurrence of secondary infection.

### **Cytokine measurement**



Plasma was frozen at – 20 °C within 4 h following blood collection and cytokine measurements were taken in batches. Plasma concentrations of IL-6, IL-10, IL-18 and TNF were measured by Simpleplex® technology using ELLA instrument (ProteinSimple®, San Jose, CA, USA), following manufacturer's instructions. Plasma concentrations of IL-1 $\beta$  and IL-1 receptor antagonist (IL-1Ra) were measured by BioPlex® technology (Bio-Rad Laboratories, Hercules, CA, USA) using Bio-Plex 200 instrument (Bio-Rad Laboratories, Hercules, CA, USA), following manufacturer's instructions. Plasma concentration of IL-1 receptor 2 (IL-1R2) was measured by MILLIPLEX® technology (Merck, Darmstadt, Germany) using Bio-Plex 200 instrument, following manufacturer's instructions.

#### **Whole blood phenotyping, flow cytometry and data acquisition**

CD4+ T cells immunophenotyping was performed on an automated volumetric flow cytometer from Beckman Coulter (Aquios CL) as previously described [31]. Monocyte HLA-DR (mHLA-DR) expression measurement was performed using the Anti-HLA-DR/Anti-Monocyte Quantibrite assay (BD Biosciences, San Jose, USA) as previously described [32]. Immature PMN (polymorphonuclear cells) and PMN-MDSCs (PMN-MDSCs: polymorphonuclear myeloid-derived suppressor cells) were monitored using following antibodies as described elsewhere [33]: KrO-labeled anti-CD45, PC7-labeled anti-CD10, PB-labeled anti-CD16 (Beckman Coulter, Brea, CA, USA), APC-labeled anti-CRTH2 (Miltenyi, Bergisch Gladbach, Germany), AF700-labeled anti-CD15, and PE-labeled anti-LOX1 or mouse IgG2a isotype control (Biolegend, San Diego, CA, USA). Immature PMN were monitored without PE-labeled anti-LOX1 or mouse IgG2a isotype control on REALISM cohort. Samples were run on a Navios flow cytometer (Beckman Coulter) and flow data were analysed using Navios software or Kaluza software 2.1 (both from Beckman Coulter). Calibration beads (Flow Check and Flow Set, Beckman Coulter) were run daily to check for routine alignment, day-to-day stability, and long-term performance validation.

## **Transcriptomic analysis**

These data were all obtained from the REALISM study, all details have previously been described [27]. RT-qPCR was used to determine the NLRP3 related genes transcriptome. Results were delivered and normalized expression values of markers were computed and used for the analyses.

## **Statistical analysis**

### *Clinical and biological characterization*

Continuous data are presented as medians and inter quartile ranges, categorical data are presented as numbers and percentages. To compare clinical and biological differences between groups, we used, for continuous data, Student's t-test (in case of normally distributed data) and Mann–Whitney U otherwise. Chi-squared tests or Fisher's exact tests were used for categorical data. Correlations were calculated using Spearman correlation tests and summarized in a correlation matrix when indicated. Kaplan–Meier survival curves were calculated in patients according to their endotype for the selected marker and the p-value of the log-rank test is given. The results were not adjusted for multiple test comparisons. All statistical analyses were performed with R (version 4.2.1).

### *Unsupervised clustering*

In order to identify patients' group with a common IL-1R2 or IL-18 mRNA dynamic over time (trajectories endotypes) we used "kml" – K-means for longitudinal data – R package 2.4.1 [34]. The number of endotypes was defined a priori. A boxcox transformation was applied to the selected marker to normalize the distribution before outlier samples detection and

exclusion based on Tukey method [35]. The KmL method pipeline works as follow, as previously described elsewhere [36]: first, marker's trajectories are clustered using k-means algorithm with a Gower adjusted Euclidean distance metric to handle missing data. Secondly, Calinski-Harabasz metric is studied to compare the different partitions; this represents the ratio between within-cluster and between-cluster dispersion and is thus a surrogate of partitioning quality. Within each cluster, missing values are imputed using linear interpolation so that imputed values follow the population mean trajectory shape. In order to avoid convergence to local minimum, the KmL method was run five thousand times, selecting the run showing the highest Calinski-Harabasz metric. As the non-parametric clustering methods used to define endotypes can identify non-linear shapes, we used the LOESS (Locally Weighted Scatterplot Smoothing) [37] non-parametric method to smooth marker measurements within each endotype. Around mean curve standard errors are also displayed, using the "stats" R package [38]. The resulting smoothed curves define mean evolution of each immunotype.

## Results

### Prospective cohort

In this prospective study, we enrolled a total of 32 patients with bacterial or viral sepsis (n=15 COVID-19 and n=17 septic shock) between December 2021 and August 2022. These patients exhibited the typical demographic and clinical features of a severe infectious ICU cohort as presented in Table 1, including elderly male patients with high severity score and morbidities. 28-day mortality was 33% among COVID-19 ICU patients and 27% in septic shock patients. Both groups of patients demonstrated a hyper-inflammatory response, with increased plasma concentrations of TNF, IL-6, and IL-10 from admission until D7, compared to controls (Fig. 1A). The acute inflammatory response observed in septic shock patients was more pronounced (Fig. 1A). The cellular response was also similar between the two groups, showing a severe lymphopenia (Fig. 1B) along with a marked decrease in mHLA-DR expression throughout the whole follow-up (Fig. 1B). This decreased mHLA-DR expression can be considered as a well-established characteristic of monocytic MDSCs (m-MDSCs) [9,39]. As expected, the clinical and immunological data indicated the occurrence of both tremendous inflammation and immune cellular deficiencies in a cohort of severely critically ill patients.

The LOX-1 PMN-MDSCs, which are characterized as immature neutrophils with immunosuppressive properties [33], displayed similar kinetics in patients with bacterial and viral sepsis, with a gradual appearance during follow-up and a peak observed at D7 (Fig. 2A). Additionally, at D7, a strong correlation was observed between LOX-1 PMN-MDSCs and the presence of immature neutrophils ( $r=0.9$ ,  $p<0.001$ , Fig. 2B). Most of immature PMN ( $CD10^{\text{low}}CD16^{\text{low}}$ ) are LOX1+ cells and thus PMN-MDSCs [33,40] (Fig 2B and additional file 1: Supplementary Figure 2).

We investigated the concentrations of IL-1 $\beta$  and IL-18 in plasma as the final products of NLRP3 inflammasome activation. While plasma levels of IL-1 $\beta$  did not significantly differ between COVID-19 patients and controls at any time points (Fig. 3A), septic shock patients showed a

significant increase at D0, which returned to normal levels for the rest of the first week (Fig. 3A). Plasma IL-18 levels were significantly higher in all septic patients compared to controls from D0 to D7 (Fig. 3A). We then explored the dynamics of soluble receptors of IL-1 $\beta$ : the IL-1 receptor 2 (IL-1R2) and the IL-1 receptor antagonist (IL-1Ra). IL-1R2 plasma concentration was increased in all patients at all time points compared to controls (except for D7 in septic shock patients). IL-1Ra concentrations increased at all time points, with a gradual decrease during the first week (Fig. 3B). As expected, in patients with bacterial sepsis, initial increased IL-1 $\beta$  concentration was associated with higher 28-day mortality (Additional file 1: Supplementary Figure 3).

We next investigated whether the persistence of NLRP3 inflammasome activation at D7 was associated with the persistence of immunosuppression markers. At this time point, we observed a negative correlation between IL-1R2 concentration and mHLA-DR expression ( $r=-0.66$ ,  $p=0.012$ ), as well as a positive correlation between the concentration of this soluble receptor and IL-10 concentration ( $r=0.57$ ,  $p=0.037$ ). Additionally, we observed a positive correlation between IL-18 and the persistence of immature PMN ( $r=0.64$ ,  $p=0.028$ ) as well as a trend with the persistence with LOX-1 PMN-MDSCs ( $r=0.57$ ,  $p=0.059$ ).

As an interim conclusion, we observed that septic patients present simultaneous occurrence of inflammatory and immunosuppressive features. In addition, for the first time, we report that the delayed occurrence of this immunosuppression, as indicated by the peak of LOX-1 PMN-MDSCs on day 7 (accompanied by high concentrations of IL-10 and low values of mHLA-DR) is correlated with the persistent activation of NLRP3. Due to the inherent limitations in sample size (common in clinical studies involving fresh whole blood analysis), we sought to validate these findings in a larger cohort of patients. Therefore we performed a reanalysis of the dataset obtained within the REALISM study. In addition to including a significant number of septic patients, this dataset allowed the exploration of mRNA levels of NLRP3-related genes combined with a complete immune phenotyping including markers of immunosuppression.

## **Inflammatory endotypes and association with immune alterations and clinical outcomes**

The REALISM study investigated patients with sepsis (n=31) and septic shock (n=76), who had similar characteristics to the previously studied septic shock patients (Additional file1: Supplementary Table 1). Among transcriptomic markers assessed in the REALISM study, we focused on IL-1R2 and the IL-18 receptor 1 (IL-18R1), both surrogates of NLRP3 inflammasome activation. The patients exhibited similar kinetics of these parameters, with an over-expression compared to controls from D0 and till D60 (Fig.4).

The Kml clustering method resulted in the definition of 2 endotypes for each parameter studied (Fig.5). Cluster 1 (Fig.5) is characterised by a mild initial inflammatory response and a rapid return to control values within 2 weeks. In contrary, cluster 2 (Fig.5) included patients characterized by a strong initial pro-inflammatory response that persisted beyond 28 days.

Importantly, during the following 28 days after admission, patients included in cluster 2 presented with more profound feature of immunosuppression (low mHLA-DR expression, increase in IL-10 plasma concentration and in the percentage of immature PMN), compared with patients from cluster 1. This was associated with a significant difference in 28-day mortality which was very low among patients from cluster 1.

In line with clustering analysis, during the whole monitoring, we observed a significant correlation between IL-1R2 mRNA, IL-18R1 mRNA and immune alterations, including a negative correlation with mHLA-DR expression and a positive correlation with IL-10 secretion and the appearance of immature PMN, which are PMN-MDSCs after D5 (Fig.6).

In this retrospective cohort, we confirmed the association of persistent NLRP3 inflammasome activation and the development of delayed sepsis-induced immunosuppression, and more precisely, the development and persistence of MDSCs. Most importantly, we reported that

patients expressing the strongest initial NLRP3 activation maintained a deeper immunosuppression and presented a higher D28-mortality.

## Discussion

Although inflammation plays a crucial role in the body's defence mechanisms, it must be tightly regulated to prevent an excessive response that can lead to cellular and organ damages as seen in sepsis. Among inflammatory mechanisms, the activation of NLRP3 inflammasome and the release of its downstream products (*i.e.* IL-1 $\beta$  and IL-18) have been observed in septic patients [6]. Additionally, in other clinical contexts like cancer, immunosuppression has been linked to the activation of the NLRP3 inflammasome, particularly in the development of MDSCs [18–24]. Given the similarities regarding immunosuppression observed in cancer and infectious diseases, this study aimed to investigate the relationship between NLRP3 related inflammation and sepsis-induced immunosuppression. In a comprehensive and dynamic approach that considers diverse sepsis etiologies and investigates markers of both immunosuppression and inflammation at various levels (including soluble factors, cellular responses, and mRNA expression), our study has yielded several important findings. Firstly, we observed simultaneous increases in NLRP3 products (IL-1 $\beta$  and IL-18) or related soluble receptors (IL-1R2) alongside markers of sepsis-induced immunosuppression. Secondly, this immunosuppression manifested in a delayed manner, as evidenced by the differed and progressive appearance of LOX-1 PMN-MDSCs and the persistence of other suppressive markers such as low mHLA-DR expression (*i.e.* m-MDSCs), elevated plasma IL-10 concentration. Thirdly, and perhaps most importantly, we showed that a strong initial NLRP3 activation as well as its persistence defines a specific hyper-inflammatory endotype of patients, characterized by marked immunosuppressive features, including m-MDSCs and PMN-MDSCs persistence, and an increased 28-day mortality.

NLRP3 inflammasome activation is responsible for IL-1 $\beta$  and IL-18 secretions as well as the induction of pyroptosis, an inflammatory form of cellular death. In the context of sepsis, there is evidence of increased initial activation of the NLRP3 inflammasome [6]. Several clinical studies have reported associations between elevated initial concentrations of components of the NLRP3 inflammasome (NLRP3, ASC, caspase-1) or its products (IL-1 $\beta$ , IL-18) and



mortality [18–24,41,42]. In addition, in some studies, persistent NLRP3 inflammasome activation has also been observed, with detectable plasma levels of IL-18 during the late phase of both bacterial and viral sepsis [20,22,42], which aligns with the present study. Additionally, mRNA related to NLRP3 inflammasome genes (*i.e.*, NLRP3, ASC, caspase-1 and IL-18) could be detected in survivors up to 1 year after sepsis [43]. While the significance of the NLRP3 inflammasome activation in sepsis pathophysiology is increasingly recognized, its association with sepsis-induced immunosuppression has not been explored. In this study, we provide evidence of an association between NLRP3 activation and increased cellular markers (m-MDSCs and LOX-1 PMN-MDSCs) as well as soluble markers (elevated plasma IL-10 concentration) of sepsis-induced immunosuppression.

Lectin-type oxidized LDL receptor 1 (LOX-1) serves as a marker for a specific subset of MDSCs (PMN-MDSCs), known for their potent immunosuppressive properties. LOX-1 expression on PMN-MDSCs has been observed in some human cancer studies. For instance, in glioblastoma, LOX-1 PMN-MDSCs inhibit CD3<sup>+</sup> T cell proliferation, negatively correlate with IFN- $\gamma$ +CD4<sup>+</sup> and IFN- $\gamma$ +CD8<sup>+</sup> T cells, and are associated with early recurrence and disease progression [44]. LOX-1 PMN-MDSCs have also been described in hepatocellular carcinoma, where they significantly reduced T cell proliferation and IFN- $\gamma$  production as well as being related to a decrease overall survival [45]. Additionally, beside cancer, these immunosuppressive cells have also been described in other clinical contexts. In trauma patients, LOX-1 PMN-MDSCs significantly inhibited T cells proliferation and decreased IFN- $\gamma$  secretion [46]. In sepsis, a previous study from our group described the ability of these cells to reduce IFN- $\gamma$  production from T cells [33]. In the present study, LOX-1 PMN-MDSCs were present in septic patients, with a progressive rise over the first week.

In cancer, numerous studies in mouse models and patients have demonstrated the association between MDSCs, immunosuppression, and clinical outcomes [47]. Animal studies have shown that the NLRP3 inflammasome has an impact on the development and immunosuppressive abilities of MDSCs. Inhibition or genetic knockout (KO) of NLRP3 [16,48,49], as well as

blocking IL-1 $\beta$  or IL-18 [49], have been shown to decrease MDSCs populations in cancer models. Furthermore, animal cancer models have shown improved tumour progression and survival following interventions such as NLRP3 KO [50,51] or IL-18 blockade [52]. Mechanistically, NLRP3 pathway, through IL-18 production, has been implicated in the signalling pathway of the PD-L1 axis [48] and contributes to its upregulation [49], leading to the induction of immunosuppressive cells. Additionally, the activation of NLRP3 in tumour-associated macrophages promoted tumour cell proliferation and triggers other immunosuppressive mechanisms [16,51], including the generation of tumour-promoting T helper type 2 cells. Furthermore, IL-18 has been shown to play a crucial role in the bone marrow environment by driving the development of functional MDSCs [52].

In septic patients, few studies mostly based on usual soluble inflammatory mediators (*e.g.*, IL-6 or CRP) have reported an association between immunosuppression and a strong inflammatory response [53,54]. Our study extended these results by showing an association between classical cellular deficiencies observed during sepsis (LOX-1 PMN-MDSCs, low mHLA-DR expression, immature PMN) and persistent NLRP3-related inflammation. Furthermore, we found by clustering analysis that the persistent elevation of the NLRP3 inflammasome was associated with decreased 28-day survival. These findings in sepsis, along with those from cancer, support the hypothesis proposed by Rincon and colleagues regarding the pivotal role of chronic inflammation following sepsis [55]. According to these authors, continuous release of inflammatory mediators during sepsis may affect bone marrow function, leading to persistent activation of myeloid cells and the subsequent development of an immunological endotype with persistent MDSCs.

Beyond pathophysiological insights, the present results highlights the possibility of using activation of the NLRP3 inflammasome pathway as a biomarker in sepsis. A study proposed NLRP3 as a biomarker for identifying high-risk patients, as higher levels of NLRP3 in serum are associated with increased mortality rates [21]. Another study utilized a transcriptomic score incorporating the IL-1R2 marker, to identify critically ill patients at higher risk of acquiring

infections in the ICU and experiencing higher ICU mortality rates [56]. Additionally, IL-1R2+ immunosuppressive neutrophils have been found to be increased in COVID-19 and sepsis [57]. These neutrophils exhibit inhibitory effects on CD4+ T cell proliferation and activation, and their presence is associated with poorer outcomes. However, this biomarker aspect warrants further exploration.

Our study has several limitations. Being a clinical observational study, we have observed associations between NLRP3-related inflammation and immune suppression alterations in septic patients, but we cannot establish a definitive causal link between these two observations. To address this aspect, further experimental studies using mouse models of sepsis are necessary. Additionally, the prospective study was constrained by its small sample size, which we addressed by validating our findings in a larger retrospective cohort.

## **Conclusions**

Our study highlighted the association between NLRP3 inflammasome activation and immunosuppression in septic patients. We found that an initial and persistent hyper inflammatory response was associated with persistent immune deficiencies and increased 28-day mortality. These findings emphasize the importance of understanding the underlying mechanisms of inflammation in sepsis and developing personalized treatment strategies based on patients' specific endotypes

## List of abbreviations

AB/C: Antibodies bound per cell

CAPS: Cryopyrin-associated periodic syndromes

ICU: Intensive care unit

IL-1Ra: IL-1 receptor antagonist

IL-1R2: IL-1 receptor 2

IL-18R1: IL-18 receptor 1

LOX-1: lectin-type oxidized LDL receptor 1

MDSCs: myeloid-derived suppressor cells

mHLA-DR: Monocytic human leukocyte antigen-DR

m-MDSCs: monocytic MDSCs

NLRP3: NACHT, leucin-rich repeat and pyrin-containing receptor 3

PAO<sub>2</sub>/FiO<sub>2</sub> ratio: Ratio of the arterial partial pressure of oxygen to the fractional inspired oxygen

PMN: polymorphonuclear cells

PMN-MDSCs: polymorphonuclear myeloid-derived suppressor cells

SAPSII: Simplified acute physiology score II

SOFA: Sequential organ failure assessment

### Authors' contributions

GM, FV, LA, MC and ACL designed the study. RC, GM, FV, MB, BFP, MG analyzed results, discussed findings and wrote the manuscript. RC, EC and LG performed immune monitoring. RC and MB performed statistical analysis. ACL, LA, MC and FB collected clinical samples. All authors read and approved the final manuscript.

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### Conflict of interest

The authors declare they have no conflict of interest.

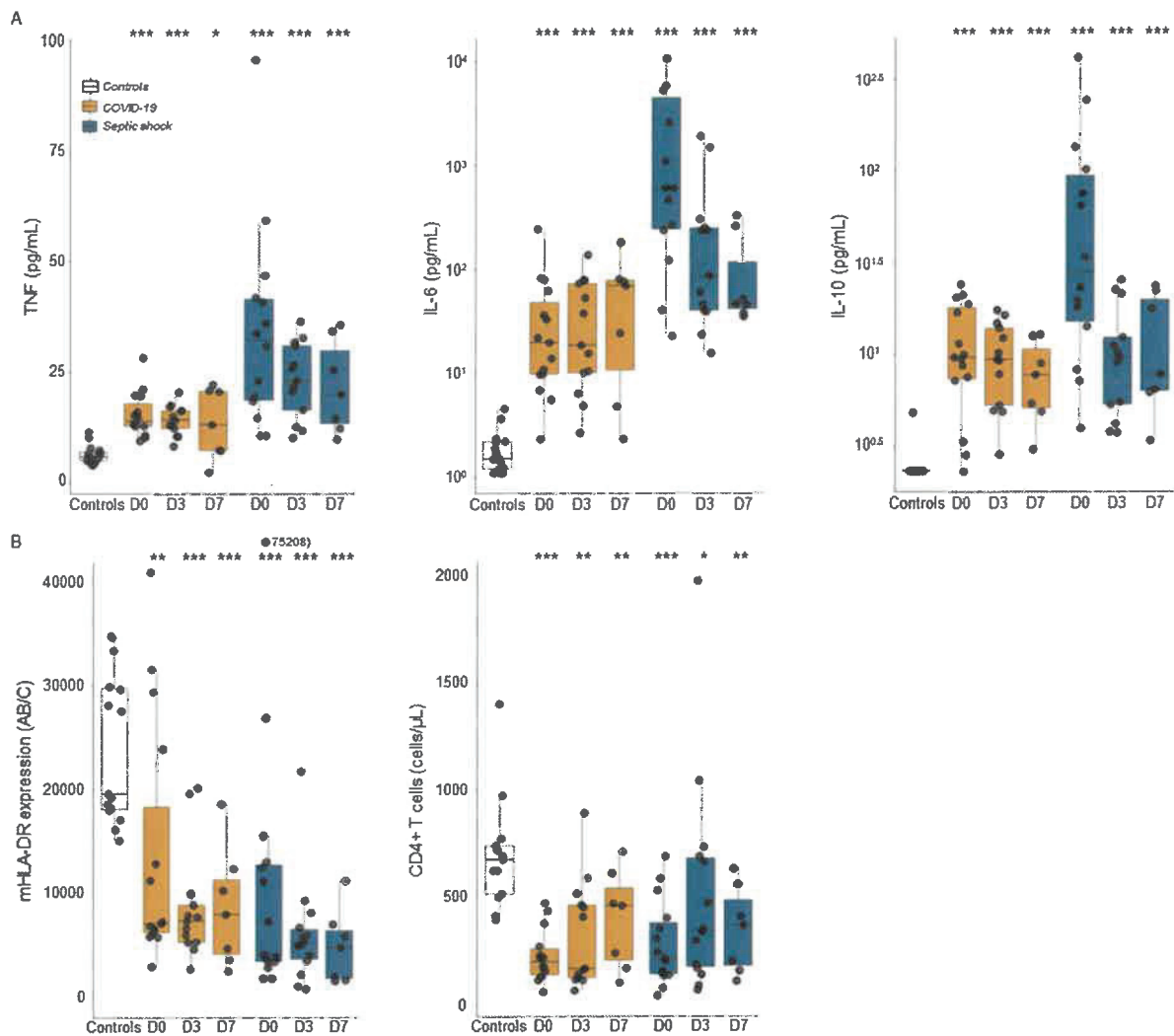
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Parameters	COVID-19 (n=15)	Septic shock (n=17)
Age at admission (years)	64 [51-71]	65 [61-69]
Gender - Male <i>n</i> (%)	11 (73)	14 (82)
<b>Main admission <i>n</i> (%)</b>		
<i>Medical</i>	15 (100)	7 (41)
<i>Surgical</i>	0 (0)	10 (59)
SAPSI score	34 [26-39]	45.5 [38-56]
SOFA score	3 [1-5]	10 [7-12]
Charlson score	0 [0-1.5]	2 [1-6]
<b>Infection diagnosis <i>n</i> (%)</b>		
<i>Microbiology</i>	15 (100)	12 (70)
<i>Surgery</i>	0 (0)	3 (18)
<i>Other</i>	0 (0)	2 (12)
<b>Microbiologically documented <i>n</i> (%)</b>		
<i>Bacilli gram -</i>	0 (0)	7 (64)
<i>Cocci gram +</i>	0 (0)	4 (36)
<i>Sars-CoV-2</i>	15 (100%)	0 (0)
<b>Respiratory dysfunction</b>		
Mechanical ventilation <i>n</i> (%)	3 (20)	15 (88)
<i>PaO<sub>2</sub>/FiO<sub>2</sub></i>	76.5 [67-93]	248 [177-294]
<b>Site of primary infection <i>n</i> (%)</b>		
<i>Pulmonary</i>	15 (100)	1 (6)
<i>Abdominal</i>	0 (0)	11 (69)
<i>Other</i>	0 (0)	4 (25)
<b>Follow-up <i>n</i> (%)</b>		
28-day non survivors	5 (33)	4 (27)
Secondary nosocomial infections	1 (7)	2 (12)
<b>Immunologic parameters (at Day 3)</b>		
<i>mHLA-DR (AB/C)</i>	7300 [5299-8842]	5161 [3613-6475]
<i>Absolute CD4+ T cell count (cells/μL)</i>	166 [126-462]	343 [174-681]

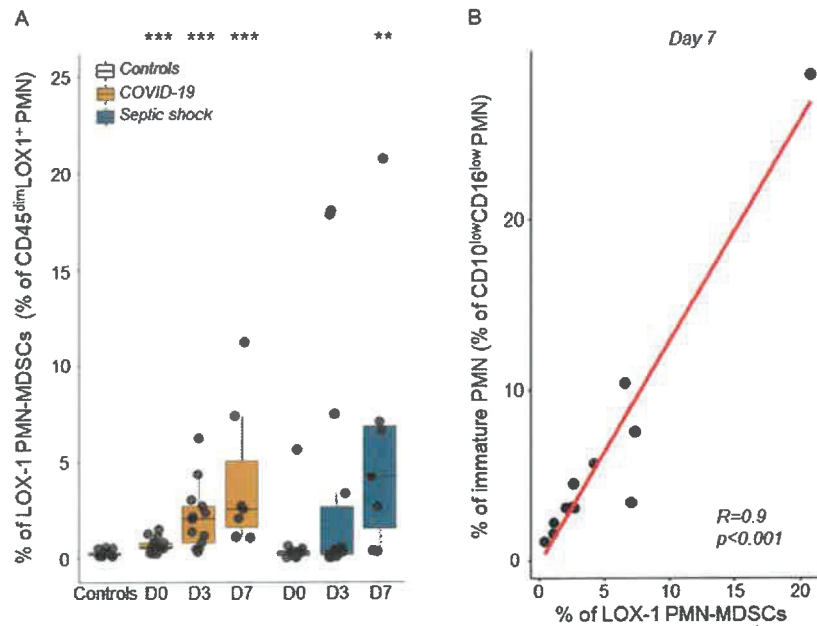
**Table. 1 Demographic, clinical, and immunologic data for viral septic (COVID-19) and bacterial septic shock patients.** Continuous data are presented as medians and interquartile ranges [Q1–Q3]. For clinical parameters, categorical data are presented as numbers of cases and percentages among the total population in brackets. SAPS II (simplified acute physiology score II) and SOFA (sequential organ failure assessment) scores were calculated at admission. Absolute CD4+ T cell count (expressed as cells/μL) was calculated on day 3 as well as mHLA-DR (expressed as numbers of anti-HLA-DR antibodies bound per monocyte, AB/C). Reference values from our routine lab: mHLA-DR: 13500-45000 AB/C, CD4+: 336–1126 cells/μL.

## Figure

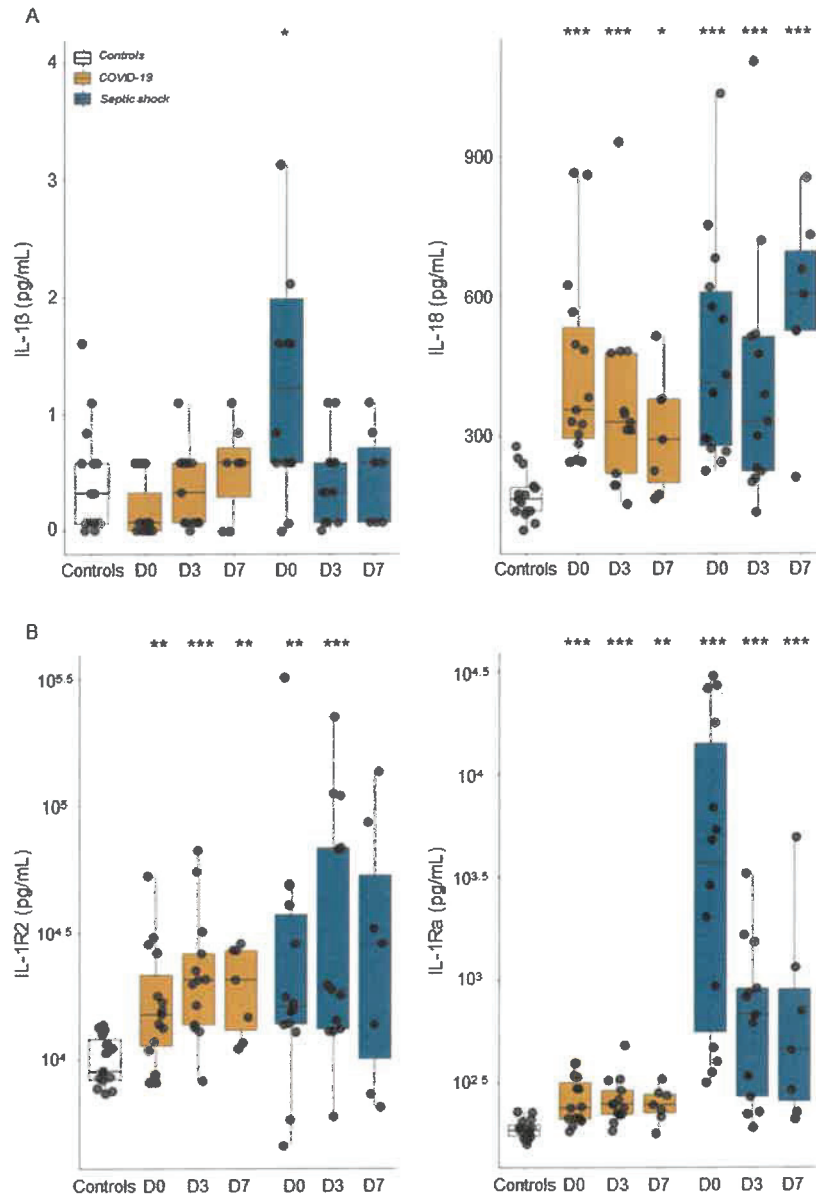


**Fig. 1** Immune response in viral septic and bacterial septic shock patients. Immune parameters were measured three times in 17 septic shock patients (D0: within the first 48 h, n=13; D3: between day 3 and day 5, n=12; D7: between D7 and D9, n=7), in 15 COVID-19 ICU patients (D0: n=15; D3: n=13; D7: n=7) and in 15 healthy controls (5 men and 10 women, median age=44 years). Patients were split in two groups based on the origin of the infection: COVID-19 infection (yellow boxes) or bacterial infection (blue boxes). **A.** Over time evolution of plasmatic inflammatory cytokines including TNF, IL-6 and IL-10 during the first week after admission is shown. Missing values corresponded to patients whose serum could not be collected (n=2 samples). **B.** The cellular immune response is shown, including mHLA-DR expression and CD4+ T cells measurement. Nonparametric Mann–Whitney U test was used to compare values between controls and patients. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

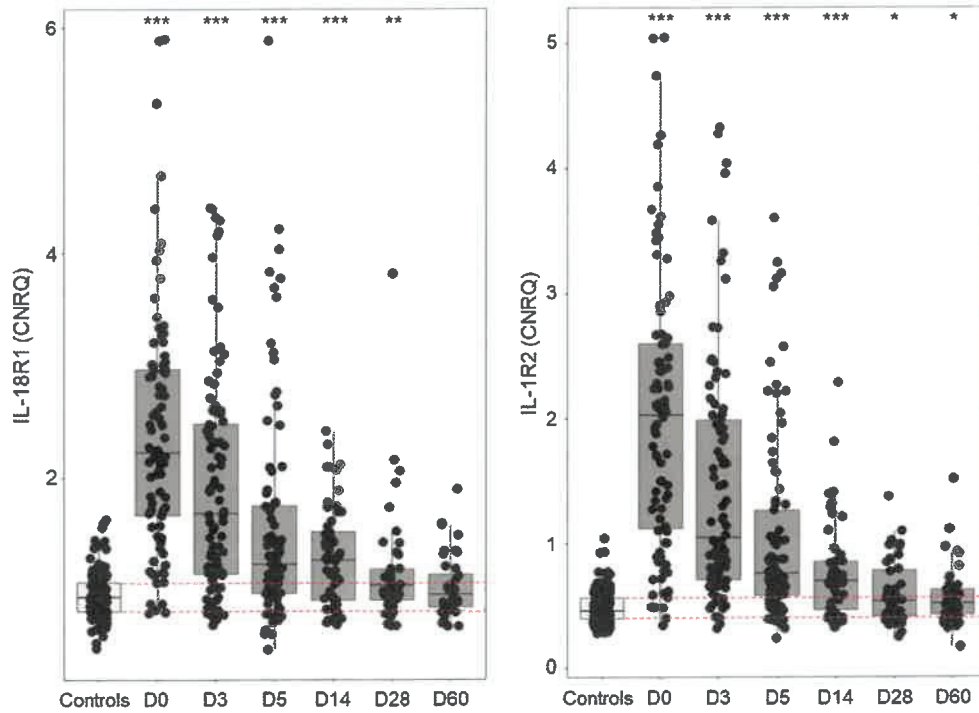




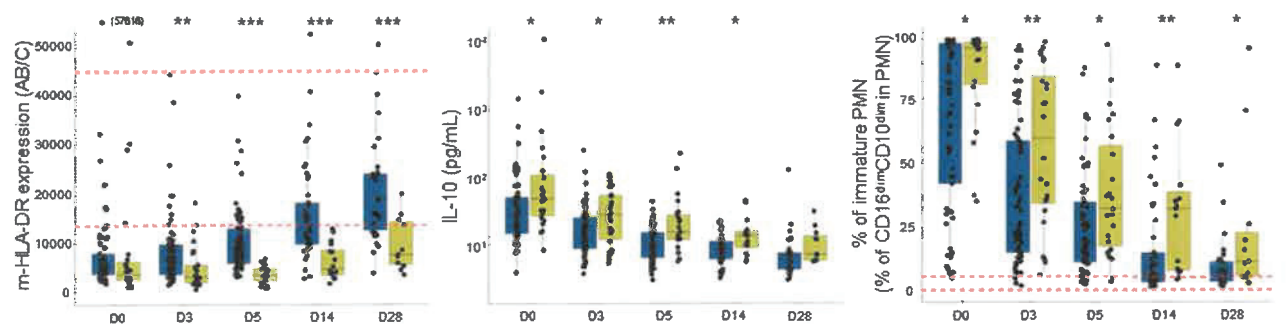
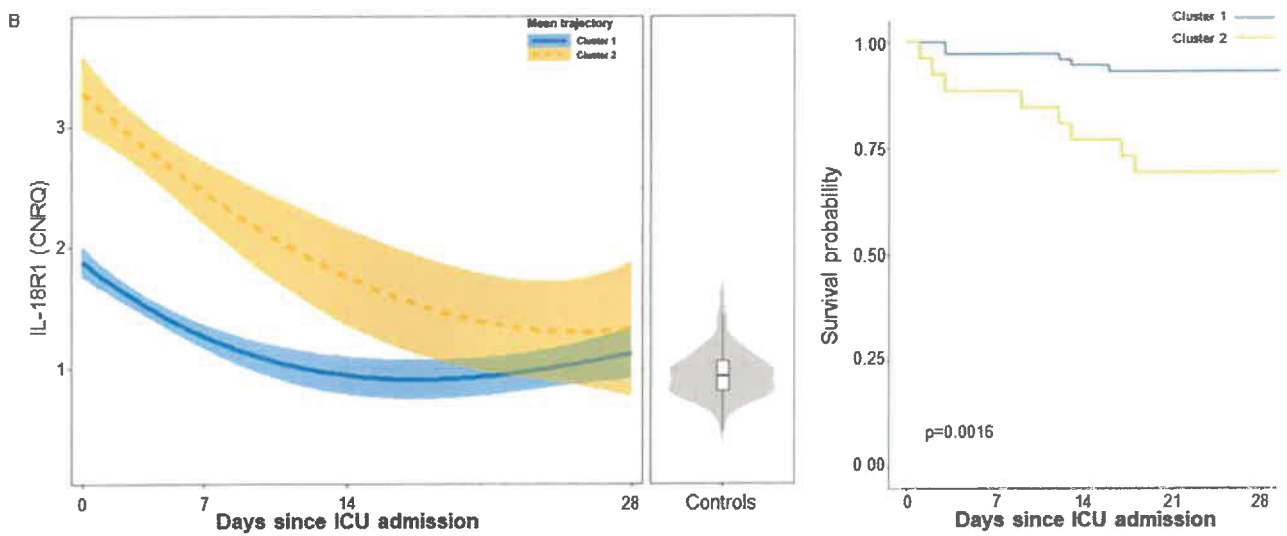
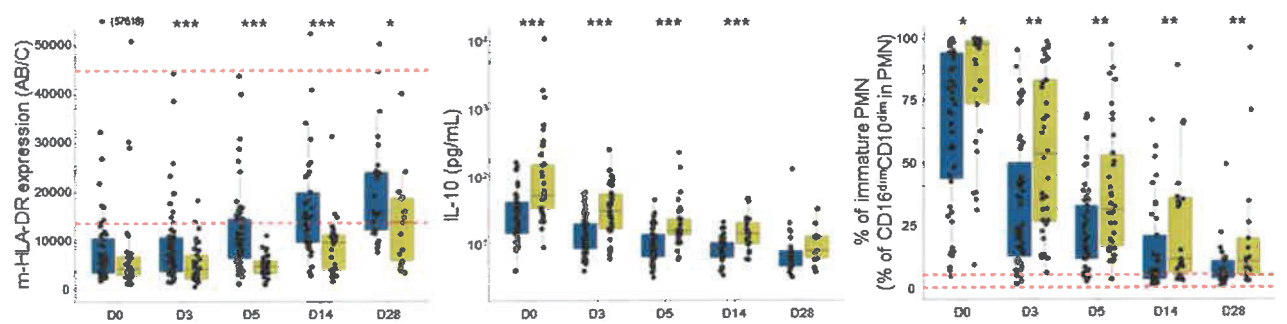
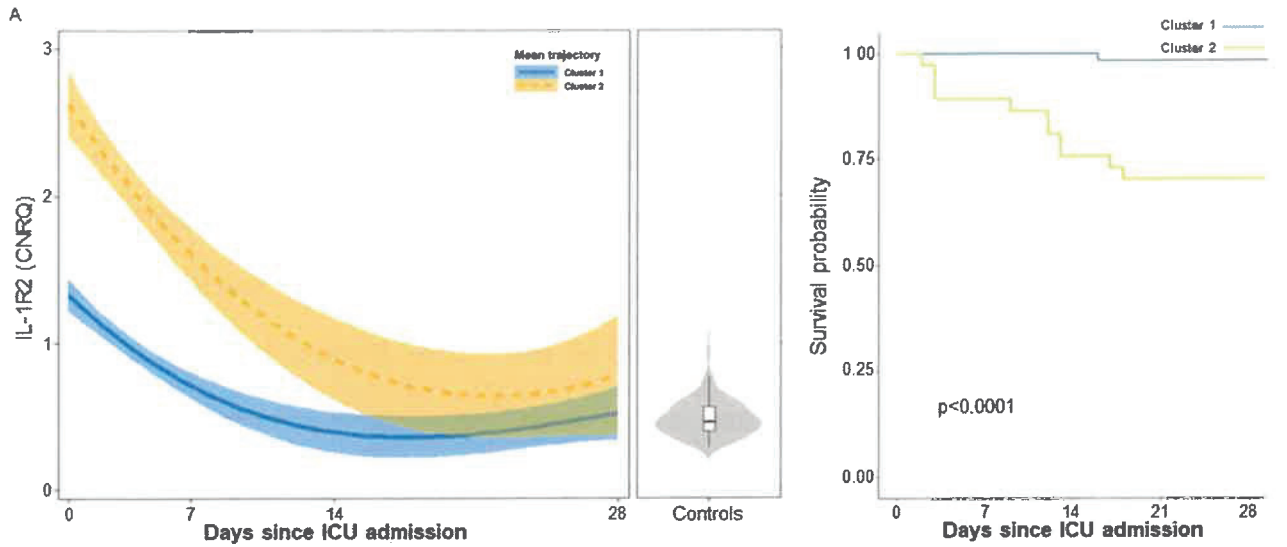
**Fig. 2 Monitoring of lectin-type oxidized LDL receptor 1 polymorphonuclear myeloid-derived suppressor cells (LOX-1 PMN-MDSCs) over time in viral septic and bacterial septic shock patients. A.** Percentages of LOX-1 PMN MDSCs among total neutrophils were measured in peripheral blood from 15 healthy controls and 15 COVID-19 ICU patients (D0: n=15; D3: n=13; D7: n=7), or 17 septic shock patients (D0: n=14; D3: n= 13; D7: n=7). **B.** Correlations between the percentage of immature PMN (% of CD10<sup>low</sup>CD16<sup>low</sup> among total neutrophils) and the percentage of LOX-1 PMN MDSCs among total neutrophils in n=12 patients at D7. One patient was excluded from the analysis due to a partial deficit in FcγRIII (CD16), and another patient sample was excluded due to an aberrant value. Nonparametric Mann–Whitney U test was used to compare values between controls and patients. Correlation was calculated using Spearman correlation test. \*\* p < 0.01, \*\*\* p < 0.001.



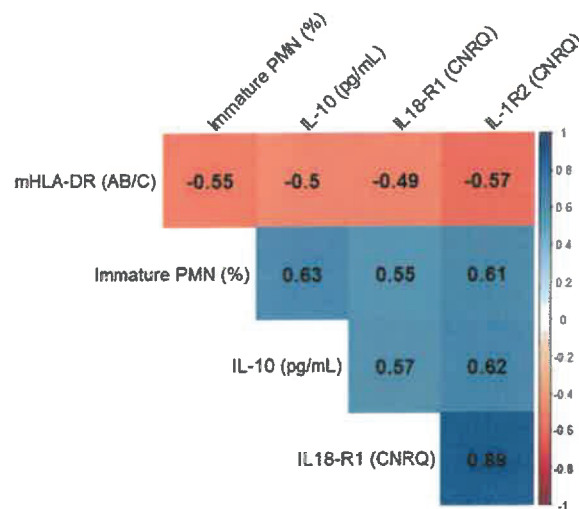
**Fig. 3 NLRP3 inflammasome activation in viral septic and bacterial septic shock patients.** Plasmatic levels of IL-1 $\beta$  and IL-18 (A) and soluble receptors that bind to IL-1 $\beta$ : the IL-1 receptor 2 (IL-1R2) and the IL-1 receptor antagonist (IL-1Ra) (B) from 15 controls and 17 septic shock patients (D0: n=13; D3: n= 12; D7: n=7), or 15 COVID-19 ICU patients (D0: n=15; D3: n=13; D7: n=7). Missing values corresponded to patients whose serum could not be collected. Nonparametric Mann–Whitney U test was used to compare values between controls and patients. \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.



**Fig. 4 Kinetics of mRNAs linked to NLRP3 inflammasome activation.** IL-1 receptor 2 (IL-1R2) and IL-18 receptor 1 (IL18-R1) mRNAs were analyzed in healthy controls (n=175, shown in white) and septic or septic shock patients (n=107, shown in grey) at various time points: D0 (n=92), D3 (n=99), D5 (n=94), D14 (n=62), D28 (n=45) and D60 (n=40). The dashed red lines represent the 1st and 3rd quartile of values observed in controls. Nonparametric Mann–Whitney U test was used to compare values between controls and patients. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .



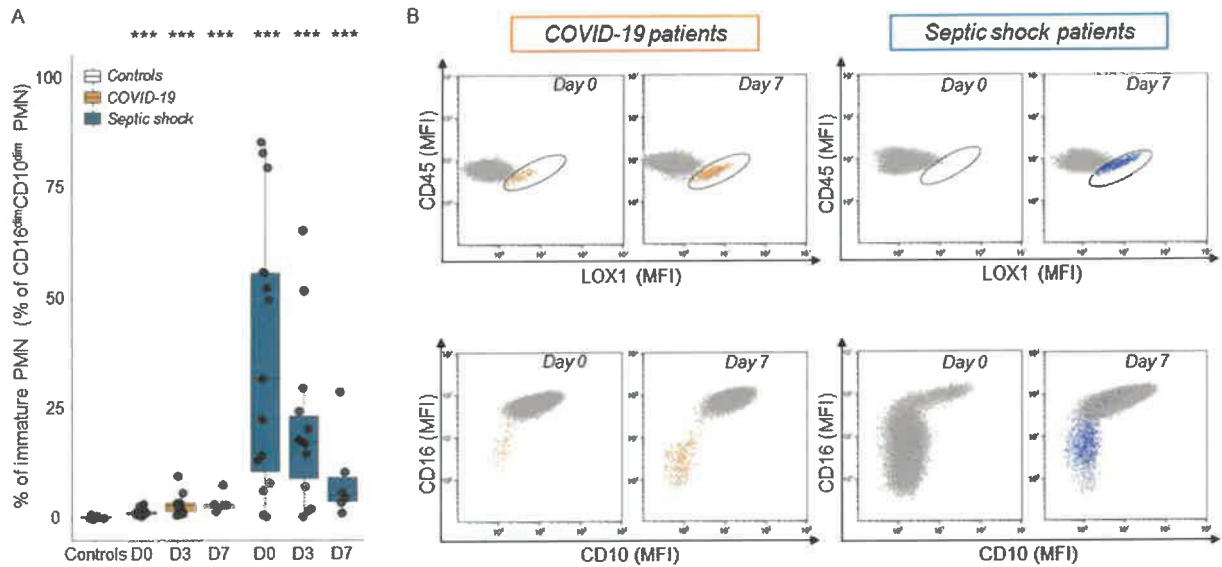
**Fig. 5 Association of endotypes linked to NLRP3 inflammasome activation with 28-day mortality and sepsis-induced immunosuppression.** Mean trajectories are drawn for each endotype: high expressors (cluster 2, yellow curve) and low expressors (cluster 1, blue). Standard errors are displayed around the mean curve. Violin plots represent data from controls (n=105). Kaplan–Meier survival curves were calculated in patients according to their endotype with the p-value of the log-rank test provided for each curve. Immune response parameters including mHLA-DR expression, plasma IL-10 level, and percentage of immature PMN are shown, stratified by endotypes for IL-1 receptor 2 (IL-1R2) (A, n=99) and for IL-18 receptor 1 (IL-18R1) (B, n=98). Red dotted lines represent reference values for mHLA-DR or immature PMN, based on 2.5th and 97.5th percentiles of healthy volunteers' values, as defined in [26]. Nonparametric Mann–Whitney U test was used to compare values between patients at D0, D3, D5, D14 and D28. \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.



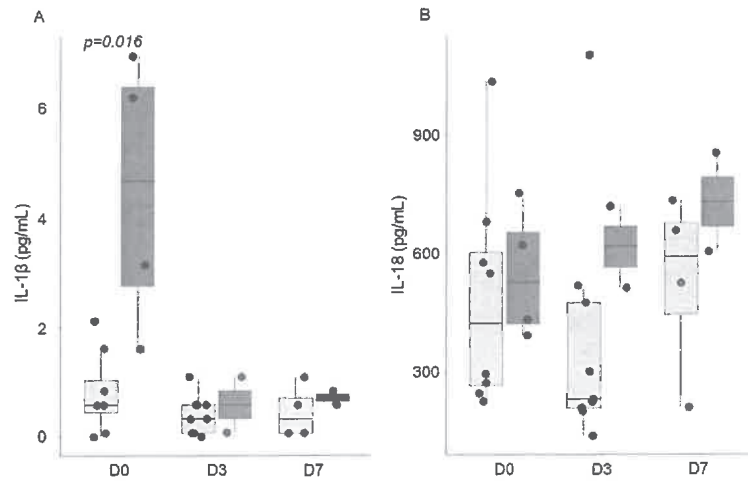
**Fig. 6 Correlation matrix of immune parameters and mRNA linked to NLRP3 inflammasome activation in septic patients.** Immune parameters and mRNA linked to NLRP3 inflammasome activation were measured during the first week of ICU-hospitalization in 107 septic or septic shock patients. Correlations were calculated using Spearman correlation tests and results are presented as a correlation matrix. Spearman correlation coefficients are indicated and cells were coloured according to the strength and trend of correlations (shades of blue = positive, shades of red = negative). All coefficients presented a p value below 0.05 after correction for multiple testing.

Category	Markers	D0/D2		D3/D5		D5/D9		D14	D28
		<i>Pro</i>	<i>Retro</i>	<i>Pro</i>	<i>Retro</i>	<i>Pro</i>	<i>Retro</i>	<i>Retro</i>	<i>Retro</i>
Sepsis-induced immune alterations	<i>mHLA-DR</i>	X	X	X	X	X	X	X	X
	<i>CD4+ T cells</i>	X	X	X	X	X	X	X	X
	<i>Immature PMN</i>	X	X	X	X	X	X	X	X
	<i>LOX-1 PMN-MDSCs</i>	X		X		X			
Non-specific inflammatory response	<i>TNF<math>\alpha</math></i>	X	X	X	X	X	X	X	X
	<i>IL-6</i>	X	X	X	X	X	X	X	X
	<i>IL-10</i>	X	X	X	X	X	X	X	X
Soluble inflammatory markers related to NLRP3 activation	<i>IL-1<math>\beta</math></i>	X		X		X			
	<i>IL-18</i>	X		X		X			
	<i>IL-1R1s</i>	X		X		X			
	<i>IL-1R2s</i>	X		X		X			
	<i>IL-1Ra</i>	X		X		X			
Transcriptomic inflamamtry markers related to NLRP3 activation	<i>IL-1R2</i>		X		X		X	X	X
	<i>IL-18R1</i>		X		X		X	X	X

**Supplementary Figure 1. Study design.** Description of the various immunological and inflammatory markers studied in the prospective cohort (pro) or in the retrospective REALISM cohort (retro) over time



Supplementary figure 2 legend: **Supplementary Figure 2. Kinetics of immature PMN and distribution of lectin-type oxidized LDL receptor 1 polymorphonuclear myeloid-derived suppressor cells (LOX-1 PMN-MDSCs) among immature PMN in viral septic and bacterial septic shock patients.** **A.** Percentages of immature PMN (% of CD16<sup>dim</sup>CD10<sup>dim</sup>) among total neutrophils were measured in peripheral blood from 15 healthy controls and 14 COVID-19 ICU patients (D0: n=14; D3: n=12; D7: n=6), or 17 septic shock patients (D0: n=14; D3: n= 13; D7: n=6). **B.** Representative example of LOX-1 PMN-MDSCs staining. LOX-1 PMN-MDSCs were gated on the biparametric CD45/LOX1 dotplot, in yellow for n=1 COVID-19 ICU patients and in blue for n=1 septic shock patients. For both patients, stainings are represented at day 0 and day 7 post ICU hospitalization. CD10 and CD16 expressions gated on the LOX-1 PMN-MDSCs cells is also represented to illustrate the immature nature of these cells. Nonparametric Mann–Whitney U test was used to compare values between controls and patients. \*\* p < 0.01 and \*\*\* p < 0.001.



Supplementary figure 3 legend: **Supplementary Figure 3. Inflammatory response in bacterial septic shock patients according to 28-day mortality.** Plasmatic levels of IL-1 $\beta$  (A) and IL-18 (B) were measured during the first week ICU-hospitalization in 17 septic shock patients (D0: n=13; D3: n= 12; D7: n=7). 28-days survivors patients were represented in white and non-survivors in grey. Missing values corresponded to patients whose serum could not be collected. Nonparametric Mann–Whitney U test was used to compare values between groups at the same time point.



Parameters	REALISM (n=107)
Age at admission (years)	68 [59-77]
Gender - Male n (%)	69 (66%)
<b>Clinical scores</b>	
SAPSII score	47 [36-54]
SOFA score	9 [7-11]
Charlson score	2 [0.7-5.3]
<b>Infection diagnosis n (%)</b>	
Microbiology	77 (73)
Surgery	8 (8)
Other	20 (19)
<b>Microbiologically documented n (%)</b>	
Bacilli gram -	26 (25)
Cocci gram +	25 (24)
Other	11 (10)
NA	43 (41)
<b>Respiratory dysfunction</b>	
Mechanical ventilation n (%)	76 (71%)
PaO <sub>2</sub> /FiO <sub>2</sub>	210 [159-292]
<b>Site of primary infection n (%)</b>	
Pulmonary	27 (26)
Abdominal	40 (38)
Other	38 (36)
<b>Follow-up n (%)</b>	
28-day non survivors	16 (15)
Secondary nosocomial infections	12 (11)
<b>Immunologic parameters (at Day 3)</b>	
mHLA-DR (AB/C)	4947 [3126-7925]
Absolute CD4+ T cell count (cells/ $\mu$ L)	325 [206-505]

Supplementary table 1 legend: **Supplementary Table 1. Demographic, clinical, and immunologic data for septic and septic shock patients from REALISM study.** Continuous data and biologic parameters are presented as medians and interquartile ranges [Q1–Q3]. For clinical parameters, categorical data are presented as numbers of cases and percentages among the total population in brackets. SAPS II (simplified acute physiology score II) and SOFA (sequential organ failure assessment) score were calculated at admission. Absolute CD4+ T cell count was calculated on day 3 as well as mHLA-DR, expressed as numbers of anti-HLA-DR antibodies bound per monocyte (AB/C). Reference values from our lab: mHLA-DR: > 15 000 ABC, CD4+: 336–1126 cells/ $\mu$ L.

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Article 3 : Monitoring NLRP3 Inflammasome Activation and Exhaustion in Clinical Samples: A Refined Flow Cytometry Protocol for ASC Speck Formation Measurement Directly in Whole Blood after Ex Vivo Stimulation (Cells. 2022 Oct 20 ; 11(20) : 3306)

Contexte et objectif

L'altération de la voie de inflammasome NLRP3 a été impliquée dans la physiopathologie de nombreuses maladies. Suite à la stimulation cellulaire, l'agrégation de la protéine ASC en un complexe multiprotéique (Specks d'ASC), a été proposée comme un marqueur de l'activation de l'inflammasome NLRP3 mesurable par cytométrie en flux. L'objectif de cette étude était de développer un protocole pour détecter la formation de speck en sang total (permettant son utilisation en recherche clinique) en incluant une étape de stimulation ex vivo sous le format d'un test fonctionnel.

Principaux résultats

Après priming avec du LPS (lipopolysaccharide) puis stimulation avec de la nigéricine, les specks d'ASC ont été détectés par cytométrie en flux. L'analyse en microscopie confocale de monocytes a montré la formation d'un unique point fluorescent correspondant au speck d'ASC. Nous avons montré qu'après lyse des globules rouges et élimination de l'étape de stimulation par le LPS, la formation des specks d'ASC pouvait être détectée directement en sang total chez des volontaires sains (n=6) après stimulation avec de la nigéricine, à la fois dans les monocytes et les neutrophiles. Comme preuve de concept, chez 4 patients septiques, nous avons montré que cette technique permettait de détecter la baisse des capacités de réactivation de l'inflammasome NLRP3 en accord avec la notion de dépression immunitaire induite par le sepsis.

Conclusion

Nous avons établi un protocole simplifié et raccourci, en sang total, qui devrait faciliter le suivi en clinique de l'activation et de l'épuisement du NLRP3 inflammasome, à la fois dans les monocytes et les neutrophiles



Article

# Monitoring NLRP3 Inflammasome Activation and Exhaustion in Clinical Samples: A Refined Flow Cytometry Protocol for ASC Speck Formation Measurement Directly in Whole Blood after Ex Vivo Stimulation

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**Abstract:** Alteration of NLRP3 inflammasome pathway including hyper-activation or exhaustion has been implicated in the pathophysiology of many diseases. Following cell stimulation, aggregation of the ASC protein into a multiprotein complex, the ASC speck, has been proposed as a specific read-out for monitoring NLRP3 inflammasome activation by flow cytometry in clinical samples. So far, only a few papers have described a technique to detect ASC speck formation directly in whole blood without any cell purification, and none included an ex vivo stimulation. The objective of this study was thus to develop a simple and shortened flow cytometry protocol to detect ASC speck formation directly in whole blood including an ex vivo stimulation step. We showed that after red blood cells lysis and removal of the LPS stimulation step, ASC speck formation can be detected in both monocytes and neutrophils from healthy donors directly in nigericin-stimulated whole blood samples. Using samples from four septic shock patients, we showed that this technique allows for the detection of NLRP3 inflammasome exhaustion in clinical samples. This novel shortened and simple whole blood protocol should facilitate day-to-day monitoring of NLRP3 inflammasome activation and exhaustion in both monocytes and neutrophils in clinical studies.

**Keywords:** NLRP3 inflammasome; ASC speck; flow cytometry; protocol; whole blood

## 1. Introduction

Inflammasomes are multiprotein platforms involved in innate immune response. They are activated following danger signal recognition in a two-step process called "priming" and "activation", leading to the cleavage of the pro-caspase-1 in activated caspase-1. NLRP3 is the most studied canonical inflammasome. It is composed of a sensor (NLRP3), recruiting a central adaptor protein known as ASC (apoptosis-associated speck-like protein containing a caspase activating and recruitment domain), which polymerizes after activation to a supramolecular signaling platform called the ASC speck. This leads to the recruitment and activation of caspase-1, responsible for pro-IL1- $\beta$  and pro-IL-18 cleavages into their pro-inflammatory active forms [1].



Aberrant activation of NLRP3 inflammasome has been implicated in the pathophysiology of many inflammatory diseases such as auto-inflammatory periodic fever syndromes, including cryopyrin-associated periodic syndromes [2] and familial Mediterranean fever [3] but also in cancer, diabetes, gout, cardiovascular [4] and neurological disorders [5]. Conversely, altered NLRP3 inflammasome activation capacity called NLRP3 inflammasome exhaustion has been described in infectious diseases such as sepsis [6] or severe COVID-19 [7] in association with deleterious outcomes. This highlights the necessity to develop robust immunomonitoring assays to evaluate NLRP3 inflammation activation and exhaustion in clinical samples.

Monitoring of NLRP3 inflammasome activation in human samples has mainly been performed through detection of IL1- $\beta$ , IL-18 secretion or caspase-1 activation after cell stimulation *ex vivo*. However, these techniques require lengthy cell purification and/or stimulation steps, which may have limited their use in clinical studies. More recently, it has been shown that NLRP3 activation can be monitored through the detection of the intracytoplasmic polymerization of the ASC protein (i.e., speck formation) [8], which might represent a more specific read-out of NLRP3 inflammasome activation than cytokine secretion measurement [9]. Indeed, before activation, a weak and diffuse ASC fluorescence is observed in cells. After activation, ASC aggregates into a single concentrated bright fluorescent speck in the perinuclear region, which can be detected by flow cytometry, while the rest of the cell is mostly depleted of any fluorescence signal [10].

So far, only a few papers have described a technique to detect ASC speck formation directly in whole blood without any cell purification. In addition, to the best of our knowledge, none of these protocols included an *ex vivo* stimulation. This is very important as such stimulation step will allow for the detection of NLRP3 inflammasome pathway exhaustion in clinical studies in addition to the monitoring of basal NLRP3 inflammasome pathway activation *in vivo*.

In this context, the objective of the current work was to define a simple flow cytometry protocol for the measurement of ASC speck formation directly in human whole blood including an *ex vivo* stimulation step. Such refined technique will help to simultaneously assess basal NLRP3 pathway activation level *in vivo* and its inhibition or exhaustion following re-activation *ex vivo* in clinical studies.

## 2. Materials and Methods

### 2.1. Reagents

The following antibodies were used: CD45-PB, CD14-APC (both from Beckman Coulter, Brea, CA, USA, clones J33 and RMO52, respectively), CD15-AF700 and anti-ASC-PE (both from Biolegend, San Diego, CA, USA, clones W6D3 and HASC-71, respectively). Following reagents were used: phosphate-buffered saline (PBS) (Eurobio Scientific, Luxembourg, Luxembourg), Ficoll (Dutscher, Bruxelles, Belgium), RPMI HEPES (Eurobio, Les Ulis, France), penicilline/streptomycine (Clinisciences, Nanterre, France), fungizone (Cheplapharm Arzneimittel, Mesekehagen, Germany), glutamine (Ozyme, Saint-Cyr-l'École, France), versalyse (Beckman Coulter), lipopolysaccharide (LPS) (Sigma-Aldrich, single mix of references L3137, L2637 and L3012, Saint-Louis, MO, USA), nigericin (InvivoGen, San Diego, CA, USA), water for injection (Aguettant, Lyon, France), Cytofix/Cytoperm, Fixation/Permeabilization Kit, stain buffer (both from BD Bioscience, Franklin Lakes, NJ, USA) and human AB serum (SAB) ("Etablissement Français du Sang", Lyon, France).

### 2.2. Blood Sample Processing

Fresh peripheral blood from healthy donors (HD) was provided by the Etablissement Français du Sang (French National Blood Bank) from Lyon using heparin anticoagulant tubes. A written non-opposition to the use of donated blood for research purposes was obtained from the HD. PBMCs were isolated using density gradient centrifugation.

Briefly, heparin blood was diluted 1:1 with PBS, carefully pipetted on the ficoll solution and centrifuged for 20 min at 770 g with low acceleration and low brake applied. The PBMCs ring was removed by pipetting and the cells washed with PBS by centrifugation for 10 min at 800 g, 3 times. After the final wash, cells were re-suspended in 1 mL of RPMI HEPES + penicilline/streptomycine (20 µg/mL) + fungizone (2.5 µg/mL + glutamine (2 mM). In addition, 4 septic shock patients, with blood drawn during the first 48 h after ICU admission, were tested to illustrate the potential of such whole blood protocol in clinical samples. Patients were included in the IMMUNOSEPSIS-4 cohort. Diagnostic criteria for septic shock was based on the Sepsis-3 definition [11]. Exclusion criteria disqualified patients under 18 years of age, subjects with aplasia or pre-existent immunosuppression, pregnant women, and institutionalized patients. This project was approved by Institutional Review Board for ethics ("Comité de Protection des Personnes Ouest II", n° RCB: 2019-A00210-57). This study is registered at the French Ministry of Research and Teaching (#DC-2008-509), at the Commission Nationale de l'Informatique et des Libertés, and on clinicaltrials.gov (#NCT04067674).

### 2.3. ASC Staining in PBMCs

In this stage, 2.5 million cells/mL in RPMI HEPES were incubated with or without LPS at 0.1 µg/mL for 3 h, at 37 °C, followed by incubation with or without nigericin at 10 µg/mL for additional 30 min, at 37 °C. Cells were washed with 1 mL of stain buffer, centrifuged for 6 min at 300 g and supernatant removed. PBMCs were then labelled with 5 µL of PB-labelled anti-CD45 antibody and 5 µL of APC-labelled anti-CD14 antibody, incubated for 15 min, at room temperature, in the dark and washed with 1 mL of stain buffer for 6 min at 300 g. Permeabilization was performed by using 100 µL of Cytoperm reagent, incubated for 20 min, at 4 °C. After two washing steps with 1 mL of 1:10 diluted Perm/Wash buffer, cells were incubated with PE-labelled anti-ASC antibody for 20 min, at room temperature, in the dark. A final washing was performed with diluted Perm/Wash buffer, supernatant removed, and cells re-suspended in 300 µL of PBS. Samples were run on a BD FACS ARIA II (BD Bioscience, Franklin Lakes, NJ, USA) and results expressed as percentage of ASC speck-positive cells among respective cell populations.

### 2.4. Cell Sorting and Confocal Microscopy

In stimulated and unstimulated conditions, monocytes were sorted on a BD FACS ARIA II cytometer (BD Bioscience). Among PBMCs, monocytes were first selected based on CD14 expression. Cells were then sorted based on ASC staining profile with a homogeneous ASC-staining profile of unstimulated monocytes and a bi-modal ASC staining in monocytes after LPS and nigericin stimulations. Sorted cells were centrifuged for 6 min at 2200 g, supernatant removed and cells re-suspended in 200 µL of SAB. Finally, cells were fixed on a glass slide using a cytospin for 5 min at 3000 rpm. Cells were imaged using Confocal Zeiss LSM980 (Carl Zeiss, Marly le Roi, France) and 10x objectives. Images were analyzed using ZEN lite software (version 3.4, Carl Zeiss, Marly le Roi, France).

### 2.5. ASC Staining in Whole Blood

In order to remove red blood cells, 100 µL of whole heparin blood was first diluted in 1 mL of lysis solution (Versalysé) for 10 min, at room temperature, in the dark. Cells were then washed with 1 mL of PBS for 10 min, at room temperature, in the dark and centrifuged for 6 min at 300 g. After supernatant removal, cells were re-suspended in 500 µL of PBS and incubated with or without LPS at 0.1 µg/mL for 3 h, at 37 °C, then with or without nigericin at 10 µg/mL for 30 min, at 37 °C. Cells were washed with 1 mL of stain buffer, centrifuged for 6 min at 300 g and supernatant removed. Cells were then labelled with 5 µL of PB-labelled anti CD45-antibody, 5 µL of APC-labelled anti-CD14 antibody and 5 µL of AF700-labelled anti-CD15 antibody, incubated 15 min, at room temperature, in the dark and washed with 1 mL of stain buffer for 6 min at 300 g. A permeabilization

step was performed by using 250  $\mu$ L of Cytoperm, incubated for 20 min, at 4 °C. After two washings steps with 1 mL of 1:10 diluted Perm/Wash buffer, cells were labelled with 5  $\mu$ L of PE-labelled anti-ASC antibody for 20 min, at room temperature, in the dark. A final wash was performed with 1 mL of diluted Perm/Wash buffer, supernatant removed and cells re-suspended in 300  $\mu$ L of PBS. Samples were run on a FACS ARIA II cytometer and results expressed as percentage of ASC speck-positive cells among respective cell population.

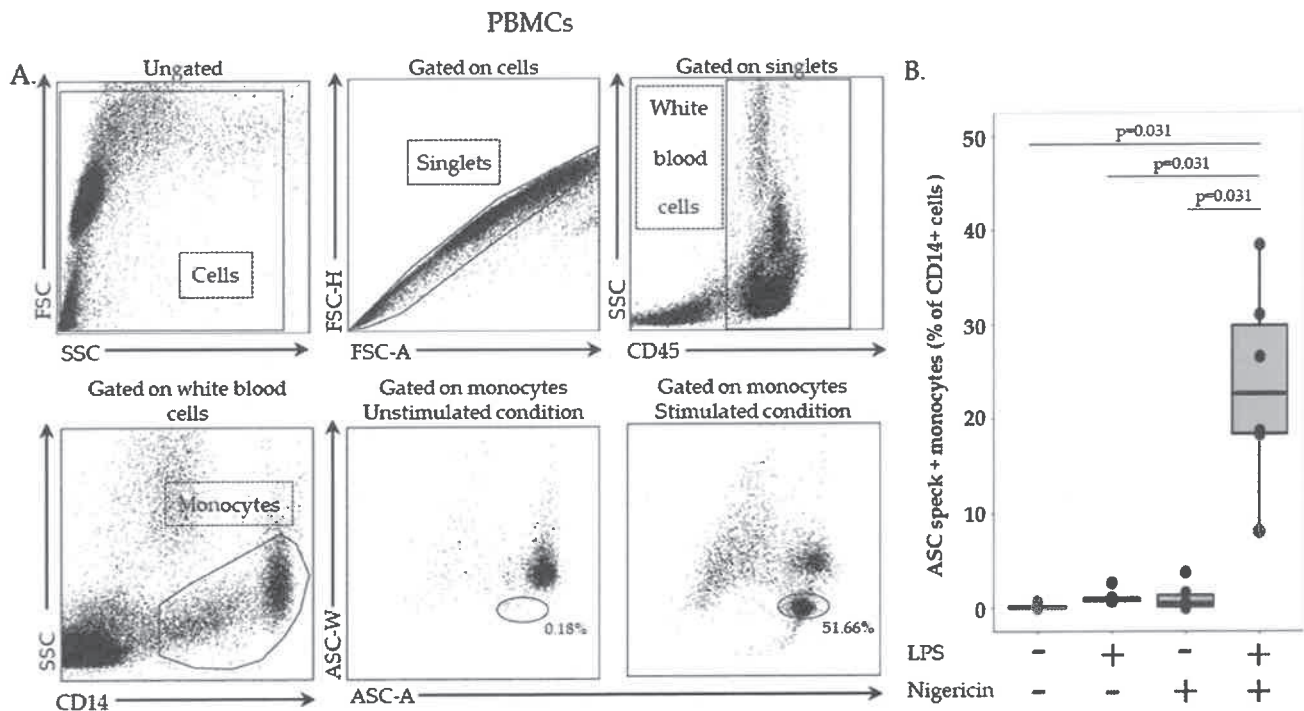
### 2.6. Statistical Analysis

Results were presented as individual values and boxplots. Statistical analysis were performed using the non-parametric Wilcoxon paired test. Data were analyzed using R Studio software (version 1.2.5001; R studio, Boston, MA, USA).

## 3. Results

### 3.1. Flow Cytometry Measurement of ASC Speck in PBMCs

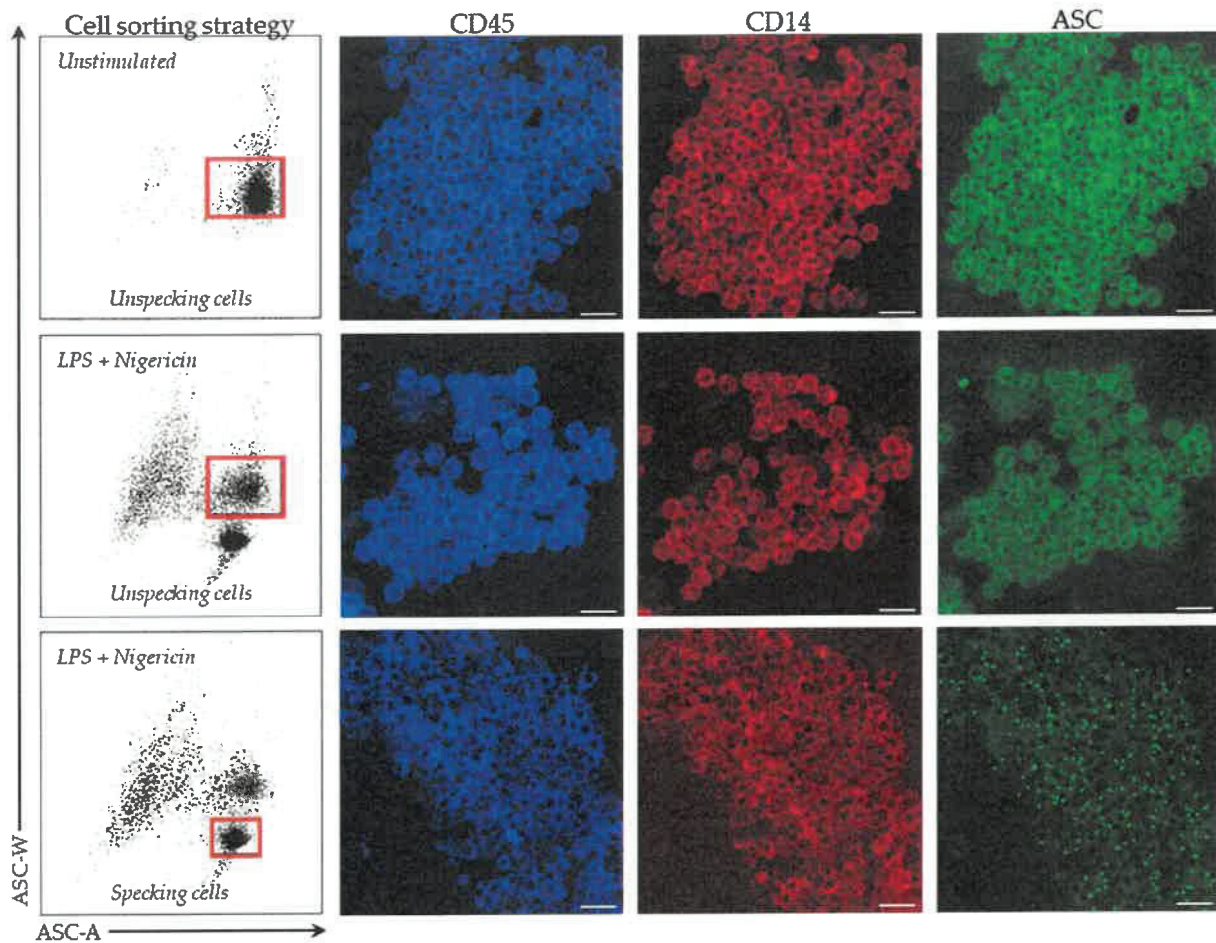
Gating strategy is presented in Figure 1A. Cells were first selected based on side scatter (SSC)/forward scatter (FSC) characteristics and doublets were then excluded using FSC-area/FSC-height histogram. Leukocytes were identified based on CD45 expression. Monocytes were selected based on CD14 expression among CD45+ leukocytes. Finally, monocytes were analyzed for ASC-width (ASC-W) and ASC-area (ASC-A) signals. Without stimulation, monocytes showed a homogenous population presenting high ASC-W and high ASC-A. After stimulation, cells with ASC speck formation could be detected based on a diminished ASC-W signal. Results obtained in six HD are depicted in Figure 1B. Without stimulation or in case of stimulation with either LPS or nigericin alone, percentages of ASC speck-positive monocytes remained low (i.e., 0.06 %, 0.85 % and 0.44 %, respectively (Figure 1B)). In sharp contrast, the percentage of ASC speck-positive monocytes increased significantly following stimulation with both LPS and nigericin (median = 22.7 %,  $p < 0.05$  vs. other conditions).



**Figure 1.** Monitoring of ASC speck-positive monocytes in PBMCs. (A) Gating strategy. After doublet exclusion, monocytes were identified based on CD45 and CD14 expressions and speck formation was assessed on a bi-parametric ASC-area (ASC-A)/ASC-width (ASC-W) histogram gated on selected cells. ASC speck-positive cells are presented with diminished ASC-width (circle) in comparison with non-specking cells. Results obtained in one representative healthy donor PBMCs without stimulation or treated for 3 h with 0.1  $\mu\text{g}/\text{mL}$  LPS prior to inflammasome induction using 10  $\mu\text{g}/\text{mL}$  nigericin for 30 min are presented. (B) Percentage of ASC speck-positive monocytes from 6 healthy donors are shown. Results of unstimulated PBMCs or after PBMCs incubation with or without LPS at 0.1  $\mu\text{g}/\text{mL}$  for 3 h, at 37 °C, followed by incubation with or without nigericin at 10  $\mu\text{g}/\text{mL}$  for additional 30 min, at 37 °C, are shown. Results were expressed as percentage of ASC speck-positive cells among CD14+ monocytes and are presented as individual values and box-plot. Statistical analysis were performed using the non-parametric Wilcoxon paired test.

### 3.2. Microscopic Detection of ASC Speck in PBMCs

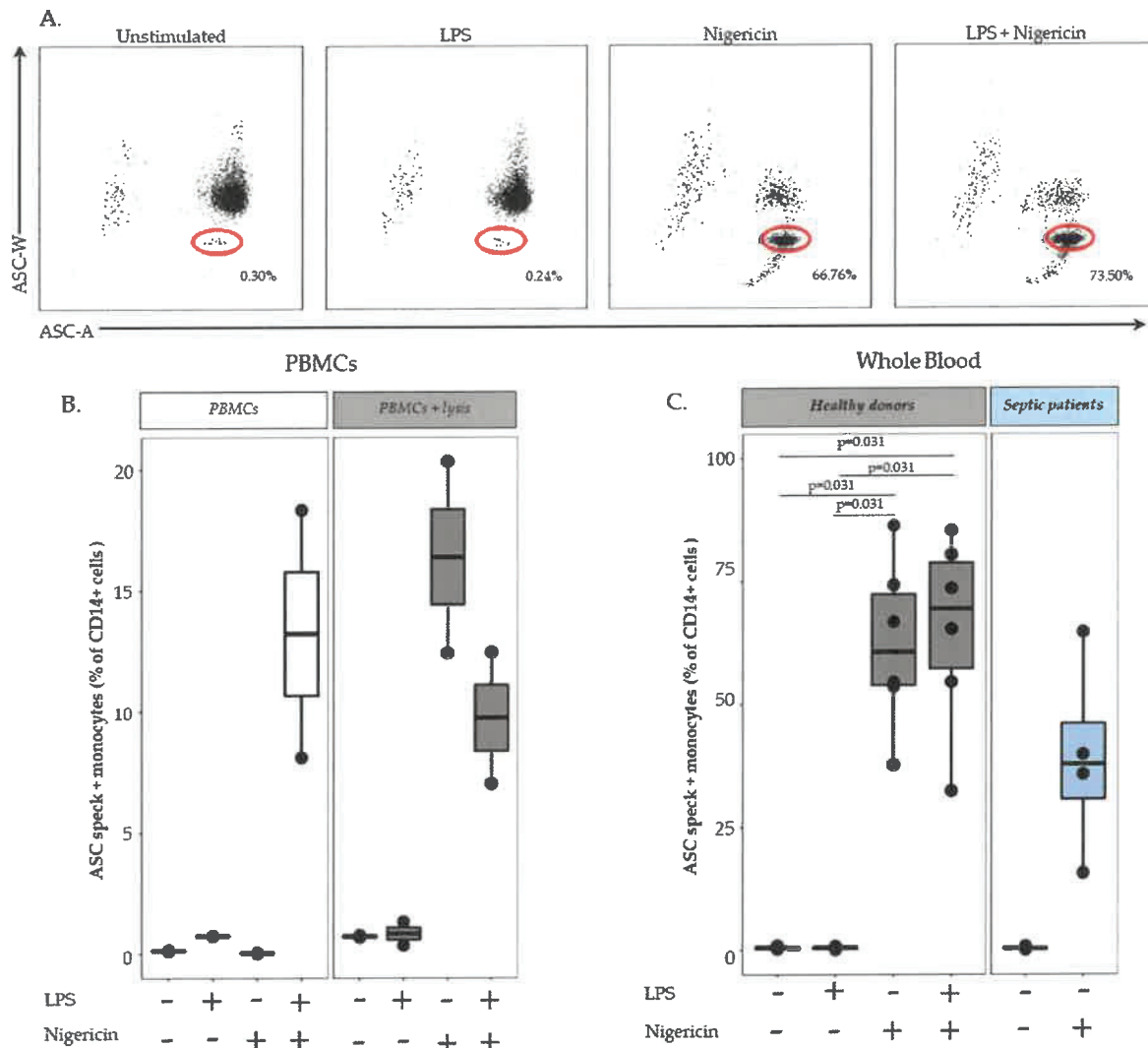
To confirm that the modification of ASC staining profile observed in monocytes after stimulation corresponded to ASC speck formation in these cells, unstimulated resting monocytes, and both ASC speck-positive and ASC speck-negative monocytes after LPS and nigericin stimulations, were purified (Figure 2 left panels) to be analyzed by confocal microscopy. By microscopy, purified monocytes are presented with clear CD45 (blue) and CD14 (red) expressions. As expected (Figure 2 right panels), a diffuse ASC fluorescence (green) was observed in sorted non-stimulated monocytes and ASC speck-negative monocytes after stimulations. In contrast, in ASC speck-positive monocytes, ASC concentrated into a single bright fluorescent speck in the perinuclear region, depleting the rest of the cell of the fluorescence signal. This confirmed the reliability of flow cytometry analysis to specifically distinguish ASC speck-positive monocytes.



**Figure 2.** ASC speck detection by confocal microscopy in sorted monocytes. Fluorescence confocal microscopic images of ASC speck formation in PBMCs purified from 1 healthy donor after flow cytometry cell sorting in unstimulated situation or after 3 h stimulation with 0.1  $\mu\text{g}/\text{mL}$  LPS prior to inflammasome induction using 10  $\mu\text{g}/\text{mL}$  nigericin for 30 min are shown. Respective sorted populations are identified by red squares. Cells were stained for CD45 (blue), CD14 (red) and ASC (green). Monocytes were identified on the basis of CD45 (blue) and CD14 (red) expressions. Scale bar, 40 $\mu\text{m}$

### 3.3. Measurement of ASC Speck-Positive Monocytes in Whole Blood by Flow Cytometry

We next attempted to transfer this protocol to whole blood samples. Thus, we added a lysis step to remove red blood cells prior to stimulation and staining (as previously described). A representative example of a flow cytometry staining in one healthy donor is shown in Figure 3A, and the results obtained in six HD are presented in Figure 3C (left panel). Similarly to previous observation in PBMCs, no monocytes with speck were detected in unstimulated condition or after stimulation with LPS alone. Unexpectedly, unlike previous results in PBMCs, stimulation with nigericin alone led to elevated percentage of ASC speck-positive monocytes (medians = 60.6 % vs. 0.44 % in PBMCs). Moreover, stimulation with both LPS and nigericin strongly increased percentages of monocytes with speck compared with results observed in PBMCs (medians = 69.4 % vs. 22.7 %, respectively).



**Figure 3.** Monitoring of ASC speck-positive monocytes in whole blood and in PBMCs. (A) Detection of ASC speck-positive monocytes. Using a similar gating strategy as described in Figure 1 for PBMCs, speck formation was assessed in whole blood. Example of a bi-parametric ASC-area (ASC-A)/ASC-width (ASC-W) histogram in one representative healthy donor is shown. Activation conditions are indicated above each panel. Briefly, 100  $\mu$ L of whole blood was treated with or without LPS at 0.1  $\mu$ g/mL for 3 h, at 37  $^{\circ}$ C, followed by incubation with or without nigericin at 10  $\mu$ g/mL for additional 30 min, at 37  $^{\circ}$ C, or left unstimulated. The percentages of ASC speck-positive cells among CD14+ monocytes (identified by red circles) in each condition are shown. (B) ASC speck formation upon stimulation in healthy donors. PBMCs from healthy donors ( $n = 2$ ) were treated (right panel) or not (left panel) with lysis solution for 10 min, then with or without LPS at 0.1  $\mu$ g/mL for 3 h, at 37  $^{\circ}$ C, followed by incubation with or without nigericin at 10  $\mu$ g/mL for additional 30 min, at 37  $^{\circ}$ C, or left unstimulated. (C) ASC formation upon stimulation in healthy donors and septic patients. Here, 100  $\mu$ L of whole blood from healthy donors ( $n = 6$ ) was treated with or without LPS at 0.1  $\mu$ g/mL for 3 h, at 37  $^{\circ}$ C, followed by incubation with or without nigericin at 10  $\mu$ g/mL for additional 30 min, at 37  $^{\circ}$ C, or left unstimulated. Then, 100  $\mu$ L of whole blood from septic patients ( $n = 4$ ) within the first 48 h after ICU admission were treated for 30 min using 10  $\mu$ g/mL nigericin or unstimulated. Results were expressed as percentage of ASC speck-positive cells among CD14+ monocytes and are presented as individual values and box-plot. Statistical analyses were performed using the non-parametric Wilcoxon paired test.

We postulated that red blood cell lysis was responsible for monocyte priming so that activation with nigericin alone was sufficient to induce ASC speck formation in monocytes. To confirm this hypothesis, PBMCs from 2 HD were treated or not with red blood

cell lysis buffer prior to nigericin stimulation (Figure 3B). In untreated samples, we confirmed that no ASC speck formation was detected in samples treated with nigericin alone, while a good response was measured in samples treated with LPS + nigericin (Figure 3B, right panel). In contrast, in PBMCs treated with cell lysis buffer, nigericin stimulation alone led to an increased percentages of monocytes forming speck equivalent to the one observed in cells stimulated with LPS and nigericin (Figure 3B, left panel). This confirmed that in this experimental setting, red blood cell lysis was sufficient to induce cell priming of NLRP3 inflammasome pathway.

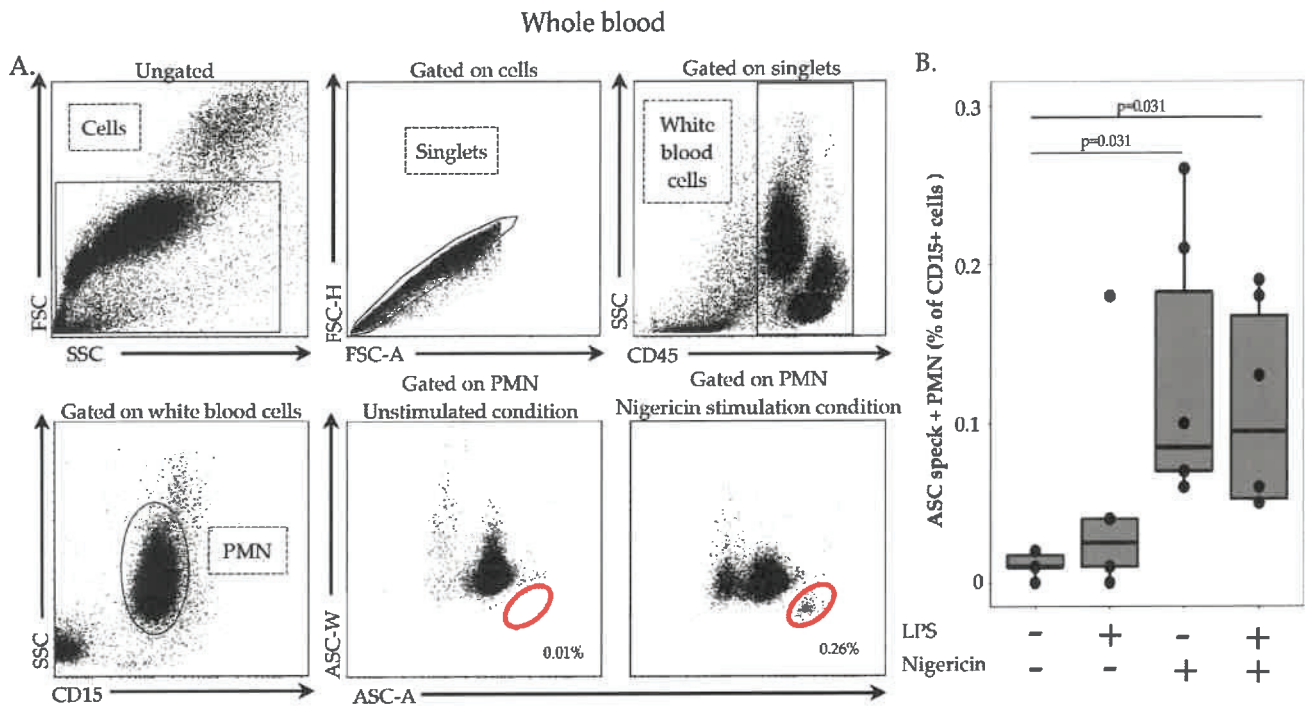
Finally, we assessed the ASC speck formation in samples from four septic shock patients sampled within the first 48 h after ICU admission (Figure 3C right panel). These patients were elderly male (median age = 64 years) and presented with high severity scores (median of Sequential Organ Failure Assessment score (SOFA) = 10.5 and median of Simplified Acute Physiology Score II (SAPS II) = 50.5). These preliminary results showed a decreased percentage of ASC speck-positive monocytes (median = 38 %) following stimulation with nigericin alone compared to HD.

Thus we have set-up a whole blood flow cytometry protocol to monitor ASC speck formation in monocytes usable in clinical samples.

#### *3.4. Measurement of ASC Speck-Positive Polymorphonuclear Neutrophils (PMN) in Whole Blood by Flow Cytometry*

In contrast to PBMCs, the use of a whole blood flow cytometry protocol enables the study of ASC speck formation in PMN; thus, we evaluated ASC speck formation in neutrophils using the current technique. The initial gating strategy for PMN identification was similar to monocytes (Figure 4A). PMN were specifically selected on their positive CD15 expression. As for monocytes, ASC speck-positive cells were identified based on their diminished ASC-width signal. In six HD, ASC speck formation in non-stimulated PMN was low (Figure 4B, median = 0.01). Upon stimulation by nigericin alone or LPS and nigericin, the percentage of ASC speck-positive PMN increased significantly (Figure 4B). However, the percentage of ASC speck-positive PMN remained low after stimulation in comparison with results observed in monocytes (Figure 4B).

Finally, preliminary results obtained in septic shock patients did not show any decrease in the percentage of ASC speck-positive PMN after stimulation compared with HD (data not shown).



**Figure 4.** Monitoring of ASC speck-positive neutrophils in whole blood. (A) Detection of ASC speck-positive neutrophils. After doublet exclusion, neutrophils were identified based on CD45 and CD15 expressions. Then, ASC speck formation was assessed in selected cells based on a bi-parametric ASC-area (ASC-A)/ASC-width (ASC-W) histogram. Specking cells presented with diminished ASC-width (red circle) in comparison with non-specking cells. Representative flow cytometry staining in one healthy donor is shown. Activation conditions are indicated above each panel. Briefly, 100  $\mu$ L of whole blood was treated with LPS at 0.1  $\mu$ g/mL for 3 h, at 37  $^{\circ}$ C, followed by incubation with nigericin at 10  $\mu$ g/mL for additional 30 min, at 37  $^{\circ}$ C, or left unstimulated. The percentages of ASC speck-positive cells among CD15+ neutrophils (identified by red circles) in each condition are shown. (B) ASC formation upon stimulation in healthy donors. Here, 100  $\mu$ L of whole blood from healthy donors ( $n = 6$ ) was treated with or without LPS at 0.1  $\mu$ g/mL for 3 h, at 37  $^{\circ}$ C, followed by incubation with or without nigericin at 10  $\mu$ g/mL for additional 30 min, at 37  $^{\circ}$ C, or left unstimulated. Results were expressed as percentage of ASC speck-positive cells among CD15+ PMN and are presented as individual values and box-plot. Statistical analysis were performed using the non-parametric Wilcoxon paired test.

**4. Discussion**

NLRP3 inflammasome is implicated in a number of highly prevalent clinical conditions and has been consequently largely investigated by different techniques both in experimental set-up and at clinical levels. A first approach consists of detecting inflammasomes activation through cytokines production (namely IL-1 $\beta$  and IL-18) commonly detected by ELISA or immunoblot analysis in plasma or after ex vivo stimulation of purified cells. However, IL-1 $\beta$  cleavage and release may occur through inflammasome-independent processes [12]. Thus, this approach may not be specific to inflammasomes. An additional method is based on the detection of caspase 1 activation through immunoblot of purified cells or by flow cytometry in whole blood [13]. This latest technology presents the benefit of studying the caspase 1 activation in specific cell types and subpopulations and has been used to investigate the NLRP3 inflammasome activation in COVID-19 patients [13]. However, as for cytokine release assays, caspase 1 activation may not be fully specific of inflammasome activation but may be confounded with other non-inflammasome-related caspase or protease activities [9]. Another way of studying inflam-



masomes activation relies on cell death detection, but the distinction of pyroptosis (specific to the inflammasome related cell death) from other forms of cell death remains challenging [14].

Overall, direct assessment of the ASC polymerization at the cellular level appears as the most specific technique to monitor inflammasomes activation. In addition, the use of nigericin stimulation provides a specific activation for NLRP3 inflammasome pathway among other inflammasomes. ASC speck formation could be assessed by microscopy [15]. However, this approach is not suitable for large-scale human studies. To circumvent microscopy practical drawbacks in clinical research, Sester and colleagues took advantage of flow cytometry to assess ASC speck polymerization in PBMCs following stimulation *ex vivo* [8]. With this approach, Martínez-García et al. reported the profoundly altered NLRP3 activation in septic patients in association with mortality [6]. Recently, after evaluation of various pre-analytical conditions (type of anticoagulant, temperature and sample storage time, etc.), Wittman and his team provided technical recommendations regarding ASC specks detection in human PBMCs [16]. In order to facilitate clinical explorations, Cui et al. reported a whole blood protocol without any subsequent *ex vivo* stimulation to study *in vivo* NLRP3 inflammasome activation level [17]. Consequently, the objective of the present study was to set up a refined whole blood protocol for ASC speck formation monitoring in primary human cells including an *ex vivo* stimulation step to follow both *in vivo* NLRP3 inflammasome activation level and *ex vivo* re-activation capacity. To the best of our knowledge, no study has, so far, described such a technique.

First, we demonstrated that the characteristic signal of ASC speck formation by flow cytometry (i.e., diminished fluorescence width) observed in PBMCs was also observed in whole blood. By using confocal microscopy on flow cytometry-sorted cells, we confirmed that cells identified by diminished ASC-width by flow cytometry show ASC speck. Based on this, we were able to monitor NLRP3 activation in whole blood from both HD and septic patients. In line with data reported by Martínez-García et al., our preliminary results showed a marked decrease in ASC speck formation in cells from patients suffering sepsis when sampled within the first 48 h after ICU admission.

In whole blood assay, high amount of ASC speck-positive monocytes were detected after LPS and nigericin stimulation as expected. Surprisingly, a similar level of ASC speck-positive monocytes was detected when stimulated with nigericin alone. Although the priming step of NLRP3 activation for cytokine release has been demonstrated to be sometimes dispensable [18,19]; priming (with LPS) preceding activation (with ATP or nigericin) considerably increases the activation of the inflammasome. It is noteworthy that we added a lysis pre-treatment step in the whole blood assay to remove red blood cells. We confirmed experimentally by using lysis buffer prior to NLRP3 inflammasome stimulation of PBMCs that cell stress caused by the initial use of lysis solution or that damage-associated molecular pattern molecules (DAMPs) released from lysed red blood cells could provide the priming signal. As a result, stimulation with nigericin alone following lysis buffer treatment enabled us to detect an increased percentages of monocytes with speck compared with results in PBMCs without lysis buffer treatment. Such a removal of the LPS stimulation step resulted in an easier and time-saving protocol.

Another asset of the described protocol is to allow the exploration of neutrophil present in whole blood but discarded upon the PBMCs purification step. Although inflammasomes were discovered and mainly studied in monocytes and macrophages, recent studies suggest that the importance of the neutrophil inflammasome has been underestimated [20]. Here, as observed in monocytes, we noticed an increase in ASC speck-positive neutrophils after stimulation. To note, although percentages of ASC speck-positive neutrophils were much lower than in monocytes upon stimulation; ASC speck formation in these cells may nevertheless be clinically relevant considering the high number of circulating neutrophils in healthy donors and their increased number in certain clinical conditions such as sepsis. In addition, these results are consistent with the study of Boucher et

al., in which neutrophils assembled less ASC speck than macrophages [21]. Thus, the current protocol should allow for the robust monitoring of ASC speck formation in neutrophils in clinical samples.

## 5. Conclusions

We developed a whole blood-based flow cytometry protocol to detect baseline ASC speck formation downstream of NLRP3 inflammasome activation and NLRP3 re-activation abilities both in monocytes and neutrophils usable in clinical samples. The removal of two time-consuming steps (i.e., ficoll purification and LPS stimulation) shortened the protocol duration by several hours, and the addition of an ex vivo stimulation step allows for the monitoring of NLRP3 inflammasome exhaustion in clinical samples. Overall, this technique should permit an easier implementation of NLRP3 studies in clinical research protocols.

**Author Contributions:** formal analysis, R.C. and M.G.; funding acquisition, G.M. and F.V.; investigation, M.G., A.-C.L. and G.M.; methodology, M.G., B.F.P., T.H., G.M. and F.V.; supervision, F.V.; writing—original draft, R.C.; writing—review and editing, B.F.P., T.H., A.-C.L., G.M. and F.V. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee ((Comité de Protection des Personnes CPP OUEST II—ANGERS) under agreement number 2019-A00210-57, 05.2019). This project was part of an ongoing prospective observational clinical study (IMMUNOSEPSIS-4).. This clinical study was registered at ClinicalTrials.gov (NCT04067674). The committee waived the need for written informed consent because the study was observational, with a low risk to patients, and no specific procedure, other than routine blood sampling, was required. Oral information and non-opposition to inclusion in the study were mandatory and were systematically obtained before any blood sample was drawn. This was recorded in patients' clinical files. If a patient was unable to consent directly, non-opposition was obtained from the patient's legally authorized representative and reconfirmed from the patient at the earliest opportunity.

**Data Availability Statement:** Data are available from corresponding author upon reasonable request.

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

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## Discussions et perspectives

En 2017, l'OMS a reconnu le sepsis comme un enjeu majeur de santé publique, nécessitant des améliorations en matière de prévention, de diagnostic et de prise en charge. Cette année-là, le sepsis a causé 11 millions de décès à l'échelle mondiale, représentant la première cause de mortalité. Pourtant, contrairement à d'autres maladies telles que le cancer ou l'infarctus du myocarde, le sepsis reste méconnu du grand public. À titre illustratif, moins de 5% des français ont déjà entendu le terme "sepsis"<sup>283</sup>. Sur le plan physiopathologique, le sepsis se caractérise, en réponse à l'infection, par la coexistence d'une réaction inflammatoire et d'une réponse anti-inflammatoire voire immunosuppressive. L'hyperinflammation initiale, déclenchée par l'organisme pour orchestrer la réponse immunitaire, lorsqu'elle n'est pas contrôlée, est à l'origine des défaillances d'organes et des décès précoces survenant lors des premiers jours du sepsis. La réponse compensatrice, développée parallèlement, vise à moduler cette réponse inflammatoire excessive. Cependant, ces mécanismes initialement bénéfiques peuvent persister chez certains patients, entraînant alors une profonde immunosuppression. Cette dernière est caractérisée par des altérations affectant l'ensemble du système immunitaire, compliquant la lutte contre l'infection initiale et favorisant le développement d'infections nosocomiales (y compris avec des germes opportunistes) et les réactivations virales. Cela contribue à la mortalité tardive, majoritaire au cours du sepsis. L'absence de traitement spécifique du sepsis rend la prise en charge de ces patients complexe. Alors que les essais cliniques ciblant l'inflammation initiale ont majoritairement échoué, les stratégies immunostimulantes font l'objet d'un intérêt croissant. Plusieurs thérapies, comme le GM-CSF, l'IFN- $\gamma$  ou l'IL-7 sont actuellement au cœur d'essais cliniques ([Tableau 5](#), page 77). Quelques cas isolés ont rapporté l'efficacité spectaculaire des thérapies anti PD-1 ou anti-PD-L1 dans le traitement compassionnel de patients très sévères<sup>138,284</sup>. Les premiers essais cliniques de phase I et II autour de ces thérapies montrent une bonne tolérance et la restauration des fonctions immunitaires.

Toutefois, une hétérogénéité des réponses immunitaires et inflammatoires est observée chez les patients septiques, tant en termes de cinétique d'apparition, d'intensité ou de durée. Cette hétérogénéité devrait conduire à l'identification de différents endotypes, chacun présentant des réponses distinctes aux thérapies appliquées (*e.g.* les thérapeutiques anti-inflammatoires vs immunostimulantes). Il paraît donc essentiel d'avoir une connaissance la plus exhaustive possible de la physiopathologie immuno-inflammatoire du sepsis pour mieux

catégoriser les patients et permettre l'utilisation de thérapies ciblées, à l'image de ce qui se fait actuellement en cancérologie.

C'est dans ce contexte que s'est inscrit mon projet de thèse. Plus précisément, ce travail visait à mieux décrire différents aspects de l'activation de l'inflammasome NLRP3 et son association avec l'immunodépression décrite au cours du sepsis. En effet, sur la base des connaissances issues de la cancérologie (et des maladies auto-inflammatoires liées à NLRP3) et de certains mécanismes immunosuppresseurs décrits dans le sepsis, nous avons formulé l'hypothèse d'un rôle de l'inflammasome NLRP3 dans la survenue de l'immunodépression induite par le sepsis, en particulier dans la survenue de MDSCs immunosuppresseives.

### *Induction de PMN-MDSC LOX-1+ au cours du sepsis*

Ce projet a débuté sur un plan méthodologique avec l'objectif de simplifier l'identification des MDSCs en recherche clinique. En effet, si les MDSCs d'origine monocyttaire sont aisément identifiables grâce à leur sous-expression de HLA-DR, il est plus complexe d'identifier les MDSCs d'origine granulocytaires. Cela nécessite une purification des PBMC (séparation par gradient de ficoll) puis un phénotypage sur la base de nombreux marqueurs (CD11b<sup>+</sup>CD14<sup>-</sup>CD15<sup>+</sup> ou CD11b<sup>+</sup>CD14<sup>-</sup>CD66b<sup>+</sup>). Néanmoins, en cancérologie, l'équipe de Gabrilovitch a découvert un marqueur spécifique exprimé par les PMN-MDSCs en sang total : le lectin-type oxidized LDL receptor 1 (LOX-1)<sup>206</sup>. Bien que ce soit l'un des récepteurs principaux des LDL (*low-density lipoprotein*) oxydées, aucun lien avec les fonctions immunosuppresseives des MDSCs n'a été formellement établi<sup>206</sup>. N'ayant jamais été décrit dans le sepsis, nous avons entrepris une étude prospective observationnelle pour rechercher la présence de ces PMN-MDSCS exprimant LOX-1 dans 2 contextes de sepsis : les chocs septiques bactériens et la COVID-19, un sepsis d'origine viral.

Après avoir développé un protocole de cytométrie en flux en sang total, nous avons initié une étude chez les patients septiques. Celle-ci a permis d'observer la présence de LOX-1 PMN-MDSCs dans 2 contextes de sepsis (choc septique bactérien et COVID-19). Ces cellules apparaissaient progressivement chez tous les patients, atteignant un pic 7 jours après l'admission en réanimation, *i.e.* durant la phase immunosuppresseive du sepsis ([Article 1](#), page 95).

Bien que l'impact sur les conséquences cliniques restent à déterminer sur une plus grande cohorte, cette étude a montré la faisabilité d'un protocole en sang total permettant de suivre le développement des LOX-1 PMN-MDSCs en sang total. D'autres études ont lié la

présence des PMN-MDSCs aux conséquences cliniques. Uhel et al ont ainsi montré que la proportion de cellules granulocytaires, exprimant l'arginase-1, retrouvées dans les PBMC et pouvant inhiber la prolifération des lymphocytes T, était significativement plus élevée chez les patients septiques développant une infection secondaire<sup>219</sup>. Ces constatations ont été corroborées par Mathias et ses collègues<sup>220</sup>. De plus, une autre étude a souligné la persistance de ces cellules jusqu'à 21 jours après un épisode de sepsis<sup>222</sup>, mettant en évidence le rôle des MDSCs dans les altérations immunitaires persistantes chez ces patients. Dans le contexte de la COVID-19, plusieurs études ont établi une association entre la présence des MDSCs et le pronostic des patients<sup>223</sup>.

Depuis la publication de nos travaux, une dizaine d'études cliniques se rapportant aux MDSCs dans le contexte du sepsis ont été publiées, soulignant l'intérêt croissant pour ces cellules. Dans l'ensemble, ces études ont rapporté une augmentation des MDSCs par rapport aux volontaires sains. Si certaines ont démontré leur effet immunosuppresseur, la plupart se sont principalement concentrées sur la caractérisation phénotypique des cellules issues des PBMC, sans pour autant confirmer leurs capacités immunosuppressives.

A ce jour, notre étude demeure la seule ayant utilisé LOX-1 comme marqueur spécifique des PMN-MDSCs dans le sepsis bactérien, tandis que quelques études ont rapportés la présence de PMN exprimant LOX-1 dans le contexte de la COVID-19, sans pour autant apporter la preuve de leurs capacités immunosuppressives<sup>165,224,285</sup>.

L'identification des PMN-MDSCs exprimant LOX-1 dans le sepsis revêt une importance particulière, facilitant ainsi leur étude en clinique. En cancérologie, domaine où les MDSCs ont été initialement identifiées, les recherches à leur sujet restent prolifiques. Une simple recherche sur PubMed avec les termes « MDSC » et « cancer » révèle plus de 1000 publications pour les années 2022 et 2023. Le recours à LOX-1 comme indicateur des PMN-MDSCs y est également présent<sup>286-289</sup>, attestant de la pertinence de ce marqueur.

Si les études sur les MDSCs liées au sepsis se multiplient, elles offrent toutefois un panorama encore fragmentaire, en particulier sur les mécanismes sous-jacents à leur apparition et persistance.

### *Association entre l'inflammation liée à l'activation de l'inflammasome NLRP3 et l'immunosuppression post-sepsis*

Pour explorer le lien entre l'immunosuppression, MDSCs et l'inflammation liées à l'activation de l'inflammasome NLRP3, nous avons mené une étude prospective sur les patients

septiques de réanimation présentant 2 étiologies différentes : le choc septique bactérien et le sepsis viral (COVID-19) ([Article 2](#), page 96).

Les patients inclus dans cette étude présentaient tous les signes caractéristiques de sepsis (*i.e.* immunosuppression et inflammation). Nous avons validé la présence des LOX-1 PMN-MDSCs chez ces patients, notant également une apparition progressive et différée de ces cellules. Au 7<sup>e</sup> jour d'hospitalisation, nos observations ont révélé une association entre l'activation de l'inflammasome NLRP3 et la présence des MDSCs, que leur origine soit monocytaire ou granulocytaire.

Cependant, le nombre limité de patients inclus dans cette étude n'a pas permis d'établir de conclusions définitives. Pour compléter ces observations, nous avons donc exploité les résultats d'une étude réalisée sur un plus grand nombre de patients septiques (n=107, étude REALISM). L'analyse de clusters basée sur l'expression des ARNm liés à l'activation de NLRP3 a permis de distinguer deux endotypes distincts pour chaque paramètre étudié. Ces endotypes partageaient les caractéristiques suivantes : un premier groupe de patients présentait une réponse inflammatoire initiale modérée et un retour rapide aux valeurs de référence, alors que le second groupe se caractérisait par une intense réponse pro-inflammatoire initiale et persistante au-delà de 28 jours. Au cours du suivi, ce 2<sup>e</sup> groupe hyperinflammatoire montrait des signes d'immunosuppression plus marqués et une différence significative en termes de mortalité à 28 jours. Grâce à cette seconde cohorte, nous avons donc pu renforcer nos premiers résultats et confirmer l'association entre l'activation, à la fois aiguë et persistante, de l'inflammasome NLRP3 et le développement d'une immunosuppression. Plus précisément, l'inflammation liée à NLRP3 était associée au développement et/ou à la persistance des MDSCs. Pour la première fois, nous avons pu observer que les patients présentant la plus forte activation initiale et persistante de NLRP3 conservaient une immunosuppression plus sévère et présentaient une mortalité plus élevée à 28 jours, confirmant l'hypothèse de ce travail.

De nombreuses études mettent en lumière l'hyperactivation de l'inflammasome NLRP3 lors du sepsis, et certaines ont établi un lien avec la sévérité de la maladie ou son issue fatale ([tableau 7](#), page 88). De même, l'immunosuppression qui survient durant le sepsis est désormais bien documentée et est associée au développement d'infections nosocomiales ainsi qu'à la mortalité<sup>20,49,52</sup>. Bien que quelques études aient établi un lien entre l'hyperinflammation initiale du sepsis et l'immunoparalysie observée chez les patients, celles-ci n'étaient pas caractérisées par les marqueurs habituellement utilisés pour définir l'immunosuppression (*e.g.* une diminution de l'expression de mHLA-DR ou une lymphopénie), mais par une diminution des capacités de sécrétions des cytokines après stimulation *ex vivo* (*e.g.* TNF- $\alpha$  ou IL-1 $\beta$ )<sup>264,265</sup>. A

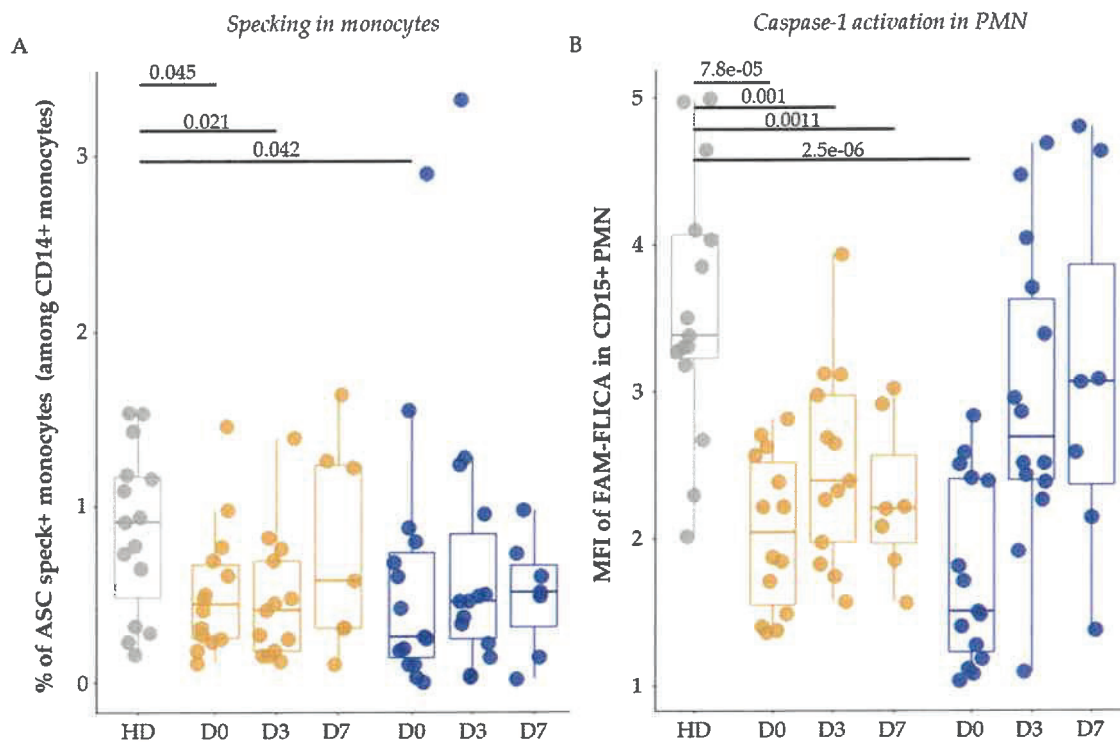
notre connaissance, cette étude est la première à associer l'inflammation, et plus spécifiquement celle liée à l'activation de l'inflammasome NLRP3, à des marqueurs cellulaires d'immunosuppression.

### Activation ex vivo de l'inflammasome NLRP3 en pratique clinique : analyse de la fonctionnalité

Pour approfondir notre étude de l'inflammasome NLRP3 chez les patients septiques, nous avons voulu explorer ses capacités d'activation *ex vivo* à travers un test fonctionnel. En recherche clinique, l'activation de l'inflammasome NLRP3 est souvent simplifiée par la mesure de ses produits finaux, *i.e.* l'IL-1 $\beta$  et l'IL-18 plasmatiques. Cependant, cette méthode manque de spécificité, d'autres inflammasomes et des processus non dépendants de ceux-ci peuvent générer ces produits<sup>290</sup>, ne fournissant donc que des informations parcellaires de l'activation de l'inflammasome NLRP3. En sang total, la détection de la capase-1 est possible grâce à une sonde fluorescente spécifique de sa forme activée. Mais cette approche souffre des mêmes limitations liées à la non-spécificité. La détection des specks d'ASC offre une autre approche pour suivre l'activation de la voie de NLRP3. Toutefois, il n'y avait pas de test fonctionnel disponible pour évaluer la formation de specks *ex vivo* en sang total. En nous appuyant sur de récentes recommandations pour la détection de ces specks<sup>291</sup>, nous avons développé un test fonctionnel en sang total pour détecter la formation des specks d'ASC *in vivo* et *ex vivo* après stimulation ([Article 3](#), page 97). L'utilisation de ce protocole chez quelques patients septiques a prouvé sa faisabilité en recherche clinique et a mis en évidence une capacité de réactivation de NLRP3 diminuée.

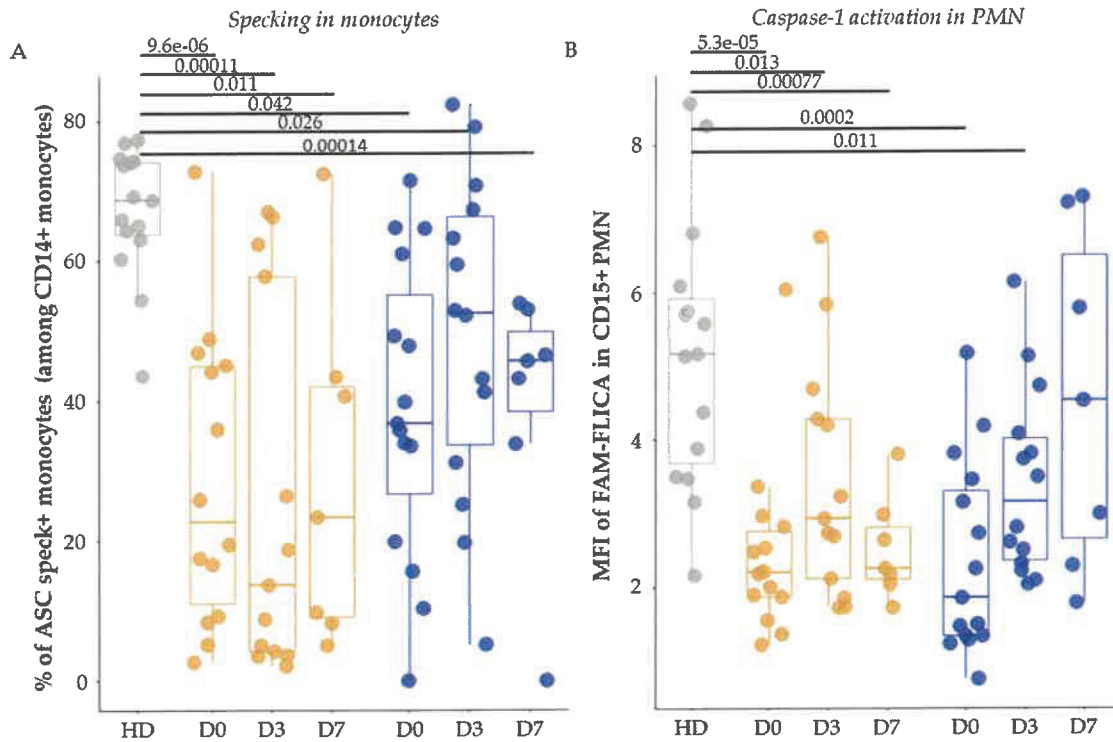
Pour approfondir notre compréhension de l'activité de NLRP3 *in vivo* (*i.e.* avant stimulation) et *ex vivo* (*i.e.* après stimulation par nigéricine), nous avons mené une étude prospective en sang total. Cette étude s'est concentrée sur la détection des specks d'ASC dans les monocytes et la détection de la caspase-1 activée dans les PMN (test FAM-FLICA). Nous avons inclus des patients souffrant de sepsis, qu'il soit d'origine bactérienne (n= 17 en choc septique) ou virale (n=15 atteints de COVID-19 en réanimation), et les avons comparés à un groupe de volontaires sains (n=15). L'analyse de ces données est toujours en cours, nous ne présenterons donc que quelques résultats préliminaires.





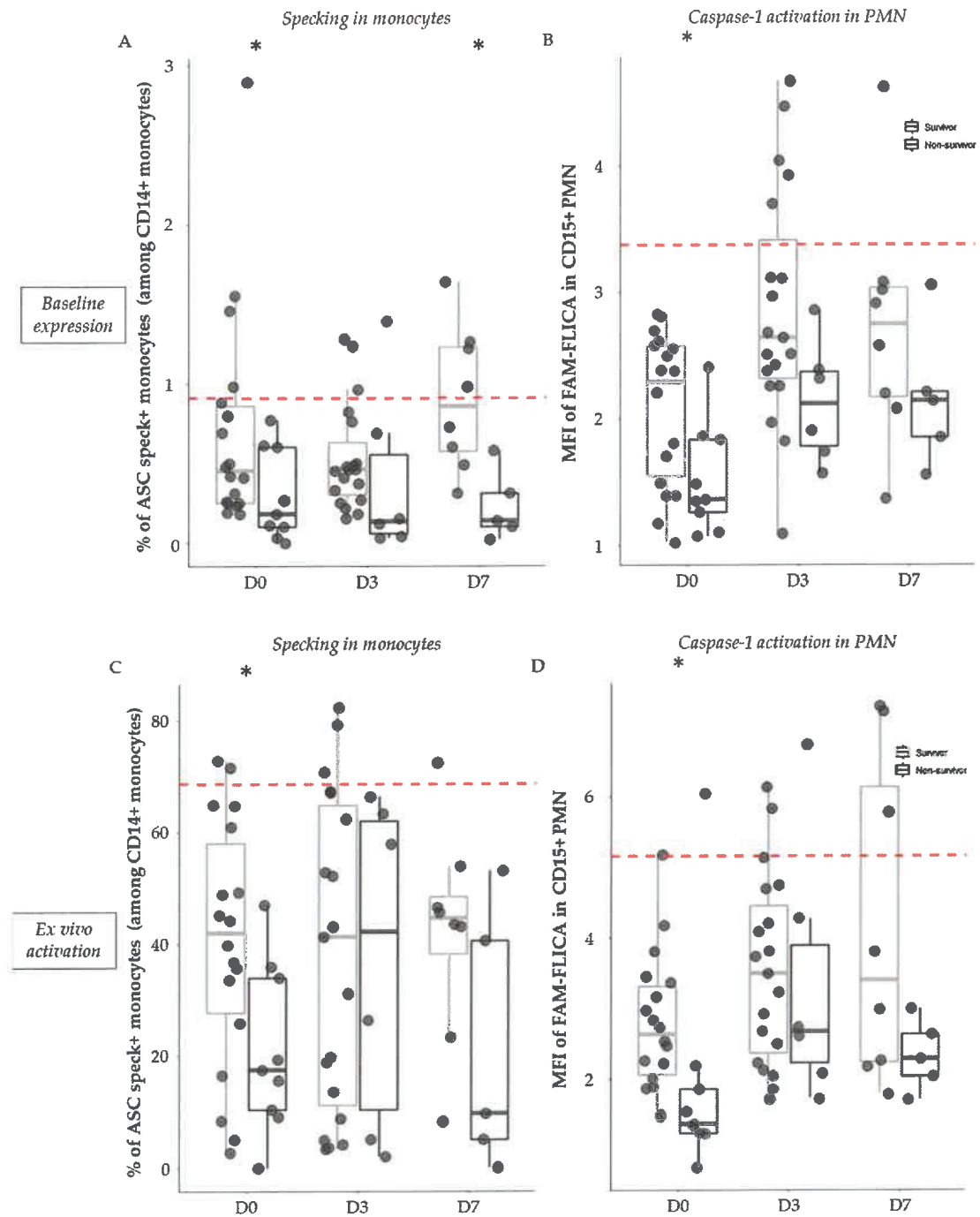
**Figure 22. Expression basale de l'inflammasome NLRP3 chez les patients septiques**  
 Le pourcentage de monocytes présentant des specks (A) et l'activité de la caspase-1 dans les PMN (B) ont été mesurés chez des volontaires sains (HD, en gris, n=15) ainsi que chez des patients atteints de COVID-19 (en doré, n=15) ou de choc septique (en bleu, n=17) lors de la première semaine d'hospitalisation. Ces analyses ont été réalisées lors des 48 premières heures d'hospitalisation chez les patients atteints de COVID-19 (D0, n=14) entre le 3<sup>e</sup> et le 5<sup>e</sup> jour d'hospitalisation (D3, n=13), et entre le 6<sup>e</sup> jour et le 9<sup>e</sup> jour d'hospitalisation (D7, n=7), ainsi que chez les patients atteints de choc septique (D0, n=15 ; D3, n=14 ; D7, n=7). Les résultats sont exprimés en pourcentage de cellules exprimant des specks parmi les monocytes ou en MFI et sont présentés sous forme de boxplots. Le test non paramétrique de Mann-Whitney a été utilisé pour comparer les valeurs entre les témoins et les patients.

Concernant l'expression basale de l'inflammasome NLRP3 (*i.e.* sans stimulation *ex vivo*), nous avons observé que les 2 groupes de patients présentaient des pourcentages plus faibles de monocytes formant des specks à J0 (**figure 22A**). Cette tendance persistait durant la première semaine, significativement chez les patients atteints de COVID-19 à J3. Concernant l'activité de la caspase-1 chez les PMN, nous avons constaté une diminution de son activité pendant toute la première semaine chez les patients hospitalisés pour COVID-19, tandis que chez les patients en choc septique, cette diminution n'était significative qu'à J1 (**figure 22B**).



**Figure 23. Activation ex vivo de l'inflammasome NLRP3 chez les patients septiques**  
 Le pourcentage de monocytes présentant des specks (A) et l'activité de la caspase-1 dans les PMN (B) ont été mesurés chez des volontaires sains (HD, en gris, n=15) ainsi que chez des patients atteints de COVID-19 (en doré, n=15) ou de choc septique (en bleu, n=17), après stimulation par de la nigéricine à 10µg/mL pendant 30 minutes, lors de la première semaine d'hospitalisation. Ces analyses ont été réalisées lors des 48 premières heures d'hospitalisation chez les patients atteints de COVID-19 (D0, n=14) entre le 3<sup>e</sup> et le 5<sup>e</sup> jour d'hospitalisation (D3, n=13), et entre le 6<sup>e</sup> jour et le 9<sup>e</sup> jour d'hospitalisation (D7, n=7), ainsi que chez les patients atteints de choc septique (D0, n=15 ; D3, n=14 ; D7, n=7). Les résultats sont exprimés en pourcentage de monocytes exprimant des specks parmi les monocytes CD14+ ou en MFI et sont présentés sous forme de boxplots. Le test non paramétrique de Mann-Whitney a été utilisé pour comparer les valeurs entre les témoins et les patients.

Ex vivo, après stimulation par la nigéricine, les monocytes des 2 groupes de patients septiques ont montré une activation réduite de l'inflammasome NLRP3. Nous avons observé une diminution importante de la formation des specks d'ASC pendant la première semaine d'hospitalisation (figure 23A). Cette diminution était particulièrement prononcée chez les patients atteints d'une infection à SARS-CoV2 (figure 23A). Une tendance similaire est observée lors de l'analyse de l'activation de la caspase-1 dans les PMN après stimulation par la nigéricine. Il y avait une forte diminution tout au long de la première semaine, bien que cette diminution n'était pas significative à J7 chez les patients souffrant de choc septique (figure 23B).



**Figure 24. Expression basale et activation ex vivo de l'inflammasome NLRP3 en fonction de la survie à 28 jours**

Le pourcentage de monocytes présentant des specks et l'activité de la caspase-1 dans les PMN ont été mesurés chez les patients septiques ( $n=32$ ) au cours des 48 premières heures d'hospitalisation (D0,  $n=27$ ) entre le 3<sup>e</sup> et le 5<sup>e</sup> jour d'hospitalisation (D3,  $n=26$ ), et entre le 6<sup>e</sup> jour et le 9<sup>e</sup> jour d'hospitalisation (D7,  $n=13$ ), à l'état basal (A et B) ou suivant une stimulation par de la nigéricine à  $10\mu\text{g/mL}$  pendant 30 minutes (C et D). Les patients ont été stratifiés en fonction de leur statut à J28 : survivants ( $n=21$ , en gris clair) ou non-survivants ( $n=9$ , gris foncé). Les données manquantes correspondent aux patients dont le statut n'était pas connu à J28 ( $n=2$ ). Les résultats sont exprimés en pourcentage de monocytes exprimant des specks parmi les monocytes CD14+ ou en MFI et sont présentés sous forme de boxplots. La ligne rouge en pointillé indique la valeur médiane observée chez les volontaires sains pour le paramètre considéré. Le test non paramétrique de Mann-Whitney a été utilisé pour comparer les valeurs entre les survivants et les non-survivants. \*  $p < 0.05$ .

Comme les deux groupes de patients septiques ont montré des réponses similaires, nous avons décidé de combiner ces données pour effectuer des analyses en lien avec la mortalité à 28 jours et augmenter la puissance statistique. Il est apparu que les patients non-survivants présentaient, dans l'ensemble, une activité moindre de cette voie inflammatoire. Qu'il s'agisse de conditions basales ou post-stimulation *ex vivo*, nous avons observé que ces patients présentaient moins de monocytes formant des specks (**figure 24A et 24C**) et une activation réduite de la caspase-1 dans les PMN (**figure 24B et 24D**). Ces constatations étaient toutes significatives à J0, avec une proportion plus faible de monocytes formant des specks *in vivo* persistante jusqu'à J7 chez les patients non-survivants (**figure 24A**).

Dans l'ensemble, nous observons que l'expression basale et l'activation *ex vivo* de l'inflammasome NLRP3 étaient réduites chez les patients au cours de la première semaine suivant le sepsis. Ces résultats renforcent nos premières observations ([Article 3](#), page 97). Les diminutions étaient particulièrement prononcées chez les patients non-survivants à 28 jours. Nos observations sont en accord avec de récentes études basées sur des techniques complémentaires.

Tout d'abord, Cui et ses collègues ont étudié la formation de specks dans les monocytes pendant la première semaine d'hospitalisation post-sepsis<sup>122</sup>. Ils n'ont observé aucune différence notable dans la proportion de monocytes formant des specks entre les volontaires sains et les patients pendant les 5 premiers jours. Toutefois, une augmentation est apparue dès le 6<sup>e</sup> jour. Nos premières observations ont plutôt indiqué une diminution globale de la formation de specks plutôt qu'une hausse tardive, bien qu'une légère augmentation soit notée à J7 par rapport à J0. Cependant, une observation plus minutieuse des données des volontaires sains montre une moyenne d'environ 0,15% de monocytes présentant des specks dans cette étude, tandis que notre étude montre une médiane de 0,91%. Cette différence explique nos interprétations divergentes. Dans cette étude, les auteurs ont aussi rapporté une diminution des monocytes exprimant des specks au 6<sup>e</sup> jour et 7<sup>e</sup> jour du sepsis chez les patients non-survivants à 90 jours par rapport aux survivants. Nous avons constaté des résultats similaires dans nos propres analyses préliminaires. Cette moindre formation des specks est d'autant plus troublante qu'elle survient pendant la phase immunosuppressive du sepsis. Il se pose donc la question de savoir si elle constitue un autre aspect de l'immunosuppression induite par le sepsis ou si elle est en relation avec son développement.

Dans une seconde étude, Martinez-Garcia *et al* ont montré que les capacités des monocytes à former des specks étaient diminuées chez certains patients septiques après stimulation *ex vivo*<sup>273</sup>. Cette diminution était observée pendant toute la première semaine suivant le

développement du sepsis. Cette altération de la formation des specks, en association avec d'autres paramètres immuno-inflammatoires, permettait de définir un phénotype « *NLRP3 immunocompromised* » qui permettait d'identifier 80% des patients non-survivants, alors que les scores de sévérité cliniques habituels (SOFA, SAPSII) ne différenciaient pas ces patients « *NLRP3 immunocompromised* » des autres patients septiques.

Nos données rapportant les capacités réduites des monocytes à former des specks, plus marquées chez les non-survivants, viennent donc corroborer ces 2 études. En exploitant une nouvelle approche basée sur un protocole en sang total, nous avons réussi à valider les résultats d'études précédentes qui utilisaient des techniques différentes.

Dans le contexte spécifique de la COVID-19, une autre étude a montré une diminution d'activation de la caspase-1 dans les neutrophiles des patients hospitalisés en réanimation, en comparaison aux neutrophiles de volontaires sains, grâce à la même technique utilisée pour notre étude. Cette étude a révélé que les patients les plus gravement atteints étaient ceux dont les neutrophiles immatures présentaient la plus faible capacité de réactivation de la caspase-1. Nos résultats préliminaires sont donc en accord avec les données actuellement disponibles dans la littérature concernant l'expression basale et l'activation *ex vivo* de l'inflammasome NLRP3.

Néanmoins, ces résultats convergents paraissent paradoxaux. Au niveau systémique, nous avons observé une augmentation de l'activité de l'inflammasome NLRP3 via des niveaux plasmatiques élevés de ses produits (IL-1 $\beta$  et IL-18) et des ARNm associés à son activation, phénomènes largement documentés dans la littérature<sup>66,122,277,279</sup>. Parallèlement, les tests fonctionnels évaluant son activation *ex vivo* sont majoritairement diminués. Il convient de présenter une récente étude rapportant des résultats contraires à nos observations. En utilisant la même technique pour mesurer l'activité de la caspase-1, Rasmussen et collègues ont signalé une augmentation de l'expression basale (*i.e.* sans stimulation *ex vivo*) dans les monocytes et les PMN de patients, durant la première semaine après l'apparition d'une bactériémie à *S.aureus* (qui, dans 65% des cas, se compliquaient d'un sepsis)<sup>123</sup>. L'augmentation de la MFI du FAM-FLICA dans les neutrophiles était plus marquée à J3 et J5 chez les non-survivants. Ces résultats semblent en contradictions avec ceux de notre étude. Cependant, il convient de noter que les populations étudiées n'étaient pas identiques. Dans notre étude, tous les patients ont développé un sepsis (contre 65% dans l'étude précédente), et plus de la moitié d'entre eux ont présenté un choc septique. Par conséquent, la gravité de la maladie pourrait être un facteur expliquant ces différences, où les patients moins graves activent leur inflammasome pour combattre l'infection, tandis que les patients les plus graves présentent déjà des altérations immunitaires liées au sepsis. Cette hypothèse est soutenue par une autre étude. En examinant l'activité de la voie de

l'inflammasome NLRP3 chez des patients septiques ainsi que chez des volontaires sains soumis à un modèle expérimental d'endotoxémie (injection intraveineuse de LPS), Giamarellos-Bourboulis *et al* ont rapporté une diminution de l'activité de la caspase-1 et de la production d'IL-1 $\beta$  chez les patients septiques<sup>269</sup>. Cette diminution de production de L'IL-1 $\beta$  n'était pas retrouvée chez les volontaires sains avant l'injection de LPS, apparaissait 2h après l'injection et s'atténuait après 8h<sup>269</sup>. Ces données illustrent le phénomène bien documenté de tolérance à l'endotoxine, qui est transitoire chez les volontaires sains, mais anormalement prolongé chez les patients septiques.

Globalement, nous avons observé de manière concomitante une diminution de l'expression basale et de l'activation *ex vivo* de l'inflammasome NLRP3. Au regard de l'inflammation systémique observée durant le sepsis, ces résultats semblent contradictoires. Seules quelques études ont montré des résultats similaires (inflammation systémique + diminution de la réactivation *ex vivo*)<sup>45,139,264,265</sup>. Les mécanismes sous-jacents de ce phénomène demeurent inexpliqués. Des études plus approfondies sont donc nécessaires pour clarifier ce paradoxe. Plusieurs hypothèses peuvent être proposées :

(i) Suite à une infection, il est possible que les signaux de danger stimulent à la fois les cellules circulantes et les précurseurs de la moelle osseuse pour produire de nouvelles cellules inflammatoires. Après avoir libéré leurs médiateurs inflammatoires, ces cellules pourraient entrer en état d'anergie, les rendant incapables de répondre à un nouveau stimulus *ex vivo*. Cette situation évoque le phénomène de tolérance à l'endotoxine observé temporairement chez les volontaires sains soumis à une endotoxémie expérimentale, un phénomène qui persiste chez les patients septiques<sup>269</sup>.

(ii) Une autre hypothèse issue de la littérature est celle de la compartimentalisation<sup>292</sup>. Elle suggère que l'inflammation est prédominante dans les tissus où l'infection ou le traumatisme a lieu, tandis que les leucocytes circulants montrent une hyporéactivité. Cette idée est soutenue par des études démontrant des concentrations accrues de cytokines, comme l'IL-1 $\beta$ , à la fois dans la circulation et localement (*e.g.* dans le LBA de patients post-trauma). Néanmoins, cette hypothèse a été remise en question par d'autres recherches qui indiquent une immunosuppression observable également au niveau des organes<sup>20</sup>.

(iii) Enfin, une autre explication pourrait résider dans le fait que le blocage de l'activation de l'inflammasome NLRP3 coexiste avec une réponse inflammatoire généralisée. Cette idée renforce la notion que, chez les patients septiques, les réponses pro-inflammatoires et anti-inflammatoires peuvent se produire simultanément.

### Limites du travail

Notre étude, bien qu'elle apporte des résultats originaux et renforce certains résultats antérieurs, présente certaines limites. D'abord, si nous avons identifié un lien entre une inflammation initiale excessive et persistante, des altérations immunitaires, et une mortalité accrue chez les patients septiques, nous n'avons pas utilisé de marqueurs directs de l'inflammasome NLRP3. En effet, nous nous sommes appuyés sur les produits tardifs de son activations (*i.e.* IL-1 $\beta$  et IL-18) et sur des marqueurs transcriptomiques liés à son activation pour démontrer ces associations. Nous aurions pu recourir à ses composants plasmatiques, comme la protéine NLRP3 circulante. Cependant, ce n'est que récemment que la concentration plasmatique de NLRP3 a été reconnue comme un outil permettant d'identifier les patients septiques les plus à risque de décès<sup>277</sup>. De plus, nous avons rapporté ici des associations entre inflammation liée à l'inflammasome NLRP3 et altérations immunitaires, et non pas des liens de causalité. Cette nuance est cependant inhérente à la nature clinique et observationnelle de notre approche. L'exploration de cette causalité est en cours sur modèles expérimentaux de sepsis et fait l'objet d'une thèse de doctorat. Si ce lien de causalité est confirmé, cela ouvrirait la voie à de nouvelles options thérapeutiques.

### Perspectives dans la prise en charge des patients septiques

L'idée d'un traitement universel pour tous les patients développant un sepsis est progressivement remplacée par des approches de médecine personnalisée, à l'image de ce qui est observé dans le traitement des cancers. Cela devient d'autant plus pertinent que malgré une baisse de la mortalité due à l'amélioration de la prise en charge précoce, la mortalité stagne depuis plusieurs années<sup>17,18</sup>. Il est donc nécessaire de repenser les stratégies de traitement et leur mise en œuvre chez les patients septiques. A cet égard, un nombre croissant d'études montrent que, bien que certains traitements n'aient pas prouvé leur efficacité pour améliorer la survie globale des patients, ils peuvent se révéler efficaces lorsqu'ils sont administrés à des sous-groupes de patients spécifiques, identifiés par des endotypes précis. Par exemple, des études post-hoc ont montré une amélioration de la survie des patients présentant une hyperinflammation lorsqu'ils sont traités par anakinra<sup>145,146</sup> ou simvastatine<sup>140</sup>. A l'avenir, la prise en charge des patients atteints de sepsis devra probablement être ajustée en fonction de leurs endotypes et, par conséquent, de leur état immuno-inflammatoire. Les essais cliniques en cours dans la prise en charge du sepsis intègrent désormais cette stratification des patients, se concentrant plus précisément sur la présence d'une hyperinflammation ou d'une immunosuppression (*e.g.* NCT05843786 et NCT04990232).

Au cœur des recherches récentes sur le sepsis, deux outils innovants ont été développés. D'une part, le marqueur LOX-1 a été pour la première fois caractérisé en sang total au cours du sepsis. Alors que les études antérieures s'intéressaient aux cellules purifiées ou l'utilisation de multiples marqueurs<sup>219,220,222</sup>, cette avancée ouvre la voie à des recherches plus approfondies visant à élucider le rôle précis de ces cellules suppressives dans le sepsis, et plus particulièrement confirmer leur rôle néfaste sur la survenue d'infections nosocomiales ou sur la mortalité. La validation d'associations entre ces cellules et ces conséquences cliniques pourrait suggérer la pertinence de traiter les patients septiques présentant une élévation anormale de LOX-1 PMN-MDSCs après la première semaine d'hospitalisation. Toutefois, pour établir avec certitude le lien entre les LOX-1 PMN-MDSCs et leurs conséquences cliniques, des études plus vastes sont nécessaires. Elles permettraient également de définir un seuil précis pour envisager l'emploi de ces cellules comme biomarqueurs en vue de traitements anti-MDSCs. D'autre part, la détection des specks d'ASC en sang total est possible via un nouveau protocole. Cette avancée a mené à l'élaboration d'un seul test, capable à la fois de mesurer l'activité initiale de l'inflammasome NLRP3 et d'évaluer sa potentialité de réactivation *ex vivo*. Ce test devrait faciliter les études cliniques autour de l'inflammasome NLRP3, notamment pour résoudre le paradoxe entre son activation systémique (augmentation de production des IL-1 $\beta$  et IL-18) et la diminution de son activation *in vivo* et *ex vivo*. Si nos observations monocentriques sur l'association entre une diminution de l'activation/réactivation de NLRP3 et la mortalité sont confirmées, cet inflammasome pourrait se retrouver au cœur des traitements thérapeutiques ciblant les patients. Dans cette optique, les futures études auraient pour mission de déterminer le marqueur le plus pertinent (qu'il s'agisse des specks d'ASC *in vivo* ou *ex vivo*, NLRP3 plasmatique, des données transcriptomiques...) et d'explorer les raisons de cette baisse d'activation et de réactivation. Si les causes sont clairement définies, la fixation d'un seuil pour le paramètre retenu pourrait ouvrir la voie à des thérapies personnalisées, telles que des traitements anti-inflammatoires en cas d'inflammation excessive.

Plusieurs directions pour les futurs traitements du sepsis peuvent ainsi être envisagées. Si on parvient à établir que les MDSCs jouent un rôle direct et néfaste dans l'immunosuppression liée au sepsis, la réorientation de certains médicaments ciblant les MDSCs pourrait s'avérer être une approche thérapeutique pertinente<sup>293</sup>. On pourrait ainsi envisager l'utilisation de traitements déjà explorés en oncologie qui visent (i) leur différenciation ou leur développement, (ii) leurs fonctions ou (iii) leur élimination. Cependant, étant donné que le sepsis est déclenché par une infection, il est primordial d'éviter les traitements susceptibles de diminuer la capacité du système immunitaire à combattre les



infections. Par conséquent, les agents cytotoxiques qui diminuent le nombre de globules blancs (e.g. gemcitabine et ou 5-FU), ne devraient pas être envisagés. En revanche, des thérapies plus sûrs comme l'acide tout-trans-rétinoïque, rapporté pour sa capacité à induire la maturation des MDSCs<sup>294</sup>, ou les inhibiteurs de la phosphodiesterase de type 5 (e.g. tadalafil), qui réduisent le nombre de MDSCs et atténuent l'immunosuppression observée dans le cancer<sup>295</sup>, pourraient être explorées.

De plus, l'identification d'un endotype de patients avec une hyperinflammation associée à l'inflammasome NLRP3 pourrait offrir une opportunité de les cibler plus spécifiquement à l'aide de thérapies axées sur cette voie inflammatoire. Ceci est d'autant plus pertinent que ces patients présentaient le taux de mortalité le plus élevé. A l'image du repositionnement de l'anakinra dans l'étude internationale ImmunoSep, cette stratégie pourrait être étudiée pour les patients ayant une forte réponse inflammatoire liée à l'inflammasome NLRP3. Récemment, un anticorps monoclonal bispécifique anti-IL-1 $\beta$ /IL-18 (MAS825) a été développé et étudié dans le traitement de l'arthrite juvénile idiopathique systémique associée à une pneumopathie interstitielle<sup>296</sup>. Cette thérapeutique a été employée dans le traitement des patients hospitalisés pour la COVID-19<sup>297</sup>. Si cet anticorps a démontré sa sûreté d'utilisation, ainsi que sa capacité à réduire l'inflammation et à accélérer l'élimination du virus chez les patients, il n'a malheureusement pas montré d'amélioration en termes de sévérité ou de mortalité. Plusieurs raisons pourraient expliquer ces résultats. D'abord, l'étude, incluant 138 participants, n'était pas suffisamment puissante pour établir une amélioration de la mortalité et n'était pas conçu dans ce but. Ensuite, elle a exclu les patients sous ventilation, souvent les plus gravement atteints, présentant la plus forte réponse inflammatoire. Enfin, il n'y a pas eu de stratification : les patients présentant la COVID-19 étaient sélectionnés sur la base des niveaux de CRP et de ferritinémie, puis randomisés pour le traitement ou un placebo. Or, nous savons que l'utilisation de thérapies anti-inflammatoires sans stratification s'est avérée décevante dans le traitement du sepsis. Cela dit, il serait pertinent de considérer cet anticorps dans le cadre d'une stratification rigoureuse des patients, axée sur des indicateurs d'activation de l'inflammasome NLRP3.

## CONCLUSION

## Conclusion

Dans le cadre de ce travail, nous avons formulé l'hypothèse selon laquelle l'activation de l'inflammasome NLRP3 pourrait jouer un rôle déterminant dans les altérations immunitaires observées lors de l'immunosuppression associée au sepsis, en particulier dans l'induction des MDSCs. Afin d'explorer cette hypothèse, nous avons initié en premier lieu un travail de recherche clinique visant à décrire conjointement ces deux paramètres (*i.e.*, activation de NLRP3 et MDSCs), chez des patients septiques hospitalisés en réanimation. Un volet plus expérimental est en cours de réalisation par ailleurs.

Notre contribution au domaine de la recherche sur le sepsis s'articule autour de deux aspects importants. Premièrement, sur un plan méthodologique, nous avons développé des outils qui permettent des explorations en recherche clinique (LOX-1 MDSCs et test fonctionnel NLRP3 / formation de specks, développés en sang total). Deuxièmement, par différentes approches mesurant différents paramètres (médiateurs solubles, ARNm) dans différentes cohortes, nous décrivons pour la première fois la coexistence d'un état pro-inflammatoire « NLRP3 », perdurant dans le temps, et d'une immunosuppression objectivée par les marqueurs de référence (mHLA-DR, IL-10) et la survenue de MDSCs. La persistance dans le temps de ce couple inflammation/immunosuppression est significativement associée à une augmentation de la mortalité à 28 jours.

Après démonstration d'une causalité délétère dans des modèles expérimentaux, interrompre le lien entre l'inflammation persistante induite par NLRP3 et l'induction de MDSCs pourrait ouvrir de nouvelles voies pour des stratégies thérapeutiques, que ce soit en bloquant la voie NLRP3 ou en inhibant l'induction de MDSCs (approches aujourd'hui disponibles dans d'autres circonstances cliniques). Ces thérapies pourraient être guidées par des biomarqueurs parmi ceux étudiés dans le cadre de cette étude, dans une approche de médecine personnalisée.

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## ANNEXES

## Annexes

**Annexe 1 : Immune monitoring of interleukin-7 compassionate use in a critically ill COVID-19 patient**, Monneret G, de Marignan D, Coudereau R, Bernet C, Ader F, Frobert E, Gossez M, Viel S, Venet F, Wallet F, Cell Mol Immunol. 2020 Sep ; 17(9) : 1001-1003

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**Annexe 5 : Longitudinal assessment of IFN-I activity and immune profile in critically ill COVID-19 patients with acute respiratory distress syndrome**, Venet F, Cour M, Rimmelé T, Viel S, Yonis H, Coudereau R, Amaz C, Abraham P, Monard C, Casalegno JS, Brengel-Pesce K, Lukaszewicz AC, Argaud L, Monneret G; RICO study group, Crit Care. 2021 Apr 12;25(1):140.

**Annexe 6 : Recombinant human interleukin-7 reverses T cell exhaustion ex vivo in critically ill COVID-19 patients**, Bidar F, Hamada S, Gossez M, Coudereau R, Lopez J, Cazalis MA, Tardiveau C, Brengel-Pesce K, Mommert M, Buisson M, Conti F, Rimmelé T, Lukaszewicz AC, Argaud L, Cour M, Monneret G, Venet F ; RICO Study Group, Ann Intensive Care. 2022 Mar 5;12(1):21

**Annexe 7 : T cell response against SARS-CoV-2 persists after one year in patients surviving severe COVID-19**, Venet F, Gossez M, Bidar F, Bodinier M, Coudereau R, Lukaszewicz AC, Tardiveau V, Brengel-Pesce K, Cheynet V, Cazalis MA, Pescarmona R, Garnier L, Ortillon M, Buisson M, Bouscambert-Duchamp M, Morfin-Sherpa F, Casalegno JS, Conti F, Rimmelé T, Argaud L, Cour M, Saadatian-Elahi M, Henaff L, Vanhems P, Monneret G; RICO study group, EBioMedicine. 2022 Apr;78:103967

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**Annexe 9 : Assessment of neutrophil subsets and immune checkpoint inhibitor expressions on T lymphocytes in liver transplantation: A preliminary study beyond the neutrophil-lymphocyte ratio**, Riff A, Haem Rahimi M, Delignette MC, Gossez M, Coudereau R, Pantel S, Antonini T, Villeret F, Zoulim F, Mabrut JY, Dumortier J, Venet F, Lebossé F, Monneret G, 2023 Front Physiol. Mar 30;14:1095723

## **Annexe 1**





## CORRESPONDENCE OPEN

## Immune monitoring of interleukin-7 compassionate use in a critically ill COVID-19 patient

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One of the immune characteristics of coronavirus disease 2019 (COVID-19) is a massive fall in lymphocyte count in which magnitude associates with mortality.<sup>1,2</sup> Recent monitoring of COVID-19 intensive care units (ICU) patients confirmed the profound lymphopenia and its remarkable stability over time.<sup>3,4</sup> While most immunomodulation approaches proposed so far in COVID-19 focused on inhibiting inflammatory cytokine response; mounting evidence indicates that this viral-induced defective lymphocyte response may play a central role in COVID-19 pathophysiology.<sup>5</sup> Interestingly, recombinant human interleukin-7 (IL-7) therapy, known to efficiently restore lymphocyte count in several viral infections was safely administered in septic shock patients<sup>6</sup> who present with similar lymphocyte alterations as observed in COVID-19.<sup>3</sup>

We report here the case of a 74-year-old patient without any comorbidity. He was admitted to our university hospital ICU (Hospices Civils de Lyon, France) for COVID-19 ARDS requiring high flow oxygen. ICU admission (thereafter corresponding to day 0) occurred 9 days after first symptom onset. SARS-CoV-2 PCR was positive (nasal swab) and CT scan was highly suspect of severe COVID-19. He was intubated 24 h after admission and ventilation was set according to guidelines for ARDS including prone positioning. PEEP was around 8–10 cm H<sub>2</sub>O for the whole ICU stay. Antibiotics were initiated at admission until bacterial samples were negative. On day 10, as the patient was still presenting with severe ARDS without any infection criteria, steroids were initiated at 1 mg/kg/day (equivalent prednisolone) but stopped 5 days later (i.e., day 15) due to ventilator associated pneumonia (VAP, *Morganella morganii* and *Aspergillus fumigatus* were identified in BALF). Antibiotics and antifungal therapies were started immediately. At day 20, an additional VAP was suspected (without any bacterial documentation) treated with meropenem. After admission, SARS-CoV-2 PCR remained positive at D10 and D16.

From day 0 to day 24, the patient remained deeply lymphopenic and presented with markedly decreased monocytic expression of HLA-DR (Fig. 1) reflecting deep immunocompromised state. Therefore, at day 24, while the patient did not show any improvement in pulmonary function, presented with several intercurrent infections, absence of negativation of SARS-CoV-2 PCR, and marked and persisting lymphopenia, compassionate use of IL-7 was initiated in order to improve immunity and consequently allow viral clearance. After inaugural injection

(3 µg/kg), the patient received IL-7 at 10 µg/kg twice a week during 4 weeks. The patient did not present any serious adverse event except for a transient skin rash at site of injection. Impressive improvement in lymphocyte count and mHLA-DR expression was rapidly observed (Fig. 1). Four days after initiation of IL-7 (day 28), results of SARS-CoV-2 detection by PCR were negative, IFN score started to decrease and circulating IFN-γ returned to normal range. In parallel, clinical condition slowly improved. Mechanical ventilation and sedation were interrupted by day 40 and the patient started to awake and opened his eyes for the first time in 6 weeks. At day 38, the patient developed a *Pseudomonas* infection successfully treated by ceftazidim and ciprofloxacin. At this time, a transient fall in both lymphocyte count and mHLA-DR was noticed. Both parameters rapidly rose again to normal values after this intercurrent infection. Unfortunately, at day 45, the patient developed an acute kidney injury that was not linked to obstructive or cardiogenic origin. At this stage, in accordance with the patient's will, his family refused dialysis that was necessary. Treatment was therefore switched to fully palliative care resulting in death of the patient on day 46.

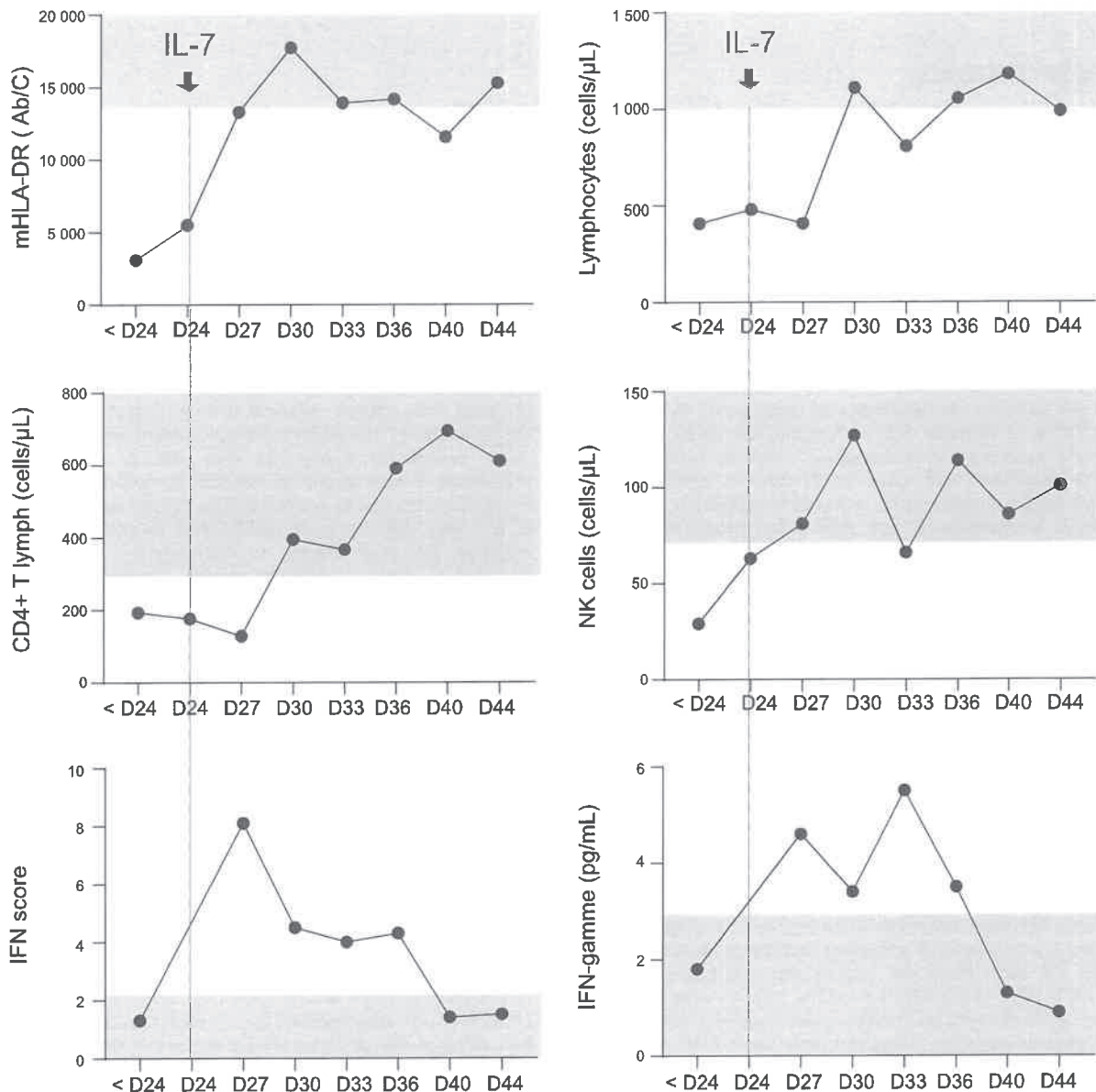
Although we cannot draw any definitive conclusion about a single case report, the present results show IL-7 beneficial effects in improving immune functions in a COVID-19 patient. Indeed, after almost 4 weeks in ICU and established protracted immunosuppression, we noticed a marked and rapid elevation of lymphocyte count and mHLA-DR toward reference ranges. This effect was not accompanied by any potential cytokine release of IL-6, IFN-γ, IL-10, or TNF-α (IL-1-β was even never detectable—Supplementary Table S1). Most importantly, this improved immune response was paralleled with negativation of SARS-CoV-2 PCR (and IFN score) and clinical improvement (switching controlled ventilation to pressure support, sedation alleviation, rapid clearance of intercurrent pseudomonas infection).

COVID-19 patients constantly present with severe lymphopenia. Meta-analyses demonstrate the independent association of this immune alteration with poor outcome.<sup>1,2</sup> In addition, lymphocyte functions were altered in COVID-19.<sup>4</sup> While viral dissemination is a prominent driver of severe disease, there is mounting evidence suggesting that such altered T-cell function and number may participate in the out of control spiraling of viral replication.<sup>5</sup> In line, negative correlation between lymphocytes count and pulmonary viral load was observed.<sup>7</sup> The absence of potent

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**Fig. 1 Immunophenotyping overtime.** Patient was sampled overtime after ICU admission. Cellular immunophenotyping was performed by flow cytometry and results are expressed as numbers of antibodies bound per monocyte (Ab/C) for monocytic HLA-DR expression and as numbers of cells per  $\mu\text{L}$  of whole blood for total lymphocyte, CD4+ T lymphocyte and NK cell counts. Plasma IFN $\gamma$  concentration was measured by Ella technology and results are expressed as pg/mL. IFN score (type I IFN-related genes mRNA levels) was measured by Nanostring technology and results are expressed as a score relative to normal values. Reference values (gray zones) are provided by routine clinical immunology laboratories at our institution. Interleukin-7 treatment was initiated at day 24 after ICU admission (D24, red arrow). Cellular measurements were performed once before IL-7 treatment initiation (<D24) and twice a week during 3 weeks after IL-7 treatment initiation: between D27 and D29 (D27), between D30 and D32 (D30), between D33 and D35 (D33), between D36 and D36 (D36), between D40 and D43 (D40), and between D44 and D48 (D44). For each sampling time, if several measurements were performed, the highest value was systematically considered.

antiviral drug along with severe immune defects therefore contribute to body's inability to normally eradicate virus<sup>8</sup> and may explain the long ICU stays reported by many authors. In agreement, first results from autopsy studies reported on the persistence of the virus at the time of death—even after few weeks in ICU.<sup>9</sup> Another indicator of deep immunosuppression is the extremely high rate of secondary infections in ICU COVID patients. This is especially true for the numerous cases of aspergillosis which are usually seen in very immunocompromised patients. Collectively, there may be a strong rationale for

considering drugs aimed at restoring T-cell function and count in the most severe COVID-19 patients. For example, thymosin alpha 1 (which shares similarities with IL-7) recently demonstrated promising results in COVID-19 patients.<sup>10</sup> The present IL-7 effects, despite unfavorable outcome, appear consistent in terms of immunological recovery as, to date, such a rapid rise in standard immune parameters has not been observed in long-standing COVID-19 patients. In this patient's case, IL-7 was used in a delayed compassionate manner (after 24 days in the ICU) in a patient likely seriously weakened by a long ICU stay. We may expect better

outcome in case of earlier administration. In conclusion, we strongly believe that IL-7 is worth trying in next trials when patients could be stratified based on marked lymphopenia.

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#### ADDITIONAL INFORMATION

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## **Annexe 2**



# Monocyte HLA-DR Measurement by Flow Cytometry in COVID-19 Patients: An Interim Review

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## • Abstract

Several months after the sudden emergence of SARS-CoV-2 and COVID-19, the understanding of the appropriate host immune response to a virus totally unknown of human immune surveillance is still of major importance. By international definition, COVID-19 falls in the scope of septic syndromes (organ dysfunction due to dysregulated host response to an infection) in which immunosuppression is a significant driver of mortality. Sepsis-induced immunosuppression is mostly defined and monitored by the measurement of decreased expression of HLA-DR molecules on circulating monocytes (mHLA-DR). In this interim review, we summarize the first mHLA-DR results in COVID-19 patients. In critically ill patients, results homogeneously indicate a decreased mHLA-DR expression, which, along with profound lymphopenia and other functional alterations, is indicative of a status of immunosuppression. © 2020 International Society for Advancement of Cytometry

## • Key terms

COVID-19; sepsis; HLA-DR; flow cytometry

## COVID-19 AS A VIRAL SEPSIS

Since the emergence of SARS-CoV-2 at the end of 2019, the total number of patients admitted to hospital with COVID-19 has never ceased to rise and related mortality, although slowly improving, has remained dramatically high. While awaiting vaccines or effective antiviral treatments, the understanding of the appropriate host immune response to a virus totally unknown of immune surveillance is of major importance. At the forefront of immune alterations described in the first cohorts of COVID-19, patients homogeneously present inflammatory response and severe lymphopenia that draw similarities with alterations seen in sepsis (1–5). By international definition, sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection (6). In COVID-19, acute respiratory distress syndrome (ARDS), an organ dysfunction, may occur in the most critically ill COVID-19 patients and therefore this should be considered as viral sepsis. While it has long been believed that sepsis alters immune homeostasis by provoking an exacerbated systemic inflammatory response, pivotal observations, mostly based on clinical flow cytometry (7), demonstrated that sepsis induces the concomitant occurrence of both pro- and anti-inflammatory mechanisms. As a result, some septic patients enter a stage of protracted immunosuppression (8, 9), but this aspect has been poorly explored in COVID-19 patients. Among immunological parameters routinely used to monitor septic patients in intensive care units (ICUs), those obtained by flow cytometry have gained interest as they are helpful to stratify septic patients for immunostimulation in randomized clinical trials (RCTs) (10, 11). Among these, monocyte HLA-DR (mHLA-DR) is currently considered as the reference (12).

### Monocyte HLA-DR measurement as a pro-/anti-inflammatory marker of the “milieu interieur”

Monocytes are by nature extremely plastic cells. They have the capacity to both detect danger and trigger inflammation and to initiate resolution and trigger anti-inflammation. Thus, throughout the progression of a disease, monocytes can be alternatively pro- and anti-inflammatory cells. The resultant of pro- and anti-inflammatory forces impacting monocytes at a given point in time is globally reflected by the level of mHLA-DR expression on their surface (12).

In sepsis, reduced mHLA-DR expression clinically illustrates the phenomenon of endotoxin tolerance characterized by altered functionality (i.e., reduced TNF release to a secondary infectious challenge) and reflects decreased antigen presentation capacity since HLA-DR molecules belong to major histocompatibility complex class II (MHC II) system (13, 14). At the time of writing, there is a consensus to consider low mHLA-DR as a marker of sepsis-induced immunosuppression (15, 16). In clinical studies, the magnitude and the persistence overtime of mHLA-DR loss has been demonstrated to be associated with worsening (9, 12), that is, increased mortality and nosocomial infections (even after multivariate analysis including confounding factors). It is of note that, beyond sepsis, mHLA-DR is progressively becoming a popular immunomonitoring tool in other ICU contexts (trauma, burns, surgery), and other clinical areas (gastroenterology, oncology, hematology, transplantation), where it also identifies patients at risk of worsening: mortality, secondary infections, cancer relapse (12, 17). Conversely, upon marked monocyte activation, extremely increased mHLA-DR values are reported in case of haemophagocytic lymphohistiocytosis or cytokine release syndrome (18). Thus, COVID-19 being a disease progressing stepwise toward increased severity until ARDS occurrence, mHLA-DR may provide useful information regarding the inflammation/immunosuppression status of patients and disease progression.

## CLINICAL RESULTS IN COVID-19 PATIENTS

### Deep Immunophenotyping Studies

Facing a totally novel disease, many exploratory nonhypothesis-driven studies have been conducted in order to decipher the immune processes at play in COVID-19 (Table 1). As a whole, these mixed various flow approaches (spectral flow, multicolor flow, time of flight mass spectrometry), transcriptomic strategies (transcriptomic signatures, single-cell RNA-seq), functional testing, and multiplex measurement of soluble mediators. Results were mostly analyzed through multi-data/-omic algorithms. Among this huge number of articles, when specifically considering monocyte and HLA-DR expression as Mesh terms in PubMed, we identified four relevant studies (19–22). Irrespective of the protocol used, these consistently reported decreased mHLA-DR in COVID-19 patients. This was the most overt phenotype. Studies also homogeneously observed that the depth of this decrease was associated with severity, that is, values were lower in patients with severe disease than those with

moderate disease. Severity was also associated with the depletion of nonclassical monocyte subset (i.e., CD14<sup>low</sup>CD16+) (21, 22). In addition, monocytes exhibited altered inflammatory cytokine release in functional testing (20, 22) indicating deactivated rather than pro-inflammatory status, which is in line with that observed by Laing et al. (19) who measured circulating cytokines and reported the absence of broad cytokine storm. Accordingly, Arunachalam et al. (20) hypothesized the pulmonary origin of circulating cytokines rather than an exacerbated systemic production. This agrees with decreased HLA-DR expression on circulating monocytes since, in case of huge monocyte activation, mHLA-DR should rise to very high levels.

Collectively, in COVID-19 patients, these data indicate an immunosuppressed phenotype of circulating monocytes.

### mHLA-DR measurement at hospital arrival (various severities)

We identified four additional articles specifically reporting on mHLA-DR measurement at hospital admission (23–26). Patients presented with various levels of severity, and routine flow cytometry was performed and results were expressed as means of fluorescence intensities (for the total CD14+ monocyte population) meaning that results cannot be strictly compared between centers. That said, by comparison with healthy controls, all studies observed lower mHLA-DR expression in COVID-19 patients. This difference was more pronounced in patients severely ill at hospital arrival. Moreover, Moratto et al. also observed that, among severe patients, those who progressed to a critical status (admission to ICU) had the lowest expression (23). In addition, as mentioned above, in COVID-19 patients, the proportion of non-classical monocytes (CD14<sup>low</sup>CD16+) was lower than in controls (23, 25).

Collectively, these results indicate that, upon hospital arrival, the magnitude of mHLA-DR is closely associated with severity of COVID-19 and may thus serve as a marker for the triage of patients.

### Kinetics of mHLA-DR monitoring in ICU patients

In this section, we identified seven articles that reported studies in which mHLA-DR was monitored in ICU patients over 1 week to 1 month. Four were based on values obtained using a standardized protocol and results are expressed as the number of antibodies bound per cell (AB/C). Most importantly, these independent studies from different groups located in different countries (Bern-CH, Nijmegen-NL, Limoges-F, Lyon-F) provided extremely homogenous results (27–30). Initial mHLA-DR values for ICU COVID-19 patients were around 10,000 AB/C, which was lower than controls (>15,000 AB/C) but not as low as that found in septic shock (around 5,000 AB/C). Later during ICU stay, values remained stable or progressively declined further still. In some studies, the lowest mHLA-DR expression levels (<4,000 AB/C) were observed in patients who died. Interestingly, mHLA-DR expression has been used for the monitoring of a critically ill COVID-19 patient who received compassionate rhIL-7 therapy (31). After more than 3 weeks in ICU and several

Table 1. Mains characteristics of cited cohorts

REFERENCE	COHORT CHARACTERISTICS	METHODS	MAIN RESULTS IN COVID PATIENTS
<i>Deep immunophenotyping studies</i>			
(19)	63 COVID patients (6 mild/26 moderate/31 severe) 55 healthy controls 10 hospitalized for non-COVID LRTIs	- RNA-sequencing (various) CyTOF - Spectral flow cytometry - Multicolour flow cytometry	↓ mHLA-DR ↓ with severity ↓ monocyte functionality ↓ CD14 <sup>low</sup> CD16 <sup>++</sup>
(20)	76 COVID patients 69 healthy controls	- transcriptomic signatures - functional testing - multiplex	monocytes
(21)	86 COVID (27 mild/16 moderate 43 severe) 72 symptomatic controls (flu-like symptoms)	measurement of soluble mediators	
(22)	53 COVID (24 mild/29 severe) 59 controls		
<i>Patients at hospital admission</i>			
(23)	36 COVID (8 moderate/28 severe)	Routine flow cytometry	↓ mHLA-DR
(24)	30 COVID (10 mild/14 moderate/6 severe) 20 healthy controls	(Results as MFI)	↓ with severity ↓ CD14 <sup>low</sup> CD16 <sup>++</sup> monocytes
(25)	40 COVID 8 healthy controls		
(26)	54 COVID (28 severe/26 moderate) 104 bacterial sepsis		
<i>Monitoring of ICU Patients</i>			
(27)	30 COVID (ICU)	Routine flow cytometry	↓ mHLA-DR
(28)	24 COVID (ICU)	Results as AB/C ( $n = 5$ )	Stable over time
(29)	13 COVID (ICU) 10 healthy controls	Results as % (of positive monocyte, $n = 2$ )	↓ with death (to be confirmed)
(30)	16 COVID (9 ICU/7 moderate)		
(31)	1 COVID (ICU)		
(32)	95 COVID (survivors) 62 COVID (nonsurvivors)		
(33)	38 COVID (ARDS) 36 Bacterial ARDS		

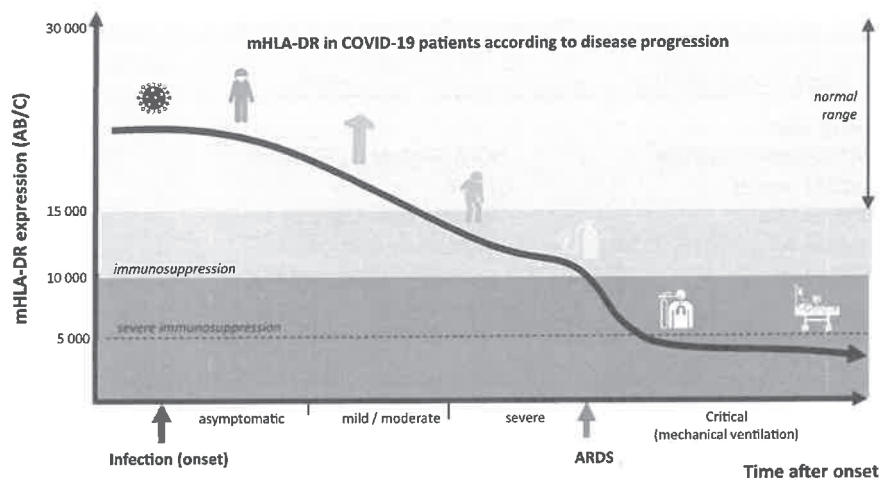
COVID: COVID-19, LRTI: low respiratory tract infections, MFI: mean of fluorescence intensities, AB/C: antibodies bound per cell, ARDS: acute respiratory syndrome.

episodes of recurrent infections along with constant SARS-CoV-2 positive PCR, mHLA-DR expression was <4,000 AB/C. After initiation of the drug, mHLA-DR expression rapidly normalized (31). In addition, although these did not report data as AB/C, two other studies are of note: Hue et al. reported a decreased mHLA-DR in patients with COVID ARDS compared with healthy controls, but this was not as low as that found in bacterial ARDS. Again, this decreased mHLA-DR expression was stable over a 6 day-period (33). At last, Wang et al. followed 157 severely ill patients for 30 days and reported a fall in mHLA-DR expression in patients who died, and this alteration was observed at each time point of the monitoring (32).

## DISCUSSION AND CONCLUSION

Overall, these data are extremely homogenous since all moderate to severely ill COVID-19 patients have a decreased mHLA-DR expression (Fig. 1). It is of note that:

- the depth of mHLA-DR fall is associated with severity at hospital admission;
- this loss of mHLA-DR expression is not as marked as in septic shock;
- in ICU low mHLA-DR is remarkably stable over time (or tends to slowly aggravate);
- the loss of mHLA-DR is associated with monocyte functional deactivation (i.e., altered inflammatory cytokine release)



**Figure 1.** Monocyte HLA-DR expression (mHLA-DR) in COVID-19 patients according to disease progression. ARDS: acute respiratory distress syndrome, AB/C: Antibodies bound per cell. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

- the loss of mHLA-DR is also accompanied by the disappearance of nonclassical monocytes (CD14<sup>low</sup>CD16<sup>++</sup>) also known as “inflammatory” monocytes;
- in some studies, the lowest mHLA-DR expression is associated with mortality in ICU although the question of the association between low mHLA-DR and mortality in long-stay ICU patients remains to be further assessed.

Based on the vast literature describing immunosuppression and its associated decreased mHLA-DR expression in sepsis, it seems thus reasonable to conclude that ICU COVID-19 patients, like septic ones, exhibit features of immunosuppression. In addition, beyond decreased mHLA-DR, these COVID-19 patients also present with profound lymphopenia, low plasmacytoid dendritic cell count, altered IFN- $\alpha$  production (3, 19, 20, 34, 35). These additional abnormalities are typical characteristics of the process of age-acquired immunosuppression (also called immunosenescence) observed in elderly people who are, by far, the main victims of COVID-19. We may hypothesize that COVID-19 is mainly a consequence of this altered immune status and that, in case of ARDS occurrence, it secondarily amplifies this immunosuppression in a vicious circle as described in sepsis in the elderly (36). The cumulated absence of antiviral drug and established memory immune response against a totally novel virus in aged immunosuppressed patients is likely to explain long ICU length of stay and stability of immune alterations in ICU COVID-19 patients in comparison with other bacterial ARDS for which antibiotics are available. Thus, despite a moderate inflammatory process that appears to be of local origin (19, 20, 37, 38), likely illustrating pulmonary spread of virus instead of systemic cytokine storm, immunostimulation in COVID-19 could be a sound approach. Only very few studies have explored this option; decreased mHLA-DR (and depth of lymphopenia) may help in guiding such therapeutic strategies.

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Literature search related to the present manuscript has been stopped on October 22, 2020. Due to the huge amount of literature on COVID-19, the authors may have missed some articles. The authors sincerely apologize for works not cited in this manuscript. The authors thank Dr P. Robinson for enthusiastic English editing. The authors are supported by Hospices Civils de Lyon and Claude Bernard Lyon I University (Lyon, France)

#### AUTHOR CONTRIBUTIONS

**IHSANE BENLYAMANI:** Conceptualization; investigation; writing-original draft. **Fabienne Venet:** Validation; writing-review and editing. **Remy COUDEREAU:** Investigation; writing-review and editing. **Morgane Gossez:** Validation; writing-review and editing. **Guillaume Monneret:** Conceptualization; investigation; supervision; validation; writing-original draft; writing-review and editing.

#### CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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## **Annexe 3**





# Characterization of Circulating IL-10-Producing Cells in Septic Shock Patients: A Proof of Concept Study

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Sepsis is a worldwide health priority characterized by the occurrence of severe immunosuppression associated with increased risk of death and secondary infections. Interleukin 10 (IL-10) is a potent immunosuppressive cytokine which plasma concentration is increased in septic patients in association with deleterious outcomes. Despite studies evaluating IL-10 production in specific subpopulations of purified cells, the concomitant description of IL-10 production in monocytes and lymphocytes in septic patients' whole blood has never been performed. In this pilot study, we characterized IL-10 producing leukocytes in septic shock patients through whole blood intracellular staining by flow cytometry. Twelve adult septic shock patients and 9 healthy volunteers were included. Intracellular tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and IL-10 productions after lipopolysaccharide stimulation by monocytes and IL-10 production after PMA/Ionomycin stimulation by lymphocytes were evaluated. Standard immunomonitoring (HLA-DR expression on monocytes, CD4+ T lymphocyte count) of patients was also performed. TNF $\alpha$  expression by stimulated monocytes was reduced in patients compared with controls while IL-10 production was increased. This was correlated with a reduced monocyte HLA-DR expression. B cells, CD4+, and CD4- T lymphocytes were the three circulating IL-10 producing lymphocyte subsets in both patients and controls. No difference in IL-10 production between patients and controls was observed for B and CD4- T cells. However, IL-10 production by CD4+ T lymphocytes significantly increased in patients in parallel with reduced CD4+ T cells number. Parameters reflecting altered monocyte (increased IL-10 production, decreased HLA-DR expression and decreased TNF $\alpha$  synthesis) and CD4+ T lymphocyte (increased IL-10 production, decreased circulating number) responses were correlated. Using a novel technique for intracellular cytokine measurement in whole blood, our results identify monocytes and CD4+ T cells as the main IL-10 producers in septic patients' whole blood and illustrate the development of a global immunosuppressive profile in septic shock. Overall, these preliminary results add to our understanding of the global increase in IL-10 production induced by septic shock.

Further research is mandatory to determine the pathophysiological mechanisms leading to such increased IL-10 production in monocytes and CD4+ T cells.

**Keywords:** sepsis, immunosuppression, flow cytometry, interleukin 10, lymphocytes, monocytes

## INTRODUCTION

In 2017, the World Health Assembly and the World Health Organization recognized sepsis as a global health priority (1). Indeed, the incidence of sepsis is high and every year sepsis is responsible for over 10 million deaths worldwide (2). While sepsis and septic shock are caused by an excessive activation of the immune system, current data indicate that after a short pro-inflammatory phase, septic shock patients develop negative regulatory mechanisms aimed at blocking initial hyper-immune activation. In some patients, this may lead to profound immunosuppression involving both innate and adaptive immunity (3).

Sepsis-induced immunosuppression prevents the efficient clearing of the primary infection, is associated with an increased risk of nosocomial infections and favors the reactivation of latent viruses (Cytomegalovirus or Herpes Simplex Virus) (3). In addition, it is reported that over 70% of total mortality after septic shock occurs in the immunosuppressive phase (i.e., after the first 3 days) (4). This is the rationale behind clinical trials based on adjunctive immunostimulation in sepsis (interferon gamma, human granulocyte-macrophage colony-stimulating factor, interleukin 7, anti-PD1/L1 checkpoint inhibitor antibodies) (5). However, a better description of sepsis-induced immune alterations is mandatory in order to improve the understanding of sepsis-induced immunosuppression pathophysiology and to identify innovative therapeutic targets and stratification biomarkers.

Interleukin 10 (IL-10) is a potent immunosuppressive cytokine which concentration is increased in the plasma of septic shock patients. IL-10 blood levels have been shown to correlate with inflammation severity and the development of organ failure in septic shock (6). Increased plasmatic IL-10 concentration has also been associated with an increase in nosocomial infections and mortality (7–9). IL-10 thus likely appears to play a major role in sepsis-induced immunosuppression.

The increased production of IL-10 by some specific cell subpopulations has only partially been described in sepsis (3–5). These data were based on *ex vivo* experiments with purified cells, which may not necessarily be representative of cytokine production status by circulating leukocytes. In addition, previous studies evaluated IL-10 production by one specific cell subset at a time and thus far, no data are available describing IL-10 production by leukocytes subpopulations simultaneously. In

this context, the aim of this exploratory study was to characterize IL-10 producing cells in sepsis through a novel whole blood intracellular staining approach by flow cytometry.

## MATERIALS AND METHODS

### Study Population

This pilot clinical study was conducted on twelve consecutive septic shock patients admitted to the intensive care unit of the Edouard Herriot Hospital (Hospices Civils de Lyon, Lyon, France). This project is part of a global study in sepsis-induced immune dysfunctions (IMMUNOSEPSIS cohort, #NCT04067674). Diagnostic criteria for septic shock was based on the Sepsis-3 definition (10). Exclusion criteria disqualified patients under 18 years of age and subjects with aplasia or pre-existent immunosuppression as defined by pre-existent immunosuppressive treatment including corticosteroids at an immunosuppressive dosage (> 10 mg equivalent prednisone/day and cumulative dose >700 mg), ongoing hematological disease or within 5 years preceding inclusion, solid tumor under chemotherapy or in remission, innate immune deficit, extracorporeal circulation within one month before inclusion (cardiac surgery or ECMO). Samples of peripheral blood were collected at day 3–4 after the onset of septic shock in heparin coated tubes. The immunosuppressive state of septic patients was verified by measuring decreased HLA-DR expression on monocytes (mHLA-DR) expressed as a number of antibodies bound per cell (AB/C, see reference for standardized laboratory protocol) and CD4+ T lymphocyte count (11). Clinical parameters were collected during the follow-up period (until 28 days).

This non-interventional study was conducted in accordance with the Declaration of Helsinki, under terms of all relevant local legislation and was approved by our Institutional Review Board for Ethics [“Comité de Protection des Personnes Ouest II - Angers” – n° RCB: 2019-A00210-57, n° CPP: 19.01.23. 71857 (2019/11)], which waived the need for informed consent, as the study was observational and performed on residual blood after the completion of routine follow-up. Patients or next-of-kin were systematically informed of the study and non-opposition to inclusion in the study was systematically obtained and registered for each patient. Residual samples were stored within a blood collection registered at French Ministry of Research and Education (#DC-2008-509) and at the “Commission Nationale de l’Informatique et des Libertés”. Peripheral blood from healthy volunteers (HV) was provided by the “Etablissement Français du Sang” (EFS) from Lyon. According to EFS standardized procedures for blood donation and to provisions of the articles R.1243–49 and following ones of

**Abbreviations:** AB/C, antibodies bound per cell; Abs, antibodies; Breg, regulatory B cell; EFS, Etablissement Français du Sang; HV, healthy volunteer; IL-10, interleukin 10; IQR, interquartile range; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; mHLA-DR, monocyte HLA-DR; SAPS, Simplified Acute Physiology Score; SOFA, Sequential Organ Failure Assessment; TNF $\alpha$ , tumor necrosis factor- $\alpha$ ; Treg, regulatory T cell.

the French public health code, a written non-opposition to the use of donated blood for research purposes was obtained from HV. The blood donors' personal data were anonymized before transfer to our research laboratory.

### Intracellular Staining Procedure

Intracellular staining protocol was optimized by Beckman Coulter Immunotech (Marseille, France).

For monocytes, 100  $\mu$ l of heparin anticoagulated whole blood was directly added to the stimulation tube (DurActive3<sup>®</sup> tube containing dry coated lipopolysaccharide (LPS) and Brefeldin A, Beckman Coulter, Brea, US) or to an empty control tube. After 3h incubation at 37°C, cells were labeled with cell surface antibodies: FITC-labeled anti-CD16, ECD-labeled anti-HLA-DR, PB-labeled anti-CD14, and KrO-labeled anti-CD45 (all from Beckman Coulter, Brea, US). Thereafter, samples were washed with PBS and treated with the IntraPrep Permeabilization Reagent set (Beckman Coulter, Brea, US) according to the manufacturer's instructions. Samples were then stained for 45min at room temperature in the dark with intracellular antibodies: PE-labeled anti-IL10 (BioLegend, San Diego, US) or Rat IgG2a PE-labeled isotype control antibody (BioLegend, San Diego, US) and AF700-labeled anti-TNF $\alpha$  (Beckman Coulter, Brea, US) or mouse IgG1 AF700-labeled isotype control antibody (BioLegend, San Diego, US). Isotype controls of anti-IL-10 and anti-TNF antibodies were used in order to evaluate non-specific binding both in stimulated and non-stimulated conditions and thus to set-up threshold of positivity when markers were expressed as percentages of positive cells.

For lymphocytes, 100  $\mu$ l of heparin anticoagulated whole blood was directly added to the stimulation tube (DurActive1<sup>®</sup> tube containing dry coated Phorbol 12-Myristate13 Acetate (PMA), Ionomycin and Brefeldin A, Beckman Coulter, Brea, US) or to an empty control tube. After 3h incubation at 37°C, cells were labeled with cell surface antibodies: PC7-labeled anti-CD19, KrO-labeled anti-CD45, PB-labeled anti-CD3 and APC-labeled anti-CD4 (all from Beckman Coulter, Brea, US). Thereafter, samples were washed with PBS and treated with the IntraPrep Permeabilization Reagent set (Beckman Coulter, Brea, US) according to the manufacturer's instructions. Samples were then stained for 45min at room temperature in the dark with intracellular antibodies: PE-labeled anti-IL10 (BioLegend, San Diego, US) or Rat IgG2a PE-labeled isotype control antibody (BioLegend, San Diego, US).

### Data Acquisition

Data acquisition was performed on a Navios Flow Cytometer (Beckman Coulter, Brea, US). Our instrument was daily calibrated with Flow Check (Beckman Coulter, Brea, US) and Flow Set (Beckman Coulter, Brea, US) calibration beads to control the optical and fluidic stability of the device and for a performance validation throughout the study. To minimize autofluorescence and the improper analysis of cell doublets, cells were first put through a forward scatter area and forward scatter height gate to identify single cells. Leukocytes were then gated out from dead cells and debris on the basis of labeling with CD45. For the monocyte panel, among the CD45+ cell

population, monocytes were identified on a CD14/SS dot-plot. Intracellular tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and mHLA-DR results were expressed as mean fluorescence intensity (MFI) of the entire monocyte subpopulation. For the lymphocyte panel, two complementary gating strategies were used. First, in order to phenotype IL-10 producing lymphocytes in stimulated tubes, on a IL-10 (x-axis) and SS (y-axis) dot-plot gated on in CD45+ leukocytes, we selected IL-10 producing lymphocytes (IL-10+ SS<sup>low</sup> cells). CD3, CD19, and CD4 expressions were then characterized on these cells based on CD4 (y-axis) and CD3 or CD19 (x-axis) dot-plots. Second, so as to evaluate the impact of sepsis on IL-10 production capacity on beforehand identified lymphocyte subpopulations, B cells were identified on a CD19/SS dot-plot and T cells on a CD3/SS dot-plot among the CD45+ cell population. Finally, CD4- and CD4+ T cells were gated among CD3+ cells on a CD3/CD4 dot-plot. The percentages of IL-10 expressing cells among these three lymphocyte subpopulations were then evaluated. Positivity threshold was defined based on isotype values set up at 1%. A minimum of 5,000 target cells (monocytes or lymphocytes) were systematically acquired to ensure robustness of results. Of note, both in patients and donors, the majority of monocytes were able to produce TNF- $\alpha$ ; which was not the case for IL-10. Thus TNF- $\alpha$  results expressed as MFI possessed a better dynamic range compared with percentages which saturated at 100%. In addition, TNF- $\alpha$  results expressed as percentages and MFI were strongly correlated (Data not shown).

### Statistical Analysis

Results are expressed as individual values and medians  $\pm$  IQR (interquartile range). Comparisons between patients and HV were made using the non-parametric Mann-Whitney U test. Comparisons between stimulated and non-stimulated tubes were made using the Wilcoxon paired test. Correlations were made with the Spearman correlation test. Statistical significance was set at  $p < 0.05$ . Statistical analyses were performed with R Studio software (version 1.2.5001; R studio, Boston, Massachusetts).

## RESULTS

### Clinical Characteristics of the Cohort

In total, 12 septic shock patients were included in this pilot study. Patients presented with usual demographic and clinical characteristics of septic shock patients (elderly patients and high severity scores, **Table 1**). These septic patients presented with signs of immunosuppression including decreased mHLA-DR and CD4+ T lymphopenia compared with reference values from the lab. In addition, nine healthy volunteers were included (median age = 53, five women - four men).

### Intracellular Cytokines in Monocytes

As reported previously, LPS stimulation induced a strong increase in intracellular TNF $\alpha$  expression in monocytes both from healthy volunteers and patients (12). However, this increase was statistically stronger in HV than in patients (MFI 61.6 [IQR:

**TABLE 1** | Demographic, clinical, and immunological data for septic shock patients.

Parameters	Septic shock Patients (n=12)
<b>Age at admission (years)</b>	69 [63–73]
<b>Gender - Male, n (%)</b>	7 (58)
<b>Main admission category</b>	
Medical, n (%)	4 (33)
Surgical, n (%)	8 (67)
<b>SAPS II score</b>	53 [50–62]
<b>SOFA score</b>	9 [8–10]
<b>McCabe score</b>	
0, n (%)	6 (50)
1, n (%)	6 (50)
<b>Infection diagnosis, n* (%)</b>	
Microbiology	10 (91)
Surgery	1 (9)
<b>Microbiologically documented, n(%)</b>	
Bacilli gram -	6 (40)
Cocci gram +	9 (60)
Other	0 (0)
<b>Site of infection, n* (%)</b>	
Pulmonary	1 (9)
Abdominal	3 (27)
<b>28-day non survivors, n (%)</b>	2 (17)
<b>Secondary nosocomial infections, n (%)</b>	3 (25)
<b>Immunological parameters</b>	
mHLA-DR (AB/C)	4044 [3,246–6,210]
Absolute CD4+ T cell count	317 [248–463]

Continuous data and biological parameters are presented as medians and interquartile ranges [Q1–Q3]. For clinical parameters, categorical data are presented as numbers of cases and percentages among the total population in brackets. SAPS II (Simplified Acute Physiology Score II) and McCabe scores were calculated after admission. SOFA (Sequential Organ Failure Assessment) score was measured after 24h of ICU stay. mHLA-DR was expressed as numbers of anti-HLA-DR antibodies bound per monocyte (AB/C). Reference values for healthy volunteers: mHLA-DR: > 15,000 AB/C, CD4+: 336–1126 cells/ $\mu$ l.

58.0–74.5] vs 23.6 [IQR: 16.8–39.9],  $p=0.0013$ ) (Figure 1A). We observed a good correlation between intracellular TNF $\alpha$  induction after stimulation and monocyte HLA-DR expression both in patients and controls, with individuals with high HLA-DR expression producing more TNF $\alpha$  than individuals with decreased HLA-DR expression ( $R=0.79$ ;  $p<0.001$ ) (Figure 1B). This was also observed at the single cell level as, in septic patients, monocytes with high HLA-DR expression also had higher expression of TNF $\alpha$  compared with HLA-DR<sup>low</sup> monocytes (MFI 27.5 [IQR: 15.3–45.7] vs 7.3 [IQR: 5.3–16.8],  $p=0.00049$ ) (Figures 1C, D).

LPS challenge also induced IL-10 expression in monocytes in both patients and controls (Figure 2A). However, in contrary to TNF $\alpha$ , the proportion of IL-10 positive monocytes was statistically higher in patients than HV (2.4% [IQR: 1.8–3.7] vs 1.2% [IQR: 0.9–1.6],  $p=0.0093$ ) (Figure 2B). The proportion of IL-10 positive monocytes was negatively correlated with HLA-DR (Figure 2C) and intracellular TNF $\alpha$  expression (Figure 2D) in these cells. Correlation coefficients were  $-0.61$  ( $p=0.0041$ ) and  $-0.67$  ( $p=0.0012$ ) respectively.

### Intracellular IL-10 Expression in Lymphocytes

In order to identify circulating lymphocyte subsets producing IL-10, we evaluated CD4, CD3 and CD19 expressions on IL-10

expressing cells after stimulation. B lymphocytes, CD4+ T cells and CD4- T cells were the three main subsets of IL-10 producing lymphocytes in both patients and HV (Figure 3A). Among the three subsets, CD4+ T cells were the main IL-10 producing lymphocytes representing more than 60% of IL-10+ lymphocytes. No significant difference was found in the relative proportions of IL-10 producing lymphocyte subsets between patients and HV and we did not observe any appearance or disappearance of an IL-10 producing lymphocyte subset after septic shock (Figure 3B).

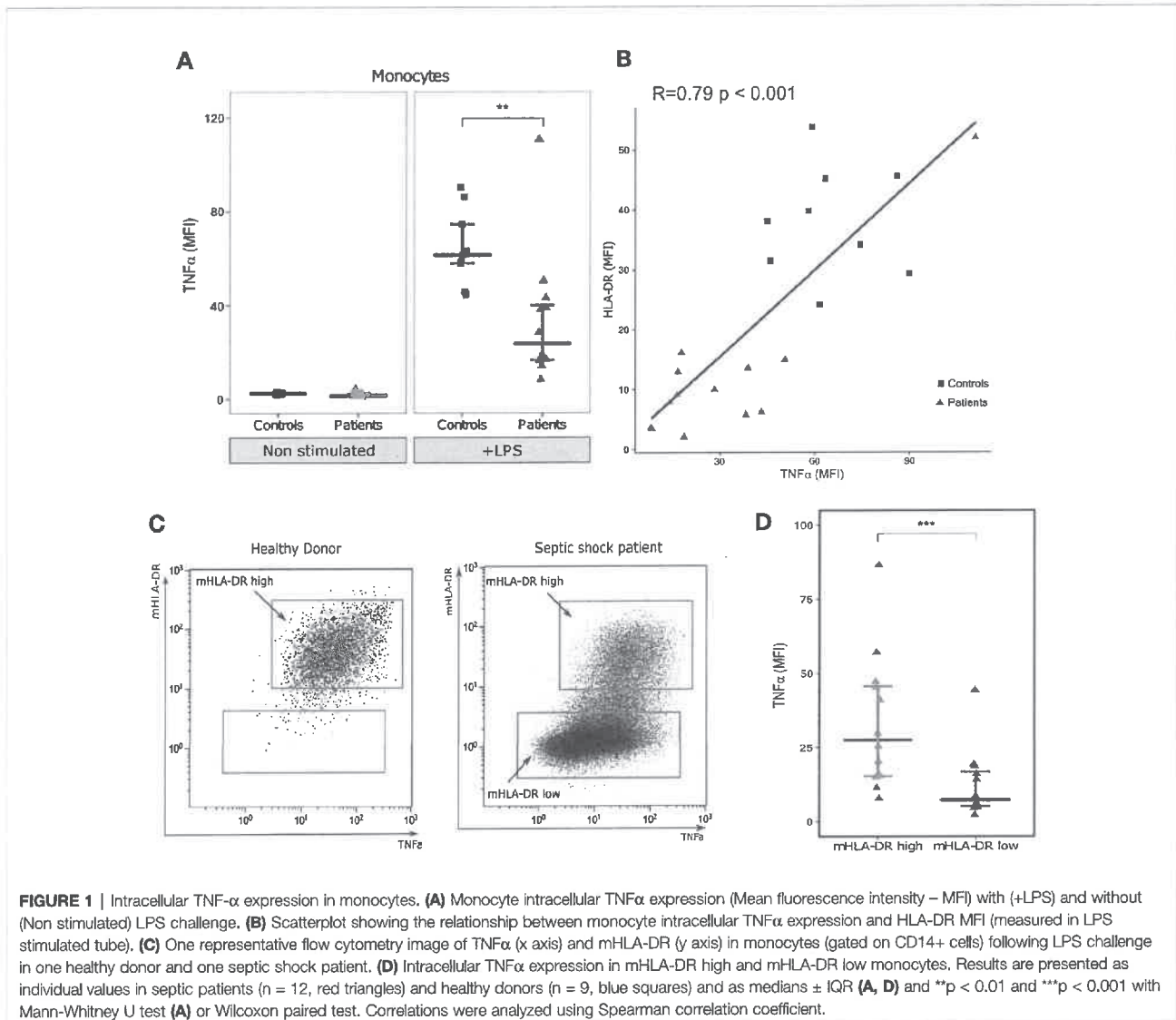
When comparing IL-10 production between patients and controls, in these beforehand identified cells no significant difference was observed in the proportion of B lymphocytes and CD4- T cells expressing IL-10. However, the proportion of CD4+ T cells expressing IL-10 was significantly higher among septic shock patients than HV (median [IQR] 2.8% [1.8–3.4] vs 1.6% [1.3–1.9],  $p=0.0077$ ) (Figure 4).

In addition, intracellular IL-10 expression in CD4+ T cells was inversely correlated to CD4+ T cell counts in patients ( $R=-0.8$ ,  $p=0.02$ ) (Figure 5A). The proportion of IL-10 positive CD4+ T cells was also negatively correlated to mHLA-DR and intracellular TNF $\alpha$  expression ( $R=-0.62$ ,  $p=0.0026$ , and  $-0.46$ ,  $p=0.034$  respectively) and positively correlated to the proportion of IL-10 positive monocytes ( $R=0.54$ ,  $p=0.012$ ) (Figures 5B–D).

## DISCUSSION

Results from this study showed that IL-10 production was induced in circulating monocytes from septic shock patients in parallel with decreased TNF $\alpha$  production and reduced HLA-DR expression. Among circulating lymphocytes, CD4+ T cells were the main IL-10 producers in circulating blood and this cytokine production was increased after septic shock. Such increase was negatively correlated with CD4+ T cell lymphopenia and positively with increased IL-10 production by monocytes. The strong correlations between parameters reflecting altered monocyte (increased IL-10 production, decreased HLA-DR expression and decreased TNF $\alpha$  synthesis) and CD4+ T lymphocyte (increased IL-10 production, decreased circulating number) responses suggest a common regulation mechanism and illustrate the development of a global immunosuppressive profile in septic shock. Overall, these preliminary results add to our understanding of the global increase in IL-10 production induced by septic shock.

Findings in the present study are consistent with sepsis-induced monocyte anergy described in the literature and the concept of leukocyte reprogramming (3–5). We replicated in whole blood results observed on purified or frozen cells showing the decreased TNF $\alpha$  but increased IL-10 productions by monocytes in septic patients characteristic of the phenomenon of endotoxin tolerance (13–15). However, we completed these observations by showing at the single cell level the correlation between decreased HLA-DR expression, decreased TNF $\alpha$  but increased IL-10 productions. Similarly, a negative correlation between mHLA-DR and IL-10 plasmatic concentrations was



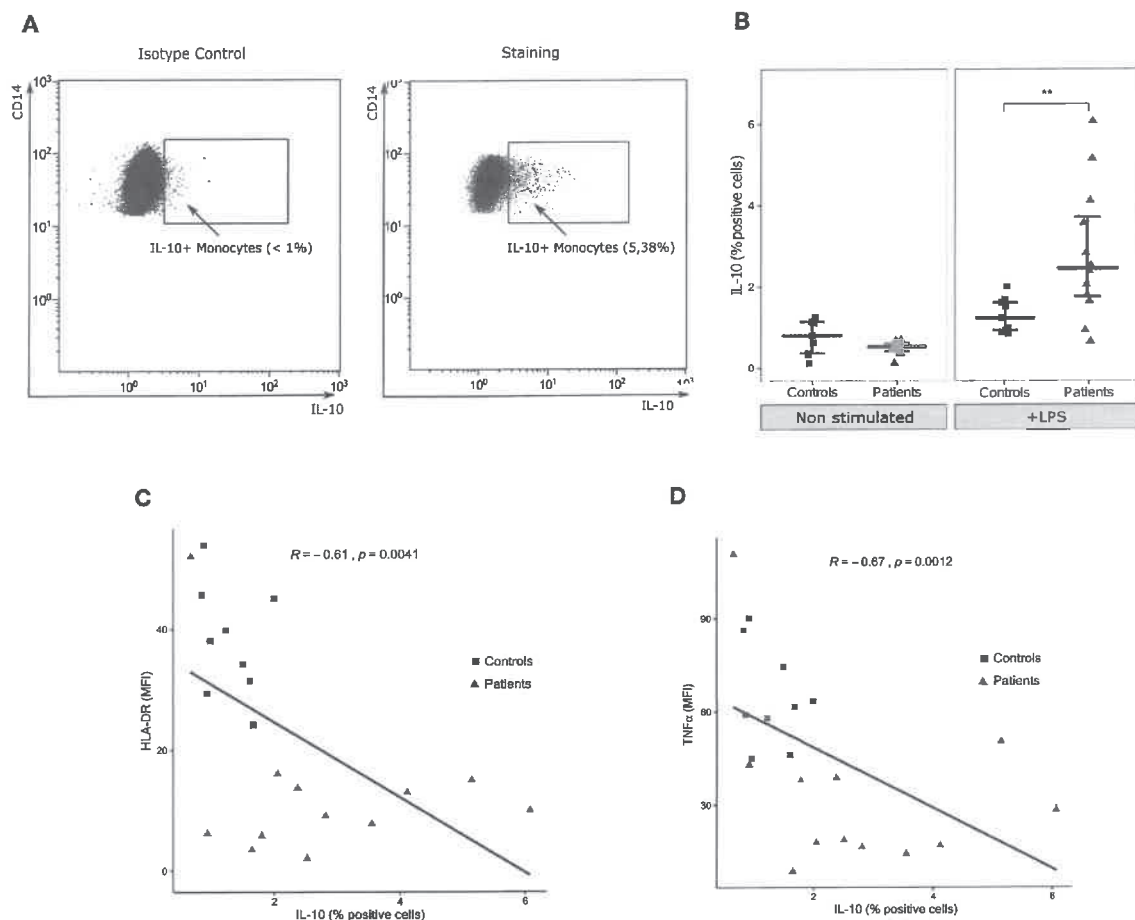
described in burned patients with sepsis (16). Together, these results call for further investigation of the theory defended by different groups suggesting that IL-10 plays a role in decreased MHC class II expression in sepsis either through intracellular sequestration of mHLA-DR or *via* inhibition of its transcription (17–19).

Three subsets of IL-10 producing lymphocytes were identified in both patients and HV: a predominant population of CD4+ T cells and two smaller populations of B lymphocytes and CD4- T cells. This was expected as IL-10 production has been reported in the literature for nearly all lymphocyte subsets (including B lymphocytes, CD4+ T cells, and CD8+ T cells) (20). It has also been described that IL-10 produced by CD4+ T cells is critical to limit inflammation in many infections that trigger adaptive immune responses (21). In addition, no new IL-10 producing lymphocyte subset emerged in septic patients. Therefore, any

difference in IL-10 production related to septic shock appears to be quantitative and not qualitative.

In other clinical contexts, IL-10 production by lymphocyte subsets such as T or B cells was proposed as a marker of regulatory cell subpopulations (22, 23). In sepsis, the issue of regulatory cells has recently grown in importance and a wide variety of regulatory cell subsets has been identified. Interestingly, different groups have reported an increase in the proportion of regulatory B cells (Breg) in septic shock patients (24, 25). In spite of the absence of consensus on the phenotype of Breg in the literature, there is consensus to attribute their immunosuppressive properties to their secretion of IL-10 (26). Contrary to data from the literature, our current results did not find a significant difference in the proportion of IL-10 positive B cells between septic shock patients and HV. This could be explained by the use of a whole blood approach or different





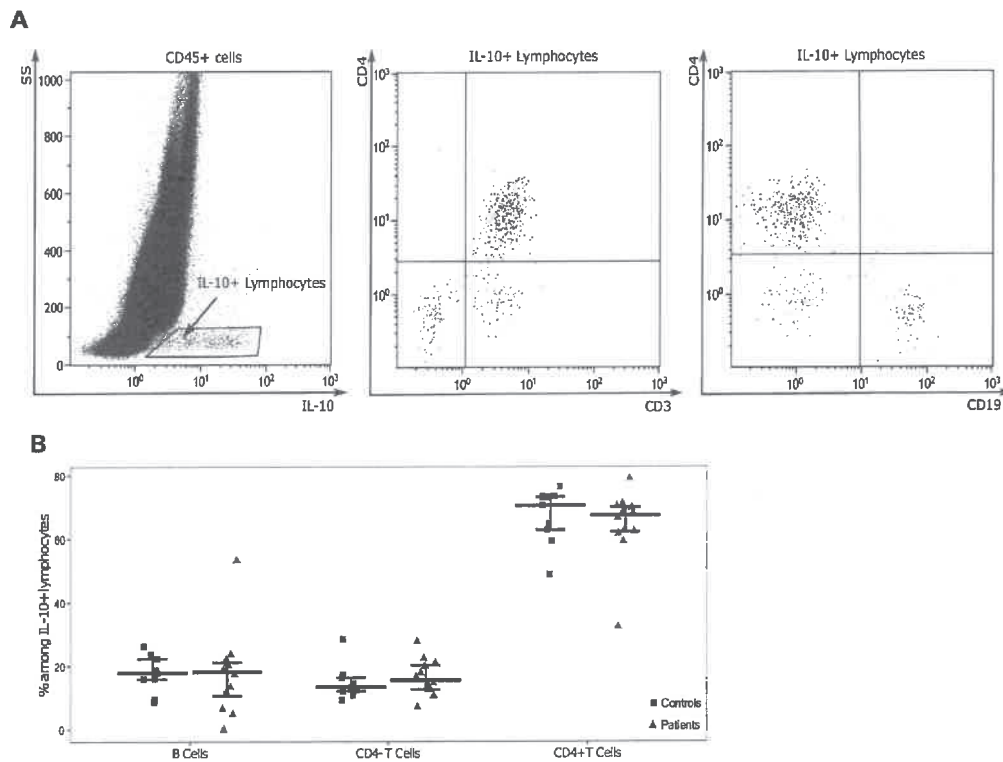
**FIGURE 2** | Intracellular IL-10 expression in monocytes. **(A)** One representative flow cytometry dot-plot and its respective isotype control of IL-10 (x-axis) and CD14 (y-axis) in monocytes (gated on CD14+ cells) following LPS challenge in a septic shock patient. Positivity threshold was defined based on isotype value below 1%. **(B)** Proportion of IL-10 positive monocytes among total monocytes with (+LPS) and without (Non stimulated) LPS challenge. Results are presented as individual values in healthy donors (n=9; blue squares) and septic patients (n = 12; red triangles) and medians  $\pm$  IQR. \*\*p < 0.01 with Mann-Whitney U test. **(C)** Scatterplot showing the relationship between monocyte intracellular IL-10 expression (% of positive monocytes, x-axis) and HLA-DR mean fluorescence intensity (MFI, y-axis). **(D)** Scatterplot showing the relationship between monocyte intracellular IL-10 expression and monocyte intracellular TNF $\alpha$  expression (MFI). Correlations were analyzed using Spearman correlation coefficient.

stimulation conditions compared with previous studies. That said, our results should not contest the importance of Breg in sepsis-induced immunosuppression but encourage further research on IL-10 independent immunoregulatory mechanisms for Breg in sepsis such as IL-35 and adenosine production, checkpoint inhibitors expression, or activation of the Fas/Fas ligand apoptotic pathway (27).

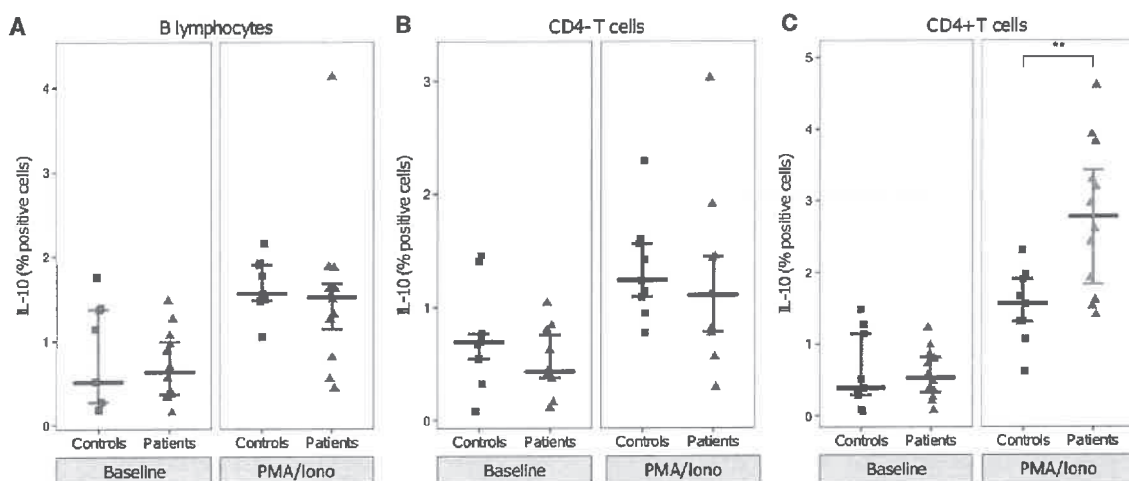
IL-10 synthesis by CD8+ T cells has been described using intracellular flow cytometry protocols in so-called CD8+ T regulatory cells (28). The immunomodulatory role of CD8+ T regulatory cells is well documented in autoimmune diseases, cancer, human transplants and certain infections (Human Immunodeficiency Virus or Epstein-Barr Virus) (29). However, to the best of our knowledge, no study has been conducted on IL-10 production by CD8+ T cells in septic shock patients. Our results did not show a significant

difference in the synthesis of IL-10 by CD8+ cells between patients and HV, although we cannot exclude that the possibility that CD4- T cells monitored in this study were double-negative. That said, CD8+ T cell exhaustion remains a hallmark of sepsis, as evidenced by the recently reported decrease in the synthesis of IL-2 and TNF $\alpha$  by CD8+ T cells (30).

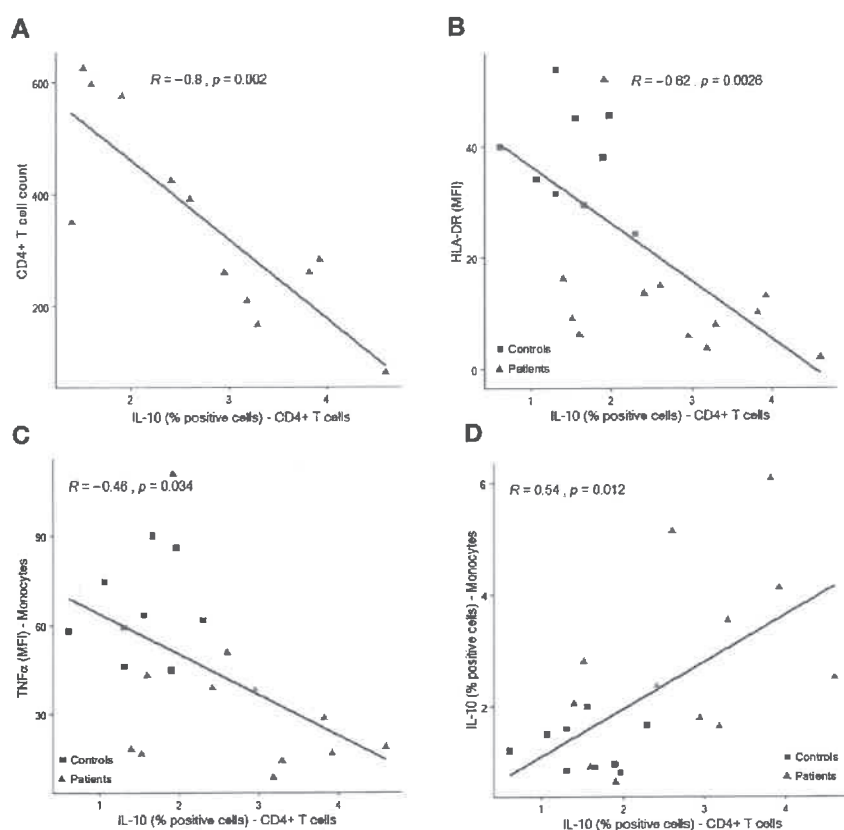
Finally, we revealed an increase in IL-10 synthesis by CD4+ T cells in septic shock patients. This finding is consistent with a considerable amount of published literature on regulatory T CD4+ cells (Treg) in sepsis and septic shock (31). Immunosuppressive properties of Treg are mediated by cell-cell contact mechanisms and IL-10 synthesis (32). In sepsis, the percentage of Treg is increased without change in their absolute value due to a selective depletion of other lymphocyte subsets (33, 34). This could imply that the increase in IL-10 production observed in our study is due to CD4+ T regs.



**FIGURE 3 |** Analysis of IL-10 producing lymphocytes. **(A)** One representative example of flow cytometry staining in a septic patient. First, on a IL-10 (x-axis) and SS (y-axis) dot-plot gated on in CD45+ leukocytes, we selected IL-10 producing lymphocytes (IL-10<sup>+</sup>SSC<sup>low</sup> cells). CD3, CD19, and CD4 expressions were then characterized on these cells based on CD4 (y-axis) and CD3 or CD19 (x-axis) dot-plots. Three populations of IL-10 producing cells were identified: CD19+CD3-CD4- cells (Green: B lymphocytes), CD19-CD3+CD4+ cells (purple: CD4+ T cells), CD19-CD3+CD4+ cells (red: CD4- T cells). **(B)** Proportions of lymphocyte subsets among IL-10 positive lymphocytes. Results are presented as individual values in healthy donors (n=9; blue squares) and septic patients (n = 12; red triangles) and medians ± IQR.



**FIGURE 4 |** Intracellular IL-10 expression in lymphocytes. Proportion of IL-10 positive B lymphocytes **(A)**, CD4- T cells **(B)**, and CD4+ T cells **(C)**. lymphocytes presented as individual values in healthy donors (n=9; blue squares) and septic patients (n = 12; red triangles) and medians ± IQR, with (PMA/Iono) and without (Non stimulated) PMA/Iono challenge. \*\*p < 0.01 with Mann-Whitney U test.



**FIGURE 5** | Correlations between IL-10 expressing CD4+ T lymphocytes and other immune parameters. Correlations between IL-10 producing CD4+ T cells (percentages among total CD4+ T cells, x-axis) and CD4+ cell counts (A, cells per  $\mu$ l, y-axis), monocyte HLA-DR expression (B, mean fluorescence intensity = MFI, y-axis), monocyte intracellular TNF $\alpha$  expression (C, MFI, y-axis) and monocyte intracellular IL-10 expression (D, percentages of positive cells among total monocytes, y-axis). Correlations were analyzed using Spearman correlation coefficient. Results from  $n = 9$  controls (blue squares) and 12 patients (red triangles) are shown.

We suggest adding an anti-Foxp3 antibody to our panel to explore this hypothesis. In addition, the negative correlation between the absolute count of CD4+ T cells and IL-10 expression in the same cells confirms previous findings by Roth et al. showing that increased IL-10 concentrations in sepsis may be due a susceptibility of Th1 T cells to apoptosis, resulting in a prevalence of Th2 T cells, known for their IL-10 production (35, 36).

To note, some authors rather described a dual role for IL-10 in human endotoxemia (37) and in COVID-19 (38) with both pro and anti-inflammatory properties. This stresses the importance to develop robust and easy to use tools to investigate IL-10 production by leukocytes.

The main limitation of our study was the small sample size and the absence of a kinetic evaluation. As it was a proof-of-concept study, only 12 patients were included, and we could not investigate association with clinical outcomes as our study was not powered for such purposes. In addition, the evaluation of potential confounding factors such as plasma LPS levels could not be performed. Results are thus preliminary and should be assessed and validated in a larger cohort of patients. In addition, in the absence of any extended phenotyping with, for example, no

specific marker of regulatory lymphocytes such as Foxp3 or of any functional test, we could not formally qualify IL-10 producing lymphocytes as regulatory cells. This specific aspect should be further confirmed in a dedicated physiopathological study.

## CONCLUSION

We demonstrated the feasibility of a novel technique for intracellular cytokine measurement in whole blood to monitor IL-10 production by circulating leukocytes in sepsis. We described sepsis-induced increase in IL-10 production by monocytes and CD4+ T cells but not B cells and CD8+ T lymphocytes. In addition, increased monocyte IL-10 production negatively correlated with mHLA-DR expression and TNF $\alpha$  production and with increased IL-10 production in CD4+ T cells and their absolute count. We therefore described the development of a global immunosuppressive profile affecting monocytes and CD4+ T cells in septic shock. Further research is now required to assess clinical significance of this profile in larger

cohorts of patients and to identify the pathophysiological mechanisms leading to its development in septic shock.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by CPP Ouest II—Anger. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

## AUTHOR CONTRIBUTIONS

AF, KK, RC, MG, GM, and FV designed and performed the experiments. AF performed the statistical analyses. PA, CM, MC,

TR, and LA included patients. All authors contributed to the article and approved the submitted version.

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

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## **Annexe 4**





# Monocyte CD169 expression in COVID-19 patients upon intensive care unit admission

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## Abstract

During the second surge of COVID-19 in France (fall 2020), we assessed the expression of monocyte CD169 (i.e., Siglec-1, one of the numerous IFN-stimulated genes) upon admission to intensive care units of 45 patients with RT-PCR-confirmed SARS-CoV2 pulmonary infection. Overall, CD169 expression was strongly induced on circulating monocytes of COVID-19 patients compared with healthy donors and patients with bacterial sepsis. Beyond its contribution at the emergency department, CD169 testing may be also helpful for patients' triage at the ICU to rapidly reinforce suspicion of COVID-19 etiology in patients with acute respiratory failure awaiting for PCR results for definitive diagnosis.

## KEYWORDS

CD169, COVID-19, monocyte, sepsis

## 1 | INTRODUCTION

The appearance of severe acute respiratory coronavirus-2 (SARS-CoV-2) has led to a rapidly spreading pandemic.<sup>1</sup> Since the first cases of coronavirus disease-19 (COVID-19), more than 73 million cases and 1.6 million deaths have been reported worldwide (by December, 16—Johns Hopkins University). In areas particularly affected by virus spread, the tremendous number of patients coming to hospital threatened to overwhelm emergency departments (ED) and intensive care

units (ICU) resources. Thus a crucial issue lied in the local capacity to rapidly test patients in order to confirm or rule out COVID-19 diagnosis and consequently optimize patients' triage. Diagnosis of COVID-19 was routinely achieved by detection of SARS-CoV-2 RNA in nasopharyngeal swabs via RT-PCR.

In response to viral infection, host cells immediately produce cytokines as a first alarm bell.<sup>2,3</sup> Among these, type-I interferons (IFN-I) are crucial components of innate immune system against viruses. IFN-I have, by themselves, antiviral properties but they also induce



the expression of hundreds of IFN-stimulated genes (ISG) turning cells into antiviral status that limits virus spread.<sup>2</sup> Among various effects, IFN-I induce sharp rise in CD169 (also known as Siglec-1) expression on monocytes (mCD169) whereas this molecule is not expressed (or at very low levels) under baseline conditions.<sup>4</sup> Such overexpression is consequently easily detectable by flow cytometry. Therefore, increased mCD169 has been proposed, by itself, as a potential host marker of viral infections (e.g., HIV, influenza). Moreover, in conjunction with concomitant assessment of neutrophil CD64 (nCD64) expression, mCD169 was used to differentiate viral from bacterial infections.<sup>5</sup> Recently, along with increased plasma IFN-I concentration, mCD169 overexpression was observed in COVID-19 patients.<sup>6</sup> Within a pandemic context, this highlighted the interest of mCD169 as a rapid marker for the triage of patients with suspected COVID-19. As this first work in COVID-19 reported on patients admitted to the ED, we investigated in prospective observational study whether CD169 overexpression would also be present in critically ill COVID-19 patients upon ICU admission.

## 2 | PATIENTS AND METHODS

### 2.1 | Patients

Critically-ill patients admitted to ICUs from university hospitals (Hospices Civils de Lyon, Lyon, France) who presented with pulmonary infection with SARS-CoV2 confirmed by RT-PCR testing were prospectively included in the study. This study was part of an ongoing prospective observational clinical study (RICO, REA-IMMUNO-COVID)<sup>7</sup> and was registered at ClinicalTrials.gov (NCT04392401). This study was approved by ethics committee (Comité de Protection des Personnes Ile de France 1—N°IRB/IORG #: IORG0009918) under agreement number 2020-A01079-30. The committee waived the need for written informed consent because the study was observational. Nevertheless, oral information and non-opposition to inclusion in the study were mandatory and were systematically obtained before any blood sample was drawn and recorded in patients' clinical files. If a patient was unable to consent directly, non-opposition was obtained from the patient's legally authorized representative and reconfirmed from the patient at the earliest opportunity. Inclusion criteria were: patients aged >18 years, diagnosis of COVID-19 confirmed by RT-PCR testing in one respiratory sample. Exclusion criteria were pregnancy, institutionalized patients, inability to obtain informed consent. As controls, patients with septic shock (bacterial infection) from the cohort IMMUNOSEPSIS<sup>8</sup> were included as well as healthy donors from Etablissement Francais du Sang (Lyon, F).

### 2.2 | Flow cytometry

EDTA-whole blood samples were processed by flow cytometry according to a newly described one-step procedure (no wash, no centrifuge protocol).<sup>9</sup> For each blood sample tested, 500  $\mu$ l of Versalyse

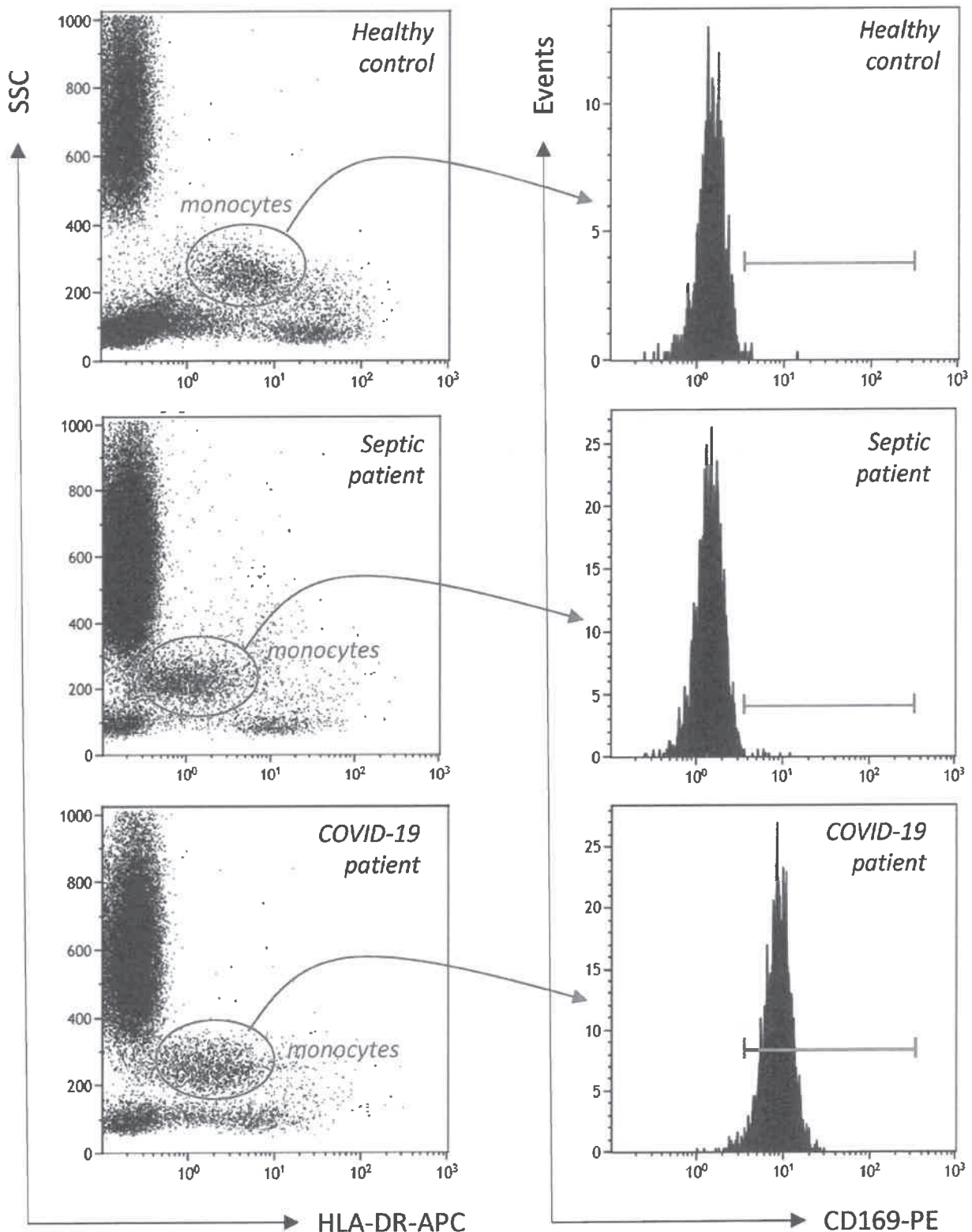
lysing solution (Beckman Coulter, Hialeah, FL), 10  $\mu$ l of blood and 10  $\mu$ l IOTest Myeloid Activation antibody cocktail (Beckman Coulter), containing three markers: anti-CD169-PE (clone 7-239), anti-CD64-PB (clone 22), anti-HLA-DR-APC (clone Immu357) were incubated for 30 min at room temperature in the dark. Afterwards, samples were directly analyzed on a three-laser, 10-color Navios flow cytometer (BeckmanCoulter) according to a compensation-free protocol. Monocytes were identified on a Side Scatter (SSC)/HLA-DR dot plot whereas neutrophils and lymphocytes were gated based on SSC and forward scatter characteristics (FSC) characteristics (Figure 1). CD169 results were expressed as mean of fluorescence intensities (MFI) and CD169 ratio (i.e., mCD169 MFI/lymphocyte CD169 MFI). In addition, to fit with results from multi-parametric paper in COVID-19,<sup>10</sup> results were also expressed as % of positive cells (positivity threshold was based on lack of CD169 expression on neutrophils). Neutrophil CD64 results were expressed as MFI.

## 3 | RESULTS

During the second surge of COVID-19 pandemic in France, between October 8th and December 8th, 2020, we enrolled 45 critically ill patients with positive SARS-CoV-2 RT-PCR. During the same period, we also included 2 ICU patients with septic shock and 12 healthy controls. Main demographic and characteristics are presented in Table 1. We observed a strong mCD169 overexpression in COVID-19 ICU patients and clear differences with septic shock or healthy donors with results expressed as MFI, percentages or ratio (Figure 2). Original data for the measurements in the three patients groups are listed in Table S1. When expressed as percentages of CD169+ monocytes, all COVID-19 patients except two expressed higher mCD169 than healthy donors and septic shock patients. Notably, in more than half of COVID-19 patients, almost all circulating monocytes expressed CD169. Regarding results as ratio, when applying the threshold recently proposed by Bedin et al. (i.e., 3.5),<sup>6</sup> 77% of COVID-19 ICU patients presented with elevated results whereas all controls and septic patients were <3.5. Of note, although nCD64 values measured in COVID-19 patients were not as high as observed in septic shock patients, they were nevertheless elevated above healthy controls (data not shown). This might be due to putative co-infections as reported in COVID-19 patients arriving at the ICU.<sup>11</sup>

## 4 | DISCUSSION

In areas particularly affected by the virus spread, the tremendous number of patients coming to hospital overwhelmed hospital resources.<sup>12</sup> As a consequence, the local capacity to rapidly test patients in order to confirm or rule out COVID-19 diagnosis and consequently optimize patients' triage has appeared as a major factor in the fight against COVID-19 pandemic. Due to the surge of patients, resources to address the diagnosis (routinely achieved by detection of SARS-CoV-2 RNA in nasopharyngeal swabs via RT-PCR) in a timely

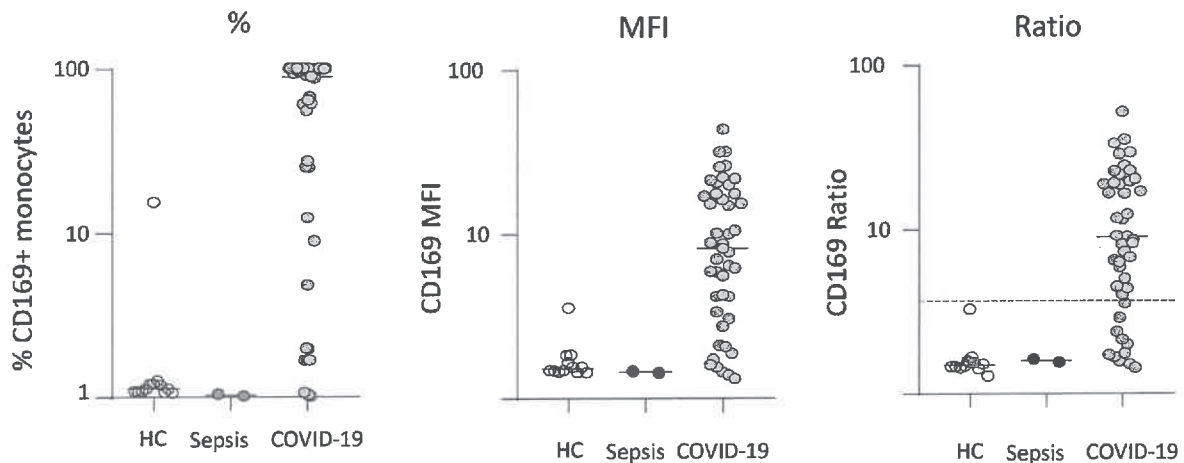


**FIGURE 1** Gating strategy (illustrative examples). Monocytes are first gated out from other cells on the basis of on SSC (side-scatter) / HLA-DR histogram (blue circle, left histogram) and CD169 is then measured on their surface (monoparametric histogram, right panel) as mean of fluorescence related to the entire monocyte population or as % of positive cells (positivity threshold, red cursor, was based on lack of CD169 expression on neutrophils). In addition, CD169 MFI was also determined on total lymphocyte population gated on SSC / FSC (forward-scatter) histogram (not shown) in order to provide results CD169 ratio (i.e., mCD169 MFI/lymphocyte CD169 MFI). One illustrative example of each group is depicted (healthy controls, septic shock, COVID-19)

	Healthy donors	COVID-19	Septic shock
Number	12	45	2
Age (years) <sup>a</sup>	40 (28.7–54.5)	65 (60.7–74.5)	37, 79
Sex (% male)	25	78	50
Invasive ventilation, n (%)	0	22 (48.9)	2 (100)

**TABLE 1** Main clinical and demographic characteristics of patients and healthy controls

<sup>a</sup>Median (interquartile range) except septic shock.



**FIGURE 2** Monocyte CD169 expression in COVID-19 ICU patients. Results are presented as percentages of positive cells (out of total monocyte population, left graph), median of fluorescence intensity in total monocyte population (MFI, middle graph), ratio monocyte CD169 MFI/lymphocyte CD169 MFI (right graph). The blue dashed line represents threshold proposed in Reference 6. Healthy controls ( $n = 8$ ) are depicted by empty circles, septic shock patients ( $n = 2$ ) are depicted by red circles and COVID-19 ICU patients ( $n = 45$ ) are depicted by orange circles

manner have been overwhelmed. Rapid antigen detection tests have been developed to identify active infection but, in their first generation, they lack sensitivity. Consequently, measurement of host marker reflecting viral infection has appeared as a sound approach. Unfortunately, IFN-I are produced at very low concentrations and most usual ELISA technologies are not able to quantify such low amount of cytokines (concentrations in fg/ml range). Only ultrasensitive digital Elisa (SIMOA) allows for the accurate IFN-I measurements but this technique is usually not available on a routine basis. The detection of ISG mRNA levels by molecular approach, although possible within the non-urgent context of auto-immunity, is not suitable to COVID-19 emergency due to extended time-to-results.<sup>13,14</sup> Alternatively, overexpression of mCD169 (siglec-1, one of ISG) by whole blood flow cytometry has been recently proposed as a sensitive and specific surrogate marker of viral infection within the context of early COVID-19.<sup>6</sup> This marker was previously described as a tool, in conjunction with nCD64 expression, to discriminate bacterial from viral infections at emergency room.<sup>5,15</sup> Specific kinetics of mCD169 expression in response to IFN-I has been described *in vitro*.<sup>4</sup>

The main result of the present study is to show that, upon ICU admission, critically ill COVID-19 patients presented with strong mCD169 overexpression. Although altered IFN-I production is known to undoubtedly concur to the most severe forms of COVID-19,<sup>16–18</sup>

mCD169 overexpression was still observed in this cohort. According to Bedin's threshold, almost 80% of patients were correctly identified as virally infected. Thus, in more severe patients, the present work reinforces the seminal description by Bedin et al.<sup>6</sup> It also extends the interest of mCD169 testing to larger cohorts of patients, especially those directly admitted to ICU for acute respiratory failure after sudden pulmonary worsening and without definitive diagnosis. Thus elevated mCD169 could help for patients triage in the ICU by reinforcing suspicion of COVID-19 etiology while awaiting for PCR results. mCD169 protocol is a one-step whole blood method that enables a streamlined flow cytometry sample preparation protocol. The simplified phenotyping procedure reduces training requirements and overcomes logistic constraints inherent to many flow cytometry applications (in particular due to the absence of compensation). Reagents costs are limited (<5 €/test) and results may be provided in less than 45 min. Consequently, mCD169 may contribute to preserving hospital medical capacities by accelerating patients' triage.

By nature, this preliminary work, conducted during the second wave of pandemic in France presents with limitations. First, a larger cohort of ICU COVID-19 patients would be desirable to confirm these results. In addition, before drawing definitive conclusion, the inclusion of a cohort of mixed ICU patients with different etiologies (surgery, trauma, sepsis, burns) needs to be tested for mCD169 to precisely

investigate specificity. Finally, the question of a potential interest of a daily mCD169 monitoring should be explored in further investigations. That given, these preliminary results in ICU patients aggregate with previously published data to underline the interest in mCD169 as a complementary tool to PCR results. As such, this could be of major help during the present pandemic. Of note, during the writing of this manuscript, another study, describing concordant mCD169 results at the ED has been released (Bourgoin et al., medRxiv 2020, doi: <https://doi.org/10.1101/2020.10.28.20221259>). Importantly, by using similar protocol (antibodies, lysis reagent), the authors shortened incubation/staining time at 15 min.

Collectively, these first results from critically ill COVID-19 patients indicate that increased mCD169 expression, an IFN-I inducible receptor, may contribute to COVID-19 patients triage in the ICU while awaiting for definitive PCR results. The development of automated flow cytometric mCD169 measurement should be considered for further viral pandemic.

#### CONFLICT OF INTEREST

This study was partially supported by Beckman Coulter through donation of the CD169 reagents used in the study. This private company had no role in the study design, or collection and interpretation of the clinical data.

#### AUTHOR CONTRIBUTIONS

**Marine Ortilon:** Formal analysis; writing-original draft. **Remy COUDEREAU:** Formal analysis; investigation; writing-review and editing. **Martin Cour:** Investigation; supervision; validation; writing-review and editing. **Thomas Rimmelé:** Investigation; supervision; validation; writing-review and editing. **Marine godignon:** Supervision; validation; writing-review and editing. **Morgane Gossez:** Validation; writing-review and editing. **hodane yonis:** Investigation; supervision; writing-review and editing. **laurent argaud:** Investigation; supervision; validation; writing-review and editing. **anne-claire lukaszewicz:** Investigation; supervision; validation; writing-original draft. **Fabienne Venet:** Investigation; methodology; supervision; validation; writing-original draft. **Guillaume Monneret:** Conceptualization; data curation; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; writing-original draft.

#### DATA AVAILABILITY STATEMENT

The authors certify that this manuscript reports original clinical research data. Individual data that underlie the results reported in this article are available from the corresponding author following publication, including the study report and study protocol. Additional data are available upon reasonable request.

#### ETHICS AND PATIENT APPROVAL

This study was part of an ongoing prospective observational clinical study (RICO, REA-IMMUNO-COVID) and was registered at ClinicalTrials.gov (NCT04392401). This study was approved by ethics committee (Comité de Protection des Personnes Ile de France 1—N°IRB/IORG #: IORG0009918) under agreement number 2020-A01079-30. The

committee waived the need for written informed consent because the study was observational. Nevertheless, oral information and non-opposition to inclusion in the study were mandatory and were systematically obtained before any blood sample was drawn and recorded in patients' clinical files. If a patient was unable to consent directly, non-opposition was obtained from the patient's legally authorized representative and reconfirmed from the patient at the earliest opportunity.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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## **Annexe 5**



RESEARCH

Open Access



# Longitudinal assessment of IFN-I activity and immune profile in critically ill COVID-19 patients with acute respiratory distress syndrome

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## Abstract

**Background:** Since the onset of the pandemic, only few studies focused on longitudinal immune monitoring in critically ill COVID-19 patients with acute respiratory distress syndrome (ARDS) whereas their hospital stay may last for several weeks. Consequently, the question of whether immune parameters may drive or associate with delayed unfavorable outcome in these critically ill patients remains unsolved.

**Methods:** We present a dynamic description of immuno-inflammatory derangements in 64 critically ill COVID-19 patients including plasma IFN $\alpha$ 2 levels and IFN-stimulated genes (ISG) score measurements.

**Results:** ARDS patients presented with persistently decreased lymphocyte count and mHLA-DR expression and increased cytokine levels. Type-I IFN response was initially induced with elevation of IFN $\alpha$ 2 levels and ISG score followed by a rapid decrease over time. Survivors and non-survivors presented with apparent common immune responses over the first 3 weeks after ICU admission mixing gradual return to normal values of cellular markers and progressive decrease of cytokines levels including IFN $\alpha$ 2. Only plasma TNF- $\alpha$  presented with a slow increase over time and higher values in non-survivors compared with survivors. This paralleled with an extremely high occurrence of secondary infections in COVID-19 patients with ARDS.

**Conclusions:** Occurrence of ARDS in response to SARS-CoV2 infection appears to be strongly associated with the intensity of immune alterations upon ICU admission of COVID-19 patients. In these critically ill patients, immune profile presents with similarities with the delayed step of immunosuppression described in bacterial sepsis.

**Keywords:** COVID-19, ARDS, Type-I IFN, Immune profile, Immunosuppression

## Introduction

The severe acute respiratory coronavirus-2 (SARS-CoV-2) is responsible for coronavirus disease-19 (COVID-19) that mostly associates with asymptomatic and mild presentations but may progress in worst cases to severe pneumonia leading to intensive care unit (ICU) admission and acute respiratory distress syndrome (ARDS) requiring respiratory support [1]. Since the onset

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of the pandemic, the number of COVID-19 patients has never ceased to rise and has ultimately outstripped hospital/ICU capacity in some areas particularly affected by virus spread. COVID-19 mortality, although currently decreasing, has remained dramatically high especially in patients requiring invasive mechanical ventilation [1].

An enormous research effort has been consented to describe and understand the mechanisms sustaining altered host immune response to a virus totally unknown of human immune surveillance. Many exploratory non-hypothesis-driven programs have been deployed in order to decipher immune processes at play in COVID-19. Through various flow cytometry approaches, transcriptomic strategies, functional testing and multiplex measurement of soluble mediators, most studies compared immune response between groups of COVID-19 patients with increasing severity, i.e., mild/severe/critical [2–5]. These works provided homogenous results describing that, upon hospital arrival, the most severe phenotype associated with inflammatory response (e.g., moderate plasma IL-6 elevation) and altered cellular immunity, i.e., decreased monocyte HLA-DR expression (mHLA-DR) and marked lymphopenia [6–11]. In addition, impaired type I interferons (IFN-I) activity has emerged as a contributor to the disease severity [12–15]. These latter cytokines are crucial components of innate immune response against viruses by ringing a “first alarm” bell. IFN-I have, by themselves, antiviral properties but they also induce the expression of hundreds of IFN-stimulated genes (ISG) inducing cellular antiviral activity, therefore, limiting virus spread [5].

In contrast, fewer studies focused on longitudinal immune monitoring in hospitalized COVID-19 patients, whereas their hospital stay may last for several weeks [1]. This is especially true in critically ill COVID-19 patients with ARDS who also present with the highest mortality [1]. To investigate this particular aspect of COVID-19 immune response, we monitored selected immunological parameters, including IFN $\alpha$ 2 measurement and IFN-stimulated genes (ISG) transcriptomic signature, in a group of 64 COVID-19 patients requiring ICU care over a 3-week period after ICU admission.

## Methods

### Clinical study design, patient population and approval

Between March and May 2020, critically ill patients admitted to three ICUs from academic hospital (Hospices Civils de Lyon, Lyon, France) who presented with pulmonary infection with SARS-CoV-2 confirmed by RT-PCR testing were prospectively included in the study. A flowchart describing the patient datasets used for the different analyses is provided in Additional file 1: Figure S1. Preliminary results from a subgroup of the cohort

were published previously [9]. This project was part of an ongoing prospective observational clinical study (RICO, REA-IMMUNO-COVID). It was approved by ethics committee (Comité de Protection des Personnes Ile de France 1 - N°IRB/IORG #: IORG0009918) under agreement number 2020-A01079-30. This clinical study was registered at ClinicalTrials.gov (NCT04392401). The committee waived the need for written informed consent because the study was observational, with a low risk to patients, and no specific procedure, other than routine blood sampling, was required. Oral information and non-opposition to inclusion in the study were mandatory and were systematically obtained before any blood sample was drawn. This was recorded in patients' clinical files. If a patient was unable to consent directly, non-opposition was obtained from the patient's legally authorized representative and reconfirmed from the patient at the earliest opportunity. Inclusion criteria were: patients aged > 18 years, diagnosis of COVID-19 confirmed by RT-PCR testing in one respiratory sample. Inclusion criteria were (1) man or woman aged 18 or over, (2) hospitalization in ICU for SARS-CoV-2 pneumopathy, (3) first hospitalization in ICU, (4) positive diagnosis of SARS-CoV2 infection carried out by PCR or by another approved method in at least one respiratory sample, (5) sampling in the first 24 h after admission to ICU (D0) feasible and (6) patient or next of kin who has been informed of the terms of the study and has not objected to participating. Exclusion criteria were pregnancy, institutionalized patients, inability to obtain informed consent.

### Patient characteristics

For each patient, demographics, comorbidities, time from onset of COVID-19 symptoms to ICU admission, initial presentation of the disease in ICU including the ratio of the arterial partial pressure of oxygen to the fractional inspired oxygen (PaO<sub>2</sub>/FiO<sub>2</sub> ratio) at admission, antiviral therapy targeting SARS-CoV-2 and organ support were documented. Organ dysfunctions according to sequential organ failure assessment (SOFA) score (range 0–24, with higher scores indicating more severe organ failures) and simplified acute physiology score II (SAPS II; range 0–164, with higher scores indicating greater severity of illness) were documented. Patients were classified in the acute respiratory distress syndrome (ARDS) group if they were invasively ventilated and met the Berlin criteria for ARDS within the first 3 days after ICU admission [16]. Follow-up included ICU length of stay, in-hospital mortality, day-28 (D28) mortality, day-90 (D90) mortality, as well as occurrence secondary infection based on recommendation from Comité technique des infections nosocomiales et des infections liées aux soins [17].

### Blood samples

Ethylene diamine tetraacetic acid (EDTA-)anticoagulated blood was drawn five times during the first month after ICU admission: within the first 48 h after admission (Day 0: D0), between 72 and 96 h after admission (D3), between D7 and D9 (D7), between D12 and D15 (D12) and between D20 and D25 (D20). Blood was stored at 4–8 °C and processed within 4 h after withdrawal. The numbers of available values for each immune parameter at each time point are presented in Additional file 1: Table S4.

### Cytokine measurement

Whole blood was sampled on EDTA tubes and plasma was frozen at – 20 °C within 4 h following blood collection. Cytokine measurement was taken by batches after 1 freeze/thaw cycle using standardized protocols fulfilling clinical and diagnostic laboratories accreditation requirements from the International Organization for Standardization. Plasma concentrations of IL-6, TNF- $\alpha$ , IFN- $\gamma$  and IL-10 were measured by Simpleplex<sup>®</sup> technology using ELLA instrument (ProteinSimple<sup>®</sup>, San Jose, CA), following manufacturer's instructions. Plasma IFN $\alpha$ 2 concentrations were determined by single-molecule Array (SIMOA<sup>®</sup>) on a HD-1 Analyzer (Quanterix) using a commercial kit for IFN- $\alpha$ 2 quantification (Quanterix, Lexington, Mass).

### IFN-stimulated genes (ISG) score calculation

Whole blood was collected on PAXgene blood RNA tubes (BD, Grenoble, France) for IFN signature and frozen at – 80 °C until RNA extraction. IFN score was obtained using nCounter<sup>®</sup> analysis technology (NanoString Technologies, Seattle, WA) by calculating the median of the normalized count of 6 ISGs using standardized protocols fulfilling clinical and diagnostic laboratories accreditation requirements from the International Organization for Standardization. As previously described, six interferon responsive genes were monitored: *SIGLEC1* (sialic acid binding Ig like lectin 1), *IFI27* (interferon alpha inducible protein27), *IFI44L* (interferon induced protein 44 like), *IFIT1* (interferon induced protein with tetratricopeptide repeats 1), *ISG15* (interferon-stimulated gene 15) and *RSAD2* (radical S-adenosyl methionine domain-containing 2). Three reference genes were also measured: *ACTB* (Actin beta), *HPRT1* (hypoxanthine phosphoribosyltransferase 1) and *POLR2A* (RNA Polymerase II Subunit A) [18].

### Flow cytometry

T lymphocyte subpopulation immunophenotyping was performed on an automated volumetric flow

cytometer from Beckman Coulter (Aquios CL) as previously described [19]. Monocyte HLA-DR expression and B and NK immunophenotyping were performed using antibodies from Beckman-Coulter and BD Biosciences. The expression of monocyte HLA-DR was determined using the Anti-HLA-DR/Anti-Monocyte Quantibrite assay (BD Biosciences, San Jose, USA). A total number of antibodies bound per cell (AB/C) were quantified using calibration with a standard curve determined with BD Quantibrite phycoerythrin (PE) beads (BD Biosciences) as described elsewhere [20]. B and NK lymphocyte immunophenotyping was performed using lyophilized antibody panel from Beckman Coulter (Duraclone kit). Data were acquired on a Navios flow cytometer (Beckman Coulter, Hialeah, FL), and flow data were analyzed using Navios software (Beckman Coulter). Enumeration of lymphocyte subpopulations as well as mHLA-DR measurement were performed using standardized protocols fulfilling clinical and diagnostic laboratories accreditation requirements from the International Organization for Standardization.

### SARS-CoV-2 detection by semiquantitative PCR

Semiquantitative values of SARS-CoV-2 viral load in upper respiratory samples at ICU admission were retrieved from clinical files for 40 patients. These results were obtained from accredited reference laboratory using RT-PCR technique with validated commercial kits (COBAS<sup>®</sup> SARS-CoV-2, Roche Diagnostics or reference technique from Pasteur Institute) based on recommendations from the Société Française de Microbiology after evaluation of analytical performances of the techniques [21]. Patients were classified into three groups according to SARS-CoV-2 PCR Ct values reflecting respiratory viral load at admission (1) A significant viral excretion (ct value  $\leq$  33) subsequently divided into two groups: high viral load (Reference Ct value  $<$  27,  $n = 12$ ) and medium viral load (Reference Ct value = [27–33],  $n = 21$ ); (2) a nonsignificant viral excretion corresponding to a low viral load (Reference Ct value = [33–37],  $n = 7$ ).

### Statistical analysis

Data are presented as numbers and percentage (qualitative variables) and medians and 25th/75th percentiles (quantitative variables). Chi square or Fisher's exact test were used for qualitative variables assessment. Quantitative variables were compared with Mann–Whitney U test. For all pairs of immune parameters, Spearman's rho correlation coefficients were estimated and summarized in a correlation matrix. Kaplan–Meier survival curves were calculated in groups of patients with or without ARDS. The p-value of the log-rank test is given. The level of significance was set at 5%. Data were analyzed using

Graphpad Prism version 5.03 (Graphpad Software, La Jolla, USA).

## Results

### Results on admission

Sixty-four patients with confirmed pulmonary SARS-CoV-2 infection admitted to three ICUs of Lyon University Hospitals (Hospices Civils de Lyon, Lyon, France) were included between March 16 and May 15, 2020. This period corresponded to the first surge in COVID-19 cases in France. Clinical characteristics are presented in Table 1. Median duration of symptoms before ICU admission was 7 [4–11] days. Forty patients (63%) presented with ARDS requiring mechanical ventilation within the first 3 days after ICU admission; 23/24 (96%) patients without ARDS needed noninvasive ventilation (including high flow nasal oxygenotherapy) for respiratory dysfunction/failure. Flowchart and the number of samples by time point are provided in Additional file 1: Figure S1A. As assessed after 28 days, mortality in this cohort was 22% with contrasting results between patients without ARDS (no mortality) and with ARDS (35%) (Additional file 1: Figure S1B).

On admission, critically ill COVID-19 patients presented with elevated plasma levels of both pro- and anti-inflammatory cytokines (Additional file 1: Table S1). Marked lymphopenia (median = 653 cells/ $\mu$ L) affecting all lymphocyte subsets (e.g., median CD4+T lymphocytes = 298 cells/ $\mu$ L, with CD4/CD8 ratio in normal range) [10, 11] and moderately decreased mHLA-DR (median = 11,125 AB/C, control values > 13,500 AB/C) [22] were also hallmarks of initial critically ill COVID-19 patients' immune profile. Collectively, these results are in agreement with previously published values in ICU cohorts [9, 23, 24]. Interestingly, we observed that IFN-I response was induced in the majority of these patients as we measured elevated levels of plasma IFN $\alpha$ 2 (median = 385 fg/mL, control values < 20 fg/mL) and ISG score (median = 40, control values < 2.3). These concentrations were consistent with the previously published results in cohorts of critically ill patients [12]. To note, for these inaugural immune parameters, no differences in their concentrations were observed based on delay between first symptoms and sampling time (data not shown).

When correlating immune parameters at ICU admission (Fig. 1a), significant correlations were noted between increased pro-inflammatory IL-6 concentration and increased TNF- $\alpha$  and IL-10 levels (positive correlations), and decreased T lymphocyte number (negative correlation). No correlation was observed between IL-6 and IFN $\alpha$ 2 levels or ISG score. In addition, initial increased IL-6 concentration was correlated with initial severity

as measured by sepsis-related organ failure assessment (SOFA) score and with intensity of pulmonary dysfunction as measured by the ratio between partial pressure of oxygen in arterial blood (PaO<sub>2</sub>) and fraction of inspired oxygen (FiO<sub>2</sub>) (PaO<sub>2</sub>/FiO<sub>2</sub> ratio, Fig. 1b). Finally, when classifying patients into three groups according to semi-quantitative levels of viremia measured in upper respiratory samples, the group of patients with the highest viral load presented not only with higher plasma IL-6 concentrations at admission but also with a higher SOFA score and increased mortality (33%, Additional file 1: Figure S2). This suggests that the immune response is positively associated with viral burden and that failure to resolve both aspects may underlie severity [8].

### Comparison between ARDS and non-ARDS patients

Considering the significant difference between ARDS and non-ARDS groups regarding D28 mortality (Additional file 1: Figure S1B), we then compared immune profiles between these 2 groups of patients (Fig. 2, Additional file 1: Table S2). As most non-ARDS patients presented with a short ICU stay of less than 1 week, we focused on the first three time points after ICU admission. Non-ARDS patients presented with a different immune profile than patients with ARDS, including higher lymphocytes count and mHLA-DR expression and lower cytokine levels and severity scores. Except for IL-6 and TNF- $\alpha$ , these differences persisted over the first week of follow-up (Fig. 2). We observed no difference between ARDS and non-ARDS patients regarding plasma IFN $\alpha$ 2 measurements or ISG score (Fig. 2). The correlation between these two parameters was statistically significant ( $r=0.7$ ,  $p<0.0001$ ). In addition, we observed a rapid decrease of IFN-I markers after ICU admission in both groups. This shows that IFN-I response was induced in critically ill COVID-19 patients and efficient in activating downstream genes. However, the intensity of this response was not impacted by the development of pulmonary dysfunction or disease severity upon ICU admission.

### Comparison between survivors and non-survivors among ARDS patients

We next focused on ARDS patients who presented with a prolonged ICU length of stay to evaluate immune profiles in regard with D28 mortality during a 3-week follow-up (Fig. 3, Additional file 1: Table S3). Clinical characteristics of survivors and non-survivors are presented in Table 2. Surprisingly, we did not observe any difference between survivors and non-survivors regarding the majority of immune parameters. In this cohort, survivors and non-survivors presented with apparent common immune responses mixing gradual return to normal ranges of cellular markers (mHLA-DR,

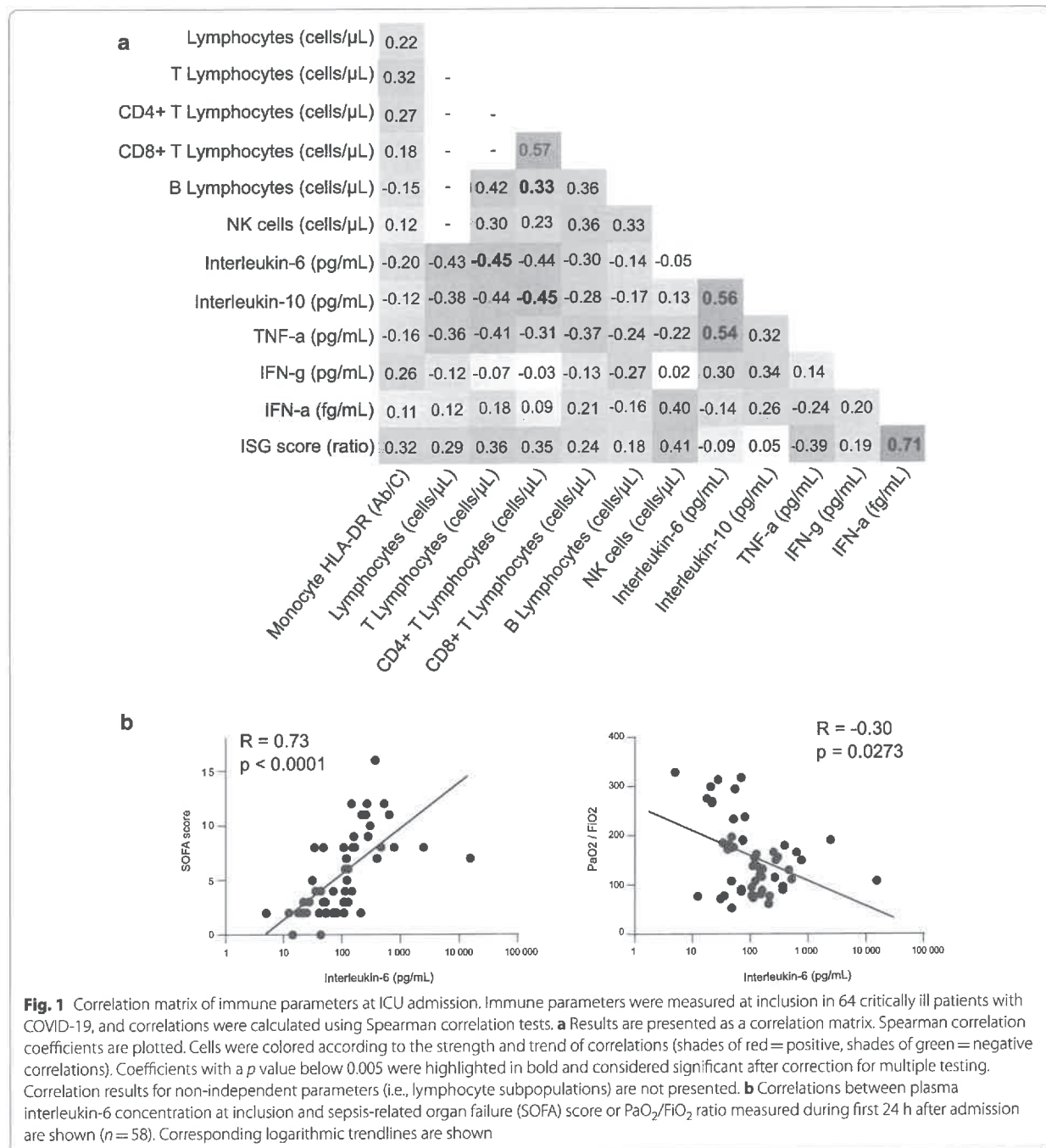
**Table 1** Clinical characteristics of critically ill patients with COVID-19 at ICU admission

	All patients (n = 64)	ARDS (n = 40)	No ARDS (n = 24)	p value
Demographics				
Age	65 [52–72]	66 [57–72]	55 [43–72]	0.0738
Gender	51 (80%)	36 (90%)	15 (63%)	<b>0.0116</b>
Body mass index (kg/m <sup>2</sup> )	28 [26–32]	29 [26–34]	28 [25–30]	0.2124
Body mass index > 30 kg/m <sup>2</sup>	23 (36%)	17 (43%)	6 (25%)	0.1874
Comorbidities				
Diabetes	16 (25%)	11 (25%)	5 (21%)	0.7693
Comorbidities				0.2979
0	36 (56%)	20 (50%)	16 (67%)	
≥ 1	28 (44%)	20 (50%)	8 (33%)	
Charlson score	0 [0–2]	1 [0–2]	0 [0–1]	<b>0.0002</b>
Admission symptoms				
Delay between first symptoms (Days)	7 [4–11]	7 [4–10]	8.5 [7–11]	0.2055
Fever	52 (81%)	34 (85%)	18 (75%)	0.3414
Cough	42 (66%)	24 (60%)	18 (75%)	0.2814
Dyspnea	40 (63%)	28 (70%)	12 (50%)	0.1208
Diarrhea	18 (28%)	10 (25%)	8 (33%)	0.5691
Diffuse pain	13 (20%)	7 (18%)	6 (25%)	0.5300
Altered general status	44 (69%)	24 (60%)	20 (83%)	0.0584
Other	28 (44%)	16 (40%)	12 (50%)	
Severity scores				
SOFA score	4 [2–8]	8 [4–9]	2 [2, 3]	<b>&lt;0.0001</b>
SAPS II score	34 [26–45]	40 [32–54]	27 [21–33]	<b>&lt;0.0001</b>
PaO <sub>2</sub> /FiO <sub>2</sub> at admission	145 [92–191]	132 [95–166]	230 [83–298]	<b>0.0152</b>
Antiviral therapy				
Hydroxychloroquine	35 (55%)	20 (50%)	15 (63%)	0.4381
Lopinavir/ritonavir	5 (8%)	3 (8%)	2 (8%)	>0.9999
Lopinavir/ritonavir + interferonβ	5 (8%)	4 (10%)	1 (4%)	>0.9999
Remdesivir	1 (2%)	1 (3%)	0 (0%)	0.5238
Organ support				
Mechanical ventilation	63 (98%)	40 (100%)	23 (96%)	0.3750
Noninvasive ventilation	23 (36%)	0 (0%)	23 (100%)	<b>&lt;0.0001</b>
Invasive ventilation	40 (63%)	40 (100%)	0 (0%)	<b>&lt;0.0001</b>
Vasoactive drugs	24 (38%)	24 (60%)	0 (0%)	<b>&lt;0.0001</b>
Renal replacement therapy	15 (23%)	15 (38%)	0 (0%)	<b>0.0004</b>
Follow-up				
Days in ICU	10 [4–30]	21 [11–58]	4 [2–7]	<b>0.003</b>
Days in hospital	21 [11–56]	33 [19–71]	13 [8–21]	<b>&lt;0.0001</b>
Hospital mortality	19 (30%)	19 (48%)	0 (0%)	<b>&lt;0.0001</b>
Day-28 mortality	14 (22%)	14 (35%)	0 (0%)	<b>&lt;0.0001</b>
Day-90 mortality	19 (30%)	19 (48%)	0 (0%)	<b>&lt;0.0001</b>
Secondary infections	32 (50%)	30 (75%)	2 (8%)	<b>&lt;0.0001</b>

The results are shown as medians and interquartile ranges [Q1–Q3] for continuous variables or numbers and percentage for categorical variables. Patients were separated into two groups based on presence of acute respiratory distress syndrome (ARDS) according to Berlin definition during the first 72 h after admission. Sepsis-related organ failure assessment (SOFA) and simplified acute physiology score II (SAPS II) scores were calculated during the first 24 h after intensive care unit (ICU) admission. Data were compared using nonparametric Mann–Whitney test for continuous variables or Fisher's exact test for categorical variables

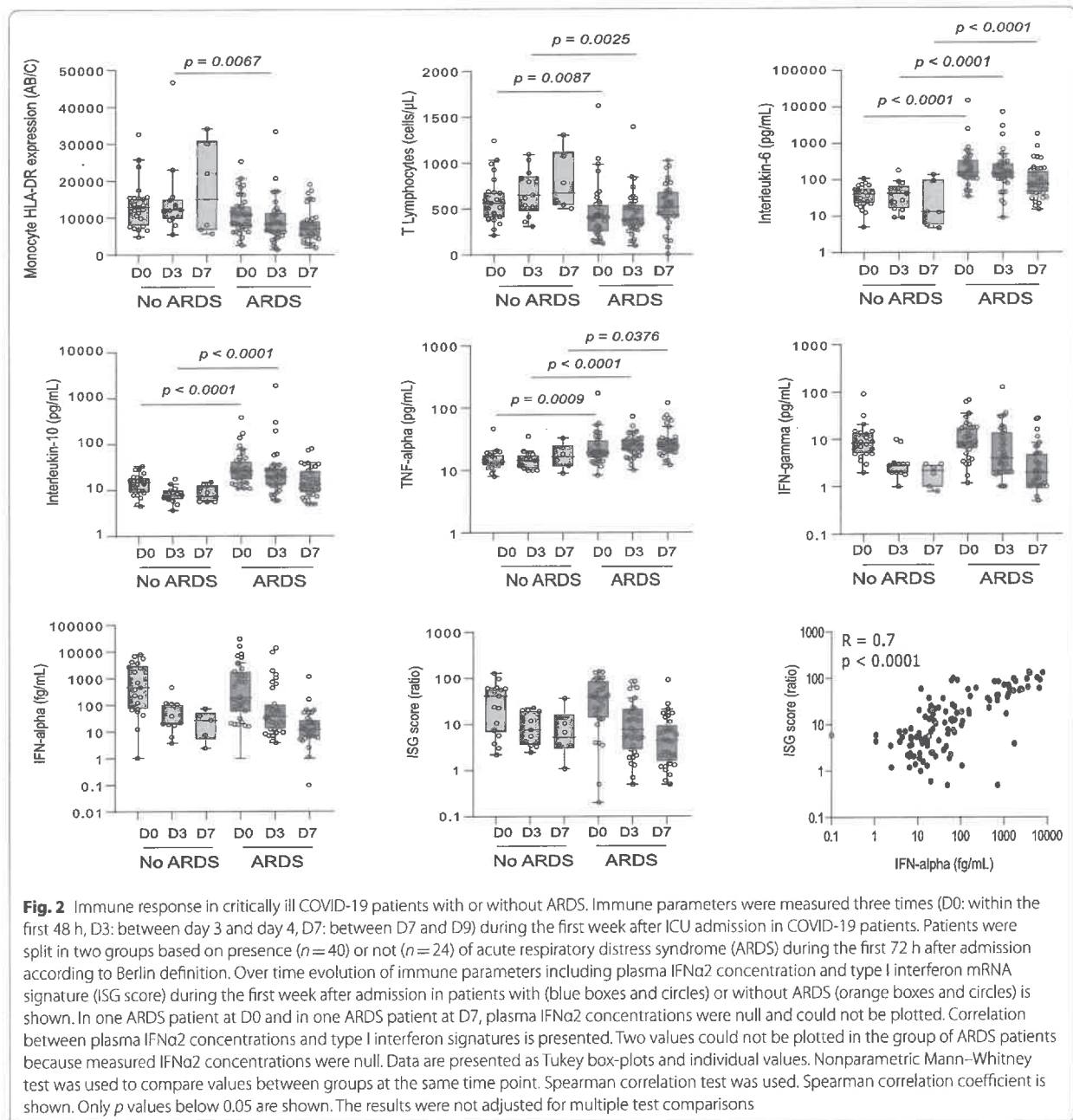
p values inferior to 0.05 are highlighted in bold

lymphocyte count) and progressive decrease of cytokines levels including IFNα2 (and related ISG score). Only plasma TNF-α presented with a specific kinetic as it was the only cytokine to slowly rise during



the follow-up. Moreover, its levels were significantly higher in non-surviving patients compared with survivors at all time points. This may reflect the high rates of secondary infections occurring in ARDS COVID-19 patients [25–27]. For example, in this cohort, 81% of survivors and 64% of non-survivors ARDS patients developed a secondary infection during their ICU

stay. The constant elevation of neutrophil count during the whole monitoring in ARDS patients probably also reflect such re-stimulation of the immune system in response to secondary infectious challenges (Additional file 1: Table S3). Last, we observed that non-survivors exhibited a significant rise in CD4/CD8 ratio compared with survivors (Fig. 3). This was mainly due

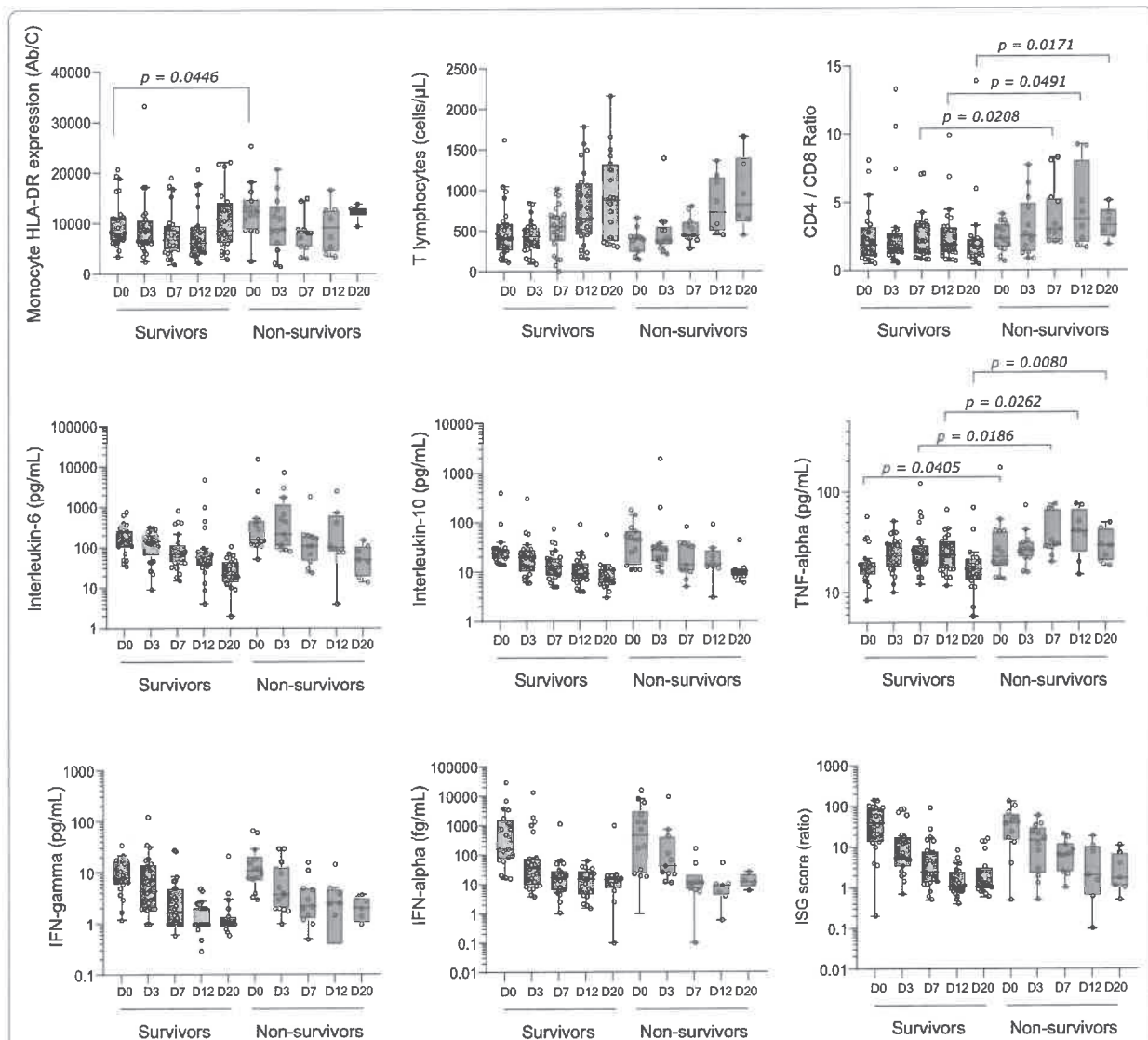


to the slower recovery of CD8+T cell numbers in this group (Additional file 1: Table S3).

### Discussion

Collectively, the present results provide an unbiased description of COVID-19 immuno-inflammatory derangements in critically ill COVID-19 patients focusing on ARDS patients who exhibit the highest mortality. We noted that, upon ICU admission, immune response

to SARS-CoV-2 infection presents with similarities with bacterial sepsis [28, 29]. These include (1) severe lymphopenia affecting all lymphocyte subsets, (2) moderately decreased mHLA-DR and (3) moderately increased plasma cytokine levels showing at the same time both inflammatory (IL-6) and immunosuppressive (IL-10) responses. In addition, we noticed increased plasma IFN $\alpha$ 2 levels and ISG score indicating the occurrence of an IFN-I response. This agrees with increased CD169



**Fig. 3** Immune response in COVID-19 ARDS patients according to status at D28. Immune parameters were measured 5 times (D0: within the first 48 h, D3: between day3 and D4, D7: between D7 and D9, D12: between D12 and D15, D20: between D20 and D25) during the first month after ICU admission in COVID-19 patients with ARDS. Patients were stratified in two groups according to their status at D28: survivors ( $n = 26$ ) or non-survivors ( $n = 14$ ). Over time evolution of immune parameters during the first month after admission in survivors (grey boxes) and non-survivors (red boxes) is shown. Regarding plasma IFN $\alpha$ 2 level, in one non-survivor patient at D0 and in one survivor patient at D7, plasma IFN $\alpha$ 2 concentration were null and could not be plotted. Data are presented as Tukey box-plots and individual values. Nonparametric Mann-Whitney test was used to compare values between groups at the same time point. Only  $p$  values below 0.05 are shown. The results were not adjusted for multiple test comparisons

expression on monocytes (aka siglec-1, one of the six genes of ISG) in COVID-19 patients upon ICU admission [30]. Thus, the present results do not support a potentially altered IFN-I response in the majority of COVID-19 patients upon ICU admission. However, as IFN $\alpha$ 2 concentrations reported elsewhere were higher in less severe/moderate patients (between 1000 and 5000 fg/mL) [12]; we cannot exclude that the incapacity to mount

a full type-I IFN response immediately following SARS-CoV2 infection in some patients may have led to their worsening and ultimately to ICU admission.

These abnormalities (along with decreased plasmacytoid cells [12, 14, 31]) are reminiscent of the process of age-acquired immunosuppression (also called immunosenescence) observed in elderly people who are, by far, the primary victims of COVID-19. We may hypothesize

**Table 2** Clinical characteristics of patients with COVID-19 with ARDS according to status at D28

	Survivors (n = 26)	Non-survivors (n = 14)	p value
Demographics			
Age	65 [55–70]	67 [58–78]	0.3076
Gender	23 (88%)	13 (93%)	> 0.9999
Body mass index (kg/m <sup>2</sup> )	30 [27–35]	28 [24–30]	0.0960
Body mass index > 30 kg/m <sup>2</sup>	14 (54%)	3 (21%)	0.0921
Delay between first symptoms (Days)	7 [4–12]	5 [4–9]	0.1909
Comorbidities			
Comorbidities			0.3202
0	15 (58%)	5 (36%)	
≥ 1	11 (42%)	9 (64%)	
Charlson score	0 [0–2]	2 [0–2]	0.1281
Severity scores			
SOFA score	8 [3–10]	8 [4–8]	0.7937
SAPS II score	41 [31–52]	40 [33–59]	0.5893
PaO <sub>2</sub> /FiO <sub>2</sub> at admission	150 [94–169]	116 [94–162]	0.2993
ARDS severity mild	1 (4%)	1 (7%)	> 0.9999
Moderate	18 (69%)	9 (64%)	> 0.9999
Severe	7 (27%)	4 (29%)	> 0.9999
Follow-up			
Days in ICU	40 [16–76]	11 [6–20]	<b>0.0008</b>
Days in hospital	64 [38–77]	15 [7–20]	<b>&lt; 0.0001</b>
Secondary infections	21 (81%)	9 (64%)	0.2777

The results are shown as medians and interquartile ranges [Q1–Q3] for continuous variables or numbers and percentage for categorical variables. COVID-19 patients with ARDS were separated in two groups based on status at D28 after admission. Sepsis-related organ failure assessment (SOFA) and simplified acute physiology score II (SAPS II) scores were calculated during the first 24 h after admission. Patients were classified in ARDS severity groups according to Berlin criteria. ICU: intensive care unit. Data were compared using nonparametric Mann–Whitney test for continuous variables or Fisher's exact test for categorical variables

p values inferior to 0.05 are highlighted in bold

that evolution of COVID-19 toward increasing severity in this population of old patients is mainly a consequence of this altered immune status [32–35]. For example, previous studies showed the negative correlation between lymphocytes count and pulmonary viral load [36, 37] while, elsewhere, the magnitude of pulmonary viral load was repeatedly associated with increased mortality [8, 38–43]. In the present work, this is also illustrated by the association between nasopharyngeal viral load and increased mortality, SOFA and IL-6 levels upon admission.

Our results emphasize that ARDS occurrence appears to be an important driver of mortality during COVID-19 progression in ICU patients [1]. In the present cohort, mortality occurred only in the group of patients with ARDS while all ICU patients without ARDS were released alive from the ICU. This observation makes a strong priority for avoiding progression toward ARDS in COVID-19 patients. After ICU admission, inflammatory response as well as IFN-I activity progressively normalized while immunological cellular parameters remained below references ranges. Thus, as

seen in bacterial sepsis, ARDS occurrence in COVID-19 patients may secondarily amplify COVID-19-induced immune alterations either by direct cytotoxic effect and/or by negative anti-inflammatory feedbacks [28, 29, 32]. This leads to the development of a torpid immunosuppressed status in ARDS patients which may last several weeks [44].

In such critically ill patients with prolonged ICU stays, this immunosuppressed status presents with deleterious consequences. First, it probably participates in the long duration necessary to eradicate SARS-CoV-2 from the lung in invasively ventilated patients as described elsewhere [43]. For example, it was shown that the viral shedding in lower respiratory tract lasted almost 30 days in median in critically ill COVID-19 patients [38, 42]. Second, it most likely contributes to the very high rates of nosocomial infections reported not only in the present study (50%) but also in many others [26, 27, 45]. In particular, COVID-19 is characterized by astonishing elevated rates of secondary aspergillosis [46–48], a fungal disease usually seen in the most immunosuppressed patients. Such secondary infectious events may explain



the persistently elevated TNF- $\alpha$  levels accompanied by increased neutrophil count in COVID-19 patients with ARDS and in non-survivors [25]. This suggests that in this second step of the disease, i.e., once ARDS occurred, immunostimulation could represent a sound approach to try to promote immune recovery and prevent secondary bacterial and fungal infections.

The most striking result of the present study was to observe similar altered immune response in both surviving and non-surviving ARDS patients over a 3-week follow-up. Thus, the duration of this COVID-19 induced immunosuppressed status remains to be defined. While a recent study observed that the onset of T cell recovery in COVID-19 ICU patients with ARDS started on day 35 [49], the 3-week ICU follow-up performed in this study may have been too short to distinguish immune trajectories according to outcomes in COVID-19 ARDS patients. Of note, in this latter work [49], patients with unfavorable outcome presented with increased CD4/CD8 ratio as observed in the present study. We may thus hypothesize that, in ARDS patients, the lack of CD8+T lymphocyte recovery could be a poor prognosis factor [35, 50].

Being exploratory, our work presents with limitations. First, SARS-CoV2 viral load could not be regularly monitored. Further studies should include strict quantitative evaluation of viral load with standardized tools throughout the monitoring to decipher the duration of viral persistence in the lung and its correlation with immune response and outcomes. Second, functional testing of immune cells was not performed. If available, this should be incorporated in addition with phenotypic markers of immune response [51–53]. This is especially true regarding CD8+T cell functionality in response to SARS-CoV-2 peptides that may help to understand which comes first: CD8 efficiency (and recovery) or virus disappearance. Three, we did not include less severe COVID-19 cohorts in order to explore (with single-molecule array—SIMOA-technology) the magnitude of IFN-I response in patients who correctly eradicated the virus. Last, at the time of first COVID-19 surge in France (corresponding to the patients reported here), optimized care protocols (oxygen, heparin, dexamethasone) were not applied. Therefore, some observations need to be confirmed under the angle of current clinical practice.

## Conclusions

In sum, upon patients' admission to the ICU, pulmonary virus spread is accompanied by an inflammatory response characterized by moderately increased circulating levels of typical inflammatory cytokines (e.g., IL-6 levels usually < 100 pg/mL) [54–57]. At this stage, no obvious observation of altered IFN-I response could be reported. If not appropriately controlled by

the immune system [33], virus replication in lungs and related inflammation may progressively lead to ARDS which appears to be one driver of mortality. Following this acute response leading to pulmonary dysfunction, inflammatory response rapidly declined. As observed in bacterial sepsis [28, 29], patients subsequently present with a marked delayed immunosuppression. This state of immunosuppression likely prevents efficient virus eradication from the lung, facilitates virus spread outside lungs as illustrated by the deleterious association of persistent viremia and mortality [58–62]. This also probably favors the occurrence of frequent secondary infections with opportunistic pathogens [26, 27, 47]. All these elements explain the long ICU stay of invasively ventilated COVID-19 patients. In the current 3-week monitoring of ARDS patients, we did not identify any immunological parameter that significantly associated with mortality. Thus, the better understanding of the mechanisms which finally permit survival after several weeks in ICU is a crucial issue for next studies.

## Abbreviations

AB/C: Numbers of antibodies bound per cell; ARDS: Acute respiratory distress syndrome; HLA-DR: Human leukocyte antigen-DR; ICU: Intensive care unit; ISG: Interferon-stimulated genes; SAPS II: Simplified acute physiology score II; SOFA: Sequential organ failure assessment score; PaO<sub>2</sub>/FiO<sub>2</sub> ratio: Ratio of the arterial partial pressure of oxygen to the fractional inspired oxygen.

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13054-021-03558-w>.

**Additional file 1. Table S1.** Immune response in critically ill COVID-19 patients, **Table S2.** Immune response in critically ill COVID-19 patients with or without ARDS, **Table S3.** Immune response in critically ill COVID-19 patients with ARDS according to status at D28, **Table S4.** Numbers of available values for each immune parameter at each time point; **Figure S1.** Flowchart and Survival Curves and **Figure S2.** Impact of viral load at admission.

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#### Authors' contributions

FV, GM, LA, TR, MC and ACL designed the study, analyzed results, discussed findings and wrote the manuscript. RC, SV, KBP and JSC performed immune monitoring experiments and viral load monitoring. RC, SV and JSC analyzed results, discussed findings and wrote the manuscript. CM, PA and HY collected clinical samples, discussed findings and wrote the manuscript. CA analyzed results and discussed findings. All authors read and approved the final manuscript.

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#### Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

#### Declarations

##### Ethics approval and consent to participate

This project was part of an ongoing prospective observational clinical study (RICO, REA-IMMUNO-COVID). It was approved by ethics committee (Comité de Protection des Personnes Ile de France 1 - N°IRB/ORG #: IORG0009918) under agreement number 2020-A01079-30. This clinical study was registered at ClinicalTrials.gov (NCT04392401). The committee waived the need for written informed consent because the study was observational, with a low risk to patients, and no specific procedure, other than routine blood sampling, was required. Oral information and non-opposition to inclusion in the study were mandatory and were systematically obtained before any blood sample was drawn. This was recorded in patients' clinical files. If a patient was unable to consent directly, non-opposition was obtained from the patient's legally authorized representative and reconfirmed from the patient at the earliest opportunity.

##### Consent for publication

Not applicable.

##### Competing interests

The authors declare no competing financial interests in relation to the work.

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## **Annexe 6**




RESEARCH

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# Recombinant human interleukin-7 reverses T cell exhaustion ex vivo in critically ill COVID-19 patients

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## Abstract

**Background:** Lymphopenia is a hallmark of severe coronavirus disease 19 (COVID-19). Similar alterations have been described in bacterial sepsis and therapeutic strategies targeting T cell function such as recombinant human interleukin 7 (rhIL-7) have been proposed in this clinical context. As COVID-19 is a viral sepsis, the objectives of this study were to characterize T lymphocyte response over time in severe COVID-19 patients and to assess the effect of ex vivo administration of rhIL-7.

**Results:** Peripheral blood mononuclear cells from COVID-19 patients hospitalized in intensive care unit (ICU) were collected at admission and after 20 days. Transcriptomic profile was evaluated through NanoString technology. Inhibitory immune checkpoints expressions were determined by flow cytometry. T lymphocyte proliferation and IFN- $\gamma$  production were evaluated after ex vivo stimulation in the presence or not of rhIL-7. COVID-19 ICU patients were markedly lymphopenic at admission. Mononuclear cells presented with inhibited transcriptomic profile prevalently with impaired T cell activation pathways. CD4<sup>+</sup> and CD8<sup>+</sup>T cells presented with over-expression of co-inhibitory molecules PD-1, PD-L1, CTLA-4 and TIM-3. CD4<sup>+</sup> and CD8<sup>+</sup>T cell proliferation and IFN- $\gamma$  production were markedly altered in samples collected at ICU admission. These alterations, characteristic of a T cell exhaustion state, were more pronounced at ICU admission and alleviated over time. Treatment with rhIL-7 ex vivo significantly improved both T cell proliferation and IFN- $\gamma$  production in cells from COVID-19 patients.

**Conclusions:** Severe COVID-19 patients present with features of profound T cell exhaustion upon ICU admission which can be reversed ex vivo by rhIL-7. These results reinforce our understanding of severe COVID-19 pathophysiology and opens novel therapeutic avenues to treat such critically ill patients based of immunomodulation approaches. Defining the appropriate timing for initiating such immune-adjuvant therapy in clinical setting and the pertinent markers for a careful selection of patients are now warranted to confirm the ex vivo results described so far.

*Trial registration* ClinicalTrials.gov identifier: NCT04392401 Registered 18 May 2020, <http://clinicaltrials.gov/ct2/show/NCT04392401>.

**Keywords:** T lymphocytes, Exhaustion, SARS-CoV-2, Critically ill, Interleukin-7, Immunostimulation

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## Introduction

The Coronavirus disease 2019 (COVID-19) pandemic caused by the highly infectious severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has now

challenged public health for almost two years. The most severe forms evolve towards acute respiratory distress syndrome (ARDS) requiring care in intensive care unit (ICU). A better understanding of the immune response in these critically ill patients is of major importance to guide therapeutics as a high mortality is still reported in this population [1] and prolonged ICU lengths of stay ultimately lead to ICU saturation in time of pandemic [2].

A fully competent immune system is mandatory to fight against infections. Evidence now suggest that severe forms of COVID-19 are associated with the development of immune alterations. For example, impaired and delayed type I and type III interferon (IFN) responses have been described in severe COVID-19 patients [3]. In addition, critically ill COVID-19 patients display features of compromised adaptive immunity mainly characterized by profound lymphopenia; which depth and persistence associate with poor prognosis [4–6].

Major lymphocyte alterations have been described in other clinical contexts such as bacterial sepsis, chronic viral infections and cancer [7]. In addition to marked lymphopenia, these patients exhibit severely altered T cell effector functions with decreased proliferation and cytokine production *ex vivo* and sustained expression of inhibitory receptors such as programmed cell death protein (PD-1) or programmed death-ligand 1 (PD-L1) [8]. These alterations of T lymphocyte response have been characterized as T cell exhaustion [9, 10]. Similar features of T cell exhaustion have been described in patients after severe SARS-CoV-2 infections [11]. However their onset of appearance and their persistence over ICU stay remain unclear.

In patients with bacterial sepsis, immunotherapies targeting T cell exhaustion have been suggested to improve T cell functions and patients' prognostic [7]. Among these, interleukin 7 (IL-7) is a pleiotropic cytokine involved in T cell survival and expansion [12]. Several *in vivo* and *ex vivo* studies reported a beneficial effect of recombinant interleukin 7 (rhIL-7) in sepsis through restoration of lymphocyte proliferation, IFN- $\gamma$  production [13–15] or metabolic reprogramming of exhausted CD4+T cells [16]. So far only few studies investigated rhIL-7 beneficial effect on T cell alterations in COVID-19 patients. One study showed that IL-7 administered *ex vivo* restored T cell IFN- $\gamma$  production [11] and compassionate use of this treatment was reported in two studies [17, 18].

In this context, the objectives of the present work were (i) to characterize T cell transcriptome, phenotype and functions over time in a cohort of critically ill COVID-19 patients and (ii) to evaluate *ex vivo* the capacity of rhIL-7 therapy to improve T cell functions in critically ill COVID-19 patients.

## Materials and methods

### Sampling and cell isolation

COVID-19 patients belong to the RICO (REA-IMMUNO-COVID) study registered at NCT04392401 and approved by local Institutional Review Board for ethics ("Comité de Protection des personnes d'Ile-de-France") [19]. All patients were prospectively included. The study group consisted in consecutive ICU-hospitalized patients in seven tertiary hospitals in France. Inclusion criteria were: (1) patients aged >18 years, (2) hospitalization in ICU for SARS-CoV-2 pneumopathy, (3) first hospitalization in ICU for COVID-19, (4) positive diagnosis of SARS-CoV-2 infection carried out by PCR or by another approved method in at least one respiratory sample, (5) sampling in the first 48 h after admission to ICU feasible and (6) patient or next of kin informed of the terms of the study and has not objected to participating. Blood samples were collected within the first 48 h after ICU admission (Day 0: D0) and between day 20 and day 25 (D20) in EDTA-anticoagulated blood bottles. Reference values were obtained from a cohort of 15 healthy volunteers (HV) after informed consent was given. Healthy volunteers' (10 women, 5 men) mean age was 38 years. Peripheral blood mononuclear cells (PBMCs) from patients and donors were isolated by Ficoll-Paque PREMIUM (GE Healthcare) density gradient centrifugation and cryopreserved in a  $-80^{\circ}\text{C}$  freezer.

### Reagents

Anti-CD2-CD3-CD28 coated beads (T cell activation/expansion kit human) were purchased from Miltenyi Biotec (Gladbach, Germany). Alexa Fluor 750 or Fluorescein isothiocyanate-conjugated anti-CD3, Allophycocyanin or Pacific Blue-conjugated anti-CD4, Krome orange-conjugated anti-CD8, Phycoerythrin-conjugated anti-CD16/56, Pacific Blue-conjugated anti-CD19, Krome Orange-conjugated anti-CD45, Allophycocyanin-conjugated anti-CD14, Phycoerythrin-Cyanin-7-conjugated anti-PD-L1, Phycoerythrin-conjugated anti-CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), were purchased from Beckman Coulter (Hialeah, FL). Allophycocyanin-conjugated anti-PD1 and Phycoerythrin-Dazzle 594 conjugated anti-TIM-3 (T-cell immunoglobulin and mucin containing protein-3) antibodies were purchased from Biolegend (San Diego, CA). rhIL-7 was purchased from R&D Systems (Minneapolis, MN). RNase-free Dnase set was purchased from Qiagen (Hilden, Germany). Click-it kit Ethynyl-2'-deoxyuridine (EdU) AF88 was purchased from ThermoFisher (Waltham, MA).

### Thawing and phenotyping of PBMCs

Cryovials containing PBMCs were thawed in water bath and quickly resuspended in RPMI medium with

RNAse-free DNase prewarmed at 37 °C. Cell number and viability were assessed via flow cytometry through propidium iodide exclusion technique. Median viability calculated on all samples after cell thawing was 80%. Percentages of B cells, NK cells, T cells and monocytes were determined by flow cytometry. PD-1, PD-L1, CTLA-4 and TIM-3 expressions were analyzed on CD3+CD4+ and CD3+CD8+ cells. Results were expressed as percentages of cells expressing each marker. Samples were run on a Navios flow cytometer (Beckman Coulter). Calibration beads (Flow Check and Flow Set, Beckman Coulter) were run daily to check for routine alignment, day-to-day stability, and long-term performance validation.

#### Gene expression analysis

Total mRNA was extracted from  $2.10^6$  PBMCs using Quick-RNA™ Microprep Kit (Zymo research) according to manufacturer's instructions. We analyzed 200 ng of total RNA from each sample using the nCounter® Pan-Cancer Immune Profiling Panel (NanoString) according to manufacturer's instructions.

Raw data were prepared for gene expression analysis as described elsewhere [20]. For each sample, eight negative probes and six serial concentrations of positive control probes were included in the panel and used in the preparation of raw data. Briefly, a first step of normalization for variation associated with the technical platform (batch effect) was performed using the internal positive controls. For each sample the geometric mean of internal positive controls was calculated. We then determined a scaling factor per sample as the ratio of the average across all geometric means and the geometric mean of the sample. For each sample, all gene counts were then multiplied by the corresponding scaling factor. Next, for each sample we calculated the background level as the median+2 standard deviations across the eight negative probe counts. For each sample, background level was subtracted to each gene.

Then, normalization for differences in RNA input used the same method as in the positive control normalization, but geometric means were calculated over three housekeeping genes (EIF2B4, HDAC3, TBP) selected from the forty candidate reference genes provided by NanoString. The number of housekeeping genes to be included was determined using geNorm method [21] and the three most relevant genes were selected using NormFinder method [22].

Finally, negative expression values were converted to zero, and genes for which more than 50% of samples presented with a null value were excluded from the analysis (i.e. 39 genes).

#### T cell proliferation assay and IFN- $\gamma$ production

The number of PBMCs per well was adjusted to  $1.10^6$  cells/ml resuspended in complete culture media (RPMI 1640 medium with HEPES, L-glutamine, 10% human AB plasma, 20 mg/ml streptomycin and 2.5 mg/ml fungizone). Cells were stimulated with anti-CD2-CD3-CD28 Abs coated beads (ratio beads/cells=2:1) and/or with rhIL-7 (100 ng/ml) for 72 h at 37 °C, 5% CO<sub>2</sub>. All samples were performed in duplicates. EdU click-it reaction was performed using EdU Click-it kit (Thermo Fisher) according to the manufacturer's instructions [23]. CD3+, CD4+ and CD8+ T cell proliferation was analyzed by monitoring EdU-AF488 incorporation into cells. Results were expressed as percentages of cells incorporating EdU.

IFN- $\gamma$  production was measured in corresponding cell culture supernatants via standardized Enzyme Linked Fluorescent Assay (VIDAS, bioMérieux). Results were expressed as international units per mL (IU/mL).

#### Statistical analysis, data visualization and software

Continuous variables were expressed as medians and interquartile ranges (IQR). Comparisons between results in patients and HV were made using non parametric Mann-Whitney U tests. Wilcoxon signed-rank test was used for paired comparisons between results obtained at D0 and D20 in patients or to assess effect of rhIL-7 within a group.

Differentially expressed genes were visualized on volcano plots and statistical differences were selected based on Log<sub>2</sub> Fold Change (i.e.  $\leq -2$  or  $\geq +2$ ) and  $-\text{Log}_{10}$  P value ( $\leq 1.3$ ). Number of significantly downregulated and upregulated genes were represented on Venn diagrams. Ingenuity Pathways Analysis tool (IPA, Ingenuity® Systems, <https://analysis.ingenuity.com>) was applied on the list of differentially expressed genes to assess canonical pathways. This analysis was performed using both the HTA and U133 gene repertoires. The significant pathways were extracted from the HTA gene repertoire because the results were similar but more complete and informative than the U133 repertoire.

Except for IPA tool, all statistical analyses were performed using R 4.0.4 (The R foundation for Statistical Computing, Vienna, Austria). A *p*-value < 0.05 was considered statistically significant. All tests realized were two-sided.

## Results

### Patients' characteristics

Twenty patients were included in the study with a median age of 71 [IQR: 66, 72] years. They were admitted in ICU 9 days [IQR: 7, 11] after symptoms onset.



**Table 1** Clinical and biological characteristics of COVID-19 patients

	All patients (n = 20)		
Demographics			
Gender, male (%)	14 (70.0)		
Age	71 [66, 72]		
Body mass index	32 [28–36]		
Comorbidities			
Comorbidity (%)	9 (50.0)		
Immunosuppressive therapy (%)	1 (5.6)		
Diabetes (%)	4 (23.5)		
Delay from symptom onset, days	9.0 [7.0, 11.0]		
Severity scores at admission			
SOFA	5.0 [4.0, 7.0]		
SAPS II	37.0 [30.3, 44.3]		
PaO <sub>2</sub> /FIO <sub>2</sub>	116.0 [68.3, 151.5]		
Organ failures during ICU stay			
Invasive mechanical ventilation (%)	18 (90)		
Vasoactive drugs (%)	15 (83.3)		
Renal replacement therapy (%)	4 (22.2)		
Follow-up			
Concurrent bacterial infection at D0 (%)	9 (45)		
ICU-acquired infection before D20 (%)	15 (75)		
SOFA score at D20	6.5 [5.00, 8.00]		
Corticotherapy in ICU (%)	18 (90)		
Mechanical ventilation duration	21.5 [16.5, 32.5]		
Days in ICU	28.0 [24.3, 39.5]		
Days in Hospital	32.5 [25.8, 45.8]		
Day-28 mortality	7 (35)		
Day-90 mortality	9 (45)		
Status at time of sampling			
	D0	D20	
Invasive mechanical ventilation	8 (40)	14 (70)	
Hospitalized in ICU	20 (100)	20 (100)	
Laboratory values			
	D0 (n = 19)	D20 (n = 18)	p-value
Lymphocyte (cells/μL)	534 [392, 751]	1 055 [713, 1 488]	0.003
CD3 + lymphocytes (cells/μL)	355 [214, 445]	760 [422, 967]	0.001
CD4 + lymphocytes (cells/μL)	189 [117, 316]	415 [228, 590]	0.003
CD8 + lymphocytes (cells/μL)	103 [82, 198]	262 [114, 430]	0.018
mHLA-DR expression (Ab/C)	6 548 [4 622, 11 704]	6 855 [5 946, 13 897]	0.741

The results are shown as medians and interquartile ranges [Q1–Q3] for continuous variables or numbers and percentages for categorical variables. SOFA: Sepsis-related organ failure assessment. SAPS II: Simplified acute physiology score II. ICU: Intensive care unit. Continuous laboratory variables were compared with paired Mann–Whitney U test and p-value reported is two-sided. Values were missing for 2 patients at D0 and one patient at D20. Normal values obtained from routine Immunology Lab for total lymphocyte count: [1000–2800] cells/μL, for T lymphocytes: [595–1861] cells/μL, for CD4 + T cells: [336–1 126] cells/μL, for CD8 + T cells: [125–780] cells/μL, for mHLA-DR: [13,500–45,000] Ab/C (number of antibodies-bound per cell)

Clinical characteristics are presented in Table 1. Overall, they presented with severe forms of COVID-19 pneumonia as 90% required invasive mechanical ventilation during their ICU stay and 28-day mortality was 35%. At D20, all selected patients were still hospitalized in ICU and 70% remained under mechanical ventilation. At this time point, SOFA score was 6.5 [5–8].

Lymphocyte absolute count was notably decreased in COVID-19 patients at D0 (534 cells/μL [IQR: 392–751]) compared to normal laboratory values (1 000–2 800 cells/μL). It significantly increased over time to ultimately normalize at D20. COVID-19-induced lymphopenia affected all T lymphocyte subsets as both CD4+ and CD8+ T cell numbers were markedly decreased in

patients at D0. CD4+ and CD8+ T cell counts also significantly increased over time to normalize at D20. Besides, mHLA-DR expression was markedly decreased in COVID-19 patients (Table 1). Overall this suggests the presence of an immunosuppressive profile in critically ill patients after SARS-Co-V2 infection.

#### Transcriptomic profile of mononuclear cells in severe COVID-19 patients

We first compared gene expression profiles of PBMCs between healthy volunteers and COVID-19 patients at ICU admission (D0) and D20. Volcano plots unveiled transcriptional differences between COVID-19 patients and HV at both time-points (Fig. 1a). Among the panel of genes analyzed with NanoString in critically ill COVID-19 patients, the number of downregulated genes ( $n=48$ ) was largely superior to the number of upregulated mRNA ( $n=21$ , Fig. 1b). The complete lists of significantly up and down regulated genes are reported in Additional file 1: Tables S1 and S2. We identified differential expressions of genes associated with immunoregulation and impaired adaptive immune response. For instance, mRNA levels of immunoregulatory molecules such as *LAG3*, *MERTK* or *IL-10* were upregulated while mRNA levels of molecules related to T cell effector functions such as *IL-2*, *IL-21*, *IL13RA2* or *RAG-1* were downregulated in patients compared with controls. In line, Ingenuity Pathway Analysis showed that pathways related to T cell activation (notably Th1, Th2 and Th17 pathways) were inhibited in patients compared to HV (Fig. 1c). The list of pathways differentially expressed at D0 and D20 compared to HV are reported in Additional file 1: Table S3.

Altogether, these data show the inhibited transcriptomic profile of mononuclear cells in COVID-19 patients. They suggest that T cell function may be impaired after severe SARS-Co-V2 infection.

#### Immune checkpoint expressions on T cells in COVID-19 patients

We next evaluated the expressions of the exhaustion markers PD-1, PD-L1, CTLA-4 and TIM-3 on CD4+ and CD8+ T cells in COVID-19 patients sampled at D0 and D20 compared to HV. Flow cytometry analyses showed that the percentages of PD-1+ and PD-L1+ cells were significantly increased among CD4+ T cells at D0 and D20 (Fig. 2a). Similarly, CD8+ T cells displayed an exhaustion phenotype as they presented sustained expressions of PD-1 and PD-L1 (Fig. 2b). CTLA-4 expression was slightly increased in patients versus controls, but the difference did not reach statistical significance. Finally, TIM-3 expression was significantly increased on CD8+ T cells at ICU admission and persisted over ICU course (Fig. 2b).

To note, we found a strong negative correlation between PD-1 expression on CD4+ T cells and the absolute CD4+ T cells count at D0 (Spearman's correlation  $\rho=-0.83$ ,  $p=0.0009$ ) and at D20 ( $\rho=-0.62$ ,  $p=0.028$ ).

Thus CD4+ and CD8+ T cells in severe COVID-19 patients presented with concomitant over-expressions of co-inhibitory molecules PD-1, PD-L1, CTLA-4 and TIM-3 in accordance with the development of a state of T exhaustion after severe SARS-Co-V2 infection.

#### T cell proliferation and IFN- $\gamma$ production in COVID-19 patients

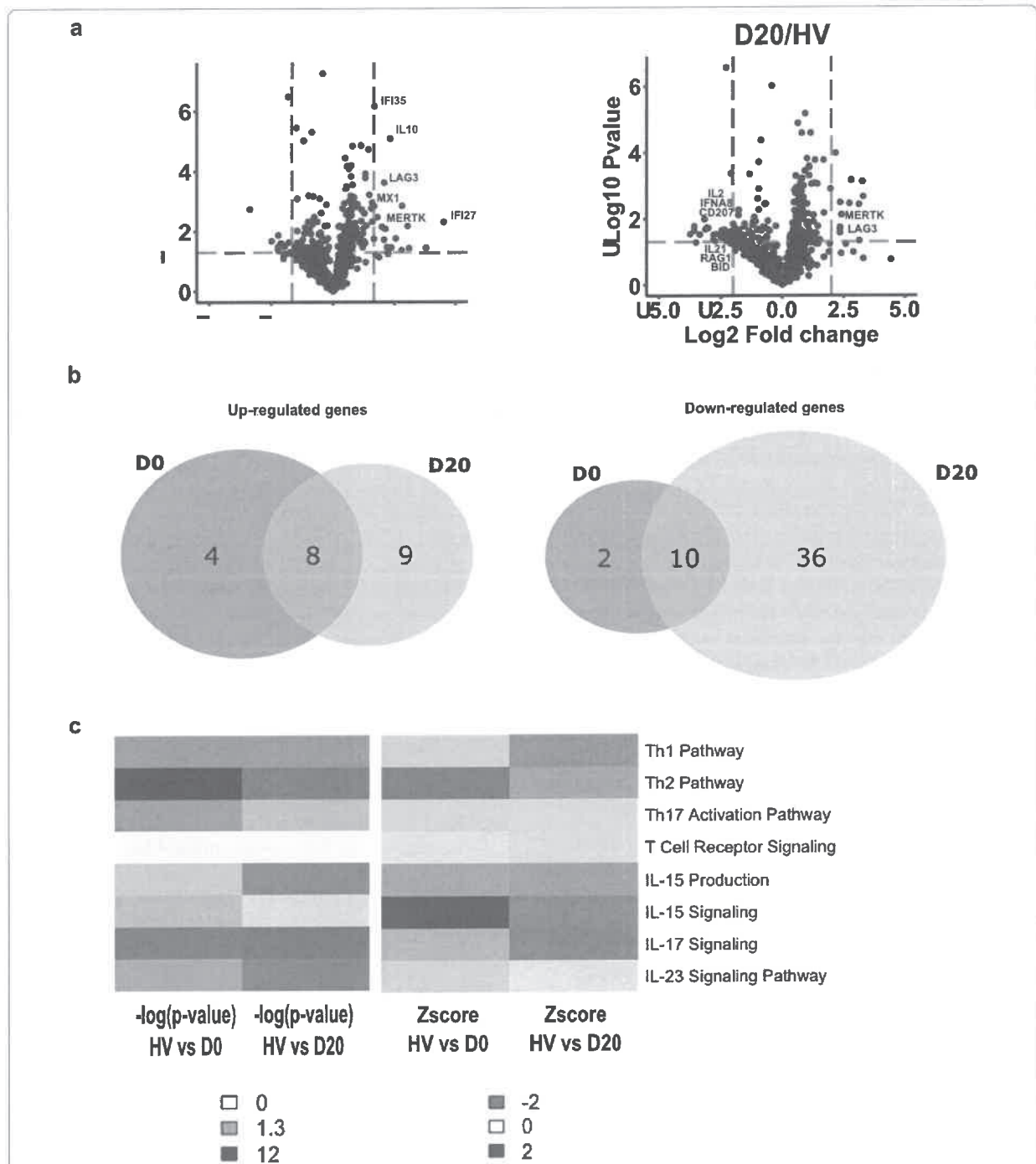
Next we assessed T cell function (i.e. proliferation and IFN- $\gamma$  production after TCR stimulation *ex vivo*) in COVID-19 patients sampled at D0 and D20 compared to HV.

Both CD4+ and CD8+ T cell proliferations were significantly decreased at ICU admission in patients compared to HV (i.e. 19.5% [IQR: 15.5–23.6] vs 27% [IQR: 24.5–28.5],  $p<0.05$  for CD4+ T cells and 19.9% [IQR: 17.3–27.4] vs 33.1% [IQR: 30.2–40.6],  $p<0.05$  for CD8+ T cells). Seven out of 10 patients presented with T cell proliferation responses below the lowest value of HV (Fig. 3a and b). In contrary, at D20, while median T cell proliferation tended to remain lower in patients compared to HV; this difference did not reach statistical significance. To note; the proliferative response was not correlated with absolute lymphocyte count of both CD4+ and CD8+ T cells (Spearman's correlation  $\rho=-0.08$  and  $\rho=-0.19$  respectively).

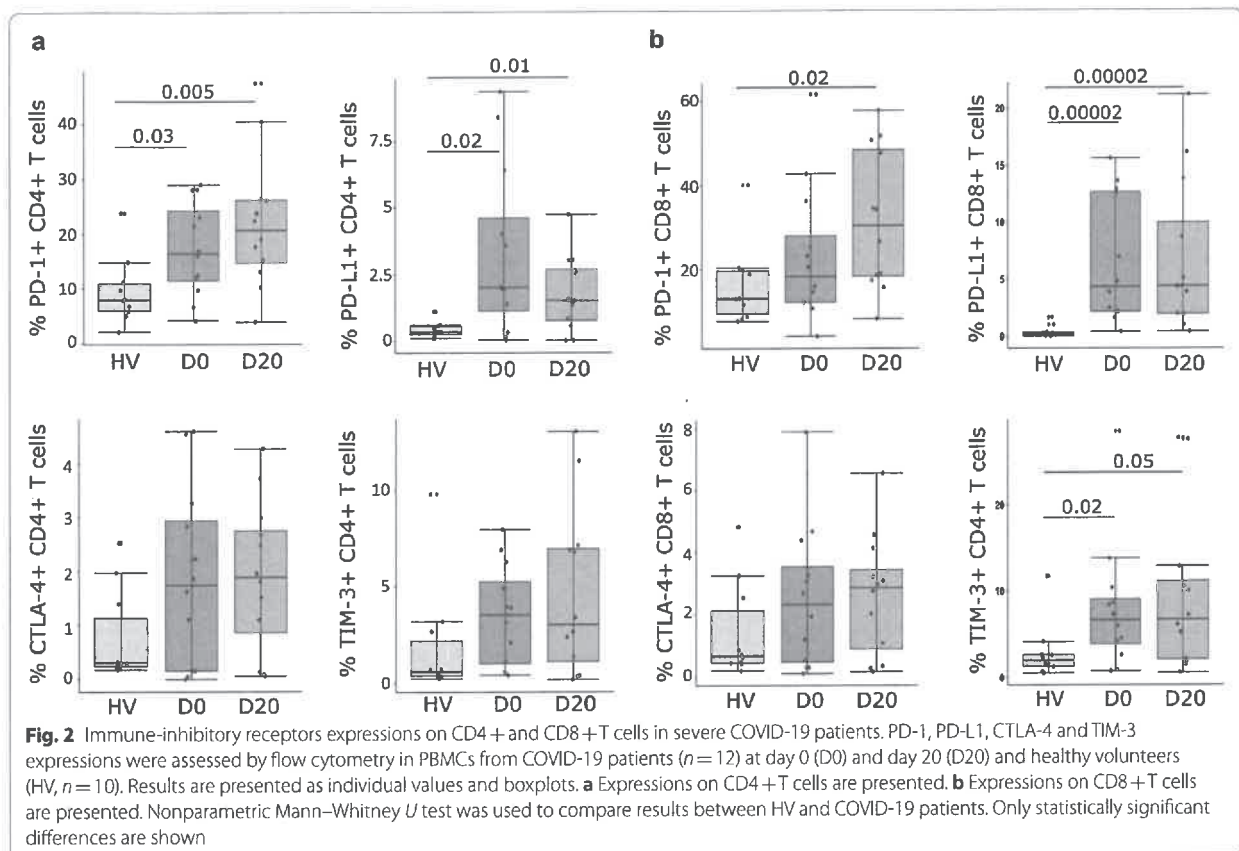
T lymphocyte function was also defined by measuring IFN- $\gamma$  production in cell culture supernatants after T cell stimulation. At D0, median natural log-transformed IFN- $\gamma$  production was dramatically decreased after TCR stimulation compared to HV (2.62 [IQR: 2.34–3.55] vs 5.99 [IQR: 5.27–6.28] log IU/mL,  $p<0.001$ , Fig. 3c). At D20, this production (5.47 [IQR: 5.12–5.63] log IU/mL) significantly increased compared with paired sampled obtained at D0 and no difference compared with normal values could be identified.

These results show that T cell function was markedly altered at ICU admission in critically ill COVID-19 patients in accordance with the development of a state of T exhaustion after severe SARS-Co-V2 infection. At D20, T cell function improved to return to normal values suggesting the COVID-19-induced T cell exhaustion might alleviate over time.

To note, we compared immune parameters between patients with or without concomitant bacterial infection at D0 and between patients with ICU-acquired infection before D20 vs patients with no secondary infections (Additional file 1: Table S4). At D0, IFN- $\gamma$  production and T cell proliferations were not different between



**Fig. 1** Transcriptomic profile of mononuclear cells in severe COVID-19 patients. RNA extracted from PBMCs of COVID-19 patients at day 0 and day 20 ( $n = 10$ ) and healthy volunteers (HV,  $n = 10$ ) was analyzed through NanoString technology. **a** Volcano plots of differentially expressed genes between patients sampled at D0 or at D20 and HV are shown. Limits of significance are illustrated by red dotted lines (i.e.  $\log_2$  Fold Change =  $-2$  or  $+2$  and  $-\log_{10} P$  value = 1.3). Selected genes are mentioned. **b** Venn diagrams of significantly up-regulated (left diagram,  $n = 21$ ) or down-regulated (right diagram,  $n = 48$ ) genes between patients and HV are shown. **c** Ingenuity Pathway Analysis was applied on the list of differentially expressed genes at D0 and D20. Heatmaps of  $\log_{10} P$ -value (from white indicating the absence of significance to dark red indicating a strong significance) and Z-score (from orange indicating a down-regulation to purple indicating an up-regulation) for pathways related to T cell activation at D0 and D20 are presented



patients with or without concomitant bacterial infection. However, patients with simultaneous bacterial and viral infections at admission presented with increased PD-1 expressions on CD4+ and CD8+ T cells, higher PD-L1 expression on CD8+ T cells and CTLA4 expression on CD4+ T cells. At D20, no differences were observed for any immune parameter between patients with or without secondary bacterial infections. Thus the simultaneous presence of bacterial and viral infections may have amplified immune dysfunctions at ICU admission but these differences did not persist overtime in patients still in the ICU at D20. Because of the small number of patients in each group, these results need to be confirmed in a larger cohort of patients.

#### Evaluation of rhIL-7 effect of T cell function ex vivo in COVID-19 patients

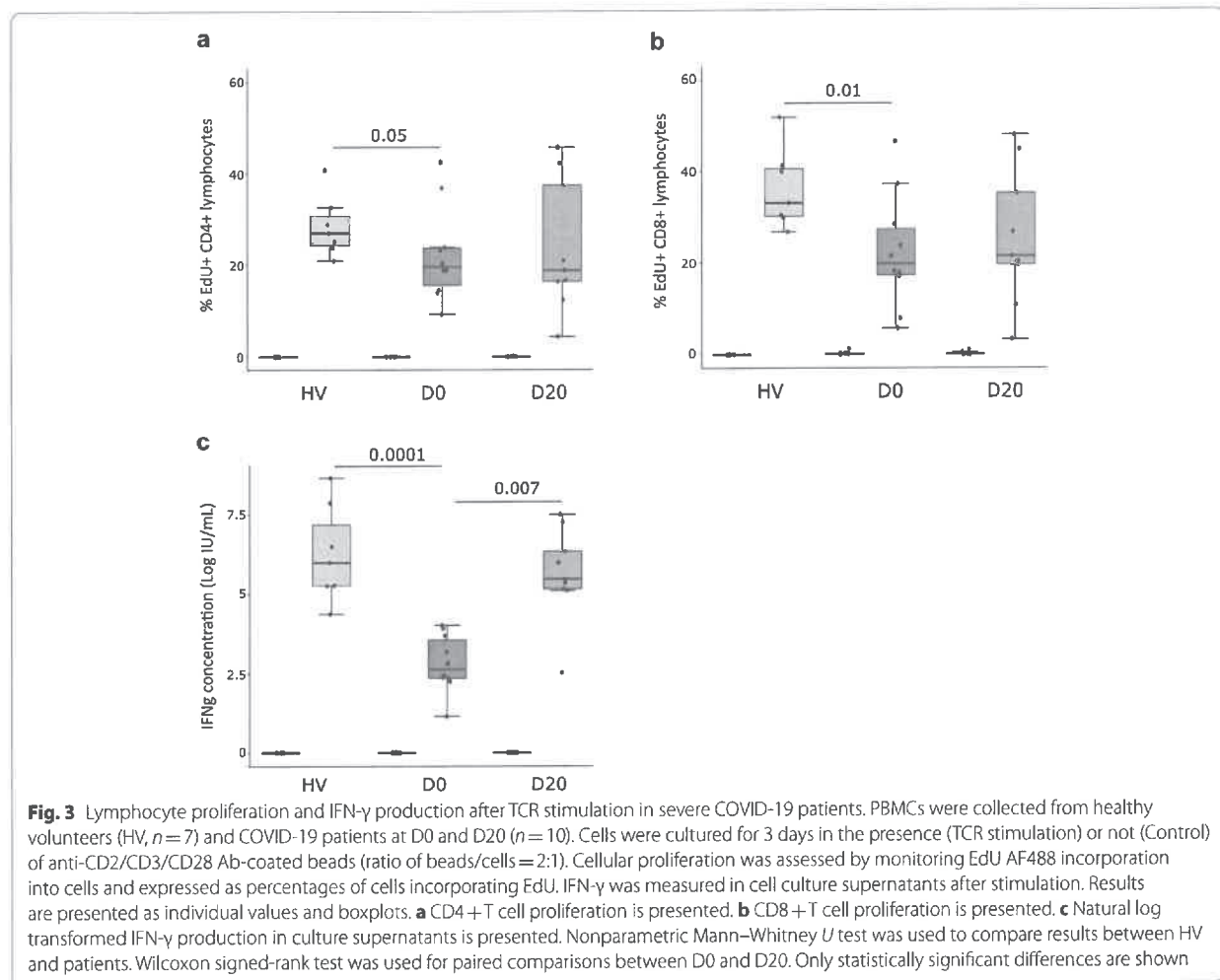
Finally, we investigated whether rhIL-7 could improve COVID-19-induced T cell exhaustion ex vivo. Based on previous results showing that major T cell alterations were identified at D0 in patients, we focused on samples obtained at ICU admission to evaluate rhIL-7 biological effect on T cell function.

We observed that rhIL-7 significantly increased both CD4+ and CD8+ T cell proliferations in critically ill COVID-19 patients. Median relative increase in proliferation was 21.8% [IQR: 11.3–33.7] and we observed a reproducible effect between patients (Fig. 4a and b). In addition, rhIL-7 showed a strong beneficial effect on IFN- $\gamma$  production as it induced a 156% [IQR: 77.3–293.0] relative increase in IFN- $\gamma$  production compared with cells stimulated without rhIL-7 (Fig. 4c).

Overall, these results showed that rhIL-7 presented with a strong beneficial effect on COVID-19-induced T cell exhaustion ex vivo in samples from COVID-19 patients.

#### Discussion

Current results demonstrate that severe COVID-19 patients present with marked lymphopenia while remaining circulating lymphocytes show (i) altered transcriptomic profile with inhibition of T cell activation pathways, (ii) increased expressions of co-inhibitory receptors and (iii) altered functional responses ex vivo with decreased proliferation and IFN- $\gamma$  production. These alterations, characteristic of T cell exhaustion,

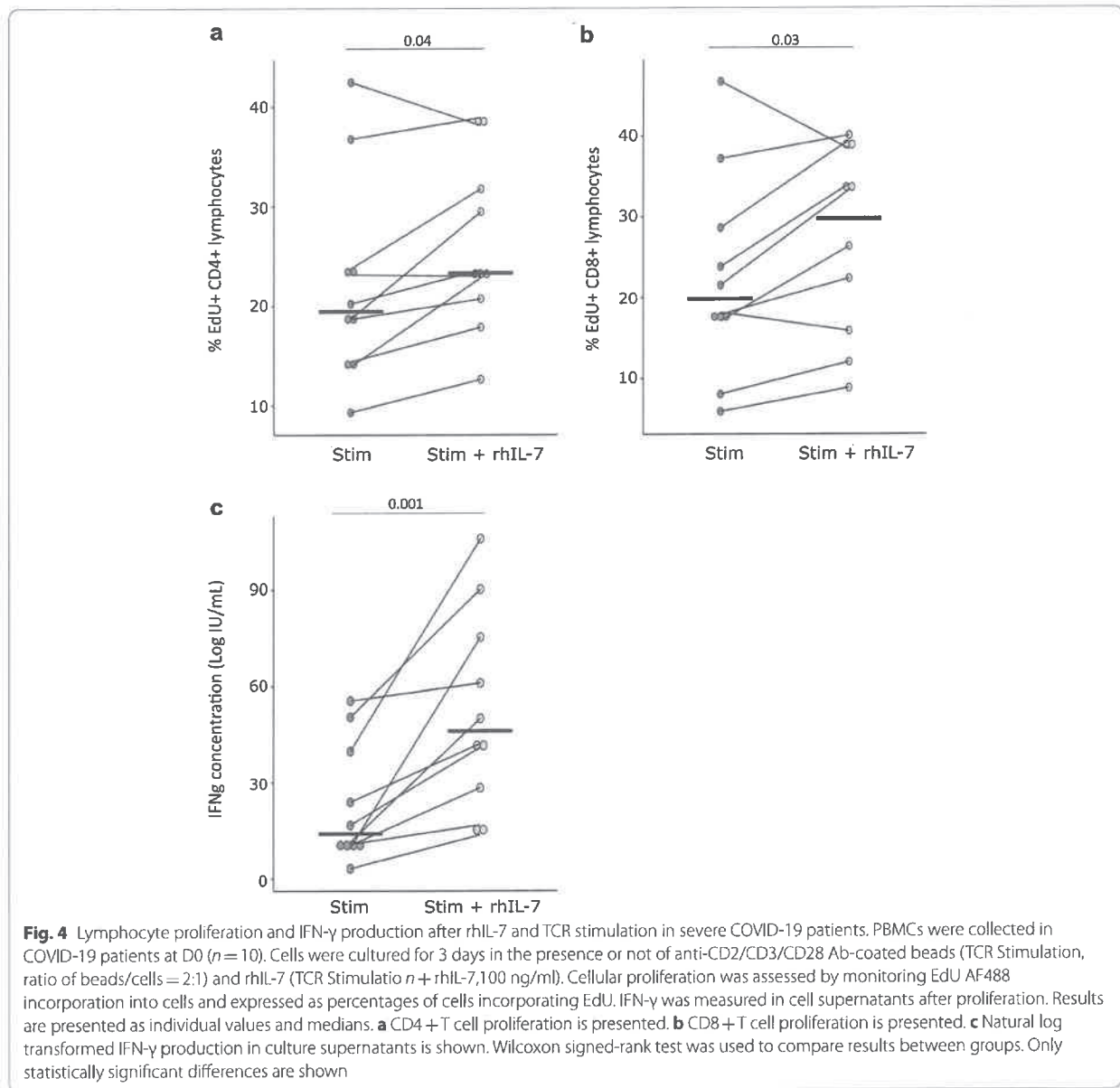


were more pronounced at ICU admission. rhIL-7 incubation *ex vivo* demonstrated a strong beneficial effect on COVID-19-induced T cell exhaustion *ex vivo* in samples from COVID-19 patients.

An increasing amount of data suggested that T-cell immunity plays an important role in eliminating SARS-CoV-2. Reports from hospitalized patients recovering without developing severe symptoms revealed emergence and rapid increase in activated CD38<sup>+</sup>HLA-DR<sup>+</sup> T cells, especially CD8<sup>+</sup> T cells, at days 7–9 after first presentation of symptoms [24]. This activation of T cells preceded the resolution of symptoms and might have prevented the evolution towards severe forms of the disease. Likewise, patients with mild symptoms who rapidly cleared the virus mounted robust T cell response, whereas only a weak and monospecific IFN- $\gamma$ -secreting cell response was detected in deadly cases [25]. More importantly, viral clearance and reduced disease severity were associated

with activation of both humoral and cellular anti-viral immune responses in COVID-19 patients as expansion of SARS-CoV-2 specific CD4+ T cells, CD8+ T cells and neutralizing antibodies have been shown to be beneficial in minimizing COVID-19 severity [26]. Overall, cell-mediated immune response seems to be of paramount importance in the fight against SARS-CoV-2 infection as a delayed specific T cell response is associated with disease severity [6].

Lymphopenia is a hallmark of severe COVID-19 and has been repeatedly associated with clinical severity and mortality [4, 27, 28]. In the present study, we confirmed the decreased lymphocyte count observed in severe COVID-19 patients affecting both CD4+ and CD8+ T cells. Similarly decreased lymphocyte number was observed in patients with bacterial sepsis [29, 30]. In septic patients also, alterations of lymphocyte count, phenotype or effector functions have been shown to be associated with deleterious outcomes [7].



In critically ill COVID-19 patients, we reported that remaining circulating T lymphocyte presented with multiple features of T cell exhaustion. This marked immunosuppressive profile was particularly present upon ICU admission. First, proliferative capacity of T cells from COVID-19 patients ex vivo was altered. Only few studies investigated this parameter so far in COVID-19. They similarly described altered T cell proliferation in severe patients [31, 32]. Several mechanisms could be involved in this reduced proliferative response: regulation through inhibitory checkpoint receptors, myeloid derived suppressor cell expansion

[32, 33] or direct pathogenic effect of the virus SARS-CoV-2 itself because the expression of its receptor, angiotensin-converting enzyme 2, is increased on CD4+ T cells after ex vivo stimulation [34]. We also observed a dramatic decrease in IFN- $\gamma$  production concomitant to this altered proliferative ability. This altered production of IFN- $\gamma$  in severe cases of COVID-19 has been described before [11, 35, 36] but was not analyzed over time. We found that this production significantly improved at D20, suggesting that the impaired adaptive immune response may be involved early in COVID-19 immunopathology. Finally, increased expression of

the co-inhibitory receptors PD-1, CTLA-4 or TIM-3 have been reported [35, 37, 38] and associated with disease severity in COVID-19 patients. We describe that this exhausted phenotype was probably related to the expression of multiple immune checkpoint inhibitors simultaneously and that this expression was sustained over time. These enhanced expressions could explain partly the lymphopenia observed in COVID-19 as increased PD-1 expression on CD4+ T cells was inversely correlated with decreased CD4+ T lymphocyte count. Similarly, in septic patients, apoptosis has been associated with increased PD-1 expression in CD4+ cells [39]. Overall, these results support the hypothesis that a dysregulated T lymphocyte response might contribute to the immunopathology of COVID-19 with the development of a state of T cell exhaustion in the most severe patients at ICU admission.

Only few studies focused on treatments to reverse COVID-19 related T cell exhaustion and rhIL-7 could be of interest in this setting [40]. IL-7 promotes lymphocyte survival and expansion but its expression declines with age-associated thymic atrophy [41]. Thus, rhIL-7 could help to overcome this pitfall by inducing increased thymopoiesis [42] and stimulating the generation of antigen-specific T cells [43]. In bacterial sepsis, preclinical studies showed that rhIL-7 treatment reduced mortality in murine models and improved cell functionality upon *ex vivo* activation of septic shock patients T lymphocytes [13–15]. A phase II clinical trial evaluating rhIL-7 in septic shock patients showed that IL-7 treatment restored T cell count in patients with severe lymphopenia in the absence of any severe side effects [44].

Herein, we report that *ex vivo* administration of rhIL-7 enhanced both CD4+ and CD8+ T cell proliferations and thus could participate in restoring the coordinated adaptive immune response required to control SARS-CoV-2 infection [26]. Furthermore, rhIL-7 improved IFN- $\gamma$  lymphocyte production which is another aspect of COVID-19 lymphocyte dysfunction. Administration of rhIL-7 was efficacious at D0 so that our results support the rationale of using rhIL-7 early in the evolution of the disease. Similarly, two recent studies showed that *ex vivo* administration of rhIL-7 was associated with increased IFN- $\gamma$  production of stimulated T cells in severe COVID-19 cases [11] and with restoration of IFN- $\gamma$  production by T cells after treatment with dexamethasone [45]. Compassionate use in 12 critically ill patients with severe lymphopenia and in a case report allowed to restore lymphocyte count without exacerbating inflammation or pulmonary injury [17, 18]. One clinical trial investigating the effect of rhIL-7 in lymphopenic patients with COVID-19 infections is ongoing in Europe and in the USA (ILIAD-7, NCT04442178, NCT04407689, NCT04379076).

In the current cohort, most patients presented with alterations of T cell function at D0 but some patients may not display such immunosuppressed profile. Thus effect of rhIL-7 in these patients may not be as worthwhile. This implies careful selection of patients who would benefit from such immune-adjuvant therapy. Functional testing could be used to monitor patients' actual adaptive immune response. However, despite representing the gold standard for functional testing, T cell proliferation assays may not be suitable to routinely monitor immune status in clinic because this test is time consuming, poorly standardized and it requires between 3 and 7 days of stimulation. Absolute lymphocyte count may not be an appropriate surrogate marker of T cell function as we did not observe any correlation between this parameter and lymphocyte proliferation in this study. Thus the appropriate stratification marker for rhIL-7 administration in critically ill COVID-19 patients remains to be determined.

Our study has some limitations that should be addressed. First, we focused on patients with the most severe forms of COVID-19 so that the T cell exhaustion profile observed may not be generalizable to all COVID-19 cases. Second, healthy donors and patients were not strictly age-matched. However we did not identify any significant correlation between increasing age of donors and PD1 expression on CD4+ T cells, PD-L1 expression on CD4+ T cells, PD-L1 expression on CD8+ T cells, CD4+ and CD8+ T cell proliferations. This argues against a strong effect of immunosenescence on immune parameters in this cohort. Third, we only assessed global T cell function and not SARS-CoV-2 specific T cell responses which may be affected in a different manner. Fourth, we did not assess whether parameters of IL-7 pathway might be altered before administering rhIL-7. Finally, the number of patients included in the cohort did not allow to define precisely which patients would respond the most to rhIL-7 therapy or if these lymphocyte alterations were associated with mortality. Both aspects now deserve to be confirmed in a larger cohort of critically ill COVID-19 patients.

## Conclusion

Our findings depict severe COVID-19 disease immunopathology as a defective T lymphocyte response related to a profound lymphopenia and exhausted T cells with decreased functionality. These alterations were present upon ICU admission and tended to improve over time. Importantly, this immunosuppressed phenotype was reversible *ex vivo* after rhIL-7 administration. Defining the appropriate timing for initiating such immune-adjuvant therapy in clinical setting and the pertinent markers for a careful selection of patients are now warranted to confirm the *ex vivo* results described so far.

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13613-022-00982-1>.

**Additional file 1.** Additional tables.

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Hospices Civils de Lyon, Croix-Rousse University Hospital, Medical intensive Care Department: Jean-Christophe Richard, Laurent Bitker, Mehdi Mezidi, Loredana Baboi.

### Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by FB, SH, MG, RC, JL, MB, MAC, MM. The first draft of the manuscript was written by F. Bidar and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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### Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

### Declarations

#### Ethics approval and consent to participate

This project was part of an ongoing prospective observational clinical study (RICO, REA-IMMUNO-COVID) performed in line with the principles of the Declaration of Helsinki. It was approved by ethics committee (Comité de Protection des Personnes Ile de France 1-N°IRB / IORG #: IORG0009918) under agreement number 2020-A01079-30. This clinical study was registered at ClinicalTrials.gov (NCT04392401). The committee waived the need for written informed consent because the study was observational, with a low risk to patients, and no specific procedure, other than routine blood sampling, was required. Oral information and non-opposition to inclusion in the study were mandatory and were systematically obtained before any blood sample was drawn. This was recorded in patients' clinical files. If a patient was unable to consent directly, non-opposition was obtained from the patient's legally authorized representative and reconfirmed from the patient at the earliest opportunity.

#### Consent for publication

All authors have read the manuscript and have consented for its publication.

### Competing interests

The authors declare no competing financial interests in relation to the work. MAC, KBP and MM are bioMérieux's employees. This private company had no role in the study design, result analysis and decision to publish this study.

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## **Annexe 7**





# T cell response against SARS-CoV-2 persists after one year in patients surviving severe COVID-19

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## Summary

**Background** In critically ill COVID-19 patients, the initial response to SARS-CoV-2 infection is characterized by major immune dysfunctions. The capacity of these severe patients to mount a robust and persistent SARS-CoV-2 specific T cell response despite the presence of severe immune alterations during the ICU stay is unknown.

**Methods** Critically ill COVID-19 patients were sampled five times during the ICU stay and 9 and 13 months afterwards. Immune monitoring included counts of lymphocyte subpopulations, HLA-DR expression on monocytes, plasma IL-6 and IL-10 concentrations, anti-SARS-CoV-2 IgG levels and T cell proliferation in response to three SARS-CoV-2 antigens.

**Findings** Despite the presence of major lymphopenia and decreased monocyte HLA-DR expression during the ICU stay, convalescent critically ill COVID-19 patients consistently generated adaptive and humoral immune responses against SARS-CoV-2 maintained for more than one year after hospital discharge. Patients with long hospital stays presented with stronger anti-SARS-CoV-2 specific T cell response but no difference in anti-SARS-CoV-2 IgG levels.

**Interpretation** Convalescent critically ill COVID-19 patients consistently generated a memory immune response against SARS-CoV-2 maintained for more than one year after hospital discharge. In recovered individuals, the intensity of SARS-CoV-2 specific T cell response was dependent on length of hospital stay.

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**Keywords:** Immune memory; T lymphocyte; SARS-CoV-2; Critically ill patients; HLA-DR; Sepsis

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### Research in context

#### *Evidence before this study*

We searched PubMed for the term (“COVID-19” or “SARS-CoV-2”) AND (“memory T cell” or “memory T lymphocyte”) AND (“critically ill” or “ICU” or “severe”) with no limit of publication date. No study was identified evaluating the long-term persistence of SARS-CoV-2 specific T cell response focusing on the subgroup of the most severe critically ill COVID-19 patients.

#### *Added value of this study*

We conducted this study to evaluate the presence of SARS-CoV-2 specific T lymphocytes in a cohort of critically ill COVID-19 patients for whom immune dysregulations occurring during ICU stay were characterized. We showed that, despite the presence of major immune alterations during ICU stay, convalescent critically ill COVID-19 patients consistently generated substantial adaptive and humoral immune responses against SARS-CoV-2 maintained for more than one year after hospital discharge. Patients with long hospital stays presented with stronger anti-SARS-CoV-2 specific T cell responses but no difference in anti-SARS-CoV2 IgG levels.

#### *Implications of all the available evidence*

Convalescent critically ill COVID-19 patients develop a robust memory immune response against SARS-CoV-2 dependent on length of hospital stay. A better description of the mechanisms underlying the establishment of protective immune memory in recovering individuals is a major concern for public health for predicting and managing potential additional waves of infections in the general population.

## Introduction

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection is still threatening public health. As of December 2021, over 275 million confirmed cases and more than 5.3 million deaths have been reported since the start of the pandemic (WHO weekly epidemiologic update). COVID-19 is primary a respiratory disease with symptoms ranging from paucisymptomatic or mild infection to severe symptoms requiring hospitalization in intensive care units (ICU).<sup>1</sup>

The development of specific adaptive immune response is crucial to control and clear viral infections. More specifically, upon infection, virus-specific T and B cells are activated, expand and differentiate into effector cells. Once the infection is cleared, neutralizing antibodies and antigen-specific memory B and T cells persist for a long time after resolution. Such memory immune response plays a central role in the prevention against reinfection and is mobilized during vaccination. It is

thus important to characterize in details the magnitude of specific adaptive immune responses in convalescent COVID-19 patients with different severities in order to understand the development and longevity of such protective immunity. A better description of the mechanisms underlying the establishment of protective immune memory in recovering individuals is a major concern for public health for predicting and managing potential additional waves of infections in the general population.

In critically ill COVID-19 patients, the initial response to SARS-CoV-2 infection is characterized by major immune dysfunctions associating a systemic inflammatory response and the development of altered innate and adaptive immune responses.<sup>1,2</sup> More specifically, T cell response is markedly affected in critically ill COVID-19 patients and severe lymphopenia, phenotypic and functional T cell alterations have been described in the most severe COVID-19 patients.<sup>1-3</sup> Thus, the capacity of these critically ill patients to mount a robust and persistent SARS-CoV-2 specific T cell response despite the presence of severe immune alterations during the ICU stay is, so far, unknown.

In this context, the goal of the current study was to monitor the immune response including memory T cells specific to SARS-CoV-2 in paired samples collected 9 and 13 months after infection in a cohort of convalescent critically ill COVID-19 patients for whom COVID-19-induced immune dysfunctions during the ICU stay were minutely characterized.

## Methods

### Clinical study design, patient population and ethics approval

Critically ill patients admitted to two ICUs (Medical and Surgical ICUs of the Edouard Herriot University Hospital) of Lyon university-affiliated hospitals (Hospices Civils de Lyon, Lyon, France) who presented with SARS-CoV-2 pulmonary infection confirmed by RT-PCR were included in the RICO (REA-IMMUNO-COVID) study.<sup>2</sup> This study was approved by ethics committee (Comité de Protection des Personnes Ile de France 1 - N°IRB / IORG #: IORG0009918) under agreement number 2020-A01079-30 and was registered at ClinicalTrials.gov (NCT04392401). The committee waived the need for written informed consent because the study was observational, with a low risk to patients, and no specific procedure, other than routine blood sampling, was required. Oral information and agreement to inclusion in the study were mandatory and were systematically obtained before any blood sample was drawn. This was recorded in patients' clinical files. If a patient was unable to consent directly, the patient's legally authorized representative were contacted and agreement was reconfirmed from the patient at the

earliest opportunity. Inclusion criteria were (i): patients aged > 18 years, (ii) hospitalization in ICU for SARS-CoV-2 pneumopathy, (iii) first hospitalization in the ICU for COVID-19, (iv) positive diagnosis of SARS-CoV-2 infection carried out by PCR in at least one respiratory sample, (v) sampling in the first 48 h after admission to the ICU feasible and (vi) patient or next of kin informed of the terms of the study and has not objected to participate.

Upon ICU release, convalescent COVID-19 individuals were requested to participate to the ancillary study: NOSO-COR-IMMUNO. The clinical protocol was approved by ethics committee of Ile de France on October 14 2020 (N° IDRCB: 2020-A02128-31). This study was registered at Clinicaltrials.gov under number NCT04637867. Inclusion criteria were (i) patients aged > 18 years included in NOSO-COR study<sup>4</sup> (ii) signed written informed consent (iii) affiliation to social security system. Exclusion criteria were: (i) opposition to participation (ii) breast feeding and pregnancy (iii) currently hospitalized patients (iv) patients under legal assignment. Patients who agreed to participate were invited for two follow-ups medical visits in an ambulatory setting in Edouard Herriot Hospital, Hospices Civils de Lyon, 9 and 13 months after SARS-CoV-2 infection. All participants provided written informed consent and the study was conducted in accordance with Good Clinical Practice.

A cohort of 15 healthy donors either non-infected and non-vaccinated ( $n = 4$ ), or after full vaccination ( $n = 4$ ) or after resolution of a non-severe SARS-CoV-2 infection ( $n = 7$ ) was concomitantly included after informed consent was given. Median age was 48 [29–55] and 10 women and 5 men were included.

#### Patient characteristics

For each patient, demographics, comorbidities, time from onset of COVID-19 symptoms to ICU admission, initial presentation of the disease in the ICU including the ratio of the arterial partial pressure of oxygen to the fractional inspired oxygen ( $\text{PaO}_2/\text{FiO}_2$  ratio) at admission, antiviral therapy targeting SARS-CoV-2 and organ support, were documented. Organ dysfunctions according to Sequential Organ Failure Assessment (SOFA) score (range 0–24, with higher scores indicating more severe organ failures), and Simplified Acute Physiology Score II (SAPS II; range, 0–164, with higher scores indicating greater severity of illness) were documented. Follow-up included ICU length of stay, in-hospital mortality, day-28 (D28) mortality, day-90 (D90) mortality.

At follow-up visits, the following clinical parameters were recorded: presence and types of persisting symptoms, presence of new symptoms, new hospitalization, contact with an infected person, presence of a positive SARS-CoV-2 PCR test and vaccination status.

#### Blood samples and cell isolation

Ethylene diamine tetra-acetic acid (EDTA)-anticoagulated blood was drawn five times after ICU admission: within the first 48 h after admission (Day 0: D0), between 72 h and 96 h after admission (D3), between D7 and D9 (D7), between D12 and D15 (D12), between D20 and D25 (D20) and then twice at follow-up visits (i.e. after 9 and 13 months after hospital discharge). Blood was stored at 4–8 °C and processed within 4 h after withdrawal for flow cytometry analyses and cell culture. Plasma was then frozen at -80 °C within 4 h following blood collection and later used for soluble markers analyses, which were performed by batches after 1 freeze / thaw cycle.

#### Cytokine measurement

Plasma concentrations of IL-6, TNF- $\alpha$ , IFN- $\gamma$  and IL-10 were measured by Simpleplex<sup>®</sup> technology using ELLA instrument (ProteinSimple<sup>®</sup>, San Jose, CA), following manufacturer's instructions.

#### Flow cytometry

T lymphocyte immunophenotyping was performed on an automated volumetric flow cytometer from Beckman Coulter (Aquios CL) as previously described.<sup>5</sup> The expression of monocyte HLA-DR was determined using the Anti-HLA-DR/Anti-Monocyte Quantibrite assay (BD Biosciences, San Jose, USA). Total number of antibodies bound per cell (AB/C) were quantified using calibration with a standard curve determined with BD Quantibrite phycoerythrin (PE) beads (BD Biosciences) as described elsewhere.<sup>6</sup> B and NK lymphocyte immunophenotyping was performed using lyophilized antibody panel from Beckman Coulter (Duraclone kit). Data were acquired on a Navios flow cytometer (Beckman Coulter, Hiialeah, FL) and flow data were analyzed using Navios software (Beckman Coulter).

#### Plasma anti-SARS-CoV-2 antibody concentration measurement

Plasmatic anti-SARS-CoV-2 immunoglobulin measurements were performed using Vidas<sup>®</sup> SARS-CoV-2 IgG *in vitro* diagnostic (IVD) assays (bioMérieux, Marcy-l'Étoile, France). Briefly, a solid-phase repository coated with the antigen (recombinant SARS-CoV-2 receptor-binding domain [RBD] of the viral spike [S] protein) served as both solid-phase and pipetting device. After a dilution step, SARS-CoV-2-specific IgG were captured on the coated antigen, and unbound components are washed out. In the second step, human IgG (Vidas<sup>®</sup> SARS-CoV-2 IgG) were specifically detected by mouse monoclonal antibodies conjugated to alkaline phosphatase and directed against human IgG, respectively. A relative fluorescence value (RFV) was generated (background reading subtracted from the final fluorescence

reading). The assay was conducted with a standard solution as well as positive and negative controls. The results were automatically calculated by the instrument, according to standard and an index value ( $i$ ) was obtained ( $i = \text{RFV}_{\text{sample}}/\text{RFV}_{\text{S1}}$ ). The test is interpreted as negative when  $i < 1.00$  and positive when  $i \geq 1.00$ .

#### Plasma SARS-CoV-2 viral load measurement

Determination of normalized viral load was performed on plasma samples by RNA extraction on the EMAG<sup>®</sup> platform (bioMérieux, Marcy-l'Étoile, France). The SARS-CoV-2 load was measured by quantitative RT-PCR, according to a scale of calibrated in-house plasmid, using the RT-PCR RdRp-IP<sub>4</sub> developed by the Institut Pasteur (Paris, France).<sup>7</sup> The amplification protocol was developed using QuantStudio 5 rtPCR Systems (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The absence of inhibitors in the specimen was checked by using the RNA Internal Control R-GENE<sup>®</sup> kit (Argene\_BioMérieux, Marcy-l'Étoile, France) on each sample. We expressed normalized SARS-CoV-2 load in log<sub>10</sub> RNA copies/mL and all viral loads strictly below 4 RNA copies/reaction were considered under the limit of detection and were reported as negative.

#### Plasma SARS-CoV-2 nucleoprotein concentration measurement

N antigenemia levels were determined with an ELISA microplate assay, COVID-Quantigene<sup>®</sup> (AAZ, Boulogne-Billancourt, France), according to manufacturer recommendations. Briefly, in each well of 96-wells microplates previously coated with anti-SARS-CoV-2 N-antibodies, 50  $\mu\text{L}$  of a solution containing a biotinylated anti-SARS-CoV-2 N antibodies and 50  $\mu\text{L}$  of sera were added. After incubation at 37 °C for 60 min, plates were washed 5 times with a phosphate buffer solution. Then, 100  $\mu\text{L}$  of a solution containing HRP-conjugated streptavidin were added, followed by incubation for 30 min at 37 °C. Plates were washed 5 times with the phosphate buffer solution, then 50  $\mu\text{L}$  of a solution containing the substrate (3,3',5,5'-tetramethylbenzidine (TMB)) and 50  $\mu\text{L}$  of a second solution containing urea were added. After 15 min at 37 °C, the colorimetric reaction was stopped by adding 50  $\mu\text{L}$  of H<sub>2</sub>SO<sub>4</sub>. Absorbance values were measured at 450 nm, with a reference set at 630 nm. In each plate, standards made of recombinant N antigens were tested, to quantify the N antigenemia levels for each patient's sample. Samples with titers above 180 pg/mL were diluted for precise quantification. Results were expressed as pg/mL.

#### Anti-SARS-CoV-2 specific T cell proliferation measurement

When sampled at 9- and 13-month follow-up visits, peripheral blood mononuclear cells (PBMCs) from

patients and donors were isolated from fresh whole blood by Ficoll-Paque PREMIUM (GE Healthcare) density gradient centrifugation and immediately processed for cell culture. The number of PBMCs per well was adjusted to 1.10<sup>6</sup> cells/mL re-suspended in complete culture media (RPMI 1640 medium with HEPES, L-glutamine, 10% human AB plasma, 20 mg/mL streptomycin and 2.5 mg/mL fungizone). Cells were then stimulated during 7 days at 37 °C, 5% CO<sub>2</sub> with SARS-CoV-2 peptide pools (JPT technology). The peptide pools consisted of 15 amino acid long peptides with 11 amino acid overlap. The following SARS-CoV-2 peptide pools were evaluated: pool of 315 peptides covering the spike (15 mers with 11-aa overlap, delivered in two sub-pools of 158 [S1 pool] and 157 [S2 pool] peptides), pool of 102 peptides covering the nucleoprotein, pool of 53 peptides covering the membrane. Peptides were used at a final concentration of 1.25  $\mu\text{g}/\text{mL}$ . All samples were performed in duplicates. EdU click-it reaction was performed using EdU Click-it kit (Thermo Fisher) as previously described.<sup>8</sup> CD3+, CD4+ and CD8+ T cell proliferations were analyzed by monitoring EdU-AF488 incorporation into cells. Results were expressed as percentages of cells incorporating EdU.

#### Statistical analyses

Data are presented as numbers and percentages (qualitative variables) and medians and 25th/75th percentiles (quantitative variables). Fisher's exact test was used for qualitative variables comparison. Quantitative variables were compared with Mann-Whitney U test. For all pairs of immune parameters, Spearman's rho correlation coefficients were estimated and summarized in a correlation matrix. Comparisons between immune parameters measured in patients with short and long hospital length of stays (hospital length of stay below or over 25 days) were limited to values measured at D<sub>0</sub>, M<sub>9</sub> and M<sub>13</sub> when all patients from both groups could be sampled. The level of significance was set at 5%. Data were analyzed using Graphpad Prism version 5.03 (Graphpad Software, La Jolla, USA) and R version 3.6.2 (R Core Team).

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	All patients (n = 16)	Short Hospital Stay (n = 7)	Long Hospital Stay (n = 9)	p-value
<b>Demographics</b>				
Age	64 [55–68]	56 [43–72]	65 [59–67]	0.6624
Gender (Male)	12 (75 %)	4 (57 %)	8 (89 %)	0.1457
Body mass index (kg/m <sup>2</sup> )	29.5 [26.4–34.3]	26.5 [25.5–28.7]	30.5 [28.7–35]	0.0907
Body mass index > 30 kg/m <sup>2</sup>	8 (50 %)	1 (14 %)	7 (78 %)	<b>0.0117</b>
<b>Comorbidities</b>				
0	13 (81 %)	7 (100 %)	6 (67 %)	0.0901
≥ 1	3 (19 %)	0 (0 %)	3 (33 %)	
Charlson score	0 [0–0]	0 [0–0]	0 [0–1]	0.2125
<b>Admission symptoms</b>				
Delay between first symptoms (Days)	9 [7–11]	9 [8–11]	9 [6–11]	0.8143
Fever	13 (81 %)	5 (71 %)	8 (89 %)	
Cough	11 (69 %)	6 (86 %)	5 (56 %)	
Dyspnea	9 (56 %)	2 (29 %)	7 (78 %)	
Diarrhea	6 (38 %)	3 (43 %)	3 (33 %)	
Diffuse pain	3 (19 %)	1 (14 %)	2 (22 %)	
Altered general status	9 (56 %)	5 (71 %)	4 (44 %)	
Other	4 (25 %)	1 (14 %)	3 (33 %)	
<b>Severity scores</b>				
SOFA score	2 [2–5]	2 [2–3]	3 [2–8]	0.2054
SAPS II score	28 [22–36]	27 [22–33]	29 [20–40]	0.8990
PaO <sub>2</sub> /FiO <sub>2</sub> at admission	162 [89–238]	238 [78–329]	147 [90–170]	0.1893
<b>Organ support</b>				
Mechanical ventilation)	16 (100 %)	7 (100 %)	9 (100 %)	
-Noninvasive ventilation)	12 (75 %)	7 (100 %)	5 (56 %)	<b>0.0417</b>
-Invasive ventilation	4 (25 %)	0 (0 %)	4 (44 %)	
Vasoactive drugs	3 (19 %)	0 (0 %)	3 (33 %)	0.0901
Renal replacement therapy	2 (13 %)	0 (0 %)	2 (22 %)	0.1824
<b>Follow-up</b>				
Days in ICU	8 [3–38]	4 [1–7]	30 [14–65]	<b>0.0031</b>
Days in Hospital	37 [12–72]	11 [7–15]	71 [44–87]	<b>0.0002</b>
Secondary infections	8 (50 %)	0 (0 %)	8 (89 %)	<b>0.0004</b>

**Table 1: Clinical characteristics of critically ill patients with COVID-19 at ICU admission. Results are shown as medians and interquartile ranges [Q1-Q3] for continuous variables or numbers and percentages for categorical variables. Patients were separated in two groups based on length of hospital stay. Sepsis-related organ failure assessment (SOFA) and Simplified acute physiology score II (SAPS II) scores were calculated during the first 24 h after intensive care unit (ICU) admission. Data were compared using non-parametric Mann-Whitney test for continuous variables or Fisher's exact test for categorical variables.**

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## Results

### Patients characteristics

Sixteen convalescent critically ill COVID-19 patients were included in this study. Demographics and clinical characteristics of patients at ICU admission are presented in Table 1. Patients were predominantly males (75%) with median age of 64 years old. Median duration of symptoms before ICU admission was 9 [7–11] days. These patients were invited for two follow-ups medical visits in an ambulatory setting at

respectively 278 [253–301] days and 382 [375–384] days after initial infection. During the first visit (i.e. after 9 months M<sub>9</sub>), nine patients presented with persistent symptoms such as dyspnea, asthenia, pain or critical illness neuropathy. Two had been re-hospitalized but with no link to the initial COVID-19 infection. At this first visit, none of the patients was vaccinated against SARS-CoV-2. During the second follow-up visit (i.e. after 13 months after initial infection – M<sub>13</sub>), ten patients had persistent symptoms and one had been re-hospitalized without any link to the COVID-19 infection. At this second visit, three patients had received a first injection and one two injections of anti-COVID-19 vaccine (BNT162b2 vaccine, Pfizer-BioNTech). All participants tested negative for SARS-CoV2 PCR at M<sub>9</sub> and M<sub>13</sub> follow-up visits.



### Non-specific immune response to infection

During the ICU stay, immune profile of critically ill COVID-19 patients recapitulated features of immune alterations as described previously.<sup>2,3,9</sup> This included decreased expression of monocyte HLA-DR expression (mHLA-DR, Figure 1A), major lymphopenia (Figure 1B) affecting both B, NK, CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Figure 1C–E, Supplementary Figure 1A, B), increased plasma concentrations of both pro (Figure 2A, C and D) and anti-inflammatory cytokines (Figure 2B). These alterations were maximal at ICU admission and tended to normalize overtime during the ICU stay.

At follow-up visits (i.e. M9 and M13), immune alterations had receded and most patients presented with immune parameters within the range of normal values. In particular, all patients presented with normal mHLA-DR levels (Figure 1A) and no evidence of general persistent lymphopenia (Figure 1B to E, Supplementary Figure 1A, B). Finally, most patients followed up at 9 and 13-month convalescence presented with normal plasmatic concentrations of pro and anti-inflammatory cytokines (Figure 2A to D) except for plasma TNF- $\alpha$  levels, which remained subnormal in some patients (Figure 2C).

### Antigenemia, viremia and anti-SARS-CoV-2 IgG response

Plasma levels of SARS-CoV-2 nucleocapsid were high upon ICU admission but decreased rapidly (Figure 2E) while anti-SARS-CoV-2 IgG concentrations increased over the first week after admission (Figure 2F). At the end of the first week, all critically ill COVID-19 patients presented an elevated concentration of anti-SARS-CoV-2 IgG and all except one tested negative for SARS-CoV-2 antigenemia. Circulating levels of SARS-CoV-2 mRNA were undetectable in plasma for most ICU patients at any time during ICU stay except for four patients who were positive for SARS-CoV-2 PCR in plasma at ICU admission with one maintaining detectable levels of viremia for the first 3 days in ICU (data not shown).

At M9 and M13, all patients remained positive for anti-SARS-CoV-2 IgG in plasma (Figure 2F). However, IgG concentrations had largely decreased compared with levels measured during the ICU stay. Only three patients who received at least one dose of anti-SARS-CoV-2 vaccine at M13 visit presented with a marked elevation of anti-SARS-CoV-2 IgG index (Figure 2F).

### Anti-SARS-CoV-2 T cell response

We included a cohort of 4 healthy donors who were not vaccinated against SARS-CoV-2 and had not been in contact with the virus. As expected, in this group of patients, we did not detect any proliferation against any of the SARS-CoV-2 antigens (Figure 3A to C). A group

of 4 donors who were fully vaccinated (BNT162b2, Pfizer BioNTech) but had not been in contact with SARS-CoV-2 was also included. In this group, we detected T cell proliferation in all patients but only after T cell stimulation with Spike peptides pool. This shows that this group of donors had developed a memory T cell response against the vaccine antigen (i.e. Spike protein, Figure 3C). Finally, we included a group of 7 donors who had been infected by SARS-CoV-2. In this last group of patients, we detected T cell proliferation in all patients against the 3 SARS-CoV-2 proteins (Figure 3A to C), which illustrated the development of a memory T cell response against several antigens from the virus.

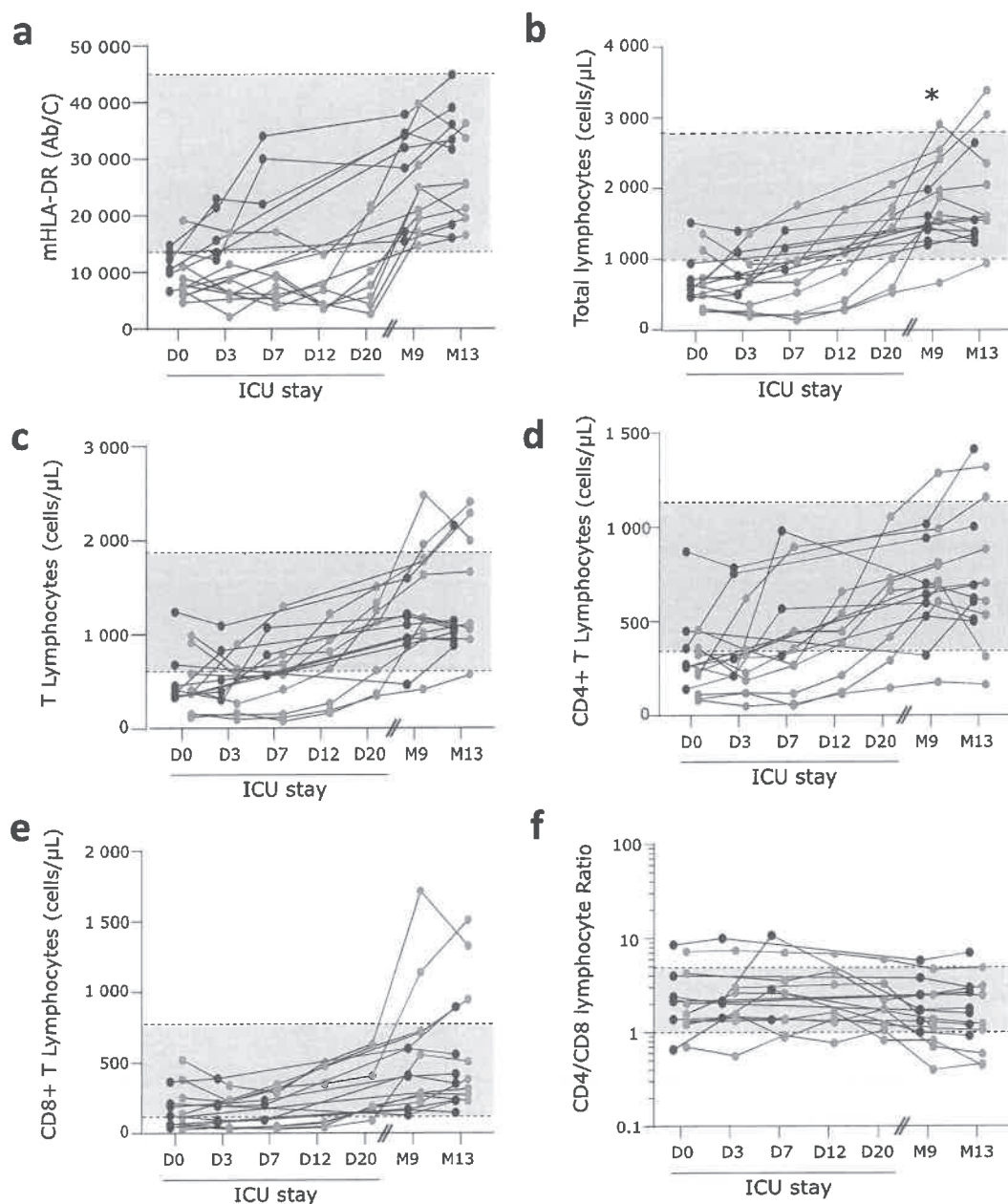
At M9 and M13, the presence of anti-SARS-CoV-2 T cells specific for SARS-CoV-2 antigens was detected in 100 % of convalescent critically ill COVID-19 patients (Figure 3A to C). Percentages of SARS-CoV-2 responding T cells in patients were heterogeneous but were mostly equivalent to levels measured in vaccinated controls or healthy individuals after non-severe SARS-CoV-2 infection. T cell proliferation was detected against the three tested SARS-CoV-2 antigens (i.e. nucleocapsid, membrane, spike). Among T cells, proliferation of both T CD4<sup>+</sup> and T CD8<sup>+</sup> lymphocytes was measured (Supplementary Figure 2A). When comparing the evolution of this specific T cell response overtime, no significant difference between results obtained at M9 and M13 in paired samples was observed (Supplementary Figure 2B).

At M9 and M13, proliferative responses against these 3 antigens were correlated but no correlation was observed between anti-SARS-CoV-2 T cell proliferative response and IgG levels (Supplementary Figure 2C). In addition, T cell response against nucleocapsid antigen was correlated with circulating levels of CD8<sup>+</sup> T cells and plasma TNF- $\alpha$  concentration (Supplementary Figure 2C).

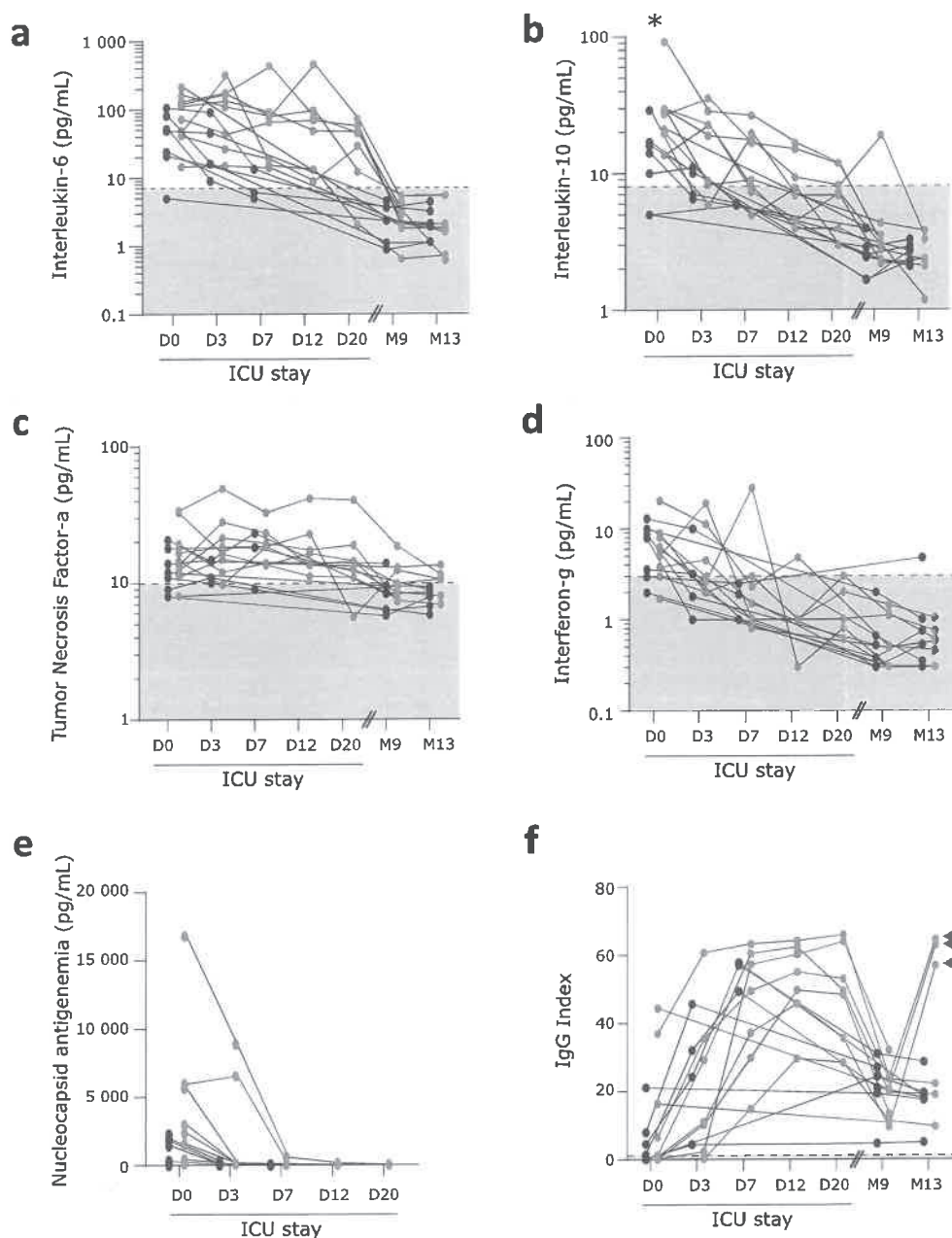
### Association between hospital length of stay and anti-SARS-CoV-2 specific immune response

As critically ill COVID-19 patients are characterized by long hospital lengths of stay (HLS),<sup>10</sup> we stratified this cohort in two groups based on their HLS. The threshold (HLS below or over 25 days) was selected to reflect the bimodal repartition of HLS in this cohort and in accordance with median ICU stay of previously published cohorts of critically ill COVID-19 patients<sup>11,12</sup>: a group of 7 patients with short HLS (ranging from 7 to 17 days) and a group of 9 patients with long HLS (ranging from 30 to 119 days).

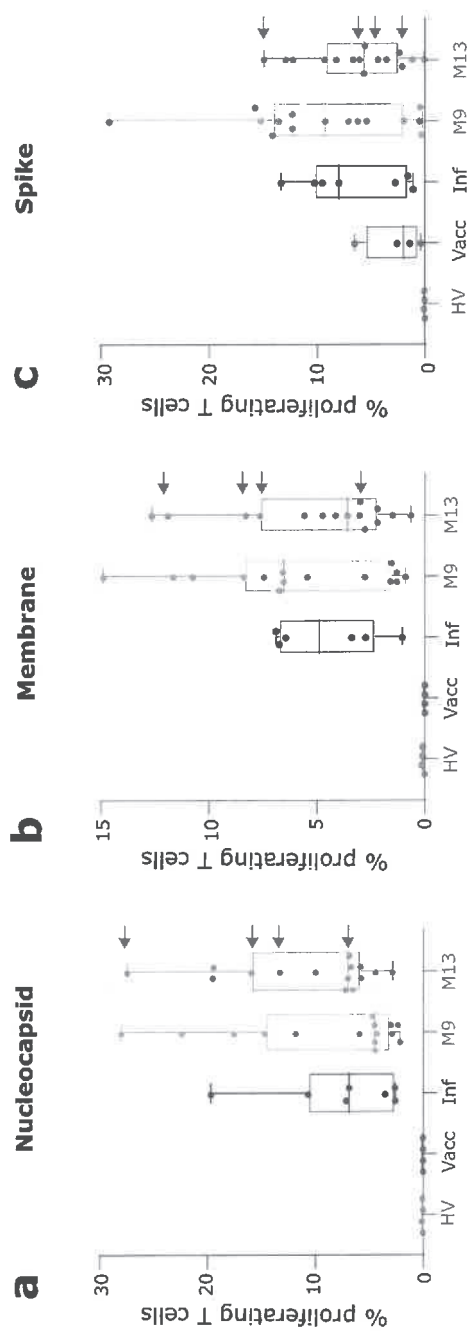
Clinical and demographic characteristics of patients with short and long HLS are presented in Table 1. Critically ill COVID-19 patients with long HLS presented more frequently with obesity (i.e. BMI superior to 30 kg/m<sup>2</sup>) but were not significantly different in terms



**Figure 1. Monitoring of COVID-19-induced cellular immune response.** Sixteen convalescent critically ill COVID-19 patients were sampled 5 times during ICU stay: within the first 48 h after admission (Day 0: D0,  $n = 15$ ), between 72 h and 96 h after admission (D3,  $n = 13$ ), between D7 and D9 (D7,  $n = 11$ ), between D12 and D15 (D12,  $n = 7$ ), between D20 and D25 (D20,  $n = 7$ ) and then twice at follow-up visits: after 9 and 13 months after hospital discharge (M9,  $n = 16$ ; M13,  $n = 15$ ). At each sampling time, the following immune parameters were monitored: **a**- monocytic expression of HLA-DR (mHLA-DR, numbers of antibodies bound per monocytes (Ab/C)), **b**- absolute counts of circulating lymphocytes (numbers of cells /  $\mu\text{L}$ ), **c**- absolute count of CD3+ T lymphocytes (numbers of cells /  $\mu\text{L}$ ), **d**- absolute count of CD4+ T cells (numbers of cells /  $\mu\text{L}$ ), **e**- absolute count of CD8+ T cells (numbers of cells /  $\mu\text{L}$ ) and **f**- ratio of numbers of CD4+ / CD8+ T cells. Results are presented as individual values. Patients with short hospital length of stay (HLS) are represented with black symbols; patients with long HLS with red symbols. Grey zones represent normal values from the routine Immunology Laboratory of Hospices Civils de Lyon for each immune parameter. At D0, M9 and M13, results were compared between patients with short and long HLS using non parametric Mann Whitney test. \*  $p < 0.05$ .



**Figure 2. Monitoring of COVID-19-induced plasmatic immune response.** Sixteen convalescent critically ill COVID-19 patients were sampled 5 times during ICU stay: within the first 48 h after admission (Day 0: D0,  $n = 15$ ), between 72 h and 96 h after admission (D3,  $n = 13$ ), between D7 and D9 (D7,  $n = 11$ ), between D12 and D15 (D12,  $n = 7$ ), between D20 and D25 (D20,  $n = 7$ ) and then twice at follow-up visits, i.e. after 9 and 13 months after hospital discharge (M9,  $n = 16$ ; M13,  $n = 15$ ). At each sampling time, the following immune parameters were monitored: **a-** plasmatic concentrations of interleukin-6 (pg/mL), **b-** plasmatic concentration of interleukin-10 (pg/mL), **c-** plasmatic concentration of tumor necrosis factor- $\alpha$  (pg/mL), **d-** plasmatic concentration of interferon- $\gamma$  (pg/mL), **e-** plasmatic concentration of nucleoprotein antigen (pg/mL) and **f-** plasmatic levels of anti-SARS-CoV-2 immunoglobulin G (IgG Index). For this last parameter, results in vaccinated patients are identified by an arrow. To note, nucleoprotein levels were only measured during ICU stay as all patients tested negative for SARS-CoV-2 at M9 and M13 visits. Results are presented as individual values. Patients with short hospital length of stay (HLS) are represented with black symbols; patients with long HLS with red symbols. Grey zones represent normal values from the routine Immunology Laboratory of Hospices Civils de Lyon or from the manufacturer when available. At D0, M9 and M13, results were compared between patients with short and long HLS using non parametric Mann Whitney test. \*  $p < 0.05$ .



**Figure 3. Monitoring of SARS-CoV-2 specific T cell proliferation.** Sixteen convalescent critically ill COVID-19 patients were sampled twice at follow-up visits (i.e. after 9 and 13 months after hospital discharge (M9,  $n = 16$ , orange symbols and boxes; M13;  $n = 15$ , red symbols and boxes; M13;  $n = 15$ ). Fifteen healthy donors either non-infected and non-vaccinated (HV,  $n = 4$ , light grey symbols), or after full vaccination (Vacc,  $n = 4$ , dark grey symbols and boxes) or after resolution of a non-severe SARS-CoV-2 infection (Inf,  $n = 7$ , black symbols and boxes) were concomitantly included. At each sampling time, the percentage of T lymphocytes that had proliferated in response to 3 SARS-CoV-2 antigens (Nucleocapsid – Panel a, Membrane – Panel b, Spike – Panel c) was monitored. Results in vaccinated patients are identified by an arrow. Results are expressed as the percentage of proliferating T cells among total T cells and are presented as individual values and as Tukey Box-plots.

of initial severity as evaluated by SAPSII and SOFA scores at ICU admission. Nevertheless, patients with long HLS were more frequently placed under invasive mechanical ventilation (44% vs 0% in patients with short HLS,  $p = 0.0417$ ). Therefore, this subgroup of patients also more frequently developed secondary / hospital acquired infections (89% vs 0% in patients with short HLS,  $p = 0.0004$ ).

The percentages of anti-SARS-CoV-2 specific proliferating T cells were significantly higher in patients with long HLS (Figure 4A to C). This difference was observed for the three SARS-CoV-2 antigens and was maintained at the three M9 and M13 follow-up visits for nucleocapsid and membrane-specific T cell responses. However, no difference regarding plasma anti-SARS-CoV-2 IgG levels was observed between patients with short and long HLS (Figure 4D).

Convalescent patients with short and long HLS did not differ for any immune parameter when measured at M9 and M13 except for the numbers of circulating total lymphocytes (Figure 1B) and of NK cells (Supplementary Figure 1A) which were higher in patients with long HLS.

Upon ICU admission, patients with short and long HLS did not significantly differ in the measurement immune parameters except for IL-10 concentration, which was increased at Do in patients with long HLS (Figure 2B). However, patients with long HLS tended to present with more profound immune alterations such as decreased mHLA-DR (Figure 1A), T cell lymphopenia (Figure 1C), higher inflammatory response (Figure 2A), higher plasmatic antigenemia (Figure 2E) and more frequently detectable plasmatic viral mRNA (0 % in patients with short HLS at Do versus 50 % in patients with long HLS) compared with patients with short HLS. In addition, the decreased MHC class II expression on monocytes (mHLA-DR) observed at Do in patients with long HLS tended to persist overtime when compared with normal values (Figure 1A).

## Discussion

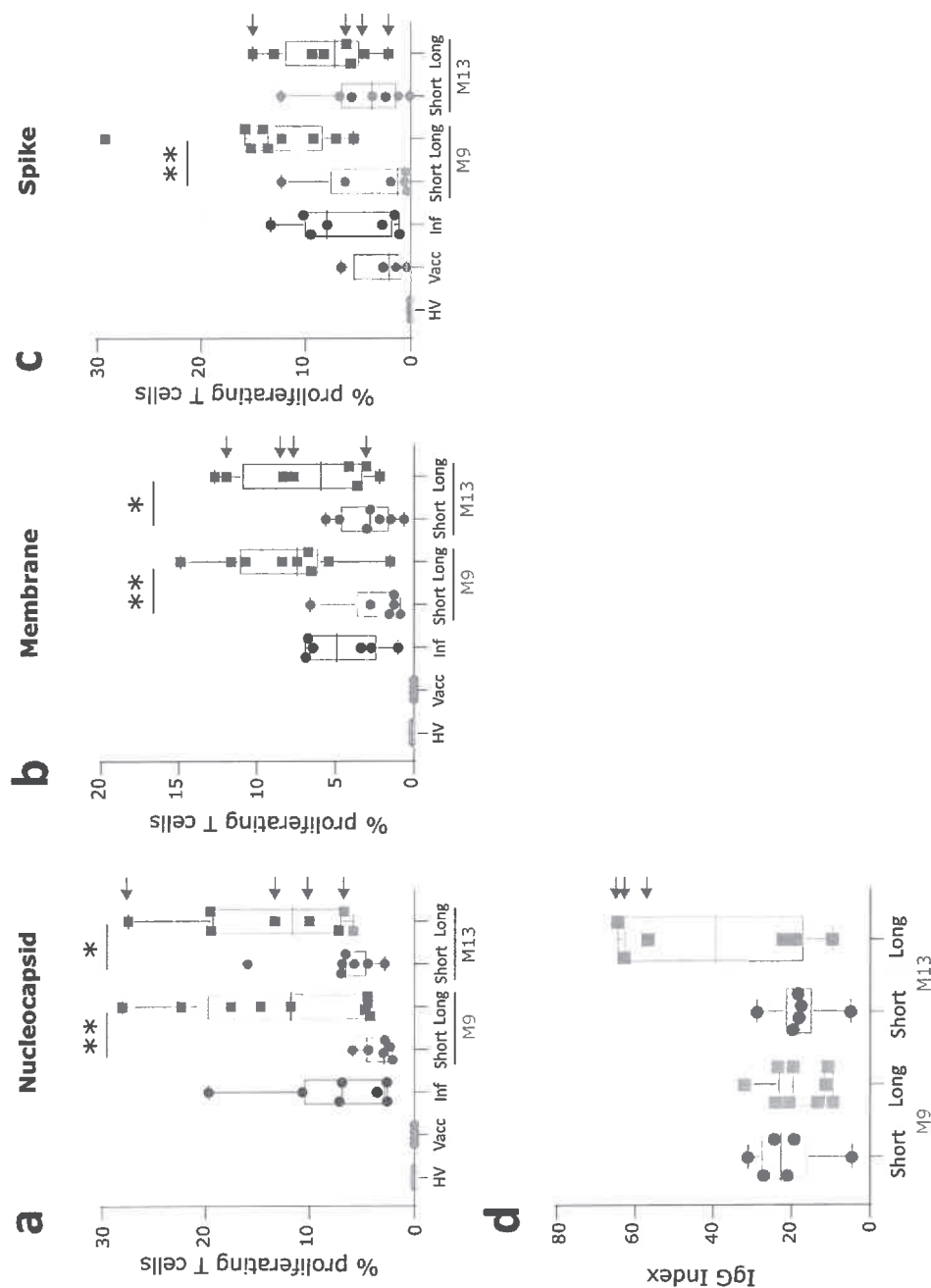
In this study, we assessed the longevity of anti-SARS-CoV-2 specific immune response in convalescent critically ill COVID-19 patients longitudinally monitored during the acute phase of the disease and 9 and 13 months after symptom onset. We showed that, despite the presence of profound immune alterations during the ICU stay (systemic inflammation, lymphopenia and decreased MHC class II expression), convalescent critically ill COVID-19 patients consistently generated a good memory T cell response against SARS-CoV-2 that was maintained for more than one year after hospital discharge. Patients with long hospital and ICU stays presented with stronger anti-SARS-CoV-2 specific T cell response but no difference in anti-SARS-CoV-2 antibody levels.

Severe COVID-19 patients develop altered immune response upon SARS-CoV-2 infection, which associates a systemic inflammatory response and innate and adaptive immune alterations.<sup>1,3</sup> More specifically, T lymphocyte response is profoundly altered in severe COVID-19 patients and the presence of an extensive lymphopenia has repeatedly been described as a prominent feature of critically ill COVID-19 patients.<sup>1,13</sup> COVID-19-induced lymphopenia affect all lymphocyte subpopulations, develop upon ICU admission and persist during ICU stay.<sup>2,14</sup> Decreased circulating numbers of lymphocytes is also associated with functional and phenotypic alterations with many similarities with T lymphocyte alterations described in patients with bacterial sepsis.<sup>3,11,15</sup> Finally, some studies associated the intensity of lymphocyte diminution and deleterious outcome.<sup>16</sup> Antigen-presentation capacity is also altered in critically ill COVID-19 patients as illustrated by the decreased expression of the MHC class II molecule HLA-DR on circulating monocytes.<sup>1,2,9,15</sup> Whether such initial alterations of immune response might persist over time upon ICU discharge and could alter the development of SARS-CoV-2 specific adaptive and humoral immune responses in the most severe survivors critically ill individuals was, so far, unknown.

Observations from the current study confirmed that critically ill COVID-19 patients developed COVID-19-induced immune alterations including increased plasma cytokine concentrations and decreased T, B and NK cell numbers in association with markedly reduced mHLA-DR during ICU stay when compared with normal values. These alterations persisted over the first week after admission with a tendency to improve over time. We completed these data by monitoring these immune parameters in the same patients 9 and 13 months after hospital discharge. We showed that COVID-19 induced immune alterations did not persist in convalescent individuals upon ICU discharge. Thus, COVID-19-induced immune alterations appear as a transitory phenomenon following acute infection. As described in other cohorts of critically ill patients, such initial response may be considered as part of the physiologic host response to stress initiated to control the overwhelming inflammatory response to infection to prevent its deleterious consequences for the patient.<sup>11</sup>

When assessing SARS-CoV-2 specific humoral immune response, we observed a strong anti-SARS-CoV-2 antibody production during ICU the stay despite the parallel presence of immune alterations. This is in agreement with previous studies and suggests that severe disease manifestations are not caused by a lack of humoral anti-SARS-CoV-2 immune response.<sup>17-20</sup>

Upon infection resolution, previous studies in non-severe COVID-19 patients described the persistence of SARS-CoV-2 neutralizing antibodies up to 6 to 8 months in 90% of recovered individuals.<sup>21,22</sup> We showed that all convalescent critically ill patients from



**Figure 4. Monitoring of SARS-CoV-2 specific immune response in patients depending on hospital length of stay.** Sixteen convalescent critically ill COVID-19 patients were sampled twice at follow-up visits (i.e. after 9 (M9,  $n = 16$ , orange symbols and boxes) and 13 months after hospital discharge (M13;  $n = 15$ , red symbols and boxes) and stratified according to length of hospital stay. Results obtained in patients with length of hospital stay inferior to 30 days ( $n = 7$  patients, short, symbols = circles) and in patients with length of hospital stay superior to 30 days ( $n = 9$  patients, Long, symbols = squares) are shown. Fifteen healthy donors either non-infected and non-vaccinated (HV,  $n = 4$ , light grey symbols), or after full vaccination (Vacc,  $n = 4$ , dark grey symbols and boxes) or after resolution of a non-severe SARS-CoV-2 infection (Inf,  $n = 7$ , black symbols and boxes) were concomitantly included. The percentages of T lymphocytes that had proliferated in response to 3 SARS-CoV-2 antigens (Nucleocapsid – Panel a, Membrane – Panel b, Spike – Panel c, expressed as percentages of proliferating T cells among total T cells) and the plasmatic concentrations of anti-SARS-CoV-2 IgG (IgG index – Panel d) were monitored. Results in vaccinated patients are identified by an arrow. Results are presented as individual values and as Tukey Box-plots. Comparison between the two groups of patients at each time point were performed using non-parametric Mann Whitney test. \*  $p < 0.05$  \*\*  $p < 0.01$ .

the current cohort had detectable anti-SARS-CoV-2 antibody titers 9 and 13 months after infection. However, we observed a substantial decline of antibody levels over time. Thus, even in patients with initially elevated anti-SARS-CoV-2 antibody titers, a marked decline in IgG concentrations was observed.

To note, in this study, four patients received at least one vaccine injection before the second follow-up visit. This was associated with a strong humoral response with elevated anti-SARS-CoV-2 IgG levels at M13. This highlights the good serological response to vaccine of these patients because, despite the substantial decline of anti-SARS-CoV-2 levels over time, humoral immune response could be re-stimulated by vaccination in convalescent critically ill COVID-19 patients.

Different read-outs are currently used in the literature to identify SARS-CoV-2 specific T cells, including expression of activation markers, IFN- $\gamma$  production by ELISPOT, IFN- $\gamma$  release assay or intracellular cytokine staining after stimulation with SARS-CoV-2 peptides.<sup>23,24</sup> For example, anti-SARS-CoV-2 specific T cell response was evaluated either through Fluorospot assay (detection of IL-2 and or IFN- $\gamma$  T cells) and through intracellular cytokine staining after stimulation with SARS-CoV-2 peptide pools in cohorts of mild to critical COVID-19 patients.<sup>25,26</sup> Both articles described the persistence of anti-SARS-CoV-2 specific T cells on a long-term basis in recovered COVID-19 patients. These techniques, with short stimulation duration, are highly efficient in monitoring SARS-CoV-2 specific T lymphocyte effector response. However, they may lack sensitivity in detecting the persistence of memory T cells when measured at distance of infection or vaccination.<sup>27</sup> On the contrary, T cell proliferation measured after one week of antigen stimulation is a sensitive technique to evaluate memory T cell responses and has been identified by experts as the reference technique for the follow-up of patients with primary immune deficiencies.<sup>28,29</sup>

Using this technique, we observed that convalescent critically ill COVID-19 patients developed a persistent memory response against SARS-CoV-2 when sampled after 9 and 13 months. This completes the data from the literature, that reported detectable SARS-CoV-2 specific T cells upon disease resolution in convalescent donors.<sup>30-33</sup> However, previous studies mostly included patients after non-severe SARS-CoV-2 infections and none used T cell proliferation to evaluate memory T cell response. Thus, to our knowledge, this is the first report of the persistence of a strong memory T cell response after more than one year in convalescent critically ill COVID-19 patients using a T cell proliferation assay. Both CD4+ and CD8+ T cells specific responses to SARS-CoV-2 were detected and responses against SARS-CoV-2 antigens were correlated showing that a broad immune response towards SARS-CoV-2 was generated.

In the present cohort, SARS-CoV-2-specific T cells could be detected up to 13 months after initial infection

and no decline was observed between results obtained after 9 and 13 months. At these late time-points, when focusing on anti-SARS-CoV-2 specific immune response, no correlations were observed between antigen-specific T cell response and anti-SARS-CoV-2 IgG levels and no increase in SARS-CoV-2 specific T cell response was observed in vaccinated patients. Thus, while anti-SARS-CoV-2 antibody levels seem to decay upon disease resolution,<sup>34-36</sup> SARS-CoV-2-specific memory T cells persist for longer time in convalescent individuals. In the present context of consecutive COVID-19 waves, the capacity of this persistent memory T cell response to prevent reinfection should be investigated.

In convalescent individuals, T cell proliferation to SARS-CoV-2 nucleocapsid was correlated with the number of circulating CD8+ T cells. This is not unexpected as cytotoxic CD8+ T lymphocytes are central to the antiviral effector response.<sup>37</sup> A significant correlation was also observed with plasma TNF- $\alpha$  levels. While TNF- $\alpha$  can have divergent effects on T cell subpopulation functions and survival<sup>37</sup> and as this cytokine is the sole to maintain subnormal levels in convalescent individuals, deciphering its role in COVID-19 pathophysiology may be of major importance.

We observed that patients with complicated clinical courses leading to long hospital stays developed significantly better memory T cell response than patients with short clinical courses. The intensity of memory T cell response did not correlate with acute COVID-19 severity as evaluated by severity score at ICU admission (SAPS II, SOFA). However, patients with long HLS tended to present with more important COVID-19 induced immune alterations upon ICU admission and higher plasmatic antigenemia and viremia. Accordingly; in the study by Vibholm et al., the magnitude of CD8+ T cell response correlated with SARS-CoV-2 copies per swab.<sup>38</sup> They also showed that persistently positive individuals had increased breath and magnitude of CD8+ specific response compared to PCR negative individuals. This is in accordance with our data, as patients with long hospital stays presented with higher and more persistent antigenemia and more frequently detectable SARS-CoV-2 mRNA in plasma compared with patients with uncomplicated clinical courses. This suggests that viral persistence resulted in an ongoing immune stimulation. Prolonged disease course and consequent larger exposure to virus experienced in hospitalized patients may provide a timeframe in which enhanced specific adaptive immune cells stimulation and maturation take place compared to shorter course and mild infections.

In addition, patients with better T cell response and long hospital stays presented with deep and persistent inflammation during the first week after admission; in addition with profound lymphopenia and decreased MHC class II expression. In this context, deciphering the mechanisms through which initial immune

response to acute infection may influence the development of specific T cell response might be important to delineate the beneficial versus deleterious effects of this initial response to stress.

Interestingly, no differences were observed in humoral immune response to SARS-CoV-2 in patients with long and short hospital stays. This suggests that the mechanisms leading to humoral *versus* adaptive memory immune responses might differ in critically ill patients. In particular, while T follicular helpers might be fully efficient in generating SARS-CoV-2 specific B cells and neutralizing antibodies; immediate cytotoxic CD8+ T lymphocyte response might be insufficient to clear the virus. In the absence of effective antiviral therapies, immunoadjuvant treatments targeting such T cell alterations might represent innovative therapies in the most severe COVID-19 patients.<sup>39–43</sup>

A first limitation of this work is the limited number of patients included. This is especially true for the analysis of patients by subgroups depending on HLS and for adjustment for potential relevant clinical confounding factors. In addition, because of the low number of patients in each group, statistical testing could only detect the most profound differences. Finally, because they left the ICU, patients with short HLS could not be monitored overtime during their ICU stays. Thus, results from this purely observational study remain preliminary and should be confirmed prospectively on a larger cohort of patients. In line, the relatively small size of the cohort did not allow us to identify patients' characteristics that would predict the maintenance of the anti-SARS-CoV-2 immune memory. In addition, as we restricted our cohort to individuals who recovered from severe COVID-19, these data did not describe the immune memory response after asymptomatic or mild COVID-19. Finally, protective efficacy of the anti-SARS-CoV-2 immune response analyzed in our study remains unclear as the exact magnitude of adaptive immune responses to SARS-CoV-2 infection required for protection from reinfection is not known so far. However, as recent longitudinal studies on larger cohorts suggest that the presence of cellular and humoral immunity was highly associated with prevention from reinfection with SARS-CoV-2,<sup>44,45</sup> we can extrapolate that convalescent critically ill COVID-19 patients may have a strong protective humoral and adaptive immunity against SARS-CoV-2 reinfection. Again, this should be confirmed in a larger dedicated study.

In conclusion, results of the present study showed that, despite the presence of major lymphopenia and decrease in MHC class II expression on monocytes during ICU stay, convalescent critically ill COVID-19 patients consistently generated a good memory immune response against SARS-CoV-2, associating adaptive and humoral responses. This response was maintained for more than one year after hospital discharge. Patients with long hospital length of stay

presented with stronger anti-SARS-CoV-2 specific T cell response but no difference in anti-SARS-CoV-2 IgG levels.

#### Contributors

FV, MG, GM, FB, RC, RP, LG, MB and MO were involved in the design, implementation, and day-to-day management of the study. ACL, TR, LA, MC, MSE, LH, PV included participants in the study. MBD, FMS, JSC were responsible for the virological analyses. FV, MG, RC, FB, CT, KBP, VC, MAC, MO, RP and LG were responsible for the immunological analyses. MB, FB and FV were involved in the statistical analyses. FB, GM and FV wrote the original draft of the manuscript, which was reviewed and edited by MG, RC, ACL, RP, TR, MSE, LH and PV. All authors have read and approved the manuscript. All authors had full access to all the data and accept responsibility for the decision to submit for publication.

#### Data sharing statement

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

#### Declaration of interests

MB, CT, KBG, VC and MAC are bioMérieux's employees. This private company had no role in the study design, result analysis and decision to publish this study. PV received consulting fees and payment for a literature review from Pfizer and Astellas. All other authors have declared no conflicts of interest.

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## Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.103967.

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## **Annexe 8**



► L'inflammasome NLRP3 est un complexe multi-protéique intracellulaire impliqué dans la réponse immunitaire innée. Après la détection de signaux de dangers, tels que ceux provenant d'agents pathogènes, ce complexe s'assemble afin d'initier la production et la sécrétion de molécules pro-inflammatoires, comme l'IL(interleukine)-1 $\beta$  et l'IL-18. L'inflammasome NLRP3 régule aussi l'activation de la gasdermine D, une protéine impliquée dans la mort cellulaire inflammatoire, ou pyroptose. Cette revue s'intéresse à l'activation et aux rôles de l'inflammasome NLRP3 dans les infections virales et plus particulièrement dans le cas de l'infection par le SARS-CoV-2. Une attention particulière est portée dans cette revue aux traitements évalués, ou en cours d'évaluation, ciblant la voie de l'inflammasome NLRP3 activée au cours de la COVID-19. ◀

Le SARS-CoV-2 (*severe acute respiratory syndrome coronavirus 2*) est un virus de la famille des coronavirus (CoV) humains qui a émergé en République populaire de Chine à la fin de l'année 2019. Il est responsable de la pandémie de COVID-19 (*coronavirus disease-2019*) et de plus de cinq millions de décès à ce jour (données de l'Organisation mondiale de la santé [OMS] au 1<sup>er</sup> janvier 2022)<sup>1</sup>. Le SARS-CoV-2 est un virus enveloppé à ARN simple brin appartenant à la famille des *betacoronavirus*. Son génome code plusieurs protéines non structurales qui permettent la réplication virale et quatre protéines structurales parmi lesquelles la protéine Spike. Cette dernière est activée sous l'action de protéases cellulaires et se lie au récepteur membranaire ACE2 (*angiotensin-converting enzyme 2*). Cela permet au virus d'entrer dans les cellules cibles et de répliquer son génome afin de créer de nouveaux virions qui seront libérés par exocytose [1].

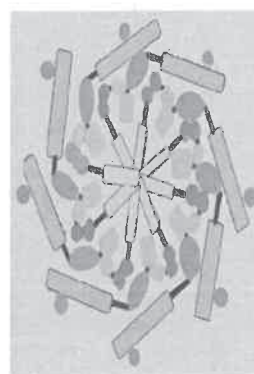
Vignette (© Thierry Jouault).

<sup>1</sup> D'après de nouvelles estimations de l'Organisation mondiale de la santé (OMS) (communiqué de presse de l'OMS du 5 mai 2022), le nombre total de décès associés directement ou indirectement à la pandémie de COVID-19 (la « surmortalité ») entre le 1<sup>er</sup> janvier 2020 et le 31 décembre 2021 était d'environ 14,9 millions (fourchette de 13,3 millions à 16,6 millions).

# L'inflammasome NLRP3 dans la physiopathologie des infections virales

## Un focus sur la COVID-19

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Les cellules des voies respiratoires supérieures constituent les cibles privilégiées du SARS-CoV-2 car elles expriment fortement ACE2. Le SARS-CoV-2 se transmet essentiellement par les gouttelettes chargées de virions émises lors d'expectorations

ou d'échanges verbaux. Parmi les sujets infectés, 80 % présentent une forme asymptomatique, légère ou modérée de la maladie, à type de syndrome grippal spontanément résolutif en six à dix jours ; 15 % développeront une forme sévère de la maladie, caractérisée par une pneumonie interstitielle atypique bilatérale nécessitant un recours à l'oxygénothérapie ; enfin, 5 % développeront une forme critique de la maladie avec, notamment, un syndrome de détresse respiratoire aiguë (SDRA), un choc septique et une défaillance multiviscérale [2].

La réponse de l'hôte face au virus joue un rôle central dans la physiopathologie de la COVID-19 [2]. En particulier, l'activation de l'inflammasome NLRP3 (*nucleotide-binding domain leucine-rich repeat [LRR] and pyrin-containing receptor 3*, nommée également cryopyrine, CIAS1 [*cold autoinflammatory syndrome 1 protein*] ou NALP3 [*NACHT, LRR and PYD domains-containing protein 3*]) pourrait participer à la phase initiale de la réponse immunitaire innée mise en place au cours de la COVID-19, particulièrement intense chez certains patients. Cette voie de signalisation pourrait ainsi représenter une cible thérapeutique innovante dans les formes les plus sévères de la maladie.

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## Physiologie et physiopathologie de l'inflammasome NLRP3

### Physiologie de l'inflammasome NLRP3

NLRP3 est une protéine de 115 kDa constituée d'un domaine LRR (*leucine-rich repeat*) C-terminal, d'un domaine central d'oligomérisation (NOD, *nucleotide-binding and oligomerization domain* ou NACHT) qui présente une activité ATPase, et d'un domaine N-terminal effecteur PYD (*pyrin domain*) capable de recruter l'adaptateur ASC (*apoptosis-associated speck-like protein containing a CARD* [*caspase recruitment domain*]). NLRP3 est un senseur cytosolique de stress qui enclenche une voie de signalisation pro-inflammatoire prenant part à la réponse immunitaire innée, première ligne de défense contre les infections. Suite à son activation (voir plus loin), NLRP3 assemble un complexe de signalisation multiprotéique dans le cytosol. Ce complexe a été appelé inflammasome en raison de son interaction avec la protéine NEK7 (*NIMA-related kinase 7*) puis du recrutement de l'adaptateur ASC via son domaine PYD, lui-même recrutant, via son domaine CARD, la caspase-1 (Figure 1). Dans le cytoplasme, l'inflammasome se concentre en une ultrastructure micrométrique, nommée *speck* (ou structure ponctiforme), visible en microscopie [3] (→).

L'inflammasome constitue une plateforme de transactivation pour la caspase-1. C'est cette caspase qui contrôle la maturation, par clivage protéolytique, des interleukines pro-inflammatoires IL-1 $\beta$  et IL-18, qui sont produites sous forme de précurseurs dans le cytosol à la suite de la stimulation de la cellule (Figure 1). La caspase-1 clive également la gasdermine D (GSDMD), une protéine cytosolique dont le domaine N-terminal, alors libéré, s'insère dans la membrane cellulaire sous forme d'oligomères, formant des pores, ce qui permet d'une part la libération de l'IL-1 $\beta$  et de l'IL-18 matures, et, d'autre part, conduit à la pyroptose, une forme de mort cellulaire pro-inflammatoire accompagnée de la libération de nombreuses alarmines [3].

La machinerie conduisant à l'activation de l'inflammasome est complexe [4] (→). Deux signaux sont nécessaires. Le premier signal, nommé *priming* (ou amorçage) est déclenché par la liaison de composants microbiens, les MAMP (*microbial-associated molecular patterns*), ou endogènes, les DAMP (*damage-associated molecular patterns*) à leurs récepteurs dédiés, les PRR (*pattern recognition receptors*). Le signal d'amorçage permet d'augmenter la concentration cellulaire de composants et de substrats de l'inflammasome, en particulier NLRP3 et la pro-IL-1 $\beta$ , dont la transcription est induite notamment par l'activation du facteur de transcription NF- $\kappa$ B (*nuclear factor kappa-B*), induite, entre autres, par les produits microbiens. Ce signal d'amorçage permet également de rendre NLRP3 compétent pour son activation, via des modifications post-traductionnelles [3]. Le second signal, ou signal d'activation, induit l'assemblage de l'inflammasome. Il correspond à l'intégration en parallèle de signaux produits lors d'un stress cellulaire [3].

(→) Voir la Synthèse de M. Gros Lambert, m/s n° 1, janvier 2018, page 47

(→) Voir la Synthèse de Y. Jamilloux, m/s n° 11, novembre 2013, page 975

### Mécanismes d'activation de l'inflammasome NLRP3 lors d'une infection virale

Lors des infections virales, l'inflammasome NLRP3 est activé. Le signal de *priming* repose alors sur la reconnaissance des acides nucléiques ou des protéines du virus par les récepteurs de l'immunité innée, les *Toll-like receptors* (TLR), qui stimulent la voie de signalisation aboutissant à l'activation de NF- $\kappa$ B [3]. Le signal d'activation, secondaire, sera, quant à lui, induit par une rupture lysosomale, qui provoque le relargage de cathepsine B dans le cytosol, par la production d'espèces réactives de l'oxygène (*reactive oxygen species*, ROS), par la détection du génome viral via l'hélicase DHX33 (*DEAH-box helicase 33*) ou la ribonucléase L (RnaseL, L pour latent), ou par des déséquilibres ioniques dus à l'activité de canaux ioniques, tels que P2X7 (*purinergic receptor P2X7*), ou de canaux formés par les viroporines virales<sup>2</sup> [5].

Pour l'infection par le virus de la grippe *Influenza A*, par exemple, le *priming* de l'inflammasome NLRP3 est induit par la reconnaissance de l'ARN viral par le TLR7. La viroporine M2 du virus crée ensuite un canal à protons au sein de l'appareil de Golgi, ce qui constitue le second signal, avec la libération de ROS par acidification du cytoplasme, activant ainsi l'inflammasome [5].

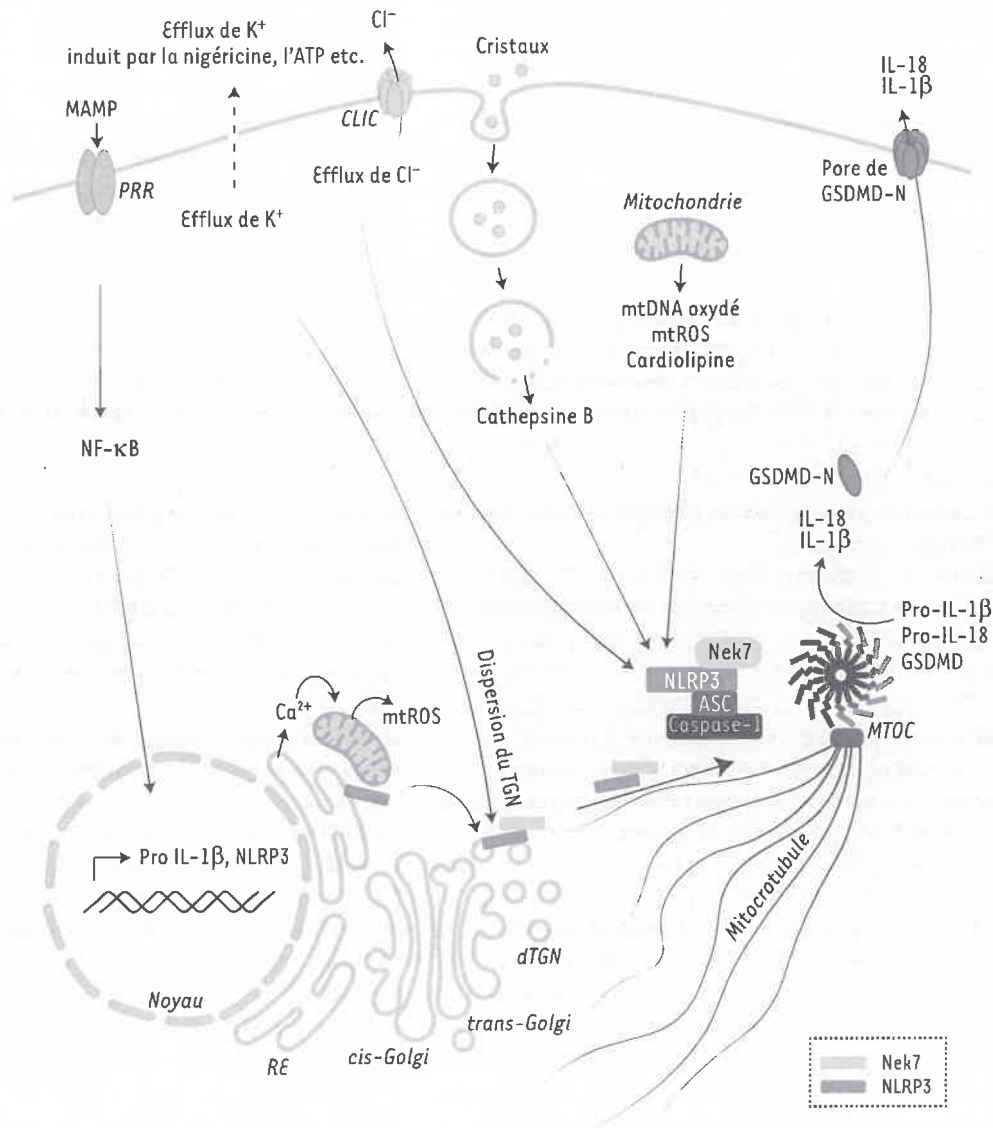
L'activation de l'inflammasome NLRP3 et la mise en place d'une réponse immunitaire pro-inflammatoire peuvent être bénéfiques ou délétères selon le stade de l'infection. Ainsi, dans un modèle murin d'infection par le virus *Influenza A*, l'inhibition tardive de l'inflammasome NLRP3 par un inhibiteur spécifique, le MCC950, empêche l'emballement du système immunitaire et est associée à une diminution de l'inflammation pulmonaire, à une diminution de la perte de poids et à une augmentation de la survie des animaux. *A contrario*, son inhibition précoce augmente la perte de poids et la mortalité des animaux infectés [6].

### Rôle de l'inflammasome NLRP3 dans la physiopathologie de la COVID-19

#### Les facteurs de risque de la COVID-19 sévère liés à l'inflammasome NLRP3

Plusieurs facteurs de risque associés aux formes les plus sévères de COVID-19 liés à l'activation de l'inflammasome NLRP3 ont été identifiés, dont le vieillissement, le diabète, l'obésité, ou l'hypertension [7]. Dans ces contextes cliniques, une activation chronique de l'inflammasome NLRP3 a en effet été décrite [7]. Par

<sup>2</sup> Petites protéines virales qui modifient les membranes plasmiques et facilitent la libération des virions par les cellules infectées.



**Figure 1. Mécanismes d'activation de l'inflammasome NLRP3.** L'activation de l'inflammasome NLRP3 requiert deux étapes. Le premier signal, ou signal de *priming*, induit l'activation de la voie NF- $\kappa$ B (nuclear factor kappa-B) et permet la transcription de NLRP3 ainsi que celle d'autres gènes codant les proformes des cytokines pro-inflammatoires (pro-IL-1 $\beta$  et pro-IL-18). Ce *priming* est déclenché par la liaison de composants microbiens MAMP (microbial-associated molecular patterns), ou endogènes DAMP (damage-associated molecular patterns) à leurs récepteurs, les PRR (pattern recognition receptors). Le second signal, ou signal d'activation, induit l'assemblage de l'inflammasome. Il correspond à l'intégration de signaux de stress cellulaires comme, par exemple, l'efflux de potassium (entraînant une perturbation du TGN [trans-Golgi network]), l'exposition à une toxine microbienne, à des cristaux, une rupture des lysosomes, ou encore un dysfonctionnement mitochondrial. Régulé par NEK7, NLRP3 s'oligomérisé et recrute la protéine adaptatrice ASC qui permet à son tour le recrutement et le clivage de la pro-caspase-1 en caspase-1. L'inflammasome ainsi formé apparaît dans la cellule sous la forme d'une structure ponctiforme appelée *speck*. La caspase-1 activée clive la gasdermine D (GSDMD) et libère son domaine N-terminal actif (GSDMD-N), et clive les pro-IL-1 $\beta$  et pro-IL-18 en leurs formes actives IL-1 $\beta$  et IL-18. La GSDMD-N va ensuite s'insérer dans la membrane plasmique pour, d'une part, permettre la sécrétion d'IL-1 $\beta$  et d'IL-18, et, d'autre part, induire la mort cellulaire inflammatoire de la cellule par pyroptose. NEK7 : NIMA-related kinase 7 ; ASC : apoptosis-associated speck-like protein containing a CARD domain ; ATP : adénosine triphosphate ; dTGN : dispersed trans-Golgi network ; IL-1 $\beta$  : interleukine 1 $\beta$  ; IL-18 : interleukine 18 ; mtDNA : ADN mitochondrial ; MTOC : microtubule-organizing center ; mtROS : mitochondrial reactive oxygen species ; NLRP3 : nucleotide-binding, LRR and PYD domains-containing protein 3 ; RE : réticulum endoplasmique ; ROS : reactive oxygen species.

exemple, chez le sujet diabétique ou obèse, l'acide urique, les cristaux de cholestérol ou l'acide palmitique sont à l'origine d'une inflammation chronique de bas grade à laquelle participe l'inflammasome NLRP3. Cette activation chronique provoque, entre autres, une fibrose pulmonaire et des cardiomyopathies qui pourraient rendre les individus plus vulnérables face aux atteintes cardio-respiratoires associées à la COVID-19.

#### Les mécanismes d'activation de l'inflammasome NLRP3 au cours de la COVID-19

La capacité des protéines de capside (protéines N) du SARS-CoV-2 à activer l'inflammasome NLRP3 a été initialement démontrée chez la souris [8], puis chez l'homme, en utilisant des monocytes sanguins isolés de volontaires sains [9, 10]. L'activation des voies de l'inflammasome NLRP3 a également été observée *ex vivo*, dans les monocytes circulants de patients atteints de COVID-19 [9, 10] de même qu'*in situ* par l'analyse de coupes histologiques de poumons réalisées chez des patients décédés de la maladie [9].

Plusieurs études se sont attachées à identifier les différents signaux participant au *priming* et à l'activation de l'inflammasome NLRP3 lors de l'infection par le SARS-CoV-2. En particulier, certaines protéines du virus pourraient jouer un rôle direct dans l'activation de l'inflammasome (Figure 2). Par exemple, une séquence de l'ARN codant la protéine Spike, riche en guanine et uracile (GU), est reconnue par le TLR8 qu'expriment les macrophages humains. Cette reconnaissance, à l'origine de la transcription de la pro-IL-1 $\beta$  et de la protéine NLRP3, représente donc un premier signal. La liaison de la protéine Spike au TLR8 induit en outre une activation alternative de l'inflammasome NLRP3 qui se traduit par l'activation de la caspase-8 [11, 12]. La protéine E du SARS-CoV, une viroporine à l'origine d'un efflux de calcium du ERGIC (*ER-Golgi intermediate compartment*), a été associée à des concentrations élevées d'IL-1 $\beta$  dans le parenchyme pulmonaire [13]. De même, l'ORF8b du génome viral, ou protéine 8b, peut également activer l'inflammasome NLRP3 en interagissant directement avec son domaine LRR. L'ORF8b induit aussi un stress du réticulum endoplasmique, connu pour être un activateur de l'inflammasome NLRP3 [14]. Une autre viroporine virale, la viroporine 3a codée par l'ORF3a, est, quant à elle, à l'origine d'un efflux de potassium dans la cellule qui pourrait être responsable de l'activation de l'inflammasome NLRP3 [15, 16]. La protéine virale N (ou nucléocapside) participerait également à l'activation de l'inflammasome en interagissant directement avec la protéine NLRP3 [8], ce qui conduirait à l'assemblage de l'inflammasome et à la sécrétion de l'IL-1 $\beta$  [8]. Enfin, la liaison de la protéine Spike à ACE2 et au TLR4 permettrait de générer le second signal d'activation [17]. En parallèle de ses capacités à activer l'inflammasome, le SARS-CoV-2 serait capable d'inhiber la pyroptose, qui dépend de l'inflammasome activé. La protéine N du virus se lie en effet directement à la GSDMD, empêchant ainsi son clivage par la caspase-1 et son activation [18]. Ainsi, le SARS-CoV-2 pourrait jouer un rôle dans la sévérité de la COVID-19 en interférant avec l'inflammasome NLRP3.

#### Conséquences cellulaires et cliniques de l'activation de l'inflammasome NLRP3 au cours de la COVID-19

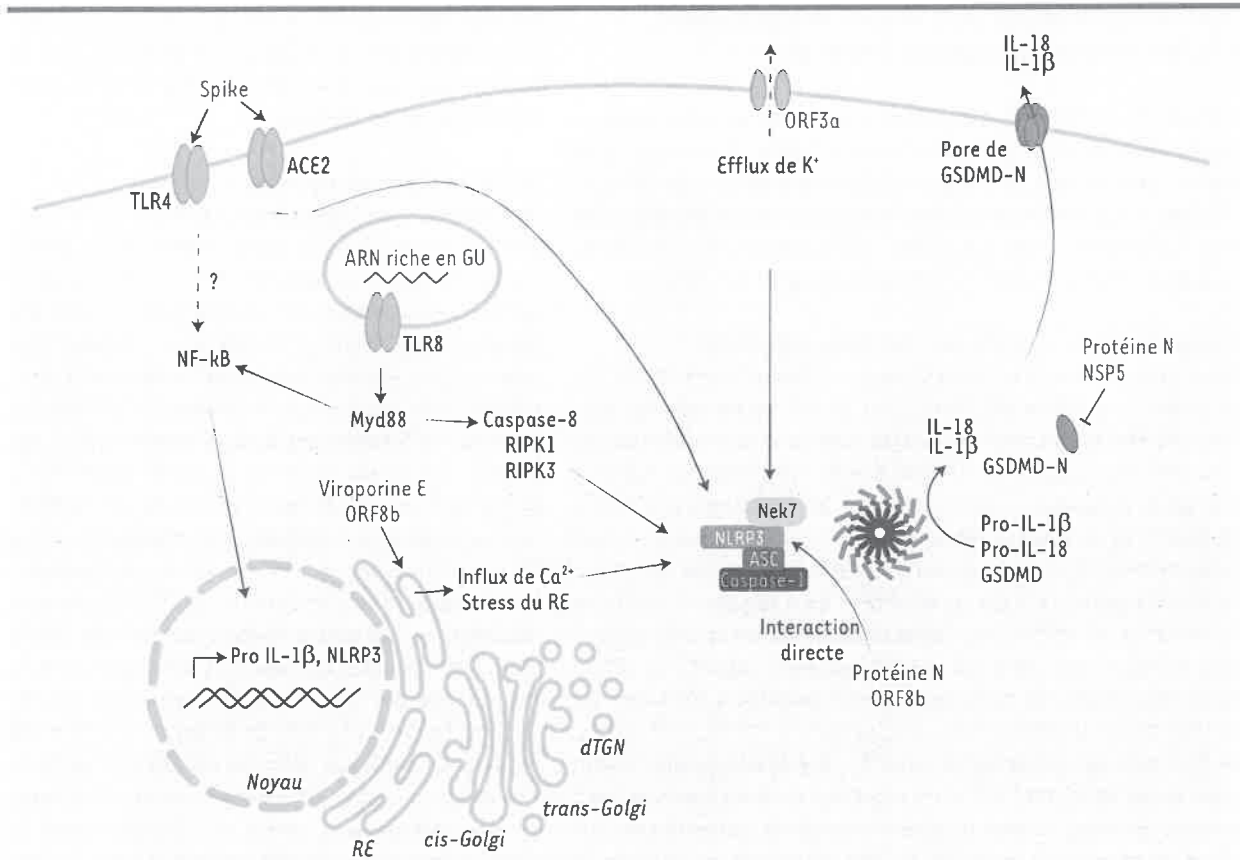
Au cours d'infections, la suractivation de l'inflammasome NLRP3 pourrait être à l'origine d'une hyperproduction des cytokines pro-inflammatoires, IL-1 $\beta$  et IL-18, ce qui conduirait, *in fine*, à l'orage cytokinique observé chez les patients atteints de COVID-19 qui nécessitent alors une prise en charge en réanimation [19]. Chez les patients les plus gravement atteints, plusieurs études ont décrit cette augmentation de concentrations de cytokines pro-inflammatoires (IL-1 $\beta$ , IL-18, IL-1RA [*interleukin-1 receptor antagonist*], IL-18Bpa [*interleukin 18-binding protein*], IL-6, IL-2R, IL-8, IL-10, TNF- $\alpha$  [*tumor necrosis factor alpha*], CXCL10 [*C-X-C motif chemokine ligand 10*], etc.) [20-23].

Localement, au niveau pulmonaire, l'IL-1 $\beta$  permettrait le recrutement de polynucléaires neutrophiles, des cellules capables de produire des pièges extracellulaires, les NET (*neutrophil extracellular traps*), constitués, entre autres, de leur ADN et d'histones. Ces pièges pourraient créer des dommages endothéliaux et épithéliaux au niveau du parenchyme pulmonaire, des facteurs de risque de défaillance pulmonaire aiguë et de syndrome de détresse respiratoire aiguë [24].

La protéine ACE2 est exprimée par un large éventail de types cellulaires. Le SARS-CoV-2 pourrait ainsi être à l'origine de dommages liés à l'activation de l'inflammasome NLRP3 dans d'autres organes : au niveau cardiaque, avec des myocardites et une diminution de la fraction d'éjection [25] ou au niveau neurologique. Des hypothèses sur l'implication de l'activation de l'inflammasome NLRP3 dans les encéphalites, les céphalées, la confusion en lien avec la COVID-19 ont en effet été émises [15, 26]. Enfin, dans les cas les plus graves, l'inflammation générale, accentuée par l'expression quasi ubiquitaire d'ACE2, pourrait conduire à des défaillances multiviscérales.

#### Des biomarqueurs liés à l'activation de l'inflammasome NLRP3

L'identification de biomarqueurs en lien avec l'activation de la voie de l'inflammasome NLRP3 pourrait conditionner la prise en charge et le devenir des patients souffrant de COVID-19. Une corrélation a en effet été observée entre des concentrations plasmatiques élevées d'IL-18, à l'admission des patients, et la sévérité de la maladie : les patients présentant les concentrations les plus importantes présentent un risque significativement augmenté de décès [9].



**Figure 2. Mécanismes d'activation et de régulation de l'inflammasome NLRP3 par les protéines du SARS-CoV-2.** La protéine Spike permet de générer, d'une part, le signal de priming en se fixant au TLR4 (Toll-like receptor 4) et, d'autre part, le signal d'activation en se liant à la fois au TLR4 et à son récepteur ACE2 (angiotensin-converting enzyme 2) pour permettre la formation de l'inflammasome NLRP3 (nucleotide-binding, LRR and PYD domains-containing protein 3). La séquence riche en GU (guanine, uracile) de l'ARN codant la protéine Spike est reconnue par le TLR8 (Toll-like receptor 8), et induit la transcription des gènes impliqués dans la signalisation de l'inflammasome NLRP3 en activant la voie de signalisation Myd88 (myeloid differentiation primary response 88). L'activation du TLR8 induit aussi une activation alternative de l'inflammasome NLRP3, impliquant la caspase-8, RIPK (receptor-interacting serine/threonine-protein kinase) 1 et RIPK3. La viroporine E et la protéine 8b du virus interagissent avec le réticulum endoplasmique (RE) et provoquent, respectivement, un efflux de calcium du RE et un stress. Comme la protéine N, la protéine 8b peut aussi interagir directement avec NLRP3 et l'activer. La viroporine 3a provoque un efflux de potassium (K<sup>+</sup>) dans la cellule responsable de l'activation de l'inflammasome NLRP3. Deux protéines du SARS-CoV-2 modulent négativement l'activation de NLRP3 : la protéine N, qui se lie à la gasdermine D (GSDMD), empêchant son activation, et NSP5 (nonstructural protein 5) qui clive la GSDMD, inhibant son ancrage dans la membrane plasmique. Les composants du SARS-CoV-2 sont indiqués en rouge. ASC : apoptosis-associated speck-like protein containing a CARD domain ; GSDMD-N : domaine N-terminal de la gasdermine D ; IL-1β : interleukine 1β ; IL-18 : interleukine 18 ; NF-κB : nuclear factor kappa-B. Pro-IL-1β : précurseur de l'interleukine 1β.

La diminution de la proportion de monocytes non classiques<sup>3</sup> dans le sang et la perte d'activation de la caspase-1 en réponse à un activateur de NLRP3, la nigéricine, dans les polynucléaires neutrophiles immatures, ont également été décrites comme des biomarqueurs associés à la sévérité et à l'évolution péjorative de la COVID-19 [23]. L'hypothèse que la suractivation de NLRP3 pourrait provenir d'un défaut de ses mécanismes

de régulation négative a été proposée [27]. Des études ont ainsi mis en évidence une régulation croisée entre les interférons de type I et l'IL-1β [28] et un défaut de production d'interférons de type I et III a été relevé dans les formes sévères de COVID-19. Cette dérégulation pourrait participer à l'excès de production d'IL-1β dans ces formes graves de la maladie [29]. Le ciblage des composants, des effecteurs, ou encore des régulateurs de l'inflammasome NLRP3, apparaît donc représenter des pistes thérapeutiques intéressantes contre la COVID-19.

<sup>3</sup> Les différentes populations de monocytes humains sont classées en fonction de l'expression de molécules de surface : CD14, le récepteur du LPS (lypopolysaccharide) et CD16 (récepteur de type IIIa de la région Fc des IgG, RFcγIIIa). Trois populations ont ainsi été identifiées : les monocytes classiques CD14<sup>+</sup> CD16<sup>neg</sup>, les monocytes non classiques CD14<sup>dim</sup> CD16<sup>+</sup> et les monocytes intermédiaires CD14<sup>+</sup> CD16<sup>+</sup>.



## L'inflammasome NLRP3 et sa cascade de signalisation : une cible thérapeutique dans la COVID-19 ?

L'efficacité de différents traitements qui ciblent des événements en amont de l'activation de l'inflammasome NLRP3, ou en aval, a été évaluée pour la prise en charge des patients souffrant de COVID-19 (Tableau 1). Ces traitements déjà utilisés ou en cours de développement pour le traitement d'autres maladies inflammatoires, ont en effet été repositionnés dans le cadre de la prise en charge de la COVID-19.

### En amont, cibler l'activation de l'inflammasome NLRP3

Il est possible d'inhiber l'activation de l'inflammasome NLRP3 soit en ciblant la protéine elle-même, soit en interférant avec des événements nécessaires à son activation, comme la polymérisation des microtubules, ou avec des conséquences de son activation, comme le clivage de la caspase 1. Deux inhibiteurs de l'inflammasome NLRP3, le DFV890 et le dapansutrile, qui sont en cours de développement respectivement pour le traitement de l'urticaire familial au froid et la crise de goutte, ont été repositionnés dans la prise en charge de la COVID-19. Le DFV890 fait l'objet d'une étude clinique de phase II afin d'évaluer son efficacité chez des patients atteints de défaillance respiratoire liée à la pneumopathie associée à l'infection par le SARS-CoV-2 (NCT04382053). Le dapansutrile (ou OLT1177) qui est un inhibiteur de l'inflammasome NLRP3, a été développé pour réduire l'activation de NF- $\kappa$ B [30]. Il est actuellement évalué dans un essai clinique de phase II dans la prise en charge de patients souffrant d'une forme modérée de COVID-19 mais présentant des facteurs de risque de développer des formes sévères (NCT04540120). L'effet de la colchicine a également été examiné. Il s'agit d'une molécule qui est notamment utilisée dans le traitement des crises de goutte, une maladie fortement dépendante de NLRP3. La colchicine inhibe la polymérisation des microtubules, nécessaire notamment à l'activation des inflammasomes auxquels participent la pyrine ou NLRP3 [31], mais également indispensable pour le recrutement des cellules inflammatoires. Deux essais randomisés réalisés avec des patients atteints de COVID-19 ont conclu à une diminution significative des hospitalisations et des décès [32], mais aussi à la prévention de la dégradation clinique des patients qui avaient été traités [33]. En revanche, une étude menée par le consortium RECOVERY n'a pas observé de différence en ce qui concerne la mortalité, ou la durée de l'hospitalisation, ou le recours à la ventilation mécanique, dans le groupe de patients hospitalisés et traités par la colchicine en comparaison du groupe de patients qui n'avaient pas été traités mais avaient reçu un placebo [34].

Une façon d'inhiber l'action des inflammasomes est de cibler les caspases responsables de la maturation des cytokines inflammatoires IL-1 $\beta$  et IL-18. L'emricasan est un inhibiteur pan-caspases (il inhibe toutes les caspases). Il a été initialement évalué pour la prise en charge de patients atteints de stéatose hépatique non alcoolique. Une étude clinique de phase I est en cours afin de déterminer la tolérance de cet inhibiteur dans la prise en charge des patients atteints de COVID-19 légère (NCT04803227) ; les laboratoires *Histogen* et *Ame-*

*immune*, en charge de l'étude, ont annoncé la résolution complète des symptômes de la COVID-19, chez 13 patients souffrant d'une forme légère de la maladie, traités avec cette molécule.

### Cibler la cascade de signalisation en aval de l'activation de l'inflammasome NLRP3

L'activation de l'inflammasome NLRP3 a pour conséquence la libération d'IL-1 $\beta$ , d'IL-18 mais aussi de nombreuses alarmines qui résultent de la mort par pyroptose des cellules. Ces médiateurs peuvent être ciblés individuellement. Une étude randomisée a montré que l'administration du canakinumab, un anticorps monoclonal recombinant humain anti-IL-1 $\beta$ , à des patients présentant une forme sévère de COVID-19, ne permettait pas d'améliorer la survie de ces derniers, comparativement à l'administration d'un placebo [35]. D'autres études ont montré néanmoins une amélioration de la fonction respiratoire [36, 37] et une augmentation de la survie à 60 jours des patients traités [36]. Ces résultats contradictoires peuvent être la conséquence des effectifs limités inclus dans les différentes études, ou résulter de différences de sévérités ou de prise en charge entre les cohortes de patients. L'anakinra, une forme recombinante de l'IL-1RA, antagoniste naturel du récepteur de l'IL-1 $\beta$ , permet de cibler indirectement l'IL-1 $\beta$  en inhibant par compétition sa fixation à son récepteur. Un essai clinique randomisé évaluant l'utilisation d'anakinra chez des patients présentant une forme modérée de COVID-19 n'a pas démontré d'effet sur l'aggravation clinique ou la mortalité des patients [38]. Le consortium CORIMUNO à l'origine de cet essai clinique évalue actuellement l'utilisation de l'anakinra chez des patients hospitalisés en réanimation (NCT04341584).

L'activation de l'inflammasome NLRP3 est largement associée à la pyroptose, une mort cellulaire hautement inflammatoire faisant intervenir la gasdermine D (GSDMD). Le disulfirame, un inhibiteur de l'ancrage de la GSDMD à la membrane et donc de la formation des pores qui en résultent, fait l'objet de deux études cliniques randomisées. Ces dernières visent à évaluer l'efficacité du disulfirame à prévenir la complication des formes modérées de la maladie (NCT04485130 et NCT04594343). Le diméthylfumarate (DMF), utilisé dans le traitement de la sclérose en plaques récurrente-rémittente, inhibe directement l'activation de la GSDMD par succinylation de ses cystéines [39]. Le consortium RECOVERY, piloté par l'université d'Oxford (Royaume-Uni), évalue actuellement l'utilisation de DMF dans la prise en charge des patients hospitalisés atteints de COVID-19 (NCT04381936).



Traitement	Cible	Mécanisme	Effet sur la COVID-19 modérée	Effet sur la COVID-19 sévère
<b>Inhibition de l'activation de l'inflammasome NLRP3</b>				
<b>Colchicine</b>	Inhibe la polymérisation des microtubules	Inhibition des inflammasomes NLRP3 et pyrine	Diminution des hospitalisations, des décès et du recours à la ventilation mécanique	Diminution des décès et du recours à la ventilation mécanique
<b>Emricasan</b>	Inhibiteur pan-caspase	Inhibition de la caspase-1	<i>En cours</i>	<i>En cours</i>
<b>DFV890</b>	NLRP3	Inhibition de NLRP3	<i>En cours</i>	<i>En cours</i>
<b>Dapansutrile</b>	NLRP3	Inhibition de NLRP3	<i>En cours</i>	<i>En cours</i>
<b>Inhibition de la cascade de signalisation en aval de l'inflammasome NLRP3</b>				
<b>Canakinumab</b> Anticorps monoclonal	IL-1 $\beta$	Inhibition de l'IL-1 $\beta$	/	Pas d'amélioration
<b>Anakinra</b> IL-1RA recombinant	IL-1 $\beta$	Inhibition de la fixation de l'IL-1 $\beta$ à son récepteur	Pas d'amélioration	Diminution de la sévérité, de la mortalité et de la durée de l'hospitalisation
<b>Disulfirame</b>	Inhibition de l'ancrage membranaire de la gasdermine D	Inhibition de la pyroptose	<i>En cours</i>	<i>En cours</i>
<b>Diméthylfumarate</b>	Inhibition de la gasdermine D	Inhibition de la pyroptose	<i>En cours</i>	<i>En cours</i>

**Tableau 1. Traitements ciblant la voie de l'inflammasome NLRP3 en cours d'évaluation dans la COVID-19.** Seuls les essais randomisés ayant inclus plus de 100 patients évaluant des traitements ciblant la voie d'activation de l'inflammasome NLRP3 utilisés ou en cours d'utilisation pour le traitement des patients atteints de la COVID-19 modérée ou sévère ont été pris en compte dans ce tableau. Les principaux résultats de ces essais cliniques sont renseignés lorsque ceux-ci sont disponibles.

### Cibler l'activation de l'inflammasome NLRP3 : trouver la bonne cinétique

La cinétique d'activation de l'inflammasome NLRP3 pourrait être un paramètre important à prendre en compte avant d'administrer un traitement ciblant cette cascade de signalisation. Par exemple, un blocage précoce de l'activation de l'inflammasome NLRP3 pourrait limiter la trop importante réponse immunitaire innée que certains patients développent. Le dosage de l'IL-18 plasmatique ou le suivi de l'activation cellulaire de l'inflammasome pourraient ainsi aider à déterminer s'il est nécessaire de bloquer cette voie de signalisation. La concentration circulante de suPAR (*soluble urokinase plasminogen activator receptor*) est associée à un risque de formes sévères de COVID-19 et est corrélée aux concentrations sanguines de calprotectine (S100A8/S100A9) et d'IL-1 $\alpha$ .<sup>4</sup> Une étude a utilisé le suPAR comme biomarqueur afin d'administrer l'anakinra, la forme recombinante de l'IL-1RA, à des patients à risque

de forme grave de la maladie. Cette étude conclut à une diminution de la sévérité, de la mortalité et de la durée d'hospitalisation des patients [40].

### Conclusion

L'immense effort de recherche effectué au cours de la pandémie de COVID-19 a permis de mettre en évidence l'activation de la voie de l'inflammasome NLRP3 au cours de l'infection par le SARS-CoV-2. Les mécanismes cellulaires et moléculaires mis en jeu doivent encore être détaillés. Néanmoins, différentes stratégies thérapeutiques ciblant cette voie sont d'ores et déjà proposées dans la prise en charge des patients infectés par le SARS-CoV-2. Les résultats des essais cliniques sont attendus afin de confirmer l'intérêt de ces stratégies thérapeutiques et la nécessité de stratifier les patients qui pourraient bénéficier au mieux de ce type de traitements.  $\diamond$

<sup>4</sup> Renieris G, Karakike E, Gkavogianni T, et al. IL-1 Mediates Tissue Specific Inflammation and Severe Respiratory Failure In Covid-19: Clinical And Experimental Evidence. medRxiv 2021.04.09.21255190; <https://doi.org/10.1101/2021.04.09.21255190>.

## SUMMARY

### Role of inflammasome NLRP3 in the pathophysiology of viral infections: A focus on SARS-CoV-2 infection

NLRP3 is one of the best characterized innate immune cytosolic sensor. As part of the innate immune response, the NLRP3 inflammasome detects a wide range of danger signals such as pathogens, tissue damages, cellular stress. The priming and activation of NLRP3 lead to the formation of an oligomeric intracellular complex and to the recruitment and activation of caspase-1. Once activated, not only this inflammasome complex controls the processing and release of pro-inflammatory factors including IL-1 $\beta$  and IL-18, but also the inflammatory cell death pyroptosis mediated by gasdermin D pores. In this review, we describe the role of the NLRP3 inflammasome activation in viral infections with a particular interest on SARS-CoV-2 infection. In addition, we present therapies evaluated or under evaluation targeting the NLRP3 inflammasome pathway as COVID-19 treatment. ◊

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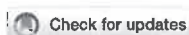
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Les auteurs déclarent n'avoir aucun lien d'intérêt concernant les données publiées dans cet article.

TIRÉS À PART  
F. Venet

## **Annexe 9**





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# Assessment of neutrophil subsets and immune checkpoint inhibitor expressions on T lymphocytes in liver transplantation: A preliminary study beyond the neutrophil-lymphocyte ratio

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**Background:** Advanced stages of cirrhosis are characterized by the occurrence of progressive immune alterations known as CAID (Cirrhosis Associated Immune Dysfunction). In advanced cirrhosis, liver transplantation (LT) remains the only curative treatment. Sepsis, shares many similarities with decompensated cirrhosis in terms of immuno-inflammatory response. In both conditions, the neutrophil-lymphocyte ratio (NLR) is associated with poor outcomes. Based on alterations in sepsis, we hypothesized that we could observe in cirrhotic and LT patients more detailed neutrophil and lymphocyte phenotypes. To this end, along with leukocyte count, we assessed immature neutrophils, LOX-1<sup>+</sup> MDSC and PD-1 and TIM-3 lymphocyte expressions in cirrhotic patients before transplantation in association with liver disease severity and during the first month after transplantation.

**Methods:** We conducted a prospective monocentric study including cirrhotic patients registered on LT waiting-list. Blood samples were collected at enrolment before LT and for 1 month post-LT. In addition to NLR, we assessed by whole blood flow cytometry the absolute count of immature neutrophils and LOX-1<sup>+</sup>

**Abbreviations:** ACLF, Acute on Chronic Liver Failure; CAID, Cirrhosis Associated Immune Dysfunction; HV, Healthy Volunteers; Lox1, Lectine-type oxidized LDL receptor 1; LT, Liver Transplantation; MELD, Model of End stage Liver Disease; PD-1, Programmed Death 1; PMN-MDSC, Polymorphonuclear Myeloid Derived Suppressor Cells; TIM3, T cell immunoglobulin and mucin domain 3.

MDSC as well as the expressions of immune checkpoint receptors PD-1 and TIM-3 on T lymphocytes.

**Results:** We included 15 healthy volunteers (HV) and 28 patients. LT was performed for 13 patients. Pre-LT patients presented with a higher NLR compared to HV and NLR was associated with cirrhosis severity. Increased immature neutrophils and LOX-1<sup>+</sup> MDSC counts were observed in the most severe patients. These alterations were mainly associated with acute decompensation of cirrhosis. PD-1 and TIM-3 expressions on T lymphocytes were not different between patients and HV. Post-LT immune alterations were dominated by a transitory but tremendous increase of NLR and immature neutrophils during the first days post-LT. Then, immune checkpoint receptors and LOX-1<sup>+</sup> MDSC tended to be overexpressed by the second week after surgery.

**Conclusion:** The present study showed that NLR, immature neutrophils and LOX-1<sup>+</sup> MDSC counts along with T lymphocyte count and checkpoint inhibitor expression were altered in cirrhotic patients before and after LT. These data illustrate the potential interest of immune monitoring of cirrhotic patients in the context of LT in order to better define risk of sepsis. For this purpose, larger cohorts of patients are now necessary in order to move forward a more personalised care of LT patients.

#### KEYWORDS

transplantation, immunosuppression, cirrhosis, immune checkpoint receptors, PD-1, LOX-1

## Introduction

Liver cirrhosis defined by annular fibrosis surrounding regenerating hepatocytes is the terminal evolution of many chronic liver diseases (Anthony et al., 1977). Advanced stages of cirrhosis are characterized by portal hypertension, hepatic insufficiency and by the occurrence of progressive immune alterations known as CAID (Cirrhosis Associated Immune Dysfunction). CAID associates both systemic inflammation and features of immunosuppression as a consequence of alterations of the gut-liver axis inducing intestinal hyper-permeability and dysbiosis (Albillos et al., 2021). This leads to a continuous immune stimulation by microbial antigens and ultimately to immune cell exhaustion (Albillos et al., 2020). As a result, both innate and adaptive immune responses are dysregulated in cirrhotic patients and dramatically worsen with cirrhosis severity such as in the highest severity stage of inflammation represented by ACLF (Acute on Chronic Liver Failure) (Arvaniti et al., 2010). In this context of advanced cirrhosis, liver transplantation (LT) remains the only curative treatment. In addition to CAID, LT amplifies the profound immunosuppressive state of patients due to major surgery, immunosuppressive drugs, and intensive care unit stay). Therefore, infections constitute a major clinical issue in pre- and post-LT patients (Tranah et al., 2022). Before LT, infections in cirrhotic patients are both more frequent and more severe in association with cirrhosis severity and they can delay the access to a graft and increase mortality risk (Finkenstedt et al., 2013). After LT, infections increase morbidity and graft dysfunction (Tranah et al., 2022). Noteworthy, infections represent the major cause of death in the first year following LT in ACLF patients (Sundaram et al., 2020).

Sepsis, a life-threatening organ dysfunction caused by a dysregulated inflammatory host response to infection, shares

many similarities with decompensated cirrhosis in terms of immuno-inflammatory response (Singer et al., 2016). It associates overwhelming inflammation and compensatory anti-inflammatory response that may lead to marked immunosuppression. Besides, immune dysfunction in ACLF has been described as a “sepsis-like” immune paralysis (Wasmuth et al., 2005). In sepsis, many immunological parameters have been demonstrated as prognostic marker of higher infectious risk/mortality (Venet and Monneret, 2018). Of them, due to lymphocyte apoptosis and emergency granulopoiesis (Venet et al., 2021) the neutrophil-lymphocyte ratio (NLR) is a widely described prognostic biomarker associated with poor outcomes (Rehman et al., 2020; Abensur Guillaume et al., 2021; Lorente et al., 2022). Moreover, on neutrophil side, additional dysfunctional subsets have been described: increased immature neutrophils (i.e., CD16<sup>low</sup>) (Rehman et al., 2020) or occurrence of LOX-1<sup>+</sup> myeloid derived suppressive cells (LOX-1<sup>+</sup> MDSC) (Coudereau et al., 2022). On lymphocyte side, several reports revealed overexpression of immune checkpoint receptors such as PD-1 (Programmed death-1) and TIM3 (T cell immunoglobulin domain and mucin domain 3) on lymphocyte surface (Guignant et al., 2011; Boomer et al., 2012). Most importantly, in septic patients, all these parameters contribute to immunosuppression and were repeatedly reported to be associated with poor outcomes (mortality, risk of secondary infections, and longer length of ICU stay) (Venet and Monneret, 2018).

So far, although NLR has been studied in cirrhosis (Cai et al., 2017; Bernsmeier et al., 2020; Liu et al., 2021; Magalhães et al., 2021) but never after LT, further phenotyping of additional cell subsets (either neutrophils or lymphocytes) has never been conducted, especially over the pre/post-transplantation period. Explorations in the field may address the unmet clinical need in early

recognition of infectious risk in cirrhotic and LT patients. Having similar NLR alterations in cirrhosis and sepsis, we hypothesize that we could observe in cirrhotic patients more detailed neutrophil and lymphocyte phenotype alterations known to be associated with immunosuppression. To this end, along with leukocyte count, we assessed immature neutrophils, LOX-1<sup>+</sup> MDSC and PD-1 and TIM3 lymphocyte expression in cirrhotic patients before transplantation in association with liver disease severity and during the first month after transplantation. We aimed to better characterize immune alterations in those patients to identify putative biomarkers that may help in defining more individualized medicine.

## Materials and methods

### Subjects

Patients registered on LT waiting for decompensated cirrhosis or for cirrhosis complicated with hepatocellular carcinoma list at Lyon University Hospital (France) were prospectively enrolled. All patients were eligible to a standard immunosuppressive protocol with administration of simulect (day 0 and day 4), corticoids (at least 7 days), tacrolimus and mycophenolate mofetil. Exclusion criteria were as follows: patients requiring multi-organ transplant, patients treated with immunosuppressors (including patients with history of previous LT) and patients without underlying cirrhosis. This protocol is an ancillary study from EdMonHG study (N<sup>o</sup>ID-RCB 2019-A00954-53, CT identifier: NCT03995537).

Patients reported in this study were included from January 2022 to September 2022. Peripheral blood samples were collected once at enrolment (within 3 months before LT). Following LT, samples were collected twice a week for 1 month or until the occurrence of infection and/or acute cellular rejection. Post-LT time points were grouped as follows: day 1 to day 3 (D1-D3), day 4 to day 6 (D4-D6), day 7 to day 13 (D7-D13), day 14 to day 20 (D14-D20), day 21 to day 27 (D21-D27) and day 28 to day 31 (D28-D31). Before LT, all clinical data related to cirrhosis severity and aetiologies were collected. All relevant clinical and biological data occurring during and after transplant surgery were recorded. Acute decompensation (AD) of cirrhosis was defined by the acute development of one or more major complications of liver disease (i.e., ascites, hepatic encephalopathy, gastrointestinal haemorrhage and/or bacterial infections) (Moreau et al., 2013). ACLF stage in pre-LT patients were defined according to Moreau's criteria (Moreau et al., 2013). Pre-LT patients were divided into two groups according to Model of End stage Liver Disease (MELD) score, a validated chronic liver disease scoring system that predicts 3-month survival on liver waiting list. A cut-off of MELD score  $\geq 30$  was chosen to identify the most severe patients. In addition, patients were stratified according to the Child-Pugh score, which is a clinico-biological scoring system used to assess prognosis of cirrhotic patients. We compared Child-Pugh A or B patients (A/B) with Child-Pugh C patients (the most severe patients).

After LT, any event of acute cellular rejection or sepsis occurrence, according to the criteria of the American Society of Transplantation (Humar et al., 2006) stopped the immune

monitoring (i.e., censored forthcoming results) since they both impact immune functions by themselves. Fifteen healthy volunteers (HV) served as controls (samples coming from French Blood Establishment). The median age of HV was 38 years and 33% were male.

### Whole blood phenotyping

At each time point, in addition to leukocyte count, we assessed immature neutrophils (CD16<sup>low</sup>) and LOX-1<sup>+</sup> MDSC (CD15<sup>+</sup>, CD45<sup>dim</sup>, LOX-1<sup>+</sup> polymorphonuclear cells) percentages as described by Coudereau et al. (2022) and immune checkpoint inhibitor (PD-1 and TIM-3) expression on CD3, CD4 and CD8 T lymphocytes. Cell staining was performed on fresh whole blood sample within 4 h after sampling. We used the following antibodies: CD45-PB, CD3-APC-AF750, CD4-FITC, CD8-Kro, CD14-PB, CD16-APC from BeckmanCoulter (Brea, CA) and: PD1-APC, TIM-3-PE-Dazzle, CD15-AF700, LOX1-PE from BioLegend (San Diego, CA). Isotype control antibodies (BioLegend) were used to determine the percentages of positive cells for PD-1, TIM-3 and LOX-1. Samples were run on Navios flow cytometer (Beckman Coulter). T lymphocytes subsets' absolute quantification was performed on Aquios flow cytometer (Beckman Coulter). Detailed protocols are presented in supplementary methods. Results were expressed as absolute counts for neutrophil subsets and T lymphocyte subsets (i.e., cells/mm<sup>3</sup>). Results were expressed as absolute cell counts for immature neutrophils and LOX-1<sup>+</sup> MDSC. Immune checkpoint inhibitor expressions on T lymphocyte subsets were expressed as percentages of positive cells based on isotype controls.

### Statistics

Statistical analyses were performed with the software RStudio (2021.09.2 + 382 version). Data are presented on boxplot graph with medians, interquartile ranges and individual values. Non-parametric Mann-Whitney, Fisher's exact test and  $\chi^2$  tests were used to assess differences between groups. When appropriate, ANOVA test was used to assess differences between more than 2 independent groups. If ANOVA assumptions were not verified Kruskal-Wallis test was performed. Spearman coefficient was used to assess correlation between quantitative data. Statistical significance was assumed at  $p < 0.05$ . Due to relatively low number of transplanted patients, we did not perform statistical analysis after LT. Given the exploratory nature of the present observational study, no power analysis was performed.

## Results

### Patients' characteristics

During the study period, 28 cirrhotic patients were enrolled in this study. Clinical characteristics are presented in Table 1. Briefly, the median age was 58 years and 86% were male. Alcohol-related liver disease represented 53% of the cirrhosis aetiology. 7% of



TABLE 1 Patients characteristics of whole cohort and according to MELD score.

Patients characteristics	All patients (n = 28)	Patients with MELD <30 (n = 20)	Patients with MELD ≥30 (n = 8)	p
<b>Demographic characteristics</b>				
Age (years)	58 [37–68]	61.5 [48–68]	55 [37–61]	<0.01
Sex (male)	24 (86)	17 (85)	7 (88)	NS
<b>Cirrhosis Aetiology</b>				
Alcohol	15 (53)	10 (50)	5 (63)	NS
Dysmetabolic	2 (7)	2 (10)	0 (0)	
HCV	1 (4)	1 (5)	0 (0)	
Mixed cirrhosis				
Alcohol/dysmetabolic	5 (18)	5 (25)	0 (0)	
Alcohol/viruses	2 (7)	1 (5)	1 (13)	
Others	3 (11)	1 (5)	2 (25)	
<b>Decompensation stages</b>				
Compensated	8 (29)	8 (40)	0 (0)	NS
Chronic decompensation (CD)	8 (29)	8 (40)	0 (0)	
Acute decompensation (AD)	12 (43)	4 (20)	8 (100)	
Aetiology of AD				
Infection	8 (67)	4 (100)	4 (50)	
AAH	1 (8)	0 (0)	1 (13)	
HBV reactivation	1 (8)	0 (0)	1 (13)	
Wilson disease	1 (8)	0 (0)	1 (13)	
Alcohol intake	1 (8)	0 (0)	1 (13)	
<b>Clinical parameters</b>				
Active smokers	10 (36)	8 (40)	2 (25)	NS
Diabetes	9 (32)	9 (45)	0 (0)	0.03
HBP	12 (42)	10 (50)	2 (25)	NS
Chronic ascitis	10 (36)	7 (35)	3 (38)	NS
HE (at inclusion)	7 (25)	4 (20)	3 (38)	NS
AKF (at inclusion)	4 (14)	0 (0)	4 (50)	<0.001
<b>Biologic markers</b>				
Bilirubin	70 [5.8–679]	51.8 [5.8–330]	468 [71–679]	<0.001
ALP	108.5 [63–250]	113 [63–250]	69.5 [63–177]	NS
GGT	61.5 [21–273]	68 [21–273]	53 [28–246]	NS
ALT	36.5 [14–139]	33.5 [14–74]	61.5 [23–139]	0.02
AST	54 [15–286]	50.5 [15–130]	81.5 [54–286]	<0.001
Albumin	34.8 [21.5–45.7]	35.7 [22.5–45.7]	24.9 [21.5–39.4]	NS
Sodium	137 [128–142]	136.5 [130–142]	137.5 [128–140]	NS
PT	37.5 [14–100]	45.5 [26–100]	26.5 [14–52]	<0.01
INR	2.02 [1–5.5]	1.79 [1–2.99]	2.88 [1.65–5.5]	<0.01
Factor V	37 [10–123]	59 [21–123]	28.5 [10–76]	0.04
Creatinine	68 [36–275]	63.5 [36–127]	166 [41–275]	NS
Platelets (G/L)	82.5 [12–243]	108 [12–243]	64 [22–216]	NS
Hemoglobin (mg/dL)	9.5 [5.7–16.4]	10.6 [5.7–16.4]	8.7 [6.0–12.1]	0.03
CRP (mg/dL)	15.7 [0.5–50.9]	8.9 [0.5–50.9]	22.9 [19.6–44.7]	0.008
<b>Pronostic scores</b>				
MELD score	24 [6–40]	18 [6–27]	35 [30–40]	<0.001
Child-Pugh score	10 [5–14]	8 [5–13]	11 [10–14]	<0.01
Child-Pugh C	16 (57)	8 (40)	8 (100)	<0.01
SOFA score	4.5 [0–15]	3 [0–10]	8 [6–15]	<0.001
ACLF	10 (36)	2 (10)	8 (100)	<0.001
<b>Immunologic parameters</b>				
Neutrophils (G/L)	4.1 [1.6–20.9]	3.4 [1.6–8.5]	4.9 [2.8–20.9]	0.008
Monocytes (G/L)	0.65 [0.33–1.89]	0.72 [0.33–1.48]	0.59 [0.35–1.89]	NS

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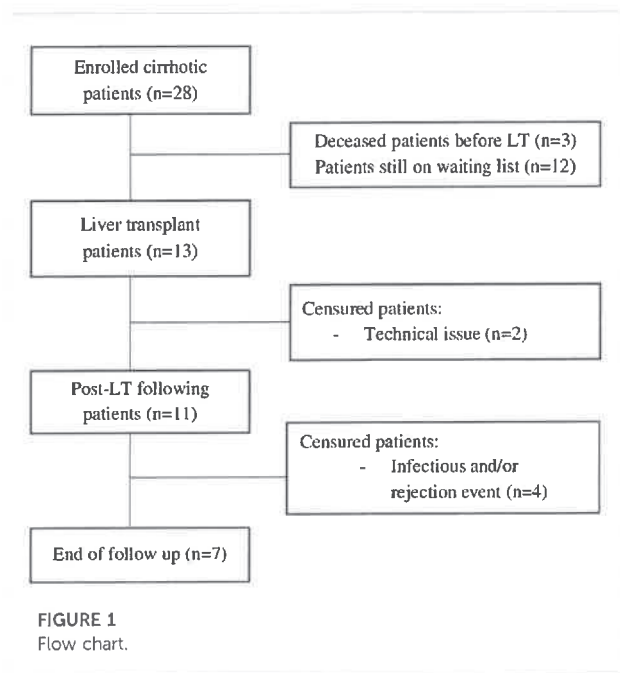
TABLE 1 (Continued) Patients characteristics of whole cohort and according to MELD score.

Patients characteristics	All patients (n = 28)	Patients with MELD <30 (n = 20)	Patients with MELD ≥30 (n = 8)	p
T lymphocytes (cells/μL)	507 [79–1,479]	737 [79–1,479]	372 [204–1,395]	0.03
NLR	3.1 [1.6–40.2]	2.4 [1.6–8.2]	8.9 [4.5–40.2]	<0.001
Death on waiting list	3 (11)	0 (0)	3 (38)	NS

Quantitative data are presented as medians with minimum and maximum value within square brackets [min–max]. Qualitative data are presented as numbers of cases and percentage among the total population or subpopulation in brackets (%). Prognostic scores and immunologic parameters were calculated the day of patients' inclusion. *p*-values were calculated using Mann-Whitney, Fisher and  $\chi^2$  tests when appropriate. AAH (acute alcoholic hepatitis). ACLF (acute on chronic liver failure). AD (acute decompensation of cirrhosis). AKF (acute kidney failure). ALP (alkaline phosphatase). ALT (alanine aminotransferase). AST (alanine aminotransferase). CD (chronic decompensation of cirrhosis). CRP (c-reactive protein). MELD (Model of End Stage Liver Disease). NLR (neutrophils to lymphocytes ratio). HBP (high blood pressure). HCV (hepatitis C virus). HE (hepatic encephalopathy). GGT (gamma glutamyl transferase). INR (international standardization ratio). PT (prothrombin time). SOFA (Sequential Organ Failure Assessment).

TABLE 2 Characteristics of transplanted patients.

Patients characteristics	Transplant patients (n = 13)
<b>Decompensation stages</b>	
Compensated	3 (23)
Chronic decompensation (CD)	3 (23)
Acute decompensation (AD)	7 (53)
<b>Pronostic scores</b>	
MELD score ≥30	4 (30)
Child-Pugh C	9 (69)
ACLF	6 (46)
<b>Liver surgery</b>	
Surgery time (minutes)	450 [248–525]
Cold ischaemia (minutes)	420 [278–560]
Red cells transfusion	2 [0–10]
<b>Post-transplant outcomes (during the first month post LT)</b>	
Intensive care length of stay (days)	6 [4–79]
Total duration of vasopressors (days)	0 [0–8]
Total duration of MV (days)	0 [0–29]
Surgical revision	4 (31)
Graft dysfunction at day 7*	6 (46)
Infectious event	5 (38)
Reject	1 (8)
One month survival	13 (100)

FIGURE 1  
Flow chart.

3 presented with sepsis, and last one presented both infection and rejection. Patient's flow chart is presented in Figure 1. Events of infection and reject are summarised in Table 3.

patients had a dysmetabolic cirrhosis and 25% had a mixed cirrhosis (5 patients had a cirrhosis related to dysmetabolic syndrome and alcohol intake and 2 patients had a cirrhosis related to HCV or HBV infection and alcohol intake). One patient had a post hepatitis C cirrhosis. The two patients with background of hepatitis C obtained a viral clearance years before inclusion. The patient with hepatitis B had a patent HBV reactivation at inclusion. 26% of patients had MELD score ≥30 (n = 8) and 43% were in AD (n = 12). Among AD patients, 83% met ACLF criteria (n = 10). All the patients with a MELD score ≥30 were in AD and met ACLF criteria. The causes of AD were infections (n = 8), acute alcoholic hepatitis (AAH) (n = 1), alcohol consumption without AAH (n = 1), HBV reactivation (n = 1) and Wilson's disease exacerbation (n = 1). 38% of patients with a MELD score ≥30 died on waiting list (n = 3). In this cohort, 46% of patients (n = 13) underwent LT (table 2). Of them, 11 were monitored over post-LT period (2 were missing due to mistakes in protocol guidance). Seven patients completed the whole follow-up,

## Pre-transplantation results

### Total neutrophil count and neutrophil-lymphocyte ratio

Neutrophil count was not significantly different in pre-LT patients (whole cohort) in comparison to HV (Figure 2A). Nevertheless, increased neutrophils were associated with more severe cases according to MELD score (Figure 2B) and were associated with decompensation stages of cirrhosis (Figure 2C). NLR was significantly increased in pre-LT patients in comparison to HV (Figure 2D). Importantly, NLR was higher in patients with MELD score ≥30 and Child-Pugh score C (Figure 2E). Moreover, NLR was significantly associated with decompensation stages of cirrhosis as it was

TABLE 3 Infectious and graft rejection outcomes.

Patients	MELD score	ACLF	Clinical events	Identified germ	Post-transplant days	Intensive care unit stay	One month survival
1	19	No	Pneumoniae and acute cellular rejection	<i>Klebsiella pneumoniae</i>	D5 (infection) D6 (reject)	7	yes
2	30	No	Peritonitis	No	D12	72	yes
3	27	No	Pneumoniae	<i>Pseudomonas aeruginosa</i>	D10	68	yes
4	36	Yes	Infectious pleuritis	<i>Enterococcus faecium</i>	D17	still in ICU at Ms submission (i.e., 85 days)	yes

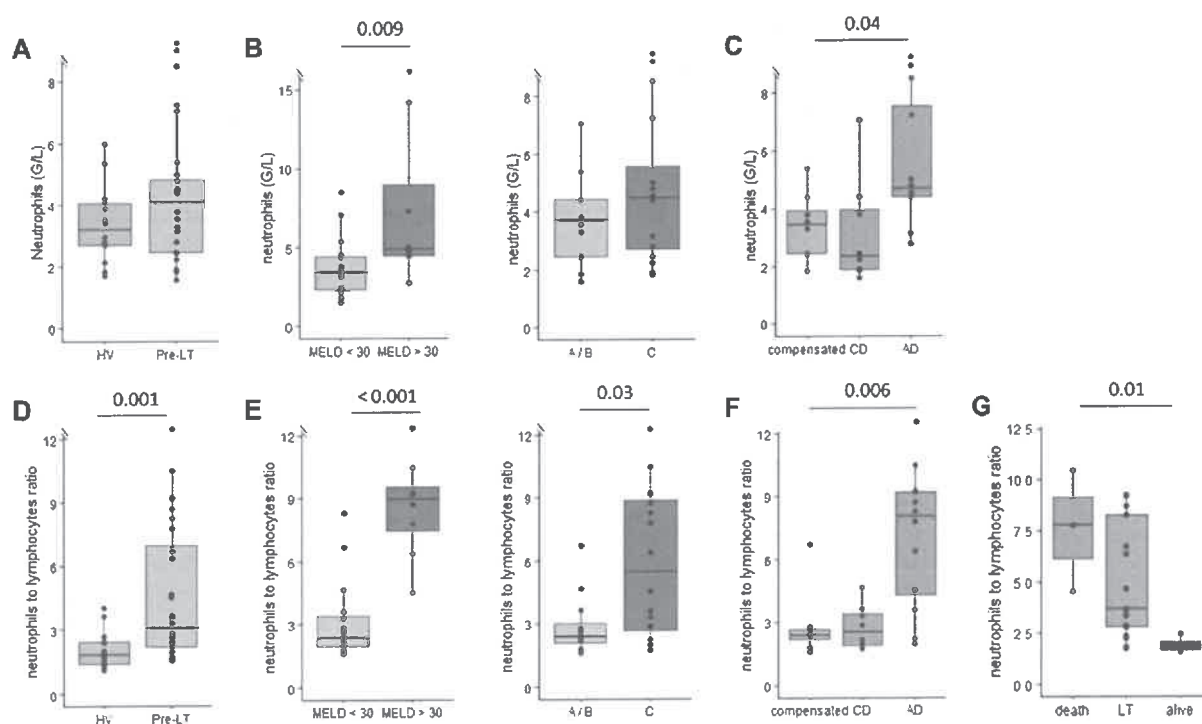


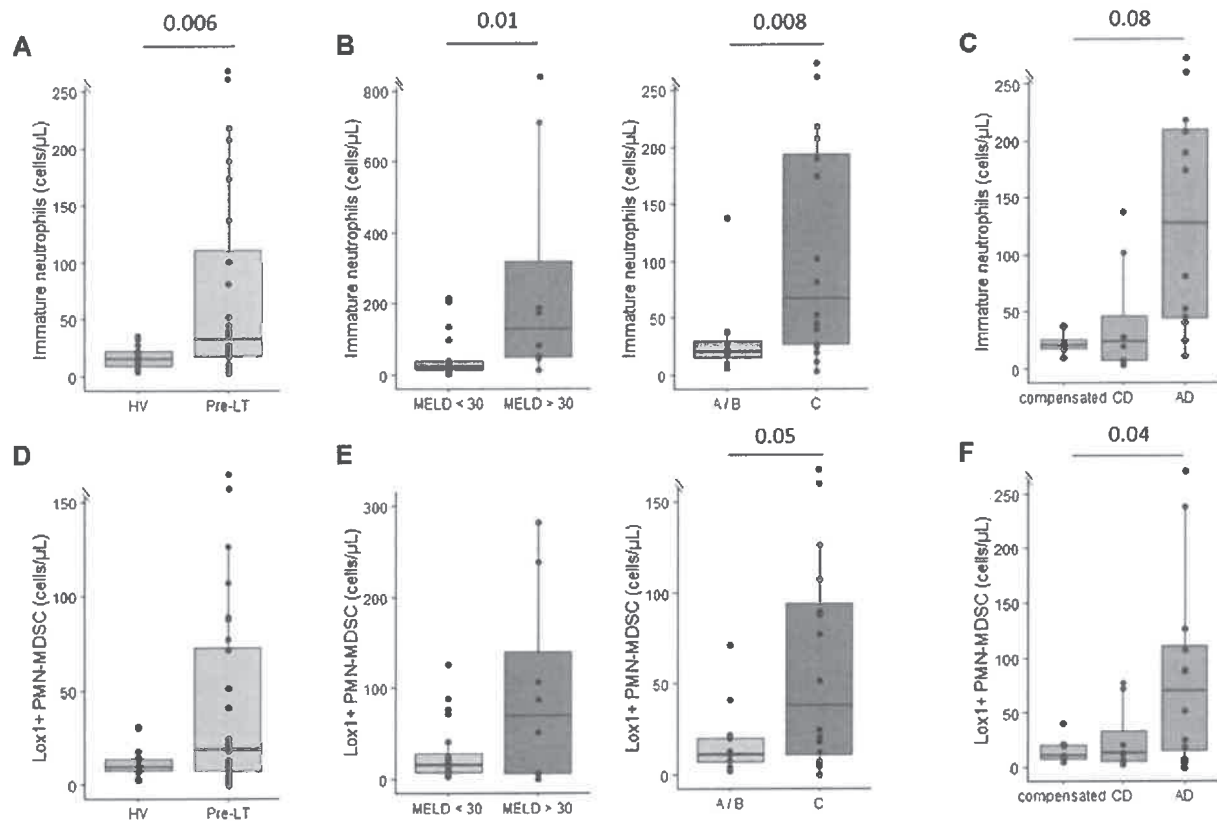
FIGURE 2

Monitoring of neutrophils count and neutrophils to lymphocytes ratio (NLR) in peripheral blood of pre-transplant patients. (A) Neutrophils count in healthy volunteers (HV,  $n = 15$ ) and pre-transplant patients (pre-LT,  $n = 28$ ). (B) Neutrophils count in patients with MELD score  $< 30$  ( $n = 20$ ) or with MELD score  $\geq 30$  ( $n = 8$ ) and in patients with Child-Pugh score A or B (A/B,  $n = 12$ ) or with Child-Pugh score C ( $n = 16$ ). (C) Neutrophils count in patients with compensated cirrhosis ( $n = 8$ ), chronic decompensated cirrhosis (CD,  $n = 8$ ) and acute decompensated cirrhosis (AD, with  $n = 10$  or without ACLF  $n = 2$ ). (D) NLR in healthy volunteers (HV) and pre-transplant patients (pre-LT). (E) NLR in patients with or without MELD score  $< 30$  and in patients with Child-Pugh A/B or C. (F) NLR in patients with compensated, chronic decompensated and acute decompensated cirrhosis. (G) NLR according to three months evolution post inclusion. The nonparametric Wilcoxon test was used to assess differences between patients and HV and between patients' subgroups determined by MELD and Child-Pugh scores. ANOVA or Kruskal-Wallis tests were used to assess differences between more than 2 independent groups.

predominantly increased in AD patients (Figure 2F). There was a positive correlation between NLR and MELD score ( $r = 0.7$ ;  $p < 0.001$ ) and between NLR and CRP ( $r = 0.74$ ;  $p = 0.001$ ). Interestingly, NLR was significantly associated with patients' survival 3 months after inclusion (Figure 2G). There was no transplant free survival in patients with NLR  $> 4$ . The cause of death was multiple organ failure syndrome secondary to uncontrolled infection for the three patients who died on waiting list.

## Neutrophil subsets

Immature CD16<sup>low</sup> neutrophil counts were significantly increased in pre-LT patients in comparison with HV (Figure 3A). Increased immature neutrophils count was associated with cirrhosis severity according to MELD and Child-Pugh scores (Figure 3B). Moreover, AD patients tended to show increased immature neutrophils count in comparison with compensated and CD patients (Figure 3C). In addition, we found a positive and significant correlation between



**FIGURE 3**

Monitoring of immature neutrophils (CD16<sup>low</sup>) and lectine-type oxidized LDL receptor 1 polymorphonuclear myeloid-derived suppressor cells (LOX1+ PMN-MDSC) in peripheral blood of pre-transplant patients. (A) Immature neutrophils count in healthy volunteers (HV,  $n = 15$ ) and pre-transplant patients (pre-LT,  $n = 28$ ). (B) Immature neutrophils count in patients with a MELD score  $< 30$  ( $n = 20$ ) or with a MELD score  $\geq 30$  ( $n = 8$ ) and in patients with Child-Pugh score A or B (A/B,  $n = 12$ ) or with Child-Pugh score C ( $n = 16$ ). (C) Immature neutrophils count in patients with compensated cirrhosis ( $n = 8$ ), chronic decompensated cirrhosis (CD,  $n = 8$ ) and acute decompensated cirrhosis (AD, with  $n = 10$  or without ACLF  $n = 2$ ). (D) Number of LOX1+ MDSC in healthy volunteers (HV) and pre-transplant patients (pre-LT). (E) Number of LOX1+ MDSC in patients with or without MELD score  $< 30$  and in patients with Child-Pugh A/B or C. (F) Number of LOX1+ MDSC in patients with compensated, chronic decompensated, and acute decompensated cirrhosis. The nonparametric Wilcoxon test was used to assess differences between patients and HV and between patients' subgroups determined by MELD and Child-Pugh score. Kruskal-Wallis test was used to assess differences between more than 2 independent groups.

immature neutrophil counts and CRP ( $r = 0.60$ ,  $p = 0.016$ ) and MELD score ( $r = 0.56$ ,  $p = 0.0039$ ). Although clearly elevated in some patients, LOX-1<sup>+</sup> MDSC counts were not significantly different between patients and HV (Figure 3D). Regarding association with pre-LT severity, solely AD patients presented with significantly elevated values (Figure 3F). Importantly, immature neutrophils and LOX-1<sup>+</sup> MDSC counts were significantly correlated to NLR ( $r = 0.57$ ,  $p = 0.002$ ; and  $r = 0.4$ ,  $p = 0.034$  respectively). Noteworthy, immature neutrophils and LOX-1<sup>+</sup> MDSC counts were not increased neither in patients with active hepatocellular carcinoma ( $n = 4$ ) nor with patients transplanted for hepatocellular carcinoma ( $n = 10$ ) (data not shown).

## T lymphocyte counts

We observed a profound T lymphopenia in cirrhotic patients in comparison to HV. This affected both CD4<sup>+</sup> (median: 496 CD4<sup>+</sup> cells/mm<sup>3</sup>, Figure 4A) and CD8<sup>+</sup> (median: 148 CD8<sup>+</sup> cells/mm<sup>3</sup>,

Figure 4D) T lymphocyte subsets in pre-LT patients. Lymphopenia was significantly more important in patients with MELD score  $\geq 30$  compared to patients with MELD score  $< 30$  (Figures 4B, E). Interestingly, CD8<sup>+</sup> T cells count was significantly decreased in compensated patients in comparison to HV ( $p = 0.002$ ). Moreover, lymphopenia tended to accentuate during decompensated stages of cirrhosis (Figures 4C, F). CD3<sup>+</sup> T cells count was negatively correlated to CRP ( $r = -0.73$ ;  $p = 0.002$ ).

## Immune checkpoint inhibitor expressions on T lymphocyte subsets

PD-1 and TIM3 expressions on CD3<sup>+</sup> T lymphocytes were not different between HV and pre-LT patients (Figures 5A, D). Overall, PD-1 and TIM3 expressions were not associated with cirrhosis severity according to MELD and Child-Pugh scores (Figures 5B, E) or with decompensation stages of cirrhosis (Figures 5C, F). These results were similar on CD8<sup>+</sup> and CD4<sup>+</sup> T cells (data not shown).

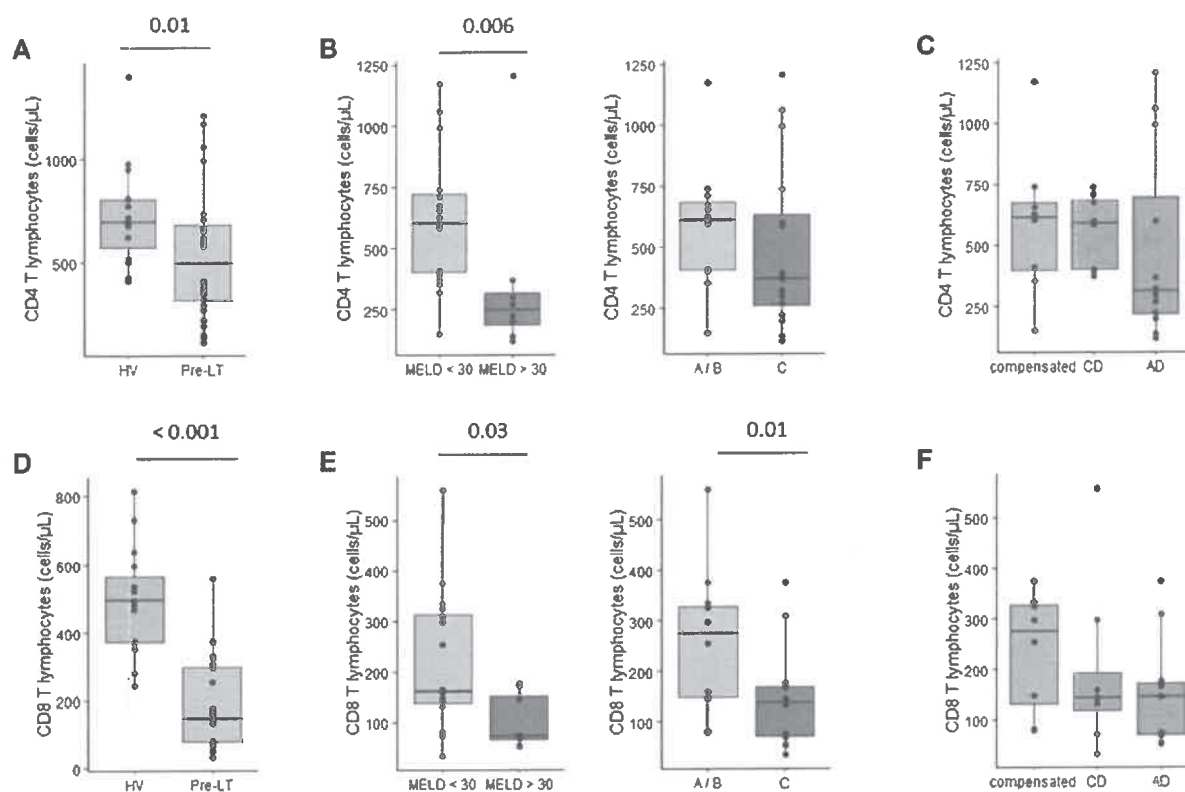


FIGURE 4

Monitoring of CD4+ and CD8+ T cell counts in peripheral blood of pre-transplant patients. (A) CD4+ T lymphocytes count in healthy volunteers (HV,  $n = 15$ ) and pre-transplant patients (pre-LT,  $n = 28$ ). (B) CD4+ T lymphocytes count in patients with a MELD score  $< 30$  ( $n = 20$ ) or with a MELD score  $\geq 30$  ( $n = 8$ ) and in patients with Child-Pugh score A or B (A/B,  $n = 12$ ) or with Child-Pugh score C ( $n = 16$ ). (C) CD4+ T lymphocytes count in patients with compensated cirrhosis ( $n = 8$ ), chronic decompensated cirrhosis (CD,  $n = 8$ ). (D) CD8+ T lymphocytes count in healthy volunteers (HV) and pre-transplant patients (pre-LT). (E) CD8+ T lymphocytes count in patients with or without MELD score  $< 30$  and in patients with Child-Pugh A/B or C. (F) CD8+ T lymphocytes count in patients with compensated, chronic decompensated, and acute decompensated cirrhosis. The nonparametric Wilcoxon test was used to assess differences between patients and HV and between patients' subgroups determined by MELD and Child-Pugh score.

Importantly, as alcohol is able to induce PD-1 and TIM3 expressions *in vitro* (Markwick et al., 2015), we verified that immune checkpoint receptors were not differently expressed in alcohol consumer patients ( $n = 5$ ) compared non-alcoholic and weaned patients ( $n = 23$ , data not shown). Moreover, as immune checkpoint receptors might be overexpressed in cancer, we addressed this aspect but noticed that PD-1 and TIM3 were not differently expressed in patients with active hepatocellular carcinoma ( $n = 4$ ). In addition, there were no differences between patients enrolled on waiting list for hepatocellular carcinoma ( $n = 10$ ) and patients without medical history of hepatocellular carcinoma ( $n = 18$ , data not shown).

## Post-transplant results

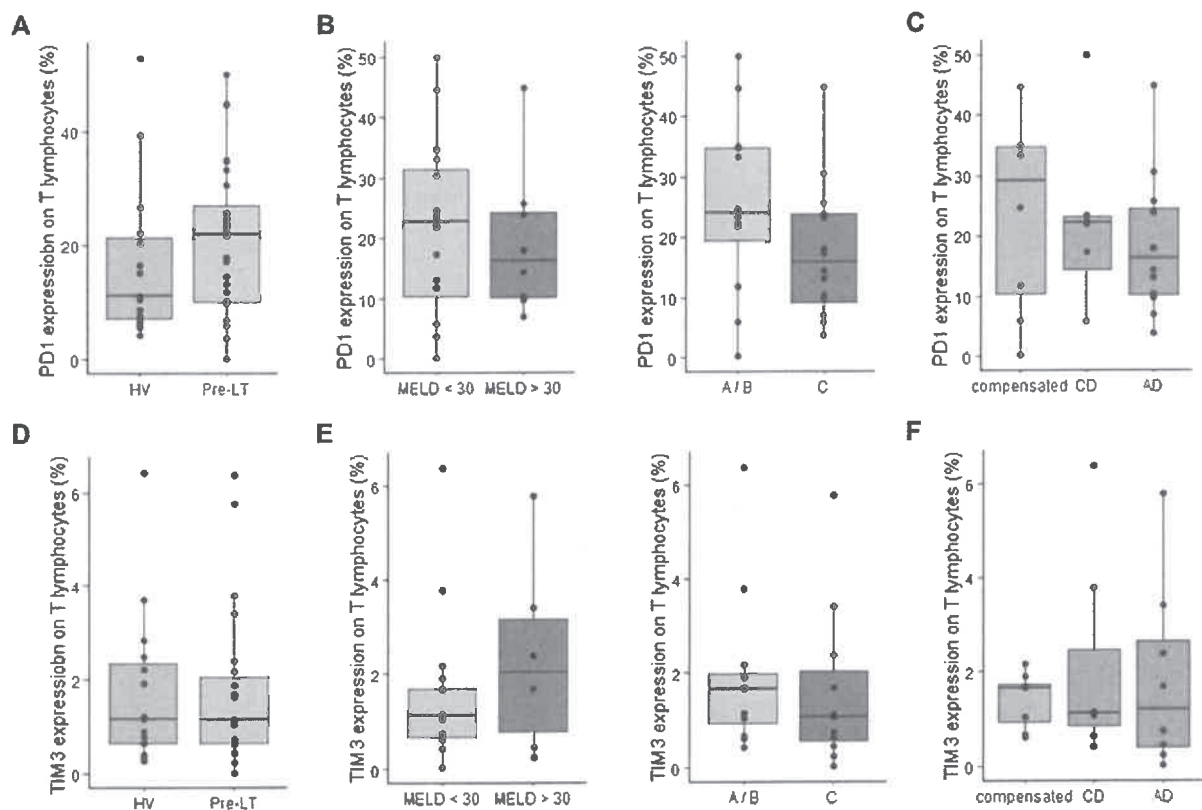
### Total neutrophil count, neutrophil-lymphocyte ratio and neutrophil subsets

After LT, we observed a tremendous increase of neutrophils count at D1-D3 post-LT. Then, neutrophils count decreased and reached pre-LT values during the third week post-LT (Figure 6A). In accordance, we

observed an important rise of NLR at D1-D3 following LT (Figure 6B). However, this elevation was transitory and decreased at D4-D6 post-LT and remained stable until 1-month post-LT. However, throughout this follow-up, NLR remained higher than that from HV controls. According to total neutrophil count, immature neutrophils count peaked at D1-D3 after LT and then returned to pre-LT values at D4-D6 (Figure 7A). At the end of follow-up, immature neutrophils count remained slightly higher (median: 37 cells/mm<sup>3</sup>) than HV value (median: 15 cells/mm<sup>3</sup>). In contrast, LOX-1<sup>+</sup> MDSC count presented with a different kinetic. LOX-1<sup>+</sup> MDSC count remained stable during the first week after LT (Figure 7B) but reached a maximum during the second week post-LT (D7-D13). This elevation was transitory as LOX-1<sup>+</sup> MDSC rapidly went back down to low values (median: 20 cells/mm<sup>3</sup>) similar to those observed in HV controls (median: 9 cells/mm<sup>3</sup> in HV).

### T lymphocyte counts

Despite being already low before LT, lymphopenia amplified after transplantation (Figure 8A). Nadir was



**FIGURE 5**

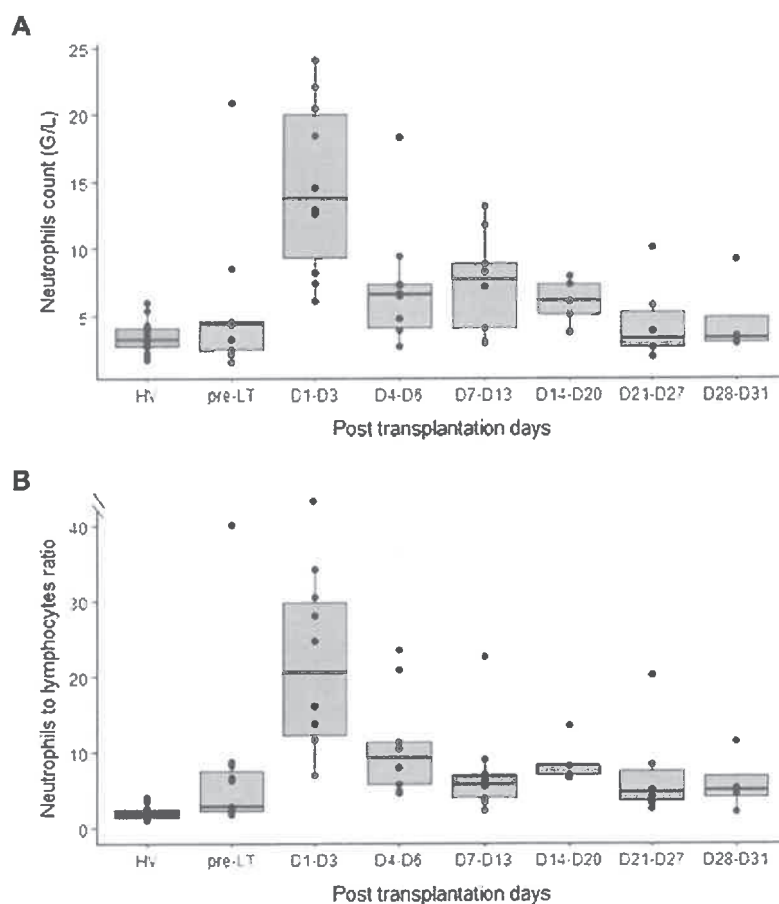
Monitoring of PD-1 and TIM3 expression on T cells in peripheral blood of pre-transplant patients. (A) Percentage of PD-1 expression on CD3+ T lymphocytes in healthy volunteers (HV,  $n = 15$ ) and pre-transplant patients (pre-LT,  $n = 28$ ). (B) Percentage of PD-1 expression on CD3+ T lymphocytes in patients with a MELD score  $< 30$  ( $n = 20$ ) or with a MELD score  $\geq 30$  ( $n = 8$ ) and in patients with Child-Pugh score A or B (A/B,  $n = 12$ ) or with Child-Pugh score C ( $n = 16$ ). (C) Percentage of PD-1 expression on CD3+ T lymphocytes in patients with compensated cirrhosis ( $n = 8$ ), chronic decompensated cirrhosis (CD,  $n = 8$ ) and acute decompensated cirrhosis (AD, with  $n = 10$  or without ACLF  $n = 2$ ). (D) Percentage of TIM3 expression on CD3+ T lymphocytes in healthy volunteers (HV) and pre-transplant patients (pre-LT). (E) Percentage of TIM3 expression on CD3+ T lymphocytes in patients with compensated, chronic decompensated, and acute decompensated cirrhosis. (F) Percentage of PD-1 expression on CD3+ T lymphocytes in patients with or without a MELD score  $< 30$  and in patients with Child-Pugh A/B or C. The nonparametric Wilcoxon test was used to assess variations between patients and HV and between patients' subgroups determined by MELD and Child-Pugh score. Kruskal-Wallis test was used to assess differences between more than 2 independent groups.

observed at D1-D3 post-LT. This profoundly affected all T cells subsets (medians as follows: CD3+ T cells: 192 cells/mm<sup>3</sup>, CD4+ T cells: 131 cells/mm<sup>3</sup>, CD8+ T cells: 50 cells/mm<sup>3</sup>). Thereafter, T lymphocytes increased at levels similar to pre-LT values during the second week post-surgery. However, at the end of follow-up, patients still presented with marked lymphopenia (Figure 8A). In parallel, we observed a progressive over expression of both TIM-3 and PD1 checkpoint inhibitor expressions on circulating T lymphocytes, TIM3 expression reached a maximum around 2–3 weeks post-LT and then remained stable (Figure 8B). Even if it was less clear, PD-1 tended to follow same pattern of expression (Figure 8C). Similar results were observed on both CD4+ and CD8+ T lymphocytes (data not shown).

## Discussion

To the best of our knowledge, this preliminary study is the first to present a detailed neutrophils and T lymphocytes immune phenotyping overtime in cirrhotic patients before and after liver transplantation. These results provide valuable additional information and markers (LOX-1, TIM-3, PD-1) to complete previous results obtained in cirrhotic patients solely based on NLR.

NLR is believed to be associated with cirrhosis severity and mortality. Cai et al. reported that this parameter was an independent predictors of hospital-acquired bacterial infections in decompensated cirrhosis (Cai et al., 2017). They also demonstrated that cirrhotic patients presenting with NLR



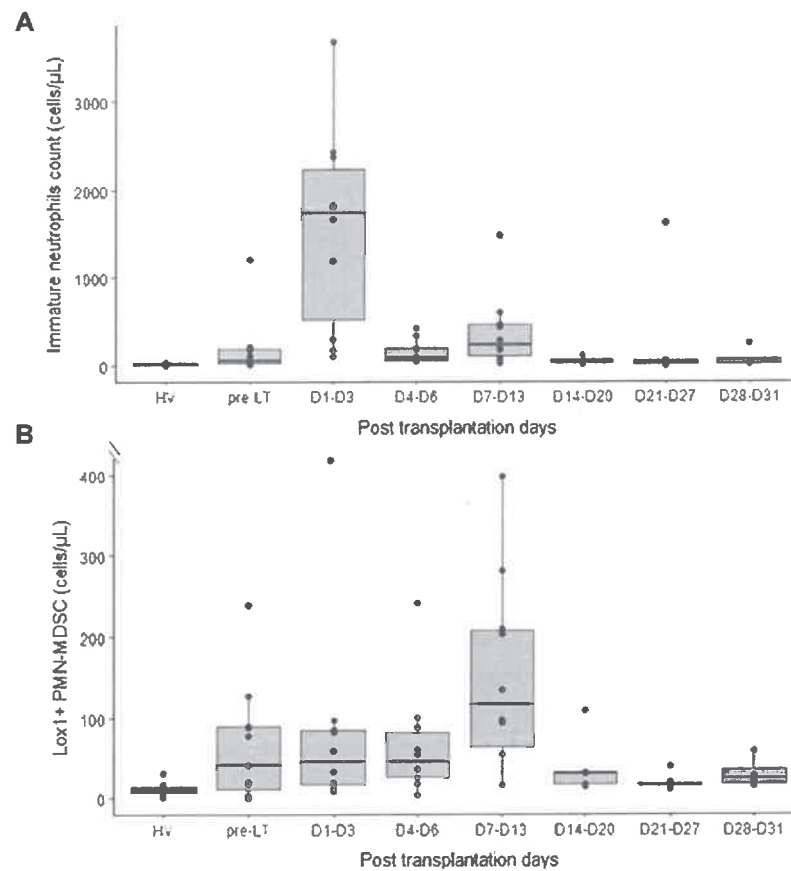
**FIGURE 6**

Monitoring of neutrophils count and neutrophils to lymphocytes ratio (NLR) before and after liver transplantation. (A) Neutrophils count in healthy volunteers (HV,  $n = 15$ ), pre-transplant patients (pre-LT,  $n = 11$ ) and after liver transplantation at different time points (day 1 to day 3,  $n = 10$ ; day 4 to day 6,  $n = 10$ ; day 7 to day 13,  $n = 10$ ; day 14 to day 20,  $n = 5$ ; day 21 to day 27,  $n = 6$ ; day 28 to day 31,  $n = 4$ ). (B) NLR in healthy volunteers (HV), pre-transplant patients (pre-LT) and following transplantation at different time points. Pre-transplant data only concern patients that benefited from transplantation.

superior or equal to 4.33 had a significantly lower survival. Others studies reported that NLR was associated with mortality in cirrhosis, both in patients with MELD score < 20 (Kalra et al., 2017) and in ACLF patients (Bernsmeier et al., 2020). The present results thus confirmed those previous findings. This composite biomarker reflects the balance between granulopoiesis induced by inflammation and lymphopenia. Whereas massive rise in neutrophils occurred in the most severe cirrhotic patients (i.e., at a time of tremendous inflammation), lymphopenia seems to be an earlier event in cirrhosis pathophysiology as it appeared in patients even at compensated stage of cirrhosis. Defect of thymopoiesis and activation-driven cell-death induced by bacterial translocation have been demonstrated to sustain this lymphopenic process (Lario et al., 2013). We extended these results by showing that mostly immature neutrophils and to a lower extent immunosuppressive LOX-1<sup>+</sup> MDSC contributed to neutrophil rise before LT. This suggests that neutrophil and NLR rise before LT was mainly due to massive inflammatory response

and emergency granulopoiesis (including immature cells) in ACLF patients. In contrast, MDSC, usually released in a more chronic manner are less elevated. This may explain why LOX-1<sup>+</sup> MDSC are less correlated to severity than neutrophils (and subsequently NLR) and immature neutrophils. Overall, the present neutrophil results completed previous studies reporting on neutrophil dysfunction in cirrhotic patients including alterations of migration, oxidative burst and phagocytic capacity (Fiuza et al., 2000; Panasiuk et al., 2005; Tritto et al., 2011). Two studies also described reduced CD16 expression on neutrophils (Taylor et al., 2014; Markwick et al., 2015) which characterizes immature neutrophils, cells known to be less efficient in opsonisation and bacteria lysis (Drifte et al., 2013).

Consequently, as observed in sepsis, the most severe cirrhotic patients with marked neutrophil phenotypic may be at higher risk of infection. In line, we observed that patients who died due to sepsis occurrence before LT presented with significantly higher NLR compared with patients who



**FIGURE 7**

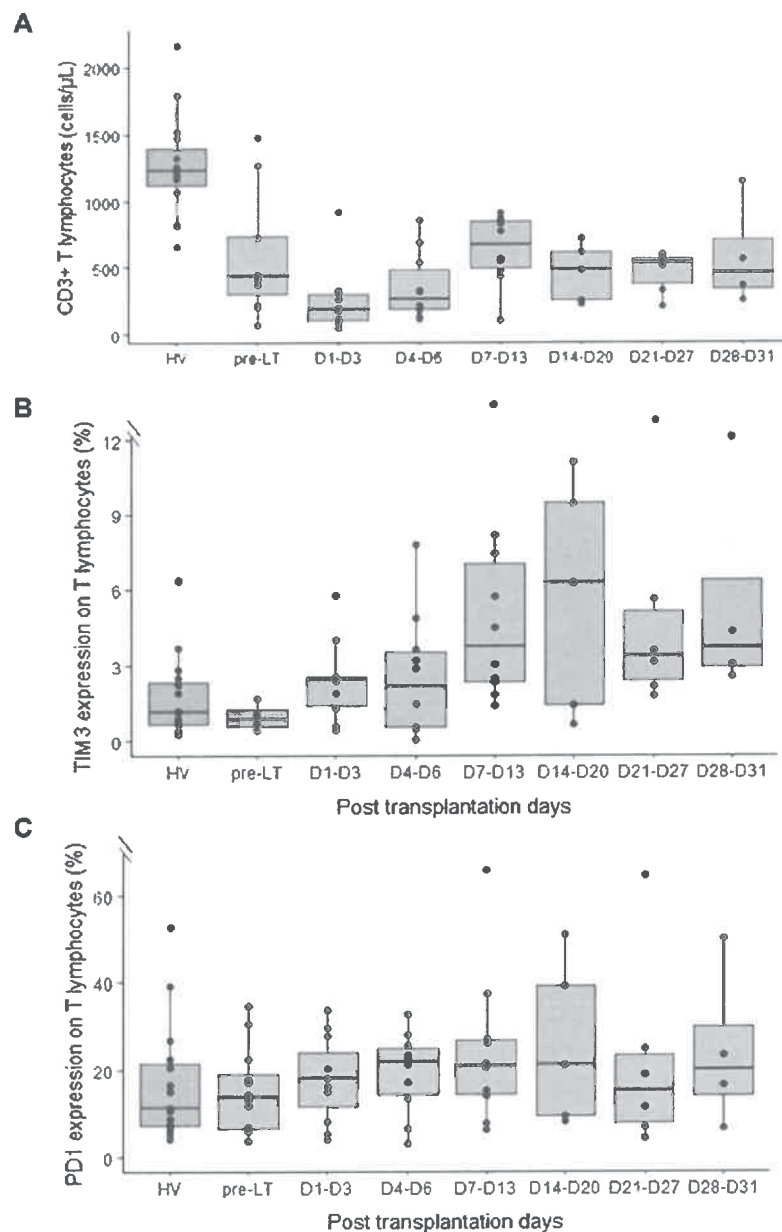
Monitoring of immature neutrophils (CD16<sup>low</sup>) and lectine-type oxidized LDL receptor 1 polymorphonuclear myeloid-derived suppressor cells (LOX1+ PMN-MDSC) in peripheral blood before and after liver transplantation. (A) Immature neutrophils count in healthy volunteers (HV,  $n = 15$ ), pre-transplant patients (pre-LT,  $n = 11$ ) and after liver transplantation at different time points (day 1 to day 3,  $n = 10$ ; day 4 to day 6,  $n = 10$ ; day 7 to day 13,  $n = 10$ ; day 14 to day 20,  $n = 5$ ; day 21 to day 27,  $n = 6$ ; day 28 to day 31,  $n = 4$ ). (B) Number of Lox1+ PMN-MDSC in healthy volunteers (HV), pre-transplant patients (pre-LT) and following transplantation at different time points. Pre-transplant data only concern patients that benefited from transplantation.

survived. In addition, we may hypothesize a role for LOX-1<sup>+</sup> MDSC. Indeed, MDSC are immature neutrophils with immunosuppressive properties as they are potent repressors of T-cell response (Gabrilovich, 2017). They expand under pathological conditions associated with acute or chronic inflammation such as sepsis (Schrijver et al., 2019), cancers (Cassetta et al., 2020), or chronic infections (Pallett et al., 2015). In these contexts, the presence of PMN-MDSC respectively promoted nosocomial infections, cancer progression and persistent viral infections. In the present work, we focused on LOX-1<sup>+</sup> MDSC since LOX-1 is the sole marker of granulocytic MDSC measurable in whole blood (Condamine et al., 2016; Coudereau et al., 2022). Thus, we likely underestimated the total number of MDSC. In hepatology, only one study reported of granulocytic MDSC in alcohol cirrhosis, especially in Child-Pugh B and C patients (Gao et al., 2019). In agreement, the present results showed increased LOX-1<sup>+</sup> MDSC in Child-Pugh C patients. More studies are required to assess the potential role of MDSC in

the pathophysiology of cirrhosis associated immune suppression.

Immune checkpoint receptors are co-inhibitory molecules expressed on immune cells that downregulate the immune response in order to promote homeostasis after immune activation. Engagement of PD-1 and TIM3 pathways on T lymphocytes leads to the inhibition of the second signal of T cell activation. High and sustained expression of the co-inhibitory molecules during persistent antigen stimulation has been shown to promote immune cells exhaustion in cancer, sepsis (Rienzo et al., 2022) and chronic hepatitis B and C (Osuch et al., 2020; Li et al., 2022). Several studies described a slight increase in PD-1 and/or TIM-3 lymphocyte expressions in acute alcoholic hepatitis/cirrhosis (Markwick et al., 2015; Lebossé et al., 2019; Riva et al., 2021; Fadriqela et al., 2022). However, in the present work, PD-1 and TIM3 expressions on T lymphocytes were not significantly different between HV and pre-LT patients and were not associated with cirrhosis severity according to MELD and Child-Pugh scores or with decompensation stages of cirrhosis.





**FIGURE 8**

Monitoring of CD3+ T lymphocytes count and PD-1 and TIM3 expression on CD3+ T lymphocytes in peripheral blood before and after liver transplantation. (A) CD3+ T lymphocytes count in healthy volunteers (HV,  $n = 15$ ), pre-transplant patients (pre-LT,  $n = 11$ ) and following transplantation at different time points (day 1 to day 3,  $n = 10$ ; day 4 to day 6,  $n = 10$ ; day 7 to day 13,  $n = 10$ ; day 14 to day 20,  $n = 5$ ; day 21 to day 27,  $n = 6$ ; day 28 to day 31,  $n = 4$ ). (B) Percentage of TIM3 expression on CD3+ T lymphocytes in healthy volunteers (HV), pre-transplant patients (pre-LT) and following transplantation at different time points. (C) Percentage of PD-1 expression on CD3+ T lymphocytes in healthy volunteers (HV), pre-transplant patients (pre-LT) and following transplantation at different time points. Pre-transplant data only concern patients that benefited from transplantation.

Taken together, before LT, results indicated that out of viral induced cirrhosis, infectious risk in cirrhotic patients would be more induced by immature/suppressive neutrophil subsets and profound lymphopenia rather than by increased immune checkpoint inhibitors expressions.

Regarding post-LT results, the immediate augmentation of NLR after LT is most likely the sum of multiple causes mixing both inflammatory signals and accentuated lymphopenia

induced by immunosuppressant regimen, surgery, ischemia-reperfusion injury and per operative bleeding. This point needs further explorations including a larger number of patients in order to perform multiparametric analyses. As immature neutrophil count rapidly decreased after LT, it most likely does not participate to post-LT infection risk. Interestingly, LOX-1+ MDSC count increased 1 week after surgery. Condamine et al. revealed that these cells accumulated as the result of two

groups of signals: those promoting myelopoiesis (mainly by inflammatory cytokines) and suppressive signals as occurring after transplantation (Condamine et al., 2016). In addition, as MDSC have a role in tissue repair, we may hypothesize that hepatic recruitment of these cells may contribute to counteract liver damage due to ischemia-reperfusion injury. Further exploration would be of utmost interest to associate these observations with liver dysfunction/rejection after transplantation. Not surprisingly, lymphopenia worsened days after transplantation and remained at low values throughout follow-up. Most importantly, we observed a progressive over expression of checkpoint inhibitor expressions on both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. TIM3 expression reached a maximum around 2–3 weeks post-LT and then remained stable. In line, Mysore et al. showed that patients who developed infection during the first year post-LT had elevated co-expressions of PD-1 and TIM3 on T lymphocytes 30 days after LT (Mysore et al., 2018). Accordingly, another study revealed that PD-1 expression on CMV-specific CD8 T cells was elevated preceding CMV reactivation in LT patients (La Rosa et al., 2008). On the opposite side, checkpoint inhibitors might also contribute to immune tolerance in order to prevent graft rejection (Gong et al., 2017). Noteworthy, we noticed that during post-LT follow-up, LOX-1<sup>+</sup> MDSC count and TIM-3 expression tended to peak at the same time (around 2 weeks after LT). One may hypothesize a common inducer for both mechanisms which remained to be investigated. Overall, the current preliminary data deserve further evaluations as they may provide novel understanding of immunosuppression occurring after LT.

Although the present study presents novelties regarding NLR by concomitantly assessing neutrophil (CD16<sup>low</sup>, LOX1<sup>+</sup>) and T lymphocyte (PD-1, TIM-3) subsets before and after transplantation, we acknowledge some limitations of this study. First, as a preliminary study, the number of included patients was low, especially in post-transplant period which did not allow us to associate immune parameters with clinical events after LT (sepsis, rejection). Second, only one single sample was performed pre-LT sample whereas elapsed time until transplantation was heterogeneous. This aspect should be better controlled in forthcoming studies. Lastly, along with cell count and checkpoint inhibitor expression, T cell and neutrophil functionality testing was not performed but may contribute to better understanding of post-LT immunosuppression.

In conclusion, the present study showed that NLR, immature neutrophils and LOX-1<sup>+</sup> MDSC counts along with T lymphocyte count and checkpoint inhibitor expression were altered in cirrhotic patients before and after LT. These data illustrate the potential interest of immune monitoring of cirrhotic patients in the context of LT in order to better define risk of sepsis or rejection. For this purpose, larger cohorts of patients, including phenotypic and functional testing, are now necessary in order to move forward a more personalised care of LT patients.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

## Ethics statement

The studies involving human participants were reviewed and approved by Comité de Protection des Personnes Ile de France XI. The patients/participants provided their written informed consent to participate in this study.

## Author contributions

MC-D, FV, FL, and GM, conceived the original idea. SP, TA, FV, FZ, J-YM, JD, FL, and AR included patients. AR, MH, MC-D, and RC performed all flow cytometry staining and analysis. AR, FV, FL, and GM wrote the manuscript. All authors contributed to the article and approved submitted version.

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## Conflict of interest

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

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2023.1095723/full#supplementary-material>

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**Rôle de l'inflammasome NLRP3 dans l'immunodépression induite par le sepsis : étude chez les patients de réanimation**

**RESUME** : Le sepsis représente un problème de santé mondial ayant un impact significatif sur la morbidité et la mortalité à l'échelle planétaire. Les experts prévoient que l'impact du sepsis sur la santé publique pourrait en faire "la pathologie du 21<sup>e</sup> siècle". Ainsi, en 2017, l'Organisation mondiale de la santé (OMS) a déclaré la nécessité urgente de considérer le sepsis comme une priorité majeure de la santé publique. Le sepsis est une condition complexe impliquant concomitamment une hyper-inflammation et une immunodépression marquée. Bien que les mécanismes à l'origine de ces dérégulations aient été partiellement décrits, il subsiste de nombreuses inconnues quant au lien causal entre ces deux facettes de la même maladie, tant en termes d'aspects mécanistiques que temporels.

Dans le cadre de ce travail, en se basant sur des similitudes entre le cancer et le sepsis en ce qui concerne les mécanismes induisant un état d'immunodépression, nous avons formulé l'hypothèse que **NLRP3 pourrait jouer un rôle dans l'induction des cellules suppressives myéloïdes (MDSCs) au cours du sepsis**. À travers différentes études observationnelles distinctes et complémentaires, pour lesquelles nous avons développé de nouveaux outils adaptés à la recherche clinique, nous avons mis en évidence l'association entre l'activation de NLRP3 et l'apparition de MDSCs (ainsi que les marqueurs habituels de l'immunodépression). Après démonstration d'une causalité délétère dans des modèles expérimentaux (travail en cours), interrompre le lien entre l'inflammation persistante induite par NLRP3 et l'induction de MDSCs pourrait ouvrir de nouvelles voies pour des stratégies thérapeutiques, que ce soit en bloquant la voie NLRP3 ou en inhibant l'induction de MDSCs (stratégies thérapeutiques aujourd'hui disponibles dans d'autres circonstances cliniques). Ces thérapies pourraient reposer sur des biomarqueurs parmi ceux étudiés dans le cadre de cette étude, dans une approche de médecine personnalisée.

**MOTS CLES** : Sepsis, inflammasome NLRP3, immunosuppression, MDSCs, cytométrie en flux, ASC speck

**ABSTRACT**: Sepsis stands as a pressing global health concern, exerting a significant toll on morbidity and mortality across the globe. Experts predict that the projected burden of sepsis on public health could make it "the quintessential medical disorder of the 21st century." In 2017, the World Health Organization (WHO) recognized the urgency of addressing sepsis as a paramount priority in public health. Sepsis involves the intricate interplay of inflammation and profound immunosuppression. Despite comprehensive elucidation of the primary mechanisms driving excessive inflammation and profound immunosuppression, a substantial gap in understanding persists concerning the intricate causal relationship uniting these dual facets within the same disease. Both mechanistic and temporal dimensions of this connection require further exploration.

In the present study, leveraging parallels between cancer and sepsis concerning the mechanisms sustaining immunosuppression, a hypothesis emerged implicating NLRP3 in the induction of MDSCs. Through an array of distinct and complementary observational studies, novel tools tailored for clinical research were designed. These endeavors collectively illuminated the correlation between NLRP3 activation and the emergence of MDSC along with its accompanying immunosuppression. With ongoing work focusing on establishing a conclusive causal relationship through experimental models, there is potential to unravel the detrimental connection between persistent NLRP3-driven inflammation and MDSCs induction. This revelation opens avenues for innovative therapeutic strategies. The disruption of the link between enduring NLRP3-induced inflammation and MDSCs induction emerges as a promising approach. This could be achieved by intervening in the NLRP3 pathway or inhibiting MDSCs generation. Such therapeutic modalities, potentially guided by biomarkers scrutinized within the framework of this study, could manifest as a cornerstone of personalized medicine.

**KEY WORDS**: Sepsis, NLRP3 inflammasome, immunosuppression, MDSCs, flow cytometry, ASC speck

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