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Diagnostic profiling of MDSCs in sepsis

Schrijver Irene

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Irene T. Schrijver

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Jury

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Scientific summary

With 50 million cases and 11 million deaths per year, sepsis is one of the leading causes of death worldwide. Sepsis is caused by a dysregulated host response to an infection, which consists of concurrent inflammatory and immunosuppressive reactions. Myeloid-derived suppressor cells (MDSCs) are immature myeloid cells characterized by their immunosuppressive properties, rising in inflammatory diseases. MDSCs are subdivided into polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs) based on their relationship with mature PMNs and monocytes. It is unknown how their level varies over time in human sepsis. We hypothesized that MDSCs have diagnostic and prognostic potential in sepsis patients. In this thesis, we aimed to illustrate the dynamic profile of MDSCs in critically ill patients in relation to sepsis severity, secondary infections, and mortality.

Four study cohorts were used: 1) healthy subjects infused with endotoxin, 2) non-infectious, critically ill patients without antibiotic therapy (PIPOVAP study), 3) hospitalized coronavirus 2019 (COVID-19) patients and, 4) sepsis patients with multi-organ dysfunction syndrome (MODS) (INCLASS study). Serial samples of peripheral blood were used to quantify MDSCs by flow cytometry and FlowSOM to avoid gating biases. M-MDSCs were defined as HLA-DR^{low} monocytes, and PMN-MDSCs as CD16^{low} granulocytes. Furthermore, we quantified up to 49 cytokines by multiplex bead assay, and analysed the functionality of monocytes and dendritic cells (DCs) in ex vivo stimulated whole blood by intracellular cytokine staining and flow cytometry.

Endotoxin infusion in healthy subjects induced a quick, massive, and transient accumulation of MDSCs in peripheral blood. PMN-MDSCs increased 40-fold to reach up to 43% of all granulocytes 6-8 hours after infusion. M-MDSCs increased 10-fold 8 hours after endotoxin infusion comprising more than half of total monocytes. After 24 hours, PMN-MDSCs and M-MDSCs returned to baseline levels. In patients enrolled in the PIPOVAP, COVID-19, and INCLASS studies, the blood concentration of PMN-MDSCs and M-MDSCs were high at study inclusion and correlated to disease severity. In the PIPOVAP study, high levels of M-MDSCs also correlated to the development of secondary gram-negative infections. High MDSCs were associated with mortality in non-infectious critically ill patients (PIPOVAP), but with survival in sepsis patients with MODS (INCLASS). MDSCs correlated with the concentrations of blood cytokines, chemokines, and growth factors in the PIPOVAP and COVID-19 studies. Finally, 3 months after the COVID-19 diagnosis, irrespective of initial disease severity, MDSCs were back to normal levels whilst the production of cytokines by blood cells was still largely affected.

Altogether, we report that MDSCs quickly rise during endotoxemia and are associated with sepsis severity. Thus, MDSCs might represent valuable tools to screen vulnerable patients in the intensive care. Additionally, we show for the first time that a high level of MDSCs is associated with improved outcomes in a subset of extremely ill sepsis patients with MODS. Overall, MDSCs may represent sepsis biomarkers and targets of therapy. To pursue clinical development, the quantification method of MDSCs in patients need to be standardized, and the function and plasticity of MDSCs during sepsis should be further explored.

Résumé scientific

Avec 50 millions de cas et 11 millions de décès par an, le sepsis est l'une des principales causes de décès dans le monde. Le sepsis est causé par une réponse dérégulée de l'hôte en réponse à une infection, qui consiste en des réactions inflammatoires et immunosuppressives concomitantes. Les cellules suppressives d'origine myéloïde (MDSCs) sont des cellules myéloïdes immatures caractérisées par leurs propriétés immunosuppressives. Les MDSCs sont subdivisées en MDSCs polymorphonucléaires (PMN-MDSCs) et MDSCs monocytaires (M-MDSCs) en fonction de leur relation avec les PMNs et monocytes matures. L'évolution des MDSCs au cours d'un sepsis humain est très peu documentée. Pour combler cette lacune, dans cette thèse nous avons analysé l'expression des MDSCs dans le sang en regard de la sévérité du sepsis et de paramètres immunologiques.

Quatre études de cohortes ont été utilisées: 1) des sujets sains perfusés avec de l'endotoxine, un composant de bactéries gram-négatives qui permet de mimer une infection bactérienne (**étude endotoxin**), 2) des patients non infectieux, hospitalisés aux soins intensifs (SI) (**étude PIPOVAP**), 3) des patients coronavirus 2019 hospitalisés (**étude COVID-19**) et 4) des patients sepsis présentant un syndrome de défaillance multiviscérale associé à une mortalité d'environ ~70% (**étude INCLASS**). Des échantillons sériels de sang périphérique ont été collectés pour quantifier les MDSCs. En outre, nous avons mesuré une cinquantaine de cytokines, chimiokines et facteurs de croissances et analysé la fonctionnalité des globules blancs du sang complet.

La perfusion d'endotoxine chez des sujets sains induisait une accumulation rapide, massive (pouvant aller jusqu'à 30-40% de tous les globules blancs) et transitoire de MDSCs. Après 24 heures, les MDSCs étaient revenus à un taux normal. Les MDSCs étaient élevées chez les patients inclus dans les études PIPOVAP, COVID-19 et INCLASS. Cette augmentation corrélait avec la gravité de la maladie et, dans l'étude PIPOVAP, avec le développement d'infections nosocomiales. Des taux élevés de MDSCs étaient associés à la mortalité chez les patients non-infectieux hospitalisés aux SI. Par contre, une accumulation de MDSCs était associée à un devenir favorable chez les patients sepsis ayant développé un syndrome de défaillance multiviscérale. Finalement, chez les patients COVID-19 ré-analysés 3 mois après leur hospitalisation, les MDSCs étaient normales alors que certains dysfonctionnements immunitaires étaient détectés. Ceux-ci qui pourraient être associés au syndrome de « COVID long ».

En résumé, nous rapportons que les MDSCs augmentent rapidement et fortement dans le sang d'individus exposés à un composant de bactérie, et qu'elles sont associées à la sévérité du sepsis. Ainsi, les MDSCs ont un potentiel diagnostique et pronostique chez les patients atteints de sepsis. Par ailleurs, les MDSCs pourraient représenter des cibles d'immunothérapie, d'autant plus que des traitements ciblant les MDSCs sont actuellement testées dans le cadre de cancers.

Summary for the general public

The immune system protects the human body against infections caused by microorganisms, like viruses and bacteria. White blood cells are one important part of the immune system. White blood cells kill invading organisms as well as warn and activate other white blood cells to increase host defences. However, sometimes the immune system does not respond appropriately to an infection, or it cannot control it. When that happens, white blood cells injure tissues through collateral damage while trying to contain the infection. This can lead to organ failure, which is associated with a high mortality rate. When this happens, it is called sepsis. With 50 million cases and 11 million deaths per year, sepsis is a global health priority. During sepsis, white blood cells can respond too strongly, stop functioning, or even repress the function of other cells. This is called immunosuppression. Immunosuppression can cause long-term effects in recovered sepsis patients, such as the development of new infections and heart diseases.

In this thesis, we were interested in a particular type of white blood cells called myeloid-derived suppressor cells (MDSCs). MDSCs restrict the function of other white blood cells. In short, they are immunosuppressive. Healthy people have very few MDSCs, but patients with inflammatory diseases such as cancer, have high levels of MDSCs. How fast MDSCs respond after an infection, and what type of impact these cells have during sepsis are largely unknown. We aimed to give an in-depth view of the dynamics of MDSCs in conditions of inflammation and infection.

We set up four studies to address our aim. Blood was collected from individuals at different time intervals to measure MDSCs and other immune parameters. The first study was with healthy volunteers who were injected with bacterial compounds, which trigger white blood cells mimicking bacterial sepsis. We observed that MDSC levels rise strongly 2 hours after infusion and remained high until 8 hours after the injection. MDSCs returned to normal levels after 24 hours. This tells us that MDSCs are very fast responders when the host is triggered by a bacterial compound. In the second study, we tested critically ill patients in the Intensive Care Unit (ICU) without an infection. We noticed that patients with high levels of MDSCs were more likely to become infected and to die. We assume that MDSCs suppressed the function of anti-microbial white blood cells, increasing the risks of infection. In the third study, we looked at COVID-19 patients. COVID-19 patients can develop sepsis, which is one of the reasons why some must be hospitalized. Patients admitted to the ICU had more MDSCs than patients who did not require admission to the ICU. In the fourth study, we analysed severe sepsis patients with multiple organ dysfunctions. Patients with high levels of MDSCs were more likely to survive. Most likely, MDSCs were beneficial in these severe patients because MDSCs were counterbalancing the otherwhite blood cells that were overreacting and damaging.

To conclude, this thesis shows 1) how MDSCs are modulated during sepsis, and 2) that MDSCs can be helpful or hurtful depending on the degree of sepsis severity. We propose to use MDSCs as signals of sepsis severity, and possibly as targets of therapy as currently tested in cancer patients.

Résumé destiné à un large public

Le système immunitaire protège notre organisme contre les infections par les bactéries et les virus. Les globules blancs présents dans le sang sont des éléments vitaux du système immunitaire car ils peuvent tuer les bactéries et les virus. Par ailleurs ils activent d'autres globules blancs, augmentent les défenses de l'hôte, et confèrent la mémoire immunitaire protégeant de réinfections. Il arrive que le système immunitaire ne réponde pas de façon appropriée ou ne peut pas contrôler l'infection. Dans ce cas, les globules blancs peuvent causer des dommages collatéraux aux tissus. Cela peut entraîner une défaillance d'organe, associée à un taux de mortalité élevé. C'est ce qu'on appelle un sepsis.

Avec 50 millions de cas et 11 millions de décès par an, le sepsis est une priorité sanitaire mondiale. Lors d'un sepsis, les globules blancs peuvent cesser de fonctionner ou empêcher le fonctionnement d'autres cellules. C'est ce qu'on appelle l'immunosuppression. Elle peut avoir des effets à long terme chez les patients ayant guéris d'un sepsis, comme le développement d'infections et ou de maladies cardiaques.

Dans cette thèse, nous nous sommes intéressés aux globules blancs appelés cellules myéloïdes suppressives (MDSCs). Les MDSCs restreignent la fonction des autres globules blancs, elles sont donc immunosuppressives. Les personnes en bonne santé, contrairement à celles atteintes de maladies inflammatoires y compris le cancer, ont très peu de MDSCs dans leur sang. Très peu étant connu quant à l'implication des MDSCs dans le sepsis, notre objectif était de fournir une analyse approfondie de la dynamique des MDSCs dans des conditions d'infection.

Nous avons mis en place quatre études pour répondre à notre objectif. Le sang a été prélevé à intervalles réguliers pour mesurer les MDSCs et d'autres paramètres immunologiques. La **première étude** portait sur des volontaires sains recevant une injection d'un morceau de bactérie simulant un sepsis à bactérie. Les MDSCs augmentaient rapidement, fortement, et transitoirement revenant à un niveau normal 24 heures après l'injection. Dans la **deuxième étude**, nous avons testé des patients sans infection, admis aux soins intensifs (SI). Nous avons remarqué que les patients avec beaucoup de MDSCs développaient plus d'infections nosocomiales, dont des infections mortelles. Nous pensons que les MDSCs supprimaient la fonction des globules blancs antimicrobiens, augmentant les risques d'infection. Dans la **troisième étude**, nous avons examiné des patients atteints de COVID-19. Les patients COVID-19 admis aux SI avaient plus de MDSCs que ceux n'ayant pas besoin d'être admis aux SI. Dans la **quatrième étude**, nous avons analysé des patients atteints de sepsis grave présentant une défaillance de plusieurs organes. Ceux avec beaucoup de MDSCs avaient un pronostique plus favorable. Nous pensons que, dans ce contexte particulièrement sévère (environ 70% des patients décédaient), les MDSCs contrebalançaient la réponse excessive de globules blancs et les dommages collatéraux subséquents.

Pour conclure, cette thèse décrit comment les MDSCs sont modulées pendant un sepsis, et que les MDSCs peuvent être utiles ou néfastes selon le degré de sévérité des patients infectés. Les MDSCs pourraient être utilisées comme indicateur de gravité du sepsis, et éventuellement comme cibles de traitements tels que ceux ciblant les MDSCs testés actuellement chez les patients cancéreux.

Lekensamenvatting

Het lichaam wordt door zijn eigen immuunsysteem beschermd tegen indringers zoals virussen en bacteriën. Witte bloedcellen spelen daarbij een belangrijke rol. Ze doden indringers en waarschuwen en activeren andere witte bloedcellen om de afweer te versterken. Soms reageren de witte bloedcellen te sterk of krijgen ze de infectie niet onder controle. In een poging de infectie te bestrijden beschadigen witte bloedcellen dan de weefsels. Deze weefselschade kan leiden tot uitval van organen. De kans op overlijden is hierbij groot. Dit ernstige ziektebeeld wordt sepsis genoemd. Jaarlijks ontwikkelen wereldwijd ongeveer 50 miljoen mensen sepsis waarvan er 11 miljoen overlijden. Na een doorgemaakte sepsis is er kans op langdurige gezondheidsproblemen, zoals bijvoorbeeld nieuwe infecties en hartaandoeningen. Dit komt doordat tijdens sepsis, witte bloedcellen niet alleen te sterk reageren, maar ook stoppen met functioneren en de functie van andere cellen onderdrukken. Dit laatste wordt immunosuppressie genoemd en kan lang aanhouden nadat patiënten van sepsis hersteld zijn.

In dit proefschrift onderzochten we een bepaald type witte bloedcel, namelijk “myeloid-derived suppressor cells” (MDSC's). MDSC's onderdrukken de functie van andere witte bloedcellen en werken dus immunosuppressief. In tegenstelling tot gezonde mensen hebben patiënten met ontstekingsziekten zoals kanker of auto-immuunziekten veel MDSC’s. Het is echter nog onbekend hoe snel MDSC’s reageren op een infectie en welke rol zij spelen tijdens sepsis.

We hebben vier onderzoeken uitgevoerd om hierin meer inzicht te krijgen. **De eerste studie** betrof gezonde vrijwilligers die stukjes van bacteriën toegediend kregen. Dit activeert witte bloedcellen en is daarom vergelijkbaar met bacteriële sepsis. We constateerden dat de MDSC-waarden in het bloed 2 uur na toediening sterk stegen en vervolgens verhoogd bleven tot 8 uur na toediening. Na 24 uur keerden MDSC-waarden terug naar normaal. Hieruit kunnen we concluderen dat MDSC's zeer snel reageren wanneer het lichaam wordt geïnfecteerd door een bacterie. **De tweede studie** betrof ernstig zieke patiënten die initieel geen infectie hadden op de intensive care (IC). In deze groep zagen we dat patiënten met hoge MDSC-waarden een grotere kans hadden om een infectie te ontwikkelen en hierdoor te overlijden. Waarschijnlijk onderdrukten MDSC's de functie van andere infectie-bestrijdende witte bloedcellen, waardoor het infectierisico toenam. **De derde studie** betrof COVID-19-patiënten. Sommige COVID-19-patiënten ontwikkelen sepsis en moeten worden opgenomen in het ziekenhuis. Wij constateerden dat COVID-19-patiënten die op de IC waren opgenomen, meer MDSC's hadden dan patiënten die op de verpleegafdeling konden blijven. Ten slotte, onderzochten we in **vierde studie** patiënten met ernstige sepsis bij wie meerdere organen waren uitgevallen. Hier constateerden we dat patiënten met hoge MDSC-waarden een grotere overlevingskans hadden. Hoogstwaarschijnlijk waren MDSC's beschermend bij deze ernstig zieke patiënten omdat MDSC’s de te hevig reagerende witte bloedcellen onder controle brachten.

Concluderend toont dit proefschrift hoe MDSC’s zich gedragen tijdens sepsis, en dat MDSC’s therapeutisch of ziekmakend kunnen werken, afhankelijk van de mate van ontsteking in het lichaam. MDSC-waarden kunnen in de toekomst mogelijk gebruikt worden als maat voor ernst van sepsis, en als aangrijpingspunt voor medicatie.

Abbreviations

ARDS	acute respiratory distress syndrome
ATRA	all-trans-retinoic acid
AUROC	area under the receiver operating characteristic curve
CCL	C-C motif chemokine ligand
CCR	C-C chemokine receptor
CDSs	cytosolic DNA sensors
cGAS	cyclic GMP–AMP synthase
ChiP-seq	chromatin immonopreciptation sequencing
CLP	cecal ligation and puncture
CLRs	C-type lectin receptors
CMV	cytomegalovirus
COVID-19	coronavirus disease 2019
COX	cyclooxygenase
CpG ODN	CpG Oligodeoxynucleotides
CRP	C-reactive protein
CSF	colony stimulating factor
CyTOF	cytometry by time of flight
DAMPs	danger-associated molecular patterns
DCs	dendritic cells
DC	discovery cohort
EEG	electroencephalogram
e-MDSCs	early stage MDSCs
eo-MDSCs	eosinophilic MDSCs
EWAS	epigenomic-wide association study
FoxP3	forkhead box P3
GF	growth factor
GM-CSF	granulocyte-macrophage-CSF
gp130	glycoprotein 130
Gr1	granulocyte receptor-1
GSDMD	gasdermin D
GWAS	genomic-wide association study
HAI	hospital-acquired infection
HAP	hospital-acquired pneumonia
HATs	histone acetyltransferases
HCAP	health-care-associated pneumonia
HDACs	histone deacetylases
HLA-DR	human leukocyte antigen – DR isotype
HSP	heat shock protein
ICU	intensive care unit

IFNγ	interferron-gamma
IGs	immature granulocytes
IKKs	IκB kinases
IL	interleukin
LDGs	low-density granulocytes
lncRNAs	long non-coding RNAs
LBP	lipopolysaccharide binding protein
LPG2	laboratory of genetics and physiology 2
LPS	lipopolysaccharide
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
MAS	macrophage activation syndrome
MBL	mannose binding lectin
M-CSF	macrophage-CSF
MDP	muramyl dipeptide
MDSCs	myeloid-derived suppressor cells
Mincle	macrophage inducible C-type lectin
miRNA	microRNA
MRI	magnetic resonance imaging
mRNA	messenger RNA
MDA5	melanoma differentiation associated gene 5
M-MDSCs	monocytic MDSCs
MODS	multi-organ dysfunction syndrome
MR	mannose receptor
MS	mass spectometry
MTP	muramyl tripeptide
MyD88	myeloid differentiation primary response 88
n/a	not available
NF-κB	nuclear factor-κB
NK	natural killer
NLR	neutrophil to lymphocyte ratio
NLRs	nucleotide-binding oligomerization domain-like receptors
NO	nitric oxide
Nrf2	nuclear factor erythroid-derived 2-like 2
PAMPs	pathogen associated molecular patterns
PCT	procalcitonin
PD-1	programmed cell death 1
PDC	pyruvate dehydrogenase complex
PDL-1	programmed cell death ligand 1
PDE5	phosphodiesterase type 5
PMNs	polymorphonuclear cells
PMN-MDSCs	polymorphonuclear MDSCs

PPRs	pattern recognition receptors
PRMs	pattern recognition molecules
RCT	randomized control trials
REMAP-CAP	randomised, embedded, multi-factorial, adaptive platform trial for community-acquired pneumonia
RIG-1	retinoic acid inducible gene I
RNA-seq	RNA sequencing
RNS	reactive nitrogen species
RLRs	retinoic acid-inducible gene-I-like receptors
ROS	reactive oxygen species
RTC	randomized control trial
SEB	staphylococcal enterotoxin B
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SIRS	systemic inflammatory response syndrome
SNP	single nucleotide polymorphisms
SOFA	sequential organ failure assessment
SRS	sepsis response signature
STAT	signal transducer and activator of transcription
TCA	tricarboxylic acid cycle
TGF	transforming growth factor
TLRs	Toll-like receptors
TNF	tumor necrosis factor
TRAIL-R	TNF-related apoptosis-inducing ligand receptor
Tregs	T regulatory cells
UO	urine output
VAP	ventilator-associated pneumonia
VC	validation cohort
VEGF	vascular endothelial-GF
WES	whole-exome sequencing
WGS	whole-genome sequencing

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1. Introduction

1.1 Sepsis

1.1.1 History and definition

Early records show ancient civilizations' awareness of sepsis. As early as 4000 years ago, the Egyptians stated that the intestine contained a dangerous substrate that could settle anywhere in the body. The Ancient Romans worshipped the goddess *Febris* – goddess of fever and malaria – and believed that tiny invisible animals in the blood caused "difficult diseases". Hippocrates noted that fever with poor circulation was dangerous for the patient [1-3]. Currently, the mechanisms behind sepsis are still largely unknown and thus the definition remains up for debate (see **Figure 1** for historical definitions of sepsis).

460 – 370 BC	**Hippocrates:** "when continuing fever is present, it is dangerous if the outer parts are cold, but the inner parts are burning hot." [1]
116 - 27 BC	**Varro:** "… minute creatures [*animalia minuta*], which cannot be seen by the eye, which float in the air and enter the body through the mouth and nose and there cause serious diseases." [2]
1469 - 1527	**Machiavelli:** "As the physicians say of hectic fever, that in the beginning of the malady it is difficult to detect but easy to treat, but in the course of time, having been neither detected nor treated in the beginning, it becomes easy to detect but difficult to treat." [4]
1849 - 1919	**Osler:** "Except on few occasions, the patient seems to die from the body's response to infection rather than from it." [5, 6]
1914	**Schottmueller:** "Sepsis is a state caused by microbial invasion from a local infectious source into the bloodstream which leads to signs of systemic illness in remote organs." [7]
1991/2001	**Sepsis-1/Sepsis-2: "**the term sepsis represents the systemic inflammatory response to the presence of infection", "'severe sepsis' is defined as sepsis associated with organ dysfunction, hypoperfusion or hypotension" and "'septic shock is defined as sepsis with arterial hypotension despite fluid resuscitation". [8, 9]
2016	**Sepsis-3:** "Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection." "Septic shock is a subset of sepsis in which underlying circulatory and cellular/metabolic abnormalities are profound enough to substantially increase mortality". [10]

Figure 1. Historical definitions of sepsis.

In 2016, a taskforce of nineteen specialists defined sepsis as "a life-threatening organ dysfunction caused by a dysregulated host response to infection" and defined septic shock as "a subset of sepsis in which particularly profound circulatory, cellular, and metabolic abnormalities are associated with a greater risk of mortality than with sepsis alone". Clinically, sepsis was defined as an increase by two or more points of the Sequential Organ Failure Assessment (SOFA) score (**Table 1**) and septic shock is defined as sepsis plus hyperlactatemia (> 2 mmol/L) and a need for vasopressors [10]. There have been many attempts at defining sepsis and the latest "Sepsis-3" will not be the final definition. The main difference between the Sepsis-3 and the antecedent Sepsis-1 (1991) and Sepsis-2 (2001) is the focus on organ failure versus the systemic inflammatory immune syndrome (SIRS). The SIRS criteria include tachycardia, tachypnea, hyperthermia or hypothermia, and abnormalities in peripheral white blood cell count. Sepsis-3 also dropped the term "severe sepsis" (SIRS with the addition of organ dysfunction). Using the SOFA score, used in Sepsis-3, compared with the SIRS criteria, used in Sepsis-1/2, makes the definition more specific but less sensitive for mortality [11].

Table 1. Sequential Organ Failure Assessment (SOFA) score

	Score				
Organ system	**0**	**1**	**2**	**3**	**4**
Respiration (PaO2/FiO2)	≥400	300-399	200-299	100-199*	<100*
Coagulation (platelets, x103/μL)	≥ 150	<150	<100	<50	<20
Liver (Bilirubin, mg/dL)	<1.2	1.2-1.9	2.0-5.9	6.0-11.9	>12.0
Cardiovascular	MAP ≥ 70 mm Hg	MAP <70 mm Hg	Dopamine <5 or dobutamine	Dopamine 5.1-15, (nor) epinephrine ≤0.1	Dopamine > 15, (nor) epinephrine > 0.1
Central nervous system (Glasgow Coma scale)**	15	13-14	10-12	6-9	<6
Renal (creatinine mg/dL, urine output (UO), mL/day)	<1.2	1.2-1.9	2.0-3.4	3.5-4.9 or UO < 500	>5.0 or UO < 200

Adapted from Singer et al. [10] *with respiratory support, **Glasgow coma scale scores range from 3-15; higher score indicates better neurological function, FiO2: fraction of inspired oxygen; PaO2: partial pressure of oxygen; MAP: mean arterial pressure.

1.1.2 Epidemiology

It is challenging to quantify the incidence and mortality rates of sepsis. An extensive global study performed in 2017 estimates that 48.9 million people develop sepsis every year with a mortality rate between 7-40%, which makes sepsis a leading cause of death globally [10, 12, 13]. The incidence of sepsis is increasing partly because of: 1) increasing antibiotic resistance, 2) a reporting bias due to a higher awareness of sepsis, and 3) an increase of obesity and aging (both risk factors for sepsis) in Western populations [14-16].

Pneumonia is the primary cause of sepsis (40% of sepsis patients), followed by abdominal, genitourinary, primary bacteremia, and skin or soft tissue infections [17, 18]. Risk factors for sepsis are similar to general infection development: immunosuppression (because of medication or disease), cancer, invasive catheters and tubes, and age. There is a slightly higher risk for men to develop sepsis, but gender does not influence outcome [19]. Obesity is often considered a risk factor for sepsis, however,

antibiotics are often underdosed in this patient-category, which might bias this recurrent finding [20]. Specific risk factors include host genetics. Adopted children showed higher risk to die from infections when a biological parent had died from infections. Twins and siblings studies confirm the heritability of sepsis susceptibility [21-25]. Furthermore, certain polymorphisms in essential genes, including Toll-like receptors, cytokines, and coagulation factors are associated with a higher risk of developing sepsis. However, genetic research in the sepsis field presents a high risk of bias and therefore, results should be interpreted carefully.

Survivors of sepsis often suffer from long-term psychological and physical problems [26]. More than half of patients suffer from anxiety and depression, memory loss and confusion [27, 28]. Furthermore, the ongoing dysregulation of the immune system can persist years after the initial sepsis episode. This results in an increased risk of recurrent infections, cardiovascular adverse events, hospital re-admissions, and death [29, 30].

Most research, including this thesis, focusses on sepsis and its treatment available in high-income countries but 85% of sepsis cases arise in low and middle-income countries, with a peak in sub-Saharan Africa (**Figure 2**) [26]. Thus, it should be emphasized that the cheapest and quickest way to save lives from sepsis is through infection prevention in low and middle-income countries by vaccinating, treating HIV/AIDS, and improving hygiene and safety of birth conditions [31].

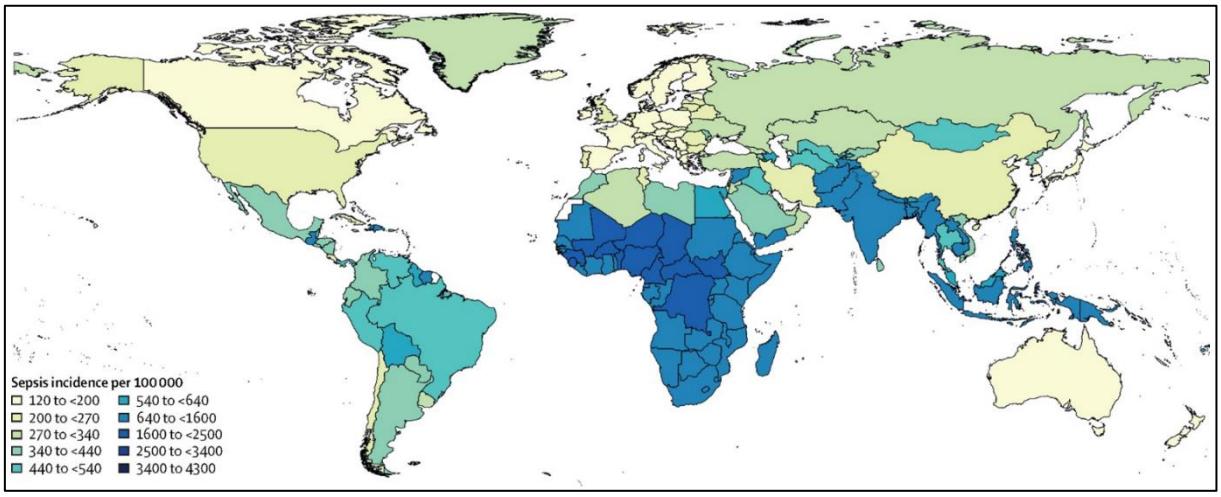

Figure 2. Global Sepsis incidence. Incidence estimated in 2017 and standardized by age. Figure from [26].

1.1.3 Pathophysiology

1.1.3.1 Immune cell activation

When a pathogen invades the human body, an array of immune cells respond. Microorganisms express conserved structures called pathogen associated molecular patterns (PAMPs), which include components of the microbial cell wall (bacterial lipopolysaccharides (LPS or endotoxin) and peptidoglycans, fungal mannose), flagellin and nucleic acids (single and double stranded RNA, DNA). PAMPs are sensed by pathogen recognition receptors (PRRs) expressed by immune cells. Furthermore, host molecules released after cellular damage or stress, such as DNA or ATP, are also sensed by PPRs.

These molecules are called danger associated molecular patterns (DAMPs) [32]. When PRRs bind to either PAMPS or DAMPs, they trigger intracellular signalling pathways that shape host response.

Richard Pfeiffer (1858–1945) noted that guinea pigs would die after infusion with heat killed *V. cholera, S.* Typhi and *H. influenzae*. He hypothesised that their death was caused by a toxic bacterial component, which he named endotoxin. Later, endotoxin was found to be an essential component the gram-negative bacterial cell wall consisting of LPS and nowadays the terms LPS and endotoxin are used in synonym. Both myeloid cells and lymphoid cells respond to LPS with a powerful immune response but for long, it was unclear which receptor –or receptors– were responsible for this recognition. The discovery of Toll in *Drosophila* by Anderson and Nusslein-Volhard –an effort later rewarded with the Nobel prize– resulted in the discovery that Toll-like receptors (TLR) 4 can recognise LPS. It is now known that the sensing of LPS involves other receptors including the LPS binding protein (LBP) and CD14, which work together to transfer LPS to TLR4, which, together with MD-2, triggers protein-tyrosine kinase signalling activating the host response (**Figure 3**) [33].

Currently, ten TLRs have been described in humans (**Table 2**). TLRs are present on the cell surface, except for TLR 3, 7, 8 and 9, which are present in the endosome where they recognise single and double strained RNA and DNA [34-36]. Furthermore, TLR4 can shuttle from the cell membrane to the endosome. Upon activation, TLRs recruit adapter molecules, either MyD88 (myeloid differentiation primary response 88) or TRIF (Toll/IL-1R (TIR)-domain-containing adaptor protein inducing IFN). MyD88 interacts with all TLRs except TLR3, while TRIF interacts with TLR3 and endosomal TLR4. MyD88 and TRIF activation set off a signalling pathway that activates NF-κB, interferon regulatory factors (IRFs), and mitogen activation protein kinases (MAPKs) (**Table 2, Figure 3**). This activation results in the production of inflammatory cytokines, chemokines and type I interferons (IFNs). The ligands and functions of TLR10 are still under debate, but, in contrast to the other TLRs, TLR10 seems to act immunosuppressive after activation [37].

Overall 5 main families of PRRs have been described including: TLRs, C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene-I (RIG)-like receptors (RLRs) and cytosolic DNA sensors (CDSs) (**Table 2, Figure 3**) [34-36]. PRRs are strategically expressed to sense microbial invasion in the extracellular milieu (TLRs, CLRs) or inside cells (NLRs, RLRs, CDSs).

The family of CLRs comprises more than 1000 different receptors, which can be either soluble or cell membrane bound. Soluble CLRs, e.g., mannose binding lectin (MBL), can opsonize pathogens to stimulate phagocytosis. Membrane-bound CLRs are mainly found on myeloid cells and dendritic cells (DCs) where they recognize carbohydrate structures, including fungal proteins (**Table 2**). Membrane-bound CLRs include mannose receptor (MR), dectin-1, dectin-2, and macrophage inducible C-type lectin (mincle) that after activation trigger SYK kinase resulting in a downstream signalling cascade that results in the production of cytokines and other mediators (**Figure 3**).

NLRs are present in the cytoplasm and oligomerize after recognizing components of intracellular bacteria, viruses and DAMPs. Some oligomerized NLRs, e.g., NOD1 and NOD2, can activate NF-κB and

MAPKs through recruiting RIPK2, which results in the production of inflammatory cytokines, chemokines and IFNs. Other oligomerized NLRs, e.g., NLRP1, NLRP3 and NLRC4, form cytosolic cellular structures called inflammasomes, which are responsible for the activation of the inflammatory caspase-1 (**Figure 3**). Caspase-1 cleaves pro-IL-1β and pro-IL-18 into IL-1β and IL-18, and gasdermin D (GSDMD) in N-and C-terminus fragments. GSDMD-Nter oligomerize in the cell membrane, which results in formation of pores that facilitate the release of IL-1β and IL-18 and cellular content. This phenomenon is called pyroptosis. Of note, cytosolic LPS from gram-negative bacteria is sensed through caspase-4/5 in human and caspase-11 in mice, called noncanonical inflammasome, to trigger a pathway similar to that activated by caspase-1 and the release of IL-1β and IL-18.

RLRs are present in the cytoplasm and recognize viral RNA. The RLR family comprises three main members: retinoic acid inducible gene I (RIG-I), melanoma differentiation associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). Activated RIG-I and MDA5 undergo a conformational change that allows binding to their adaptor protein MAVS (mitochondrial antiviral-signaling protein) that leads to downstream activation of NF-κB, MAPKs and IRFs signalling pathways. LGP2 functions as a regulatory molecule of RIG-I and MDA5 [38, 39].

CDSs, the youngest family of PRRs, are present in the cytoplasm where they recognise DNA. The two most researched CDSs are cyclic GMP–AMP synthase (cGAS) and AIM2. Both cGAS and AIM2 recognise retroviral DNA and DAMPS, e.g., mitochondrial DNA [40, 41]. Activated cGAS triggers the adapter protein STING (stimulator of interferon genes) to stimulate IFN production [42]. AIM2 activation leads to inflammasome formation and the consequential IL-1β and IL-18 secretion [43].

Generally, immune cells prevent or resolve the pathogenic intrusion. However, it can happen that the inflammatory cascade spirals out of control resulting in high level of cytokines (also called the cytokine storm), general inflammation, and sepsis (**Figure 4**).

Table 2. Examples of pattern recognition receptors *(focussed on human leukocytes)*

PPR		Ligands	Cell types *(high expression)*	Location
TLR	TLR 1	Triacyl lipopeptides, DAMPS	Macrophages, neutrophils, lymphocytes, DCs	Cell membrane
	TLR 2	Peptidoglycans, mannans, DPI, DAMPS		
	TLR4	LPS, fungal mannans, envelope proteins, DAMPs		
	TLR5	Flagellin		
	TLR6	Lipopetides, zymosan		
	TLR3	Viral dsRNA	DCs, T cells, B cells, Myeloid cells (TLR8)	Endosome membrane
	TLR7-8	ssRNA viruses		
	TLR9	CpG ODN, ds DNA viruses		
	TLR10	Potentially: LPS, flagellin and dsRNA	B cells, DCs, granulocytes	Cell/endosome membrane
CLR	Mannose receptor	Mannans	Myeloid cells, DCs	Cell membrane
	Dectin-1	β-glucans		
	Dectin-2	α-mannans		
	Mincle	α-mannans, DAMPs		
NLR	NOD1	MTP	Generally expressed	Cytosol
	NOD2	MDP		
	NLRP1	Anthrax toxin		
	NLRP3	Peptidoglycans, bacterial toxins, DAMPS among others	Basophils, monocytes, DCs	
	NLRC4	Flagellin	B cells	
RLR	RIG I	Viral short dsRNA	Generally expressed	Cytosol
	MDA5	Viral long dsRNA		
	LGP2	dsRNA		
CDS	cGAS	DNA, DAMPS	Generally expressed	Cytosol
	AIM2	DNA, DAMPS	B cells	

Abbreviations: CDS: cytosolic DNA sensor; CLR: C-type lectin receptor; CpG ODN: CpG oligodeoxynucleotides; DCs: dendritic cells; IFN: type I interferons IL: Interleukin; IRF: interferon regulator factor; MDP: muramyl dipeptide; Mincle: macrophage inducible C-type lectin; MTP: muramyl tripeptide; NLR: nucleotide-binding oligomerization domain-like receptor; RLR: retinoic acid-inducible gene-I-like receptor; ROS: reactive oxygen species; TLR: Toll-like receptor [35-37, 41, 44, 45].

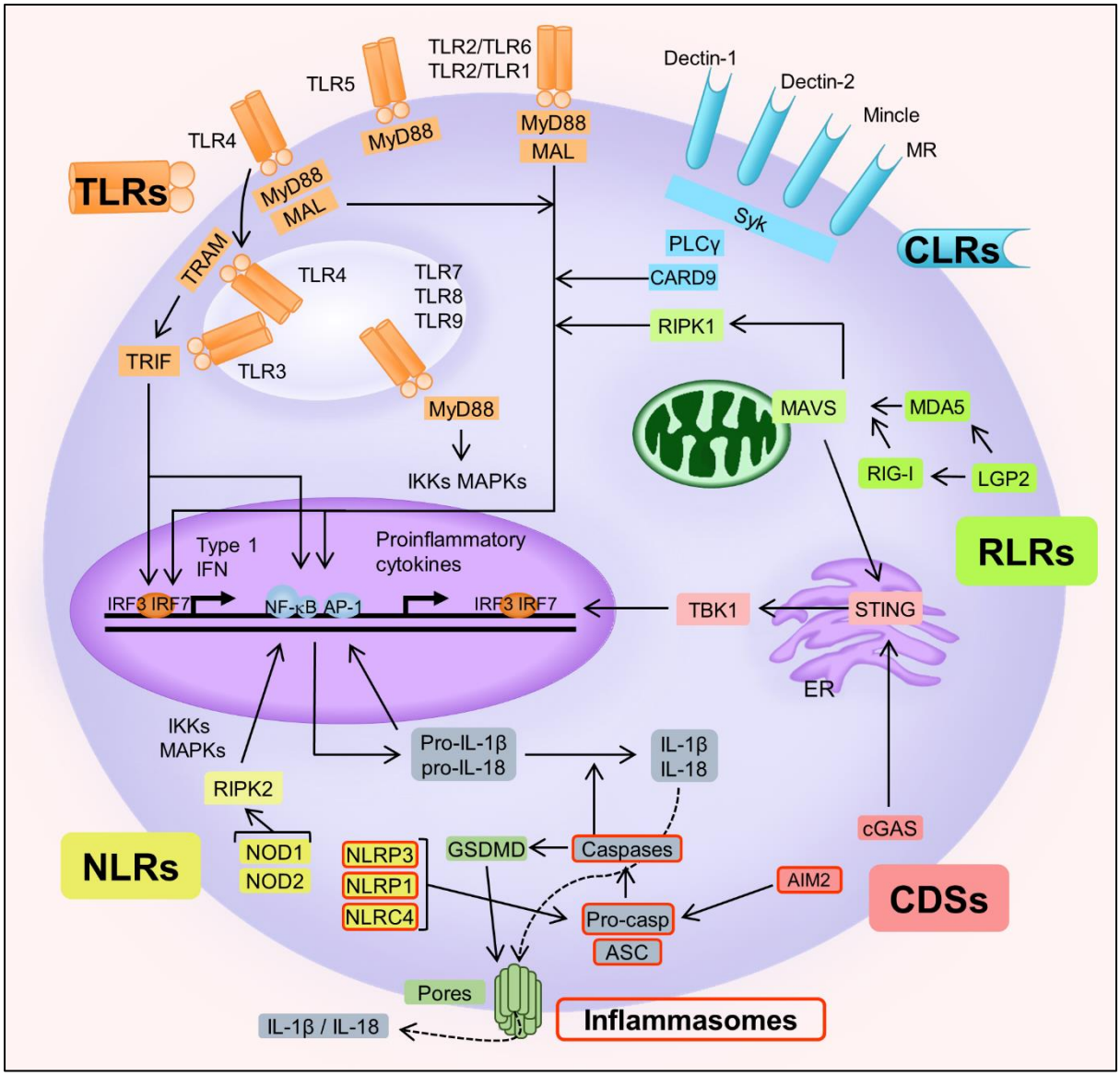

Figure 3. A simplified overview of pattern recognition pathways. Abbreviations: AP-1: activator protein 1; CDS: cytosolic DNA sensor; CLR: C-type lectin receptor; GSDMD: gasdermin D; IFN: interferon; IKKs: IκB kinases; IL: interleukin; IRF: interferon regulator factor; MAPKs: mitogen-activated protein kinases; Mincle: macrophage inducible C-type lectin; NLR: nucleotide-binding oligomerization domain-like receptor; RLR: retinoic acid-inducible gene-I-like receptor; TLR: Toll-like receptor. Adapted from [46] and a presentation slide of Thierry Roger.

1.1.3.2 The complement system

The complement system, a pivotal part of the innate immune defence system, is an inflammatory pathway consisting of 35 functionally linked proteins that responds to PAMPs and DAMPs. When the complement system is activated, it ignites a complement protein cascade that eventually leads to an increase of inflammatory molecules and the formation of the membrane attack complex, which disrupts pathogen membranes. Three different pathways activate the complement system [47-50]:

1) The classical pathway, which is activated through the binding of immune complexes consisting of antigen-antibody substrates, pentraxins (e.g., C-reactive protein (CRP)), and various DAMPs and PAMPs.
2) The lectin pathway, which is activated through the binding of MBL to various PAMPs and DAMPs, which includes mannose and LPS.
3) The alternative pathway, which is a delicate balance of continuous activation and inhibiting molecules. The alternative pathway can amplify the classical or lectin pathway or it can be activated by the presence of DAMPs (e.g., LPS), which downregulate the inhibiting molecules.

The activation of the complement pathways leads to cleavage of complement factors C3 and C5. C3 and C5, sequentially, are cleaved into an "a" and "b" fragment. The "a" fragment (also called anaphylatoxin) functions as an inflammatory, cell-activating, mediator, while the "b" fragment opsonizes pathogens and forms, together with C6, C7, C8 and C9, the membrane attack complex [51-53]. C3a and, especially, C5a are highly inflammatory molecules (C5a is 10-100 times as inflammatory as C3a) that bind to specific complement receptors present on myeloid cells. This binding triggers the production of chemokines and cytokines, and the degranulation of neutrophils, basophils, eosinophils and mast cells (**Figure 4, Table 3**) [49, 50, 54, 55]. Inhibiting complement factors or complement receptors might prevent unfavourable outcomes in sepsis. For example, anti-C5a antibodies and inhibitors of C5 cleavage were shown to lower mortality in septic shock animal models [48, 56].

Table 3. Anaphylatoxin-activation of immune cells

Anaphylatoxins	Cellular targets	Effect
C3a and C5a	Antigen presenting cells	Regulation of T cell responses
	Mast cells, eosinophils (mainly C3a) and basophils	Degranulation, chemotaxis and release of vasoactive amines → contraction of smooth muscle and increased vascular permeability
C3a	Neutrophils	Attenuating mobilisation
C5a	Neutrophils, monocytes	Chemotaxis, activation of complement receptors; increase of FcγR; assembly of ROS; cytokine production; increased survival of neutrophils
	Resident macrophages	Increased FcγR expression
	Endothelium	Increased selectins, IL-8 and IL-6 synthesis
	Hepatocytes	Acute phase protein synthesis

Abbreviations: IL: interleukin; ROS: reactive oxygen species. [49, 54, 55]

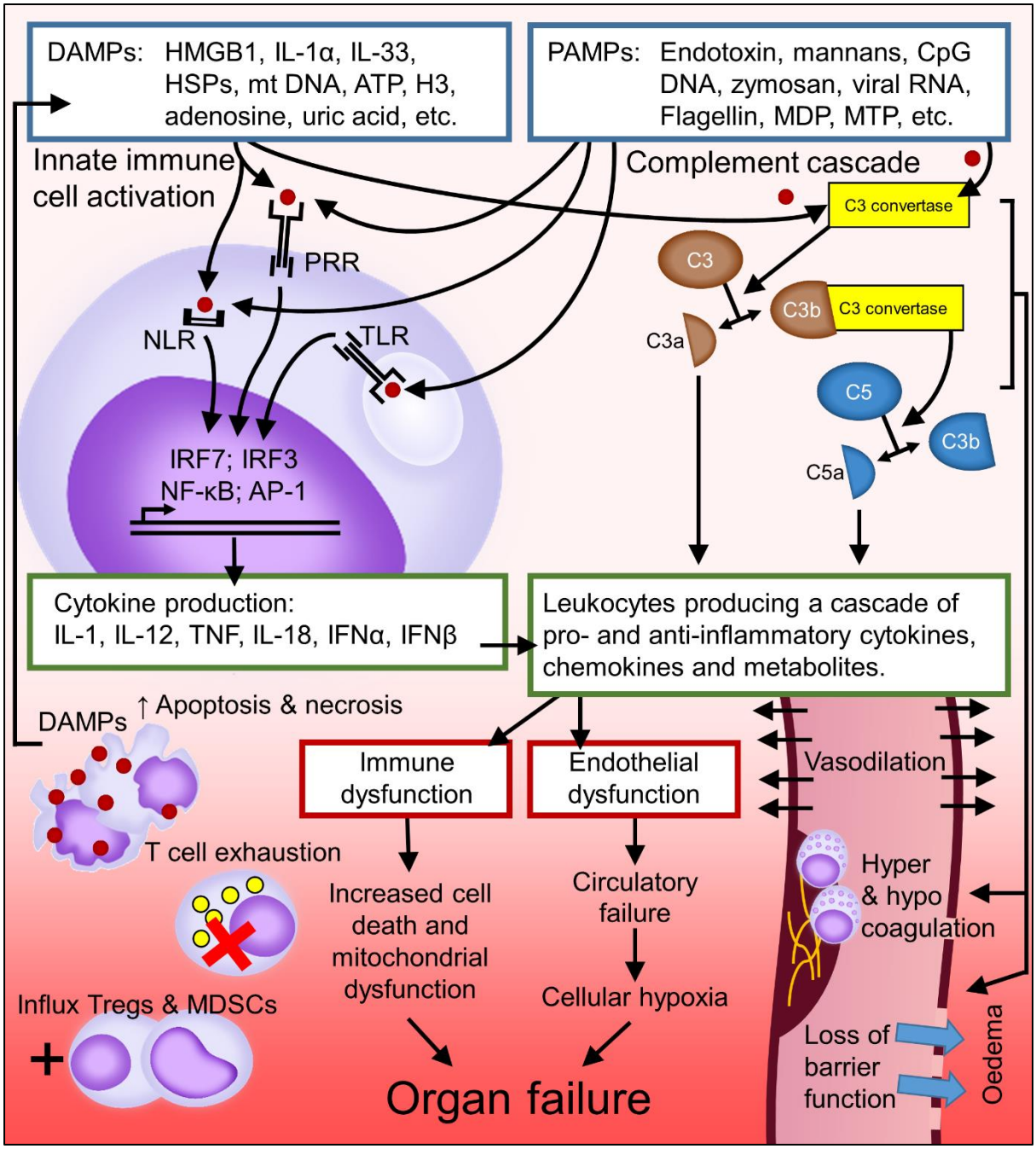

Figure 4. Pathophysiology of sepsis. DAMPS and PAMPS activate innate immune cells and the complement cascade. Immune cells produce cytokines and the complement cascade cleaves C3 and C5 into inflammatory C3a and C5a fragments. Both the cytokines and C3a/C5a activate leukocytes producing a cascade of cytokines, chemokines and metabolites, which lead to immune and endothelial dysfunction. Eventually the lack of oxygen and dysfunctionality of tissue cells can lead to organ failure as seen in sepsis. Note that the complement cascade also has a direct effect on coagulation through the production of thrombin and fibrin-like products. Abbreviations: DAMPs: danger-associated molecular patterns; H3: histone 3; HMGB1: high mobility group box 1; HSP: heat shock protein; PAMPS: pathogen-associated molecular patterns; MDP: muramyl dipeptide; MTP: muramyl tripeptide; PRR: pattern recognizing receptor; NLR: nucleotide-binding oligomerization domain-like receptor; TLR: Toll-like receptor; IL: interleukin; TNF: tumor necrosis factor; Tregs: T-regulatory cells; MDSCs: myeloid-derived suppressor cells.

1.1.3.3 Endothelial dysfunction

The endothelium is the anticoagulant layer of the blood vesicles that separates blood from the surrounding tissues. Glycocalyx is a layer consisting of glycoprotein that covers the endothelium and regulates inflammatory and coagulation homeostasis. Reactive oxygen species (ROS), TNF, cytokines, and bacterial endotoxins can shed the glycocalyx resulting in: 1) a disruption of the barrier function, 2) exposition of the selectin, integrins and fibrin clothing areas of the endothelium, and 3) an increase in sheer stress on the endothelial cells [57, 58]. With the glycocalyx shedding, the activated neutrophils and monocytes can bind to the exposed selectins and disrupt the tight junctions that connects the endothelial cells. Without the protective glycocalyx layer and intact tight junctions, plasma and intravascular proteins leak into the tissue contributing to oedema and poor tissue perfusion [57, 58].

The coagulation homeostasis is a delicate balance of thrombogenic and antithrombogenic factors [59]. During sepsis, the coagulation cascade commences through platelet and neutrophil activation, and fibrin clotting area exposition after glycocalyx shedding. In addition, the lectin pathway of the complement cascade activates coagulation by generating thrombin and fibrin-like products. This prothrombotic state leads to disseminated microvascular thrombosis preceding organ ischemia and, secondly, to uncontrolled bleeding due to excessive consumption of clotting factors and platelets [60].

Vasodilation and decreased intravascular volume are two mechanisms that lead to low blood pressure as seen in septic shock. Endothelium cells under sheer stress release nitric oxide (NO) and endothelin, which relaxes the smooth muscles covering the vascular system and consequently increases vasodilation. Mast cells, eosinophils and basophils also produce vasoactive amines further increasing vasodilation [58, 61, 62]. The combination of extravasation of the plasma due to membrane disruption and vasodilation causes septic shock (**Figure 4**). Therefore, to treat this low blood pressure, it is often necessary to administer both vasoconstrictive agents and fluids [32, 58].

1.1.3.4 Immunosuppression in sepsis

The dysregulated host response of sepsis is concurrently hyperinflammatory and immunosuppressive. During the early phase of sepsis, more cells tend to be hyperinflammatory resulting in overwhelming inflammation, organ failure and shock [29, 32, 63]. Generally, the hyperinflammative response subsides within days while the immunosuppressive response can persist for weeks to months. Immunosuppression in sepsis is characterized by: 1) an increased apoptosis and/or anergy of cells, 2) a decrease of human leukocyte antigen-DR isotype (HLA-DR) expression on antigen presenting cells (APCs), 3) an influx of myeloid-derived suppressor cells (MDSCs) and T-regulatory cells (Tregs), and 4) a mitochondrial dysfunction [64, 65].

Increased apoptosis

Apoptosis is a physiological response of cells to go into cell death. In contrast to necrosis, it does not produce inflammation and injury to other cells. It regulates cell populations and aids downregulation of the inflammatory response. However, an overshoot in the depletion of effector cells can result in an immunosuppressive state. Monocytes, macrophages, B cells, T cells, dendritic cells (DCs) and gastrointestinal epithelial cells experience apoptosis during sepsis. Furthermore, apoptosis itself can

induce immunosuppression by pushing M1 macrophages and T helper (Th) 1 cells (which are inflammatory), towards M2 macrophages and Th2 cells (which are immunosuppressive). DCs that internalise apoptotic cells decrease their secretion of pro-inflammatory cytokines, while increasing immunosuppressive cytokine production [66-72]. The degree of lymphocytic apoptosis corresponds with the severity of sepsis, secondary infections and mortality [73, 74].

The mechanisms behind excessive apoptosis of immune cells in sepsis are not completely understood, but mainly attributed to two factors: 1) the high cytokine levels present in sepsis, and 2) the upregulation of programmed cell death protein 1 (PD1) on T cells and natural killer (NK) cells, and its ligand PD-L1 on endothelial cells, DCs, and macrophages [74, 75]. PD-1 is an inhibitory receptor, which protects tissues from over activation and aids return to homeostasis [76]. When PD-1 binds to PD-L1, it activates a downstream signalling pathway that inhibits cell differentiation, cytokine production, and induces apoptosis. One year after sepsis onset, PD-1 is still upregulated in sepsis survivors [77]. Blocking the PD-1/PD-L1 receptor prevents T cell apoptosis and restores T cell cytokine production (see 4.1.6) [78].

Anergy and exhaustion

Anergy is the absence of an immune cell response induced by either a lack of co-stimulation or high co-inhibitory molecule stimulation. Exhaustion takes place when cells have been chronically activated decreasing cytokine expression and acquiring inhibitory surface molecules. Anergy and exhaustion take place within 24 hours after sepsis onset [79, 80]. In sepsis, anergy and exhaustion are mainly described in T cells but also occur in B cells, neutrophils, monocytes, DCs and NK cells [79, 81-83].

Low HLA-DR-expression on monocytes and DCs is indicative for anergy of these cell-types [84-86]. Monocytes in a septic environment decrease histone acetyltransferases (HATs) and increase histone deacetylases levels, which modify chromatin state and results in reduced HLA-DR expression and production of pro-inflammatory cytokines, and impaired antigen presentation. A decreased monocytic HLA-DR expression correlates to the development of secondary infections and mortality in septic shock patients [87-90]. DCs with low HLA-DR expression produce higher levels of immunosuppressive IL-10, have less antigen presenting abilities and are more likely to go into apoptosis [84, 91].

During sepsis, T and B cells suffer a progressive loss of function; they produce less cytokines, show impaired cytotoxicity, and proliferate less [92-94]. Furthermore, they shift toward a more immunosuppressive status by expressing inhibitory receptors and producing IL-10 [83, 92, 95]. T cells in a septic environment show low levels of transcription factors T-bet and GATA3, resulting in low levels of CD62L and CD127, which are markers associated with effector T cells. Like monocytes and DCs, B cells show decreased levels of HLA-DR, which is probably indicative of a lower antigen presenting capacity [94].

Induction of Tregs and MDSCs

The increased chemokine production in sepsis leads to emergency granulopoiesis/myelopoiesis, a rapid influx of immature myeloid and granulocytic cells in the blood stream and tissue. However, the inflammatory environment blocks the maturation of these cells promoting the egress of immature immunosuppressive cells: the myeloid-derived suppressive cells (MDSCs), into the blood. Patients with

sepsis have high levels of MDSCs, which contribute to persistent immunosuppression by producing immunosuppressive cytokines, arginase, ROS, reactive nitrogen species (RNS), and chemokines, which recruit Tregs (for more details see chapter 4.2) [96-98].

Tregs are immunosuppressive T cells that express CD25 and nuclear transcription factor Forkhead box P3 (FoxP3). In humans, they comprise 5-10% of CD4+ T cells in the circulation, which doubles during sepsis [99-101]. Tregs produce inhibitory cytokines, like transforming growth factor (TGF) β and IL-10, and induce apoptosis of effector cells, e.g., by the expression of TNF-related apoptosis-inducing ligand (TRAIL). Tregs suppress DC and NK cell maturation, and inhibit cytokine production of DCs, B cells and NK cells [102]. Tregs have a high content of mitochondria and benefit from FoxP3 reprogramming, which help them survive in septic conditions maintaining the immunosuppressive response [103]. High levels of Tregs are associated with immunoparalysis and mortality in sepsis [99, 100, 103]

Metabolic dysfunction

Endothelial dysfunction and disturbed coagulation are two drivers of cellular hypoxia and organ failure. Another cause of cellular failure is mitochondrial dysfunction. Normally, the mitochondria generates ATP through oxidative phosphorylation employing the tricarboxylic acid (TCA) cycle. However, in case of sepsis, independent of the presence of oxygen, the mitochondria shift from oxidative phosphorylation to aerobic glycolysis (also known as the Warburg effect). This shift can lead to apoptosis and organ failure. Mechanisms that are responsible for mitochondrial dysfunction are [104-109]:

1) An instability of the pyruvate dehydrogenase complex (PDC). PDC is the main enzyme to stimulate pyruvate to enter the TCA cycle. During sepsis, due to low levels of active PDC, pyruvate fails to enter the TCA cycle transforming to lactate, which results in an ATP loss.
2) High doses of ROS and RNS present in the cytoplasm, which damage the mitochondrial membrane. A damaged mitochondrial membrane leaks protons, which diminishes the proton gradient needed for ATP production through oxidative phosphorylation.
3) An accumulation of succinate. Succinate is an intermediate of the TCA cycle. If succinate accumulates in the mitochondrion during sepsis, it drives ROS production and anaerobic glycolysis.
4) Inhibition of TCA enzymes and mitochondrial enzyme complexes by NO.

Hyperlactatemia in sepsis patients indicates shock and is strongly correlated to mortality. Hyperlactatemia is often attributed to poor perfusion and microcirculatory disturbances caused by endothelial dysfunction. However, lactate also indicates the metabolic shift to glycolysis independent of circulation, which also increases mortality [104, 106, 107, 110, 111]. Targeting mitochondrial dysfunction by decreasing succinate levels, limiting ROS production and activating PDC can facilitate the return to homeostasis and increase the chance of survival. For example, inhibiting succinate with dimethyl malonate or blocking glycolysis with 2-DG decreases mortality in mouse models of sepsis [112, 113].

1.1.3.5 Organ dysfunction

Organ dysfunction is a hallmark of sepsis and is a consequence of a changed hemodynamic and cellular response [83, 104, 114-116]. The mechanisms that lead to reduced organ functionality are:

1) The leakage of plasma and intravascular proteins into tissues contributing to oedema, low blood pressure and poor tissue perfusion. The reduced blood flow decreases oxygen availability.
2) The prothrombotic state with disseminated microvascular thrombosis leading to organ ischemia.
3) An increased metabolic demand of oxygen by the inflamed cells.
4) Mitochondrial dysfunction.

The endothelial dysfunction in combination with unstable coagulation results in reduced microcirculation, which reduces oxygen levels within the organs. The inflamed immune status increases the oxygen demand resulting in hypoxia. The mitochondrial dysfunction reduces the energy potential resulting in organ cell failure. Organ failure in sepsis can target every organ system. However, the SOFA score accounts for six organ systems: the cardiovascular, hepatic, renal, neurological, respiratory, and haematological system (**Figure 5**). Other organ systems, e.g., the gut, are just as much afflicted, but biomarkers/symptoms to assess them are not available or not used in clinical practice [114].

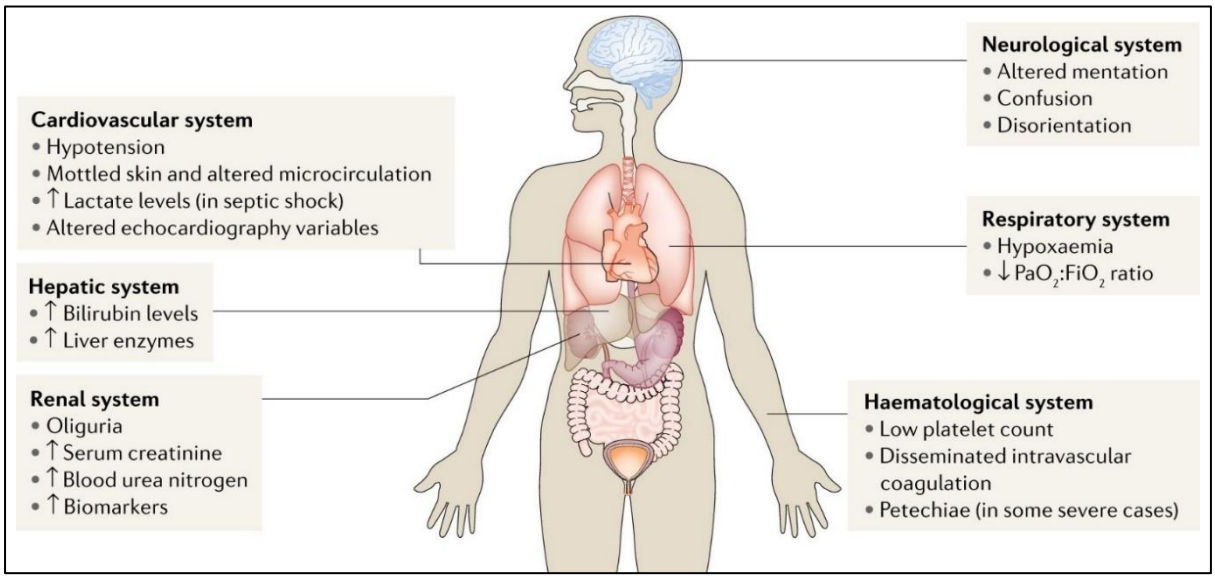

Figure 5. Signs and diagnostic criteria of major organ systems that are clinically monitored in sepsis. (Reprinted by permission from: Springer, "Nature Reviews Nephrology" [114]).

The apoptosis of the epithelial gastrointestinal tract destroys its barrier function allowing bacteria and endotoxin to enter the systemic circulation feeding inflammation [87]. The microbial diversity in the gut, also called the microbiota, is of utmost importance for a stable inflammatory situation [117]. Sepsis, and especially antibiotic treatment, disturbs the microbiota increasing inflammatory bacterial species in the gut, which predisposes patients to immunosuppression and an increased risk of organ failure [118]. Promoting microbial diversity in the gut using probiotics and faecal transplantation might increase sepsis survival [119].

Since all the processes leading towards organ failure take place on a generalized level, dysfunction of a single organ is rare. Distant organs communicate mechanistically and by excreting signalling factors to maintain homeostasis, the so-called inter-organ crosstalk. However, when one organ fails, inter-organ crosstalk further amplifies organ failure of other organ systems. For example, the kidney-lung crosstalk occurs when the kidneys fail to expel fluid resulting in increased fluid pressure. This generates lung oedema and alveolar cell apoptosis, which then results in hypoxemia and hypercapnia. The reduced lung function worsens the kidney failure resulting in a self-propagating loop [114, 120-122]. This self-propagating organ failure loop due to organ crosstalk is described between many organ systems e.g., gut-liver, lung-kidney/liver, and the kidney/liver-brain-cardiovascular system [114, 122-125]. Unsurprisingly, the number of organs affected corresponds strongly to mortality [126, 127]. Organ failure present in sepsis can be reversible but patients experience the consequences of organ failure long after the initial sepsis episode has passed [128].

1.1.3.6 Sepsis morbidity, mortality and long-term health consequences

Patients that die early after sepsis diagnosis often suffer from the hyper-inflammatory response, resulting in multiple organ failure, cardiovascular shock and death (**Figure 6**). Patients that die later are more often immunosuppressed resulting in secondary infection development, of which many are not virulent and only seen in immunosuppressed patients [83, 129]. Twenty percent of initial sepsis survivors develop secondary infections within 30 days [130]. Latent infections, like cytomegalovirus (CMV), Epstein-Barr and herpes simplex, often flare-up in sepsis patients. The viral load of these viruses corresponds to the immunosuppressive state and to mortality [131, 132]. It is estimated that more than half of the sepsis deaths are a consequence of immunosuppression [29, 133].

Sepsis patients have a higher mortality rate compared with non-septic critical ill patients or those undergoing cardiovascular surgery even ten years after being hospitalised [134]. A matched case-control study showed that 35% of septic survivors would develop sepsis again compared with 4% in the control group in the 8 years after the initial episode [135]. The immunosuppressive response leads to less immune surveillance and low-grade inflammation, which causes late infections, cardiovascular diseases, weakness, and cognitive impairment [29, 30, 136].

Sepsis affects the cardiovascular system for years after initial presentation. In the early sub-acute phase, the hyper-inflammation, mitochondrial dysfunction, endothelial dysfunction, altered production of oxygen species and altered calcium homeostasis accompanying sepsis causes cardio myocyte depression and hypotension (and possibly septic shock). Furthermore, fatty acids and glucose, the main ATP sources of the cardiac muscle, are both strongly decreased in sepsis. A decreased cardiac output in sepsis correlates to mortality [137, 138]. In people with pre-conditioned – whether or not diagnosed – cardiovascular comorbidities e.g., atherosclerosis and cardiomyopathy, sepsis can lead to complete heart failure, pre-existing plaque rupture resulting in acute myocardial infarction, and death [139]. The persistent chronic inflammation and dysregulated coagulation increase plaque formation, arterial stiffness, and reduced myocardial contractility in recovered sepsis patients. In the year after discharge, 18% of patients suffer from new-onset stroke, 7% of myocardial infarction and 8.6% of heart failure [140, 141].

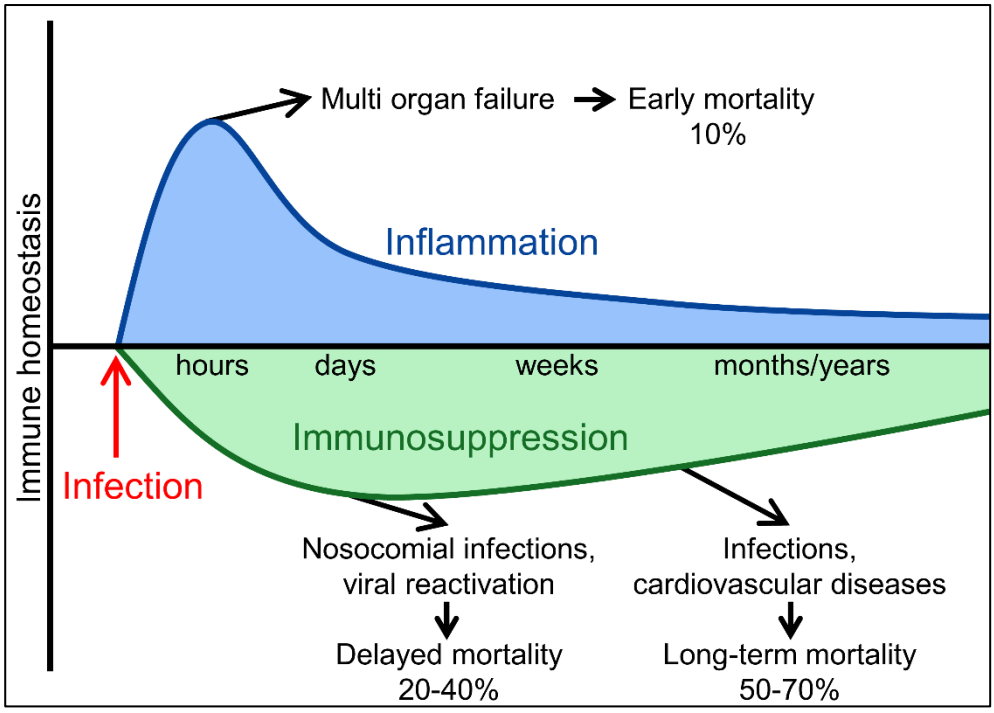

Figure 6. Sepsis over time. The dysregulation of the immune homeostasis over time. The inflammatory and immunosuppressive response are concurrently represented. However, the early deaths are mainly attributed to organ failure due to overwhelming inflammation. Late deaths are strongly associated to immunosuppression causing increased susceptibility to (nosocomial) infections, viral reactivation and cardiovascular diseases.

Unsurprisingly, the endothelial dysfunction extends to the blood brain barrier. This in combination with the oxidative stress and overwhelming inflammation induces cognitive impairment [142]. Ten percent of severe sepsis (sepsis-2) survivors develop cognitive impairment, which can persist for eight years after the initial sepsis episode [143]. In addition, even patients who are not obviously impaired show a subtle cognitive reduction of intellect, including verbal learning and memory. Sepsis induces brain atrophy and lesions seen on magnetic resonance imaging (MRI), and more low-frequency electroencephalogram (EEG)-activity indicating brain impairment, which are still present 3.5 years after the initial episode. In addition, patients that survive sepsis have more depression, anxiety, and post-traumatic stress disorder and receive more new psychoactive prescriptions the first three months after hospitalisation [30, 144-146].

In summary, sepsis is a highly complex syndrome of a dysregulated host response triggered by an infection. It can trigger organ failure in all systems causing high short-term mortality and morbidity. The dysregulated host response can hold on for years after the initial episode causing a reduced quality of life in sepsis survivors and an increased long-term risk of mortality.

1.1.4 Biomarkers and phenotypes in sepsis

Our understanding of sepsis and the tools available to diagnose sepsis are limited. The common denominators of sepsis patients are an infection and organ dysfunction but demographics, underlying conditions, pathogens, the location of primary infection and inflammatory status can be vastly different [147]. Yet, all patients are treated following the same clinical guidelines. In a 2004 nature commentary, prof. Nathan elegantly encapsulates this principle: "It makes no sense to use twenty-first century technology to develop drugs targeted at specific infections whose diagnosis is delayed by nineteenth-century methods" [148]. Sepsis researchers need to focus on identifying (semi-) homogeneous subgroups, so-called phenotypes, to facilitate targeting treatment [149]. Biomarkers ("biological markers") can aid clustering patients into phenotypes and consequently guide therapy [150-152]

The World Health Organisation defined biomarkers as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" [153]. (Sepsis-) Biomarkers can be subdivided into types based on their nature (next paragraph) and on their usage (**Figure 7**) [154]:

1) Diagnostic biomarkers, which can identify patients with and without sepsis or type of infection. A good diagnostic biomarker for sepsis could limit and target therapies (like antibiotics).
2) Prognostic biomarkers aid clinicians by risk-stratifying patients based on their outcome. Prognostic biomarkers can help guide family and clinicians to emit needless therapy and start palliative care.
3) Theragnostic biomarkers stratify patients in groups that would benefit from or harmed by a certain therapy. Current sepsis research focusses on theragnostic biomarkers that segregates patients on type of immune response.

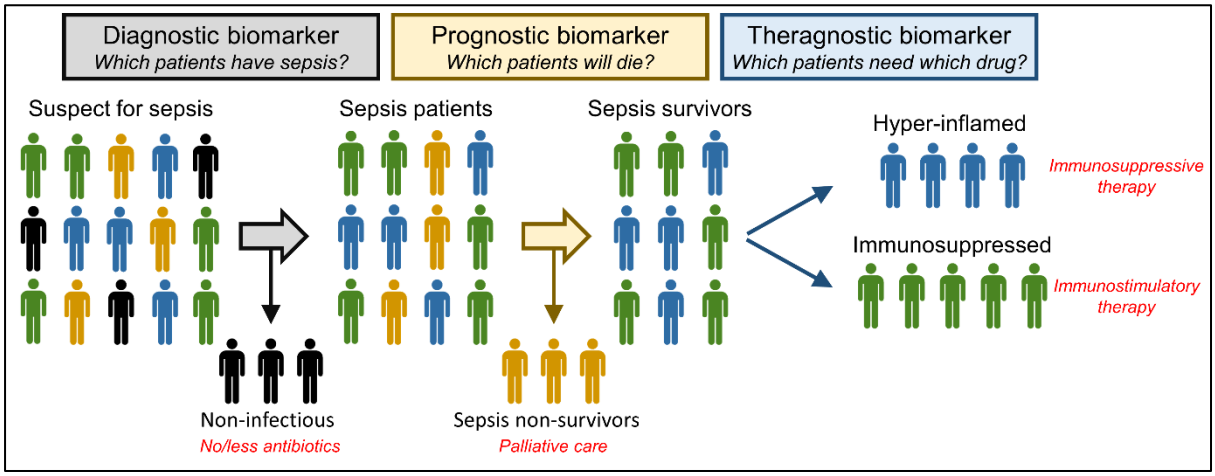

Figure 7. The utility of different biomarker types in sepsis. There are three types of biomarkers. Diagnostic biomarkers can divide the patients suspected for sepsis in a high and low risk of sepsis limiting antibiotic usage in the latter. Prognostic biomarkers can risk-stratify on mortality informing clinicians and family members steering towards palliative care. Lastly, in patients with high risk of surviving a theragnostic marker can guide targeted therapies based on inflammation status [155]

The second way to divide biomarkers is by their nature: clinical, molecular, cellular, genomic, transcriptomic, epigenomic, proteomic and metabolomic biomarkers. The biomarkers that are easiest and least invasive to measure are clinical biomarkers, like temperature and blood pressure [156]. Hypothermia at admission or a low blood pressure (mean arterial pressure < 65 mmHg) correlate to a lower survival rate [157, 158].

Lactate was the first molecular sepsis biomarker discovered in 1843. Lactate, still used today, has high specificity and low sensitivity for sepsis, and correlates strongly to mortality. Other molecular biomarkers that are used in the ICU are procalcitonin (PCT) and CRP [159, 160]. PCT is a prohormone for calcitonin and is synthesised by many cell-types. PCT rises drastically with bacterial infection and sepsis but also rises in other inflammatory diseases like trauma, cancer, and viral infections [161-163]. PCT has been vividly debated for its use to shorten antibiotic therapy. Two different meta-analyses, published in 2018 and 2019, show a reduction in mortality and antibiotic use, but the studies present a high risk of bias [164, 165]. CRP is an acute phase protein synthesized by the liver that has been well established as a biomarker for sepsis since the 1980s [6]. It is very sensitive as an indicator for inflammation, but lacks the specificity of PCT [166]. Because it is a sensitive marker, which is easy to measure, it is often used to screen for early onset sepsis/infection. Other markers that have a similar or higher sensitivity/specificity to PCT and/or CRP, but are not used in daily management, are suPAR, IL-6, sCD25 and decoy receptor 3 [167].

Techniques to analyse cellular biomarkers include cell counters (manual or automatic), microscopy, flow cytometry and cytometry in time of flight (CyTOF). The first cellular sepsis biomarker –discovered in 1927– was leucocyte count [168]. Leukocyte count is still used today even though sensitivity and specificity are low compared with CRP and PCT [6, 169]. A better, but less used cellular biomarker is the neutrophil to lymphocyte ratio (NLR), which rises quickly during acute physiologic stress and has a high sensitivity but low specificity for sepsis [169]. Not just cell count, but also the expression of specific cellular surface markers can aid sepsis diagnosis. Shankar-Hari et al. studied the discriminating ability of 47 leukocyte surface biomarkers in patients with and without acute infection, and sepsis. They showed higher expression of CD24 and CD279 on neutrophils and lower HLA-DR expression on monocytes, however, the predictive validity of these, or a combination of these, biomarkers was low. Another much-researched surface marker is CD64. Two meta-analysis performed in 2010 and 2015 showed a pooled sensitivity of 79% / 76% and specificity of 91% / 85% for the diagnosis of bacterial infection or sepsis, which would outperform CRP and PCT. However, both meta-analysis mention a high variability between the studies and a low/medium methodological quality [170].

A combination between clinical, molecular, cellular biomarkers and demographic data can aid phenotyping of sepsis patients. Garlund et al. used latent class analysis, a methodological modelling technique to identify subgroups, to define six sepsis phenotypes using 46 demographic, clinical and laboratory variables in 1696 sepsis patients. The phenotypes are, ordered from least severe to most severe based on ICU stay and mortality: “Uncomplicated Septic Shock”, “Pneumonia with acute respiratory distress syndrome (ARDS)”, “Postoperative Abdominal”, “Severe Septic Shock”, “Pneumonia with ARDS and multiple organ dysfunction syndrome (MODS)”, and “Late Septic Shock”.

Seymour et al. performed a similar study but distilled four sepsis phenotypes: phenotype α-δ, each with their own inflammatory profile and mortality rate. The "α" patients were less ill and had less mortality and inflammation compared with the other groups, which had, respectively, kidney injury, inflammation and lung injury, and liver injury and shock [150].

While molecular biomarkers are still at the forefront in sepsis biomarker research, the development of machine learning and the ability to analyse grand datasets facilitates "omics" biomarker research. This "omics" research measures an entire class of biochemical species in a systematic way. The five most described "omics" areas are: 1) genomics, which analyses DNA sequence variations like single nucleotide polymorphisms (SNP), 2) epigenomics, which analyses DNA methylation, histone modifications, and non-coding RNA, 3) transcriptomics, which analyses transcript expression in the form of messenger RNA (mRNA), 4) proteomics, which analyses protein levels and their chemical modifications, and 5) metabolomics, which analyses endogenous and exogenous chemicals (**Table 4**). Omics technologies present new opportunities to aid phenotyping sepsis patients based on shared pathophysiology [171]. In the following paragraphs, I will cover some influential "omics" research in sepsis displaying its use for phenotyping sepsis, predicting mortality and discovering new biomarkers.

Over a 1000 genetic association studies have looked at genomic biomarkers and more than 100 variants have been described in sepsis [172]. However, most of these studies in sepsis show low methodological quality, are not-reproducible, and are likely to present false positive results [173]. A more unbiased approach to discover new genomic variants in sepsis are genomic-wide association studies (GWAS), whole-exome sequencing (WES) and whole-genome sequencing (WGS). Using GWAS, Rautanen et al. discovered that common variants of the *FER* gene were strongly associated with survival. Twenty-eight day mortality was 9.5% in patients with the CC genotype (4% of patients) versus 25.3% in patients with the TT genotype (70% of patients) [174]. However, this was not confirmed by another study [175]. In 2017, Scicluna et al. used GWAS in whole blood to classify four sepsis phenotypes "MARS 1-4". The 30% of patients that were phenotyped into "Mars 1" (identified by high BPGM and TAP2 expression) had significantly higher mortality compared with the other phenotypes (hazard ratio of 1.86) [176].

Wong et al. carried out the one of the first sepsis-phenotyping transcriptomics study in children with septic shock using genome-wide microarrays in whole blood. They identified 100 genes that defined three sepsis subclasses, of which subclass A (compared with B and C) had higher illness severity, organ failure and mortality [177]. More recently, Sweeney et al. used unsupervised clustering analysis of transcriptomic data of 700 patients to identify three sepsis subtypes: the adaptive (44% of patients), inflammopathic (35% of patients) and coagulopathic subtype (21% of patients). The adaptive subtype had lower mortality rates compared with the other two (18.5 vs 29% / 31%) [178]. Davenport et al. identified two sepsis response signatures (SRS) based on the expression of seven genes. SRS1 patients, compared with SRS2 patients, showed more immunosuppression, LPS tolerance, T cell exhaustion and metabolic dysfunction, which resulted in twice the mortality risk [179]. Lastly, McHugh et al. developed a 4-gene model (CEACAM4, LAMP1, PLA2G7, PLAC8) that could discriminate between sepsis patients and non-infectious critical ill patients. This gene model is now being developed into a rapid test ("Septicyte®") but is not used in clinical practice [180].

In sepsis, more than 50 epigenetic biomarkers have been described, including changes in DNA methylation, histone modification, and non-coding RNAs (thoroughly reviewed by Beltrán-García et al. [181]). Binnie et al. showed the potential of using epigenomic-wide association studies (EWAS) in whole blood to identify sepsis biomarkers. In a nested-case control study comparing septic and non-infectious critical ill patients, they showed differences in methylated region-associated gene expression corresponding to antigen processing and presentation, methyltransferase activity, and cell adhesion and cell junctions, which correlated to severity of illness and length of ICU stay.

Wang et al. showed the potential of metabolomics in sepsis by combining 21 cohorts with in total 1287 sepsis patients and 2509 metabolite comparisons. The metabolites corresponding to several death-related metabolic pathways showed the strongest correlation to mortality, and a prospective metabolomic analysis (in the same paper) showed a prediction for mortality with an area under the receiver operating characteristic curve (AUROC) of 0.88 [182]. Using mass spectrometry (MS), DeCoux et al. showed distinct proteomic patterns between survivors and non-survivors of which many related to complement and coagulation cascades. The authors suggest that anti-thrombin-III and complement factor VIII could be possible target for therapy [183]. Thavarajah et al. aimed to find proteins in whole blood that distinguish sepsis patients from non-infectious critical ill patients using MS. They found a difference of more than 50 proteins of which SAA1 protein had the most potential as a novel biomarker in sepsis. Lastly, Langley et al. combined both metabolomics and proteomics in 152 patients with suspected community-acquired sepsis. Both the metabolome and the proteome showed a difference between non-infectious critical ill patients, septic survivors and septic non-survivors. The differences between septic survivors and non-survivors became more striking when closer to death. They created a prediction-model using four carnitine esters, age, haematocrit and lactate, which outperformed the APACHE score to predict mortality [184].

The aforementioned studies show that “omics” research can help us to identify sepsis phenotypes or novel sepsis biomarkers. However, most techniques are time consuming and high variable models are inconvenient to use in clinical practice or study inclusion. However, therapy efficacy can dependent of sepsis phenotype. For example, corticosteroids show a worse outcome in patients with the SRS2 phenotype compared with the SRS1 phenotype [185]. Of date, no successful therapy-phenotype combinations are used in the clinic, but the future holds promise [159].

Table 4. "Omic" studies in sepsis

Type of "omics"	Techniques	Examples in sepsis	Ref
Genomics: DNA sequence variations like single nucleotide polymorphisms (SNP).	Genomic-wide association studies (GWAS) Whole-exome sequencing (WES) Whole-genome sequencing (WGS)	*GWAS, whole blood*: A variant in the FER gene is protective in sepsis. *GWAS, whole blood*: Four sepsis phenotypes based on 2 genes each how different clinical characteristics and mortality rates. *GWAS, whole blood*: A 5-gene model (C14orf159, AKNA, PILRA, STOM and USP4) sorted patients in two patient classes of which one had double the mortality rate compared with the other.	Rautanen, 2015 [174] Scicluna, 2017 [176] Zhang, 2020[186]
Epigenomics: DNA methylation, histone modifications, and non-coding RNA.	Epigenomic-wide association studies (EWAS) Chromatin Immonopreciptation sequencing (ChiP-seq)	*EWAS, whole blood:* Methylation patterns of antigen processing and presentation, methyltransferase activity, cell adhesion, and cell junctions are associated with severity of illness, vasopressor need and length of stay.	Binnie, 2020 [187]
Transcriptomics: Transcript expression (mRNA).	Gene expression microarrays RNA sequencing (RNA-seq)	*Microarrays, whole blood*: A 100-gene model clusters children with septic shock in three different subclasses of which one has three times higher mortality rate. *Microarrays, whole blood:* A 4-gene model (CEACAM4, LAMP1, PLA2G7, PLAC8) could cluster sepsis from non-infectious critical ill patients with an AUC of 0.9. *Microarrays, blood leukocytes using leukoLock (Thermo Fisher Scientific):* A 7-gene model (DYRK2, CCNB1IP1, TDRD9, ZAP70, ARL14EP, MDC1, ADGRE3) show two sepsis responds signatures (SRS1/SRS2) with different mortality rate and inflammatory profile. *Microarrays and RNA-seq of 14 dataset:* A 33-gene model clusters sepsis patients in an inflammopathic, adaptive and coagulopathic phenotype. The adaptive phenotype was less severe and had a lower mortality rate.	Wong, 2009 [177] McHugh, 2015 [180] Davenport, 2016 [179] Sweeney, 2018 [178]
Proteomics: protein levels and their chemical modifications.	Gel based or liquid based MS Cross linking MS (XL-MS) MALDI-TOF	*Liquid based MS, plasma:* The 234 proteins analysed in sepsis patients showed distinct patterns differentiating between survivors and nonsurvivors. *Liquid based MS, plasma*: Sepsis patients had different proteomic signatures compared with other critical ill patients, with most strikingly a difference in SAA1 processing.	DeCoux, 2015 [183] Thavarajah, 2020 [188]
Metabolomics: endogenous and exogenous chemicals.	Nuclear magnetic resonance spectoscopy (NMR) Gas/liquid based mass spectometry (MS)	*Liquid and gas based MS, plasma:* The proteins and metabolites of non-infectious critical ill patients, sepsis survivors and non-survivor differ. An algorithm of four carnitine esters, age, haematocrit and lactate outperformed the APACHE score to predict mortality. *Meta-analyses:* Death-related metabolic pathways were the best predictor of death in sepsis with a pooled AUROC of 0.81.	Langley, 2013 [184] Wang, 2020 [182]

Abbreviation: MALDI-TOF: matrix-assisted laser desorption/ionization time-of-flight.

1.1.5 Immunomodulation and biomarker-guided therapy

Patient survival rates have improved by faster recognition and more developed supportive therapy [189]. However, therapeutics targeting the dysregulated host response have not been successful. Novel host-directed therapies successfully tested in animal models were not effective or even harmful in sepsis patients [190, 191]. Since 1976, over 100 phase II and phase III therapy trials failed, which includes the treatment with corticosteroids, ibuprofen, therapies targeting host inflammatory factors like TNF and IL-1 and PRRs (using eritoran and TAK-242 to block TLR4 signalling), and the administration of anticoagulant molecules (such as recombinant activated protein C) [191-193]. The fail of these host directed therapy trials is mainly attributed to two factors: 1) the focus on suppressing hyperinflammation instead of reversing immunosuppression, and, as mentioned before, and 2) the heterogeneity of sepsis and the lack of targeting certain sepsis phenotypes [29, 194-196].

A number of animal and phase I/II clinical studies try to reverse immunosuppression in sepsis. Granulocyte-macrophage-CSF (GM-CSF), an immune modulatory cytokine, promotes cell survival, proliferation and maturation of myeloid cells, DCs and T cells, and increases phagocytosis and bacterial killing of neutrophils and monocytes. Twelve RCTs with sepsis patients treated with GM-CSF showed increased monocytic HLA-DR expression, less nosocomial infections, shorter hospital stays and less antibiotic use [197-199]. However, two meta-analyses, performed in 2011 and 2019, did not show a difference in mortality and length of hospital stay in GM-CSF treated adults and neonates with sepsis compared with placebo [200, 201].

Another potential immunostimulatory therapy is IFNγ. In healthy volunteers infused with LPS, IFNγ partially reverses immunoparalysis, normalizing IL-10 and TNF production, and increasing monocytic HLA-DR expression [202]. IFNγ therapy in sepsis patients and patients with invasive fungal infections showed restored monocytic HLA-DR expression and monocyte function [203-205]. Furthermore, therapies targeting IL-7, PD-1 or PD-L1, showed a restoration of T cell function in *in vivo* studies and positive safety profiles in phase I/II, phase 1b and phase II clinical trials [78, 206-209]. Three meta-analyses show that therapy with thymosin alpha 1, a thymus-derived immunomodulatory peptide, in combination with ulinastatin, a protease inhibitor used for acute pancreatitis, reduces 28-day and 90-day mortality in sepsis patients and improves IL-6 and TNF levels [210-212]. Furthermore, cell therapy with intravascular administration of mesenchymal stromal cells shows a positive safety profile and lowers mortality in animal studies. In septic shock patients, it showed faster hemodynamic stabilization and faster neutropenic recovery compared with patients without treatment [213-215]. Lastly, phase I/II studies showed that extracorporeal cell therapy with donor granulocytes yielded lower bacterial endotoxin concentration, a reduced need for noradrenaline, lower CRP and PCT, and higher monocytic HLA-DR values [216, 217]. However, all aforementioned studies need randomized control trials to confirm their results.

Host directed therapy research in sepsis is shifting from doing big (unsuccessful) RCTs towards biomarker/phenotype-guided targeted therapy, also known as theragnostics or precision medicine (**Table 5**). As mentioned before, current sepsis phenotyping require slow "omics" techniques and/or many variables complicating trial enrolment [218]. Therefore, many current trials try to enrich patient groups based on one or two biomarkers by either pre-enriching (by including only biomarker positive patients) or pre-specifying subgroup analyses.

Some first considered negative trials showed a positive treatment effect in subgroup analysis, such as anakinra (recombinant IL-1 receptor antagonist, IL-1RA), which targets IL-1 signaling, in patients with macrophage activation syndrome (MAS) [219], or afelimomab, which targets TNF, in patients with high IL-6 levels [220]. Combinations of biomarker and therapy now in phase I/II development include (**Table 5**): recombinant IL-7 (CYT107) and nivolumab (anti-programmed death-1 (PD-1) monoclonal antibody) in patients with low leukocyte count, anakinra in patients with high ferritin and low monocytic HLA-DR (clinical features of MAsargramostim (human recombinant GM-CSF) in patients with low monocytic HLA-DR, and CytoSorb (an extracorporeal cytokine adsorber, also called polymyxin B hemoperfusion) in patients with high IL-6 [198, 208, 209, 221, 222]. However, the published studies were mainly done to testify for treatment safety and did not show any definitive benefit regarding mortality or ICU stay. Larger-scale ongoing trails should show if the aforementioned biomarker-targeted therapies are beneficial for sepsis patients.

Table 5. Examples of biomarker guided therapy

Targeting excessive inflammation			
Target/drug	**Biomarker enrichment**	**Summary of results**	**References**
Anti-TNF	High IL-6	Reduce of 28-day mortality, and less severity of organ dysfunction	Panacek, 2004 [220]
IL-1R antagonist, anakinra	Low monocytic HLA-DR and high ferritin	In patients with MAS (defined as high ferritin and low monocytic HLA-DR): lower mortality and less septic shock. – *post hoc analyses*	Shakoory, 2016 [219]
Targeting immunosuppression			
Target/drug	**Biomarker enrichment**	**Summary of results**	**References**
PD-1 inhibitors PD-L-1 inhibitors	Low leukocyte count	Phase IB trial showed increase in monocytic HLA-DR expression but no difference in pro-inflammatory cytokines. Phase 1/2 trials showed increase in monocytic HLA-DR expression and absolute lymphocyte counts. Both studies showed a positive safety profile.	Hotchkiss, 2019 [207] Watanabe, 2020 [223]
GM-CSF	Low monocytic HLA-DR	RCT with 38 patients showed increase in monocytic HLA-DR expression and shorter mechanical ventilation times and improved APACHE score.	Meisel, 2009 [198]
IL-7	Low leukocyte count	Patients that received CYT107 therapy had 3- to 4-fold increase in absolute lymphocyte counts and T cells, which lasted 2-4 weeks after study inclusion.	Francois, 2018 [209]

Abbreviations: APACHE: acute physiology and chronic health evaluation; CRP: C-reactive protein; IL: interleukin; PCT: procalcitonin; MAS: macrophage activation syndrome.

Ongoing clinical trials enriching for one or two biomarkers might be inefficient to target subsets of sepsis patients. Sorting patients into specific phenotypes might be more efficient, but as mentioned before, this is difficult to incorporate into clinical trials. Biomarker adaptive enrichment trials (first used in the cancer field) use a real-time updating strategy to sort patients during the study into the statistically more promising treatment-subgroup combinations [224]. If a treatment-subgroup combination reached superiority/inferiority or harm, that arm would be promoted/dropped (**Figure 8**) [224-226]. This ensured that efficiency is preserved while trials are flexible and can test multiple drugs in multiple subgroups [226]. Note that the design and operation of these trials are complex and simulation-intensive. It is easy to concede to investor-driven bias when not designing precise trial conditions beforehand [227]. One of the first adaptive trials in sepsis shock patients was looking at the effect of selepressin, a selective vasopressor. However, it has subsequently been terminated due to futility [228, 229]. Another adaptive trial, now ongoing, is the "randomised, embedded, multi-factorial, adaptive platform trial for community-acquired pneumonia" (REMAP-CAP), which is enrolling ICU patients with community-acquired pneumonia and is to be finished by the end of 2023. The first result of this trial show slight superiority for the treatment of hydrocortisone versus placebo in sepsis patients infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [230, 231].

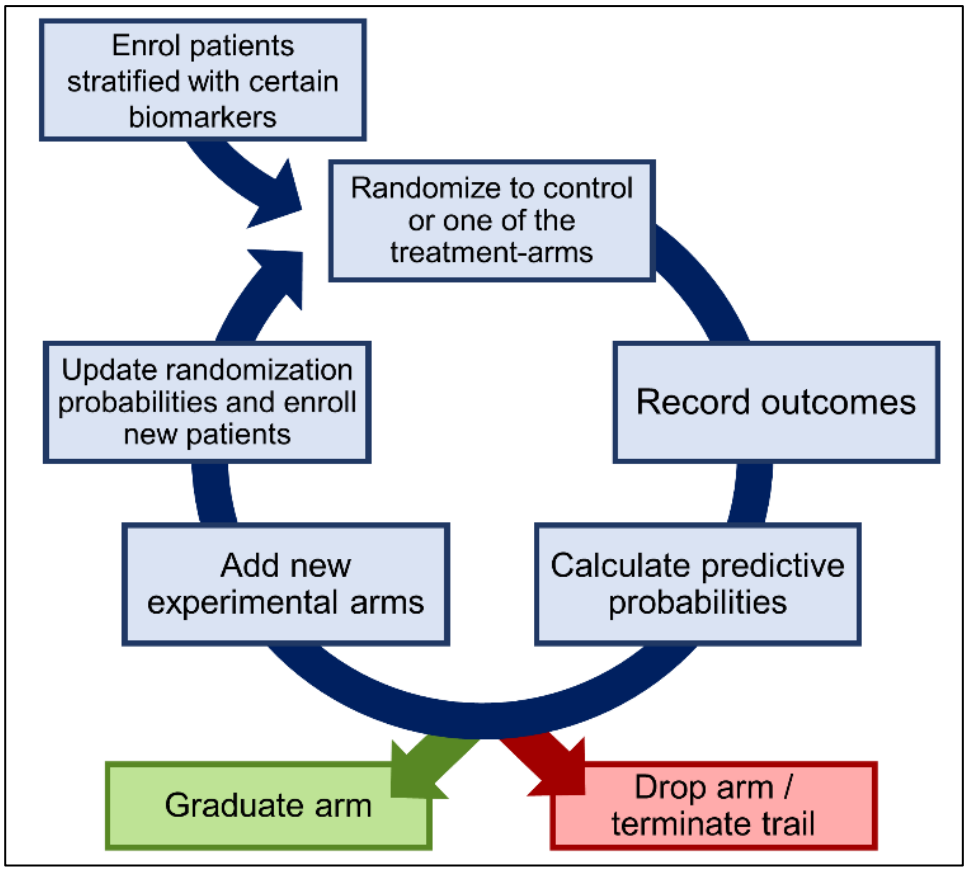

Figure 8. Adaptive trial design. Adaptive trial design is similar to conventional trial design regarding enrolling patients, setting outcomes and recording trial data. However, in adaptive trial design this information is used real-time to adjust the randomization towards the more beneficial outcomes and to drop non-beneficial arms, which can differ per subgroup [225, 226].

1.2 Myeloid-derived suppressor cells in sepsis

Part of this chapter has been published in Schrijver et al. Front. Immunol 2019 Feb 27;10:327. Reuse of text is in accordance to the current thesis guidelines of the University of Lausanne, the journal guidelines, and we have permission from all co-authors.

MDSCs are immature myeloid cells defined by their immunosuppressive function that expand during chronic and acute inflammatory conditions. Ideally, MDSCs are defined using phenotypic markers (mainly analysed using by flow cytometry) in combination with functional analyses of immunoregulatory activity (the golden standard is inhibition of T cells). However, a definite, consensual phenotyping scheme is lacking [232]. Generally, MDSCs are divided into two subtypes: monocytic MDSCs (M-MDSCs) and polymononuclear MDSCs (PMN-MDSCs), named after their morphological and phenotypical homologies with monocytes and PMNs [232-234].

In mice, MSDCs are defined as $Gr1^+$ $CD11b^+$ cells (Gr1: granulocyte receptor-1 antigen, consisting of Ly-6G and Ly-6C antigens). PMN-MDSCs are $CD11b^+$ $Ly6G^+$ $Ly6C^{low}$ cells and M-MDSCs $CD11b^+$ $Ly6G^-$ $Ly6C^{high}$ cells. In humans, the M-MDSCs are, phenotypically, HLA-DR low/neg monocytes. PMN-MDSCs are described as either low density granulocytes (as assessed by ficol paque) or as CD11b+, CD33+, CD15+ / CD66b+ cells. Furthermore, PMN-MDSCs present lower CD16 levels compared withPMNs [235, 236]. Other MDSC subsets have been described, including early MDSCs (e-MDSCs) and eosinophilic MDSCs (eo-MDSCs) [232, 237, 238]. Single cell RNA sequencing in late sepsis confirmed the presence of e-MDSCs, PMN-MDSCs and M-MDSCs but did not show gene expression described in MDSCs present in cancer, like arginase 1 (ARG1), CD274, COX2, PGE2 and NOS2 [238].

1.1.6 MDSCs activation and expansion

Hematopoietic stem cells differentiate into common myeloid progenitors, giving rise to immature myeloid cells. An inflammatory environment, as observed in sepsis, stimulates the egress of immature myeloid cells from the bone marrow into the blood stream and the gain of immunosuppressive functions (**Figure 9**) [97, 234]. The identification of mediators and molecular mechanisms underlying the expansion and the immunosuppressive functions of MDSCs may pinpoint to original therapeutic targets for various diseases. Most of our knowledge comes from disease conditions other than sepsis. In sepsis, the most relevant studies analyse the impact of gene specific knockout or the infusion of MDSCs in mice exposed to polymicrobial sepsis induced by cecal ligation and puncture (CLP).

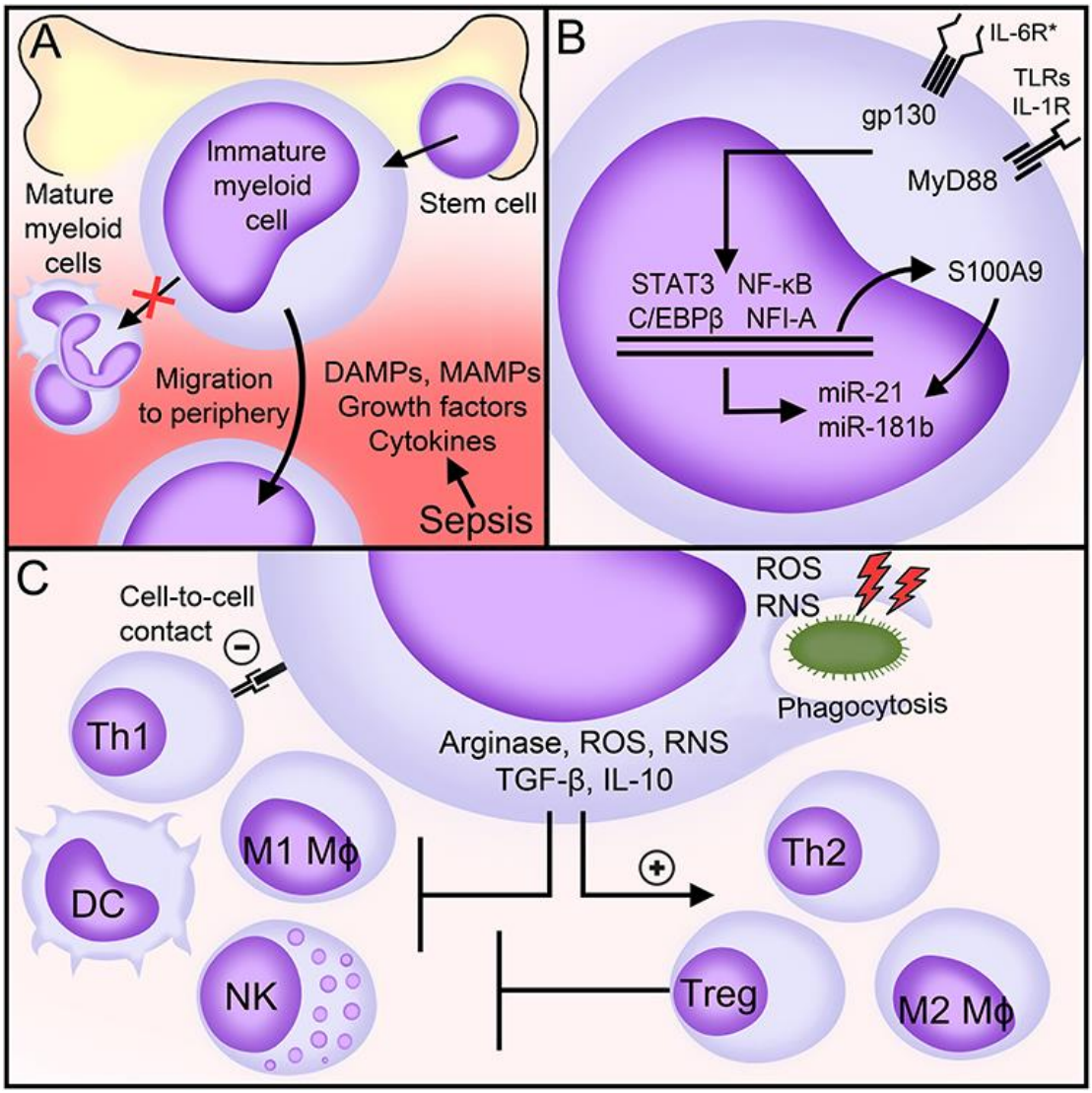

Figure 9. MDSCs in sepsis. A) Factors generated during sepsis induce the expansion and egress of MDSCs from the bone marrow into the peripheral blood. **B)** Main signalling pathways involved in the expansion and the immunosuppressive functions of MDSCs during sepsis. **C)** Biological functions of MDSCs during sepsis. From Schrijver et al., *Front. Immunol* 2019 Feb 27;10:327 [97].

The rise of MDSCs appears to be a complex and progressive process that involves expansion and activation of immature myeloid cells. Many factors supply signals for the expansion and the activation of MDSCs. The expansion and maturation of immature myeloid cells is primarily mediated by the action of growth factors (GF), colony stimulating factors (CSF), such as vascular endothelial-GF (VEGF), GM-CSF, macrophage-CSF (M-CSF), stem cell factor (SCF), DAMPs (S100 calcium-binding protein A8/A9, S100A8/9), and possibly chemokines (CXCL1, CXCL2). MAMPs, DAMPs, cytokines, and acute phase proteins (α2-macroglobulin, serum amyloid A) activate pathogenic MDSCs [234, 239-241].

MyD88, glycoprotein 130 (gp130), and nuclear factor I A (NFIA, a transcription factor) control the expansion and the immunosuppressive functions of MDSCs. MyD88 is an adaptor molecule that initiates intracellular signalling pathways through the IL-1 receptor and all TLRs except TLR3. gp130 is a signal transducer co-receptor for IL-6 family cytokines that cooperates with signal transducer and

activator of transcription (STAT3) and C/EBPβ, to upregulate MDSCs [82, 241]. MDSCs do not expand in MyD88$^{-/-}$ germline mice and in hepatocyte-specific gp130$^{-/-}$ and myeloid-specific Nfia$^{-/-}$ mice subjected to CLP [82, 239]. Additionally, Gr1^{+} CD11b^{+} MDSCs lacking NFI-A lose their immunosuppressive functions and stop differentiating into mature myeloid cells. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activated mice regulate metabolic reprogramming of MDSCs resulting in an increase of splenic MDSCs, similar to LPS-infused mice [242].

During CLP, the triggering of a NF-κB/C/EBPβ/ STAT3-axis upregulates the expression of S100A9 (also known as calgranulin B). S100A9 translocates into the nucleus to upregulate the transcription of microRNAs miR-21 and miR-181b, which fine-tune the expansion and the functions of MDSCs. Mice lacking S100A9 have less splenic and bone marrow MDSCs especially during late sepsis and are protected from death [243]. *In vivo* blockade of miR-21 and miR-181 decreases bone marrow MDSCs and improves sepsis survival [244]. The opposite is true for miR-150, miR-150 decreases during human and murine sepsis and replenishing miR-150 results in less MDSC expansion and a reduction of MDSC-induced immunosuppression [245]. The adoptive transfer of Gr-1^{+} CD11b^{+} MDSCs or PMN-MDSCs protects mice from acute endotoxemia, rapidly lethal CLP and pseudomonas airway infection [241, 246, 247]. However, the transfer of early Gr-1^{+} CD11b^{+} MDSCs (collected three days post-infection) increases early mortality from CLP, while the transfer of late MDSCs (collected 10-12 days post-infection) decreases or has no change on mortality [246, 248]. This might be caused by the instability of the functionality of MDSCs, as MDSCs seem to become more immunosuppressive after the initial sepsis episode in both mice and humans [246, 248, 249].

1.1.7 Immunosuppressive functions of MDSCs

MDSCs suppress the activity of immune cells through various mechanisms involving the downregulation of L-arginine, the production of ROS and RNS, the secretion of anti-inflammatory/immunosuppressive cytokines IL-10 and TGF-β as well as the activation of Tregs (**Figure 9**).

L-arginine becomes a semi-essential amino acid during sepsis because of increased usage due to inflammation and reduced production. L-arginine shortage is sustained by the production of arginase by MDSCs, which metabolizes L-arginine into L-ornithine and urea. L-arginine depletion limits the function of T cells and the activity of NK cells. ROS, RNS, IL-10 and TGF-β skew the polarization of monocytes/macrophages and T cells towards anti-inflammatory/pro-resolving M2, Th2 and regulatory phenotypes. Furthermore, they impair TCR and IL-2 receptor signalling, NK cell activity and DC maturation and antigen presentation [82, 246, 250]. MDSCs suppress Th1 responses though direct cell-to-cell contact, partly though the upregulation of PD-L1 [82, 93, 250]. Together with C-C motif chemokine ligand (CCL) 5 (RANTES) and CCL4 (MIP-1β), RNS, IL-10 and TGF-β promote the recruitment and the immunosuppressive activity of Tregs, at least in cancer and in neonates [82, 250]. The interaction between MDSCs and Tregs in sepsis is unknown. Of note, MDSCs can also help fight infections. Indeed, MDSCs efficiently phagocytose *E. coli* and group B streptococci [251] and clear bacteria during late sepsis through a robust production of ROS [246]. More work is required to understand to what extent these biological variations reflect the accumulation or the differentiation of different MDSCs subpopulations during sepsis.

1.1.8 Epigenetic changes of MDCSs in sepsis

MDSCs are highly plastic cells that shift in function and phenotype. Epigenetic changes, such as DNA methylation, histones modifications, and non-coding RNAs like micro RNAs (miRNAs) and long non-coding RNAs (lncRNAs), mediate the development and transition of MDSCs [252].

Histone deacetylase (HDACs) 11, a regulator of C/EBP-β, inhibits expansion and function of MDSCs [253-256]. In mice and human, we see many changes in miRNA in MDSCs of sepsis patients. The two most described upregulated miRNAs in human and mouse MDSCs are miR-21 and miR-181 [244, 249, 257]. These miRNAs, through a synergistic mechanism, regulate MDSCs differentiation and maturation, and enhance MDSC accumulation [240, 244]. The lncRNA Hotairm1 supports MDSC expansion in septic mice by increasing S100A9 [258]. Therapeutics targeting epigenetics in MDSCs limiting their function are developed in the oncology field and they might be useful in sepsis as well [252, 259].

1.1.9 Diagnostic and prognostic value of MDSCs for sepsis

MDSCs make up an important proportion of immature myeloid cells. Multiple retrospective and prospective observational studies showed that immature granulocytes (IGs) levels discriminate between infected and uninfected patients and is associated with disease severity, clinical deterioration, and mortality [260, 261]. Few reports demonstrate the immunosuppressive functions of immature myeloid cells in relation with sepsis and/or monitor MDSCs subpopulations using advanced flow cytometry. Since cell preparation (whole blood, with and without ficoll purification) and flow cytometry strategies are not standardized, the phenotype of MDSCs, PMN-MDSCs and M-MDSCs differs between studies (**Table 6**).

The frequency of PMN-MDSCs (SSC^{high} $CD16^{+}$ $CD15^{+}$ $CD33^{+}$ $CD66b^{high}$ $CD114^{+}$ $CD11b^{+/low}$ LDG) and M-MDSCs (SSC^{low} $CD14^{+}$ $CD11b^{+}$ $CD16^{-}$ $CD15^{+}$) does not differ between non-infectious critical ill patients and sepsis patients [262]. However, high levels of PMN-MDSCs link to nosocomial infections and sepsis development. In a first study, PMN-MDSCs ($CD14^{-}$ $CD15^{+}$ low-density granulocytes, LDG) representing more than 36% of white blood cells (WBC) in blood sampled from ICU patients within three days of study inclusion predicts the subsequent occurrence of nosocomial infections [263]. Patients that develop nosocomial infections have 2.5 times more PMN-MDSCs than patients that do not. Lastly, in patients with resected esophageal cancer, the level of PMN-MDSCs corresponds to the development of sepsis [264]. ICU surgical patients (at days 1, 4, 7, 14, 21 and 28 or until discharge of ICU) with continuously high proportions of $CD33^{+}$ $CD11b^{+}$ $HLA\text{-}DR^{-/low}$ MDSCs have a longer stay in the ICU, more nosocomial infections, and poor functional status at discharge [265]. The percentage of total MDSCs in patients with severe sepsis/septic shock raises comprising 45% of WBCs, and a high proportion of MDSCs at diagnosis is associated with early mortality [263, 265, 266]. Results are conflicting about the association of M-MDSCs with mortality [264, 267]. M-MDSC levels correlate to gram-negative infections in sepsis patients [263, 266]. Of note, MDSCs from sepsis patients dose dependently suppress IFNγ, IL-4 and IL-10 production by T cells more efficiently than MDSCs from healthy subjects, while healthy and disease-associated MDSCs suppress T cell proliferation alike [265].

In summary, MDSCs seem to play a role during sepsis, and some clinical studies associate high proportions of blood MDSCs with clinical worsening, occurrence of nosocomial infections and mortality of sepsis patients. However, which MDSC subtypes causally relate to unfavourable outcomes is not clear. One limitation of current clinical studies, not limited to the sepsis field, resides in the uneven phenotypic classification of MDSCs.

Table 6. MDSCs as a biomarker in sepsis

Subjects	Cells/phenotypes	Observations	Reference
56 sepsis patients and 18 healthy controls.	M-MDSCs: $CD14^+$ $CD64^+$ $HLA\text{-}DR^-$ PMN-MDSCs: LDG $CD33^+$ $CD14^{neg/low}$ $CD64^{low}$ $CD15^{+/low}$	High % of M-MDSCs in all sepsis, but particularly in gram-negative sepsis patients. Prominent PMN-MDSCs in gram-positive sepsis. PMN-MDSCs suppress T cell proliferation *in vitro*.	Janols, 2014 [266]
67 surgical patients with severe sepsis/septic shock, 18 healthy controls.	MDSCs: $CD33^+$ $CD11b^+$ $HLA\text{-}DR^-$ M-MDSCs: $CD14^+$ PMN-MDSCs: $CD14^-$ $CD15^+$	High % of MDSCs at admission correlates with early mortality. Decreasing levels of MDSCs correlate with short ICU stay. Sustained levels of MDSCs (>30% of WBC) predict nosocomial infections.	Mathias, 2017 [265]
14 sepsis and 8 uninfected critically ill patients, 15 healthy controls.	M-MDSCs: SSC^{low} $CD14^+$ $CD11b^+$ $CD16^-$ $CD15^+$ PMN-MDSCs: $SSC^{high}CD16^+$ $CD15^+CD33^+CD66b^{high}$ $CD114^+CD11b^{+/low}$	M-MDSCs but not PMN-MDSCs increase at day 13-21 post-sepsis. Similar % of M-MDSCs and PMN-MDSCs in sepsis and non-septic critical ill patients.	Patera, 2016 [262]
94 sepsis patients, 11 severity-matched ICU patients, 67 health donors.	M-MDSCs: Lin^- $CD14^+$ $HLA\text{-}DR^{-/low}$ PMN-MDSCs: LDG $CD14^-$ $CD15^+$ (Excluding eosinophils)	High % of PMN-MDSCs in sepsis patients. M-MDSCs are higher in gram-negative than gram-positive sepsis. PMN-MDSCs > 36% WBC at entry are associated with higher risk of nosocomial infections. PMN- and M-MDSCs suppress T cell proliferation *in vitro*.	Uhel, 2017 [263]
301 septic shock patients and 18 healthy controls	M-MDSCs: $CD14^+$, $HLA\text{-}DR^-$	First time point do not correlate to outcomes (mortality and HAI). Levels of M-MDSCs on day 6-8 are associated with HAI and mortality.	Waeckel, 2020 [267]
68 esophageal cancer resection patients	MDSCs: $CD33^+$ $CD11b^+$ $HLA\text{-}DR^-$ M-MDSCs: $CD14^+$ PMN-MDSCs: $CD15^+$	Higher PMN-MDSC levels correlated to developing sepsis after surgery. Patients that developed sepsis with > 82.5% of PMN-MDSC had more mortality. M-MDSCs did not predict mortality.	Xu 2020 [264]

Abbreviations: HAI: hospital acquired infection; ICU: intensive care unit; MDSCs: Myeloid-derived suppressor cells; PMN-MDSCs: Polymorphonuclear MDSCs; M-MDSCs: monocytic MDSCs. Table adapted from [97]

1.1.10 MDSCs as a therapeutic target

Since MDSCs have such a versatile arsenal in immunosuppressing their environment, targeting them is an attractive and high impact option to combat the immunosuppressive phase of sepsis. Most studies targeting MDSCs occur in the oncology field, which ranges from animal studies to phase II clinical trials [268-271]. There are four different ways to inhibit MDSCs (**Figure 10**) [269, 270, 272-276]:

1) Blocking MDSC recruitment. The easiest way to block the recruitment of MDSCs is through chemokine inhibitors. CCL2, CCL5, CXCR2 and CSF-1R antagonists showed promise reducing MDSC populations in tumor and bloodstream, inducing T cell activity and enhancing therapeutic efficacy of other cancer therapies.
2) Inducing MDSC apoptosis. Depleting MDSCs from the circulation can be achieved with low dose chemotherapy, tyrosine kinase inhibitors (like Sunitinib) or DS-8273a (TRAIL receptor 2 antibody that mediates apoptosis of MDSCs), which showed success in oncological mice and patients.
3) Differentiating MDSCs in more mature, less immunosuppressive, cells. Low doses of chemotherapy and vitamins like vitamin D3 and all-trans-retinoic acid (ATRA) induce MDSC differentiation.
4) Inhibiting MDSC activity. Inhibiting the function of MDSCs can be reached by disrupting signalling of COX-2, STAT3/5, though epigenetic reprogramming by HDAC inhibitors or through directly targeting ARG1 expression and ROS production.

All mentioned therapies showed an increased T cell reactivity and improved clinical outcome in oncology patients. Furthermore, a combination of therapies might be ideal due to its synergistic effect. However, in the sepsis field, it is not yet clear which patients can benefit from MDSC-targeted therapy.

The pathophysiology between cancer and sepsis regarding the immune system overlap significantly. Both originate by activation of the immune system through persistent exposure of PAMPs and DAMPs, have recruitment of immune cells and have a severe immunosuppressive response through the presence of MDSCs, heightened apoptosis and T cell exhaustion [277]. Therefore, it is likely that successful therapies targeting MDSCs in oncological patients can make the translation to sepsis patients.

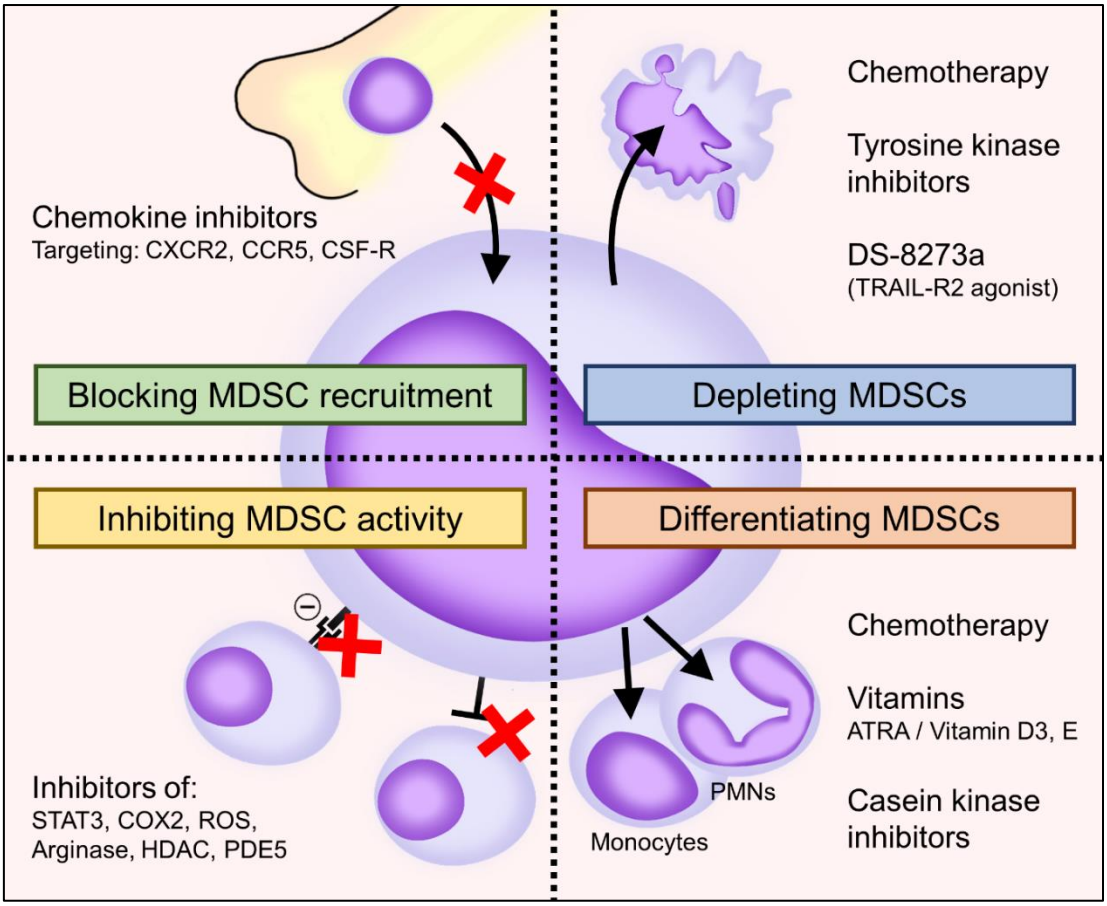

Figure 10. Strategies to target myeloid-derived suppressor cells (MDSCs). MDSCs inhibition can be achieved by: 1) targeting MDSC recruitment by inhibiting chemokines, 2) depleting MDSCs by inducing their apoptosis, 3) inhibiting MDSC activity by blocking signalling and cytokine, arginase and ROS production and 4) by inducing MDSC differentiation towards mature phenotypes. Abbreviations: ATRA: all-*trans*-retinoic acid; CCR: C-C chemokine receptor; COX: cyclooxygenase; CSF-R: colony stimulating factor receptor; HDAC: histone deacetylase; PDE5: phosphodiesterase type 5; PMNs: polymorphonuclear cells; STAT3: signal transducer and activator of transcription 3; TRAIL-R2: TNF-related apoptosis-inducing ligand receptor 2 [269, 270, 275].

Sepsis research directly targeting MDSCs has not yet reached the clinical trial stage. However, several mouse studies targeted MDSCs in sepsis (**Table 7**) [264, 278-282]. Immunosuppression was induced in two ways: 1) by CLP creating polymicrobial sepsis, and 2) by administering multiple, increasing LPS doses over a longer time period. ATRA is the most popular treatment to combat immunosuppression induced by MDSCs. Two studies by Martire-Greco et al. looked at the effect of ATRA in LPS infused mice. They observed improved functional immune responses and lower numbers of MDSCs in both spleen and blood stream [279, 280]. Xu et al. showed that ATRA improved survival by 50% in mice first implanted with Lewis lung cancer cells and afterwards infected by CLP [264].

Hu et al. showed that LDK378, an anaplastic lymphoma kinase inhibitor, inhibited the migration of MDSCs to the spleen by blocking the upregulation of CCR2 and the G-protein-coupled receptor kinase-2. This resulted in more CD4+ and CD8+ T cells and reduced Tregs and overall improved survival from 40% to 80% in CLP inflicted mice [282]. Lui et al. looked at YCP, an α-glucan that interacts with TRL2 and TRL4 [281, 283]. In CLP septic mice, the YCP treatment group had decreased MDSCs in lung and liver, and improved survival from 39% to 72% [281]. Lastly, Xu et al. showed that ferumoxytol, an

intravenous iron preparation, is internalized by MDSCs resulting in increased MDSC maturation, reduced numbers of MDSCs in the spleen and a reduced production of ROS in MDSCs in LPS challenged mice [278].

Overall, MDSCs seem to be a promising target of therapy in sepsis patients. However, more research is needed to establish which subtypes of MDSCs are mainly involved in the induction and maintenance of the immunosuppressive phase of sepsis, which patient populations would benefit from MDSC targeting therapies, and, importantly, if it would be safe to use MDSC targeted therapies in sepsis since these patients are at risk for agranulocytosis.

Table 7. Therapies inhibiting MDSC in sepsis

Drug	Target Mechanism	Type of model	Outcome	Reference
ATRA	Induction of differentiation and depletion of MDSCs	Immunosuppressed BALB/c mice by LPS infusion	Decreased amount of functional MDSCs and restored T cell proliferation	Martire-Greco 2014 [280]
		Immunosuppressed BALB/c mice by LPS infusion	Decreased number of CD34+ precursor cells. Less MDSCs in spleen and restoration of T lymphocyte proliferation	Martire-greco 2017 [279]
		Cancer-sepsis model: Lewis lung cancer cell, and 3 weeks later CLP. Male C57BL/6 mice.	Improved survival by 50% in mice treated with ATRA.	Xu 2020 [264]
LDK378	Inhibition of the recruitment of MDSCs	CLP induced septic mice	Improved survival of septic mice, more CD4+ and CD8+ T cells Less MDSCs in the spleen	Hu 2019 [282]
α-glucan: YCP	Inhibition of the expansion of MDSCs	CLP induced septic C57BL/6J male mice	Improved survival rate from 39% to 72%. Decreased MDSC in long, liver and bone marrow. However, function is increased.	Liu 2017, [281]
Ferumoxytol	Induction of the differentiation of MDSCs and inhibition of their function	Ex-vivo and in LPS challenged mice	Downregulated Arginase-1, S100A8, S100A9 and ROS production. Lower percentages of PMN-MDSCs in blood and spleen.	Xu 2019, [278]

Abbreviations: ATRA: all-*trans*-retinoic acid; CLP: cecal ligation and puncture; LPS: lipopolysaccharide; MDSCs: myeloid-derived suppressor cells; ROS: reactive oxygen species.

2. Aim

Sepsis is one of the leading causes of mortality and morbitiy worldwide, yet there is no targeted treatment available. Sepsis patients simultaneously show overwhelming inflammation and long-lasting immunosuppression. We hypothesized that MDSCs play a dual role in sepsis, where on the one hand they limit overwhelming inflammation, organ failure and short term mortality, and on the other, they sustain the immunosuppressive responses that result in secondary infections and long term mortality. Furthermore, since MDSCs are virtually absent in healthy subject, and present a diverse arsenal of immunosuppressive functions, they could present interesting targets of therapy.

The aim of this thesis was to investigate the behavior of MDSC subpopulations and general immune cell populations during the course of sepsis, and to assess their diagnostic, prognostic and theragnostic potential.

To achieve our aims, we set up four studies (**Table 8**), where we analyzed MDSCs in relation to illness severity, secondary infections, and mortality. We standardized the identification of MDSCs by flow cytometry and automatic clustering using FlowSOM to avoid gating bias [284].

Table 8. Clinical studies used in this thesis

	Endotoxin	PIPOVAP	LUH-COVID-19	INCLASS
Type of study	Experimental	Prospective cohort	Prospective cohort	Interventional
Type and number of patients	8 healthy male volunteers	32 non-infectious critical-ill patients without antibiotics	56 hospitalized PCR confirmed COVID19 patients	48 pneumonia-based sepsis patients with MODS
Intervention	2 endotoxin infusions, 7 days apart	n/a	n/a	Clarithromycin
Time points, blood drawing	15 time points	ICU admission and ICU discharge	During hospital stay and 3 months later	1, 5 and 10 days after inclusion
MDSC identification	Customized duraclone tubes*	Pipetted	Pipetted	Customized duraclone tubes*
Location	Radboud UMC, Nijmegen	Lausanne University Hospital	Lausanne University Hospital	Attikon university hospital, Athens
Study duration	June 2018 – August 2018	July 2018 – May 2019	April 2020 – January 2021	December 2017 – September 2019

*In collaboration with Beckman Courter (IN, USA). Abbreviations: PIPOVAP: Profile, Interaction, and PrOgnosis in Ventilator Associated Pneumonia; LUH: Lausanne University Hospital; COVID-19: Coronavirus Disease 2019; INCLASS: Benefit of Clarithromycin in Patients With Severe Infections Through Modulation of the Immune System; MODS: Multi-organ dysfunction syndrome.

3. Results

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3.2 “Myeloid-derived suppressor cells are elevated in healthy subjects infused with endotoxin and predict outcome in non-infected critical care patients.”
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3.1. Myeloid-derived suppressor cells in sepsis

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Summary:

Myeloid-derived suppressor cells (MDSCs) are immature myeloid cells characterized by their immunosuppressive functions. MDSCs expand during chronic and acute inflammatory conditions, the best-described being cancer. Recent studies uncovered an important role of MDSCs in the pathogenesis of infectious diseases along with sepsis. Here we discuss the mechanisms underlying the expansion and immunosuppressive functions of MDSCs, and the results of preclinical and clinical studies linking MDSCs to sepsis pathogenesis. Strikingly, all clinical studies to date suggest that high proportions of blood MDSCs are associated with clinical worsening, the incidence of nosocomial infections, and/or mortality. Hence, MDSCs are attractive biomarkers and therapeutic targets for sepsis, especially because these cells are barely detectable in healthy subjects. Blocking MDSC-mediated immunosuppression and trafficking or depleting MDSCs might all improve sepsis outcome. While some key aspects of MDSCs biology need in-depth investigations, exploring these avenues may participate to pave the way toward the implementation of personalized medicine and precision immunotherapy for patients suffering from sepsis.

My contribution to this work:

I reviewed the literature, drafted the paper and designed the figure and table.

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Myeloid-Derived Suppressor Cells in Sepsis

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Myeloid-derived suppressor cells (MDSCs) are immature myeloid cells characterized by their immunosuppressive functions. MDSCs expand during chronic and acute inflammatory conditions, the best described being cancer. Recent studies uncovered an important role of MDSCs in the pathogenesis of infectious diseases along with sepsis. Here we discuss the mechanisms underlying the expansion and immunosuppressive functions of MDSCs, and the results of preclinical and clinical studies linking MDSCs to sepsis pathogenesis. Strikingly, all clinical studies to date suggest that high proportions of blood MDSCs are associated with clinical worsening, the incidence of nosocomial infections and/or mortality. Hence, MDSCs are attractive biomarkers and therapeutic targets for sepsis, especially because these cells are barely detectable in healthy subjects. Blocking MDSC-mediated immunosuppression and trafficking or depleting MDSCs might all improve sepsis outcome. While some key aspects of MDSCs biology need in depth investigations, exploring these avenues may participate to pave the way toward the implementation of personalized medicine and precision immunotherapy for patients suffering from sepsis.

Keywords: sepsis, infectious disease, innate immunity, myeloid-derived suppressor cells, biomarker, immunosuppression, inflammation, personalized medicine

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INTRODUCTION

Sepsis is one of the leading causes of preventable death. Sepsis is defined as a "life-threatening organ dysfunction caused by a dysregulated host response to infection" (1). The mortality rate of sepsis accounts for five-to-six million deaths of ∼30 million cases per year worldwide. Sepsis incidence is rising due to the aging of the population, the burden of chronic diseases, the increasing number of immunocompromised patients, and the resistance of microorganisms to antimicrobials (2). In 2017, the World Health Assembly and the World Health Organization made sepsis a global health priority by adopting a resolution to improve the prevention, diagnosis, and management of sepsis.

Innate immune cells, such as monocytes/macrophages, dendritic cells (DCs), and neutrophils, sense microbial and danger-associated molecular patterns (MAMPs produced by microorganisms, and DAMPs released by injured or stressed cells) through pattern recognition receptors (PRRs). PRRs are grouped into five main families: toll-like receptors (TLRs), NOD-like receptors, C-type lectins, scavenger receptors, RIG-I-like receptors, and intra-cytosolic DNA sensors (3). The interaction between PRRs and MAMPs or DAMPs triggers intracellular signaling pathways that coordinate gene expression, the development of the inflammatory response, the establishment of antimicrobial cellular and humoral responses, and the restoration of homeostasis once pathogens have been contained or eradicated. Sepsis is characterized by an early exacerbation of antimicrobial defense mechanisms, the so-called hyper-inflammatory "cytokine storm," mediating

tissue injury, organ dysfunctions and early mortality, and a concomitant shift toward inflammation resolution and tissue repair. Sepsis-induced immunoparalysis (or immunosuppression) favors the development of secondary infections and long-term immune disabilities accounting for late mortality (4–8).

During the last decades, early goal-directed therapy decreased early mortality from sepsis, which contributed to shift the sepsis ICU population toward a population suffering from chronic critical illness (CCI). Indeed, a subset of ICU patients surviving sepsis develop CCI characterized by long-lasting immunosuppression associated with a persistent, low-grade, inflammation maintained by the continuing release of DAMPs. The underlying inflammation is associated with catabolism and malnutrition. The term persistent inflammation-immunosuppression and catabolism syndrome (PICS) has been proposed to characterize this degraded state. PICS is associated with long-term morbidity, late multiple organ failures and late mortality (9–11).

Clinical trials testing adjunctive therapy to dampen inflammation-related dysfunctions in sepsis have not been conclusive (12). Several reasons may account for these failures, among them the large heterogeneity of the sepsis syndrome. Nowadays, the prevalent view is that restoration of immune capacities using immuno-stimulants might be more efficient than anti-inflammatory therapies. In any case, personalized medicine should be used to define at an individual level whether inflammatory cytokines, immunoparalysis, or metabolism has to be targeted (4, 7, 13–17). In that perspective, significant efforts are devoted to the identification of genetic, molecular, and cellular biomarkers to stratify patients for clinical studies and treatment based on clinical condition and disease stage.

We poorly understand what is responsible for a dysregulated host response and the delay returning to homeostasis in sepsis patients (4–8, 18). Growing interest focuses on a subpopulation of leukocytes called myeloid-derived suppressor cells (MDSCs). MDSCs are involved in the regulation of the immune response in many pathological situations, the best-studied being cancer. A number of comprehensive reviews discusses MDSCs in the context of cancer, autoimmunity and infectious diseases [see for example (19–26)]. Interestingly, recent data suggest that MDSCs are involved in immune dysfunctions observed in sepsis. In this review, we summarize and discuss our current knowledge about the role played by MDSCs during sepsis and the potential of using MDSCs as biomarkers and therapeutic targets of sepsis.

MYELOID-DERIVED SUPPRESSOR CELLS (MDSCs)

MDSCs are immature myeloid cells that expand during chronic and acute inflammatory conditions. The premises of MDSC discovery date back more than a century when tumor progression was associated with extra-medullary haematopoiesis and neutrophilia. In the mid-1960s, Lappat and Cawein reported that subcutaneously transplanted A-280 tumor cells generate factors involved in a leucocytosis response that sustains tumor growth (27). Subsequently, leucocytosis was involved in the expansion of cells of myeloid origin with immunosuppressive activity (24). These cells express reduced levels of conventional markers for mature myeloid and lymphoid cells and were named natural suppressor cells, null cells, immature myeloid cells, or myeloid suppressor cells. In 2007, "myeloid-derived suppressor cells" was adopted as a unifying term to minimize the confusion prevailing in the literature (28).

MDSCs are defined primarily by their immunosuppressive functions. Within sepsis, one may predict that MDSCs play a dual role depending on disease progression. On the one hand, MDSCs may be beneficial by limiting hyper-inflammation during the early stages of sepsis, hence protecting from early organ dysfunction. On the other hand, MDSCs may be detrimental by amplifying long-term immunosuppression and contributing to CCI and/or PICS (8, 10). As discussed later, these two facets have been highlighted in experimental models, while clinical studies all pointed to a deleterious role of MDSCs.

Minimal phenotypic characteristics of MDSCs have been proposed, but a definite, consensual phenotyping scheme is lacking (29, 30). Two main subpopulations of MDSCs are usually considered: polymorphonuclear MDSCs (PMN-MDSCs, previously called granulocytic-MDSCs) and monocytic MDSCs (M-MDSCs), so-called because of their morphological and phenotypical homologies with PMNs and monocytes (26, 29–32). In mice, MSDCs are defined as $Gr1^+$ $CD11b^+$ cells (Gr1: granulocyte receptor-1 antigen, consisting of Ly-6G and Ly-6C antigens). PMN-MDSCs are $CD11b^+$ $Ly6G^+$ $Ly6C^{low}$ cells and M-MDSCs $CD11b^+$ $Ly6G^-$ $Ly6C^{high}$ cells. In humans, PMN-MDSCs are $CD11b^+$ $CD14^-$ $CD33^+$ ($CD15^+$ or $CD66^+$) cells and M-MDSCs $CD11b^+$ $CD14^+$ $HLA\text{-}DR^{low/-}$ $CD15^-$ cells. PMN-MDSCs overlap phenotypically with mature neutrophils but contrary to PMNs, MDSCs sediment within the PBMC fraction in ficoll gradients after density separation of whole blood. Whether low density gradient (LDGs) PMNs and PMN-MDSCs are the same entity is unclear, albeit the terms is used interchangeably in the literature. The identification of PMN-MDSCs by density gradient is further limited by the rise of not only low-density neutrophils, but also high-density $CD62L^{dim}$ neutrophils that suppress T cells in the blood of healthy humans infused with endotoxin (33). Additional markers are proposed to differentiate MDSCs from monocytes or granulocytes, for example high expression of lectin-type oxidized LDL receptor-1 (LOX-1) by PMN-MDSCs when compared to granulocytes in whole blood (33, 34).

Complicating the picture, other MDSC subsets have been described, among others early-stage MDSCs (e-MDSCs) and eosinophilic MDSCs (eo-MDSCs) (29, 35). In addition, tumor-associated macrophages (TAMs), which unlike their name suggests are present in inflammatory conditions bedsides cancer, can be considered as one of the members making up the MDSC spectrum (36, 37). Finally, MDSCs are highly plastic. They can differentiate into osteoclasts and non-suppressive mature myeloid cells, and M-MDSCs can differentiate into TAMs and PMN-MDSCs (38–41). Overall, to this day, identifying MDSCs based on cell surface phenotyping usually ends up with a mixed population, eventually containing other myeloid cell types, that

does not take into account the hallmark immunosuppressive function of MDSCs.

Adding to the above caveats, improper cell separation through density gradient and freezing whole blood or PBMC samples before flow cytometry analyses affects the detection of MDSCs, especially PMN-MDSCs. Hence, an objective of future studies is to optimize and harmonize sample handling and flow cytometry strategies (labeling, gating, and analyses) to quantify MDSCs in whole blood. This will facilitate the comparison of results from different studies to determine whether MDSCs are reliable disease biomarkers (32, 42). Strategies to identify cell surface markers discriminating MDSCs from other leukocytes using unbiased high discriminating techniques like RNA sequencing and mass cytometry analyses are starting to be used and have not yet improved the immuno-phenotyping of MDSCs (43). To summarize, the analysis of MDSCs and comparing results from different studies is complicated mainly because of: (1) the functional definition of MDSCs, (2) the lack of a defined phenotype(s) of MDSCs, and (3) the plasticity of MDSCs.

MDSCs EXPANSION AND ACTIVATION

Hematopoietic stem cells differentiate into common myeloid progenitors giving rise to immature myeloid cells. An inflammatory environment, as observed in sepsis, stimulates the egress of immature myeloid cells from the bone marrow into the blood stream and the gain immunosuppressive functions (26, 44) (**Figure 1**). The identification of mediators and molecular mechanisms underlying the expansion and the immunosuppressive functions of MDSCs may pinpoint to original therapeutic targets for various diseases. Most of our knowledge comes from disease conditions other than sepsis. In sepsis, most relevant studies analyse the impact of gene specific knockout or the infusion of MDSCs in mice exposed to polymicrobial sepsis induced by cecal ligation and puncture (CLP).

In mice subjected to CLP, MDSCs accumulate in secondary lymphoid organs, in which they represent as much as 10–20% of all leukocytes (45). In the spleen, MDSCs expand within 3–5 days, culminate after 10–14 days and stay high for at least 12 weeks. The rise of MDSCs appears to be a complex and progressive process that involves expansion and activation of immature myeloid cells through many factors. These factors are not specific to sepsis and can be redundant. The expansion of immature myeloid cells is primarily mediated by the action of growth factors (GF) and colony stimulating factors (CSF) [such as vascular endothelial-GF (VEGF), granulocyte-macrophage-CSF, macrophage-CSF (M-CSF) and stem cell factor (SCF)], DAMPs (S100 calcium-binding protein A8/A9, S100A8/9), and possibly chemokines (CXCL1, CXCL2). Activation of pathogenic MDSCs is induced by MAMPs (LPS, staphylococcal enterotoxins), DAMPs (HMGB1), cytokines (IFNγ, IL-1β, IL-4, IL-6, IL-7, IL-10, IL-13, TNF, CXCL3), and acute phase proteins (α2-macroglobulin, serum amyloid A) (26, 42, 46–56). These same factors may induce the maturation of MDSCs, with possible different outcomes. For example, M-MDSCs exposed to R848 (a TLR7/8 agonist), TNF and IFNγ differentiate into inflammatory macrophages that produce TNF and IL-12, while M-MDSCs exposed to Pam_3CSK_4 (a TLR1/2 agonist) differentiate into immunosuppressive macrophages producing IL-10 (47, 57).

Myeloid differentiation primary response 88 (MyD88), glycoprotein 130 (gp130) and nuclear factor I A (NFIA, a transcription factor) control the expansion and the immunosuppressive functions of MDSCs (**Figure 1**). MyD88 is an adaptor molecule that initiates quick nuclear factor-κB (NF-κB) signaling through the IL-1 receptor and all TLRs except TLR3. gp130 is a signal transducer co-receptor for IL-6 family cytokines that cooperates with signal transducer and activator of transcription (STAT3) and C/EBPβ to upregulate MDSCs (45, 54). MDSCs do not expand in $MyD88^{-/-}$ germline mice and in hepatocyte-specific $gp130^{-/-}$ and myeloid-specific $Nfia^{-/-}$ mice subjected to CLP (25, 45, 49, 58, 59). Additionally, $Gr1^+$ $CD11b^+$ MDSCs lacking NFI-A lose their immunosuppressive functions and stop differentiating into mature myeloid cells. The expansion of MDSCs is normal in myeloid-specific $Cebpb^{-/-}$ septic mice, but $Cebpb^{-/-}$ MDSCs produce reduced levels of IL-10 (52, 60). During CLP, triggering of a NF-κB/C/EBPβ/STAT3 axis upregulates the expression of S100A9 (also known as calgranulin B). S100A9 translocates into the nucleus to upregulate the transcription of microRNAs miR-21 and miR-181b that fine tune the expansion and the functions of MDSCs. Mice lacking S100A9 have less splenic and bone marrow MDSCs especially during late sepsis and are protected from death (61, 62). *In vivo* blockade of miR-21 and miR-181 decreases bone marrow MDSCs and improves sepsis survival (63). Recent work suggest that Nfe2l2 (nuclear factor, erythroid derived 2, Like 2; also known as NRF2) contributes to increase the metabolic activity and the expansion of $Gr1^+$ $CD11b^+$ MDSCs during endotoxemia (64).

The molecules mentioned above are not specific to MDSCs, and their genetic ablation can influence other arms of the defenses systems. To bypass this limitation, MDSCs isolated from sepsis mice are infused into wild-type recipient mice subjected to microbial insults. The adoptive transfer of Gr-1^+ $CD11b^+$ MDSCs or PMN-MDSCs harvested from septic donor-mice into recipient mice protects the later from acute endotoxemia, rapidly lethal CLP and *Pseudomonas* airway infection (54, 60, 65–68). Two studies compare the benefits provided by the infusion of Gr-1^+ $CD11b^+$ MDSCs taken either quickly or late after the onset of infection (i.e., 3 vs. 10–12 days post-infection). Interestingly, the transfer of early MDSCs increases while the transfer of late MDSCs decreases or does not change mortality (65, 69). Supported by additional *in vivo* and *in vitro* data (65, 69), this can be explained by the fact that, during the course of sepsis, MDSCs evolve to a more immature and anti-inflammatory state. More work will be required to appraise how much the maturation stage of MDSCs, the timing of expansion and/or infusion of MDSCs and the severity of the infectious models tip the balance toward a beneficial or a detrimental impact of MDSCs on sepsis outcome.

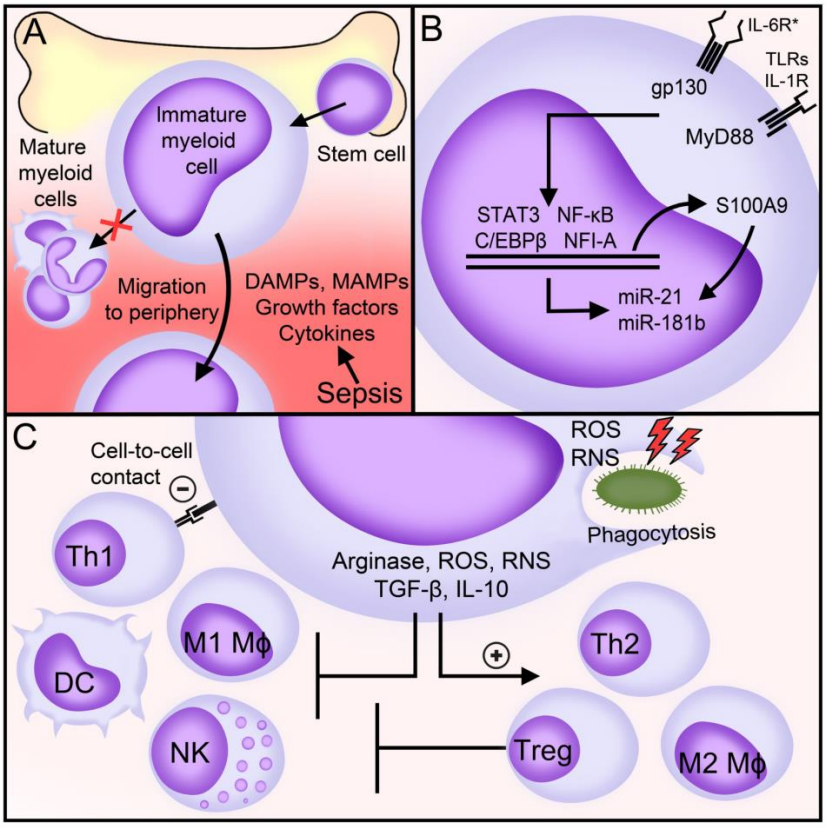

FIGURE 1 | MDSCs in sepsis. **(A)** Factors generated during sepsis induce the expansion and egress of MDSCs from the bone marrow into the peripheral blood. **(B)** Main signaling pathways involved in the expansion and the immunosuppressive functions of MDSCs during sepsis. **(C)** Biological functions of MDSCs during sepsis. See body text for detailed explanations. DAMPs, danger-associated molecular patterns; MAMPs, microbial-associated molecular patterns; IL-6R*, interleukin (IL)-6 receptor family of cytokines (commonly referred to as gp130 cytokines); gp130, glycoprotein 130; TLRs, toll-like receptors; IL-1R, interleukin-1 receptor; MyD88, Myeloid differentiation primary response 88; NF-κB, nuclear factor-κB; NFI-A, nuclear factor I A; STAT, signal transducer and activator of transcription; miR, microRNA; Mφ, macrophage; DC, dendritic cell; Th, T helper; NK, natural killer; Treg, T regulatory; IFNγ, interferon γ; ROS, reactive oxygen species; RNS, reactive nitrogen species; TGF-β, transforming growth factor-β; IL-10, interleukin-10.

As we will see in the last paragraph, the picture is clearer in clinical settings where high proportions of MDSCs indicate a poor prognosis.

The main epigenetic mechanisms, i.e., DNA methylation, histones methylation and acetylation, miRNAs and long non-coding RNAs (LncRNAs), have been implicated in the development of MDSCs with different outcomes (70). For example, inhibition of the DNA methyltransferases (DNMTs) 3a and 3b promotes the suppressive functions of MDSCs while inhibition of the histone methyltransferase SETD1B limits their suppressive function (71, 72). Pan-inhibitors of histone deacetylases (HDACs) 1–11 elicit robust expansion of M-MDSCs (73), in agreement with the observation that HDAC11 itself acts as a negative regulator of expansion and function of MDSCs (74). Interestingly, HDAC2 drives the phenotypic differentiation of M-MDSCs into PMN-MDSCs in tumor bearing mice (75), suggesting that individual HDACs have discrete, specific impact on MDSCs. Remarkably, combination therapies of inhibitors of either DNMTs or HDACs and checkpoint inhibitors (anti-PD-1 or anti-CTLA-4 antibodies) allow the eradication of checkpoint inhibitor resistant metastatic cancers by suppression of MDSCs (76). Finally, miRNAs both positively and negatively regulate the accumulation and functions of MDSCs (for instance miR-9, 17-5p, 21, 34a, 155, 181b, 210, 494, 690 vs. miR-9, 146a, 147a, 185-5p, 223, 185, 424) (70, 77). These observations, obtained in cancer models, are particularly interesting because cancer and sepsis share certain epigenetic features. Therefore, it is no surprise that oncolytic epigenetic drugs have a strong impact on innate immune responses and sepsis development (78–81). Numerous epigenetic drugs are tested in oncologic clinical trials while some are already approved for clinical applications. Altogether, these observations open a fascinating area to test epigenetic drugs targeting the expansion and/or function of MDSCs during sepsis.

IMMUNOSUPPRESSIVE FUNCTIONS OF MDSCs

MDSCs suppress the activity of immune cells through various mechanisms involving the degradation of L-arginine, the production of reactive oxygen and reactive nitrogen species (ROS, RNS), the secretion of anti-inflammatory/immunosuppressive cytokines like IL-10 and transforming growth factor (TGF)-β and the activation of T regulatory cells (Tregs) (**Figure 1**).

L-arginine becomes a semi-essential amino acid during sepsis because of increased usage and reduced production. L-arginine shortage is sustained by the production by MDSCs of arginase that metabolizes L-arginine into L-ornithine and urea (82). L-arginine depletion affects the function of T cells through a decreased expression of the CD3 zeta-chain, which is essential for T-cell receptor (TCR) signaling (50, 83). A lack of arginase also limits the activity of natural killer (NK) cells (84). ROS, RNS, IL-10, and TGF-β skew the polarization of monocytes/macrophages and T cells toward anti-inflammatory/pro-resolving M2, Th2 and regulatory phenotypes (45, 65, 85) and impair TCR and IL-2 receptor signaling, NK cell activity and DC maturation and antigen presentation (86–89) (**Figure 1**). MDSCs suppress Th1 responses though direct cell-to-cell contact, but how precisely this occurs remains to be determined (45, 85). Together with CCL5/RANTES and CCL4/MIP-1β, RNS, IL-10, and TGF-β promote the recruitment and the immunosuppressive activity of Tregs, at least in cancer and in neonates (45, 85, 90, 91). The interaction between MDSCs and Tregs in sepsis is unknown.

Splenic MDSCs harvested from CLP mice early (3–5 days) and late (10 days) after sepsis onset inhibit T cell proliferation. Early MDSCs secrete less S100A9 than late MDSCs (61) and, in response to LPS and IL-6, less TNF, IL-6, IL-10, ROS, and arginase I (65). However, in response to GM-CSF, early MDSCs produce RNS and proinflammatory cytokines while late MDSCs produce arginase, IL-10 and TGF-β (69). Of note, MDSCs can also help fight infections. Indeed, MDSCs efficiently phagocytose *E. coli* and group B streptococci (92) and clear bacteria during late sepsis through a robust production of ROS (65). Thus, MDSCs have diverse biological outputs according to their surrounding milieu and sepsis progression (54, 65). More work is required to fully understand to which extend these biological variations reflect the accumulation or the differentiation of different MDSCs subpopulations during sepsis.

DIAGNOSTIC AND PROGNOSTIC VALUES OF IMMATURE GRANULOCYTES AND MDSCs IN HUMAN SEPSIS

MDSCs make up an important proportion of immature myeloid cells. Thus, we will discuss reports analyzing immature granulocytes (IG) in adult sepsis and then move forward to studies that used more elaborated immuno-phenotyping strategies to identify MDSCs. **Table 1** provides details about the design and the main observations of these studies.

Accumulation of immature myeloid cells is one of the criteria established more than 25 years ago to characterize SIRS (systemic inflammatory response syndrome) and sepsis (107). The assessment of immature cells remained laborious up to the advent of automated cell counters. In an earliest study using automated IG counting on a small number of patients, the percentage of IG was higher in infected than in uninfected patients and was proposed to be a predictor of sepsis (93). Retrospective and prospective observational studies confirmed that IG proportion discriminates between infected and uninfected patients and is associated with disease severity (94–99) (**Table 1**). Automated cell counters can determine a delta neutrophil index (DNI), which reflects the number of immature neutrophils in the blood. A meta-analysis of ten Korean and one Egyptian studies including 1,822 sepsis patients suggests that an elevated DNI (i.e., an increased proportion of immature granulocytes) is associated with mortality (100).

Few reports demonstrate the immunosuppressive functions of immature myeloid cells in relation with sepsis and/or monitor MDSCs subpopulations using advanced flow cytometry. Since cell preparation (whole blood, with and without ficoll purification) and flow cytometry strategies are not standardized, the phenotype of MDSCs, PMN-MDSCs and M-MDSCs differs between studies (**Table 1**).

Gradient density interphase neutrophils arise during sepsis and their proportion correlates with disease severity in ICU patients. Cells isolated from septic shock patients deplete arginine and impair T cell functions *in vitro*, suggesting that they represent PMN-MDSCs (50). High levels of circulating $CD10^{dim}$ $CD16^{dim}$ IG are predictive of clinical deterioration and mortality (101, 102). This population contains a subset of $CD14^{-}$ $CD24^{+}$ myeloid suppressor cells that kill activated T cells *in vitro* (101).

The frequency of PMN-MDSCs (SSC^{high} $CD16^{+}$ $CD15^{+}$ $CD33^{+}$ $CD66b^{high}$ $CD114^{+}$ $CD11b^{+/low}$ LDG) and M-MDSCs (SSC^{low} $CD14^{+}$ $CD11b^{+}$ $CD16^{-}$ $CD15^{+}$) does not differ between non-infectious critical ill patients and sepsis patients (103). However, high levels of MDSCs are linked to nosocomial infections (**Table 1**). In a first study, PMN-MDSCs ($CD14^{-}$ $CD15^{+}$ low-density granulocytes, LDG) representing more than 36% of WBC in ICU patients sampled within 3 days of study inclusion predicts the subsequent occurrence of nosocomial infections (104). Patients that develop nosocomial infections have 2.5 times more PMN-MDSCs than patients that do not. In a second study, a close follow-up of ICU surgical patients (at days 1, 4, 7, 14, 21, and 28 or until discharge of ICU) reveals that patients with continuously high proportions of $CD33^{+}$ $CD11b^{+}$ HLA-$DR^{-/low}$ MDSCs have a longer stay in the ICU, more nosocomial infections and poor functional status at discharge (105). The percentage of total MDSCs in patients with severe sepsis/septic shock raises up to 45% of WBC, and a high proportion of MDSCs at diagnosis is associated with early mortality. Comparing cell-sorted enriched $CD33^{+}$ $CD11b^{+}$ HLA-$DR^{-/low}$ MDSCs from the blood of healthy subjects and septic patients reveals that pathogenic MDSCs dose dependently suppress IFNγ, IL-4, and IL-10 production by T cells more efficiently than MDSCs from

TABLE 1 | Studies investigating immature granulocytes and MDSCs in adults with sepsis.

Subjects	Cells/phenotypes	Observations	References
142 ED patients, 29 uninfected outpatients.	IG (automate-based determination)	Higher % in infected patients, predictor of sepsis.	(93)
70 consecutive ICU patients (51 infected, 19 uninfected).	IG (automate-based determination)	Higher % in infected patients, unrelated to day-21 and in-hospital mortality.	(94)
184 sepsis patients.	IG (automate-based determination)	Increase % associated with severity, but not predictive of mortality.	(95)
136 consecutive ICU patients.	IG (morphology and staining)	Higher % in sepsis than in uninfected patients. Unrelated to mortality.	(96)
35 sepsis and 22 non-septic consecutive burn patients, 19 healthy controls.	IG (flow cytometry)	Increase % post-burn, associated with reduced neutrophil function. Remaining elevated levels (day 7–28) associated with sepsis development	(97)
781 sepsis patients, 20 control outpatients.	IG (flow cytometry)	High % at admission related to organ failure and day-7 and day-28 mortality.	(98)
47 uninfected and 17 infected cardiac surgery patients.	IG (flow cytometry)	Increase % postoperative. Highest levels associated with secondary infection complications.	(99)
Meta-analysis (11 studies) of 1'822 sepsis patients.	Delta neutrophil index (DNI, automate-based determination)	Elevated DNI associated with mortality.	(100)
24 sepsis ICU patients, 12 hospital controls.	Interphase neutrophils (flow cytometry)	Present only in sepsis patients, proportional to sepsis severity. Suppress T-cell activity *in vitro*.	(50)
177 sepsis patients.	IG (flow cytometry)	Increase % at 48 h predictive of clinical deterioration. High % of $CD10^{dim}$ and $CD16^{dim}$ IG correlates with mortality. Kill activated T cells *in vitro*.	(101)
43 septic shock patients, 23 healthy controls.	IG (flow cytometry)	Increased % of $CD10^{dim}$ and $CD16^{dim}$ IG at days 3–4 and 6–8. Patients with lower % have better survival.	(102)
14 sepsis and 8 uninfected critically ill patients, 15 healthy controls.	M-MDSCs: SSC^{low} $CD14^{+}$ $CD11b^{+}$ $CD16^{-}$ $CD15^{+}$ PMN-MDSCs: SSC^{high} $CD16^{+}$ $CD15^{+}$ $CD33^{+}$ $CD66b^{high}$ $CD114^{+}$ $CD11b^{+/low}$	M-MDSCs but not PMN-MDSCs increase at day 13-21 post-sepsis. Similar % of M-MDSCs and PMN-MDSCs in sepsis and non-septic critical ill patients.	(103)
94 sepsis, 11 severity-matched ICU patients, 67 health donors.	M-MDSCs: Lin^{-} $CD14^{+}$ $HLA\text{-}DR^{-/low}$ PMN-MDSCs: LDG $CD14^{-}$ $CD15^{+}$ (Excluding eosinophils)	High % of PMN-MDSCs in sepsis patients. M-MDSCs are higher in gram-negative than gram-positive sepsis. PMN-MDSCs > 36% WBC at entry are associated with higher risk of nosocomial infections. PMN- and M-MDSCs suppress T-cell proliferation *in vitro*.	(104)
67 surgical patients with severe sepsis/septic shock, 18 healthy controls.	MDSCs: $CD33^{+}$ $CD11b^{+}$ $HLA\text{-}DR^{-}$ M-MDSCs: $CD14^{+}$ PMN-MDSCs: $CD14^{-}$ $CD15^{+}$	High % of MDSCs at admission correlates with early mortality. Decreasing levels of MDSCs correlate with short ICU stay. Sustained levels of MDSCs (>30% of WBC) predict nosocomial infections.	(105)
56 sepsis patients and 18 healthy controls.	M-MDSCs: $CD14^{+}$ $CD64^{+}$ $HLA\text{-}DR^{-}$ PMN-MDSCs: LDG $CD33^{+}$ $CD14^{neg/low}$ $CD64^{low}$ $CD15^{+/low}$	High % of M-MDSCs in all sepsis, but particularly in gram-negative sepsis patients. Prominent PMN-MDSCs in gram-positive sepsis. PMN-MDSCs suppress T-cell proliferation *in vitro*.	(106)

ED, emergency department; ICU, intensive care unit; IG, immature granulocytes; LDG, low density granulocytes; Lin, lineage; WBC, white blood cells.

healthy subjects, while healthy and disease MDSCs suppress T cell proliferation alike (105).

The proportion of PMN-MDSCs and M-MDSCs, defined as $CD14^{neg/low}$ $CD64^{low}$ $CD15^{+/low}$ LDG and $CD14^{+}$ $CD64^{+}$ $HLA\text{-}DR^{neg}$ leukocytes, may vary according to causative agent leading to sepsis (**Table 1**). M-MDSCs increase in all sepsis patients, predominantly in gram-negative cases, while PMN-MDSCs increase prominently in gram-positive sepsis (106). A subsequent study confirmed that M-MDSCs (Lin^{-} $CD14^{pos}$ $HLA\text{-}DR^{low/neg}$) are enriched during gram-negative sepsis, but PMN-MDSCs ($CD14^{-}$ $CD15^{+}$ LDG) do not differ according to the gram of the causative bacteria (104). Larger studies are required to ascertain that the microbial origin of sepsis shapes the pattern of MDSCs (108). This is an important parameter since M-MDSCs are more potent immunosuppressive than PMN-MDSCs on a per cell basis (109).

CONCLUDING REMARKS

MDSCs play a dual role during infection and sepsis. MDSCs expanding along emergency erythropoiesis provide a first barrier against microbial invasion by producing high amounts of bactericidal molecules like ROS and RNS and counteract the hyperinflammatory response associated with early organ dysfunctions. However, MDSCs are also detrimental by supporting the establishment and/or the maintenance of a late

protracted immunosuppressive environment. In line with a deletary role of MDSCs, all clinical studies to date associate high proportions of blood MDSCs with clinical worsening, occurrence of nosocomial infections and mortality of sepsis patients. Hence, MDSCs are attractive biomarkers, especially since these cells are barely detectable in healthy subjects. One limitation of clinical studies, not limited to the sepsis field, resides in the uneven phenotypic classification of MDSCs. One important future objective is to harmonize sample handling and flow cytometry strategies. Besides being attractive biomarkers, MDSCs are attractive therapeutic targets for sepsis. Inhibiting MDSCs-mediated immunosuppression or MDSCs trafficking or depleting MDSCs themselves (by normalizing myelopoiesis or inducing the differentiation of MDSCs into mature myeloid cells) would positively influence patient outcome. Interestingly, more than 30 clinical trials are running targeting MDSCs directly or indirectly in cancer patients (22). If ever envisaged for sepsis, these therapies will need specific evaluation since targeting MDSCs aggressively may put critically ill patients at risk of agranulocytosis. The results arising from these oncological studies, added to those from current or future studies in the field of sepsis, will give invaluable information onto whether and how MDSCs might be used to implement sepsis personalized medicine and precision immunotherapy.

AUTHOR CONTRIBUTIONS

IS and TR conceived and structured the manuscript. IS drafted the manuscript and the figure. CT revised the manuscript. TR finalized and edited the manuscript.

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3.2. Myeloid-derived suppressor cells are elevated in healthy subjects infused with endotoxin and correlate to outcome in non-infected critical care patients.

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Manuscript in preparation

Summary:

In this study, we described the dynamics of MDSCs in well-defined inflammatory conditions using a model of endotoxin infusion in eight healthy human subjects (endotoxin study). Then we investigated MDSCs in 32 non-infected critical care patients who developed nosocomial infections (PIPOVAP study). MDSCs were quantified in blood by flow cytometry followed by automatic clustering (FlowSOM). A multiplex bead assay was used to quantify 49 mediators including cytokines, chemokines and growth factors. PMN-MDSCs and M-MDSCs levels increased 10-40-fold 4 to 8 hours after endotoxin infusion and returned to baseline levels after 24 hours. PMN-MDSCs and M-MDSCs levels were high at admission in non-infected intubated ICU patients and they were normalized at time of ICU discharge. M-MDSCs levels correlated with inflammatory mediators in the blood. High levels of $CD15^+$ MDSCs at admission correlated with the occurrence of gram-negative bacteria nosocomial infections and with overall mortality. Overall, MDSCs strongly increased in blood during endotoxemia. High levels of M-MDSCs at admission in ICU correlated with the development of gram-negative bacterial infection, while high levels of PMN-MDSCs and $CD15^+$ M-MDSCs were independently associated with mortality of ICU patients. These results suggest that immunoprofiling at ICU admission time might be useful to initiate targeted supportive care.

My contribution to this work:

I participated into the design of the endotoxin study and the drafting of the protocol. I performed flow cytometry experiments and analysed the data. I drafted the article.

Myeloid-derived suppressor cells are elevated in healthy subjects infused with endotoxin and correlate to outcome in non-infected critical care patients

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Short title: MDSCs in endotoxemia and critical care patients

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Table: 1

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Background: Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of immature myeloid cells usually divided into polymorphonuclear and monocytic MDSCs (PMN-MDSCs and M-MDSCs). MDSCs are rare in the blood of healthy subjects, but increase under inflammatory conditions. Here, we first describe the dynamics of MDSCs in well-defined inflammatory conditions using a model of endotoxin infusion in healthy subjects. We then investigate whether MDSCs are predictive in non-infected critical care patients who develop nosocomial infections.

Methods: We sampled blood from eight healthy volunteers infused for 0-168 hours (nine time points) with endotoxin, as well as from 32 non-infected intubated ICU patients at their admission to and discharge from the ICU (n=17). Blood was analyzed by flow cytometry based on the detection of 11 cell surface markers chosen to detect MDSCs. Data were processed by automatic clustering using FlowSOM. A multiplex bead assay was used to quantify 49 mediators in the blood of patients at their admission in the ICU.

Results: PMN-MDSCs and M-MDSCs levels increased 10-40 fold between 4 to 8 hours after endotoxin infusion, and returned to baseline levels after 24 hours. PMN-MDSCs and M-MDSCs levels were high at admission in non-infected intubated ICU patients, and were normalized at time of ICU discharge. M-MDSCs levels correlated with inflammatory mediators in the blood. High levels of $CD15^+$ MDSCs at admission correlated with the occurrence of gram-negative bacterial nosocomial infections and with overall mortality. ROC curve analyses performed after stratifying patients based on high- and low-MDSC levels revealed acceptable, and excellent, predictive mortality performance by PMN-MDSCs (AUC 0.70, 95% CI: 0.40-1), and $CD15^+$ M-MDSCs (AUC 0.86, 95% CI: 0.62-1), respectively.

Conclusions: MDSCs strongly increase in blood during endotoxemia. High levels of M-MDSCs at admission in ICU correlate with the development of gram-negative bacterial infection, while highest levels of PMN-MDSCs and $CD15^+$ M-MDSCs are independent predictors of mortality of ICU patients. Immunoprofiling of critically ill patients at their ICU admission time might be useful to initiate targeted supportive care.

Keywords: ICU, Infection, Endotoxemia, Myeloid derived suppressor cell, Flow cytometry

Introduction

In-hospital mortality of intensive care unit (ICU) patients ranges from 7% to 40%, and frequently results from nosocomial infections and sepsis [1-3]. Timely intervention is key to saving patients, but it remains difficult to identify the most appropriate intervention. ICU scoring systems of morbidity and mortality probability, like the SOFA, APACHE II and SAPS II scores, are calculated using multiple parameters, which may delay decision-making [4-6]. Rapidly measurable prognostic biomarkers would be invaluable to risk-stratify critically ill patients in ICUs to select and/or adapt therapy [7, 8].

Sepsis is a heterogeneous syndrome defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection [3]. Sepsis is accompanied by immune alterations affecting the innate and adaptive arms of the immune system. While septic patients show concomitant signs of exacerbated proinflammatory and anti-inflammatory mechanisms, proinflammatory responses mediating tissue injury and organ dysfunctions are primarily involved in early mortality, while sustained immune dysfunctions and immunoparalysis favor the development of secondary infections involved in long-term mortality.

The name myeloid-derived suppressor cells (MDSCs) was recently adopted to unify a heterogeneous group of immature myeloid cells that display immunosuppressive functions [9]. MDSCs are commonly divided into two subtypes that are phenotypically and morphologically similar to neutrophils and monocytes: polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs). PMN-MDSCs are defined as low-density granulocytes (following centrifugation over a ficoll gradient) or as $CD11b^{+}$ $CD14^{-}$ $CD16^{low/intermediate}$ $CD33^{+}$ $CD15^{+}/CD66b^{+}$ cells, while M-MDSCs are defined as $CD11b^{+}$ $CD14^{+}$ $HLA\text{-}DR^{low/negative}$ cells [9, 10]. A third subpopulation of early-stage MDSCs (eMDSCs) that do not express any lineage markers have been reported [10], but eMDSCs may largely overlap with basophils [11].

MDSCs are barely detectable in the peripheral blood of healthy subjects. MDSCs increase substantially in the blood under conditions stimulating myelopoiesis including inflammation. MDSCs are therefore considered as attractive biomarkers and targets for a number of disease conditions [12], where the greatest advances are in the field of cancer research. The role of MDSCs in sepsis is poorly understood. The common view is that MDSCs, owing to their immunosuppressive functions, can be detrimental to host defenses. Indeed, MDSCs may support the establishment and/or maintenance of a late protracted immunosuppressive environment contributing to chronic critical illness, secondary infections, and long-term morbidity and mortality on the ICU [13-16]. However, MDSCs are proficient phagocytic cells that produce high amounts of bactericidal molecules, such as reactive oxygen species and reactive nitrogen species [12, 13]. In this way, MDSCs may participate in host defense mechanisms against microbial invasion. Additionally, through their regulatory functions, MDSCs may counterbalance detrimental inflammatory responses as observed in sepsis patients.

The aim of this study was to analyze the expression of MDSCs during a well-controlled acute inflammation, and to challenge the detrimental role of MDSCs in non-infectious critically ill patients. MDSCs were monitored in peripheral blood by standardized flow cytometry, using samples collected in

two clinical studies. The first study consisted of eight healthy subjects infused with endotoxin, which mimics early events of host response to gram-negative bacteria. The second study consisted of 32 non-infected intubated ICU patients. Overall, our data indicate that MDSCs represent a dynamic population that increase strongly in peripheral blood during endotoxemia. Further, high levels of M-MDSCs at admission in ICU were associated with the occurrence of gram-negative bacteria nosocomial infections and with the overall mortality of critically ill patients.

Materials and Methods

Ethics, subjects and study design

The endotoxin study was conducted at the Radboud university medical centre in Nijmegen, the Netherlands. Eight healthy male volunteers (**Table 1**) were infused with a single dose of 2 ng per kilogram of bodyweight of endotoxin from *Escherichia coli* O:113 (Lot #94332B1, National Institutes of Health, Bethesda, MD) following a standard protocol as described elsewhere [17]. Blood was collected in EDTA tubes just before endotoxin administration (baseline), as well as 1, 2, 3, 4, 6, 8, 24 hours, and 7 days after endotoxin administration. This study was approved by the local ethics review board (CMO Arnhem-Nijmegen; reference no. 2017–3607) and was conducted in compliance with the declaration of Helsinki [18], International Conference on Harmonization Good Clinical Practice guidelines, and the rulings of the Dutch Medical Research Involving Human Subjects Act. Written informed consent was obtained from all study participants.

The PIPOVAP (Profile, Interaction, and PrOgnosis in Ventilator Associated Pneumonia) study is a prospective, observational study conducted at the Lausanne Hospital University, Switzerland. Ethical approval was obtained from the Commission cantonale d'éthique de la recherche sur l'être humain, Canton de Vaud, Switzerland (CER-VD, reference 2017-01820). Thirty-two non-infected intubated patients admitted to the ICU with an anticipated length of mechanical ventilation over 48 hours were included in the study (**Table 1**). Exclusion criteria were treatment with an immunosuppressive agent and/or treatment with antibiotics. Blood was collected in EDTA tubes within 24 hours after admittance to the ICU, and at discharge from the ICU. This study was conducted in compliance with the declaration of Helsinki, the Essentials of Good Epidemiological Practice issued by Public Health Schweiz (EGEP), the Swiss law and Swiss regulatory authority's requirements as applicable. Written informed consent was obtained from study participants or their legal representatives.

Flow cytometry analysis

For the endotoxin study, 100 µL blood were added to custom-made tubes of lyophilized antibodies for flow cytometry (Duraclone, Beckman Coulter, Brea, CA). The tubes contained antibodies directed against the following antigens (clone name and labelling in brackets): CD11b (Bear1, PE-Cy7), CD16 (3G8, ECD), CD14 (RMO52, APC-AF750), CD3 (UCHT1, APC-AF700), CD56 (NKH-1, APC-AF700), CD19 (J3-119, APC-AF700), CD33 (D3HL60.251, APC), CD15 (80H5, Pacific Blue), CD124 (G077F6, PE), HLA-DR (Immu-357, FITC), and CD45 (J33, Krome Orange). For the PIPOVAP study, 100 µL blood was added to an antibody mixture identical to the one described above, with the difference that the anti-CD3/CD56/CD19 antibodies were labelled with AF700. After 20 minutes of incubation at room temperature in the dark, 900 µL of 1 x BD FACS™ lysing solution (BD) was added to the reactions. Tubes were vortexed and frozen at -80°C until analysis. For the analysis, samples were thawed at 37°C for one minute, washed once with PBS with 0.5% BSA and sodium azide 0.02%, and acquired on the Attune NxT Flow Cytometer (Thermo Fisher scientific, Waltham, MA). For analyses, we excluded debris, doublets and non-hematopoietic cells using manual gating (FlowJo™ Software version 10.6.2, Ashland, OR: Becton, Dickinson and Company; 2019, **Supplementary Figure 1**), and performed FlowSOM clustering using the biexponential transformed and normalized expression levels of CD45,

CD11b, CD33, CD14, HLA-DR, CD16, CD124, CD15, and the lineage markers (CD3, CD19, CD56). The metaclusters were set on 30 populations and manually merged into populations based on biological knowledge as represented in the tSNE plots (**Figures 1A and 2A**).

Measurement of serum mediators by multiplex bead assay

Serum samples were obtained from patients at their admission in the ICU (PIPOVAP study). The concentration of 49 mediators were determined by the clinical laboratory of the Division of Immunology and Allergy of Lausanne University Hospital using the Luminex xMAP Technology (Luminex Corporation, Austin, TX) and a BioPlex 200 array reader (Bio-Rad Laboratories, Hercules, CA). The mediators measured were cytokines (IL-1α, IL-1RA, IL-1β, IL-2 , IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27, IL-31, IFN-α, IFN-β, TNF), chemokines (CCL2, CCL3, CCL4, CCL5, CCL11, CXCL1, CXCL8, CXCL9,CXCL10, CXCL12, CXCL13) and growth factors (TNF-β, NGF-β, BDNF, EGF, FGF-2, HGF, LIF, PDGF-BB, PlGF-1, SCF, VEGF-A, VEGF-D, BAFF, GM-CSF, and G-CSF).

Statistical analysis

Baseline comparisons were performed using chi-square exact test, Mann-Whitney U test and Kruskal-Wallis test, as appropriate. The relationship between population percentages and clinical data was assessed using the Mann-Whitney U test. Expression levels of MDSCs >1.3% and ≤1.3% (cutoff value based on tertile) were categorized as high and low levels, which were used to analyze mortality over time. Statistical analyses of survival curves were performed using the log-rank test. Luminex data were analyzed using Spearman's rank correlation controlling for False Discovery Rate using the BH step-up procedure. Mediators with a coefficient of correlation greater than 0.3 with at least one population of MDSCs are reported. Statistical analyses and figure design were performed using R software v.3.6.0 (R Foundation for Statistical Computing, Vienna, Austria). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

Results

Baseline characteristics of subjects

Peripheral blood was obtained from subjects enrolled in the endotoxin and PIPOVAP studies (**Table 1**). The endotoxin cohort consisted of eight healthy male volunteers infused with *E. coli* O113:H10 endotoxin. Median age of subjects was 23.5 years (Interquartile range-IQR: 22-27). Baseline leukocyte counts were 5.9 [IQR: 5.5-7.4] x 10^9 cells/mL. Blood was collected before (baseline), 1, 2, 3, 4, 6, 8, 24 and 168 hours after endotoxin infusion. Leukocyte counts dropped 1 hour after endotoxin infusion, then the cell counts began to increase over the next 8 hours, before returning close to baseline values after 24 hours (**Supplementary Figure 2**).

Thirty-two non-infected intubated ICU patients were enrolled in the PIPOVAP study. Twenty-four patients (75%) developed an infection during their ICU stay, of which 58% of ventilator associated pneumonia (VAP). Four patients (12.4%) died. Survivors and non-survivors had a similar SOFA score, but non-survivors had a lower APACHE II score (**Table 1**). Blood samples were collected at ICU admission for all patients and at ICU discharge from 17 patients.

PMN-MDSCs are strongly induced by endotoxin infusion

A standardized flow cytometry panel, based on the detection of CD3, CD14, CD15, CD11b, CD16, CD19, CD33, CD45, CD56, CD124 and HLA-DR, was designed to analyze MDSCs in whole blood (see *Materials and Methods*). An automatic clustering based on the expression of the different cell surface markers, as well as the side scatter-A (SSC-A) and forward scatter-A (FSC-A), allowed the identification of eight leukocyte populations in samples from the endotoxin study (**Figure 1A-B**): basophils, eosinophils, neutrophils, classical monocytes, intermediate/non-classical monocytes, lineage$^+$ cells/DCs, and M-MDSCs and PMN-MDSCs.

MDSCs were barely detectable in the blood of healthy subjects. PMN-MDSCs were at 0.02 [0.01-0.04] x 10^6 cells/mL at baseline (**Figure 1C**). They increased 1-2 hours after endotoxin infusion, plateaued at 1.9 [1.5-2.9] x 10^6 cells/mL after 4-8 hours and returned to baseline levels after 24 hours (**Figure 1D**). In proportion with total leukocyte counts, PMN-MDSCs increased from 0.6% [0.4-1.4] at baseline to 36% [28-43] 4 hours after infusion (**Figure 1D**). In parallel, Neutrophil counts declined 1 hour after endotoxin infusion, increased at 2-8 hours, and returned to baseline levels after 168 hours. Neutrophils constituted 55-65% of total leukocytes, except for at 1 hour after endotoxin infusion when they represented 40% of total leukocytes (**Figure 1D**).

M-MDSCs were present at 0.02 [0.01-0.05] x 10^6 cells/mL at baseline (**Figure 1E**). M-MDSCs decreased 1 hour after endotoxin infusion, increased to 0.13 [0.12-0.17] x 10^6 cells/mL after 8 hours, and returned to baseline levels after 24 hours. Monocytes were strongly affected by endotoxin infusion. They dropped from 0.24 [0.20-0.29] x 10^6 cells/mL at baseline to 2.88 cells [1.86-4.54] x 10^3 cells/mL 1 hour after endotoxin infusion, then steadily increased to reach baseline levels after 8-24 hours (0.31 [0.27-0.37] and 0.33 [0.20-0.50] x 10^6 cells/mL, respectively). Consequently, M-MDSCs, which represented 0.6% [0.4-1.6] of all monocytic cells at baseline, constituted around 50% of these cells 2-8

hours after endotoxin infusion (**Figure 1E**, right panel). Overall, endotoxin infusion induced a quick, massive and transient accumulation of PMN-MDSCs, while it impacted M-MDSCs more subtly.

MDSCs are increased in patients admitted in the ICU and are normalized at discharge

Automatic clustering of samples from the PIPOVAP study identified basophils, eosinophils, $CD15^{high}$ neutrophils, $CD15^{low}$ neutrophils, classical monocytes, intermediate/non-classical monocytes, $lineage^{+}$ cells/DCs, $CD14^{high}$ $CD15^{+}$ and $CD14^{low}$ $CD15^{-}$ M-MDSCs (later called $CD15^{+}$ and $CD15^{-}$ M-MDSCs) and PMN-MDSCs (**Figure 2A-B**).

The absolute counts and frequencies of PMNs and monocytes, but not of MDSCs, were stable in between ICU admission and ICU discharge (**Figure 2C** and **Supplementary Figure 3**). PMN-MDSCs and M-MDSCs were at 0.24 [0.05-1.6] and 0.13 [0.07-0.20] x 10^6 cells/mL at ICU admission, and at 0.08 [0.01-0.13] and 0.04 [0.02-0.11] x 10^6 cells/mL at ICU discharge, respectively (admission *versus* discharge: P=0.008 and P=0.007). $CD15^{+}$ MDSCs were more abundant than $CD15^{-}$ M-MDSCs at ICU admission (0.059 *versus* 0.036 x 10^6 cells/mL). The two populations similarly decreased at discharge (0.024 *versus* 0.018 x 10^6 cells/mL). To evaluate the consequences of MDSCs in ICU patients, we compared PMN-MDSCs and M-MDSCs counts from the PIPOVAP study to those measured in the endotoxin study. We selected the 0, 4 and 8 hour time-points for comparison, which were the peaks of MDSC levels during endotoxemia (**Figure 3**). Compared with baseline levels in healthy subjects, PMN-MDSCs strongly increased in non-infectious patients at ICU admission (P=0.0016), albeit less than during endotoxemia. M-MDSCs also increased in the ICU patients compared with baseline levels in healthy subjects (P=0.00016). At ICU discharge, PMN-MDSCs and M-MDSCs counts were similar to the counts measured in healthy subjects (P=0.17 and P=0.67, respectively).

Inflammatory cytokines/chemokines and growth factors are known to stimulate the expansion of immature myeloid cells and the generation of MDSCs (13). Therefore, we used multiplex bioassays to quantify the levels of 23 cytokines, 11 chemokines, and 15 growth factors in serum collected from patients at admission and discharge in the ICU, and performed correlation analyses between MDSCs, as well as between MDSCs and mediator expression levels (**Figure 4**). PMN-MDSCs and $CD15^{+}$ M-MDSCs expression levels were correlated with each other (ρ=0.43, P=0.03). PMN-MDSCs were inversely correlated with platelet-derived growth factor-BB (PDGF-BB) (**Figure 4**). M-MDSCs were positively correlated with IL-6, monocyte chemoattractant protein-1 (MCP-1/CCL2), macrophage inflammatory protein (MIP-1β/CCL4), eotaxin (CCL11), IL-8 (CXCL8), stromal cell-derived factor 1 (SDF-1) and hepatocyte growth factor (HGF), and were inversely correlated with IL-13, IL-15 and IL-31. The correlations were significant between $CD15^{+}$ M-MDSCs and IL-6, as well as $CD15^{-}$ M-MDSCs and, SDF and HGF.

Levels of CD15$^+$ M-MDSCs at admission correlate with the development of gram-negative infection

PMN-MDSCs and M-MDSCs at ICU admission (percentage of total leukocytes and absolute counts) did not correlate with the development of hospital acquired infection (HAI) and VAP (**Figure 5A**). They were also not correlated with time to develop infection, nor with the occurrence of sepsis or septic shock. None of the other cell populations analyzed in this study (**Figure 2A-B**) correlated with infection rate. Twelve ICU patients developed a gram-negative infection, and six developed a gram-positive infection. The frequency (% of total leukocytes) and the absolute counts of PMN-MDSCs at admission were similar between patients, independent of infection type (**Figure 5B**). By contrast, M-MDSCs levels at admission were 4-fold higher in patients who developed gram-negative bacterial infections (2.8% [1.1-3.8]) than in patients who developed gram-positive bacterial infections (0.7% [0.6-1.5]) (P=0.02). The increase was largely driven by higher levels of CD15$^+$ MDSCs levels (gram-negative bacterial infection: 1.0% [0.7-2.0], gram-positive bacterial infection: 0.4% [0.3-0.5], P=0.011) (**Figure 5B**).

Levels of PMN-MDSCs and M-MDSCs at ICU admission correlate with outcome

PMN-MDSCs and M-MDSCs levels at ICU admission were higher in patients who later died during the hospital stay, though results were only significant for CD15$^+$ M-MDSCs (Survivors: 0.7% [0.5-1.3], non-survivors: 2.1% [1.8-2.4], P=0.02) (**Figure 6A-B**). We stratified patients into low and high MDSC levels based on cutoff values corresponding to the highest tertile, which was 10% for PMN-MDSCs and 1.3% for CD15$^+$ M-MDSCs. Using this segregation method, all patients with low PMN-MDSCs (n=20) survived, while 33% of patients with high PMN-MDSCs (n=12) died (P=0.0046) (**Figure 6C**). Similarly, all patients with low CD15$^+$ M-MDSCs (n=21) survived, while 36% of patients with high M-MDSCs (n=11) died (P=0.014, **Figure 6D**). Sorting patients with low PMN-MDSCS and/or low CD15$^+$ M-MDSCs in one group (n=24) and comparing them with high PMN-MDSCs and high CD15$^+$ M-MDSCs in the other group (n=8) presented a mortality rate in the high expression group of 50% (P=0.0014) (**Figure 6E**). Receiver operating characteristics (ROC) and area under the ROC curve (AUC) analyses were used to assess the predictive survival performance of MDSCs (**Figure 6F**); PMN-MDSC levels (AUC 0.70, 95% CI: 0.40-1) and to a superior extent CD15$^+$ M-MDSC levels (AUC 0.86, 95% CI: 0.62-1) predicted mortality well.

Discussion

Our data show that the generation of MDSCs is strongly stimulated by endotoxin infusion, and that MDSCs can risk-stratify non-infected critically ill patients. M-MDSCs levels at admission correlated with the development of gram-negative infections, while the highest levels of PMN-MDSCs and $CD15^+$ M-MDSCs were predictors of mortality for ICU patients.

Minimal phenotypic characteristics of MDSCs have been proposed, but there is no specific marker for these cells. New cell surface molecules are occasionally proposed to ease the identification of MDSCs [10, 11, 19]. Further, it is difficult to compare data between studies using different biological specimen, antibody cocktails for cell labelling, gating strategies and flow cytometer apparatus. Consequently, MDSC levels may differ between studies, showing as much as 10-fold differences in healthy subjects [13-16, 20-23]. The quantification of MDSCs in whole blood by flow cytometry for routine analyses remains an important, yet challenging task. Here, we used whole blood staining and automatic clustering of the acquired data to minimize variations due to cell preparation and gating strategies.

Our strategy allowed us to capture $CD15^+$ and $CD15^-$ MDSCs in ICU patients. CD15 is a cell adhesion molecule (known as Lewis X antigen) known to be expressed by granulocytes, but also by monocytes, macrophages, mast cells and normal myeloid precursor cells. A study in the late 90's described a minor population of monocytes with high SSC parameter, CD15 expression and production of ROS [24], which appears to be similar to M-MDSCs. Our study shows that a subdivision on CD15 for M-MDSCs is useful in a critical care setting. This subdivision might also be useful for other diseases. Interestingly, $CD15^+$ M-MDSCs expression levels correlated with mortality, suggesting that these cells might be a useful marker in other pathologies.

Human experimental endotoxemia induced by intravenous administration of *E. coli* LPS is used to study pathophysiological changes observed in septic patients. Experimental endotoxemia induces a well-controlled systemic inflammatory response accompanied by high blood levels of proinflammatory and anti-inflammatory cytokines [25]. These cytokines, chemokines and growth factors stimulate bone marrow production of MDSCs, which we observed during endotoxemia. Interestingly, PMN-MDSCs and M-MDSCs differentially responded to endotoxin infusion. PMN-MDSCs increased quickly and sharply, while M-MDSCs showed an initial drop before a progressive increase from 3-4 hours onwards, a time at which M-MDSCs became a dominant monocytic population. These fluctuations reflected, to some extent, the temporary neutrophilia and monocytopenia characterizing human endotoxemia [26].

We did not measure cytokines in healthy subjects infused with endotoxin, but it is very likely that cytokine levels would correlate with MDSCs influx in blood. In patients admitted in the ICU, M-MDSCs correlated with blood levels of IL-6, chemokines (IL-8, MIP-1β, MCP-1 and eotaxin) and growth factors (SDF-1α, HGF), while PMN-MDSCs inversely correlated with PDGF-BB. This may reflect the fact that the accumulation of MDSCs in critically ill patients, particularly of PMN-MDSCs, were initiated before ICU admission. Therefore at ICU inclusion we may not capture the entire spectra of mediators influencing the expression of MDSCs.

IL-6 is a pivotal regulator of the accumulation and activation of MDSCs, and chemokines are chemoattractants for MDSCs in the tumor environment [27-31]. Targeting MIP-1β and HGF decreased MDSCs accumulation in tumors. It follows that these molecules might represent targets for MDSCs in critically ill patients [32, 33]. MCP-1 and eotaxin, which are potent chemoattractant for regulatory T cells (Treg) and eosinophils, are produced by M-MDSCs in tumorigenic mice [34]. The inverse correlation of MDSCs with IL-13 and IL-31 is more puzzling, as these cytokines are mainly produced by T helper 2 (Th2) cells, a cell type induced by MDSCs [13]. It is possible that high levels of IL-6 inducing MDSCs accumulation also skewed T helper cell polarization away from Th2 cells [35]. We observed an inversed correlation between PMN-MDSCs and growth factor PDGF-BB. This was unexpected as MDSCs from tumorigenic mice secrete PDGF-BB [36]. However, in sepsis patients, PDGF-BB levels correlated with survival [37].

MDSCs were elevated in patients admitted in ICU, and normalized at ICU discharge. Accordingly, all studies comparing MDSC levels in ICU patients with levels in healthy controls showed elevated levels of MDSCs in critically ill patients [14-16, 20, 21, 23]. We did not find a correlation between MDSCs levels and nosocomial infections. However, high levels of M-MDSCs, and especially $CD15^+$ MDSCs, correlated with the occurrence of gram-negative infection. Moreover, M-MDSCs increased up to 10-fold during endotoxemia. In line with these observations, high M-MDSCs levels were linked to gram-negative sepsis [14, 20].

Importantly, all non-survivors presented with high levels of PMN-MDSCs and $CD15^+$ M-MDSCs at ICU admission. In accordance with these observations, high levels of PMN-MDSCs at admission correlated with mortality in patients with severe sepsis/septic shock, septic patients after esophageal cancer resection, non-surgical ICU patients and patients with COVID-19 [16, 22, 23, 38]. These data suggest that MDSCs expression levels could be used as stratifying markers to identify patients who might benefit from aggressive targeted therapy [39]. In fact, preclinical sepsis studies targeting MDSCs showed promising results lowering MDSC levels and increasing T-cell function, where phase II clinical trials targeting MDSCs in oncologic patients are ongoing [22, 40-45].

A limitation of our work is that we defined MDSCs by their phenotype and not by their function. However, previous studies have shown the functionality of MDSCs isolated based on phenotype [14, 21, 46-49]. Moreover, MDSCs levels positively correlated with levels of well-known inducers and regulators of MDSCs, such as IL-6, HGF and SDF-1α, and inversely correlated with interleukins produced by effector cells. Another limitation of this study is the limited number of ICU patients included in this study, particularly of patients with unfavorable outcomes.

In conclusion, we show that MDSCs are highly responsive to endotoxin, and correlate with the development of gram-negative nosocomial infections, length of ICU stay and outcome. M-MDSCs levels correlated with cytokines, chemokines and growth factors, which may present targets for MDSC targeted therapies in critically ill patients. Finally, our results suggest that monitoring MDSCs in patients admitted to the ICU might provide prognostic biomarkers for outcome, and might therefore aid clinicians by risk-stratifying patients.

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Author contributions statement

IS, JH, TC and TR designed the study. GL and MK designed the endotoxin study with IS, JH, TC and TR, and collected samples. AK and BG designed the PIPOVAP study and recruited the patients. PB and AH setup the Duraclone tubes. MP and GP performed multiplex analyses. IS and CT analyzed the samples. All the authors interpreted the data. IS, TC and TR wrote the manuscript. All the authors revised the manuscript.

Conflict of Interest Statement

The authors do not have any conflict of interest regarding this manuscript.

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Tables and figures

Table 1. Patient characteristics

	Endotoxin study	PIPOVAP study	
	Baseline	Survivors (28 day)	Non-survivors (28 day)
Number of subjects/patients	7	28	4
Gender, male	7 (100%)*	15 (54%)	3 (75%)
Age (years)	23.5 [22-27]*	64 [52-68]	63 [53-67]
Severity of illness at admission:			
Mechanical ventilation	-	28 (100%)	4 (100%)
APACHE II score	-	22 [19-31]	15 [10.5-21.0]**
SOFA score	0	11 [10-14]	9.5 [6.8-11.5]
Developed a secondary infection	-	22 (69%)	2 (50%)
Type of secondary infection:			
VAP/HAP	-	13 (59%)	1 (50%)
Non-VAP HAI	-	9 (41%)	1 (50%)
ICU stay (days)	-	8.5 [6.00-16.25]	7 [5.50-9.25]
CRP (mg/L)	-	23.5 [6.5-74.5]	-
Leukocytes (x 10^9/L)	5.9 [5.5-7.4]	12.5 [10.6-16.0]	14.4 [11-20]
Lactate (mmol/L)	-	1.7 [0.90-3.28]	2.3 [1.43-3.93]

*Medians [IQR] or n (%). **, P<0.05 (survivors *versus* non-survivors).

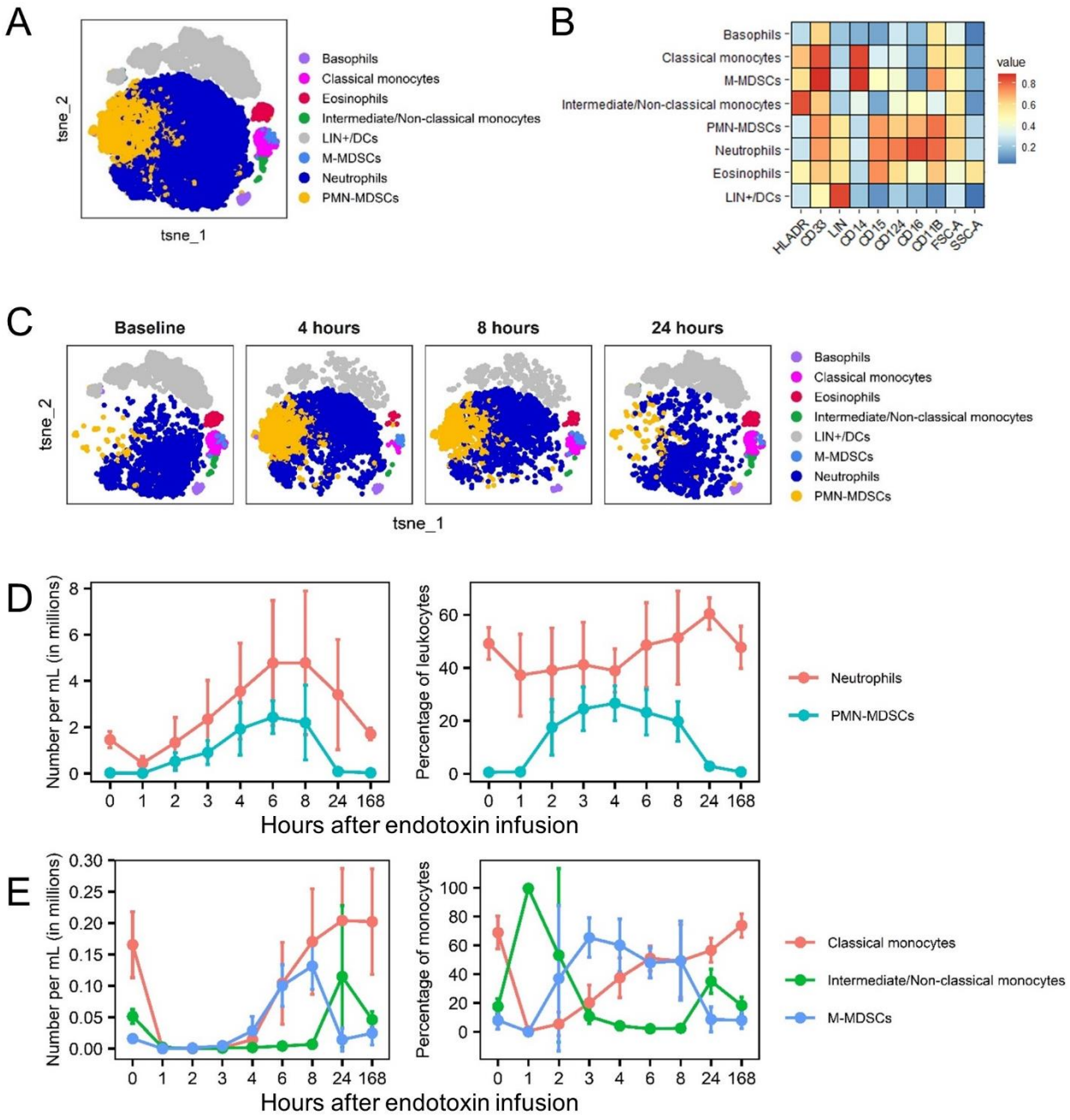


Figure 1. MDSCs in the blood of healthy subjects challenged with endotoxin. Blood collected from eight healthy subjects before and 1, 2, 3, 4, 6, 8, 24 and 168 hours after endotoxin infusion was added to Duraclone tubes containing antibodies directed against CD3, CD14, CD15, CD11b, CD16, CD19, CD33, CD45, CD56, CD124 and HLA-DR, and analyzed using flow cytometry (see *Materials and Methods*). A) t-SNE plots of leukocyte populations. B) Expression levels of cell surface markers and FSC-A/SSC-A characteristics of leukocyte populations identified in A. C) t-SNE plots of leukocyte populations over time. D) Absolute counts (left panel) and percentage in leukocytes (right panel) of PMN-MDSCs and neutrophils. E) Absolute counts (left panel) and percentage in monocytic cells (right panel) of M-MDSCs, classical monocytes and intermediate/non-classical monocytes. Graphs show median with standard deviation. LIN: lineage (i.e., positive for CD3, CD56 or CD19), DCs: dendritic cells, M-MDSCs: monocytic MDSCs, PMN-MDSCs: polymorphonuclear MDSCs**.**

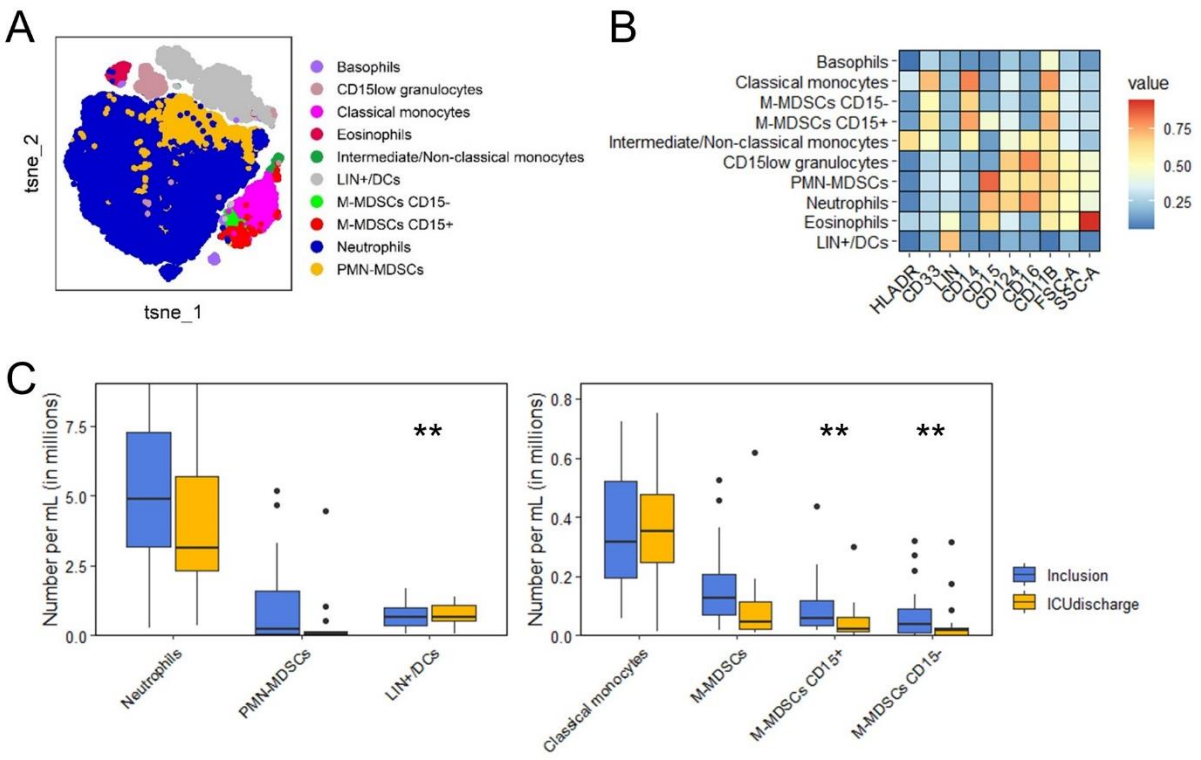


Figure 2. MDSCs in the blood of intubated ICU patients. Blood was obtained at study inclusion (n=32) and at ICU discharge (n=17) from intubated ICU patients without infection. Blood was incubated with fluorescently labelled antibodies directed against CD3, CD14, CD15, CD11b, CD16, CD19, CD33, CD45, CD56, CD124 and HLA-DR and analyzed by flow cytometry (see *Materials and Methods*). **A**) t-SNE plots of leukocyte populations. **B**) Expression levels of cell surface markers and FSC-A/SSC-A characteristics of leukocyte populations identified in **A**. **C**) Counts of leukocyte populations at study inclusion and at ICU discharge. Boxplots show median, upper and lower quartiles. The whiskers show the 5 to 95 percentiles. * P<0.05, ** P<0.01.

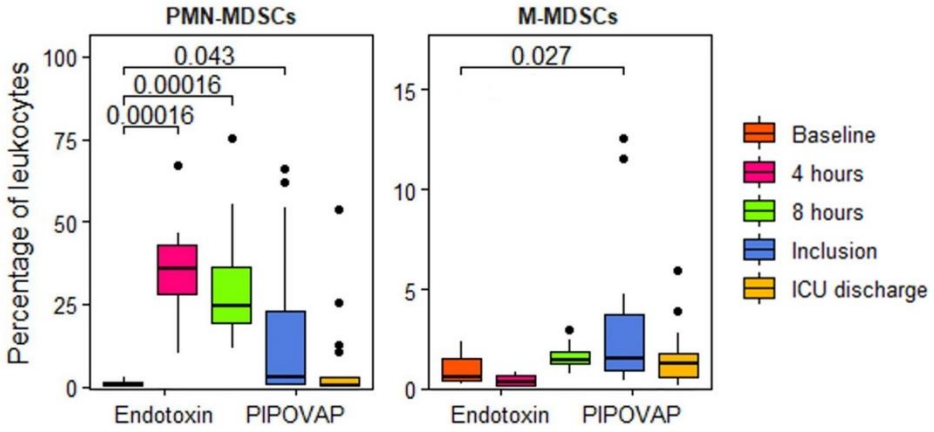


Figure 3. Comparison of MDSCs levels between healthy subjects infused with endotoxin and intubated ICU patients. Percentages in leukocytes of PMN-MDSCs and M-MDSCs in healthy subjects infused for 0, 4 and 8 hours with endotoxin, and in intubated ICU patients at study inclusion and at ICU discharge. Statistics between baseline endotoxin patients and other groups. Boxplots show median, upper and lower quartiles. The whiskers show 5 to 95 percentiles.

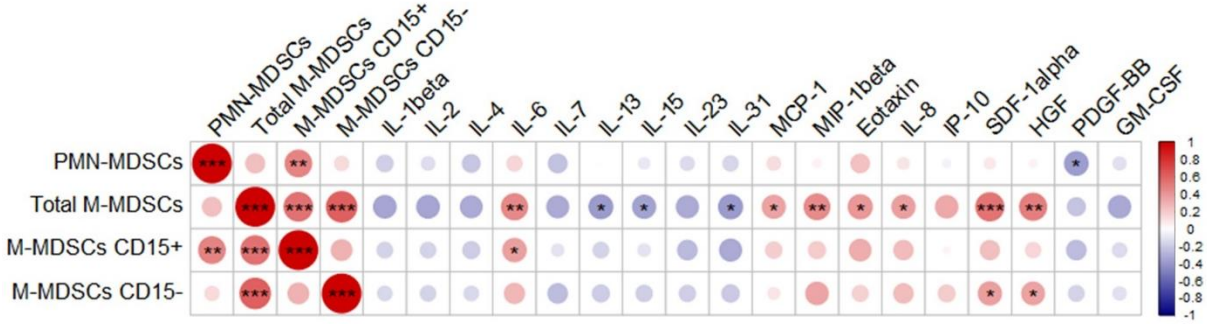


Figure 4. Correlation analysis between the expression levels of MDSCs, cytokines, chemokines and growth factors. Blood was collected from patients at their admission in the ICU to quantify 23 cytokines, 11 chemokines, and 15 growth factors by multiplex bioassay and MDSCs by flow cytometry. Correlations were calculated using Spearman's rank correlation controlled for False Discovery Rate using the BH step-up procedure. The correlation plot depicts mediators with a correlation coefficient greater than 0.3 with at least one population of MDSCs. * P<0.05, ** P<0.01, *** P<0.001.

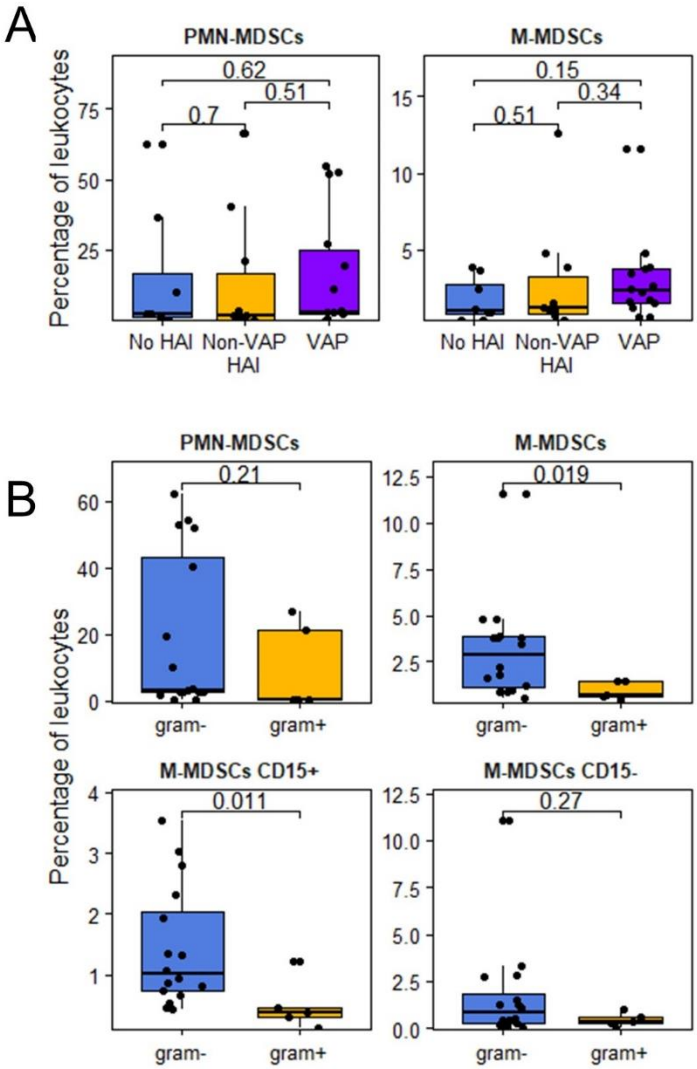


Figure 5. MDSCs at admission correlate with the development of gram-negative infection in intubated ICU patients. A) Percentages of PMN-MDSCs and M-MDSCs in leukocytes of patients that do not develop an infection (No HAI, n=8), hospital-acquired infection (HAI, n=10) and ventilator-associated pneumonia (VAP, n=14). **B**) Percentage of MDSCs at admission in patients who developed gram negative (gram-) or gram positive (gram+) infections. Boxplots show median, upper and lower quartiles. The whiskers show 5 to 95 percentiles. Each dot represents an individual sample.

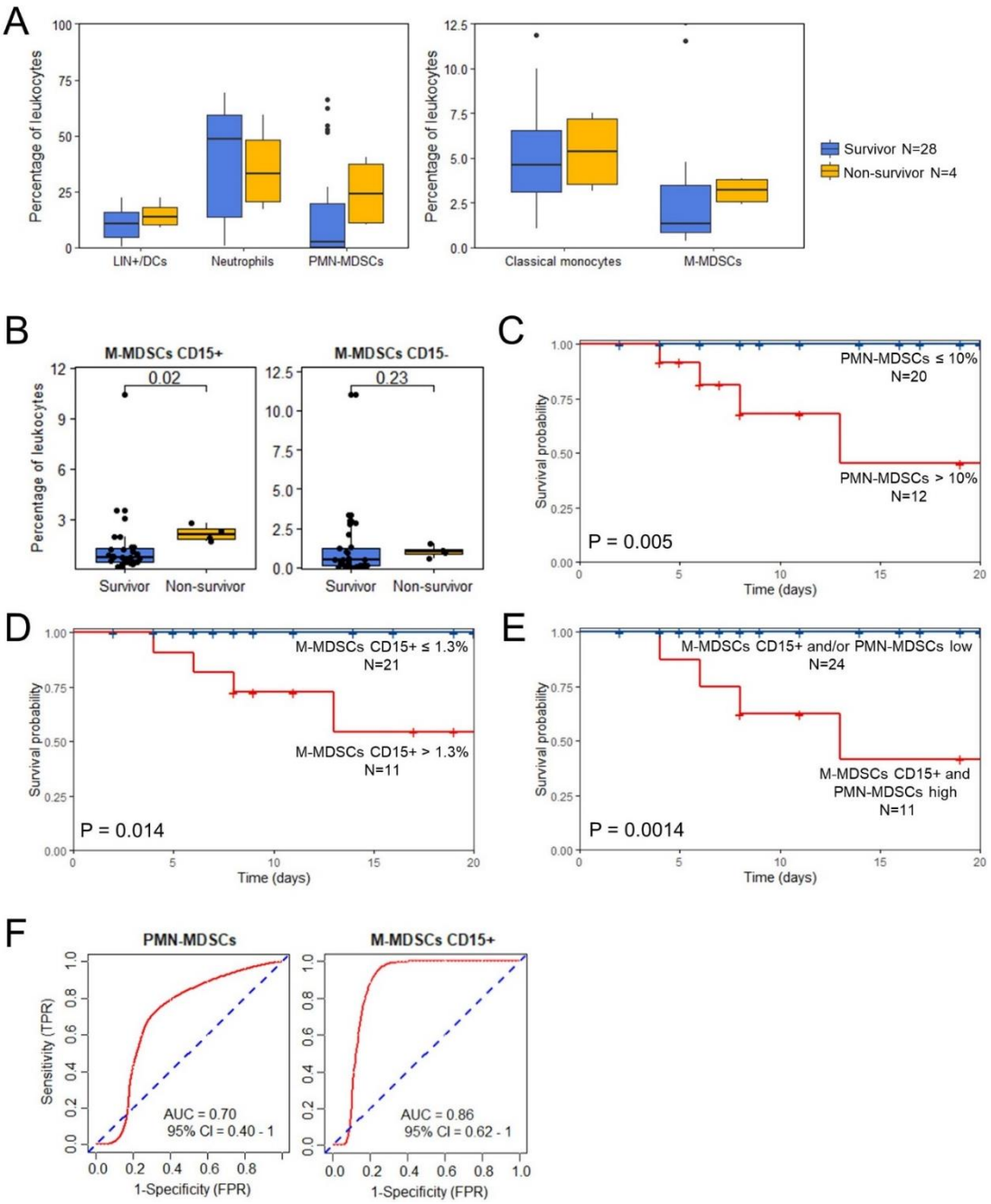


Figure 6. High MDSCs levels at admission are associated with a worse outcome for intubated ICU patients. A) Leukocyte populations expressed as percentage for the intubated ICU patients, comparing survivors (n=28) and non-survivors (n=4). **B**) Boxplot showing $CD15^-$ MDSCs and $CD15^+$ MDSCs levels in survivors and non-survivors. **C**) Kaplan-Meier 28 day survival curve of patients with low (<10%) and high (>10%) levels of PMN-MDSCs. **D**) Kaplan-Meier 28 day survival curve of patients with low (<1.3%) and high (>1.3%) levels of $CD15^+$ M-MDSCs. **E**) Kaplan-Meier 28 day survival curve for patients split on low and/or high of either PMN-MDSCs (split on 10%) and CD15+ M-MDSCs (split on 1.3%), and high levels of both PMN-MDSCs and $CD15^+$ M-MDSCs. **F**) ROC curves of PMN-MDSCs and $CD15^+$ M-MDSCs for patient mortality. The area under the curve (AUC) and the confidence of intervals (CI) are given. Boxplots show median, upper and lower quartiles. The whiskers show 5 to 95 percentiles. Each dot represents an individual sample. The association of MDSCs with survival was assessed using the Mann-Whitney U and the Kruskal-Wallis. The cutoff values of MDSCs used to segregate high and low levels for the event curve were based on the highest tertile and statistical differences were assessed using the log-rank test.

Supplementary Figures

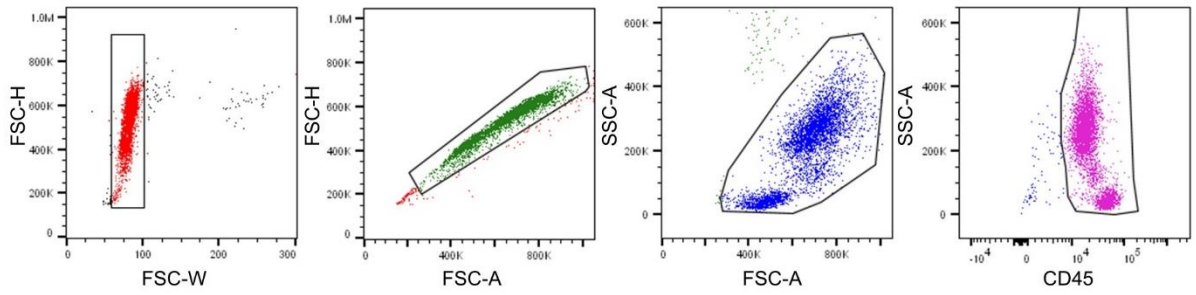
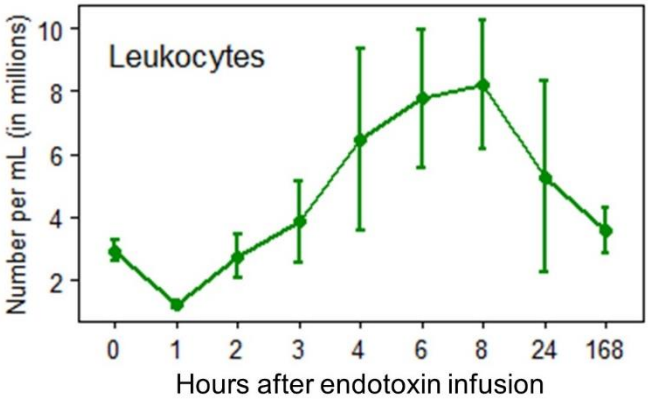


Figure S1. Gating strategy to exclude debris, doublets and non-hematopoietic cells when analyzing blood leukocytes by flow cytometry.



Hours of infusion	0	1	2	3	4	5	8	24	168
Median (x 10^6/mL)	2.93	1.23	2.74	3.85	6.45	7.75	8.19	5.27	3.55
IQR	2.79-3.65	1.17-1.69	2.38-3.32	2.94-4.43	4.54-7.93	6.66-10.07	7.62-10.86	3.04-6.59	3.37-4.33

Figure S2. Leukocytes in the blood of healthy subjects challenged with endotoxin. Blood collected from eight healthy subjects before and 1, 2, 3, 4, 6, 8, 24 and 168 hours after endotoxin infusion. $CD45^+$ cells were quantified by flow cytometry.

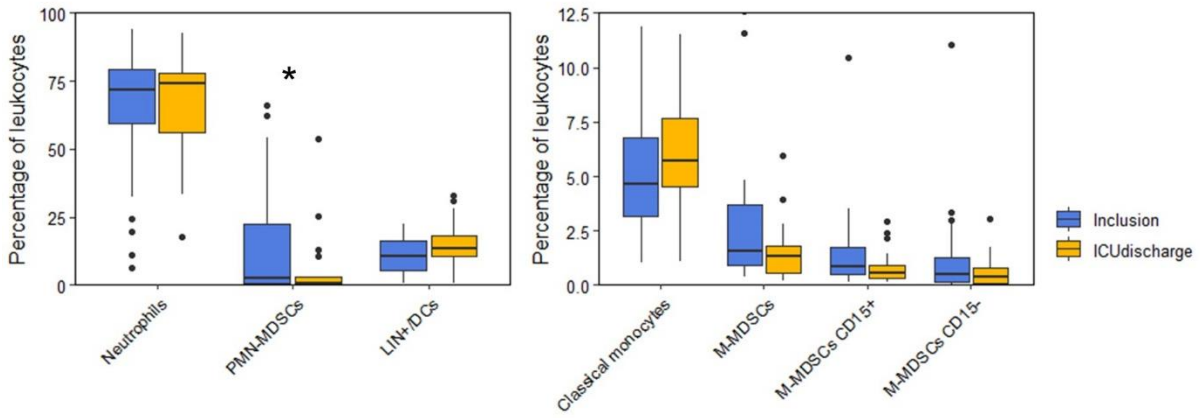


Figure S3. MDSCs in the blood of intubated ICU patients. Blood was obtained at study inclusion (n=32) and at ICU discharge (n=17) from intubated ICU patients without infection. Blood was analyzed by flow cytometry as described in *Materials and Methods*. Data are presented as the percentage of leukocytes. Boxplots show median, upper and lower quartiles. The whiskers show 5 to 95 percentiles. LIN: lineage (i.e., positive for CD3, CD56 or CD19), DCs: dendritic cells. * P<0.05.

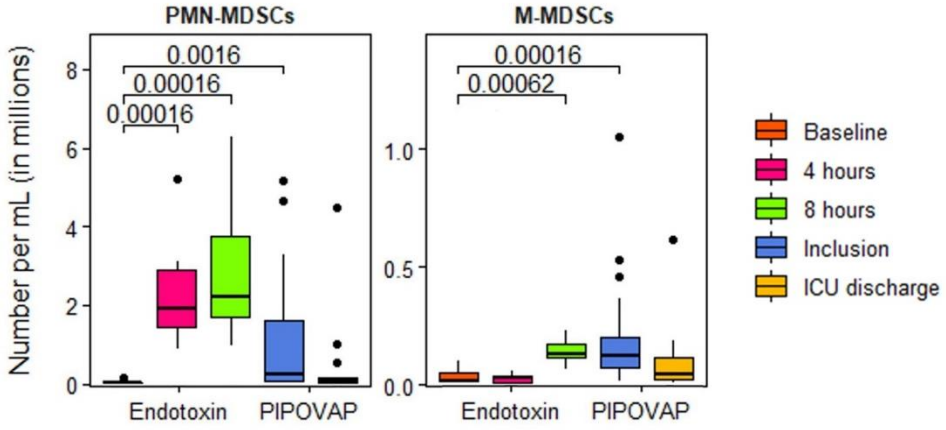


Figure S4. Comparison of MDSCs levels between healthy subjects infused with endotoxin and intubated ICU patients. Absolute counts of PMN-MDSCs and M-MDSCs in healthy subjects infused for 0, 4 and 8 hours with endotoxin, and in intubated ICU patients at study inclusion and at ICU discharge. Statistics between baseline endotoxin patients and other groups. Boxplots show median, upper and lower quartiles. The whiskers show 5 to 95 percentiles.

3.3. Rapid increase of myeloid-derived suppressor cells, and prolonged innate immune dysfunctions in patients with COVID-19.

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Manuscript in preparation

Summary:

The COVID-19 pandemic gave us the unique opportunity to set up a cohort with sepsis and non-sepsis patients caused by one specific pathogen, SARS-CoV-2. Like patients with bacterial sepsis, COVID-19 patients show concurrent signs of exuberant inflammation and immunosuppression. The objective of the study was to define whether the expression of MDSCs and the production of cytokines by innate immune cells in blood were affected by COVID-19 and, if so, whether these effects persisted over time. Blood was collected from 10 healthy controls and 56 COVID-19 patients at study inclusion and 3 months later. Blood was stimulated with Toll-like receptor ligands. Leukocytes, including PMN-MDSCs and M-MDSCs, and up to 49 mediators (cytokines, chemokines and growth factors) were analyzed by flow cytometry, mass cytometry, multiplex bead assay, and ELISA. PMN-MDSCs and M-MDSCs were 2-3.7-fold higher in COVID-19 patients than in controls ($P < 0.05$) and were associated with disease severity ($P < 0.01$). MDSCs inversely correlated with T cell counts, and positively correlated with blood cytokines. The proportion of monocytes and DCs producing TNF and IL-6, and the concentrations of cytokines were reduced in *ex vivo*-stimulated whole blood from COVID-19 patients. Three months after COVID-19 diagnosis, irrespective of initial disease severity, MDSCs were back to normal levels while the production of cytokines by blood cells was still largely affected. These data suggest that COVID-19 induces rapid and long-standing innate immune dysregulation.

My contribution to this work:

I participated into the design of the study. I performed flow cytometry experiments, analysed the data, and performed correlation studies. I drafted the article.

Rapid increase of myeloid-derived suppressor cells, and prolonged innate immune dysfunctions in patients with COVID-19

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Short title: MDSCs and innate immune response in patients with COVID-19

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Number of figures: 8

Number of tables: 1

Number of supplementary tables and figures: 8

Background. Acute COVID-19 has been associated with exuberant inflammation, but COVID-19 patients also exhibit immunosuppression. The objective of the study was to define whether the expression of myeloid-derived suppressor cells (MDSCs) and the production of cytokines in blood are affected by COVID-19, and whether these effects persist over time.

Methods. Blood was collected from 10 healthy controls and 56 (45 moderate, 11 severe) COVID-19 patients at study inclusion and 3 months later. Blood was stimulated with Toll-like receptor ligands. Leukocytes including polymorphonuclear and monocytic MDSCs (PMN-MDSCs and M-MDSCs), and up to 49 cytokines (including chemokines and growth factors) were analyzed by flow cytometry and mass cytometry, multiplex bead assay and ELISA.

Results. PMN-MDSCs and M-MDSCs were 2-3.7-fold higher in COVID-19 patients than in controls ($P < 0.05$) and were associated with disease severity ($P < 0.005$). MDSCs inversely correlated with T cell counts, and positively correlated with blood cytokines. The proportion of monocytes and DCs producing TNF and IL-6, and the concentrations of cytokines were reduced in *ex vivo*-stimulated whole blood from COVID-19 patients. Three months after COVID-19 diagnosis, irrespective of initial disease severity, MDSCs were back to normal levels while the production of cytokines by blood cells was still largely affected.

Conclusions. PMN-MDSCs and M-MDSCs were elevated and correlated with disease severity and blood cytokine concentrations in COVID-19 patients. Innate immune response of whole blood was strongly impaired in patients and largely persisted for up to 3 months. These data suggest that COVID-19 induces rapid and long-standing innate immune dysregulation.

Key words: COVID-19, SARS-CoV-2, innate immunity, myeloid-derived suppressor cells, cytokine, monocyte, dendritic cell.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) quickly spread worldwide following its discovery in December 2019, with more than 170 million cases (June 1st, 2021) of infection reported worldwide. SARS-CoV-2 is responsible for Coronavirus disease 2019 (COVID-19), which presents with a wide clinical spectrum. Most cases of COVID-19 are asymptomatic or involve mild symptoms, but 10% to 20% of patients develop pneumonia that can progress into acute respiratory distress syndrome (ARDS) resulting in high in-hospital mortality [1-3]. Numerous factors including the SARS-COV-2 strain and patient's demographics, underlying conditions, and immune status influence outcome (4-11).

SARS-CoV-2 is an enveloped, positive-sense, single-stranded RNA coronavirus that binds to angiotensin-converting enzyme 2 (ACE2) expressed on nasal, bronchial and alveolar cells to promote infection [12]. Innate immune sensing of the virus relies on the expression of pattern recognition receptors (PRRs) such as Toll-like receptor (TLR) 3, TLR7, retinoic acid inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA-5) [13-17]. Triggering of these PRRs results in the production of type I interferons (IFNs), cytokines and chemokines that promote anti-viral responses and SARS-CoV-2 elimination. However, uncontrolled overwhelming cytokine production can be pathogenic, leading to a cascade of immune-related manifestations including organ failure (sepsis), and death [3, 11, 18, 19].

The "cytokine storm", which defines the release of large amounts of cytokines that trigger systemic inflammation involved in multi-organ failure, is commonly associated with the pathogenesis of severe COVID-19. However, COVID-19 patients with ARDS have relatively low blood concentrations of cytokines when compared to patients with ARDS unrelated to COVID-19 and to patients with bacterial sepsis [20-22]. Patients with COVID-19 show severe lymphopenia and impaired immune effector cell functions associated with secondary infections and death [3, 18]. Thus, besides hyperinflammation, immunosuppression may play a significant role in COVID-19 morbidity and mortality [23-26]. However, the persistence of immune dysfunctions in COVID-19 pathogenesis remains unclear.

The replication-competent SARS-CoV-2 is typically no longer detectable after 4 weeks, signifying the end of acute COVID-19. However, a fraction of patients exhibits so-called long-COVID with persistent symptoms including tiredness, myalgia, cough, shortness of breath, and loss of taste and/or smell [27-30]. Recent estimates suggest that as much as 10-22% of individuals still have symptoms 5-12 weeks after SARS-CoV-2 infection, and 70% of COVID-19 hospitalized patients still experience shortness-of-breath one month after discharge [30, 31]. Furthermore, abnormalities persisting beyond 12 weeks after acute COVID-19 indicate a post-acute COVID-19 syndrome [29]. Long COVID and post-acute COVID-19 syndrome are similar to post-sepsis syndrome. This debilitating chronic phase of physical and psychological long-term effects following sepsis is characterized by immunosuppression associated with a persistent low-grade inflammation which can remain for months or years [32, 33].

Myeloid-derived suppressor cells (MDSCs) are immature-like myeloid cells that suppress innate and adaptive immune responses through different mechanisms. MDSCs expand during sterile and infectious inflammation through the induction of inflammatory and danger signals. MDSCs can be

generated through the expansion and egress of immature myeloid cells with suppressive functions into the circulation. An alternative proposition is the generation of MDSCs though the conversion of neutrophils and monocytes into so-called pathologically-activated MDSCs [34]. MDSCs are usually divided into two major groups of granulocytic/polymononuclear MDSCs and monocytic MDSCs (PMN-MDSCs and M-MDSCs) [34-41]. The role of MDSCs is mainly studied in cancer, where these cells accumulate in tumor micro-environments to suppress anti-tumor immunity and rise in blood circulation to one of the main leukocyte subtypes [42, 43]. MDSCs are usually associated with poor clinical outcomes, and viewed as biomarkers for a number of pathological conditions including cancer and autoimmune diseases [33-35, 40, 41, 44].

The aim of this study was to determine the dynamics of MDSCs and the innate immune response in COVID-19 patients. To assess this aim we used highly discriminative technologies applied to healthy controls and 56 hospitalized COVID-19 patients sampled during hospitalization and 3 months later. We observed that MDSCs were elevated in patients and correlated with disease severity and immunological parameters. Our results suggest that COVID-19 induces rapid and long-standing innate immune dysregulation.

Materials and methods

Subjects and ethic statement

Fifty-six hospitalized PCR-confirmed SARS-COV2 infected adult patients were enrolled in the Lausanne University Hospital (LUH) COVID-19 cohort study. The exclusion criterion for study enrolment was pregnancy. Blood samples were collected at study inclusion and 3 months later. Moderate COVID-19 was defined as hospital admission without the need for intubation, while severe COVID-19 was defined as hospital admission with intubation or death. A control group was comprised of 10 age- and sex-matched healthy individuals. Exclusion criteria were prior diagnosis of SARS-COV2 infection, acute or chronic viral hepatitis, autoimmune disease, immunodeficiency and use of immunomodulatory drugs. This study was approved by the Commission cantonale d'éthique de la recherche sur l'être humain, Canton de Vaud, Switzerland (CER-VD). Study participants provided written informed consent.

Detection of MDSCs in whole blood by flow cytometry

One hundred µL of EDTA-anticoagulated blood were incubated for 20 minutes at room temperature in the dark with a cocktail of antibodies directed against CD3, CD7, CD11b, CD14, CD15, CD16, CD19, CD33, CD45, CD56, CD135 and HLA-DR (**supplementary Table 1**). Samples were diluted with 2 mL 1 x 1-step Fix/Lyse solution (eBioscience™), washed once with cell stain medium (CSM: PBS containing 0.5% BSA and 0.02% sodium azide) and acquired using an Attune NxT Flow Cytometer (Thermo Fisher Scientific, Waltham, MA, USA). For analysis, debris, doublets and non-hematopoietic cells were excluded using manual gating (**Supplementary Figure 1**), followed by FlowSOM unsupervised clustering using the biexponential transformed expression levels of CD11b, CD14, CD15, CD16, CD33, CD45, HLA-DR and lineage markers (CD3, CD7, CD19, CD56). Metaclustering was set on 30 populations manually merged into populations based on biological knowledge as represented in tSNE plots (**Figure 1**). PMN-MDSCs were identified based on their relatively low expression levels of CD16 and CD11b when compared to mature neutrophilic granulocytes, while M-MDSCs were identified based on low expression levels of HLA-DR.

Profiling of blood T cell populations and quantification of serum mediators

T cell populations were profiled through flow cytometry and mass cytometry (detailed in [45]). The concentrations of cytokines (IL-1α, IL-1RA, IL-1β, IL-2 , IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27, IL-31, IFN-α, IFN-β, TNF), chemokines (CCL2, CCL3, CCL4, CCL5, CCL11, CXCL1, CXCL8, CXCL9,CXCL10, CXCL12, CXCL13) and growth factors (TNF-β, NGF-β, BDNF, EGF, FGF-2, HGF, LIF, PDGF-BB, PlGF-1, SCF, VEGF-A, VEGF-D, BAFF, GM-CSF, and G-CSF) were determined by multiplex bead assay using the Luminex xMAP Technology (Luminex Corporation, Austin, TX) and a BioPlex 200 array reader (Bio-Rad Laboratories, Hercules, CA).

Whole blood stimulation assay

To quantify cytokine production by monocytes and DCs, 300 µL of EDTA-anticoagulated blood was incubated for 4 hours at 37°C with or without 100 ng/mL *Escherichia coli* 055:B5 ultrapure ultrapure lipopolysaccharide (LPS), 5 µg/mL R848, 100 ng/mL $Pam_3CysSerLys_4$ (Pam_3CSK_4), and 5 µg/mL CpG ODN 2216 (Invivogen, San Diego, CA). Brefeldin A (5 µg/mL, Invitrogen, Carlsbad, CA) was added

during the incubation. To analyze monocytic cells, 100 µL of reaction mixtures were incubated with LIVE/DEAD™ reagent and antibodies directed against CD14, CD16, CD19, CD33, CD56, HLA-DR and PD-L1/CD274. To analyze on DCs, 200 µL of reaction mixtures were incubated with LIVE/DEAD™, Anti-Human Lineage Cocktail 2 (Lin-2, i.e. anti-CD3, CD14, CD19, CD20 and CD56 antibodies), and anti-CD1c, CD11c, CD16, CD123, HLA-DR and PD-L1/CD274 antibodies. After 20 minutes of incubation at room temperature in the dark, samples were diluted with 2 mL 1x 1-step Fix/Lyse Solution (eBioscience™), washed with CMS, incubated for 10 minutes with CMS containing 0.3% saponin (Sigma-Aldrich, Saint Louis, MI), incubated for 20 minutes with CMS containing antibodies directed against TNF, IL-6 and IL-10, washed and acquired using an Attune NxT Flow Cytometer. Reagents are described in **supplementary Table 2**. Samples were analyzed after excluding debris, doublets and dead cells (LIFE/DEAD™). To analyze monocytic cells, SSC-A intermediate, $CD33^{+}$, $CD3^{-}$ and $CD20^{-}$ cells were selected by manual gating. FlowSOM automatic unsupervised clustering (metacluster set on 20) was performed based on the expression of HLA-DR, CD14, CD16, CD33, CD56 and SSC-A. Monocytic cells were selected for a second round of clustering to distinguish classical monocytes, (HLA-DR^{+} $CD14^{+}$ $CD16^{-}$), non-classical and intermediate monocytes (HLA-DR^{+} $CD14^{+/-}$ $CD16^{+}$), and M-MDSCs (HLA-$DR^{+/-}$ $CD14^{+}$ $CD16^{-}$). FlowSOM unsupervised clustering (metacluster set on 12) based on intracellular cytokine labelling (TNF, IL-6 and IL-10) was applied to quantify cytokine-producing monocytic cells. To analyze DCs, HLA-DR^{+} and $Lin2^{-}$ cells were selected. Patients with very low counts of DCs (< 30 cells/mL) were excluded. FlowSOM (metacluster set on 12) based on the intracellular cytokine labelling (IL-6, IL-10 and TNF) and PD-L1 expression was applied to quantify DCs producing cytokines and expressing PDL-1.

To quantify cytokine release by whole blood, 30 µL of EDTA-anticoagulated blood was incubated for 24 hours at 37°C with or without LPS, Pam_3CSK_4, R848 and CpG as described above. Supernatants were collected and used to quantify mediators by multiplex bead assay using the Luminex xMAP Technology and TNF and IL6 by TNF ELISA Set (555212, Becton Dickinson, NJ, USA) and Human IL-6 ELISA MAX set (B214736, BioLegend, CA, USA).

Statistics and software

Manual gating was performed on FlowJo™ Software version 10.6.2 (Ashland, OR). Statistical analyses and figure design were performed using R v.3.6.0 (R Foundation for Statistical Computing, Vienna, Austria). Baseline characteristic comparisons were made using Mann-Whitney U, Chi square or Kruskall-Wallis tests for skewed variables and student's t-test or Chi square for normal distributed variables. Cytokine and flow cytometry data were compared using the Kruskall-Wallis test, Mann-Whitney U, or Spearman's rank correlation controlling for False Discovery Rate (FDR) using the BH step-up procedure. PMN-MDSCs and M-MDSCs levels were split into high (> 0.86 and > 0.12 x 10^6/mL), intermediate (0.12-0.86 and 0.05-0.12 x 10^6/mL) and low (< 0.12 and < 0.05 x 10^6/mL) levels based on cutoff values corresponding to tertiles for further analyses. For Luminex and Elisa samples analysis, we normalized both data using a root-mean-square normalization, merged the data, and linearly scaled the merged data making the maximum value one and minimum value zero. A 2-tailed $P < 0.05$ was considered statistically significant.

Results

MDSCs increase transiently in COVID-19 patients

We analyzed 56 hospitalized PCR-confirmed SARS-COV2 infected adult patients included in the Lausanne University Hospital COVID-19 cohort study (**Table 1**). Forty-five patients (80%) developed moderate COVID-19 and did not require intubation. Eleven patients (20%) developed severe COVID-19, of which 2 died (3.5%). The groups of moderate and severe COVID-19 patients were similar in gender, age, immunosuppressive drug medication and number of comorbidities. Severe COVID-19 patients had a longer hospital stay than moderate COVID-19 patients (moderate *vs* severe: 3.5 [IQR: 1.8-6.5] *vs* 24 [21-27] days, $P < 0.001$) and higher leukocyte counts (3.0 [2.4-4.7] *vs* 7.0 [3.3-10.9] x 10^9 cells/mL, $P = 0.024$) (**Table 1**).

Blood was collected at study inclusion and 3 months later for 21 patients (14 moderate and 7 severe COVID-19). Immune profiling targeted at MDSCs was performed by flow cytometry followed by automatic unsupervised clustering with FlowSOM based on the expression of CD3, CD7, CD11b, CD14, CD15, CD16, CD19, CD33, CD45, CD56, CD135 and HLA-DR (**Figure 1A-B**). PMNs were at 1.7 [1.1-2.3] x 10^6 cells/mL, representing around 50% [38-63%] of total leukocytes irrespective of infection status and timing of blood collection (**Figure 2**). Lineage (CD3, CD7, CD19 or CD56) positive cells and DCs (Lin^+/DCs) represented the second largest group, followed by $CD14^+$ $CD16^-$ classical monocytes and PMN-MDSCs (**Figure 2**). Patients with an active SARS-CoV-2 infection had less Lin^+/DCs and monocytes than healthy controls, but 4-fold more PMN-MDSCs (control *vs* COVID-19: 0.11 [0.07-0.23] *vs* 0.40 [0.09-1.6] x 10^6 cells/mL, $P = 0.031$) and 2-fold more M-MDSCs (control *vs* COVID-19: 0.038 [0.021-0.045] *vs* 0.076 [0.040-0.15] x 10^6 cells/mL, $P = 0.01$) (**Figure 2**). The counts of PMN-MDSCs and M-MDSCs, as well as the counts of leukocytes, were normalized in blood collected from patients 3 months after study inclusion (**Figure 2**).

MDSCs counts correlate with COVID-19 severity and blood cytokine levels

PMN-MDSCs and M-MDSCs counts were 10- and 4-fold higher in severe COVID-19 patients compared with moderate COVID-19 patients, respectively ($P = 0.0013$ and $P = 0.0014$) (**Figure 3**). In contrast, PMNS, eosinophils, basophils, Lin^+/DCs and monocytes were similarly expressed in severe and moderate COVID-19 patients (**Figure 3**). PMN-MDSCs and M-MDSCs levels correlated with each other (correlation coefficient (ρ) = 0.43, $P = 0.03$) (**Figure 4A**). They did not correlate with the use of immunosuppressive drugs ($P = 0.47$ and $P = 0.28$).

Lymphocytopenia and high serum cytokine levels at time of hospitalization were predictors of COVID-19 severity (3, 18, 46). We took advantage of the measurements of lymphocyte populations and 49 cytokines, chemokines, and growth factors in the blood of COVID-19 patients at study admission ((45)) to perform correlation studies between the absolute counts of MDSCs and immunological parameters. PMN-MDSCs and M-MDSCs inversely correlated with lymphocyte, T cells, $CD4^+$ T cells, $CD8^+$ T cells and T regulatory cells (Tregs) counts. The strongest association was between PMN-MDSCs and lymphocytes ($\rho = -0.37$, $P = 0.025$) (**Figure 4A-B**). B cell numbers were not affected by SARS-CoV2 infection in our cohort study ($P > 0.05$), and correlation with MDSCs was not considered.

PMN-MDSCs and M-MDSCs had a positive correlation with the 33 mediators detectable in the serum of patients (positive *vs* negative association: red *vs* blue dots, 53/66 *vs* 3/66 associations) (**Figure 5A**). Eight statistically-significant positive associations were identified after correction for multiple comparisons. PMN-MDSCs and M-MDSCs correlated with epidermal growth factor (EGF) and hepatocyte growth factor (HGF) (PMN-MDSCs: $\rho = 0.47$, $P = 0.01$ and $\rho = 0.42$, $P = 0.02$; M-MSDCs: $\rho = 0.44$, $P = 0.02$ and $\rho = 0.46$, $P = 0.01$), M-MDSCs correlated with IL-1β ($\rho = 0.42$, $P = 0.03$), IL-7 ($\rho = 0.38$, $P = 0.05$), platelet-derived growth factor-BB (PDGF-BB) ($\rho = 0.56$, $P < 0.0001$) and vascular endothelial growth factor (VEGF) ($\rho = 0.40$, $P = 0.03$) (**Figure 5A**). Normalized values per patient are depicted in **supplementary Figure 2**.

To have a more detailed view about the relationship between the expression of MDSCs and the expression of serum mediators, we partitioned PMN-MDSCs and M-MDSCs into high, intermediate, and low expression levels (high: > 0.86 and > 0.12, intermediate: 0.12-0.86 and 0.05-0.12, and low: < 0.12 and < 0.05 x 10^6 cells/mL, respectively; cutoffs based on tertiles) (**Figure 5B**). Twenty-three mediators were differentially expressed in the 6 groups constituted (high, intermediate and low PMN-MDSCs and M-MDSCs). High expression levels of PMN-MDSCs and M-MDSCs were in general associated with high concentrations of mediators (15/23 and 16/23 of associations, respectively) (**Figure 5B**). Low expression levels of M-MDSCs were associated with high levels of MCP-1, CXCL9, CXCL10 and CXCL13.

COVID-19 durably impairs cytokine response of blood monocytes and dendritic cells

MDSCs drive immunosuppressive functions on both innate and adaptive immune cells (34, 35). COVID-19 has persistent effects on the adaptive immune system, but the long-term impact on the innate immune system is largely unknown. Thus, we investigated the consequence of COVID-19 on innate immune functions. We exposed whole blood from COVID-19 patients (at study inclusion and after 3 months) and from healthy subjects to LPS, R848, Pam_3CSK_4 and CpG (*i.e.* ligands of TLR4, TLR7/8, TLR1/2 and TLR9). After for 4 hours, we performed intracellular cytokine staining (ICS) followed by flow cytometry analysis of TNF and IL-6 expression by monocytes and DCs.

In healthy controls, 0.02% [0-0.04] and 4.3% [3.0-5.0] of monocytes produced TNF and IL-6 at baseline, 24% [17-25] and 17% [14-20] in response to LPS, and 79% [67-81] and 46% [32-50] in response to R848, respectively. The percentage of blood monocytes producing TNF and IL-6 in response to LPS and R848 was 1.3-4.9-fold lower in COVID-19 patients (LPS: $P < 0.001$, R848: $P < 0.05$) (**Figure 6A-B**). The reduction was more striking in severe than in moderate COVID-19 patients (**Figure 6C-D**). The impaired response of monocytes persisted up to 3 months in COVID-19 patients (**Figure 6A-B**). A similar pattern was observed when subdividing monocytes into classical and intermediate/non-classical monocytes (**Supplementary Figure 3A-D**). Of note, few M-MDSCs produced TNF and IL-6 in response to LPS and R848 (**Supplementary Figure 3A-D**). The percentages of monocytes producing TNF and IL-6 in response to Pam_3CSK_4 and CpG and IL-10 in response to LPS, R848, Pam_3CSK_4 and CpG were, nonetheless lower in COVID-19 patients. The decrease was essentially normalized in patients analyzed after 3 months (**supplementary Figure 4 and 5**).

DCs presented a similar response pattern to that observed with monocytes. In healthy controls, 0.6% [0.5-1.6] and 0.6% [0.2-0.6] of DCs produced TNF and IL-6 at baseline, 38% [36-45] and 36% [34-40] in response to LPS, and 68% [48-77] and 58% [47-75] in response to R848, respectively. TNF and IL-6 response by DCs was 2.1-5.1-fold lower in COVID-19 patients ($P < 0.001$) (**Figure 7A-B**), more impaired in severe than in moderate COVID-19 patients (**Figure 7C-D**), and still reduced after 3 months. The percentage of DCs producing TNF and IL-6 upon stimulation with Pam_3CSK_4 and CpG, and IL-10 at baseline and upon stimulation was too low (< 0-5%) to reliably detect an effect of COVID-19. PD-L1 expression by DCs was decreased in COVID-19 patients and was normalized to healthy control levels after 3 months (**Supplementary Figure 3B**).

Finally, we assessed whether the defects observed in monocytes and DCs impacted the production of cytokines/chemokines/growth factors by whole blood. The concentrations of 24 mediators were measured by multiplex bead assay, and that of TNF and IL-6 by ELISA in the supernatants of blood incubated for 24 hours with LPS and R848 (**Figure 8**). Upon stimulation, 71% and 54% (17/24 and 13/24) of mediators were secreted at lower concentrations in blood of COVID-19 patients tested at inclusion, while 25% and 29% (6/24 and 7/24) were at lower concentrations in blood of patients tested after 3 months, respectively (**Figure 8A**). The concentrations of TNF induced by LPS and R848 and of IL-6 induced by LPS were lower in COVID-19 patients tested at inclusion. Moderate and severe COVID-19 were similarly affected (**Figure 8B-E**).

Discussion

We analyzed immune parameters in whole blood of hospitalized patients with moderate and severe COVID-19, and healthy subjects. Along with cytokine levels, PMN-MDSCs and M-MDSCs were increased in patients and were associated with T cell depletion and worse outcomes. *Ex vivo* production of cytokines by innate immune cells in response to stimulation with TLR agonists was downregulated, particularly in severe COVID-19 patients. We are the first to report that MDSCs returned to normal levels while innate immune dysregulation was persisting over 3 months from disease symptoms. MDSCs may represent early markers of COVID-19 severity.

Massive release of cytokines has been associated with the pathophysiology of COVID-19, but its consequences on the dysregulated response affecting adaptive and innate arms of the immune system remain poorly understood. Systemic inflammation fuels widespread tissue damage and organ dysfunction. However, it also stimulates myelopoiesis and consequently the generation of MDSCs [47-53]. MDSCs represented 10-15% of blood leukocytes in COVID-19 patients. This situation is reminiscent of that observed in sepsis patients, in whom MDSCs strongly increased and eventually exceeded 40% of total leukocytes [54-56]. Interestingly, M-MDSCs levels in blood, but not in airways, correlated to COVID-19 severity [49], confirming that peripheral blood is a reliable source of biomarkers of COVID-19 disease.

We show that PMN-MDSCs were 4-10-fold more abundant than M-MDSCs. This mirrors measurements in other COVID-19 studies and other pathologies, though subtypes of MDSCs were not always differentiated [48-52]. Of note, there are discrepancies in MDSCs numbers in between studies, illustrated by one measuring a 90% PMN-MDSC proportion of total blood mononuclear cells in patients with severe COVID-19 [47], while in another showed similar frequencies of M-MDSCs and PMN-MDSCs [52]. Discrepancies reported in the literature may involve patient heterogeneity, but can also be related to different labelling and gating strategies to define MDSCs by flow cytometry since there is no specific marker allowing indisputable delineation of MDSCs. To rule out problems associated with manual gating, we applied automatic unsupervised clustering to analyze our flow cytometry data. We did not establish the immunosuppressive function of MDSCs. However, several studies demonstrated that PMN-MDSCs and M-MDSCs of COVID-19 patients inhibited the proliferation and cytokine production by T cells [47-49, 51]. Of note, in all but one of these studies, MDSCs were isolated though magnetic cell isolation technology, and therefore may not fully reflect the populations detected using more sophisticated flow cytometry analyses.

Single cell RNA sequencing (scRNA-seq) and single cell multi-omics analyses have been used to profile immune cells in the blood of COVID-19 patients [57-59]. Unfortunately, MDSCs were not identified by dimensional reduction of transcriptomic data. This was rather disappointing since in one of these studies, immunosuppressive myeloid cells were documented functionally. Low density gradient neutrophils and immature HLA-DR^{low} CD14^{+} monocytes suppressed T cell activity, and to a higher extent in severe patients who patient survived than in those who deceased [57]. Moreover, comparative gene profiling of PMN-MDSCs and neutrophils on the one hand, and of M-MDSCs and monocytes on the other hand, revealed great differences in cancer patients [40]. Similarly, a pilot scRNA-Seq study of

MDSCs in sepsis patients suggested the existence unique transcriptional responses [60]. Further work will be required to identify the mechanisms driving the expansion of MDSCs or the differentiation of neutrophils and monocytes into MDSCs and markers that could be used to discern MDSCs from classical monocytes and neutrophils by flow cytometry.

High levels of MDSCs at study inclusion, i.e. at hospital admission for virtually all patients, were associated with high circulating levels of cytokines and worse outcomes, and were inversely associated with lymphocytopenia. In addition, monocytes and DCs of COVID-19 patients showed severely reduced cytokine production in response to stimulation with TLR ligands, a dysfunction that was extreme in most severe patients. These observations supported the assumption that exuberant immune response to SARS-CoV-2 infection exacerbates the development of an immunosuppression environment limiting anti-microbial defenses. This is congruent with reports showing that severe COVID-19 patients have an increased risk of contracting nosocomial bacterial and fungal infections [61, 62].

Lymphopenia and high expression levels of cytokines (including IL-1β, IL-6, IL-7, IL-8, IL-10, IL-12, IL-1Ra, IFNγ, TNF, CCL2, CCL3, CXCL9, CXCL10, CXCL11, CXCL13, G-CSF and HGF) are hallmarks of severe COVID-19 [3, 18, 63]. Strengthening this notion, a retrospective study identified high IL-6, IL-8 and TNF levels at time of hospital admission as a signature predicting COVID-19 severity and survival [46]. PMN-MDSCs and M-MDSCs levels correlated with blood concentrations of EGF and HGF, and M-MDSCs also with PDGF-BB, VGEF, IL-1β and IL-7. Growth factors are involved in the expansion of MDSCs, and EGF and HGF act as chemoattractant of MDSCs in the tumor environment [64, 65]. IL-1β stimulates myelopoiesis [53, 66], while IL-7 sustained the expansion and T cell-suppressing activity of MDSCs as well as the expression of IL-10-producing B cells following sepsis [67, 68]. Targeting EGF and HGF pathways with tyrosine kinase inhibitors might be viable therapies in COVID-19, especially since inhibitors are used in the field of cancer [69, 70].

PMN-MDSCs and M-MDSCs as well as monocytes, DCs and lineage positive leukocytes were back to healthy control levels in patients screened 3 months after study inclusion. However, monocytes and DCs were still harboring reduced functionality (i.e. cytokine production), indicative for long-term immune disturbances. In a similar way, patients recovering from COVID-19 showed signs of profound cellular abnormalities, including signs of T cell activation/exhaustion several months after infection [71-75]. Failure to restore immune homeostasis, as observed in the post-sepsis syndrome [32, 33], may be one of the drivers of long-COVID and post-acute COVID-19 syndrome [27-30].

Taken together, our findings demonstrate that MDSCs rapidly and strongly expanded in COVID-19 patients and correlated to disease severity, suggesting that MDSCs may represent interesting markers to stratify SARS-CoV-2 infected patients. MDSCs recovered to baseline levels, but monocytes and DCs showed persistent signs of dysfunction over 3 months from disease onset. Therefore, while immunotherapies have been focusing on reducing inflammation, adjunct therapies targeting MDSCs or immune dysfunctions might be useful to counterbalance immunosuppression, reduce nosocomial and long-term infections and decrease late mortality in severe COVID-19 patients.

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Author contributions statement

IS, CT, and TR designed the study. MP, GP, TC and TR designed the LUH-COVID19 cohort. IS, CT, NA and MP analyzed the samples. All the authors interpreted the data. IS, TC and TR wrote the manuscript. All the authors revised the manuscript.

Conflict of Interest Statement

The authors do not have any conflict of interest regarding this manuscript.

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Tables and figures

Table 1. Patient's characteristics

Characteristic	Control	Moderate COVID-19	Severe COVID-19	3 months after study inclusion
Number of subjects	10	45	11	21
Gender, male	7 (70%)	31 (70%)	7 (64%)	15 (71%)
Age (years)	58 (55-65)	62 (53-74)	60 (48-63)	61 (54-75)
Charlson comorbidity index	-	3 (1.8-6)	2 (1-4.5)	2 (1-6)
Immunosuppressive drugs	-	7 (16%)	1 (%)	5%
Days of symptoms before inclusion	-	7 (5-10)	7 (5-11)	9 (6.5-11)
Length of hospital stay[#]	-	3.5 (1.8-6.5)	24 (21-27)***	5 (2.5-17)
Death	-	-	2 (18%)	-
Leukocytes (x 10^9 cells/L)	3.4 (3.2-3.7)	3.0 (2.4-4.7)	7.0 (3.3-10.9)*	3.9 (3.2-4.8)
PMN-MDSCs (x 10^9 cells /L)	0.1 (0.07-0.2)	0.2 (0.08-1.0)	2.3 (0.6-8.6)**	0.1 (0.06-0.2)
M-MDSCs (x 10^9 cells /L)	0.04 (0.02-0.05)	0.05 (0.03-0.1)	0.22 (0.11-0.31)**	0.04 (0.02-0.07)

Data are medians (IQR) or N (%). [#]Excluding non-survivors, from moment of inclusion to hospital discharge. Statistics between moderate and severe COVID-19 patients: * P < 0.05, ** P < 0.01, *** P < 0.001.

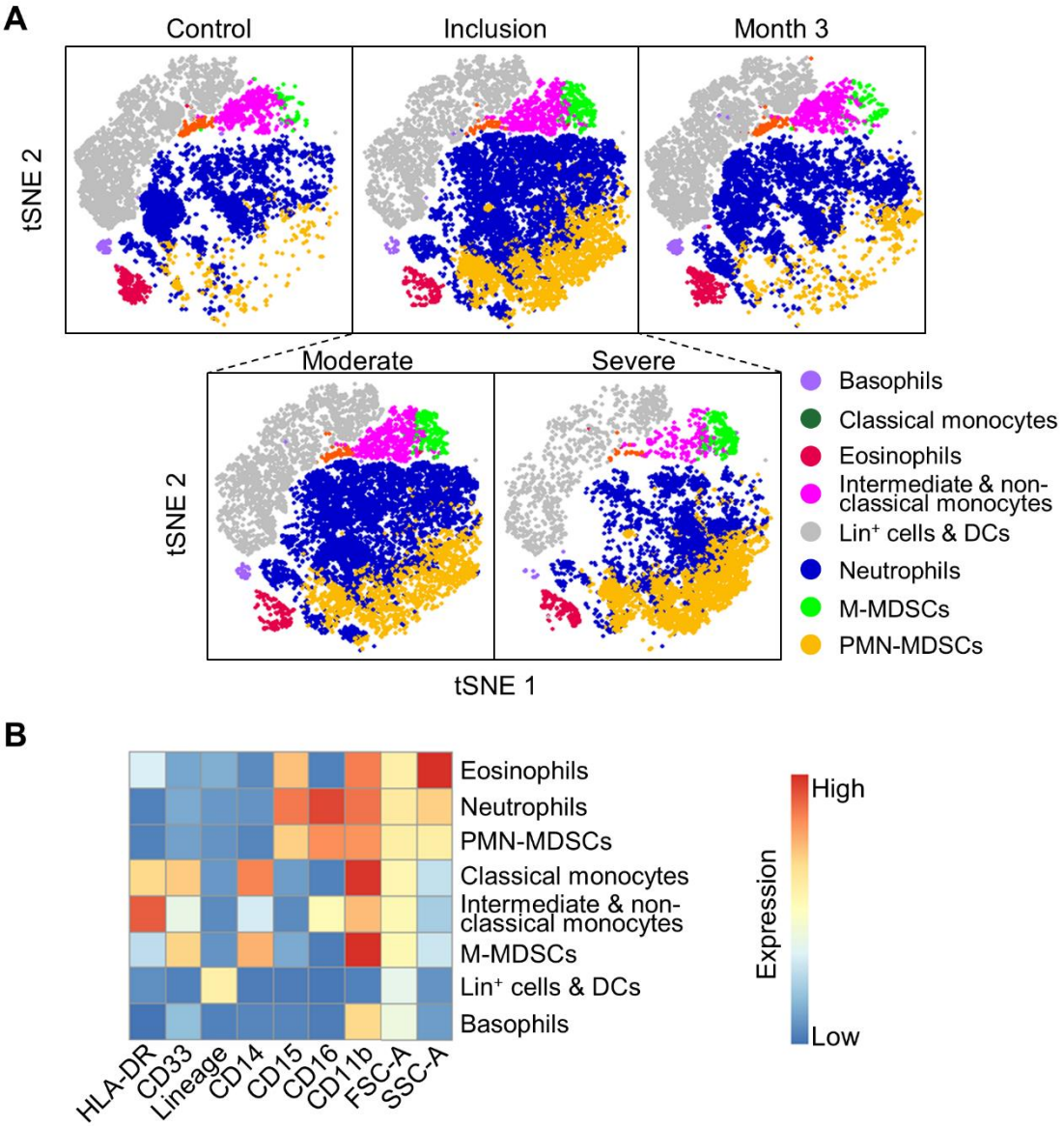


Figure 1. Identification of MDSCs in the blood of COVID-19 patients. A) Blood was obtained from 10 healthy subjects and 56 COVID-19 patients (46 moderate and 11 severe COVID-19) at study inclusion and after 3 months (N=17), stained with antibodies directed against CD3, CD14, CD15, CD11b, CD16, CD19, CD33, CD45, CD56, CD124 and HLA-DR, and analyzed by flow cytometry (see *Materials and Methods*). **A**) t-SNE plots of leukocyte populations. **B**) Expression levels of cell surface markers and FSC-A/SSC-A of leukocyte populations. PMN-MDSCs: polymorphonuclear-MDSCs, M-MDSCs: monocytic-MDSCs, LIN: lineage (i.e. positive for CD3, CD56 or CD19), DCs: dendritic cells.

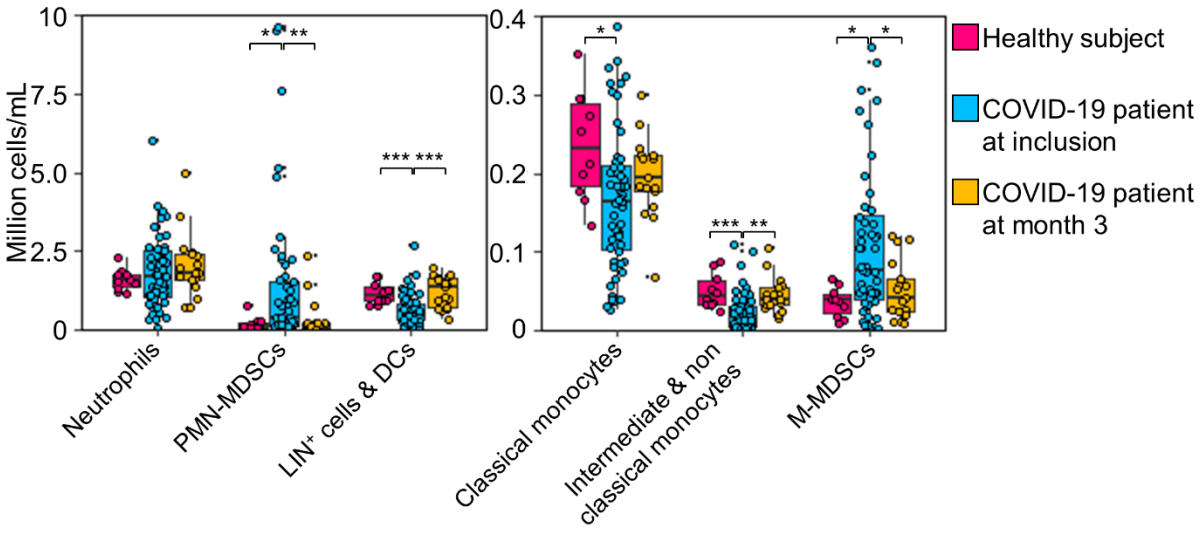


Figure 2. MDSCs increased rapidly in the blood of COVID-19 patients. Counts in million cells/mL of leukocyte populations identified as described in *Figure 1*. Boxplots show median, upper and lower quartiles. The whiskers show 5 – 95 percentiles. Each dot represents an individual sample. N = 10 healthy subjects, 56 COVID-19 patients at inclusion and 17 COVID-19 patients at month 3. * P < 0.05, ** P < 0.01, *** P < 0.001.

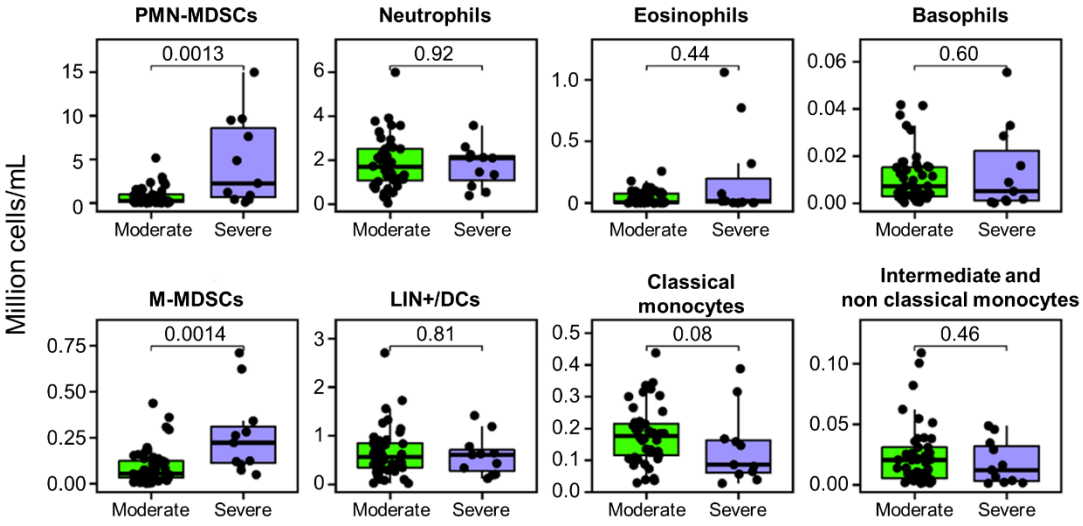


Figure 3. MDSCs are increased in patients with severe COVID-19. Counts in million cells/mL of leukocyte populations. Boxplots show median, upper and lower quartiles. The whiskers show 5 – 95 percentiles. Each dot represents an individual sample. N = 45 moderate and 11 severe COVID-19 patients. ** P < 0.01.

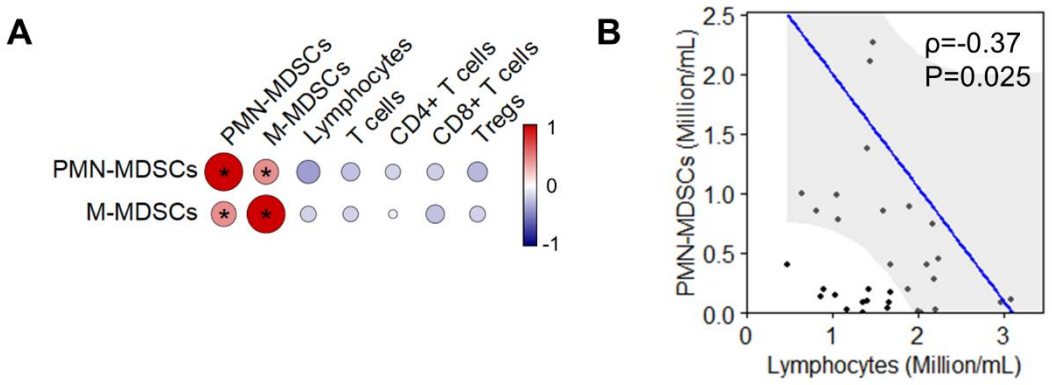


Figure 4. High MDSCs levels inversely correlate with lymphocytopenia. A) Correlation plot matrix for MDSCs and lymphocyte populations in 48 patients. Correlations were calculated using Spearman's rank correlation controlled for False Discovery Rate (FDR). * P < 0.05. **B**) Scatterplot showing an inverse correlation between PMN-MDSCs and total lymphocytes.

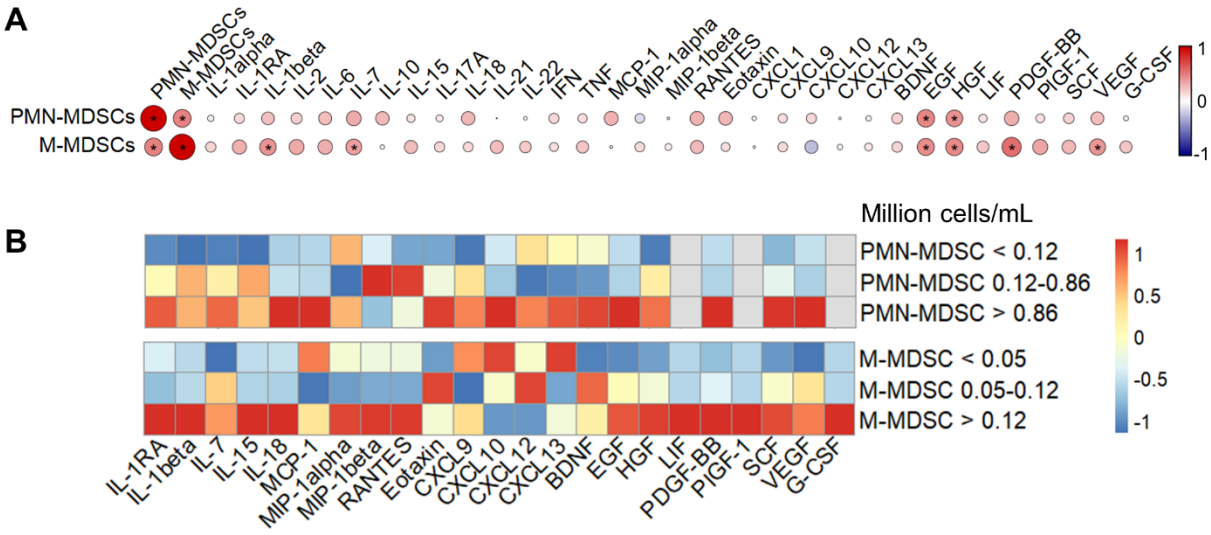


Figure 5. Correlation between MDSCs, cytokines, chemokines and growth factors expression levels. A) Correlation plot matrix for PMN-MDSCs and M-MDSCs (counts measured by flow cytometry) and 33 serum mediators (14 cytokines, 10 chemokines and 9 growth factors) measured in 36 patients. Correlations were calculated using Spearman's Rank-Order correlation controlled for FDR. * P < 0.05. **B**) Heatmap scaled expression plot divided on high, intermediate and low levels of PMN-MDSCs and M-MDSCs (high: > 0.86 and > 0.12, intermediate: 0.12-0.86 and 0.05-0.12, and low: < 0.12 and < 0.05 x 10^6 cells/mL, respectively; cutoffs based on tertiles). Mediators showing differences between subgroups are depicted.

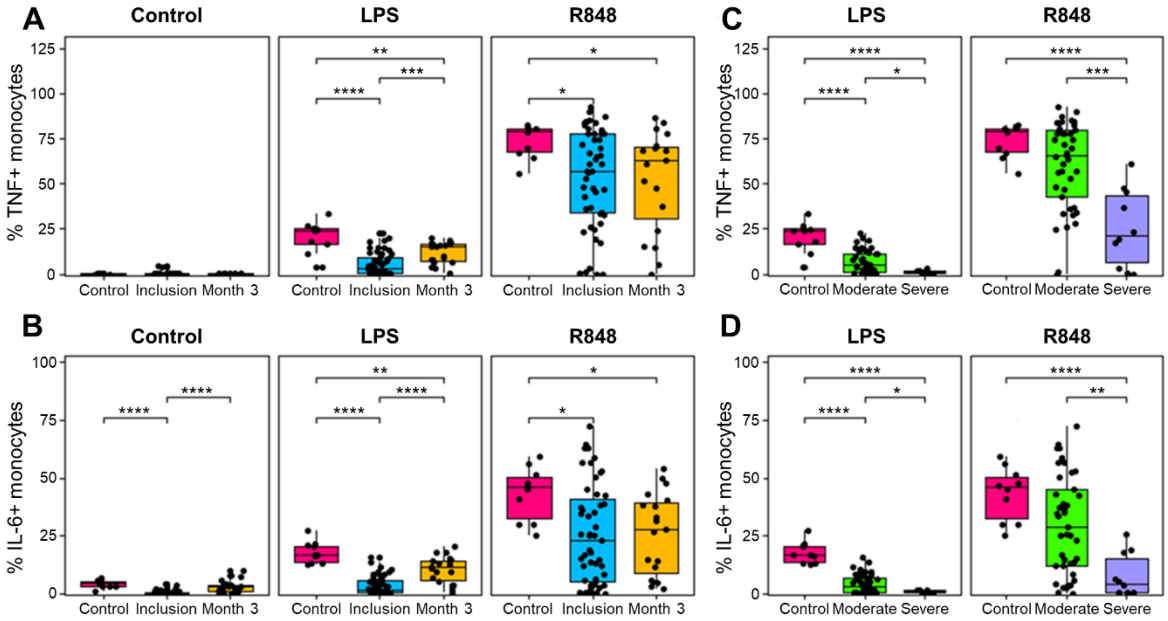


Figure 6. Long-lasting reduced cytokine response by monocytes of COVID-19 patients. Blood was obtained from 10 healthy subjects and COVID-19 patients at study inclusion (N = 50) and after 3 months (N = 19), exposed for 4 hours to LPS (100 ng/mL) and R848 (5 µg/mL), and analyzed by intracellular cytokine staining (ICS) followed by flow cytometry analysis of TNF and IL-6 expression by monocytes (see *Materials and Methods*). **A-B**) Percentage of TNF^+ and $IL\text{-}6^+$ monocytes in healthy subjects and COVID-19 patients tested at study inclusion and after 3 months. **C-D**) Percentage of TNF^+ and $IL\text{-}6^+$ monocytes in healthy subjects, moderate (N = 41) and severe (N = 9) COVID-19 patients tested at study inclusion. Boxplots show median, upper and lower quartiles. The whiskers show 5 – 95 percentiles. Each dot represents an individual sample. * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001.

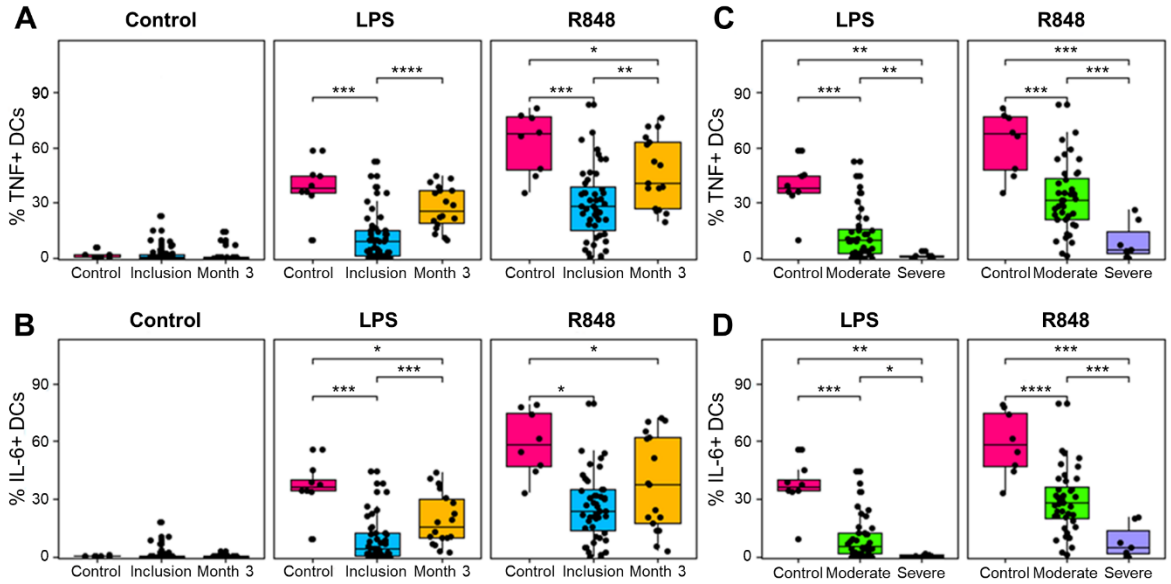


Figure 7. Long-lasting reduced cytokine response by DCs of COVID-19 patients. Blood from healthy subjects and COVID-19 patients was exposed for 4 hours to LPS and R848, and analyzed by ICS followed by flow cytometry analysis of TNF and IL-6 expression by DCs. **A-B**) Percentage of TNF^+ and $IL\text{-}6^+$ DCs in healthy subjects (N = 8) and COVID-19 patients tested at study inclusion (N = 49) and after 3 months (N = 18). **C-D**) Percentage of TNF^+ and $IL\text{-}6^+$ DCs in healthy subjects, moderate (N = 42) and severe (N = 7) COVID-19 patients tested at study inclusion. Boxplots show median, upper and lower quartiles. The whiskers show 5 – 95 percentiles. Each dot represents an individual sample. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

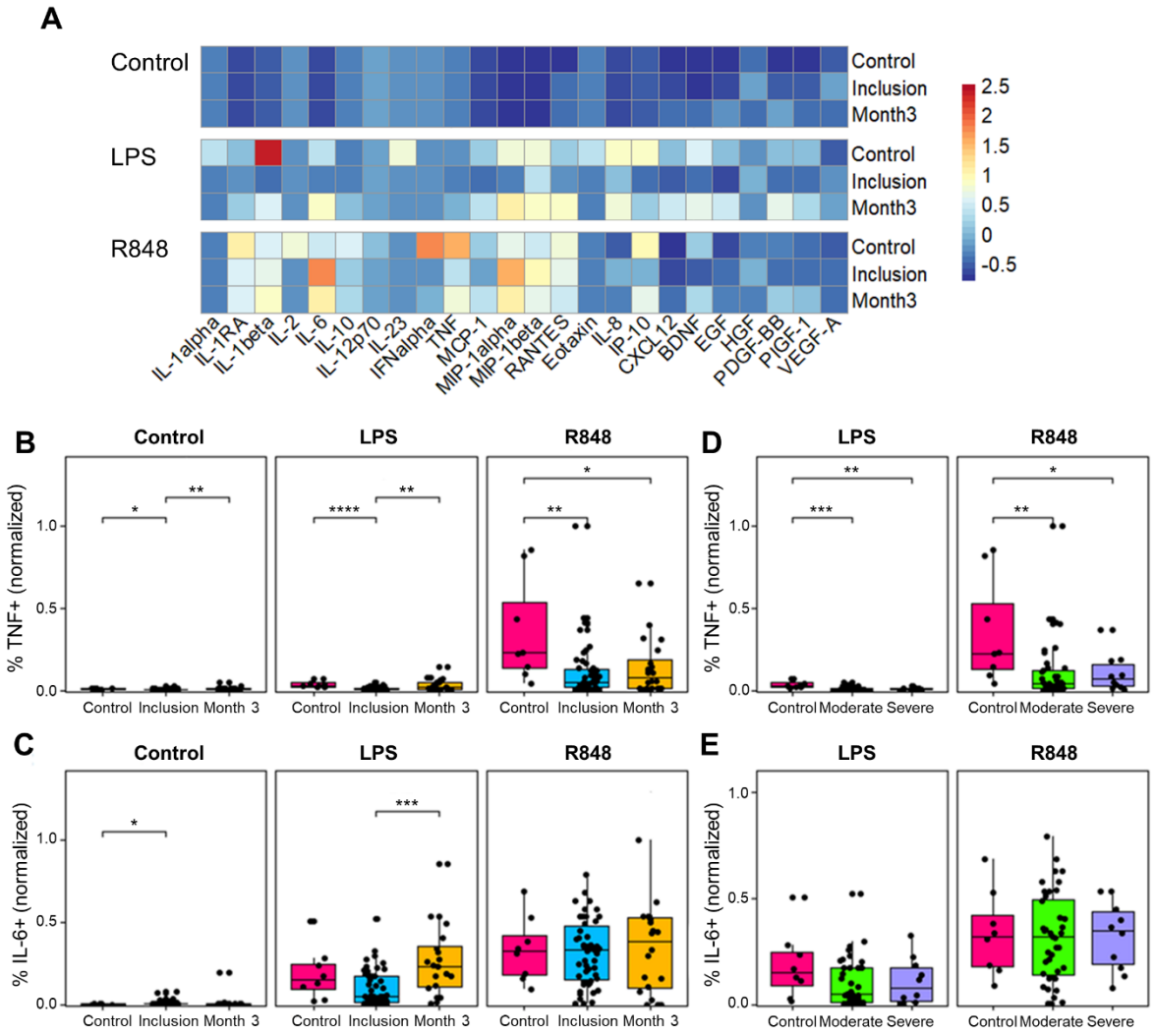


Figure 8. Production of cytokines, chemokines and growth factors by whole blood. Blood was incubated for 24 hours with LPS (100 ng/mL) and R848 (5 µg/mL). Supernatants were collected to quantify serum mediators by multiplex bead assay (**A**) and TNF and IL-6 by ELISA (**B-E**). **A**) Heat map scaled expression plot of serum mediators in healthy controls (N = 5) and COVID-19 patients at inclusion (N = 13) and after 3 months (N = 12). **B-E**) TNF and IL-6 concentrations were measured by multiplex bead assay and ELISA (N = 4 healthy controls, 38 patients at inclusion, and 10 patients after 3 months). Results were normalized to the highest value set at 1. Boxplots show median, upper and lower quartiles. The whiskers show 5 – 95 percentiles. Each dot represents an individual sample. * P < 0.05; ** P < 0.01; *** P < 0.001.

Supplementary tables and figures

Supplementary Table 1: Antibodies used in flow cytometery targeting MDSC

Target	Clone	Fluorochrome	Company	Reference number
CD3	UCHT1	AlexaFluor 700	eBioscience	56-0038-42
CD7	M-T701	AlexaFluor 700	BD	561603
CD11b	Bear1	PC-7	Beckman Coulter	A54822
CD135	BV10A4H2	PE	Biolegend	313305
CD14	RMO52	APC-AF750	Beckman Coulter	B92421
CD15	80H5	Pacific Blue	Beckman Coulter	B49218
CD16	3G8	ECD	Beckman Coulter	B49216
CD19	J3.119	AlexaFluor 700	Beckman Coulter	B76284
CD33	D3HL60.251	APC	Beckman Coulter	IM2471
CD45	J33	Krome orange	Beckman Coulter	B36294
CD56	HCD56	AlexaFluor 700	Biolegend	318316
HLA-DR	Immu-357	FITC	Beckman Coulter	IM1638U

Supplementary Tables 2. Antibodies used in flow cytometery targeting dendritic cells and monocytes

Dendritic cells

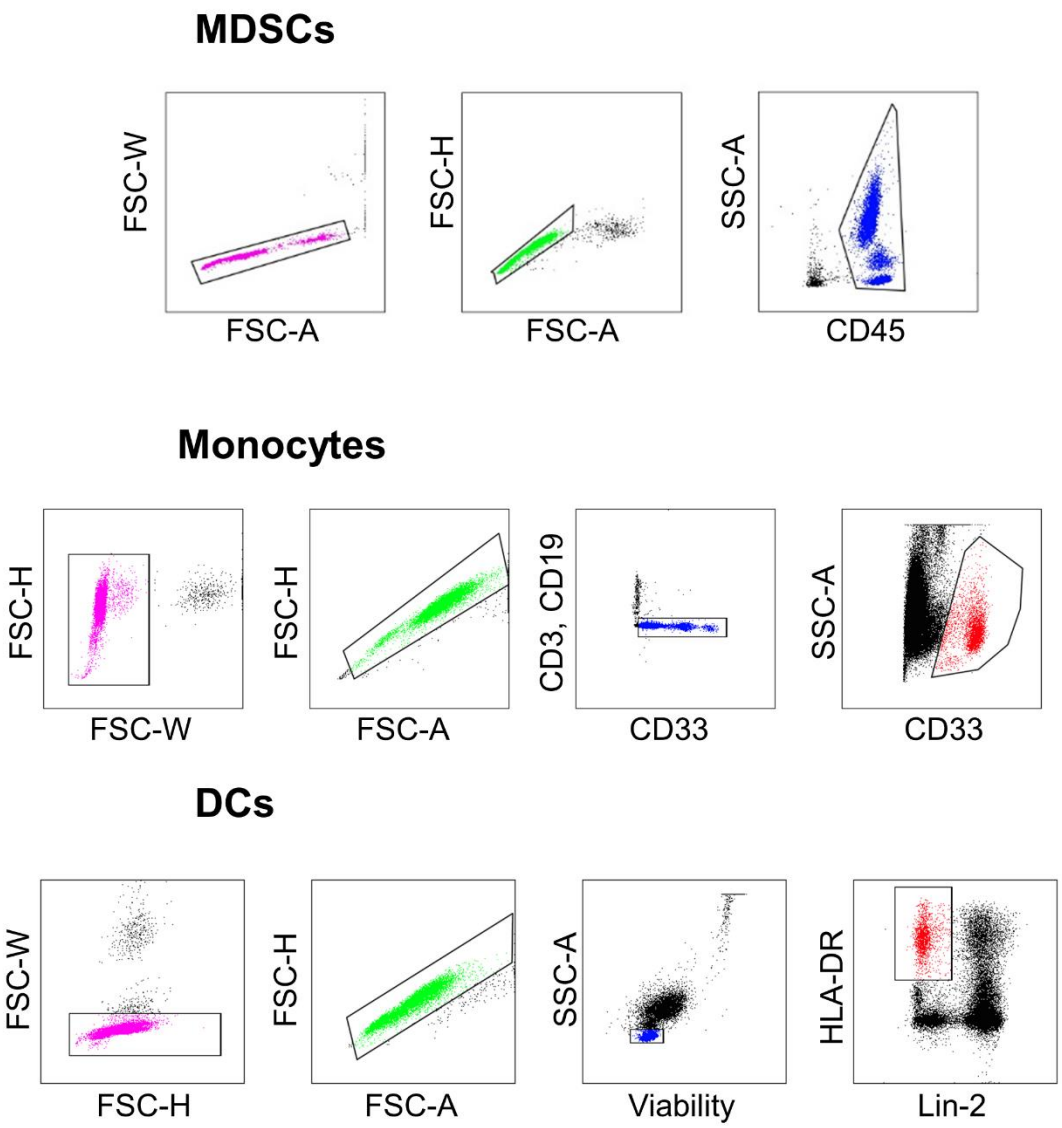
Target	Clone	Fluorochrome	Company	Reference number
LIVE/DEAD™		Fixable Aqua	Invitrogen™	L34957
CD1c	L161	AF700	biolegend	331530
CD11c	B-ly6	PE-TXR	BD Pharmingen	562393
CD123	6H6	BV711	biolegend	306030
CD16	3G8	PB	BD Pharmingen	558122
CD274	MIH1	PE-Cy™7	BD Pharmingen	558017
HLA-DR	REA332	APC-Vio770	Miltenyi Biotec	130-104-871
Lin-2	multiple	FITC	BD	643397

Monocytes

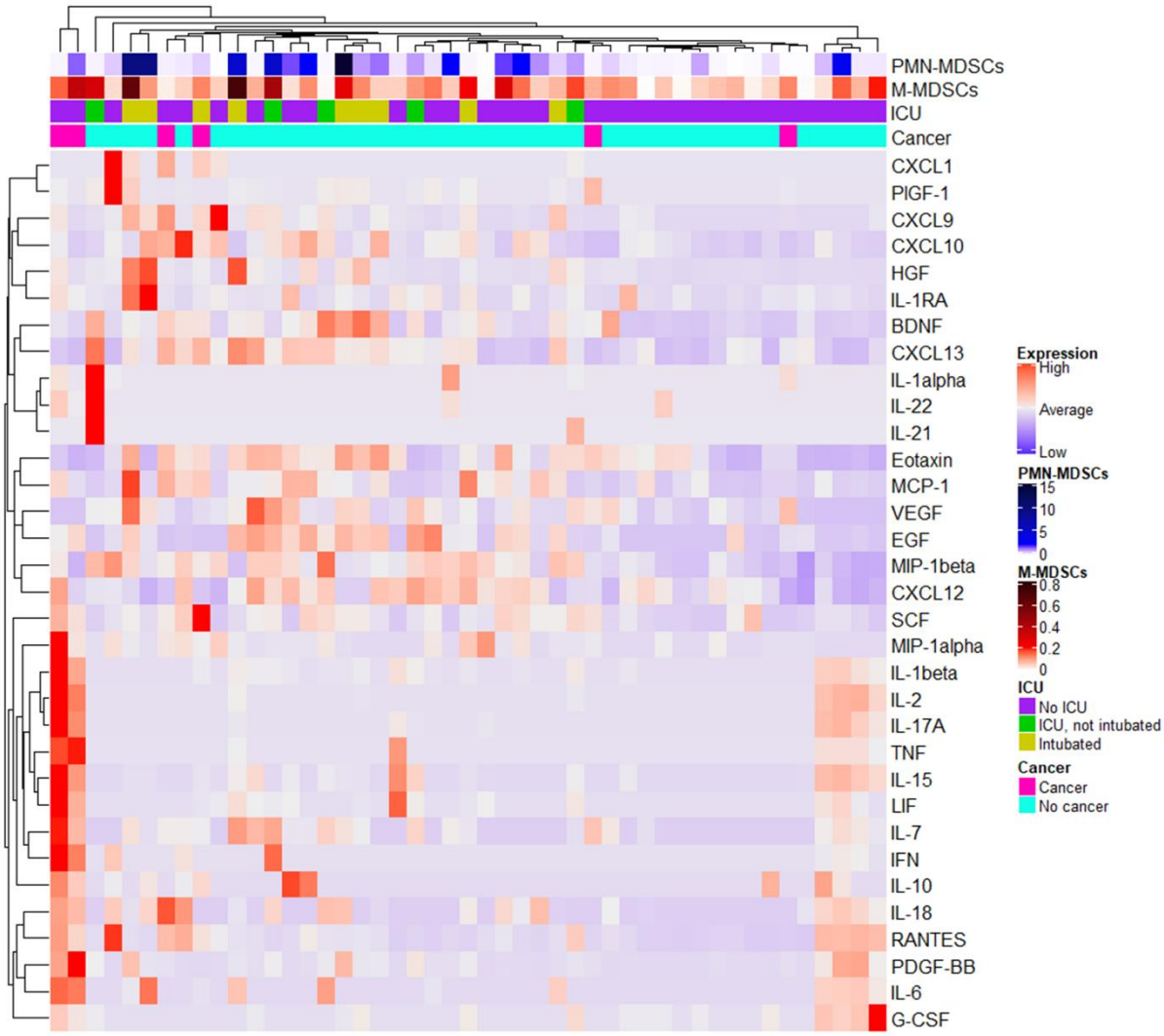
Target	Clone	Fluorochrome	Company	Reference number
LIVE/DEAD™		Fixable Aqua	Invitrogen™	L34957
CD3	SP34	APC-C7	BD Pharmingen	557757
CD14	18D11	FITC	ImmunoTools	21620143
CD16	3G8	PB	BD	558122
CD19	SJ25C1	APC-C7	BD Pharmingen	557791
CD274	MIH1	PE-Cy™7	BD Pharmingen	558017
CD33	WM33	BV711	BD Pharmingen	563171
CD56	HCD56	AF700	Biolegend	318316
HLA-DR	Immu-357	PE-TXR	Beckman Coulter	B94238

Intracellular staining monocytes/DCs

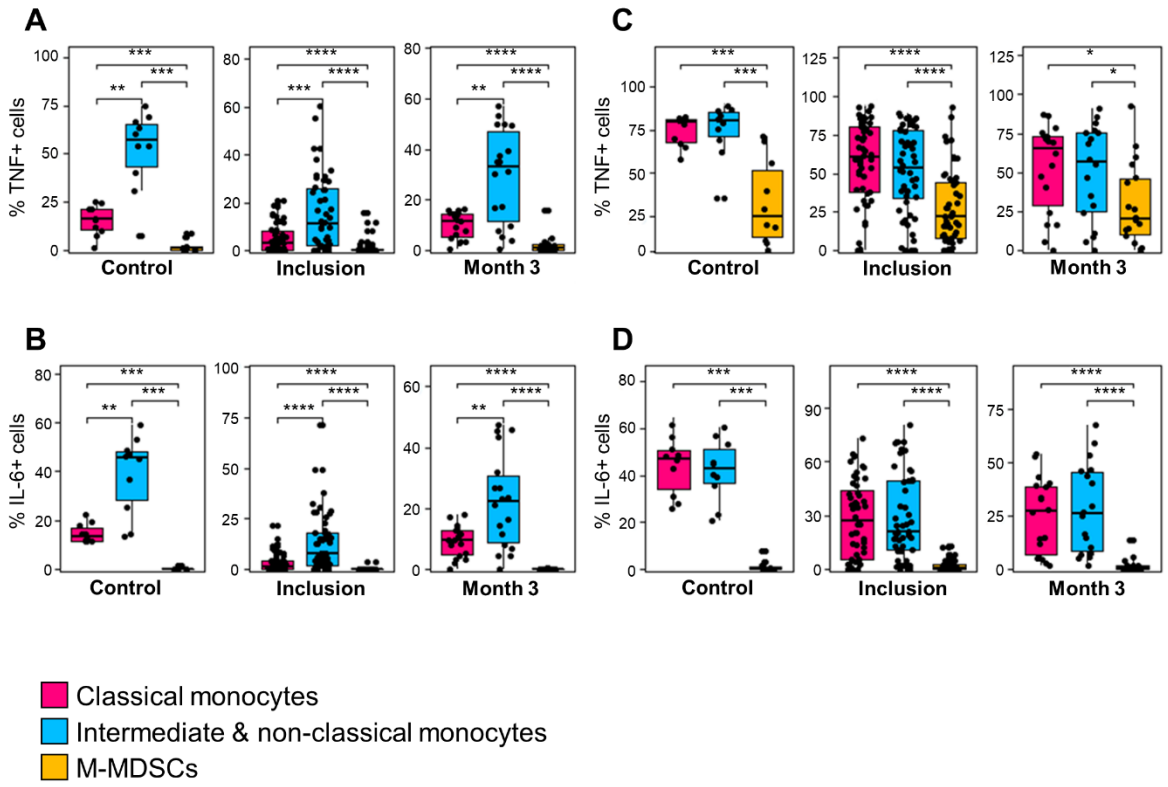
Target	Clone	Fluorochrome	Company	Reference number
IL-6	MQ2-13A5	PerCP/Cy5.5	Biolegend	501117
IL-10	JES3-9D7	PE	BD Pharmingen	559337
TNF-a	MAb11	APC	biolegend	307626



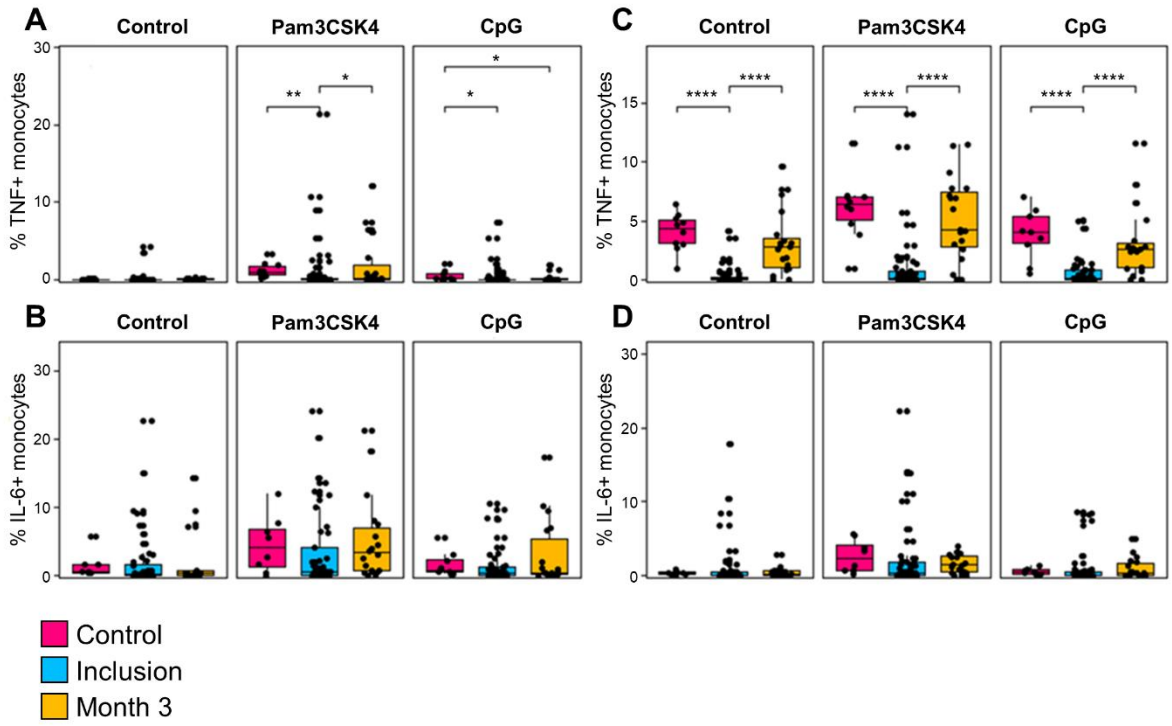
Supplementary Figure 1. Gating strategy to exclude debris, doublets and non-hematopoietic cells to analyze blood leukocytes by flow cytometry.



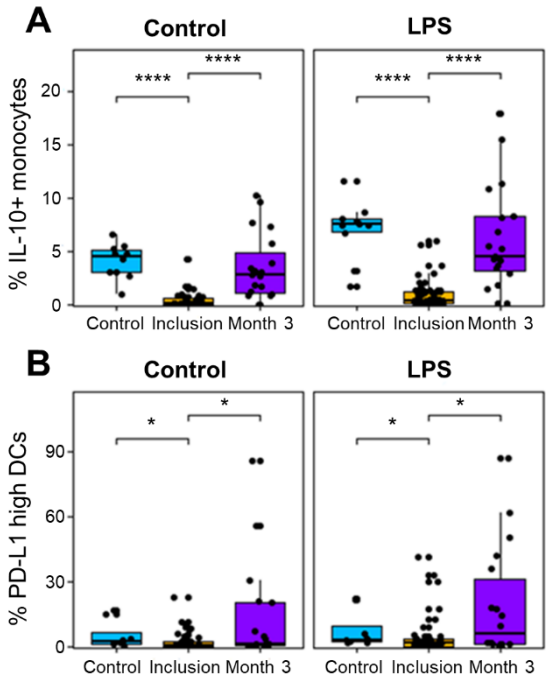
Supplementary Figure 2. Heatmap and hierarchical clustering of cytokine, chemokine and growth factor expression levels per patients at inclusion.



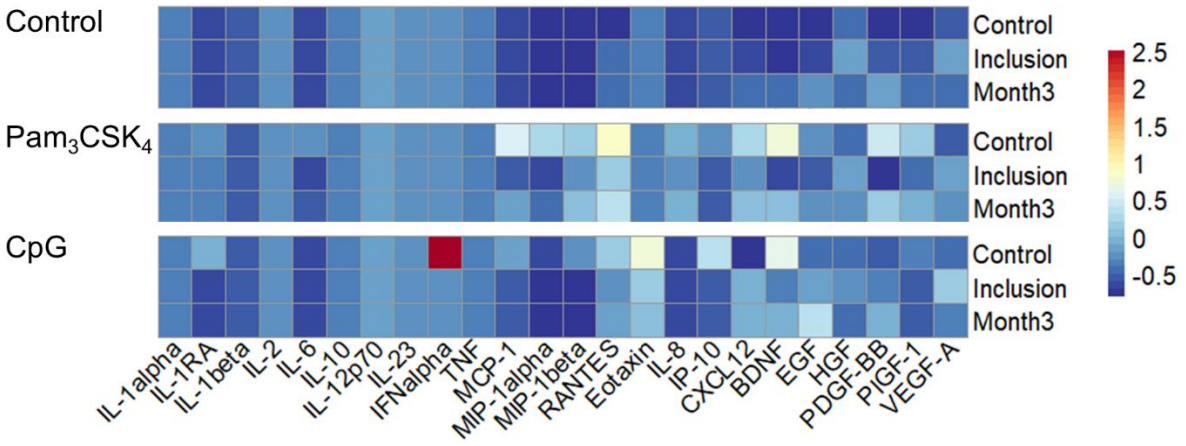
Supplementary Figure 3. Long-lasting reduced cytokine response by LPS and R848-stimulated monocytic subpopulations of COVID-19 patients. Blood from healthy subjects and COVID-19 patients was exposed for 4 hours to LPS (100 ng/mL) and R848 (5 (µg/mL) and analyzed by intracellular cytokine staining (ICS) followed by flow cytometry analysis of TNF and IL-6 expression by classical monocytes, intermediate/non-classical monocytes, and M-MDSCs (see *Materials and Methods*). **A-C**) Percentage of TNF^+ and $IL\text{-}6^+$ cells in healthy subjects (N = 10) and COVID-19 patients tested at study inclusion (N = 41 moderate and 9 severe COVID-19) and after 3 months (N = 19). Boxplots show median, upper and lower quartiles. The whiskers show 5 – 95 percentiles. Each dot represents an individual sample. * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001.



Supplementary Figure 4. Long-lasting reduced cytokine response by Pam_3CSK_4 and CpG-stimulated monocytes of COVID-19 patients. Blood from healthy subjects and COVID-19 patients was exposed for 4 hours to Pam_3CSK_4 (100 ng/mL) and CpG (5 µg/mL), and analyzed by ICS followed by flow cytometry analysis of TNF and IL-6 expression by DCs. **A-B**) Percentage of TNF^+ and $IL\text{-}6^+$ monocytes in healthy subjects (N = 10) and COVID-19 patients tested at study inclusion (N = 50) and after 3 months (N = 19). Boxplots show median, upper and lower quartiles. The whiskers show 5 – 95 percentiles. Each dot represents an individual sample. * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001.



Supplementary Figure 5. IL-10 production by monocytes and PD-L1 expression by DCs of COVID-19 patients. Blood from healthy subjects and COVID-19 patients was exposed for 4 hours to LPS, R848, Pam_3CSK_4 and CpG, and analyzed by ICS followed by flow cytometry analysis of IL-10 and PD-L1 by monocytes and DCs. **A**) Percentage of IL-10^+ monocytes. **B**) Percentage of PD-$L1^+$ DCs. Boxplots show median, upper and lower quartiles. The whiskers show 5 – 95 percentiles. Each dot represents an individual sample. Stimulation did not influence IL-10 and PD-L1 expression, therefore only LPS is shown. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.



Supplementary Figure 6. Production of cytokines, chemokines and growth factors by whole blood. Blood was incubated for 24 hours with Pam_3CSK_4 (100 ng/mL) and CpG (5 µg/mL). Supernatants were collected to quantify serum mediators by multiplex bead assay. Results were represented with a heat map scaled expression plot of serum mediators in healthy controls (N = 5) and COVID-19 patients at inclusion (N = 13) and after 3 months (N = 12). The control panel is identical to the one presented in **Figure 8**.

3.4. High levels of monocytic myeloid-derived suppressor cells correlate with improved outcome in sepsis patients with multi-organ dysfunction syndrome.

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Manuscript in preparation

Summary:

For this study, we aimed to assess whether MDSCs correlated with disease severity in critically ill sepsis patients with MODS. We conducted a prospective observational study embedded in the INCLASS study (NCT03345992) conducted in eight Greek hospitals. Adult sepsis patients with pneumonia were included when they presented a SOFA score of at least 7, combining respiratory failure (PiO2/FiO2 < 200), and any other organ system failure. Blood collected at days 1, 5, and 10 from inclusion were added into lyophilized antibody tubes designed to detect MDSCs by flow cytometry. FlowSOM was used for clustering and identification of leukocyte populations. Forty-eight patients were included, of which 34 (71%) died within 90 days. At study inclusion, M-MDSCs and PMN-MDSCs represented 3% and 22% of leukocytes, respectively. M-MDSCs were higher in survivors than in patients who died within 28-days (P = 0.028). Stratification of patients based on MDSCs levels revealed that M-MDSCs^{high} patients had improved 90-day survival (high versus low M-MDSCs: 53% versus 16% survival, P = 0.003, hazard ratio (HR) = 3.2). The effect was even more pronounced in patients who had a low APACHE II score (high *versus* low M-MDSCs: 80% versus 20% survival, P = 0.0096, HR: 7.2). Lastly, high levels of PMN-MDSCs were associated with secondary infections and new sepsis episodes. Overall, high levels of M-MDSCs were associated with the survival of sepsis patients with MODS, especially in patients with a low APACHE II score. This is the first study to attribute a beneficial role to MDSCs in sepsis. Further investigations are needed to assess whether MDSCs can be used as prognostic and/or theragnostic biomarkers in sepsis patients.

My contribution to this work:

I was a sub-investigator of the INCLASS study. I participated into the design of the study and to organize blood collection. I performed flow cytometry experiments, and analysed the data. I drafted the article.

High levels of monocytic myeloid-derived suppressor cells are associated with favorable outcome in sepsis patients with multi-organ failure

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Keywords: Sepsis, multi-organ dysfunction syndrome, myeloid-derived suppressor cells, critically ill

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Abstract

Objectives: Myeloid derived suppressor cells (MDSCs) are immature myeloid cells with immunosuppressive functions subclassified into polymorphonuclear and monocytic MDSCs (PMN-MDSCs and M-MDSCs). Clinical studies have reported increased levels of MDSCs that were associated with poor outcome in sepsis patients. Since sepsis patients exhibit signs of inflammation and immunosuppression, MDSCs may provide benefit by dampening deleterious inflammation in some patients. To challenge this hypothesis, we measured MDSCs in critically ill sepsis patients with multi-organ dysfunction syndrome (MODS) and a high likelihood of death.

Design: Multi-center prospective observational study.

Setting: Eight ICUs in Athens and Thessaloniki, Greece.

Patients: Critically ill patients with pneumosepsis and MODS.

Measurements and main results: Forty-eight patients were included, of whom 34 died within 90 days. Blood MDSCs were quantified by flow cytometry and unsupervised clustering. At study inclusion, PMN-MDSCs and M-MDSCs were increased in sepsis patients when compared to healthy subjects (22% vs 2.1% and 3.07% vs 0.96% of leukocytes, respectively; $p < 10^{-4}$). High levels of PMN-MDSCs were associated with secondary infections ($p = 0.024$) and new sepsis episodes ($p = 0.036$). Interestingly, M-MDSCs were more abundant in survivors than in patients who died within 28-days ($p = 0.028$). Stratification of patients according to M-MDSC levels revealed that high levels of M-MDSC were associated with reduced 90-day mortality (high vs low M-MDSCs: 47% vs 84% mortality, $p = 0.003$, hazard ratio = 3.2). Combining high M-MDSC levels with low APACHE II score improved further patient stratification (high-M-MDSCs/low-APACHE II vs low-M-MDSCs/low-APACHE II: 20% vs 80% 90-day mortality, $p = 0.0096$, hazard ratio = 7.2).

Conclusions: This is the first study to associate high levels of M-MDSCs with improved survival in sepsis patients. Additional investigations will be required to assess whether MDSCs are prognostic and/or theragnostic biomarkers in sepsis.

Introduction

Sepsis is defined as a dysregulated host response to an infection resulting in life-threatening organ dysfunction. Exuberant proinflammatory responses during the early phase of sepsis are implicated in tissue damage, organ dysfunctions and early mortality [1]. A compensatory anti-inflammatory response promotes immunosuppression that can persist for weeks to months [2]. Immunosuppression is associated with adverse effects, and is responsible for more than half of all sepsis deaths. Hence, immunomodulatory therapies in sepsis should target inflammation or immunosuppression depending on patient's status [1, 2]. Theranostics approaches hold promise for monitoring immune status and selecting the most appropriate host-directed immunotherapy to be implemented in a personalized manner.

Myeloid derived suppressor cells (MDSCs) are immunosuppressive immature myeloid cells, rare in blood at homeostasis and expanding in inflammatory conditions. MDSCs are subdivided into polymorphonuclear and monocytic MDSCs (PMN-MDSCs and M-MDSCs), yet additional subtypes have been proposed [3]. MDSCs are mainly studied in the field of cancer, a condition in which these cells are enriched in tumor environment and impair anti-tumor immunity. Clinical trials targeting MDSCs are running to counterbalance tumor-associated immunosuppression in cancer patients [4].

In the field of infection and sepsis, clinical studies have shown an association between high levels of PMN-MDSCs and M-MDSCs in the blood and development of nosocomial infections and mortality [5]. These observations led to the proposal that MDSCs sustain immunosuppression, and could be targeted to reverse immunosuppression in septic patients. However, clinical studies included a limited number of patients [2, 5]. Moreover, MDSCs may have context-dependent impacts depending on disease progression [5]. Hence, we conducted a prospective clinical study in patients with sepsis due to pneumonia, multi-organ failure and high likelihood of poor outcome to characterize MDSCs in severely ill sepsis patients.

Materials and Methods

Between December 2017 and February 2019, 48 adult patients with pneumonia were prospectively recruited from 8 hospitals in Athens and Thessaloniki, Greece (**Table 1**). This was part of the INCLASS study (Benefit of clarithromycin in patients with severe infections through modulation of the immune system study; registered at ClinicalTrials.gov, reference NCT03345992). Inclusion criteria were a Sequential Organ Failure Assessment (SOFA) score ≥ 7, including respiratory failure (PiO_2/FiO_2 < 200), and any other organ system failure with SOFA score ≥ 3. Exclusion criteria were pregnancy, allergy to macrolides, macrolide treatment, corticosteroid intake, neutropenia (< 1000/mm^3), HIV infection (with $CD4^+$ T cells < 200/mm^3), transplantation or neoplasm. EDTA-anticoagulated blood samples were collected at study inclusion and 5 and 10 days later. Patients were followed-up for 28 days, recording all-cause mortality and incidence of secondary infections and new sepsis episodes. A late assessment of mortality at 90 days was performed. The study was conducted in compliance with the declaration of Helsinki, and was approved by the central Ethics committee (52086/2017) and the

National organization for Medicines-EOF (51239/01-06-2017) in Athens, Greece. Written informed consent was obtained from study participants or legal representatives prior to enrollment. Eighteen healthy volunteers served as controls (**Table 1**).

One hundred microliter of blood were added to tubes containing lyophilized antibodies (clone/fluorochrome) directed against CD3 (UCHT1/APC-AF700), CD11b (Bear1/PE-Cy7), CD14 (RMO52/APC-AF750), CD15 (80H5/Pacific Blue), CD16 (3G8/ECD), CD19 (J3-119/APC-AF700), CD33 (D3HL60.251/APC), CD45 (J33/Krome Orange), CD56 (NKH-1/APC-AF700), CD124 (G077F6/PE) and HLA-DR (Immu-357/FITC) (DURAClone, Beckman Coulter, Brea, CA). After 20 minutes, 900 µL of 1 x BD FACS™ lysing solution (BD Biosciences, San Jose, CA) were added. Samples were vortexed, washed and acquired using an Attune NxT Flow Cytometer (Thermo Fisher Scientific, Waltham, MA, USA). Debris, doublets and CD45 negative cells were excluded by manual gating using FlowJo™ (v10.6.2, Ashland, OR). We applied FlowSOM for unsupervised clustering using the biexponential transformed and normalized expression levels of cell surface markers. Metaclusters were set on 30 populations, manually merged into 8 populations based on biological knowledge and marker expression, as represented in tSNE and heatmap plots (**Figure 1A**).

Baseline patient characteristics were compared using chi-square exact test, Mann-Whitney U test, and Kruskal-Wallis test as appropriate. Correlations between cell populations and clinical data were evaluated using the Mann-Whitney U test. M-MDSCs ≤ 4.3% and > 4.3% of leukocytes were considered as low and high percentages, respectively. The cutoff value was based on highest tertile of % M-MDSCs in sepsis patients. APACHE II scores ≤ 20 and > 20 (cutoff values based on median) were considered as low and high. Statistical differences between survival and event curves were assessed with the log-rank test. Statistics and figure design were performed using R v.3.6.0 (R-Foundation for Statistical Computing, Vienna, Austria). p values < 0.05 were considered to be statistically significant.

Results

Forty-eight patients with sepsis due to pneumonia were included in the study, of whom 23 (48%) died within 28 days and 34 (71%) died within 90 days (**Table 1**). A targeted flow cytometry approach (using DURAClone tubes, see Materials and Methods) combined to unsupervised automatic clustering was used to identify PMN-MDSCs and M-MDSCs in blood samples (**Figure 1A**). PMN-MDSCs and M-MDSCs represented 2.1% [0.7-3.1] and 0.96% [0.46-1.5] of leukocytes in healthy individuals (median, [IQR]). In sepsis patients analyzed at study inclusion, PMN-MDSCs and M-MDSCs represented 22% [7.9-43.0] and 3.1% [2.0-4.9] of leukocytes ($p < 10^{-4}$ vs healthy individuals). These percentages remained stable over a 10-day follow-up period, and subsequent analyses were done using levels at study inclusion.

Twenty-six (66.7%) patients developed a secondary infection, among which 23 (47%) were associated with a new sepsis episode. These patients presented higher levels of PMN-MDSCs than patients that did not develop a secondary infection (31% [13-46] vs 11% [7-26]; $p = 0.03$) and new sepsis episode (33%

[14-45] vs 11% [7-26]; $p = 0.04$) (**Figure 1B**). No difference was observed for M-MDSCs. PMN-MDSCs and M-MDSCs were similarly represented in patients with gram-negative ($n = 23$) and gram-positive ($n = 8$) infections.

At study inclusion, sepsis survivors and non-survivors (90-days) had similar SOFA scores ($p = 0.41$) and leukocyte counts ($p = 0.32$), while survivors were younger (57 [47-74] vs 75 [67-86]; $p = 0.0014$) and had a lower APACHE II score (15.5 [14-20.8] vs 23 [18-27]; $p = 0.009$), Charlson comorbidity index (4 [1-5] vs 6 [5-9]; $p = 0.002$) and lactate levels ($p = 0.02$). Survivors and early and late deaths (i.e. ≤ 28 and >28 days) expressed similar levels of PMN-MDSCs (**Figure 1C**). In contrast, survivors expressed 1.64-fold more M-MDSCs (4.6% [2.6-6.7]) than early deaths (2.8% [1.5-3.6], $p = 0.028$), and 1.55-fold more M-MDSCs than late deaths (3.0% [2.3-4.7], $p = 0.19$) (**Figure 1C**).

We then stratified patients according to the expression of M-MDSCs (low and high levels: ≤ 4.3% and > 4.3%) and the APACHE II score (low and high: ≤ 20 and > 20) (see Materials and Methods). Ninety-day mortality was decreased in patients with high levels of M-MDSCs (high vs low MDSCs: 47% vs 84% mortality, $p = 0.003$, hazard ratio = 3.2, 95%CI 1.4-7.2) (**Figure 1D, left**). Combining M-MDSCs and APACHE II score in the analysis increased patient stratification. The 90-day mortality rate was 20% in patients with high M-MDSCs and low APACHE II score, while it was 71-88% in the three other groups (overall comparison: $p = 0.0062$; high M-MDSCs/low APACHE II vs low M-MDSCs/low APACHE II: 20% vs 80%; $p = 0.0096$, hazard ratio = 7.2, 95%CI 1.6-32) (**Figure 1D, right**).

Discussion

To our knowledge, this is the first study reporting that high expression levels of M-MDSCs are associated with improved outcome of sepsis patients.

Experimental investigations and all clinical studies to date suggested that MDSCs are detrimental during sepsis [2, 5-8]. For example, high levels of MDSCs at admission correlated with early mortality of surgical septic shock patients [7], and high levels of MDSCs on days 6-8 correlated with mortality and secondary infections in septic shock patients [8]. In the present cohort, high levels of PMN-MDSCs were associated with the occurrence of secondary infections and new sepsis episodes. These data confirmed that MDSCs may drive negative effects in sepsis patients.

However, MDSCs help fighting infections through phagocytosis and killing of microorganisms. MDSCs may also dampen systemic or local inflammation induced by molecular patterns of pathogen or endogenous origin, the latter being released upon stress or during tissue injury. Accordingly, MDSCs harvested from septic mice protected recipient mice from acute infections [5]. Remarkably, in patients with multi-organ failure and high likelihood of mortality, highest levels of M-MDSCs were associated with reduced 90-day mortality independently from the APACHE II score. Considering that M-MDSCs are more potent immunosuppressive cells than PMN-MDSCs on a per cell basis [5], increased M-MDSCs might drive beneficial effects through dampening inflammation-induced organ dysfunction in

severely ill sepsis patients. A limitation of our study is the characterization of MDSCs by phenotypic and not functional analyses, and the absence of immunological correlates. Yet, several studies reported the immunosuppressive function of MDSCs based on their phenotype [6, 9, 10].

To conclude, this represents the first report of an association between high levels of M-MDSCs and improved outcome of sepsis patients. It should incite additional studies to appreciate the role of MDSCs especially in patients with severe sepsis and multi-organ failure.

Author contributions statement

ITS, EK, EJGB, TC and TR conceptualized and designed the study. ITS, PB, AH and TR developed custom-made DURAClone tubes. EK and EJGB designed the clinical study and organized blood sampling. ITS and CT analyzed the samples. All the authors interpreted the data. ITS and TR wrote the manuscript. All the authors revised the manuscript.

Conflict of Interest Statement

The authors do not have any conflict of interest regarding this manuscript.

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Table 1. Patient characteristics

Characteristic	Healthy controls	Survivors (90 days)	Non-survivors (90 days)	*p**
Number of patients	18	14	34	
Gender, male	15 (83%)	11 (79%)	24 (71%)	
Age (years)	53 [25-58]	57 [47-74]	75 [67-86]	0.0014
Type of infection:	-			
VAP/HAP	-	11 (79%)	22 (65%)	0.35
HCAP	-	3 (21%)	12 (35%)	
Severity of illness at admission:				
APACHE II score	-	16 [14-21]	23 [18-27]	0.009
SOFA score	-	10 [8.8-11]	10 [9-12]	0.41
Secondary infections	-	9 (64%)	17 (50%)	0.36
New sepsis episode	-	7 (50%)	16 (47%)	0.85
Charlson Comorbidity Index	-	4 [1-5]	6 [5-9]	0.002
Length of hospital stay	-	36 [24-48]	14 [8-28]	0.003
Length of ICU stay	-	26 [13-37]	15 [8-28]	0.16
Leukocytes (x 10^9/L)	-	13.9 [7.5-16.0]	13.8 [10.6-20.4]	0.32
M-MDSCs (% of leukocytes)	0.96 [0.46-1.5]	4.6 [2.6-6.5]	2.9 [1.8-4.1]	0.052
PMN-MDSCs (% of leukocytes)	2.1 [0.74-3.1]	22 [6-44]	22 [8-37]	0.96
Lactate (mmol/L)	-	1.4 [0.85-2.2]	2.8 [1.6-2.8]	0.02

Data are medians [IQR] or *n* (%). VAP: ventilator associated pneumonia, HAP: hospital acquired pneumonia, HCAP: healthcare-associated pneumonia, M-MDSCs: monocytic-myeloid-derived suppressor cells, PMN-MDSCs: polymorphonuclear-MDSCs. **p* values comparing survivors and non-survivors at 90 days.

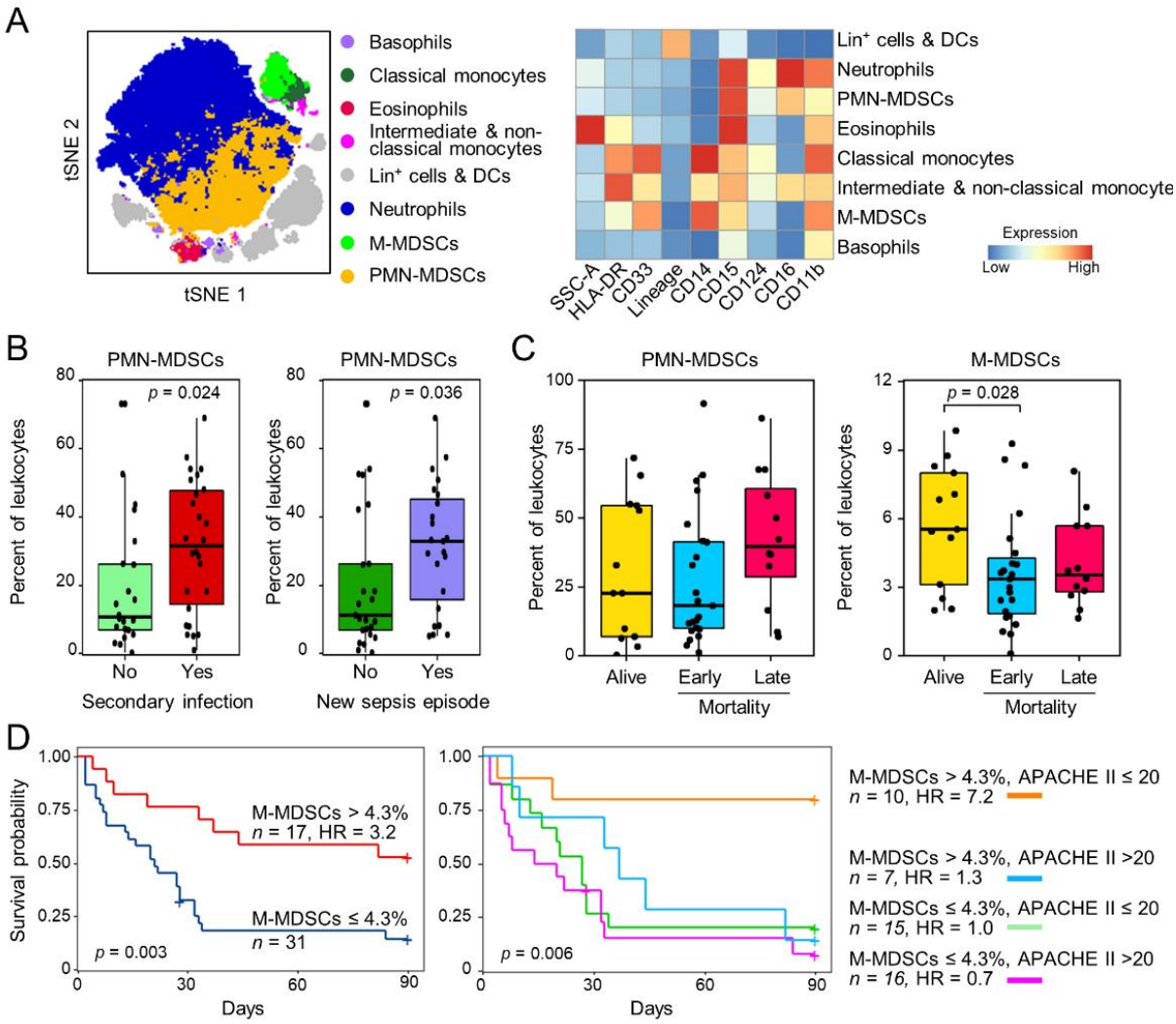

Figure 1: MDSCs in sepsis patients with MODS. Blood collected at study inclusion was analyzed by flow cytometry and unsupervised clustering (see Materials and Methods). **A**) t-SNE plots of leukocyte populations and relative SSC-A and expression levels of surface markers. LIN: lineage (CD3, CD19, CD56), DCs: dendritic cells. **B**) PMN-MDSCs in relation with secondary infection and new sepsis episode. **C**) PMN-MDCs and M-MDSCs in survivors (alive, *n* = 14), early deaths (≤ 28 days, *n* = 23) and late deaths (> 28 days, *n* = 12). Boxplots show median, upper and lower quartiles. Whiskers show 5 to 95 percentiles. Each dot represents an individual sample. **D**) Kaplan Meier 90-day survival curve based on M-MDSC levels and APACHE II score. Statistical differences were assessed using the log-rank test.

4. Discussion and perspectives

The goal of this thesis was to elucidate the behaviour of MDSCs during sepsis because we were interested in the diagnostic, prognostic, and theragnostic potential of MDSCs. We utilized four cohorts: 1) healthy volunteers infused with endotoxin as a model of early sepsis (Endotoxin study); 2) non-infectious critical-ill patients without antibiotics who were admitted to the intensive care unit (PIPOVAP study), with the hypothesis that patients with high levels of MDSCs would contract more nosocomial infections; 3) patients hospitalized with COVID-19 (LUH-COVID study), as the COVID-19 pandemic presented the unique opportunity to study sepsis and non-sepsis hospitalized patients infected with SARS-COV-2; and 4) sepsis patients with MODS (INCLASS study) (**Figure 11**).

Few publications have focused on the role of MDSCs during sepsis. Furthermore, results from individual studies are difficult to compare due to differences between methodologies and markers used to identify MDSCs, illustrated by the huge variation of MDSC levels measured in healthy subjects (**Result section 2.1**). To limit this problem, we applied a uniform protocol across the Endotoxin, PIPOVAP, LUH-COVID-19, and INCLASS studies. We showed that MDSCs closely correspond to inflammation and were associated with the development of secondary infections and mortality. Interestingly, and following our overall hypothesis, our data suggest that MDSCs can have either a beneficial or a detrimental role on the patient's outcome, depending on their state of inflammation (an overall summary of the results can be found in **Figure 11**).

The dynamic profile of blood MDSCs

Endotoxin infusion is a model used to mimic the early events of Gram-negative sepsis, which results in clinical symptoms and metabolic responses similar to sepsis. However, endotoxin given as a bolus infusion is cleared by the liver within a few minutes. Cytokines and chemokines IL-6, IL-1β, IL-8, and TNF levels peak in blood 2 hours after endotoxin infusion and return to baseline levels 6 hours later [285, 286]. This allowed us to assess the longevity of MDSCs but contrasts with the more progressive development of sepsis, which is characterised by prolonged exposure to endotoxin and the rise of cytokines and chemokines. We observed that PMN-MDSCs levels increased quickly and M-MDSCs levels increased more gradually after endotoxin infusion. After 24 hours, the percentage and absolute count of MDSCs returned to baseline levels indicating that both types of MDSCs respond quickly to inflammatory mediators, as well as also disappear quickly (**Figure 11, Result section 2.2**).

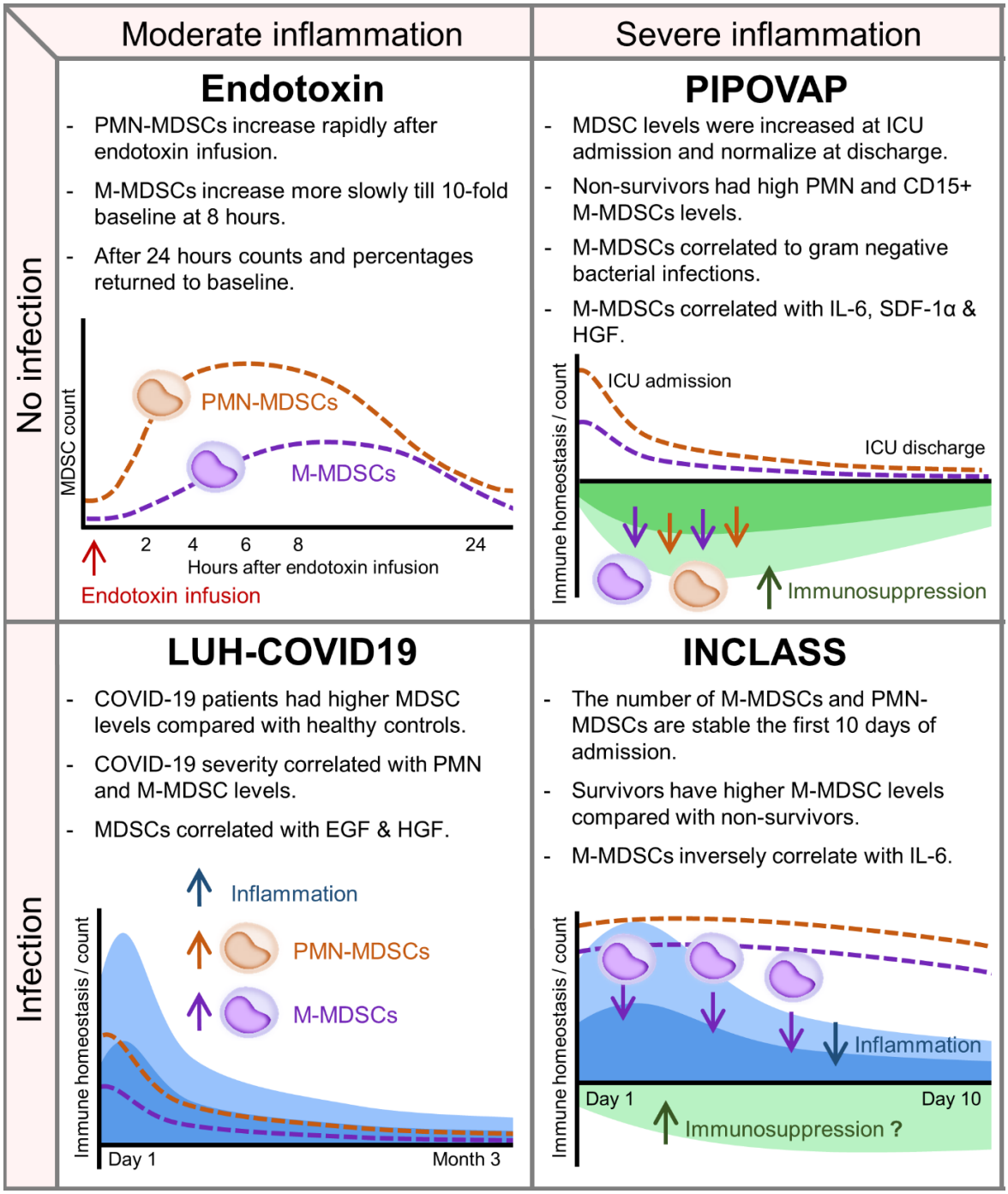

Figure 11. Summary of results: the behaviour and effect of MDSCs in four different cohorts. Dotted lines represent changes of PMN-MDSCs (orange) and M-MDSCs (purple) over time. Blue areas represent inflammation. Green areas represent immunosuppression. The x-axis represents time, with the origin starting at time of inclusion to the study. PIPOVAP: Profile, Interaction, and PrOgnosis in Ventilator Associated Pneumonia; LUH: Lausanne University Hospital; COVID-19: Coronavirus Disease 2019; INCLASS: Benefit of Clarithromycin in Patients With Severe Infections Through Modulation of the Immune System; PMN-MDSCs: Polymorphonuclear MDSCs; M-MDSCs: monocytic-MDSCs; ICU: Intensive Care Unit; EGF: Epidermal growth factor; HGF: Hepatic growth factor; IL: Interleukin.

In the blood, human granulocytes and monocytes have lifespans is 2-5 days and 1-2 days, respectively. These lifespans increase when exposed to endotoxin and other DAMPs [287-289]. This caused us to question the fate of MDSCs during endotoxemia. The rapid reduction of MDSCs after an initial increase post-endotoxin infusion may be due to a short half-life of these cells. For example, PMN-MDSCs in cancer patients showed a shorter life span compared with their mature counterparts [290]. Alternatively, the decrease in cytokines and growth factors during the resolution of inflammation might allow MDSCs to differentiate into mature myeloid cells such as neutrophils, DCs, and macrophages, therefore reducing the proportion of blood MDSCs. This is supported by the fact that MDSCs began differentiating into mature cell populations after the blockage of TNF with the TNF antagonist Entanercept [291]. Finally, MDSCs may have migrated into other tissues such as the spleen, lymph nodes, and lungs, which has been reported to occur in mouse models of for endotoxemia and sepsis [292].

The dynamic profile of MDSCs in human endotoxemia showed that MDSCs are quick to rise and then to dissipate. However, this may not be the case in critically ill patients, where inflammatory mediators that impact the fate of MDSCs tend to persist. We therefore hypothesized that prolonged elevation of MDSCs could induce and sustain long-lasting immunosuppression in sepsis patients [97]. To challenge this hypothesis, we measured MDSCs in the PIPOVAP, COVID-19, and INCLASS studies. We observed that MDSCs levels increased in critically ill patients. MDSCs returned to baseline levels in critically ill patients discharged from the ICU (between 2 and 22 days), and after 3 months in COVID-19 patients (**Result section 2.2 and 2.3**). Yet 3 months after the initial COVID-19 infection, monocytes and DCs showed reduced cytokine production after TLR stimulation (**Result section 2.3**). This suggests that mechanisms besides those involving MDSCs, affect innate immune responses in the long-term, for instance, through the exhaustion of monocytes and DCs. Interestingly, in patients with MODS (**Result section 2.4**), we did not observe a decrease of MDSCs over the first 10 days, and patients with high levels of PMN-MDSCs had a higher risk of secondary infections and sepsis occurrence. This may indicate that a long-lasting immunosuppressive response sustained by MDSCs occurs during bacterial sepsis. This is supported by the presence of immunosuppressive MDSCs for 21 days in sepsis patients compared to control patients [238]. Alternatively, as hypothesized for endotoxemia, MDSCs might migrate from the bloodstream to tissues where they can sustain an immunosuppressive state [82, 293, 294]. Future studies should follow up on the levels and functionality of MDSCs in sepsis patients with MODS for months, or even years.

MDSCs as a biomarker and therapeutic target

One of the current challenges in the diagnostics of sepsis is the differentiation between gram-positive and gram-negative infections. In current practice, Gram staining and blood cultures have limitations; gram staining provides diagnostic results in 30-40% of patients, and blood cultures can take days before turning positive [295, 296]. M-MDSCs have been reported to increase to a higher degree in gram-negative sepsis, as opposed to gram-positive sepsis [263, 266]. Accordingly, we observed that the levels of M-MDSCs at ICU inclusion correlated with subsequent gram-negative, but not gram-positive, nosocomial infections (**Results 2.2**). Monocytic HLA-DR expression paralleled the counts of M-

MDSCs and currently represents one of the most advanced biomarkers in the sepsis field [267]. The development of an automated bedside flow cytometer for monocytic HLA-DR determination could provide a fast and practical way to assess the status of sepsis patients [297]. In surgical sepsis patients, lower monocytic HLA-DR expression was associated with gram-negative infection [87]. Yet, another study showed that monocytic HLA-DR expression was lower in sepsis patients infected with *S. pneumoniae* and *S. aureus* compared with gram-negative pathogens [298]. However, *S. pneumoniae* and *S. aureus*-infected patients were more severely ill, which may have affected HLA-DR expression and biased conclusions of the study. Indeed, a recent study based on similar outcomes did not show differences between pathogens and monocytic HLA-DR expression [88]. Despite conflicting observations, it appears that M-MDSCs counts or monocytic HLA-DR expression have potential as diagnostic biomarkers for gram-positive and gram-negative sepsis guiding antibiotic therapy. As described in the introduction, whole blood transcriptome profiles have been used to discriminate sterile inflammation from sepsis, viral from fungal and bacterial infections and to correlate with outcome in infected patients [177-180]. Combining different innovative approaches will certainly benefit patient stratification.

Predicting mortality at admission to the ICU can aid clinicians to manage patients, such as by initiating aggressive therapy in the most severe cases, yet robust mortality scores are currently lacking [154]. MDSCs were associated with mortality (**Result section 2.2** and **2.4**) and are frequently considered as promising therapeutic targets in cancer patients [274]. In addition, we hypothesized that MDSCs may have predictive values in critically ill patients. In non-infectious critically ill patients, we observed that M-MDSCs and IL-6 levels were associated, and that high MDSCs levels correlated with mortality rates (**Result section 2.2**). However, in sepsis patients with MODS (INCLASS study), high levels of M-MDSCs were associated with lower mortality rates (**Result section 2.4**) and inversely correlated with IL-6 levels in non-survivors (**Figure 12**). IL-6 is an important regulator of MDSCs accumulation and activation [299]. The contrasting MDSCs/IL-6 correlations in PIPOVAP and INCLASS studies most likely reflect differences in patient inclusion. Non-infectious critical care patients were studied at ICU admission. In these patients, high IL-6 levels potentially stimulated the generation of MDSCs reaching the bloodstream. In contrast, sepsis patients with MODS were on a dynamic immunosuppressive path, downregulating IL-6 levels. It is possible that combining IL-6 and MDSCs will help identify patients with worse predicted outcomes, particularly since IL-6 is an often-mentioned predictive biomarker for mortality [300, 301].

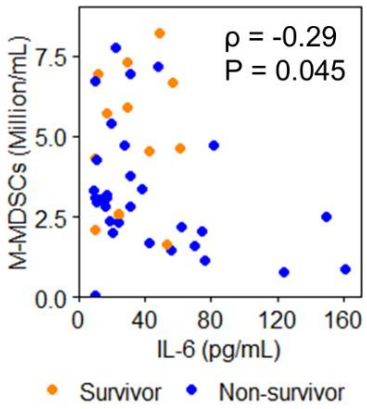

Figure 12. Correlation between IL-6 and M-MDSCs in peripheral blood of sepsis patients with MODS.

Targeting MDSCs to treat malignant tumours has advanced from the preclinical test phase but they are not yet used in clinical practice. There are multiple ways to target MDSCs (**Figure 10**), yet it is not clear which therapy is most beneficial in sepsis. Preclinical murine studies showed that ATRA (all-trans retinoic acid, which induces the differentiation of MDSCs into mature cells), LDK378 (an inhibitor of anaplastic lymphoma kinase-ALK), and YCP (an α-glucan, purified from the marine fungus *Phoma herbarum*, which has displayed antitumor activity) decreased MDSC levels and improved survival rates in sepsis models [264, 279-282] (**Introduction 1.1.10**). We observed that PMN-MDSCs and M-MDSCs correlated with blood concentrations of hepatocyte growth factor (HGF) in non-infectious critically ill patients and COVID-19 patients (PIPOVAP: ρ = 0.08, ρ = 0.38, COVID-19: ρ = 0.42, ρ = 0.46). HGF acts as a pleiotropic cytokine that can stimulate cell motility, morphogenesis and limit endothelial injury in sepsis [302-304]. In tumorous mice, HGF upregulated the expansion of MDSCs [305, 306]. Targeting HGF to limit MDSCs might have counterproductive effects such as increasing endothelial injury. However, HGF treatment in endotoxemic mice protected them from multiple organ injuries and death [307]. Therefore, an active-formed HGF might be the ideal treatment option for limiting inflammation severe sepsis patients.

Caveats prior to clinical implication targeting MDSCs, and ongoing studies

We have shown that the levels of MDSCs increased rapidly during endotoxemia, that they were associated with severity, secondary infections, and mortality in critically ill patients. Therefore, MDSCs might represent potential biomarkers in critically ill patients. However, before MDSC sepsis research can make the transition from “bench to bedside”, two caveats need to be resolved. First, a standardized MDSCs identification protocol needs to be defined. Second, understanding the functional plasticity of MDSCs in acute diseases like sepsis is required. Currently, the differentiation between PMN-MDSCs from mature neutrophils, as well as M-MDSCs from mature monocytes, is based on the expression of cell surface markers that are gradually expressed according to cell maturation [98]. This generates gating bias and makes it difficult to compare results between studies. A cell surface marker or combination of surface markers unique for MDSCs is imperative to be able to standardize research and, more importantly, to develop future biomarkers for use in clinical care. For example, in patients with cancer, LOX-1 presents on the surface of PMN-MDSCs. Other markers, including CD1d, CD13 and

CD39, have been described for MDSCs [308-311]. Recently, LOX-1 PMN-MDSCs have also been described in sepsis shock patients, where they peaked one week after ICU admission [312]. To illuminate the expression of these and other markers on MDSCs in sepsis patients, we constructed a mass cytometry panel of 39 surface markers of which 25 have been described specifically to aid identifying MDSCs (**Table 9**). This approach has been used in the INCLASS and PIPOVAP study (*data not yet analysed*) giving us the opportunity to better delineate surface markers that are exclusive to MDSCs in sepsis, that point towards discrete MDSCs subpopulations, and to correlate these markers with other immune cell populations.

Table 9. Mass cytometry panel targeted on MDSCs

Antibody	Ions	Antibody	Ions	Antibody	Ions
Caspase 3	142Nd	**CD32**	152Eu	CD86	146Nd
CD1c	143Nd	**CD33**	197AU	**CD115**	174Yb
CD1d	154Sm	CD34	156Gd	**CD123 (IL3r)**	162Dy
CD3	148Sm	CD38	194Pt	**CD135**	158Gd
CD4	144Nd	**CD39**	173Yb	**CD192 (CCR2)**	165Ho
CD7	169Tm	**CD45**	089Y	**CD195 (CCR5)**	153Eu
CD8	145Nd	CD45RA	166Er	**CD197 (CCR7)**	150Nd
CD11b	141Pr	**CD48**	168Er	**CD274 (PD-L1)**	171Yb
CD11c	163Dy	CD56	159Tb	CD279 (PD1)	164Dy
CD13	198Pt	**CD62L**	172Yb	**HLA-DR**	170Er
CD14	160Gd	CD64	175Lu	**LOX1**	167Er
CD16	209Bi	**CD66b**	149Sm	**slan**	151Eu
CD20	147Sm	**CD74**	176Yb		
CD27	155GD	**CD80**	161Dy		

Bold and underlined surface markers have been described to identify (subpopulations of) MDSCs.

The high plasticity of MDSCs increases the complexity of MDSCs definition. It has been proposed that MDSCs can mature and lose their suppressive function, differentiate into osteoclasts, tolerogenic dendritic cells, and tumour-associated macrophages, or can shift between MDSC-subtypes [313, 314]. Most research (including ours) portrays two separate entities of MDSCs, but it is more likely that MDSCs shift between a range of subtypes with more or less suppressive states (**Figure 12**) [313]. This is illustrated by a murine CLP-induced sepsis study that showed increased levels of IL-10, IL-6, ARG1 and less TNF in MDSCs collected 12 days after CLP, compared with MDSCs collected after 3 days [248]. This change in cytokine expression during CLP pathogenesis, shows that MDSCs shift towards a more immunosuppressive state [248]. Furthermore, studies have shown that the adoptive transfer of CLP-induced MDSCs in CLP-subjected mice can either protect against or exacerbate mortality depending on the moment at which MDSCs have been harvested from sick mice. This suggests that transferred MDSCs differentiate to become more immunosuppressive cells [241, 246, 248].

Our results support the assumption that MDSCs might likewise shift functionality, depending on disease development. We observed different behaviours of MDSCs in sepsis patients with MODS, compared with non-infectious critically ill patients (**Result section 2.4** *versus* **2.2**). Furthermore, we are in the process of better defining phenotypical and immune functional differences over time in the endotoxin, PIPOVAP, and INCLASS studies. In addition to the mass cytometry studies, we analysed the

response of whole blood to stimulation with microbial ligands by flow cytometry. We observed that in the Endotoxin study, in comparison with mature neutrophils, PMN-MDSCs expressed less IL-6, ARG1, and MPO levels at baseline and did not respond to stimulation. These PMN-MDSCs are most likely very immature cells that will become more actively immunosuppressive over time (see preliminary **Supplementary results 6.1**: "Neutrophil granulocyte and polymorphonuclear myeloid-derived suppressor cell tolerance in human endotoxemia"). This aligns with observations obtained in mouse studies and as illustrated in **Figure 13** [246, 248]. Our ongoing functional studies in the PIPOVAP and INCLASS studies will provide more insights into the plasticity of MDSCs in sepsis and critically ill patients.

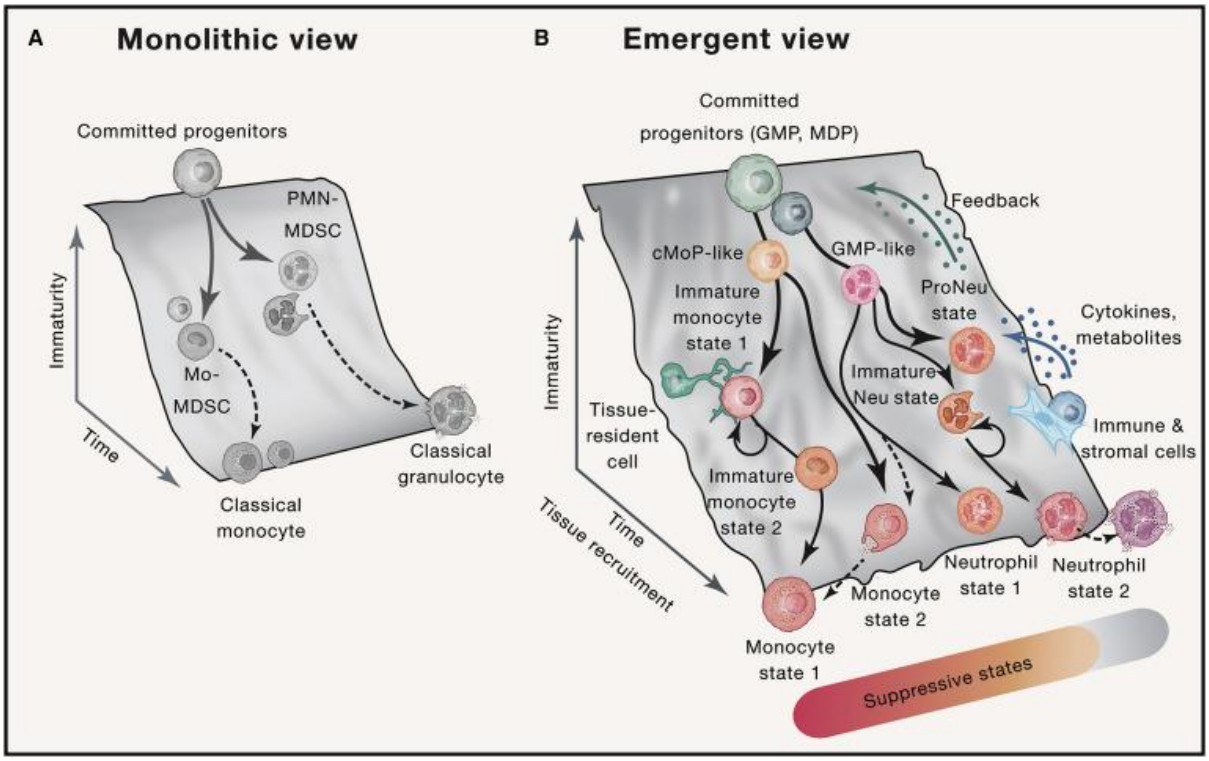

Figure 13. Myeloid suppressive states are highly heterogeneous and context dependent. A) The dominant view of MDSC phenomena defines two major subtypes of suppressive cells (M-MDSC and PMN-MDSC) with ontological and functional differences. **B**) The emergent view of MDSCs as a facet of emergency haematopoiesis captures the heterogeneity and plasticity of these myeloid cell states in a more "ruffled" ontogenetic landscape. Abbreviations: GMP, granulocyte-monocyte progenitor; MDP, monocyte-dendritic cell progenitor; cMoP, common monocyte progenitor; proNeu, neutrophil progenitor. Reprinted by permission from: Elsevier, "Immunity" [313].

The main limitation of our studies, as in most studies in the field, is the lack of immunosuppressive functional conformation of the MDSCs, which is the "gold standard" in the field. However, all studies published to date have shown that cells with a similar phenotype have immunosuppressive properties in patients with cancer, COVID-19, and sepsis [236, 263, 266, 315, 316]. The in- and exclusion criteria of our studies were very strict, as we aimed to include specific subpopulations: a relatively homogeneous sepsis population (INCLASS study) and, to include critically ill patients without antibiotics, to diminish

antibiotic influence on secondary infections (PIPOVAP study). The advantage of this approach is that we collected distinct cohorts, which allowed us to observe a unique, often hypothesized consequence of MDSCs levels, which could have been overlooked in larger but less selected patient populations. However, by adhering to these strict criteria this also resulted in smaller cohorts, which could be considered a limitation of these studies.

Conclusions and perspectives

Evolutionarily, MDSCs may have developed to protect the host from overwhelming inflammation, in particular during pregnancy and the early neonatal period. [317]. In pregnant women, MDSCs accumulate in the circulation during early gestation and remain elevated until they return to baseline levels several few days postpartum [318, 319]. Furthermore, maternal PMN-MDSCs are highly present in the placenta, where they play a major role in maternal-foetal tolerance by suppressing T cells and NK cells that respond to the foetus [320, 321]. This is exemplified by the increased risk of spontaneous abortion in pregnant women with low levels of functional MDSCs. In addition, women with high MDSC levels have an increased chance of successful *in vitro* fertilisation [322-324]. Neonates also present with increased levels of MDSCs during their first weeks and months of life [321, 325, 326]. MDSCs might protect neonates from overwhelming inflammation when exposed to commensal bacteria, and, as MDSCs are highly bactericidal, from neonatal infections [327]. This is signified by the inverse correlation of MDSCs with the development of necrotizing enterocolitis in prematurely born infants [328, 329].

Here, we report a dual role of MDSCs in critically ill patients depending on inflammation levels (**Figure 11**). The dual role becomes more apparent when comparing between different diseases. In cancer patients - where immunosuppression drives tumour growth and spread, higher levels of MDSCs correlate with metastasis risk, resistance to therapy, and higher mortality [330, 331]. In transplantation patients – where immunosuppression is desirable, higher levels of MDSCs corresponded to less graft-versus-host disease, less antibody-mediated rejection, and longer graft survival [332]. In autoimmune diseases, the relation between MDSCs and outcome is more nuanced. Generally, more severe autoimmune diseases have higher levels of MDSCs, but this is most likely a compensatory response to limit inflammation [98]. Overall, immunosuppressive MDSCs might be beneficial or detrimental depending on inflammation and pathogenesis.

Research in sepsis and other fields reported promising observations for the use of MDSCs as a biomarker and targeted therapy (**Text box 1**). However, several challenges must be overcome before MDSCs can be used in clinical practice. In particular, we need specific standardized phenotypic protocols for MDSCs, specifically for PMN-MDSCs. In the meantime, monocytic HLA-DR expression can be used as a proxy for M-MDSCs levels, but more research is needed to confirm this. Single-cell RNA sequencing (scRNA-seq) and single-cell multi-omics analyses could lead to the discovery of new MDSC surface markers and possibly uncover new MDSC subtypes.

In cancer patients, a fairly unique transcriptomic profile for PMN-MDSCs has been obtained based on increased gene expression associated with cell cycle and autophagy, and decreased gene expression associated with NF-κB signalling. Moreover, transcriptomics guided the discovery of a novel surface marker e.g., LOX-1 to identify MDSC Unfortunately, the transcriptomic profiles of MDSCs in cancer patients may differ from those of MDSCs in sepsis patients. Indeed, a pilot scRNA-seq study in two sepsis patients reported that MDSCs from sepsis patients differ from MDSCs from cancer patients, even though cells from both groups were phenotypically similar. For example, the expression of Arg1 and PD-L1 was low on MDSCs found in sepsis patients when compared to MDSCs found in cancer patients [238]. Interestingly, we observed using flow cytometry that there was a similar low Arg1 expression in PMN-MDSCs from patients infused with endotoxin (**Supplementary result 6.1**). Two recent multi-omic studies in COVID-19 patients illuminated the diversity of myeloid cells [333, 334]. A subgroup of immunosuppressive neutrophils similar to PMN-MDSCs presented high expression of *PD-L1* and *ZC3H12A* (an MCP1 induced protein acting as an inflammatory regulator) [334]. However, this was not repeated in a second study [333], which reported high expression levels of *CEACAM8* (CD66b) and *DEFA3* (human alpha defensin 3) in a subgroup of immunosuppressive neutrophils reminiscent of immature neutrophils [333]. These results illustrate that a multi-omics approach can aid the discovery of unique MDSCs profiles in sepsis.

To conclude, many hurdles exist to assess MDSCs in sepsis. MDSCs lack a clear phenotypic definition and there is a lack of knowledge of their functionality and plasticity in sepsis. Sepsis, as mentioned in the introduction, is a heterogeneous syndrome without clearly defined phenotypes, and lacks long-term follow-up. MDSCs in sepsis research shows promising results and the advances in high-dimensional research could help unravel the heterogeneity in MDSCs and sepsis. Following this, we suggest two short-term (points 1 and 2) and two long-term (points 3 and 4) future lines of studies:

1) To assess the cellular landscape in critically ill patients to aid the characterization of MDSCs in sepsis (performed in INCLASS and PIPOVAP study) employing high-dimensional phenotyping.
2) To provide insights into the functionality and plasticity of MDSCs in critically ill patients (performed in INCLASS and PIPOVAP study) in whole blood stimulated cells assessed by multi-colour flow cytometry.
3) To exhaustively characterize myeloid cells in sepsis patients by integrating multi-omics data, for example by combining mass cytometry, scRNA-seq, single-cell metabolomics (using mass-cytometry-based metabolome profiling), and functionality testing.
4) To identify patients that could profit from targeting MDSCs by advancing sepsis phenotyping studies in relation to MDSCs levels (as extensively discussed in **Introduction 1.1.4**).

Overall, this thesis explored the modulation of MDSCs in sepsis and supports the use of MDSCs as biomarkers in sepsis. Improving the understanding of sepsis and the role of MDSCs in sepsis could aid the identification of patients who would benefit from MDSCs-targeted therapy; particularly as such therapies are advanced in cancer patients. This suggests that MDSCs may play a leading role in future clinical (post-) sepsis care, both as a biomarker and as a target for therapy.

Textbox 1. Potential applications for MDSCs in sepsis

MDSCs as biomarkers

- Diagnostic**:**
  - PMN-MDSCs as markers for severity of inflammation.
  - M-MDSCs as markers for gram-negative bacterial infections.
- Prognostic:
  - Predictor of outcome, specifically secondary infections and mortality.
- Theragnostic:
  - Potentially, MDSC in combination with IL-6 for MDSCs targeted therapy.
  - Guiding antibiotic therapy (orientating towards gram-positivity/negativity).

MDSCs as targets for therapy

- Limiting inflammation:
  - By expanding MDSCs in sepsis patients with MODS, potentially by using an active-formed HGF.
- Reversing immunosuppression:
  - Prevention of long-term infections and mortality in sepsis-induced immunosuppressed patients with high levels of MDSCs by treatment with e.g., ATRA, LDK378 and YCP.

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6. Supplementary files

6.1 “Preliminary results: Neutrophil granulocyte and polymorphonuclear myeloid-derived suppressor cell tolerance in human endotoxemia.”
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6.2 “High-dimensional mass cytometry reveals persistent HCV-mediated immune dysfunction in patients successfully treated with direct-acting antiviral agents.”
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6.1. Preliminary results: Neutrophil granulocyte and polymorphonuclear myeloid-derived suppressor cell tolerance in human endotoxemia.

Preliminary results.

Summary:

Polymorphonuclear neutrophils (PMNs) are the most abundant leukocytes in human blood. PMNs are professional phagocytic cells that play a critical in resolving bacterial and fungal infections. Upon infection, inflammatory mediators (cytokines, growth factors) and microbial products such as endotoxin present in the blood stimulate the pool of granulocytes residing in the bone marrow to migrate into the blood and the production of newly generated granulocytes to restore homeostatic steady state. This orchestrated process is called emergency granulopoiesis. However, the striking neutrophilia in sepsis patients does not protect from infections, indicating that these might be fully. This study aimed to profile cell populations, and especially neutrophils, during human endotoxemia. We infused healthy subjects twice at one week apart with endotoxin and analysed whole blood by mass cytometry (CyTOF) on 15 different time points. One hour after endotoxin infusion neutrophils were 0.5 fold lower compared to baseline, but after 4 hours they increased reaching up to 3.5 times baseline. This was mainly due to the increase of CD11c low, CD32 low granulocytes, a population resembled PMN-MDSCs, which was barely present at baseline. After the second endotoxin infusion, this population only increased by 15%. We assessed neutrophil function by flow cytometry focussing on MDSC-functionality by stimulating whole blood for 4 hours with different Toll-like receptor ligands. We observed that granulocytes drawn 4 hours after endotoxin infusion, had a reduced IL-6 response after stimulation, which is not yet normalized 24 hours after endotoxin infusion, but return to baseline levels after 7 days. Comparing PMN-MDSCs and mature neutrophils, we observe that the former show less baseline expression of IL-6, are non-responsive to stimulation and, contrary to our expectation, do not produce more ARG1 and MPO. With this study, we provide insight into the effect of endotoxin and endotoxin tolerance on granulocytic neutrophils. Our results suggest that neutrophils might be interesting targets and biomarkers for therapy in sepsis.

My contribution to this work:

I participated into the design of the endotoxin study and the drafting of the protocol. I performed experiments, analysed the data, and drafted the preliminary results.

Preliminary results: Polymorphonuclear neutrophils (PMNs), PMN-myeloid-derived suppressor cells (PMN-MDSCs) and tolerance in experimental human endotoxemia.

Background

Polymorphonuclear neutrophils (PMNs) are the most abundant leukocytes in human blood. PMNs are professional phagocytic cells that play a critical in resolving bacterial and fungal infections [1]. Upon infection, inflammatory mediators (cytokines, growth factors) and microbial products such as endotoxin present in the blood stimulate the pool of granulocytes residing in the bone marrow to migrate into the blood and the production of newly generated granulocytes to restore homeostatic steady state. This orchestrated process is called emergency granulopoiesis [1-4]. However, the striking neutrophilia in sepsis patients does not protect from infections, suggesting that newly generated granulocytes might not be fully functional [5]. PMN-myeloid-derived suppressor cells (PMN-MDSCs) are a neutrophil subtype triggered by emergency granulopoiesis notorious for their immunosuppressive function. PMN-MDSCs are immature PMNs phenotypically characterized as $CD11b^{+}$ $CD14^{-}$ $CD15^{+}$/$CD66b^{+}$ and $CD16^{low}$ $CD33^{low}$ when compared with their mature counterparts [3, 6]. Others and we have shown that PMN-MDSCs rise in patients with sepsis (**Result sections 2.2, 2.3 and 2.4**) [3, 5].

The model of endotoxin (LPS) infusion in healthy subjects mimics early events of host response to gram-negative bacteria infection. LPS induces tolerance, defined as a refractory state of response to LPS of innate immune cells previously exposed to minute amounts of LPS. Tolerance has mainly been studied for monocytes/macrophages [7-10]. Tolerance impacts not only on cell to LPS, but also on cell response to other microbial stimuli, what is commonly termed cross-tolerance. Clinically, tolerance may affect monocytes/macrophages in sepsis patients, contributing to immunosuppression and increasing susceptibility to nosocomial infections.

Our aim was to profile cell populations, and especially neutrophils, during human endotoxemia. We infused healthy subjects twice at one week apart with endotoxin and analysed whole blood cells by mass cytometry (CyTOF). Since we detected major modifications in a cell population resembling PMN-MDSCs, we subsequently assessed the function of neutrophilic cells by flow cytometry, focussing on MDSCs.

Materials and methods

Study design.

Seven healthy male volunteers were enrolled at the Radboud University Medical Center, the Netherlands. Volunteers were infused twice at 1 week apart with a bolus of 2 ng/kg endotoxin (from *Escherichia coli* O:113, Lot #94332B1, National Institutes of Health, Bethesda, MD) following a standardized protocol [11]. Blood was drawn in K-EDTA tubes 0, 1, 2, 3, 4, 6, 8 and 24 hours after the first infusion of endotoxin, and 1, 2, 3, 4, 6, 8 hours after the second infusion of endotoxin. This study was approved by the local ethics review board (CMO Arnhem-Nijmegen; reference no. 2017-3607) and was conducted in compliance with the declaration of Helsinki (Forteleza, 2013); International Conference on Harmonisation Good Clinical Practice guidelines, and the rulings of the Dutch Medical Research Involving Human Subjects Act. Written informed consent was obtained from study participants.

Sample processing.

One mL blood was incubated for 10 minutes at 20°C with 1.4 mL of Smart Tube Proteomic Stabilizer (Smart Tube Inc.), and frozen at -80°C. Additionally, 1 mL blood collected 0, 4, 24, and 168 hours after endotoxin infusion was incubated for 4 hours at 37°C with or without 10 ng/mL *Escherichia coli* O55:B5 ultrapure lipopolysaccharide (LPS), 1 mg/mL $Pam_3CysSerLys_4$ (Pam_3CSK_4), 1 μM R848. Brefeldin A (5 μg/mL, Invitrogen, Carlsbad, CA) was added during the incubation. After 4 hours, samples were treated with Smart Tube Proteomic Stabilizer as mentioned above.

For mass cytometry analyses, samples were thawed for 6 minutes at 10°C, and incubated twice for 5 minutes at 20°C with 25 mL Lyse Buffer (Smart Tube Inc.). Samples were resuspended in 1 mL inactivated FCS and filtered through a 85-μm filter (Sefar, Nitex). Non-stimulated samples were counted and aliquoted per 2.5 million cells in tubes and washed three times in ice-cold PBS. Samples were barcoded with palladium and indium labelled anti-CD45 monoclonal antibodies (mAbs) (**supplementary Table 1**) in ice-cold PBS containing 0.02% saponin [12, 13]. Barcoded samples were incubated for 15 min at 4°C, washed with cell staining medium (CSM: PBS, 0.5% BSA, 0.02% sodium azide) and pooled. Pooled samples were incubated for 30 min at 20°C with 20 μL Fc-block (Beriglobin® diluted 500 ug/mL) and mAbs directed against CCR2, CCR5, CD1c, CD1d, CD3, CD4, CD7, CD8, CD11b, CD11c, CD14, CD16, CD20, CD26, CD32, CD33, CD36, CD38, CD45, CD45RA, CD56, CD62L, CD64, CD66b, CD74, CD80, CD86, CD123, CD141, CD223, CX3CR1, HLA-DR, PD1, PD-L1, Slan (**supplementary Table 1**) in 100 μL CSM, washed with CSM, and incubated for 30 min at 20°C with streptavidin-gold. Samples were washed with PBS, and incubated for 30 minutes at 20°C with intercalation solution (PBS, 0.3% saponin, 1% formaldehyde, 125 nM iridium CellID (Fluidigm)). Samples were acquired on a Helios apparatus (CyTOF System, Fluidigm) at a flow rate of 0.030 mL/min.

For flow cytometry analyses, samples were thawed as described above, transferred into a 96 well plate, washed once with CSM and incubated for 30 min at 20°C with Fc-block and mAbs directed against CD3, CD7, CD11c, CD16, CD19, CD32, CD33, CD56 and CD66b (**supplementary Table 2**) in 50 μL CSM. Samples were incubated for 20 min at 20°C with 200 μL PBS, 2.4% formaldehyde, washed with CSM

and CSM containing 0.03% saponin, and intubated for 30 min on 20°C with mAbs directed against arginase 1 (Arg1), caspase-3, IL-6, IL-10 and myeloperoxidase (MPO) (**supplementary Table 2**). Samples were washed twice with CSM and acquired on an Attune NxT Flow Cytometer (Thermofisher scientific, Waltham, MA, USA).

Data processing and analysing.

Mass cytometry data files were normalized using MATLAB normalizer [14]. Debris, doublets, apoptotic cells, and $CD45^-$ cells were manually gated out and files were de-barcoded. A high caspase-3 level was used to remove apoptotic cells. Viability staining based on intact cell membrane was not feasible since cells were fixated before freezing and transportation to Switzerland [15]. The expression levels were scaled and biexponential transformed as described previously (Herderschee et al., in revision, supplementary file section 6.3). Cells were clustered using FlowSOM (metaclusters set on 40) based on the biexponential transformed expression levels of all surface markers and merged into populations based on biological knowledge, as depicted in tSNE plots and heatmap representation (**Figure 1**) [16, 17]. For expression levels of granulocytes over time, $CD11c^{low}$ granulocytes and mature granulocytes were analysed together. For flow cytometry data, debris, doublets and dead cells were excluded by manual gating. Neutrophilic granulocytes were selected based on SSC-A and FSC-A parameters (**Supplementary Figure 1**) before applying automated FlowSOM clustering (metaclusters set on 20) based on the expression of CD molecules. Clusters that were non-granulocytic were removed, and FlowSOM clustering reapplied to identify PMN-MDSCs (based on $CD11c/CD16^{low}$ expression) and mature PMNs. Intracellular cytokine expression was expressed as the median expression of the population depicted as a fold change with the non-stimulated, baseline sample set as 1. FlowSOM clustering was used to distinguish granulocytes producing or not Arg1, IL-6, IL-10 and MPO. Arg1 and IL-10 showed minimal differences between clusters, and were excluded from the analyses of the percentage or positive cells.

Statistics and software.

Cytokine and flow cytometry data were compared using the Kruskall-Wallis test and Mann-Whitney U. A 2-tailed $P < 0.05$ was considered statistically significant. Manual gating was performed on FlowJo™ Software version 10.6.2 (Ashland, OR: Becton, Dickinson and Company; 2019). Statistical analyses and figure design were performed using R statistical software version 3.6.0 (R Foundation for Statistical Computing, Vienna, Austria).

Results

We included seven healthy male subjects with a median age of 23.5 years [interquartile range (IQR): 22-27] and baseline leukocyte count of 5.9 x 10^9 cells/mL [5.5-7.4]. Subjects were infused twice at 1 week apart with a bolus of 2 ng/kg *E. coli* endotoxin. Blood was drawn 0, 1, 2, 3, 4, 6, 8, 24 and 168 hours after the first infusion of endotoxin, and 1, 2, 3, 4, 6, 8 hours after the second infusion of endotoxin. Samples from 6 subjects were analysed by mass cytometry, and samples from 7 subjects by flow cytometry.

Characterization of leukocyte populations over time by mass cytometry

At baseline, neutrophils represented 57% [56-59] of leukocytes (2.2 x 10^6 cells/mL measured by flow cytometry). One hour after the first infusion of endotoxin, neutrophils represented 48% [39-54] (0.7 x 10^6 cells/mL [0.6-0.9]) of leukocytes (**Figure 2, Supplementary Figure 2 and Tables 3 and 4**). They increased sharply to reach 94% [93-96] (7.6 x 10^6 cells/mL [6.5-8.6]) of leukocytes at 4 hours. After 7 days, neutrophils were back to baseline levels. Following the second infusion of endotoxin, neutrophils increased to represent 66% [59-76] (2.5 x 10^6 cells/mL [1.8-3.6]) of leukocytes after 1 hour. They started to decline after 8 hours.

The sharp rise in neutrophils was caused by a subgroup of cells expressing low levels of CD11c (integrin αX) and CD32 (FcγRII), named $CD11c^{low}$ neutrophils (**Figure 1 and 2B**). $CD11c^{low}$ neutrophils increased from 0.14 x 10^6 cells/mL [0.08-0.20] (3% [2-4] of leukocytes) at baseline to 1.1 x 10^6 cells/mL [0.8-1.3] (29% [24-29]) at 2 hours, peaked at 3.0 x 10^6 cells/mL (37% [31-39]) at 4 hours (**Figure 2, Supplementary Figure 2 and Tables 3 and 4**). $CD11c^{low}$ neutrophils normalized after 24 hours. Following the second endotoxin infusion, $CD11c^{low}$ neutrophils peaked at 2 hours at 1.0 x 10^6 cells/mL (16% [13-19]).

Non-granulocytic subpopulations including classical, intermediate and non-classical monocytes, monocytic myeloid-derived suppressor cells (M-MDSCs), $CCR2^+$ and $CD45RA^+$ $CD4^+$ and $CD8^+$ T cells, B cells and $CD56^{high}$ and $CD56^{low}$ NK cells and DCs decreased after endotoxin infusion. Notably, classical monocytes, which represent the largest monocytic subtype in peripheral blood, decreased from 1.5 x 10^3 cells/mL [1.2-2.0] at baseline to 0.02 x 10^3 cells/mL [0.02-0.02] 1 hour after endotoxin infusion (**Supplementary Figure 3**). Classical monocytes were back to baseline levels after 24 hours. Following the second endotoxin infusion, monocytic subpopulations similarly declined and returned to baseline levels within 6 hours for classical monocytes. Intermediate monocytes and M-MDSCs were 1.5 times baseline values, 6 hours after the second infusion (baseline *versus* 6 hours for intermediate monocytes 0.9 x 10^3 cells/mL [0.5-2.1] to 1.7 x 10^3 cells/mL [1.4-1.8], and M-MDSCs: 0.56 x 10^3 cells/mL [0.44-0.73] to 0.83 x 10^6 cells/mL [0.63-0.91]). Lymphocytes showed a similar decline after the first and second endotoxin infusion.

A close look at neutrophils (neutrophils and $CD11c^{low}$ neutrophils) revealed that 2 to 8 hours after the first infusion of endotoxin the expression levels of CD11c, CD16, CD32, CD62L and CD66b decreased while that of CD195 increased (**Figure 3**). The second infusion of endotoxin modestly affected the

expression of these molecules. These data indicated that neutrophils were impacted by endotoxin infusion, possibly showing signs of tolerance.

Impact of endotoxin infusion on neutrophil response to TLR ligands

We tested whether neutrophils in subjects infused with endotoxin were tolerized. Blood collected 0, 4, 24, and 168 hours after endotoxin infusion was incubated for 4 hours with medium (control), LPS, Pam_3CSK_4, and R848. We then measured by intracellular flow cytometry the production of Arg1, IL-6, IL-10 and MPO by neutrophils, which was expressed as median expression levels (**Figure 4**), and as relative proportion of positive cells (**Figure 5**). In blood collected before endotoxin infusion, neutrophils upregulated IL-6 and MPO in response to stimulation with LPS and Pam_3CSK_4 ($P < 0.001$ for both), while they downregulated Arg1 in response Pam_3CSK_4 and R848 ($P < 0.001$ and $P = 0.02$, respectively) (**Figure 4AB**). Infusion with endotoxin increased baseline expression of Arg1 (4 and 168 hours), reduced baseline expression of Arg1 (4 hours) and IL-6 (4 and 24 hours), and did not modify MPO. In blood drawn 4 hours after endotoxin infusion, neutrophils did not upregulate IL-6 after stimulation with LPS and R848. The upregulation of IL-6, and MPO in response to LPS, Pam_3CSK_4 and R848 was normalized in blood collected 168 hours after endotoxin infusion (**Figure 4AB**).

A similar picture was obtained when looking at the percentage of neutrophils that expressed the molecules of interest (**Figure 5**). After stimulation with LPS and Pam_3CSK_4, the percentage of neutrophils that produced IL-6 increased from 23% [20-28] (baseline) to 32% [30-36] and 31% [30-34], respectively. Four and 24 hours after endotoxin infusion, 15% [13-16] and 17.9% [14-21] of neutrophils produced IL-6. This proportion did not increase after *ex vivo* stimulation in blood collected 4 hours after endotoxin infusion. It increased to 25% [23-29] LPS-stimulated blood collected 24 hours after endotoxin infusion (**Figure 5A**). At baseline, MPO^{high} neutrophils increased from 56% [54-61] to 77% [65-80], 72% [73-76] and 62% [48-63] upon LPS, Pam_3CSK_4 and R848 stimulation. This did not change significantly in blood collected 4 and 24 hours after endotoxin infusion. However, 168 hours after infusion, MPO^{high} neutrophils increased from 59% [57-68] to 80% [74-82], 79% [79-80] and 69% [62-79] upon LPS, Pam_3CSK_4 and R848 stimulation (significant for Pam_3CSK_4) (**Figure 5B**).

PMN-MDSCs raised transiently following endotoxin infusion

PMN-MDSCs rise in the blood in conditions of systemic inflammation and can be viewed as an "immature" subset of neutrophilic granulocytes. Therefore, we quantified mature neutrophils and PMN-MDSCs by flow cytometry (**Figure 6**). PMN-MDSCs differentiated from mature neutrophils primarily based on strongly low levels of CD16. Additionally, PMN-MDSCs expressed lower levels of CD11c, CD32, CD33 and CD66b cells (**Figure 6B**). PMN-MDSCs raised from 4% [1-15] at baseline, to 35% [7-47] 4 hours after endotoxin infusion. PMN-MDSCs returned to baseline levels 24 hours after endotoxin infusion (**Figure 6C**).

Finally, we compared the expression of Arg1, IL-6 and MPO by PMN-MDSCs and neutrophils in whole blood incubated with medium (control), LPS, Pam_3CSK_4, and R848. Overall, PMN-MDSCs expressed lower levels of Arg1 and IL-6 but not of MPO when compared to neutrophils (**Figure 7A**). PMN-MDSCs and neutrophils responded similarly to stimulation regarding the expression of Arg1 and MPO

(although on a lower level for Arg1). PMN-MDSCs did not increase IL-6 expression upon stimulation (**Figure 7B**).

Conclusions

We found that neutrophilic granulocytes rise strongly after LPS infusion mainly due to the increase of $CD11c^{low}$ granulocytes. After the second infusion of endotoxin, we see a similar increase of neutrophilic granulocytes but these consist of less $CD11c^{low}$ granulocytes. Generally, we see less expression of CD11c, CD32, CD16, CD62L and CD66b by neutrophil granulocyte and upregulation of CD195 after the first infusion of endotoxin. This disappears after the second infusion suggesting that neutrophilic granulocytes develop a type of endotoxin tolerance. When stimulating neutrophilic granulocytes with TLR ligands, we observed an upregulation of IL-6 and MPO, and a downregulation of Arg1. Four hours after endotoxin infusion, granulocytes showed a cross tolerance regarding IL-6. This result is subtle but we see a similar trend looking at median expression and percentage of cells producing the mediators. When splitting granulocytes in PMN-MDSC-like cells and more mature neutrophils, we observed that the former show less baseline expression of IL-6, are non-responsive to stimulation and, contrary to expectation, do not produce more Arg1 and MPO. With this study, we provide insights on the effect of endotoxin and endotoxin tolerance on granulocytic neutrophils.

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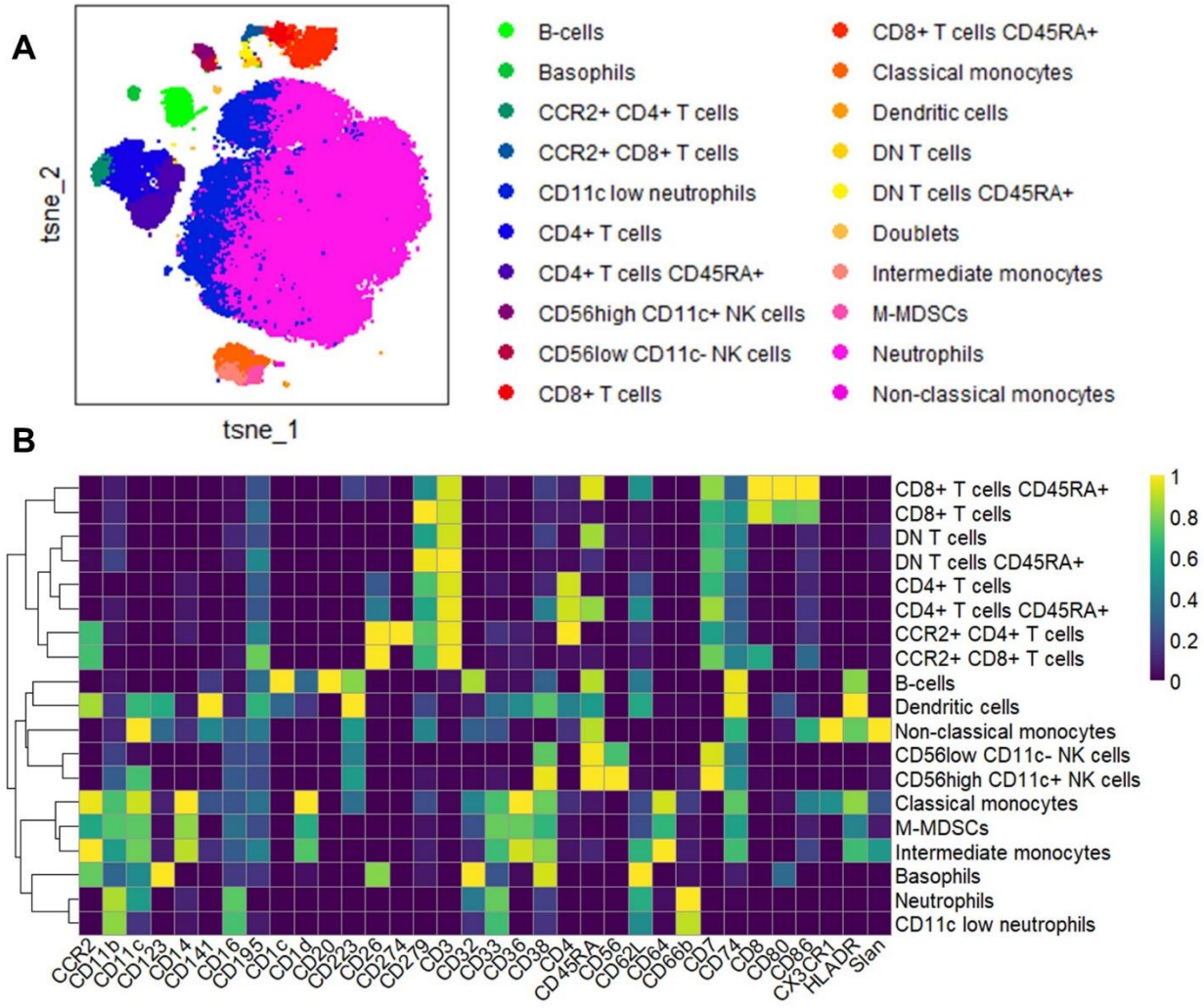


Figure 1. Identification by mass cytometry of leukocyte populations in the blood healthy subjects infused with endotoxin. Six healthy subjects were infused with endotoxin (bolus of 2 ng/kg) twice at 1 week apart. Blood was drawn from 0, 1, 2, 3, 4, 6, 8, 24 and 168 hours after the first infusion, and 1, 2, 3, 4, 6, 8 hours after the second infusion. Samples were stained and analysed by mass cytometry as described in *Materials and Methods*. **A**) t-SNE plots of leukocyte populations. **B**) Expression levels of cell surface markers. DN: double negative.

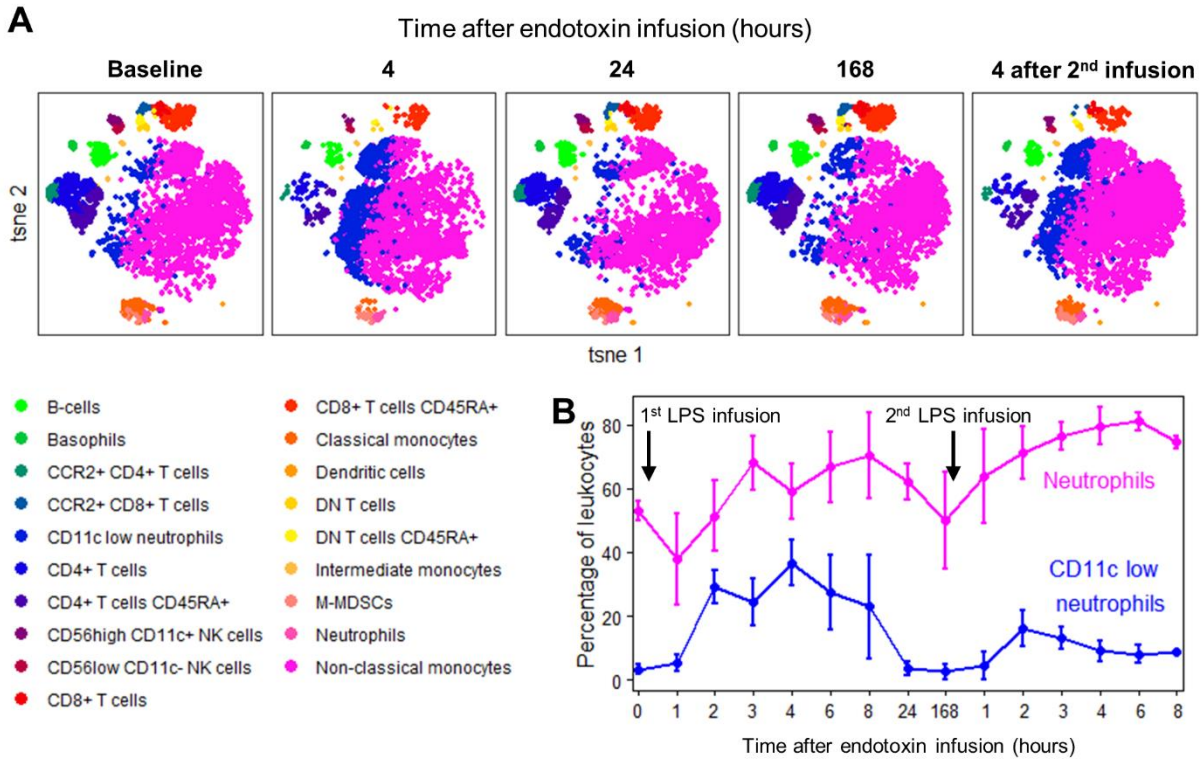


Figure 2. Dynamic changes of leukocyte populations in the blood of healthy subjects infused with endotoxin. Six healthy subjects were infused with endotoxin (bolus of 2 ng/kg) twice at 1 week apart. Blood was drawn from 0, 1, 2, 3, 4, 6, 8, 24 and 168 hours after the first infusion, and 1, 2, 3, 4, 6, 8 hours after the second infusion. Samples were stained and analysed by mass cytometry as described in *Materials and Methods*. **A**) t-SNE plots of leukocyte populations over time. **B**) Percentage of $CD11c^{low}$ neutrophils and mature neutrophils within leukocytes. Line graphs describe the medians, bars represent the median absolute deviation.

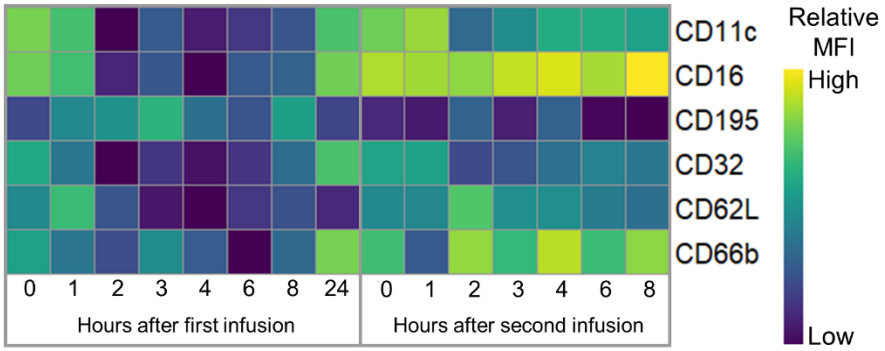


Figure 3. Expression of CD11c, CD16, CD195, CD32, CD62L and CD66b by neutrophils in the blood of healthy subjects infused with endotoxin. Six healthy subjects were infused with endotoxin (bolus of 2 ng/kg) twice at 1 week apart. Blood was drawn from 0, 1, 2, 3, 4, 6, 8, 24 and 168 hours after the first infusion, and 1, 2, 3, 4, 6, 8 hours after the second infusion. Samples were stained and analysed by mass cytometry as described in *Materials and Methods*. The heat map shows normalized expression of the marker by mature neutrophils and $CD11c^{low}$ neutrophils. h = hours after endotoxin infusion.

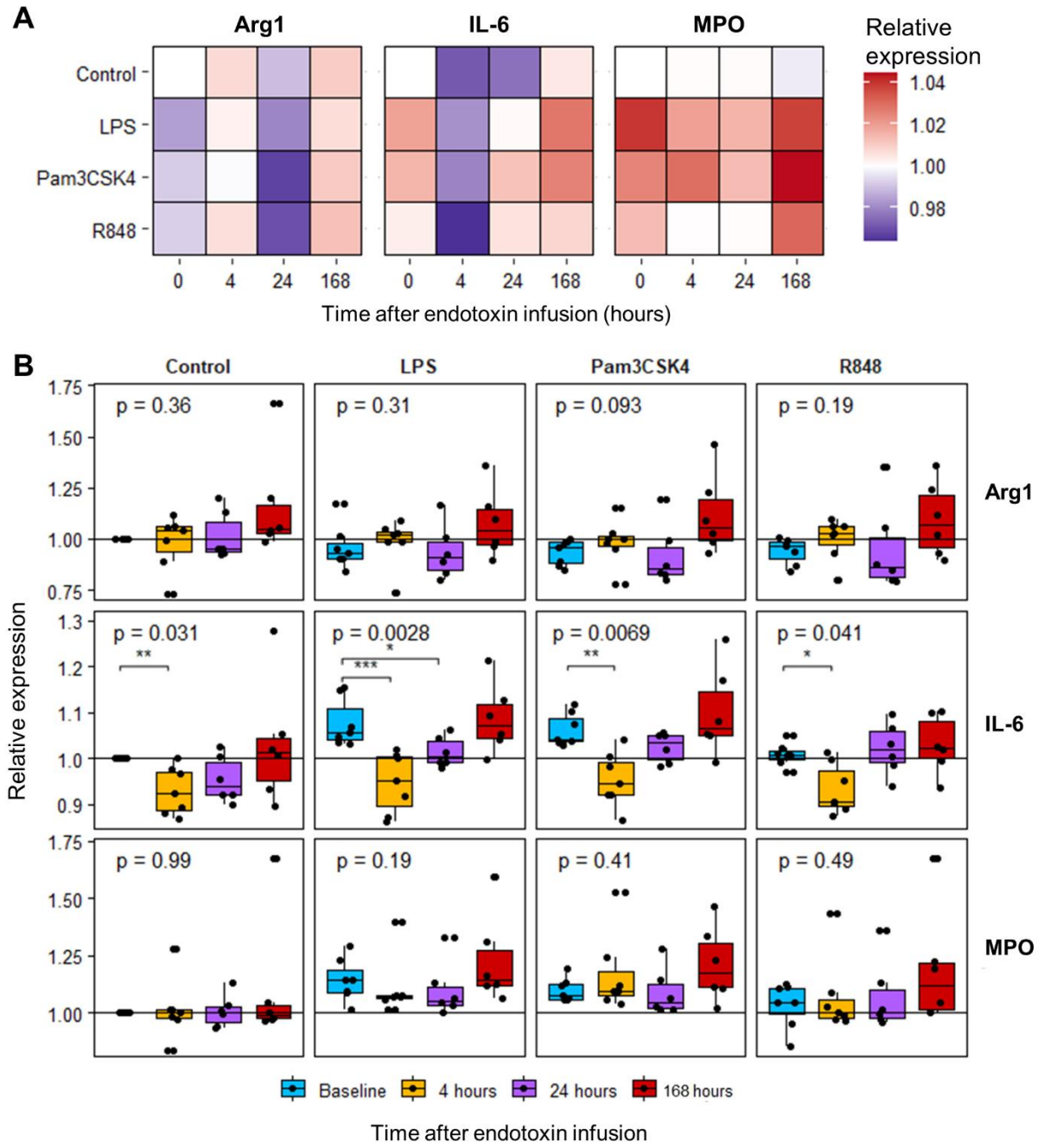


Figure 4. Reduced IL-6 production by neutrophils in blood collected 4 h after endotoxin infusion. Blood was collected from 7 healthy subjects 0, 4, 24, and 168 hours after endotoxin infusion, exposed for 4 hours to 10 ng/ml LPS, 1 µg/mL Pam_3CSK_4 and 1 µM R848, and analyzed by intracellular staining followed by flow cytometry analysis of Arg1, IL-6 and MPO by neutrophils (see *Materials and Methods*). Expression was set as fold change from unstimulated baseline conditions (before endotoxin infusion). **A**) Heatmap and, **B**) Boxplots showing the expression of Arg1, IL-6 and MPO. Boxplots show median, upper and lower quartiles. The whiskers show 5-95 percentiles. Each dot represents an individual sample. * P < 0.05; ** P < 0.01; *** P < 0.001.

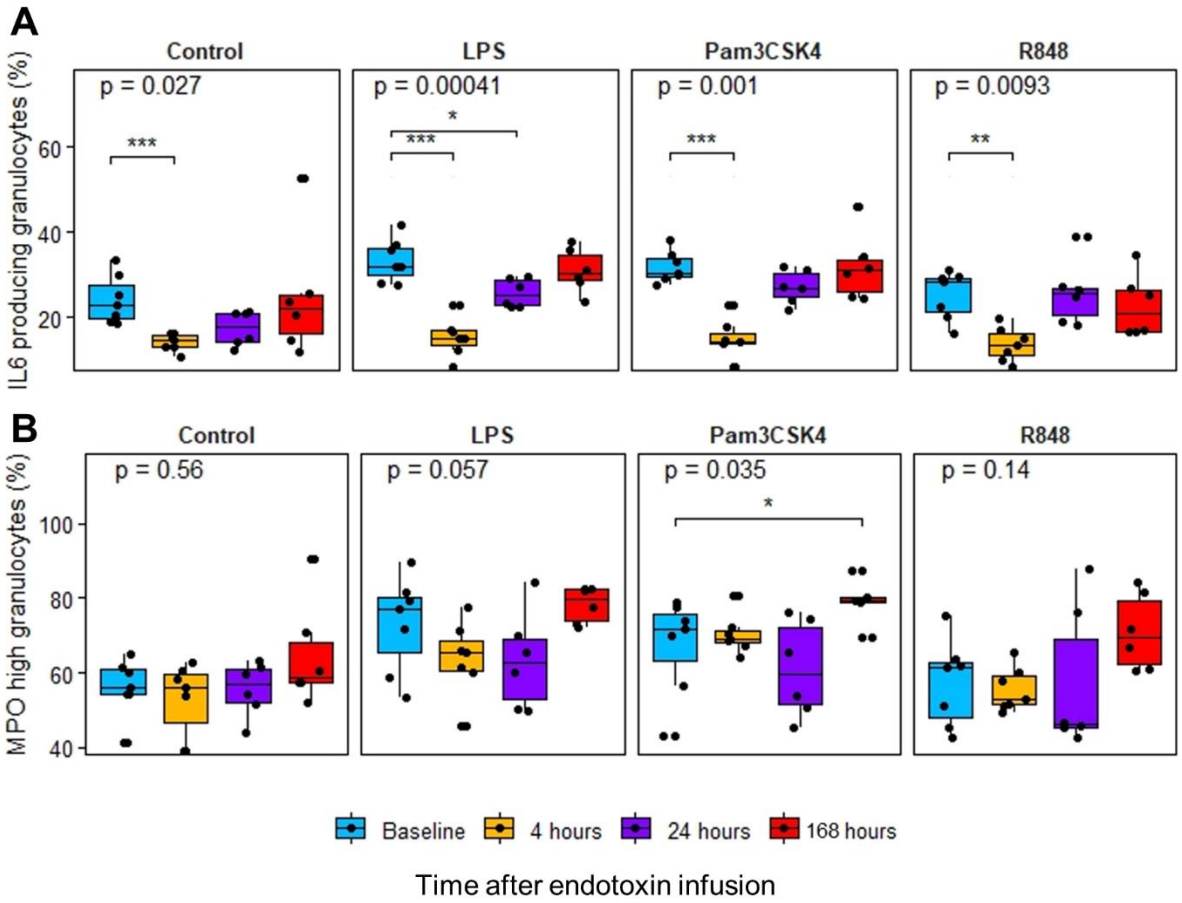


Figure 5. Reduced frequency of neutrophil responding to TLR ligands in blood collected 4 h after endotoxin infusion. Blood was collected from 7 healthy subjects 0, 4, 24, and 168 hours after endotoxin infusion, exposed for 4 hours to 10 ng/ml LPS, 1 µg/mL Pam_3CSK_4 and 1 µM R848, and analyzed by intracellular staining followed by flow cytometry analysis of Arg1, IL-6 and MPO by neutrophils (see *Materials and Methods*). **A-B**) Percentage of $IL\text{-}6^+$ and MPO^{high} neutrophils, 0, 4, 24 and 168 hours after endotoxin infusion. Boxplots show median, upper and lower quartiles. The whiskers show 5-95 percentiles. Each dot represents an individual sample. * P < 0.05; ** P < 0.01; *** P < 0.001.

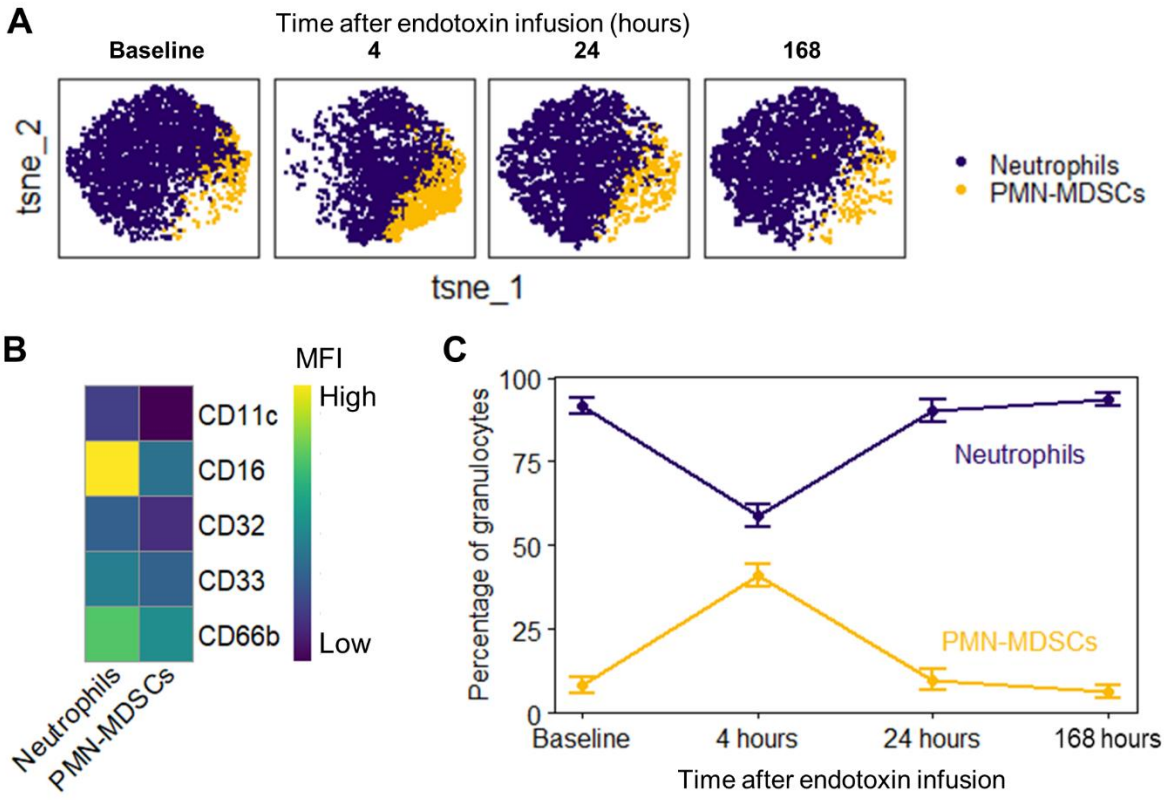


Figure 6. Dynamic changes of neutrophils and PMN-MDSCs in the blood of healthy subjects challenged with endotoxin. Blood was collected from 7 healthy subjects 0, 4, 24, and 168 hours after endotoxin infusion and analyzed by flow cytometry (see *Materials and Methods*). **A**) t-SNE plots of neutrophilic populations over time. **B**) Expression level of cell surface markers. **C**) Percentage of $CD11c^{low}$ neutrophils and (mature) neutrophils within leukocytes. PMN-MDSCs: polymorphonuclear-MDSCs. Line graphs describe the medians, bars represent the median absolute deviation.

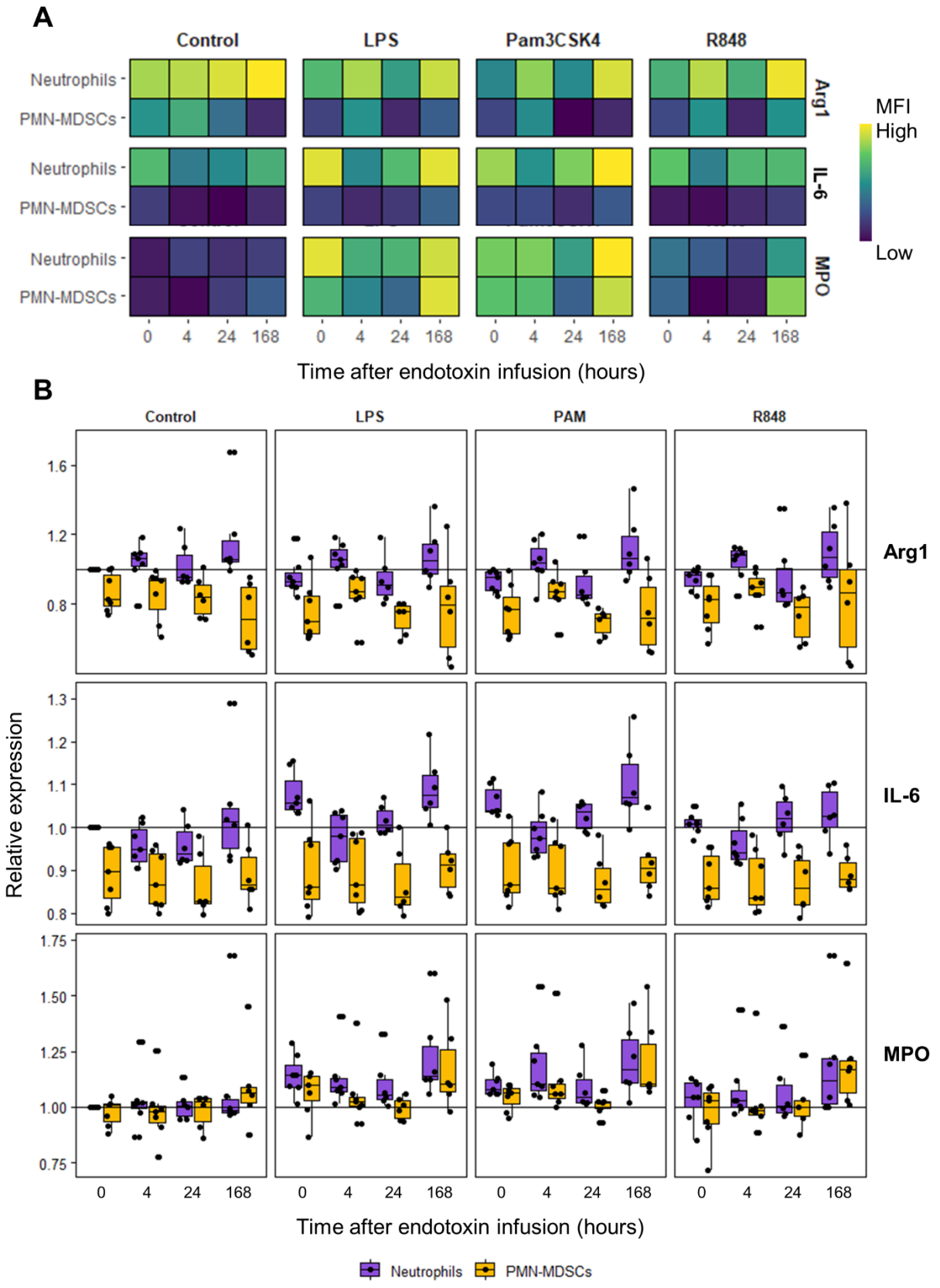

A
Control
LPS
Pam3CSK4
R848
Neutrophils
PMN-MDSCs
Arg1
IL-6
MPO
MFI
High
Low
0
4
24
168
Time after endotoxin infusion (hours)
B
Control
LPS
PAM
R848
Arg1
IL-6
MPO
Relative expression
Time after endotoxin infusion (hours)
Neutrophils
PMN-MDSCs

Figure 7. Differential expression of Arg1, IL-6 and MPO by neutrophils and PMN-MDSCs in the blood of healthy subjects challenged with endotoxin. Blood was collected from 7 healthy subjects 0, 4, 24, and 168 hours after endotoxin infusion, exposed for 4 hours to 10 ng/ml LPS, 1 µg/mL Pam_3CSK_4 and 1 µM R848, and analyzed by intracellular staining followed by flow cytometry analysis of Arg1, IL-6 and MPO by neutrophils (see *Materials and Methods*). **A**) Heat map of relative MFI by neutrophils and PMN-MDSCs per time point, and per stimuli. **B**) Boxplots of relative expression of neutrophils and PMN-MDSCs. Expression was calculated as fold change from values obtained in unstimulated neutrophils from blood collected before endotoxin infusion. Boxplots show median, upper and lower quartiles. The whiskers show 5-95 percentiles. Each dot represents an individual sample.

Supplementary tables and figures.

Supplementary Table 1. Antibodies and barcodes used in mass cytometry

Target	Clone	Manufacturer	Isotope
Barcode	Scn-BN-EDTA	In lab	104Pd
Barcode	Scn-BN-EDTA	In lab	105Pd
Barcode	Scn-BN-EDTA	In lab	106Pd
Barcode	Scn-BN-EDTA	In lab	108Pd
Barcode	Scn-BN-EDTA	In lab	110Pd
Barcode	mDOTA	In lab	113In
Barcode	mDOTA	In lab	115In
CCR2	B27	Biolegend	165Ho
CCR5	Rea245	Miltenyi	153Eu
CD1c	L161	Biolegend	143Nd
CD1d	51.1	Biolegend	154Sm
CD3	UCHT1	Biolegend	148Nd
CD4	RPA-T4	Biolegend	144Nd
CD7	CD7-6B7	Biolegend	169Tm
CD8	SK1	Biolegend	145Nd
CD11b	ICRF44	Biolegend	141Pr
CD11c	Bu15	Biolegend	163Dy
CD14	HCD14	Biolegend	160Gd
CD16	3G8	Fluidigm	209Bi
CD20	2H7	Biolegend	147Sm
CD26	BA5b	Biolegend	173Yb
CD32	FUN2	Biolegend	152Sm
CD33	REA775	Miltenyi	197AU
CD36	5-271	Biolegend	174Yb
CD38	HIT2	Biolegend	142Nd
CD45	HI30	Fluidigm	89Y
CD45RA	HI100	Biolegend	166Er
CD56	R19-760	BD	159Tb
CD62L	DREG-56	Biolegend	172Yb
CD64	10.1	Biolegend	175Lu
CD66b	REA306	Miltenyi	158Gd
CD74	5-329	Miltenyi	176Yb
CD80	2D10	Biolegend	161Dy
CD86	IT2.2	Biolegend	146Nd
CD123	6H6	Biolegend	162Dy
CD141	M80	Biolegend	168Er
CD223	7H2C65	Biolegend	167Er
CX3CR1	2A9-1	Biolegend	156Gd
HLA-DR	L243	Biolegend	170Er
PD1	EH12.2H7	Biolegend	164Dy
PD-L1	29E.2A3	Biolegend	171Yb
Slan	DD1	Miltenyi	151Eu

Supplementary Table 2. Antibodies used in flow cytometry

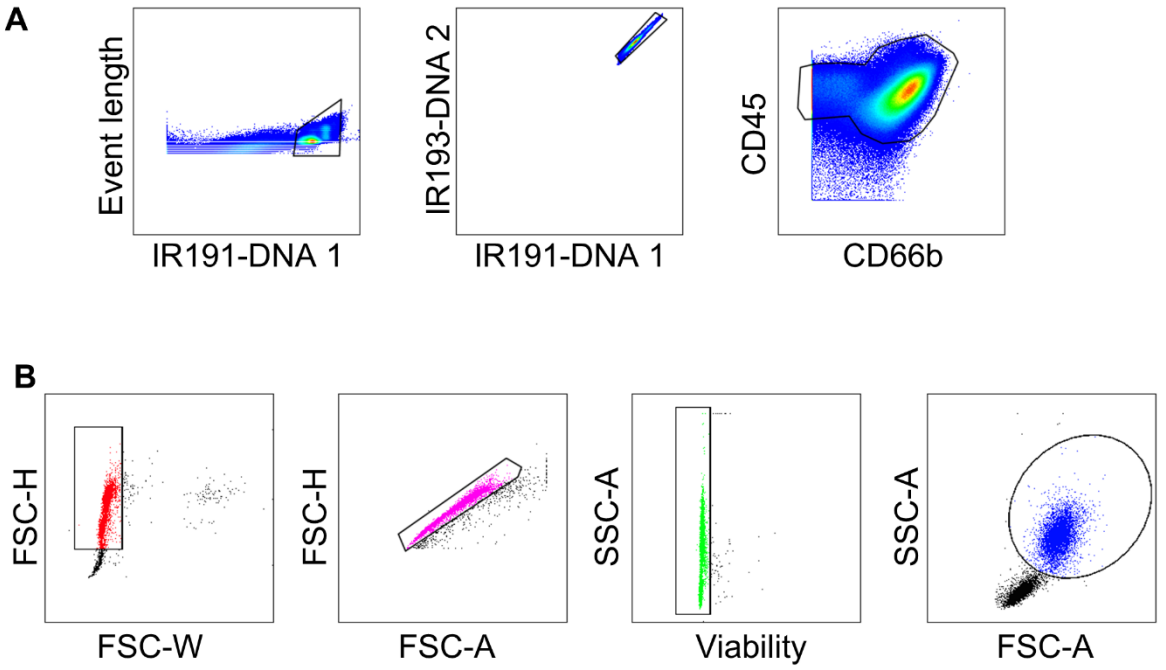
Target	Clone	Fluorochrome	Company	Reference number
Arg1	125D2C253	APC	Biolegend	369706
Caspase-3	C92-605	PE	BD	55082
CD3	UCHT1	AlexaFluor 700	eBioscience	56-0038-42
CD7	M-T701	AlexaFluor 700	BD	561603
CD11c	Bu15	PB	Biolegend	337212
CD16	3G8	PE-CF594	Beckman Coulter	B49216
CD19	J3.119	AlexaFluor 700	Beckman Coulter	B76284
CD32	FUN2	APC-F750	Biolegend	303220
CD33	WM33	BV711	BD	563171
CD56	HCD56	AlexaFluor 700	Biolegend	318316
CD66b	G10F5	PE-CY7	Biolegend	305116
IL-10	JES3-9D7	BV650	BD	6E+06
IL-6	MQ2-13A5	PerCP-EF710	Biolegend	501117
MPO	Rea491	FITC	miltenyi	130-120-241

Supplementary table 3. Cell population (%) per time point (Median [IQR])

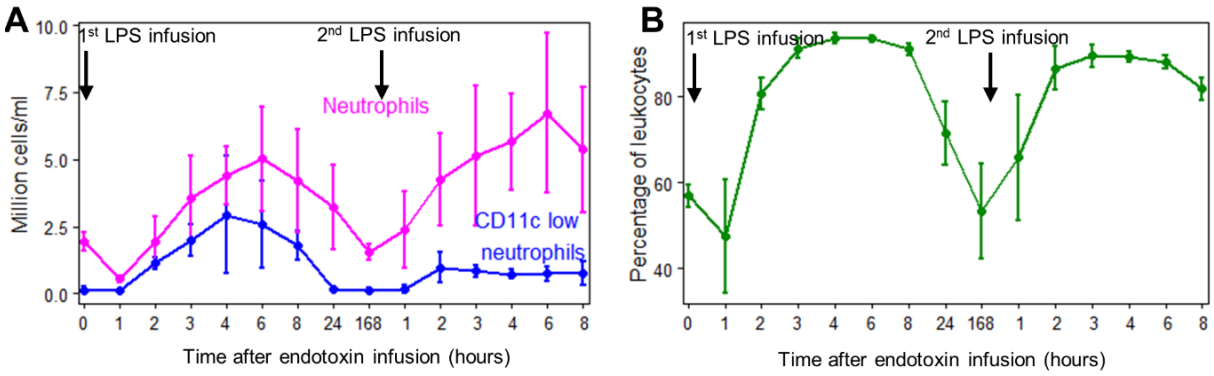
Hours after first infusion	0	1	2	3	4	6	8	24
Neutrophils	53 (51, 54)	38 (32, 48)	51 (43, 55)	68 (58, 72)	59 (55, 64)	67 (55, 68)	70 (56, 74)	62 (59, 69)
CD11c low neutrophils	3 (2, 4)	5 (4, 6)	29 (24, 29)	24 (22, 32)	37 (31, 39)	27 (26, 38)	23 (18, 35)	4 (2, 4)
Total neutrophils	57 (56, 59)	48 (39, 54)	81 (77, 81)	91 (90, 92)	94 (93, 95)	94 (93, 94)	91 (90, 92)	72 (66, 72)
Hours after second infusion	**0**	**1**	**2**	**3**	**4**	**6**	**8**	
Neutrophils	50 (41, 58)	64 (54, 71)	71 (63, 76)	76 (71, 77)	79 (73, 82)	81 (78, 81)	74 (65, 76)	
CD11c low neutrophils	3 (2, 5)	4 (2, 7)	16 (13, 19)	13 (12, 16)	9 (8, 13)	8 (7, 10)	9 (8, 9)	
Total neutrophils	53 (48, 60)	66 (59, 76)	87 (83, 89)	90 (88, 91)	89 (88, 90)	88 (87, 88)	82 (81, 83)	

Supplementary table 4. Cell population (Million cells/ml) per time point (Median [IQR])

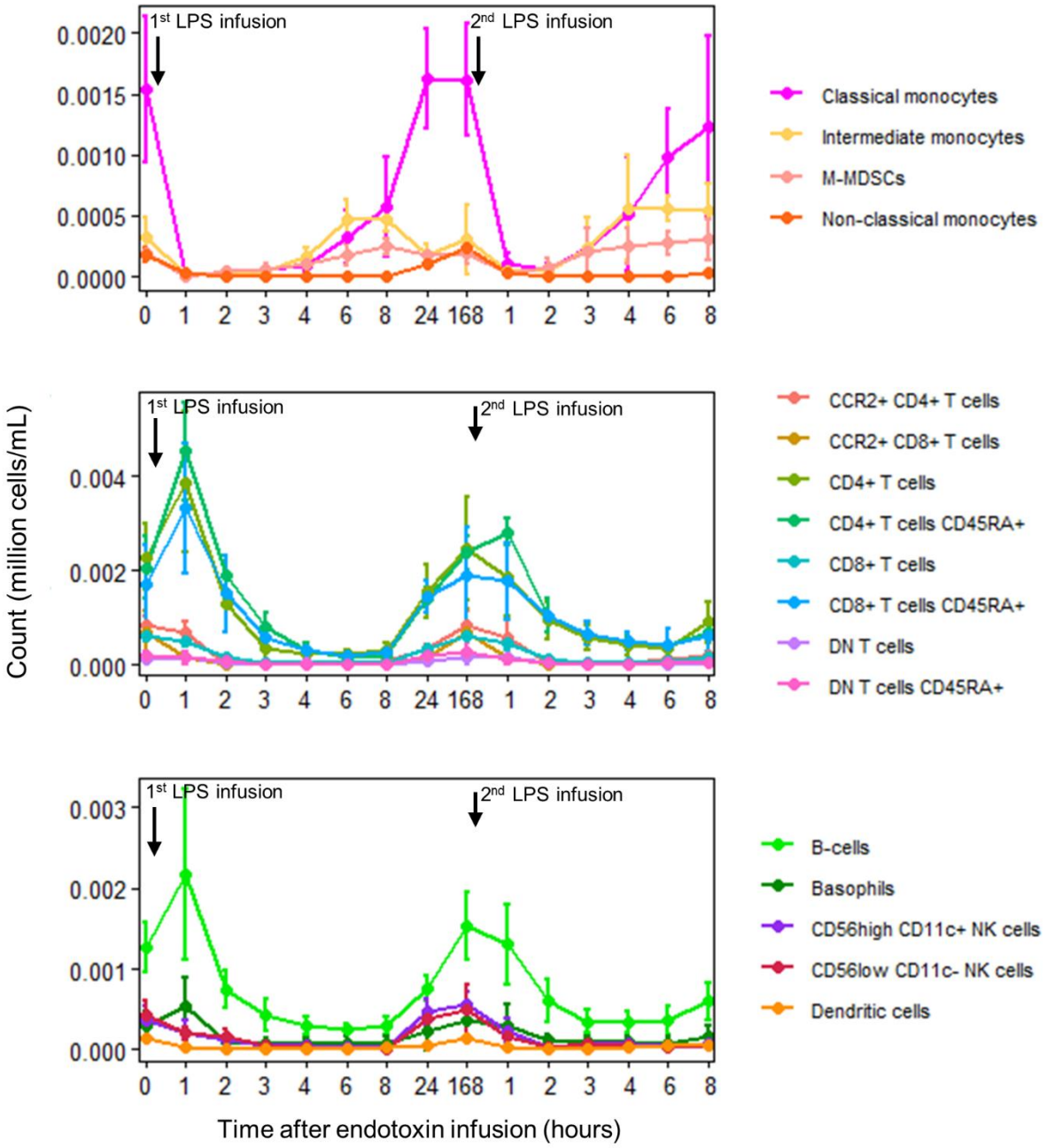
Hours after first infusion	0	1	2	3	4	6	8	24
Neutrophils	1.9 (1.8, 2.2)	0.5 (0.5, 0.8)	1.9 (1.1, 2.1)	3.6 (2.9, 5.5)	4.4 (3.8, 5.1)	5.0 (3.7, 6.0)	4.2 (3.2, 5.3)	3.2 (2.1, 3.3)
CD11c low neutrophils	0.1 (0.1, 0.2)	0.1 (0.0, 0.1)	1.1 (0.8, 1.3)	2.0 (1.4, 2.3)	3.0 (2.3, 4.8)	2.6 (1.4, 3.3)	1.8 (1.1, 2.1)	0.1 (0.1, 0.2)
Total neutrophils	2.2 (2.0, 2.3)	0.7 (0.6, 0.9)	2.8 (1.9, 3.2)	5.0 (4.5, 7.6)	7.6 (6.5, 8.6)	7.7 (6.8, 8.3)	5.5 (5.2, 5.8)	3.3 (2.6, 3.6)
Hours after second infusion	**0**	**1**	**2**	**3**	**4**	**6**	**8**	
Neutrophils	1.5 (1.5, 2.3)	2.4 (1.7, 3.4)	4.3 (3.2, 5.4)	5.1 (3.5, 6.7)	5.7 (4.5, 6.8)	6.7 (4.7, 8.3)	5.4 (3.9, 6.7)	
CD11c low neutrophils	0.1 (0.1, 0.5)	0.2 (0.1, 0.2)	1.0 (0.7, 1.7)	0.8 (0.7, 2.0)	0.7 (0.6, 0.8)	0.7 (0.5, 0.9)	0.8 (0.4, 0.9)	
Total neutrophils	1.9 (1.6, 2.5)	2.5 (1.8, 3.6)	5.5 (4.4, 6.2)	6.4 (5.0, 7.5)	6.9 (5.5, 7.5)	7.6 (5.0, 9.0)	6.0 (4.9, 7.5)	



Supplementary figure 1. Gating strategy to exclude debris, doublets and non-hematopoietic cells to analyze blood leukocytes by: A) mass cytometry, and B) flow cytometry.



Supplementary figure 2. Dynamic changes of neutrophils in the blood of healthy subjects challenged with endotoxin. Six healthy subjects were infused with endotoxin (bolus of 2 ng/kg) twice at 1 week apart. Blood was drawn from 0, 1, 2, 3, 4, 6, 8, 24 and 168 hours after the first infusion, and 1, 2, 3, 4, 6, 8 hours after the second infusion. **A**) Absolute counts of CD11c^{low} neutrophils and mature neutrophils. **B**) Percentage of neutrophils within leukocytes. Line graphs describe the medians, bars represent the median absolute deviation.



Supplementary figure 3. Dynamic change of leukocyte populations in the blood of healthy subjects challenged with endotoxin. Six healthy subjects were infused with endotoxin (bolus of 2 ng/kg) twice at 1 week apart. Blood was drawn from 0, 1, 2, 3, 4, 6, 8, 24 and 168 hours after the first infusion, and 1, 2, 3, 4, 6, 8 hours after the second infusion. From top to bottom: absolute counts of monocytic, T cell, B-cells, basophils, NK cells and dendritic cells populations. Line graphs describe the medians, bars represent the median absolute deviation.

6.2. High-dimensional mass cytometry reveals persistent HCV-mediated immune dysfunction in patients successfully treated with direct-acting antiviral agents.

Jacobus Herderschee[1], Tytti Heinonen[1], Craig Fenwick[2], Irene Schrijver[1], Khalid Ohmiti[2], Darius Moradpour[3], Matthias Cavassini[1], Giuseppe Pantaleo[2,4], Thierry Roger[1]*, Thierry Calandra[1]*, and the Swiss HIV Cohort Study

[1.]Infectious Diseases Service, [2.]Division of Immunology and Allergy, and [3.]Division of Gastroenterology and Hepatology, Department of Medicine, Lausanne University Hospital and University of Lausanne, Lausanne.

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Manuscript submitted.

Summary:

Chronic hepatitis C virus (HCV) infection affects the immune system. Whether elimination of HCV with direct-acting antivirals (DAA) restores immunity is unclear. To address that question, we used mass cytometry to get a broad and in-depth assessment of blood cell populations of patients with chronic HCV before and after DAA therapy. Before and 12 weeks after sustained virological response to DAA therapy (SVR12), blood was collected from 10 healthy controls and 20 patients chronically infected with HCV with (10) or without human immunodeficiency virus (HIV) (10) infection. We analyzed 22 blood cell populations by mass cytometry. Chronic HCV infection caused profound alterations in the frequency of 14 out of 22 (64%) blood cell populations. At baseline, the fraction of intermediate and non-classical monocytes, conventional dendritic cells type 2, and CD56dim natural killer cells were reduced by 35% to 65%, particularly in HCV/HIV co-infected patients. In contrast, the fraction of activated double-negative T cells, CD4 and CD8 T cells increased by 1.4 to 3.5 times. Upon stimulation with Toll-like receptor ligands ex vivo, innate immune cells of HCV-infected and particularly of HIV/HCV co-infected patients displayed up-regulated pro-inflammatory cytokine expression profile. Remarkably, most immune cell alterations persisted long after viral clearance (i.e. up to SVR12). To conclude, chronic HCV and HCV/HIV infections induce profound and durable perturbations of innate and adaptive immune homeostasis.

My contribution to this work:

I processed part of the samples, revised the figures and the manuscript.

High-dimensional immune phenotyping of blood cells by mass cytometry in patients infected with hepatitis C virus

Jacobus Herderschee[1], Tytti Heinonen[1], Craig Fenwick[2], Irene Schrijver[1], Khalid Ohmiti[2], Darius Moradpour[3], Matthias Cavassini[1], Giuseppe Pantaleo[2,4], Thierry Roger[1$], Thierry Calandra[1$#], and the Swiss HIV Cohort Study

[1]Infectious Diseases Service, [2]Division of Immunology and Allergy, and [3]Division of Gastroenterology and Hepatology, Department of Medicine, Lausanne University Hospital and University of Lausanne, Lausanne; [4]Swiss Vaccine Research Institute, Lausanne, Switzerland.

[$]These authors contributed equally to this work.

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Abstract

Objectives

Chronic hepatitis C virus (HCV) infection affects the immune system. Whether elimination of HCV with direct-acting antivirals (DAA) restores immunity is unclear. We used mass cytometry to get a broad and in-depth assessment of blood cell populations of patients with chronic HCV prior to and after DAA therapy.

Methods

Before and 12 weeks after sustained virological response to DAA therapy (SVR12), 22 cell populations were analysed by mass cytometry in blood collected from 10 healthy controls and 20 HCV patients with (10) or without human immunodeficiency virus (HIV) (10) infection.

Results

HCV infection altered the frequency of 14/22 (64%) blood cell populations. At baseline, the frequencies (median [IQR]; control, HCV, HCV/HIV) of intermediate monocytes (1.2 [0.47-1.46], 1.76 [0.83-2.66], 0.78 [0.28-1.77]), non-classical monocytes (1.11 [0.49-1.26], 0.9 [0.18-0.99], 0.54 [0.28-1.77]), conventional dendritic cells type 2 (0.55 [0.35-0.59], 0.31 [0.16-0.38], 0.19 [0.11-0.36]) and $CD56^{dim}$ natural killer cells (8.08 [5.34-9.79], 4.72 [2.59-6.05], 3.61 [2.98-5.07]) were reduced by 35% to 65%, particularly in HCV/HIV co-infected patients. In contrast, activated double-negative T cells (0.07 [0.06-0.10], 0.10 [0.09-0.19], 0.19 [0.12-0.25]), activated CD4 T cells (0.28 [0.21-0.36], 0.56 [0.33-0.77], 0.40 [0.22-0.53]) and activated CD8 T cells (0.23 [0.14-0.42], 0.74 [0.30-1.65], 0.80 [0.58-1.16]) were increased 1.4 to 3.5 times. Upon stimulation with Toll-like receptor ligands, the expression of cytokines was up-regulated in 7/9 (78%) and 17/19 (89%) of the conditions in HCV and HCV/HIV patients, respectively. Most alterations persisted at SVR12.

Conclusions

Chronic HCV and HCV/HIV infections induces profound and durable perturbations of innate and adaptive immune homeostasis.

Introduction

Infections with hepatitis C virus (HCV) and human immunodeficiency virus (HIV) are leading causes of morbidity and mortality worldwide [1]. In 2015, a study estimated that 71 million people were chronically infected with HCV. Among the 37.9 million people living with HIV, 2.3 million were coinfected with HCV [1, 2]. Patients with HCV and HIV coinfection have higher mortality rates than patients with HCV or HIV monoinfection [3]. The ability of HIV and HCV to subvert and evade the host immune response explains why the diseases follow a chronic course [4, 5].

HCV evades the host innate immune system efficiently, preventing a successful adaptive immune response, resulting in chronic infection in up to 80% of patients [1, 5]. In infected hepatocytes, HCV proteins inhibit transduction of signals from pattern recognition receptors and cap-dependent mRNA translation, limiting the production of type I interferon (IFN) and other cytokines, thereby preventing the establishment of an antiviral state [6]. After infection, HCV titers rise within days, reach a plateau of 10^5–10^7 IU/mL and decrease after 4 to 8 weeks contemporaneously with the appearance of HCV-specific CD8 T cells [7]. HCV replication results in prolonged antigenic stimulation associated with disappearance of HCV-specific CD4 T cells and exhaustion of CD8 T cell responses [6, 8]. In HCV/HIV coinfected patients, HCV-specific immunity is impaired further by the depletion of CD4 T cells. Depletion of mucosal CD4 T cells also impairs gut barrier function, leading to increased microbial translocation and production of pro-fibrotic cytokines by Kupffer cells [6]. Thus, HIV infection alters the course of HCV disease *via* combined effects on innate and adaptive immunity.

For two decades, studies of the innate immune responses during HCV therapy were hampered by the use of pegylated-IFNα that exerts widespread effects on cellular immune functions. This changed with the introduction of direct-acting antivirals (DAA) for the treatment of hepatitis C. In this study, we used high-dimensional mass cytometry to get a broad and in-depth qualitative and quantitative assessment of blood cells before, during and after DAA-based therapy in patients with chronic hepatitis C with or without HIV coinfection.

Results

Characteristics of patients and controls

We conducted a longitudinal, non-interventional study in 10 patients infected with HCV, 10 patients infected with HCV and HIV (HCV/HIV), and 10 healthy subjects (controls) (Figure 1a and Table S1). Patients and controls were recruited by the Infectious Diseases Service and Division of Gastroenterology and Hepatology of Lausanne University Hospital (Switzerland). HCV patients were slightly older than controls (p=0.001) and HCV/HIV patients (p=0.042). Baseline HCV loads and viral load reductions were comparable in HCV and HCV/HIV patients. All subjects achieved sustained virologic response 12 weeks after the end of therapy and completed the follow-up period (Figure 1b). Whole blood was collected from healthy controls and patients just before, during and after DAA therapy and used immediately to perform in-depth immunoprofiling (Figure 1a).

HCV infection causes extensive and sustained changes in populations of circulating immune cells

Twenty-two cell populations were identified in whole blood by mass cytometry and FlowSOM clustering (Figure 1c,d, Figs S1-S3). It included 3 granulocyte clusters (basophils, $CD11b^{high}$ and $CD11b^{low}$ neutrophils), intermediate and non-classical monocytes, type 2 conventional DCs (cDC2), plasmacytoid DCs (pDCs), classical, $CD56^{high}$ and $CD56^{dim}$ NK cells, $CD1c^{+}$, $CD11c^{+}$ and $CD1c^{-}/CD11c^{-}$ B cells, CD4 and CD8 double-negative (DN), single-positive (SP) and double-positive (DP) T cells. T cell subsets included activated and non-activated T cells according to CD38 and HLA-DR expression.

We compared the frequency of merged clusters at baseline in controls and patients using generalized mixed linear models [9]. The full dataset of the normalized frequencies of immune cell populations prior to antiviral therapy and at SVR12 is shown in Figure 2a. The proportion of the main subsets of innate and adaptive immune cells is reported in Table S2 and Table S3. Overall, both HCV monoinfection and HCV/HIV coinfection were associated with significant changes in the frequency of 14 of 22 (64%) immune cell populations (Figure 2b). Prior to therapy, innate immune cells were markedly reduced. The reduction was 35% to 51% for intermediate and non-classical monocytes (HCV/HIV, p=0.038), 44% to 65% for cDC2 (HCV, p=0.007; HCV/HIV, p<0.001) and 42% to 55% for circulating $CD56^{dim}$ NK cells (HCV, p=0.074; HCV/HIV, p=0.012). In contrast, the frequencies of activated DN T cells (HCV, p=0.042; HCV/HIV, p<0.001), activated CD4 T cells (HCV, p=0.059), CD8 T cells (HCV/HIV, p<0.001) and activated CD8 T cells (HCV, p=0.023; HCV/HIV, p=0.025) were increased by a factor ranging from 1.4 to 3.5. The frequencies of B cells and $CD11c^{+}$ B cells were increased 2.1 and 3-fold in HCV/HIV coinfected patients (p=0.125 and p=0.042). Except for cDC2 (HCV patients) and activated DN cells (HCV and HCV/HIV patients), changes in innate and adaptive cell populations persisted up to SVR12 (Figs 2 and 3). Consistent with this observation, SVR12 samples tended to cluster with baseline samples from the same group.

Impact of HCV infection on innate immune responses

To investigate the effect of HCV on innate immune function, we stimulated whole blood with TLR agonists (LPS and R848) and analysed the expression of pro-inflammatory (TNF, IL-1α, IL-1β, IL-6, IL-8, IL-12p40, IFNα and MCP-1) and anti-inflammatory (IL-1RA and IL-10) cytokines by mass cytometry (Figures S4-S9). The full dataset of cytokine expression is shown in Figure 4a, Figure 5, Table S4 and Table S5. Cytokine expression induced by LPS and R848 clustered by cell type (Figure 4a). Classical, intermediate and non-classical monocytes and cDC2 had the highest frequencies of TNF, IL-1α, IL-1β and IL-8-positive cells after LPS stimulation. All classes of innate immune cells had a high frequency of TNF-positive cells after R848 stimulation, while cDC2, classical, intermediate and non-classical monocytes had the highest frequencies of IL-1β and IL-8-positive cells and pDCs had the highest frequency of IFNα-positive cells. Overall, the frequency of TNF, IL-1α, IL-1β, IL-6 and IL-8 cytokine-positive cells was higher in classical, intermediate and non-classical monocytes especially in HCV/HIV patients after stimulation with R848 (Figure 4b). The trend was less striking in cDC2. The frequency of IFNα-positive cells was also higher in HCV/HIV patients than in controls. In contrast, the expression of IL-1RA and IL-10 by monocytes and DCs did not differ between patients and controls. The pro-

inflammatory signature of monocytes persisted up to SVR12 in HCV/HIV patients and to a lesser degree in HCV patients (Figure 5).

Inflammatory signals enhance the expression of CD80 and CD86 co-stimulatory molecules and PD-1 and PD-L1 immune checkpoint molecules by antigen presenting cells [10]. Considering the pro-inflammatory signature of HCV patients, we analysed the expression CD80, CD86, PD-1 and PD-L1 by monocytes, cDC2 and pDCs. At baseline or 24 hours after LPS stimulation, we found no differences in the expression of these molecules by innate immune cells of patients and controls (cDC2 are shown in Figure S10).

Discussion

Patients infected with HCV with or without HIV display an immune signature characterized by altered innate and adaptive immune cell profiles and cytokine response by monocytes and DCs. Innate immunity was severely dysregulated. Chronic HCV caused a profound and widespread decrease of intermediate and non-classical monocytes, cDC2 and $CD56^{dim}$ NK cells. Yet, *ex vivo* production of pro-inflammatory cytokines by innate immune cells was upregulated particularly in HCV/HIV patients. Adaptive immune signatures were upregulated, with increased frequencies of activated DN, CD4, and CD8 T lymphocytes. This HCV-related immune dysregulation persisted up to 12 weeks after HCV clearance.

Previous studies on the effects of HCV used fresh or cryopreserved peripheral blood mononuclear cells (PBMCs) or monocyte-derived DCs (moDCs) and a limited number of phenotypic markers [11-19]. Isolation, cryopreservation and differentiation alter the phenotype and function of PBMCs and moDCs. To avoid these limitations, we analysed blood immediately after drawing and mass cytometry with clustering strategies allowing the identification of a broad spectrum of immune cells and cytokines. Studies of innate immunity in HCV patients yielded mixed results. Some studies reported a clear reduction in the frequency and function of pDCs and cDCs, while others did not [11-18, 20]. Our results are unambiguous with striking reduction of intermediate and non-classical monocytes, cDC2 and $CD56^{dim}$ NK cells. The broad and persistent reduction in circulating innate immune cells is an important hallmark of acute hepatitis C and possibly part of a strategy whereby HCV evades innate immune responses and prevents the development of an effective adaptive immune response. HCV-infected patients do not suffer from opportunistic infections, nor do they appear to be protected against other infections. The response to vaccination, such as against HBV, appears to be intact in patients with HCV infection, although some authors have found a reduced response [21].

Cytokine analysis at the single cell level revealed a pro-inflammatory profile in patients. One cannot exclude a restoration of immune homeostasis beyond SVR12. However, the persistence of immune alterations may be a systemic signature consistent with reports of ongoing liver inflammation documented long after SVR in patients with normal levels of transaminases [22-24]. If sustained, the inflammatory signature may contribute to the development of HCV-associated complications such as hepatocellular carcinoma, cardiovascular disease and diabetes. Along this line, increased cytokine response by innate immune cells were more pronounced in HCV/HIV patients than in HCV patients, indicating that HIV coinfection amplified immunophenotypic alterations. This observation may explain

the progression of liver disease, including cirrhosis, liver failure and hepatocellular carcinoma in HCV/HIV patients. Of note, coinfected patients had HIV viral loads below the lower limit of detection. Coinfected patients receiving HIV treatment progress more slowly than untreated patients. Yet, disease progression in the context of an undetectable HIV viral load remains incompletely understood [6].

HCV infection has been associated with broad-spectrum alterations of adaptive immunity [5]. We confirmed and extended these findings by demonstrating increased frequencies of activated DN T cells at baseline that normalized after viral clearance. We also observed an increase in activated CD8 T cells in HCV and HCV/HIV at baseline that persisted up to SVR12. A recent study reported a molecular signature of exhaustion that persisted in HCV-specific CD8 T cells after DAA-mediated cure of HCV [8]. This illustrates the strength of the clustering approach to look at a broad range of cell populations. As we did not examine antigen specificity of T cells, we did not check for T cell exhaustion in chronically infected patients. We cannot differentiate between the expansion of HCV-specific T cells driven by the high HCV mutation rate or the expansion of non-HCV-specific T cells linked to a general pro-inflammatory status or secondary to other patient characteristics such as fibrosis. Chronic hepatitis C is characterized by an increased risk of progressive liver fibrosis and immune complex-mediated autoimmunity [1]. The increased frequencies of activated T cells in patients with chronic HCV infection may facilitate the development of these features.

Our study has several strengths and limitations. We performed whole blood analysis of 22 cell populations and conducted *ex vivo* functional studies by mass cytometry in patients before, during and up to 12 weeks after DAA-mediated virological cure. The limited number of subjects may have limited our ability to detect more subtle changes possibly linked to HCV genotype or host genetic or risk factors features. An HIV mono-infected group could have been included. Finally, all patients were under ART and had low or undetectable HIV viral loads, precluding to detect interactions between HIV with HCV infections.

High-dimensional immune profiling of blood cells provided a broad and in-depth picture of the systemic immune dysregulation induced by chronic HCV. A salient finding was the observation of a profound derangement of the homeostasis of the immune system triggered by HCV characterized by a pro-inflammatory innate immune signature extending well beyond the clearance of HCV, especially in HCV/HIV patients. This inflammatory phenotype may contribute to the pathogenesis of systemic complications of chronic HCV infection.

Materials and Methods

Subjects

We conducted a longitudinal, non-interventional study in 10 patients with HCV, 10 patients with HCV and HIV (HCV/HIV), and 10 healthy subjects (controls) (Figure 1, Table S1). Ethical approval was obtained from the Commission cantonale d'éthique de la recherche sur l'être humain, Canton de Vaud, Switzerland (CER-VD, PB_2016-01464). The project was approved by the Scientific Board of the Swiss HIV Cohort Study (SHCS, project number 788). Study participants provided written informed consent. HCV-infected patients received IFNα-free, DAA-based therapy. Depending on the duration of DAA treatment, follow-up ranged from 6 to 9 months. HCV viral loads were measured at baseline, one or two

weeks after initiation of treatment, at the end of therapy, and 12 weeks thereafter to determine whether the patient achieved a sustained virologic response (SVR). For immune profiling, blood was collected in heparin tubes at baseline (within 4 weeks prior to the initiation of DAA-based therapy) and 12 weeks after therapy (SVR12) (#1 and #4, Figure 1a). To avoid bias linked to cell isolation or cryopreservation, blood was immediately either stabilized for frequency analysis or stimulated as described in Supplementary Materials and Methods. Blood was collected once in healthy controls. HCV or HIV viral loads were determined by the clinical laboratory of the Division of Immunology and Allergy of Lausanne University Hospital.

Processing and clustering of CyTOF data

Information about conjugation of antibodies, mass-tag barcoding, sample staining for CyTOF and data analysis are presented in the Supplementary Materials and Methods and Tables S6-8. Samples were acquired on a CyTOF 2 (Fluidigm) (25). FCS files were normalized using the MATLAB normalizer [26]. Debris were gated out manually and barcoded files were deconvoluted using OpenCyto-based boolean gating [27]. FlowSOM clustering was performed on 99.5th percentile scaled, hyperbolic arcsine transformed expression levels, using co-factor 5, for the markers CD1c, CD3, CD4, CD7, CD8, CD11b, CD11c, CD14, CD16, CD20, CD38, CD56, CD66b, CD123, CD141, HLADR and Slan [28]. We overclustered the data to 40 metaclusters, manually merged into 22 populations based on biological knowledge [9]. For dimensionality reduction, we performed Uniform Manifold Approximation Projection (UMAP) with a minimal distance of 0.2 on a dataset down-sampled to 5000 randomly selected cells per sample [29].

Statistical analysis

Data were analyzed in R with the lme4 package using generalized linear mixed models [9]. To control for batch effects, random effects were added for sample and batch. Thresholds for cytokine positivity were determined as the 99th percentile levels of matched unstimulated samples that were processed simultaneously and identically as stimulated sample in a subject and time point dependent fashion [30]. Median signal intensities (MSI) of cytokines and co-stimulatory or inhibitory markers were calculated and compared with linear models. For all methods, *p* values were extracted and corrected for multiple testing using the false discovery rate (FDR). We used a threshold of 5% to indicate statistical significance and of 15% to indicate a trend. Only FDR adjusted *p* values are shown. Statements regarding percentage increase or decrease are based on medians. For boxplots show the 75th and 25th percentile, the horizontal line the median and the whiskers hinges to 1.5-times the interquartile range. Heatmaps show normalized frequencies where the frequency of each population is scaled 0-1.

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Author contributions statement

JH, TR and TC designed the study. MC and DM recruited the patients. JH, TH and IS processed the blood samples. CF and GP gave advice and access to the CyTOF. JH performed CyTOF studies. JH and KO performed computational analysis of the data. JH, TR and TC analysed and interpreted the data. JH, TR and TC wrote the manuscript. All the authors revised the manuscript.

Members of the Swiss HIV Cohort Study

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Conflict of Interest Statement

The authors do not have any conflict of interest regarding this manuscript.

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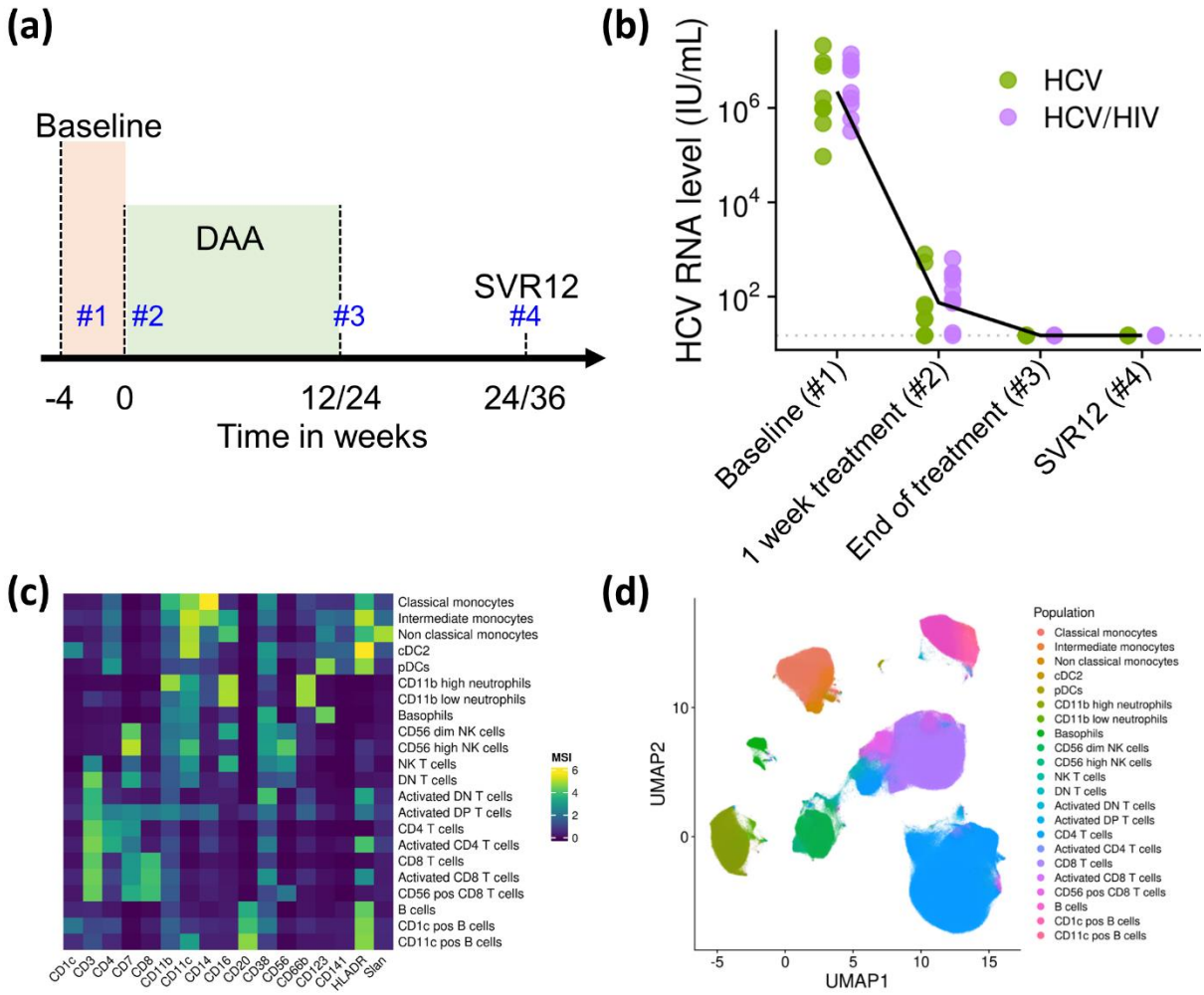

Figure 1. Study design, HCV viral loads and analyses of blood cell populations with mass cytometry workflow. (a) Blood samples were obtained within 4 weeks of the start of therapy (baseline, #1), after 1 week of treatment (#2), at the end of treatment (#3), and 12 weeks after the end treatment and sustained virologic response (SVR12) (#4). Four blood samples (#1 to 4) were collected for measurement of HCV viral loads and two blood samples (#1 and #4) were collected for immune profiling. **(b)** HCV viral loads. Each dot represents one single patient (some dots overlap). The solid line connects the median viral loads of each time point. RNA levels were similar in patients with HCV or HCV/HIV infections. The horizontal dotted line indicates the lower limit of detection of the test (15 IU/ml). **(c)** Heatmap showing expression of markers and blood cell populations. Each row corresponds to a manually annotated immune cell type based on the profile identified with the markers (columns) used for clustering. Data are expressed as median of hyperbolic arcsine transformed signal intensities (MSI). **(d)** Dimensionality reduction with UMAP performed with a random subset of 5000 cells per sample. Colors correspond to merged and annotated FlowSOM clusters (populations).

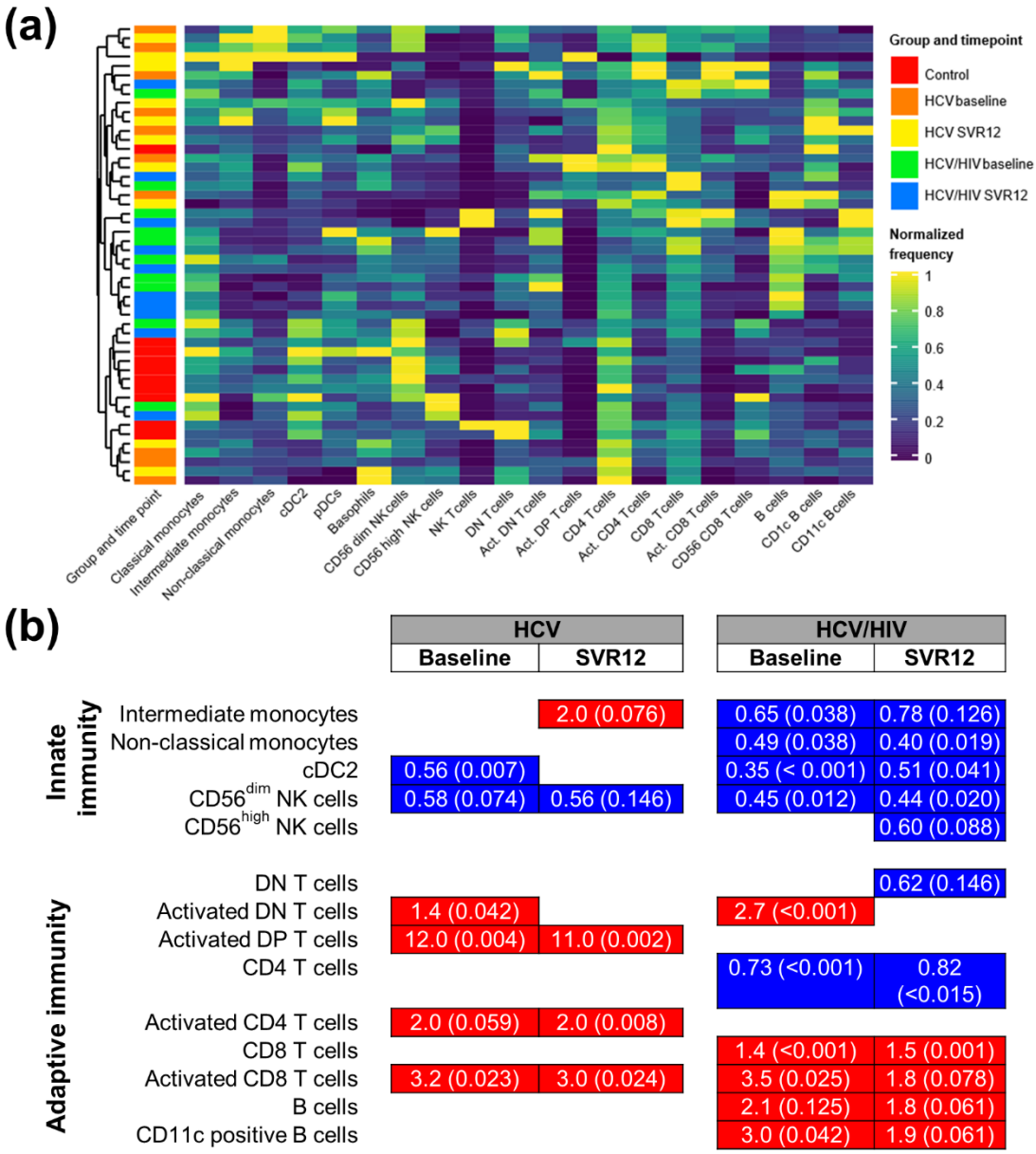

Figure 2. Changes of immune cell populations in patients infected with HCV or HCV and HIV. **(a)** Heatmap showing normalized frequencies of cell subsets in unstimulated samples at baseline and at SVR12. The scale of normalized frequencies ranges from 0 to 1. Row order was determined by hierarchical clustering. The group and timepoint column serve as a legend and was not used for clustering. **(b)** Summary of statistically significant changes in immune cell populations observed at baseline and at SVR12 when compared to healthy controls (N=10 in each group). Red, white and blue squares show increased, unchanged or decreased frequencies of the immune cell populations shown on the y-axis. Numbers in cells indicate fold-change and p value (in brackets). Medians of the frequency of cytokine positive cells and p values are provided in Tables S2 and S3.

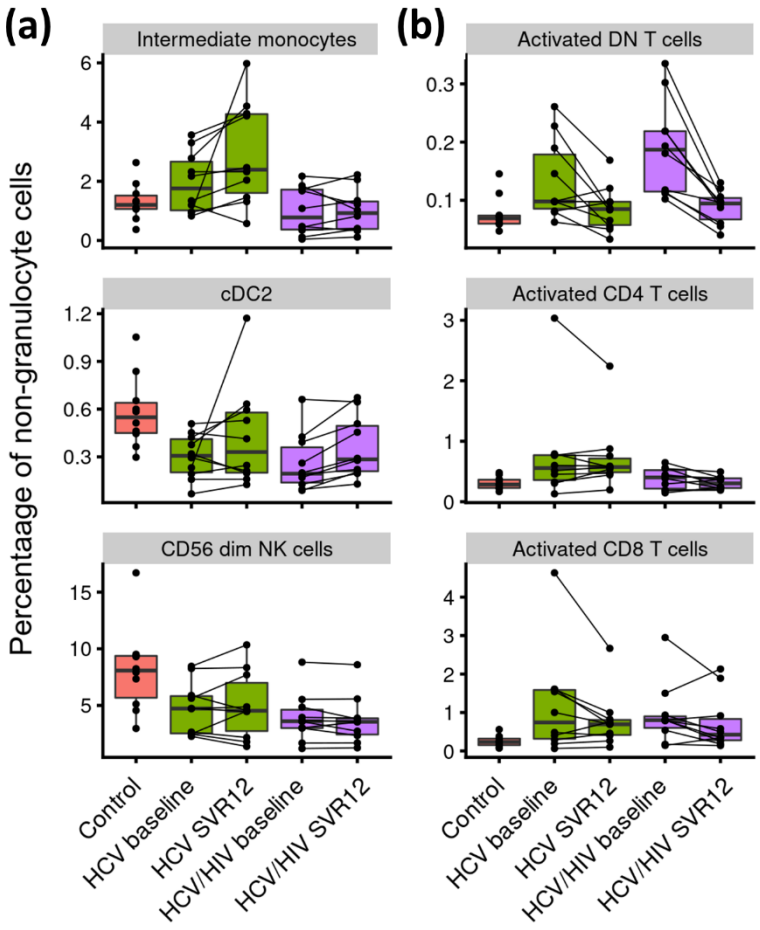

Figure 3. Proportions of innate and adaptive immune cells before and after HCV therapy. Box plots of the frequencies of intermediate monocytes, type 2 conventional DC (cDC2) and CD56^{dim} NK cells **(a)** and activated double negative (DN) T cells, activated CD4 T cells and activated CD8 T cells **(b)** at baseline and at SVR12. N=10 in each group. Each dot represents one individual subject. Statistical analyses were performed using mixed linear models. P values corrected for multiple testing using the false discovery rate are provided in Tables S2 and S3.

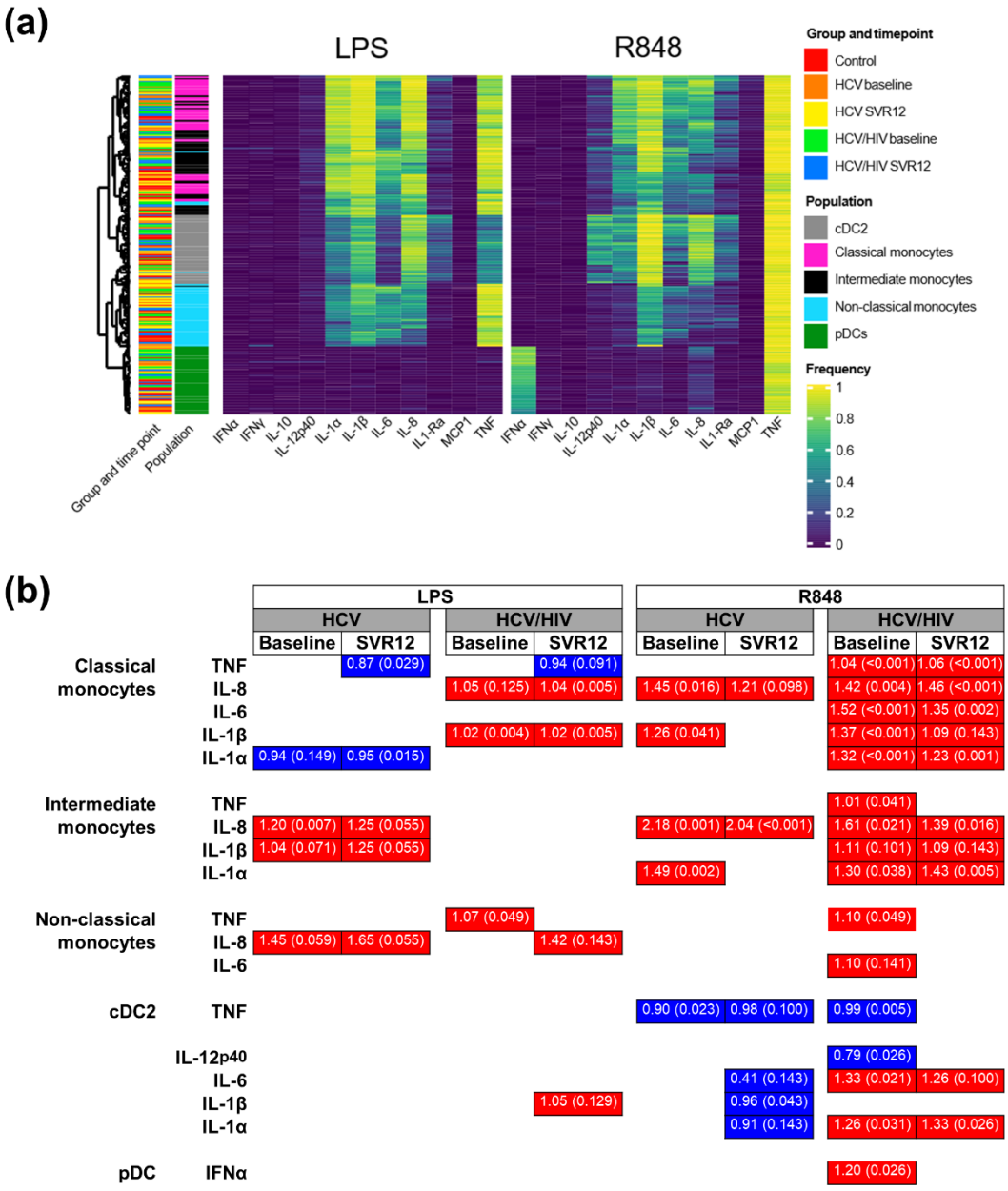

Figure 4. Cytokine response of innate immune cells in patients infected with HCV or HCV and HIV. (a) Frequency of cytokine-positive innate immune cells following stimulation with LPS or R848 of blood samples collected at baseline and SVR12. Row order was determined by hierarchical clustering. Left two columns serve as a legend and have not been used for clustering. **(b)** Summary of statistically significant changes in the frequency of cytokine-positive innate immune cells at baseline and at SVR12 when compared to healthy controls (N=10 in each group). Red, white and blue squares illustrate increased, unchanged or decreased frequencies. Numbers in each cell indicate fold-change and p value (in brackets). Medians of the frequency of cytokine positive cells and p values are provided in Tables S4 and S5.

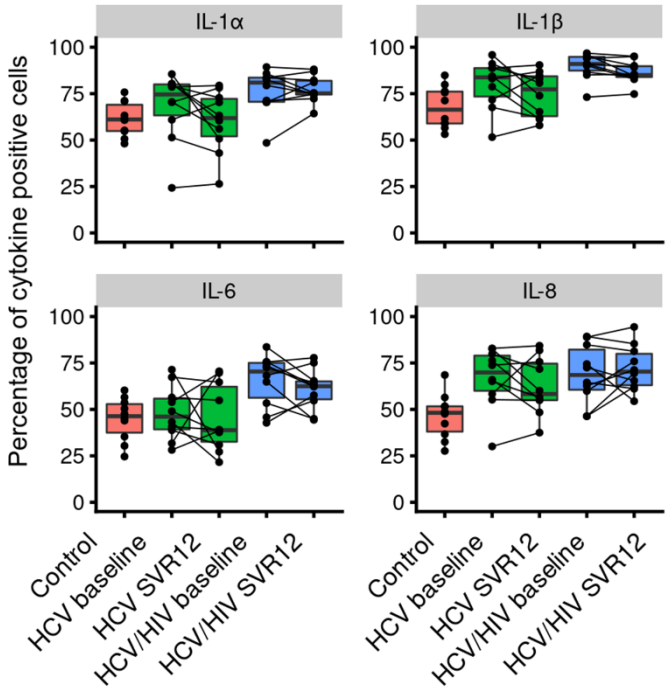

Figure 5. Proportion of cytokine-positive classical monocytes after stimulation with a TLR7/8 agonist. Box plots of the frequencies of classical monocytes positive for IL-1α, IL-1β, IL-6 and IL-8 after stimulation with R848 at baseline and at SVR12. N=10 in each group. Statistical analysis was performed using mixed linear models. P values corrected for multiple testing using the false discovery rate are provided in Tables S4 and S5.

The following supplementary files are available upon request:

Supplementary files:

1. Supplementary methods.

Supplementary Tables:

2. Table S1. Characteristics of patients and healthy subjects.
3. Table S2 Proportion of immune cell populations in the blood of healthy controls and HCV and HCV/HIV infected patients at baseline.
4. Table S3. Proportion of immune cell populations in the blood of healthy controls and HCV and HCV/HIV infected patients at SVR12.
5. Table S4. Cytokine expression by TLR ligand-stimulated innate immune blood cells isolated from healthy controls and from HCV and HCV/HIV infected patients (baseline).
6. Table S5. Cytokine expression by TLR ligand-stimulated innate immune blood cells isolated from healthy controls and from HCV and HCV/HIV infected patients (SVR12).
7. Table S6. Antibodies and barcodes.
8. Table S7. Reagents.
9. Table S8. Softwares.

Supplementary figures:

1. Figure S1. Analyses of blood cell populations with mass cytometry.
2. Figure S2. Staining pattern in ungated cells.
3. Figure S3. Minimal gating example.
4. Figure S4. Cytokine expression in healthy controls after LPS stimulation.
5. Figure S5. Cytokine expression in HCV infected patients after LPS stimulation.
6. Figure S6. Cytokine expression in HCV/HIV infected patients after LPS stimulation.
7. Figure S7. Cytokine expression in healthy controls after R848stimulation.
8. Figure S8. Cytokine expression in HCV infected patients after R848stimulation.
9. Figure S9. Cytokine expression in HCV/HIV infected patients after R848stimulation.
10. Figure S10. Baseline and induced expression of inhibitory and co-stimulatory molecules in cDC2.
11. Figure S11. Depletion of CD15 positive cells does not affect the frequency of other immune subsets.

6.3. Impact of the Timeliness of Antibiotic Therapy on the Outcome of Patients with Sepsis and Septic Shock.

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Summary:

With this research, we aimed to review the impact of the timeliness of antibiotic therapy on the outcome of patients with sepsis or septic shock. We searched MEDLINE, EMBASE, the Cochrane Library, Web of Science, Open-SIGLE databases, ClinicalTrials.gov, and the metaRegister of Controlled Trials on July 27, 2020, for relevant studies on the timing of antibiotic therapy in adult patients with sepsis or septic shock. The primary outcome measure was all-cause crude or adjusted mortality at reported time points. We included 35 sepsis studies involving 154,330 patients. Nineteen studies (54%) provided information on the appropriateness of antibiotic therapy in 20,062 patients of whom 16,652 patients (83%) received appropriate antibiotics. Twenty-four studies (68.6%) reported an association between time-to-antibiotics and mortality. Time thresholds associated with patient's outcome varied considerably between studies consisting of a wide range of time cut-offs (1 h, 125 min, 3 h or 6 h) in 14 studies, hourly delays (derived from the analyses of time intervals ranging from to 1 to 24 h) in 8 studies or time-to-antibiotic in 2 studies. Analyses of subsets of studies that focused on patients with septic shock (11 studies, 12,756 patients) or with sepsis (6 studies, 24,281 patients) yielded similar results. To conclude, two-thirds of sepsis studies reported an association between early administration of antibiotic therapy and patient outcome but the time-to-antibiotics metrics varied significantly across studies and no robust time thresholds emerged.

My contribution to this work:

I analysed part of the data, created figures, and revised the manuscript.

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Review

Impact of the timeliness of antibiotic therapy on the outcome of patients with sepsis and septic shock

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SUMMARY

Objectives: To review the impact of the timeliness of antibiotic therapy on the outcome of patients with sepsis or septic shock.
Methods: We searched MEDLINE, EMBASE, the Cochrane Library, Web of Science, Open-SIGLE databases, ClinicalTrials.gov and the metaRegister of Controlled Trials on July 27, 2020 for relevant studies on the timing of antibiotic therapy in adult patients with sepsis or septic shock. The primary outcome measure was all-cause crude or adjusted mortality at reported time points.
Results: We included 35 sepsis studies involving 154,330 patients. Nineteen studies (54%) provided information on the appropriateness of antibiotic therapy in 20,062 patients of whom 16,652 patients (83%) received appropriate antibiotics. Twenty-four studies (68.6%) reported an association between time-to-antibiotics and mortality. Time thresholds associated with patient's outcome varied considerably between studies consisting of a wide range of time cutoffs (1 h, 125 min, 3 h or 6 h) in 14 studies, hourly delays (derived from the analyses of time intervals ranging from to 1 to 24 h) in 8 studies or time-to-antibiotic in 2 studies. Analyses of subsets of studies that focused on patients with septic shock (11 studies, 12,756 patients) or with sepsis (6 studies, 24,281 patients) yielded similar results.
Conclusions: While two-thirds of sepsis studies reported an association between early administration of antibiotic therapy and patient outcome, the time-to-antibitiocs metrics varied significantly across studies and no robust time thresholds emerged.

Introduction

Current global estimates indicate that 49 million cases of sepsis occur annually worldwide with about 11 million deaths.[1] Sepsis results in mortality rates of about 10% in patients with sepsis and of more than 40% in patients with septic shock.[2] Administration of appropriate antimicrobial agents is a cornerstone of sepsis management guidelines and bundles.[3–8] However, there is considerable controversy on the target for time-to-antibiotic therapy in patients with sepsis.[9–14] Several studies indicated that early administration of antibiotics reduces sepsis mortality.[3–5,15–17] Yet, other studies found no association between early antibiotic therapy and patient's outcome.[18–21] This prompted us to review the literature on the timeliness of antibiotic therapy on the outcome of patients with sepsis with the aim to identify targets for time-to-antibiotics associated with favorable outcomes.

Materials and methods

Search strategy

The literature search was performed on July 27, 2020 in MEDLINE, EMBASE.com, and the Cochrane Library Wiley for relevant studies published any year and in any language. We also searched proceedings of conference from 2008 to 2020 using the Web of Science and unpublished studies using Open-SIGLE databases, the US National Institutes of Health Ongoing Trials Register ClinicalTrials.gov (https://clinicaltrials.gov) and the metaRegister of Controlled Trials (www.controlled-trials.com). We also reviewed the reference lists of articles for additional relevant studies. The search criteria used the following Medical Subject Headings terms: (sep-

Abbreviations: ICU, Intensive care units; ED, Emergency Department; LOS, Length of hospital stay; RR, Risk ratio; OR, Odds ratio; CI, Confidence interval; IQR, Interquartile range; SOFA Score, Sequential Organ Failure Assessment, IRB, Institute for Research in Biomedicine.

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sis OR severe sepsis OR septic shock) AND (antimicrobial agents OR antibacterial agents OR antibiotics) AND (Time-factor OR time-to-treatment OR time-to-antibiotic OR timing).

Data extraction and eligibility criteria

Two authors (SAA and TC) independently reviewed the articles selected for full-text screening of eligibility criteria. We included studies of adult patients (≥ 18 years) with sepsis, severe sepsis or septic shock as defined in the 1991 ACCP/SCCM Consensus Conference definitions (retrospectively labelled Sepsis-1), the 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference (retrospectively labelled Sepsis-2), The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3), codes (995.92 and 785.52) of the International Classification of Diseases, 9th Edition and the predisposition, infection, response and organ failure (PIRO) score.[2,22–24] For the analyses of the impact of the timing of antibiotic therapy in subgroups of sepsis patients, we assumed that patients with "severe sepsis" (according to Sepsis-1 and Sepsis-2) and the patients with "sepsis" (according to Sepsis-3) had similar grades of sepsis severity. Eligible studies included randomized controlled trials, observational cohort studies and analyses of prospectively collected datasets providing information on the timing of antibiotic therapy. We excluded animal studies, case reports, case studies, meta-analyses, editorials and letters to the editor.

Patient characteristics

We recorded information on the selection of patients, inclusion criteria, the duration and time period of the study, the setting (emergency department versus intensive care units versus wards), the study design and the total number of patients in each sepsis severity stratum. We also extracted data on other key study characteristics such as the set point (i.e. time zero), the time cutoffs or intervals used for assessing the timing and impact of antibiotic therapy, the assessment of the appropriateness of antibiotic therapy and the study endpoints. Criteria used for the analysis of antibiotic appropriateness were based on *in vitro* susceptibility of causative pathogens in case of microbiologically-documented infections or on antibiotic therapy management guidelines in case of clinically-documented infections.[25]

Subgroup analyses

We performed subgroup analyses to investigate the effects of sepsis severity strata (sepsis versus septic shock) on study endpoints. We also conducted post-hoc stratified analyses of studies that addressed the appropriateness of antibiotic administration.

Outcome measures

The primary outcome was all-cause mortality at the time points reported in the study. All but five studies[26–30] adjusted mortality using multivariable analyses which included covariates considered to be potential confounders such as age, gender, Acute Physiology and Chronic Health Evaluation II (APACHE II), Charlson index measures, site of infection, hypotension, SOFA score, lactate clearance, fluid resuscitation and the use of vasopressors. Secondary outcomes included admission to the ICU, length of ICU or of hospital stay (LOS), progression from sepsis/severe sepsis to septic shock and mortality at fixed time-points (one, three or twelve months) after hospital discharge. The principal summary measures for dichotomous outcomes were odds ratio (OR) with 95% confidence interval (CI) (as provided by the authors or calculated based on available data) and risk ratios (RR). Medians with interquartile range (IQR) were reported for continuous outcomes. Given that we did not perform a systematic review with meta-analysis, we do not provide information on cumulative OR, RR or on the heterogeneity of studies (I^2). Statistical analyses and figure design were performed using IBM SPSS Statistics version 26.0 (IBM Corp., New York, USA) and R statistical software 3.6.0 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Selection of studies

Fig. 1 shows the flow diagram for the selection of studies. We screened the abstracts of 4409 records and selected 94 articles for full-text screening. The Cohen's kappa coefficient for agreement between the two reviewers was 0.78.[23] After full-text screening, we included 35 studies, of which 34 were observational cohorts (retrospective: 20 studies, prospective: 14 studies) and one was an analysis of patients enrolled in a randomized controlled trial.

Study characteristics

Table 1 shows a summary of the 35 studies that included 154,330 patients (median: 1058 patients per study, range: 117 to 49,331). The enrolment period ranged from 1989 to 2020 (Fig. 2). The median duration of studies was 2.1 years (range: 0.4 to 15.5). The criteria used for the selection of patients varied between studies (Supplementary Table 1). The set point or "time zero", the time cutoffs (1, 3, 6, 12, 24 or 48 h) and the time intervals (hourly increments up to 24 h) used for assessing the timing of antibiotic therapy varied considerably between studies (Table 1). The settings were emergency departments in 21 studies, ICUs in 8, hospital wards in 1 and a combination of emergency departments, ICUs and wards in 5 (Table 1). Septic shock and sepsis with or without organ dysfunction occurred in 51,094 patients (33.1%), 61,094 patients (39.6%) and 13,444 patients (8.7%), respectively. In nine studies, 28,698 patients (18.6%) with sepsis, severe sepsis and septic shock were grouped together. All but one study used mortality endpoints, which was adjusted mortality in 29 studies (83%), all-cause crude mortality in five (14.3%) and a combination of adjusted and unadjusted mortality in one. The time points for assessing mortality were the end of the ICU or the hospital stay in 24 studies (68.6%), day 28 or day 30 in 7 (20.0%), various time points in three (8.6%) and one year in one (2.8%).

Impact of the timeliness of antibiotic therapy on mortality

In 24 studies (68.6%) time-to-antibiotics was associated with in-hospital mortality (18 studies),[3–5,15–17,27,28,31–40] mortality at other time points (5 studies)[38,41–44] and the ICU or hospital length of stay (one study)[30] (Table 1). The time thresholds associated with patient's outcome consisted of various time cutoffs (1 h, 125 min, 3 h or 6 h) in 14 studies, hourly delays (based on the analyses of time intervals ranging from to 1 to 24 h) in 8 studies and time-to-antibiotic in two studies. Fig. 3 shows the odds ratio plots for mortality according to three time-to-antibiotics parameters (hourly delays, 1 h and 3 h time cutoffs). Arrival or registration in the emergency department (ED) was identified as time zero in 10 studies,[16,20,27,29,34,36,38,45–47] of which 4 reported an association between time cutoffs (hourly delays, 1 h, 3 h) and patients outcome in a multivariable analysis.[16,34,36,38] Recognition of symptoms or signs of sepsis was considered as time zero in 9 studies,[17,26,31,33,40,41,44,48,49] of which 5 reported an association between time cutoffs (1 h, 125 min, 3 h) and mortality in multivariable analyses.[17,31,33,40,44]

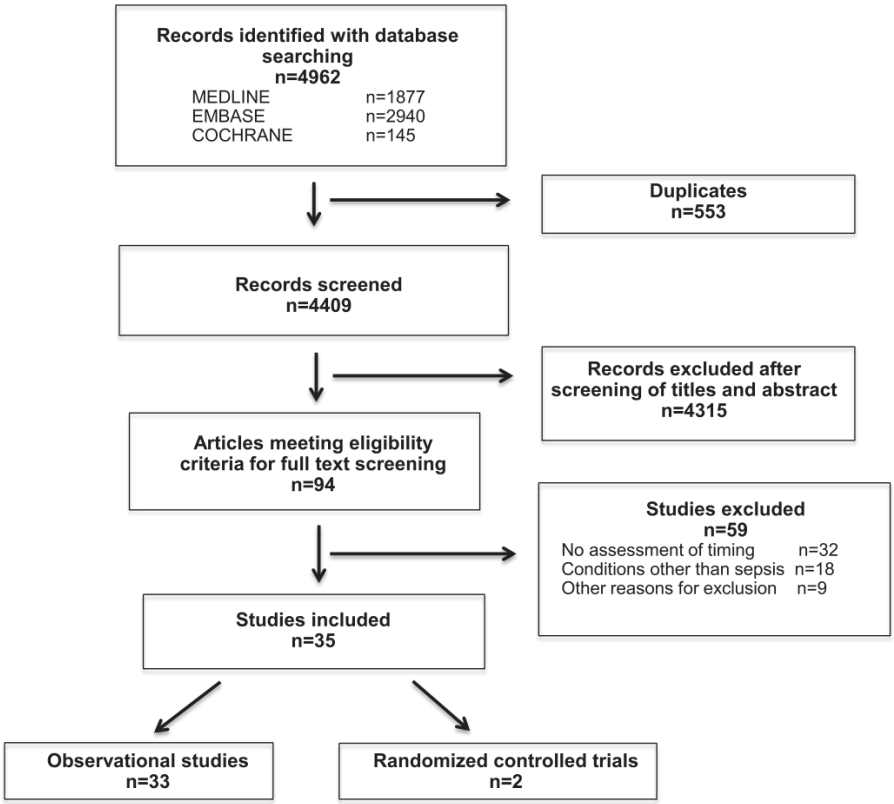

Fig. 1. Flowchart of study selection
Fifty-nine studies were excluded after full text screening. Thirty-two studies did not evaluate the timing of antimicrobial therapy, 18 reported on conditions other than sepsis (bacteremia in 10, febrile neutropenia in 5 and pneumonia in 3). Nine studies were excluded as they focused on predictors for delayed antibiotic administration ($n=5$) or enrolled pediatric patients ($n=4$).

Appropriateness of antibiotic therapy

Nineteen studies (54%) provided information on the appropriateness of antibiotic therapy in 20,062 patients (13.0% of the total patient population) of whom 16,652 patients (83%) received appropriate antibiotics. Among these 19 studies, 11 based the assessment on *in vitro* susceptibility criteria and 8 on combined clinical and microbiological criteria. Ten of these 19 studies reported an association between time-to-antibiotics and mortality with hourly delays or using various timecutoffs ranging from 1 h, 3 h or 6 h delays and onwards. Three studies reported on associations between one-hour delay or hourly delays and mortality among patients with septic shock.[3,30,50] All but one study[30] conducted multivariable analyses.

Septic shock and sepsis studies

We then examined the effects of the timing of antibiotic therapy in studies that included patients with septic shock or with sepsis.

Septic shock

Twenty-one studies (60%) enrolled patients with septic shock, of which 11 specifically analysed the impact of the time-to-antibiotic on mortality in 12,756 patients.[3,16,18,26,30,40,41,46–48,50] In five studies that included 79.6% of the patients, the in-hospital mortality or the length of stay in the ICU increased significantly with each hour delay in the administration of antibiotics[3,16,30,40] or with a start of antibiotics more than 3 h after triage in the emergency department.[50] No association between early antibiotic therapy and outcome was noted in the other six studies.[18,26,41,46–48]

Sepsis

Twenty studies (57.1%) enrolled patients with sepsis and organ dysfunction but without shock, of which six analysed the relationship between time-to-antibiotic and mortality in 24,281 patients.[16,21,26,32,37,47] In three studies that included 92.0% of the patients, mortality increased with an hourly delay in antibiotic therapy[16], a longer time-to-antibiotic[32] or a delay of more than 6 h in the administration of empirical antibiotic therapy.[37] Of note, one of these studies reported an 8.0% risk of progression to septic shock when antibiotic therapy was delayed.[32] There was no association between early antibiotic therapy and mortality in the other three studies.

Discussion

Overall, two-thirds of the studies included in this review reported an association between time-to-antibiotics and mortality (Table 1). Yet, the time metrics for antibiotic delivery associated with patient's outcome varied considerably among studies and the time thresholds were wide ranging from one to six hours. The studies that identified an increase in mortality with each hour delay in the start of antibiotics used risk-adjusted linear models over time intervals of 6 to 12 h, which are likely to be influenced by the

Table 1
Study characteristics.

Author, year, reference	Setting	Study design, number of centers or units	Number of patients included	Classification by sepsis severity (number of patients)	Set point for assessing timing of antibiotic therapy	Time cutoffs and intervals	Primary endpoint	Assessment of antibiotic therapy (percent with appropriate therapy)	Main findings
Abe, 2019[48]	ICU	Prospective observational, 59 centers	1124	Severe Sepsis (421) Septic shock (703)	Recognition of sepsis at the ED, ward or ICU	0–60, 61–120, 121–180,181–240, 241–360, 361–1440 min and continuous variable	In-hospital mortality	No	No association between time to antibiotic administration (1 h or 3 h cutoffs or time as continuous variable) and in-hospital mortality
Alam, 2018[26]	ED	Randomized controlled open-label trial, 34 centers	2631*	Sepsis (1003) Severe sepsis (1525) Septic shock (103)	Recognition of sepsis or shock by paramedics	Hourly up to 4 h and more than 4 h	All-cause crude mortality at day 28	No	No impact of prehospital antibiotic administration or of time to antibiotics administration on day 28 mortality
Ascuntar, 2020[46]	ED and ICU	Prospective observational cohort study, 3 centers	2454	Sepsis, septic shock (869)	Admission to the ED	1 h, 3 h	In-hospital mortality	Yes (76.5%)	No association between antibiotic administration within 1 h or 3 h and in-hospital mortality
Ballester, 2018[27]	ED	Retrospective observational study, single center	153	Sepsis, severe sepsis and septic shock (153)	ED arrival	Median door-to-antibiotic time	In-hospital crude mortality	No	Door-to-antibiotic time were associated with mortality.
Bloos-1, 2014[19]	ICU	Prospective observational multicenter cohort study, 44 centers	1011	Severe sepsis (379) Septic shock (632)	Documentation of first infection-related organ dysfunction	0 to 1 h, 1 to 3 h, 3 to 6 h, more than 6 h	All-cause crude mortality at day 28	Yes (58%)	No linear association between time to antibiotic and day 28 mortality
Bloos-2, 2017[42]	ICU	Prospective multicenter cohort study 44 centers	4183	Severe sepsis (1001); septic shock (3182)	Documentation of first infection-related organ dysfunction	Hourly (time range not specified)	All-cause day 28 mortality	Yes	2% increase in 28-day mortality for each 1 h delay in initiation of antimicrobial therapy
Castaño, 2019[45]	ED	Prospective cohort study, three centers	705	Severe sepsis (632) Septic shock (73)	ED arrival	Hourly (time range not specified)	Inpatient mortality and length of hospital stay	Yes (75% - 85.6% dependent upon criteria used)	No association between hourly delay in antibiotic administration and in-patient mortality
De Groot, 2015[20]	ED	Prospective observational cohort study, three centers	1168	Sepsis (1168) PIRO scores (1–7: 413; 8–14: 532; >14: 223)	Registration in the ED	0 to 1 h, 1 to 3 h, more than 3 h	Number of surviving days outside the hospital at day 28	Yes (76%)	No association between time to antibiotics and surviving days outside hospital or mortality.
Ferrer-1, 2009[33]	ICU	Prospective observational study, 77 ICUs	2796	Severe sepsis and septic shock (2796)	Symptom recognition	0-1 h, 1–3 h, 3–6 h, no antibiotic in the first 6 h	Propensity-adjusted hospital mortality	No	Early antibiotic therapy (within 1 h vs. no treatment within 6 h) associated with lower hospital mortality
Ferrer-2, 2014[15]	ED or ICU	Retrospective analysis of a prospectively collected dataset	17,990	Severe sepsis (6432) and septic shock (11,558)	Triage (ED) or symptoms recognition (ICU)	One-hour time period up to 6 h	Adjusted hospital mortality	No	More than 2-h delay in antibiotic administration associated with increase hospital mortality
Gaieski, 2010[4]	ED	Retrospective cohort study, single center	261	Severe sepsis (126) Septic shock (135)	ED triage	One-hour time point up to 5 h	Adjusted in-hospital mortality	Yes (85%)	Association between time (one hour) from triage to appropriate antibiotic administration and in-hospital mortality.
Husabo, 2020[43]	ED	Retrospective cohort study, 24 centers	1559	Sepsis (1559)	ED triage	0-1 h, 1-2 h, 2-3 h, 3-4 h and > 4 h; time to first antibiotic dose	30-day all-cause mortality	No	Lower mortality rates when antibiotics administred between 2 and 3 h after ED admission compared to ⟨2 h or ⟩ 3 h
Jalili, 2013[29]	ED	Prospective cohort study, one single center;	145	Sepsis (145)	ED arrival	0 to 1 h, 1 to 2 h, more than 2 h	Crude sepsis-related death or discharge	Yes	Association between door-to-antibiotic time and mortality, but only in patients with APACHE II scores of 21 or higher

(*continued on next page*)

Table 1 (*continued*)

Author, year, reference	Setting	Study design, number of centers or units	Number of patients included	Classification by sepsis severity (number of patients)	Set point for assessing timing of antibiotic therapy	Time cutoffs and intervals	Primary endpoint	Assessment of antibiotic therapy (percent with appropriate therapy)	Main findings
Joo, 2014[34]	ED	Retrospective cohort study (sepsis registry), single center	591	Severe sepsis and septic shock (591)	ED arrival	0–3 h versus greater than 3 h	Adjusted in-hospital mortality	No	Association between timely (within 3 h) antibiotic administration and improved outcome (survival, reversal of organ failure and shorter length of stay)
Kim, 2018[35]	ED	Retrospective, observational cohort study, single center	117	Sepsis and septic shock (117)	Time from triage to antibiotic(s)	One-hour intervals up to 3 h and >3 h	Unadjusted and adjusted in-hospital mortality	No	Association between time from triage to antibiotic administration and in-hospital mortality
Ko, 2020[50]	ED	Prospective observational cohort study, 10 centers	2229	Septic shock (2229)	ED triage	0 - 1 h, 1-2 h, 2-3 h, > 3 h and 0–3 h	In-hospital mortality	Yes	Early antibiotic (<1 h) associated with lower in-hospital mortality with propensity score analysis. No linear association between hourly delays and in-hospital mortality.
Kumar, 2006[3]	ICU	Retrospective analysis of three cohorts, 10 centers (14 ICUs)	2154	Septic shock (2154)	Initial onset of recurrent or persistent hypotension	One-hour time point up to 6 h, plus 4 additional time cutoffs up to more than 36 h	Adjusted survival to hospital discharge	Yes (100%)	Association between hourly delay in appropriate antibiotic administration and mortality
Liu, 2017[16]	ED	Retrospective study, 21 hospitals	35,000	Sepsis (12,122) Severe sepsis (18,210) Septic shock (4668)	ED registration	30-min increment from 0 to 6 h	Risk-adjusted hospital mortality	No	For each sepsis severity stratum, hourly delays in antibiotic administration (up to 6 h) associated with increased adjusted odds ratios for hospital mortality
Londoño, 2018[36]	ED	Prospective cohort study, three hospitals	884	Severe sepsis and septic shock (884)	ED arrival	Less than 1 h or 3 h, hourly up to 24 h	Adjusted in-hospital mortality	Yes (83%)	Administration of antibiotic within 1 or 3 h associated with reduced mortality
Lueangarum 2012[44]	Medical wards	Retrospective cohort study, single center	229	Sepsis (31) Severe sepsis (58) Septic shock (140)	Sepsis recognition	0 to 1 h, 1 to 6 h, more than 3 or 6 h	Crude 28-day and overall mortality	Yes (70%)	More than 3-h delay in antibiotic administration associated with higher overall mortality
Nygard, 2014[37]	Infectious diseases or cardiology ward and ICUs	Prospective observational study, single center	220	Severe sepsis (220)	Hospital admission	< 6 h, ≥ 6h	In-hospital mortality	Yes (76–82%)	Delay in administration of antibiotics of 6 h or more associated with increased mortality
Peltan, 2019[38]	ED	Retrospective cohort study, fours centers	10,811	Sepsis and septic shock (10,811)	ED arrival	Door-to-antibiotic time, ≤ 1 h vs. > 1 h, ≤ 1 h vs. each hour beyond first hour up to 6 h, ≤ 3 h vs. > 3 h	Adjusted one-year mortality, plus in-hospital, 30-day, 90-day and 1-year mortality	No	Door-to-antibiotic time (3-h cutoff) associated with increased adjusted odds of one-year mortality
Peng, 2018[31]	ICU	Retrospective study, single center	541	Sepsis (382) Septic shock (159)	Diagnosis of sepsis	≤ 1 h, 1–24 h, 24–48 h, > 48 h	ICU and hospital mortality	Yes	Delayed appropriate antibiotic therapy associated with higher ICU and in-hospital mortality

(*continued on next page*)

Table 1 (*continued*)

Author, year, reference	Setting	Study design, number of centers or units	Number of patients included	Classification by sepsis severity (number of patients)	Set point for assessing timing of antibiotic therapy	Time cutoffs and intervals	Primary endpoint	Assessment of antibiotic therapy (percent with appropriate therapy)	Main findings
Pruinelli, 2018[17]	All hospitalized patients	Retrospective cohort study, six hospitals	5072	Severe sepsis and septic shock (5072)	Identification of sepsis (ICD-9 codes)	0 to 6 h	Adjusted in-hospital mortality	No	Association between antibiotic delivery delays above 125 min and mortality
Puskarich, 2011[18]	ED	Pre-planned analysis of RCT, 3 centers	291	Septic shock (291)	Triage in the ED and shock recognition	Hourly up to 6 h	Adjusted in-hospital mortality	Yes (91%)	No association between time to antibiotics (up to 6 h) and in-hospital mortality
Ryoo, 2015[41]	ED	Retrospective cohort study, single center	426	Septic shock (426)	Shock recognition	One-hour increment up to 5 h	Adjusted 28-day mortality	Yes (98%)	No association between hourly delay in antibiotic administration and mortality
Seok, 2020[47]	ED	Prospective cohort study, single center	482	High grade infection/sepsis (279), septic shock (203)	ED arrival	0-1 h, 0–3 h, hourly	Day 7, day 14 and day 28 mortality	Yes (77.8%)	No association between time to antibiotics and outcomes in overall and subgroup analyses including (patients with septic shock or with appropriate antibiotics)
Seymour-1, 2017[5]	ED	Retrospective cohort study, 149 hospitals	49,331	Severe sepsis (26,995) Septic shock (22,336)	First medical contact (prehospital) and in ED	Hourly up to 12 h	Risk-adjusted in-hospital mortality	No	Longer time to the administration of antibiotics associated with higher risk-adjusted in-hospital mortality
Seymour-2, 2017[39]	ED	Retrospective cohort study, nine hospitals (21 EMS)	2683	Sepsis and septic shock (2683)	First medical contact	0 to 6 h, > 6 to 12 h, > 12 h	Risk-adjusted in-hospital mortality	No	ED antibiotic delay associated with in-hospital mortality
Suberviola Canas, 2015[40]	ICU	Prospective observational cohort study	342	Septic shock (342)	Documentation of septic shock	0 to 1 h, 1 to 6 h, > 6 h	ICU and in-hospital mortality	Yes (88%)	Association between antibiotic treatment delay and increased mortality
Tan, 2019[28]	ED	Retrospective chart review, single center	261	Sepsis (41), severe sepsis (76) and septic shock (144)	Sepsis recognition	≤ 3 h, > 3 h	In-hospital mortality	No	Lower in-hospital mortality in patients who received antibiotics within 3 h
Whiles, 2017[32]	ED	Retrospective cohort study	3929	Severe sepsis (3929)	ED triage or ED arrival time	One-hour time point up to 5.99 h, 6 to 8.99 h, 9 to 11.99 h, 12 to 17.99 and 18 to 24 h	Adjusted in-hospital mortality. Progression from severe sepsis to septic shock	No	Longer time to initial antimicrobial administration associated with mortality and progression to septic shock
Wisdom, 2015[21]	ED	Retrospective review of patients presenting to ED with sepsis, single center	220	Sepsis (102) Severe sepsis (118)	ED triage by nurse	≤1 h, 1 to 3 h, 3 to 6 h, > 6 h	Adjusted in-hospital mortality	No	No association between time from triage to administration of antibiotic and mortality in the entire cohort; trend in patients with severe sepsis who received antibiotics after 6 h
Yokota, 2014[49]	ICU	Retrospective cohort study, single center	1279	Severe sepsis (403) Septic shock (876)	Severe sepsis recognition	0-1 h	Adjusted in-hospital mortality	Yes (74%)	No association between antibiotic administration and reduced mortality
Zhang, 2015[30]	All hospitalized patients	Retrospective cohort study, single center	1058	Severe sepsis (311) Septic shock (747)	Documentation of *in-vitro* susceptibility to antibiotics	0-1 h, hourly up to 24h	ICU and hospital length of stay	Yes (70%)	Association between time to appropriate antibiotic therapy (1-h increments) and ICU or hospital LOS

Abbreviations: APACHE II: Acute Physiology, Age, Chronic Health Evaluation II. ED: emergency department. EMS: emergency medical services. H: hour(s). ICU: intensive care unit. LOS: length of hospital stay. Min: minutes. PIRO: predisposition, infection, response, and organ dysfunction. RCT: randomized controlled trial.

* after exclusion of 41 patients with other diagnoses.

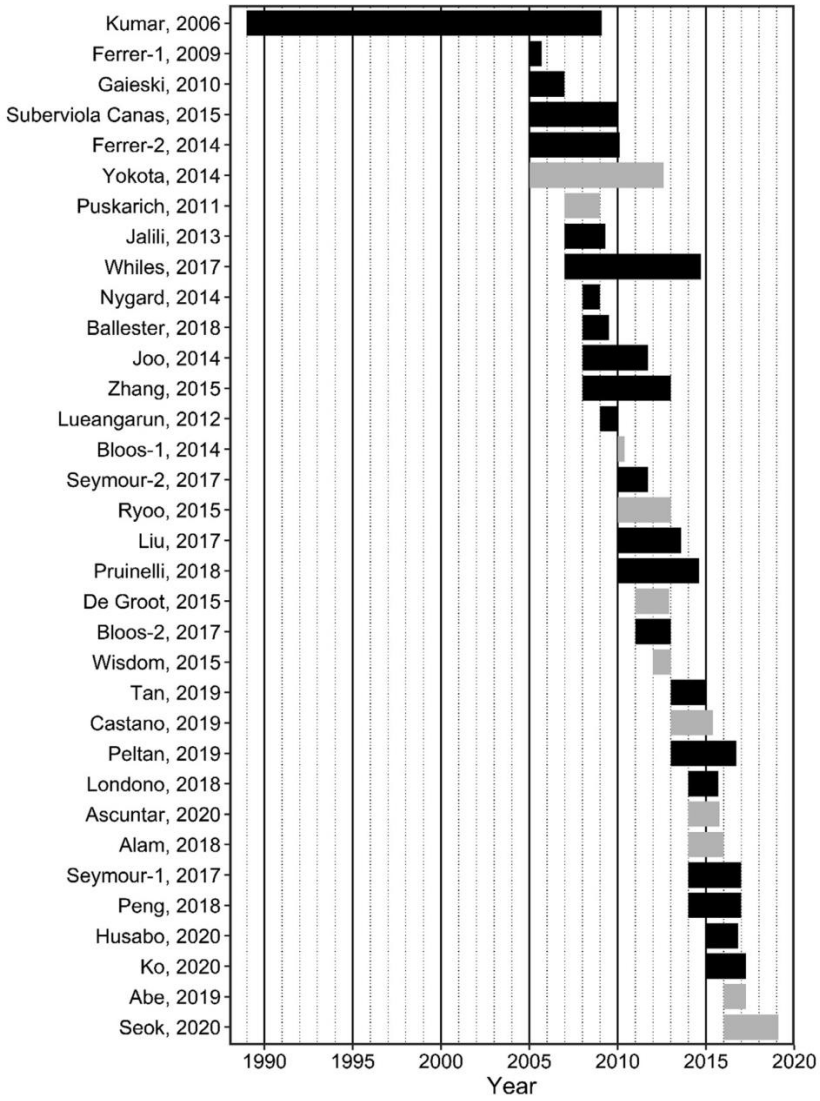

Fig. 2. Study enrolment periods
The graph displays the patient's enrolment period of the 34 studies that provided this information. The black or gray filling color symbolizes studies that found (black) or did not find (gray) an association between time-to-antibiotics and patient's outcome.

increased odds of mortality associated with long delays in the initiation of antibiotic therapy (Fig. 3).[3,5,16,38,42] Of note, the largest studies did not find associations between early antibiotic delivery and patient's outcome.[19,20,45–48] Therefore, the available data do not allow making recommendation on the timing of antibiotic dispensation with a great level of precision in patients with sepsis.[6,8–11]

With a 3-fold larger number of included studies, this review supports the results of two systematic reviews and meta-analyses.[51,52] In a review of 11 studies, Sterling et al. reported no survival benefit with dispensation of antibiotics within one hour of detection of severe sepsis or septic shock or within three hours of triage in the emergency department.[51] Likewise, a meta-regression analysis of 13 studies concluded that there was no difference in mortality between patients receiving antibiotics within one or three hours after the onset of sepsis.[52] In contrast, in a meta-analysis of 10 studies Johnston et al. found a 33% reduction in the odds of mortality among patients in whom antibiotics were administred within one hour.[53] Yet, their findings were largely influenced by one study that reported a 7.5% linear increase in the risk of mortality after adjusting for numerous covariables including geographic locations.[15] Other limitations are the inclusion of studies that did not provide information on the type of antibiotics administered and tying together patients who received antibiotics over long time intervals (more than one hour to six hours) after the arrival in the emergency department.

The need for stratifying recommendations for time-to-antibiotic according to the severity of sepsis is well recognized.[10,11] Given the extremely high mortality rates of septic shock, antibiotics should be administered immediately in patients with septic shock. Sepsis is a continuum with no clear-cut zone of rarity,[54] hence the difficulty of providing treatment with robust predictive validity across a broad range of disease probability. Notwithstanding that delays were also associated with increased mortality among patients with

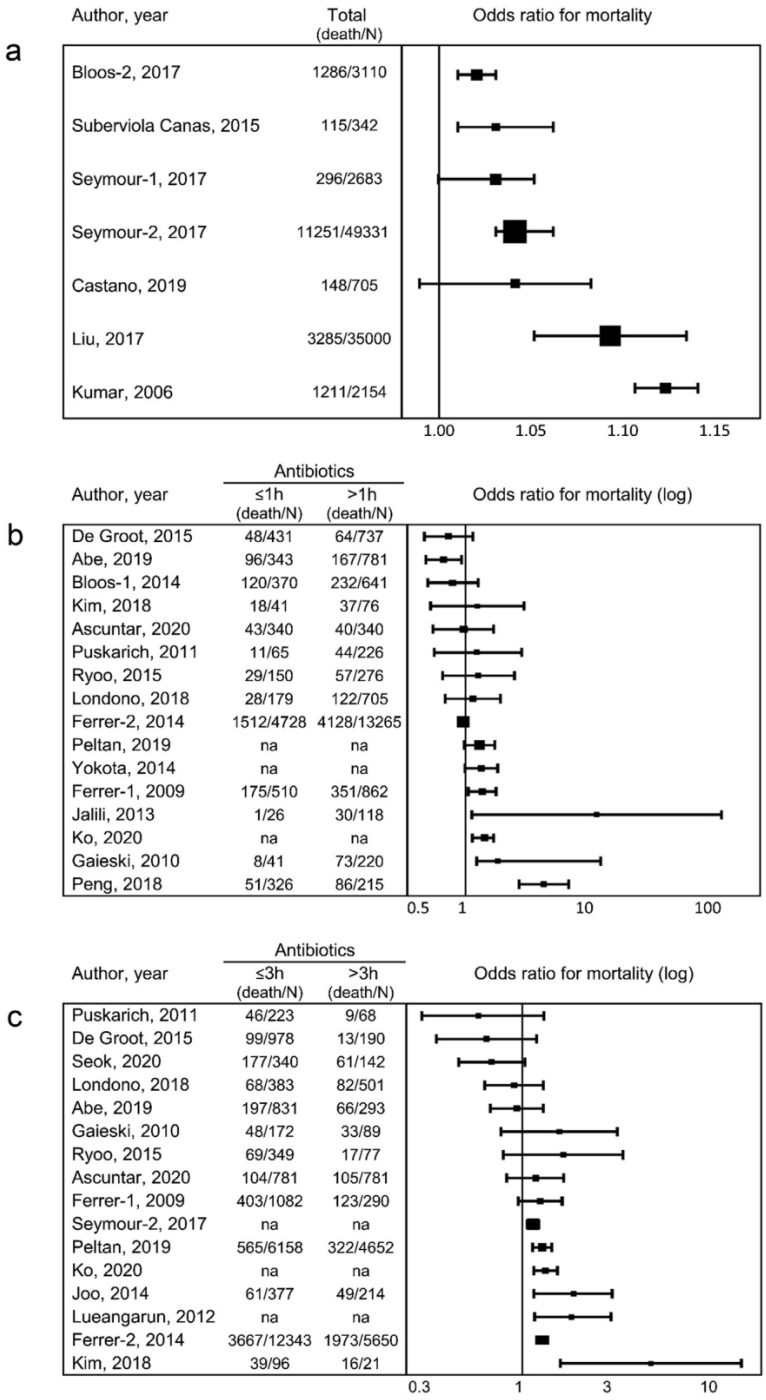

Fig. 3. Timeliness of antibiotic therapy and mortality of sepsis. Odds ratio plots for mortality with 95% confidence intervals according to the time-to-antibiotics reported as hourly delays (panel a), 1 h (panel b) or 3 h (panel c) time cutoffs. Data were available from 28 studies.

sepsis, no clear time threshold emerged from available data for this subgroup. Conceivably, time thresholds for the administration of antibiotics in patients with suspicion of sepsis could be tailored to the likelihood of infection. This approach is supported by a recent review by Naucler et al. on the outcome of patients with bacterial infections of different sources and degrees of severity.[55] While prompt antibiotic therapy was recommended for patients with septic shock and bacterial meningitis, the authors did not find evidence of worse outcome when initiation of therapy was delayed (e.g. by 4 to 8 h) in patients with less severe infections. A perilous tradeoff of delayed therapy is the risk of progression from sepsis to septic shock. This was addressed in only one of the

studies reviewed here and was found to be 8%.[32] Assuming a 10% risk of death in sepsis and 40% in septic shock, an 8% risk of transition from sepsis to septic shock would result in an increase of mortality from 10 to 12.4%. Finding an equipoise between potential morbidity criteria benefit and an increased risk of mortality is a delicate balancing act. Immunocompetent, non-neutropenic patients with suspected sepsis without shock and a low probability of infection could be a suitable patient population to investigate whether watchful clinical observation and prompt initiation of antibiotic upon documentation of infection is feasible and safe.

Strengths of the current review are a large sample size, a rigorous assessment of eligibility increasing the reliability of the results, subgroup analyses according to sepsis severity defined *a priori* and post hoc analyses of studies addressing the appropriateness of antibiotic therapy. Limitations predominantly relate to the risk of bias in the included studies inherent to their observational design. A high degree of heterogeneity regarding the inclusion of patients with various duration of sepsis and degrees of sepsis severity, the use of differents definitions of time zero and the lack of information on time-to-adequate antibiotic therapy may also impact on the robustness of the findings. Few studies provided information on the number of patients in each sepsis severity stratum, considerably limiting the additional value of cumulative odd ratios or relative risks. Of note, no study provided information on source control and therapeutic drug monitoring. Future studies on this topic should provide information on these critical parameters.

Conclusions

Two-thirds of studies included in this review reported an association between early antibiotics and mortality. However, the time metrics linking early allocation of antibiotics with mortality varied significantly across studies and no robust time threshold emerged from the overall study population or from subsets of studies that included patients with sepsis or septic shock.

Declarations of Competing Interest

None.

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Author's contributions

Conception and design of the study: SAA and TC. Analyses of the data: SAA, FD, ITS and TC. Contribution of materials/analysis tools: SAA, FD, ITS and TC. Writing of the manuscript: SAA, FD, ITS and TC. All authors read and approved the final version of the manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2021.03.003.

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