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**Impact de la modulation β_1 -adrénergique sur la balance lymphocytaire dans
le choc septique expérimental**

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Abbreviations

AC: adenylate cyclase	CXCR2: chemokine (C-X-C motif) receptor
ACCP: american college of chest physicians	2
AKT: protein kinase B	DAMP: damage-associated molecular
ANS: autonomic nervous system	pattern
APACHE: acute physiology and chronic	DC: dendritic cell
health evaluation	DIC: disseminated intravascular
AR: adrenoreceptor	coagulation
ATP: adenosine tri-phosphate	DIL-AC-LDL: human dil acetylated low
Bax: bcl-2–associated X	density lipoprotein
Bcl-2: B-cell lymphoma 2	DNA: deoxyribonucleic acid
C.: control	dP/dtmax: derivative of pressure over
CaMKII: protein kinase Calcium /	time maximum
calmodulin	dP/dtmin: derivative of pressure over time
CAMP: cathelicidin anti-microbial peptide	minimum
cAMP: cyclic adenosine monophosphate	E.: esmolol
CD: cluster of differentiation	ECE-1: endothelin converting enzyme-1
cGMP: cyclic guanosine monophosphate	EDTA: ethylenediaminetetraacetic acid
CI: cardiac index	eNOS: endothelial nitric oxide synthase
CLP: caecal ligation and puncture	ES: embryonic stem
CO: cardiac output	ET-1: endothelin-1
CPI: cardiac power index	FasL: Fas Ligand
CVP: Central venous pressure	FBS: fetal bovin serum

GATA-3: trans-acting T-cell-specific

transcription factor

GBD: global burden of disease

GM-CSF: granulocyte-macrophage colony-stimulating factor

G_s stimulatory G proteins

GSH: reduced glutathione

GVHD: graft-versus-host disease

HEPES: acide 4-(2-hydroxyéthyl)-1-pipérazine éthane sulfonique

HIV: human immunodeficiency virus

HLA-DR: human leukocyte antigen-DR isotype

HMGB-1: high-mobility group box-1

HMGB1: high-mobility group box 1 protein

HMVEC: human cardiovascular endothelial cells

HO-1: heme oxygenase-1

HR: heart rate

HSP: heat shock protein

ICON: intensive care over nations

ICU: intensive care unit

IFN- γ : interferon- γ

Ig: immunoglobulin

IL: interleukine

IL-1RA: IL-1 receptor antagonist

iNOS: inducible nitric oxide synthase

IP: intraperitoneal

IV: intravenous

JNK: c-jun N-terminal protein kinase

KO: knock out

L-NAME: L-NG-nitroarginine methyl ester

L-NMMA: L-N-monomethyl arginine

LBP: LPS binding protein

LPS: lipopolysaccharide

LV: left ventricular

LVEF: left ventricular ejection fraction

MAP: mean arterial pressure

MAPK: mitogen-activated protein kinase

MCP-1: monocyte chemo-attractant protein 1

MHC: major histocompatibility complex

MMP-1: matrix metalloproteinase 1

mRNA: messenger ribonucleic acid

MyD88: myeloid differentiation factor 88

NADPH: nicotinamide adenine dinucleotide phosphate

NE: norepinephrine

NF- κ B: nuclear factor- κ B	PMA: phorbol myristate acetate
NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells	PRR: pathogen recognition receptor
NF- κ B: nuclear factor- kappa B	qSOFA: quick sequential organ failure assessment
NK: natural killer	RIG-I: retinoic acid-inducible gene-I
NLR: NOD-like receptor	RLR: RIG-I-like receptor
nNOS: neuronal nitric oxide synthase	ROR- γ t: Retinoic-acid-receptor-related orphan nuclear receptor gamma
NO: nitric oxide	ROS: reactive oxygen species
NOS: nitric oxide synthase	RPMI: Roswell Park Memorial Institute medium
ONOO ⁻ : peroxynitrite	RV: right ventricular
p-eNOS: phosphorylated endothelial nitric oxide synthase	SAPS: simplified acute physiology score
PAMP: pathogen-associated molecular pattern	SC: subcutaneous
PAR-1: proteinase-activated receptor 1	SCCM: society of critical care medicine consensus conference
PBS: phosphate buffered saline	SIRS: systemic inflammation response syndrome
PCR: polymerase chain reaction	SNP: sodium nitroprusside
PD-1: programmed cell death 1	SNS: sympathetic nervous system
PFA: paraformaldehyde	SSC: Surviving Sepsis Campaign
PHA: phytohemagglutinin	SOFA: sequential organ failure assessment
PKA: protein kinase A	SV: stroke volume
PKC: protein kinase C	SvcO ₂ : central venous oxygen saturation
PLB: phospholamban	
PLC: phospholipase C	

SVR: systemic vascular resistance

T-bet: T-box transcription factor

TBE: tris-borate-EDTA

TCR: T cell receptor

TFG- β : transforming growth factor- β

Th: T helper

TIR: toll interleukin receptor

TIR: toll-interleukin 1 receptor

TIRAP: TIR-associated protein

TLR: toll-like receptor

TNF- α : tumor necrosis factor- α TNF- α

TRAIL: targeting TNF-related apoptosis-inducing ligand

Treg: regulatory T lymphocyte

UV: ultra-violet

VEGF: vascular endothelial growth factor

VIT: velocity integral time

VSMC: vascular smooth muscle cell

vWB: von Willebrand

WT: wild type

β -ARK: β -adrenoreceptor kinase

Legends

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Résumé de la thèse

Situation du sujet

L'histoire naturelle d'une infection grave est un continuum qui débute par le sepsis et qui évolue en choc septique. Le choc septique se définit par un état infectieux associé à une hypotension, marqueur d'une dysfonction d'organe, persistante malgré l'expansion volémique et requérant l'administration de drogues vasoactives. Son évolution conduit, en l'absence de prise en charge, à un dysfonctionnement multi-organes potentiellement létal.

Le sepsis et le choc septique sont l'une des principales causes d'admission en unité de soins intensifs et sont reconnus par l'Organisation Mondiale de la Santé comme un problème de santé publique majeur [1]. En 2017, les données mondiales, provenant principalement des pays à revenu élevé, estimaient à 19,4 millions le nombre de cas de sepsis grave et de chocs septiques, entraînant 5,3 millions de décès par an [2]. Ces statistiques étaient sans doute sous-estimées de par le manque d'informations provenant des pays à faible revenu. En effet, en 2020, une étude incluant un plus grand nombre de données provenant de pays à faible revenu et à revenu intermédiaire a estimé à 48,9 millions le nombre de cas de sepsis graves et de chocs septiques avec en conséquence un nombre de décès deux fois plus élevé que décrit précédemment [3].

Malgré des progrès dans sa prise en charge, le choc septique est toujours associé à une mortalité de 40% [4]. Aucune thérapeutique spécifique testée au cours de ces quatre dernières décennies n'a montré, pour l'heure, une quelconque efficacité sur la mortalité. Les essais cliniques de phase III pour le traitement du choc septique tels que la protéine C activée, les anticorps anti-TNF, anti-LPS ou l'antithrombine III, ont tous échoué à améliorer la survie

malgré des études positives de phase II et des études pré-cliniques hautement favorables [5-7]. L'amélioration des processus de prise en charge, bien qu'ayant largement diminué la mortalité du choc septique, montre aujourd'hui ses limites. Il y a donc un besoin autant sur le plan individuel qu'en terme de santé publique de développer de nouvelles stratégies thérapeutiques [8].

Le choc septique est associé à une activation physiologique du système nerveux sympathique (SNS). L'activation du SNS, en libérant de la noradrénaline et de l'adrénaline, est une réponse essentielle à l'infection à la phase initiale de tout processus septique. Ces effets bénéfiques sont une augmentation de l'inotropisme, une tachycardie et une vasoconstriction, qui contrecarrent les effets toxiques et/ou inflammatoires initiaux du processus infectieux [9]. Néanmoins, la persistance de cette activation dans le temps à un haut niveau d'intensité peut aussi être délétère et entraîner un syndrome de dysautonomie sympathique. Cette dernière est marquée par une perte de la variabilité cardiovasculaire, une désensibilisation des adrénorécepteurs et une immunosuppression adaptative à l'origine d'une surmortalité [10-14].

L'immunosuppression induite par le choc septique est caractérisée par une déplétion et une dysfonction des cellules immunitaires conduisant à l'incapacité du patient à répondre à une seconde infection [15]. Cette dysfonction touche les cellules de l'immunité innée, caractérisée par une diminution de la production cytokinique, de la cytotoxicité et de la capacité d'orchestrer la réponse adaptative. Ces cellules de l'immunité adaptative sont également impactées. Les lymphocytes présentent une diminution de leurs capacités prolifératives, des sécrétions de cytokines et un dérèglement de leurs fonctions.

Les lymphocytes T CD4 sont plus particulièrement touchés par l'immunosuppression

septique. Le nombre de lymphocytes T CD4 circulants diminue drastiquement lors d'un choc septique. L'analogie avec le VIH peut être pertinente car dans cette pathologie chronique, source d'un déficit T CD4, les infections opportunistes font le lit du pronostic défavorable de cette maladie. Contrairement aux autres sous populations lymphocytaires, plusieurs études décrivent une augmentation du pourcentage de lymphocytes T régulateurs (Tregs) circulants [16-19]. Cette augmentation est observée immédiatement après le début du sepsis et persiste chez les patients non survivants [16, 20]. L'augmentation des proportions de Tregs participe à la diminution des capacités prolifératives des lymphocytes, induit la production de cytokines anti-inflammatoires et réprime les fonctions de cellules de l'immunité innée jouant un rôle majeur dans l'immunosuppression.

L'adrénaline et la noradrénaline, deux inopresseurs majeurs ayant principalement des effets adrénergiques α_1 et β_1 adrénergiques (β_1 -AR), ont été récemment confirmés comme étant des facteurs cruciaux dans la genèse de l'immunosuppression [21]. Lors d'un choc septique, la noradrénaline favorise la sécrétion de cytokines anti-inflammatoires et est associée à des effets inhibiteurs de la prolifération des lymphocytes T et de la production de cytokines [22-24].

Ainsi, la modulation du SNS pourrait être une approche thérapeutique intéressante dans la prise en charge du choc septique. Pour moduler l'interaction entre le SNS et la réponse inflammatoire, l'utilisation d'un traitement β_1 -bloquant semble la plus justifiée.

Les premières études chez l'homme ont principalement porté sur l'effet bénéfique de la réduction de la fréquence cardiaque par le blocage β_1 -AR sur la fonction cardiovasculaire lors d'un choc septique [25]. Cependant, les effets cardiovasculaires protecteurs des β_1 -bloquants semblent plutôt associés aux effets anti-inflammatoires de ces molécules qu'à la

réduction de la fréquence cardiaque. En effet, l'adjonction de doses non-chronotropes d'un β_1 -AR bloquant dans un modèle expérimental de choc septique est associée à une cardioprotection *in vivo* et à une meilleure vasoréactivité *ex vivo* [26]. Ces effets sont probablement en partie médiés par une régulation de l'inflammation.

Parallèlement, il a été démontré que les cellules de l'immunité innée et adaptative exprimaient des β -AR [27-31]. Des études expérimentales ont exploré l'effet inflammatoire du blocage β_1 -AR et ont retrouvé une pondération globale des cytokines plasmatiques pro- et anti-inflammatoires ainsi qu'une diminution de l'inflammation tissulaire chez des animaux septiques β_1 -AR bloqués [32]. Cependant, l'effet de ce blocage sur la réponse immunitaire adaptative, et plus particulièrement sur les dysfonctions lymphocytaires T CD4 survenant au cours du choc septique n'a jamais été étudié.

Objectifs du travail

L'objectif général de cette thèse est d'explorer l'impact de la modulation β_1 -AR sur l'hémodynamique et le système inflammatoire et plus particulièrement sur les lymphocytes T CD4 dans un modèle murin de sévère de sepsis induit par ligature et ponction du caecum (LPC).

Méthodologie générale

En utilisant un modèle de sepsis polymicrobien sévère induit par LPC les effets hémodynamiques et inflammatoires du blocage β_1 -AR ont été étudiés :

- Chez des souris sauvages (WT), auxquelles un β_1 -AR bloquant (esmolol, 18 mg/kg/h) est administré ou non trois heures après l'opération via une mini-pompe osmotique sous cutanée.

- Chez des souris knock-out (KO) pour le β_1 -AR.

Six groupes ont été définis :

- 1) WT sham,
- 2) WT sham + esmolol,
- 3) WT LPC,
- 4) WT LPC + esmolol,
- 5) KO Sham,
- 6) KO LPC.

Dix-huit heures après l'induction du choc septique, quatre séries d'expériences ont été réalisées :

1) Caractérisation hémodynamique et inflammatoire (n=74) :

- La fonction cardiaque *in vivo* a été évaluée par échocardiographie afin de mesurer la fréquence (FC), le débit cardiaque (DC) et la puissance myocardique de chaque souris,
- La pression artérielle moyenne (PAM) *in vivo* a été mesurée par cathétérisation de la carotide,
- L'infiltration des leucocytes dans le myocarde a été évaluée par immunohistochimie (marquage CD45+),
- Le dosage plasmatique de 46 médiateurs pro- et anti-inflammatoires a été réalisé par Luminex[®],
- Les différentes populations lymphocytaires de la rate (T CD3+, T CD8+, T CD4+, Th1, Th2, Th17, Treg) ont été immunophénotypées par cytométrie en flux.

2) Un test de prolifération des lymphocytes T CD4 sur 72 heures en présence d'un agent mitogène a été réalisé et analysé par cytométrie en flux (n=44).

- 3) La prolifération et la mortalité des lymphocytes T CD4 en présence de Tregs pré-activés par des catécholamines (dobutamine, β_1 -AR agoniste ; phényléphrine, α_1 -AR agoniste ; noradrénaline, α_1 - et β_1 -AR agoniste) provenant de splénocytes de souris non septiques a été mesurée *in vitro* en temps réel pendant cinq jours (Incucyte®) (n=4).
- 4) Une étude de survie sur cinq jours a été réalisée dans les groupes de souris suivant : WT LPC, WT LPC + esmolol administré pendant 24 heures et KO LPC (n=60).

Résultats obtenus

Cette étude confirme que l'induction d'une septicémie par LPC reproduisait les caractéristiques clinico-biologiques usuelles de cette pathologie :

- 1) Cliniquement : une hypotension artérielle, une diminution du DC et une diminution de la puissance cardiaque ;
- 2) Biologiquement : une augmentation de l'ensemble des médiateurs pro et anti-inflammatoires, une diminution des proportions de lymphocytes T CD4 corrélée à une augmentation des proportions de Tregs dans la rate ainsi qu'une diminution des capacités prolifératives des lymphocytes T CD4.

Dans ce travail, il était retrouvé que le blocage β_1 -AR lors du choc septique, qu'il soit pharmacologique ou génétique, avait pour conséquence une préservation de la proportion de lymphocytes T CD4 et de la proportion des Tregs, restant à un niveau comparable à celui des souris sham. De plus, ce blocage β_1 -AR permettait le maintien des capacités prolifératives des lymphocytes T CD4.

Nous avons également démontré le rôle direct de la stimulation des β_1 -ARs sur les fonctions immunorégulatrices des Tregs. En effet, les capacités prolifératives des lymphocytes T CD4 tendent à diminuer en présence de Treg pré-stimulé à la dobutamine, un β_1 -AR agoniste.

Ces résultats se retrouvaient au travers d'une diminution des cytokines plasmatiques pro- et anti-inflammatoires. De plus, malgré une FC diminuée chez les souris β_1 -AR bloquées, la PAM, le DC ainsi que la puissance myocardique étaient maintenus.

L'étude de survie sur cinq jours a également montré un taux de survie de 60% chez les souris β_1 -AR KO en choc septique. Chez les souris WT, le taux de survie était de 36% chez les souris septiques, et de 37% lorsqu'un β_1 -AR bloquant leur était administré pendant les 24 premières heures.

Discussion

Caractéristiques du modèle

Pour étudier le rôle immunitaire et cardiovasculaire des β_1 -ARs dans le sepsis, nous avons délibérément choisi un modèle murin de LPC non réanimé, afin d'éviter toute interférence entre l'administration de catécholamines exogènes et le système sympathique. De ce fait le terme choc septique ne peut pas être utilisé. Ainsi, les animaux ont tous présenté un sévère sepsis typique caractérisé par une hypotension et un bas débit cardiaque associé à une augmentation des concentrations plasmatiques de médiateurs pro- et anti-inflammatoires. Ce modèle reproduit au niveau splénique de nombreuses altérations immunitaires observées au niveau périphérique chez les patients en choc septique, caractéristique de l'immunosuppression septique : une diminution des proportions des lymphocytes T CD4 corrélée à une augmentation des proportions de Tregs dans la rate a été retrouvée. De plus, les capacités de proliférations des lymphocytes T CD4 étaient altérées.

La stimulation β_1 -AR induit un dysfonctionnement des cellules T CD4

Les cellules de l'immunité innée comme adaptative expriment les récepteurs adrénergiques. Cependant leur caractérisation, particulièrement pour les β -AR, est moins bien définie. Plusieurs études ont démontré la présence de β -AR sur les différentes sous-populations lymphocytaires, à l'exception des lymphocytes Th2. Cependant, jusqu'à récemment, seule la présence de β_2 -AR et non β_1 -AR sur les lymphocytes a été étudiée. De ce fait, le β_1 -AR n'est pas considéré comme un acteur de la réponse inflammatoire. Toutefois, une étude publiée en 2020 apporte une preuve de l'expression de β_1 -AR à la surface des lymphocytes T CD4 dans la rate.

Dans cette étude, l'impact des β_1 -AR bloquants sur le dysfonctionnement des cellules T CD4 chez des souris cirrhotiques est examiné. Ainsi, il a été démontré qu'un traitement à la dobutamine, β_1 -AR agoniste, est capable d'activer les Tregs, suggérant la présence de β_1 -AR à leur surface [33]. De la même manière, notre étude démontre que la prolifération *in vitro* des lymphocytes T CD4 tend à réduire en présence de Tregs préalablement traités à la dobutamine, révélant l'impact direct de la stimulation β_1 -AR sur la fonction inhibitrice des Tregs.

Le blocage β_1 -AR réduit l'immunosuppression septique

Dans de nombreuses maladies telles que l'insuffisance cardiaque aiguë, les brûlures, l'insuffisance hépatique chronique ou le choc septique, le blocage β_1 -AR est associé à des effets protecteurs hémodynamiques et inflammatoires [26, 34]. De façon similaire, dans cette étude, nous avons trouvé une pondération globale des médiateurs pro-inflammatoires et anti-inflammatoires chez les animaux en état de choc septique traités avec un β_1 -AR bloquant. Cependant, les mécanismes sous-jacents ne sont toujours pas élucidés. Il a été observé chez

des souris infectées par *Listeria monocytogenes* qu'un stress aigu, substitut de la stimulation adrénergique sympathique, inhibe la réponse des lymphocytes T et augmente la production de cytokines. Dans ce modèle, le blocage pharmacologique β_1 - mais pas β_2 -AR a permis de diminuer cet état pro-inflammatoire et de raccourcir le temps de clairance bactérienne [35]. Dans notre étude, le blocage β_1 -AR chez les souris en choc septique, qu'il soit pharmacologique ou génétique, préserve les proportions physiologiques de lymphocytes T CD4 et de Tregs dans la rate. Ceci étant associé à un maintien des capacités prolifératives des T CD4 chez les souris traitées suggérant ainsi que l'activation β_1 -AR participe à l'immunosuppression induite par le sepsis. Le blocage β_1 -AR pourrait donc contrer l'immunosuppression induite par le sepsis et favoriser le passage d'un état immunosuppresseur à un état homéostatique.

Le blocage β_1 -AR maintient l'hémodynamique

Hormis l'impact immunitaire de la modulation β_1 -AR observé lors d'un choc septique expérimental, il est important de rappeler que le blocage β_1 -AR a également des effets cardiovasculaires majeurs, principalement dus à ses effets chronotropes négatifs. En accord avec la plupart des études expérimentales publiées précédemment, la réduction de la FC chez les souris septiques β_1 -AR bloquée n'a pas modifié l'index cardiaque ni la pression artérielle [25, 26]. De fait, il a été constaté que la puissance cardiaque, un marqueur du travail cardiaque externe, était similaire dans les groupes traités et non traités avec un β_1 -AR bloquant. Chez les animaux β_1 -AR bloqués la FC, déterminant majeur de la consommation en oxygène des tissus, était réduite en comparaison avec les animaux non-traités, notre étude suggère ainsi une meilleure efficacité cardiaque lorsqu'un traitement β_1 -AR bloquant est administré.

Limitations de l'étude

Si des études expérimentales suggèrent que l'administration précoce de β_1 -AR bloquant est bénéfique en termes hémodynamique, inflammatoire et pour la survie, les études cliniques traitant des patients en choc septique avec des β_1 -AR bloquants sont néanmoins peu concluantes ou méthodologiquement fragiles.

Dans notre modèle, les animaux septiques n'ont pas été réanimés et aucun antibiotique n'a été administré, contrairement aux recommandations habituelles pour les patients en choc septique. L'objectif était de limiter l'administration d'agonistes adrénergiques qui, bien que recommandée dans le traitement du choc septique, aurait interféré avec nos objectifs.

Les études expérimentales reportées sur l'administration de β_1 -AR bloquant sont principalement réalisées sur de petits animaux, souris ou rats, pour lesquels la FC au repos est entre 300 et 400 par minute. Le volume et le débit cardiaque sont dans ces modèles, étroitement dépendant de la FC. Cependant, dans notre étude et dans bien d'autres, tous les modèles murins de sepsis ne sont pas tachycardes [36, 37]. L'induction d'une septicémie chez la souris est le plus souvent associée à une diminution marquée du métabolisme énergétique avec pour conséquence une diminution de la FC [37]. À l'inverse, chez l'homme, le métabolisme est le plus souvent augmenté, entraînant une tachycardie adaptative précoce. De plus, le moment d'administration du β -bloquant est toujours défini expérimentalement et est souvent précoce. Au contraire, le traitement β -bloquant est débuté chez l'homme le plus souvent tardivement. Finalement, si le modèle du petit animal est intéressant sur le plan conceptuel, l'expérimentation chez gros animal et plus encore chez l'homme reste indispensable pour vérifier les constatations chez le petit animal.

Perspectives

Les β_1 -ARs, mais également les β_2 -ARs sont exprimés par les cellules immunitaires et notamment par les lymphocytes [30]. La stimulation β_2 -AR semble avoir des effets anti-inflammatoires et favoriser la différenciation des lymphocytes Th2 [38]. Pour soutenir cette hypothèse, il a été démontré chez des patients en choc septique que certains polymorphismes β_2 -ARs étaient associés à une surmortalité [39]. Dans la présente étude, l'effet du blocage β_1 -AR sur les lymphocytes T CD4 a été analysé. Une suite logique serait de s'intéresser à l'impact de la modulation β_2 -AR sur l'immunosuppression septique, grâce à un blocage pharmacologique (ICI 118,551) et à un modèle de souris β_2 -AR KO que nous possédons au laboratoire.

L'étude du rôle des β_3 -ARs, exprimés par les cellules endothéliales, durant le choc septique pourrait également être prometteur. En effet, il est démontré que l'administration de nébivolol, un agoniste des β_3 -ARs, favorise la vasorelaxation [40]. De plus, une étude préliminaire a révélé dans la graisse brune chez le rat que l'esmolol pourrait être un antagoniste des β_3 -ARs [41]. Il sera donc intéressant de regarder si les β_1 -ARs bloquants ont également des effets sur les β_3 -ARs, exprimés par les cellules endothéliales. Plusieurs études expérimentales et cliniques ont montré que l'administration d'un β_1 -AR bloquant diminuait l'hyporéactivité vasculaire aux catécholamines, alors que ce récepteur n'est pas exprimé dans les vaisseaux [25, 32]. L'éventuel effet antagoniste d'un β_1 -AR bloquant sur les β_3 -ARs pourrait expliquer l'amélioration de la réactivité vasculaire aux catécholamines lors d'un choc septique expérimental.

Conclusion

Durant le processus septique, la pondération de la dysautonomie adrénergique par l'administration d'un β_1 -AR bloquant est associée à une amélioration de la fonction immunitaire adaptative. Ces résultats sont dans notre étude, pour la première fois, aussi retrouvés chez les souris génétiquement invalidées pour le récepteur β_1 -AR. Ainsi, au lieu de considérer le blocage de β_1 -AR comme un simple traitement hémodynamique, ces résultats ouvrent la voie à une approche immuno-régulatrice rétablissant l'homéostasie lymphocytaire T CD4 en cas de septicémie.

Background

Definition of septic shock

The definition of sepsis has evolved in the past few years to improve the diagnosis and the management provided to patients. Septic shock was first defined by the American College of Chest Physicians (ACCP) and Society of Critical Care Medicine (SCCM) consensus conference in 1991 [42]. This definition included a continuous worsening process starting with systemic inflammation response syndrome (SIRS) (**Table 1**). SIRS with presumed or proven infection is defined as sepsis. Finally, septic shock is referred to severe sepsis with signs of persistent hypoperfusion despite adequate fluid resuscitation.

Table 1: Criteria for SIRS, sepsis, severe sepsis, and septic shock based on the 1991 ACCP/SCCM Consensus Conference.

According to the reference: [42]

Term	Criteria
SIRS	Two of the four following criteria: Temperature $>38^{\circ}\text{C}$ or $<36^{\circ}\text{C}$ Heart rate $>90\text{ min}^{-1}$ Hyperventilation evidenced by respiratory rate $>20\text{ min}^{-1}$ or arterial CO_2 lower than 32 mmHg White blood cell count $>12000\text{ cells.}\mu\text{l}^{-1}$ or $<4000\text{ cells.}\mu\text{l}^{-1}$
Sepsis	SIRS criteria with presumed or proven infection
Severe sepsis	Sepsis with organ dysfunction
Septic shock	Sepsis with hypotension despite adequate fluid resuscitation

However, this definition has many limitations. Some patients may not have symptoms meeting two SIRS criteria despite having an infection and develop organ failure. Kaukonen et al., in a retrospective study from 2000 to 2013 with more than 1 million of patients with infection and organ failure, confirmed that the SIRS criteria rule missed one patient in eight with severe sepsis [43].

An improvement in our understanding of sepsis pathophysiology, such as the early activation of both pro- and anti- inflammatory responses, led the Sepsis Definition Task Force members to update sepsis definitions between January 2014 and January 2015 [44]. The updated definition includes both pathophysiological and clinical heterogeneities of septic shock. Thus, sepsis is defined as organ dysfunction induced by an infection. Organ dysfunction can be summarised with the Sequential Organ Failure Assessment (SOFA) score of 2 points or more. This score aims to describe the degree of organ dysfunction or failure over time in patients by assessing the respiratory, cardiovascular, coagulation, renal, hepatic, and central nervous systems. A SOFA score of 2 or more associated with a suspected or documented infection defines sepsis.

Patients with septic shock are treated with a vasopressor aiming to maintain mean arterial pressure above 65 mmHg and have a serum lactate level greater than 2 mmol.l⁻¹ despite adequate volume resuscitation (**Table 2**). The terminology of severe sepsis has been removed.

Table 2: Criteria for sepsis and septic shock based on the Third International Consensus.

According to the reference: [44]

	Sepsis	Septic shock
Definition	Sepsis is 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
Clinical criteria	Suspected or documented infection and an acute increase of ≥ 2 SOFA points (a proxy for organ dysfunction)	Sepsis and vasopressor therapy needed to elevate MAP ≥ 65 mmHg and lactate >2 mmol.l ⁻¹ (18 mg.dl ⁻¹) despite adequate fluid resuscitation

In order to facilitate the identification of sepsis at the bedside, patients with a suspected infection who are likely to have a prolonged intensive care unit (ICU) stay or to die during hospital stay can be promptly identified at the bedside if they have at least two of the quick SOFA (qSOFA) clinical criteria. The latter is a surrogate of SOFA score. The qSOFA score calculation just needs the respiratory, the consciousness and the systolic blood pressure (**Table 3**). However, Raith *et al.* demonstrates that, among adults with suspected infection admitted to an ICU, an increase in the SOFA score of 2 or more points had greater prognostic accuracy for in-hospital mortality than qSOFA, suggesting that qSOFA may have limited use for predicting mortality in an ICU setting [45].

Table 3: Quick SOFA criteria.

According to the reference:[44]

qSOFA (quick SOFA) criteria
Respiratory rate $\geq 22 \text{ min}^{-1}$
Altered mentation
Systolic blood pressure $\leq 100 \text{ mmHg}$

Epidemiology of septic shock

Incidence

The description of septic shock epidemiology is crucial as 31,5 million cases of sepsis and 19,4 million cases of severe sepsis were estimated each year in high-income-countries. Fleischmann *et al.* in a meta-analysis, including 27 studies from seven countries, showed that the incidence was 288 hospital-treated cases of sepsis and 148 hospital-treated cases of severe sepsis per 100,000 person-years between 1979 to 2015. In the last decade, the incidence was 437 cases of sepsis and 270 cases of severe sepsis per 100,000 person-years [2].

These results are in accordance with other studies describing an increasing incidence of sepsis among hospitalised patients in the two last decades [46, 47].

Several fundamental characteristics have altered the population pattern with septic shock patients treated in ICU, which may explain this increase: the aging of the population, the development of better diagnostic tools, the impact of septic shock guidelines, or the increasing use of invasive procedures. These elements justify a regular update of epidemiological data on septic shock. In France, 14 epidemiological studies published from 1995 to 2020 described the change in septic shock populations (**Table 4**).

Table 4: Epidemiology of septic shock in France from 1995 to 2020.

According to the references: [4, 48].

Authors	Type of study, duration	Type of ICU	Population	Onset of sepsis	Mortality
Brun-Buisson <i>et al.</i> 1995[49]	Prospective multicentric study, 31 days in 1993	170 ICUs 62% medical-surgical 20% surgical 18% medical	11,828 patients 1,064 severe sepsis	Prevalence 9% CI (8.5-9.5)	28 day mortality: 56% CI (52-60)
Brun-Buisson <i>et al.</i> 1996[50]	Prospective multicentric study, 31 days in 1993	24 ICUs	85,750 patients 842 bacteraemia, 221 severe sepsis with bacteraemia	Prevalence bacteraemia: 9.8 IC (9.2-10.5) severe sepsis and bacteraemia: 2.6(IC2.2-2.9) pour 1000 admissions	28 day mortality: bacteraemia: 25% CI (23-29) bacteraemia and severe sepsis with bacteraemia 54% CI (48-61)
Alberti <i>et al.</i> 2002[51]	Prospective multicentric international study, 1997 to 1998	27 ICUs in 8 counties	14,364 admissions 1,115 sepsis 944 severe sepsis 1,180 septic shock	Overall prevalence: 21%	ICU mortality: sepsis: 26.8% CI (20.1-33.5) severe sepsis 33.6% CI (25.7- 41.4) septic shock: 57.4% CI (54.4- 71.1)
Annane <i>et al.</i> 2003[52]	Retrospective multicentric study, 1993 to 2000	22 ICUs	100,554 admissions 8,251 septic shock	Prevalence 8.2% Incidence 1993: 7% 2000: 9.7%	Overall mortality: 60.1% 1993: 62.7% CI (59.8-65.6) 2000: 55.6% CI (53.4- 57.8)
Brun-Buisson <i>et al.</i> 2004[53]	Prospective multicentric study, 2 weeks	213 ICUs 95 in university hospitals 97 in general hospitals 14 in semi-private hospitals	3,738 patients 621 severe sepsis	Prevalence 16.6%	30 day mortality: 35% Two month mortality: 46.7%

Guidet <i>et al.</i> 2005[54]	Retrospective multicentric study, 1997 to 2001	35 ICUs 23 in university hospitals 8 in general hospitals	96,193 admissions 20,963 severe sepsis	Prevalence severe sepsis 21.4% incidence 1997: 17.2% 2001: 18.7%	ICU mortality: severe sepsis 5.5% septic shock 1997: 44.7% 2001: 40.1%
Adrie <i>et al.</i> 2007[55]	Retrospective multicentric case control study, 1997 to 2005	12 ICUs 7 in university hospitals 5 in general hospitals	4,860 patients 1,692 severe sepsis	Prevalence severe sepsis 34% septic shock 10%	Hospital mortality severe sepsis 29%
Zahar <i>et al.</i> 2011[56]	Retrospective multicentric study, 1996 to 2009	12 ICUs 7 in university hospitals 5 in general hospitals	11,992 patients 3,588 severe sepsis	Prevalence severe sepsis 30% septic shock 10.7%	Hospital mortality severe sepsis 30.4% septic shock 42%
Quenot <i>et al.</i> 2013[4]	Multicentric prospective study, 2009 to 2011	10 ICUs 5 in university hospitals 5 in general hospitals	10,941 patients 1,495 septic shock	Incidence 13.5 %	ICU mortality: 39.5% 28 day mortality 42% hospital mortality 48.7%
Nessler <i>et al.</i> 2013[57]	Observational study, 2008 to 2018	1 ICU in a university hospital	93 septic shock patients	Not reported	28 day mortality: 25.8% 90 day mortality: 39.8% 180 day mortality: 45.2%
Tolsma <i>et al.</i> 2014[58]	Retrospective multicentric study, 1997 to 2011	11 ICUs	14,419 patients 1,981 severe sepsis and septic shock patients	Overall prevalence 13.7%	28 day mortality: 29.6%
Chauvet <i>et al.</i> 2015[59]	Prospective study, 2008 to 2010	1 ICU in a university hospital	962 patients 218 septic shock	Overall prevalence 22.7%	ICU mortality: 30.3% 28 day mortality: 38%
Contou <i>et al.</i> 2016[60]	Prospective study, 2014 to 2015	10 ICUs	508 patients in septic shock	Not reported	ICU mortality: 36% overall mortality: 46%
Jamme <i>et al.</i> 2017[61]	Retrospective study, 2008 to 2015	1 ICU in a university hospital	801 patients in septic shock	Not reported	3 day mortality: 14.1% ICU mortality: 37.3% hospital mortality: 41.3%

Causes of septic shock

Data from a worldwide audit conducted in 2012 by the Intensive Care Over Nations (ICON) were analyzed by Sakr *et al.* They published a prospective study performed in 730 ICUs from 84 countries and demonstrated that the overall prevalence of Gram negative bacteria (67.1%) was higher than that of Gram positive bacteria (49.8%). The most common isolated Gram negative bacteria were *Pseudomonas* spp. and *Escherichia coli*, while the most common

Gram positive strain was *Staphylococcus aureus*. Microbiological patterns varied around the globe; for example, in south Asia Gram-positive infections are much less frequent (21.4%) than in other regions. The prevalence of bacterial species also varies according to continents. *Pseudomonas* spp. was most common in South America (20.4%) and eastern Europe (21.1%) and infection with fungal organisms was found in 14.5% and 14.8 % of cases, respectively, in western and eastern Europe, but only 5.1% in North America [1]. Similarly, Vincent *et al.* showed that 70% of infected patients have positive microbial samples, 62% of them were Gram negative organisms, 47% were Gram⁺, and 19% were fungi [62].

Site of infection

The primary origin of sepsis remains virtually unchanged over time. Thus, in 1997-1998, in decreasing order of frequency, the infected sites were from respiratory, digestive tract, and urinary; bacteraemia represented more than 80% of the infectious aetiologies of patients with septic shock [51]. Thereafter, a retrospective cohort including 32 ICUs in the Paris area including 31,251 patients in septic shock found that the most common site of infection was still pulmonary (50.9%) in the period from 1998 to 2010 [63]. This was confirmed in many further studies [4, 60, 64].

Risk factors

Risk factors of death in septic shock patients are well identified. Regardless of the time period studied, severity scores are reliable prognostic markers. Thus, the SOFA score is systematically found as a prognostic marker. Age and sex are also strongly associated with mortality, as well as the presence of at least one comorbidity [4, 65].

Historically, Vincent *et al.* showed that Simplified Acute Physiology Score (SAPS) II score was independently associated with mortality and a higher risk of infection [62]. However, the clinical pattern at admission is continuously evolving over time. Thus, new scores are being implemented, such as SAPS III or APACHE III scores. The latter improve risk prediction of poor outcome compared to APACHE II or SAPS II [66, 67]. Moreover, specific subgroups deserve an independent analysis and have been studied. Galbois *et al.*, investigated septic shock patients with cirrhosis. Thus, from 1998 to 2010, the ICU mortality of cirrhotic patients with septic shock markedly decreased, from 84.7% to 68.5% for patients receiving mechanical ventilation and from 91.2% to 78.4% for those with renal replacement therapy [63].

Diabetes mellitus is also a common comorbidity in septic shock patients. Diabetes is associated with chronic organ dysfunction. One epidemiologic study from 1979 to 2003 including 2,070,459 septic patients with diabetes are less likely to develop an acute respiratory failure and more likely to have an acute kidney injury compared to non-diabetic patients. Interestingly, there was no difference in mortality between septic patients with and without diabetes [68].

Mortality

Sepsis accounts for more than five million deaths around the world each year, and it is accepted that sepsis is the leading cause of death in ICUs around the world [62, 69]. A study published by Fleishman *et al.* estimated the global burden of sepsis at 19.4 million incident sepsis cases and 5.3 million sepsis-related deaths annually in seven high-income countries for the years 1979 to 2015 [2]. More recently, Rudd *et al.* used data from 109 million individual death records to assess mortality related to sepsis in 2017. They estimated the incident cases

of sepsis at 48.9 million and the number of sepsis-related deaths at 11.0 million, representing 19.7% of deaths worldwide. The estimated number of sepsis-related deaths was two-fold higher than previously described [2]. This increase could be accountable to the inclusion of more data from low-income and middle-income countries for which the incidence and mortality were considerably higher and from which data were previously lacking [3].

Several studies have found a decrease in mortality related to sepsis in the three last decades [46, 47]. From 1990 to 2017, Rudd *et al.* estimated that sepsis mortality was reduced by 52.8% worldwide [3]. In a retrospective study from Australia and New Zealand performed between 2000 to 2012, it was found that absolute septic mortality decreased from 35.0% to 18.4%, representing an annual decrease rate of 1.3% [70]. Furthermore, Stevenson *et al.*, in a comparative meta-analysis, observed a reduction in mortality from 46.9% from 1991-1995 to 29% from 2006-2009, representing an annual mortality decrease of 3.0% [71]. Since there is no new efficient therapy for sepsis introduced in the last for decades, it can be assumed that mortality decrease in high income countries could be attributed to the early detection and to the prompt management of septic patients [72].

Clinical management of patients

The Surviving Sepsis Campaign consortium established guidelines for the management of sepsis and septic shock. They were first published in 2004 [73] and revised in 2008 [74], 2012 [75], and 2016 [8]. This consensus defined the best practice for managing septic and septic shock patients. The first principle was that the success in treating septic shock is time-dependent. Management of sepsis and septic shock patients can be summarised in three points which must be handled simultaneously: infection source control, hemodynamic stabilisation, and the management of organ failure.

The early diagnosis of the infection source is essential based on a clinical examination, bacteriological samples, imaging such as echography of chest X-ray. As soon as possible after the diagnosis, broad-spectrum antibiotic therapy should be administered. Kumar *et al.* have shown that a delay in the initiation of effective antibiotic therapy is associated with increased mortality [76]. Similarly, prescription of an inappropriate antibiotic regimen is also associated with increased mortality [77].

Simultaneously, patients urgently need an hemodynamic stabilisation. Initial resuscitation starts at the onset of diagnosis. At least 30 ml/kg of IV crystalloid fluid should be infused within the first 3 hours. Patients who are still hypotensive despite fluid resuscitation, vasopressors are required vasopressors aiming to restore a mean arterial pressure (MAP) of 65 mmHg. Norepinephrine (NE) is the first recommended vasopressor choice. This α_1 and β_1 -adrenergic agent increases vascular resistance and inotropism with little change in heart rate (HR) compared to dopamine or adrenaline which are also α_1 , β_1 , and β_2 adrenergic agents.

The last part of the SSC guidelines is about supportive care, which is also crucial. For instance, treatments of respiratory, kidney, or coagulation failure are most often required during the septic shock management. For more detail, readers should refer to the guidelines [8].

Cardiovascular physiopathology of septic shock

Cardiac physiopathology

Myocardial dysfunction

Left ventricular dysfunction

The failure of the cardiovascular system due to sepsis involves both peripheral vascular and myocardial dysfunction, with as both cause and consequence an increase in circulating catecholamine and cytokine levels [78, 79]. Sepsis-induced myocardial dysfunction occurs in approximately 50% of septic patients, and both diastolic and systolic dysfunction are observed [80]. A study by Parker *et al.* published in 1984 was the first to demonstrate that myocardial dysfunction is of major importance in septic shock patients [81]. In this work, using the cineangiography technique and pulmonary artery catheterisation, left ventricular (LV) ejection dysfunction (<40%) was initially found in 10 of 13 surviving patients and was completely resolved 7 to 10 days later [81]. Then, Ellrodt *et al.* and Ognibene *et al.* showed that the LV stroke work index was depressed in 94% of septic patients [82, 83].

Using cardiac ultrasound measurements, Jardin *et al.* also found a decrease in the LV ejection fraction (LVEF) and preload dependence, despite fluid resuscitation, in non-survivors [80]. Recent studies also confirmed these findings [84, 85]. Interestingly, Sanfilippo *et al.*, in a meta-analysis, found that a decrease in myocardial strain was a good predictor of adverse outcome in severe sepsis or septic shock, while such association was not found for LVEF [85].

Diastolic dysfunction, while less characterised and much more difficult to diagnosed, is also frequently reported in septic shock patients. Barraud *et al.* reported an increase in end-diastolic pressure resulting from a reduction in LV compliance in anaesthetised endotoxaemic rabbits [86]. Landersberg *et al.*, in a cohort of 262 ICU patients with severe sepsis and septic

shock, found that 9.1% of patients had systolic dysfunction, 14.1% had combined systolic and diastolic dysfunction, and 38% had isolated diastolic dysfunction [87]. This study demonstrated a six-fold higher mortality in septic patients with diastolic dysfunction. Further studies also confirmed the strength of this association [88, 89].

Right ventricular dysfunction

Right ventricular (RV) dysfunction in septic patients was discovered early but received far less attention and investigation than LV dysfunction [90, 91]. RV dysfunction is reported in 30-60% of septic patients and is most often associated with LV dysfunction [92]. Thus, Vallabhajosyula *et al.* reported that, in a cohort of 388 patients admitted to the ICU for severe sepsis or septic shock, RV dysfunction was diagnosed in 55%. Forty-seven percent of them had isolated RV dysfunction and 53% combined bi-ventricular dysfunction [93]. During septic shock, RV dysfunction can be characterised by either dilation or a decreased ejection fraction, or both [91, 94]. The latter is multifactorial and can be due to an increase in RV afterload, or sepsis-related myocardial fibre depression. In acute respiratory distress syndrome with septic shock, RV failure is most often worsened by hypoxaemia, hypercapnia, and the high positive end-expiratory pressure level, which increases the RV afterload [95].

Molecular mechanism of cardiac dysfunction

Pathogen-associated molecular patterns and Toll-like receptors

Intramyocardial inflammation, involving many pathways of the inflammatory response, is a major cause of myocardial dysfunction [96]. The Toll-like receptors (TLRs) expressed on cardiomyocytes are activated by pathogen-associated molecular patterns (PAMPs) originating from the pathogen [97] and by endogenous damage-associated

molecular patterns (DAMPs) [98]. TLR stimulation leads to nuclear factor- κ B (NF- κ B) activation and the subsequent release of pro-inflammatory cytokines [99]. Indeed, the administration of endotoxin (molecule released by the lysis of Gram negative bacteria) in healthy volunteers is responsible for a reduction in both LVEF and LV performance, leading to the typical hemodynamic pattern of sepsis-related myocardial dysfunction [100]. TLR-4, by itself, plays a pivotal role in sepsis-induced myocyte dysfunction. Taverner *et al.* demonstrated that TLR-4 was required to induce myocyte and mitochondrial dysfunction in mice with endotoxaemia. and Zhou *et al.* reported that septic TLR-4 knock-out (KO) mice were protected against sepsis-induced cardiac dysfunction due to decreased myocardial neutrophils infiltration, attenuated cardiomyocytes apoptosis, and decreased pro-inflammatory cytokines release in heart tissue [98, 101].

Cytokines

The inflammatory cytokines released during septic shock play a pivotal role in cardiac dysfunction. Kumar *et al.* showed that tumour necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) decreased myocardial cell contraction *in vitro* [102]. Another study using rabbit ventricular myocytes demonstrated *in vitro* that myocytes exposed to high doses of TNF- α showed decreased contractility [103]. Kumar *et al.* showed that TNF- α and IL-1 β were responsible for early sepsis-induced cardiac dysfunction, while prolonged effects were associated with the excessive production of nitric oxide (NO) [103-105].

Nitric oxide

Nitric oxide is a widely expressed intracellular messenger. It is a small lipophilic molecule produced through the metabolism of L-arginine by the activity nitric oxide synthase (NOS)[106]. The effects of NO are divided into direct and indirect effects Direct effects induce the activation or inhibition of haem enzymes. NO induces the activation of guanylate cyclase, a second messenger, that initiates several physiological effects resulting, for instance, in the relaxation of vascular smooth muscle cells, inhibition of platelet aggregation, and activation or inhibition of leukocyte adhesion to the vascular endothelium [107-109]. Indirect effects of NO occur with the release of oxygen species such as nitrous oxide. These specific molecules have a strong affinity for thiol groups and have inhibitory effects on glycolysis (inhibition of glyceraldehyde-3-phosphate dehydrogenase by nitrosylation) [106]. However, the major indirect effect of NO is the formation of peroxynitrite (ONOO⁻), produced by the interaction between NO and the superoxide anion (O₂⁻). The peroxynitrite molecule, including during septic processes, has deleterious effects by inducing both necrosis and apoptosis and promoting pro-inflammatory cellular responses [110-112].

Nitric oxide is considered as to be beneficial and detrimental to cardiac function. NO can be produced by three forms of NOS: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) [113, 114]. The activity of eNOS is dependent on the cytosolic calcium concentration and results in low levels of NO, which is involved in several physiological processes. Ichinose *et al.* showed that over-expression of cardiomyocyte-specific eNOS prevented endotoxin- or sepsis-induced myocardial dysfunction in mice [115]. In this study, increased NO level was associated with reduced reactive oxygen species (ROS) production, with increased sarcoplasmic reticulum Ca²⁺ load and with increased myofilament sensitivity to Ca²⁺. Conversely, inflammatory or infectious stimuli (LPS, lipoteichoic acid, cytokines, free

radicals, etc.) leads to the activation, in a Ca^{2+} independent manner, of iNOS, which produces high concentrations of NO [116]. The inflammatory process leads to an increase in iNOS expression by a large set of cells such as cardiomyocytes, smooth muscle cells, endothelial cells, or organs such as the kidney and liver. Ullrich *et al.* demonstrated that endotoxin-induced myocardial dysfunction was attenuated in iNOS deficient mice [117]. In addition, iNOS-induced hypovasoreactivity during endotoxaemic stimulation by LPS has also been observed in iNOS knock-out mice [118].

Low levels of NO have beneficial effects under physiological conditions, but in pathological states, the increase in NO production becomes deleterious and participates in multiorgan dysfunction in septic shock. NOS inhibition has been tested both experimentally and clinically. An in vitro study found that administration of non-selective NOS inhibitors such as methylene blue and L-NG-nitroarginine methyl ester (L-NAME) moderated sepsis-induced myocardial depression [119]. However, clinical data on NO inhibition by L-N-monomethyl arginine (L-NMMA), a competitive inhibitor of NOS (selective or non-selective), demonstrated an increase in mortality in the treated group, resulting in the early termination of the study [120].

Mitochondrial dysfunction

Mitochondria fills 30% to 50% of the cardiomyocyte cytoplasmic volume and are the major source of energy for cardiomyocytes. Mitochondrial injuries play a pivotal role in cardiac dysfunction, and is associated with severity and outcome in septic shock [121-123]. Damage to the mitochondrial ultrastructure in cardiomyocytes has been demonstrated in several animal models and in humans [124-128]. In a study of 25 patients with severe sepsis or septic shock, Soriano *et al.* demonstrated that patients who presented with cardiac dysfunction also

exhibited histologic alterations in mitochondrial structures [128]. Also, in a post mortem analysis of 44 human septic hearts, compared to 26 non-septic hearts, Takasu *et al.* found hydropic mitochondria (oedema of the mitochondrial matrix) only in septic hearts [129]. Mitochondrial injuries result in decreased production of adenosine tri-phosphate (ATP) and a persistent low level of ATP, leading to apoptotic signalling pathway activation [129]. The reduction in ATP level could be compensated for by an increase in glycolysis; however, this is not sufficient to deliver the required levels of ATP [130].

Autonomic dysregulation

Autonomic dysregulation, including catecholamine resistance and HR variability loss, plays a pivotal role in sepsis-induced cardiac dysfunction. Sharshar *et al.* showed, in septic patients, that cerebral damage and specific autonomic neuronal apoptosis resulted in inadequate autonomic control of the cardiovascular system [131, 132]. High level of circulating catecholamines is systematically found in both experimental and human septic shock [133-136]. This increase in catecholamines is responsible for inadequate tachycardia and an increase in oxygen requirements, both of which are associated with a higher mortality rate [137]. Several experimental studies have demonstrated in septic rats that these high catecholamine levels were associated with a desensitisation process at the cardiomyocyte level, characterised by the downregulation of β -adrenergic receptors (β -AR) [138, 139]. This desensitisation process is described in another section.

In addition to the decrease in myocardial adrenoceptor density, signal transduction is also disrupted by an increase in the expression of inhibitory G-proteins, while stimulatory G proteins (G_s) are reduced (**Figure 1**) [140-142].

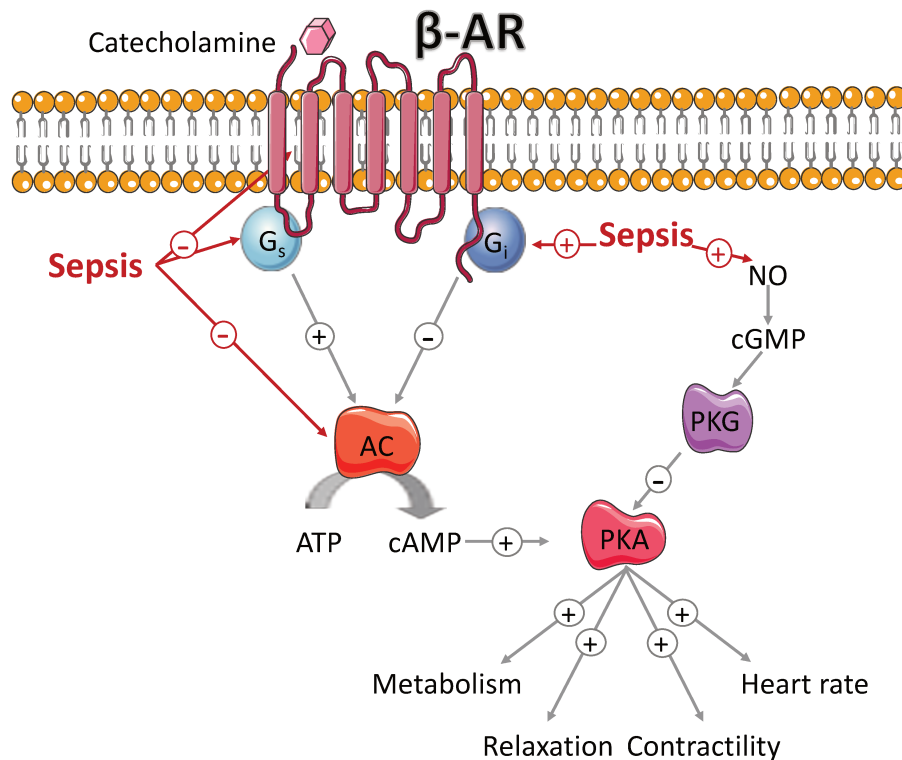


Figure 1: Influence of sepsis in adrenergic signalling.

Catecholamines bind to β -AR, inducing the activation of adenylate cyclase (AC) which transforms adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). cAMP activates PKA, which phosphorylates key enzymes influencing metabolism, relaxation, contraction, and HR. G proteins associated with the receptor, i.e. stimulatory G_s or inhibitory G_i, modify signal transduction. Sepsis induces a disruption in adrenergic signal transduction directly at β -AR, G proteins, AC, cAMP, PKA, and via cyclic guanosine monophosphate (cGMP). cGMP is produced by guanylate cyclase. The latter is activated by nitrogen monoxide (NO) produced in large quantities during sepsis. According to the reference: [143].

Vascular pathophysiology

Vascular hyporeactivity

In septic shock, hemodynamic injuries include vascular hyporesponsiveness characterised by endothelial and microvascular impairment and a decrease in vascular tone [144]. These alterations result in an abnormal cellular oxygen metabolism and in a vascular hyporesponsiveness to vasopressors [145].

Vascular hyporeactivity is defined by the inability to increase blood pressure despite the administration of vasopressor agents in septic shock patients. Thus, the constriction of vascular smooth muscle cells is not able to maintain transparietal pressure and thus arterial blood pressure. Experimentally, vascular reactivity can be assessed in myography chambers by exposing segments of isolated vessels to vasopressor or vasodilator agents [146, 147]. Dose-response studies with phenylephrine, a pure alpha agonist, allow for the detection of vessel contractility, while dose-response studies with acetylcholine allow for the detection of endothelium-dependent relaxation capacity and those with sodium nitroprusside (SNP) allow for the detection of the endothelium-independent relaxation capacity of the vessel. In clinical practice, dose-response studies with phenylephrine can also be performed; in this specific case, vascular hyporesponsiveness can be defined as a lower increase in arterial pressure for the same dose of phenylephrine or NE administered to patients compared to healthy volunteers [148].

There is considerable clinical evidence of vascular hyporeactivity during septic shock. First, despite a higher level of endogenous and exogenous-administered catecholamines, patients in septic shock who have received adequate fluid resuscitation still present arterial hypotension reflecting hypovasoreactivity [133]. Indeed, NE level is higher in non-survivor than survivor patients [149]. Bellisant *et al.* confirmed the decrease in vascular contractility to phenylephrine in septic compared to healthy patients [148]. In addition, hypotension associated with vascular hyporeactivity was reported as a risk factor of mortality [150].

Endothelial dysfunction

Endothelial cells form an inner cell monolayer in blood and lymphatic vessels and are the interface between the circulation and parenchymal cells. The endothelium plays a crucial

role in controlling blood flow and vascular tone and participates in immune responses [151]. Septic shock is associated with severely altered endothelial function, affecting all organs. During septic shock, the leakage in the endothelial layer increases due to alterations in cell-cell junctions. The loss of barrier function leads to leakage in the extravascular space and to life-threatening oedema. Bannerman *et al.* demonstrated that lipopolysaccharide (LPS) directly contributes to dysfunction of the endothelial barrier by caspase-mediated cleavage of junction proteins [152]. Matrix metalloproteinase 1 (MMP-1) is also involved in endothelial dysfunction. Tressel *et al.* demonstrated that increased plasma MMP-1 levels were correlated with worse outcomes in patients and that the inhibition of MMP-1 activity in mice decreased endothelial dysfunction in a proteinase-activated receptor 1 (PAR-1) dependent manner [153]. Numerous other molecules have been implied in sepsis-induced endothelial dysfunction, including vascular endothelial growth factor (VEGF) and angiopoietin-1 [154, 155].

Vascular endothelial cells are layered by the glycocalyx which is a complex network of macromolecules, including endothelial cell-bound proteoglycans and glycoproteins. This structure is also altered in septic shock. Glycocalyx degradation by ubiquitination compromises endothelial permeability and promotes the development of oedema [156].

Mechanism of vascular dysfunction

α_1 -adrenergic receptor desensitisation

When blood pressure decreases, the baroreflex of the sympathetic nervous system is activated, leading to the release of NE. Physiologically, the stimulation of α_1 -AR expressed on vessels by NE induces the contraction of arteries which maintains, or increases, blood pressure. However, during septic shock, several studies have reported α_1 -AR desensitisation

that contributes to vascular hyporeactivity and subsequently favours circulatory failure [157]. Carcillo *et al.* demonstrated a decrease in the number of α_1 -AR in the aorta of septic rats [158]. Moreover, Bucher *et al.* reported downregulation of the α_1 -AR gene in several organs such as the aorta, caused by the activation of NF- κ B [159, 160 , 161].

Reactive oxygen species

During sepsis and septic shock, excessive production of NO and superoxide anions (O_2^-) productions induce large quantities of peroxynitrite. In an *in vivo* rat model of endotoxaemic shock, an increase in the production of peroxynitrite was demonstrated [162]. Furthermore, Lundberg *et al.* showed that the inhibition of peroxynitrite formation was able to reverse vascular hyporesponsiveness in an endotoxic and hemorrhagic shock model [163].

Superoxide anions are also involved in vascular hyporesponsiveness. Superoxide anions can oxidise catecholamines [164], while in an experimental model of septic shock, Macarthur *et al.* demonstrated that vascular hyporesponsiveness was due in part to the deactivation of catecholamines by superoxide anions [164, 165]. Indeed, administration of superoxide dismutase removed the excess of superoxide, restored vascular contractility, and corrected hypotension in septic rats [165].

Immune response in septic shock

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection characterised by deep circulatory, cellular, and metabolic abnormalities. Historically, an excessive pro-inflammatory response was thought to lead to an early mortality in the first days of sepsis, and the compensatory anti-inflammatory response was thought to induce mortality later by immune suppression and long-term organ failure (**Figure 2, Theory 1**) [166, 167].

However, new insights gathered from septic patients have identified that both pro-inflammatory and anti-inflammatory responses occur early and simultaneously during sepsis [168]. The first part is a dominant hyper-inflammatory response characterised by hemodynamic instability, fever, and hyper-metabolism, mediated by the release of pro-inflammatory cytokines from innate immune cells. In the first few days, the early death is mainly due to a hyper-inflammatory cytokine storm [15]. In the second theory, it is postulated that the primary infection persists and that mortality is associated with a late dysregulated immune-inflammatory state. Alterations in the adaptive immune response could occur simultaneously with the persistent activation of inflammation and innate immunity (**Figure 2, Theory 2**) [169].

In the third theory, which is the most accepted one, the pro-inflammatory state predominates in the first days and then decreases. Failure of both innate and adaptive immune responses occurs in the second state, and death is due to the immune inability to cope with a second infection (**Figure 2, Theory 3**) [170].

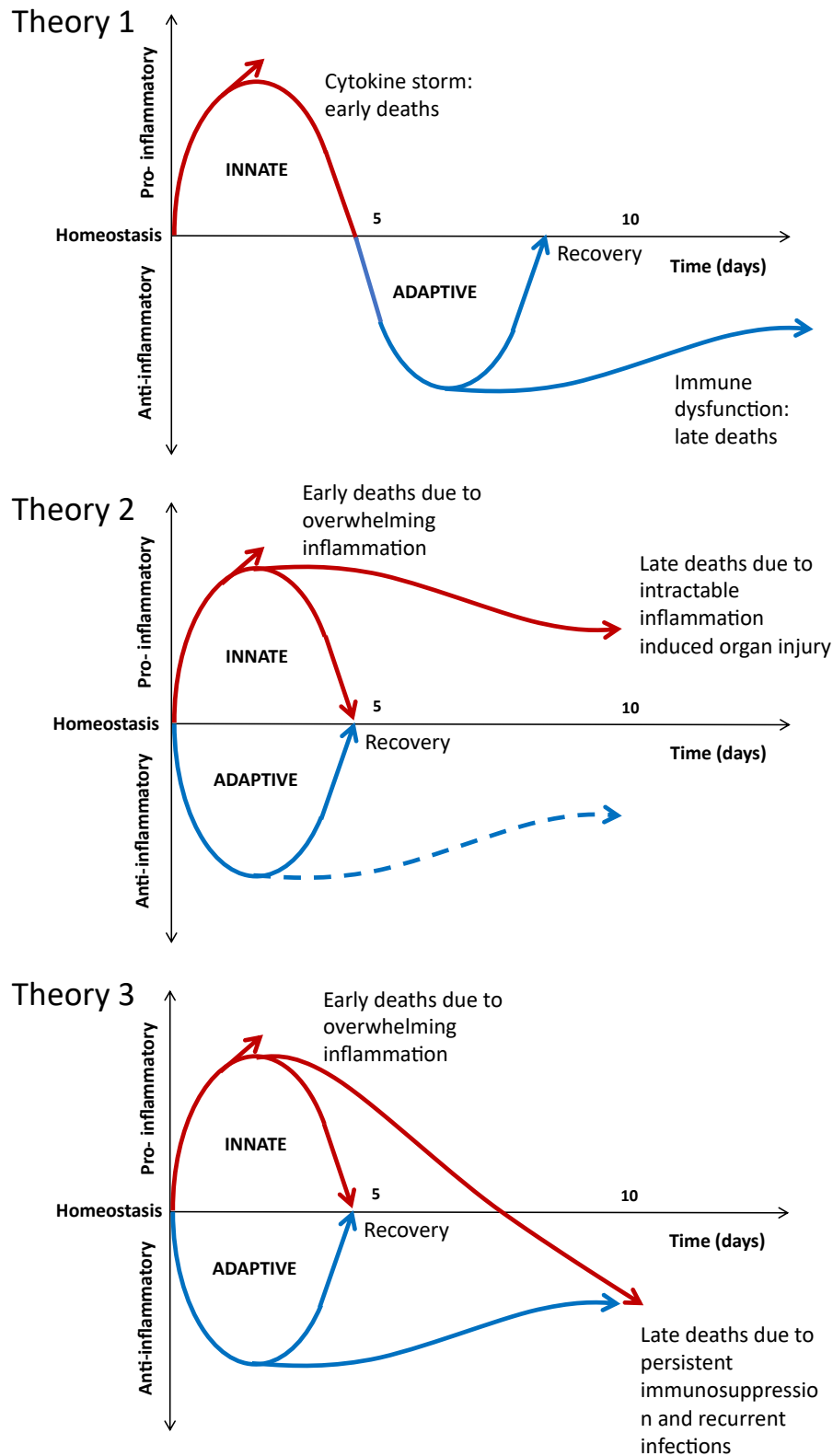


Figure 2: Theories of the host immune response in sepsis.

Theory 1: After the onset of sepsis, the hyper-inflammatory response dominates; this state is called the 'cytokine storm'. In the second step, an anti-inflammatory compensatory syndrome occurs and may cause immunosuppression. If patient does not achieve homeostasis, he frequently dies from a secondary infection.

Theory 2: After the onset of sepsis, activation of both the pro-and anti-inflammatory response occurs. This first stage can be the cause of an early death (top red line, day 3), typically due to a global hyper-inflammatory ‘cytokine storm’ response. The intensity of this response, induced by innate immunity cells, depends on the cause of the infection, patient comorbidities, and genetic factors. In most patients, recovery can take five days. In this theory, the late death of septic patients results from a persistent activation of the innate immune response, associated with adaptive responses causing intractable inflammation and organ injuries.

Theory 3: The first stage is similar to that of the second theory, thereafter, patients enter a profound immunosuppressive state characterised by inability of the immune system to cope a new infection. Most of patients will die from a nosocomial infection.

According to the references: [15, 167].

Activation of the immune innate system

The innate immune system is mainly based on physical and chemical barriers such as the skin, vascular endothelium, mucosal surfaces, and ciliated respiratory epitheliums. Cells of innate immunity include antigen presentation dendritic cells (DCs), macrophages and granulocytes and also, natural killer (NK), and $\gamma\delta$ T lymphocytes.

After the onset of an infection, the pathogen is recognised by the host innate immune system, mediated by the interaction between PAMPs and pathogen recognition receptors (PRRs). PRRs are proteins expressed in the cytoplasm and on the membrane of innate immune cells. Their role is to identify two classes of molecules: PAMPs and DAMPs. PAMPs are conserved motifs expressed by pathogens, not found on eukaryotic cells, including bacterial and viral nucleic acids, peptidoglycan, LPS, fungal β -glucan, α -mannan, and the bacterial protein flagellin. DAMPs are intracellular proteins or mediators that are released from dying cells, also known as ‘alarmins’, including high-mobility group box 1 protein (HMGB1), IL-1 α , heat shock protein (HSP), and uric acid [171].

PRRs are located on macrophages and DC, but also on various non-immune cells such as epithelial cells [172]. Four classes of PRRs have been identified: TLRs and C-type lectin

receptors, which are transmembrane proteins, and retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs), which are cytoplasmic proteins [173]. The recognition of PAMPs and DAMPs by PRRs induces the transcription of genes involved in the inflammatory response.

During sepsis, high levels of PAMPs and DAMPs lead to overstimulation of immune cells, resulting in a 'cytokine storm' state.

Toll-like receptors

TLRs are PRRs that have been most studied in the context of sepsis. TLRs are transmembrane proteins whose ectodomains contain leucine-rich domain repeats that mediate the recognition of PAMPs; the intracellular TIR (toll-interleukin 1 receptor) domain is required to downstream the signal transduction, leading to inflammatory mediator transcription [174]. Thirteen TLRs have been identified: 12 receptors in mice and 10 in humans.

To describe the signalling pathway, we will take the classic example of TLR4 (**Figure 3**). TLR4 responds to LPS, a component of the outer membrane of Gram negative bacteria. In the blood, LPS combines with LPS binding protein (LBP). The LPS/LBP complex is recognised by the cluster of differentiation CD14 [175]. TLR4 is associated with MD-2 protein, which binds to the extracellular domain of the receptor. All of these are essential to activate TLR4 downstream signalling [176]. The cytoplasmic portion of TLR4 activates adaptors such as myeloid differentiation factor 88 (MyD88) and TIR-associated protein (TIRAP) to the TIR-domain of the receptor, leading to I κ B α / β ubiquitination and degradation by the proteasome complexes. The consequence is the release of NF- κ B, which is translocated of into the nucleus. The latter initiates the transcription of pro-inflammatory cytokines such as IL1- β and TNF- α [99].

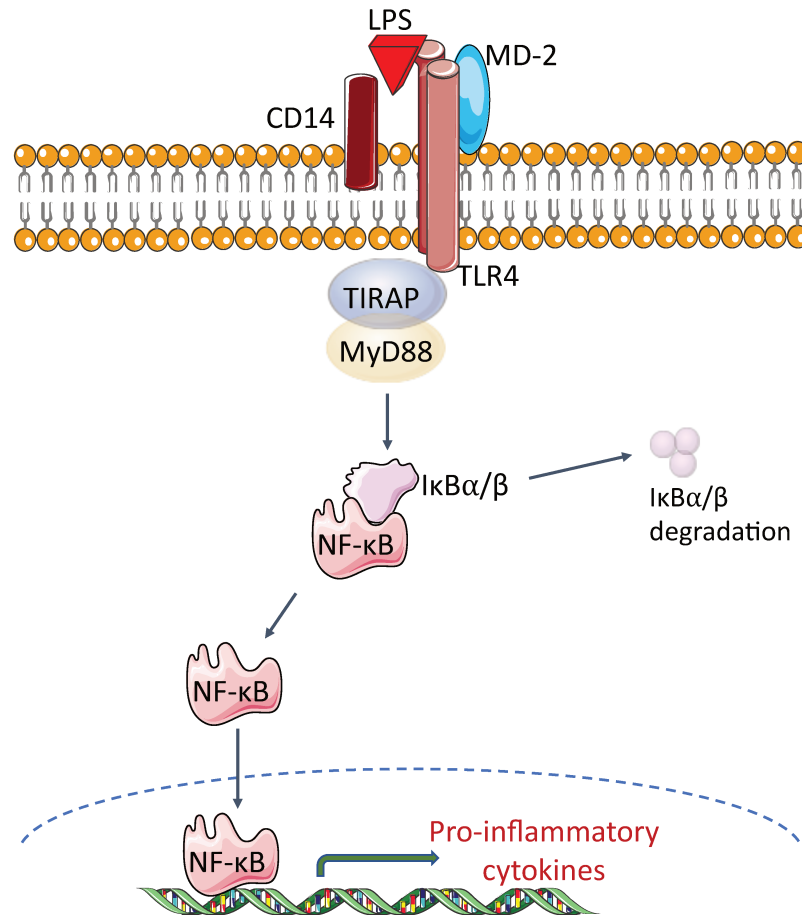


Figure 3: TLR4 signalling pathway.

TLR4 stimulation by LPS recruits TIR-domain adaptors including MyD88 and TIRAP, which finally results in IκBα/β degradation via activation of a series of signal molecules. Freed NF-κB is translocated into the nucleus, promoting the transcription of pro-inflammatory cytokines.

Inflammatory mediators

Cytokine storm

The release of inflammatory cytokines induces the production and release of new cytokines, which in turn causes cells and organs injuries. This phenomenon is commonly called the 'cytokine storm' [177]. The term 'cytokine storm' was first used by Ferrara *et al.* in 1993, describing the effects of graft-versus-host disease (GVHD) [178].

The cytokine storm involves many mediators, the best-known of which are IL-1 and TNF-α. The increase in cytokines is associated with both organ failure and with poor outcomes.

[179-181]. Targeted therapies such as antibodies and anti-TNF- α , anti-IL-1, or anti IL-1 receptor antagonist (IL-1Ra) have been tested, and have provided interesting preclinical results in terms of reducing plasma cytokine levels in mouse models [182]. However, these treatments consistently failed to reduce overall sepsis mortality in the clinic [7, 183, 184]. Osuchowski *et al.*, using a caecal ligation and puncture (CLP) mouse model of septic shock, observed an elevation at 24 hours of both pro- and anti-inflammatory cytokines. The pro-inflammatory cytokines peak was resolved within 48 hours, while the anti-inflammatory peak persisted for several days. Mortality was associated with the degree of both pro- and anti-inflammatory cytokine levels [185].

The most investigated anti-inflammatory interleukins are IL-1Ra and IL-10. IL-1Ra, a specific inhibitor of IL-1, is secreted by immune and epithelial cells and blocks the action of IL-1 α or IL-1 β inflammatory signal [186]. IL-1Ra treatment in mice with polymicrobial peritonitis or Gram negative pneumonia reduces the acute hemodynamic instability and decreases mortality [183, 187]. IL-10, a pleiotropic cytokine produced by both T cells and macrophages, is the main central anti-inflammatory cytokine [188]. High levels of IL-10 have been found 15 days after the onset of sepsis and may contribute to or amplify sepsis-induced immunosuppression [189]. In a two-hit model of sepsis, Muenzer *et al.* showed that blocking IL-10 can reverse sepsis immunosuppression. In this study, four days after CLP, mice were challenged with *Pseudomonas aeruginosa* bacteria and treated with an anti-IL-10 antibodies. Mice treated with the anti-IL-10 antibody showed a recovery of interferon- γ (IFN- γ) production, an increase in pro-inflammatory cytokines, and a decrease in bacterial growth [190].

Nitric oxide

Please refer to chapter 'Cardiovascular physiopathology of septic shock', page XX.

Coagulation

Coagulation abnormalities may occur in 50 to 70% of patients with septic shock [191]. In septic patients, various mechanisms at different sites act simultaneously, resulting in a procoagulant state that can lead to disseminated intravascular coagulation (DIC). The latter is associated with organ dysfunction and/or hemorrhage [192]. Pro-inflammatory cytokines are the main mediators that disrupt the coagulation system [193]. The interaction between inflammatory cytokines and tissue factor trigger the pro-coagulant state. Tissue factor, the main starter of thrombin generation, is expressed on endothelial cells and mononuclear phagocytes such as monocytes and macrophages [194]. Its activation leads to the production of thrombin. The combination of activated platelets to thrombin induces blood clot formation [195]. In addition, downregulation of fibrinolysis by endothelial cells promotes intravascular fibrin deposition [196]. The pathogenic pathways involved in coagulation alteration during septic shock are described in **Figure 4**.

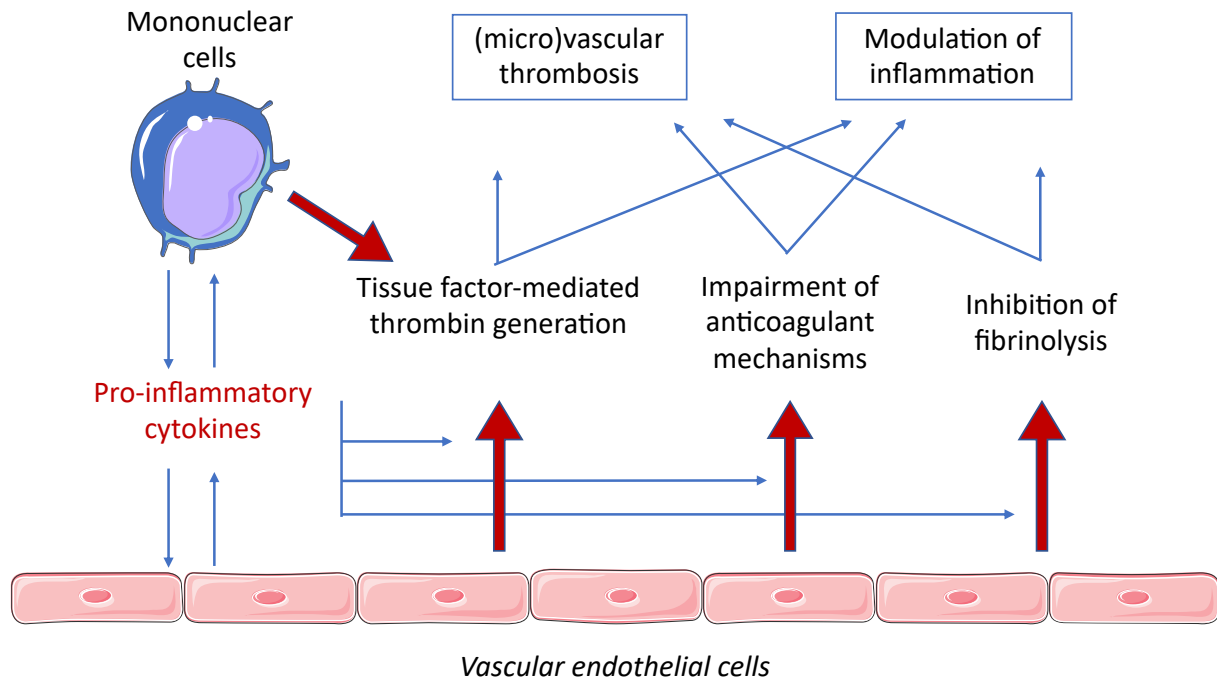


Figure 4: Pathogenic pathways involved in the activation of coagulation in sepsis.

During sepsis, both damaged endothelial cells and activated circulating mononuclear cells produce pro-inflammatory cytokines that mediate the activation of coagulation system. Tissue factor expression by the endothelium and activated mononuclear cells initiates the activation of coagulation. In addition, impairment of anticoagulant mechanisms and inhibition of fibrinolysis by the endothelium promote intravascular fibrin deposition. The latter results in a procoagulant state in septic shock patients. According to the reference: [196]

Immunoparalysis

Innate immune cells

Polynuclear neutrophils

Neutrophils are the first line of defence in protecting the host against infection. Under usual conditions, neutrophils have a short half-life (7-12 hours *in vivo*) while during septic shock an extended lifespan is observed [197-199]. This increase is beneficial on the one hand, giving more time for neutrophils to resolve sepsis, while on the other hand it is detrimental, by increasing neutrophil-dependent inflammation and tissue damage [92]. Delayed neutrophil apoptosis is also associated with decreased ROS secretion, potentially due to impaired TLR

signalling [200]. Taken as a whole, these alterations are associated with an increased risk of death after septic shock [201].

Multiple mechanisms contribute to sepsis-induced impairment of neutrophils migration [202]. For instance, proportionally to the severity of sepsis, pro-inflammatory cytokine release increases neutrophil cell membranes rigidity and reduces cellular deformability [203]. Thus, Lu *et al.* demonstrated a reduction in the neutrophil chemotaxis velocity to LPS in septic patients compared to healthy controls [204]. Furthermore, Raymond *et al.* showed a decrease in spontaneous neutrophil migration velocity in whole blood samples from septic adults [205]. One explanation might be the reduction in surface expression of chemokine (C-X-C motif) receptor 2 (CXCR2) and the adhesion molecule CD11b [205]. Interestingly, neutrophil phagocytosis capacity seemed to be preserved [206].

Monocytes/macrophages

During sepsis, monocytes undergo phenotypic and functional re-programming that allows them to transit from an inflammatory to an immunosuppressive state. Two of the most well studied markers of monocyte dysfunction in sepsis are *ex vivo* LPS-induced TNF- α production and monocyte cell surface expression of the human leukocyte antigen DR isotype (HLA-DR) [207]. Typically, monocytes from septic patients have a diminished capacity to release pro-inflammatory cytokines such as TNF- α , IL-1 α , IL-6, and IL-12, while the production of anti-inflammatory mediators such as IL-1ra and IL-10 is enhanced [208]. Moreover, during septic shock, monocytes are reprogrammed and develop a kind of tolerance to endotoxin [209]. HLA-DR expression by monocytes is reduced in septic patients and has become a hallmark of monocyte anergy and sepsis-induced immunosuppression [210, 211]. Decreased HLA-DR expression is associated with an increased in nosocomial infections and death [212,

213]. Re-expression of HLA-DR is a therapeutic strategy under investigation to reduce repeated super-infections in mechanically ventilated patients [214].

Dendritic cells

DCs are antigen-presenting cells that play a major role in the activation of adaptive immunity. During sepsis, the number of DCs is decreased in both the spleen and the circulation [215-217]. This decrease in septic shock patients during the first 24 hours is associated with disease severity and could predict a poor outcome [218]. This loss of DCs may significantly impair B and T cell function and participate in sepsis-induced immunosuppression [89].

Indeed, DC populations derived from septic patient monocytes are not able to induce a robust effector T cell response, but rather induce either T cell anergy or Treg cell proliferation [219]. In addition, DCs from septic patients show reduced surface HLA-DR expression, reduced secretion of pro-inflammatory cytokines, and increased synthesis of IL-10 [220, 221].

Natural killer lymphocytes

NK cells are granular lymphocytes largely recognised for their role in tumour surveillance and in the host response to viral infections. A significant decrease in the proportion of NK cells to the total lymphocyte count is observed in septic patients and is associated with an increase in mortality [222-224]. Several studies have reported that both CD56^{hi} and CD56^{low} NK cell subsets are affected during sepsis [225, 226]. In addition, there is a significant decrease in NK cell cytotoxicity and a profound reduction in their INF- γ release capacity [227, 228]. The decrease in the production of IFN- γ by NK cells could contribute to the incidence of secondary infections [229].

$\gamma\delta$ T cells

$\gamma\delta$ T cells are a distinct subset of lymphocytes that reside in the intestinal mucosa and display capacity of both innate and adaptive immune cells. Due to their location, $\gamma\delta$ T cells may be responsible of the first line of defence against infection [230]. There is a marked reduction in $\gamma\delta$ T cells in the circulation of septic patients (by around 80%), which translates into an increase in patient mortality [231, 232]. However, the consequences of the decreased number of $\gamma\delta$ T cells are still not fully elucidated [233]. In $\gamma\delta$ T cell-deficient mice, septic shock induces a decrease in inflammatory cytokines and an increase in mortality [234, 235]. One study suggested that the V γ 4+ subset of $\gamma\delta$ T cells contributed to the protective immune response of septic mice and could delay mortality [235].

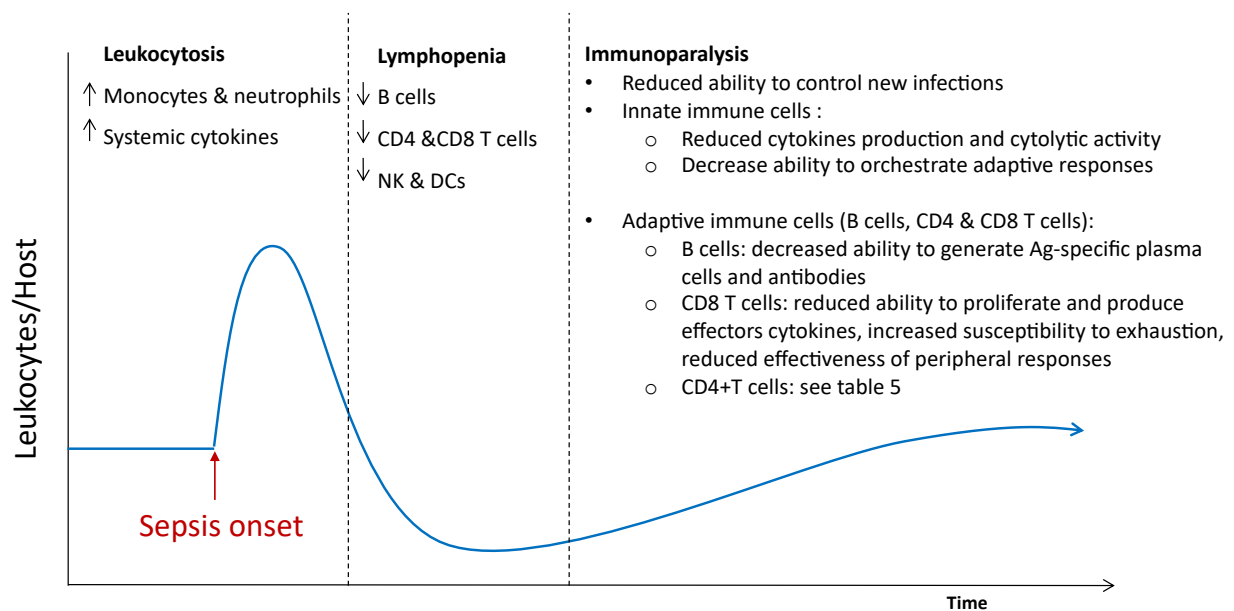


Figure 5: Effects of septic shock on immune cell subsets.

The immune system develops a state of leukocytosis for the first 2-4 days after the onset of septicaemia, characterised by an increase in circulating neutrophils and monocytes and an increased in levels of both circulating pro- and anti-inflammatory cytokines. Thereafter lymphopenia takes hold with a marked decrease in the number of adaptive immune cells such as B cells, CD4 and CD8 T cells, as well as innate immune cells including NK cells and DCs. For most patients, one month is required a full recovery. However, septic shock patients suffer from a long-lasting state of immunoparalysis despite a normal number of leukocytes. The state of immunoparalysis is characterised by a reduced ability in the control of new infections and by functional alterations in both innate and adaptive immune cells. According to the reference: [236].

Adaptive immune cells

B lymphocytes

B lymphocytes play a pivotal role in both innate and adaptive immune responses. Their key role in the humoral response, *i.e.* immunoglobulin synthesis, is the best known. As antigen-presenting cells, B lymphocytes can activate T cells through the expression of major histocompatibility complex (MHC) class II [237]. B cells recognise a wide variety of antigens (such as proteins, lipids, polysaccharides, nucleic acids, and chemicals) that bind to surface immunoglobulin M (IgM) or IgD and cause their activation and cytokine production [238, 239]. Moreover, a subpopulation of regulatory B cells with immunoregulatory properties has been described [240].

In the specific setting of septic shock, the phenotype and function of B cells during the immunosuppressive phase have been poorly studied. Kelly-Scumpia *et al.* demonstrated that, in B cell deficient mice, the induction of septic shock decreased inflammatory cytokine and chemokine production and increased mortality rate [239]. In septic shock patients, in the immunosuppressive state, the absolute B cell number is reduced due to an increase in apoptosis, whereas the B cell proportion is increased compared to the whole lymphocyte count [225, 241, 242]. The functions of B cells are also altered. B cells from septic patients present decreased in HLA-DR expression, a deficiency in IgM synthesis, and a decrease in activation, proliferation, and IL-10 production capacities [242].

CD8 T lymphocytes

CD8 T lymphocytes recognise and respond to pathogen through the MHC class I molecules expressed on infected cells and antigen-presenting cells. Activated CD8 T cells clear infected cells by cytotoxicity (via granzyme B and perforin production) or recruit and activate

other immune cells (via IFN- γ and TNF- α secretion) [243, 244]. Following sepsis, alterations in the number and functions of CD8 T cells occur. Post-septic apoptosis affects both pathogen-specific naïve and memory CD8 T cells [245, 246]. The recovery of naïve CD8 T cells following sepsis is slow and most often partial, resulting in the loss of some precursor specificities [247]. Memory CD8 T cells show an impairment in both Ag-dependent and independent functions with a reduction in Ag-sensitivity, proliferative capacity, and in the ability to produce cytokines [248]. Defective naïve and memory CD8 T cells contribute to the increased susceptibility of septic shock survivors to new infections [247, 248].

Several studies also demonstrate, in CLP models, increased expression of inhibitory receptors such as programmed cell death 1 (PD-1) and 2B4, a reduced ability to produce effector cytokines (IFN- γ and TNF- α), and a reduced ability to clear the infection, indicating the exhaustion of CD8 T cells [249-251]. The latter plays a major role in lingering viral infection but is less pronounced than CD4 T cell exhaustion in sepsis-induced immunosuppression.

CD4 T lymphocytes

CD4 T cell loss and recovery

The number of CD4 T cells is considerably reduced after the onset of sepsis [252-254]. Thus, T cell apoptosis plays a crucial role in reducing the number of circulating T cells [255, 256]. Lymphocyte apoptosis during sepsis results from both the extrinsic (death receptor-mediated) and intrinsic (mitochondria-mediated) pathways [257]. The extrinsic pathway is initiated by the binding of inducers to surface death receptors. For instance, Ayala *et al.* demonstrated that survival was improved by the administration of Fas death receptor-mediated pathway inhibitors in polymicrobial sepsis [258]. Conversely, the intrinsic pathway is triggered by an inversion of the balance between pro- and anti-apoptotic proteins within

mitochondria. Several studies have found that mice overexpressing the anti-apoptotic B-cell lymphoma 2 (Bcl-2) protein, localised on the mitochondrial membrane, were resistant to sepsis-induced apoptosis [259].

For most patients, a one-month delay is required for a post-septic recovery in absolute CD4 T cell numbers. The failure to normalise CD4 T cell functions is associated with a poor outcome [252, 253, 260]. In addition, advanced age has been shown to increase lymphopenia and to prolong CD4 T cell recovery in elderly septic patients [253]. Finally, Cabrera-Perez *et al.*, demonstrated an incomplete recovery of the antigen-specific T cell repertoire after sepsis, secondary to the attrition of antigen-specific T cell receptor (TCR) diversity. This is associated with impaired CD4 T cell responses after sepsis [254].

CD4 T cell functional defects

Many early studies have shown that cytokine production by CD4 T cells is altered, and suggest that septic shock induces a phenotypic change in the CD4 T cell balance from Th1 to Th2 [261-266]. A recent study published by Boomer *et al.* investigated freshly isolated post-mortem spleen and lung cytokine production in patients who died from septic shock [170]. They found an inability of stimulated splenocytes to produce IFN- γ , TNF- α , IL-6, and IL-10 and an increase in the expression of some inhibitory receptors. These results provide evidence of the functional unresponsiveness of T cells, which appears to be a state of cell exhaustion [253, 266, 267]. This point is strengthened by several studies showing a decrease in the mRNA transcription levels of the T-bet, GATA3, and ROR γ t transcription factors that regulate differentiation into the Th1, Th2, and Th17 CD4 T cells subsets, respectively [254, 268, 269].

The inability to proliferate and the over-expression of inhibitory receptors are surrogates of functional alterations. PD-1 is known to induce an inhibitory signal in T cells

characterised by cell cycle arrest, the inability to proliferate, and reduced cytokine synthesis [270]. In septic shock, several studies have shown an increase in the expression of PD-1 in CD4 T cells, which is associated with increased mortality and the occurrence of secondary nosocomial infections [271, 272]. Moreover, a role for the receptor TRAIL (targeting TNF-related apoptosis-inducing ligand) in immune suppression during septic shock has been identified. Targeting the TRAIL/TRAIL receptor pathway could be a potential target for therapeutic intervention for restoring T cell functions [273, 274]. Thus, septic shock is responsible of global functional defects regulating in CD4 T cell responses, which are summarised **Table 5**.

Changes in CD4 T cells subsets: increased regulatory T cell proportions

One of the characteristics of CD4 T cells is their ability to differentiate into subtypes. The latter have specific functions best fitted to fight against specific threats [275]. As described in the previous sections, sepsis disrupts both numbers and function of CD4 T cells, including the Th1, Th2, Th17, and Treg subsets.

A number of studies have described an increased percentage of circulating Treg cells in septic shock patients [16-19]. This increase was observed immediately after the onset of sepsis but persisted only in septic shock patients who subsequently died [16, 20]. The increased number of circulating Treg cells could be the result of preferential loss of other subsets (*i.e.*, Th1, Th2, and Th17) [276, 277]. This elevated proportion of the Treg subset might be involved in decreasing T cell proliferation [278, 279]. Stieglitz *et al.* demonstrated that specific depletion of Treg cells improved impaired T cell proliferation in septic mice, illustrating the participation of Treg in sepsis-induced immunosuppression [279]. In addition, IL-33, release during widespread tissue injury, has an essential function in the expansion of Treg

cells; IL-33 induces the polarisation of M2 macrophages, thus enhancing expansion of the Treg cell population through IL-10 secretion and finally contributes to the development of long-term sepsis-induced immunosuppression [280].

Treg cells can also have a suppressive effect on innate immune cells. In the setting of LPS activation, Treg cells act on monocytes by inhibiting their survival through a pro-apoptotic mechanism involving the Fas/FasL pathway [281]. Furthermore, Treg cells inhibit IFN- γ production by $\gamma\delta$ T cells in response to *Mycobacterium tuberculosis* [282].

Table 5: Effects on sepsis on CD4 T cells.

According to the reference: [236]

Category	Effects	References
Loss and recovery	CD4 T cell number is dramatically reduced	[252-254]
	Apoptosis results from both the intrinsic and extrinsic pathways	[257-259]
	One month is required to recover the pre-septic number of T cells	[252, 253, 260]
	There is an incomplete recovery for some epitope specificities	[254]
Functional defects	Global anergy:	
	• Reduced ability to produce cytokines	[170, 253, 266, 267]
	• Reduced ability to proliferate	[254, 261, 269]
	• Increased expression of inhibitory receptors	[271-274]
Changes in subset representation	There is a decreased representation of Th1, Th2, and Th17 subsets	[276, 277]
	There is a decreased transcript levels of T-bet, GATA3, and ROR γ t	[254, 268, 269]
	There is an increased Treg cell representation leading to decreased in T cell proliferation and innate cells	[16-19, 278, 279]

Experimental therapies to alleviate sepsis-induced immunoparalysis

Therapies designed to alleviate sepsis-induced immunoparalysis have been focused on reducing immune cell death, on increasing the number of surviving immune cells, and on restoring their functions. At first sight, studies investigating anti-apoptotic molecules, *i.e.* Bcl-2 or caspase inhibitors, to block lymphocyte apoptosis showed a survival benefit in septic mouse models [283-286]. However, the therapeutic potential has not been widely adopted because of their impact on other cellular processes [236]. Thereafter, research has been redirected towards investigation of immunostimulant strategies [287]. Among them, the four most studied immunomodulating agents are GM-CSF, INF- γ , PD-1, and IL-7 [288]. Meisel *et al.* demonstrated that treatment using GM-CSF is safe and restores monocytic immunocompetence in patients with sepsis. This tended to improve the severity of the disease, although 28-day mortality was not increased [289]. Two studies have shown that INF- γ treatment had a beneficial effect on monocyte reactivation, characterised by an increase in HLA-DR expression [290, 291].

Another approach to reversing immunosuppression in sepsis involves the blockade of the co-inhibitory molecules PD1 and PDL1. To date, there have been no randomised controlled trials on PD1 blockade in septic shock. Preliminary experimental studies showed that blockade of PD1-PDL1 signalling could improved survival in clinically-relevant animal models [292].

IL-7 is also a potential immunomodulating agent. Studies in mouse septic models and in T lymphocytes from humans showed that IL-7 administration might be able to restore T lymphocyte changes [293]. In addition, a phase II clinical trial conducted in patients with septic shock and severe lymphopenia also demonstrated that IL-7 reversed the marked loss of CD4 and CD8 T cells [294].

Dysregulation of the sympathetic nervous system following septic shock

Sympathetic nervous system

The sympathetic nervous system (SNS) is one of the three components of the autonomic nervous system (ANS), the other two being the parasympathetic and enteric systems. The main function of the SNS is to maintain both basal and stress-related homeostasis [295]. The sympathetic system is located in the locus coeruleus and comprises four anatomical levels (cervical, thoracic, lumbar, and pelvic), with two neurons connected in series at each level by a para-spinal ganglion fibre that innervates all parts of the body.

The SNS is activated by different inputs from sensory afferent nerves (via the hypothalamus), or by central nervous stimuli (cortical areas, limbic system). Conceptually, activation of the sympathetic nervous system releases NE by the second post-ganglionic sympathetic neuron in specific organs. Stimulation by acetylcholine released from splanchnic pre-ganglionic fibres on the adrenal gland turns on the production and release by the medulla of NE and epinephrine in the bloodstream [295]. NE and epinephrine bind to AR, a G-protein coupled receptor. There are two main classes of AR: α and β . The distribution, signalling pathways, effects of activation and inhibition of these AR on hemodynamics and inflammation are detailed in **Table 6**.

Table 6: Classification, localisation, signalling, agonist, antagonist, hemodynamic effects and inflammatory effects of main adrenoreceptors.

According to the references: [296 , 297 , 298]

Adreno-receptor	Localisation	Signalling	Agonist	Antagonist	Hemodynamic effects	Inflammatory effects
α_1	Heart Blood vessels Lung Digestive system	$G\alpha_q$ protein $\rightarrow \nearrow PLC$ $\rightarrow \nearrow Ca^{2+} \nearrow PKC$ $\rightarrow \nearrow$ Eicosanoids, $\nearrow Ca^{2+}$ channels, $\nearrow MAPK$ signals, Regulation of K^+ channels	Phenylephrine Methoxamine	Prazosin Doxazosin Terazosin	Vasoconstriction Smooth muscle contraction	Pro-inflammatory
α_2	Blood vessels Digestive system Nervous system	$G\alpha_i$ and $G\alpha_o$ protein $\rightarrow \searrow AC$ $\rightarrow \searrow cAMP, \searrow PKA,$ $\nearrow K^+$ channel activity	Clonidine	Yohimbine Idazoxan Atipamezole	Vasoconstriction	Pro-inflammatory
β_1	Heart Kidney Adipose cells	$G\alpha_s$ protein $\rightarrow \nearrow AC$ $\rightarrow \nearrow cAMP,$ $\nearrow PKA, \nearrow Ca^{2+}$ channel activity	Isoproterenol Dobutamine	Propranolol Metoprolol Atenolol Esmolol Landiolol	Positive inotropic and chronotropic effects	Pro-inflammatory
β_2	Heart Blood vessels Lung Digestive system Liver Urinary system Reproductive system Nervous system	$G\alpha_s$ and $G\alpha_i$ protein $\rightarrow \nearrow AC$ $\rightarrow \nearrow cAMP,$ $\nearrow PKA, \nearrow Ca^{2+}$ channel activity	Isoproterenol Terbutamine Albuterol Salmeterol	Propranolol Butaxamine	Vasodilatation Smooth muscle relaxation	Anti-inflammatory
β_3	Adipose cells Heart	$G\alpha_s$ and $G\alpha_i$ protein $\rightarrow \nearrow AC$ $\rightarrow \nearrow cAMP,$ $\nearrow PKA, \nearrow Ca^{2+}$ channel activity	Isoproterenol Mirabegron Amibegron Solabegron	Propranolol	Negative inotropic effects	Anti-inflammatory

Abbreviations: AC: adenylate cyclase; cAMP: cyclic adenosine monophosphate; MAPK: mitogen-activated protein kinase; PLC: phospholipase C; PKA: protein kinase A; PKC: protein kinase C; \rightarrow : induced; \nearrow : increased; \searrow : decreased.

When homeostasis is disrupted or threatened by internal or external challenges, the SNS is activated, resulting in an increased plasma level of catecholamines, which play a crucial role in maintaining the homeostasis. In a shock state, activation of the SNS increases HR, cardiac inotropism, and vascular contractility. These effects aim to preserve and/or restore organ perfusion. In addition, SNS activation has much broader effects, including bronchodilation, relaxation of smooth muscle and the intestine sphincter, gluconeogenesis, glycolysis and lipolysis, and anti-inflammatory regulation [298]. This schematic representation of SNS activation is closely associated with other response systems, such as the parasympathetic and hypothalamic-pituitary-adrenal systems.

β -AR and their signalling pathways

The β -AR belongs to the G-protein-coupled seven-transmembrane domain receptor superfamily. There are three β -AR receptor subtypes: β_1 -AR, β_2 -AR, and β_3 -AR. The β_1 -AR gene is on chromosome 10, the β_2 -AR gene is on chromosome 5, and the β_3 -AR gene is on chromosome 8. β_1 -AR and β_2 -AR are widely expressed. In the heart, β_1 -AR is predominant (β_1 -AR: 75-80% of cardiac β -AR, β_2 -AR: 20-25%); conversely, in smooth muscles such as in the bronchi and vessels, β_2 -AR are predominant [297]. β_3 -AR is the least studied isotype; it is expressed in adipose tissue, but also in the myocardium, in the urinary tract, and in endothelial cells [299, 300].

Following activation by specific agonists such as NE, epinephrine or dopamine, β_1 -AR binds to the G_s protein. The G_s protein activates adenylate cyclase, which causes an increase in the second messenger cyclic AMP (cAMP). Protein kinase A (PKA) is subsequently activated by cAMP, which phosphorylates numerous targets in cytoplasm. In cardiomyocytes, PKA activates L-type Ca^{2+} channels, which increases the influx of Ca^{2+} , phospholamban (PLB), and

the phosphorylation of troponin I, strengthening cardiac contraction [301]. This modulation also favours the occurrence of pro-apoptotic processes. Thus, in response to β_1 -AR stimulation, an increase in intracellular calcium is observed, which activates the protein kinase calcium/calmodulin (CaMKII). This protein promotes the mitochondrial apoptotic pathway through the release of cytochrome C, leading to the activation of caspase-9 and cell death [302, 303].

Unlike β_3 -AR, prolonged stimulation of the β_1 -AR or β_2 -AR is associated with desensitisation due to the phosphorylation of the C-terminal end of receptors. Adrenoreceptor desensitisation requires three steps: 1. phosphorylation of the receptor by PKA and β -AR kinase (β -ARK), leading to G_s uncoupling; 2. sequestration of the receptor by translocation of the β -AR from the plasma membrane to a vesicular compartment deficient in G_s ; 3. Downregulation of the receptor, resulting in its degradation through a lysosomal pathway [139, 304]. The β_3 -AR does not have these phosphorylation sites and is therefore not subjected to a desensitisation process [305]. The β_3 -AR signalling pathway is still poorly understood. Nevertheless, it is probably involved in regulation processes during sympathetic overstimulation [306].

Interaction between the sympathetic system and immune system

Many organs, such as the heart and the gastrointestinal tract, receive both sympathetic and parasympathetic innervation. However, in both primary (bone marrow and thymus) and secondary (spleen and lymph nodes) lymphoid organs, sympathetic innervation is predominant [307, 308]. A recent study demonstrated, with immunofluorescent staining of neurofilaments, the innervation of splenic sub-compartments, including splenic nodules, marginal zones, and red pulp [309]. The findings supported the location of sympathetic nerves

in lymphoid organs and prompted the analysis of AR expression on the surface of immune cells. It was demonstrated that ARs are expressed on the cell membranes of neutrophils, NK cells, DCs, macrophages, B lymphocytes, and T lymphocytes [27-31].

The effect of NE mediated through β -AR was recently confirmed as a crucial factor involved in immunoparalysis [24, 296]. The activation of PKA through the increase in cAMP due to β -AR activation inhibits NF- κ B and results in reduced expression of pro- inflammatory cytokine genes (**Figure 6**) [21]. Moreover, the cAMP/PKA pathway enhances the synthesis of IL-10 [310]. In human blood, Van der poll *et al.* demonstrated that NE infusion decreased the production of TNF- α and IL-6 and increased IL-10. These effects were reversed by the administration of a β -AR blocker [311, 312].

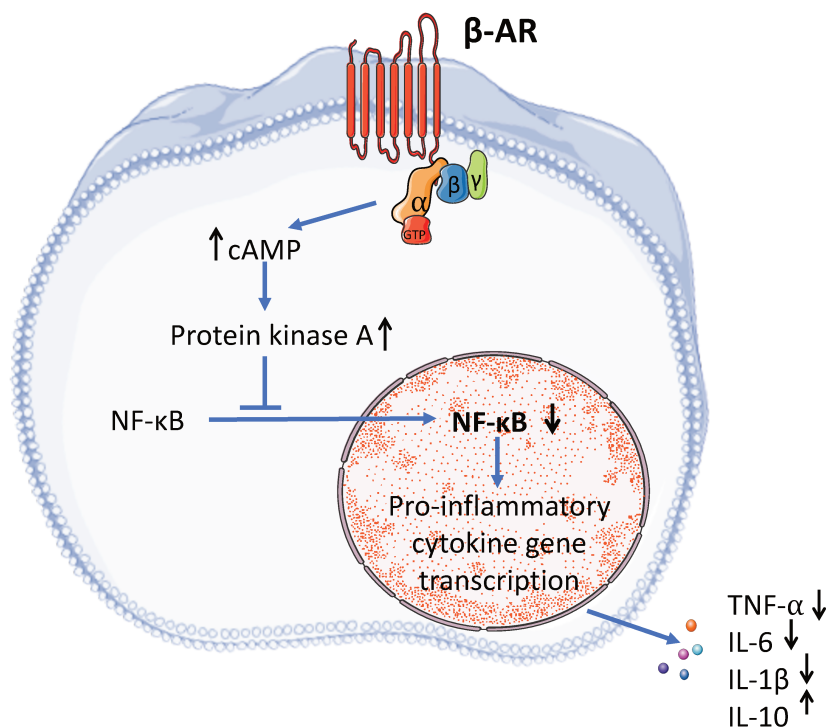


Figure 6: Intracellular mechanisms behind the effects of β -AR stimulation on cytokine production.

Stimulation of β -AR results in increased intracellular cAMP, which activates PKA, in turn resulting in blocked translocation of NF- κ B to the nucleus. Consequently, reduced pro-inflammatory cytokine transcription or production and increased production of IL-10 were observed. Abbreviations: cAMP: cyclic adenosine monophosphate; IL: interleukin; NF- κ B: nuclear factor kappa light chain enhancer of activated B cells; PKA: protein Kinase A; TNF- α : tumour necrosis factor-alpha.

Adrenergic regulation of innate immune cells

Recent findings provide evidence that the SNS regulates the innate immune response, which is summarised in **Figure 7**. NE treatment of neutrophils, *in vitro* and *in vivo*, inhibits their migratory function, activation, and phagocytosis capacity [313-315]. Similarly, the consequences of β -AR stimulation on monocytes are usually anti-inflammatory. β -AR activation can also inhibit oxygen radical production, TNF- α production, and LPS-induced MIP-1, IL-18, and IL-12 production [316-319]. Under certain conditions (*i.e.* in unstimulated cells), β -AR stimulation may result in a pro-inflammatory response in monocytes, including an increased production of IL-18 or an increase in metalloproteinases (MMP-1) in both circulating monocytes and macrophages [320, 321]. NE and epinephrine decrease NK cell cytotoxicity and cytokine production [322-325]. Dendritic cells also express β -AR [326]. Several studies have demonstrated that β_2 -AR activation on bone marrow DCs impaired the production of TNF- α and IL-6, reduced the ability of DCs to present antigens, and enhanced IL-10 production by CD4 T cells [22, 38, 327]. In addition, independently of IL-10 receptor activation and transforming growth factor- β (TFG- β) release, activation of β_2 -AR on DCs leads to an increase in Treg cells [22].

Adrenergic regulation of adaptive immune cells

Numerous studies demonstrated that almost all lymphocyte subtypes express β -AR and that adrenergic agents can suppress T cell activity and proliferation [23, 38, 328-330]. Treatment with NE is able to suppress cytokine production by T cells, illustrating its immunosuppressive capability [23]. Furthermore, several studies have demonstrated in a

cancer context that adrenergic signalling impaired the antitumor CD8 T cell response, which was reversed by a β -AR blocker administration [331-333].

To date, mostly the β_2 -AR subtype has been described on T cells: naive T cells, Th1, Th17, and Treg cells predominantly express β_2 -AR, while Th2 cells do not express β_2 -AR [330, 334-336]. However, catecholamines drive a Th2 shift at the level of both antigen presenting cells and Th1 cells. β_2 -AR stimulation on CD4 T cells inhibits IFN- γ production by Th1 cells, but does not change IL-4 production by Th2 cells, while β_2 -AR antagonists are able to restore Th1 cytokine production [295, 334, 337, 338].

While SNS-mediated modulation of CD4 T cell immune responses has been focused on the Th1/Th2 dichotomy, very little is known on the effect of adrenergic stimulation on other subsets such as Th17 and Treg cells. Liu *et al.* recently demonstrated that β_2 -AR are expressed on Th17 cells and that β_2 -AR/cAMP/PKA signalling in Th17 cells reduces their inflammatory response in the context of rheumatoid arthritis [339]. Another study showed a suppressive effect of NE on IL-17 and IFN- γ production by human leukocytes [340]. In acute stress, two studies showed a decrease in the number of peripheral of Treg cells, while Guerreschi *et al.* found that noradrenaline treatment enhanced the suppressive activity of Treg cells [330, 335, 341].

Until recently, β_1 -ARs were far less reported on [335, 338, 342]. A recent study investigating the impact of β -AR blockers on CD4 T cell dysfunction in cirrhotic mice reported that β_1 -AR were overexpressed by spleen CD4 T cells. In addition, chronic β -AR blocker treatment was accompanied by the redistribution of T cell subsets, leading to the normalisation of Th2 and Treg levels in the spleen [33].

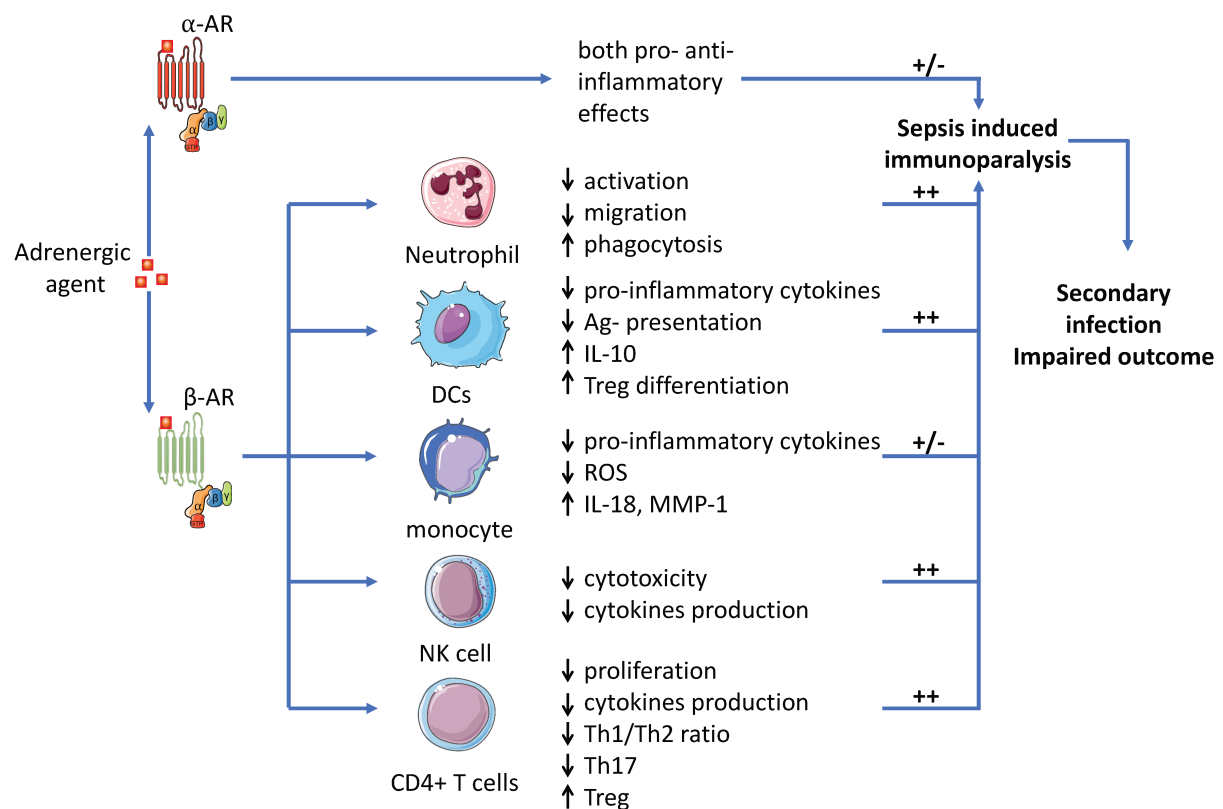


Figure 7: Several adrenergic mechanisms contribute to sepsis-induced immunoparalysis.

Abbreviations: DCs: dendritic cells; IL: interleukin; MMP: matrix metalloproteinase; NK: natural killer; ROS: reactive oxygen species; TNF: tumour necrosis factor; Th: T helper. According to the reference: [21]

Sympathetic dysautonomia

Dysautonomia, also called autonomic dysfunction, defines all deleterious events caused by the persistence over time of high-level activation of the SNS [342]. The biological markers of this over-activation are a concentration of epinephrine and NE 5 to 100 times higher than physiological activation [9]. High plasma levels of catecholamines are associated with an impairment of cardiovascular, coagulation, and inflammation function (**Figure 8**).

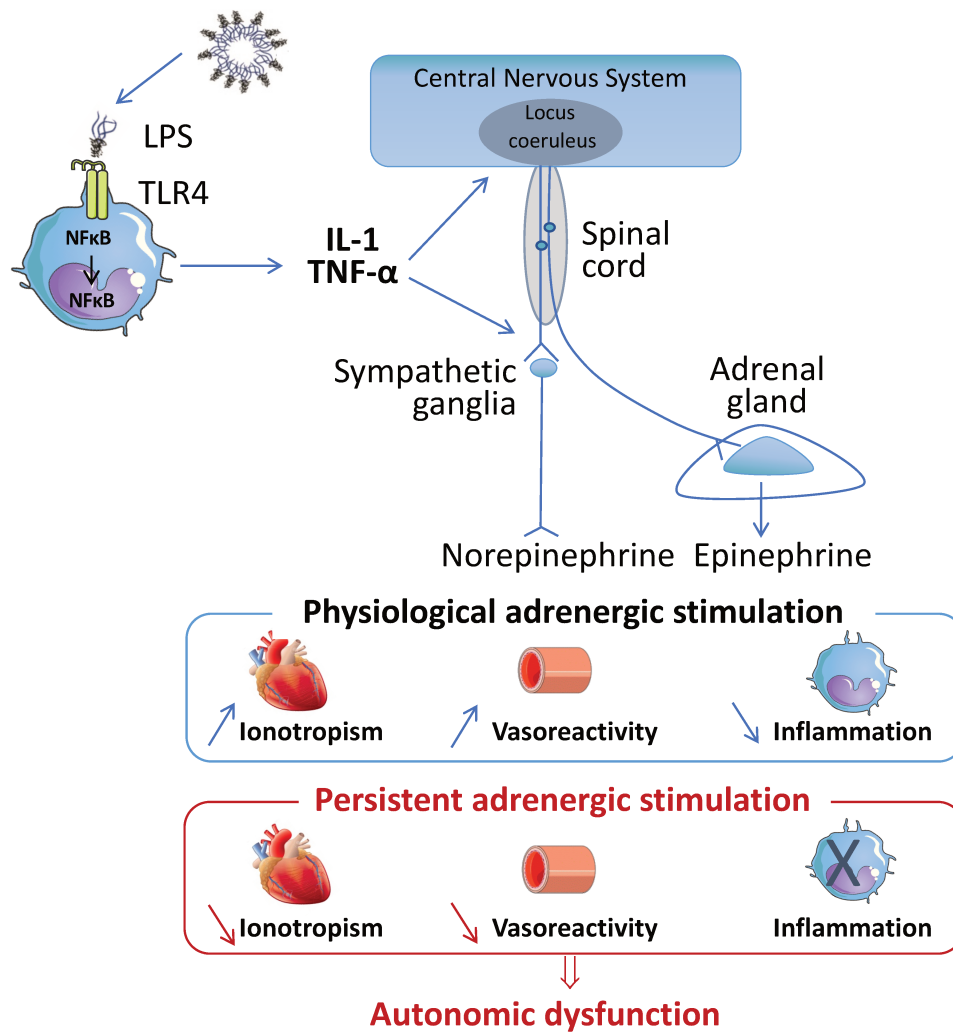


Figure 8: Sympathetic nervous system activation during septic shock.

During an infection, LPS challenge (a major component of the bacterial membrane) activates Toll-like receptor 4 (TLR4) present on innate immune cells. TLR4 activation induces NF-κB nuclear translocation. The latter drives pro-inflammatory cytokine expression, i.e. IL-1 and TNF-α. Cytokines activate the sympathetic nervous system at the central level in the locus coeruleus and at the peripheral level via sympathetic ganglia, which induces a massive release of catecholamines. Catecholamines activate adrenergic receptors and lead, under physiological conditions, to an increase in inotropism and in vasoreactivity, and a reduction in inflammation. When this activation persists over time, it becomes pathological and leads to a decrease in inotropism and vasoreactivity as well as the induction of immunosuppression. This phenomenon happens during septic shock and is called dysautonomia.

Modulation of the sympathetic system by β_1 -AR blocker

Which molecules could be used to modulate the sympathetic nervous system during septic shock?

The peripheral therapeutic target regarding SNS overactivation seems naturally to be β -AR. β -AR blockers are therefore the molecules indicated in this context. Nevertheless, non-cardioselective β -AR blockers, *i.e.* β_1/β_2 -AR blockers, are probably less suitable than cardioselective β_1 -AR blockers. Indeed, β_2 -AR inhibition is counterintuitive since β_2 -AR activation has a key role in the inflammatory balance [295]. Both experimental and clinical studies showed that its inhibition and its genetic polymorphisms were associated with a poor prognosis in septic shock [39, 343]. Conversely, in an experimental setting, several studies have demonstrated that β_2 -AR stimulation has beneficial effects in terms of reducing organ failure [344]. However, these results were never translated to the clinic [345].

Esmolol, a cardioselective β_1 -AR blocker with a half-life of two minutes and a half-life elimination of nine minutes, could be used. The main advantages of this molecule are its very short duration of action, its short half-life, and its intravenous administration, which make its use possible in the management of septic shock. Other β_1 -AR blockers could be used, such as landiolol, which has a higher β_1 -cardioselectivity and an even shorter elimination half-life (three to four minutes).

A central approach could also be added to the abovementioned peripheral approach to the modulation of the SNS. Central modulation of the SNS is also an approach to regulating adrenergic overstimulation. Similarly to the peripheral approach, this is associated with a recovery of the contractile, cardiac, and vascular response to catecholamines through the re-expression of AR on cardiac and vascular smooth muscle cells [346]. Thus, the stimulation of

α_2 -ARs in the locus coeruleus drives a decrease in the peripheral sympathetic adrenergic response [295]. Dexmedetomidine or clonidine, used for light sedation and alcohol withdrawal, respectively, are the two best-known and most tested α_2 -AR agonist molecules. Although there is yet no clinical evidence, numerous preclinical studies have demonstrated that, during experimental septic shock, central modulation of the sympathetic nervous system was associated with improved vasoreactivity, microcirculation, and a reduction in tissue and plasma inflammation [347-349]. The combination of the peripheral approach with esmolol and the central approach with dexmedetomidine has also been experimentally evaluated, demonstrating the feasibility of this combination. This strategy needs to be confirmed in patients in a randomized trial [350]. Indeed, it should be remembered that it is counterintuitive in septic shock to reduce the main factors of arterial oxygen transport, which are HR and inotropism. It is therefore appropriate, as a first step, to accumulate experimental and clinical data assessing the tolerance to and the clinical effects of such an approach.

Pharmacology of esmolol

Esmolol is an ultra-short acting cardioselective β_1 -blocker. The structure of esmolol (methyl 3-[4-(2-hydroxy-3-[isopropylamino] propoxy) phenyl] propionate) is shown in **Figure 9**. Esmolol is a well-tolerated therapy in patients with tachycardia and critical cardiovascular conditions that require a β_1 -AR blocker with fast-acting and above all fast-reversible properties.

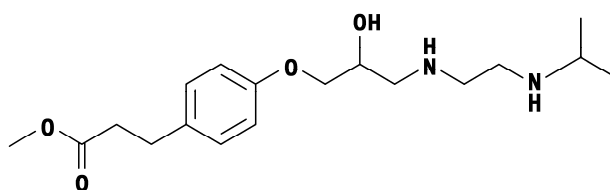


Figure 9: Structural formula of esmolol.

Esmolol is an ultra-short-acting β_1 -AR blocker with a half-life of distribution of 2 minutes and an elimination half-life of 9 minutes [351] [352]. Esmolol-induced β_1 -AR blockade can be maintained as long as the infusion is continued. This molecule is hydrolysed in blood by esterases and metabolised in the liver by forming its main metabolites, which are methanol and propionic acid. The metabolites, cleared by the renal system, have 1500-fold less β_1 -blocking activity than esmolol. Thus, no further effect is detected 30 minutes after treatment has stopped [353]. The concentration of methanol, which is a metabolite of esmolol, remains within the range of the normal endogenous level [354].

Search and selection of studies investigating the effect of β_1 -AR blockers on septic shock

We conducted a systematic literature search of experimental and clinical studies on the hemodynamic and inflammatory effects of β_1 -AR blocker treatment in septic shock in the period ranging from January 1, 1980 through September 30, 2020.

The following MeSH terms were applied on the PubMed website:

'shock, septic' [MH] OR ('shock, septic' [TW] OR 'septic shock' [TW] OR 'toxic shock' [TW] OR 'endotoxic shock' [TW] OR 'toxic shock syndromes' [TW] OR 'syndrome, toxic shock' [TW] OR 'toxic shock syndrome' [TW] OR 'septic shock' [TW] OR 'septic shock (disorder)' [TW] OR 'septicaemic shock' [TW] OR 'sepsis-associated hypotension' [TW] OR 'septicaemic shock' [TW])) OR ('sepsis' [MH] OR ('sepsis' [TW] OR 'blood poisoning' [TW] OR 'septicaemias' [TW] OR 'blood poisonings' [TW] OR 'sepsis, severe' [TW] OR 'pyohaemia' [TW] OR 'septicaemia' [TW] OR 'pyaemia' [TW] OR 'severe sepsis' [TW] OR 'pyaemia' [TW] OR 'poisoning, blood' [TW]

OR 'pyaemias' [TW] OR 'septicaemia' [TW] OR 'unspecified septicaemia' [TW] OR 'unspecified septicaemia' [TW] OR 'bacteraemia' [TW] OR 'SIRS' [TW] OR 'systemic inflammatory response syndrome' [TW])) AND ('adrenergic beta-antagonists' [MH] OR ('adrenergic beta-antagonists' [TW] OR 'adrenergic beta receptor blockaders' [TW] OR 'blockers, beta-adrenergic' [TW] OR 'beta adrenergic receptor blockaders' [TW] OR 'beta adrenergic blocking agents' [TW] OR 'beta adrenergic blockers' [TW] OR 'adrenergic beta-blockers' [TW] OR 'beta-adrenergic blockers' [TW] OR 'adrenergic beta-receptor blockaders' [TW] OR 'beta-adrenergic blocking agents' [TW] OR 'beta-adrenergic receptor blockaders' [TW] OR 'adrenergic beta antagonists' [TW] OR 'beta blockers' [TW] OR 'beta-adrenergic antagonist' [TW] OR 'beta-blocker poisoning' [TW])) NOT Review [ptyp] AND ('1990/01/01' [PDAT]: '2020/09/30' [PDAT])

The initial search strategy identified 218 studies, of which 199 were fully reviewed. One hundred and twenty were clinical studies and 79 were experimental studies. Editorials and case reports were excluded as were studies in which using β_1 -AR blocker treatment in sepsis was not the main topic. Twelve clinical studies and 24 experimental studies were included as described in the PRISMA flow chart (**Figure 10**).

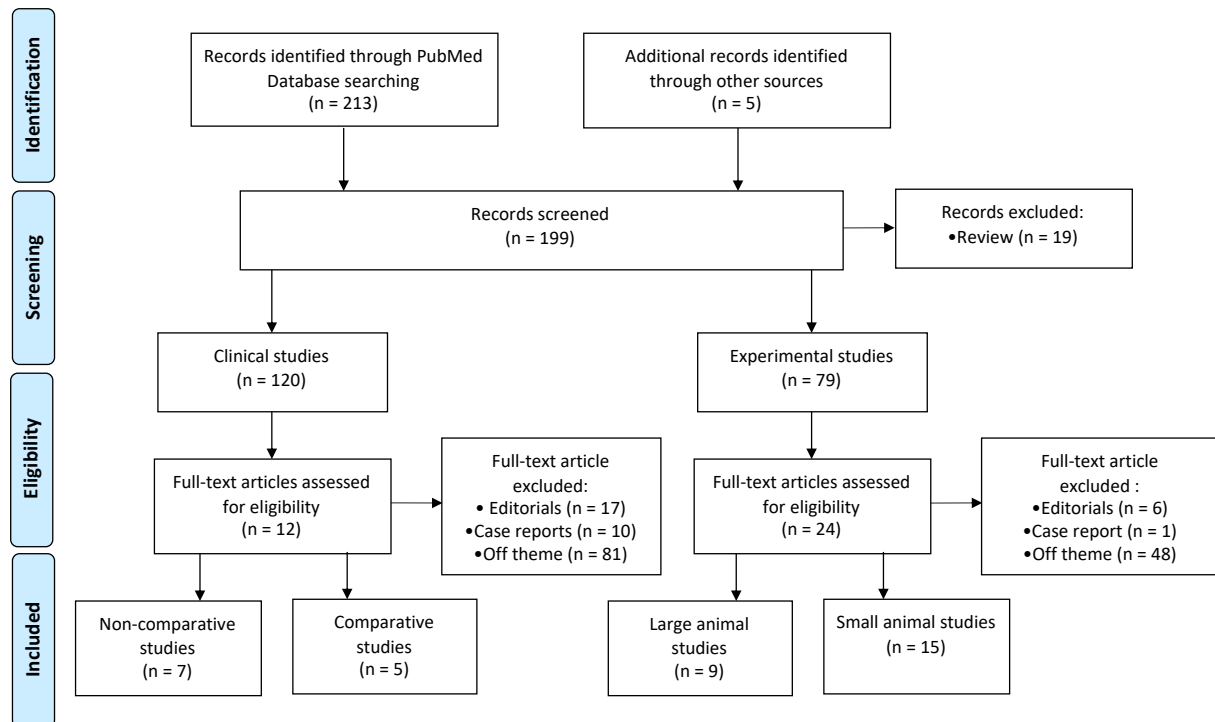


Figure 10: Flow diagram of researched studies.

Experimental support for β -AR blocker use in septic shock

Experimental studies in small and large animals assessed two aspects of β -AR blockade in septic shock: cardiovascular and inflammatory effects. The models used were mainly polymicrobial by CLP or endotoxaemia induced by the injection of LPS, leading to various sepsis-like phenotypes, ranging from mild to severe sepsis (**Table 7**). Most of the studies administered a β_1 -AR blocker, with as the main goal a 20% reduction in HR.

Table 7: Summary of experimental studies

Author	Model	Severity	β-blocker	Time of assessment		Survival	Assessment: cardiac function	Assessment: vasoreactivity	Lactate	Assessment: immunity function		
										Systemic	Tissue	
SMALL ANIMAL STUDIES												
Suzuki <i>et al.</i> , 2005 [36]	Rat Male CLP	+++	E. (10 mg/kg/h or 20 mg/kg/h) to reduce HR Injection: during CLP	24h	after induction	NA	<i>In vivo</i> : ↘ MAP for E. 10 mg/kg/h = MAP for E. 20mg/kg/h <i>Ex vivo</i> *: ↗ CO ↗ SV ↗ dP/dt _{max} , dP/dt _{min}	NA	=	↘ Markers: TNF-α ↘, no effect on white blood cells count	NA	
Schmitz <i>et al.</i> , 2007 [355]	Mice Male CLP	++	Propranolol (0.5 mg/k, SC, every 2h) Injection: 20 min before CLP	48h	after induction	↘	NA	NA	NA	NA	↗ Markers: apoptosis ↗ proliferation↘, IL-6 ↗ and INF-γ ↘ in splenocytes	
Hagiwara <i>et al.</i> , 2009 [356]	Rat Male LPS	+	Landiolol (0.1 mg/kg/min) to reduce HR by 25% Injection: before LPS	12h	after induction	NA	<i>In vivo</i> : = MAP <i>Ex vivo</i> *: ↗ dP/dt _{max} , dP/dt _{min}	NA	NA	↘ Markers: peak circulating level of TNF-α, IL-6 and HMGB-1 ↘	↘ Markers: inflammatory cell infiltration↘, oedema↘, lung DNA binding activity of NF-κB ↘	
Ackland <i>et al.</i> , 2010 [34]	Rat Male CLP or LPS	+	Metoprolol (100 mg/kg, IP), Atenolol (6 mg/kg, IP) to reduce HR by 20% Injection: 2 days before	24 to 48h	after induction	↗	<i>In vivo</i> : = CO = SV ↗ MAP ↗ dP/dt _{max} , dP/dt _{min}	NA	NA	↘ Marker: IL-6 ↘	↘ Markers: hepatic cytokines ↘ (TNF-α, IL-1β, IL-6, INF-γ) cardiac inflammation ↘ (MCP-1, IL-18)	
Mori <i>et al.</i> , 2011 [357]	Rat Male CLP	++	E. (15 mg/kg/h) to reduce HR by 20% Injection: 1h after CLP	18h	after induction	↗	<i>In vivo</i> : = MAP	NA	=	NA	↘ Markers: intraperitoneal TNF α ↘, injury score of the gut mucosa ↘	
Wilson <i>et al.</i> , 2013 [358]	Rat Male CLP	+	Propranolol (10 mg/kg, IP) to reduce Injection: 30 min before CLP then every 8h	24h	after induction	↗	<i>In vivo</i> : = MAP	NA	=	↘ Marker: TNF-α ↘	↘ Marker: lung HO-1 expression ↗	
Ibrahim-Zada <i>et al.</i> , 2014 [359]	Mice Male LPS	+	E. (6.67 μg/kg/min) Injection: 24 h before LPS	48h	after induction	↗	<i>In vivo</i> : = VTI	NA	NA	NA	↘ Marker: CAMP gene expression ↗	
Seki <i>et al.</i> , 2014 [360]	Rat Male LPS	+	Landiolol (100 μg/kg/min) Injection: 15 min before LPS	3h	after induction	NA	<i>In vivo</i> : = MAP	NA	↘	= Markers: no difference on TNF-α, IL-6 and ET-1	↘ Marker: endothelin converting enzyme ECE-1 ↘	
Abdel <i>et al.</i> , 2015 [361]	Rat Male CLP	+++	Carvedilol (2 mg/kg, IP) Injection: 30 min before CLP	18h	after induction	↗	<i>In vivo</i> : = MAP	NA	NA	↘ Markers: TNF-α et IL-6↘	↘	

											Markers: kidneys injury score ↘, kidneys anti-oxidative activity (NADP-H, GSH) ↗
Kimmoun <i>et al.</i> , 2015 [32]	Rat Male CLP	+++	E. (300 µg/kg/min) to reduce HR Injection: 4h after CLP	18h after induction	↗	<i>In vivo</i> : = CO ↗ SV = MAP	↗ vasoreactivity	↘	NA	↘ Markers: p-AKT and p-eNO ₃ expression ↗, NF-κB and iNO ₃ expression ↘	
Wang <i>et al.</i> , 2016 [362]	Mice Male LPS	+	E. (6.7 µg/kg/min) Injection: directly after LPS	6h after induction	NA	<i>In vivo</i> : = CO ↗ MAP ↗ dP/dtmax	NA	NA	NA	↘ Markers: myocytes apoptosis (Caspase3, Bax, JNK, p38) ↘	
Wei <i>et al.</i> , 2016 [26]	Rat Male CLP	+++	E. (1; 5 or 18 mg/kg/h) Injection: 4 h after CLP	18h after induction	NA	<i>In vivo</i> : ↗ CO ↗ SV ↗ MAP	↗ vasoreactivity	↘	↘ Markers: IL-6 et IL-10 ↘	↘ Markers: p-AKT and p-eNO ₃ expression ↗, NF-κB and iNO ₃ expression ↘	
Lu <i>et al.</i> , 2017 [363]	Rat Male CLP	+++	E. (1.75; 2.6 or 3.5 mg/kg/h) Injection: 1 h after CLP	6 to 24h after induction	NA	NA	NA	NA	↘ Markers: IL-6, TNF-α ↘, IL-10 ↗	↘ Markers: liver and kidney inflammatory cells infiltration ↘, Bcl2 expression↗	
Mathieu <i>et al.</i> , 2018 [364]	Rat Male + female CLP	+++	Landiolol Injection: 1 h after CLP	18h after induction	NA	<i>In vivo</i> : Male: = CO, ↗ SV = MAP Female: ↘ CO, = SV = MAP	NA	=	= Markers: no difference on TNF-α, IL-6, IL-1β	↘ Markers: p-AKT and p-eNO ₃ expression ↗	
Bedet <i>et al.</i> , 2019 [365]	Mice Male CLP	+++	Atenolol (3 µg/12 h) Injection: 2 h after CLP	20 hours after induction	=	<i>In vivo</i> ↘ CO = MAP = dP/dtmax = SV	NA	NA	NA	NA	
LARGE ANIMAL STUDIES											
Aboab <i>et al.</i> , 2011 [366]	Pig Male LPS	+	E. to reduce HR by 20% Injection: 30 after LPS	300 min after induction	NA	<i>In vivo</i> : = CO ↗ Stroke index = MAP	↗ systemic vascular resistance	NA	NA	NA	
Calzavacca <i>et al.</i> , 2014 [367]	Sheep Male <i>E. coli</i>	+	Atenolol (10 mg IV bolus then 0.125 mg/kg/h) to reduce HR by 20% Injection: 1 day before LPS	24h after induction	NA	<i>In vivo</i> : ↘ CO ↗ SV = MAP	NA	=	↘ Markers: no difference in TNF-α; IL-6, IL-10 ↘	NA	
Jacquet-Lagrèze <i>et al.</i> , 2015 [368]	Pig Male <i>P. aeruginosa</i>	+	E. (7.5 µg/kg/min) to reduce HR between 80-90/min Injection: after stabilisation period	300 min after induction	NA	<i>In vivo</i> : ↘ CO = Stroke index = MAP	↗ systemic vascular resistance	=	NA	NA	

Hernandez <i>et al.</i> , 2016 [350]	Sheep Male LPS	+	E. (15 mg/h) to reduce HR by 20- 30% Injection: after stabilisation period	Every hour during 3h	NA	<i>In vivo</i> : = CO ↗ MAP	↘ norepinephrine requirement	↘	NA	NA
Hosokawa <i>et al.</i> , 2017 [369]	Sheep Female CLP	++	E. to reduce HR by 80-100/min Injection: 2 h after CLP	During after induction	24h =	<i>In vivo</i> : = CO ↗ SV = MAP	NA	=	NA	NA
Du <i>et al.</i> , 2017 [370]	Dog Male <i>E. coli</i>	+	E. (25 mg/h) to reduce HR by 10-15% Injection: after stabilisation period	Every hour during 2h	↗	<i>In vivo</i> : = CO ↗ SV = MAP	NA	↘	NA	NA
Uemura <i>et al.</i> , 2018 [371]	Dog Male <i>E. coli</i>	+	Landiolol (1 to 10 µg/kg/min) Injection: 1 h after <i>E. coli</i>	During after induction	4h NA	<i>In vivo</i> : = CO = MAP	= systemic vascular resistance	=	= Marker: no difference on IL-6	NA
Boselli <i>et al.</i> , 2018 [372]	Pig Male <i>P. aeruginosa</i>	+	E. (7.5 µg/kg/min) to maintain a HR<90/min	Every 30 min during 3 h	NA	<i>In vivo</i> : ↘ CO = SV = MAP	= systemic vascular resistance	NA	NA	NA
Van loon <i>et al.</i> , 2018 [373]	Sheep Female LPS	+	E. (50 µg/kg/min) to reduce HR by 30%	From the beginning to 30 min after reaching targeted HR	NA	<i>In vivo</i> : ↘ CO = SV ↘ MAP ↗ CVP	↗ norepinephrine requirement	=	NA	NA

Abbreviations: AKT: protein kinase B; Bax: bcl-2-associated X; CAMP: cathelicidin anti-microbial peptide; CLP: caecal ligation and puncture; CVP: Central venous pressure; CO: cardiac output; dP/dtmax: derivative of pressure over time maximum; dP/dtmin: derivative of pressure over time minimum; ECE-1: endothelin converting enzyme-1; E.: esmolol; ET-1: endothelin-1; GSH: reduced glutathione; HMGB-1: high-mobility group box-1; HO-1: haem oxygenase-1; HR: heart rate; IL: interleukin; INF-γ: interferon-γ; iNOS: inducible nitric oxide synthase; IP: intraperitoneal; IV: intravenous; JNK: c-Jun N-terminal protein kinase; LPS: lipopolysaccharide; MAP: mean arterial pressure; MCP-1: monocyte chemo-attractant protein 1; NADPH: nicotinamide adenine dinucleotide phosphate; NF-κB: nuclear factor- kappa B; p-eNOS: phosphorylated endothelial nitric oxide synthase; SC: subcutaneous; SV: stroke volume; TNF-α: tumour necrosis factor-α; VIT: velocity integral time; ↗: increased; ↘: decreased; =: equal.

Severity definition: +++: CLP model with an immediately below the ileocaecal valve ligature, non-reanimated model, low MAP, mortality >50% at 24h; ++: CLP model with a 75% of the caecum ligature, reanimated model, mortality <50% at 24h; +: LPS model or CLP with a 30% of the caecum ligature, short time of the experiment.

*Experiment performed with a Langendorff isolated heart perfusion apparatus.

Overall, the 20% HR reduction target was always achieved. Regardless of the animal model and the administration route of the β_1 -AR blocker, a cardioprotective effect was found. Ackland *et al.* showed that administration of metoprolol or atenolol, both cardioselective β_1 -AR blockers, in a moderately severe septic shock rodent model resulted in a decrease of HR, an increased of systolic ejection volume and a maintained cardiac output. In contrast, intracerebral administration of a β_1 -AR blocker did not improve survival in rats with septic shock, suggesting a peripheral rather than a central β_1 -AR mechanism [34].

All studies performed in rodents showed either a maintenance or an increase in cardiac output despite the decrease in HR. One possible explanation could be an increase in diastole duration in these chronodependant animals secondary to the HR reduction. As a result, left ventricular filling was increased, allowing a similar cardiac output despite the reduction in HR. External myocardial work was reduced, which led to better overall myocardial efficiency at a lower energy cost [36].

This result was surprisingly associated with an improvement in vascular function. Theoretically, β_1 -ARs are not expressed on vessels, so β_1 -AR blockers should have no direct effect on vasoreactivity. However, an improvement in vasoreactivity was found during septic shock, in both animal models and humans after β_1 -AR blockade [32, 374]. In the pig, a large animal model, two studies demonstrated that the administration of esmolol resulted in an increase in systemic vascular resistance *in vivo* [366, 368]. Similarly, in a model of severe septic shock in rats, Wei *et al.* found that, *ex vivo*, the administration of esmolol induced a restoration of vascular contractility capacity to phenylephrine in dose response studies. In the same work, low concentrations of esmolol, without a direct hemodynamic effect, led to a similar improvement in vasoreactivity [26]. An improved prognosis in animals with septic

shock receiving a β_1 -AR blocker was also found in all survival studies conducted in small animals [32, 34, 357-359, 361].

Another crucial point is effects on inflammation induced by the the use of β_1 -AR blockers in septic shock. In the early phase of septic shock, the 'cytokine storm' is consistently observed, characterised by a significant increase in the level of pro-inflammatory cytokines, and anti-inflammatory cytokines, which promotes organ failure and participates in the sepsis-induced immunosuppressed state [15].

The administration of β_1 -AR blockers in animal models of septic shock induces an overall decrease in inflammation, at both the systemic and tissue levels. Hagiwara *et al.* showed that early administration of landiolol in a rodent LPS model significantly decreased cytokine production, including IL-6 and TNF- α , allowing a reversal of cardiac inflammation and contractile dysfunction [356]. Regardless of the model and the time of assessment, administration of a β_1 -AR blocker is most often associated with an overall pro- and anti-inflammatory systemic and tissue weighting (**Table 7**). Mori *et al.* similarly showed that intraperitoneal TNF- α production was decreased by the administration of esmolol in CLP rodent model of septic shock. They also demonstrated a decrease in functional and structural alterations of the intestinal barrier in treated rats [357]. Other studies showed similar results on the kidney, liver, vessels, and lung [26, 361-363].

Clinical indication of the utility of β_1 -AR blockers in septic shock

The clinical data in **Tables 8, 9** are limited to the period from 1990 to September 2020

Table 8: Summary of non-comparative clinical studies.

Author	Type of study design	Method	Time of assessment	Patients number	Heart rate (/min) mean±SD or median [interquartile]	Stroke volume and cardiac output	Vascular resistance	Mortality	Tissue perfusion	Additional results
Gore <i>et al.</i> , 2006 [375]	Prospective, interventional, monocentric, before-after	Sepsis without shock E. to reduce HR by 20%	Before and after 3 h of E. administration	6	114± 15 vs. 91± 12	SV: ↗ CO: ↘	=	NA	=	Not very severe patients No anabolic effect on energy metabolism
Schmittinger <i>et al.</i> , 2008 [376]	Retrospective, monocentric	Septic shock Metoprolol <i>per os</i> to reduce HR <95/min +/- milrinone if CI <2.5 l/min/m ²	Before metoprolol administration and at 6,12, 24, 72 and 96 h	40	101± 18 vs. 84± 13	SV: ↗ CO: =	=	33% at 28 days	=	Cardiac output decrease in 7 patients with milrinone administration
Balik <i>et al.</i> , 2012 [377]	Prospective, monocentric	Bolus of E. followed by continuous 24h infusion to reduce HR by 20%	Before E. administration and at 2,6, 12, 24 and 30 h	10	142± 11 vs. 112± 9	SV: = CO: =	=	10% at 28 days	=	Very mild patients
Chen <i>et al.</i> , 2013 [378]	Prospective, monocentric, before-after	Severe sepsis Continuous infusion of E. to reduce HR by 20%	Before E. administration and at 3 h	16	114 ± 15 vs. 91 ± 13	SV: = CO: ↘	=	NA	=	
Morelli <i>et al.</i> , 2013 [374]	Prospective, interventional, monocentric, before-after	Septic shock Continuous infusion of E. to reduce HR between 80 and 94/min	At 24 h	25	117 [112-126] vs. 86 [80-89]	SV: = CO: ↘	NA	NA	=	Improvement of microvascular flow index Norepinephrine requirements were significantly reduced with E. therapy
Morelli <i>et al.</i> , 2016 [379]	Prospective, monocentric, before-after	Septic shock Continuous infusion of E. to reduce HR between 80 and 94/min	Before E. administration and at 4 h	45	115 ± 11 vs. 88 ± 9	SV: = CO: ↘	↘	51% at 28 days	=	Ventricular-arterial decoupling improvement
Du <i>et al.</i> , 2016 [380]	Prospective, monocentric, before-after	Septic shock hemodynamically stable for at least 3h with HR >100/min Continuous infusion of E. to reduce HR by 10-15%	Before E. administration and 2 h after achieving the targeted HR.	63	107.8 ± 8.7 vs. 86.2 ± 10.2	SV: ↗ CO: ↘	NA	6.3% at 28 days	=	Very low mortality Target blood pressure higher than recommended in surviving sepsis campaign

Abbreviations: CI: cardiac index; CO: cardiac output; E.: esmolol; HR: heart rate; SV: stroke volume; vs.: *versus*; ↗: increased; ↘: decreased; =: same.

Table 9: Summary of comparative clinical studies.

Author	Type of study design	Goal	Inclusion	Method	Patients number	Main result	Secondary results	Remark
Morelli <i>et al.</i> , 2013 [25]	Randomised, open-label, comparative, monocentric, phase II	HR reduction between 80 et 94 /min	Stabilised septic shock for 24 hours with HR >95/min	2 groups:E. vs. C. Levosimendan administration if SvO2<65% Assessment before and at 24, 48, 72, 96 h	154 (77 in each group)	HR (/min)* E.: -28 [-37 - -21] vs. C.: -6 [-14 - 0]	Increased in E., vs. C. group: SV* and SVR* 28 day mortality* E.: 49.4% vs.C.: 80.5%	The need for levosimendan rescue therapy did not differ between groups (E.: 49.9% vs. C.: 40.3%)
Yang <i>et al.</i> , 2014 [381]	Randomised, controlled, monocentric	Not clearly defined. General hemodynamic assessment	Septic shock	Randomisation within 6 h 2 groups: E. vs. C. HR reduction <100/min within 2 h after inclusion in the E. group. Assessment before and at 12, 24, 48 and 72 h	41 E.: 21 vs. C.: 20	HR (/min)* E.: 93 ± 4 vs. C.: 118 ± 13 CI (l/min/m ²)* E.: 3.3 ± 8 vs. C.: 4.5 ± 6	The ScvO ₂ was not decrease in both group Troponin I significantly decrease in E. group vs. C. group	Target blood pressure higher than recommended in surviving sepsis campaign
Wang <i>et al.</i> , 2015 [382]	Randomised, controlled, open-label, monocentric, phase II	HR reduction between 75 et 94 /min	Sepsis with HR>95/min	3 groups: C. vs. milrinone vs. E. -milrinone Bolus of milrinone followed by continuous administration Continuous administration of E. Assessment before and at 12, 24, 48 and 72 h	90 (30 in each group)	Successful control*: C.: 43.3% vs. milrinone: 53.3% vs. E. -milrinone: 100%	HR Increase in E. -milrinone vs. C. group: SV* and CI* Decrease in E.-milrinone vs. C. group: TNF-α and IL-6 28 day mortality*: C.: 73% vs. milrinone: 67% vs. E. -milrinone: 40%	
Xinqiang <i>et al.</i> , 2015 [383]	Randomised, controlled, double-blinded, monocentric	28 day mortality and length of stay in ICU	Stabilised septic shock for 6 hours with HR>95/min	E. continuous administration to obtain a HR <100/min Assessment before and at 24, 48 and 72 h	48 (24 in each group)	28 day mortality *: E.: 25% vs.C.: 62.5% Length of stay in ICU (days)*: E.: 13.75 ± 8.68 vs. C.: 21.7 ± 6.06	Decrease at 24 h in E. vs. C. group: HR* and at 48 hours: lactate* Increase at 24 in E. vs. C. group: SVR*, ScvO ₂ * No difference at 24 h in E. vs. C. group: arterial blood pressure, central venous pressure, CI	
Shang <i>et al.</i> , 2016 [384]	Prospective, cohort, non-randomised comparison between C. and E.	Not clearly defined. General hemodynamic assessment	Severe sepsis with HR>100/min	E. continuous administration or natural saline in C. group Assessment before and at 24, 48 and 72 h	151 E.: 75 vs. C.: 76	At 24 h HR (/min)*: E.: 101.96 ± 7.36 vs. C.: 110.12 ± 8.59	At 24 hours: ScvO ₂ *: E.: 0.8 ± 0.05 vs. C.: 0.76 ± 0.05 28 day mortality: E.: 5.3%vs. C.: 7.9%	No randomisation in this work Low mortality (no septic shock)

Abbreviations: C.: control; CI: cardiac index; E: esmolol; HR: heart rate; IL: interleukin; SvcO₂: central venous oxygen saturation; SV: stroke volume; SVR: systemic vascular resistance; TNF-α: tumour necrosis factor-α;

vs.: versus; * p<0.05.

Of note, the clinical studies in **Tables 8, 9** sought to assess the efficiency in reducing HR and tolerance for patients initially with tachycardia > 90/minutes. Prior to β_1 -AR blocker administration, in all studies, patients were optimally resuscitated and considered hemodynamically stable.

The first work was published in 2006 by Gore *et al.* This prospective before-and-after study in six non-severe septic patients assessed the physiological effects of a 20% decrease in HR by esmolol. The authors demonstrated that the reduction in HR was associated with a decrease in cardiac output with an increase in systolic ejection volume without a hypoxic effect or a change in vascular resistance. They observed good tolerance to this treatment [375]. It was also found that the reduction in HR did not induce a decrease in cardiac output [376, 377]. In one of the two publications, milrinone (a phosphodiesterase type 3 inhibitor, adrenoceptor-independent positive inotropic) was co-administered to counteract the negative inotropic effects of the β_1 -AR blocker [376].

Only one major clinical study was published in 2013 by Morelli *et al.* [25]. This monocentric, prospective, randomised, open-label, esmolol vs. placebo study included a population of 154 patients in septic shock, who remained with a tachycardia above 95 beats per minute 24 hours after admission. The primary objective was to determine the efficiency of esmolol administration in reducing HR between 80-94/minutes and to assess the associated safety. The primary outcome was an effective reduction of HR in the esmolol group within the target range. Cardiac output was broadly maintained (although decreased in the first 24 hours) despite the reduction in HR. The explanation for this was an increase in systolic ejection volume secondary to the prolongation of the diastolic period in the esmolol group. Levosimendan (an inotropic calcium sensitiser, independent of adrenoceptors) was also

administered in a similar manner in both groups (esmolol: 49.4%, placebo: 40.3%, $p=0.39$). The required dose of NE was lower in the esmolol group than in the placebo group and vascular resistance was increased in the esmolol group compared to the placebo group. Moreover, mortality was reduced from 80% in the control group to 31% in the esmolol group compared to 80% mortality in the control group. Considering the dramatic decrease in mortality, these results were the subject of an intense debate [385-391].

Four points should be highlighted concerning the effects of esmolol treatment:

- The effectiveness of HR reduction;
- The excellent tolerance to esmolol, bearing in mind that 50% of the population included had received an inotropic agent (levosimendan);
- The vasoactive effects, as the reduction in NE doses in the esmolol group is unexplained;
- The increase in survival rate, which should be moderated by the unexplained high mortality rate in the control group.

Based on the results of Morelli *et al.*, further studies aimed to confirm the tolerance and physiological effects of this treatment. However, in each further study, the guidelines for septic shock management were systematically not followed. For instance, arterial pressure was most often beyond the recommended values [381-384].

Finally, given the methodological weaknesses of these clinical studies, it is premature to recommend the use of esmolol during septic shock, mainly because the targeted population, in whom this treatment could be beneficial, is not clearly defined. Indeed, a feasibility study, including patients with usual baseline characteristics, showed that only 10/186 patients were eligible to receive esmolol [392].

Objective

β_1 -AR blockade is one therapeutic approach currently under investigation, aimed at downregulating the excessive adrenergic stimulation induced by septic shock. Early human studies have mainly shown the hemodynamic benefits of lowering HR through β_1 -AR blockade, on the improvement of cardiac efficiency, with one also finding survival benefit [25, 376, 377]. Some experimental studies have investigated the inflammatory effects of β_1 -AR blockade in septic shock and demonstrated a global decrease in both pro- and anti-inflammatory plasma cytokines [26, 361, 363]. However, little is known about the effects of β_1 -AR blockade on the early adaptive immune response, specifically on CD4 T cells expressing β -AR [393, 394]. Accordingly, the present study aimed to address this question in a murine model of high-grade sepsis.

Methodology

Animals

β_1 -AR (C57BL/6J, DBA/2, 129Sv) knockout mice ($\beta_1^{-/-}$) and wild type littermates ($\beta_1^{+/+}$), obtained free of charge from the Institute of Pharmacology and Toxicology of Munich, Germany, were used for this study. The French Animal Care Committee, in accordance with European regulations, has approved all animal protocols (n°APAFIS#4636). Both males and females were used.

$\beta_1^{-/-}$ mice were generated by the department of molecular pharmacology of Roche Bioscience [395]. Briefly, the targeting vector of the endogenous β_1 -AR genomic locus was constructed using sequence that had been cloned from a 129sv mouse genomic library. The gene of the β_1 -AR was disrupted in the targeting vector by placing Escherichia coli lacZ gene encoding b-galactosidase and neomycin resistance gene. Embryonic stem (ES) cells were electroporated and selected for neomycin resistance. ES where homologous recombination occurred were injected into C57Bl/6 blastocysts to obtain germ line chimeras. Chimeric males were then mated to DBA/2 female mice to screen for germ line transmission of the ES cell DNA. Then heterozygote mice were intercrossed to generate $\beta_1^{+/+}$ and $\beta_1^{-/-}$ mice.

After obtaining $\beta_1^{-/-}$ mice in the laboratory, heterozygotes were generated by backcross with C57BL/6J mice. Mice were genotyped and two strains, $\beta_1^{-/-}$ and these littermates $\beta_1^{+/+}$ were used in this study.

Genotyping of mouse ear DNA via PCR

DNA extraction

Mice were tattooed for identification and a piece of ear was cut between 14-21 days of age. Sample was stored at -80°C. DNA was extracted using KAPA Mouse Genotyping Kit (KAPA BIOSYSTEMS, Sigma Aldrich, Saint-Louis, MO) which contains a thermostable protease, designed for rapid sample lysis. A mix was prepared in 0,2 ml tube containing the piece of ear. Tubes were placed in thermocycler MyiQ™ (BioRad, Hercules, CA) 10 minutes at 75°C followed by 5 minutes at 95°C. DNA samples were stored at -20°C.

Polymerase Chain Reaction (PCR)

Principle

PCR uses the DNA polymerase that drives the synthesis of DNA from deoxynucleotide substrates on a single-stranded DNA template. When upstream and downstream primers are annealed to a longer template DNA, DNA polymerase adds nucleotides to their 3' end to extend primers. Under the action of DNA polymerase, each primer is elongated in the direction 5' to 3' of a sequence complementary to the template strand. For an exponential amplification of the target gene sequence, the denaturation, the annealing and the elongation cycles are repeated.

Procedure

Extracted DNA was amplified using KAPA Mouse Genotyping Kit (KAPA BIOSYSTEMS, Sigma Aldrich) containing DNA polymerase, dNTPs, MgCl₂ and stabilizers, 1µl of DNA and 1 µl of primers (forward and reverse primers) were added to the solution. The primers used are described in **Table 10**. DNA amplification was realised with a real-time PCR Detection System

MyiQ™ (BioRad). A cycle of amplification was composed of 3-step runs after pre-heating DNA for 10 minutes at 94°C.

- 1) Denaturation: the DNA was heated at 94°C for 30 seconds to render it single- stranded;
- 2) Annealing: 62°C for 15 seconds for both primers to bind to the appropriate complementary strand;
- 3) Extension: 72°C for 30 seconds for DNA polymerase extending the primer by polymerase activity.

This cycle was repeated 30 times followed by a step at 72°C for 10 min.

An agarose gel was prepared (1,5% to separate 200-400 kDa DNA fragments) by dissolving the agarose powder in Tris-Borate-EDTA buffer (TBE 1%). Ethidium bromide (10 µl, Sigma Aldrich) was added to the agarose solution before it had gelled to visualise DNA under UV light. Amplified DNA samples were loaded in the wells. Agarose gel electrophoresis was done horizontally in a submarine mode whereby the slab gel was completely submerged in TBE buffer during electrophoresis (100 volts, 45 minutes). The gel was viewed in UV light with a Gel Doc™ XR+ (BioRad), the DNA stained with ethidium bromide appeared as glowing bands (Figure 11).

Table 10: Primers sequence used for mouse genotyping.

Gene	Primers sequence forward (F) and reverse (R)
β ₁ -AR	F: ATG GCC TTC GTGT ACC TGC R: AGA GCC ACG AGG CGC GAC
Neomycine	F: TAT CAG GAC ATA GCG TTG G R: ATA GAG CCC ACC GCA TCC

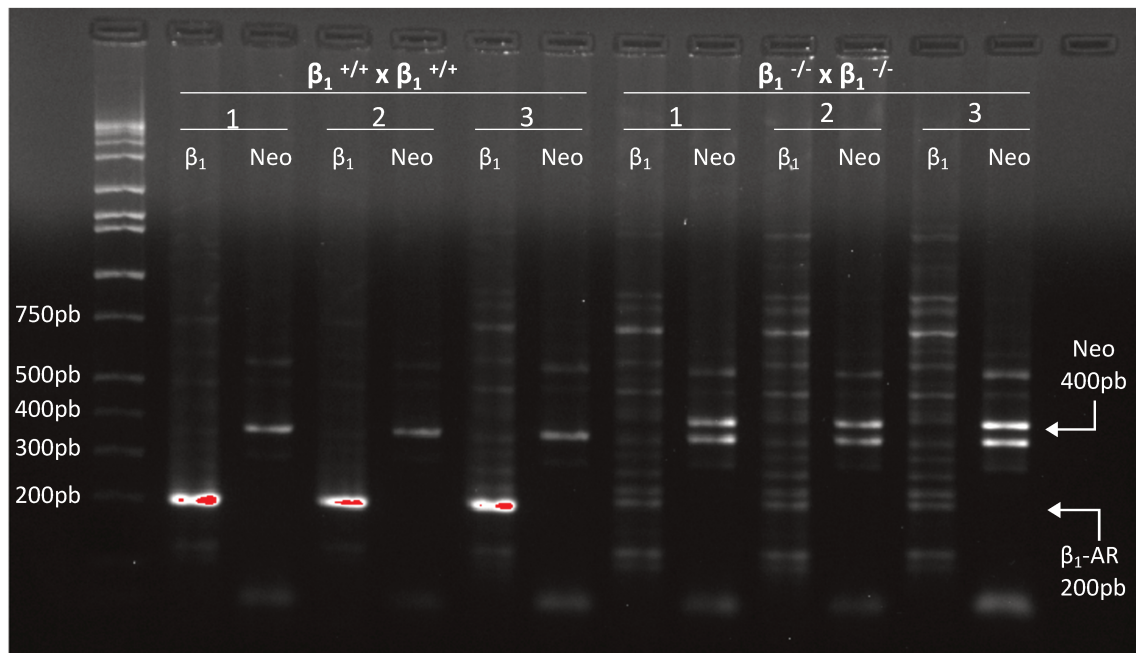


Figure 11: Genotyping of mouse DNA by PCR.

Mouse ear DNA was amplified with primers for neomycine (Neo, 400pb) and β_1 -AR (600pb). PCR products were separated on 1.5% agarose gels. Three mice of $\beta_1^{+/+}$ and $\beta_1^{-/-}$ genotypes were represented. $\beta_1^{+/+}$ mice expressed β_1 -AR but not Neo, while $\beta_1^{-/-}$ mice did not expressed β_1 -AR but expressed Neo.

Caecal ligation and puncture model

High-grade sepsis was induced by caecal ligation and puncture (CLP) in mice aged 12-16 weeks, weighing 22-32 grams. Mice were anaesthetised with 3.5% isoflurane (Forene®, Abbvie, North Chicago, Ill) and subsequently maintained with 2% isoflurane in a prewarmed (38°C) electric heat pad. A laparotomy was performed and 75% of the caecum (large ligature) is being ligated and then punctured with an 18-gauge needle (high-grade sepsis) (**Figure 12**) [396]. The caecum was re-inserted into the abdomen and the incision closed by suture. Sham-operated mice underwent the same procedure except for the caecal ligation and puncture. An incision was performed between the scapulae in all mice and a mini-osmotic pump (2001D, Alzet, Cupertino, CA) implanted subcutaneously in animals to be treated with the selective β_1 -AR blocker, esmolol. After 20 minutes, mice received subcutaneous analgesia with

buprenorphine 0.2 mg/kg (Buprécare®, Axience, Pantin, France) in physiological saline solution (0.9% NaCl; 5ml/100g; Baxter, Guyancourt, France). The mouse was then returned in its cage and kept until recovery with water and food freely available.

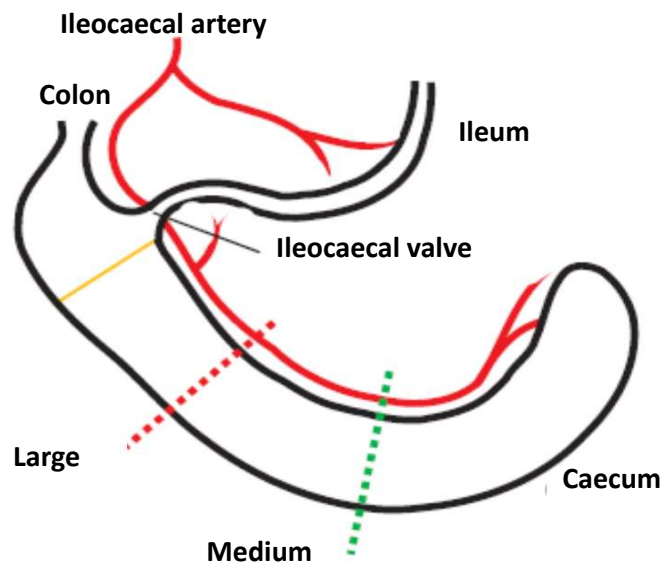


Figure 12: Anatomical locations for caecal ligation of mice.

Schematic illustration of caecal ligation position to induce mid-grade sepsis (dotted green line) and high-grade sepsis (dotted red line). The yellow line represents the basis of the caecum, below the ileocaecal valve used as a reference to ligature the caecum at the same length during each surgery. According to the reference: [396].

Experimental design

First, the dose of esmolol (a selective β_1 -AR blocker) responsible for a 20% reduction HR was determined in $\beta_1^{+/+}$ mice (n=25). Then, $\beta_1^{+/+}$ mice were randomly assigned to four groups: sham, sham + esmolol, CLP or CLP + esmolol. Esmolol (Esmocard®, Amomed, Meudon, France) was infused at 18 mg/kg/h (8 μ l/h volume) through the subcutaneous mini-osmotic pump which was activated 3 hours post-insertion. $\beta_1^{-/-}$ mice were randomly assigned to two groups: sham or CLP. Eighteen hours after surgery, echocardiography was performed, plasma

collected and spleens immunophenotyped (n=74). Splenic CD4 T cell proliferation assays were also performed (n=44). In $\beta_1^{+/+}$ non-septic mice, Treg cells and CD4 T cells were isolated and co-cultured. A 5-day survival study was performed in 3 groups of mice: $\beta_1^{+/+}$ CLP (n=20), $\beta_1^{+/+}$ CLP + esmolol (n=20), and $\beta_1^{-/-}$ CLP mice (n=20). The number and reasons for failed measurements and excluded mice are provided in **Table 11**.

Table 11: Number and reasons for failed measurements and excluded values.

Experiment	n=	Measure failed	Reason	Excluded value	Reason
Immunophenotyping	T cells	11	Reagent expired	4	No staining
	Treg cells	11	Reagent expired	1	No staining
Echocardiography	74	NA		NA	
Mean arterial pressure measurement		4	Catheterization failed	NA	
Plasma level of inflammatory mediators		NA		37*	Not measured
Proliferation assay	44	NA		NA	
CD4 T and Treg cells co-culture	4	1 well	Contaminated	NA	
Survival study	60	NA		1	Mouse dead before the first count

* Due to covid-19 pandemia, Magnetic Luminex® Performance Assay – Mouse XL Discovery Premixed kit (Bio-Techne, Minneapolis, MN) distribution was stopped by the manufacturer. Thus 37 plasma samples were analyzed.

Echocardiography

Transthoracic echocardiography was performed 18 hours after CLP with a VEVO770 (FUJIFILM VisualSonics, Toronto, Canada) and 30 MHz mechanical probe. Mice were placed on a prewarmed (38 °C) electric heat pad, anaesthetized with 1.5% isoflurane and kept at rest for 5 minutes before the echocardiographic assessment. Ascending aortic diameter (AD) was obtained during systole from the left parasternal long axis view. The aortic velocity time integral (VTI) was measured in the aortic arch from suprasternal view. Stroke volume (SV), cardiac output (CO) and cardiac index (CI) were calculated with the following formulas:

- Aortic root area: $A = \pi \times (\text{Aortic root diam})^2 / 4 \text{ (cm}^2\text{)}$
- Stroke volume: $SV = VTI \times A \text{ (ml)}$
- Cardiac output: $CO = VTI \times HR \times A \text{ (ml/min)}$
- Cardiac index: $CI = CO / \text{animal weight (ml/min/g)}$
- Cardiac power index: $CPI = MAP \times CI / 451$

All measurements were averaged over three consecutive cardiac cycles. The **Figure 13** represent a representative example of the VTI measurement.

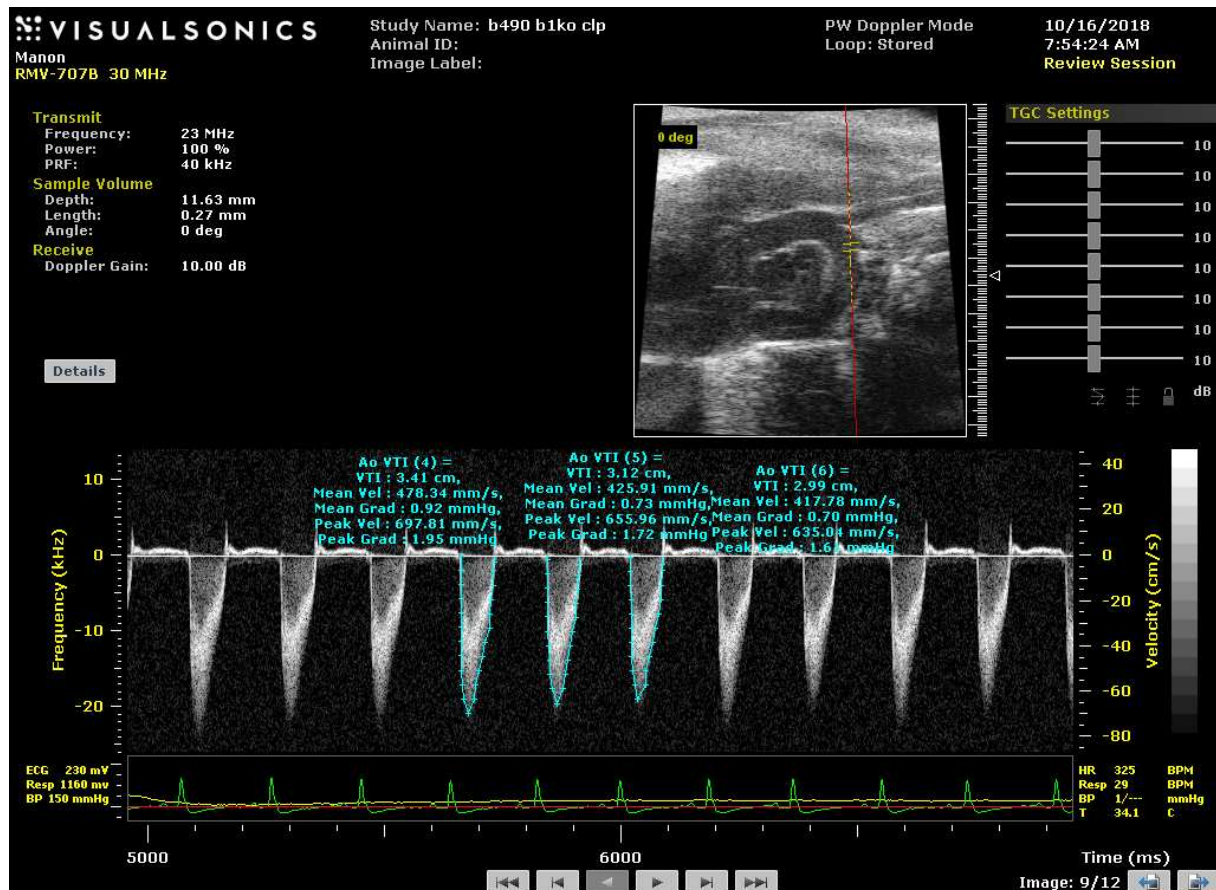


Figure 13: Representative measurement of Velocity Time Integral by echocardiography.

The VTI was measured in the aortic arch from suprasternal view. Measurement was the averaged over three cardiac cycles between two breaths in Doppler mode.

Intra-arterial blood pressure measurement

Principle

The invasive intra-arterial blood pressure measuring systems consist of a column of fluid directly connecting the arterial system to a pressure transducer (hydraulic coupling). The pressure waveform of the arterial pulse is transmitted via the column of fluid, to a pressure transducer where it is converted into an electrical signal. This electrical signal is then processed, amplified and converted into a visual display by a microprocessor.

Procedure

Anesthesia was maintained by 1.5% Isoflurane. Temperature was maintained by a plate heated at 37°C. After shaving the fur and disinfecting the skin, an incision was performed in the middle of the anterior cervical region, followed by dissection of the muscles and isolating the right carotid artery. The distal end of the carotid artery was ligated with a suture and the proximal end was clamped with an artery clamp. A hole was cut with a micro-scissor, then a catheter (PE-50, Braintree Scientific®, Braintree, MA) was inserted towards the proximal end. The catheter was connected to the pressure transducer (P23XL, BD Bioscience, San Jose, CA) and filled with heparinized saline (50 UI/ml). The catheter was fixed with a suture and the clamp was removed. Blood pressure was monitored with Ponemah P3Plus software for about 5-10 minutes until it became stable and mean arterial pressure (MAP) was reported.

Sample Collection

Blood was collected via the catheter into the carotid in EDTA tubes (BD vacutainer®, BD Bioscience) and centrifuged 10 minutes at 1750 rpm for plasma extraction. Heart tissues were removed and rinsed in PSB for immunohistochemistry and spleen was removed and rinsed in PBS for immune cells analysis.

Quantification of cytokines

In 5–6 mice from each group, the plasma levels of 46 inflammatory mediators were measured in duplicate using the Magnetic Luminex® Performance Assay – Mouse XL Discovery Premixed kit (Bio-Techne, Minneapolis, MN) according to the manufacturer's instructions. Results were expressed as pg/ml

Immunohistochemistry

Hearts were sectioned in a transverse slice and embedded in optimal cutting temperature compound (CellPath, Newtown, UK), frozen in liquid nitrogen and stored until use at -80°C. Hearts were cut by Leica CM1950 cryostat (LeicaBiosystem, Nussloch, Germany) into 7 µm thick sections at -20°C. The sections were rinsed with PBS and fixed in 4% paraformaldehyde (PFA, Sigma Aldrich). Heart section were stained in Autostainer Link 48 Dako (Agilent, Santa Clara, CA) with rabbit polyclonal primary antibody to CD45 (1/150, ab10558, Abcam, Cambridge, UK). Staining was visualised using the appropriate secondary antibody (goat anti-rabbit HRP polymer, ab214880, Abcam) and 3,3'-diaminobenzidine with peroxidase substrate (Dako Real DAB Chromogen, Agilent). The sections were counterstained with hematoxylin. For each heart section the CD45 positives cells were count using a 40x objective lens in 10 fields of the microscope, in blind, by three different experimenters.

Splenocytes isolation

Spleens were harvested and immediately placed in cold PBS (Sigma Aldrich) and gently grounded between frosted slides to produce a single-cell suspension. Suspensions were filtered through 70-µm nylon filters (Biologix, Shandong, China) and centrifuged at 300g for 10 minutes at 4°C. Red blood cells were lysed using the BD PharmLyse™ (BD Bioscience) solution. Then, splenocytes were washed twice in PBS before being resuspended in RPMI 1640 medium (Gibco™, ThermoFisher Scientific, San Diego, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma Aldrich). The preparation was left overnight at 4°C.

Immunophenotyping of lymphocytes

Splenocyte Fc receptors were blocked (CD16/CD32-purified antibodies, eBioscience™, ThermoFisher Scientific) and cell surface markers used to identify total T cells (CD3+), CD4 T cells (CD3+ CD4+), CD8 T cells (CD3+ CD4-) and Treg cells (CD3+ CD4+ CD25^{hi} CD127^{low}). Cells surface antigens were stained with 100 µl of PBS containing surface markers antibodies PerCP Cy5.5-CD3, BV421-CD4, PE-CD25, APC-CD127 (all from BD Pharmigen, BD Bioscience) and Fixable Viability Dye eFluor™ 506 (eBioscience™, Thermo Fisher Scientific). Cells were incubated 30 minutes in the dark at 4°C, washed with PBS+ 1% FBS and resuspended in PBS + 1% FBS for flow cytometric analysis. The gating strategy of Treg cells identification are described in the **Figure 14**.

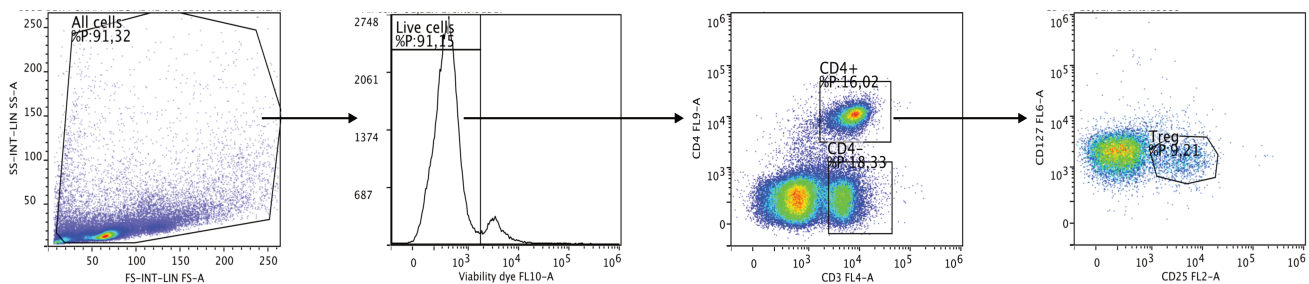


Figure 14: Gating strategy for the identification of T cells subsets by flow cytometry.

Total mouse splenocytes were analyzed. Live/dead discrimination was determined using Fixable Viability Dye eFluor 506. CD4 T cells were distinguished by CD3+ and CD4+ expression. CD8 T cells were distinguished by CD3+ and CD4- expression. Treg cells sub-population was defined by CD127^{low} and CD25^{high} expressions into CD4 T cells. Frequencies of cells in each sub-gate are expressed as a percentage of the previous gate.

Immunophenotyping of Th1, Th2 and Th17

Lymphocytes stimulation

Splenocytes was stimulated during 4 hours at 37°C with phorbol myristate acetate (PMA, 250 ng/10⁶cells, Sigma Aldrich) and ionomycin (500ng/10⁶cells, Sigma Aldrich) in

conjunction with a protein transport inhibitor containing Brefeldin A (BD GolgiPlug, BD Bioscience) resulting in the accumulation of cytokines in the golgi complex.

Cell surface staining

Cells were washed with PBS and Fc receptors were blocked with antimouse CD16/CD32-purified antibodies (eBioscience™, Thermo Fisher Scientific) for 15 min. Stimulated cells (2×10^6 /tube) were stained 30 minutes at 4°C with 100 µl of PBS containing surface markers antibodies PerCP Cy5.5-CD3, BV421-CD4, PE-Cy7-CD8 (all from BD Pharmingen, BD Bioscience) and Fixable Viability Dye eFluor™ 506 (eBioscience™, ThermoFisher Scientific) according to the manufacturer's instructions.

Intracellular staining

Cells were washed with PBS + 1% FBS and resuspended in 400 µl of Fixation/Permeabilization solution (BD Cytofix/ Cytoperm™, BD Bioscience) during 20 minutes at 4°C and washed twice in BD Perm/Wash™ (BD Bioscience) buffer. Fixed/permeabilised cells were resuspended in 100 µl of BD Perm/Wash™ buffer containing intracellular antibodies AF700-INF-γ (Biolegend, San Diego, CA), APC-IL4 and PE-IL-17A (both from BD Pharmingen, BD Bioscience) and incubated at 4°C during 30 minutes in the dark. Cells were washed and resuspended in BD Perm/Wash™ (BD Bioscience) buffer prior to flow cytometric analysis.

Flow cytometry was carried out using a Gallios analyzer (Bekman Coulter, Brea, CA). Blind analysis of the data was performed using the Flowlogic™ software (MiltenyiBiotec, Bergisch Gladbach, Germany). The gating strategy of the Th1, Th2, Th17 lymphocyte subpopulations are describe in the **Figure 15**.

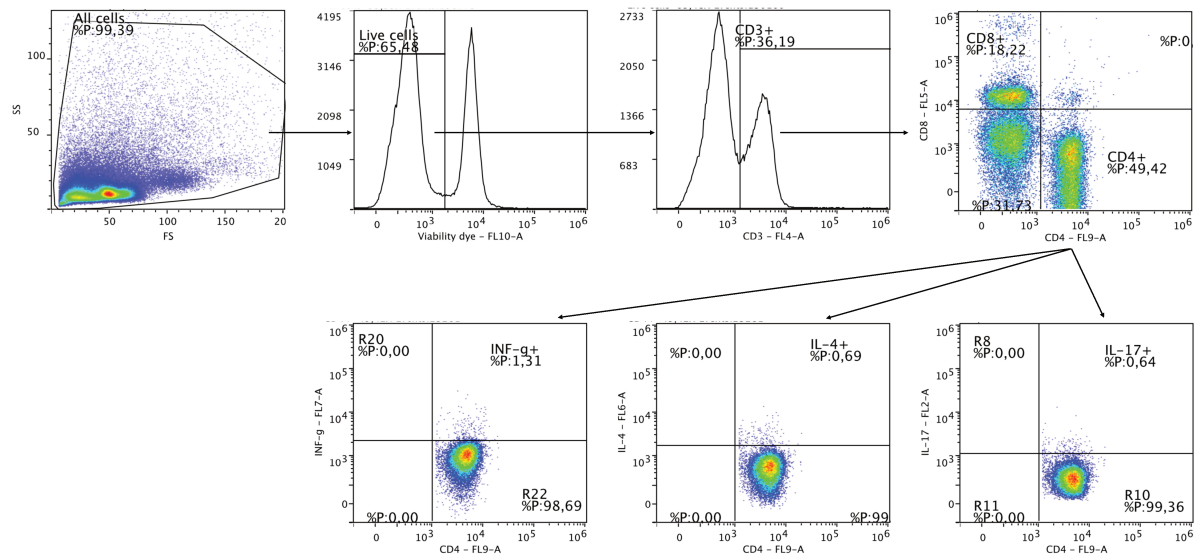


Figure 15: Gating strategy for the identification of Th1, Th2 and Th17 subsets by flow cytometry.

Total mouse splenocytes were analyzed. Live/dead discrimination was determined using Fixable Viability Dye eFluor 506. CD4 T cells were sub-gated into CD3+ T cells. Th1 subset were distinguished by INF- γ expression, Th2 by IL-4 expression and Th17 by IL-17 expression. Frequencies of cells in each sub-gate are expressed as a percentage of the previous gate.

Immunophenotyping of monocytes

Non-stimulated splenocytes were washed with PBS and Fc receptors were blocked with anti-mouse CD16/CD32-purified antibodies (eBioscienceTM, Thermo Fisher Scientific) for 15 min. To visualised monocytes (CD11b+ Ly6G- Ly6C+), cells were stained with 100 μ l of PBS containing surface markers antibodies PE-Cy7-CD11b, PE-Ly6G (BD Pharmigen, BD Bioscience), APC-Ly6C (NovusBio, Bio-Techne) and Fixable Viability Dye eFluorTM 506 (eBioscienceTM, Thermo Fisher Scientific) according to the manufacturer's instruction. Cells were incubated 30 minutes in the dark at 4°C, washed with PBS + 1% FBS and resuspended in PBS + 1% FBS for flow cytometric analysis. The gating strategy of the monocytes identification are described in the **Figure 16**.

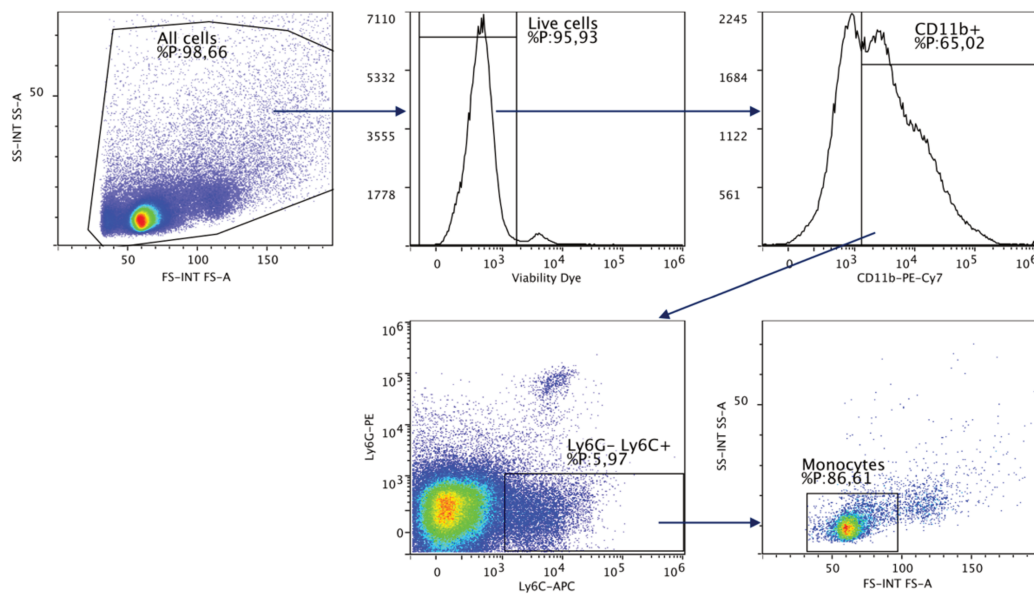


Figure 16: Gating strategy for the identification of monocytes by flow cytometry.

Total mouse splenocytes were analyzed. Live/dead discrimination was determined using Fixable Viability Dye eFluor 506. Ly6G- Ly6C+ cells were sub-gated in CD11b+ cells. Monocytes were gated based on FS and SS characteristics. Frequencies of cells in each sub-gate are expressed as a percentage of the previous gate

Proliferation Assay

The proliferative capacities of splenocytes were assessed using a Proliferation and Cell Tracking Dye. Splenocytes were diluted at a density of 1×10^6 cells/ml in PBS and stained with the Tag-it Violet™ Proliferation and Cell Tracking Dye ($5\mu\text{M}/10^6$ cells; Biolegend) for 20 minutes at 37°C . Staining was then quenched by the addition of culture medium containing 10% FBS. Cells were then centrifuged (300g, 5 minutes), resuspended in RPMI 1640 medium (supplemented with 10% FBS, 2 mM L-glutamine, 20 U/ml penicillin and 20 $\mu\text{g}/\text{ml}$ streptomycin) and incubated for 10 minutes at 37°C . Thereafter, splenocytes were seeded (200 000 cells per well) in 96-well plates and stimulated with phytohemagglutinin (PHA; $4\mu\text{g}/10^6$ cells; Remel, ThermoFisher Scientific) for 72 hours at 37°C . Cells were stained with CD4-BV421 (BD Pharmingen, BD Bioscience) and propidium iodide (MilenyiBiotec). Tag-It Violet signal was assessed in live CD4 T cells by flow cytometry.

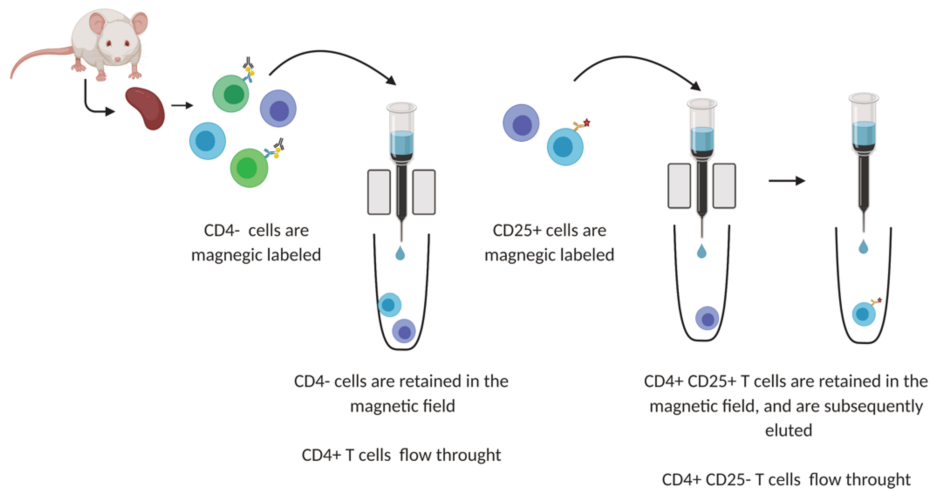
Purification of CD4⁺ CD25⁺ Treg and CD4⁺ CD25⁻ T cells

CD4 T cell effectors and Treg cells were isolated in a two-step procedure following the manufacturer's instructions (CD4⁺ CD25⁺ Regulatory T Cell Isolation Kit, MiltenyiBiotec) in $\beta_1^{+/+}$ non-septic mice. First CD4 T cells were negatively selected by depletion of unwanted cells. CD4⁻ cells were magnetically labeled with a biotin-conjugated antibodies cocktail against CD8a, CD11b, CD45R, CD49b, and Ter-119 (10 μ L per 10^7 cells in adapted buffer: PBS, 0,5% BSA, 2mM EDTA, pH 7,2) at 4°C for 10 min. Then cells were incubated at 4°C for 15 minutes in the dark with anti-biotin monoclonal antibodies conjugated to microbeads (20 μ L per 10^7 cells) and anti CD25-PE antibody (10 μ L per 10^7 cells). The magnetically labeled cells were flowed through LD MACS[®] Column (MiltenyiBiotec), which is placed in the magnetic field of a MACS[®] Separator (MiltenyiBiotec). Labeled CD4⁻ cells were retained in the column and CD4 T cells passed through (**Figure 17, panel A**).

Then CD4⁺ CD25⁺ Treg cells were positively selected from the pre-enriched CD4 T cells fraction. CD4 T cells was centrifuged and incubated with anti-PE microbeads (10 μ L per 10^7 cells) at 4°C during 15 minutes in the dark. Cells were flowed through MD MACS[®] Column placed in the magnetic field of a MACS[®] Separator. The CD4⁺ CD25⁺ Treg cells were retained in the column, while the flow- through CD4⁺ CD25⁻ T cells were collected.

A sample of the two fractions were stained with CD4-BV421 (BD Pharmigen, BD Bioscience) and Fixable Viability Dye eFluor[™] 506 (eBioscience[™], Thermo Fisher Scientific) antibodies and analyzed by flow cytometer to validate the isolation of Treg cells (**Figure 17, panel B**).

A



B

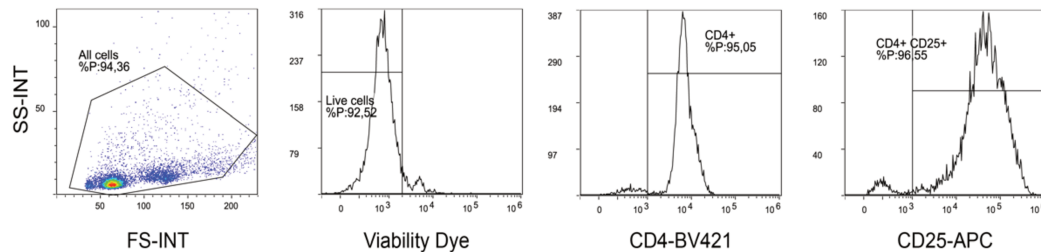


Figure 17: Isolation of CD4 T cells and Treg cells.

(A) Isolation of CD4 and Treg cells from the spleen in a two-step procedure. Firstly, CD4 T cells were negatively selected by depleting unwanted cells. Then, CD4⁺ CD25⁺ Treg cells were positively selected from the CD4⁺ fraction. (B) After magnetic separation, Treg cells were analyzed by flow cytometry in order to assess the purity of the cell population. The cell viability, the percentage of CD4 T cells and the percentage of CD25⁺ cells were analyzed.

Co-culture of CD4 T cell and Treg cells stimulated with catecholamines

Treg cells were stimulated with 1 μ M dobutamine (a β_1 -AR agonist), norepinephrine (a mixed α_1 - and β_1 -AR agonist), phenylephrine (α_1 -AR agonist); (all from Sigma-Aldrich) or PBS for 30 minutes, then washed twice to remove the catecholamines. Pre-treated Treg cells were co-cultured with CD4 T cells at a 1:4 ratio in complete RPMI 1640 medium (supplemented with 10% FBS, 2 mM L-glutamine, 20 U/ml penicillin, 20 μ g/ml streptomycin, 1% sodium pyruvate,

1% HEPES, 50 μ M 2-mercaptoethanol and 30 U/ml IL-2) in 96-well plates coated with 50 μ L of 0.01% poly-L-ornithine solution (Sigma Aldrich). Poly-L-ornithine supporting proliferation and providing a uniform cell distribution for imaging.

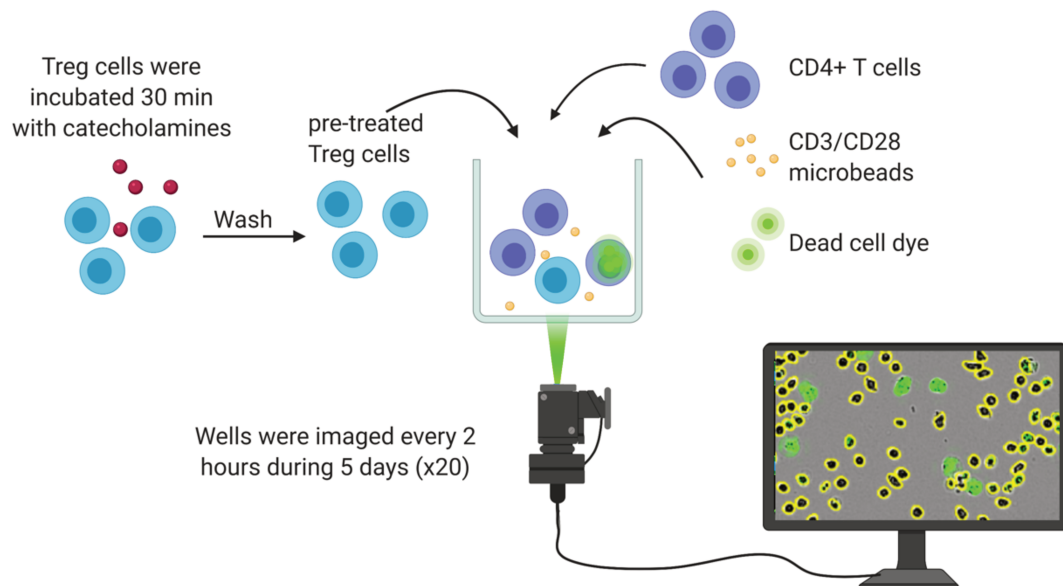


Figure 18: Co-culture of CD4 T cells and Treg cells pre-treated with catecholamines.

Treg cells were stimulated with catecholamines 30 minutes before being co-culture with CD4 T cells. T cell were stimulated to proliferate using anti-CD3 and anti-CD28 beads. Dead cells were identified with a green fluorescent dye. All wells were imaged every 2 h. The total number of cells and the number of green dead cells were counted.

Cells were activated with anti-CD3/CD28 beads (bead-to-cell ratio 1:1; T cell activation/expansion kit, MiltenyiBiotec) and imaged every 2 hours for 5 days (37 °C, 5% CO₂) using the 20× objective of the IncuCyte® S3 Live-Cell Analysis System (Sartorius, Goettingen, Germany). Proliferation was measured by cell counting in the phase contrast mode. Dead cells were identified using the green fluorescent Cytotox Green dye (Sartorius; final concentration, 250 nM) (**Figure 18**). The experiment was performed in duplicate, in four mice. Analysis was performed using the IncuCyte™ Basic Software.

Survival study

Survival was studied in $\beta_1^{+/+}$ CLP mice, $\beta_1^{+/+}$ CLP + esmolol (18 mg/kg/h) mice, and $\beta_1^{-/-}$ CLP mice. Census of animals was undertaken twice a day for 5 days. Administration of esmolol (Esmocard®, Amomed) was started 3 hours post-CLP and continued for a total infusion period of 24 hours. The one animal that died before the first count was considered a technical failure and excluded from the study.

Statistical analysis

Results are expressed as mean and standard deviation (mean \pm SD). Results in $\beta_1^{-/-}$ mice were compared using a Mann-Whitney test while the Kruskal-Wallis test was used to compare $\beta_1^{+/+}$ groups. When the Kruskal-Wallis test was significant, *post hoc* comparisons were performed between sham, CLP, and CLP + esmolol groups using Dunn's multiple comparisons and a Bonferroni correction.

Variations were calculated as follows: $100 - ((\text{mean group 1} / \text{mean group 2}) \times 100)$.

To analyze the effect of catecholamines on Treg cells co-cultured with CD4 T cells, we firstly assessed the interaction between mouse and catecholamine at each time point. Considering the mouse has a random effect, the p-value of the test for the treatment effect was calculated. Only the last p-value (obtained at 132 hours) is shown.

For measurement of plasma inflammatory mediators, groups were compared using the Mann-Whitney test. A Benjamini-Hochberg adjustment was performed for multiple comparisons.

Kaplan–Meier curves were drawn. Significance was assessed using the log-rank test.

P values <0.05 were considered statistically significant.

Statistical analysis was performed using R, version 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria), using Graph-Pad Software 6.0, (San Diego, CA) and using NCSS Statistical Software 9 (East Kaysville, UT).

Results

Esmolol dosage

The dosage of esmolol responsible for 20% HR reduction was tested. Esmolol was infused in $\beta_1^{+/+}$ CLP mice at 1, 5 and 18 mg/kg/h. The **Figure 19** showed that administration of 18 mg/kg/h of esmolol in CLP mice allowed a HR reduction of 32% ($p=0,01$) compared to sham mice and 19% ($p=0,28$) compared to CLP mice. Esmolol at 18 mg/kg/h was ultimately chosen for subsequent study.

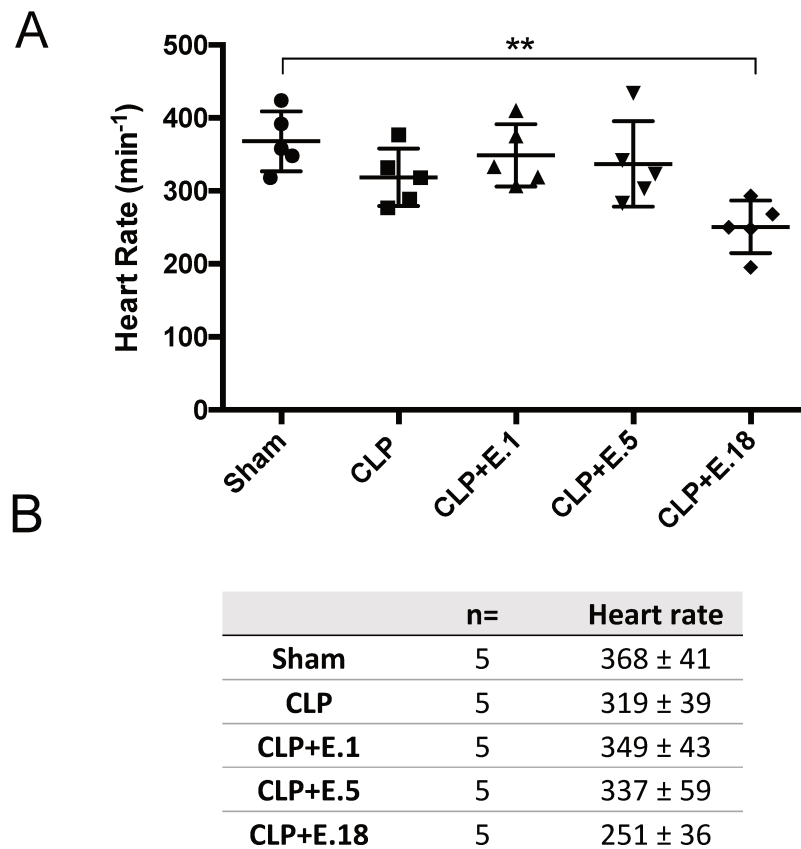


Figure 19: Effect of esmolol on heart rate in septic mice.

Esmolol (E.) at 1, 5 or 18 mg/kg/h was administrated three hours after the onset of sepsis in $\beta_1^{+/+}$ mice. (A) Heart rate was measured by echocardiography at 18 hours after surgery. (B) Details of HR variations compared to the sham group and CLP group. Data are expressed as mean \pm SD. ** $p<0,01$.

β_1 -AR blockade does not change lymphocytes proportion and proliferation in non-septic mice

Male and female mice were used in equal numbers for all experiments (sex ratio =1.02). In $\beta_1^{+/+}$ sham mice, pharmacological blockade of β_1 -AR by esmolol had no impact on CD4 T cells and Treg cells proportions as well as genetic blockade of β_1 -AR in $\beta_1^{-/-}$ sham mice (**Figure 20, panels A, B**). Furthermore, the lymphocytes proliferative capacity was not modified by the pharmacologic or genetic blockade of β_1 -AR (**Figure 20, panel C**).

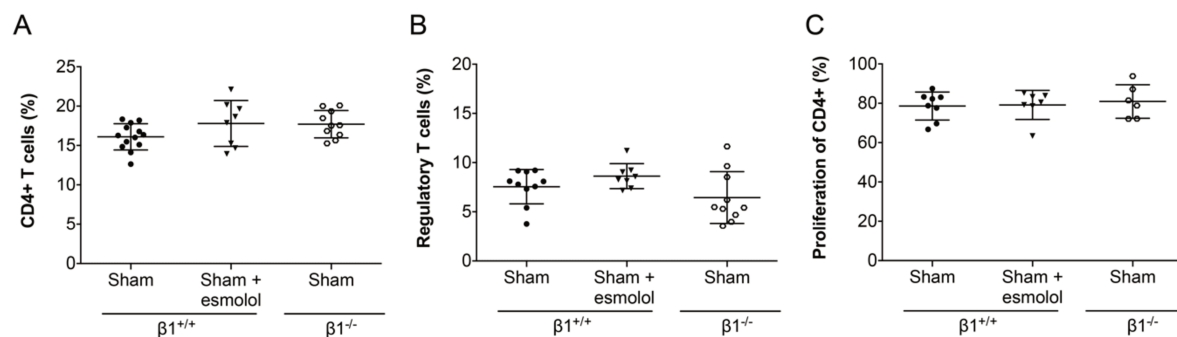


Figure 20: β_1 -AR blockade in sham treated mice does not change lymphocyte proportions and functions.

The proportions of CD4 T cells in live cells (A), the proportion of Treg cells (CD25^{hi} CD127^{low}) in CD4 T cells (B), and the CD4 T cell proliferative capacity (C) were assessed in sham mice where β_1 -AR was pharmacologically or genetically blocked. Data are expressed as mean \pm SD.

β_1 -AR blockade does not change monocytes proportions

No change was observed in septic mice compared to simulated mice for the proportion of monocytes in the spleen. Furthermore, pharmacological and genetic blockade of 1-AR had no influence on the proportions of monocytes in the spleen (**Figure 21**).

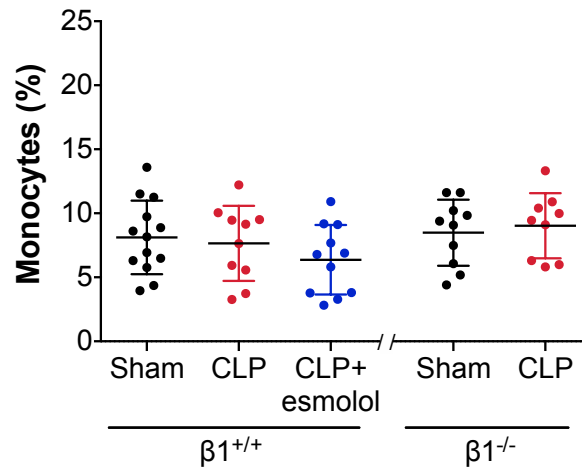


Figure 21: β_1 -AR does not change monocytes proportions in sepsis.

Splenocytes were immunophenotyped 18 hours after high-grade sepsis induction. The proportion of monocytes was determined. Data are expressed as mean \pm SD.

β_1 -AR contributes to post-septic immunosuppressive phenotype

Eighteen hours after surgery, the spleens of $\beta_1^{+/+}$ and $\beta_1^{-/-}$ mice were harvested and immunophenotyped to study T lymphocyte subpopulations. **Figure 22, panel A** shows that, compared to the $\beta_1^{+/+}$ sham group, the percentage of CD3 T cells decreased in the $\beta_1^{+/+}$ CLP (-24% vs. sham, $p=0.002$) and $\beta_1^{+/+}$ CLP + esmolol groups (-20% vs. sham, $p=0.004$). In $\beta_1^{-/-}$ mice, the percentages of CD3 T cells were similar in the sham and CLP groups (-8% vs. sham, $p=0.06$). **Figure 22, panel C** shows that in $\beta_1^{+/+}$ mice, high-grade sepsis induced a significant decrease in CD4 T cell proportions (-34% vs. sham, $p<0.001$) counteracted by β_1 -AR blockade (+37% vs. CLP, $p=0.03$). In $\beta_1^{-/-}$ mice, sepsis significantly decreased the proportion of CD4 T cells (-17% vs. sham, $p=0.02$), however no change in CD8 T cells proportion was observed between groups (**Figure 22, panel B**).

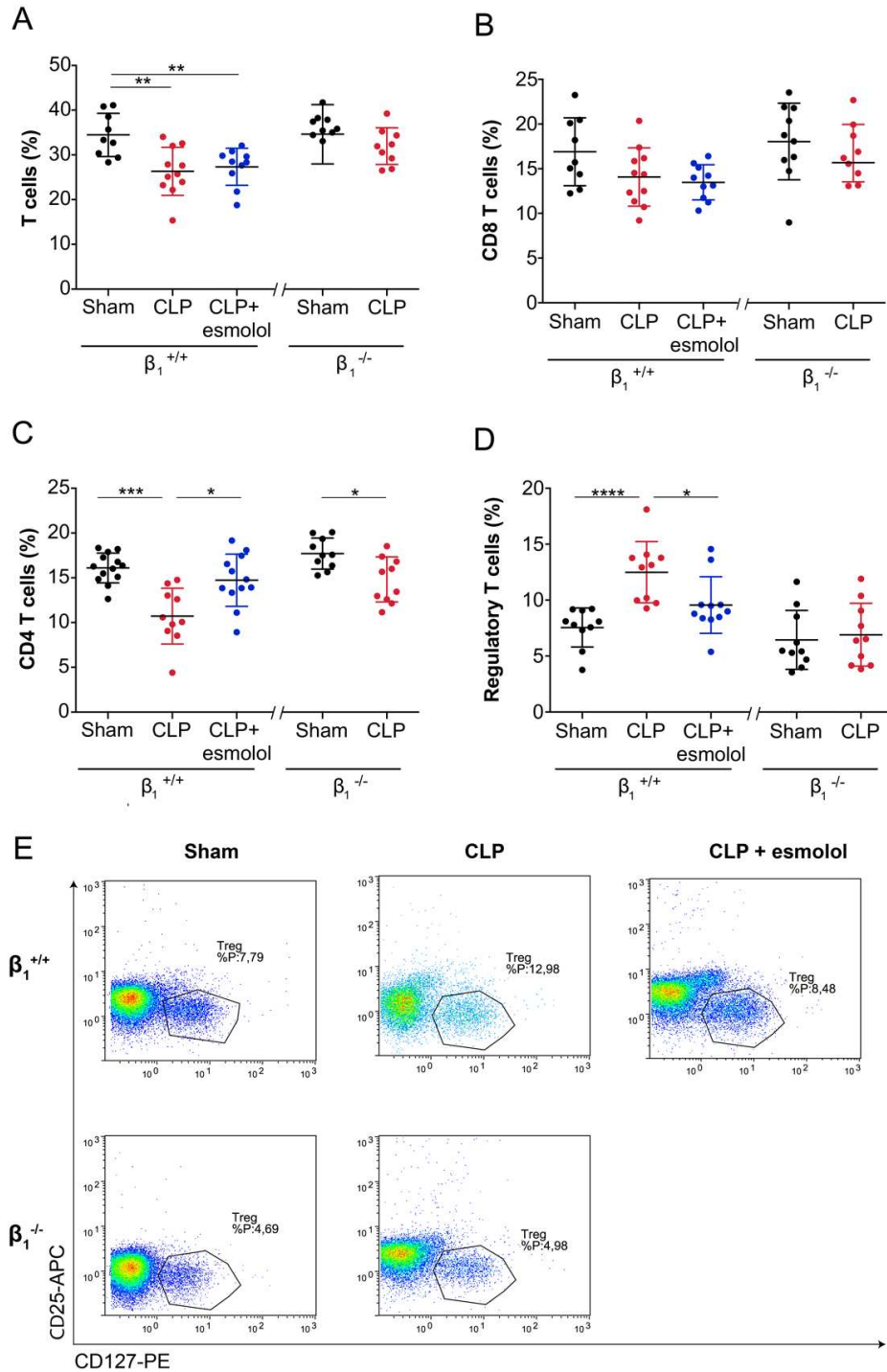


Figure 22: β_1 -AR contributes to the post-septic immunosuppressive phenotype.

Splenocytes were immunophenotyped eighteen hours after high-grade sepsis induction. The proportion of T cells in live splenocytes (A), the proportion of CD8 T cells in live splenocytes (B), the proportion of CD4 T cells in live splenocytes (C), and the proportion of Treg cells (CD25^{hi} CD127^{low}) (D) were assessed. Density plots of Treg cells for one representative mouse per group are showed in panel (E). Data are expressed as mean \pm SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

With respect to Treg cells (CD25^{hi} CD127^{low}), in $\beta_1^{+/+}$ mice, high-grade sepsis induced an increase in Treg cell proportions (+65% vs. sham, $p < 0.001$), inhibited by β_1 -AR blockade (-19% vs. CLP, $p = 0.04$) **Figure 22, panels D, E**. In $\beta_1^{-/-}$ septic mice, the proportion of Treg cells was unchanged (+7% vs. sham, $p = 0.68$). No modification was observed in other CD4 T cells subsets (Th1 (INF- γ), Th2 (IL-4) and Th17 (IL-17)) in these experimental groups (**Figure 23**). These findings were not caused by a difference in cell viability among groups (**Figure 24**).

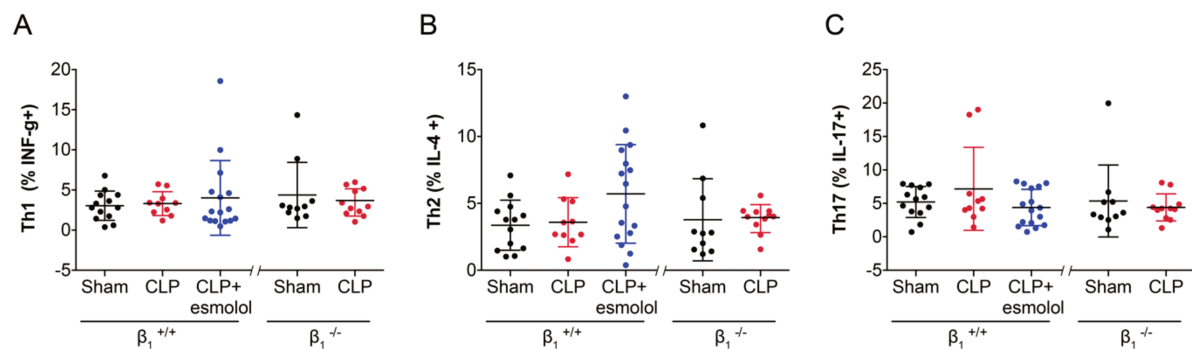


Figure 23: β_1 -AR does not change the CD4 T, Th1, Th2, Th17 subpopulation proportions in sepsis.

Splenocytes were immunophenotyped 18 hours after high-grade sepsis induction. The proportion of Th1 (INF- δ +) (A), Th2 (IL-4+) (B) and Th17 (IL-17+) (C) cells among CD4 T cells were determined. Data are expressed as mean \pm SD.

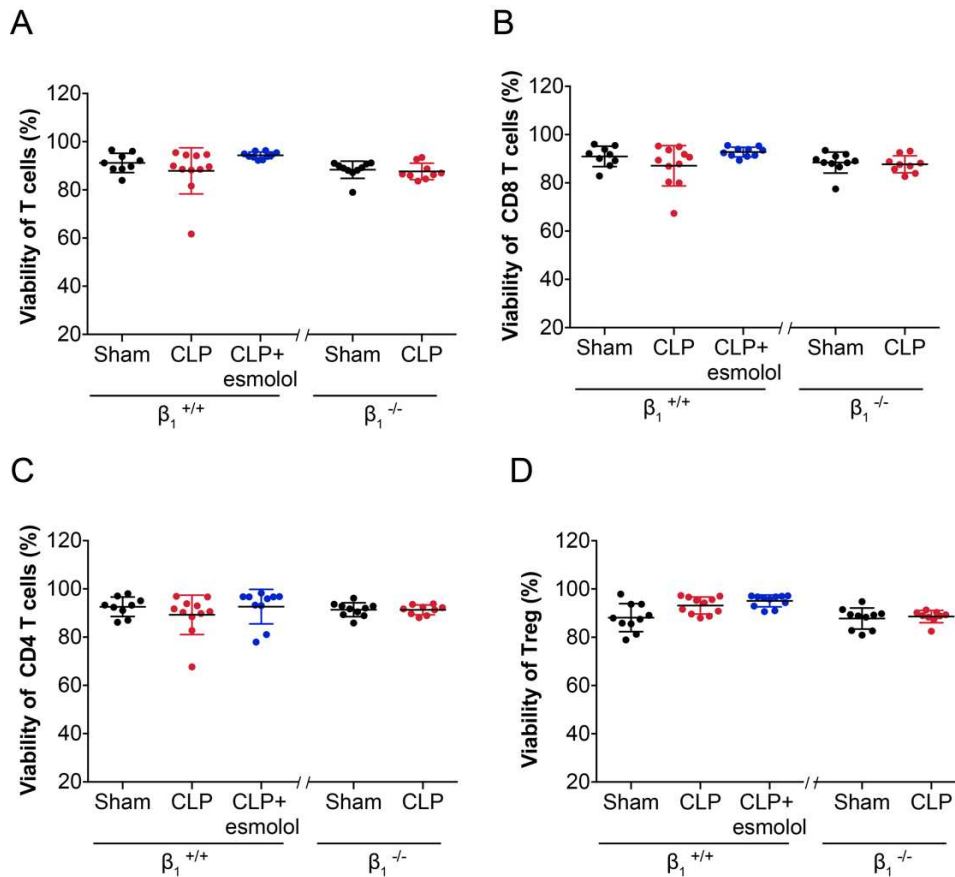


Figure 24: β_1 -AR does not change lymphocyte viability in sepsis.

Splenocytes were immunophenotyped 18 hours after high-grade sepsis induction. The viabilities of T cells (A), CD8 T cells (B), CD4 T cells (C) and Treg cells (D) were determined in each group. Data are expressed as mean \pm SD.

β_1 -AR activation induces a decrease in CD4 T cell proliferation during sepsis

After culturing splenocytes for 72 hours with PHA stimulation, the proliferative response of CD4 T cells was measured to assess sepsis-induced immune suppression (**Figure 25, panel A**). In $\beta_1^{+/+}$ mice, high-grade sepsis significantly reduced the proliferative capacity of CD4 T cells (-15% vs. sham, $p=0.04$). However, administration of a β_1 -AR blocker limited this effect (-8% vs. sham, $p=0.38$). In $\beta_1^{-/-}$ mice, sepsis did not significantly decrease the proliferative capacities of CD4 T cells (-4% vs. sham, $p=0.71$) (**Figure 25, panel B**). These results were not caused by variations in cell viability among groups (**Figure 25, panel C**).

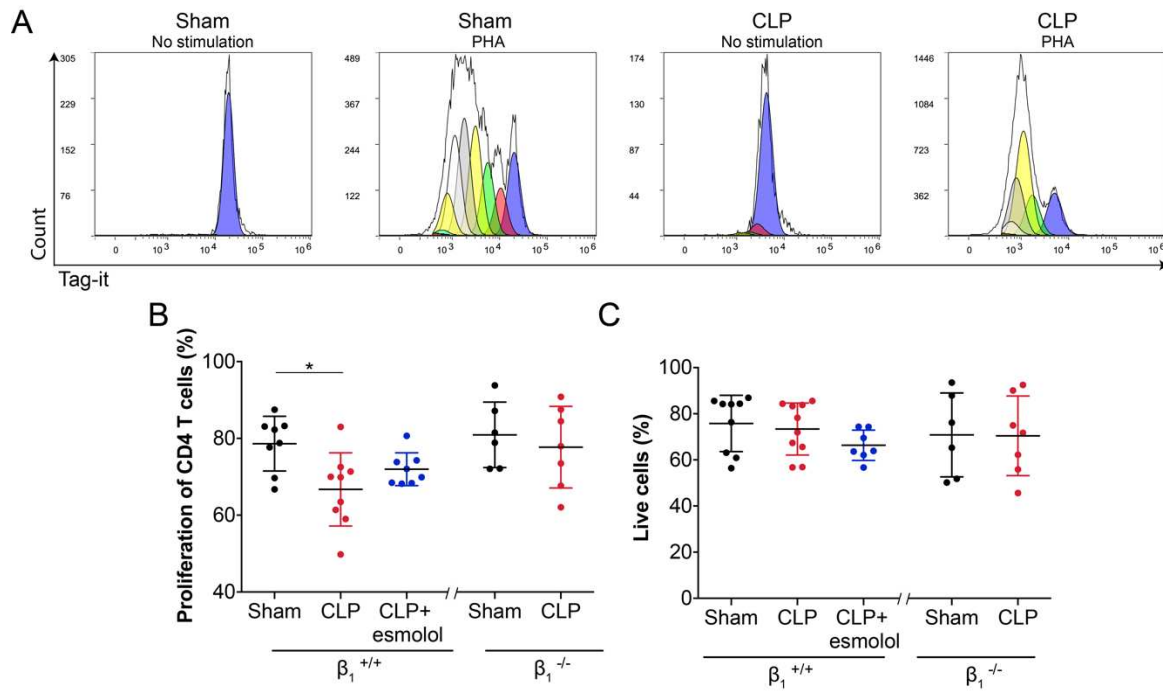


Figure 25: β_1 -AR activation decreases CD4 T cell proliferation during sepsis.

Splenocytes harvested 18 hours after sepsis induction were cultured for 72 hours with a mitogen (PHA) to assess their proliferative response. (A) Histograms of the proliferation marker in non-stimulated and PHA stimulated splenocytes from one representative $\beta_1^{+/+}$ sham and CLP mouse. Each peak has been fitted and colored by FlowLogic software. (B) Proliferation of CD4 T cells according to cell tracking dye staining. (C) Proportion of live cells in each group. Data are expressed as mean \pm SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

β_1 -AR stimulation of Treg cells increases their immunosuppressive capacities

The direct effect of β_1 -AR stimulation on the immunosuppressive functions of Treg cells was assessed. CD4 T cell counts tended to decrease after five days of co-culture with Treg cells pre-treated with dobutamine, in comparison to untreated Treg cells ($p = 0.08$, **Figure 26, panel A**). This lower cell count, noted when co-culture was performed with dobutamine pre-treated Treg cells, was not caused by an increase in cell death (**Figure 26, panel B**).

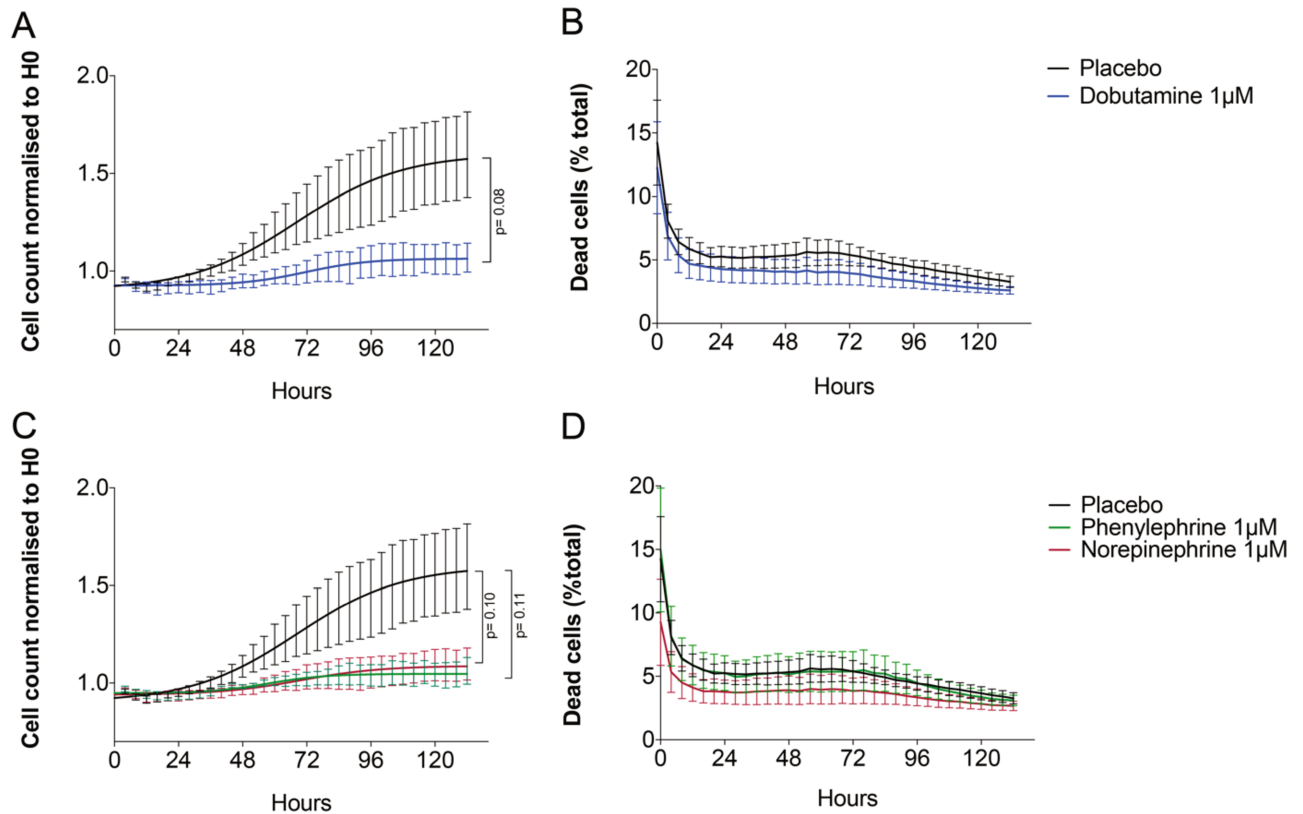


Figure 26: Treg cell stimulation by catecholamines increases their immunosuppressive capacities.

Treg cells isolated from $\beta_1^{+/+}$ mice were treated with dobutamine (A,B) phenylephrine or norepinephrine (C,D) for 30 min. Pre-treated Treg cells were then co-cultured with CD4 T cells at a 1:4 ratio in the presence of anti-CD3 and anti-CD28 beads for 5 days. A fluorescent green reagent was added for counting dead cells. (A,C) shows the real-time cell count normalized to the first count at H0, and (B,D) shows the dead cell count as a percentage of the total cells. Data shown are the means of 4 images per well in duplicate with 4 mice per time point \pm SEM (total 32 images/time/group). Images were taken at 20 \times magnification on the IncuCyte[®] imager.

Similar results were found when co-culture assays of CD4 T cells were performed in the presence of norepinephrine and phenylephrine pre-treated Treg cells. Catecholamines pre-treatment tend to decrease the CD4T cells proliferation without increased cell death (Figure 26, panels C, D).

β_1 -AR blockade, despite reduced heart rate, did not alter hemodynamic characteristics in sepsis

Hemodynamic parameters were assessed by echocardiography (**Table 12**). In $\beta_1^{+/+}$ septic mice, administration of a β_1 -AR blocker induced a decrease in HR (-24% vs. sham, $p<0.001$; -14% vs. CLP, $p=0.07$). In $\beta_1^{-/-}$ mice, sepsis did not affect HR (+9% vs. sham, $p=0.24$). Compared to $\beta_1^{+/+}$ sham, high-grade sepsis induced a decrease in MAP (-27% vs. sham, $p<0.001$), SV (-40% vs. sham, $p=0.004$), CI (-49% vs. sham, $p<0.001$), and CPI (-64% vs. sham, $p<0.001$). These parameters remained unaltered by the administration of a β_1 -AR blocker (MAP: +8% vs. CLP, $p=0.51$; SV: +6% vs. CLP, $p=0.99$; CI: +2% vs. CLP, $p=0.99$; CPI: +25% vs. CLP, $p=0.99$). In $\beta_1^{-/-}$ mice, sepsis induced a decrease in MAP (-19% vs. sham, $p=0.04$), SV (-47% vs. sham, $p<0.001$), CI (-37% vs. sham, $p=0.002$) and CPI (-50% vs. sham, $p<0.001$).

Table 12: Despite reduced heart rate, β_1 -AR blockade did not alter hemodynamic characteristics in sepsis.

Hemodynamic characteristics were measured by echocardiography 18 hours after surgery. Data are expressed as mean \pm SD.

Variable	$\beta_1^{+/+}$					$\beta_1^{-/-}$		
	Sham	CLP	CLP + esmolol	P value	Adjusted P value	Sham	CLP	P value
	n=13	n=13	n=18			n=10	n=12	
Female (%)	54%	46%	56%			50%	50%	
Weigh (g)	27.5 \pm 5.0	28.4 \pm 4.1	26.8 \pm 3.0	0.53	ns	29.1 \pm 4.4	28.4 \pm 5.8	0.61
Heart rate (min ⁻¹)	360 \pm 48	318 \pm 31	273 \pm 45	<0.001	0.23: Sham vs. CLP <0.001: Sham vs. CLP + esmolol 0.07: CLP vs. CLP + esmolol	250 \pm 27	274 \pm 45	0.24
Mean arterial pressure (mmHg)	84.8 \pm 6.9	62.2 \pm 11.8	67.4 \pm 17.3	<0.001	<0.001: Sham vs. CLP 0.01: Sham vs. CLP + esmolol 0.51: CLP vs. CLP + esmolol	80.9 \pm 15.9	65.2 \pm 11.9	0.04
Cardiac index (ml/min/g)	0.59 \pm 0.09	0.30 \pm 0.13	0.29 \pm 0.17	<0.001	<0.001: Sham vs. CLP <0.001: Sham vs. CLP + esmolol 0.99: CLP vs. CLP + esmolol	0.43 \pm 0.04	0.27 \pm 0.12	0.002
Cardiac power index (W/g)	0.11 \pm 0.02	0.04 \pm 0.02	0.05 \pm 0.03	<0.001	<0.001: Sham vs. CLP <0.001: Sham vs. CLP + esmolol 0.99: CLP vs. CLP + esmolol	0.08 \pm 0.02	0.04 \pm 0.02	<0.001
Stroke volume (μ L)	44.5 \pm 8.1	26.5 \pm 10.2	28.1 \pm 15.9	0.001	0.004: Sham vs. CLP 0.01: Sham vs. CLP + esmolol 0.99: CLP vs. CLP + esmolol	50.3 \pm 10.7	26.9 \pm 10.5	<0.001

Sepsis does not influence leukocyte infiltration in the heart

Leukocyte infiltration into cardiac tissue was analyzed by immunohistochemistry and no changes were observed in septic mice compared to the sham mice. In addition, the pharmacological and genetic blockade of β_1 -AR had no influence on the infiltration of leukocytes into the heart (**Figure 27**).

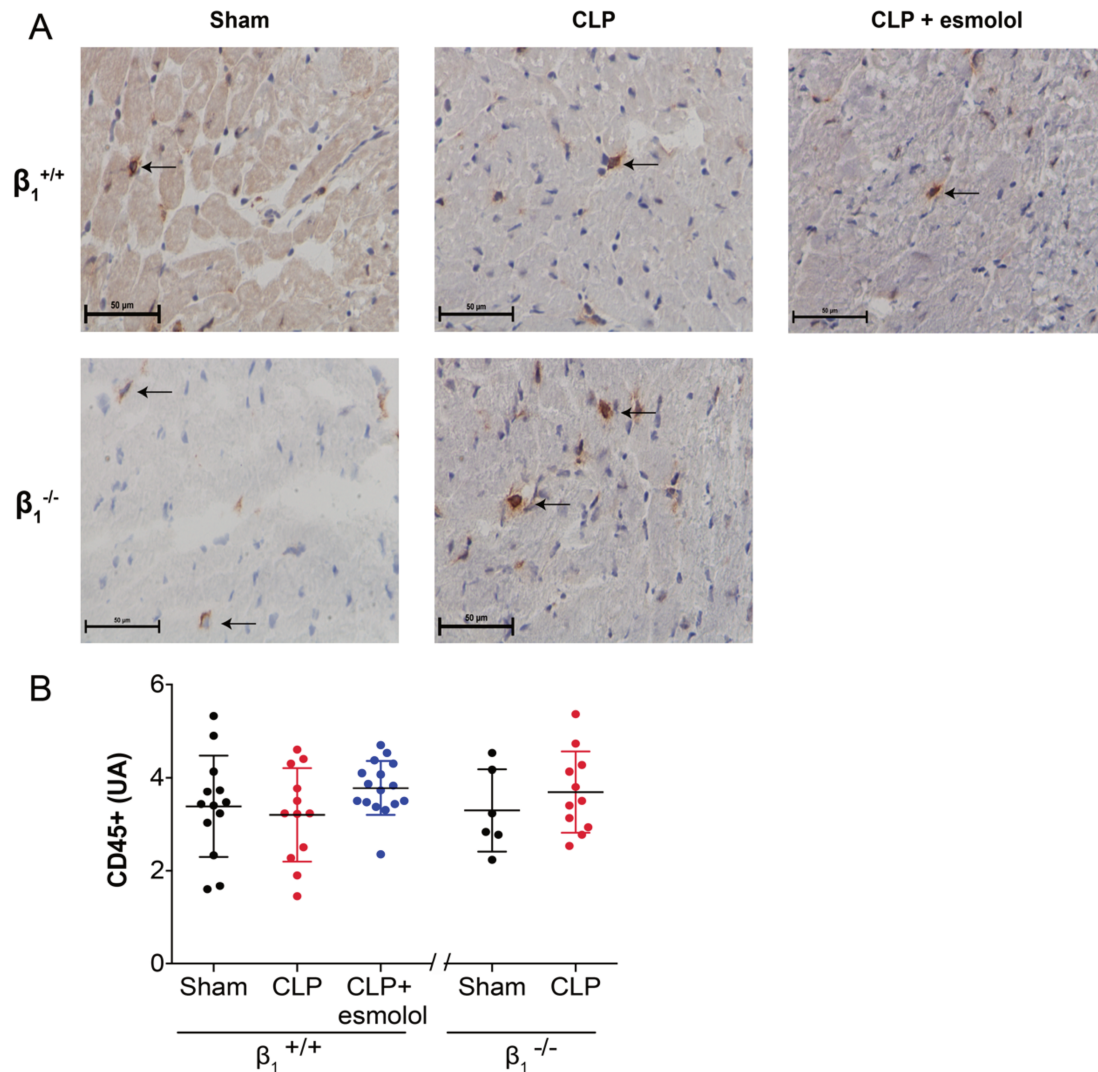


Figure 27: Leukocytes infiltration is not modified by the sepsis in the heart.

(A) Leukocytes infiltration was evaluated by immunohistochemistry in myocardial samples. Leukocytes was stained with anti-CD45 antibodies. One representative image for each group was presented. (B) The number of CD45+ cells was quantified by 3 experimenters on 10 fields with a x40 lens and averaged for each mouse.

β_1 -AR modulates the concentration of both pro- and anti-inflammatory circulatory mediators

The plasma concentrations of 31 pro-inflammatory and 15 anti-inflammatory mediators were measured. Briefly, all inflammatory mediators were increased in $\beta_1^{+/+}$ CLP mice compared to sham controls (**Table 13**). Compared to $\beta_1^{+/+}$ CLP mice, esmolol-treated CLP mice showed an overall decrease in plasma concentrations of 58% (18/31) and 60% (9/15) for pro- and anti-inflammatory mediators, respectively (**Figure 28**). For instance, compared to $\beta_1^{+/+}$ CLP mice, administration of a β_1 -AR blocker reduced plasma TNF- α by 66% vs. CLP ($p=0.02$), and IL-10 by 82% vs. CLP ($p=0.01$). In $\beta_1^{-/-}$ mice, sepsis induced an overall increase in both pro- and anti-inflammatory mediators compared to sham animals (**Table 13**).

Figure 28: β_1 -AR modulates the concentration of both pro- and anti-inflammatory circulatory mediators.

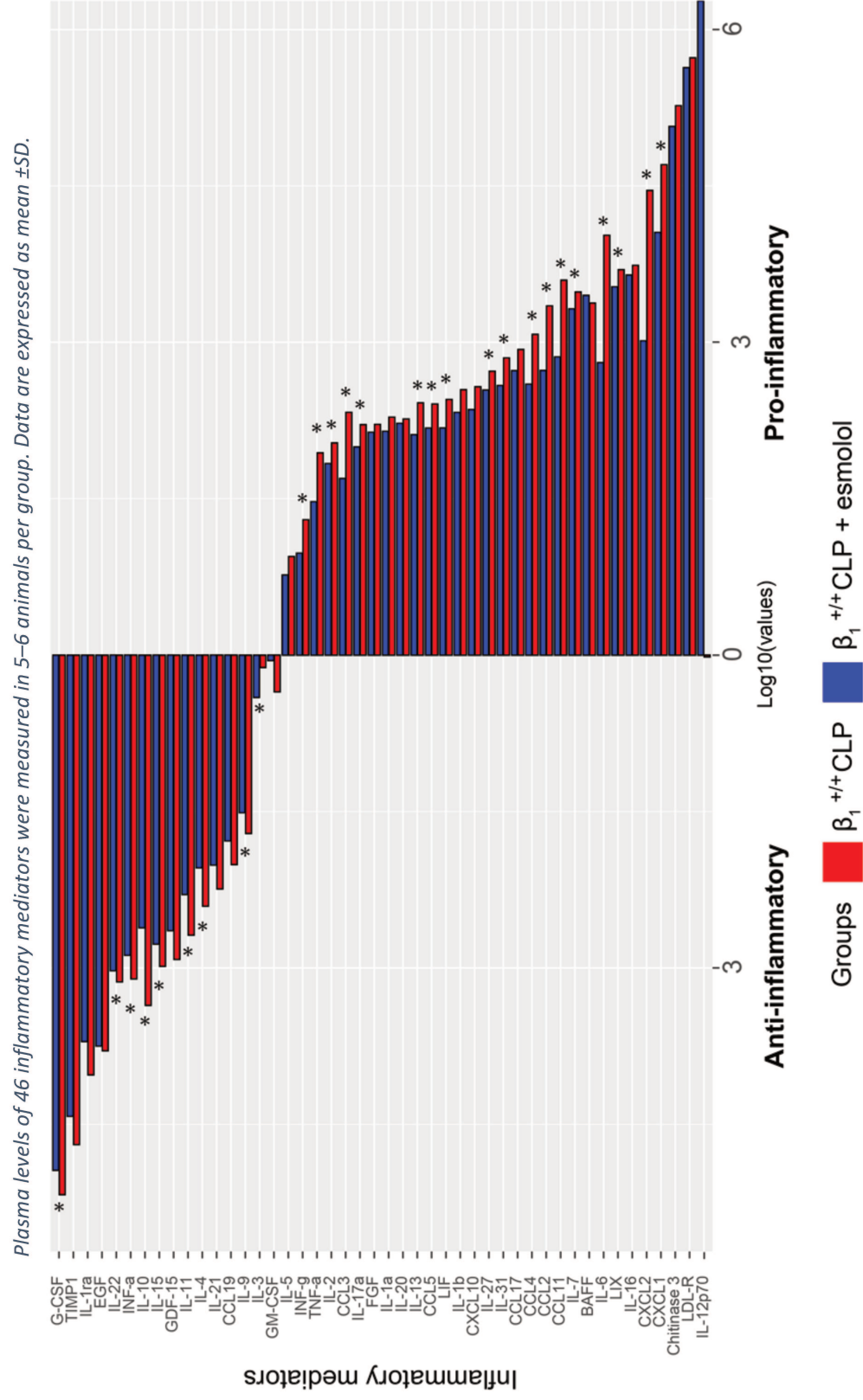


Table 13: Sepsis induces an increase in both pro- and anti-inflammatory mediators in $\beta_1^{+/+}$ and $\beta_1^{-/-}$ mice.

Data are expressed as mean \pm SD.

Cytokine (pg/ml)	$\beta_1^{+/+}$			$\beta_1^{-/-}$		
	Sham n=5	CLP n=6	P adjusted	Sham n=5	CLP n=5	P adjusted
BAFF	2007 \pm 559	2381 \pm 441	0.352	2434 \pm 825	3953 \pm 1554	0.165
CCL2	59 \pm 27	2233 \pm 3098	0.008	62 \pm 51	1629 \pm 1298	0.014
CCL3	4.2 \pm 2.2	212 \pm 278	0.009	1.9 \pm 2.1	122 \pm 54	0.014
CCL4	148 \pm 26	1188 \pm 1746	0.008	100 \pm 30	751 \pm 439	0.014
CCL5	74 \pm 17	255 \pm 48	0.008	73 \pm 11	233 \pm 90	0.014
CCL11	380 \pm 64	3939 \pm 2277	0.008	384 \pm 114	2244 \pm 1810	0.014
CCL17	49 \pm 22	852 \pm 425	0.008	53 \pm 42	1101 \pm 717	0.014
CCL19	59 \pm 80	102 \pm 40	0.137	17 \pm 10	84 \pm 30	0.014
Chitinase 3	13360 \pm 2334	186017 \pm 11300	0.008	19289 \pm 9545	227808 \pm 114392	0.014
CXCL1	304 \pm 223	50267 \pm 29822	0.008	343 \pm 279	54595 \pm 57326	0.014
CXCL2	10 \pm 6	28489 \pm 60858	0.008	15 \pm 8	33145 \pm 66852	0.014
CXCL10	111 \pm 46	375 \pm 170	0.008	58.3 \pm 26	581 \pm 360	0.014
EGF	7219 \pm 5897	6233 \pm 5486	0.645	5292 \pm 4928	6705 \pm 6349	1
FGF	85 \pm 9	163 \pm 17	0.009	76 \pm 12	167 \pm 17	0.014
G-CDF	542 \pm 392	149309 \pm 0	0.008	1287 \pm 1948	106311 \pm 58876	0.014
GDF-15	117 \pm 85	830 \pm 480	0.008	117 \pm 77	1133 \pm 820	0.014
GM-CSF	0.4 \pm 0.1	2.3 \pm 0.8	0.009	0.4 \pm 0.2	1.8 \pm 0.8	0.014
INF- α	401 \pm 85	1274 \pm 255	0.008	345 \pm 134	1035 \pm 245	0.014
INF- γ	2.9 \pm 1.5	20 \pm 11	0.008	0.7 \pm 0.8	17 \pm 8	0.014
IL-1 α	26 \pm 4	191 \pm 65	0.009	38 \pm 33	188 \pm 31	0.014
IL-1 β	104 \pm 37	352 \pm 333	0.008	61 \pm 9	320 \pm 150	0.014
IL-1ra	202 \pm 71	10603 \pm 10074	0.008	156 \pm 76	11724 \pm 9628	0.014
IL-2	22 \pm 1	108 \pm 13	0.009	22 \pm 11	110 \pm 19	0.014
IL-3	0.2 \pm 0.2	0.8 \pm 0.2	0.009	0.2 \pm 0.2	0.4 \pm 0.5	0.692
IL-4	41 \pm 41	255 \pm 43	0.009	11 \pm 24	251 \pm 57	0.014
IL-5	119 \pm 120	8.8 \pm 4.9	0.092	65 \pm 48	12 \pm 14	0.036
IL-6	53 \pm 27	10583 \pm 15012	0.008	55 \pm 36	7587 \pm 7756	0.014
IL-7	457 \pm 153	3041 \pm 298	0.008	419 \pm 283	2969 \pm 958	0.014
IL-9	19 \pm 6	51 \pm 8	0.009	16 \pm 10	47 \pm 1	0.014
IL-10	41 \pm 7	2281 \pm 1223	0.009	42 \pm 15	1595 \pm 1679	0.014
IL-11	88 \pm 43	486 \pm 220	0.008	31 \pm 47	391 \pm 115	0.014
IL-12p70	0.0 \pm 0.0	1.0 \pm 2.4	0.476	0.0 \pm 0.0	7 \pm 15	0.453
IL-13	76 \pm 9	262 \pm 40	0.008	48 \pm 44	254 \pm 38	0.014
IL-15	226 \pm 78	965 \pm 182	0.008	201 \pm 60	906 \pm 142	0.014
IL-16	2100 \pm 585	5450 \pm 806	0.008	1755 \pm 238	4762 \pm 1584	0.014
IL-17a	67 \pm 13	162 \pm 59	0.009	70 \pm 14	146 \pm 52	0.014
IL-20	234 \pm 107	183 \pm 150	0.448	225 \pm 139	197 \pm 136	0.86

IL-21	44 ± 14	175 ± 31	0.008	21 ± 26	143 ± 27	0.014
IL-22	656 ± 148	1361 ± 115	0.009	582 ± 69	1333 ± 195	0.014
IL-27	152 ± 17	527 ± 59	0.009	142 ± 43	501 ± 101	0.014
IL-31	215 ± 66	707 ± 114	0.008	138 ± 92	593 ± 81	0.014
LDL R	227140 ± 24317	532897 ± 130243	0.008	189990 ± 30388	626815 ± 128731	0.014
LIF	31 ± 9	283 ± 126	0.009	40 ± 20	218 ± 48	0.014
LIX	659 ± 332	4954 ± 677	0.008	1060 ± 730	4889 ± 626	0.014
TIMP1	5910 ± 1524	49443 ± 39078	0.008	6117 ± 1523	59504 ± 39477	0.014
TNF- α	10 ± 3	87 ± 73	0.009	11 ± 3	62 ± 27	0.014

β_1 -AR modulates survival during sepsis.

The survival rate was documented in $\beta_1^{+/+}$ CLP, $\beta_1^{+/+}$ CLP + esmolol and $\beta_1^{-/-}$ CLP mice, and shown in **Figure 29**. Survival at 110 hours decreased from 60% in $\beta_1^{-/-}$ CLP mice, to 37% in $\beta_1^{+/+}$ CLP + esmolol mice and to 35% in $\beta_1^{+/+}$ CLP mice (log rank $p = 0.13$).

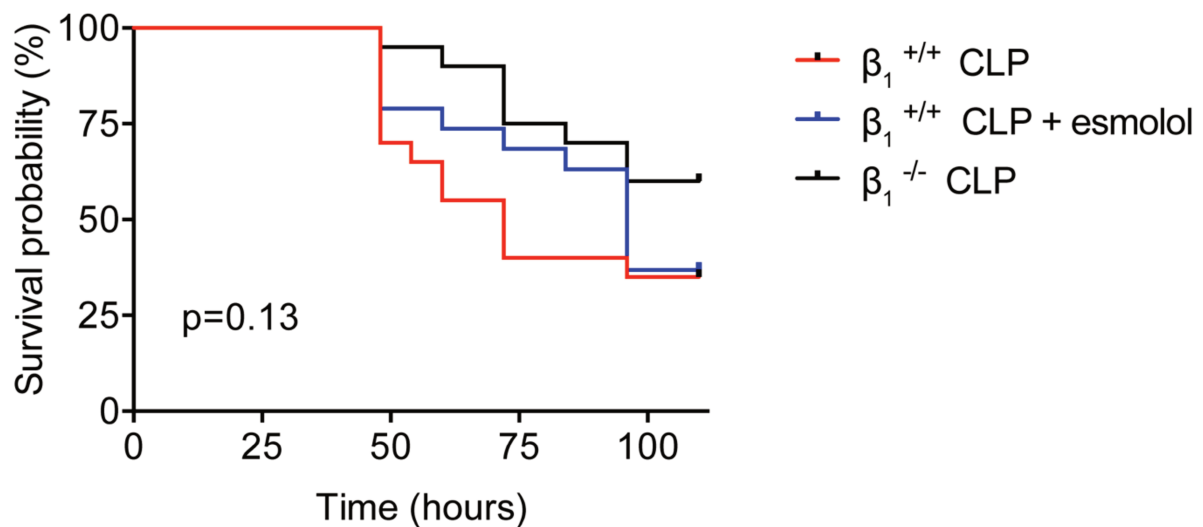


Figure 29: β_1 -AR blockade modulates survival during SS.

Survival curve of $\beta_1^{+/+}$ CLP mice, $\beta_1^{+/+}$ CLP mice receiving esmolol for 24 hours, and $\beta_1^{-/-}$ CLP mice.

Sex does not affect the β_1 -AR blockade effect

Sensitivity analyses were performed on results obtained for major hemodynamic parameters (**Figure 30**) and immune effects (**Figure 31**) according to sex. No difference was found between male and female mice.

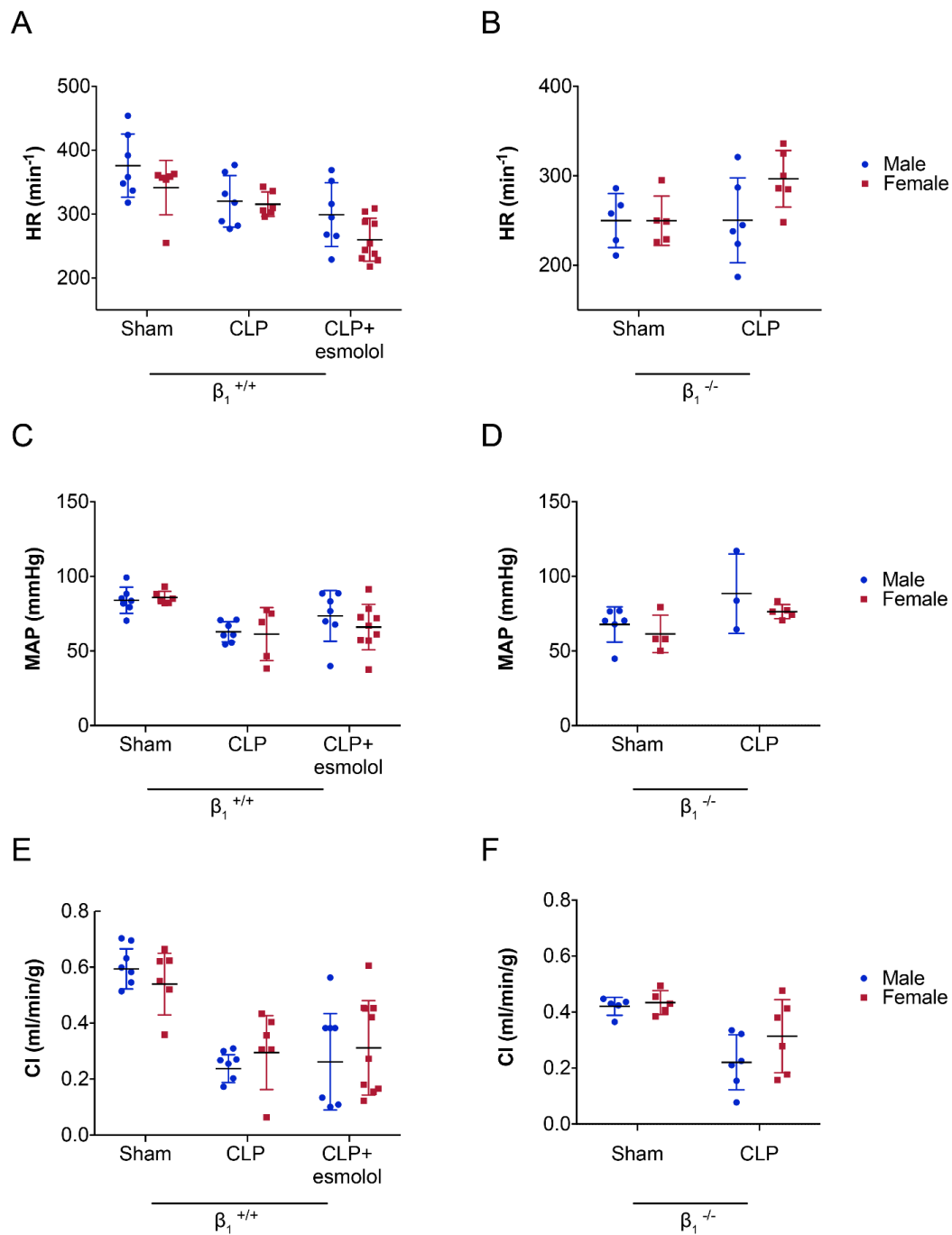


Figure 30: sex does not affect the hemodynamic effects of β_1 -AR.

Heart rate (A,B), mean arterial pressure (C,D) and cardiac index (E,F) are presented according to the sex of the animals in the $\beta_1^{+/+}$ (A,C,E) and $\beta_1^{-/-}$ (B,D,E) groups. Data are expressed as mean \pm SD.

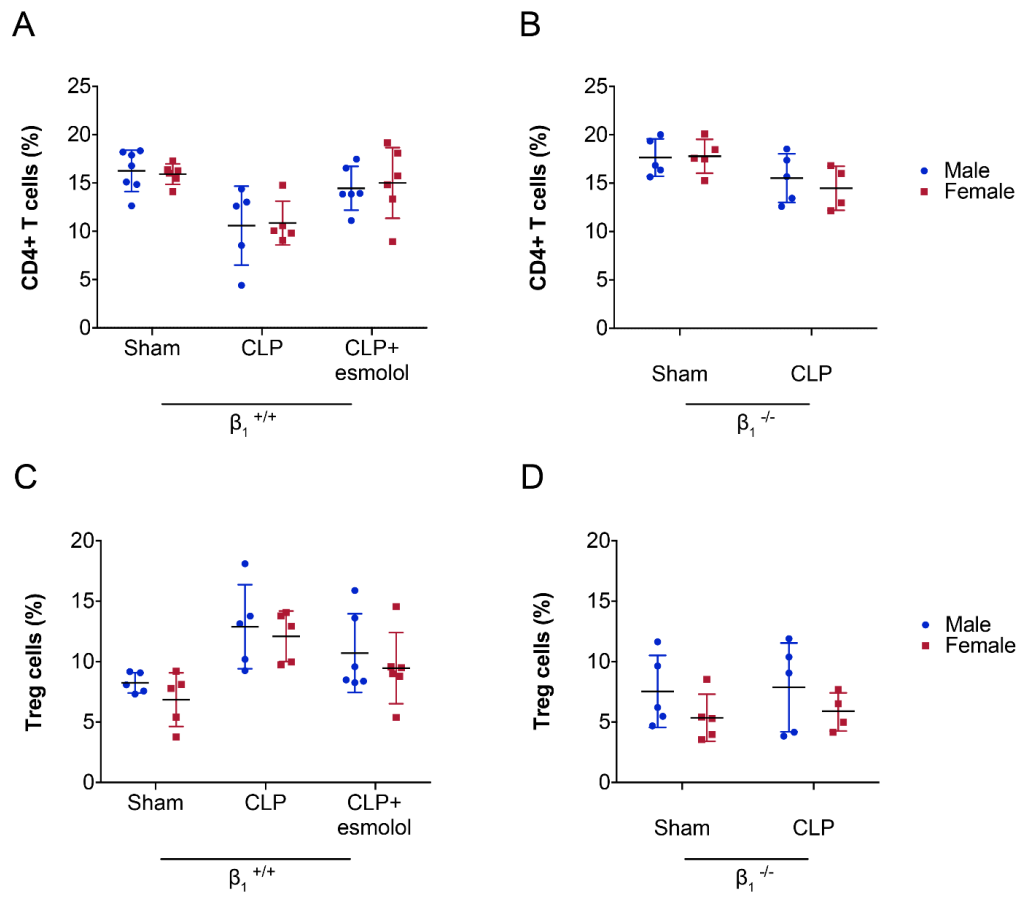


Figure 31: Sex does not affect the immune effects of β_1 -AR.

CD4 T cell (A,B) and Treg cell (C,D) proportions are presented according to the sex of the animals in the $\beta_1^{+/+}$ (A,C) and $\beta_1^{-/-}$ (B,D) groups. Data are expressed as mean \pm SD.

Discussion

In the present study, we confirm that experimental high-grade sepsis induces three hallmark features of sepsis-induced immunosuppression: a significant decrease in splenic CD4 T cells, an increase in splenic Treg cells, and a decrease in CD4 T cell proliferation capacity [15]. A notable finding was the marked increase in Treg cells in the spleen of septic mice, which could be counteracted by pharmacologically or genetically blocking β_1 -AR signalling. This observed decrease in Treg cells in treated animals was associated with a simultaneous increase in CD4 T cells and a global decrease in circulating pro- and anti-inflammatory mediators, indicating the involvement of β_1 -AR in the septic-immune phenotype. These results also translated into an *in vivo* global improvement in cardiac efficiency.

Model characteristics

To investigate the immune and cardiovascular roles of β_1 -AR in sepsis, we deliberately chose a non-resuscitated murine model of CLP to avoid any interference between exogenous catecholamine administration and the immune system, therefore the term septic shock cannot be used [397]. This model depicts all hemodynamic and survival characteristics of a typical high-grade sepsis, with an increase in both plasma pro- and anti-inflammatory mediators and a decrease in CD4 T cell proportion [37, 396, 398, 399]. Analysis of CD4+ subtypes revealed that early after onset of sepsis, the percentages of Th1, Th2 and Th17 cells were unchanged. Many studies usually report a switch from Th1 to Th2 phenotypes, and a decrease in Th17 cell percentages [262, 264, 400, 401]. However, other studies found that sepsis could also disrupt in a time-dependent manner both the representation and the

function of CD4 T cell subsets, including Th1, Th2, Th17 [170, 225, 254, 268]. Unlike other lymphocyte populations, Treg cells are increased during trauma in both animals and humans, as well as during sepsis and septic shock (as seen in our study) [170, 225]. This increase in Treg cells contributes to lymphocyte paralysis and, more broadly, enhances the post-septic immunosuppressive process [236].

β -AR stimulation contributes to CD4 T cell dysfunction

During sepsis and septic shock, overstimulation of the sympathetic system is associated to the massive release of plasma β and α adrenergic agonists such as norepinephrine and epinephrine [342]. Norepinephrine is known to enhance IL-10 concentration, to decrease the release of pro-inflammatory cytokines and to contribute to sepsis-induced immunoparalysis [24]. If in both innate and adaptive immune cells, adrenergic receptors are widely expressed, although they are not well characterised, particularly for the β subtype [295]. Numerous studies demonstrated the presence of β -AR on almost all lymphocyte subtypes [38, 330, 334]. However, until recently, only β_2 -AR but not β_1 -AR subtypes were found on T cells [335, 342]. As a consequence, β_1 -AR stimulation was not considered as a major actor of inflammation. Indeed, in an early study, a short β_1 -AR stimulation in healthy volunteers challenged with endotoxins did not influence the inflammatory pattern [402]. It is only very recently that β_1 -AR were found to be widely expressed on splenic CD4 T cells. In a study investigating the impact of β -AR blockers on CD4 T cell dysfunction in cirrhotic mice, dobutamine, a β_1 -AR agonist, was able to re-activate inhibited Treg cells, suggesting the presence of β_1 -AR on their surface [33]. In accordance, we too found that *in vitro* CD4 T cell proliferation tended to be reduced by Treg cells pre-treated with dobutamine.

What is the best balance between stimulation and inhibition of β -ARs?

Cardioselective β_1 -AR blockers seem to be the natural target to treat SNS overactivation. However, this strategy may appear counterintuitive. Indeed, the recommended treatment for fluid-unresponsive sepsis-related hypotension remains the prescription of adrenergic agents. To date, norepinephrine, an α_1 -AR and β_1 -AR agonist, is the main inopressor used for the treatment of both cardiac and vascular failures. By stimulating the α_1 -AR widely expressed on VSMCs, norepinephrine agent is consistently confirmed as a very potent and reliable vasopressor. The latter also increases the cardiac work by increasing MAP without any concomitant increase in heart rate [403].

The adrenergic system is also a powerful modulator of cell immunity. Norepinephrine displays numerous adverse effects on immune, metabolic and coagulation functions [21, 24, 404]. Overall, *in-vitro* effects of catecholamines can be summed-up as a roughly inhibition of adaptive immunity, characterised by a lymphopenia (inhibition of T CD4, T CD8 and B cell proliferations), a decreased production of pro-inflammatory cytokines such as TNF- α , an increased production of the anti-inflammatory cytokine IL-10, and an enhanced bacterial growth. [405-409]. However, no clinical study has specifically investigated the detrimental effects of norepinephrine-induced immunosuppression as this therapeutic agent is meanwhile the cornerstone of the hemodynamic stabilisation at the onset of the shock [385, 410].

Several non-adrenergic vasopressors have been explored notably vasopressin or angiotensin II [411, 412]. Despite no overall outcome benefit compared to norepinephrine, low dose of vasopressin reduces norepinephrine requirement and may improve the survival rate of less severe shocked patients [413].

Another option, which we have investigated in the present study, is the use of β -blockers. In this work, we found that some of the adverse immunologic effects of norepinephrine could be attenuated by blocking, at least partially, β_1 -AR. This strategy, in experimental models, prevents organ dysfunctions due to the sympathetic overstimulation.

β -AR blockade reversed sepsis-induced dysautonomia

In numerous conditions, such as acute heart failure, burn injuries, chronic hepatic failure, the peri-operative period and septic shock, β_1 -AR blockade has been consistently associated with protective effects on inflammation and hemodynamics [26, 414, 415]. As others, we too found a global decrease in pro- and anti-inflammatory mediators in animals with high-grade sepsis treated by β_1 -AR blockade [26, 34, 36]. Underlying mechanisms are still not elucidated. In *Listeria monocytogenes* infected mice acute cold or restraint stress (surrogates of sympathetic adrenergic stimulation) inhibited the host T cell response and increased cytokine production [416]. In this model, pharmacological blockade of the β_1 -AR but not β_2 -AR could downregulate this pro-inflammatory state and shorten time to bacterial clearance [35].

In β_1 -AR blocked animals with high-grade sepsis, we found the decrease in Treg cells in the spleen was associated with a concomitant increase in CD4 T cells, as reported previously [417]. In addition, we found that proliferative capacity of splenic CD4 T cells in septic animals was maintained when the β_1 -AR was blocked either pharmacologically or genetically. This suggests that β_1 -AR activation contributes to sepsis-induced immunosuppression, which could be reversed by β_1 -blockade. By comparison, volunteers challenged with lipopolysaccharide, and then being infused with norepinephrine for 5 hours showed a shift to an anti-inflammatory state (decreased TNF- α /IL-10 ratio) which could be counterbalanced by β -AR

blockade (increased TNF- α /IL-10 ratio) [24]. β_1 -AR blockade in our septic animals reduced the inflammatory burden and, taken as a whole, helped to shift from an immunosuppressive state to a state of immune homeostasis.

Beside this newly observed immune rebalancing in experimental sepsis, it is of utmost importance to reiterate that β_1 -AR blockade also has major cardiovascular effects, mostly driven by its negative chronotropic effects. In agreement with most previously published experimental or clinical studies, the septic mice with β -AR blockade did not show an alteration in cardiac power, despite a decrease in HR, suggesting a better cardiac efficiency [25, 26, 32, 36]. In fact, in the present study, it was found a similar cardiac power, a surrogate of external cardiac work, in non-treated and treated groups [418]. Considering that HR, a major determinant of oxygen consumption, was reduced in treated animals, our result suggests a better cardiac efficiency in esmolol treated animals.

While the association between an increased Treg cell percentage during sepsis and an immunosuppressive state is well proven, effects on survival remain inconclusive [419, 420]. The benefits of β_1 -AR blockade on immune and cardiovascular function should improve the survival rate. However, in the present study, and without any treatment (antibiotics, fluid or vasopressors), no improvement was seen in survival. In a similar CLP surgery model, Ackland *et al.* also found that metoprolol infusion, commenced 6 hours after the onset of fecal peritonitis, did not confer any benefit in improving survival whereas pretreatment was effective [34]. A dose effect may be relevant as the β_1 -AR blocker was infused only during the first 24 hours after CLP surgery. In a single-center study prolonged administration of esmolol in septic shock patients, titrated to maintain heart rate between 80-94 bpm, was associated with increased survival [25].

Study limitations

In our model, CLP animals were not resuscitated, preventing us from mentioning a model of septic shock, and no antibiotic was administered in contrast to the usual management of sepsis. Models of resuscitated septic mice have already been described using miniaturised equipment to reproduce the ICU environment, *i.e.* catheterisation of the femoral artery for arterial pressure measurements, catheterisation of the external jugular vein for fluid resuscitation, and drug administration and tracheotomy for mechanical ventilation [421]. In our model, we aimed to limit the consequence of highly aggressive ICU procedures and thus to limit confounding factors. In addition, administration of adrenergic agonists, recommended for sepsis management, would have interfered with our goals. Nevertheless, our results will require further confirmation in resuscitated models.

Our animal model also presents conceptual limits. Indeed, in mice for which the HR is greater than 400 bpm at rest, sepsis induction is most often associated with a marked decrease in metabolism with, as a surrogate, a decrease in HR [37]. In this setting, HR in wild type septic mice was lower compared to previous studies [422]. Thus, the use of β_1 -AR blockade could appear counterintuitive. Moreover, the reduced HR in this model could have been the consequence of the mouse strain. Wild type littermates $\beta_1^{+/+}$ were used as the closest comparators to $\beta_1^{-/-}$ mice. The complex genetic background comes from three different strains, which might explain the unexpected reduction in HR observed in the untreated groups. Moreover, sepsis-induced hypothermia is also known to reduce HR in murine models of sepsis [37].

In this model, the percentages of spleen Th1, Th2, and Th17 lymphocytes were unmodified while several studies found a shift from Th1 to Th2 in sepsis. One explanation could be the timing of the assessment. Indeed, in mice, the immune cell pattern depends on

several factors, for instance, the mouse strain chosen, the method for inducing sepsis, the delay between sepsis induction, and the measurement or the method used to collect immune cells could have impacted the immune cell pattern [423-425]. It seems that the shift from Th1 to Th2 in mice occurs later compared to our time of assessment which was at 18 hours post-surgery. Most studies investigating the Th1/Th2 balance have used a 48-hour sepsis model [426]. Moreover, to date, no study has assessed the immunologic pattern of the $\beta_1^{-/-}$ mouse strain.

Future researches, should firstly assess the tolerance pattern of this treatment in septic patients, secondly should define the best endpoints (HR reduction, inflammation modulation), thirdly, should precise which population is the most likely to benefit from this approach. Finally, experimental studies demonstrated the inflammatory weighting and the reduced sepsis-induced immunosuppression following β_1 -AR blockers administration in sepsis models. This effect has never been investigated in human septic shock and might not require high doses of β -AR blockers which could be extremely interesting in hemodynamically unstable patients [4, 25]. Studies aiming to define more precisely the safety pattern in septic shock patients are ongoing (NCT02120404, NCT02369900).

Conclusion

In summary, in the murine model tested, the β_1 -AR contributes to sepsis-induced immunosuppression through modulation of regulatory T cell inhibitory functions. β_1 -AR blockade, while restoring CD4 T cell homeostasis, also decreased global systemic inflammation and improved cardiac efficiency. Thus, instead of considering β_1 -AR blockade as only a hemodynamic treatment, we suggest an immunoregulatory mechanism by which β_1 -AR blockers may also be beneficial in sepsis.

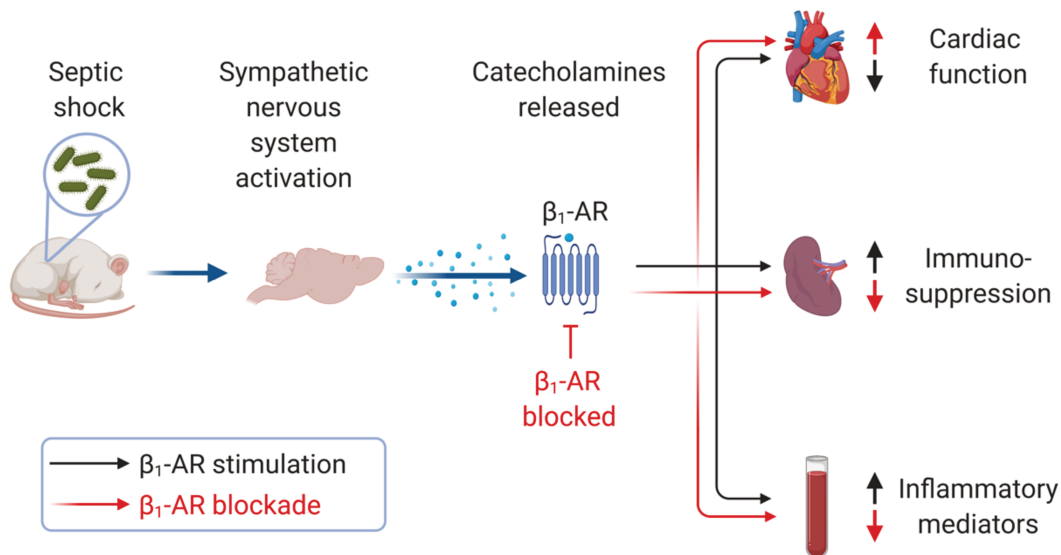


Figure 32: Graphical summary of the involvement of β_1 -AR in sepsis-induced dysautonomia and the beneficial effects of receptor blockade.

Perspectives

Impact of β_2 -AR modulation on inflammatory pattern in experimental sepsis

β_1 -AR but also β_2 -AR are widely expressed on both innate and adaptive immune cells. Specifically, it has been reported that naïve CD4 T cells, Th1, Th17, and Treg cells, but not Th2, express β_2 -AR [30, 334]. β_2 -AR stimulation promotes a shift from a Th1 to Th2 inflammatory pattern and could activate Treg suppressive functions [330, 338]. Reports have also found that β_2 -AR stimulation enhances the macrophage M2 anti-inflammatory phenotype and favours IL-10 production by CD4 T cells [38]. The beneficial effects of β_2 -AR modulation remain unclear and very little is known about β_2 -AR signalling in CD4 T cells during sepsis. In a murine model of endotoxemia, β_2 -AR blockade reduced survival and, in septic pigs, amplified sepsis-induced hepatic injuries [38, 427, 428]. In humans, it has been reported that, during sepsis, β_2 -AR polymorphism is associated with increased mortality [39]. In the present study, we only investigated the impact of β_1 -AR blockade on the alteration of CD4 T cell patterns in the spleen. Therefore, it will be crucial to investigate, first in a murine model of sepsis, the impact of β_2 -AR blockade on hemodynamic and CD4 T cells, and secondly the impact of non-selective β -AR blockade in this setting.

Methodology

To achieve this goal, similarly to the model used in the present study, the hemodynamic and inflammatory effects of β_2 -AR blockade will be studied using a CLP polymicrobial model of sepsis. Two strains of mice will be used: β_2 -AR (C57BL/6J, CD-1, FVB/N) knockout mice ($\beta_2^{-/-}$) and wild type littermates ($\beta_2^{+/+}$), to which a β_2 -AR blocker (CI 118,551) or

non-selective β_1/β_2 -AR blocker (propranolol) will be administered 3 hours after the surgery via a subcutaneous mini-osmotic pump.

Hemodynamic assessment:

- Echocardiography and mean arterial pressure measurements will be performed.
- Cardiac pressure-volume loop will be analyzed using a conductance catheter.

Immune function assessment:

- The immunophenotyping of lymphocyte subpopulations will be investigated by flow cytometry.
- β -AR receptors on lymphocytes will be quantified by quantitative PCR and western blot.
- Cytokines will be measured in β -AR stimulated lymphocyte culture medium,
- Lymphocyte function will be assessed.

Impact of β -AR modulation on vascular leakage in experimental sepsis

During sepsis, dysautonomia is also responsible for the desensitisation of β_1 -AR and β_2 -AR. Their distribution vary according to organ and species [297]. Classically, β_2 -AR are predominant in vessels, and β_1 -AR are rarely found [429]. In all cases, excessive and persistent β -AR stimulation leads to vascular hyporesponsiveness to catecholamines, associated with AR desensitisation [430]. Nevertheless, β_3 -AR, which are widely present on endothelial cells, are not affected by this desensitisation process [159]. The activation of β_3 -AR is consistent during sepsis and seems to be associated with vasorelaxant effects.

Several studies have demonstrated that β_1 -AR blockers can also have effects on β_3 -AR, depending on the molecule used. Thus, nebivolol, used in heart failure, is a β_3 -AR agonist

promoting vasorelaxation, a beneficial effect in heart failure [40]. Conversely, one preliminary study found that esmolol might be a β_3 -AR antagonist in brown fat in rats [41].

To date, β_1 -AR blockers have not been found act directly on vascular function since β_1 -AR are poorly expressed in vessels. Nevertheless, in the clinical study conducted by Morelli *et al.*, septic shock patients in the esmolol group received less norepinephrine than patients in the placebo group [25]. Moreover, administration of esmolol in CLP rats treated with norepinephrine did not change MAP and decreased the hyporesponsiveness to vasopressor treatment and the sepsis-induced endothelial dysfunction [32].

Given the short time between the administration of the treatment and the observation of the effect, these results probably cannot be explained only by an inflammatory pathway. Other mechanisms, acting earlier, are probably involved in the improvement of vascular function during esmolol-treated sepsis. Therefore, it will be interesting to assess if β_1 -AR blockers, also have effects on β_3 -AR expressed on endothelial cells. The β_3 -AR antagonistic effect of esmolol could explain the improvement in vascular responsiveness to catecholamines during experimental sepsis.

Methodology

In vitro study of the antagonistic effects of esmolol on β_3 -AR

Endothelial cell culture

Two cell types will be studied: primary aortic endothelial cells from $\beta_1^{+/+}$ and $\beta_1^{-/-}$ mice and human aortic endothelial cells (HAEC).

- 1/ The β_1 -AR, β_2 -AR and β_3 -AR receptors will be quantified by quantitative PCR and western blot in control and LPS conditions.

- Cells will be fixed and labelled by immunochemistry in order to visualize the β -AR and cells differentiation markers of endothelial cells types by confocal microscopy (Dil-ac-LDL, vWB, CD31, eNOS, iNOS).
- 2/ These cells will be cultured in the presence or not of β_3 -AR agonist (CL316243) and β_3 -AR antagonists (SR59230A) in control and LPS conditions.
- 3/ These cells will be cultured in the presence or not of β_1 -AR antagonists (esmolol)+ β_2 -AR antagonist (ICI118551) in control and LPS conditions.
- 4/ Effect of β_3 -AR agonist (CL316243) and β_3 -AR antagonists (SR59230A) in control and LPS will be tested on cells culture 3/ to rule out the involvement of these two receptors.

For 2/ 3/ and 4/ NO, eNOS, cGMP and cAMP will be measured.

Endothelial permeability assay

Endothelial cells will be challenged with LPS and the effect of β -AR blockers on the endotoxin-mediated barrier disruption will be evaluated in the presence or not of β_1 -AR (esmolol) and β_3 -AR (CL316243) antagonists.

- The barrier function will be evaluated by measuring the leakage of the Evans blue dye through an endothelial monolayer.
- The quantity of NO will be measured.
- Adherents junctions markers (vascular endothelial (VE)-cadherin) and tight junctions markers (occludins and claudins) will be measured.

In vivo study of antagonistic effects of esmolol on β_3 -AR

High-grade sepsis will be induced in mice by CLP. Animals will be randomly divided into five groups and assessed 18 hours after the onset of sepsis: sham, CLP, CLP + esmolol, CLP + CL316243, and CLP + esmolol + CL316243.

Parameter evaluated:

- The arterial pressure will be measured by carotid catheterization.
- Left ventricular function will be analyzed by echocardiography.
- Vasoreactivity to vasoconstrictors and vasodilators of aortic and mesenteric rings will be analysis by wire myography.
- The lung wet-to-dry ratio will be assessed to measure the total amount of lung water.
- The blood vessel permeability will be measured the leak of Evans blue dye in tissues.
- The cytokines profile will be analyzed in plasma.

In summary, the precise contribution of each subtype of β -AR on the dysfunction of different organs and systems occurring in the context of sepsis remains to be studied. A better understanding of β -AR mechanisms and their interactions on the cardiac, vascular, and immune processes could actually be one key feature for new therapeutic strategies for sepsis.

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Annexes

Annexe 1: β -bloquants dans la prise en charge du choc septique

β -bloquants dans la prise en charge du choc septique

β -Blocker in Septic Shock Management

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Résumé Les adrénorécepteurs α et en particulier β sont les principales cibles de l'adrénaline et de la noradrénaline libérées par le système sympathique activé. Durant le choc septique, la dysautonomie est une stimulation prolongée à un haut niveau d'intensité du système nerveux sympathique à l'origine d'une altération de la contractilité, de la vasoréactivité et d'une immunodépression. Ainsi, l'administration précoce d'un traitement β -bloquant lors du choc septique pourrait pondérer les effets délétères de cette surstimulation sympathique. Néanmoins, si les preuves expérimentales sont en faveur de cette approche, l'accumulation des preuves cliniques reste encore insuffisante.

Mots clés Choc septique · Sepsis · β -bloquant · Hémodynamique · Récepteur adrénergique

Abstract Adrenoreceptors α and particularly β are the main targets of epinephrine and norepinephrine released by the sympathetic system. During septic shock, dysautonomia could be defined as a prolonged over-activation of the sympathetic system. Dysautonomia is among other abnormalities, responsible for cardiac and vascular hypo-contraction and immunosuppression. Thus, during septic shock, early administration of β -blockers might limit the deleterious

effects of sympathetic over-stimulation. However, if experimental data are convincing, formal clinical evidences are still lacking.

Keywords Sepsis · Septic shock · β -blocker · Hemodynamic · Adrenergic receptor

Introduction

L'histoire naturelle d'une infection grave est un continuum qui débute par le sepsis simple et qui évolue en choc septique. Schématiquement, pour la forme la plus sévère de l'infection, l'invasion de l'organisme par un agent pathogène est associée à une réponse inflammatoire exagérée à l'origine d'une vasodilatation et d'une coagulopathie prothrombotique. L'ensemble de ces phénomènes est responsable des dysfonctions d'organes de l'état de choc septique. Les réponses de l'organisme face à cette agression visent à assurer, en premier lieu, le transport en oxygène. Les structures impliquées pour le maintien de ce transport sont nombreuses, mais incluent principalement les systèmes : nerveux sympathique et parasympathique, corticosurrénalien, posthypophysaire antidiurétique et rénine-angiotensine-aldostérone [1].

En l'absence de traitement, l'évolution vers le choc septique se caractérise par l'importance de la réponse pro-inflammatoire initiale qui va sidérer, à différents degrés, les systèmes neuroendocriniens et endocriniens périphériques précités et aggraver l'hémodynamique [2].

L'activation du système nerveux sympathique, en libérant de la noradrénaline et de l'adrénaline, est une réponse essentielle à l'agression à la phase initiale de tout processus septique. Ces effets sont une augmentation de l'inotropisme, une tachycardie et une vasoconstriction, qui contrecarrent les effets toxiques et/ou inflammatoires initiaux du processus infectieux. Néanmoins, la persistance de cette activation dans le temps à un haut niveau d'intensité peut aussi être délétère et entraîner un syndrome de dysautonomie sympathique [3].

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Durant le choc septique, cette dysautonomie survient de façon précoce, et son intensité, reflet de la suractivation sympathique adrénérergique, est associée à la gravité clinique et à la mortalité [4,5]. Pour le clinicien, ce paradigme est contre-intuitif, car il suggère que l'excès de catécholamines est délétère alors que la pierre angulaire du traitement du choc septique tel qu'il est recommandé, outre l'expansion volémique et le contrôle infectieux, repose sur l'administration exogène de catécholamines (noradrénaline, adrénaline, dobutamine).

Ainsi, l'hypothèse de l'administration d'un traitement visant à diminuer le stress adrénérergique périphérique pourrait se justifier par la diminution précoce des effets délétères de la suractivation sympathique et éventuellement améliorer le pronostic des patients en choc septique. Ce concept de rééquilibration adrénérergique ou de « décatécholaminisation », en particulier évalué avec des β -bloquants cardiosélectifs, repose sur des bases scientifiques de plus en plus robustes [6,7].

Dans ce travail, nous ferons la synthèse des travaux scientifiques, expérimentaux et cliniques ayant évalué l'efficacité d'un traitement par β -bloquants pour améliorer la prise en charge du choc septique. Une recherche suivant les recommandations PRISMA a été réalisée via Pubmed en utilisant, à partir du constructeur de requêtes bibliographiques médicales, les termes Mesh suivants « choc septique » ou « sepsie », traduction française de « sepsis » et « propanolamines » sur la

période de 1990 à 2018 incluse (Fig. 1). Toutes les langues ont été prises en compte. La sélection des travaux s'est concentrée sur les termes suivants lors de la lecture de l'abstract : hémodynamique, survie et inflammation.

Présentation du système nerveux sympathique et concept dysautonomique

Système nerveux sympathique

Le système sympathique, situé au niveau du *locus caeruleus* dans le tronc cérébral, comprend anatomiquement quatre niveaux (cervical, thoracique, lombaire et pelvien) avec deux neurones en série à chaque niveau, connectés par un relais ganglionnaire paravertébral. De façon schématique, l'activation du système nerveux sympathique va, par l'intermédiaire du deuxième neurone postganglionnaire sympathique, libérer de la noradrénaline sur les organes cibles. La libération de l'adrénaline dans la circulation sanguine via la médullosurrénale est secondaire à la stimulation de la glande par l'acétylcholine libérée des fibres préganglionnaires splanchniques [8].

La réponse à ces deux hormones est médiée par des protéines G couplées à des adrénorécepteurs. Ces derniers

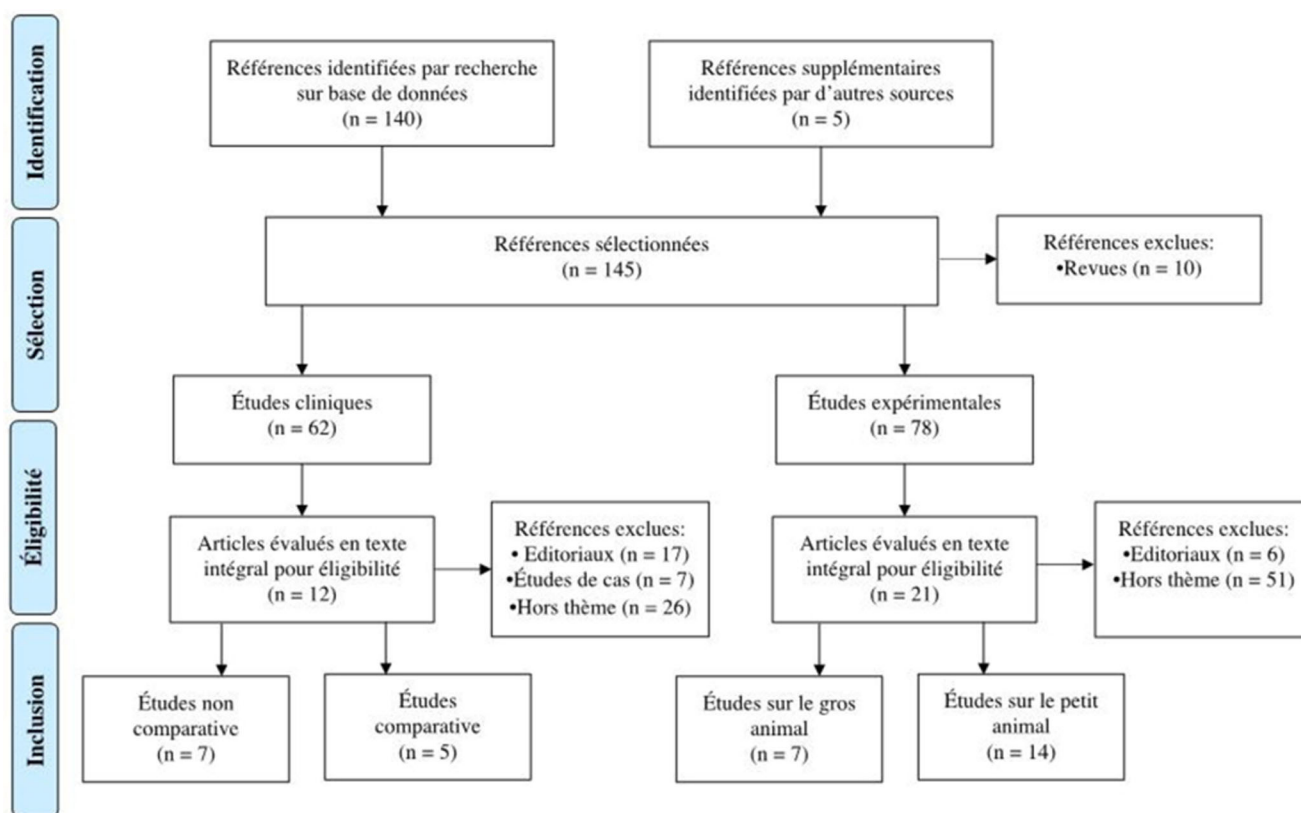


Fig. 1 Diagramme de flux des études recherchées.

comprennent deux classes : α et β . La répartition, les voies de signalisation et les effets de l'activation et de l'inhibition de ces adrénorécepteurs sur l'hémodynamique et l'inflammation sont détaillés dans le tableau 1.

Les effets cliniques hémodynamiques de cette activation les plus connus se résument à une tachycardie, une augmen-

tation de l'inotropisme cardiaque ainsi qu'une vasoconstriction permettant de maintenir la stabilité cardiovasculaire. Les effets sont en réalité beaucoup plus larges et incluent aussi une bronchodilatation, une relaxation des muscles lisses intestinaux et des sphincters, une augmentation de la néoglucogénèse, de la glycolyse, de la lipolyse et une régulation

Tableau 1 Classification, localisation, signalisation, agonistes, antagonistes, effets hémodynamiques et inflammatoires des principaux adrénorécepteurs (d'après Scanzano et Cosentino, de Montmollin et al. et Tank et Lee Wong [9,10,11])

Adrénorécepteurs	Localisation	Signalisation	Agoniste	Antagonistes	Effets hémodynamiques	Effets inflammatoires supposés
$\alpha 1$	Cœur Vaisseaux Poumon Tube digestif	Activation protéine $G\alpha_q$ → \nearrow PLC → \nearrow Ca_2^+ \nearrow PKC → \nearrow eicosanoïdes, \nearrow canaux Ca_2^+ , \nearrow voie des MAPK, régulation des canaux K^+	Phényléphrine Méthoxamine	Prazosine Doxazosine Térazosine	Vasoconstriction Contraction des muscles lisses	Pro-inflammatoire
$\alpha 2$	Vaisseaux Tube digestif Système nerveux central	Activation protéine $G\alpha_i$ et $G\alpha_o$ → \searrow AC → \searrow cAMP, \searrow PKA, \nearrow activité des canaux K^+	Clonidine	Yohimbine Idazoxan Atipamézole	Vasoconstriction	Pro-inflammatoire
$\beta 1$	Cœur Reins Cellules adipeuses	Activation protéine $G\alpha_s$ → \nearrow AC → \nearrow cAMP, \nearrow PKA, \nearrow activité des canaux Ca_2^+	Isoprotérénol Dobutamine	Propranolol Métoprolol Aténolol Esmolol Landiolol	Effet inotropique et chronotropique positif	Pro-inflammatoire
$\beta 2$	Cœur Vaisseaux Poumon Tube digestif Foie Systèmes urinaire et reproducteur Système nerveux central	Activation protéine $G\alpha_s$ et $G\alpha_i$ → \nearrow AC → \nearrow cAMP, \nearrow PKA, \nearrow activité des canaux Ca_2^+	Isoprotérénol Terbutamine Albuterol Salmétérol	Propranolol Butaxamine	Vasodilatation Relaxation des muscles lisses	Anti-inflammatoire
$\beta 3$	Cellules adipeuses Cœur	Activation protéine $G\alpha_s$ et $G\alpha_i$ → \nearrow AC → \nearrow cAMP, \nearrow PKA, \nearrow activité des canaux Ca_2^+	Isoprotérénol Mirabegron Amibegron Solabegron	Propranolol	Effet inotropique négatif	Anti-inflammatoire

AC : adénylate cyclase ; cAMP : adénosine monophosphate cyclique ; MAPK : *mitogen-activated protein kinase* ; PLC : phospholipase C ; PKA : *protein kinase A* ; PKC : *protein kinase C* ; → : induit ; \nearrow : augmentation ; \searrow : diminution.

anti-inflammatoire [11]. La représentation schématisée de ce système de réponse suraiguë à l'agression ne doit pas faire perdre de vue qu'il est relié et dépendant d'autres systèmes de réponse à l'agression, comme par exemple le système parasympathique (beaucoup moins bien connu) ou encore le système hypothalamo-hypophyso-corticosurrénalien.

Dysautonomie sympathique (Fig. 2)

La dysautonomie est un terme générique qui englobe l'ensemble des effets considérés comme délétères liés à la persistance dans le temps d'un haut niveau d'activation du système nerveux sympathique [6]. Les marqueurs biolo-

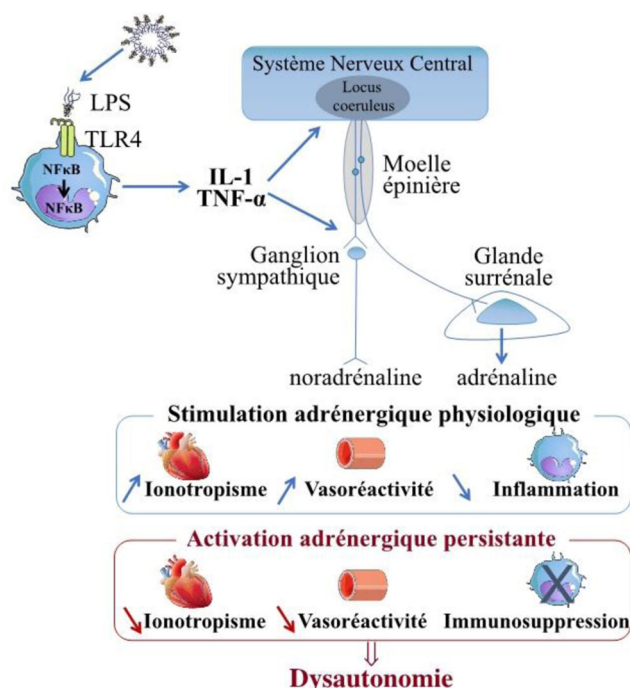


Fig. 2 Activation du système nerveux sympathique au cours du choc septique. Lors d'une infection, les lipopolysaccharides (LPS), un composant majeur de la membrane bactérienne, activent les *toll-like receptors 4* (TLR4) présents sur les cellules de l'immunité innée. L'activation des récepteurs TLR4 induit la translocation de NFκB dans le noyau, permettant la transcription de cytokines pro-inflammatoires telles que IL-1 ou TNF-α. Ces cytokines activent le système nerveux sympathique au niveau central, dans le *locus coeruleus* et au niveau périphérique via les ganglions sympathiques, induisant une libération massive de catécholamines. Ces catécholamines activent les récepteurs adrénergiques conduisant, dans des conditions physiologiques, à une augmentation de l'ionotropisme, de la vasoréactivité et une diminution de l'inflammation. Lorsque cette activation persiste dans le temps, elle devient pathologique et implique une diminution de l'ionotropisme, de la vasoréactivité et une immunosuppression. Ce phénomène, se produisant lors d'un choc septique, est appelé dysautonomie.

giques de cette suractivation sont les concentrations d'adrénaline et de noradrénaline de 5 à 100 fois plus élevées par rapport à un système non suractivé [4]. Ces hautes concentrations sont associées à des altérations des fonctions cardiaque et vasculaire, de la coagulation et de l'inflammation.

Participation dysautonomique à la cardiomyopathie septique

La noradrénaline mais surtout l'adrénaline vont, par la stimulation β_1 -adrénergique, augmenter la consommation en adénosine triphosphate (ATP) de la cellule cardiomyocytaire afin d'augmenter l'inotropisme et le chronotropisme cardiaque. Sur le plan fonctionnel, l'excès de stimulation dans le temps va altérer la réponse β_1 -adrénergique d'une part, par une internalisation des β_1 -adrénorécepteurs et, d'autre part, par une altération de la fonction énergétique mitochondriale diminuant ainsi globalement l'effet des catécholamines [12–14]. Sur le plan morphologique, l'excès de stimulation adrénergique est associé à des lésions histologiques (apoptose cardiomyocytaire, œdème interstitiel), à des nécroses de bandes de contraction et à une infiltration macrophagique [15,16]. Néanmoins, cette atteinte est bien plus fonctionnelle que morphologique, étant donné la constante réversibilité de la cardiomyopathie septique en rapport avec l'élimination des facteurs dépresseurs myocardiques comme l'interleukine-1 (IL-1), le *tumor necrosis factor-α* (TNF-α) et le monoxyde d'azote (NO) [17].

Participation dysautonomique à la vasoplégie

L'altération de la vasoréactivité durant l'état de choc septique comprend deux versants :

- une diminution de la réponse contractile vasculaire à l'augmentation de la stimulation catécholaminergique vasopressive ;
- une altération de la relaxation vasculaire à l'augmentation des doses d'acétylcholine.

Les mécanismes sont aujourd'hui assez bien caractérisés avec la place centrale du monoxyde d'azote [18]. Similairement au myocarde, l'hypovasaréactivité contractile est en partie médiée par des mécanismes de désensibilisation des α -adrénorécepteurs situés sur les cellules musculaires lisses vasculaires [19].

Participation dysautonomique à l'immunoparalysie (Fig. 3)

La dysautonomie est aussi responsable d'une altération de la réponse immunitaire. En effet, les cellules de l'immunité présentent à leur surface des adrénorécepteurs principalement de type β_2 [8]. Schématiquement, la stimulation sympathique

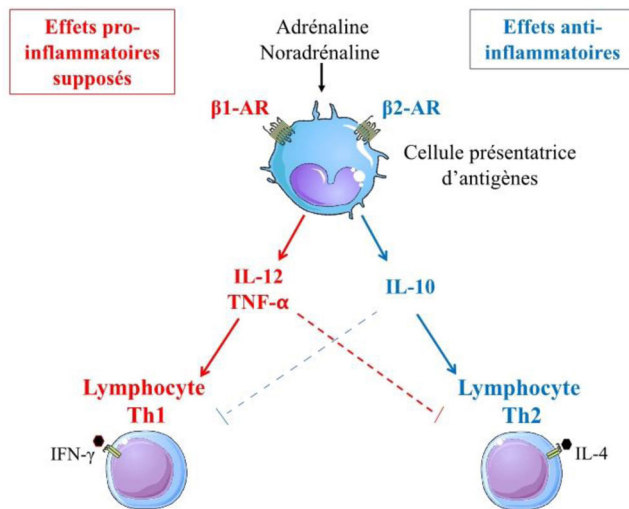


Fig. 3 Effet des catécholamines sur la balance lymphocytaire Th1/Th2. Les catécholamines activent les récepteurs adrénergiques présents sur les cellules présentatrices d'antigènes. La stimulation des récepteurs β_2 -adrénergiques entraîne la libération de cytokines anti-inflammatoires induisant la différenciation en lymphocytes Th2 et réprimant la différenciation de lymphocytes Th1. Inversement, il semblerait que la stimulation des récepteurs β_1 -adrénergiques entraîne la libération de cytokines pro-inflammatoires induisant la différenciation de lymphocytes Th1 et réprimant la différenciation de lymphocytes Th2, cela restant à mettre en évidence plus précisément [21].

adrénergique qui initialement durant le processus septique a un rôle bénéfique de régulation anti-inflammatoire entraîne, lorsqu'elle se poursuit dans le temps à un haut niveau d'activité, une immunoparalysie pouvant favoriser la survenue d'une deuxième infection [20]. Les β_2 -adrénorécepteurs situés sur les cellules présentatrices d'antigènes (cellules dendritiques, monocytes...) et les lymphocytes T *helper 1* (Th1) vont, lorsqu'ils sont activés, entraîner un déplacement de la réponse inflammatoire Th1 vers une réponse préférentielle Th2. Les monocytes et les lymphocytes Th2 ainsi stimulés vont libérer des cytokines telles que l'IL-4, l'IL-6, l'IL-10 et l'IL-13 qui inhibent la réponse Th1 ; prises ensemble, la réponse immunitaire semble s'orienter vers une réponse humorale de type anti-inflammatoire. Sa persistance dans le temps est une des explications de l'immunoparalysie secondaire du choc septique [21].

Participation dysautonomique à la mortalité

L'impact de la dysautonomie sympathique sur la mortalité des patients en choc septique a pu être évalué à partir de l'étude de la variabilité cardiovasculaire. De façon simplifiée, la variabilité cardiovasculaire explore dans les domaines temporels et fréquentiels (ou spectraux) la fréquence

cardiaque (FC), les pressions artérielles systoliques, diastoliques et moyennes. Ces variables sont intimement liées au système nerveux sympathique. Durant le choc septique, la perte de la variabilité cardiovasculaire et un haut niveau de catécholamines endogènes ou exogènes sont constamment associés à un pronostic défavorable, tandis que le maintien de la variabilité est associé à un sevrage plus précoce des catécholamines [4,5,22,23].

Ainsi, le système nerveux sympathique est un système central de la réponse précoce à l'agression septique. Néanmoins, s'il est indispensable à la phase initiale, de très nombreux experts considèrent qu'au regard de la littérature sa persistance dans le temps est délétère et, donc, que sa modulation pourrait être bénéfique [3,24]. Regroupée sous différents termes comme la « décatécholaminisation », la modulation du système nerveux sympathique, qu'elle soit centrale ou périphérique, est une voie thérapeutique envisageable, mais qui, comme nous allons le voir par la suite, reste encore au stade de la preuve de concept [7].

Quelles molécules seraient utilisables pour moduler le système nerveux sympathique dans le choc septique ?

La cible thérapeutique sur le système nerveux sympathique semble naturellement être les récepteurs β -adrénergiques. Les β -bloquants sont donc, a priori, les molécules indiquées dans ce cadre. Néanmoins, les β -bloquants non cardiosélectifs, c'est-à-dire avec une activité à la fois β_1 et β_2 bloquante, sont probablement moins adaptés que les β_1 -bloquants cardiosélectifs. En effet, l'inhibition β_2 est contre-intuitive étant donné que l'activation β_2 -adrénergique semble centrale dans la balance inflammatoire [8]. Les travaux expérimentaux autant que cliniques montrent que son inhibition ou l'existence d'un certain polymorphisme du gène codant pour cet adrénorécepteur semble associée à une moins bonne évolution et à un moins bon pronostic lors du choc septique [25,26]. À l'inverse, sur le plan expérimental, sa stimulation semble présenter des effets plutôt bénéfiques en terme de réduction des défaillances d'organes [27].

L'esmolol (*methyl 3-[4-(2-hydroxy-3-[isopropylamino]propoxy) phenyl] propionate*) est un β_1 -bloquant cardiosélectif dont la demi-vie de distribution est de deux minutes et la demi-vie d'élimination de neuf minutes. Cette molécule est hydrolysée dans le sang par des estérases et métabolisée dans le foie en formant ses principaux métabolites, que sont le méthanol et l'acide propionique. Si la demi-vie d'élimination de ces métabolites est beaucoup plus longue, seul l'esmolol a des effets β_1 -bloquants. Ainsi, aucun effet mesurable n'est retrouvé à 30 minutes de l'arrêt du traitement par esmolol [28]. Les principaux avantages de ce type de molécules sont : son très court délai d'action, sa courte demi-vie et son

administration par voie intraveineuse qui rendent son utilisation éventuellement possible lors de la prise en charge d'un état de choc septique. D'autres molécules de ce type existent comme le landiolol, possédant une forte cardiosélectivité et dont la demi-vie d'élimination est encore plus courte (trois à quatre minutes).

Cette approche périphérique de la modulation du système nerveux sympathique pourrait être complétée aussi par une approche centrale. Théoriquement, il se pourrait que la modulation centrale du système nerveux sympathique permette de pondérer l'hyperactivation adrénergique. Cette pondération serait associée à une restauration de la réponse contractile, cardiaque et vasculaire aux catécholamines via une réexpression des adrénorécepteurs sur les cellules musculaires cardiaques et vasculaires [29]. Ainsi, la stimulation des α_2 -adrénorécepteurs présents au sein du *locus caeruleus* entraîne une diminution centrale de la réponse adrénergique sympathique [8]. La dexmédétomidine ou la clonidine sont les deux molécules α_2 -agonistes les plus connues et les plus testées pour cette approche. Sans qu'il existe encore de preuves cliniques, de nombreux travaux ont démontré que durant le choc septique expérimental, la modulation centrale du système nerveux sympathique était associée à une amélioration de la vasoréactivité, de la microcirculation et une diminution de l'inflammation tissulaire et plasmatique [30–32]. L'association d'une approche périphérique par esmolol et centrale par dexmédétomidine a aussi été évaluée expérimentalement, démontrant que l'approche combinée est envisageable [33].

Malgré ces concepts intéressants, il faut tout de même rappeler qu'il est contre-intuitif lors du choc septique de réduire les principaux facteurs du transport artériel en oxygène, que sont la FC et l'inotropisme. Il convient donc, dans un premier temps, de synthétiser les données expérimentales et cliniques, évaluant la tolérance et les effets cliniques de l'administration d'un traitement β_1 -bloquant chez le patient en choc septique, en se concentrant plus spécifiquement sur la diminution du débit cardiaque, la vasoréactivité, la mortalité et l'inflammation.

Preuves expérimentales de l'intérêt des β -bloquants dans le choc septique

Le diagramme de la recherche systématique des articles expérimentaux est détaillé sur la figure 1.

Les études expérimentales chez le petit et le gros animal ont évalué deux aspects de l'effet des β -bloquants dans des modèles de choc septique : les effets sur la fonction cardiovasculaire et les effets inflammatoires. Les modèles utilisés sont principalement polymicrobiens par ligature et ponction du cæcum (CLP) ou endotoxinique par injection de lipopolysaccharides (LPS) et conduisent à des sepsis de sévérité

variable, allant du sepsis peu grave au choc septique sévère (Tableau 2). La majorité des travaux ont administré un β_1 -bloquant, avec pour objectif une diminution de 20 % de la FC.

Globalement, l'objectif de diminution de la FC de 20 % était toujours atteint. Quels que soient le modèle animal et la modalité d'administration du β_1 -bloquant, un effet cardioprotecteur était retrouvé. Par exemple, Ackland et al. ont montré que l'administration de métoprolol ou d'aténolol, des β_1 -bloquants cardiosélectifs, chez des rats en choc septique moyennement sévère, avait pour conséquence une augmentation du volume d'éjection systolique combiné au maintien du débit cardiaque, tout en réduisant la FC. En revanche, l'administration de β_1 -bloquant en intracérébral, contrairement à l'administration en intrapéritonéal dans cette étude, n'améliore pas la survie des rats en choc septique, suggérant ainsi un mécanisme β_1 -adrénergique périphérique plutôt que central [37].

L'ensemble des études réalisées chez les rongeurs montrent soit un maintien, soit une augmentation, du débit cardiaque malgré la diminution de la FC. Une explication possible serait, chez ces animaux chronodépendants, un allongement de la diastole secondaire à la réduction de la FC. Du fait de cet allongement, le remplissage ventriculaire gauche est augmenté permettant un maintien du débit cardiaque. Le travail externe myocardique est diminué, ce qui permet une meilleure efficacité myocardique globale pour un moindre coût énergétique [34].

Ce résultat s'associe, de façon étonnante, à une amélioration de la fonction vasculaire. Théoriquement, les β_1 -adrénorécepteurs n'étant pas présents sur les vaisseaux, les β_1 -bloquants n'ont aucun effet direct sur la vasoréactivité. Cependant, une amélioration de la vasoréactivité est retrouvée lors du choc septique, aussi bien dans les modèles animaux que chez l'homme [43,54]. Dans un modèle de gros animal tel que le cochon, deux études ont démontré que l'administration d'esmolol permettait *in vivo* une augmentation des résistances vasculaires systémiques [48,50]. De même, dans un modèle de choc septique sévère chez le rat, Wei et al. ont retrouvé que l'administration d'esmolol permet, *ex vivo*, de restaurer la contractilité vasculaire à l'augmentation des doses de phényléphrine. Dans ce même travail, des concentrations faibles d'esmolol, sans effet hémodynamique direct, conduisaient à une amélioration de la vasoréactivité [45].

Ces améliorations hémodynamiques sont associées à une diminution de la lactatémie qui pourrait être le reflet d'une demande en oxygène moins importante par les tissus et donc un meilleur métabolisme oxydatif [33]. Une amélioration du pronostic chez les animaux en choc septique recevant un β_1 -bloquant est aussi retrouvée dans toutes les études de survie réalisées chez le petit animal.

Tableau 2 Résumé des études expérimentales

Auteurs	Modèle	Sévérité	β-bloquant	Moment de l'évaluation	Survie	Évaluation : fonction cardiaque	Évaluation : vasoréactivité	Lactate	Évaluation : fonction immunitaire		
									Systémique	Tissulaire	
Étude sur le petit animal											
Suzuki et al. 2005 [34]	Rat Mâle CLP	+++	E. (10 mg/kg/h ou 20 mg/kg/h) pour réduire la FC Injection : lors de la CLP	24 h après induction	NA	In vivo : ⬇ PAM pour E. 10 mg/kg/h = PAM pour E. 20 mg/kg/h Ex vivo ^a : ⬆ DC ⬆ VES ⬆ dP/dt _{max} , dP/dt _{min}	NA	=	⬇ Marqueurs : TNF-α ⬇, nombre de globules blanc ne varie pas	NA	
Schmitz et al. 2007 [35]	Souris Mâle CLP	++	Propranolol (0,5 mg/k en SC tous les 2 h) Injection : 20 min avant CLP	48 h après induction	⬇	NA	NA	NA	NA	⬆ Marqueurs : apoptose ⬆, prolifération ⬇, IL-6 ⬆ et INF-γ ⬇ dans les splénocytes	
Hagiwara et al. 2009 [36]	Rat Mâle LPS	+	Landiolol (0,1 mg/kg/min) pour réduire de 25 % la FC Injection : avant les LPS	12 h après induction	NA	In vivo : = PAM Ex vivo ^a : ⬆ dP/dt _{max} , dP/dt _{min}	NA	NA	⬇ Marqueurs : pic de TNF-α, IL-6 et HMGB-1 ⬇	⬇ Marqueurs : infiltration des cellules inflammatoires ⬇, œdème ⬇, liaison NF-κB à ADN ⬇ dans les poumons	
Ackland et al. 2010 [37]	Rat Mâle CLP ou LPS	+	Métoprolol (100 mg/kg en IP), Aténolol (6 mg/kg en IP) pour réduire de 20 % la FC Injection : 2 jours avant	24 à 48 h après induction	⬆	In vivo : = DC = VES ⬆ PAM ⬆ dP/dt _{max} , dP/dt _{min}	NA	NA	⬇ Marqueur : IL-6 ⬇	⬇ Marqueurs : cytokines hépatiques ⬇ (TNF-α, IL-1β, IL-6, INF-γ), inflammation cardiaque ⬇ (MCP-1, IL-18)	
Mori et al. 2011 [38]	Rat Mâle CLP	++	E. (15 mg/kg/h) pour réduire de 20 % la FC Injection : 1 h après CLP	18 h après induction	⬆	In vivo : = PAM	NA	=	NA	⬇ Marqueurs : TNF-γ intrapéritonéal ⬇, sévérité des lésions de la muqueuse intestinale ⬇	
Wilson et al. 2013 [39]	Rat Mâle CLP	+	Propranolol (10 mg/kg en IP) pour réduire la FC Injection : 30 min avant CLP puis tous les 8 h	24 h après induction	⬆	In vivo : = PAM	NA	=	⬇ Marqueur : TNF-α ⬇	⬇ Marqueurs : expression HO-1 dans les poumons ⬆	
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Tableau 2 (suite)

Auteurs	Modèle	Sévérité	β-bloquant	Moment de l'évaluation	Survie	Évaluation : fonction cardiaque	Évaluation : vasoréactivité	Lactate	Évaluation : fonction immunitaire	
									Systémique	Tissulaire
Ibrahim-Zada et al. 2014 [40]	Souris Mâle LPS	+	E. (6,67 µg/kg/min) Injection : 24 h avant LPS	48 h après induction	↗	In vivo : = ITV aortique	NA	NA	NA	↘ Marqueurs : expression du gène <i>CAMP</i> ↗
Seki et al. 2014 [41]	Rat Mâle LPS	+	Landiolol (100 µg/kg/ min) Injection : 15 min avant LPS	3 h après induction	NA	In vivo : = PAM	NA	↘	= Marqueurs : TNF-α, IL-6 et ET-1 ne varient pas	↘ Marqueurs : enzyme de conversion de l'endothéline ECE-1 ↘
Abdel Kawy 2015 [42]	Rat Mâle CLP	+++	Carvédilol (2 mg/kg) en IP Injection : 30 min avant CLP	18 h après induction	↗	In vivo : = PAM	NA	NA	↘ Marqueurs : TNF-α et IL-6 ↘	↘ Marqueurs : score d'inflammation des reins ↘, activité antioxydante rénale (NADP-H, GSH) ↗
Kimmoun et al. 2015 [43]	Rat Mâle CLP	+++	E. (300 µg/kg/min) pour réduire la FC Injection : 4 h après CLP	18 h après induction	↗	In vivo : = DC ↗ VES = PAM	↗ vasoréactivité	↘	NA	↘ Marqueurs : expression de p-AKT et p-eNOS ↗ et NF-κB et iNOS ↘
Wang et al. 2016 [44]	Souris Mâle LPS	+	E. (6,7 µg/kg/min) Injection : immédiatement après LPS	6 h après induction	NA	In vivo : = DC ↗ PAM ↗ dP/dtmax	NA	NA	NA	↘ Marqueurs : apoptose des myocytes (Caspase3, Bax, JNK, p38) ↘
Wei et al. 2016 [45]	Rat Mâle CLP	+++	E. (1 ; 5 ou 18 mg/kg/h) Injection : 4 h après CLP	18 h après induction	NA	In vivo : ↗ DC ↗ VES ↗ PAM	↗ vasoréactivité	↘	↘ Marqueurs : IL-6 et IL-10 ↘	↘ Marqueurs : expression de p-AKT et p-eNOS ↗ et NF-κB et iNOS ↘
Lu et al. 2017 [46]	Rat Mâle CLP	+++	E. (1,75 ; 2,6 ou 3,5 mg/ kg/h) Injection : 1 h après CLP	6 et 24 h après induction	NA	NA	NA	NA	↘ Marqueurs : IL-6, TN-α ↘, IL-10 ↗	↘ Marqueurs : infiltration des cellules inflammatoires dans le foie et les reins ↘, expression de Bcl2 ↗
Mathieu et al. 2018 [47]	Rat Mâle + femelle CLP	+++	Landiolol Injection : 1 h après CLP	18 h après induction	NA	In vivo : Mâle : = DC, ↗ VES = PAM Femelle : ↘ DC, = VES = PAM	NA	=	= Marqueurs : TNF-, IL-6, IL-1β ne varient pas	↘ Marqueurs : expression de p-AKT et p-eNOS ↗

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Tableau 2 (suite)										
Auteurs	Modèle	Sévérité	β-bloquant	Moment de l'évaluation	Survie	Évaluation : fonction cardiaque	Évaluation : vasoréactivité	Lactate	Évaluation : fonction immunitaire	
									Systémique	Tissulaire
Études sur le gros animal										
Aboab et al. 2011 [48]	Cochon Mâle LPS	+	E. pour réduire de 20 % la FC Injection : 30 min après LPS	300 min après induction	NA	In vivo : = DC ↗ VES indexé = PAM	↗ résistance vasculaire systémique	NA	NA	NA
Calzavacca et al. 2014 [49]	Mouton Mâle E. Coli	+	Bolus d'aténolol puis 0,125 mg/kg/h pour réduire de 20 % la FC Injection : 1 jour avant LPS	24 h après induction	NA	In vivo : ↘ DC ↗ VES = PAM	NA	=	↘ Marqueurs : TNF-α et IL-6 ne varient pas, IL-10 ↘	NA
Jacquet-Lagrece et al. 2015 [50]	Cochon Mâle P. aeruginosa	+	E. (7,5 µg/kg/min) pour réduire la FC entre 80 et 90/min Injection : après la période de stabilisation	300 min après induction	NA	In vivo : ↘ DC = VES indexé = PAM	↗ résistance vasculaire systémique	=	NA	NA
Hernandez et al. 2016 [33]	Mouton Mâle LPS	+	E. (15 mg/h) pour réduire de 20 à 30% la FC Injection : après la période de stabilisation	Toutes les heures pendant 3 h	NA	In vivo : = DC ↗ PAM	↘ besoin de norépinéphrine	↘	NA	NA
Hosokawa et al. 2017 [51]	Mouton Femelle CLP	++	E. pour réduire la FC entre 80 et 100/min Injection : 2 h après CLP	Pendant 24 h après induction	=	In vivo : = DC ↗ VES = PAM	NA	=	NA	NA
Du et al. 2017 [52]	Chien Mâle E. Coli	+	E. (25 mg/h) pour réduire de 10 à 15 % la FC Injection : après la période de stabilisation	Toutes les heures pendant 2 h	↗	In vivo : = DC ↗ VES = PAM	NA	↘	NA	NA
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Tableau 3 Résumé des études cliniques non comparatives

Auteurs	Type d'étude	Méthode	Moment de l'évaluation	Nombre de patients	FC (/min) moyenne \pm ET ou médiane [interquartile]	VOLUME d'éjection systolique et débit cardiaque	Résistances vasculaires	Mortalité	Perfusion tissulaire	Résultats additionnels
Gore et Wolfe 2006 [55]	Prospective, interventionnelle, monocentrique, avant-après	Sepsis sans choc E. pour réduire la FC de 20 %	Avant et après 3 heures d'administration d'E.	6	114 \pm 15 vs. 91 \pm 12	VES : \nearrow DC : \searrow	=	NA	=	Patients peu sévères Aucun effet anabolique sur le métabolisme énergétique
Schmittinger et al. 2008 [56]	Rétrospective, monocentrique	Choc septique Métoprolol per os pour réduire la FC < 95/min \pm Milrinone si IC < 2,5 l/min/m ²	Avant l'administration de métoprolol puis à 6, 12, 24, 72 et 96 heures	40	101 \pm 18 vs. 84 \pm 13	VES : \nearrow DC : =	=	33 % à 28 jours	=	Diminution du débit cardiaque chez 7 patients avec administration de milrinone
Balik et al. 2012 [57]	Prospective, monocentrique	Bolus d'E. puis perfusion continue	Avant l'administration d'E. puis à 2,6, 12, 24 et 30 heures	10	142 \pm 11 vs. 112 \pm 9	VES : = DC : =	=	10 % à 28 jours	=	Patients très peu sévères
Chen et al. 2013 [58]	Prospective, monocentrique, avant-après	Sepsis sévère E. en perfusion continue pour réduire la FC de 20 %	Avant l'administration d'E. puis à 3 heures	16	114 \pm 15 vs. 91 \pm 13	VES : = DC : \searrow	=	NA	=	
Morelli et al. 2013 [54]	Prospective, interventionnelle, monocentrique, avant-après	Choc septique E. en perfusion continue pour réduire la FC entre 80 et 94/min	À 24 heures	25	117 [112–126] vs. 86 [80–89]	VES : = DC : \searrow	NA	NA	=	Amélioration de l'index de débit microvasculaire en OPS Diminution sous E. de la posologie de noradrénaline
Morelli et al. 2016 [59]	Prospective, monocentrique, avant-après	Choc septique E. en perfusion continue pour réduire la FC entre 80 et 94/min	Avant l'administration d'E. puis à 4 heures	45	115 \pm 11 vs. 88 \pm 9	VES : = DC : \searrow	\searrow	51 % à 28 jours	=	Amélioration du couplage ventriculoartériel

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Tableau 3 (suite)										
Auteurs	Type d'étude	Méthode	Moment de l'évaluation	Nombre de patients	FC (/min) moyenne \pm ET ou médiane [interquartile]	Volume d'éjection systolique et débit cardiaque	Résistances vasculaires	Mortalité	Perfusion tissulaire	Résultats additionnels
Du et al. 2016 [60]	Prospective, monocentrique, avant-après	Choc septique stabilisé depuis 3 heures avec FC > 100/min E. en perfusion continue pour réduire la FC de 10–15 % par rapport à la valeur basale	Avant l'administration d'E. et dans les 2 heures qui suivent la réduction de la FC sous E.	63	107,8 \pm 8,7 vs. 86,2 \pm 10,2	VES : ➤ DC : ➤	NA	6,3 % à 28 jours	=	Mortalité très faible Objectif de pression artérielle supérieur à ce qui est recommandé dans la <i>surviving sepsis campaign</i>
DC : débit cardiaque ; E. : esmolol ; FC : fréquence cardiaque ; IC : index cardiaque ; OPS : <i>orthogonal polarization spectral</i> ; VES : volume d'éjection systolique ; vs. : versus ; ➤ : augmentation ; ➤ : diminution ; = : identique ; ET : écart-type.										

augmentation du volume d'éjection systolique sans effet hypoxique associé ni modification des résistances vasculaires. Ils concluaient à la bonne tolérance de ce traitement [55]. Ensuite, les travaux ont essentiellement été monocentriques, observationnels, sur de petites populations légèrement plus sévères de choc septique, et ont retrouvé que la réduction de la FC n'induisait pas de diminution du débit cardiaque avec des résistances vasculaires qui restaient similaires [56,57]. Dans une des deux publications, la milrinone (inhibiteur des phosphodiesterase de type 3, inotrope positif indépendant des adrénorécepteurs) était coadministrée pour contrecarrer les effets inotropes négatifs du β -bloquant administré [56].

Une seule étude clinique d'envergure a été publiée en 2013 par Morelli et al. [61]. Ce travail monocentrique, prospectif, randomisé, esmolol versus placebo en ouvert incluait une population de 154 patients en choc septique 24 heures après l'admission qui restait tachycarde à plus de 95 battements par minute. L'objectif principal portait sur l'efficacité de l'administration de l'esmolol à diminuer la FC entre 80–94/min et sur l'évaluation de la tolérance qui y était associée. Le résultat principal est une réduction efficace de la FC dans la cible visée dans le groupe esmolol. Le débit cardiaque est globalement non diminué malgré la réduction de la FC, en partie grâce à l'augmentation du volume d'éjection systolique secondaire à l'allongement de la période diastolique dans le groupe esmolol. Du lévosimendan (un inotrope calcium sensibilisateur, indépendant des adrénorécepteurs) était administré de façon similaire dans les deux groupes (esmolol : 49,4 %, placebo : 40,3 %, $p = 0,39$). La posologie de noradrénaline requise était inférieure dans le groupe esmolol par rapport au groupe placebo. Les résistances vasculaires étaient augmentées par rapport au groupe placebo. La mortalité était réduite de 31 % dans le groupe esmolol avec 80 % de mortalité dans le groupe témoin. Ces résultats ont suscité d'intenses débats dans la littérature [66–72]. On retiendra surtout quatre points :

- l'efficacité sur la réduction de la FC est certaine ;
- la tolérance semble excellente, mais la prise en charge est en dehors des pratiques usuelles, car près de 50 % de la population reçoit du lévosimendan ;
- la réduction de la dose de noradrénaline dans le groupe esmolol est inexpliquée ;
- la diminution de la mortalité est à pondérer à la mortalité du groupe témoin qui est inhabituellement très élevée et en dehors de ce qui était attendu en 2013.

Fondés sur les résultats de Morelli et al., les travaux qui ont suivi ont cherché à confirmer la tolérance et les effets physiologiques de ce traitement. Néanmoins, les populations incluses ne correspondent jamais aux recommandations de prise en charge du choc septique : soit les patients ont des objectifs de pression artérielle trop élevés, soit les patients ont une prise en charge non usuelle ou un niveau de sévérité qui est trop faible [62–65].

Tableau 4 Résumé des études cliniques comparatives

Auteur	Type d'étude	Objectif principal	Inclusion	Méthode	Nombre de patients	Résultat principal	Résultats secondaires	Commentaires
Morelli et al. 2013 [61]	Randomisée, comparative en ouvert, monocentrique, de phase II	Réduction de la FC entre 80 et 94/min	Choc septique stabilisé depuis 24 heures avec FC > 95/min	2 groupes : E. vs. C. Administration de lévosimendan si SvO ₂ < 65 % Évaluation avant puis à 24, 48, 72, 96 heures	154 (77 dans chaque groupe)	FC (/min)* E. : -28 [-37 – -21] vs. C. : -6 [-14-0]	Augmentation dans le groupe E., vs. C. : VES* et des RVS* Mortalité à 28 j* E. : 49,4 % vs. C. : 80,5 %	49,4 % d'administration de lévosimendan dans le groupe E. vs. 40,3 % dans le groupe C.
Yang et al. 2014 [62]	Randomisée, contrôlée, monocentrique	Non clairement défini. Évaluation hémodynamique générale	Choc septique	Randomisation dans les 6 heures. 2 groupes : E. vs. C. Réduction de la FC < 100/min dans les 2 heures suivant l'inclusion dans le groupe E. Évaluation avant puis à 12, 24, 48 et 72 heures	41 E. : 21 vs. C. : 20	FC (/min)* E. : 93 ± 4 vs. C. : 118 ± 13 IC (l/min/m ²)* E. : 3,3 ± 8 vs. C. : 4,5 ± 6	Pas de différence dans le temps et entre les groupes de la SvcO ₂ Diminution significative de la troponine dans E. vs. C.	Objectif de pression artérielle supérieur à ce qui est recommandé dans la <i>surviving sepsis campaign</i>
Wang et al. 2015 [63]	Randomisée, contrôlée en ouvert, monocentrique, de phase II	Réduction de la FC entre 75 et 94/min	Sepsis avec FC > 95/min	3 groupes : C. vs. milrinone vs. E.-milrinone Milrinone administrée en continu après bolus E. administré en continu Évaluation avant puis à 12, 24, 48, 72 et 96 heures	90 (30 dans chaque groupe)	FC dans l'objectif * : C. : 43,3 % vs. Milrinone : 53,3 % vs. E.-milrinone : 100 %	Augmentation dans le groupe E.-milrinone vs. C. : VES* et IC* Diminution dans le groupe E.-milrinone vs. C. : TNF-α et IL-6 Mortalité à 28 j* C. : 73 % vs. Milrinone : 67 % vs. E.-milrinone : 40 %	

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Tableau 4 (suite)								
Auteur	Type d'étude	Objectif principal	Inclusion	Méthode	Nombre de patients	Résultat principal	Résultats secondaires	Commentaires
Xinqiang et al. 2015 [64]	Randomisée, contrôlée, en aveugle, monocentrique	Mortalité à 28 jours et durée de séjour en réanimation	Choc septique stabilisé depuis 6 heures avec FC > 95/min	E. administré en continu pour obtenir une FC < 100/min Évaluation avant puis à 24, 48 et 72 heures	48 (24 dans chaque groupe)	Mortalité à 28 j* : E. : 25 % vs. C. : 62,5 % Durée de séjour (jours)* : E. : 13,75 ± 8,68 vs. C. : 21,7 ± 6,06	Diminution à 24 heures dans le groupe E. vs. C. : FC* et à 48 heures : lactate* Augmentation à 24 heures dans le groupe E. vs. C. : RVS*, SvcO ₂ * Pas de différence à 24 heures dans le groupe E. vs. C. : pression artérielle, pression veineuse centrale, index cardiaque	
Shang et al. 2016 [65]	Prospective, cohorte, comparaison non randomisée entre C. et E.	Non clairement défini. Évaluation hémodynamique générale	Sepsis sévère avec FC > 100/min	Administration en continu d'E. ou sérum salé dans le groupe C. Évaluation avant puis à 24, 48 et 72 heures	151 E. : 75 vs. C. : 76	À 24 heures : FC (/min)* E. : 101,96 ± 7,36 vs. C. : 110,12 ± 8,59	À 24 heures : SvcO ₂ * : E. : 0,8 ± 0,05 vs. C. : 0,76 ± 0,05 Mortalité à 28 j : E. : 5,3 % vs. C. : 7,9 %	Pas de randomisation dans ce travail Mortalité très faible (pas de choc septique)
C. : contrôle ; E : esmolol ; FC : fréquence cardiaque ; IC : index cardiaque ; IL : interleukine ; RVS : résistance vasculaire systémique ; SvcO ₂ : saturation veineuse centrale en oxygène ; TNF- α : <i>tumor necrosis factor-α</i> ; VES : volume d'éjection systolique ; vs. : versus ; * $p < 0,05$.								

Finalement, étant donné les faiblesses méthodologiques des travaux présentés, il est prématuré de recommander l'utilisation d'esmolol durant le choc septique, essentiellement en raison de l'absence d'identification d'une population à qui l'administrer. En effet, une étude de faisabilité, incluant des patients d'une population comparable à celle que nous avons dans nos services, démontre que seuls 10/186 étaient éligibles à l'administration d'esmolol [73].

Les β -bloquants ont-ils une place dans la prise en charge du choc septique ?

Si les études expérimentales suggèrent que l'administration précoce intraveineuse d'un β_1 -bloquant est bénéfique en terme hémodynamique et pour un certain nombre d'études sur la survie, les travaux cliniques sont néanmoins soit peu concluants, soit méthodologiquement fragiles. Ces différences de résultat peuvent s'expliquer par le modèle et les conditions expérimentales appliqués. Ainsi, les études expérimentales sont réalisées essentiellement chez le petit animal, les souris ou les rats, dont les FC au repos sont supérieures à 450/min. Le débit cardiaque est donc en cas de choc septique étroitement relié à la FC et à la durée de la diastole et donc au remplissage ventriculaire en fin de diastole. Ainsi, dans un travail chez la souris en choc septique par ligature et ponction caecale, la survie était améliorée lorsque le ventricule gauche se dilatait permettant ainsi un meilleur remplissage ventriculaire [74]. Les modèles de choc septique utilisant les petits animaux sont d'ailleurs loin d'être tous tachycardes, et plus particulièrement chez la souris, où l'induction d'un choc septique provoque une régulation négative du métabolisme conduisant à une diminution de la FC [75]. Au contraire, chez l'homme, la survenue d'un choc septique induit le plus souvent une tachycardie dès la phase initiale. De même, le moment d'administration du β -bloquant est toujours défini lors d'une étude expérimentale, le plus souvent précoce alors qu'il est par définition hautement variable chez l'homme et le plus souvent tardif [61,76]. Finalement, si le modèle du petit animal est intéressant sur le plan conceptuel, la transposition des méthodes appliquées, chez le gros animal et plus encore chez l'homme, ne produit pas des résultats équivalents.

En pratique, les résultats de la seule étude randomisée sur le sujet restent encore à confirmer de façon multicentrique. Plus précisément, la tolérance clinique reste sujette à caution au regard de la mortalité de la population en choc septique du groupe non traité (80,5 %) incluse dans l'étude princeps, différente de celle rencontrée habituellement (42 %) [61,77].

Par ailleurs, des études tendent à démontrer que les critères d'application de ce traitement, c'est-à-dire le patient cliniquement stable à 24 heures mais présentant une tachycardie persistante supérieure à 95/min, ne sont que rarement

rencontrés [73]. De plus, il n'existe pas d'indicateur simple permettant de différencier une tachycardie adaptative (une hypovolémie par exemple) d'une tachycardie dysautonomique, c'est-à-dire qui est le reflet d'un hyperadrénergisme central inadapté [78,79].

Au regard de la littérature, il n'existe pas de preuves cliniques suffisantes pour recommander l'administration d'un traitement β_1 -bloquant chez le patient en choc septique, stabilisé mais toujours tachycarde avec pour objectif la réduction de la FC en vue de l'amélioration de son pronostic vital. Les études qui permettront de mieux définir le profil de tolérance des patients en choc septique pouvant éventuellement bénéficier d'un traitement bradycardisant par β_1 -bloquant sont actuellement en cours (NCT02120404, NCT02369900).

Si les données précliniques révèlent des effets intéressants sur le plan de la rééquilibration inflammatoire, une meilleure compréhension des mécanismes sous-jacents à cette régulation est un prérequis nécessaire à une meilleure définition des patients qui éventuellement pourraient bénéficier, par la suite, de ce traitement.

Conclusion

La dysautonomie, surstimulation du système sympathique, est une complication précoce du choc septique associée à une altération des fonctions cardiaque et vasculaire et une immunosuppression. Son association à la mortalité semble avérée. Sa modulation est un enjeu qui pourrait se faire via l'inhibition des adrénorécepteurs β_1 par un traitement β_1 -bloquant. Néanmoins, si les preuves expérimentales sont en faveur de cette approche, l'accumulation des preuves cliniques n'est pas encore suffisante.

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Annexe 2: The β_1 -adrenergic receptor contributes to sepsis-induced immunosuppression through modulation of regulatory T cell inhibitory function



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The β_1 -adrenergic receptor contributes to sepsis-induced immunosuppression through modulation of regulatory T cell inhibitory function

Short title: **β_1 -AR blockade decreases sepsis immunosuppression**

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Abstract

Rationale: Sepsis typically induces dysautonomia, an over-activation of the sympathetic nervous system. This can lead to impaired cardiovascular function and immunosuppression, both of which are associated with poor outcome. Beta₁-adrenoreceptor blockade downregulates dysautonomia. Although cardiovascular benefits of β_1 -adrenoreceptor blockade have been described in sepsis, little is known about its impact on the adaptive immune response, specifically CD4 T cells.

Objective: To investigate the effects of β_1 -adrenoreceptor modulation on CD4 T cell function in a murine model of sepsis.

Methods and results: High-grade sepsis was induced by caecal ligation and puncture (CLP) in wild-type mice ($\beta_1^{+/+}$) with or without esmolol (a selective β_1 -adrenoreceptor blocker), or in β_1 -adrenoreceptor knockout mice ($\beta_1^{-/-}$). At 18 hours, $\beta_1^{+/+}$ mice exhibited characteristics of high-grade sepsis and three surrogate markers of immunosuppression, namely decreased splenic CD4 T cells, reduced CD4 T cell proliferation and increased Treg cell proportions. Pharmacologic and genetic β_1 -adrenoreceptor blockade reversed the impact of sepsis on CD4 T and Treg proportions and maintained CD4 T cell proliferative capacity. β_1 -adrenoreceptor blocked mice also exhibited a global decrease in both pro- and anti-inflammatory mediators and improved *in-vivo* cardiovascular efficiency with a maintained cardiac power index despite the expected decrease in heart rate.

Conclusion: β_1 -adrenoreceptor activation enhances Treg inhibitory function and thus contributes to sepsis-induced immunosuppression. This can be attenuated by β_1 -adrenoreceptor blockade, suggesting a potential immunoregulatory role for this therapy in the treatment of sepsis.

Keywords: Septic shock - adrenoreceptor - beta blocker - lymphocytes - immunosuppression

Abbreviations:

AR: adrenergic receptor

CD: cluster of differentiation

CI: cardiac index

CLP: caecal ligation and puncture

CO: cardiac output

CPI: cardiac power index

ELISA: enzyme-linked immunosorbent assay

FBS: fetal bovine serum

IL: interleukin

MAP: mean arterial pressure

PBS: phosphate-buffered saline

PHA: phytohemagglutinin

PMA: phorbol myristate acetate

RMPI: Roswell Park Memorial Institute

SNS: sympathetic nervous system

SV: stroke volume

Th: T helper cell

TNF: tumor necrosis factor

Treg: regulatory T lymphocyte

Introduction

Sepsis and septic shock are a leading cause of intensive care unit admission and recognized by the World Health Organization as a major healthcare issue ¹. Data estimate the number of sepsis cases globally at 48.9 million, resulting in 11.0 million deaths in 2017 ². Unfortunately, despite significant effort, all therapeutic research conducted over the last four decades has systematically failed³.

Sepsis is the consequence of a highly complex and dysregulated inflammatory host response to infection leading to life-threatening organ dysfunction ⁴. After initial activation, the immune system – both innate and adaptive – is downregulated, leading to immunosuppression that may be long-lasting and predispose the patient to secondary infection. A disproportionate and prolonged activation of the sympathetic nervous system (dysautonomia) also occurs, characterized by loss of cardiovascular variability, tachycardia, and excessive release of catecholamines such as epinephrine and norepinephrine ⁵. These hormones, mainly possessing α_1 and β_1 adrenergic effects, are crucial factors underlying immunosuppression ⁶. Sepsis-induced immunosuppression is characterized by immune cell depletion; dysregulated cytokine production and compromised T helper cell (CD4 T cells) function. There is an associated increase in regulatory T cells (Treg cells) leading to a decreased ability to respond to subsequent infectious insults ⁷. In this context, norepinephrine favors an anti-inflammatory cytokine imbalance, associated with inhibitory effects on T cell growth and cytokine production ⁸⁻¹⁰.

Beta-1 adrenoreceptor (β_1 -AR) blockade is a therapeutic approach aimed at downregulating the excessive adrenergic stimulation induced by sepsis. Initial single-center studies in human

septic shock have mainly reported hemodynamic benefits with improvements in cardiac efficiency, with one also finding survival benefit ¹¹⁻¹³. Multicenter trial will begin soon (EudraCT: 2017-002138-22). Some experimental studies have investigated the inflammatory effects of β_1 -AR blockade in sepsis and demonstrated a global decrease in both pro- and anti-inflammatory plasma cytokines ¹⁴⁻¹⁶. However, little is known about the effects of β_1 -AR blockade on the early adaptive immune response, specifically on CD4 T cells ^{17, 18}. The present study aimed to address this question in a murine model of high-grade sepsis.

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Methods

Sepsis model

β_1 -AR (C57BL/6J, DBA/2, 129Sv) knockout mice ($\beta_1^{-/-}$) and wild type littermates ($\beta_1^{+/+}$), obtained free of charge from the Institute of Pharmacology and Toxicology of Munich, Germany, were used for this study. The French Animal Care Committee, in accordance with European regulations, approved all protocols (n°APAFIS#4636). Sepsis was induced by caecal ligation and puncture (CLP) in both male and female mice aged 12-16 weeks, weighing 22-32 grams. Anesthesia was induced with 3.5% isoflurane (Forene®, Abbvie, North Chicago, Ill) and subsequently maintained through the procedure with 2% isoflurane. A laparotomy was performed, 75% of the caecum is being ligated and then punctured with an 18-gauge needle (high-grade sepsis) ¹⁹. The caecum was re-inserted into the abdomen and the incision closed by suture. Sham-operated mice underwent the same procedure except for the caecal ligation and puncture. An incision was performed between the scapulae in all mice and a mini-osmotic pump (2001D, Alzet, Cupertino, CA) implanted subcutaneously in animals to be treated with the selective β_1 -AR blocker, esmolol (Esmocard®, Amomed, Meudon, France). After 20 minutes, mice received subcutaneous analgesia with buprenorphine 0.2 mg/kg (Buprécare®, Axience, Pantin, France) in physiological saline solution (0.9% NaCl; 5ml/100g; Baxter, Guyancourt, France).

Experimental design

$\beta_1^{+/+}$ mice were randomly assigned to four groups: sham, sham + esmolol, CLP or CLP + esmolol. Esmolol was infused at 18 mg/kg/h (8 μ l/h volume) through the subcutaneous mini-osmotic pump which was activated 3 hours post-insertion. Esmolol dosage was chosen according to literature and preliminary experiments to reducing heart rate (HR) by 20%

(Figure S1). $\beta_1^{-/-}$ mice were randomly assigned to two groups: sham or CLP. Eighteen hours after surgery, echocardiography was performed, plasma collected and spleens immunophenotyped (n=74). Splenic CD4 T cell proliferation assays were also performed (n=44). In $\beta_1^{+/+}$ non-septic mice, Treg cells and CD4 T cells were isolated and co-cultured (n=4). A 5-day survival study was performed in 3 groups of mice: $\beta_1^{+/+}$ CLP (n=20), $\beta_1^{+/+}$ CLP + esmolol (n=20), and $\beta_1^{-/-}$ CLP mice (n=20). The number and reasons for failed measurements and excluded mice are provided in **Table S1**.

Splenocyte isolation

Spleens were harvested, immediately placed in cold PBS (Sigma Aldrich, Saint-Louis, MO), gently ground between frosted slides, then dispersed through 70 μ m nylon filters (Biologix, Shandong, China). Red blood cells were lysed using BD PharmLyse™ (BD Bioscience, San Jose, CA) solution. Splenocytes were then washed twice in PBS before being resuspended in RPMI 1640 medium (Gibco, ThermoFisher Scientific, San Diego, CA) supplemented with 10% FBS.

Immunophenotyping

Splenocyte Fc receptors were blocked (CD16/CD32-purified antibodies, eBioscience™, ThermoFisher Scientific) and cell surface markers used to identify total T cells (CD3+), CD4 T cells (CD3+ CD4+), CD8 T cells (CD3+ CD4-) and Treg cells (CD3+ CD4+ CD25^{hi} CD127^{low}). Th1 (INF- γ +), Th2 (IL-4+) and Th17 (IL-17+) status was characterized by intracellular staining of CD4 T cells. Cell surface antigens were stained for 30 minutes in the dark. For intracellular staining, cells were stimulated for 4 hours at 37 °C with phorbol myristate acetate (PMA, 250 ng/10⁶ cells) and ionomycin (500 ng/10⁶ cells) (both Sigma Aldrich) and Brefeldin A (BD

GolgiPlug, BD Bioscience), and then fixed and permeabilised with BD Cytofix/Cytoperm™ solution (BD Bioscience).

The following monoclonal antibodies were used according to the manufacturer's recommendations: PerCP Cy5.5-CD3, BV421-CD4, PE-Cy7-CD8, PE-CD25, APC-CD127, APC-IL4, PE-IL-17A (all BD Pharmingen, BD Bioscience), and AF700-INF- γ (Biolegend, San Diego, CA). Dead cells were excluded using Fixable Viability Dye eFluor™ 506 (eBioscience™, ThermoFisher Scientific). Flow cytometry was carried out using a Gallios analyzer (Beckman Coulter, Brea, CA). Blind analysis of the data was performed using Flowlogic™ software (MiltenyiBiotec, Bergisch Gladbach, Germany).

Proliferation assay

Splenocytes (1×10^6 cells/ml in PBS) were stained with Tag-it Violet™ Proliferation and Cell Tracking Dye ($5 \mu\text{M}/10^6$ cells; Biolegend) for 20 minutes at 37°C . Staining was then quenched by addition of culture medium containing 10% FBS. Cells were then centrifuged (300g, 5 minutes), resuspended in RPMI 1640 medium (supplemented with 10% FBS, 2 mM L-glutamine, 20 U/ml penicillin and $20 \mu\text{g}/\text{ml}$ streptomycin) and incubated for 10 minutes at 37°C . Thereafter, splenocytes were seeded (2×10^5 cells per well) in 96-well plates and stimulated with phytohemagglutinin (PHA; $4 \mu\text{g}/10^6$ cells; Remel, ThermoFisher Scientific) for 72 hours at 37°C . Cells were stained with CD4-BV421 (BD Pharmingen) and propidium iodide (MiltenyiBiotec). The Tag-it Violet signal was assessed in live CD4 T cells by flow cytometry.

Purification of CD4⁺ CD25⁻ T cells and CD4⁺ CD25⁺ Treg cells

CD4 T and Treg cells were isolated in a two-step procedure following the manufacturer's instructions (CD4⁺ CD25⁺ Regulatory T Cell Isolation Kit, MiltenyiBiotec). Briefly, non-CD4 T cells were magnetically labelled with a biotin-conjugated antibody cocktail against CD8a, CD11b, CD45R, CD49b, and Ter-119, then flowed through a magnetic field. CD4⁺ T cells were retained by the magnetic field and CD4 T cells collected. CD4⁺ CD25⁺ Treg cells were then magnetically labelled with a biotin-conjugated anti-CD25 antibody. The flow-through containing CD4⁺ CD25⁻ T cells, while CD4⁺ CD25⁺ Treg cells were retained by the magnetic field and then were collected (**Figure S2**).

Co-culture of CD4 T cells with catecholamine-stimulated Treg cells

Treg cells were stimulated with 1 μ M dobutamine (a β_1 -AR agonist), norepinephrine (a mixed α_1 - and β_1 -AR agonist), phenylephrine (α_1 -AR agonist); (all from Sigma-Aldrich) or PBS for 30 minutes, then washed twice to remove the catecholamines. Pre-treated Treg cells were co-cultured with CD4 T cells at a 1:4 ratio in complete RPMI 1640 medium (supplemented with 10% FBS, 2 mM L-glutamine, 20 U/ml penicillin, 20 μ g/ml streptomycin, 1% sodium pyruvate, 1% HEPES, 50 μ M 2-mercaptoethanol and 30 U/ml IL-2) in 96-well plates coated with 50 μ L of 0.01% poly-L-ornithine solution (Sigma Aldrich). Cells were activated with anti-CD3/CD28 beads (bead-to-cell ratio 1:1; T cell activation/expansion kit, MiltenyiBiotec) and imaged every 2 hours for 5 days (37 °C, 5% CO₂) using the 20 \times objective of the IncuCyte[®] S3 Live-Cell Analysis System (Sartorius, Goettingen, Germany). Proliferation was measured by cell counting in the phase contrast mode. Dead cells were identified using the green fluorescent Cytotox Green dye (Sartorius; final concentration, 250 nM). Analysis was performed using IncuCyte[™] Basic Software (**Figure S3**).

Echocardiography

Transthoracic echocardiography was performed 18 hours after CLP with a VEVO770 (FUJIFILM VisualSonics, Toronto, Canada) and a 30 MHz mechanical probe. The aortic diameter, and velocity integral time were averaged over three consecutive cardiac cycles and used to calculate all parameters : stroke volume (SV), cardiac index (CI), and cardiac power index (CPI). Mean arterial pressure (MAP) was measured by a blood pressure transducer (Emka, Paris, France) inserted through the left carotid artery. Details of the *in vivo* hemodynamic procedure are provided as **supplementary data**.

Quantification of inflammatory mediators

In 5–6 mice from each group, the plasma levels of 46 inflammatory mediators were measured in duplicate using the Magnetic Luminex® Performance Assay – Mouse XL Discovery Premixed kit (Bio-Techne, Minneapolis, MN) according to the manufacturer's instructions. Results were expressed as pg/ml.

Survival study

Survival was studied in $\beta_1^{+/+}$ CLP mice, $\beta_1^{+/+}$ CLP + esmolol (18 mg/kg/h) mice, and $\beta_1^{-/-}$ CLP mice. Census of animals was undertaken twice a day for 5 days. Administration of esmolol (Esmocard®, Amomed) was started 3 hours post-CLP and continued for a total infusion period of 24 hours. The one animal that died before the first count was considered a technical failure and excluded from the study.

Statistical analysis

Results are expressed as mean and standard deviation (mean \pm SD). Results in $\beta_1^{-/-}$ mice were compared using a Mann-Whitney test while the Kruskal-Wallis test was used to compare $\beta_1^{+/+}$ groups. When the Kruskal-Wallis test was significant, *post hoc* comparisons were performed between sham, CLP, and CLP + esmolol groups using Dunn's multiple comparisons and a Bonferroni correction.

Variations were calculated as follows: $100 - ((\text{mean group 1} / \text{mean group 2}) \times 100)$.

To analyze the effect of catecholamines on Treg cells co-cultured with CD4 T cells, we firstly assessed the interaction between mouse at each time point. Considering the mouse has a random effect, the p-value of the test for the treatment effect was calculated. Only the last p-value (obtained at 132 hours) is shown.

For measurement of plasma inflammatory mediators, groups were compared using the Mann-Whitney test. A Benjamini-Hochberg adjustment was performed for multiple comparisons.

Kaplan–Meier curves were drawn. Significance was assessed using the log-rank test.

P values <0.05 were considered statistically significant. Statistical analysis was performed using R, version 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria), using Graph-Pad Software 6.0, (San Diego, CA) and using NCSS Statistical Software 9 (East Kaysville, UT).

Results

β_1 -AR contributes to post-septic immunosuppressive phenotype.

Male and female mice were used in equal numbers for all experiments (sex ratio=1.02). Eighteen hours after surgery, the spleens of $\beta_1^{+/+}$ and $\beta_1^{-/-}$ mice were harvested and immunophenotyped to study T lymphocyte subpopulations. **Figure 1, panel A** shows that in $\beta_1^{+/+}$ mice, sepsis induced a significant decrease in CD4 T cell proportions (-34% vs. sham, $p<0.001$) counteracted by β_1 -AR blockade (+37% vs. CLP, $p=0.03$). In $\beta_1^{-/-}$ mice, sepsis significantly decreased the proportion of CD4 T cells (-17% vs. sham, $p=0.02$), however no change in CD8 T cells proportion was observed between groups (**Figure 1, panel B**).

With respect to Treg cells (CD25^{hi} CD127^{low}), in $\beta_1^{+/+}$ mice, sepsis induced an increase in Treg cell proportions (+65% vs. sham, $p<0.001$), inhibited by β_1 -AR blockade (-19% vs. CLP, $p=0.04$) (**Figure 1, panels C, D**). In $\beta_1^{-/-}$ septic mice, the proportion of Treg cells was unchanged (+7% vs. sham, $p=0.68$). Of note, β_1 -AR blockade in $\beta_1^{+/+}$ sham mice had no impact on CD4 T cell and Treg cell proportions (**Figure S4, panels A, B**). No modification was observed in other CD4 T cells subsets (Th1 (INF- γ), Th2 (IL-4) and Th17 (IL-17)) in these experimental groups (**Figure S5**). These findings were not caused by a difference in cell viability among groups (**Figure S6**).

β_1 -AR activation induces a decrease in CD4 T cell proliferation during sepsis.

After culturing splenocytes for 72 hours with PHA stimulation, the proliferative response of CD4 T cells was measured to assess sepsis-induced immune suppression (**Figure 2, panel A**). In $\beta_1^{+/+}$ mice, sepsis reduced the proliferative capacity of CD4 T cells (-15% vs. sham, $p=0.04$). However, administration of a β_1 -AR blocker limited this effect (-8% vs. sham, $p=0.38$). In $\beta_1^{-/-}$

mice, sepsis did not decrease the proliferative capacities of CD4 T cells (-4% vs. sham, $p=0.71$) (**Figure 2, panel B**). These results were not caused by variations in cell viability among groups (**Figure S7**). Of note, β_1 -AR blockade in $\beta_1^{+/+}$ sham mice had no impact on CD4 T cell proliferation (**Figure S4, panel C**).

β_1 -AR stimulation of Treg cells increases their immunosuppressive capacities.

The direct effect of β_1 -AR stimulation on the immunosuppressive functions of Treg cells was assessed. CD4 T cell counts tended to decrease after five days of co-culture with Treg cells pre-treated with dobutamine, in comparison to untreated Treg cells ($p=0.08$, **Figure 3, panel A**). This lower cell count, noted when co-culture was performed with dobutamine pre-treated Treg cells, was not caused by an increase in cell death (**Figure 3, panel B**). Similar results were found when co-culture assays of CD4 T cells were performed in the presence of norepinephrine and phenylephrine pre-treated Treg cells (**Figure S8**).

β_1 -AR blockade, despite a reduced heart rate, did not alter hemodynamic characteristics in sepsis.

Hemodynamic parameters were assessed by echocardiography (**Table 1**). In $\beta_1^{+/+}$ septic mice, administration of a β_1 -AR blocker induced a decrease in HR (-24% vs. sham, $p<0.001$; -14% vs. CLP, $p=0.07$). In $\beta_1^{-/-}$ mice, sepsis did not affect HR (+9% vs. sham, $p=0.24$).

Compared to $\beta_1^{+/+}$ sham, high-grade sepsis induced a decrease in MAP (-27% vs. sham, $p<0.001$), SV (-40% vs. sham, $p=0.004$), CI (-49% vs. sham, $p<0.001$), and CPI (-64% vs. sham, $p<0.001$). These parameters remained unaltered by the administration of a β_1 -AR blocker (MAP: +8% vs. CLP, $p=0.51$; SV: +6% vs. CLP, $p=0.99$; CI: +2% vs. CLP, $p=0.99$; CPI: +25% vs. CLP, $p=0.001$).

CLP, $p=0.99$). In $\beta_1^{-/-}$ mice, sepsis induced a decrease in MAP (-19% vs. sham, $p=0.04$), SV (-47% vs. sham, $p<0.001$), CI (-37% vs. sham, $p=0.002$) and CPI (-50% vs. sham, $p<0.001$).

β_1 -AR modulates the concentration of both pro- and anti-inflammatory circulatory mediators.

The plasma levels of 31 pro-inflammatory and 15 anti-inflammatory mediators were measured. Briefly, all inflammatory mediators were increased in $\beta_1^{+/+}$ CLP mice compared to sham controls (**Table S2**). Compared to $\beta_1^{+/+}$ CLP mice, esmolol-treated CLP mice showed an overall decrease in plasma concentrations of 58% (18/31) and 60% (9/15) for pro- and anti-inflammatory mediators, respectively (**Figure 4**). For instance, compared to $\beta_1^{+/+}$ CLP mice, administration of a β_1 -AR blocker reduced plasma TNF- α by 66% vs. CLP ($p=0.02$), and IL-10 by 82% vs. CLP ($p=0.01$). In $\beta_1^{-/-}$ mice, sepsis induced an overall increase in both pro- and anti-inflammatory mediators compared to sham animals (**Table S2**).

β_1 -AR modulates survival during sepsis.

The survival rate was documented in $\beta_1^{+/+}$ CLP, $\beta_1^{+/+}$ CLP + esmolol and $\beta_1^{-/-}$ CLP mice (**Figure 5**). Survival at 110 hours decreased from 60% in $\beta_1^{-/-}$ CLP mice, to 37% in $\beta_1^{+/+}$ CLP + esmolol mice and 35% in $\beta_1^{+/+}$ CLP mice (log rank $p=0.13$).

Sex does not affect the β_1 -AR effect.

Sensitivity analyses were performed on results obtained for major hemodynamic parameters (**Figure S9**) and immune effects (**Figure S10**) according to sex. No differences were found between male and female mice.

Discussion

In the present study, we confirm that experimental high-grade sepsis induces three hallmark features of sepsis-induced immunosuppression: a significant decrease in splenic CD4 T cells, an increase in splenic Treg cells, and a decrease in CD4 T cell proliferation capacity⁷. A notable finding was the marked increase in Treg cells in the spleen of septic mice, which could be counteracted by pharmacologically or genetically blocking β_1 -AR signaling. This observed decrease in Treg cells in treated animals was associated with a simultaneous increase in CD4 T cells and a global decrease in circulating pro- and anti-inflammatory mediators, indicating the involvement of β_1 -AR in the septic-immune phenotype. These results also translated into an *in vivo* global improvement in cardiac efficiency. **Figure 6** shows a graphical summary of the effect of β_1 -AR blockade on cardiac and immune function during sepsis induced-dysautonomia.

Model characteristics

To investigate the immune and cardiovascular roles of β_1 -AR in sepsis, we deliberately chose a non-resuscitated murine model of CLP to avoid any interference between exogenous catecholamine administration and the immune system²⁰. This model depicts all hemodynamic and survival characteristics of a typical high-grade sepsis, with an increase in both plasma pro- and anti-inflammatory mediators and a decrease in CD4 T cell proportion^{19, 21-23}. Analysis of CD4 T subtypes revealed that early after onset of sepsis, the percentages of Th1, Th2 and Th17 cells were unchanged. Many studies usually report a switch from Th1 to Th2 phenotypes, and a decrease in Th17 cell percentages²⁴⁻²⁷. However, other studies found that sepsis could also disrupt in a time-dependent manner both the representation and the function of CD4 T cell subsets, including Th1, Th2, Th17²⁸⁻³¹. Unlike other

lymphocyte populations, Treg cells are increased during trauma in both animals and humans, as well as during sepsis and septic shock (as seen in our study)^{29, 30}. This increase in Treg cells contributes to lymphocyte paralysis and, more broadly, enhances the post-septic immunosuppressive process³².

β -adrenergic stimulation contributes to CD4 T cell dysfunction

In both innate and adaptive immune cells, adrenergic receptors are widely expressed though not well characterized, particularly for the β subtype³³. Numerous studies have demonstrated the presence of β -AR on almost all lymphocyte subtypes³⁴⁻³⁶. However, until recently, only β_2 -AR but not β_1 -AR subtypes were found on T cells^{37, 38}. As a consequence, β_1 -AR stimulation was not considered to have a major role in inflammation³⁹. β_1 -AR were found to be widely expressed on splenic CD4 T cells only recently. In cirrhotic mice, the β_1 -AR agonist dobutamine, could re-activate inhibited Treg cells, suggesting the presence of β_1 -AR on their surface⁴⁰. In accordance, we too found that *in vitro* CD4 T cell proliferation tended to be reduced by Treg cells pre-treated with dobutamine.

β -adrenoreceptor blockade reversed sepsis-induced dysautonomia

In numerous conditions, such as acute heart failure, burn injuries, chronic hepatic failure, the peri-operative period and septic shock, β_1 -AR blockade has been consistently associated with protective effects on inflammation and hemodynamics^{15, 41, 42}. As others, we too found a global decrease in pro- and anti-inflammatory mediators in animals with high-grade sepsis treated by β_1 -AR blockade^{15, 43, 44}. Underlying mechanisms are still not elucidated. In *Listeria monocytogenes* infected mice acute cold or restraint stress (surrogates of sympathetic adrenergic stimulation) inhibited the host T cell response and increased cytokine production

⁴⁵. In this model, pharmacological blockade of the β_1 -AR but not β_2 -AR could downregulate this pro-inflammatory state and shorten time to bacterial clearance ⁴⁶.

In β_1 -AR blocked animals with high-grade sepsis, we found the decrease in Treg cells in the spleen was associated with a concomitant increase in CD4 T cells, as reported previously ⁴⁷. In addition, we found that proliferative capacity of splenic CD4 T cells in septic animals was maintained when the β_1 -AR was blocked either pharmacologically or genetically. This suggests that β_1 -AR activation contributes to sepsis-induced immunosuppression, which could be reversed by β_1 -blockade. By comparison, volunteers challenged with lipopolysaccharide, and then being infused with norepinephrine for 5 hours showed a shift to an anti-inflammatory state (decreased TNF- α /IL-10 ratio) which could be counterbalanced by β -AR blockade (increased TNF- α /IL-10 ratio) ¹⁰. β_1 -AR blockade in our septic animals reduced the inflammatory burden and, taken as a whole, helped to shift from an immunosuppressive state to a state of immune homeostasis.

Beside this immune rebalancing in experimental high-grade sepsis, it is important to reiterate that β_1 -AR blockade also has major cardiovascular effects. In agreement with most other experimental or clinical studies, septic mice receiving β -AR blockade showed no alteration in cardiac power, despite a decrease in HR, suggesting improved cardiac efficiency ^{13, 15, 43, 48}.

While the association between an increased Treg cell percentage during sepsis and an immunosuppressive state is well proven, effects on survival remain inconclusive ^{49, 50}. The benefits of β_1 -AR blockade on immune and cardiovascular function should improve the survival rate. However, in the present study, and without any treatment (antibiotics, fluid or

vasopressors), no improvement was seen in survival. In a similar CLP surgery model, Ackland *et al.* also found that metoprolol infusion, commenced 6 hours after the onset of fecal peritonitis, did not confer any benefit in improving survival whereas pretreatment was effective⁴⁴. A dose effect may be relevant as the β_1 -AR blocker was infused only during the first 24 hours after CLP surgery. In a single-center study prolonged administration of esmolol in septic shock patients, titrated to maintain heart rate between 80-94 bpm, was associated with increased survival¹³.

Perspectives

β_1 -AR and β_2 -AR are widely expressed on both innate and adaptive immune cells. Specifically, it has been reported that naïve CD4 T cell subtypes Th1, Th17 and Treg cells, but not Th2, express β_2 -AR^{34, 51}. β_2 -AR stimulation promotes a shift from Th1 to Th2 anti-inflammatory patterns and could activate Treg suppressive functions^{35, 52}. β_2 -AR stimulation may enhance the macrophage M2 anti-inflammatory phenotype, favoring IL-10 production by CD4 T cells³⁶. Beneficial effects of β_2 -AR modulation remain unclear and little is known about β_2 -AR signaling in CD4 T cells during sepsis. In a murine endotoxemia model, β_2 -AR blockade reduced survival while, in septic pigs, it amplified sepsis-induced hepatic injury^{36, 53, 54}. In humans, it has been reported that β_2 -AR polymorphism is associated with increased mortality in septic shock⁵⁵. In this study, we only investigated the impact of β_1 -AR blockade on alteration of CD4 T cell patterns. The impact of selective β_2 -AR modulation as well as non-specific β -AR blockade on CD4 T cells during sepsis merits investigation.

In summary, in the murine model tested, the β_1 -AR contributes to sepsis-induced immunosuppression through modulation of regulatory T cell inhibitory functions. β_1 -AR

blockade, while restoring CD4 T cell homeostasis, also decreased global systemic inflammation and improved cardiac efficiency. Thus, instead of considering β_1 -AR blockade as only a hemodynamic treatment, we suggest an immunoregulatory mechanism by which β_1 -AR blockers may also be beneficial in sepsis.

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Authors' contributions.

MD, AK: study concept and design. MD, HL: Experiments (MD: involved in all experiments, HL involved in flow cytometry supervision. CL, AK with the help of François Husson: statistical analysis, AK, MD, PA, JPF, MS, BGC: Drafting of the manuscript. All authors were involved in critical revision of the manuscript. All authors read and approved the final manuscript.

Availability of supporting data.

Raw data and statistical analyses of all presented results are available at the following: <https://osf.io/nz5u9/> or DOI 10.17605/OSF.IO/NZ5U9.

MD takes the responsibility of the integrity of the data.

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Supplemental Materials:

Expanded Materials & Methods

Tables S1 & S2

Figure S1 – S10

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Tables

Table 1: Despite reduced heart rate, β_1 -AR blockade did not alter hemodynamic characteristics in high-grade sepsis. Hemodynamic characteristics were measured by echocardiography 18 hours after surgery. Data are expressed as mean \pm SD.

Variable	$\beta_1^{+/+}$					$\beta_1^{-/-}$		
	Sham	CLP	CLP + esmolol	P value	Adjusted P value	Sham	CLP	P value
	n=13	n=13	n=18			n=10	n=12	
Female (%)	54%	46%	56%			50%	50%	
Weight (g)	27.5 \pm 5.0	28.4 \pm 4.1	26.8 \pm 3.0	0.53	ns	29.1 \pm 4.4	28.4 \pm 5.8	0.61
Heart rate (min ⁻¹)	360 \pm 48	318 \pm 31	273 \pm 45	<0.001	0.23: Sham vs. CLP <0.001: Sham vs. CLP + esmolol 0.07: CLP vs. CLP + esmolol	250 \pm 27	274 \pm 45	0.24
Mean arterial pressure (mmHg)	84.8 \pm 6.9	62.2 \pm 11.8	67.4 \pm 17.3	<0.001	<0.001: Sham vs. CLP 0.01: Sham vs. CLP + esmolol 0.51: CLP vs. CLP + esmolol	80.9 \pm 15.9	65.2 \pm 11.9	0.04
Cardiac index (ml/min/g)	0.59 \pm 0.09	0.30 \pm 0.13	0.29 \pm 0.17	<0.001	<0.001: Sham vs. CLP <0.001: Sham vs. CLP + esmolol 0.99: CLP vs. CLP + esmolol	0.43 \pm 0.04	0.27 \pm 0.12	0.002
Cardiac power index (W/g)	0.11 \pm 0.02	0.04 \pm 0.02	0.05 \pm 0.03	<0.001	<0.001: Sham vs. CLP <0.001: Sham vs. CLP + esmolol 0.99: CLP vs. CLP + esmolol	0.08 \pm 0.02	0.04 \pm 0.02	<0.001
Stroke volume (μ L)	44.5 \pm 8.1	26.5 \pm 10.2	28.1 \pm 15.9	0.001	0.004: Sham vs. CLP 0.01: Sham vs. CLP + esmolol 0.99: CLP vs. CLP + esmolol	50.3 \pm 10.7	26.9 \pm 10.5	<0.001

Figures and Figure Legends

Figure 1: β_1 -AR contributes to the post-septic immunosuppressive phenotype, which is reversed by β_1 -AR blockade. Splenocytes were immunophenotyped 18 hours after sepsis induction. The proportion of CD4 T cells in live cells (A), the proportion of CD8 T cells in live cells (B), and the proportion of Treg cells (CD25^{hi} CD127^{low}) in CD4 T cells (C) were assessed. Density plots of Treg cells for one representative mouse per group are showed in panel (D). Data are expressed as mean \pm SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 2: β_1 -AR activation decreases CD4 T cell proliferation during sepsis. Splenocytes harvested 18 hours after sepsis induction were cultured for 72 hours with a mitogen (PHA) to assess their proliferative response. (A) Histograms of the proliferation marker in non-stimulated and PHA stimulated splenocytes from one representative $\beta_1^{+/+}$ sham and CLP mouse. Each peak has been fitted and colored by FlowLogic software. (B) Proliferation of CD4 T cells according to cell tracking dye staining. Data are expressed as mean \pm SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 3: β_1 -AR stimulation of Treg cells increases their immunosuppressive capacities. Treg cells isolated from $\beta_1^{+/+}$ mice were treated with dobutamine or PBS for 30 minutes. Pre-treated Treg cells were then co-cultured with CD4 T cells at a 1:4 ratio in the presence of anti-CD3 and anti-CD28 beads for 5 days. A fluorescent green reagent was added for counting dead cells. (A) shows the real-time cell count normalized to the first count at H0, and (B) shows the dead cell count as a percentage of total cells. Data shown are the means of 4 images per well in duplicate with 4 mice per time point \pm SEM (total 32 images/time/group). Images were taken at 20 \times magnification on the IncuCyte[®] imager.

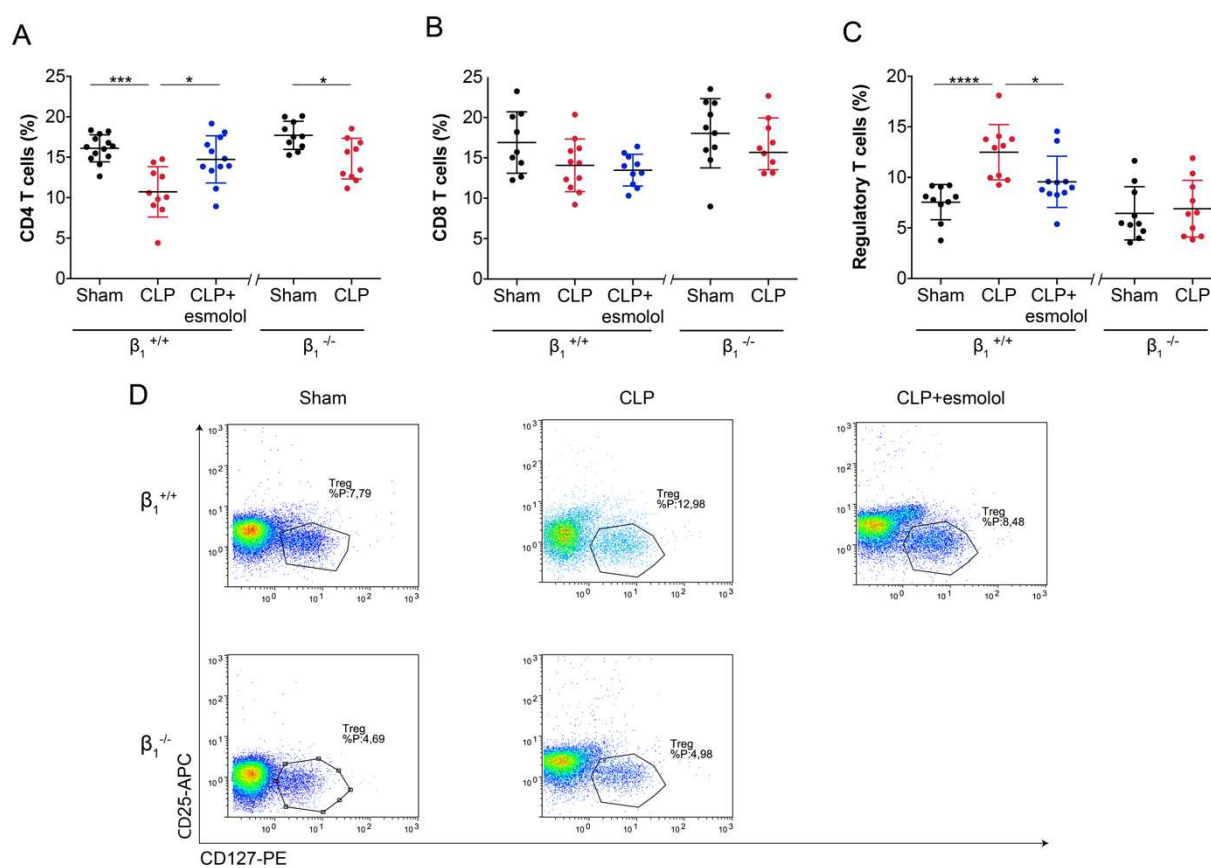
Figure 4: β_1 -AR modulates the level of both pro- and anti-inflammatory circulatory mediators. Plasma levels of 46 inflammatory mediators were measured in 5–6 animals per group. Data are expressed as Log10(mean).

Figure 5: β_1 -AR blockade modulates survival during sepsis. Survival curve of $\beta_1^{+/+}$ CLP mice, $\beta_1^{+/+}$ CLP mice receiving esmolol for 24 hours, and $\beta_1^{-/-}$ CLP mice.

Figure 6: Graphical summary of the involvement of β_1 -AR in sepsis-induced dysautonomia and the beneficial effects of receptor blockade.

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Figure 1



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Figure 2

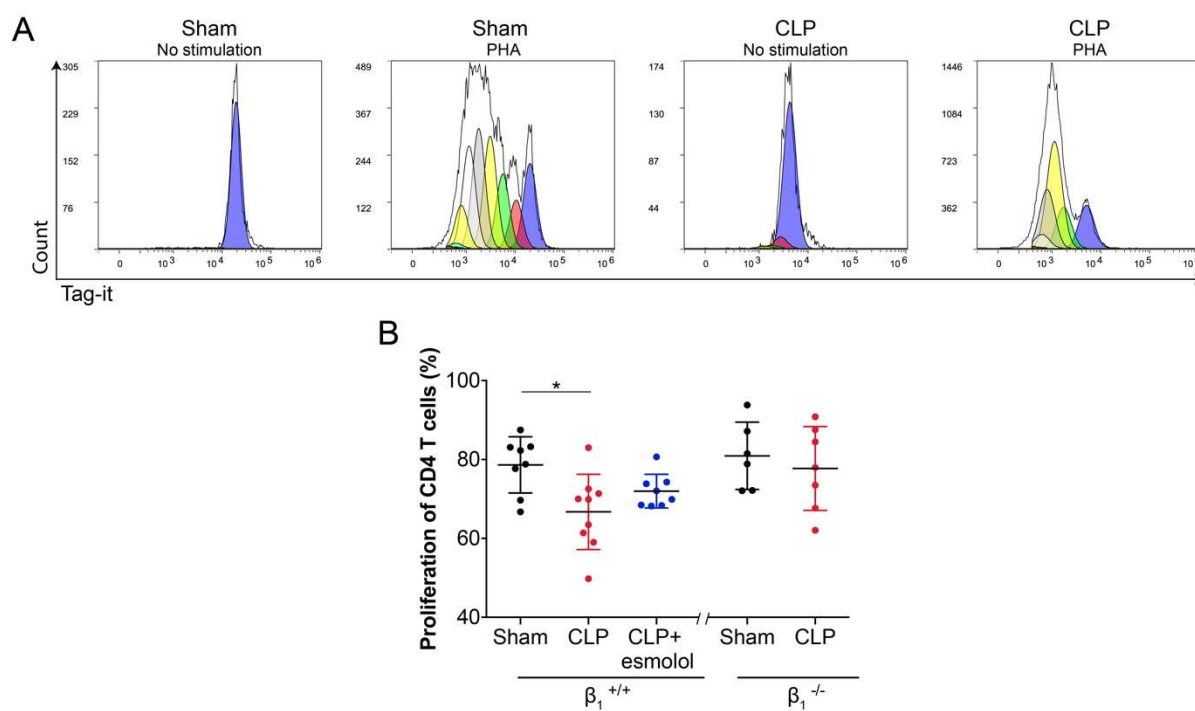


Figure 3

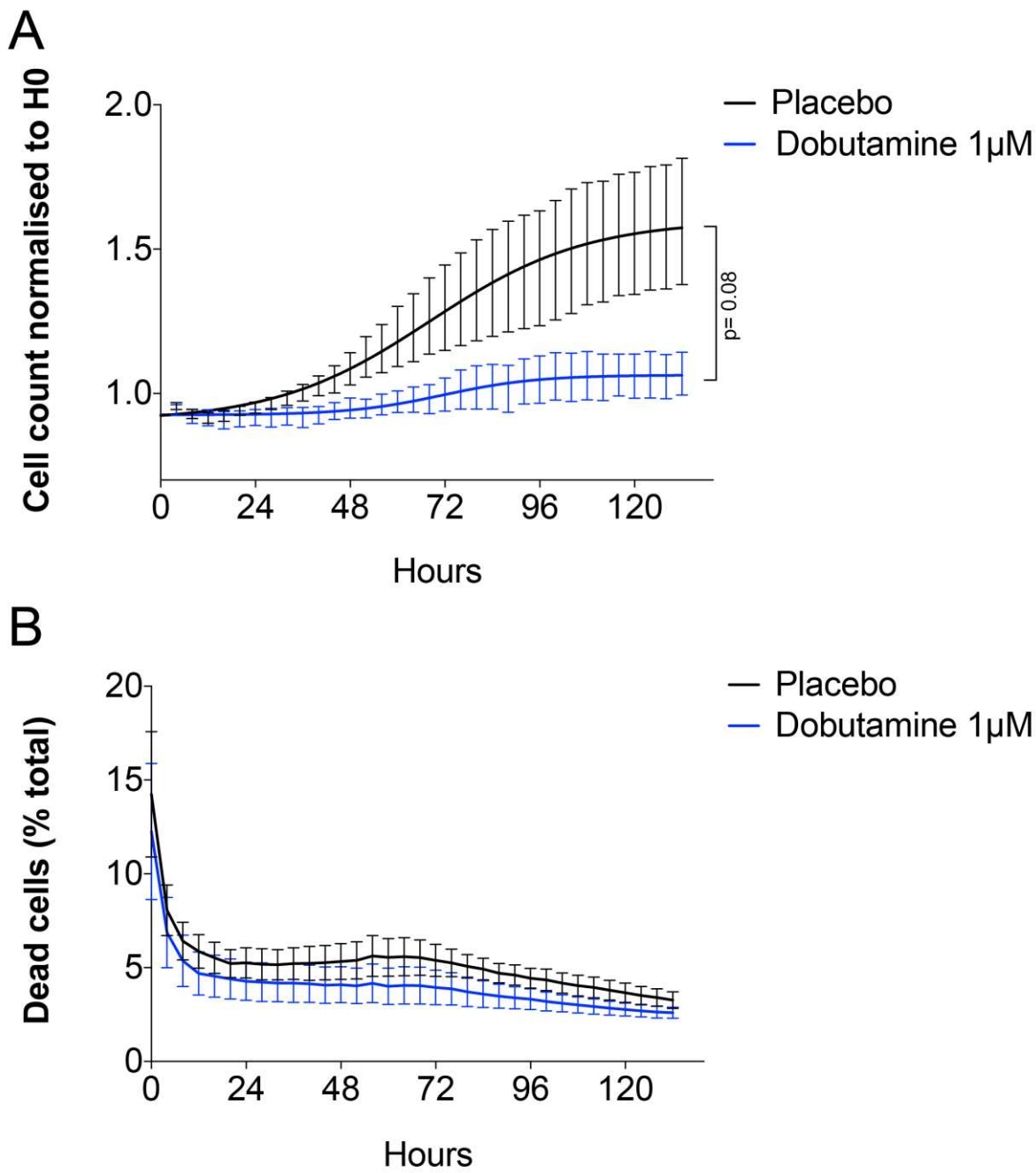


Figure 4

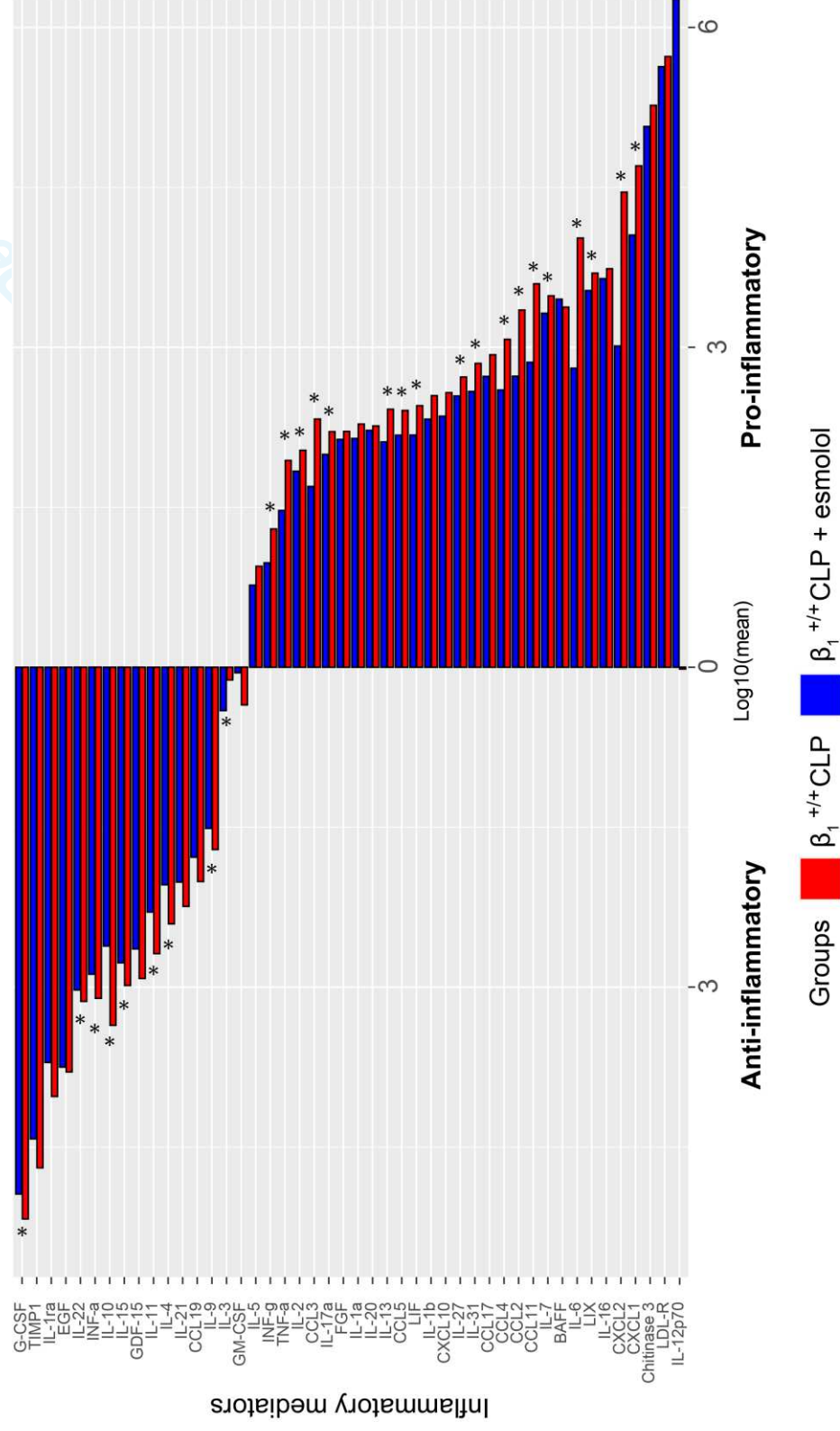
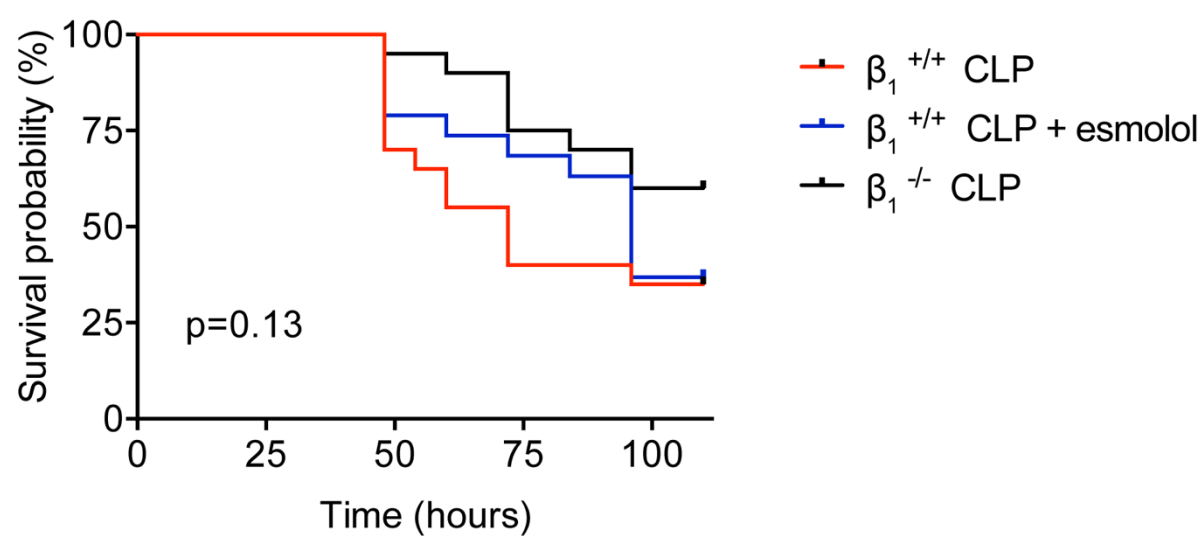
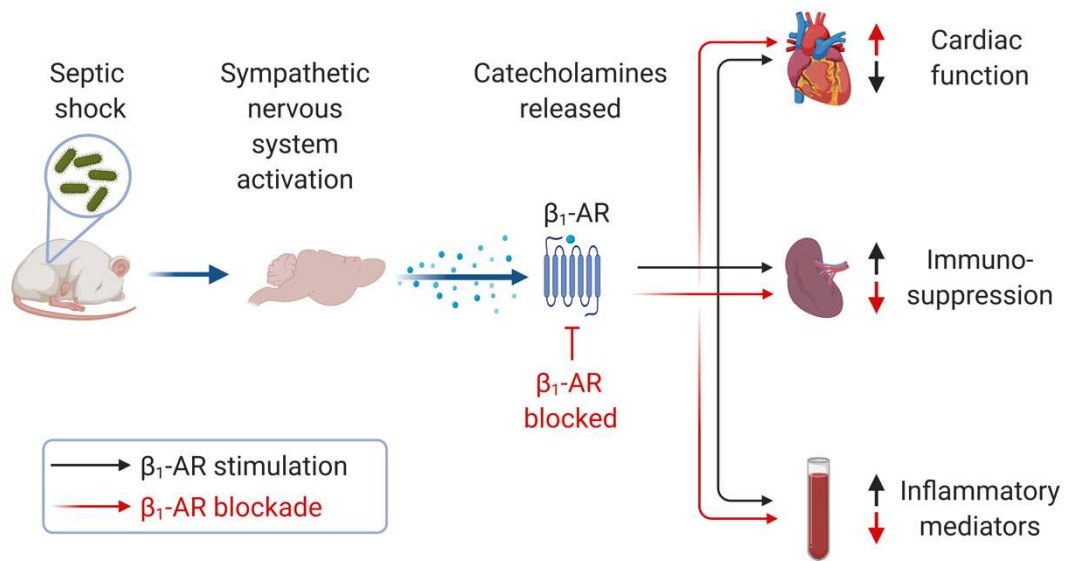


Figure 5



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Figure 6:



SUPPLEMENTAL MATERIAL

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Detailed Methods

Echocardiography: Mice were placed on a prewarmed (38 °C) electric heat pad, anaesthetized with 1.5% isoflurane and kept at rest for 5 minutes before the echocardiographic assessment.

Ascending aortic diameter (AD) was obtained during systole from the left parasternal long axis view. The aortic velocity time integral (VTI) was measured from the aortic arch.

Stroke volume (SV) and cardiac output (CO) were calculated with the following formulas:

$$SV = VTI \times \pi \times AD^2 / 4$$

$$CO = HR \times SV$$

The CO was divided by the weight of the mouse to define the cardiac index (CI). The cardiac power index (CPI) was calculated with the following formula: $CPI = MAP \times CI / 451$. All measurements were averaged over three consecutive cardiac cycles. Mean arterial pressure (MAP) was measured by a blood pressure transducer (Emka, Paris, France) inserted through the left carotid artery.

Supplemental Tables

Table S1: Number and reasons for failed measurements and excluded values.

Experiment	n=	Measure failed	Reason	Excluded value	Reason
Immunophenotyping	T cells	11	Reagent expired	4	No staining
	Treg cells	11	Reagent expired	1	No staining
Echocardiography	74	NA		NA	
Mean arterial pressure measurement		4	Catheterization failed	NA	
Plasma level of inflammatory mediators		NA		37*	Not measured
Proliferation assay	44	NA		NA	
CD4 T and Treg cells co-culture	4	1 well	Contaminated	NA	
Survival study	60	NA		1	Mouse dead before the first count

*Due to covid-19 pandemia, Magnetic Luminex® Performance Assay – Mouse XL Discovery Premixed kit (Bio-Techne, Minneapolis, MN) distribution was stopped by the manufacturer. Thus 37 plasma samples were analyzed.

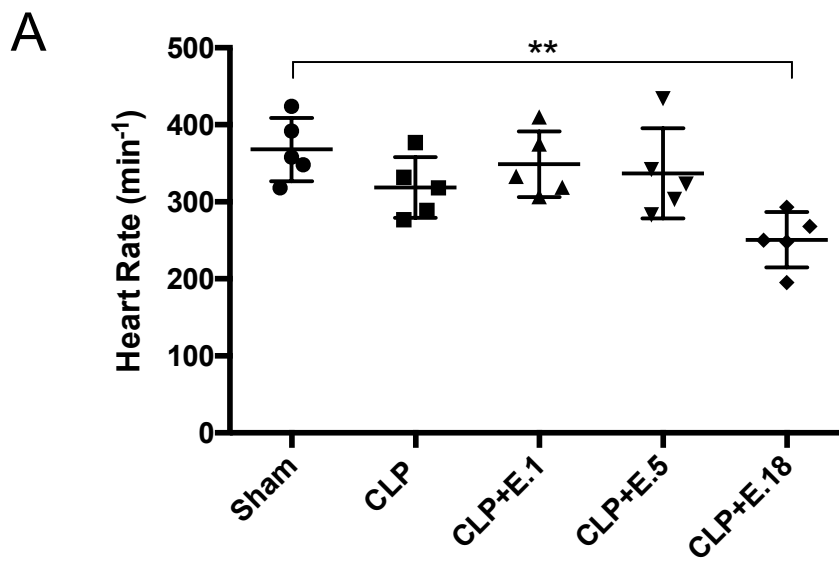
Table S2: Sepsis induces an increase in both pro- and anti-inflammatory mediators in

$\beta_1^{+/+}$ and $\beta_1^{-/-}$ mice. Data are expressed as mean \pm SD (pg/ml)

Inflammatory mediators (pg/ml)	$\beta_1^{+/+}$			$\beta_1^{-/-}$		
	Sham n=5	CLP n=6	P adjusted	Sham n=5	CLP n=5	P adjusted
BAFF	2007 \pm 559	2381 \pm 441	0.352	2434 \pm 825	3953 \pm 1554	0.165
CCL2	59 \pm 27	2233 \pm 3098	0.008	62 \pm 51	1629 \pm 1298	0.014
CCL3	4.2 \pm 2.2	212 \pm 278	0.009	1.9 \pm 2.1	122 \pm 54	0.014
CCL4	148 \pm 26	1188 \pm 1746	0.008	100 \pm 30	751 \pm 439	0.014
CCL5	74 \pm 17	255 \pm 48	0.008	73 \pm 11	233 \pm 90	0.014
CCL11	380 \pm 64	3939 \pm 2277	0.008	384 \pm 114	2244 \pm 1810	0.014
CCL17	49 \pm 22	852 \pm 425	0.008	53 \pm 42	1101 \pm 717	0.014
CCL19	59 \pm 80	102 \pm 40	0.137	17 \pm 10	84 \pm 30	0.014
Chitinase 3	13360 \pm 2334	186017 \pm 11300	0.008	19289 \pm 9545	227808 \pm 114392	0.014
CXCL1	304 \pm 223	50267 \pm 29822	0.008	343 \pm 279	54595 \pm 57326	0.014
CXCL2	10 \pm 6	28489 \pm 60858	0.008	15 \pm 8	33145 \pm 66852	0.014
CXCL10	111 \pm 46	375 \pm 170	0.008	58.3 \pm 26	581 \pm 360	0.014
EGF	7219 \pm 5897	6233 \pm 5486	0.645	5292 \pm 4928	6705 \pm 6349	1
FGF	85 \pm 9	163 \pm 17	0.009	76 \pm 12	167 \pm 17	0.014
G-CDF	542 \pm 392	149309 \pm 0	0.008	1287 \pm 1948	106311 \pm 58876	0.014
GDF-15	117 \pm 85	830 \pm 480	0.008	117 \pm 77	1133 \pm 820	0.014
GM-CSF	0.4 \pm 0.1	2.3 \pm 0.8	0.009	0.4 \pm 0.2	1.8 \pm 0.8	0.014
INF- α	401 \pm 85	1274 \pm 255	0.008	345 \pm 134	1035 \pm 245	0.014
INF- γ	2.9 \pm 1.5	20 \pm 11	0.008	0.7 \pm 0.8	17 \pm 8	0.014
IL-1 α	26 \pm 4	191 \pm 65	0.009	38 \pm 33	188 \pm 31	0.014
IL-1 β	104 \pm 37	352 \pm 333	0.008	61 \pm 9	320 \pm 150	0.014
IL-1ra	202 \pm 71	10603 \pm 10074	0.008	156 \pm 76	11724 \pm 9628	0.014
IL-2	22 \pm 1	108 \pm 13	0.009	22 \pm 11	110 \pm 19	0.014
IL-3	0.2 \pm 0.2	0.8 \pm 0.2	0.009	0.2 \pm 0.2	0.4 \pm 0.5	0.692
IL-4	41 \pm 41	255 \pm 43	0.009	11 \pm 24	251 \pm 57	0.014
IL-5	119 \pm 120	8.8 \pm 4.9	0.092	65 \pm 48	12 \pm 14	0.036
IL-6	53 \pm 27	10583 \pm 15012	0.008	55 \pm 36	7587 \pm 7756	0.014
IL-7	457 \pm 153	3041 \pm 298	0.008	419 \pm 283	2969 \pm 958	0.014
IL-9	19 \pm 6	51 \pm 8	0.009	16 \pm 10	47 \pm 1	0.014
IL-10	41 \pm 7	2281 \pm 1223	0.009	42 \pm 15	1595 \pm 1679	0.014
IL-11	88 \pm 43	486 \pm 220	0.008	31 \pm 47	391 \pm 115	0.014
IL-12p70	0.0 \pm 0.0	1.0 \pm 2.4	0.476	0.0 \pm 0.0	7 \pm 15	0.453
IL-13	76 \pm 9	262 \pm 40	0.008	48 \pm 44	254 \pm 38	0.014
IL-15	226 \pm 78	965 \pm 182	0.008	201 \pm 60	906 \pm 142	0.014
IL-16	2100 \pm 585	5450 \pm 806	0.008	1755 \pm 238	4762 \pm 1584	0.014
IL-17a	67 \pm 13	162 \pm 59	0.009	70 \pm 14	146 \pm 52	0.014
IL-20	234 \pm 107	183 \pm 150	0.448	225 \pm 139	197 \pm 136	0.86
IL-21	44 \pm 14	175 \pm 31	0.008	21 \pm 26	143 \pm 27	0.014
IL-22	656 \pm 148	1361 \pm 115	0.009	582 \pm 69	1333 \pm 195	0.014
IL-27	152 \pm 17	527 \pm 59	0.009	142 \pm 43	501 \pm 101	0.014
IL-31	215 \pm 66	707 \pm 114	0.008	138 \pm 92	593 \pm 81	0.014
LDL R	227140 \pm 24317	532897 \pm 130243	0.008	189990 \pm 30388	626815 \pm 128731	0.014
LIF	31 \pm 9	283 \pm 126	0.009	40 \pm 20	218 \pm 48	0.014
LIX	659 \pm 332	4954 \pm 677	0.008	1060 \pm 730	4889 \pm 626	0.014
TIMP1	5910 \pm 1524	49443 \pm 39078	0.008	6117 \pm 1523	59504 \pm 39477	0.014
TNF- α	10 \pm 3	87 \pm 73	0.009	11 \pm 3	62 \pm 27	0.014

Supplemental Figures and Figure Legends

Figure S1: Esmolol at 18 mg/kg/h decrease heart rate by 20% in septic mice. Esmolol infusion (E.) at 1, 5 or 18 mg/kg/h was started 3 hours post-infection in $\beta_1^{+/+}$ mice. (A) HR was measured by echocardiography at 18 hours after surgery. (B) Details of HR. Data are expressed as mean \pm SD. ** $p < 0.01$.

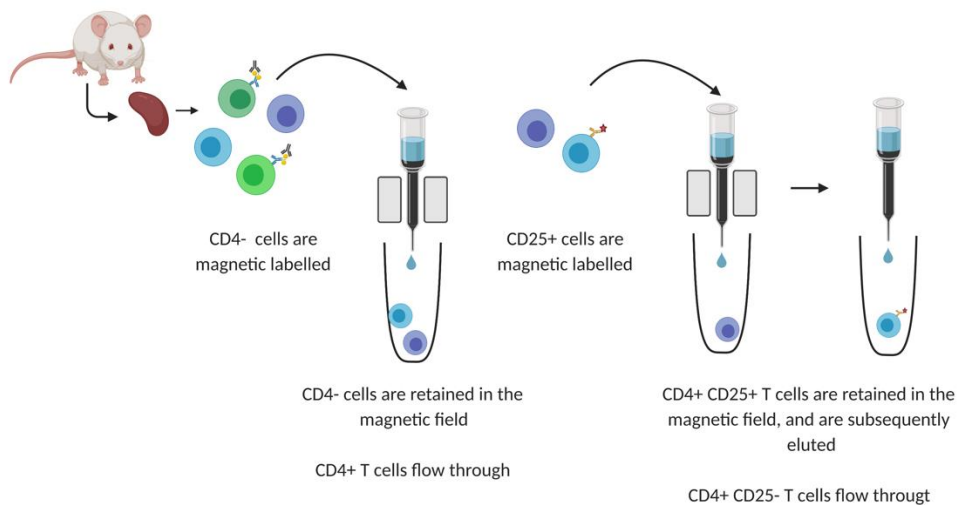


B

	n=	Heart rate
Sham	5	368 ± 41
CLP	5	319 ± 39
CLP+E.1	5	349 ± 43
CLP+E.5	5	337 ± 59
CLP+E.18	5	251 ± 36

Figure S2: Isolation of CD4 T cells and Treg cells. (A) Isolation of CD4⁺ and Treg cells in a two-step procedure. Firstly, CD4 T cells were negatively selected by depleting unwanted cells with magnetic beads bound to biotin-conjugated antibodies against CD8a, CD11b, CD45R, CD49b, and Ter-119. Labelled cells were retained in the magnetic field. CD4 T cells flow through. Then, CD25⁺ cells were positively selected from the CD4⁺ fraction. The CD4⁺ CD25⁻ T cells flow through the magnetic field, the CD4⁺ CD25⁺ Treg cells are retained and subsequently eluted. (B) After magnetic separation, Treg cells were analyzed by flow cytometry in order to assess the purity of the cell population. The cell viability, percentage of CD4⁺ cells and percentage of CD25⁺ cells were analyzed.

A



B

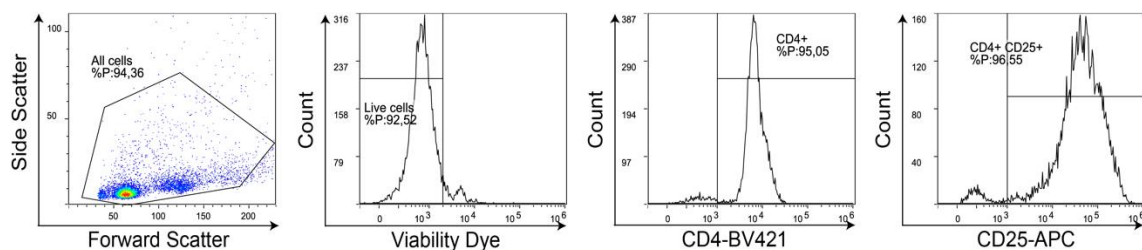


Figure S3: Co-culture of CD4 T cells and Treg cells pre-treated with catecholamines. Treg cells were stimulated with catecholamines 30 minutes before being co-culture with CD4 T cells. T cell were stimulated to proliferate using anti-CD3 and anti-CD28 beads. Dead cells were identified with a green fluorescent dye. All wells were imaged every 2 h. The total number of cells and the number of green dead cells were counted.

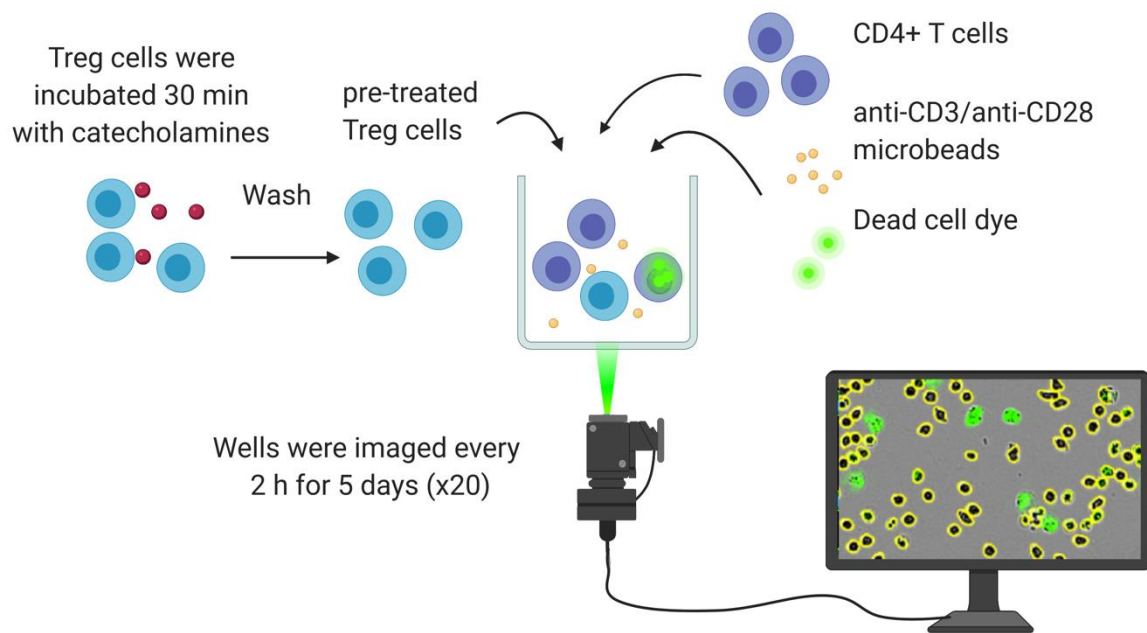


Figure S4: β_1 -AR blockade in sham treated mice does not affect lymphocyte proportions and functions. The proportions of CD4 T cells in live cells (A), the proportion of Treg cells (CD25^{hi} CD127^{low}) (B) in CD4 T cells, and the CD4 T cell proliferative capacity (C) were assessed in sham mice where β_1 -AR was pharmacologically or genetically blocked. Data are expressed as mean \pm SD.

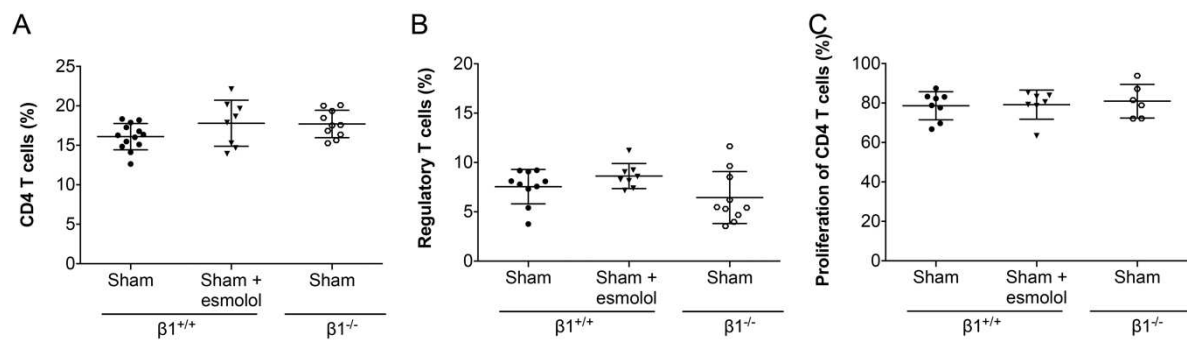


Figure S5: β_1 -AR does not affect the Th1, Th2, Th17 subpopulation proportions in sepsis.

Splenocytes were immunophenotyped 18 hours after sepsis induction. Proportion of Th1 (INF- δ +) (A), Th2 (IL-4+) (B) and Th17 (IL-17+) (C) cells in CD4 T cells were determined. Data are expressed as mean \pm SD.

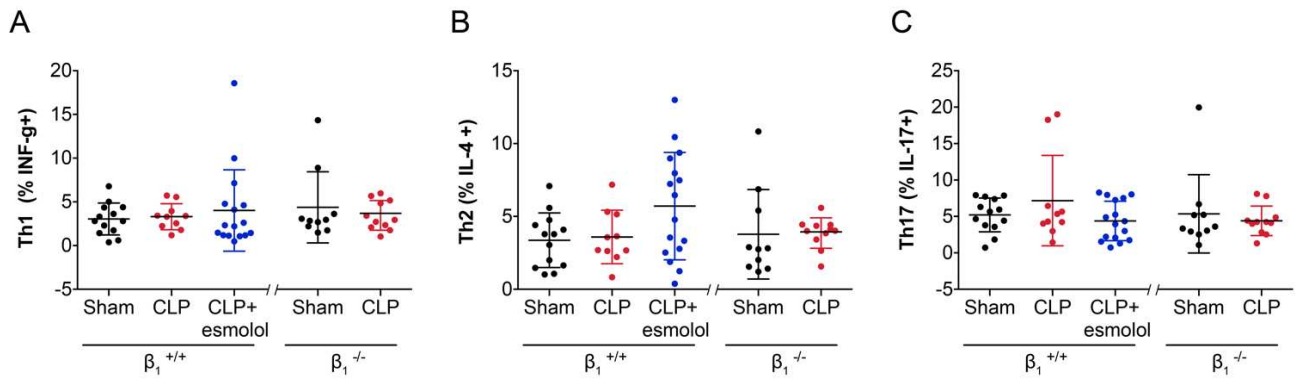


Figure S6: β_1 -AR does not affect lymphocyte viability in sepsis. Splenocytes were immunophenotyped 18 hours after sepsis induction. The viabilities of CD4 T cells (A), CD8 T cells (B) and Treg cells (C) were determined in each group. Data are expressed as mean \pm SD.

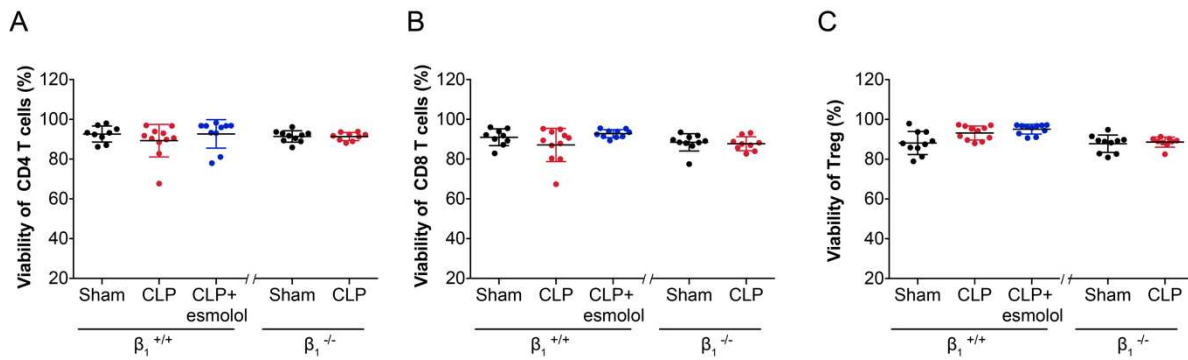


Figure S7: β_1 -AR does not affect splenocyte viability after a proliferation assay. Eighteen hours after sepsis induction, splenocytes were harvested and subjected to a proliferation assay by culturing them for 72 hours in the presence of a mitogen (PHA). At the end of this experiment, the proportion of live cells was measured in each group. Data are expressed as mean \pm SD.

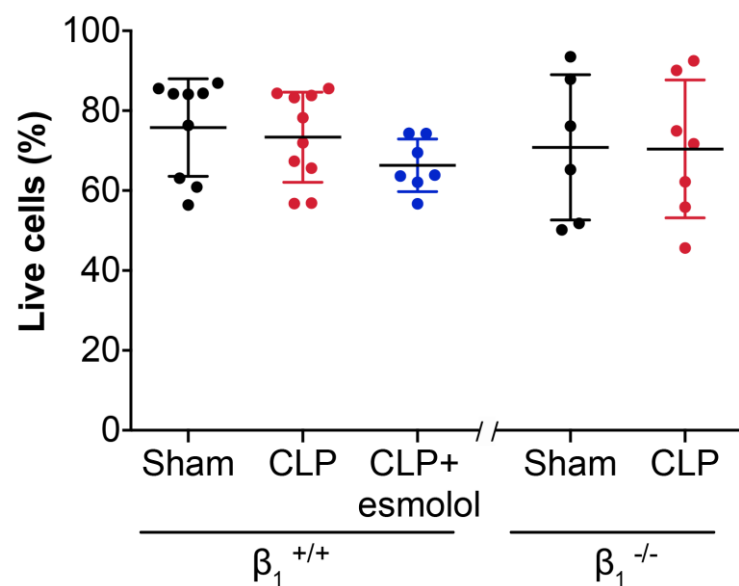


Figure S8: Treg cell stimulation by catecholamines increases their immunosuppressive capacities. Treg cells isolated from $\beta_1^{+/+}$ mice were treated with phenylephrine, norepinephrine or PBS for 30 minutes. Pre-treated Treg cells were then co-cultured with CD4 T cells at a 1:4 ratio in the presence of anti-CD3 and anti-CD28 beads for 5 days. A fluorescent green reagent was added for counting dead cells. (A) shows the real-time cell count normalized to the first count at H0, and (B) shows the dead cell count as a percentage of the total cells. Data shown are the means of 4 images per well in duplicate with 4 mice per time point \pm SEM (total 32 images/time/group). Images were taken at 20 \times magnification on the IncuCyte[®] imager.

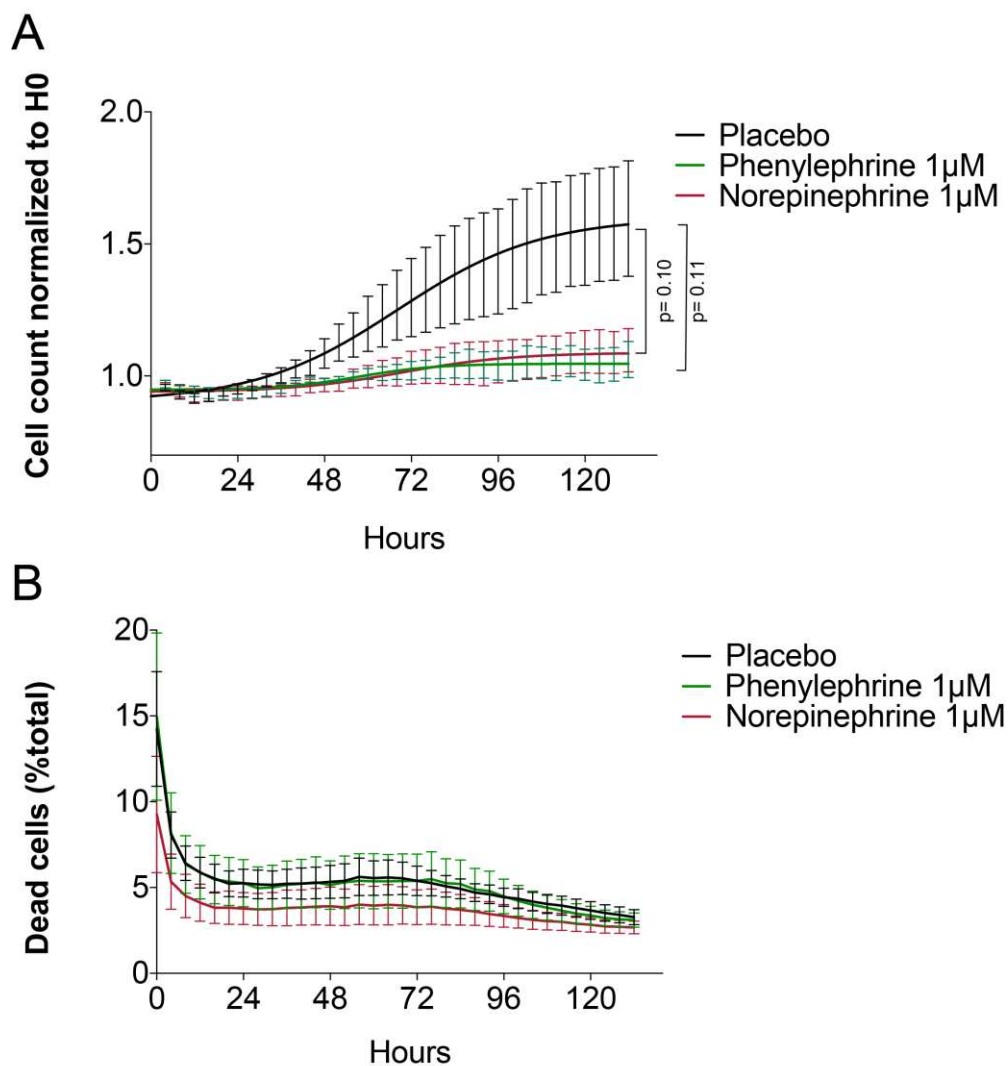


Figure S9: Sex does not affect the hemodynamic effects of β_1 -AR. Heart rate (A,B), mean arterial pressure (C,D) and cardiac index (E,F) are presented according to the sex of the animals in the $\beta_1^{+/+}$ (A,C,E) and $\beta_1^{-/-}$ (B,D,F) groups. Data are expressed as mean \pm SD.

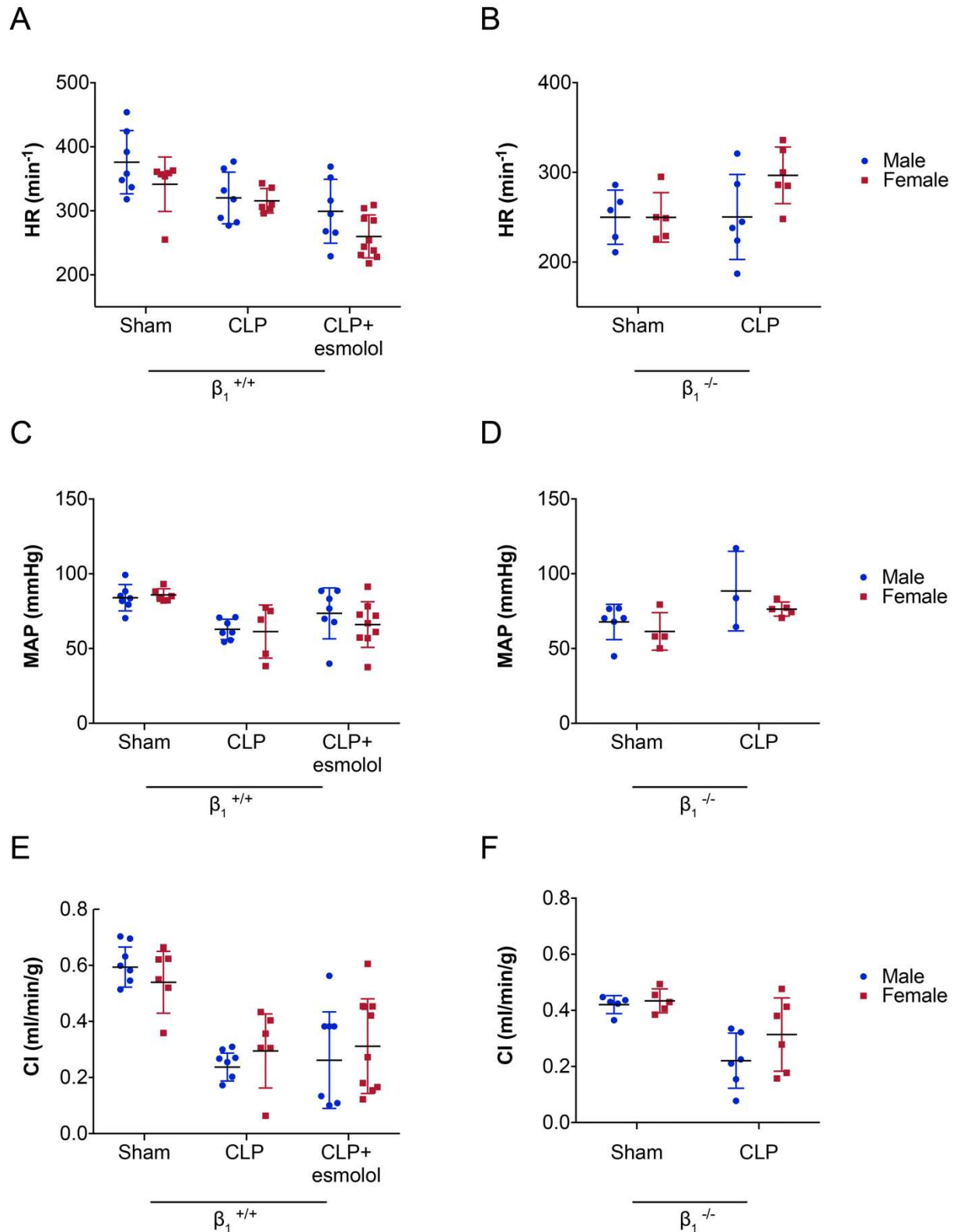
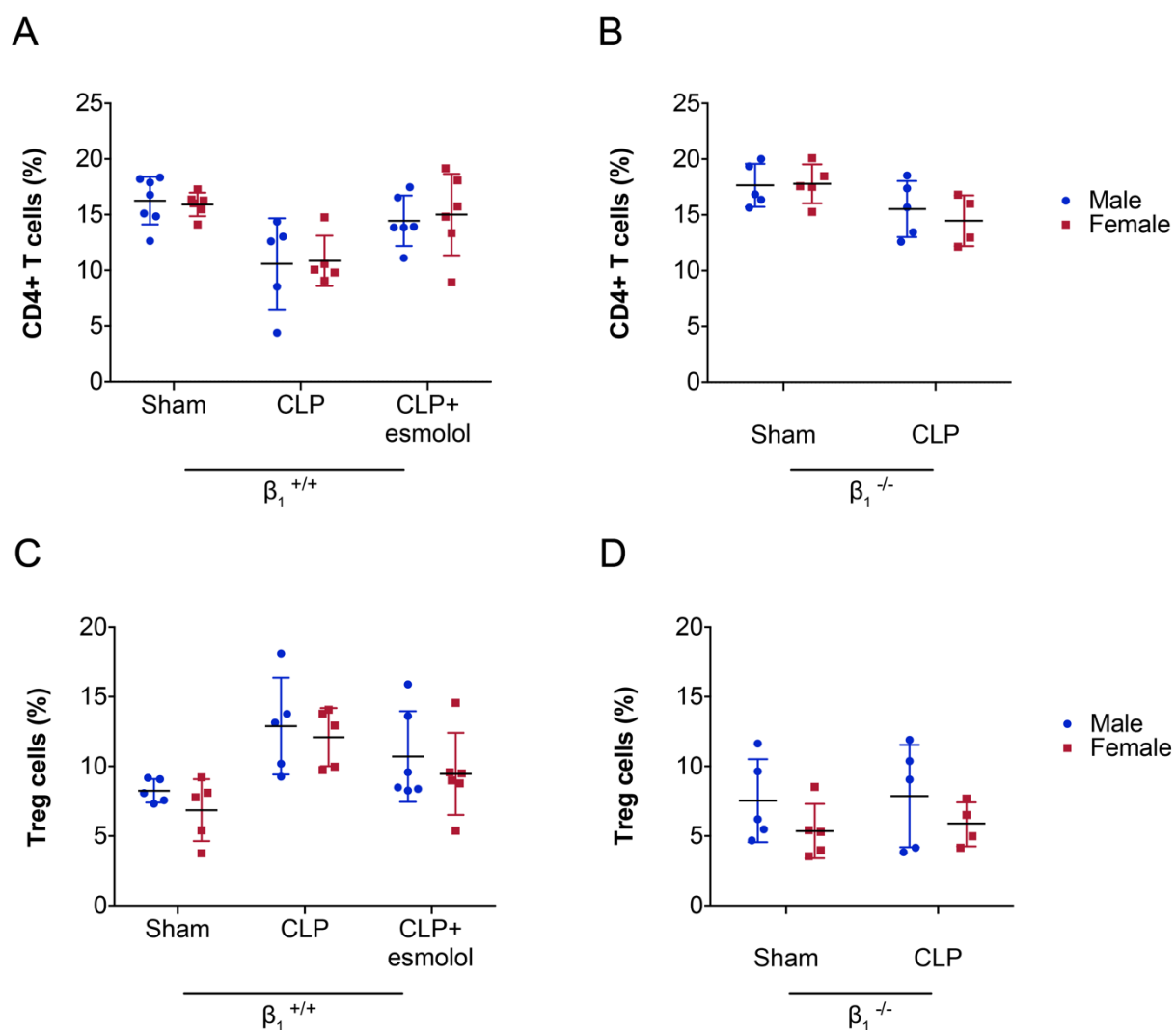


Figure S10: Sex does not affect the immune effects of β_1 -AR. CD4 T cell (A,B) and Treg cell (C,D) proportions are presented according to the sex of the animals in the $\beta_1^{+/+}$ (A,C) and $\beta_1^{-/-}$ (B,D) groups. Data are expressed as mean \pm SD.



Résumé

Contexte : La dysautonomie sympathique est une complication sepsis à l'origine d'une altération des fonctions cardiovasculaires, d'une immunosuppression et d'une surmortalité. Lors d'une septicémie, la modulation du système sympathique par le blocage des β_1 -adrénorécepteurs protège les fonctions cardiovasculaires. En revanche, l'effet du blocage β_1 -adrénergique sur la réponse immunitaire adaptative n'a que très peu été étudiée.

Objectifs : Cette étude vise à étudier les effets de la modulation β_1 -AR sur les dysfonctions lymphocytaires T CD4 (LT CD4) dans un modèle murin sévère de sepsis.

Méthodes et résultats : La septicémie est induite par ligature et ponction du caecum chez des souris knock-out pour le β_1 -adrénorécepteur et des souris sauvages traitées ou non par un β_1 -AR bloquant (esmolol). Dix-huit heures après la chirurgie, les souris présentaient toutes les caractéristiques d'un sévère sepsis et notamment une diminution des proportions des LT CD4 et de leurs capacités prolifératives, ainsi qu'une augmentation des proportions des LT régulateurs (Tregs), trois marqueurs caractéristiques de l'immunoparalysie. Le blocage pharmacologique et génétique des β_1 -adrenorecepteurs a permis de rétablir des proportions de LT CD4 et de Treg similaires aux souris non-septiques, mais également de maintenir les capacités prolifératives des LT CD4. Le blocage β_1 -adrénergique entraîne également une pondération globale des cytokines inflammatoires circulantes ainsi qu'une amélioration de l'efficacité myocardique in vivo.

Conclusion : Le renforcement des fonctions inhibitrices des Tregs survenant lors du sepsis est réduit pour le blocage β_1 -adrénergique. Dès lors, bloquer ces récepteurs pourrait avoir un réel intérêt thérapeutique pour contrer l'immunosuppression post-septique en rétablissant l'homéostasie des LT CD4.

Abstract

Rationale: Sepsis typically induces dysautonomia, an over-activation of the sympathetic nervous system. This can lead to impaired cardiovascular function and immunosuppression, both of which are associated with poor outcome. Beta1-adrenoreceptor blockade downregulates dysautonomia. Although cardiovascular benefits of β_1 -adrenoreceptor blockade have been described in sepsis, little is known about its impact on the adaptive immune response, specifically CD4 T cells.

Objective: To investigate the effects of β_1 -adrenoreceptor modulation on CD4 T cell function in a murine model of sepsis.

Methods and results: High-grade sepsis was induced by caecal ligation and puncture (CLP) in wild-type mice (β_1 +/+) with or without esmolol (a selective β_1 -adrenoreceptor blocker), or in β_1 -adrenoreceptor knockout mice (β_1 -/-). At 18 hours, β_1 +/+ mice exhibited characteristics of high-grade sepsis and three surrogate markers of immunosuppression, namely decreased splenic CD4 T cells, reduced CD4 T cell proliferation and increased Treg cell proportions. Pharmacologic and genetic β_1 -adrenoreceptor blockade reversed the impact of sepsis on CD4 and Treg proportions and maintained CD4 T cell proliferative capacity. β_1 -adrenoreceptor blocked mice also exhibited a global decrease in both pro- and anti-inflammatory mediators and improved in-vivo cardiovascular efficiency with a maintained cardiac power index despite the expected decrease in heart rate.

Conclusion: β_1 -adrenoreceptor activation enhances Treg inhibitory function and thus contributes to sepsis-induced immunosuppression. This can be attenuated by β_1 -adrenoreceptor blockade, suggesting a potential immunoregulatory role for this therapy in the treatment of sepsis.