UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

Alterações na expressão de receptores de peptídeos formilados, citocinas e monócitos que auxiliam no colapso vascular e imunológico da sepse

Patrícia Terra Alves

Uberlândia – MG

2017

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Tese apresentada a Universidade Federal de Uberlândia como parte dos requisitos para obtenção do Título de Doutor em Genética e Bioquímica (Área: Genética)

Uberlândia – MG

2017

Dados Internacionais de Catalogação na Publicação (CIP)
Sistema de Bibliotecas da UFU, MG, Brasil.

A474a
2017

Alves, Patrícia Terra, 1986-
Alterações na expressão de receptores de peptídeos formilados, citocinas e monócitos que auxiliam no colapso vascular e imunológico da sepse / Patrícia Terra Alves. - 2017.
110 f. : il.

Orientador: Luiz Ricardo Goulart.
Tese (doutorado) - Universidade Federal de Uberlândia, Programa de Pós-Graduação em Genética e Bioquímica.
Disponível em: http://dx.doi.org/10.14393/ufu.te.2017.19
Inclui bibliografia.

1. Genética - Teses. 2. Choque séptico - Teses. 3. Citocinas - Teses. 4. Sepse - Diagnóstico - Teses. I. Goulart, Luiz Ricardo. II. Universidade Federal de Uberlândia. Programa de Pós-Graduação em Genética e Bioquímica. III. Título.

CDU: 577.1

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Data da defesa: 16/10/2017

As sugestões da Comissão Examinadora e as Normas da PGGB para o formato da tese foram contempladas.

_____

Luiz Ricardo Goulart

Dedico este trabalho...

Aos meus pais, ***Joaquim e Ivanilde****,*

...meu porto seguro, minha base e responsáveis por tudo que me tornei!

As minhas irmãs, ***Larissa e Letícia****,*

... pela amizade, carinho e alegrias durante todos esses anos de nossas vidas.

“A tarefa não é tanto ver aquilo que ninguém viu,

mas pensar o que ninguém pensou sobre aquilo que todo mundo vê.”

Arthur Schopenhauer

AGRADECIMENTOS

Teve choro, teve risos... momentos em que o desespero bateu a porta, mas que a cada resultado positivo me mostrava o porquê me aventurei nesta etapa! Hoje olho para atrás e relembro com gratidão as pessoas que fizeram a diferença durante esta caminhada, fica aqui registrado o meu sincero MUITO obrigada!

À Deus por caminhar ao meu lado, me capacitar, permitir que os meus sonhos se tornem reais e colocar pessoas especiais em minha vida;

Aos meus pais, Joaquim Faria Alves e Ivanilde Terra Alves, por toda paciência, dedicação, amor, cuidado, cumplicidade, por idealizarem essa etapa e me ajudarem a concluí-la! Vocês são verdadeiros anjos na minha vida!

À toda minha família, pelo apoio, por compreenderem as minhas ausências, pelas orações e pelo amor que tem por mim! Vocês são essenciais na minha caminhada!

Ao Prof. Dr. Luiz Ricardo Goulart, pela oportunidade, por visualizar o meu potencial e despertar a pesquisadora que existe em mim! Estudar a sepse foi um grande desafio! Hoje percebo o quanto aprendi e amadureci nestes anos. Sou grata pelas oportunidades que tive de trabalhar com tantas técnicas, interagir com diferentes projetos e me tornar a profissional que sou hoje!

Ao Dr. Thulio Marquez Cunha, por abraçar a causa, por prover as amostras biológicas e principalmente por ser um médico com alma de cientista! A ciência necessita de médicos como você!

À equipe fantástica do Thulio: Augusto Ramos, Eduarda Lemes, Guilherme Leonel, Guilherme Teodoro e Matheus Pereira; por madrugarem no Hospital de Clínicas e permitirem que as amostras estivessem tão cedo em minhas mãos, vocês contribuíram de forma ímpar para realização deste estudo!

Aos voluntários que participaram, meus sinceros agradecimentos e respeito.

Ao Prof. Dr. Luiz Cláudio Cameron e ao Dr. Gustavo Souza por auxiliarem com a Proteômica;

À Larissa Prado, pela amizade e por aceitar se aventurar nos estudos com sepse me auxiliando na citômetria;

À Emília Rezende, Bruna Matias e Cláudia Mendonça pelas coletas de sangue e grandes momentos descontração;

Ao João Pedro Goulart, pela parceria e por auxiliar na tentativa de construção do biosensor para detecção de infecção;

Ao Douglas Eulálio Antunes por aprimorar os meus conhecimentos com estatística;

À Luciana Machado e a Hebreia Almeida pelas pessoas incríveis que são, pela energia que transmitem e a calma que trazem!

À Karina Marangoni, Aline Gomes e Victor Bastos pela amizade e os diversos momentos que compartilhamos: rindo, chorando e tendo insight que se tornaram verdadeiras eurekas! ...como é divertido e produtivo quando somos uma equipe, Padrão Ouro sempre!

À Léa Duarte que no último ano se tornou uma grande amiga!

Aos amigos de Pós-Graduação em Genética e Bioquímica, pela parceria, discussões científicas e momentos compartilhados;

Aos demais amigos leais, que o espaço não coube citar nomes, quero agradecer por toda palavra amiga, por torcerem e vibrarem comigo a cada conquista, por orarem por mim e principalmente florearem a minha caminhada... vocês sabem que são especiais!

A todos os Professores que encontrei ao longo desta caminhada que foram verdadeiros mestres na arte de ensinar;

Em especial gostaria de agradecer ao Prof. Dr. José Roberto Mineo por ter adotado a 1ª Turma de Biomedicina da UFU e independente do tempo sempre esteve pronto a ajudar; e ao Prof. Dr. Tiago Mineo por ter sido um bom conselheiro durante estes anos de pós-graduação;

As agências de fomento CNPq, CAPES e FAPEMIG por financiarem a pesquisa, em especial ao CNPq pela bolsa de auxílio financeiro oferecida;

À secretária da pós-graduação, Janaína de Souza, pela amizade e disposição em ajudar;

Aos funcionários do departamento, pela ajuda indispensável e atenção;

À Universidade Federal de Uberlândia, que tem sido a minha casa nos últimos 10 anos. Muito obrigada pela minha formação, por oferecer ensino e pesquisa de qualidade!

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LISTA DE ABREVIATURAS E SIGLAS

ACAD9: Membro da família Acyl-CoA desidrogenase 9, mitocondrial

ACP1: Proteína fosfotirosina fosfatase de baixo peso molecular

ADGRD1: Adesão ao receptor D1 acoplado a proteína G

AGES: Produtos de glicação avançada

ALB: Soro albumina

ALX: Lipoxina A4

AMIB: Associação de médicos intesivistas Brasileiros

ANAPC5: Subunidade complexa promotora de anafase 5

ANXA3: Anexina A3

APACHE: Fisiologia aguda e avaliação da saúde crônica

ATAD2B: Proteína 2B contendo domínio AAA da família ATPase

ATP: Adenosina trifosfato

ATP13A3: Provável transportador de cations ATPase 13A3

ATP6V1B1: Subunidade B da ATPase do protótipo do tipo V, isoforma renal

basic FGF: Fator de crescimento básico de fibroblasto

BCR: Região proteica próxima ao ponto de interrupção

C8B: Componente C8 da cadeia beta do complemento

CARS: Síndrome da Resposta Anti-Inflamatória Compensatória

CCT8: Proteína do complexo T subunidade 8

CD: Diferenciação de Cluster

CD40L: Ligante de CD40

cDNA: DNA complementar

CEACAM6: Antígeno carcinoma embriogênico relacionado com a molécula 6 de adesão celular

CEBPA: CCAAT/proteína alfa de ligação ao *enhancer*

CEBPB: CCAAT/ proteína beta de ligação ao *enhancer*

CEBPE: CCAAT/proteína epsilon de ligação ao *enhancer*

CHTF8: Isoforma homóloga de proteína 8 de fidelidade de transmissão cromossômica

CID: Dissociação induzida por colisão

CLCN4: H(+)/Cl(-) Transportador de câmbio 4

CLR: Receptores de Lectina tipo-C

COG4: Subunidade 4 de oligomérico complexo de golgi conservado

CTSG: Catepsina G

DAMP: Padrão Molecular Associado ao Dano

DLC1: Proteína 7 de ativação de Rho GTPase

DNA: Ácido Desoxirribonucleico

dsDNA: DNA dupla fita

EDTA: Ácido Etilenodiamino Tetra-Acético

EFTUD2: Pequeno componente ribonucleoproteína nuclear U5 de 116 kDa

EMRT: Tempo de Retenção de massa exato

EPRS: Bifuncional glutamato/prolina tRNA ligase

ESRP2: Proteína 2 regulatória do *splicing* epitelial

FAM167A: Proteína FAM167A

FasL: Ligante de Faz

FDR: False Discovery Rate

FGF2: Fibroblast growth factor 2

FIO2: Fração inspirada de Oxigênio

FLNB: Filamina-B

fMLP: N-formil-metionil-leucil-fenilalanina

FPR: Receptor de Peptídeo Formilado

FPR1: Receptor de Peptídeo Formilado 1

FPR2: Receptor de Peptídeo Formilado 2

FPR3: Receptor de peptídeo Formilado 3

GAPDH: Gliceraldeído 3-fosfato desidrogenase

G-CSF: Fator estimulante de colônia de granulócito

GM-CSF: Fator estimulante de colônia de granulócito e macrófago

GO: Gene Ontology

GRHL2: Fator de transcrição 2 similar a Grainyhead

GXYLT2: Glucoside xylosyltransferase 2

HIF-1: Hypoxia-inducible factor 1-alpha

HIST1H2AC: Histona H2A tipo 1-C

HIST1H2BB: Histona H2B tipo 1-B

HIST1H2BK: Histona H2B tipo 1-K

HIST1H4A: Histone H4

HLA-DR: Antígeno Leucocitário humano

HMGB1: Proteína B1 de grupo de alta mobilidade

HMGB2: Proteína B2 do grupo de alta mobilidade

HPSS: Proteínas de choque térmico

HYDIN: Proteína homologa indutora de hidrocefalia

ICU: Unidade de Cuidades Intensivos

IFN: Interferon

IGSF10: Imunoglobulina membro da superfamília 10

IL: Interleucina

IL1-ra: Receptor antagonista de IL-1

ILAS: Instituto Latino Americano de Sepse

ILK: Integrina tipo kinase

iNOS: Indutor de Oxido Nítrico Sintase

CXCL10: Proteína 10 induzida por interferon gama

ITGA3: Integrina alfa 3

ITGB3: Integrina beta-3

kDa: quilo daltons

KIR2DL2: Receptor tipo imunoglobulina de células *killer*

KLB: Beta-klotho

KRT1: Citoqueratina 1, tipo II

KRT10: Citoqueratina 10, tipo I

KRT9: Citoqueratina 9, tipo I

LC-MS/MS: Cromatografia líquida de espectrometria de massa em tandem

LCN2: Lipocalina associada a gelatinase de neutrófilos

LIGHT: Homológo de linfotoxina

LTF: Lactotransferrina

LZTR1: Regulador transcricional de Leucine-Zipper-like 1

MAP: Média da Pressão Arterial

MATR3: Matrin-3

MCP-1: Proteína 1 quimioatraente de monócitos

M-CSF: Fator estimulante de colônia de macrófago

MDA5: Proteína 5 associada a diferenciação de melanoma

MHC: Complexo de histocompatibilidade

MIP: Proteínas inflamatórias de macrófagos

MLH3: Proteína de reparo de incompatibilidade de DNA MLH3

MPO: Myeloperoxidase

mtDNA: DNA mitocondrial

MYH10: Miosina 10

MYO5: Miosina Va

mRNA: RNA mensageiro

NALP: Proteína contendo os domínios NACHT, LRR e PYD

NFX1: Fator de transcrição Nuclear

NK: *Natural killer*

NLR: Receptores semelhantes à NOD

NNMT: Nicotinamida N-metiltransferase

NOD: Receptores com domínio oligomerização de ligação a nucleotídeo

NRCAM: Molécula de adesão a célula neuronal

NUDC: Proteína de migração nuclear nudC

OSM: Oncostatina M

PaCO2: Pressão parcial de dióxido de carbono

PAMP: Padrão molecular associado ao patógeno

PaO2: Pressão arterial parcial de Oxigênio

PBMC: Células mononucleares do sangue periférico

PCR: Reação da Cadeia da Polimerase

PDGF-bb: Fator de crescimento derivado de plaquetas

PDPK1: Proteína quinase 1 dependente de 3 fosfoinositideo

PGK2: Quinase 2 fosfoglicerato

PICS: Síndrome do catabolismo, inflamação e imunossupressão persistente

PLAT: Ativador Plasmogênico tecidual

PPP1CB: Subunidade catalítica beta da proteína PP1 serina/treonina fosfatase

PRR: Receptor de Reconhecimento Padrão

PSMC4: Subunidade regulatória 6B da protease 26S

PTPN1: Proteína tirosina fosfatase tipo não receptor

qPCR: Reação da Cadeia da Polimerase quantitativa

qSOFA: SOFA rápido

RAGE: Receptores de produtos finais com glicação avançada

RETN: Resistina

RIG-I: Receptores semelhantes ao gene I induzível de ácido retinoico

RNA: Ácido ribonucleico

RNASE3: Proteína catiônica eosinofílica

RPM: Rotação por minuto

ROS: Espécie Reativa de Oxigênio

RT-qPCR: Reação transcrição reversa seguida de PCR quantitativa

S100A3: Proteína S100-A3

S100A8: Proteína S100-A8

S100A9: Proteína S100-A9

SAA: Soro Albumina A

SACM1L: Fosfatidilinositide fosfatase SAC1

SAPS-3: Escore fisiológico agudo

SEPSIS-3: Terceiro Consenso Internacional de definição de Sepse e Choque Séptico

SH3TC2: Domínio SH3 contendo a proteína 2 com tetratricopeptídeo

SIRS: Síndrome da Resposta inflamatória sistêmica

SOFA: Escore de avaliação de falência de órgãos sequenciais

ssRNA: RNA fita simples

sTNFR: Receptor solúvel do TNF

TGFA: Fator de crescimento transformante

TLN2: Talina-2

TLR: Receptores semelhantes a Toll

TNC: Tenascina

TNF: Fator de Necrose Tumoral

TRAIL: Ligante indutor de apoptose relacionado ao TNF

TUBGCP5: Componente do complexo gama tubulina

UTI: Unidade de Terapia Intensiva

VEGF: Fator de crescimento do endotélio vascular

WDR90: Proteína 90 contendo repetido WD

ZEB2: Zinc finger E-box-binding homeobox 2

ZFHX2: Zinc finger homeobox protein 2

ZNF133: Zinc finger protein 133

ZNF677: Zinc finger protein 677

RESUMO

A sepse é uma síndrome clínica grave responsável por grande parte dos óbitos nas Unidades de Terapia Intensiva. Há uma diversidade de causas primárias que podem evoluir para um quadro clínico de sepse o que dificulta o diagnóstico precoce e a descoberta de drogas eficientes para tratamento. Os altos custos dos tratamentos e as taxas de letalidade mundial demonstram a necessidade de uma melhor compreensão desta síndrome. Este estudo buscou avaliar a expressão gênica dos Receptores de Peptídeos Formilados (FPR) em leucócitos totais, análise de marcadores sistêmicos e o perfil proteínas globais dos monócitos para averiguar as alterações presentes no organismo do indivíduo com a síndrome da sepse. Foi realizado a caracterização das alterações sistêmicas responsáveis pelo óbito de pacientes com sepse através da quantificação simultânea de 27 biomarcadores; verificou-se o silenciamento da expressão gênica do FPR1 e o FPR2 em pacientes com sepse e choque, relatando as possíveis disfunções imunológicas provocadas pela ausência desses FPRs; e a análise proteômica dos monócitos de pacientes com choque séptico permitiu averiguar a contribuição dessa célula no colapso desta síndrome, sua influência na lesão vascular, endotoxemia, inflamação e trombose. Esta investigação identificou pela primeira vez alvos biológicos relevantes os quais podem desempenhar papeis importantes no diagnóstico, prognóstico e terapêutica.

Palavras chaves: sepse, choque séptico, monócitos, citocinas, receptores de peptídeos formilados.

ABSTRACT

Sepsis is a serious clinical syndrome responsible for most of the deaths in the Intensive Care Units. There are a number of primary causes that may develop for a clinical picture of sepsis, making it difficult to diagnose early and find effective treatment medications. The high costs of treatments and global lethality rates demonstrate the need for a better understanding of this syndrome. This study aimed to evaluate the gene expression of Formulated Peptide Receptors (FPR) in total leukocytes, analysis of systemic markers and the profile of monocyte global proteins to ascertain the changes present in the body of the individual with sepsis syndrome. The characterization of the systemic changes responsible for the death of patients with sepsis was carried out through the simultaneous quantification of 27 biomarkers. The silencing of the gene expression of FPR1 and FPR2 in patients with sepsis and septic shock, reporting as possible immunological dysfunctions caused by the absence of these FPRs. The proteomic analysis of the monocytes of patients with septic shock allowed us to investigate the contribution of this cell in the collapse of this syndrome, its influence on vascular injury, endotoxemia, inflammation and thrombosis. This research identified for the first time, what is more important for the diagnosis, prognosis and therapeutics.

Palavras chaves: sepsis, septic shock, monocytes, biomarkers, formyl peptide receptors

APRESENTAÇÃO

A sepse é uma síndrome clínica grave responsável por grande parte dos óbitos nas Unidades de Terapia Intensiva. Há uma diversidade de causas primárias que podem evoluir para um quadro clínico de sepse o que dificulta o diagnóstico precoce e a descoberta de drogas eficientes para tratamento. Os altos custos dos tratamentos e as taxas de letalidade mundial demonstram a necessidade de uma melhor compreensão desta síndrome.

A sepse é caracterizada como uma resposta desregulada do hospedeiro frente à infecção. Os patógenos por muitos anos foram considerados os principais vilões desta síndrome. Entretanto conhecimentos atuais das alterações provocadas no funcionamento dos órgãos, morfologia, biologia celular e na circulação sanguínea tem sugerido que a desregulação imunológica em resposta à infecção é o principal evento patogênico na sepse.

A resposta imunológica nesta síndrome inicia-se com exacerbação e posteriormente pode evoluir para um quadro de imunossupressão, caracterizado como uma tentativa de o organismo silenciar a resposta exagerada, uma vez que o sistema imunológico deixou de ser um mecanismo protetor e se tornou autodestrutivo.

A ativação exacerbada deve-se a presença de trauma e infecção, os quais liberam várias moléculas na circulação sanguínea que são capazes de promover a ativação sistêmica da imunidade inata.

Os receptores de peptídeos formilados reconhecem algumas moléculas advindas do trauma e da lesão. Aos serem ativados desencadeiam cascatas que protegem o organismo contra à infecção como ativam e atraem células imunológicas à região afetada. No entanto também podem causar lesão endotelial, formação de trombos, entre outros efeitos que colaboram com a gravidade da sepse.

A ativação intensa e posteriormente a imunossupressão compromete o funcionamento de diversas células do organismo. Os monócitos são células fagocitárias, as quais se apresentam na primeira linha de defesa contra os microrganismos. Há relatos que a sepse desencadeia neste tipo celular um estado de anergia no qual a célula deixa de ter uma resposta eficaz contra os patógenos.

As citocinas coordenam as respostas imunológicas ao avaliar o perfil sistêmico pode-se inferir se o paciente está conseguindo combater a infecção e proporcionar um tratamento mais eficaz ao intervir em situações que seja diagnosticado respostas imunológica extremas: exacerbação ou imunossupressão.

Esta tese teve como objetivos:

1. Avaliar a expressão gênica da família dos receptores de peptídeos formilados em leucócitos totais de amostras de indivíduos saudáveis, sepse e choque séptico, uma vez que há uma alta quantidade de agonistas para esses receptores na circulação e cada receptor (FPR1, FPR2 e FPR3) possui uma função distinta.

2. Verificar o perfil de vinte e sete marcadores no plasma de indivíduos saudáveis, sepse e choque séptico para determinar o padrão predominante em cada grupo analisado; identificar as moléculas que podem servir como preditoras da doença e/ou de desfecho clínico: sobrevivência ou óbito; verificar através destes marcadores as possíveis alterações imunológicas responsáveis pelos pacientes morrerem.

3. Analisar o perfil de proteínas totais expresso nos monócitos de indivíduos saudáveis e de pacientes com choque séptico. Para determinar as alterações presentes nas células dos pacientes e qual a contribuição destas no colapso da sepse, uma vez que o choque é o quadro clínico mais grave desta síndrome.

CAPÍTULO I

FUNDAMENTAÇÃO TEÓRICA

SEPSE – EPIDEMIOLOGIA E CLÍNICA

A sepse é uma síndrome de anormalidades patológicas, fisiológicas e bioquímicas. É classificada como uma das síndromes mais antigas e indescritíveis da medicina sendo um grave problema de saúde e a principal causa de morte nas Unidades de Terapia Intensiva - UTI (Angus e Van Der Poll, 2013).

Estima-se que ocorram entre 15 a 30 milhões de casos anualmente de sepse no mundo com potencial de 5,3 milhões de mortes a cada ano (Fleischmann *et al.*, 2016). A incidência de sepse adulta na população brasileira corresponde a 290 casos por 100.000 adultos, que se traduz em 419.047 pacientes adultos com sepse tratados em UTI por ano no Brasil, dos quais 233.409 morrem no hospital (Machado *et al.*, 2017).

A sepse se correlaciona como uma disfunção orgânica potencialmente fatal causada por uma resposta imune desregulada a uma infecção (Seymour *et al.*, 2016). Quando há presença de profundas anormalidades circulatórias e celulares / metabólicas, capazes de aumentar a mortalidade substancialmente, o quadro de sepse evoluiu para choque séptico (Shankar-Hari *et al.*, 2016).

Toda situação com sepse é grave, sua evolução para choque séptico significa que há uma forma generalizada de falência circulatória aguda a qual é ameaçadora a vida e representa uma utilização inadequada de oxigênio pelas células, motivo pelo qual há aumento da probabilidade de óbito dos pacientes (Schultz *et al.*, 2017).

Atualmente os critérios clínicos para diagnóstico de sepse se baseiam na presença ou suspeita de infecção e dois pontos ou mais no escore de SOFA (*Sequential Organ Failure Assessment Score* – Escore de Avaliação sequencial de falha de órgãos). O diagnóstico do choque séptico é caracterizado com sepse, presença de lactato acima de 2 mmol/L (18 mg/dL) e a necessidade de utilização de vasopressores para elevar a pressão arterial média acima de 65mmHg após reanimação volêmica adequada (Seymour *et al.*, 2016; Shankar-Hari *et al.*, 2016).

O escore de SOFA está discriminado na tabela 1, ele gradua anormalidades em diferentes sistemas do organismo considerando as intervenções clínicas (Seymour *et al.*, 2016).

Para identificar de forma rápida os pacientes que estão com suspeita de infecção sob maior risco de desfecho adversos, utiliza-se na beira do leito o escore de SOFA simplificado denominado de qSOFA (*quick SOFA)*. No qual os critérios utilizados são Pressão Sistólica de ≤100mmHg, frequência respiratória ≥22 / incursões por minuto e alteração do estado mental (escore segundo a escala de coma de Glasgow inferior a 15). Cada variável possui um ponto no escore, portanto ele vai de 0 a 3. Uma pontuação igual ou maior que 2 indica maior risco de mortalidade e permanência prolongada na UTI (Seymour *et al.*, 2016).

Os critérios atuais para diagnóstico de sepse foram estabelecidos no Terceiro Consenso Internacional de Definição de Sepse e Choque Séptico (SEPSIS-3) o qual ocorreu em 2016 e teve participação das Sociedades Americana e Europeia de Medicina Intensivista (Singer *et al.*, 2016).

A nova definição deve-se ao melhor entendimento dos mecanismos fisiopatológicos responsáveis pelas disfunções celulares e moleculares relacionadas a esta síndrome, bem como o maior número de recursos de suporte a vida disponíveis nas UTIs atuais (Abraham, 2016).

O Instituto Latino Americano de Sepse (ILAS) e a Associação de Medicina Intensiva Brasileira (AMIB) acreditam que os critérios de sepse estabelecidos a partir do SEPSIS-3 aumentaram a especificidade a custo da diminuição da sensibilidade, podendo haver um atraso no diagnóstico e identificação de pacientes em situações muito graves, o que se torna preocupante uma vez que a incidência no Brasil é extremamente alta e muitos hospitais são precários (Machado *et al.*, 2016).

Tabela 1. Escore de Avaliação sequencial de falha de órgãos relacionado a sepse – SOFA

	ESCORE				
SISTEMA	0	1	2	3	4
Respiratório					
PaO_2/FIO_2	>400	≤400	≤300	≤200 com suporte respiratório	≤100 com suporte respiratório
Coagulação					
Plaquetas $x10^3/mm^3$	≥ 150	<150	<100	<50	<20
Fígado					
Bilirrubina, mg/dL	<1.2	1.2-1.9	2.0-5.9	6.0-11.9	>12
Cardiovascular	Ausência de hipotensão	MAP <70 mm Hg	Dopamina ≤ 5 mg/kg/min ou Dobutamina (qualquer dose)	Dopamina ≥ 5 mg/kg/min ou epinefrina ou noradrenalina ≤ 0,1 mg/kg/min	Dopamina > 15 mg/kg/min ou epinefrina ou noradrenalina > 0,1 mg/kg/min
Neurológico					
Glasgow	15	13-14	10-12	6-9	<6
Renal					
Creatinina, mg/dL	<1.2	1.2-1.9	2.0-3.4	3.5-4.9	>5.0
Débito urinário, mL/d				<500	<200

Adaptado de Vicent, et al., 1996 (Vincent *et al.*, 1996).

O ILAS e AMIB aconselham aos hospitais brasileiros não abandonarem os fatores antigos para diagnóstico de sepse como a presença de hipotensão (MAP <70) e critério de Glasgow (13-14) ambas condições se enquadram em SOFA 1 que não é considerado sepse para os critérios atuais. Entretanto essas situações são características de quadro clínico de pacientes com alto risco ou presença de sepse. Outro fator deve-se aos critérios de SOFA serem desconhecidos para profissionais não intensivistas e muitos pacientes com sepse são identificados em outros setores do hospital e posteriormente encaminhados para as UTIs (Machado *et al.*, 2016).

As manifestações clínicas provocadas pela sepse são diversas, os sinais de infecção e disfunção de órgãos podem ser sutis, para facilitar o diagnóstico desta síndrome os critérios antigos resumidos na tabela 2 são avaliados.

A resposta humana à infecção varia de acordo com o quadro clínico do indivíduo, dependendo da gravidade da doença e do grau de disfunção de múltiplos órgãos. Os sintomas como a febre resulta do controle do hipotálamo para reajustar a temperatura corporal; taquipnea em pacientes com sepse pode ser atribuída ao aumento da ativação respiratória provocada por mediadores inflamatórios ou uma resposta compensatória a acidose metabólica. Taquicardia representa um importante mecanismo compensatório para manter a perfusão em resposta ao déficit do volume intravascular, da redução da contractilidade cardíaca e da vasodilatação. No choque séptico há hipotensão arterial apesar da ressuscitação de fluídos apropriada (Iskander *et al.*, 2013).

Os patógenos (bactérias, fungos, vírus, protozoários e parasitas) por muitos anos foram considerados os principais vilões da sepse, no entanto conhecimentos atuais das alterações provocadas por esta síndrome no funcionamento dos órgãos, morfologia, biologia celular e na circulação sanguínea tem sugerido que a desregulação imunológica em resposta à infecção é o principal evento patogênico na sepse (Bermejo-Martin *et al.*, 2016; Singer *et al.*, 2016).

Tabela 2. Critérios para diagnóstico de Sepse, Sepse grave e Choque Séptico

Sepse (infecção documentada ou suspeita ≥1 critério abaixo)

Variáveis gerais

Temperatura: Febre (>38.3°C) ou Hipotermia (<36°C);

Batimentos cardíacos: elevação (>90 batimentos por minuto) ou taquipenia;

Alteração do estado mental;

Edema substancial ou balanço de fluido positivo (>20mL/kg do peso corporal em 24horas);

Hiperglicemia (glicose plasma >120mg/dL) na ausência de diabetes;

Variáveis inflamatórias

Leucocitose (contagem de células brancas >12000/mm^3);

Leucopenia (contagem de células brancas <4000/mm^3);

Contagem de células brancas normal com >10% de formas imaturas;

Elevação de Proteína C Reativa no plasma (>2 desvios padrão acima do limite superior do intervalo normal);

Elevação Procalcitonina no plasma (>2 desvios padrão acima do limite superior do intervalo normal);

Variáveis hemodinâmicas

Hipotensão arterial (pressão sistólica <90 mm Hg; média pressão arterial, <70mm Hg; ou diminuição da pressão sistólica de >40mm Hg ou para >2 desvios padrão abaixo do limite inferior normal para idade);

Elevação da saturação venosa de oxigênio (>70%);

Índice cardíaco elevado (>3.5 litros/min/metro quadrado de área corporal);

Variável disfunção de órgão

Hipoxemia arterial (Relação da pressão parcial de oxigênio arterial à fração de oxigênio inspirado <300);

Oligúria aguda (Débito urinário, <0.5ml/kg/hr ou 45ml/hr durante pelo menos 2h);

Aumento do nível da creatinina >0.5 mg/dl (>44µmol/L);

Anormalidades da coagulação (relação normalizada internacional > 1,5 ou tempo de tromboplastina parcial ativada > 60 segundos);

Trombocitopenia (contagem de plaquetas <100000/mm^3);

Hiperbilirrubinemia (bilirrubina total do plasma >4mg/dl);

Variáveis de Perfusão tecidual

Hiperlactemia (lactato >1mmol/L)

Diminuição volume capilar do vaso

*Sepse grave (Fatores associados a sepse mais disfunção de órgãos), definição em desuso, este quadro foi incorporado a sepse;

**Choque séptico (Sepse mais hipotensão arterial e hiperlactatemia).

Tabela adaptada de Angus & Poll, 2013 (Angus e Van Der Poll, 2013).

RESPOSTA IMUNOLÓGICA

RECONHECIMENTO DE PATÓGENOS E SINAIS DE DANOS

A resposta imunológica é desencadeada por sucessivos mecanismos inatos e adaptativos, no qual há um predomínio da imunidade inata nas fases iniciais da infecção evoluindo para uma resposta mais específica na imunidade adaptativa (Kourilsky e Truffa-Bachi, 2001).

O sistema imune inato é a primeira linha de defesa do organismo à infecção e a lesão. O reconhecimento da presença de patógenos ocorre através da identificação de componentes específicos estruturalmente conservados, produzidos por um amplo grupo de microrganismos potencialmente patogênicos. Estes componentes são assinaturas moleculares dos microrganismos e popularmente conhecido como PAMPs (*Pathogen-Associated Molecular Pattern*: Padrões Moleculares Associados a Patógenos) (Pugin *et al.*, 1994; Chun e Seong, 2010).

Essas assinaturas são moléculas conservadas que fazem parte do ciclo de vida dos patógenos, nelas incluem os ácidos nucleicos com características únicas a microrganismos, como RNA de fita dupla encontrado em replicação de vírus e sequências CpG de DNA não metiladas encontradas em bactérias; proteínas típicas de bactérias que possuem sítios de iniciação com N-formilmetionina; complexos de lipídeos e carboidratos que são sintetizados por microrganismos mas não por células de mamíferos, tais como lipopolissacarídeos, ácidos teicóicos em bactérias; e oligossacarídeos ricos em manose encontrados em glicoproteínas microbianas, mas não em mamíferos (Janeway e Medzhitov, 2002; Underhill, 2003; Abbas, 2007).

Já os sinais de danos teciduais são denominados Padrões Moleculares Associados a Danos (*Damage-associated Molecular Patterns*: DAMPs) são moléculas endógenas capazes de ativar a imunidade inata e gerar processos inflamatórios semelhantes aos PAMPs.

Os DAMPs são liberados após a morte celular não programada, situações de estresse fisiológico e na ativação da resposta imune, sendo considerados verdadeiros “alarmes” do organismo. Neste contexto as células imunológicas secretam as alarminas / DAMPs para recrutar e ativar diretamente ou indiretamente a imunidade adaptativa. As principais alarminas já descritas são HMGB1 *(High*

mobility group box 1), S100, HPSs *(Heat shock proteins)*, ácido úrico, DNA mitocondrial (Bianchi, 2007; Raymond *et al.*, 2017).

Os DAMPs e os PAMPs são reconhecidos por Receptores do Reconhecimento de Padrões (PRR) (Pasare e Medzhitov, 2004). Os quais são constitutivamente expressos na superfície celular, em compartimentos intracelulares e/ou secretados na corrente sanguínea e em fluídos teciduais (Qian e Cao, 2013).

Os principais receptores descritos na literatura que reconhecem PAMPs e DAMPs estão listados na tabela 3 e incluem os Receptores semelhantes à Toll (*TLR*), Receptores de Lectina tipo-C (CLRs), Receptores com domínio oligomerização de ligação a nucleotídeo (NOD), Receptores semelhantes à NOD (NLRs), Receptores semelhantes ao gene I induzível de ácido retinoico (RIG-1) e Receptores de produtos finais com glicação avançada (RAGE) (Raymond *et al.*, 2017).

A ativação dos PRRs desencadeia cascatas de sinalização que tem papel essencial de sobrevivência em resposta à infecção e a lesão, bem como função patológica na lesão de tecidos e órgãos comumente associada à sepse e ao trauma (Raymond *et al.*, 2017).

As principais funções destes receptores são induzir a opsonização, ativação do sistema complemento e da cascata de coagulação, fagocitose, mecanismos microbicidas através de produção de espécies reativas de oxigênio e de nitrogênio, e o estimulo e produção de citocinas inflamatórias e quimiocinas, as quais tem a função de recrutar e ativar células que regem o desenvolvimento da imunidade adaptativa (Medzhitov, 2001; Underhill, 2003).

Tabela 3. Lista de PRRs que são reconhecidos por PAMPs e DAMPs. Produtos microbianos e sinais de danos endógenos que são reconhecidos por receptores de reconhecimento padrão e desencadeiam resposta comum no hospedeiro.

PRRs	LOCALIZAÇÃO	PAMPs	DAMPs
TLR1	Membrana	Lipoproteínas	
TLR2	Membrana	Lipoproteínas Peptideoglicano Ácido lipoteícoico	HMGB1 Hsp70 S100
TLR3	Endossomo	dsRNA viral	
TLR4	Membrana	Lipopolissacarídeo	HMGB1 Hsp70, S100 Ácido hialurônico
TLR5	Membrana	Flagelina	
TLR6	Membrana	Lipoproteínas	
TLR7	Endossomo	ssRNA viral	
TLR8	Endossomo	ssRNA viral	
TLR9	Endossomo	DNA patogênico	mtDNA, nDNA
Dectina	Membrana	Betaglicanos	
NOD1	Citoplasma	Peptideoglicano	
NOD2	Citoplasma	Peptideoglicano	
NALP3	Citoplasma	dsDNA viral ssRNA viral	mDNA, ATP Ácido úrico
RIG-1	Citoplasma	dsRNA viral	
MDA5	Citoplasma	dsRNA viral	
RAGE	Membrana		AGEs, HMGB1 S100

Tabela adaptada (Raymond *et al.*, 2017).

RECEPTORES DE PEPTÍDEOS FORMILADOS (FPR)

Os PRR capazes de reconhecerem os peptídeos formilados são denominados de Receptores de Peptídeos Formilados: FPR (*Formyl Peptide Receptors*). Os peptídeos formilados ativam o sistema imunológico sendo considerados como PAMPs quando advém de bactérias e DAMPs quando sua origem são as mitocôndrias celulares (Migeotte *et al.*, 2006).

Os FPRs apresentam sete domínios transmembrânicos acoplados à proteína G e foram identificados em 1976 como um sítio de ligação de alta afinidade para o peptídeo N-formil-metionil-leucil-fenilalanina (fMLP) na superfície de neutrófilos (Le *et al.*, 2002; Migeotte *et al.*, 2006).

Já foram descritos três FPRs em humanos (FPR1, FPR2/ALX e FPR3), os quais são codificados por três genes agrupados nas regiões cromossômicas 19q13.3-19q13.4. O FPR1 apresenta 69% de identidade dos aminoácidos com FPR2 e 56% com FPR3, enquanto que FPR2 apresenta 83% de identidade com FPR3 (Ye *et al.*, 2009).

A alta identidade entre os FPRs não confere similar sensibilidade a agonistas comuns. A ampla diversidade de ligantes (proteínas, peptídeos e lipídeos) faz com que sejam vulgarmente conhecidos como receptores promíscuos (Migeotte *et al.*, 2006; Ye *et al.*, 2009; Dufton e Perretti, 2010).

O FPR1 é o receptor que apresenta maior afinidade a peptídeos formilados podendo ser ativados por eles em baixa concentração. Entre seus agonistas endógenos se destacam a catepsina G, anexina A1 e uma família de citocinas com similaridade na sequência 19 (FAM19A4) (Dorward *et al.*, 2015; Wang *et al.*, 2015).

O FPR2/ALX (ALX: Receptor de Lipoxin A) é considerado o receptor mais promiscuo desta família (He *et al.*, 2014). Reconhece com baixa sensibilidade os peptídeos formilados (fMLP), no entanto há vários agonistas endógenos caracterizados, a exemplo tem-se a lipoxina A4, soro amiloide A (SAA), anexina A1e β- amiloide (Dufton e Perretti, 2010; Wan *et al.*, 2011; Cooray *et al.*, 2013).

O FPR3 é relativamente insensível a peptídeos formilados, poucas moléculas ligantes têm sido identificadas. O F2L é o agonista específico deste receptor, sendo essa molécula um peptídeo derivado da clivagem da proteína 1 de ligação ao grupo

heme pela catepsina D (He *et al.*, 2014). Este receptor também pode ser ativado pela anexina A1 a qual é considerada um pan-FPR, devido a capacidade de interagir com os três FPRs (Pupjalis *et al.*, 2011).

Os FPRs estão amplamente distribuídos em diferentes tipos celulares, incluindo células da resposta imunológica, como monócitos, neutrófilos e linfócitos, e também em células endoteliais e epiteliais (Le *et al.*, 2002; Migeotte *et al.*, 2006). Os monócitos/macrófagos expressam: FPR1, FPR2 e FPR3 (Le *et al.*, 2002); neutrófilos: FPR1 e FPR2 (Kim *et al.*, 2010), células *Natural Killer (NK)*: FPR1 e FPR2 (Kim *et al.*, 2009), células dendríticas: imaturas FPR1 e FPR3; maturas: FPR3 (Yang *et al.*, 2002), células T *naive* e células dos perfis Th1, Th2 e Th17 expressam FPR2 (Lee *et al.*, 2017; Nagaya *et al.*, 2017).

Nas células do sistema imune estes receptores ao serem ativados desencadeiam cascatas de sinalização que promovem migração celular, fagocitose, degranulação, geração de espécies reativas de oxigênio, citocinas, quimiocinas, entre outros efeitos (Chen *et al.*, 2017; Skvortsov e Gabdoulkhakova, 2017). O resultado da ativação é dependente do tipo celular e do receptor a ser ativado, o qual pode estar em hetero ou homodimero.

A ativação destes receptores não está apenas correlacionada a combater patógenos ou fagocitar células mortas, estudos tem demonstrado a relevância na diferenciação das células imunes, como monócitos em macrófagos e células dendríticas (Yang *et al.*, 2001); na polarização do macrófago para o perfil M2 (Li *et al.*, 2011); e diferenciação das células T *helper* (Nagaya *et al.*, 2017);

A presença de altas quantidades de peptídeos formilados mitocondriais na circulação ativa os FPRs e são capazes de desencadearem cascatas que resultam em efeitos sistêmicos como hipotensão grave, vasodilatação, disfunção endotelial, lesão vascular, coágulo sanguíneo e lesão pulmonar (Wenceslau *et al.*, 2015).

As vias de sinalização desencadeadas pelos FPRs podem estar contribuindo para melhora ou piora dos pacientes com sepse. Na sepse normalmente o paciente possui DAMPs na circulação devido a lesões, traumas e/ou outros fatores, associado a isto há presença de infecção a qual gera PAMPs, ambas as situações

produzem grandes quantidades de agonistas desses receptores na circulação podendo ativar constantemente o sistema imune inato (Wiersinga *et al.*, 2014).

A resposta imune na sepse é exacerbada. Há ativação maciça do perfil pro-inflamatório e anti-inflamatório os quais se não forem controlados podem desencadear uma exaustão da imunidade inata e adaptativa. Nesta situação o paciente entra em estado de anergia onde suas células não respondem de forma eficaz contra os patógenos (Hotchkiss *et al.*, 2013). A situação do paciente com sepse é grave, a ativação imunológica sistêmica e posteriormente o quadro de imunossupressão contribuem para os desfechos clínicos adversos (Mira, Gentile*, et al.*, 2017).

CITOCINAS

As citocinas são uma ampla categorias de pequenas proteínas (<40kDa) as quais são produzidas e lançadas com objetivo final a sinalização celular (Dinarello, 2007). Estas moléculas podem exercer funções autocrina, paracrina, endócrinas e imunomoduladoras (Chousterman *et al.*, 2017).

As citocinas são divididas em diversas categorias como interleucinas (IL), interferons (IFN), quimiocinas, fator de necrose tumoral (TNF) e fatores de crescimento.

As interleucinas são secretadas principalmente por leucócitos e células endoteliais. São capazes de promover a sinalização celular gerando a ativação, proliferação, morte e/ou migração das células imunes. Podem ser classificadas em dois perfis: pró-inflamatórias e anti-inflamatórias. As pró-inflamatórias são responsáveis por ativação celular, dano tecidual e necrose, enquanto que as anti-inflamatórias tem a função de diminuir ou reverter o efeito dos processos inflamatórios. Há ainda interleucinas que podem ser pleiotrópicas, isto é dependendo do contexto possuem ação pró-inflamatória ou anti-inflamatória (Chousterman *et al.*, 2017).

Os interferons são classificados em três tipos principais o Interferon tipo I: Interferon alfa (IFN-α) e Interferon beta (IFN-β); IFN tipo II: Interferon gama (IFN-γ),

e o IFN tipo III: Interferon lambda (IFN –λ) (Schroder *et al.*, 2004; Schoenborn e Wilson, 2007).

O INF-α/β é produzido principalmente por células epiteliais e fibroblastos. Apresentam potentes atividades antivirais, induzem uma variedade de efeitos imuno modulatórios como indução de moléculas de MHC classe I, ativação da citotoxicidade de células NK, maturação e função de células dendriticas, indução de células T $CD8^+$ de memória, regulação na produção de citocinas e expressão de receptores citocinas (Biron, 2001).

O INF-γ é produzido por linfócitos T $CD4^+$, células T $CD8^+$, NK, células B e células apresentadoras de antígenos. Tem efeito anti-viral, anti-proliferativo, apoptótico e pode desencadear várias funções efetoras anti-microbianas como indução de produção de intermediários de óxido nítrico (*Inducible Nitric Oxide Synthase:* iNOS) e espécies reativas de oxigênio (*Reactive Oxygen Species*: ROS).

O INF-γ possui a habilidade de coordenar a transição da imunidade inata para adaptativa por mecanismos que incluem o desenvolvimento de uma resposta Th1; além disso ele atua na regulação dos leucócitos com o endotélio (Schroder *et al.*, 2004). O INF- λ apresentam propriedades anti-proliferativas e anti-virais. As células dendriticas plasmocitoides são as principais produtoras deste IFN (Yin *et al.*, 2012).

As quimiocinas são pequenas moléculas (8-12kDa), capazes de recrutar e ativar células imunes. A quimioatração necessita de um gradiente de concentração das quimiocinas. Há quatro tipos de quimiocinas as quais são classificadas de acordo com a sua sequência de aminoácidos e o espaçamento entre os dois resíduos de cisteína: CC, CXC, CX3C e XC. Em contraste com outras citocinas que têm efeitos amplos em muitas células, as quimiocinas geralmente são células específicas. Por exemplo, MCP-1 ou CX3CL1 são quase específicos para monócitos, CXCL1 e CXCL2 para neutrófilos e CXCR3 para linfócitos T (Robben *et al.*, 2005; Cai *et al.*, 2010).

A família dos fatores de necrose tumoral inclui o fator de necrose tumoral alfa (TNF-α), fator de necrose tumoral beta (TNF-β), ligante de CD40 (*CD40 ligand*: CD40L), ligante de Fas (*Fas Ligand*: FasL), ligante indutor de apoptose relacionado

ao TNF (*TNF-related apoptosis inducing ligand*: TRAIL) e LIGHT o qual é um homologo para linfotoxina. Essas moléculas estão envolvidas em diversos processos fisiológicos, inflamação sistêmica, lise tumoral, apoptose e no início de reação de fase aguda. A principal célula produtora de TNF-α são os macrófagos enquanto que o TNF-β é principalmente produzido por linfócitos T, outras células expressam essas duas moléculas mas em menor quantidade (Chu, 2013).

Os fatores de crescimento são moléculas que podem auxiliar na autoamplificação da produção de citocinas podendo gerar o "*cytokine storm"*. Os principais fatores hematopoiéticos estimulantes de colônia são o fator estimulante de colônia de macrófagos e granulócitos (*Granulocyte-Macrophage Colony-Stimulating Factor*: GM-CSF), fator estimulante de colônia de macrófagos (*Macrophage Colony-Stimulating fator*: M-CSF) e o fator estimulante de colônia de granulócitos (*Granulocyte Colony-Stimulating Factor*: G-CSF). Os fatores de crescimento induzem a proliferação, diferenciação e ativação de células mieloides. Acredita-se que eles amplificam a resposta de moléculas de fase inicial como IL-1β e TNF-α (Hamilton, 2008).

As citocinas orquestram a resposta imune, ao avaliar os níveis sistêmicos é possível determinar o padrão de resposta de um paciente frente a um patógeno e averiguar se ele está conseguindo combater ou não a infecção (Moscovitz *et al.*, 1994).

RESPOSTA IMUNOLÓGICA NA SEPSE

O paciente com sepse pode apresentar alguns perfis críticos de resposta imunológica como a síndrome da resposta inflamatória sistêmica (*Systemic Inflammatory Response Syndrome: SIRS*), síndrome da resposta anti-inflamatória compensatória (*Compensatory Anti-inflammatory Response Syndrome: CARS*) e a síndrome do catabolismo, imunossupressão e inflamação persistentes *(Persistent Inflammation, Immunosuppression, and Catabolism Syndrome: PICS)* (Gentile *et al.*, 2012; Mira, Brakenridge*, et al.*, 2017).

Na situação de SIRS o paciente apresenta exacerbada resposta da imunidade inata que inicialmente é benéfica para combater o patógeno. No entanto

se prolongada pode gerar lesão endotelial, perfusão inadequada e danos teciduais os quais estão associados a disfunção em múltiplos órgãos (Bone *et al.*, 1997). Os critérios de diagnóstico de SIRS são temperatura anormal (>38°C ou <36°C), aumento dos batimentos cardíacos (>90 batimentos por minuto), aumento na taxa respiratória (>20 respirações por minuto) ou diminuição $Paco_2$ (<32 mm Hg) e a quantidade anormal de células brancas na circulação sanguínea (>12000/μL ou <4000/ μL) (Kaukonen *et al.*, 2015).

CARS foi caracterizada primeiramente como uma desativação sistêmica do sistema imunológico a qual objetiva restaurar a homeostasia após o quadro clínico de SIRS. Essa situação compensatória caracteriza-se por apresentar apoptose e/ou disfunção dos linfócitos (redução na proliferação e/ou produção de citocinas pró-inflamatórias), a diminuição da expressão de receptores HLA bem como a desativação de monócitos, os quais reduzem a produção de citocinas pró-inflamatórias e aumentam a produção de citocinas anti-inflamatórias como IL-10, IL-6 e TGF-β bem como de antagonistas de citocinas (IL1-ra e sTNFR1). Fatores clínicos associados a CARS são anergia cutânea, hipotermia, leucopenia, susceptibilidade à infecções e falha na eliminação da infecção (Bone, 1996; Ward *et al.*, 2008).

Acreditava-se que SIRS e CARS eram eventos que ocorriam em momentos distintos, no entanto Osuchowski e colaboradores reportaram que em modelos de sepse polimicrobiana a produção de citocinas pró e anti-inflamatórias ocorriam simultaneamente (Osuchowski *et al.*, 2006). Posteriormente análise genômica de leucócitos de pacientes com trauma grave confirmou esta afirmação, demonstrando que nestas células houve indução da imunidade inata (expressão de genes pró e anti-inflamatórios) e supressão de genes da imunidade adaptativa (Xiao *et al.*, 2011).

A evolução do quadro clínico SIRS e CARS pode resultar na melhora do paciente quando o sistema imunológico é capaz de retornar a homeostasia. Entretanto caso a disfunção imunológica persista a doença evolui para uma situação crônica crítica na qual há um prolongado tempo de disfunção orgânica,

imunossupressão, inflamação e catabolismo de proteínas somáticas, situação caracterizada como PICS (Gentile *et al.*, 2012).

Os marcadores clínicos utilizados para identificar pacientes com PICS são: indivíduos doentes críticos os quais estão a mais de 14 dias na UTI; apresentam persistente inflamação (níveis de proteína C reativa >50µg/dL) e imunossupressão (contagem de linfócitos totais <0.8 x 10^9/L). Estes pacientes estão em catabolismo o qual pode ser identificado pela quantificação de proteínas no sangue como a presença de soro albumina <3.0g/dL, pré-albumina <10mg/dL e o índice de creatinina <80%. Outra característica do catabolismo é a perda de peso, sendo considerado os níveis que estão maiores que 10% e/ou índice de massa corporal menor que 18 durante os dias de hospitalização (Mira, Brakenridge*, et al.*, 2017).

A grave disfunção da imunidade inata e adaptativa ou de processos pró-inflamatórios e anti-inflamatórios são os principais eventos prejudiciais na sepse (Figura 1). As alterações impactam diretamente em diversas células, sendo capaz de alterar a vida útil, produção e a função das células efetoras responsáveis pela homeostasia (Gentile *et al.*, 2012).

DISFUNÇÃO CELULAR CAUSADA PELA SEPSE

As células da imunidade inata (neutrófilos, monócitos/macrófagos, *Natural Killer* e células dendriticas) e adaptativa (linfócitos T, linfócitos Treg e linfócitos B) são diretamente afetadas pela sepse e podem ter suas funções exacerbadas e/ou imunossuprimidas.

Os neutrófilos são componentes cruciais da resposta imune inata, liberam importantes citocinas regulatórias, quimiocinas e leucotrienos, engolfam patógenos invasores e matam microrganismos (Kovach e Standiford, 2012). A sepse promove diferentes alterações neste tipo celular e a apoptose tardia é uma delas. Por mecanismos não esclarecidos os neutrófilos nesta síndrome sobrevivem por mais de 24 horas após serem liberados pela medula, no entanto com o passar do tempo apesar de não sofrerem apoptose eles se tornam disfuncionais, na tentativa de suprir essa resposta celular a medula libera neutrófilos imaturos na circulação (Hotchkiss e Nicholson, 2006; Drifte *et al.*, 2013). A disfunção celular resulta no

comprometimento da migração dirigida para focos infecciosos e respostas antimicrobianas inadequadas nesses locais (Alves-Filho *et al.*, 2010) como diminuição na produção de ROS e *clearance* bacteriano (Grailer *et al.*, 2014).

A disfunção dos monócitos e macrófagos contribuem para a imunossupressão da imunidade inata e adaptativa (Munoz *et al.*, 1991). As alterações provocadas pela sepse fazem com que estas células apresentem dificuldades de realizar o *clearance* bacteriano, apresentar antígenos e produzir citocinas pró-inflamatórias, resultando em um quadro de anergia celular. Situação na qual a célula se torna incapaz de produzir uma resposta eficaz contra o patógeno, devido o desenvolvimento de tolerância à endotoxinas (Ayala e Chaudry, 1996; Docke *et al.*, 1997).

A tolerância resulta de uma reprogramação epigenética na qual as células adquirem fenótipos imunossupressores, diminuindo a capacidade de secretar citocinas pró-inflamatórias (TNF, IL-1α, IL-6 e IL12) sem alterar a secreção de mediadores anti-inflamatórios (IL-10 e o antagonista do receptor de IL-1: IL-1ra). Associada a estes fatores as células com estes fenótipos apresentam redução de moléculas apresentadoras de antígeno (HLA-DR) (Cavaillon e Adib-Conquy, 2006; Landelle *et al.*, 2010; Carson *et al.*, 2011; Hotchkiss *et al.*, 2013).

As células dendriticas são cruciais no reconhecimento e indução de uma resposta imune específica. São células apresentadoras de antígenos profissionais que desempenham um papel vital na resposta imune através da ativação de linfócitos T (Fan *et al.*, 2015).

Na sepse há uma redução significativa dessas células devido ao aumento da apoptose. A disfunção celular se caracteriza por diminuição da capacidade de apresentação de antígenos já que possuem redução da expressão de moléculas HLA-DR e há uma alteração no perfil de resposta dessas células à estímulos externos e/ou sinais de danos, no qual a expressão de IL-12 é suprimida e há o aumento na produção de IL-10, configurando uma resposta anti-inflamatória (Hotchkiss *et al.*, 2002; Pastille *et al.*, 2011).

A sepse é caracterizada por diminuição da concentração de células NK no sangue (Venet *et al.*, 2010). Sendo o desenvolvimento de tolerância à endotoxinas

e a diminuição da citotoxicidade as principais alterações observadas nestas células em resposta à esta síndrome (Limaye *et al.*, 2008; Chiche *et al.*, 2012).

Nas células T as alterações relacionam-se com exaustão celular, aumento da apoptose e a diminuição da diversidade de receptores de células T. As células T $CD4^+$ apresentam também diminuição da expressão de moléculas de adesão e aumento da polarização para o perfil de resposta Th2; e as células T $CD8^+$ diminuição da função citotóxica e secreção de citocinas (Hotchkiss *et al.*, 2013).

A sepse ocasiona um aumento na quantidade células T regulatórias (Treg) no sangue, bem como potencializa a sua função imunossupressora (Monneret *et al.*, 2003; Cao *et al.*, 2015). As Treg desempenham um papel essencial no controle da resposta imune inata e adaptativa, sendo capaz de reduzir as atividades efetoras mediadas por células T $CD4^+$, T $CD8^+$, NK, dendríticas e células B (De Pablo *et al.*, 2014). A regulação da resposta efetora é importante para obtenção de uma resposta homeostática à infecção, contribuindo para o término de respostas inflamatórias e citotóxicas. Entretanto as Treg podem mediar a imunossupressão se sua atividade for persistente e prolongada o que se correlaciona com imunoparalisia grave e tem sido associado com piores prognósticos (Cao *et al.*, 2015; Huang *et al.*, 2015).

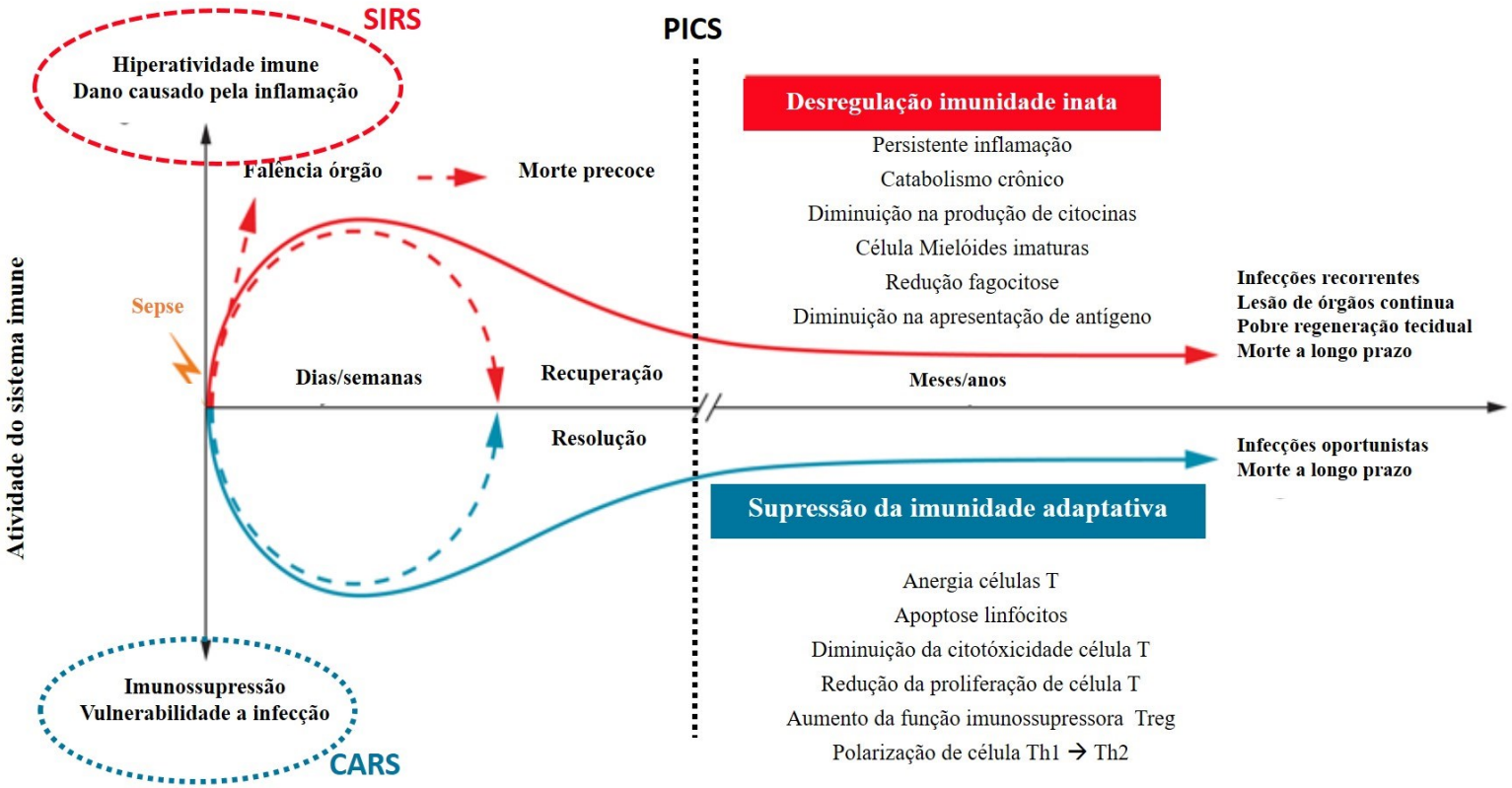
Os pacientes com sepse apresentam uma redução no número de células B devido ao aumento da apoptose, além disso as células que sobrevivem apresentam diminuição na produção de anticorpos específicos a antígenos, redução na produção de citocinas o que dificulta a realização do *clearance* de patógenos por outras células (Kelly-Scumpia *et al.*, 2011; Monserrat *et al.*, 2013).

As alterações nas células endoteliais também colaboram com a disfunção imunológica da sepse. Estas células são capazes de reconhecer diferentes PAMPs e DAMPs, e ativam cascatas de sinalização intracelular que culminam na produção e liberação de citocinas e quimiocinas as quais auto amplificam a resposta imunológica (Faure *et al.*, 2000; Opitz *et al.*, 2009).

As alterações nas células endoteliais se manifestam em diversos processos patológicos, incluindo extravasamento vascular, alteração no tônus vasomotor e trombose microvascular (Colbert e Schmidt, 2016). Uma consequência adicional do dano as células endoteliais é a liberação de altas quantidades do fator de von

Willebrand, o qual promove a agregação plaquetária fazendo com que essas se aderem à camada subendotelial e formem trombos patológicos (Mikacenic *et al.*, 2015).

A sepse é uma síndrome complexa e apesar de muitos estudos continua sendo um grande desafio para a ciência. Entender as alterações celulares e suas contribuições para o colapso desta síndrome pode auxiliar os cientistas a desenvolverem moléculas capazes de parar a evolução clínica e principalmente restaurar organismo garantindo uma maior sobrevida aos pacientes que adquirem essa síndrome.



Adaptado de Delano, M.J.& Peter A. W. (Delano e Ward, 2016)

Figura 1. Disfunção imunológica na sepse. Resumo da evolução do paciente associado as alterações imunológicas: SIRS (Síndrome da Resposta Inflamatória Sistêmica), CARS (Síndrome da Resposta Anti-Inflamatória Compensatória) e PICS (Síndrome do Catabolismo, Inflamação e Imunossupressão Persistentes).

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CAPÍTULO II

DOWNREGULATION OF FORMYL PEPTIDE RECEPTORS IN LEUKOCYTES DURING SEPSIS PROGRESSION

(*Short communication* escrito de acordo com as normas da revista Immunobiology)

DOWNREGULATION OF FORMYL PEPTIDE RECEPTORS IN LEUKOCYTES DURING SEPSIS PROGRESSION

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ABSTRACT

Sepsis is a potentially fatal clinical syndrome that presents a major challenge to science. Many studies have sought to understand the mechanisms that trigger the immune dysfunction in this syndrome. This study aimed to evaluate the gene expression of the Formyl Peptide Receptors (FPR) and Annexin A1 (ANXA 1) in leukocytes from healthy individuals, and those from cases of sepsis and septic shock. Relative quantification in both patient groups has revealed a silencing in the production of mRNA of FPR1 and FPR2, which can be more than 100 times lower than the levels found in healthy individuals ($p<0.05$). ANXA 1 and FPR3 do not present a uniform profile in patients; however, 40% of sepsis patients show upregulation in ANXA 1 expression. Downregulation of FPR1/2 in leukocytes can contribute significantly to the collapse of the immune response in sepsis. Once these receptors are activated, they trigger several signaling pathways that control cell differentiation, fight infection and regulate pro- and anti-inflammatory activity.

Key Words: Sepsis, Formyl Peptide Receptor, immune dysfunction, Leukocytes

INTRODUCTION

Sepsis is a clinical syndrome in which the individual presents relevant hemodynamic and immunological changes [1]. It has been defined as a life-threatening organ dysfunction resulting from infection [2]. Classified as a serious health problem and is the main cause of deaths in Intensive Care Units (ICUs) [3].

It is estimated that 32 million sepsis cases occur annually worldwide with 5.3 million potential deaths [4]. In Brazil, the incidence of adult sepsis in the Brazilian population is high, corresponding to 290 cases per 100,000 adults, which translate into 419,047 adult sepsis patients treated in ICUs per year in Brazil, of which 233,409 die in the hospital [5].

Infection and trauma (tissue injury) generate excess exogenous (PAMPs – Pathogen-Associated Molecular Patterns) and endogenous (DAMPs - Damage-Associated Molecular Patterns) immunological stimuli, triggering cascades of events that exacerbate the immune response in sepsis patients [6].

In this context, the Formyl Peptide Receptors (FPR) are considered key molecules of the immune response, and are activated by different PAMPs and DAMPs [7]. FPRs are part of a family of G protein-coupled receptors composed of FPR1, FPR2 and FPR3. They were first described in immune cells, however is now known that epithelial, endothelial, liver, spleen, lung and skeletal muscle cells can express these receptors [8, 9].

In leukocytes the expression profiles of FPRs are cell-type-dependent, characterized by their presence in the Monocyte/Macrophage (FPR1, FPR2 and FPR3) [8], Neutrophils (FPR1 and FPR2) [10], Natural Killer (FPR1 and FPR2) [11],

Dendritic cells (immature: FPR1 and FPR3; mature: FPR3) [12], Naive T, Th1, Th2 and Th17 cells (FPR2) [13, 14].

The activation of FPRs trigger intracellular signaling cascades that can culminate not only in cell migration, phagocytosis, degranulation, but also in the generation of ROS, cytokines and chemokines, among other functions [15, 16]. The effect of activation depends on the cell type and the receptor to be activated.

The annexin A1 (ANXA 1) is the only endogenous pan-FPR agonist known to date [17]. This protein is regulated by glucocorticoid and presents pro- and anti-inflammatory activities, being able to activate the phagocytes for the clearance of apoptotic cells [18].

This study aimed to evaluate the gene expression of FPR1, FPR2, FPR3 and ANXA 1 in leukocytes in order to understand the contribution of FPRs expression in the immunological dysregulation of patients with sepsis.

MATERIALS AND METHODS

STUDY POPULATION

Blood samples were obtained from patients of the Clinics' Hospital at the Federal University of Uberlandia (UFU) in 2015-2016. All procedures were approved by the UFU Ethics Committee on Human Research (N. 153.331/2012); informed consent was obtained from all participants. Blood samples were collected EDTA Vacutainer tubes (BD Vacutainer, USA).

The 20 healthy control subjects were comprised of 12 women and 8 men (age range, 19-55 y; median age, 27y). Donors with HIV infection, autoimmune disease or those otherwise not fully healthy were excluded.

Samples were collected from sepsis patients within 48 hours after the sepsis diagnosis [2]. The relevant clinical and laboratory data for the 64 sepsis patients are displayed in Table 1. Samples were analyzed from 34 sepsis and 30 septic shock cases. The patients groups presented the same median age.

GENE EXPRESSION ANALYSIS FOR FPR1, FPR2/ALX, FPR3 AND ANXA I.

RNA from blood samples was extracted using the Trizol LS (Life Technologies, USA) according to the manufacturer's protocols. The concentration, purity, and integrity of the RNA samples were determined by ultraviolet absorbance and electrophoresis. Complementary DNA (cDNA) was generated by reverse transcription (MMLV, Life Technologies, USA) using 1µg of RNA, according to the manufacturer's protocol. RT-qPCR was performed on ABI PRISM 7300 (Applied Biosystems, USA) using TaqMan Universal PCR Master Mix to quantify mRNA levels of FPR1, FPR2/ALX, FPR3 and ANXA in blood samples. The reaction volume was 12 µL, including 6 µL master mix (Applied Biosystems, USA), 5 µL cDNA template, 0.2 µL probe, and 0.8 µL water. The following probes were used: FPR1: Hs04235426_s1, FPR2/ALX: Hs02759175_s1, FPR3: Hs01574392_m1, ANXA1: Hs00167549_m1 and the endogenous control GAPDH: Hs03929097_g1 (Applied Biosystems, USA). Cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All reactions were performed in duplicate. The expression of the target gene transcripts was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and relative quantification was done using the $2^{-\Delta\Delta Ct}$ method, using the control group as calibrator. In addition,

we used $-1/2^{-ddCt}$ in the values between +1 and -1 to demonstrate better down-regulation.

STATISTICAL ANALYSIS

Statistical analyses were performed using the software GraphPad Prism version 7.0 for Windows (GraphPad Software, San Diego, CA, USA). The results were expressed as median and interquartile range. Differences' between controls, sepsis and septic shock were analyzed by one-way analysis of variance (ANOVA) followed by the Kruskal-Wallis multiple comparisons test. Differences' between groups were considered statistically significant at *p<0.05, ***p<0.001, **** p <0.0001.

DETERMINATION OF THE TRANSCRIPTIONAL PROFILE OF FPR1, FPR2 / ALX, FPR3, ANXA1

Figure 1 displays the gene expression of FPRs and ANXAI. The analysis of transcriptional expression revealed an exacerbated decrease of FPR1 and FPR2 mRNA in whole blood patients with sepsis and shock septic in relation to the controls. The median expression of FPR1 was reduced approximately 80-fold, with patients declining up to 272 times in the sepsis group and 353 times in the shock group (p <0.0001). The reduction of FPR2 expression was more drastic; the mean decline in sepsis group was 150 times and septic group 60 times (p<0.0001).

The profile expressed by the receptor 3 is not uniform; there are patients presenting positive regulation and others showing negative regulation.

The ANXAI expression in most patients remained constant, producing levels similar to those in healthy individuals. However, 40% of the individuals with sepsis

presented an increase of ANXAI. A systemic immunologic deactivation of FPRs in sepsis syndrome is observed irrespective of the clinical outcome of the patients (data not shown).

DISCUSSION

This is the first study to demonstrate deep downregulation of the FPR1/FPR2 genes suggesting being a key molecular event during sepsis. Many agonists, PAMPs and DAMPs of the FPRs are present in the circulation of the sepsis patient [6, 7, 9]. However, once the FPR is activated, it becomes non-responsable for subsequent activation by the same stimulus. Receptors undergo homologous desensitization and internalization [19].

The evaluated sepsis and septic shock patients presented downregulation of the FPR1 and FPR2 gene expression when compared to the healthy individuals. Decreased expression of these receptors may be a protective mechanism for the evolution of sepsis.

Wenceslau et al. demonstrated that mitochondrial formyl peptides may provoke serious symptoms common to this syndrome, such as severe hypotension that generates hyporeactivity to vasoconstrictors, vasodilatation, endothelial dysfunction, vascular injury, blood clot and lung injury. These effects are responses of the exacerbated activation of FPR1/2 culminating in the release of histamine and nitric oxide [20].

FPR2 plays a key role in sepsis, and can be activated by lipids, proteins and peptides [21]. Some protective effects were described by the activation of the receptor FPR2, which in sepsis is responsible for diminishing lung injury whereas in

myocardial infarction its activation aids in mobilizing angiogenic cells to the affected region, thus reducing the area of injury [10, 22].

Reduction of FPR2 expression in patients with sepsis may contribute to the immune dysfunction of this syndrome. This receptor is important for the differentiation of several immune cells such as macrophage polarization into the M2 profile [23], monocyte into macrophage and dendritic cells [24] and T helper cells [13]. In addition, neutrophil dysfunction in relation to the ability to migrate to infection sites has been correlated with decreased FPR2 [16].

The effect of decreasing FPR1 is unknown. Molloy, et al., demonstrated that FPR1-deficient mice showed large microbial translocation, resulting in increased mortality compared to wild-type mice [25]. The low expression of FPR1 in patients may be leading to the same phenomenon observed by Molloy et al.

The increase in ANXA 1 expression in individuals with sepsis may be a result of the host's response to infection. This protein is a pan-FPR agonist that is capable of activating all FPRs to promote in immune cells the migration, phagocytosis, degranulation, ROS generation, and the release of cytokines and chemokines to combat pathogens [16]. The patient with shock exhibits ANXA 1 and FPR3 gene expression levels similar to the control.

CONCLUSION

Our study has limitations, such as the low sample size and the results are based on gene expression. However, the conclusions drawn from our data demonstrate that patients presented similar expression to the control of FPR3 and ANXAI genes with profound reduction in the regulation of FPR1 and FPR2 genes expression that may be corroborating with the immune dysfunction of this syndrome.

COMPETING INTERESTS

The authors declare that they have no competing interests.

ACKNOWLEDGEMENTS

The authors thank the all the individuals who participated in this work, including the medical staff from the Intensive Unit Care of the University Hospital for providing the biological samples and clinical parameters, and are grateful for the financial support from the National Council for Scientific and Technological Development (CNPq), Coordination for the Improvement of Higher Education Personnel (CAPES), and Foundation for Research Support of Minas Gerais (FAPEMIG).

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FIGURE AND TABLES

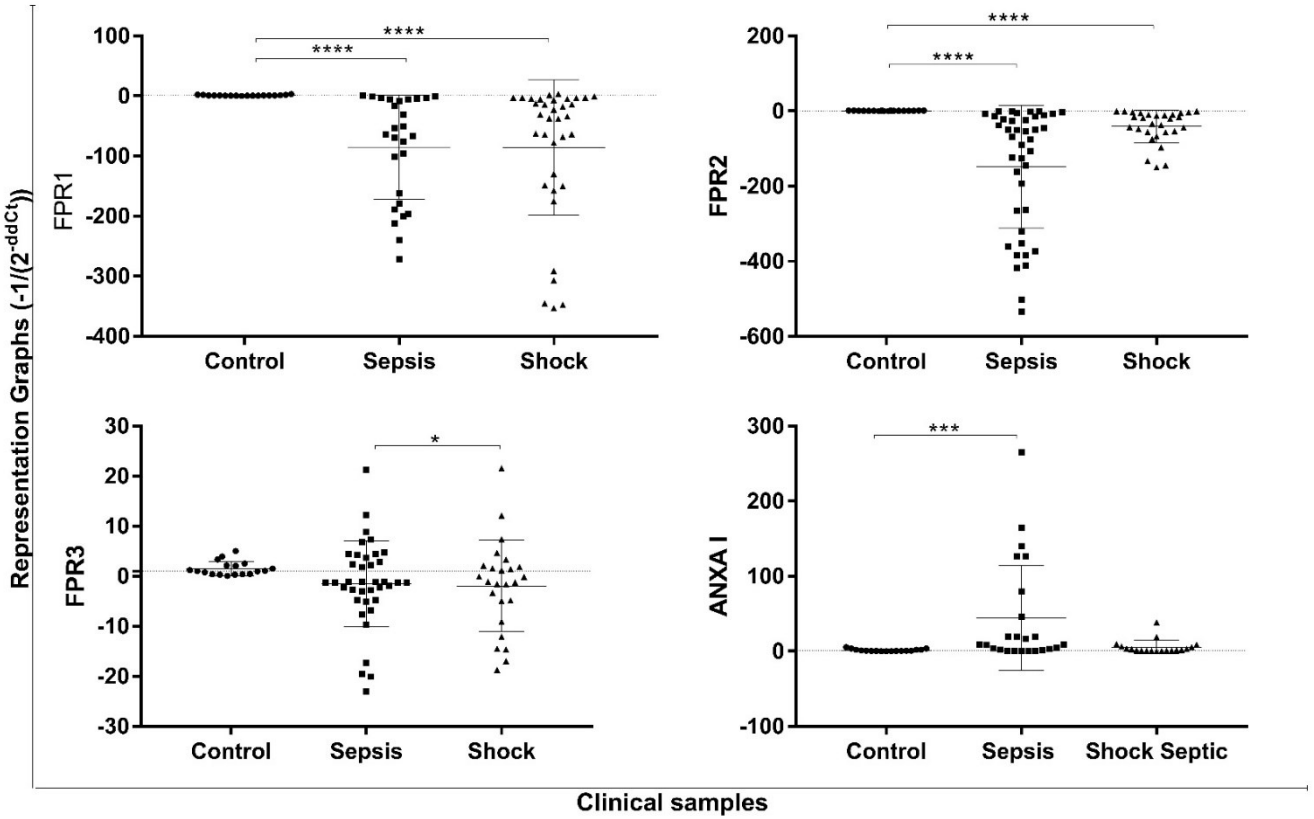

Figura 1 . Relative expression of FPRs and ANXA1 mRNA in whole blood

Erro! Indicador não definido.. Relative expression of RNAm from FPR1, FPR2/ALX, FPR3 and ANXA1 in sepsis, septic shock and controls. The transcription levels were normalized with the GAPDH gene and calibrated with the control group. * p <0.05, *** p <0.001, **** p <0.0001

Table 1. Characteristics of patients with sepsis

	SEPSIS	SEPTIC SHOCK
Patients (n)	34	30
Age (years)[a]	49 (36-59)	50 (30-73)
Gender (Male/Female)	19/15	15/15
Type of hospitalization		
Surgical (%)	30	42
Traumatic (%)	27	15
Neurological (%)	24	25
Clinical (%)	19	18
Microbiological data[b]		
Gram-negative bacteria	5 (15%)	4 (13%)
Gram-positive bacteria	4 (12%)	5 (17%)
Polymicrobial	15 (44%)	15 (50%)
Fungi	5 (15%)	7 (23%)
Positive cultures	27 (80%)	24 (80%)
Positive blood cultures	10 (30%)	13 (44%)
Mechanical ventilation[C]	13.5(11-20.25)	14 (10.5-22.75)
AKF (%)	36	46
Hemodialysis (%)	29	40
SAPS3 score[a]	61 (54.25-69)	65 (53-76)
% Expected Mortality[a]	38 (24.5-56.25)	48 (58-69)
SOFA score[a]	8 (6-9)	9 (7-11)
Days of ICU stay[a]	24 (19-39)	21.5 (10.75- 38.25)
Clinical outcome		
Survivor (%)	68	37

[a] Median (interquartile), [b] The percentages demonstrated in sepsis and septic shock refers to the population whereas those found in survivors and non-survivors refer to the total of the microorganisms analyzed. [c] Days of mechanical ventilation are expressed as median (interquartile); SOFA, Sequential Organ Failure Assessment; AKF, Acute Kidney Failure

CAPÍTULO III

PLASMA CYTOKINE SIGNATURE ASSOCIATED WITH DEATH IN SEPSIS

(Artigo no formato da revista *Critical Care)*

PLASMA CYTOKINE SIGNATURE ASSOCIATED WITH DEATH IN SEPSIS

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ABSTRACT

Sepsis is defined as a highly lethal condition of organ dysfunction caused by a dysregulated host response to infection. Cytokines, chemokines and regulatory molecules orchestrate the immune response. This study aimed to identify and determine specific alterations in cytokines' signatures in the plasma of patients with sepsis that survive and died. We have used a high definition multiplex system for simultaneous detection of 27 cytokines. We identified significant increases of IL6, IL10, IL8, CCL4 and CXCL10, and decrease of CCL5 in patient samples in relation to the profile expressed in controls. IL5, IL9, IL13, IL17 and TNFα were differentially expressed as sepsis versus septic shock. Sepsis non-survivors presented lower expressions of IL5, IL7, TNFα and IFNγ and higher levels of CCL4 and CXCL10 than the survivors. In terms of predicting sepsis, cytokines IL6 and IL8 were the best markers analyzed. IFNγ and TNFα are protective factor molecules while CCL4 and CXCL10 are able to predict the risk of death in sepsis. In this exploratory analysis using a cytokine panel, we have identified a specific cytokine profile associated with death during sepsis progression, and putative pathways involved in immunological silencing.

Key Words: Sepsis, cytokines, chemokines, CXCL10, path analysis, biomarkers

Abbreviations:

ICU (Intensive Care Units), ILAS (Latin American Sepsis Institute), IL (Interleukin), Basic FGF (Basic Fibroblast Growth Factor), G-CSF (Granulocyte Colony-Stimulating Factor), GM-CSF (Granulocyte-Macrophage Colony-Stimulating Factor), IFN-γ (Interferon Gamma), CXCL10 (IP10:Interferon Gamma-Induced Protein 10), CCL2 (MCP1:Monocyte Chemoattractant Protein-1), PDGF-bb (Platelet-Derived Growth Factor), TNFα (Tumor Necrosis Factor Alpha) and VEGF (Vascular Endothelial Growth Factor).

INTRODUCTION

Sepsis is a clinical syndrome in which the individual undergoes relevant hemodynamic and immunological changes [1]. It is classified as one of the oldest and most enigmatic syndromes of medicine, and constitutes a serious health problem and the main cause of death in the Intensive Care Units (ICUs) [2]. It is estimated that 32 million sepsis cases occur annually worldwide with a potential of 5.3 million deaths each year [3]. The incidence of adult sepsis in the Brazilian population corresponds to 290 cases per 100,000 adults, which translates into 419,047 adult sepsis patients treated in ICUs per year in Brazil, of which 233,409 die in the hospital [4].

Sepsis is associated with potentially fatal infection that produces an organic dysfunction caused by a dysregulated host response to infection, which increases morbidity and mortality [5].

The immune system performs the function of recognizing an infectious agent, controlling its spread, limiting inflammation and repairing damaged tissues. For the execution of this process, this system has components of the humoral and cellular response. Immunological activation results in the increase and release of pro- and anti-inflammatory mediators at the lesion site [6].

The immune response in sepsis is exacerbated by the occurrence of cytokine storms in both profiles, which may promote an exhaustion in innate and adaptive immunity. This situation is worrisome given that in the exhaustion the individual does not produce an effective response against the pathogen, making the organism susceptible to secondary infections [7]. Therefore, the patient's sepsis condition is

severe, with initially persistent inflammation and subsequent immunosuppressive factors contributing to adverse clinical outcomes [8].

Time is a critical factor for sepsis patients. Thus, rapid diagnosis, surveillance under the clinical evolution and the correct treatment of these patients may be determining factors in the attempt to ensure their survival. Cytokines, chemokines and regulatory molecules orchestrate the immune response; this study aimed to evaluate 27 markers to identify the profile expressed by patients and to ascertain the changes that correlate with their clinical outcome (survival or death).

MATERIALS AND METHODS

STUDY POPULATION

The Ethics Committee of Federal University of Uberlândia (UFU) (protocol number: 153.331/2012) approved this prospective observational trial protocol. Prior to blood collection, written informed consent was obtained from all controls and patients or their guardians. The samples were collected in 2015-2016.

Sepsis patients. Patients who were in treatment at the Intensive Care Unit in the Clinical Hospital – UFU (Uberlândia –Brazil), were aged more than 18 years, and fulfilled the defining criteria of sepsis were selected for inclusion in this study. The sample was taken within 48 hours after the diagnosis [5].

Healthy volunteers. Healthy control subjects consisted of 20 individuals, comprising 12 women and 8 men (age range, 19-55 y; median age, 27y). Donors with HIV infection, autoimmune disease or otherwise not fully healthy were excluded. Written consent for blood draws was obtained from each donor.

MULTIPLEX CYTOKINE ASSAY

Blood samples were collected in an EDTA tube between 6 and 7am. The plasma was separated by centrifugation at 800G for 15 min at 4°C, aliquoted and stored at -70°C until analysis. A multiplex cytokine (Bio-PLex Pro-human Cytokine standard 27-Plex) which contains IL1β, IL1Ra, IL2, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL12p70, IL13, IL15, IL17A, CCL26, Basic FGF (Basic fibroblast growth factor), G-CSF (granulocyte colony-stimulating Factor), GM-CSF (Granulocyte-macrophage colony-stimulating factor), IFNγ, CXCL10, CCL2, CCL3, CCL4, CCL5, PDGF-bb (Platelet-derived growth factor), TNFα and VEGF was obtained, and the assay was performed in accordance with the manufacturer's instructions (Bio-Rad, Hercules, CA, USA).

In brief, the appropriate cytokine standards and samples (50 µl), diluted in plasma dilution buffer, were added to wells of a filtered plate. The samples were incubated with 50 µl of the antibody-coupled microsphere set (2,000 beads/well) at room temperature for 30 min on a plate shaker (set to 300 rpm) in the dark and filter-washed three times with 100 µl wash buffer. Freshly diluted secondary/detection antibody (25 µl/well) was added to the wells and then incubated at room temperature on a plate shaker for 30 min in the dark and filter-washed three times with 100 µl wash buffer. Fifty microliters of streptavidin-PE (16 µg/ml in assay buffer) was added to the wells, and incubation at room temperature continued for the first 10 min on a plate shaker. Unbound analytes were filtered through the wells using the vacuum manifold; the bound beads were washed three times with 100 µl/wash buffer. After the last wash step, 125 µl of assay buffer was added to each well and the plate

placed for 1 min on a plate shaker set at 500 rpm and then for 3 min at the reduced speed of 300 rpm. Fifty microliters of sample was analyzed on the Bio-Plex system (Bio-Rad) in accordance with the manufacturer's instructions.

STATISTICAL ANALYSIS

Statistical analyses were performed using the software GraphPad Prism version 7.0 for Windows (GraphPad Software, San Diego, CA, USA). Cytokines data are represented as percentages to identify the signature expressed by each patient in on a heat map, since the basal level of each cytokine is different. After elimination of the outliers samples, the maximum quantification for each cytokine was verified, being this value considered 100% and the percentage of each sample calculated in relation to this value. Numeric variables are expressed as median (interquartile range) and were assessed via the Mann-Whitney U-test and Kruskal-Wallis test. The cut-off value was established by sensitivity and specificity parameters. After determining the cut off value the contingency analysis was performed between the groups by the Fisher test to obtain the odds ratio value

RESULTS

CLINICAL AND BIOLOGICAL PARAMETERS

The relevant clinical and laboratory data for the 64 patients are in Table 1. Samples of 34 sepsis and 30 septic shock cases were analyzed. The patients groups had the same median age ($p>0.05$). The clinical outcome of the patients were 33 survivors and 31 deaths.

SIGNATURE PROFILE OF CYTOKINES

The baseline quantification of each cytokine found in plasma is different. The data are represented as percentages to identify a molecular signature present in each evaluated group independent of its concentration. Twenty-seven molecules were evaluated; VEGF and IL2 were excluded from the analyses because they were detected in few samples.

The maximum quantifications obtained from the molecules in pg/ml were: IL1β (12), IL1ra (2181), IL4 (12), IL5 (80), IL6 (257), IL7 (49), IL8 (141), IL9 (106), IL10 (60), IL12p70 (3848), IL13 (1012), IL15 (2855), IL17A (9468), TNFα (187), IFNγ (500), CXCL10 (4263), CCL2 (174), CCL3 (10.05), CCL4 (303), CCL5 (21023), Basic-FGF (30101), PDGF-BB(4353), GM-CSF (110), G-CSF (321) and CCL11 (6566).

Figure 1 represents the expression pattern of these molecules per individual (line:cytokine; column:sample). The heat map result reveals the presence of a molecular signature of the predominant cytokine expression. Most healthy cases have a cytokine expression grade of 50% or less except IL15, IL17A, FGF-basic and CCL26. In sepsis there is increased expression of most molecules; however, in septic shock these quantifications are reduced. Patients in this study who survived had a higher cytokine response profile than the fatalities (Fig.1).

The heterogeneity of individuals in each group reflects the variety of primary causes that have evolved into sepsis. Despite the diversity, thirteen cytokines were statistically differentiable among the groups: IL6, IL8, IL10, CXCL10 and CCL4 are

more highly expressed in patients than healthy controls; only CCL5 decreased. IL5, IL9, IL13, IL17 and TNFα are able to distinguish the degree of sepsis (Table 2).

The non-survivors were found to have decreased expression of IL5, IL7, IFNγ and TNFα with increased chemokines CCL4 and CXCL10 compared to survivors (Table 3). Sepsis patients with levels greater than 86.24 pg/ml TNFα and 225.2 pg/ml IFNγ had a 4-fold chance of survival, whereas the CXCL10 and CCL4 molecules were predictive of the death rate when their levels exceeded 1688 pg/ml and 85.2 pg/ml, respectively. The best biomarker able to predict death risk is the ratio of CXCL10 to IFNγ; when this ratio is over 9.8, the patient faces a 49-fold greater chance of death from sepsis syndrome (Table 4).

DISCUSSION

The cytokine expression profile in sepsis is variable suggesting that the host response to this syndrome is not a simple model but rather a dynamic process in which the thin balance in the cytokine and chemokine pathways is crucial for the elimination of invading pathogens, control of injury and survival of the individual [9]. In this report we found that in sepsis the pattern of IL6, IL10, IL8, CCL4, CCL3, CXCL10 and CCL5 in the plasma contrasts with that of healthy individuals.

We have shown that IL6 is an accurate predictor of sepsis but could not be correlated with the severity of the syndrome due to the protective and deleterious effects of this molecule in sepsis. IL6 is a pleiotropic molecule whose anti-inflammatory effect is associated with inhibiting the release of pro-inflammatory cytokines such as TNFα and IL1β and inducing the release of anti-inflammatory mediators: IL1ra, sTNFRs, IL10, TGFβ and cortisol, to control local and systemic

inflammation [10-13]. However, this cytokine not only causes fever and mediation of the acute phase response, but also induces systemic reaction to an inflammatory stimulus and myocardial depression. Myocardial depression in septic shock can trigger impaired tissue perfusion, organ failure and death [9, 14].

Expression of IL10 is increased in sepsis patients. IL10's role in septic immune dysfunction is correlated with anti-inflammatory or immunosuppressive activity, which triggers protective or harmful effects whose impact is determined by the timing and localization of release [15]. Anti-inflammatory effects focus on the ability of IL10 to suppress gene expression and synthesis of pro-inflammatory cytokines [16]. The mechanism by which IL10 promotes immunosuppression is correlated with downregulation of co-stimulatory molecules of antigen presentation [17], deletion of T cell responses [18] and macrophage apoptosis [19].

Chemokines play a key role in the action of immune effector cells against bacterial infection. High levels of circulating chemokines contribute to deactivation of circulating leukocytes and limit their recruitment to the inflammatory focus in sepsis. High levels of this chemokine might be useful in predicting disease severity [20]. In the evaluated patients the chemokines IL8, CCL4, CCL3 and CXCL10 are increased.

Corroborating our findings, Kraft, R. et. al. was able to predict sepsis through the concentration of IL8 in plasma. Although we did not find significant differences in the levels between the survivors and those patients who died, the patients analyzed in the Kraft, R. et al. study represent the group with low IL8 levels and mortality [21]. IL8 is one of the major mediators of the inflammatory response, activation of

neutrophils, tissue repair mechanisms such as angiogenesis, and cell proliferation [22-24].

The expressions of CCL4 and CXCL10 are exacerbated in patients with sepsis, and are predictors of disease and death. Although CCL4 plays a major role in the recruitment of leukocites to infection sites, the function of this chemokine in sepsis has not yet been elucidated [25].

CXCL10 is a pleiotropic molecule capable of exerting different biological functions, including chemotactic activity, apoptosis induction and the regulation of cell growth and proliferation [26]. The action of CXCL10 protects against or promotes infection depending on the immune status of the host, whereas high levels of this molecule are able to predict the severity of the disease [26-28]. The mechanisms for exacerbation of CXCL10 expression in non-surviving patients are induced by microorganisms since the expression of IFNγ is decreased and the ratio between these molecules is an apt predictor of death [26].

CCL5 is a proinflammatory chemokine that acts on differents immune cells, mediating not only trafficking and homing, but also inducing the release of proinflammatory cytokines [29]. Low CCL5 levels in sepsis compared with the healthy controls were observed by us and by Pablo, R. et. al.. Platelets are the main secretors of CCL5; patients with sepsis might have low and dysfunctional platelets, leading to impaired secretion of this chemokine [20]. In animal models, CCL5 blockade diminished the cellular responses and increased mice susceptibility to lethal pneumococcal disease [30].

Individuals who had septic shock presented a decrease in the expression of the cytokines IL5, IL9, IL13, IL17 and TNFα compared to the expression of patients

with sepsis. Septic shock shows the main alterations present in this syndrome, containing the changes present in sepsis and hypotension despite fluid resuscitation [31]. David, S. et. al. demonstrated dysfunction of the vascular endothelium in the presence of elevated levels of cytokines, but by mechanically decreasing the amount of IL9, IL10, IL13 and TNFα, among other molecules, reestablished vascular integrity [32].

In the sepsis syndrome there is an exacerbation of the pro-inflammatory and anti-inflammatory response, which generates an immune exhaustion in which the individual stops producing an effective response [7, 33, 34]. This mechanism may have been responsible for the decrease in the expressions of the pro-inflammatory (IL17 and TNFα) and anti-inflammatory proteins (IL9, IL10 and IL13), which are molecules related to the improvement of vascular reactivity and hemodynamics in animal models of hemorrhagic shock [35].

The clincial outcome was characterized by non-survival rates of 32% in sepsis and 63% in septic shock. These patients had a low expression of IL5, IL7, TNFα and IFNγ with exacerbation of CCL4 and CXCL10 when compared with the individuals who survived. The effect of this change is summarized in Supplementary Figure 1 (S.Fig.1).

Linch, S.N. et. al. demonstrated the relevance of IL5 in sepsis, reporting that, in monocytes, IL5 performs a function similar to that already described in eosinophils, which is to stimulate the production of cytokines, mobilization of intracellular calcium, augmentation of phagocytosis and delay of cellular death. The loss of IL5 increases mortality, tissue damage, bacteremia (bacterial load) during

sepsis; this explains why the patient that did not survive has lower IL5 levels that those that survived [36].

The decrease of IL7 in patients who died shows the relevance of this cytokine in this syndrome. This molecule plays a key role in the immune response by acting to block apoptosis of CD4 and CD8 T cells, restore IFNγ production, and activate recruitment of immune effector cells to the infected site, a situation that is impaired in sepsis [37].

The pro-inflammatory cytokines TNFα and IFNγ have pivotal importance in generating an appropriate bactericidal response to infection [38]. The low expression of these molecules can be responsible for fatal secondary nosocomial infections. IFNγ is IL7 induced and inhibited by IL6 anti-inflammatory pathways; this molecule is important in the dysfunction of sepsis because it is able to restore the production of cytokines and stimulate the expression of HLA-DR by antigen-presenting cells thus activating cells [37, 39, 40].

Studies have shown that administration of specific cytokines are capable of reversing exhaustion as therapy with IFNγ and GM-CSF that restores inflammatory functions by inducing TNFα production and surface expression of HLA-DR in monocytes [41].

CONCLUSION

The evaluation of 27 markers in the plasma of healthy individuals, sepsis and septic shock allowed the identification of candidate molecules to predict disease and clinical outcome. This evaluation of multiple cytokines can identify the pattern of cytokine response in surviving and non-surviving patients and contributed to

understand the immunological changes responsible for the clinical outcome of the patients. This approach is important for monitoring sepsis patients, identifying immune depletion, and activating the system to avoid a fatal outcome.

COMPETING INTERESTS

The authors declare that they have no competing interests.

ACKNOWLEDGEMENTS

The authors thank the all the individuals who participated in this work, including the medical staff from the Intensive Unit Care of the University Hospital for providing the biological samples and clinical parameters, and are grateful for the financial support by the National Council for Scientific and Technological Development (CNPq), Coordination for the Improvement of Higher Education Personnel (CAPES), and Foundation for Research Support of Minas Gerais (FAPEMIG).

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FIGURE LEGENDS

Fig 1. Heat map of differentially expressed cytokine in plasma from control and sepsis patients. The normalized pg/ml values were expressed in percentage each cytokine. The expression levels was represent by the color: Red is high, black medium and green low expression levels. The expression pattern of these molecules has represented the individual (line:cytokine; column:sample).

S. Fig. 1 Effect of altered cytokine expression in non-surviving patients.

The correlation between the cytokines are expressed in R^2 and *p-value*. There is a decrease in IL5, IL7, TNFα and INFγ expression (blue) and increase in CCL4 and CXCL10 (red) in non-surviving patients. The low expression of IL7 promove lymphocyte apoptosis. The absence of these cells decreases the secretion of INFγ and TNFα, these cytokines are important for activating antigen-presenting cells (APCs) and monocytes. Co-stimulatory molecule expression are decrease in low IL7 and IL5 expression deactivating APC cells. CXCL10 induce lymphocytes apoptosis and block the APC . This cytokine induces monocytes activation however high levels of circulating chemokines (CXCL10 and CCL4) contribute to deactivation of circulating leukocytes. In short, this change promove disfunction of monocytes, lymphocytes and APC, cells important in fight the pathogen.

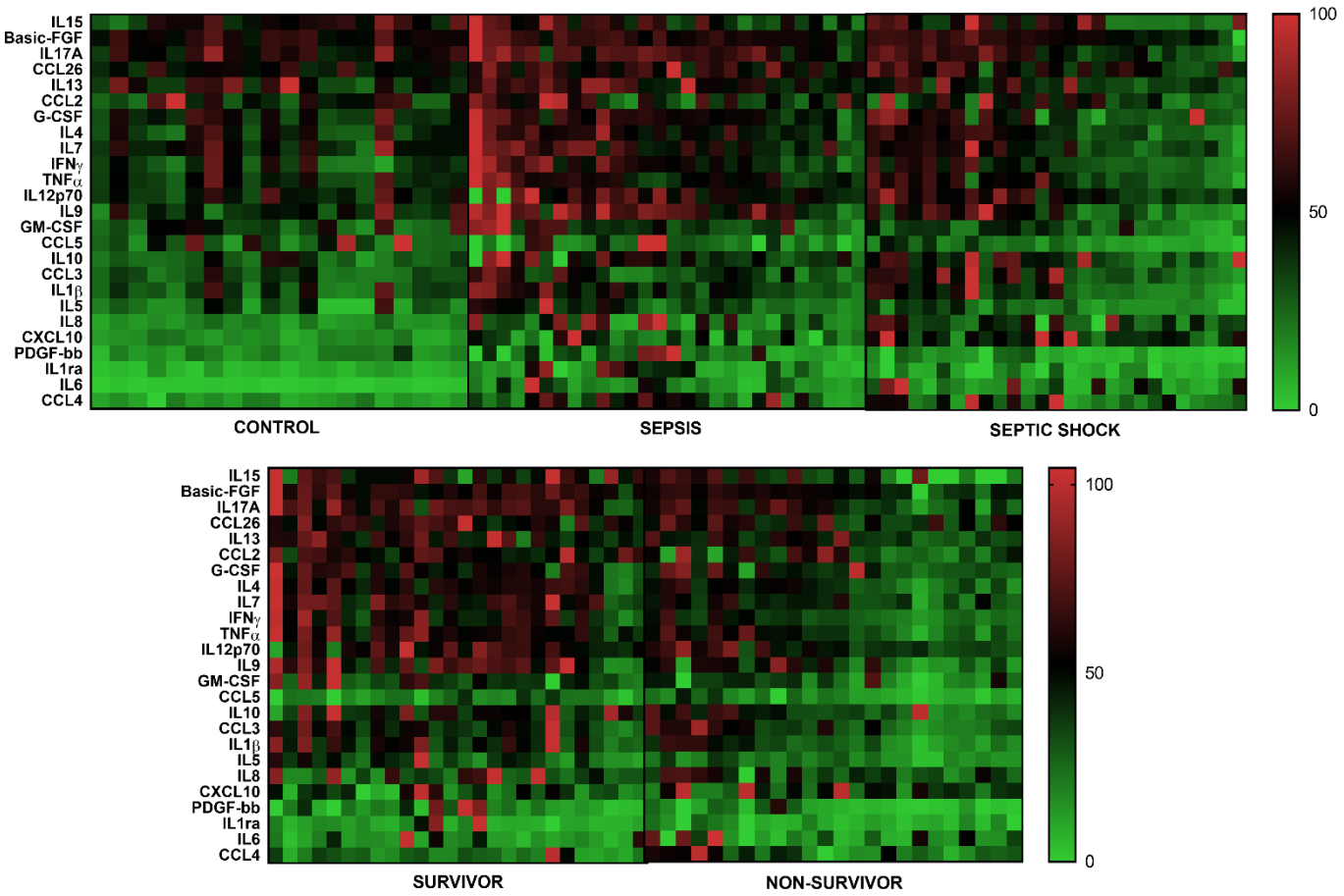
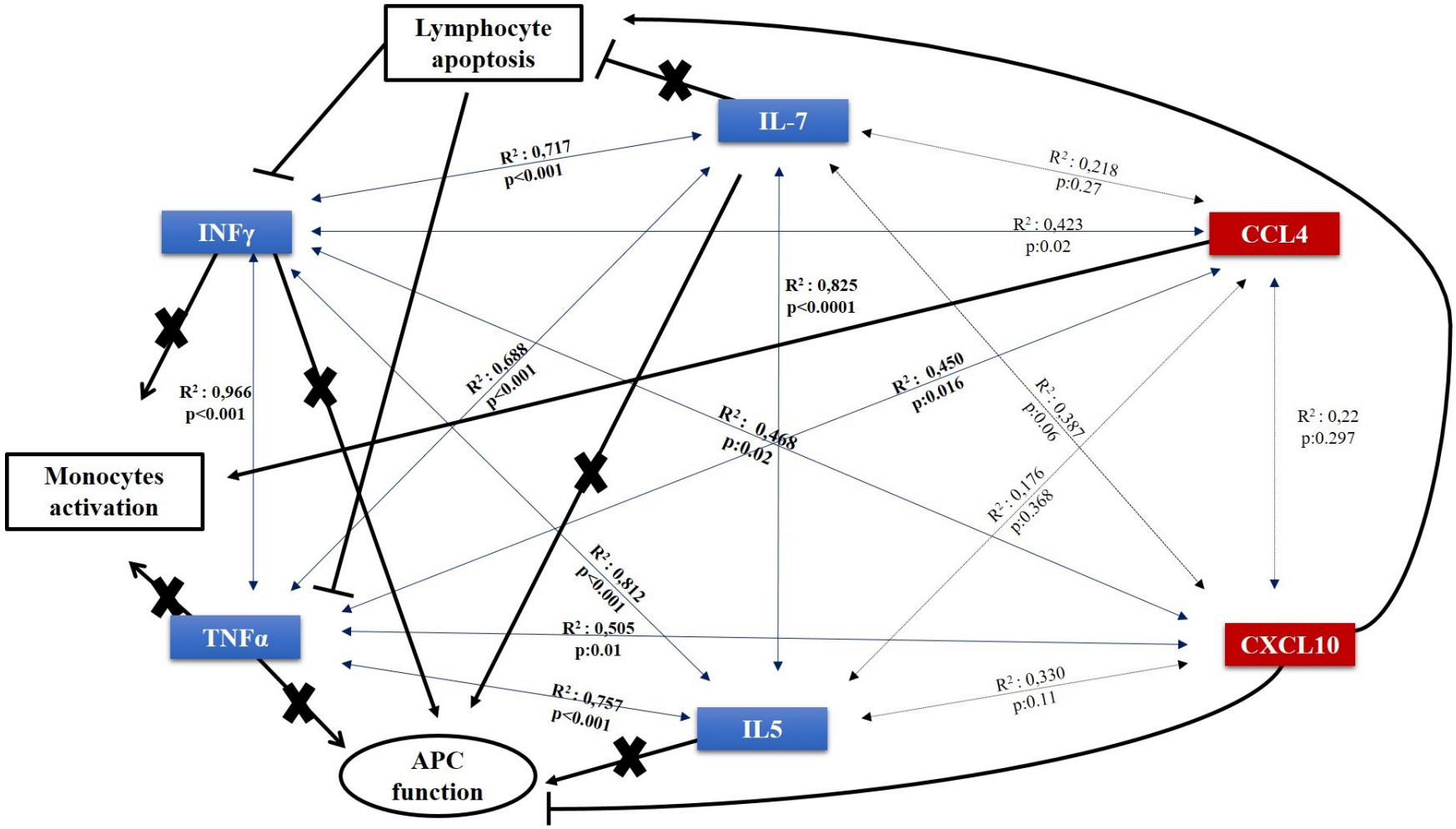

IL15
Basic-FGF
IL17A
CCL26
IL13
CCL2
G-CSF
IL4
IL7
IFNγ
TNFα
IL12p70
IL9
GM-CSF
CCL5
IL10
CCL3
IL1β
IL5
IL8
CXCL10
PDGF-bb
IL1ra
IL6
CCL4
CONTROL
SEPSIS
SEPTIC SHOCK
100
50
0
SURVIVOR
NON-SURVIVOR

Fig.1


Lymphocyte apoptosis
IL-7
INFγ
CCL4
Monocytes activation
TNFα
CXCL10
IL5
APC function
R² : 0,717 p<0.001
R² : 0,218 p:0.27
R² : 0,423 p:0.02
R² : 0,825 p<0.0001
R² : 0,966 p<0.001
R² : 0,688 p<0.001
R² : 0,450 p:0.016
R² : 0,468 p:0.02
R² : 0,387 p:0.06
R² : 0,22 p:0.297
R² : 0,176 p:0.368
R² : 0,812 p<0.001
R² : 0,505 p:0.01
R² : 0,757 p<0.001
R² : 0,330 p:0.11

S.Fig.1

Table 1. Characteristics of patients with sepsis

	SEPSIS	SEPTIC SHOCK	SURVIVORS	NON-SURVIVORS
Patients (n)	34	30	33	31
Age (years)[a]	49 (36-59)	50 (30-73)	43.5 (58-89)	55 (38.5-72.5)
Gender (Male/Female)	19/15	15/15	18/15	15/16
Type of hospitalization				
Surgical (%)	30	42	31	28
Traumatic (%)	27	15	19	18
Neurological (%)	24	25	28	31
Clinical (%)	19	18	22	23
Microbiological data[b]				
Gram-negative bacteria	5 (15%)	4 (13%)	7 (78%)	2 (12%)
Gram-positive bacteria	4 (12%)	5 (17%)	3 (34%)	6 (67%)
Polymicrobial	15 (44%)	15 (50%)	12 (40%)	18 (60%)
Fungi	5 (15%)	7 (23%)	6 (50%)	6 (50%)
Positive cultures	27 (80%)	24 (80%)	25 (49%)	26 (51%)
Positive blood cultures	10 (30%)	13 (44%)	9 (39%)	14 (61%)
Mechanical ventilation[C]	13.5(11-20.25)	14 (10.5-22.75)	13 (10.25-19.75)	14(12-28)
AKF (%)	36	46	26	52
Hemodialysis (%)	29	40	23	38
SAPS3 score[a]	61 (54.25-69)	65 (53-76)	59 (52-68)	67 (57-77)
%Expected Mortality [a]	38 (24.5-56.25)	48 (58-69)	35 (21.5-52.5)	52 (31.5-69.5)
SOFA score[a]	8 (6-9)	9 (7-11)	7.5 (5-9)	9 (7.5-11)
Days of ICU stay[a]	24 (19-39)	21.5 (10.75- 38.25)	22 (17.5-34.5)	24 (17.5-42.5)
Clinical outcome				
Survivor (%)	68	37	100	0

[a] Median (interquartile), [b] The percentage demonstrated in sepsis and septic shock refers to the population whereas that demonstrated in survivors and non-survivors refers to the total of the analyzed microorganism [c] Days of mechanical ventilation expressed as median (interquartile); SOFA, Sequential Organ Failure Assessment; AKF, Acute Kidney Failure.

Table 2. Plasma cytokine concentrations: control, sepsis and septic shock

CYTOKINE	CONTROL	SEPSIS	SEPTIC SHOCK	p-*value*[1]	*p-value*[2]	*p-value*[3]
IL15	1537 (1206-1807)	1570 (110-1980)	1601 (980-1930)	>0.99	>0.99	>0.99
BASIC-FGF	1643 (1498-1934)	1729 (1558-1950)	1719 (1477-1900)	>0.99	>0.99	>0.99
IL17	5092 (4291-6076)	5996 (4547-6810)	4747 (3078-5869)	0.68	0.38	**0.04**
CCL26	3126 (2768-3515)	3785 (3122-4188)	3477 (2561-4084)	0.09	0.63	0.43
IL13	485.4 (357-624)	549 (434-638)	410.6 (348-527)	0.34	>0.99	**0.02**
CCL2	77.9 (44.8-101)	91.8 (64.5-123)	73.8 (49.4-102.5)	0.18	>0.99	0.10
G-CSF	126.9 (113-173)	154.6 (134-189)	133.8 (93.2-202)	0.52	>0.99	0.58
IL4	4.6 (3.14-6.18)	5.8 (4.6-7)	4.5 (3.2-6.2)	0.13	>0.99	0.13
IL7	15.2 (12.3-18)	16.7 (14-23)	13.6 (10.2-18.3)	0.81	>0.99	0.07
IFNγ	181.6 (132-243)	206.8 (154-287)	168.7 (128-255)	0.71	>0.99	0.26
TNFα	74.2 (60.6-93)	97 (75.4-116)	79 (49.4-99.4)	0.05	>0.99	**0.04**
IL12p70	1709 (1369-1925)	2031 (1630-2651)	1884 (1369-2507)	0.05	0.87	0.45
IL9	41.9 (36.05-52)	67 (37-81)	38.2 (19.5-61)	0.06	0.88	**0.009**
GM-CSF	35.9 (25.4-54.8)	40.4 (31-54.6)	38.9 (21.7-46.8)	>0.99	>0.99	0.56
CCL5	6149 (5277-9974)	3674 (2950-4795)	3546 (2160-5071)	**<0.001**	**<0.001**	>0.99
IL10	15.9 (13-18)	25.5 (19.2-33)	21.8 (17-39)	**0.001**	**0.007**	>0.99
CCL3	3.2 (2.4-3.5)	4.7 (3.2-5.6)	3.3 (2.5-6.05)	**0.01**	0.342	0.60
IL1β	3.9 (2.5-4.7)	4.9 (3.3-6.2)	3.3 (2.3-5.9)	0.25	>0.999	0.17
IL5	18.7 (8.8-27)	22.4 (17.5-33)	14.7 (11.6-24.7)	0.10	>0.99	**0.01**
IL8	16.8 (14.3-22)	35.9 (25-67.39)	39.4 (27.7-59.3)	**<0.001**	**<0.001**	**>**0.99
CXCL10	641.7 (478- 746)	1386 (876-2089)	1621 (1266-2068)	**<0.001**	**<0.001**	0.92
PDGF-bb	663 (505-959)	798.1 (350-1303)	296.4 (168-742)	>0.99	0.12	0.05
IL1ra	145 (109-221)	196.1 (148-367)	212.8 (152-396)	0.16	0.09	>0.99
IL6	6.2 (3.55-7.19)	40 (30.9-54.6)	39.7 (23-124)	**<0.001**	**<0.001**	>0.99
CCL4	39.5 (30.15-55.3)	78.2 (47.5-95.9)	79.7 (51.6-158)	**0.01**	**<0.001**	0.66

Values are in pg/ml, and as expressed as median (interquartile). Results of comparison test *p-value*[1] (control vs. sepsis), *p-value* [2](control vs. shock septic), *p-value* [3] (sepsis vs. shock septic). IL-6, IL-8, IL-10, CCL4, CXCL10 and CCL5 cytokines are differentially expressed in patients compared to the control group. CCL3 is higher in sepsis than control. IL-5, IL-9, IL-13, IL-17 and TNF-α are expressed less in shock than sepsis. BASIC-FGF, basic fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; IFN, interferon; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; TNF, tumor necrosis factor.

Table 3. Plasma cytokine/chemokine levels in patients by clinical outcome

CYTOKINE	SURVIVORS	NON-SURVIVORS	*p-value*[4]
IL15	1537 (1306-1980)	1603 (980.8-1909)	0.17
BASIC-FGF	1790 (1532-2029)	1687 (1491-1883)	0.64
IL17	5869 (3841-6797)	5268 (3164-6345)	0.08
CCL26	3785 (3228-4154)	3462 (2683-4154)	0.19
IL13	476.8 (385-634.2)	445.2 (363.8-634.2)	0.66
CCL2	87.9 (71.8-112.9)	82 (52.7-120.3)	0.61
G-CSF	164.9 (130.3-193.8)	140.8 (98.5-188.8)	0.45
IL4	5.9 (3.969-6.785)	4.952 (3.2-6)	0.07
IL7	18.3 (12.75-22.03)	15.2 (10.3-17.5)	**0.03**
INFγ	231.3 (152.1-315)	175.2 (128.1-228.3)	**0.01**
TNFα	98.2 (74.13-119.5)	83.8 (56.3-101.8)	**0.03**
IL12p70	2031 (1677-2570)	1855 (1388-2514)	0.34
IL9	62.6 (31.61-77.19)	53.7 (19.9-67.0)	0.19
GM-CSF	38.2 (29.08-46.26)	39.6 (22.8-48.5)	0.74
CCL5	4109 (3383-4949)	3136 (2160-5051)	0.13
IL10	25.5 (20.66-34.67)	21.8 (14.7-32.8)	0.59
CCL3	4.2 (3.1-5.4)	4.6 (2.6-6.0)	0.72
IL1β	5.0 (3.3-6.8)	4 (2.4 -5.5)	0.32
IL5	24.7 (14.7-32.9)	16.1 (11.6-24.7)	**0.02**
IL8	34.3 (25.3-71)	41.8 (28.1-62.6)	0.33
CXCL10	1262 (636-1775)	1898 (1535-2449)	**0.04**
PDGF-bb	571.7 (251.1-962.4)	490.8 (176.4-1215)	0.63
IL1ra	196.1 (151.5-357.2)	221.1 (149 - 434)	0.56
IL6	35.94 (27-48.3)	43.8 (33.3 - 88.6)	0.18
CCL4	57.5 (41.9-85.2)	91.3 (54-157.6)	**0.005**

Values are in pg/ml, and expressed as median (interquartile). [4]Mann-Whitney rank sum test.

Table 4. Predictor of sepsis disease and sepsis survival or non-survival determined by cytokine expression

CYTOKINE	CUTOFF	PREDICTOR	O.R	95%CI	*p-value*
IL6	>10.0	Disease	Infinity	4.658 to 135.1	<0.0001
IL8	> 27.9	Disease	55.73	8.921 to 591.7	<0.0001
CXCL10	> 788.3	Disease	18.67	5.033 to 57.64	<0.0001
CCL5	< 5196	Disease	10.62	3.432 to 30.93	<0.0001
CCL4	>58.8	Disease	8.81	2.406 to 30.41	0.0005
IL10	> 21.5	Disease	6	1.8 to 16.66	0.0017
TNFα	> 86.2	Survivor	0.46	0.2154 to 0.883	0.0167
IFNγ	> 225.2	Survivor	0.23	0.0826 to 0.752	0.0080
CXCL10	> 1688	Death	3.9	1.201 to 12.49	0.0205
CCL4	>85.2	Death	3.6	1.224 to 10.93	0.0225
CXCL10 / IFNγ	>9.8	Death	49	8.97 to 267.52	<0.0001

O.R: Odds Ratio; CI: Confindence Interval.

CAPÍTULO IV

PROTEOMIC ANALYSIS PREDICTS THE FEASIBLE MONOCYTE FUNCTION IN THE PRESENCE OF THE SEPTIC SHOCK COLLAPSE

(Artigo a ser submetido para a *Plos Medicine*)

PROTEOMIC ANALYSIS PREDICTS THE FEASIBLE MONOCYTE FUNCTION IN THE PRESENCE OF THE SEPTIC SHOCK COLLAPSE

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ABSTRACT

Sepsis is a great challenge for science due to its lethal condition associated with multiple organ dysfunction and immune response impairment. Among several factors affecting sepsis, monocytes have played a major role; however, their phenotype, proteomic profile and function in septic shock are still unknown. Our aim was to fully characterize the sub-population and proteomic profiles of monocytes in septic shock and discuss their possible impact on the disease. Peripheral blood monocytes subpopulations were phenotyped based on CD14/CD16 expression by flow cytometry, and proteins were extracted from monocytes of individuals with septic shock (SS) and healthy controls (HC) to ascertain changes in global protein expression in these cells. We were able to demonstrate that there was a profound depletion (50-fold) of immature monocytes (C14++/CD16-) in SS, whereas CD14dim/CD16+ had an 8-fold increase, indicating that the proteomic data was mainly due to the later phenotype. A 3-fold cutoff on the protein expression during global proteomic analysis of monocytes through 2D-nanoUPLC-UDMS^{E} demonstrated that 67 proteins were differentially expressed in SS patient compared to controls, in which 44 were up-regulated and 23 down-regulated. These alterations were related to proteins that were involved in monocytes' reprogramming, immune dysfunction, severe hypotension, hypo-responsiveness to vasoconstrictors, vasodilation, endothelial dysfunction, vascular injury, and blood clotting, which explain the disease severity and failure of therapies during septic shock. This investigation identified for the first time important biological targets that could play major roles in diagnosis, prognosis and therapy, which are discussed herein.

Key Words: Septic shock, monocytes, proteomic, systemic dysfunction

INTRODUCTION

Sepsis is a potentially fatal severe clinical syndrome in which the patient has organ dysfunction caused by the host's deregulated response to infection [1]. This syndrome evolves to septic shock when the pathophysiological changes are intensified in which circulatory, cellular and metabolic abnormalities are deep enough to disturb, being associated with an increased risk of decease [2].

Arterial hypotension is characteristic of septic shock resulting from hemodynamic changes that include degrees of hypovolemic response, decreased vascular tone and myocardial depression [3]. One of the characteristics of this alteration is the increase in generalized vascular permeability, generating progressive subcutaneous and cavity edemas that result in blood hypovolemia. Endothelial injury, in addition to causing vascular leakage and edema, may be responsible for shock, microvascular thrombosis and organic failure [4].

In septic shock, the systemic vascular dysfunction is observed as described above, however, these pivotal changes are essential to control the infection. The endothelial lesion enables inflammatory activation and coagulation. Both are important to activate an immune response against the pathogens and to compartmentalize the infection avoiding a systemic response. Subsequently the rolling and adhesion of the white blood cells and the increase of the vascular permeability allow these cells to enter the tissues and fight the invading microorganisms [3].

In this context, monocytes display an essential role, since they are cells present in the systemic blood circulation. These cells are also part of the immunity first line of scouting defense with the function to combat microorganisms, perform dead cells clearance and undertaking injured tissue regeneration [5].

Monocytes are present in three distinct populations: Classical (CD14high:CD16low), Non-classical (CD14low:CD16high) and Intermediate (CD14high:CD16high). Classical monocytes that are considered pro-inflammatory: perform phagocytosis and release large amounts of reactive oxygen species. Non-classical monocytes are considered to be anti-inflammatory, acting primarily on tissue regeneration, immunological surveillance and vascular endothelial patrol. Intermediate monocytes have been considered monocytes in transition from the classic to the non-classical profile, and can display both profiles [5, 6].

The comprehension about how the deep biological and clinical function, at septic shock collapse, linked to monocytes is still entwined. Many researches has focused their findings and efforts on the cell response to microorganism i.e. phagocytic capacity, microbicide, antigen presentation (HLA-DR) and cytokines production in order to determine if the monocytes has any dysfunction [7-9].

In this present study, we intended to evaluate the global protein profile from patients circulating monocytes with septic shock, to determine the alterations existent in these cells and to investigate the possible contribution in the collapse .

MATERIALS AND METHODS

STUDY POPULATION

The Ethics Committee of the Federal University of Uberlândia (UFU) under protocol number approved this study: 153.331. Prior to blood collection, a written informed consent was obtained from all controls and patients or their guardians.

Healthy volunteers. HC subjects consisted of 10 individuals, comprising 5 women and 5 men (age range, 19-55 y; median age, 27y). Donors with HIV infection, autoimmune disease or otherwise not fully healthy were excluded.

Patients. Five SS, which were in treatment at the Intensive Care Unit in the Clinical Hospital – Federal University of Uberlândia (Uberlândia –Brazil), were aged more than 18 years, and fulfilled the defining criteria of septic shock were selected for inclusion in this study. The sample was taken within 48 hours after the diagnosis [2]. The relevant clinical and laboratory data for the septic shock patients are displayed in Supplementary Table 1.

PERIPHERAL BLOOD MONOCYTES ISOLATION

About 40 mL of blood was collected in tubes containing Heparin (anti-coagulant) from patients and control subjects. The fresh blood sample was immediately processed for isolation of peripheral blood mononuclear cells (PBMC) using density gradient centrifugation through Histopaque-1077 (Sigma, Catalog n° H1077-1). Subsequently the monocytes were isolated from PBMC using the kit Dynabeads untouched Human monocytes, (Thermo Fisher Scientific, Catalog n°11350D) according to the manufacturer's. The kit contain antibodies mix and magnetic beads which capture T cells, B cells, NK cells, dendritic cells, erythrocytes, granulocytes and macrophages, allowing isolating monocytes untouched and free of surface-bound antibodies and beads. Monocyte population in PBMC was characterized by flow cytometry using the labeling for CD14/CD16 (Biolegend: CD14 PECy7, catalog: 325618/400126 e CD16 Alexa Fluor 647, catalog: 302020/400130).

PROTEIN EXTRATION AND SAMPLE PREPARATION

Monocytes total proteins were extract using Complete Lysis-M, EDTA-free (Roche, catalog: 04719964001) following the manufacturer's recommendation. Proteins were concentrated using Amicon ultra-filtration devices with dialysis membranes of 10 kDa (Merck-Millipore, catalog: UFC501096) and quantified by Micro

BCA Protein assay Kit (Thermo Fisher Scientific, catalog: 23235). Samples were adjusted to the same protein concentration and digested with trypsin as described below. Each pooled sample (50 µg of total protein) was buffer exchanged in 50-mM ammonium bicarbonate, denatured in the presence of 0.2% Rapi*GEST* SF (Waters, Milford, USA) at 80°C for 15 min in a dry bath reduced with 100-mM dithiothreitol at 60°C for 60 min, then alkylated with 300-mM iodoacetamide for 30 min in the dark at room temperature. The samples were digested with a modified trypsin (Promega, USA) at a ratio of enzyme:protein equal to 1:100 (w/w) at 37 °C and incubated overnight. The enzyme reaction was stopped using 10 µL of 5% (V/V) trifluoroacetic acid (TFA), mixed, incubated for 90 min at 37 °C. The samples were centrifuged at 14,000 rpm at 4 °C for 30 min [10]. The supernatant was recovered and then reconstituted in ammonium hydroxide (NH_4OH) previously prepared at 1 N and then transferred to a Waters Total Recovery vial (Waters) prior to 2D-nanoUPLC-UDMS^E analyses. Tryptic digested peptides from yeast alcohol dehydrogenase (ADH) (Waters, Milford, USA) were added to a final concentration of 25 fmol.µL^{-1}. ADH internal standard to estimate the amount of each sample injected at the nanocolumn for absolute quantification and column loading capacity estimation. The quantitative method utilized at the above estimation is described in details at Jeffrey Silva et. al. (2006) and was performed accordingly [11].

Each sample has been injected as a “scouting” runs for stoichiometry purposes between the conditions using a integrated total ion current as previously described [12].

MASS SPECTROMETRY OF COMPLEX DIGESTED SAMPLES

Proteomic analyses were performed in a 2D-nanoUPLC-tandem nanoESI-UDMS^{E} instrument platform by multiplexed data-independent acquisition experiments [13]. 2D-RP/RP nanoACQUITY UPLC chromatography system (Waters Corporation, USA) coupled to a Synapt G2-S mass spectrometer (Waters Corporation, UK) platform was used.

The samples were fractionated using a dual reversed-phase (RP) approach. In first-dimension chromatography, peptides (5 µg) were loaded into an M-Class BEH C18 Column (130 Å, 5 µm, 300 µm X 50 mm, Waters Corporation). The fractionation was performed through ten discontinuous steps of acetonitrile (8.7 %, 11.4 %, 13.2 %, 14.7 %, 16.0 %, 17.4 %, 18.9 %, 20.7 %, 23.4 %, 50 %) and high pH fractionation over 10 min at a flow rate of 2 µL.min^{-1}. After each step, peptide loads were carried to second dimension separation in a nanoACQUITY UPLC HSS T3 Column (1.8 µm, 75 µm x 150 mm, Waters Corporation).

Peptide elution was achieved using a continuous acetonitrile gradient from 7% to 40% (V/V) for 54 min at a flow rate of 0.4 µL.min^{-1} directly into a Synapt G2-S HDMS. For every measurement, the mass spectrometer was operated in resolution mode with a scan time (0.5s) adjusted such at least 10 points per chromatographic peak were obtained for each low and elevated energies ($\sigma_{10\%}$20). A *m/z* resolving power was approximately 1,800,000 FWHM considering a conjoined stacked ring ion guide such as the T-wave ion mobility, operating with a cross-section resolving power of at least 40 Ω/ΔΩ. LC–MS/MS multiplex data, were collected using ion mobility enhanced MS^{E} [14, 15].

The exact mass retention time (EMRT) signals from multiplexed ion-mobility DIA scanning (UDMS^{E}) were detected in an alternating low energy and elevated

energy acquisition mode. In the low energy mode, data were collected at 6 eV. In the elevated collision energy, *quasi m/z*-specific collision energies were applied at the travelling-wave stacked ring ion guide transfer lens (TWIG) to the different drift time bins [16]. This were used to fragment precursor ions prior to orthogonal acceleration time-of-flight (*oa*TOF) analysis, at the transfer TWIG cell filled with argon gas, by the use of collision-induced dissociation (CID) [17].

The mass spectrometer was calibrated with an MS/MS spectrum of human [Glu^1]-Fibrinopeptide B (Glu-Fib) that was delivered every 30 s through the reference sprayer of the NanoLock Spray source. Quadrupole profile were adjusted such *m/z* less than 400 arose from dissociations in the collision cell. Global quality control of the obtained data and the figures of merit (FOM's) were displayed as described at Souza et al. 2017 (S1.Fig) [17].

DATABASE SEARCHING AND BIOINFORMATICS

Proteins were identified and quantified by the use of dedicated algorithms and searched against the UniProt human proteomic database, version 2016_02 [18, 19]. For proper spectra processing and database searching conditions, we used Progenesis QI for Proteomics software package with Apex3D, Peptide 3D, and Ion Accounting informatics (Waters Corporation).

The processing parameters used were 150 counts for the low-energy threshold, 50.0 counts for the elevated energy threshold, 750 counts for the intensity threshold as default, such as peak picking and alignment processing as well [20]. Moreover, the following parameters were considered for protein identification and quantitation: 1) Digestion by trypsin with at least one missed cleavage; 2) variable modifications by oxidation (M) and fixed modification by carbamidomethyl (C); 3) false discovery rate (FDR) less than 1%. At least 3 peptides per protein with 1 unique, and quantitation

based on non-conflicted peptides per condition, were used. Also, only reproducible proteins were investigated across all replicates obtained. Identifications and quantitative values that did not satisfy these criteria were rejected.

The raw files and the database search Tables were deposited in the Proteome X change repository under accession number PXD004696. Functional annotations (gene ontology and chromosome annotations) of the dataset were performed using DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/home.jsp).

Gene Ontology (GO) functional classifications were analyzed with Blast2GO software (www.blast2go.org), and GO enrichment analysis was performed to identify GO terms that were significantly enriched in differentially expressed proteins. We used FUNRICH software (http://www.funrich.org/) to evaluate the interatome [21]. The Pathway Studio Software (www.pathwaystudio.com) was used to explore the associations between differentially expressed proteins and their effects on monocyte and systemic disturbed in septic shock.

RESULTS

MONOCYTES PROFILE

After the PBMC extraction, three different populations of monocytes were detected CD14high:CD16low, CD14high:CD16high and CD14low:CD16high. The controls present about 50% CD14high:CD16 low of the monocytes population evaluated, 12% CD14high:CD16high and 12% CD14low:CD16high. Shock patients in this study have the CD14low:CD16high monocyte population as the most abundant in peripheral blood (approximately 70%), only 2% of the cells were CD14high:CD16low and 3% CD14high:CD16low (S.2_Fig).

MASS SPECTROMETRY AND BIOINFORMATICS

In our study, 3472 proteins (1705 proteins in the control sample and 1727 in the shock) were collectively identified, with an average of 7 peptides per protein. For the entire study, the coefficients of variance for all quantitative protein measurements were less than 6%. 67 proteins were differentially expressed in the monocytes of the individuals with shock in relation to the control with at least 3 folds. The alteration corresponds to 48 proteins increased and 23 decreased, the description of the protein, statistical value and the cellular location of these are in the Table 1.

We used Gene ontology, FunRich and Pathway Studio to interpret the changes present in the monocyte of the patient with septic shock. The identification of the major cellular components and biological processes that are involved in altered proteins was determined by the analysis of these in FunRich by Gene Ontology database; and they are shown in Fig. 1.

Differentially expressed proteins do not correlate with each other. However, they participate in common pathways requiring only one molecule for the communication of a differentially expressed protein to binds to another forming an Interactome (Fig.2). Supplementary Table 2 describes the proteins that are interconnected.

The major regulatory molecules of proteins that are augmented are nuclear proteins with Enhancer-binding proteins action, such as CEBPA, CEBPE e CEBPP; the activation of the inflammasome and S100A3 protein in the cytoplasm and signaling pathways activated by HIF-1a, interleukin 17 and lactoferrin (Table 2). Proteins that are decreased are influenced mainly by transcriptional factors GRHL2 and NFX1; growth factors: TGFA and FGF2; the integrins ITGA3 and ILK; and PLAT and Oncostatina M proteins, with actions described in the Table 3.

To determine if protein changes are influencing the cell profile, it was investigated which proteins would have an effect under the phenotype, cellular activation, phagocytosis, migration, senescence, cell viability and death (apoptosis / necrosis); At the 67 proteins 46 (29 up and 17 down) present these functions which can be evaluated as a up and down regulated proteins and it is not possible to determine which alteration is most relevant for monocyte dysfunction (S.3_Table).

The Resistin, S100A8, S100A9, MPO, LCN2, PDPK1, CSTG, LTF, PTP1 and EPRS are proteins that may be acting directly in the collapse of sepsis. Since they are increased in septic shock and can be secreted by monocytes. These proteins have been correlated with direct effect in situations of endotoxemia, chronic inflammation, vascular damage and thrombosis as described in Table 4.

DISCUSSION

Monocytes are believed to orchestrate the host immune response and contribute to the pathogenesis of sepsis. In the early stages of this syndrome they aid in the exacerbation of the immune response with cytokine storm and when the disease progresses they participate in the immunological exhaustion and adopt an immunosuppressive phenotype being incapable of responding to secondary infections [22].

Classical, intermediate, and nonclassic monocytes have distinct predominant functions. The disturbance in the proportion of monocytes in the blood has been correlated with several serious pathologies with poor clinical outcomes [23].

Patients with septic shock evaluated have a predominance of non-classical monocytes in the circulation. Some authors have reported the increase of this profile in patients with clinical symptoms of sepsis and negative blood culture while the

increased intermediate profile correlates with positive cultures [24, 25]. Thus, it is feasible a non-classical profile ability to control the intermediate infection disruption.

Patients with high non-classical and low classic monocytes profile have impaired endothelial function, as such it is recognized that high levels of this cellular profile increase the production of superoxide vascular leading to dysfunction [26].

Although the patients presented predominance in non-classical monocytes, which were observed by immunophenotyping, the S100A9, CTSG, MPO and RNAS3 proteins that are increased were quantified as up regulated in the classical profile of the proteomic study with healthy subjects [27]. The large increase of non-classical monocytes in the circulation of the patients analyzed may be the result of a rapid migration without complete differentiation.

The Enhancer-binding protein, HIF-1a and inflammasome are major regulatory molecules of the increased proteins (Table 2) and perform key mechanisms in monocyte regulation.

Enhancer-binding protein regulates almost all listed proteins; in addition they have been associated as essential proteins for the differentiation of classic monocytes into non-classical [28]. On the other hand, the role of Hypoxia-Inducible Factor-1a (HIF1a) is to induce immunosuppression and repairing characteristics in classic monocytes and intermediates of patients with active sepsis [8].

Inflammasome is a crowded multiprotein complex that finely regulates caspase-1 to induce pre-inflammatory proteins. At the period of immune exacerbation is activated; therefore in septic shock this function is decreased contributing to deactivation of the monocyte [29].

Many studies have sought to understand the biological reprogramming of the monocyte in sepsis, there are reports of dysfunction of this cell type in septic shock

due to changes in the ability to present antigens and produce cytokines, but it is known that phagocytic and microbicide function remains conserved [7-9]. In an attempt to combat pathogens monocytes can secrete molecules that have a direct effect on the collapse of septic shock (Table 4).

Defense mechanisms are beneficial when activated locally, but harmful when systemically activated. Septic shock results from generalized consequences of the immune response to infection, which correlates with the cascade of events involving endotoxemia, inflammation, vascular damage, and thrombosis [30, 31].

Endotoxemia is correlated with the infection *per se*, in which components of the pathogens cell wall are present in an exacerbated abundance at the systemic circulation. The presence of gram-negative bacteria is frequently associated, however, endotoxin levels were also found in the plasma of patients with septic shock caused by gram-positive variations or candida [32].

The fact is: the presence of endotoxins activates the immune system cells. This process leads to utter inflammation disturbance of pre-inflammatory cytokines, such as TNF-α, IL-6, IL-1β, IL-8, IL-12, platelet activation factor, reactive oxygen species, and microbicidal components (myeloperoxidase, cationic proteins, acid hydrolases, cathepsin G and lactoferrin), which struggles against microorganisms [33]. Nevertheless, exacerbation of these factors generates damage to the vascular endothelium [34].

Prolonged inflammation and the presence of endothelial injury pushes homeostasis toward a prothrombotic and anti-fibrinolytic state, which can lead to disseminated microvascular thrombosis, organ ischemia and multiple organ dysfunction syndrome, common and well know circumstances that ensues the septic shock [35, 36].

Our study has limitations like pool samples and absence of separation of the monocytes in subtypes; however, we demonstrated the possible contribution of the monocyte in the collapse of the septic shock.

CONCLUSION

Monocyte changes in sepsis shock correlate with a sort of “biological reprogramming” greater than an ordinary biological function forfeiture. In this study, we were able to present robust evidence of data on monocyte alteration in septic shock. There are several proteins secreted by these cells that are with increased expression that contribute directly to the vascular lesion, endotoxemia, inflammation and thrombosis, situations severe which responsible by poor clinical outcome

COMPETING INTERESTS

The authors declare that there are no competing interests.

ACKNOWLEDGEMENTS

The authors would like to thank the all the individuals who participated in this work, the medical staff from the Intensive Unit Care of the University Hospital for providing the biological samples and the clinical parameters, and the financial support by the National Council for Scientific and Technological Development (CNPq), Coordination for the Improvement of Higher Education Personnel (CAPES), and Foundation for Research Support of Minas Gerais (FAPEMIG).

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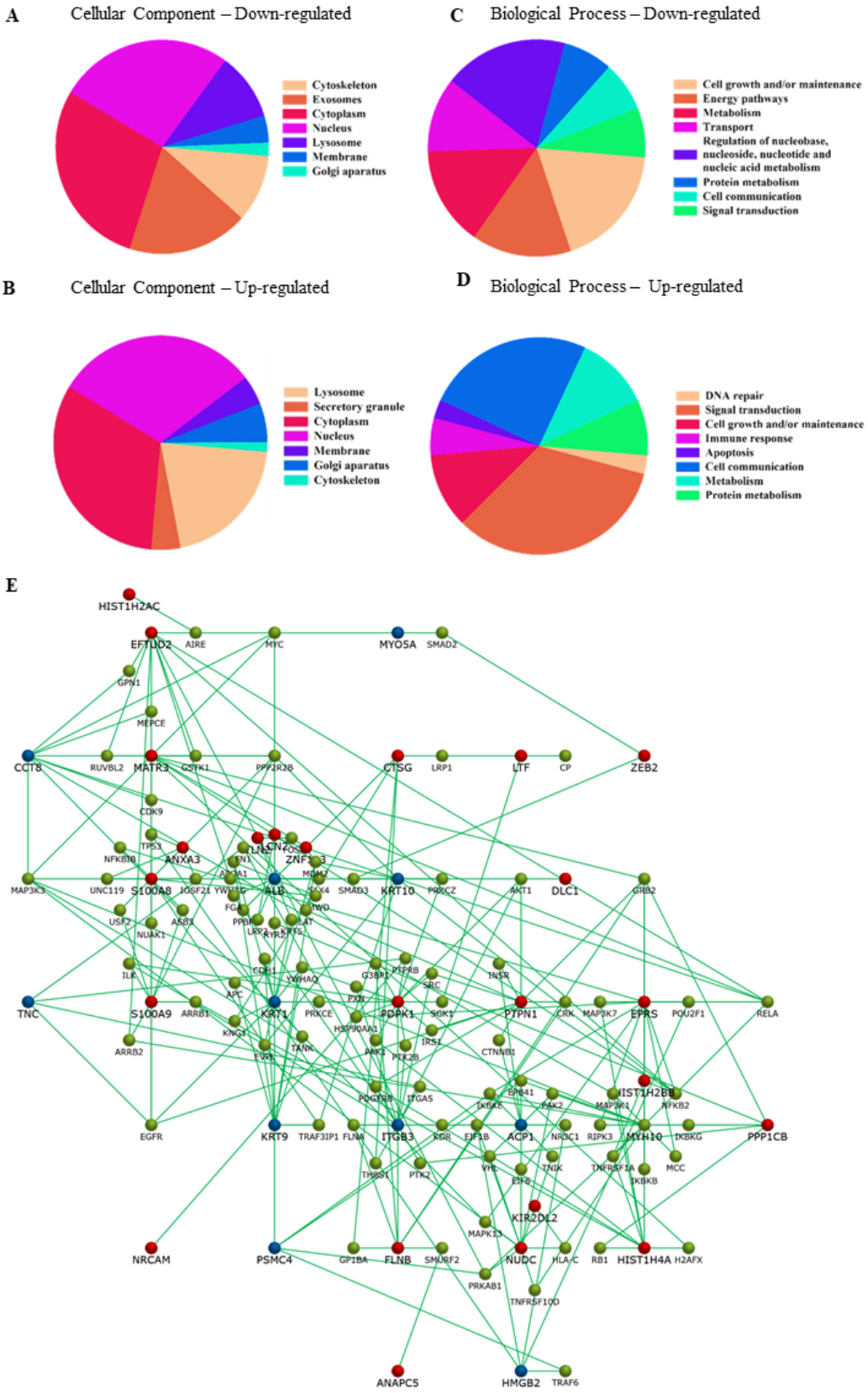
FIGURE AND LEGENDS

Fig 1. Biological components and interaction of differentially expressed proteins. Major cellular components involved by down regulated **(A)** and up regulated **(B)**. Main biological processes participating differentially expressed proteins **(C)** down and **(D)** up regulated. The interaction of differentially expressed proteins is not direct. However, they influence several molecules that communicate directly. Blue is representing down regulated genes, red up regulated genes and green interaction genes of FunRich data base **(E).**

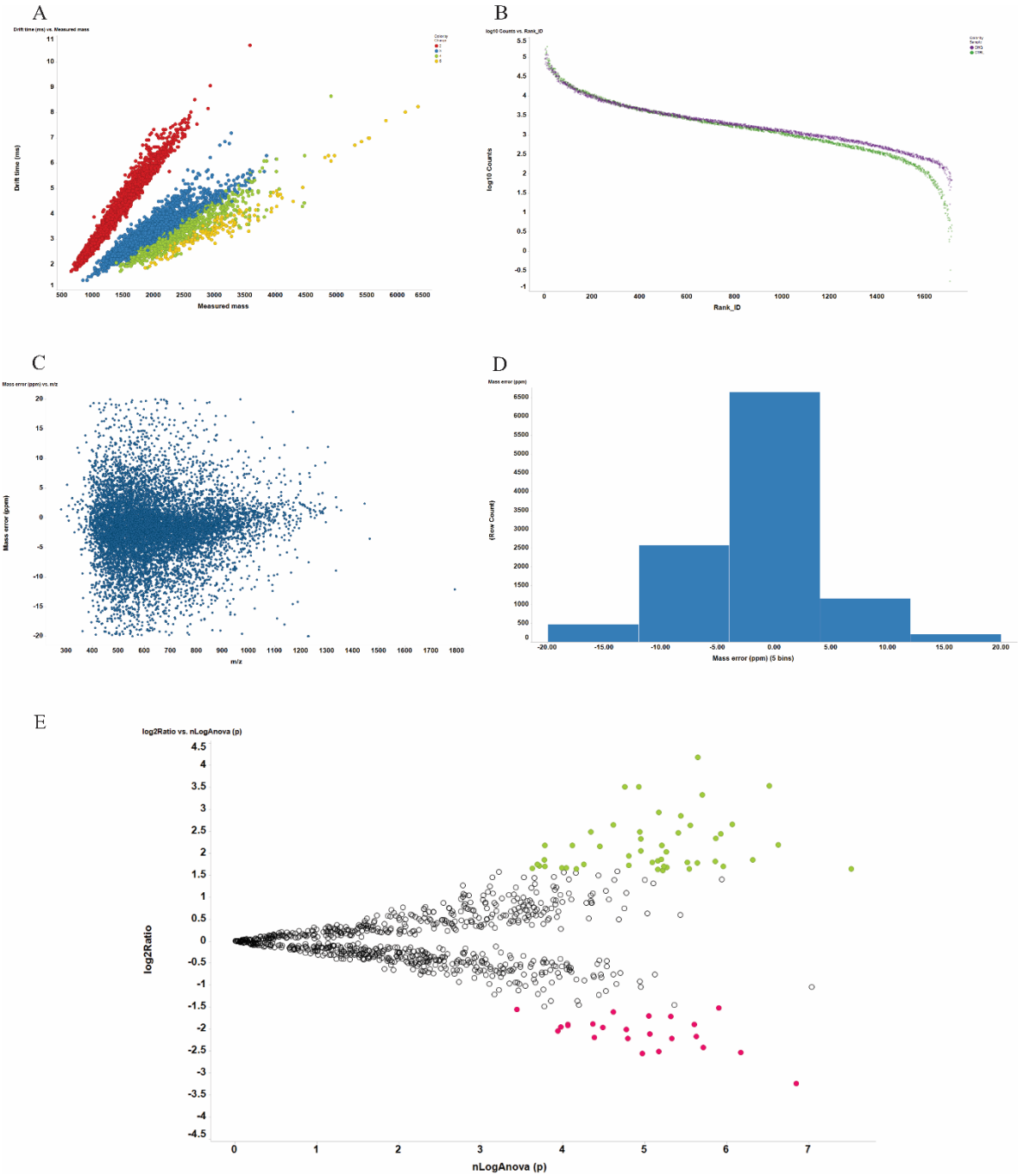
S.1_FIG. Mass spectrometry: analytical and physico-chemical atributes for quality control (proteomics samples) – fom's. Fig.s **A** to **E**. Fig. **A:** peptide precursor mass (Da) and drift time (dt) separation of molecular species across different intrinsec charges and conformations ("chimericity") [37] discriminated by ion mobility separation – legend color by charge state; Red: $[M + 2H]^{2+}$; Blue: $[M + 3H]^{3+}$; Green: $[M + 4H]^{4+}$; Yellow: $[M + 5H]^{5+}$. **B**: Dynamic ranges of the quantified proteins in the study. Both conditions displays a comparable profile due to similar protein quantitation fulfilled by the previous stoichiometry total ion count detection integrated area (σTIC) normalization. **C**: Mass error distribution of all peptide measurements, displaying the most dense and compact multitude of point across 0 ppm over m/z range. **D**: Mass error distribuition frequency displaying a normal distribution profile across 0 ppm. **E**: Volcano plot (log_2fold vs. ANOVA ($-log_{10}$p) of up (green), unchanged (black) and down-regulated (red) proteins.

S.2 FIG. Monocytes profile. Percentage of classical (CD14high:CD16low), intermediate (CD14high:CD16high), non-classical (CD14low:CD16high) monocytes expression in PBMC from patients and controls evaluated.

FIG.1


A
Cellular Component – Down-regulated
Cytoskeleton
Exosomes
Cytoplasm
Nucleus
Lysosome
Membrane
Golgi aparatus
C
Biological Process – Down-regulated
Cell growth and/or maintenance
Energy pathways
Metabolism
Transport
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
Protein metabolism
Cell communication
Signal transduction
B
Cellular Component – Up-regulated
Lysosome
Secretory granule
Cytoplasm
Nucleus
Membrane
Golgi aparatus
Cytoskeleton
D
Biological Process – Up-regulated
DNA repair
Signal transduction
Cell growth and/or maintenance
Immune response
Apoptosis
Cell communication
Metabolism
Protein metabolism
E
HIST1H2AC
EFTUD2
AIRE
MYC
MYO5A
SMAD2
GPN1
MEPCE
CCT8
RUVBL2
MATR3
G3BP1
PPP2R2B
CTSG
LRP1
LTF
CP
ZEB2
CDK9
TP53
NFKBIB
ANXA3
LCN2
ZNF183
MAP3K3
UNC119
S100A8
IGSF21
ALB
SMAD3
KRT10
PRKCZ
AKT1
DLC1
GRB2
USF2
NUAK1
ILK
CDH1
YWHAQ
TNC
S100A9
ARRB1
KRT1
PRKCE
PDPK1
SGK1
PTPN1
CRK
MAPBK7
EPRS
POU2F1
RELA
ARRB2
KNG1
TANK
PAK1
IRS1
CTNNB1
EGFR
KRT9
TRAF3IP1
FLNA
ITGB3
KDR
EIF1B
ACP1
NR3C1
RIPK3
MYH10
IKBKG
PPP1CB
THBS1
PTK2
VHL
EIF0
TNIK
MCC
IKBKB
KIR2DL2
MAPK13
NRCAM
PSMC4
GP1BA
FLNB
SMURF2
NUDC
HLA-C
RB1
HIST1H4A
H2AFX
PRKAB1
TNFRSF10D
ANAPC5
HMGB2
TRAF6
Interacting genes not from dataset
Genes Up-regulated
Genes Down-regulated

S.1_FIG


A
Drift time (ms) vs. Measured mass
Drift time (ms)
Measured mass
B
log10 Counts vs. Rank_ID
log10 Counts
Rank_ID
C
Mass error (ppm) vs. m/z
Mass error (ppm)
m/z
D
Mass error (ppm)
(Row Count)
Mass error (ppm) (5 bins)
E
log2Ratio vs. nLogAnova (p)
log2Ratio
nLogAnova (p)

S.2_FIG

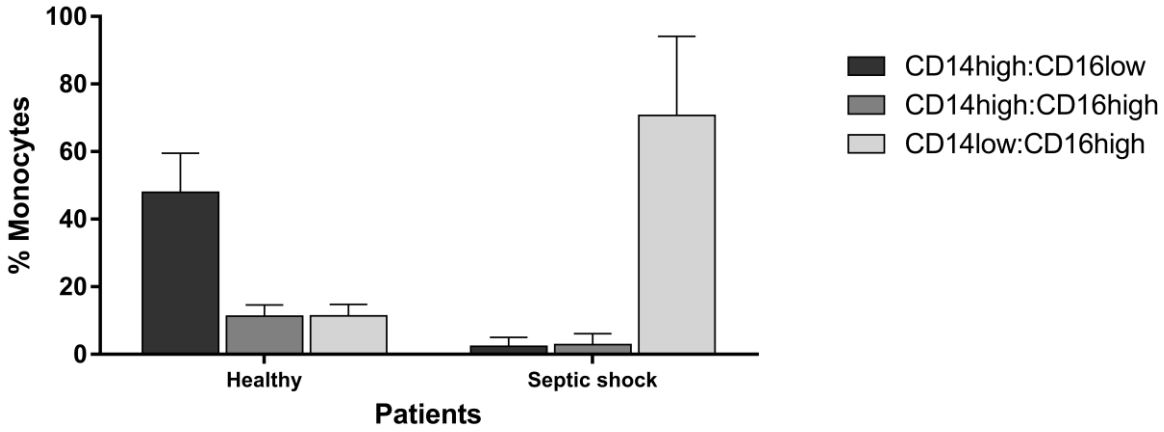

100
80
60
40
20
0
% Monocytes
Healthy
Septic shock
Patients
CD14high:CD16low
CD14high:CD16high
CD14low:CD16high

Table 1. Differential expressed proteins identified in the monocytes of patients with shock septic compared with healthy control.

GENE	ACESSION	DESCRIPTION	SCORE	ANOVA (p)	Ratio	N	C	M	S	R
ANXA3	P12429	Annexin A3	171.36	6.04E-06	3.046		■	■	■	+
HIST1H2AC	Q93077	Histone H2A type 1-C	44.50	7.00E-06	3.087	■				+
FLNB	O75369	Filamin-B	155.76	3.045E-08	3.121		■			+
GXYLT2	A0PJZ3	Glucoside xylosyltransferase 2	9.12	6.82E-05	3.122			■		+
IGSF10	Q6WRI0	Immunoglobulin superfamily member 10	63.37	2.88E-06	3.125				■	+
WDR90	Q96KV7	WD repeat-containing protein 90	46.32	0.0002	3.146	■				+
ZEB2	O60315	Zinc finger E-box-binding homeobox 2	7.80	0.0001	3.167		■			+
EPRS	P07814	Bifunctional glutamate/proline--tRNA ligase	19.40	9.10E-05	3.168			■		+
HYDIN	Q4G0P3	Hydrocephalus-inducing protein homolog	91.08	5.43E-06	3.187					+
ADGRD1	Q6QNK2	Adhesion G-protein coupled receptor D1	13.29	5.89E-06	3.239			■		+
RETN	Q9HD89	Resistin	24.98	0.0001	3.251	■			■	+
LCN2	P80188	Neutrophil gelatinase-associated lipocalin	53.16	1.11E-06	3.255				■	+
PDPK1	O15530	3-phosphoinositide-dependent protein kinase 1	15.67	0.0001	3.284	■	■	■		+
NNMT	P40261	Nicotinamide N-methyltransferase	13.64	1.56E-05	3.286		■			+
HIST1H2BK	O60814	Histone H2B type 1-K	68.54	5.53E-05	3.356	■	■			+
RNASE3	P12724	Eosinophil cationic protein	35.48	0.0002	3.359				■	+
ATP6V1B1	C9JL73	V-type proton ATPase subunit B, kidney isoform	45.81	2.28E-06	3.436			■		+
DLC1	Q96QB1	Rho GTPase-activating protein 7	10.11	3.05E-06	3.452		■	■		+
HIST1H2BB	P33778	Histone H2B type 1-B	75.93	6.93E-06	3.540	■	■			+
MLH3	Q9UHC1	DNA mismatch repair protein Mlh3	36.53	4.81E-07	3.599	■				+
ZNF133	P52736	Zinc finger protein 133	7.84	0.0001	3.603	■				+
SACM1L	Q9NTJ5	Phosphatidylinositide phosphatase SAC1	25.19	6.30E-06	3.625		■			+
TLN2	Q9Y4G6	Talin-2	139.60	1.56E-05	3.833		■	■		+
LTF	P02788	Lactotransferrin	207.35	5.46E-06	4.066	■	■		■	+
PPP1CB	P62140	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	36.26	1.12E-05	4.134	■	■			+
S100A8	P05109	Protein S100-A8	68.05	3.52E-05	4.458		■	■	■	+
PTPN1	B4DSN5	Tyrosine-protein phosphatase non-receptor type	25.30	0.0001	4.506			■		+
KLB	Q86Z14	Beta-klotho	15.35	6.19E-06	4.515			■		+
ANAPC5	F5H0F9	Anaphase-promoting complex subunit 5	14.87	7.71E-05	4.525	■		■	■	+
CTSG	P08311	Cathepsin G	137.54	2.36E-07	4.539	■		■	■	+
CHTF8	P0CG12	Chromosome transmission fidelity protein 8 homolog isoform 2	23.38	1.11E-05	5.026	■			■	+
C8B	P07358	Complement component C8 beta	13.87	1.36E-06	5.059			■	■	+
MPO	P05164	Myeloperoxidase	350.67	1.19E-06	5.399	■	■		■	+
EFTUD2	Q15029	116 kDa U5 small nuclear ribonucleoprotein component	51.23	3.93E-06	5.491	■	■	■		+
	K7ERQ8	Uncharacterized protein (Fragment)	18.18	1.15E-05	5.579	■		■		+
TUBGCP5	Q96RT8	Gamma-tubulin complex component	9.59	4.57E-05	5.580	■		■		+

FAM167A	Q96KS9	Protein FAM167A	15.10	2.78E-06	6.212					+
NRCAM	C9JYY6	Neuronal cell adhesion molecule	8.18	2.43E-05	6.232					+
S100A9	P06702	Protein S100-A9	141.73	3.65E-06	7.217					+
MATR3	P43243	Matrin-3	31.47	6.77E-06	7.618					+
HIST1H4A	P62805	Histone H4	132.30	2.00E-06	10.050					+
CEACAM6	P40199	Carcinoembryonic antigen-related cell adhesion molecule 6	28.45	1.75E-05	11.350					+
KIR2DL2	Q8N742	KIR2DL2	14.60	1.18E-05	11.410					+
NUDC	Q9Y266	Nuclear migration protein nudC	15.40	3.04E-07	11.518					+
SH3TC2	E9PDF1	SH3 domain and tetratricopeptide repeat-containing protein 2	28.98	2.27E-06	18.110					-
KRT9	P35527	Keratin, type I cytoskeletal 9	10.62	1.42E-07	9.427					-
ACP1	P24666	Low molecular weight phosphotyrosine protein phosphatase	9.94	1.08E-05	5.852					-
KRT1	P04264	Keratin, type II cytoskeletal 1	57.80	6.76E-07	5.782					-
KRT10	P13645	Keratin, type I cytoskeletal 10	137.21	6.77E-06	5.695					-
LZTR1	Q8N653	Leucine-zipper-like transcriptional regulator 1	11.91	4.68E-06	4.618					-
ATP13A3	Q9H7F0	Probable cation-transporting ATPase 13A3	28.15	1.61E-05	4.614					-
ZNF677	Q86XU0	Zinc finger protein 677	7.78	4.14E-05	4.543					-
ITGB3	P05106	Integrin beta-3	174.14	2.37E-06	4///.476					-
TNC	P24821	Tenascin	36.37	8.73E-06	4.307					-
ZFHX2	Q9C0A1	Zinc finger homeobox protein 2	14.11	0.0001	4.104					-
ESRP2	Q9H6T0	Epithelial splicing regulatory protein 2	16.19	1.7E-05	4.011					-
MYH10	P35580	Myosin-10	76.79	3.25E-05	3.879					-
COG4	Q9H9E3	Conserved oligomeric Golgi complex subunit 4	14.50	0.0001	3.870					-
PSMC4	P43686	26S protease regulatory subunit 6B	10.90	8.76E-05	3.762					-
ATAD2B	Q9ULI0	ATPase family AAA domain-containing protein 2B	13.66	8.72E-05	3.703					-
PGK2	P07205	Phosphoglycerate kinase 2	94.21	2.49E-06	3.701					-
ALB	P02768	Serum albumin	164.05	4.34E-05	3.670					-
ACAD9	Q9H845	Acyl-CoA dehydrogenase family member 9, mitochondrial	14.60	4.86E-06	3.262					-
CCT8	P50990	T-complex protein 1 subunit theta	76.19	8.92E-06	3.255					-
HMGB2	P26583	High mobility group protein B2	63.46	2.43E-05	3.059					-
CLCN4	P51793	H(+)/Cl(-) exchange transporter 4	8.30	0.0003	2.927					-
MYO5	G3V394	Unconventional myosin-Va	128.70	1.25E-06	2.855					-

* Cellular Location: N (nucleus); M (membrane); C (cytoplasm); S (secreted)

** Protein Regulation: + (up-regulation); - (down-regulation)

Table 2. Main regulation of proteins with up expression in septic shock

Protein	Protein Effector	Effect	Reference
LTF	CEBPE	CEBPE is involved in the positive regulation of lactoferrin gene expression	[38]
	CEBPA	C/EBPα binds to the C/EBP site in the lactoferrin promoter in induced myeloid cells	[39]
MPO	CEBPE	Up-regulation of CBPE induces expression of myeloperoxidase	[40]
	LTF	LTF decreased the serum C-reactive protein level, inducible nitric oxide synthase (iNOS) and myeloperoxidase (MPO) gene expression levels	[41]
	HIF-1	Activation of HIF-1 by P4HA2 gene silencing attenuated myeloperoxidase expression in myocardium following ischemia-reperfusion	[42]
	IL1-RA	Interleukin-1ra and anti-TNF-α also significantly lowered MPO levels	[43]
	CEBPA	C/EBPα inhibits monocyte/macrophage differentiation and initiates granulocyte differentiation by inducing myeloperoxidase gene expressions	[44]
RETN	CEBPE	Human leukocyte resistin expression depends on the myeloid specific nuclear transcription factor CEBPE	[45]
	CEBPB	Resistin stimulates expression of chemokine genes in chondrocytes via combinatorial regulation of C/EBPβ and NF-κB.	[46]
	HIF-1	Hypoxia inducible factor-1 results in an increased production of leptin, resistin, TNF, and IL-6.	[47]
	IL1-RA	IL-1Ra treatment reduction in leptin and resistin levels	[48]
	CEBPA	Resistin gene promoter carries the C/EBP-α binding site, which is necessary and sufficient for transcription from the resistin gene promoter	[49]
LCN2	CEBPE	Stable inducible expression of CEBPE in the murine fibroblast cell line NIH 3T3 activated expression of mRNA LCN2	[50]
	INFLAMMASOME	Inflammasome-mediated production of antimicrobial peptides, including Reg3β, Reg3γ, S100A8, S100A9, and LCN2	[51]
	CEBPB	LCN2 promoter region contains the binding sites of several transcription factors such as NF-κB, STAT1, STAT3,CEBPB	[52]
	IL17	IL17 positively regulate LCN2 expression.	[53]
	CEBPA	IL6 and LCN2 promoters require both NF-κB and C/EBP elements	[54]
S100A8 S100A9	S100A3	S100A3 co-expression inhibits AP-1 and NF-κB-dependent transcription upon S100A8 and S100A9 over-expression	[55]
	INFLAMMASOME	Inflammasome-mediated production of antimicrobial peptides, including Reg3β, Reg3γ, S100A8, S100A9, and LCN4	[51]
	LTF	Lactoferrin induces the production of chemokines (MIP-1a, MCP-1, and S100A9)	[56]
	CEBPB	IL-1α-induced S100A9 expression is signaled through the IL-1 receptor and p38 MAPK pathways, resulting in the binding of CEBPB to the upstream S100A9 promoter	[57, 58]
	HIF-1	Hypoxia and HIF-1 increase S100A8 and S100A9 expression	[59]
	IL1-RA	IL-1Ra inhibited interleukin-1 α-induced S100A8 and S100A9 gene expression	[60]
	IL17F	IL-17 stimulate expression of human beta-defensin- 2, S100A9 and enhanced the expression of S100A7 and S100A9	[61]
	CEBPA	Increase in reactive oxygen species and the expression levels of the transcription factors Klf-5 and CEBPA in neutrophils both of which promote S100A8/S100A9 expression	[62]
PDPK1	LTF	Lactotransferrin downregulates the level of 3-phosphoinositide-dependent protein kinase 1 (PDK1) transcription and subsequently inhibits	[63]
	HIF-1	Repression of hypoxia-inducible factor-1 activity, attenuate PDK-1 expression	[64]
HIST4H4	CEBPB	CEBPB can significantly transactivate the expression of HIST4H4	[65]
NNMT	CEBPB	NNMT promoter region also contains the consensus sequences for signal transducers and activators of transcription binding elements	[66]
CEACAM6	HIF-1	Hypoxia-inducible factor-1 transcription factor increases CEACAM6 expression in intestinal epithelial cells	[67]

Table 3. Main regulation of proteins with low expression in septic shock

Protein	Protein effector	Function Protein effector	Effect	Reference
KRT1	GRHL2	Transcription factor	Down regulate KRT1 and KRT10	[68]
	NFX1	Nuclear transcription factor	Up regulate KRT1 and KRT10	[69]
	BCR	Breakpoint cluster region protein	Loss of BCR reduces expression of KRT1 and KRT10	[70]
	PLAT	Plasminogen activator, tissue	Plays a role in the expression of KRT1 and KRT10	[71]
	OSM	Oncostatin M	Down regulate mRNA expression	[72]
KRT10	GRHL2	Transcription fator	Down regulate KRT1 and KRT10	[68]
	NFX1	Nuclear transcription fator	Up regulate KRT1 and KRT10	[69]
	COLLAGEN	Matrix Protein	Down regulate the mRNA expression KRT10	[73]
	BCR	Breakpoint cluster region protein	Loss of BCR reduces expression of KRT1 and KRT10	[70]
	TGFA	Transforming grown fator	Suppress KRT10 expression, promoted late terminal differentiation	[74]
	FGF2	Fibroblast growth factor 2	Down regulate the KRT10 expression	[75]
	PLAT	Plasminogen activator, tissue	Plays a role in the expression of KRT1 and KRT10	[71]
	ITGA3	Integrin alpha 3	Inhibition KRT10 production	[76]
	OSM	Oncostatin M	Decrease expression	[77]
ABL	GRHL2	Transcription fator	Inhibits exppression ABL	[78]
	COLLAGEN	Matrix Protein	Inhibit albumin production at short times, but enhances albumin production at longer times	[79]
	TGFA	Transforming grown fator	Stimulate albumin synthesis	[80]
	FGF2	Fibroblast growth factor 2	Induce expression	[81]
	ILK	Integrin like kinase	ALB mRNA expression is down regulated by ILK	[82]
	OSM	Oncostatin M	Up-regulate production	[83]
ITGB3	COLLAGEN	Matrix Protein	Enhance β3 integrin tyrosine phosphorylation by adhesion platelets to collagen	[84]
	TGFA	Transforming grown fator	Stimulate ITGB3 expression	[85]
	ITGA3	Integrin alpha 3	Enhance the expression	[86]
TNC	COLLAGEN	Matrix Protein	Tenascin-C mRNA expression is reduced by native collagen and is upregulated by denatured collagen	[87]
	FGF2	Fibroblast growth factor 2	Up regulator of tenascin expression and activation	[88]
	ILK	Integrin like kinase	Induce expression TNC	[89]
	OSM	Oncostatin M	Inhibit mRNA expression	[90]
MYH10	FGF2	Fibroblast growth factor 2	Decrease MYH10 expression	[91]
	ILK	Integrin like kinase	Regulate MYH10 expression	[92]
ESRP2	FGF2	Fibroblast growth factor 2	Repress the levels of ESRP2 mRNA	[93]
PGK2	FGF2	Fibroblast growth factor 2	Modulate transcription of PGK-2 genes	[94]

Table 4. Systemic effect of secreted proteins up-regulation on septic shock

Protein	Effect	Action	Reference
RETN	**Inflammation**	Mediated chronic inflammation can lead to atherosclerosis, and other cardiometabolic diseases	[95]
	Vascular damage	Major inducer of endothelial damage through the induction of permeability.	[96]
	Thrombosis	Resistin, an adipokine associated with the metabolic syndrome is believed to have a role in thrombotic conditions.	[97]
S100A8	**Endotoxemia**	S100A8 administration attenuated inflammation and injury in a mouse model of endotoxemia	[98]
	Inflammation	S100A8 important mediators of various processes during chronic inflammation	[99]
	Vascular damage	High S100A8 expression leads to endothelial damage by inducing the apoptosis and death of endothelial cells	[100]
	Thrombosis	S100A8 which is secreted by trophoblast cells probably regulates the level of macrophage activation and procoagulant factors	[101]
S100A9	**Inflammation**	S100A9, an important pro-inflammatory mediator in acute and chronic inflammation	[102]
	Vascular damage	S100A8/S100A9 is released in high amounts at sites of inflammation, S100A8/S100A9-induced endothelial damage	[103]
	Thrombosis	Platelet-derived S100 family member myeloid-related protein-14 regulates thrombosis	[104]
MPO	**Endotoxemia**	MPO can aggravate this inflammatory response in rodent models of endotoxemia	[105]
	Inflammation	MPO plays an important role in the initiation and progression of acute and chronic inflammation.	[106]
	Vascular damage	MPO consumes nitric oxide, leading to vasoconstriction and promoting endothelial damage	[107]
	Thrombosis	Hemoglobin-Hp2-2 complex may promote a proinflammatory macrophage phenotype via oxidative mechanisms (MPO) leading to plaque destabilization and atherothrombosis.	[108]
LCN2	**Endotoxemia**	Acute endotoxemia is associated with upregulation of lipocalin 24p3/Lcn2 in lung and liver	[109]
	Inflammation	LCN2 is involved in chronic inflammation	[110]
	Vascular damage	LCN2 supposedly mediates vascular damage and plaque rupture.	[111]
	Thrombosis	LCN2 could have an important role in thrombotic events associated with polycythemia vera and essential thrombocythemia	[112]
PDPK1	**Inflammation**	Deletion of PDPK1 induces chronic inflammation of the intestine	[113]
	Thrombosis	PDK1 is required for Ca(2+)-dependent platelet activation on stimulation of collagen receptor glycoprotein VI, arterial thrombotic occlusion, and ischemic stroke in vivo	[114]
CTSG	**Inflammation**	CTSG is thought to contribute to self-propagating, chronic inflammation.	[115]
	Vascular damage	CTSG cause the activation of bystander platelets, enhance vascular damage and inhibit fibrinolysis	[116]
	Thrombosis	CTSG is a potent platelet activator and promotes intravascular thrombosis, thus contributing to the formation of a thrombus clot	[117]
LTF	**Endotoxemia**	Lactoferrin suppresses endotoxemia by interfering with lipopolysaccharide-dependent TLR4 activation	[118]
	Inflammation	Persistent production of lactoferrin in pediatric patients may contribute to chronic inflammation in the rectum	[119]
	Thrombosis	Lactoferrin may play a role in the pathogenesis of disseminated intravascular coagulation and thrombotic complications	[120]
PTPN1	**Endotoxemia**	PTP1 not protect lipopolysaccharide-induced inflammation, hyperinsulinemia, and endotoxemia through an IL-10 STAT3-dependent mechanism.	[121]
	Vascular damage	Cytokines, ROS, and COX trigger an acute inflammatory response and induce vascular damage that may be reduced by PTP1B deletion	[122]
EPRS	**Endotoxemia**	EPRS inhibition has beneficial effects against organ dysfunction due to reperfusion injury and endotoxemia	[123]
	Vascular damage	PARS activation plays a role in the pathogenesis of endothelial injury in endotoxin shock.	[124]

S. 1. Table Characteristics of patients with septic shock

	P1	P2	P3	P4	P5
Age	71	68	51	61	32
Gender	M	M	F	M	M
Days of ICU stay	23	49	10	57	9
Type of hospitalization	Traumatic	Neurologic	Surgical	Clinical	Surgical
Microbiological	Gram-negative	Polymicrobial	Gram-negative	Polymicrobial	-
Positive culture	Hemoculture	Tracheal secretion	Hemoculture	Hemoculture	Negative
Mechanical ventilation	13	17	10	13	0
AKF	No	Yes	No	yes	No
Hemodialysis	No	Yes	No	yes	No
SAPS3 score	102	61	51	80	50
% Expected Mortality	92	38	19	74	17
SOFA score	16	11	14	11	17
Clinical outcome	Non-survive	Survivor	Non-survive	Survivor	Survivor

AKF, Acute Kidney Failure; SAPS3, Simplified Acute Physiology Score III; SOFA, Sequential Organ Failure Assessment;

S.2 _Table. List of protein interactions

PROTEIN	INTERACTING PARTNERS
PPP1CB	RIPK3; SH2D4A; PPP1R11; TMEM33; BRCA1; PPP1R8; PPP1R2; IKBKG; PPP1R12C; PPP1R12B; RB1; SMARCB1; BCL2; PPP1R7; ZFYVE9; PTK2; RYR2; PPP1R12A; CDC34; WBP11; GRB2; MAX; CCND1; PPP1R15A; CDKN2A; PRKAR2A; PPP1R9B; AKAP11; CCND3; NCL; TLX1;
RIPK3	PPP1CB; MYH10; EPRS;
PDPK1	CDAN1; PKN1; MAPK8; AKT1; PRKCD; YWHAQ; PRKACA; PKN2; SGK1; STRAP; PRSS23; RPS6KB1; PTPRC; PRKCI; PTPRB; IRS1; TBL2; RPS6KA1; PTPN12; ITGB3; PTPRG; RPS6KA3; PRKCZ; PTK2B; PRKCE; PAK1; HSP90AA1; SLC9A3R2; SGK3; MTOR; PXN; AKT2; SND1; ILK; YWHAH; PNN; PRKCB; PTPN22; SRC; G3BP1; RPS6KB2; RALGDS; SGK2; PTPRK; PTPRJ; AKTIP; PTPRO; PA2G4; AKT3; CARD11;
EPRS	NELFCD; EEF1D; TFE3; IARS; ATG12; POU2F1; MAP3K14; RARS; MCC; ARL4D; IKBKE; MAPK13; AIMP2; RELB; HSP90AA1; DUS2; SYNCRIP; NFKBIB; IKBKB; MAP3K7; ETS1; RIPK3; NFKB2; NFKBIE; RELA;
FLNB	ATF7IP; NCK1; FBLIM1; GRB2; TSHR; NPHP1; PIK3R1; SMURF2; G3BP1; ITGB1; ITGB6; PLCG1; FLNA; OTUD1; PSEN2; CRK; PSEN1; GP1BA; ITGB3; PLEKHO1;
EFTUD2	DFFA; YWHAB; SNRNP40; USP39; PHLDA3; GSTK1; ARPC3; MYC; CD2BP2; ARRB2; AIRE; PTP4A3; PRPF8; SNRPB; RELA; YWHAG; RNPS1; MEPCE; TOP1; GOLM1; PRKAB1; NFKB2; SREK1; RPAP1; RPAP3; GPN1; RUVBL2;
PTPN1	LTK; JAK2; CRK; IGF1R; PIN1; CTNNB1; PIAS1; INSR; CDH2; GHR; CDK1; PDGFRB; STAT5A; GRB2; NTRK3; IRS1; BCAR1; MAPK1; CAV1; CSNK2A2; RRAS2; CLK1; BCR; TYK2; AKT1; STAT5B; ESR1; ACTN1; GSK3B; EGFR; TRPV6; CAPN2; NTRK2; NTRK1; GLRX; SRC; NFKBIA; CSNK2A1; CLK2; LAT;
ILK	ITGB3; PDPK1; S100A9;
ITGB3	ILK; FBLN2; PECAM1; NID1; AKT1; TLN1; KDR; VTN; CAPN1; DOK1; ITGAV; PDGFRA; PDPK1; DAB2; PTK2; NUMB; ITGB3BP; SHC1; FN1; THY1; ITGA2B; TNS2; TGM2; SRC; DAB1; FGA; FGG; CD36; THBS1; CIB1; FLNB; COL1A2; PTK2B; VWF; ANGPTL3; PXN; FLNA; PDGFRB; ITGA5; EPS8; P2RY2;
ANXA3	EMG1; UNC119; IGSF21; TP53; REG3A; UBR1;
COG4	COG7; COG5; COG2; COG1; COG3;
APOA1	ALB; KRT1; KRT9;
ALB	APOA1; SLX4; NPHS1; ST13; CDCP1; LUC7L2; ZNF232; AHSG; KIAA0232; DMWD; PDZRN4; SLA2; CUBN; CRB1; MYLK3; ATM; FCGRT; UIMC1; HP; APOC3; KIAA1551; QTRTD1; SCAF1; USP37; DICER1; RLF; PON1; C4A; SLC25A13; CABLES1; RANBP3; KLK3; APOE; SPATA31A7; KRT6A; RANBP2; KRT10; CROT; GRAP2; GFAP; LAT; AP4E1; CST3; GJC2; APOA2; SACS; RBP4; PALB2; AMPD3; ADRA1B; DCD; SGOL2; TF; CTAGE5; PLA2G4F; ITGA2; ORM1; SRGN; CRYAA; PRSS3; PPP2R2B; ETF1; TRAPPC11; APOC2; TTR; KRT5; AGA; KCNMA1; CACNA1I; TSC22D1; HPX; PLAG1; RYR2; BBC3; SH3BP5; KRT13; EXOC6; PDE4B; GABBR1; AMBP; LRP2; LDB3; ZNF292; PF4V1; CNOT1; THRAP3; SCN5A; PCDH1; DMD; DDB1; GSN; SLC9A8; F2; OBSL1; F7; CFB; CAMTA1; KRT16; KRT6B; ITIH1; FN1; ITGB5; DERL1; DGKG; CLCA2; CEP44; MYL4; SETX; IGDCC4; OR8D2; PPBP; OR2T6; TTPAL; NLRC4; DCC; CDC45; SORBS3; NR5A2; TLN2; TTN; CFH; CFD; PEG3; CTSL; FAM71E2; OR3A2; KRT9; GCN1; JARID2; SLC1A5; TIAM1; KRT1; SERPING1; NCOA3; PHC3; CHKB; AP1M1; FGA; HNF1A; PCED1B; SPAST; PRSS1; APOC1; YWHAG; KRT14;
SLX4	ALB; S100A8;
KRT1	APOA1; PRKCE; IVL; TANK; FANCA; LOR; MDM2; MBL2; CSTA; F12; FANCC; DSP; PI3; EVPL; PPP2R2B; CALB2; YWHAQ; KNG1; KRT5; APC; EGFR; ALB; CDH1; MAPK11;
HIST1H4A	CENPA; NASP; PAK1; HDAC4; GRB2; PTMA; HJURP; HIST1H3F; MAP3K7; MCM3; MAP3K3; SETD8; HIST1H1E; DNAJA2; HDAC5; H2AFX; ARRB1; RBBP7; HIST1H3E; ASF1A; MCM5; MCM4; HIST1H3B; MCM7; TBL1X; NPM1; MCM2; FKBP14; LIN54; HDAC6; HIRIP3; HIST1H3H; HIST1H3I; LIN37; HAT1; DAXX; DEK; RBBP4; HDAC1; KAT7; HIST1H3G; HIST1H3J; MAP3K1; MCM6; HIST1H3A; RB1; L3MBTL1; HIST1H3C; TNFRSF1A;
CCT8	PPP2CB; PPP2R2D; PPP4C; TCP1; PPP2CA; CTTNBP2; PPP2R2B; TBK1; IGBP1; STRN4; ACTB; STRN3; MYC; PPP2R4; MAP3K3; TRAF3IP3; TP63; RFWD2; PPP2R2C; STRN; MAP3K1; MAPK13; DOCK5; MOB4; CDK9; MEPCE; STK24; RELA; GPN1;
PSMC4	KRT33B; UCHL5; PSMD11; PSMD10; PSMC5; PAAF1; PSMD13; BAG2; RIOK3; TRIP6; USP14; PSMC2; PSMC3; PSMC6; HTR1E; PSMD1; PSMD2; ATG4C; PRKAB1; PAICS; EPB41; PSMD7; PSMD6; CMYA5; PSMC1; IKBKE; MYC; PSMD5; RORB; TRAF6;
NUDC	HLA-B; PAFAH1B1; VDAC1; HLA-C; PRKAB1; MAP3K3; LXN; BTRC; EIF6; ATG5; TIMP2; PLK1; TNIK; TNFRSF10D; PAK2; DGKE; FBXW11; WIPI2; EIF1B; ARF6; MAPK13; ELF3; VHL;
S100A9	S100A8; PPP2R2B; USF2; TAGLN; ARRB2; EGFR; PAK7; ASB3; ARRB1; NUAK1; CFTR; PPP2R1A; ILK;
S100A8	S100A9; NCF2; MOB1A; LRIF1; PRMT1; C14orf1; PPIA; PDCD11; CHGB; SLX1B; IGSF21; MAP3K3; ASB3; RAB17; NUAK1; SLX1A; USF2; PPM1B; CDK2; CEACAM3; UNC119; KLC2; SLX4; PPP2R2B; TP53; LRRK1; TUBA4A; TRAF3IP1; GDF9; NFKBIB; RIF1; DMWD;
EIF1B	ACP1; NUDC; HMGB2;
ACP1	EIF1B; IKBKE; EPHB1; NR3C1; FYN; TNIK; CTNNB1; SFMBT1; EPHB2; SPTAN1; FNBP1L; EPB41; INSR; PAK2; EIF6; EPHA2; FABP4; LCK; VHL; ZAP70; KDR; MRPL20;
MYO5A	BMF; RAB27A; MYC; MLPH; TRIM2; DYNLL2; DYNLL1; SMAD2; SHANK2; PRPH; RAB11A; TRIM3; DLGAP1; NEFL;

FOS	ZNF133; MATR3; TLN2;
ZNF133	FOS; MDM2;
ANAPC5	SMURF1; CDC16; ANAPC16; CDC20; CDC23; ANAPC13; CREBBP; ANAPC1; ZBTB16; CDC27; ANAPC2; PABPC1; ANAPC7; ANAPC4; EP300; APC2; MED19; FZR1; CDT1; SMURF2; BUB3; CDC26; TGFBR1; BUB1B;
CRK	PTPN1; FLNB;
MYH10	ARRB2; COPS5; TNFRSF10D; PRKAB1; SVIL; PBX1; TNFRSF1A; IKBKE; RELA; PAK2; GRB2; MYH9; IKBKG; LLGL1; BCAP31; TNFRSF1B; EPB41; MCC; RIPK3; MAP3K1; S100A4; IKBKB; MARK4; USP45; RIPK2; CHUK; MAP3K3;
ARRB2	MYH10; S100A9; EFTUD2;
IKBKE	ACP1; MYH10; EPRS; PSMC4;
AKT1	PDPK1; ITGB3; KRT10; PTPN1; DLC1;
MATR3	RELA; NFKB2; GFI1B; GSTK1; NR4A1; CDK9; GRB2; MYC; PCBP1; DISC1; EGFR; FOS; TTF2; RUVBL2; MAP3K3; HNRNPK; MEPCE; PPP2R2B; H2AFX;
RELA	MATR3; EFTUD2; MYH10; CCT8; EPRS;
PRKCE	KRT1; PDPK1;
LZTR1	TUBGCP4; BMPR1B;
YWHAQ	PDPK1; KRT9; KRT1;
TANK	KRT9; KRT1;
KRT9	TANK; PPP2R2B; MDM2; TRAF3IP1; APC; SH3GL3; CDH1; YWHAQ; ALB; APOA1;
MLH3	MLH1; MSH4;
LTF	LRP1; MUC7; CD14; SGK1; CP; CDK5RAP3; CALM1; ITLN1; CEL; LCN1; LYZ;
LRP1	LTF; CTSG;
CTNNB1	PTPN1; ACP1;
NR3C1	ACP1; HMGB2;
TLN2	PIP5K1C; FOS; ALB;
HMGB2	POU2F2; PRKDC; PGR; RAG1; CSNK1A1; GZMA; POU3F1; PON2; SET; NFKB1; EIF1B; AR; POU2F1; APEX1; TP53; TRAF6; NR3C1; POU5F1;
C8B	C5; C8A; CLU;
ATP6V1B1	STX1A; ACTN4; SLC9A3R1;
CHTF8	DSCC1; EIF4EBP1; CHTF18; PCNA; RFC2; RFC3;
SGK1	PDPK1; LTF;
UNC119	ANXA3; S100A8;
DMWD	ALB; S100A8;
PPP2R2B	S100A9; KRT9; KRT10; CCT8; ALB; S100A8; KRT1; MATR3;
POU2F1	EPRS; HMGB2;
TNC	NCAN; FN1; EGFR; CNTN1; PTPRB; ITGA5;
KDR	ITGB3; ACP1;
CTSG	C3; SERPINA1; PPBP; SELPLG; SDC1; CASP7; CXCL12; GP1BA; F2RL2; CAMP; KNG1; F2R; IGFBP3; PARP1; F5; CXCR4; VCAM1; SERPIND1; THBS1; SERPINB13; LRP1; F2RL1; SERPINA3; AGT;
IKBKG	PPP1CB; MYH10;
PAK1	HIST1H4A; PDPK1;
SMAD2	ZEB2; MYO5A;
ZEB2	SMAD2; SMAD9; SMAD5; SMAD1; SMAD3; COPS6; CTBP2; SCHIP1; CTBP1;
TNFRSF10D	MYH10; NUDC;

GRB2	FLNB; HIST1H4A; PTPN1; MATR3; MYH10; PPP1CB;
PRKAB1	MYH10; NUDC; PSMC4; EFTUD2;
TRAF3IP1	KRT10; KRT9; S100A8;
KRT10	TRAF3IP1; PPP2R2B; TJP1; EVPL; AKT1; RPS9; ALB; PRKCZ; SMAD3; MDM2; NFKB2; GLE1; MME;
INSR	PTPN1; ACP1;
GSTK1	EFTUD2; MATR3;
TNIK	ACP1; NUDC;
MYC	MYO5A; EFTUD2; MATR3; CCT8; PSMC4;
USF2	S100A9; S100A8;
HLA-C	NUDC; KIR2DL2;
NFKB2	MATR3; HIST1H2BB; KRT10; EFTUD2; EPRS;
PTPRB	PDPK1; TNC; NRCAM;
RB1	PPP1CB; HIST1H4A;
IRS1	PDPK1; PTPN1;
MAP3K3	NUDC; HIST1H4A; S100A8; CCT8; MATR3; MYH10;
PDGFRB	PTPN1; ITGB3;
MAP3K7	HIST1H4A; EPRS;
EVPL	KRT10; KRT1;
EGFR	S100A9; TNC; PTPN1; MATR3; KRT1;
SMURF2	FLNB; ANAPC5;
NRCAM	CNTN2; MAGI3; MACF1; HSPA12A; ANK2; NFASC; PTPRB;
G3BP1	FLNB; PDPK1;
LCN2	MMP2; MMP9; HGF; LRP2;
TUBGCP5	TUBG1; TUBGCP3; MZT1; MZT2B;
CDK9	MATR3; CCT8;
MDM2	KRT1; ZNF133; KRT9; KRT10;
EIF6	NUDC; ACP1;
FN1	TNC; ITGB3; ALB;
SMAD3	ZEB2; KRT10;
KIR2DL2	HLA-C; CD93;
IGSF21	S100A8; ANXA3;
AIRE	EFTUD2; HIST1H2AC;
HIST1H2BB	TNFRSF1A; UHRF1; NFKB2;
TNFRSF1A	HIST1H2BB; MYH10; HIST1H4A;
MCC	EPRS; MYH10;
HIST1H2AC	TFAP2B; AIRE;
PPBP	CTSG; ALB;
ASB3	S100A8; S100A9;
PRKCZ	PDPK1; KRT10;
PTK2B	PDPK1; ITGB3;

EPB41	ACP1; PSMC4; MYH10;
DEFA1	PFDN1; DEFA3; SERPINF2; C1QB;
YWHAG	EFTUD2; ALB;
LAT	ALB; PTPN1;
PTK2	PPP1CB; ITGB3;
H2AFX	HIST1H4A; MATR3;
PAK2	MYH10; ACP1; NUDC;
ARRB1	HIST1H4A; S100A9;
FLNA	FLNB; ITGB3;
NUAK1	S100A8; S100A9;
RYR2	PPP1CB; ALB;
MAPK13	EPRS; CCT8; NUDC;
MEPCE	EFTUD2; CCT8; MATR3;
SRC	ITGB3; PTPN1; PDPK1;
CEACAM6	CEACAM8; CEACAM1;
HSP90AA1	PDPK1; EPRS;
KRT5	ALB; KRT1;
FGA	ITGB3; ALB;
CP	LTF; MPO;
VHL	ACP1; NUDC;
GP1BA	CTSG; FLNB;
DLC1	TNS3; AKT1;
TP53	S100A8; ANXA3; HMGB2;
PXN	PDPK1; ITGB3;
LRP2	ALB; LCN2;
THBS1	ITGB3; CTSG;
KNG1	CTSG; KRT1;
MAP3K1	MYH10; CCT8; HIST1H4A;
SACM1L	COPG1; COPB2; COPA;
RUVBL2	MATR3; EFTUD2;
NFKBIB	EPRS; S100A8;
APC	KRT9; KRT1;
IKBKB	EPRS; MYH10;
CDH1	KRT9; KRT1;
ITGA5	TNC; ITGB3;
TRAF6	HMGB2; PSMC4;
GPN1	EFTUD2; CCT8;

S.3 _Table. The major proteins correlation cell function

Protein	Phenotype	Activation	Phagocytosis	Migration	Senescence	Death	Viability
ANXA3			■	■		■	
FLNB				■		■	
ZEB2	■			■		■	
EPRS	■	■				■	
RETN		■					
LCN2	■			■		■	
PDPK1					■		
NNMT	■					■	■
RNASE3					■		■
DLC1	■			■	■		
SACMIL	■						■
TLN2				■			
LTF	■	■	■	■		■	
PPP1CB	■						
S100A8	■	■	■	■		■	
PTPN1						■	
KLB	■				■	■	
CTSG	■	■	■	■			
MPO	■	■	■	■		■	
EFTUD2							■
NRCAM	■			■		■	
S100A9	■	■	■	■		■	
MATRA3						■	
CEACAM 6	■		■			■	
KIR2DL2			■				
NUDC	■			■		■	
SH3TC2				■			
HIST1H2AC	■						
ACP1	■	■		■		■	
ITGB3	■	■	■				
TNC	■	■	■	■		■	
ALB	■	■	■	■			
HMGB2	■	■					■
MYH10	■			■		■	
MYO5A	■		■	■		■	
ESRP2	■			■			
PSMC4						■	
KRT1	■	■					
KRT10	■	■				■	
ZFHX2	■					■	
LZTR1						■	
CLCN4	■			■			
CCT8				■			
ATP13A3					■		
ATAD2B	■						
KRT9	■						