Anti-inflammatory effects of Human Serum Albumin and its mechanism of action in decompensated cirrhosis

Mireia Casulleras i Oliver

Anti-inflammatory effects of Human Serum Albumin and its mechanism of action in decompensated cirrhosis

---

Memory presented by:

Mireia Casulleras i Oliver

For the degree of Doctor by University of Barcelona

Work performed under the direction of **Dr. Joan Clària i Enrich** in the group of inflammation and liver disease, in Institut D'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS) and Biochemistry and molecular genetic service of Hospital Clínic.

Mireia Casulleras Oliver

PhD Candidate

Joan Clària Enrich

Director

PhD program in Medicine and Translational Research

Faculty of Medicine and Health Science, University of Barcelona

June, 2022

I, Professor Joan Clària Enrich, director of the doctoral thesis of Mireia Casulleras Oliver, give my approval for the presentation of this doctoral thesis entitled: Anti-inflammatory effects of Human Serum Albumin and its mechanism of action in decompensated cirrhosis.

Joan Clària Enrich 77111365R

Digitally signed by Joan Clària Enrich 77111365R
Date: 2022.05.26 16:37:20 +02'00'

Professor Joan Clària Enrich

MIREIA CASULLERAS OLIVER - DNI 45987542P

Firmado digitalmente por MIREIA CASULLERAS OLIVER - DNI 45987542P
Nombre de reconocimiento (DN): c=ES, sn=CASULLERAS OLIVER, givenName=MIREIA, serialNumber=IDCES-45987542P, cn=MIREIA CASULLERAS OLIVER - DNI 45987542P
Fecha: 2022.05.26 18:44:56 +02'00'

Mireia Casulleras Oliver

“La meva feina és indagar. Busco l’origen de les coses. Intento entendre per què passen, com funcionen. D’aquesta feina en diem ser un investigador. Un científic. Indagar és buscar respostes a tota mena de preguntes. Millor encara: fer-se preguntes que encara no tenen resposta.”

Salvador Macip. 2020

A la Marta i el Roger, els meus companys d'aventura

TABLE OF CONTENTS

FIGURE INDEX

TABLE INDEX

ACRONYMS

AA	Arachidonic acid
ACLF	Acute-on-chronic liver failure
AD	Acutely decompensated cirrhosis
AKI	Acute kidney injury
ATL	Aspirin-triggered LXs
AP-1	Activator protein 1
APC	Antigen presenting cells
ASK	Apoptosis-associated speck-like protein containing a caspase recruitment domain
ATP	Adenosine triphosphate
BCR	B cell receptors
BTM	Blood transcription modules
CO	Carbon monoxide
COX	Cyclooxygenase
CpG	DNA rich in unmethylated cytosine-phosphate-guanine motif
DAMP	Damage associated molecular patterns
DARC	Duffy antigen receptor for chemokines
DC	Dendritic cells
DD	Death domain
DHA	Docosahexaenoic acid
dsRNA	Double-strand RNA
EPA	Eicosapentaenoic acid
FA	Fatty acids
FcRn	Neonatal Fc receptor
FITC	Fluorescein isothiocyanate
FLAP	5-LOX activating protein
G-CSF	Granulocyte colony-stimulating factor
GPCR	G protein-coupled receptor
HMA	Mercaptoalbumin
HNA	Nonmercaptoalbumin
HPGD	15-hydroxyPG dehydrogenase
HSA	Human serum albumin

HS	Healthy subjects
HV	Healthy subjects
Ig	Immunoglobulins
IL	Interleukin
IL-6R	Ligand-binding IL-6 receptor
INF	Interferon
iNOS	inducible nitric oxide synthase
IRF	Interferon regulatory factor
LGR6	Leucine-rich repeat containing G protein-coupled receptor 6
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LT	Leukotrienes
LX	Lipoxins
MAL	MyD88-adaptor-like
MaR	Maresins
MARS	Molecular Adsorbent Recirculating System
MBCD	Methyl-β-cyclodextrin
MCP-1	Monocyte chemoattractant protein-1
MD-2	Myeloid differentiation factor-2
MHC	Histocompatibility complex
mTNFα	Transmembrane TNFα
MUFA	Monounsaturated fatty acids
MyD88	Myeloid differentiation primary response 88
NET	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa B
NKT	Natural killer T cells
NO	Nitric oxide
NPD	Neuroprotectin
NSAID	Nonsteroidal anti-inflammatory drugs
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBMDM	Peripheral blood monocyte-derived macrophages

PD	Protectin
PG	Prostaglandin
PLA_2	Phospholipase A_2
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear leukocytes
PRR	Pattern recognition receptors
PUFA	Polyunsaturated fatty acids
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Rv	Resolvins
SBP	Spontaneous bacterial peritonitis
sIL-6R	Soluble form of IL-6R
SMT	Standard medical therapy
SPM	Specialized pro-resolving mediators
ssRNA	Single-strand RNA
sTNFα	Soluble TNFα
S1P	Sphingosine-1-phosphate
TCR	T-cell receptors
TGF-β	Transforming growth factor beta
TIR	Toll/interleukin-1 receptor domain
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRACE	TNFα-converting enzyme
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein inducing INFβ
VCAM-1	Vascular cell adhesion protein 1
WT	Wild type
5-HETE	5-hydroxyeicosatetranoic acid
14-HDHA	14-hydrodocosahexanoic acid
17-HDHA	17-hydroxydocosahexaenoic acid
18-HEPE	18-hydroxyeicosapentanoic acid

LIST OF THE ARTICLES THAT COMPRISE THE THESIS

Thesis in the form of a collection of published articles. This thesis comprises eleven objectives and two articles:

- **Casulleras M**, Flores-Costa R, Duran-Güell M, Alcaraz-Quiles J, Sanz S, Titos E, López-Vicario C, Fernández J, Horrillo R, Costa M, de la Grange P, Moreau R, Arroyo V, Clària J. Albumin internalizes and inhibits endosomal TLR signaling in leukocytes from patients with decompensated cirrhosis. Sci Transl Med. 2020 Oct; 12(566):eaax5135. doi: 10.1126/scitranslmed.aax5135.

  IF: 17.992
  Quartile: Q1
  Subject and category: Medicine, Research and Experimental

- **Casulleras M**, Flores-Costa R, Duran-Güell M, Zhang IW, López-Vicario C, Curto A, Fernández J, Arroyo V, Clària J. Albumin Lipidomics Reveals Meaningful Compositional Changes in Advanced Cirrhosis and Its Potential to Promote Inflammation Resolution. Hepatol Commun. 2022 Feb. doi: 10.1002/hep4.1893.

  IF: 5,073
  Quartile: Q2
  Subject and category: Gastroenterology and Hepatology

THESIS SUMMARY

Aquesta tesi esta dividida en dos estudis englobats sota l'objectiu d'elucidar els mecanismes moleculars de l'efecte de l'albúmina en leucòcits, en el context de la Cirrosis hepàtica avançada. En el primer estudi, es van estudiar els mecanismes moleculars pels quals l'albúmina exerceix les propietats immunomodulatòries. L'albúmina sèrica humana (HSA) s'utilitza com a tractament per prevenir un excés de la inflamació sistèmica i la fallada orgànica en pacients amb cirrosis descompensada (AD). L'administració de HSA en pacients amb AD cirrosis que tenien nivells circulants elevats de ADN bacterià ric en motius CpG, es va associar a una reducció de la concentració de citocines en plasma. En leucòcits aïllats, la HSA va ser capaç d'erradicar l'expressió i l'alliberament de citocines induïdes per CpG independentment de les seves propietats oncòtiques i de segrest. Es van observar efectes antiinflamatoris similars amb albúmina humana recombinant. A més, HSA provoca canvis en el transcriptoma de les cèl·lules immunes, específicament en gens relacionats amb les citocines i la resposta a interferó tipus I. Les nostres dades van revelar que els leucòcits eren capaços de captar HSA i internalitzar-la en vesícules intracel·lulars, en endosomes on HSA colocalitza amb CpG, ja que el seu receptor tipus Toll 9 (TLR9) es troba en aquestes vesícules. A més, HSA també era capaç d'inhibir les respostes inflamatòries induïdes per poly (IC) i LPS, que són exclusives de la senyalització endosomal TLR3 i TLR4, respectivament. Les accions immunomoduladores de HSA no van comprometre els mecanismes defensius dels leucòcits, com la fagocitosi, l'eferocitosi i la producció d'espècies reactives d'oxigen intracel·lular. Els efectes immunomoduladors in vitro de HSA es van confirmar in vivo en ratolins humanitzats analbuminèmics. Aquestes troballes indiquen que HSA s'internalitza a les cèl·lules immunitàries i modula les respostes a través de la interacció amb la senyalització de TLR endosomal, fet que proporciona un mecanisme per als beneficis de les infusions d'HSA en pacients amb cirrosi.

Al segon projecte, es va estudiar el lipidoma de l'albúmina en aquests pacients, ja que l'albúmina té una alta afinitat pels lípids. A més vam explorar els efectes de l'albúmina en l'alliberament de mediadors lipídics bioactius per part dels leucòcits. Els mediadors lipídics es van mesurar mitjançant cromatografia líquida unida a espectrometria de masses en tàndem en fraccions de plasma enriquides amb albúmina i empobrides en albúmina separades per cromatografia d'afinitat i en incubacions de leucòcits de 18 pacients amb cirrosi i 10 subjectes sans. El lipidoma plasmàtic associat amb la cirrosi es va caracteritzar per la supressió generalitzada de totes les classes de lípids excepte els àcids grassos (FA). A diferència del que veiem en subjectes sans (HS), l'albúmina dels pacients tenia un contingut més baix de FA poliinsaturats (PUFA), especialment

dels omega-3-PUFA. D'acord amb això, el panorama de mediadors lipídics derivats de PUFA de l'albúmina dels pacients va estar dominat per un contingut més baix de precursors de mediadors lipídics antiinflamatoris i pro-resolutius (és a dir, àcid 15-hidroxieicosatetraenoic, 15-HETE). A més, a l'albúmina dels pacients amb AD la prostaglandina (PG) E2 es trobava en menor concentració, cosa que suggereix que aquesta PG pro inflamatòria viatja principalment dissociada a l'albúmina en aquests pacients. La incubació de leucòcits amb albúmina exògena va reduir la producció de PG mentre induïa l'alliberament de 15-HETE. Finalment, els mediadors de lípids també es van mesurar en 41 pacients amb AD inclosos en un assaig clínic de teràpia amb albúmina. Els nivells de PG van ser més baixos en els pacients amb AD que van rebre tractament amb albúmina, mentre que els nivells de 15-HETE estaven augmentats després del tractament amb albúmina en comparació del valor inicial. Les nostres troballes indiquen que la composició lipídica de l'albúmina està severament desorganitzada a la cirrosi descompensada i que l'administració d'albúmina exògena té el potencial de redirigir la biosíntesi de leucòcits de mediadors lipídics pro inflamatoris a pro-resolutius.

INTRODUCTION

1. ALBUMIN

The name albumin evolved from the more general term, "albumen", the early German word for protein. Its origin was Latin, "albus" (white), the color of that part of an egg surrounding the yolk when it is cooked. Whereas the -in ending refers to the specific protein from blood plasma or protein with similar properties (Peters TJ. Academic Press, 1995).

Human serum albumin (HSA) is the most abundant protein in the human plasma. It constitutes the 50-60% of the plasma proteins. It is synthesized in the liver (10-15 g/day) by hepatocytes and released into the intravascular space. Approximately, between 30-40% of the albumin produced remains in intravascular compartment, whereas the rest travels to the interstitial space through capillaries and back to the systemic circulation where it is able to reversibly bind a wide variety of molecules with high affinity (Peters TJ. Academic Press, 1995). Albumin concentration depends on its synthesis and degradation and distribution between the intravascular and extravascular compartments (Nicholson JP, et al. BJA, 2000). Although albumin is a prototypic extracellular molecule, it is taken up by many cell types by endocytosis and catabolized through liposomal degradation (Garcia-Martinez R, et al. Hepatology, 2013). The half-life of albumin in healthy subject is between 12-19 days; although its life span is altered in many disease conditions. Due to its long half-life, albumin commonly suffers posttranslational modifications, as the result of oxidation, glycation, and truncation. These modifications contribute to the micro heterogeneity of circulating HSA and may alter its biological activity under disease conditions. Albumin is a protein with high stability, although more than 75 isoforms have been reported (Watanabe H, et al. J Phar Sci, 2017).

1.1. CHEMICAL AND STRUCTURAL PROPERTIES

Albumin protein has a molecular weight of 66,5 kDa and consist of a single polypeptide chain of 585 amino acids. This protein is composed by three domains of similar size arranged in a heart shape and formed by eight α-helices. Due to its structural similarity the domains can be divided in sub-domains (IA, IB, IIA, IIB, IIIA and IIIB) that have functional relevance because they define the binding sites of many albumin ligands (see Figure 1). Albumin is a multi-domain protein stabilized by 17 disulfide bounds that confer a remarkable stability to its structure (He XM, et al. Nature, 1992). Despite its large size and stability, the albumin molecule can be refolded from the fully reduced state, a

property that facilitates the synthesis of this molecule in the laboratory and the study of its structural and biophysical properties (Mao H, et al. Protein Expr Purif, 2000).

Many of the structural properties of albumin rely on its ability to reversibly bind a wide range of ligands to increase their solubility to plasma or transport them. The most important binding sites for ligands reported in albumin are commonly known as Sudlow's sites I and II which are found in subdomains IIA and IIIA, respectively (Sudlow G, et al. Mol Pharmacol, 1975). Albumin is the main transporter of long chain fatty acids since its molecule has up to seven sites able to bind fatty acids (FA1 to FA7) including both Sudlow's sites, with site I corresponding to FA7 and site II corresponding to FA3 and FA4 (Bhattacharya AA, et al. J Mol Biol, 2000).

Albumin has a free cysteine (Cys-34) residue involved in disulfide bounds that is exposed and plays an important role in albumin antioxidant activity due to a presence of a thiol group that trap free radicals. In healthy conditions, albumin exists predominantly (70%-80%) in its reduced form, known as mercaptoalbumin (HMA). A small fraction of albumin (20%-30%) is reversibly oxidized by binding small thiol molecules as cysteine, homocysteine, and glutathione, known as nonmercaptoalbumin 1 (HNA1). The remaining 5% of the total albumin circulates in the form of nonmercaptoalbumin 2 (HNA2), with the Cys-34 residue is irreversibly oxidized to sulfidic or sulfonic acids (Watanabe H, et al. J Phar Sci, 2017). A consequence of these modifications is a decrease in the anti-oxidant properties as well as alterations of albumin functions such as its ability to bind molecules in Sudlow's sites (Jalan R, et al. Hepatology, 2009).

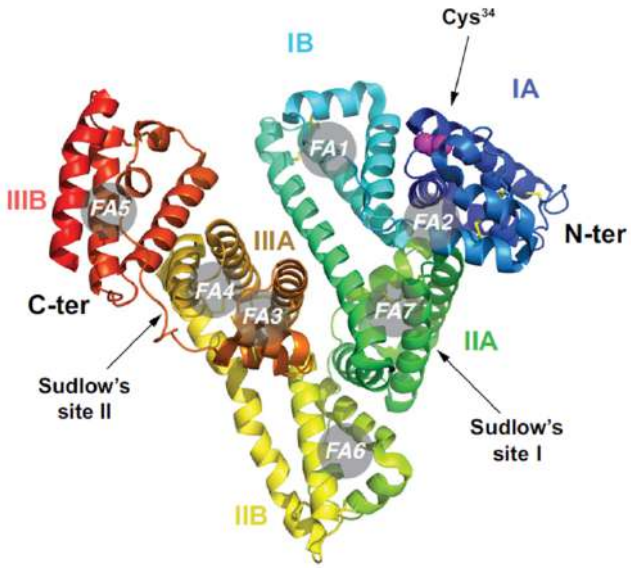


Figure 1. Molecular structure of Human Serum Albumin. Cristal structure (pdb code 1e7i) of HSA with its subdomains labeled as IA, IB, IIA, IIB, IIIA and IIIB, Sudlow's sites I and II, Fatty acids binding sites labeled as FA1 to FA7 and Cys-34 shown as purple spheres (Arroyo V, et al. J Hepatol, 2014).

1.2 BIOLOGICAL PROPERTIES

Albumin molecule is considered a pleiotropic protein with a wide range of biological functions that will be deeply explained in this section.

1.2.1 Plasma oncotic pressure

This is the most well-known property of albumin due to its high concentration in plasma and its negative charge. Albumin contributes up to 80% of the normal oncotic pressure of about 25 mm Hg. This is due that albumin concentration is higher than other plasma proteins. In fact, the osmotic effect of albumin represents about the 60% of the oncotic pressure exerted by albumin. The remaining 40% of albumin oncotic pressure is related to its negative charge, which translate into the intravascular retention of positively charged solute particles (Effect known as Gibbs-Donnan). Albumin water-solubility and negative charge plays a significant role in regulation of tissue fluid distribution avoiding the accumulation of fluids in the tissues (Nicholson JP, et al. BJA, 2000).

1.2.2 Solubilization and transport

Albumin has the capacity to bind a diverse range of molecules. The negative charge of albumin facilitates electrostatic binding of other substances, acting as a storage and vehicle. In parallel, albumin transporting functions are secondary to the binding sites present in its tertiary structure (Garcia-Martinez R, et al. Hepatology, 2013).

Fatty acids are the most common substances transported by albumin. Since the solubility of fatty acids in aqueous solutions such as blood plasma is very low, fatty acid-binding molecule is needed to guarantee the transport of fatty acids from adipose tissue to their consuming organs. Among Albumin fatty acid binding sites, 2, 4 and 5 are known as primary sites because they offer the most favorable conditions for high-affinity binding

and 1, 3, 6 and 7 are the secondary binding sites (Van der Vusse GJ, et al. Drug Metab Pharmacokinet, 2009).

Albumin also binds and carries a great variety of other endogenous hydrophobic metabolites as bilirubin, bile acids, eicosanoids, steroids, vitamin D and folate and other less hydrophobic with lower affinity as tryptophan and also, exogenous molecules as drugs, with consequent implications on solubilization transport and metabolism of these substances. (Bernardi M, et al. J Clin Exp Hepatol, 2014 and Nicholson JP, et al. BJA, 2000). Depending on the molecule it is bind in different sites of albumin. Site I tends to bind relatively large heterocyclic compounds. This site is large and able to bind endogenous substances, including bilirubin and porphyries. By contrast, site II is smaller and less flexible in nature, because binding is more stereo-specific, such as, benzodiazepines (Quinlan GJ, et al. Hepatology, 2005).

1.2.3 Antioxidant

Albumin is considered the most important extracellular antioxidant due to two different mechanisms of action. The first one is because of its thiol group in the Cys-34 residue that traps free radicals and works as a scavenger for reactive oxygen species (ROS) and reactive nitrogen species (RNS) derived from oxidative stress (Quinlan GJ, et al. Hepatology, 2005). The second mechanism is the capacity of albumin to bind and inactivate free metals such as cooper, cobalt, nickel, zing and iron, to its N-terminal portion and with high affinity binding, which catalyze the formation of aggressive ROS (Bernardi M, et al. J Clin Exp Hepatol, 2014). Finally, albumin bound to bilirubin inhibits lipid peroxidation that represents an indirect antioxidant effect of the molecule (Neuzil J, et al. J Biol Chem, 1994).

1.2.4 Endothelial stabilizing

Vascular endothelium produces substances that maintain vascular homeostasis by modulating vascular tone, Thrombogenesis, fibrogenesis and maintenance of tissue integrity, such as glycocalyx. Albumin has a net negative charge that promotes a binding to the glycocalyx with the effect of reducing hydraulic conductivity across the vascular barrier, resisting glycocalyx degradation and contributing to the maintenance of vascular integrity and normal capillary permeability (Aldecoa C. et al, Ann. Intensive Care, 2020).

Moreover, albumin has an important role in delivering sphingosine-1-phosphate (S1P) to the endothelial cell surface where it functions in maintaining normal vascular permeability Thuy AV, et al. Cell Physiol Biochem, 2014). It is demonstrated that albumin also contributes to protecting endothelial cells against oxidative-mediated injury through activation of the oxidant-sensitive transcription of pro-inflammatory proteins. Also, it decreases endothelial nitric oxide (NO) synthase activity, in endothelial cells and increases their glutathione levels maintaining endothelial cells function (Kremer H, et al. Crit Care Med, 2011).

1.2.4 Antithrombotic and anticoagulant

Albumin might have antithrombotic and anticoagulant effects related to its capacity to bind NO at the Cys-34 site with the subsequent formation of nitro albumin preventing the rapid inactivation of NO and promoting vasodilatation and inhibition of platelet aggregation (Evans TW. et al, Aliment Pharmacol Ther, 2002). Some clinical studies demonstrate that hypoalbuminemia is directly related to the hyper aggregation of platelets (Kim SB. et al, Am J Kidney Dis, 1999) and that albumin modification (nitro albumin) has an impact on platelet aggregation and coagulation.

On the other hand, albumin has another effect on coagulation acting as heparin, due to the similarities in the structures of both molecules. Heparin has many negative charged groups that bind to positive charged groups on antithrombin III, exerting an anticoagulant effect. Albumin, which is a negative charged molecule, exerts a heparin-like action (Nicholson JP. et al, BJA, 2000). There is a negative correlation between albumin concentration and heparin requirement in patients in hemodialysis (Jorgensen KA. et al, Thromb Res, 1979).

1.2.5 Anti-inflammatory and immunomodulatory

Many of the immunomodulatory effects of albumin rely on its ability to bind pro-inflammatory substances and mediators of inflammation. Albumin binds several bacterial products such as lipopolysaccharide (LPS), Lipoteichoic acid and peptidoglycan that activate the innate immune system through Toll-like receptor 4 (TLR4) and induce inflammation (Jurgens G, et al. J Endotoxin Res, 2002). In vivo studies showed that endotoxin activity decreases in the presence of albumin concentrations (Kitano H, et al. Alcohol Clin Exp Res, 1996). Moreover, albumin is able to reduce oxidative and nitrosative stress in a mouse model induced by LPS (Meziani F, et al. Am J Pathol, 2007).

Therefore, albumin could also play a role in moderating the inflammatory response to bacterial infections.

Although the beneficial effects of albumin in models of endotoxemia may be mediated by the capacity to bind LPS other mechanisms are also involved. Albumin is able to inhibit tumor necrosis factor alpha (TNFα) gene expression in macrophages stimulated with LPS. This effect is mediated by the attenuation of nuclear factor kappa B (NF-κB) activation (Aubin E, et al. Vox Sang, 2011). In addition, ex vivo studies have shown that albumin increases intracellular glutathione and regulates NF-kb activation (Cantin AM, et al. Am J Respir Crit Care Med, 2000). Similarly, albumin inhibits TNFα induced upregulation of Vascular cell adhesion protein 1 (VCAM-1) and NF-kB activation in endothelial cells (Zhang WJ, et al. Cardiovasc Res, 2002). This suggests that albumin could promote the intracellular protection against inflammatory and oxidative stress damage. Finally, albumin is able to reduce the circulating levels of prostaglandin E_2 (PGE_2), a pro-inflammatory and immune-suppressive eicosanoid involved in the inflammatory response (O'Brien AJ, et al. Nat Med, 2014). Albumin can also modulate immune cell function. For instance, albumin has been shown to upregulate the expression of the histocompatibility complex (MHC) and other related genes in antigen presenting cells (APC), thus facilitating the activation of T lymphocytes (Aubin E, et al. Vox Sang, 2011). Additionally, albumin has been shown to prevent neutrophil dysfunction in vitro, and therefore to ameliorate the risk of infections (Jaisson S, et al. FEBS Lett, 2007).

2 INFLAMMATION AND IMMUNE RESPONSE

2.1 IMMUNE CELLS

Immune cells, also known as leukocytes, are the first line of defense of the body. All white blood cells are derived from common hematopoietic stem cell progenitors that generate myeloid and lymphoid cells after differentiation: Myeloid cells comprise the majority of the cells of innate immune system, which are basically phagocytes, including granulocytes (neutrophils, basophils and eosinophils), monocytes/macrophages and dendritic cells. On the other hand, lymphoid cells comprise T-lymphocytes, B-lymphocytes, the central players in adaptive immune system, and natural killer T (NKT) cells, which share innate and adaptive immune properties (Delves PJ, John Wiley & Sons, 2017).

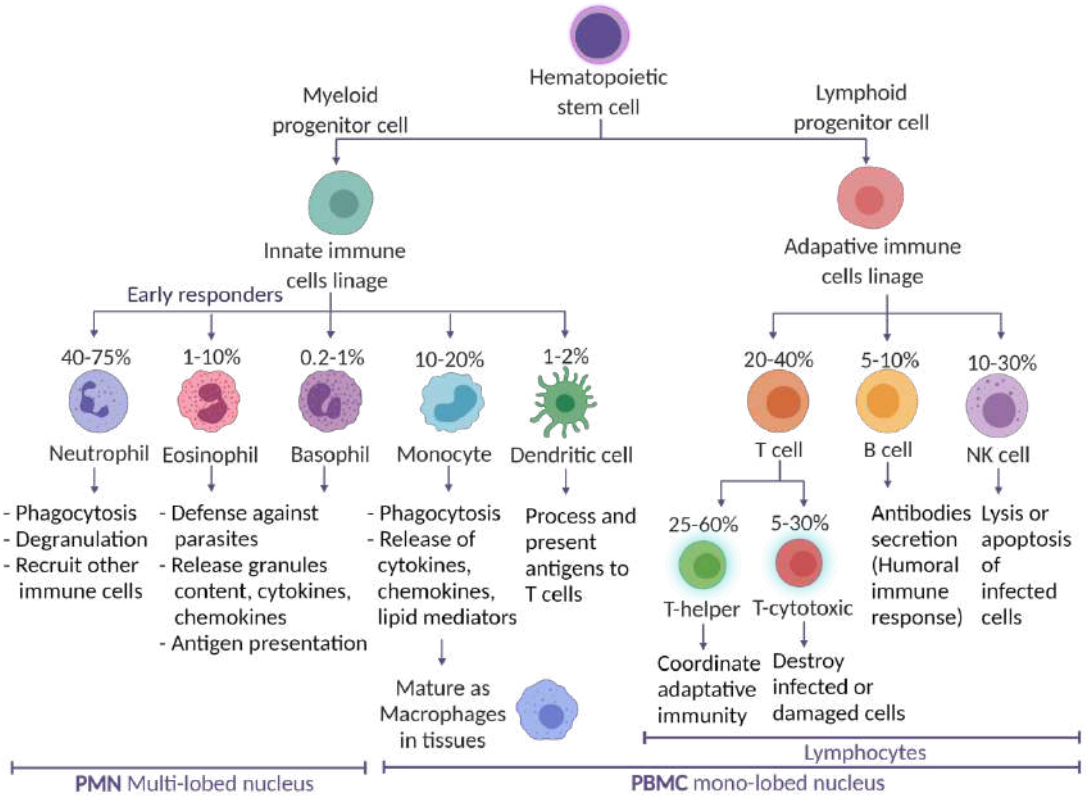


Figure 2. Immune cells linage. Scheme of individual blood immune cells derived from a common pluripotent cell with their specific functions. Classification of leukocytes can be based on their origin (myeloid or lymphoid) or on the morphology of their nucleus, multi-lobed nucleus as polymorphonuclear leukocytes (PMN) or mono-lobed nucleus as peripheral blood mononuclear cells (PBMC). Created with BioRender.com

- Granulocytes are a family of cells formed by neutrophils and their close relatives, basophiles and eosinophils. All of them circulate in the bloodstream however and under some signals they entry to the peripheral tissues. They have relative short half-lives (i.e. about a day or two). Granulocytes are also known as polymorphonuclear leukocytes (PMN) due to its multi-lobed nucleus (Delves PJ, John Wiley & Sons, 2017).

  Neutrophils are the most abundant leukocytes and are the first cells recruited to sites of infection or inflammatory injury. They produce large quantities of ROS that are cytotoxic to bacterial pathogens and possess phagocytic capabilities that permit them to sequester microbes internally with its subsequent degradation. Moreover, they contain granules in their cytoplasm with enzymes with a potent effect on the elimination of pathogens that are released in a process called degranulation, forming Neutrophil extracellular traps (NETs). Moreover, these cells produce cytokines and chemokines that help in the recruitment of other cells at the site of infection (Chaplin et al, J Allergy Clin Immunol. 2010 and Kennedy et al, Immunol Res 2009).

  Basophils and eosinophils play an important role in the destruction of parasites that are often too large to be phagocytized. Instead, these cells attack them with a bombardment of degrading enzymes stored in their granules. They also produce important amounts of lipid mediators that stimulate tissue inflammation. (Marshall et al. Allergy Asthma Clin Immunol 2018). In addition, these cells are important sources of cytokines that have a role in adaptive immune responses (Delves et al, John Wiley & Sons, 2017).

- **Monocytes** and **macrophages**: Monocytes are innate immune cells that account for 10 % of mono-nucleated blood cells (PBMC) (Auffray et al, Ann Rev Immunol, 2009). They appear to be mobilized shortly after the recruitment of neutrophils and they persist for long periods at sites of chronic inflammation and infection (Chaplin et al, J Allergy Clin Immunol. 2010). During homeostatic or inflammatory conditions monocytes migrate into tissue, they become the so-called macrophages. Both play a key role in anti-microbial immunity through direct responses such as phagocytosis. Additionally, monocytes are equipped with a large array of scavenger receptors that recognize microorganisms (pathogen associated molecular patterns, PAMPs) but also lipids and dying cells (damage associated molecular patterns, DAMPs). Stimulated monocytes can produce large quantities of effector molecules involved in the defense against pathogen like cytokines, chemokines, lipid mediators and other which can activate or regulate other cells of the innate and adaptive immune systems (Geissmann et al, Science, 2010 and Cormican et al, Front Immunol, 2020).

Nowadays, monocytes are subdivided into 3 subsets, classical, intermediate, and non-classical, as defined by expression of CD14 and CD16 in their membranes (Ziegler-Heitbrock et al, Blood, 2010). Classical monocytes are the most abundant (80-90%), have cytokine production and high phagocytic capacity and ROS release. Intermediate are considered pro-inflammatory with high cytokine production and phagocytic capacity but without ROS release. Finally, non-classical monocytes are about 10-20% and are considered the most inflammatory with high production of pro-inflammatory cytokines (Cros et al, Immunity, 2010 and Cormican et al, Front Immunol, 2020).

- **Dendritic cells (DCs)** are tissue resident cells with similar phagocytic capacity to macrophages. DCs have characteristic morphology with multiple long arms (dendrites) that enable them to maximize contact with their environment. They are the principal link between innate and adaptive immunity. DC are known as APC, their primary role is sampling of the environment for the detection and internalization of PAMPs through pinocytosis. Once PAMPs are detected, DCs undergo maturation changing their properties to a low phagocytic but highly migratory cell equipped with class I and class II MHC used for the presentation of processed antigen efficiently to T-cells in local lymph nodes (Delves PJ, John Wiley & Sons, 2017 and Chaplin et al, J Allergy Clin Immunol. 2010).

- **Lymphocytes:** Constitute the major cell type within PBMC (about 80%). T and B lymphocytes are the main players in adaptive immunity and have the ability to generate highly specific cell surface receptors for particular antigens (Delves PJ, John Wiley & Sons, 2017). **Lymphocytes T** have specific receptors called T-cell receptors (TCR) that recognize specific antigen presented by APC. Once detected, they have the capacity to rapidly proliferate and differentiate into cytotoxic T cells or T helper cells. Cytotoxic T cells are activated by the interaction of TCR with an antigen bound to MHC class I. They are involved in the destruction of cells infected by pathogens. Helper T cells are activated by antigens bound to MHC class II and play an important role in establishing and maximizing the immune response, thus, they mediate the immune response by directing other cells (Bonilla et al, J Allergy Clin Immunol, 2010 and Marshall et al. Allergy Asthma Clin Immunol 2018). **Lymphocytes B** are phenotypically defined by the expression of B cell receptors (BCR), which are Immunoglobulins (Igs) anchored in their membranes. The principal function of B cells is the production of antibodies against foreign antigens. They can recognize antigens directly without the need of APC; in fact, they can act as APC for

T cells activation. Once activated, B cells undergo proliferation and differentiate into antibody-secreting plasma cells or memory B cells that will respond quickly eliminating an antigen upon re-exposure (Kwak et al, Nat Immunol, 2019 and Marshall et al. Allergy Asthma Clin Immunol 2018)

- **NKT cells** are also lymphocytes, although they can play a role in innate immunity. They control several types of tumors and microbial infections by limiting their spread and subsequent tissue damage. NKT cells use receptors different to that of T and B cells with the ability to kill cells that express abnormal MHC receptor profiles as well as some viruses with MHC molecule expression. NKT cells are regulatory cells interacting with dendritic cells, macrophages, T cells and endothelial cells that make them limit or exacerbate immune responses (Vivier et al, Nat Immunol, 2008). Moreover, NKT cells have receptors specific for antibodies IgG so they can use it to display the antibody in their surface and find and kill infected cells, a process called antibody-dependent cellular toxicity (Delves PJ, John Wiley & Sons, 2017).

2.2 TOLL-LIKE RECEPTORS (TLRs)

The first TLR was discovered in 1991 in Drosophila melanogaster. On 1985, Christiane Nüsslein-Volhard saw a weird-looking fly larva in which the ventral portion of the body was underdeveloped. Her spontaneous comment was "Das war ja toll!" meaning "That was weird!" and she named "Toll" for the mutated gene (Anderson et al, Cell, 1985). Some years later, it was reported that Toll not only controls dorsoventral polarity *but also has a role in the immune defense in Drosophila (Schneider et al, Genes Dev, 1991). With this discovery, Toll-like receptors were, for the first time, associated with host defense.*

TLRs are functional and effective biomolecules in the front line of immune defense system. They are transmembrane proteins expressed in leukocytes and other non-immune cells, such as endothelial cells and fibroblasts, that belong to the pattern recognition receptors (PRRs) considered the initial sensing of infection (Takeuchi et al, Cell, 2010). Specifically, TLRs detect a wide range of ligands that once activated, the intracellular signaling cascades lead to transcriptional expression of inflammatory mediators such as cytokines, chemokines and type I interferons, which increase effector functions such as phagocytosis and capacity to present antigens to T cells. Therefore, TLR are crucial for innate immune response and also a requisite for the induction of

adaptive response (Krishnan et al, Exp Mol Med, 2007 and Behzadi et al, J Immunol Res, 2021).

2.2.1 TLR structure and types

TLRs are highly conserved through evolution. They are composed of 3 segments comprising an extracellular structure known as N-terminal ligand recognition domain that adopt a horseshoe-shaped structure composed by tandem short motifs of leucine-rich repeats (LRR). For the ligand binding, two extracellular domains form an m-shaped dimer sandwiching the ligand molecule (Bell et al, Proc Natl Acad Sci U S A, 2003). TLRs are also composed by a single transmembrane helix and finally, a C-terminal cytoplasmic domain known as Toll/interleukin (IL)-1 receptor (TIR) domain. This domain share homology with the signaling domains of IL-1 family and mediates the interaction between TLRs and TIR-containing adapters. The TIR domain is essential for cellular signaling (Botos et al, Structure, 2011).

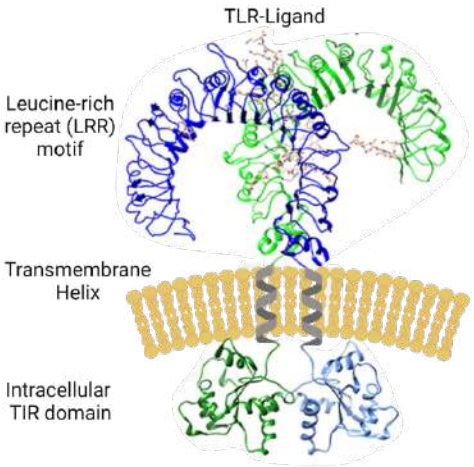


Figure 3. Representative structure of heterodimer TLR1-TLR2. The structural features of all TLRs consist of three critical components. 1. Leucine-rich repeat (LRR) motif; 2. Transmembrane helix; 3. Intracellular TIR domain. The LRR structure is based on the model of TLR1-TLR2 heterodimer interacting with their ligand, Pam3CSK4; whereas the TIR domain homology model is based on TLR2 TIR structure (Modified from Jin et al, Cell, 2007).

Up to date, 10 TLRs has been identified in humans, each of them having their specific molecular structures, characteristics and abilities involved in different diseases. TLRs can be subdivided by membrane TLRs (1, 2, 4, 5 and 6) and intracellular TLRs (3, 7, 8 and 9), which likely signal from acidic endosomes. These TLRs in endosomes recognize

basically nucleic acids from virus and bacteria, the subcellular location is very important for the discrimination of viral nucleic acid from self-nucleic acid (Barton et al, Nat Immunol, 2006). Human TLRs are listed in the following table with their properties and signaling pathway:

TLRs	Cell type	Location	TLR active form	Ligands	Adaptors
TLR1	• Monocytes[1] • DC[3] • B cells[2] • T cells[1]	Cell surface	Heterodimer TLR1-TLR2	Triacyl-lipoprotein, Pam_3CSK_4	MyD88 and MAL
TLR2	• Monocytes • Neutrophils[4] • DC • T Cells	Cell surface	Heterodimer TLR2-TLR1,6,10 Homodimer TLR2-TLR2	HSPs, HMGB1, LPS, glycoinositolphospholipids, glycolipids, lipoproteins, Zymosan, peptidoglycan, Pam_3CSK_4	MyD88 and MAL
TLR3	• Monocytes • DC • B cells • T cells	Endosomal membrane and ER	Homodimer TLR3-TLR3	Viral double-stranded (ds)RNA, Poly(I:C), Poly(A:U)	TRIF
TLR4	• Monocytes • Neutrophils • DC • T Cells • B cells	Cell surface and endosomal translocation	Homodimer TLR4-TLR4 Heterodimer TLR4-TLR6	LPS, HSPs, amyloid-β peptides, oxidized LDL, Hyaluronic acid, Fibronectin, Lipid A	MyD88 and MAL TRIF and TRAM
TLR5	• Monocytes • DC • B cells	Cell surface	Homodimer TLR5-TLR5	HMGB1, Flagellin	MyD88
TLR6	• Monocytes • B cells	Cell Surface	Heterodimer TLR6-TLR2,4	amyloid-β peptides, oxidized LDL, LTA, Zymosan, MALP2, Pam_2CSK_4	MyD88 and MAL
TLR7	• DC • B cells	Endosomal membrane and ER	Homodimer TLR7-TLR7	Viral Single-stranded (ss)RNA, Imidazoquinolinone, oligodeoxynucleotides (ODNs)	MyD88
TLR8	• Monocytes • Some DC	Endosomal membrane and ER	Homodimer TLR8-TLR8	Viral Single-stranded (ss) RNA, Imidazoquinolinone	MyD88
TLR9	• Monocytes • DC • B cells • T cells	Endosomal membrane and ER	Homodimer TLR9-TLR9	A, B and C classes of CpG-ODNs, Chromatin IgG immune complexes, mitochondrial DNA, DNA-RNA hybrids	MyD88
TLR10[5]	• Monocytes • B cells	Cell surface, Endosomal membrane and ER	Heterodimer TLR10-TLR1,2 Homodimer TLR10-TLR10	Diacyl-lipoprotein, triacyl lipoprotein, viral glycoproteins, dsRNA	MyD88

Table 1. Summary of human TLR properties: location and pathway of activation. DC, Dendritic cells; Pam3CSK4, synthetic triacylated lipopeptide; MyD88, Myeloid differentiation primary response 88; MAL, MyD88-adaptor-like; HSP, Heat shock proteins; HMGB1, High-mobility group box 1; LPS, Lipopolysaccharide; ER, Endoplasmic reticulum; TRIF, TIR-domain-containing adaptor protein inducing INFβ; LDL, low-density lipoproteins; TRAM, TRIF-related adaptor molecule; LTA, lipoteichoic acid; MALP2, macrophage-activating lipopeptide 2. (Table modified from Behzadi et al, J Immunol Res, 2021 and Krishnan et al, Exp Mol Med, 2007 and complemented with [1]Dasari et al, Pediatr Allergy Immunol, 2011; [2]Buchta et al, Immunol Res, 2014; [3]Kadowaki et al, J Exp Med, 2001; [4]Sabroe et al, Clin Infect Dis, 2005; [5]Chuang et al, Biochim Biophys Acta, 2001)

2.2.2 TLR signaling

TLRs are activated by different molecules named pathogen-associated molecular patterns (PAMPs), which are bacterial components that can activate TLRs, such as LPS peptidoglycan or bacterial DNA. Moreover, TLRs recognize not only PAMPs, but also host molecules released by dying cells called danger-associated molecular patterns (DAMPs), such as adenosine triphosphate (ATP), formyl peptides or mitochondrial DNA (Bianchi et al, J Leukoc Biol, 2007). TLR signaling is initiated with the dimerization of two TLRs, which could be heterodimer complexes (such as TLR1 and 2) or homodimer complexes with two TLRs of the same kind (such as TLR4 or TLR9). TLR dimers are pre-assembled before ligand binding however once the ligand binds, a conformational change is produced and brings the two TIR domains of each receptor closer (Ozinsky et al, Proc Natl Acad Sci U S A, 2000). These domains are the key for the initiation of the specific signaling cascade because they are located in the TLRs and also in the adaptor molecules and the TIR domains together create a platform necessary for adaptor recruitment. The main protein adaptors are four: myeloid differentiation primary response 88 (MyD88), MyD88-adaptor-like (MAL, also known as TIRAP), TIR-domain-containing adaptor protein inducing INFβ (TRIF) and TRIF-related adaptor molecule (TRAM) (O'Neill and Bowie, Nat Rev Immunol, 2007). MyD88 is the universal adapter protein recruited by all TLRs except TLR3, for this reason the signaling pathways of TLRs are divided in MyD88-dependent and independent. Briefly, MyD88-dependent signaling pathway goes through the activation of NF-κB or MAPK that triggers the activation of the transcription factors NF-κB or activator protein 1 (AP-1), respectively, which together govern the production of large amount of pro-inflammatory cytokines and chemokines (Medzhitov et al, Mol Cell, 1998). On the other hand, mostly in endosomal signaling, MyD88 activates the protein Interferon regulatory factor 7 (IRF7) that activates type I

interferon (INF) genes and the production of IFN-alpha (Kawai et al, Nat Immunol. 2004). Focusing now on MyD88-independent signaling, it is controlled by TRIF or TRIF/TRAM which is only activated by TLR3 and TLR4. TRIF regulates three different signaling pathways, NF-κB activation with the production of pro-inflammatory cytokines; IRF3 activation with the production of type I IFN genes and INF beta; and apoptosis via caspase-8 (Yamamoto et al, Science, 2003 and Krishnan et al, Exp Mol Med, 2007). The different signaling pathways for TLRs are represented in Figure 4.

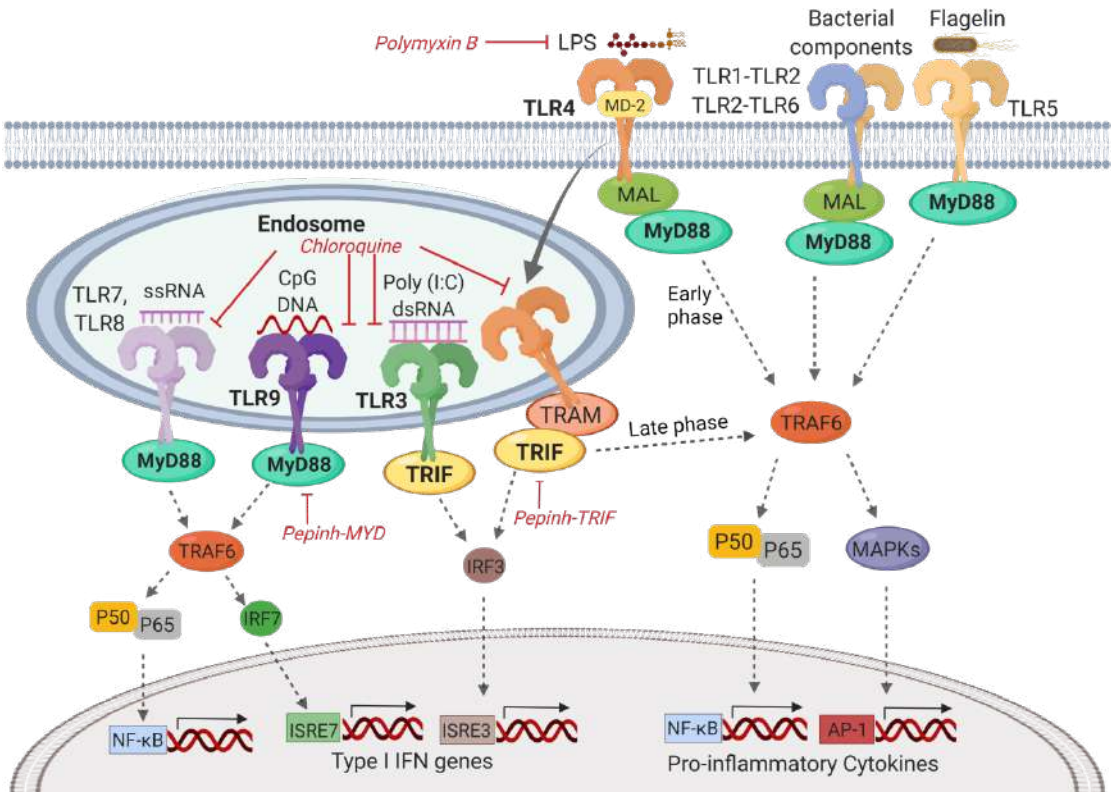


Figure 4. Schematic diagram of TLRs signaling. TLRs are represented in their locations and with their most representative ligand bound to them. Each TLR has their specific adaptor protein such as, MyD88, MAL, TRIF and TRAM that activate a particular signaling cascade and finally induce the release of pro-inflammatory cytokines or type I interferon genes. In red some inhibitors of TLRs are represented. Polymixin B: inhibitor of LPS-binding to TLR4; Chloroquine: inhibitor of endosomal acidification/endosomal TLR signaling; Pepinh-MyD: peptide inhibitor of MyD88 homodimerization; Pepinh-TRIF: interfering peptide of the TLR-TRIF interaction. Created with BioRender.com

The specific signaling for TLR4, TLR9 and TLR3 are detailed in the following paragraphs.

- TLR4 signaling:

TLR4 is known as the first TLR identified in mammals (Ulevitch et al, Nature, 1999), has specific characteristics and a wide range of functions considered as critical TLR among others. TLR4 could bind a wide range of ligands, including PAMPs and DAMPs however gram-negative bacterial LPS is their main activator. Moreover, it could be found in the cell membrane or within the cell endosomes and each location activates a different signaling pathway that will trigger a specific response (Behzadi et al, J Immunol Res, 2021). The active form of TLR4 is forming homodimers, with the help of Myeloid differentiation factor-2 (MD-2) co-receptor molecule. MD-2 is a glycoprotein that interacts with TLR4 and these adopt a new configuration building an internal pocket. This new configuration provides a complex with TLR4 homodimer and two co-receptors of MD-2 that will bind to LPS or other ligands. Therefore, MD-2 has a vital role in the homodimerization of TLR4 and ligand attachment (Shimazu et al, J Exp Med, 1999).

Once the complex is formed with LPS, the activation signal occurs in two waves (Covert et al, Science, 2005). The first wave is upon the cell membrane activating MyD88-dependent pathway. In this particular case, MyD88 does not bind the TIR domain of TLR4 directly due to both TIR domains are electro-positive, however, TIR domain of TLR4 could interact with MAL adaptor which its TIR domain is electro-negative (Dunne et al, J Biol Chem, 2003). Then MAL adaptor recruits MyD88 activating the signaling pathway. As explained before, MyD88 activation results in the expression of NF-κB and the production of inflammatory cytokines as the final product. The second delayed wave of activation is mediated through endosomal compartments and TRIF-dependent signaling pathway is recruited. Again, TIR domain of TLR4 does not recognize TRIF directly and TRAM adaptor serves as a link between TIR domains of TLR4 and TRIF (Yamamoto et al, Nat Immunol 2003). Subsequently, the signaling pathway is activated leading to the expression of IRF3 that results in the expression of type 1 IFN genes and IFN-beta production and the expression of late phase NF-κB with the production of inflammatory cytokines.

- Endosomal TLR9 and TLR3 signaling:

Endosomal TLRs are specific for the recognition of microbial nucleic acids. Viruses and bacteria typically enter cells through endocytic or phagocytic pathways which can lead to their degradation and release of nucleic acids. Endosomal TLRs recognize specific nucleic acid structures, such as, TLR3 that recognizes double-strand RNA (dsRNA) for example Poly (I:C) (Alexopoulou et al, Nature, 2001) or TLR9 that recognizes bacterial

DNA (Hemmi et al, Nature, 2000). The most known ligand for TLR9 is bacterial DNA rich in unmethylated cytosine-phosphate-guanine motif (CpG).

Numerous regulatory mechanisms exist to ensure endosomal TLR-activation only with microbial nucleic acids. The principal mechanism is the location. Nucleic acid sensing TLRs are within endosomes, which is useful for two reasons. Firstly, for limiting immune responses to self-nucleic acids circulating in the bloodstream. Secondly, because nucleic acids released from engulfed microbes within particles are protected from nucleases, found in the extracellular environment, and can activate TLRs signaling (Bettina et al, Trends Cell Biol, 2014). Endosomal location is also necessary for the function and activation of these receptors. For example, neutralizing the pH of endosomes with drugs such as chloroquine inhibits the activation of nucleic acid sensing TLRs (Rutz et al, Eur J Immunol, 2004).

Unlike cell membrane TLRs, once single-strand RNA (ssRNA) or bacterial DNA interact with TLR3 and TLR9 homodimers, respectively, the ectodomain of these receptors are proteolytically processed by endosomal proteases such as Cathepsins (Ewald et al, J Exp Med, 2011). This cleavage separates a part of the ectodomain from the rest of the TLR, resulting in truncated TLR that becomes functional. Surprisingly, proteolytic processing is not required for ligand binding, but it is necessary to initiate downstream signals. Cleavage of TLR9 or TLR3 facilitate a conformational change that reorient the cytosolic TIR domains and promote MyD88 or TRIF recruitment (Ewald et al, Nature, 2008).

Focusing on TLR9 signaling, once MyD88 is recruited, two signaling pathways are activated: NF-κB that triggers the production of proinflammatory cytokines and IRF7 that results in the antiviral gene expression of type 1 IFN and the release of IFN-alpha (Kawai et al, Nat Immunol, 2004). These two different pathways are likely depending of the ligand activating TLR9 and the cell type. For example, different classes of synthetic CpG oligonucleotides are likely to activate more pro-inflammatory cytokines or IFN I genes (Vollmer et al, Eur J Immunol, 2004).

TLR3 is the only TLR that not use MyD88 as adaptor. After homodimerization and cleavage, TRIF adaptor is recruited and activates IRF3 pathway which lead to the expression of type I IFN genes and IFN-beta release. Moreover, TLR3 activates a late phase of NF-κB with the production of pro-inflammatory cytokines (Alexopoulou et al, Nature, 2001).

2.3 CYTOKINES AND CHEMOKINES

Cytokines are key modulators of the innate immune response and inflammation, participating in acute and chronic inflammation via a complex network of interactions. They are low molecular weight proteins that upon damage or stress are released by immune cells to trigger an inflammatory cascade (Turner et al, Biochim Biophys Acta, 2014).

Many cytokines have multiple and sometimes unrelated functions that depend on the target cell or on the presence or absence of other cytokines. Among the many functions of cytokine there are the control of cell proliferation and differentiation, the regulation of angiogenesis and immune and inflammatory responses, although immunomodulatory functions are their major feature. Cytokines are known to be primarily responsible for systemic inflammation, not only promoting a proinflammatory environment but also amplifying the inflammatory process through a positive feedback process (Tisoncik et al, Microbiol Mol Biol Rev, 2012).

It is possible to classify cytokines in 5 different groups represented in Table 2.

Type	Actions	Example
Interleukins	Growth and differentiation of leukocytes, the majority are pro-inflammatory.	IL-1β, IL-6
Interferons	Regulation of innate immunity, activation of antiviral properties and anti-proliferative effects.	IFN-β, IFN-2α,
Tumor necrosis factor	Proinflammatory, activates cytotoxic T lymphocytes	TNFα
Chemokines	Control of Chemotaxis, leukocyte recruitment. Many are proinflammatory.	CXCL10, MIP1-α (CCL3)
Colony-stimulating factors	Stimulation of hematopoietic progenitor, cell proliferation and differentiation.	G-CSF, GM-CSF

Table 2. Major actions and representative examples of the 5 groups of cytokines. (Table modified from Tisoncik et al, Microbiol Mol Biol Rev, 2012)

2.3.1 Chemokines

Chemokines are a large family of small secreted proteins. Chemotaxis is the cardinal feature of chemokines; however, their physiological role is more complex, with many additional homeostatic functions in hematopoiesis and initiation of adaptive immune response (Hughes et al, FEBS J, 2018). The majority of chemokines are considered pro-inflammatory and they are released by a variety of immune cells in response to infection or injury. Chemokine recruitment of immune cells can be highly selective for specific cell types. For example, CXCL8 (IL-8) is a chemoattractant factor for neutrophils or CCL2 (Monocyte chemoattractant protein-1, MCP-1) for monocytes (Tisoncik et al, Microbiol Mol Biol Rev, 2012).

Chemokines can be split in four groups depending of the configuration of two cysteine residues: CXC, CC, (the majority of chemokines are contained in these two subfamilies), C subfamily and CX3C that in humans only has one member (CXC3L1 or fractalkine) (Zlotnik et al, Immunity, 2000). Chemokine signals are transduced through binding to members of the seven-transmembrane G protein-coupled receptor (GPCR) superfamily (Hughes et al, FEBS J, 2018). In addition, there are decoy receptors for chemokines that are not coupled to G proteins and cannot signal, such as, Duffy antigen receptor for chemokines (DARC). They play a role in reducing the immune response, leading the resolution of inflammation. Although, these receptors can also mediate transcytosis, enchasing leukocyte migration across monolayers (Pruenster et al, Nat Immunol, 2009).

2.3.2 Pro-inflammatory cytokines

IL-1β, IL-6 and TNFα are key pro-inflammatory cytokines in the immune response. Their functions and signaling pathways are discussed below.

- IL-1β:

IL-1β is one of 11 members of the IL-1 family and acts as a crucial regulator of inflammation and host responses to infection. IL-1β is the most well-studied member, considered as a potent pro-inflammatory cytokine that was originally identified as a pyrogen. Nowadays, more functions related to this cytokine are known, such as, the increase of acute-phase signaling, trafficking of immune cells to the site of infection,

epithelial cell activation and secondary cytokine production (Dinarello, Annu Rev Immunol, 2009).

It is synthesized in multiply cell types including monocytes, macrophages and neutrophils and it has been found that promote T cells responses (Santarlasci et al, Front Immunol, 2013), having a stimulatory effect on differentiation into T helper and in addition, increasing their expansion and survival. The increase in T cell number enhances antibody production by B cells (Nakae et al, J Immunol, 2001). IL-1β serves as a mechanism to amplify the danger message to cell populations that cannot recognize microbial products directly. So, this cytokine is a crucial link in translating innate immune responses to adaptive responses (Sims et al, Nat Rev Immunol, 2010).

IL-1β expression is induced mainly in response to microbial molecules detected by the TLRs in the immune cells, although it can also stimulate its own expression (Dinarello, Annu Rev Immunol, 2009). This cytokine is produced as an inactive pro form by myeloid cells in response to activation of membrane TLR by PAMPs and remain as cell-associated until a second stimuli, which is typically a DAMP, triggers their processing and secretion. This is performed in a specialized intracellular complex named Inflammasome, which is a large intracellular protein complex that comprise a cytosolic PRR that sense injured cells and require two signals for its activation (Broz et al, Nat Rev Immunol, 2016). The best characterized Inflammasome is the NLRP3 Inflammasome that once activated, recruits the adaptor molecule apoptosis-associated speck-like protein containing a caspase recruitment domain (ASK) to form a complex. The ASK molecule forms a large prion-like filamentous structures known as specks that recruit pro-caspase-1 and activate the enzyme caspase-1 which cleaves inactive pro- IL-1β to a mature form that is secreted by the cell (Daniels et al, Int J Mol Sci, 2017).

IL-1β has affinity for two IL-1 receptors, IL-R1 and IL-1R2 however is necessary a heterodimer complex between IL-1R and IL-1R accessory protein (IL-1RAcP) to be functional (O'Neill et al, Immunol Rev, 2008). By this mechanism, following ligand binding, the adaptor molecule MyD88 leads to activation of both MAPKs and NF-kB resulting in pro-inflammatory cytokine expression that amply the inflammatory response. On the other hand, IL-1R2 could have an inhibitory role, act as a decoy receptor sequestering IL-1β as an anti-inflammatory mechanism (Dinarello, Annu Rev Immunol, 2009).

- IL-6:

IL-6 is a pleiotropic cytokine that exerts multiple functions in the body. It was characterized by its ability to promote expansion and activation of T cells, differentiation of B cells and regulating the secretion of acute phase proteins by the liver, which it does in cooperation with IL-1. Physiological concentrations of IL-6 in human serum are relatively low (1-5 pg/ml), but are rapidly elevated in inflammatory conditions (Hunter et al, Nature Immunol, 2015).

IL-6 is expressed by an array of cells including fibroblasts, monocytes, macrophages, T cells, B cells and hepatocytes. IL-6 synthesis and secretion is induced during inflammatory conditions after stimulation of TLRs by PAMPs or stimulation by IL-1 or TNFα that triggers to a massive release of IL-6 and other cytokines (Schmidt-Arras et al, J Hepatol, 2016). IL-6 signals through a ligand-binding IL-6 receptor (IL-6R) and the signal transducing component gp130 (Skiniotis et al, Nat Struct Mol Biol, 2005). Functional IL-6R requires the formation of IL6-IL6R-gp130 complex that is clustered in a dimer structure. Following IL-6 binding, the signal is transduced to gp130 chains to activate JAK-STAT signaling and result in STAT3 nuclear translocation and expression of IL-6 gene. However, this signaling is very controlled and inhibitors of STATs are suppressors of cytokine signaling (Heinrich et al, Biochem J, 2003).

IL-6 uses two mechanisms to mediate its biological effect, due to IL-6R is restricted to lymphocytes and hepatocytes, only IL-6R expressing cells can directly respond to IL-6. The “Classical” mechanism is defined by when IL-6 signaling is performed through membrane-bound IL-6R and occurs only in cells that express both receptor subunits (Hunter et al, Nature Immunol, 2015). In contrast, the “trans-signaling” mechanism is defined by binding of secreted IL-6 by a soluble form of IL-6R (sIL-6R) to form a complex that increase circulating half-life of IL-6 and any cell type that express pg130 could have a response to this cytokine (Peters et al, J Exp Med, 1996). sIL-6R is considered as a alarmin that promotes a danger response to disease that affects innate and adaptive immunological outcomes (Jones et al, J Immunol, 2005).

- TNFa:

TNFα is a potent pro-inflammatory cytokine central in the inflammatory response of innate immune system, including induction of cytokine production, activation of adhesion molecules and causes inflammatory, antiviral and immunoregulatory effects (Turner et al, Biochim Biophys Acta, 2014). TNFα is a member of the TNF superfamily that includes

30 receptors and 19 associated ligands. It is secreted mainly by activated macrophages, although it may also be secreted by monocytes, T cells, NKT cells, fibroblasts and neurons (Aggerwal et al, Nat Rev Immunol, 2003).

It is known that TNFα is produced in two forms. Initially as a transmembrane protein (mTNFα) which is transported to the cell surface, where it is cleavage by TNFα-converting enzyme (TRACE) and the result is the soluble TNFα (sTNFα) that constitute a potent ligand which activate TNF receptors (Kalliolias et al, Nat Rev Rheumatol, 2016).

Molecular actions of TNFα typically occur through binding to one of two receptors: TNFR1 and TNFR2, which have distinct biological effects. TNFR1 is widely expressed in different cell types, it is expressed by both soluble and transmembrane TNFα. This receptor has an intracellular death domain (DD) that is able to drive either apoptosis or production of pro-inflammatory cytokines through interaction with associated adaptor molecules. TNFR1 mainly promotes inflammation and tissue degeneration (Kalliolias et al, Nat Rev Rheumatol, 2016). On the other hand, TNFR2 is expressed predominantly on leukocytes and endothelial cells and it is activated primarily by mTNFα. TNFR2 lacks DD so it is unable to induce apoptosis, its signaling is activated by a motif that activates NF-kB expression. This receptor mediates cell survival and tissue regeneration (Grell et al, Cell, 1995). Interestingly, the binding of TNFα to these receptors causes the release of a soluble form of the extracellular part of the receptor that could have biological role, such as, limiting cytokine availability to other cells (Müllberg et al, Eur Cytokine Netw, 2000).

2.4 LIPID MEDIATORS

Lipid mediators are potent signaling molecules that regulate a multitude of cellular responses including inflammation and immune response. They are generated from polyunsaturated fatty acids (PUFAs) that serve as precursors for their conversion through specific enzymatic pathways, predominantly expressed in immune cells (Dennis et al, Nat Rev Immunol, 2015 and Quehenberger et al, N Engl J Med, 2011). The PUFAs are composed of two distinct families: the omega-6 and the omega-3, both located in the phospholipid membrane and released by the activation of phospholipase A_2 (PLA_2) (Dennis et al, Chem Rev, 2011). Arachidonic acid (AA), the most representative omega-6-PUFA, is converted to prostaglandins (PGs) through the cyclooxygenase (COX) enzymatic pathway or into leukotrienes (LTs) through the lipoxygenase (LOX) pathway.

Both, PGs and LTs are potent pro-inflammatory and immunosuppressive lipid mediators (Funk et al, Science, 2001). Alternatively, AA give rise to lipoxins (LXs), considered pro-resolutive, through interaction of COX and LOX pathways (Serhan et al, Nature, 2014). On the other hand, the omega-3-PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are converted by the same enzymatic pathways into an array of anti-inflammatory lipid mediators involved in the resolution of inflammation, known as specialized pro-resolving mediators (SPMs) which comprises resolvins protectins, and maresins (Serhan et al, Nature, 2014). The biosynthetic pathways generating lipid mediators are represented in Figure 5.

An equilibrium between omega-6 and omega-3 derived lipid mediators is crucial for the regulation of inflammatory processes and maintenance of tissue homeostasis. Consequently, an altered lipid composition can lead to immune-metabolic dysregulation and uncontrolled inflammation (López-Vicario et al, J Hepatol, 2020).

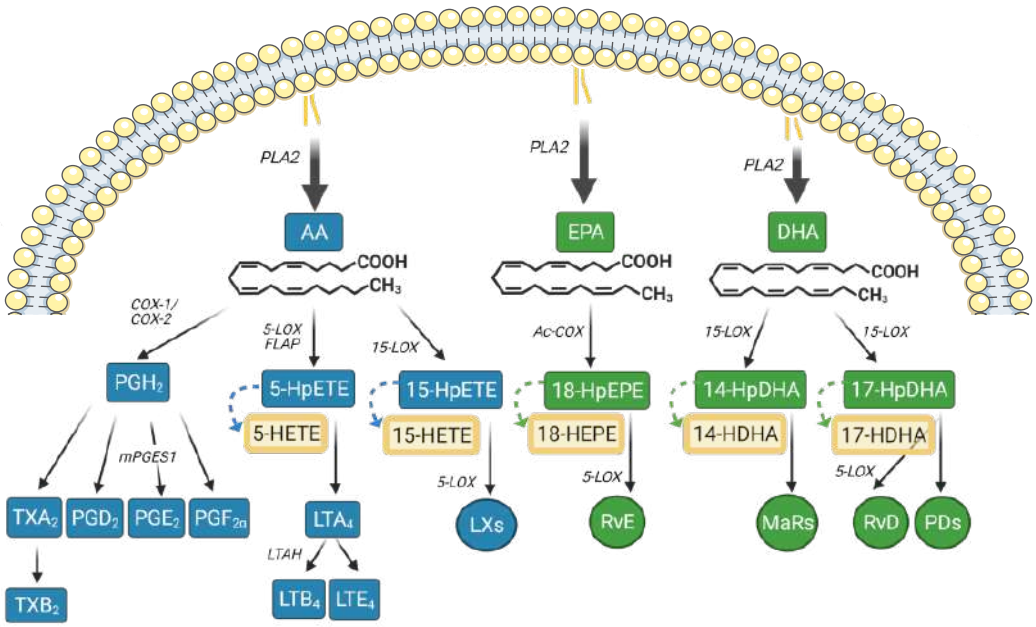


Figure 5. Lipid mediator biosynthetic pathways. Omega-6 PUFA AA biosynthesis pathway is represented in blue and Omega-3 PUFA (EPA and DHA) pathways are represented in green. Then, from each precursor, following the arrows, bioactive lipid mediators are represented in order of release with the specific enzymes involved in the conversion of each lipid. Specialized pro-resolving mediators (SPMs) families are represented with a circle. Created with BioRender.com

2.4.1 Omega-6-derived lipid mediators

AA is the main precursor of omega-6 lipid mediators. AA can be metabolized via three principal pathways, orchestrated by the enzymes COX, LOX or cytochrome P450, which generate an important family of products called eicosanoids (Bennett et al, Microbiol Spectr. 2016).

- Prostanoids

The COX pathway gives rise to PGs and thromboxane (collectively term as Prostanoids). There are two COX isoforms involved in the metabolism of AA: COX-1, which is constitutively expressed in most tissues and cells, and COX-2, which is rapidly induced when cells are challenged with inflammatory stimuli (Dubois et al, FASEB J, 1998). COX-1 is involved in cellular homeostatic functions necessary for normal physiological activity while COX-2 acts at sites of inflammation, is induced by inflammatory mediators such as IL-1, TNFα or LPS, and participates in the inflammatory response (Clària et al, Curr Pharm Des, 2003).

COX begins catalyzing AA to form PGG_2, which is subsequently reduced by COX to form PGH_2. PGH_2 is the common precursor of bioactive prostanoids which are generated through the action of specific synthases. The COX pathway is shown in Figure 5. (Bennett et al, Microbiol Spectr. 2016). The differential expression of these synthase in cells determines the profile of prostanoids production. For example, from PGH_2, PGD synthase produce PGD_2 in brain and mast cells, also PGH_2 could be converted to PGF2α by PGF synthase which is mainly expressed in the uterus or platelets release TXA_2 produced by TX synthase (Clària et al, Curr Pharm Des, 2003). PGE_2 is predominantly produce in macrophages but is also formed in many cell types by the action of PGE synthase that have three isoforms cPGES, mPGES-1 and mPGES-2. The first two isoforms are constitutively expressed, whereas mPGES-1 is an inducible isoform linked with COX-2, which plays a critical role in the final steps of PGE2 production without altering the levels of other PGs (Maione et al, Pharmacol Res, 2020). The biological effects of prostanoids is initiated by binding to specific cell surface receptors. There are 9 types of prostanoid receptors that belong to the GPCR superfamily (Funk et al, Science, 2001). Prostanoids modulate a wide range of biological processes related with inflammation, such as vascular permeability, vasodilation, hyperalgesia, fever and platelet aggregation (Bennett et al, Microbiol Spectr. 2016). As well as these pro-inflammatory properties, many prostanoids also exert immunosuppressive effects. For example, PGE2 reduces the ability of leukocytes to phagocytose and kill microorganisms as well as inhibits the production of inflammatory mediators (Aronoff et al, J Immunol,

2004). The clinical importance of prostanoids is emphasized by the fact that COX is the target of nonsteroidal anti-inflammatory drugs (NSAIDs), one of the most used agents to treat chronic inflammation (Clària et al, Curr Pharm Des, 2003).

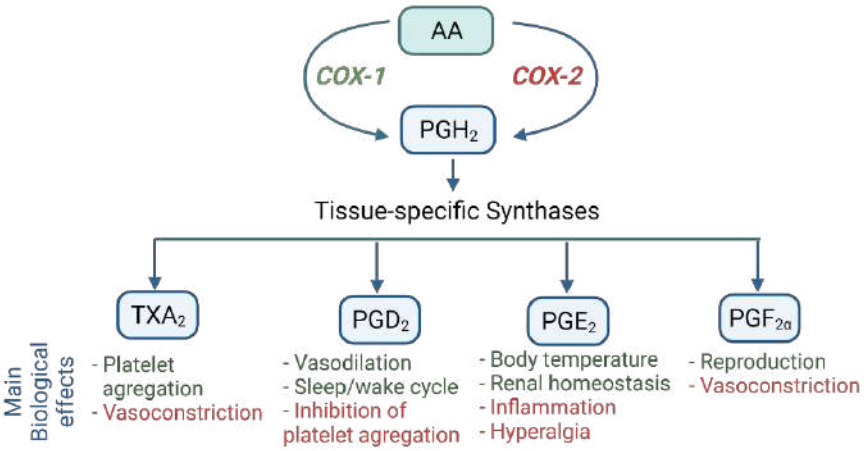


Figure 6. COX-1 and COX-2 effects on prostanoids. Biosynthesis and main biological effects of prostanoid mediators formed by COX-1 or COX-2 enzymes. Modified from Tomic et al, Academic Press, 2017 and Created with BioRender.com

- Leukotrienes and lipoxins

Leukotrienes and lipoxins are the most prominent eicosanoids generated through the LOX pathway. Specifically, 5-LOX converts AA into a 5-hydroperoxyeicosatetranoic acid hydroperoxide (5-HpETE), which is then converted to LTA_4 by 5-LOX aided by the 5-LOX activating protein (FLAP). FLAP is a small resident integral protein in the nucleus membrane that acts as an AA transfer protein and facilitates its presentation to 5-LOX. Subsequently LTA_4 is converted to LTB_4 or in LTC_4, LTD_4 and LTE_4 (Funk et al, Science, 2001) Alternatively, 5-HpETE can be reduced to 5-hydroxyeicosatetranoic acid (5-HETE). The LOX pathway is shown in Figure 5.

LTs are generated in sites of infection and inflammation primarily by inflammatory cells, including PMNs and macrophages. They play a critical role in the inflammatory response and each of them has a distinct role in driving different phases of inflammation. For example, LTB_4 attracts and activate innate immune cells in the site of inflammation (Ott et al, Nat Immunol, 2003). Four subtypes of receptors located in the membrane of inflammatory cells, all of which are involved in both acute and chronic inflammation with specific functions (Sasaki et al, Int Immunol, 2019). Despite their pro-inflammatory role, LTs are important players in the host response against pathogens, since LT deficiency

is a feature of various clinical conditions that are associated with impaired microbial clearance (Peters-Golden et al, J Immunol, 2005).

Another LOX enzyme, the 15-LOX, converts AA to a set of anti-inflammatory and pro-resolutive lipid mediators called lipoxins. Despite LX are produced from AA, they are considered pro-resolving mediators or SPM (Serhan et al, Nature, 2014). This can be achieved by two major routes. In the first pathway, AA is converted by 15-LOX into 15-HpETE which is taken up by PMNs or monocytes and is rapidly converted to bioactive LXA_2 or LXB_2 (Serhan et al, Biochim Biophys Acta, 1989). The second major route occurs in LTA_4-dependent manner. Leukocyte 5-LOX converts AA to LTA_4 which is taken up by platelets and is transformed to LXA_2 or LXB_2 via the LX synthase activity of human 12-LOX (Romano et al, Biochemistry, 1992). Moreover, exist a third route of LX production which occurs after exogenous administration of aspirin. This one irreversibly acetylates COX-2 that cause the transformation of AA into 15R-HETE and is rapidly metabolized in a transcellular manner by 5-LOX to form aspirin-triggered LXs (ATLs) that carry the R-configuration rather than 15S native LX. ATLs share many of the anti-inflammatory and pro-resolutive characteristics of LX (Clària et al, Proc Natl Acad Sci U S A. 1995).

LX control both, granulocytes and monocytes in the sites of inflammation. The major role of LXs is promote the resolution of inflammation with actions such as, Inhibiting the transmigration of neutrophils, promoting no inflammatory infiltration of monocytes required for the resolution of inflammation, stimulate macrophages to ingest and clear apoptotic neutrophils, elevate the levels of anti-inflammatory cytokines and improve tissue remodeling (Bennett et al, Microbiol Spectr. 2016).

2.4.1 Omega-3-derived lipid mediators

Omega-3 PUFA are known to be important for maintaining organ function and also for reducing the incidence of infection and inflammation. An array of compounds derived from EPA and DHA are responsible for these specific and potent anti-inflammatory and pro-resolving actions (Serhan et al, Nature, 2014, López-Vicario et al, Eur J Pharm, 2016).

- Resolvins (Rv)

Rvs can be generated from EPA or DHA and are therefore categorized as E-series or D-series. Biosynthesis of E-series Rv occur with the conversion of EPA to 18-hydroxyeicosapentanoic acid (18-HEPE), then can be released from endothelial cells to

leukocytes for the conversion by 5-LOX to RvE1 (Serhan et al, J Exp Med, 2000). In addition, reduction of 5-LOX via a peroxidase leads to the formation of RvE2 (Tjonahen et al, Chem Biol, 2006) and through 12/15-LOX pathway, RvE3 is produced (Isobe et al, J Biol Chem, 2012). All SPMs evoke specific bioreactions mediated in the nano molar range by their binding to specific GPCRs. ChemR23 is the GPCR that signals for RvE1 (Arita et al, Biochem Biophys Res Commun, 2005). In addition, a polymorphism in ChemR23 gene is related with an increased ERV1/ChemR23 protein expression and reduced levels of the inflammatory cytokine IL-6 (López-Vicario et al, Sci Rep. 2017). RvE1 is also an endogenous receptor antagonist for leukotriene B4 receptor, BLT_1, which explains its ability to regulate PMN trafficking to sites of inflammation (Arita et al, J Immunol, 2007). RvE1 has a wide range of biological functions involved in resolution of inflammation such as, decrease PMN infiltration and T-cell migration, reduce TNFα and INFγ secretion, inhibit chemokine formation, block IL-1-induced NF-kB activation, stimulate macrophages phagocytosis of apoptotic PMN and is a potent counter-regulator of L-selectin expression (Bannenberg et al, Biochim Biophys Acta, 2010; Schwab et al, Nature, 2007 and Dona et al, Blood, 2008).

On the other hand, from DHA are produced the D-Series Rvs. The biosynthesis of D-series Rvs is initiated by 15-LOX which transforms DHA into 17-hydroxydocosahexaenoic acid (17-HDHA) that is converted by 5-LOX to this pro-resolving family of 6 members, RvD1, RvD2, RvD3, RvD4, RvD5 and RvD6 (Serhan et al, Nature, 2014). Endothelial cells expression COX-2 acetylated by aspirin also transform DHA into 17R-HDHA which give rise to aspirin-triggered (AT)-resolvins (Serhan et al, Chem Rev, 2011). Like E-series Rv, D-series Rv exert anti-inflammatory properties through binding to their GPCR receptors. Two receptors (ALX/FPR2) transmit RvD1 signals (Krishnamoorthy et al, Proc Natl Acad Sci U S A, 2010). Of note, GPR32 is the receptor for RvD3 and RvD5 (Dalli et al, Chem Biol, 2013). Similar to RvE1, RvD1 and RvD2 reduce inflammatory pain, block IL-1β release in microglial cells, are potent regulators limiting PMN infiltration into inflamed brain, skin and peritoneum (Sun et al, J Biol Chem, 2007 Hong et al, J Biol Chem, 2003) and reduce cytokine levels while enhancing bacterial clearance (Spite et al, Nature, 2009). Recently, it has been reported that RvD2 inhibits the priming and the deactivation of the NLRP3 Inflammasome in macrophages during the resolution process (Lopategi et al, J Leukoc Biol. 2019).

- Protectins (PD)

DHA also serves as a precursor for the biosynthesis of PDs that are enzymatically converted by 15-LOX from 17-HpDHA. One specific compound is called protectin D1

(PD1) and has tissue-specific bioactivity. This SPM is also known as neuroprotectin (NPD1) when is formed in neural tissues (Bazan et al, J Lipid Res, 2009). PD1 is synthetized in microglial, monocytes and T cells (Serhan et al, Chem Rev, 2011). PD1 displays specific binding to PMN and human epithelial cells in which no other SPM competes for its specific binding, indicating that PD1 actions are likely mediated by separate receptors (Serhan et al, Curr Opin Pharmacol, 2013). PD1 exerts potent immunomodulatory effects that include neutrophil migration and toll-like receptor mediated activation, suppression of inflammatory cytokines and lipid mediators and blockage of T cells migration and promotes their apoptosis (Serhan et al, Chem Rev, 2011 and Ariel et al, J Biol Chem, 2005).

- Maresins (MaR)

MaRs were identified in macrophages as mediators that possess potent anti-inflammatory and pro-resolving properties. MaR are generated by the conversion of DHA into 14-hydroperoxydocosahexanoic acid (14-HpDHA) via 12-LOX followed by reduction to 14-HDHA to generate MaR1 and MaR2 (Serhan et al, J Exp Med, 2009). MaR1 is an activator for human leucine-rich repeat containing G protein-coupled receptor 6LGR6), expressed in phagocytes (Chiang et al, J Clin Invest, 2019). Their main functions are very similar to Rvs and PDs, for example, MaR1 inhibits PMN infiltration in vivo, and stimulates macrophage phagocytosis and efferocytosis (Serhan et al, J Exp Med, 2009). In addition, MaR1 exerts potent tissue regenerative actions (Serhan et al, FASEB J, 2012), such as, prevents lipotoxic-triggered endoplasmic reticulum stress and hypoxia-induced inflammation in liver tissue and enhance Kupffer cells phagocytic capacity (Rius et al, FASEB J, 2017).

3. LIVER CIRRHOSIS

Cirrhosis is a neologism derived from Greek word "kirrhos" that mean yellowish, tawny referring to the orange-yellow color of the liver in this pathology. The suffix -osis, means condition. Although the disease was known before, on 1819 the French physician René-Théophile Laennec gave it this name (Roguin et al, Clin Med Res, 2006).

3.1 DEFINITION AND EPIDEMIOLOGY

Liver cirrhosis is a late-stage liver disease in which healthy liver parenchyma is replaced by scar tissue, thereby the liver is permanently damaged and cannot perform the homeostatic functions of this organ.

The most frequent causes that lead to cirrhosis are alcohol, hepatitis B and C or fat accumulation in the liver (non-alcoholic fatty liver disease). These conditions damage the liver causing cell death and inflammation that the liver itself tries to repair causing the formation of scar tissue. As cirrhosis progresses over months and years, more scar tissue is formed, blocking the flow of blood through the liver, increasing portal hypertension and slowing its ability to process nutrients, hormones, drugs and toxins. It also reduces the production of proteins such as albumin, coagulation factors, lipoproteins and other molecules exclusively synthesized by the liver. For this reason, the late-stages of cirrhosis are life-threatening and associate with high mortality.

In its initial stages, there are often no symptoms, but as it progresses, symptoms such as tiredness, swelling in the legs, yellow skin, spider-like blood vessels and fluid accumulation in the abdomen start to occur. Finally, in the most severe cases, liver cirrhosis may lead to hepatic encephalopathy or liver cancer. Though liver damage is irreversible, treatment can slow down the progression of the disease and reduce complications (Bowman, Foster Academics, 2019).

Cirrhosis and other chronic liver diseases are a major cause of morbidity and mortality globally. On 2017, there were registered 132.5 cases of cirrhosis per 100.000 population globally and it caused more than 1.32 million deaths, Affecting more men than women in both prevalence and death. Cirrhosis is the 11th most common cause of death globally, which the most commune cause is Hepatitis (57%) followed by alcohol related liver disease (27.3%) (GBD 2017 Cirrhosis Collaborators, Lancet Gastroenterol Hepatol, 2020). In Europe, liver cirrhosis is responsible for 170.000 deaths per year, with large inter-country variation, in which alcohol related liver disease is the strongest factor. That

means more than 5.500 liver transplants performed in Europe per year (Blachier et al, J Hepatol, 2013).

3.2 CLINICAL PROGRESION

The clinical course of cirrhosis cannot be considered unidirectional and is determined by the increase of multiple conditions related with the disease such as portal hypertension, hyperdynamic circulation, bacterial translocation and activation of systemic inflammation. Cirrhosis is typically classified as compensated or decompensated, based on the absence or presence of variceal bleeding, ascites, jaundice or encephalopathy. Although theses disease states do not follow a predictable sequence, clinical states enable the classification of patients according to increasing mortality risk. In Addition, another state of the disease is Acute-on-chronic liver failure or ACLF which may occur either in decompensated and compensated cirrhosis and is always associated with high short-term mortality (D'Amico et al, J Hepatol, 2018). In Figure 7 several clinical conditions associated to the clinical progression of the disease are represented.

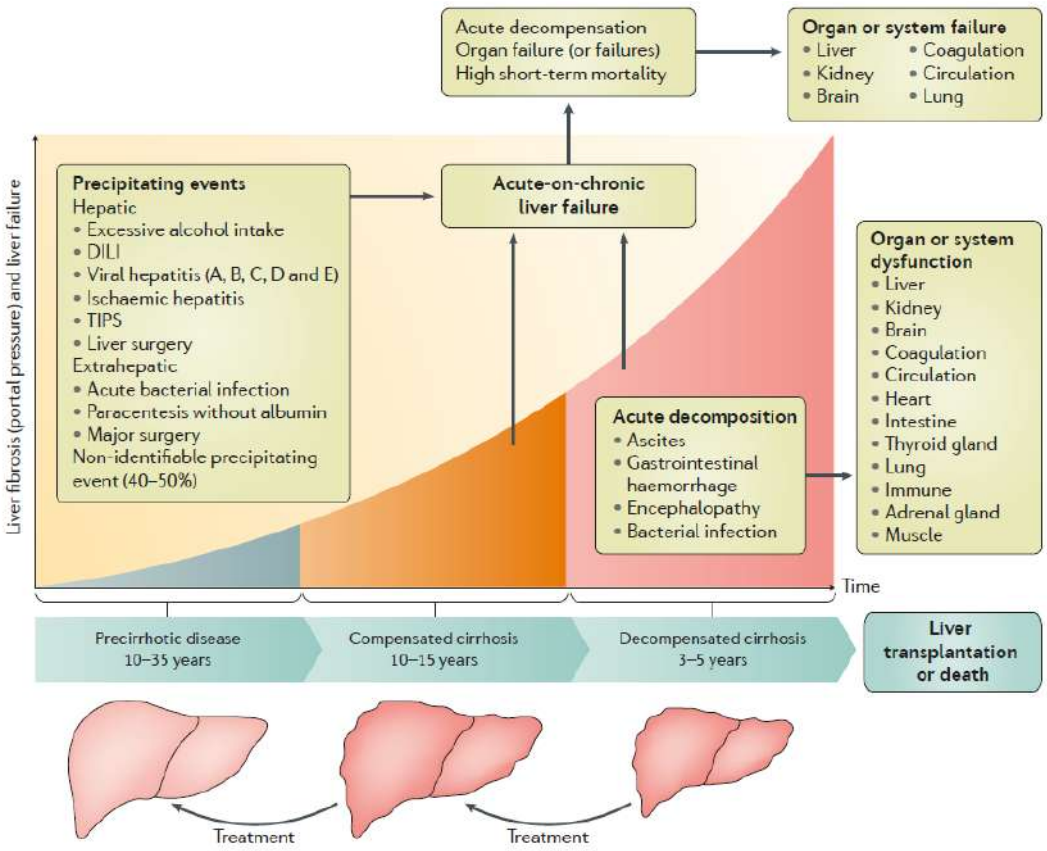


Figure 7. The clinical course of cirrhosis. Cirrhosis and their main hepatic or extrahepatic precipitating events are represented from an initial state of fibrosis or pre-cirrhotic disease until acute decompensated state with multi-organ failure characteristic of Acute-on-chronic liver failure (ACLF). A considerable proportion of patients have no identifiable triggering event. In Addition, acute decompensation events that define decompensation and trigger to organ failure such as bacterial translocation and impaired immune system are also represented. Paracentesis means (>5 litres), DILI, drug-induced liver injury; TIPS, transjugular intrahepatic portosystemic shunt (Arroyo et al, Nat Rev Dis Primers 2016).

3.2.1 Compensated cirrhosis

Compensated cirrhosis defines the period between the onset of cirrhosis (Alcohol, viral hepatitis, fat accumulation, etc) and the first major complication. During this period, which is very long (more than 10 years), in most patients, the symptoms are absent or minor however liver lesions and portal hypertension progress (Arroyo et al, Nat Rev Dis Primers 2016). Compensated cirrhosis would comprise two sub-stages depending on the development of gastroesophageal varices. State 1 is named compensated cirrhosis without varices which is considered the earliest clinical state with a low incidence of decompensation and very low mortality. These patients have portal Hypertension between 5 mmHg and 10 mmHg (Garcia-Tsao et al, Hepatology, 2010) and liver stiffness measurement ≥20 kPa alone or in combination with platelet count and spleen size (Berzigotti et al, Gastroenterology, 2013). State 2 of compensated cirrhosis is determined in patients that have clinically significant portal hypertension (> 10 mmHg) and are at risk of variceal bleeding and further decompensation. So, they require a specific treatment according to the severity of risk (D'Amico et al, J Hepatol, 2018 and Villanueva et al, Hepatology, 2016).

3.2.2 Acutely decompensated cirrhosis (AD)

AD cirrhosis is the symptomatic stage of the disease, associated with short-term survival, about 3-5 years. It is defined by the development of disease-related complications such as ascites, variceal bleeding, hepatic encephalopathy or jaundice, which require the portal hypertension to be above 10 mmHg (Garcia-Tsao et al, Hepatology, 2017). Traditional observations indicate portal hypertension with splanchnic vasodilatation and hyperdynamic circulation as central mechanism of acute decompensation. However, recent studies demonstrated that systemic inflammation, mitochondrial dysfunction,

oxidative stress and metabolic changes can have a role in decompensation and impairment of extrahepatic organs (Engelmann et al, J Hepatol. 2021).

The most commune decompensating event is ascites (18–27%), which is considered the hallmark of decompensation, followed by bleeding (9.5-18%), encephalopathy (2-7%) or jaundice (1.5%). Sometimes, more than one decompensating event at once occur and the most frequent combination is bleeding with ascites which has a five-years mortality prognosis (D'Amico et al, J Hepatol, 2018). AD cirrhosis is characterized by impairment in the function of liver but also extrahepatic organs and systems including Brain (encephalopathy), Kidneys (impaired renal function), coagulation (impairment in hepatic synthesis of coagulant and anticoagulant factors), intestines (increase translocation of bacteria or bacterial products to circulation) and immune system (systemic inflammation and impaired leukocytes function) (Arroyo et al, Nat Rev Dis Primers 2016).

Treatments for AD cirrhosis are focused in treating infections before patients develop organ failure. Treatments include administration of antibiotics, intravenous administration of albumin, which is highly effective preventing hepatorenal syndrome (Sort et al, N engl J Med, 1999) and reduce systemic inflammation (Fernández et al, Gastroenterology, 2019). Other potential treatments could be Norfloxacin administration, which reduce the rate of spontaneous bacterial peritonitis and modulate immune response to bacterial translocation (Fernández et al, Gastroenterology, 2007), or administration of Granulocyte colony-stimulating factor (G-CSF) plus Darbepoetin, which improve liver function and reduce incidence of sepsis (Kedarisetty et al, Gastroenterology, 2015).

3.2.3 Acute-on-chronic liver failure (ACLF)

ACLF is defined as a syndrome that develops in patients with cirrhosis and is characterized by acute decompensation, organ failure and high short-term (28-days) mortality. This definition was consolidated in 2009 by the European Association of the Study of Liver Failure (EASL-CLIF) with the CANONIC study, aimed to define ACLF in cirrhosis and assess the clinical course of the syndrome (Moreau et al, Gastroenterology, 2013).

ACLF could be develop at any phase of the disease from compensated to early or late AD cirrhosis and is defined by organ failure normally as a consequence of a precipitating event. The most commune precipitating events are active alcoholism and bacterial infections although in approximately 40% of patients that develop ACLF no precipitating event can be identified (Moreau et al, Gastroenterology, 2013). Organ failure is the

feature that differentiates ACLF from AD cirrhosis without ACLF. It is defined by intense impairment in the function of six specific organs or systems. The most frequently affected organs or systems were kidneys, followed by the liver, coagulation, the brain, circulation and lungs (Arroyo et al, Nat Rev Dis Primers 2016). Organ failure is the feature used to stratify decompensated patients with ACLF in three groups of severity:

- ACLF grade 1: include patients with single kidney failure (most prevalent) or single liver, brain, coagulation, circulatory or lung failure associated with creatinine levels of 1.5-1.9 mg/dl.
- ACLF grade 2: diagnosed when there are two organ failures of any combination.
- ACLF grade 3: diagnosed when there are 3 or more organ failures of any combination.

The worldwide mortality of ACLF correlates closely with the number of organ failures. In Europe the average of mortality is 28 days without liver transplantation (Moreau et al, Gastroenterology, 2013).

Medical management of ACLF consists of early recognition, treatment the precipitating event and supportive care (Ginès et al, J Hepatol, 2012). Liver transplantation is the only definitive therapeutic option for patients with ACLF. However, data indicate that less than half of patients are listed and the procedure is performed in only 10-25% of patients, as the others die in the waiting list (Chan et al, Hepatol Int, 2015).

4. SISTEMIC INFLAMMATION IN CIRRHOSIS

Systemic inflammation is a well-recognized feature in AD cirrhosis. Recent studies aimed to understand the pathophysiological basis of AD cirrhosis and ACLF have established the systemic inflammatory hypothesis, by which a hyper-inflammatory state in the systemic circulation is the mechanism leading the progression from the compensated to the decompensated forms of cirrhosis as well as the development of organ failures and ACLF (Bernardi et al, J Hepatol, 2015 and Arroyo et al, J Hepatol. 2021). This inflammatory state can be perceived by the increase in the circulating levels of C-reactive protein, the increase of white blood cell count and the persistent activation of the immune cells among them neutrophils and monocytes (Moreau et al, Gastroenterology 2013 and Papp et al, Liver Int, 2012). As consequence of their activation, immune cells produce a massive release of inflammatory mediators such as cytokines, chemokines, bioactive lipid mediators and ROS that lead to immune-mediated tissue damage (Clària et al, Hepatology 2020 and López-Vicario et al, J Hepatol, 2020). The degree of inflammation parallels the severity of liver, circulatory and renal dysfunction and hepatic encephalopathy.

4.1 ORIGIN OF SYSTEMIC INFLAMMATION IN CIRRHOSIS

Systemic inflammation in AD cirrhosis is due to two major mechanisms. Firstly, in these patients, bacterial translocation is produced by bacterial overgrowth and dysbiosis of the microbiota, that change the gut microbiome (Acharya et al, JCI Insight, 2017) and increase gut permeability causing the translocation of live bacteria from intestinal lumen into systemic circulation. In fact, bacterial infections are a characteristic feature of cirrhosis, leading to a hyper-inflammatory response that ultimately leads to encephalopathy, hepatorenal syndrome and ACLF (Bernardi et al, J Hepatol, 2015). In addition to infections, translocation of bacterial products (PAMPs) such as LPS and bacterial DNA are common findings in patients with cirrhosis (Bernardi et al, J Hepatol, 2015). These PAMPs favor the continue activation of immune cells interacting with their TLRs and leading to constant release of pro-inflammatory cytokines and chemokines responsible of inflammation (Wiest et al, J Hepatol, 2014). In the liver, PAMPs activate TLRs in Kupffer cells, which are the resident macrophages, and stimulate the production of TNFα and IL-8 inducing hepatocyte death and liver inflammation (Hansen et al, Hepatology,1994 and Moreau et al, Semin Liver Dis, 2016).

Secondly, in patients with AD cirrhosis, there is a massive release of DAMPs resulting from live tissue damage and hepatocyte death, leading to acute inflammatory bursts. For example, necrotic hepatocytes release markers of cell death such as Keratin 18 which is a marker of disease progression and severity of inflammation (Macdonald et al, Hepatology, 2018). These DAMPs contribute to activate the immune cells through TLRs aggravating the hyper-inflammatory state in AD cirrhosis (Kubes et al, Gastroenterology, 2012).

Both, PAMPs and DAMPs stimulate inflammatory responses that contribute to progressive recruitment of immune cells to the liver. This persistent inflammatory response and dysfunctional immune regulation can worsen tissue damage and lead to decompensation or organ failure (Engelmann et al, J Hepatol. 2021). In Figure 8 this process is represented as a diagram.

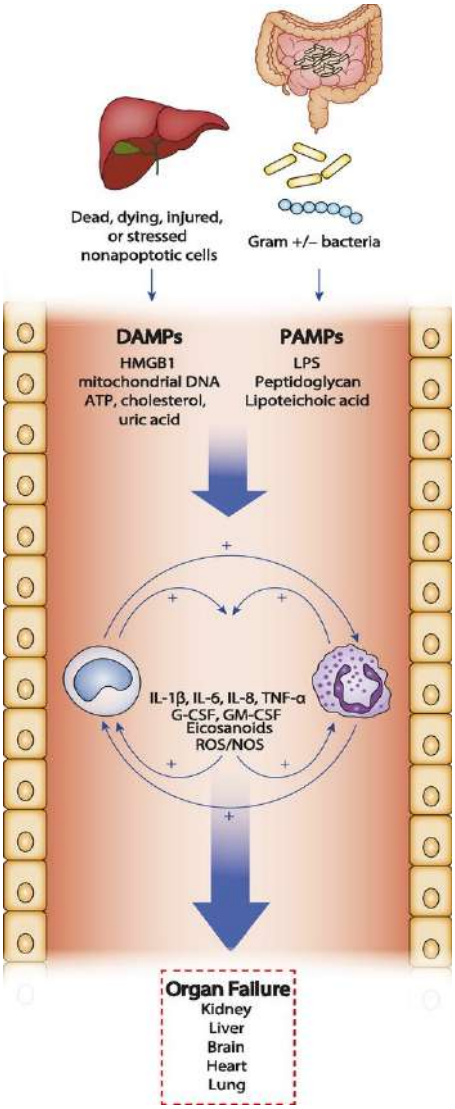


Figure 8. Schematic diagram summarizing the origin of systemic inflammation in decompensated cirrhosis. DAMPs and PAMPs produced from dying liver cells and translocated bacteria respectively, activate innate immune cells, such as monocytes and neutrophils resulting in a massive release of inflammatory mediators such as cytokines (IL-1β, IL-6, IL-8, TNF-α), hematopoietic factors (G-CSF, GM-CSF), Eicosanoids (lipid mediators with inflammatory properties) and reactive oxygen (ROS) and nitrogen species (NOS) that cause a hyper-inflammatory state and immune paralysis. In patients with decompensated cirrhosis the action of these inflammatory mediators may cause organ failure and ACLF. (Clària et al, J Immunol, 2016).

4.2 ROLE OF INFLAMMATION IN THE PATHOGENESIS OF THE DISEASE

4.2.1. Cellular effectors of inflammation in cirrhosis

The direct consequence of the hyper-inflammatory state present in cirrhosis is the immune paralysis. It is produced as a consequence of a pro-inflammatory state prolonged in time together with an impairment of liver functions (Albillos et al, J Hepatol, 2014).

In early stages of the disease, DAMPs produced from liver tissue and necrotic hepatocytes due to alcohol, or hepatitis activate immune cells evoking and inflammatory response. Cirrhosis progression from compensated to decompensated impairs liver capacity to produce proteins involved in the immune response (Homann et al, Gut, 1997), as well as impaired production of albumin. Neutrophils secrete large quantities of pro-inflammatory cytokines (TNFα, IL-1β and IL-6) and increase the expression of adhesion markers, such as CD11b (Rosenbloom et al, JAMA, 1995). Moreover, Macrophages or Kupffer cells play a major role in the progression of the disease becoming inflammatory macrophages (also named M1) that persistently release inflammatory mediators that cause more tissue damage rather than adopt a regulatory phenotype (M2 macrophages) driving tissue repair (Tacke et al, J Hepatol, 2017). This early and persistent immune activation promotes disease progression.

As already discussed, in AD cirrhosis, bacterial translocation occurs at high rate and PAMPs released from the gut activates also immune system and aggravate systemic inflammation. Then, immune response reprograming occurs after constant PAMPs and DAMPs pressure and the predominantly pro-inflammatory state switches to anti-inflammatory co-existing both conditions in the same patient that triggers immune cells switch into a non-responsive phenotype causing an Immunodeficiency state (Albillos et al, J Hepatol, 2014). AD cirrhosis is characterized by lymphopenia affecting T helper and T cytotoxic subsets and disrupted T cell compartment that causes an impairment in the *de novo* production of T cells (Albillos et al, J Hepatol, 2014). B cells are also profoundly affected by cirrhosis with diminished absolute counts, especially memory B cells which show an impaired response to TLR9 activation as well as impaired TNFα and IgG production (Doi et al, Hepatology, 2012). Circulating NKT cells are also defective with poor response to cytokine stimulation. At the intrahepatic level, NKT cells play an important role in alleviating liver fibrosis (Tian et al, Hepatology, 2013). In contrast to lymphocytes, patients with AD cirrhosis present neutrophilia, although the function of these immune cells appears to be impaired (Liu et al, Cell Mol Immunol, 2021). For

example, neutrophils from these patients show reduced CXCR1/2 expression, which is essential for migration, due to constant exposure at IL-8 (Xu et al, Sci Rep. 2016). Moreover, neutrophils in decompensated alcoholic cirrhosis exhibit a deficient p38-MAPK signaling which triggers to deficient myeloperoxidase release, respiratory burst and phagocytic activity. These defects increase susceptibility to bacterial infections (Boussif et al, J Hepatol, 2016). Monocyte counts are also increase in patients with AD cirrhosis. Circulating monocytes (CD14+) displayed increased expression of both activating and inhibitory surface markers and become non-responsive to pathogens (Weichselbaum et al, J Hepatol. 2020). In addition, in the liver, resident macrophages, Kupffer cells are constantly activated by DAMPs from dying hepatocytes and continue secreting pro-inflammatory cytokines triggering to more tissue damage (Kolios et al, World J Gastroenterol, 2006).

Severe immune response reprogramming is observed in patients with ACLF, who develop a state of immune paralysis similar to patients with sepsis. In ACLF patients, plasma levels of cytokines involved in the immune adaptive response were not different to those in patients with AD without ACLF, suggesting that the innate immune system plays a more prominent role in ACLF development (Clària et al, Hepatology, 2016). In ACLF, absolute neutrophil counts are higher, probably induced by higher circulating G-CSF levels (Khanam et al, Front Immunol, 2017). Moreover, neutrophils display reduced phagocytic capacity which is clinically relevant, as impaired phagocytic function correlate with high risk of organ failure and mortality (Taylor et al, Aliment Pharmacol Ther, 2014). The inflammatory phenotype in Monocytes is defined by persistently reduced HLA-DR expression, as a marker of dysfunction, which correlates with the risk of secondary infections. Also, the inability of monocytes to produce TNFα in response to LPS (Bernsmeier et al. Gut, 2018). Functional alterations are also evident in Monocytes subsets, for example, Intermediate monocytes ($CD14^+$, $CD16^+$) display attenuated pro-inflammatory cytokine production and higher production of anti-inflammatory cytokines, such as IL-10 (Bernsmeier et al. Gut, 2018). In addition, classical monocytes ($CD14^+$, $CD16^-$) have a severely impaired phagocytic capacity and oxidative burst response in ACLF compared with AD patients (Korf et al, Gut, 2019). Finally, In the liver, activated Kupffer cells release soluble CD163 and CD206, which increases with severity of ACLF and predicts mortality (Nielsen et al, Cells, 2020).

Figure 9 represents schematically all these factors mentioned above involved in immune paralysis in cirrhosis.

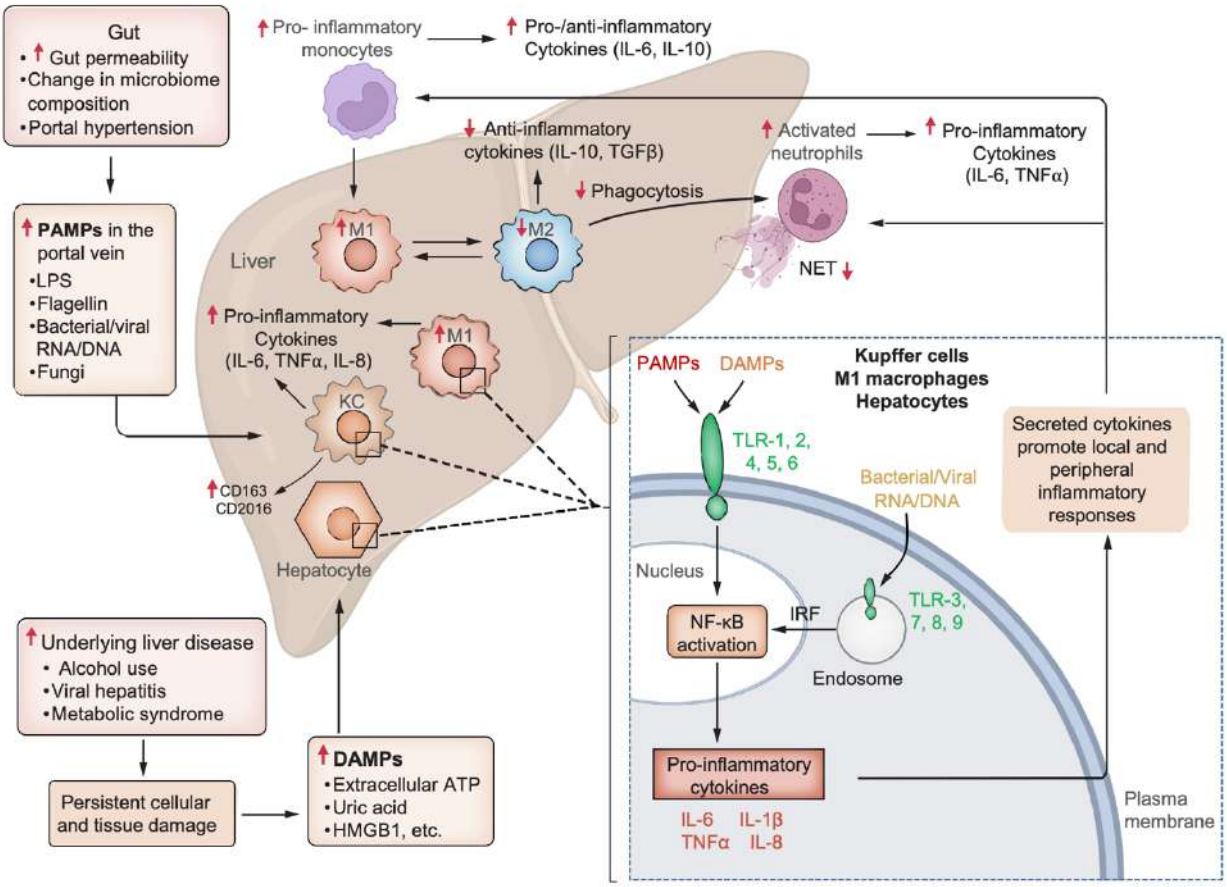


Figure 9. Schematic representation of immune paralysis in cirrhosis. The constant influx of DAMPs and PAMPs produced from dying liver cells and translocated bacteria activate the immune cells in the liver, which start producing a large amount of cytokines. These ones promote local and peripheral inflammatory responses that activate neutrophils, monocytes and pro-inflammatory macrophages. In this context, immune paralysis is produced and monocytes start producing pro and anti-inflammatory cytokines with less capacity to respond against pathogens; Neutrophils has less phagocytic activity and less capacity to produce NETs; M2 macrophages are decreased with low capacity to revert tissue damage and M1 macrophages or Kupfer cells increase producing more pro-inflammatory cytokines and increasing tissue damage (Modified from Engelman et al, J Hepatol, 2021).

4.2.2. Molecular effectors of inflammation in cirrhosis

The dysfunctional immune system in cirrhotic patients is basically due to small molecules which are cytokines, chemokines and lipid mediators that initiate, amplify and mediate inflammation and immune response in cirrhosis.

- Cytokine storm:

Exacerbated circulating levels of cytokines in patients with AD cirrhosis and ACLF can be categorized within the term “Cytokine storm” which defines the massive production of these mediators as a consequence of over activated immune system without control. Comprehensive characterization of these cytokines in AD cirrhosis and ACLF and their correlation with disease severity were described recently (Clària et al, Hepatology, 2016 and Trebicka et al, Front Immunol, 2019). As commented before, cytokines increased in AD cirrhosis and ACLF are the ones activated by the innate immune system, in contrast these patients display low levels of those cytokines derived from adaptive immune system, such as IFN-γ or IL-17 (Clària et al, Hepatology, 2016). ACLF patients show a profile of pro-inflammatory cytokines (IL-6, TNFα, IL-1β) and chemokines (IL-8) but also a profile of anti-inflammatory cytokines (IL-10) which their levels in circulation and balance have a direct effect on the prognosis, severity and mortality of the disease (Clària et al, Hepatology, 2016). In addition, genetic factors such as a single nucleotide polymorphism in the IL-1 gene protect patients with AD cirrhosis from inflammation and reduce predisposition to develop ACLF (Alcaraz-Quiles et al, Hepatology, 2017).

Pro-inflammatory cytokines have a very important role acting at different stages of the disease with different etiologies. For example, IL-6 is one of the major stimuli for the release of hepatic acute phase proteins and levels of IL-6 together with TNFα strongly correlates with the development of renal impairment in cirrhotic patients with bacterial peritonitis (Navasa et al, Hepatology, 1998). In early stages of alcoholic hepatitis, IL-6 has also a protective effect on hepatocytes inhibiting apoptosis (Hong et al, Oncogene, 2002), whereas IL-1b and TNFα cause hepatocytes necrosis and apoptosis (Tilg et al, Hepatology 2016 and Hansen et al, Hepatology, 1994). In this condition, Kupffer cells release IL-6 and other cytokines, such as the anti-inflammatory cytokine IL-10, which both together keep the inflammatory response at bay preventing hepatic steatosis (Kawaratani et al, Mediators Inflamm, 2013). However, IL-1β cause neutrophils infiltration and Transforming growth factor beta (TGF-β, a pro-fibrotic cytokine) secretion, which drive to fibrosis and posterior cirrhosis (Tilg et al, Hepatology 2016). Moreover, IL-1β and TNFα favors intestinal permeability breaking the intracellular bindings of intestinal mucosa and contributing to the release of PAMPs and bacterial translocation from the gut to systemic circulation (Al-Sadi et al, J Immunol, 2008 and Wiest et al, J Hepatol, 2014).

- Eicosanoid storm:

Similar to cytokines, eicosanoids are massively released by leukocytes in response to infections or tissue injury originating also a "Eicosanoid storm" (Dennis et al, Nat Rev Immunol, 2015). Cirrhotic patients exhibit a clear imbalance between omega-6 PUFA and omga-3 PUFA, where omega-6 PUFA-derived lipid mediators are increased and has a pathophysiological relevance since AA is the precursor of potent inflammatory lipid mediators (Lopez-Vicario et al, J Hepatol, 2020). For example, the prostanoid PGE_2 drives immunosuppression and increases the risk of infection in AD cirrhosis patients (O'Brien et al, Nat Med, 2014). Indeed, inflammatory lipid mediators have a predictive role, as specific lipids profile in plasma in these patients is associated with 3-month mortality (Becares et al, Clin Gastroenterol Hepatol. 2020). In addition, elevated levels of PGE_2 and leukotriene E_4 (LTE4), both pro-inflammatory, in parallel with decreased levels of pro-resolving lipoxin A_5 (LXA5) compose a specific signature in AD cirrhosis that predicts the severity of the disease (Lopez-Vicario et al, J Hepatol, 2020). Leukotrienes have an important role in cirrhosis from time ago, as increased levels of urinary LTE_4 were found in patients with AD cirrhosis and hepatorenal syndrome (Moore et al, J Hepatol, 1990) and the inhibition of 5-LOX reduce production of LT, which avoids cell damage in cirrhotic rats (Titos et al, J Leukoc Biol, 2005). Recently, it has been described that in ACLF patients LTE_4 levels strongly correlated with IL-8 and the necrosis/apoptosis marker K18 (Lopez-Vicario et al, J Hepatol, 2020). In addition, the same study demonstrates that pro-resolving LXA_5 had a negative correlation with inflammation and cell death. Finally, some lipid mediators derived from linoleic acid, which are indicators of active bactericidal activity, were remarkably suppressed in ACLF patients (Lopez-Vicario et al, J Hepatol, 2020). So, imbalance formation of inflammatory/pro-resolving lipid mediators has an important role in systemic inflammation.

- Other effectors: albumin oxidized forms

Albumin could suffer some post-translational modifications that are reversible or irreversible like oxidation in cysteine-34 residue, glycosylation, N- and C-terminal truncations or homodimerization (Oettl et al, Biochim Biophys Acta, 2008; Domenicali et al, Hepatology, 2014 and Baldassarre, Sci Rep, 2016). Patients with advanced cirrhosis not only have reduced serum albumin levels, also present a reduction in the serum abundance of naive albumin (Garcia-Martinez, Hepatology, 2013). In addition, the ability of albumin to transport protein-bound substances and drugs and act as detoxification

agent is severely compromised in cirrhotic patients and even more reduced in acute-on-chronic liver failure (ALCF) (Jalan et al, Hepatology, 2009). However, altered albumin also exerts a pro-inflammatory activity. Both HNA-1 and HNA-2 are increased in patients with severe alcoholic hepatitis and this hyper-oxidized albumin activate neutrophils and induce respiratory burst (Das et al, Hepatology, 2017). In addition, a study published in our group reveals that HNA-1 and HNA-2 are significantly increased in patients with decompensated cirrhosis and ACLF and specially HNA-1 is able to induce the expression and release pro-inflammatory cytokines and eicosanoids through the phosphorylation of p38 MAPK pathway (Alcaraz-Quiles et al, Hepatology, 2018). These results suggest that in patients with advanced cirrhosis is necessary to replace the albumin because it is severely modified, and these modified forms are inflammatory.

4.2.1 Role of inflammation in extrahepatic organ failures in cirrhosis

Following systemic inflammation hypothesis, organ dysfunction and further organ failure characteristics of advance cirrhosis and ACLF do not occur only as a consequence of failure in system circulation or organ hypo-perfusion rather for innate immune system activation and the loss of tissue and cell homeostasis (Bernardi, J Hepatol, 2015). In figure 10 are represented the most common extrahepatic organ dysfunction and failure as a consequence of systemic inflammation.

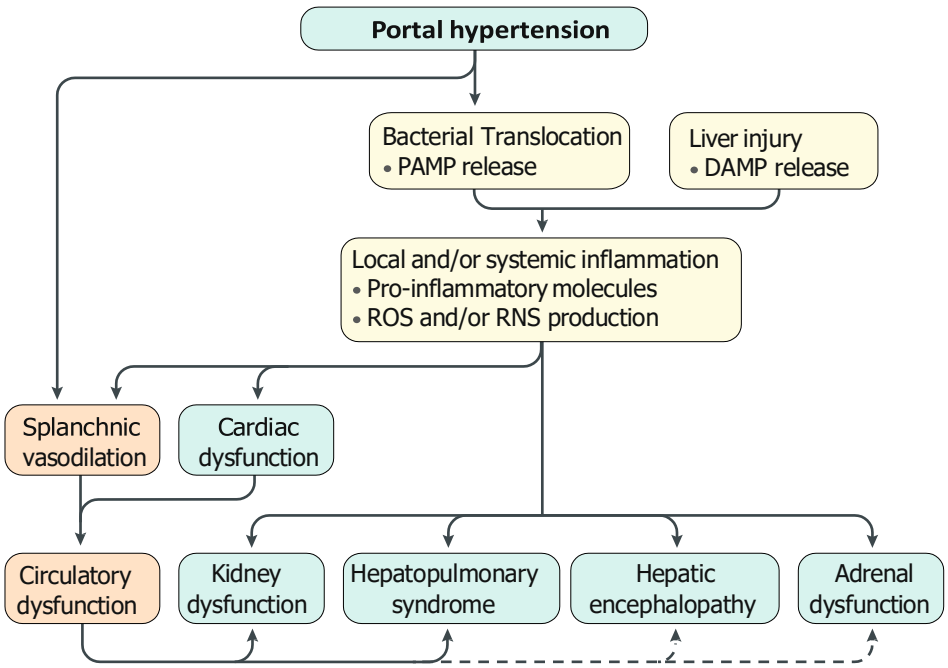


Figure 10. Mechanisms leading to extrahepatic organ failure in advanced cirrhosis. Portal Hypertension is the first sign of decompensation followed by a systemic spread of PAMPs from bacterial translocation and DAMPs from the damaged liver activate innate host immunity with a consequence of pro-inflammatory mediators and oxidative and nitrosative species that leads to cardiac dysfunction and splanchnic vasodilation, induced also by portal hypertension, that trigger circulatory dysfunction. The direct effects of systemic inflammation and circulatory dysfunction lead to multiorgan dysfunction, such as Hepatorenal syndrome, hepatopulmonar syndrome, Hepatic encephalopathy and adrenal dysfunction (Bernardi et al, Nat Rev Gastroenterol Hepatol, 2018).

- Cirrhotic cardiomyopathy: This complication appears when splanchnic vasodilatation leads to the hyperdynamic circulatory syndrome, which occurs in patients with portal hypertension and is characterized by increased cardiac output and heart rate, and decreased systemic vascular resistance with low arterial blood pressure (Møller et al, Eur Heart J, 2013). However, the pathophysiology of cardiac dysfunction is complex and the role of inflammation is becoming more important. It is known that NO, which is a vasodilator substance, has a relevant role due to its overproduction leads to arterial vasodilation (Sogni et al, J Hepatol 1995). Moreover, the oxidative stress produced by NO and TNFα present in cirrhosis, induce activation of NF-kB and inducible nitric oxide synthase (iNOS) pathway that produce and impairment in ventricular contractility (Bortoluzzi et al, Hepatology, 2013).

- Hepatorenal syndrome: Renal failure is defined as kidney functional failure in cirrhotic patients, normally with ascites. Often the precipitated event to develop acute kidney injury (AKI) are bacterial infections (Fasolato et al, Hepatology, 2007), therefore, inflammation represent an important factor in AKI development. Cirrhosis infection and followed inflammation leads to renal TLR4 activation by bacterial translocation, increasing the transcription of pro-inflammatory cytokines leading to apoptotic tubular damage, suggesting that TLR4 mediates renal injury (Shah et al, J Hepatol, 2012 and Shah et al, Liver Int, 2013).

- Hepatopulmonary syndrome: This syndrome is caused by intrapulmonary vasodilatation, which is likely multifactorial but the involvement of bacterial translocation induced inflammation is considered a precipitating event. Hepatopulmonary syndrome is strongly related to increase NO release in pulmonary circulation (Rodríguez-Roisin et al, N Engl J Med, 2008). Pro-inflammatory response induced by bacterial translocation, lead to macrophage accumulation in lung microvasculature (Thenappan et al, Am J Respir

Crit Care Med, 2011) and activate fractalkine (CX3CL1) a chemokine that enhance monocyte adherence. Monocytes express iNOS that promotes carbon monoxide (CO) production and increase vasodilatation (Carter et al, Am J Physiol Lung Cell Mol Physiol, 2002). In addition, fractalkine produced by circulating monocytes also contribute to angiogenesis and intrapulmonary vasodilatation pathogenic factor (Zhang et al, Am J Pathol, 2014).

- Hepatic encephalopathy: It is a clinical manifestation of a low-grade cerebral edema, which is exacerbated in response to ammonia and other precipitating factors (Häussinger et al, Gut, 2008). Systemic inflammation activates resident brain immune cells (microglial cells) to produce pro-inflammatory cytokines, such as TNFα, that exacerbate astrocyte ammonia-damage and oxidative stress (Romero-Gómez et al, J Hepatol, 2015).

- Adrenal dysfunction: Adrenal insufficiency is frequent in decompensated cirrhosis with severe sepsis and is associated with increased mortality (Tsai et al, Hepatology, 2006). Factors such as liver failure and systemic inflammation are involved with impaired cholesterol synthesis, so adrenal gland cannot synthetize adequate quantities of cortisol, and enhanced levels of pro-inflammatory cytokines and LPS. Pro-inflammatory cytokines contribute to decrease levels of cholesterol resulting in limited delivery to adrenal glands (Karagiannis et al, World J Hepatol, 2015). In addition, TNFα reduce the secretion of adrenocorticotropic hormone from the pituitary gland that results in a glucocorticoid deficiency.

5. ALBUMIN AS A THERAPY IN ACUTELY DECOMPENSATED CIRRHOSIS

The administration of HSA is one of the most frequent therapies in patients with decompensated cirrhosis.

Since years ago, the oncotic properties of HSA have been used for plasma volume expansion with the ultimate goal to improve effective volemia. In the case of advanced liver disease, HSA infusions are clinically indicated in patients with decompensated cirrhosis to prevent spontaneous bacterial peritonitis (SBP)-induced renal dysfunction (Sort et al, Hepatology, 1999), to prevent paracentesis-induced circulatory dysfunction (Bernardi et al, Hepatology, 2012) and to prevent, in combination with Terlipressin, the development of hepatorenal syndrome (Ortega et al, Hepatology 2002). In all of these indications, albumin administration results in a reduced rate of complications and a short-term survival benefit.

Evidence has emerged that the beneficial effects of albumin therapy are also related to its non-oncotic properties, unrelated with fluid regulation. For example, in patients with cirrhosis and SBP, HSA reduces plasma levels of coagulation factor VIII and circulating nitric oxide, suggesting that HSA attenuates endothelial activation (Fernández et al, Hepatology, 2005). In addition, O'Brien et al. has demonstrated that HSA is able to revert PGE_2-induced immune dysfunction in patients with AD cirrhosis and ACLF (O'Brien et al, Nat Med, 2014). These authors have also described a method based on the profile of lipids to classify the inflammatory response in patients with AD/ACLF, a method that allows the identification of patients most likely to respond to HSA treatment (China et al, Clin Gastroenterol Hepatol. 2018). Moreover, the same group has determined that plasma IL-4 levels could serve as a good marker of improvement in the degree of systemic inflammation after HSA treatment of AD patients and ACLF (Becares et al, Clin Gastroenterol Hepatol, 2020). At the experimental level, it has been demonstrated in rats with cirrhosis and ascites, that HSA improves left ventricular contractility and counteracts the negative effect of oxidative stress and TNFα activation (Bortoluzzi et al, Hepatology, 2013). Moreover, in cirrhotic mice, a recent study has demonstrated that HSA is able to protect hepatocytes from TNFα-induced apoptosis through mechanisms related to reduced lysosomal cathepsin B release. This study has also demonstrated that HSA increase the population of $Ly6C^{low}$ monocytes, which promote tissue repair (Duran-Güell et al, FASEB J, 2021).

The combined oncotic and non-oncotic properties, make HSA a multi-target agent able to exert beneficial effects at different points of the disease. Some clinical trials have been performed with long-term albumin administration aimed to test albumin efficiency to avoid further complications in patients with decompensated cirrhosis. The first one was published in 1999 and the authors demonstrated that the administration of 25g of albumin for three years reduced the development of ascites and hospital readmission (Gentilini et al, J Hepatol, 1999). Further, the same group demonstrated that long-term albumin administration (84 months) resulted in more time without liver transplant in these patients (Romanelli et al, World J Gastroenterol, 2006). However, no conclusive results were obtained in any of these clinical trials regarding albumin administration. In recent years, more clinical trials with conflicting results about albumin have been published. The ANSWER study compared the effectiveness of HSA, at higher dose (40g), plus standard medical therapy (SMT) with SMT alone for 18 months in patients with cirrhosis and uncomplicated ascites (Caraceni et al, Lancet, 2018). Results show that the incidence of complications such as infections, ascites, SBP, hepatic encephalopathy or hepatorenal syndrome were reduced in this group, moreover, the hazard ratio of 18-months mortality was also reduced (Caraceni et al, Lancet, 2018). However, conflicting results emerged from MATCh study, another clinical trial published in the same year (Solà et al, J Hepatol, 2018). It is a Multicenter, randomized trial performed in patients with AD cirrhosis awaiting liver transplantation. Patients received Midodrine, an alpha-adrenergic vasoconstrictor, together with intravenous albumin (40g/15 days) or placebo for 1 year until liver transplantation. Results showed that treatment with Midodrine and albumin, slightly suppressed the activity of vasoconstrictor systems, but did not prevent complications of cirrhosis or improve survival (Solà et al, J Hepatol, 2018). One year later, another clinical trial with two different studies were published. The pilot-PRECIOSA study was aimed to identify the optimal HSA dosage (1g/kg or 1,5g/kg) for 12 weeks (log-term treatment) in patients with AD cirrhosis. Also, the randomized controlled trial INFECIR-2 was aimed to compare the efficacy of adding HSA as short-term treatment (1 week) at higher dosage in combination with SMT and antibiotics (Fernández et al, Gastroenterology, 2019). Both studies have demonstrated that HSA reduced systemic inflammation and cardiac dysfunction, in addition, long-term and high-dose HSA was associated with normalization of serum level of albumin, improved stability of the circulation and left ventricular function, and reduced plasma levels of cytokines without significant changes in portal pressure (Fernández et al, Gastroenterology, 2019). Then, the ATTIRE clinical trial was performed in hospitalized patients with decompensated cirrhosis who had a serum albumin level of less than 30 g per liter. Patients received either 20% human albumin solution (until increasing the albumin level to ≥30 g/L) for up

to 14 days or standard care. Results of this study demonstrated that albumin infusions were useful to increase the albumin level to a target of 30 g per liter or more but no beneficial effects regarding severe or life-threatening serious adverse events were observed in comparison as the current standard care (China et al, N Engl J Med, 2021). Finally, the most recent clinical trial, the ALPS trial was performed in patients with cirrhosis and sepsis-induced hypotension that received either 20% albumin (0.5-1.0gm/kg) or Plasmalyte (30ml/kg). Results demonstrated that albumin improves the hemodynamics with a decrease in arterial lactate, lesser proportion of dialysis, and a higher time to initiation of dialysis. However, the 28-day mortality was not different between albumin and Plasmalyte group (Maiwall et al, J Hepatol, 2022).

These divergent results might be explained by the differences in the patients included in the different studies: despite of sharing AD cirrhosis, they differ in the other complications and disease-related syndromes. In addition, the differing dosage and time of albumin administration and the combination with different drugs may also have some influence in the results obtained.

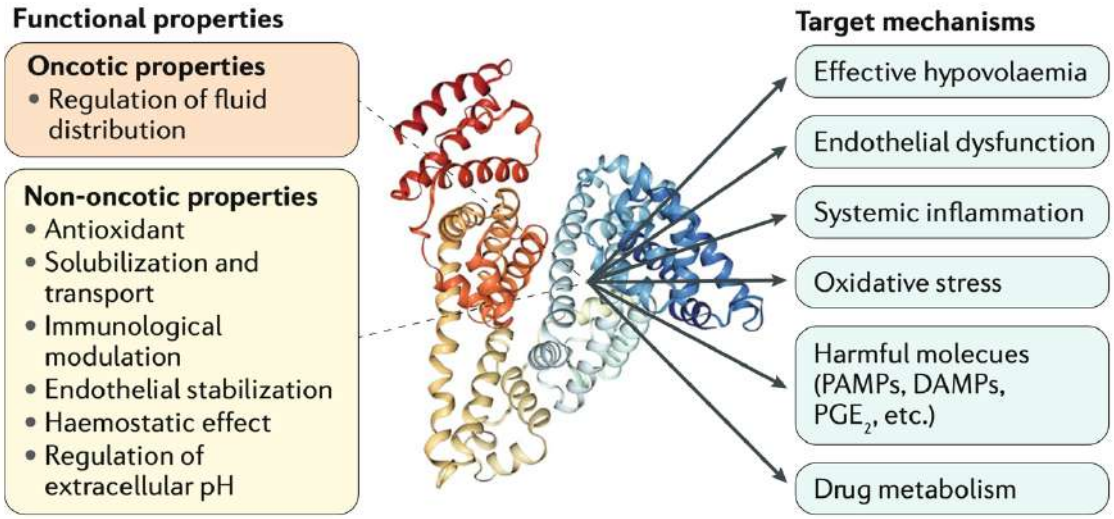


Figure 11. Functional properties of albumin and potential target mechanisms. Human albumin can exert a range of oncotic and non-oncotic effects that convert this molecule a suitable treatment for multiple different process in cirrhosis. (Bernardi et al, Nat Rev Gastroenterol Hepatol, 2018).

Although the most effective therapy in ACLF patients is liver transplantation, this treatment is limited by the restricted number of liver donors. As alternative to liver

transplantation, several clinical centers have tested the value of extracorporeal liver support systems as albumin dialysis or plasma exchange. The most common is MARS system (Molecular Adsorbent Recirculating System) which is based on albumin dialysis (Saliba et al, Crit Care, 2006). Several trials demonstrated that albumin dialysis is able to improve cholestasis, liver and kidney function and hemodynamics in patients with acute liver injury and advanced cirrhosis (Heemann et al, Hepatology, 2002 and Hassanein et al, Hepatology, 2007). Moreover, in the case of ACLF patients, the RELIEF trial, which compare MARS with SMT performed provides temporary support of organ failure but no differences were found between MARS and SMT at 28 or 90 days in these patients (Bañares R, Hepatology, 2013). A recent randomized control trial assessed the effect of albumin dialysis with MARS in ACLF patients resulting with an improve survival (Bañares R, Therap Adv Gastroenterol. 2019). Another study combines intermittent albumin dialysis with continuous venous hemodialysis in patients with severe ACLF and renal insufficiency, which also shown an improved 28- and 90-days survival and several key clinical and laboratory parameters (Niewinski G, Artif Organs, 2020). Regarding therapies with plasma exchange, a recent study demonstrated that this therapy displayed lower mortality risk and significantly reversed organ failures in patients with ACLF (Yang Z, Hepatol Int, 2020). Interestingly, it has been demonstrated that both systems, albumin dialysis and plasma exchange, are able to temporary modify the oxidative state of albumin from HNA1 to naïve albumin HMA in patients with ACLF, which reduce oxidative albumin circulating and recover functional albumin. However, HNA2 oxidative state was not changed with this technology (Oettl et al, Ther Apher Dial, 2009). In summary, the recent trials using liver support systems show encouraging results regarding survival on ACLF patients.

HYPOTESIS AND AIMS

Systemic inflammation has become the most important pathogenic factor for the transition from compensated to decompensated liver cirrhosis and for the subsequent development of organ dysfunction and failure (i.e. ACLF), a condition characterized by high short-term mortality. Increased circulating levels of cytokines, inflammatory lipid mediators, acute phase proteins and altered albumin molecular forms in these patients lead to persistent systemic inflammation with an overstimulation of immune cells, which results in immune paralysis and increased susceptibility to infections.

Albumin is synthetized in the liver and is continuously secreted to the circulation. It is the major contributor to plasma osmotic pressure and is commonly used as plasma volume expander in hypovolemic conditions and in those situations where its biosynthesis is impaired. Consistent with this, albumin infusions are considered an effective therapy for patients with decompensated cirrhosis. Indeed, it has been demonstrated that both short- and long-term albumin administration are useful for the prevention of renal dysfunction and development of ascites and to lessen hospital readmissions and mortality. Moreover, recent clinical trials have reported that albumin administration is associated with a reduction of systemic inflammation in patients with decompensated cirrhosis. The anti-inflammatory effects of albumin are closely related to its antioxidant properties by acting as a free radical scavenger, together with its ability to interact with a number of circulating molecules including bioactive lipids, especially FAs, which are transported in the circulation attached to its molecule. However, not all the anti-inflammatory effects of albumin can be simply explained by its ability to bind and sequester soluble inflammatory mediators, and a cellular and molecular mechanism of albumin anti-inflammatory actions is at present lacking.

The running hypothesis of this thesis dissertation is that albumin exerts immunomodulatory and anti-inflammatory actions by interacting with leukocytes in the systemic circulation leading to a modulation of the release of inflammatory mediators by these immune cells.

To test this hypothesis, this project has two primary objectives comprising each one a number of secondary objectives:

PRIMARY OBJECTIVE 1: Obtain a comprehensive view of the immunomodulatory properties of HSA and investigate its potential mechanisms of action at the cellular and molecular levels.

Secondary objectives:

1.1 Investigate the ability of HSA to modulate inflammation in PAMP-elicited leukocytes isolated from blood of healthy subjects and patients with decompensated cirrhosis patients.

1.2 Evaluate the anti-inflammatory actions of HSA independently from its oncotic and scavenger properties.

1.3 Identify the transcriptional profile of isolated leukocytes from patients with decompensated cirrhosis incubated with HSA.

1.4 Elucidate the cellular mechanisms of HSA immunomodulatory actions in isolated leukocytes challenged with inflammatory stimuli and treated with HSA.

1.5 Elucidate the molecular mechanisms of HSA anti-inflammatory actions in leukocytes challenged with inflammatory stimuli and treated with HSA.

1.6 Verify that HSA anti-inflammatory effects do not interfere in leukocytes function and defense mechanisms.

PRIMARY OBJECTIVE 2: Characterize the albumin lipidome in patients with decompensated cirrhosis and investigate the effects of this protein on the release of bioactive lipid mediators by isolated leukocytes. The specific aims of this study are the following.

Secondary objectives:

1.1 Evaluate the plasma lipid profile in patients with decompensated cirrhosis

1.2 Optimize a method to split the albumin-bound lipids from the rest of lipids of the plasma.

1.3 Compare the lipidomics profile of the plasma fractions from patients with decompensated cirrhosis and healthy subjects.

1.4 Assess the effect of HSA on the biosynthesis and release of bioactive lipid mediators by isolated leukocytes form healthy subjects and patients with decompensated cirrhosis

1.5 Validate changes in the plasma levels of lipid mediators in patients with decompensated cirrhosis included in a randomized clinical trial evaluating the effects of albumin on infections and survival.

RESULTS

STUDY 1: *Albumin internalizes and inhibits endosomal TLR signaling in leukocytes from patients with decompensated cirrhosis*

The aim of this study was to obtain a comprehensive view of the immunomodulatory properties of HSA and investigate its potential mechanisms of action at the cellular and molecular levels. Specifically, we investigated the effects of HSA on isolated human PBMC and PMN challenged with bacterial DNA rich in unmethylated CpGs. CpGs are common in bacterial and mitochondrial DNA, and trigger inflammation by acting as either a PAMP or a DAMP, respectively. CpGs are able to activate immune cells and the release of inflammatory mediators through the activation of endosomal TLR9. Moreover, we investigated the signaling pathways involved in the anti-inflammatory and immunomodulatory properties of HSA and to ascertain its effects on leukocyte defense functions.

Patients with decompensated cirrhosis show increased circulating levels of CpGs, which are potent stimulus for the activation of blood leukocytes

CpG levels were measured in plasma form healthy subjects (HV), patients with compensated cirrhosis (CC) and patients with AD cirrhosis. CpG concentrations were significantly higher in AD patients and their concentrations remain the same during HSA treatment. To mimic the conditions that immune cells encounter in the systemic circulation of patients with cirrhosis, PBMC and PMN were challenged with different PAMPs and DAMPs or the combination of both, such as LPS, N-formyl-methionyl-leucyl-phenylalanine (fMLP), ATP and CpGs. The results show that CpGs produce the highest expression of IL-1β.

HSA is able to inhibit PBMC and PMN responses to CpGs at the mRNA and protein level.

To investigate the ability of albumin to modulate CpG inflammatory response, PBMC and PMN were isolated from blood of HV and then pre-treated with HSA at 10 mg/ml and 15 mg/ml, which is the equivalent concentration to that therapeutically infused to correct hypoalbuminemia in patients with cirrhosis (from 25 to 30 mg/ml to 35 to 45 mg/ml). Then, cells were stimulated with CpGs. Both PBMC and PMN stimulated with CpGs showed up-regulation of *IL1B, IL6* and *TNF* expression as well as and increased release of IL-1β, IL-6, and TNFα. HSA was able to prevent cytokine stimulation at both concentrations and also at both RNA and protein level.

To confirm that the inhibitory effect was produced by HSA, the same experiments were reproduced using a recombinant HSA (rHSA) produced in *Oryza sativa.* Again, rHSA was able to prevent cytokine induction by CpG.

HSA anti-inflammatory effect is independent of its oncotic and scavenging properties

To rule out the possibility that the changes in cytokine expression with HSA incubation were due to variations in the oncotic pressure of the cell media, PBMC of HV were pre-incubated with HSA at increasing concentrations followed by CpG stimulation. Results show that the inhibitory potential of HSA on cytokine expression was observed from the lowest concentration. In contrast, the inhibitory effect was not observed with mannitol, a well-known oncotic agent. On the other hand, to discard the option that the effects of HSA were due to a possible CpG quenching by this molecule, experiments using treatment mode were performed. Results showed that HSA was also effective in inhibiting the expression of cytokines induced by CpG. Therefore, these results reinforce the view that the anti-inflammatory effects of albumin are independent of their oncotic and scavenging capacities.

HSA inhibits the inflammatory response produced by CpGs in leukocytes from patients with AD and ACLF

Next, the same experiments performed in HV were repeated in PBMC and PMN isolated from blood of patients with AD cirrhosis and ACLF to ascertain that HSA effects were also valid in patients. PBMC from patients showed higher baseline cytokine expression and more intense response to CpG. In ACLF more than AD patients and both were higher compared to HV. HSA was able to reduce cytokine expression overproduction in leukocytes from both AD and ACLF patients.

HSA inhibits CpG-stimulated transcriptomic profile of PBMC from AD patients.

To gain a wider perspective of the immunomodulatory actions of HSA we next assessed the global changes in the transcriptional profile of PBMC from AD patients stimulated with CpGs. One hundred thirty-three genes were differentially expressed in PBMC stimulated with CpGs in comparison to vehicle and 343 in those stimulated with CpGs plus HSA in comparison with CpGs alone. Then, genes were grouped according to blood transcription modules (BTM) and those with a *P* value below 0,05 were selected. This analysis resulted in the selection of 17 BTM with a set of genes of interest. CpG up-regulated genes involved in regulation of innate immunity, adaptive immunity and cytokines and chemokines modules. These modules together with platelet activation and

degranulation modules, were down-regulated by HSA plus CpG when comparing with CpG alone.

HSA uptake by PBMC colocalizes with early endosomes, as well as CpGs

As HSA inhibits the effects of CpGs, which signals by endosomal TLR9, we next assessed whether HSA may reach endosomal compartment by incubating PBMC with fluorescein isothiocyanate (FITC)–labeled HSA with or without CpG and observed by confocal microscopy. FITC-HSA was internalized by PBMC in vehicle conditions; however, the uptake was higher in PBMC challenged with CpG. To elucidate the percentage of cells that internalize albumin, PBMC were analyzed by flow cytometry, which revealed that HSA uptake was produced in about 11% in vehicle conditions and 28,4% in CpG conditions. Moreover, HSA-internalized PBMC were double positive for CD45 and CD14 indicating that myeloid cells are the responsible for the internalization. Then, with a time-course from 10 to 120 min, we observed that HSA colocalizes with early endosomes at early time periods. Finally, performing a triple immunofluorescence assay, we demonstrated that HSA colocalizes with CpG in early endosomes, mainly at 10 min of HSA incubation. Together, these results suggest that HSA blocks the inflammatory actions of CpG by interfering with TLR9 signaling pathway in endosomes.

HSA inhibits endosomal TLR9, TLR3 and TLR4 signaling

To confirm that HSA acts on endosomal TLR signaling, PBMC were exposed to HSA or chloroquine, which inhibits endosomal acidification necessary for TLRs signaling, and challenged with CpG. Chloroquine produced a similar blockage of CpG-induced IL6 expression to that of HSA. Interestingly, the combination of chloroquine and HSA reveled a greater reduction of IL-6 expression. Therefore, this results suggest that HSA acts in a similar manner as chloroquine. Next, same experiments were performed using an inhibitor of MyD88 (pepinh-MyD), which blocks TLR9 downstream signaling. Pepinh-MyD blocked the CpG–induced IL6 expression, an effect that was comparable to that of HSA. Subsequently, we wondered if HSA also modulates other endosomal TLR. PBMC were incubated with a dsRNA poly(I:C), which activate TLR3 or LPS which stimulate TLR4 in addition of HSA and specific inhibitors for each pathway. Poly(I:C) was able to upregulate the expression of CXCL10, IFNβ, IFN-stimulated gene oligoadenylate synthetase 2 (OAS2) and IRF3 phosphorylation. All of them were considerably reduced by HSA. On the other hand, HSA was also able to ameliorate the late phase of TLR4 activation by LPS and reduced the expression of CXCL10, OAS2 and the phosphorylation of IRF3. Collectively, these findings identify HSA as a potent modulator of endosomal TLR signaling.

HSA internalization is partially mediated by FcRn and preferentially involves caveolae-mediated endocytic pathways

To investigate if HSA internalization were produced through its neonatal Fc receptor (FcRn), PBMC were incubated with a HSA, CpG and FcRn blocking antibody (ADM31). The inhibitory actions of HSA on CpG-induced cytokine expression were only partially reversed in the presence of ADM31. Additionally, we investigated if internalization was related to caveolin-mediated endocytosis. Because caveolae forms invaginations of the plasma membrane enriched in cholesterol, PBMC were incubated with methyl-β-cyclodextrin (MBCD), which have depleted cholesterol content, completely block the inhibitory actions of HSA on CpG-induced cytokine expression. These findings indicate that HSA is internalized predominantly through caveolin-mediated endocytic pathways.

HSA does not affect leukocyte defensive functions

Next, we investigate whether HSA anti-inflammatory effects compromise the defense mechanisms of leukocytes against pathogens. The impact of HSA on phagocytosis, efferocytosis and ROS production were assessed. Results demostrated that HSA incubation in monocyte-derived macrophages (PBMDM) increase phagocytosis of zymosan particles and enhanced efferocytosis of apoptotic PMNs. In addition, HSA did not impaired PMN capacity to produce ROS in response to phorbol 12-myristate 13-acetate (PMA) or *Escherichia coli*.

In vivo translation of the in vitro HSA anti-inflammatory effects

Finally, experiments were performed in humanized analbuminemic mice expressing the human FcRn receptor (Alb−/−FcRn−/−hFcRn+/+). Leukocytes from wild type (WT) and analbuminemic mice were stimulated with CpG and treated with HSA. Compared to WT the CpG-induction of *Il1b*, *Il6* and *Tnf* expression was more pronounced in analbuminemic mice. Moreover, the response to CpG-induced cytokines was attenuated by HSA. These findings suggest that albumin is essential to regulate excessive cytokine production in response to circulating PAMPs and DAMPs.

CIRRHOSIS

Albumin internalizes and inhibits endosomal TLR signaling in leukocytes from patients with decompensated cirrhosis

Mireia Casulleras[1,2], Roger Flores-Costa[2], Marta Duran-Güell[1,2], José Alcaraz-Quiles[2], Silvia Sanz[2]*, Esther Titos[2,3], Cristina López-Vicario[1,2], Javier Fernández[1,4], Raquel Horrillo[5], Montserrat Costa[5], Pierre de la Grange[6], Richard Moreau[1,7], Vicente Arroyo[1], Joan Clària[1,2,3]†

Human serum albumin (HSA) is an emerging treatment for preventing excessive systemic inflammation and organ failure(s) in patients with acutely decompensated (AD) cirrhosis. Here, we investigated the molecular mechanisms underlying the immunomodulatory properties of HSA. Administration of HSA to patients with AD cirrhosis with elevated circulating bacterial DNA rich in unmethylated cytosine-phosphate-guanine dideoxynucleotide motifs (CpG-DNA) was associated with reduced plasma cytokine concentrations. In isolated leukocytes, HSA abolished CpG-DNA–induced cytokine expression and release independently of its oncotic and scavenging properties. Similar anti-inflammatory effects were observed with recombinant human albumin. HSA exerted widespread changes on the immune cell transcriptome, specifically in genes related to cytokines and type I interferon responses. Our data revealed that HSA was taken up by leukocytes and internalized in vesicles positively stained with early endosome antigen 1 and colocalized with CpG-DNA in endosomes, where the latter binds to Toll-like receptor 9 (TLR9), its cognate receptor. Furthermore, HSA also inhibited polyinosinic:polycytidylic acid– and lipopolysaccharide-induced interferon regulatory factor 3 phosphorylation and TIR domain–containing adapter-inducing interferon-β–mediated responses, which are exclusive of endosomal TLR3 and TLR4 signaling, respectively. The immunomodulatory actions of HSA did not compromise leukocyte defensive mechanisms such as phagocytosis, efferocytosis, and intracellular reactive oxygen species production. The in vitro immunomodulatory effects of HSA were confirmed in vivo in analbuminemic humanized neonatal Fc receptor transgenic mice. These findings indicate that HSA internalizes in immune cells and modulates their responses through interaction with endosomal TLR signaling, thus providing a mechanism for the benefits of HSA infusions in patients with cirrhosis.

INTRODUCTION

Systemic inflammation has emerged as the most important factor linking acutely decompensated (AD) cirrhosis with acute-on-chronic liver failure (ACLF), which is characterized by single or multiple extrahepatic organ failures and high short-term mortality (*1*–*3*). Peripheral blood leukocytosis, a predictor of organ failure(s) and death, and increased release of cytokines and other inflammatory mediators, which are markers of intense episodes of inflammation, are common findings in patients with AD cirrhosis and ACLF (*1*, *4*). In addition, persistent immune cell stimulation in these patients leads to immune paralysis, resulting in an increased frequency of bacterial infections that may precipitate organ dysfunction and ACLF (*5*). Therefore, systemic inflammation in advanced cirrhosis is at the crossroads of immune dysfunction, tissue immunopathology, and the development of extrahepatic organ failure(s) (*3*).

Infusion of human serum albumin (HSA) is an effective therapy in the management of patients with AD cirrhosis (*6*). In these patients, short- and long-term HSA administration prevents circulatory and renal dysfunction and reduces the number of hospital readmissions and mortality (*7*, *8*). In addition, a recent open-label randomized trial demonstrated that HSA administration resulted in a 38% reduction in the mortality hazard ratio, further consolidating the use of HSA infusions as disease-modifying treatment in patients with AD cirrhosis (*9*). Furthermore, the pilot-PRECIOSA study, aimed at identifying the optimal HSA dosage able to normalize HSA concentrations in patients with AD cirrhosis, and the randomized controlled INFECIR-2 study, aimed at comparing the efficacy of adding HSA to standard medical therapy with antibiotics (SMT) in patients with AD cirrhosis and active bacterial infection, recently reported that both short- and long-term HSA treatments induce immunomodulatory effects in these patients (*10*).

HSA is synthesized in the liver and continuously secreted into the bloodstream, where it is the most abundant protein (*6*, *11*). HSA is the main contributor to plasma oncotic pressure and is clinically used in conditions of hypovolemia when its synthesis is impaired such as liver cirrhosis (*6*, *11*). In addition, HSA is the main transporter of fatty acids and plays major roles in the binding of drugs and free radicals (*6*, *11*). In sharp contrast with its oncotic and scavenging properties, there is a clear lack of understanding of the mechanism of action underlying the anti-inflammatory properties of HSA. The aims of this study were to obtain a comprehensive view of the immunomodulatory properties of HSA and to investigate its potential mechanisms at cellular and molecular levels. In particular, we investigated the effects of HSA upon stimulation of human leukocytes with DNA rich in unmethylated cytosine-phosphate-guanine (CpG) dideoxynucleotide motifs (CpG-DNA), which is highly present in bacterial and mitochondrial DNA, and therefore acts as a

[1]European Foundation for the Study of Chronic Liver Failure (EF Clif), 08021 Barcelona, Spain. [2]Biochemistry and Molecular Genetics Service, Hospital Clínic-IDIBAPS-CIBERehd, 08036 Barcelona, Spain. [3]Department of Biomedical Sciences, University of Barcelona, 08036 Barcelona, Spain. [4]Liver Unit, Hospital Clínic, 08036 Barcelona, Spain. [5]Bioscience Research Group, Grifols, 08150 Barcelona, Spain. [6]GenoSplice Technology, 75005 Paris, France. [7]Centre de Recherche sur l'Inflammation (CRI), INSERM, Université Paris Diderot, CNRS, 75018 Paris, France.
*Present address: EMBL Barcelona, Carrer Dr. Aiguader 88, Barcelona 08003, Spain.
†Corresponding author. Email: jclaria@clinic.cat

pathogen- and/or damage-associated molecular pattern (PAMP and/or DAMP) (*12*, *13*). CpG-DNA triggers the activation of the immune system and the secretion of proinflammatory cytokines by its binding to intracellular (endosomal) Toll-like receptor 9 (TLR9) (*12*, *13*). We also sought to obtain more in-depth knowledge of the intracellular signaling pathways involved in the immunomodulatory properties of HSA and to ascertain its effects on leukocyte defense functions against pathogens, including phagocytosis, efferocytosis, and production of radical oxygen species (ROS).

RESULTS

Study patients

Table S1 shows the characteristics of the patients with AD cirrhosis from the INFECIR-2 study included in this investigation. Patients with AD cirrhosis showed baseline hypoalbuminemia (HSA concentration, <34 g/liter) and signs of systemic inflammation determined by elevated concentrations of interleukin-6 (IL-6), IL-1β, tumor necrosis factor α (TNFα), and C-reactive protein. After treatment, plasma IL-6 and TNFα concentrations were reduced in AD patients receiving HSA in addition to SMT but not in those receiving SMT alone (fig. S1A). Plasma IL-1β did not change after treatment (fig. S1A). These findings confirm recent studies reporting that the administration of HSA can ameliorate systemic inflammation in patients with AD cirrhosis (*10*). The reduction in plasma cytokines by HSA treatment was not the consequence of the dilution of cytokine concentrations secondary to correction of hypovolemia because other soluble protein factors present in the plasma of these patients were not altered (table S2).

Intense response to CpG-DNA in blood leukocytes and increased concentrations of CpG-DNA in patients with cirrhosis

To investigate the mechanisms underlying the systemic anti-inflammatory effects of HSA, we performed experiments in peripheral blood leukocytes isolated from healthy donors (HV) and patients with AD cirrhosis. To mimic the conditions encountered by circulating immune cells in patients with cirrhosis, peripheral blood mononuclear cells (PBMCs) were challenged with PAMPs, including lipopolysaccharide (LPS) and *N*-formyl-methionyl-leucyl-phenylalanine (*f*MLP), DAMPs, such as adenosine triphosphate (ATP), and a PAMP in combination with a DAMP (LPS and ATP). PBMCs were also challenged with CpG-DNA, which is common in bacterial and mitochondrial DNA. As shown in fig. S1 (B and C), among the different PAMPs and DAMPs, CpG-DNA produced the most intense and reproducible stimulatory effect on cytokine expression in PBMCs and polymorphonuclear leukocytes (PMNs), and therefore, this PAMP/DAMP was selected for subsequent experiments. Of note, plasma CpG-DNA concentrations were significantly ($P < 0.05$) higher in patients with compensated and AD cirrhosis relative to HV (fig. S1D). Plasma CpG-DNA concentrations did not decline during HSA treatment (fig. S1E).

HSA inhibits the leukocyte responses to CpG-DNA

We next investigated the ability of HSA to modulate CpG-DNA–induced cytokine expression in PBMCs and PMNs from HV. The HSA concentrations used in these experiments (10 to 15 mg/ml) were equivalent to those therapeutically infused to correct hypoalbuminemia (from 25 to 30 mg/ml to 35 to 45 mg/ml) in patients

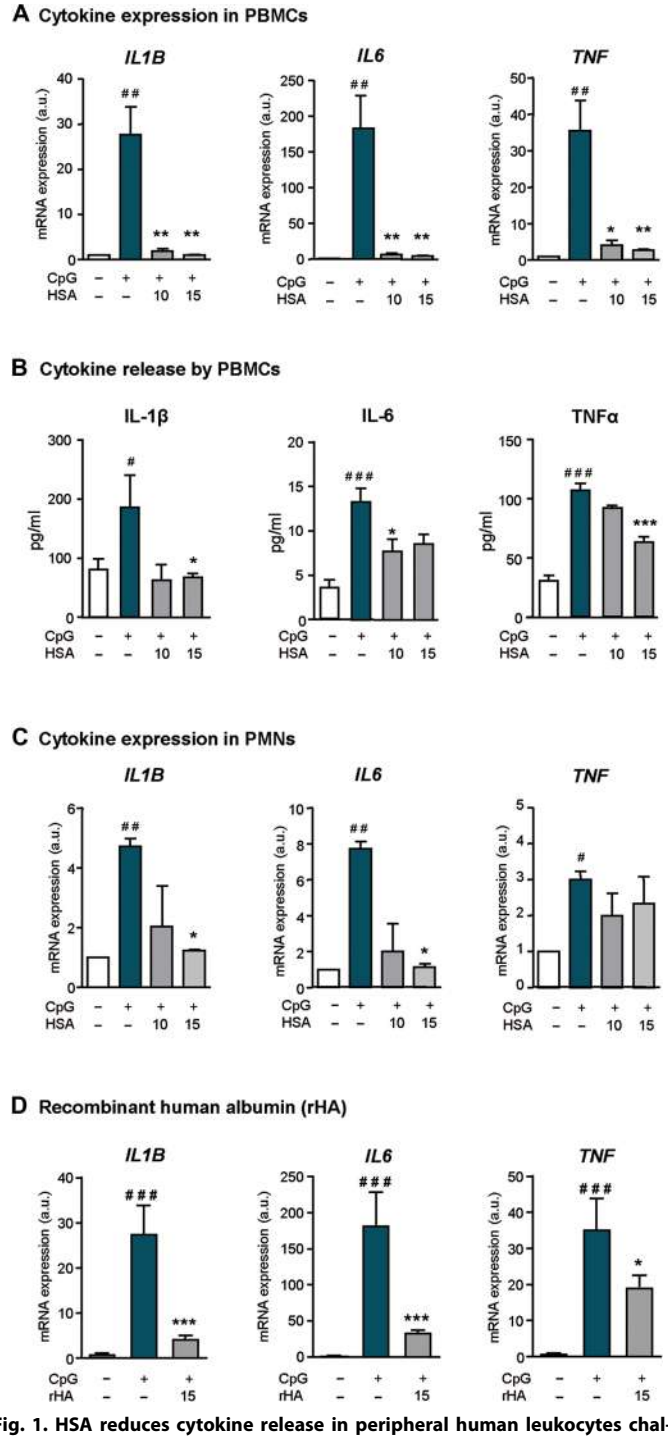


Fig. 1. HSA reduces cytokine release in peripheral human leukocytes challenged with CpG-DNA. (**A**) mRNA expression for *IL1B*, *IL6*, and *TNF* in human PBMCs from HV incubated in the absence or presence of HSA at concentrations of 10 and 15 mg/ml for 30 min and then challenged with either vehicle or CpG-DNA (2 μM) for 2 hours. (**B**) IL-1β, IL-6, and TNFα concentrations in the supernatants of experiments described in (A). (**C**) PMNs isolated from HV were incubated at the same conditions described in (A). (**D**) PBMCs from HV were incubated with rHA (15 mg/ml) for 30 min and then challenged with CpG-DNA as described in (A). Results are expressed as means ± SEM of three separate experiments performed in duplicate. $^{\#}P < 0.05$, $^{\#\#}P < 0.005$, and $^{\#\#\#}P < 0.001$ for CpG-DNA versus vehicle. $^{*}P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.001$ for CpG-DNA plus HSA versus CpG-DNA alone. a.u., arbitrary units.

with AD cirrhosis (*6*, *11*). As shown in Fig. 1A, PBMCs stimulated with CpG-DNA showed a dramatic up-regulation in *IL1B*, *IL6*, and *TNF* expression and this inflammatory reaction was prevented when PBMCs were preincubated with HSA. These inhibitory actions of HSA were confirmed at the protein level by measuring the concentrations of IL-1β, IL-6, and TNFα released into the cell supernatants using a Luminex assay (Fig. 1B). It is worth mentioning that in vitro HSA significantly ($P < 0.05$) reduced IL-1β expression at the protein level, which was not observed in patients (fig. S1A). HSA also reduced CpG-DNA–induced macrophage inflammatory protein–1α (MIP-1α) and interferon 2α (IFN2α) in PBMC incubations (fig. S2A). The anti-inflammatory actions of HSA were not specific of mononuclear leukocytes because similar effects were seen in PMNs elicited by CpG-DNA, although the response of these cells to CpG-DNA and the inhibitory effects of HSA were less intense to those seen in PBMCs (Fig. 1C). Time-course experiments revealed that the anti-inflammatory actions of HSA were sustained over time for at least 240 min (end of experiment) (fig. S2B). Last, to exclude the possibility that the anti-inflammatory actions of HSA were dependent on the presence of potential anti-inflammatory serum factors bound to HSA, we repeated the experiments with recombinant human albumin (rHA) expressed in *Oryza sativa*. As shown in Fig. 1D and fig. S2C, the inhibitory actions of rHA on CpG-DNA–induced cytokine expression were similar to those of HSA.

The effects of HSA are independent of its oncotic properties

HSA is the main determinant of oncotic pressure in the extracellular space (*14*). To rule out the possibility that changes in cytokine expression in response to HSA were due to variations in the oncotic pressure of the cell media, we used PBMCs from HV and challenged them with CpG-DNA in the presence of increasing concentrations of HSA. As shown in Fig. 2A, the inhibitory actions of HSA on CpG-DNA–induced cytokine expression were observed at concentrations as low as 0.05 mg/ml and over a wide range of HSA concentrations in the cell media. In contrast, inhibition of CpG-DNA–induced cytokine expression was not observed in the presence of increasing concentrations of mannitol, a chemically inert oncotic agent (Fig. 2B), thus dissociating the immunomodulatory actions of HSA from its oncotic properties.

The effects of HSA are independent of its scavenging properties

HSA is also a major molecule implicated in transportation and scavenging of a variety of compounds, having an impact on their delivery and efficacy (*11*). To rule out the possibility that the anti-inflammatory effects of HSA observed in the pretreatment experiments might reflect the ability of this protein to quench CpG-DNA, we next performed experiments in a treatment mode, in which HSA was added to PBMCs 2 hours after CpG-DNA stimulation. As shown in fig. S3A, HSA was also effective in the treatment mode and blocked CpG-DNA–induced cytokine expression in PBMCs to a similar extent to that observed in pretreatment experiments. Anti-inflammatory actions of HSA after the treatment strategy were also seen in PMNs (fig. S3B). These findings, together with the use of an intracellular readout not susceptible to being quenched by HSA such as monitoring of changes in mRNA expression, support the view that the immunomodulatory actions of HSA are independent of its scavenging capacities.

HSA inhibits the response of leukocytes from patients with AD cirrhosis and ACLF to CpG-DNA

To ascertain whether the immunomodulatory effects of HSA observed in leukocytes from HV were also valid in patients with advanced liver disease, we next repeated the experiments in PBMCs isolated from patients with AD cirrhosis with and without ACLF. As shown in Fig. 2C, PBMCs from AD patients showed higher baseline cytokine expression and a more intense response to CpG-DNA than those from HV (Fig. 1A for comparison), suggesting either overactivation or priming of mononuclear leukocytes in this condition. The inflammatory response to CpG-DNA was even more exacerbated in PBMCs from patients with ACLF (Fig. 2D). In both conditions, HSA blocked CpG-DNA–induced cytokine overexpression (Fig. 2, C and D) to a similar extent to that seen in HV (Fig. 1A). Similar effects were observed in PMNs from patients with AD cirrhosis and ACLF (fig. S4).

HSA inhibits extensive CpG-DNA–induced gene expression in leukocytes from patients with AD cirrhosis

To gain a wider perspective of the impact of HSA on immune cells, we next assessed the global changes in the transcriptional profile of leukocytes from patients with AD cirrhosis stimulated with CpG-DNA and incubated with either HSA or its vehicle using oligonucleotide microarrays. Among more than 30,000 genes analyzed, 133 were found to be differentially expressed (85 up-regulated and 48 down-regulated) in leukocytes stimulated by CpG-DNA and 343 (146 up- and 197 down-regulated) in those treated with CpG-DNA and HSA (fig. S5). Then, we used the recently described 346 blood transcription modules (BTMs), which have been shown to be a very useful tool for analysis of blood immune cell transcriptome (*15*, *16*). We performed pairwise comparisons between CpG-DNA versus vehicle and between CpG-DNA plus HSA versus CpGNA alone and selected the BTMs with a *P* value of below 0.05 in at least one of the two comparisons and drew a ring plot according to the calculated *t* score. This analysis resulted in the selection of 17 BTMs, each containing a set of genes of interest (tables S3 and S4). According to this analysis, CpG-DNA had extensive immunostimulatory effects on leukocytes from patients with AD cirrhosis, resulting in the induction of genes involved in innate immunity [innate activation of cytosolic DNA sensing, neutrophil recruitment, myeloid, dendritic cell activation via NF-κB (nuclear factor κB), and natural killer cells], adaptive immunity [CD4 T cell surface signature T helper 1 (T_H1)–stimulated and B cell development], and cytokines and chemokines (myeloid cell cytokines, metallopeptidases and laminins, cytokine receptor cluster, and chemokine cluster) (Fig. 3). The modules related to immune responses (cytokines-receptors cluster, activated dendritic cells, type I IFN responses, and platelet activation and degranulation) were markedly affected by HSA. The genes that were up-regulated or down-regulated ($P < 0.05$) by HSA in these conditions are shown in tables S5 and S6, respectively.

HSA uptake by leukocytes colocalizes in early endosomes

Because HSA inhibited the effects of CpG-DNA, which predominantly signals through endosomal TLR9 (*12*, *13*, *17*), we asked whether HSA may reach the endosomal compartment by incubating PBMCs with fluorescein isothiocyanate (FITC)–labeled HSA and examined its uptake by confocal microscopy. As shown in Fig. 4A, FITC-labeled HSA was internalized in resting PBMCs (top), a response that was enhanced in leukocytes challenged with CpG-DNA

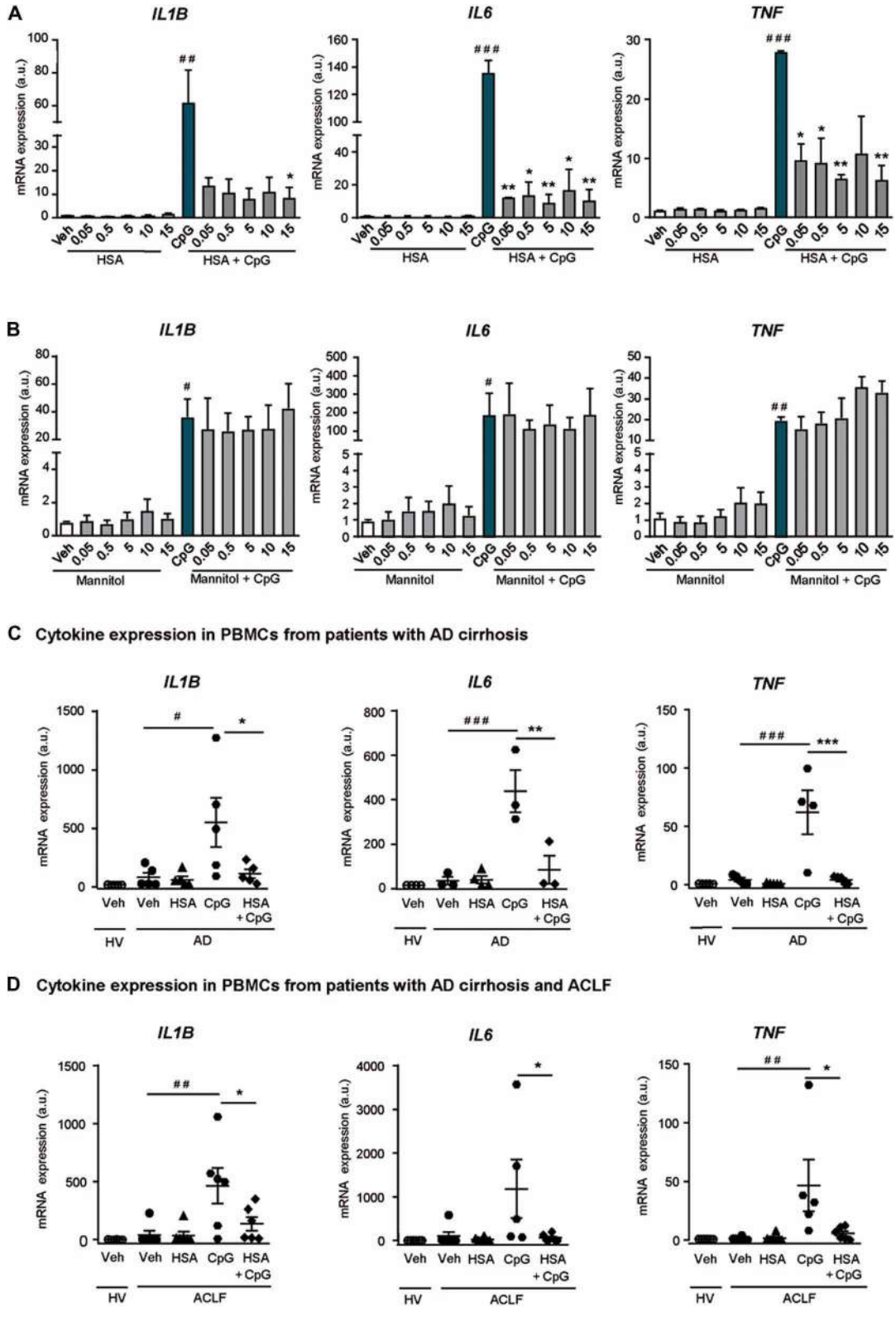


Fig. 2. The immunomodulatory actions of HSA are independent of their oncotic properties and are reproduced in PBMCs from patients with AD cirrhosis with and without ACLF. (**A**) *IL1B*, *IL6*, and *TNF* mRNA expression in PBMCs from HV that were incubated with increasing concentrations (from 0.05 to 15 mg/ml) of HSA in the absence or presence of CpG-DNA. (**B**) Experiments were performed as described in (A) but with increasing concentrations (0.05 to 15 mg/ml) of mannitol. (**C**) PBMCs isolated from HV ($n = 5$) and patients with AD cirrhosis ($n = 5$) and incubated with either vehicle or CpG-DNA for 2 hours and then with HSA or vehicle for 2 more hours. (**D**) Experiments were performed as described in (C) but with PBMCs from patients with ACLF ($n = 5$). Results are expressed as means ± SEM and are representative of three to six experiments performed in duplicate. $^{\#}P < 0.05$, $^{\#\#}P < 0.005$, and $^{\#\#\#}P < 0.001$ for CpG-DNA versus vehicle. $^{*}P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.001$ for CpG-DNA plus HSA versus CpG-DNA alone.

(bottom). Flow cytometry analysis revealed that about 11% of the cells within the PBMC fraction were loaded with FITC-labeled HSA (Fig. 4B), with this percentage being consistent with the proportion of myeloid cells (~10 to 20%) populating a typical PBMC suspension. Among the cells loaded with FITC-labeled HSA, 71.8% were double positive for CD45/CD14, indicating their myeloid origin (Fig. 4B). The percentage of PBMCs (28.4%) and double-positive CD45/CD14 cells (82.5%) loaded with FITC-labeled HSA was also enhanced by CpG-DNA (Fig. 4B). We next performed a time-course study of the intracellular trafficking of FITC-conjugated HSA and demonstrated that after its uptake, HSA is retained in intracellular vesicles for a period from 10 to 120 min. By performing immunofluorescence staining with an Alexa Fluor 555–labeled secondary antibody against a primary anti-EEA1 antibody, which specifically recognizes early endosomes, we found that FITC-labeled HSA colocalized with EEA1-positive stained vesicles, mainly at early time periods (10 min) (Fig. 4C).

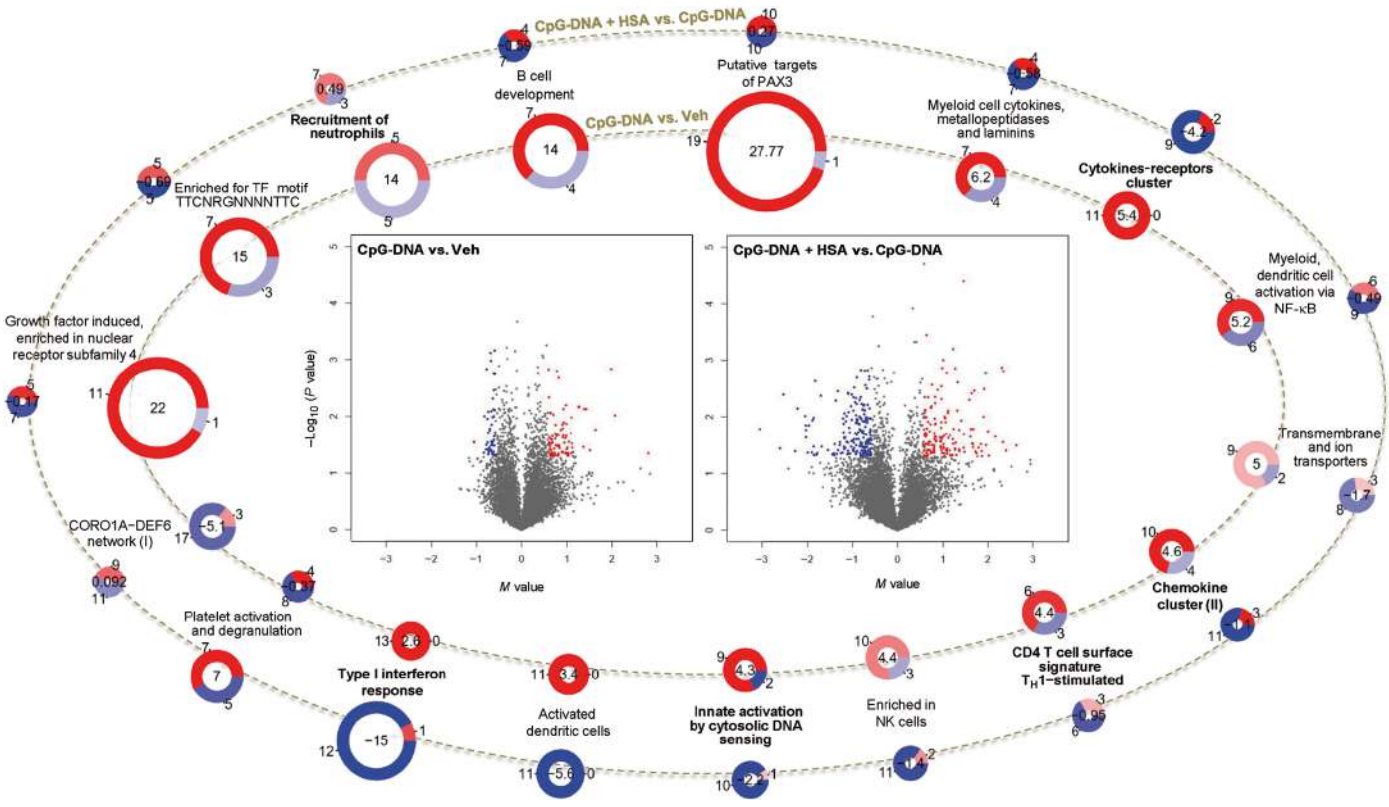


Fig. 3. HSA modulates the transcriptional profile of leukocytes from patients with AD cirrhosis stimulated with CpG-DNA. Transcriptomic analysis using oligonucleotide microarrays was performed in PBMCs from patients with AD cirrhosis ($n = 10$) incubated with either vehicle (Veh), CpG-DNA (4 μM), or CpG-DNA plus HSA (15 mg/ml) for 24 hours. Volcano plots indicate up-regulated (red) and down-regulated (green) genes with a fold change of ≥1.5 and a P value of <0.05 in PBMCs stimulated with CpG-DNA in comparison to vehicle (left graph) and in PBMCs incubated with CpG-DNA plus HSA compared with CpG-DNA alone (right graph). Circular charts surrounding volcano plots represent 17 BTMs of interest and pairwise comparisons between CpG-DNA and vehicle (inner dashed line) or between CpG-DNA plus HSA and CpG-DNA alone (outer dashed line) with a P value of below 0.05 in at least one of the two comparisons. Each BTM is shown as an open circle, and the number at the center of the circle corresponds to the value of t score for the corresponding pairwise comparison. The size of the circle depends on the P value of the comparison: the more significant the P value, the wider the circle. In addition, the number of up- and down-regulated genes is given for every BTM and illustrated by giving to the circular chart a red color and a blue color, respectively.

HSA and CpG-DNA colocalize in early endosomes

We next performed a time-dependent analysis of the intracellular trafficking of FITC-conjugated CpG-DNA, and with the use of an EEA1 antibody labeled with Alexa Fluor 555, we were able to demonstrate that CpG-DNA was a major and persistent cargo of endosomes (fig. S6), the cell compartment where CpG-DNA binds to TLR9 (*12*, *13*, *17*). By using an HSA-specific antibody recognized by a secondary antibody labeled with Alexa Fluor 647, we demonstrated that HSA colocalized with FITC–CpG-DNA in EEA1-positive endosomes, an observation that was more evident at 10 min of HSA incubation (Fig. 5). Together, these results suggest that HSA blocks the inflammatory actions of CpG-DNA by interfering with the CpG-DNA–TLR9 signaling pathway in the endosomal compartment.

HSA inhibits endosomal TLR9 signaling

To gain further confirmation that HSA acts on endosomal TLR signaling, PBMCs were exposed to chloroquine, which inhibits endosomal acidification and blocks the activation of endosomal TLRs by nucleic acids (fig. S7). As shown in Fig. 6A, chloroquine produced a similar blockage of CpG-DNA–induced *IL6* expression (selected as readout of proinflammatory cytokines) to that of HSA. Of note, combination of HSA and chloroquine was associated with a greater reduction in *IL6* expression (Fig. 6A), suggesting that changes in endosomal acidification cannot account for HSA actions in a fully explainable manner. This is consistent with the finding that chloroquine was ineffective when added 2 hours after the addition of CpG-DNA, whereas the combination of HSA with chloroquine was still able to produce some changes in CpG-DNA–induced *IL6* expression under these conditions (Fig. 6B). To narrow down the HSA actions to the endosomal CpG-DNA–TLR9 signaling pathway, we next used the compound pepinh-MyD, a specific peptide inhibitor of the adaptor myeloid differentiation primary response 88 (MyD88), which links TLR9 signaling in the endosome to NF-κB activation. As expected, pepinh-MyD completely blocked the CpG-DNA–induced *IL6* expression, an effect that was comparable to that of HSA (fig. S8A).

The inhibitory actions of HSA are not exclusive to TLR9 but common to other endosomal TLRs

We next asked whether, in addition to TLR9, HSA also modulates other TLRs localized in the endosomal compartment (fig. S7). This is the case of TLR3, which recognizes pathogen double-stranded RNA in the endosome. However, in contrast to TLR9 that transduces signals through MyD88, TLR3 uses TIR domain–containing

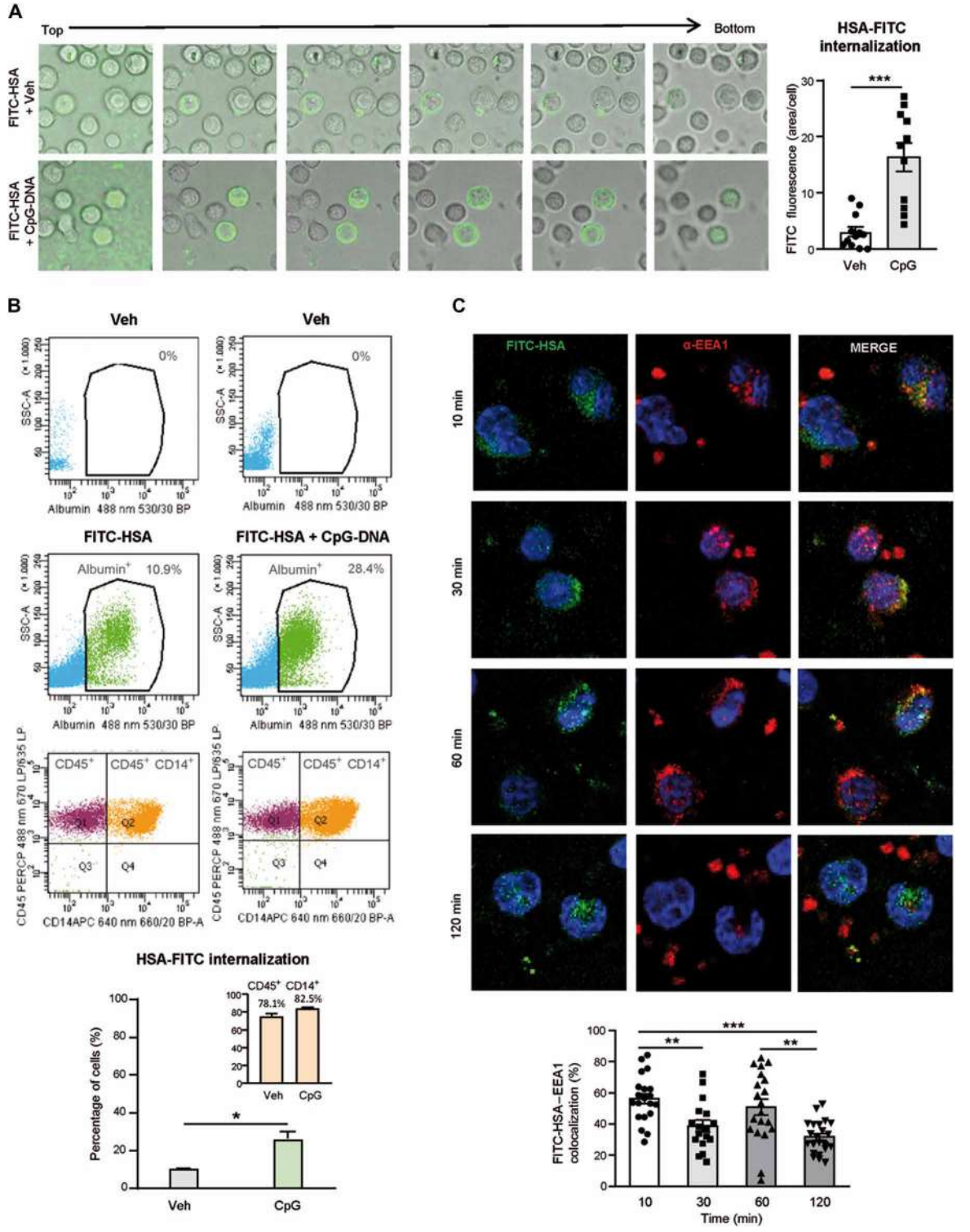


Fig. 4. HSA is internalized in endosomes by human leukocytes. (**A**) Representative images segmented in different *Z* stacks from the top to the bottom of the plate captured with confocal microscopy of PBMCs from HV and incubated with FITC-labeled HSA (green) (5 mg/ml) alone (top) or in combination with CpG-DNA (2 μM) (bottom) for 2 hours. (**B**) Representative flow cytometry plots of PBMCs incubated with vehicle (top) or FITC-labeled HSA (5 mg/ml) (middle) in the presence or absence of CpG-DNA (2 μM) for 2 hours. The bottom panel shows the population of FITC-HSA–positive PBMCs that was double positive for CD45 and CD14. (**C**) Representative confocal images of PBMCs incubated with FITC-labeled HSA (green) for 10 to 120 min and then immunostained with an Alexa Fluor 555–labeled antibody (red) against a primary antibody that recognizes EEA1, a marker of early endosomes. Nuclei were stained with Hoechst 33342 (blue). Images were taken at 63× oil objective, zoom 3. Results are representative of two to three independent experiments. **P* < 0.05, ***P* < 0.005, and ****P* < 0.001 for CpG-DNA versus vehicle or between the different time points.

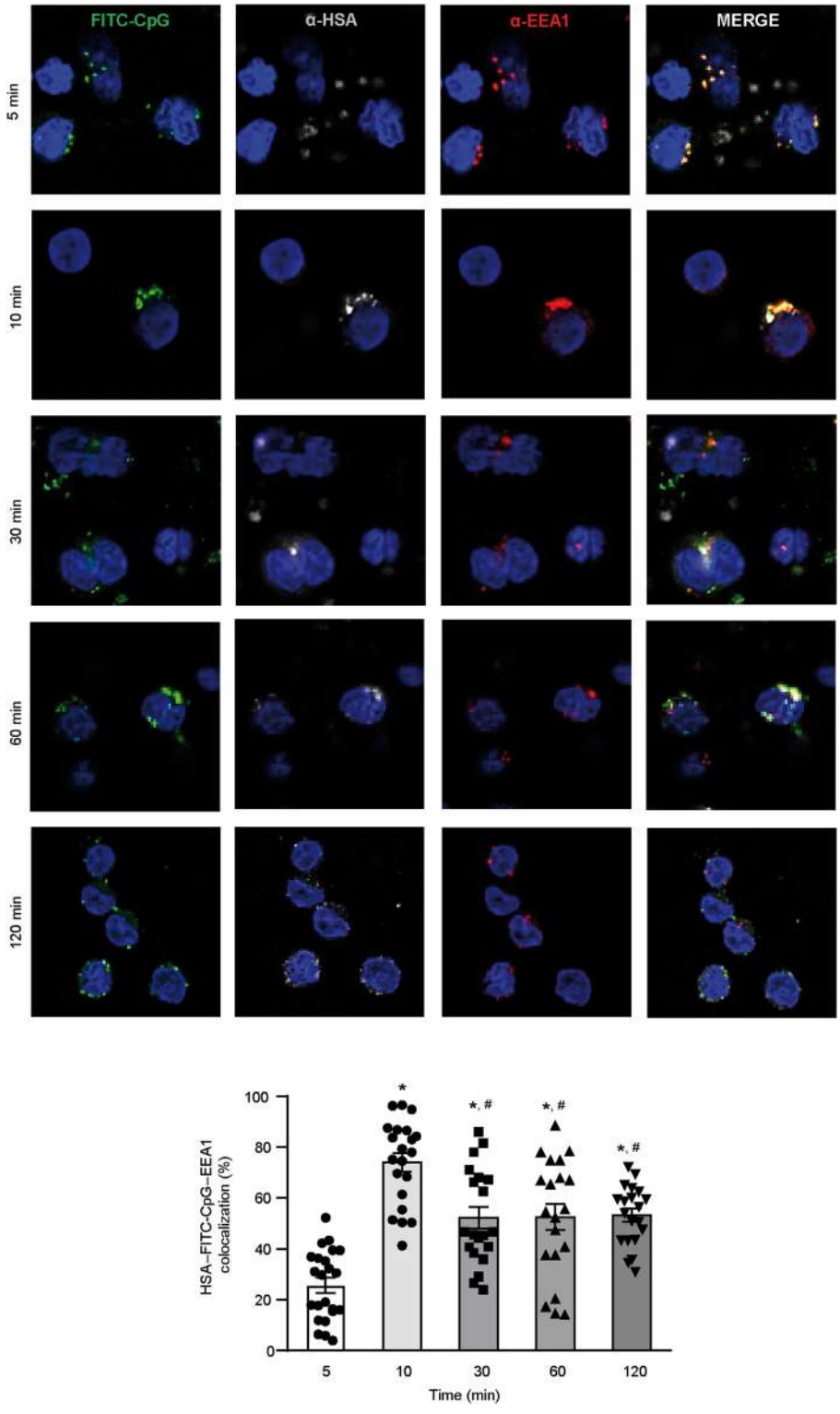


Fig. 5. HSA colocalizes with CpG-DNA in early endosomes. Representative confocal images of triple colocalization of CpG-DNA, HSA, and EEA1 (early endosomes) in PBMCs. Cells were incubated with FITC–CpG-DNA (2 μM) (green) and HSA (5 mg/ml) for 5 to 120 min and then incubated with a primary anti-HSA antibody that was stained with a secondary antibody labeled with Alexa Fluor 647 (gray). Cells were also stained intracellularly with anti-EEA1 with the secondary antibody Alexa Fluor 555 (red). Nuclei were stained with Hoechst 33342 (blue). Images were taken at 63× oil objective, zoom 3 and are representative of two to three independent experiments. $^{*}P < 0.001$ and $^{\#}P < 0.005$ with respect to 5 and 10 min, respectively.

adapter-inducing IFN-β (TRIF), leading to the induction of type I IFN genes mediated by IFN regulatory factor 3 (IRF3) (*18*). To address this question, we performed experiments in PBMCs stimulated with polyinosinic:polycytidylic acid [poly(I:C)] (a synthetic double-stranded RNA agonist of TLR3). As shown in Fig. 6 (C to E), PBMCs stimulated with poly(I:C) exhibited induction of TRIF-dependent cytokines including C-X-C motif chemokine 10 (*CXCL10*) and interferon beta 1 (*IFNB1*) as well as induction of the prototypic IFN-stimulated gene 2′-5′-oligoadenylate synthetase 2 (*OAS2*), an induction that was considerably reduced by HSA. Consistent with these findings, HSA decreased poly(I:C)-induced IRF3 phosphorylation (Fig. 6F). The inhibition of the poly(I:C)-induced *CXCL10* expression was of higher magnitude in PBMCs treated with HSA in combination with the specific TRIF inhibitor pepinh-TRIF than in PBMCs treated with HSA alone (fig. S8B). We finally explored whether HSA also interferes with endosomal TLR4 signaling. TLR4 is a cell surface receptor that, after recognizing LPS, engages MyD88 for the early phase of NF-κB activation (fig. S7). There is also a MyD88-independent pathway of TLR4 signaling that involves the translocation of the TLR4-LPS complex from the cell membrane to the endosome, where it recruits the TRIF adaptor to trigger the production of type I IFN–inducible genes (*19*). As shown in Fig. 6G, HSA was also able to ameliorate the late phase of TLR4 activation by LPS and reduced the expression of the TRIF-dependent cytokine *CXCL10*. Changes in the expression of the IFN-stimulated gene *OAS2* did not reach statistical significance (Fig. 6H). A reduction in LPS-induced IRF3 phosphorylation was also observed in PBMCs incubated with HSA (Fig. 6I). To characterize the relative contribution of HSA inhibition of TLR4 signaling in the endosome (TRIF pathway) versus TLR4 signaling in the cell membrane (MyD88 pathway), we compared the effects of chloroquine on *CXCL10* gene expression with those of polymyxin B (which disrupts LPS binding to TLR in cell membranes) on *IL6* expression in PBMCs exposed to LPS. As shown in fig. S8 (C and D), HSA exhibited similar inhibitory effect on LPS-induced *CXCL10*

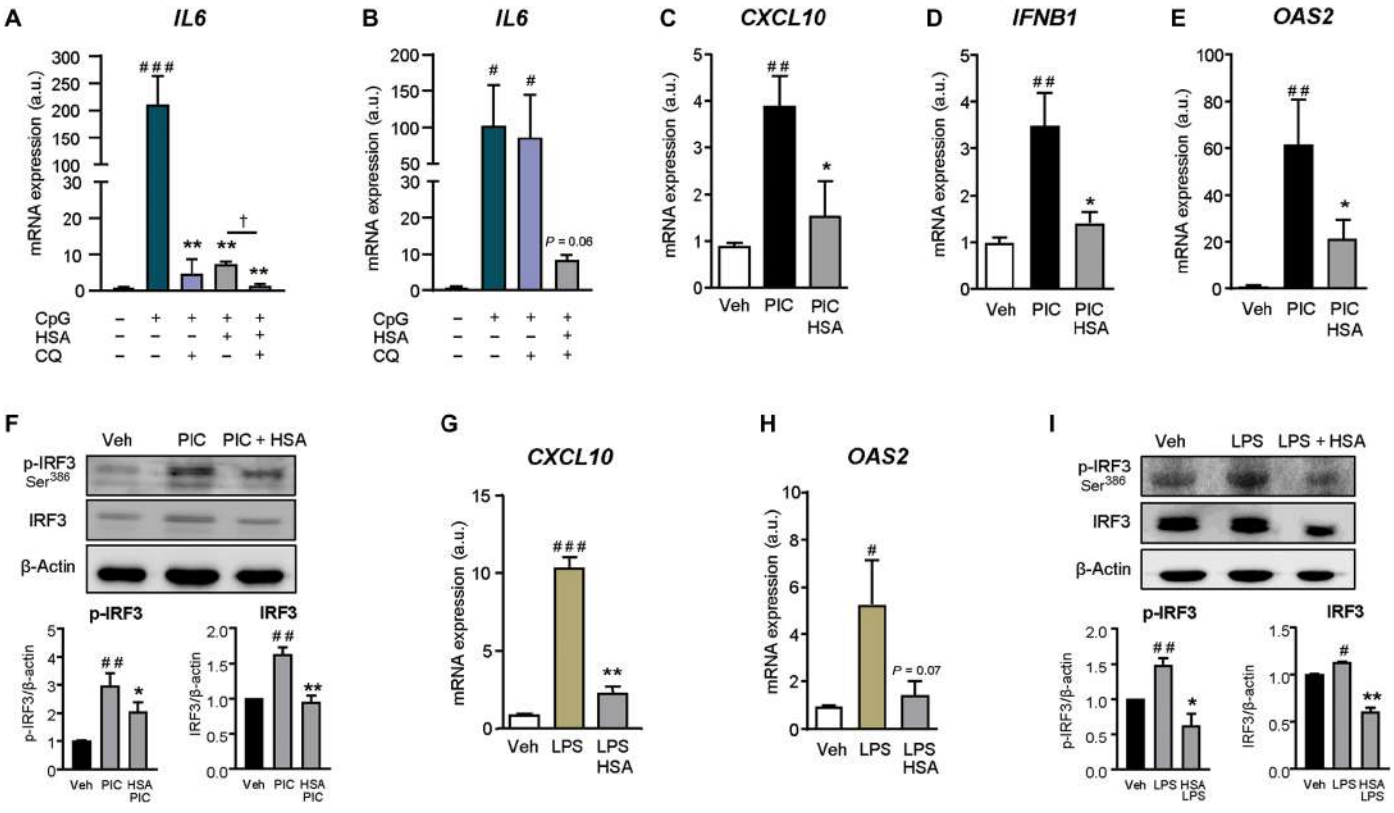


Fig. 6. HSA blocks endosomal TLR signaling. (**A**) Effects of chloroquine (CQ) on CpG-DNA–induced *IL6* mRNA expression. PBMCs were incubated with vehicle or chloroquine (50 μM) for 30 min before addition of vehicle or HSA (15 mg/ml) for an additional 30 min and then challenged with CpG-DNA (4 μM) for 2 hours. (**B**) Changes in CpG-DNA–induced *IL6* mRNA expression in PBMCs treated as described in (A), except that chloroquine was added 1 hour after incubation with HSA and CpG-DNA. (**C**) *CXCL10* mRNA expression in PBMCs incubated with poly(I:C) (PIC; 50 μg/ml) for 5 hours in the presence or absence of HSA (15 mg/ml) for 30 min. (**D**) *IFNB1* mRNA expression in PBMCs treated as in (C). (**E**) *OAS2* mRNA expression in conditions described in (C) and (D). (**F**) Protein expression of phosphorylated IRF3 (p-IRF3), IRF3, and β-actin as determined by Western blot in PBMCs incubated for 30 min with PIC (50 μg/ml) in the presence or absence of HSA (15 mg/ml). (**G**) *CXCL10* mRNA expression in PBMCs incubated with LPS (10 ng/ml) for 4 hours in the presence or absence of HSA (15 mg/ml) for 30 min. (**H**) *OAS2* mRNA expression in PBMCs treated as in (G). (**I**) Protein expression of p-IRF3, IRF3, and β-actin in PBMCs incubated for 60 min with LPS (10 ng/ml) in the presence or absence of HSA (15 mg/ml). Results are expressed as means ± SEM of $n = 2$ to 4 experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.005$, and $^{\#\#\#}P < 0.001$ for stimulus versus vehicle. $^{*}P < 0.05$ and $^{**}P < 0.001$ for stimulus plus HSA or chloroquine versus stimulus alone. $^{\dagger}P < 0.05$ for HSA plus chloroquine versus HSA alone.

expression to that of chloroquine, whereas HSA was less effective than polymyxin B in inhibiting LPS-induced *IL6* expression. Collectively, these findings identify HSA as a potent modulator of endosomal TLR signaling.

HSA internalization is partially mediated by FcRn binding and preferentially involves clathrin-independent, caveolae-mediated endocytic pathways

To investigate whether the immunomodulatory effects of HSA were dependent on its binding to neonatal Fc receptor (FcRn), we incubated PBMCs with HSA and CpG-DNA in the presence of a blocking FcRn antibody (ADM31) or its isotype control (IgG2b). As shown in Fig. 7A, the inhibitory actions of HSA on CpG-DNA–induced cytokine expression were only partially reversed by disruption of FcRn-HSA interactions with ADM31. We next explored whether HSA internalization was related to caveolin-mediated endocytosis, which is another major endocytic pathway. Because caveolae forms flask-shaped invaginations of the plasma membrane in association to lipid rafts enriched in cholesterol (*20*), we depleted cell membrane cholesterol content with methyl-β-cyclodextrin (MBCD) (fig. S9). As shown in Fig. 7B, exposure of leukocytes to MBCD completely abrogated the inhibitory actions of HSA on CpG-induced cytokine expression. Together, these findings indicate that HSA is internalized by leukocytes predominantly through clathrin-independent, caveolin-mediated endocytic pathways.

HSA does not impair leukocyte defensive functions

We next sought to investigate whether HSA effectively reduces the release of cytokines by leukocytes without compromising their defense mechanisms. To test this, we assessed the impact of HSA on phagocytosis, efferocytosis, and ROS production, three critical leukocyte defensive functions against pathogens. As shown in Fig. 7C, HSA did not impair, but rather increased, the phagocytosis of fluorescently labeled zymosan particles by peripheral blood monocyte–derived macrophages (PBMDMs). In addition, HSA enhanced efferocytosis of apoptotic PMNs by M2-polarized PBMDMs (Fig. 7D and fig. S10). Furthermore, HSA did not impair the PMN capacity to intracellularly produce ROS in response to phorbol 12-myristate 13-acetate (PMA) or *Escherichia coli* (Fig. 7E).

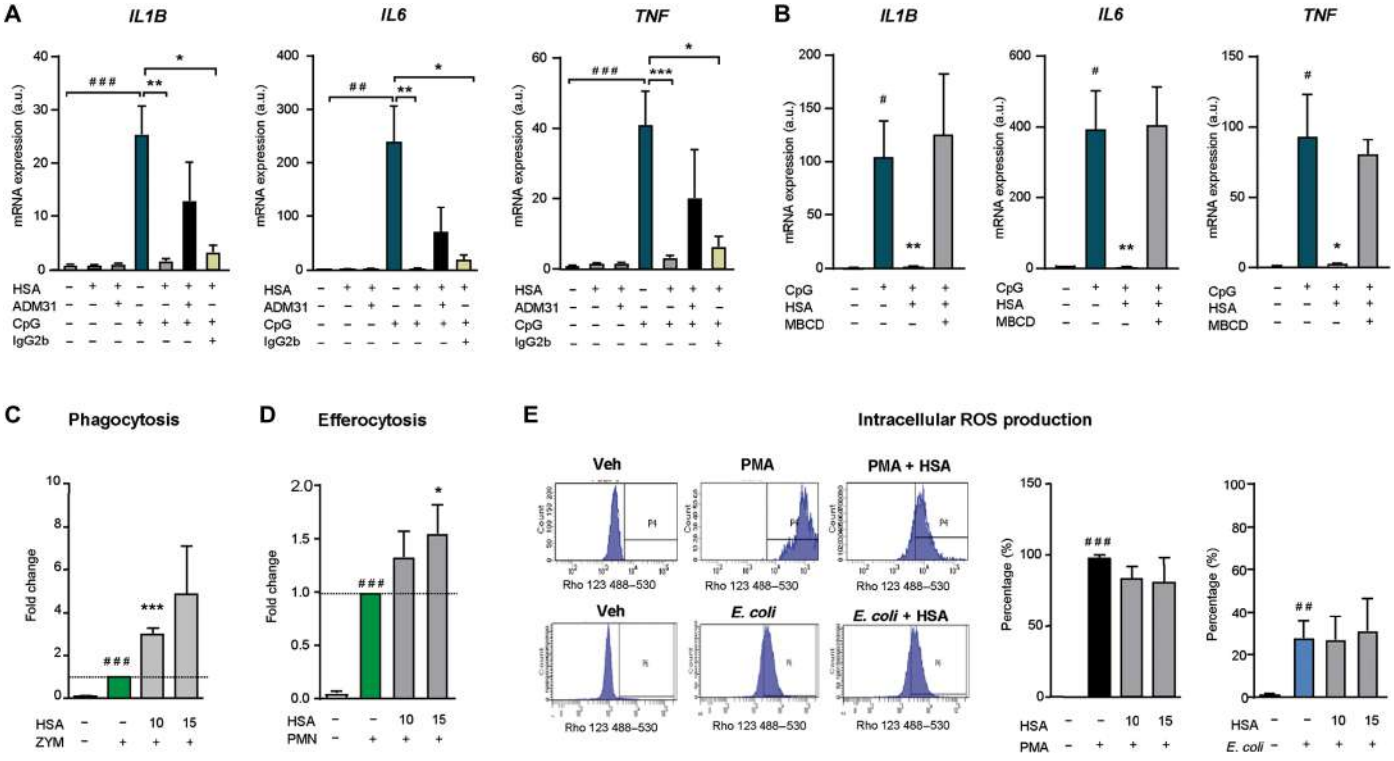


Fig. 7. HSA internalization involves clathrin-independent and caveolae endocytic pathways. Effects of HSA on cell defense mechanisms. (**A**) *IL1B*, *IL6*, and *TNF* mRNA expression in human PBMCs incubated with a blocking FcRn antibody (ADM31) or its isotype control (IgG2β) at 10 μg/ml for 20 min before adding CpG-DNA alone or CpG-DNA plus HSA for 2 hours. (**B**) PBMCs incubated with MBCD (10 mM) for 30 min before the addition of HSA (15 mg/ml) for an additional 30 min and challenged with CpG-DNA (4 μM) for 2 hours. (**C**) Phagocytic capacity of human PBMDMs incubated with FITC-conjugated zymosan bioparticles for 60 min in the presence or absence of HSA (10 or 15 mg/ml). The response to zymosan alone was considered as the maximum (100%) phagocytic capacity. (**D**) Efferocytosis assay in M2-polarized BMDM incubated with 5-Carboxyfluorescein diacetate (CFDA)–stained apoptotic neutrophils for 60 min in the presence or absence of HSA (10 or 15 mg/ml). (**E**) Representative flow cytometry plots of intracellular ROS production in DHR123 (dihydrorhodamine 123)–loaded human neutrophils stimulated with PMA (200 nM) or *E. coli* (1×10^9 to 2×10^9 bacteria/ml) in the presence or absence of HSA (10 and 15 mg/ml) for 2 hours. Quantification of intracellular ROS production is shown on the right. Results are expressed as means ± SEM of three independent experiments performed in duplicate (A, B, and E) and five independent experiments performed in quadruplicate (C and D). $^{\#}P < 0.05$, $^{\#\#}P < 0.005$, and $^{\#\#\#}P < 0.001$ for stimulus versus vehicle. $^{*}P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.001$ for stimulus plus HSA versus stimulus alone.

In vivo translation of the in vitro HSA anti-inflammatory effects

To translate in vivo our in vitro observations, we finally performed experiments in humanized analbuminemic mice expressing the human FcRn receptor ($Alb^{-/-}FcRn^{-/-}hFcRn^{+/+}$). Under resting conditions, leukocytes from $Alb^{-/-}FcRn^{-/-}hFcRn^{+/+}$ mice showed similar inflammatory tone to that of wild-type mice, assessed by the expression of *Il1b*, *Il6*, and *Tnf* (Fig. 8A). However, as compared to wild type, the response to CpG-DNA was more pronounced in leukocytes from analbuminemic mice for *Il1b* and *Il6* but not for *Tnf* (Fig. 8A). The response in these transgenic mice was attenuated by HSA (Fig. 8B). These findings suggest that albumin is essential to regulate excessive cytokine production in response to circulating PAMPs and DAMPs.

DISCUSSION

The results of the current study provide a mechanism of action for the immunomodulatory properties of HSA in human leukocytes stimulated with CpG-DNA. This mechanism is mediated by the internalization of HSA into the cytoplasm of leukocytes and its interaction with TLR trafficking and/or signaling in the endosomal compartment. This mechanism is independent of its oncotic and scavenging properties and is unrelated to any serum factor that might be bound to HSA. Our results are consistent with previously described effects of HSA independent of its role as plasma volume expander in experimental cirrhosis, involving its capacity to counteract the negative effects of TNFα and oxidative stress on cardiac tissue (*21*). It is worth mentioning that the concentrations of HSA used in our study were equivalent to those clinically used to correct hypoalbuminemia in patients with cirrhosis (*6*, *9*, *11*). However, we observed immunomodulatory actions in vitro at very low concentrations of HSA, a finding that is consistent to that reported by previous studies evaluating the effects of this molecule in hepatocytes (*22*). The reason why in the clinical practice hypoalbuminemia needs to be corrected to see the immunomodulatory actions of HSA is at present unknown, but it is likely related to the fact that infusions of exogenous HSA, which is enriched in its reduced (non-oxidized) form, not only correct hypoalbuminemia but also reduce the proinflammatory environment present in patients with AD cirrhosis. Patients with AD cirrhosis have abundant oxidized HSA forms, such as nonmercaptoalbumin-1, in the circulation (*4*), which is a potent inducer of leukocyte activation and cytokine secretion (*23*), something that does not occur in vitro in isolated cells. Collectively, the results of the present study contribute to the understanding of

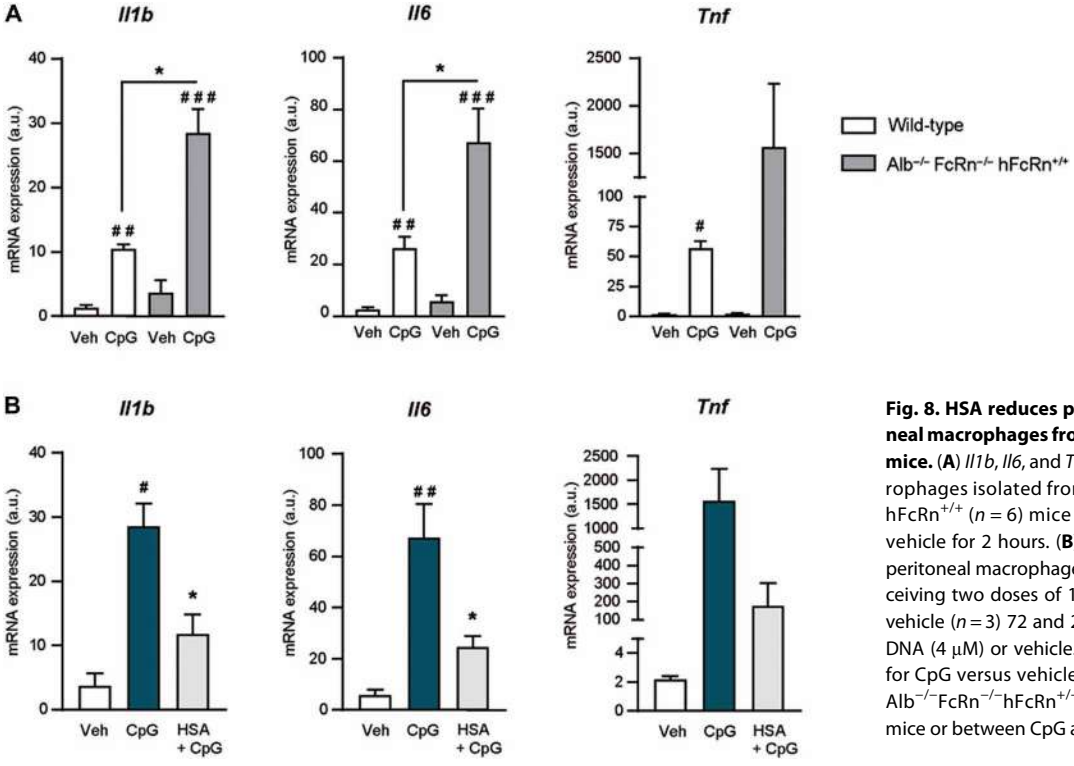


Fig. 8. HSA reduces proinflammatory cytokines in peritoneal macrophages from humanized Alb$^{-/-}$FcRn$^{-/-}$hFcRn$^{+/+}$ mice. (**A**) *Il1b*, *Il6*, and *Tnf* mRNA expression in peritoneal macrophages isolated from wild-type ($n = 6$) and Alb$^{-/-}$FcRn$^{-/-}$hFcRn$^{+/+}$ ($n = 6$) mice challenged with CpG-DNA (4 μM) or vehicle for 2 hours. (**B**) *Il1b*, *Il6*, and *Tnf* mRNA expression in peritoneal macrophages from Alb$^{-/-}$FcRn$^{-/-}$hFcRn$^{+/+}$ mice receiving two doses of 1.5 g/kg body weight of HSA ($n = 3$) or vehicle ($n = 3$) 72 and 24 hours before stimulation with CpG-DNA (4 μM) or vehicle. $^{\#}P < 0.05$, $^{\#\#}P < 0.005$, and $^{\#\#\#}P < 0.001$ for CpG versus vehicle. $^{*}P < 0.05$ for the response to CpG in Alb$^{-/-}$FcRn$^{-/-}$hFcRn$^{+/+}$ mice compared to that in wild-type mice or between CpG and HSA and CpG alone.

the immunomodulatory effects of HSA in patients with AD cirrhosis, in whom HSA therapy is associated with a reduction in the circulating concentrations of cytokines (*10*). Moreover, because systemic inflammation is a driver of severity in cirrhosis (*4*), our findings would explain the improvement in outcomes (in particular, survival) observed in patients with AD cirrhosis receiving long-term HSA therapy (ANSWER trial) (*9*).

Our study demonstrates HSA internalization in human leukocytes. Previous reports have described the uptake of HSA in rat alveolar type II epithelial cells and astrocytes (*24*, *25*), mouse hepatocytes (*22*), and several cell lines (*26*, *27*). In our study, we demonstrated that HSA is internalized by leukocytes by tracking the fate of fluorescently labeled HSA. Ten minutes after the incubation of leukocytes with labeled HSA, confocal microscopy showed the appearance of punctate fluorescent signals in the cytoplasm. Subsequent immunofluorescence assays revealed that the intracellular HSA signal colocalized with a marker of early endosomes, indicating that, similar to previous studies in other cell types (*26*, *27*), HSA preferentially targets this cellular compartment in leukocytes. Although the precise intracellular trafficking of HSA in leukocytes was not fully described in the present study, it is likely that HSA could undergo either recycling or lysosomal degradation after its internalization.

Uptake of HSA can be mediated by clathrin-dependent (*24*, *28*) or clathrin-independent, caveolin-mediated (*27*, *29*) endocytic routes, depending on each cell type. There are, however, specific cell types using both clathrin-dependent and clathrin-independent mechanisms to uptake HSA (*30*). Our results indicate that HSA uptake by leukocytes is predominantly mediated by a clathrin-independent, caveolae-mediated pathway. This endocytic route relies on the formation of caveolin-1–enriched smooth invaginations of the plasma membrane in close association with lipid rafts (*19*). Disruption of lipid rafts in leukocytes produced a complete inhibition of the immunomodulatory actions of HSA. However, lipid rafts are not exclusive of caveolin-mediated endocytosis and also participate in clathrin-mediated endocytosis. In this regard, the immunomodulatory actions of HSA in leukocytes were partially inhibited by a FcRn blocking antibody. Considering that HSA binding to FcRn is linked to the activation of clathrin-mediated endocytosis (*22*, *31*, *32*), these findings suggest that clathrin-dependent routes might also be involved.

A major finding of our study was the observation that once internalized into endosomes, HSA was able to block the production of cytokines by leukocytes in response to CpG-DNA. Bacterial DNA, as well as mitochondrial DNA, is enriched in unmethylated CpG dinucleotides in its sequence and has the ability to trigger the immune response by acting like a PAMP and/or DAMP (*12*, *13*, *33*). PAMPs and DAMPs are sensed by intracellular receptors, of which TLR3, TLR7, TLR8, and TLR9 are localized in endosomes (*34*). In particular, CpG-DNA is internalized via clathrin-mediated endocytosis in early endosomes, where it binds to its cognate receptor, TLR9 (*17*), to induce a MyD88-mediated signaling, resulting in the induction of inflammatory cytokines. This process is of high complexity because after CpG-DNA moves into early endosomes and is subsequently transported to a tubular lysosomal compartment, TLR9 needs to be redistributed from the endoplasmic reticulum (ER) to the Golgi and then interact with the CpG-DNA–containing structures, which also recruit MyD88 (*35*). Because inappropriate activation of endosomal TLRs has the potential to trigger autoimmune diseases through interaction with self-nucleic acids, their intracellular trafficking is tightly controlled (*36*). For instance, TLR9 trafficking and signaling is controlled by the insulin-regulated aminopeptidase, which retains this TLR in the ER (*37*). In the present

study, HSA inhibition of CpG-DNA–stimulated cytokine production in leukocytes was likely mediated by the accumulation of this protein in the endosomal compartment and the blockage of TLR9 activation and signaling, although exact mechanisms remain to be elucidated. In any case, the view that HSA exerts immunomodulatory actions in leukocytes after its internalization is supported by five main observations. First, in leukocytes incubated with HSA, this protein was rapidly internalized in endosomes. Second, in the endosomes, HSA colocalized with CpG-DNA. Third, HSA not only modulated TLR9 signaling but also reduced TLR3 activation by poly(I:C) as well as the late wave of TLR4 activation by LPS, being events that are exclusively localized in endosomes. Fourth, exposure of leukocytes to chloroquine, which inhibits endosomal acidification and, consequently, activation of TLRs by nucleic acids, produced similar effects to those of HSA on CpG-DNA–induced cytokine expression. Moreover, our data indicate that HSA blocks CpG-DNA actions through the TLR9-MyD88 axis. Fifth, the reduction of cytokine production was independent of HSA scavenging properties because this phenomenon was observed not only in leukocytes first incubated with HSA and later challenged with CpG-DNA but also in leukocytes in which HSA was added after the CpG-DNA challenge. HSA effects on cytokine production were also independent of its oncotic properties and were not seen with another volume expander such as mannitol. Moreover, HSA induced a reduction in cytokine mRNA expression, which was used as an intracellular readout. It is important to mention that the intracellular HSA signal was higher and persisted for longer periods of time when leukocytes were stimulated with CpG-DNA, suggesting enhanced uptake and/or distinct intracellular trafficking of HSA in activated leukocytes.

There were some limitations in our study. First, we were not able to completely dissect the signaling pathways underlying the anti-inflammatory actions of HSA. For example, the mechanism by which HSA blocks CpG-DNA–induced cytokine expression and at which point of the signaling cascade is HSA acting (modifying endosomal acidification, blocking TLR9 dimerization, or affecting the MyD88 complex formation or any other factor within the signaling route) were not completely elucidated in the current investigation. Second, future studies are required to fully understand the endocytic route(s) involved in HSA uptake by leukocytes. Last, future studies are also needed to fully understand the intracellular trafficking routes of HSA after its uptake by leukocytes.

In summary, our findings provide a mechanism by which HSA modulates the excessive production of cytokines by leukocytes without affecting the defense functions of these cells against pathogens. This mechanism is independent of the scavenging and oncotic properties of HSA and is related to its ability to modulate intracellular (endosomal) pathways involved in the production of cytokines. Therefore, the observation that HSA is internalized by leukocytes offers an alternative perspective for understanding the anti-inflammatory effects associated with the infusion of HSA to patients with advanced liver disease.

MATERIALS AND METHODS

Study design

The objective of this study was to elucidate the mechanism by which HSA infusions exert anti-inflammatory effects in patients with AD cirrhosis at risk of developing ACLF. Experiments included measurements in plasma samples (n = 16) from the INFECIR-2 study, which compared the effects of HSA infusions to SMT on hospital mortality in patients with AD cirrhosis and active infections. Data inclusion/exclusion criteria, sample size justification, rules for stopping data collection, outlier, and selection of end points of this phase 4, randomized, open-label, multicenter investigation (ClinicalTrials.gov: NCT02034279) are described elsewhere (*10*). The INFECIR-2 study was approved by the corresponding Ethics Committee of each participating hospital, and the informed consent included the potential use of biobanking material for research purposes. Experiments were also performed in leukocytes from peripheral blood of HV (n = 30) from the Blood Bank of the Hospital Clínic and from patients with AD cirrhosis (n = 17) of whom 5 had ACLF recruited at the Liver Intensive Care Unit of this hospital. Table S7 shows the number of patients assigned to each experiment and their origin. Leukocytes were challenged with CpG-DNA in the presence or absence of HSA. Cytokine gene expression was measured by real-time polymerase chain reaction (PCR) and oligonucleotide microarrays, cytokine release was assessed by Luminex technology, and protein phosphorylation was assessed by Western blot. HSA internalization and endosomal trafficking were assessed by confocal microscopy and flow cytometry, and functional assays of defense mechanisms were performed using fluorimetric and flow cytometry assays. Last, data were collected from in vivo experiments in humanized analbuminemic mice expressing the human FcRn receptor ($Alb^{-/-}FcRn^{-/-}hFcRn^{+/+}$, n = 6) and C57BL/6J wild-type mice (n = 6). Study size was selected on the basis of previous experience with animal models. Studies were conducted in accordance with the criteria of the Investigation and Ethics Committee of the Universitat de Barcelona (protocol number 139/19).

Cell isolation and incubations

PBMCs and PMNs were isolated from 20 ml of blood by the Ficoll-Hypaque method. Incubations with HSA (Albutein, Grifols) at concentrations of 10 and 15 mg/ml were performed in both pretreatment and treatment intervention modes.

Measurement of cytokines and CpG-DNA

Cytokine and CpG concentrations were determined by multiplexed Milliplex bead–based immunoassay and ELISA (enzyme-linked immunosorbent assay), respectively.

RNA isolation, real-time PCR, and microarray analysis

Isolation of total RNA from cells was performed by TRIzol, and real-time PCR analysis was performed in the 7900HT Fast System (Applied Biosystems). Microarray analysis was performed according to the Affymetrix WT PLUS Reagent Kit, and single-stranded complementary DNAs (cDNAs) were hybridized on GeneChip Human gene 2.1ST 24-array plates. Results were analyzed using BTMs (*15*, *38*). Raw data have been deposited at GEO (Gene Expression Omnibus) under the dataset ID GSE146462.

Phagocytosis, efferocytosis, and ROS production assays

To assess phagocytosis, human PBMDMs were obtained from PBMCs and incubated with opsonized fluorescein conjugate zymosan bioparticles (Molecular Probes). To assess efferocytosis, PBMDMs were polarized to M2 phenotype (fig. S10) and incubated with apoptotic PMNs stained with 5-carboxyfluorescein diacetate. Fluorescent intensities were read in a microplate reader (FLUOstar Optima). Respiratory burst was assessed using a Neutrophil/Monocyte

Respiratory Burst assay kit (Cayman Chemical) in a flow cytometer (BD FACSCanto II).

Preparation of FITC-labeled HSA, visualization, and immunofluorescence assay

HSA was labeled using a fluorescein-EX protein labeling kit (Molecular Probes). PBMCs incubated with FITC-HSA were visualized in an LSM 880 Zeiss confocal microscope. For the immunofluorescence assay, PBMCs were incubated with vehicle, unlabeled HSA (5 mg/ml), or HSA plus FITC–labeled CpG-DNA (2 μM). Cells were fixed, permeabilized, and stained with anti-albumin and anti-EEA1 antibodies recognized by secondary A647 and A555 antibodies, respectively, and visualized by confocal microscopy.

In vivo experiments in humanized $Alb^{-/-}FcRn^{-/-}hFcRn^{+/+}$ mice

Mice received two HSA doses of 1.5 g/kg body weight 72 and 24 hours before collection of peritoneal macrophages, which were stimulated with CpG-DNA with or without HSA.

Statistical analysis

Continuous variables were analyzed using one-way analysis of variance (ANOVA) and paired and unpaired Student's *t* tests with Welch's correction, if necessary, or one-way ANOVA corrected with Tukey's post hoc test when comparing more than two groups. All analyses were performed using GraphPad Prism software with α set at 0.05 and two-tailed test. Results were expressed as means ± SEM, and a *P* value of <0.05 was considered statistically significant.

SUPPLEMENTARY MATERIALS

stm.sciencemag.org/cgi/content/full/12/566/eaax5135/DC1
Material and Methods
Fig. S1. Plasma concentrations of cytokines and CpG-DNA in patients with AD cirrhosis under HSA therapy.
Fig. S2. HSA reduces cytokine release in PBMCs challenged with CpG-DNA.
Fig. S3. HSA anti-inflammatory effect is independent of its scavenging properties.
Fig. S4. HSA reduces cytokine expression in peripheral PMNs from patients with AD cirrhosis and ACLF challenged with CpG-DNA.
Fig. S5. Changes in the leukocyte transcriptome in response to CpG-DNA and HSA.
Fig. S6. CpG-DNA localizes in early endosomes at early times.
Fig. S7. Endosomal TLR signaling.
Fig. S8. Inhibition of MyD88 and TRIF pathways.
Fig. S9. Depletion of cell membrane cholesterol.
Fig. S10. Expression of M2 markers in differentiated PBMDMs.
Table S1. Characteristics of patients from the INFECIR-2 study whose samples were analyzed in this investigation.
Table S2. Plasma concentrations of soluble protein factors in patients with AD cirrhosis under HSA therapy.
Table S3. BTMs of interest with corresponding *P* values and *t* scores.
Table S4. Genes included in each BTM of interest.
Table S5. Genes up-regulated by HSA in CpG-stimulated PBMCs from patients with AD cirrhosis.
Table S6. Genes down-regulated by HSA in CpG-stimulated PBMCs from patients with AD cirrhosis.
Table S7. Number of patients with AD cirrhosis assigned to each experiment and their origin.
Data file S1. Individual data from patients (provided as separate Excel file).

View/request a protocol for this paper from *Bio-protocol*.

REFERENCES AND NOTES

1. R. Moreau, R. Jalan, P. Gines, M. Pavesi, P. Angeli, J. Cordoba, F. Durand, T. Gustot, F. Saliba, M. Domenicali, A. Gerbes, J. Wendon, C. Alessandria, W. Laleman, S. Zeuzem, J. Trebicka, M. Bernardi, V. Arroyo; CANONIC Study Investigators of the EASL–CLIF Consortium, Acute-on-chronic liver failure is a distinct syndrome that develops in patients with acute decompensation of cirrhosis. *Gastroenterology* **144**, 1426–1437.e1-9 (2013).
2. J. Clària, V. Arroyo, R. Moreau, The acute-on-chronic liver failure syndrome, or when the innate immune system goes astray. *J. Immunol.* **197**, 3755–3761 (2016).
3. V. Arroyo, R. Moreau, P. S. Kamath, R. Jalan, P. Ginès, F. Nevens, J. Fernández, U. To, G. García-Tsao, B. Schnabl, Acute-on-chronic liver failure in cirrhosis. *Nat. Rev. Dis. Primers* **2**, 16041 (2016).
4. J. Clària, R. E. Stauber, M. J. Coenraad, R. Moreau, R. Jalan, M. Pavesi, À. Amorós, E. Titos, J. Alcaraz-Quiles, K. Oettl, M. Morales-Ruiz, P. Angeli, M. Domenicali, C. Alessandria, A. Gerbes, J. Wendon, F. Nevens, J. Trebicka, W. Laleman, F. Saliba, T. M. Welzel, A. Albillos, T. Gustot, D. Benten, F. Durand, P. Ginès, M. Bernardi, V. Arroyo; CANONIC Study Investigators of the EASL-CLIF Consortium and the European Foundation for the Study of Chronic Liver Failure (EF-CLIF), Systemic inflammation in decompensated cirrhosis: Characterization and role in acute-on-chronic liver failure. *Hepatology* **64**, 1249–1264 (2016).
5. A. Albillos, M. Lario, M. Álvarez-Mon, Cirrhosis-associated immune dysfunction: Distinctive features and clinical relevance. *J. Hepatol.* **61**, 1385–1396 (2014).
6. M. Bernardi, P. Angeli, J. Claria, R. Moreau, P. Gines, R. Jalan, P. Caraceni, J. Fernandez, A. L. Gerbes, A. J. O'Brien, J. Trebicka, T. Thevenot, V. Arroyo, Albumin in decompensated cirrhosis: New concepts and perspectives. *Gut* **69**, 1127–1138 (2020).
7. P. Sort, M. Navasa, V. Arroyo, X. Aldeguer, R. Planas, L. Ruiz-del-Arbol, L. Castells, V. Vargas, G. Soriano, M. Guevara, P. Ginès, J. Rodés, Effect of intravenous albumin on renal impairment and mortality in patients with cirrhosis and spontaneous bacterial peritonitis. *N. Engl. J. Med.* **341**, 403–409 (1999).
8. P. Ginès, L. Titó, V. Arroyo, R. Planas, J. Panés, J. Viver, M. Torres, P. Humbert, A. Rimola, J. Llach, S. Badalamenti, W. Jiménez, J. Gaya, J. Rodés, Randomized comparative study of therapeutic paracentesis with and without intravenous albumin in cirrhosis. *Gastroenterology* **94**, 1493–1502 (1988).
9. P. Caraceni, O. Riggio, P. Angeli, C. Alessandria, S. Neri, F. G. Foschi, F. Levantesi, A. Airoldi, S. Boccia, G. Svegliati-Baroni, S. Fagiuoli, R. G. Romanelli, R. Cozzolongo, V. Di Marco, V. Sangiovanni, F. Morisco, P. Toniutto, A. Tortora, R. De Marco, M. Angelico, I. Cacciola, G. Elia, A. Federico, S. Massironi, R. Guarisco, A. Galioto, G. Ballardini, M. Rendina, S. Nardelli, S. Piano, C. Elia, L. Prestianni, F. M. Cappa, L. Cesarini, L. Simone, C. Pasquale, M. Cavallin, A. Andrealli, F. Fidone, M. Ruggeri, A. Roncadori, M. Baldassarre, M. Tufoni, G. Zaccherini, M. Bernardi; ANSWER Study Investigators, Long-term albumin administration in decompensated cirrhosis (ANSWER): An open-label randomised trial. *Lancet* **391**, 2417–2429 (2018).
10. J. Fernández, J. Clària, A. Amorós, F. Aguilar, M. Castro, M. Casulleras, J. Acevedo, M. Duran-Güell, L. Nuñez, M. Costa, M. Torres, R. Horrillo, L. Ruiz-del-Árbol, C. Villanueva, V. Prado, M. Arteaga, J. Trebicka, P. Angeli, M. Merli, C. Alessandria, N. K. Aagaard, G. Soriano, F. Durand, A. Gerbes, T. Gustot, T. M. Welzel, F. Salerno, R. Bañares, V. Vargas, A. Albillos, A. Silva, M. Morales-Ruiz, J. C. García-Pagán, M. Pavesi, R. Jalan, M. Bernardi, R. Moreau, A. Páez, V. Arroyo, Effects of albumin treatment on systemic and portal hemodynamics and systemic inflammation in patients with decompensated cirrhosis. *Gastroenterology* **157**, 149–162 (2019).
11. V. Arroyo, R. García-Martinez, X. Salvatella, Human serum albumin, systemic inflammation, and cirrhosis. *J. Hepatol.* **61**, 396–407 (2014).
12. A. M. Krieg, A. K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky, D. M. Klinman, CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546–549 (1995).
13. C. Fang, X. Wei, Y. Wei, Mitochondrial DNA in the regulation of innate immune responses. *Protein Cell* **7**, 11–16 (2016).
14. C. M. Mendez, C. J. McClain, L. S. Marsano, Albumin therapy in clinical practice. *Nutr. Clin. Pract.* **20**, 314–320 (2005).
15. S. Li, N. Rouphael, S. Duraisingham, S. Romero-Steiner, S. Presnell, C. Davis, D. S. Schmidt, S. E. Johnson, A. Milton, G. Rajam, S. Kasturi, G. M. Carlone, C. Quinn, D. Chaussabel, A. K. Palucka, M. J. Mulligan, R. Ahmed, D. S. Stephens, H. I. Nakaya, B. Pulendran, Molecular signatures of antibody responses derived from a systems biology study of five human vaccines. *Nat. Immunol.* **15**, 195–204 (2014).
16. E. Weiss, P. E. Rautou, M. Fasseu, M. Giabicani, M. de Chambrun, J. H. Wan, C. Minsart, T. Gustot, A. Couvineau, R. Maiwall, M. Hurtado-Nedelec, N. Pilard, D. Lebrec, D. Valla, F. Durand, P. de la Grange, R. C. Monteiro, C. Paugam-Burtz, S. Lotersztajn, R. Moreau, Type I interferon signaling in systemic immune cells from patients with alcoholic cirrhosis and its association with outcome. *J. Hepatol.* **66**, 930–941 (2017).
17. H. Hemmi, O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, S. Akira, A Toll-like receptor recognizes bacterial DNA. *Nature* **408**, 740–745 (2000).
18. L. Alexopoulou, A. C. Holt, R. Medzhitov, R. A. Flavell, Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature* **413**, 732–738 (2001).
19. C. Watts, Location, location, location: Identifying the neighborhoods of LPS signaling. *Nat. Immunol.* **9**, 343–345 (2008).
20. S. Xu, B. Z. Olenyuk, C. T. Okamoto, S. F. Hamm-Alvarez, Targeting receptor-mediated endocytotic pathways with nanoparticles: Rationale and advances. *Adv. Drug Deliv. Rev.* **65**, 121–138 (2013).

21. A. Bortoluzzi, G. Ceolotto, E. Gola, A. Sticca, S. Bova, F. Morando, S. Piano, S. Fasolato, S. Rosi, A. Gatta, P. Angeli, Positive cardiac inotropic effect of albumin infusion in rodents with cirrhosis and ascites: Molecular mechanisms. *Hepatology* **57**, 266–276 (2013).
22. M. Pyzik, T. Rath, T. T. Kuo, S. Win, K. Baker, J. J. Hubbard, R. Grenha, A. Gandhi, T. D. Krämer, A. R. Mezo, Z. S. Taylor, K. McDonnell, V. Nienaber, J. T. Andersen, A. Mizoguchi, L. Blumberg, S. Purohit, S. D. Jones, G. Christianson, W. I. Lencer, I. Sandlie, N. Kaplowitz, D. C. Roopenian, R. S. Blumberg, Hepatic FcRn regulates albumin homeostasis and susceptibility to liver injury. *Proc. Natl. Acad. Sci. U.S.A.* **114**, E2862–E2871 (2017).
23. J. Alcaraz-Quiles, M. Casulleras, K. Oettl, E. Titos, R. Flores-Costa, M. Duran-Güell, C. López-vicario, M. Pavesi, R. E. Stauber, V. Arroyo, J. Clària, Oxidized albumin triggers a cytokine storm in leukocytes through P38 mitogen-activated protein kinase: Role in systemic inflammation in decompensated cirrhosis. *Hepatology* **68**, 1937–1952 (2018).
24. M. Ikehata, R. Yumoto, K. Nakamura, J. Nagai, M. Takano, Comparison of albumin uptake in rat alveolar type II and type I-like epithelial cells in primary culture. *Pharm. Res.* **25**, 913–922 (2008).
25. A. Bento-Abreu, A. Velasco, E. Polo-Hernández, P. L. Pérez-Reyes, A. Tabernero, J. M. Medina, Megalin is a receptor for albumin in astrocytes and is required for the synthesis of the neurotrophic factor oleic acid. *J. Neurochem.* **106**, 1149–1159 (2008).
26. E. G. W. Schmidt, M. L. Hvam, F. Antunes, J. Cameron, D. Viuff, B. Andersen, N. N. Kristensen, K. A. Howard, Direct demonstration of a neonatal Fc receptor (FcRn)-driven endosomal sorting pathway for cellular recycling of albumin. *J. Biol. Chem.* **292**, 13312–13322 (2017).
27. E. Dobrinskikh, K. Okamura, J. B. Kopp, R. B. Doctor, J. Blaine, Human podocytes perform polarized, caveolae-dependent albumin endocytosis. *Am. J. Physiol. Renal Physiol.* **306**, F941–F951 (2014).
28. R. Yumoto, H. Nishikawa, M. Okamoto, H. Katayama, J. Nagai, M. Takano, Clathrin-mediated endocytosis of FITC-albumin in alveolar type II epithelial cell line RLE-6TN. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **290**, L946–L955 (2006).
29. M. Chatterjee, E. Ben-Josef, R. Robb, M. Vedaie, S. Seum, K. Thirumoorthy, K. Palanichamy, M. Harbrecht, A. Chakravarti, T. M. Williams, Caveolae-mediated endocytosis is critical for albumin cellular uptake and response to albumin-bound chemotherapy. *Cancer Res.* **77**, 5925–5937 (2017).
30. H. H. Li, J. Li, K. J. Wasserloos, C. Wallace, M. G. Sullivan, P. M. Bauer, D. B. Stolz, J. S. Lee, S. C. Watkins, C. M. S. Croix, B. R. Pitt, L. M. Zhang, Caveolae-dependent and -independent uptake of albumin in cultured rodent pulmonary endothelial cells. *PLOS ONE* **8**, e81903 (2013).
31. K. M. Knudsen Sand, M. Bern, J. Nilsen, H. T. Noordzij, I. Sandlie, J. T. Andersen, Unraveling the interaction between FcRn and albumin: Opportunities for design of albumin-based therapeutics. *Front. Immunol.* **5**, 682 (2015).
32. W. He, M. S. Ladinsky, K. E. Huey-Tubman, G. J. Jensen, J. R. McIntosh, P. J. Björkman, FcRn-mediated antibody transport across epithelial cells revealed by electron tomography. *Nature* **455**, 542–546 (2008).
33. D. M. Klinman, Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat. Rev. Immunol.* **4**, 249–258 (2004).
34. B. L. Lee, G. M. Barton, Trafficking of endosomal Toll-like receptors. *Trends Cell Biol.* **24**, 360–369 (2014).
35. E. Latz, A. Schoenemeyer, A. Visintin, K. A. Fitzgerald, B. G. Monks, C. F. Knetter, E. Lien, N. J. Nilsen, T. Espevik, D. T. Golenbock, TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat. Immunol.* **5**, 190–198 (2004).
36. A. Engel, G. M. Barton, Compartment-specific control of signaling from a DNA-sensing immune receptor. *Sci. Signal.* **3**, pe45 (2010).
37. J. Babdor, D. Descamps, A. C. Adiko, M. Tohmé, S. Maschalidi, I. Evnouchidou, L. R. Vasconcellos, M. De Luca, F. X. Mauvais, M. Garfa-Traore, M. M. Brinkmann, M. Chignard, B. Manoury, L. Saveanu, IRAP$^+$ endosomes restrict TLR9 activation and signaling. *Nat. Immunol.* **18**, 509–518 (2017).
38. P. de la Grange, M. Dutertre, N. Martin, D. Auboeuf, FAST DB: A website resource for the study of the expression regulation of human gene products. *Nucleic Acids Res.* **33**, 4276–4284 (2005).

Acknowledgments: We thank the technical support of A. I. Martínez-Puchol and A. Salvatella and the research nurse F. Aziz. We also thank M. Calvo of the University of Barcelona Advanced Microscopy Service for assistance and S. Ozcoz of the IDIBAPS Cytomics Core Facility for technical help. **Funding:** Supported by Spanish MEC (SAF15-63674-R and PID2019-105240RB-I00 to JC) under European Regional Development Funds (ERDF) and EF-Clif, a nonprofit private organization aimed at stimulating research in cirrhosis. Our laboratory is a Consolidated Research Group recognized by the Generalitat de Catalunya (2017SGR1449 to J.C.). This study was carried out at the Center Esther Koplowitz, IDIBAPS, which is part of the CERCA Programme/Generalitat de Catalunya. Our laboratory is receiving funding from the EU Horizon 2020 program (nos. 825694 and 847949). **Author contributions:** M. Casulleras designed, conducted, and analyzed data from patients, mice, and cell experiments; J.A.-Q., R.F.-C., M.D.-G., S.S., E.T., and C.L.-V. contributed to acquisition of data and analysis of in vivo and in vitro experiments; J.F. contributed to patients' recruitment and provided clinical insights; P.d.l.G. performed the analysis of the transcriptomics data; R.M., V.A., M. Costa, and R.H. contributed to data interpretation and provided feedback; J.C. conceived and supervised the study; and M. Casulleras and J.C. wrote the paper. **Competing interests:** M. Costa and R.H. are full-time employees of Grifols, which develops Albutein, and have no other competing interests to declare. All other authors declare no competing interests. **Data and materials availability:** The microarray data have been deposited in the Gene Expression Omnibus. The dataset ID is GSE146462. All the other data are present in the main text or in the Supplementary Materials.

Submitted 29 March 2019
Resubmitted 2 December 2019
Accepted 24 June 2020
Published 21 October 2020
10.1126/scitranslmed.aax5135

Citation: M. Casulleras, R. Flores-Costa, M. Duran-Güell, J. Alcaraz-Quiles, S. Sanz, E. Titos, C. López-Vicario, J. Fernández, R. Horrillo, M. Costa, P. de la Grange, R. Moreau, V. Arroyo, J. Clària, Albumin internalizes and inhibits endosomal TLR signaling in leukocytes from patients with decompensated cirrhosis. *Sci. Transl. Med.* **12**, eaax5135 (2020).

Science Translational Medicine
AAAS

stm.sciencemag.org/cgi/content/full/12/566/eaax5135/DC1

Supplementary Materials for

Albumin internalizes and inhibits endosomal TLR signaling in leukocytes from patients with decompensated cirrhosis

Mireia Casulleras, Roger Flores-Costa, Marta Duran-Güell, José Alcaraz-Quiles, Silvia Sanz, Esther Titos, Cristina López-Vicario, Javier Fernández, Raquel Horrillo, Montserrat Costa, Pierre de la Grange, Richard Moreau, Vicente Arroyo, Joan Clària*

*Corresponding author. Email: jclaria@clinic.cat

Published 21 October 2020, *Sci. Transl. Med.* **12**, eaax5135 (2020)
DOI: 10.1126/scitranslmed.aax5135

The PDF file includes:

Table S7. Number of patients with AD cirrhosis assigned to each experiment and their origin. Legend for data file S1

Other Supplementary Material for this manuscript includes the following:

(available at stm.sciencemag.org/cgi/content/full/12/566/eaax5135/DC1)

Data file S1. Individual data from patients (provided as separate Excel file).

Material and Methods

Cell Isolation

PBMC and PMN were isolated from 20 mL of blood in EDTA tubes from 30 healthy donors and 17 patients with AD cirrhosis (of whom 5 had ACLF) recruited at the Hospital Blood Bank and at the Liver Intensive Care Unit of the Hospital Clínic of Barcelona, respectively. **Table S7** shows the number of patients assigned to each experiment and their origin. Blood samples were centrifuged at 200 *g* for 10 min to collect plasma and sedimented cells were diluted with Dulbecco's Phosphate-Buffered Saline (DPBS) without calcium and magnesium ($DPBS^{--}$) up to a volume of 20 mL. Diluted blood was layered over 13.3 mL of Ficoll-Hypaque PLUS (GE Healthcare Life Sciences) and centrifuged at 500 *g* for 25 min with the break on. PBMC were obtained from the mononuclear cell layer, washed with $DPBS^{--}$ and centrifuged again at 400 *g* for 5 min. Pelleted PMN were incubated with pre-warmed ammonium-chloride-potassium lysis buffer for 10 min at room temperature to remove red blood cells and then centrifuged at 400 g for 5 min. The red blood lysis procedure was repeated two times and the resultant pellet was washed with $DPBS^{--}$. The purity of PMN preparations was ≈ 90% as assessed by flow cytometry. Isolated PBMC and PMN were enumerated and resuspended in RPMI 1640 medium containing penicillin (100 U/mL), streptomycin (100 U/mL) and L-glutamine (4 mM) without fetal bovine serum (FBS).

Flow cytometry analysis

For purity assessment, PBMC and PMN suspensions were centrifuged at 400 *g* for 5 min at 4ºC and incubated with FcBlock for 15 min at 4ºC. Thereafter, cells were stained with LIVE/DEAD fixable dead cell stain kit (L34963, Thermo Fisher Scientific) and specific cell type markers (PerCP anti-human CD45 (368505, BioLegend), APC anti-human CD14 (325607, BioLegend), FITC anti-human CD15 (394706, BioLegend) and PE anti-human

CD66b (305106, BioLegend) antibodies for 20 min at 4ºC in dark conditions. Cells were centrifuged and resuspended with FACS buffer (2% FBS, 0,01% NaN3 in PBS) and analyzed in a FORTESSA 5L Cytometer. For assessment of intracellular HSA loading, PBMC were incubated with FITC-labeled HSA at 5 mg/mL for 2 hours and then centrifuged at 400 g for 5 min at 4ºC. Cells were stained with the LIVE/DEAD fixable dead cell stain kit, PerCP anti-human CD45 or APC anti-human CD14 for 20 min at 4ºC in dark conditions. Cells were centrifuged and resuspended with FACS buffer (2% FBS, 0,01% of NaN3 and PBS) and analyzed in a FORTESSA Cytometer.

Cell Incubations

To test the optimal stimulus, leukocytes were incubated with vehicle, LPS (TLR4 agonist, 10 ng/mL) for 4 hours, ATP (5 mM) for 5 hours, LPS+ATP, fMLP (bacterial peptide agonist of formyl peptide receptors, 1 μM) for 4 hours or CpG-DNA (oligodeoxyribonucleotides ODN 2395, Class C, InvivoGen, 4 μM) for 2 hours at 37ºC in a 5% CO_2 atmosphere. CpG-DNA contains unmethylated CpG dinucleotides in particular sequence contexts (CpG motifs), which are present at a 20-fold greater frequency in bacterial DNA compared to mammalian DNA. CpG-DNA is recognized by TLR9 leading to strong immunostimulatory effects. CpG-DNA is also common in mitochondrial DNA. Incubations with HSA were performed in both pre-treatment and treatment intervention modes. Pre-treatment experiments were performed in leukocytes seeded at a density of $1.5x10^6$ cells/mL and incubated in the absence or presence of HSA (Albutein, Grifols) at concentrations of 10 and 15 mg/mL for 30 min and then challenged with either vehicle or CpG-DNA (2 μM) for 2 hours. Endotoxin concentration in the HSA solution used in these experiments was less than 1.3 IU per mL. Treatment experiments were performed in leukocytes incubated with either vehicle or CpG-DNA for 2 hours and then incubated with HSA or vehicle for 2 more hours. Concentration- (0.05, 0.5, 5,

10 and 15 mg/mL of HSA) and time- (30, 120 and 240 min) dependent experiments were also performed under these conditions. In some experiments, PBMC were pre-incubated with MyD88 inhibitory peptide, pepinh-MyD (50 μM) (Invivogen) for 60 min before the addition of HSA for 30 min and then incubated with CpG-DNA (4 μM) for 2 hours. In other experiments, PBMC were activated with CpG-DNA (4 μM) for 2 hours, exposed to chloroquine (Invivogen) for 30 min and finally incubated with HSA for 90 min. For microarray analysis, PBMC were seeded at a density of $1.0x10^6$ cells/mL and incubated with HSA (15 mg/ml) for 24 hours in the presence of CpG-DNA (4 μM) or vehicle for the last 4 hours. Some experiments were performed with recombinant human albumin (rHA, A9731, Sigma-Aldrich) as well as with mannitol (M1902, Sigma-Aldrich). PBMC were also incubated with high molecular weight polyinosinic-polycytidylic acid (poly(I:C)) (double-stranded RNA, ligand of TLR3, InvivoGen) (50 μg/mL) or LPS (10 ng/ml) for 5 or 4 hours, respectively. Inhibitory experiments in pre-treatment mode were performed in PBMC pre-incubated with chloroquine (tlrl-chq, Invivogen) at 50 μM and polymyxin B (tlrl-pmb, Invivogen) at 20 μg/ml, for 30 min or MyD88 inhibitory peptide, pepinh-MYD (tlrl-pimyd, Invivogen) and TRIF inhibitory peptide, pepinh-TRIF (tlrl-pitrif) at 50 μM, for 60 min. Then HSA was added for 30 min followed by the stimulus (CpG-DNA at 4 μM for 2 hours, poly(I:C) at 50 μg/mL for 5 hours or LPS at 10 ng/ml for 4 hours). Inhibitory experiments in treatment mode were performed in PBMC stimulated with CpG-DNA (4 μM) for 2 hours, then exposed to chloroquine for 30 min and finally incubated with HSA for additional 90 min. At the end of the incubation periods, samples were centrifuged at 1500 *g* for 5 min at 4ºC and supernatants and cell pellets were collected for further analysis.

Plasma Samples from Patients with AD cirrhosis

Biobanked plasma samples were obtained from 16 patients with AD cirrhosis included in the INFECIR-2 study, a phase 4, randomized, open-label, parallel, multicenter investigation promoted by the Fundació Clínic (Hospital Clínic, University of Barcelona, Spain) (ClinicalTrials.gov: NCT02034279). Data inclusion/exclusion criteria, rules for stopping data collection, outlier and selection of endpoints are described elsewhere *(10)*. The INFECIR-2 study was approved by the corresponding Ethics Committee of each participating hospital and the informed consent included the potential use of biobanking material for measuring plasma cytokine concentrations. Samples were collected at baseline and during standard medical treatment with antibiotics (SMT) (n=8) or SMT plus HSA treatment (n=8). Criteria for sample selection were: 1) samples from patients with signs of intense systemic inflammation and bacterial infection; and 2) samples in which plasma concentrations of IL-6, IL-1β, TNFα were detectable.

Measurement of Cytokines and Soluble Protein Factors

Cytokine concentrations in plasma and supernatants from PBMC cultures stimulated with CpG-DNA or CpG-DNA plus HSA were determined by multiplexed bead-based immunoassay (MilliplexMAP Human Cytokine/Chemokine Magnetic Bead Plex Kit, Merck Millipore). Concentrations of soluble protein factors in plasma were assessed using the MilliplexMAP Human Sepsis Magnetic bead panel 1 (Merk Millipore). Briefly, 25 µL of each sample was incubated overnight at 4ºC with shaking in a 96-well plate and then washed and incubated with detection antibodies for 1 hour. Thereafter, the plate was washed again, and samples were incubated with 25 µL of streptavidin-phycoerythrin for 30 min. Finally, the plate was washed twice, the beads re-suspended with 100 µL of sheath fluid and then signals were read in a Luminex 100 Bioanalyzer (Luminex Corp.). A five-parameter logistic

regression model was used to create standard curves (pg/mL) and to calculate the concentration of each sample with Milliplex Analyst software (Merck Millipore).

Measurement of Plasma CpG-DNA Concentrations

Plasma CpG-DNA concentrations were measured with a human CpG oligodeoxynucleotide ELISA kit (E01C0809, BlueGene Biotech) following the manufacturer's instructions. The measurements were performed in plasma from healthy donors (n=8), patients with compensated cirrhosis (n=8) and in patients with AD cirrhosis (n=8, selected from the 17 patients hospitalized at the Liver Intensive Care Unit of the Hospital Clínic). The concentration of CpG-DNA was also determined in 11 out of the 16 patients from the INFECIR-2 study.

RNA Isolation, Reverse Transcription and Real-Time PCR

Isolation of total RNA from PBMC and PMN was performed using the TRIzol reagent following the manufacturer's instructions. RNA concentrations were assessed in a NanoDrop-1000 spectrophotometer (NanoDrop Technologies). cDNA synthesis from 200 to 1000 ng of total RNA was performed using the High-Capacity cDNA Archive Kit (Applied Biosystems). Real-Time PCR analysis of human *IL1B* (Hs01555410_m1, encoding IL-1β), *IL6* (Hs00985639_m1, encoding IL-6), *TNF* (Hs01113624_g1, encoding TNFα), and *CXCL10* (Hs01124251_g1, encoding interferon (IFN)-γ inducible protein 10), *OAS2* (Hs00942643_m1, encoding 2'-5'-oligoadenylate synthetase 2), *IFNB1* (Hs01077958_s1, encoding IFNβ), *TGM2* (Hs01096681_m1, encoding transglutaminase 2), *MRC1* (Hs00267207_m1, encoding for mannose receptor, C type 1, CD206) and *CCL22* (Hs01574247_m1, encoding C-C motif chemokine ligand 22) was performed in a 7900HT Fast System (Applied Biosystems) using *ACTB* (Hs99999903_m1, encoding for β-actin) as

endogenous control. For mouse experiments, *Il1b* (Mm01336189_m1), *Tnf* (Mm00443258_m1) and *Il6* (Mm00446190_m1) were analyzed using *Actb* (Mm00607939_s1) as endogenous control. Quantitative PCR results were analyzed with Sequence Detector 2.1 Software (Applied Biosystems). Relative quantification of gene expression was performed using comparative Ct method. The amount of target gene, normalized to *ACTB* and relative to a calibrator, was determined by the arithmetic equation $2^{-\Delta\Delta Ct}$, as described in the comparative Ct method (http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf).

Microarray Analysis

RNA was isolated and quantified as described above and RNA integrity was re-checked in the Bioanalyzer 2100 before performing microarray hybridization. The average RIN value of total RNAs was 9.05 (from 8 to 9.5 range). Processing of RNA samples, fragmentation and labelling of single-stranded-cDNA were prepared according to Affymetrix WT PLUS Reagent Kit user guide in an automated system (Beckman FX robot). Following fragmentation and terminal labeling, single-stranded-cDNAs were hybridized for 17 hours at 45ºC on the GeneChip Human gene 2.1ST 24 - array plates, using the automated GeneTitan System, which includes the hybridization oven, the fluidic station and the scanner. Microarray data analysis and visualization were performed using EASANA (GenoSplice technology), which is based on GenoSplice FAST DB® annotations. Exon Array data were normalized using quantile normalization. Background corrections were made with antigenomic probes, and the probes were selected as described previously. Only probes targeting exons annotated from FAST DB® transcripts were selected to focus on well-annotated genes whose mRNA sequences are in public databases *(38)*. Bad-quality selected probes such as probes labeled by Affymetrix as 'cross-hybridizing' and probes which low intensity signal compared to

antigenomic background probes with the same GC content were excluded from the analysis. Only probes with a DABG P value <0.05 in at least half of the arrays were considered for statistical analysis *(38)*. Only genes expressed in at least one comparison condition were analyzed. To be considered as expressed, at least half of the gene probes had to have a DABG P-value <0.05. Comparison of gene intensities among the different biological replicates was performed using the paired Student's t-test. Genes were considered significantly regulated with a fold-change ≥1.5 and with a P value <0.05. In this data analysis, we also used the recently described 346 blood transcription modules (BTMs), which are a very useful tool for analysis of blood immune cell transcriptome *(15,16)*. Each BTM includes a set of genes which are co-expressed in PBMC across various immunological stimuli. For each BTM and for each condition (vehicle, CpG-DNA and CpG-DNA plus HSA), we calculated a BTM activity which was taken as the mean value of its member genes and then compared BTM activities in two pairwise comparisons (CPG-DNA versus vehicle and CpG-DNA plus HSA versus CpG-DNA) and selected the BTMs with a P value below 0.05 in at least one of the two comparisons.

Analysis of protein expression by Western blot

Total protein was extracted with lysis buffer containing 50 mM HEPES, 20 mM β-glycerophosphate, 2 mM EDTA, 1% Igepal, 10% (v/v) glycerol, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 150 mM NaCl, supplemented with protease (Complete Mini) and phosphatase (PhosSTOP) inhibitor mixtures (Roche Diagnostics). Homogenates were incubated in ice for 15 min with frequent vortexing. Homogenates were centrifuged at 16.000 *g* for 20 min at 4°C, and supernatants were collected. A total of 10 μg of protein was resuspended in SDS-containing Laemmli sample buffer and heated for 5 min at 95°C, and proteins separated for 90 min at 120 V 4°C in 10% SDS-PAGE. Transfer was performed by the iBlot Dry Blotting

System (Invitrogen) onto PVDF membranes at 20 V over 7 min. Membranes were then soaked for 45 min at room temperature in TBS (20 mM Tris/HCl pH 7.4 and 0.5 M NaCl) containing 0.1% (v/v) Tween 20 (0.1% TBST) and 5% (w/v) non-fat milk powder. Blots were washed three times for 5 min each with 0.1% TBST and subsequently treated overnight at 4°C with primary rabbit anti-human IRF3 (dilution 1:500; Cell Signaling Technology), phospho-IRF3 (SER396) (dilution 1:250; Cell Signaling Technology), phospho-IRF3 (SER386) (dilution 1:250; Abcam) or β-actin (dilution 1:1000; Cell Signaling Technology) antibodies in 0.1% TBST. After washing the blots three times for 5 min each with 0.1% TBST, membranes were incubated for 1 hour at room temperature with a HRP-linked donkey anti-rabbit secondary antibody (dilution 1:2000; Abcam) in 0.1% TBST containing 5% BSA. Bands were visualized using the EZ-ECL chemiluminescence detection kit (Biological Industries) in a LAS 4000 imaging system (GE Healthcare Life Sciences) and quantified using Image GE ImageQuant TL analysis software.

Phagocytosis Assay

Human PBMDM were obtained from PBMC seeded at a density of $5x10^5$ cells/ml with RPMI 1640 medium supplemented with 10% FBS in black-walled 96-well plates. After 2 hours of incubation at 37ºC in a 5% CO_2 atmosphere, non-adhered cells were discarded and fresh RPMI medium was supplemented with 10% pooled human serum from 5 healthy donors. The medium was changed after 2-3 days and replaced with fresh RPMI with 10% pooled serum. After 5 days of culture in these conditions PBMDM were grown overnight with FBS-free RPMI medium. On the day of experimentation, 50 µL of opsonized fluorescein conjugate zymosan bioparticles (BioParticles®, Molecular Probes) were added to each well (ratio cells/bioparticles, 1:10) with a final volume of 200 µL and incubated at 37ºC for 60 min. Cells were then washed with sterile $DPBS^{--}$ and 100 µL trypan blue solution (diluted 1/10 in sterile

DPBS^{--}) was added to quench fluorescence of extracellular bioparticles. Plates were finally centrifuged for 5 min at 400 *g* at room temperature and excess trypan blue was carefully aspirated. The fluorescent intensity of each well was read in a microplate reader (FLUOstar Optima).

Efferocytosis assay

For the efferocytosis assay, PBMDM were polarized to M2 phenotype by differentiation with macrophage colony-stimulatory factor (M-CSF) (216-MC, R&D Systems) for 7 days in the presence of IL-4 (204-IL, R&D Systems) for the last 2 days. M2 phenotype was confirmed by real time PCR analysis of *TGM2*, *MRC1* and *CCL22*. For the preparation of apoptotic PMN, once isolated from blood, these cells ($5x10^6$ cells/ml) were stained with 5-carboxyfluorescein diacetate (CFDA) (C4916, Sigma-Aldrich) at 2 µM for 10 min at room temperature, seeded in a culture plate and incubated for 48 hours at 37ºC (**Figure S10**). The efferocytosis assay was performed in M2 macrophages incubated with HSA (10 or 15 mg/ml) for 30 min before the addition of CFDA-labeled apoptotic PMN at 3:1 ratio and incubated for 1 hour at 37ºC. Trypan blue solution (diluted 1/10 in sterile DPBS^{--}) was added to quench extracellular fluorescence and signal intensity was read in a microplate reader (FLUOstar Optima).

Apoptosis Assay Annexin V

Isolated PMN were seeded at a density of $2x10^6$ cell/ml and maintained in culture (37ºC, 5% CO_2) for 0, 24 or 48 hours. Then, apoptosis assay was performed using FITC-Annexin V Apoptosis Detection Kit with PI (BioLegend), following manufacturer's instructions. Briefly, PMN were pelleted and resuspended with 100 µl of annexin buffer, then 5 µl of FITC-annexin were added and incubated for 15 min at room temperature in dark conditions. PMN

were centrifuged to avoid the excess of annexin and resuspended with annexin buffer. All samples were analyzed in a FACS canto II cytometer.

ROS Production Assay

Respiratory burst was assessed using the Neutrophil/Monocyte Respiratory Burst assay kit (Cayman Chemical) following the manufacturer's instructions. Briefly, 10 µl of dihydrorhodamine 123 was added to 100 µl of whole blood collected in a polypropylene EDTA tube in a water bath at 37ºC for 15 min, and then incubated with HSA (10 and 15 mg/ml) for 30 min at 37ºC. At the end of this incubation, cells were challenged with phorbol 12-myristate 13-acetate (PMA) or *Escherichia coli* (stabilized and opsonized, non-labeled, E.coli suspension, 1-2x10^9 bacteria/ml, Phagoburst, 341058, BD biosciences) and incubated again for 45 min at 37ºC. Finally, cell suspensions were washed twice with red blood cell lysis buffer, incubated at 37ºC for 10 min, centrifuged at 500 *g* for 10 min and analyzed by flow Cytometry (BD FACSCanto II, BD biosciences).

Preparation of Fluorescein Isothiocyanate (FITC)-Labeled HSA

HSA was labeled using the fluorescein-EX protein labeling Kit (F10240, Molecular Probes), following the manufacturer's instructions. Briefly, at an initial concentration of 20 mg/ml, HSA was incubated with fluorescein-5-EX succinimidyl ester for 1 hour at room temperature. The mixture was purified in a resin column and FITC-labeled HSA was collected. The concentration of labeled HSA was determined by measuring the absorbance of the conjugate solution at 280 and 494 nm in a spectrophotometer (Ultrospec 3300 Pro, GE Healthcare Life Sciences).

Visualization of FITC-HSA Cell Distribution

Isolated PBMC were seeded at a concentration of $5x10^5$ cells/well and incubated with FITC-HSA (5 mg/ml) alone or with CpG-DNA (2 μM) for 2 hours. Cell suspensions were placed on round micro cover glasses (25 mm diameter, Electron Microscopy Sciences (EMS)) previously coated with 200 μL of 0.01% poly-L-lysine, incubated overnight at 37ºC (5% CO_2) and washed with $DPBS^{--}$ twice. Cells were visualized in a LSM880 Zeiss confocal microscope (Zeiss).

HSA Immunofluorescence Assay

Isolated PBMC were seeded (250.000 cells/well) in 8-well flat-bottom culture glass slides with polystyrene vessels (BD biosciences) previously coated with poly-L-lysine (0.01%, Sigma Aldrich) and incubated with vehicle, unlabeled HSA (5 mg/ml) or HSA plus FITC-labeled CpG-DNA (2 μM) for 5, 10, 30, 60 and 120 min. Cells were fixed with 4% paraformaldehyde diluted in PBS at pH 7.4 for 20 min and permeabilized with 0.1% triton X-100 for 10 min at room temperature. Thereafter, cells were blocked with 1% gelatin type A skin porcine (Sigma Aldrich) for 45 min at room temperature. Blocking solution was used to dilute primary antibodies (anti-albumin rabbit monoclonal antibody [EPR20195] (ab207327, Abcam) (dilution 1:500) and anti-EEA1 antibody [1G11] (early endosome marker, ab70521, Abcam) (dilution 1:200)). Cells were incubated with primary antibodies and incubated overnight at 4ºC in a humid chamber. After washing with PBS, cells were incubated with 2.5 μg/ml of goat anti-mouse IgG (H+L) secondary antibody Alexa-Fluor Plus 555 (A32727, Thermo Fisher Scientific) (dilution, 1:1000) or goat anti-rabbit IgG (H+L) highly cross-absorbed secondary antibody Alexa-Fluor 647 (A21245, Thermo Fisher Scientific). Finally, cells were washed again and incubated with 5 μM of Hoechst 33342 (H3570, Thermo Fisher Scientific) for nuclei staining and visualized in a LSM880 confocal microscope (Zeiss).

FcRn Blockade

Isolated PBMC were seeded at a concentration of $1x10^6$ cells/well and incubated with the FcRn blocking antibody, ADM31 (Aldevron) or its isotype control, IgG2b (ABIN964473, Antibodies online), both at 10 μg/ml, for 20 min. Thereafter, HSA (15 mg/mL) was added for 30 min and finally CpG-DNA (2 μM) for 2 hours. At the end of the incubation period, samples were centrifuged at 1500 *g* for 5 min at 4ºC and supernatants and cell pellets were collected for further analysis.

Lipid Raft Disruption and Filipin Staining

For lipid raft disruption, PBMC were treated with 10 mM methyl-β-cyclodextrin (MBCD, C4555, Sigma-Aldrich) for 30 min after incubation with HSA (15 mg/ml) for 30 min and CpG-DNA (4 μM) for 2 hours. For filipin staining, PBMC were seeded at $2.5x10^5$ cells/ml in 8-well flat-bottom culture glass slides with polystyrene vessels (BD biosciences) previously coated with poly-L-lysine and treated with either vehicle or MBCD for 30 min. Cells were subsequently fixed with 4% paraformaldehyde diluted in PBS at pH 7.4 for 15 min at room temperature, washed three times with PBS cells and stained with filipin solution (Filipin Complex from Streptomyces filipinensis, F9765, Sigma-Aldrich) at 0.33 mg/ml for 20 min at ambient temperature and protected from light. Thereafter, cells were washed again with PBS and visualized under a fluorescent microscope (Nikon e600) (340-380 nm excitation) using a 405 nm fluorescence filter.

In Vivo Experiments in Humanized $Alb^{-/-}FcRn^{-/-}hFcRn^{+/+}$ mice

Humanized male $Alb^{-/-}FcRn^{-/-}hFcRn^{+/+}$ (Cg-Tg(FCGRT)32Dcr $Alb^{em12Mvw}$ $Fcgrt^{tm1Dcr}$/MvwJ mice, The Jackson Laboratory) and wild-type C57BL/6J mice were housed on woodchip bedding in cages with 50–60% humidity and a 12 hour light/dark cycle and given ad libitum

access to food and water. To test the effects of HSA infusions, two HSA doses of 1.5 g/kg b.w. or $HBSS^{--}$ were administered 72 and 24 hours prior to administer an isoflurane overdose to collect peritoneal exudates by peritoneal lavage with 7 ml of ice-cold $DPBS^{--}$. To isolate peritoneal macrophages, exudates were centrifuged at 500 *g* for 5 min and the pellet resuspended in DMEM supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml), L-glutamine (2 mM) and 5% FBS. Cells were seeded in 24-well plates and allowed to adhere in a humidified 5% CO_2 incubator at 37°C during 2 hours. Non-adherent cells were removed by washing twice with $DPBS^{--}$. In some experiments, peritoneal macrophages from wild-type and humanized $Alb^{-/-}FcRn^{-/-}hFcRn^{+/+}$ mice were treated with either vehicle, CpG-DNA (4 μM) or CpG-DNA plus HSA (15 mg/ml) for 2 hours. All studies were conducted in accordance with the criteria of the Investigation and Ethics Committee of the Universitat de Barcelona (UB) (protocol number: 139/19) and the European Community laws governing the use of experimental animals.

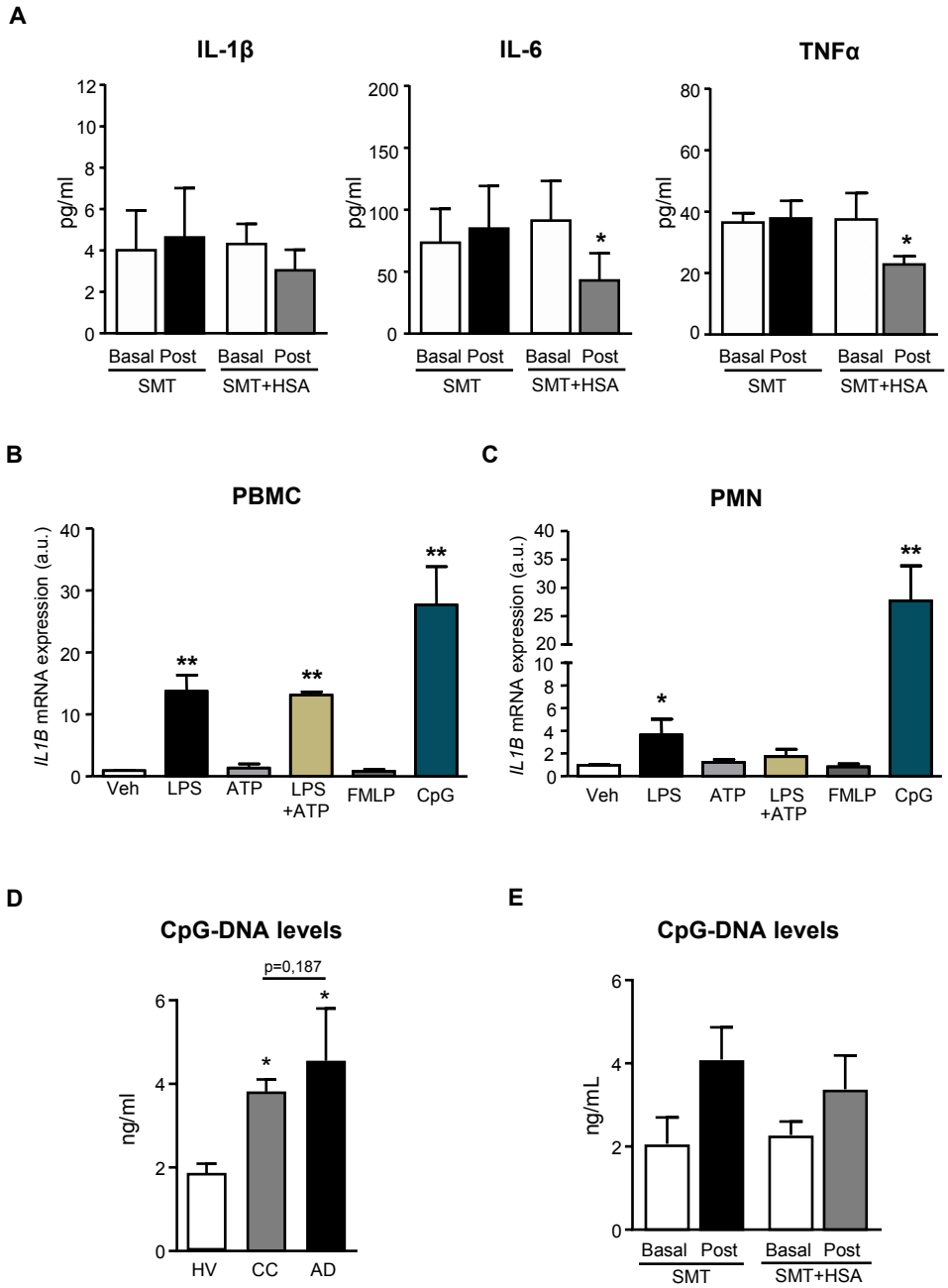


Figure S1. Plasma levels of cytokines and CpG-DNA in patients with AD cirrhosis under HSA therapy. (**A**) Plasma levels of IL-1β, IL-6 and TNFα in patients with AD cirrhosis under basal conditions and after (post) receiving either standard medical therapy (SMT) (n=8) or SMT plus HSA (n=8). (**B**) IL1B mRNA expression in human PBMC isolated from healthy volunteers incubated with either vehicle, LPS (10 ng/mL), ATP (5 mM), LPS+ATP, FMLP (1 μM) or CpG-DNA (4 μM) for 2-5 hours at 37ºC in a 5% CO2 atmosphere. Data are representative of 3 experiments performed in duplicate. (**C**) Results from PMN under the conditions described in B. (**D**) Plasma CpG-DNA levels in healthy volunteers (HV) and in patients with compensated (CC) and decompensated (AD) cirrhosis (n=8 in each group). (**E**) Plasma CpG-DNA levels in patients with AD cirrhosis before (basal) and after (post) receiving either SMT (n=8) or SMT plus HSA (n=8). Results are expressed as mean ± SEM. *P<0.05 and **P<0.001 versus basal, vehicle or HV.

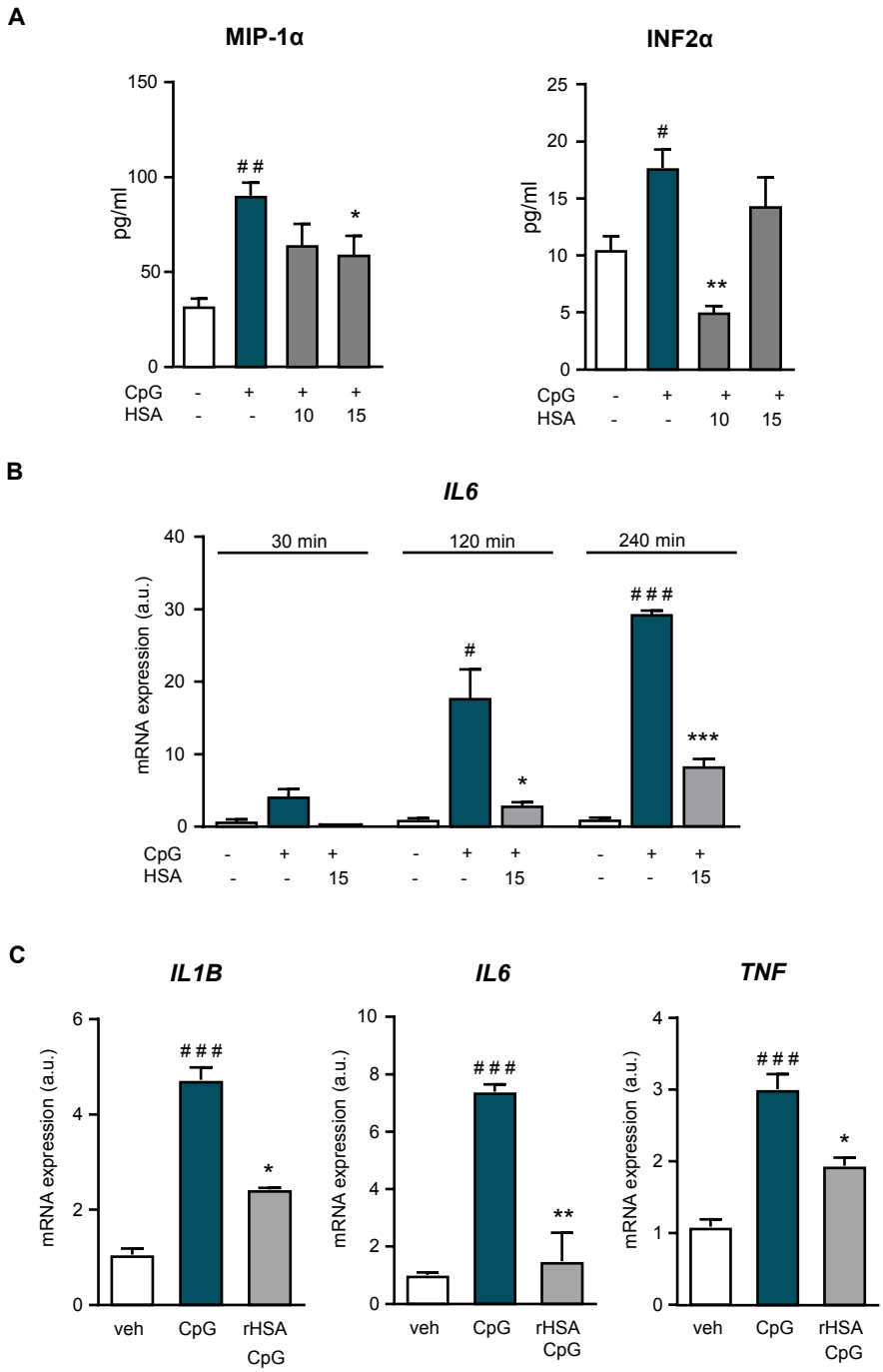


Figure S2. (A) HSA reduces cytokine release in PBMC challenged with CpG-DNA. Levels of MIP-1α and INF2α in human PBMC isolated from healthy volunteers and challenged with CpG (4 μM) in the presence or absence of HSA (10 or 15 mg/ml) for 2 hours. **(B) Time-course in IL-6 expression.** PBMC were incubated for increasing periods of time (30-240 min) with CpG-DNA in the presence or absence of HSA (15 mg/ml). **(C) Effects of recombinant human albumin (rHA) in PMN.** IL1B, IL6 and TNF mRNA expression in PMN from healthy donors incubated with rHA at 15 mg/ml for 30 min and then, challenged with CpG-DNA (4μM) for 2 hours. Results are expressed as mean ± SEM of 3 different experiments performed in duplicate. #, P<0.05, # #, P<0.005 and # # #, P<0.001 for CpG-DNA versus vehicle. *,P<0.05, **, P<0.005 and ***, P<0.001 for CpG-DNA + HSA versus CpG-DNA alone.

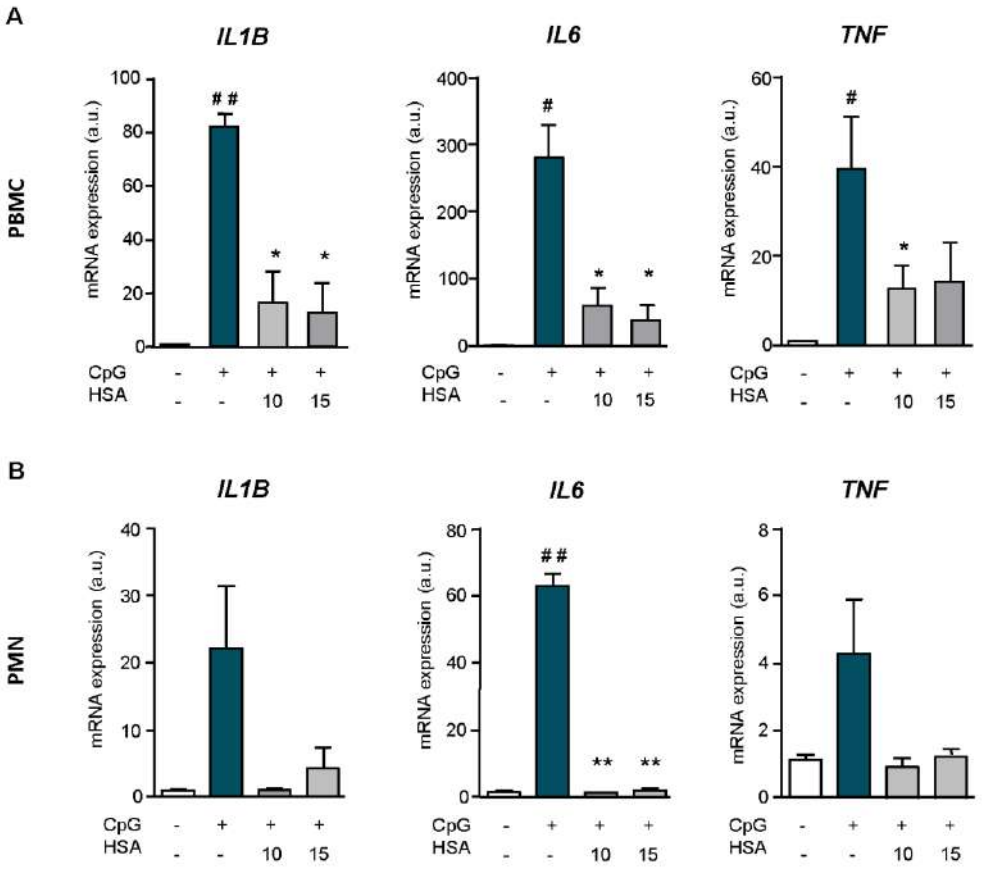


Fig. S3. HSA anti-inflammatory effect is independent of its scavenging properties. mRNA expression of *IL1B*, *IL6* and *TNF*, determined by real-time PCR, in PBMC (**A**) and PMN (**B**) isolated from healthy volunteers and incubated with CpG-DNA (4 μM) for 2 hours before the addition of HSA (10 mg/ml and 15 mg/ml) for 2 additional hours. Results are expressed as mean ± SEM from n=3 in duplicate. $^{\#}$, P<0.05 and $^{\#\#}$, P<0.005 for CpG-DNA versus vehicle. *, P<0.05 and **, P<0.005 for CpG-DNA plus HSA versus CpG-DNA alone.

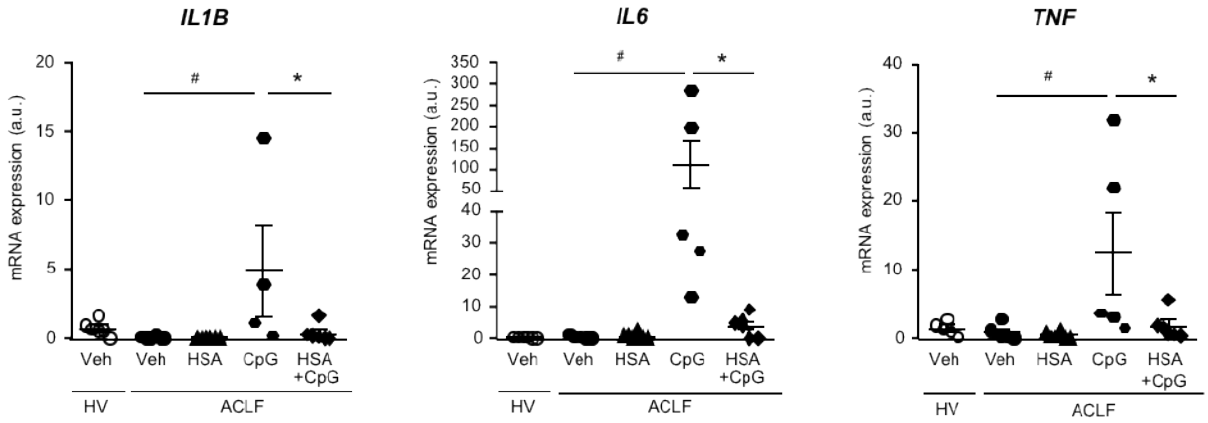


Fig. S4. HSA reduces cytokine expression in peripheral PMNs from patients with AD cirrhosis and ACLF challenged with CpG-DNA. mRNA expression of *IL1B*, *IL6* and *TNF*, determined by real-time PCR, in human PMN isolated from healthy volunteers (HV) and patients with AD cirrhosis and ACLF incubated with Veh, HSA, CpG-DNA or HSA plus CpG-DNA using a pre-treatment mode. Results are expressed as mean ± SEM of n=5 patients in duplicate. $^{\#}$, P<0.05 for CpG-DNA versus vehicle. *, P<0.05 for CpG-DNA plus HSA versus CpG-DNA alone.

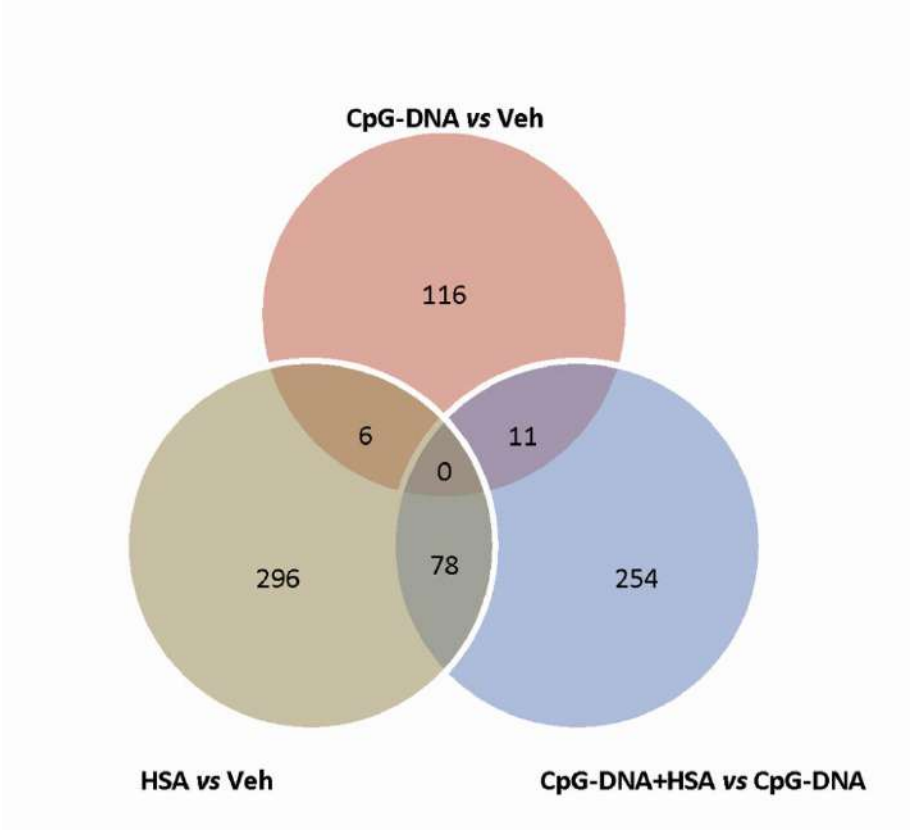


Fig. S5. **Changes in the leukocyte transcriptome in response to CpG-DNA and HSA.** The Venn diagram shows the number of genes differentially expressed in PBMC from patients with AD cirrhosis after CpG-DNA challenge with respect to vehicle (Veh) (upper circle), in PBMC treated with HSA with respect to Veh (bottom left circle) and in PBMC receiving HSA during CpG-DNA challenge with respect to those that did not receive HSA during CpG-DNA stimulation (bottom right circle).

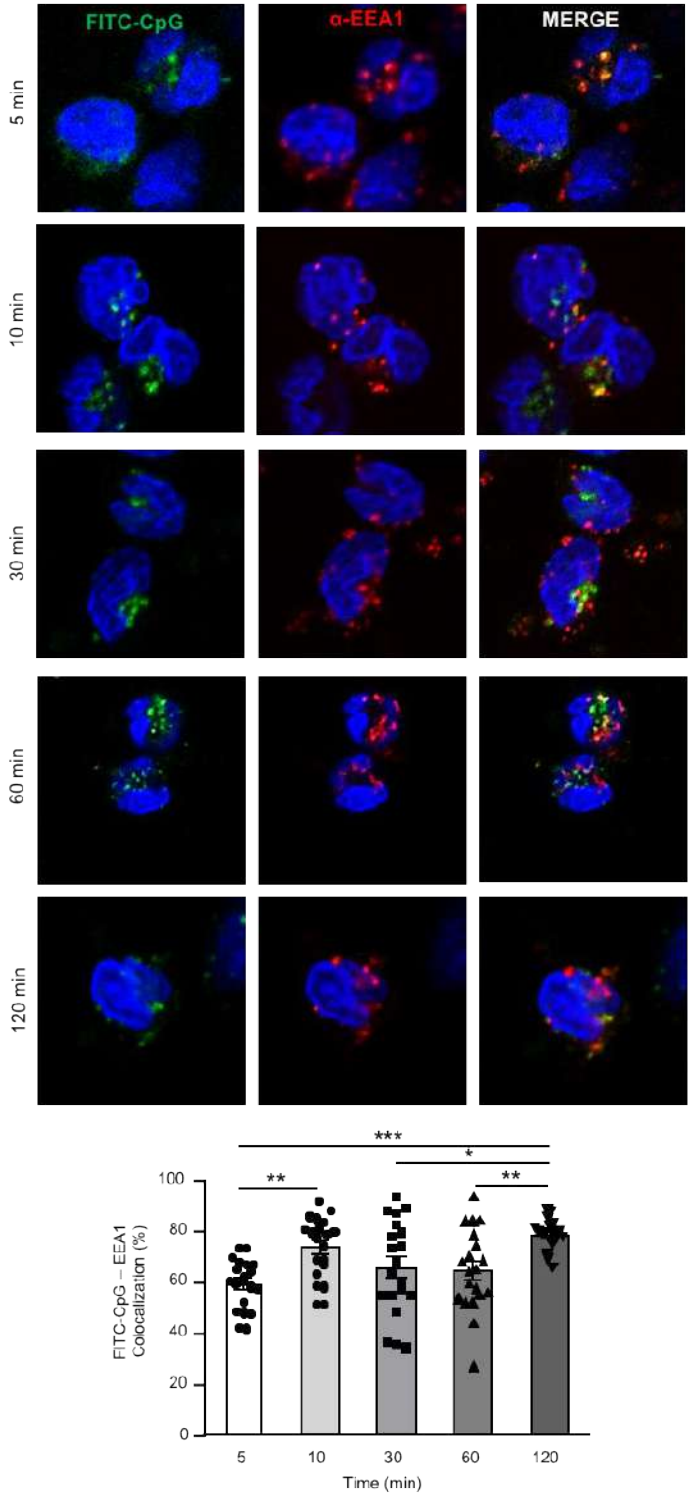


Fig. S6. CpG-DNA localizes in early endosomes at early times. Representative confocal images of human PBMC isolated from healthy volunteers incubated with FITC-CpG-DNA (green) at 2 µM from 5 to 120 min and stained intracellularly with anti-EEA1 primary antibody with Alexa Fluor 555 secondary antibody (red). Nuclei were stained with Hoechst 33342 (blue). Visualized under confocal microscope at 63x oil objective. Quantitative data are expressed as mean ± SEM. *P<0.05, **P<0.005 and ***P<0.001.

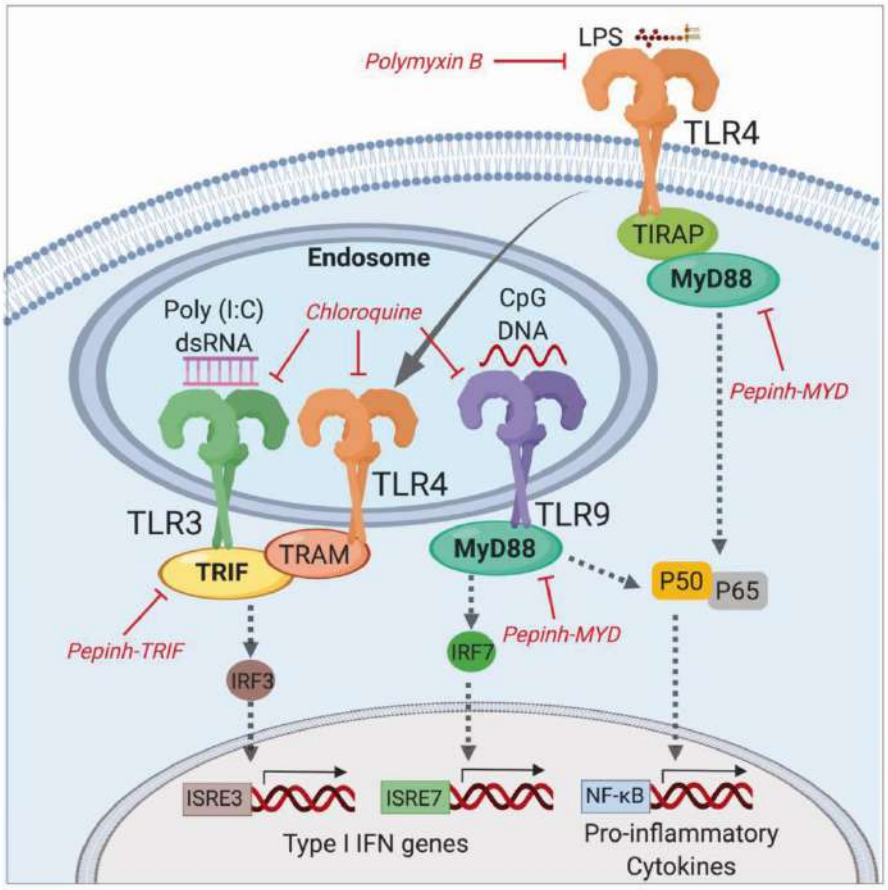


Fig. S7. Endosomal TLR signaling. TLR9 signaling is activated when single-strand CpG-DNA binds to its receptor TLR9, localized in endosomes. The signal is initiated with the recruitment of myeloid differentiation primary response gene 88 (MyD88) culminating in the upregulation of transcription factors and the activation of genes coding for pro-inflammatory cytokines and type I IFN genes. TLR3 is also an endosomal receptor and its activated when double-strand RNA such as poly (I:C) binds to this receptor. TLR3 signaling is activated through the TIR-domain-containing adapter-inducing interferon-β (TRIF) pathway, which culminates in the activation of type I IFN genes, resulting in the late-phase response in the transcription of pro-inflammatory cytokines. Finally, LPS binds to TLR4 located in the cell membrane, where it recruits MyD88 leading to the upregulation of genes coding for pro-inflammatory cytokines. In addition, after binding to LPS, TLR4 translocates to the endosome where it activates the TRIF pathway and the expression of type I IFN genes.

Polymyxin B: inhibitor of LPS-binding to TLR4.
Chloroquine: inhibitor of endosomal acidification/endosomal TLR signaling.
Pepinh-MyD: peptide inhibitor of MyD88 homodimerization.
Pepinh-TRIF: interfering peptide of the TLR-TRIF interaction.

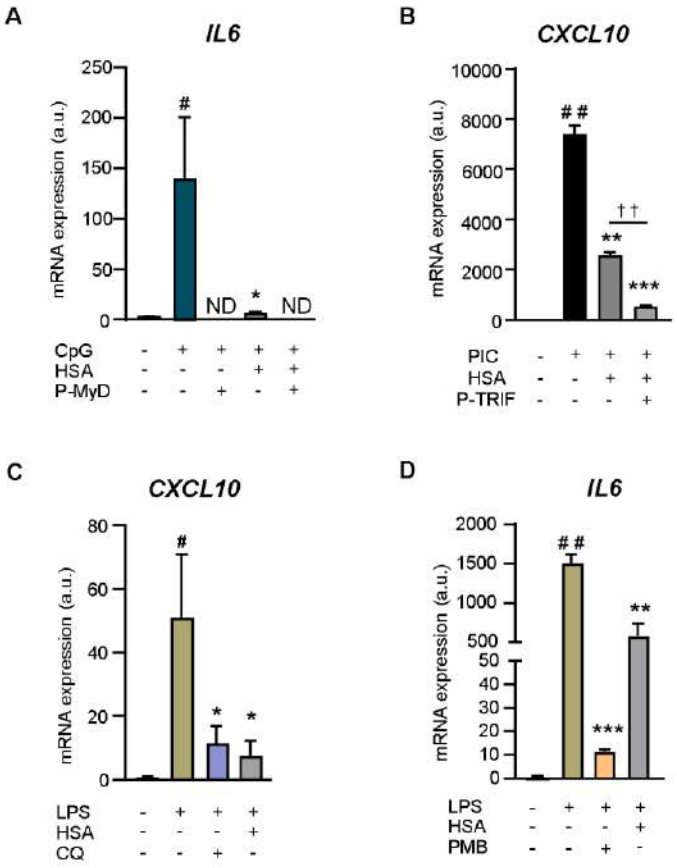


Fig. S8. Inhibition of MyD88 and TRIF pathways. (**A**) *IL6* mRNA expression in PBMC incubated with pepinh-MyD (P-MyD, 50µM) for 1 hour before addition of HSA (15 mg/mL) for 30 min and then challenged with CpG-DNA (4µM) for 2 hours. (**B**) *CXCL10* mRNA expression in PBMC incubated with pepinh-TRIF (P-TRIF, 50µM) for 1 hour before addition of HSA (15 mg/mL) for 30 min and poly (I:C) (PIC, 50 µg/mL) for 5 hours. (**C**) *CXCL10* mRNA expression in PBMC incubated with chloroquine (CQ, 50µM) for 30 min before the addition of HSA (15 mg/ml) for additional 30 min and then challenged with LPS (10 ng/ml) for 4 hours. (**D**) *IL6* mRNA expression in PBMC incubated with polymyxin B (PMB, 20 µg/ml) for 30 min before the addition of HSA (15 mg/mL) for additional 30 min and then challenged with LPS (10 ng/mL) for 4 hours. Results are expressed as mean ± SEM. $^{\#}$, P<0.005 and $^{\#\#}$, P<0.001 for stimulus versus vehicle; *, P<0.05, **, P<0.005 and ***P<0.001 for stimulus plus HSA versus stimulus alone and †, P<0.005 for PIC+HSA+P-TRIF versus PIC+HSA.

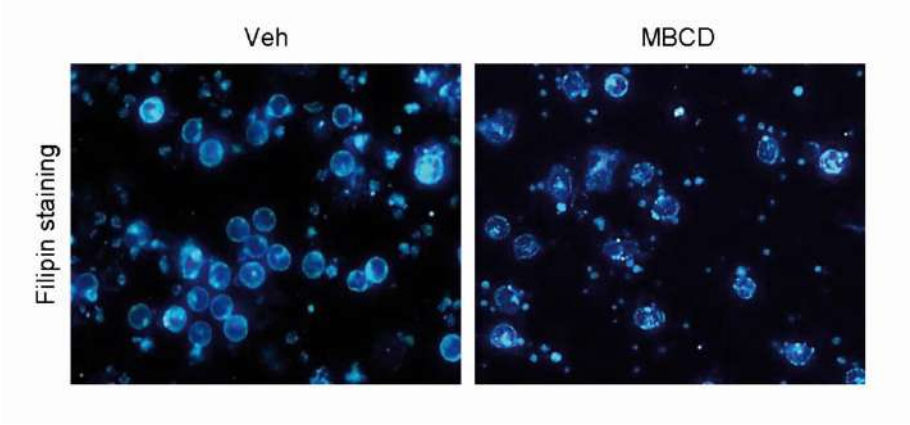


Fig. S9. Depletion of cell membrane cholesterol. Representative images of human PBMC isolated from healthy volunteers incubated with vehicle (Veh) or methyl-β-cyclodextrin (MBCD) for 30 min and stained with filipin (blue). Visualized under microscope at 40x objective with a 405 nm fluorescence filter.

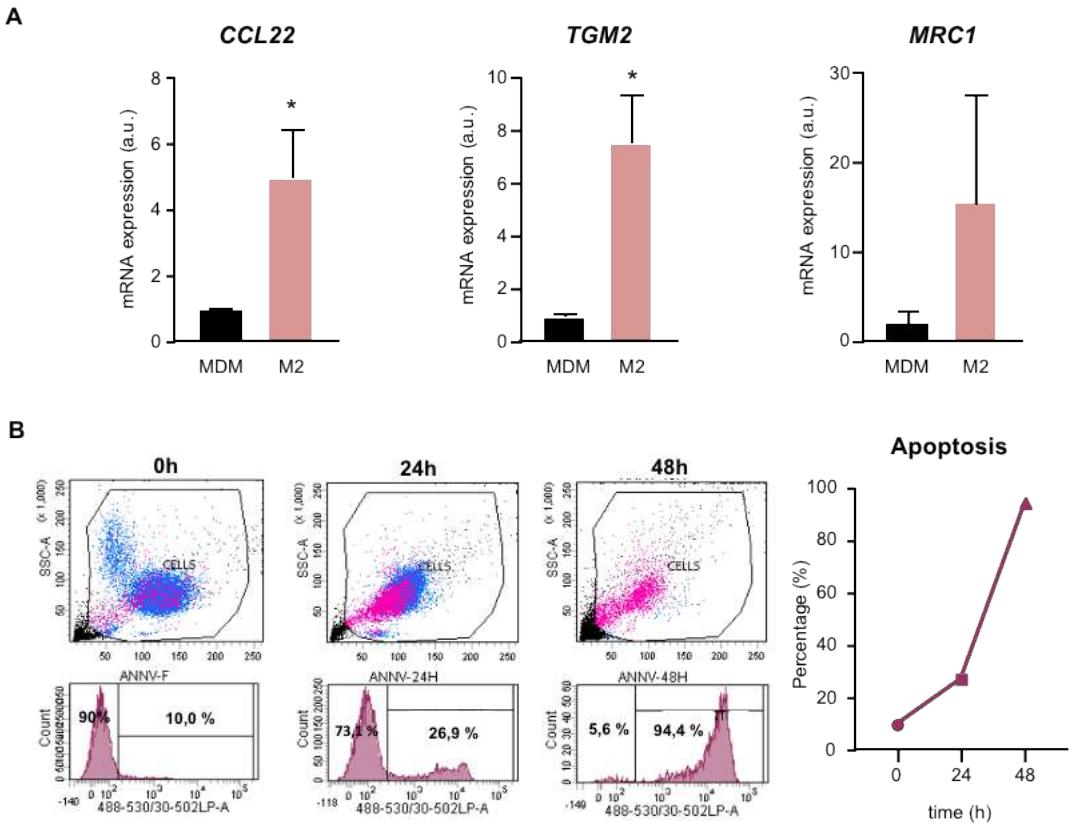


Fig. S10. Expression of M2 markers in differentiated PBMDMs. (**A**) Expression of *CCL22, TGM2* and *MCR1*, determined by real-time PCR, in human peripheral blood monocyte-derived macrophages isolated from healthy volunteers after 7 days of differentiation to M2 with macrophage colony-stimulating factor (M-CSF) and IL-4. MDM indicates monocytes incubated with RPMI 1640 (vehicle) for 7 days. Results are expressed as mean ± SEM. *, P<0.05 versus MDM. **(**B) Induction of PMN apoptosis. Flow cytometry plots after incubating PMN for 0, 24 and 48 hours at 37ºC and stained with Annexin V.

Table S1. Characteristics of patients from the INFECIR-2 study whose samples were analyzed in this investigation.

	Patients (N=16)	Normal range values
Baseline data		
Age (years)	59.3 ± 8.0	-
Male sex, n (%)	11 (68.8%)	-
Alcoholic cirrhosis, n (%)	14 (87.5%)	-
Previous variceal bleeding, n (%)	6 (37.5%)	-
Previous SBP, n (%)	2 (12.5%)	-
Long-term norfloxacin prophylaxis, n (%)	1 (6.3%)	-
Previous hepatic encephalopathy, n (%)	11 (68.8%)	-
Diabetes mellitus, n (%)	5 (31.3%)	-
HCC, n (%)	2 (12.5%)	-
Ascites, n (%)	12 (75.0%)	-
Blood leukocyte count, $x10^9$/L	9.9 ± 5.5	4.00 - 11.00
Hematocrit, %	30.1 ± 5.6	36 - 51
Platelet count, $x10^9$/L	119 ± 113	130 - 400
Serum bilirubin, mg/dL	6.3 ± 4.5	< 1.2
Serum albumin, g/L	25.7 ± 7.9	34 - 48
INR	1.8 ± 0.6	1 - 2
Serum creatinine, mg/dL	1.4 ± 0.5	0.3 - 1.3
BUN, mg/dL	50.2 ± 38.1	6 - 25
Serum sodium, mEq/L	133.3 ± 7.0	135 - 145
Serum C-reactive protein, mg/L	8.1 ± 8.9	0.8-3.0
IL-1β, pg/ml	4.21 ± 4.2	< 3.2
IL-6, pg/ml	78.14 ± 80	< 5.0
TNFα, pg/ml	37.23 ± 18	< 9.0
Child-Pugh score, points	9.6 ± 1.5	-

SBP: Spontaneous bacterial peritonitis; HCC: hepatocellular carcinoma; INR: international normalized ratio; BUN: Blood urea nitrogen; IL: Interleukin; TNF: Tumor necrosis factor; MELD: model for end-stage liver disease.

* Data are presented as mean ± SD or number of patients (%).

Table S2. Plasma concentrations of soluble protein factors in patients with AD cirrhosis under HSA therapy. Plasma concentrations of protein markers of endothelial and platelet dysfunction in patients with AD cirrhosis under basal conditions and after (post) receiving either standard medical therapy (SMT) or SMT plus HSA. Results are expressed as mean ± SEM. No significant P values are reported.

	SMT (n=8)		**SMT + HSA (n=8)**	
	Basal	Post	Basal	Post
PAI-1 (ng/ml)	57±4	60±6	81±13	80±10
sCD40L (pg/ml)	400±76	488±129	366±119	332±63
sICAM-1 (ng/ml)	557±50	527±54	568±54	574±76
sVCAM-1 (ng/ml)	1730±7106	1662±116	1977±145	1946±116

Table S3. BTMs of interest with corresponding *P* values and *t* scores.

BTM	BTM ID	CpG vs. VEH		CpG+HSA vs. CpG	
		P-value	t-score	P-value	t-score
Growth factor induced, enriched in nuclear receptor subfamily 4	M94	2,01E-03	22,00	8,79E-01	-0,17
Enriched for TF motif TTCNRGNNNNTTC	M172	4,38E-03	15,00	5,63E-01	-0,69
Recruitment of neutrophils	M132	5,08E-03	14,00	6,72E-01	0,49
B cell development	M9	5,29E-03	14,00	6,18E-01	-0,59
Putative targets of PAX3	M89.0/M89.1	1,29E-03	27,77	8,12E-01	0,27
Myeloid cell cytokines, metallopeptidases and laminins	M78	2,48E-02	6,20	6,21E-01	-0,58
Cytokines - receptors cluster	M115	3,22E-02	5,40	4,34E-02	-4,20
Myeloid, dendritic cell activation via NFkB (I)	M43.0	3,52E-02	5,20	6,72E-01	-0,49
Transmembrane and ion transporters (II)	M224	3,85E-02	5,00	2,26E-01	-1,70
Chemokine cluster (II)	M27.1	4,33E-02	4,60	4,03E-01	-1,10
CD4 T cell surface signature Th1-stimulated	S6	4,76E-02	4,40	4,42E-01	-0,95
Enriched in NK cells (III)	M157	4,90E-02	4,40	3,00E-01	-1,40
Innate activation by cytosolic DNA sensing	M13	4,90E-02	4,30	1,63E-01	-2,20
Activated dendritic cells	M67	7,68E-02	3,40	3,00E-02	-5,60
Type I interferon response	M127	1,23E-01	2,60	4,45E-03	-15,00
Platelet activation and degranulation	M85	7,49E-01	-0,37	1,99E-02	7,00
CORO1A-DEF6 network (I)	M32.2	3,67E-02	-5,10	9,35E-01	0,09

Table S4. Genes included in each BTM of interest.

Module ID	Module title	Module member genes
M115	cytokines - receptors cluster	IL10
M115	cytokines - receptors cluster	IL15RA
M115	cytokines - receptors cluster	IL2RA
M115	cytokines - receptors cluster	IL23A
M115	cytokines - receptors cluster	IFNG
M115	cytokines - receptors cluster	CSF3
M115	cytokines - receptors cluster	LIF
M115	cytokines - receptors cluster	IL15
M115	cytokines - receptors cluster	IL7R
M115	cytokines - receptors cluster	CSF2
M115	cytokines - receptors cluster	IL6
M127	type I interferon response	IFIT1
M127	type I interferon response	IFITM1
M127	type I interferon response	IRF7
M127	type I interferon response	RSAD2
M127	type I interferon response	IFIH1
M127	type I interferon response	STAT1
M127	type I interferon response	USP18
M127	type I interferon response	TAP1
M127	type I interferon response	PARP9
M127	type I interferon response	PLSCR1
M127	type I interferon response	HERC5
M127	type I interferon response	DDX60
M127	type I interferon response	TAP1
M13	innate activation by cytosolic DNA sensing	AIM2
M13	innate activation by cytosolic DNA sensing	IRF7
M13	innate activation by cytosolic DNA sensing	NFKBIA
M13	innate activation by cytosolic DNA sensing	PYCARD
M13	innate activation by cytosolic DNA sensing	CCL4
M13	innate activation by cytosolic DNA sensing	CCL5
M13	innate activation by cytosolic DNA sensing	IL1B
M13	innate activation by cytosolic DNA sensing	ZBP1
M13	innate activation by cytosolic DNA sensing	CXCL10
M13	innate activation by cytosolic DNA sensing	IL6
M13	innate activation by cytosolic DNA sensing	DDX58
M132	recruitment of neutrophils	PTAFR
M132	recruitment of neutrophils	GPR109B
M132	recruitment of neutrophils	C5AR1
M132	recruitment of neutrophils	FPR2
M132	recruitment of neutrophils	FPR1
M132	recruitment of neutrophils	CXCR1
M132	recruitment of neutrophils	CCR3

M132	recruitment of neutrophils	PROK2
M132	recruitment of neutrophils	F2RL1
M132	recruitment of neutrophils	GNG10
M157	enriched in NK cells (III)	C1orf21
M157	enriched in NK cells (III)	KLRC3
M157	enriched in NK cells (III)	GPR18
M157	enriched in NK cells (III)	RASGRP1
M157	enriched in NK cells (III)	CCL4
M157	enriched in NK cells (III)	CD7
M157	enriched in NK cells (III)	MATK
M157	enriched in NK cells (III)	PTPN4
M157	enriched in NK cells (III)	PPP1R16B
M157	enriched in NK cells (III)	TIGIT
M157	enriched in NK cells (III)	CX3CR1
M157	enriched in NK cells (III)	PVRIG
M157	enriched in NK cells (III)	GIMAP6
M172	enriched for TF motif TTCNRGNNNNTTC	PLAU
M172	enriched for TF motif TTCNRGNNNNTTC	PHLDA2
M172	enriched for TF motif TTCNRGNNNNTTC	NRIP3
M172	enriched for TF motif TTCNRGNNNNTTC	GJB2
M172	enriched for TF motif TTCNRGNNNNTTC	FOSB
M172	enriched for TF motif TTCNRGNNNNTTC	CXCL3
M172	enriched for TF motif TTCNRGNNNNTTC	EDN1
M172	enriched for TF motif TTCNRGNNNNTTC	AG2
M172	enriched for TF motif TTCNRGNNNNTTC	EGR3
M172	enriched for TF motif TTCNRGNNNNTTC	NR4A3
M224	transmembrane and ion transporters (II)	GRIK3
M224	transmembrane and ion transporters (II)	CALCA
M224	transmembrane and ion transporters (II)	KCNA5
M224	transmembrane and ion transporters (II)	NTF3
M224	transmembrane and ion transporters (II)	ATP7B
M224	transmembrane and ion transporters (II)	SLC1A6
M224	transmembrane and ion transporters (II)	JPH2
M224	transmembrane and ion transporters (II)	DMD
M224	transmembrane and ion transporters (II)	CACNA1F
M224	transmembrane and ion transporters (II)	SLC24A2
M224	transmembrane and ion transporters (II)	SLC4A3
M27.1	chemokine cluster (II)	CCL2
M27.1	chemokine cluster (II)	CCL4
M27.1	chemokine cluster (II)	CCL5
M27.1	chemokine cluster (II)	CCL20
M27.1	chemokine cluster (II)	CCR5
M27.1	chemokine cluster (II)	CCR1
M27.1	chemokine cluster (II)	CXCL6
M27.1	chemokine cluster (II)	CXCL13

M27.1	chemokine cluster (II)	CXCL5
M27.1	chemokine cluster (II)	CXCL9
M27.1	chemokine cluster (II)	CXCL10
M27.1	chemokine cluster (II)	CXCL11
M27.1	chemokine cluster (II)	XCL1
M27.1	chemokine cluster (II)	ANXA1
M32.2	CORO1A-DEF6 network (I)	SIPA1
M32.2	CORO1A-DEF6 network (I)	TSC2
M32.2	CORO1A-DEF6 network (I)	SH2B1
M32.2	CORO1A-DEF6 network (I)	ACAP1
M32.2	CORO1A-DEF6 network (I)	HGS
M32.2	CORO1A-DEF6 network (I)	CBX4
M32.2	CORO1A-DEF6 network (I)	ATP5D
M32.2	CORO1A-DEF6 network (I)	STXBP2
M32.2	CORO1A-DEF6 network (I)	TECR
M32.2	CORO1A-DEF6 network (I)	CLPTM1
M32.2	CORO1A-DEF6 network (I)	FAM108A1
M32.2	CORO1A-DEF6 network (I)	AES
M32.2	CORO1A-DEF6 network (I)	TGFB1
M32.2	CORO1A-DEF6 network (I)	APEH
M32.2	CORO1A-DEF6 network (I)	H1FX
M32.2	CORO1A-DEF6 network (I)	BRD2
M32.2	CORO1A-DEF6 network (I)	DEF6
M32.2	CORO1A-DEF6 network (I)	DEF6
M32.2	CORO1A-DEF6 network (I)	CCDC22
M32.2	CORO1A-DEF6 network (I)	CORO1A
M43.0	myeloid, dendritic cell activation via NFkB (I)	VCAM1
M43.0	myeloid, dendritic cell activation via NFkB (I)	MAP3K8
M43.0	myeloid, dendritic cell activation via NFkB (I)	NFKB2
M43.0	myeloid, dendritic cell activation via NFkB (I)	IL23A
M43.0	myeloid, dendritic cell activation via NFkB (I)	CCL5
M43.0	myeloid, dendritic cell activation via NFkB (I)	EBI3
M43.0	myeloid, dendritic cell activation via NFkB (I)	BCL3
M43.0	myeloid, dendritic cell activation via NFkB (I)	RELB
M43.0	myeloid, dendritic cell activation via NFkB (I)	NFKBID
M43.0	myeloid, dendritic cell activation via NFkB (I)	SAMSN1
M43.0	myeloid, dendritic cell activation via NFkB (I)	CD83
M43.0	myeloid, dendritic cell activation via NFkB (I)	TNF
M43.0	myeloid, dendritic cell activation via NFkB (I)	CTGF
M43.0	myeloid, dendritic cell activation via NFkB (I)	FUT7
M43.0	myeloid, dendritic cell activation via NFkB (I)	ICAM1
M67	activated dendritic cells	C1QC
M67	activated dendritic cells	SERPING1
M67	activated dendritic cells	BIRC3
M67	activated dendritic cells	IRF7

M67	activated dendritic cells	RSAD2
M67	activated dendritic cells	IFIH1
M67	activated dendritic cells	PLSCR1
M67	activated dendritic cells	CD38
M67	activated dendritic cells	DDX60
M67	activated dendritic cells	C2
M67	activated dendritic cells	DDX58
M78	myeloid cell cytokines, metallopeptidases and laminins	LAMC2
M78	myeloid cell cytokines, metallopeptidases and laminins	IL10
M78	myeloid cell cytokines, metallopeptidases and laminins	LAMB3
M78	myeloid cell cytokines, metallopeptidases and laminins	IL23A
M78	myeloid cell cytokines, metallopeptidases and laminins	MMP19
M78	myeloid cell cytokines, metallopeptidases and laminins	IL1RN
M78	myeloid cell cytokines, metallopeptidases and laminins	MMP9
M78	myeloid cell cytokines, metallopeptidases and laminins	EDN1
M78	myeloid cell cytokines, metallopeptidases and laminins	IL6
M78	myeloid cell cytokines, metallopeptidases and laminins	GSN
M78	myeloid cell cytokines, metallopeptidases and laminins	SFN
M85	platelet activation and degranulation	F3
M85	platelet activation and degranulation	F3
M85	platelet activation and degranulation	VWF
M85	platelet activation and degranulation	SERPINA1
M85	platelet activation and degranulation	THBS1
M85	platelet activation and degranulation	COL1A1
M85	platelet activation and degranulation	FN1
M85	platelet activation and degranulation	SPARC
M85	platelet activation and degranulation	COL1A2
M85	platelet activation and degranulation	SERPINE1
M85	platelet activation and degranulation	CLU
M85	platelet activation and degranulation	TIMP1
M89.0/M89.1	putative targets of PAX3	RGS1
M89.0/M89.1	putative targets of PAX3	G0S2
M89.0/M89.1	putative targets of PAX3	ATF3
M89.0/M89.1	putative targets of PAX3	EGR2
M89.0/M89.1	putative targets of PAX3	NR4A1
M89.0/M89.1	putative targets of PAX3	DUSP2
M89.0/M89.1	putative targets of PAX3	NR4A2
M89.0/M89.1	putative targets of PAX3	SIK1
M89.0/M89.1	putative targets of PAX3	MAFF
M89.0/M89.1	putative targets of PAX3	CXCL2
M89.0/M89.1	putative targets of PAX3	EGR1
M89.0/M89.1	putative targets of PAX3	PLK2
M89.0/M89.1	putative targets of PAX3	HBEGF
M89.0/M89.1	putative targets of PAX3	DUSP1
M89.0/M89.1	putative targets of PAX3	CD83

M89.0/M89.1	putative targets of PAX3	CDKN1A
M89.0/M89.1	putative targets of PAX3	INHBA
M89.0/M89.1	putative targets of PAX3	EGR3
M89.0/M89.1	putative targets of PAX3	GEM
M89.0/M89.1	putative targets of PAX3	IL8
M9	B cell development	HHEX
M9	B cell development	SPI1
M9	B cell development	CEBPE
M9	B cell development	CBFA2T3
M9	B cell development	TNFSF13
M9	B cell development	CCR7
M9	B cell development	LIF
M9	B cell development	LTA
M9	B cell development	TNF
M9	B cell development	LYN
M9	B cell development	TLR4
M94	growth factor induced, enriched in nuclear receptor subfamily 4	CYR61
M94	growth factor induced, enriched in nuclear receptor subfamily 4	EPHA2
M94	growth factor induced, enriched in nuclear receptor subfamily 4	NR4A1
M94	growth factor induced, enriched in nuclear receptor subfamily 4	PPP1R15A
M94	growth factor induced, enriched in nuclear receptor subfamily 4	NR4A2
M94	growth factor induced, enriched in nuclear receptor subfamily 4	ID1
M94	growth factor induced, enriched in nuclear receptor subfamily 4	PLK2
M94	growth factor induced, enriched in nuclear receptor subfamily 4	DUSP1
M94	growth factor induced, enriched in nuclear receptor subfamily 4	CDKN1A
M94	growth factor induced, enriched in nuclear receptor subfamily 4	IL6
M94	growth factor induced, enriched in nuclear receptor subfamily 4	EGR3
M94	growth factor induced, enriched in nuclear receptor subfamily 4	NR4A3
S6	CD4 T cell surface signature Th1-stimulated	SV2A
S6	CD4 T cell surface signature Th1-stimulated	LAG3
S6	CD4 T cell surface signature Th1-stimulated	NDFIP2
S6	CD4 T cell surface signature Th1-stimulated	GPR114
S6	CD4 T cell surface signature Th1-stimulated	TSPAN5
S6	CD4 T cell surface signature Th1-stimulated	F2R
S6	CD4 T cell surface signature Th1-stimulated	TMEM200A
S6	CD4 T cell surface signature Th1-stimulated	C6orf129
S6	CD4 T cell surface signature Th1-stimulated	GPR19

Table S5. Genes up-regulated by HSA in CpG-stimulated PBMCs from patients with AD cirrhosis.

Gene Symbol	Regulation	Fold-Change	P-Value	Gene Name	Ensemble ID
EREG	up	6,22	3,19E-02	epiregulin	ENSG00000124882
VCAN	up	5,26	3,94E-02	versican	ENSG00000038427
DNER	up	5,06	1,56E-03	delta/notch like EGF repeat containing	ENSG00000187957
HTRA1	up	5,03	2,80E-02	HtrA serine peptidase 1	ENSG00000166033
TMEM45B	up	4,94	1,36E-03	transmembrane protein 45B	ENSG00000151715
PDK4	up	4,48	2,23E-02	pyruvate dehydrogenase kinase 4	ENSG00000004799
PDPN	up	4,06	4,85E-02	podoplanin	ENSG00000162493
PID1	up	4,01	1,80E-02	phosphotyrosine interaction domain containing 1	ENSG00000153823
MT1X	up	3,88	3,15E-02	metallothionein 1X	ENSG00000187193
PINLYP	up	3,87	1,70E-03	phospholipase A2 inhibitor and LY6/PLAUR domain containing	ENSG00000234465
FLRT2	up	3,77	3,22E-03	fibronectin leucine rich transmembrane protein 2	ENSG00000185070
NT5E	up	3,74	1,00E-02	5'-nucleotidase ecto	ENSG00000135318
SLC30A1	up	3,65	4,95E-02	solute carrier family 30 member 1	ENSG00000170385
GPR68	up	3,64	4,05E-02	G protein-coupled receptor 68	ENSG00000119714
MYO10	up	3,59	3,04E-02	myosin X	ENSG00000145555
FGFR1	up	3,46	3,50E-02	fibroblast growth factor receptor 1	ENSG00000077782
FGF2	up	3,37	4,40E-02	fibroblast growth factor 2	ENSG00000138685
EPB41L3	up	3,3	4,10E-02	erythrocyte membrane protein band 4.1 like 3	ENSG00000082397
WFDC21P	up	3,25	3,44E-03	WAP four-disulfide core domain 21, pseudogene	ENSG00000261040
SPINT1	up	3,2	1,65E-02	serine peptidase inhibitor, Kunitz type 1	ENSG00000166145
PHLDA1	up	3,19	3,43E-02	pleckstrin homology like domain family A member 1	ENSG00000139289
SPRED1	up	3,18	3,04E-02	sprouty related EVH1 domain containing 1	ENSG00000166068
CAMK1	up	3,14	2,96E-02	calcium/calmodulin dependent protein kinase I	ENSG00000134072
XYLT1	up	3,12	3,82E-03	xylosyltransferase 1	ENSG00000103489
SPRY2	up	3,09	1,65E-02	sprouty RTK signaling antagonist 2	ENSG00000136158
TMEM52B	up	3,06	2,24E-02	transmembrane protein 52B	ENSG00000165685
TGM3	up	3,01	4,98E-03	transglutaminase 3	ENSG00000125780
SAMD1	up	2,8	4,17E-02	sterile alpha motif domain containing 1	ENSG00000141858
MIR4261	up	2,77	2,02E-02	microRNA 4261	ENSG00000265418
TRPS1	up	2,76	4,00E-05	transcriptional repressor GATA binding 1	ENSG00000104447
DLG4	up	2,76	3,52E-02	discs large MAGUK scaffold protein 4	ENSG00000132535

RFX2	up	2,66	1,17E-02	regulatory factor X2	ENSG00000087903
CD36	up	2,57	3,77E-02	CD36 molecule	ENSG00000135218
COL23A1	up	2,57	1,70E-02	collagen type XXIII alpha 1 chain	ENSG00000050767
CLEC5A	up	2,52	2,46E-03	C-type lectin domain containing 5A	ENSG00000258227
GLIS3	up	2,51	7,78E-03	GLIS family zinc finger 3	ENSG00000107249
CD300E	up	2,47	4,73E-02	CD300e molecule	ENSG00000186407
TMEM170B	up	2,45	2,87E-02	transmembrane protein 170B	ENSG00000205269
EVC2	up	2,43	1,40E-03	EvC ciliary complex subunit 2	ENSG00000173040
SGMS2	up	2,42	3,62E-02	sphingomyelin synthase 2	ENSG00000164023
ACADVL	up	2,42	3,83E-02	acyl-CoA dehydrogenase very long chain	ENSG00000072778
OLR1	up	2,37	1,63E-02	oxidized low density lipoprotein receptor 1	ENSG00000173391
DGAT2	up	2,36	3,00E-02	diacylglycerol O-acyltransferase 2	ENSG00000062282
CYP1B1	up	2,35	6,40E-04	cytochrome P450 family 1 subfamily B member 1	ENSG00000138061
CRISPLD2	up	2,34	2,49E-02	cysteine rich secretory protein LCCL domain containing 2	ENSG00000103196
RMI1	up	2,33	4,72E-02	RecQ mediated genome instability 1	ENSG00000178966
PCGF6	up	2,27	4,89E-02	polycomb group ring finger 6	ENSG00000156374
YBX3	up	2,24	2,13E-02	Y-box binding protein 3	ENSG00000060138
RAB34	up	2,23	4,70E-02	RAB34, member RAS oncogene family	ENSG00000109113
OR5B21	up	2,22	3,98E-02	olfactory receptor family 5 subfamily B member 21	ENSG00000198283
HIPK2	up	2,17	7,80E-03	homeodomain interacting protein kinase 2	ENSG00000064393
IGF2BP2	up	2,17	2,34E-02	insulin like growth factor 2 mRNA binding protein 2	ENSG00000073792
AREG	up	2,15	8,56E-03	amphiregulin	ENSG00000109321
SRD5A3	up	2,13	3,67E-02	steroid 5 alpha-reductase 3	ENSG00000128039
MCEMP1	up	2,13	4,48E-02	mast cell expressed membrane protein 1	ENSG00000183019
ST7	up	2,11	1,68E-03	suppression of tumorigenicity 7 // ST7 overlapping transcript 3	ENSG00000004866
EGR1	up	2,11	3,75E-02	early growth response 1	ENSG00000120738
SSBP3	up	2,11	1,99E-02	single stranded DNA binding protein 3	ENSG00000157216
CHRFAM7A	up	2,1	3,85E-02	CHRNA7 (exons 5-10) and FAM7A (exons A-E) fusion	ENSG00000166664
P4HA1	up	2,08	1,24E-02	prolyl 4-hydroxylase subunit alpha 1	ENSG00000122884
TCF7L2	up	2,06	1,06E-02	transcription factor 7 like 2	ENSG00000148737
RGCC	up	2,05	9,98E-03	regulator of cell cycle	ENSG00000102760
ANPEP	up	2,04	4,89E-02	alanyl aminopeptidase, membrane	ENSG00000166825
MITF	up	2,03	4,34E-02	melanogenesis associated transcription factor	ENSG00000187098

MMP10	up	2,02	3,61E-02	matrix metallopeptidase 10	ENSG00000166670
PPDPF	up	2,02	3,05E-02	pancreatic progenitor cell differentiation and proliferation factor	ENSG00000125534
SASH1	up	2,01	3,42E-02	SAM and SH3 domain containing 1	ENSG00000111961
HSDL2	up	2	1,00E-03	hydroxysteroid dehydrogenase like 2	ENSG00000119471

Table S6. Genes down-regulated by HSA in CpG-stimulated PBMCs from patients with AD cirrhosis.

Gene Symbol	Regulation	Fold-Change	P-Value	Gene Name	Ensemble ID
SPINK1	down	8,34	1,64e-02	serine peptidase inhibitor, Kazal type 1	ENSG00000164266
CSF3	down	6,11	3,59e-02	colony stimulating factor 3	ENSG00000108342
PLPP3	down	5,78	4,02e-03	phospholipid phosphatase 3	ENSG00000162407
GJB2	down	4,85	4,04e-02	gap junction protein beta 2	ENSG00000165474
MYLIP	down	4,16	9,74e-03	myosin regulatory light chain interacting protein	ENSG00000007944
WARS	down	4,12	1,42e-02	tryptophanyl-tRNA synthetase	ENSG00000140105
MIR3945	down	4,11	2,30e-02	microRNA 3945	ENSG00000266698
CLEC12A	down	4,00	4,29e-02	C-type lectin domain family 12 member A	ENSG00000172322
CD38	down	3,93	1,41e-02	CD38 molecule	ENSG00000004468
MIR3945HG	down	3,76	4,26e-02	MIR3945 host gene	ENSG00000251230
MUCL1	down	3,72	1,15e-02	mucin like 1	ENSG00000172551
SNORA50C	down	3,66	3,34e-02	small nucleolar RNA, H/ACA box 50C	ENSG00000277887
LY9	down	3,66	1,04e-02	lymphocyte antigen 9	ENSG00000122224
IFIT3	down	3,57	2,34e-03	interferon induced protein with tetratricopeptide repeats 3	ENSG00000119917
CMPK2	down	3,50	4,69e-02	cytidine/uridine monophosphate kinase 2	ENSG00000134326
MRAS	down	3,46	4,36e-02	muscle RAS oncogene homolog	ENSG00000158186
RSAD2	down	3,27	4,24e-03	radical S-adenosyl methionine domain containing 2	ENSG00000134321
LAIR1	down	3,23	3,36e-02	leukocyte associated immunoglobulin like receptor 1	ENSG00000167613
DNAJB9	down	2,91	3,87e-02	DnaJ heat shock protein family (Hsp40) member B9	ENSG00000128590
GDF15	down	2,72	4,62e-02	growth differentiation factor 15	ENSG00000130513
UGCG	down	2,67	4,42e-02	UDP-glucose ceramide glucosyltransferase	ENSG00000148154
MS4A4A	down	2,64	4,76e-02	membrane spanning 4-domains A4A	ENSG00000110079
TBC1D2	down	2,63	3,04e-02	TBC1 domain family member 2	ENSG00000095383
IFIT2	down	2,59	5,17e-03	interferon induced protein with tetratricopeptide repeats 2	ENSG00000119922
IL36G	down	2,55	4,51e-02	interleukin 36 gamma	ENSG00000136688
HDAC9	down	2,50	3,87e-02	histone deacetylase 9	ENSG00000048052
ADGRE1	down	2,50	3,51e-03	adhesion G protein-coupled receptor E1	ENSG00000174837
WNT5A	down	2,43	3,16e-02	Wnt family member 5A	ENSG00000114251
IL12B	down	2,42	2,82e-02	interleukin 12B	ENSG00000113302
DRAM1	down	2,39	4,38e-02	DNA damage regulated autophagy modulator 1	ENSG00000136048

SNORA80E	down	2,33	4,30e-02	small nucleolar RNA, H/ACA box 80E	ENSG00000207475
ST6GAL1	down	2,31	1,07e-02	ST6 beta-galactoside alpha-2,6-sialyltransferase 1	ENSG00000073849
ZC3H12C	down	2,29	1,32e-02	zinc finger CCCH-type containing 12C	ENSG00000149289
UBTD2	down	2,28	2,35e-02	ubiquitin domain containing 2	ENSG00000168246
SNORD92	down	2,28	3,72e-02	small nucleolar RNA, C/D box 92	ENSG00000264994
TM4SF1	down	2,26	2,41e-02	transmembrane 4 L six family member 1	ENSG00000169908
ADCY3	down	2,26	8,69e-03	adenylate cyclase 3	ENSG00000138031
MIR155HG	down	2,24	2,30e-02	MIR155 host gene	ENSG00000234883
ZC3H12D	down	2,23	3,55e-02	zinc finger CCCH-type containing 12D	ENSG00000178199
UBXN8	down	2,21	1,19e-02	UBX domain protein 8	ENSG00000104691
SNORA75	down	2,19	9,72e-03	small nucleolar RNA, H/ACA box 75	ENSG00000206885
SNORD70	down	2,19	2,76e-02	small nucleolar RNA, C/D box 70	ENSG00000212534
BABAM2	down	2,17	4,56e-02	BRISC and BRCA1 A complex member 2	ENSG00000158019
SUCNR1	down	2,15	2,85e-02	succinate receptor 1	ENSG00000198829
SDF2L1	down	2,15	3,99e-02	stromal cell derived factor 2 like 1	ENSG00000128228
IFIT1	down	2,14	3,80e-03	interferon induced protein with tetratricopeptide repeats 1	ENSG00000185745
SLC39A10	down	2,13	8,28e-03	solute carrier family 39 member 10	ENSG00000196950
TCF4	down	2,13	3,91e-03	transcription factor 4	ENSG00000196628
PMAIP1	down	2,11	1,56e-02	phorbol-12-myristate-13-acetate-induced protein 1	ENSG00000141682
MANF	down	2,09	1,89e-02	mesencephalic astrocyte derived neurotrophic factor	ENSG00000145050
LINC00944	down	2,05	6,51e-03	long intergenic non-protein coding RNA 944	ENSG00000256128
PCSK6	down	2,05	3,37e-02	proprotein convertase subtilisin/kexin type 6	ENSG00000140479
SEC61A1	down	2,03	4,02e-03	Sec61 translocon alpha 1 subunit	ENSG00000058262
LMAN1	down	2,03	2,71e-02	lectin, mannose binding 1	ENSG00000074695
DHX58	down	2,03	1,13e-02	DExH-box helicase 58	ENSG00000108771
SNORD116	down	2,00	2,63e-02	small nucleolar RNA, C/D box 116-1	ENSG00000207063

Table S7. Number of patients with AD cirrhosis assigned to each experiment and their origin.

	Number	Origin
PBMC and PMN *in vitro* experiments	n=17	Liver Intensive Care Unit, Hospital Clínic of Barcelona
Assessment of plasma cytokines	n=16	INFECIR-2 study, Fundació Clínic
Assessment of plasma CpG-DNA	n=8 n=16	Liver Intensive Care Unit, Hospital Clínic of Barcelona INFECIR-2 study, Fundació Clínic

Data file S1. Individual data from patients (provided as separate Excel file).

STUDY 2: *Albumin Lipidomics Reveals Meaningful Compositional Changes in Advanced Cirrhosis and Its Potential to Promote Inflammation Resolution*

The aim of this study was to characterize the albumin lipidome in patients with AD cirrhosis and to investigate its modulatory effects on the production and release of bioactive lipid mediators. We performed a lipidomics investigation of the albumin molecule by comparing the lipid composition of albumin-enriched and albumin-depleted plasma fractions from patients with AD cirrhosis with that of HS. In addition, we assessed the effects of exogenous albumin on the biosynthesis and release of FA-derived lipid mediators by PBMCs and PMNs.

Plasma lipid levels are different in AD patients than in HS

The circulating lipid profile of patients with AD cirrhosis was determined by liquid chromatography coupled to tandem mass spectrometry (LC/MS-MS) and compared with that of HS. Results show a generalized lipid suppression of all subclasses in plasma from AD patients compared to HS, except for FA, which were significantly increased. In these patients, FA become the third most commune family of lipids in the plasma. Moreover, comparing individual lipids values between AD patients and HS in a Cleveland plot, 8 out of 15 lipids with the highest fold change increases were FA. This increase was primarily attributed to monounsaturated FAs (MUFAs), especially palmitoleic, oleic, and gadoleic acids together with the omega-9 PUFA mead acid.

Optimization of a fractionation method to identify lipids in the albumin molecule

To investigate the albumin lipidome in AD patients, plasma was fractionated by affinity chromatography into albumin-enriched and albumin-depleted fractions and assessed their lipid composition. Proper separation of the two fractions was confirmed by western blot analysis and albumin concentration was measured by polyethyleneglycol–enhanced immunoturbidimetry in both, HS and patients with AD. Furthermore, AA was selected as a representative lipid species and prove the ability of our system to detect lipids in both albumin fractions.

Lipid distribution in albumin-enriched and albumin-depleted fractions varied between HS and AD patients

Lipids were primarily found in the albumin-enriched fraction in both HS and AD patients. Mirroring what was found in total plasma, the lipid profile of albumin-enriched and albumin-depleted fractions was characterized by a generalized suppression in AD

patients, except for FAs. In AD patients, although FA are mainly found in albumin-enriched fraction, higher content of MUFAs and PUFAs were detected in the albumin-depleted fraction compared to HS. Moreover, a shift in the distribution of omega-6 and omega-3 PUFAs between the two fractions was observed. In HS the most abundant FA was the omega-3 DHA, whereas in AD patients this leading position was taken by the omega-6-PUFAs adrenic acid and AA.

Altered plasma profile of PUFA-derived lipid mediators in AD cirrhosis

Bioactive lipid mediators, recognized endogenous cell signaling molecules, were measured in total plasma from HS and AD patients. The overall plasma concentration of lipid mediators was significally reduced in AD patients, mostly attributed to the suppression of monohydroxy FA and TXB_2, which could be related to the thrombocytopenia present in cirrhosis as all those lipid mediators are derived from platelets. PGs showed a decreased trend in plasma from AD patients but an increased expression of PG-degrading enzyme 15-hydroxyPG dehydrogenase (HPGD), suggesting an increased degradation of PGs. In contrast, pro-inflammatory LTs showed an increase trend in patients with AD and SPMs levels were similar in HS and AD patients. The ratio between LTs and SPMs was higher in patients with AD, likely reflecting a preponderance of inflammatory environment.

Differential partitioning of PUFA-derived lipid mediators in AD cirrhosis

We next compared the distribution of lipid mediators in albumin-enriched and albumin-depleted fractions. Results showed that in HS, the albumin fraction was enriched with monohydroxy FAs, which are precursors of SPM, such as 17-HDHA, 14-HDHA, 15-HETE and 18-HEPE, in addition with the protectin PDX. On the other hand, albumin from patients with AD had a lower content of precursors of SPMs and in these patients, a significantly higher content of PGE_2 was found in the albumin-depleted fraction. Moreover, PGD_2 and $PGF_{2\alpha}$ showed the same trend in AD patients. These results suggest that the distribution of monohydroxy FAs, and SPMs between the albumin-enriched and albumin-depleted fractions was altered in patients with AD cirrhosis and proinflammatory PGs primarily travels disassociated to albumin in the circulation of AD patients.

Exogenous HSA modulates the biosynthesis of lipid mediators in leukocytes

As albumin lipidome was altered in AD patients we wanted to investigate if the administration of exogenous HSA could modulate the release of lipid mediators in leukocytes. PBMC and PMN were incubated with HSA and lipid mediators release were

measured. Reduced levels of PGE_2, PGD_2, $PGF_{2\alpha}$ and TXB_2 were observed after albumin treatment, also under leukocyte challenged with LPS+fMLP, a classical eicosanoid activation model that mimics the inflammatory conditions encountered in patients with AD. Unexpectedly, albumin triggered the release of the anti-inflammatory and pro-resolutive precursors 15-HETE and 18-HEPE, also under LPS+fMLP-primed PBMCs. Consistent with this, HSA up-regulated 15-LOX2 expression in PBMCs. Then, same experiments were performed with PBMC isolated from AD patients. When albumin was added to these incubations, a prominent inhibition of the LPS+fMLP-induced release of PGE2, PGD2, PGF2α, and TXB2 was observed, in addition, albumin triggered the formation of 15-HETE and 18-HEPE in a concentration-dependent manner in LPS/fMLP-elicited PBMCs.

Modulation of the plasma levels of lipid mediators in AD patients receiving albumin therapy

Finally, levels of lipid mediators were measured in plasma samples from AD patients SMT alone or STM plus albumin included in the INFECIR-2 clinical trial. The results of this measurements showed that patients receiving albumin had lower levels of PGE_2 and PGD_2 in the circulation as compared with those receiving SMT alone. In addition, patients receiving SMT plus albumin presented a significant increase in the plasma concentration of the pro-resolving monohydroxy FA 15-HETE. These results suggest that our previous results of HSA modulating the production of lipid mediators might have some translation in patients with AD cirrhosis receiving albumin therapy.

Albumin Lipidomics Reveals Meaningful Compositional Changes in Advanced Cirrhosis and Its Potential to Promote Inflammation Resolution

Mireia Casulleras ,[1,2] Roger Flores-Costa ,[1,2] Marta Duran-Güell,[1,2] Ingrid W. Zhang,[1,2] Cristina López-Vicario,[1,2] Anna Curto,[1] Javier Fernández,[1,3] Vicente Arroyo,[1] and Joan Clària [1,2,4]

Albumin infusions are therapeutically used to revert hypoalbuminemia and to replace the extensively oxidized albumin molecule circulating in patients with acutely decompensated (AD) cirrhosis. Because albumin has high affinity for lipids, here we characterized the albumin lipidome in patients with AD and explored the albumin effects on the release of fatty acid (FA)–derived lipid mediators by peripheral leukocytes. Lipids and lipid mediators were measured by liquid chromatography–tandem mass spectrometry in albumin-enriched and albumin-depleted plasma fractions separated by affinity chromatography and in leukocyte incubations from 18 patients with AD and 10 healthy subjects (HS). Lipid mediators were also measured in 41 patients with AD included in an albumin therapy trial. The plasma lipidome associated with AD cirrhosis was characterized by generalized suppression of all lipid classes except FAs. In contrast to HS, albumin from patients with AD had lower content of polyunsaturated FAs (PUFAs), especially of the omega-3-PUFA docosahexaenoic acid. Consistent with this, the PUFA-derived lipid mediator landscape of albumin from patients with AD was dominated by lower content of monohydroxy FA precursors of anti-inflammatory/pro-resolving lipid mediators (i.e., 15-hydroxyeicosatetraenoic acid [15-HETE]). In addition, albumin from patients with AD was depleted in prostaglandin (PG) E_2, suggesting that this proinflammatory PG primarily travels disassociated to albumin in these patients. Incubation of leukocytes with exogenous albumin reduced PG production while inducing 15-lipoxygenase expression and 15-HETE release. Similar effects were seen under lipopolysaccharide plus *N*-formylmethionyl-leucyl-phenylalanine-stimulated conditions. Finally, PG levels were lower in patients with AD receiving albumin therapy, whereas 15-HETE was increased after albumin treatment compared with baseline. *Conclusion:* Our findings indicate that the albumin lipid composition is severely disorganized in AD cirrhosis and that administration of exogenous albumin has the potential to redirect leukocyte biosynthesis from pro-inflammatory to pro-resolving lipid mediators. (*Hepatology Communications* 2022;0:1-14).

Lipids not only represent an important energy source and are essential structural components of cell membranes, but they play critical roles in organelle homeostasis, cell metabolism, signaling and survival, and interorgan communication.[1-4] It is also recognized that lipids, especially fatty acids (FAs), govern immune responses.[3,4] For example, polyunsaturated FAs (PUFAs) serve as parent precursors for conversion through specific enzymatic pathways, predominantly expressed in immune cells, into a wide array of bioactive lipid mediators.[2] The PUFAs consist of two distinct families: the omega-6 and the omega-3. The omega-6-PUFAs, of which arachidonic acid (AA) is the best representative, is the common substrate of the

Abbreviations: 14-HDHA, 14-hydroxydocosahexaenoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid; 17-HDHA, 17-hydroxydocosahexaenoic acid; 18-HEPE, 18-hydroxyeicosapentaenoic acid; AA, arachidonic acid; AD, acute decompensation; COX, cyclooxygenase; DHA, docosahexaenoic acid; DPBS, Dulbecco's phosphate-buffered saline; EPA, eicosapentaenoic acid; FA, fatty acid; fMLP, N*-formylmethionyl-leucyl-phenylalanine; HS, healthy subjects; HSA, human serum albumin; LC/MS-MS, liquid chromatography coupled to tandem mass spectrometry; LOX, lipoxygenase; LPS, lipopolysaccharide; LT, leukotriene; LX, lipoxin; MUFA, monounsaturated FA; PBMC, peripheral blood mononuclear cell; PG, prostaglandin; PMN, polymorphonuclear neutrophil; PUFA, polyunsaturated FA; SMT, standard medical therapy; SPM, specialized pro-resolving mediator; TXB_2, thromboxane B_2.*

Received September 30, 2021; accepted December 22, 2021.
Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1893/suppinfo.

cyclooxygenase (COX) and lipoxygenase (LOX) pathways for the biosynthesis of potent pro-inflammatory and immunosuppresive lipid mediators such as prostaglandin (PG) and leukotrienes (LTs).[2,5] On the other hand, the omega-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are converted by the same COX and LOX pathways into an array of anti-inflammatory lipid mediators involved in the resolution of inflammation, designated as specialized pro-resolving mediators (SPMs).[6] AA can also be a precursor of SPMs, such as lipoxins (LXs).[6] Fine equilibrium between omega-6-derived and omega-3-derived lipid mediators is critical for the regulation of immune and inflammatory processes and maintenance of tissue homeostasis.[7] Consequently, an altered lipid composition can lead to immune-metabolic dysregulation and uncontrolled inflammation.

Albumin has high affinity for lipids, especially FAs, which are transported in the circulation attached to its molecule.[8] Albumin, which is synthesized in the liver, is the most abundant protein in the bloodstream, is the main contributor to plasma colloid osmotic pressure, and is clinically used in conditions of hypovolemia and hypoalbuminemia.[9] Indeed, infusion of exogenous albumin is an effective therapy in patients with acutely decompensated (AD) liver cirrhosis.[9,10] Specifically, in these patients, short-term and long-term albumin administration prevents circulatory and renal dysfunction, the development of ascites, and reduces the number of hospital readmissions and mortality.[9,10] In addition to acting as a plasma volume expander, albumin is a potent immunomodulatory molecule, and its internalization by peripheral leukocytes from patients with AD cirrhosis has been shown to regulate endosomal toll-like receptor signaling and immune response to pathogens.[11] However, increased oxidative stress and high frequency of post-translational modifications of the albumin molecule are common findings in patients with AD cirrhosis.[12,13] These features, on top of hypoalbuminemia, likely affect the lipid content of the albumin molecule and alter the circulatory lipid profile of patients with AD, which could contribute to the hyperinflammatory status and immune dysregulation characteristic of this condition. Taking all of these into account, in the current study we performed a lipidomics investigation of the albumin molecule by comparing the lipid composition of albumin-enriched and albumin-depleted plasma fractions from patients with AD cirrhosis with that of healthy subjects (HS). In addition, because albumin infusions are therapeutically used in patients with AD, we assessed the effects of exogenous albumin on the biosynthesis and release of FA-derived lipid mediators by peripheral blood mononuclear cells (PBMCs) and polymorphonuclear leukocytes (PMNs). Finally, we evaluated changes in the plasma levels of lipid mediators in patients with AD cirrhosis included in a therapeutic albumin trial.

Supported by the Agëncia de Gestiû d'Ajuts Universitaris i de Recerca, (2017SGR1449) and Ministerio de Ciencia e Innovaciûn (PID2019-105240RB-I00).

View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep4.1893

Potential conflict of interest: J.F. is on the speakers' bureau of Grifols SA. V.A. advises Yaqrit.

ARTICLE INFORMATION:

From the [1]European Foundation for the Study of Chronic Liver Failure, Barcelona, Spain; [2]Biochemistry and Molecular Genetics Service, Hospital Clínic-IDIBAPS-CIBERehd, Barcelona, Spain; [3]Liver Unit, Hospital Clínic, Barcelona, Spain; [4]Department of Biomedical Sciences, University of Barcelona, Barcelona, Spain.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Joan Clària, PhD
Biochemistry and Molecular Genetics Service
Hospital Clínic/IDIBAPS/EF-Clif
Villarroel 170
08036 Barcelona, Spain
E-mail: jclaria@clinic.cat
Tel.: +1-34-932-271-400

Experimental Procedures

PATIENTS WITH AD CIRRHOSIS AND HS

Eighteen patients with AD cirrhosis from the Liver Intensive Care Unit (ICU) of the Hospital Clinic (Barcelona, Spain) were enrolled in this study. Ten of these patients were included in plasma albumin fractionation experiments, and 8 were included in the experiments with isolated leukocytes. Samples and cells from HS (n = 10) aged 30-60 years were obtained from the Blood Bank of the Hospital Clinic of Barcelona integrated within the Blood and Tissue Bank of the Catalan Department of Health. In addition, biobanked plasma samples were obtained from patients with AD cirrhosis (n = 41) included in the INFECIR-2 study, a phase 4, randomized, open-label, parallel, multicenter therapeutic study aimed at comparing the effect of standard medical therapy with antibiotics (SMT) versus SMT plus human serum albumin (HSA) treatment on hospital mortality in patients with AD cirrhosis and infections other than spontaneous bacterial peritonitis (ClinicalTrials.gov: NCT02034279).[14] Samples were collected before (basal) and after treatment (post) (SMT alone or SMT plus HSA). The selection of patients for these measurements among the whole cohort of the INFECIR-2 study was based on the availability of plasma samples at baseline and after treatment from the same patients. The INFECIR-2 study was approved by the corresponding Ethics Committee of each participating hospital, and informed consent included the potential use of biobanking material for plasma measurements. All participating patients provided signed informed consent. Approval for the use of biological samples from patients in the ICU was obtained from the Hospital Clinic internal review board (#HCB/2020/0138).

PREPARATION OF ALBUMIN-ENRICHED AND ALBUMIN-DEPLETED PLASMA FRACTIONS

Albumin depletion was performed with a Pierce Albumin depletion kit (Thermo Fisher Scientific, Waltham, MA) based on dye-ligand affinity chromatography. Briefly, a spin column was loaded with 200 μL of albumin depletion agarose resin and centrifuged for 1 minute at 12,000*g*. Then, 200 μL of binding buffer were added to the column, centrifuged, and the flow-through discarded before the addition of 50 μL of plasma from HS or patients with AD to the column. Samples were incubated for 2 minutes at room temperature and again centrifuged. The flowthrough was collected, and 50 μL of binding buffer was again added to the column before centrifugation; this process was repeated four times. After that, the first albumin-depleted fraction (albumin-depleted fraction 1) was obtained. The albumin-enriched fraction retained in the column was placed in a new collection tube and washed four times with 200 μL of elution buffer (20 mM Na_3HPO_4 and 250 mM NaSCN; pH 7.2) by centrifugation, retaining the flowthrough in all cases. Because preliminary experiments showed that the albumin-depleted fraction 1 contained some albumin leftover; this fraction was added to a new column followed by the addition of binding buffer (50 μL) to the column and centrifugation. After repeating this process four times, the second albumin-depleted fraction (albumin-depleted fraction 2) was collected. The albumin-enriched fraction retained in this second column was also eluted as described previously. This methodological process is shown in Supporting Fig. S1.

AA MEASUREMENT BY COMPETITIVE ENZYME-LINKED IMMUNOSORBENT ASSAY

To test whether the preparation of albumin-enriched and albumin-depleted fractions by dye-ligand affinity chromatography affects the binding of lipids to this molecule, we monitored AA concentrations by enzyme-linked immunosorbent assay (see Supporting Materials).

ANALYSIS OF ALBUMIN LEVELS IN ALBUMIN-ENRICHED AND ALBUMIN-DEPLETED PLASMA FRACTIONS

The albumin levels in the different plasma fractions were assessed by western blot and polyethylene glycol–enhanced immunoturbidimetry (see Supporting Materials).

CELL ISOLATION AND INCUBATIONS

PBMCs and PMNs were isolated from 20 mL of peripheral blood collected in ethylene diamine tetraacetic acid tubes as described.[11] Blood samples were centrifuged at 200*g* for 10 minutes, plasma collected, and sedimented cells diluted with Dulbecco's phosphate-buffered saline (DPBS) without magnesium and calcium ($DPBS^{-/-}$) up to a volume of 20 mL. Diluted blood was coated over 13.3 mL of Ficoll-Hypaque PLUS (GE Healthcare Lifescience, Chicago, IL) and centrifuged at 500*g* for 25 minutes. The mononuclear cell layer was collected and washed with $DPBS^{-/-}$ and centrifuged again at 400*g* for 5 minutes. The pellet containing the PMN was incubated with prewarmed ammonium-chloride-potassium lysis buffer for 10 minutes at room temperature to remove red blood cells and then centrifuged at 400*g* for 5 minutes. The red blood lysis procedure was repeated twice, and the resultant pellet was washed with $DPBS^{-/-}$. Isolated PBMCs and PMNs were enumerated and resuspended in Roswell Park Memorial Institute 1640 medium with penicillin (100 U/mL) and streptomycin (100 U/mL) and L-glutamine (4 mM) without fetal bovine serum. Cells were seeded at a density of 1.5×10^6 cells/mL and incubated with vehicle or human serum albumin (Albutein 20%; Grifols, Barcelona, Spain) at concentrations of 10 and 15 mg/mL for 30 minutes and then exposed to vehicle or lipopolysaccharide (LPS) from Escherichia coli O111:B4 (1 μg/mL; Merck, Darmstadt, Germany) for 30 minutes plus *N*-formyl-methionyl-leucyl-phenylalanine (fMLP, Merck, Darmstadt, Germany) (1 μg/mL) for 15 minutes. At the end of the incubation period, samples were centrifuged at 1,500*g* for 5 minutes at 4°C, and supernatants and cell pellets were collected for further analysis.

LIPIDOMICS

Untargeted Lipidomics for the Analysis of Structural Lipids

Lipid extraction was accomplished by fractionating the plasma samples and fractions into pools of species with similar physicochemical properties, and analysis was performed using mass spectrometry coupled with ultrahigh performance liquid chromatography (see Supporting Materials).

Targeted Lipidomics for the Analysis of FA-Derived Lipid Mediators in Cell Supernatants

A total of 200 μL of cell supernatants were spiked with an internal standard in acetonitrile, water, and 2,6-di-*tert*-butyl-4-methylphenol, and shaken vigorously. After centrifugation, supernatants were submitted to solid-phase extraction (SPE) in Bond Elute Certify II columns (Agilent Technologies, Santa Clara, CA). The eluate was evaporated and solid residues dissolved in 70 μL acetonitrile/H_2O (50/50, vol/vol) and analyzed using an Agilent 1290 high-performance liquid chromatography (HPLC) system coupled with an Agilent 6495 Triplequad mass spectrometer with an electrospray ionization (ESI) source. The liquid chromatography coupled with tandem mass spectrometry (LC/MS-MS) conditions used to profile arachidonic acid–derived metabolites are described in Supporting Table S1. Quantification was performed using calibration curves with synthetic standards for each of the lipid mediators included in the analysis.

Targeted Lipidomics for the Analysis of FA-Derived Lipid Mediators and SPMs in Plasma

The extraction protocol and analysis of bioactive lipid mediators were performed as described by Le Faouder et al.,[15] adapted by the Ambiotis SAS (Toulouse, France) standard operating procedure. Briefly, plasma (1 mL) was mixed with 0.4 mL of ice-cold methanol and held at −80°C for protein precipitation. Samples were then centrifuged and supernatants collected. After removal of the organic solvent under a stream of nitrogen, samples were suspended in methanol and rapidly acidified to pH 3.5 with HCl. Acidified samples were then loaded into C-18 SPE cartridges (Waters, Milford, MA), rapidly neutralized, and eluted with methyl formate. Eluate solvents were evaporated under a stream of nitrogen and residues suspended in mobile phase for LC/MS-MS analyses using an Exion LCAD U-HPLC system coupled with a Sciex QTRAP 6500+ MS-MS system (AB Sciex, Framingham, MA), equipped with an ESI source in negative ion mode. Quantification was performed using calibration curves with synthetic standards for each of the SPMs included in the analysis.

RNA ISOLATION, REVERSE TRANSCRIPTION, AND REAL-TIME POLYMERASE CHAIN REACTION

Details for the isolation of total RNA from PBMCs and PMNs and analysis by real-time polymerase chain reaction are provided in the Supporting Materials.

STATISTICAL ANALYSIS

Continuous variables were analyzed using unpaired Student *t* tests with Welch's correction, if necessary, or one-way analysis of variance corrected with Tukey's correction if necessary, when comparing more than two groups. Data were expressed as mean ± SEM. Data analyses were performed using GraphPad Prism software version 9 with α set at 0.05 and two-tailed test. Supervised heatmaps representing mean values for each group of the study were represented. Log_2 fold changes comparing groups were calculated and represented in Cleveland, and volcano plots together with $-log_{10}$ transformed *P* values. An unpaired Student *t* test was used for single comparisons and Spearman correlation to assess the correlation of variables. Missing values of some lipid species were set to 0 to approximate no detection and added 0.01 before log_2 transform to assess normal distribution. Statistical analysis was performed using R version 4.0.2 (R-Foundation for statistical computing, www.Rproject.org).

Results

CHANGES IN PLASMA LIPID LEVELS IN PATIENTS WITH AD CIRRHOSIS

The clinical characteristics and standard laboratory data of the patients included in the plasma fractionation by affinity chromatography are given in Supporting Table S2. The circulating lipid profile of patients with AD cirrhosis was determined by LC/MS-MS and compared with that of HS. Consistent with a recent study,[4] the variation in the plasma lipidome associated with AD cirrhosis was characterized by generalized lipid suppression, especially in the glycerophospholipid family (Fig. 1A). FAs were the only lipid class that was not suppressed but rather significantly increased (Fig. 1A), probably reflecting the intense lipolysis present in these patients.[16]The increased abundance of FAs in the circulation of patients with AD can also be appreciated by plotting the percentual contribution of each lipid class to the total plasma lipid pool (Fig. 1B). The analysis of the lipid abundance expressed as arbitrary units (corresponding to the chromatographic peak areas) revealed that, in patients with AD, FAs escalated from the last to the third position of the lipid class rank order (Supporting Fig. S2A). We next compared the plasma levels of each individual lipid species in a pairwise manner between patients with AD and HS and ranked their fold changes in a Cleveland plot. As shown in Fig. 1C, most lipids were reduced in patients with AD, being the 15 lipids with the highest fold-change reductions (phosphatidylethanolamines [PEs] and phosphatidylcholines containing the omega-6-PUFA AA (20:4) together with cholesteryl esters and PEs containing the omega-3 PUFAs EPA [20:5] and DHA [22:6]). Members of the sphingolipid family (i.e., sphingomyelin [d18:2/23:0] and ceramide [d18:1/23:0]) were also among the lipids more severely suppressed in plasma from patients with AD cirrhosis (Fig. 1C). In contrast, a small group of lipids were increased in patients with AD, among them FAs, which accounted for 8 of the 15 lipids with the highest fold-change increases (Fig. 1C). (See Supporting Table S3 for details of the FAs detected in our study.) This increase was primarily attributed to monounsaturated FAs (MUFAs), especially palmitoleic, oleic, and gadoleic acids together with the omega-9 PUFA mead acid (Fig. 1D and Supporting Fig. S2B). Of interest, the percent contribution of PUFAs to the plasma FA pool decreased in patients with AD (Supporting Fig. S2C).

SETUP OF A PLASMA FRACTIONATION METHOD TO IDENTIFY THE CONTENT OF LIPIDS IN THE ALBUMIN MOLECULE

Albumin has high avidity for lipids.[8] Patients with AD cirrhosis present qualitative and quantitative

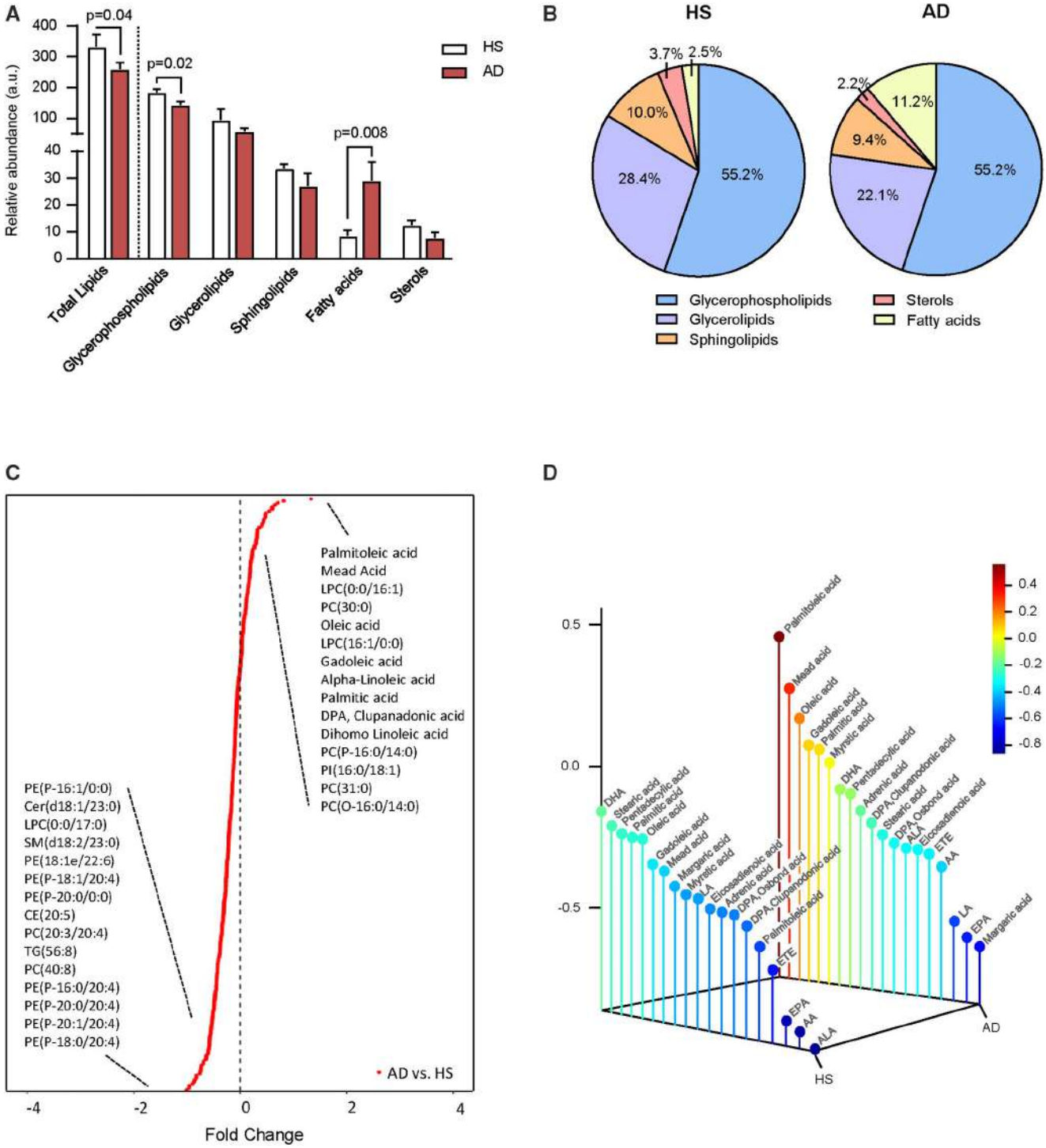


FIG. 1. Lipidomic profile in patients with AD cirrhosis compared to HS. (A) Levels of total lipids and the five lipid classes measured by LC/MS-MS in total plasma from patients with AD cirrhosis (n = 10) and HS (n = 5). Results are expressed as mean ± SEM. (B) Pie charts representing the percentage of each lipid class with respect to total plasma lipid content in patients with AD cirrhosis and HS. (C) Cleveland plot of the whole set of 342 lipids measured in total plasma ranked according to the fold changes between patients with AD versus HS. Upper-right inset: zooming on the 15 lipids more abundant in AD compared to HS. Lower left inset: zooming on the 15 lipids with the highest fold-change reductions in AD compared to HS. (D) Three-dimensional plot of log10-converted values of fatty acids measured by LC/MS-MS in total plasma from HS and patients with AD ranked by their abundance. Abbreviations: ALA, Alpha-Linolenic acid; CE, Cholesteryl Ester; Cer, Ceramide; DPA, Docosapentaenoic acid; ETE, Eicosatrienoic acid; LA, Linoleic acid; LPC, Lysophosphatidylcholine; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PI, Phosphatidylinositol; SM, Sphingomyelin; TG, Triacylglycerol.

changes in the albumin molecule. For example, these patients present extensive posttranslational albumin modifications[12,13] together with hypoalbuminemia (Supporting Fig. S3A), features that likely contribute to altering their plasma lipidome. To investigate this, we fractionated the patient's plasma by affinity chromatography into albumin-enriched and albumin-depleted fractions and assessed their lipid composition. As described previously, the first albumin-depleted fraction contained some albumin leftover; therefore, the depletion process was repeated (see Materials and Methods and Supporting Fig. S1 for details). Following this procedure, proper separation of the two fractions was achieved as confirmed by western blot analysis (Supporting Fig. S3B). This fractionation process was specific for albumin, as other proteins abundant in serum such as immunoglobulin Gs were detected in the albumin-depleted fraction but not in the enriched one (Supporting Fig. S3C). The quantification of the albumin concentration by polyethylene glycol–enhanced immunoturbidimetry also confirmed proper separation in both HS and patients with AD (Supporting Fig. S3D). Indeed, albumin enrichment was 99.96% and 98.27% in HS and patients with AD, respectively (Supporting Fig. S3E). Furthermore, we selected AA as a representative lipid species to prove the ability of our system to detect lipids in the two albumin fractions (Supporting Fig. S3F).

LOWER ABUNDANCE OF FAS IN THE ALBUMIN-ENRICHED FRACTION FROM PATIENTS WITH AD CIRRHOSIS

We next performed a supervised cluster analysis of the lipids detected in the albumin-enriched and albumin-depleted fractions. As shown in Fig. 2A,B and Supporting Fig. S4, lipids were primarily found in the albumin-enriched fraction in both HS and patients with AD. The only lipid that was not primarily found in this fraction was the steroid hormone cortisol (Supporting Fig. S4). Mirroring that described in total plasma (Fig. 1A), the lipid profile of the albumin-enriched and albumin-depleted fractions from patients with AD was characterized by a generalized suppression (Fig. 2A,B). The distribution of FAs between albumin-enriched and albumin-depleted fractions varied between HS and patients with AD. In HS, saturated FAs were similarly distributed between the two fractions, whereas MUFAs and PUFAs were found primarily in the albumin-enriched fraction (Fig. 2C). In patients with AD, higher content of MUFAs and PUFAs was detected in the albumin-depleted fraction, although these FAs appeared to travel mostly associated with albumin in the bloodstream of these patients (Fig. 2D). Moreover, a shift in the distribution of omega-6 and omega-3 PUFAs between the two fractions was observed in patients with AD. Specifically, whereas in HS the omega-3 DHA was the most significant PUFA in the albumin-enriched fraction, this leading position was taken by the omega-6-PUFAs adrenic acid and AA in patients with AD (Fig. 2E,F).

ALTERED PLASMA PROFILE OF PUFA-DERIVED LIPID MEDIATORS IN AD CIRRHOSIS

Bioactive lipid mediators are recognized as important endogenous cell signaling molecules. These lipid mediators originate primarily from PUFAs released from plasma membrane phospholipids into the cytoplasm, where they are converted by COX and LOX pathways into PGs, LTs and LXs in the case of AA, and into resolvins, protectins, and maresins in the case of DHA and EPA (Fig. 3A). PGs and LTs carry potent inflammatory properties, whereas LXs, resolvins, protectins, and maresins have anti-inflammatory and pro-resolution activities and are collectively known as SPMs.[5,6] In our study, the overall plasma concentrations of lipid mediators were significantly reduced in patients with AD cirrhosis—a reduction mostly attributed to the suppression of the monohydroxy FAs 12-hydroxyeicosatetraenoic acid (12-HETE) and 14-hydroxydocosahexaenoic acid (14-HDHA) as well as thromboxane B_2 (TXB_2) (Fig. 3B,C). This observation could be related to the characteristic thrombocytopenia prevalent in cirrhosis, as all of these lipid mediators are derived from platelets.[17] PGs showed a decrease trend in leukocytes from patients with AD, which was not associated with changes in PG-generating enzymes such as *PTGS1* but rather with increased expression of the PG-degrading enzyme 15-hydroxyPG dehydrogenase (Fig. 3B,D). In contrast, plasma levels of pro-inflammatory LTs showed an increase trend in patients with AD, which was not associated with changes in *ALOX5* expression

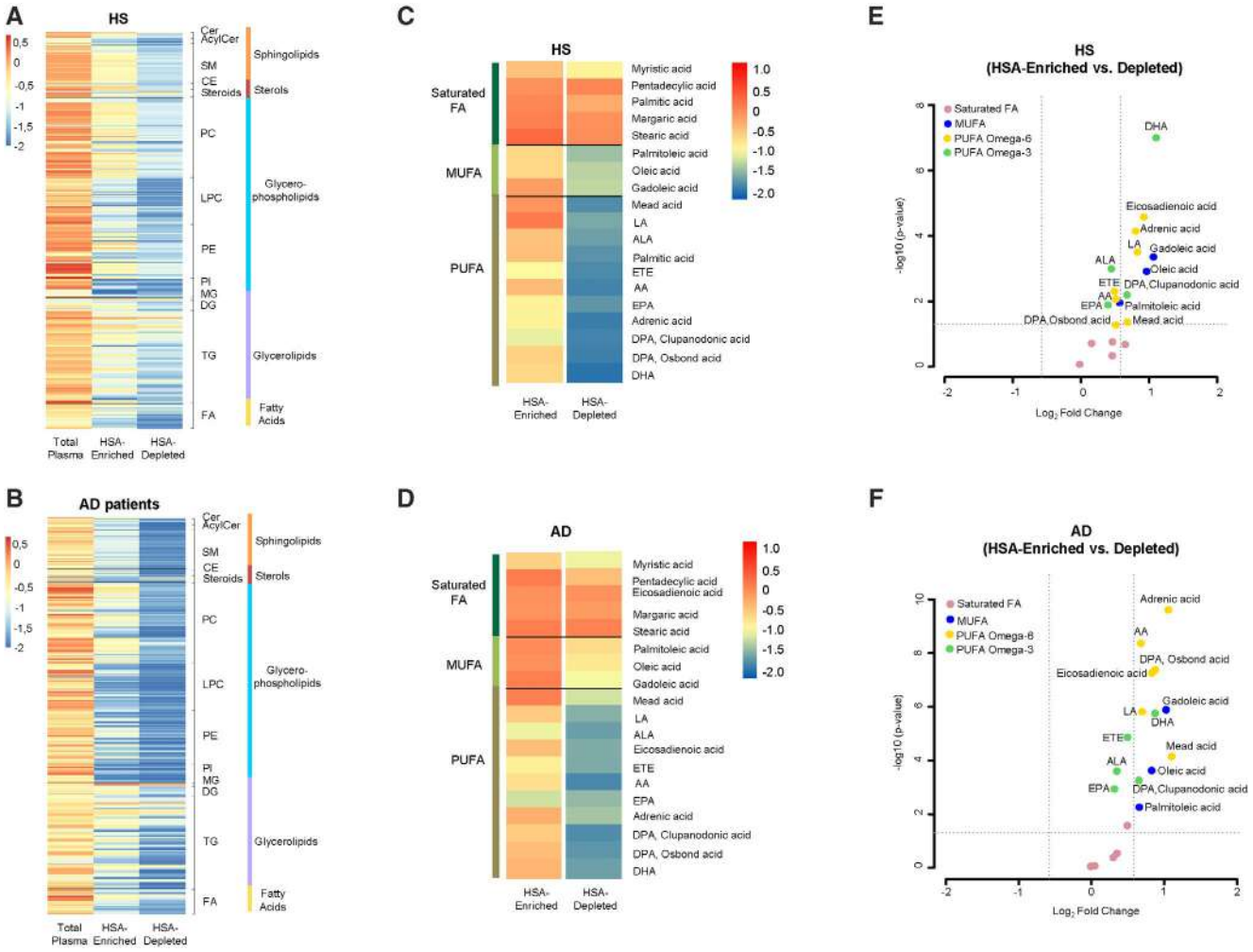


FIG. 2. Lipidomic profile after plasma albumin fractionation in patients with AD and HS. (A) Supervised heatmap of lipids in total plasma and in albumin-enriched and albumin-depleted fractions in HS (n = 5). Mean values of log10-converted amount of lipids are represented. (B) Same as (A) but for lipids in patients with AD (n = 10). (C) Zooming in the supervised heatmap of FAs in plasma albumin fractions represented by log10-transformed mean values for HS. (D) Same as (C) but for FAs in patients with AD. (E) Volcano plot of FAs in albumin-enriched versus albumin-depleted fractions from HS. (F) Same as (E) but for FAs in patients with AD. Log_2-fold changes comparing groups were calculated together with $-log_{10}$-transformed *P* values, considered as significant when a log_2-fold change was >0.5 and a $-log_{10}$ *P* value was > 1.5. Abbreviations: Acylcer, Monohexosylceramide; ALA, Alpha-Linolenic acid; CE, Cholesteryl Ester; Cer, Ceramide; DG, Diacylglycerol; ETE, Eicosatrienoic acid; DPA, Docosapentaenoic acid; DPA, Docosapentaenoic acid; LA, Linoleic acid; LPC, Lysophosphatidylcholine; MG, Monoacylglycerol; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PI, Phosphatidylinositol; SM, Sphingomyelin; TG, Triacylglycerol.

(Fig. 3B,E). Finally, SPM levels were similar in HS and patients with AD (Fig. 3B), but the ratio between LTs and SPMs was higher in patients with AD cirrhosis (Supporting Fig. S5A), likely reflecting a preponderance of an inflammatory environment in this condition. To understand to what extent the circulating lipid mediator network was altered in AD cirrhosis, we created a supervised clustered correlation matrix of lipid mediators. As shown in Supporting Fig. S5B, two different large clusters, including SPMs, were identified in HS. One cluster was associated with PGs and the other with LTs, reinforcing the view that lipid mediators that promote the onset of inflammation (i.e., PGs and LTs) also dictate its resolution (i.e., SPMs) as previously described by Serhan and Savill.[18] Of note, these SPM clusters were not clearly seen in patients with AD, suggesting a profound alteration of the lipid mediator networks in these patients.

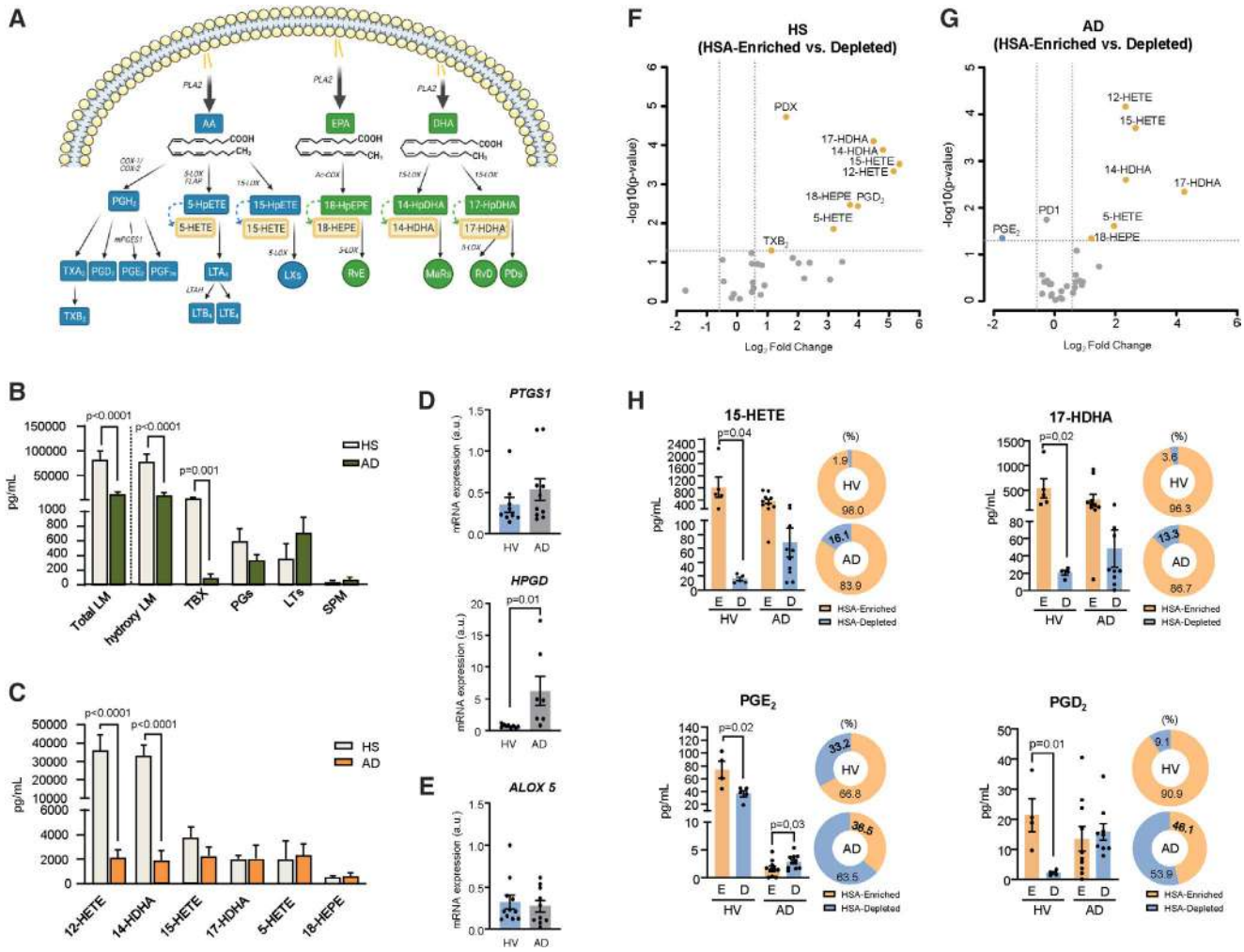


FIG. 3. Distribution of bioactive lipid mediators between albumin-enriched and albumin-depleted fractions. (A) Schematic diagram summarizing the lipid mediator biosynthetic pathways from AA (omega-6-PUFA) (blue) and EPA and DHA (omega-3-PUFA) (green). (B) Concentrations of total lipid mediators and lipid mediator families were obtained by LC/MS-MS in total plasma form HS (n = 5) and patients with AD cirrhosis (n = 10). (C) Detail of the monohydroxy FA family. (D) Messenger RNA (mRNA) expression of *PTGS1* (COX-1) and *15-hydroxyPG dehydrogenase* in PBMCs from HS (n = 9) and patients with AD (n = 9). (E) Same as in (D) but for *ALOX5* (5-LOX) mRNA expression. (F) Volcano plot of lipid mediators in albumin-enriched versus albumin-depleted fraction from HS (n = 5). Log_2 fold changes comparing groups were calculated together with $-log_{10}$-transformed *P* values, considered as significant with a log_2 fold change >0.5 and a $-log_{10}$ *P* value > 1.5. (G) The same as in (F) but for patients with AD (n = 10). (H) Absolute concentrations and percent distribution of 15-HETE, 17-HDHA, PGE_2, and PGD_2 in albumin-enriched (denoted as "E") and albumin-depleted (denoted as "D") fractions from HS (n = 5) and patients with AD (n = 10). Results are expressed as mean ± SEM. Abbreviation: *HPGD*, 15-hydroxyPG dehydrogenase. Abbreviations: ALOX5, arachidonate 5-lipoxygenase (5-LOX); PTGS1, prostaglandin-endoperoxide synthase 1 (COX-1); LM, Lipid mediators; Mar, Maresins; PD, Protectins; RvD, D-series Resolvins; RvE, E-series Resolvins; 5-HETE, 5-Hydroxyeicosatetraenoic acid; 12-HETE, 12-Hydroxyeicosatetraenoic acid.

DIFFERENTIAL PARTITIONING OF PUFA-DERIVED LIPID MEDIATORS IN AD CIRRHOSIS

We next compared the distribution of lipid mediators into the albumin-enriched and albumin-depleted plasma fractions, and the results were plotted in volcano plots comparing fold changes between the two fractions in HS on one side and in patients with AD on the other. In HS, the albumin fraction was enriched with monohydroxy FAs, which are intermediate precursors and/or pathway markers of the biosynthesis of SPMs such

as 17-hydroxydocosahexaenoic acid (17-HDHA), 14-HDHA, 15-hydroxyeicosatetraenoic acid (15-HETE), and 18-hydroxyeicosapentaenoic acid (18-HEPE) (Fig. 3F). The lipid mediator that reached the highest statistical significance was PDX, a 17-HDHA-derived SPM (Fig. 3F). Other AA-derived eicosanoids were present at a higher extent in the albumin-enriched fraction. including 12-HETE, PGD_2, 5-HETE, and TXB_2 (Fig. 3F). On the other hand, albumin from patients with AD had a lower content of precursors/markers of SPMs (Fig. 3G). Notably, in these patients, a significantly higher content of PGE_2 was found in the albumin-depleted fraction, suggesting that this proinflammatory and immunosuppressive PG primarily travels disassociated to albumin in the circulation of patients with AD (Fig. 3G). Figure 3H shows the absolute values and the percent distribution between the two plasma fractions of two SPM precursors (15-HETE and 17-HDHA) and two PGs (PGE_2 and PGD_2), reinforcing the view of profound changes in the composition of lipid mediators in the albumin molecule from patients with AD cirrhosis. In fact, the clustering of PGs with SPMs was severely disturbed in the albumin-enriched fraction from patients with AD cirrhosis (Supporting Fig. S6). The distribution of other monohydroxy FAs, prostanoids, and SPMs between the albumin-enriched and albumin-depleted fractions was also altered in patients with AD cirrhosis (Supporting Fig. S7).

ALBUMIN MODULATES THE BIOSYNTHESIS OF PUFA-DERIVED LIPID MEDIATORS BY HUMAN LEUKOCYTES

A recent study demonstrated that albumin inhibits the release of cytokines by peripheral leukocytes from patients with AD cirrhosis.[11] Therefore, we wondered whether albumin could also modulate the release of lipid mediators by peripheral leukocytes. To investigate this, we incubated PBMCs and PMNs from HS with exogenous albumin. The lipid composition and the content of lipid mediators of the commercial albumin used in our experiments are described in Supporting Table S4 (see separate Excel file) and Supporting Tables S5 and S6. The lipid mediator 17-HDHA, which levels in the commercial albumin, exceeded those present in the leukocyte culture media and was excluded from the analysis. As shown in Supporting Fig. S8A, incubation of resting PBMCs with albumin translated into reduced PGE_2, PGD_2, $PGF_{2\alpha}$, and TXB_2 levels in the supernatant. Unexpectedly, albumin triggered the release of 15-HETE and, to a lower extent, 18-HEPE, which are precursors of anti-inflammatory SPMs (Supporting Fig. S8A). Consistent with this, albumin predominantly up-regulated 15-LOX-2 expression in PBMCs (Supporting Fig. S8B). The effects of albumin on the release of lipid mediators were confirmed in LPS/fMLP-primed PBMCs—a condition that mimics the inflammatory conditions encountered by peripheral leukocytes in patients with AD cirrhosis. In comparison to HS, stimulation of PBMCs from patients with AD cirrhosis with LPS/fMLP induced a higher prostanoid production with no significant changes in the levels of SPM-forming monohydroxy-FAs (Supporting Fig. S8C). When albumin was added to these incubations, a prominent inhibition of the LPS/fMLP-induced release of PGE_2, PGD_2, $PGF_{2\alpha}$, and TXB_2 was observed in PBMCs from both HS and patients with AD (Fig. 4A). Furthermore, albumin triggered the formation of 15-HETE and 18-HEPE in a concentration-dependent manner in LPS/fMLP-elicited PBMCs, independently of whether these cells were isolated from either HS or patients with AD (Fig. 4B). The effects of albumin on the release of lipid mediators by PMN followed a similar trend to that observed in PBMCs from HS, but the magnitude of changes was lower (Supporting Fig. S9).

MODULATION OF THE PLASMA LEVELS OF PUFA-DERIVED LIPID MEDIATORS IN PATIENTS WITH AD CIRRHOSIS RECEIVING ALBUMIN THERAPY

We finally determined the levels of lipid mediators in plasma samples from patients with AD cirrhosis receiving albumin therapy included in the INFECIR-2 study. The baseline clinical characteristics and standard laboratory data from these patients are described in Fernández et al.[14] The results of these measurements are shown in Fig. 5. Under baseline conditions, plasma levels of PGE_2, PGD_2, and $PGF_{2\alpha}$ were not statistically different between patients of the SMT group and patients who received SMT plus albumin (Fig. 5A). However, patients who received albumin showed lower levels of PGE_2 in the circulation as compared with those receiving SMT alone (Fig. 5A). If expressed as percent change versus basal, PGE_2 levels increased after SMT, something that was not observed in patients receiving SMT

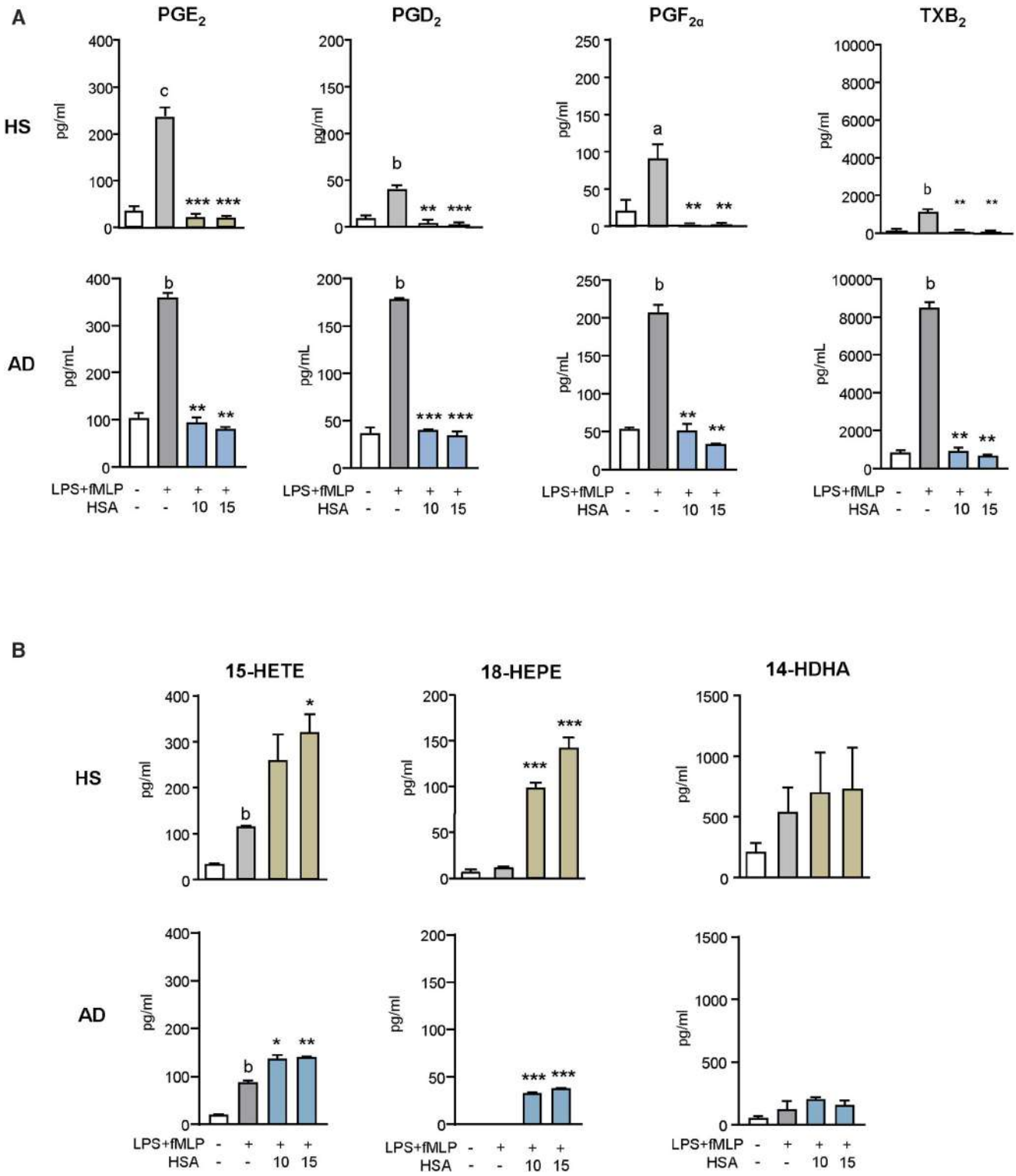


FIG. 4. Effect of exogenous albumin on the release of lipid mediators by PBMCs. (A) PGE_2, PGD_2, $PGF_{2\alpha}$, and thromboxane TXB_2 levels measured by LC/MS-MS in supernatants of PBMCs from HS (upper panel) and patients with AD (lower panel) incubated with albumin (HSA, 10 and 15 mg/mL) for 30 minutes and then stimulated with LPS (1 μg/mL for 30 minutes) plus fMLP (1 μg/mL for 15 minutes) (n = 3 in duplicate). (B) 15-HETE, 18-HEPE, and 14-HDHA levels measured by LC/MS-MS in the same experimental conditions as in (A). Results are expressed as mean ± SEM. $^{a}P < 0.05$, $^{b}P < 0.005$, $^{c}P < 0.001$ as LPS + fMLP versus vehicle, and $^{*}P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.001$ as LPS + fMLP plus HSA versus LPS + fMLP alone.

plus albumin (Supporting Fig. S10A). Patients who received SMT plus albumin also had significantly lower levels of PGD_2 (Fig. 5A). In addition, patients receiving SMT plus albumin presented a significant increase in the plasma concentration of the pro-resolving monohydroxy FA 15-HETE (Fig. 5B). The percent change data versus basal is also shown in Supporting Fig. S10B. Nevertheless, the response of patients with AD to albumin administration was very heterogeneous, indicating that these finding need to be confirmed in other studies, including a larger number of patients with AD cirrhosis as well as with other commercial albumins that might have different lipid compositions.

Discussion

The results of the current study support the concept that albumin has the ability to modulate the lipid composition of human plasma—an effect that is relevant to patients with AD cirrhosis in whom albumin infusions are used as therapy. The most important findings of our study are the following: (1) Circulating lipid levels in patients with AD cirrhosis are remarkably suppressed in comparison to HS. This suppression affects all major lipid families except FA, which are increased. (2) In patients with AD, albumin shows a characteristic lipid composition, especially of PUFAs and PUFA-derived lipid mediators. In particular, in comparison to HS, the albumin-enriched fraction of patients with AD has a lower content of pro-resolving monohydroxy FAs (i.e., 15-HETE). (3) In contrast, the albumin-depleted fraction of patients with AD has an increased content of PGE_2, suggesting that this proinflammatory and immunosuppressive PG primarily travels disassociated to albumin in the circulation of patients with AD. (4) Albumin modulates the biosynthesis of lipid mediators by

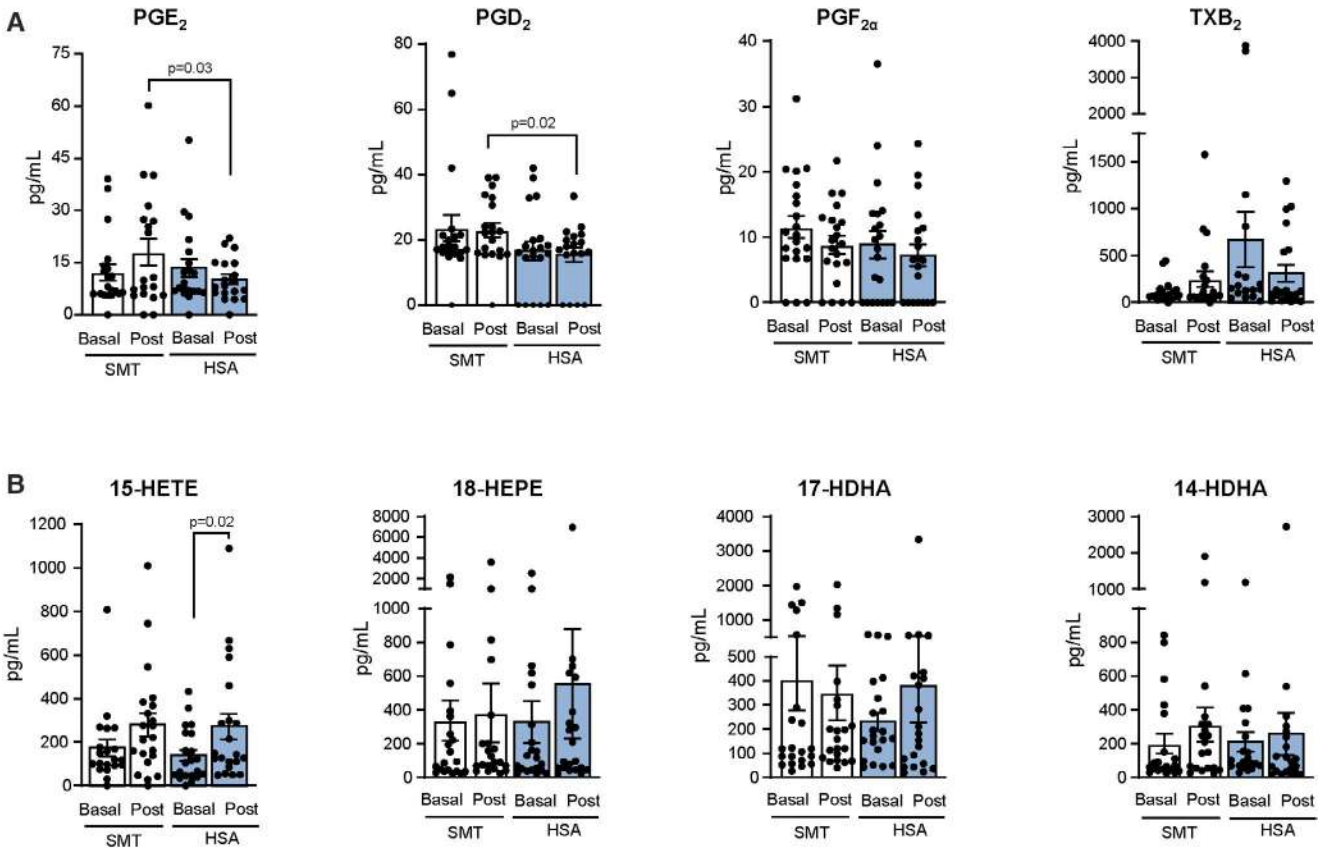


FIG. 5. Plasma levels of lipid mediators in patients with AD cirrhosis receiving albumin therapy. (A) PGE_2, PGD_2, and $PGF_{2\alpha}$ concentrations were measured by LC/MS-MS in plasma from patients with AD included in the INFECIR-2 study under basal conditions and after 7 days of receiving SMT (n = 21) alone or in combination with albumin infusions (HSA, n = 21). (B) 15-HETE, 18-HEPE, and 17-HDHA concentrations in the same conditions as in (A). Results are expressed as mean ± SEM.

circulating leukocytes by reducing pro-inflammatory PG production, while inducing the release of pro-resolving monohydroxy FAs, such as 15-HETE. (5) Lower plasma PG levels and higher concentrations of 15-HETE were observed in patients with AD cirrhosis receiving albumin therapy.

Many of the physiological functions of albumin are based on its ability to reversibly bind soluble molecules, contributing to their transportation in the plasma to different tissues and organs.[9,10] In particular, the albumin molecule contains up to seven binding sites for long chain FAs, some of which are located in the critical Sudlow's sites I and II.[19] Albumin can also bind esterified FAs in phospholipids, especially lysoPC.[20] This is important because the major part of the FA pool in the human plasma is esterified with a minor proportion actually free in solution.[21] Albumin can also bind the sphingolipid sphingosine-1-phosphate[22] (Supporting Table S4). Although the binding of acylglycerols by albumin has been previously reported,[20] in our analysis of commercial human serum albumin we did not detect any member of this lipid family (Supporting Table S4). Any alteration of the albumin chemical structure has a direct impact on its ligand-binding function.[23] Although there is no direct evidence that links the structural alterations of albumin with its ability to bind FAs, oxidation of the Cys-34 residue in the albumin molecule has been shown to reduce the binding of molecules in the Sudlow's sites, where some of the FA binding sites are located.[23] This fact, together with the presence of lower concentrations of albumin, which in addition is highly oxidized in patients with AD cirrhosis,[10,13] might explain the existence of higher levels of free FAs in the bloodstream of these patients. This is particularly relevant because albumin binds omega-6 and omega-3 PUFAs, especially AA and DHA.[24] Of note, commercial albumin is more enriched in DHA than in AA (Supporting Table S5). Moreover, albumin binds biologically active lipid mediators derived from PUFAs, including PGs,[25] LTs,[26,27] and monohydroxy Fas.[28,29] All of these data can help to understand the observed changes in the lipid composition of the albumin molecule in patients with AD cirrhosis.

Albumin has been reported to sequester PGs and accelerate the decomposition kinetics of these lipid mediators—an effect that is dose-dependent.[28] Our finding that albumin inhibits PGE_2 production by leukocytes from both HS and patients with AD *in vitro* is consistent with previous studies demonstrating that albumin reduces PGE_2 levels in patients with AD cirrhosis.[30,31] PGE_2 is a lipid mediator that drives immunosuppression, and therefore is relevant to AD cirrhosis because it can potentially increase the risk of infection in these patients. In our study, we could not confirm that PGE_2 levels were increased in patients with AD cirrhosis, but we observed that PGE_2 levels were reduced in the albumin-enriched fraction of patients with AD. On the other hand, albumin has been reported to favor lipoxygenation of PUFAs and to increase the formation of LOX-derived products.[29,32] Consistent with this, we detected a significant increase in the 15-LOX-derived product 15-HETE in leukocytes from HS and patients with AD incubated with albumin. The enhanced formation of 15-HETE cannot be attributed to the presence of this monohydroxy FA in the commercial albumin used in our experiments because its levels in cell supernatants further exceeded those attached to the commercial albumin preparation (Supporting Table S6). Importantly, increased 15-HETE levels were observed in plasma from patients with AD cirrhosis after receiving albumin therapy. Together, our findings provide evidence that albumin is not merely transporting lipid mediators in the bloodstream but rather a molecule that modulates their biosynthesis and release by circulating immune cells.

Our study has some limitations. For instance, the results of our study do not allow us to bring any conclusion on the source of the circulating FAs in patients with AD cirrhosis. However, similar to other liver disease conditions, such as metabolic-associated fatty liver disease, circulating FAs in patients with AD patients are likely to be derived from white adipose tissue lipolysis. In addition, at present, there is scarce information about whether altered lipid levels in the systemic circulation of patients with AD is related to impaired lipoprotein production by the diseased liver of these patients. Finally, we recognize the preliminary nature of our results in patients with AD cirrhosis receiving albumin therapy. Because the response of patients with AD to albumin administration was very heterogeneous in terms of changes in lipid mediators, it is necessary to confirm these findings in other studies, including a larger number of patients and with proper stratification. Furthermore, these findings need to be confirmed with other commercial albumins that may have a different lipid composition.

In summary, this investigation provides a comprehensive analysis of the composition of lipids and lipid mediators in the albumin molecule from patients with AD cirrhosis, identifying a characteristic pattern of

PUFA and PUFA-derived lipid mediators in these patients. Furthermore, we provide evidence that exogenous albumin modulates the biosynthesis of lipid mediators in circulating leukocytes by redirecting the production of pro-inflammatory and immunosuppressive PGs to the activation of endogenous pro-resolving lipid mediator pathways.

Acknowledgment: The authors thank María Belén Sánchez, Fatima Aziz, and Bryan Javier Contreras for their assistance in the management of samples.

REFERENCES

1) Phillips R, Ursell T, Wiggins P, Sens P. Emerging roles for lipids in shaping membrane-protein function. Nature 2009;459:379-385.
2) Quehenberger O, Dennis EA. The human plasma lipidome. N Engl J Med 2011;365:1812-1823.
3) Ertunc ME, Hotamisligil GS. Lipid signaling and lipotoxicity in metaflammation: indications for metabolic disease pathogenesis and treatment. J Lipid Res 2016;57:2099-2114.
4) **Clària J**, **Curto A**, **Moreau R**, Colsch B, López-Vicario C, Lozano JJ, et al. Untargeted lipidomics uncovers lipid signatures distinguishing severe versus moderate forms of acutely decompensated cirrhosis. J Hepatol 2021;75:1116-1127.
5) Samuelsson B. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. Science 1983;220:568-575.
6) Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology. Nature 2014;510:92-101.
7) López-Vicario C, Checa A, Urdangarin A, Aguilar F, Alcaraz-Quiles J, Caraceni P, et al. Targeted lipidomics reveals extensive changes in circulating lipid mediators in patients with acutely decompensated cirrhosis. J Hepatol 2020;73:817-828.
8) Spector AA. Fatty acid binding to plasma albumin. J Lipid Res 1975;16:165-179.
9) Arroyo V, García-Martinez R, Salvatella X. Human serum albumin, systemic inflammation, and cirrhosis. J Hepatol 2014;61:396-407.
10) Bernardi M, Angeli P, Claria J, Moreau R, Gines P, Jalan R, et al. Albumin in decompensated cirrhosis: new concepts and perspectives. Gut 2020;69:1127-1138.
11) Casulleras M, Flores-Costa R, Duran-Güell M, Alcaraz-Quiles J, Sanz S, Titos E, et al. Albumin internalizes and inhibits endosomal TLR signaling in leukocytes from patients with decompensated cirrhosis. Sci Transl Med 2020;12:eaax5135.
12) **Clària J**, **Stauber RE**, Coenraad MJ, Moreau R, Jalan R, Pavesi M, et al. Systemic inflammation in decompensated cirrhosis: characterization and role in acute-on-chronic liver failure. Hepatology 2016;64:1249-1264.
13) Alcaraz-Quiles J, Casulleras M, Oettl K, Titos E, Flores-Costa R, Duran-Güell M, et al. Oxidized albumin triggers a cytokine storm in leukocytes through P38 mitogen-activated protein kinase: role in systemic inflammation in decompensated cirrhosis. Hepatology 2018;68:1937-1952.
14) **Fernández J**, **Clària J**, Amorós A, Aguilar F, Castro M, Casulleras M, et al. Effects of albumin treatment on systemic and portal hemodynamics and systemic inflammation in patients with decompensated cirrhosis. Gastroenterology 2019;157:149-162.
15) Le Faouder P, Baillif V, Spreadbury I, Motta J-P, Rousset P, Chêne G, et al. LC–MS/MS method for rapid and concomitant quantification of pro-inflammatory and pro-resolving polyunsaturated fatty acid metabolites. J Chromatogr B Anal Technol Biomed Life Sci 2013;932:123-133.
16) **Moreau R**, **Clària J**, Aguilar F, Fenaille F, Lozano JJ, Junot C, et al. Blood metabolomics uncovers inflammation-associated mitochondrial dysfunction as a potential mechanism underlying ACLF. J Hepatol 2020;72:688-701.
17) Laffi G, Tarquini R, Marra F. Thrombocytopenia in chronic liver disease: Lessons from transplanted patients. J Hepatol 2007;47:625-629.
18) Serhan CN, Savill J. Resolution of inflammation: the beginning programs the end. Nat Immunol 2005;6:1191-1197.
19) Bhattacharya AA, Gru T, Curry S. Crystallographic analysis reveals common modes of binding of medium and long-chain fatty acids to human serum albumin. J Mol Biol 2000;303:721-732.
20) Belgacem O, Stu G, Allmaier G, Buchacher A, Pock K. Isolation of esterified fatty acids bound to serum albumin purified from human plasma and characterised by MALDI mass spectrometry. Biologicals 2007;35:43-49.
21) Morgenthaler JJ, Baillod P, Friedli H. Fatty acid patterns during plasma fractionation. Vox Sang 1980;38:288-293.
22) Hammad SM, Al Gadban MM, Semler AJ, Klein RL. Sphingosine 1-phosphate distribution in human plasma: associations with lipid profiles. J Lipids 2012;2012:1-8.
23) Kawakami A, Kubota K, Yamada N, Tagami U, Takehana K, Sonaka I, et al. Identification and characterization of oxidized human serum albumin: a slight structural change impairs its ligand-binding and antioxidant functions. FEBS J 2006;273:3346-3357.
24) Savu L, Benassayag C, Vallette G, Christeff N, Nunez E. Mouse α1-fetoprotein and albumin A comparison of their binding properties with estrogen and fatty acid ligands. J Biol Chem 1981;256:9414-9418.
25) Raz A. Interaction of prostaglandins with blood plasma proteins. Comparative binding of prostaglandins A2, F2α and E2 to human plasma proteins. Biochem J 1972;130:631-636.
26) Zsila F, Bikádi Z, Lockwood SF. In vitro binding of leukotriene B4 (LTB4) to human serum albumin: evidence from spectroscopic, molecular modeling, and competitive displacement studies. Bioorg Med Chem Lett 2005;15:3725-3731.
27) Fitzpatrick FA, Waynalda MA. Albumin-lipid interactions: prostaglandin stability as a probe for characterizing binding sites on vertebrate albumins. Biochemistry 1981;20:6129-6134.
28) Dadaian M, Westlund P. Albumin modifies the metabolism of hydroxyeicosatetraenoic acids via 12-lipoxygenase in human platelets. J Lipid Res 1999;40:940-947.
29) Ek-von Mentzer BA, Zhang F, Hamilton JA. Binding of 13-HODE and 15-HETE to phospholipid bilayers, albumin, and intracellular fatty acid binding proteins. Implications for transmembrane and intracellular transport and for protection from lipid peroxidation. J Biol Chem 2001;276:15575-15580.
30) O'Brien AJ, Fullerton JN, Massey KA, Auld G, Sewell G, James S, et al. Immunosuppression in acutely decompensated cirrhosis is mediated by prostaglandin E2. Nat Med 2014;20:518-523.
31) China L, Maini A, Skene SS, Shabir Z, Sylvestre Y, Colas RA, et al. Albumin counteracts immune-suppressive effects of lipid mediators in patients with advanced liver disease. Clin Gastroenterol Hepatol 2018;16:738-747.
32) Broekman MJ, Eiroa AM, Marcus AJ. Albumin redirects platelet eicosanoid metabolism toward 12(S)-hydroxyeicosatetraenoic acid. J Lipid Res 1989;30:1925-1932.

Author names in bold designate shared co-first authorship.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1893/suppinfo.

SUPPLEMENTARY MATERIAL

Albumin lipidomics reveals meaningful compositional changes in advanced cirrhosis and its potential to promote inflammation resolution.

Mireia Casulleras, Roger Flores-Costa, Marta Duran-Güell, Ingrid W. Zhang, Cristina López-Vicario, Anna Curto, Javier Fernández, Vicente Arroyo and Joan Clària.

Table of contents:

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Analysis of structural lipids

Lipid extraction was accomplished by fractionating the plasma samples and fractions into pools of species with similar physicochemical properties, using appropriate combinations of organic solvents. The following methods were used according to the target lipid chemical classes: Platform 1: Fatty acyl, steroid and lysoglycerophospholipid profiling. Proteins were precipitated from the defrosted samples of plasma and albumin-enriched and albumin-depleted fractions by adding 4 volumes of methanol on ice. Samples were then vortexed and incubated overnight at -20˚C. Supernatants were collected after centrifugation at 18,000 g for 15 min at 4ºC and dried under vacuum. The dried extracts were reconstituted in methanol before being transferred to vials for mass spectrometry coupled to ultra-high performance liquid chromatography (UHPLC-MS) analysis. Platform 2: Glycerolipid, cholesteryl ester, sphingolipid and glycerophospholipid profiling. Plasma and albumin-enriched and albumin-depleted fractions were mixed with sodium chloride (50 mM) and chloroform/methanol (2:1) on ice. Samples were then vortexed and incubated for 1 hour at -20˚C. After centrifugation at 18,000 g for 15 min at 4ºC, the organic phase was collected and the solvent discarded. Then, acetonitrile/isopropanol (1:1) was added to the organic phase and after a brief vortex, samples were transferred to vials for UHPLC-MS analysis. For each analytical platform, randomized sample injections were performed with each of the quality control calibration and validation reference extracts uniformly interspersed throughout the entire batch run.

Targeted lipidomics for the analysis of FA-derived lipid mediators in cell supernatants

Two-hundred µl of cell supernatants were spiked with an internal standard consisting of 15-HETE-d8, LTB4-d4, PGE2-d2 (Cayman Chemical, Ann Arbor, MI) (5 ng each) in 500 µL acetonitrile, 100 µL water and 5 µL 2,6-di-tert-butyl-4-methylphenol (BHT 10 mg/mL) and shaken vigorously. The samples were brought to pH 6 with 2 mL phosphate buffer. After centrifugation, supernatants were submitted to solid-phase extraction (SPE) in Bond Elute Certify II columns (Agilent Technologies, Santa Clara, CA) preconditioned with 3 mL methanol, followed by 3 mL of 0.1 mol/L phosphate buffer containing 5% methanol (pH 6) in a SPE Vacuum Manifold. The SPE-columns were then washed with 3 mL methanol/H2O (50/50, vol/vol) and eluted with 2 mL of n-hexane/ethyl acetate (25:75, vol/vol) with 1% acetic acid. The eluate was evaporated on a heating block at 40°C under a stream of nitrogen. The solid residues were dissolved in 70 µL acetonitrile/H2O (50/50, vol/vol) and analyzed using an

Agilent 1290 high-performance liquid chromatography (HPLC) system with binary pump, multisampler and column thermostat with a Zorbax Eclipse plus C-18 column (2.1 x 150 mm, 1.8 μm) using a solvent system of aqueous acetic acid (0.05%) and acetonitrile/methanol (50:50, vol/vol). The elution gradient was started with 5% organic phase, which was increased within 0.5 min to 56%.The flow rate was set at 0.3 mL/min and the injection volume was 15 μLin a HPLC coupled with an Agilent 6495 Triplequad mass spectrometer (Agilent Technologies) with an electrospray ionization (ESI) source. Analysis was performed with multiple reaction monitoring in negative mode with the following source parameters (drying gas: 115 °C/16 L/min, sheath gas: 390°C/12 L/min, capillary voltage: 4300 V, nozzelvoltage: 1950 V and nebulizer pressure: 35 psi). The liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) conditions used to profile arachidonic acid derived metabolites are described in **Supplementary Table 1**. Quantification was performed using calibration curves with synthetic standards for each of the lipid mediators included in the analysis.

AA measurement by competitive ELISA

To test whether the preparation of albumin fractions by dye-ligand affinity chromatography was affecting their lipid content, we monitored the AA concentrations in albumin-enriched and albumin-depleted fractions using a human arachidonic acid ELISA kit (reference NBP2-59872, Novus Biologicals, CO). Reagents, samples and standards were prepared following the manufacturer's protocol. Briefly, 50 μL of each sample or standards were added to a 96-well plate pre-coated with antibody specific for AA. Thereafter, 50 μL of horseradish peroxidase conjugated AA were added, and the plate was incubated for 40 min at 37ºC. At the end of the incubation period, the plate was washed 5 times and 90 μL of 3,3′,5,5′-tetramethylbenzidine were added to each well and the plate was incubated for 20 min at 37ºC, protected from light. Finally, 50 μL of stop solution were added and the plate was read at 450 nm in a microplate reader (FLUOstar Optima). A four-parameter logistic regression curve-fit was used to create the standard curve and to calculate the concentration of each sample.

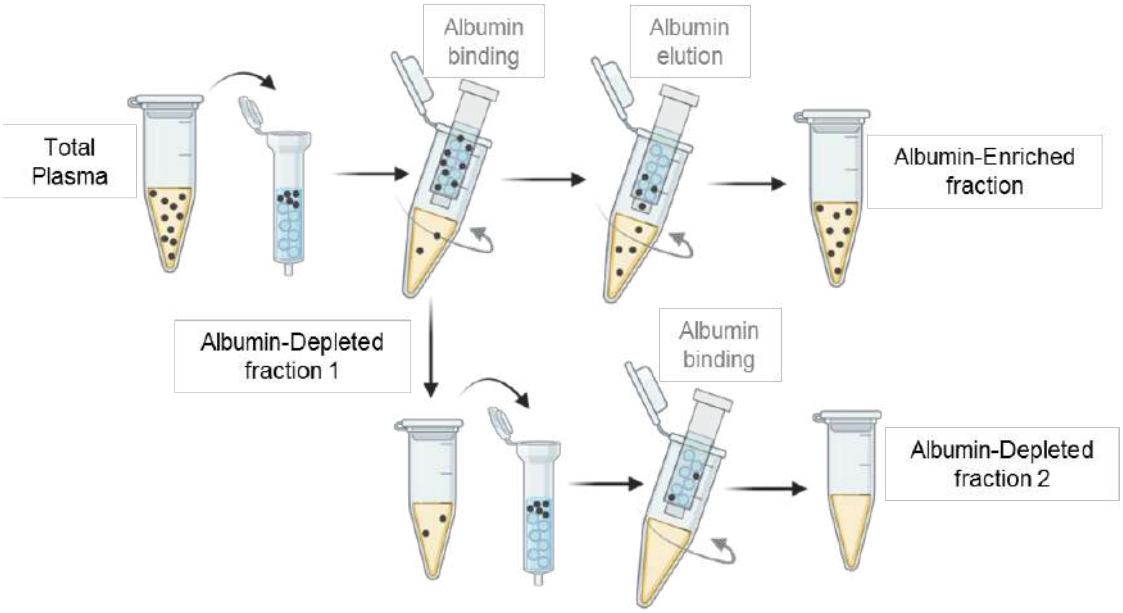
Analysis of albumin levels in albumin-enriched and albumin-depleted plasma fractions

To assess albumin levels in the different plasma fractions, plasma samples were dissolved with 2x Laemmli buffer, heated for 5 min at 95°C, and separated by 10–15% (vol/vol) SDS-PAGE for 90 min at 120 V. Albumin levels were assessed by Western blot using a mouse monoclonal anti-human albumin (dilution 1:500) (HYB 192-01-02, Thermo Fisher Scientific). Gel bands were visualized using the EZ-ECL chemiluminescence detection kit in a LAS 4000 imaging

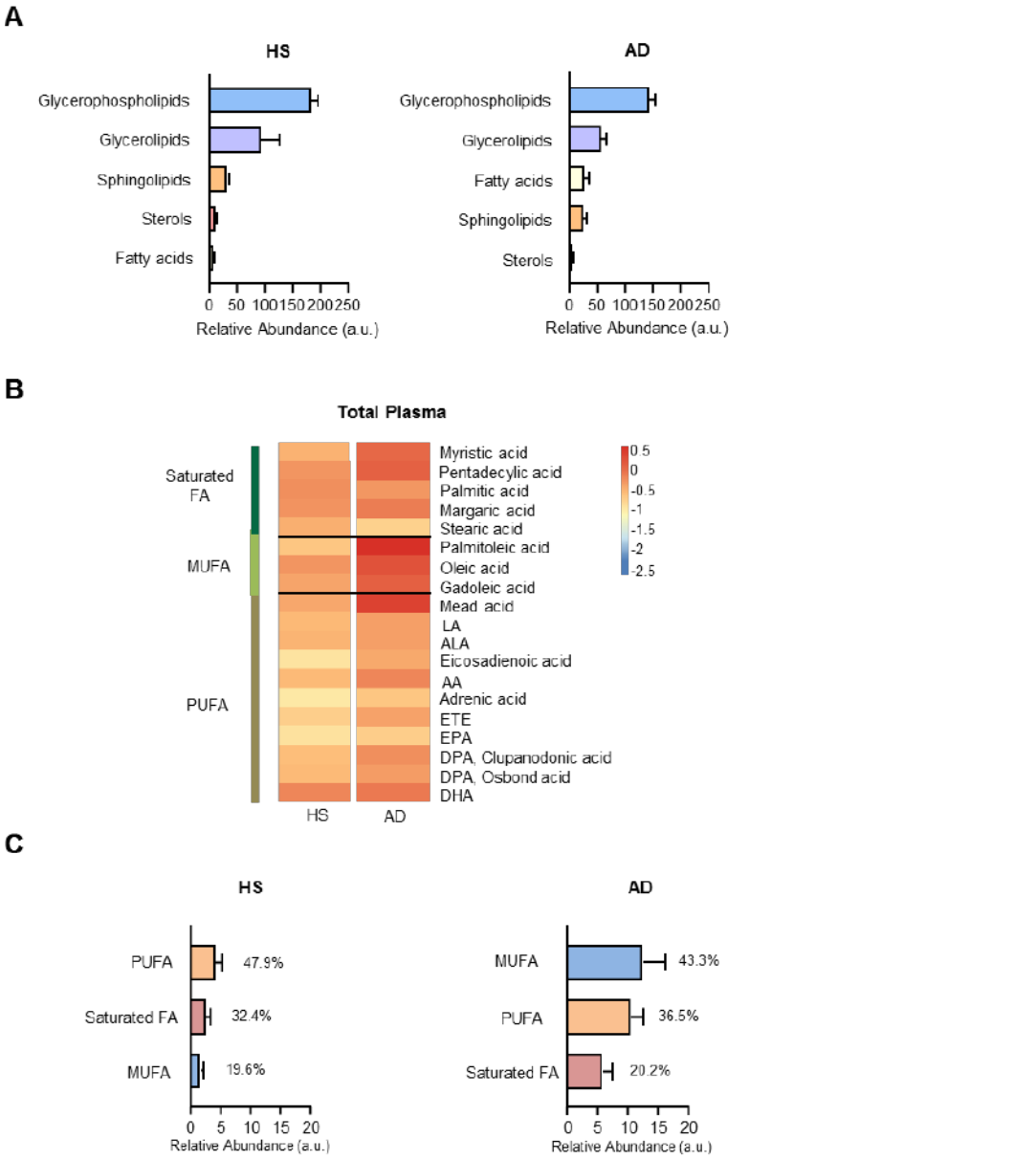
system, as described above. The band signals were quantified using Image GE ImageQuant TL analysis software (GE Healthcare Life Sciences). Albumin concentrations in plasma fractions were also determined by polyethylene glycol-enhanced immunoturbidimetry at the Hospital Clínic Biomedical Diagnostic Center.

RNA isolation, reverse transcription and real-time PCR

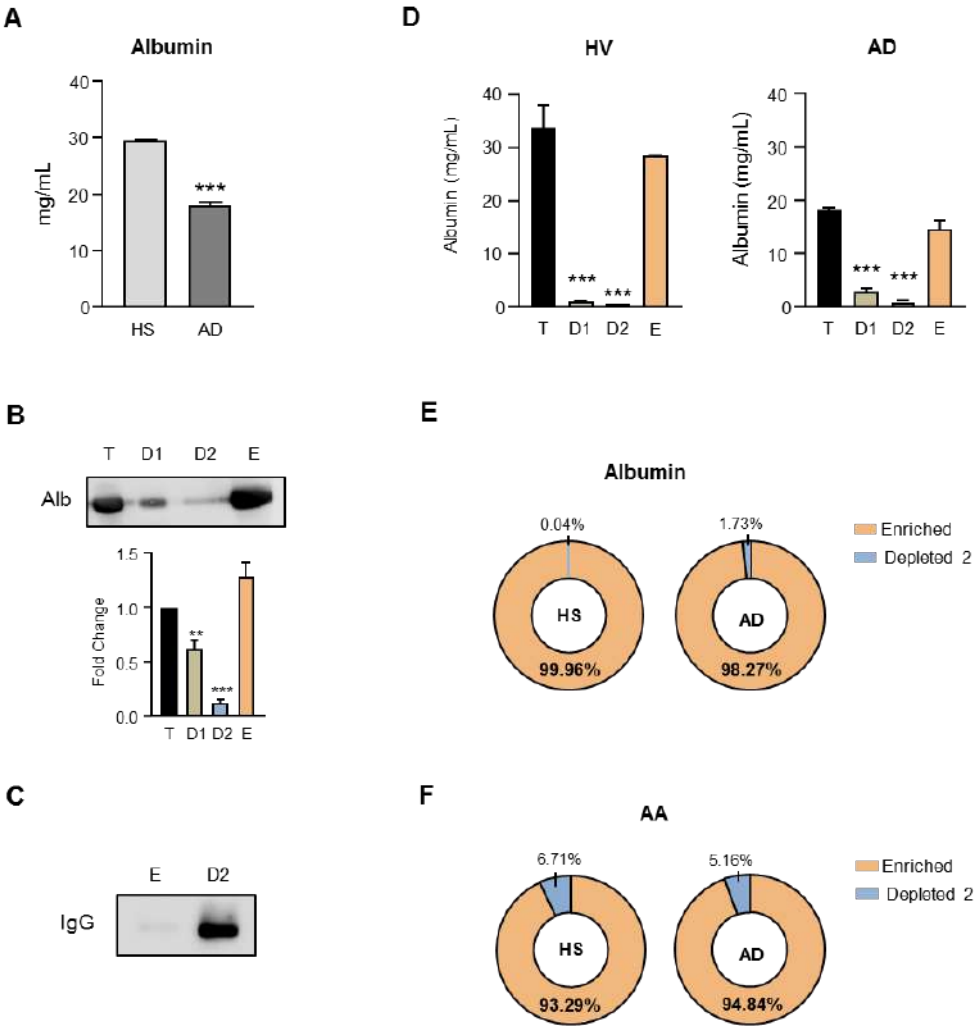
Isolation of total RNA from PBMCs and PMNs was performed using the TRIzol reagent following the manufacturer's instructions. RNA concentrations were assessed in a NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). cDNA synthesis from 200 to 1000 ng of total RNA was performed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Real-Time polymerase chain reaction (PCR) analysis of PTGS2 (COX-2, Hs00153133_m1), PTGS1 (COX-1, Hs00377726_m1), ALOX5AP (Flap, Hs00970921_m1), ALOX5 (5-LOX, Hs00167536_m1), ALOX15 (15-LOX-1, Hs00993765_g1), ALOX15B (15-LOX-2, Hs00153988_m1), mPGES-1 (Hs01115610_m1) and HPGD (Hs00960586_g1) was performed in a 7900HT Fast System (Applied Biosystems) using β-actin (Hs99999903_m1) as endogenous control. Quantitative PCR results were analyzed with Sequence Detector 2.1 Software (Applied Biosystems). Relative quantification of gene expression was performed using a comparative Ct method. The amount of target gene, normalized to β-actin and relative to a calibrator, was determined by the arithmetic equation $2^{-\Delta\Delta Ct}$, as described in the comparative Ct method (http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf).



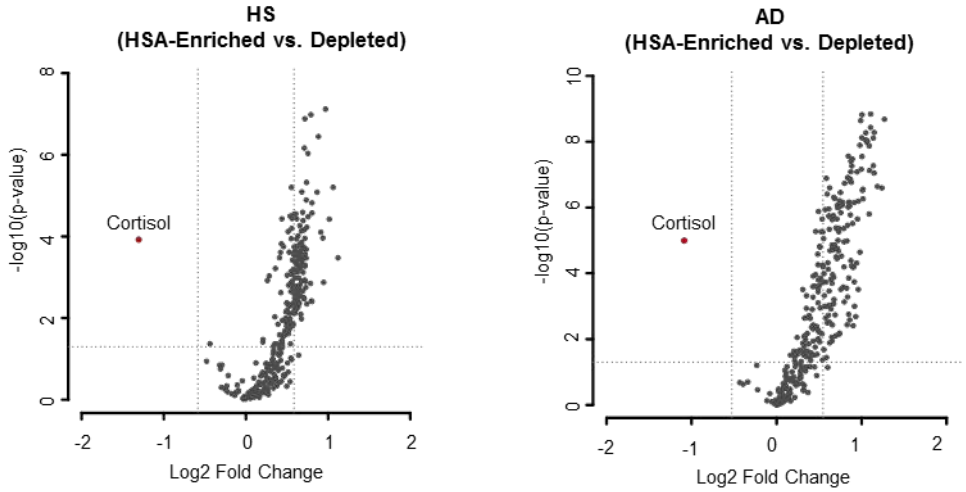
Supplementary Figure 1. Schematic diagram of the separation method used to prepare the plasma albumin fractions by dye-ligand affinity chromatography. Total plasma from HS or AD patients was added to Cibacron blue dye agarose resin columns which have high affinity for albumin. Then, binding buffer was added to the column and the flow-through was collected as the albumin-depleted fraction 1. Then, albumin elution buffer was added to the column and albumin attached to resin was eluted in a new tube labeled as albumin-enriched fraction. Finally, the albumin-depleted fraction 1 was added to a new column together with the binding buffer and the flow-through collected and labeled as albumin-depleted fraction 2.



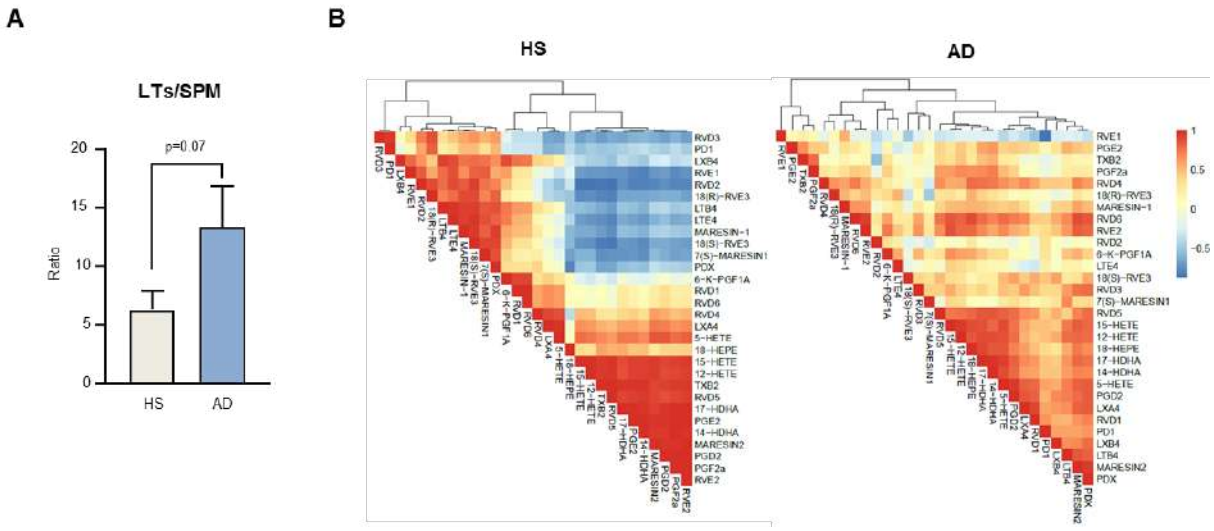
Supplementary Figure 2. Levels of lipids and fatty acids (FAs) in total plasma from HS and AD patients. (**A**) Levels of the five lipid classes measured by LC-MS/MS in plama from HS (n=5) and patients with AD cirrhosis (n=10). (**B**) Supervised heatmap of the FAs categorized for their saturation status in HS and AD patients. (**C**) Plasma levels of the three FA subclasses and their percentage of the total FA pool in HS and patients with AD cirrhosis. Results are expressed as mean ± SEM.



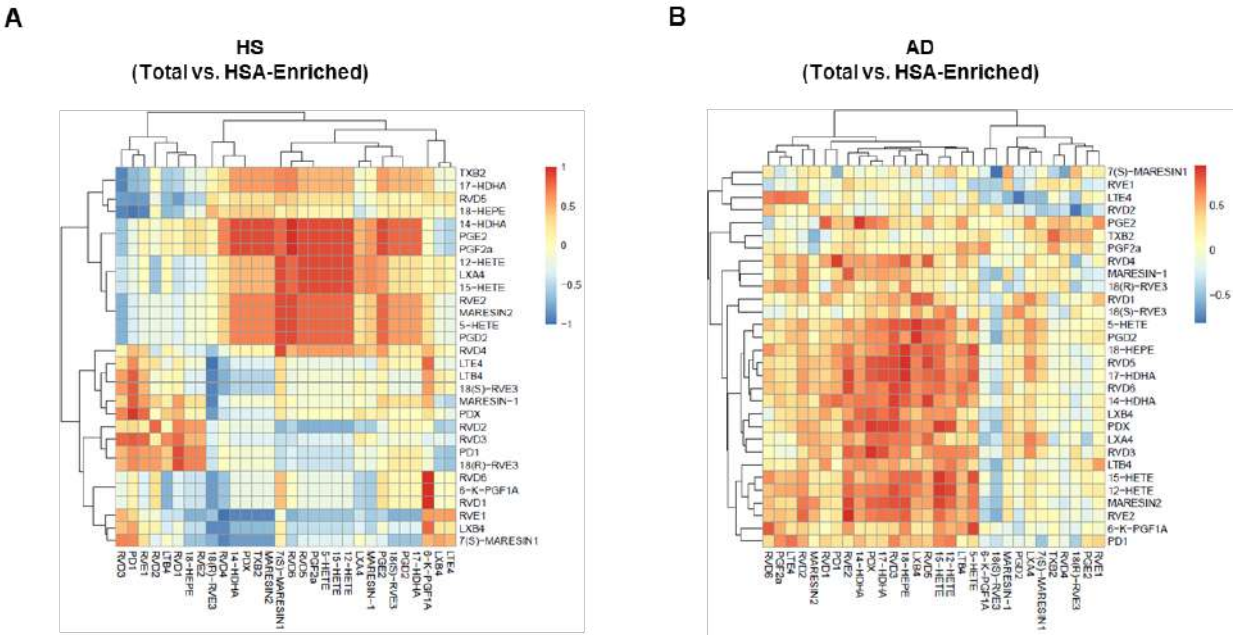
Supplementary Figure 3. Plasma albumin fractionation. (**A**) Plasma albumin concentrations in HS (n=5) and AD patients (n=10) were measured by bromocresol green colorimetry. (**B**) Albumin levels determined by Western blot in total plasma (T), albumin-depleted fraction 1 (D1), albumin-depleted fraction 2 (D2) and albumin-enriched fraction (E). The densitometry analysis of the bands is shown at the bottom. (**C**) Western blot of IgG levels in albumin-enriched fraction (E) and albumin-depleted fraction 2 (D2). (**D**) Concentration of albumin in plasma fractions (total, T; albumin-depleted, D and albumin-enriched, E) from HS and AD patients determined by polyethylene glycol-enhanced immunoturbidimetry. (**E**) Percentage of albumin in albumin-enriched and albumin-depleted fraction 2 after plasma separation in HS and AD patients. (**F**) Same as in E but with the percentage of arachidonic acid (AA). Results are expressed as mean ± SEM of n = 3 experiments. ***P < 0.001 for AD patients versus HS or for albumin-depleted fractions versus total plasma.



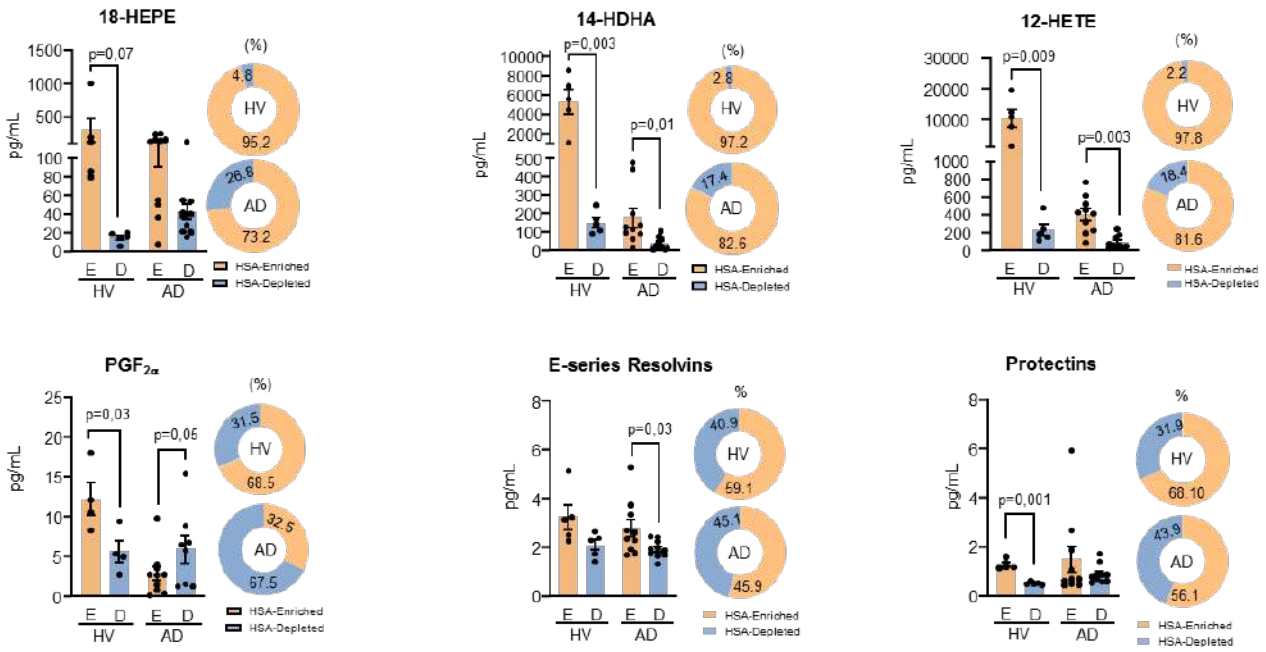
Supplementary Figure 4. Levels of lipids in the albumin fractions in HS and in AD patients. Volcano plots of all lipids detected in the albumin-enriched versus albumin-depleted fractions from HS (n=5) and AD patients (n=10). Log2 fold changes comparing groups were calculated together with -log10 transformed p-values, considering significance when log2 fold change > 0.5 and -log10 p-value > 1.5.



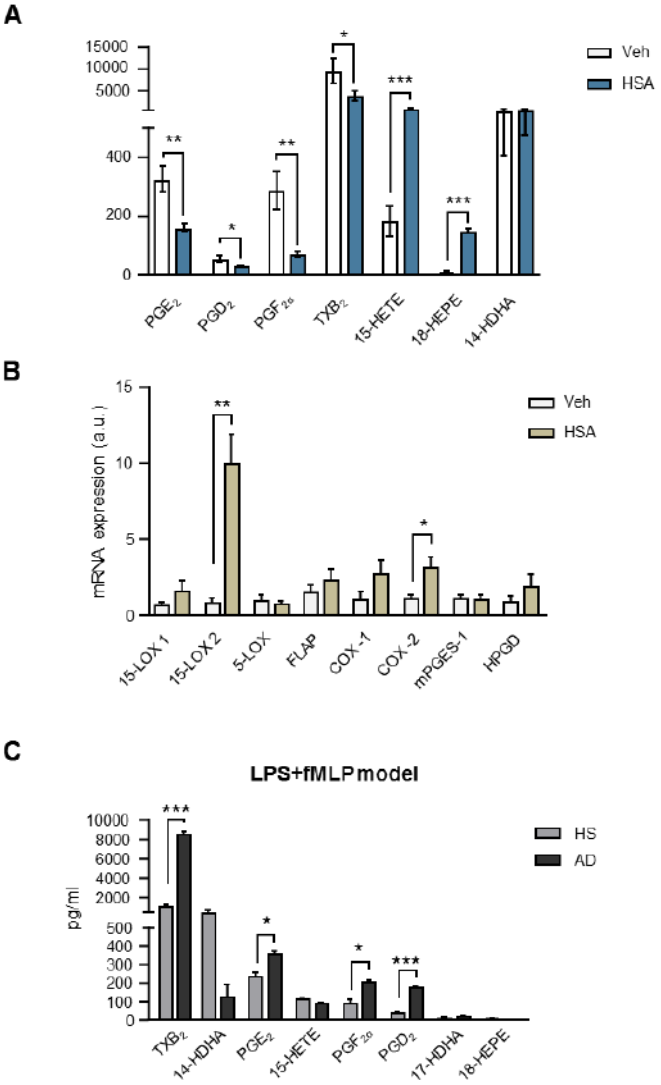
Supplementary Figure 5. Lipid mediators in total plasma from HS and AD patients. (**A**) LT/SPM ratio in total plasma of HS (n=5) and patients with AD cirrhosis (n=10). (**B**) Supervised correlation matrix of log10 transformed lipid mediator levels in total plasma from HS and AD cirrhosis. Results are expressed as mean ± SEM.



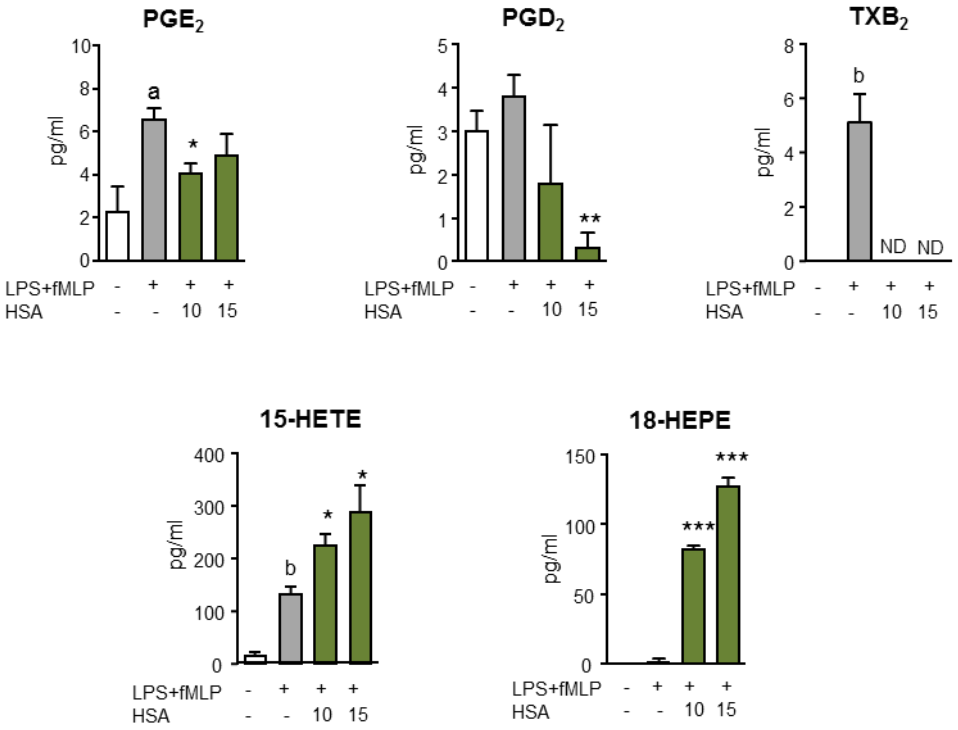
Supplementary Figure 6. Correlation of lipid mediators detected in total plasma and in the albumin-enriched fraction in HS and AD patients. (**A**) Supervised correlation matrix between log10 transformed lipid mediator levels in total plasma and those in the albumin enriched fraction in HS. (**B**) Supervised correlation matrix between log10 transformed lipid mediator levels in total plasma and those in the albumin enriched fraction in AD patients. Results are expressed as mean of HS (n=5) and AD (n=10) patients.



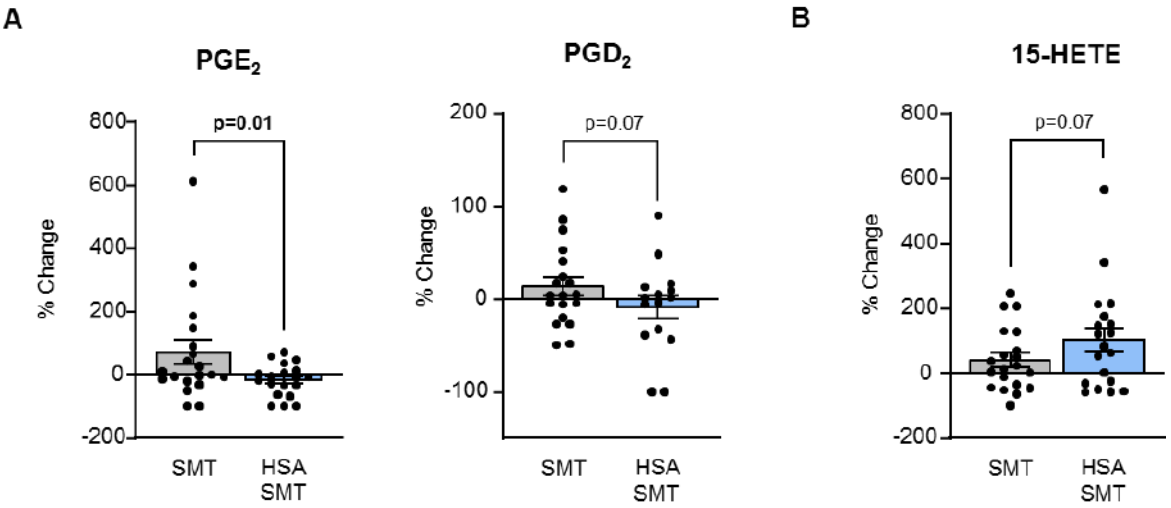
Supplementary Figure 7. Distribution of lipid mediators between albumin-enriched and albumin-depleted fractions. Concentration and percent distribution of 18-HEPE, 14-HDHA, 12-HETE, $PGF_{2\alpha}$ and SPMs (E-series resolvins and protectins) determined by LC-MS/MS in the albumin-enriched (E) and albumin-depleted (D) fractions from HS (n=5) and AD patients (n=10). Results are expressed as mean ± SEM and as percentage of each lipid mediator in the two fractions.



Supplementary Figure 8. Release of bioactive lipid mediators by PBMCs. (**A**) Lipid mediators were measured by LC-MS/MS in supernatants of PBMC sfrom HS incubated with vehicle or albumin (HSA, 15 mg/ml) for 2 hours (n=3 in duplicate). (**B**) mRNA expression of the enzymes involved in the biosynthesis of lipid mediators, determined by real-time PCR, in human PBMC from HS incubated with vehicle or albumin (HSA, 15 mg/ml) for 2 hours (n=3 in duplicate). (**C**) Concentrations of lipid mediators measured by LC-MS/MS in supernatants of PBMCs from HS and AD patients stimulated with LPS (1 µg/ml for 30 min) plus fMLP (1 µg/ml for 15 min) (n=3 in duplicate). Results are expressed as mean ± SEM. *P<0.05 and ***P<0.001 for HSA versus Veh or for AD versus HS.



Supplementary Figure 9. Effect of exogenous albumin on the release of lipid mediators by PMN. Concentrations of lipid mediators measured by LC-MS/MS in supernatants of PMN from HS incubated with albumin (HSA, 10 and 15 mg/ml) for 30 min and then stimulated with LPS (1 µg/ml for 30 min) plus fMLP (1 µg/ml for 15 min) (n=3 in duplicate). Results are expressed as mean ± SEM. a, $P<0.05$ and b, $P<0.005$ for LPS+fMLP versus vehicle and *$P<0.05$, **$P<0.005$ and ***$P<0.001$ for LPS+fMLP plus HSA versus LPS+fMLP alone.



Supplementary Figure 10. Percent change in the plasma levels of lipid mediators in patients with AD cirrhosis after receiving therapy. (**A**) Percent changes with respect to baseline in the plasma levels of PGE_2 and PGD_2 after receiving standard medical treatment (SMT, n=21) alone or in combination with albumin infusions (HSA, n=21). (**B**) Same analysis as in A but for 15-HETE levels. Results are expressed as mean ± SEM.

Supplementary Table 1. LC-ESI-MS/MS conditions used to determine arachidonic acid-derived metabolites.

Compound name	Precursor ion	Product ion	Collision energy	Retention time* (min)
TXB2	367.2	169.1	16	4.109
TXB2	367.2	195.1	10	4.109
PGE2-D4*	355.2	275.2	18	5.289
PGF2α	353.2	193.1	26	5.389
PGF2α	353.2	291.2	21	5.389
PGE2	351.2	315.2	10	5.459
LTB4-D4*	339.2	197.1	16	10.346
LTB4	335.2	129.0	20	10.476
LTB4	335.2	195.1	16	10.476
15-HETE-D8*	327.2	226.2	11	15.26
5-HETE	319.2	115.1	14	16.74
5-HETE	319.2	191.2	14	16.74

*Used as internal standards.

Supplementary Table 2. Characteristics of the patients with acute decompensation of cirrhosis included in the plasma fractionation experiments.

	Patients (n=10)	**Normal range values**
Baseline data		
Age (years)	57.9 ± 10.6	-
Male sex, n (%)	8 (80%)	-
Body Mass Index	27.6 ± 1.3	-
ALT (U/L)	76.2 ± 40.5	5 - 40
AST (U/L)	47.4 ± 36.9	5 - 40
Serum bilirubin (mg/dL)	14.3 ± 13.2	<1.2
Cholesterol HDL (mg/dL)	37 ± 7.1	>40
Cholesterol LDL (mg/dL)	64.5 ± 0.7	<160
Serum C-reactive protein (mg/dL)	23.3 ± 20.4	< 1.0
Blood leukocyte count (x10^9/L)	10.7 ± 7.6	4.00 - 11.00

ALT: Alanine transaminase; AST: Aspartate transaminase; HDL: High density lipoprotein; LDL: Low density lipoprotein.
* Data are presented as mean ± SD or number of patients (%).

Supplementary Table 3. Fatty acids detected in plasma.

Subfamily	Common name	Individual notation
Saturated FA	Myristic acid	14:0
Saturated FA	Pentadecylic acid	15:0
Saturated FA	Palmitic acid	16:0
Saturated FA	Margaric acid	17:0
Saturated FA	Stearic acid	18:0
MUFA	Palmitoleic acid	16:1n-7
MUFA	Oleic acid	18:1n-9
MUFA	Gadoleic acid	20:1n-6
PUFA	Linoleic acid (LA)	18:2n-6
PUFA	Alpha-Linolenic acid (ALA)	18:3n-3
PUFA	Eicosadienoic acid	20:2n-6
PUFA	Mead acid	20:3n-9
PUFA	Arachidonic acid (AA)	20:4n-6
PUFA	Eicosatrienoic acid (ETE) Dihomo Linoleic acid	20:3n-3
PUFA	Eicosapentaenoic acid (EPA)	20:5n-3
PUFA	Docosapentaenoic acid, DPA (Clupanodonic acid)	22:5n-3
PUFA	Adrenic acid	22:4n-6
PUFA	Docosapentaenoic acid, DPA (Osbond acid)	22:5n-6
PUFA	Docosahexaenoic acid (DHA)	22:6n-3

Supplementary Table 5: Omega-6 and omega-3 PUFAs detected in commercial human serum albumin.

PUFA Class	Fatty acid	% Lipid / PUFA class
Omega-6 FA	**8**	**17.7 % family / total FAs**
	18:2n-6 (Linoleic acid)	**62.87**
	20:4n-6 (Arachidonic acid)	**29.72**
	22:4n-6 (Adrenic acid)	**2.53**
	18:3n-6 (Gamma-Linolenic acid)	**2.12**
	20:2n-6 (Eicosadienoic acid)	**1.42**
	20:1n-6 (Gadoleic acid)	**1.08**
	18:2n-6 trans (Linoelaidic acid)	**0.25**
Omega-3 FA	7	**16.95 % family / total FAs**
	22:6n-3 (Docosahexaenoic acid)	**44.06**
	18:3n-3 (Alpha-Linolenic acid)	**27.11**
	20:3n-3 (Eicosatrienoic acid)	**10.23**
	20:5n-3 (Eicosapentaenoic acid)	**8.87**
	22:5n-3 (DPA. Clupanodonic acid)	**8.44**
	20:4n-3 (Eicosatetraenoic acid)	**0.64**
	18:4n-3 (Stearidonic acid)	**0.64**
Total PUFAs	15	**34.65 % / total FAs**

Supplementary Table 6: Content of lipid mediators in commercial human serum albumin.

Lipid mediator	mean (pg/mL)	SD
5-HETE	19430.92	1745.51
12-HETE	15440.30	1734.23
12,13-EpOME	14268.07	561.94
9,10-EpOME	6379.65	541.06
17-HDHA	2126.95	199.82
8-HETE	1652.79	132.07
14-HDHA	1440.50	56.41
15-HETE	1148.25	107.37
8-HDHA	1072.50	113.85
20-HETE	893.68	56.39
5-HEPE	687.44	128.55
12-HEPE	672.41	75.34
trans-EKODE-(E)-Ib-(9,10-DiHOME-)	637.02	53.10
7-HDHA	405.65	54.50
18-HEPE	258.94	21.80
15-HEPE	130.21	11.90
TXB_2	21.82	2.26

DISCUSSION

The first study of this dissertation provides a mechanism of action for the immunomodulatory properties of HSA in human leukocytes stimulated with CpG. In fact, the results of this study demonstrate that albumin exerts anti-inflammatory and immunomodulatory effects against PAMPs and DAMPs in leukocytes from HS and AD patients. These results are consistent with previously described effects of HSA in experimental cirrhosis, independent of its role as plasma volume expander, involving its capacity to counteract the effects of TNFα and oxidative stress on cardiac tissue (Bortoluzzi et al, Hepatology, 2013). It is worth mentioning that the concentrations of HSA used in our study were equivalent to those clinically employed to correct hypoalbuminemia in patients with cirrhosis (Bernardi et al, Gut, 2020 and Caraceni et al, Lancet, 2018). In addition, we observed immunomodulatory actions of HSA in vitro at very low concentrations, a finding that is consistent to that reported by previous studies evaluating its effects in hepatocytes (Pyzik et al, Proc Natl Acad Sci U S A, 2017). Recent findings demonstrated that HSA therapy is associated with a reduction in the circulating concentrations of cytokines (Fernández et al, Gastroenterology, 2019). Due to systemic inflammation is a driver of severity in cirrhosis (Clària et al, Hepatology, 2016), our findings would explain the improvement in outcomes and survival observed in patients with AD cirrhosis receiving long-term HSA therapy, such as ANSWER trial (Caraceni et al, Lancet, 2018)

Moreover, our study elucidates the mechanism of action for the anti-inflammatory effects of HSA by its internalization in human leukocytes. Previous reports have described HSA internalization in rat alveolar type II epithelial cells and astrocytes (Ikehata et al, Pharm Res, 2008 and Bento-Abreu et al, J Neurochem, 2008), mouse hepatocytes (Pyzik et al, Proc Natl Acad Sci U S A, 2017) and several cell lines (Schmidt et al, J Biol Chem, 2017 and Dobrinskikh et al, Am J Physiol Renal Physiol, 2014). In our study, we demonstrated that HSA is internalized by leukocytes by tracking the fluorescently-labeled HSA from ten minutes to two hours after the incubation of leukocytes with labeled HSA. Subsequent immunofluorescence assays with or without CpG stimulated leukocytes revealed that the intracellular HSA signal colocalized with a marker of early endosomes, indicating that, similar to previous studies in other cell types (Schmidt et al, J Biol Chem, 2017 and Dobrinskikh et al, Am J Physiol Renal Physiol, 2014), HSA preferentially targets this cellular compartment in leukocytes. Although the precise intracellular trafficking of HSA in leukocytes was not fully described in the present study, it is likely that HSA could undergo either recycling or lysosomal degradation following its internalization.

Uptake of HSA can be mediated by clathrin-dependent (Ikehata et al, Pharm Res, 2008 and Yumoto et al, Am J Physiol Lung Cell Mol Physiol, 2006) or clathrin-independent, caveolin-mediated (Dobrinskikh et al, Am J Physiol Renal Physiol, 2014 and Chatterjee et al, Cancer Res, 2017) endocytic routes, depending on each cell type. There are, however, specific cell types using both clathrin-dependent and clathrin-independent mechanisms to uptake HSA (Li et al, PLoS One, 2013). In our study we observed that HSA uptake by leukocytes is predominantly mediated by a caveolae-mediated pathway. This endocytic route relies on the formation of caveolin-1-enriched smooth invaginations of the plasma membrane in close association with lipid rafts (Watts et al, Nat Immunol, 2008). Indeed, disruption of lipid rafts in leukocytes produced a complete inhibition of the immunomodulatory actions of HSA. However, lipid rafts are not exclusive of caveolin-mediated endocytosis and also participate in clathrin-mediated endocytosis. In this regard, the immunomodulatory actions of HSA in leukocytes were partially inhibited by a FcRn blocking antibody. Considering that HSA binding to FcRn is linked to the activation of clathrin-mediated endocytosis Pyzik et al, Proc Natl Acad Sci U S A, 2017; Sand et al, Front Immunol, 2015 and He et al, Nature, 2008), these findings suggest that clathrin-dependent routes might also be involved. However, a specific study inhibiting both pathways, clathrin and caveolin-dependent proteins is necessary to clarify the internalization pathway of HSA in leukocytes.

A major finding of our study was the observation that once internalized into endosomes, HSA was able to block the production of cytokines by leukocytes in response to CpG. Bacterial DNA, as well as mitochondrial DNA, are enriched in unmethylated CpG dinucleotides, which has the ability to trigger the immune response by acting like a PAMP and/or DAMP (Krieg et al, Nature, 1995 and Fang et al, Protein Cell, 2016 and Klinman et al, Nat Rev Immunol, 2004). PAMPs and DAMPs that are nucleic acids are mainly sensed by intracellular receptors located in endosomes which are TLR3, TLR7, TLR8 and TLR9 (Lee et al, Trends Cell Biol, 2014). In particular, CpG is internalized via clathrin-mediated endocytosis in early endosomes, where it binds to its cognate receptor, TLR9 (Hemmi et al, Nature, 2000), to induce a MyD88-mediated signaling resulting in the induction of inflammatory cytokines. Since inappropriate activation of endosomal TLRs has the potential to trigger autoimmune diseases through interaction with self-nucleic acids, their intracellular trafficking is tightly controlled (Engel et al. Sci Signal, 2010). In the present study, HSA inhibition of CpG-stimulated cytokine production in leukocytes was likely mediated by the accumulation of this protein in the endosomal compartment and the blockage of TLR9 activation and signaling, although exact mechanisms remain to be elucidated. In any case, the view that HSA exerts

immunomodulatory actions in leukocytes following its internalization is supported by fourth main observations. First, HSA incubated in leukocytes was rapidly internalized in endosomes. Second, in the endosomes, HSA co-localized with CpG. Third, HSA not only modulated TLR9 signaling but also reduced TLR3 activation by poly(I:C) as well as the late wave of TLR4 activation by LPS. And fourth, exposure of leukocytes to chloroquine, which inhibits endosomal acidification and consequently, activation of TLRs by nucleic acids, produced similar effects to those of HSA on CpG-induced cytokine expression. It is important to mention that the intracellular HSA signal was higher and persisted for longer periods of time when leukocytes were stimulated with CpG, suggesting enhanced uptake and/or distinct intracellular trafficking of HSA in activated leukocytes.

In summary, the findings of the first study provide a mechanism by which HSA modulates the excessive production of cytokines by leukocytes without affecting the defense functions of these cells against pathogens. This mechanism is independent of the scavenging and oncotic properties of HSA and is related to its ability to modulate intracellular (endosomal) pathways involved in the production of cytokines. Therefore, the observation that HSA is internalized by leukocytes offers an alternative perspective for understanding the anti-inflammatory effects associated with the infusion of HSA to patients with advanced liver disease.

The second study of this dissertation provides evidence that albumin has the ability to modulate the lipid composition of human plasma, an effect that is relevant to patients with AD cirrhosis in whom albumin infusions are used as therapy.

Many of the physiological functions of albumin are based on its ability to reversibly bind soluble molecules, contributing to their transportation in the plasma to different tissues and organs (Arroyo et al, J Hepatol, 2014 and Bernardi et al, Gut, 2020). In particular, albumin molecule contains up to seven binding sites for long chain FAs, some of which are located in the critical Sudlow's sites I and II (Bhattacharya et al, J Mol Biol, 2000). Any alteration of the albumin chemical structure has a direct impact on its ligand-binding function (Kawakami et al, FEBS J, 2006). Although there is no direct evidence that links the structural alterations of albumin with its ability to bind FAs, oxidation of the Cys-34 residue in the albumin molecule has been shown to reduce the binding of molecules in the Sudlow's sites, where some of the FA binding sites are located (Kawakami et al, FEBS J, 2006). This fact together with the presence of lower concentrations of albumin, which in addition, is highly oxidized in patients with AD cirrhosis (Bernardi et al, Gut, 2020 and Alcaraz-Quiles et al, Hepatology, 2018) ,might explain the existence of higher

levels of free FAs in the bloodstream of these patients. This is particularly relevant because albumin can bind esterified FAs in phospholipids, especially lysoPC (Belgacem et al, Biologicals, 2007) and the major part of the FA pool in the human plasma is esterified with a minor proportion actually free in solution (Morgenthaler et al, Vox Sang, 1980). Albumin also binds omega-6- and omega-3-PUFAs, especially AA and DHA (Savu et al, J Biol Chem, 1981) and biologically active lipid mediators derived from PUFAs, including PGs (Raz et al, Biochem J, 1972), LTs (Zsila et al, Bioorg Med Chem Lett, 2005 and Fitzpatrick et al, Biochemistry, 1981) and monohydroxy FAs (Dadaian et al, J Lipid Res, 1999 and Ek-von Mentzer, J Biol Chem, 2001). All these data can help to understand that the observed changes in the lipid composition could be due to changes in the albumin molecule with less capacity to bind lipids in patients with AD cirrhosis.

In addition, it has been reported the ability of albumin to sequester PGs and accelerate the decomposition kinetics of these lipid mediators, an effect that is dose dependent (Dadaian et al, J Lipid Res, 1999). Our finding that albumin inhibits PGE_2 production by leukocytes from both HS and AD patients in vitro is consistent with previous studies demonstrating that albumin reduces PGE_2 levels in patients with AD cirrhosis (O'Brien et al, Nat Med, 2014 and China et al, Clin Gastroenterol Hepatol, 2018). PGE_2 is a lipid mediator that drives immunosuppression, and therefore is relevant to AD cirrhosis because it can potentially increase the risk of infection in these patients. In this study, we could not confirm that PGE_2 levels were increased in patients with AD cirrhosis, but we observed that PGE_2 levels were reduced in the albumin-enriched fraction of AD patients. On the other hand, albumin has been reported to favor lipoxygenation of PUFAs and to increase the formation of LOX-derived products (Ek-von Mentzer, J Biol Chem, 2001 and Broekman et al, J Lipid Res, 1989). Consistent with this, we detected a significant increase in the 15-LOX-derived product 15-HETE in leukocytes from HS and AD patients incubated with albumin. The enhanced formation of 15-HETE cannot be attributed to the presence of this monohydroxy FA in the commercial albumin employed in our experiments because its levels in cell supernatants further exceeded those attached to the commercial albumin preparation. Importantly, increased 15-HETE levels were also observed in plasma from patients with AD cirrhosis after receiving albumin therapy. However, the response to albumin administration in these patients was very heterogeneous in terms of changes in lipid mediators and it is necessary to confirm these findings in other studies including a larger number of patients with proper stratification. Together, these findings provide evidence that albumin is not merely transporting lipid

mediators in the bloodstream but rather a molecule that modulates their biosynthesis and release by circulating immune cells.

In summary, the data collected in the second study provides a comprehensive analysis of the composition of lipids and lipid mediators in the albumin molecule from patients with AD cirrhosis, identifying a characteristic pattern of PUFA and lipid mediators in these patients. Furthermore, we provide evidence that exogenous albumin modulates the biosynthesis of lipid mediators in circulating leukocytes by redirecting the production of pro-inflammatory and immunosuppressive PGs to the activation of endogenous pro-resolving lipid mediator pathways.

In conclusion, results obtained in both studies of the present dissertation provide convincing data of the pro-resolutive and immunomodulatory mechanisms that HSA exert over leukocytes to counteract the exacerbated inflammatory environment found in patients with advance liver diseases. HSA directly modulate the pro-inflammatory effectors present in this disease such as cytokines and lipid mediators to potentiate resolution of inflammation.

CONCLUSIONS

The conclusions that can be drawn from the two studies included in the current dissertation are the following:

1. HSA is able to inhibit cytokine production and release in leukocytes isolated from healthy subjects and patients with advance cirrhosis challenged with Bacterial DNA rich in CpG.
2. CpG-cytokine production by leukocytes is inhibited also by recombinant HSA
3. HSA anti-inflammatory effect is independent of its oncotic and scavenging properties
4. HSA inhibits CpG up-regulation of genes involved in innate, adaptive immunity and cytokine production in PBMC from patients with advanced cirrhosis.
5. HSA is internalized by leukocytes in early endosomes mainly at early time periods where it colocalizes with CpG.
6. Anti-inflammatory effects of HSA are due to inhibition of endosomal TLR9 signaling and in extension, endosomal TLR3 and late phase of TLR4.
7. HSA internalization is partially produced by its cognate receptor FcRn and is preferentially mediated by caveolae endocytic pathways
8. Albumin reduction of cytokines does not compromise leukocyte defense mechanisms rather increase their phagocytic and efferocytosis functions without affecting oxidative burst capacity.
9. HSA effects were translated to in vivo model with analbuminemic humanized mice that show HSA is essential to regulate CpG-induced cytokine production.
10. Circulating lipid levels in patients with AD cirrhosis are remarkably suppressed in comparison to HS, except FA, which are increased.
11. In AD patients, albumin shows a characteristic lipid composition, especially of fatty acids and PUFA-derived lipid mediators.
12. Albumin-enriched fraction in AD patients has less content of pro-resolving monohydroxy FAs and albumin-depleted fraction has increased content of PGs.

13. Exogenous albumin addition to leukocytes modulates the biosynthesis of lipid mediators by reducing pro-inflammatory PG production while inducing the release of pro-resolving monohydroxy FAs.

14. Lower plasma PG levels and higher concentrations of 15-HETE are observed in patients with AD cirrhosis receiving albumin therapy.

BIBLIOGRAPHY

A

- Acharya C, Sahingur SE, Bajaj JS. *Microbiota, cirrhosis, and the emerging oral-gut-liver axis*. JCI Insight. 2017; 2:e94416.
- Aggarwal BB. *Signalling pathways of the TNF superfamily: a double-edged sword.* Nat Rev Immunol. 2003; 3:745-756.
- Albillos A, Lario M, Álvarez-Mon M. *Cirrhosis-associated immune dysfunction: distinctive features and clinical relevance*. J Hepatol. 2014; 61:1385-1396.
- Alcaraz-Quiles J, Casulleras M, Oettl K, Titos E, Flores-Costa R, Duran- Guell M, et al. *Oxidized albumin triggers a cytokine storm in leukocytes through P38 mitogen-activated protein kinase: role in systemic inflammation in decompensated cirrhosis.* Hepatology 2018; 68:1937– 1952.
- Alcaraz-Quiles J, Titos E, Casulleras M, Pavesi M, López-Vicario C, Rius B, et al. *Polymorphisms in the IL-1 gene cluster influence systemic inflammation in patients at risk for acute-on-chronic liver failure*. Hepatology. 2017; 65:202-216.
- Aldecoa C, Llau JV, Nuvials X and Artigas A. *Role of albumin in the preservation of endothelial glycocalyx integrity and the microcirculation: a review*. Ann Intensive Care 2020; 10: 85.
- Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. *Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3*. Nature. 2001; 413:732-738.
- Al-Sadi R, Ye D, Dokladny K, Ma TY. *Mechanism of IL-1beta-induced increase in intestinal epithelial tight junction permeability*. J Immunol. 2008; 180:5653-5661.
- Anderson KV, Bokla L, Nüsslein-Volhard C. *Establishment of dorsal-ventral polarity in the Drosophila embryo: the induction of polarity by the Toll gene product.* Cell 1985; 42:791-798.
- Ariel A, Li PL, Wang W, Tang WX, Fredman G, Hong S. *The docosatriene protectin D1 is produced by TH2 skewing and promotes human T cell apoptosis via lipid raft clustering*. J Biol Chem. 2005; 280:43079-43086.
- Arita M, Clish CB, Serhan CN. *The contributions of aspirin and microbial oxygenase to the biosynthesis of anti-inflammatory resolvins: novel oxygenase products from omega-3 polyunsaturated fatty acids.* Biochem Biophys Res Commun. 2005; 338:149-57.

- Arita M, Ohira T, Sun YP, Elangovan S, Chiang N, Serhan CN. *Resolvin E1 selectively interacts with leukotriene B4 receptor BLT1 and ChemR23 to regulate inflammation.* J Immunol. 2007; 178:3912-3917.
- Arroyo V, Angeli P, Moreau R, Jalan R, Clària J, Trebicka J, et al. *The systemic inflammation hypothesis: Towards a new paradigm of acute decompensation and multiorgan failure in cirrhosis.* J Hepatol. 2021; 74:670-685.
- Arroyo V. García-Martinez R and Salvatella X. *Human serum albumin, systemic inflammation and cirrosis.* J Hepatol 2014; 61:396-407.
- Arroyo V, Moreau R, Kamath PS, Jalan R, Ginès P, Nevens F, et al. *Acute-on-chronic liver failure in cirrhosis.* Nat Rev Dis Primers. 2016; 2:16041.
- Aronoff DM, Canetti C, Peters-Golden M. *Prostaglandin E2 inhibits alveolar macrophage phagocytosis through an E-prostanoid 2 receptor-mediated increase in intracellular cyclic AMP.* J Immunol. 2004; 173:559-565.
- Auffray C, Sieweke MH, Geissmann F. *Blood monocytes: development, heterogeneity, and relationship with dendritic cells*. Ann Rev Immunol. 2009; 27:669-692.
- Aubin É, Roberge C, Lemieux R, Bazin R. *Immunomodulatory effects of therapeutic preparations of human albumin.* Vox Sang 2011; 101:131-137.

B

- Bannenberg G, Serhan CN. *Specialized pro-resolving lipid mediators in the inflammatory response: An update.* Biochim Biophys Acta. 2010; 1801:1260-1273.
- Bañares R, Ibáñez-Samaniego L, Torner JM, Pavesi M, Olmedo C, Catalina MV, et al. *Meta-analysis of individual patient data of albumin dialysis in acute-on-chronic liver failure: focus on treatment intensity.* Therap Adv Gastroenterol. 2019; 12:1756284819879565.
- Bañares R, Nevens F, Larsen FS, Jalan R, Albillos A, Dollinger M, et al. *Extracorporeal albumin dialysis with the molecular adsorbent recirculating system in acute-on-chronic liver failure: the RELIEF trial.* Hepatology. 2013; 57:1153-1162.
- Barton GM, Kagan JC, Medzhitov R. *Intracellular localization of toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA*. Nat Immunol 2006; 7:49-56.

- Baldassarre M, Domenicali M, Naldi M, Laggetta M, Giannone FA, Biselli M, et al. *Albumin homodimers in patients with cirrhosis: clinical and prognostic relevance of a novel identified structural alteration of the molecule*. Sci Rep 2016; 6:35987.
- Bazan NG. *Neuroprotectin D1-mediated anti-inflammatory and survival signaling in stroke, retinal degenerations, and Alzheimer's disease.* J Lipid Res. 2009; 50 Suppl: S400-S405.
- Becares N, Härmälä S, China L, Colas RA, Maini AA, Bennet K, et al. *Immune Regulatory Mediators in Plasma from Patients With Acute Decompensation Are Associated With 3-Month Mortality.* Clin Gastroenterol Hepatol. 2020; 18:1207-1215.
- Behzadi P, García-Perdomo HA, Karpinski TM. Toll-Like Receptors: General Molecular and Structural Biology. J Immunol Res. 2021; 9914854.
- Bell JK, Botos I, Hall PR, Askins J, Shiloach J, Segal DM, et al. *The molecular structure of the Toll-like receptor 3 ligand-binding domain*. Proc. Natl. Acad. Sci. U.S.A. 2005; 102:10976-10980.
- Belgacem O, Stübiger G, Allmaier G, Buchacher A, Pock K. *Isolation of esterified fatty acids bound to serum albumin purified from human plasma and characterised by MALDI mass spectrometry.* Biologicals. 2007; 35:43-49.
- Bennett M, Gilroy DW. *Lipid Mediators in Inflammation*. Microbiol Spectr. 2016; 4.
- Bento-Abreu A, Velasco A, Polo-Hernández E, Pérez-Reyes PL, Tabernero A, Medina JM. *Megalin is a receptor for albumin in astrocytes and is required for the synthesis of the neurotrophic factor oleic acid.* J Neurochem. 2008; 106:1149-1159.
- Bernardi M, Angeli P, Claria J, Moreau R, Gines P, Jalan R, et al. *Albumin in decompensated cirrhosis: new concepts and perspectives.* Gut. 2020; 69:1127-1138.
- Bernardi M, Caraceni P, Navickis RJ, Wilkes MM. *Albumin infusion in patients undergoing large-volume paracentesis: a meta-analysis of randomized trials.* Hepatology. 2012; 55:1172-1181.
- Bernardi M, Ricci CS and Zaccheriniet G. *Role of Human Albumin in the Management of Complications of Liver Cirrhosis.* J Clin Exp Hepatol 2014; 4:302-311.

- Bernardi M, Moreau R, Angeli P, Schnabl B, Arroyo V. *Mechanisms of decompensation and organ failure in cirrhosis: From peripheral arterial vasodilation to systemic inflammation hypothesis*. J Hepatol. 2015; 63:1272-1284.
- Bernsmeier C, Triantafyllou E, Brenig R, Lebosse FJ, Singanayagam A, Patel VC, et al. *CD14+ CD15- HLA-DR- myeloid-derived suppressor cells impair antimicrobial responses in patients with acute-on-chronic liver failure.* Gut. 2018; 67:1155-1167.
- Berzigotti A, Seijo S, Arena U, Abraldes JG, Vizzutti F, García-Pagán JC, et al. *Elastography, spleen size, and platelet count identify portal hypertension in patients with compensated cirrhosis.* Gastroenterology. 2013; 144:102-111.
- Bhattacharya AA, Grüne T and Curry S. *Crystallographic analysis reveals common modes of binding of medium and long-chain fatty acids to human serum albumin.* J Mol Biol 2000; 303:721-732.
- Bianchi ME. *DAMPs, PAMPs and alarmins: all we need to know about danger*. J Leukoc Biol. 2007; 81:1-5.
- Blachier M, Leleu H, Peck-Radosavljevic M, Valla DC, Roudot-Thoraval F. *The burden of liver disease in Europe: a review of available epidemiological data*. J Hepatol. 2013; 58:593-608.
- Bonilla FA, Oettgen HC. *Adaptive immunity*. J Allergy Clin Immunol. 2010; 125:S33-40.
- Bortoluzzi A, Ceolotto G, Gola E, Sticca A, Bova S, Morando F, et al. *Positive cardiac inotropic effect of albumin infusion in rodents with cirrhosis and ascites: molecular mechanisms*. Hepatology. 2013; 57:266-276.
- Botos I, Segal DM, Davies DR. *The structural biology of Toll-like receptors*. Structure. 2011; 19:447-59.
- Boussif A, Rolas L, Weiss E, Bouriche H, Moreau R, Périanin A. *Impaired intracellular signaling, myeloperoxidase release and bactericidal activity of neutrophils from patients with alcoholic cirrhosis.* J Hepatol. 2016; 64:1041-1048.
- Broekman MJ, Eiroa AM, Marcus AJ. *Albumin redirects platelet eicosanoid metabolism toward 12(S)-hydroxyeicosatetraenoic acid.* J Lipid Res. 1989; 30:1925-1932.
- Broz P, Dixit VM. *Inflammasomes: mechanism of assembly, regulation and signalling*. Nat Rev Immunol. 2016; 16: 407-420.

- Buchta CM, Bishop GA. *Toll-like receptors and B cells: functions and mechanisms*. Immunol Res. 2014; 59:12-22.

C

- Cantin AM, Paquette B, Richter M, Larivee P. *Albumin-mediated regulation of cellular glutathione and nuclear factor kappa B activation*. Am J Respir Crit Care Med 2000; 162:1539-1546.

- Caraceni P, Riggio O, Angeli P, Alessandria C, Neri S, Foschi FG, et al. *Long-term albumin administration in decompensated cirrhosis (ANSWER): an open-label randomised trial*. Lancet. 2018; 391:2417-2429.

- Carter EP, Hartsfield CL, Miyazono M, Jakkula M, Morris KG Jr, McMurtry IF. *Regulation of heme oxygenase-1 by nitric oxide during hepatopulmonary syndrome*. Am J Physiol Lung Cell Mol Physiol, 2002; 283:L346-L353.

- Chan AC, Fan ST. *Criteria for liver transplantation in ACLF and outcome*. Hepatol Int. 2015; 9:355-359.

- Chatterjee M, Ben-Josef E, Robb R, Vedaie M, Seum S, Thirumoorthy K, et al. *Caveolae-Mediated Endocytosis Is Critical for Albumin Cellular Uptake and Response to Albumin-Bound Chemotherapy.* Cancer Res. 2017; 77:5925-5937.

- Chiang N, Libreros S, Norris PC, de la Rosa X, Serhan CN. *Maresin 1 activates LGR6 receptor promoting phagocyte immunoresolvent functions.* J Clin Invest. 2019; 129:5294-5311.

- China L, Freemantle N, Forrest E, Kallis Y, Ryder SD, Wright G, et al. *ATTIRE Trial Investigators. A Randomized Trial of Albumin Infusions in Hospitalized Patients with Cirrhosis.* N Engl J Med. 2021; 384:808-817.

- China L, Maini A, Skene SS, Shabir Z, Sylvestre Y, Colas RA, et al. *Albumin Counteracts Immune-Suppressive Effects of Lipid Mediators in Patients With Advanced Liver Disease.* Clin Gastroenterol Hepatol. 2018; 16:738-747

- Chuang T, Ulevitch RJ. *Identification of hTLR10: a novel human Toll-like receptor preferentially expressed in immune cells.* Biochim Biophys Acta. 2001; 1518:157-161.

- Clària J, Arroyo V, Moreau R. *The Acute-on-Chronic Liver Failure Syndrome, or When the Innate Immune System Goes Astray*. J Immunol. 2016; 197:3755-3761.
- Clària J, Serhan CN. *Aspirin triggers previously undescribed bioactive eicosanoids by human endothelial cell-leukocyte interactions*. Proc Natl Acad Sci U S A. 1995; 92:9475-9479.
- Clària J, Stauber RE, Coenraad MJ, Moreau R, Jalan R, et al. *Systemic inflammation in decompensated cirrhosis: Characterization and role in acute-on-chronic liver failure.* Hepatology. 2016; 64:1249-64.
- Cormican S, Griffin MD, *Human Monocyte Subset Distinctions and Function: Insights From Gene Expression Analysis.* Front. Immunol. 2020; 11:1070.
- Cros J, Cagnard N, Woollard K, Patey N, Zhang S-Y, Senechal B, et al. *Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors.* Immunity 2010 33:375–86.
- Covert MW, Leung TH, Gaston JE, Baltimore D. *Achieving stability of lipopolysaccharide-induced NF-kappaB activation*. Science. 2005; 309:1854-1857.

D

- Dadaian M, Westlund P. *Albumin modifies the metabolism of hydroxyeicosatetraenoic acids via 12-lipoxygenase in human platelets*. J Lipid Res. 1999; 40:940-947.
- D'Amico G, Morabito A, D'Amico M, Pasta L, Malizia G, Rebora P, et al. *Clinical states of cirrhosis and competing risks*. J Hepatol. 2018; 68:563-576.
- Dallas Bowman. *Liver Cirrhosis: From Pathophysiology to Disease Management*. FOSTER ACADEMICS, 2019.
- Dalli J, Winkler JW, Colas RA, Arnardottir H, Cheng CY, Chiang N, et al. *Resolvin D3 and aspirin-triggered resolvin D3 are potent immunoresolvents.* Chem Biol. 2013; 20:188-201.
- Daniels et al, Daniels MJ, Brough D. *Unconventional Pathways of Secretion Contribute to Inflammation*. Int J Mol Sci. 2017; 18:102.

- Das S, Maras JS, Hussain MS, Sharma S, David P, Sukriti S, et al. *Hyper- oxidized albumin modulates neutrophils to induce oxidative stress and inflammation in severe alcoholic hepatitis.* Hepatology 2017;65:631– 646.
- Dasari P, Zola H, Nicholson IC. *Expression of Toll-like receptors by neonatal leukocytes*. Pediatr Allergy Immunol. 2001; 22:221-228.
- Delves PJ, Martin SJ, Burton DR, Roitt IM. *Roitt's Essential Immunology, 13th Edition*. John Wiley & Sons, 2017.
- Dennis EA, Cao J, Hsu YH, Magrioti V, Kokotos G. *Phospholipase A2 enzymes: physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention*. Chem. Rev. 2011; 111:6130-6185.
- Dennis EA, Norris PC. *Eicosanoid storm in infection and inflammation*. Nat Rev Immunol. 2015; 15:511-523.
- Dinarello, C. A. *Immunological and inflammatory functions of the interleukin-1 family*. Annu. Rev. Immunol. 2009; 27:519-550.
- Domenicali M, Baldassarre M, Giannone FA, Naldi M, Mastroroberto M, Biselli M, et al. *Posttranscriptional changes of serum albumin: clinical and prognostic significance in hospitalized patients with cirrhosis.* Hepatology 2014; 60:1851–1860.
- Dona M, Fredman G, Schwab JM, Chiang N, Arita M, Goodarzi A, et al. *Resolvin E1, an EPA-derived mediator in whole blood, selectively counterregulates leukocytes and platelets.* Blood. 2008; 112:848-855.
- Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB, et al. *Cyclooxygenase in biology and disease.* FASEB J. 1998; 12:1063-1073.
- Dunne A, Ejdeback M, Ludidi PL, O'Neill LA, Gay NJ. *Structural complementarity of Toll/interleukin-1 receptor domains in Toll-like receptors and the adaptors Mal and MyD88*. J Biol Chem. 2003; 278:41443-41451.
- Duran-Güell M, Flores-Costa R, Casulleras M, López-Vicario C, Titos E, Díaz A, et al. *Albumin protects the liver from tumor necrosis factor α-induced immunopathology*. FASEB J. 2021; 35:e21365.

E

- Ek-Von Mentzer BA, Zhang F, Hamilton JA. *Binding of 13-HODE and 15-HETE to phospholipid bilayers, albumin, and intracellular fatty acid binding proteins. implications for transmembrane and intracellular transport and for protection from lipid peroxidation.* J Biol Chem. 2001; 276:15575-15580.
- Engel A, Barton GM. *Compartment-specific control of signaling from a DNA-sensing immune receptor.* Sci Signal. 2010; 3:pe45.
- Engelmann C, Clària J, Szabo G, Bosch J, Bernardi M. *Pathophysiology of decompensated cirrhosis: Portal hypertension, circulatory dysfunction, inflammation, metabolism and mitochondrial dysfunction.* J Hepatol. 2021; 75:S49-S66.
- Evans TW. et al, Review article: *albumin as a drug: biological effects of albumin unrelated to oncotic pressure*. Aliment Pharmacol Ther 2002; 16:6-11.
- Ewald SE, Engel A, Lee J, Wang M, Bogyo M, Barton GM. *Nucleic acid recognition by Toll-like receptors is coupled to stepwise processing by cathepsins and asparagine endopeptidase*. J Exp Med. 2011; 208:643-651.
- Ewald SE, Lee BL, Lau L, Wickliffe KE, Shi GP, Chapman HA, et al. *The ectodomain of Toll-like receptor 9 is cleaved to generate a functional receptor.* Nature. 2008; 456:658-662.

F

- Fang C, Wei X, Wei Y. *Mitochondrial DNA in the regulation of innate immune responses.* Protein Cell. 2016; 7:11-16.
- Fasolato S, Angeli P, Dallagnese L, Maresio G, Zola E, Mazza E, et al. *Renal failure and bacterial infections in patients with cirrhosis: epidemiology and clinical features.* Hepatology. 2007; 45:223-229.
- Fernández J, Clària J, Amorós A, Aguilar F, Castro M, Casulleras M, et al. *Effects of Albumin Treatment on Systemic and Portal Hemodynamics and Systemic Inflammation in Patients With Decompensated Cirrhosis.* Gastroenterology. 2019 Jul;157(1):149-162.

- Fitzpatrick FA, Waynalda MA. *Albumin-lipid interactions: prostaglandin stability as a probe for characterizing binding sites on vertebrate albumins.* Biochemistry. 1981; 20:6129-6134.
- Funk CD. *Prostaglandins and leukotrienes: advances in eicosanoid biology*. Science. 2001; 294:1871-1875.

G

- Garcia-Martinez R, Caraceni P, Bernardi M, Gines P, Arroyo V and Jalan R. *Albumin: pathophysiologic basis of its role in the treatment of cirrhosis and its complications.* Hepatology 2013; 58:1836-1846.
- Garcia-Tsao G, Abraldes JG, Berzigotti A, Bosch J. *Portal hypertensive bleeding in cirrhosis: Risk stratification, diagnosis, and management: 2016 practice guidance by the American Association for the study of liver diseases.* Hepatology. 2017; 65:310-335.
- Garcia-Tsao G, Friedman S, Iredale J, Pinzani M. *Now there are many (stages) where before there was one: In search of a pathophysiological classification of cirrhosis.* Hepatology. 2010; 51:1445-1449.
- GBD 2017 Cirrhosis Collaborators. *The global, regional, and national burden of cirrhosis by cause in 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017*. Lancet Gastroenterol Hepatol. 2020; 5:245-266.
- Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. *Development of monocytes, macrophages, and dendritic cells*. Science. (2010) 327:656-61.
- Gentilini P, Casini-Raggi V, Di Fiore G, Romanelli RG, Buzzelli G, Pinzani M, et al. *Albumin improves the response to diuretics in patients with cirrhosis and ascites: results of a randomized, controlled trial*. J Hepatol. 1999; 30:639-645.
- Ginès P, Fernández J, Durand F, Saliba F. *Management of critically-ill cirrhotic patients*. J Hepatol. 2012; 56:S13-S24.
- Grell M, Douni E, Wajant H, Löhden M, Clauss M, Maxeiner B,et al. *The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor*. Cell. 1995; 83:793-802.

H

- Hansen J, Cherwitz DL, Allen JI. *The role of tumor necrosis factor-alpha in acute endotoxin-induced hepatotoxicity in ethanol-fed rats.* Hepatology. 1994; 20:461-474.
- Hassanein TI, Tofteng F, Brown RS Jr, McGuire B, Lynch P, Mehta R, et al. *Randomized controlled study of extracorporeal albumin dialysis for hepatic encephalopathy in advanced cirrhosis*. Hepatology. 2007; 46:1853-1862.
- Häussinger D, Schliess F. *Pathogenetic mechanisms of hepatic encephalopathy.* Gut. 2008; 57:1156-1165.
- He XM, Carter DC. *Atomic structure and chemistry of human serum albumin.* Nature 1992; 358:209-215.
- He W, Ladinsky MS, Huey-Tubman KE, Jensen GJ, McIntosh JR, Björkman PJ. *FcRn-mediated antibody transport across epithelial cells revealed by electron tomography*. Nature. 2008; 455:542-546.
- Heemann U, Treichel U, Loock J, Philipp T, Gerken G, Malago M, et al. *Albumin dialysis in cirrhosis with superimposed acute liver injury: a prospective, controlled study.* Hepatology. 2002; 36:949-958.
- Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. *A Toll-like receptor recognizes bacterial DNA*. Nature. 2000; 408:740-745.
- Homann C, Varming K, Høgåsen K, Mollnes TE, Graudal N, Thomsen AC, et al. *Acquired C3 deficiency in patients with alcoholic cirrhosis predisposes to infection and increased mortality.* Gut. 1997; 40:544-549.
- Hong S, Gronert K, Devchand PR, Moussignac RL, Serhan CN. *Novel docosatrienes and 17S-resolvins generated from docosahexaenoic acid in murine brain, human blood, and glial cells. Autacoids in anti-inflammation.* J Biol Chem. 2003; 278:14677-14687.
- Hughes CE, Nibbs RJB. *A guide to chemokines and their receptors*. FEBS J. 2018; 285:2944-2971.

I

- Ikehata M, Yumoto R, Nakamura K, Nagai J, Takano M. *Comparison of albumin uptake in rat alveolar type II and type I-like epithelial cells in primary culture.* Pharm Res. 2008; 25:913-922.
- Isobe Y, Arita M, Matsueda S, Iwamoto R, Fujihara T, Nakanishi H, et al. *Identification and structure determination of novel anti-inflammatory mediator*

resolvin E3, 17,18-dihydroxyeicosapentaenoic acid. J Biol Chem. 2012; 287:10525-10534.

J

- Jaisson S, Delevallee-Forte C, Toure F, Rieu P, Garnotel R, Gillery P. *Carbamylated albumin is a potent inhibitor of polymorphonuclear neutrophil respiratory burst*. FEBS Lett 2007; 581:1509-1513.
- Jalan R, Schnurr K, Mookerjee RP, Sen S, Cheshire L, Hodges S, et al. *Alterations in the functional capacity of albumin in patients with decompensated cirrhosis is associated with increased mortality.* Hepatology 2009; 50:555-564.
- Jones SA. *Directing transition from innate to acquired immunity: defining a role for IL-6.* J Immunol. 2005; 175:3463-3468.
- Jorgensen KA, Stoffersen E, et al. *Heparin like activity of albumin*. Thromb Res 1979; 16:596-574.
- Jurgens G, Muller M, Garidel P, Koch MH, Nakakubo H, Blume A, et al. *Investigation into the interaction of recombinant human serum albumin with re-lipopolysaccharide and lipid A*. J Endotoxin Res 2002; 8:115-126.

K

- Kadowaki N, Ho S, Antonenko S, Malefyt RW, Kastelein RA, Bazan F, Liu YJ. *Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens*. J Exp Med. 2001; 194:863-869.
- Kalliolias GD, Ivashkiv LB. *TNF biology, pathogenic mechanisms and emerging therapeutic strategies.* Nat Rev Rheumatol. 2016; 12:49-62.
- Karagiannis AK, Nakouti T, Pipili C, Cholongitas E. *Adrenal insufficiency in patients with decompensated cirrhosis.* World J Hepatol. 2015; 7:1112-1124.
- Kawai T, Sato S, Ishii KJ, Coban C, Hemmi H, Yamamoto M, et al. *Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6*. Nat Immunol. 2004; 5:1061-1068.
- Kawakami A, Kubota K, Yamada N, Tagami U, Takehana K, Sonaka I, et al. *Identification and characterization of oxidized human serum albumin. A slight structural change impairs its ligand-binding and antioxidant functions.* FEBS J. 2006; 273:3346-3357.

- Kawaratani H, Tsujimoto T, Douhara A, Takaya H, Moriya K, Namisaki T, et al. *The effect of inflammatory cytokines in alcoholic liver disease.* Mediators Inflamm. 2013; 2013:495156.
- Kedarisetty CK, Anand L, Bhardwaj A, Bhadoria AS, Kumar G, Vyas AK, et al. *Combination of granulocyte colony-stimulating factor and erythropoietin improves outcomes of patients with decompensated cirrhosis.* Gastroenterology. 2015; 148:1362-1370.
- Khanam A, Trehanpati N, Riese P, Rastogi A, Guzman CA, Sarin SK. *Blockade of Neutrophil's Chemokine Receptors CXCR1/2 Abrogate Liver Damage in Acute-on-Chronic Liver Failure.* Front Immunol. 2017; 8:464.
- Kim SB, Chi HS, Park JS, Hong CD, Yang WS, et al. *Effect of increasing serum albumin on plasma D-dimer, von Willebrand factor, and platelet aggregation in CAPD patients.* Am J Kidney Dis 1999; 33:312-317.
- Kitano H, Fukui H, Okamoto Y, Kikuchi E, Matsumoto M, Kikukawa M, et al. *Role of albumin and high-density lipoprotein as endotoxinbinding proteins in rats with acute and chronic alcohol loading.* Alcohol Clin Exp Res 1996; 20:73-76.
- Klinman DM. *Immunotherapeutic uses of CpG oligodeoxynucleotides. Nat Rev Immunol.* 2004; 4:249-58.
- Kolios G, Valatas V, Kouroumalis E. *Role of Kupffer cells in the pathogenesis of liver disease*. World J Gastroenterol. 2006; 12:7413-7420.
- Korf H, du Plessis J, van Pelt J, De Groote S, Cassiman D, Verbeke L, et al. *Inhibition of glutamine synthetase in monocytes from patients with acute-on-chronic liver failure resuscitates their antibacterial and inflammatory capacity*. Gut. 2019; 68:1872-1883.
- Kremer H, Baron-Menguy C, Tesse A, Gallois Y, Mercat A, Henrion D, et al. *Human serum albumin improves endothelial dysfunction and survival during experimental endotoxemia: concentration-dependent properties.* Crit Care Med 2011; 39:1414-1422.
- Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, et al. *CpG motifs in bacterial DNA trigger direct B-cell activation.* Nature. 1995; 374:546-549.
- Krishnamoorthy S, Recchiuti A, Chiang N, Yacoubian S, Lee CH, Yang R, et al. *Resolvin D1 binds human phagocytes with evidence for proresolving receptors.* Proc Natl Acad Sci U S A. 2010; 107:1660-1665.

- Krishnan J, Selvarajoo K, Tsuchiya M, Lee G, Choi S. *Toll-like receptor signal transduction.* Exp Mol Med 2007; 39:421-38.
- Kubes P, Mehal WZ. *Sterile inflammation in the liver.* Gastroenterology. 2012; 143:1158-1172.
- Kwak K, Akkaya M, Pierce SK. *B cell signaling in context.* Nat Immunol 2019; 20:963-969.

L

- Lee BL, Barton GM. *Trafficking of endosomal Toll-like receptors.* Trends Cell Biol. 2014; 24:360-369.
- Li HH, Li J, Wasserloos KJ, Wallace C, Sullivan MG, Bauer PM, et al. *Caveolae-dependent and -independent uptake of albumin in cultured rodent pulmonary endothelial cells.* PLoS One. 2013; 8:e81903.
- Liu K, Wang FS, Xu R. *Neutrophils in liver diseases: pathogenesis and therapeutic targets.* Cell Mol Immunol. 2021; 18:38-44.
- Lopategi A, Flores-Costa R, Rius B, López-Vicario C, Alcaraz-Quiles J, Titos E, et al. *Frontline Science: Specialized proresolving lipid mediators inhibit the priming and activation of the macrophage NLRP3 inflammasome.* J Leukoc Biol. 2019; 105:25-36.
- López-Vicario C, Checa A, Urdangarin A, Aguilar F, Alcaraz-Quiles J, Caraceni P, et al. *Targeted lipidomics reveals extensive changes in circulating lipid mediators in patients with acutely decompensated cirrhosis*. J Hepatol. 2020; 73:817-828.
- López-Vicario C, Rius B, Alcaraz-Quiles J, García-Alonso V, Lopategi A, Titos E, et al. *Pro-resolving mediators produced from EPA and DHA: Overview of the pathways involved and their mechanisms in metabolic syndrome and related liver diseases.* Eur J Pharmacol. 2016; 785:133-143.
- López-Vicario C, Rius B, Alcaraz-Quiles J, González-Périz A, Martínez-Puchol AI, Casulleras M, et al. *Association of a variant in the gene encoding for ERV1/ChemR23 with reduced inflammation in visceral adipose tissue from morbidly obese individuals*. Sci Rep. 2017; 7:15724.

M

- Macdonald S, Andreola F, Bachtiger P, Amoros A, Pavesi M, Mookerjee R, et al. *Cell death markers in patients with cirrhosis and acute decompensation.* Hepatology. 2018; 67:989-1002.
- Maione F, Casillo GM, Raucci F, Iqbal AJ, Mascolo N. *The functional link between microsomal prostaglandin E synthase-1 (mPGES-1) and peroxisome proliferator-activated receptor γ (PPARγ) in the onset of inflammation.* Pharmacol Res. 2020; 157:104807.
- Maiwall R, Kumar A, Pasupuleti SSR, Hidam AK, Tevethia H, Kumar G, et al. *A randomized-controlled trial comparing 20% albumin to plasmalyte in patients with cirrhosis and sepsis-induced hypotension [ALPS trial]*. J Hepatol. 2022; S0168-8278(22)00239-2.
- Mao H, Gunasekera AH, Fesik SW. *Expression, refolding and isotipic labeling of human serum albumin domains for NMR spectroscopy.* Protein Expr Purif 2000; 20:492-499.
- Medzhitov R, Preston-Hurlburt P, Kopp E, Stadlen A, Chen C, Ghosh S, et al. *MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways.* Mol Cell. 1998; 2:253-258.
- Meziani F, Kremer H, Tesse A, Baron-Menguy C, Mathien C, Mostefai HA, et al. *Human serum albumin improves arterial dysfunction during early resuscitation in mouse endotoxic model via reduced oxidative and nitrosative stresses*. Am J Pathol 2007; 171:1753-1761.
- Møller S, Bernardi M. *Interactions of the heart and the liver*. Eur Heart J. 2013; 34:2804-2811.
- Moore KP, Taylor GW, Maltby NH, Siegers D, Fuller RW, Dollery CT, et al. *Increased production of cysteinyl leukotrienes in hepatorenal syndrome.* J Hepatol. 1990; 11:263-271.
- Moreau R, Jalan R, Gines P, Pavesi M, Angeli P, Cordoba J, et al. *Acute-on-chronic liver failure is a distinct syndrome that develops in patients with acute decompensation of cirrhosis*. Gastroenterology. 2013; 144:1426-1437.
- Moreau R. *The Pathogenesis of ACLF: The Inflammatory Response and Immune Function.* Semin Liver Dis. 2016; 36:133-140.

- Morgenthaler JJ, Baillod P, Friedli H. *Fatty acid patterns during plasma fractionation*. Vox Sang. 1980; 38:288-293.
- Müllberg J, Althoff K, Jostock T, Rose-John S. *The importance of shedding of membrane proteins for cytokine biology*. Eur Cytokine Netw. 2000; 11:27-38.

N

- Nakae S, Asano M, Horai R, Sakaguchi N, Iwakura Y. *IL-1 enhances T cell-dependent antibody production through induction of CD40 ligand and OX40 on T cells.* J Immunol. 2001; 167:90-97.
- Navasa M, Follo A, Filella X, Jiménez W, Francitorra A, Planas R, et al. *Tumor necrosis factor and interleukin-6 in spontaneous bacterial peritonitis in cirrhosis: relationship with the development of renal impairment and mortality.* Hepatology. 1998; 27:1227-1232.
- Neuzil J, Stocker R. *Free and albumin-bound bilirubin are efficient co-antioxidants for alpha-tocopherol, inhibiting plasma and low density lipoprotein lipid peroxidation.* J Biol Chem 1994; 269:16712-16719.
- Nicholson JP, Wolmarans MR and Park GR. *The role of albumin in critical illness.* Br J Anaesth 2000; 85:599-610.
- Nielsen MC, Hvidbjerg Gantzel R, Clària J, Trebicka J, Møller HJ, Grønbæk H. *Macrophage Activation Markers, CD163 and CD206, in Acute-on-Chronic Liver Failure*. Cells. 2020; 9:1175.
- Niewinski G, Raszeja-Wyszomirska J, Hrenczuk M, Rozga A, Malkowski P, Rozga J. *Intermittent high-flux albumin dialysis with continuous venovenous hemodialysis for acute-on-chronic liver failure and acute kidney injury*. Artif Organs. 2020; 44:91-99.

O

- O'Brien AJ, Fullerton JN, Massey KA, Auld G, Sewell G, James S, et al. *Immunosuppression in acutely decompensated cirrhosis is mediated by prostaglandin E2*. Nat Med 2014; 20:518-523.
- Oettl K, Stadlbauer V, Krisper P, Stauber RE. *Effect of extracorporeal liver support by molecular adsorbents recirculating system and Prometheus on redox state of albumin in acute-on-chronic liver failure.* Ther Apher Dial. 2009; 13:431-436.

- Oettl K, Stadlbauer V, Petter F, Greilberger J, Putz-Bankuti C, Hallstrom S, et al. *Oxidative damage of albumin in advanced liver disease.* Biochim Biophys Acta 2008; 1782:469–473.

- O'Neill LA. *The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress*. Immunol Rev. 2008; 226:10-18.

- O'Neill LA, Bowie AG. *The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling.* Nat Rev Immunol. 2007; 7:353-364.

- Ortega R, Ginès P, Uriz J, Cárdenas A, Calahorra B, De Las Heras D, et al. *Terlipressin therapy with and without albumin for patients with hepatorenal syndrome: results of a prospective, nonrandomized study.* Hepatology. 2002; 36:941-948.

- Ott VL, Cambier JC, Kappler J, Marrack P, Swanson BJ. *Mast cell-dependent migration of effector CD8+ T cells through production of leukotriene B4.* Nat Immunol. 2003; 4:974-981.

- Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, et al. *The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors.* Proc. Natl. Acad. Sci. U.S.A. 2000; 97:13766-13771.

P

- Papp M, Vitalis Z, Altorjay I, Tornai I, Udvardy M, Harsfalvi J, et al. *Acute phase proteins in the diagnosis and prediction of cirrhosis associated bacterial infections.* Liver Int. 2012; 32:603-611.

- Peters-Golden M, Canetti C, Mancuso P, Coffey MJ. *Leukotrienes: underappreciated mediators of innate immune responses.* J Immunol. 2005; 174:589-594.

- Peters M, Jacobs S, Ehlers M, Vollmer P, Müllberg J, Wolf E, et al. *The function of the soluble interleukin 6 (IL-6) receptor in vivo: sensitization of human soluble IL-6 receptor transgenic mice towards IL-6 and prolongation of the plasma half-life of IL-6*. J Exp Med. 1996; 183:1399-1406.

- Peters T Jr. *All about albumin*. Academic Press 1995.

- Pruenster M, Mudde L, Bombosi P, Dimitrova S, Zsak M, Middleton J, et al. *The Duffy antigen receptor for chemokines transports chemokines and supports their promigratory activity*. Nat Immunol. 2009; 10:101-108.
- Pyzik M, Rath T, Kuo TT, Win S, Baker K, Hubbard JJ, et al. *Hepatic FcRn regulates albumin homeostasis and susceptibility to liver injury*. Proc Natl Acad Sci U S A. 2017; 114:E2862-E2871.

Q

- Quehenberger O, Dennis EA. *The human plasma lipidome*. N Engl J Med. 2011; 365:1812-1823.
- Quinlan GJ, Martin GS and Evans TW. *Albumin: Biochemical Properties and Therapeutic Potential.* Hepatology 2005; 41:1211-1219.

R

- Raz A. *Interaction of prostaglandins with blood plasma proteins. Comparative binding of prostaglandins A 2 , F 2 and E 2 to human plasma proteins.* Biochem J. 1972; 130:631-636.
- Rius B, Duran-Güell M, Flores-Costa R, López-Vicario C, Lopategi A, Alcaraz-Quiles J, et al. *The specialized proresolving lipid mediator maresin 1 protects hepatocytes from lipotoxic and hypoxia-induced endoplasmic reticulum stress.* FASEB J. 2017; 31:5384-5398.
- Rodríguez-Roisin R, Krowka MJ. *Hepatopulmonary syndrome--a liver-induced lung vascular disorder.* N Engl J Med. 2008; 358:2378-2387.
- Roguin A. *Rene Theophile Hyacinthe Laënnec (1781-1826): the man behind the stethoscope.* Clin Med Res. 2006; 4:230-235.
- Romanelli RG, La Villa G, Barletta G, Vizzutti F, Lanini F, Arena U, et al. *Long-term albumin infusion improves survival in patients with cirrhosis and ascites: an unblinded randomized trial.* World J Gastroenterol. 2006; 12:1403-1407.
- Romano M, Serhan CN. *Lipoxin generation by permeabilized human platelets.* Biochemistry. 1992; 31:8269-8277.
- Romero-Gómez M, Montagnese S, Jalan R. *Hepatic encephalopathy in patients with acute decompensation of cirrhosis and acute-on-chronic liver failure.* J Hepatol. 2015; 62:437-447.

- Rosenbloom AJ, Pinsky MR, Bryant JL, Shin A, Tran T, Whiteside T. *Leukocyte activation in the peripheral blood of patients with cirrhosis of the liver and SIRS. Correlation with serum interleukin-6 levels and organ dysfunction.* JAMA. 1995; 274:58-65.
- Rutz M, Metzger J, Gellert T, Luppa P, Lipford GB, Wagner H, et al. *Toll-like receptor 9 binds single-stranded CpG-DNA in a sequence- and pH-dependent manner*. Eur J Immunol. 2004; 34:2541-2550.

S

- Sabroe I, Dower SK, Whyte MK. *The role of Toll-like receptors in the regulation of neutrophil migration, activation, and apoptosis*. Clin Infect Dis. 2005; 41:S421-6.
- Saliba F. *The Molecular Adsorbent Recirculating System (MARS) in the intensive care unit: a rescue therapy for patients with hepatic failure.* Crit Care. 2006; 10:118.
- Sand KM, Bern M, Nilsen J, Noordzij HT, Sandlie I, Andersen JT. *Unraveling the Interaction between FcRn and Albumin: Opportunities for Design of Albumin-Based Therapeutics.* Front Immunol. 2015; 5:682.
- Santarlasci V, Cosmi L, Maggi L, Liotta F, Annunziato F. *IL-1 and T Helper Immune Responses*. Front Immunol. 2013; 4:182.
- Sasaki F, Yokomizo T. *The leukotriene receptors as therapeutic targets of inflammatory diseases.* Int Immunol. 2019; 31:607-615.
- Savu L, Benassayag C, Vallette G, Christeff N, Nunez E. *Mouse alpha 1-fetoprotein and albumin. A comparison of their binding properties with estrogen and fatty acid ligands.* J Biol Chem. 1981; 256:9414-9418.
- Schmidt-Arras D, Rose-John S. *IL-6 pathway in the liver: From physiopathology to therapy*. J Hepatol. 2016; 64:1403-1415.
- Schwab JM, Chiang N, Arita M, Serhan CN. *Resolvin E1 and protectin D1 activate inflammation-resolution programmes.* Nature. 2007; 447:869-874.
- Serhan CN. *On the relationship between leukotriene and lipoxin production by human neutrophils: evidence for differential metabolism of 15-HETE and 5-HETE.* Biochim Biophys Acta. 1989; 1004:158-168.

- Serhan CN. *Pro-resolving lipid mediators are leads for resolution physiology.* Nature. 2014; 510:92-101.
- Serhan CN, Chiang N. *Resolution phase lipid mediators of inflammation: agonists of resolution.* Curr Opin Pharmacol. 2013; 13:632-640.
- Serhan CN, Clish CB, Brannon J, Colgan SP, Chiang N, Gronert K. *Novel functional sets of lipid-derived mediators with antiinflammatory actions generated from omega-3 fatty acids via cyclooxygenase 2-nonsteroidal antiinflammatory drugs and transcellular processing.* J Exp Med. 2000; 192:1197-204.
- Serhan CN, Petasis NA. *Resolvins and protectins in inflammation resolution*. Chem Rev. 2011; 111:5922-5943.
- Serhan CN, Yang R, Martinod K, Kasuga K, Pillai PS, Porter TF, et al. *Maresins: novel macrophage mediators with potent antiinflammatory and proresolving actions.* J Exp Med. 2009; 206:15-23.
- Shah N, Dhar D, El Zahraa Mohammed F, Habtesion A, Davies NA, Jover-Cobos M, et al. *Prevention of acute kidney injury in a rodent model of cirrhosis following selective gut decontamination is associated with reduced renal TLR4 expression*. J Hepatol. 2012; 56:1047-1053.
- Shah N, Mohamed FE, Jover-Cobos M, Macnaughtan J, Davies N, Moreau R, et al. *Increased renal expression and urinary excretion of TLR4 in acute kidney injury associated with cirrhosis*. Liver Int. 2013; 33:398-409.
- Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, el al. *MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4*. J Exp Med. 1999; 189:1777-1782.
- Sims JE, Smith DE. *The IL-1 family: regulators of immunity*. Nat Rev Immunol. 2010; 10:89-102.
- Skiniotis G, Boulanger MJ, Garcia KC, Walz T. *Signaling conformations of the tall cytokine receptor gp130 when in complex with IL-6 and IL-6 receptor*. Nat Struct Mol Biol. 2005; 12:545-551.
- Sogni P, Moreau R, Gadano A, Lebrec D. *The role of nitric oxide in the hyperdynamic circulatory syndrome associated with portal hypertension.* J Hepatol. 1995; 23:218-224.

- Solà E, Solé C, Simón-Talero M, Martín-Llahí M, Castellote J, Garcia-Martínez R, et al. *Midodrine and albumin for prevention of complications in patients with cirrhosis awaiting liver transplantation. A randomized placebo-controlled trial.* J Hepatol. 2018; 69:1250-1259.
- Sort P, Navasa M, Arroyo V, Aldeguer X, Planas R, Ruiz-del-Arbol L, et al. *Effect of intravenous albumin on renal impairment and mortality in patients with cirrhosis and spontaneous bacterial peritonitis*. N Engl J Med. 1999; 341:403-409.
- Spite M, Norling LV, Summers L, Yang R, Cooper D, Petasis NA, et al. *Resolvin D2 is a potent regulator of leukocytes and controls microbial sepsis.* Nature. 2009; 461:1287-1291.
- Sudlow G, Birkett DJ, Wade DN. *The characterization of two specific drug binding sites on human serum albumin.* Mol Pharmacol 1975; 11:824-832.
- Sun YP, Oh SF, Uddin J, Yang R, Gotlinger K, Campbell E, et al. *Resolvin D1 and its aspirin-triggered 17R epimer. Stereochemical assignments, anti-inflammatory properties, and enzymatic inactivation.* J Biol Chem. 2007; 282:9323-9334.

T

- Tacke F. *Targeting hepatic macrophages to treat liver diseases.* J Hepatol. 2017; 66:1300-1312.
- Takeuchi O, Akira S. *Pattern recognition receptors and inflammation*. Cell 2010; 140:805-820.
- Taylor NJ, Manakkat Vijay GK, Abeles RD, Auzinger G, Bernal W, Ma Y, et al. *The severity of circulating neutrophil dysfunction in patients with cirrhosis is associated with 90-day and 1-year mortality*. Aliment Pharmacol Ther. 2014; 40:705-715.
- Thenappan T, Goel A, Marsboom G, Fang YH, Toth PT, Zhang HJ, et al. A central role for CD68(1) macrophages in hepatopulmonary syndrome: reversal by macrophage depletion. Am J Respir Crit Care Med. 2011; 183:1080–1091.
- Thuy AV, Reimann CM, Hemdan NY, Gräler MH. *Sphingosine 1-phosphate in blood: function, metabolism, and fate*. Cell Physiol Biochem 2014; 34:158-71.
- Tian Z, Chen Y, Gao B. *Natural killer cells in liver disease*. Hepatology. 2013; 57:1654-1662.

- Tilg H, Moschen AR, Szabo G*. Interleukin-1 and inflammasomes in alcoholic liver disease/acute alcoholic hepatitis and nonalcoholic fatty liver disease/nonalcoholic steatohepatitis.* Hepatology. 2016; 64:955-965.
- Tisoncik JR, Korth MJ, Simmons CP, Farrar J, Martin TR, Katze MG. *Into the eye of the cytokine storm*. Microbiol Mol Biol Rev. 2012 Mar;76(1):16-32.
- Titos E, Clària J, Planagumà A, López-Parra M, González-Périz A, Gaya J, et al. *Inhibition of 5-lipoxygenase-activating protein abrogates experimental liver injury: role of Kupffer cells*. J Leukoc Biol. 2005; 78:871-878.
- Tjonahen E, Oh SF, Siegelman J, Elangovan S, Percarpio KB, Hong S, et al. *Resolvin E2: identification and anti-inflammatory actions: pivotal role of human 5-lipoxygenase in resolvin E series biosynthesis.* Chem Biol. 2006; 13:1193-1202.
- Tomić M, Micov A, Pecikoza U, Stepanović-Petrović R. *Chapter 1 - Clinical Uses of Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) and Potential Benefits of NSAIDs Modified-Release Preparations.* Microsized and Nanosized Carriers for Nonsteroidal Anti-Inflammatory Drugs, Academic Press. 2017.
- Trebicka J, Amoros A, Pitarch C, Titos E, Alcaraz-quiles J, Schierwagen R, et al. *Addressing Profiles of Systemic Inflammation Across the Different Clinical Phenotypes of Acutely Decompensated Cirrhosis.* Front. Immunol. 2019; 10: 476.
- Tsai MH, Peng YS, Chen YC, Liu NJ, Ho YP, Fang JT, et al. *Adrenal insufficiency in patients with cirrhosis, severe sepsis and septic shock.* Hepatology. 2006; 43:673-681.
- Turner MD, Nedjai B, Hurst T, Pennington DJ. *Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease.* Biochim Biophys Acta. 2014; 1843:2563-2582.

U

- Ulevitch RJ. *Toll gates for pathogen selection*. Nature. 1999; 401:755-756

V

- Van der Vusse GJ. *Albumin as fatty acid transporter.* Drug metab pharmacokinet 2009; 24:300-307.

- Villanueva C, Albillos A, Genescà J, Abraldes JG, Calleja JL, Aracil C, et al. *Development of hyperdynamic circulation and response to β-blockers in compensated cirrhosis with portal hypertension.* Hepatology. 2016; 63:197-206.
- Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. *Functions of natural killer cells*. Nat Immunol. 2008; 9:503–510.
- Vollmer J, Weeratna R, Payette P, Jurk M, Schetter C, Laucht M, et al. *Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities*. Eur J Immunol. 2004; 34:251-262.

W

- Watanabe H, Imafuku T, Otagiri M, and Maruyamaet T. *Clinical Implications Associated With the Posttranslational ModificationeInduced Functional Impairment of Albumin in Oxidative StresseRelated Diseases.* J phar sci 2017; 106:2195-2203.
- Watts C. *Location, location, location: identifying the neighborhoods of LPS signaling*. Nat Immunol. 2008; 9:343-345.
- Weichselbaum L, Azouz A, Smolen KK, Das J, Splittgerber M, Lepida A, et al. Epigenetic basis for monocyte dysfunction in patients with severe alcoholic hepatitis. J Hepatol. 2020; 73:303-314.
- Wiest R, Lawson M, Geuking M. *Pathological bacterial translocation in liver cirrhosis.* J Hepatol. 2014; 60:197-209.

X

- Xu R, Bao C, Huang H, Lin F, Yuan Y, Wang S, et al. *Low expression of CXCR1/2 on neutrophils predicts poor survival in patients with hepatitis B virus-related acute-on-chronic liver failure.* Sci Rep. 2016; 6:38714.

Y

- Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, et al. *Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway.* Science 2003; 301:640-643.
- Yamamoto M, Sato S, Hemmi H, Uematsu S, Hoshino K, Kaisho T, et al. *TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway*. Nat Immunol. 2003; 4:1144-1150.

- Yang Y, Tang LQ, Wei W. *Prostanoids receptors signaling in different diseases/cancers progression.* J Recept Signal Transduct Res. 2013; 33:14-27.
- Yang Z, Zhang Z, Cheng Q, Chen G, Li W, Ma K, et al. *Plasma perfusion combined with plasma exchange in chronic hepatitis B-related acute-on-chronic liver failure patients.* Hepatol Int. 2020; 14:491-502.
- Yumoto R, Nishikawa H, Okamoto M, Katayama H, Nagai J, Takano M. *Clathrin-mediated endocytosis of FITC-albumin in alveolar type II epithelial cell line RLE-6TN.* Am J Physiol Lung Cell Mol Physiol. 2006; 29:L946-L955.

Z

- Zhang J, Yang W, Hu B, Wu W, Fallon MB. *Endothelin-1 activation of the endothelin B receptor modulates pulmonary endothelial CX3CL1 and contributes to pulmonary angiogenesis in experimental hepatopulmonary syndrome*. Am J Pathol. 2014; 184:1706-1714.
- Zhang WJ, Frei B. *Albumin selectively inhibits TNF alpha-induced expression of vascular cell adhesion molecule-1 in human aortic endothelial cells*. Cardiovasc Res 2002; 55:820-829.
- Zlotnik A, Yoshie O. *Chemokines: a new classification system and their role in immunity.* Immunity. 2000; 12:121-127.
- Zsila F, Bikádi Z, Lockwood SF. *In vitro binding of leukotriene B4 (LTB4) to human serum albumin: evidence from spectroscopic, molecular modeling, and competitive displacement studies.* Bioorg Med Chem Lett. 2005; 15:3725-3731.

ANNEX I: MATERIAL TABLES

PROTEIN	DILUTION	HOST	REFERENCE	TECHNIQUE
ADM31	10 μg/ml		Aldevron	In vitro
Albumin	1:500	Rabbit	ab207327, Abcam	IF
β-actin	1:1000	Rabbit	5125 CST	WB
CD14 (APC Conjugated)	1:20	Mouse	325607 BioLegend	FC
CD15 (FITC Conjugated)	1:20	Mouse	394706 BioLegend	FC
CD45 (PerCP Conjugated)	1:20	Mouse	368505 BioLegend	FC
CD66b (PE Conjugated)	1:20	Mouse	305106 BioLegend	FC
EEA1	1:200	Rabbit	ab70521, Abcam	IF
Human Serum Albumin	1:500	Mouse	HYB 192-01-02, Thermo Fisher Scientific	WB
IgG				WB
IgG2b	10 μg/ml	Rabbit	ABIN964473, Antibodies online	In vitro
IRF3	1:500	Rabbit	CST	WB
P-IRF3 (SER396)	1:250	Rabbit	Abcam	WB

Annexed Table 1: Antibodies used in the experimental part of the thesis.
IF: immunofluorescence; WB: Western Blot; FC: Flow Cytometry; In vitro: used in experimental conditions in vitro.

GENE	PROTEIN	SPECIE	REFERENCE
ACTB	β-actin	Human	Hs99999903_m1
Actb	β-actin	Mouse	Mm00607939_s1
ALOX15	15-LOX-1	Human	Hs00993765_g1
ALOX15B	15-LOX-2	Human	Hs00153988_m1
ALOX5	5-LOX	Human	Hs00167536_m1
ALOX5AP	FLAP	Human	Hs00970921_m1
CCL22	CCL22	Human	Hs01574247_m1
CXCL10	CXCL10	Human	Hs01124251_g1
HPGD	HPGD	Human	Hs00960586_g1
IFNB1	IFNβ	Human	Hs01077958_s1
IL1B	IL-1β	Human	Hs01555410_m1
Il1b	IL-1β	Mouse	Mm01336189_m1
IL6	IL-6	Human	Hs00985639_m1
Il6	IL-6	Mouse	Mm00446190_m1
MRC1	CD206	Human	Hs00267207_m1
mPGES-1	mPGES-1	Human	Hs01115610_m1
OAS2	OAS2	Human	Hs00942643_m1
PTGS1	COX-1	Human	Hs00377726_m1
PTGS2	COX-2	Human	Hs00153133_m1
TGM2	TGM2	Human	Hs01096681_m1
TNF	TNFα	Human	Hs01113624_g1
Tnf	TNFα	Mouse	Mm00443258_m1

Annexed Table 2: Real-time PCR probes used in the experimental part of the thesis. All the probes are validated and pre-designed Taqman Gene Expresion Assays acquired from Thermofisher scientific.

ANNEX II: OTHER PUBLICATIONS

Publications included in this thesis:

- **Casulleras M**, Flores-Costa R, Duran-Güell M, Alcaraz-Quiles J, Sanz S, Titos E, López-Vicario C, Fernández J, Horrillo R, Costa M, de la Grange P, Moreau R, Arroyo V, Clària J. Albumin internalizes and inhibits endosomal TLR signaling in leukocytes from patients with decompensated cirrhosis. Sci Transl Med. 2020; 12:eaax5135.

- **Casulleras M**, Flores-Costa R, Duran-Güell M, Zhang IW, López-Vicario C, Curto A, Fernández J, Arroyo V, Clària J. Albumin Lipidomics Reveals Meaningful Compositional Changes in Advanced Cirrhosis and Its Potential to Promote Inflammation Resolution. Hepatol Commun. 2022.

Other Publications:

- Sánchez-Rodríguez MB, Téllez É, **Casulleras M**, Borràs FE, Arroyo V, Clària J, Sarrias MR. Reduced Plasma Extracellular Vesicle CD5L Content in Patients With Acute-On-Chronic Liver Failure: Interplay With Specialized Pro-Resolving Lipid Mediators. Front Immunol. 2022; 13:842996.

- Zhang IW, Curto A, López-Vicario C, **Casulleras M**, Duran-Güell M, Flores-Costa R, Colsch B, Aguilar F, Aransay AM, Lozano JJ, Hernández-Tejero M, Toapanta D, Fernández J, Arroyo V, Clària J. Mitochondrial dysfunction governs immunometabolism in leukocytes of patients with acute-on-chronic liver failure. J Hepatol. 2022; 76:93-106.

- Duran-Güell M, Flores-Costa R, **Casulleras M**, López-Vicario C, Titos E, Díaz A, Alcaraz-Quiles J, Horrillo R, Costa M, Fernández J, Arroyo V, Clària J. Albumin protects the liver from tumor necrosis factor α-induced immunopathology. FASEB J. 2021; 35:e21365.

- **Casulleras M**, Zhang IW, López-Vicario C, Clària J. Leukocytes, Systemic Inflammation and Immunopathology in Acute-on-Chronic Liver Failure. Cells. 2020; 9:2632.

- Flores-Costa R, Duran-Güell M, **Casulleras M**, López-Vicario C, Alcaraz-Quiles J, Diaz A, Lozano JJ, Titos E, Hall K, Sarno R, Masferrer JL, Clària J. Stimulation of soluble guanylate cyclase exerts antiinflammatory actions in the liver through a VASP/NF-κB/NLRP3 inflammasome circuit. Proc Natl Acad Sci U S A. 2020; 117:28263-28274.

- Fernández J, Clària J, Amorós A, Aguilar F, Castro M, **Casulleras M**, Acevedo J, Duran-Güell M, Nuñez L, Costa M, Torres M, Horrillo R, Ruiz-Del-Árbol L, Villanueva C, Prado V, Arteaga M, Trebicka J, Angeli P, Merli M, Alessandria C, Aagaard NK, Soriano G, Durand F, Gerbes A, Gustot T, Welzel TM, Salerno F, Bañares R, Vargas V, Albillos A, Silva A, Morales-Ruiz M, Carlos García-Pagán J, Pavesi M, Jalan R, Bernardi M, Moreau R, Páez A, Arroyo V. Effects of Albumin Treatment on Systemic and Portal Hemodynamics and Systemic Inflammation in Patients With Decompensated Cirrhosis. Gastroenterology. 2019; 157:149-162.

- López-Vicario C, Titos E, Walker ME, Alcaraz-Quiles J, **Casulleras M**, Durán-Güell M, Flores-Costa R, Pérez-Romero N, Forné M, Dalli J, Clària J. Leukocytes from obese individuals exhibit an impaired SPM signature. FASEB J. 2019; 33:7072-7083.

- Alcaraz-Quiles J, **Casulleras M**, Oettl K, Titos E, Flores-Costa R, Duran-Güell M, López-Vicario C, Pavesi M, Stauber RE, Arroyo V, Clària J. Oxidized Albumin Triggers a Cytokine Storm in Leukocytes Through P38 Mitogen-Activated Protein Kinase: Role in Systemic Inflammation in Decompensated Cirrhosis. Hepatology. 2018; 68:1937-1952.

- Flores-Costa R, Alcaraz-Quiles J, Titos E, López-Vicario C, **Casulleras M**, Duran-Güell M, Rius B, Diaz A, Hall K, Shea C, Sarno R, Currie M, Masferrer JL, Clària J. The soluble guanylate cyclase stimulator IW-1973 prevents inflammation and fibrosis in experimental non-alcoholic steatohepatitis. Br J Pharmacol. 2018; 175:953-967.

- López-Vicario C, Rius B, Alcaraz-Quiles J, González-Périz A, Martínez-Puchol AI, **Casulleras M**, Duran-Güell M, Ibarzabal A, Corcelles R, Laguna-Fernández A, Back M, Titos E, Clària J. Association of a variant in the gene encoding for ERV1/ChemR23 with reduced inflammation in visceral adipose tissue from morbidly obese individuals. Sci Rep. 2017; 7:15724.

- Rius B, Duran-Güell M, Flores-Costa R, López-Vicario C, Lopategi A, Alcaraz-Quiles J, **Casulleras M**, Lozano JJ, Titos E, Clària J. The specialized proresolving lipid mediator maresin 1 protects hepatocytes from lipotoxic and hypoxia-induced endoplasmic reticulum stress. FASEB J. 2017; 31:5384-5398.

- Alcaraz-Quiles J, Titos E, **Casulleras M**, Pavesi M, López-Vicario C, Rius B, Lopategi A, de Gottardi A, Graziadei I, Gronbaek H, Ginès P, Bernardi M, Arroyo V, Clària J. Polymorphisms in the IL-1 gene cluster influence systemic inflammation in patients at risk for acute-on-chronic liver failure. Hepatology. 2017; 65:202-216.

cells

MDPI

Review

Leukocytes, Systemic Inflammation and Immunopathology in Acute-on-Chronic Liver Failure

Mireia Casulleras [1,2], Ingrid W. Zhang [1,2], Cristina López-Vicario [1,2,*] and Joan Clària [1,2,3,*]

1 Biochemistry and Molecular Genetics Service, Hospital Clínic-IDIBAPS, 08036 Barcelona, Spain; mcasulleras@clinic.cat (M.C.); iwzhang@clinic.cat (I.W.Z.)
2 European Foundation for the Study of Chronic Liver Failure (EF Clif) and Grifols Chair, 08021 Barcelona, Spain
3 Department of Biomedical Sciences, School of Medicine and Health Sciences, Universitat de Barcelona, 08036 Barcelona, Spain
* Correspondence: clopezv@clinic.cat (C.L.-V.); jclaria@clinic.cat (J.C.); Tel.: +34-932275400 (ext. 4536) (C.L.-V.); +34-932275400 (ext. 4784) (J.C.)

Received: 18 October 2020; Accepted: 1 December 2020; Published: 8 December 2020

Abstract: Acute-on-chronic liver failure (ACLF) is a complex syndrome that develops in patients with cirrhosis and is characterized by acute decompensation, organ failure(s) and high short-term mortality. ACLF frequently occurs in close temporal relationship to a precipitating event, such as acute alcoholic, drug-induced or viral hepatitis or bacterial infection and, in cases without precipitating events, probably related to intestinal translocation of bacterial products. Dysbalanced immune function is central to its pathogenesis and outcome with an initial excessive systemic inflammatory response that drives organ failure and mortality. This hyperinflammatory state ultimately impairs the host defensive mechanisms of immune cells, rendering ACLF patients immunocompromised and more vulnerable to secondary infections, and therefore to higher organ dysfunction and mortality. In this review, we describe the prevailing characteristics of the hyperinflammatory state in patients with acutely decompensated cirrhosis developing ACLF, with special emphasis on cells of the innate immune system (i.e., monocytes and neutrophils), their triggers (pathogen- and damage-associated molecular patterns [PAMPs and DAMPs]), their effector molecules (cytokines, chemokines, growth factors and bioactive lipid mediators) and the consequences on tissue immunopathology. In addition, this review includes a chapter discussing new emerging therapies based on the modulation of leukocyte function by the administration of pleiotropic proteins such as albumin, Toll-like receptor 4 antagonists, interleukin-22 or stem cell therapy. Finally, the importance of finding an appropriate intervention that reduces inflammation without inducing immunosuppression is highlighted as one of the main therapeutic challenges in cirrhosis.

Keywords: advanced liver disease; systemic inflammation; immunosuppression; immunometabolism; mononuclear phagocytes; cytokines; bioactive lipid mediators

1. Acute-on-Chronic Liver Failure (ACLF)

ACLF is a severe syndrome evolving in patients with acutely decompensated (AD) liver cirrhosis. ACLF is characterized by the manifestation of organ dysfunctions and failures across the six major organ systems (liver, kidney, brain, coagulation, circulation, and respiration) resulting in high short-term mortality (28-day mortality of 32%) [1–3]. The liver and the kidney are the most commonly affected organ systems followed by coagulation, brain, circulation and respiration. ACLF is classified in three grades of severity (ACLF-1, -2 and -3) according to the number of organ failures and may exhibit a variable course during hospitalization as it can follow a steady course or resolve, improve or worsen

within a few days. The CANONIC study, a prospective observational investigation in 1343 patients hospitalized for acute decompensation of cirrhosis, provided the first evidence-based definition of ACLF which includes the presence of organ failure(s) and a 28-day mortality risk of 15% or higher [1]. In Western countries, ACLF is particularly prevalent among young patients with alcoholic cirrhosis and in 60% of the cases develops in close association with potential precipitating events, mainly bacterial infections or active alcoholism. In Asian countries, ACLF is more commonly diagnosed in patients with hepatitis B-related cirrhosis who exhibit lower prevalence of extrahepatic organ failures.

2. Systemic Inflammation and Immunopathology Are Major Drivers of ACLF

Recent advances in our understanding of the pathophysiological basis of ACLF indicate that a systemic hyperinflammatory state is the main driver of widespread tissue and organ injury in patients with AD cirrhosis developing ACLF [4,5]. This hyperinflammatory state is produced by the massive release of inflammatory mediators such as cytokines, chemokines, growth factors and bioactive lipid mediators (see below) that lead to immune-mediated tissue damage, a process that is also known as immunopathology. For example, in the microvasculature of vital organs, proinflammatory cytokines damage the endothelium glycocalyx and trigger neutrophil and monocyte adhesion to endothelial cells and their transmigration into tissues [6]. Activated immune cells, in turn, release mediators such as proteases, oxidative molecules, cytotoxic cytokines, prostaglandins (PGs) and leukotrienes (LTs) (see below), which further intensify tissue damage.

At present little is known about the identity of the triggers (either of infectious or noninfectious origin) leading to immune cell activation and immunopathology in patients with AD cirrhosis evolving to ACLF. Bacterial infections are present in 33% of cases of ACLF and therefore pathogen-associated molecular patterns (PAMPs) released by infecting bacteria are likely contributing [7]. In addition, circulating PAMPs can be the result of the translocation of bacterial products from the intestinal lumen to the systemic circulation. In fact, bacterial overgrowth, increased permeability of the intestinal mucosa, and impaired function of the intestinal innate immune system are common in AD patients developing ACLF [8,9]. PAMPs are unique conserved molecular structures that are recognized by the host via dedicated receptors called pattern-recognition receptors (PRRs), including among others Toll-like receptors (TLRs) present at the cell surface or in the endosomal compartment and NOD-like receptors (NLRs), present in the cytosol of the cells [10]. These receptors recognize nucleic acids and protein, lipid and carbohydrate components characteristic of bacteria and viruses. The engagement of PRRs results in the stimulation of signaling cascades that activate transcription factors such as nuclear factor (NF)-kB or activator protein 1 [11], which in turn induce the expression of a battery of genes encoding for molecules involved in inflammation (i.e., interleukin 6 (*IL6*) and tumor necrosis factor α (*TNF*). Lipopolysaccharide (LPS) from the cell wall of Gram-negative bacteria, which engages TLR4-mediated activation of multiple downstream signaling pathways that result in the synthesis of cytokines and interferons, is a prime example of PAMPs [11].

Systemic inflammation can occur in patients with AD cirrhosis and ACLF in the absence of bacterial infections and/or bacterial translocation as the result of the release of damage-associated molecular patterns (DAMPs) from injured organs and tissues. DAMPs are released by dead, dying or injured cells and originate from several cellular compartments, especially from the nucleus (high mobility group box 1 (HMGB1) and histones), mitochondria (mitochondrial DNA and formyl peptides) and the cytosol (adenosine triphosphate (ATP)) [10]. Apart from necrosis, other immunogenic forms of cell-death such as necroptosis and pyroptosis are common in advanced liver disease and contribute to the enhanced release of DAMPs in this condition [12]. Similar to PAMPs, DAMPs initiate an immune response by binding to specific PRRs. In certain cases, inflammatory cytokines such as IL-1α and IL-33 can act as DAMPs and trigger inflammation through binding to their respective MyD88-coupled cognate receptors.

The intensity of systemic inflammation and the response of the immune system to PAMPs and DAMPs may depend on host genetic factors. For example, single-nucleotide variants might

modulate the release of inflammatory molecules by innate immune cells or might induce changes in the expression of PRRs, such as TLRs. Consistent with this, genetic variants in genes coding for receptors of the innate immune system such as nucleotide-binding oligomerization domain 2 (NOD2) or ligands as mannan-binding lectin (MBL) and MBL-associated serine proteases (MASP) 2 have been shown to associate with increased short-term mortality in AD and ACLF patients [13]. Moreover, single nucleotide polymorphisms within the IL-1 gene cluster have been reported to protect patients with AD cirrhosis from uncontrolled systemic inflammation and to reduce the predisposition of these patients to develop ACLF [14].

3. Immunosuppression Is a Common Feature in ACLF

The hyperinflammatory response in patients with ACLF frequently occurs in parallel with the presence of dysfunctional innate immune system at the humoral, physical and cell-mediated level [8,15,16]. Due to hepatocellular insufficiency, cirrhotic patients commonly display reduced humoral anti-defense capacities as a result of decreased production of acute phase proteins, hypoalbuminemia and defective complement system [17–19]. Additionally, the physical barrier in cirrhosis is impaired, and even more so in ACLF, with gut leakage and dysfunction of the vasculature and sinusoidal endothelium being the most prominent features.

Taking all these components of the innate immune system into account, the overall immune status in patients with ACLF ranges in the spectrum from immunosuppressive/immunoregulatory/tolerogenic to exuberantly hyperinflammatory, and these extremes are not mutually exclusive. Rather the contrary, these two conditions frequently coexist in the same patient, as a constant and persistent hyperinflammatory milieu characterized by increased circulating proinflammatory and anti-inflammatory mediators (e.g., galectin-3, IL-6, TNFα, IL-10) [4,20] and lipid mediators such as PGE_2 can cause the downplaying of innate immune defensive responses and the expansion of regulatory immune cells leading to immunosuppression [21,22], probably in an attempt to keep the proinflammatory response at bay. The predominance of one or the other depends on temporal and spatial aspects: immune cells in the circulation may behave differently (inflammatory phenotype with production of cytokines) than their counterparts in the liver for example (more tolerogenic phenotype), and the immunodeficient phenotype tends to assume greater importance with increasing disease severity [23].

Bernsmeier et al. proposed a model which harmonizes these two extremes: Circulating inflammatory mediators, released by immune cells in an excessive manner in cirrhosis in response to PAMPs and DAMPs induce the formation of immunoregulatory monocytes/macrophages [20]. Thereupon, these cells migrate across endothelia facilitated by endothelial dysfunction into inflamed tissues. In ACLF, tissue macrophages tend to display functionally endotoxin tolerant/immunoregulatory features. Due to their enhanced migratory potential, these cells reverse migrate into the circulation, where they further expand into other tissues and lymph nodes, contributing to the immunosuppressive phenotype in ACLF [20]. The clinical consequence is increased susceptibility to bacterial infections as major precipitating event of organ failure(s), which is the discriminant feature of ACLF [1].

4. Portal Hypertension, Endothelial Activation and the Interplay with the Innate Immune System

The PREDICT study, a European-wide prospective study, recently identified portal hypertension as the second major pathophysiological mechanism in ACLF [24]. It is well-recognized that increased shunting in the context of portal hypertension leads to insufficient clearance of bacterial products and escape of bacteria from the reticuloendothelial system [25]. Therefore, innate immune activation is intricately linked to portal hypertension, as the latter favors bacterial translocation. For instance, bacterial translocation leads to stimulation of TLR4-mediated signaling in hepatic stellate cells, Kupffer cells, and liver sinusoidal endothelial cells and to a pre-activation of the innate immune system facilitating an exaggerated inflammatory response [26,27]. Interestingly, systemic inflammation can be dampened by reduction of the portal pressure via insertion of a transjugular intrahepatic portosystemic shunt, although systemic inflammation often still persists thereafter [28,29].

The interplay between the immune system and endothelial activation is illustrated by the fact that the inflammatory microenvironment favors the formation of microthrombi in the microvasculature of different organs [30] and targets the endothelium to release procoagulant factors [31,32], thereby contributing to immunopathology. Indirect markers of endothelial activation such as angiopoietin 2 [33], which recruits inflammatory cells and promotes cytokine-induced vascular leakage, and vascular cell adhesion molecule 1 (VCAM-1) [34] are markedly elevated in the circulation of patients with AD cirrhosis and ACLF. VCAM-1 participates in the adhesion of leukocytes to the endothelium and trans-endothelial migration. It is associated with multiorgan failure and in-hospital mortality in patients with severe sepsis [35]. Moreover, microparticles of leuko-endothelial, lymphocyte and hepatocyte origin, whose release is stimulated by LPS [36] are increased in plasma of patients with cirrhosis and correlate with their severity [37].

5. Immunometabolism Also Plays a Critical Role in ACLF

Under normal conditions, mammalian cells obtain the vast majority of energy from mitochondrial oxidative phosphorylation (OXPHOS), which combines electron transport with cell respiration and ATP synthesis [38]. However, upon inflammatory conditions, mitochondria become dysfunctional and cells shift from producing ATP by OXPHOS to aerobic glycolysis [38–40]. Aerobic glycolysis (also known as Warburg effect) ultimately produces lactate and generates 2 ATP molecules per glucose molecule, thus is less efficient than OXPHOS, which generates about 36 ATPs per each molecule of glucose [38–40]. An important aspect to consider is that lactate as end-product of glycolysis is secreted in high amounts by innate immune cells upon activation, and that this metabolite acts as a negative feedback to limit inflammation by decreasing cytokine production and migration of monocytes and macrophages [41,42]. Nevertheless, a clear disadvantage of glycolysis is that it highly depends on glucose as a sole fuel source, whereas mitochondrial OXPHOS has more metabolic flexibility and can use fatty acids and amino acids for example as carbon sources [40]. Patients with AD cirrhosis also exhibit increased blood levels of intermediates of the pentose phosphate pathway, which branches off from glycolysis at the first committed step of glucose metabolism, suggesting that cytosolic glucose metabolism through alternative routes to glycolysis is also common in these patients [43]. At early stages of injury, energy homeostasis is still attainable through diverting energy production from OXPHOS to glycolysis. However, this is a short-term solution because cells are unable to sustain high energy production from glycolysis at more advanced stages of chronic liver disease [44]. Furthermore, impaired OXPHOS is also linked to an enhanced production of reactive oxygen species (ROS) [45].

The prevailing metabolic alteration in patients with AD cirrhosis and ACLF is also characterized by intense proteolysis and lipolysis releasing high amounts of amino acids and fatty acids, respectively, as well as by severe amino acid catabolism [43,46]. Similar catabolic processes are observed in other critical illnesses associated with systemic inflammation and multiorgan failure, such as sepsis or trauma [47], pointing to the systemic hyperinflammatory state present in patients with AD cirrhosis as the origin of their metabolic alteration, although the contribution of microbiota and metabolites of microbial origin cannot be excluded [43,48]. Nevertheless, the finding that the higher the plasma levels of inflammatory markers, the higher the intensity of the metabolic alteration positions systemic inflammation as its major driving force [43]. The goal of the intense catabolic metabolism in patients with AD cirrhosis is to provide nutrients to the energetically expensive inflammatory response, which must face with the production of inflammatory mediators, immune cell proliferation and migration, respiratory burst, and production of acute-phase proteins [47]. This energetically expensive systemic inflammatory response requires reallocation of stored nutrients to fuel immune activation. To cope with this, immune cells compete for energy with other maintenance programs, including those ensuring proper functioning of peripheral organs. Ultimately, the energetic trade-off between immune activation and organ function homeostasis may lead to peripheral organ hypometabolism and organ dysfunction and failure in these patients.

6. Cells of the Innate Immune System: Role in ACLF

Ongoing studies are currently attempting to decipher the relative role of the innate versus the adaptive immune system in the pathogenesis of systemic inflammation in patients with AD cirrhosis evolving to ACLF. Although this dichotomy has not been resolved yet, plasma levels of cytokines involved in reshaping the adaptive immune system (i.e., IFN-γ, IFN-α2 and IL-17A) were not statistically different in patients with ACLF and patients with AD cirrhosis [4], suggesting that, similar to sepsis [49], the innate immune system plays a major contributory role to ACLF development. Consistent with this view, ACLF patients display increased leukocyte count compared to those with AD cirrhosis [1], in particular increased neutrophil and monocyte counts but accompanied by lymphopenia [50]. Information about other cell types of the innate immune system such as dendritic cells (DCs) and natural killer cells (NKs) is scarce, but decreased number of NK cells and attenuated function (i.e., cytotoxicity and killing activity) have been shown to predispose patients with HBV-related ACLF to infection [51]. Because of their predominance in the circulation and their primary role in protecting cirrhotic patients against secondary infections [52,53], in the following paragraphs we will focus our discussion on monocytes and neutrophils.

6.1. Mononuclear Phagocytes

Cirrhosis is commonly associated with the presence of monocytosis [54]. In 2005, before the existence of a widely accepted definition of ACLF, Wasmuth et al. had already described impaired antigen-presentation capacities and functional deactivation of monocytes from patients with AD liver cirrhosis [55]. In contrast to this finding, Albillos et al. observed signs of cellular activation in peripheral blood monocytes of patients with decompensation of cirrhosis, indicated by increased number of CD14+ cells expressing HLA-DR and CD80 co-stimulatory molecules, and increased spontaneous and LPS-stimulated TNFα-expression [56]. These seemingly contradictory findings might be explained by the high heterogeneity of the patients, with inclusion of patients displaying more severe decompensations requiring ICU admissions in the former study. Taken together, these two studies illustrate that the immune phenotype changes dynamically with disease progression, and that cross-sectional studies are only able to capture a snapshot.

More recent studies have further detailed that immunoparesis in ACLF is attributed to the expansion of MER receptor tyrosine kinase positive (MERTK+) monocytes (CD14+HLA-DR+MERTK+) [20], monocytic myeloid-derived suppressor cells (M-MDSCs) characterized by CD14+CD15-CD11b+HLA-DR- [21], and intermediate CD14++CD16+ monocytes [57]. MERTK+ monocytes have an impaired response to LPS stimulation and possibly contribute to secondary infections. Intermediate suppressive monocyte subsets are characterized by attenuated pro-inflammatory cytokine production in response to TLR2, TLR4 and TLR9 stimulation and higher production of anti-inflammatory/immunosuppressive cytokines such as IL-10 [20,21]. Functional alterations are also evident in classical CD14++CD16- monocytes of patients with ACLF. This subset of monocytes, which is considered to be highly phagocytic [58], exhibits reduced expression of TLR2 and TLR4 and more severely impaired phagocytic capacity and oxidative burst response compared to patients with AD cirrhosis [57]. The latter was found to be a result of diminished expression of interferon regulatory factor 8 (*IRF8*), a transcriptional activator of the oxidative burst response, which was only detected in ACLF but not in AD monocytes. In the same study, transcriptome analysis of the classical monocyte subset revealed upregulation of genes associated with immune dampening responses such as scavenger receptors (*CD163*, *MRC1*, *CD36*), suppressive cytokines (*IL-10*), chemokines (*CCL22*) as well as *MERTK*. Interestingly, the authors demonstrated that the phagocytic capacity of ACLF monocytes can be partially restored by targeting immunometabolism via inhibition of glutamine synthetase [57]. Another feature of monocytes from patients with AD cirrhosis is the downregulation of T-cell immunoglobulin domain and mucin domain-containing molecule-3 (Tim-3), most probably elicited by endotoxemia [59]. This finding was associated with a hypersensitive response to LPS challenge, decreased HLA-DR expression and reduced phagocytic activity. Alterations in monocyte function are clinically relevant, since persistently reduced HLA-DR

expression as a surrogate marker of monocyte dysfunction was correlated with secondary infection and mortality [21,60].

6.2. Neutrophils

Neutrophils account for 55–70% of all circulating white blood cells and are among the first line of immune cells to be recruited to the site of infection. Whereas cirrhotic patients often present neutropenia [61], patients with ACLF have higher absolute neutrophil counts compared to healthy controls [62], probably induced by higher circulating granulocyte colony-stimulating factor (G-CSF) levels [4]. Neutrophils in patients with advanced cirrhosis display high levels of activation markers such as CD11b, a β_2-integrin that mediates firm adhesion of neutrophils to cytokine-activated endothelium, and epidermal growth factor-like molecule containing mucin-like hormone receptor [63]. However, this is often combined with defects in their core functions, i.e., phagocytosis, respiratory burst and degranulation (exocytosis) [64]. These observations are suggestive of chronic intravascular activation of neutrophils, eventually leading to an exhausted phenotype.

In keeping with elevated molecules necessary for cell adhesion (e.g., VCAM-1, CD11b), and chemotactic signals such as IL-8 produced by immune cells, vascular endothelial cells and hepatocytes, neutrophils of patients with cirrhosis display increased adhesion to microvascular endothelium [65], but impaired ex vivo and in vivo transendothelial migration. The underlying mechanisms probably involve attenuated IL-33/ST2 signaling [66] as well as diminished levels of CD62L (L-selectin) due to activation-induced shedding [65].

It is also long-known that neutrophils from patients with alcoholic cirrhosis (active alcoholism is the second most frequent trigger of ACLF) display reduced phagocytic capacity towards Gram-positive and Gram-negative bacteria [67]. The reduced phagocytic capacity of neutrophils is independent of cirrhosis etiology [62]. In terms of ROS production, which is needed for respiratory burst, neutrophils of patients with ACLF produce higher basal levels of ROS, indicating a primed state of neutrophils [62]. On the other hand, ROS production upon fMet-Leu-Phe (fMLP)-stimulation in patients with alcoholic cirrhosis is reduced [68]. The diminished production of ROS was attributed to markedly reduced phospholipase C activity [69], deficient phosphorylation [64] as well as decreased baseline protein expression of components of the NADPH oxidase complex [68]. The latter could be explained by a defective mTOR-dependent translational machinery and degradation of gp 91phox/NOX2 through plasma elastase, which is present in high levels in plasma of patients with advanced cirrhosis. Together with defective myeloperoxidase exocytosis, which is possibly a consequence of decreased activation of AKT and p38-MAP-kinase, impaired ROS production contributes to deficient bactericidal activity [64,67].

Apart from impaired bacterial killing, CD11b+CD16+ neutrophils of patients with ACLF overexpress CXCR1 and CXCR2, the chemokine receptors recognizing IL-8, and induce hepatocyte death in vitro by direct contact and by release of inflammatory mediators. This finding provides another link between impaired neutrophil function and immunopathology [62], whereby dying hepatocytes release DAMPs which further activate the innate immune system. Neutrophil dysfunction is highly clinically relevant, as impaired respiratory burst and phagocytic activity correlate with higher risk of organ failure and mortality [70,71]. Elevated neutrophil-to-lymphocyte ratio, which can be easily calculated, predicts poor survival in patients with ACLF and proved to be higher in the ACLF than AD group [72].

6.3. Macrophages

Since ACLF is predominantly a systemic disease, information on immune cells such as macrophages residing in tissue and organs is scarce compared to their circulatory counterparts. This is mostly true for extrahepatic organs although there is some direct evidence on the role of macrophages in the liver. For instance, it has been reported that an expansion of hepatic macrophages expressing high levels of MERTK and CD163 occurs in ACLF patients undergoing liver transplantation [20,57]. These authors also provide evidence that MERTK+ macrophages accumulate in mesenteric lymph nodes, where these

macrophages are phenotypically MERTKhigh/CD163high/HLA-DRhigh and functionally endotoxin tolerant. Furthermore, since soluble CD163, which is shed by activated macrophages, increases with severity of ACLF and predicts mortality, it was proposed that Kupffer cells, the liver resident macrophages which constitutively express high levels of scavenger receptors and IL-10, play an important role in the development and course of ACLF [73].

7. Mediators of Inflammation in ACLF

The dysfunctional innate immune system in ACLF patients is characterized by increased circulating levels of small proteins (cytokines, chemokines and growth factors) and lipids (bioactive lipid mediators) that signal for exuberant inflammatory and immune responses.

7.1. Cytokines

Cytokines are low-molecular-weight glycoproteins that orchestrate the effectiveness of innate immunity by inducing local inflammation and systemic acute responses [74,75]. The production of cytokines by leukocytes is one of the initial steps of the inflammatory cascade. Once released, cytokines bind specific receptors in their target cells [75–77]. Although leukocytes are the major source of cytokines, parenchymal cells are increasingly recognized to also produce inflammatory cytokines and to interact with leukocytes to optimize immune responses [78]. Cytokines are also important in initiating, amplifying and mediating adaptive immunity [79]. Cytokines can be classified in different families including TNF, IL-6 families and interferon (α, β, and γ) families. Cytokines can also be categorized according to their role as pro-inflammatory or anti-inflammatory. TNF-α, IL-1β and IL-6 are well-characterized as pro-inflammatory cytokines, whereas IL-4, IL-10 and IL-1 receptor antagonist (IL-1ra) are considered anti-inflammatory [79]. The presence of increased circulating levels of TNFα and IL-6 in patients with cirrhosis without infections were described decades ago [80,81], but comprehensive characterization of these inflammatory mediators in AD cirrhosis and ACLF and their correlation with disease severity and organ failures were described more recently [4,5]. Circulating levels of cytokines in patients with ACLF are of similar magnitude to those reported in patients with sepsis. Therefore, the term "cytokine storm", which defines the exacerbated production of these mediators as consequence of overactivated immune system accompanied by systemic inflammation, commonly observed in sepsis-like diseases [59] is also pertinent to ACLF.

IL-6 is a pleiotropic cytokine produced in response to infections and tissue injuries. IL-6 synthesis and secretion is induced upon stimulation of TLR4 by LPS, IL-1β or TNF-α and is one of the major stimuli for the release of hepatic acute phase proteins. IL-6 strongly correlates with the development of renal impairment and mortality in patients with cirrhosis and bacterial peritonitis [82]. The inhibition of IL-6 for the prevention or treatment of inflammatory diseases has been extensively approached [83]. For example, the neutralization of IL-6 signal by using the humanized anti-IL-6 receptor antibody tocilizumab, has been used in patients with systemic hyperinflammatory response including sepsis and macrophage activation syndrome in rheumatoid arthritis [84,85]. However, tocilizumab long-term trials have shown significant increments of transaminase levels requiring the need for regular monitorization and dose adjustments [86]. More recently, tocilizumab in combination with remdesivir, chloroquine and others has been repurposed for the treatment of the cytokine release syndrome in cirrhotic patients with Covid-19 [87].

7.2. Chemokines

Chemokines consists of a superfamily of chemoattractant-related ligands and receptors that participate in the regulation of the immune system and inflammatory responses [88]. Based on the structural criteria, chemokines are classified into four subfamilies: CXC, CC, (X)C, and a single member of the CX3C subfamily (CX3CL1 or fractalkine). The pro-inflammatory chemokine CXCL8 (also known as IL-8) is produced by liver cells, including hepatocytes, stellate cells, and Kupffer cells and serves as

predictor of mortality in ACLF [62]. Of interest, circulating CXCL10 levels have been shown to predict ACLF development and survival in cirrhotic patients with portal hypertension receiving TIPS [29].

7.3. Growth Factors

Growth factors such as granulocyte-macrophage colony stimulating factor (GM-CSF) and G-CSF are implicated in hematopoiesis and proliferation of hepatic progenitor cells in liver failure [89]. Indeed, G-CSF has been considered for the treatment of ACLF with improvement of liver function and survival [90] (see below). The transforming growth factor β (TGF-β) is also associated with ACLF severity and survival and increased levels have been reported in non-survivor patients with hepatitis B infection [91].

7.4. Lipid Mediators

Lipid mediators are bioactive lipids generated from structural lipid species (i.e., phospholipids containing polyunsaturated fatty acids (PUFA)), which compose the lipid bilayer of cell membranes [92]. Most lipid mediators are derived from omega-6 and omega-3 PUFAs, which are released on demand in response to an inflammatory stimulus from cell membrane into the cytosol by phospholipase A_2 [93,94]. In the cytosol, PUFA are readily converted by cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) enzymatic pathways into an array of biologically active lipid mediators, which are released to exert their actions as autacoids [93,95]. The essential omega-6 PUFA arachidonic acid (AA) is the major substrate for the intracellular biosynthesis of eicosanoids. The eicosanoid family consists of PGs, thromboxane A_2 (TXA_2), LTs, lipoxins (LXs) and epoxyeicosatrienoic acids (EETs). With the exception of LXs, the majority of eicosanoids have pro-inflammatory properties [96] and in fact, PGs and TXA_2 are the prime targets for non-steroidal anti-inflammatory drugs (NSAIDs) [97]. Similar to cytokines, eicosanoids are massively released by leukocytes in response to infections or tissue injury originating the so-called "eicosanoid storm" [92]. In contrast to AA, the omega-3 PUFAs eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids are converted by the COX, LOX and CYP pathways into potent anti-inflammatory lipid mediators [98]. These mediators are generically known as "specialized pro-resolving mediators" or SPM (i.e., resolvins, protectins and maresins), which have attracted much attention in recent years because they do not only act as 'braking signals' of unremitting inflammation, but also play critical roles in the dynamic resolution of tissue inflammation [97].

Little is known about the role of lipid mediators in ACLF. O'Brien et al. provided evidence that PGE_2 drives immunosuppresion and increases the risk of infection in AD cirrhosis [22]. Subsequently, China et al. described that ACLF patients can be differentially categorized into two lipid mediator phenotypes (i.e., hyperactivated and hypoactivated) in their therapeutic response to human serum albumin (HSA) infusions [99]. The same authors reported that patient survival was associated with a shifted profile in the levels of SPMs [100]. More recently, our laboratory performed targeted analysis of 100 bioactive lipid mediators in more than 200 patients with AD cirrhosis with and without ACLF [101]. This study revealed elevated levels of pro-inflammatory and vasoconstrictor eicosanoids including LTE_4 and $PGF_{2\alpha}$, in parallel with decreased levels of the pro-resolving SPM LXA_5 in patients with ACLF [101] (Figure 1). In these patients, LTE_4 levels were strongly correlated with IL-8 and the necrosis/apoptosis marker K18, whereas the pro-resolving LXA_5 had a negative correlation with inflammation and cell death [101]. On the other hand, some lipid mediators derived from linoleic acid, 9(10)-epoxy-9Z-octadecenoic acid (EpOME) and 12(13)-EpOME, which are indicators of effective bactericidal activity, were remarkably suppressed in ACLF patients [101] (Figure 1). Together, these data suggest that systemic inflammation in ACLF can be driven by an imbalance formation between pro-inflammatory and anti-inflammatory/pro-resolving lipid mediators.

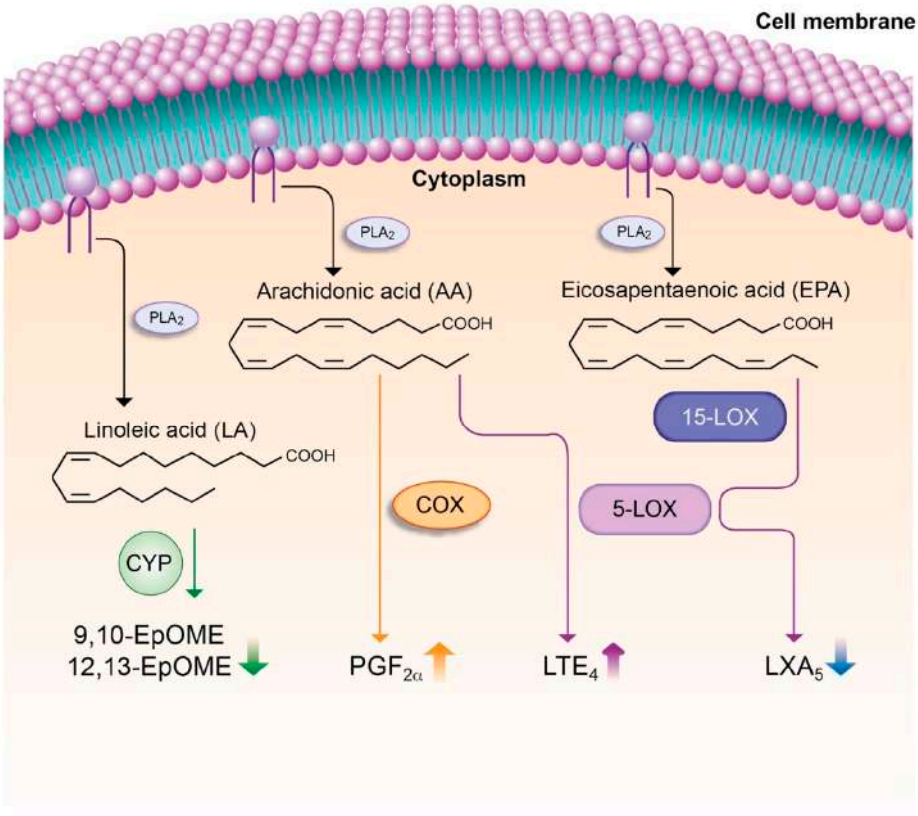


Figure 1. Imbalanced formation between pro-inflammatory and anti-inflammatory lipid mediators in leukocytes from patients with AD cirrhosis and ACLF. In response to inflammatory stimulus, omega-6 (linoleic acid (LA) and arachidonic acid (AA)) and omega-3 (eicosapentaenoic acid (EPA)) polyunsaturated fatty acids (PUFA) are released from cell membrane phospholipids into the cytosol by phospholipase A_2 (PLA_2). In the cytosol, unesterified free PUFA serve as available substrates for cyclooxygenase (COX), lipoxygenase (5-LOX/15-LOX) and cytochrome P450 (CYP) enzymatic pathways, which generate an array of bioactive lipid mediators. Patients with AD cirrhosis and ACLF present elevated levels of AA-derived pro-inflammatory and vasoconstrictor lipid mediators such as LTE_4 and $PGF_{2\alpha}$, in parallel with decreased levels of the EPA-derived product LXA_5, which is an anti-inflammatory and pro-resolving lipid mediator. In addition, the CYP-products derived from LA, 9,10-EpOME and 12,13-EpOME, which are produced by leukocytes during oxidative burst, were remarkably suppressed in ACLF patients, indicating poor bactericidal activity.

8. Therapeutic Approaches to Limit Systemic Inflammation in ACLF

The most effective therapy for ACLF patients is liver transplantation. However, the availability of suitable organ donors is a major limitation. Therefore, finding a cure to prevent organ failures by limiting excessive systemic inflammation without inducing immunosuppression is an unmet need in patients with AD cirrhosis at risk of developing ACLF. At present, short- and long-term infusions of HSA are one of the few approved systemic therapies for the prevention of paracentesis-induced circulatory dysfunction and development of ascites and hepatorenal syndrome during an episode of spontaneous bacterial peritonitis (SBP) in patients with AD cirrhosis [102,103]. The therapeutic use of HSA in these patients was recently corroborated in a multicenter, randomized study, in which the long-term prophylactic administration of HSA was effective in reducing hospital readmissions and prolonging survival [104]. More recently, the pilot-PRECIOSA study aimed at identifying the optimal HSA dosage able to normalize HSA concentrations in patients with AD cirrhosis, and the randomized controlled INFECIR-2 study aimed at comparing the efficacy of adding HSA to standard medical therapy with

antibiotics in patients with AD cirrhosis and active non-SBP bacterial infection, have demonstrated that both short- and long-term HSA treatments induce significant systemic immunomodulatory effects [105]. Specifically, HSA was shown to act as a disease-modifying anti-inflammatory agent with the ability to reduce the circulating levels of inflammatory cytokines in patients with AD cirrhosis [105]. Previously, O'Brien et al. had demonstrated that HSA was able to revert PGE_2-induced immune dysfunction in patients with AD cirrhosis and ACLF [22]. This laboratory also demonstrated that plasma IL-4 levels served as a good marker of improvement in the degree of systemic inflammation after HSA treatment in these patients [100]. Other therapies based on HSA but alternative to infusions are extracorporeal liver support systems based on albumin dialysis or plasma exchange. This is the case of recent studies showing that patients treated with extracorporeal liver support systems had improved survival [106–108].

Although traditionally the therapeutic effects of HSA in patients with AD cirrhosis were attributed to its oncotic, antioxidant and scavenging properties [103], new mechanisms have been recently described that contribute to understand the immunomodulatory and anti-inflammatory properties of the albumin molecule. In particular, ex vivo experiments in isolated leukocytes from patients with AD and ACLF have provided evidence that HSA abolish cytokine expression and release induced by bacterial DNA rich in unmethylated CpG-DNA [109]. These anti-inflammatory effects of HSA were independent of its oncotic and scavenging properties and were reproduced by incubating the leukocytes with recombinant human albumin. In addition, HSA exerted widespread changes on the leukocyte transcriptome, specifically in genes related to the endosomal compartment involved in cytosolic DNA sensing and type I interferon responses. Consistent with this, flow cytometry and confocal microscopy analyses revealed that HSA was taken up by leukocytes and internalized in endosomes, the compartment where CpG-DNA binds to TLR9, its cognate receptor (Figure 2). In this compartment, HSA also inhibited poly(I:C)- and LPS-induced interferon regulatory factor 3 (IRF3) phosphorylation and TIR-domain-containing adapter-inducing interferon-β (TRIF)-mediated responses, which are exclusive of endosomal TLR3 and TLR4 signaling, respectively (Figure 2). Importantly, the immunomodulatory actions of HSA did not compromise leukocyte defensive mechanisms such as phagocytosis, efferocytosis and intracellular ROS production, and thus appear to not induce immunosuppression. Together, these findings indicate that similar to that reported for other cell types such as hepatocytes and endothelial cells, albumin internalizes in leukocytes and modulates the responses to PAMPs through interaction with endosomal TLR signaling pathways [109].

Other therapies currently explored to treat excessive systemic inflammation and restore the immunological response in patients with AD cirrhosis and ACLF are G-CSF, TLR4 antagonists, IL-22 and stem cell therapy. Therapy with G-CSF, which mobilizes bone marrow stem cells and impacts on hepatocyte proliferation as it also acts as a growth factor, has been tested in patients with ACLF in four randomized clinical trials [110–112] including the GRAFT study, which was stopped after interim analysis because lack of additional effects to standard medical treatment [113]. In any case, administration of G-CSF subcutaneously was shown to be safe in patients with ACLF, to increase the number of CD34+ cells and to promote hepatic regeneration. Additionally, a recent study has demonstrated that autologous infusion of G-CSF-induced CD34+ cells effectively improves liver function and HSA levels up to one year although this benefit was not sustained at the long-term [114]. Moreover, G-CSF treatment has been shown to prevent hepatorenal syndrome, hepatic encephalopathy and sepsis [110,112]. Another therapeutic target in ACLF is TLR4, which plays an essential role in mediating organ injury in this condition. Recently, TAK-242, TLR4 antagonist was experimentally tested in vivo in different rodent models of ACLF and in vitro in monocyte and hepatocyte cell lines. The results obtained indicated that in addition to reduce cytokine levels and hepatocyte cell death, TAK-242 was able to increase survival in mice experimentally induced to ACLF [115]. A recent study in a mouse model mimicking the key features of ACLF has provided evidence that IL-22 treatment reprograms impaired regenerative pathways and protects against bacterial infection [116], although significant discrepancies between the data reported by these authors and data reported earlier

in human ACLF have been raised [24]. Finally, stem cell therapy has been tested in two open-label controlled studies in patients with ACLF, who received umbilical cord-derived mesenchymal stem cells (MSC) [117] or allogenic bone marrow-derived MSC [118]. In these studies, MSC infusions increased survival rate, improved liver function and increased serum albumin, although none of them addressed the effects on systemic inflammation.

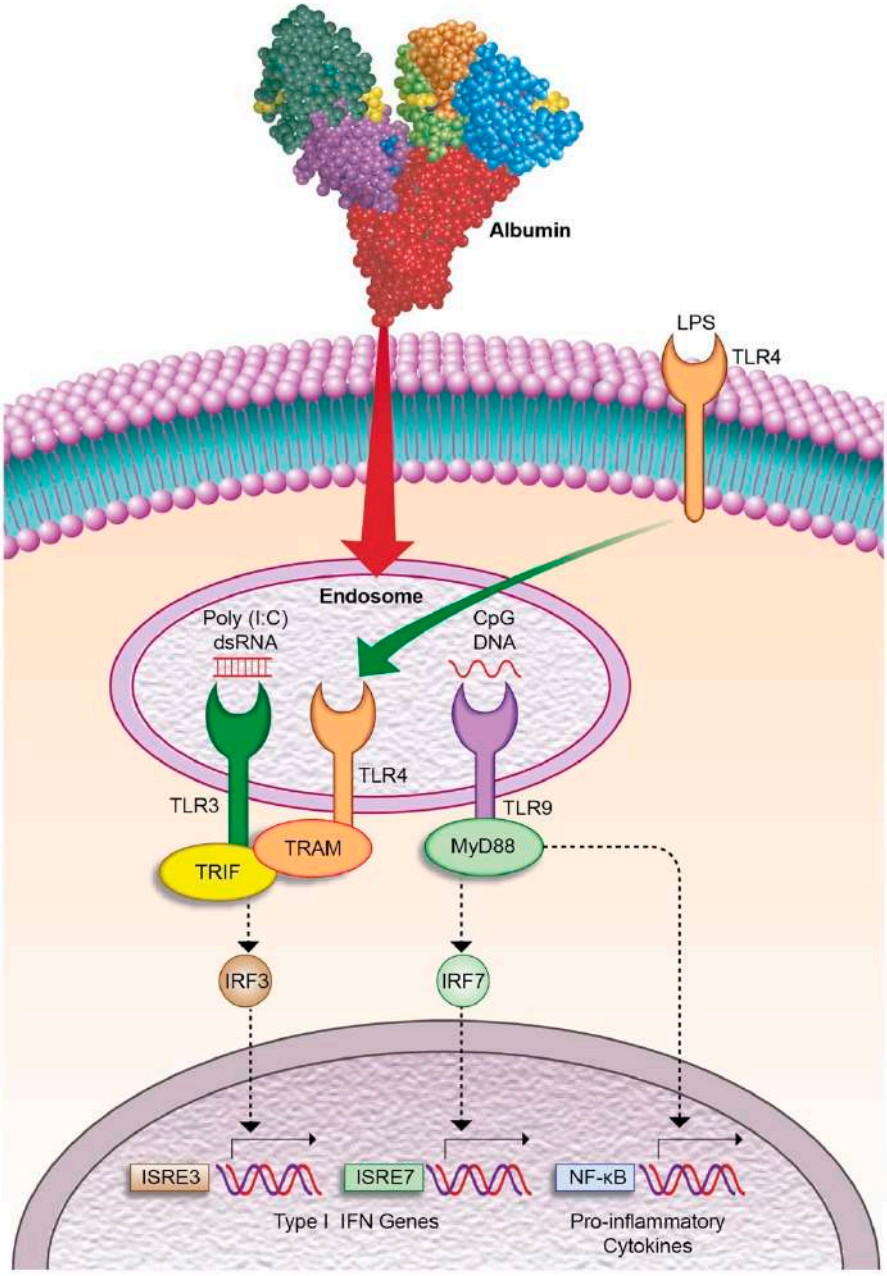


Figure 2. Human serum albumin (HSA) exerts immunomodulatory effects in leukocytes by blocking Toll-like receptor (TLR) signaling pathways in the endosomal compartment. HSA is internalized by leukocytes and in the endosomes inhibits inflammatory cytokine production induced by bacterial single-strand CpG-DNA, which binds to its cognate receptor TLR9 and triggers the signaling by recruitment of myeloid differentiation primary response gene 88 (MyD88). HSA also inhibits other endosomal TLRs such as TLR3, which is activated by double-strand RNA (i.e., poly (I:C)) and TLR4, which, after binding to LPS, translocates to the endosome. Both TRL3 and TLR4 signal via TIR-domain-containing adapter-inducing interferon-β (TRIF), which mediates type I interferon (IFN) responses.

9. Conclusions

The ACLF syndrome represents a new paradigm among the diseases characterized by the presence of an excessive systemic inflammatory response leading to organ failure(s). The ACLF syndrome thus is a suitable disease condition to investigate the mechanisms underlying systemic inflammation and tissue damage. At present, little is known about the individual participation of each immune cell type to this process, but ongoing studies using cutting edge technologies such as single blood cell-RNA seq will likely provide new insights soon. The relative contribution of the innate and adaptive immune systems to systemic inflammation and immunopathology in patients with AD cirrhosis evolving to ACLF also needs to be elucidated. Future studies are also needed for the identification of the triggers of the systemic inflammatory response that leads to end-organ dysfunction in the context of ACLF. Finally, it is of utmost importance to find an appropriate intervention that would reduce inflammation without inducing immunosuppression to these patients.

Author Contributions: Conceptualization and design, J.C. and C.L.-V.; writing review and editing, M.C., I.W.Z., C.L.-V. and J.C. All authors have read and agreed to the published version of the manuscript.

Funding: Our laboratory is a Consolidated Research Group recognized by the Generalitat de Catalunya (2017SGR1449) and is supported by the Spanish Ministerio de Ciencia e Innovacion (PID2019-105240RB-I00), the European Union's Horizon 2020 research and innovation program (grant agreements N° 825694 and 847949) and by EF Clif, a non-profit private organization that receives unrestricted donations from Cellex Foundation and Grifols. Ingrid W. Zhang is supported by the Sheila Sherlock Post Graduate Programme of the European Association for the Study of the Liver (EASL).

Conflicts of Interest: The authors declare no competing interest.

References

1. Moreau, R.; Jalan, R.; Gines, P.; Pavesi, M.; Angeli, P.; Cordoba, J.; Durand, F.; Gustot, T.; Saliba, F.; Domenicali, M.; et al. Acute-on-chronic liver failure is a distinct syndrome that develops in patients with acute decompensation of cirrhosis. *Gastroenterology* **2013**, *144*, 1426–1437. [CrossRef]
2. Arroyo, V.; Moreau, R.; Kamath, P.S.; Jalan, R.; Ginès, P.; Nevens, F.; Fernández, J.; To, U.; García-Tsao, G.; Schnabl, B. Acute-on-chronic liver failure in cirrhosis. *Nat. Rev. Dis. Prim.* **2016**, *2*, 1–18. [CrossRef]
3. Arroyo, V.; Moreau, R.; Jalan, R. Acute-on-Chronic Liver Failure. *N. Engl. J. Med.* **2020**, *382*, 2137–2145. [CrossRef]
4. Clària, J.; Stauber, R.E.; Coenraad, M.J.; Moreau, R.; Jalan, R.; Pavesi, M.; Amorós, À.; Titos, E.; Alcaraz-Quiles, J.; Oettl, K.; et al. Systemic inflammation in decompensated cirrhosis: Characterization and role in acute-on-chronic liver failure. *Hepatology* **2016**, *64*, 1249–1264. [CrossRef]
5. Trebicka, J.; Amoros, A.; Pitarch, C.; Titos, E.; Alcaraz-quiles, J.; Schierwagen, R.; Deulofeu, C.; Fernandez-Gomez, J.; Piano, S.; Caraceni, P.; et al. Addressing Profiles of Systemic Inflammation Across the Different Clinical Phenotypes of Acutely Decompensated Cirrhosis. *Front. Immunol.* **2019**, *10*, 476. [CrossRef]
6. Van Der Poll, T.; Van De Veerdonk, F.L.; Scicluna, B.P.; Netea, M.G. The immunopathology of sepsis and potencial therapeutic targets. *Nat. Immunol.* **2017**, *17*, 407–420. [CrossRef]
7. Fernández, J.; Acevedo, J.; Wiest, R.; Gustot, T.; Amoros, A.; Deulofeu, C.; Reverter, E.; Martínez, J.; Saliba, F.; Jalan, R.; et al. Bacterial and fungal infections in acute-on-chronic liver failure: Prevalence, characteristics and impact on prognosis. *Gut* **2018**, *67*, 1870–1880. [CrossRef]
8. Wiest, R.; Lawson, M.; Geuking, M. Pathological bacterial translocation in liver cirrhosis. *J. Hepatol.* **2014**, *60*, 197–209. [CrossRef]
9. Chen, P.; Stärkel, P.; Turner, J.R.; Ho, S.B.; Schnabl, B. Dysbiosis-induced intestinal inflammation activates TNFRI and mediates alcoholic liver disease in mice. *Hepatology* **2015**, *61*, 883–894. [CrossRef]
10. Bianchi, M.E. DAMPs, PAMPs and alarmins: All we need to know about danger. *J. Leukoc. Biol.* **2007**, *81*, 1–5. [CrossRef]
11. Takeuchi, O.; Akira, S. Pattern Recognition Receptors and Inflammation. *Cell* **2010**, *140*, 805–820. [CrossRef] [PubMed]
12. Aizawa, S.; Brar, G.; Tsukamoto, H. Cell Death and Liver Disease. *Gut Liver* **2020**, *14*, 20–29. [CrossRef]

13. Schaapman, J.J.; Amoros, À.; Van Der Reijden, J.J.; Laleman, W.; Zeuzem, S.; Bañares, R.; Jalan, R.; Arroyo, V.; Clària, J.; Verspaget, H.W.; et al. Genetic variants of innate immunity receptors are associated with mortality in cirrhotic patients with bacterial infection. *Liver Int.* **2020**, *40*, 646–653. [CrossRef] [PubMed]
14. Alcaraz-Quiles, J.; Titos, E.; Casulleras, M.; Pavesi, M.; López-Vicario, C.; Rius, B.; Lopategi, A.; de Gottardi, A.; Graziadei, I.; Gronbaek, H.; et al. Polymorphisms in the IL-1 gene cluster influence systemic inflammation in patients at risk for acute-on-chronic liver failure. *Hepatology* **2017**, *65*, 216–220. [CrossRef] [PubMed]
15. De Meo, A.N.; Andersen, B.R.; English, D.K.; Peterson, J. Defective Chemotaxis Associated with a Serum Inhibitor in Cirrhotic Patients. *N. Engl. J. Med.* **1972**, *286*, 735–740. [CrossRef] [PubMed]
16. Li, Q.; Lu, Q.; Zhu, M.; Huang, C.; Yu, K.; Huang, Y.; Zhao, X.; Luo, X.-G.; Zheng, J.-M. Lower level of complement component C3 and C3a in the plasma means poor outcome in the patients with hepatitis B virus related acute-on-chronic liver failure. *BMC Gastroenterol.* **2020**, *20*, 1–9. [CrossRef]
17. Bernardi, M.; Maggioli, C.; Zaccherini, G. Human albumin in the management of complications of liver cirrhosis. *Crit. Care* **2012**, *16*, 211. [CrossRef] [PubMed]
18. Homann, C.; Varming, K.; Hogasen, K.; Mollnes, T.E.; Graudal, N.; Thomsen, A.C.; Garred, P. Acquired C3 deficiency in patients with alcoholic cirrhosis predisposes to infection and increased mortality. *Gut* **1997**, *40*, 544–549. [CrossRef]
19. Jenne, C.N.; Kubes, P. Immune surveillance by the liver. *Nat. Immunol.* **2013**, *14*, 996–1006. [CrossRef]
20. Bernsmeier, C.; Pop, O.T.; Singanayagam, A.; Triantafyllou, E.; Patel, V.C.; Weston, C.J.; Curbishley, S.; Sadiq, F.; Vergis, N.; Khamri, W.; et al. Patients With Acute-on-Chronic Liver Failure Have Increased Numbers of Regulatory Immune Cells Expressing the Receptor Tyrosine Kinase MERTK. *Gastroenterology* **2015**, *148*, 603–615. [CrossRef]
21. Bernsmeier, C.; Triantafyllou, E.; Brenig, R.; Lebosse, F.J.; Singanayagam, A.; Patel, V.C.; Pop, O.T.; Khamri, W.; Nathwani, R.; Tidswell, R.; et al. CD14+ CD15− HLA-DR− myeloid-derived suppressor cells impair antimicrobial responses in patients with acute-on-chronic liver failure. *Gut* **2018**, *67*, 1155–1167. [CrossRef] [PubMed]
22. O'Brien, A.J.; Fullerton, J.N.; Massey, K.A.; Auld, G.; Sewell, G.; James, S.; Newson, J.; Karra, E.; Winstanley, A.; Alazawi, W.; et al. Immunosuppression in acutely decompensated cirrhosis is mediated by prostaglandin E2. *Nat. Med.* **2014**, *20*, 518–523. [CrossRef] [PubMed]
23. Xing, T.; Li, L.; Cao, H.; Huang, J. Altered immune function of monocytes in different stages of patients with acute on chronic liver failure. *Clin. Exp. Immunol.* **2006**, *147*, 184–188. [CrossRef] [PubMed]
24. Trebicka, J.; Fernandez, J.; Papp, M.; Caraceni, P.; Laleman, W.; Gambino, C.; Giovo, I.; Uschner, F.E.; Jimenez, C.; Mookerjee, R.; et al. The PREDICT study uncovers three clinical courses of acutely decompensated cirrhosis that have distinct pathophysiology. *J. Hepatol.* **2020**, *73*, 842–854. [CrossRef]
25. Garcia-Tsao, G.; Albillos, A.; Barden, G.E.; West, A.B. Bacterial translocation in acute and chronic portal hypertension. *Hepatology* **1993**, *17*, 1081–1085. [CrossRef]
26. Lozano-ruiz, B.; Bachiller, V.; García-martínez, I.; Zapater, P.; Gómez-hurtado, I.; Moratalla, A.; Giménez, P.; Bellot, P.; Francés, R.; Such, J.; et al. Absent in melanoma 2 triggers a heightened inflammasome response in ascitic fluid macrophages of patients with cirrhosis. *J. Hepatol.* **2015**, *62*, 64–71. [CrossRef]
27. Michelena, J.; Altamirano, J.; Abraldes, J.G.; Affò, S.; Morales-Ibanez, O.; Sancho-Bru, P.; Dominguez, M.; García-Pagán, J.C.; Fernández, J.; Arroyo, V.; et al. Systemic inflammatory response and serum lipopolysaccharide levels predict multiple organ failure and death in alcoholic hepatitis. *Hepatology* **2015**, *62*, 762–772. [CrossRef]
28. Berres, M.; Asmacher, S.; Lehmann, J.; Jansen, C.; Görtzen, J.; Klein, S.; Meyer, C.; Strunk, H.M.; Fimmers, R.; Tacke, F.; et al. CXCL9 is a prognostic marker in patients with liver cirrhosis receiving transjugular intrahepatic portosystemic shunt. *J. Hepatol.* **2015**, *62*, 332–339. [CrossRef]
29. Lehmann, J.M.; Claus, K.; Jansen, C.; Pohlmann, A.; Schierwagen, R.; Meyer, C.; Thomas, D.; Manekeller, S.; Clària, J.; Strassburg, C.P.; et al. Circulating CXCL10 in cirrhotic portal hypertension might reflect systemic inflammation and predict ACLF and mortality. *Liver Int.* **2018**, *38*, 875–884. [CrossRef]
30. Levi, M.; Keller, T.T.; van Gorp, E.; ten Cate, H. Infection and inflammation and the coagulation system. *Cardiovasc. Res.* **2003**, *60*, 26–39. [CrossRef]
31. Coughlin, S.R. Thrombin signalling and protease-activated receptors. *Nature* **2000**, *407*, 258–264. [CrossRef] [PubMed]

32. Plessier, A.; Denninger, M.-H.; Consigny, Y.; Pessione, F.; Francoz, C.; Durand, F.; Francque, S.; Bezeaud, A.; Chauvelot-Moachon, L.; Lebrec, D.; et al. Coagulation disorders in patients with cirrhosis and severe sepsis. *Liver Int.* **2003**, *23*, 440–448. [CrossRef] [PubMed]
33. Allegretti, A.S.; Parada, X.V.; Ortiz, G.A.; Long, J.; Krinsky, S.; Zhao, S.; Fuchs, B.C.; Sojoodi, M.; Zhang, D.; Karumanchi, S.A.; et al. Serum Angiopoietin-2 Predicts Mortality and Kidney Outcomes in Decompensated Cirrhosis. *Hepatology* **2019**, *69*, 729–741. [CrossRef] [PubMed]
34. Solé, C.; Solà, E.; Morales-ruiz, M.; Fernàndez, G.; Huelin, P.; Graupera, I.; Moreira, R.; de Prada, G.; Ariza, X.; Pose, E.; et al. Characterization of Inflammatory Response in Acute-on-Chronic Liver Failure and Relationship with Prognosis. *Sci. Rep.* **2016**, *6*, 32341. [CrossRef] [PubMed]
35. Amalakuhan, B.; Habib, S.A.; Mangat, M.; Reyes, L.F.; Rodriguez, A.H.; Hinojosa, C.A.; Soni, N.J.; Gilley, R.P.; Bustamante, C.A.; Anzueto, A.; et al. Endothelial adhesion molecules and multiple organ failure in patients with severe sepsis. *Cytokine* **2016**, *88*, 267–273. [CrossRef]
36. Gauley, J.; Pisetsky, D.S. The release of microparticles by RAW 264.7 macrophage cells stimulated with TLR ligands. *J. Leukoc. Biol.* **2010**, *87*, 1115–1123. [CrossRef]
37. Rautou, P.; Bresson, J.; Sainte-Marie, Y.; Vion, A.; Paradis, V.; Renard, J.M.; Devue, C.; Heymes, C.; Letteron, P.; Elkrief, L.; et al. Abnormal Plasma Microparticles Impair Vasoconstrictor Responses in Patients with Cirrhosis. *Gastroenterology* **2012**, *143*, 166–176. [CrossRef]
38. Lodish, H.; Berk, A.; Zipursky, S.L.; Matsudaira, P.; Baltimore, D.; Darnell, J. *Molecular Cell Biology*, 4th ed.; W.H. Free: New York, NY, USA, 2000; ISBN 0-7167-3136-3.
39. Wang, A.; Luan, H.; Medzhitov, R. An Evolutionary Perspective on Immunometabolism. *Science* **2019**, *363*, 6423. [CrossRef]
40. Mills, E.L.; Kelly, B.; O'Neill, L.A.J. Mitochondria are the powerhouses of immunity. *Nat. Immunol.* **2017**, *18*, 488–498. [CrossRef]
41. Goetze, K.; Walenta, S.; Ksiazkiewicz, M.; Kunz-schughart, L.A.; Mueller-klieser, W. Lactate enhances motility of tumor cells and inhibits monocyte migration and cytokine release. *Int. J. Oncol.* **2011**, *39*, 453–463. [CrossRef]
42. Ratter, J.M.; Rooijackers, H.M.M.; Hooiveld, G.J.; Hijmans, A.G.M.; de Galan, B.E.; Tack, C.; Stienstra, R. In vitro and in vivo Effects of Lactate on Metabolism and Cytokine Production of Human Primary PBMCs and Monocytes. *Front. Immunol.* **2018**, *9*, 2564. [CrossRef] [PubMed]
43. Moreau, R.; Clària, J.; Aguilar, F.; Fenaille, F.; Lozano, J.J.; Junot, C.; Colsch, B.; Caraceni, P.; Trebicka, J.; Pavesi, M.; et al. Blood metabolomics uncovers inflammation-associated mitochondrial dysfunction as a potential mechanism underlying ACLF. *J. Hepatol.* **2020**, *72*, 688–701. [CrossRef] [PubMed]
44. Nishikawa, T.; Bellance, N.; Damm, A.; Bing, H.; Zhu, Z.; Yovchev, M.I.; Sehgal, V.; Moss, T.J.; Oertel, M.; Pipinos, I.I.; et al. A switch in the source of ATP production and a loss in capacity to perform glycolysis are hallmarks of hepatocyte failure in advance liver disease. *J. Hepatol.* **2014**, *60*, 1203–1211. [CrossRef] [PubMed]
45. Chan, D.C. Mitochondria: Dynamic Organelles in Disease, Aging, and Development. *Cell* **2006**, *125*, 1241–1252. [CrossRef] [PubMed]
46. Zaccherini, G.; Aguilar, F.; Caraceni, P.; Clària, J.; Lozano, J.; Fenaille, F. Addressing the role of amino acids in systemic inflammatory responses and organ failures in patients with ACLF. *J. Hepatol.* **2020**, in press. [CrossRef]
47. Van Wyngene, L.; Vandewalle, J.; Libert, C. Reprogramming of basic metabolic pathways in microbial sepsis: Therapeutic targets at last? *EMBO Mol. Med.* **2018**, *10*, 1–18. [CrossRef]
48. Bajaj, J.S.; Reddy, K.R.; Leary, J.G.O.; Vargas, H.E.; Lai, J.C.; Kamath, P.S.; Tandon, P.; Wong, F.; Subramanian, R.M.; Thuluvath, P.; et al. Serum Levels of Metabolites Produced by Intestinal Microbes and Lipid Moieties Independently Associated With Acute-on-Chronic Liver Failure and Death in Patients With Cirrhosis. *Gastroenterology* **2020**, *159*, 1715–1730. [CrossRef]
49. Hotchkiss, R.S.; Monneret, G.; Payen, D. Sepsis-induced immunosuppression: From cellular dysfunctions to immunotherapy. *Nat. Rev. Immunol.* **2013**, *13*, 862–874. [CrossRef]
50. Weiss, E.; de la Grange, P.; Defaye, M.; Lozano, J.J.; Aguilar, F.; Hedge, P.; Jolly, A.; Moga, L.; Baweja, S.; Agarwal, B.; et al. RNA Identification of Dysregulated Blood Immune Cells Playing a Pathophysiological Role in Critically Ill Patients With ACLF. *Front. Immunol.* **2020**, in press.
51. Liu, F.; Duan, X.; Wan, Z.; Zang, H.; You, S.; Yang, R.; Liu, H.; Li, D.; Li, J.; Zhang, Y.; et al. Lower number and decreased function of natural killer cells in hepatitis B virus related acute-on-chronic liver failure. *Clin. Res. Hepatol. Gastroenterol.* **2016**, *40*, 605–613. [CrossRef]

52. Stephan, F.; Yang, K.; Tankovic, J.; Soussy, C.-J.; Dhonneur, G.; Duvaldestin, P.; Brochard, L.; Brun-Buisson, C.; Harf, A.; Delclaux, C. Impairment of polymorphonuclear neutrophil functions precedes nosocomial infections in critically ill patients. *Crit. Care Med.* **2002**, *30*, 315–322. [CrossRef] [PubMed]
53. Vergis, N.; Khamri, W.; Beale, K.; Sadiq, F.; Aletrari, M.O.; Moore, C.; Atkinson, S.R.; Bernsmeier, C.; Possamai, L.A.; Petts, G.; et al. Defective monocyte oxidative burst predicts infection in alcoholic hepatitis and is associated with reduced expression of NADPH oxidase. *Gut* **2017**, *66*, 519–529. [CrossRef] [PubMed]
54. Albillos, A.; Lario, M.; Álvarez-Mon, M. Cirrhosis-associated immune dysfunction: Distinctive features and clinical relevance. *J. Hepatol.* **2014**, *61*, 1385–1396. [CrossRef] [PubMed]
55. Wasmuth, H.E.; Kunz, D.; Yagmur, E.; Timmer-strangho, A.; Vidacek, D.; Siewert, E.; Bach, J.; Geier, A.; Purucker, E.A.; Gressner, A.M.; et al. Patients with acute on chronic liver failure display 'sepsis-like' immune paralysis. *J. Hepatol.* **2005**, *42*, 195–201. [CrossRef] [PubMed]
56. Albillos, A.; de la Hera, A.; Reyes, E.; Monserrat, J.; Muñoz, L.; Nieto, M.; Prieto, A.; Sanz, E.; Alvarez-Mon, M. Tumour necrosis factor-alpha expression by activated monocytes and altered T-cell homeostasis in ascitic alcoholic cirrhosis: Amelioration with norfloxacin. *J. Hepatol.* **2004**, *40*, 624–631. [CrossRef] [PubMed]
57. Korf, H.; Plessis, J.; Van Pelt, J.; De Groote, S.; Cassiman, D.; Verbeke, L.; Ghesquière, B.; Fendt, S.; Bird, M.J.; Talebi, A.; et al. Inhibition of glutamine synthetase in monocytes from patients with acute-on-chronic liver failure resuscitates their antibacterial and inflammatory capacity. *Gut* **2019**, *68*, 1872–1883. [CrossRef]
58. Sampath, P.; Moideen, K.; Ranganathan, U.D.; Bethunaickan, R. Monocyte Subsets: Phenotypes and Function in Tuberculosis Infection. *Front. Immunol.* **2018**, *9*, 1–8. [CrossRef]
59. Shi, Y.; Wu, W.; Yang, Y.; Yang, Q.; Song, G.; Wu, Y.; Wei, L.; Chen, Z. Decreased Tim-3 expression is associated with functional abnormalities of monocytes in decompensated cirrhosis without overt bacterial infection. *J. Hepatol.* **2015**, *63*, 60–67. [CrossRef]
60. Berry, P.A.; Mcphail, M.J.W.; Davies, E.T.; Wendon, J.A.; Vergani, D. Severity of the compensatory anti-inflammatory response determined by monocyte HLA-DR expression may assist outcome prediction in cirrhosis. *Intensive Care Med.* **2011**, *37*, 453–460. [CrossRef]
61. Kusaba, N.; Kumashiro, R.; Ogata, H.; Sata, M.; Tanikawa, K. In Vitro Study of Neutrophil Apoptosis in Liver Cirrhosis. *Intern. Med.* **1998**, *37*, 11–17. [CrossRef]
62. Khanam, A.; Trehanpati, N.; Riese, P.; Rastogi, A.; Guzman, C.A.; Sarin, S.K. Blockade of Neutrophil's Chemokine Receptors CXCR1/2 Abrogate Liver Damage in Acute-on-Chronic Liver Failure. *Front. Immunol.* **2017**, *8*, 464. [CrossRef] [PubMed]
63. Huang, C.; Jeng, W.; Ho, Y.; Teng, W.; Hsieh, Y.; Chen, W.-T.; Lin, H.-H.; Sheen, I.-S.; Lin, C.-Y. Increased EMR2 expression on neutrophils correlates with disease severity and predicts overall mortality in cirrhotic patients. *Sci. Rep.* **2016**, *6*, 38250. [CrossRef] [PubMed]
64. Boussif, A.; Rolas, L.; Weiss, E.; Bouriche, H.; Moreau, R.; Périanin, A. Impaired intracellular signaling, myeloperoxidase release and bactericidal activity of neutrophils from patients with alcoholic cirrhosis. *J. Hepatol.* **2016**, *64*, 1041–1048. [CrossRef] [PubMed]
65. Fiuza, C.; Salcedo, M.; Clemente, G.; Tellado, J.M. Granulocyte Colony-Stimulating Factor Improves Deficient In Vitro Neutrophil Transendothelial Migration in Patients with Advanced Liver Disease. *Clin. Diagn. Lab. Immunol.* **2002**, *9*, 433–439. [CrossRef]
66. Artru, F.; Bou Saleh, M.; Maggiotto, F.; Lassailly, G.; Ningarhari, M.; Demaret, J.; Ntandja-Wandji, L.-C.; Pais de Barros, J.-P.J.L.; Drumez, E.; Helou, D.G.; et al. IL-33/ST2 pathway regulates neutrophil migration and predicts outcome in patients with severe alcoholic hepatitis. *J. Hepatol.* **2020**, *72*, 1052–1061. [CrossRef]
67. Rajkovic, I.A.; Williams, R. Abnormalities of Neutrophil Phagocytosis, Intracellular Killing and Metabolic Activity in Alcoholic Cirrhosis and Hepatitis. *Hepatology* **1986**, *6*, 252–262. [CrossRef]
68. Rolas, L.; Boussif, A.; Weiss, E.; Lettéron, P.; Haddad, O.; El-benna, J.; Rautou, P.; Moreau, R.; Périanin, A. NADPH oxidase depletion in neutrophils from patients with cirrhosis and restoration via toll-like receptor 7/8 activation. *Gut* **2018**, *67*, 1505–1516. [CrossRef]
69. Garfia, C.; García-Ruiz, I.; Solís-Herruzo, J.A. Deficient phospholipase C activity in blood polimorphonuclear neutrophils from patients with liver cirrhosis. *J. Hepatol.* **2004**, *40*, 749–756. [CrossRef]
70. Mookerjee, R.P.; Stadlbauer, V.; Lidder, S.; Wright, G.A.K.; Hodges, S.J.; Davies, N.A.; Jalan, R. Neutrophil Dysfunction in Alcoholic Hepatitis Superimposed on Cirrhosis is Reversible and Predicts the Outcome. *Hepatology* **2007**, *46*, 831–840. [CrossRef]

71. Taylor, N.J.; Vijay, G.K.M.; Abeles, R.D.; Auzinger, G.; Bernal, W.; Ma, Y.; Wendon, J.A.; Shawcross, D.L. The severity of circulating neutrophil dysfunction in patients with cirrhosis is associated with 90-day and 1-year mortality. *Aliment. Pharmacol. Ther.* **2014**, *40*, 705–715. [CrossRef]
72. Moreau, N.; Wittebole, X.; Fleury, Y.; Forget, P.; Laterre, P.-F.; Castanares-Zapatero, D. Neutrophil-to-Lymphocyte Ratio Predicts Death in Acute-on-Chronic Liver Failure Patients Admitted to the Intensive Care Unit: A Retrospective Cohort Study. *Shock* **2018**, *49*, 385–392. [CrossRef] [PubMed]
73. Grønbæk, H.; Rødgaard-Hansen, S.; Aagaard, N.K.; Arroyo, V.; Moestrup, S.K.; Garcia, E.; Solà, E.; Domenicali, M.; Piano, S.; Vilstrup, H.; et al. Macrophage activation markers predict mortality in patients with liver cirrhosis without or with acute-on-chronic liver failure (ACLF). *J. Hepatol.* **2016**, *64*, 813–822. [CrossRef] [PubMed]
74. Rittirsch, D.; Flierl, M.A.; Ward, P.A. Harmful molecular mechanisms in sepsis. *Nat. Rev. Immunol.* **2008**, *8*, 776–787. [CrossRef] [PubMed]
75. Mantovani, A.; Dinarello, C.A.; Molgora, M.; Garlanda, C. Interleukin-1 and Related Cytokines in the Regulation of Inflammation and Immunity. *Immunity* **2019**, *50*, 778–795. [CrossRef]
76. Eleftheriadis, T.; Georgios Pissas, V.L.; Stefanidis, I.; Lawson, B.R. Toll-like receptors and their role in renal pathologies. *Inflamm Allergy Drug Targets* **2012**, *11*, 464–477. [CrossRef] [PubMed]
77. Clària, J.; Arroyo, V.; Moreau, R. The Acute-on-Chronic Liver Failure Syndrome, or When the Innate Immune System Goes Astray. *J. Immunol.* **2016**, *197*, 3755–3761. [CrossRef]
78. Gabay, C.; Kushner, I. Acute-Phase Proteins and Other Systemic Responses to Inflammation. *N. Engl. J. Med.* **1999**, *340*, 448–454. [CrossRef]
79. Borish, L.C.; Steinke, J.W. 2. Cytokines and chemokines. *J. Allergy Clin. Immunol.* **2003**, *2*, 460–475. [CrossRef]
80. Byl, B.; Roucloux, I.; Crusiaux, A.; Dupont, E.; Devière, J. Tumor necrosis factor α and interleukin 6 plasma levels in infected cirrhotic patients. *Gastroenterology* **1993**, *104*, 1492–1497. [CrossRef]
81. Albillos, A.; de la Hera, A.; González, M.; Moya, J.-L.; Calleja, J.-L.; Monserrat, J.; Ruiz-del-Arbol, L.; Alvarez-Mon, M. Increased lipopolysaccharide binding protein in cirrhotic patients with marked immune and hemodynamic derangement. *Hepatology* **2003**, *37*, 208–217. [CrossRef]
82. Navasa, M.; Follo, A.; Filella, X.; Jiménez, W.; Francitorra, A.; Planas, R.; Rimola, A.; Arroyo, V.; Rodés, J. Tumor Necrosis Factor and Interleukin-6 in Spontaneous Bacterial Peritonitis in Cirrhosis: Relationship With the Development of Renal Impairment and Mortality. *Hepatology* **1998**, *27*, 1227–1232. [CrossRef] [PubMed]
83. Schmidt-arras, D.; Rose-john, S. IL-6 pathway in the liver: From physiopathology to therapy. *J. Hepatol.* **2016**, *64*, 1403–1415. [CrossRef] [PubMed]
84. Tanaka, T.; Narazaki, M.; Kishimoto, T. Immunotherapeutic implications of IL-6 blockade for cytokine storm. *Immunotherapy* **2016**, *8*, 959–970. [CrossRef] [PubMed]
85. Tanaka, T.; Narazaki, M.; Kishimoto, T. IL-6 in Inflammation, Immunity, and Disease. *Cold Spring Harb. Perspect. Biol.* **2014**, *6*, a016295. [CrossRef]
86. Genovese, M.C.; Kremer, J.M.; van Vollenhoven, R.F.; Alten, R.; Scali, J.J.; Kelman, A.; Dimonaco, S.; Brockwell, L. Transaminase Levels and Hepatic Events During Tocilizumab Treatment. Pooled Analysis of Long-Term Clinical Trial Safety Data in Rheumatoid Arthritis. *Arthritis Rheumatol.* **2017**, *69*, 1751–1761. [CrossRef]
87. Boettler, T.; Newsome, P.N.; Mondelli, M.U.; Maticic, M.; Cordero, E.; Cornberg, M.; Berg, T. Care of patients with liver disease during the COVID-19 pandemic: EASL-ESCMID position paper. *J. Hepatol. Reports* **2020**, *2*, 100113. [CrossRef]
88. Zlotnik, A.; Yoshie, O. The Chemokine Superfamily Revisited. *Immunity* **2012**, *36*, 705–716. [CrossRef]
89. Lu, W.; Bird, T.G.; Boulter, L.; Tsuchiya, A.; Cole, A.M.; Hay, T.; Guest, R.V.; Wojtacha, D.; Man, T.Y.; Mackinnon, A.; et al. Hepatic progenitor cells of biliary origin with liver repopulation capacity. *Nat. Cell Biol.* **2015**, *17*, 971–983. [CrossRef]
90. Simonetto, D.; Shah, V.; Kamath, P. Improving survival in ACLF: Growing evidence for use of G-CSF. *Hepatol. Int.* **2017**, *11*, 473–475. [CrossRef]
91. Yu, X.; Guo, R.; Ming, D.; Deng, Y.; Su, M.; Lin, C.; Li, J.; Lin, Z.; Su, Z. The Transforming Growth Factor β1/Interleukin-31 Pathway Is Upregulated in Patients with Hepatitis B Virus-Related Acute-on-Chronic Liver Failure and Is Associated with Disease Severity and Survival. *Clin. Vaccine Immunol.* **2015**, *22*, 484–492. [CrossRef]

92. Dennis, E.A.; Norris, P.C. Eicosanoid storm in infection and inflammation. *Nat. Rev. Immunol.* **2015**, *15*, 13–16. [CrossRef] [PubMed]
93. Buczynski, M.W.; Dumlao, D.S.; Dennis, E.A. An integrated omics analysis of eicosanoid biology. *J. Lipid Res.* **2009**, *50*, 1015–1038. [CrossRef] [PubMed]
94. Clària, J.; Romano, M. Pharmacological intervention of cyclooxygenase-2 and 5-lipoxygenase pathways. Impact on inflammation and cancer. *Curr. Pharm. Des.* **2005**, *11*, 3431–3447. [CrossRef] [PubMed]
95. Stables, M.J.; Gilroy, D.W. Old and new generation lipid mediators in acute inflammation and resolution. *Prog. Lipid Res.* **2011**, *50*, 35–51. [CrossRef]
96. Claria, J.; Serhan, C.N. Aspirin triggers previously undescribed bioactive eicosanoids by human endothelial cell-leukocyte interactions. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 9475–9479. [CrossRef]
97. López-vicario, C.; Rius, B.; Alcaraz-quiles, J.; García-alonso, V.; Lopategi, A.; Titos, E.; Clària, J. Pro-resolving mediators produced from EPA and DHA: Overview of the pathways involved and their mechanisms in metabolic syndrome and related liver diseases. *Eur. J. Pharmacol.* **2016**, *785*, 133–143. [CrossRef]
98. Serhan, C.N. Pro-resolving lipid mediators are leads for resolution physiology. *Nature* **2014**, *510*, 92–101. [CrossRef]
99. China, L.; Maini, A.; Skene, S.S.; Shabir, Z.; Sylvestre, Y.; Colas, R.A.; Ly, L.; Salles, N.B.; Belloti, V.; Dalli, J.; et al. Albumin Counteracts Immune-Suppressive Effects of Lipid Mediators in Patients With Advanced Liver Disease. *Clin. Gastroenterol. Hepatol.* **2018**, *16*, 738–747. [CrossRef]
100. Becares, N.; Härmälä, S.; Colas, R.A.; Maini, A.A.; Bennet, K.; Skene, S.S.; Shabir, Z.; Dalli, J.; O'Brien, A. Immune Regulatory Mediators in Plasma from Patients With Acute Decompensation Are Associated With 3-Month Mortality. *Clin. Gastroenterol. Hepatol.* **2020**, *18*, 1207–1215. [CrossRef]
101. López-Vicario, C.; Checa, A.; Urdangarin, A.; Aguilar, F.; Alcaraz-Quiles, J.; Caraceni, P.; Amorós, A.; Pavesi, M.; Gómez-Cabrero, D.; Trebicka, J.; et al. Targeted lipidomics reveals extensive changes in circulating lipid mediators in patients with acutely decompensated cirrhosis. *J. Hepatol.* **2020**, *73*, 817–828. [CrossRef]
102. Sort, P.; Navasa, M.; Arroyo, V.; Aldeguer, X.; Planas, R.; Ruiz-del-Arbol, L.; Castells, L.; Vargas, V.; Soriano, G.; Guevara, M.; et al. Effect of intravenous albumin on renal impairment and mortality in patients with cirrhosis and spontaneous bacterial peritonitis. *N. Engl. J. Med.* **1999**, 403–409. [CrossRef] [PubMed]
103. Bernardi, M.; Angeli, P.; Claria, J.; Moreau, R.; Gines, P.; Jalan, R.; Caraceni, P.; Fernandez, J.; Gerbes, A.L.; Brien, A.J.O.; et al. Albumin in decompensated cirrhosis: New concepts and perspectives. *Gut* **2020**, *69*, 1127–1138. [CrossRef] [PubMed]
104. Caraceni, P.; Riggio, O.; Angeli, P.; Alessandria, C.; Neri, S.; Foschi, F.G.; Levantesi, F.; Airoldi, A.; Boccia, S.; Svegliati-Baroni, G.; et al. Long-term albumin administration in decompensated cirrhosis (ANSWER): An open-label randomised trial. *Lancet* **2018**, *391*, 2417–2429. [CrossRef]
105. Fernández, J.; Clària, J.; Amorós, A.; Aguilar, F.; Castro, M.; Casulleras, M.; Acevedo, J.; Duran-Güell, M.; Nuñez, L.; Costa, M.; et al. Effects of Albumin Treatment on Systemic and Portal Hemodynamics and Systemic Inflammation in Patients With Decompensated Cirrhosis. *Gastroenterology* **2019**, *157*, 149–162. [CrossRef] [PubMed]
106. Bañares, R.; Ibáñez-samaniego, L.; Torner, J.M.; Pavesi, M.; Olmedo, C.; Catalina, M.V.; Albillos, A.; Larsen, F.S.; Nevens, F.; Hassanein, T.; et al. Meta-analysis of individual patient data of albumin dialysis in acute-on-chronic liver failure: Focus on treatment intensity. *Therap. Adv. Gastroenterol.* **2019**, *12*, 1–12. [CrossRef]
107. Niewinski, G.; Raszeja-Wyszomirska, J.; Hrenczuk, M.; Rozga, A.; Malkowski, P.; Rozga, J. Intermittent high-flux albumin dialysis with continuous venovenous hemodialysis for acute-on-chronic liver failure and acute kidney injury. *Artif. Organs* **2020**, *44*, 91–99. [CrossRef] [PubMed]
108. Yang, Z.; Zhang, Z.; Cheng, Q.; Chen, G.; Li, W.; Ma, K.; Guo, W.; Luo, X.; Chen, T.; Ning, Q. Plasma perfusion combined with plasma exchange in chronic hepatitis B-related acute-on-chronic liver failure patients. *Hepatol. Int.* **2020**, *14*, 491–502. [CrossRef]
109. Casulleras, M.; Flores-Costa, R.; Duran-Güell, M.; Alcaraz-quiles, J.; Sanz, S.; Titos, E.; López-vicario, C.; Fernández, J.; Horrillo, R.; Costa, M.; et al. Albumin internalizes and inhibits endosomal TLR signaling in leukocytes from patients with decompensated cirrhosis. *Sci. Transl. Med.* **2020**, *12*, eaax5135. [CrossRef]
110. Garg, V.; Garg, H.; Khan, A.; Trehanpati, N.; Kumar, A.; Sharma, B.C.; Sakhuja, P.; Sarin, S.K. Granulocyte Colony–Stimulating Factor Mobilizes CD34+ Cells and Improves Survival of Patients With Acute-on-Chronic Liver Failure. *Gastroenterology* **2012**, *142*, 505–512. [CrossRef]

111. Duan, X.; Liu, F.; Tong, J.; Yang, H.; Chen, J.; Liu, X.; Mao, Y.; Xin, S.-J.; Hu, J.-H. Granulocyte-colony stimulating factor therapy improves survival in patients with hepatitis B virus-associated acute-on-chronic liver failure. *World J. Gastroenterol.* **2013**, *19*, 1104–1110. [CrossRef]
112. Saha, B.K.; Al Mahtab, M.; Akbar, S.M.F.; Noor-E-Alam, S.M.; Al Mamun, A.; Hossain, S.M.S.; Alam, M.A.; Moben, A.L.; Khondaker, F.A.; Chowdhury, F.I.; et al. Therapeutic implications of granulocyte colony stimulating factor in patients with acute-on-chronic liver failure: Increased survival and containment of liver damage. *Hepatol. Int.* **2017**, *11*, 540–546. [CrossRef] [PubMed]
113. Engelmann, C.; Brosteanu, O.; Tenckhoff, H.; Splith, K.; Schmelzle, M.; Berg, T. Granulocyte colony stimulating factor (G-CSF) to treat acute-on-chronic liver failure: A multicenter randomized trial (GRAFT STUDY). *J. Hepatol.* **2015**, *62*, S847. [CrossRef]
114. Sharma, M.; Kulkarni, A.; Sasikala, M.; Kumar, P.; Jaggaiahgari, S.; Pondugala, K.; Jaishetwar, G.; Darisetty, S.; Jagtap, N.; Gupta, R.; et al. Long-term Outcome of Autologous Hematopoietic Stem Cell Infusion in Cirrhosis: Waning Effect over Time. *J. Clin. Transl. Hepatol.* **2020**, *9*, 1–6. [CrossRef]
115. Engelmann, C.; Sheikh, M.; Sharma, S.; Kondo, T.; Loeffler-Wirth, H.; Zheng, Y.B.; Novelli, S.; Hall, A.; Kerbert, A.J.C.; Macnaughtan, J.; et al. Toll-like receptor 4 is a therapeutic target for prevention and treatment of liver failure. *J. Hepatol.* **2020**, *73*, 102–112. [CrossRef]
116. Xiang, X.; Feng, D.; Hwang, S.; Ren, T.; Wang, X.; Trojnar, E.; Matyas, C.; Mo, R.; Shang, D.; He, Y.; et al. Interleukin-22 ameliorates acute-on-chronic liver failure by reprogramming impaired regeneration pathways in mice. *J. Hepatol.* **2020**, *72*, 736–745. [CrossRef]
117. Shi, M.; Zhang, Z.; Xu, R.; Lin, H.; Fu, J.; Zou, Z.; Zhang, A.; Shi, J.; Chen, L.; Lv, S.; et al. Human Mesenchymal Stem Cell Transfusion Is Safe and Improves Liver Function in Acute-on-Chronic Liver Failure Patients. *Stem. Cells Transl. Med.* **2012**, *1*, 725–731. [CrossRef]
118. Lin, B.-L.; Chen, J.-F.; Qiu, W.-H.; Wang, K.-W.; Xie, D.-Y.; Chen, X.-Y.; Liu, Q.-L.; Peng, L.; Li, J.-G.; Mei, Y.-Y.; et al. Allogeneic Bone Marrow–Derived Mesenchymal Stromal Cells for Hepatitis B Virus–Related Acute-on-Chronic Liver Failure: A Randomized Controlled Trial. *Hepatology* **2017**, *66*, 209–219. [CrossRef]

ANNEX III: CONFERENCE PRESENTATIONS

Results derived from this following thesis have been presented at the following conferences:

Mireia Casulleras, Roger Flores-Costa, Marta Duran-Güell, Ingrid W. Zhang, Cristina López-Vicario, Anna Curto, Javier Fernández, Vicente Arroyo and Joan Clària. El análisis lipidómico de la molécula de albumina de pacientes con cirrosis hepática identifica importantes alteraciones en su composición lipídica y una menor abundancia de mediadores pro-resolutivos. 47º Congreso anual de la Asociación Española para el Estudio del Hígado (AEEH). Madrid, Spain. May 25-27, 2022. **Poster**.

Mireia Casulleras, Roger Flores-Costa, Marta Duran-Güell, Anna Curto, Ingrid W. Zhang, Cristina López-Vicario, Esther Titos, Javier Fernández, Vicente Arroyo and Joan Clària**.** Albumin lipidomics reveals meaningful compositional changes in decompensated cirrhosis and the potential of exogenous albumin to promote inflammation resolution. The AASLD Liver Meeting. Digital Experience (Los Angeles, CA, USA). November 12-15, 2021. **Oral presentation.**

Mireia Casulleras, José Alcaraz-Quiles, Marta Duran-Güell, Roger Flores-Costa, Esther Titos, Cristina López-Vicario, Raquel Horrillo, Montserrat Costa, Richard Moreau, Javier Fernández, Vicente Arroyo, Joan Clària. Albumin modulates endosomal TLR9 signaling in human peripheral leukocytes. A mechanism for its anti-inflammatory role in ACLF. The International Liver Congress 2019 (EASL). Viena, Austria. April 10-45, 2019. **Poster (ePoster oral presentation)**

Mireia Casulleras, José Alcaraz-Quiles, Marta Duran-Güell, Roger Flores-Costa, Esther Titos, Cristina López-Vicario, Raquel Horrillo, Montserrat Costa, Richard Moreau, Javier Fernández, Vicente Arroyo, Joan Clària. La albúmina modula la señalización vía TLR9 en los endosomas de leucocitos periféricos humanos. Un mecanismo para su papel antiinflamatorio en ACLF. 44º Congreso anual de la Asociación Española para el Estudio del Hígado (AEEH). Madrid, Spain. February 20-22, 2019. **Poster**.

Mireia Casulleras, José Alcaraz-Quiles, Marta Duran-Güell, Roger Flores-Costa, Esther Titos, Cristina López-Vicario, Raquel Horrillo, Montserrat Costa, Richard Moreau, Vicente Arroyo, Joan Clària. Albumin modulates lipid mediator biosynthesis in human peripheral leukocytes. 7th European Workshop on Lipid Mediators (7EWLM). Brussels, Belgium. September 12-14, 2018. **Poster.**

Mireia Casulleras, José Alcaraz-Quiles, Marta Duran-Güell, Roger Flores-Costa, Esther Titos, Cristina López-Vicario, Raquel Horrillo, Montserrat Costa, Richard Moreau, Vicente Arroyo and Joan Clària. Albumin modulates TLR9 and FPR1 signaling in human peripheral leukocytes. 25nd Annual Scientific meeting of the European society for clinical investigation 2018 (ESCI). Barcelona, Spain. May 30 - June 1, 2018. **Oral presentation.**