UNIVERSITAT DE BARCELONA

Effects of human serum albumin on TNFα-induced cell death and mitochondrial dysfunction in the liver cells

Marta Duran Güell

EFFECTS OF HUMAN SERUM ALBUMIN ON TNFα-INDUCED CELL DEATH AND MITOCHONDRIAL DYSFUNCTION IN LIVER CELLS

Doctoral thesis dissertation presented by:

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to apply for the degree of Doctor at the University of Barcelona

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Dr. Joan Clària, Professor at the School of Medicine, Department of Biomedical Sciences, with Identity Card: 77111365R,

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The thesis memory presented by Marta Duran Güell with title "Effects of human serum albumin on TNFα-induced cell death and mitochondrial dysfunction in liver cells", has been developed under my supervision and I authorize the deposit for being defended and judged by a tribunal.

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"No és el que diem o el que pensem
el que ens defineix, sinó allò que fem."

Jane Austen

Al meu avi Pere

A vosaltres Mireia i Roger, que m'heu acompanyat fins aquí

AGRAÏMENTS

He deixat pel final la redacció dels agraïments tot i ser un dels apartats més importants de la tesi i l'apartat que tothom llegeix primer! Tot aquest treball només ha sigut possible gràcies a l'ajuda de tots els meus companys de feina i gràcies a tot el suport dels meus amics i de la meva família.

En primer lloc, vull agrair al Dr. Joan Clària, director de la tesi i IP del grup, la oportunitat de fer el doctorat en aquest grup de recerca. Recordo que quan vaig entrar a fer les pràctiques del màster no tenia molt clar si volia fer el doctorat però els companys em deien: aquest és un grup molt bo, amb el Joan publicaràs segur i faràs una bona tesi. Realment tenien raó i estic molt agraïda de tota la formació rebuda, sobretot de la teva implicació en cada projecte, en escriure els papers i en donar-me la oportunitat d'ensenyar els meus resultats en diferents congressos. En conclusió, vull donar-te les gràcies per la teva guia i bon criteri durant tota la meva tesi doctoral i sobretot per transmetre la teva passió per la ciència i per la feina ben feta.

En segon lloc, vull agrair a tots i cada un dels membres del grup que en major i menor mesura han contribuït en aquesta tesi i han aportat el seu granet de sorra.

Esther, vas ser la primera persona que vaig conèixer del grup, i vull agrair-te tota la dedicació que has posat per tal que el grup funcioni a la perfecció sense perdre el teu sentit de l'humor. Moltes gràcies per saber veure el millor de cada persona i per aconseguir treure el millor de cada persona. La teva experiència i trajectòria dins del grup han sigut un factor clau per mi (i crec que per tots), recordo venir-te a preguntar tots els dubtes i dissenys experimentals amb la confiança cega de que ho "saps tot" encara que ens vulguis fer creure el contrari (com per exemple quan dius que no tens un bon nivell d'anglès, que ja sabem que és mentida). Gràcies també per no perdre el contacte amb nosaltres, seguir-nos motivant i fer-nos riure sempre amb les teves històries!

Bibi, fa temps que vas marxar del laboratori però vas ser la meva supervisora i penso que gràcies a tu vaig decidir fer la tesi. La veritat és que la teva empenta i alegria s'encomanen i agraeixo molt haver après els primers passos amb tu (a fer els PCLS i els aïllaments d'hepatòcits!).

Cristina, has estat al laboratori des del primer dia que vaig entrar fins avui. Recordo la teva amabilitat amb tothom qui entra al grup, fas que tothom se senti acollit. Et vull agrair sobretot la teva valentia que et fa tirar endavant qualsevol projecte i qualsevol embarcada

que et proposis. Admiro també moltíssim la teva mà esquerra amb els animals (ho havia vist ja amb els ratolins i ho he pogut comprovar amb les rates en aquest últim projecte), per mi ets el flautista d'Hamelin del lab. Estic molt contenta d'haver-te tingut de companya en el projecte de les rates, hem fet un molt bon equip i estic molt agraïda per l'ajuda que sempre estàs disposada a donar. Finalment també et vull agrair la teva manera de fer les coses amb aquesta alegria (i paraules inventades) que ens fan riure a totes i desconnectar de les misèries. Gràcies per ser una companya de feina i una amiga.

José, crec que t'ho he dit molts cops però és que és digne de menció: la primera ensenyança que recordo teva va ser a la sala de cultius on em vas dir: "esto es como una discoteca, vamos todos así vestidos (con bata) y hay alcohol". Moltes gràcies per totes les teves píndoles de saviesa i per la teva manera d'explicar les coses tan clara i entenedora, i per tots els teus consells aplicables tan al lab com a fora. Gràcies per ser un amic també, espero poder seguir compartint molts més moments amb tu.

Belén, han sigut moltes les nostres converses sobre pisos, hipoteques, PIAS, sobre la gent molt social i la gent no tan social, etc. Estic molt agraïda per totes les teves ensenyances sobre temes legals i temes de la vida en general. Admiro molt la teva pulcritud a l'hora de treballar i la teva atenció als detalls. Gràcies també per haver sigut una gran veïna de pupitre, va ser per poc temps però va estar molt bé. Gràcies per ajudar-me en les crítiques quan veies que ho necessitava sense que jo t'ho demanés. Gràcies també per totes les vegades que he pensat que "no trigaria tant" a retolar tres mil tubs i m'has ajudat a acabar la feina molt més de pressa. En general, moltes gràcies per tot el teu suport i per recordar-me que les coses es fan pas a pas i d'una en una.

Albert, vam entrar a la vegada i hem anat creixent junts dins del grup. Moltes gràcies per la teva inestimable ajuda amb tots els ratolins i rates, per tots els teus consells sobre com agafar millor el ratolí, per compartir les penes de les rates i per treure-li ferro a les situacions complicades que semblen molt més fàcils quan ho expliques tu. A veure si alguna vegada arribem a coincidir pel Pirineu!

Bryan, el predoc del grup! Estic molt agraïda pel teu recolzament diari, pel teu interès per aprendre, fer coses noves i conèixer gent nova (ja saps que faig el seguiment de les teves converses a la impressora). Moles gràcies també per comprar xocolatines quan veus que algú està en un moment crític (com jo amb les crítiques, rates, paper, tesi, etc), realment es

tot un detall i de gran ajuda! Et queda un gran camí per endavant i estic segura que assoliràs tots els teus objectius!

Berta, la predoc del grup! El futur està en vuestras manos (teves i del Bryan). Estic molt agraïda d'haver fet equip de rates amb tu també, el teu entusiasme i la teva passió s'encomanen i van fer que les dificultats fossin molt més suportables. Espero que no perdis mai el teu bon humor i el teu optimisme i segueixis confiant en la "teva floreta" que mai et falla. Moltes gràcies també per la teva empatia i per saber de seguida qui té un mal dia i intentar-hi posar remei. Molts ànims també amb el teu camí cap al doctorat!

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Sílvia, la meva altra veïna de pupitre! Moltíssimes gràcies per alegrar-me els dies, per comentar la jugada, per admirar els teus nebot (que ara ja tenen un cosinet guapíssim), per planejar "scape rooms" i idear la foto guanyadora del concurs de fotografia (amb el millor nom possible: "els 7 nans").

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Vull agrair també a totes les persones que han passat pel laboratori: Aritz, Mònica, Maria, Ewa, John, Pandelis, Thomas, Andrea, Can i l'última incorporació Maria-Fernanda, gràcies a totes per haver contribuït. Glòria Garrabou, et vull agrair haver-me acollit al teu laboratori i haver-me transmès la teva passió pels mitocondris.

Pepa, que ets la millor lab manager de l'edifici no ho dic jo, és el que diu tothom. Moltes gràcies per totes les comandes, per les teves històries en "momentos valle" i pels préstecs de les coses més inversemblants que guardes! Sense tu aquesta tesi hagués sigut el doble de complicada!

Ara ve el torn de dos pilars fonamentals per aquesta tesi com són la Mireia i el Roger. No em puc imaginar la tesi sense vosaltres i estic infinitament agraïda de que la vida ens hagi posat junts en el mateix grup de recerca fent el doctorat, perquè em sembla increïble haver conegut a dos companys de feina amb els qui hagi connectat tant i compartit tantíssimes coses, em sembla increïble que tinguem tantes coses en comú i em sembla increïble també l'amistat que ha sorgit entre nosaltres.

Mireia, Pili, per on començar! Hem anat "a la par", recordo que el primer dia que vas venir jo encara no et coneixia però de seguida vam congeniar bé, després ens vam assentar juntes i ja no hem parat de passar 8 hores diàries una al costat de l'altra durant aquests 6 anys, realment és molt fort la de coses que hem compartit! Moltes gràcies per la teva ajuda dins la feina, per les incomptables hores que has dedicat a donar-li voltes als meus problemes, al projecte, als experiments que a vegades no sortien, preparació de presentacions etc i també per la teva ajuda fora de la feina. No m'imagino aquesta tesi sense tu al meu costat comentant i opinant sobre tot el que passa, fent pinya davant les dificultats o fent el burro pel laboratori (i fora del laboratori també). Des del primer congrés a Frankfurt on, sense conèixer-nos massa, vam haver de dormir juntes fins ara que estic en els moments finals de la tesi no has deixat de fer-me costat, hem preparat les presentacions al EF-CLIF, els pòsters pels congressos, el viatge a França (el tour de França), el calendari mes a mes (amb la vella quaresma), la setmana santa a Olot, els viatges a Eivissa, també hem planejat viatges que mai hem fet (com a los Angeles, quina pena), esquiades, les incursions a les festes de Sanfe i a les festes d'Esparreguera on podia posar cara a tots els teus amics que tant he sentit mencionar! No puc escriure la teva part sense mencionar també al Bernat! Moltes gràcies Bernat per estar sempre atent a tots els detalls i fer que tothom se senti cuidat, i per fer-nos riure amb les teves imitacions (a destacar les imitacions de velociraptor)! En conclusió Pili, que t'estimo molt, moltíssimes gràcies per formar una part tant important de la meva vida i espero seguir compartint molts més moments juntes!

Roger, el meu germà perdut, es diu que ens van separar al néixer. Fa quasi un any que vas marxar del lab i et trobo a faltar cada dia! Penso que la nostra amistat va sorgir en un lloc molt concret com és la sala d'animals fent aïllament d'hepatòcits. Des de llavors hem compartit moltíssimes coses més que estones de feina, gràcies per totes les converses profundes sobre la vida, per veure les coses de la mateixa manera i per entendre'ns tan fàcilment. Estic molt agraïda d'haver-te conegut, gràcies per saber escoltar i per tenir sempre una resposta apunt. Tinc molt clar que sense tu aquesta tesi no hagués sigut possible, he pogut comptar amb tu dins i fora el laboratori i no has dubtat mai en donar-me

un cop de mà, en tranquil·litzar-me abans de les presentacions importants i en recolzar-me sempre. Gràcies per fer-nos els dies al lab més divertits amb les teves bromes i sobretot cantant cançons que després ens quedaven tot el dia enganxades al cap. Ets capaç d'aconseguir l'equilibri perfecte entre feina-oci, admiro la teva professionalitat i organització impol·luta quan treballes (especialment la teva agenda-full de paper). Realment espero que seguim compartint tantes coses junts des de concerts, viatges (espero que el pròxim inclogui un sac de dormir que abrigui molt), birres, cafès, converses de ressaca, gales d'eurovision i festes varies ja siguin a Sanfe, a Mataró o Esparreguera. En resum, t'estimo molt i moltíssimes gràcies per ser com ets i formar part de la meva vida! Saps que pots comptar amb mi.

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Finalment vull agrair a totes les persones que no estan dins del laboratori però a la seva manera han contribuït en aquesta tesi, és a dir la meva família i els meus amics.

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sempre en mi. Gràcies per tots els cops que m'has dit: "ah però ja te'n vas?" quan estic a casa vostra, que fan que em quedi un ratet més a comentar la jugada tot berenant. Gràcies papa per dir-me que no es pot tenir tot en aquesta vida tot i que jo no ho entengui mai i sempre ho vulgui tot. Gràcies a tots dos per aguantar totes les meves queixes i problemes que em duren una tarda i que fan que us preocupeu més vosaltres que jo! En resum, moltes gràcies per tot el que heu fet per mi i per haver-me donat tot el suport del món. Us estimo molt. També vull agrair al meu germà Jaume per entendre'm amb algunes coses que només entenem nosaltres, i ser el meu còmplice quan ens posem d'acord en contra dels papes. Sempre et dic que quan erets petit erets molt divertit però la veritat és que ara també ho ets, gràcies per fer-me riure i pel teu suport silenciós!

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MOLTES GRÀCIES!

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ABBREVIATIONS AND ACRONYMS

AA	amino acid
ACLF	acute-on-chronic liver failure
AD	acute decompensation
AIF	apoptosis-inducing factor
ALT	alanine aminotransferase
ATF3	activating transcription factor 3
ATP	adenoside triphosphate
AST	aspartate aminotransferase
BAs	bile acids
Bid	BH3 interacting-domain death agonist
B.w.	body weight
Caspase	cysteine-dependent aspartate specific protease
CCCP	carbonyl cyanide m-chlorophenyl hydrazone
CPT1	carnitine palmitoyltransferase
DAMPs	damage-associated molecular patterns
D-gal	D-galactosamine
DISC	death-inducing signaling complex
ER	endoplasmic reticulum
ETC	electron transport chain
FA	fatty acid
FADD	fas-associated protein with death domain
FADH	flavin adenine dinucleotide
FAO	fatty acid oxidation
FcRn	neonatal crystallisable fragment receptor
H_2O_2	hydrogen peroxide
HIF-1α	hypoxia-inducible factor-1α
HMA	mercaptoalbumin
Hmox1	heme oxygenase-1

HNA	nonmercaptoalbumin
HSA	human serum albumin
IDH	isocitrate dehydrogenase
IFN	interferon
IL	interleukin
IMM	inner mitochondrial membrane
IRG1	immune responsive gene 1
i.p.	intraperitoneal
JNKs	c-Jun N-terminal kinases
LDH	lactate dehydrogenase
LMP	lysosomal membrane permeabilization
LPS	lipopolysaccharide
MOMP	mitochondrial outer membrane permeabilization
mtDNA	mitochondrial DNA
NADH	nicotinamide adenine dinucleotide
NASH	non-alcoholic steatohepatitis
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
NRF2	nuclear factor erythroid 2 related factor 2
O_2	oxygen
OAA	oxaloacetate
OI	4-octyl itaconate
OXPHOS	oxidative phosphorylation
PAMPs	pathogen-associated molecular patterns
PRR	pattern-recognition receptor
RIPK1	receptor-interacting serine/ threonine-protein kinase 1
RNS	reactive nitrogen species
ROS	reactive oxygen species

ROX	residual oxygen consumption
SBP	spontaneous bacterial peritonitis
SDH	succinate dehydrogenase
SMAC/DIABLO	second mitochondrial activator of caspases/direct IAP binding protein with low PI
TCA	tricarboxylic acid
TNF	tumor necrosis factor
TRADD	adaptor molecule TNF-R1-associated death domain protein
TRAF1	TNFR-associated factor 1
TUNEL	TdT-mediated dUTP nick end labelling
VCAM-1	vascular cell adhesion molecule-1
XIAP	X-chromosome linked inhibitor of apoptosis protein
α-KG	α-ketoglutarare

LIST OF ARTICLES IN THE THESIS

Thesis in compendium of publications format. This thesis comprises eleven objectives and two articles:

Marta Duran-Güell, Roger Flores-Costa, Mireia Casulleras, Cristina López-Vicario, Esther Titos, Alba Díaz, José Alcaraz-Quiles, Raquel Horrillo, Montserrat Costa, Javier Fernández, Vicente Arroyo, Joan Clària. Albumin protects the liver from tumor necrosis factor α-induced immunopathology. FASEB J. 2021, 35(2):e21365. DOI: 10.1096/fj.202001615RRR.

IF: 5,192
Quartile: Q1
Subject and category: Biochemistry and molecular biology

Marta Duran-Güell, Glòria Garrabou, Roger Flores-Costa, Mireia Casulleras, Cristina López-Vicario, Ingrid W. Zhang, Judith Cantó-Santos, Bryan J. Contreras, María Belén Sánchez-Rodríguez, Berta Romero-Grimaldo, Raquel Horrillo, Montserrat Costa, Vicente Arroyo and Joan Clària. Essential role for albumin in preserving liver cells from TNFα-induced mitochondrial injury. FASEB J. 2023, 37:e22817. DOI: 10.1096/fj.202201526R.

IF: 5,192
Quartile: Q1
Subject and category: Biochemistry and molecular biology

THESIS SUMMARY

Títol: Efectes de l'albúmina sèrica humana sobre la mort cel·lular i la disfunció mitocondrial induïda per TNFα en les cèl·lules del fetge

Introducció: l'albúmina exerceix accions pliotròpiques a part del seu poder oncòtic, que inclouen la unió, el transport i la desintoxicació de molècules endògenes i exògenes, activitat antioxidant i la modulació de les respostes immunes i inflamatòries. En particular, estudis recents han demostrat que l'albúmina redueix la producció de citocines en leucòcits. En el primer estudi investiguem si l'albúmina té la capacitat de protegir els teixits davant les accions perjudicials dels mediadors inflamatoris. Hem circumscrit la nostra investigació al factor de necrosi tumoral (TNF)α, que exemplifica la connexió entre immunitat i lesions dels teixits.

La inflamació induïda per citocines i l'estrès oxidatiu mitocondrial són factors clau de lesions del teixit hepàtic. En el segon estudi, modelem les condicions inflamatòries hepàtiques en què la fuita de plasma condueix a l'alliberament de grans quantitats d'albúmina a l'interstici i a les superfícies parenquimàtiques, per explorar si aquesta proteïna té un paper en la preservació mitocondrial dels hepatòcits contra les accions perjudicials de la citocina citotòxica TNFα.

Hipòtesi: L'albúmina protegeix les cèl·lules hepàtiques contra la immunopatologia i la disfunció mitocondrial induïda per la citocina pliotròpica TNFα, donant lloc a l'homeòstasi del teixit i evitant la disfunció dels òrgans present en la malaltia hepàtica avançada.

Objectius: L'objectiu del primer estudi és determinar els efectes protectors de l'albúmina en el teixit hepàtic en un model de mort cel·lular governat per lesions induïdes per TNFα, imitant la mort cel·lular present al fetge de pacients amb malaltia hepàtica avançada. A més, el primer estudi també pretén dilucidar els mecanismes implicats en la protecció de l'albúmina en les cèl·lules hepàtiques.

L'objectiu del segon estudi és determinar el paper homeostàtic de l'albúmina en la preservació de les cèl·lules hepàtiques contra la lesió mitocondrial induïda pel TNFα, una patologia comuna que contribueix a la progressió de la malaltia hepàtica avançada.

Mètodes: Es realitzen experiments *in vitro* en hepatòcits incubats amb albúmina i amb TNFα. Es realitzen experiments *ex vivo* en rodanxes de fetge tallades amb precisió (PCLS) sota les condicions descrites anteriorment. Els efectes de l'albúmina es comproven *in vivo* en ratolins doble-transgènics que expressen el gen humà per l'albúmina i el receptor neonatal Fc (FcRn), (h*Alb*$^{+/+}$/h*FcRn*$^{+/+}$), que són induïts a la cirrosi incipient mitjançant l'administració de tetraclorur de carboni (CCl_4), d'altra banda s'indueix *acute-on-chronic liver failure* mitjançant la injecció de lipopolisacàrid (LPS) més D-galactosamina (D-gal).

L'apoptosi s'avalua per activitat de caspasa-3 en assajos de luminescència i els nivells de catepsina B es mesuren en assajos fluoromètrics. L'alliberament del citocrom c dels mitocondris al citosol es visualitza per microscòpia confocal i es valida per Western blot. L'expressió gènica i proteica es determina mitjançant PCR en temps real i Western Blot, respectivament. S'avalua l'F4/80 hepàtic, el receptor de fractalquina CX3CR1 i la immunotinció de la caspasa 3 per immunohistoquímica i el nombre de monòcits Ly6C+Cd11b+ de sang perifèrica es determina mitjançant citometria de flux.

Els hepatòcits i els PCLS es cultiven en absència o presència d'albúmina al medi cel·lular i després s'exposen a lesions mitocondrials amb la citocina TNFα. El paper homeostàtic de l'albúmina també s'investiga en un model de ratolí de lesió hepàtica induïda per TNFα a través de LPS+D-gal. La ultraestructura mitocondrial, el consum d'oxigen, la generació d'ATP i espècies reactives d'oxigen (ROS), la β-oxidació d'àcids grassos (FAO) i els fluxos metabòlics s'avaluen mitjançant microscòpia electrònica de transmissió (TEM), respirometria d'alta resolució, assaigs de luminescència-fluorimètric-colorimètric i producció de NADH/$FADH_2$ a partir de diversos substrats, respectivament.

Principals resultats: Els experiments *in vivo* en ratolins analbuminèmics demostren que aquests ratolins presenten una resposta més pronunciada a un model de lesió hepàtica per TNFα induït per l'administració de LPS+ D-gal. També s'observa una acció protectora dels teixits contra la lesió hepàtica LPS+D-gal durant l'administració d'albúmina humana a ratolins humanitzats (h*Alb*$^{+/+}$/h*FcRn*$^{+/+}$) amb cirrosi precoç preestablerta induïda per CCl_4. Les accions citoprotectores de l'albúmina contra la lesió induïda per TNFα es confirmen *ex vivo*, en PCLS, i *in vitro*, en hepatòcits primaris en cultiu. Les accions protectores de l'albúmina són independents de les seves propietats oncòtiques i de segrest, i es reprodueixen utilitzant albúmina humana recombinant expressada en *Oryza sativa*. La citoprotecció de l'albúmina contra la lesió de TNFα es relaciona amb la inhibició de l'alliberament de catepsina B lisosòmica acompanyada de la reducció de l'alliberament del citocrom c mitocondrial i de l'activitat de la caspasa-3.

L'anàlisi TEM revela que en absència d'albúmina, els hepatòcits són més susceptibles a les accions perjudicials del TNFα i mostren més mitocondris de forma rodona amb menys crestes intactes que els hepatòcits cultivats amb albúmina. En absència d'albúmina al medi cel·lular, els hepatòcits també mostren un augment de la generació de ROS mitocondrial i FAO. Les accions protectores de l'albúmina als mitocondris contra el dany del TNFα s'associa amb la restauració d'un punt de ruptura entre l'isocitrat i el α-cetoglutarat en el cicle dels àcids tricarboxílics i la regulació del factor activador de transcripció antioxidant 3

(ATF3). La implicació d'ATF3 en les accions protectores de l'albúmina contra la disfunció mitocondrial induïda per citocina es confirma *in vivo* en ratolins amb lesió hepàtica induïda per LPS/D-gal.

Conclusions: En el primer estudi, els nostres resultats demostren la capacitat de l'albúmina com a protectora dels hepatòcits contra els efectes citotòxics de la citocina TNFα. Aquestes accions protectores de l'albúmina es duen a terme a través de la inhibició de la fuita de catepsina cisteïna proteasa B dels lisosomes, acompanyades d'una reducció de l'alliberament de citocrom c dels mitocondris. En conjunt, aquestes troballes proporcionen proves de que l'albúmina no només és capaç de reduir la producció de citocines, sinó que també és capaç d'evitar danys als teixits per les accions dels mediadors inflamatoris.

En el segon estudi, les nostres dades proporcionen proves *in vitro, ex vivo* i *in vivo* del paper essencial de l'albúmina en la preservació dels mitocondris de cèl·lules hepàtiques contra les accions perjudicials del TNFα. En conjunt, les nostres dades destaquen la importància de mantenir els nivells d'albúmina dins del rang normal no només al compartiment intravascular sinó també a l'espai extravascular i intersticial, especialment en condicions de malaltia hepàtica avançada en què la hipoalbuminèmia és freqüent.

INTRODUCTION

1. Advanced liver disease and organ failure

1.1 Definition and epidemiology

Advanced liver disease mainly refers to the stage when liver fibrosis progresses to liver cirrhosis. Liver fibrosis is the exacerbated accumulation of extracellular matrix proteins that occurs in most types of liver diseases which can progress to cirrhosis, liver failure and portal hypertension (Bataller R et al, J Clin Invest. 2005). The term cirrhosis involves all the morphological and vascular changes in the liver that happen as a consequence of chronic liver inflammation and hepatic fibrosis, in which the normal hepatic architecture is replaced by regenerative hepatic nodules that may result in liver failure (Ginès P et al, Lancet 2021). The morphological and vascular changes are responsible for the hepatocellular dysfunction and a markedly increased portal pressure. In the clinical perspective, it can be classified into compensated cirrhosis, defined as asymptomatic cirrhosis in which portal hypertension is the predominant physiopathology, or decompensated cirrhosis. The most common clinical presentation of patients with decompensated cirrhosis is ascites (that is, the accumulation of large amounts of fluid within the peritoneal cavity), variceal bleeding, hepatic encephalopathy, jaundice and weight loss, which is associated to advanced liver disease.

The common causes to develop advanced liver diseases include viral infections (Hepatitis B and C virus represent around 40 and 10 % of the cirrhotic patients, respectively around the world, but not in the Western countries like our), alcohol consumption, non-alcoholic fatty liver disease, genetic disorders, autoimmune and biliary diseases, vascular obstructions and drug-related damage (Ginès P et al, Lancet 2021).

The exact prevalence of advanced liver disease around the word is unknown but about 2 million deaths worldwide annually are due to liver disease, 1 million of them attributable to cirrhosis (Asrani SK et al, J Hepatol. 2019). Only around 30% of all liver disease-related deaths are woman, as compared to men. Moreover, cirrhosis is the 11th most common cause of death, and it is the third leading cause of death in people aged 45-64 years (GBD 2017 Cirrhosis Collaborators.Lancet Gastroenterol Hepatol. 2020).

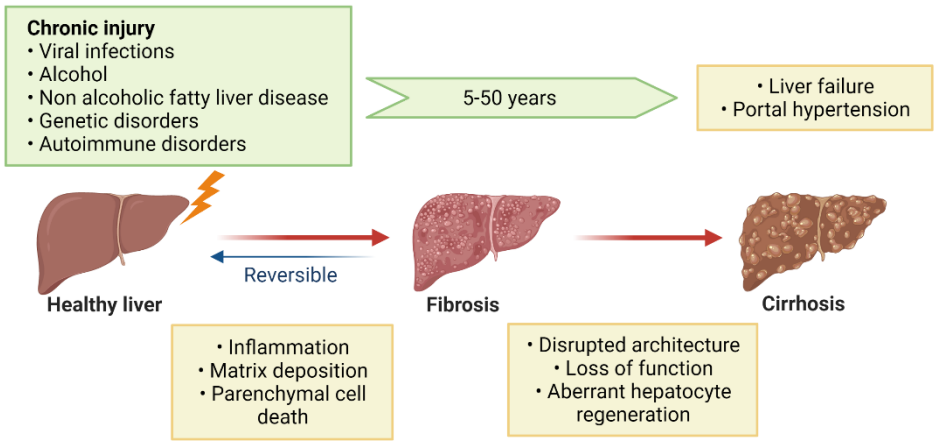

Figure 1. Development of chronic liver disease. Constant injury in the liver triggers inflammatory damage, matrix deposition and parenchymal cell death leading to fibrosis. The scar matrix accumulates very slowly but when cirrhosis is established it decreases the potential for reversing this process and complications can develop. Currently, liver transplantation is the only available treatment for liver failure. (Modified from Pellicoro, A. et al, Nat Rev Immunol. 2014)

1.2 Clinical progression

Understanding cirrhosis as advanced liver disease, it can be classified into two stages: compensated cirrhosis and decompensated cirrhosis. Although the clinical progression of cirrhosis is not predicable; it is parallel to the inflammatory, haemodynamic and functional state of the liver and it also parallels an increasing mortality risk (D'Amico G et al, J Hepatol. 2018).

Compensated cirrhosis is defined as asymptomatic cirrhosis in which portal hypertension is the predominant physiopathology of these patients. However, during this period that can last 10 years, symptoms are absent or minor in most compensated patients. (Arroyo et al, Nat. Rev. Dis. Prim. 2016).

On the other hand, decompensated cirrhosis defines the period following the development of ascites, variceal hemorrhage and/or hepatic encephalopathy. This period is associated with high short-term survival (3-5 years) and in addition, 30% of patients develop extrahepatic organ failures and acute-on-chronic liver failure (ACLF) (Arroyo et al, Nat. Rev. Dis. Prim. 2016) (Arroyo et al, N. Engl. J. Med. 2020) (Engelmann C et al, J Hepatol. 2021). ACLF is characterized by the manifestation of organ failures in the six major organ systems (liver, kidney, brain, coagulation, circulation and respiration), which presents high short-

term mortality (28-day mortality of 32%) (Moreau et al, Gastroenterol. 2013) (Arroyo et al, Nat. Rev. Dis. Prim. 2016) (Arroyo et al, N. Engl. J. Med. 2020).

The complexity of the disease is emphasized by the unpredictable disease onset, the variability of the disease dynamics as well as the combinations of extrahepatic organ failures in patients with ACLF.

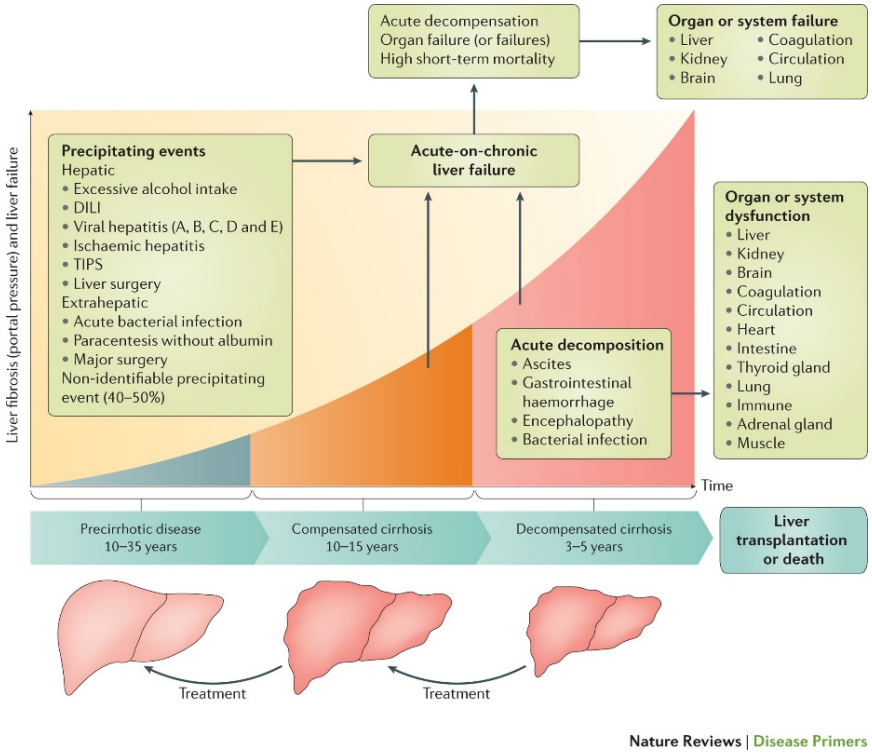

Figure 2. Clinical progression of cirrhosis. Cirrhosis can be classified into compensated cirrhosis or, with the presence of precipitating events, decompensated cirrhosis. ACLF can develop at any stage of cirrhosis triggered by a hepatic or extrahepatic event. Acute decompensation leads to organ or system dysfunction. DILI, drug-induced liver injury; TIPS, transjugular intrahepatic portosystemic shunt. (From Arroyo V. et al, Nat Rev Dis Prim 2016)

1.3 Systemic inflammation

Inflammation is the response of the immune system to defend the body from pathogens or from damaged cells. In normal homeostasis, inflammation is eventually resolved as a result of precise self-regulatory mechanisms. Nonetheless, in advanced liver disease, these regulatory circuits are ineffective, leading to decompensation and functional failure

(Engelmann C et al, J Hepatol. 2021). Hepatic inflammation is usually useful to protect liver cells from pathogens and tissue injury, to induce the repair of tissue damage and re-establish homeostasis. However, systemic inflammation present in advanced liver disease fails to resolve resulting in an excessive inflammatory response (Schattenberg JM. et al, Liver Int. 2006). In fact, patients from acute decompensated (AD) cirrhosis present elevated levels of white blood cell count, C-reactive protein, pro-inflammatory and anti-inflammatory cytokines and chemokines and oxidative stress markers, emphasizing the important role of the exacerbated inflammatory response in these patients (Moreau R. et al, Gastroenterology 2013) (Clària J. et al, Hepatology 2016) (Tilg H. et al, Gastroenterology 1992) (Solé C. et al, Sci Rep. 2016).

To understand the factors that could lead to decompensation in cirrhosis, initially, the hypothesis of the peripheral arterial vasodilation was presented by Schrier et al, in 1988. This hypothesis postulated that the impairment of circulatory function related to portal hypertension was the major pathogenic event in the development of complications of cirrhosis such as ascites and renal dysfunction (Schrier RW. et al, Hepatology 1988). Nevertheless, this hypothesis could not explain other physiological effects occurring during cirrhosis, for instance the multiorganic failure associated with AD.

Then, after decades of research, systemic inflammation caused by the activation of innate immune cells by pathogen-associated molecular patterns (PAMPs), arose as a major contributor in the induction of decompensation and the main hypothesis to explain most of the pathophysiology of the disease (Arroyo V. et al, J Hepatol. 2021). The conclusions from the CANONIC study indicate that exacerbated systemic inflammation together with the impairment of the circulatory system present in cirrhotic patients are the main cause of organ failure. Therefore, organ hypoperfusion together with the negative effect of the inflammatory mediators would lead to cell death and organ dysfunction (Bernardi M. et al, J Hepatol. 2015). Recent findings highlight as well, the contribution of mitochondrial dysfunction, oxidative stress and metabolic changes, along with systemic inflammation as key contributors in the process of AD. (Engelmann C et al, J Hepatol. 2021) (Moreau R et al, J Hepatol. 2020).

Systemic inflammation in advanced liver disease can be triggered by two major sources. In the first place, there is an increased gut permeability that results in translocation of gram-negative bacteria across the intestinal barrier, inducing the release of bacterial byproducts known as PAMPs (such as lipopolysaccharide [LPS]) into the systemic circulation that

trigger the activation of immune cells (Clària J. et al, J Immunol. 2016) (Acharya C. et al, JCI Insight 2017) (Albillos A. et al, J Hepatol. 2020). Second, the liver is constantly exposed to the PAMPs derived from translocated bacteria and it is also exposed to damaging factors underlying liver disease itself (such as alcohol and viral hepatitis) that initiate hepatocyte cell death and tissue injury resulting in the release of damage-associated molecular patterns (DAMPs) (Clària J. et al, Hepatology 2016) (Casulleras M. et al, Cells 2020). At their turn, DAMPs will also interact and activate immune cells, contributing to the release of pro inflammatory cytokines, chemokines, lipid mediators and reactive oxygen species (ROS) nitrogen species (RNS).

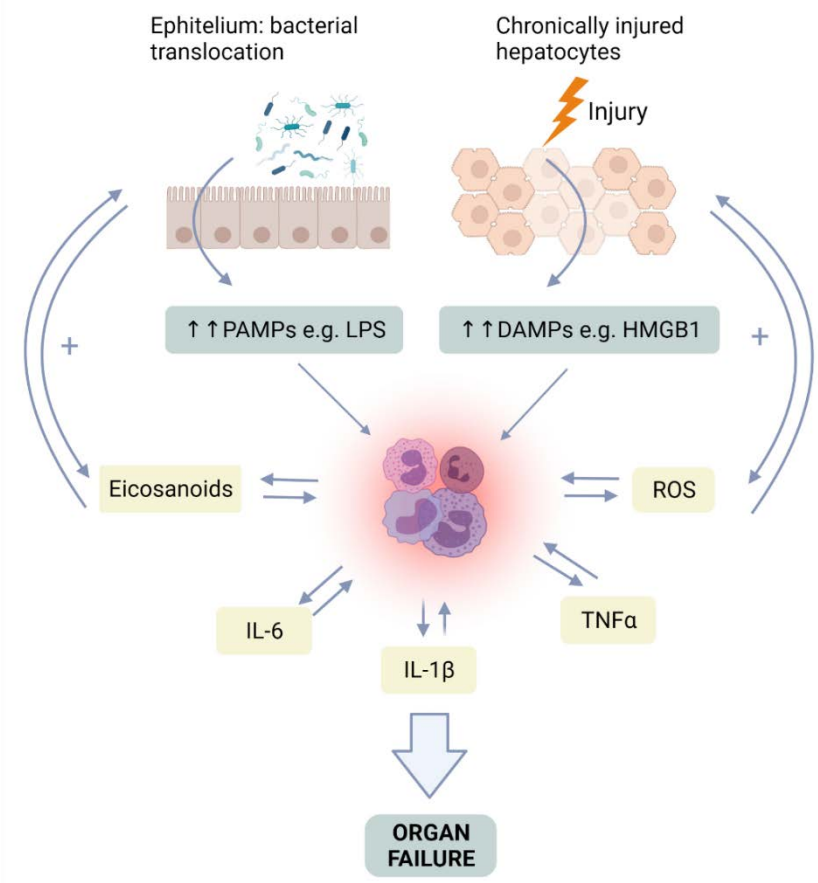

Figure 3. Systemic inflammation in cirrhosis. PAMPs derived from bacterial translocation and DAMPs derived from liver death cells activate immune cells into the release of inflammatory mediators (TNFα, IL-1β and IL-6), lipid mediators (eicosanoids) and ROS and reactive nitrogen species (RNS). At its turn, these mediators will amplify the inflammatory response to a systemic level. In decompensated cirrhotic patients and patients with ACLF, the combined action of inflammatory mediators can lead to organ failure.

1.4 Cytokine storm

PAMPs are conserved molecular structures that are recognized by the innate immune cells receptors called pattern-recognition receptors (PRRs). This interaction results in the activation of signals that mediate the inflammatory response, including interleukin (IL)-1, tumor necrosis factor (TNF)-α, IL-6, type I interferons (IFNs) and various chemokines (Medzhitov R, Curr Opin Immunol. 1997). Although this is the first response of the organs against pathogens, in advanced liver disease, the immune system is dysregulated and the inflammatory response is exacerbated and not resolved, resulting in a large and progressive generation of cytokines and chemokines that initiate, amplify and mediate inflammation and immune response. This effect, instead of being beneficial for the organism, becomes harmful, excessive and prolonged over time, leading to cell death and organ failure. This is called "cytokine storm" and the term refers to an excessive activation of the innate inflammatory system together with exacerbated systemic inflammation. Characterization of the cytokines present in AD cirrhotic and ACLF patients and the correlation with the severity of the disease were described recently (Clària J. et al, Hepatology 2016) (Clària J. et al, J Immunol. 2016).

One of the key cytokine involved in the cytokine storm present in advanced liver disease is TNFα. TNFα is a pleiotropic cytokine that exerts different effects on tissue homeostasis as it can mediate contrary cellular responses such as cell death and inflammation on the one hand, and cell survival and proliferation on the other hand (Kalliolias GD. et al, Nat Rev Rheumatol. 2016). It is produced by macrophages/monocytes during acute inflammation and LPS originated from the bacterial translocation in the gut is able induce immune cells to produce more TNFα (Wiest R. et al, J Clin Invest. 1999)(Ashkenazi A. et al, Science 1998) (Idriss HT. et al, Microsc Res Tech. 2000). Moreover, TNFα can disrupt intestinal tight junctions augmenting gut permeability resulting in the release of PAMPs (Wiest R. et al, J Hepatol. 2014) and in the liver, it mediates hepatocyte cell death, causing the disruption of the liver architecture, the liberation of DAMPs and eventually organ failure (Tiegs G, Semin Immunopathol. 2022).

1.5 Cell death and organ failure

Cell death is the final stage of a cell which stop doing its functions. It can be the result of old cells dying and being replaced by new ones or it may result from tissue injury. There are different types of regulated or programmed cell death including apoptosis, necroptosis, ferroptosis and pyroptosis. All these processes involve cell membrane permeabilization and

release of cellular components, which induce strong inflammatory responses and contribute to the recruitment of immune cells (Gautheron J. et al, J Hepatol. 2020). In contrast, it also exists a poorly controlled cell death, resulting in the spilling of the cellular contents into the surrounding tissues. This process is called necrosis and was first described by Kerr et al. (Kerr JF. et al, Br J Cancer 1972).

Regulated or programmed cell death is strongly associated with inflammatory liver disease. However, in the beginning, cell death was not associated with inflammation, the two phases in the development of liver disease were never connected. Nowadays, a link between these two pathological events in advanced liver disease has been well established (Canbay A. et al Hepatology 2004). In fact, under pathological conditions in the liver, both resident liver cells (such as Kupffer cells, hepatic stellate cells and sinusoidal endothelial cells) and recruited cells in response to injury (such as monocytes, macrophages, dendritic cells and natural killer cells) release pro-inflammatory signals including cytokines, chemokines, lipid mediators and ROS that contribute to apoptosis (programmed cell death) and necrosis of hepatocytes. This process results in and in an irreversible damage to the liver parenchyma that will evolve into organ failure (Brenner C, J Hepatol. 2013).

Not only the liver undergoes organ failure due to systemic inflammation present in advanced liver disease but there are usually some extrahepatic organ failures which are the following:

- Hepatorenal syndrome: Hepatorenal syndrome is defined by a renal dysfunction that is a consequence of portal hypertension secondary to liver cirrhosis (Gupta K. et al, World J Gastroenterol. 2021). Bacterial infection is usually the precipitating event, especially when an exacerbated inflammatory response develops (Follo A. et al, Hepatology 1994) (Cazzaniga M. et al, J Hepatol. 2009).

- Cardiac dysfunction: Cirrhotic cardiomyopathy includes a wide spectra of hepatic disorders occurring under right-sided heart failure (Møller S. et al, Eur Heart J. 2013). It is characterized by a reduced systemic vascular resistance and arterial blood pressure, that leads to increased heart rate. This causes an hyperdinamic circulation in cirrhotic patients as a result of peripheral and splenic vasodilatation (Møller S. et al, Gut 2008).

- Hepatopulmonary syndrome: Systemic inflammation may also be involved in the pathogenesis of hepatopulmonary syndrome. This syndrome is caused by

intrapulmonary vasodilatation related to increased nitric oxide, a potent vasodilator (Machicao VI. et al, Hepatology. 2014). Bacterial translocation and inflammation induce macrophage recruitment in the lung vasculature, which at their turn will enhance monocyte adherence promoting carbon monoxide production and vascular endothelial growth factors that contribute to vasodilatation and angiogenesis (Zhang J, J Hepatol. 2012).

- Hepatic encephalopathy: It may be manifested as a spectrum, ranging from minimal disturbances in mental function to coma in cirrhotic patients. Hyperammonemia, systemic inflammation and oxidative stress, together with modifications in the glutaminase gene are thought to be key factors in the developing of hepatic encephalopathy. (Romero-Gómez M. et al, J Hepatol. 2015).

- Adrenal insufficiency: It is defined by impaired cholesterol metabolism, dysregulated inflammatory pathways, vascular changes and centralized dysfucntion of the hypothalamic-pituitary-adrenal axis (Wentworth BJ. et al, Liver Int. 2021).

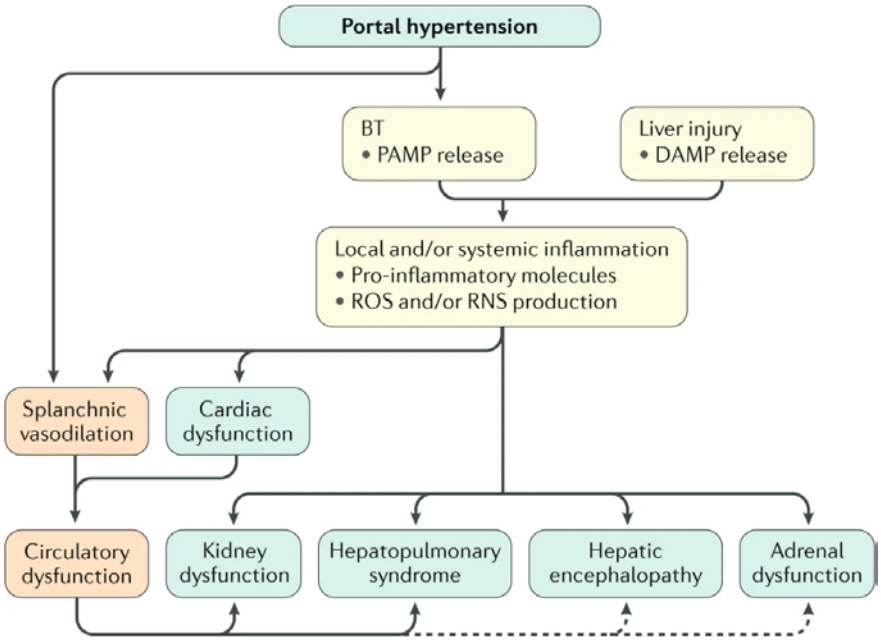

Figure 4. Extrahepatic organ failures in cirrhotic patients. Portal hypertension is a common symptom in cirrhotic patients, that together with PAMPs and DAMPs induce systemic inflammation. The consequent release of pro-inflammatory cytokines and ROS and RNS that contribute to circulatory dysfunction. Eventually, the direct effects of systemic inflammation and circulatory abnormalities will trigger organ failure. BT, bacterial translocation; PAMPs, pathogen-associated molecular patterns; RNS, reactive nitrogen species (Bernardi M. et al, Nat Rev Gastroenterol Hepatol. 2018) (From Arroyo et al, N. Engl. J. Med. 2020).

2. Human serum albumin (HSA)

Albumin is the most abundant protein in the human plasma (3.5-5.0 g/dL, which represents approximately 50% of all plasma proteins) and in extracellular fluids (Bernardi M. et al, Gut 2020). This protein is synthetized in the liver, exclusively by hepatocytes, where it is translated as preproalbumin, cleaved of its N-terminal propeptide in the endoplasmic reticulum, transported to the Golgi and continuously secreted into the bloodstream (Strauss AW. et al, Proc Natl Acad Sci U S A 1977). 30-40% of total albumin in the body can be found in the intravascular compartment, whilst the rest is going to the interstitial space through the large capillary gaps and returned to systemic circulation through the lymphatic system (Peter T.Jr, Academic Press 1995).

Under physiological conditions, 10-15 g of HSA is synthetized daily, and its half-life is around 12-19 days. Given its prolonged half-life, this molecule can suffer from post transcriptional modifications such as oxidation and glycosylation, which contribute to the heterogeneity of albumin (Garcia-Martinez R. et al, Hepatology 2013).

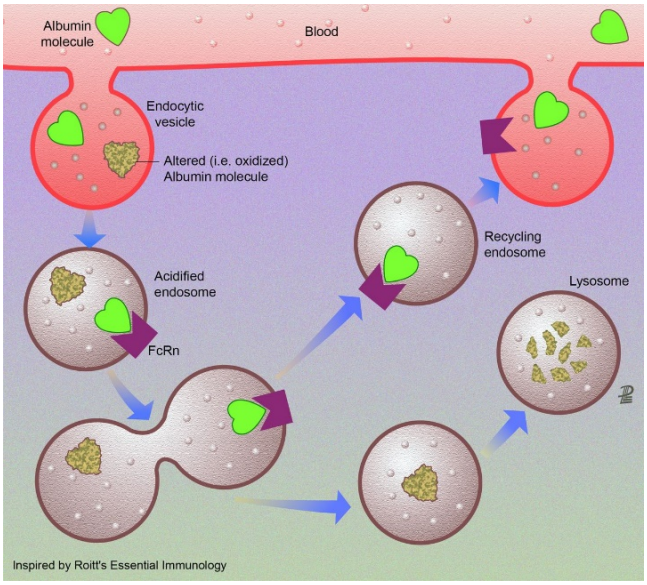

Figure 5. Albumin recycling by endothelial cells. In order to maintain the proper concentration of albumin in serum, endothelial cells recycle albumin. The total amount of albumin in healthy subjects is 360 g, 120 of which are in the intravascular space and 240g are in the extravascular space. Albumin is recycled in the endosomal compartment, in which the low pH promotes the link of healthy albumin with FcRn. After this, when the recycling endosome contacts the higher plasma pH, albumin is released to the systemic circulation (From Bernardi M. et al, Gut 2020).

In the intravascular space it is constantly exchanged in a rate of 4-5%/h (Jagdish RK. et al, Hepatology 2021) (Arroyo V. et al, J Hepatol. 2014), moreover, the constant presence in the circulation is a consequence of from its continuous uptake and recycling by hepatocytes: albumin is transported through the intravascular space, and is internalized by surface cell receptors forming an endocytotic vesicle. Chemically modified albumin (such as oxidized albumin) is degraded in the lysosomes, but another type of receptor called neonatal crystallisable fragment receptor (FcRn) is able to bind albumin and rescue it from lysosomal degradation contributing to extend albumin half-life (represented in Figure 5) (Kim J. et al, Am J Physiol Gastrointest Liver Physiol. 2006) (Andersen JT. et al J Biol Chem. 2011) (Chaudhury C. et al, J Exp Med. 2003).

2.1 Structural and functional properties

Albumin is a hearth shaped protein, negatively charged, with a single polypeptide chain of 585 amino acid (AA) residues with a molecular mass of 66.5 kDa. In 1989, Carter DC et al, were the first to discover the structure of HSA at low resolution (6 Å) and then, He XM et al, from the same laboratory, discovered the high resolution atomic structure (2.8 Å) three years later (Carter DC. et al, Science. 1989) (He XM. et al, Nature 1992). These studies uncovered a structure formed by 34 cysteine residues stabilized by 17 disulfide bonds. It also possesses a free cysteine (Cys-34) residue which accounts for a free thiol group.

The structure of albumin comprises three different domains of similar size formed by eight α-helices, that can be divided in sub-domains (IA, IB, IIA, IIB, IIIA and IIIB) (Peter T.Jr, Academic Press 1995). Domains I and III, which are lobes in the structure, can fold, but domain II, which is the core, cannot fold, providing a framework for the allosteric regulation of albumin. Owing to the molecular structure of albumin, it appears to be a very flexible protein that allows the binding of a wide range of ligands, including small molecules like hormones, fatty acids, bilirubin and heme as well as metals such as Cu, Fe and Hg; but also more complex substances such as LPS (Arroyo V. et al, J Hepatol. 2014) (Bhattacharya AA. et al, J Mol Biol. 2000).

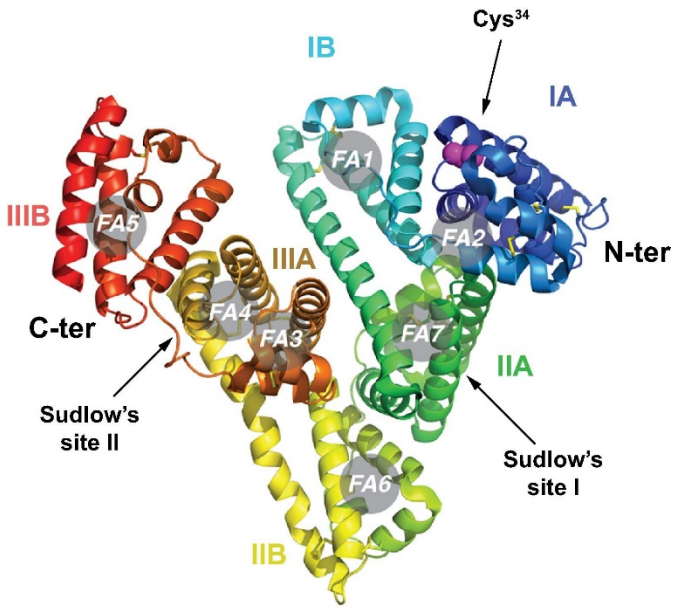

Figure 6. Human serum albumin molecular structure. Human serum albumin molecule with the three homologous domains and subdomains (IA, IB, IIA, IIB, IIIA an IIIB), and main binding sites: fatty acid (FA) 1 to 7, Cys34 represented as purple spheres and Sudlow sites I and II (From Arroyo V. et al, J Hepatol. 2014).

The most important binding sites for ligands that have been reported in albumin are known as Sudlow's sites I and II, which are found in subdomains IIA and IIIA, respectively (Sudlow G. et al, Mol Pharmacol. 1975). Another important binding site includes the multimetal binding site, the Cys-34 site and seven long-chain fatty acid (FA) binding sites (FA1-FA7), as represented in Figure 6. Albumin mainly circulates in a reduced state (mercaptoalbumin, HMA) but the cys-34 residue can be reversible or irreversible oxidized, producing two molecular forms of oxidized albumin called nonmercaptoalbumin (HNA) 1 and HNA2 (Oettl K. et al, J Hepatol. 2013), each of them presenting different grades of pro-inflammatory actions.

Apart from being able to bind exogenous and endogenous ligands, the main function of albumin is to maintain the oncotic pressure of plasma in the blood: albumin contributes to 80% of the oncotic pressure of plasma (25–33 mmHg). It relies on the elevated albumin levels in plasma and its net negative charge, which enables the attraction of sodium and water (Jagdish RK. et al, Hepatology 2021). This property let to the introduction of albumin in medical practice as a plasma volume expander, used in different patients for circulatory support weather they had liver disease or not (Peter T.Jr, Academic Press 1995).

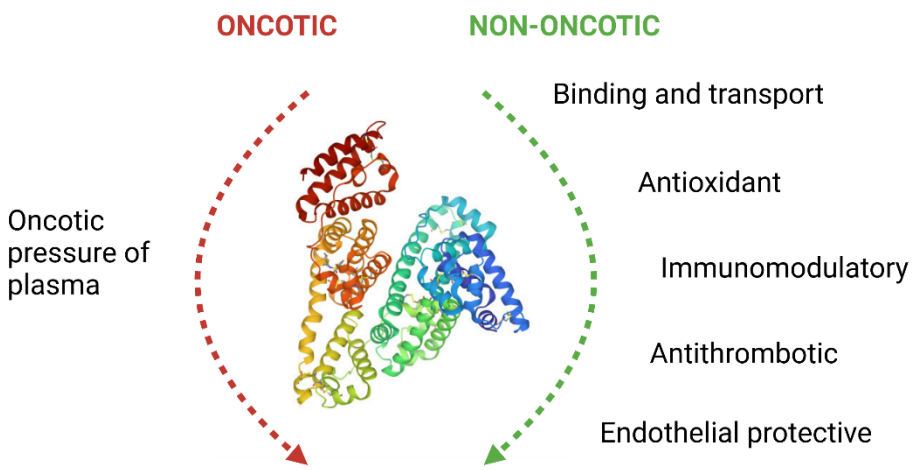

Figure 7. Oncotic and non-oncotic properties of albumin. Beside regulating the oncotic pressure of plasma, albumin has other properties such as binding and transport, antioxidant, immunomodulatory, antithrombotic and endothelial protective.

2.2 Non-oncotic properties of albumin

2.2.1 Binding and transport

Albumin is capable to reversibly bind an extensive range of exogenous and endogenous molecules, allowing solubilisation and transport to distant organs. Due to its structure, it enables the binding at various sites but specially sites I and II (Fasano M. et al, IUBMB Life 2005). Moreover, its negative charge facilitates electrostatic binding of many substances, acting as a storage and vehicle for many compounds.

Structural changes may have functional consequences for albumin because the different ligands can alter the affinity of distant sites through changes in its tertiary structure or compete with each other for a binding site of albumin (Spinella R. et al, Hepatol Int. 2016).

Between the compounds transported by albumin, there are a large number of drugs, bilirubin, bile acids, hormones, metals, anions, L thyroxine, nitric oxide, endotoxin, (Kitano H. et al, Alcohol Clin Exp Res. 1996). and other bacterial products such as the protein G-like albumin binding molecule (Lejon S. et al, J Biol Chem. 2004). Binding to albumin allows to decrease toxicity and increase half-life, which makes albumin a modulator of the pharmacokinetics and pharmacodynamics of many drugs (Fanali G. et al, Mol Aspects Med.

2012). Especial attention is required for fatty acids, due to their low solubility in plasma they need a fatty-acid binding molecule to transport them. Albumin present 7 sites able to bind fatty acids (FA1 to FA7). In fact, a recent study demonstrate that albumin lipid composition is severely impaired in AD cirrhosis patients, and the administration of exogenous albumin to these patients has the ability to modulate the synthesis of pro-resolving lipid mediators (Casulleras M. et al, Hepatol Commun. 2022).

2.2.2 Antioxidant

Albumin is predominantly circulating in a reduced state characterized by the presence of the free thiol group in the Cys-34 residue. This free thiol group acts as a potent free radical scavenger for ROS and reactive nitrogen species (RNS) such as hydrogen peroxide (H_2O_2) and peroxynitrite (Oettl K. et al, J Hepatol. 2013) (Quinlan GJ. et al, Hepatology 2005). Furthermore, albumin also provides antioxidant function by neutralizing free metals at the N-terminal site such as copper and iron, ions involved in catalyzing the production of free radicals (Loban A. et al, Clin Sci (Lond). 1997).

Albumin also exerts a further but less direct antioxidant effect based on inhibition of lipid peroxidation by the binding of albumin and bilirubin (Neuzil J. et al, J Biol Chem. 1994) (Stocker R. et al, Proc Natl Acad Sci USA 1987).

Other studies demonstrated that albumin may also modulate cellular responses to oxidative stress mediating the glutathione redox system (Cantin AM. et al, Am J Respir Crit Care Med. 2000) (Seidkhani-Nahal A. et al, Biotechnol Appl Biochem. 2019) and in addition, in a study of cirrhotic rats with ascites, albumin counteracted the negative effects of oxidative stress on cardiac contractility (Bortoluzzi A. et al, Hepatology 2013).

Therefore, albumin potentially reduces the effects of oxidative stress, especially during systemic inflammation and subsequent organ failure by diverse antioxidant activities.

2.2.3 Immunomodulatory

Albumin plays an important role in modulating the signaling pathways of inflammatory cells and the release of pro inflammatory substances and mediators of inflammation (Garcia-Martinez R. et al, J Hepatol. 2015). On one hand, this ability is related to its endotoxin-binding capacity contributing to the binding of mediators like LPS and other bacterial

antigens (Jürgens G. et al, J Endotoxin Res. 2002) (Dziarski R. et al, J Biol Chem. 1994) (David, S.A. et al, J Endotoxin Res. 1995).

On the other hand, it has recently been demonstrated by Casulleras et al, that albumin is internalized by leukocytes and it inhibits endosomal Toll-like receptor signaling, modulating the immune cell transcriptome. This effect is independent of the scavenging and binding capacity of albumin, thus reaffirming that albumin has intracellular beneficial effects (Casulleras M. et al, Sci Transl Med. 2020).

In addition, albumin inhibits TNFα-induced upregulation of vascular cell adhesion molecule-1 (VCAM-1) and monocyte adhesion via NF-κB inhibition in human aortic endothelial cells, further supporting the hypothesis that albumin enhances intracellular protection against inflammatory and oxidative stress damage (Zhang WJ. et al, Cardiovasc Res. 2002).
Albumin may also be involved in the prevention of neutrophil dysfunction, associated with higher risk of infection, in cirrhotic patients with acute alcoholic hepatitis (Mookerjee RP. et al, Hepatology 2007) (Stadlbauer V. et al, Am J Physiol Gastrointest Liver Physiol. 2009) (Jaisson S. et al, FEBS Lett. 2007). Moreover, in clinical studies from patients with decompensated cirrhosis, albumin treatment reduced systemic inflammation and cardiocirculatory dysfunction (Fernández J., Clària J. et al, Gastroenterology 2019) (Fernández J. et al, Clin Gastroenterol Hepatol. 2020). Furthermore, in patients with cirrhosis and bacterial peritonitis, albumin was associated with a reduction of pro-inflammatory cytokines and endotoxin (Chen TA. et al, Scand J Gastroenterol. 2009). Finally, albumin has been able to reduce circulating prostaglandin E_2 levels, a pro-inflammatory eicosanoid, attenuating immune suppression and reducing the risk of infection in patients with acutely decompensated cirrhosis (O'Brien AJ. et al, Nat Med. 2014).

2.2.4 Antithrombotic

Albumin is capable to bind nitric oxide (NO) at the Cys-34 position preventing rapid inactivation of NO, thus enhancing the vasodilator effect and preventing platelet aggregation (Kim SB. et al, Am J Kidney Dis. 1999) (Tsiountsioura M. et al, Biomedicines 2022).

2.2.5 Endothelium protective

Albumin contributes to the stabilization of the endothelium by physiologically binding within the glycocalyx, thus preserving its integrity. The glicocalyx has antithrombotic and anti-inflammatory functions, plays a key role in vascular homeostasis, regulates vascular permeability and cell adhesion, and acts as a mechanosensor for hemodynamic shear stresses (Aldecoa C. et al, Ann Intensive Care 2020).

In addition, albumin can inhibit neutrophil adhesion mediated by inflammation and oxidative stress, which could provide protection from endothelial dysfunction (Lang JD Jr et al, Anesthesiology 2004); and it helps to maintain endothelial permeability by interaction with the instersitial matrix (Qiao R. et al, Am J Physiol. 1993).

In an *in vivo* study with analbuminemic rats with chronic liver failure, they showed worse hemodynamics associated with markers of endothelial dysfunction and inflammation (Garcia-Martinez R. et al, J Hepatol. 2015). Finally, in a randomized clinical study among cirrhotic patients with spontaneous bacterial peritonitis (SBP), those patients receiving albumin showed as well, an improvement in systemic hemodynamics and endothelial dysfunction markers (Fernández J. et al, Hepatology 2005). These findings highlight the importance of non-oncotic properties of albumin on endothelial stabilization.

2.3 Albumin as treatment for patients with acutely decompensated cirrhosis

It has become evident during the last decade that the persistent systemic inflammation present in advanced cirrhosis induces structural, conformational and molecular changes of albumin that affect its capacity of binding, transport and detoxify. In fact, alterations in temperature, pH and chemical environment present in AD cirrhotic patients results in denaturation of albumin (Yamasaki K. et al, Biochim Biophys Acta 2013) (Das S. et al, Clin Gastroenterol Hepatol. 2018). Oxidative damage of the cys-34 represents the most frequent post-translational alteration, and these oxidized molecules have been demonstrated to activate immune cells and promote inflammation (Das S. et al, Hepatology 2017) (Alcaraz-Quiles J. et al, Hepatology 2018).

Moreover, the constant release of pro-inflammatory cytokines such as TNFα, IL-6 and IL-1β that are shown to inhibit albumin synthesis, and together with a significant loss of hepatocyte mass results in a marked hypoalbuminemia (Jagdish RK. et al, Hepatology. 2021).

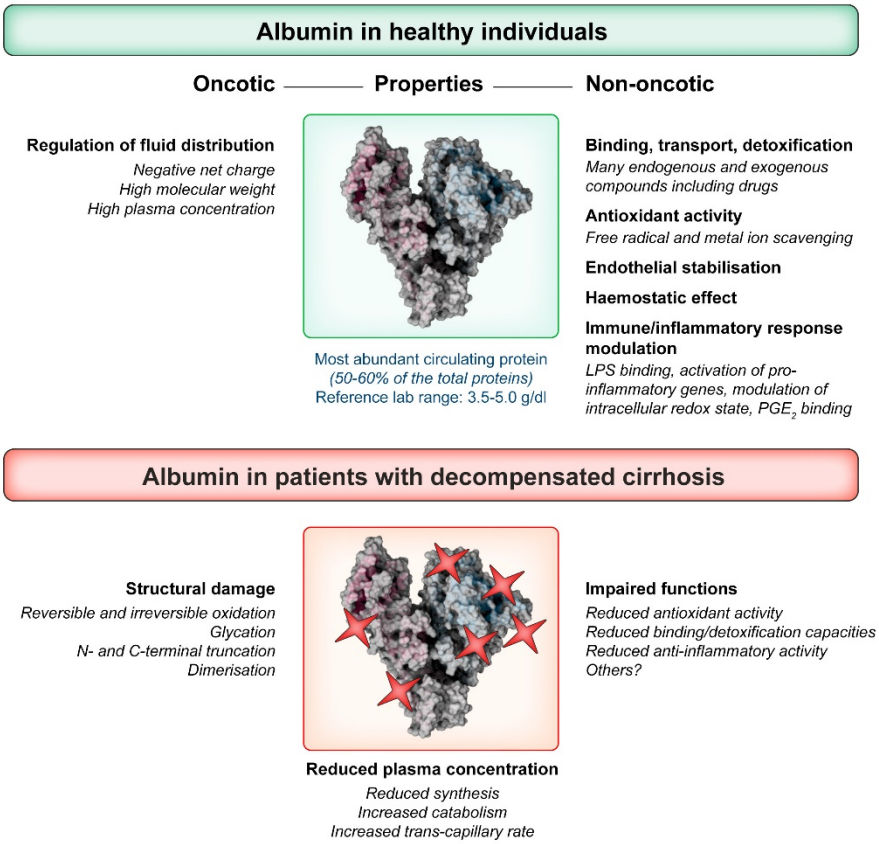

Figure 8. Albumin properties. Comparison between albumin in healthy individuals versus albumin in patients with decompensated cirrhosis. LPS, lipopolysaccharide; PGE2, prostaglandin E2 (From Caraceni P. et al, J Hepatol. 2022).

The administration of albumin started in 1940, when a long-term stable substitute of blood was used to treat shock on the battlefield during the World War II (Peter T.Jr, Academic Press 1995). However, the first indications of albumin in cirrhotic patients were published between 1946 and 1949 and they assessed the effect of short- and long-term intravenous (i.v) infusion of albumin in cirrhotic patients with ascites (Thorn GW. et al, J Clin Invest. 1946) (Kunkel HG. et al, J Clin Invest. 1948) (Faloon WW. et al, J Clin Invest. 1949). Serum albumin concentration and urine volume increased and peripheral edema was improved. However, only some patients improved from ascites.

Later on, a study in 1987 demonstrated that paracentesis (4 liters/day) in association with albumin treatment (8 g per liter of ascitic fluid removed) was an effective therapy for treating ascites (Ginés P. et al, Gastroenterol. 1987). Since then, many studies have been published using albumin for its properties on volume expansion in cirrhotic patients with ascites. In fact, albumin has been shown to improve hypovolemia, reduce the activity of vasoconstrictor systems and increase arterial pressure in patients enduring paracentesis,

having SBP or hepatorenal syndrome (Ginès P. et al, Gastroenterology 1988) (Ginès A. et al, Gastroenterology 1996) (Ortega R. et al, Hepatology 2002).

After this, clinical data and experimental data have showed that albumin has other beneficial properties beyond volume expansion. Several studies have observed the effect of long-term albumin administration in cirrhotic patients, the first one of them was performed by Wilkinson and Sherlok (Wilkinson P, Sherlock S. Lancet 1962) and stated that repeated albumin infusions improved the oncotic pressure in patients with cirrhosis. Recent studies implementing long-term us of albumin in decompensated patients are the following: Pilot-PRECIOSA, ANSWER and MACHT.

The pilot-PRECIOSA (Fernández J., Clària J. et al, Gastroenterology 2019) administered albumin for 12 weeks comparing two dosages of albumin (1,5g/kg weekly versus 1g/kg every two weeks). High doses of albumin were associated with normalization of albumin levels, left ventricular circulatory stability and reduced levels of inflammatory cytokines, without significant changes in portal pressure.
The ANSWER study (Caraceni P. et al, Lancet 2018) compared patients with cirrhosis and uncomplicated ascites that received wither standard medical treatment (SMT) or SMT plus albumin (40 g twice a week for 2 weeks and then 40 g weekly) for up to 18 months. The patients receiving albumin presented a marked reduction in ascites, hepatorenal syndrome, encephalophaty, infections and hospital admissions, which resulted in an amelioration of quality of life and an improvement in survival. This benefits in survival were corroborated by Di Pascolli et al. (Di Pascoli M. et al, Liver Int. 2019) in a non-randomized trial in which 70 patients received albumin infusions.
In contrast, the MACHT study (Solà E. et al, J Hepatol. 2018) that assessed the role of long-term administration of miodrine (an agent that raises the blood pressure) and albumin (40g every 15 days) in decompensated patients, showed no reduction in acute kidney failure, infections, hepatic encephalophaty, gastrointestinal bleeding and mortality at one year.

Afterwards, the ATTIRE study assessed the use of repeated albumin infusions in patients hospitalized for decompensation. Those patients presented a serum albumin level minor than 30 g per liter and they received either 20% of human albumin solution for up to 14 days or standard care. The results showed that albumin infusions increased the concentration of albumin in those patients but apart from that no beneficial effects were found regarding acute kidney injury, infections or death. Furthermore, this study reported

an increase of adverse effects in patients treated with albumin infusions, such as fluid overload and pulmonary edema (China L. et al, N Engl J Med. 2021).

The most recent clinical trial with albumin (ALPS trial) assessed the administration of 20% albumin (0.5-1.0 g/kg over 3 hours) versus the administration of plasmalyte (a family of balanced crystalloid solutions that have beneficial effects on volume and electrolyte deficit while addressing acidosis, 30 ml/kg over 3 hours). The results indicated that albumin administration is linked to a faster improvement in hemodynamics and lactate clearance than plasmalyte, while 28-day survival was similar. However, patients administered with albumin were more prone to have pulmonary complications (Maiwall R. et al, J Hepatol. 2022).

These divergent results highlight that albumin is effective in some AD cirrhotic patients but not others. The ANSWER, the MACHT and the ATTIRE study contrasted in design, patient characteristics, dosage and timing of albumin administration and length. The conclusions of this comparisons would be that short-term albumin treatment has been clearly demonstrated to be effective in patients enduring SBP (Fernández J. et al, Hepatology 2005) or hepatorenal syndrome (Wong F. et al, N Engl J Med. 2021). On the other side, long-term human albumin administration arises as a safer and more effective way of treating cirrhotic patients, but there is the clinical need to assess larger-scale studies in order to maximize the benefits of albumin treatment.

Finally, there are other clinical studies that use albumin in extracorporeal liver assist devices for patients with ACLF. The molecular adsorbent recirculating system (MARS) uses albumin dialysis and the Prometheus system uses fractionated plasma separation and provide detoxification by removal of protein-bound and water soluble substances (Nevens F. et al, Best Pract Res Clin Gastroenterol. 2012). Such devices have been shown to improve haemodynamics and reduce portal pressure. Specially MARS ameliorated hepatic encephalopathy and improved individual organ dysfunction but no significant survival benefit was shown (Jalan R, Williams R. Liver Transpl. 2001) (Bañares R. et al, Hepatology 2013). The Prometheus system showed similar results in terms of survival in patients with ACLF (Kribben A. et al, Gastroenterology 2012).

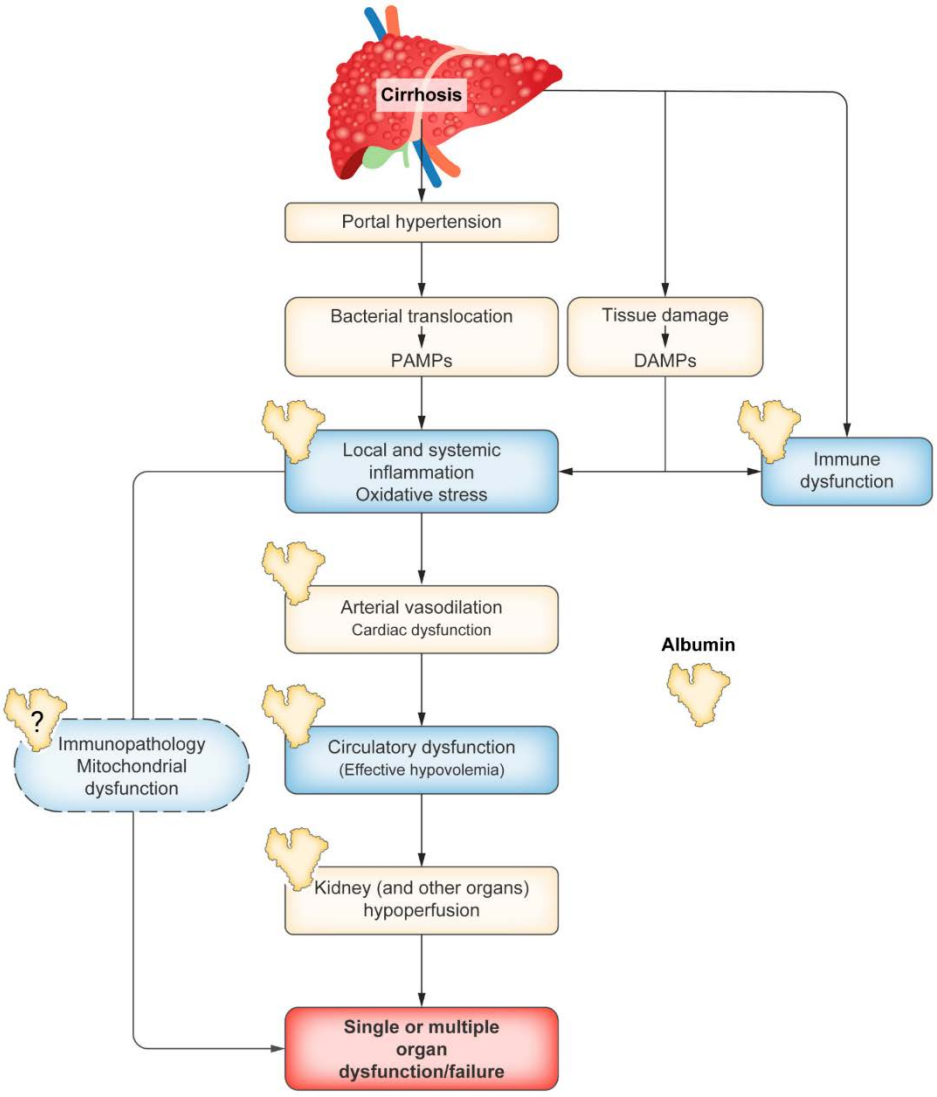

Figure 9. Albumin effects in cirrhotic patients. The oncotic and non-oncotic properties of the albumin molecule exert different beneficial effects on the pathophysiological events present in cirrhotic patients with ascites (Modified from Caraceni P. et al, J Hepatol. 2022).

Nonetheless, the latest studies regarding albumin dialysis and plasma exchange in cirrhotic patients, showed an improvement in survival (Bañares R. et al, Therap Adv Gastroenterol. 2019) and highlight their important as a bridge therapy whilst the patients are waiting for liver transplantation.

A device called DIALIVE aims to remove and replace the damaged albumin in cirrhotic patients and also remove PAMPs and DAMPs (Lee KC. et al, J Hepatol. 2015). It has been

tested in an acetaminophen-induced acute liver failure in pigs and it reduced the risk of death and was associated with a decrease in circulating HNA2.

In summary, there are a high number of clinical and therapeutic applications for albumin that call attention to the importance to the understanding of albumin biology and function in cirrhosis.

3. Cytokine-induced liver cell death

3.1. TNFα-induced cell death

TNFα was first identified in 1975 by Lloyd Old and Carswell as an endotoxin-induced serum factor that caused the necrosis of tumors in a murine study (Carswell EA, Old LJ. et al, Proc Natl Acad Sci USA.1975). Since then, many studies were needed to characterize and isolate this cytokine that is involved not only in inflammatory responses but also in cellular communication, cell differentiation and cell death.

TNFα is a pleiotropic cytokine produced mainly by activated macrophages, T-lymphocytes, and natural killer cells (Jang DI. et al, Int J Mol Sci. 2021). However, a wide range of other cells can also release this cytokine, including B-lymphocytes, neutrophils, endothelial cells, etc. (Jang DI. et al, Int J Mol Sci. 2021). TNFα is primarily produced in stable trimers as a type II transmembrane protein of 26 kDa with its N-terminus inside the cytoplasm and C-terminus outside the cell. This pro-TNFα is released via proteolytic cleavage by the metalloprotease TNFα converting enzyme (TACE, also known as ADAM-17) becoming a mature soluble 17 kDa protein. Both the cell-associated 26 kDa and secreted 17 kDa forms require trimerization and present both overlapping and distinct biological activities (Kriegler M. et al, Cell. 1988) (Tang P. et al, Biochemistry. 1996) (Wajant H. et al, Cell Death Differ. 2003) (Palladino MA. et al, Nat Rev Drug Discov. 2003).

TNFα exerts its biological functions through the binding by two membrane receptors: TNF-R1 and TNF-R2. These receptors share the same structure and function in the extracellular domains, but their intracellular domains are different and induce distinct signaling pathways. The main feature that distinguishes the intracellular domains is the presence of a death domain in the TNF-R1, which is absent in TNF-R2. TNF-R1 is expressed constitutively in all cell types, in fact, no cell type in the body has yet been described to not express TNF-R1. Moreover, TNF-R1 can be activated by both soluble and transmembrane TNFα. Expression of TNF-R2 instead is restricted to specific cell types, mainly immune cells and endothelial cells, and it is activated by transmembrane TNFα (Kalliolias GD. et al, Nat Rev Rheumatol. 2016).

Some studies propose that TNF-R1 primarily promotes inflammation and tissue degeneration, whereas TNF-R2 plays homeostatic roles such as cell survival and tissue regeneration. In fact, activation of TNF-R1 can induce apoptosis in sensitized cells but

mostly it is no cytotoxic and induces direct pro-inflammatory signaling (Probert L. Neuroscience 2015). Other studies suggest that the two TNFRs transduce their signals cooperatively, TNF-R2 can trigger more induction of TNFα and then the signaling pathway through TNF-R1 is increased (Aggarwal BB. et al, Nat Rev Immunol. 2003).

The death domain present in TNF-R1 enables the recruitment of the adaptor molecule TNF-R1-associated death domain protein (TRADD), a key component of the TNF-R1 signaling complex, which is not recruited by TNF-R2. In contrast, TNF-R2 recruits TNFR-associated factor 1 (TRAF1) and TRAF2. All these signaling pathways, TNF-R1-TRADD and TNF-R2 trough TRAF1 and TRAF2, can trigger nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation. Activation of NF-κB leads to target genes that are important in inflammation, host defense, and cell proliferation and survival (Kalliolias GD. et al, Nat Rev Rheumatol. 2016) (Brenner D. et al, Nat Rev Immunol. 2015). Nonetheless, the engagement of TNF-R1-TRADD may result in either cell survival or cell death depending on cellular context, whilst TNF-R2-TRAF will never be able to trigger cell death.

In advanced liver disease, large amounts of TNFα are released in response to LPS and other bacterial products. This cytokine in the liver can restore liver mass by inducing hepatocyte proliferation but it is also a mediator of hepatotoxicity (Schwabe RF. et al, Am J Physiol Gastrointest Liver Physiol. 2006).

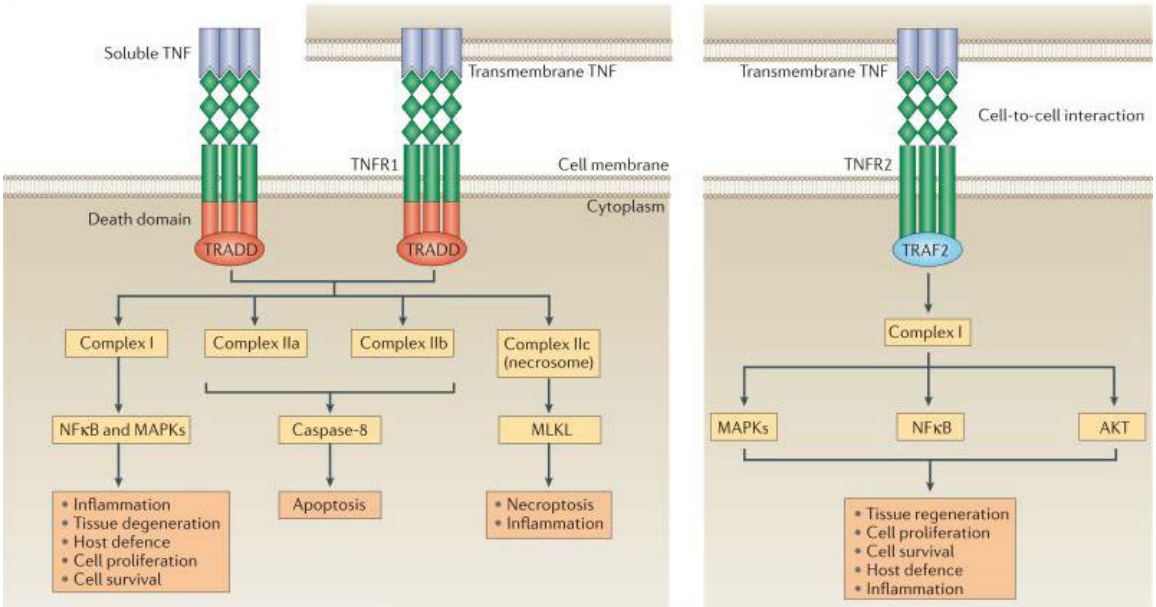

Figure 10. TNFα signalling. TNF receptor 1 (TNF-R1) can be activated by soluble and transmembrane TNFα. TNFR1 have a death domain that allows the recruitment of the adaptor molecule TNF-R1-associated death domain protein (TRADD) that trigger the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation. Activation of NF-κB leads to target genes that are important in inflammation, host defense, and cell proliferation and survival. Ligation of TNF-R1 also leads to the activation of caspase 8 and it is associated with programmed cell death. Ligation of TNF-R2 with transmembrane TNFα recruits TNFR-associated factor 2 (TRAF2) mediating tissue regeneration, cell proliferation and cell survival via the activation of NF-κB. This pathway can also induce inflammation and host defense against pathogens (From Kalliolias GD. et al, Nat Rev Rheumatol. 2016).

3.2. Transcriptional arrest

TNFα does not induce cell death unless protein synthesis is blocked, suggesting the presence of cellular factors that can inhibit the apoptotic pathway induced by TNFα (Ashkenazi A. et al, Science 1998). When TNFα interacts with its receptor, NF-κB is activated and it mediates the transcription of pro-survival genes such as those encoding cellular FLICE-like inhibitory protein (c-FLIP), apoptosis regulator BCL-XL (also known as BCL2L1) or X-chromosome linked inhibitor of apoptosis protein (XIAP) (Van Antwerp DJ. et al, Trends Cell Biol. 1998) (Wajant H. et al, Cell Death Differ. 2003) (Schwabe RF, Luedde T. Nat Rev Gastroenterol Hepatol. 2018). This process is tightly regulated and cell death pathways in response to TNF signaling are controlled by diverse post-transcriptional modification steps, which means that the first response of the cell to TNFα would be cell survival and not cell death.

An important molecular switch involved in mediating cell survival or apoptosis is the ubiquitylation status of receptor-interacting serine/ threonine-protein kinase 1 (RIPK1). The polyubiquitylation of RIPK1 bound to TRADD is essential for activation of NF-κB and its subsequential expression of pro-survival genes. It also prevents cell death induction, such that the cell survives (Li, H. et al, J. Biol. Chem. 2006). In contrast, when RIPK1 is not ubiquitylated, there is a stop in NF-κB activation, leading to the formation of protein complexes that promote the apoptotic signaling pathway and the cell dies (Brenner D. et al, Nat Rev Immunol. 2015).

To induce cell death, it is necessary to inhibit the transcription of the pro-survival genes induced by NF-κB. In in vitro experiments with TNFα, it is very common the use of actinomycin D, which binds to the DNA pt within the transcriptional complex and immobilizes the complex, interfering with the elongation of growing RNA chains, as an inhibitor of the transcription actinomycin D (Sobell HM. Proc Natl Acad Sci USA. 1985) (Polunovsky VA. et al, Exp Cell Res. 1994) (Beyaert R. et al, FEBS Lett. 1994) (Kirstein M., Baglioni C. J Biol Chem. 1986) (Roulston A. et al, J Biol Chem. 1998). The result of this blockade of the transcription prevent the NF-κB-mediated induction of pro-survival genes.

3.3. Hepatocytes and cell death

Hepatocytes represent 70–85% of the liver volume (Zhou Z. et al, Cell Mol Immunol. 2016). Between its endless functions, they are in charge of the synthesis and secretion of plasma proteins, especially albumin, coagulation factors and acute phase proteins. Hepatocytes metabolize and store gut-derived nutrients and they are able to generate glucose under conditions of starvation. Hepatocytes are also a key component of the regulation of lipid metabolism and they are the only cell type in the body that can synthesize bile acids (BAs) by *de novo* synthesis from cholesterol. These cells also provide a function of detoxification of endogenous and exogenous compounds (Guicciardi ME. et al, Compr Physiol. 2013).

Under physiological conditions, liver cells are in standby in G0 phase with little turnover and almost no cell death. In advanced liver disease, the presence of bacterial byproducts, cytotoxic molecules and pro-inflammatory cytokines between others, can perturbate this state of the cells thus inducing hepatocellular cell death, followed by inflammation (Schwabe RF, Luedde T. Nat Rev Gastroenterol Hepatol. 2018). The presence of high levels of TNFα produced by immune cells, together with the perturbated state of the cells results in massive loss of hepatocytes hence causing an irreversible damage to the liver parenchyma. Each cell in the liver is susceptible to different type pf damage and play a role in clinical syndromes, specially hepatocyte injury, which results in liver dysfunction (Malhi H. et al, Physiol Rev. 2010).

TNF-induced liver cell death occurs mainly by apoptosis, although other forms of cell death such as necroptosis and pyroptosis may also occur. Apoptosis is a programmed cell death that is characterized by chromatin condensation, DNA fragmentation, plasma membrane blebbing and finally cell fragmentation into apoptotic bodies (Guicciardi ME. et al, Compr Physiol. 2013). In the cell, apoptosis is executed by the activation of different caspases,

which kill cells through the cleavage of proteins and subsequent activation of nucleases that at its turn will cleave DNA into fragments (McIlwain DR. et al, Cold Spring Harb Perspect Biol. 2013). In contrast, necrosis is a form of cell death that is caused by external factors such as infection that results in a passive and uncontrolled autolytic loss of cellular integrity (Majno G, Joris I. Am J Pathol. 1995). Apoptosis may provide beneficial effects to the organism but necrosis is usually detrimental and damaging.
Cell death in the liver ensure efficient cell regeneration, however, in the setting of advanced liver disease where there is an acute hepatocellular injury, chronic hepatocyte death and the consequent inflammation present in the liver are closely associated with the development of fibrosis and cirrhosis (Schwabe RF, Luedde T. Nat Rev Gastroenterol Hepatol. 2018).

Disturbances of hepatic function in advanced liver diseases are very common and are linked to the activation of the innate immune system that can cause liver stress, compromising hepatocyte survival thus increasing cell death (Malhi H. et al, Physiol Rev. 2010). In fact, several studies strongly associate hepatocyte apoptosis with inflammation and fibrosis present in advanced liver diseases (Takehara T. et al, Gastroenterology) (Vick B. et al, Hepatology 2009).

3.4. TNFα induced-apoptotic pathway

Apoptosis can be mediated by the extrinsic pathway, which is triggered by the binding of death receptors to their ligands, or by the intrinsic pathway that can be induced by different intracellular stress inducers such as DNA damage, oxidative stress, toxins and endoplasmic reticulum (ER) stress. Intrinsic apoptosis pathways converge at the mitochondria, inducing mitochondrial dysfunction and mitochondrial outer membrane permeabilization (MOMP) (Guicciardi ME. et al, Compr Physiol. 2013).

Apoptosis induced by TNFα is included in extrinsic apoptosis due to the need of the binding of death receptor (TNF-R1) to its ligand (TNFα). Binding of TNFα to TNF-R1 results in the recruitment TRADD, which at its turn will interact with Fas-associated protein with death domain (FADD) and with RIPK1, which when it is not ubiquitylated trigger the activation of apoptotic pathway through the formation of a platform for the activation of caspase 8. Active caspase 8 then starts a proteolytic cascade that finishes in the activation of the effector caspases: caspase 3, 6 and 7, leading to the cleavage and degradation of cellular proteins thus inducing cell death (Micheau O, Tschopp J. Cell. 2003) (Brenner C. et al, J

Hepatol. 2013). In this stage, there is a classification of cells into type I death receptor signaling (such as lymphocytes) and type II signaling (such as hepatocytes and cholangiocytes). Type I cells activate substantial amounts of caspase 8 that directly induces the apoptotic pathway through the cleavage of procaspase 3 by caspase 8 directly. In opposition to type I cells, type II cells activate less caspase 8 and they need the caspase 8-mediated cleavage of BH3 interacting-domain death agonist (Bid) and subsequent signal amplification through the mitochondrial apoptotic pathway to activate enough caspase 3 to kill the cell (Meng XW. et al, J Biol Chem. 2011) (Luo X. et al, Cell. 1998).

The mitochondrial apoptotic pathway activates effector caspases after MOMP that results in the release of cytochrome c and second mitochondrial activator of caspases/direct IAP binding protein with low PI (SMAC/DIABLO) (Guicciardi ME, Gores GJ. L FASEB J. 2009). SMAC/DIABLO is able to bind and inhibit XIAP, which is an inhibitor of effector caspases, thus allowing the apoptotic cascade to proceed. Meanwhile, the cleavage of Bid by caspase 8 mediates mitochondrial dysfunction, generating a truncated fragment of Bid (tBid) that cooperates with the proapoptotic proteins Bax and Bak to induce more MOMP. At its turn, cytochrome c forms a complex called apoptosome with procaspase 9 that induces caspase 9 activation followed by the activation of the effector caspases 3, 6 and 7 (Guicciardi ME. et al, Compr Physiol. 2013).

TNFα-induced apoptosis has also other signaling pathways. TNFR1 can also activate -c-Jun N-terminal kinase (JNK) which is a mitogen-associated protein kinase strongly associated with hepatocyte injury (Seki E. et al, Gastroenterology. 2012). JNK activation is associated with cell survival and proliferation, but TNFα induction of ROS contribute to prolonged activation of JNK and shifts the balance towards cell death (Schwabe RF. et al, Am J Physiol Gastrointest Liver Physiol. 2006).

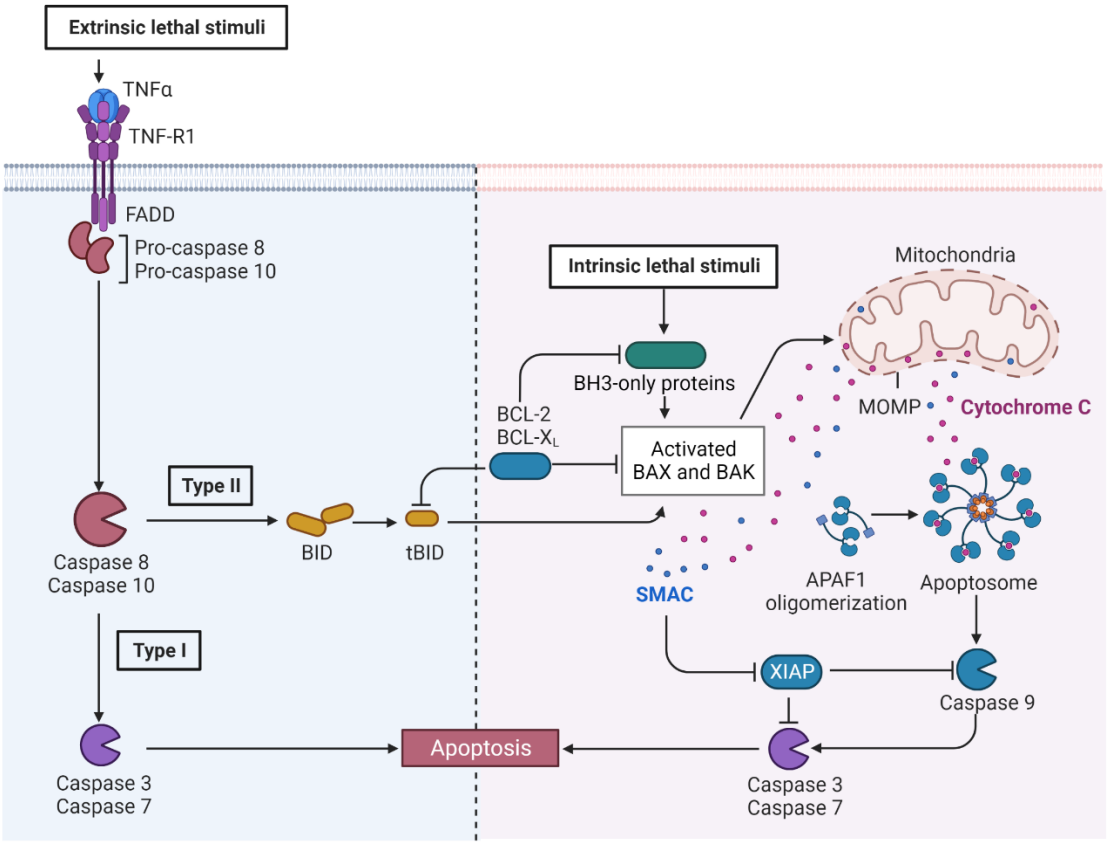

Figure 11. TNFα-induced apoptosis. Binding of TNFα to TNF-R1 results in the recruitment TRADD and FADD that trigger the activation of the extrinsic apoptotic pathway by the activation of caspase 8. Type I cells induce directly the apoptotic pathway through the cleavage of procaspase 3 by caspase 8. Instead, type II cells (hepatocytes and cholangiocytes) need the caspase 8-mediated cleavage of BH3 interacting-domain death agonist (Bid) and subsequent signal amplification through the mitochondrial apoptotic pathway to activate enough caspase 3 to kill the cell. Intrinsic apoptotic stimuli can lead to apoptosis directly through the mitochondrial apoptotic pathway, that induces mitochondrial outer membrane permeabilization (MOMP) through the activation of the proapoptotic proteins Bax and Bak. The mitochondria release cytochrome c, which forms the apoptosome and eventually activates caspase 3. Created with Biorender.com.

3.4.1. The lysosomal system in cell death

The endocytic pathway is specialized in the internalization of compounds from the cell microenvironment for their recycling or degradation. Internalized molecules are driven

inside early endosomes where they are sorted. Some molecules are recycled back to the plasma membrane to be reused, but some are transported to late endosomes and lysosomes for degradation (Gruenberg, J. Nat Rev Mol Cell Biol 2001). Endosomes can signal the presence of pathogens through Toll-like receptors, they can generate antigenic peptides and they can also kill pathogens, between other functions in fighting infections (Gruenberg J, van der Goot FG. Nat Rev Mol Cell Biol. 2006).

Lysosomes were first discovered by de Duve in 1955 (de Duve C. et al, Biochem J. 1955) which established these organelles as degradative and metabolic centers in the cells. Lysosomes have a single membrane that contain hundreds of integral and peripheral membrane proteins. The lysosomal lumen is acidic, its low pH (4.5-5.5) allows the activation of hydrolases that digest macromolecules such as proteins, nucleic acids, lipids and carbohydrates (Yang C. et al, J Cell Biol. 2021). Because of the high content of proteases, de Duve called lysosomes "suicide bags".

The role of lysosomes in cell death is discovered in necroptosis and necrosis, where lysosomal membrane permeabilization (LMP) is a late process that together with the cellular disintegration phase caused by proteolysis contribute to the generation of PAMPs, which activate the immune system (Vanden Berghe T. et al, Cell Death Differ. 2010). Nonetheless, LMP can also be involved in apoptosis, either initiating or amplifying the process. There is a relationship between the amount of lysosomal break and the mode of cell death induced by LMP: modest injury implies a limited release of lysosomal content into the cytosol leading to apoptosis. In contrast, stronger injury results in a complete release of lysosomal contents leading to necrosis (Kågedal K. et al, Biochem J. 2001) (Turk B, Turk V. J Biol Chem. 2009). Therefore, lysosomes play a very interesting and regulated role in inducing cell death.

As described above, apoptotic cell death can be mediated by the extrinsic (or death receptor) pathway, and the intrinsic or mitochondrial pathway, which is induced by metabolic stress, UV radiation and DNA damage. Regularly, LMP can trigger the intrinsic apoptotic pathway because it is induced usually by numerous agents and molecules of endogenous or synthetic origin. To begin with, ROS are possibly the most important endogenous LMP inducer (Kurz T. et al, Biochim Biophys Acta. 2008). As a result of oxidative stress, hydrogen peroxide is produced in the mitochondria and it is not completely detoxified by the cell. This hydrogen peroxide is then able to diffuse into the lysosomes, which would cause peroxidation of membrane lipids and result into the release of the

lysosomal content. Moreover, lysosomal enzymes promote ROS generation acting upon mitochondria, thus creating a loop that leads to more lysosomal damage (Česen MH. et al, Exp Cell Res. 2012).

LMP can also be triggered by lysosomotropics, which are compounds that accumulate in the lysosomes and when they reach a high concentration inside the lysosome, they are able to induce permeabilization and release of the lysosomal constituents (Miller DK. et al, J Cell Biol. 1983). Among the lysosomotropics compounds, the most important is sphingolipid sphingomyelin, which in the lysosomes is converted to sphingosine. Interestingly, the generation of sphingosine is increased in TNFα-induced apoptosis (Schütze S. et al, J Biol Chem. 1999) which is associated with lysosomal permeabilization (Guicciardi ME. et al, J Clin Invest. 2000).

TNFα, as an inducer of the extrinsic apoptotic pathway, is therefore, also involved in lysosomal permeabilization. Several mechanisms have been proposed regarding the induction of LMP in TNFα-induced apoptosis. As described in section **3.4**, TNFα recruits and activates caspase 8 that starts apoptotic cascades via different mechanisms. It can directly activate effector caspases 3, 6 and 7 leading by a direct route to apoptosis. In addition, caspase 8 can cleave Bid and induce cytochrome c release from the mitochondria, resulting in the formation of the apoptosome and subsequent activation of effector caspases. Furthermore, there is also another pathway, in which TNFα-associated cytotoxic signaling results in permeabilization of lysosomes, which induces the release of cathepsin B in the cytosol and cathepsin B then initiates the mitochondrial pathway of apoptosis (Werneburg NW. et al, Am J Physiol Gastrointest Liver Physiol. 2002) (Foghsgaard L. et al, J Cell Biol. 2001). Gyrd-Hansen et al, propose that when TNFα activates caspase 8, it cleaves caspase-9, that is the caspase that induce lysosomal permeabilization (Gyrd-Hansen M. et al, Mol Cell Biol. 2006). Cathepsins released from the lysosome cleave the BCL-2 family member Bid into tBid, that promotes the oligomerization of proapoptotic Bax and Bak protein, which in turn triggers MOMP (Repnik U. et al, Cold Spring Harb Perspect Biol. 2013).

Several studies have demonstrated the contribution of the TNFα-induced lysosomal release of cathepsin B to the lysosomal-mediated pathway of apoptosis in hepatocytes (Guicciardi ME. et al, J Clin Invest. 2000) (Guicciardi ME. Et al, Am J Pathol. 2001) and in hepatic injury and fibrosis (Canbay A. et al, J Clin Invest. 2003), strongly suggesting that cathepsin B-mediated apoptosis have a key role in the induction and progression of liver diseases. The most intriguing finding in the liver is that lysosomal permeabilization is mediated by members of the Bcl-2 protein family that regulate apoptosis. Evidence show the

involvement of Bax, Bim, Mcl-1 and Bid in lysosomal permeabilization in different models of liver injury. Feldestein et al showed data demonstrating that free FA (FFA) treatment of liver cells resulted in Bax translocation to lysosomes and lysosomal destabilization, resulting in the release of cathepsin B (Feldstein AE. et al, Hepatology 2004). Werneburg et al also demonstrate in different studies that TNFα-mediated lysosomal permeabilization is caspase 8/Bid dependent (Werneburg N. et al, Am J Physiol Gastrointest Liver Physiol. 2004), Bim and Bax are also involved in lysosomal permeabilization (Werneburg NW. et al, J Biol Chem. 2007) and the translocation of this proteins to the lysosomes is mediated by phosphofurin acidic cluster sorting protein-2 (PACS-2), a protein that regulates membrane trafficking (Werneburg NW. et al, J Biol Chem. 2012).

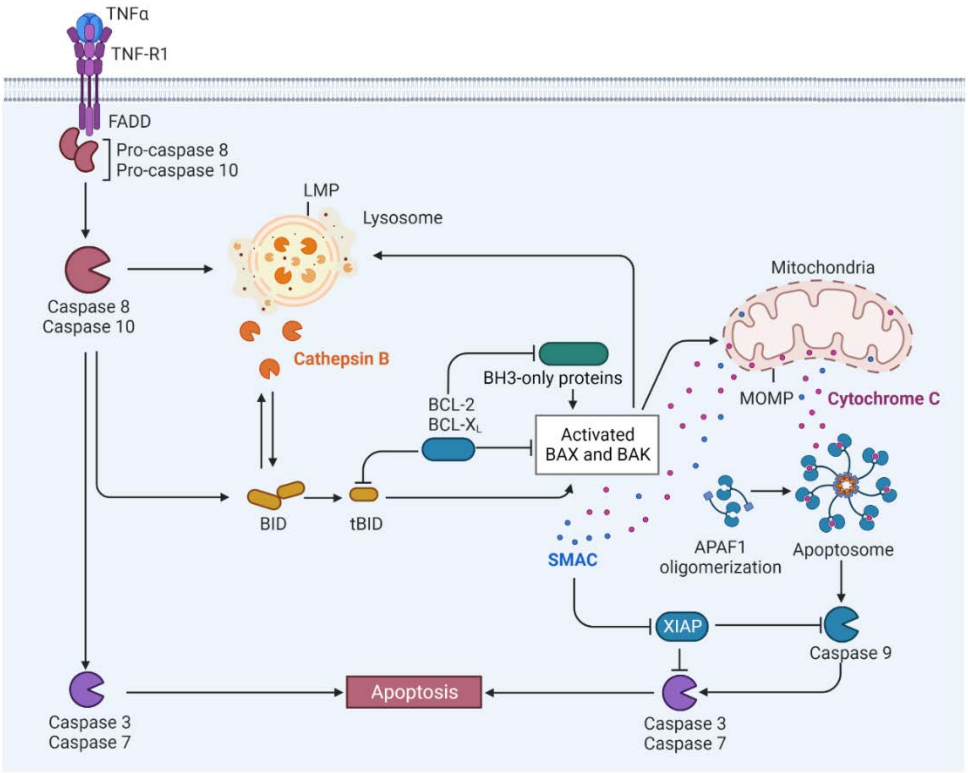

Figure 12. The lysosomal system in TNFα-induced apoptosis. Summary of the pathway of apoptosis induced by TNFα adding the lysosomal pathway. Created with Biorender.com.

3.4.1.1. Cathepsin B

Cathepsins are proteases, enzymes that degrade proteins and are in charge of the digestion of all the major cellular macromolecules. They are activated by the low pH found in

lysosomes. Cysteine protease cathepsin B specially, plays a major role in the execution of the apoptotic dell death (Boya P, Kroemer G. Oncogene. 2008) (Repnik U. et al, Biochim Biophys Acta. 2012).

Cathepsin B contributes to toxic bile salt-induced hepatocyte apoptosis (Roberts LR. et al, Gastroenterology 1997) (Jones B. et al, Am J Physiol. 1998). Another study demonstrates that pharmacological inhibition of cathepsin B blocks apoptosis induced by cytotoxic agents (Lotem J. et al, Proc Natl Acad Sci USA. 1996) in myeloid leukemia cells. In the same line, Canbay et al showed that inhibition of cathepsin B significantly reduced hepatocyte apoptosis and liver injury (Canbay A. et al, J Clin Invest. 2003). Finally, Guicciardi et al, defined cathepsin B apoptotic pathway highlighting the role of this protease promoting mitochondrial release of cytochrome c in TNFα-mediated hepatocyte apoptosis (Guicciardi ME. et al, J Clin Invest. 2000).

3.4.2. Caspase cascade

Apoptosis is initiated and executed by the activation of caspases, a cysteine-dependent aspartate specific protease enzyme that are synthetized as zymogens (an inactive enzymatic precursor). From several years, caspases have been divided into pro-inflammatory or apoptotic but some of the caspases attributed to be apoptotic (caspase 2, 3, 6, 7, 8, 9 and 10) have at least one non-apoptotic role. The apoptotic group of caspases was then classified into initiators or apical caspases versus executioners or effector or downstream caspases, distinguishing the caspases that activate the cascade (caspase 8, 9 and 10) than the caspases activated by the initiators to execute apoptosis (caspase 3, 6 and 7) (Pop C, Salvesen GS. J Biol Chem. 2009).

Initiator caspases are activated by dimerization, they are monomers that require homodimerization for activation. This process is facilitated by platforms of recruitment with adaptor molecules that specifically bind caspases. Activation of initiator caspases is strongly regulated and the assembly of the activation complexes is required. For caspase 8 and 10, which is activated via death receptor ligation, the activating complex is called death-inducing signaling complex (DISC), and for the assembly of caspase 9, the activating complex is called the apoptosome (Langlais C. et al, Cold Spring Harb Protoc. 2015).

Executioner or effector caspases are activated by cleavage of the catalytic domain. The dimerization that is also key to activation, has already occurred after their synthesis and the

zymogens are controlled by a short linker that separated the large and the small subunits of the catalytic domain. The cleavage of intersubunit linkers, made of aspartate residues is what induces the activation (Pop C, Salvesen GS. J Biol Chem. 2009).

Following activation, both initiator and executioner caspases would need additional proteolytic events in order to mature the caspases to more stable forms.

3.4.3. Mitochondrial cytochrome c leakage

The mitochondrial apoptotic pathway is closely regulated by the Bcl-2 family of proteins, which act upstream and downstream and at the level of the mitochondria to integrate death and survival signals (Guicciardi ME. et al, Compr Physiol. 2013). This family of proteins are classified into three main subclasses comprising the antiapoptotic proteins that promote cell survival (Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1), the proapoptotic proteins (Bac, Bak and Bok) and finally another type of proapoptotic proteins which function is activate Bax and Bak (Bid, Bim, Bad, Bik, Bmf, Hrk, Noxa and Puma, named BH3-only proteins) (Youle RJ, Strasser A. Nat Rev Mol Cell Biol. 2008).

After different intracellular stress signals, the BH3-only proteins are activated and they activate at its turn Bax and Bak. Bax and Bak experiment conformational changes when they are activated, which allow them to insert in the outer mitochondrial membrane and perform oligomerization into large molecular complexes to form pores, resulting in MOMP (Nechushtan A. et al, J Cell Biol. 2001). The loss of the mitochondrial outer membrane integrity results in the release of several proapoptotic proteins into the cytosol: cytochrome c, SMAC/DIABLO, apoptosis-inducing factor (AIF) and endonuclease G. Cytochrome C and SMAC/DIABLO contribute to the apoptotic cascade by activating the effector caspases, and AIF and endonuclease G translocate to the nucleus mediating DNA fragmentation (Guicciardi ME. et al, Compr Physiol. 2013).

In the liver, it has been found that the Bcl-2 protein members Bcl-xL and Mcl-1 are highly expressed in hepatocytes (Takehara T. et al, Gastroenterology) and apoptosis caused by its deficiency is found to be dependent on Bid truncation (Hikita H. et al, Hepatology. 2009). Also, several studies demonstrate that Bid contributes to TNFα-induced hepatocyte apoptosis (Kaufmann T. et al, Immunity 2009) (Guicciardi ME. et al, Gastroenterology 2005).

3.5. Cell death in patients with cirrhosis

Cell death is present in patients with advanced liver disease. Indeed, commonly used biomarkers of liver injury are based on the measurement of alanine transaminase (ALT), and AST, which are released into the serum after hepatocyte death. (Guicciardi ME, Gores GJ. Semin Liver Dis. 2010). The ACLF syndrome, the most advanced and severe stage of live cirrhosis is driven by systemic inflammation induced by cytokine storm, oxidative stress, immune dysfunction and bacterial infections (Moreau R. et al, Gastroenterology 2013). ACLF is defined by the failure of hepatic and extrahepatic organs, therefore cell death plays an important role. To begin with, cell death residues (apoptotic bodies) constitute DAMPs that drive more inflammation which directly perpetuate cell death and mediate additional organ failures (Macdonald S. et al, Hepatology 2018). Moreover, one of the cytokines that the immune cells release under conditions of systemic inflammation is TNFα, an important cell death ligand which also contributes to more hepatocyte cell death (Clària J. et al, Hepatology 2016). Finally, apoptotic cells can also induce inflammation, particularly when apoptotic cells escape efferocytosis, which is the process of removal of dead cells by phagocytic cells (Ravichandran KS. Immunity 2011).

As the mechanisms that drive apoptosis are well defined, the methods of detection of cell death in the liver of patients can be driven by histological tests. For instance, the detection of cleaved caspase 3 is very common to use as a detection marker of cell death in human and mouse liver tissue. Another test for cell death detection is via recognition of cells with fragmented DNA with a technique called TdT-mediated dUTP nick end labelling (TUNEL) test. Finally, there are mediators of apoptosis such as cleavage products of caspases: cytokeratin 18 (CK18) that can be detected in the serum of patients and have been proposed multiple times as biomarkers in liver disease (Schwabe RF, Luedde T. Nat Rev Gastroenterol Hepatol. 2018) (Macdonald S. et al, Hepatology 2018).

There are not many clinical studies assessing cell death despite its fundamental role in all kinds of liver disease. An initial trial with a caspase inhibitor for the treatment of liver disease was published in 2003 (Valentino KL. et al, Int J Clin Pharmacol Ther. 2003), and since then, this inhibitor has been tested in several clinical trials improving liver enzyme levels, especially in non-alcoholic steatohepatitis (NASH) (Baskin-Bey ES. et al, Am J Transplant. 2007) (Pockros PJ. et al, Hepatology. 2007) (Shiffman ML. et al, Aliment Pharmacol Ther. 2010).

Finally, a study performed in samples from the CANONIC (CLIF Acute-on-Chronic Liver Failure in Cirrhosis) study, determined the role of cell death in patients with AD cirrhosis and ACLF using the biomarker CK18 and cleaved CK18. They confirmed that hepatic cell death is a very important feature of AD and ACLF patients and its magnitude correlates with clinical severity (Macdonald S. et al, Hepatology 2018).

4. Cytokine-induced mitochondrial damage and oxidative stress in the liver

4.1. The mitochondria

Mitochondria are organelles that have evolved from an endosymbiotic process consisting in an alpha-proteobacterium engulfed by a eukaryotic progenitor (Yang D. et al, Proc Natl Acad Sci USA. 1985) (Nunnari J, Suomalainen A. Cell. 2012). They maintain similarities with their bacterial ancestor: mitochondria are shaped by two distinct membranes (the outer (OM) and the inner (IM) membranes) that encapsulate the intermembrane space and matrix compartments. These organelles also contain a double-stranded circular mitochondrial DNA (mtDNA), which encodes 13 polypeptides of the electron transport chain complexes and adenoside triphosphate (ATP) synthase, 22 transfer RNAs and 2 ribosomal RNAs required for intra-mitochondrial translation. The other mitochondrial proteins are encoded by nuclear DNA, synthesized in the cytoplasm and transported into the mitochondria (Mansouri A. et al, Gastroenterology. 2018).

Despite being classically appreciated for their role as the powerhouse of the cell, the metabolic functions of mitochondria reach far beyond bioenergetics (Spinelli JB, Haigis MC. Nat Cell Biol. 2018). Firstly, mitochondria coordinate the cell adaptation to stress, for instance, nutrient deprivation, oxidative stress, DNA damage and ER stress. Secondly, in addition to the production of ATP, mitochondria also generate metabolic precursors of macromolecules such as lipids, proteins and nucleic acids. Finally, mitochondria produce metabolic by-products such as ROS and ammonia and they are important in processes of clearance or utilization of waste products. In summary, mitochondria exert several homeostatic functions including metabolic functions as bioenergetic powerhouses, biosynthetic centers and hubs of reducing equivalents and waste management (Spinelli JB, Haigis MC. Nat Cell Biol. 2018).

4.2. The tricarboxylic acid (TCA) cycle

Most of the ATP generated by cells is provided by an aerobic processing of the fuel molecules and their oxidation to CO_2. This oxidation consists in a series of chemical reactions in a closed loop that forms a metabolic engine within cells. This metabolic cycle was first described by Hans Adolf Krebs between 1933 and 1937, and now the cycle bears his name, although it is also known as the citric acid cycle or the tricarboxylic acid (TCA) cycle (Martínez-Reyes I, Chandel NS. Nat Commun. 2020) (Krebs HA, Johnson WA. FEBS Lett.

1980). In eukaryotic organisms, the reactions of the TCA cycle occur inside the mitochondrial matrix (Ryan DG, O'Neill LAJ. Annu Rev Immunol. 2020).

The TCA cycle is the central metabolic hub of the cell, and acts as a link for the integration of anabolic (gluconeogenesis and lipid synthesis) and catabolic (glycolisys and β-oxidation) pathways. In fact, it is the central biochemical process in eukaryotic life (Ryan DG, O'Neill LAJ. Annu Rev Immunol. 2020).

TCA cycle starts with a two-carbon acetyl CoA that has been generated from fatty acids, amino acids or pyruvate oxidation. This acetyl CoA combines with a four-carbon oxaloacetate (OAA) to generate the six-carbon molecule citrate. The second step converts citrate into isocitrate and is catalyzed by aconitase. This step comprises two reactions: the dehydration of citrate to cis-aconitate and the rehydration of cis-aconitate to isocitrate (Lloyd SJ. et al, Protein Sci. 1999). Then, after two oxidative decarboxylation, isocitrate is converted into the five carbon α-ketoglutarare (α-KG), catalyzed by isocitrate dehydrogenase (IDH) that produces the first molecule of NADH and one carbon unit is lost in the form of CO_2. After this, in the fourth step of the cycle, α-KG is converted to succinyl-CoA, and this yields the second molecule of NADH while another carbon unit is lost as CO_2 (Ryan DG, O'Neill LAJ. Annu Rev Immunol. 2020). In the fifth reaction, succinyl-CoA is converted to succinate, coupled with the phosphorylation of a purine nucleoside diphosphate (usually GDP but also ADP). The final stage of the TCA cycle, which is composed of three reactions, consist in the regeneration of OAA. First, there is the oxidation to succinate to fumarate by succinate dehydrogenase (SDH), also an important part of the electron transport chain (ETC) as the complex II (Yankovskaya V. et al, Science. 2003). In this step, $FADH_2$ is produced because two hydrogen atoms are transferred to FAD. Secondly (and seventh step of the cycle), fumarate is converted to malate and finally, the last step of the cycle consists in the oxidation of malate to OAA in order to start the cycle again (Martínez-Reyes I, Chandel NS. Nat Commun. 2020).

The TCA cycle is an essential function for the maintenance of cellular homeostasis, nonetheless, recently it has been demonstrated that the metabolites involved in the TCA cycle can also control cell fate and function (Martínez-Reyes I, Chandel NS. Nat Commun. 2020). In fact, some of the metabolites can act as signals. For instance, succinate plays an important role in inflammatory and metabolic signaling, while itaconate, which comes from another intermediate, cis-aconitate, has an anti-inflammatory role (Murphy MP, O'Neill LAJ. Cell. 2018).

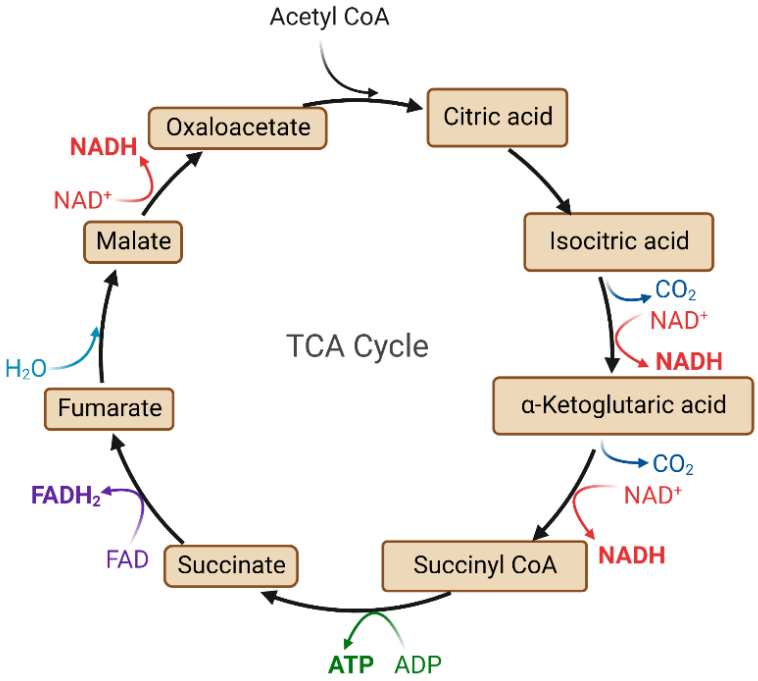

Figure 13. Representation of the TCA cycle. Created with BioRender.com.

In recent years, it has been proposed that the activation of macrophages toward a pro-inflammatory phenotype indices two metabolic breakpoints in the TCA cycle (O'Neill LA. Immunity. 2015). These two breakpoints have also been identified in leukocytes from patients with ACLF (Zhang IW. et al, J Hepatol. 2022). The first breakpoint occurs at IDH level, where citrate can be converted to itaconate, via cis-aconitate. The mitochondrial enzyme in charge of this reaction is the immune responsive gene 1 (IRG1). Increased levels of itaconate result in the inhibition of complex II (SDH) and impair the succinate oxidation. Moreover, itaconate can activate the antioxidant and anti-inflammatory transcription factors nuclear factor erythroid 2 related factor 2 (NRF2) and activating transcription factor 3 (ATF3) (Mills EL. et al, Nature. 2018) (Bambouskova M. et al, Nature. 2018). The second metabolic breakpoint in the TCA cycle is proposed to occur at SDH level, leading to the accumulation to succinate.

Succinate has emerged as a key player in macrophage activation, acting on different pathways to exert its immunomodulatory properties. In macrophages, succinate can be transported and accumulated outside the mitochondria and stabilize hypoxia-inducible

factor-1α (HIF-1 α), produce IL-1β and drive the generation of mitochondrial ROS (mtROS) from complex I (Tannahill GM. et al, Nature. 2013) (Mills EL. et al, Cell. 2016).

4.2.1. IRG1-NRF2-ATF3 axis

In response to oxidative stress, Kuppfer cells and other cells of the liver produce inflammatory mediators that attract inflammatory cells into this organ, thus contributing to hepatocyte cell death. In order to maintain redox homeostasis, the liver has tightly regulated antioxidant systems that represents an important protective mechanism to prevent liver injury during acute or chronic oxidative stress (Yi Z. et al, Hepatology. 2020).

One of the mechanisms of protection involves the remodeling of the TCA cycle in order to synthesize more itaconate through IRG1, which is one of the most upregulated genes under pro-inflammatory conditions (Degrandi D. et al, J Interferon Cytokine Res. 2009).
Itaconate exerts anti-inflammatory actions in immune cells counteracting the pro-inflammatory signals of succinate in the mitochondria by inhibiting SDH (Lampropoulou V. et al, Cell Metab. 2016) (Cordes T. et al, J Biol Chem. 2016) (Domínguez-Andrés J. et al, Cell Metab. 2019). Therefore, itaconate can be generated to counteract and resolve the pro-inflammatory pathways activated by succinate. Moreover, itaconate is a key metabolite for the activation of the anti-inflammatory transcription factor NRF2 via the alkylation of the protein KEAP1, which leads to the increase of the expression of downstream genes with antioxidant and anti-inflammatory capacities in macrophages activated by LPS (Mills EL. et al, Nature. 2018). Recent studies also demonstrate the protective role of itaconate and IRG1 in activating NRF2 anti-inflammatory pathway in microglia (Ni L. et al, Cell Death Dis. 2022) and in the antigen-presenting dendritic cells (Jaiswal AK. et al, Mucosal Immunol. 2022). In addition, in the liver, IRG1 protects against injury induced by concanavalina A also activating NRF2 and its subsequent activation of the antioxidant and anti-inflammatory downstream genes such as heme oxygenase-1 (Hmox1) (Yang W. et al, Free Radic Biol Med. 2022).

Itaconate has also been shown to be involved in ATF3 induction in macrophages, as another mechanism of protection (Bambouskova M. et al, Nature. 2018). ATF3 is a stress induced transcription factor that can lead to the repression of inflammatory genes in multiple cell types and diseases (Kwon JW. et al, Sci Rep. 2015), including liver inflammation (Zhu Q. et al, Cell Death Dis. 2018) and also it has been demonstrated that hepatic ATF3 can protect against atherosclerosis (Xu Y. et al, Nat Metab. 2021). Interestingly, Rao J. established that

the disruption of ATF3 decreases NRF2/Hmox1 signaling, resulting in the exacerbation of liver injury (Rao J. et al, Am J Transplant. 2015).

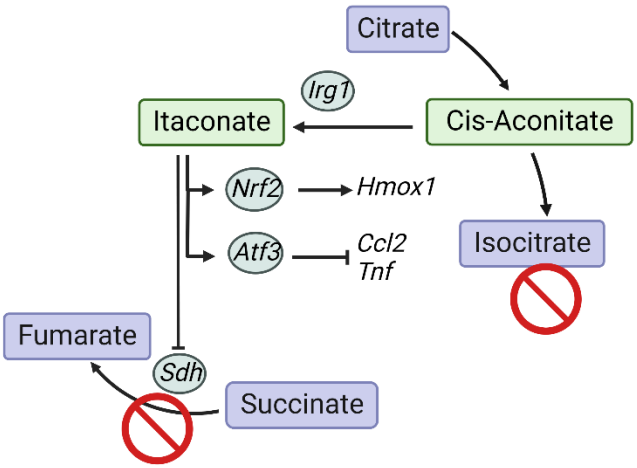

Figure 14. Remodeling of the TCA cycle. Scheme of the TCA cycle under inflammatory conditions. Cis-aconitate is converted to itaconate by IRG1, subsequently activating the anti-inflammatory and antioxidant pathways of Nrf2 and Atf3 plus the inhibition of Sdh. Created with BioRender.com

4.3. Electron transport chain

The electron transport chain (ETC) has the purpose to generate ATP, which is the principal donor of energy in the cells. The turnover of ATP in the body is very elevated and this is possible thanks to the combination of the complexes involved in the ETC, which synthesize ATP from ADP and inorganic phosphate, in a process called oxidative phosphorylation (OXPHOS) (Schultz BE, Chan SI. Annu Rev Biophys Biomol Struct. 2001) (Saraste M. Science. 1999) (Ryan DG, O'Neill LAJ. Annu Rev Immunol. 2020). The ETC is located in the inner mitochondrial membrane (IMM) and it stablishes a proton (H+) gradient out of the mitochondrial matrix into the intermembrane space that generates a pH gradient and transmembrane electrical potential. This is able to synthesize ATP when protons flow back to the mitochondrial matrix through ATP synthase (ATPase or complex V). The transfer of electrons from NADH and $FADH_2$ to O_2 occurs at the IMM and leads to the pumping of protons. The ETC consists of three electron-driven proton pumps: NADH-Ubiquinone oxidoreductase (complex I), cytochrome c oxidoreductase (complex III) and cytochrome oxidase (complex IV). There are also electron carriers such as CoQ (it shuttles electrons from complex I to complex III) and cytochrome c, a small protein that shuttles electrons

from complex III to complex IV, which it is also the reduction site of O_2 to H_2O. Complex II (or SDH) also interacts with CoQ to generate the proton force and it is directly associated with the TCA cycle. Finally, the last stage of OXPHOS is driven through ATP synthase where the protons are back into the mitochondrial matrix enabling the conversion of ADP to ATP (Schultz BE, Chan SI. Annu Rev Biophys Biomol Struct. 2001) (Saraste M. Science. 1999) (Ryan DG, O'Neill LAJ. Annu Rev Immunol. 2020).

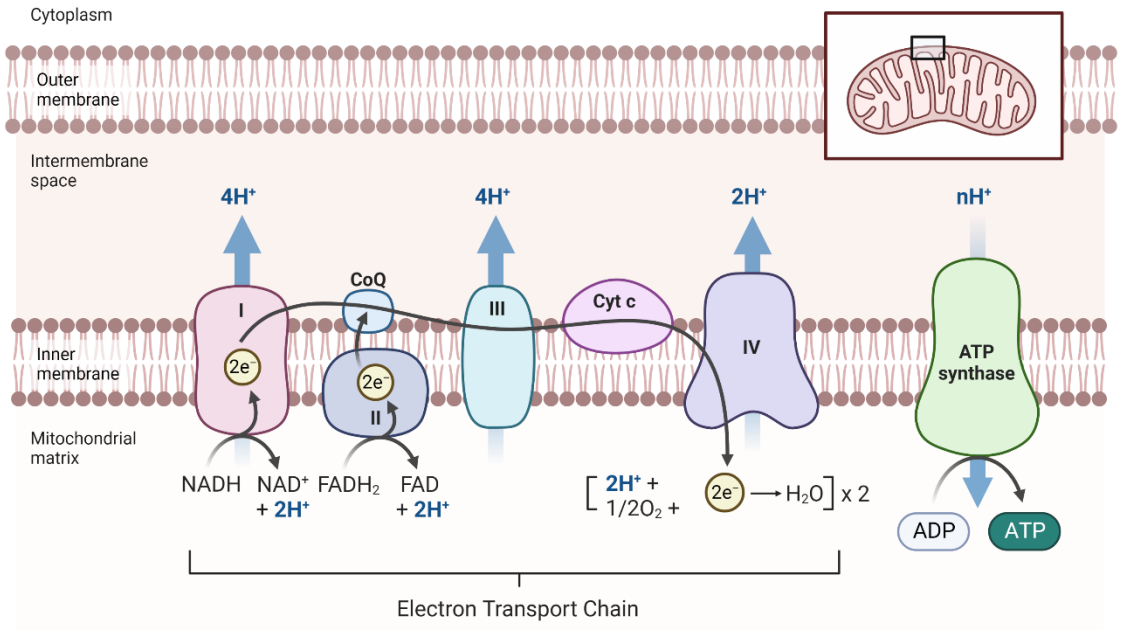

Figure 15. Oxidative phosphorylation (OXPHOS). Diagram representing the electron transport chain (ETC), which is located in the mitochondrial inner membrane. The ETC is associated with the production of ATP by ATP synthase (or complex V) in a process called oxidative phosphorylation (OXPHOS). Created with BioRender.com

4.4. β-oxidation

FAs represent an important source of energy for the cells, specially under starvation and nutrient stress (Spinelli JB, Haigis MC. Nat Cell Biol. 2018). Mitochondria is the place for the oxidation of plasma free FAs or triglycerides, however it arose the need for a transport system into the mitochondria before FA are oxidized. When FAs translocate through the cell membrane, they are rapidly converted to acyl-CoAs, but the mitochondrial membrane is impermeable to acyl-CoAs. Therefore, for their import into mitochondria, they benefit from a carnitine shuttle. The first step is achieved by carnitine palmitoyltransferase (CPT) 1 that

converts acyl-CoA into an acylcarnitine, which is able to cross the mitochondrial membrane. Once inside the mitochondria, CPT2 is able to reconvert the acylcarnitines into acyl-CoA again, leading to the start of fatty acid oxidation (FAO) or β-oxidation (Houten SM, Wanders RJ. J Inherit Metab Dis. 2010). The β-oxidation pathway consists in a cyclic process in which the two carboxi-terminal carbon atoms of the acyl-CoA are released as acetyl-CoA units each time a cycle is completed (Houten SM, Wanders RJ. J Inherit Metab Dis. 2010). This Acetyl-CoA is used then for the TCA cycle (Spinelli JB, Haigis MC. Nat Cell Biol. 2018).

4.5. TNFα-induced mitochondrial dysfunction

Given that ROS are byproducts of the normal functioning of the ETC, mitochondria are the largest source of ROS within the cell (Zorov DB. et al, Physiol Rev. 2014). The electrons donated to the respiratory chain usually react with protons and oxygen to form water through complex IV of the ETC. Nonetheless, some of these electrons react directly with oxygen at complex I and complex III leading to the formation of the superoxide anion radical (O_2-), which generates subsequently hydrogen peroxide (H_2O_2). Mitochondria has its own mechanisms to clear ROS but if it is excessive, the generation of ROS can damage mtDNA, proteins and lipids, thus inducing more mitochondrial ROS (Mansouri A. et al, Gastroenterology. 2018). This is a common pathological factor in many diseases, including liver disease, that can lead to tissue injury and organ dysfunction.

Oxidative stress is often associated with inflammation, in fact, the release of inflammatory cytokines by immune cells can directly trigger oxidative stress (Tilg H, Diehl AM. N Engl J Med. 2000). Exposure to TNFα produced by monocytes and macrophages, is known to induce damage to the mitochondria and increase mitochondrial ROS production (Kastl L. et al, FEBS Lett. 2014) (Schulze-Osthoff K. et al, J Biol Chem. 1992). Moreover, previous studies demonstrated that the mechanisms of TNFα cytotoxicity led to a degeneration of the mitochondrial ultrastructure and to an inhibition of the ETC (Schulze-Osthoff K. et al, J Biol Chem. 1992). In the liver, TNFα treatment of hepatocytes trigger an increase of mitochondrial O_2 consumption, which reflects the high energy demand that require the cells under pro-inflammatory conditions (Kastl L. et al, FEBS Lett. 2014).
In conclusion, TNFα produced by immune cells under systemic inflammation conditions, is able to increase the mitochondrial ROS production, inducing damage in the mitochondria.

4.6. Mitochondrial dysfunction in cirrhotic patients

Recent studies postulate that beyond the traditional mechanisms in the process of acute decompensation in cirrhotic patients (portal hypertension and systemic vasodilatation), there are new mechanisms that contribute to the progression of advanced liver disease. These mechanisms include systemic inflammation, oxidative stress, metabolic changes and mitochondrial dysfunction (Engelmann C et al, J Hepatol. 2021) (Moreau R et al, J Hepatol. 2020) (Arroyo et al, N. Engl. J. Med. 2020) (Zhang IW. et al, J Hepatol. 2022).

According to Zhang IW et al, patients with ACLF use mitochondria-based pathways in a less efficiently way and the immune cell responses are dictated by the alteration in energy production that occurs in peripheral blood leukocytes (Zhang IW. et al, J Hepatol. 2022). This study provides direct mechanistic evidence of impaired mitochondrial function in peripheral leukocytes from patients with AD and ACLF, highlighting the importance of bioenergetics failure as an emerging aspect in the pathophysiology of this disease. Importantly, these findings showed that there was not a general shutdown of mitochondrial function in leukocytes from patients with ACLF, but rather break-points in the TCA cycle. Specially, they identified two break points, one at the isocitrate and the other at the succinate level (Zhang IW. et al, J Hepatol. 2022), revealing the need for anaplerotic pathways to feed the TCA cycle in ACLF patients. Moreover, the first isocitrate break-point can lead to the accumulation of cis-aconitate, which as its turn formats itaconate, a metabolite known to connect cell metabolism with immune responses (Zhang IW. et al, J Hepatol. 2022).

In patients with cirrhosis, the metabolic alteration is characterized by a reduced oxidative glucose metabolism in the mitochondria and a shift from producing ATP by OXHPOS to aerobic glycolysis, which produces lactate and it is less efficient than OXPHOS (Mills EL. et al, Nat Immunol. 2017). In fact, as systemic inflammation is energetically expensive, it requires reallocation of nutrients to maintain immune activation, which competes for energy with the programs to ensure the proper functioning of peripheral organs. In order to deal with this, there is an energetic trade-off between immune activation and organ homeostasis that may result into peripheral organ hypo metabolism and organ dysfunction (Engelmann C et al, J Hepatol. 2021) (Ganeshan K. et al, Cell. 2019). In peripheral organs, systemic inflammation can lead to the inhibition of the translocation of FAs into the mitochondria and subsequent β-oxidation, which is translated to a decreased ATP production. Moreover, OXPHOS is also impaired, which is related to an enhanced production

of ROS. Altogether, it causes a disruption of mitochondrial metabolism that results in an altered functioning of the organ, thus contributing to the progression of the disease (Arroyo et al, N. Engl. J. Med. 2020).

HYPOTHESIS AND OBJECTIVES

Albumin is the most abundant protein in human plasma. Albumin exerts pleiotropic actions and besides its oncotic power, the albumin molecule also binds, transports, and detoxifies endogenous and exogenous substances, plays antioxidant activity, and modulates the immune and inflammatory responses. Low albumin levels are a sign of advanced liver disease and more so in ACLF, a condition characterized by persistent systemic inflammation leading to multi-organ failure. Beyond systemic inflammation other processes contribute to the progression of the disease including oxidative stress, impaired metabolism and mitochondrial dysfunction.

In the clinical setting, albumin infusions are used to improve circulatory dysfunction and survival in patients with AD cirrhosis. Moreover, recent studies have demonstrated that albumin administration to these patients is associated with a decrease in circulating levels of cytokines. Mechanistic studies in immune cells isolated from patients with AD cirrhosis have provided evidence of the internalization of the albumin molecule by mononuclear cells and its ability to block TLR signaling in the endosomal compartment. However, at present it is unknown whether in addition to reduce the production of cytokines by immune cells, the albumin molecule also has the ability to protect the organs and tissues against cytokine-induced damage (immunopathology) and mitochondrial dysfunction.

The running hypothesis of this thesis dissertation is that albumin protects liver cells from immunopathology and mitochondrial dysfunction induced by the cytokine TNFα, avoiding organ dysfunction and failure in conditions where albumin synthesis is compromised as is the case of advanced liver disease.

To test this hypothesis, this thesis consists of two general objectives subdivided into several specific aims:

OBJECTIVE 1: Determine the protective effects of albumin in liver tissue in a cell death injury model induced by TNFα and elucidate the mechanisms underlying these tissue protective effects.

1.1. Determine the apoptotic effect of TNFα in murine primary hepatocytes and evaluate the protective effects of albumin.

1.2. Investigate whether the cytoprotective effects of albumin are independent from its scavenging properties and also from any serum factor bound to the albumin molecule.

1.3. Elucidate the molecular mechanisms underlying the cytoprotective effects of albumin.

1.4. Investigate the protective effects of albumin in the liver tissue using an *in vivo* model of transgenic mice lacking the albumin gene.

1.5. Assessing the effects of albumin *in vivo* on the recruitment of inflammatory cells into the injured liver.

OBJECTIVE 2: Determine the homeostatic role of albumin in preserving liver cells from TNFα-induced mitochondrial injury and oxidative stress, and elucidate the mechanisms underlying these tissue protective effects.

2.1. Investigate whether albumin preserves the mitochondrial ultrastructure of liver cells against TNFα-induced damage.

2.2. Determine the ability of albumin in reducing mitochondrial oxidative stress in hepatocytes challenged with TNFα.

2.3. Identify whether albumin modulates mitochondrial respiration in hepatocytes challenged with TNFα.

2.4. Asses the effects of albumin on mitochondrial fatty acid oxidation and the TCA cycle in hepatocytes challenged with TNFα.

2.5. Explore mechanisms underlying the mitochondrial protective actions of albumin.

RESULTS

STUDY I: Albumin protects the liver from tumor necrosis factor α-induced immunopathology.

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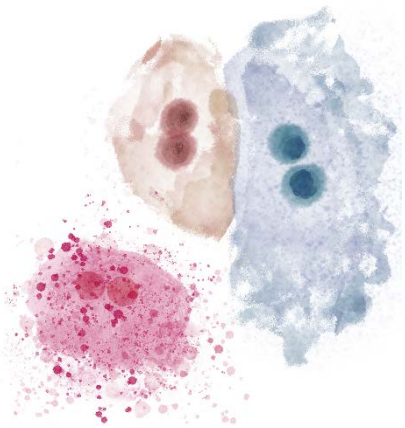

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RESEARCH ARTICLE

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Albumin protects the liver from tumor necrosis factor α-induced immunopathology

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Abstract

Besides its oncotic power, albumin exerts pleiotropic actions, including binding, transport, and detoxification of endogenous and exogenous molecules, antioxidant activity, and modulation of immune and inflammatory responses. In particular, recent studies have demonstrated that albumin reduces leukocyte cytokine production. Here, we investigated whether albumin also has the ability to protect tissues from the damaging actions of these inflammatory mediators. We circumscribed our investigation to tumor necrosis factor (TNF) α, which exemplifies the connection between immunity and tissue injury. In vivo experiments in analbuminemic mice showed that these mice exhibit a more pronounced response to a model of TNFα-mediated liver injury induced by the administration of lipopolysaccharide (LPS) and D-galactosamine (D-gal). A tissue protective action against LPS/D-gal liver injury was also observed during the administration of human albumin to humanized mice expressing the human genes for albumin and neonatal Fc receptor (h$Alb^{+/+}$/h$FcRn^{+/+}$) with preestablished carbon tetrachloride (CCl_4)-induced early cirrhosis. The cytoprotective actions of albumin against TNFα-induced injury were confirmed ex vivo, in precision-cut liver slices, and in vitro, in primary hepatocytes in culture. Albumin protective actions were independent of its scavenging properties and were reproduced by recombinant human albumin expressed in *Oryza sativa*. Albumin cytoprotection against TNFα injury was related to inhibition of lysosomal cathepsin B leakage accompanied by reductions in mitochondrial cytochrome c release and caspase-3 activity. These data provide evidence that in addition to reducing cytokines, the albumin molecule also has the ability to protect tissues against inflammatory injury.

KEYWORDS

albumin, cytokine-induced tissue injury, liver cells, liver injury, mitochondrial damage

Abbreviations: Atf3, activating transcription factor 3; Ccl2, monocyte chemotactic protein 1; CCl_4, carbon tetrachloride; cK18, caspase-cleaved K18; D-gal, D-galactosamine; Ddit3, DNA damage inducible transcript 3; H_2O_2, hydrogen peroxide; hAlb, human albumin; hFcRn, human neonatal Fc receptor; IHC, immunohistochemistry; IL, interleukin; K18, keratin 18; LPS, lipopolysaccharide; PCLS, precision-cut liver slices; rHA, recombinant human albumin; ROS, radical oxygen species; TLR, Toll-like receptor; TNFα, tumor necrosis factor alpha; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

1 | INTRODUCTION

Inflammation is a first-line response of the innate immune system to danger signals from pathogens or damaged cells, the so-called pathogen- and damage-associated molecular patterns (PAMPs and DAMPs).[1] Under homeostatic conditions, inflammation is rapidly induced and later resolved in a timely manner, but if this regulatory circuit is inefficient and the inflammatory response is intense, it leads to an overwhelming production of cytokines by the cells of the innate immune system, which can directly trigger tissue injury.[2] The tumor necrosis factor (TNF) family is one of the best examples of the link between the cytokine storm and tissue pathology, a process commonly referred as immunopathology.[3] The most representative member of this family, TNFα, is a pleiotropic cytokine predominantly produced by monocytes/macrophages with a major role in immune-mediated cytotoxicity.[4] In the liver, particularly, the foremost role of TNFα after binding to its cognate receptor is the engagement of the cell death program in hepatocytes experiencing global transcriptional arrest.[5-7]

Albumin is a 66.5 kDa negatively charged protein consisting of 585 amino acids forming a single polypeptide sequence.[8] Albumin has high solubility and stability and a relative abundance of charged amino acids such as lysine, arginine, glutamine, and aspartic acid.[8] Albumin is the most abundant protein in plasma as well as in the interstitial space, and is the main contributor to the maintenance of oncotic pressure.[9] Besides its oncotic power, albumin exerts pleiotropic actions, including binding, transport, and detoxification of endogenous and exogenous molecules, antioxidant activity, contribution to endothelial stabilization and vascular integrity, and modulation of immune and inflammatory responses.[8-10] Since albumin is synthesized in the liver, it is not surprising that patients with cirrhosis who have impaired hepatocellular function exhibit reduced albumin synthesis.[11] Of interest, pro-inflammatory cytokines such as TNFα inhibit the hepatic albumin synthesis, whereas albumin inhibits the pro-inflammatory consequences of TNFα signaling.[12,13] However, whether albumin exerts cytoprotective actions and specifically whether albumin protects liver cells against the damaging effects of TNFα is at present unkown.

In the current study, we undertook a multimodal approach to collect evidence at different experimental levels (in vivo, ex vivo, and in vitro) of the actions of albumin against TNFα-induced liver cell death and tissue damage. Specifically, we performed in vivo experiments using a model of TNFα-mediated acute liver injury induced by the administration of lipopolysaccharide (LPS) and D-galactosamine (D-gal). This model of TNFα-mediated liver injury was also tested in mice lacking the albumin gene and in humanized mice expressing the human albumin (h*Alb*) and human neonatal Fc receptor (h*FcRn*) genes (h$Alb^{+/+}$/h$FcRn^{+/+}$mice) chronically induced to early cirrhosis with carbon tetrachloride (CCl_4). In addition, we performed experiments in precision-cut liver slices (PCLS), an ex vivo model that preserves cell-cell interactions in the original three-dimensional hepatic architecture and is a reliable tool for studying the response of liver tissue to injury. Finally, we carried out mechanistic in vitro studies in hepatocytes incubated with TNFα.

2 | MATERIAL AND METHODS

2.1 | Animal models

Double transgenic humanized C57BL/6J male mice expressing the human albumin (h*Alb*) and human neonatal Fc receptor (h*FcRn*) genes (h$Alb^{+/+}$/h$FcRn^{+/+}$) (genOway, Lyon, France) (n = 13) and analbuminemic male mice ($Alb^{-/-}$) (n = 8) were from The Jackson Laboratory (Bar Harbor, ME). Wild-type (WT) C57BL/6J male mice (n = 7) were from Charles River Laboratories (Saint-Aubin-les-Elbeuf, France). Animals were housed in cages with woodchip bedding at 50%-60% humidity and a 12-hour light/dark cycle and given free access to food and water. Mice received an intraperitoneal (i.p.) injection of LPS (20 μg/Kg body weight [b.w.]) and D-gal (600 mg/Kg b.w.), as a model of TNFα-mediated acute liver injury. Six hours later, mice were euthanized by an overdose of anesthetic (a mixture of 0.1 mg ketamine/g b.w. and 0.01 mg xylazine/g b.w.) and peripheral blood was collected and plasma obtained by centrifugation at 800 *g* for 10 minutes. Liver tissue was excised and rinsed in Dulbecco's phosphate-buffered saline (DPBS) with calcium and magnesium ($DPBS^{++}$) and either fixed in 10% of formalin and embedded in paraffin or immersed in optimal cutting temperature compound in cold 2-methylbutane on dry ice or kept at −80°C or snap-frozen in N_2 for further analysis. To test the effects of albumin on liver injury, two doses of human serum albumin (Albutein, Grifols, Barcelona, Spain) of 1.5 g/kg b.w. or its control stabilizer were administered 72 and 24 hours prior to injection of LPS + D-gal. When indicated, mice were induced to early cirrhosis by i.p. administration (twice weekly) of CCl_4 (1 μL/g b.w. in olive oil) for 6 weeks, as described elsewhere.[14] All animal studies were conducted in accordance with the criteria of the Investigation and Ethics Committee of the University of Barcelona (Barcelona, Spain) following the EU laws governing the use of experimental animals.

2.2 | Precision-cut liver slices

Control C57BL/6J mice (n = 15) were anesthetized with ketamine/xylazine. The inferior cava vein was cutoff and blood was allowed to drain for 1 minute. The liver was

excised and placed into ice-cold DPBS without calcium and magnesium ($DPBS^{-/-}$). Blocks (0.5 cm^3) from the main lobe were cut with a scalpel and embedded in 4% of UltraPure low-melting agarose (Invitrogen, Waltham, MA) diluted in Hanks balanced salt solution without calcium and magnesium. An agarose cube with the tissue inside was cut into 250-μm-thick slices using a vibrating-blade microtome (VT1000S; Leica Microsystems, Wetzlar, Germany) as described.[15,16] PCLS were individually transferred to P-35 Petri dishes containing Millicell cell culture inserts (EMD Millipore, Burlington, MA) and pre-balanced for 20 minutes with warmed William's E medium containing GlutaMAX-I, 25 mM of D-glucose, and 50 μg/mL of gentamicin. PCLS were maintained at 37°C in a 5% CO_2 incubator for 120 minutes before being incubated for 12 hours with vehicle or TNFα (10 ng/mL). To test the effects of albumin, experiments were carried out in pretreatment (incubating PCLS with albumin, 15 mg/mL, for 30 minutes before the addition of TNFα for 12 hours) or treatment (PCLS exposed to TNFα for 1 hour followed by the addition of albumin [15 mg/mL] for 12 hours) modes. The concentration of albumin was selected considering that the interstitial concentration of albumin has been described to be between 4 and 15 mg/mL.[17] All incubations with TNFα were performed in the presence of the transcriptional inhibitor actinomycin D (10 μg/mL).[18] In another set of experiments, PCLS were exposed to hydrogen peroxide (H_2O_2, 0.75 mM) for 4 hours in the presence or absence of albumin (experiments were performed in pretreatment mode as described above). PCLS were also incubated with unmethylated CpG DNA (5 μg/mL) or adenosine triphosphate (ATP, 100 μM) for 4 or 18 hours, respectively, in the presence or absence of albumin. At the end of the incubation period, PCLS were snap-frozen in N_2 for further analysis.

2.3 | Isolation and characterization of primary hepatocytes

Hepatocytes were isolated from C57BL/6J mice (n = 26) by a three-step in situ perfusion procedure using 0.04% of collagenase IV (Sigma, St. Louis, MO) through the cava vein and posterior digestion with 0.001% of DNAse (Sigma).[15,16] The cell suspension was filtered through a 100-μm cell strainer (Becton Dickinson (BD), Franklin Lakes, NJ) and subsequently washed by centrifugation at 70 *g* for 2 minutes at 4°C with cold William's E medium. The viability of pelleted hepatocytes was determined by trypan blue exclusion and the cell number was assessed in an automated cell counter (Countess II, Life Technologies, Carlsbad, CA). Isolated hepatocytes were seeded on collagen I-coated 12-well plates (4×10^5 cells/well) or white-walled 96-well plates (4×10^4 cells/well) with William's E medium supplemented with 10% of fetal bovine serum (FBS), 200 mM of L-glutamine, 50 U/mL of penicillin, 50 μg/mL of streptomycin, 1 μM of insulin, 15 mM of Hepes, and 50 μM of β-mercaptoethanol. Four hours later, hepatocytes were washed three times with $DPBS^{+/+}$ and visualized by phase contrast microscopy. Thereafter, hepatocytes were grown in a humidified 5% CO_2 incubator at 37°C in 1% of FBS-William's E medium overnight. For further characterization studies, hepatocytes were plated on 8-well Nunc Lab-Tek (Thermo Fisher Scientific, Waltham, MA) and grown overnight. After three washes with PBS, cells were fixed with 4% of paraformaldehyde for 10 minutes. Then, cells were permeabilized with 0.1% of triton X-100 for 10 minutes and washed with PBS three more times. The cells were incubated with 0.2% of gelatin-PBS for 30 minutes to block unspecific binding of the antibodies and later incubated 30 minutes at room temperature with a rabbit anti-mouse albumin antibody (dilution 1/250, Abcam, Cambridge, UK), and a mouse anti-mouse hepatocyte nuclear factor 4 alpha (HNF4α) antibody (dilution 1/500, Abcam). For albumin and HNF4α immunostaining, cells were incubated in 0.2% of gelatin-PBS for 1 hour at room temperature in the dark with a goat anti-rabbit IgG secondary antibody labeled with Alexa Fluor 488 and goat anti-mouse IgG secondary antibody labeled with Alexa Fluor 555 (both from Thermo Fisher Scientific), respectively. Finally, the cells were stained with Hoechst (300 ng/mL) (Thermo Fisher Scientific) for 1 minute, mounted with a drop of ProLong Gold Antifade Mountant and visualized in a LSM880 Zeiss Confocal Microscope (Zeiss, Jena, Germany).

2.4 | Incubation of primary hepatocytes

To test the effects of albumin on TNFα-induced apoptosis, experiments were carried out in pretreatment and treatment modes (pretreatment: hepatocytes received albumin [15 mg/mL] for 30 minutes before the addition of TNFα (20 ng/mL) for 6 hours; treatment: hepatocytes exposed to TNFα (20 ng/mL) for 1 hour before the addition of albumin [15 mg/mL] for 5 hours). Pretreatment experiments were repeated with recombinant human albumin (15 mg/mL) expressed in *Oryza sativa* (Sigma). In some experiments, hepatocytes were pretreated with the cathepsin B inhibitor CA-074-methyl ester (Me) (Calbiochem, San Diego, CA) at a concentration of 10 mM for 30 minutes before inducing apoptosis with TNFα for 6 hours. All experiments with TNFα were performed in the presence of actinomycin D (250 ng/mL).[18] In another set of experiments, hepatocytes were exposed to oxidant injury with H_2O_2 (0.2-1.5 mM) for 4 hours in the presence or absence of albumin (experiments were performed in a preventive mode as described

above). At the end of the incubation period, supernatants were collected, and hepatocytes were scraped for further assessments.

2.5 | Assessment of cytochrome c localization by confocal microscopy

Hepatocytes were grown in 8-well Nunc Lab-Tek (Thermo Fisher Scientific) and treated with TNFα and actinomycin D in the presence or absence of albumin for 4 hours and fixed with 4% of paraformaldehyde for 10 minutes. After three washes with PBS, cells were permeabilized with 0.1% of triton X-100 for 10 minutes and washed with PBS three more times. The cells were incubated with 10% of goat serum for 30 minutes to block unspecific binding of the antibodies and later incubated overnight at 4ºC with a rabbit anti-mouse ATP5a antibody (dilution 1/200, Abcam) and Alexa Fluor 555-labeled mouse anti-cytochrome c antibody (dilution 1/200, BD). The cells were then washed three times in PBS and incubated with a goat anti-rabbit IgG secondary antibody labeled with Alexa Fluor 488 (Thermo Fisher Scientific) in 10% of goat serum for 1 hour at room temperature in the dark. Finally, the cells were stained with Hoechst (300 ng/mL) for 1 minute, mounted with a drop of ProLong Gold Antifade Mountant and visualized in a LSM880 Zeiss Confocal Microscope (Zeiss). Colocalization analyses were performed for both ATP5a (mitochondrial marker) and cytochrome c.

2.6 | Histology and immunohistochemistry analysis

Liver samples fixed in 10% of formalin and embedded in paraffin, were cut into 5 μm sections and stained with hematoxylin-eosin and Sirius red. For Sirius red staining, sections were incubated in 0.5% of thiosemicarbazide for 10 minutes followed by incubation with 0.1% of Sirius red F3B in saturated picric acid for 1 hour and by a wash with 0.5% of acetic acid solution. immunohistochemistry (IHC) assessment of cleaved-caspase-3 (a marker of the programed cell death process), F4/80 (a marker of total tissue macrophages), and CX3CR1 (a marker of monocyte-derived recruited macrophages) was carried out on 5 μm-thick deparaffinized sections. Cleaved-caspase-3 and CX3CR1 were detected with nonspecies-specific rabbit polyclonal antibodies from Cell Signaling Technology (CST, Danvers, MA) and Abcam, respectively. F4/80 was detected with a primary rat anti-mouse F4/80 antibody (Bio-Rad, Hercules, CA). Sections were visualized at x200 magnification in a Nikon Eclipse E600 microscope (Kawasaki, Japan).

2.7 | Terminal deoxynucleotidyl transferase dUTP nick end labeling assay

Paraffin-embedded tissues were deparaffinized in xylene, and then, rehydrated in a graded series of ethanol and staining was performed using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit-HRP-DAB from Abcam, according to the manufacturer's specifications. Briefly, slides were permeabilized with a proteinase K solution, and covered with TdT equilibration buffer. Tissues were then labeled with a TdT labeling reaction mixture for 1.5 hours and immersed in a conjugate solution for 30 minutes. DAB was added for 15 minutes, and then, slides were washed with H_2O_2. Slides were counterstained with methyl green and mounted with DPX Mountant (Sigma) and sections were visualized at x200 magnification in a Nikon Eclipse E600 microscope.

2.8 | Biochemical analyses

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatinine concentrations in plasma and lactate dehydrogenase (LDH) levels in PCLS supernatants were determined by automated standard laboratory procedures at the Hospital Clínic Biomedical Diagnostic Center.

2.9 | Protein expression analysis by Western blot in tissue and cells

Total protein from liver tissue and PCLS was extracted using lysis buffer containing 50 mM of Hepes, 20 mM of β-glycerophosphate, 2 mM of EDTA, 1% of Igepal, 10% (vol/vol) glycerol, 1 mM of $MgCl_2$, 1 mM of $CaCl_2$, and 150 mM of NaCl supplemented with a mixture of protease (Complete Mini) and phosphatase (PhosStop) inhibitors (Roche Diagnostics, Basel, Switzerland). For protein isolation from hepatocytes, cells were scraped into ice-cold DPBS and resuspended in 150 μL of lysis buffer. Homogenates were incubated on ice for 10-15 minutes and centrifuged at either 9300 *g* for 12 minutes (tissue and PCLS) or 8000 *g* for 8 minutes (cells) at 4ºC. The protein pellet was dissolved in 2X Laemmli buffer, heated for 5 minutes at 95°C, and separated by 10%-15% (vol/vol) SDS-PAGE for 90 minutes at 120 V. Transfer was performed using the iBlot Dry Blotting System (Invitrogen) onto PVDF membranes at 20 V over 5-7 minutes, and the efficiency of the transfer was visualized by Ponceau S staining. The membranes were then soaked for 1 hour at room temperature in tris-buffered saline containing 0.1% of Tween 20 (T-TBS) and 5% (wt/vol) nonfat dry milk. Blots were washed three times for 5 minutes each

with T-TBS and rabbit polyclonal phospho-SAPK/c-Jun N-terminal kinase (JNK) (Thr183/Tyr185, 1:250 dilution, 9251, CST), rabbit polyclonal cleaved-caspase-3 (Asp175) (1:500 dilution, 9661, CST), and rabbit anti-mouse β-actin (1:1000 dilution, 5125, CST) in 0.1% of T-TBS containing 5% of BSA. Thereafter, the blots were washed three times for 5 minutes each with 0.1% of T-TBS and incubated for 1 hour at room temperature with a horseradish-peroxidase-linked donkey anti-rabbit antibody (1:2000 dilution) in 0.1% of T-TBS containing 5% of nonfat dry milk. The bands were visualized with the EZ-ECL chemiluminescence detection kit (Biological Industries, Haemek, Israel) in ImageQuant LAS 4000 equipment (GE Healthcare Life Sciences, Little Chalfont, UK). To assess total JNK protein expression, membranes were stripped at 37ºC for 15 minutes in Restore Western blot stripping buffer (Thermo Fisher Scientific) and reblotted overnight at 4ºC with rabbit monoclonal SAPK/JNK (56G8) (1:250 dilution, 9258, CST). Membranes were visualized as described above. To assess cytochrome c levels, proteins were extracted using 20% of trichloroacetic acid, precipitated on ice for 1 hour and centrifuged at 16 000 *g* for 10 minutes at 4ºC. Pellets were washed with ice-cold acetone and dried at room temperature and resuspended in Laemmli buffer, and then, processed as described above before incubating overnight at 4°C with primary mouse anti-mouse cytochrome c (1:200 dilution) (Abcam).

2.10 | Assessment of circulating human albumin in mice

To assess circulating human albumin levels in h*Alb*$^{+/+}$/h*FcRn*$^{+/+}$ and WT mice, plasma samples were dissolved with 2× Laemmli buffer, heated for 5 minutes at 95°C, and separated by 10%-15% (vol/vol) SDS-PAGE for 90 minutes at 120 V. Levels of human albumin in mouse plasma were assessed by Western blot using a mouse monoclonal antihuman 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).

2.11 | Gene expression analysis by real-time PCR

Isolation of total RNA from hepatocytes and PCLS was performed using the TRIzol reagent. RNA concentration was assessed in a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific). cDNA synthesis from 0.5 to 1 μg of total RNA was performed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Real-time PCR analysis for interleukin (IL) 6 (*Il6,* Mm00446190_m1), monocyte chemotactic protein 1 (*Ccl2*, Mm00441242_m1), TNFα (*Tnf*, Mm00443258_m1), DNA damage inducible transcript 3 (*Ddit3*, Mm001135937_g1), activating transcription factor 3 (*Atf3*) (Mm00476032_m1), and C-X-C motif chemokine ligand 1 (*Cxcl1*) (Mm04207460_m) was performed using validated and predesigned *TaqMan* Gene Expression Assays purchased from Applied Biosystems in a 7900HT Fast Real-Time PCR System using β-actin (*Actb*; Mm00607939_s1) as endogenous control (Table S1). The PCR results were analyzed with Sequence Detector Software version 2.1 (Applied Biosystems). Relative quantification of gene expression was performed using the comparative C_t method. The amount of target gene normalized to β-actin and relative to a calibrator was determined by the arithmetic equation $2^{-\Delta\Delta Ct}$ described in the comparative C_t method.

2.12 | Caspase-3 and caspase-8 activities

Caspase-3 and caspase-8 activities in hepatocytes were determined using the Caspase-Glo 3/7 (G8091) and the Caspase-Glo 8 (G8201) Assay Systems (Promega, Madison, WI), respectively. Briefly, hepatocytes (40 000 cells/well in 96-well plates) were allowed to equilibrate to room temperature for 30 minutes and Caspase-Glo reagent (50 μL) was added to each well and gently mixed with a plate shaker at 300-500 rpm for 30 seconds. For assessment of caspase-3 in PCLS, total protein was extracted with a hypotonic extraction buffer containing 25 mM of Hepes, 5 mM of $MgCl_2$, 1 mM of EGTA pH 8, and supplemented with 1 μg/mL of protease inhibitor (Complete Mini, Roche Diagnostics), centrifuged at 15 700 *g* for 15 minutes at 5ºC, and the supernatant was recovered. The Bradford method was used to quantify the proteins and the concentration was adjusted to 100 μg/mL of cytosolic protein from PCLS, which was diluted up to 50 μg/mL with hypotonic buffer before the addition of Caspase-Glo 3/7 to reach a 1:1 ratio with the volume of sample. The luminescence signal was measured in a plate-reading luminometer (FLUOstar Optima, BMG Labtech, Germany) with parameters of a 15-min lag time and 0.5 seconds/well-reading time.

2.13 | Measurement of cytosolic cathepsin B levels

Hepatocytes were seeded on 100 mm-Petri dishes and treated with TNFα and actinomycin D for 6 hours in the presence or absence of albumin. The cells were subsequently washed twice in cytosolic solution (KCl 120 mM, $CaCl_2$ 0.15 mM,

K_2HPO_4 10 mM, Hepes 25 mM, EGTA 2 mM, $MgCl_2$ 5 mM, and ATP 2 mM; pH adjusted to 7.4 with 1 N KOH) and centrifuged at 300 *g* for 5 minutes. The cells were then resuspended in permeabilization solution (cytosolic buffer with 0.4 IU/mL streptolysin O) for 20 minutes on ice, and then, centrifuged for 15 minutes at 16 000 *g* to collect the cytosolic fraction in the supernatant. A total of 10-15 μg of protein (determined by micro BCA) was used to analyze cathepsin B levels on the cytosol with the cathepsin B activity assay kit (ab65300, Abcam). Briefly, cytosol fractions were incubated with reaction buffer and Ac-RR-AFC substrate for 1-2 hours at 37ºC protected from light, and the output was read on a fluorescent microplate reader at Ex/Em = 400/505 nm.

2.14 | Flow cytometry

Two hundred microliter of blood were extracted from mice and red blood cells were lysed for 15 minutes at room temperature with Lysis Buffer 1X (Biolegend, San Diego, CA) with vortexing every 5 minutes twice to extract peripheral blood cells. A total of 5×10^5 cells were used for each condition and incubated in Mouse BD Fc Block (diluted 1/100, BD) in $DPBS^{-/-}$ for 15 minutes at 4ºC in the dark. Then, the cells were incubated with APC-labeled anti-mouse Cd11b antibody and live/death Pacific Blue (Invitrogen), FITC-labeled anti-mouse Ly-6C antibody (Biolegend) and V450-labeled mouse anti-mouse CD45.2 (BD) for 20 minutes at 4ºC in the dark. Two milliliter of FACS buffer containing 1% of NaN_3 and 2% of FBS diluted in 1X PBS were added, and the cells were filtered into 5 mL polypropylene tubes and centrifuged 400 *g* for 5 minutes at 4ºC. The cells were then fixed with 0.01% of paraformaldehyde and 1% of NaN_3 in PBS for 15 minutes at 4ºC in the dark and washed with PBS. Cells were resuspended in 250 μL of FACS buffer and stored at 4ºC in the dark for further analysis in a BD LSRFortessa cytometer. BD FACSDiva software was used for correct sorting of the cells into the different populations ($Ly6C^{low}$ and $Ly6C^{high}$).

2.15 | Assessment of reactive oxygen/nitrogen species

The OxiSelect In Vitro reactive oxygen/nitrogen species (ROS/RNS) Assay Kit (Cell Biolabs Inc, San Diego, CA) containing the reactive probe dichlorodihydrofluorescein (DCF) DiOxyQ (DCFH-DiOxyQ) was used to measure the total amount of ROS/RNS by fluorescence in hepatocytes exposed to H_2O_2 (0.2-1.5 mM) for 6 hours in the presence or absence of albumin. Briefly, 50 μL of the sample was incubated with 50 μL of the catalyst reagent of the assay kit for 5 minutes, and then, with 100 μL of DCFH-DiOxyQ solution in the dark for 30 minutes at room temperature. The generated fluorescent product DCF was measured in a FLUOstar Optima fluorescence plate reader (BMG Labtech) at excitation/emission 480/530 nm wavelengths. The ROS/RNS concentrations were determined fluorometrically against the DCF standards.

2.16 | Statistics

The statistical analysis was performed with GraphPad Prism software (San Diego, CA). Statistical differences of the endpoints were evaluated with the unpaired t test with Welch's correction or one-way ANOVA for multiple comparisons corrected with Tukey posttest. Results were expressed as mean ± standard error of the mean (SEM) and differences considered significant at P values $< .05$.

3 | RESULTS

3.1 | Evidence of the tissue protective effects of albumin against liver injury

To achieve a proof of concept that endogenous albumin is essential to counteract excessive tissue damage, we first assessed the response to LPS/D-gal (a model of TNFα-mediated acute liver injury) in an analbuminemic mouse strain (mice lacking the albumin gene, $Alb^{-/-}$ mice) compared to that in wild-type (WT) mice, using cleaved-caspase-3 as a proxy for apoptotic cell death. The phenotype of this mouse strain is given in Table S2 and Figure S1, indicating that at baseline, $Alb^{-/-}$ mice showed no clear evidence of enhanced liver injury. However, $Alb^{-/-}$ mice showed a more pronounced response to LPS/D-gal-induced apoptotic injury since the immunostaining for cleaved-caspase-3 protein was significantly higher in these mice as compared to WT (Figure 1A). We next assessed the ability of the administration of exogenous albumin to modulate LPS/D-gal-induced tissue damage. Since the administration of human albumin to murine models results in incomplete effectiveness in the maintenance of its circulating levels due to the different affinity of human and mouse recycling receptors for human albumin,[19] we used double transgenic humanized mice expressing the genes coding for human albumin (h*Alb*) and its human h*FcRn* receptor ($hAlb^{+/+}$/$hFcRn^{+/+}$). Phenotypically, $hAlb^{+/+}$/$hFcRn^{+/+}$ mice were similar to WT mice at baseline, except that these mice appeared to have higher body weight and white adipose tissue to body weight ratio (Table S2). $hAlb^{+/+}$/$hFcRn^{+/+}$ mice had constitutive levels of human albumin in the systemic circulation, as determined by the use of a human-specific antibody against this protein in plasma samples from these mice (Figure 1B). As compared to WT, $hAlb^{+/+}$/$hFcRn^{+/+}$

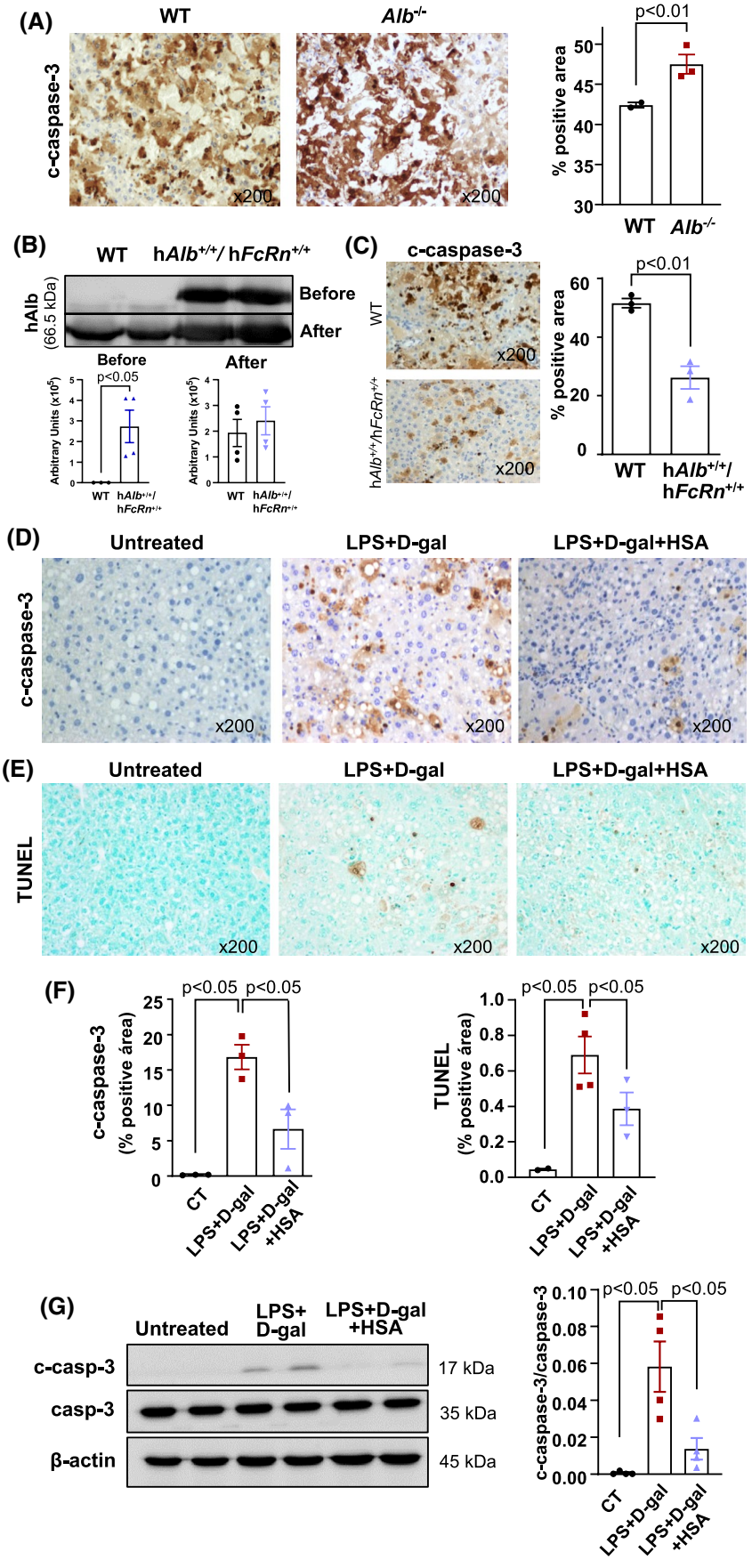

FIGURE 1 Experimental evidence that albumin exerts tissue protective effects. A, Representative photomicrographs (200× magnification) of liver sections from WT (n = 3) and $Alb^{-/-}$ (n = 4) mice challenged with LPS and D-galactosamine (LPS + D-gal) and stained for cleaved-caspase-3 (c-caspase-3). B, Detection of human albumin (hAlb) in plasma from humanized h$Alb^{+/+}$/h$FcRn^{+/+}$ and WT mice before (upper blot) and after (lower blot) the infusion of exogenous human serum albumin (HSA). The densitometric analysis of the bands is shown at the bottom. C, Representative photomicrographs (×200 magnification) of c-caspase-3-stained liver sections from WT and h$Alb^{+/+}$/h$FcRn^{+/+}$ mice receiving HSA, and then, challenged with LPS + D-gal. D, Representative photomicrographs (x200 magnification) of liver sections from h$Alb^{+/+}$/h$FcRn^{+/+}$ mice with preexisting CCl_4-induced early cirrhosis challenged with LPS + D-gal receiving or not HSA. E, Results from TUNEL assay in the mouse groups described in panel D. F, Quantitation of c-caspase-3 staining and TUNEL from panel D and E, respectively. G, Protein levels of c-caspase-3 and total caspase-3 in the mouse groups described in panel D. Results are expressed as mean ± SEM. Two-tailed unpaired t test was used for statistical differences of the end-points and one-way ANOVA was used for statistical analysis for multiple comparisons corrected with Tukey post hoc test

mice appeared to better maintain circulating human albumin levels after its exogenous administration, but differences did not reach statistical significance (Figure 1B). Importantly, h$Alb^{+/+}$/h$FcRn^{+/+}$ mice exhibited superior efficacy in the ability of HSA to lessen LPS/D-gal-induced injury than WT mice (Figure 1C). These protective effects against LPS/D-gal injury were also seen in h$Alb^{+/+}$/h$FcRn^{+/+}$ mice chronically induced to early cirrhosis by CCl_4 administration. (Figures 1D and S2). Indeed, the immunostaining for cleaved-caspase-3 protein in response to LPS/D-gal was significantly reduced in h$Alb^{+/+}$/h$FcRn^{+/+}$ mice with preexisting early cirrhosis receiving albumin than in those not receiving it. This inhibitory action was further confirmed by measuring the level of fragmented DNA of apoptotic cells by TUNEL assay (Figure 1E). Quantification of cleaved-caspase-3 and fragmented DNA in these experiments is given in Figure 1F. Further confirmation was obtained by measuring the protein levels of cleaved-caspase-3 by Western blot (Figure 1G).

3.2 | Effects of albumin on inflammatory cell infiltration into the injured liver

Since inflammatory cell infiltration into the injured liver is a hallmark of the LPS/D-gal model,[20] we next assessed whether albumin administration impacted the recruitment of macrophages into liver tissue. As shown in Figure 2A, h$Alb^{+/+}$/h$FcRn^{+/+}$ mice with early cirrhosis that were administered albumin showed less extensive hepatic staining for the macrophage marker F4/80 in response to LPS/D-gal challenge. Furthermore, the signal for the fractalkine receptor CX3CR1, which is a marker of infiltrating monocyte-derived macrophages, in the

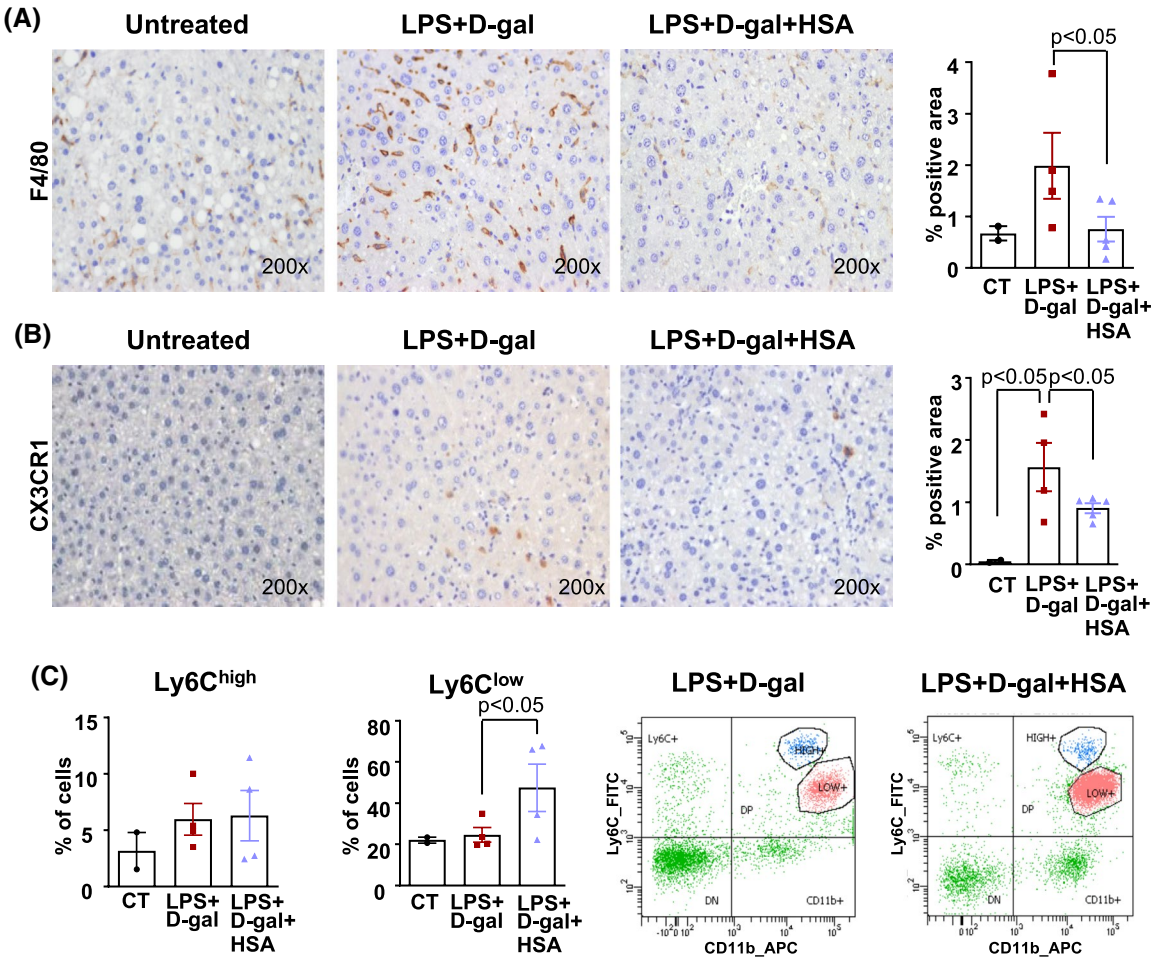

FIGURE 2 Effects of albumin on inflammatory cell infiltration into the injured liver. A, Representative photomicrographs (×200 magnification) of F4/80-stained liver sections from h$Alb^{+/+}$/h$FcRn^{+/+}$ mice with preexisting CCl_4-induced early cirrhosis, exposed to LPS + D-gal injury and receiving or not human serum albumin (HSA). B, Representative photomicrographs (×200 magnification) of CX3CR1-stained liver sections from the mouse groups described in panel A. C, Ly6C expression in peripheral monocytes from h$Alb^{+/+}$/h$FcRn^{+/+}$ mice with cirrhosis exposed to LPS + D-gal injury and receiving HSA. Right side of the panel: representative flow cytometry plots using APC-labeled anti-mouse CD11b and FITC-labeled anti-mouse Ly6C antibodies. Results are expressed as mean ± SEM. Two-tailed unpaired t test was used for statistical differences of the end-points and one-way ANOVA was used for statistical analysis for multiple comparisons corrected with Tukey post hoc test. CT, control placebo for LPS + D-gal

liver of h$Alb^{+/+}$/h$FcRn^{+/+}$ mice after LPS/D-gal challenge was significantly reduced by albumin administration (Figure 2B). We also assessed whether albumin administration could impact the phenotype of circulating monocytes, limiting their ability to infiltrate into the injured liver. As shown in Figure 2C, LPS/D-gal-challenged h$Alb^{+/+}$/h$FcRn^{+/+}$ mice receiving albumin had a larger proportion of peripheral monocytes with low Ly6C expression (Ly6C^{low}), which is a marker of "patrolling" monocytes with a more pro-resolutive phenotype and less capacity to infiltrate tissues.[21] Representative flow cytometry plots are also shown in Figure 2C.

3.3 | Evidence of the cytoprotective actions of albumin in cultured hepatocytes

To get an in vivo-in vitro translation of our findings, we next performed experiments in hepatocytes challenged with TNFα in the presence of the transcriptional inhibitor actinomycin D, which is required to unveil the cell death process induced by this cytokine in hepatocytes.[18] Figure 3A shows a characteristic phase contrast microscopy image of the hepatocytes in culture (left image). The center image shows the characterization of hepatocytes by immunofluorescence using an antibody against albumin and the right image shows a representative hepatocyte labeled with anti-albumin and anti-HNF4α antibodies (see Material and Methods for details). Figure 3B shows changes in caspase-3 activity in these cultures of hepatocytes when they were treated with TNFα in the absence or presence of albumin. As shown, incubation of hepatocytes with TNFα resulted in increased caspase-3 activity, whereas addition of albumin for 30 minutes prior to TNFα challenge protected these cells by reducing caspase-3 activity as compared to hepatocytes exposed to TNFα alone (Figure 3B). To exclude the possibility that the addition of albumin could interfere with the binding of TNFα to hepatocytes, the experiments were repeated following a treatment mode, in which hepatocytes were first exposed to TNFα and subsequently incubated with albumin. As shown in Figure 3C, under these conditions, albumin reduced TNFα-induced caspase-3 activity in a similar manner to that observed in the pretreatment mode. To exclude the possibility that the effect of albumin was dependent on the presence of serum

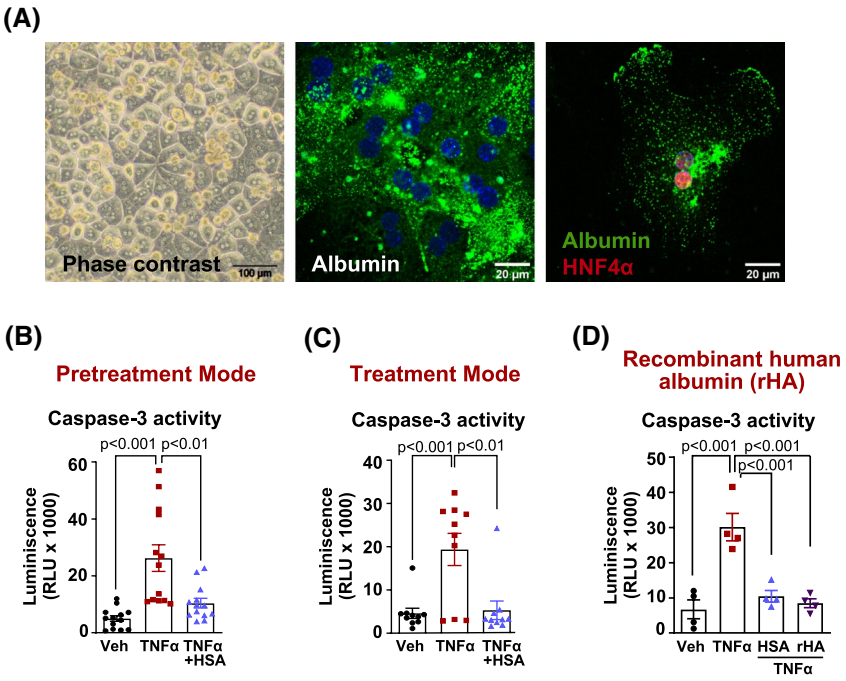

FIGURE 3 Cytoprotective effects of albumin in cultured hepatocytes. A, Left: Representative image of cultured hepatocytes captured by phase contrast microscopy. Middle: Representative image of hepatocytes stained with Alexa Fluor 488-labeled secondary antibody (green) against a primary antibody that recognizes albumin and visualized by confocal microscopy. Right: Representative image of an individual hepatocyte incubated with primary antibodies that recognize albumin (green) or HNF4α (red) and stained, respectively, with Alexa Fluor 488 and Alexa Fluor 555-labeled secondary antibodies. Nuclei were stained with Hoechst (blue). Images were taken at ×63 oil objective, zoom 1. B, Luminescent signals corresponding to the measurement of caspase-3 activity in primary hepatocytes incubated in the presence or absence of human serum albumin (HSA) (15 mg/mL) for 30 minutes, and then, exposed to vehicle (Veh) or TNFα (20 ng/mL) + actinomycin D (250 ng/mL) for 6 hours. C, Caspase-3 activity in primary hepatocytes exposed to Veh or TNFα plus actinomycin D for 1 hour, and then, incubated in the presence or absence of HSA for a total of 6 hours. D, Caspase-3 activity in primary hepatocytes pretreated for 30 minutes with HSA or recombinant human albumin (rHA) from *Oryza sativa* before inducing damage with TNFα plus actinomycin D for 6 hours. Results are expressed as mean ± SEM from a minimum of three independent experiments assayed in duplicate. Two-tailed unpaired t test was used for statistical differences of the end-points and one-way ANOVA was used for statistical analysis for multiple comparisons corrected with Tukey post hoc test. RLU, relative light units

factors with potential caspase-3 inhibitory properties bound to its molecule, we repeated the experiments with recombinant human albumin expressed in *Oryza sativa*. As shown in Figure 3D, the protective effects of recombinant albumin were similar to those of albumin. These findings indicate that the modulatory actions of albumin on caspase-3 activity were unrelated to its scavenging properties and were independent of serum factors potentially bound to this molecule.

3.4 | Mechanistic aspects of the cytoprotective actions of albumin in cultured hepatocytes

Since albumin is taken up and internalized by hepatocytes,[22] we hypothesized that albumin might modulate the intracellular TNFα signaling pathway in these liver cells. The first mechanism that we explored was whether albumin had the ability to modulate the mitochondrial release of cytochrome c, which plays a central role in TNFα-induced caspase-3 activation in hepatocytes.[23] For this purpose, we conducted confocal microscopy experiments in hepatocytes treated with TNFα and actinomycin D in the presence or absence of albumin. As shown in Figure 4A, cytochrome c was localized within the mitochondria in resting vehicle-treated hepatocytes (upper panel) and was released into the cytoplasm during TNFα incubation (middle panel), an effect that was not observed when albumin was present in the hepatocyte culture medium (lower panel). Quantification of the colocalization of cytochrome c and the mitochondrial marker ATP5a is given in Figure 4B.

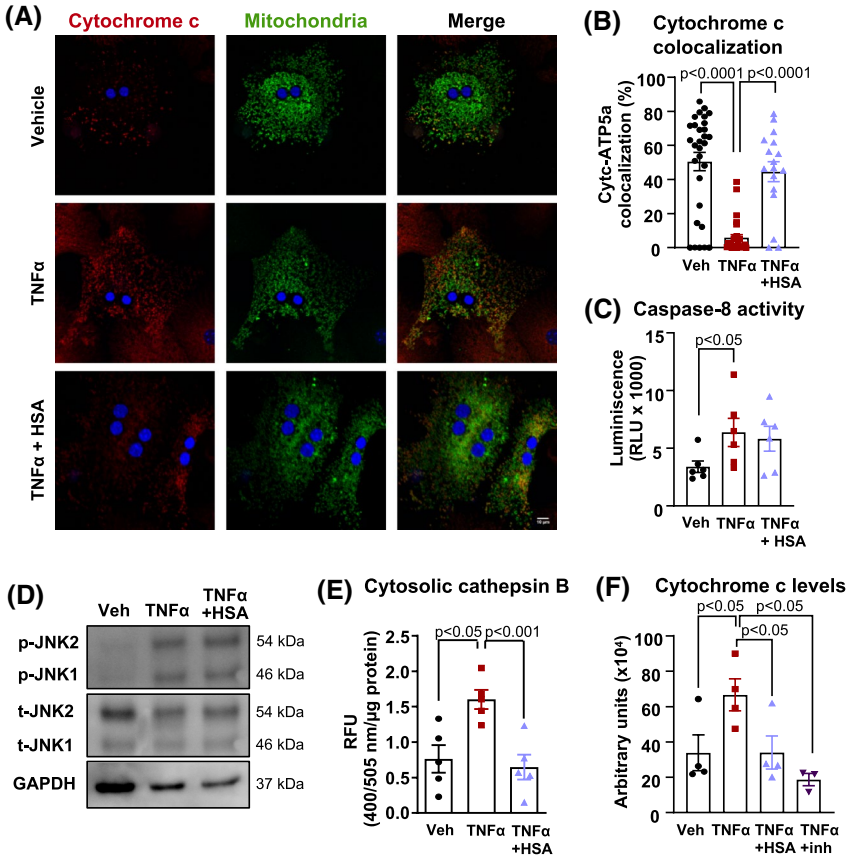

FIGURE 4 Mechanisms underlying the cytoprotective actions of albumin in cultured hepatocytes. A, Representative images captured by confocal microscopy of primary hepatocytes treated with vehicle (upper panel), TNFα (20 ng/mL) plus actinomycin D (middle panel), or TNFα plus actinomycin D in the presence of human serum albumin (HSA) (15 mg/mL) (lower panel) for 4 hours. Mitochondria (green) were stained with Alexa Fluor 488-labeled secondary antibody against a primary antibody that recognizes the mitochondrial membrane marker ATP5a. Cytochrome c (red) was stained with a primary antibody anti-cytochrome c conjugated with Alexa Fluor 555. Nuclei were stained with Hoechst (blue). Images were taken at ×63 oil objective, zoom 3. B, Quantitation of cytochrome c and ATP5a colocalization in hepatocytes incubated with the experimental conditions described in A. C, Luminescent signals corresponding to the measurement of caspase-8 activity in hepatocytes incubated with Veh or HSA for 30 minutes, and then, challenged with TNFα plus actinomycin for 6 hours. D, Representative Western blot of p-JNK and JNK levels in hepatocytes incubated as above. E, Fluorescence signals corresponding to the measurement of cytosolic levels of cathepsin B in hepatocytes incubated with the experimental conditions described in C. F, Cytochrome c levels in hepatocytes incubated as described above but including pretreatment with the cathepsin B inhibitor CA-074 Me (inh). Results are expressed as mean ± SEM from a minimum of three independent experiments assayed in duplicate. Two-tailed unpaired t test was used for statistical differences of the end-points and one-way ANOVA was used for statistical analysis for multiple comparisons corrected with Tukey post hoc test. RLU, relative light units

We next explored the intracellular routes linking HSA to decreased mitochondrial cytochrome c release and reduced caspase-3 activity in hepatocytes challenged with TNFα. The first route we assessed was caspase-8, which is the most proximal caspase activated following interaction of TNFα with its death receptor.[24] As shown in Figure 4C, albumin was ineffective in reducing TNFα-induced caspase-8 activity. We then wondered whether albumin would interfere with JNKs, which are mitogen-associated protein kinases strongly associated with hepatocyte injury.[25] As shown in Figure 4D, phosphorylation of JNK1 and JNK2 in response to TNFα was not affected by albumin. We finally explored the possibility that the inhibitory actions of albumin could be mediated by cathepsin B, which leakage from the lysosome to the cytosol triggers mitochondrial cytochrome c release and caspase-3 activity in hepatocytes incubated with TNFα.[26] As shown in Figure 4E, hepatocytes treated with TNFα had increased cytosolic content of cathepsin B, an effect that was completely reversed by the presence of albumin in the culture medium. Of note, the effect of albumin on TNFα-induced cytochrome c release from the mitochondria was of similar magnitude to that of the selective cathepsin B inhibitor CA-074-Me (Figure 4F). A schematic diagram of how albumin could affect the TNFα apoptotic signaling pathway in hepatocytes is shown in Figure 5, which proposes that albumin protects hepatocytes from TNFα-induced caspase-3 activation by blocking the lysosomal cathepsin B leakage and the subsequent release of cytochrome c from the mitochondria to the cytosol.

3.5 | The protective effects of albumin against liver injury were confirmed ex vivo in PCLS

The protective effects of albumin observed in vivo (mice) and in vitro (hepatocytes) were further confirmed ex vivo in PCLS. The advantage of the PCLS model is that it preserves cell-cell interactions in the original three-dimensional hepatic architecture and is a reliable tool for studying the response of liver tissue to injury.[15,16] Therefore, it is useful to monitor not only the response of hepatocytes, but also the contribution of other liver cells types (ie, Kupffer cells) to inflammatory injury. In preliminary experiments, we observed that gene expression was maintained stable in PCLS for periods up to 32 hours (Figure S3A), although LDH leakage (a signature marker of damage/permeabilized hepatocyte membrane) followed a clear upward trend after 20 hours of culture (Figure S3B). To perform the experiments in the most optimal conditions, studies in PCLS were always performed within a 12-hour time frame. As shown in Figure 6A, caspase-3 activity was higher in PCLS incubated with TNFα (always in the presence of actinomycin D) than in those exposed to vehicle. Similar to what was observed in cultures of hepatocytes, preincubation with albumin for 30 minutes rendered PCLS resistant to TNFα-induced caspase-3 activation (Figure 6A). In addition, PCLS in which albumin was added 1 hour after the TNFα challenge (treatment mode) also exhibited reduced caspase-3 activity in comparison to PCLS receiving TNFα alone (Figure 6B). In these experiments, LDH release was not significantly affected by either TNFα or albumin (Figure S4).

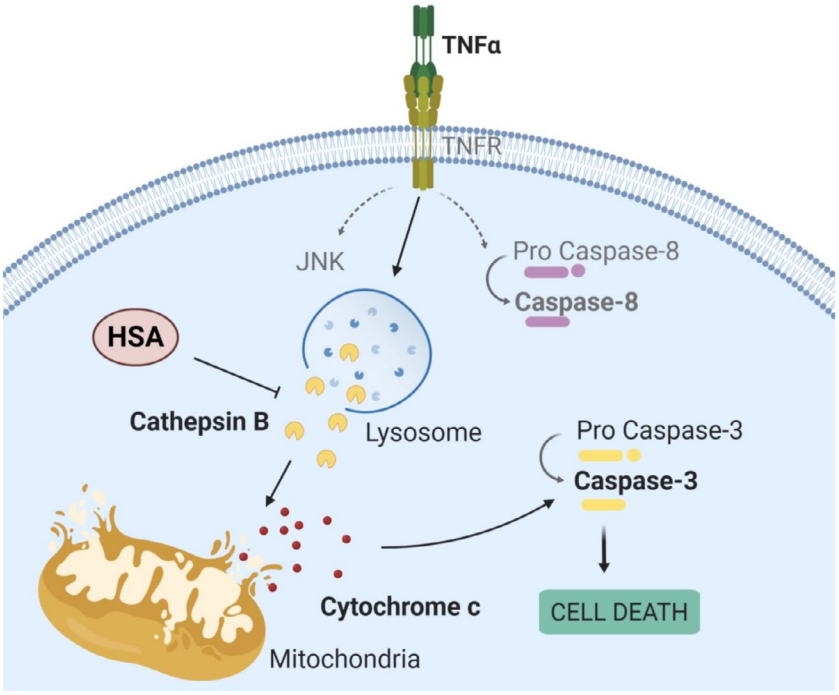

FIGURE 5 Schematic diagram of the potential modulatory effect of albumin on the TNFα apoptotic signaling pathway in hepatocytes. Among the different pathways leading to hepatocyte cell death, human serum albumin (HSA) appears to specifically inhibit the leakage of cathepsin B from lysosomes to the cytosol in response to TNFα, an effect that was associated with decreased mitochondrial cytochrome c release and reduced caspase-3 activity. JNK, c-Jun N-terminal kinase; TNFR, TNFα receptor

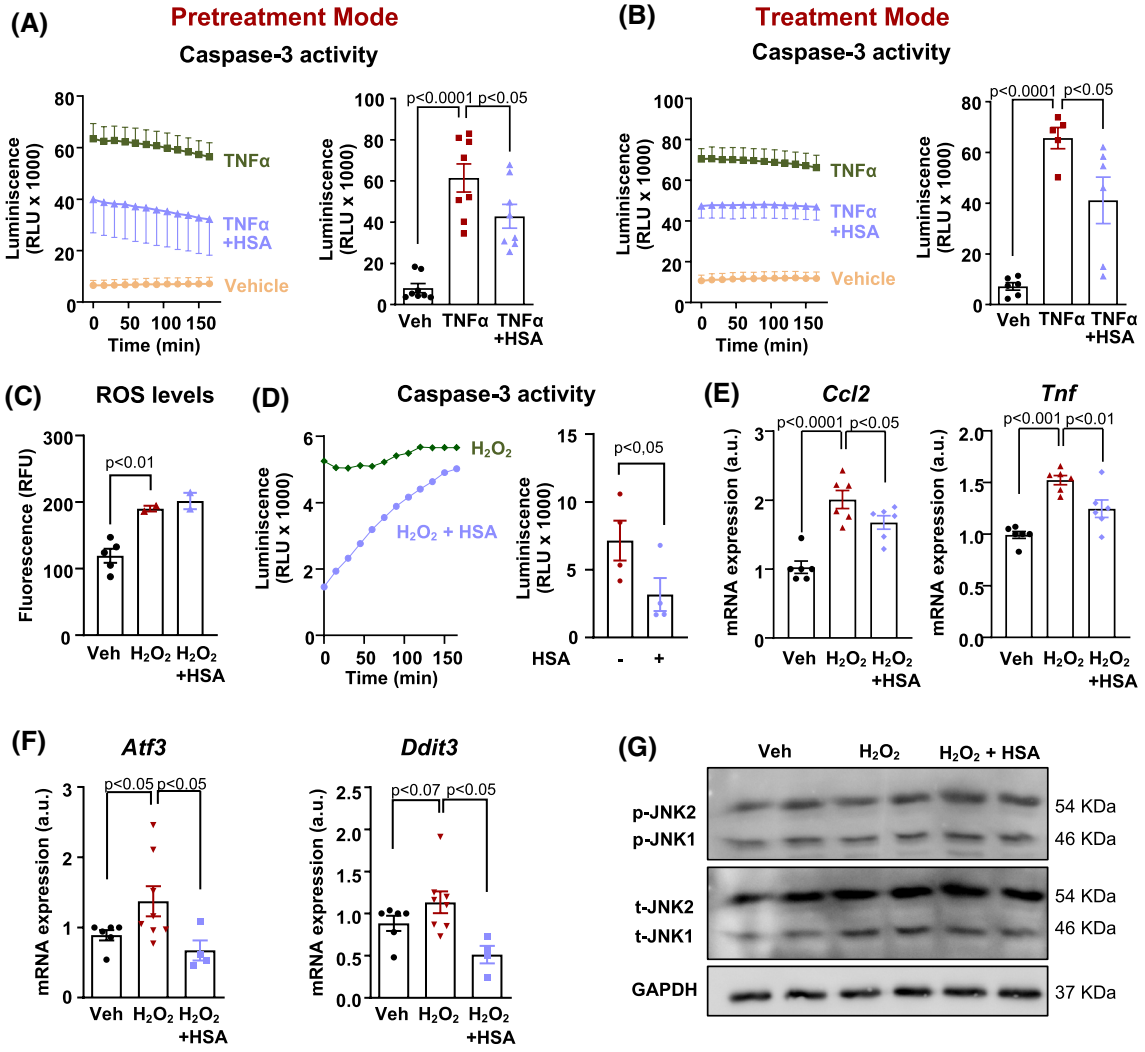

FIGURE 6 Cytoprotective effects of albumin in PCLS and effects on the H_2O_2-induced oxidative stress model. A, Luminescent signals corresponding to the measurement of caspase-3 activity in PCLS incubated in the presence or absence of human serum albumin (HSA) (15 mg/mL) for 30 minutes, and then, exposed to Veh or TNFα (10 ng/mL) + actinomycin D (10 μg/mL) for 12 hours. B, Caspase-3 activity in PCLS exposed to Veh or TNFα plus actinomycin D for 1 hour, and then, incubated in the presence or absence of HSA for a total of 12 hours. C, Effects of HSA on H_2O_2-induced ROS levels. D, Luminescent signals corresponding to the measurement of the impact of HSA on H_2O_2-induced caspase-3 activity. E, Effect of HSA on H_2O_2-induced *Ccl2* and *Tnf* mRNA expression. F, Effect of HSA on H_2O_2-induced *Atf3* and *Ddit3* mRNA expression. G, Effect of HSA on p-JNK1, p-JNK2, and total JNKs (t-JNK1 and t-JNK2) protein levels determined by Western blot. Results are expressed as mean ± SEM from 3 to 4 independent experiments assayed in duplicate. Two-tailed unpaired t test was used for statistical differences of the end-points and one-way ANOVA was used for statistical analysis for multiple comparisons corrected with Tukey post hoc test. RFU, relative fluorescence units; RLU, relative light units

3.6 | The cytoprotective effects of albumin were not limited to the TNFα-induced liver injury model

To explore whether the effects of albumin were restricted to the TNFα-induced tissue injury model or had a broader scope, we next exposed hepatocytes and PCLS to H_2O_2, which is a radical oxygen species (ROS) producing oxidative stress-induced injury model.[27] Although albumin was not able to reduce ROS levels in hepatocytes incubated with H_2O_2 (Figure 6C), this protein exerted cytoprotection against H_2O_2-induced caspase-3 activity (Figure 6D). While the TNFα model requires transcriptional arrest, and therefore, the absence of changes in gene expression (see, eg, Figure S5 for the expression of inflammatory genes such as *Tnf* and *Ccl2*), the H_2O_2 model is associated with an early adaptive response characterized by the induction of genes involved in inflammation.[27] Consistent with this view, H_2O_2 significantly upregulated *Ccl2* and *Tnf* expression, effects that were modestly, but significantly prevented by albumin (Figure 6E). In addition, albumin significantly ameliorated the H_2O_2 induction of genes involved in ER stress and DNA damage

such *Atf3* and *Ddit3* (Figure 6F). Finally, no changes in JNK phosphorylation were observed in this model (Figure 6G). These data indicate that albumin not only ameliorates TNFα-induced cell death, but might also modulate tissue injury and the induction of inflammatory genes in response to oxidative damage.

4 | DISCUSSION

The results of the current study provide evidence that albumin is able to ameliorate cytokine-induced liver tissue damage. This conclusion was reached from data collected at multimodal experimental levels, including in vitro and ex vivo studies in hepatocytes and PCLS challenged with TNFα and in vivo studies in double transgenic humanized h$Alb^{+/+}$/h$FcRn^{+/+}$ mice with preexisting CCl_4-induced early cirrhosis challenged with LPS/D-gal, a model of TNFα-mediated acute liver injury. Collectively, our data support the view that albumin, in addition to ameliorate cytokine production, might also protect tissues from cytokine-induced immunopathology.

A number of studies have previously demonstrated that albumin is the major antiapoptotic signaling component in the serum.[28-33] In particular, Zoellner et al,[28] Barbaro et al[29] and Tabernero et al[30] were among the first to describe that albumin at physiological concentrations was a specific inhibitor of programed cell death in human endothelial cells, human pancreatic islets, and rat neurons, respectively. Similar findings were reported in human and murine cell lines.[31-33] Although these studies provided little information on the mechanisms underlying the antiapoptotic effects of albumin and none specifically referred to TNFα-induced hepatocyte death, several potential pathways were enumerated. Among these, modulation of the AKT signaling pathway,[31] attenuation of intracellular calcium levels,[28] and prevention of mitochondrial membrane depolarization.[32,33] Mitochondria, indeed, play a key role in TNFα-induced hepatocyte apoptosis by releasing cytochrome c into the cytosol.[5,23,34] Cytochrome c is a 12-kDa protein that functions in the mitochondrial electron transport chain diffusing between the inner and the outer membranes within complexes III and IV.[35] However, in response to TNFα, cytochrome c is released into the cytosol where it forms the apoptosome complex resulting in the activation of caspase-3, which is a potent executioner of hepatocyte apoptosis.[34] In line with these data, in our experiments, we observed a reduction in cytochrome c release from mitochondria to the cytosol accompanied by decreased caspase-3 activity after incubating primary hepatocytes with albumin, indicating that the mitochondrial pathway is an essential component underlying the protective actions of albumin in the liver.

Our results also provided a mechanistic basis by which albumin reduced the mitochondrial release of cytochrome c in response to TNFα. Binding of TNFα to its death receptor results in recruitment of adaptor proteins and initiator procaspase-8 to form a large multiprotein complex known as death inducing signaling complex or DISC.[23,34] However, in our conditions, albumin was ineffective in reducing TNFα-induced caspase-8 despite affecting caspase-8 downstream signals such as cytochrome c release. HSA did not affect TNFα-induced JNK either. In view of these findings, we explored alternative pathways leading to mitochondrial cytochrome c release and focused our attention on the endolysosomal system. A variety of death stimuli produce lysosomal membrane permeabilization and trigger the release of lysosomal enzymes into the cytosol. Among the different lysosomal enzymes, the cysteine protease cathepsin B plays a prominent role in the execution of the apoptotic cascade upstream of the mitochondria.[23,26,34] In fact, our data on primary hepatocytes indicated the ability of albumin to inhibit the leakage of cathepsin B from lysosomes to the cytosol in response to TNFα, an effect that was associated with decreased mitochondrial cytochrome c release and reduced caspase-3 activity. Together, these findings propose that albumin modulates TNFα-induced injury by preventing lysosomal membrane permeabilization and the release of cathepsin B.

Our study poses some limitations worth noting. First, in the current investigation, we did not elucidate the mechanism by which albumin prevents lysosomal membrane permeabilization in hepatocytes, although we could speculate that this process is related to the ability of cells to internalize albumin and its preferential accumulation in the endosome-lysosomal compartment. Indeed, intracellular albumin uptake has been demonstrated in hepatocytes and leukocytes as well as in endothelial and epithelial cell lines using fluorescently labeled albumin and confocal microscopy or flow cytometry approaches.[10,22,36,37] Importantly, in these cells, the intracellular albumin signal was shown to colocalize with markers of the endosome-lysosomal compartment.[10,37] In this subcellular compartment, albumin is not a mere bystander but rather is an effector molecule able to block endosomal TLR signaling and the production of cytokines by leukocytes in response to bacterial DNA.[10] The intricate mechanisms by which albumin modulates endosome-lysosomal functions, and specifically how albumin might prevent lysosomal membrane permeabilization and the leakage of cathepsin B, are difficult to delineate, but a plausible hypothesis is that albumin stabilizes the lysosomal membrane due to its lipophilic properties.[8] The second limitation of our study was the short duration (6 weeks) of the CCl_4 treatment in our model, which does not completely reproduce advanced stages of liver cirrhosis. Although we provided evidence that our mice had histologically proven early cirrhosis after 6 weeks of CCl_4 treatment, more appropriate models with longer duration of CCl_4 exposure combined with LPS injection, such as that one described by Carl et al[38] are needed to fully understand the effect of albumin in more advanced stages of liver cirrhosis.

In summary, the results of the current study provide evidence of the cytoprotective actions of albumin in the liver. In particular, our findings demonstrate the ability of albumin to protect hepatocytes from the cytotoxic effects of the cytokine TNFα. These albumin protective actions were mediated by inhibition of the leakage of the cysteine protease cathepsin B from lysosomes accompanied by a reduction in cytochrome c release from mitochondria. Taken together, these findings provide evidence that albumin is not only able to reduce cytokine production, but also to prevent the tissue damaging actions of these inflammatory mediators.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST/DISCLOSURE

The authors have no conflicts of interest.

AUTHOR CONTRIBUTIONS

M. Duran-Güell Performed the experiments; M. Duran-Güell, J. Clària conceived and designed the experiments; M. Duran-Güell acquisition and analysis of data; E. Titos, J. Alcaraz-Quiles, A. Díaz, and C. López-Vicario supervised procedures; M. Casulleras, R. Flores-Costa contributed to acquisition of data; M. Duran-Güell, J. Clària drafting of the manuscript; M. Duran-Güell, J. Clària writing of the final manuscript; V. Arroyo, J. Fernández, R. Horrillo, M. Costa participated in critically revising the draft of the article; J. Clària study supervision.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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SUPPLEMENTARY DATA

Albumin Protects the Liver from Tumor Necrosis Factor α-Induced Immunopathology

Marta Duran-Güell, Roger Flores-Costa, Mireia Casulleras, Cristina López-Vicario, Esther Titos, Alba Díaz, José Alcaraz-Quiles, Raquel Horrillo, Montserrat Costa, Javier Fernández, Vicente Arroyo and Joan Clària

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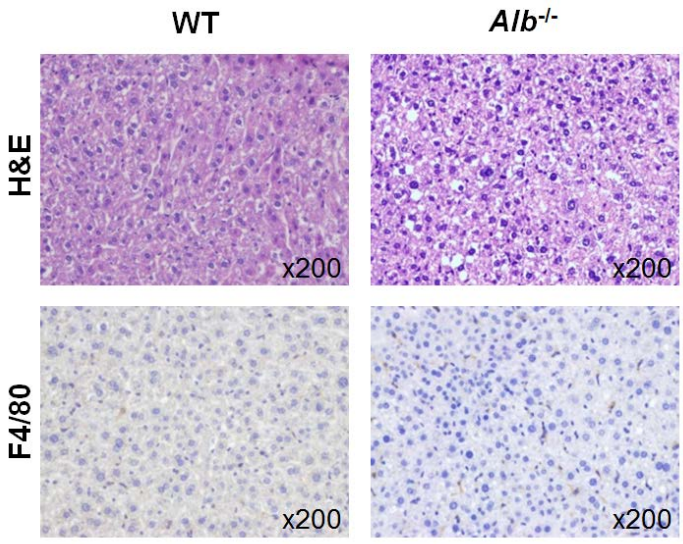
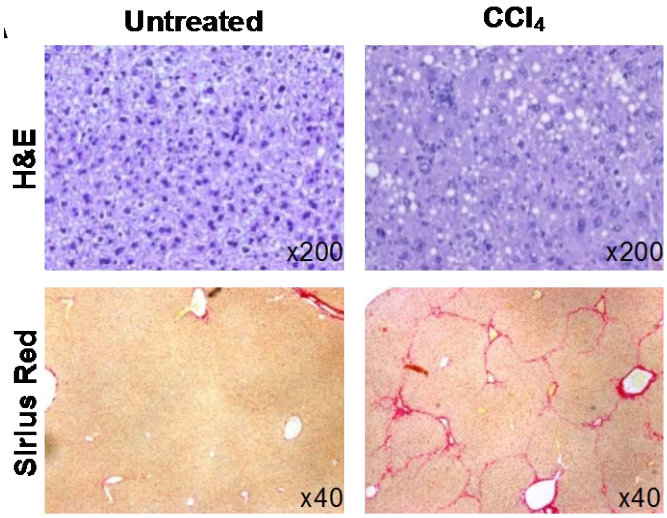
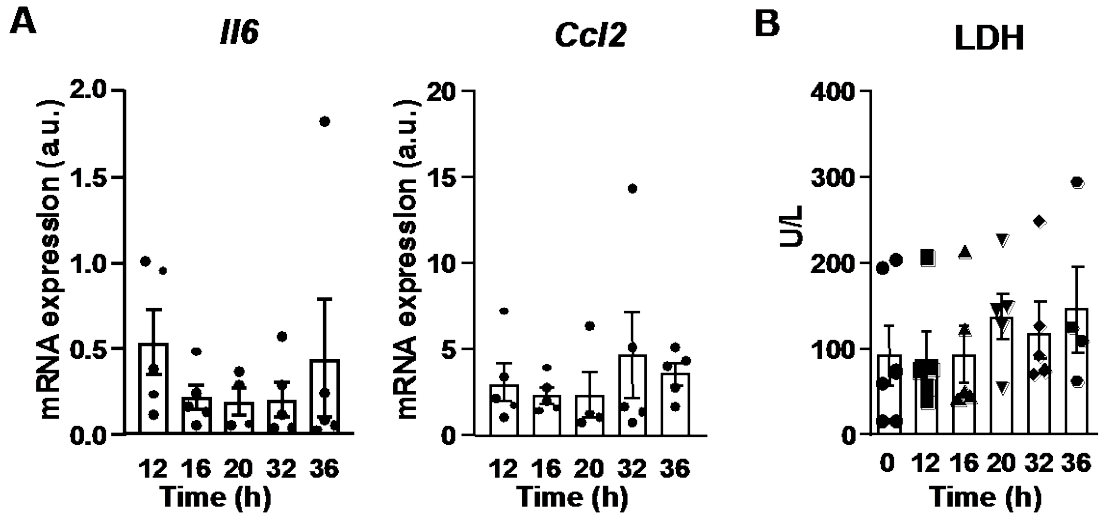


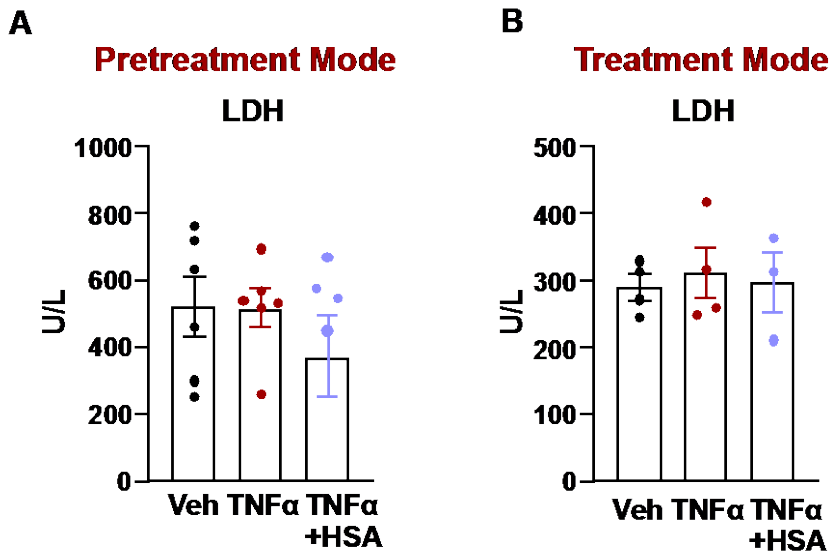
Supplemental Figure 1. Representative photomicrographs (x200 magnification) of liver sections stained with hematoxylin and eosin (H&E) and F4/80 from analbuminemic ($Alb^{-/-}$) and wild type (WT) mice.



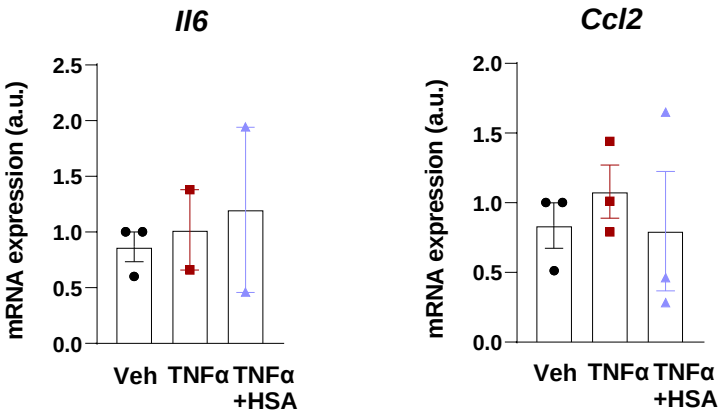
Supplementary Figure 2. Representative photomicrographs of liver sections stained with hematoxylin and eosin (H&E) (x200 magnification) and Sirius red (x40 magnification) from untreated h*Alb*$^{+/+}$/h*FcRn*$^{+/+}$ mice and from h*Alb*$^{+/+}$/h*FcRn*$^{+/+}$ mice receiving CCl_4 for 6 weeks.



Supplementary Figure 3. (A) *Il6* and *Ccl2* mRNA expression determined by real-time PCR in PCLS maintained in culture from 12 to 36 hours. **(B)** LDH levels in PCLS supernatants of the experiments described in A. Results are expressed as mean±SEM from a minimum of 3 independent experiments assayed in duplicate. One-way ANOVA was used for statistical analysis for multiple comparisons corrected with Tukey post-hoc test.



Supplementary Figure 4. (A) LDH levels in PCLS supernatants incubated in the presence or absence of human serum albumin (HSA) (15 mg/mL) for 30 min and then exposed to vehicle (Veh) or TNFα (10 ng/ml) + actinomycin D (10 µg/mL) for 12 hours. **(B)** LDH levels in PCLS supernatants exposed to Veh or TNFα (10 ng/ml) + actinomycin D (10 µg/mL) for 30 min and then incubated with HSA (15 mg/mL) for 12 hours. Results are expressed as mean±SEM from a minimum of 3 independent experiments assayed in duplicate. One-way ANOVA was used for statistical analysis for multiple comparisons corrected with Tukey post-hoc test.



Supplementary Figure 5. *Il6* and *Ccl2* mRNA expression in PCLS incubated with TNFα and actinomycin D for 12 hours in the presence or absence of human serum albumin (HSA). Results are expressed as mean±SEM from a minimum of 3 independent experiments assayed in duplicate. One-way ANOVA was used for statistical analysis for multiple comparisons corrected with Tukey post-hoc test.

Supplementary Table 1. Real-time PCR probes used for the assessment of gene expression. All the probes are validated and pre-designed Taqman Gene Expression Assays.

Gene	Protein	Location	Reference
Actb	β-ACTIN	Chr.5: 142903116 - 142906724	Mm00607939_s1
Atf3	ATF3	Chr.1: 191170296 - 191183333	Mm00476032_m1
Ccl2	MCP-1	Chr.11: 82035577 - 82037452	Mm00441242_m1
Cxcl1	CXCL1	Chr.5: 90891245 - 90893121	Mm04207460_m1
Ddit3	CHOP	Chr.10: 127290793 - 127296288	Mm001135937_g1
Il6	IL-6	Chr.5: 30013161 - 30019968	Mm00446190_m1
Tnf	TNF-α	Chr.17: 35199367 - 35202007	Mm00443258_m1

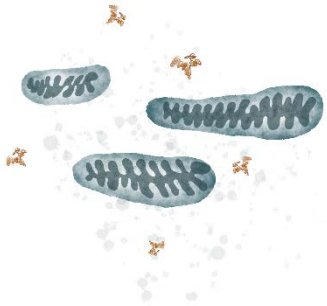
Supplementary Table 2. Baseline phenotype of albumin deficient mice (*Alb*$^{-/-}$) and transgenic humanized mice expressing the human genes for albumin (h*Alb*) and its FcRn receptor (h*FcRn*) as compared to wild type (WT) mice.

Parameter	**WT**	***Alb*$^{-/-}$**	**h*Alb*$^{+/+}$/h*FcRn*$^{+/+}$**
Body weight (g)	32.8 ± 1.7	41.25 ± 3.1	44.0 ± 3.0*
Liver to body weight ratio	0.04 ± 0.01	0.04 ± 0.003	0.04 ± 0.01
WAT to body weight ratio	0.015 ± 0.002	0.06 ± 0.009*	0.047 ± 0.002*
AST (U/L)	101.0 ± 18.8	79.7 ± 38.7	81.0 ± 1.0
ALT (U/L)	21.8 ± 4.1	17.7 ± 3.4	29.0 ± 4.0
Creatinine (mg/dL)	0.20 ± 0.02	0.15 ± 0.01	0.15 ± 0.01

WAT: white adipose tissue; AST: aspartate aminotransferase; ALT: alanine aminotransferase. Data are presented as mean ± SEM. *p<0.05 vs. WT

STUDY II: Essential role for albumin in preserving liver cells from TNFα-induced mitochondrial injury.

Marta Duran-Güell, Glòria Garrabou, Roger Flores-Costa, Mireia Casulleras, Cristina López-Vicario, Ingrid W. Zhang, Judith Cantó-Santos, Bryan J. Contreras, María Belén Sánchez-Rodríguez, Berta Romero-Grimaldo, Raquel Horrillo, Montserrat Costa, Vicente Arroyo and Joan Clària. FASEB J. 2023, 37:e22817. DOI: 10.1096/fj.202201526R.



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RESEARCH ARTICLE

The FASEB Journal

Essential role for albumin in preserving liver cells from TNFα-induced mitochondrial injury

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Graphical Abstract

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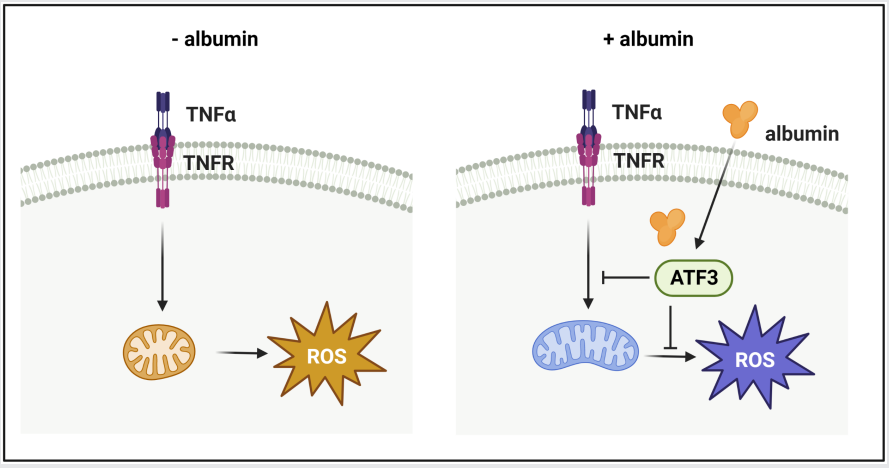

Albumin modulates TNFα -induced mitochondrial oxidative stress in hepatocytes. After binding to its membrane receptor, TNFα induces mitochondrial injury and the release of radical oxygen species (ROS). In the presence of albumin, the mitochondrial release of ROS is ameliorated through mechanisms involving the antioxidant-activating transcription factor 3. TNFR, TNFa receptor.

Abbreviations: ATF3, activating transcription factor 3; ATP, adenosine triphosphate; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; Ccl2, monocyte chemotactic protein 1; D-gal, D-galactosamine; ETC, electron transport chain; FADH, flavin adenine dinucleotide; FAO, fatty acid β-oxidation; GSH, glutathione; HO-1, heme oxygenase-1; HSA, human serum albumin; IRG1, immunoresponsive gene 1; LPS, lipopolysaccharide; NADH, nicotinamide adenine dinucleotide; NRF2, nuclear factor erythroid-2-related factor 2; OI, 4-octyl itaconate; OXPHOS, oxidative phosphorylation; PCLS, precision-cut liver slices; ROS, radical oxygen species; ROX, residual oxygen consumption; SDH, succinate dehydrogenase complex; TAG, triacylglycerol; TCA, tricarboxylic acid; TEM, transmission electron microscopy; TNFα, tumor necrosis factor alpha.

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RESEARCH ARTICLE

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Essential role for albumin in preserving liver cells from TNFα-induced mitochondrial injury

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Abstract

Cytokine-induced inflammation and mitochondrial oxidative stress are key drivers of liver tissue injury. Here, we describe experiments modeling hepatic inflammatory conditions in which plasma leakage leads to large amounts of albumin to reach the interstitium and parenchymal surfaces to explore whether this protein plays a role in preserving hepatocyte mitochondria against the damaging actions of the cytotoxic cytokine tumor necrosis factor alpha (TNFα). Hepatocytes and precision-cut liver slices were cultured in the absence or presence of albumin in the cell media and then exposed to mitochondrial injury with the cytokine TNFα. The homeostatic role of albumin was also investigated in a mouse model of TNFα-mediated liver injury induced by lipopolysaccharide and D-galactosamine (LPS/D-gal). Mitochondrial ultrastructure, oxygen consumption, ATP and reactive oxygen species (ROS) generation, fatty acid β-oxidation (FAO), and metabolic fluxes were assessed by transmission electron microscopy (TEM), high-resolution respirometry, luminescence-fluorimetric-colorimetric assays and NADH/$FADH_2$ production from various substrates, respectively. TEM analysis revealed that in the absence of albumin, hepatocytes were more susceptible to the damaging actions of TNFα and showed more round-shaped mitochondria with less intact cristae than hepatocytes cultured with albumin. In the presence of albumin in the cell media, hepatocytes also showed reduced mitochondrial ROS generation and FAO. The mitochondria protective actions of albumin against TNFα damage

Abbreviations: ATF3, activating transcription factor 3; ATP, adenosine triphosphate; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; Ccl2, monocyte chemotactic protein 1; D-gal, D-galactosamine; ETC, electron transport chain; FADH, flavin adenine dinucleotide; FAO, fatty acid β-oxidation; GSH, glutathione; HO-1, heme oxygenase-1; HSA, human serum albumin; IRG1, immunoresponsive gene 1; LPS, lipopolysaccharide; NADH, nicotinamide adenine dinucleotide; NRF2, nuclear factor erythroid-2-related factor 2; OI, 4-octyl itaconate; OXPHOS, oxidative phosphorylation; PCLS, precision-cut liver slices; ROS, radical oxygen species; ROX, residual oxygen consumption; SDH, succinate dehydrogenase complex; TAG, triacylglycerol; TCA, tricarboxylic acid; TEM, transmission electron microscopy; TNFα, tumor necrosis factor alpha.

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were associated with the restoration of a breakpoint between isocitrate and α-ketoglutarate in the tricarboxylic acid cycle and the upregulation of the antioxidant activating transcription factor 3 (ATF3). The involvement of ATF3 and its downstream targets was confirmed in vivo in mice with LPS/D-gal-induced liver injury, which showed increased hepatic glutathione levels, indicating a reduction in oxidative stress after albumin administration. These findings reveal that the albumin molecule is required for the effective protection of liver cells from mitochondrial oxidative stress induced by TNFα. These findings emphasize the importance of maintaining the albumin levels in the interstitial fluid within the normal range to protect the tissues against inflammatory injury in patients with recurrent hypoalbuminemia.

KEYWORDS

hepatocytes, liver injury, mitochondrial dysfunction, mitochondrial oxidative stress, mitochondrial respiration, tricarboxylic acid cycle

1 | INTRODUCTION

The generation of reactive oxygen species (ROS) is inherent to living cells and characteristic ROS, such as superoxide anions, are byproducts of the normal functioning of the mitochondrial respiratory chain.[1] If this process is excessive and not properly controlled, it can give rise to oxidative stress, which is a common pathological factor in many complex diseases including liver disease, that leads to cell and tissue injury and organ dysfunction.[2] Oxidative stress is frequently associated with inflammation and both processes are mutually synergizing. For example, the production of inflammatory cytokines by cells of the innate immune system can directly trigger oxidative stress and tissue injury.[3] Specifically, exposure to the cytokine tumor necrosis factor (TNF)α, predominantly produced by monocytes/macrophages, is known to damage the mitochondria and to increase mitochondrial ROS production.[4,5]

Albumin is the most abundant protein in the bloodstream and is the major contributor to the maintenance of the plasma oncotic pressure.[6] In the intravascular compartment and besides its oncotic power, albumin exerts pleiotropic effects such as detoxification, and endothelial stabilization, with these effects being related to the ability of this protein to bind endogenous and exogenous molecules.[6–9] Apart from these scavenging properties, albumin is internalized in the endosomal compartment of peripheral leukocytes where it regulates toll-like receptor signaling and immune response to pathogens.[10] Likewise, in the extravascular compartment and interstitium, albumin exhibits non-oncotic properties in addition to maintaining the osmotic gradient.[11,12] For instance, in the liver parenchyma, albumin protects hepatocytes from TNFα-induced cell-death under conditions of actinomycin D transcriptional arrest.[13] The antiapoptotic actions of albumin on liver cells were demonstrated to be unrelated to the scavenging properties of this molecule,[13] placing albumin as a key element in liver tissue homeostasis.

In the current study, we investigated in vitro, ex vivo and in vivo how important is albumin in the maintenance of mitochondrial homeostasis when liver cells are exposed to an inflammatory microenvironment. Specifically, we cultured hepatocytes and precision-cut liver slices (PCLS) in the absence or presence of albumin and then induced mitochondrial injury with the cytokine TNFα. In our experiments, we employed albumin concentrations close to those encountered in the interstitial fluid.[12] In addition, we performed experiments with and without actinomycin D-induced transcriptional arrest to compare the mitochondrial damaging actions of this cytokine when hepatocyte survival is not compromised. We also investigated the homeostatic role of albumin in a mouse model of TNFα-mediated liver injury. Our data indicate that when hepatocytes are growing in an inflammatory milieu, the albumin molecule is required to preserve the ultrastructure and mitochondrial function in these cells from the damaging actions of TNFα. Importantly, the presence of albumin in the hepatocyte media is required for protecting these cells from mitochondrial oxidative stress induced by TNFα. These findings uncover the importance of maintaining the albumin levels within the normal range to have protection against inflammatory tissue injury in conditions characterized by recurrent hypoalbuminemia, such is the case of advanced liver disease.

2 | MATERIALS AND METHODS

2.1 | Animal models

Wild-type C57BL/6J male mice (Charles River Laboratories) were housed in cages with woodchip bedding at 50%–60% humidity and a 12-h light/dark cycle with free access to food and water. For in vitro and ex vivo experiments, mice ($n = 45$) were anesthetized with ketamine and xylazine before collection of hepatocytes or PCLS, as described below. For in vivo experiments, mice ($n = 15$) received an intraperitoneal (i.p.) injection of lipopolysaccharide (LPS, 20 μg/kg body weight [b.w.]) and D-galactosamine (D-gal, 600 mg/kg b.w.) as a model of TNFα-mediated liver injury. To test the effects of albumin, two doses of human serum albumin (Albutein, Grifols) of 1.5 g/kg b.w. or its control stabilizer were administered via i.p. 72 and 24 h prior to injection of LPS+D-gal. Six hours later, mice were euthanized by an overdose of anesthetic (a mixture of 0.1 mg ketamine/g b.w. and 0.01 mg xylazine/g b.w.), and peripheral blood was collected and serum obtained by centrifugation at 1200 ***g*** for 10 min. Liver tissue was excised and rinsed in Dulbecco's phosphate-buffered saline with calcium and magnesium ($DPBS^{++}$) and either fixed in 10% formalin and embedded in paraffin or kept at −80°C or snap-frozen in N_2 for further analysis. Animal studies were conducted in accordance with the criteria of the Investigation and Ethics Committee of the University of Barcelona following EU laws.

2.2 | Hepatocyte isolation

Hepatocytes were isolated from mice by a three-step in situ perfusion procedure using 0.04% of collagenase IV (Sigma) through the vena cava and posterior digestion with 0.001% of DNAse (Sigma)[14,15] (Figure S1). The cell suspension was filtered through a 100-μm cell strainer (Becton Dickinson) and subsequently washed by centrifugation at 600 ***g*** for 10 min with cold Hank's balanced salt solution (HBSS) followed by a centrifugation at 70 ***g*** for 2 min at 4°C. The pelleted cells were resuspended in cold William's E medium supplemented with 10% fetal bovine serum (FBS), L-glutamine (200 mM), penicillin (50 U/mL), streptomycin (50 μg/mL), insulin (1 μM), Hepes (15 mM), and β-mercaptoethanol (50 μM). The viability of hepatocytes was determined by trypan blue exclusion and the cell number was determined in a Countess II cell counter (Life Technologies). Isolated hepatocytes were seeded on collagen I-coated 12-well (5×10^5 cells/well), 6-well (9×10^5 cells/well), or 96-well (4×10^4 cells/well) plates with William's E medium. Four hours later, hepatocytes were washed three times with $DPBS^{++}$ and visualized by phase contrast microscopy. Thereafter, hepatocytes were grown overnight in a humidified 5% CO_2 incubator at 37°C in 1% FBS William's E medium before incubation with TNFα and/or albumin. To test the effects of albumin on TNFα-induced injury, experiments were carried out in pretreatment (hepatocytes were incubated first with albumin [15 mg/mL] 30 min before TNFα [20 ng/mL] addition) and treatment (hepatocytes were incubated with albumin [15 mg/mL] 1 h after the addition of TNFα [20 ng/mL]) modes. Some experiments were performed with mannitol (15 mg/mL, M1902, Sigma-Aldrich). In other experiments, hepatocytes were pre-treated with 4-octyl itaconate (OI, 125 μM) for 2 h or tempol (3 mM, Sigma-Aldrich) for 30 min. When indicated, experiments were performed in the presence of actinomycin D (250 ng/mL) to induce transcriptional arrest. At the end of the incubation period (5 h), supernatants and hepatocytes were collected for further assessments.

2.3 | PCLS and transmission electron microscopy (TEM)

For PCLS preparation, the inferior vena cava of anesthetized mice was cut off and blood was allowed to drain for 1 min. The liver was excised and placed into ice-cold DPBS without calcium and magnesium ($DPBS^{--}$). Blocks (0.5 cm^3) were cut from the main lobe with a scalpel and embedded in 4% UltraPure low-melting agarose (Invitrogen) diluted in HBSS. An agarose cube with the tissue inside was cut into 250-μm-thick slices using a vibrating blade VT1000S microtome (Leica Microsystems) as described elsewhere.[14,15] PCLS were individually transferred to P-35 Petri dishes containing Millicell cell culture inserts (EMD Millipore) and prebalanced for 20 min with warmed William's E medium containing GlutaMAX-I, D-glucose (25 mM), and gentamicin (50 μg/mL). PCLS were maintained at 37°C in a 5% CO_2 incubator for 120 min and then incubated with albumin (15 mg/mL) for 30 min before the addition of vehicle or TNFα (10 ng/mL) for 22 h. At the end of the incubation period, PCLS were collected and fixed for 30 min at room temperature with 2% paraformaldehyde and 2.5% glutaraldehyde in sodium phosphate buffer (0.1 M, ph 7.4) and kept at 4°C. The samples were post-fixed with 1% osmium tetroxide and 0.8% potassium ferrocyanide, dehydrated in acetone, and embedded in Spurr's epoxy resin. Four ultrathin sections were obtained from each sample, which were then post-stained with uranyl acetate and lead citrate and examined under a JEOL J1010 TEM.

2.4 | Measurement of mitochondrial oxygen consumption

High-resolution respirometry was performed using Oroboros Oxygraph-2k system. Hepatocytes were seeded in 6-well plates under the conditions described above. A minimum of 300 000 hepatocytes were trypsinized and resuspended in mitochondrial respiration medium (MiR05) and added into the oxygraph chamber at a final volume of 2 mL. Oxygen consumption was measured at 37°C under basal conditions and following the sequential addition of oligomycin (2 μM), repeated additions of uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP), rotenone, and antimycin A, all at 1 μM. Oligomycin inhibits adenosine triphosphate (ATP) synthase allowing the assessment of the proton leak across the inner mitochondrial membrane. CCCP renders the mitochondrial inner membrane permeable to protons, leading to rapid oxygen consumption without ATP generation, allowing the assessment of uncoupled maximal respiration. Rotenone and antimycin A are inhibitors of mitochondrial I and III complexes and allow the assessment of residual oxygen consumption (ROX) or non-mitochondrial respiration.

2.5 | Measurement of ATP levels

ATP levels in hepatocytes incubated under the conditions described above were determined using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) according to the manufacturer's instructions. Briefly, hepatocytes were seeded in 96-well opaque plates at a concentration of 40 000 cells/well and equilibrated to room temperature for 30 min before the addition of CellTiter-Glo® Reagent for 2 min to induce cell lysis. Luminescence was recorded in a plate reading luminometer (FLUOstar Optima, BMG Labtech) and background signal from control wells was subtracted.

2.6 | Assessment of fatty acid oxidation (FAO)

A non-radioactive FAO kit from Biomedical Research Service Center based on the oxidation of octanoyl-CoA and generation of nicotinamide adenine dinucleotide (NADH), which is coupled to the reduction of iodonitrotetrazolium to formazan, was used. Briefly, hepatocytes (500 000 cells/well in 12-well plates) incubated under the conditions described above were washed with $DPBS^{--}$, lysed with 100 μL of Cell Lysis Solution, and incubated on ice for 5 min. Lysates were centrifuged at 16 000 ***g*** for 5 min, supernatants were collected and protein concentrations were determined by the micro-BCA method. Fifty microliters of FAO Assay solution or control solution was added to 10 μL of the protein sample and the plate was kept in a non-CO_2 incubator for 60 min at 37°C. Optical density was read at 492 nm with a FLUOstar Optima spectrophotometer. Blank readings were subtracted and enzyme activities were corrected by the amount of protein.

2.7 | Assessment of mitochondrial oxidative stress

Hepatocytes were seeded in 96-well black-walled plates at a concentration of 40 000 cells/well and mitochondrial oxidative stress was determined using MitoSOX™ Red mitochondrial superoxide indicator (Molecular Probes) according to the manufacturer's protocol. MitoSOX is a fluorogenic dye and superoxide indicator specifically targeted to mitochondria in live cells. Oxidation of the MitoSOX reagent by mitochondrial superoxide is widely used to detect mitochondrial ROS, especially superoxide. Briefly, the MitoSOX reagent was dissolved in 13 μL of dimethyl sulfoxide to prepare a 5 mM stock solution and subsequently a 5 μM working solution by dilution with HBSS. Then, the MitoSOX reagent was added to the cells for 10 min at 37°C and 5% CO_2 protected from light. Cells were then washed once with $DPBS^{++}$ to remove background fluorescence and the plate was then read with a monochromator fluorescence reader (Infinite 200 PRO, TECAN Life Sciences) at 510 and 580 nm as excitation and emission wavelengths.

2.8 | Assessment of mitochondrial membrane potential

Hepatocytes were seeded in 96-well black-walled plates at a concentration of 50 000 cells/well, and following the incubations described above, the supernatants were removed and cells were exposed to the JC-1 dye (2 μM) (MitoProbe™, Molecular Probes) for 30 min at 37°C. After washing with warmed PBS, fluorescence was measured in the FLUOstar Optima microplate reader, first at 550/600 nm and later at 485/535 of excitation/emission wavelengths to calculate the ratio between the red and green signals. CCCP at 50 μM was used as a positive control.

2.9 | Assessment of mitochondrial metabolic function

Metabolic flux analyses were performed using MitoPlate S-1 (Biolog) based on the production of NADH and flavin

adenine dinucleotide ($FADH_2$) from various substrates, which feed electrons to the electron transport chain (ETC).[16] A tetrazolium-based redox dye acts as an electron acceptor at the distal end of the ETC and the color formation, which indicates the utilization of the substrate present in each well, is read kinetically. For the preparation of the substrates, the MitoPlate S-1 plates were solubilized with 30 µL per well of a solution containing 2× Biolog Mitochondrial Assay Solution, 6× tetrazolium redox dye MC reagent and saponin (30 µg/mL) for 1 h at 37°C. Thereafter, 30 µL of each of the solubilized substrates was added to a 96-well half area plate with hepatocytes seeded at a concentration of 40 000 cells/well, and the optical density at 590 nm was monitored at 37°C for 12 h with a kinetic reading of 5 min intervals in the FLUOstar Optima microplate reader. The value of the area under the curve was used as the measurement unit.

2.10 | Measurement of glutathione (GSH) levels

GSH levels in liver tissue were determined by the GSH-Glo™ Glutathione Assay (Promega) according to the manufacturer's instructions. Liver tissue (10 mg) was homogenized in 1 mL of PBS containing 2 mM EDTA, centrifuged at 16 000 ***g*** for 20 min at 4°C and 50 µL of the supernatant was incubated with 50 µL of prepared GSH-Glo™ Reagent 2× at room temperature in a 96-well plate. After 30 min, 100 µL of reconstituted Luciferin Detection Reagent wasadded and the plate was incubated for 15 min at room temperature. Luminescence was recorded in a plate reading luminometer (FLUOstar Optima) and readings were corrected by the tissue weight.

2.11 | Measurement of triacylglycerol (TAG) levels

Liver tissue was homogenized in NP40 Substitute Assay Reagent (Cayman Chemicals) containing protease inhibitors (Complete Mini; Roche Diagnostics) whereas hepatocytes were resuspended in 500 µL of cold diluted Standard Diluent and sonicated 20 times at 1 s bursts. Tissue and cell homogenates were centrifuged 10 min at 10 000 ***g*** at 4°C and supernatants assayed using a TAG colorimetric assay (Cayman Chemicals), according to the manufacturer's instructions.

2.12 | Measurement of succinate levels

Hepatocytes were seeded onto 96-well plates at a concentration of 40 000 cells/well. Extracellular levels of succinate were determined using the EnzyChrom™ Succinate Assay Kit (Bioassay Systems) according to the manufacturer's instructions. Briefly, 20 µL of cell supernatants was assayed in duplicate, one with the internal standard (1 mM succinate standard), and the other without. Then, 80 µL of working reagent of the proprietary kit was added to the cells for 30 min at room temperature and optical density was read at 570 nm in the microplate reader FLUOstar Optima. Intracellular levels of succinate in hepatocytes were measured with the Succinate Colorimetric Assay kit (Sigma) after homogenizing the cells on ice-cold Succinate Assay Buffer, centrifugation at 10 000 ***g*** for 5 min, and collection of supernatant. Thereafter, 50 µL of the diluted samples was added onto 96-well plates containing 50 µL of the Reaction Mix of the proprietary kit for 30 min at 37°C. Optical density was read at 450 nm in the microplate reader FLUOstar Optima.

2.13 | Analysis of gene expression by real-time polymerase chain reaction (PCR)

Isolation of total RNA from tissue and hepatocytes was performed using the TRIzol reagent and RNA concentration was assessed in a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific). cDNA synthesis from 0.5 to 1 µg of total RNA was performed using the High-Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR analysis for immunoresponsive gene 1 (*Irg1*, Mm01224532_m1), succinate dehydrogenase complex subunits (A, *Sdha*, Mm01352360_m1; B, *Sdhb* Mm00458272_m1; C, *Sdhc* Mm00481172_m1; and D, *Sdhd* Mm00546511_m1), activating transcription factor 3 (ATF3) (*Atf3*, Mm00476032_m1), nuclear factor erythroid-2-related factor 2 (NRF2) (*Nrf2*, Mm00477784_m1), isocitrate dehydrogenases (NADP+ *Idh1*, Mm00516030_m1; *Idh2* Mm00612429_m1; *Idh3* alpha [*Idh3a*], Mm00499674_m1; and *Idh3* beta, *Idh3b*, Mm00504589_m1), TNFα (*Tnf*, Mm00443258_m1), monocyte chemoattractant protein-1 (MCP-1) (*Ccl2*, Mm00441242_m1), interleukin (IL) 6 (*Il6*, Mm00446190_m1), and heme oxygenase-1 (HO-1) (*Hmox1*, Mm00516005_m1) was performed using validated and predesigned *TaqMan* Gene Expression Assays purchased from Applied Biosystems in a 7900HT Fast Real-Time PCR System using β-actin (*Actb*, Mm00607939_s1) as endogenous control. The PCR results were analyzed with Sequence Detector Software version 2.1 (Applied Biosystems). Relative quantification of gene expression was performed using the comparative C_t method. The amount of target gene normalized to β-actin and relative to a calibrator was determined by the arithmetic equation $2^{-\Delta\Delta Ct}$ described in the comparative C_t method.

2.14 | Analysis of protein expression

Total protein from tissue was extracted with RIPA buffer (Sigma) supplemented with a mixture of protease (Complet Mini) and phosphatase (PhosStop) inhibitors (Roche Diagnostics). Total protein from hepatocytes was extracted by scraping the cells into 150 μL of RIPA buffer (Sigma) supplemented with the same mixture of inhibitors. Homogenates were incubated on ice for 10–15 min and centrifuged at 16 000 ***g*** for 20 min at 4°C. The supernatant was dissolved in 2× Laemmli buffer, heated for 5 min at 95°C, and separated by 10% (vol/vol) SDS-PAGE for 90 min at 120 V. Transfer was performed using the iBlot Dry Blotting System (Invitrogen) on PVDF membranes at 20 V over 7 min. The membranes were then soaked for 1 h at room temperature in Tris-buffered saline containing 0.1% of Tween 20 (T-TBS) and 5% (wt/vol) nonfat dry milk. Blots were washed three times for 5 min each with T-TBS and incubated overnight at 4°C with primary monoclonal rabbit anti-mouse NRF2 antibody (12721, Cell Signaling Technology (CST), 1:1000 dilution), ATF3 (33593 CST, 1:1000 dilution), and β-actin (5125 CST, 1:1000 dilution) in T-TBS containing 5% of BSA. Thereafter, the blots were washed three times for 5 min each with 0.1% of T-TBS and incubated for 1 h at room temperature with a horseradish-peroxidase-linked donkey anti-rabbit antibody (406401, BioLegend, 1:2000 dilution) in 0.1% of T-TBS containing 5% of nonfat dry milk. For the assessment of IκB and JNK phosphorylation, blots were incubated overnight at 4°C with primary polyclonal rabbit anti-mouse phospho-SAPK/JNK (Thr183/Tyr185) antibody (9251 CST, 1:250 dilution) and mouse monoclonal anti-mouse phospho-IκB-α (9246 CST, 1:500 dilution). To assess total JNK and total IκB-α protein expression, membranes were stripped at 37°C for 15 min in Restore Western blot stripping buffer (Thermo Fisher Scientific) and reblotted overnight at 4°C with rabbit monoclonal SAPK/JNK (56G8) (9258 CST, 1:250 dilution) and polyclonal IκB-α (9242 CST, 1:1000). The bands were visualized with the EZ-ECL chemiluminescence detection kit (Biological Industries) in ImageQuant LAS 4000 equipment (GE Healthcare Life Sciences).

2.15 | Statistics

The statistical analysis was performed with GraphPad Prism software version 9.0.1. Statistical differences of the endpoints were evaluated with the unpaired *t* test with Welch's correction or one-way ANOVA for multiple comparisons corrected with Tukey posttest. Results were expressed as mean ± SEM and differences were considered significant at $p < .05$.

3 | RESULTS

3.1 | Albumin is required to preserve the mitochondrial ultrastructure of liver cells in front of TNFα-induced damage

Structural mitochondrial damage accompanies the cytotoxic effects of the cytokine TNFα on liver cells.[5] Figure 1 shows TEM images of the hepatocyte mitochondria ultrastructure at two different magnifications in PCLS incubated for 22 h with vehicle (left panels), TNFα in the absence of albumin (middle panels), and TNFα in the presence of albumin (right panels). Unless indicated, all experiments described in the following paragraphs were performed in the absence of transcriptional arrest (i.e., PCLS or hepatocytes were not exposed to actinomycin D), avoiding the induction of cell death and merely focusing on the mitochondrial damaging actions of TNFα. As compared to vehicle control, in the absence of albumin, more mitochondria per cell were present in hepatocytes treated with TNFα and these mitochondria were of smaller size and abnormally shaped with cristae rarefication (Figure 1). These TNFα-induced mitochondrial disturbances were less evident in PCLS incubated in the presence of albumin (Figure 1).

3.2 | Albumin is required to prevent mitochondrial oxidative stress in hepatocytes challenged with TNFα

We next determined changes in hepatocyte mitochondrial membrane potential, which is essential for energy production during oxidative phosphorylation.[17] Whereas the uncoupling agent CCCP, used as positive control, induced a severe alteration in mitochondrial membrane potential monitored by the JC-1 fluorescence assay (Figure S2A), no changes in this parameter were observed with TNFα in the presence or absence of albumin (Figure 2A). We also determined changes in mitochondrial oxidative stress using the Mitosox dye assay. As shown in Figure 2B, in the absence of albumin, TNFα-induced mitochondrial oxidative stress in hepatocytes, an effect that was not seen when these cells were pre-treated with albumin. To exclude the possibility that albumin could interfere with the binding of TNFα to hepatocytes, the experiments were repeated adding albumin 1 h after the stimulation of hepatocytes with TNFα (a condition in which this cytokine has already interacted and activated its membrane receptors). As shown in Figure 2B, under these conditions, albumin also reduced TNFα-induced mitochondrial oxidative stress. Of interest, albumin reduced TNFα-induced mitochondrial oxidative stress to a similar extent to tempol,

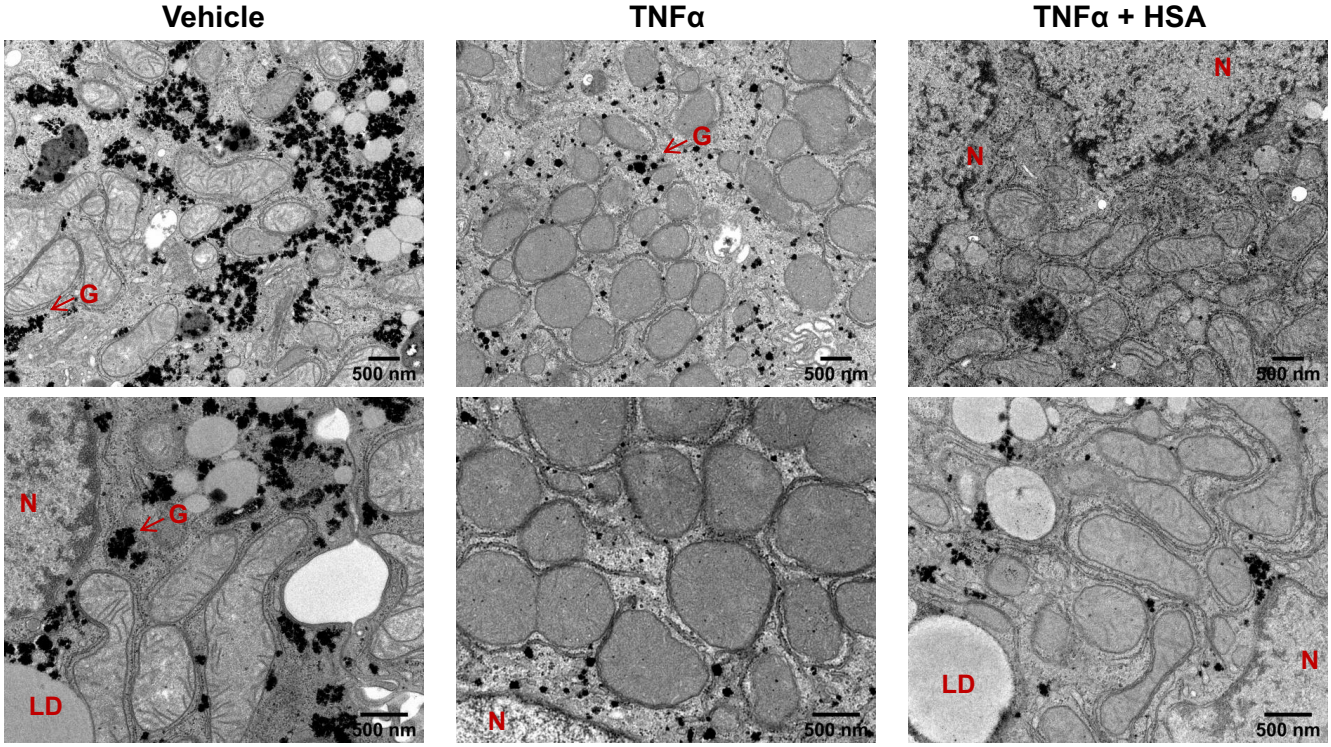

FIGURE 1 Influence of albumin in preserving hepatocyte mitochondrial ultrastructure. Representative electron microscopy images of PCLS incubated for 22 h with vehicle (left panels), TNFα (20 ng/mL) (middle panels), and albumin (HSA, 15 mg/mL) for 30 min and then challenged with TNFα (right panels). Images were taken at ×20 000 and ×30 000. G, glycogen; N, nucleus; LD, lipid droplet.

an intracellular antioxidant that preserves mitochondria against oxidative damage (Figure 2B). The antioxidant effects of albumin were not mirrored by mannitol, another oncotic agent, and were not related to the stabilizer contained in the albumin solution, which did not affect TNFα-induced mitochondrial oxidative stress (Figure S2B). We also observed that albumin disrupted the hepatocyte intracellular signaling of TNFα and significantly reduced the induction of IκB-α phosphorylation produced by this cytokine (Figure 2C). In these cells, JNK1 and JNK2 phosphorylation remained unchanged in response to both TNFα and albumin (Figure 2D).

3.3 | Albumin is not required to preserve mitochondrial respiration in hepatocytes challenged with TNFα

To investigate the mechanisms by which hepatocytes growing in the presence of albumin had reduced TNFα-induced mitochondrial oxidative stress, we monitored changes in mitochondrial ETC, one of the major sources of ROS.[18] In particular, we measured oxygen (O_2) consumption by high-resolution respirometry in the presence and absence of protein uncouplers and inhibitors of the ETC, as schematized in Figure S3. We first established that incubation for 5 h with TNFα was enough to induce significant changes in basal hepatocyte O_2 consumption (Figure 2E) and this time-period was selected for subsequent experiments. As compared to vehicle and in the absence of albumin, hepatocytes exposed to TNFα showed significantly increased basal and uncoupled O_2 consumption (Figure 2F,G) as well as maximal and residual (ROX) respiration (Figure S4A). Increased mitochondrial respiration induced by TNFα did not translate into higher ATP production (Figure 2H). Albumin did not modify the increased respiratory response to TNFα (Figure 2F,G and Figure S4A). Also, albumin per se did not induce changes in resting hepatocyte respiration or ATP levels (Figure S4B,C). Similarly, ATP levels were not affected by shorter incubation time periods (2 h) (Figure S4D). In contrast, albumin significantly reduced the induction in mitochondrial FAO produced by TNFα, as assessed by a non-radioactive assay based on the oxidation of octanoyl-CoA (Figure 2I).

3.4 | Albumin is required for the homeostatic control of the hepatocyte TCA cycle in front of TNFα

We next performed functional flux analysis with MitoPlates S-1 to monitor changes in mitochondrial metabolism that can lead to the generation of the reducing agents NADH and $FADH_2$. The results were graphed in lollipop charts

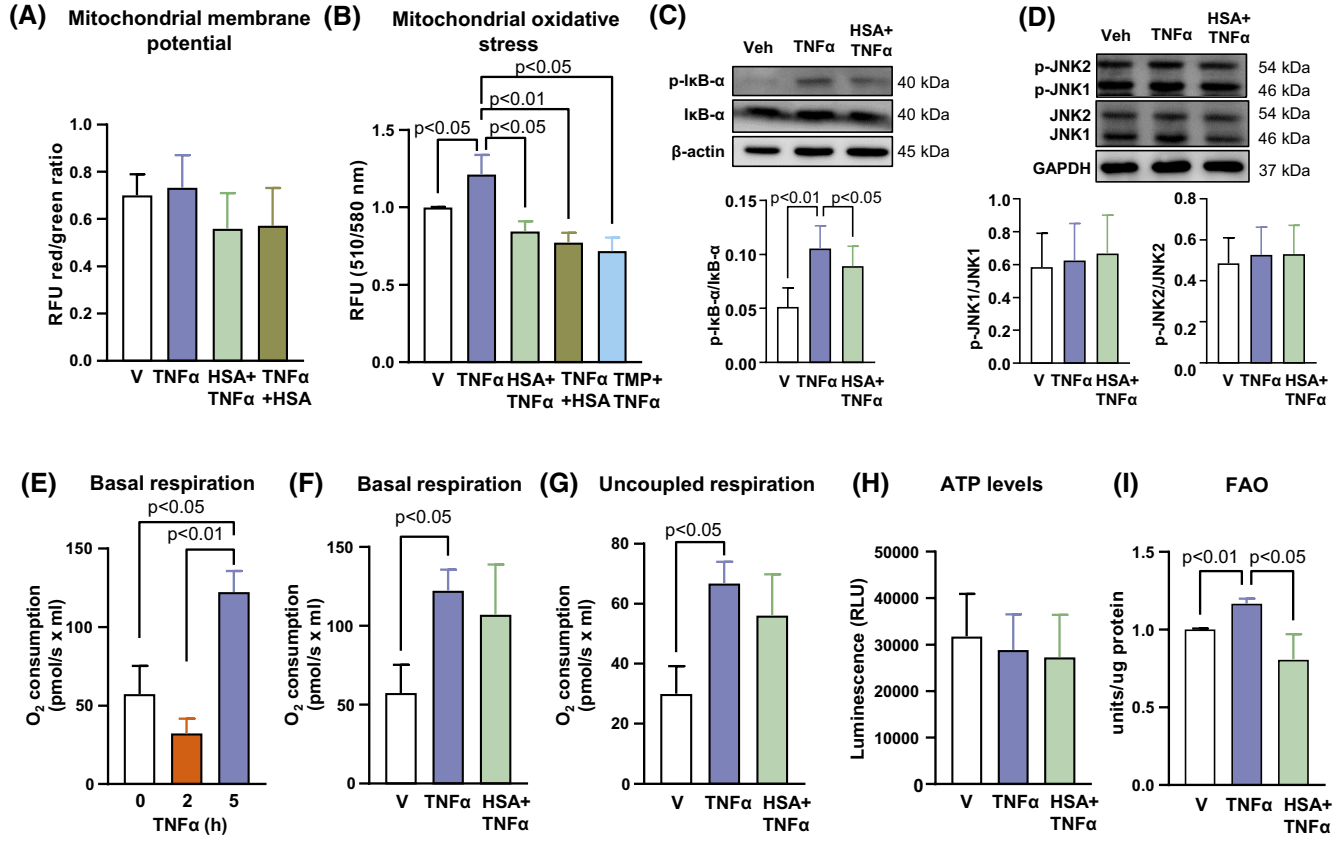

FIGURE 2 Albumin reduces mitochondrial oxidative stress and modulates fatty acid β-oxidation (FAO). (A) Mitochondrial membrane potential in hepatocytes incubated with vehicle (V) or albumin (HSA) for 30 min and then challenged with TNFα for 5 h or treated with HSA 1 h after TNFα addition. (B) Mitochondrial oxidative stress in hepatocytes incubated with V, has, or tempol (TMP) for 30 min and then challenged with TNFα for 5 h or treated with HSA 1 h after TNFα addition. (C) Representative Western blot of p-IκB-α and IκB-α protein expression in hepatocytes incubated with V or HSA for 30 min and then challenged with TNFα for 5 h. (D) Representative Western blot of p-JNK1, p-JNK2, JNK1, and JNK2 protein expression in hepatocytes incubated with V or HSA for 30 min and then challenged with TNFα for 5 h. (E) Basal respiration in hepatocytes challenged with TNFα for 2 or 5 h. (F) Basal respiration in hepatocytes incubated with or without HSA and challenged with TNFα for 5 h. (G) Uncoupled respiration from hepatocytes incubated as described in (F) in the presence of the ATP synthase inhibitor oligomycin (2 μM). (H) ATP levels in hepatocytes incubated as described in (F). (I) FAO in hepatocytes incubated as described in (F). Results from three or more independent experiments assayed in duplicate and expressed as mean ± SEM. RLU, relative luminescence units; RFU, relative fluorescence units.

using three pairwise comparisons: TNFα versus vehicle (Figure 3A), HSA+TNFα versus vehicle (Figure S5A), and HSA+TNFα versus TNFα (Figure 3D). Acetylcarnitine was the most favored mitochondrial substrate in hepatocytes exposed to TNFα, whereas isocitrate was the mitochondrial substrate less utilized by hepatocytes stimulated by this cytokine (Figure 3A). The utilization of these substrates in hepatocytes incubated with TNFα with respect to that of resting hepatocytes (incubated with vehicle) is shown in Figure 3B. These findings are consistent with the view that TNFα modulates mitochondrial FAO and induces a breakpoint in the TCA cycle between isocitrate and α-ketoglutarate leading to less utilization of isocitrate (Figure 2I and Figure 3C). The TNFα-induced mitochondrial overutilization of acetylcarnitine and the impairment in mitochondrial utilization of isocitrate were restored in hepatocytes in which albumin was present in the cell culture medium (Figure 3D). The utilization of these substrates in hepatocytes incubated with TNFα and albumin with respect to that of hepatocytes incubated with TNFα alone is shown in Figure 3E. The utilization of these substrates in hepatocytes incubated with TNFα and albumin with respect to that of hepatocytes incubated with vehicle alone is shown in Figure S5B,C. The presence of the isocitrate breakpoint in hepatocytes incubated with TNFα alone suggests that anaplerotic reactions might replenish the TCA cycle under inflammatory injury conditions. Indeed, the utilization of the amino acid glutamine, which enters the TCA cycle at the α-ketoglutarate level (Figure 3C), was higher in hepatocytes exposed to TNFα alone than in hepatocytes exposed to TNFα in the presence of albumin (Figure S6A). The restoration by albumin of the breakpoint

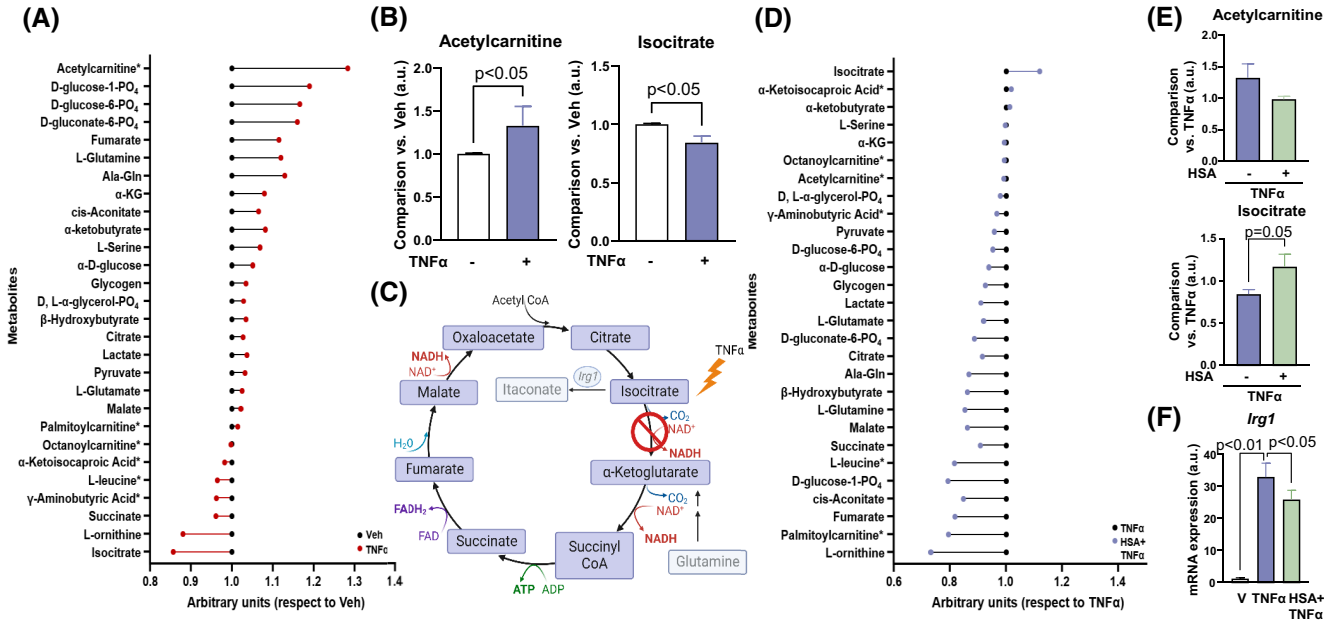

FIGURE 3 Albumin modulates the tricarboxylic acid (TCA) cycle. (A) Utilization of different energy substrates comparing hepatocytes incubated with TNFα for 5 h from hepatocytes incubated with vehicle (Veh). (B) Acetylcarnitine and isocitrate utilization in hepatocytes incubated with TNFα for 5 h in comparison to vehicle. (C) Schematic diagram of the damaging actions of TNFα on the TCA cycle. (D) Utilization of different energy substrates comparing hepatocytes incubated with TNFα and albumin (HSA) for 5 h from hepatocytes incubated with TNFα but without HSA. (E) Acetylcarnitine and isocitrate utilization in hepatocytes incubated with or without HSA for 30 min and then challenged with TNFα for 5 h. (F) *Irg1* expression in hepatocytes incubated as described in (E). Results from three independent experiments and expressed as mean ± SEM.

at the isocitrate level induced by TNFα was consistent with a decrease in the expression of the *Irg1* gene, a proxy of the diversion of the isocitrate metabolic intermediate cis-aconitate into itaconate[19] (Figure 3F). The reduced utilization of isocitrate in hepatocytes challenged with TNFα in the absence of albumin was neither related to changes in the expression of isocitrate dehydrogenase enzymes, which convert isocitrate to α-ketoglutarate (Figure S6B) nor in the expression of genes coding for the dehydrogenation of the biologically active TCA metabolite succinate (Figure S6C). No changes in the utilization of glycogen or the ketone body β-hydroxybutyrate were observed after TNFα stimulation, although the latter was significantly reduced by albumin treatment (Figure S7A,B). Finally, no lipid (i.e., TAG) changes were observed in hepatocytes exposed to TNFα in the absence or presence of albumin (Figure S7C).

3.5 | Albumin is also essential for the homeostatic control of mitochondrial TCA cycle when hepatocytes stimulated with TNFα are submitted to transcriptional arrest

When hepatocytes are under transcriptional arrest, TNFα-induced mitochondrial injury translates into programmed cell death (apoptosis).[13,20] To investigate how important is the albumin molecule in this situation, we repeated the mitochondrial flux analysis in the presence of actinomycin D. Figure 4A shows a lollipop chart of the pairwise comparisons of the utilization of energy substrates between hepatocytes incubated with TNFα+ actinomycin D in comparison to vehicle. Unlike hepatocytes treated with TNFα alone (Figure 3A), acylcarnitines were not the top utilized mitochondrial substrates in hepatocytes exposed to TNFα+ actinomycin D (Figure 4A). In these conditions, succinate replaced isocitrate as the mitochondrial substrate less utilized by these hepatocytes (Figure 4A,B). No changes in the mitochondrial utilization of isocitrate were observed (Figure 4C). These findings indicate that in the absence of albumin and under transcriptional arrest, TNFα triggers a breakpoint in the TCA cycle between succinate and fumarate leading to less utilization of succinate (Figure 4D). In contrast, in the presence of albumin, the TNFα+ actinomycin D-induced succinate breakpoint was not observed but rather the utilization of this substrate was enhanced (Figure 4E,F). Albumin did not modify the mitochondrial utilization of isocitrate (Figure 4G). Unlike intracellular, extracellular levels of succinate were reduced in hepatocytes treated with TNFα+ actinomycin D in the presence of albumin (Figure S8A,B).

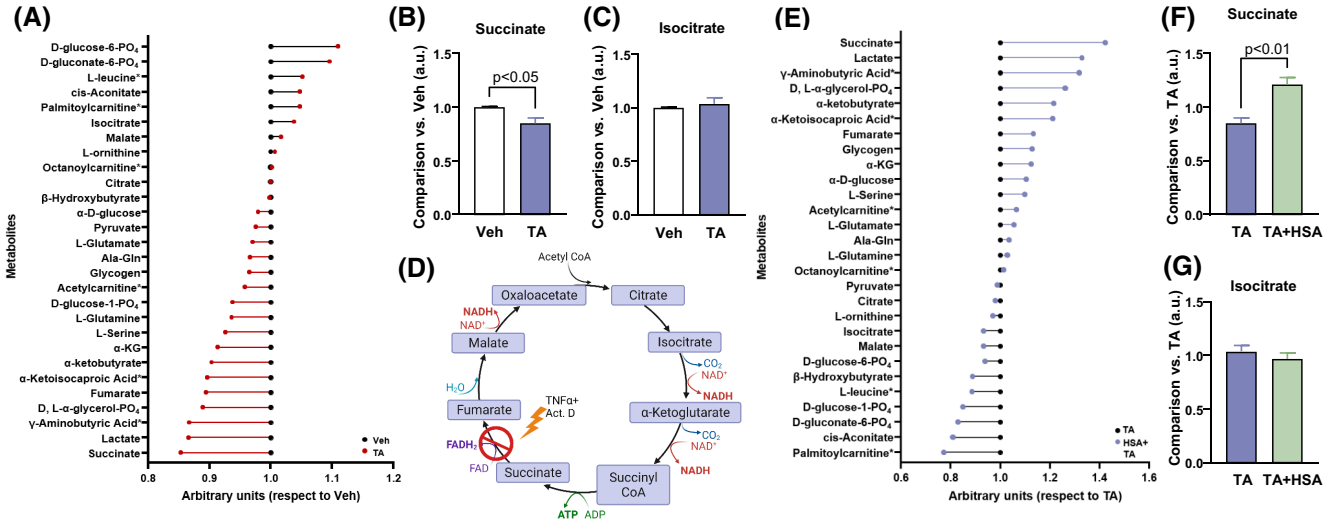

FIGURE 4 Effects of TNFα and albumin on the TCA cycle in hepatocytes growing under transcriptional arrest. (A) Utilization of different energy substrates comparing hepatocytes incubated with TNFα plus actinomycin D (TA) for 5 h from hepatocytes incubated with vehicle (Veh). (B) Succinate utilization in hepatocytes challenged with TA for 5 h in comparison to Veh. (C) Isocitrate utilization in hepatocytes incubated as described in (B). (D) Schematic diagram of TCA cycle and the damaging effects of TA. (E) Utilization of different energy substrates comparing hepatocytes incubated with TA plus albumin (HSA) for 5 h to hepatocytes incubated with TA but without HSA. (F) Succinate utilization in hepatocytes incubated with or without HSA for 30 min and then challenged with TA for 5 h. (G) Isocitrate utilization in hepatocytes incubated as described in (F). Results are from a minimum of three independent experiments assayed in duplicate and expressed as mean ± SEM.

3.6 | The homeostatic role of albumin is related to the stimulation of the antioxidant ATF3 pathway

To explore by which mechanism albumin prevents mitochondrial oxidative stress in response to TNFα, we compared the expression of antioxidant pathways, including NRF2, HO-1, and ATF3 in hepatocytes growing in the presence of albumin with respect to those growing in its absence. Expression of *Nrf2*, the gene coding for NRF2, was equal in both groups (Figure 5A). Similar findings were obtained at the protein level, in which OI was added as positive control (Figure 5B). Expression of *Hmox1*, the gene coding for HO-1, was higher in hepatocytes growing in the presence of albumin, but changes did not reach statistical significance (Figure 5C). In contrast, expression of *Atf3*, the gene coding for ATF3, was significantly higher in hepatocytes incubated with TNFα in the presence of albumin in comparison to those cultures not containing albumin in the cell media (Figure 5D). The stimulatory actions of albumin on the expression of the *Atf3* gene were confirmed at the protein level (Figure 5E). Albumin also modulated the expression of ATF3 downstream targets including *Ccl2*, *Tnf*, and *Il6* in hepatocytes exposed to TNFα (Figure 5F). Furthermore, the stimulatory actions of albumin on ATF3 and downstream targets were confirmed in vivo in mice treated with LPS and D-gal, as an experimental model of TNFα-mediated acute liver injury (Figure 5G,H). Importantly, in the in vivo model, hepatic glutathione levels were increased by albumin, indicating a reduction in oxidative stress (Figure 5I). Neither TNFα nor albumin-modified hepatic TAG levels (Figure S9). The protective role of albumin against TNFα-induced mitochondrial oxidative stress was not observed in hepatocytes under actinomycin D-induced transcriptional arrest (Figure S8C), suggesting that in our experiments the mitochondrial antioxidant properties of the albumin molecule might occur at the intracellular level. Together, these findings suggest that the presence of albumin in the culture media of hepatocytes is able to counteract TNFα-induced mitochondrial oxidative stress by mechanisms related to the activation of the ATF3 antioxidant pathway.

4 | DISCUSSION

Albumin is primarily produced by hepatocytes and is one of the most abundant proteins in the bloodstream.[6,9] Albumin is responsible for the maintenance of the oncotic pressure not only in the intravascular compartment but also in the extravascular and interstitial compartment.[6,8,9] In recent years, albumin has been described to exert pleiotropic actions beyond its oncotic properties, including immunomodulatory and cytoprotective effects.[8–10,13] In the current study, we expanded the non-oncotic properties of this protein by describing how albumin

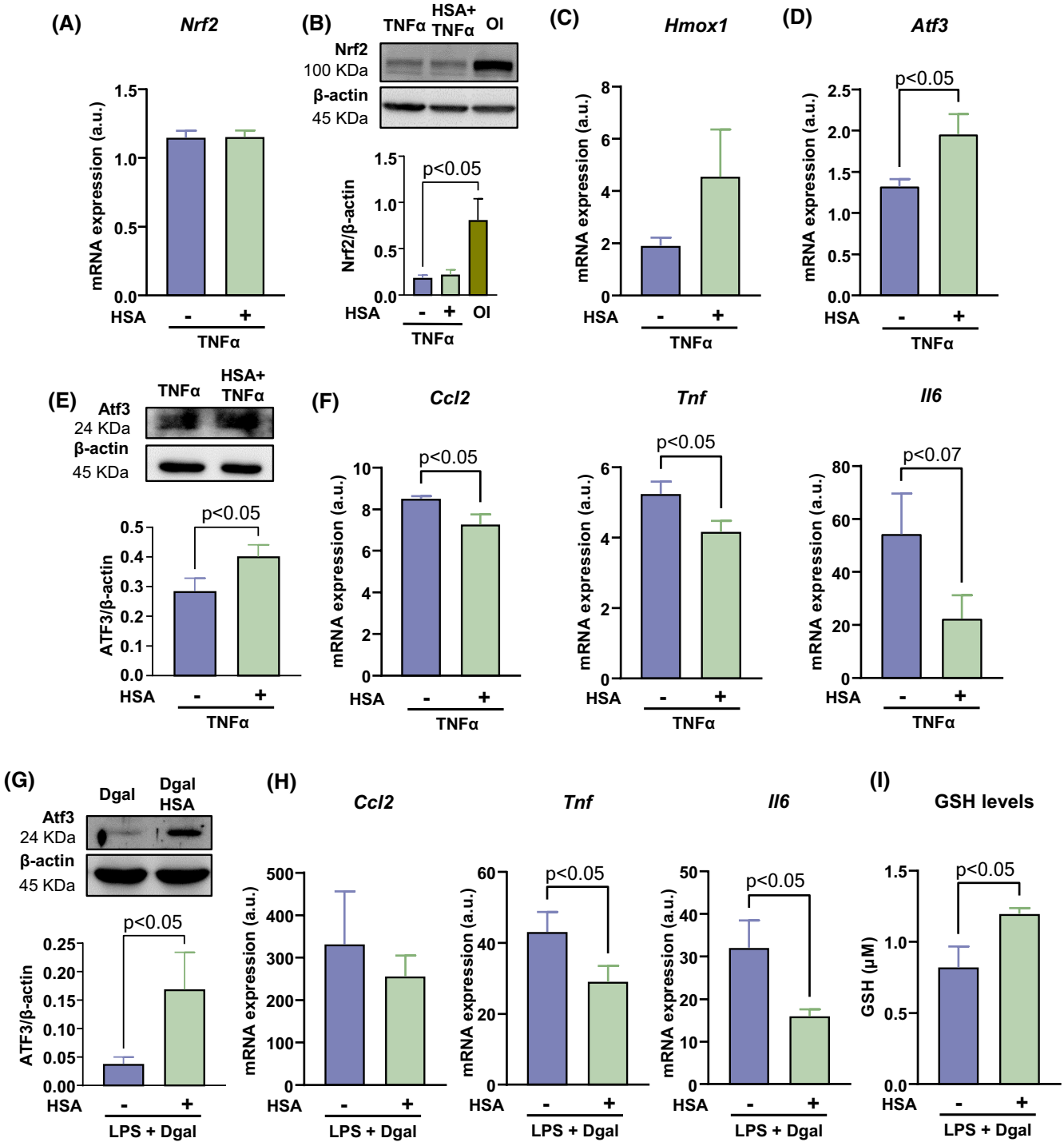

FIGURE 5 Albumin activates the antioxidant signaling ATF3 pathway in hepatocytes. (A) *Nrf2* expression in hepatocytes challenged with TNFα for 5 h and incubated with or without albumin (HSA) for 30 min. (B) Representative Western blot of NRF2 protein expression in hepatocytes incubated as described in (A). OI (125 μM, 2 h) was used as positive control. The densitometric analysis is shown below. (C) *Hmox1* expression in hepatocytes incubated as described in A. (D) *Atf3* expression in hepatocytes incubated as described in (A). (E) Representative Western blot of ATF3 protein expression in hepatocytes incubated as described in (A). The densitometric analysis is shown below. (F) *Ccl2*, *Tnf*, and *Il6* mRNA expression in hepatocytes incubated as described in (A). (G) Representative Western blot of liver ATF3 protein expression in mice treated with LPS+D-galactosamine (LPS+Dgal) receiving HSA ($n = 5$) or placebo ($n = 5$). The densitometric analysis is shown below. (H) *Ccl2*, *Tnf*, and *Il6* mRNA expression in mice treated as described in (G). (I) Glutathione levels in mice treated as described in (G). Results from hepatocytes are from a minimum of three independent experiments assayed in duplicate. All results are expressed as mean ± SEM.

preserves mitochondrial function in parenchymal liver cells and protects them from the damaging actions of pro-inflammatory and cytotoxic cytokines. Specifically, in this study, we provide evidence that albumin counteracts excessive TNFα-induced mitochondrial ROS production in ex vivo and in vitro models that mimic the extravascular and interstitial microenvironment of liver cells. TNFα is a common pro-inflammatory and cytotoxic cytokine that plays a major role in perturbing mitochondrial morphology and function during hyperinflammatory conditions.[4,5] TNFα is invariably elevated within the broad spectrum of liver diseases, from the mildest forms of non-alcoholic fatty liver disease, to the most severe, such as those manifested in patients with advanced liver cirrhosis in whom cytokine-induced tissue injury increases the risk of developing organ failure.[21,22] Therefore, our findings are of clinical relevance because mitochondrial dysfunction is a prime pathogenic factor in liver disease,[16] since these cell organelles are the main source of ROS in the cell and excessive ROS production leads to oxidative damage of cellular components such as lipids, proteins and mitochondrial DNA, promoting cytotoxicity, and tissue injury.[23] Our findings are also of relevance to the conditions in which liver patients exhibit remarkable hypoalbuminemia, especially in patients with decompensated cirrhosis with risk to progress to ACLF, in whom tissue injury and mitochondrial dysfunction leads to organ failure.[24]

Our study provides evidence that albumin is essential to preserve the antioxidant homeostasis in the liver cell microenvironment. In the peripheral circulation, albumin has long been regarded as a potent antioxidant molecule due to its free radical scavenging activities and its ability to bind pro-inflammatory cues.[9] In our study, the antioxidant properties of albumin in the liver cell microenvironment appeared to be independent of its interference with the binding of TNFα to their receptors. This view is supported by the observation that the addition of albumin 1 h after the stimulation of hepatocytes with TNFα (an approach in which TNFα has already interacted and activated its membrane receptors) produced the same protection against mitochondrial oxidative stress as pre-incubating first the hepatocytes with albumin and subsequently stimulate with TNFα. In addition, the antioxidant properties of albumin are unrelated to changes in the osmotic pressure in the cell culture since other oncotic agents such as mannitol were not able to reduce TNFα-induced mitochondrial oxidative stress in hepatocytes. Moreover, the observation that albumin produced expression changes at the gene and protein level together with the finding that the reduction in mitochondrial oxidative stress was abrogated under transcriptional arrest, suggest that the mitochondrial antioxidant properties of albumin against TNFα in liver cells occur at the intracellular level. Consistent with this, it is well known that albumin is taken up and internalized by hepatocytes[25] as well as by endothelial and immune cells.[9,10] It is also well characterized that once internalized by cells, albumin is not a mere bystander but rather an effector molecule able to block endosomal toll-like receptor signaling, lysosomal cathepsin B leakage, mitochondrial cytochrome c release and caspase-3 activity.[10,13]

Our study provides some mechanistic data underlying the antioxidant protective actions of albumin in hepatocytes. Previous studies have reported that TNFα treatment of hepatocytes leads to increased mitochondrial O_2 consumption,[4] a response likely reflecting the high energy demand required by cells exposed to pro-inflammatory stimuli.[26] However, in our experiments in which hepatocytes were incubated with albumin, the protection from TNFα-induced mitochondrial oxidative stress was not related to either changes in mitochondrial respiration or in mitochondrial membrane permeability. On the contrary, the protection from TNFα-induced mitochondrial oxidative stress by albumin was associated with a normalization of mitochondrial FAO, which when accelerated might cause excessive electron flux in ETC and ROS overproduction.[27] In addition, in these experiments, the presence of albumin prevented the formation of a breakpoint between isocitrate and α-ketoglutarate in the mitochondrial TCA cycle in response to TNFα. More importantly, following TNFα stimulation, we observed that the levels of the endogenous antioxidant ATF3 pathway were significantly higher in hepatocytes growing with albumin than in those without this molecule. Although ATF3 was initially described in macrophages, this transcription factor has been recognized in other cell types and tissues including hepatocytes and the liver, where its activation results in protection against oxidative injury.[28–30] Therefore, our data point to the common stress-responsive transcription factor ATF3 as a mechanism by which albumin preserves hepatocyte mitochondria from cytokine-induced damage.

In summary, our data provide in vitro, ex vivo, and in vivo evidence of the essential role of albumin in the preservation of liver cell mitochondria from the damaging actions of TNFα. In aggregate, our data highlight the importance of maintaining the albumin levels within the normal range not only in the intravascular compartment but also in the extravascular and interstitial space, especially in conditions such as advanced liver disease in which hypoalbuminemia is common.

AUTHOR CONTRIBUTIONS

Marta Duran-Güell and Joan Clària conceived and designed the experiments; Marta Duran-Güell, Cristina López-Vicario, Mireia Casulleras, Ingrid W. Zhang, Roger Flores-Costa, María B. Sánchez-Rodríguez, Bryan J. Contreras, and Berta Romero-Grimaldo performed

experiments; Marta Duran-Güell, Judith Cantó-Santos, and Glòria Garrabou performed and designed respirometry measurements; Raquel Horrillo, Montserrat Costa and Vicente Arroyo revised the manuscript; Marta Duran-Güell and Joan Clària wrote the manuscript.

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DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article and the online supporting information.

DISCLOSURES

RH and MC are full-time employees of Grifols.

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SUPPORTING INFORMATION

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

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SUPPLEMENTARY DATA

Essential role for albumin in preserving liver cells from TNFα-induced mitochondrial injury

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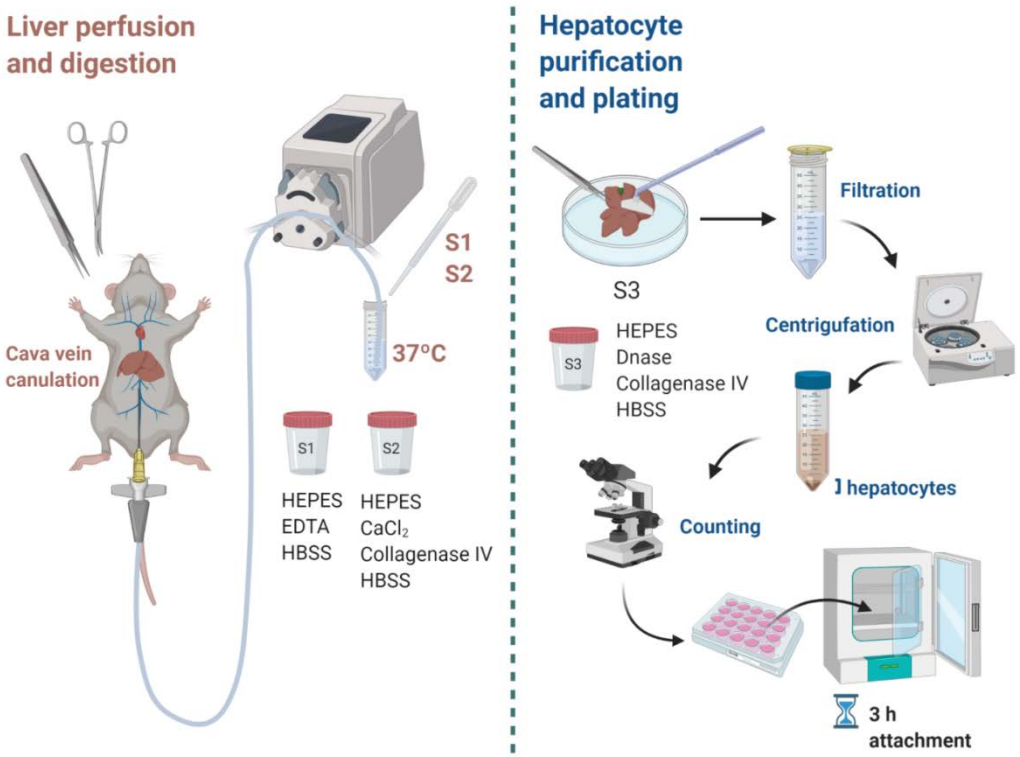
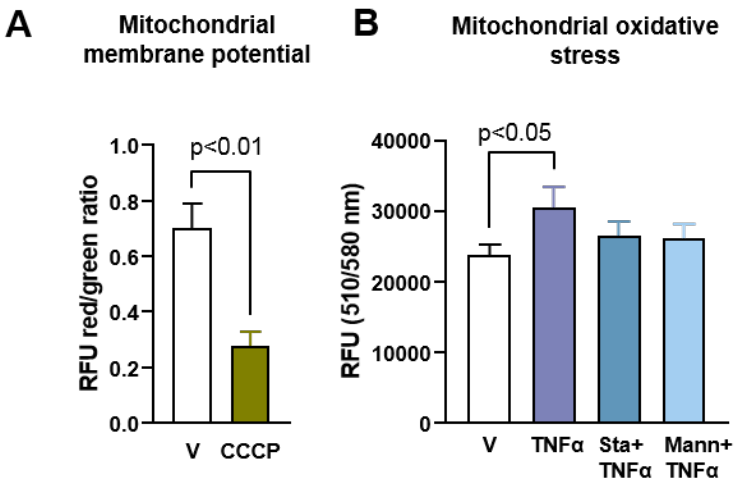
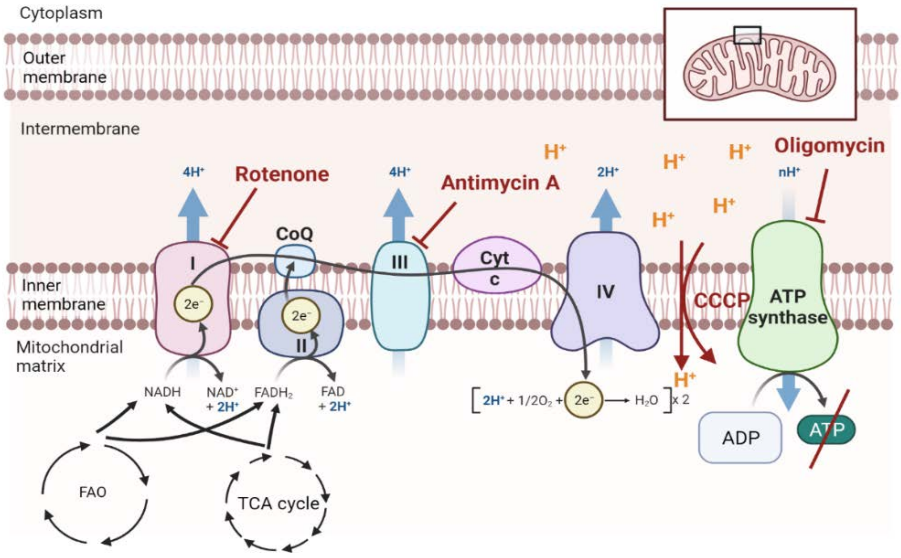


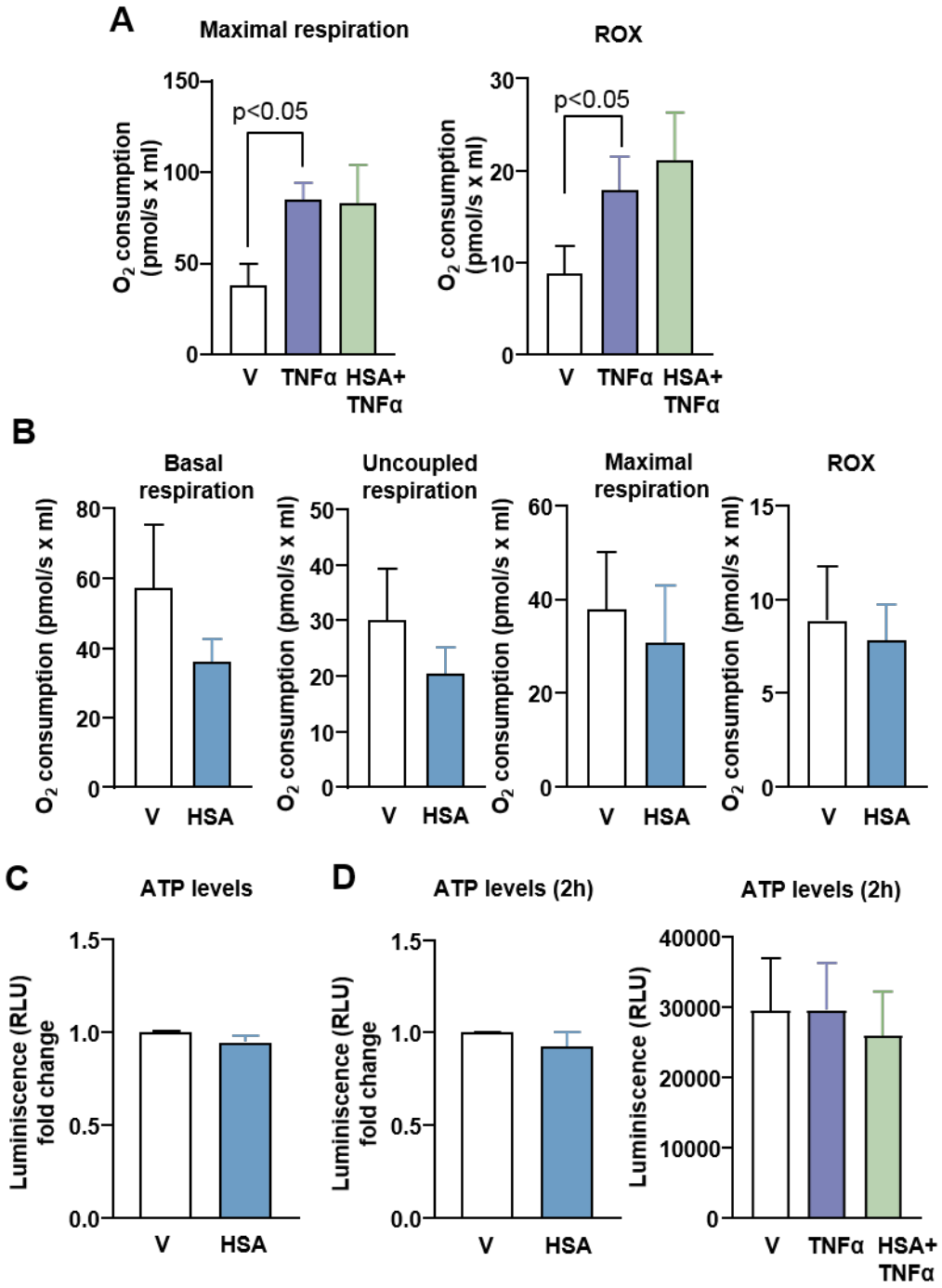
Supplementary Figure 1. Schematic diagram of mouse primary hepatocyte isolation.



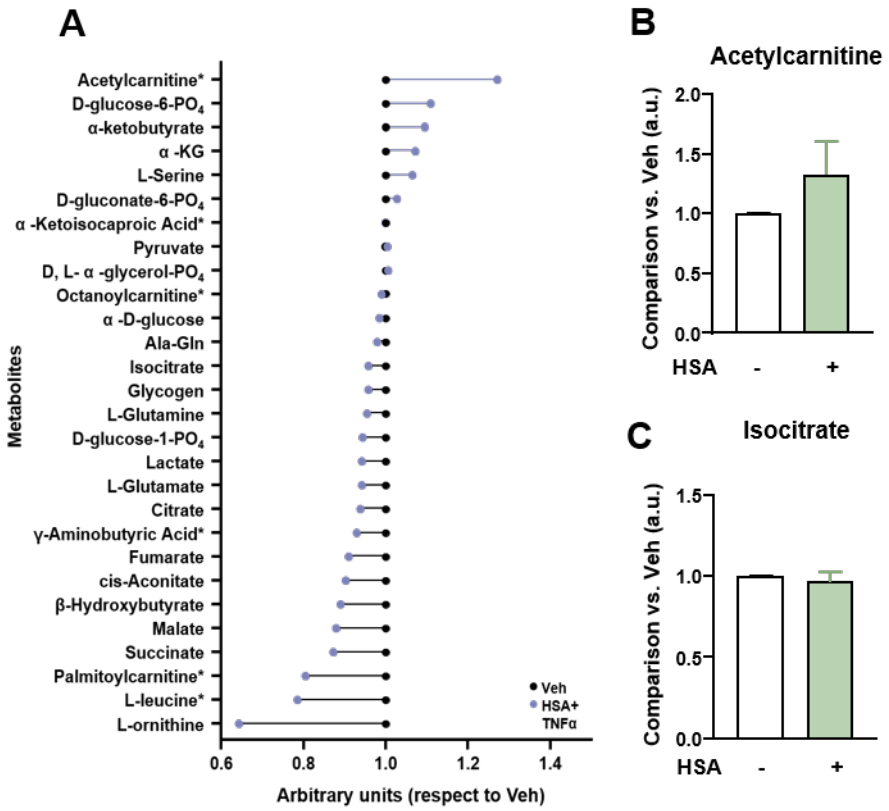
Supplementary Figure 2. (A) Fluorescence signals corresponding to the measurement of mitochondrial membrane potential by the JC-1 fluorescence assay in hepatocytes incubated with vehicle (V) or the uncoupler reagent carbonyl cyanide m-chlorophenyl hydrazone (CCCP), used as positive control. (B) Mitochondrial oxidative stress in hepatocytes incubated with vehicle, stabilizer (Sta) or mannitol (Mann) for 30 min and then challenged with TNFα for 5 hours. RFU, relative fluorescence units.



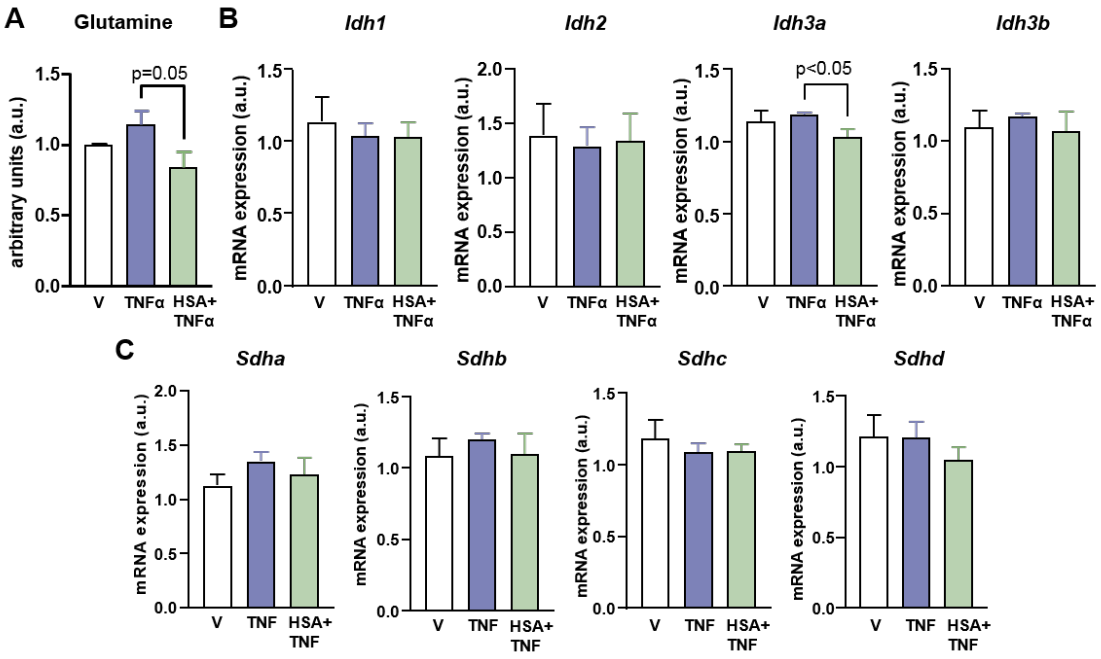
Supplementary Figure 3. Schematic diagram of the protein uncouplers and inhibitors of mitochondrial electron transfer chain used in our experiments.



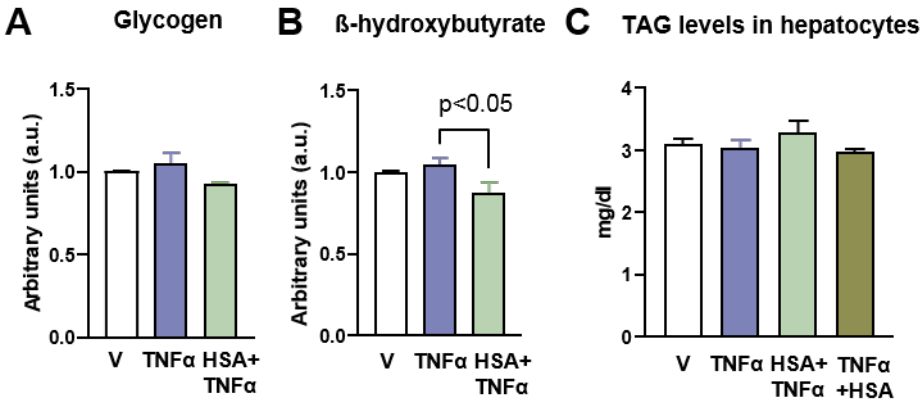
Supplementary Figure 4. (A) Maximal respiration and residual oxygen respiration (ROX) in hepatocytes incubated with vehicle (V) or albumin (HSA) for 30 min and then challenged with TNFα for 5 hours in the presence of the uncoupler CCCP (1 μM) or rotenone and antimycin A (1 μM). (B) HSA effects on basal, uncoupled, maximal and ROX respiration. (C) Normalized luminescent signals corresponding to the measurement of ATP levels in hepatocytes incubated with vehicle or HSA for 5 hours. (D) Normalized luminescent signals corresponding to the measurement of ATP levels in hepatocytes incubated with vehicle or HSA for 30 min and then challenged with TNFα for 2 hours. Results are expressed as mean±SEM and are from 3 or more independent experiments assayed in duplicate. RLU, relative luminescence units; RFU, relative fluorescence units.



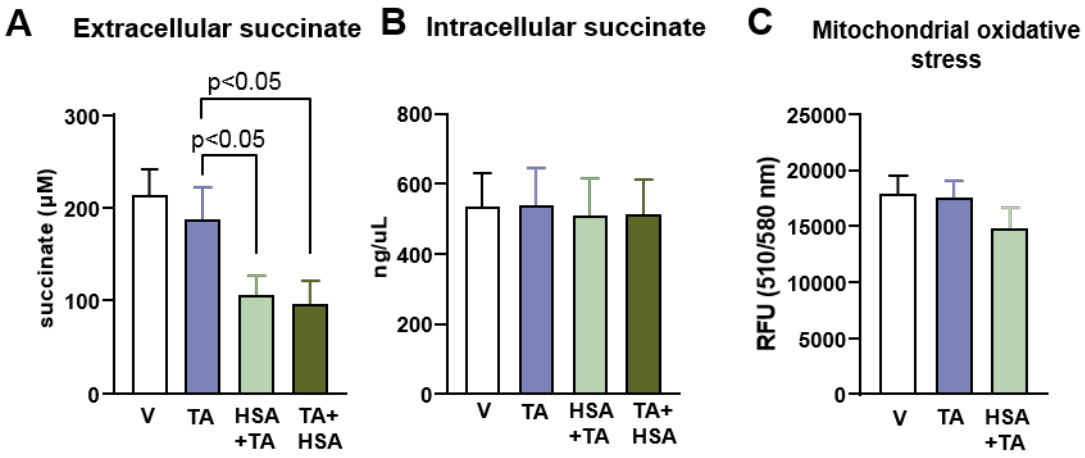
Supplementary Figure 5. (A) Utilization of different energy substrates comparing hepatocytes incubated with TNFα and albumin (HSA) for 5 hours from hepatocytes incubated with vehicle (Veh). (B) Acetylcarnitine utilization in hepatocytes incubated with HSA for 30 min and then challenged with TNFα for 5 hours in comparison to Veh. (C) Isocitrate utilization in hepatocytes incubated as described in B.



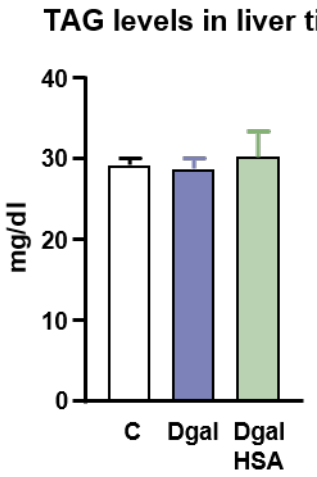
Supplementary Figure 6. (A) Colorimetric signals from glutamine utilization in hepatocytes incubated with vehicle (V) or albumin (HSA) for 30 min and then challenged with TNFα for 5 hours. (B) Effect of HSA and TNFα on *Idh1*, *Idh2*, *Idh3a* and *Idh3b* gene expression in hepatocytes incubated as described in A. (C) Effect of HSA and TNFα on *Sdha, Sdhb*, *Sdhc* and *Sdhd* gene expression in hepatocytes incubated as described in A.



Supplementary Figure 7. (A) Colorimetric signals from glycogen utilization in hepatocytes incubated with vehicle (V) or albumin (HSA) for 30 min and then challenged with TNFα for 5 hours. (B) Colorimetric signals from β-hydroxybutyrate utilization in hepatocytes incubated as described in A. (C) Colorimetric signals corresponding to triacylglycerol (TAG) levels in hepatocytes incubated with V or HSA for 30 min and then challenged with TNFα for 5 hours or treated with HSA one hour after TNFα addition.



Supplementary Figure 8. (A, B) Colorimetric signals corresponding to extracellular and intracellular levels of succinate from hepatocytes incubated with vehicle (V) or albumin (HSA) for 30 min and then challenged with TNFα and actinomycin D (TA) for 5 hours or treated with HSA one hour after the addition of TA to the culture media. (C) Mitochondrial oxidative stress in hepatocytes incubated with V or HSA for 30 min and then challenged with TA for 5 hours. RFU, relative fluorescence units.



Supplementary Figure 9. Triacylglycerol (TAG) levels in liver tissue from mice treated with LPS and D-galactosamine.

DISCUSSION

Albumin is primarily produced by hepatocytes and is one of the most abundant proteins in the bloodstream (Bernardi M. et al, Gut 2020) (Arroyo V. et al, J Hepatol. 2014). Albumin is responsible for the maintenance of the oncotic pressure not only in the circulation but also in the interstitial space (Bernardi M. et al, Gut 2020) (Arroyo V. et al, J Hepatol. 2014) (Garcia-Martinez R. et al, J Hepatol. 2015). In recent years, albumin has been described to exert pleiotropic actions beyond its oncotic properties, including immunomodulatory and cytoprotective effects (Arroyo V. et al, J Hepatol. 2014) (Garcia-Martinez R. et al, J Hepatol. 2015) (Casulleras M. et al, Sci Transl Med. 2020).

The results of the first study provide evidence that albumin is able to ameliorate cytokine-induced liver tissue damage. This conclusion was reached from data collected at multimodal experimental levels, including *in vitro* and *ex vivo* studies in hepatocytes and PCLS challenged with TNFα and *in vivo* studies in double transgenic humanized h*Alb*$^{+/+}$/h*FcRn*$^{+/+}$ mice with pre-existing chronic CCl_4-induced cirrhosis challenged with LPS/D-gal, a model of TNFα-mediated acute liver injury. Collectively, our data support the view that albumin, in addition to ameliorate cytokine production, might also protect tissues from cytokine-induced immunopathology.

A number of studies have previously demonstrated that albumin is the major antiapoptotic signaling component in the serum. In particular, Zoellner et al, (Zoellner H. J Cell Sci. 1996), Barbaro et al (Barbaro B. Artif Cells Blood Substit Immobil Biotechnol. 2008) and Tabernero et al (Tabernero A. J Neurochem. 2002) were among the first to describe that albumin at physiological concentrations was a specific inhibitor of programed cell death in human endothelial cells, human pancreatic islets, and rat neurons, respectively. Similar findings were reported in human and murine cell lines (Jones DT. Blood 2003) (Gallego-Sandín S. Eur J Pharmacol. 2005) (Liu SY. Apoptosis 2012). Although these studies provided little information on the mechanisms underlying the antiapoptotic effects of albumin and none specifically referred to TNFα-induced hepatocyte death, several potential pathways were enumerated. Among these, modulation of the AKT signaling pathway, (Jones DT. Blood 2003) attenuation of intracellular calcium levels (Zoellner H. J Cell Sci. 1996), and prevention of mitochondrial membrane depolarization (Gallego-Sandín S. Eur J Pharmacol. 2005) (Liu SY. Apoptosis 2012). Mitochondria, indeed, play a key role in TNFα-induced hepatocyte apoptosis by releasing cytochrome c into the cytosol (Leist M. Gastroenterology 1997) (Guicciardi ME. et al, Compr Physiol. 2013) (Luedde T. Gastroenterology 2014). Cytochrome c is a 12-kDa protein that functions in the mitochondrial electron transport chain diffusing between the inner and the outer membranes within complexes III and IV

(Cortese JD. Biochim Biophys Acta. 1993). However, in response to TNFα, cytochrome c is released into the cytosol where it forms the apoptosome complex resulting in the activation of caspase-3, which is a potent executioner of hepatocyte apoptosis (Luedde T. Gastroenterology 2014). In line with these data, in our experiments, we observed a reduction in cytochrome c release from mitochondria to the cytosol accompanied by decreased caspase-3 activity after incubating primary hepatocytes with albumin, indicating that the mitochondrial pathway is an essential component underlying the protective actions of albumin in the liver.

Our results also provided a mechanistic basis by which albumin reduced the mitochondrial release of cytochrome c in response to TNFα. Binding of TNFα to its death receptor results in recruitment of adaptor proteins and initiator procaspase-8 to form a large multiprotein complex known as death inducing signaling complex or DISC (Guicciardi ME. et al, Compr Physiol. 2013) (Luedde T. Gastroenterology 2014). However, in our conditions, albumin was ineffective in reducing TNFα-induced caspase-8 despite affecting caspase-8 downstream signals such as cytochrome c release. Albumin did not affect TNFα-induced JNK either. In view of these findings, we explored alternative pathways leading to mitochondrial cytochrome c release and focused our attention on the endolysosomal system. A variety of death stimuli produce lysosomal membrane permeabilization and trigger the release of lysosomal enzymes into the cytosol. Among the different lysosomal enzymes, the cysteine protease cathepsin B plays a prominent role in the execution of the apoptotic cascade upstream of the mitochondria (Guicciardi ME. et al, Compr Physiol. 2013) (Guicciardi ME. et al, J Clin Invest. 2000) (Luedde T. Gastroenterology 2014). In fact, our data on primary hepatocytes indicated the ability of albumin to inhibit the leakage of cathepsin B from lysosomes to the cytosol in response to TNFα, an effect that was associated with decreased mitochondrial cytochrome c release and reduced caspase-3 activity. Together, these findings propose that albumin modulates TNFα-induced injury by preventing lysosomal membrane permeabilization and the release of cathepsin B.

In summary, the results of the first study provide evidence of the cytoprotective actions of albumin in the liver. In particular, our findings demonstrate the ability of albumin to protect hepatocytes from the cytotoxic effects of the cytokine TNFα. These albumin protective actions were mediated by inhibition of the leakage of the cysteine protease cathepsin B from lysosomes accompanied by a reduction in cytochrome c release from mitochondria. Taken together, these findings provide evidence that albumin is not only able to reduce cytokine

production, but also to prevent the tissue damaging actions of these inflammatory mediators.

In the second study, we expanded the non-oncotic properties of albumin by describing how albumin preserves mitochondrial function in parenchymal liver cells and protects them from the damaging actions of pro-inflammatory and cytotoxic cytokines. Specifically, in this study we provide evidence that albumin counteracts excessive TNFα-induced mitochondrial ROS production in liver cells. TNFα is a common pro-inflammatory cytokine involved in systemic inflammation. TNFα is also a cytotoxic cytokine that plays a major role in perturbing mitochondrial morphology and function during hyperinflammatory conditions (Kastl L. et al, FEBS Lett. 2014) (Schulze-Osthoff K. et al, J Biol Chem. 1992). TNFα is invariably elevated within the broad spectrum of liver diseases, from the mildest forms of non-alcoholic fatty liver disease, to the most severe, such as those manifested in patients with advanced liver cirrhosis in whom cytokine-induced tissue injury increases the risk of developing organ failure (Bradham CA. Am. J. Physiol.- Gastrointest. Liver Physiol. 1998) (Clària J. et al, Hepatology 2016). Therefore, our findings are of clinical relevance because mitochondrial dysfunction is a prime pathogenic factor in liver disease (Zhang IW. J. Hepatol. 2022), since these cell organelles are the main source of ROS in the cell and excessive ROS production leads to oxidative damage of cellular components such as lipids, proteins and mitochondrial DNA, promoting cytotoxicity and tissue injury (Balaban RS. Cell. 2005). Our findings are also of relevance to the conditions in which liver patients exhibit remarkable hypoalbuminemia, especially in patients with decompensated cirrhosis with risk to progress to ACLF, in whom tissue injury and mitochondrial dysfunction leads to organ failure (Engelmann C et al, J Hepatol. 2021).

The second study provides evidence that albumin is essential to preserve the antioxidant homeostasis in the liver cell microenvironment. In the peripheral circulation, albumin has long been regarded as a potent antioxidant molecule due to its free radical scavenging activities and its ability to bind pro-inflammatory cues (Arroyo V. et al, J Hepatol. 2014). In this study, the antioxidant properties of albumin in the liver cell microenvironment appeared to be independent of its interference with the binding of TNFα to their receptors. This view is supported by the observation that the addition of albumin one hour after the stimulation of hepatocytes with TNFα (an approach in which TNFα has already interacted and activated its membrane receptors) produced the same protection against mitochondrial oxidative stress as pre-incubating first the hepatocytes with albumin and subsequently stimulate with TNFα. In addition, the antioxidant properties of albumin are

unrelated to changes in the osmotic pressure in the cell culture since other oncotic agents such as mannitol were not able to reduce TNFα-induced mitochondrial oxidative stress in hepatocytes. Moreover, the observation that albumin produced expression changes at the gene and protein level together with the finding that the reduction in mitochondrial oxidative stress was abrogated under transcriptional arrest, suggest that the mitochondrial antioxidant properties of albumin against TNFα in liver cells occur at the intracellular level. Consistent with this, it is well known that albumin is taken up and internalized by hepatocytes (Pyzik M. Proc. Natl. Acad. Sci. USA 2017) as well as by endothelial and immune cells (Arroyo V. et al, J Hepatol. 2014) (Casulleras M. et al, Sci Transl Med. 2020). It is also well characterized that once internalized by cells, albumin is not a mere bystander but rather an effector molecule able to block endosomal toll-like receptor signaling, lysosomal cathepsin B leakage, mitochondrial cytochrome c release and caspase-3 activity (Casulleras M. et al, Sci Transl Med. 2020).

Our study provides some mechanistic data underlying the antioxidant protective actions of albumin in hepatocytes. Previous studies have reported that TNFα treatment of hepatocytes leads to increased mitochondrial O_2 consumption (Kastl L. et al, FEBS Lett. 2014), a response likely reflecting the high energy demand required by cells exposed to pro-inflammatory stimuli (Ganeshan K. et al, Cell. 2019). However, in our experiments in which hepatocytes were incubated with albumin, the protection from TNFα-induced mitochondrial oxidative stress was not related to either changes in mitochondrial respiration or in mitochondrial membrane permeability. On the contrary, the protection from TNFα-induced mitochondrial oxidative stress by albumin was associated with a normalization of mitochondrial FAO, which when accelerated might cause excessive electron flux in ETC and ROS overproduction (Serra D. Antioxidants Redox Signal. 2013). In addition, in these experiments, the presence of albumin prevented the formation of a breakpoint between isocitrate and α-ketoglutarate in the mitochondrial TCA cycle in response to TNFα. More importantly, following TNFα stimulation, we observed that the levels of the endogenous antioxidant ATF3 pathway were significantly higher in hepatocytes growing with albumin than in those without this molecule. Although ATF3 was initially described in macrophages, this transcription factor has been recognized in other cell types and tissues including hepatocytes and the liver, where its activation results in protection against oxidative injury (Hoetzenecker W. Nature Med. 2011) (Rao J. Am. J. Transplant. 2015) (Liu Y. Front. Pharmacol. 2021). Therefore, our data point to the common stress responsive transcription factor ATF3 as a mechanism by which albumin preserves hepatocyte mitochondria from cytokine-induced damage.

In summary, our data provide *in vitro, ex vivo* and *in vivo* evidence of the essential role of albumin in the preservation of liver cell mitochondria from the damaging actions of TNFα. In aggregate, our data highlight the importance of maintaining the albumin levels within the normal range not only in the intravascular compartment but also in the extravascular and interstitial space, especially in conditions such as advanced liver disease in which hypoalbuminemia is common.

CONCLUSIONS

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

1. Albumin administration protects the liver tissue against a model of TNFα-mediated acute liver injury in mice.

2. Albumin administration impacted the recruitment of macrophages into liver tissue, reducing inflammatory cell infiltration into the injured liver.

3. Albumin exerts cytoprotective effects against TNFα-induced apoptosis in cultured hepatocytes.

4. The cytoprotective effects of albumin are independent from its oncotic and scavenging properties.

5. Albumin effects are related to the modulation of the intracellular TNFα signaling pathway in liver cells reducing the mitochondrial release of cytochrome c.

6. The inhibitory actions of albumin are mediated by cathepsin B, which leakage from the lysosome to the cytosol triggers mitochondrial cytochrome c release and induces apoptosis.

7. The cytoprotective effects of albumin are also effective against H_2O_2-induced caspase 3 activity.

8. Albumin preserves the mitochondrial ultrastructure of liver cells in from the damaging actions of TNFα.

9. Albumin prevents mitochondrial oxidative stress in hepatocytes challenged with TNFα.

10. Albumin contributes to the homeostasis of mitochondrial fatty acid oxidation and tricarboxylic acid cycle against TNFα damaging actions.

11. Albumin in the culture media of hepatocytes is able to counteract TNFα-induced mitochondrial oxidative stress by mechanisms related to the activation of the ATF3 antioxidant pathway.

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ANNEX I: OTHER PUBLICATIONS

Publications included in the thesis:

- **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(2):e21365.

- **Duran-Güell M**, Garrabou G, Flores-Costa R, Casulleras M, López-Vicario C, Zhang IW, Cantó-Santos J, Contreras BJ, Sánchez-Rodríguez MB, Romero-Grimaldo B, Horrillo R, Costa M, Arroyo V and Clària J. Essential role for albumin in preserving liver cells from TNFα-induced mitochondrial injury. FASEB J. 2023, 37:e22817.

Other publications:

- Romo M, López-Vicario C, Pérez-Romero N, Casulleras M, Martínez-Puchol AI, Sánchez B, Flores-Costa R, Alcaraz-Quiles J, **Duran-Güell M**, Ibarzábal A, Espert JJ, Clària J, Titos E. Small fragments of hyaluronan are increased in individuals with obesity and contribute to low-grade inflammation through TLR-mediated activation of innate immune cells. Int J Obes. 2022;46:1960-1969.

- López-Vicario C, Sebastián D, Casulleras M, **Duran-Güell M**, Flores-Costa R, Aguilar F, Lozano JJ, Zhang IW, Titos E, Kang JX, Zorzano A, Arita M, Clària J. Essential lipid autacoids rewire mitochondrial energy efficiency in metabolic dysfunction-associated fatty liver disease. Hepatology 2022.

- 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;6:1443-1456.

- 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.

- Zhang IW, López-Vicario C, **Duran-Güell M**, Clària J. Mitochondrial Dysfunction in Advanced Liver Disease: Emerging Concepts. Front Mol Biosci. 2021;8:772174.

- Clària J, Flores-Costa R, **Duran-Güell M**, López-Vicario C. Proresolving lipid mediators and liver disease. Biochim Biophys Acta Mol Cell Biol Lipids. 2021;1866:159023.

- 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(566):eaax5135.

- 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(45):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(1):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 Jun;33(6):7072-7083. doi: 10.1096/fj.201802587R.

- 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 Nov;68(5):1937-1952. doi: 10.1002/hep.30135.

- 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 Mar;175(6):953-967. doi: 10.1111/bph.14137.

- 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 Nov 16;7(1):15724. doi: 10.1038/s41598-017-15951-z.

- 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 Dec;31(12):5384-5398. doi: 10.1096/fj.201700394R.

ANNEX II: CONFERENCE PRESENTATIONS

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

Marta Duran-Güell, Glòria Garrabou, Roger Flores-Costa, Mireia Casulleras, Cristina López-Vicario, Ingrid W. Zhang, María Belén Sánchez-Rodríguez, Bryan J. Contreras, Berta Romero-Grimaldo, Raquel Horrillo, Montserrat Costa, Vicente Arroyo and Joan Clària. Albumin preserves hepatocyte mitochondria from cytokine-induced damage through the common stress responsive transcription factor ATF3. The AASLD Liver Meeting 2022. Washington DC, USA, November 4-8, 2022**. Poster**

Marta Duran-Güell, Roger Flores-Costa, Mireia Casulleras, Cristina López-Vicario, Esther Titos, José Alcaraz-Quiles, Raquel Horillo, Montserrat Costa, Vicente Arroyo and Joan Clària Enrich. Albumin protects TNF-alpha-induced hepatocyte apoptosis by preventing lysosomal cathepsin b release. The AASLD Liver Meeting 2019. Boston, MA, USA, November 8-12, 2019. **Poster**

Marta Duran-Güell, Mireia Casulleras, Roger Flores-Costa, Cristina López-Vicario, Esther Titos, José Alcaraz-Quiles, Raquel Horrillo, Montserrat Costa, Vicente Arroyo, Joan Clària. Albumin protects the liver from tumor necrosis factor alpha-induced cell death. The International Liver Congress 2019 (EASL). Viena, Austria. April 10-14, 2019. **Oral presentation**

Marta Duran-Güell, Mireia Casulleras, Roger Flores-Costa, Cristina López-Vicario, Esther Titos, José Alcaraz-Quiles, Raquel Horrillo, Montserrat Costa, Vicente Arroyo y Joan Clària. Efectos protectores de la albúmina frente al daño hepático inducido por TNF-alfa. 44º Congreso anual de la Asociación Española para el Estudio del Hígado (AEEH). Madrid, Spain. February 20-22, 2019. **Poster**

Marta Duran-Güell, Mireia Casulleras, Roger Flores-Costa, Cristina López-Vicario, Esther Titos, José Alcaraz-Quiles, Raquel Horrillo, Montserrat Costa, Vicente Arroyo and Joan Clària. Albumin protects liver cells from TNF-α-induced apoptosis. The european club for liver cell biology - 8 (ECLCB-8). Bonn, Germany. October 4-6, 2018. **Oral presentation**