Programa de Doctorado en Señalización Celular

"MECANISMOS MOLECULARES DE LA RESISTENCIA A LA INSULINA ASOCIADA A LA INFLAMACIÓN EN ESTADOS DE OBESIDAD Y NAFLD"

Tesis Doctoral presentada por

VIRGINIA PARDO MARQUÉS

Directoras:

DRA/ ÁNGELA Mª MARTÍNEZ VALVERDE

DRA/ ÁGUEDA GONZÁLEZ RODRÍGUEZ

Alcalá de Henares, 2014

Universidad de Alcalá

FACULTAD DE MEDICINA
Campus Universitario
28871 Alcalá de Henares (Madrid)
Teléfono: 91 885 45 13
Fax: 91 885 45 85

DEPARTAMENTO DE BIOLOGÍA DE SISTEMAS

ANTONIO JIMÉNEZ RUIZ, Director del Departamento de Biología de Sistemas de la Universidad de Alcalá,

INFORMA:

Que **Dª Virginia Pardo Marqués** ha realizado la Tesis Doctoral titulada **"Mecanísmos moleculares de la resistencia a la insulina asociada a la inflamación en estados de obesidad y NAFLD"** bajo la dirección de las doctoras **Dª Ángela Mª Martínez Valverde** y **Dª Águeda González Rodríguez** en el Instituto de Investigaciones Biomédicas "Alberto Sols", dentro del programa de Doctorado "Señalización Celular" del Departamento de Biología de Sistemas de la Universidad de Alcalá y, a su juicio, cumple todos los requisitos necesarios para proceder a su defensa pública.

Alcalá de Henares, a 10 de Octubre de 2014

Dr. Antonio Jiménez Ruiz

Universidad de Alcalá

FACULTAD DE MEDICINA
Campus Universitario
28871 Alcalá de Henares (Madrid)
Teléfono: 91 885 45 13
Fax: 91 885 45 85

DEPARTAMENTO DE BIOLOGÍA DE SISTEMAS

La **Dra. Ángela Mª Martínez Valverde**, Investigadora Científica del Instituto de Investigaciones Biomédicas "Alberto Sols" (Centro mixto CSIC/UAM), y la **Dra. Águeda González Rodríguez**, Investigadora Contratada del Instituto de Investigaciones Biomédicas "Alberto Sols" (Centro mixto CSIC/UAM),

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Dra. Ángela M. Martínez Valverde

Dra. Águeda González Rodríguez

“La ignorancia afirma o niega rotundamente; la ciencia duda.”

François Marie Arouet, Voltaire

“Hay una fuerza motriz más poderosa que el vapor, la electricidad o la energía atómica: la voluntad”.

Albert Einstein

AGRADECIMIENTOS

En primer lugar, quiero agradecer a mis directoras Ángela y Águeda el haberme dado la oportunidad de realizar esta Tesis. Muchas gracias a las dos por vuestra confianza desde el principio, por enseñarme todo lo que he aprendido de investigación, por vuestra dedicación y por haberme guiado durante este largo camino.

Quiero agradecer también a todas mis compis del labo (de abajo y de arriba ;)) su constante e incondicional ayuda. Muchas gracias chicas por todo el tiempo que habéis dedicado a enseñarme cosas nuevas. Gracias por estar ahí siempre, tanto en los momentos buenos como en los malos, porque sé que siempre estabais ahí y podía contar con vuestra ayuda en todo momento. Para mí es muy importante tener buen ambiente en el trabajo, por eso, nunca olvidaré todos los buenos ratos que hemos pasado.

Gracias también a todo el personal del iib, sobre todo al personal de cultivos y de los dos animalarios (que es dónde más tiempo me he tirado) porque siempre que lo he necesitado he podido contar con vuestra ayuda.

Quiero dar las gracias también a la gente de Alcalá, sobre todo a Borja y Angélica, por haber tenido tanta paciencia conmigo y ayudarme a solucionar todas mis dudas.

Por último, dar las gracias a quien realmente ha estado a mi lado día a día, a toda mi familia, gracias por vuestra constante preocupación y sobre todo gracias por animarme a seguir adelante en los días "reguleros". Javi, gracias por apoyarme y animarme en todo momento, porque todo el tiempo que he estado encerrada escribiendo esta Tesis tú has estado sentado a mi lado con tu ordenador, aunque hiciese un sol espléndido y te apeteciese salir a la calle. Y finalmente, a todos mis amigos, a los que a pesar de tener un poco abandonados estos últimos meses sé que están y siempre estarán ahí.

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ABREVIATURAS

AO Ácido Oleico *(Oleic Acid)*

AP Ácido Palmítico *(Palmitic Acid)*

ATF6 Factor de transcripción activador-6 *(Activating Transcription Factor-6)*

ATM Macrófagos del tejido adiposo *(Adipose Tissue Macrophages)*

BMI Índice de masa corporal *(Body Mass Index)*

DAG Diacilglicerol *(Diacylglycerol)*

ERK Quinasa regulada por señales extracelulares *(Extracellular signal-regulated kinase)*

ER stress Estrés del retículo endoplásmico *(Endoplasmic Reticulum stress)*

FATP Proteína transportadora de ácidos grasos *(Fatty Acid Transporter Protein)*

FFA Ácido graso libre *(Free Fatty Acid)*

HFD Dieta alta en grasa *(High Fat-Diet)*

IKK Quinasa de IκB *(Inhibitor of nuclear factor κB kinase)*

IκB Proteína inhibitoria de NFκB (*Inhibitor of nuclear factor κB)*

iNOS Óxido nítrico sintasa inducible *(inducible Nitric Oxide Synthase)*

IRS-1/2 Sustrato del receptor de insulina 1/2 *(Insulin Receptor Sustrate 1/2)*

JNK Quinasa del N-terminal de c-Jun *(c-Jun-N-terminal kinase)*

LDL Lipoproteína de baja densidad *(Low-Density Lipoprotein)*

LPS Lipopolisacárido *(Lipopolysaccharide)*

LT Leucotrieno *(Leukotriene)*

MAPK Proteína quinasa activada por mitógenos *(Mitogen-Activated Protein Kinase)*

M-CSF Factor estimulante de colonias de macrófagos

MPO Mieloperoxidasa *(Myeloperoxidase)*

nPKC Proteína quinasa C novel *(novel Protein Kinase C)*

NAFLD Enfermedad del hígado graso no alcohólico *(Non-Alcoholic Fatty Liver Disease)*

NASH Esteatohepatitis no alcohólica *(Nonalcoholic Steatohepatitis)*

NFκB Factor nuclear κB *(Nuclear Factor κB)*

PAMPs Patrones moleculares asociados a patógenos *(Pathogen-Associated Molecular Patterns)*

PERK Quinasa del retículo endoplásmico similar a PRKR *(PRKR-like Endoplasmic Reticulum Kinase)*

PG Prostaglandina *(Prostaglandin)*

PKC Proteina quinasa C *(Protein Kinase C)*

polyI:C Ácido poliriboinosinico:poliribocitidilico *(polyriboinosinic:polyribocytidylic acid)*

PP2A Proteína fosfatasa 2A *(Protein Phosphatase 2A)*

PTP1B Proteína tirosina fosfatasa 1B *(Protein Tyrosine Phosphatase 1B)*

RI Receptor de insulina *(Insulin Receptor)*

RNIs Especies reactivas de nitrógeno (*Reactive Nitrogen Intermediates*)

ROIs Especies reactivas de oxígeno (*Reactive Oxygen Intermediates*)

SM Síndrome Metabólico *(Metabolic Syndrome)*

SOCS Supresor de señalización de citoquinas *(Suppressor Of Cytokine Signaling)*

TNFα Factor de necrosis tumoral *(Tumor Necrosis Factor-α)*

TGs Triglicéridos *(Triglycerides)*

TNF-R1 Receptor 1 de TNFα *(TNF-receptor 1)*

TPA Tetradecanoilforbol acetato *(Tetradecanoylphorbol Acetate)*

UPR Respuesta a proteínas mal plegadas *(Unfolded Protein Response)*

X-BP1 Proteína de unión a X-box *(X-box Binding Protein 1)*

RESUMEN

La inflamación crónica de bajo grado característica de la obesidad está directamente implicada en la patogénesis de la resistencia a la insulina, así como en las complicaciones que padecen los pacientes diabéticos. Durante la obesidad se produce una hiperplasia e hipertrofia del tejido adiposo blanco, el cual sufre una disfunción caracterizada por una lipólisis exacerbada. Como consecuencia, se incrementa la liberación de ácidos grasos libres al torrente circulatorio, y estos ácidos grasos son los primeros responsables de activar la respuesta proinflamatoria del sistema inmune. Tras un largo periodo de inflamación de bajo grado y daño crónico en los tejidos periféricos responsables del control de la homeostasis glucídica, se acaba instaurando una resistencia a la insulina.

Aunque la importancia de la activación de los macrófagos en los mecanismos moleculares que desencadenan la resistencia a la insulina en el músculo esquelético y el tejido adiposo ya se han descrito por otros grupos, el impacto de las moléculas liberadas por los macrófagos sobre la señalización de la insulina en el hígado todavía no ha sido explorado.

Por tanto, en esta Tesis Doctoral se han descrito, en primer lugar, los efectos inducidos por el ácido palmítico (AP) sobre las vías de estrés que conducen a la lipoapoptosis y la resistencia a la insulina en hepatocitos, así como su reversión por parte del ácido oleico (AO) y los mecanismos moleculares asociados, ya que estos son los ácidos grasos mayoritarios tanto en la dieta como en la circulación sanguínea.

Asimismo, se han evaluado los efectos de los productos secretados por los macrófagos tras su activación con el AO o el AP, sobre las vías de señalización en respuesta a estrés, la lipoapoptosis y sobre la cascada de señalización de la insulina en los hepatocitos.

Para llevar a cabo este objetivo se ha puesto a punto un sistema *"in vitro"* de medios condicionados (MC) de macrófagos estimulados con los dos ácidos grasos de interés. Estos MC, que contienen tanto ácidos grasos libres como citoquinas, quimioquinas y demás especies lipídicas secretadas por los macrófagos tras su activación, se han

utilizado para estimular los hepatocitos y posteriormente analizar en ellos las vías de estrés del retículo endoplásmico (RE), la lipoapoptosis y la señalización de la insulina.

Tras realizar dichos experimentos, se observó que el AP produce una activación clásica o proinflamatoria de los macrófagos, los cuales aumentan la secreción de citoquinas y quimioquinas proinflamatorias, y estos productos derivados de los macrófagos, a su vez, producen lipoapoptosis y resistencia a la insulina en los hepatocitos. Sin embargo, el AO induce una activación alternativa o antiinflamatoria en los macrófagos la cual mejora la sensibilidad a la insulina en los hepatocitos.

Por otro lado, también se propuso estudiar el papel de la proteína tirosina fosfatasa 1B (PTP1B) en la respuesta inflamatoria, ya que la PTP1B es el modulador negativo más importante de la cascada de señalización de la insulina, por su función de defosforilar al receptor de insulina. De esta manera, los inhibidores de PTP1B están ampliamente reconocidos por sus efectos beneficiosos sobre la recuperación de la resistencia a la insulina, sin embargo, no se conoce cómo la inhibición de esta fosfatasa modula las respuestas inflamatorias del sistema inmune.

Los experimentos llevados a cabo en ratones deficientes en PTP1B han demostrado que la inhibición de esta enzima en todos los tejidos del organismo produce una respuesta proinflamatoria exacerbada por parte del sistema inmune, una mayor tasa de mortalidad, y un incremento en la expresión de genes de la rutas de p53 y de escisión y reparación del ADN.

Por lo tanto, se puede concluir que es necesario un control minucioso de las acciones de los inhibidores de la PTP1B si se propone su aplicación terapéutica para tratar la resistencia a la insulina ya que estos compuestos podrían ejercer efectos perjudiciales sobre la regulación de las repuestas inflamatorias del sistema inmune.

INTRODUCCIÓN

Hoy en día, el estilo de vida está caracterizado por una abundancia calórica, actividad física reducida y aumento de la esperanza de vida, lo cual ha disparado durante las últimas décadas la incidencia de obesidad a proporciones epidémicas, especialmente en países desarrollados, y siendo particularmente alarmante en la población infantil [2-4]. El incremento en la incidencia de obesidad lleva asociado un conjunto de patologías metabólicas entre las que se incluyen la diabetes mellitus tipo 2 (DMT2), la enfermedad del hígado graso no alcohólico (en inglés, *Nonalcoholic Fatty Liver Disease*, NAFLD) y la ateroesclerosis, que han motivado la investigación dedicada a esclarecer los mecanismos moleculares que las desencadenan [13].

El primer paso que realiza el organismo en respuesta a la abundancia nutricional es un excesivo acúmulo de lípidos en el tejido adiposo. Se sabe que a partir de cierto nivel de almacenamiento lipídico, el adipocito comienza a tener signos de estrés, entre los que se incluyen: hipoxia, disrupción de la función mitocondrial, producción de especies reactivas de oxígeno, estrés del retículo endoplásmico (RE), apoptosis, incremento de lipolisis y liberación de ácidos grasos e inflamación [14-16].

Recientemente, se ha descrito que la obesidad, aparte de ser un trastorno metabólico, está acompañada de una inflamación crónica de los tejidos grasos, que acaba desencadenando resistencia a la insulina en los tejidos periféricos diana de la insulina como el hígado o el músculo esquelético. En este sentido, la investigación de las interacciones entre los procesos metabólicos y el sistema inmunológico constituye una nueva disciplina de estudio denominada inmunometabolismo. De hecho, se han publicado diversos estudios que demuestran que la inmunología podría estar más relacionada con la resistencia a la insulina de lo que se creía hasta ahora [17-20].

1. El macrófago como célula clave en el inmunometabolismo.

En el proceso inflamatorio participan diferentes tipos de células efectoras que varían a medida que evoluciona la respuesta inflamatoria. Uno de los tipos celulares directamente implicado desde el inicio hasta la resolución de la inflamación es el macrófago.

Los macrófagos son células especializadas que forman parte del sistema inmune con un papel esencial en la respuesta primaria a patógenos, así como en el mantenimiento de la homeostasis tisular, inflamación e inmunidad. Los macrófagos derivan de las células madre hematopoyéticas de la médula ósea, que tras diferenciarse a monocitos migran por el torrente circulatorio a diferentes tejidos y órganos, diferenciándose a macrófagos específicos de cada tejido: macrófagos de tejido adiposo (en inglés, *adipose tissue macrophages*, ATMs), células de Kupffer en el hígado, microglía en el sistema nervioso central, osteoclastos en el hueso, histiocitos en el tejido conjuntivo, etc. [21,22]. Los macrófagos permanecen inactivos en los tejidos y pueden activarse tras reconocer estímulos como el lipopolisacárido de la pared de bacterias Gram negativas (en inglés, *lipopolysaccharide*, LPS), citoquinas o secuencias de reducido tamaño asociadas a patógenos llamadas patrones moleculares asociados a patógenos (en inglés, *pathogen-associated molecular patterns*, PAMPs), a través de los receptores transmembrana conocidos como receptores tipo Toll (en inglés, *Toll-like receptors*, TLRs), desencadenando así la respuesta inmunitaria [23,24].

Se han atribuido diversas funciones a los macrófagos durante el proceso inflamatorio. Los macrófagos son células dinámicas que, junto a los monocitos circulantes, pertenecen al sistema fagocítico mononuclear, cuya principal función es la **fagocitosis**, eliminando microorganismos invasores, células extrañas, y tejidos muertos [25,26]. Por otra parte, los macrófagos, también son **células secretoras** de mediadores como las citoquinas (IL-1, IL-6, TNF-α) y quimioquinas (MCP-1), que promueven la respuesta inflamatoria ya que estimulan la fagocitosis, activan a los

linfocitos T, estimulan la hematopoyesis, atraen a otras células inmunes hacia el foco inflamatorio, etc. [27,28]. Estas células, una vez activadas, además de secretar factores quimiotácticos, liberan también otros mediadores proinflamatorios, como son los lípidos bioactivos, y mediadores citotóxicos, como los radicales libres de nitrógeno y de oxígeno. Otra de las funciones fundamentales del macrófago activado es su **capacidad presentadora de antígenos**. Tras la fagocitosis, los macrófagos degradan las proteínas y procesan los antígenos para presentarlos en los complejos mayores de histocompatibilidad de la superficie celular, permitiendo que los linfocitos T puedan reconocer estos compuestos como extraños. Por su capacidad destructiva, los macrófagos guían el curso de la respuesta inflamatoria e intervienen en la síntesis y reparación del tejido dañado durante el proceso inflamatorio, participando de forma activa en la **resolución de la inflamación** [29].

1.1 Polarización de los macrófagos

Se ha descrito una heterogeneidad fenotípica en el macrófago dependiendo del grado de diferenciación, distribución tisular y respuesta a estímulos endógenos y exógenos [30]. Los macrófagos tienen capacidad para modificar su perfil funcional en respuesta a una variedad de estímulos y polarizarse en distintos fenotipos. Recientemente, haciendo referencia a la dicotomía de linfocitos T colaboradores 1 y 2 (en inglés, *T helper cells 1/2,* Th1/Th2), se han propuesto dos subtipos de macrófagos según sean activados por la vía clásica (M1) o por la vía alternativa (M2) [31]. Estos diferentes modos de polarización van a depender del entorno y del daño que se haya producido.

La **activación clásica** es fundamental para el inicio y mantenimiento del proceso inflamatorio, así como para la respuesta frente a patógenos y respuesta inmune. La activación clásica o **M1** es producida por la interacción de TLR4 con los PAMPs como el LPS de la pared de bacterias Gram negativas, o por determinadas citoquinas como

el TNF-α o el IFN-γ. Este grupo de macrófagos activados de forma clásica produce cantidades elevadas de citoquinas proinflamatorias como TNF-α, interleuquinas (IL-1, IL-6, IL-12), quimioquinas proinflamatorias como MCP-1 y óxido nítrico (ON), promoviendo la activación de la respuesta inmune [30].

En presencia de citoquinas secretadas por linfocitos Th2, como la IL-4 o la IL-13, se induce en el macrófago una **activación alternativa** característica de los macrófagos **M2** [32]. Estos macrófagos M2 tienen escasa capacidad presentadora de antígenos, mientras que secretan altos niveles de citoquinas antiinflamatorias como la IL-10. A diferencia de la activación clásica, estos macrófagos no son capaces de producir óxido nítrico a partir de L-arginina y tampoco consiguen controlar el crecimiento de patógenos intracelulares [33]. Sin embargo, son capaces de producir una elevada cantidad de arginasa 1, enzima que metaboliza la L-arginina para producir prolina, glutamato y poliaminas favoreciendo la reparación tisular. La prolina actúa como precursora del colágeno [34] y las poliaminas han sido ampliamente relacionadas con procesos de proliferación y diferenciación [35]. Dentro de este contexto antiinflamatorio, estos macrófagos también pueden producir TGF-β, que inhibe la generación de especies reactivas de oxígeno e intermediarios del nitrógeno [36,37]. Además, también se ha descrito que los macrófagos M2 liberan factores proangiogénicos (VEGF, MMP9) [38].

Se han descrito varias subpoblaciones M2 (a y b). La M2a interviene en la cicatrización de heridas, angiogénesis y remodelado tisular tras la estimulación de IL-4 o IL-13, activando la vía de STAT6 (*Signal Transducer and Activator of Transcription 6*). Por otra parte, la subpoblación M2b participa en la modulación y finalización de la respuesta inmune tras la unión de complejos inmunes con los ligandos de TLR y ligandos del receptor de IL-10, respectivamente [30,39,40] (Figura 1).

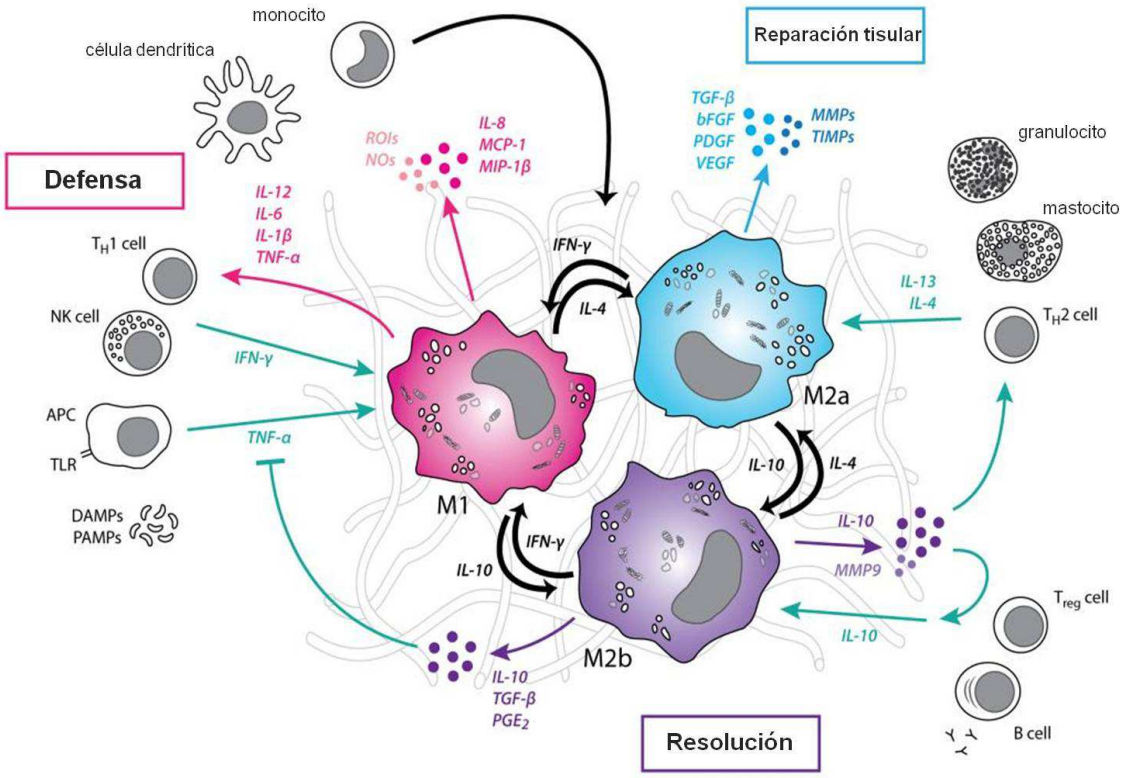

Figura 1. Activación y polarización de los macrófagos M1 y M2 (Adaptado de S. L. M. van Loon, Calcific Aortic Valve Disease, Chapter 8, 2013)

1.2 Rutas de señalización en el macrófago activo

- **Receptores tipo Toll (TLRs)**

Los TLRs constituyen una familia de once proteínas (en el ser humano) y cada una está codificada por un gen diferente. Todos comparten un dominio citoplasmático similar, que se encuentra también presente en receptores de IL-1, razón por la cual se le denominó dominio TIR (Toll/IL-1 receptor). En la Tabla 1 pueden observarse los diferentes receptores de patrones Toll-Like, cada uno con su ligando característico, el agente que reconoce, el tipo de localización celular y las células en las que puede encontrarse. Después de unirse a sus ligandos, los TLRs forman heterodímeros u homodímeros, lo que resulta esencial para su activación. Estos receptores pueden desencadenar la migración de las células presentadoras hacia los tejidos linfáticos secundarios, así como provocar el incremento de la expresión de moléculas coestimuladoras para la respuesta inmune como CD80/86. En células con capacidad

fagocítica, los TLR pueden actuar estimulando la fagocitosis además de aumentar el potencial microbicida e inducir la producción y secreción de quimioquinas y citoquinas.

Receptores celulares	Localización	Ligando	Procedencia	Activan
TLR1, TLR2, TLR6	Membrana	Lipoproteínas, peptidoglicanos, partículas virales	Bacterias Virus	Linfocitos T
TLR3	Endosoma	RNA viral de doble cadena	Virus	IFNα,β
TLR4, (CD14)	Membrana	Lipopolisacárido Partículas virales	Bacterias Virus	Macrófagos IL-6, IL-1, IL-12, IL-8, MCP-1
TLR5	Membrana	Flagelina	Bacterias	Linfocitos T
TLR7 **TLR8**	Endosoma	ARN monocatenario vírico	Virus	Macrófagos
TLR9	Endosoma	CpG bacterias virus sin metilar	Bacterias Virus	Macrófagos

Tabla 1. Receptores de reconocimiento de patógenos tipo *Toll Like Receptor*

- **El factor de transcripción NFκB**

Uno de los principales factores de transcripción implicados en la regulación de la respuesta inflamatoria es el factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas (en inglés, *nuclear factor kappa B,* NF-κB). La activación de este factor es necesaria para el desarrollo de la respuesta inmune al inducir la expresión de genes de primera defensa frente a microorganismos [41-43].

En condiciones basales, el NF-κB está presente en el citoplasma de forma inactiva unido a las proteínas inhibitorias κB (IκB). La familia de proteínas IκB se caracteriza por la presencia de unas secuencias de repetición de anquirina y está compuesta por siete miembros: IκBα, IκBβ, IκBε, IκBγ, IκBζ, IκBns y Bcl3 (*B-cell*

lymphoma 3). Las proteínas IκBα, β, ε y γ se localizan en el citoplasma y se encuentran unidas a distintas unidades de NF-κB. Estas cuatro proteínas se degradan tras ser fosforiladas, lo cual no ocurre con el resto de miembros (IκBζ, IκBns y Bcl3) que se localizan principalmente en el núcleo [44,45].

En mamíferos, la familia de proteínas NF-κB comprende cinco proteínas, p65 (RelA), RelB, c-Rel, p105/p50 (NFκB1) y p100/52 (NFκB2) [46] que se asocian entre sí formando homodímeros y heterodimeros para actuar como complejos transcripcionales activos (Figura 2).

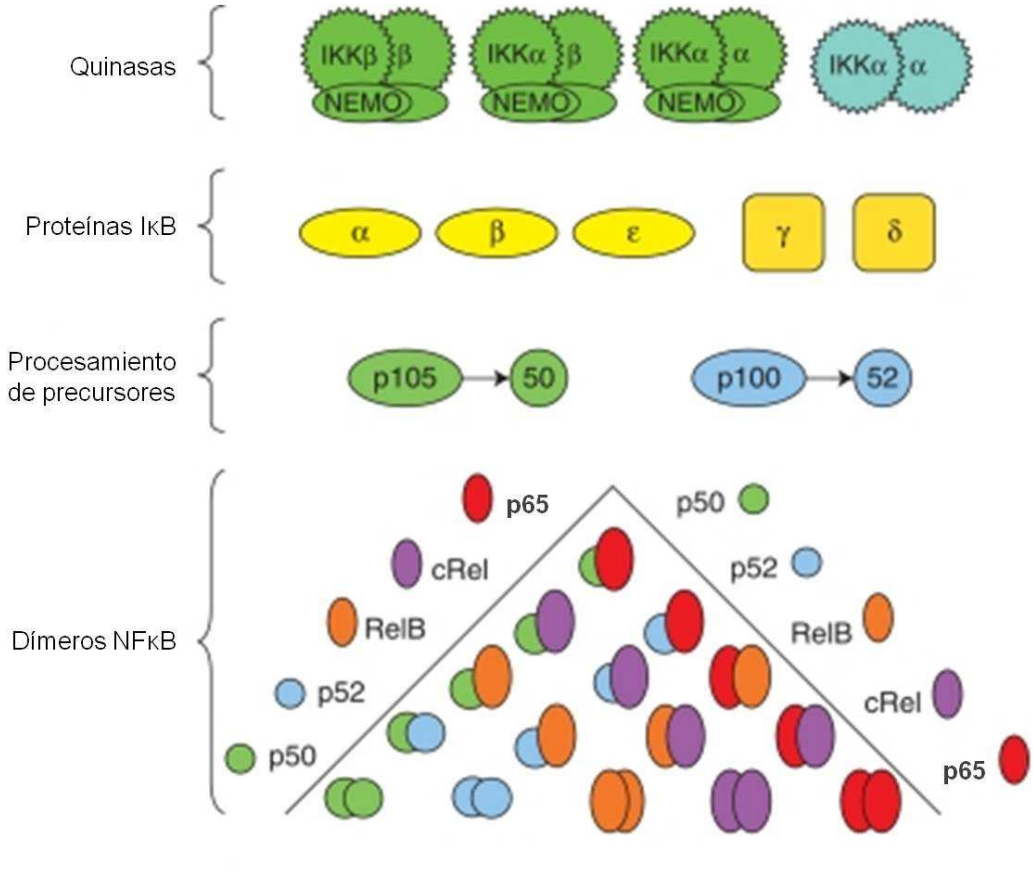

Figura 2. Componentes moleculares de la vía de activación IKK-IκB-NFκB. (Adaptado de referencia [11])

Se han descrito varias vías de activación del factor de transcripción NF-κB. La activación clásica comienza en respuesta a diferentes señales extracelulares como son las citoquinas proinflamatorias (TNF-α e IL-1), mitógenos, LPS o por activación del

receptor de células T. En esta vía, las proteínas IκBα son fosforiladas por las proteínas IκB quinasas o complejo IKK, formado por dos subunidades catalíticas (IKKα e IKKβ) y una reguladora (IKKγ, también llamada NEMO) [47]. Tras esta fosforilación se lleva a cabo la poliubiquitinación de IκBα y su posterior degradación por la subunidad 26S del proteasoma. Como consecuencia de la degradación de IκB, el complejo p65/p50 se transloca al núcleo donde va a regular la transcripción de numerosos genes proinflamatorios, como aquellos que codifican para la expresión de citoquinas, receptores implicados en la adhesión y migración leucocitaria y enzimas que producen mediadores inflamatorios secundarios como COX-2 y NOS-2 [48,49] (Figura 3).

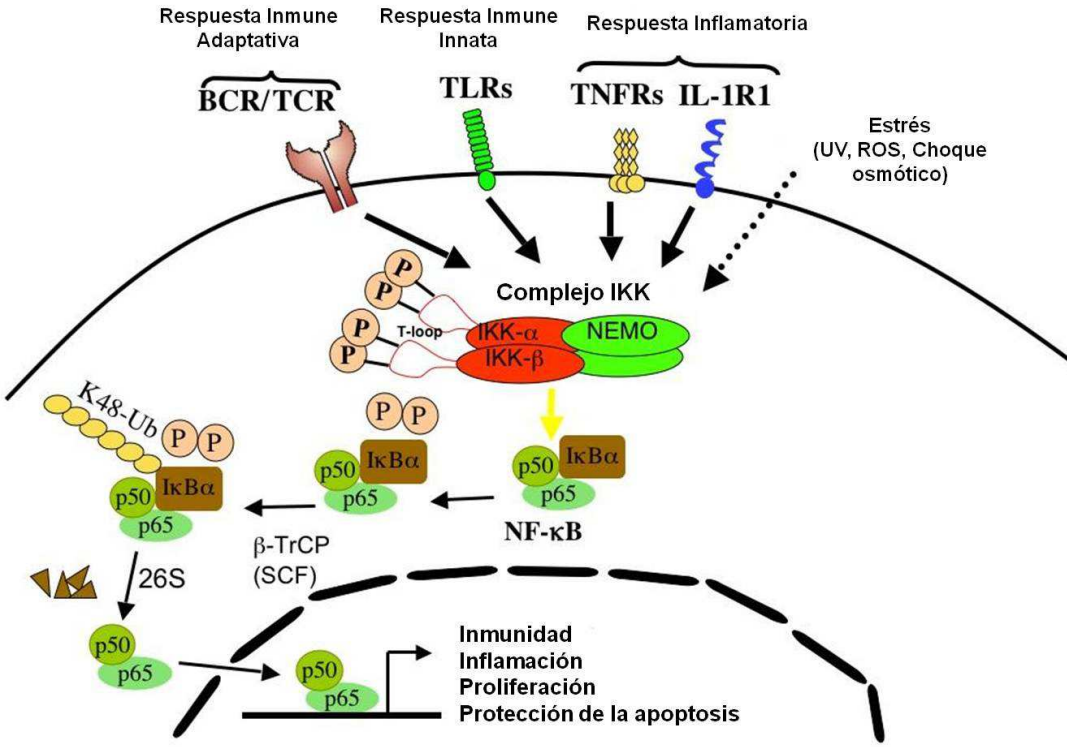

Figura 3. Vía de señalización del factor de transcripción NFκB. (Adaptado de referencia [5])

- **Activación de las MAPK en la inflamación**

En la respuesta inmune están implicados un gran número de tipos celulares que pueden actuar como iniciadores, reguladores y efectores en la transducción de señales que regula la expresión génica y función inmunológica. La cascada de señalización de las proteínas quinasas activadas por mitógenos (en inglés*, Mitogen-Activated Protein Kinase,* MAPK) es una de las vías conocidas más antiguas y conservadas en eucariotas a lo largo de la evolución, implicadas en numerosos procesos de la respuesta inmune y otros procesos fisiológicos tales como proliferación, estrés celular y apoptosis [50].

Existen tres subfamilias bien definidas de MAPK: las proteínas quinasas reguladas por señales extracelulares (ERK, p42 y p44), las quinasas del extremo amino terminal de c-Jun (JNK), también conocidas como MAPK activadas por estrés, y por último, las quinasas p38 [51].

El proceso de activación se lleva a cabo por una serie de fosforilaciones en cascada comenzando con la activación de las MAPK quinasas quinasas (MAPKKK). Cuando las MAPK se activan, son capaces de fosforilar un amplio rango de proteínas implicadas en numerosas vías de señalización intracelular (Figura 4). Además, se pueden translocar al núcleo y fosforilar proteínas que estabilizan la cromatina, o bien factores de transcripción, tales como la proteína activadora 1 (AP-1) [51]. AP-1 es un importante factor de transcripción que regula la expresión de genes proinflamatorios (al igual que NFκB). Su actividad es regulada por JNK, que se une al dominio de activación amino terminal de c-Jun fosforilándolo en los residuos Ser-63 y Ser-73 [52,53]. Cuando c-Jun es fosforilado, forma homodímeros o heterodímeros con otras proteínas como c-Fos constituyendo el factor de transcripción AP-1 activo.

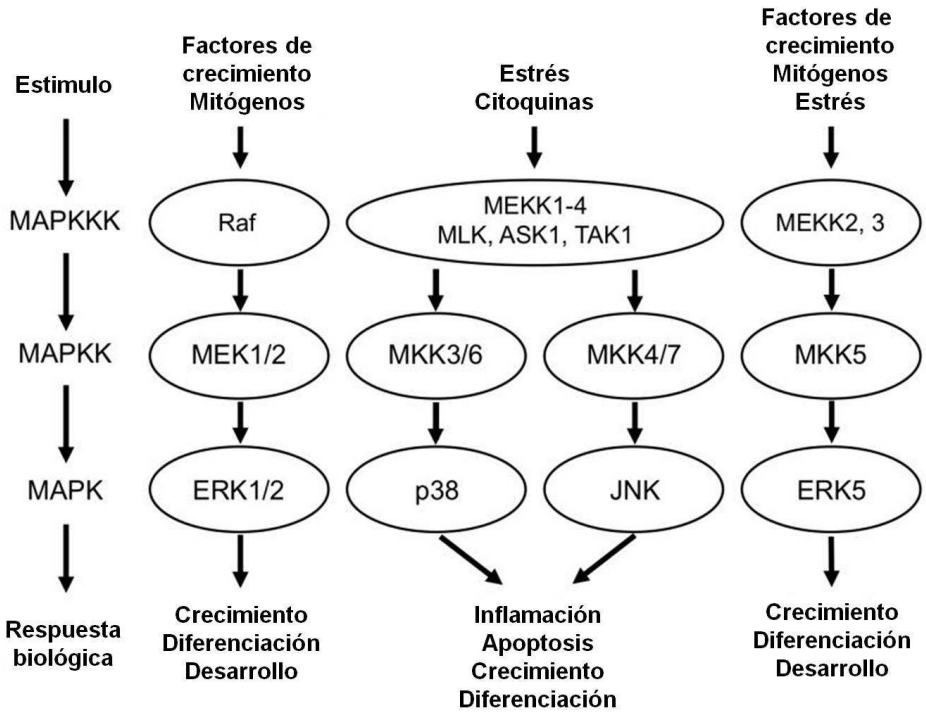

Figura 4. Rutas de activación de las MAPK (Adaptado de Maki Urushihara, Glomerulopathies - Etiology and Pathogenesis, Capítulo 11)

1.3 Mediadores de la respuesta inflamatoria

La citoquinas son proteínas de bajo peso molecular implicadas en la regulación de la respuesta inmune innata y adaptativa y en la hematopoyesis; actúan también como moduladores inflamatorios, participando tanto en la inflamación aguda como en la crónica. Las células endoteliales, los macrófagos (inmunidad innata) y los linfocitos T (inmunidad adaptativa) son los tipos celulares que liberan mayor cantidad de citoquinas [54]. Generalmente actúan de forma sinérgica entre ellas o junto a otros estímulos pudiendo inducir a su vez la síntesis de otras citoquinas.

Las principales **citoquinas proinflamatorias** son **IL-1β, IL-6** y **TNF-α,** producidas principalmente por macrófagos. Las citoquinas IL-1β y TNF-α actúan conjuntamente en el foco inflamatorio estimulando la expresión de enzimas proinflamatorias, como la ciclooxigenasa (COX), lipooxigenasa (LOX) y la oxido nítrico sintasa-2 (NOS-2 ó iNOS), responsables de la producción de prostaglandinas (PGs),

leucotrienos (LTs) y óxido nítrico (NO), respectivamente. Además actúan como agentes quimiotácticos e inducen la expresión de moléculas de adhesión (ICAM-1, VCAM-1 y selectina E) [55-57].

El **IFN-γ** es otra citoquina proinflamatoria (también llamada linfoquina) sintetizada por los macrófagos, linfocitos T activados y células Natural Killer (NK) desempeñando un importante papel en los mecanismos de defensa al inducir la producción de NO y aumentar la actividad del TNF-α.

Por otro lado, existe otro grupo de citoquinas capaces de inhibir la expresión de genes inflamatorios o impedir la acción de las citoquinas proinflamatorias. Entre las principales **citoquinas antiinflamatorias** destacan las IL-4, IL-6, IL-10, IL-13 y el factor de crecimiento transformante-β (TGF-β). La IL-6 es una citoquina pleiotrópica que, en función del entorno, actuará como anti o proinflamatoria con un amplio espectro de actividades en la regulación inmune, hematopoyesis, inflamación y oncogénesis [58]. Cuando esta citoquina actúa como antiinflamatoria disminuye la liberación de IL-1β, TNF-α e IFN-γ y potencia la síntesis de las citoquinas antiinflamatorias IL-10 y TGF-β [59]. Por su parte, la IL-10 es una citoquina esencial para la regulación de la respuesta inmune y el mantenimiento de la homeostasis tisular; es sintetizada mayoritariamente por los linfocitos T reguladores, monocitos, macrófagos y células dendríticas y su función principal es la de inhibir la síntesis de IFN-γ e IL-2, además de bloquear la translocación al núcleo del factor de transcripción NF-κB [60].

Las **quimioquinas** del tipo Cxcl5, Cxcl9 o Ccl17 son liberadas principalmente por macrófagos, células endoteliales, linfocitos T, fibroblastos y monocitos, cuya función principal es controlar la adhesión y quimiotaxis, es decir, la atracción y activación de leucocitos polimorfonucleares, monocitos y linfocitos hacia el tejido inflamado [55].

En el desarrollo del proceso inflamatorio también se ha descrito la participación de otros mediadores proinflamatorios entre los que se incluyen distintos **lípidos**

bioactivos (eicosanoides, prostanoides, resolvinas, protectinas y lipoxinas). En la fase inicial de la inflamación, los neutrófilos y macrófagos liberan *eicosanoides,* mediadores lipídicos derivados del metabolismo oxidativo del ácido araquidónico (AA) de las membranas celulares. El AA es sustrato de diferentes enzimas, como las del sistema citocromo P-450 o las lipoxigenasas (LOX) y ciclooxigenasas (COX), responsables de la síntesis de leucotrienos (LTs) y prostaglandinas (PGs), respectivamente [61] (Figura 5). Además, la mayoría de estos metabolitos derivados de ácidos grasos se consideran factores relevantes en la patogénesis de la obesidad, la resistencia a la insulina y el síndrome metabólico (SM) [62-64].

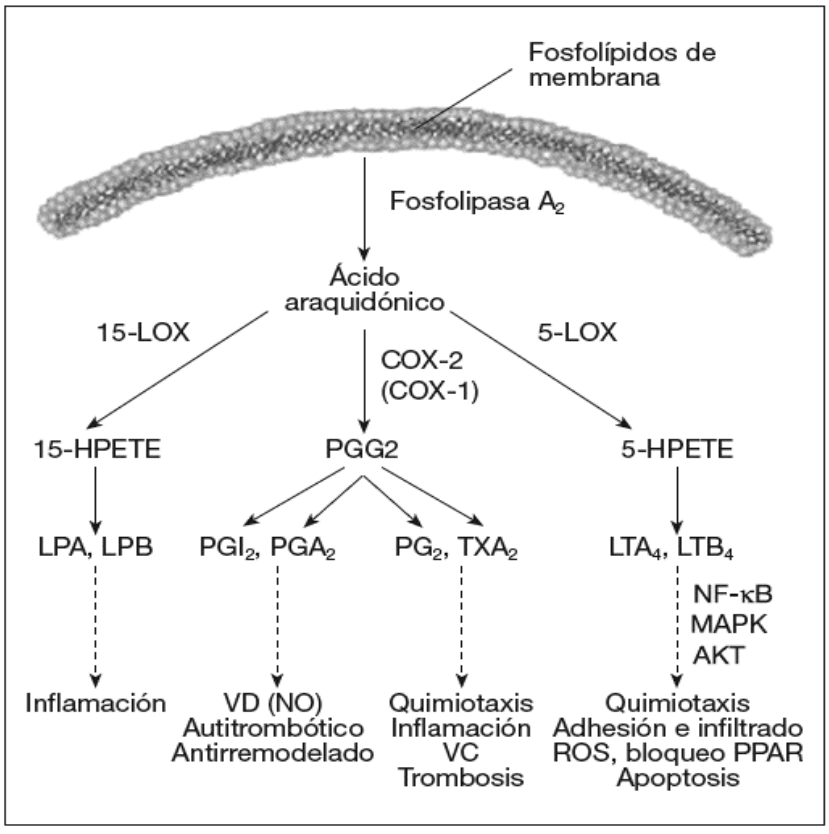

Figura 5. Metabolismo de PGs y LTs a partir del Ácido Araquidónico de la membrana plasmática. (Adaptado de Óscar Lorenzo, Clin Invest Arterioscl., 2009)

1.4 Los macrófagos y su papel en obesidad

El descubrimiento hace más de dos décadas de que la citoquina proinflamatoria conocida como factor de necrosis tumoral alfa (TNF-α) se encontraba altamente elevada en el tejido adiposo de ratones obesos, fue la primera evidencia de que los mediadores inflamatorios estaban asociados a la obesidad [65,66]. Posteriormente se ha verificado que la infiltración de células del sistema inmune (sobre todo macrófagos) en el tejido adiposo y el aumento de la secreción de adipoquinas y citoquinas proinflamatorias son características primordiales en la resistencia a la insulina inducida por obesidad y la enfermedad metabólica asociada, tanto en modelos animales como en humanos [67,68]. En bases a estos hallazgos en la actualidad se define la obesidad como un estado de inflamación crónica de bajo grado. De hecho, más del 40 % del total de células del tejido adiposo en roedores y humanos obesos son macrófagos, lo que contrasta con el 10 % en el caso de animales y sujetos delgados (Figura 6). Además, estos macrófagos son los responsables de la mayor parte de la producción y secreción de citoquinas proinflamatorias como TNF-α, IL-6, IL-1β y quimioquinas como MCP-1, la cual regula la migración e infiltración de monocitos circulantes [69,70].

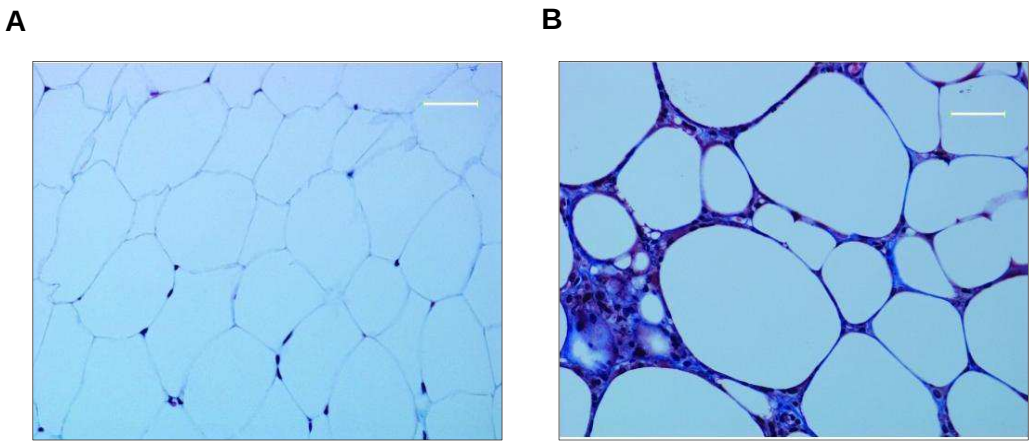

Figura 6. Infiltrados de macrófagos en el tejido adiposo. Secciones del tejido adiposo de ratones delgados (A) y obesos (B). (Adaptado de referencia [1])

Durante la obesidad, la polarización de los macrófagos infiltrados en el tejido adiposo (ATMs) es trascendental [71]. En ratones delgados, los macrófagos residentes están polarizados hacia un estado M2 (altos niveles de IL-10 y de arginasa 1) que mantiene la función del adipocito, promueve la reparación tisular e induce angiogénesis. Cuando hay un exceso de nutrientes, los adipocitos aumentan de tamaño e incrementan la secreción de adipoquinas y quimioquinas, reclutando así monocitos $CCR2^{+}$ (que contienen receptores de CCL2, también llamada MCP-1), y que posteriormente se diferencian a macrófagos M1, generando altos niveles de TNF-α, IL-6, etc. La activación "alternativa" de los macrófagos, podría prevenir la acumulación de macrófagos M1 [72], y como consecuencia proteger a las células colindantes frente a los efectos perjudiciales del TNF-α y demás citoquinas proinflamatorias [73]. En concordancia con esta idea, en estudios realizados en ratones deficientes (*knock out*) en $CCR2^{-/-}$ y $CCL2^{-/-}$ y bajo condiciones de obesidad, se ha observado que sus ATMs poseen una reducida capacidad inflamatoria [74,75].

El incremento en la exposición a ácidos grasos, bien por un aumento en el contenido de grasa de las dietas modernas o por una lipolisis aberrante de los adipocitos hipertróficos, se ha propuesto como un factor clave, tanto en la desregulación del metabolismo, como en la señalización inmune durante la inflamación [76] (Figura 7). Incluso, algunos autores definen con el término de "metainflamación" a la respuesta inmunológica modulada por nutrientes y un excedente metabólico [16].

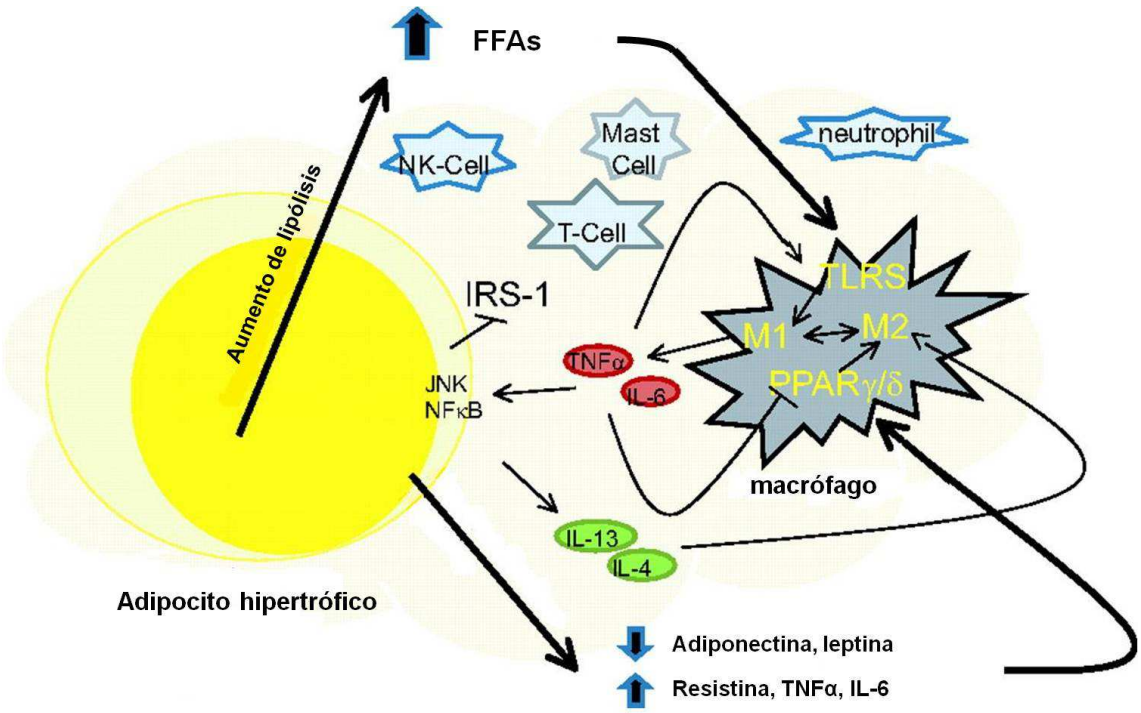

Figura 7. Modulación de la actividad de células inmunes por señales metabólicas en el tejido adiposo obeso. (Adaptado de referencia [10])

En estados de obesidad se produce hiperplasia e hipertrofia de los adipocitos, lo cual contribuye a una expansión del tejido adiposo. Esta expansión puede ocasionar múltiples efectos, como hipoxia, muerte de los adipocitos, aumento de la secreción de adipoquinas y quimioquinas y desregulación del flujo de ácidos grasos libres (en inglés, *free fatty acids*, FFAs) [1]. Los FFAs, que normalmente están almacenados en forma de triglicéridos (TG) en el tejido adiposo, son liberados por los adipocitos hipertróficos tras una lipolisis aberrante. Asimismo, la liberación de FFAs al torrente sanguíneo aumenta debido a un incremento en la expresión de lipoproteína lipasa (LPL) que ocurre en el adipocito hipertrófico por la activación del complejo conocido como inflamosoma que conduce al aumento de IL-1β [77]. De esta forma, los ATM se encuentran en un ambiente enriquecido en lípidos, y numerosos estudios han demostrado que los ácidos grasos son capaces de modular la respuesta inflamatoria

en macrófagos [78]. Por otro lado, parte de los FFAs liberados al torrente sanguíneo, son desviados al hígado y almacenados en forma de gotas lipídicas. Los FFAs de cadena corta (4-6 átomos de carbono) y de cadena media (8-10 átomos de carbono) pueden penetrar en las células a través de las membranas plasmáticas por simple difusión debido a su pequeño tamaño y sus características hidrosolubles, mientras que los FFAs de cadena larga (C12-C20) entran en el hepatocito gracias a proteínas transportadoras de ácidos grasos (en inglés, *Fatty Acid Transporter Protein,* FATPs) y una vez dentro, su metabolismo puede producir distintos intermediarios lipídicos como diacilglicerol (DAG) o ceramidas, los cuales tienen conocidas funciones como segundos mensajeros en rutas de señalización implicadas en la patogénesis de la resistencia a la insulina inducida por lípidos [79,80].

Sin embargo, los FFAs también pueden actuar como ligandos de los receptores TLR4, de esta manera activan la respuesta inflamatoria clásica, vía JNK y factor de transcripción NFκB, en el contexto de alta concentración de lípidos extracelulares [81]. Estudios en modelos animales han reforzado estos conceptos. La expresión de TLR4 en macrófagos aumenta con la obesidad, mientras que una disminución en su expresión conlleva una reducción en la respuesta inflamatoria inducida por FFAs saturados, tanto en macrófagos como en adipocitos, hepatocitos y células de músculo esquelético [82-84]. Igualmente, se ha demostrado que el incremento de ácidos grasos circulantes por infusión de lípidos, produce resistencia a insulina en ratones de genotipo salvaje, mientras que en ratones deficientes en TLR4 este efecto no tiene lugar [85]. Además, los ratones deficientes en TLR4 ganan menos peso que los ratones control cuando son alimentados con una dieta alta en grasa [86].

Consideraciones estructurales y ensayos de unión directa argumentan en contra de la posibilidad de que los FFAs saturados sean ligandos directos de TLRs [87]. Sin embargo, hay varios mecanismos por los cuales los FFAs saturados podrían activar la señalización mediada por TLRs. Una posibilidad es a través de su interacción con

proteínas de unión a FFAs saturados, que actúan como correceptores de los TLRs. Un precedente para este tipo de mecanismo es proporcionado por la capacidad de CD36, un transportador de FFAs y receptor de LDL oxidadas, para interaccionar con TLR2 y/o TLR4 e inducir la señalización en respuesta a LDL oxidadas [88,89]. Por otro lado, también se ha visto que los FFAs saturados son capaces de inducir la expresión génica dependiente de TLR4 en macrófagos, promoviendo la dimerización de TLR4, un paso necesario para la activación del receptor, en dominios lipídicos especializados llamados “lipid rafts” [90]. Una tercera posibilidad es que los FFAs saturados pueden estimular la producción o liberación de señales de daño tisular, moléculas endógenas liberadas de células dañadas o necróticas, las cuales son captadas por los TLRs [91].

2. Progresión de la enfermedad del hígado graso no alcohólico

La enfermedad del hígado graso no alcohólico (en inglés, *Nonalcoholic Fatty Liver Disease*, NAFLD) es la principal manifestación hepática del síndrome metabólico (SM) y la principal causa de enfermedad hepática crónica en el mundo occidental. La prevalencia de la NAFLD entre individuos obesos se encuentra en torno al 75-100% frente al 20-30% de la población general, por lo que es considerada una de las principales manifestaciones del SM [92]. Mientras que la mayoría de los pacientes con NAFLD presentan la enfermedad de manera asintomática, el 20% de los individuos acaban desarrollando inflamación hepática crónica o esteatohepatitis no alcohólica (en inglés, *Nonalcoholic Steatohepatitis*, NASH), que a su vez puede conducir hacia un estado de cirrosis, hipertensión portal e incluso carcinoma hepatocelular (en inglés, *Hepatocellular Carcinoma*, HCC) [93].

El tejido adiposo visceral posee una gran influencia sobre el desarrollo de la NAFLD, mediante la secreción de FFAs y de una gran variedad de citoquinas y adipoquinas, como TNF-α, leptina, adiponectina, resistina y visfatina, que pueden

actuar directamente sobre el hígado. Los FFAs juegan un papel fundamental en el desarrollo de esteatosis simple, mientras que las adipoquinas y citoquinas proinflamatorias inducen apoptosis e inflamación en el hígado, favoreciendo así la progresión de esteatosis simple a NASH [9] (Figura 8). La combinación de acumulación lipídica y apoptosis en hepatocitos es conocida como lipoapoptosis, la cual es el mecanismo crucial en el desarrollo de NASH [94,95].

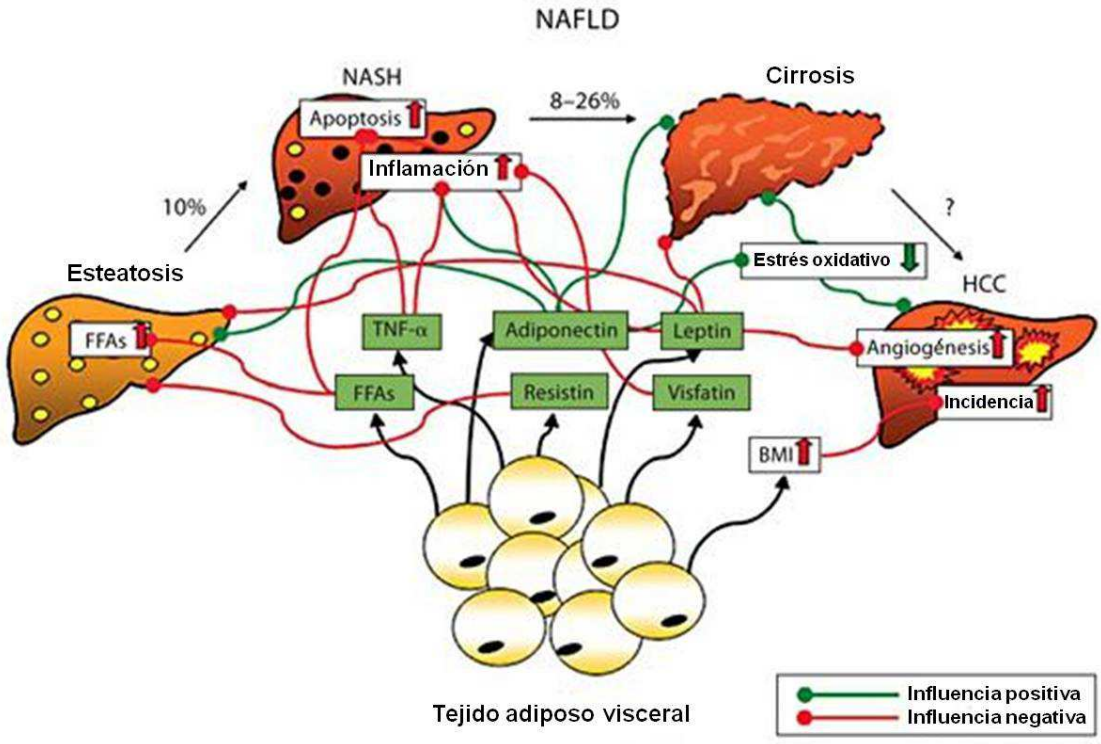

Figura 8. Influencia del tejido adiposo visceral en la progresión de la NAFLD (Adaptado de referencia [9])

2.1 Procesos implicados en la progresión de la NAFLD

Existen varios procesos biológicos con un papel fundamental en la progresión de la NAFLD como son el estrés oxidativo, la disfunción mitocondrial, el estrés del RE, la activación de las quinasas de estrés, la apoptosis, la autofagia, etc. En nuestro caso nos vamos a centrar en el estrés del RE.

- **Respuesta a la acumulación de proteínas mal plegadas y estrés del RE**

El RE juega un papel esencial en la biosíntesis de proteínas destinadas a la secreción o inserción en membranas de células eucariotas. El RE es el lugar donde las proteínas, todavía inmaduras, completarán su ensamblaje. Estos procesos son supervisados por diversos sistemas de control y las proteínas incapaces de plegarse adecuadamente son translocadas al citosol, donde son degradadas por el proteosoma en un proceso denominado ERAD (en inglés*, Endoplasmic Reticulum-Associated Degradation)* [96]. Las agresiones biológicas tales como las infecciones, la interrupción nutricional, la exposición a toxinas o un exceso de lípidos, pueden alterar la homeostasis del RE, provocando un mal ensamblaje proteico y causando una acumulación de estas proteínas mal plegadas, dando como resultado a una sobrecarga proteica (estrés). Para combatir esta sobrecarga, el RE inicia un programa transcripcional denominado respuesta a mal ensamblaje proteico (en inglés, *Unfolded Protein Reponse*, UPR) diseñado específicamente para disminuir o detener la síntesis de proteínas mal plegadas y para promover su rápida degradación restableciendo de esta manera la homeostasis celular [97].

Este complejo sistema de respuestas a la sobrecarga del RE se compone de 3 rutas distintas que están controladas por proteínas de transmembrana presentes en el propio RE: la proteína dependiente de inositol 1 (en inglés, *inositol requiring enzyme 1*, IRE-1), la quinasa eIF2α (en inglés, *PKR-like endoplasmic reticulum kinase eIF2α*, PERK) y el factor de transcripción activador 6 (en inglés, *activating transcription factor 6,* ATF6).

El RE contiene varias chaperonas para facilitar el plegamiento correcto de las proteínas, incluyendo la proteína de unión a inmunoglobulina (en inglés, *binding Ig protein,* BiP), también conocida como GRP78 (en inglés, *glucose regulated protein 78*), que se une de modo transitorio a proteínas sintetizadas [98]. En condiciones normales,

BiP se encuentra también unido a los dominios del lumen reticular de IRE1, ATF6 y PERK, pero cuando en el RE aumentan las proteínas mal ensambladas, BiP se une preferentemente a estas últimas liberando sus dominios y permitiendo su activación.

La ruta de **PERK** en la UPR media la inhibición de la traducción proteica mediante la fosforilación de la subunidad α del factor de transcripción eIF2α [96]. Esto produce una disminución de la producción celular de proteínas en un intento de reducir la carga de ensamblaje proteico en el RE. Además, esta vía incrementa la producción del factor de transcripción ATF4, que a su vez está involucrado en la inducción de genes de la respuesta antioxidante y en el transporte de aminoácidos. La activación de PERK también aumenta la expresión de CHOP, un factor de transcripción de la familia C/EBP. En condiciones normales, la expresión basal de CHOP es baja; sin embargo, su expresión aumenta notoriamente cuando el estrés de RE persiste y no es eliminado. La expresión de CHOP da lugar a apoptosis celular por diferentes mecanismos, como la inhibición del factor de protección frente a la apoptosis Bcl-*2*, la activación de caspasas y la translocación de la proteína proapoptótica Bax desde el citosol hacia la mitocondria [99].

La activación de **IRE-1** supone su dimerización y aumento de su actividad endorribonucleasa, produciendo un procesamiento alternativo (splicing) del RNA mensajero de la proteína de unión a X-box 1 (en inglés*, X-box Binding Protein 1,* XBP-1) que cambia su marco de lectura ribosómico, lo que da lugar a la forma activa XBP-1s, que se transloca al núcleo activando la expresión de chaperonas de RE y proteínas involucradas en la ERAD.

La tercera vía canónica de la respuesta UPR está formada por el factor de transcripción **ATF6** que, al activarse, se transloca al aparto de Golgi donde es cortado para producir un fragmento amino-terminal activo que viaja al núcleo donde regula la expresión de proteínas chaperonas (Figura 9).

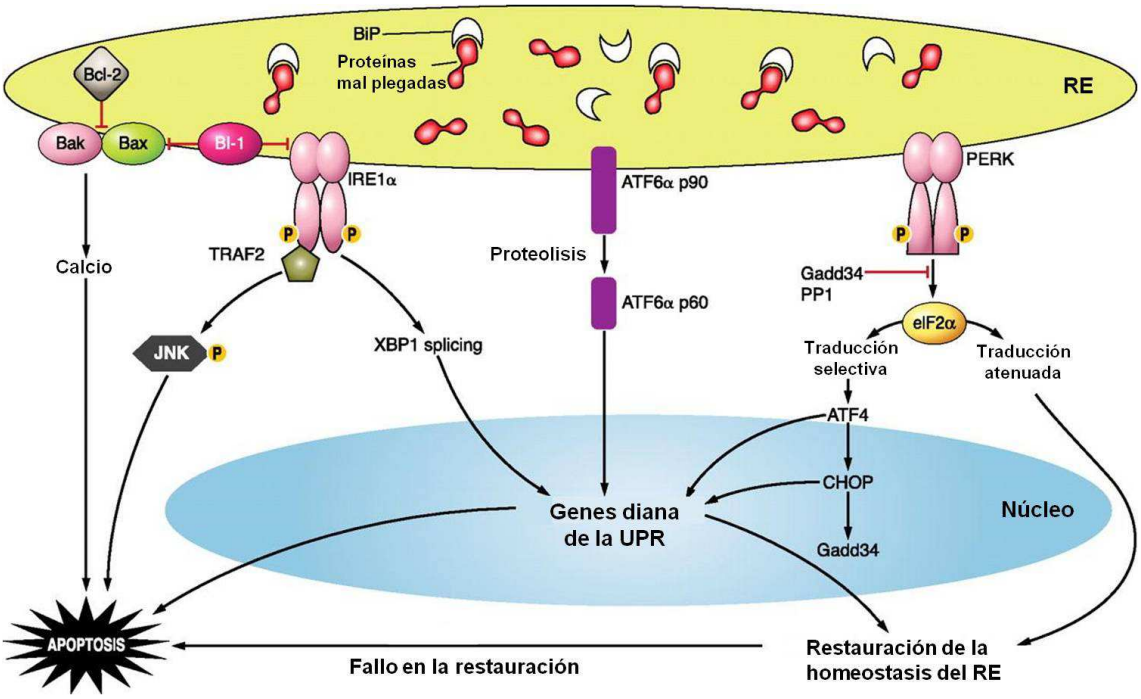

Figura 9. Estrés de RE y activación de la respuesta UPR (Adaptado de referencia [12])

En los últimos años varios estudios han demostrado que un incorrecto funcionamiento de la respuesta a proteínas mal ensambladas (UPR) y el consiguiente estrés del RE, desempeñan un papel clave en la desregulación inmunometabólica, es decir, en la inflamación de bajo grado asociada a enfermedades metabólicas crónicas, incluyendo obesidad, resistencia a insulina y diabetes. Específicamente, la obesidad produce un aumento de estrés de RE, particularmente en hígado y tejido adiposo de ratón [100,101]. Asimismo, un aumento de la expresión de mediadores de la UPR se correlacionó significativamente con el índice de masa corporal y con la resistencia a la insulina en el tejido adiposo de pacientes obesos comparados con individuos delgados

[102,103]. Por otra parte, la pérdida de peso inducida por la cirugía de bypass gástrico demostró que mejora la sensibilidad a la insulina y reduce el estrés de RE en individuos obesos [101].

2.2 Resistencia a insulina asociada a la NAFLD

La dieta occidental actual, rica en grasas saturadas y fructosa, juega un papel primordial en la relación entre NAFLD, obesidad y resistencia a insulina. Como se ha mencionado anteriormente, la disfunción del tejido adiposo visceral en obesidad tiene como resultado en una gran liberación de FFAs a la circulación, como consecuencia de un incremento en la lipolisis, y un aumento de la expresión de adipoquinas y citoquinas proinflamatorias por parte de adipocitos y ATMs. De esta forma, todos estos mediadores proinflamatorios pueden actuar de manera autocrina sobre las propias células secretoras, paracrina sobre las células adyacentes en el tejido o endocrina tras su salida al torrente circulatorio y llegada a otros órganos diana como hígado, músculo o páncreas. Tanto los FFAs como las citoquinas proinflamatorias son capaces de producir un estado de daño y estrés celular en estos tejidos diana, y, como consecuencia, producir una resistencia periférica a la insulina [10,16,100] (Figura 10).

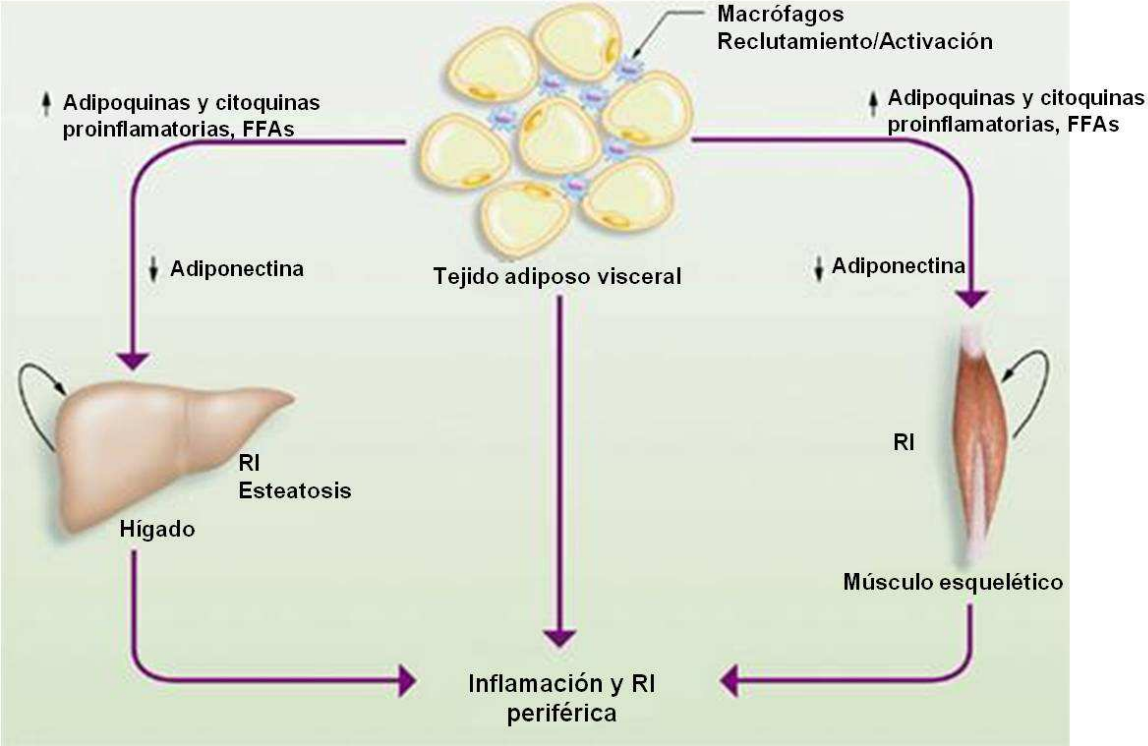

Figura 10. Resistencia a insulina (RI) periférica asociada a obesidad y NAFLD

(Adaptado de referencia [7])

El mecanismo molecular subyacente a la resistencia a insulina hepática implica una disminución de la ruta de señalización del receptor de insulina (en inglés, *insulin receptor*, IR). La cascada comienza cuando el IR es activado por autofosforilación en varios residuos de tirosina que conducen al reclutamiento y la fosforilación en tirosina de los sustratos del IR, IRS1 e IRS2. De este modo, los IRSs fosforilados se unen a proteínas que contienen dominios de homología Src 2 como la fosfatidilinositol 3 quinasa (PI3K), que tiene un papel central en las acciones metabólicas producidas por la insulina [104]. Por debajo de PI3K, la proteína quinasa B (PKB o también llamada AKT) es otra proteína crítica implicada en la vía de señalización de la insulina. Esta serina/ treonina quinasa fosforila al factor de transcripción forkhead box (FoxO1) evitando así su entrada en el núcleo y, como consecuencia, la transcripción de genes gluconeogénicos tales como glucosa 6 fosfatasa y fosfoenolpiruvato carboxiquinasa

[105]. De esta manera, la insulina regula negativamente la producción de glucosa hepática.

En estados de obesidad, la resistencia periférica a la insulina está asociada a un incremento de la expresión de mediadores proinflamatorios y una acumulación masiva de lípidos intracelulares en los tejidos diana de la insulina [106-108]. Numerosos estudios han demostrado que las infusiones de emulsiones lipídicas, que aumentan los niveles de FFAs circulantes, producen un descenso notable en la sensibilidad a la insulina [109,110] (Figura 11).

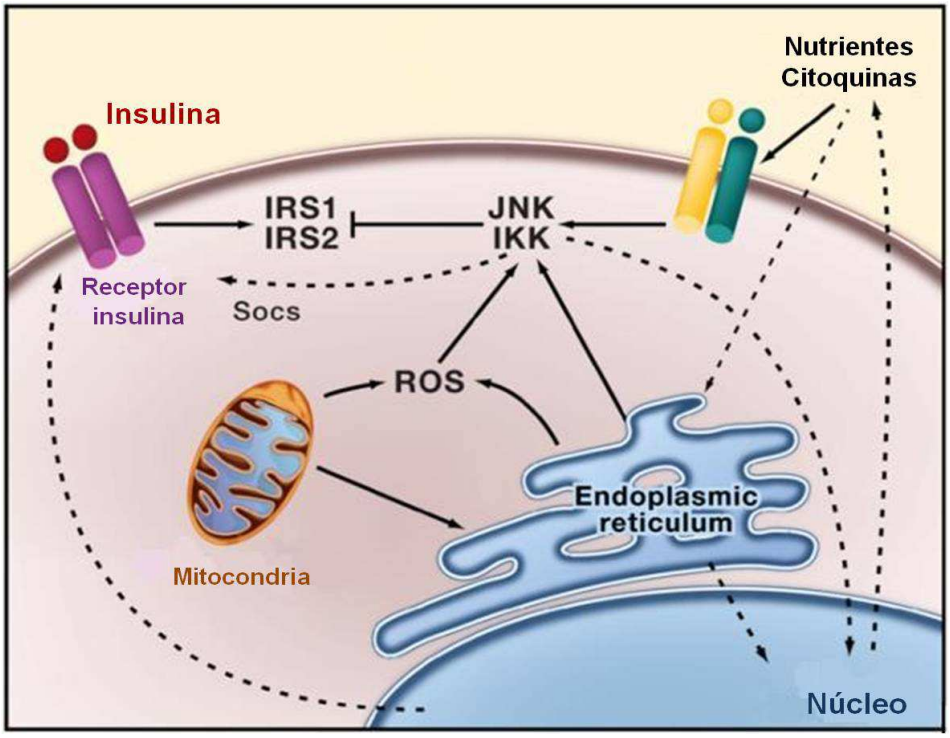

Figura 11. Señalización de la insulina, inflamación y señales de estrés

(Adaptado de referencia [8])

A nivel molecular, los FFAs saturados son capaces de activar las cascadas proinflamatorias de señalización de IKKβ/NFκB y JNK/AP1 en hepatocitos, a través de la estimulación de TLR4/TLR2 o de la activación de las rutas de estrés del RE

[87,111,112]. La activación de estas quinasas de estrés por FFAs o por diferentes mediadores proinflamatorios conlleva a la fosforilación en serina y posterior degradación en el proteosoma de IRS1 e IRS2, lo que desemboca en la inhibición de la cascada de señalización de la insulina. [83,113,114] (Figura 12).

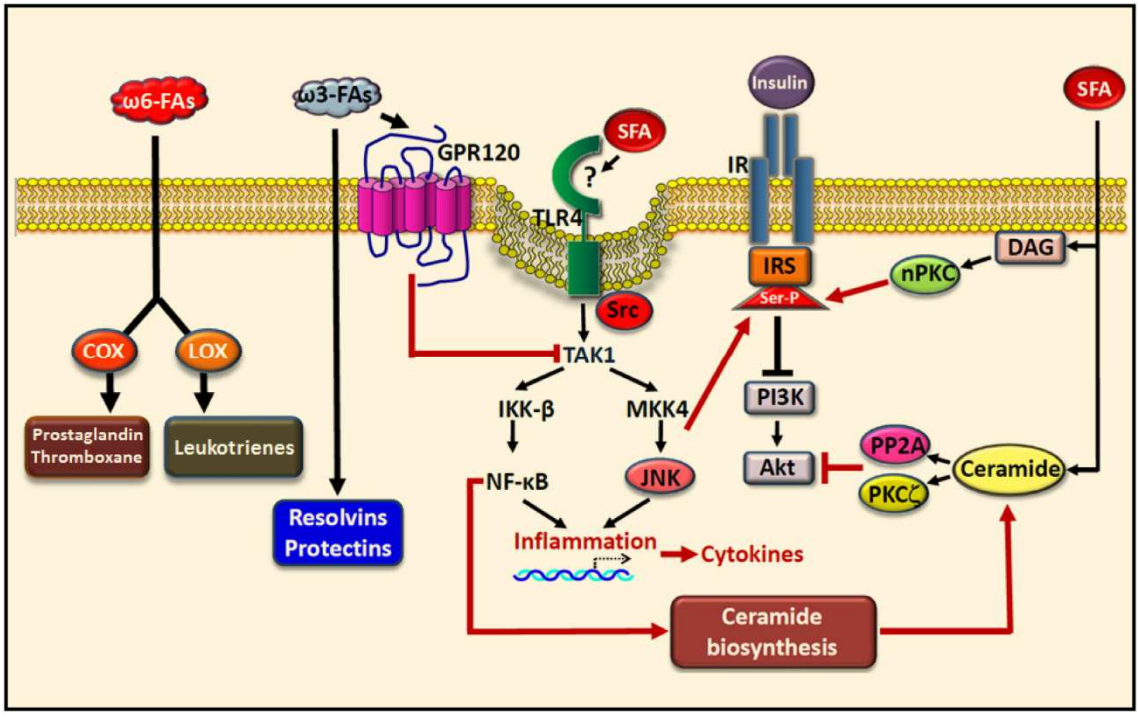

Figura 12. Efectos de los ácidos grasos saturados (SFA) en la inflamación y resistencia a insulina (Adaptado de referencia [6]).

A demás de las fosforilaciones en serina y la degradación de las proteínas IRSs, la ruta de señalización de la insulina tiene otros moduladores negativos, como ciertas fosfatasas específicas. Entre ellas, cabe destacar a la proteína tirosina fosfatasa 1B (PTP1B) que es el mayor regulador negativo de la cascada de señalización de la insulina, ya que actúa defosforilando directamente los residuos tirosina del receptor de insulina impidiendo su activación [115,116]. Sin embargo, también hay otras fosfatasas capaces de modular dicha vía, como PTEN (en inglés, *phosphatase and tensin*

homologue,) que actúa a nivel de la PI3K hidrolizando el grupo fosfato del carbono 3 del fosfatidilinositol-3,4,5-trisfosfato, y PP2A (en inglés *protein phosphatase 2A*) que defosforila directamente el residuo serina 473 de AKT, bloqueando también de esta forma la señalización de la insulina.

En conjunto, estos mecanismos podrían explicar los efectos de la "lipotoxicidad" inducida por FFAs que se produce en situaciones de obesidad y NAFLD, mediante la cual se promueve un estado de inflamación crónica que puede acabar ocasionando resistencia a la insulina en el hígado.

HIPÓTESIS Y OBJETIVOS

En el primer capítulo de esta tesis se abordan los mecanismos moleculares característicos de la progresión de la NAFLD, desde la esteatosis inicial por el acúmulo de ácidos grasos libres en el hígado, hasta la lipotoxicidad inducida por éstos que provoca la disfunción y apoptosis en los hepatocitos.

Posteriormente, en el segundo capítulo se pretende desarrollar una aproximación mas fisiológica de inflamación asociada a la obesidad, y para ello se ha puesto a punto un sistema *"in vitro"* de medios condicionados (MC) de macrófagos estimulados con nuestros dos ácidos grasos de interés. Estos MC, que contienen tanto ácidos grasos libres como citoquinas, quimioquinas y demás especies lipídicas secretadas por los macrófagos tras su activación, se han utilizado para estimular los hepatocitos y posteriormente analizar en ellos las vías de estrés del RE, la lipoapoptosis y la señalización de la insulina. Con este sistema de MC se pretende simular el ambiente de inflamación ("inflammatory milieu") que existe en el hígado de las personas obesas, donde llegan a través del torrente circulatorio los ácidos grasos libres y los productos derivados de los macrófagos sistémicos (sobre todo del tejido adiposo blanco). Además, los hepatocitos reciben los productos derivados de los macrófagos residentes (células de Kupffer). Por tanto, este sistema de MC nos ha permitido estudiar la relación endocrina y paracrina que existe entre los macrófagos y los hepatocitos.

Finalmente, se ha propuesto estudiar el papel de la proteína tirosina fosfatasa 1B (PTP1B) en el inmunometabolismo, ya que PTP1B es el modulador negativo más importante en la cascada de señalización de la insulina porque bloquea la actividad del receptor de insulina, defosforilando las tirosinas activadoras dentro del dominio catalítico. A raíz de trabajos previos de nuestro grupo de investigación realizados en animales deficientes en PTP1B en los que se describen numerosas mejoras en la sensibilidad a la insulina periférica y en la regeneración hepática de estos ratones [117-122], se ha propuesto el estudio de las posibles consecuencias moleculares de la

pérdida de PTP1B en otros procesos fisiológicos, como son las respuestas inflamatorias del sistema inmune. Para llevar a cabo este objetivo, en primer lugar se ha puesto a punto el modelo tratando los macrófagos y los ratones con estímulos proinflamatorios (LPS, PolyI:C) y antiinflamatorios (IL-4, IL-13) propios del sistema inmune, aunque la idea posterior es poder tratarlos con ácidos grasos para simular el estado de inflamación crónica asociada a la obesidad en el modelo de deficiencia de PTP1B.

Los **objetivos específicos** propuestos para el desarrollo de este trabajo han sido los siguientes:

- Determinar el mecanismo molecular involucrado en el efecto protector del ácido oleico sobre la lipotoxicidad y resistencia a la insulina inducidas por ácido palmítico en los hepatocitos.

- Evaluar los efectos del ácido oleico y el ácido palmítico sobre la activación de los macrófagos y sus posteriores interacciones endocrinas y/o paracrinas sobre las vías de estrés y la cascada de señalización de la insulina en los hepatocitos.

- Estudiar las posibles consecuencias fisiológicas de la pérdida de PTP1B en la regulación de las respuestas proinflamatorias y antiinflamatorias del sistema inmune.

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CAPÍTULO I

CAPÍTULO I

EVALUACIÓN DEL EFECTO PROTECTOR DEL ÁCIDO OLEICO SOBRE LA LIPOAPOPTOSIS Y LA RESISTENCIA A LA INSULINA INDUCIDAS POR ÁCIDO PALMÍTICO EN LOS HEPATOCITOS.

En este primer capítulo de la tesis doctoral se exponen los efectos que provoca el ácido palmítico sobre las vías de estrés que conducen a la lipoapoptosis y la resistencia a la insulina en hepatocitos humanos y murinos, así como su reversión por parte del ácido oleico y los mecanismos moleculares asociados.

Desde hace tiempo se sabe que el exceso de ácidos grasos libres saturados, como el ácido palmítico (AP), inducen lipotoxicidad en los hepatocitos, y que este daño celular está directamente implicado en el desarrollo de la NAFLD y de la resistencia a la insulina en el hígado. Por otro lado, el ácido oleico (AO), ácido graso libre monoinsaturado, es capaz de atenuar los efectos del AP, aunque los mecanismos moleculares están todavía sin elucidar. Por este motivo, en este primer trabajo nos hemos propuesto evaluar si el AP está directamente relacionado con la progresión de la resistencia a la insulina y la lipoapoptosis tanto en hepatocitos murinos como en humanos, así como el efecto del AO sobre los mecanismos moleculares que median ambos procesos.

En síntesis, hemos demostrado que en cultivos primarios de hepatocitos humanos, el AP a una concentración lipotóxica es capaz de: inducir la activación de quinasas relacionadas con el estrés del RE, aumentar la expresión del factor de transcripción CHOP, activar la caspasa 3 e incrementar el porcentaje de células apoptóticas. Todos estos efectos concuerdan con la posterior resistencia a la insulina reflejada por el descenso de la fosforilación de la mayoría de las proteínas de su ruta de señalización. En

hepatocitos inmortalizados de ratón se obtuvieron resultados similares. Sin embargo, en el caso del tratamiento de los hepatocitos con AO no se observó ni estrés de RE ni resistencia a la insulina, y además, tras el cotratamiento con AP, era capaz de prevenir los efectos perjudiciales de éste.

Intentando averiguar cuál podría ser el mecanismo molecular por el cual el AO actúa inhibiendo los efectos deletéreos del AP en los hepatocitos, encontramos que la proteína quinasa S6K1, diana del complejo mTORC1, se activaba tras el tratamiento de los hepatocitos con AP y curiosamente su fosforilación disminuía cuando se trataba a las células con ambos ácidos grasos. Además, esta protección del AO frente a la lipoapoptosis y la resistencia a insulina inducidas por el AP también fue emulada inhibiendo la S6K1 mediante procedimientos farmacológicos o genéticos.

En resumen, de este primer trabajo se puede concluir que la activación de la proteína S6K1 por AP es un mecanismo común por el cual se induce estrés del RE y lipoapoptosis en los hepatocitos, y que la inhibición de esta quinasa por el AO previene de la lipoapoptosis y la resistencia a la insulina en hepatocitos.

Estos resultados han dado lugar al artículo titulado: **“Oleic acid protects against endoplasmic reticulum stress-mediated lipoapoptosis and insulin resistance induced by palmitic acid in hepatocytes: role of S6K1”** el cual ha sido enviado a la revista “***The Journal of Nutritional Biochemistry”.***

OLEIC ACID PROTECTS AGAINST ENDOPLASMIC RETICULUM STRESS-MEDIATED LIPOAPOPTOSIS AND INSULIN RESISTANCE INDUCED BY PALMITIC ACID IN HEPATOCYTES: ROLE OF S6K1

RUNNING TITLE: Oleic acid protects against ER-stress mediated lipoapoptosis and insulin resistance in hepatocytes

Virginia Pardo[1,2], Águeda González-Rodríguez[1,2,*], Jordi Muntané[3,4], Sara C Kozma[5] and Ángela M. Valverde[1,2,*]

[1]Instituto de Investigaciones Biomédicas Alberto Sols (CSIC/UAM), 28029 Madrid, Spain. [2]Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), ISCIII, Spain. [3]Oncology Surgery, Cell Therapy and Transplant Organs, Institute of Biomedicine of Seville (IBiS)/Virgen del Rocio Universitary Hospital/CSIC/University of Seville, Seville, Spain. [4]Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Instituto de Salud Carlos III, Spain.[5]

[*]Corresponding authors:

Águeda González-Rodríguez and Ángela M. Valverde. Instituto de Investigaciones Biomédicas Alberto Sols (CSIC/UAM). C/ Arturo Duperier 4. 28029 Madrid. Spain. Tel 34-91 5854497; Fax 34-915854401.

Emails: aguedagr@iib.uam.es and avalverde@iib.uam.es.

Keywords. Insulin signaling; lipoapoptosis; hepatocytes; free fatty acids; ER stress.

ABSTRACT

The excess of saturated free fatty acids, such as palmitic acid, that induces lipotoxicity in hepatocytes, has been implicated in the development of non-alcoholic fatty liver disease also associated with insulin resistance. On the other hand, oleic acid, a monounsaturated fatty acid, attenuates the effects of palmitic acid. We have evaluated whether palmitic acid is directly associated with both insulin resistance and lipoapoptosis in mouse and human hepatocytes and the impact of oleic acid in the molecular mechanisms that mediate both processes. In human primary hepatocytes palmitic acid at a lipotoxic concentration triggered early activation of endoplasmic reticulum (ER) stress-related kinases (JNK, PERK, eIF2α), induced the apoptotic transcription factor CHOP, activated caspase 3 and increased the percentage of apoptotic cells. Similar results were found in immortalized mouse hepatocytes. In both cellular models, these effects concurred with decreased IR/IRS1/Akt insulin pathway. Neither ER stress nor insulin signaling were directly modulated by oleic acid but, importantly, it suppressed the deleterious effects of palmitic acid on both ER stress activation and induction of apoptotic markers, and prevented the impairment of insulin signaling. Moreover, oleic acid suppressed palmitic acid-induced activation of S6K1, a novel regulator of ER stress-mediated lipoapoptosis. This protection was mimicked by pharmacological or genetic inhibition of S6K1 in hepatocytes. In conclusion, this is the first study highlighting ER stress-mediated signaling and the activation of S6K1 by palmitic acid as a common mechanism by which its inhibition by oleic acid prevents both lipoapoptosis and insulin resistance in hepatocytes.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is an emergent public health problem since is the most common cause of chronic liver disease in Western countries. NAFLD is strongly associated with overweight/obesity, insulin resistance, type 2 diabetes (T2DM) and cardiovascular complications; therefore, it is considered the hepatic manifestation of the metabolic syndrome [1-3]. NAFLD is characterized by the accumulation of ectopic fat in the liver triggering firstly in a benign steatosis. However, 10% of NAFLD patients develop more severe liver injuries such as non-alcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma [4]. In fact, elevated levels of circulating free fatty acids (FFAs) are related with the progression of NAFLD and the pathogenesis of the insulin resistance [5, 6]. The excess of circulating FFAs is particularly harmful for the liver since saturated FFAs induce apoptosis in hepatocytes, a phenomenon known as lipoapoptosis [7]. Indeed, lipoapoptosis is increased in correlation with the severity of NAFLD [8, 9].

Different mechanisms contribute to hepatocyte dysfunction such as endoplasmic reticulum (ER) stress, oxidative stress and metabolic alterations [10, 11]. Among them, ER stress is activated when the unfolded protein response (UPR) cannot re-establish the homeostasis in the ER. ER stress signaling involves three main proteins, X-box binding protein 1 (X-BP1), PRKR-like endoplasmic reticulum kinase (PERK) and activating transcription factor-6 (ATF6), which cooperate to alleviate ER stress. These proteins remain inactive while sensors are bound to the intraluminal chaperone glucose-regulated protein 78 (GRP78). If the cell fails to adapt, oxidative and inflammatory signaling pathways are triggered leading to apoptosis through the expression of PERK-mediated C/EBP homologous protein (CHOP), IRE1α-mediated recruitment of tumor necrosis factor receptor associated factor 2 (TRAF2) and signal regulated kinase 1 (ASK1)/C-jun N

terminal kinase (JNK) [12]. In fact, the activation of JNK has been revealed as a central mediator of FFA-induced hepatic lipoapoptosis [13, 14].

Activation of ER stress signaling induced by FFAs plays also an important role in the hepatic insulin resistance associated to the progression of NAFLD [10]. It has been demonstrated that chemical chaperones (tauroursodeoxycholic acid (TUDCA) and 4-phenylbutryate), which mitigate ER stress through protein stabilization, improve systemic glucose homeostasis [15]. Moreover, obese subjects treated with TUDCA showed increased insulin sensitivity in liver and skeletal muscle by approximately 30% compared to those receiving placebo therapies [16].

At a molecular level, FFAs can impair insulin action through the down-regulation of early mediators of the insulin signaling cascade such as the insulin receptor (IR) or insulin receptor substrate 1 (IRS1). In this regard, it has been demonstrated that ER stress induces IR down-regulation and dysfunction and contributes to obesity-induced insulin resistance in peripheral tissues [17]. Particularly, palmitate, a representative saturated FFA, inhibits insulin signaling acting at the level of IR expression in two well-differentiated hepatocyte cell lines [18]. Moreover, after saturated FFA challenge in hepatic cells, the activation of stress kinases such as JNK, ribosomal protein S6 kinase 1 (S6K1), IκB kinase (IKK) and also protein kinase C (PKC) increases serine/threonine phosphorylation of IRS1, reducing its tyrosine (stimulatory) phosphorylation and targets it for proteosomal degradation [19-22].

The most abundant FFAs present in the diet and in serum are palmitic acid (PA), a saturated FA, and oleic acid (OA), a monounsaturated one [23]. Some studies have demonstrated that monounsaturated FFAs are less toxic and attenuate palmitate-induced

toxicity in hepatocytes [24, 25]. Moreover, it has been demonstrated that oleic acid improves insulin sensitivity in human diet treatments [26]. However, the molecular mechanism implicated in the protective role that oleic acid elicits in the liver remains unclear. Taking together, in this study we have investigated the effects of PA and OA on lipotoxicity and insulin signaling in hepatocytes elucidating which mechanisms are involved.

MATERIALS AND METHODS

Reagents. Fetal bovine serum (FBS) (#10270), culture media DMEM (#41966-029) and 4, 6-diamidino-2-phenylindole (DAPI) (D1306) were obtained from Invitrogen (Gran Island, NY). Palmitic acid (P0500), oleic acid (O1008), bovine serum albumin (BSA) (A6003), rapamycin (37094), tunicamycin (T7765), puromycin (8833), polybrene (S2667) and insulin (I0516) were from Sigma Aldrich (St. Louis, MO). Bradford reagent, acrylamide, immunobloting PVDF membrane and Immobilon Western Chemiluminescent HRP Substrate were purchased from Bio-Rad (Hercules, CA).

Cell culture. Human hepatocytes were isolated by the two-step collagenase procedure from non-tumour areas of liver biopsies from patients submitted to a surgical resection for liver tumours after obtaining patients' written consent [27]. The generation and characterization of immortalized mouse hepatocyte cell line has been previously described [28]. Briefly, pooled (4-6) livers from wild-type and S6K1-deficient neonates (3.5-4 days old) were digested and hepatocytes were isolated. Viral Bosc-23 packaging cells were transfected at 70% confluence with 3 μg/6 cm-dish of the puromycin-resistance retroviral vector pBabe encoding SV40 Large T antigen (provided by J. de Caprio, Dana Farber Cancer Institute, Boston, MA). Neonatal hepatocytes (60% confluence) were infected with polybrene (4 μg/ml)-supplemented virus and maintained in culture medium for 72 h before selection with puromycin (1 μg/ml) for 10-15 days. Pools of infected cells rather than individual clones were selected to avoid potential clone-to-clone variations. These pools of immortalized neonatal hepatocytes were further cultured with arginine-free medium supplemented with 10% FBS for at least 2 weeks to avoid growth of non-parenchymal cells. Cells were grown in DMEM plus 10% heat inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine.

Cell treatments. Palmitic acid (PA), that was dissolved in isopropyl alcohol at a stock concentration of 80 mM, and oleic acid (OA), that was dissolved in chloroform at a stock concentration of 3540 mM, were added to DMEM containing 5% FBS and 1% bovine serum albumin to assure a physiological ratio between bound and unbound FFA in the medium [29]. The concentration of PA used in the experiments was similar to the fasting PA plasma concentrations observed in human non-alcoholic steatohepatitis [30, 31] and has been used before for mimicking hepatic lipoapoptosis [32]. Confluent cells were treated with PA (200-800 μM) and/or OA (400 μM) for the indicated time periods.

Preparation of protein extracts and Western blot. To obtain total cell lysates, attached cells were scraped off and incubated for 10 min on ice with lysis buffer (25 mM HEPES, 2.5 nM EDTA, 0.1% Triton X-100, 1 mM PMSF and 5 μg/ml leupeptin). After protein content determination with Bradford reagent, total protein were boiled in Laemmli sample buffer and submitted to 8-15% SDS-PAGE. Proteins were transferred to Immunoblot PVDF membrane and, after blocking with 3% BSA or 5% non-fat dry milk, membranes were incubated overnight with several antibodies as indicated. Immunoreactive bands were visualized using the ECL Western blotting protocol. Densitometric analysis of the bands was performed using Image J software. The anti-phospho-PERK (Thr 980) (#3179), anti-phospho-eIF2α (Ser 51) (#9721), anti-phospho-JNK (#9251), anti-phospho-STAT3 (#9131), anti-phospho-MAPK (#9101), anti-S6K1 (#9202), anti-active caspase 3 (#9661) and anti-MAPK (#4695) antibodies were from Cell Signaling Technology (Danvers, MA). The anti-JNK (sc-571), anti-phospho-S6K1 (Thr 389) (sc-11759) anti-IRβ (sc-711), anti-PERK (sc-13073), anti-eIF2α (sc-11386), anti-GRP78 (sc-376768), anti-CHOP (sc-7351), anti-phospho-p38 (sc-17852), anti-p38 (sc-9212), anti-IRS1 (sc-559), anti-IRS2 (sc-8299), anti-phospho-IR (sc-25103), anti-phospho-AKT (Thr 308) (sc-

16646), anti-phospho-AKT (Ser 473) (sc-7985) and anti-AKT (sc-8312) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-α-tubulin (T5168) antibody (A5441) was from Sigma Aldrich (St. Louis, MO).

Quantification of Apoptosis. Cells were grown in glass coverslips and treated as described above. Then, cells were washed twice with PBS, fixed in p-formaldehyde (4%) for 10 min and characteristic morphological changes of apoptosis were assessed by staining nuclei with DAPI followed by the analysis by fluorescence microscopy.

Analysis of caspase 3 activity. Cells were scraped off, collected by centrifugation at 2.500 x g for 5 minutes and lysed at 4ºC in 5 mM Tris/HCl pH 8, 20 mM EDTA, 0.5% Triton X-100. Lysates were clarified by centrifugation at 13.000 x g for 10 min. Reaction mixture contained 25 µl cellular lysates, 325 µl assay buffer (20 mM HEPES pH 7.5, 10% glycerol, 2 mM dithiothreitol) and 20 µM caspase 3 substrate (Ac-DEVD-AMC). After 2 h incubation in the dark, enzymatic activity was measured in a luminiscence spectrophotometer (Perkin Elmer LS-50, Norwalk, CT) (λ excitation, 380 nm; λ emission, 440 nm).

Statistical analysis. Data are presented as mean ± SEM, and were compared by using the Bonferroni *ANOVA* test. All statistical analyses were performed using the IBM SPSS Statistics 21.0 (SPSS Inc. IBM, Armonk, NY) software with 2-sided tests. Differences were considered statistically significant at $p<0.05$.

RESULTS

Palmitic acid induces lipoapoptosis and insulin resistance in immortalized mouse hepatocytes.

In order to elucidate the molecular mechanisms involved in the lipotoxicity induced by FFAs in the liver, we assessed whether both primary human hepatocytes and immortalized mouse hepatocytes, generated in our laboratory from wild-type 3-5 day-old wild-type mice [28], exhibited the same responses to palmitic acid (PA), a saturated FFA that is elevated in obesity [33]. For this goal, we first treated primary human hepatocytes with 600 μM PA, dose at which lipoapoptosis was previously reported [13], for 8 h. As depicted in Figure 1A, the phosphorylation of PERK, eiF2α and JNK was enhanced in PA-treated human primary hepatocytes as compared to control cells (treated with vehicle). After 24 h, PA increased expression of CHOP, an ER stress marker, and induced the activation of caspase 3, detected by the presence of the active fragment (Figure 1B). Moreover, the analysis of nuclear morphology by fluorescence microscopy following DAPI staining showed an increase in the incidence of condensed and/or fragmented nuclei (Figure 1C). Altogether, these results demonstrate that human primary hepatocytes are sensitive to lipotoxic doses of PA. Next, we assessed these responses in immortalized mouse hepatocytes treated with PA at different concentrations (200-800 μM). In this cellular system, maximal phosphorylation of JNK, the induction of CHOP and the activation of caspase 3 was detected at 600 μM concentration (Figure 2A).

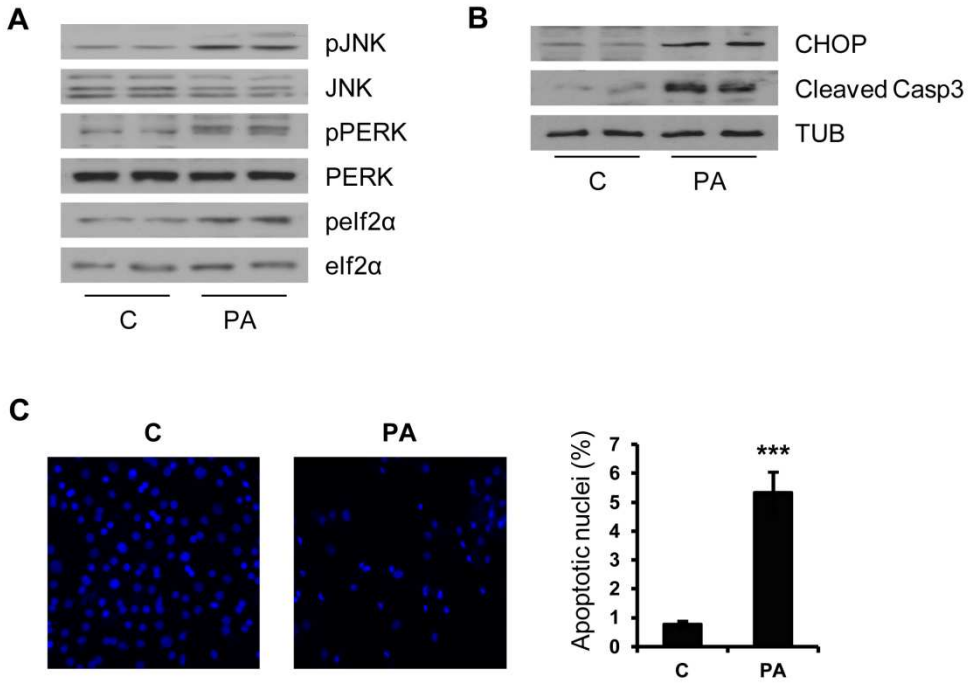

Figure 1. Palmitic acid (PA) induces ER stress and lipoapoptosis in primary human hepatocytes. Primary human hepatocytes were treated with 600 µM PA for several time periods (A, 8 h; B and C, 24 h). **A, B.** Total protein was analyzed by Western blot using the indicated antibodies. Representative blots are shown (n=3). **C.** Representative nuclear morphology images after staining of DNA with DAPI. After quantification of apoptotic nuclei from all images, results are expressed as % of number of apoptotic nuclei and are mean ± SEM (n=3), ***p<0.001, PA vs C.

Next, we characterized the PA-mediated lipoapoptosis in immortalized mouse hepatocytes at the molecular levels since this cell line was not previously used for *in vitro* studies of lipotoxicity. We already detected the phosphorylation of kinases related to ER stress (PERK and eiF2α) and inflammation-linked stress kinases (STAT3, JNK, p38 MAPK and ERK) in hepatocytes treated with PA for only 6 h (Figure 2B). Notably, activation of these kinases was not detected at early time-periods (results not shown). After 16 h, PA triggered an elevation of CHOP expression and cleavage of caspase 3, which correlated with increased caspase 3 enzymatic activity (Figure 2C). In the light of this, phase-contrast microscopy revealed many hepatocytes with morphological characteristics of apoptotic cells after 16 h of PA treatment (Figure 2D).

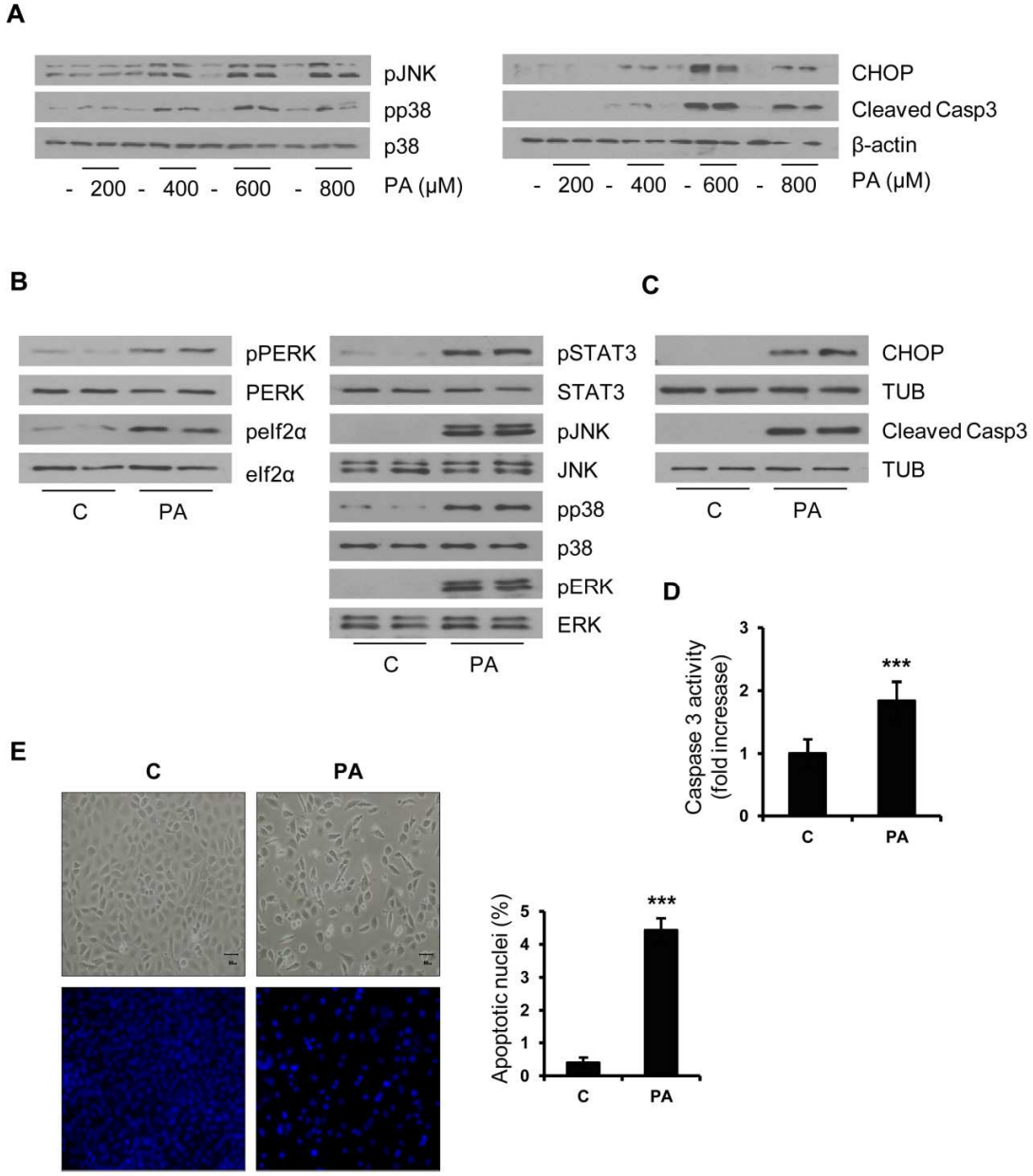

Figure 2. Palmitic acid (PA) triggers ER stress and lipoapoptosis in immortalized mouse hepatocytes. **A.** Immortalized mouse hepatocytes were treated with 200-800 μM PA for several time periods (left panel, 6 h; right panel, 16 h). Representative blots are shown (n=3). **B, C.** Immortalized mouse hepatocytes were treated with 600 μM PA for several time periods (B, 6 h; C, 16 h). Representative blots are shown (n≥4). **D.** Analysis of caspase-3 enzymatic activity. Results are expressed as fold increase relative to C condition (1) and are mean ± SEM (n=3), ***p<0.001. **E.** *(upper panel)* Representative phase-contrast microscopy images. *(lower panel)* Representative nuclear morphology images after staining of DNA with DAPI. After quantification of apoptotic nuclei from all images, results are expressed as % of number of apoptotic nuclei and are mean ± SEM (n=3), ***p<0.001, PA vs C.

Activation of ER stress has been shown to interfere with the early steps of insulin signaling by multiple feed-back mechanisms [10]. On that basis, we explored if the impact of PA on the insulin signaling cascade was similar in primary human hepatocytes and in our immortalized mouse cell line. For this goal, primary human and immortalized mouse hepatocytes were pretreated with 600 μM PA for 24 and 16 h, respectively, and then, stimulated with insulin for a further 10 min. As described in Figure 3, insulin-induced IR tyrosine phosphorylation was reduced in both kinds of hepatocytes pretreated with PA compared to hepatocytes treated with vehicle. Likewise, PA induced IR and IRS1 degradation and decreased Akt phosphorylation at both catalytic (Thr308) and regulatory (Ser473) phosphorylation sites. Notably, IRS2 levels were not affected after PA treatment. Altogether, these results indicate that immortalized neonatal hepatocytes are a suitable cellular model to study the responses to PA overload since they recapitulate all of the qualitative features of lipotoxicity and insulin resistance observed in primary human hepatocytes.

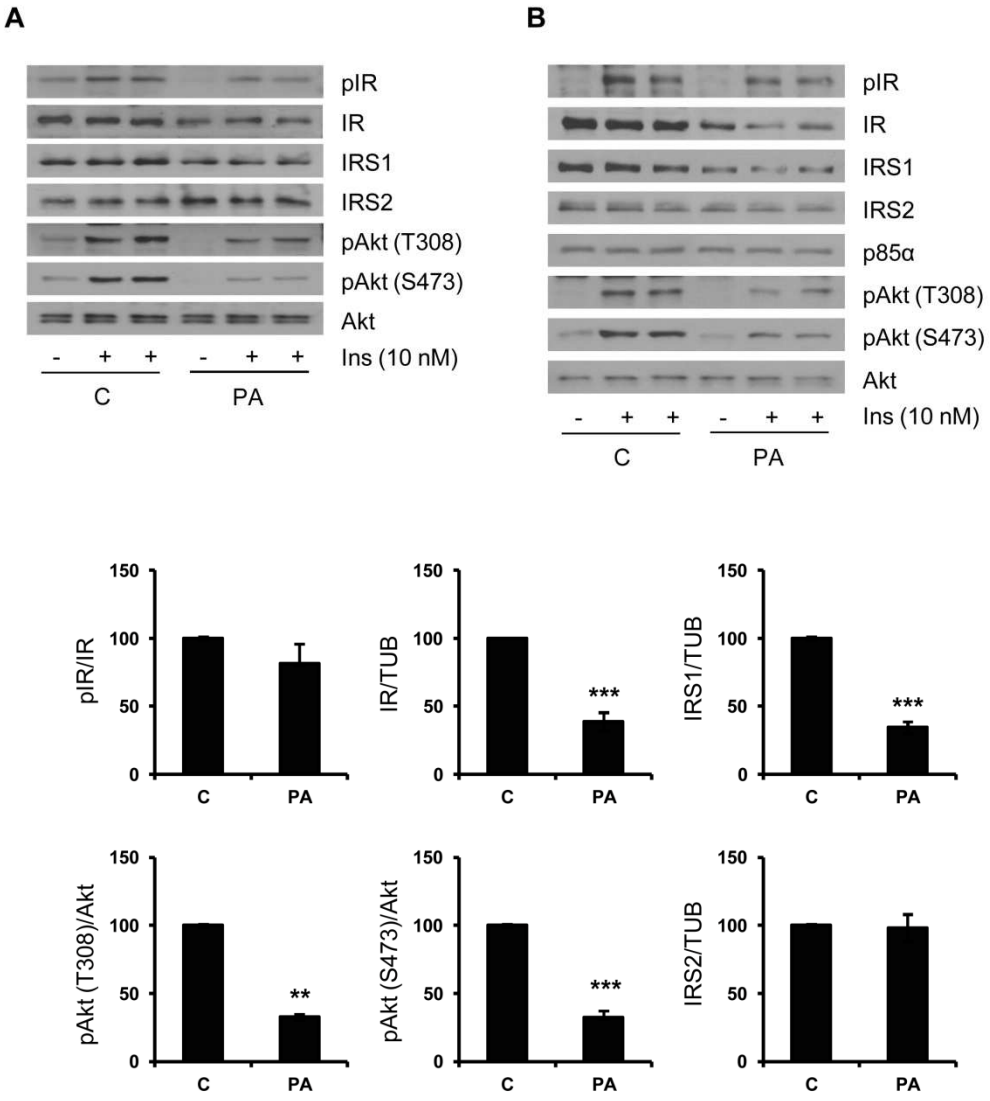

Figure 3. Palmitic acid (PA) induces insulin resistance in primary human and immortalized mouse hepatocytes. **A**. Primary human hepatocytes were treated with 600 µM PA for 16 h, followed by serum starvation (2 h) and insulin stimulation (10 min, 10 nM). Total protein was analyzed by Western blot using the indicated antibodies. Representative blots are shown (n=3). **B.** Immortalized mouse hepatocytes were treated with 600 µM PA for 16 h, followed by serum starvation (2 h) and insulin stimulation (10 min, 10 nM). After quantification of all blots, results are expressed as % of insulin stimulation or % of protein expression relative to the C condition (100%) and are mean ± SEM (n≥6), **p<0.01, ***p<0.001, PA vs C.

Oleic acid prevents ER stress and lipoapoptosis induced by palmitic acid in immortalized neonatal hepatocytes.

It is well known that the two most abundant dietary fatty acids, oleic acid (OA) and PA, play a different role in hepatic metabolism [24, 34, 35]. This prompted us to investigate whether OA was able to prevent PA-induced ER stress and lipoapoptosis. First, we evaluated the differential effects of OA and PA on ER stress. Of note, OA itself did not induce the activation of ER stress-mediated signaling in hepatocytes (Figure 4A). Interestingly, co-treatment with 400 μM OA was sufficient to abolish PA-induced PERK and eiF2α phosphorylation and also significantly blocked JNK and p38 MAPK phosphorylation (Figure 4A). Moreover, co-treatment of PA and OA prevented the increase of CHOP expression and the accumulation of the active fragment of caspase 3 (Figure 4B).

Next, we investigated the role of OA in PA-induced lipoapoptosis in hepatocytes. Phase-contrast microscopy revealed that OA did not elicit any effect on cell morphology. Moreover, after 16 h of co-treatment with PA and OA, most of the immortalized hepatocytes remained intact whereas as stated above, hepatocytes treated with PA alone displayed an apoptotic phenotype (Figure 4C). These results strongly suggest that OA elicits a protective effect against lipoapoptosis induced by PA.

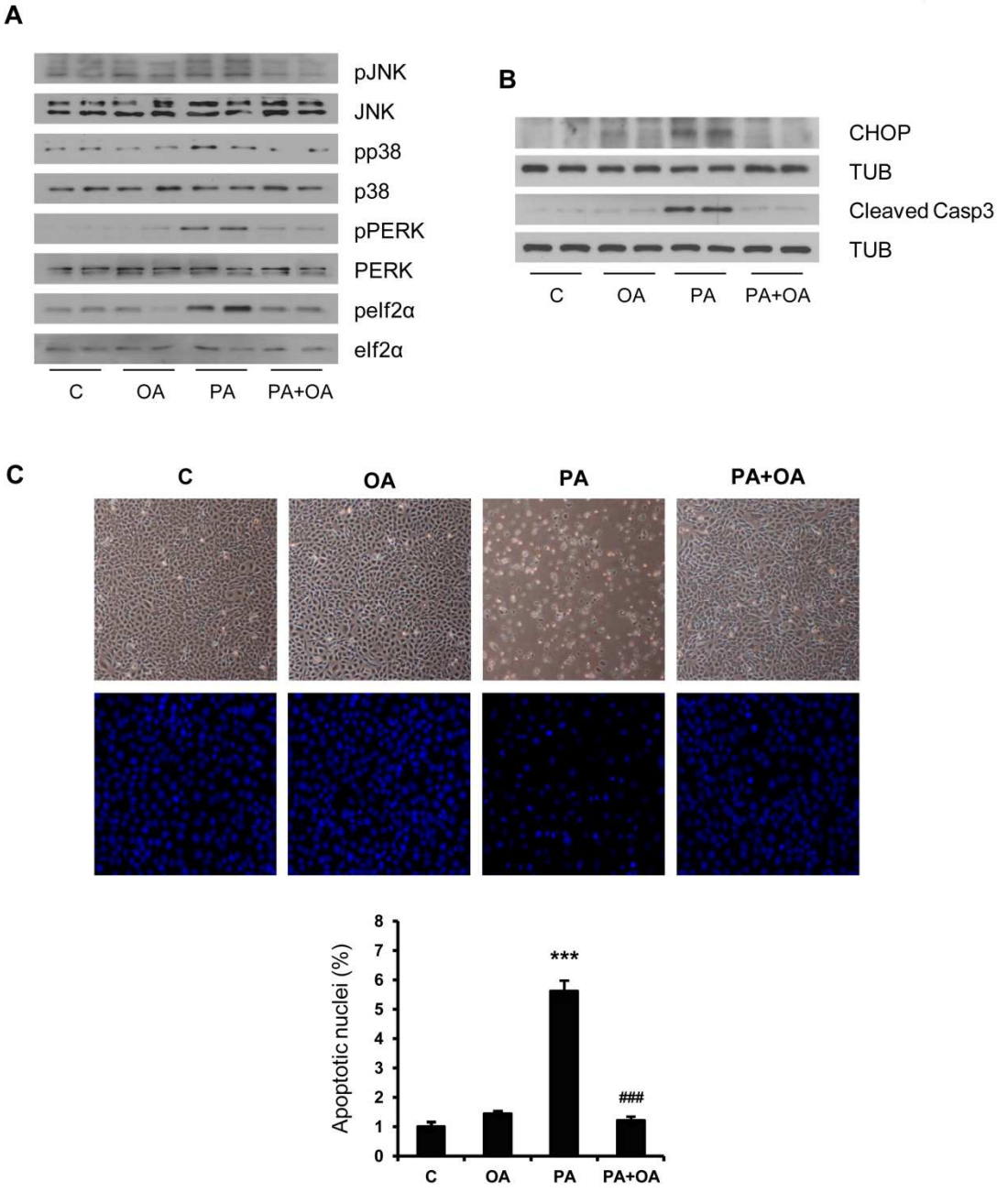

Figure 4. Oleic acid (OA) prevents ER stress and lipoapoptosis induced by palmitic acid (PA) in immortalized mouse hepatocytes. Immortalized mouse hepatocytes were treated with 600 µM PA, 400 µM OA and 600 µM PA plus 400 µM OA for 6 h (**A**) and 16 h (**B, C**). **A. B.** Total protein was analyzed by Western blot using the indicated antibodies. Representative blots are shown (n≥3). **C.** *(upper panel)* Representative phase-contrast microscopy images. *(lower panel)* Representative nuclear morphology images after staining of DNA with DAPI. After quantification of apoptotic nuclei from all DAPI staining images, results are expressed as % of number of apoptotic nuclei and are mean ± SEM (n≥4), ***p<0.001, PA vs C; $^{###}$p<0.001, PA+OA vs PA.

Insulin resistance induced by palmitic acid is prevented by oleic acid in immortalized neonatal hepatocytes.

Regarding insulin sensitivity, we investigated whether OA protects against the inhibitory effects of PA. As observed in Figure 5A, OA alone did not affect insulin-induced IR and Akt phosphorylation in immortalized neonatal hepatocytes compared to cells treated with vehicle. Next, hepatocytes were incubated with PA in the presence or absence of OA before insulin stimulation. As depicted in Figure 5B, both IR and Akt phosphorylation were recovered in hepatocytes incubated with PA plus OA as compared to cells treated exclusively with PA. In fact, co-treatment with PA and OA induced similar phosphorylation levels to those detected in control hepatocytes treated with vehicle. Moreover, after co-treatment with PA and OA, the expression of IR and IRS1 was maintained at similar levels of those detected in the control condition. Altogether, these results indicate that OA enhanced insulin signaling by relieving PA-induced ER stress.

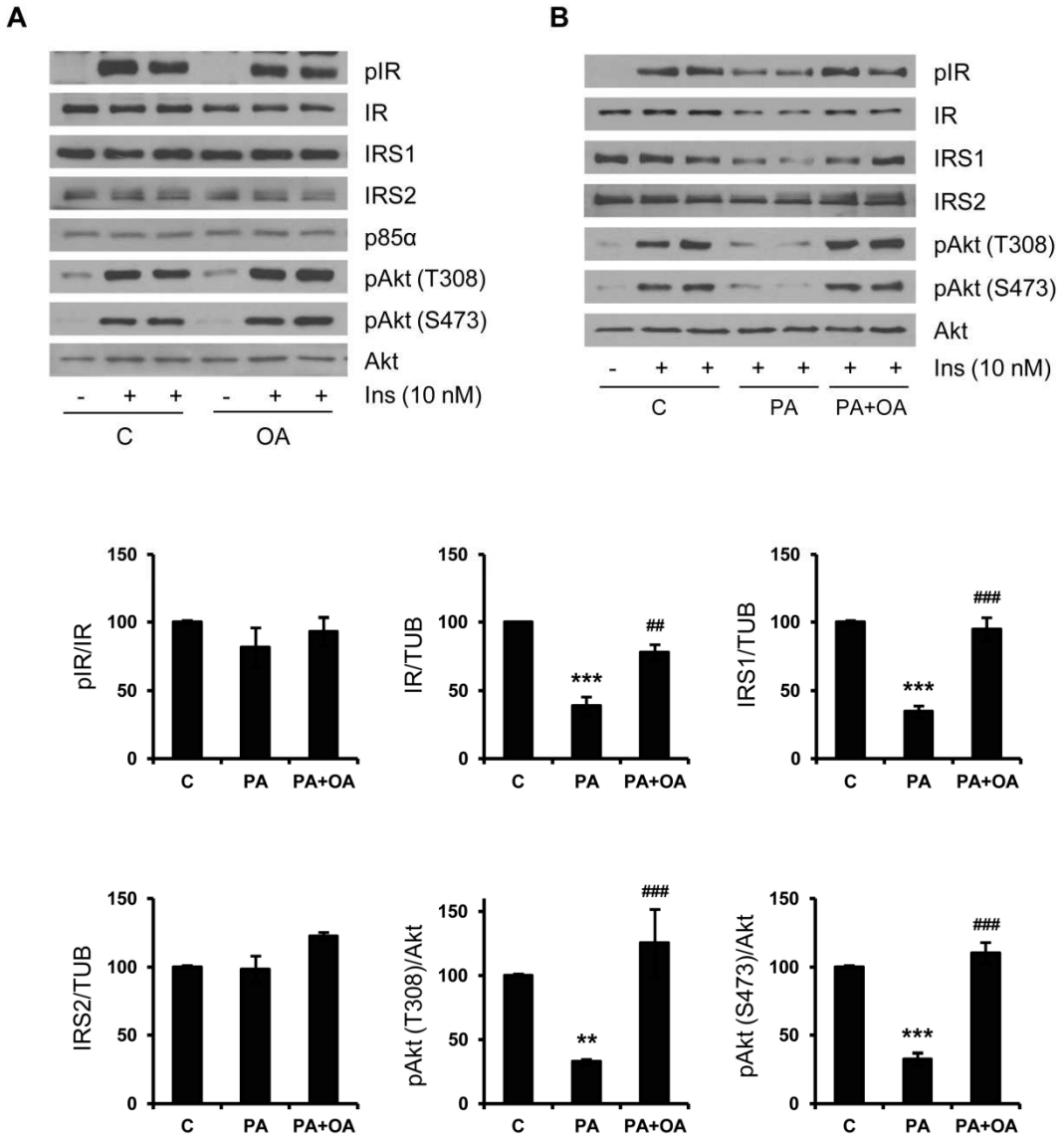

Figure 5. Insulin resistance induced by palmitic acid (PA) is prevented by oleic acid (OA) in immortalized mouse hepatocytes. Immortalized mouse hepatocytes were treated with 400 μM OA (**A**), with 600 μM PA and 600 μM PA plus 400 μM OA (**B**) for 16 h, followed by serum starvation (2 h) and insulin stimulation (10 min, 10 nM). Total protein was analyzed by Western blot using the indicated antibodies. Representative blots are shown. After quantification of all blots, results are expressed as % of insulin stimulation or % of protein expression relative to the C condition (100%) and are mean ± SEM (n≥3), **p<0.01, ***p<0.001, PA vs C; $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001, PA+OA vs PA.

S6K1 inhibition reduces ER stress induced by palmitic acid in immortalized neonatal hepatocytes.

In addition to stress kinases such as JNK and p38 MAPK, S6K1, an mTORC1 target, also modulates insulin signaling after saturated FFA challenge in hepatic cells by increasing serine phosphorylation of IRS1 and targeting it for proteosomal degradation [19]. Interestingly, phosphorylation of S6K1 was detected in hepatocytes treated with PA for 6 h and this event was prevented in the presence of OA (Figure 6A), suggesting that S6K1 might be involved in PA-induced lipoapoptosis and insulin resistance in immortalized neonatal hepatocytes.

To delineate the role of S6K1 in hepatic lipoapoptosis, we used two different cellular models of S6K1 inhibition and checked levels of CHOP and active caspase 3 as markers of ER stress and apoptosis, respectively. Firstly, we treated immortalized hepatocytes with PA combined with rapamycin, a well-known inhibitor of mTORC1/S6K1 signaling pathway. As it was expected, rapamycin inhibited S6K1 phosphorylation (Figure 6B). Regarding lipotoxicity, PA treatment increased CHOP expression and the accumulation of the active fragment of caspase 3 in hepatocytes compared to the untreated controls. Co-treatment with PA and rapamycin (25 ng/ml) significantly reduced these effects induced by PA. Next, we confirmed these data using an immortalized neonatal hepatocyte cell line with genetic ablation of S6K1 generated from livers of neonatal S6K1-deficient mice. We have previously used this cellular model to investigate whether S6K1 deficiency alters the susceptibility of hepatocytes to undergo apoptosis apoptosis induced by death receptor activation or trophic factors withdrawal [28]. After 16 h of PA treatment, CHOP expression was reduced in S6K1-deficient hepatocytes compared to control cells. Moreover, cleavage of caspase 3 was increased in immortalized neonatal hepatocytes after

PA challenge, but not in those lacking S6K1 (Figure 6C). These data suggest that PA-induced ER stress is suppressed in S6K1-deficient hepatocytes. In fact, S6K1 deficiency protects against ER stress in response to tunicamycin, a classical ER stress-inducing agent. Figure 6D shows that both CHOP and active caspase 3 were almost undetectable in S6K1-deficient hepatocytes after 16 h of tunicamycin (10 μg/ml) treatment compared to control cells. Taking together, these results indicate that inhibition of S6K1 confers protection against ER stress in hepatocytes regardless of the ER stressor.

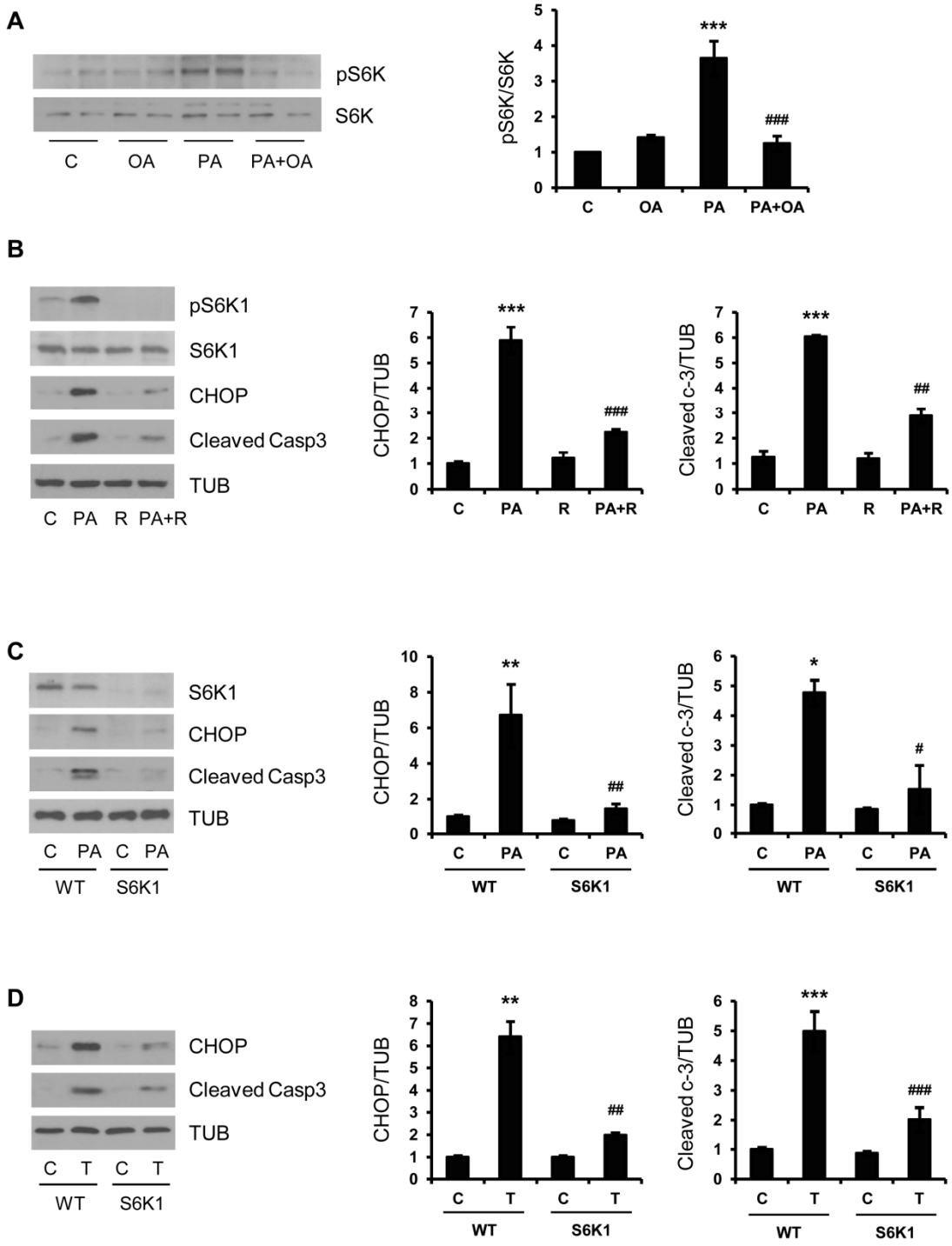

Figure 6. S6K1 inhibition reduced ER stress produced by PA in immortalized mouse hepatocytes. Immortalized mouse hepatocytes were treated with 400 µM OA, 600 µM PA and 400 µM OA plus 600 µM PA for 6 h (**A**) or co-treated with 600 µM PA and rapamycin (R, 25 ng/ml) for 16 h (**B**). Wild-type (WT) and S6K1-deficient (S6K1) immortalized mouse hepatocytes were treated with 600 µM PA (**C**) and with tunicamycin (T, 10 µg/ml) (**D**) for 16 h. Total protein was analyzed by Western blot using the indicated antibodies. Representative blots are shown. After quantification of all blots, results are expressed as % of protein expression relative to the C condition (100%) and are mean ± SEM (n=3), *p<0.05, **p<0.01, ***p<0.001, PA vs C and T vs C; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001, PA+R vs PA and S6K1 vs WT.

DISCUSSION

The excess of circulating FFAs is related with the progression of NAFLD and the pathogenesis of insulin resistance [5, 6]. As OA and PA are the most abundant FFAs present in the diet and serum and, on the other hand, OA and PA play different roles in hepatic metabolism, in this study we have investigated the effects of these two FFAs on lipotoxicity and insulin signaling in hepatocytes elucidating which mechanisms are involved in both processes.

Firstly, we have demonstrated that immortalized neonatal hepatocytes are a suitable cellular model to study steatogenesis since their response to PA overload is similar to that observed in primary human hepatocytes; both type of hepatic cells exhibited significant lipoapoptosis and insulin resistance. Consequently, immortalized neonatal hepatocyte cell line has allowed us to perform this study without the experimental limitations of the human primary hepatocytes.

Next, we explored the effects of treatment with OA, PA or their combination on ER stress, lipoapoptosis and insulin signaling. Our data showed that while OA itself neither activated ER stress nor affected cell viability in immortalized neonatal hepatocytes, PA triggered ER stress-mediated signaling leading to lipoapoptosis. It has been demonstrated that several molecular mechanisms are involved in PA-associated apoptosis such as ER stress, reactive species generation, ceramide production, nuclear factor-kB activation, etc [7, 36]. Among them, perturbed ER homeostasis has been postulated as a major initiator of lipid-induced toxicity [37]. In this regard, CHOP is a transcription factor that is strongly expressed in response to ER stress and plays an important role in ER stress-induced apoptosis through the activation of both intrinsic and extrinsic classical pathways of

apoptosis [38]. Moreover, the reduction of CHOP expression by genetic ablation or by siRNA approaches decreases hepatocyte toxicity triggered by PA [39] [40]. Taking all these findings together, CHOP can be considered a key marker of ER-stress-induced lipotoxicity in hepatic cells. In the same way, activation of the effector caspase 3 is a hallmark of hepatic lipoapoptosis since this effect is markedly reduced by caspase inhibitors [13, 41-43]. On that basis, in this study caspase 3 has been analyzed as a molecular marker to monitor the apoptotic process.

Regarding insulin effects, PA, but not OA, impaired insulin signaling cascade in primary human and immortalized neonatal hepatocytes, suggesting that PA-induced ER stress may mediate insulin resistance. ER stress has been involved in the association between inflammation and insulin resistance produced by saturated FFAs [12]. In liver cells, attenuation of ER stress using chemical chaperones protected against PA-induced impairment of insulin signaling [44]. To support this, metformin also significantly inhibited lipoapoptosis triggered by PA in HepG2 hepatic cells by blocking the induction of ER stress and, as a result, insulin signaling was restored [45]. Interestingly, our results in immortalized hepatocytes have demonstrated that OA elicits a protective effect on ER-stress activated by PA and, as a consequence, ameliorates the lipoapoptosis. Concerning insulin responses, OA prevented the blockade of the early events of the insulin signaling cascade by PA, maintaining the response to insulin in inducing IR and Akt phosphorylation. It has been reported that OA prevents PA-induced ER stress and insulin resistance in skeletal muscle [46, 47]. Previously, Ricchi et al. [24] described that apoptosis and impaired insulin signaling were lower in hepatic cells after combined treatment of OA and PA compared to those treated only with PA but these authors did not elucidate the molecular mechanisms involved. In the light of these previous studies, this is

the first time that a study involves ER stress-mediated signaling as a common mechanism by which its inhibition by OA prevents both PA-induced lipoapoptosis and insulin resistance in hepatocytes.

Activation of mTORC1 by PA induces insulin resistance in C2C12 myotubes [48]. In hepatocytes, we and others have shown that downstream of mTORC1, its target S6K1 is phosphorylated at Thr389 in response to PA [19, 40]. These *in vitro* findings have relevance in the development of insulin resistance *in vivo* since phosphorylation of S6K1 is elevated in the liver of *ob/ob* mice and, on the other hand, global or liver-specific deletion of S6K1 in mice protects against the attenuation of insulin signaling in the liver in a model of diet-induced obesity. At the molecular level, this protection was due to the suppression of a negative feed-back loop elicited by S6K1 on IRS1 Ser307 phosphorylation thereby blocking its activation by the IR [49, 50]. This molecular mechanism was also reported by our group in a model of apoptosis induced by trophic factors withdrawal in hepatocytes [28]. In addition to these previous findings, in this study we have demonstrated for the first time that OA prevents PA-induced S6K1 phosphorylation, suggesting that targeting S6K1 is an attractive strategy against insulin resistance and ER-mediated lipoapoptosis. In this regard, our results clearly show that the inhibition of S6K1 by both pharmacological (rapamycin) or genetic (S6K1 deletion) approaches mimicked the effects of oleic acid since both strategies reduced both CHOP levels and the activation of caspase 3 in response to PA treatment. Likewise, S6K1 deficiency blocked the effects of tunicamycin, a classical ER stressor. This is the first study in which S6K1 is directly linked with the lipoapotosis induced by persistent ER stress in hepatocytes. Previously, Gores and co-workers reported that silencing JNK1 in hepatocytes, that also elicits a feed-back on insulin signaling [51],

inhibits PA-induced lipoapoptosis by a mechanism involving the proapoptotic proteins PUMA and Bax [13].

In conclusion, our results have elucidated the molecular interplay between signaling pathways triggered by persistent ER stress, insulin resistance and lipoapoptosis in hepatocytes treated with PA. All these deleterious effects were prevented by OA, including the activation of S6K1 which for the first time has been implicated in lipoapoptosis induced by persistent ER stress in hepatic cells.

Acknowledgements

This work was supported by grants SAF2012-33283, (Ministerio de Economía y Competitividad, Spain), Comunidad de Madrid S2010/BMD-2423 (Spain), EFSD and Amylin Paul Langerhans Grant, Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM, Instituto de Salud Carlos III, Spain). A. G-R receives support from CIBERDEM.

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Este primer estudio se ha centrado en describir los efectos que desencadenan los dos FFAs mayoritarios de la dieta y la circulación sanguínea (AO y AP) sobre la apoptosis y la señalización de la insulina en los hepatocitos con objeto de elucidar los mecanismos moleculares implicados en ambos procesos.

En primer lugar, se ha demostrado que los hepatocitos inmortalizados neonatales se pueden utilizar como un modelo adecuado de esteatogénesis, ya que su respuesta a una sobrecarga lipídica producida por el AP es similar a la observada en hepatocitos primarios humanos, de esta forma se evitan las limitaciones experimentales de los hepatocitos primarios humanos.

A continuación, se exploraron los efectos del tratamiento con AO, AP o la combinación de ambos sobre el estrés del RE, la lipoapoptosis (características principales de la progresión de la NAFLD) y la señalización de la insulina. Nuestros datos muestran que, mientras que el AO no activa el estrés del RE ni afecta a la supervivencia celular en los hepatocitos neonatales inmortalizados, el AP induce estrés de RE y una posterior lipoapoptosis en estas células. La perturbación de la homeostasis del RE se ha postulado como un importante desencadenante de la toxicidad provocada por lípidos [35]. En este sentido, CHOP es un factor de transcripción que se expresa en respuesta al estrés del RE y juega un papel importante en la apoptosis inducida por este daño de lipotoxicidad, tanto a través de la activación de la vía intrínseca como extrínseca de la apoptosis [36]. De hecho, la reducción de la expresión de CHOP mediante deficiencia genética o RNA de interferencia (siRNA) disminuye la toxicidad provocada por AP en los hepatocitos [37, 38]. De la misma manera, la activación de la caspasa 3 es otra característica de la lipotoxicidad hepática ya que la apoptosis inducida por el AP se reduce utilizando inhibidores de caspasas [13, 41-43]. Por consiguiente, en este estudio se han analizado fundamentalmente estos dos marcadores para monitorizar el proceso lipoapoptótico.

En cuanto a los efectos diferenciales de los ácidos grasos sobre la respuesta a la insulina, el AP, pero no el AO, disminuye la activación de la cascada de señalización de esta hormona en los hepatocitos neonatales inmortalizados, lo que sugiere que el estrés del RE inducido por AP puede mediar esta resistencia a la insulina en las células hepáticas.

Curiosamente, nuestros resultados han demostrado que el AO provoca un efecto protector sobre el estrés del RE inducido por el AP, aliviando la consiguiente lipoapoptosis en hepatocitos neonatales inmortalizados. En cuanto a la señalización de la insulina, el AO revirtió el efecto inhibidor del AP sobre la cascada de señalización de la insulina, recuperando la fosforilación del RI y de AKT tras la estimulación con insulina. Estos resultados concuerdan con los anteriormente descritos en músculo esquelético, donde el AO impide tanto el estrés del RE como la resistencia a la insulina que provocan el AP en el músculo esquelético [41, 42]. Con respecto al hígado, hay estudios que describen que el tratamiento combinado de AO y AP mejora la apoptosis y la señalización de la insulina en las células hepáticas en comparación con aquellas tratadas sólo con AP, pero estos autores no esclarecen los mecanismos moleculares implicados [23]. Por esto, se puede concluir que el AO previene tanto la resistencia a la insulina como la lipoapoptosis inducidas por AP, mediante la inhibición del estrés del RE en los hepatocitos.

En hepatocitos, la activación de S6K1, diana de mTORC1, es relevante en el desarrollo de resistencia a la insulina ya que su fosforilación está incrementada en el hígado de ratones obesos *ob/ob* y, por otro lado, los ratones deficientes en S6K1 están protegidos contra la resistencia a la insulina inducida por obesidad. Esto es debido a que en ausencia de S6K1 se suprime un bucle de retroalimentación negativa provocado por esta proteína, que fosforila en residuos serina al IRS1 bloqueando así su activación por el RI [49,50]. Además de estos hallazgos previos, en este estudio se ha demostrado por

primera vez que el AO impide la fosforilación de la S6K1 inducida por el AP, lo que sugiere que la S6K1 podría ser una estrategia atractiva contra la resistencia a la insulina y la lipoapoptosis mediadas por el AP. Por ello, en este trabajo se ha inhibido la activación de la S6K1 en hepatocitos utilizando una estrategia farmacológica mediante el tratamiento con rapamicina y otra genética empleando hepatocitos deficientes en S6K1. Mediante ambos abordajes experimentales, encontramos que tanto la expresión de CHOP como la activación de la caspasa 3 tras en tratamiento con AP o con un inductor clásico de estrés del RE (tunicamicina), se reducen tras la inhibición de la S6K1. En conclusión, estos resultados han dilucidado la interacción molecular entre las vías de estrés y lipoapoptosis con la resistencia a la insulina en los hepatocitos tratados con AP. El AO previene de todos estos efectos deletéreos desencadenados por el AP, incluyendo la activación de la S6K1 que por primera vez ha sido implicada en la lipoapoptosis inducida por el estrés persistente del RE en hepatocitos.

* Las referencias de esta parte corresponden a las del artículo I

CAPÍTULO II

CAPÍTULO II

ESTUDIO DE LOS EFECTOS OPUESTOS PRODUCIDOS POR EL ÁCIDO OLEICO O EL ÁCIDO PALMÍTICO SOBRE LA SEÑALIZACIÓN DE LA INSULINA EN HEPATOCITOS A TRAVÉS DE LA ACTIVACIÓN DEL SISTEMA INMUNE.

La inflamación crónica de bajo grado que se produce por la activación de las células inmunes, sobre todo y en primera instancia, en el tejido adiposo blanco durante la obesidad está asociada con alteraciones en la cascada de señalización de la insulina en los tejidos periféricos. Por ello en este segundo capítulo se ha evaluado el efecto del ácido palmítico (AP) y del ácido oleico (AO) sobre la respuesta inmune de los macrófagos. Asimismo, se han estudiado los efectos que los productos secretados por los macrófagos producen sobre la cascada de señalización de la insulina en hepatocitos humanos y murinos.

Para llevar a cabo este objetivo, se estimularon macrófagos murinos RAW 264.7 y células primarias de Kupffer con AO y AP con el fin de analizar los niveles de citoquinas expresadas y el perfil lipidómico de eicosanoides. En los macrófagos tratados con AP se observó un aumento de citoquinas proinflamatorias, mientras que en el caso del tratamiento con AO se observó una disminución de los niveles de leucotrieno B_4 (LTB_4) secretado al medio. Posteriormente, se trataron hepatocitos murinos y humanos con el medio condicionado de macrófagos RAW 264.7 o células de Kupffer estimulados previamente con AO (MC-O) o con AP (MC-P), y se analizaron las rutas de estrés y la señalización de la insulina. Tras la estimulación con el MC-P se observó una reducción de la señalización de la insulina y un aumento de la apoptosis en los hepatocitos,

mientras que tras la estimulación de los hepatocitos con el MC-O se observó un aumento en la señalización mediada por el receptor de insulina y una reducción en la expresión de dos fosfatasas reguladoras negativas de la ruta, PTP1B y PTEN.

A nivel molecular, el MC-P desencadena una activación temprana de quinasas de estrés, particularmente de estrés del RE en hepatocitos, pero estos efectos están ausentes en los hepatocitos tratados con el MC-O. Sorprendentemente, cuando se tratan hepatocitos con el MC-O suplementado con LTB_4, producto derivado de los macrófagos que disminuye tras las estimulación de los mismos con AO, se observa una supresión de la hipersensibilidad que se producía cuando eran tratados únicamente con el MC-O y, además, aumentaron los niveles de proteína tanto de PTP1B como de PTEN.

Por lo tanto se puede concluir que el ácido oleico y el ácido palmítico ejercen efectos paracrinos y/o endocrinos opuestos en la relación entre macrófagos y hepatocitos. El MC-P interfiere bloqueando las primeras fases de la señalización de la insulina, mientras que el MC-O aumenta la sensibilidad a la insulina posiblemente reduciendo la secreción de LTB_4 por parte de los macrófagos.

Los resultados derivados de este estudio han dado lugar al manuscrito **"Opposite cross-talk by oleate and palmitate on insulin signaling in hepatocytes through macrophage activation"**, que está en revisión en la revista ***"The Journal of Biological Chemistry"***

Opposite cross-talk by oleate and palmitate on insulin signaling in hepatocytes through macrophage activation*

Virginia Pardo[1,2], Águeda González-Rodríguez[1,2*], Carlos Guijas[2,3], Jesús Balsinde[2,3], and Angela M. Valverde[2,3*]

[1]Instituto de Investigaciones Biomédicas Alberto Sols (CSIC/UAM), 28029 Madrid, Spain.

[2]Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Instituto de Salud Carlos III, 28029 Madrid, Spain.

[3]Instituto de Biología y Genética Molecular (CSIC), 47003 Valladolid, Spain.

*Running title: *Paracrine cross-talk between macrophages and hepatocytes*

To whom correspondence should be addressed**:** Águeda González-Rodríguez and Ángela M. Valverde, Instituto de Investigaciones Biomédicas Alberto Sols, C/ Arturo Duperier 4, 28029 Madrid, Spain. Emails: aguedagr@iib.uam.es; avalverde@iib.uam.es. Tel: 34-915854497. Fax: 34-915854401

Keywords: Cytokine, eicosanoid, endoplasmic reticulum stress (ER stress), fatty acid, hepatocyte, macrophage, inflammation, insulin resistance, Type 2 diabetes, signal transduction.

Background: Chronic low-grade inflammation during obesity is associated to impairments in the insulin signaling cascade.

Results: Oleate and palmitate elicit opposite effects in insulin signaling in hepatocytes through macrophage stimulation.

Conclusion: An endocrine/paracrine cross-talk between macrophages/Kupffer cells and hepatocytes modulates insulin signaling.

Significance: Switching macrophages/Kupffer cells polarization will be of benefit against insulin resistance in the liver.

ABSTRACT

Chronic low-grade inflammation in adipose tissue during obesity is associated to impairments in the insulin signaling cascade. In this study we have evaluated the impact of palmitate or oleate overload of macrophage/Kupffer cells in triggering stress-mediated signaling pathways and lipoapotosis, as well as in the cross-talk with the insulin signaling cascade in mouse and human hepatocytes. RAW 264.7 or Kupffer cells were stimulated with oleate or palmitate to analyze levels of cytokines and the lipidomic profile of eicosanoids. Whereas proinflammatory cytokines were elevated in macrophages/Kupffer cells stimulated with palmitate, lower leukotriene B_4 (LTB_4) levels were detected in macrophages stimulated with oleate. Then, mouse or human hepatocytes were treated with conditioned medium from RAW 264.7 or Kupffer cells loaded with oleate (CM-O) or palmitate (CM-P) prior to insulin stimulation. Whereas insulin signaling was impaired and lipoapoptosis was detected in hepatocytes treated with CM-P, enhanced insulin receptor (IR)-mediated signaling and reduced levels of the phosphatases protein tyrosine phosphatase (PTP) 1B and phosphatase and tensin homolog (PTEN) were found in hepatocytes treated with CM-O. At the molecular level, CM-P triggered an early activation of stress kinases and endoplasmic reticulum (ER) stress signaling in hepatocytes, but these effects were absent in hepatic cells treated with CM-O. However, supplementation of CM-O with LTB_4 suppressed insulin hypersensitivity in hepatocytes and up-regulated PTP1B and PTEN. In conclusion, oleate and palmitate elicit an opposite cross-talk between macrophages/kupffer cells and hepatocytes. Whereas CM-P interferes at the early steps of insulin signaling, CM-O increases insulin sensitization possibly by reducing LTB_4.

INTRODUCTION

Evidences from clinical and epidemiological studies have clearly established that obesity is the most common cause of insulin resistance, type 2 diabetes mellitus (T2DM) and non-alcoholic fatty liver disease (NAFLD). In fact, insulin resistance in peripheral tissues such as liver and skeletal muscle is an early metabolic abnormality in the development of T2DM (1). Although the precise molecular mechanisms underlying insulin resistance associated to obesity have not been completely elucidated, one major contributor is the chronic low-grade systemic inflammation state which interferes with the early steps of the insulin signaling cascade possibly through the effects of free fatty acids (FFAs) and cytokines secreted by the overgrowing white adipose tissue (WAT) (2-4).

Proinflammatory cytokines impair insulin action by activating stress kinases such as c-jun (NH2) terminal kinase (JNK), IκB kinase (IKK) and also protein kinase C (PKC)-mediated pathways (5,6). In addition, cytokines down-regulate early key mediators of the insulin signaling cascade such as the insulin receptor (IR) or insulin receptor substrate 1 (IRS1) (7,8),

as well as increase the expression of negative modulators of this pathway such as the protein tyrosine phosphatase 1B (PTP1B) (9,10). On the other hand, circulating FFAs, which are usually increased in insulin resistant states (11-13), also activate these proinflammatory pathways boosting the defects in peripheral insulin actions (14-16).

In addition to the inflammatory effects, the activation of endoplasmic reticulum (ER) stress-mediated signaling pathways by FFAs has been linked to obesity-associated immunometabolic dysregulation and insulin resistance (17, 18). ER stress is sensed by three main proteins: X-box binding protein 1 (X-BP1), PRKR-like endoplasmic reticulum kinase (PERK) and activating transcription factor-6 (ATF6), which cooperate to mitigate ER stress by reducing protein translation, stabilizing proteins by chaperones and activating ER-associated protein degradation (19). Chemical chaperones (tauroursodeoxycholic acid (TUDCA) and 4-phenylbutryate) that alleviate ER stress through protein stabilization, improve systemic glucose homeostasis, increase glucose uptake in adipose and skeletal muscle and reduce hepatic glucose production (20). Moreover, a study in obese subjects has revealed that treatment with TUDCA increases insulin sensitivity in liver and skeletal muscle by approximately 30% compared to placebo therapy (21).

FFAs-derived metabolites are relevant factors in the pathogenesis of the metabolic syndrome (MS) (22). Eicosanoids (prostaglandins (PGs), tromboxanes (TXs), leukotrienes (LTs) and cytochrome P450 metabolites) are bioactive lipids synthesized from polyunsaturated FFAs such as proinflammatory omega-6 arachidonic acid (AA) or antiinflammatory omega-3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) through the cyclooxigenases (COX), lipoxygenases (LOX) and cytochrome P450 (CYP450) pathways. The functional role of eicosanoids in obesity and insulin resistance has recently been studied in humans and animal models. For instance, an accumulation of polyunsaturated FFAs, leukotriene B4 (LTB_4) and 6-keto-prostaglandin-$F_{1\alpha}$ was observed in plasma from patients with MS (23). Moreover, the development of insulin resistance in patients affected by MS leads to increased synthesis of thromboxane A_2. Regarding studies in cellular and animal models, it was shown that PGE_2 aggravates insulin resistance induced by interleukin 6 (IL6) in hepatocytes (24) whereas LTB_4 receptor 1 (BLT1)-deficient mice are resistant to high fat-diet (HFD)-induced obesity and insulin resistance (25).

Several studies have reported that besides adipocytes, adipose tissue resident macrophages, which migrate and accumulate in WAT, have a relevant role in obesity-induced chronic inflammation (26-28) through their polarization toward the M1-like state (29). Although the importance of macrophages in the molecular mechanisms triggering insulin resistance in skeletal muscle and adipose tissue has been explored (29-31), it remains unclear whether the

inflammatory milieu impact insulin signaling in hepatocytes. On that basis, in this study we have investigated for the first time the effect of macrophages activated by two distinct FFAs, palmitate (saturated) and oleate (unsaturated), on stress-mediated pathways, lipoapoptosis and the insulin signaling cascade in hepatocytes.

EXPERIMENTAL PROCEDURES

Reagents. Fetal bovine serum (FBS) (#10270) and culture media DMEM (#41966-029) were obtained from Invitrogen (Gran Island, NY). TRIzol reagent (#T9424), sodium palmitate (#P9767), sodium oleate (#O7501), bovine serum albumin (BSA) (#A6003), fatty acid free-BSA endotoxin free (#A8806) and insulin (#I0516) were from Sigma Aldrich (St. Louis, MO). Bradford reagent, acrylamide, immunobloting PVDF membrane and Immobilon Western Chemiluminescent HRP Substrate were purchased from Bio-Rad (Hercules, CA).

Free fatty acid preparation. 2.5 mM free fatty acid (FFA) stock solutions were prepared by modification of Spector method (32). Briefly, cold sodium palmitate or sodium oleate were dissolved in 0.1M NaOH by heating at 70°C while 0.5 mM BSA solution was prepared by dissolving fatty acid free-BSA in NaCl 0.9% by heating at 50°C (at maximum). Once BSA and FFA solutions were completely dissolved, palmitate and oleate solutions were diluted 10-times in the BSA solution and mixed by pipetting to achieve a final molar ratio of 5:1. Control BSA was prepared by adding the same amount of 0.1M NaOH into 0.5 mM BSA solution. All preparations were filtered, aliquoted and stored at –20°C.

Culture of RAW 264.7 murine macrophages. Murine RAW 264.7 macrophage cell line, kindly provided by Dr. Tarín (CNIC, Madrid, Spain), were cultured in RPMI supplemented with 10% heat inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine. The generation and characterization of immortalized mouse hepatocyte cell line has been previously described (33). Cells were grown in DMEM plus 10% heat inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine. Confluent macrophages were treated with BSA or FFAs solutions (750 µM conjugated oleate/BSA or 750 µM conjugated palmitate/BSA) for 24 h to obtain the corresponding conditioned media (CM) (31). CM were centrifuged to remove dead cells and added to hepatocytes for several time periods.

Isolation and culture of Kupffer cells. For Kupffer cells (KC) isolation, the supernatant from the first centrifugation of the hepatocyte isolation protocol was collected and centrifuged twice at 50 x g for 5 min to discard the pellet with remaining hepatocytes. The latest supernatant was centrifuged at 500 x g for 5 min at 4ºC and the pellet containing the KC was resuspended in attachment media. Cells were mixed by inversion with 50% percoll and centrifuged at 1.059 x g

for 30 min without acceleration or brake at room temperature. Finally, KC pellet was washed with PBS 1X, centrifuged twice at 500 x g for 10 min at 4ºC to wash out the residual percoll solution and cells were resuspended in RPMI supplemented with 10% heat inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine. Cells were then plated on 12 well-plates and maintained for 24 h before treatments. Conditioned media was prepared as described in RAW 264.7 cells.

Hepatocyte cell culture. Human hepatocytes were isolated by the two-step collagenase procedure from non-tumor areas of liver biopsies from patients submitted to a surgical resection for liver tumours after obtaining patients' written consent (34). Primary mouse hepatocytes were isolated from non-fasting male C57BL/6 mice (10-12-week-old) by perfusion with collagenase as described (35). Cells were seeded on 6 well-plate (Corning, New York, NY) and cultured in media containing Dulbecco's modified Eagle's medium and Ham's F-12 medium (1:1) with 10% FBS, supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 mM sodium pyruvate (attachment media) and maintained for 24 h before treatments. The generation and characterization of immortalized mouse hepatocyte cell line has been previously described (33). Cells were grown in DMEM plus 10% heat inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine.

Analysis of FFAs-derived metabolites. 0.01% (w/v) of butylated hydroxytoluene in methanol was added to supernatants to prevent metabolites degradation. CM collected from macrophages treated with BSA or FFAs solutions were melted slowly and 400 pmol of deuterated PGE_2 and LTB_4 were added as internal standards before solid phase extraction. Lipid metabolites were extracted using Bond Elut Plexa solid phase extraction columns (Agilent) as indicated by manufacturer´s. Columns were homogenized with 3 ml of methanol followed by 3 ml of water. Supernatants were acidified with 0.5% acetic acid and 10% methanol was also added before samples loading. Samples were washed with 3 ml of 10% methanol and finally, lipid products were eluted with 2 x 1.5 ml of 100% methanol. Lipid metabolites were concentrated under vacuum and redissolved in 100 µl of solvent A (water, acetonitrile/acetic acid, 70/30/0.02 by vol) for their analysis by HPLC/MS/MS. Chromatographic protocol was adapted from Dumlao D.S. et al., (36). Quantification was carried out by the integration of chromatographic peaks of the previously identified species comparing with an external calibration curve made with analytical standards. The software analyst 1.5.2 was employed in this process.

Preparation of protein extracts and Western blot. To obtain total cell lysates, attached cells were scraped off and incubated for 10 min on ice with lysis buffer (25 mM HEPES, 2.5 nM EDTA, 0.1% Triton X-100, 1 mM PMSF and 5 µg/ml leupeptin). After protein content

determination with Bradford reagent, total protein were boiled in Laemmli sample buffer and submitted to 8-15% SDS-PAGE. Proteins were transferred to Immunoblot PVDF membrane and, after blocking with 3% BSA or 5% non-fat dry milk, membranes were incubated overnight with several antibodies as indicated. Immunoreactive bands were visualized using the ECL Western blotting protocol. Densitometric analysis of the bands was performed using Image J software. The anti-phospho-PERK (Thr 980) (#3179), anti-phospho-eIF2α (Ser 51) (#9721), anti-phospho-JNK (#9251), anti-phospho-STAT3 (#9131), anti-phospho-MAPK (#9101) and anti-MAPK (#4695) antibodies were from Cell Signaling Technology (Danvers, MA). The anti-JNK (sc-571), anti-IR β (sc-711), anti-PERK (sc-13073), anti-eIF2α (sc-11386), anti-GRP78 (sc-376768), anti-CHOP (sc-7351), anti-phospho-p38 (sc-17852), anti-p38 (sc-9212), anti-IRS1 (sc-559), anti-IRS2 (sc-8299), anti-phospho-IR (sc-25103), anti-phospho-AKT (Thr 308) (sc-16646), anti-phospho-AKT (Ser 473) (sc-7985), anti-AKT (sc-8312), anti-PTP1B (sc-1718) and anti-PTEN (sc-7974) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-α-tubulin (T5168) and anti-β-actin antibodies (A5441) were from Sigma Aldrich (St. Louis, MO).

RNA isolation and qPCR. Total RNA was isolated using Trizol reagent and was reverse transcribed using a SuperScriptTM III First-Strand Synthesis System for qPCR following manufacturer's indications. qPCR was performed with an ABI 7900 sequence detector using the SyBr Green method and d(N)6 random hexamer with primers purchased from Invitrogen. PCR thermocycling parameters were 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Each sample was run in triplicate and normalized to 18S RNA. Fold changes were determined using the ΔΔCt method. Primer sequences are available upon request.

Quantification of apoptosis. Cells were grown in glass coverslips and treated as described above. Then, cells were washed twice with PBS, fixed in p-formaldehyde (4%) for 10 min and characteristic morphological changes of apoptosis were assessed by staining nuclei with DAPI followed by the analysis by fluorescence microscopy.

Statistical analysis. Data are presented as mean ± SEM, and were compared by using the Bonferroni *ANOVA* test. All statistical analyses were performed using the IBM SPSS Statistics 21.0 (SPSS Inc. IBM, Armonk, NY) software with 2-sided tests. Differences were considered statistically significant at $p<0.05$.

RESULTS

Conditioned medium from macrophages stimulated with palmitate (CM-P) impairs insulin signaling in mouse hepatocytes. The liver is composed primarily of hepatocytes, but also contains blood and lymph vessels, nerves and immune cells. Since all these cell types can potentially respond to a high fat-mediated inflammatory environment *in vivo*, we performed a cell culture-based approach to investigate the specific cross-talk between macrophages and hepatocytes in the context of fatty acid overload. For this goal, RAW 264.7 macrophages were treated with palmitate, a typical saturated fatty acid found in western diets, for 24 h. Then, culture media (conditioned medium) was removed. Conditioned medium was hereafter called CM-P (collected from RAW 264.7 cells treated with palmitate) or CM-B (from RAW 264.7 cells treated only with BSA as a control). CM-B or CM-P were added to immortalized mouse hepatocytes generated and validated in our laboratory (33) for a further 24 h and then, cells were stimulated with 10 nM insulin for 10 min. As depicted in Figure 1A, IR tyrosine phosphorylation was reduced in hepatocytes preincubated with CM from palmitate-treated macrophages compared to those treated with control CM-B. Likewise, levels of IRS1 decreased only in hepatocytes treated with CM-P in parallel with decreases in Akt Ser473 and Thr308 phosphorylation in response to insulin. Notably, we found similar impairment of the insulin signaling in hepatocytes treated with CM collected from RAW 264.7 cells stimulated with palmitate for 8 h and then reefed with fresh medium for a further 16 h (Figure 1B). These results indicate that the components of the CM-P mediate the negative effects observed in insulin signaling in hepatocytes.

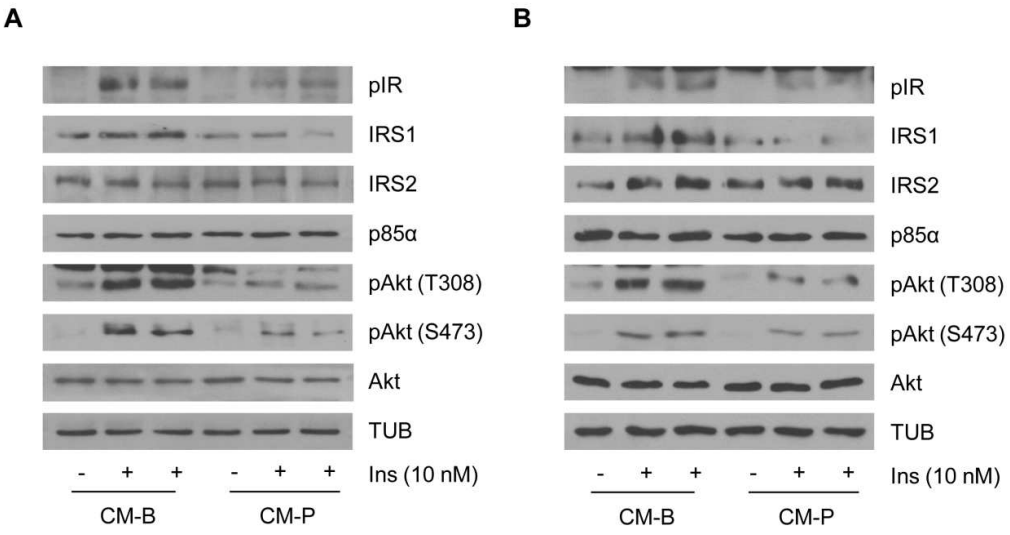

Figure 1. Proinflammatory components of the conditioned medium from RAW 264.7 macrophages stimulated with palmitate are mediators of the impairment in insulin signaling in hepatocytes.

Hepatocytes were treated 24 h with conditioned media collected from RAW 264.7 cells treated with BSA (CM-B) or palmitate (CM-P) for 24 h (**A**) or with conditioned media collected from RAW 264.7 cells treated with BSA (CM-B) or palmitate (CM-P) for 8 h and then reefed with fresh medium for a further 16 h (**B**). Then, cells were stimulated with 10 nM insulin for 10 min. Total protein was analyzed by Western blot using the indicated antibodies. Representative blots are shown. ($n \geq 3$).

Insulin signaling is opposite modulated in mouse and human hepatocytes pretreated with conditioned medium from macrophages stimulated with palmitate or oleate. Next, we checked that the effects of CM-P on insulin signaling in hepatocytes were specific of palmitate treatment and not due to the FFA overload. For this goal, we obtained conditioned medium from RAW 264.7 cells treated with oleate, a well nontoxic monounsaturated FFA. Conditioned medium was hereafter called CM-O (collected from RAW 264.7 cells treated with oleate). After addition of CM-B, CM-O or CM-P for 24 h, immortalized mouse hepatocytes were stimulated with 10 nM insulin for 10 min. As depicted in Figure 2A, insulin induced IR tyrosine phosphorylation was enhanced in hepatocytes preincubated with CM-O as compared to control hepatocytes preincubated with control CM-B. As stated above (Figure 1A), IR tyrosine and Akt phosphorylation was reduced in presence of CM-P (Figure 2A). Moreover, protein levels of IR and IRS1 decreased only in hepatocytes treated with CM-P. In agreement with enhanced IR tyrosine phosphorylation and maintained of intact levels of IR and IRS1, the response to insulin in Akt phosphorylation at both residues was increased in hepatocytes treated with CM-O compared to those treated with CM-B. Notably, IRS2 levels were not affected by treatment of hepatocytes with either CM. Then, the differential effect of both types of CM was assessed in human primary hepatocytes. Figure 2B shows that the decreased IR/IRS1/Akt-mediated insulin signaling was also observed in human primary hepatocytes pretreated with CM-P whereas a significant enhancement of this signaling pathway was found in human hepatocytes pretreated with CM-O. Altogether, these results indicate opposite effects of palmitate and oleate in triggering cross-talks between macrophages and hepatocytes with relevant effects in insulin signaling.

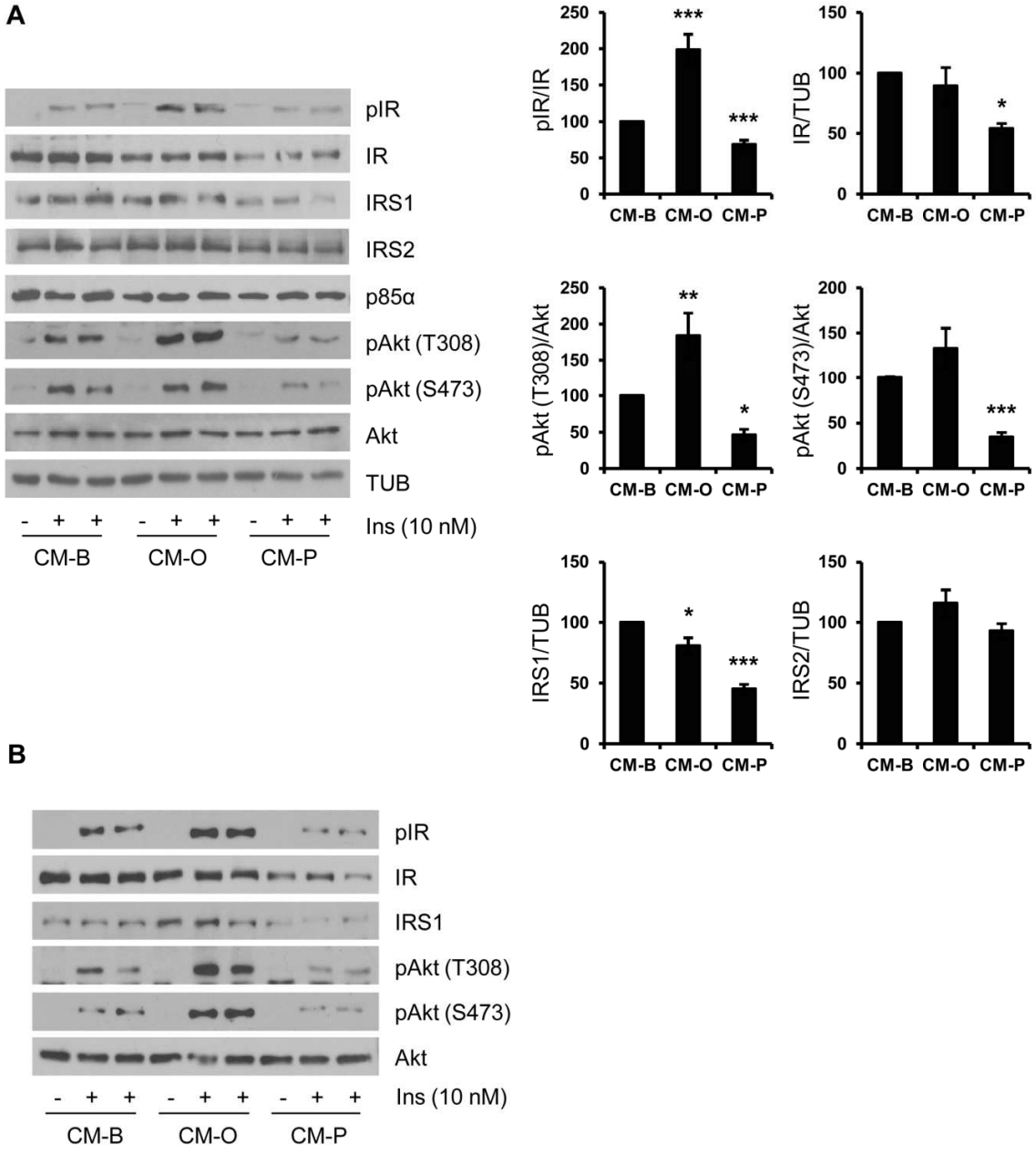

Figure 2. Insulin signaling is differentially modulated in hepatocytes pretreated with conditioned medium from RAW 264.7 macrophages stimulated with oleate or palmitate. **A**. Conditioned medium from RAW 264.7 macrophages treated with BSA (CM-B), oleate (CM-O) or palmitate (CM-P) was added to immortalized mouse hepatocytes for 24 h. Then, cells were stimulated with 10 nM insulin for 10 min. Total protein was analyzed by Western blot using the indicated antibodies. Representative blots are shown. After quantification of all blots, results are expressed as % of insulin stimulation or % of protein expression relative to the CM-B condition (100%) and are mean ± SEM (n≥6), *p<0.05, **p<0.01, ***p<0.001, CM-O or CM-P, respectively, vs CM-B. **B**. Human primary hepatocytes were treated with conditioned medium from RAW 264.7 macrophages treated with BSA, oleate or palmitate as described in A. Total protein was analyzed by Western blot using the indicated antibodies. Representative blots are shown (n=4).

Differential effects of palmitate and oleate in the content of proinflammatory cytokines and eicosanoids secreted by macrophages. In an attempt to characterize the molecules released by macrophages to the CM, we assessed the expression of proinflammatory cytokines characteristic of M1 macrophage polarization by real-time PCR. Treatment of RAW 264.7 macrophages with palmitate, but not oleate, increased IL6, IL1β, TNFα and MCP1 mRNA levels (Figure 3A). Moreover, the induction of iNOS, a key proinflammatory marker, was only detected in RAW 264.7 cells treated with palmitate (Figure 3B). On the other hand, oleate treatment significantly up-regulated mRNA levels of arginase 1, a relevant M2 antiinflammatory cytokine. Notably, no significant differences were observed in other M2 polarization markers such as IL10, Mcr1 and Mgl1.

Next, we performed a broad lipidomic screening of the CM collected from RAW 264.7 cells treated with oleate or palmitate (Figure 4). Total amount of eicosanoids detected in the CM-P was higher compared to CM-O or control CM-B, including COX-derived products such as PGE_2, PGD_2, $PGF_{2\alpha}$, TX_{B2}, 12-HHT and 11-HETE as well as PGD_2 dehydration metabolites like 15d-PGD_2, 15d-PGJ_2 and 15k-PGE_2. Moreover, a greater increase of LOX-derived molecular species such as 15-HETE, prostaglandin D_2 and leucoxene B_4 (LXB_4) was observed in CM of palmitate-treated macrophages as compared to CM from macrophages stimulated with oleate. Surprisingly, levels of leukotriene B_4 (LTB_4) were lower in CM-O compared to those found in CM-P or CM-BSA.

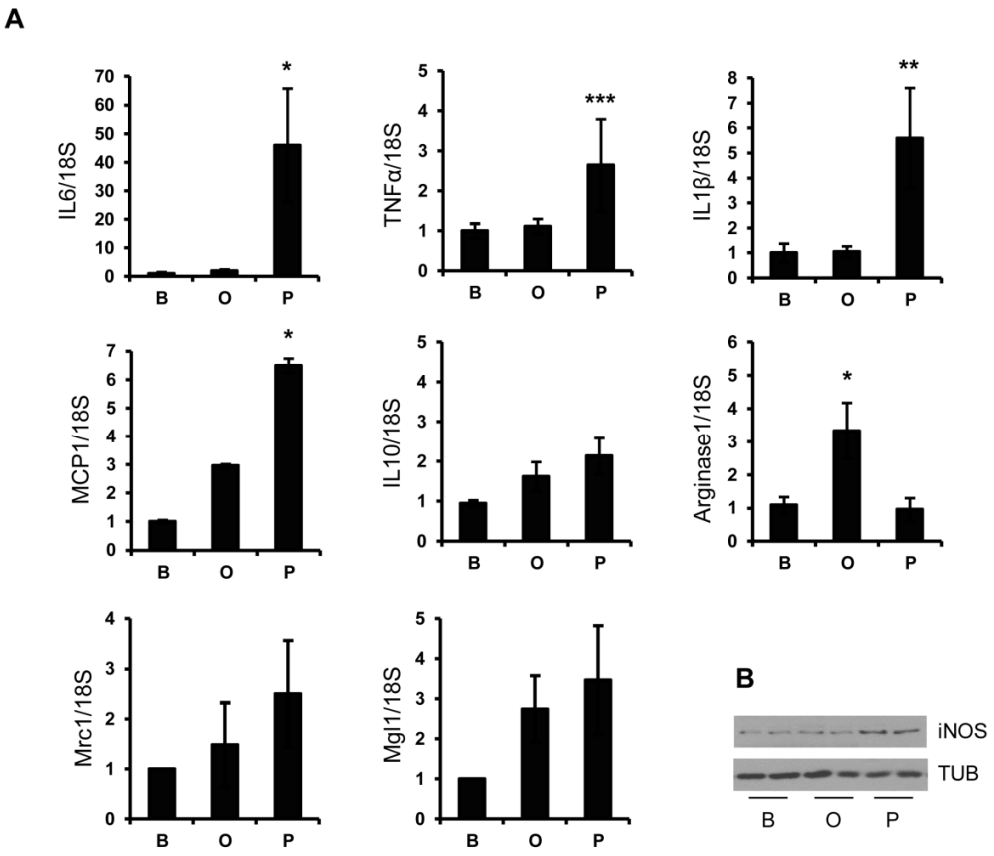

Figure 3. Palmitate, but not oleate, treatment induces proinflammatory cytokine and chemokine gene expression in RAW 264.7 macrophages. RAW 264.7 macrophages were treated with BSA (B), oleate (O) or palmitate (P) for 24 h. **A**. TNFα, IL-6, IL-1β, MCP1, IL-10, arginase 1, Mcr1 and Mgl1 mRNA levels were analyzed by qRT-PCR. Results are expressed as fold increase relative to the BSA condition (1) and are mean ± SEM (n≥4), *p<0.05, **p<0.01, O or P, respectively, vs B. **B**. Total protein was analyzed by Western blot using the indicated antibodies. Representative blots are shown (n≥4).

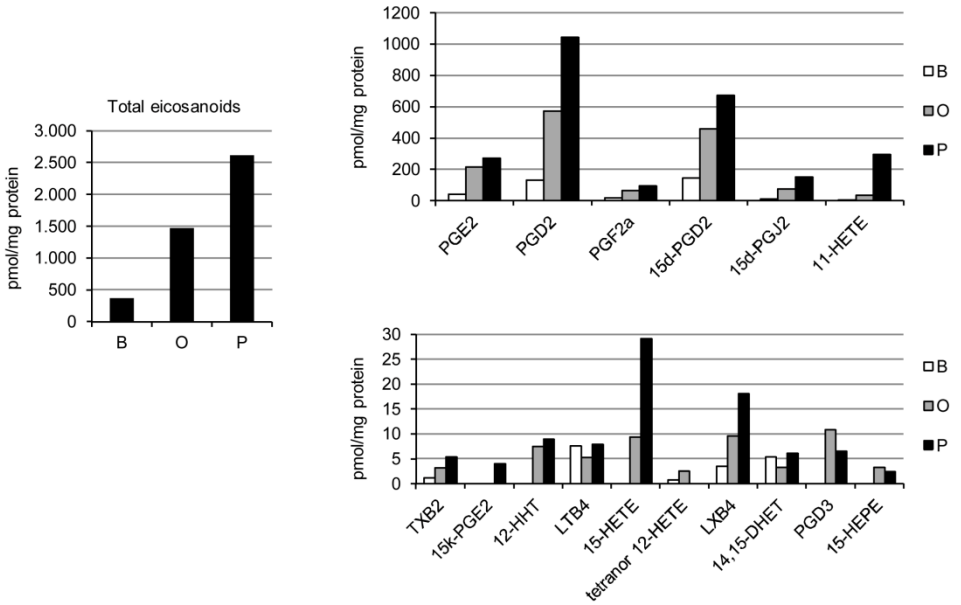

Figure 4. Differential effect of palmitate and oleate in total eicosanoids content in RAW 264.7 macrophages. Analysis of eicosanoids from the conditioned medium (CM) from RAW 264.7 macrophages treated with BSA (B), oleate (O) or palmitate (P) by high performance liquid chromatography/tamdem mass spectrometry. Results are expressed as pmol/mg protein. Conditioned media (CM) collected from 3 independent experiments performed in triplicate were pooled and the results showed in the graphs correspond to the global analysis of pooled CM.

Conditioned medium from RAW 264.7 macrophages stimulated with palmitate, but not oleate, activates stress kinases, ER stress signaling and induces lipoapoptosis in hepatocytes. It is well known that cytokines secreted by macrophages such as TNFα and IL6 induce insulin resistance in hepatic cells through the activation of proinflammatory signaling pathways (37,38). Moreover, FFAs and their metabolites have been involved in the activation of stress-mediated signaling pathways linked to chronic metabolic low grade inflammation (39). On that basis, we tested whether oleate or/and palmitate activate stress kinases in hepatocytes through their effects in macrophages. As depicted in Figure 5A, the phosphorylation of inflammation-linked stress kinases (STAT3, JNK, p38 MAPK and ERK) and kinases related to ER stress (PERK and eiF2α) was rapidly detected (at 30 min) in hepatocytes stimulated with CM-P, but not with CM-O, the latter remained at similar basal levels than that of hepatic cells stimulated with CM-B. This pattern of kinases activation concurred with an increase in the expression of GRP78 and CHOP, two relevant ER stress markers, and with the detection of active caspase 3 fragment after 24 h of CM-P challenge (Figure 5B). Conversely, neither increased GRP78 and CHOP expression nor active caspase 3 fragment were detected in hepatocytes treated with CM-O and in control cells treated with CM-B. Notably, in hepatocytes treated with CM-P the activation of caspase 3 correlated with increased apoptosis (Figure 5C).

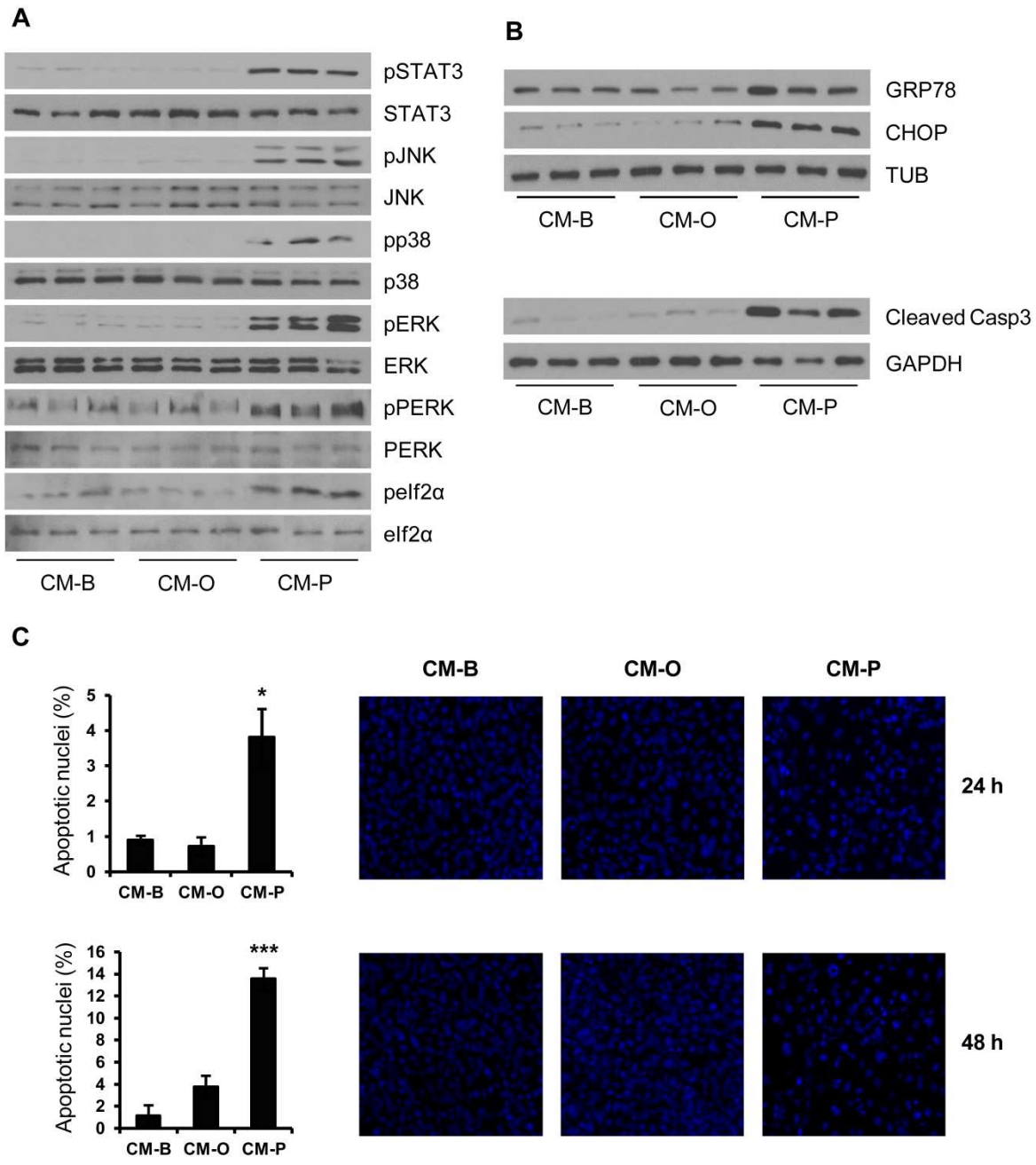

Figure 5. Conditioned medium from RAW 264.7 macrophages treated with palmitate activates stress kinases, ER stress and induces lipoapoptosis in hepatocytes. Conditioned medium from RAW 264.7 macrophages treated with BSA (CM-B), oleate (CM-O) or palmitate (CM-P) was added to immortalized mouse hepatocytes for several time periods (**A**, 30 min **B**, 24 h, **C**, 24 h and 48h). **A, B.** Total protein was analyzed by Western blot using the indicated antibodies. Representative blots are shown (n≥4). **C**. Representative images after DAPI staining. After quantification of apoptotic nuclei from all images, results are expressed as % of number of apoptotic nuclei and are mean ± SEM (n≥3), *p<0.05, ***p<0.001, CM-P vs CM-B.

Conditioned medium from Kupffer cells stimulated with palmitate activates stress-mediated signaling pathways and induces insulin resistance in primary mouse hepatocytes. In order to confirm the differential effects of oleate and palmitate in a physiological context, we isolated and cultured Kupffer cells from C57/BL6 mice and these resident macrophages were stimulated with oleate or palmitate for 24 h. In agreement with the results found in RAW 264.7 cells, an increase of IL6, IL1β, TNFα and MCP1 mRNA levels was observed only in palmitate-stimulated Kupffer cells (Figure 6A). By contrast, oleate increased mRNA levels of M2 polarization markers (IL10, Mcr1, Mgl1 and arginase 1). We confirmed these data analyzing the activation of stress kinases in primary mouse hepatocytes treated with CM from Kupffer macrophages stimulated with oleate or palmitate (CMK-O or CMK-P, respectively). As a control, hepatocytes were stimulated with CM from Kupffer cells loaded with BSA (CMK-B). As shown in Figure 6B, phosphorylation of STAT3, p38 MAPK, JNK, PERK and eiF2α were observed exclusively in hepatocytes treated with CMK-P. In the light of these data, CHOP and the active fragment of caspase 3, indicators of apoptosis, were detected in hepatocytes treated with CMK-P (Figure 6C).

Next, we analyzed the effects of Kupffer cells-derived products on insulin signaling in hepatocytes. For this goal, primary hepatocytes were treated with CMK-P or CMK-O for 24 h and subsequently stimulated with 10 nM insulin for 10 min. As depicted in Figure 6D, insulin-induced tyrosine phosphorylation of the IR and Akt phosphorylation at both Ser473 and Thr308 residues was enhanced in hepatocytes pretreated with CMK-O whereas these responses were decreased in hepatocytes pretreated with CMK-P. These results also reflect an opposite paracrine cross-talk between hepatocytes and resident macrophages.

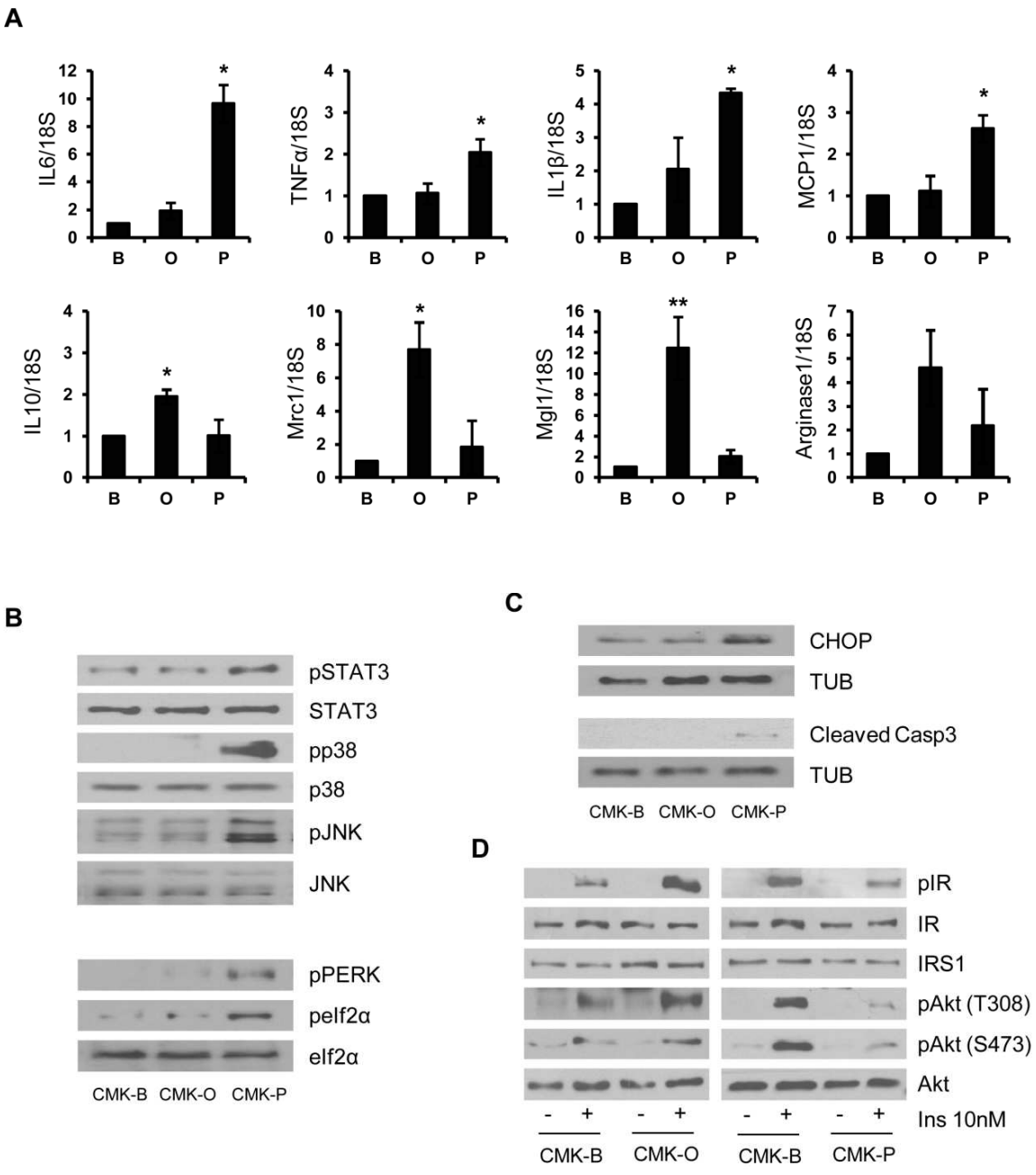

Figure 6. Paracrine effects of primary Kupffer cells stimulated with palmitate or oleate in stress and insulin-mediated signaling pathways in primary mouse hepatocytes. **A**. Primary Kupffer cells were treated with BSA (B), oleate (O) or palmitate (P) for 24 h. TNFα, IL-6, IL-1β, MCP1, IL-10, arginase 1, Mcr1 and Mgl1 mRNA levels were analyzed by qRT-PCR. Results are expressed as fold increase relative to BSA condition (1) and are mean ± SEM (n=3), *p<0.05, **p<0.01, O or P, respectively, vs B. **B, C**. Conditioned medium from primary Kupffer cells treated with BSA (CMK-B), oleate (CMK-O) or palmitate (CMK-P) was added to primary mouse hepatocytes for several time periods (**B**, 30 min and **C**, 24 h). Total protein was analyzed by Western blot using the indicated antibodies. Representative blots are shown (n=3). **D**. CMK was added to primary hepatocytes for 24 h. Then, cells were stimulated with 10 nM insulin for 10 min. Total protein was analyzed by Western blot using the indicated antibodies. Representative blots are shown (n=3).

Lower levels of PTP1B and LTB_4 contribute to the insulin sensitization induced by CM-O in hepatocytes. We evaluated the possibility that changes in the expression of negative modulators of the early steps of the insulin signaling could account for the insulin sensitization induced by CM-O or CMK-O in hepatocytes. Among them, PTP1B was a potential candidate given its ability to directly dephosphorylate tyrosine residues of the IR (40). In addition, PTEN, a lipid phosphatase, limits Akt activation by decreasing levels of phosphatidylinositol 3,4,5-triphosphate (41). Consistent with this hypothesis, we measured the expression of these phosphatases in hepatocytes incubated with CM from RAW 264.7 cells treated with BSA or oleate. As depicted in Figure 7A and 7B, PTP1B and PTEN protein content was decreased in hepatocytes treated with CM-O without changes at their mRNA levels.

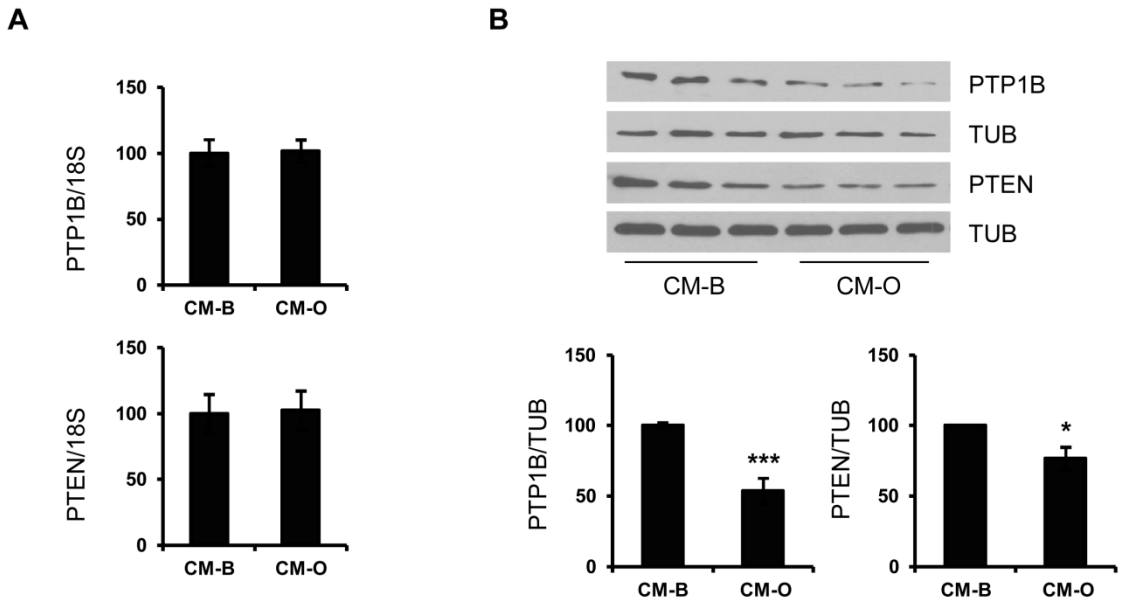

Figure 7. Lower levels of PTP1B and PTEN contribute to the insulin sensitization induced by CM-O in hepatocytes. Conditioned medium from RAW 264.7 macrophages treated with BSA (CM-B) or oleate (CM-O) was added to immortalized mouse hepatocytes for 24 h. **A**. PTP1B and PTEN mRNA levels were analyzed by qRT-PCR. Results are expressed as fold increase relative to CM-B condition (1) and are mean ± SEM (n≥3). **B**. Total protein was analyzed by Western blot using the indicated antibodies. Representative blots are shown. After quantification of all blots, results are expressed as % of protein expression relative to CM-B condition (100%) and are mean ± SEM (n≥3), *p<0.5, ***p<0.001 CM-O vs CM-B.

Finally, as we detected lower levels of LTB_4 in CM-O compared to those found in CM-P or control CM-BSA, we analyzed the involvement of this lipid specie in the effects of oleate in insulin sensitization in hepatocytes. LTB_4 is a pro-inflammatory lipid mediator generated from arachidonic acid through the activities of 5-5-LOX, 5-LOX-activating protein (FLAP) and leukotriene A_4 hydrolase (42,43). Firstly, we checked whether LTB_4 directly modulates stress

kinases and insulin sensitivity in hepatocytes. As depicted in Figure 8A *(left panel)*, LTB_4 activated STAT3 and p38 MAPK phosphorylation, as well as decreased in insulin-induced Akt phosphorylation in a dose-dependent manner *(right panel)*. This result indicates that LTB_4 *per se* induces insulin resistance in hepatocytes. Next, hepatocytes were treated with CM-O in the absence or presence of LTB_4 and then, stimulated with insulin. As shown in Figure 8B, both IR and Akt phosphorylation were decreased in hepatocytes incubated with CM-O plus LTB_4 compared to hepatocytes treated with CM-O. Moreover, after CM-O challenge in the presence of LTB_4, the expression of PTP1B and PTEN was increased and reached levels detected in the CM-B condition (Figure 8C). This effect was particularly evident in the modulation of PTP1B levels.

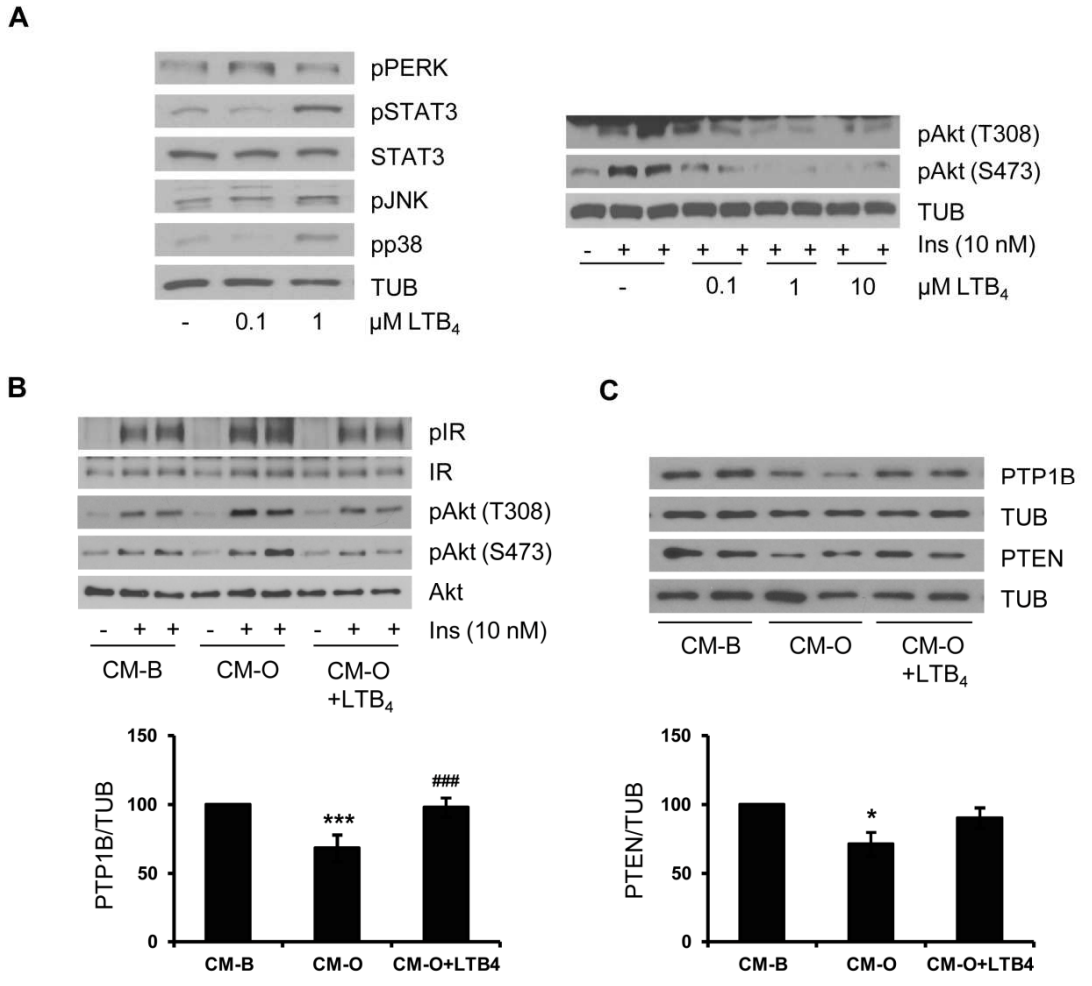

Figure 8. LTB_4 impairs the enhancement of insulin signaling induced by CM-O in hepatocytes. **A**. Immortalized hepatocytes were treated with various doses of LTB_4 for 1 h *(left panel)* or for 24 h followed by stimulation with 10 nM insulin for 10 min *(right panel)*. Representative blots are shown (n=3). **B**. Conditioned medium from RAW 264.7 macrophages treated with BSA (CM-B) or oleate (CM-O) was added to immortalized mouse hepatocytes for 24 h, in the presence or absence of LTB_4 (0.5 µM) followed by insulin stimulation (10 nM for 10 min). Representative blots are shown (n=4). **C**. Conditioned medium from RAW 264.7 macrophages treated with BSA (CM-B) or oleate (CM-O) was

added to immortalized mouse hepatocytes for 24 h, in the presence or absence of LTB_4 (0.5 μM). Representative blots are shown. After quantification of all blots, results are expressed as % of protein expression relative to CM-B condition (100%) and are mean ± SEM (n≥3), *p<0.05,***p<0.001, CM-O vs CM-B; $^{###}$p<0.001, CM-O plus LTB_4 vs CM-O.

DISCUSSION

In obesity-associated insulin resistance, M1-like macrophages polarization state has been associated with the enhancement of the proinflammatory milieu by the ability to secrete proinflammatory cytokines. This effect is in part mediated through high local concentrations of FFAs released by elevated lipolysis of the surrounding insulin resistant adipocytes that trigger proinflammatory signaling pathways in macrophages trough their binding to Toll-like receptors (TLRs) (44-46). However, we know now that beyond the interplay between adipocytes and adipose tissue resident macrophages, these inflammatory signals also dysregulate key metabolic responses in peripheral tissues, thereby exacerbating insulin resistance (3).

The liver is a target organ of the inflammatory mediators. In obesity, the hepatic lipid accumulation (first hit) together with the proinflammatory input (second hit) trigger the necroinflammatory changes that are recognized histopahologically as steatohepatitis (NASH) (47). Of relevance, adipose tissue inflammation has been correlated with hepatic steatosis in humans (48). In this study, we have dissected for the first time the molecular cross-talk between signals emerging from macrophage-derived products in response to fatty acid overload and insulin signaling in hepatocytes. A step further, we attempted to compare the responses of hepatocytes to the milieu containing macrophage-secreted cytokines and lipid metabolites derived from oleate or palmitate, that represent unsaturated or saturated circulating FFAs. Interestingly, an opposite response in insulin-mediated IR tyrosine phosphorylation, the earliest event in the insulin signaling cascade, was found in both mouse and human hepatocytes exposed to the CM from macrophages treated with oleate or palmitate, with a significant increase or decrease, respectively, as compared to control hepatocytes (treated with CM-BSA). This opposite response was also evidenced in Akt phosphorylation (at both Ser473 and Thr308), a critical node of insulin´s metabolic actions in hepatic cells (49). Thus, these results suggested that oleate and palmitate induce different secretory responses in macrophages and this might differentially modulate insulin signaling in liver cells. In the light of these findings, the M1 polarization state induced by palmitate, reflected by elevated TNFα, IL6, IL1β and iNOS in agreement to Samokhvalov et al. (30), was not observed in RAW 264.7 macrophages loaded with oleate. The absence of M1 polarization is critical to understand the modulation of insulin signaling by oleate in hepatocytes as will be discussed below. In fact, increased arginase 1 levels reflects a M2 profile of RAW 264.7 macrophages after oleate challenge in agreement

with recent results reported by Camell et al. that demonstrated the role of dietary oleic acid in M2 macrophages polarization (50).

The ER plays a central role in the determination of cell fate under conditions of stress. Increased ER stress has been shown to contribute to the development of NAFLD (17). In this regard, CM-P-treated hepatocytes rapidly activated the PERK branch of UPR by inducing PERK, eiF2α and JNK phosphorylation, resulting in increased CHOP expression. Under these experimental conditions, STAT3 phosphorylation was also increased, this response is probably mediated by the proinflammatory cytokines IL6 and IL1β. Moreover, TNFα-mediating signaling might also boost JNK and p38 MAPK activation. The convergence of all these proinflammatory signaling cascades leads to a negative-cross talk with insulin signaling resulting in the degradation of both IR and IRS1 (Figure 9A) that agrees with results reported in hepatocytes treated with palmitate (51,52). Notably, IRS2 levels were not affected by either CM-P or CM-O in human and mouse hepatocytes indicating that IRS2 might be more resistant to postranslational degradation compared to IRS1 as reported in primary mouse hepatocytes (53). Therefore, lower IR and IRS1 levels detected in hepatocytes stimulated with CM-P evidence the contribution of the early activation of stress kinases in the reduced insulin-mediated Akt phosphorylation. Neither the early activation of stress kinases nor CHOP expression were detected in hepatocytes treated with CM-O, highlighting the absence of oleate-mediated proinflammatory responses in the macrophage-hepatocyte axis.

Activation of Kupffer cells, the hepatic resident macrophages, to secrete proinflammatory mediators is a key event in the initiation of NAFLD, and limiting their polarization into an M1 phenotype is considered an attractive strategy against chronic liver inflammation (54-57). Depletion of Kupffer cells using gadolinium chloride attenuated the development of hepatic steatosis and hepatic insulin resistance, suggesting an important early role for Kupffer cells in diet-induced alterations in hepatic insulin resistance (55). The differential effect of palmitate and oleate in proinflammatory cytokine secretion in RAW 264.7 macrophages was also found in primary Kupffer cells and, importantly, similar differences were observed in the activation of stress kinases and in the modulation of insulin signaling in primary hepatocytes after CM challenge. Of particular relevance is the enhancement of insulin signaling in primary hepatocytes incubated with CM from Kupffer cells loaded with oleate (summarized in Figure 9B) that reinforces the beneficial effects on insulin sensitivity in a paracrine manner.

In addition to inflammation, FFAs-induced lipotoxicity contributes to the pathogenesis of NAFLD, being saturated FFAs the more toxic lipid species (58-60). Although the evaluation of the apoptotic responses under inflammatory conditions was not the major goal of this study, we detected cleavage of caspase 3 together with an increase in the percentage of apoptotic cells in hepatocytes treated with CM-P, suggesting that the signals derived from the macrophage M1 polarization are likely involved in lipoapoptosis. This interesting issue deserves future research.

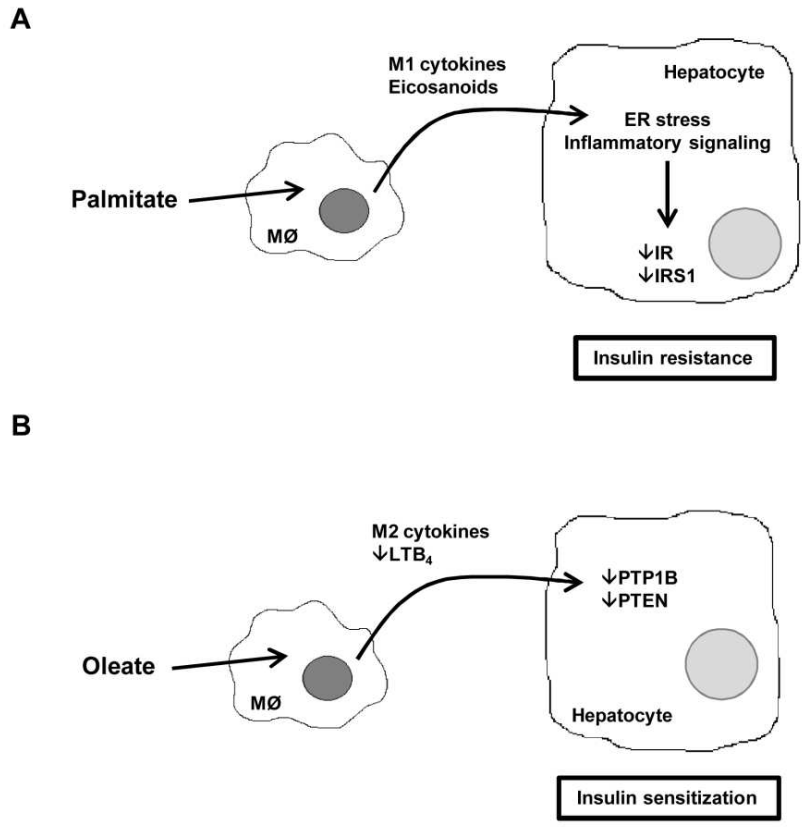

Figure 9. **A.** Schematic representation of palmitate-induced M1 polarization of macrophages that leads to a negative cross-talk with hepatic insulin signaling. **B**. Schematic representation of the beneficial effects of oleate in switching macrophages polarization by inducing M2 state to enhance insulin sensitivity in hepatocytes.

The fact that CM-O pretreatment enhanced insulin signaling in hepatocytes reaching levels of IR and Akt phosphorylation above the controls (treated with CM-B) together with the observation that the levels of proinflammatory cytokines in CM-O did not decrease below those found in the controls, prompted us to investigate additional macrophage-secreted molecules that could account for insulin sensitization. In a broad lipidomic analysis of eicosanoid species we noted that levels of LTB_4 decreased in CM-O as compared to CM-B or CM-P. LTB_4 is involved in sustaining chronic inflammation and insulin resistance in obesity (61,62) and, more recently,

deficiency of LTB_4 receptor in mice (BLT1-deficient mice) has been reported to confer protection against systemic insulin resistance and diet-induced obesity (25). Notably, livers of BLT1-deficient mice showed decreased hepatic triglyceride accumulation and enhanced Akt phosphorylation upon insulin injection. Preincubation of mouse hepatocytes with LTB_4 increased STAT3 and p38 MAPK phosphorylation and in agreement with previous data decreased insulin-induced Akt phosphorylation. Moreover, when LTB_4 was added to CM-O insulin sensitization was abolished in hepatocytes as manifested by decreased IR tyrosine and Akt Ser473 and Thr308 phosphorylations. Importantly, the supplementation of CM-O with LTB_4 upregulated PTP1B protein levels in hepatocytes. Previous findings have evidenced that macrophage-derived factors in response to LPS enhanced the effect of insulin in muscle cells by increasing Akt phosphorylation, GLUT4 translocation to the plasma membrane and glucose uptake due to an elevation of IL10 (30). Since no differences in IL10 levels were found between macrophages loaded with oleate or BSA, our data pinpoint firstly LTB_4 as one of the macrophage-secreted lipid species which is decreased by oleate leading to a positive effect in the macrophage-hepatocyte axis and, secondly, PTP1B as a critical node of the insulin signaling that is modulated by levels of LTB_4. Although PTP1B has been involved in obesity and inflammation (63-65), this is the first study showing the modulation of PTP1B protein levels by a macrophage-derived lipid product from oleate with a parallel enhancement of insulin-mediated IR tyrosine phosphorylation in hepatocytes. These data might be of relevance since PTP1B has emerged as a therapeutic target against obesity-mediated insulin resistance by its ability to regulate peripheral (muscle and liver) insulin sensitivity (64,66-71) as well as the central control of appetite and energy expenditure (63,72,73). Besides PTP1B, CM-O decreased PTEN content which boosts the enhancement of insulin signaling at Akt level and this effect was also reverted by addition of LTB_4. As recent data have revealed that unsaturated FFAs up-regulate microRNA-21 to induce PTEN degradation (74), the contribution of this phosphatase to the modulation of insulin signaling in hepatocytes under pro or antiinflammatory conditions should also be considered.

In summary, we have demonstrated an endocrine/paracrine cross-talk from macrophages/Kupffer cells to hepatocytes, which bears opposite differences depending on the factors secreted by the immune cells when loaded with palmitate or oleate. LTB_4 was identified as a lipid product that mediates this cross-talk since reduced secretion of LTB_4 by macrophages concurs with the enhancement of insulin sensitivity in hepatocytes. To our knowledge and as summarized in Figure 9B, this is the first study providing data of the beneficial effects of oleate in switching macrophages/Kupffer polarization to increase insulin sensitization in hepatocytes.

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Acknowledgements We acknowledge J. Muntané for supplying human primary hepatocytes. A. G-R holds a CIBERDEM (ISCIII) postdoctoral contract.

Footnotes

* This work was supported by SAF2012-33283, (Ministerio de Economía y Competitividad, Spain), Comunidad de Madrid S2010/BMD-2423 (Spain), EFSD and Amylin Paul Langerhans Grant, Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM, Instituto de Salud Carlos III, Spain). A. G-R receives support from CIBERDEM.

To whom correspondence should be addressed: Águeda González-Rodríguez and Ángela M. Valverde, Instituto de Investigaciones Biomédicas Alberto Sols, C/ Arturo Duperier 4, 28029 Madrid, Spain. Emails: aguedagr@iib.uam.es; avalverde@iib.uam.es. Tel: 34-915854497. Fax: 34-915854401

Abbreviations: PTP1B, protein tyrosine phosphatase 1B, PTEN, phosphatase and tensin homolog, JNK, c-jun (NH2) terminal kinase, IR, insulin receptor, IRS, insulin receptor substrate, FBS, fetal bovine serum, CM, conditioned medium.

En este segundo capítulo se han descrito las respuestas de los hepatocitos a las citoquinas y especies lipídicas secretadas por los macrófagos cargados con dos FFAs de interés, AO y AP, que, como se ha mencionado anteriormente, son los FFAs mayoritarios tanto en la dieta como en la circulación sanguínea. Asimismo, se ha analizado por primera vez la interacción molecular entre las diferentes señales procedentes de los macrófagos y la señalización de la insulina en los hepatocitos. Curiosamente, encontramos efectos opuestos sobre la señalización de la insulina, tanto en hepatocitos murinos como en hepatocitos humanos expuestos al medio condicionado de macrófagos tratados con AO (MC-O) o AP (MC-P), con un aumento significativo o disminución, respectivamente, en comparación con los hepatocitos control (tratados con medio condicionado de macrófagos tratados con BSA (MC-B)). Esta respuesta opuesta se evidenció tanto en la fosforilación en tirosina del RI como en la fosforilación de AKT tras la estimulación con insulina. Estos resultados sugieren que los ácidos grasos libres insaturados y saturados provocan respuestas inflamatorias distintas en los macrófagos y esto podría modular de manera diferencial la señalización de la insulina en las células hepáticas. De hecho, el AP, y no el AO, induce un estado de polarización M1, que se refleja por el aumento de los marcadores TNFα, IL-6, IL1 e iNOS en macrófagos RAW 264.7. La ausencia de polarización al estado M1 es fundamental para entender la modulación de la señalización de la insulina por AO en hepatocitos como se discutirá más adelante. De hecho, el aumento de los niveles de arginasa 1 refleja un perfil M2 de los macrófagos RAW 264.7 tras su exposición a AO.

En cuanto al estrés del RE, en los hepatocitos tratados con el MC-P rápidamente se activa la UPR induciéndose la fosforilación de PERK, eiF2α y JNK, lo cual conlleva a un aumento de la expresión de CHOP. Bajo estas condiciones experimentales, también aumentó la fosforilación de STAT3, factor de transcripción que activa la

transcripción de genes proinflamatorios, probablemente debido al aumento de la citoquina proinflamatoria IL-6. La convergencia de todas estas cascadas de señalización proinflamatorias conduce a una regulación negativa de la cascada de señalización de la insulina, mediante la degradación tanto del RI como del IRS1.

La activación de las células de Kupffer, macrófagos residentes en el hígado, y la consiguiente secreción de mediadores proinflamatorios es un evento clave en el inicio de la NAFLD. Por tanto, la limitación de su polarización hacia un estado M1 se considera una estrategia para combatir la inflamación crónica del hígado (53-56). El efecto diferencial producido por el AP y el AO en la secreción de citoquinas pro-inflamatorias por parte de los macrófagos RAW 264,7 también se encontró en las células de Kupffer y, sobre todo, se observaron diferencias similares en la activación de las quinasas de estrés y en la modulación de la señalización de la insulina en hepatocitos primarios tras estimularlos con el MC de las células de Kupffer tratadas con AO ó AP.

El hecho de que el MC-O mejore la señalización de insulina en los hepatocitos sin reducir los niveles de citoquinas proinflamatorias en comparación con el MC control (MC-B), nos llevó a investigar si el efecto de otras moléculas secretadas por los macrófagos como son las especies lipídicas derivadas de los ácidos grasos, pudiera explicar esta sensibilización a la insulina. Tras un amplio análisis lipidómico, se observó que los niveles de LTB_4 disminuyeron en el MC-O en comparación con MC-B o MC-P. Se sabe que el LTB_4 está involucrado en la inflamación crónica y en la resistencia a la insulina inducida en estados de obesidad (53,54) y nuestros datos confirman que el tratamiento de hepatocitos con LTB_4 aumenta la fosforilación de STAT3 y p38, y reduce la fosforilación de AKT inducida por insulina. Por otra parte, cuando añadimos LTB_4 al MC-O, encontramos que se perdía esa hipersensibilización a la insulina producida por el MC-O en los hepatocitos, y este efecto en parte, podría

explicarse por la recuperación de los niveles de PTP1B hasta alcanzar niveles similares a la situación control.

En resumen, en este segundo trabajo se ha demostrado una relación endocrina/paracrina entre macrófagos/células de Kupffer, respectivamente, y hepatocitos, que conlleva a diferentes respuestas dependiendo de los factores secretados por las células inmunes cuando se cargan con AP o AO. Asimismo, el LTB_4 se ha identificado como uno de los productos lipídicos que pueden mediar esta relación ya que la reducción en la secreción de LTB_4 por los macrófagos tratados con AO coincide con la mejora de la sensibilidad a la insulina en los hepatocitos tratados con ese MC.

* Las referencias de esta parte corresponden a las del artículo II

CAPÍTULO III

CAPÍTULO III

CARACTERIZACIÓN DEL PAPEL DE LA PROTEÍNA TIROSINA FOSFATASA 1B (PTP1B) EN LA MODULACIÓN DE LAS RESPUESTAS PRO Y ANTIINFLAMATORIAS DE LOS MACRÓFAGOS.

Diversos estudios indican que la inhibición de la proteína tirosina fosfatasa PTP1B se puede sugerir como una importante estrategia terapéutica para mejorar la sensibilidad a la insulina en diferentes tejidos. En este tercer capítulo se describe el papel de la PTP1B sobre la respuesta inmune de los macrófagos. Para ello, por un lado, se han aislado macrófagos primarios de ratones de genotipos salvaje y deficientes en PTP1B y, por otro, se han obtenido macrófagos humanos y se han transfectado con RNAs de interferencia (siRNA) dirigidos frente a la PTP1B. En ambos sistemas, observamos que la deficiencia de PTP1B incrementa la respuesta inmune de los macrófagos frente a estímulos proinflamatorios, a la vez que disminuye la respuesta alternativa M2 ó antiinflamatoria.

Además, la ausencia de PTP1B induce una pérdida de la viabilidad en los macrófagos en reposo. Tras un análisis genómico de muestras de macrófagos salvajes y deficientes en PTP1B obtenidas tras un tratamiento de 24 h con estímulos proinflamatorios, se observó que los macrófagos deficientes en PTP1B presentaban un aumento de la expresión de genes implicados en las vías de p53, reparación del DNA y señalización de la insulina.

Por otro lado, en el modelo animal de sobrecarga de LPS y D-galactosamina para revelar respuestas inflamatorias *"in vivo"*, los ratones deficientes en PTP1B exhiben una mayor tasa de mortalidad.

En resumen, los resultados de este tercer trabajo indican que, aunque la inhibición de PTP1B tiene potentes beneficios en el tratamiento de la diabetes, por otro lado puede producir efectos no deseados por el riesgo de una respuesta proinflamatoria exacerbada comprometiendo, al menos, la viabilidad de los macrófagos.

Estos resultados dieron lugar a la publicación del artículo "**Pivotal role of protein tyrosine phosphatase 1B (PTP1B) in the macrophage response to pro-inflammatory and anti-inflammatory challenge"** en la revista **"*Cell Death and Disease*"** (2014) 5, e1125; doi:10.1038/cddis.2014.90

OPEN

Citation: Cell Death and Disease (2014) 5, e1125; doi:10.1038/cddis.2014.90

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Pivotal role of protein tyrosine phosphatase 1B (PTP1B) in the macrophage response to pro-inflammatory and anti-inflammatory challenge

PG Través[1,5], V Pardo[1,2,5], M Pimentel-Santillana[1,5], Á González-Rodríguez[1,2], M Mojena[1], D Rico[3], Y Montenegro[1], C Calés[1], P Martín-Sanz[1,4], AM Valverde*[,1,2] and L Boscá*[,1,4]

Inhibition of protein tyrosine phosphatase 1B (PTP1B) has been suggested as an attractive target to improve insulin sensitivity in different cell types. In the present work, we have investigated the effect of PTP1B deficiency on the response of human and murine macrophages. Using *in vitro* and *in vivo* approaches in mice and silencing PTP1B in human macrophages with specific siRNAs, we have demonstrated that PTP1B deficiency increases the effects of pro-inflammatory stimuli in both human and rodent macrophages at the time that decreases the response to alternative stimulation. Moreover, the absence of PTP1B induces a loss of viability in resting macrophages and mainly after activation through the classic pathway. Analysis of early gene expression in macrophages treated with pro-inflammatory stimuli confirmed this exacerbated inflammatory response in PTP1B-deficient macrophages. Microarray analysis in samples from wild-type and PTP1B-deficient macrophages obtained after 24 h of pro-inflammatory stimulation showed an activation of the p53 pathway, including the excision base repair pathway and the insulin signaling pathway in the absence of PTP1B. In animal models of lipopolysaccharide (LPS) and D-galactosamine challenge as a way to reveal *in vivo* inflammatory responses, animals lacking PTP1B exhibited a higher rate of death. Moreover, these animals showed an enhanced response to irradiation, in agreement with the data obtained in the microarray analysis. In summary, these results indicate that, although inhibition of PTP1B has potential benefits for the treatment of diabetes, it accentuates pro-inflammatory responses compromising at least macrophage viability.
Cell Death and Disease (2014) **5,** e1125; doi:10.1038/cddis.2014.90; published online 13 March 2014
Subject Category: Immunity

Protein tyrosine phosphatase 1B (PTP1B) is a ubiquitously expressed phosphatase that has emerged as a relevant modulator of signaling pathways initiated by the activation of the tyrosine kinase receptor superfamily.[1] Clinically relevant, PTP1B is a critical node of insulin signaling due to its ability to dephosphorylate and inactivate the insulin receptor, thereby switching off insulin signaling.[2,3] Indeed, PTP1B-deficient mice are a unique model of insulin hypersensitivity due to enhanced insulin action.[4–6] These mice are protected against diet[4,6] and age-induced obesity and insulin resistance linked to a low grade of chronic inflammation in white adipose tissue.[7,8] PTP1B is also involved in the control of immune cell signaling,[9,10] controlling cytokine signaling pathways by dephosphorylation of janus kinase 2 (JAK2), non-receptor tyrosine-protein kinase 2 (TYK2) and signal transducer and activator of transcription 5 (STAT5).[11,12] Moreover, it has been reported that interleukin-4 (IL-4) induced PTP1B mRNA in a phosphatidylinositol 3-kinase (PI3K)-dependent manner and enhanced PTP1B protein stability to suppress IL-4-induced STAT6 signaling.[13]

PTP1B-deficient mice exhibited an increase in monocyte/macrophages in the spleen and the bone marrow.[14] This was due to a decreased threshold of response to macrophage colony-stimulating factor (M-CSF) by enhancing tyrosine phosphorylation of the activation loop of its receptor (M-CSF1R). In addition to lineage specification, PTP1B

[1]Instituto de Investigaciones Biomédicas Alberto Sols (Centro Mixto CSIC-UAM), Madrid, Spain; [2]Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (Ciberdem), ISCIII, Madrid, Spain; [3]Structural Biology and Biocomputing Programme, Spanish National Cancer Research Center (CNIO), ISCIII, Madrid, Spain and [4]Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (Ciberehd), ISCIII, Madrid, Spain
*Corresponding author: AM Valverde or L Boscá, IB-Alberto Sols, Arturo Duperier 4, Madrid 28029, Spain. Tel: +34 91585400; Fax: +34 915854401; E-mail: avalverde@iib.uam.es (AMV) or Tel/Fax: +34 914972747; E-mail: lbosca@iib.uam.es (LB)
[5]These authors contributed equally to this work.
Keywords: protein tyrosine phosphatase; cell viability; p53; radiation sensitivity
Abbreviations: ALT, alanine aminotransferase; AKT, protein kinase B; Arg-1, arginase-1; CXCL, chemokine (C-X-C motif) ligand; COX-2, cyclooxygenase-2; DE, differentially expressed; D-GalN, D-galactosamine; ERK, extracellular signal-regulated kinase; F4/80, EGF-like module-containing mucin-like hormone receptor-like 1; FDRs, false discovery rates; GSEA, gene set enrichment analysis; HO-1, heme oxygenase 1; IFN, interferon; IL, interleukin; i.p., intraperitoneal; IRF-3, IFN regulated factor 3; JAK/STAT, janus kinase/signal transducer and activator of transcription; JNK, c-Jun N-terminal kinase; KEGG, Kyoto encyclopedia of genes and genomes; LPS, lipopolysaccharide; KO, knockout; LTA, lipoteichoic acid; MAPK, mitogen-activated protein kinase; M-CSF, macrophage colony-stimulating factor; MPO, myeloperoxidase; NF-κB, nuclear factor κB; NO, nitric oxide; NOS-2, nitric oxide synthase-2; PGE_2, prostaglandin E_2; PI3K, phosphatidylinositol 3-kinase; polyI:C, polyriboinosinic:polyribocytidylic acid; PVDF, polyvinylidene fluoride; q(RT)-PCR, quantitative real-time (RT) PCR; POV, peroxovanadate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TGF-β, transforming growth factor β; TLRs, Toll-like receptors; TNF-α, tumor necrosis factor-α; TPA, tetradecanoylphorbol acetate; TYK, non-receptor tyrosine-protein kinase; WT, wild type
Received 27.11.13; revised 31.1.14; accepted 10.2.14; Edited by T Brunner

modulates activation of monocytes/macrophages. In fact, in the absence of PTP1B tissue macrophages display an activated phenotype assessed by the increased expression of CD80 that has a role in the maintenance of inflammation. However, this effect might require developmental adaptations in macrophages since short-term treatment of mice with the PTP1B inhibitor suramin protects against apoptotic liver damage induced by CD95 and against endotoxic shock mediated by tumor necrosis factor-α (TNF-α).[15]

In this work, we have characterized the effect of PTP1B deficiency on the early signaling in response to pro-inflammatory challenge in murine and human macrophages. Our data show that in the absence of PTP1B both classic and innate responses (M1 polarization) are overactivated at the time that alternative polarization (M2) is reduced.[16,17] Studies using *in vivo* models provide support to these enhanced pro-inflammatory responses. However, these data also suggest that the broad use of PTP1B inhibitors, although with potential benefits over the insulin signaling pathway, might exert undesirable effects on response to stressors of the immune system including the fine tuning of the pro-inflammatory and pro-resolution balance.

Results

PTP1B deficiency results in enhanced M1 responses and attenuated activation by M2 stimuli. Peritoneal macrophages from wild-type (WT) and PTP1B-deficient mice were activated with lipopolysaccharide (LPS) and polyriboinosinic:polyribocytidylic acid (polyI:C) (M1 stimuli) and the expression of genes characteristic of the innate immune response was determined. PTP1B deficiency results in an enhancement in the levels of these M1 representative genes (Figure 1a). In the same way, challenge with combinations of IL-4 and IL-13 (M2 cytokines) decreased heme oxygenase 1 (HO-1) and arginase-1 (Arg-1) expression reflecting attenuation of M2 responses in PTP1B knockout (KO) macrophages (Figure 1b). Interestingly, PTP1B levels were upregulated upon M1 or M2 challenge (Figure 1c). The accumulation of nitric oxide (NO), prostaglandin E2 (PGE_2) and TNF-α in the culture medium (Figure 1d), and the time course of the rise in the mRNA levels of IL-6 and nitric oxide synthase-2 (NOS-2) (Figure 1e) were potentiated in PTP1B KO macrophages upon M1 activation. Moreover, a significant loss of viability was observed in PTP1B-deficient cells after 48–72 h of culture (Figure 1f).

Early pro-inflammatory signaling in PTP1B-deficient peritoneal macrophages. To evaluate the impact of PTP1B deficiency on the early response of macrophages to LPS, the nuclear factor-κB (NF-κB) pathway was analyzed. The degradation of IκBα was more efficient in PTP1B KO *versus* the WT counterparts, at the time that the late recovery by resynthesis was impaired (Figure 2a). This was also confirmed when the kinetics of the IκBα mRNA levels was determined (Figure 2b). Similar results were observed for IκBβ. Regarding the interferon (IFN) regulated factor 3 (IRF-3) pathway, our data show a lesser upregulation in PTP1B KO macrophages, confirming previous work.[18] Finally, the absence of PTP1B resulted in a higher translocation of p65 from the cytosol to the nucleus (Figure 2c).

The phosphorylation state of protein kinase B (AKT) and the mitogen-activated protein kinases (MAPKs), two well-known targets activated after LPS challenge, was investigated. As Figure 3a shows, AKT phosphorylation was sustained in cells lacking PTP1B. The same response was evidenced in the MAPKs extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK). Since PI3K has a role in the attenuation of the response to LPS in macrophages,[19,20] cells lacking PTP1B were incubated with the PI3K inhibitor LY294002 and then activated with LPS. As Figure 3b shows, AKT phosphorylation was suppressed whereas the phosphorylation of p38 and JNK was significantly attenuated. However, the levels of phospho-ERK remained unchanged regardless the presence of LY294002. Since PI3K inhibition results in enhanced IκBα degradation in response to LPS,[20,21] the contribution of this pathway to the phenotype observed in PTP1B-deficient cells was investigated.[19,21–23] Macrophages challenged with low doses of LPS exhibited a similar response in terms of IκBα degradation with negligible recovery at 45 min after PI3K inhibition regardless the presence of PTP1B (Figure 3c). In the same regard, treatment with recombinant PTP1B of extracts from PTP1B WT or KO macrophages activated for 30 min with LPS resulted in a robust dephosphorylation of all MAPKs but not of AKT (Figure 3d).

Mini-array and microarray analysis of pro-inflammatory responses in macrophages from PTP1B-deficient mice. Because PTP1B-deficient macrophages have an enhanced response to pro-inflammatory stimuli a mini-array analysis of chemokine and receptors was performed. As Supplementary Figure S1 summarizes the levels of Ccl2, Ccl5, Ccl7 and Ccl9, chemokines that recruit inflammatory cells, were significantly elevated in PTP1B KO macrophages. To better understand these results, we performed microarray gene expression analysis in PTP1B WT or KO macrophages. In all, 276 probes were differentially expressed (DE, false discovery rate (FDR) < 0.05; Figure 4a) in WT *versus* PTP1B KO macrophages stimulated with LPS. Among the DE genes, we identified chemokines (IL-6, CCL2 and CCL17), which showed significantly higher expression in PTP1B KO cells, in agreement with qPCR and mini-array results. Additionally, chemokine (C-X-C motif) ligand 5 (CXCL5) also showed higher expression in PTP1B-deficient macrophages. However, an important fraction of DE genes tend to show lower upregulation after 24 h of LPS challenge in PTP1B KO cells (Figure 4a, *heatmap of only DE genes*). Interestingly, Toll-like receptor 4 (TLR4) was downregulated at 24 h in PTP1B KO macrophages, suggesting a complex scenario where deregulation of the inflammatory response in PTP1B-deficient macrophages is occurring at different levels of the regulatory network. To get biological insights about the pathways altered, we performed gene set enrichment analysis (GSEA) using Kyoto encyclopedia of genes and genomes (KEGG) and REACTOME pathways (Figure 4b). Four KEGG pathways were significantly upregulated (FDR < 0.25) in PTP1B KO macrophages ('p53 signaling', 'Base excision repair', 'Propanoate metabolism' and 'Allograft rejection') and one REACTOME pathway

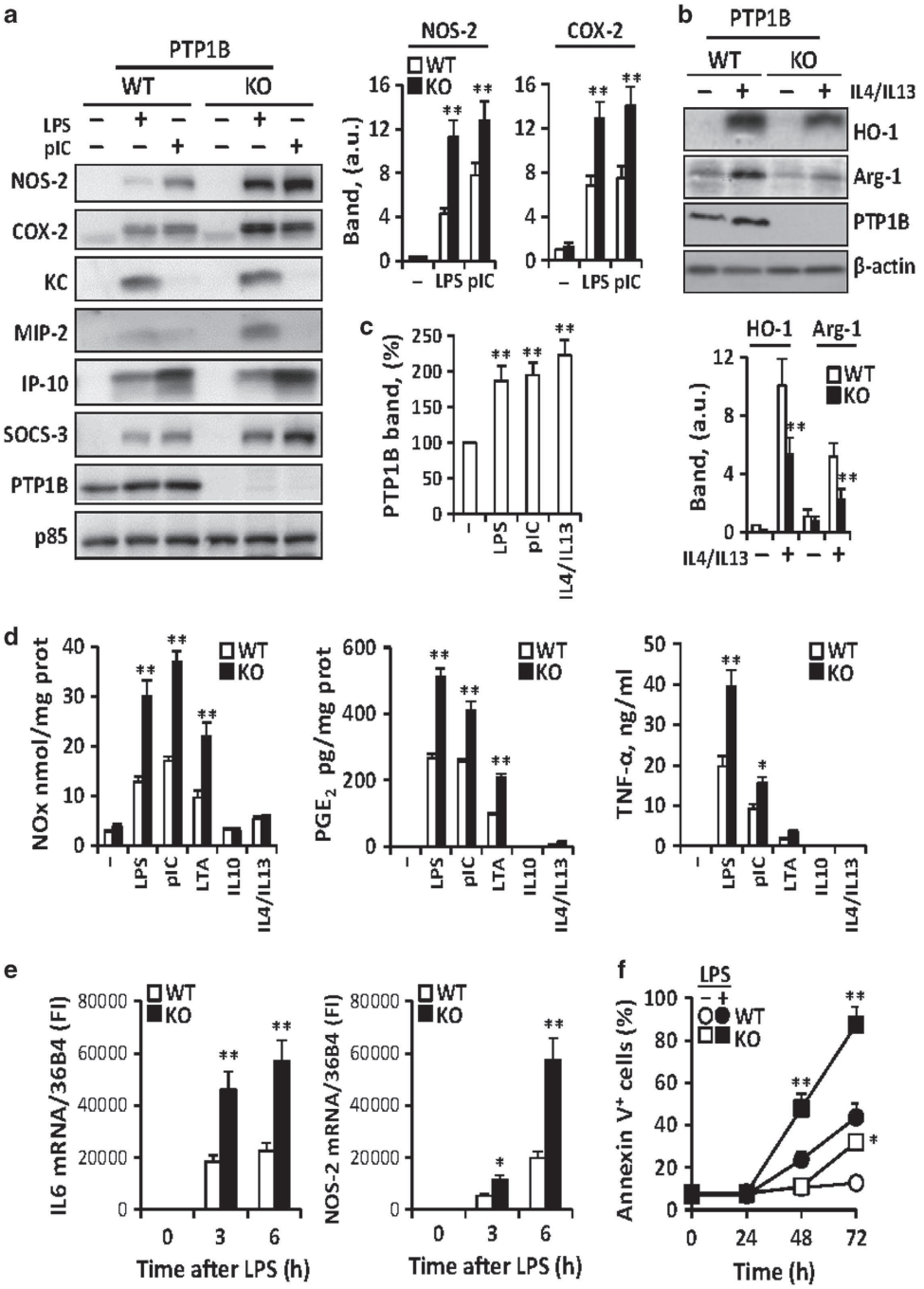

Figure 1 Effect of PTP1B deficiency on the polarization of macrophages. Peritoneal macrophages from wild-type (WT) or PTP1B-deficient (KO) mice were isolated after thioglycollate eliciting. Cells were stimulated with 200 ng/ml of LPS or 25 μg/ml polyI:C (M1 stimuli) and the levels of the indicated proteins were determined at 24 h (**a**). The response to M2 stimuli was analyzed after challenge for 24 h with a mixture of 20 ng/ml each IL-4 + IL-13 (**b**). The levels of PTP1B in M1 and M2 polarized macrophages were determined (**c**). The accumulation of NO, PGE_2 and TNF-α in the incubation medium was determined after 24 h of activation with 200 ng/ml LPS, 25 μg/ml polyI:C, 5 μg/ml LTA, 20 ng/ml IL-10 or 20 ng/ml of IL-4 + IL-13 (**d**). The time course of mRNA levels of IL-6 and NOS-2 after activation with 200 ng/ml LPS (**e**) and of cells undergoing apoptosis by annexin V binding criteria (**f**) were determined. Results show the mean ± S.D. of three independent experiments. *$P<0.05$; **$P<0.01$ *versus* the same condition in the WT cells or non-treated cells (**c**). (**a**–**c**) The band intensities of NOS-2, COX-2 (**a**), HO-1, Arg-1 (**b**) and PTP1B (**c**) after normalization using p85 and β-actin, respectively, are shown

('Insulin Synthesis and Processing'). The alterations in insulin synthesis reflect the known role of PTP1B in insulin signaling,[1–3,5,7,12,24] while the DE in propanoate metabolism genes suggests a possible novel role for PTP1B in this pathway. In fact, propionic acid has been associated with lipogenesis and glucose uptake and has an anti-inflammatory effect on human macrophages.[25,26] The so-called 'Allograft rejection' pathway included PTP1B upregulated inflammatory genes such as perforin 1, IL-2, CD40LG and IL-12A. Interestingly, the alteration in p53 and base-excision

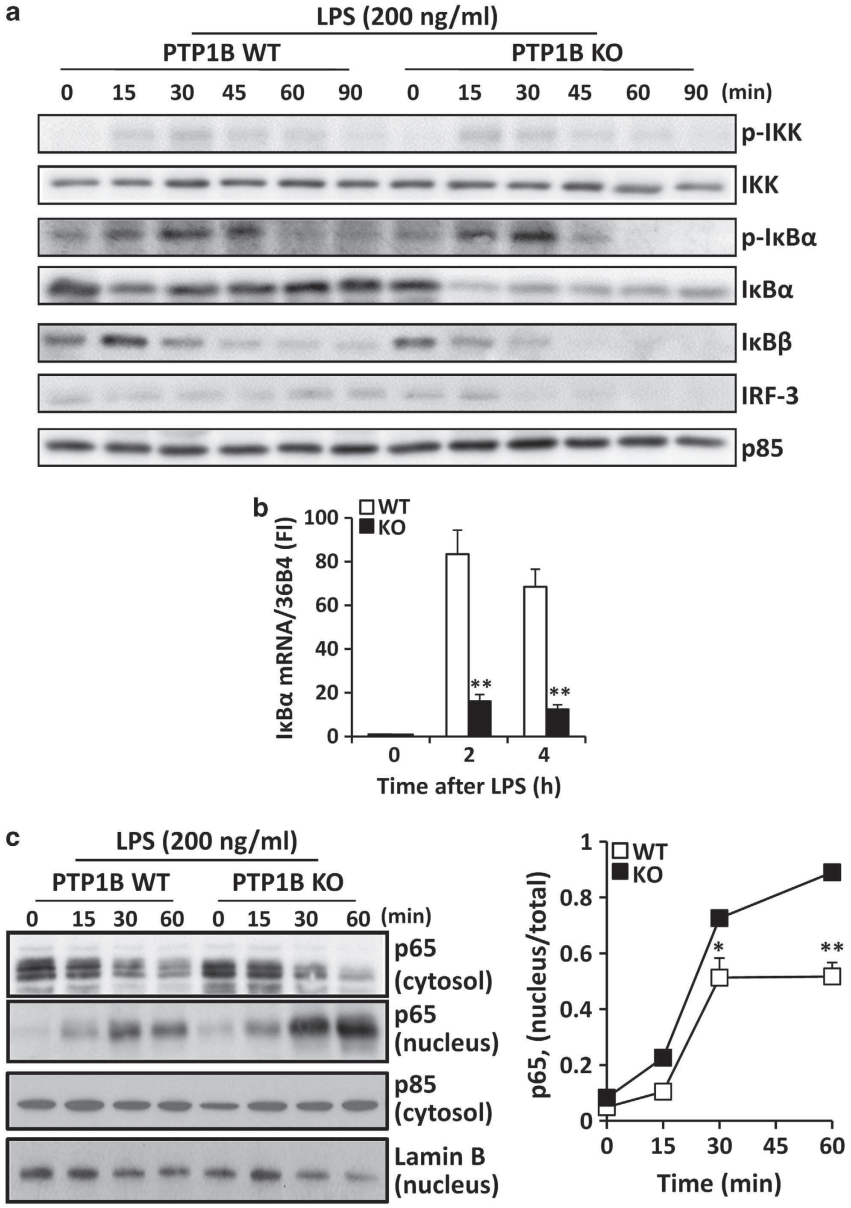

Figure 2 PTP1B deficiency enhances IκBα degradation and impairs IκBα recovery. Macrophages from WT and PTP1B KO mice were stimulated with 200 ng/ml of LPS for the indicated times and the NF-κB and IRF3 pathways were analyzed (**a**). The upregulation of IκBα mRNA expression as a sensor of resetting of NF-κB was determined by qPCR (**b**). The nuclear translocation of p65 was determined by western blotting using p85 and lamin B as markers of the corresponding cytosolic and nuclear fractions, respectively (**c**). Results show the mean ± S.D. of four independent experiments (**b**) or a representative blot out of four (**a**, **c**). $^{*}P<0.05$; $^{**}P<0.01$ *versus* the same condition in the WT cells

pathways could be directly related with the higher sensitivity of PTP1B-deficient mice to high doses of irradiation. Notably, PTP1B KO macrophages showed upregulation of many genes activated after DNA damage (i.e., PCNA, ATR, CHEK2, GADD45A and BAX) and this exacerbated response could explain the apoptosis of these cells at 48 h (Figure 1e).

Effect of silencing human PTP1B on the response of human monocytes/macrophages to pro-inflammatory stimulation. PTP1B siRNA efficiently decreased the levels of PTP1B at the time that moderately increased the expression of cyclooxygenase-2 (COX-2) after pro-inflammatory challenge (Figure 5a). In addition to this, the response to LPS + IFN-γ resulted in a decrease in IκBα (without recovery as in the murine counterparts) and accelerated IκBβ degradation (Figure 5b). The phosphorylation of AKT and MAPKs was notably enhanced after silencing PTP1B (Figure 5b). Also, TNF-α and PGE_2 levels measured in the culture medium increased in cells treated with PTP1B siRNA (Figure 5c). This enhanced response to pro-inflammatory challenge in cells with reduced PTP1B levels resulted in a higher susceptibility to apoptosis in response to LPS and staurosporine (Figure 5d). Notably, the bright-field morphology

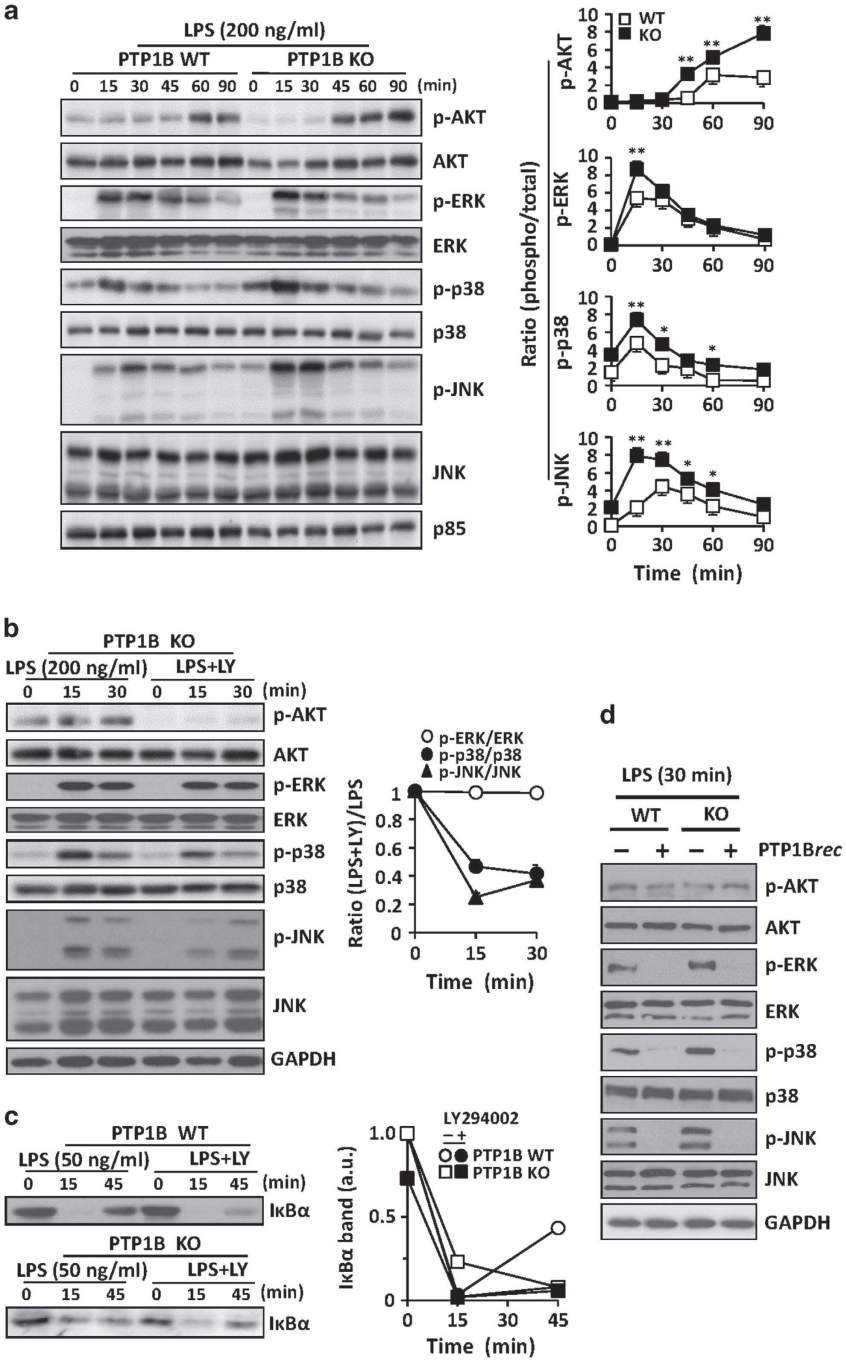

Figure 3 PTP1B deficiency enhances AKT and MAPK activation. Macrophages from WT and PTP1B KO mice were stimulated with 200 ng/ml of LPS and the phosphorylation of AKT, ERK, p38 and JNK was determined at the indicated times (**a**). To evaluate the effect of the PI3K pathway in the modulation of AKT and MAPK activities, macrophages from PTP1B KO mice were treated for 10 min before LPS activation with 10 μM LY29004. At the indicated times, the phosphorylation state of these proteins was determined (**b**). In parallel to the precedent assays, the levels of IκBα were determined using a lower LPS concentration (50 ng/ml) as stimulus (**c**). The effect of recombinant PTP1B on the dephosphorylation of extracts from WT and PTP1B macrophages activated for 30 min with 200 ng/ml of LPS was assayed *in vitro* after 30 min of incubation at 30 °C with recombinant PTP1B (**d**). Results show the mean ± S.D. of three independent experiments, or a representative dephosphorylation experiment (**d**). *$P<0.05$; **$P<0.01$ *versus* the same condition in the WT cells

of murine and human macrophages lacking PTP1B was very similar, exhibiting a more fusiform shape than the corresponding WT cells (Supplementary Figure S2A). Finally, inhibition of PTP activity with peroxovanadate (POV) showed a similar profile to that observed after PTP1B silencing, including the lack in recovery of IκBα, a higher translocation of p65 to the nucleus (Supplementary Figure S2B), and a loss in viability, a process that was partially inhibited by the caspase inhibitor z-VAD (Supplementary Figure S2C).

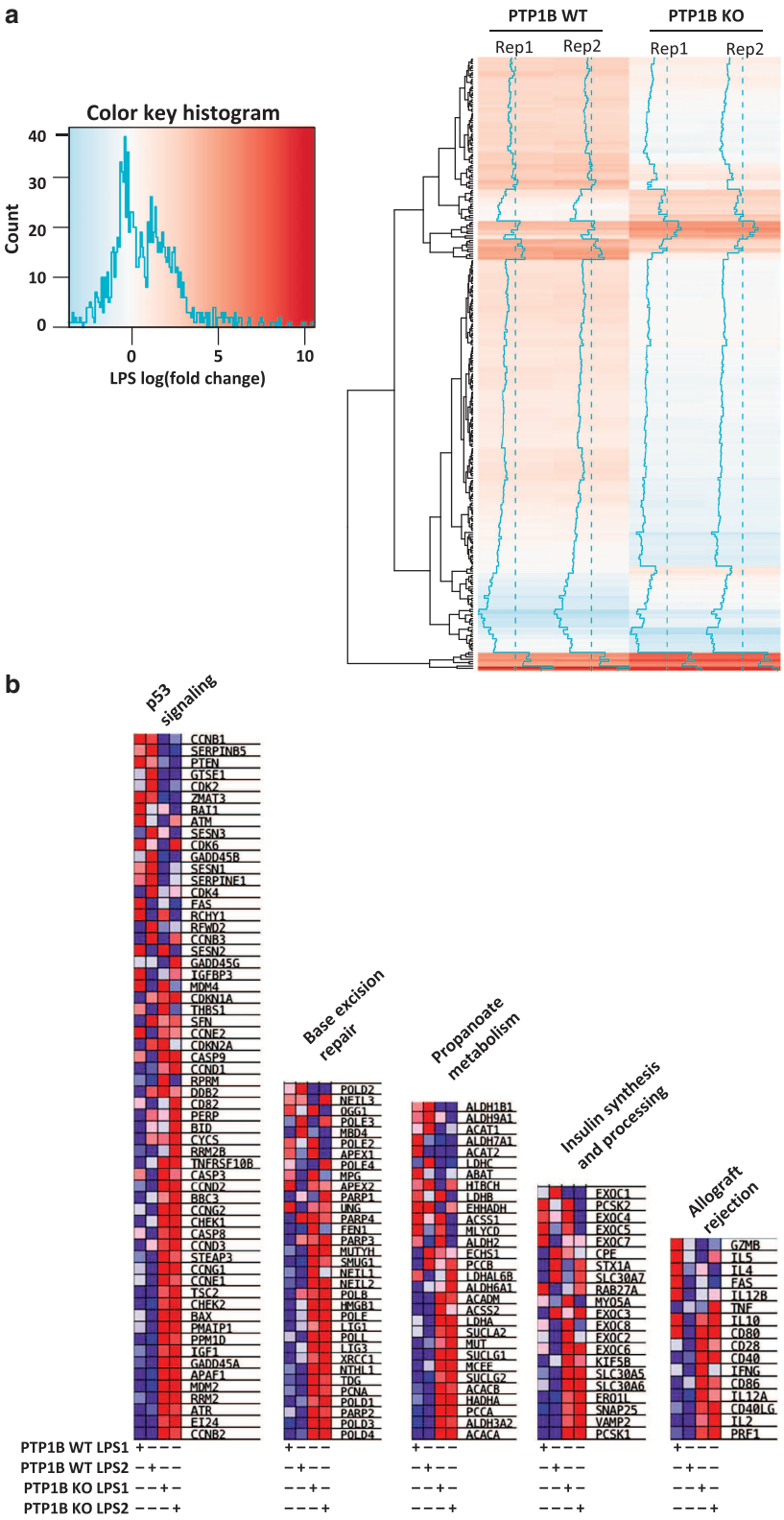

Figure 4 Gene expression profiling of WT and PTP1B-deficient macrophages treated with LPS. Macrophages were activated with 200 ng/ml of LPS for 24 h and the RNA was extracted, analyzed for quality in a Bioanalyzer and submitted to microarray hybridization (Agilent). Heatmap showing the microarray probes with differential response (FDR < 0.05) to LPS between WT and PTP1B KO macrophages (**a**). Gene set enrichment analysis (GSEA) of KEGG and REACTOME pathways in macrophages from PTP1B KO mice after pro-inflammatory stimulation (**b**). The genes in significantly enriched pathways (FDR < 0.25) are shown and the color code corresponds to the standardized gene expression per row (not LPS fold change as in **a**). Red indicates higher expression and blue lower expression

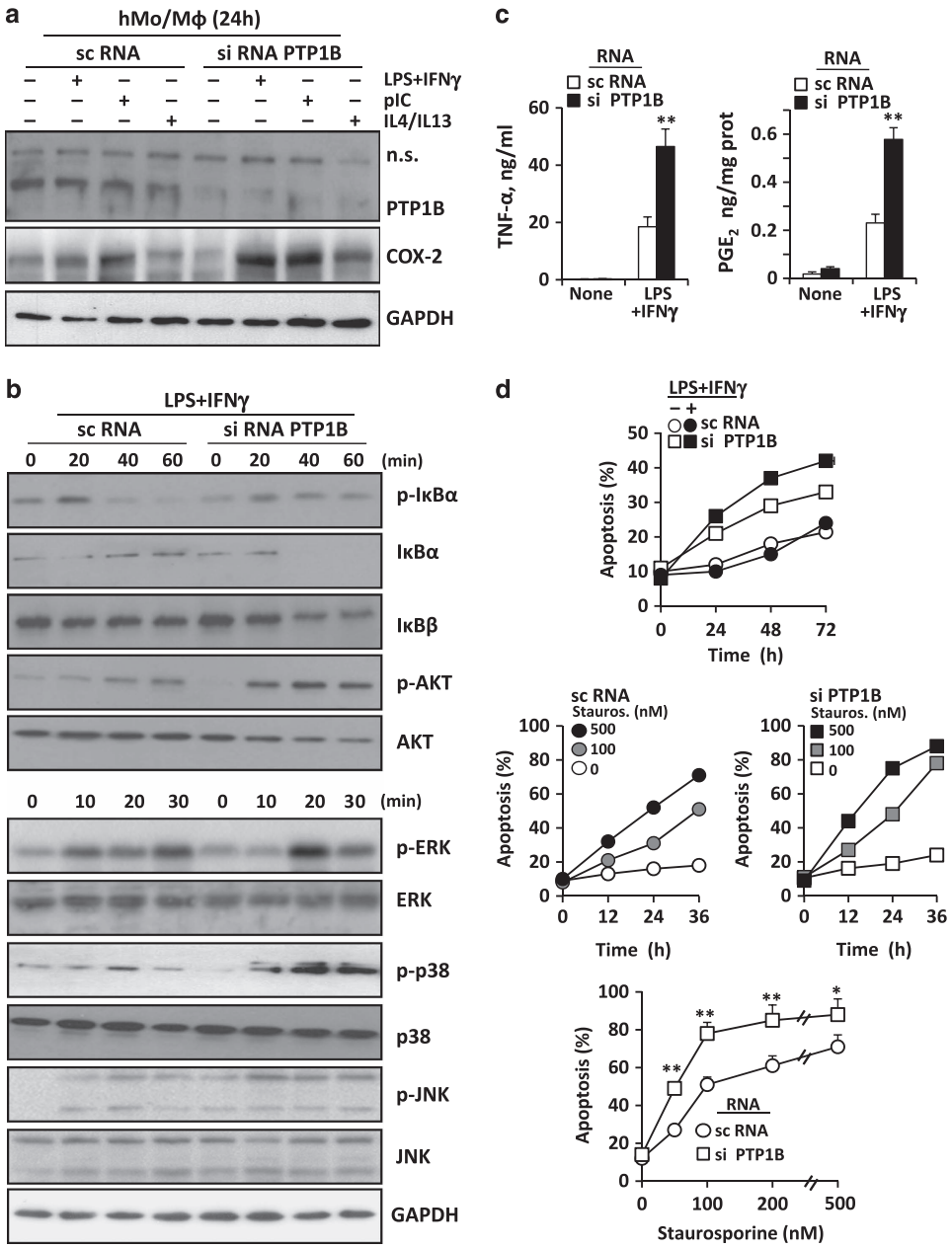

Figure 5 Effect of PTP1B silencing on the response of human macrophages. Human monocytes from buffy coats were differentiated into macrophages and treated with a mixture of sc- or siRNA oligonucleotides to silence PTP1B. After incubation for 24 h with 200 ng/ml of LPS plus 20 ng/ml of human IFN-γ, 25 μg/ml of polyI:C or 20 ng/ml of each human IL-4 + IL-13, the levels of PTP1B and COX2 were determined (**a**). The time course of the phosphorylation and total levels of proteins related to NF-κB, AKT and MAPKs were determined (**b**). The release of TNF-α and PGE_2 to the culture medium was determined with specific kits (**c**). The induction of apoptosis, measured as the annexin V-positive population, was determined in both resting and LPS + IFN-γ activated cells (**d**). In addition to this, to determine the sensitivity of cells silenced for PTP1B to apoptosis in response to pro-inflammatory dependent stimuli the time course and the sensitivity at 36 h to different concentrations of staurosporine (as an stressor to promote mitochondrial-dependent apoptosis) were determined (**d**). Results show a representative experiment or the mean ± S.D. of three independent experiments. *$P<0.05$; **$P<0.01$ *versus* the same condition in cells treated with scRNA

In vivo effects of pro-inflammatory activation in PTP1B-deficient mice. To determine the *in vivo* contribution of PTP1B to pro-inflammatory responses, the basal levels of TNF-α in serum (Figure 6a), the development of ear edema (Figures 6b and c), the rise in TNF-α levels in serum after administration of LPS or zymosan (Figure 6d), the sensitivity to irradiation (Figure 6e) and the response to LPS/D-galactosamine (D-GalN) challenge (Figures 6f–i) were analyzed. The basal levels of TNF-α in serum were significantly lower in PTP1B KO mice than in the WT counterparts. However, in the ear pro-inflammatory model induced after tetradecanoylphorbol acetate (TPA) application, PTP1B KO mice exhibited a higher accumulation of neutrophils, as measured by the myeloperoxidase (MPO) activity criteria and edema (Figures 6b and c). Also, the TNF-α levels measured in serum after i.p. challenge with LPS

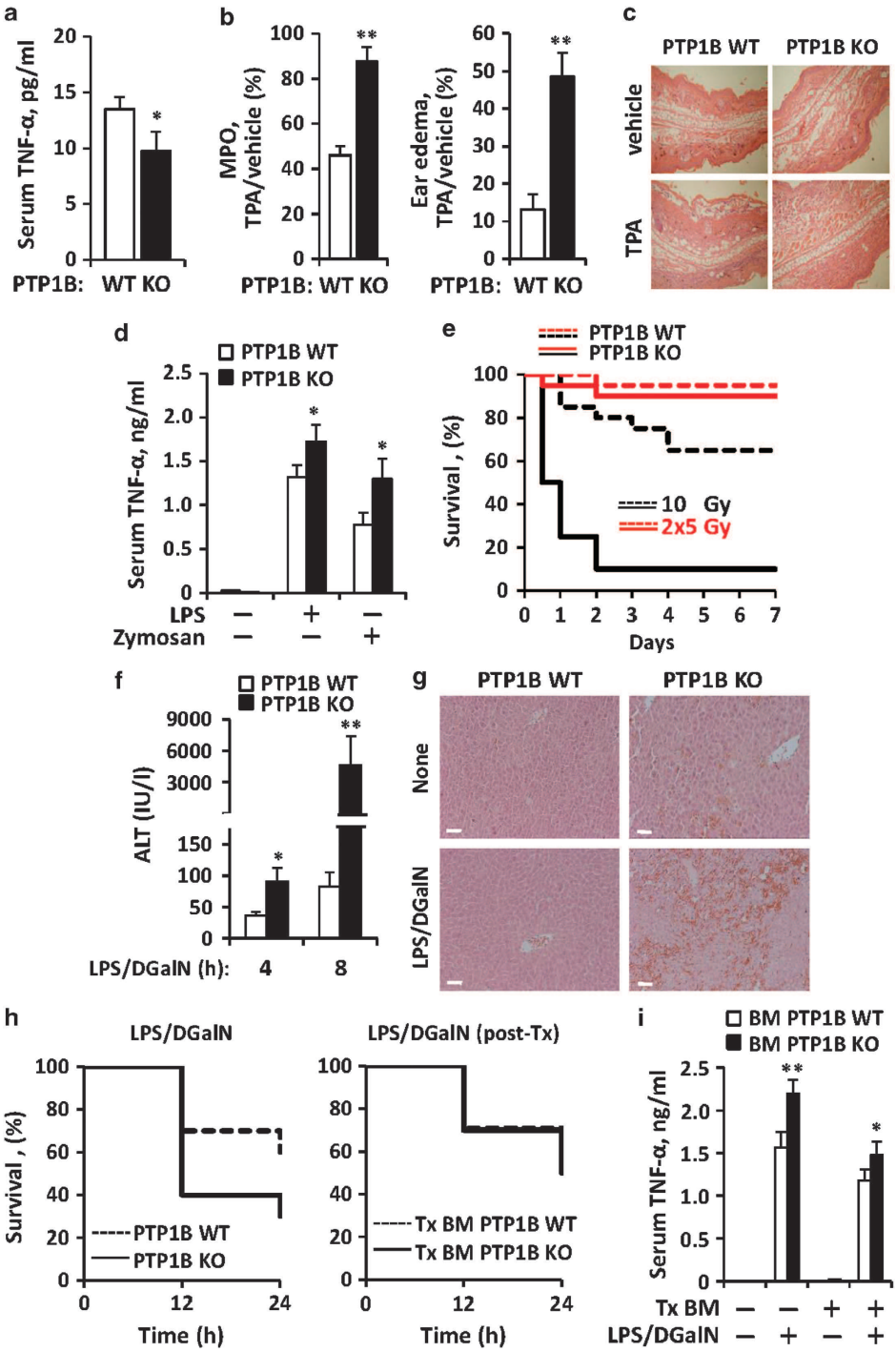

Figure 6 *In vivo* effect of PTP1B deficiency on the response of mice to different pro-inflammatory challenges. WT or PTP1B KO male mice matched in age were used in these experiments. The serum levels of TNF-α of untreated animals ($n = 12$) were determined (**a**). The ear MPO activity and edema ($n = 10$ animals per condition) after topic administration of TPA was calculated as ratio *versus* the ear treated with vehicle (**b**). Fixed ears were stained with eosin/hematoxylin to show the structure and with (sagittal sections; **c**). The serum levels of TNF-α of untreated animals or i.p. injected LPS (1 mg/kg body weight) or zymosan (30 mg/kg body weight) were determined at 1 h ($n = 7$ animals per condition) (**d**). The sensitivity to irradiation after a unique dose of 10 Gy or two doses of 5 Gy administered in an interval of 4 h ($n = 15$ per group) was evaluated as survival after 7 days post transplantation (Tx) of the corresponding bone marrow; $P = 0.0005$ *versus* the WT at 10 Gy (**e**). The liver injury, determined by the measurement of the ALT activity in the serum (**f**) and structure (eosin/hematoxylin staining at 4 h; white bar = 100 μm; **g**), and the survival of animals after i.p. administration of a single dose of LPS/D-GalN ($n = 12$ animals per condition) were determined; $P = 0.074$ *versus* the WT (**h**, *left*). Alternatively, a series of WT animals underwent irradiation (10 Gy) as depicted in (**e**), and were subsequently submitted to bone marrow transplantation from WT or PTP1B KO mice. After restitution of the immune system (12 weeks), these animals were submitted to LPS/D-GalN challenge and survival was determined (**h**, *right*). The TNF-α levels after 1 h of treatment with LPS/D-GalN were determined in serum from animals of (**i**). Results show the mean ± S.D. of the indicated number of animals and/or the Kaplan–Meier representation of survival (**e** and **h**). $^*P < 0.05$; $^{**}P < 0.01$ *versus* the same condition in the WT animals or samples

or zymosan were significantly higher in the PTP1B KO counterparts. Interestingly, these animals were very sensitive to irradiation and at least 90% died in <2 days when treated with 10 Gy, linking this response to the activation of the p53 and excision base repair pathways observed in the microarray (Figure 4b). Opposite to this situation 70% of the WT mice were alive. When the same quantity of irradiation was administered in two doses in a 4-h interval, $>90\%$ of the animals survived regardless the absence of PTP1B.

PTP1B KO mice challenged with LPS/D-GalN exhibited a higher liver injury than the WT counterparts as assessed by the rapid rise in serum alanine aminotransferase (ALT) activity (Figure 6f) and the loss of liver integrity (Figure 6g). These PTP1B KO mice exhibited a higher rate of death upon LPS/D-GalN administration (Figure 6h, *left*), that was abrogated when the experiments were repeated in irradiated WT animals reconstituted with bone marrow from PTP1B KO donor mice (Figure 6h, *right*), suggesting that resident liver macrophages (Kupffer cells?), or even hepatocytes, have a key role in the liver-induced animal death. Interestingly, the TNF-α levels in the serum of these animals at 1 h reflected a minimal, but statistically significant increase when the circulating monocytes were from PTP1B KO mice (Figure 6i). In addition to this approach, mice were treated with clodronate to remove resident macrophages. Under these conditions, reconstitution of WT-irradiated mice with the bone marrow from PTP1B KO animals failed to show differences in survival after LPS/D-GalN challenge; however, the levels of TNF-α in serum after LPS/D-GalN were significantly lower than those obtained in the above reported experimental models (avoiding clodronate treatment), which might explain the absence of differences in survival between both groups (Supplementary Figure S3A and B). Regarding the quality of the macrophage replenishment, RT-PCR analysis ensured that the liver population after transplantation was from the PTP1B KO donors (Supplementary Figure S3C) and, in the absence of transplantation, all F4/80-positive liver population was removed after 48 h of clodronate treatment (Supplementary Figure S3D).

Discussion

Macrophages respond very efficiently and regulate several signaling pathways in a strict temporal pattern in which resolution is a key component.[27,28] Among the systems that modulate this response, protein phosphatases exert a significant role.[29] Several reports suggested that PTP1B is an important modulator of the activation process of macrophages restricting the time of pro-inflammatory signaling in response to TLR and to both type I and type II IFN signaling.[14,18,30–32] Also, PTP1B could be increased or activated by inflammatory factors such as TNF-α.[33] Intriguingly, these observations contrast with results obtained in myeloid-cell (LysM)-PTP1B KO mice, despite to show enhanced NOS-2 expression and accumulation of nitrite in the medium upon LPS challenge,[34] and in a murine model of experimental colitis.[35] Opposite to these results, other studies have identified an essential role for PTP1B in obesity-induced inflammation, in regulating leukocyte recruitment during allergic inflammation and in macrophage development.[30,32,36] At the same time, evidence from the area of diabetes has suggested that inhibition of PTP1B might be envisaged as a way to prolong insulin action.[3,5,12,36] Keeping in mind these ideas, we have investigated whether the deficiency of PTP1B in murine and human macrophages affects the regulation of the response of these cells to pro-inflammatory and anti-inflammatory stimuli. Our data show that peritoneal macrophages from PTP1B KO mice presented an exacerbated inflammatory response *versus* the WT, with increased production of M1 polarization markers. Moreover, this enhanced activation in PTP1B KO macrophages was not restricted to TLR4 signaling but also occurred in response to TLR2 (lipoteichoic acid, LTA) and TLR3 (polyI:C) challenge, confirming preliminary data in the Raw 264.7 macrophage cell line.[18] Conversely, in the absence of PTP1B, macrophages show an impaired response to IL-4 plus IL-13 resulting in attenuated M2 polarization that suggests a dual role for PTP1B in the regulation of M1/M2 commitment. It is well known that induction of target genes upon TLR stimulation requires a combination of downstream signaling events, including transcriptional activation *via* NF-κB factors and activation of the PI3K/AKT and MAPK pathways.[37–39] The enhanced signaling of these three pathways in PTP1B-deficient macrophages suggests that PTP1B acts as a common negative regulator of NF-κB, AKT and MAPK activities. In this regard, the more efficient degradation of IκBα together with its delayed resynthesis in PTP1B KO macrophages resulted in a persistent and augmented p65 translocation to the nucleus and, therefore, of NF-κB DNA binding activity consistently with the observed exacerbated inflammatory responses.

Several studies have shown that the PI3K/AKT pathway exerts a key role in the regulation of the innate immune responses under pro-inflammatory conditions and against different pathogens.[20–23,39] In agreement with previous reports performed in hepatocytes,[7,36] in this study we have shown that PTP1B deficiency induces an increased and persistent AKT phosphorylation in human and mouse macrophages. This AKT activation was dependent on PI3K activity as far as it was suppressed by the broad PI3K inhibitor LY294002. Moreover, in the presence of this inhibitor the levels of phospho-p38 and phospho-JNK were significantly reduced whereas the phosphorylation of ERK remained unaffected, indicating a complex regulatory network at early steps of the pro-inflammatory signaling, and orchestrated by the PI3K/AKT pathway. In this regard, the inhibition of PI3K/AKT resulted in an almost indistinguishable overactivation of the NF-κB pathway with a delayed resetting of IκB levels, regardless the presence or absence of PTP1B (see Figure 3c). PTP1B dephosphorylates downstream substrates through its phosphatase activity and we found that PTPT1B deficiency significantly increased the phosphorylation of MAPKs and AKT; however, the treatment of extracts from WT or PTP1B KO macrophages with recombinant PTP1B lead to a significant dephosphorylation of all MAPKs but not of AKT. These findings suggest that MAPKs are direct substrates of PTP1B and, therefore, PTP1B negatively regulates LPS responses in macrophages through its phosphatase activity.

Another relevant aspect in PTP1B biology is its involvement in the endocytosis of IFN-α/β receptor chain 1 in human but not in murine cells[31] and therefore, restricting antiviral therapies. However, in terms of regulation of inflammatory responses both human and murine macrophages behave similarly. Indeed, we observed that silencing PTP1B in human macrophages significantly reduced cell viability, counteracting in this way the potential beneficial aspects of the use of PTP1B inhibitors.[40–42] Moreover, this loss of viability due to reduced PTP1B activity, even under basal conditions, was also reproduced by broad and transient PTP inhibitors, such as POV, indicating that the activity of these PTPs is required at specific moments of cell activation to maintain cell viability. Therefore, caution should be taken in the development of strategies of inhibition of PTP1B activity, at least under circumstances in which maintenance of cell viability is a requisite (i.e., phagocytosis). In addition to this, the mini-array data obtained after short periods of pro-inflammatory activation confirmed the enhanced pro-inflammatory responses expected from targets downregulated by this PTP. However, when we performed a microarray analysis at 24 h after LPS challenge we realized that the main differences in gene expression were not related to pro-inflammatory genes, but to very specific pathways: the p53 and DNA excision/repair pathways and the propanoate metabolism pathway. Interestingly, the link between the p53 pathway and the DNA excision/repair pathway fits with the observed loss of viability of PTP1B-deficient cells. Moreover, when we irradiated the animals to perform bone marrow transplantation, we were surprised by the extreme sensitivity of the PTP1B KO mice, compatible with an enhanced DNA injury. This role of PTP1B in protecting mice against irradiation injury is a relevant issue of the present work and we reasoned that perhaps strategies to improve transient PTP1B activity in the course of irradiation will have beneficial effects. Moreover, the levels of PTP1B are consistently increased after pro-inflammatory challenge in macrophages, perhaps as a way to improve cell viability under conditions of intense synthesis of reactive oxygen and/or nitrogen species that may damage the DNA.

Regarding the *in vivo* experiments in PTP1B-deficient animals, according to our results and those from other groups,[30] we observed an enhanced ear edema in these animals, compatible with an increased leukocyte trafficking, as deduced by the higher levels of MPO in the ear samples. In addition to this, we evaluated the effects of PTP1B deficiency in the LPS/D-GalN model that evidences TNF-α toxicity in the liver. In this way, under identical conditions, animal death (mainly by liver hemorrhage) was significantly more prominent in the PTP1B KO mice, together with minimally, but statistically significant higher serum levels of TNF-α in PTP1B KO mice. However, when we performed bone marrow transplantation and reintroduced the PTP1B KO myeloid system into the WT animals, the rates of survival were identical between both groups, suggesting that perhaps the main contribution to the animal death upon LPS/D-GalN challenge was not the circulating monocyte/macrophages, but the tissue resident population of the liver. Using clodronate administration as a strategy to deplete tissue macrophages and to replenish the organs with the myeloid cells coming from the transplanted bone marrow,[43] we analyzed the effect of reintroduction of myeloid cells from PTP1B KO mice into irradiated WT counterparts and then performed the LPS/D-GalN challenge; however, again it was impossible to distinguish between the two groups. One possible explanation is that the levels of TNF-α reached in both models were very similar and lower than those measured in the absence of clodronate depletion. Alternatively, the fact that our PTP1B KO mice lack of this enzyme in all tissues, including the liver, cannot exclude a contribution of the hepatocytes as a result of a paracrine effect to the increased sensitivity to LPS/D-GalN observed in the intact PTP1B KO model. Finally and in an intriguing way, it has been reported that PTP1B deficiency confers resistance against apoptosis induced by pro- and anti-inflammatory stimuli such as transforming growth factor β (TGF-β), TNF-α or resveratrol in hepatocytes and brown adipocytes.[8] Our observation that even under basal conditions the viability of human and murine macrophages is reduced in the absence of PTP1B suggests the existence of alternative pathways linking cell viability to its presence in immune cells. In fact, a cross-talk between different groups of PTP members has been described as a regulatory network to ensure sufficient immunity against pathogen challenge and in preventing adverse autoimmune responses.[29] In conclusion, our data show a previously unrecognized genetic damage in myeloid cell upon pro-inflammatory activation, and suggest that the broad use of PTP1B inhibitors, although with potential benefits over the insulin signaling pathway, might exert undesirable effects in response to stressors of the immune system including the fine tuning of the pro-inflammatory and pro-resolution balance.

Materials and Methods

Materials. Common reagents including z-VAD.FMK were from Sigma-Aldrich (St Louis, MO, USA) or Roche (Darmstadt, Germany). Murine or human cytokines were obtained from PeproTech (London, UK). Antibodies were from Ambion (Austin, TX, USA), Santa Cruz Biotech (Santa Cruz, CA, USA) or Cell Signaling (Danvers, MA, USA). Reagents for electrophoresis were from Bio-Rad (Hercules, CA, USA) and Sigma-Aldrich. Tissue culture dishes were from Falcon (Lincoln Park, NJ, USA), and serum and culture media were from Invitrogen and Gibco (Life Technologies/Thermo Fisher, Madrid, Spain).

Animal care and preparation of macrophages. Male and female PTP1B heterozygous (HET) mice, maintained on a mixed genetic background (C57BL/6 $\times$ 129sv), were intercrossed to yield the three genotypes of mice (WT, HET and KO). In this study, we used 8- to 12-week-old WT and PTP1B KO male mice housed under 12 h light/dark cycle and food and water was provided *ad libitum*. Animals were cared for according to a protocol approved by the Ethical Committee of our institution (following directive 2010/63/EU of the European Parliament). PTP1B-deficient mice were used as previously described.[36] Elicited peritoneal macrophages were obtained from male mice 4 days after intraperitoneal (i.p.) administration of 2.5 ml of 3% thioglycollate broth essentially as described previously.[44] Cells were seeded in 6-multiwell plates at a density of 4×10^6 cells/plate and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) at 37 °C in a humidified atmosphere with 5% CO_2. After 2 h of incubation, non-adherent cells were removed. Adherent cells were maintained in these culture conditions and used within 2 days after plating. After overnight serum reduction, cells were challenged with LPS, polyI:C and LTA (as M1 stimuli) or with IL-4, IL-13 and IL-10 (as M2 stimuli).

Isolation of human monocytes. PBMCs were isolated from enriched buffy coats of healthy donors by separation on Ficoll-Hypaque Plus (GE Healthcare, Madrid, Spain) following the manufacturers' protocol. After washing the upper layer, $CD14^+$ cells were obtained using immunomagnetic isolation

(Miltenyi Biotec, Bergisch, Germany). Cells were maintained for 4 h at 1×10^6 cells/ml in DMEM supplemented with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). After this period, the supernatant was removed and adherent cells were cultured in the same medium supplemented with 10% heat-inactivated FCS. Cells were maintained overnight with this medium and differentiated into macrophages with hM-CSF (20 ng/ml; PeproTech) for 7 days.

Silencing of PTP1B in human monocyte/macrophages. Human differentiated macrophages were incubated with Opti-Mem (Gibco) supplemented with 10% FCS and after 4 h in this medium were treated with 50 nM scrambled (sc)- or siRNAs specific for human PTP1B (Dharmacon, Madrid, Spain). Transfection was accomplished with lipofectamine RNAiMAX (Invitrogen) following the instructions of the supplier. After 12 h, the dishes were supplemented with 2 vol of complete medium containing 10% FCS and antibiotics and maintained for an additional 24-h period. Cell viability was ensured after this incubation and the plates were washed twice with PBS and incubated with fresh medium containing 2% FCS. Cells were used 12 h after this step.

Preparation and treatment of murine or human macrophages with POV. A fresh solution of POV was used to mimic broad PTP inhibition. Briefly, POV was prepared by incubating 1 mM vanadate in PBS with 1 mM H_2O_2 for 10 min at 30 °C.[45] The mixture was treated with purified recombinant catalase (200 μg/ml) to remove the residual H_2O_2 and was used in the next 10 min by adding the solution to the culture medium. As a control, cells received a solution prepared in parallel, but lacking vanadate (this preparation was referred to as vehicle in the same set of experiments).

Determination of ALT activity and NO, TNF-α and PGE_2 levels. The ALT activity was determined in serum samples using a Reflotron plus device (Roche). The amount of nitrate and nitrite in the culture medium was measured after reduction of nitrate to nitrite, and quantification of nitrite using a previous protocol.[44] The accumulation of TNF-α or PGE_2 in the culture medium was measured using specific immunoassay kits (GE Healthcare). The levels of TNF-α in serum were measured under similar conditions following the instructions of the immunoassay kit supplier.

Measurement of cell viability. Macrophage viability was determined by flow cytometry as follows: cells were seeded in TPP dishes that favor its detachment with cold PBS. After centrifugation at 4 °C for 5 min at $1000 \times g$, cells were resuspended in 10 mM HEPES; pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$ and labeled with annexin V–FITC (Invitrogen) solution and/or propidium iodide (PI) (10 μg/ml) for 15 min at RT in the dark and then analyzed in a BD-Canto flow cytometer (BD Biosciences, Madrid, Spain). PI is impermeable to living but stains necrotic and apoptotic dying cells with impaired membrane integrity in contrast to annexin V, which stains early apoptotic cells.

Preparation of total protein cell extracts. Macrophages were homogenized in a buffer containing 10 mM Tris-HCl, pH 7.5; 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 0.5% CHAPS, 1 mM β-mercaptoethanol and 0.1 mM PMSF and a protease and phosphatase inhibitor cocktail (Sigma-Aldrich). The extracts were vortexed for 30 min at 4 °C and after centrifuging for 20 min at $13\,000 \times g$, the supernatants were stored at -20 °C. Protein levels were determined using Bradford reagent (Bio-Rad).

Western blotting. Protein extracts were boiled in loading buffer (250 mM Tris-HCl; pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol and 2% β-mercaptoethanol) and 30 μg of protein were subjected to 8–10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis gels. Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (GE Healthcare). Membranes were incubated for 1 h with low-fat milk powder (5%) in PBS containing 0.1% Tween-20. Blots were incubated for 2 h or overnight at 4 °C with primary antibodies against NOS-2, COX-2, KC, MIP2, IP10, SOCS3, PTP1B, HO-1, Arg-1, IRF-3, p65, and the specific anti-phospho- or total AKT, ERK, p38 and JNK at the dilutions recommended by the suppliers. The blots were developed with ECL Advance protocol (GE Healthcare) and different exposure times were performed for each blot with a Charged Coupling Device camera in a luminescent image analyzer (Molecular Imager; Bio-Rad) to ensure the linearity of the band intensities. Blots were normalized for lane charge using antibodies against p85-PI3K, GAPDH or β-actin. Values of densitometry were determined using the Quantity One software (Bio-Rad).

RNA isolation and RT-PCR analysis. RNA was extracted with TRIzol Reagent (Invitrogen) and reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit for RT-PCR following the indications of the manufacturer (Roche). Real-time PCR was conducted with SYBR Green Master (Roche) on a MyiQ Real-Time PCR System (Bio-Rad). Primer sequences for mouse IL-6, NOS-2, IκBα and PTP1B are available on request. Validation of amplification efficiency was performed for each pair of primers.[44] PCR thermocycling parameters were 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Each sample was run in duplicate and was normalized *versus* 36B4. The fold induction (FI) was determined in a $\Delta\Delta$Ct-based fold-change calculation.

Mini-array and microarray analysis. Differential expression at 0 and 3 h was tested using the 'Chemokine and Receptors' 96-well PCR mini-array (Qiagen, Madrid, Spain; ref. PAMM-022Z) and RNA samples of peritoneal macrophages from WT and PTP1B KO mice challenged with LPS. RNA extraction and expression analysis was performed according to the instructions of the supplier (Qiagen). In addition to this, normalized expression data in macrophages after 24 h of stimulation were obtained in our core facility, using a mouse microarray platform (Agilent-014868 Whole Mouse Genome Microarray 4×44K G4122F). Quality of the samples was ensured in a Bioanalyzer 2100. Processing, normalization and differential expression were performed using Limma Bioconductor package.[46] Enrichment of gene sets of interest from KEGG and REACTOME was accomplished using the GSEA software as described by Mootha *et al.*[47]

Bone marrow transplantation and clodronate treatment. Eight- to nine-week-old C57BL/6 $\times$ 129sv WT male mice were irradiated with a dose of 1000 rads (10 Gy) by a cesium γ-source. One-week prior and 2-week post bone marrow transplantation, 100 mg/l neomycin and 10 mg/l polymyxin B sulfate (Sigma-Aldrich) were added to the acidified water. The bone marrow was collected from the femurs and tibias of 8- to 9-week-old donor male PTP1B WT or KO mice (C57BL/6 $\times$ 129sv) by flushing femurs with phosphate-buffered saline. Each recipient mouse was injected with about $5–6 \times 10^6$ bone marrow cells by retroorbital injection 4 h after irradiation. In another set of animals, 2 weeks after irradiation, mice were injected 200 μl of clodronate- or PBS-loaded liposomes (www.clodronateliposomes.com) by retroorbital injection.[43] Twelve weeks after transplantation, genomic DNA was harvested from whole blood and liver for use in PCR-based genotyping of WT and PTP1B-deficient alleles.[36] In addition to this, liver sections were prepared and stained with F4/80-FITC and laminin to ensure the depletion of Kupffer and resident macrophages.

***In vivo* evaluation of sensitivity to LPS/D-GalN treatment.** To evaluate the *in vivo* response to inflammatory challenge, PTP1B WT and KO animals were injected (i.p.) a low dose of LPS (2 μg/kg) in combination with D-GalN (800 mg/kg) and animal survival was evaluated. In addition to this, WT mice transplanted with bone marrow from PTP1B KO or WT donors with or without treatment with clodronate liposomes to decrease resident macrophage populations were also used for applying the LPS/D-GalN protocol. Serum from these animals was prepared from blood obtained by retroorbital puncture. Liver sections were prepared and stained with eosin/hematoxylin to determine the macroscopic integrity of the tissue upon LPS/D-GalN administration.

Measurement of ear edema and MPO activity. TPA (2.5 μg) dissolved in 20 μl acetone was applied in 10 μl volumes to both the inner and the outer surfaces of the left ear of mice (20–25 g), and the same procedure was repeated in the right ear with the vehicle. Animals were killed after 4 h, and equal-sized samples of both ears were punched out and weighed. Edema was measured as the weight difference between the right and left ear samples. These ear samples were homogenized in 500 μl of ice-old PBS, snap frozen and thawed four times, subsequently submitted to sonication (twice for 10 s) and centrifuged at $10\,000 \times g$ for 15 min at 4 °C. MPO activity was measured in the supernatants as a marker for neutrophil infiltration. Aliquots of supernatants (50 μl) were assayed in a reaction mixture that contained 110 μl PBS, 20 μl of 0.22 M NaH_2PO_4 (pH 5.4), 20 μl of 0.026% (v/v) H_2O_2, and 20 μl of 18 mM tetramethylbenzidine in 8% (v/v) aqueous dimethylformamide. After 10 min of reaction at 37 °C, 30 μl sodium acetate (1.5 M; pH 3) was added, and the absorbance at 620 nm was read in a microtiter plate reader. The activity was expressed as mU/mg protein.[48,49] In addition to this, ears

were fixed with paraformaldehyde and stained with eosin/hematoxylin to visualize their structure and extension of the edema.

Analysis of PTP1B-mediated dephosphorylation. Macrophages from PTP1B KO mice were stimulated with LPS (200 ng/ml) for 30 min and immediately homogenized in buffer containing 20 mM imidazole-HCl, 2 mM EGTA and 2 mM EDTA (pH 7.0) supplemented with protease inhibitors (10 mg/ml leupeptin, 10 mg/ml aprotinin and 1 mM PMSF). Samples were sonicated 3×15 s at 1.5 mA and lysates were clarified by centrifugation at $12\,000 \times g$ for 10 min. After protein content determination, 60 μl of cell lysates (1 mg/ml) was incubated with 10 units of recombinant PTP1B (Upstate, Merck-Millipore, Darmstadt, Germany) for 30 min at 37 °C. The reaction was stopped by adding Laemmli's buffer and samples were analyzed by western blotting.

Statistical analysis. The values in graphs correspond to the means ± S.D. The statistical significance was determined with Student's *t* test for unpaired observation. Data were analyzed by the SPSS for Windows statistical package, v21 (IBM, Madrid, Spain).

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. This work was supported by grants BFU2011-24760 and SAF2012-33283 from MINECO, S2010/BMD-2378 and S2010/BMD-2423 from Comunidad de Madrid and FIS-RIC RD06/0014/0025 and EFSD and Amylin Paul Langerhans Grant. Ciberdem and Ciberehd are funded by the Instituto de Salud Carlos III.

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Supplementary Information accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)

SUPPLEMENTARY MATERIAL

Pivotal role of protein tyrosine phosphatase 1B (PTP1B) in the macrophage response to pro-inflammatory and anti-inflammatory challenge

Short Title: *PTP1B modulation of macrophage responses*

[1]Paqui G. Través*, [1,2]Virginia Pardo*, [1]María Pimentel-Santillana*, [1,2]Águeda González-Rodríguez, [1]Marina Mojena, [3]Daniel Rico, [1]Yenny Montenegro, [1]Carmela Calés, [1,4]Paloma Martín-Sanz, [1,2]Angela M. Valverde# and [1,4]Lisardo Boscá#

[1]Instituto de Investigaciones Biomédicas Alberto Sols (Centro Mixto CSIC-UAM), Arturo Duperier 4, 28029 Madrid, Spain.

[2]Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (Ciberdem), ISCIII; Spain

[3]Structural Biology and Biocomputing Programme, Spanish National Cancer Research Center (CNIO), ISCIII, Madrid, Spain.

[4]Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (Ciberehd), ISCIII; Spain

*Contributed equally to the work

#Address for correspondence:

Lisardo Boscá. IIB-Alberto Sols. Arturo Duperier 4. 28029 Madrid. Spain

Fax: 34 914972747; e-mail: lbosca @iib.uam.es

and

Angela M Valverde. IIB-Alberto Sols. Arturo Duperier 4. 28029 Madrid. Spain

Fax: 34 915854401; e-mail: avalverde @iib.uam.es

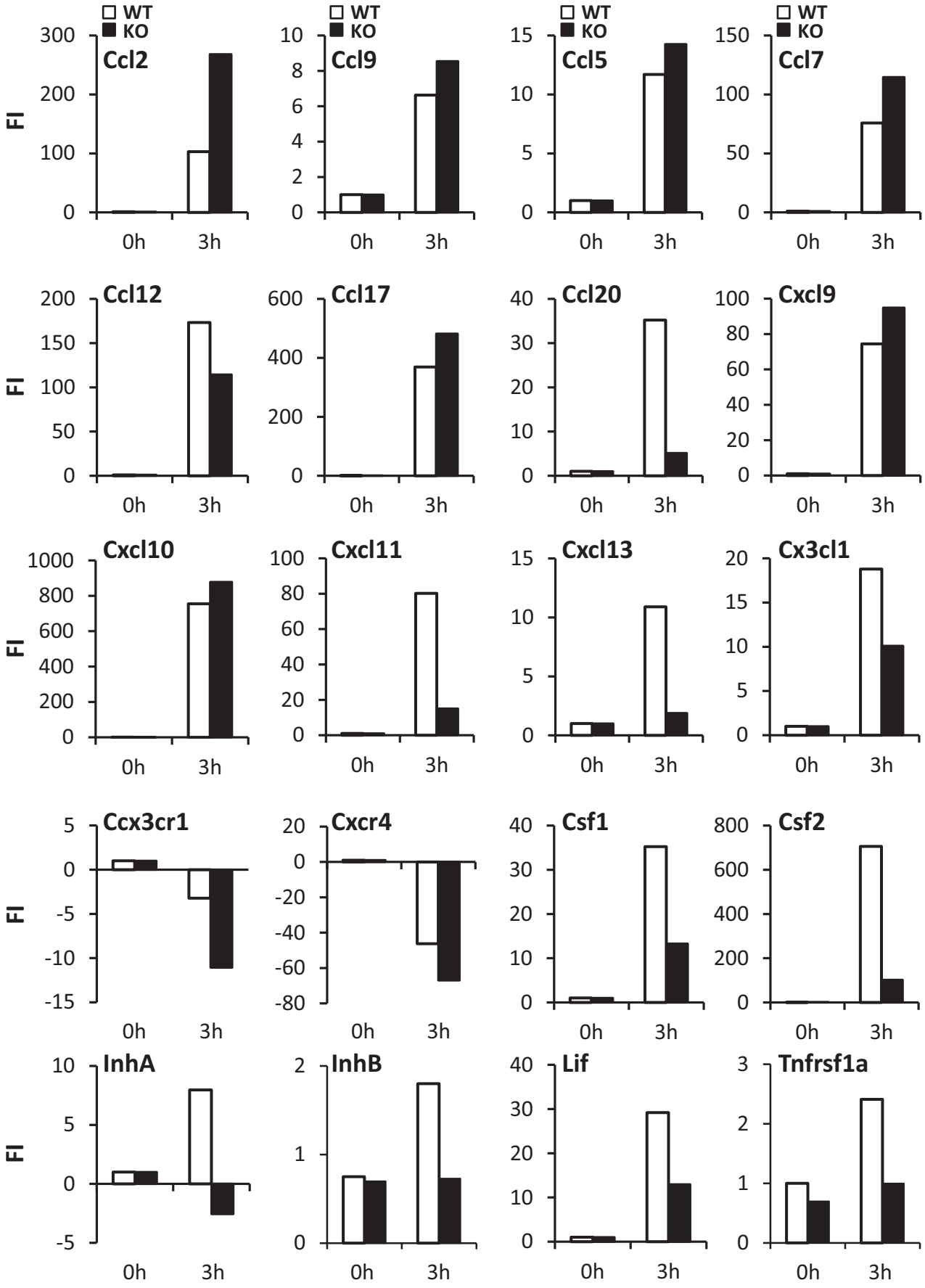
Supplementary Figures:

Figure S1. ***RT-PCR mini-array analysis of the response to LPS in PTP1B-deficient macrophages.*** Macrophages from WT and PTP1B KO mice were challenged for the indicated periods of time with 200 ng/ml of LPS and the levels of mRNA were determined using a 96-well Superarray test for 'Chemokines and receptors'. Samples were processed as indicated by the supplier and a selection of the genes was represented. Results show the mean of three experiments combining the macrophages of at least 4 animals in each experiment.

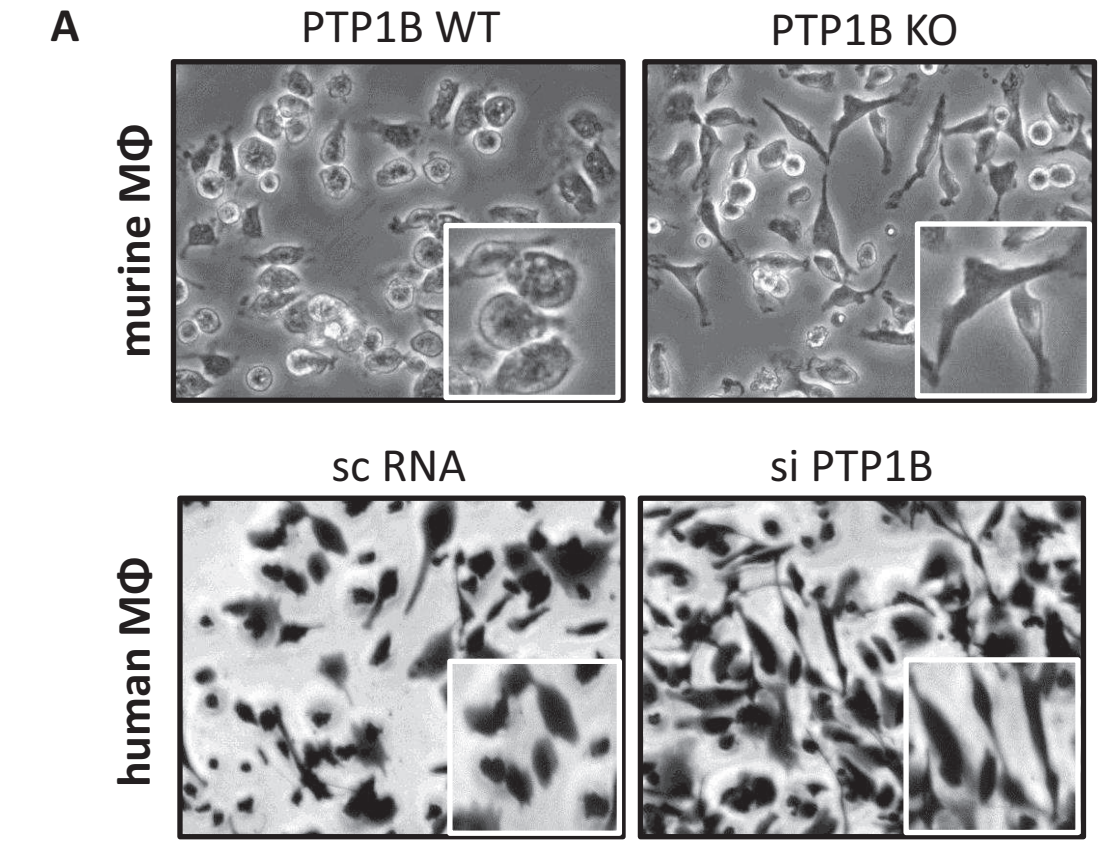
Figure S2. ***Deficiency of PTP1B or silencing of PTP1B with specific siRNA induces morphological changes in murine and human macrophages (A), and effect of peroxovanadate (POV) on the response to LPS+IFNγ of human macrophages (B,C).*** Peritoneal macrophages from WT or PTP1B KO mice or human macrophages treated with a mixture of sc- or siRNA oligonucleotides to silence PTP1B were plated as indicated in Methods and clear field images (murine cells) or after staining with crystal violet (human) were obtained. Insets show representative morphologies of the corresponding cells (A). Human monocytes were differentiated into macrophages and treated with a fresh solution of POV or the same mixture lacking vanadate (vehicle). After 10 min cells were challenged with LPS+IFNγ as in Fig. 5 and samples were collected to determine the protein levels of PTP1B, IκBα, IκBβ and the cytosolic/nuclear distribution of p65 at the indicated times (B). To evaluate the effect of PTP inhibition by POV on cell viability, macrophages were incubated with the indicated stimuli (as in Fig. 5) and viability was determined as the population negative for PI and annexin V staining. z-VAD was used at 50 μM (C). Results show a representative blot out of three (B) or the mean of three experiments (C).

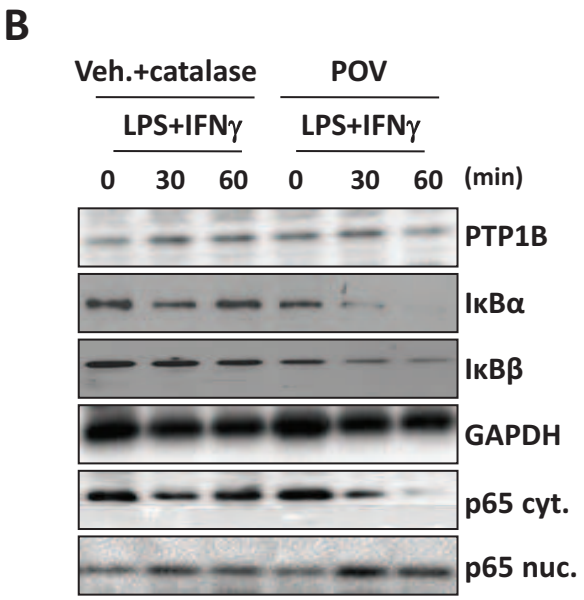
Figure S3. ***Analysis of animal viability and measurement of TNF-α serum levels in mice submitted to bone marrow transplantation (Tx) and treated with clodronate liposomes.*** WT recipient mice (8-10 per group) were transplanted with bone marrow from WT or PTP1B KO donors. Two weeks later, mice were i.p. injected 200 μl of clodronate liposomes, and 12 weeks after transplantation mice were injected LPS/D-GalN. Kaplan-Meier curves for survival after LPS/D-Gal administration (A). The serum levels of TNF-α were determined at 1h after LPS/D-GalN challenge (B). The replenishment of PTP1B-deficient macrophages in the liver and blood after bone marrow transplantation was determined by RT-PCR after finishing the LPS/D-GalN protocol (C). The effectiveness of clodronate treatment was evaluated at 48h after administration of the clodronate-liposomes in liver sections stained with F4/80-FITC, laminin and DAPI; white bar= 100 μm (D). Results show the mean±SD of the TNF-α measurements (B). *$P<0.05$ *vs.* the same condition in animals transplanted with WT bone marrow, or three

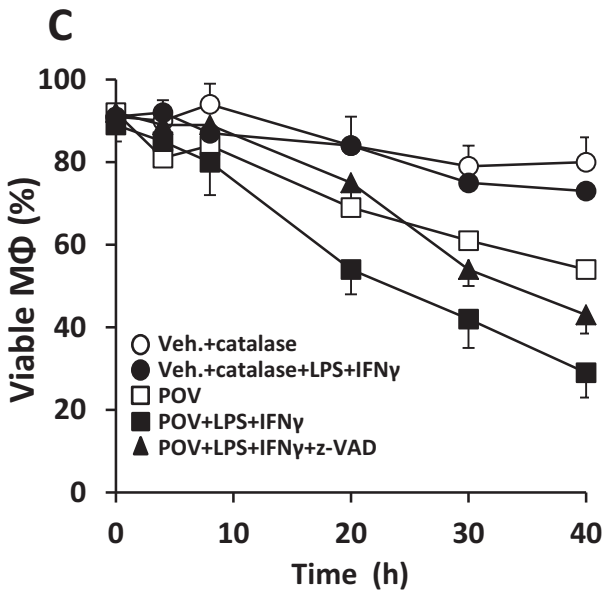
representative animals of each group after bone marrow transplantation. The blood from a heterozygote animal was used as a control (C).



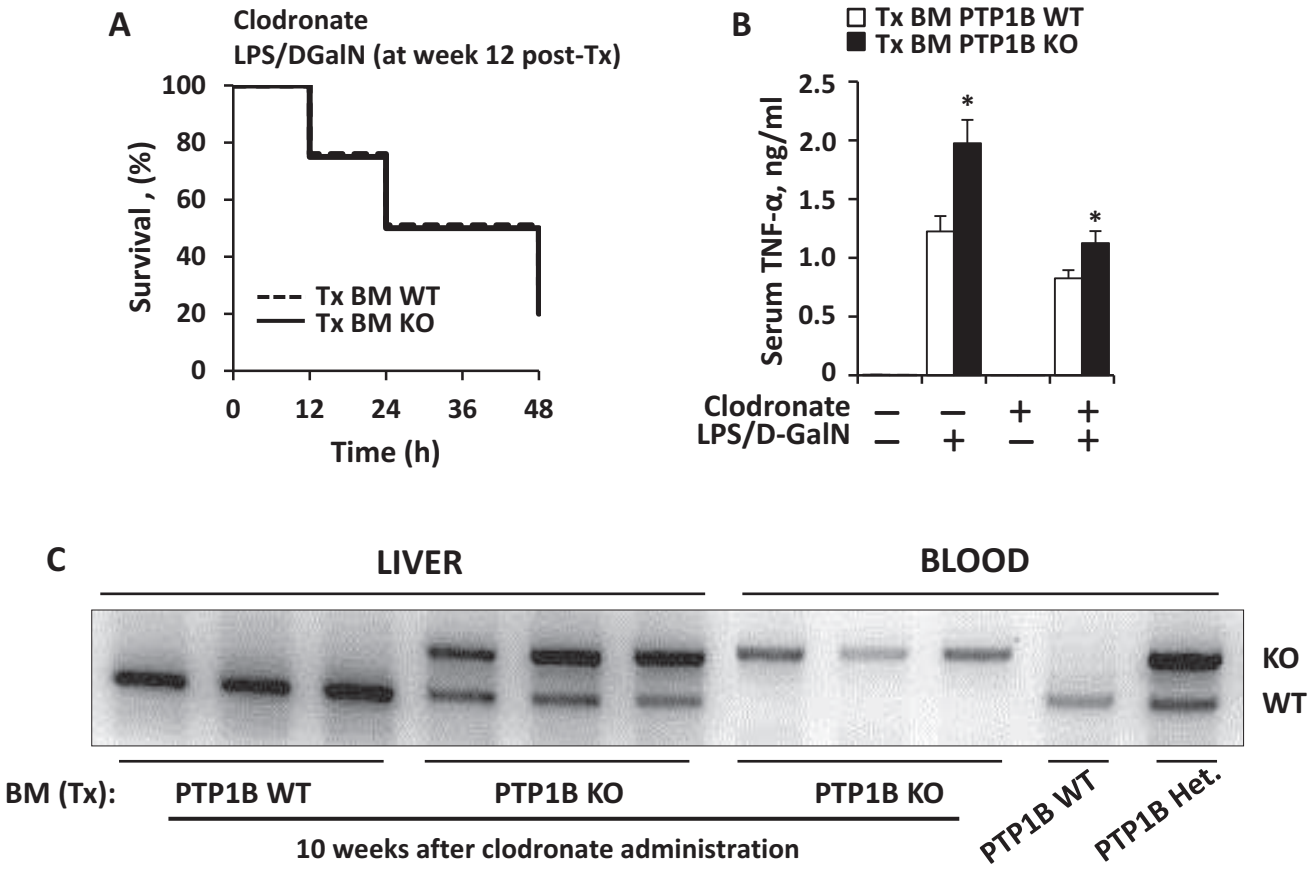
Supplementary Fig. S1







Supplementary Fig. S2



Supplementary Fig. S3

En este tercer trabajo se ha descrito el papel de la fosfatasa PTP1B sobre las repuestas inflamatorias de los macrófagos. Entre los sistemas que modulan la activación de los macrófagos, las proteínas fosfatasas ejercen un papel relevante (29). Además, la PTP1B podría aumentar su expresión o activarse por factores inflamatorios, tales como TNF-α (33). De hecho, otros estudios han identificado un papel esencial para la PTP1B en la inflamación inducida por la obesidad, en la regulación del reclutamiento de leucocitos durante la inflamación alérgica y en el desarrollo de los macrófagos (30,32, 36).

Nuestros datos muestran que los macrófagos peritoneales procedentes de ratones deficientes en PTP1B (PTP1B KO) presentan una respuesta inflamatoria exacerbada frente a los macrófagos procedentes de ratones de genotipo salvaje (WT), que queda reflejada por el aumento de la producción de marcadores de polarización M1. Además, este aumento de la activación encontrada en los macrófagos PTP1B KO no se limita a la señalización vía TLR4, sino que también se produjo en respuesta a ligandos del TLR2 (ácido lipoteicoico, LTA) y TLR3 (polyI:C), confirmando los datos preliminares observados en la línea celular de macrófagos RAW 264.7 (18). Por el contrario, en ausencia de PTP1B, los macrófagos muestran una disminución en la respuesta a IL-4 e IL-13 resultando en una polarización M2 atenuada, lo que sugiere un papel dual de esta fosfatasa en la regulación de la relación M1/M2. Asimismo, el aumento de la señalización a través de NF-kB, PI3K/AKT y MAPKs en los macrófagos PTP1B KO sugiere que la PTP1B actúa como un regulador negativo común de sus actividades.

Otro aspecto relevante en las acciones de PTP1B es que al reducir sus niveles en macrófagos humanos mediante siRNA, se reduce significativamente la viabilidad celular, contrarrestando de esta forma los posibles aspectos beneficiosos del uso de inhibidores de esta fosfatasa (40-42). De esta manera, los datos obtenidos del mini-array tras períodos cortos de activación con LPS confirmaron la respuesta

proinflamatoria exacerbada debida a la deficiencia de PTP1B. Sin embargo, cuando se realizó un análisis de microarray tras 24 h de estimulación con LPS, observamos que las diferencias principales en la expresión de genes no estaban relacionados con genes proinflamatorios, sino con vías muy concretas: p53, vías de escisión y reparación de ADN y la vía del metabolismo del propanoato. Curiosamente, la conexión entre la ruta de p53 y la vía de escisión y reparación de ADN encaja con la pérdida de viabilidad observada en las células deficientes en PTP1B. Por otra parte, cuando irradiamos los animales para realizar el trasplante de médula ósea, nos sorprendió la extrema sensibilidad de los ratones PTP1B KO a la irradiación, compatible con un aumento en el daño del ADN. De hecho, los niveles de PTP1B aumentan tras el tratamiento de los macrófagos con estímulos proinflamatorios, tal vez como una forma de mejorar la viabilidad celular en condiciones de intensa síntesis de especies reactivas de oxígeno y nitrógeno que pueden dañar el ADN.

En cuanto a los experimentos *in vivo* en animales deficientes en PTP1B y de acuerdo con nuestros resultados y los de otros grupos (30), se observó un mayor edema de oreja en estos animales tras la exposición a TPA, compatible con un aumento del tráfico de leucocitos, como se deduce de los elevados niveles de mieloperoxidasa en las muestras de oreja. Igualmente, se evaluaron los efectos de la falta de PTP1B en el modelo de LPS/D-GalN que evidencia la toxicidad del TNF-α en el hígado. De esta manera, bajo condiciones idénticas, la muerte del animal (principalmente por hemorragia hepática) fue significativamente más prominente en los ratones PTP1B KO, junto con un pequeño pero estadísticamente significativo aumento de los niveles séricos de TNF-α. Sin embargo, cuando se realizó un trasplante de médula ósea y se reintrodujo el sistema mieloide de los ratones PTP1B KO en los animales WT, las tasas de supervivencia fueron idénticas entre ambos grupos, lo que sugiere que tal vez la principal contribución a la muerte del animal tras ser tratado con

LPS/D-GalN no eran los monocitos circulantes, sino la población de macrófagos residentes del hígado (las células de Kupffer).

En conclusión, nuestros datos muestran que la falta de PTP1B produce unas alteraciones genéticas no descritas previamente en células mieloides tras la activación proinflamatoria, y sugieren que los inhibidores de PTP1B, aunque tengan efectos beneficiosos sobre la vía de señalización de la insulina, podrían ejercer consecuencias indeseables sobre el fino equilibrio que existe entre la respuesta proinflamatoria y de resolución de la inflamación, en respuesta a factores de estrés del sistema inmunológico.

* Las referencias de esta parte corresponden a las del artículo III

CONCLUSIONES

1. El ácido oleico previene la activación del estrés del retículo endoplásmico, la lipoapoptosis y la resistencia a la insulina inducidos por el ácido palmítico en hepatocitos humanos y murinos.

2. La activación de la proteína S6K1 por ácido palmítico es un mecanismo común por el cual se induce estrés del retículo endoplásmico, lipoapoptosis y resistencia a la insulina en los hepatocitos.

3. El medio condicionado de los macrófagos tratados con ácido palmítico produce una activación de las vías de estrés y, como consecuencia, una resistencia a la insulina en hepatocitos humanos y murinos.

4. Los macrófagos tratados con ácido oleico expresan marcadores de actividad antiinflamatoria y además, secretan al medio una menor cantidad de LTB_4 en comparación con los macrófagos control y los tratados con ácido palmítico.

5. El medio condicionado de macrófagos tratados con ácido oleico provoca una disminución en la expresión de las fosfatasas PTP1B y PTEN, aumentando de este modo la sensibilidad a la insulina en los hepatocitos, efecto que se revierte añadiendo LTB_4 al medio condicionado.

6. Las células mieloides de los ratones deficientes en PTP1B poseen una mayor capacidad de respuesta inmune ante estímulos proinflamatorios.

7. Los ratones deficientes en PTP1B presentan exacerbadas las respuestas inflamatorias ya que manifiestan un mayor edema de oreja tras la exposición a TPA y un aumento de los niveles séricos de TNF-α junto con una mayor tasa de mortalidad tras la inyección de LPS/D-GalN, con respecto a los ratones de genotipo salvaje.

REFLEXIÓN FINAL

Los resultados de esta Tesis Doctoral ponen de manifiesto el impacto del inmunometabolismo sobre el desarrollo de la resistencia a la insulina y apoyan la utilización de estrategias terapéuticas moduladoras de las respuestas del sistema inmune para tratar patologías metabólicas como la diabetes.

ANEXOS

ANEXO I

Otras publicaciones

- **Studies of naturally occurring friedelane triterpenoids as insulin sensitizers in the treatment type 2 diabetes mellitus.**
  Ardiles AE, González-Rodríguez A, Núñez MJ, Perestelo NR, **Pardo V**, Jiménez IA, Valverde AM, Bazzocchi IL.

  Phytochemistry. 2012 Dec; 84:116-24.doi:10.1016/j.phytochem.2012.07.025. Epub2012 Aug 25.

- **Loss of protein tyrosine phosphatase 1B increases IGF-I receptor tyrosine phosphorylation but does not rescue retinal defects in IRS2-deficient mice.**
  Arroba AI, Revuelta-Cervantes J, Menes L, González-Rodríguez Á, **Pardo V**, de la Villa P, Burks DJ, Valverde ÁM.

  Invest Ophthalmol Vis Sci. 2013 Jun 19; 54(6):4215-25. doi: 10.1167/iovs.12-11438.

- **Impaired autophagic flux is associated with increased endoplasmic reticulum stress during the development of NAFLD.**
  González-Rodríguez A, Mayoral R, Agra N, Valdecantos MP, **Pardo V**, Miquilena-Colina ME, Vargas-Castrillón J, Lo Iacono O, Corazzari M, Fimia GM, Piacentini M, Muntané J, Boscá L, García-Monzón C, Martín-Sanz P, Valverde ÁM.

  Cell Death Dis. 2014 Apr 17;5:e1179. doi: 10.1038/cddis.2014.162

- **Essential role of nrf2 in the protective effect of lipoic acid against lipoapoptosis in hepatocytes.**
  M. Pilar Valdecantos, Pedro Luis Prieto-Hontoria, **Virginia Pardo**, Teresa Módol, Beatriz Santamaría, Jordi Muntané, Antonio Cuadrado, María Jesús Moreno-Aliaga, J Alfredo Martínez and Ángela M. Valverde.

  Enviado a la revista *Antioxidants & Redox Signaling* (Octubre 2014).

ANEXO II

Participaciones en congresos

- **IX Spanish-Italian Symposium on Organic Chemistry.**
  Póster: *"Study of the anti-diabetic activity of Friedelane Triterpenoids from Celastraceae species"*. Universidad de La Laguna, Tenerife (Febrero 2012).

- **48th Annual Meeting European Association for the Study of Diabetes (EASD).**
  "Resveratrol treatment restores peripheral insulin action in diabetic IRS2-deficient mice in a Sirt1-independent manner". Berlín (Octubre 2012).

- **III Simposio sobre la Investigación en Diabetes y Enfermedades Metabólicas Asociadas.** CIBERDEM y MSD Cardiometabólico. Barcelona (Octubre 2012)

- **23rd Annual Meeting Eye Complications (EASD).**
  "Loss of protein tyrosine phosphatase 1B increases IGF-1 receptor tyrosine phosphorylation but does not rescue retinal defects in IRS2-deficient mice". Barcelona (Mayo 2013).

- **XI Congreso de Red de Apoptosis (APORED).**
  Exposición oral: *"Molecular cross-talk between fatty acid-induced inflammation, insulin resistance and hepatocyte cell death"*. Fuengirola, Málaga. (Junio 2013).

- **49th Annual Meeting European Association for the Study of Diabetes (EASD).**
  "Inhibition of autophagic flux is associated with increased endoplasmic reticulum stress during the development of non-alcoholic fatty liver disease". Barcelona (Septiembre 2013).
- **XII International Symposium on Insulin Receptors and Insulin Action (IR).**
  Póster: *"Opposite cross-talk by oleate and palmitate on insulin signaling in hepatocytes by direct effects or through macrophage activation".* Barcelona (Noviembre 2013).
- **74th Scientific Sessions American Diabetes Association (ADA).**
  Póster: *"Beneficial Effects of a Dual Acting GLP-1R/ Glucagón Receptor Co-Agonist in the Treatment of Hepatic Regeneration in NAFLD".* San Francisco (Junio 2014).