UNIVERSITAT DE BARCELONA

Papel del Polietilenglicol 35 en la Modulación de los Procesos Inflamatorios Asociados al Páncreas

Ana Ferrero Andrés

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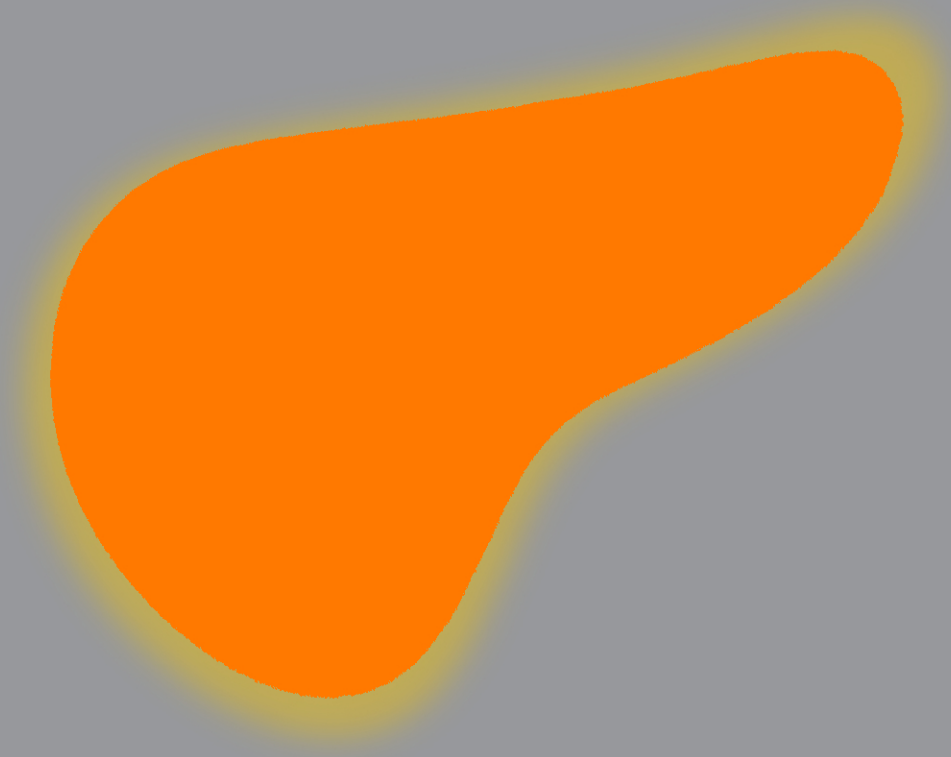

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Barcelona 2020

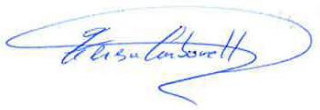
PAPEL DEL POLIETILENGLICOL 35 EN LA MODULACIÓN DE LOS PROCESOS INFLAMATORIOS ASOCIADOS AL PÁNCREAS

Programa de Doctorado en Biomedicina

Universidad de Barcelona

Tesis Doctoral para optar al título de Doctora por la Universidad de Barcelona presentada por:

Ana Ferrero Andrés



Directora: Dra. Emma Folch Puy Tutora: Dra. Teresa Carbonell Camós

Barcelona, 2020

“Per aspera, ad astra.”

Lucio Anneo Séneca

AGRADECIMIENTOS

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"I know now. Folk in those stories had lots of chances of turning back only they didn't. Because they were holding on to something."

Samsagaz Gamyi, *El Señor de los Anillos, Las Dos Torres*

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ABREVIATURAS

ACh: Acetilcolina

AMPc: Adenosín-monofosfato cíclico

APACHE II: *Acute Physiology and Chronic Health Examination II*

Apaf-1: Factor de activación de la proteasa – 1

BCL-2: Linfoma de células B

CCK: Colecistoquinina

CLPA: Colecciones Líquidas Peripancreáticas Agudas

CNA: Colecciones Necróticas Agudas

CXCL2: Quimioquina del ligando 2

CXCR2: Receptor de CXCL2

DAMP: Patrones moleculares asociados a daño

ERO: Especies Reactivas de Oxígeno

FDA: *Food and Drug Administration*

Gp130: Glucoproteína 130

Grb2: Receptor de crecimiento unido a proteína 2

ICAM: Molécula de adhesión intercelular

IKB: Iκ-B quinasa

IL-1α: Interleuquina – 1 alpha

IL-1β: Interleuquina – 1 beta

IL-1R: Receptor de IL-1

IL-6: Interleuquina – 6

IL-6Rs: Receptor soluble de IL-6

IL-33: Interleuquina – 33

I-R: Isquemia-reperfusión

JAK/STAT: Janus quinasa/señal de activadores y transductores

LFA: Antígeno asociado a la función de linfocitos

LPS: Lipopolisacárido

MAPK: Proteínas quinasas activadoras por mitógenos

MLKL: Proteína de dominio quinasa de linaje mixto

NLRP3: Proteína 3 que contiene dominio de pirina NLR

NFκB: Nuclear Factor kappa B

NO: Óxido nítrico

PA: Pancreatitis Aguda

PAEI: Pancreatitis Aguda Edematosa Intersticial

PAN: Pancreatitis Aguda Necrotizante

PAMP: Patrones moleculares asociados a patógenos

PC: Pancreatitis Crónica

PEG: Polietilenglicol

PI3K: Fosfatidil inositol 3 quinasa

PIT: Péptido Inhibidor de Tripsina

PMN: Polimorfonucleares

PP: Polipéptido-pancreáticas

PRR: Receptores de Reconocimiento de Patrones

Ras: Guanosín. trifosfato de unión a membrana

RER: Retículo Endoplasmático Rugoso

RIP: Receptor de interacción con proteínas

RIPK: Receptor de interacción con proteínas quinasas

RLO: Radicales Libres de Oxígeno

SOFA: *Sequential Organ Failure Assessment*

SRAC: Síndrome de Respuesta Antiinflamatoria Compensatoria

SRIS: Síndrome de Respuesta Inflamatoria Sistémica

TC: Tomografía Computarizada

TLR: Receptores tipo Toll

TNF-α: Factor de Necrosis Tumoral – alpha

TNF-R1: Receptor de Factor de Necrosis Tumoral

UCI: Unidad de Cuidados Intensivos

XAD: Xantina Deshidrogenasa

XAO: Xantina Oxidasa

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INTRODUCCIÓN

1. EL PÁNCREAS

El término “páncreas” proviene del griego “pan” (todo) y “kreas” (carne), acuñado por primera vez por Rufo de Éfeso, médico de la Antigua Grecia, para hacer referencia a un órgano presente en la anatomía humana sin tejido cartilaginoso ni óseo.

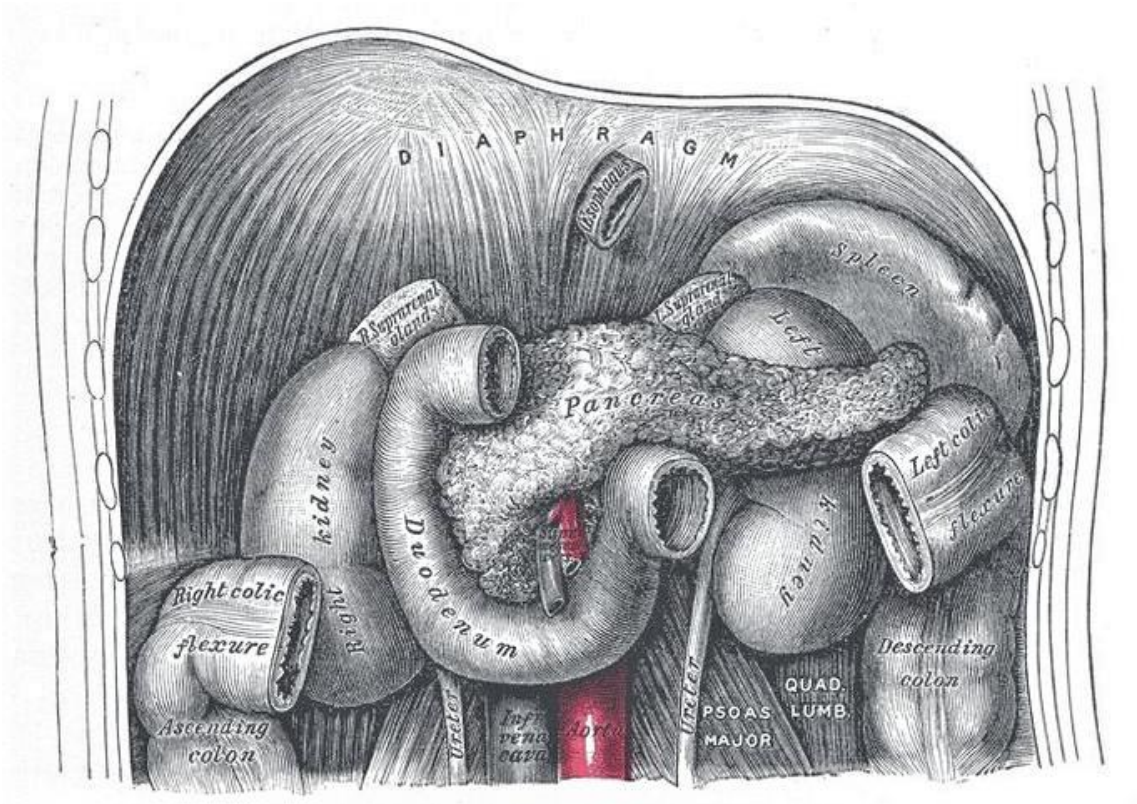

Figura 1. Localización anatómica del páncreas en relación a otros órganos de la cavidad abdominal (199).

1.1 Anatomía pancreática

El páncreas es una glándula digestiva mixta de tono rosáceo – amarillento localizada en el cuadrante superior izquierdo de la cavidad retroperitoneal del abdomen, por detrás del estómago y del colón transversal (1). En los humanos adultos tiene una medida de aproximadamente 15 cm de largo, 2 a 9 cm de ancho y unos 3 cm de grosor, con un peso de entre 70 – 110 gramos (2).

Es un órgano de apariencia lobulada dividido en cuatro regiones principales: la cabeza, el cuello, el cuerpo y la cola. La cabeza es la porción más amplia situada en la curvatura del duodeno próxima al píloro y orientada ligeramente hacia delante y a la derecha. En su parte baja y hacia el extremo izquierdo, la cabeza se curva formando el

llamado “páncreas de Winslow”, el proceso unciforme, que está atravesado por la vena mesentérica superior (3). A continuación, el páncreas se estrecha constituyendo el cuello o istmo del páncreas, que une la cabeza con el cuerpo de la glándula. Este se va alargando hacia la parte lateral superior izquierda, y, finalmente, termina en la cola del páncreas, que limita con el hilio del bazo (4). En la cola del páncreas está ubicado el origen del conducto pancreático principal o de Wirsung que se extiende hasta la cabeza terminando, junto con el colédoco, en la ampolla hepatopancreática o de Váter que se introduce en la papila mayor duodenal rodeada por una capa de músculo liso, que constituye el esfínter de la papila o de Oddi (5). El conducto pancreático accesorio o de Santorini se separa del conducto pancreático principal a nivel de la cabeza y se dirige hacia el duodeno donde drena por la papila menor duodenal, localizada por encima de la papila mayor (5).

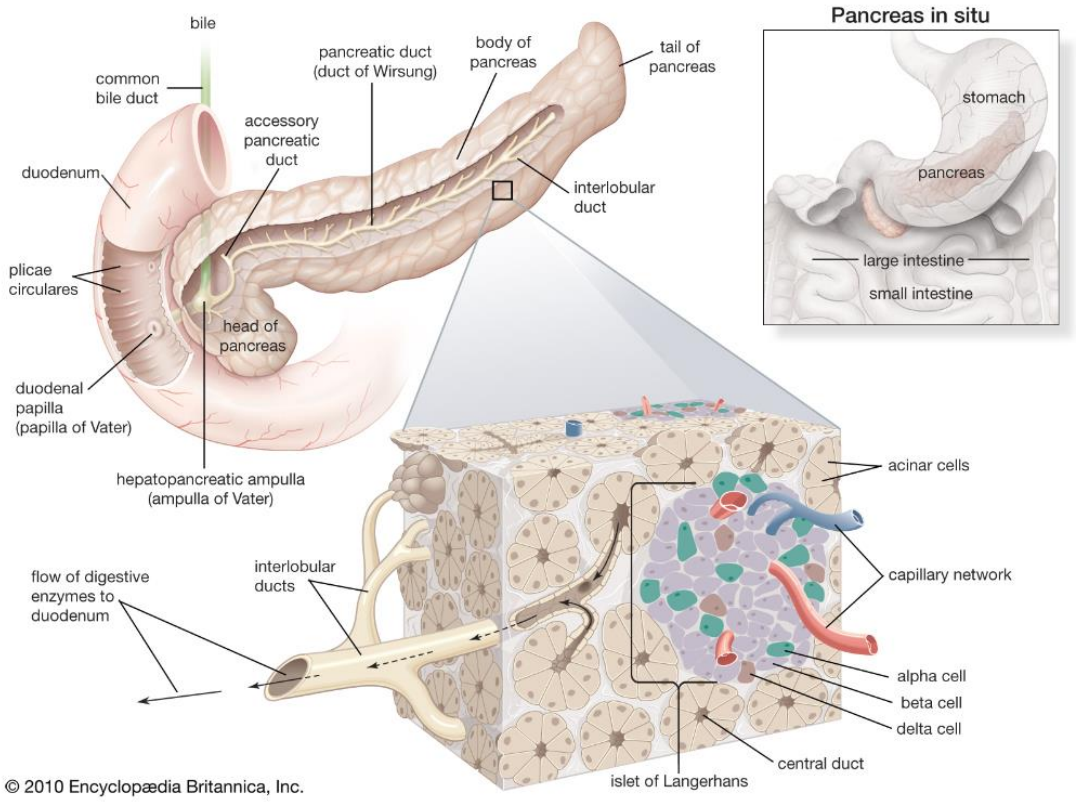

Figura 2. Estructura anatómica e histológica del páncreas (200).

Debido a su carácter de glándula mixta, el páncreas está constituido por una parte endocrina y otra exocrina. La glándula de secreción exocrina está representada por los

acinos glandulares, en cuyo centro se localiza un conducto excretor, mientras que la glándula de secreción endocrina la constituyen los islotes pancreáticos o de Langerhans, situados entre los acinos y extensamente vascularizados (3). Los vasos arteriales que irrigan el páncreas proceden principalmente de la ramificación de la arteria mesentérica superior y del tronco celíaco originados desde la arteria aorta abdominal (6). Desde la vena porta, se ramifican las venas que acompañan a las arterias en su recorrido alrededor del páncreas para asegurar una correcta irrigación, siendo las principales la vena mesentérica superior, las venas pancreaticoduodenales y la vena esplénica (3).

El sistema linfático del páncreas está distribuido en cuatro zonas marcadas, dividiendo los ganglios en: ganglios pancreáticos superiores, ganglios pancreáticos inferiores, ganglios esplénicos y ganglios pancreatoduodenales (3). El sistema de nodos linfáticos del páncreas cubre cada una de sus regiones (cabeza, cuello, cuerpo y cola) asegurando la recogida local de líquido intersticial (7).

El páncreas es un órgano inervado tanto por el sistema nervioso simpático como por el sistema nervioso parasimpático (3). El sistema nervioso simpático, cuya excitación se distribuye por este órgano por el nervio esplácnico y sus ramificaciones, tiene un efecto difuso sobre las funciones pancreáticas. Su estimulación influye sobre todo en la presión y el flujo sanguíneos. La excitación nerviosa que provoca la secreción pancreática tanto exocrina como endocrina procede, en mayor medida, de la estimulación del nervio vago (8).

1.2 Histología pancreática

El páncreas presenta dos tipos de tejido: exocrino y endocrino, cada uno con una conformación característica, rodeados por una delgada capa de tejido conjuntivo que divide el parénquima formando septos (9). La parte exocrina está representada principalmente por las células acinares que constituyen entre un 80 – 85% de la masa total del páncreas, un 10 – 15% corresponde a la matriz extracelular, los vasos y las células ductales, y tan sólo alrededor de un 2% está representado por las células endocrinas (10).

1.2.1 PÁNCREAS EXOCRINO

Es una estructura tubuloacinosa dividida en lóbulos formados por los septos de tejido conjuntivo laxo (9). Se distingue, por lo tanto, una unidad funcional básica que es el lóbulo pancreático y un sistema ductal ramificado. El lóbulo pancreático está constituido por numerosos acinos formados por un conjunto de células dispuestas alrededor de una luz acinar en la que se localizan los conductos intralobulares que convergen en los conductos interlobulares, los cuales desembocan finalmente en los conductos pancreáticos principales: el de Wirsung y el de Santorini (10).

En el páncreas exocrino podemos encontrar tres tipos celulares: células secretoras acinares, células centroacinares y células ductales. Las células que conforman los acinos propiamente dichos son las células secretoras acinares, las que rodean la luz del acino y se encargan de secretar enzimas digestivos. Son células epiteliales con forma piramidal y polarizadas que presentan el núcleo en posición basal y, en el citoplasma, un retículo endoplasmático rugoso (RER) bastante desarrollado que les confiere un carácter basófilo (10). También muestran polirribosomas y un gran número de mitocondrias (10). En la zona apical de las células acinares se localiza el aparato de Golgi, también bastante desarrollado, y los gránulos acidófilos secretores de zimógeno, en cuyo interior se encuentran las enzimas pancreáticas: proteasas, lipasa, amilasa y nucleasas, que constituyen la secreción pancreática (9). Comunicando los acinos con los conductos se encuentran las células centroacinares, que marcan el comienzo del conducto intercalar. Las células centroacinares son más aplanadas, cuboideas, no presentan gránulos de zimógeno y tienen un aparato de Golgi y un retículo endoplasmático rugoso menos desarrollado. Por otro lado, los conductos que constituyen el sistema ductal están revestidos por las células ductales, cuyos orgánulos presentan características similares a los de las células centroacinares (10).

A parte de una función de revestimiento epitelial, estas células también secretan sustancias como agua y electrolitos (10).

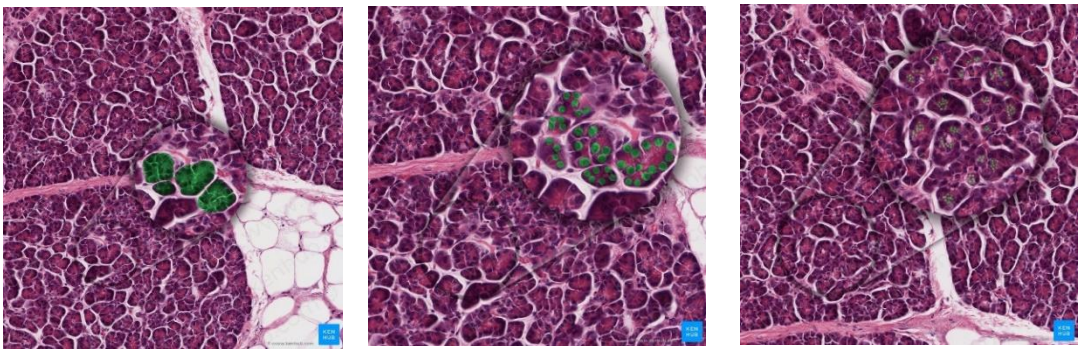

Figura 3. Histología del páncreas exocrino, de izquierda a derecha: acinos pancreáticos, células acinares pancreáticas y gránulos de zimógeno (201).

1.2.2 PÁNCREAS ENDOCRINO

El páncreas endocrino está representado por unas estructuras redondeadas denominadas islotes de Langerhans. Estas estructuras quedan rodeadas por los acinos pancreáticos, contienen abundantes vasos sanguíneos y están separadas entre ellas por fibras de reticulina (9). Se localizan esencialmente en la cola del páncreas y cada uno de ellos está formado por cientos de células endocrinas, aunque solo ocupen un 2% del volumen total pancreático (11).

Las células endocrinas que conforman los islotes de Langerhans se dividen en células β, localizadas en el centro del islote constituyendo cerca de un 60% de su masa total, y células α, que forman un 30% de la estructura, células δ, células ϵ y células polipéptido-pancreáticas (PP) que representan un 10% del volumen del islote y forman un manto a su alrededor (12).

Cada una de las células endocrinas se encarga de producir y secretar un tipo diferente de hormonas. Las células α producen glucagón, las células β son las responsables de segregar insulina, las células δ, somatostatina, las células ϵ liberan grelina, y las células PP segregan el polipéptido pancreático (13). Todas ellas se encuentran polarizadas, situándose los gránulos secretores en la zona apical por donde discurren capilares fenestrados y, al igual que las células

acinares exocrinas, presentan un RER y un aparato de Golgi muy desarrollados, característica típica de cualquier célula secretora de proteínas (9).

1.3 Desarrollo embrionario histológico y funcional del páncreas

El desarrollo del páncreas comienza a partir de la quinta semana de gestación. Se originan dos brotes: uno dorsal, a partir del endodermo intestinal anterior y otro ventral, desde el divertículo hepatovesicular. A partir del esbozo dorsal se forma el cuello, el cuerpo y la cola del páncreas, así como la mayor parte del sistema ductal mientras que desde el esbozo ventral se origina la cabeza del páncreas, el proceso unciforme y la parte terminal del conducto pancreático (14). Durante el desarrollo embrionario, el esbozo pancreático ventral rota hacia la posición dorso-medial fusionándose con la yema dorsal e integrándose con el conducto biliar, futuro colédoco, y el duodeno (15).

Ambos esbozos, dorsal y ventral, están formados por una serie de conductos o cordones primitivos. En el esbozo dorsal, a partir de estos conductos, se desarrolla una red luminal que da lugar a los futuros acinos y sistema ductal (14). Los islotes de Langerhans se forman a partir del desarrollo de células epiteliales de los conductos pancreáticos primitivos que se van agrupando y separando y se disponen rodeando los acinos (9). El conducto pancreático principal o de Wirsung se origina por anastomosis entre el segmento terminal de la yema ventral y el conducto de la yema dorsal. Una parte de este conducto puede continuar como conducto accesorio dando lugar al conducto de Santorini (9).

1.4 Secreciones pancreáticas exocrinas

La parte exocrina del páncreas secreta un jugo pancreático constituido por dos tipos de secreciones: enzimática e hidroelectrolítica. La primera, producida por las células acinares, es la encargada de digerir las proteínas, lípidos y glúcidos contenidos en los alimentos, mientras que la hidroelectrolítica, secretada por las células centroacinares y ductales, sirve de transporte a las enzimas digestivas pancreáticas y neutraliza la acidez del quimo procedente del estómago, necesario para que estas enzimas actúen (16).

1.4.1 SECRECIÓN HIDROELECTROLÍTICA

Está compuesta principalmente por agua, en un 98%, y por iones bicarbonato, cloro, potasio y sodio. En menor proporción podemos encontrar iones de magnesio y calcio (17). Esta secreción es importante porque garantiza el transporte de las enzimas digestivas pancreáticas desde los acinos y conductos hasta el duodeno. Una vez en el duodeno, las enzimas pueden llevar a cabo su acción gracias al pH ligeramente alcalino proporcionado por esta solución acuosa de iones. Además, esta solución, permite la formación de micelas necesarias para la absorción de las grasas y protege la mucosa duodenal de la aparición de úlceras.

1.4.2 SECRECIÓN ENZIMÁTICA

En las células acinares pancreáticas se produce la mayor parte de la síntesis proteica. Gracias al considerable desarrollo de su RER se sintetiza una gran cantidad de enzimas que son transportadas en vesículas hasta el aparato de Golgi (18). El RER también proporciona almacén de calcio intracelular que colabora como regulador de la secreción de enzimas almacenadas en el sistema ductal (19). Desde el aparato de Golgi, las enzimas son transportadas hacia los gránulos de zimógeno que se dirigen a la membrana apical de las células para secretar las enzimas pancreáticas al lumen del acino mediante un proceso de exocitosis (10). Este proceso de exocitosis engloba tres pasos principales: el desplazamiento de los gránulos de zimógeno hacia la zona apical de la célula, el reconocimiento de un lugar específico para la fusión con la membrana apical y la fusión con la misma (18).

Los distintos tipos de enzimas digestivas pancreáticas son sintetizadas y secretadas como proenzimas inactivas para evitar la autodigestión del páncreas, activándose tras alcanzar la luz intestinal. Además, su almacenamiento en los gránulos de zimógeno con ambiente ácido impide su activación hasta ser liberadas al llegar a la membrana apical de las células acinares (20). Otro mecanismo que impide la activación temprana de las proenzimas digestivas es la secreción del péptido inhibidor de tripsina (PIT) desde las células glandulares del páncreas (10). Distinguimos cuatro grupos principales de enzimas, dependiendo de la naturaleza

de las biomoléculas que se encarguen de degradar: proteolíticas, glucolíticas, lipolíticas y nucleolíticas (21).

Las enzimas proteolíticas son las más abundantes y pueden dividirse en dos tipos: endopeptidasas, que hidrolizan enlaces peptídicos internos, y exopeptidasas, cuya acción se dirige a romper los enlaces situados en los extremos de las cadenas peptídicas. Las enzimas endopeptidasas son la tripsina, la quimotripsina y la elastasa, cuyas formas inactivas son el tripsinógeno, el quimotripsinógeno y la proelastasa. Llegadas a la mucosa duodenal, el tripsinógeno se transforma en tripsina por la acción de otra enzima presente en las criptas de Lieberkühn del duodeno, la enterocinasa o enteropeptidasa, que se secreta cuando el quimo alcanza esta zona (10). Al transformarse el tripsinógeno en tripsina, se produce una cascada de activación del resto de endopeptidasas a partir de esta tripsina. Las enzimas exopeptidasas son las carboxipeptidasas A y B, cuyas formas inactivas son las procarboxipeptidasas A y B, que deben este nombre a su acción de hidrolizar extremos carboxilos de las cadenas peptídicas (18).

Dentro de las enzimas glucolíticas cabe destacar la amilasa, encargada de la degradación de polisacáridos, como el glucógeno y el almidón (22).

Las enzimas lipolíticas se ocupan de la digestión de los lípidos. Entre ellas, la principal es la lipasa, que requiere de los ácidos biliares y de la unión a la colipasa para poder ejercer su acción digestiva, permitiendo la degradación de triacilglicéridos a ácidos grasos y monoglicéridos. La tripsina interviene indirectamente en la activación de la lipasa debido a que produce la activación de la procolipasa, forma inactiva de la colipasa (10). Otras enzimas lipolíticas son la colesterolesterasa, que hidroliza los ésteres de colesterol y retinol, y la fosfolipasa A_2 que produce la degradación de los ácidos grasos en fosfolípidos (22).

Por último, las enzimas pancreáticas encargadas de degradar los ácidos nucleicos son las ribonucleasas y desoxirribonucleasas, que hidrolizan los enlaces fosfodiésteres del ácido ribonucléico y desoxirribonucléico, respectivamente (10).

1.4.3 REGULACIÓN DE LA SECRECIÓN

En la regulación de la secreción pancreática se combinan estímulos hormonales y neurales, junto con otros que provocan una retroalimentación enzimática y mecanismos intracelulares. Existe, además, un efecto potenciador de unos estímulos sobre otros cuando actúan todos al mismo tiempo. La secreción pancreática se produce, por lo tanto, de la interacción de varios de estos estímulos (22).

1.4.3.1 HORMONAS Y AGENTES PARACRINOS

El control hormonal de la secreción pancreática viene dado, esencialmente por la secreción de secretina, colecistoquinina (CCK) y gastrina por parte de las células de la mucosa del duodeno, del yeyuno y del antro del estómago.

La secretina es el regulador principal de la secreción hidroelectrolítica que se activa cuando se produce un descenso en el pH del duodeno con la llegada del quimo ácido. La secretina neutraliza esta acidez creando un medio básico/neutro favorable a la secreción enzimática, que está controlada por la liberación de CCK (23). La gastrina es una hormona con una estructura similar a la CCK que ejerce un efecto estimulador más débil (23).

Otra hormona que tiene un efecto sobre la secreción enzimática exocrina es el polipéptido pancreático, que parece tener un efecto inhibitorio en respuesta a la CCK y a la secretina, así como a la presencia de alimento en el duodeno (24).

Las hormonas de la parte endocrina del páncreas, insulina y glucagón, también tienen efectos sobre la secreción exocrina pancreática. La insulina estimula la secreción de CCK y de secretina, por lo tanto, favorece la secreción enzimática e hidroelectrolítica, mientras que el glucagón las inhibe (25). La somatostatina se ha descrito también como otro inhibidor de esta secreción, pues impide la liberación de secretina desde las células duodenales (26).

1.4.3.2 NERVIOS, NEUROTRANSMISORES Y NEUROPÉPTIDOS

Debido a la extensa irrigación nerviosa, simpática y parasimpática, del páncreas, es lógico pensar que existirá una regulación de la secreción

pancreática en la que intervengan estas conexiones neurales. Las fibras parasimpáticas estimulan la secreción exocrina mediante la modulación de la secreción de CCK y secretina (27). Los nervios del sistema simpático inhiben la secreción pancreática indirectamente causando una vasoconstricción que disminuye el flujo sanguíneo debido a la liberación de norepinefrina estimulada desde el sistema nervioso central (28).

Sin embargo, el estímulo neural más importante en la regulación de la secreción pancreática es la liberación de acetilcolina (ACh). Este neurotransmisor tiene un efecto estimulante sobre la secreción de las células acinares pancreáticas. La secreción hidroelectrolítica también está estimulada positivamente bajo la acción de este neurotransmisor, pero en menor medida (22).

1.4.3.3 RETROALIMENTACIÓN ENZIMÁTICA

Parte de la regulación de la secreción pancreática puede deberse también a una retroalimentación negativa de determinadas enzimas y hormonas. La tripsina duodenal inhibe el incremento de CCK y, por lo tanto, de la secreción enzimática después de las ingestas (29).

A nivel del páncreas endocrino, también existe un mecanismo de retroalimentación negativa que actúan sobre las células PP de los islotes de Langerhans, inhibiendo la liberación de ACh y, con ello, la secreción pancreática exocrina (30).

1.4.3.4 MECANISMOS INTRACELULARES

En la inducción de los gránulos de zimógeno también participan mecanismos de regulación intracelulares dependientes de la liberación de Ca^{2+} extracelular y de la presencia de adenosín-monofosfato cíclico (AMPc) citosólico. El aumento del AMPc en el citosol produce una activación de la adenilato ciclasa en las células acinares que estimula la secreción granular por parte de estas (31).

El papel del ion Ca^{2+} como regulador intracelular de las secreciones pancreáticas ha sido ampliamente estudiado desde que se describió por primera

vez su relación con el páncreas exocrino en unos estudios de Hokin en 1966 (32). La activación por parte de la CCK y la ACh de otra proteín-quinasa, la proteín-quinasa C, regula la liberación de Ca^{2+} necesaria para el mantenimiento de la secreción de enzimas pancreáticas desde los canales iónicos de las células acinares (33).

2. PATOLOGÍAS PANCREÁTICAS EXOCRINAS

Son muchas las patologías que pueden afectar a la zona exocrina del páncreas, desde aquellas que presentan un componente hereditario, como la fibrosis quística, hasta deformaciones congénitas, neoplasias o cáncer, así como distintos desórdenes inflamatorios, como la pancreatitis aguda y la crónica. Cada una de ellas engloba un conjunto de eventos fisiológicos que acaba provocando la insuficiencia funcional de la glándula.

2.1 Pancreatitis aguda

La primera acepción nosológica de la pancreatitis aguda fue elaborada en 1889 por el patólogo Reginald H. Fitz, que, además, inquirió en la importancia del estudio y caracterización de esta afección, pues era mucho más frecuente de lo que se creía (34).

La pancreatitis aguda (PA) es un proceso inflamatorio del páncreas, generalmente leve, pero en aproximadamente un tercio de los pacientes está asociado a la aparición de complicaciones locales y sistémicas que, frecuentemente, resultan en una disfunción orgánica sistémica (35). La reincidencia de episodios de PA a lo largo del tiempo relacionada con determinados factores de riesgo, especialmente el abuso de alcohol y el tabaco, puede dar paso a una pancreatitis crónica (PC) en la que se produce un deterioro del parénquima pancreático, apareciendo una fibrosis que se extiende por todo el tejido con la pérdida de la funcionalidad de las células acinares (36), (37).

La PA se caracteriza, principalmente, por una activación prematura de las enzimas pancreáticas digestivas en las células acinares lo que lleva a la autodigestión de la glándula desencadenando la activación de un gran número de monocitos, diferenciados

en macrófagos, que inducen citoquinas proinflamatorias (38). En la clínica está caracterizada por la aparición de fuertes dolores abdominales y una elevación de los niveles de enzimas pancreáticas en suero (39).

1.2.3 EPIDEMIOLOGÍA

La PA es la principal causa de los ingresos hospitalarios relacionados con enfermedades gastrointestinales (40) presentando una elevada incidencia en Estados Unidos, varias regiones del oeste del Pacífico y el Norte y Este de Europa (41). El índice de hospitalizaciones debidas a este trastorno ha ido aumentando, sólo durante el 2009 se produjeron 275.000 ingresos, más del doble que en 1988 (40). Este incremento en la incidencia de la PA se debe, indirectamente, al aumento de la obesidad en la población que facilita la formación de cálculos biliares, una de las principales causas de la aparición de esta enfermedad inflamatoria (40). En España, entre los años 2008 y 2018, se identificaron 72 casos de cada 100.000 personas que ingresaban con PA, la mayoría debida a esta aparición de cálculos en la vesícula biliar (42).

A nivel global, los casos de PA se elevan a 34 de cada 100.000 habitantes por año, sin que existan unas diferencias determinantes entre hombres y mujeres, pero sí por edad, siendo más común entre la población de mediana y tercera edad (43). Si bien es cierto que la letalidad asociada a este trastorno ha descendido, la ratio de mortalidad de 1.16 casos de cada 100.000 por año se mantiene constante (43). Un dato curioso relacionado con la mortalidad asociada a esta enfermedad es que tras el primer episodio de PA, la mortalidad desciende. Se cree que puede deberse a que el daño sufrido en el parénquima pancreático tras el primer episodio aumenta la respuesta inflamatoria del páncreas (44). Existe, por tanto, una menor mortalidad entre los pacientes que ingresan con una PA recurrente, de los cuales cerca de un 36% evolucionarán a una PC, siendo más susceptibles aquellos que desarrollaron una PA alcohólica (45).

El índice de mortalidad de la PA es similar en cada una de las etiologías asociadas a ella (46). El fallo multiorgánico persistente, principalmente el que

afecta a los pulmones, es la mayor causa de muerte asociada a la PA, junto con otros factores que pueden incrementar el riesgo como la diabetes mellitus (47), la infección hospitalaria (48) o la edad avanzada (≥ 70 años) (49).

1.2.4 ETIOLOGÍA

La PA es un proceso inflamatorio que lleva asociada una extensa etiología, siendo la causa más frecuente la obstrucción del conducto pancreático o colelitiasis, que ocupa un 40% de los casos, seguida del consumo excesivo y prolongado de alcohol, que abarca hasta un 30% (50).

En los últimos diez años se han estudiado un gran número de estímulos que pueden producir la inflamación pancreática: metabólicos, anormalidades anatómicas y funcionales, distintas drogas, traumas, infecciones y predisposición genética. Los casos de PA que se registran debidos a estas causas se agrupan en un porcentaje de alrededor de un 15 – 20% (51). Sin embargo, sigue existiendo alrededor de un 10 – 15% de casos cuya causa no ha sido claramente identificada y se clasifican como PA idiopáticas (52).

1.2.4.1 CAUSAS OBSTRUCTIVAS

El desarrollo más común de PA es por obstrucción del conducto pancreático o colelitiasis ocasionada por la presencia de un cálculo a nivel de la papila de Váter o bien por la aparición de un edema papilar transitorio o por relajación del orificio de la papila que permite el reflujo duodenopancreático (46).

La activación del tripsinógeno ha sido tradicionalmente considerada como el evento patogénico central de la pancreatitis. La obstrucción del conducto pancreático provoca la acumulación de gránulos de zimógenos en las células apicales y activación del tripsinógeno en tripsina, produciéndose la autodigestión pancreática y desencadenando una respuesta inflamatoria en el parénquima. Sin embargo, la evidencia experimental apunta al papel de la activación de la tripsina en el daño temprano de las células acinares (53) pero no en la respuesta inflamatoria de la PA, que recientemente se ha demostrado que es inducida por la activación del nuclear factor kappa B (NFκB) (54).

Otro tipo de obstrucción puede deberse a la aparición de pólipos por la región periampular (alrededor de la papila de Váter), o por divertículos duodenales intraluminales que obstruyen el duodeno y pueden provocar la entrada del quimo al conducto pancreático (53). El carcinoma pancreático, el páncreas anular o una disfunción en el esfínter de Oddi pueden ocasionar también una obstrucción que desarrolle una PA (46). De hecho, se ha estimado que aproximadamente el 5 – 14% de los pacientes con tumores pancreatobiliares benignos o malignos son diagnosticados inicialmente como PA idiopática.

1.2.4.2 CAUSAS TÓXICAS

El abuso en el consumo de alcohol tiene un efecto tóxico sobre las células acinares pancreáticas. No está estimada la cantidad exacta que ha de ingerirse para desarrollar una PA, puesto que parecen intervenir también otros factores ambientales y genéticos (55).

La ingesta de alcohol produce un aumento del número de enzimas digestivos y lisosomales, facilitando su activación y contribuyendo al proceso de autodigestión del páncreas. El metabolismo del alcohol genera radicales libres que producen un estrés oxidativo en el interior de las células acinares, donde también aumenta los niveles de Ca^{2+} produciendo una disfunción mitocondrial (56).

Otras drogas como el tabaco o determinados medicamentos también se han establecido como posibles factores de riesgo de esta enfermedad inflamatoria (55). Existen diversos estudios sobre el efecto potenciador del consumo de tabaco en los casos de PA alcohólica en los que se observó como la nicotina presente en los cigarrillos afectaba directamente tanto a las células acinares pancreáticas como a las ductales (57).

1.2.4.3 OTRAS CAUSAS

En un conjunto más amplio de causas menos frecuentes se pueden englobar las mutaciones genéticas, determinados desórdenes metabólicos como la

hiperlipemia o hipercalcemia o problemas vasculares que suelen acompañar a una PA idiopática (46).

1.2.5 CLASIFICACIÓN

La clasificación de la PA siempre ha sido un tema complicado de abordar por la gran variabilidad que presenta en su diagnóstico clínico. Desde la antigüedad, cuando a todas las inflamaciones del páncreas se las englobaba dentro del término "cirrosis pancreáticas", hasta nuestros días, pancreatólogos y médicos e investigadores especializados han establecido varias clasificaciones, tanto clínicas como patológicas (58).

El Simposio Internacional de Atlanta de 1992 estableció una clasificación basada en la clínica en la que dividieron la pancreatitis en: PA, leve y severa, y PC (58). En el 2013, se realizó una revisión de esta clasificación teniendo en cuenta las mejoras en las técnicas de diagnóstico clínico y los nuevos conceptos que habían ido surgiendo en los últimos años. La clasificación se basó principalmente en discriminar la severidad de las distintas formas de PA y definir los conceptos de fallo orgánico y complicaciones locales (59). De esta forma se dividieron dos tipos de PA:

1.2.5.1 PANCREATITIS AGUDA EDEMATOSA INTERSTICIAL (PAEI)

Engloba el 80 – 90% de los casos clínicos de PA y es considerada la forma más leve de esta enfermedad inflamatoria que suele resolverse en la primera semana (60). Sus características histológicas principales son:

- Aparición de edema intersticial.
- Colecciones peripancreáticas de fluido con paredes poco definidas.
- Inflamación del parénquima pancreático y peripancreático.
- Ausencia de tejido necrótico observable.

1.2.5.2 PANCREATITIS AGUDA NECROTIZANTE (PAN)

Alrededor del 20% de los pacientes con PA desarrollan PAN, la forma más agresiva de la enfermedad que lleva asociado un elevado índice de mortalidad (61). Se caracteriza por presentar:

- Necrosis en el parénquima pancreático y/o en el tejido graso peripancreático, además de la característica inflamación.
- Pseudoquistes y abscesos pancreáticos.
- Aparición de colecciones necróticas agudas.
- Posible hemorragia intrapancreática.

La PAN puede presentarse a su vez como infecciosa o estéril, basándose en el diagnóstico de la aparición de sepsis y evaluando el tejido necrótico pancreático y peripancreático en el que puede aparecer necrosis infectada. Este tipo de PAN suele presentarse tras una intervención quirúrgica y eleva el riesgo de mortalidad (60).

La revisión de Atlanta también elaboró una clasificación de la severidad de este trastorno basada en la evaluación clínica, en la cual estableció tres grados principales de severidad:

- PA leve: no existe fallo orgánico ni aparecen complicaciones locales y/o sistémicas.
- PA moderadamente severa: fallo orgánico transitorio que suele resolverse en menos de 48 horas y pueden aparecer complicaciones locales o sistémicas.
- PA severa: persistencia del fallo orgánico, múltiple o simple, por encima de las primeras 48 horas.

En el desarrollo de la PA se pueden distinguir dos fases: una temprana que puede alcanzar hasta las dos semanas de duración, a lo largo de la cual pueden desarrollarse complicaciones sistémicas y/o el síndrome de respuesta compensatoria antiinflamatoria. Si cualquiera de ellas, o ambas, persisten en el tiempo, esta fase puede desembocar en un fallo orgánico. La segunda fase, la

fase tardía, puede extenderse hasta meses de duración, se caracteriza porque el fallo orgánico, o multiorgánico, se vuelve persistente, acompañado de complicaciones tanto locales como sistémicas (60).

1.2.6 COMPLICACIONES LOCALES Y SISTÉMICAS

A partir de la clasificación actualizada de Atlanta, se definieron los distintos tipos de colecciones que podían aparecer asociadas a la PA. Estas representan las complicaciones locales más comunes asociadas a esta enfermedad pancreática junto con el edema intersticial, característico de la PAEI, y la necrosis del tejido en la PAN:

- *Colecciones líquidas peripancreáticas agudas (CLPA)*: homogéneas, presentan líquido en su interior, pero no se distingue inflamación en las paredes ni necrosis. Aparecen adyacentes al páncreas y se desarrollan durante la primera semana (61).
- *Pseudoquistes peripancreáticos*: colecciones de fluido más definidas que las CLPA, en las aparece inflamación en las paredes, pero sin signos de necrosis (61).
- *Colecciones necróticas agudas (CNA)*: aparte del contenido líquido, presentan tejido necrótico y ciertos componentes sólidos otorgándoles un aspecto más heterogéneo (60). Pueden formarse tanto intrapancreáticas como extrapancreáticas y son complicadas de distinguir de las CLPA durante la primera fase de la PA pues la necrosis comienza a definirse más clara a partir de la segunda semana (61).
- *Necrosis pancreática encapsulada*: presentan una pared inflamatoria bien definida con necrosis pancreática y peripancreática. Aparecen a partir de las cuatro semanas desde el inicio de la PAN y se puede observar en su interior líquido heterogéneo con restos necróticos (61). Más del 80% de las muertes por PA se atribuyen a la aparición de este tipo de colecciones (38).

- *Edema interstitial*: inflamación aguda del parénquima pancreático y peripancreático sin necrosis (53). La formación de edema es una característica típica de los procesos inflamatorios que se produce debido a la liberación de mediadores inflamatorios que aumentan la permeabilidad de los vasos y disminuyen la tensión superficial de las membranas celulares (62).

Otras complicaciones locales asociadas a la PA son trombosis de la vena esplénica o porta manifestada por la aparición de ascitis, hemorragia pancreática o retroperitoneal, aneurismas en la arteria y fístulas pancreáticas (63).

En las complicaciones locales, la respuesta inflamatoria se concentra en el área pancreática y es controlada por el sistema inmune del huésped. Sin embargo, en los casos más graves de PA, los mediadores inflamatorios son liberados a la circulación sanguínea y, a través de ella, alcanzan otros órganos, desarrollándose el llamado síndrome de respuesta inflamatoria sistémica (SRIS) (64). El fallo multiorgánico es una de las complicaciones sistémicas más comunes, afectando, sobre todo, a los riñones y a los pulmones por la extensa red de capilares sanguíneos que rodea a estos órganos, así como al sistema cardiovascular (60). En ocasiones, la respuesta antiinflamatoria del organismo puede sobrecompensar y se produce el síndrome de respuesta antiinflamatoria compensatoria (SRAC) que puede ocasionar el desarrollo de una sepsis multiorgánica en la fase tardía de la PA (64).

3. RESPUESTA INFLAMATORIA ASOCIADA A LA PANCREATITIS AGUDA

El daño pancreático comienza con la activación del tripsinógeno en el interior de las células acinares produciendo una respuesta inflamatoria por parte de estas. En este proceso, se desencadena la secreción de determinadas citoquinas proinflamatorias que provocan la migración de neutrófilos hacia el tejido dañado. Si la respuesta inflamatoria

se mantiene y/o intensifica, la migración de neutrófilos puede extenderse hacia otros órganos favoreciendo la progresión de la PA hacia la forma más severa (65).

3.1 Citoquinas y quimioquinas

En la primera fase o fase temprana de la PA las células acinares pancreáticas expresan citoquinas y quimioquinas en respuesta a la activación prematura de las enzimas pancreáticas. Estas moléculas proinflamatorias van a promover el desarrollo de la PA, activando y atrayendo hacia el tejido dañado a las células inflamatorias circulantes, los polimorfonucleares (PMN) (66). Las citoquinas y quimioquinas proinflamatorias también promueven la adherencia y extravasación de neutrófilos aumentando la permeabilidad capilar y, de esta manera, pueden agravar el daño pancreático (67).

1.2.7 FACTOR DE NECROSIS TUMORAL – ALPHA

El factor de necrosis tumoral – α (TNF-α) es una citoquina pleiotrópica proinflamatoria secretada por macrófagos y células acinares pancreáticas que promueve la expresión de otras citoquinas. Presenta dos receptores de membrana: TNF-R1 o p-55 y TNF-R2 o p-75, a los cuales se une para desencadenar la regulación inflamatoria (67). Durante la PA, la concentración de esta citoquina aumenta tanto en el interior del páncreas como en los pulmones y en el hígado, por lo que se podría decir que contribuye a aumentar la respuesta inflamatoria local y el progreso hacia la respuesta sistémica relacionada con esta enfermedad (68). En los pulmones, el TNF-α activa los macrófagos alveolares y promueve la infiltración de neutrófilos. Los factores que facilitan esta infiltración, tanto en tejido pulmonar como pancreático, son el aumento de la permeabilidad vascular y la liberación de mediadores moleculares como el óxido nítrico (NO), la fosfolipasa A-2, los radicales libres de oxígeno (RLO) y el factor activador de plaquetas (68).

Esta citoquina también aparece relacionada con el estrés oxidativo producido en las células en respuesta a estímulos inflamatorios. Unos mayores niveles de TNF-α colaboran en la oxidación de xantina deshidrogenasa (XAD) a xantina oxidasa (XAO), enzima que presenta la capacidad de producir especies reactivas de oxígeno (ERO) que

provocan cambios en la membrana endotelial perdiendo la permeabilidad selectiva característica (69).

La unión de TNF-α a su receptor transmembrana TNF-R1 desencadena el proceso de muerte celular programada por apoptosis, por necroptosis o daño inflamatorio (70). Tras el ensamblaje entre TNF-α y TNF-R1 se forma un primer complejo molecular, complejo I, que activa la vía de NFκB desencadenando, posteriormente, la liberación de otras citoquinas y quimioquinas proinflamatorias (70). El complejo I presenta en su estructura un receptor que interactúa con proteínas (RIP-1) y que puede sufrir desubiquitinización por medio de la enzima CYLD (71). De esta manera, el complejo I pasa a convertirse en otro complejo, complejo II, que puede seguir dos vías dependiendo de la activación o inhibición de la caspasa-8. Cuando esta caspasa se activa, se produce la ruptura proteolítica de dos moléculas de la familia de las RIPs, RIPK-1 y RIPK-3, provocando su silenciamiento y llevando a la célula a un estado de apoptosis producido por la activación de la caspasa-3 (72).

Por otro lado, la inhibición de la caspasa-8 permite que RIPK-1 y RIPK-3 permanezcan ensambladas, provocando su fosforilación y la formación de una nueva estructura denominada necrosoma (73). Este nuevo complejo origina el reclutamiento y fosforilación de la proteína tipo dominio quinasa de linaje mixto (MLKL) que forma tetrámeros que atraviesan la membrana plasmática e inicia el flujo de Ca^{2+} hacia el exterior celular (74). En estas condiciones, RIPK-3 produce la liberación de ERO desde el interior mitocondrial hacia el citoplasma provocando, finalmente, la ruptura de la membrana plasmática e iniciando el proceso de necroptosis (71).

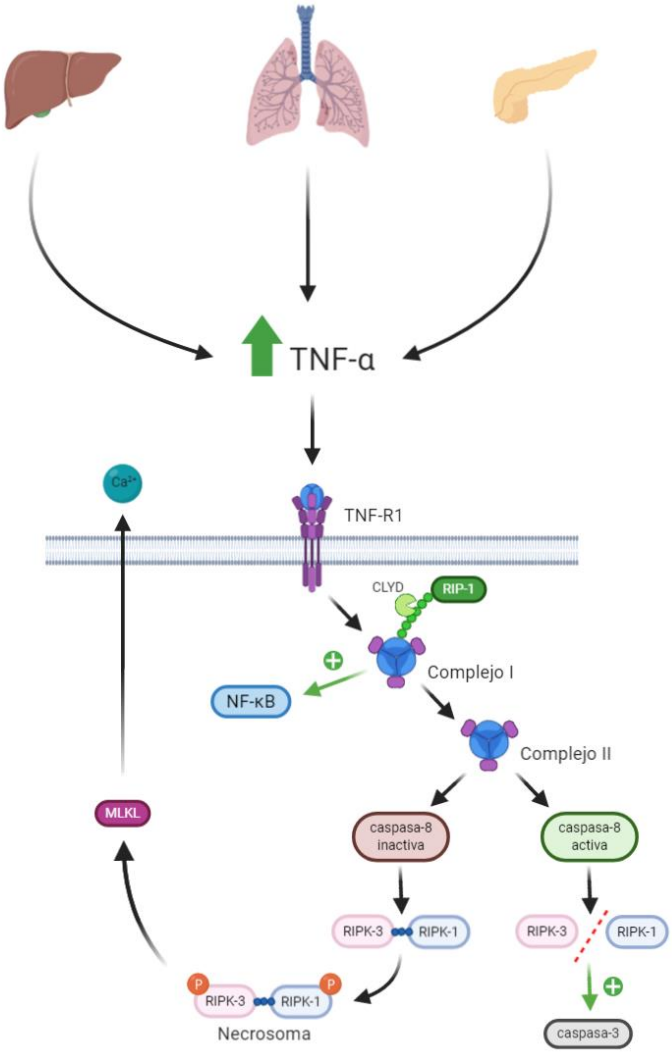

Figura 4. Vía de señalización de TNF-α durante la PA.

1.2.8 INTERLEUQUINA – 6

La interleuquina – 6 (IL-6) es una citoquina pleiotrópica proinflamatoria sintetizada en fibroblastos, monocitos, macrófagos, células T y endoteliales (75). La secreción de esta citoquina es inducida por la estimulación de receptores Toll – 4 (TLR-4) por la llegada de toxinas como el lipopolisacárido, o por la secreción de otras citoquinas como TNF-α o interleuquina – 1 (IL-1) (76). IL-6 media la secreción de proteínas de fase aguda, moléculas antiinflamatorias implicadas en el inicio de la respuesta inmune del organismo, y también regula el paso hacia procesos inflamatorios crónicos (77).

Los niveles de esta citoquina aumentan considerablemente en suero durante la PA, por lo que se le ha relacionado como un buen biomarcador para evaluar la severidad de este proceso inflamatorio, contando con un rango de sensibilidad de hasta el 90% durante las primeras 24 horas (77). Además, IL-6 promueve la secreción de otros mediadores moleculares asociados a severidad de la respuesta inflamatoria, como son el NO, moléculas de adhesión, y otras citoquinas (78). Un prolongado incremento de IL-6 puede llevar asociada la aparición de una respuesta inflamatoria sistémica con afectación pulmonar (79).

Esta citoquina puede seguir dos vías de activación dependiendo de la presencia o no del receptor de membrana de IL-6. Si este receptor no está presente en la membrana celular, IL-6 se une a su receptor soluble (IL-6Rs). El ensamblaje de IL-6 con su receptor forma un complejo que se une a una glucoproteína de membrana (gp130) (80). Este complejo produce la activación de la vía Janus quinasa/señal de activadores y transductores (JAK/STAT) (81). A través de la fosforilación de JAK se pueden inducir tres vías de activación de quinasas: STAT3, fosfatidilinositol-3-quinasa (PI3K) y las proteínas quinasas activadoras por mitógenos (MAPK) (91). La fosforilación de STAT3, que es un mediador de apoptosis, angiogénesis, inflamación y respuesta de fase aguda, contribuye al desarrollo de complicaciones sistémicas. STAT3 induce la expresión génica de citoquinas y quimioquinas proinflamatorias (82). La vía de PI3K representa una protección celular frente a la apoptosis asociada a procesos inflamatorios, en la cual en último lugar se produce la translocación de NFκB al núcleo. La activación de la cascada de MAPKs regula la expresión de determinados genes en el núcleo que desencadenan señales de supervivencia y proliferación celular (83). La activación de gp130 al formar el complejo con el receptor de IL-6 induce la unión de dos proteínas, proteína Shc y receptor de factor de crecimiento unido a proteína 2 (Grb2), que activan la proteína guanosín-trifosfatasa de unión a membrana (Ras), desencadenando la fosforilación de las MAPKs (84).

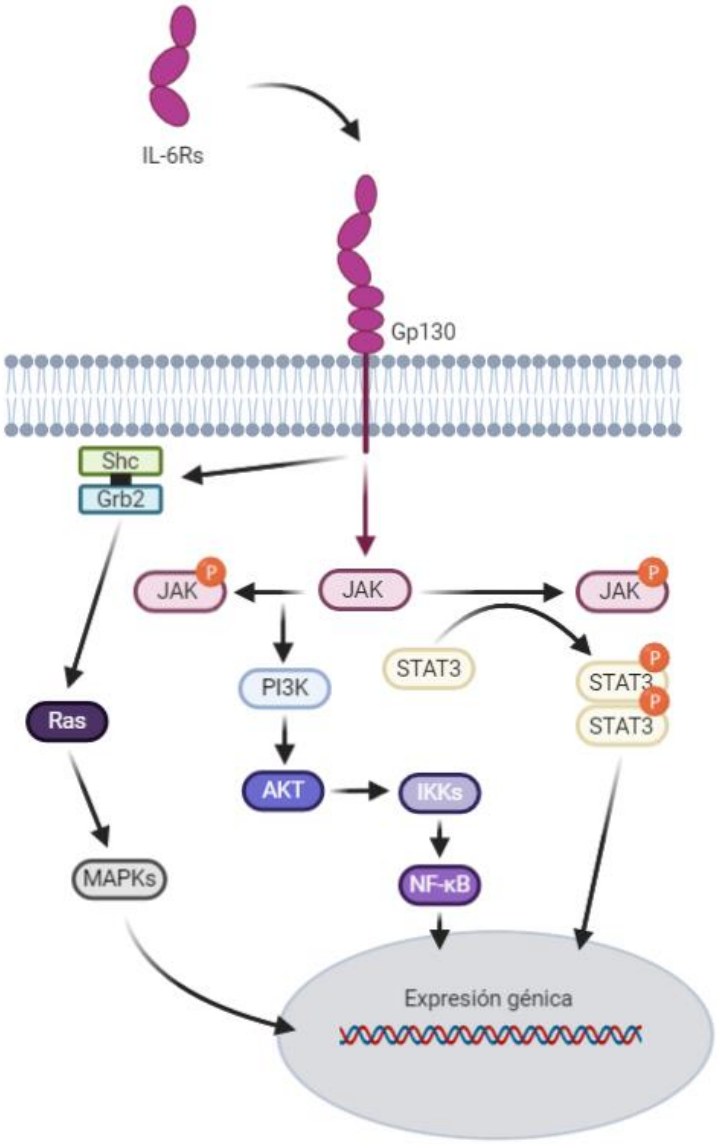

Figura 5. Vía de señalización de IL-6 durante la PA.

3.1.3 INTERLEUQUINA – 1B

Interleuquina – 1 β (IL-1β) es una citoquina proinflamatoria integrada en la familia de IL-1, junto con otras citoquinas como interleuquina – 33 (IL-33) e interleuquina – 1 alpha (IL-1α). Esta última comparte receptor con IL-1β, ambas se unen al receptor de membrana IL-1R. Sin embargo, IL-1β presenta una mayor afinidad por la parte soluble de este receptor, IL-1R2 (85). IL-1β se libera, principalmente, en monocitos, macrófagos y células dendríticas, activada por la llegada de patrones moleculares asociados a patógenos (PAMPs) o asociados a daño (DAMPs) también llamados “alarminas” (86).

Para que IL-1β sea secretada se requiere la proteólisis de la enzima, procaspasa-1, asociada al inflamosoma NLRP3, un complejo multiproteico de reciente nomenclatura, responsable de la activación de procesos inflamatorios como parte del sistema inmune innato. El inflamosoma NLRP3 está constituido por tres dominios proteicos: un dominio central, NACHT, un extremo carboxi-terminal rico en leucina, LRR, y un dominio de pirina, PYD (87). El ensamblaje del inflamasoma NLRP3 provoca la activación de la procaspasa-1 en caspasa-1, que actúa sobre la procitoquina IL-1β (proIL-1β) induciendo la transformación en su forma madura IL-1β, que será secretada al exterior celular (88).

La llegada a la célula de un estímulo externo a través de receptores de reconocimiento de patrones (PRRs) como TLR-4 puede activar factores de transcripción de genes relacionados con la respuesta inflamatoria del organismo. Una de las principales familias de factores de transcripción es la de NFκB. La fosforilación de una de las proteínas de NFκB, IκB, localizada en el citoplasma por medio de la IκB quinasa (IKB) desencadena la traslocación al núcleo de las subunidades de NFκB, p-50 y p-65. Estas son las que estimularan la producción de proIL1-β induciendo la transcripción de genes implicados en inflamación (89). La secreción de IL-1β es otro marcador de la inflamación pancreática. Esta citoquina aparece sobreexpresada en los primeros estadios de la PA, estando relacionada no solo con la inflamación local sino también con el desarrollo de complicaciones sistémicas asociadas, tales como daño hepático y pulmonar (90). Los niveles de IL-1β en suero provocan la activación de la tripsina en tripsinógeno y el descenso de la viabilidad de las células acinares pancreáticas estimulando la autofagia celular. También induce procesos de estrés en el retículo endoplasmático al producirse la liberación de Ca^{2+} al citosol por la activación de IL-1β, que altera la homeostasis intracelular de este ion (91).

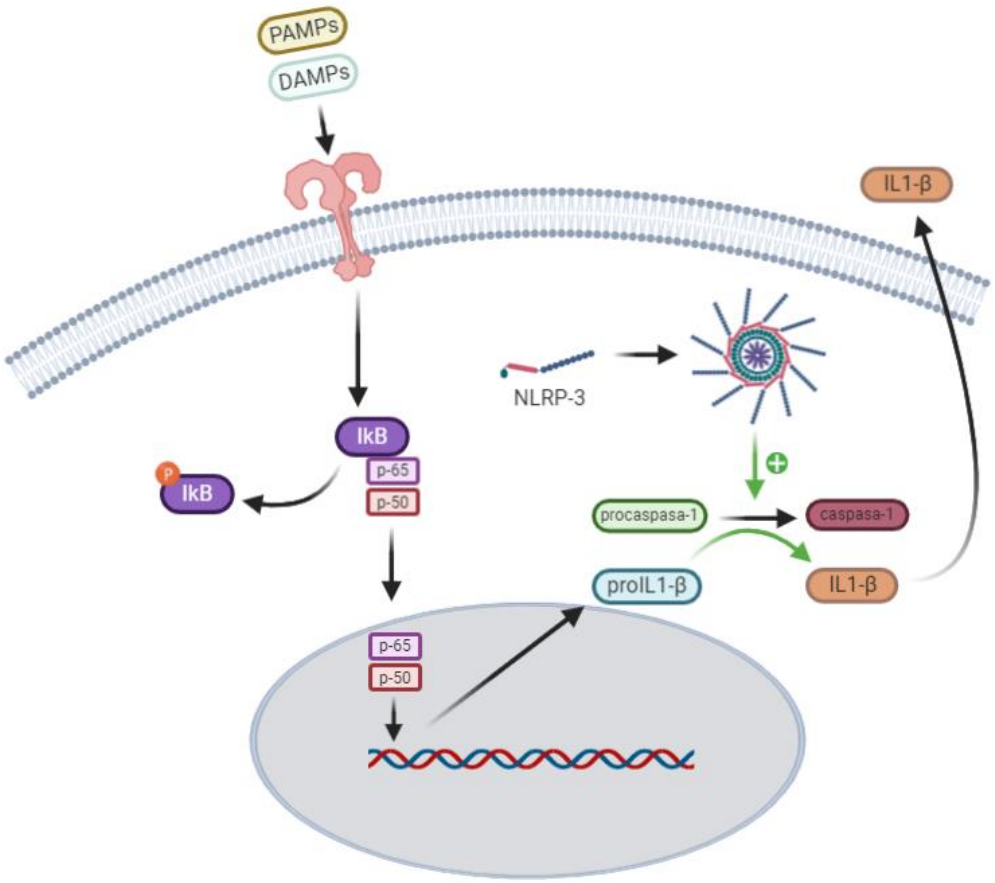

Figura 6. Vía de señalización de IL1-β e inflamosoma NLRP3 durante la PA.

3.1.4 QUIMIOQUINA DEL LIGANDO 2

Las quimioquinas son una familia de pequeñas citoquinas que promueven la atracción quimiotáctica de los monocitos y neutrófilos a los tejidos dañados.

La quimioquina del ligando 2 (CXCL-2) es producida en las células acinares pancreáticas durante la fase temprana de la PA actuando como inductora de la cascada inflamatoria al atraer neutrófilos hacia el tejido pancreático (92). La señal de transcripción de esta quimioquina llega a través de la activación de los TLRs por la presencia de DAMPs.

CXCL-2 presenta un receptor en la membrana de los neutrófilos, CXCR-2, cuya unión permite la extravasación leucocitaria (93). Además, recientemente, se ha implicado esta

unión entre CXCL-2 y su receptor transmembrana con la activación del inflamosoma NLRP3 en el interior de los macrófagos (94).

CXCL-2 juega un papel importante en la progresión de la inflamación al estar relacionada con procesos de migración celular (95). Los efectos de esta quimioquina se han estudiado en diversos modelos experimentales de enfermedades inflamatorias (96).

La liberación de las citoquinas y quimioquinas es necesaria para atraer las células inmunes (monocitos, macrófagos, neutrófilos y linfocitos) hacia las células acinares del páncreas donde se activa la respuesta inflamatoria.

3.2 Moléculas de adhesión

En el proceso de activación de la respuesta inflamatoria tienen un papel fundamental las moléculas de adhesión, glicoproteínas de la membrana celular, que permiten la migración celular, la proliferación y la transducción de la señal inflamatoria (97).

Las moléculas de adhesión intervienen en el mantenimiento de la homeostasis y de la estructura celular. Su función principal es la adhesión de los leucocitos y su "rodamiento" a través del endotelio de los vasos sanguíneos (98). Entre las principales moléculas de adhesión que intervienen en procesos inflamatorios están las selectinas y las moléculas de adhesión intercelulares (ICAMs).

Las selectinas pueden ser de tres tipos dependiendo de célula en la que se expresen: endoteliales (E-selectinas), leucocitarias (L-selectinas) y plaquetarias (P-selectinas). Las E-selectinas y las P-selectinas participan en el desarrollo de la necrosis celular en la PA. La P-selectina en concreto ha sido relacionada en múltiples estudios con el daño pulmonar asociado a la PA (99).

Las ICAMs promueven la unión entre leucocitos y células endoteliales para iniciar la migración transendotelial. Están relacionadas con la aparición de necrosis en el tejido pancreático, aunque no permiten diferenciar entre una necrosis infectada o estéril (97). También están involucradas en la progresión del daño pulmonar asociado a la PA (99).

3.2.1 P – SELECTINA

La P-selectina es una molécula de adhesión que transloca a la membrana de las células endoteliales bajo un estímulo inflamatorio. Esta glicoproteína se encuentra almacenada en los gránulos α de las plaquetas y en los cuerpos de Weibel-Palade de las células endoteliales. Cuando estas células se activan por la presencia de mediadores como la histamina o la trombina, la P-selectina se redistribuye a lo largo de la superficie celular (100) y se inicia el proceso de adhesión leucocitaria (101). La P-selectina se une a su ligando y esta interacción media el inicio del reclutamiento y rodamiento leucocitario (102).

Si la activación del endotelio vascular se mantiene puede desencadenarse una sobreexpresión de esta molécula de adhesión y, por lo tanto, un incremento de la extravasación de leucocitos y de la presencia de estos en el tejido afectado (103).

La sobreexpresión de la P-selectina, al estar relacionada con la persistencia de la respuesta inflamatoria, la convierte en una posible diana terapéutica para paliar las complicaciones asociadas a las enfermedades inflamatorias. También se ha estudiado como un posible biomarcador de inflamación para una detección precoz que permita administrar un tratamiento adecuado para mejorar la morbilidad y mortalidad del paciente (101).

3.2.2 ICAM – 1

La ICAM-1 es una glicoproteína transmembrana de la superfamilia de las inmunoglobulinas que actúa como molécula de adhesión expresándose en la membrana de varios tipos celulares, entre ellos los leucocitos y las células endoteliales (104).

Existen numerosos estudios que han investigado su función en la migración transendotelial, así como su participación en la sinapsis inmunológica, la unión entre las células presentadoras de antígenos y las células T (104). Estas dos funciones se desencadenan a raíz de la interacción entre ICAM-1 y su principal ligando, el antígeno asociado con la función de linfocitos (LFA-1) (105).

ICAM-1 tiene un importante papel en la respuesta inmune tanto innata como adaptativa. Por este motivo, terapias que inhiban la sobreexpresión de esta glicoproteína podrían evitar la propagación de la respuesta inflamatoria, limitando las complicaciones sistémicas. Además, ICAM-1 presenta una forma soluble en el plasma cuyos niveles aumentan de manera proporcional a la severidad de la enfermedad, constituyendo su medición como una posible herramienta de diagnóstico clínico (106).

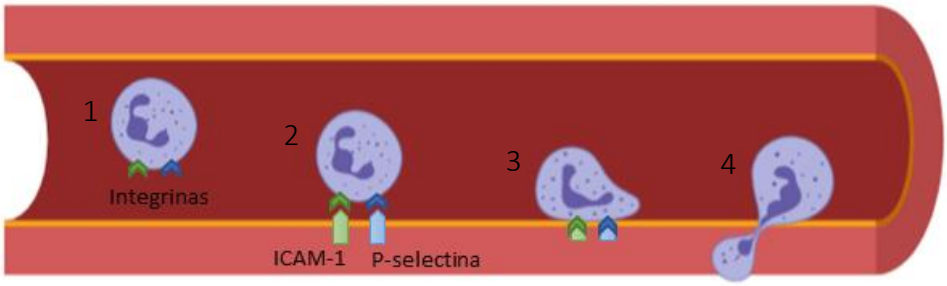

Figura 7. Proceso de extravasación leucocitaria en el que intervienen las moléculas de adhesión p-selectina e ICAM-1. 1: Neutrófilo e integrinas. 2: Captación del neutrófilo por atracción de las moléculas de adhesión y rodamiento. 3: Adhesión del neutrófilo al endotelio. 4: Extravasación del neutrófilo hacia el tejido dañado.

3.3 Muerte celular inducida por inflamación

Tanto la necrosis como la apoptosis son formas de muerte celular que han sido relacionadas con la inflamación pancreática. Las células necróticas producen la liberación de DAMPs y otras moléculas que activan la respuesta inflamatoria, mientras que la apoptosis desarrolla su papel inmunológico formando compartimentos en el interior celular que se degradan por autofagia, aunque también pueden participar en la liberación de estos DAMPs (107).

3.3.1 NECROSIS

El proceso de necrosis celular corresponde a una autodigestión de la propia célula por medio de sus enzimas que produce una inflamación, una ruptura de la membrana celular y la consecuente liberación del contenido intracelular al espacio intersticial dañando a las células vecinas (108). Este proceso siempre se había descrito como un suceso descontrolado que se desencadena en las células debido a la presencia de un determinado estímulo. Sin embargo, recientemente, se ha descrito un proceso celular

necrótico “programado” llamado necroptosis. Los estudios que han secundado esta nueva forma de “necrosis regulada” se basan en la presencia de receptores de muerte celulares que activan distintas vías de señalización y que están regulados por genes (109) (71). Los primeros receptores implicados en este tipo de muerte celular son RIPK-1 y RIPK-3, los cuales, como se ha descrito anteriormente, forman un complejo denominado necrosoma que se encarga de fosforilar la enzima MLKL, que actúa como efector en este proceso de muerte celular. Una vez fosforilada, MLKL transloca a la membrana celular provocando un desequilibrio osmótico al influir sobre el intercambio iónico (110). A parte de este evento, durante la necroptosis también tiene lugar la producción de ERO (111). La necrosis en las células acinares pancreáticas es una de las complicaciones más serias de la PA, relacionada con la forma más severa de esta enfermedad.

3.3.2 APOPTOSIS

La apoptosis es un proceso de muerte celular programada que depende de receptores intracelulares y extracelulares. Morfológicamente, durante la apoptosis las células sufren un encogimiento, se produce una condensación de su cromatina y fragmentación del ADN, y se forman los característicos cuerpos apoptóticos (112). Existen dos vías apoptóticas principales: una extrínseca en la que se desencadena una cascada de activación de caspasas por medio de la inducción inicial de un receptor celular de la caspasa-8. A partir de esta, se comienzan a activar otras caspasas, como la caspasa-3 que rompe residuos proteicos (112). La otra vía apoptótica, intrínseca a la célula, se inicia con la activación de determinados factores que regulan la permeabilización de la membrana mitocondrial, como la familia de las proteínas BCL-2. Cuando la integridad de la membrana mitocondrial se pierde, se produce la liberación al citosol de factores pro-apoptóticos, como el citocromo C, que forma un complejo llamado apoptosoma junto con la procaspasa-9 y con el factor de activación de la proteasa-1 (Apaf-1), activando la caspasa-9 (112). Dentro de la familia de las proteínas BCL-2, también existen bloqueadores que impiden la liberación del citocromo C porque bloquean el proceso de permeabilización (113). Estos son algunos de los principales efectores apoptóticos que ejercen un papel protector al inducirse en las células acinares pancreáticas. El lipopolisacárido (LPS), la ceruleína y varias citoquinas proinflamatorias inducen vías de señalización apoptóticas en la PA (107).

Durante los procesos de necrosis y apoptosis se dan lugar en la célula diferentes eventos que promueven la activación de la respuesta inflamatoria (114). Una de las características de la apoptosis celular es la creación de una inmunotolerancia, desencadenándose procesos antiinflamatorios en la célula mientras que en la necrosis se activan vías proinflamatorias que producen la activación de una respuesta autoinmune (115). Existe, por lo tanto, un enlace entre las vías de muerte celular e inflamación. Dentro de la ruta extrínseca apoptótica, la caspasa-8, al igual que la caspasa-1, puede activar el complejo del inflamosoma NLRP3 para inducir la muerte por apoptosis. Asimismo, a través de la ruta necroptótica de fosforilación de la MLKL se puede inducir la activación de NLRP3 (116). MLKL activa transloca a la membrana celular y provoca un flujo de potasio, desequilibrando la homeostasis celular y permitiendo el ensamblaje de NLRP3. Otro estímulo activador de este inflamasoma es la unión de RIPK-1 y RIPK-3 durante la necroptosis celular (117). La formación de este complejo desencadena la activación de la caspasa-1 y, en respuesta a estímulos infecciosos, induce NLRP3 a través de las ERO liberadas desde la mitocondria (118). La disrupción en la membrana celular producida durante el proceso de necroptosis también facilita la liberación de IL-1β, otro indicador principal de inflamación (119).

3.4 Exosomas

Como ya se ha descrito anteriormente, los pulmones son uno de los órganos que aparece dañado más frecuentemente cuando se produce el SRIS. De hecho, cerca del 60% de las muertes asociadas a la PA que aparecen durante la primera semana de ingreso son debidas a una disfunción pulmonar grave (120). Recientemente, se ha descrito la relación de los exosomas con esta complicación pulmonar asociada a la PA (121).

Los exosomas son nanovesículas extracelulares con una membrana formada por una bicapa lipídica, de origen endocítico, y en su interior contienen biomoléculas de diversa naturaleza. Estas nanovesículas son secretadas por varios tipos celulares tanto en situaciones fisiológicas normales como patológicas (122).

Durante la PA, las células pancreáticas liberan exosomas al medio extracelular. Por un lado, estos exosomas se acumulan en el líquido ascítico y una parte de ellos se degrada por hidrólisis enzimática, mientras que la otra llega al hígado a través del sistema porta, donde se encuentran el resto de exosomas procedentes del páncreas (123). Los exosomas son retenidos en el tejido hepático que actúa como un filtro, pero no presentan afinidad por ningún órgano específico. Este hecho puede estar más relacionado con la distribución del flujo sanguíneo local y la posición anatómica del páncreas respecto al hígado (121). Las células hepáticas, respondiendo a este estímulo, también son capaces de generar exosomas que se liberan al sistema circulatorio para alcanzar los compartimentos alveolares de los pulmones, junto con aquellos de origen pancreático que no hayan sido filtrados (123). Estos exosomas son capaces de atravesar la barrera endotelial alveolar para ser captados por los macrófagos. Bajo este estímulo provocado por la captación de los exosomas, los macrófagos alveolares responden activándose y produciendo citoquinas proinflamatorias que causan daños en el tejido pulmonar (124). Este es uno de los hechos que confirmó la participación de los macrófagos alveolares en el progreso de la PA y sus complicaciones sistémicas (125).

La liberación de exosomas durante la PA es capaz de activar los macrófagos alveolares y estos generan mediadores que forman parte de la respuesta inflamatoria asociada a esta enfermedad. De esta manera, se ha confirmado la participación de los exosomas en enfermedades inflamatorias como la PA y su influencia en la propagación sistémica de la misma.

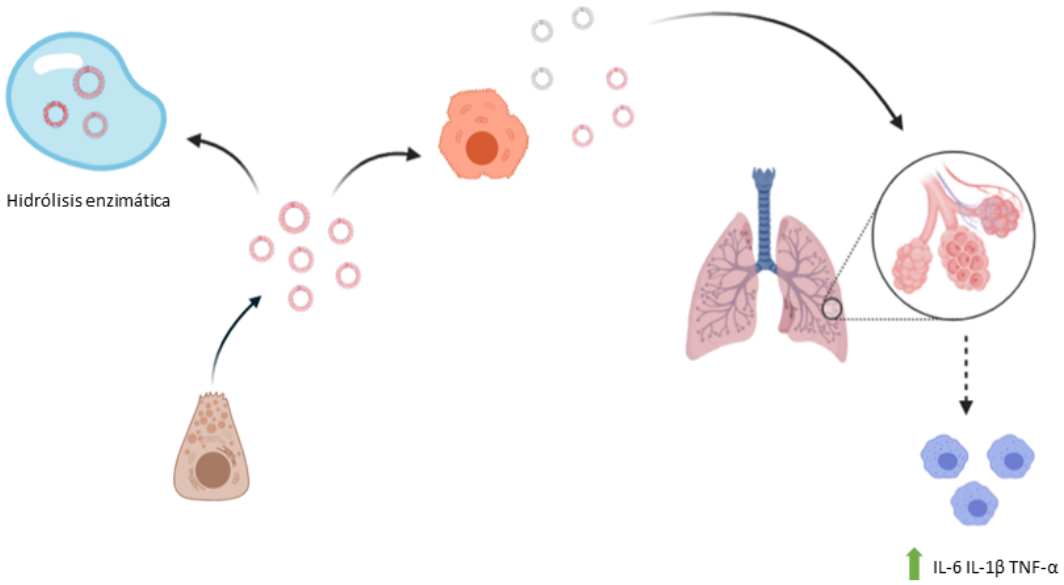

Figura 8. Liberación y transporte sistémico de los exosomas desde las células acinares pancreáticas hasta los alveolos pulmonares donde inducen una respuesta inflamatoria al producir la activación de macrófagos alveolares.

4. MANEJO Y DIAGNÓSTICO CLÍNICO

El diagnóstico preciso de la PA es crucial durante las primeras 48 – 72 horas para asignar un tratamiento acorde a la severidad de ésta y evitar, en la medida de lo posible, una evolución más grave, así como la aparición de posibles recidivas.

4.1 Técnicas de diagnóstico y evaluación de la severidad de la pancreatitis aguda

El diagnóstico clínico de este proceso inflamatorio se realiza cuando se cumplen al menos dos de los siguientes criterios: dolor abdominal en la región periumbilical y/o epigástrica que irradia hacia la espalda, niveles en suero tres veces superiores al normal de las enzimas pancreáticas, lipasa y amilasa, siendo especialmente determinante la lipasa, y el estudio de imágenes de la sección transversal del abdomen, en las que pueden aparecer reflejadas complicaciones, tanto locales como sistémicas, características de la PA (64).

La sintomatología característica de ingreso de los pacientes con PA se inicia con un fuerte dolor abdominal que aparece súbitamente y que puede llevar asociado otros síntomas físicos como vómitos, náuseas, fiebre, equimosis, hipotensión, taquicardias o taquipnea (126). En los primeros estadios puede ayudar al diagnóstico la evaluación de la presencia o no de determinados factores de riesgo como la edad, el historial de consumo periódico de alcohol, la obesidad y/o enfermedades comórbidas (cáncer, infartos, enfermedades crónicas en hígado o riñón) (127).

Alrededor de un 15 – 20% de casos clínicos de PA evolucionan con complicaciones severas y un elevado índice de mortalidad, por lo que es de extrema importancia la evaluación del grado de severidad de la PA en los primeros estadios (128). Para ello se usan distintos sistemas de puntuación que ayudan al pronóstico. El criterio de Ranson fue el primero en utilizarse, pero no permite evaluar determinadas medidas antes de las 48 horas del ingreso hospitalario. Actualmente, dos de los sistemas más utilizados son el "Sequential Organ Failure Assessment" (SOFA) y el "Acute Physiology and Chronic Health Examination II" (APACHE II). Ambos son buenos predictores de la mortalidad asociada a la PA permitiendo un monitoreo constante (129).

Unidas a estas escalas de graduación de la severidad de la PA, se utilizan técnicas de imágenes que permiten confirmar el diagnóstico previo con mayor fidelidad, así como detectar posibles complicaciones locales y/o sistémicas. En los últimos años se han ampliado y mejorado permitiendo también una clasificación clínica más ajustada de este trastorno. Las modalidades tradicionales incluyen la radiología simple, la ecografía abdominal, la tomografía computarizada (TC), la colangiopancreatografía retrógrada endoscópica, y las más actuales son la ecografía endoscópica y la colangiopancreatografía por resonancia magnética (130). Además del diagnóstico por imágenes, también puede ayudar a la evaluación de la severidad de la PA el análisis de otros biomarcadores. La determinación de la amilasa y la lipasa son las pruebas de laboratorio por excelencia. Otros parámetros que pueden analizarse y ayudar a los médicos en la diagnosis de la posible necrosis pancreática son los niveles de

hematocrito, la presencia de nitrógeno en sangre y la presencia de la proteína C reactiva. Sin embargo, la medición de estos parámetros moleculares no determina un diagnóstico preciso por ellos solos, han de ir acompañados de la observación de imágenes, generalmente por TC (126).

4.2 Tratamiento y terapias actuales

Una vez diagnosticada la etiología y severidad de la PA se inicia un tratamiento básico en la sala de emergencia en el que se prevé de analgesia, soporte nutricional y reemplazo intravenoso de líquidos (61). Los pacientes que presentan SRIS en la fase temprana o fallo multiorgánico ingresan directamente en la unidad de cuidados intensivos (UCI). El reemplazamiento adecuado de fluidos durante las primeras 72 horas es determinante para evitar este ingreso durante la fase tardía y está relacionado con un descenso de la mortalidad asociada a la PA (127). Para mitigar los daños producidos durante un SRIS se puede realizar una administración intravenosa de inhibidor de proteasas o hemofiltración (128).

El control del dolor abdominal con analgésicos es esencial desde el inicio del ingreso y también el aporte nutricional, preferiblemente por vía enteral al ser una técnica menos invasiva (53). La administración de nutrición enteral es recomendada antes de que transcurran las primeras 48 horas tras la hospitalización y está relacionada con un menor riesgo de desarrollo de fallo multiorgánico e infecciones (129). Si aparecen complicaciones que agravan la fisiopatología de la PA relacionadas con SRIS o una intolerancia a la vía oral es posible pasar a una nutrición parenteral (127).

El uso de antibióticos en el tratamiento de la PA viene indicado exclusivamente para los casos en los que aparecen infecciones locales o sistémicas, por lo que es conveniente confirmar la presencia de estas a través de una TC que pueda mostrar tejido pancreático o peripancreático necrótico infectado (131).

En las PA causadas por una obstrucción biliar, es recomendable la realización de una endoscopia para disminuir el riesgo de una posible recaída (38). La intervención quirúrgica está indicada para aquellos casos en los que la PA se produce por la presencia de cálculos biliares, o la existencia de una necrosis infectada que no remite con el tratamiento de antibióticos durante más de cuatro semanas (132).

El diagnóstico y el tratamiento de la PA continúan siendo retos clínicos importantes debido, en parte, al aumento de ingresos hospitalarios en las últimas décadas (133) y a la gran variabilidad de formas clínicas que puede presentar (127). El avance en la comprensión fisiopatológica de este trastorno y en la investigación biomédica son pilares básicos para ampliar y mejorar la diagnosis y prognosis e intervención clínica.

5. POLIETILENGLICOLES

5.1 Estructura, nomenclatura y propiedades fisicobioquímicas

Los polietilenglicoles (PEGs) son compuestos líquidos o sólidos de bajo punto de fusión formados por la polimerización de "n" unidades de etilenglicol, también conocido como óxido de etileno, cuya estructura química es HO-(CH_2-CH_2-O-)$_n$-H. Pueden formar estructuras lineales y tridimensionales, sin carga eléctrica (134). Dependiendo de sus distintos pesos moleculares, variarán las propiedades físicas que presenten estos polímeros y su estructura química definirá también su actividad biológica (135).

Los PEGs son compuestos solubles en agua y en disolventes orgánicos debido a la presencia de moléculas de agua en su estructura que forman enlaces de hidrógeno, lo cual les confiere biocompatibilidad y procesabilidad (136). La formación de estos enlaces, unida a la flexibilidad y movilidad que presentan las cadenas de etilenglicol, puede impedir la adsorción de proteínas a las membranas celulares (137).

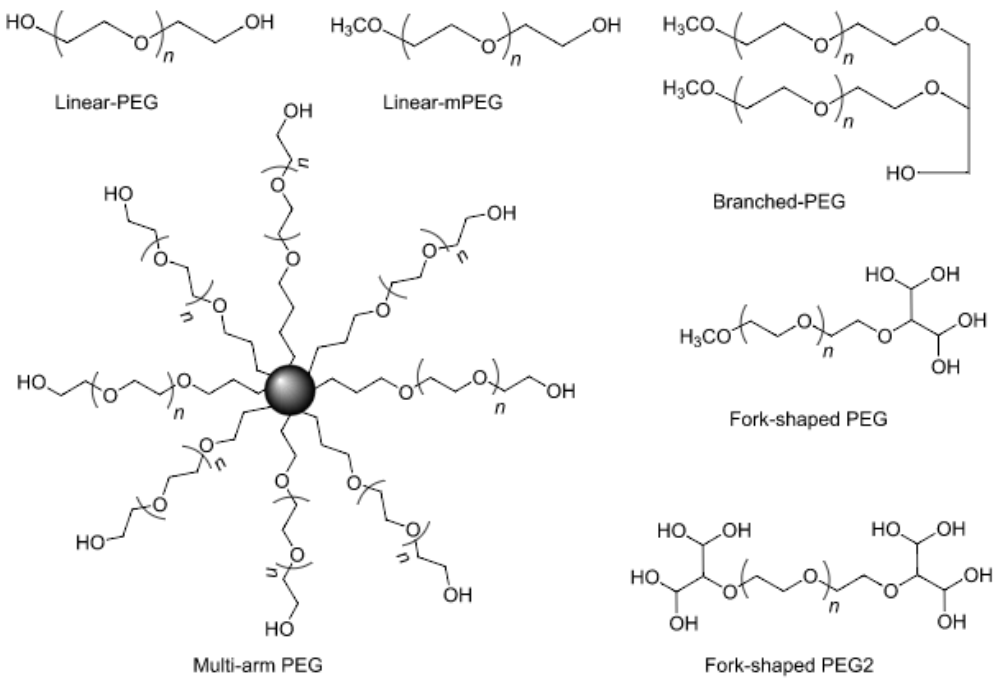

Figura 9. Estructura y tipos de PEGs.

Los PEGs presentan la capacidad de establecer enlaces covalentes con biomacromoléculas, proceso conocido como PEGilación. Esta unión les confiere mayor estabilidad física y térmica, aumento de la solubilidad, reducción del aclaramiento plasmático, reducción de la inmunogenicidad y de la antigenicidad y mejoría del perfil de toxicidad (138). Además, son fácilmente desechables por el organismo a través del riñón o del hígado, dependiendo de su peso molecular (139).

Desde que se describió por primera vez en 1977 la propiedad de PEGilación, se ha considerado que los PEGs carecen de inmunogenicidad y antigenicidad (140). Recientemente, esta propiedad asociada a los PEGs se ha puesto en duda en algunos estudios en los que se habla de la formación de anticuerpos frente a esta familia de polímeros (141). Sin embargo, no se ha llegado a un consenso claro sobre estos estudios de antigenicidad, pues en la literatura científica podemos encontrar cierta controversia al respecto (142).

5.2. Efectos de los PEGs en condiciones fisiopatológicas

El uso de los PEGs se ha estudiado en las últimas décadas en varios modelos experimentales asociados a diferentes patologías.

En los procesos inflamatorios, la liberación de citoquinas afecta a la estructura del glicocálix, exudado que rodea la membrana celular compuesto por glicoproteínas y glicolípidos que contribuye a la comunicación intercelular. También tiene efectos sobre las propias células inflamatorias, macrófagos y neutrófilos, que segregan enzimas y ERO que desestabilizan las membranas. Estos mediadores inflamatorios permiten la unión de los neutrófilos a moléculas de adhesión produciéndose la extravasación leucocitaria a través de la membrana endotelial dañada (143). La permeabilidad de la membrana, que regula el flujo de agua y proteínas entre el interior del vaso y el intersticio, se ve alterada produciéndose un desequilibrio osmótico debido a los cambios en las presiones hidrostática y oncótica que da lugar a la acumulación de líquido intersticial, llamado edema (144).

Por su estructura y su naturaleza química los PEGs presentan la capacidad de formar coloides (145). Estas estructuras coloidales aumentan la presión oncótica y evitan la desestabilización del glicocálix. De esta manera, los PEGs limitan los efectos deletéreos del edema que aparece como una complicación asociada a los procesos de preservación de órganos para trasplantes (146).

Los PEGs, al interactuar con los glicerofosfolípidos de las membranas celulares, son capaces de “enmascarar” determinados receptores evitando la unión con sus ligandos (136). Con la aparición de complicaciones inflamatorias locales y sistémicas en el organismo, el glicocálix pierde su función al desestructurarse (143). La interacción con la membrana celular les confiere a estos polímeros un efecto citoprotector al ayudar a preservar su integridad (147).

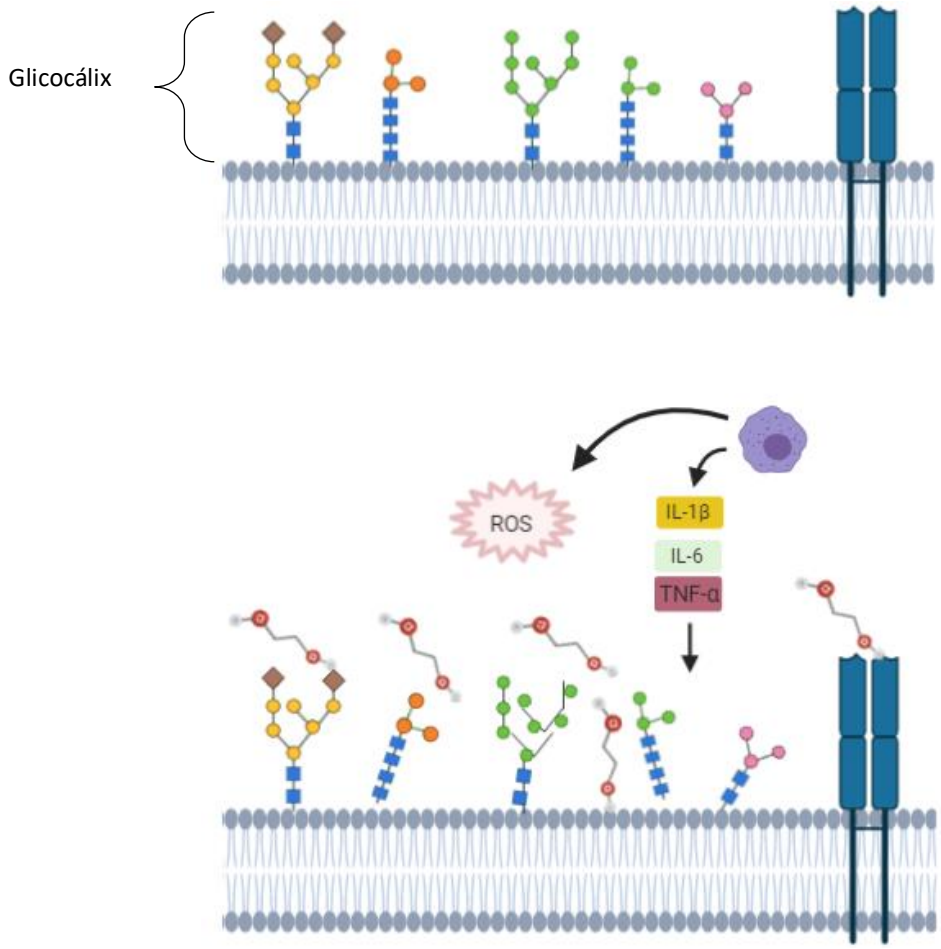

Figura 10. Estructura del glicocálix. Arriba: estructura de la membrana celular con la bicapa lipídica y el glicocálix. Abajo: llegada de un estímulo inflamatorio que desestabiliza la estructura y unión de las moléculas de PEG que enmascaran receptores transmembrana y reorganizan el glicocálix.

Una de las consecuencias de la isquemia-reperfusión (I-R) orgánica es la alteración del glicocálix endotelial, que se asocia con la formación de edema, la adhesión de neutrófilos y una pérdida de la respuesta vascular, todos ellos eventos característicos de la respuesta inflamatoria del organismo (148), (149). Los PEGs pueden recuperar la integridad de la membrana para que pueda ejercer su efecto citoprotector y también contribuyen al inmunocamuflaje celular enmascarando lugares de unión a antígenos mediante enlaces covalentes con la membrana lipídica (150).

Otro de los efectos que tienen estos polímeros es la prevención de la generación de ERO, evitando el daño celular por estrés oxidativo característico de las condiciones de hipoxia-reoxigenación a las que se someten las células en procesos de isquemia (151).

El efecto antiinflamatorio de los PEGs ha sido probado en varios modelos experimentales en los que se utilizaron soluciones de polietilenglicol de distintos pesos moleculares. En un modelo experimental de colitis en ratas, PEG de 4000 Da fue administrado vía oral para reforzar la barrera epitelial y disminuir la respuesta inflamatoria en el colón (152). En otro estudio de un modelo experimental de inflamación postraumática, el PEG fue usado para recubrir la superficie peritoneal de las ratas y, de esta manera, reducir la presencia de leucocitos (153). Asimismo, el PEG de 8000 Da administrado con la dieta, en un modelo experimental de cáncer de colón en ratas, inhibía la formación de tumores debido a su efecto antiinflamatorio en el tejido colónico (154).

En concreto, nuestro grupo de investigación ha explorado los beneficios del PEG de 35 kDa (PEG35) en diferentes modelos experimentales de I-R fría y caliente en ratas. La adicción de PEG35 a la solución de preservación de órganos reduce la vulnerabilidad del injerto hepático al daño por isquemia-reperfusión, evitando la autofagia (155). Por otro lado, nuestro grupo de investigación también evaluó la administración profiláctica intravenosa de PEG35 en un modelo experimental de I-R hepática en ratas. El PEG35 mantenía la función mitocondrial, proporcionaba protección al citoesqueleto e inducía vías de señalización citoprotectoras (146).

5.3 Aplicaciones de los PEGs

Una de las características que hace a los PEGs más interesantes desde el punto de vista del desarrollo de aplicaciones biológicas es que son compuestos de baja toxicidad, cuyo uso ha sido aceptado por la “Food and Drug Administration” (FDA) en sectores como la industria alimentaria, farmacéutica y cosmética (156). De esta manera, pueden aparecer como componentes de distintos productos comerciales, tales como:

- Surfactantes, agentes de limpieza, dispersantes y solventes dentro de la industria química (157).

- Cremas, dentífricos y lubricantes.
- Impermeabilizantes de objetos y/o superficies.

En cuanto a sus usos farmacéuticos, los PEGs son importantes agentes de revestimiento de pastillas, utilizados en fármacos de liberación controlada, y buenos excipientes en formulaciones orales, tópicas, oftalmológicas y rectales. También está extendido su uso como supositorios y laxantes (158).

En medicina, estos polímeros, bajo su estructura de hidrogeles, pueden formar adhesivos para el cierre y tratamiento de heridas. En técnicas más complejas e innovadoras de medicina regenerativa, como es la ingeniería tisular, se han utilizado para la formación de aloinjertos. Hoffman et al. (159) diseñaron un periostio utilizando hidrogeles de PEGs hidrolíticamente degradables para trasplantar y localizar células madre mesenquimales en las superficies del aloinjerto. El tratamiento con PEG aumentó la vascularización del injerto y la capacidad de formación de tejido óseo endocondral.

Por otro lado, debido a su capacidad de formar matrices y cubiertas, los hidrogeles de PEGs también se han utilizado en cultivos celulares formando superficies de soporte para el crecimiento de las células cultivadas (160). En un modelo experimental *in vitro* de producción de magnetosomas se probaron los efectos de la adicción de PEG de varios pesos moleculares (200, 6000, 20000 y 500000 Da) al medio de cultivo. La proliferación de estos magnetosomas, estructuras que presentan importantes aplicaciones en biotecnología y nanotecnología, aumentaba un 24% con la agregación de PEG200. La presencia de este polímero mejoraba la síntesis de los magnetosomas, por lo que podría presentarse como un avance interesante para la producción en masa de estas estructuras en biorreactores (161). Otra aplicación reciente de esos polímeros en técnicas de investigación es su utilización en un nuevo protocolo de tratamiento de muestras para microscopía confocal, el Clear^{T2}. La inmersión de las muestras en soluciones de concentraciones crecientes de PEG permite mantener la integridad y estabilidad de las partículas marcadas con fluorescencia, favoreciendo la duración de esta (162).

Asimismo, más relacionado con su propiedad de “PEGilación”, se usan como vehículo transmembranal de otras moléculas (liposomas, nanopartículas, micelas,

dendrímeros) en tratamientos farmacológicos, pues aumentan el tiempo de circulación y la estabilidad de estas (163).

Dentro de las aplicaciones clínicas de los PEGs, estos se han empleado como aditivos en soluciones de preservación orgánica debido a su capacidad para atenuar el daño asociado a la I-R fría durante los trasplantes de riñón, páncreas e hígado. Por su efecto oncótico, anteriormente mencionado, limitan la aparición de edema celular y permiten aumentar los tiempos de isquemia, como se ha podido apreciar en diversos modelos experimentales de trasplante en animales (164), (165), (166), (167). Concretamente en trasplante hepático, se ha comprobado que durante el tiempo de preservación en frío promueve la activación de vías protectoras de señalización celular y aumenta los niveles de óxido nítrico, como respuesta del órgano a la privación de oxígeno (168).

En un estudio retrospectivo realizado en Europa, a lo largo de 10 años, evaluando diversos hígados trasplantados, se comparó la solución de preservación con PEG35 y otra solución de la Universidad de Wisconsin sin coloides en su composición. Se observó un mayor porcentaje de supervivencia entre los injertos preservados con una solución que contiene PEG35 (169) en comparación con los injertos preservados en la solución Wisconsin, considerada la solución de preservación “gold standard” para la mayoría de los órganos trasplantados. Además, otros estudios experimentales observaron que esta solución de preservación producía un aumento en mediadores moleculares que promueven la regeneración hepática (170).

En relación con el trasplante de páncreas, la preservación orgánica en la solución con PEG35 permite aumentar el tiempo de isquemia de 13 a 17 horas. En cinco páncreas humanos preservados bajo estas condiciones, se observó que no existía pérdida del injerto por rechazo o trombosis e independiente de insulina exógena (171).

Finalmente, en industria alimentaria, se utilizan en el procesamiento de alimentos para prevenir la espumosidad y, recientemente, se estudian como posibles materiales para el envasado (163).

OBJETIVOS

A pesar de las extensas investigaciones durante las últimas décadas, la PA continúa siendo un reto para el manejo clínico, con una importante carga de morbilidad, mortalidad y coste, cuando la enfermedad desencadena la disfunción de órganos y sistemas distantes. Por ello, cualquier tratamiento para reducir la severidad de la enfermedad podría mejorar el pronóstico de los pacientes.

En este sentido, la presente Tesis Doctoral se ha centrado en la búsqueda de nuevas estrategias terapéuticas para la protección del órgano pancreático.

El objetivo principal de esta Tesis Doctoral es estudiar el efecto protector del PEG35 en distintos modelos experimentales *in vivo* e *in vitro* de inflamación pancreática y, más concretamente:

1. Caracterizar los mecanismos de protección del PEG35 en un modelo experimental de pancreatitis aguda necrotizante en rata.

2. Analizar los efectos de la administración de PEG35 en un modelo experimental de pancreatitis aguda edematosa intersticial en rata.

3. Evaluar la respuesta del PEG35 frente a la inflamación y la muerte celular en células acinares pancreáticas.

4. Analizar el efecto del PEG35 en la modulación de la inflamación mediada por exosomas.

INFORME DE LA DIRECTORA

Barcelona, 7 de setembre de 2020

La Dra. Emma Folch Puy, com a directora de la tesi doctoral que porta per títol "Papel del Polietilenglicol 35 en la modulación de los procesos inflamatorios asociados al páncreas" presentada per la doctoranda Ana Ferrero Andrés, fa constar que la doctoranda ha participat activament en la preparació dels articles presentats en aquesta tesi. La doctoranda ha dut a terme el treball experimental, ha establert els models d'experimentació animal i processat les mostres obtingudes. També, ha contribuït de manera molt activa en l'anàlisi crític de les dades i resultats i ha participat en la redacció dels articles, que es presenten a continuació:

1- *Polyethylene Glycol 35 (PEG35) Protects against Inflammation in Experimental Acute Necrotizing Pancreatitis and Associated Lung Injury*. Ferrero-Andrés A, Panisello-Roselló A, Serafín A, Roselló-Catafau J, Folch-Puy E. *Int J Mol Sci*. 2020;21(3):917.

   Factor impacte: 4.556. Q1, Biochemistry and molecular biology (índex SJR)

2- *NLRP3 Inflammasome-Mediated Inflammation in Acute Pancreatitis.* Ferrero-Andrés A, Panisello-Roselló A, Roselló-Catafau J, Folch-Puy E. *Int J Mol Sci*. 2020;21(15):5386.

   Factor impacte: 4.556. Q1, Biochemistry and molecular biology (índex SJR)

3- *Polyethylene glycol 35 ameliorates pancreatic inflammatory response in cerulein-induced acute pancreatitis in rats*. Ferrero-Andrés A, Panisello-Roselló A, Roselló-Catafau J, Folch-Puy E. *World J Gastroenterol*. 2020. En premsa. Acceptat per publicació 12 d'Agost 2020.

   Factor impacte: 3.534. Q1, Gastroenterology (índex SJR)

4- *PEG35 modulates cellular uptake and function of exosomes*. Ferrero-Andrés A, Closa D, Folch-Puy E. No enviat.

Per últim, declara que cap dels articles ha estat utilitzat per a l'elaboració d'altres tesis doctorals.

Dra. Emma Folch Puy

PUBLICACIONES

ESTUDIO 1. EFECTOS PROTECTORES DEL PEG35 EN UN MODELO EXPERIMENTAL DE PANCREATITS AGUDA NECROTIZANTE

En este estudio, evaluamos el efecto de la administración profiláctica y terapéutica del PEG35 en la reducción de la severidad de la PAN y el daño pulmonar asociado a esta enfermedad.

Para ello, inducimos una pancreatitis aguda necrotizante en ratas siguiendo un modelo experimental basado en la inyección de una solución de taurocolato sódico al 5% a través del conducto biliopancreático. Esta concentración induce una PA que desarrolla una lesión pulmonar a las 3 horas de la inducción, provocando la muerte del animal en el 100% de los casos. El PEG35 se administró, tanto de manera profiláctica como terapéutica, vía intravenosa en una única dosis. Pasadas tres horas de la inducción de la PAN, se recogieron las muestras para su posterior análisis.

Evaluamos indicadores de daño tisular pancreático y pulmonar, así como distintos marcadores de inflamación y muerte celular.

Los resultados obtenidos de este estudio nos permiten elucidar el papel protector del PEG35 frente al proceso inflamatorio sistémico asociado a la pancreatitis aguda necrotizante y presentarlo como un potencial tratamiento para las complicaciones letales que presenta esta enfermedad.

International Journal of *Molecular Sciences*

MDPI

Article

Polyethylene glycol 35 (PEG35) protects against inflammation in experimental Acute Necrotizing Pancreatitis and Associated Lung Injury

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Abstract: Acute pancreatitis is an inflammatory disorder of the pancreas. Its presentation ranges from self-limiting disease to acute necrotizing pancreatitis (ANP) with multiorgan failure and a high mortality. Polyethylene glycols (PEGs) are non-immunogenic, non-toxic and water-soluble chemicals composed of repeating units of ethylene glycol. The present article explores the effect of PEG35 administration on reducing the severity of ANP and associated lung injury. ANP was induced by injection of 5% sodium taurocholate into the biliopancreatic duct. PEG35 was administered intravenously either prophylactically or therapeutically. Three hours after ANP induction, pancreas and lung tissue samples and blood were collected and ANP severity was assessed. To evaluate the inflammatory response, gene expression of pro-inflammatory cytokines and chemokine and the changes in the presence of myeloperoxidase and adhesion molecule levels were determined in both the pancreas and the lung. To evaluate cell death, LDH activity and apoptotic cleaved caspase-3 localization were determined in plasma and in both the pancreatic and lung tissue respectively. ANP-associated local and systemic inflammatory processes were reduced when PEG35 was administered prophylactically. PEG35 pre-treatment also protected against acute pancreatitis-associated cell death. Notably, therapeutic administration of PEG35 significantly decreased associated lung injury, even when the pancreatic lesion was equivalent to that in the untreated ANP-induced group. Our results support a protective role of PEG35 against the ANP-associated inflammatory process and identify PEG35 as a promising tool for the treatment of the potentially lethal complications of the disease.

Keywords: inflammation; pulmonary injury; polyethylene glycols; cytokines; neutrophil infiltration.

1. Introduction

Acute pancreatitis, an inflammatory disorder of the pancreas, is the main cause of hospitalizations related to gastrointestinal diseases and the fifth most common cause of death in hospitals [1]. This disease entity can be divided into two morphological subtypes [2]: interstitial edematous pancreatitis, and necrotizing pancreatitis. Interstitial edematous acute pancreatitis represents 80-90% of cases and the clinical symptoms usually resolve within the first week. However, up to 20% of patients develop acute necrotizing pancreatitis (ANP), the more severe form, which is associated with high rates of morbidity and mortality; lung failure is the main contributing factor to early death within the first week after admission [3]. At present, there is no specific medical treatment for ANP; the management of the disease is mainly supportive and targeted to prevent and treat systemic complications.

Polyethylene glycols (PEGs) are non-immunogenic, non-toxic and water-soluble polymers composed of repeating units of ethylene glycol [4]. One of their most positive characteristics is their low toxicity regardless of the route of administration [5]. In fact, PEGs are currently the only water-soluble polymers that are widely accepted and approved by the Food and Drug Administration for use in food, cosmetics and pharmaceuticals [6]. In clinical settings, PEGs are used as additives to organ preservation solutions to attenuate the damage associated with cold ischemia-reperfusion of kidney, pancreas and liver transplantation [7].

In recent years, several experimental studies have focused on the protective effects of PEGs. In lung endothelial cells, treatment with 15-20 kDa molecular weight PEG (PEG 15-20) was found to enhance cell function by activating endothelial cell-barrier signal transduction pathways and by contributing to cytoskeleton reorganization [8]. In addition, pre-treatment with PEG 15-20 in cultured ventricular myocytes subjected to hypoxia-reoxygenation reduced oxidative stress and apoptosis and increased cell survival [9]. The protective effects of PEGs have also been reported in *in vivo* models, although reports of its use in the context of inflammatory processes are scarce. In a murine model of lethal gut-derived sepsis, therapeutic administration of low molecular weight PEG provided protection against bacterial infections and reduced mortality [10]. Likewise, prophylactic oral administration of 4-kDa molecular weight PEG in experimental colitis strengthened the epithelial barrier and reduced inflammation of the colon [11]. Another study found that coating the peritoneal surfaces of the rat with PEG was a highly effective measure to limit the number of leukocytes in a model of post-traumatic inflammation [12]. Lastly, in colon carcinogenesis, animals receiving a diet with 8-kDa molecular weight PEG presented reduced colonic inflammation [13].

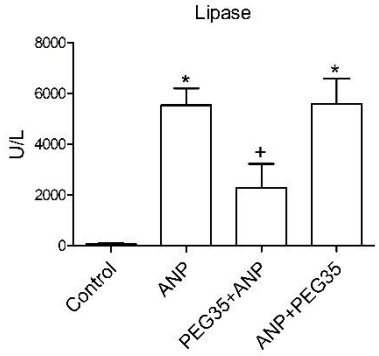
In previous work, our group explored the benefits of using 35-kDa molecular weight PEG (PEG35) in experimental models of cold and warm liver ischemia-reperfusion in the rat, finding that the addition of PEG35 to the organ preservation solution decreased liver graft vulnerability to cold ischemia-reperfusion injury [14]. Furthermore, prophylactic intravenous administration of PEG35 to rats reduced the ischemia-reperfusion-induced hepatic injury associated with the preservation of the mitochondrial status, cytoskeleton protection, and the induction of cyto-protective signaling pathways [15]. Intravital microscopy studies demonstrated the location of PEG35 adsorbed in the liver vascular bed after ischemia-reperfusion.

Given the numerous benefits of PEGs just described, and in view of the fact that they are water-soluble and above all non-toxic, the objective of this paper was to study the potentially protective effects of PEG35 in an experimental model of ANP. Our results suggest that PEG35 strongly reduces ANP severity and improves the associated inflammatory process in the lung.

2. Results

2.1. Effects of PEG35 on plasma lipase levels

Sodium taurocholate-induced ANP in rats was associated with significant increases in the plasma levels of lipase reflecting the degree of pancreatic injury in this experimental model

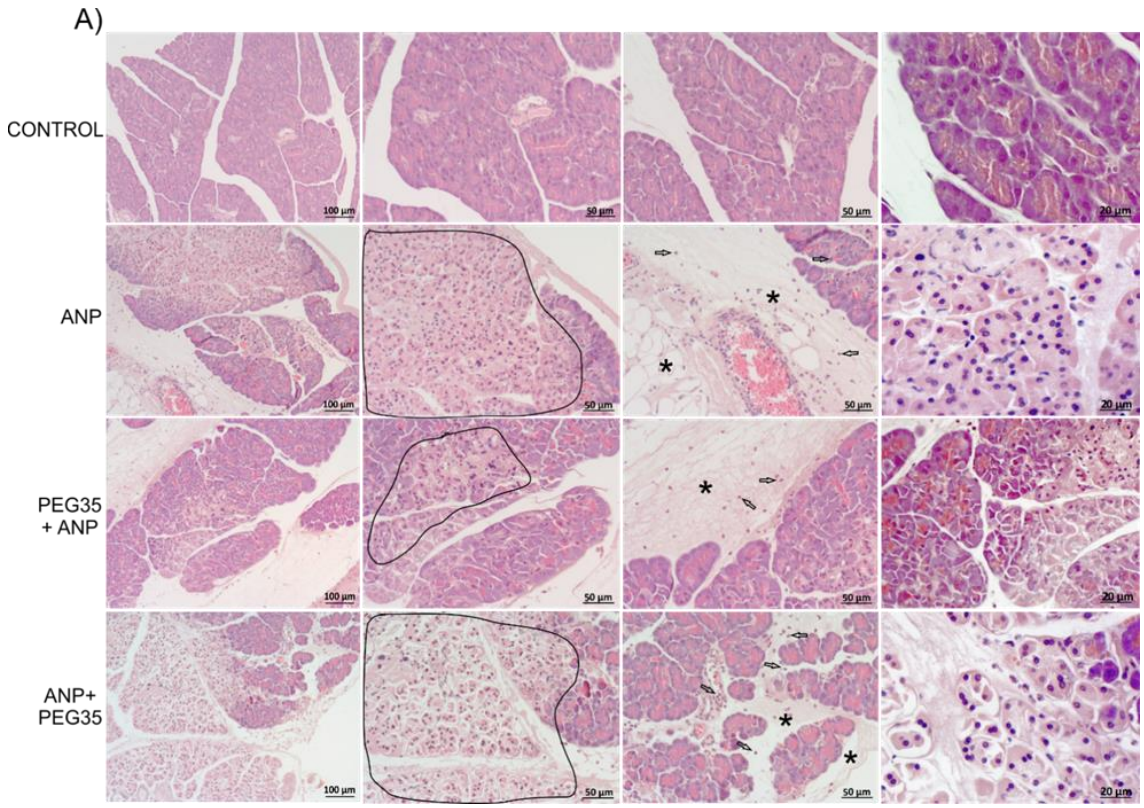


(Figure 1). This increase was significantly reduced in the rats pretreated with 10mg/kg of PEG35. In contrast, therapeutic PEG35 administration had no effect on the pancreatic injury associated with ANP.

Figure 1. **Effect of PEG35 treatment on plasma lipase activity in sodium taurocholate-induced ANP**. Plasma lipase levels in U/L. Bars represent mean values of each group ± SEM. **P* < 0.05 versus Control, +*P* < 0.05 versus ANP. ANP, Acute Necrotizing Pancreatitis. PEG35, Polyethylene glycol 35. Each determination was carried out in triplicate.

2.2. Prophylactic and therapeutic PEG35 reduced systemic tissue damage associated with ANP

Intraductal administration of 5% sodium taurocholate in the rats produced a severe hemorrhagic pancreatitis with large areas of interstitial edema, necrosis and neutrophil infiltration in the pancreas (Figure 2A and Table 1). In the PEG35-treated groups, only when the animals were treated prophylactically were there consistent reductions in pancreatic interstitial edema, leukocyte infiltration and acinar cell necrosis. Histological evaluation of the lungs showed significant edema, leukocyte infiltration and alveolar septal thickening (Figure 2B) associated with ANP. However, these findings were less marked when the animals were treated either prophylactically or therapeutically with PEG35.


A)
CONTROL
ANP
PEG35
+ ANP
ANP+
PEG35
100 µm
50 µm
20 µm

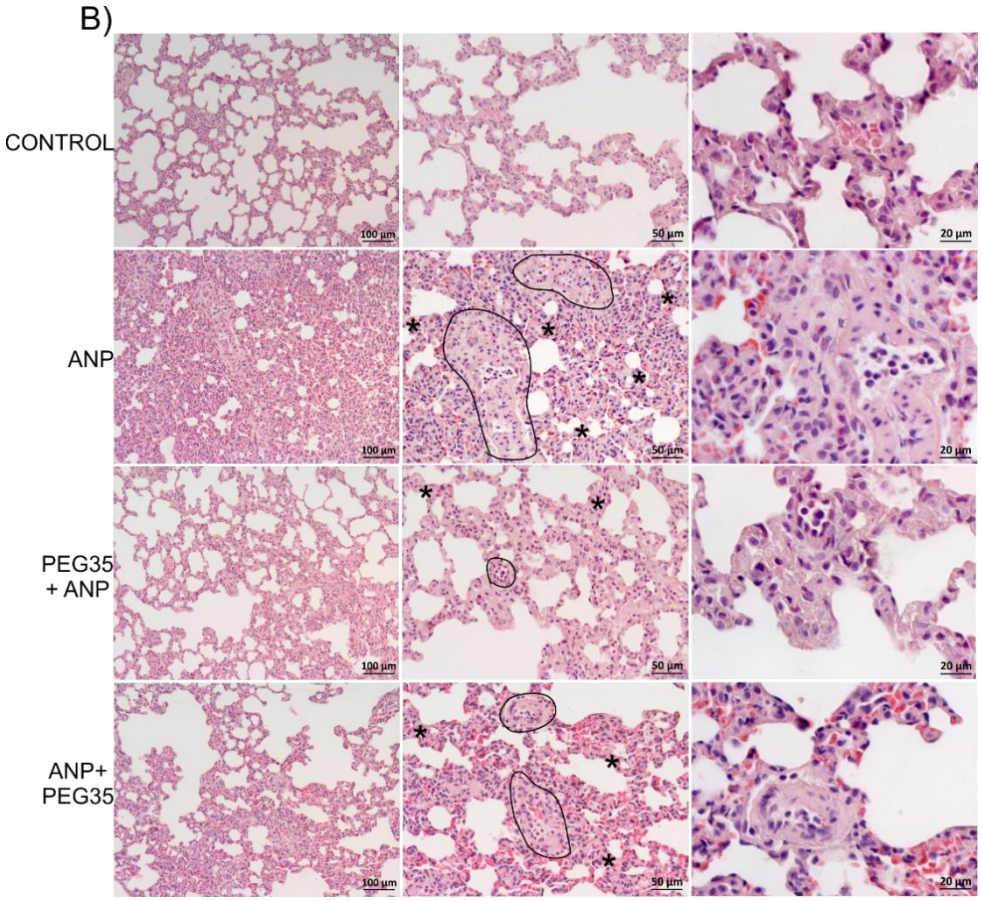

B)
CONTROL
ANP
PEG35
+ ANP
ANP+
PEG35
100 µm
50 µm
20 µm

Figure 2. **Effect of PEG35 treatment on histological changes in experimental acute necrotizing pancreatitis and associated acute lung injury.** A) Representative images of hematoxylin and eosin-stained pancreatic sections for each experimental group. Control group showed normal pancreas structure. ANP and ANP+PEG35 groups presented large areas of necrosis (under area), infiltrated polymorphonuclear neutrophils (indicated by empty arrows) and interstitial edema (indicated by an asterisk). Prophylactic administration of PEG35 significantly reduced these features. B) Representative images of hematoxylin and eosin-stained lung sections for each experimental group. Control group showed normal alveolar structure. In the ANP group, a marked alveolar septal thickening (indicated by an asterisk) with infiltrated neutrophils, and the presence of vessel neutrophils (under area) were seen. Both prophylactic and therapeutic PEG35 treatment normalized alveolar septal thickening and neutrophils infiltration. ANP, Acute Necrotizing Pancreatitis. PEG35, Polyethylene glycol 35. Scale bar, 100, 50 and 20 μM.

Table 1. Pancreatic and pulmonary lesions in all experimental groups

	Pancreas			Lung		
	Edema	Inflammation	Necrosis	Alveolar wall thickening	Infiltrated neutrophils	Vessel neutrophils
Control	+/-	-	-	+/-	+/-	+/-
ANP	+++	+++	+++	+++	+++	+++
PEG35+ANP	+/-	+	+/-	+/-	+/-	+
ANP+PEG35	+++	+++	+++	+	+	+++

Abbreviations: ANP, Acute Necrotizing Pancreatitis. PEG35, Polyethylene glycol 35.

2.3. PEG35 abrogated ANP-induced Interleukin 6 (IL6) expression in plasma

IL6 is an important multifunctional cytokine with many roles in inflammation, and its serum levels reflect the magnitude of the inflammatory response. This cytokine has been reported to have prognostic value for acute pancreatitis upon admission [16, 17]. We therefore measured its levels in plasma and, as expected, a significant increase was detected after ANP induction (Figure 3A). Prophylactic and therapeutic treatment with PEG35 resulted in a significant reduction in systemic IL6 levels.

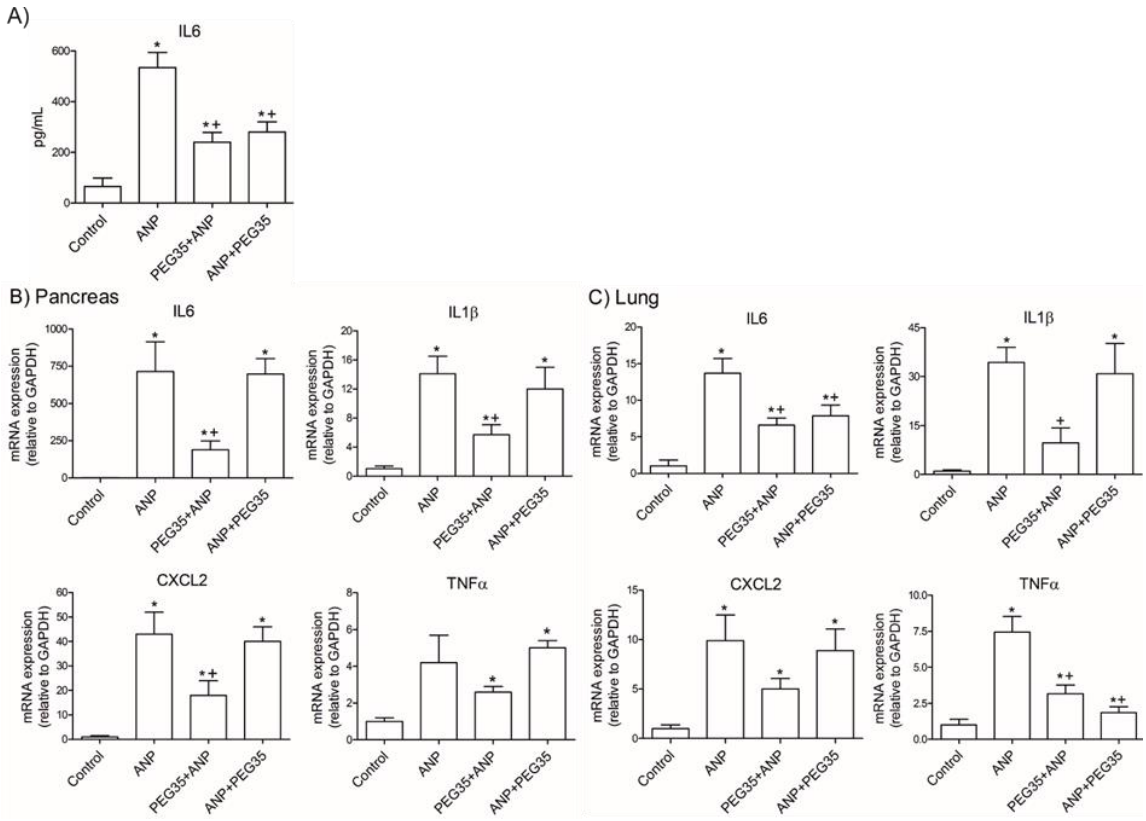


Figure 3. **Role of PEG35 on the modulation of pro-inflammatory cytokines and chemokines expression in sodium taurocholate acute necrotizing pancreatitis and associated acute lung injury.** A) IL6 expression levels in plasma. B) Pancreatic tissue gene expression of IL6, IL1β, CXCL2 and TNFα by real-time qRT-PCR. C) Lung tissue gene expression of IL6, IL1β, CXCL2 and TNFα by real-time qRT-PCR. In all cases, mRNA induction levels were normalized to GAPDH mRNA expression. Bars represent mean values of each group ± SEM. **P* < 0.05 versus Control, +*P* < 0.05 versus ANP. ANP, Acute Necrotizing Pancreatitis. PEG35, Polyethylene glycol 35. Each determination was carried out in triplicate.

2.4. Prophylactic and therapeutic PEG35 improved ANP-induced expression of pro-inflammatory cytokines in the lung

Next, we explored whether PEG35 administration might improve inflammatory response after ANP induction. To do so, we measured the gene expression of pro-inflammatory markers IL6, Interleukin 1β (IL1β), Tumor Necrosis Factor α (TNFα) and chemokine (C-X-C motif) ligand 2 (CXCL2) in both pancreas and lung. Pancreatic tissue levels of these mediators rose markedly three hours after ANP induction compared with control animals (Figure 3B), except for TNFα. As expected, only prophylactic treatment with PEG35 was able to significantly reduce the ANP-induced increases in these cytokines. Regarding the inflammatory process in the lung, ANP induction raised expression levels of IL6, IL1β, TNFα and CXCL2 (Figure 3C). PEG35 administration prior to the induction of ANP significantly reduced IL6, IL1β and TNFα levels, and therapeutic administration significantly reduced the levels of IL6 and TNFα in the lung.

2.5. PEG35 abrogated ANP-related adhesion molecules expression in the lung

The recruitment of leukocytes is a hallmark of inflammation. The process is controlled by complex interactions between surface receptors on neutrophils and their corresponding endothelial cell ligands [18]. To further study the protective function of PEG35 in ANP, we focused on the expression of two of the main adhesion molecules involved in this inflammatory disease: P-selectin and Intercellular Adhesion Molecule-1 (ICAM-1) [19]. A significant up-regulation in both adhesion molecules was evident in the pancreas and lung three hours after ANP induction compared to control-operated mice (Figure 4A and B). Prophylactic administration of PEG35 helped to reduce the gene expression of P-selectin and ICAM-1 expression in both these tissues, while its therapeutic administration significantly reduced their expression only in the lung. Accordingly, immunoblot assay of ICAM-1 protein confirmed that PEG35 abrogates the inflammatory process in the lung when is administered either prophylactically or therapeutically (Figure 4C and D).

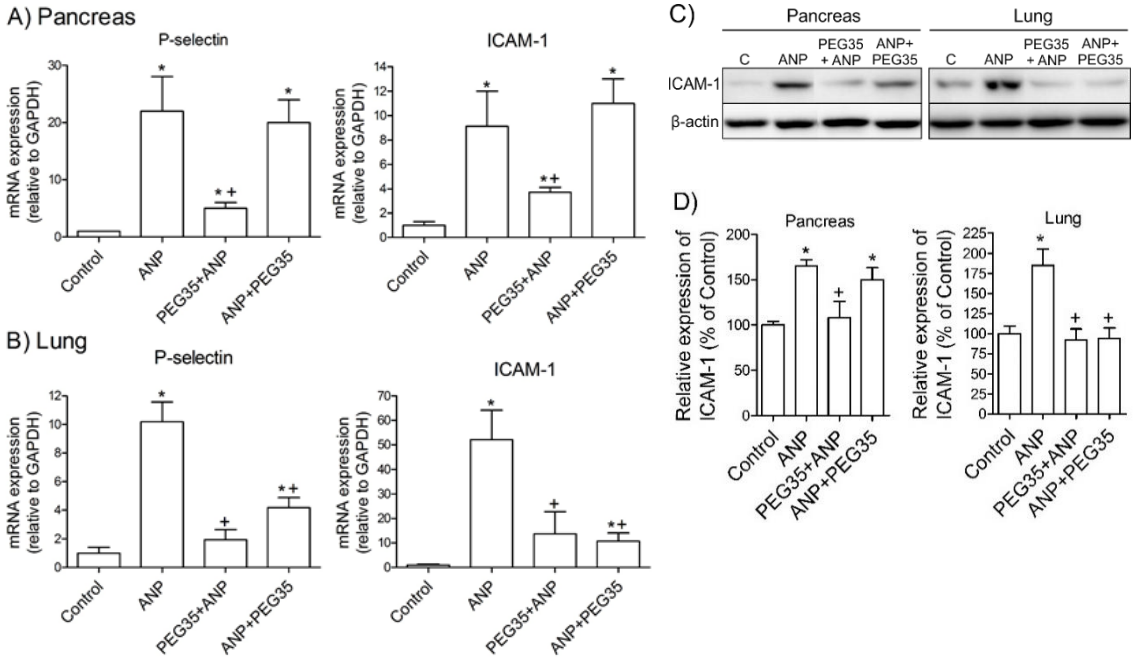
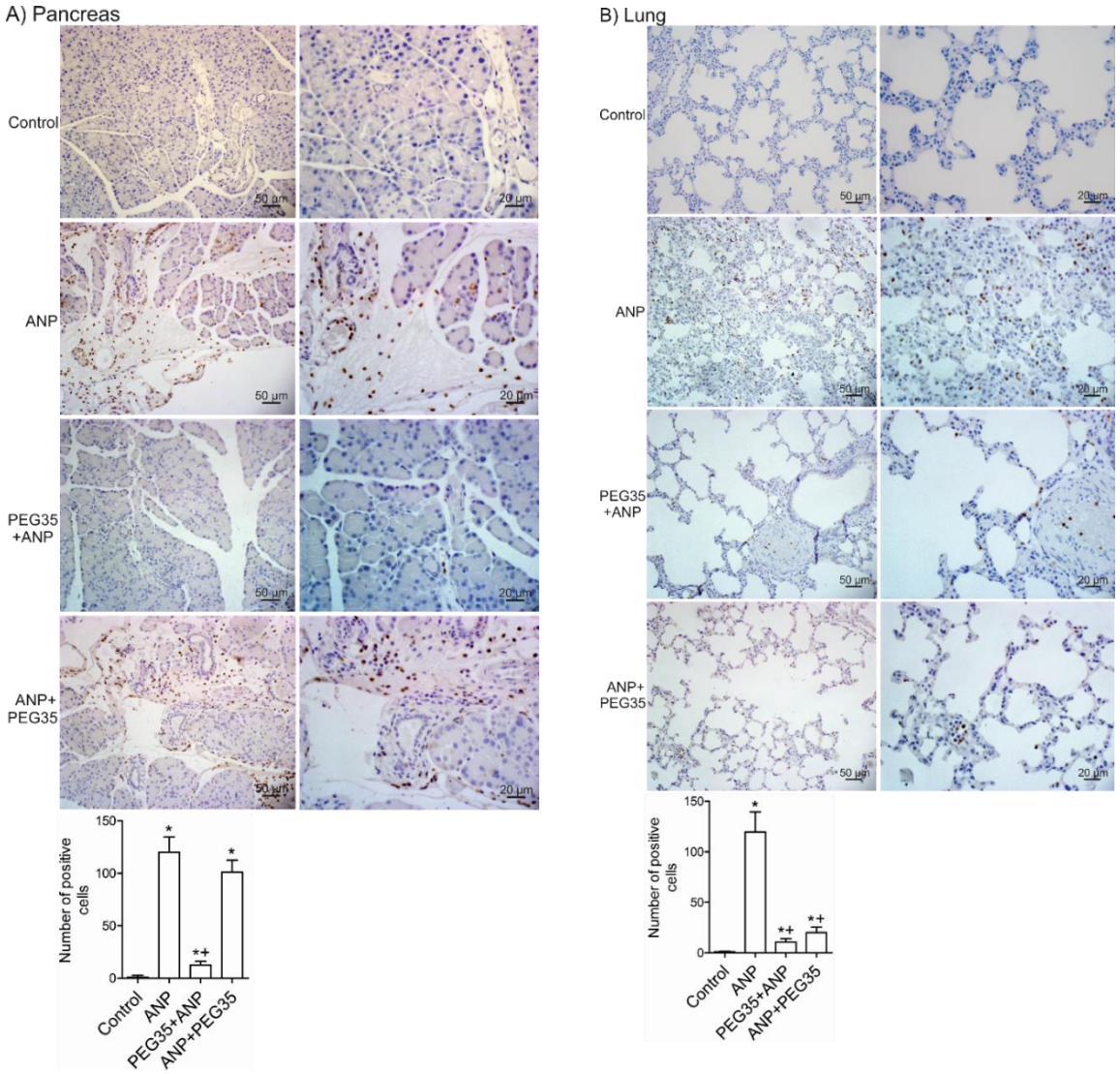


Figure 4. **Effects of PEG35 administration on the expression of adhesion molecules in acute necrotizing pancreatitis and associated acute lung injury**. A) Pancreatic tissue gene expression of P-selectin and ICAM-1 by real-time qRT-PCR. B) Lung tissue gene expression of P-selectin and ICAM-1 by real-time qRT-PCR. In all cases, mRNA induction levels were normalized to GAPDH mRNA expression. C) Pancreatic and pulmonar protein expression of ICAM-1 assessed by Western Blot analysis. B-actin expression was used as loading control. Data shown are representative blots for each group. D) Densitometry quantification of Western blot for ICAM-1 in pancreatic and lung tissue. Bars represent mean values of each group ± SEM. **P* < 0.05 versus Control, **P* < 0.05 versus Control, +*P* < 0.05 versus ANP. C, Control. ANP, Acute Necrotizing Pancreatitis. PEG35, Polyethylene glycol 35. Each determination was carried out in triplicate.

2.6. Prophylactic and therapeutic administration of PEG35 reduced the pulmonary neutrophil infiltration associated with ANP

Increased numbers of neutrophils in both pancreas (Figure 5A) and lung (Figure 5B) marked the inflammatory response following ANP induction. Additionally, areas of intense cell infiltration with extravasation of leukocytes to the interacinar space were found. Pretreatment

with PEG35 significantly attenuated the infiltration of leukocytes into the pancreas. In addition to local pancreatic neutrophil recruitment, a significant increase in myeloperoxidase (MPO) positive cells in the lung was noted in the ANP-induced animals compared to sham-operated



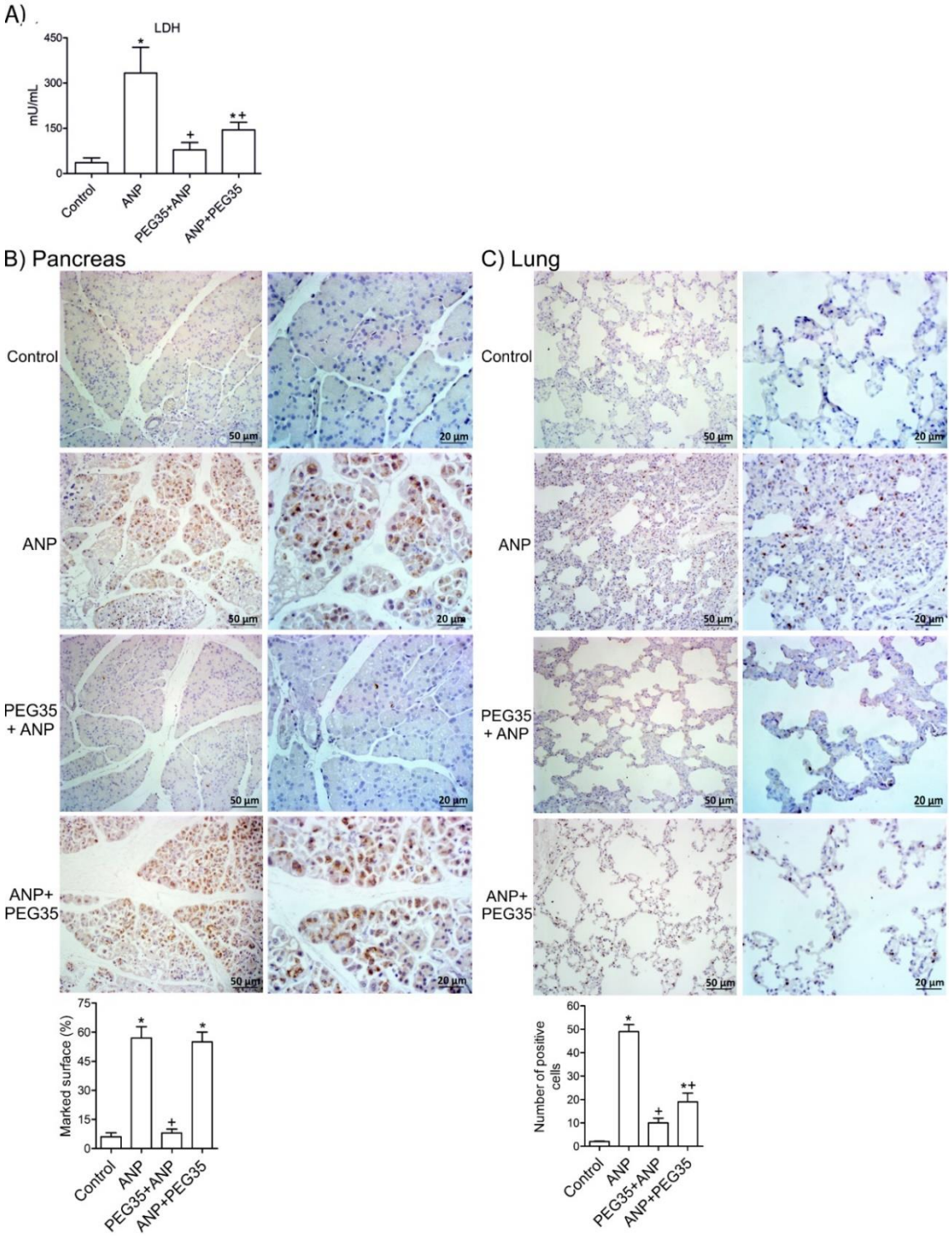
mice. By contrast, both prophylactic and therapeutic intravenous administration of PEG35 lessened pulmonary neutrophil recruitment and extravasation.

Figure 5. Effects of PEG35 treatment on acute necrotizing pancreatitis-induced myeloperoxidase expression. A) Top, Representative images of pancreatic sections stained with anti-MPO antibody (brown). Bottom, Pancreas MPO immunostaining quantification represented as the average number of positive cells per field. B) Top, Representative images of lung sections stained with anti-MPO antibody (brown). Bottom, Pulmonary MPO immunostaining quantification represented as the average number of positive cells per field. ANP, Acute Necrotizing Pancreatitis. PEG35, Polyethylene glycol 35. Scale bar, 50 and 20 µM.

2.7 Effect of PEG35 treatment on inflammation-induced cell death

To further explore the potential protective effects of PEG35 on pancreas and lung, cell necrosis and apoptosis were determined through LDH release and caspase 3 activity respectively. As illustrated in Figure 6A, a significant increase in LDH activity in plasma occurred three hours after ANP induction. Similarly, cleaved caspase-3 levels were markedly higher following ANP induction both in the pancreas and in the lung, compared with the control group (Figure 6B and

C). Levels of both necrotic and apoptotic cell markers were significantly reduced under conditions of prophylactic administration with PEG35 in both these tissues. Therapeutic administration of PEG35 significantly lessened LDH levels as well as cleaved caspase-3



expression in the lung. Taken together, these results suggest that PEG35 exerts both anti-necrotic and anti-apoptotic effects, protecting against inflammation-induced cell death following ANP.

Figure 6. **Effects of PEG35 treatment on ANP-induced cell death.** A) Plasma lactate dehydrogenase activity three hours after acute necrotizing pancreatitis induction expressed as mU/mL. B) Top: Representative images of pancreatic sections stained with anti-cleaved caspase-3 antibody (brown). Bottom: Pancreas cleaved caspase-3

immunostaining quantification represented as the percentage of marked surface per field. C) Top: Representative images of lung sections stained with anti-cleaved caspase-3 antibody (brown). Bottom: Pulmonary cleaved caspase-3 immunostaining quantification represented as the average number of positive cells per field. Bars represent mean values of each group ± SEM. *$P < 0.05$ versus Control, *$P < 0.05$ versus Control, +$P < 0.05$ versus ANP. ANP, Acute Necrotizing Pancreatitis. PEG35, Polyethylene glycol 35. Each determination was carried out in triplicate.

3. Discussion

Despite extensive research in recent decades, ANP continues to present a significant burden in terms of morbidity, mortality and financial cost, and its management remains a major challenge. Increases in the annual incidence of this disease have been observed in most recent studies and no pharmacological therapies are as yet available to improve the disease course, especially in patients who develop a systemic inflammatory response syndrome.

Many studies have reported the beneficial effects of PEGs in tissue injury [8, 9, 11], but the role of these polymers in acute pancreatitis has not yet been elucidated. The present paper aims to establish whether the intravenous administration of a 35-kDa molecular weight PEG in a single non-toxic dose of 10mg/kg could protect pancreatic and lung tissue against the deleterious effects of ANP. In the study, the prophylactic administration of PEG35 significantly abrogated the severity of acute pancreatitis in sodium taurocholate-treated rats, as indicated by the decreased activity of lipase in plasma. Histopathologic evaluation of the pancreas and systemic lung also revealed a marked reduction in overall histopathology score in the PEG35 pre-treated animals. Surprisingly, the therapeutic administration of PEG35 significantly reduced lung injury, even when the pancreatic lesion was equivalent to that of the untreated ANP-induced group.

Serum levels of pro-inflammatory cytokines and chemokines rise over the course of ANP. IL6 is an important inflammatory mediator of the acute-phase response that has been experimentally associated with distant organ complications [20]. In addition, in the clinical setting, it is considered a reliable early marker for predicting the severity of acute pancreatitis [21, 22]. We found that both prophylactic and therapeutic use of PEG35 was able to significantly abrogate the up-regulated levels of systemic IL6 following ANP induction. Similar results were found regarding the presence of pro-inflammatory cytokines and chemokines locally in the pancreas and in the lung. The gene expressions of IL6, IL1β, and CXCL2 (though not TNFα) were found to be significantly elevated in the pancreas three hours after ANP induction, and prophylactic PEG35 administration abrogated these up-regulated cytokines and chemokine levels. As expected, therapeutic administration of PEG35 did not have any protective effect on the injured pancreas; in ANP, once the pro-inflammatory cascade is triggered, the process is exceedingly difficult to reverse. With regard to the inflammatory process in the lung, the induction of ANP increased the levels of expression of IL1β, TNFα and CXCL2 compared with the control-operated group. PEG35 administration prior to the induction of ANP significantly reduced IL6, IL1β and TNFα mRNA levels. Interestingly, the therapeutic administration of PEG35 was able to downregulate IL6 and TNFα, the main pro-inflammatory cytokines involved in ANP.

Endothelial P-selectin and ICAM-1 are major adhesion molecules that are highly overexpressed during acute pancreatic inflammation and their blockade has been associated with reductions in pancreatic and lung damage [23, 24]. In the present study, levels of P-selectin and ICAM-1 expression rose significantly in the pancreas and in the lung three hours after ANP induction. As expected, pre-treatment of rats with PEG35 abrogated the increased levels of those

adhesion molecules in both tissues. Therapeutic administration of PEG35 was unable to reverse the inflammatory process in the pancreas, but was able to do so in the lung.

These changes in pro-inflammatory processes brought about by PEG35 administration were further emphasized by the marked reduction in neutrophil recruitment and extravasation both in the pancreas and in the lung when PEG35 was administered previously to ANP. As measured by the presence of MPO, PEG35 pre-treatment reduced the number of MPO positive cells in both tissues while (as in the case of the histological score) therapeutic administration of PEG35 only reduced the levels of MPO positive cells in the lung. In fact, neutrophils were found in high numbers within the lung endothelial vessels even though they did not extravasate into the surrounding tissue. In this regard, PEG35 may exert at least part of its protective function through the endothelial cell coating, which may stop neutrophils in the microcirculation entering the interstitium and infiltrating the lung tissue.

Furthermore, both prophylactic and therapeutic use of PEG35 reduced cell death by lowering plasmatic LDH activity and tissue cleaved-caspase-3 expression both in the pancreas and in the lung. These findings are consistent with those of a previous study which found that PEGs protected against apoptosis when administered intravenously in an animal model of spinal cord injury (25). PEG 15–20 also has a potent protective antiapoptotic effect in cardiac myocytes exposed to ischemia-reperfusion injury (9). Additionally, different molecular weight PEGs have been found to protect renal cells against cold-induced cellular necrosis (26), PEG35 being the most effective. All these findings demonstrate that PEG35 may notably alleviate the severity of ANP and protect against inflammation-induced cell death.

By the time of presentation of ANP, pancreatic necrosis is already non-reversible, so the aim is to minimize the systemic inflammatory response syndrome in order to reduce rates of organ failure, morbidity, and mortality. PEG compounds with different molecular weights have been applied topically, orally, and systemically with notable efficacy in a variety of experimental models. Our data highlight the potential therapeutic use of PEG35 to modulate the progression of ANP towards a lethal outcome. We have demonstrated that prophylactic PEG35 improves the inflammatory response in the lung as a direct consequence of attenuating the initial pancreatic injury. In addition, PEG35 exerts a substantial anti-inflammatory role by directly lowering the lung inflammatory response subsequent to acute pancreatitis when administered therapeutically. This feature is particularly relevant in the clinical setting where new therapeutic treatments are urgently required.

4. Materials and Methods

4.1. Experimental animals

Male Wistar rats weighting 200-250 g were housed in a controlled environment with free access to standard laboratory pelleted formula (A04; Panlab, Barcelona, Spain) and tap water. A period of one week was allowed for animals to acclimatize before any experimentation. All procedures were conducted in accordance with European Union regulatory standards for animal experimentation (Directive 2010/63/EU on the protection of animals used for scientific purposes). The Ethical Committee for Animal Experimentation (CEEA, Directive 396/12, University of Barcelona) approved the animal experiments.

4.1.1. Animal model of ANP

The rats were anesthetized with an intraperitoneal injection of pentobarbital at a dose of 50mg/kg. After a midline laparotomy, a polyethylene catheter connected to an infusion pump was

inserted through the duodenum, via the Ampulla of Vater, and 3–4 mm into the biliopancreatic duct. A bulldog clamp was applied to the proximal biliopancreatic duct (near the liver) to prevent infusion into the liver. The experimental model of ANP was induced in the rats (n=8) by retrograde injection of 5% sodium taurocholate in saline solution at 1mL/Kg/1min for 5 min. using an infusion pump (Harvard Instruments, Edenbridge, UK). Control animals (n=8) received saline solution (NaCl 0.9%). This model represents the reference standard of biliary acute pancreatitis, the most common cause of ANP in humans. As previously reported by our group, the infusion of this bile salt at 5% results in lung injury after 3 hours of induction. This lung failure is the main contributing factor to early death in patients with ANP [25]. PEG35 was administered intravenously through the penile vein in a single dose of 10mg/kg either prophylactically (10 minutes before ANP induction) or therapeutically (10 minutes after ANP induction) (n=8 for each group). PEG35 was selected based on the literature review. PEG polymers of high molecular weight (≥4000 Da) have been reported to be suitable as potential therapeutic agents. In addition, our group has wide experience in the study of the protective role of PEG35, which is a high molecular weight PEG currently added to preservation solutions for pancreas, liver and kidney transplantation with optimal results for protecting the tissue from ischemia-reperfusion injury. Buprenorphine (0.05 mg/Kg) was intravenously administered as an analgesic immediately before surgery. Three hours after ANP induction, animals were euthanized and blood was collected in heparinized syringes from the vena cava. Harvested blood was centrifuged, the plasma removed and stored at −80°C. Three tissue samples from each animal were taken from the head of the pancreas and from the lung. One portion of each tissue was fixed in 10% phosphate-buffered formalin for histological analysis, another portion was frozen and immediately stored at -80ºC for western blot analysis and the last portion was stored in RNAlater solution for real-time PCR analysis.

4.2. Histopathological examination

Pancreatic and lung tissue were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Sections of 3µm thickness were mounted on glass slides. Slides were dewaxed and rehydrated before staining with hematoxylin and eosin. Then, a pathologist examined multiple randomly chosen microscopic fields from each experimental group in a blinded manner. Sections of pancreas tissue were scored for the severity of pancreatitis based on edema, leukocyte infiltration and necrosis graded on a semi-quantitative scale from no lesion to intense lesion. Lung injury was assessed histologically using a semi-quantitative scale from no lesion to intense lesion for interstitial and intra-alveolar leukocyte infiltration and alveolar septal thickening. The semi-quantitative scale was the mean of the lesions in each group: -, no lesion; +, slight lesion; +/-, slight lesion in some sections; ++, moderate lesion; +++, intense lesion.

4.3. Biochemical determinations

4.3.1. Lipase activity

Plasma lipase activity were determined using a commercial turbidimetric assay kit from Randox (County Antrim, UK), according to the supplier's specifications. Briefly, the degradation of triolein by the pancreatic lipase results in decreased turbidity, which was measured in the sample at 340 nm using an automated microplate reader (iEMS Reader MF; Labsystems, Helsinki, Finland). The activity of the samples was obtained in U/L. All samples were run in duplicate.

4.3.2. IL6 immunoassay

Interleukin-6 (IL6) in plasma was measured using a commercially available ELISA Kit (R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer's instructions. Briefly,

standards, control, and samples reacted with a specific antibody against IL6 immobilized in a microplate. Another antibody specific for rat IL6 was then added to the wells. After washing, a substrate solution was added, yielding a yellow product. The intensity of the color measured is in proportion to the amount of IL6. The optical density was measured at 450 nm using an automated microplate reader (iEMS Reader MF; Labsystems, Helsinki, Finland). IL6 levels were obtained in pg/mL. All samples were run in duplicate.

4.3.3. Lactate dehydrogenase activity

Lactate dehydrogenase (LDH) is a soluble cytosolic enzyme present in most eukaryotic cells, and is released upon cell death due to damage to the plasma membrane. LDH activity was measured in samples of plasma using the Lactate Dehydrogenase Assay Kit (Abcam; Cambridge, UK). In this assay, LDH reduces NAD to NADH which then interacts with a specific probe to produce color. Changes in absorbance due to NADH formation were recorded at 450 nm at 37 °C using an automated microplate reader (iEMS Reader MF; Labsystems, Helsinki, Finland). The activity of the samples was expressed in milliunits per milliliter (mU/mL). All samples were run in duplicate. The lower limit of detection for ELISA ranged from 14 to 36 mU/mL.

4.3.4. Real-time qRT-PCR

Total RNA from the pancreas and lungs was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA concentration and quality were measured with the OD A260/A280 ratio and OD A260/A230 ratio respectively, and the integrity of 18S and 28S ribosomal bands for all RNA preparations was verified by running a 1% agarose gel electrophoresis. Reverse transcription was conducted on a 1 μg RNA sample using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Subsequent PCR amplification was conducted using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) on a CFX Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using 10μL of amplification mixtures containing 50 ng of reverse-transcribed RNA and 250 nM of the corresponding forward and reverse primers. PCR primers for the detection of Interleukin 6 (IL6), Interleukin 1β (IL1β) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were experimentally validated primers from BioRad (Hercules, CA, USA). PCR primers for Chemokine (C-X-C motif) ligand 2 (CXCL2), Tumor necrosis factor α (TNFα), P-selectin and Intercellular adhesion molecule-1 (ICAM-1) were designed with Primer3.0 plus [26]. The sequences were as follows: CXCL2 forward, 5′-TGCTCAAGACTCCAACCACTC-3′ and reverse 5′-CACAACAACCCCTGTACCCTG-3′; TNFα forward, 5′- ATGGGCTCCCTCTCATCAGT-3′ and reverse 5′-GCTTGGTGGTTTGCTACGAC-3′; P-Selectin forward, 5′-TCTCCTGCAACGAGGAGTTT-3′ and reverse 5′-GGTGTCGACAGGACATTGTG-3′; and ICAM-1 forward, 5′-GAGCGACATTGGGGAAGACA-3′ and reverse 5′-CACTCGCTCTGGGAACGAATA-3′. The specificity of the amplicons was determined by melting curve analysis. Reactions were carried out in duplicate and threshold cycle values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression. The ratio of the relative expression of target genes to GAPDH was calculated by the DCt formula.

4.3.5. Immunohistochemistry

Pancreatic and lung tissue were fixed and embedded in paraffin slices. Sections of 3μm thickness were then deparaffinized in xylene, rehydrated with graded ethanol, and washed in Tris-buffered saline. After quenching endogenous peroxidase activity and blocking non-specific binding, antigen retrieval was conducted by incubating samples with 10 mM sodium citrate buffer (pH 6.0). Then, the tissue sections were incubated overnight with the myeloperoxidase antibody

(1:100 dilution; reference ab9535 Abcam; Cambridge, UK) and the rabbit cleaved caspase-3 (Asp175) antibody (1:800 dilution, reference #9661 Cell signaling, Leiden, The Netherlands). Sections were then incubated with the appropriate dilution of the corresponding biotinylated secondary antibody for 1 h at room temperature. After further washing with Tris-buffered saline, sections were incubated with Vectastain Elite ABC Reagent (Dako, Inc., USA) for 30 min at room temperature. Chromogenic immunolocalization was conducted using 0.05% 3,3′-diaminobenzidine (DAB). All sections were counterstained with hematoxylin, dehydrated and mounted. Negative controls were included by replacing the primary antibody with non-immune serum. Images were taken with a Nikon Eclipse E1000 microscope (Nikon, Amsterdam, Netherlands) and analyzed using cellSens imaging software (Olympus, Hamburg, Germany). The mean number of peroxidase-positive cells was counted in six randomly chosen microscopic high-power fields (20X) per animal in a blinded fashion. For pancreatic cleaved caspase-3 analysis, the percentage of the thresholded area occupied by DAB staining was measured using the Image J program.

4.3.6. Western Blot

Pancreas and lung tissue were homogenized in ice-cold RIPA buffer (50mM Tris-HCl, 150mM NaCl, 0.05% Triton X-100, 1mM EDTA, 1mM DTT, 1mM PMSF, 1 mM NaF, 1mM Na3VO4, 1µg/mL Aprotinin, 1µg/mL Leupeptin; pH 7.4). Lysates were then centrifuged at 15,000 g for 20 min at 4ºC, and the supernatants were collected. Protein concentration of the supernatants was determined by the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). SDS-PAGE was performed on a 10% gel on which 40 µg of total protein per well was loaded. After SDS-PAGE, the proteins were transferred to a polyvinylidene difluoride membrane. Immunoblotting was performed using the mouse monoclonal ICAM-1 antibody conjugated to HRP (dilution 1:100, Santa Cruz Biotechnology, sc-8439 HRP) and β-actin-HRP conjugated (dilution 1:20000, Sigma, A3854). The bound antibody was detected using enhanced chemiluminescence (ECL) detection (Bio-Rad Laboratories, Hercules, CA, USA), and the bands were analyzed using ChemiDoc™ Touch Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). For quantification, protein expression of ICAM-1 was normalized to β-actin.

4.4. Statistical analysis

All data were exported into Graph Pad Prism 4 (GraphPad Software, Inc.) and were presented as means ± SEM. Statistical analyses were carried out by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test to determine the significance between pairs. The minimal level of significance was considered at $P < 0.05$.

Author Contributions: Conceptualization, E.F-P. and J.R-C.; formal analysis, A.F-A., A.P-R. and E.F-P.; investigation, A.F-A., A.P-R., A.S., J.R-C. and E.F-P.**;** methodology, A.F-A., A.P-R., A.S. and E.F-P; visualization, A.F-A. and E.F-P.; supervision, J.R-C. and E.F-P.; writing—original draft preparation, A.F-A, J.R-C. and E.F-P.; writing—review and editing, J.R-C. and E.F-P.; funding acquisition, E.F-P.; project administration, E.F-P.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ANP	Acute Necrotizing Pancreatitis

PEG35	Polyethylene glycol 35
IL6	Interleukin 6
TNFα	Tumor Necrosis Factor α
IL1β	Interleukin 1β
CXCL2	Chemokine (C-X-C motif) ligand 2
ICAM-1	Intercellular Adhesion Molecule -1
LDH	Lactate Dehydrogenase
MPO	Myeloperoxidase

References

1. Lankisch, P.G.; Apte, M.; Banks, P.A. Acute pancreatitis. Lancet **2015**, 386, 85-96. DOI: 10.1016/S0140-6736(14)60649-8
2. Banks, P.A.; Bollen, T.L.; Dervenis, C.; Gooszen, H.G.; Johnson, C.D.; Sarr, M.G.; Tsiotos, G.G.; Vege, S.S. Acute Pancreatitis Classification Working G: Classification of acute pancreatitis--2012: revision of the Atlanta classification and definitions by international consensus. Gut **2013**, 62, 102-11. DOI: 10.1136/gutjnl-2012-302779.
3. Akbarshahi H, Rosendahl AH, Westergren-Thorsson G, Andersson R. Acute lung injury in acute pancreatitis--awaiting the big leap. Respir Med. 2012; 106(9):1199-210. doi: 10.1016/j.rmed.2012.06.003.
4. Turecek PL, Bossard MJ, Schoetens F, Ivens IA. PEGylation of Biopharmaceuticals: A Review of Chemistry and Nonclinical Safety Information of Approved Drugs. J Pharm Sci. 2016 ; 105(2):460-475. doi: 10.1016/j.xphs.2015.11.015.
5. Ivens IA, Achanzar W, Baumann A, Brändli-Baiocco A, Cavagnaro J, Dempster M, Depelchin BO, Rovira AR, Dill-Morton L, Lane JH, Reipert BM, Salcedo T, Schweighardt B, Tsuruda LS, Turecek PL, Sims J. PEGylated Biopharmaceuticals: Current Experience and Considerations for Nonclinical Development. Toxicol Pathol. 2015; 43(7):959-83. doi: 10.1177/0192623315591171.
6. Knop K, Hoogenboom R, Fischer D, Schubert US. Poly(ethylene glycol) in drug delivery: pros and cons as well as potential alternatives. Angew Chem Int Ed Engl. 2010; 49(36):6288-308. doi: 10.1002/anie.200902672.
7. Pasut. G.; Panisello, A.; Folch-Puy, E.; Lopez, A.; Castro-Benítez, C.; Calvo, M.; Carbonell, T.; García-Gil, A.; Adam, R.; Roselló-Catafau, J. Polyethylene glycols: An effective strategy for limiting liver ischemia reperfusion injury. World J. Gastroenterol. **2016**, 22, 6501-8. DOI: 10.3748/wjg.v22.i28.6501
8. Chiang, E.T.; Camp, S.M.; Dudek, S.M.; Brown, M.E.; Usatyuk, P.V.; Zaborina, O.; Alverdy, J.C.; Garcia, J.G. Protective effects of high-molecular weight polyethylene glycol (PEG) in human lung endothelial cell barrier regulation: role of actin cytoskeletal rearrangement. Microvasc. Res. **2009**, 77, 174-86. DOI: 10.1016/j.mvr.2008.11.007
9. Malhotra, R.; Valuckaite, V.; Staron, M.L.; Theccanat, T.; D'Souza, K.M.; Alverdy, J.C.; Akhter, S.A. High-molecular-weight polyethylene glycol protects cardiac myocytes from hypoxia- and reoxygenation-induced cell death and preserves ventricular function. Am. J. Physiol. Heart Circ. Physiol. **2011**, 300, H1733-42. DOI: 10.1152/ajpheart.01054.2010
10. Ackland, G.L.; Gutierrez Del Arroyo, A.; Yao, S.T.; Stephens, R.C.; Dyson, A.; Klein, N.J.; Singer, M.; Gourine, A.V. Low-molecular-weight polyethylene glycol improves survival in experimental sepsis. Crit. Care Med. **2010**, 38, 629-36. DOI: 10.1097/CCM.0b013e3181c8fcd0.
11. Videla, S.; Lugea, A.; Vilaseca, J.; Guarner, F.; Treserra, F.; Salas, A.; Crespo, E.; Medina, C.; Malagelada, J.R. Polyethylene glycol enhances colonic barrier function and ameliorates experimental colitis in rats. Int. J. Colorectal Dis. **2007**, 22, 571-80. DOI: 10.1007/s00384-006-0232-4
12. Nagelschmidt, M.; Minor, T.; Saad, S. Polyethylene glycol 4000 attenuates adhesion formation in rats by suppression of peritoneal inflammation and collagen incorporation. Am. J. Surg. **1998**, 176, 76-80. DOI: 10.1016/s0002-9610(98)00102-0
13. Karlsson, P.C.; Hughes, R.; Rafter, J.J.; Bruce, W.R. Polyethylene glycol reduces inflammation and aberrant crypt foci in carcinogen-initiated rats. Cancer Lett. **2005**, 223, 203-9. DOI: 10.1016/j.canlet.2004.10.029

14. Zaouali, M.A.; Bejaoui, M.; Calvo, M.; Folch-Puy, E.; Pantazi, E.; Pasut, G.; Rimola, A.; Ben Abdennebi, H.; Adam, R.; Roselló-Catafau, J. Polyethylene glycol rinse solution: an effective way to prevent ischemia-reperfusion injury. World J. Gastroenterol. **2014**, 20, 16203-14. DOI: 10.3748/wjg.v20.i43.16203
15. Bejaoui, M.; Pantazi, E.; Calvo, M.; Folch-Puy, E.; Serafín, A.; Pasut, G.; Panisello, A.; Adam, R.; Roselló-Catafau, J. Polyethylene Glycol Preconditioning: An Effective Strategy to Prevent Liver Ischemia Reperfusion Injury. Oxid. Med. Cell. Longev. **2016**, 2016, 9096549. DOI: 10.1155/2016/9096549
16. Jain S, Midha S, Mahapatra SJ, Gupta S, Sharma MK, Nayak B, Jacob TG, Shalimar1, Garg PK. Interleukin-6 significantly improves predictive value of systemic inflammatory response syndrome for predicting severe acute pancreatitis. Pancreatology. 2018. pii: S1424-3903(18)30086-3. doi: 10.1016/j.pan.2018.05.002.
17. Gregoric, P.; Sijacki, A.; Stankovic, S.; Radenkovic, D.; Ivancevic, N.; Karamarkovic, A.; Popovic, N.; Karadzic, B.; Stijak, L.; Stefanovic, B.; Milosevic, Z.; Bajec, D. SIRS score on admission and initial concentration of IL-6 as severe acute pancreatitis outcome predictors. Hepatogastroenterology **2010**, 57, 349-53.
18. Rossaint J, Margraf A, Zarbock A. Role of Platelets in Leukocyte Recruitment and Resolution of Inflammation. Front Immunol. 2018; 9:2712. doi: 10.3389/fimmu.2018.02712
19. Sato T, Shibata W, Maeda S. Adhesion molecules and pancreatitis. J Gastroenterol. 2019; 54(2):99-107. doi: 10.1007/s00535-018-1500-0.
20. Yong Zhang, Jinxiang Zhang, Sebastian Korff, Faez Ayoob, Yoram Vodovotz, and Timothy R. Billiar. Delayed Neutralization of IL-6 Reduces Organ Injury, Selectively Suppresses Inflammatory Mediator and Partially Normalizes Immune Dysfunction following Trauma and Hemorrhagic Shock. Shock. 2014; 42(3): 218–227. doi: 10.1097/SHK.0000000000000211
21. Sternby, H.; Hartman, H.; Johansen, D.; Thorlacius, H.; Regnér, S. IL-6 and CRP are superior in early differentiation between mild and non-mild acute pancreatitis. Pancreatology **2017**, 17, 550-4. DOI: 10.1016/j.pan.2017.05.392
22. Rao SA, Kunte AR. Interleukin-6: An Early Predictive Marker for Severity of Acute Pancreatitis. Indian J Crit Care Med. 2017; 21(7):424-428. doi: 10.4103/ijccm.IJCCM_478_16.
23. Hackert T, Büchler MW, Werner J. Targeting P-selectin in acute pancreatitis. Expert Opin Ther Targets. 2010; 14(9):899-910. doi: 10.1517/14728222.2010.504717.
24. Zhang X, Wu D, Jiang X. Icam-1 and acute pancreatitis complicated by acute lung injury. JOP. 2009; 10(1):8-14.
25. Luo J, Shi R. Polyethylene glycol inhibits apoptotic cell death following traumatic spinal cord injury. Brain Res. 2007;1155:10-6.
26. Dutheil D, Rioja-Pastor I, Tallineau C, Goujon JM, Hauet T, Mauco G, Petit-Paris I. Protective effect of PEG 35,000 Da on renal cells: paradoxical activation of JNK signaling pathway during cold storage. Am J Transplant. 2006;6(7):1529-40.
27. Gutierrez PT, Folch-Puy E, Bulbena O, Closa D. Oxidised lipids present in ascitic fluid interfere with the regulation of the macrophages during acute pancreatitis, promoting an exacerbation of the inflammatory response. Gut. 2008; 57(5):642-8. doi: 10.1136/gut.2007.127472.
28. Untergasser, A.; Cutcutache, I.; Koressaar, T.; Ye, J.; Faircloth, B.C.; Remm, M.; Rozen, S.G. Primer3--new capabilities and interfaces. Nucleic Acids Res. **2012**, 40, e115. DOI: 10.1093/nar/gks596

ESTUDIO 2. PAPEL DEL PEG35 FRENTE AL DAÑO PANCREÁTICO ASOCIADO A LA PANCREATITIS AGUDA INDUCIDA POR CERULEÍNA EN MODELOS EXPERIMENTALES *IN VIVO* E *IN VITRO*

En un segundo estudio, analizamos el efecto del tratamiento con PEG35 en modelos experimentales *in vivo* e *in vitro* de inflamación pancreática.

La administración de dosis supramáximas de ceruleína, análogo de la CCK, es una práctica común para la reproducción experimental de un modelo de PA leve caracterizado por la formación de edema, que lleva asociada también la activación de distintos procesos inflamatorios y necróticos en las células pancreáticas. Este modelo, al igual que sucede con la PAEI en humanos, sigue un curso leve y autolimitado. Así, utilizando este modelo experimental en rata, evaluamos el efecto de la administración profiláctica vía intravenosa de PEG35.

Los resultados obtenidos *in vivo* fueron corroborados *in vitro* en células acinares pancreáticas (AR42J) bajo diferentes estímulos: TNF- α, ceruleína y estaurosporina.

Polyethylene Glycol 35 ameliorates pancreatic injury in Cerulein-induced Acute Pancreatitis in Rats

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Abstract

BACKGROUND

Acute pancreatitis (AP) is an inflammatory process of the pancreas that may also involve surrounding tissues and/or remote organs. Inflammation and parenchymal cell death are common pathological features of this condition and determinants of disease severity. Polyethylene glycols (PEGs) are non-immunogenic, non-toxic and water-soluble polymers widely used in biological, chemical, clinical and pharmaceutical settings.

AIM

To evaluate the protective effect of PEG35 on the pancreatic damage associated to cerulein-induced acute pancreatitis *in vivo* and *in vitro*.

METHODS

Wistar rats were randomly assigned to a control group, a cerulein–induced AP group and a PEG35 treatment group. AP was induced by five hourly intraperitoneal injections of cerulein (50 μg/kg/bw) while the control animals received saline solution. PEG35 was administered intraperitoneally 10 minutes before each cerulein injection in a dose of 10mg/kg. After AP induction, samples of pancreatic tissue and blood were collected for analysis. AR42J pancreatic acinar cells were treated with increasing concentrations of PEG35 prior to exposure with TNFα, staurosporine or cerulein. The severity of AP was assessed on the basis of plasma levels of lipase, lactate dehydrogenase activity and pancreatic edema. To evaluate the extent of the inflammatory response, the gene expression of inflammation-associated markers was determined in the pancreas and in AR42J-treated cells. Inflammation-induced cell death was also measured in both the *in vivo* and the *in vitro* models of pancreatic damage.

RESULTS

Administration of PEG35 significantly improved pancreatic damage through reduction on lipase levels and tissue edema in cerulein-induced AP rats. The increased associated inflammatory response caused by cerulein administration was attenuated by means of a decrease in inflammation-related cytokines and iNOS enzyme gene expression in the pancreas. By contrast, pancreatic tissue mRNA expression of IL10 was markedly increased. PEG35 treatment also protected against inflammation-induced cell death by attenuating lactate dehydrogenase activity and modulating the pancreatic levels of apoptosis regulator proteins, caspase-3 and BCL-2 in cerulein hyperstimulated rats. Furthermore, the activation of pro-inflammatory markers and inflammation-induced cell death in TNFα, cerulein or staurosporine-treated pancreatic acinar cells was significantly regulated under PEG35 treatment in a dose-dependent manner.

CONCLUSIONS

PEG35 ameliorated pancreatic damage in cerulein-induced AP and AR42J-treated cells through the attenuation of the inflammatory response and associated cell death. PEG35 may be a valuable option in the management of AP.

Key words: acute pancreatitis, inflammation, polyethylene glycols, cytokines, AR42J cells, cell death.

Core tip: Acute pancreatitis (AP) is an acute inflammatory condition of the pancreas with variable involvement of peri-pancreatic tissues and/or remote organ systems. This disease is a major clinical challenge since currently no specific pharmacological therapy exists. The manuscript describes the protective role of PEG35 on cerulein-induced AP. PEG35 treatment was able to lessen the inflammatory process in the pancreas and associated cell death in both cerulein-induced AP *in vivo* and *in vitro* models of pancreatic damage.

INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease of the exocrine pancreas characterized by abnormal intracellular activation of proteolytic enzymes. The parenchymal injury, pancreatic acinar cells death and an intense inflammatory reaction are common pathological features of this condition and determine the severity of the disease[1]. A majority of patients presenting with AP have the mild form of the disease; this is mostly self-limited and consists of the appearance of edema and inflammation of the pancreas[2]. In this group, organ failure and local complications are generally not observed and the disease usually resolves in the first week. Conversely, between 20% and 30% develop a severe form requiring intensive care unit admission, which is often associated with local and systemic complications and, in some occasions, leads to the death of the patient[3]. Up to date, there is no drug to prevent or treat the condition and the improved clinical outcomes are mostly due to continuous advancement of various supportive treatments.

Although pancreatic inflammation may be firstly triggered by intra-acinar events such as trypsinogen activation, it ultimately depends on the subsequent activation of components of the innate immune system. The initial acinar cell

damage triggers the release of pro-inflammatory cytokines and chemokines leading to increase of microvascular permeability and subsequent formation of interstitial edema[4]. Then, activation of inflammatory cells provokes the production of additional cytokines and other mediators that initiate the inflammatory response. These mediators recruit different types of leukocytes (neutrophils, and then macrophages, monocytes, and lymphocytes) to the pancreas. In parallel to the pro-inflammatory response, an anti-inflammatory response is also released[5]. If the anti-inflammatory response is adequate, the local inflammation resolves at this stage. However, in some cases, an overwhelming pro-inflammatory response drives the migration of inflammatory mediators into systemic circulation leading to distant organ dysfunction[6].

Polyethylene glycols (PEGs) are hydrophilic polymers composed of repeating ethylene glycol units[7]. PEGs have several physicochemical properties that make it useful in various biological, chemical and pharmaceutical settings, especially in view of its low toxicity. For instance, these polymers have been found to exert beneficial effects in several *in vivo* and *in vitro* models of cell and tissue injury[8–10]

There are very few studies linking PEGs of different molecular weight with an anti-inflammatory activity. In a model of traumatic inflammation, the intraperitoneal administration of PEG of 4 kDa prevented the formation of initial adhesions and reduced the leukocytes number in the peritoneal cavity as a consequence of an inflammatory peritoneal reaction[11]. The prophylactic oral administration of 4-kDa molecular weight PEG in experimental colitis reinforced the epithelial barrier function and reduced inflammation in the colon[12]. Likewise, in two different models of gut-derived sepsis, therapeutic administration of low molecular weight PEG reduced inflammatory cytokine expression and activation of neutrophils[13]. Additionally, our group has recently demonstrated an anti-inflammatory role for PEG35 in an experimental model of severe necrotizing AP. In this sense, the therapeutic administration of PEG35

notably alleviated the severity of AP and protected against the associated lung inflammatory response[14].

Taking into account the protective features of PEGs, in this paper we evaluated the effect of PEG35 in experimental models of pancreatic damage *in vivo* and *in vitro*.

MATERIALS AND METHODS

Experimental animals and model of cerulein-induced AP

All experimental animals' procedures were conducted in accordance with European Union regulatory standards for animal experimentation (Directive 2010/63/EU on the protection of animals used for scientific purposes). The Ethical Committee for Animal Experimentation (CEEA, ethic approval number: 211/18, University of Barcelona, 11/04/2018) approved the animal experiments.

The protocol was designed to minimize pain or discomfort to the animals. Male Wistar rats weighting 200-250 g were purchased from Charles River (Boston, MA, USA). They were then housed in a controlled environment with free access to standard laboratory pelleted formula (A04; Panlab, Barcelona, Spain) and tap water. The rats were kept in a climate-controlled environment with 12-h light/12-h dark cycle for a one-week period and then were fasted for 12 h with free access to drinking water prior to the induction of AP.

The rats were randomly divided into three equal experimental groups: saline-treated control rats (Control, n=7), rats with cerulein-induced AP (Cer-AP, n=7) and rats pretreated with PEG35 before and during induction of AP by cerulein administration (PEG35+Cer-AP, n=7). Buprenorphine (0.05 mg/Kg) was intravenously administered as an analgesic immediately before first cerulein injection. Cerulein (Sigma-Aldrich, St. Louis, MO) was dissolved with phosphate-buffered saline and administered intraperitoneally at a supramaximal stimulating concentration of 50 μg/kg/bw at 1-hour intervals (total of 5 injections) while control animals received intraperitoneal saline solution. The use of this

supramaximal doses of cerulein induce a transient form of interstitial edematous AP characterized by marked hyperamylasemia, pancreatic edema, and neutrophil infiltration within the pancreas and pancreatic acinar cell vacuolization and necrosis[15].

PEG35 was administered intraperitoneally in a dose of 10mg/kg, 10 minutes before each cerulein injection. Immediately after the last injection of cerulein or saline, animals were euthanized by intravenous injection of sodium pentobarbital and blood was collected in heparinized syringes from the vena cava. Harvested blood was centrifuged, the plasma removed and stored at −80°C for analysis. Tissue samples from each animal were taken from the head of the pancreas and appropriately stored until further analysis. A sample of tissue was immediately weighted and oven dried for the wet-to-dry weight ratio calculation.

Cell lines and treatments

Rat pancreatic acinar AR42J cell line was obtained from Sigma (St. Louis, MI, USA). The cells were grown at 37ºC in RPMI medium supplemented with 100 mL/L fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/mL) in a humidified atmosphere of 50 mL/L CO_2. The acinar cells were plated at a density of $3x10^5$/well in 12-well culture plates or at a density of $2x10^4$/well in 96-well plates and allowed to attach for 24 or 48 hours. The cells were pretreated with PEG35 diluted in PBS, at a concentration of 0.5, 1, 2, 4, and 6% for 30 minutes prior to the treatment of appropriate stimuli: two and 4 μM staurosporine, 100 ng/ml TNFα or 10 nM cerulein. All three reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). The time points of stimulation were 3 hours for TNFα treatment and 24 hours for the rest of stimuli.

Lipase activity

Plasma lipase activity was determined using a commercial turbidimetric assay kit from Randox (County Antrim, Crumlin, UK), according to the supplier's specifications. Briefly, the degradation of triolein by the pancreatic lipase results in decreased turbidity, which was measured in the sample at 340 nm using an automated microplate reader (iEMS Reader MF; Labsystems, Helsinki, Finland). The activity of the samples was obtained in U/L. All samples were run in duplicate.

Pancreas wet-to-dry weight ratio

Pancreatic edema was evaluated by measuring the wet-to-dry weight ratio. A segment of the pancreas was weighed. The water content was determined by calculating the wet-to-dry weight ratio from the initial weight (wet weight) and its weight after incubation in an oven at 60 °C for 48 h (dry weight).

Lactate dehydrogenase activity

Lactate dehydrogenase (LDH) is a soluble cytosolic enzyme present in most eukaryotic cells, and is released upon cell death due to damage to the plasma membrane. LDH activity was measured in samples of plasma using the Lactate Dehydrogenase Assay Kit (Abcam; Cambridge, UK). In this assay, LDH reduces NAD to NADH, which then interacts with a specific probe to produce color. Changes in absorbance due to NADH formation were recorded at 450 nm at 37 °C using an automated microplate reader (iEMS Reader MF; Labsystems, Helsinki, Finland). The activity of the samples was expressed in milliunits per milliliter (mU/mL). All samples were run in duplicate. The lower limit of detection for ELISA ranged from 14 to 36 mU/mL.

MTT cell proliferation assay

The cell proliferation was determined by measuring cellular metabolic activity through the reduction of the tetrazolium dye MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to its insoluble formazan. AR42J cells were seeded in triplicates in 96-well plates at a density of 2×10^4 cells/well in 100 μL of culture medium with or without compounds to be tested. The cells were cultured in a CO_2 incubator at 37°C for 24 hours. MTT reagent was added to each well and incubated for 2 h at 37°C. Then, the formazan produced in the cells appeared as dark crystals in the bottom of the wells. The culture medium from each well was aspirated and crystal-dissolving solution was added to dissolve the formazan crystals. The absorbance of each sample was quantified at 570 nm using an automated microplate reader (iEMS Reader MF; Labsystems, Helsinki, Finland). All samples were run in duplicate. The absorbance intensity was proportional to the number of viable cells.

Real-time qRT-PCR

Total RNA from the pancreas and cultured cells was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA concentration and quality were measured with the OD A260/A280 ratio and OD A260/A230 ratio respectively, and the integrity of 18S and 28S ribosomal bands for all RNA preparations was verified by running an agarose gel electrophoresis. Reverse transcription was conducted on a 1 μg RNA sample using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Subsequent PCR amplification was conducted using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) on a CFX Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using 10μL of amplification mixtures containing 50 ng of reverse-transcribed RNA and 250 nM of the corresponding forward and reverse primers.

PCR primers for the detection of Interleukin 6 (IL6), Interleukin 1β (IL1β) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were experimentally

validated primers from BioRad (Hercules, CA, USA). PCR primers for Tumor necrosis factor α (TNFα) were designed with Primer3.0 plus[16]. The sequences were as follows: TNFα forward, 5'- ATGGGCTCCCTCTCATCAGT-3' and reverse 5'-GCTTGGTGGTTTGCTACGAC-3'. The specificity of the amplicons was determined by melting curve analysis. Reactions were carried out in duplicate and threshold cycle values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression. The ratio of the relative expression of target genes to GAPDH was calculated by the DCt formula.

Western Blot

Pancreatic tissue was homogenized in ice-cold RIPA buffer (50mM Tris-HCl, 150mM NaCl, 0.5 mL/L Triton X-100, 1mM Ethylenediamine Tetraacetic Acid, 1 mM Dithiothreitol, 1mM Phenylmethylsulfonyl fluoride, 1 mM NaF, 1mM Na3VO4, 1 μg/mL Aprotinin, 1 μg/mL Leupeptin; pH 7.4). Lysates were then centrifuged at 15,000 g for 20 min at 4 °C, and the supernatants were collected. Protein concentration of the supernatants was determined by the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). SDS-PAGE was performed on a 10% gel on which 40 μg of total protein per well was loaded. After SDS-PAGE, the proteins were transferred to a polyvinylidene difluoride membrane.

Immunoblotting was performed using the following antibodies: rabbit polyclonal cleaved caspase-3 (Asp175) antibody (1:800 dilution, reference #9661) from Cell Signaling, rabbit polyclonal BCL-2 (1:500 dilution, reference #59348) from Abcam (Cambridge, UK) and β-actin-HRP conjugated (1:20000 dilution, reference A3854) from Sigma (Sigma-Aldrich, St. Louis, MO). The bound antibody was detected using enhanced chemiluminescence (ECL) detection (Bio-Rad Laboratories, Hercules, CA, USA), and the bands were analyzed using ChemiDoc™ Touch Imaging System (Bio-Rad Laboratories, Hercules, CA, USA).

For quantification, protein expression of cleaved caspase-3 and BCL-2 were normalized to β-actin.

Statistical analysis

All data were exported into Graph Pad Prism 4 (GraphPad Software, Inc.) and were presented as means ± SEM. Statistical analyses were carried out by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test to determine the significance between pairs. The minimal level of statistical significance was considered at $P < 0.05$.

RESULTS

PEG35 reduced the release of lipase associated with cerulein-induced AP

Cerulein-induced AP in rats was associated with significant increases in the plasma levels of lipase, compared with the control group, reflecting the degree of pancreatic injury (Figure 1A). This increase was significantly reduced in the rats treated with intravenous PEG35 at 10mg/kg.

PEG35 abrogated pancreatic edema following cerulein-induced AP

As cerulein-induced pancreatitis is characterized by a progressive interstitial edema development, we analyzed the pancreas wet-to-dry weight ratio (Figure 1B). In control-treated rats, the wet-to-dry weight ratio was 2.76 ± 0.28. After AP induction with cerulein a significant increase in the pancreas wet-to-dry weight ratio to 7.865 ± 0.86 was observed. Treatment with PEG35 resulted in a significant reduction in the wet-to-dry weight ratio to 3.8 ± 0.85.

PEG35 ameliorated the expression of inflammatory markers in cerulein-induced AP and AR42J-treated cells

Next, we explored whether PEG35 administration might improve inflammatory response after cerulein hyperstimulation in rats by measuring the

gene expression of inflammatory mediators in the pancreas. Pancreatic tissue levels of IL6, IL1β, TNFα, IL10 and iNOS had risen markedly after AP induction compared with control animals (Figure 2). Treatment with PEG35 was able to significantly reduce the AP-induced increases in IL1β, IL6, and iNOS. No significant effect on TNFα was observed, although there was a tendency toward a decrease in expression. In accordance with the anti-inflammatory role for IL10, this cytokine did not reduce its gene expression in PEG35-treated animals.

Furthermore, a direct anti-inflammatory effect of PEG35 was observed in cultured AR42J cells. Using an *in vitro* model of cerulein-induced inflammation in the acinar cells, PEG35 was able to attenuate the gene expression of pro-inflammatory IL1β and TNFα in a dose-dependent manner (Figure 3A). Additionally, TNFα-treated cells induced the production of TNFα itself and iNOS which both were markedly reduced after the treatment with increasing concentrations of PEG35 (Figure 3B).

PEG35 lessened inflammation-associated cell death in cerulein-induced AP

To investigate the potential protective effects of PEG35 on the pancreas, cell death was determined through LDH release and expression of apoptosis-related proteins BCL-2 and cleaved-caspase-3 by Western blot. As illustrated in Figure 4A, a significant increase in LDH activity in plasma occurred in cerulein AP-induced animals. Levels of this necrotic marker were significantly reduced under conditions of PEG35 treatment.

In addition, pancreatic levels of BCL-2 were markedly higher following cerulein-induced AP compared with the control group while the increase in pro-apoptotic cleaved-caspase-3 failed to reach statistical significance (Figure 4B and C). The administration of PEG35 promoted a further increase in the levels of anti-apoptotic BCL-2 as compared with cerulein hyperstimulated rats.

PEG35 reduced inflammation-associated cell death in models of pancreatic damage in vitro

AR42J cells are well-established cell model for studying intracellular mechanisms involved in the cell death and inflammatory responses of acute pancreatitis. Thus, the effect of PEG35 on the cell viability of AR42J cells was examined in the presence of the pro-inflammatory stimulus TNFα (Figure 4D). In this sense, TNFα-induced cell death was reduced by PEG35 in a dose-dependent manner. As well, PEG35 markedly prevented the AR42J apoptotic cell death process induced by staurosporine dose-dependently (Figure 4E). These results suggest that PEG35 exerts a protective role against inflammation-induced cell death *in vitro* and *in vivo.*

DISCUSSION

Acute pancreatitis (AP) is an inflammatory disease that can have a mild to severe course. We have recently reported an anti-inflammatory role for PEG35 in a severe necrotizing AP experimental model. To further investigate the effect of this polymer in a milder form of the disease, we used a model of cerulein-induced mild edematous pancreatitis mainly characterized by a dysregulation of the production and secretion of digestive enzymes, interstitial edema formation, infiltration of neutrophil and mononuclear cells within the pancreas and cytoplasmic vacuolization and the death of acinar cells[17]. Our findings were that PEG35 reduced the course of cerulein-induced AP by inhibiting the inflammatory response as well as inflammation-induced cell death. In the study, the administration of PEG35 significantly abrogated the severity of cerulein AP, as indicated by the decreased activity of lipase in plasma and edema formation in the PEG35-treated animals.

A sudden inflammatory response in the pancreas contributes to the development of AP, primarily through the release of inflammatory cytokines. TNFα has long been considered as one of the initial triggers of the inflammatory

cascade in experimental pancreatitis[18]. In this setting, stimulation of pancreatic acinar cells by TNFα have been reported to cause a direct activation of pancreatic enzymes contributing to premature protease activation and cell necrosis [19]. Increased accumulation of TNFα promotes the production of other inflammatory cytokines, including IL1β and IL6, which result in the activation of an inflammatory cascade leading to widespread tissue damage in multiple tissues and organs. Indeed, the levels of TNFα, IL1β and IL6 have been correlated with the severity of AP[20–23]. In the current study, the treatment with PEG35 was able to significantly reduce the AP-induced increases in pro-inflammatory IL1β and IL6. However, no significant effect on TNFα was observed. This fact could be explained by the levels of IL10 found in PEG35-treated animals, which were similar to that found in cerulein-induced rats. Since IL10 plays an important role in the attenuation of the cytokine response during acute inflammation, the significant increase of IL10 found in hyperstimulated rats may contribute to slow TNFα production, observing a tendency toward a decrease in its expression. Indeed, in an experimental model of cerulein-AP intraperitoneal IL10 administration attenuated TNFα production, which was associated with dramatically lessened pancreatitis severity and mortality[24].

Furthermore, a direct anti-inflammatory effect of PEG35 was observed in cultured AR42J cells. In an *in vitro* model of cerulein-induced inflammation, PEG35 was able to attenuate the gene expression of pro-inflammatory IL1β and TNFα in a dose-dependent manner. Moreover, PEG35 reduced the levels of TNFα in AR42J cells stimulated with TNFα.

Pro-inflammatory cytokines are known to activate the inducible isoform of NOS (iNOS) and the subsequent production of nitric oxide thus contributing to the pathophysiology of AP. In fact, the degree of pancreatic inflammation and tissue injury of cerulein-induced AP has been found to be markedly reduced in iNOS-deficient mice[25]. In our study, we observed an increased mRNA expression of iNOS following cerulein hyperstimulation in rats and a significant

reduction after PEG35 treatment. Likewise, PEG35 was able to abrogate TNFα-induced iNOS expression in acinar cells in a dose-dependent manner. Altogether, these results suggested that PEG35 treatment reduced pancreatic inflammation in pancreatitis by suppressing the expression of pro-inflammatory mediators.

These changes in inflammatory processes brought about by PEG35 administration were further emphasized by a reduction in pancreatic cell death. The treatment with PEG35 reduced cell death by lowering plasmatic LDH activity. In addition, the increased release of LDH observed in TNFα-treated acinar cells *in vitro* was reverted upon incubation with increasing concentrations of PEG35.

In the pancreas, inflammation is associated with injured acinar cells that can go through necrosis or apoptosis. Thus, we measured the apoptosis index in pancreatic tissue following cerulein-induced AP. Injured pancreatic tissue induced the increase in caspase-3 and BCL2 apoptotic proteins compared to respective controls although caspase-3 did not reach significance. Following treatment with PEG35, anti-apoptotic BCL-2 further increased as compared with cerulein-treated animals. Collectively, these findings suggested that PEG35 has anti-apoptotic and anti-necrotic properties for cerulein-induced pancreatitis.

In conclusion, results from this study supported that PEG35 exerts anti-inflammatory effects to alleviate experimental cerulein-induced AP by inhibiting the inflammatory response as well as inflammation-induced cell death. Because of the low toxicity and its proved biocompatibility, PEG35 could be used as a new therapeutic strategy to resolve the cellular damage associated to mild AP.

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REFERENCES

1. Gukovskaya AS, Gukovsky I, Algül H, Habtezion A. Autophagy, Inflammation, and Immune Dysfunction in the Pathogenesis of Pancreatitis. *Gastroenterology* 2017;**153**:1212–26 [DOI: 10.1053/j.gastro.2017.08.071]

2. Banks PA, Bollen TL, Dervenis C, Gooszen HG, Johnson CD, Sarr MG, Tsiotos GG, Vege SS. Classification of acute pancreatitis--2012: revision of the Atlanta classification and definitions by international consensus. *Gut* 2013;**62**:102–11 [PMID: 23100216 DOI: 10.1136/gutjnl-2012-302779]

3. van Santvoort HC, Bakker OJ, Bollen TL, Besselink MG, Ahmed Ali U, Schrijver AM, Boermeester MA, van Goor H, Dejong CH, van Eijck CH, van Ramshorst B, Schaapherder AF, van der Harst E, Hofker S, Nieuwenhuijs VB, Brink MA, Kruyt PM, Manusama ER, van der Schelling GP, Karsten T, Hesselink EJ, van Laarhoven CJ, Rosman C, Bosscha K, de Wit RJ, Houdijk AP, Cuesta MA, Wahab PJ, Gooszen HG. A conservative and minimally invasive approach to necrotizing pancreatitis improves outcome. *Gastroenterology* 2011;**141**:1254–63 [PMID: 21741922 DOI: 10.1053/j.gastro.2011.06.073]

4. Garber A, Frakes C, Arora Z, Chahal. P. Mechanisms and management of acute pancreatitis. Gastroenterol. Res. Pract.2018;**2018** [PMID: 29736167 DOI: 10.1155/2018/6218798]

5. Sendler M, van den Brandt C, Glaubitz J, Wilden A, Golchert J, Weiss FU, Homuth G, De Freitas Chama LL, Mishra N, Mahajan UM, Bossaller L, Völker U, Bröker BM, Mayerle J, Lerch MM. NLRP3 Inflammasome Regulates Development of Systemic Inflammatory Response and Compensatory Anti-Inflammatory Response Syndromes in Mice With Acute Pancreatitis. *Gastroenterology* 2020;**158**:253-269.e14 [PMID: 31593700 DOI: 10.1053/j.gastro.2019.09.040]

6. Kylänpää ML, Repo H, Puolakkainen PA. Inflammation and immunosuppression in severe acute pancreatitis. *World J Gastroenterol* 2010;**16**:2867–72 [DOI: 10.3748/wjg.v16.i23.2867]

7. Pasut G, Panisello A, Folch-Puy E, Lopez A, Castro-Benítez C, Calvo M, Carbonell T, García-Gil A, Adam R, Roselló-Catafau J. Polyethylene glycols: An effective strategy for limiting liver ischemia reperfusion injury. World J. Gastroenterol.2016;**22**:6501–8 [PMID: 27605884 DOI: 10.3748/wjg.v22.i28.6501]

8. Chen H, Quick E, Leung G, Hamann K, Fu Y, Cheng JX, Shi R. Polyethylene glycol protects injured neuronal mitochondria. *Pathobiology* 2009;**76**:117–28 [PMID: 19468251 DOI: 10.1159/000209389]

9. Malhotra R, Valuckaite V, Staron ML, Theccanat T, D'Souza KM, Alverdy JC, Akhter SA. High-molecular-weight polyethylene glycol protects cardiac myocytes from hypoxia- and reoxygenation-induced cell death and preserves ventricular function. *Am J Physiol - Hear Circ Physiol* 2011;**300** [PMID: 21335476 DOI: 10.1152/ajpheart.01054.2010]

10. Chiang ET, Camp SM, Dudek SM, Brown ME, Usatyuk P V., Zaborina O, Alverdy JC, Garcia JGN. Protective effects of high-molecular weight Polyethylene Glycol (PEG) in human lung endothelial cell barrier regulation: Role of actin cytoskeletal rearrangement. *Microvasc Res* 2009;**77**:174–86 [DOI: 10.1016/j.mvr.2008.11.007]

11. Nagelschmidt M, Minor T, Saad S, Nagelschmidt M. Polyethylene glycol 4000 attenuates adhesion formation in rats by suppression of peritoneal inflammation and collagen incorporation. *Am J Surg* 1998;**176**:76–80 [DOI: 10.1016/S0002-9610(98)00102-0]

12. Videla S, Lugea A, Vilaseca J, Guarner F, Treserra F, Salas A, Crespo E, Medina C, Malagelada JR. Polyethylene glycol enhances colonic barrier function and ameliorates experimental colitis in rats. *Int J Colorectal Dis* 2007;**22**:571–80 [DOI: 10.1007/s00384-006-0232-4]

13. Ackland GL, Gutierrez Del Arroyo A, Yao ST, Stephens RC, Dyson A, Klein NJ, Singer M, Gourine A V. Low-molecular-weight polyethylene glycol improves survival in experimental sepsis. *Crit Care Med* 2010;**38**:629–36 [DOI: 10.1097/CCM.0b013e3181c8fcd0]

14. Ferrero-Andrés A, Panisello-Roselló A, Serafín A, Roselló-Catafau J, Folch-Puy E. Polyethylene glycol 35 (PEG35) protects against inflammation in experimental acute necrotizing pancreatitis and associated lung injury. *Int J Mol Sci* 2020;**21** [PMID: 32019239 DOI: 10.3390/ijms21030917]

15. Hyun JJ, Lee HS. Experimental models of pancreatitis. Clin. Endosc.2014;**47**:212–6 [DOI: 10.5946/ce.2014.47.3.212]

16. Untergasser A , Cutcutache I, Koressaar T, Ye J,Faircloth BC, Remm M RS. Primer3--new capabilities and interfaces. *Nucleic Acids Res* 2012;**40**:e115

17. Ding SP, Li JC, Jin C. A mouse model of severe acute pancreatitis induced with caerulein

and lipopolysaccharide. *World J Gastroenterol* 2003;**9**:584–9 [PMID: 12632523 DOI: 10.3748/wjg.v9.i3.584]

18. Surbatovic M, Radakovic S. Tumor necrosis factor-α levels early in severe acute pancreatitis: Is there predictive value regarding severity and outcome? *J Clin Gastroenterol* 2013;**47**:637–43 [PMID: 23470643 DOI: 10.1097/MCG.0b013e31828a6cfc]

19. Sendler M, Dummer A, Weiss FU, Krüger B, Wartmann T, Scharffetter-Kochanek K, Rooijen N Van, Malla SR, Aghdassi A, Halangk W, Lerch MM, Mayerle J. Tumour necrosis factor α secretion induces protease activation and acinar cell necrosis in acute experimental pancreatitis in mice. *Gut* 2013;**62**:430–9 [DOI: 10.1136/gutjnl-2011-300771]

20. Sathyanarayan G, Garg PK, Prasad HK, Tandon RK. Elevated level of interleukin-6 predicts organ failure and severe disease in patients with acute pancreatitis. *J Gastroenterol Hepatol* 2007;**22**:550–4 [DOI: 10.1111/j.1440-1746.2006.04752.x]

21. Pooran N, Indaram A, Singh P, Bank S. Cytokines (IL-6, IL-8, TNF): Early and reliable predictors of severe acute pancreatitis. *J Clin Gastroenterol* 2003;**37**:263–6 [DOI: 10.1097/00004836-200309000-00013]

22. Silva-Vaz P, Abrantes AM, Castelo-Branco M, Gouveia A, Botelho MF, Tralhão JG. Multifactorial scores and biomarkers of prognosis of acute pancreatitis: Applications to research and practice. Int. J. Mol. Sci.2020;**21** [PMID: 31947993 DOI: 10.3390/ijms21010338]

23. Mayer J, Rau B, Gansauge F, Beger HG. Inflammatory mediators in human acute pancreatitis: clinical and pathophysiological implications. *Gut* 2000;**47**:546–52 [PMID: 10986216 DOI: 10.1136/gut.47.4.546]

24. Rongione AJ, Kusske AM, Kwan K, Ashley SW, Reber HA, McFadden DW. Interleukin 10 reduces the severity of acute pancreatitis in rats. *Gastroenterology* 1997;**112**:960–7 [DOI: 10.1053/gast.1997.v112.pm9041259]

25. Cuzzocrea S, Mazzon E, Dugo L, Serraino I, Centorrino T, Ciccolo A, Van De Loo FAJ, Britti D, Caputi AP, Thiemermann C. Inducible nitric oxide synthase-deficient mice exhibit resistance to the acute pancreatitis induced by cerulein. *Shock* 2002;**17**:416–22 [DOI: 10.1097/00024382-200205000-00013]

Footnotes

Institutional animal care and use committee statement: All experimental animals' procedures were conducted in accordance with European Union regulatory standards for animal experimentation (Directive 2010/63/EU on the protection of animals used for scientific purposes). The Ethical Committee for Animal Experimentation (CEEA, ethic approval number: 211/18, University of Barcelona, 11/04/2018) approved the animal experiments.

Conflict-of-interest statement: The authors have disclosed that they do not have any conflict of interest.

Data sharing statement: No additional data are available.

FIGURE LEGENDS

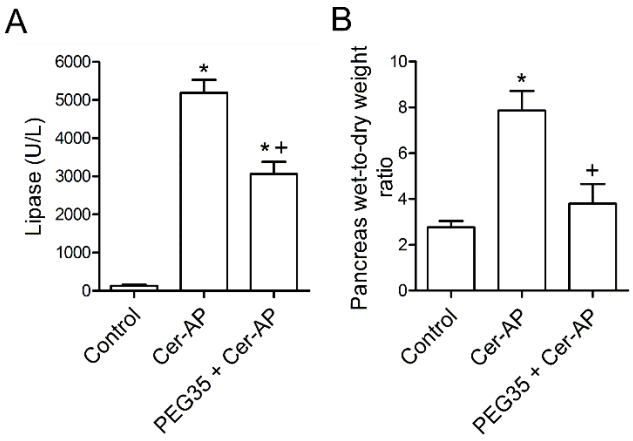

Figure 1. Effect of PEG35 treatment on plasma lipase activity and pancreatic edema in cerulein-induced AP. A: Plasma lipase levels in U/L; B: Pancreatic wet-to-dry weight ratio. Bars represent mean values of each group ± SEM. *$P < 0.05$ versus Control, +$P < 0.05$ versus Cer-AP. Cer-AP, Cerulein-induced Acute Pancreatitis. PEG35, Polyethylene glycol 35. Each determination was carried out in triplicate.

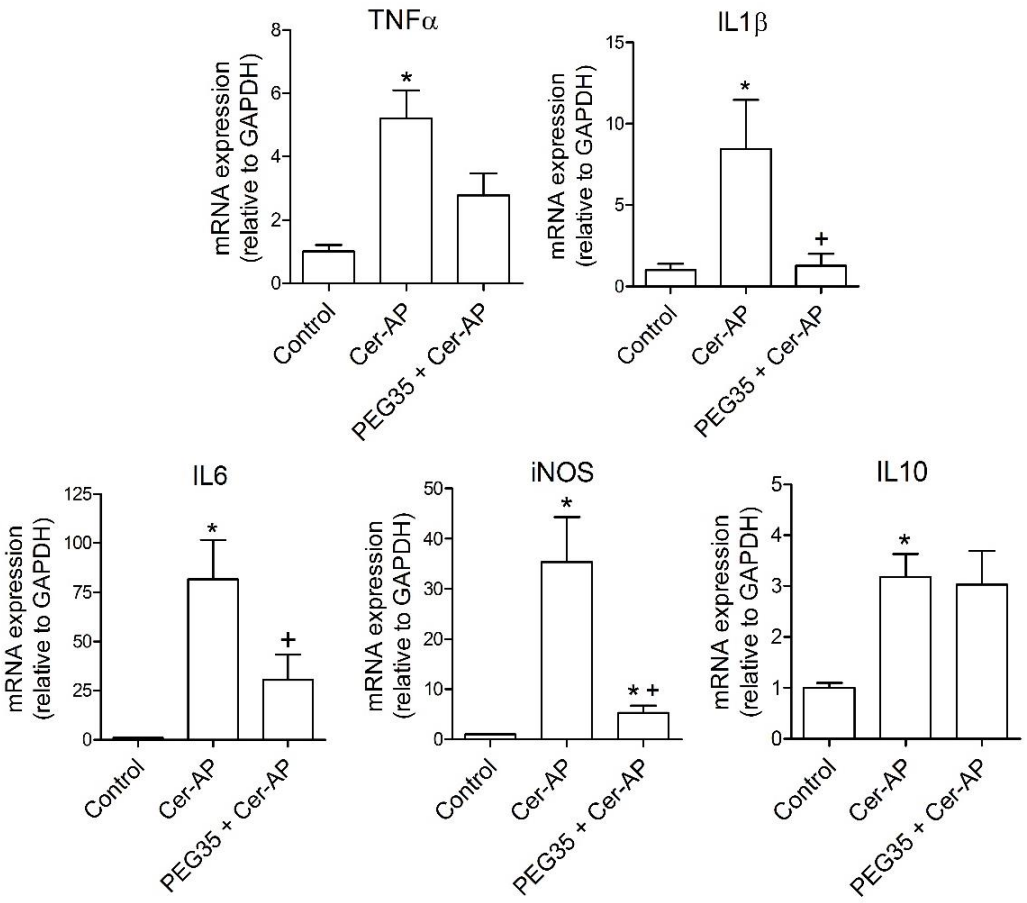

Figure 2. Role of PEG35 on the modulation of inflammation-associated cytokines and iNOS enzyme expression in cerulein-induced AP. Pancreatic tissue gene expression of TNFα, IL1β, IL6, iNOS and IL10 by real-time qRT-PCR. Bars represent mean values of each group ± SEM. *P < 0.05 versus Control, +P < 0.05 versus Cer-AP. Cer-AP, Cerulein-induced Acute Pancreatitis. PEG35, Polyethylene glycol 35. Each determination was carried out in triplicate.

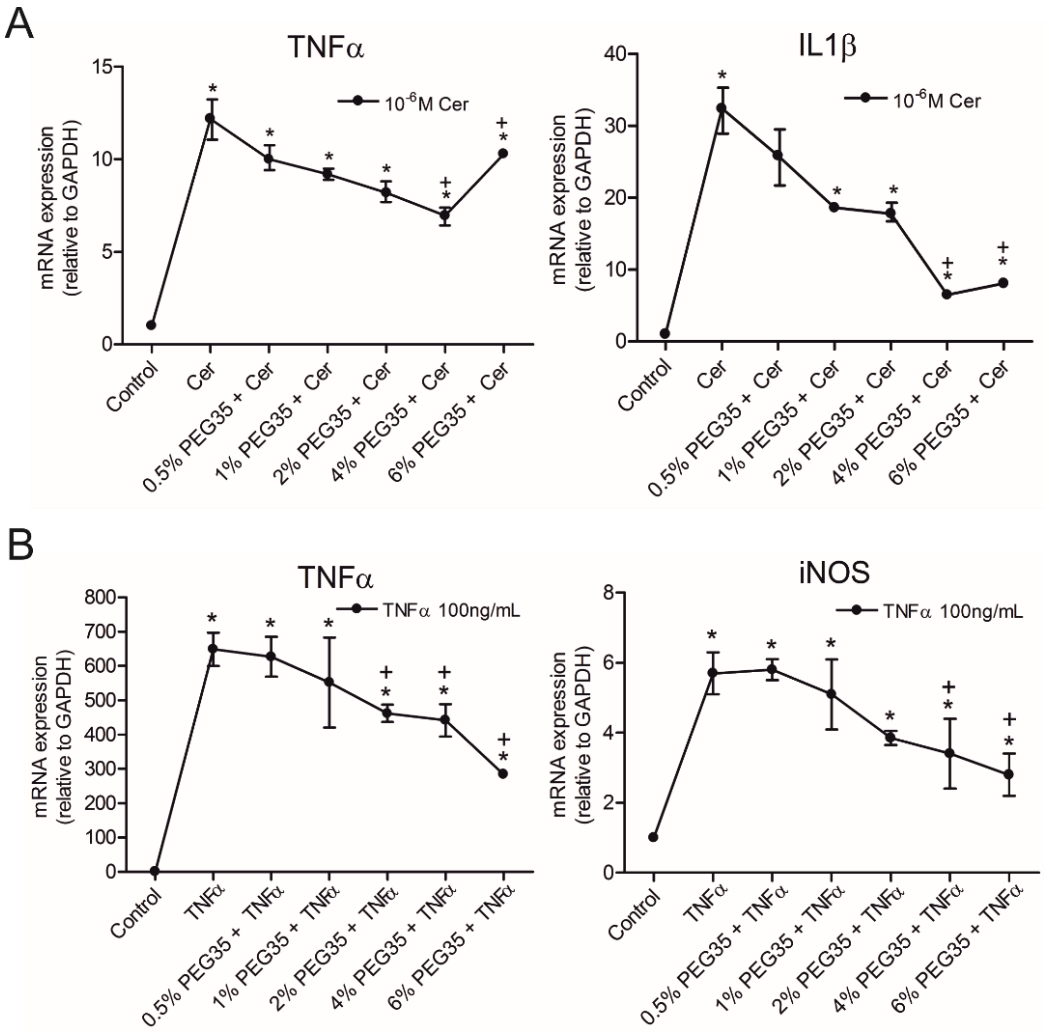

Figure 3. Gene expression of inflammatory markers in AR42J-treated cells. A: Gene expression by real-time qRT-PCR of TNFα and IL1β in cerulein-treated AR42J cells subjected to increasing concentrations of PEG35; B: Gene expression by real-time qRT-PCR of TNFα and iNOS in TNFα-treated AR42J cells subjected to increasing concentrations of PEG35. In both cases, mRNA induction levels were normalized to GAPDH mRNA expression. Bars represent mean values of each group ± SEM. *P < 0.05 versus Control, +P < 0.05 versus Cer or TNFα. Cer, Cerulein. PEG35, Polyethylene glycol 35. TNFα, Tumor Necrosis Factor α. Each determination was carried out in triplicate.

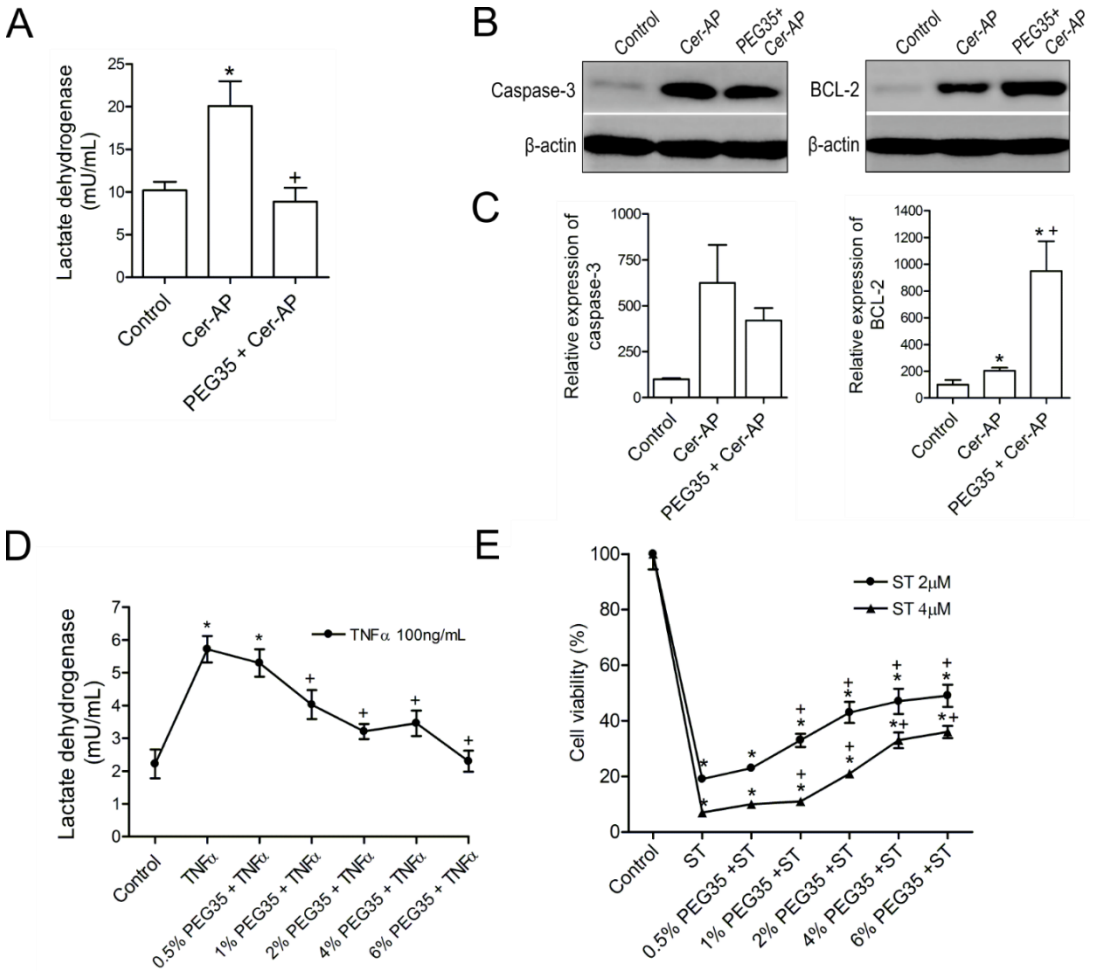

Figure 4. Effect of PEG35 on inflammation-induced cell death in cerulein-induced AP and cultured pancreatic acinar AR42J cells. A: Plasma LDH activity after cerulein-induced AP expressed as mU/mL; B: Pancreatic protein expression of cleaved caspase-3 and BCL-2 assessed by Western Blot analysis. β-actin expression was used as loading control. Data shown are representative blots for each group; C: Densitometry quantification of Western blot for cleaved caspase-3 and BCL-2 in pancreatic tissue; D: Cell death rate measured through LDH activity. AR42J cells pre-treated with increasing concentrations of PEG35 (0. 5, 1, 2, 4, and 6%) for 30 minutes and then co-incubated with or without 100ng/mL of TNFα for another 2.5 hours; E: Cell viability rate determined by MTT assay. AR42J cells were pre-treated with increasing concentrations of PEG35 (0. 5, 1, 2, 4, and 6%) for 30 minutes and then co-incubated with or without 2 and 4μM staurosporine for another 24 hours. The values shown represent the mean ± SEM. *P < 0.05 versus Control, +P < 0.05 versus Cer-AP, TNFα or ST. Cer-AP, Cerulein-induced Acute Pancreatitis. PEG35, Polyethylene glycol 35. TNFα, Tumor Necrosis Factor α. ST, Staurosporine. Each determination was carried out in triplicate.

ESTUDIO 3. INFLAMOSOMA NLRP3 EN EL PROCESO INFLAMATORIO ASOCIADO A LA PANCREATITIS AGUDA

Recientemente se ha descrito el concepto de inflamosoma como un complejo citosólico multiproteico responsable de la activación de procesos inflamatorios como parte del sistema inmune innato. El más caracterizado de todos ellos es el inflamosoma NLRP3, cuya activación se ha relacionado con diversas enfermedades inflamatorias.

Esta revisión presenta una actualización en el progreso de la investigación del mecanismo de acción del inflamosoma NLRP3 en la inflamación aguda del páncreas.

La activación de este inflamosoma está estrechamente ligada a la regulación de la secreción de citoquinas de la familia IL-1, que intervienen en el desarrollo de las complicaciones sistémicas que aparecen como consecuencia del progreso de la PA.

La relación del ensamblaje de este complejo proteico con la estimulación de la inflamación pancreática y el estudio de sus inhibidores, puede abrir una puerta al control de la respuesta inflamatoria asociada esta enfermedad.

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Review

NLRP3 Inflammasome-Mediated Inflammation in Acute Pancreatitis

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Abstract: The discovery of inflammasomes has enriched our knowledge in the pathogenesis of multiple inflammatory diseases. The NLR pyrin domain-containing protein 3 (NLRP3) has emerged as the most versatile and well-characterized inflammasome, consisting of an intracellular multi-protein complex that acts as a central driver of inflammation. Its activation depends on a tightly regulated two-step process, which includes a wide variety of unrelated stimuli. It is therefore not surprising that the specific regulatory mechanisms of NLRP3 inflammasome activation remain unclear. Inflammasome-mediated inflammation has become increasingly important in acute pancreatitis, an inflammatory disorder of the pancreas that is one of the fatal diseases of the gastrointestinal tract. This review presents an update on the progress of research into the contribution of the NLRP3 inflammasome to acute pancreatic injury, examining the mechanisms of NLRP3 activation by multiple signaling events, the downstream interleukin 1 family of cytokines involved and the current state of the literature on NLRP3 inflammasome-specific inhibitors.

Keywords: inflammation; inflammasome; pancreatitis; immune system; interleukins; DAMPs; SIRS; NLRP3.

1. Acute pancreatitis: initiating events and disease progression

Acute pancreatitis (AP), a sudden inflammatory condition of the pancreas, is one of the leading causes of hospital admission for digestive diseases [1]. Nowadays, the most commonly used classification system for AP is the 2012 revision of the Atlanta classification, with definitions based on international consensus. This classification defines three degrees of severity: mild, moderately severe, and severe AP [2]. The mild form of pancreatitis is identified by the absence of organ failure. When organ failure is present within the first 24 hours but resolves within 48 hours the patient is classified as having moderately severe AP. If the patient develops persistent organ failure, they are classified as having severe AP. In such cases, acute lung injury is the most serious complication associated, since it accounts for the majority of deaths in untreated patients and in hospitalized patients who die within a week of the onset of AP [3]. Currently, apart from

the supportive care, there is still no specific pharmacological therapy against this severe form of the disease.

Although the etiology of AP is complex, almost all cases are due to a sterile factor that initiates pancreatic damage. The presence of gallstones obstructing the outflow of pancreatic fluid and excessive ethanol consumption causes of AP in over 80% of cases regardless of the geographical distribution of the population [4]. Whatever the causative factor, in response to a predisposing insult the pancreatic acinar cell undergoes a series of alterations that begin with the premature activation of pancreatic proteolytic enzymes, leading to cell damage and gland self-digestion. Injured acinar cells trigger an inflammatory process by releasing inflammatory cytokines and chemokines that mediate the recruitment and activation of circulating neutrophils and macrophages. The activated neutrophils release high concentrations of oxidants and cytotoxic agents, which further worsen the local pancreatic damage. As inflammation continues, neutrophils transmigrate across endothelial cells and cause the local inflammation to evolve into what is known as the systemic inflammatory response syndrome (SIRS), leading to a dysfunction of vital organs and, in some occasions, to organ failure and death [5].

These tissue-infiltrating neutrophils are the main producers of pro-inflammatory cytokines and chemokines, which are important mediators in the function of the innate immune system. As a major driver of the inflammatory response in AP, nuclear factor-kappa B (NF-κB) is a central transcription factor that regulates the expression of a large array of genes involved in inflammation. Among them, the cytokines interleukins (IL) 1β, 6, 8, 18 and tumor necrosis factor alpha (TNFα) or its soluble receptor, have been studied as markers of severity of AP [6]. Unlike other cytokines, cellular IL1β and IL18 are synthesized as precursor proteins and need to be cleaved to generate their biologically active forms. This process is dependent upon the assembly of a multi-protein signaling platform: the inflammasome.

1.1. Pattern recognition receptors contributing to inflammation in acute pancreatitis

The innate immune and inflammatory cells express specialized receptors known as Pattern Recognition Receptors (PRRs), capable of recognizing molecules frequently found in pathogens (known as Pathogen-Associated Molecular Patterns—PAMPs), and endogenous molecules released from damaged cells, named Damage-Associated Molecular Patterns (DAMPs) [7]. Five different types of PRR have been identified to date expressed on the cell surface or in intracellular compartments, but they can be secreted into the blood stream and tissue fluids as well [8]. Transmembrane proteins toll-like receptors (TLRs) and C-type lectin receptors (CLRs) induce inflammatory responses through the recognition of their extracellular ligands. By contrast, cytoplasmic proteins including retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), absence in melanoma 2 (AIM2)-like receptors (ALRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) recognize intracellular ligands. Following ligand recognition or cellular disruption, these receptors activate downstream signaling pathways resulting in the upregulation of pro-inflammatory cytokines and chemokines which are important in inflammatory and antimicrobial responses. Both ALRs and NLRs induce an inflammatory response starting with the formation of the inflammasome complex.

TLRs and NLRs are major contributors to inflammation in AP. The deficiency of toll-like receptor 4 (TLR4) in mice has demonstrated the key role of this receptor for full tissue injury in AP, while its involvement in AP-associated acute lung injury appears to be important only when the disease is worsened by sepsis [9-11]. Toll-like receptor 9 (TLR9) also promotes the development of pancreatic injury, since TLR9-deficient mice had lower rates of edema, leukocyte infiltration and IL1β gene expression in the pancreas after cerulein-administration. In the same study, mice treated with an antagonist of TLR9 after AP induction through taurolithocholic acid 3-sulphate (a more severe model of AP) reduced serum amylase, pancreatic necrosis and inflammatory cell infiltration in the systemic lungs [12]. Evaluating the role of the NLR nucleotide-binding oligomerization domain-containing protein 1 (NOD1) in pancreatitis

pathogenesis, Tsuji and colleagues found that this signaling is essential to the development of pancreatitis; more interestingly, they showed that the activation of NOD1 stimulated by translocated commensal organisms is an indispensable element in sustaining and widening the inflammatory process in the pancreas [13]. More recently, the receptor for advanced glycation end-products (RAGE) has been recognized. RAGE was initially characterized and named for its ability to bind to advanced glycation end-products whose concentrations are known to increase in conditions such as diabetes, as well as during ageing. However, it is now widely accepted that RAGE functions as a PRR, since it binds with numerous PAMPS and DAMPS ligands. The interaction between RAGE and its ligands mainly results in a pro-inflammatory response, and can lead to stress events often favoring mitochondrial dysfunction or cellular senescence. In this regard, Kang et al. has provided evidence that RAGE mediates the nucleosome-induced AIM2 inflammasome activation in macrophages [14]. In addition, genetic deletion of RAGE protects against local and systemic lung injury in L-arginine-induced AP in mice.

Hence, pancreatic inflammation can activate these sensing components, TLR4, TLR9, RAGE and NOD1 expressed in pancreatic acinar, ductal and/or immune cells [10, 11]. These components sense DAMPs and PAMPs as the first signals in the cells of the affected pancreas, inducing the formation of the nuclear factor-kappa B (NF-κB) complex and its activation, which stimulates the expression of pro-IL1β and pro-IL18 [15]. The second signal that is activated by sensing components causes the assembly of inflammasome complex and effectors. Taken together, there is no doubt that while pancreatic inflammation may be initially triggered by intra-acinar events such as trypsinogen activation, it ultimately depends on the subsequent immune responses induced by the activation of components of the innate immune system.

2. Inflammasomes

In the early 2000s, Tschopp and colleagues introduced the concept of the inflammasome, a group of cytosolic multi-protein complexes expressed in myeloid cells responsible for activation of inflammatory processes as part of the innate immune system [15]. Inflammasomes consist of an upstream sensor protein (belonging to the NLR or the ALR family), and an adaptor protein – the apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). ASC serves as a bridge, connecting the sensor to the downstream effector, the cysteine protease caspase-1 (formerly known as IL1β converting enzyme, ICE) [12]. Active caspase-1 cleaves the precursor cytokines pro-IL1β and pro-IL18, generating their biologically active forms (IL1β and IL18 respectively). Active caspase-1 is also able to induce an inflammatory form of cell death known as pyroptosis, which involves cell swelling, membrane rupture, and release of the cytoplasmic content into the extracellular space [16]. This pathway is known today as the canonical inflammasome. By contrast, more recently, Kayagaki et al. described a new non-canonical inflammasome pathway that is independent of caspase-1 [17].

2.1. The NLRP3 inflammasome

Since the discovery of the founding member of the NLR family, NOD1, 22 distinct NLR proteins have been identified in humans and 34 in mice [18]. To date, the best characterized NLR inflammasome is the NLR pyrin domain-containing protein 3 (NLRP3), also known as cryopyrin or NALP3. NLRP3 is a cytosolic receptor expressed in monocytes, neutrophils, dendritic cells, lymphocytes, osteoblasts and epithelial cells [19]. It is composed by three domains: a carboxy-terminal leucine-rich-repeat (LRR), a central nucleotide-binding and oligomerization (NACHT) domain, and an amino terminal pyrin domain (PYD). This last domain allows the joining to the pyrin domain of ASC through PYD-PYD interactions, and then the assembly with pro-caspase-1 by the caspase activation and recruitment domain (CARD)-CARD interactions, whose active form caspase-1 initiates the activation of proinflammatory cytokines IL1β and IL18 [16]. The ASC domain acts as a cell-to-cell communication signal and so can intensify the inflammasome response, processing IL1β in the extracellular space and sending a danger signal. Every domain

has a function inside the inflammasome assembly: LRR triggers the danger signal response and the autoregulation of the inflammasome, the PYD domain recruits the adaptor molecule ASC, and NACHT carries out oligomerization and hydrolyses the ATP binding to it. This domain has ATPase activity, which is important for regulating the activation of inflammasome [20].

The NLRP3 inflammasome is considered a general sensor of cellular damage that responds to both PAMPS and DAMPs. However, it is the recognition of DAMPS that confers particular importance on NLRP3 in the context of the sterile inflammatory responses observed in many human diseases such as AP. Here, we present a comprehensive review of current developments in the study of the mechanism of action of the NLRP3 inflammasome in acute inflammation of the pancreas.

2.2. Signals of action of the NLRP3 inflammasome in the immune response

Canonical NLRP3 inflammasome is activated in two parallel and independent steps: priming, and activation. The first step is regulated by innate immune signaling in which a stimulus joins to TLR, NLR or cytokine receptors stimulating NF-κB activation, and this produces the upregulation of the mRNA and protein expression of NLRP3 and pro-IL1β [21]. Toll-like receptor (TLR)-adaptor molecules myeloid differentiation primary response 88 (MyD88) and toll/IL1 receptor homology-domain-containing adaptor-inducing interferon-β (TRIF) mediates this step in response to TLRs receptors. All TLRs except TLR3 can activate the MyD88-dependent pathway which results in the transcription of pro-inflammatory genes through the activation of NF-kB. Priming signal upregulates the expression of NLRP3, which is thought to exist at concentrations that are inadequate for initiating inflammasome activation under resting conditions, and pro-IL1β, which is not constitutively expressed. In contrast, priming signals do not appear to affect the expression levels of ASC, pro-caspase-1, or pro-IL18.

Recently, several studies have provided strong evidence that the priming step is not limited to the transcriptional upregulation of NLRP3, since post-translational modifications (ubiquitination and phosphorylation, sumoylation and ribosylation) of NLRP3 protein also play significant roles in NLRP3 inflammasome regulation [22-25]. After priming, canonical NLRP3 inflammasome activation requires a second signal (activation signal) that results in NLRP3 inflammasome oligomerization, leading to caspase-1 activation and, in turn, IL1β and IL18 processing and release. This step depends on different stimuli: re-localization of NLRP3 to the mitochondria, releasing of mitochondrial (mt) reactive oxygen species (ROS) or DNA (mtDNA) or cardiolipin into the cytosol, increasing potassium efflux, the release of cathepsin from damaged lysosomal membranes, extracellular ATP, pore-forming particulate matter, pathogen-associated RNA and bacterial and fungal toxins and components [26]. Independently of IL1β maturation, caspase-1 activation also promotes pyroptosis through the cleavage of the pore-forming protein gasdermin-D (GSDM-D). Pyroptosis then promotes the release of additional cytosolic proteins, such as high mobility group box 1 (HMGB1), a pro-inflammatory mediator that plays a significant role in the pathogenesis of several inflammatory diseases such as AP [27].

Besides canonical NLRP3 inflammasome activation, a non-canonical caspase-11-dependent NLRP3 activation has been characterized, mainly in relation to Gram-negative bacteria [28]. In the first step, Gram-negative bacteria activate the TLR4–MyD88 and TRIF pathways, leading to nuclear translocation of NF-κB, which in turn promotes the transcription of IL1β, IL18, and NLRP3 as well as interferon regulatory factor (IRF)-3 and IRF7 genes. Subsequently, the IRF3–IRF7 complex leads to activation of the JAK/STAT pathway and the consequent transcription of caspase-11 gene in mice (or its human analogues caspase-4 and caspase-5) [29]. Conversely, when LPS is in abundance or has entered the cytosol through other processes, caspase-11 binds to this intracellular LPS independently of TLR4 (the well-known extracellular receptor for LPS). Direct interaction between the lipid A portion of LPS and the CARD domain of caspase-11 catalyzes the oligomerization of the caspase-11-LPS complex. This complex induces pyroptosis through the

cleavage of GSDM-D and leads to a leakage of its N-terminal domain, forming pores in the plasma membrane through which IL18 and IL1β mature forms are released outside the cytosol.

Both processes of canonical and non-canonical NLRP3 inflammasome activation occur independently. However, non-canonical caspase-11 enhances canonical caspase-1 processing and IL1β/IL18 production in the presence of specific stimuli (e.g., cholerae toxin or *E. coli*) [30]. Additionally, another protease required in transcriptional priming and activation of canonical and non-canonical NLRP3 inflammasome is caspase-8, best characterized as an initiator caspase involved in death receptor-mediated apoptosis in response to external stimuli [31].

3. The NLRP3 inflammasome in the pathogenesis of acute pancreatitis and associated lung injury

Patients with AP exhibit elevated serum levels of pro-inflammatory cytokines such as IL1β, TNFα, IL6 and IL18 [32, 33]. As we noted before, the precursor form of IL1β and IL18 cytokines is converted into an active form via the NLRP3 inflammasome, and so the NLRP3 inflammasome is likely to play an important role in AP. For that very reason, Hoque and Mehal elucidated the molecular mechanisms of NLRP3 inflammasome contributing to the initial inflammation in AP and progression [12]. They demonstrated that the NLRP3 inflammasome is notably activated during AP and that components of this inflammasome are required for full pancreatic injury. In an experimental model of cerulein-induced AP in mice, the absence of caspase-1, ASC or NLRP3 substantially reduced edema and inflammation. Furthermore, in a more severe model of AP in mice, TLR9 inhibition decreased both pancreatic IL1β expression and lung inflammation. Another study using NLRP3-deficient mice or the NLRP3 inhibitor INF-39 found suppression of the maturation and release of IL1β and further prevention of the inflammatory cascade in a cerulein plus LPS-induced AP model [34].

The involvement of TLR4 in the initiation of the disease was also demonstrated by Hoque using the metabolic intermediate lactate to block this receptor [35]. In a mouse model of cerulein plus LPS-induced AP, the administration of lactate reduced TLR4-mediated activation of NLRP3 inflammasome via Gi-protein-coupled receptor 81 signaling. This finding is concordant with clinical studies showing an anti-inflammatory effect of Ringer's lactate solution for fluid resuscitation in patients with AP [36, 37]. Other TLR4 modulators (e.g., a natural activator of cofactor NAD^+ or carbon monoxide) have been used as well, demonstrating a clear role of the NLRP3 inflammasome in AP [38, 39].

Further definitive support for the involvement of NLRP3 inflammasome in the development of lung injury secondary to pancreatitis comes from a recent study in exosome research. In that study, the plasma-derived exosomes triggered NLRP3 inflammasome activation and pyroptosis in alveolar macrophages, thus leading to pulmonary dysfunction in the progression of pancreatitis [40]. This study is the first to describe the process of pyroptosis, an inflammasome-induced programmed cell death, as a driver of AP-associated lung injury. Notably, depletion of exosomes only partially abrogated the pyroptosis-inducing effect of AP-conditioned plasma indicating that, besides exosomes, other components are probably needed to promote alveolar macrophage pyroptosis in the progression of AP.

Until now, there has been little clinical information supporting the experimental results regarding inflammasome activation in AP. A study from Algaba-Chueca et al. identified increased levels of AIM2 and NLRP3 inflammasomes and derived IL1β and IL18 in the early course of AP [41]; furthermore, AIM2 expression was increased in patients who developed moderate or severe AP. In a very recent study, a rise in free ASC and IL18 was found in parallel with increased AP severity, suggesting a clear correlation between inflammasome activation and the progression of systemic complications in patients suffering from this disease [42]. In this elegant study, the authors propose a new model to understand the progression of inflammation associated with AP in which both SIRS and compensatory anti-inflammatory response syndrome (CARS) phases are initiated early during AP and progress in parallel. This proposal challenges

the previously assumed sequential activation of SIRS and CARS. Interestingly, both pathways are regulated by the NLRP3 inflammasome-derived IL18. This cytokine plays a pivotal role by inducing a pro-inflammatory response of the innate immune system and acting as a Th2-cell mediator for the adaptive immune system.

3.1. NLRP3 inflammasome activation in acute pancreatitis

3.1.1. Extracellular DAMPs

We have emphasized that, differently from other inflammasomes which only respond to few specific PAMPs, NLRP3 Inflammasome is activated by a wide variety of stimuli. Among them, DAMPs have attracted increased interest because of their impact in the pathogenesis of many human diseases. Apart from immune cells, several types of non-immune cells, such as epithelial cells, endothelial cells and fibroblasts, can be activated by DAMPs.

HMGB1 is s nuclear molecule constitutively expressed in nearly all cells. Its presence seems indispensable for life since HMGB1-deficient animal die shortly after birth [43]. As a nuclear protein, HMGB1 acts as a DNA chaperone and facilitates the binding of transcription factors to DNA. Under a variety of stressful situations, however, HMGB1 translocate to the cytosol where it sustains autophagy, and then it is released into the extracellular space. Outside the cell, HMGB1 may function as a DAMP with the ability to trigger inflammatory mediators [44].

Serum levels of HMGB1 are elevated in several inflammatory diseases, including sepsis, mechanical trauma, acute myocardial infarction, acute respiratory distress syndrome, hepatic injury, rheumatoid arthritis and stroke [45]. During AP, damaged pancreatic acinar cells release different intracellular contents, including DAMPs, which in turn promote NLRP3 inflammasome activation and trigger the inflammatory response. The circulating HMGB1 levels in AP are significantly increased and positively correlate with the severity of the disease both in humans and in experimental animal models [46-49]. As well, the use of antibodies against HMGB1, the pharmacological blockade or delayed therapeutic delivery confers protection against injury in experimental AP [50-54].

Extracellular HMGB1 signaling induces and enhances sterile inflammatory responses through TLR4 and TLR9 [55]. TLR4 is a well-known LPS-recognizing receptor, but responds to several DAMPs as well. In this sense, HMGB1 activates the TLR4-mediated NF-κB signaling pathway to induce pancreatic injury in AP while this pancreatic injury is significantly reduced in TLR4-deficient mice [56]. Extracellular HMBG1 it is also known to complex with nucleic acid DAMPs released from necrotic cells and promote TLR9 recognition [57]. A role for TLR9 as sensor of DAMPs in AP has been established by Hoque et al. since TLR9 inhibition could decrease both pancreatic IL1β expression and lung inflammation in experimental AP [12]. The potential mechanism responsible for these phenomena is that the increased release of mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) during pancreatic injury activates TLR9 as well as NLRP3 inflammasome pathways. Conversely, a more recent study demonstrated that HMGB1 is involved in the activation of another inflammasome, the AIM2 inflammasome, mediated by the receptor RAGE [14]. Thus, mice lacking RAGE did not develop an inflammatory response after AP induced by L-arginine or cerulein.

Heat shock proteins (HSP) are highly conserved proteins found in all prokaryotes and eukaryotes which intracellular levels markedly increase in front of a wide variety of stressful and biological alterations [58, 59]. Heat shock protein 70 (HSP70) has been well characterized as an intracellular molecular chaperone although, in the recent years, there has been a rise in its role as a DAMP in the extracellular environment [60-61]. For instance, extracellular HSP70 (eHSP70) has been found to modulate the immune response via the MyD88/IRAK/NF-κB signal transduction pathway through TLRs and CD14 interaction [61-62].

The expression of HSP70 is upregulated in experimental AP and contributes in heat stress induced protection [63]. Further evidence for the functional significance of HSP70 in the

modulation of AP damage came from a clinical study that revealed that the HSP70.2 gene polymorphism expression was linked to the severity of pancreatitis [64]. To date, a role for eHSP70 in AP has been reported in one study where the administration of recombinant HSP70 in mice aggravated cerulein-induced AP in a TLR4-dependent manner [65]. As well, a recent work from Somensi et al. suggested that eHSP70 is able to induce NF-κB gene activation through RAGE ligation and ERK signaling pathway in a lung carcinoma cell line [66]. In this context, it would be interesting to evaluate if eHSP70 could also modulate AP-associated immune response via RAGE interaction.

Although ATP is well recognized as a source of high energy phosphate bonds to support cellular metabolism, once is released from cells following cellular damage, it acts as a DAMP signal. Cellular necrosis and apoptosis trigger the release of ATP and other nucleotides into the extracellular space. These can prompt pro-inflammatory immune responses via cell-surface P2X7 purinergic receptor [67, 68]. In a model of cerulein-induced AP in mice, extracellular ATP binds to P2X7, one of the most potent activators of the NLRP3 inflammasome, and results in NLRP3 assembly, caspase-1 activation and IL1β secretion [12]. In addition, the neutralization or blockage of P2X7 limited systemic injury but did not ameliorate the local pancreatic injury in different experimental models of AP of varying severity [69].

3.1.2. Bacterial translocation

Infection and bacterial colonization of the inflamed pancreas occurs in severe forms of AP being one of the most feared complications of the disease [70]. Bacterial translocation in AP is supported by studies showing that antibiotic treatment reduces or prevents pancreatic inflammation, infection, and mortality in various experimental pancreatitis models [13, 71-73] and limiting gram-negative colonization of the digestive tract significantly reduce mortality in humans with severe AP [74, 75].

TLR4 activation is one of the mechanisms by which bacterial translocation may account for the development of severe experimental AP [9]. As in the case of TLR4, commensal organisms as well as pathogens can induce NOD1 signaling. In this sense, the simultaneous administration of low doses of cerulein with a NOD1 ligand (neither of which is able to induce pancreatitis on its own) in mice demonstrated that NOD1 signaling is essential to the development of pancreatitis [13]. Moreover, NOD1-deficient mice were almost completely resistant to cerulein-induced AP.

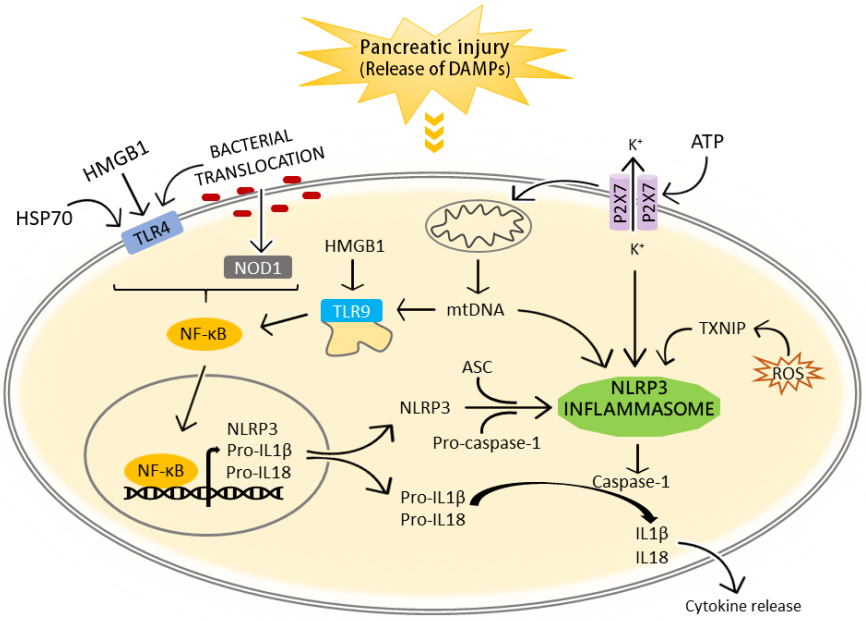

Figure 1. **Schematic diagram illustrating the mechanisms of NLRP3 inflammasome activation during acute pancreatitis.** DAMPs and gut bacteria have been recognized for their crucial role in

the initial onset of pancreatic inflammation. Prototypical DAMPs derived from pancreatic injured cells include the HMGB1, HSP70 and purine metabolites, such as ATP. HMGB1, HSP70 and the translocation of intestinal bacteria can act through TLR4 in acute pancreatitis stimulating the NF-κB activation, and further upregulation of the mRNA and protein expression of NLRP3, pro-IL1β and pro-IL18. As well, the stimulation of intracellular NOD1 by translocated bacteria is an indispensable element to sustain the inflammatory process in the pancreas. Extracellular ATP, released by damaged cells, interacts with P2X7 inducing mitochondrial dysfunction and intracellular K^+-depletion which results in NLRP3 assembly, caspase-1 activation, maturation of pro-IL1β and pro-IL18 and IL1β and IL18 secretion. Some intracellular damage-associated events have also been suggested to initiate NLRP3 inflammasome assembly in acute pancreatitis: mitochondrial DNA directly binds to NLRP3, and ROS production detaches TXNIP from thioredoxin and enables NLRP3 activation. Additionally, TLR9 senses intracellular HMGB1 and mtDNA with subsequent activation of NF-κB. LPS, lipopolysaccharide; HSP70, heat shock protein 70; HMGB1, high mobility group box 1; TLR4, toll like receptor 4 ; TLR9, toll like receptor 9; ROS, reactive oxygen species; ATP, adenosine triphosphate; NLRP3, NLR pyrin domain containing protein 3; ASC, caspase recruitment domain; NOD1, nucleotide-binding oligomerization domain 1; NF-kB, nuclear factor-kappa B; mtDNA, mitochondrial DNA.

3.2. The IL1 family of cytokines as effectors of the NLRP3 inflammasome: their role in acute pancreatitis

The IL1 superfamily of cytokines are important regulators of innate and adaptive immunity, playing key roles in the host defense against infection, inflammation, injury, and stress. IL1α and IL1β were the founding members of this family of cytokines which comprise eleven members, the others being IL18, IL33, IL36α, β and γ, IL1 receptor (IL1R) agonist, IL36Ra, IL37, and IL38 [76]. This section highlights recent advances in the understanding of the molecular and cellular mechanisms of AP associated with NLRP3 inflammasome-regulated IL1 cytokines, focusing particularly on IL1, IL33, and IL18.

3.2.1. IL1β

Among all the IL1 cytokines, IL1β is the one that is most active in mediating inflammation in processes of sterile necrosis, an important event in AP [77]. Indeed, the discovery of the inflammasomes was an essential step in improving our understanding of the molecular mechanisms of IL1β-mediated inflammation, since this multi-protein complex plays a critical role in the regulation of IL1β maturation.

It is widely recognized that the development of AP depends on pro-inflammatory cytokine responses secreted by leukocytes accumulated in the inflamed pancreas [78]. TNFα and IL1β are considered primary cytokines mediating early-phase inflammation and propagation to extra pancreatic tissues during AP. As mentioned, there is significant evidence that inflammasomes and IL1β maturation are required for the induction of inflammation in AP [79]. IL1β is transcribed by monocytes, macrophages, and dendritic cells following TLR activation by PAMPs or by the cytokines TNFα, IL18, IL1α or IL1β itself. In fact, IL1β self-induction is a part of the mechanism of autoinflammation [80]. Thus, it is important to stress that activation of IL1β can also occur via alternative mechanisms independently of the inflammasomes. For instance, neutrophil-derived serine proteases and pathogen-released enzymes can also process and activate IL1β, and these processes have important effects during inflammation and infection [81].

3.2.2. IL18

IL18 is best known for its capacity to induce Interferon γ (IFNγ). It is expressed by macrophages, epithelial cells and dendritic cells and is stored in the cytoplasm [82]. As occurs with IL1β, the NLRP3 inflammasome-caspase-1-dependent mechanism of IL18 maturation and release is the most common, although some caspase-1-independent mechanisms of cleavage have also been reported. IL18 functions by ligation of IL18 receptors (IL18R) α and β, and the complex

recruits MyD88. Then, MyD88-induced events result in the activation of NF-κB and mitogen-activated protein kinase (MAPK) via association with the signal adaptors IL1R-associated kinase (IRAK) 1-4 and tumor necrosis factor receptor-activated factor 6 (TRAF6) respectively [83].

In humans, serum levels of IL18 have been widely associated with the pathogenesis of AP and are known to correlate with disease severity [6, 84-86]. Additionally, induced IL18 levels have been reported in pancreas and lungs of rats with AP [87, 88]. These findings contrast with those of study showing that genetic deficiency of IL18 in mice resulted in significantly exacerbated pancreatic injury in a model of cerulein-induced AP [89]. The controversy could be due to differences in the model and in the severity of the disease. Notably, IL18 plus IL12 administration is a well-characterized model of experimental pancreatitis in obese mice, emphasizing the importance of IL18 in the pathophysiology of the disease [90].

The involvement of inflammasome-derived IL18 in AP was first indicated in a 2007 study in which the therapeutic effects of caspase-1 inhibitors in acute lung injury associated with AP were related with the inhibition of IL1β and IL18 [88]. Sendler and co-workers have completed the picture in a very recent study which suggests a clear correlation of inflammasome activation and AP severity [42]. IL18 deficiency in mice resulted in a complete absence of Th2 response and a reduction of Th1 after the onset of AP. These findings underline the importance of IL18 for T-cell activation during AP and challenges the notion of the sequential activation of SIRS and CARS, proposing that both responses occur in parallel during severe AP.

3.2.3. IL33

IL33, the latest addition to the IL1 superfamily, was discovered over a decade ago and is now clearly defined as a key component of innate and adaptive immune responses. IL33 is constitutively expressed in epithelial and endothelial cells, and following translation is stored as a biologically active molecule in the nucleus where it binds to chromatin [91]. When cells undergo necrosis or are stressed, nuclear IL33 is immediately available to act as an early signal of damage. Unlike other members of the IL1 family, IL33 does not require processing through an inflammasome in order to achieve its biological activity and, in fact, it is inactivated by caspase cleavage [92]. However, cleavage by neutrophil elastase and cathepsin G proteases, which are found in the microenvironment during inflammation, can increase its potency [93]. Upon release of IL33 by DAMPs and PAMPs, IL33 specifically binds to the ST2 receptor in target cells and undergoes conformational changes resulting in the recruitment of IL1 receptor accessory protein (IL1RAcP) forming a ternary complex. In most cell types, and similar to IL18, IL33 signaling activates the MyD88/IRAK/TRAF6 axis resulting in the activation of downstream NF-κB and MAPK signaling pathways [94].

Although several lines of evidence demonstrate a role for IL33 in fibrogenesis during chronic pancreatitis [95], investigations into the potential function of IL33 in the pathogenesis of AP are limited. As in IL1β and IL18, serum levels of this cytokine are increased in patients at the early stage of AP and correlate with AP severity. The same findings have been reported using experimental animal models of AP [96-98]. Thus, in a bile duct ligation model of AP, exogenous IL33 administration exacerbated pancreatic inflammation and mast cell degranulation [98]. Similarly, in experimental sodium taurocholate-induced AP, IL33/ST2 signaling was found to be a major mediator of the disease through TNFα [96]. Conversely, data from other experimental studies in IL33 receptor ST2 deficiency suggested a protective role of IL33 in AP [99-100]. Although the effects of IL33 have been shown to be either pro- or anti-inflammatory depending on the disease and the model, many questions about its potential dual effect in AP remain to be resolved. Further additional studies are clearly needed on this issue and also on the role of inflammasome in IL33 processing and release during AP, which is not currently supported in the literature.

4. Inhibitors of the NLRP3 inflammasome

The association of the NLRP3 inflammasome with a myriad of human diseases has encouraged researchers to search for effective inhibitors of this multi-protein complex. To date, a wide variety of NLPR3 inflammasome inhibitors have been studied in experimental models of human diseases, but clinical application of this knowledge remains limited.

4.1. The clinical application of NLRP3 inflammasome inhibitors: the IL1 antagonists

The currently available clinical treatment for NLRP3-related diseases involve the agents that target IL1β, including the recombinant IL1 receptor antagonist anakinra, the soluble decoy IL1β receptor rilonacept, and the neutralizing IL1β antibody canakinumab. The effectiveness of these inhibitors in AP has been demonstrated in the case of anakinra which significantly decreased cerulein-related pancreatic tissue injury and pancreatic apoptosis in rats [101].

Anakinra is a recombinant form of the IL1R antagonist approved for the treatment of rheumatoid arthritis in 2001. However, its clinical indications were extended, in 2003, to other conditions such as cryopyrin-associated periodic syndrome (CAPS), a group of rare inherited autoinflammatory diseases generally caused by autosomal-dominant mutations in the NLRP3 gene [102]. Because of the safety and rapid onset of action, IL1 inhibition with anakinra occupies an important position in IL1 therapeutics [103]. The development of Rilonacept was later introduced in 2008. Rilonacept is a fusion protein that incorporates the extracellular domains of the IL1R components required for IL1 signaling and the Fc portion of immunoglobulin G [104]. As anakinra, it was indicated for the treatment of CAPS caused by dysregulated IL1 production.

The third therapeutic option, canakinumab, is a humanized monoclonal antibody against IL1β which does not cross react with other members of the IL1 family [105]. Canakinumab was first authorized by the FDA in 2009 for the treatment of CAPS and active systemic juvenile idiopathic arthritis [106]. In 2016, it received approval as first-line treatment for TNF receptor associated periodic syndrome, familial Mediterranean fever and mevalonate kinase deficiency which are also linked to the activation of the pyrin inflammasome.

All agents reduce or even resolve clinical symptoms, decrease the biochemical activity markers associated and improve quality of life in CAPS patients [107]. In addition, they are currently applied in other inflammatory disorders and as antitumor drugs – as one might expect, given that inflammation is a hallmark of early tumorigenic events.

4.2. NLRP3 inflammasome inhibitors in acute pancreatitis

The evidence of NLRP3 inhibition in AP comes from experimental data since no clinical studies have yet explored the benefits of the inhibition of IL1β or other inflammasome components. Here, we present an overview of the inhibitors of the inflammasome pathway in experimental AP reported to date (Table 1).

Table 1. Inhibitory compounds of NLRP3 inflammasome activation in acute pancreatitis.

Compound	**Type**	**Target**	**References**
Glyburide	Sulphonylurea	NLRP3 (ATP-sensitive K^+ channels)	[109]
MCC950	Diarylsulphonylurea	NLRP3 (ASC oligomerization)	[42]
Emodin	Anthraquinone	Nrf2/ NF-κB/ NLRP3/ P2X7	[116, 117]

Dansensu	Phenolic acid	NF-κB/ STAT3/ NLRP3	[121]
Fraxinellone	Limonoid	NLRP3 (CARD, caspase-1, IL1β, IL18)	[123]
Withaferin A	Alkaloid	NF-κB/ NLRP3	[127]
Rutin	Flavonoid	NLRP3 (ASC, caspase-1)	[129]
Sulforaphane	Isothiocyanate	Nrf2/ NLRP3	[131]
Cordycepin	Adenosine analogue	NF-κB/NLRP3	[137]
Indomethacin	COX-2 inhibitor	NLRP3 (ASC, IL1β)	[143]
Iguratimod	COX-2 inhibitor	NF-κB/ NLRP3	[144]
Apocynin	NOX inhibitor	NF-κB/ NLRP3	[145]
INT-777	Bile acid receptor agonist	ROS/ NLRP3	[148]

NLRP3, NLR pyrin domain-containing protein 3; ASC, caspase recruitment domain; CARD, caspase activation and recruitment domain; NF-κB, nuclear factor kappa B; Nrf2, nuclear factor erythrocyte-2 associated factor-2; COX2, cyclo-oxygenase-2; NOX, NADPH oxidase; ROS, reactive oxygen species.

4.2.1. Sulphonylureas drugs

Glyburide, also known as glibenclamide, is an NLRP3 inhibitor of the class of sulphonylureas drugs widely used for the treatment of type 2 diabetes. Glyburide is not a direct inhibitor of the NLRP3 inflammasome since it interferes with signaling events upstream of the NLRP3 assembly. Specifically, it blocks ATP-sensitive potassium (K^+) channels on the pancreatic β cell membrane preventing a cellular efflux of K^+, a known danger signal for inflammasome activation [108]. The administration of glyburide in a mouse model of cerulein AP significantly reduced serum levels of IL6, lipase, and amylase and lowered the IL1β release in cultured peritoneal cells treated with LPS [109].

MCC950 is a diarylsulfonylurea-containing compound considered one of the most potent and selective inhibitors of the NLRP3 inflammasome [110]. MCC950 functions by blocking both canonical and non-canonical NLRP3 inflammasome activation by abrogating ASC oligomerization instead of K^+ efflux, Ca^{2+} flux or NLRP3-ASC interactions. Notably, MCC950 had no inhibitory effect on the activation of other inflammasomes complexes such as AIM2, NLRC4, or NLRP1 [111]. MCC950 has previously been involved as a potential treatment in an increasing number of inflammatory diseases, including atherosclerosis, nonalcoholic fatty liver disease, Alzheimer's disease and myocardial infarction [112]. The study by Sendler et al. is the only one to have assessed the use of MCC950 in experimental AP [42]. The therapeutic administration of this small molecule lowered IL18 levels and significantly reduced disease severity and systemic injury.

4.2.2. Natural products from plants and fungi

A large number of compounds of plant origin, including polyphenols, terpenes, alkaloids, glycosides, quinones and flavonoids, have been explored for the treatment of a variety of pancreatic disorders including diabetes and pancreatitis. Here, we describe a large number of NLRP3-blocking compounds that may be able to deal with the inflammatory challenge of AP.

Emodin is an anthraquinone derivative (1,3,8-trihydroxy-6-methyl-anthraquinone) that can be isolated from rhubarb, buckthorn, and Japanese knotweed and has proven antitumoral, anti-inflammatory and immunomodulatory effects [113]. Previous experimental studies have confirmed that emodin alleviates lung injury associated to AP [114, 115], but the involvement of NLRP3 inflammasome on the protective effects of emodin have been recently investigated. Zhang et al. found that this natural compound delayed the progression of AP through P2X7/NLRP3 signaling pathway inhibition, thereby improving the associated systemic inflammation [116]. Moreover, in the same experimental model of severe AP, emodin exerted its protective effect by inhibiting NLRP3 inflammasome activation via Nuclear factor erythrocyte-2 associated factor 2 (Nrf2) pathway [117].

Danshensu is a water-soluble ingredient of danshen, an active constituent of Salvia miltiorrhiza generally known to exert cardioprotective function in myocardial ischemic injury [118, 119]. Danshensu has been reported to attenuate LPS-induced inflammatory responses and exhibit anti-oxidative effects in cultured macrophages [120]. In a mouse model of cerulein-induced AP, Danshensu directly suppressed the pancreatic activation of the NLRP3 inflammasome and NF-kB and STAT3 signaling pathways [121].

Fraxinellone is a natural component isolated from the plant Dictamnus dasycarpus and has been reported to exert anti-inflammatory activity through the suppression of NF-κB in macrophages [122]. Its inhibitory effect on the activation of the NLRP3 inflammasome cascade has been observed in three different experimental models of AP [123]. The treatment of mice with fraxinellone significantly attenuated the severity of AP by inhibiting the pancreatic activation of multiple inflammasome molecules such as NLRP3, PY-CARD, caspase-1, IL18, and IL1β. Additionally, fraxinellone inhibited the infiltration of macrophages and neutrophils into the pancreas through the suppression of inflammasome signaling.

Withaferin A is an alkaloid isolated from ginseng (Withania somnifera), with pharmacological properties ranging from antitumoral to anti-diabetic and anti-inflammatory effects [124-126]. Withaferin A effectively suppresses the sustained activation of endoplasmic reticulum stress and inhibits NLRP3 inflammasome activation during cerulein-induced AP in mice [127].

Rutin is a flavonoid found in certain vegetables and fruits. In an L-arginine-induced AP model in mice, rutin decreased the pancreatic injury but also furthered catalase and superoxide dismutase antioxidant activities [128]. Rutin treatment in rats with cerulein-induced AP and fed on ethanol reduced pancreatic inflammation and modulated the NLRP3 inflammasome by attenuating the expression of ASC, caspase-1 and IL1β [129].

Sulforaphane, a naturally occurring isothiocyanate, is mainly present in vegetables and plays an important role in the maintenance of cellular redox balance [130]. Sulforaphane is the most widely studied member of isothiocyanates, in both in vivo and in vitro models of different diseases, (mainly diabetes and cancer). In a mouse model of cerulein-induced AP, sulforaphane protected from pancreatic damage by exerting antioxidant and anti-inflammatory effects through the Nrf2 pathway and the NLRP3 inflammasome, respectively [131]. Nevertheless, sulforaphane is not specific to the NLRP3 inflammasome since it has shown inhibitory activity for the AIM2 or NLRC4 inflammasome and NF-κB activation [132].

Cordycepin, a natural product derived from the medicinal fungus Cordyceps militaris, possesses various pharmacological properties, including tumor inhibition, suppression of inflammation and immunomodulation [133-135]. In a macrophage cell line, cordycepin notably inhibited LPS-induced activation of the NLRP3 inflammasome and the ERK1/2 signaling

pathway [136]. In cerulein- induced AP in mice, cordycepin protected against pancreatic inflammatory processes by directly inhibiting NF-κB and NLRP3 inflammasome activation via AMPK [137].

4.2.3. Non-steroidal anti-inflammatory drugs and other antioxidants

The detrimental effects of oxidative stress and excessive inflammatory cascade reaction in the pathogenesis of AP have been extensively investigated [138]. Recent studies have suggested that cyclo-oxygenase-2 (COX-2) inhibitors are potent modulators of the inflammatory response and NLRP3 inflammasome activation [139]. Thus, the use of non-steroidal anti-inflammatory drugs (NSAIDs) appears a viable option for inflammasome inhibition.

Indomethacin, one of the most common NSAIDs and COX-2 inhibitors, has an evident anti-inflammatory effect and is widely used in rheumatoid arthritis and other diseases [140, 141]. Clinical experience with NSAIDS for treating AP has centered mostly on the prevention of post-endoscopic retrograde cholangiopancreatography pancreatitis and has shown that patients who received indomethacin were less likely to develop this form of pancreatitis [142]. In an experimental AP model induced by cerulein combined with LPS in mice, indomethacin has recently been found to protect against pancreatic damage through inhibition of the NLRP3 inflammasome components [143].

Another selective COX-2 inhibitor, Iguratimod, has been reported to play an anti-inflammatory role against cerulein plus LPS-induced AP in mice through inhibition of the NF-κB signaling pathway and NLRP3 inflammasome activity [144].

The use of apocynin, an inhibitor of NADPH oxidase, mitigated AP-induced acute lung injury as well as pancreatic injury [145]. The mechanism underlying these protective effects includes the inhibition of the activation of NLRP3 inflammasome associated proteins NLRP3, pro-caspase-1 and IL1β.

4.2.4. Bile acids

In addition to their role as metabolic regulators, bile acids are involved in the modulation of inflammatory responses and the maintenance of immune homeostasis [146]. INT-777, a bile acid receptor agonist, has a protective effect against many inflammatory diseases [147]. Recent studies have found that INT-777 plays a regulatory role in the ROS/NLRP3 signaling. In the context of AP, INT-777 effectively alleviated the inflammation and pancreatic acinar cell injury by blockading the ROS/NLRP3 pathway [148].

4.2.5. Antibiotics

Dysfunctional intestinal homeostasis in the early stage of AP has been shown to enhance intestinal bacterial translocation, subsequently exacerbating AP. A very recent publication by Jia et al. revealed the beneficial effect of prophylactic antibiotic treatment on AP development [149]. In a mouse model of cerulein-induced AP, combinatorial antibiotic treatment reduced pancreatic inflammation and decreased gut bacteria translocation to the pancreas through colonic TLR4/NLRP3 inflammasome inhibition. This procedure improved pancreatic inflammation and delayed the progression of AP into a systemic inflammatory response.

5. Concluding remarks and perspectives

In 1925, Lord Moynihan's description of AP reflected its dramatic nature: "the most terrible of all calamities that occurs in connection with the abdominal viscera. The suddenness of its onset, the illimitable agony which accompanies it, and the mortality attendant upon it, all renders it the most formidable of catastrophes". Almost a century later, AP continues to be a devastating gastrointestinal disease with high morbidity and mortality, relatively little scope for action, and an often unpredictable outcome. Fundamental insights into the pathophysiology of AP have

notably increased our knowledge of this disease in recent years, but there are still no effective treatment options available. The definition of early disease management strategies would help to mitigate the associated systemic complications, which are the main cause of death in these patients.

No studies have explored the clinical benefits of NLRP3 inhibition in patients with AP. We do know, however, that the levels of NLRP3 and AIM2 inflammasomes are increased in the early course of AP [41]; therefore, clinical studies that investigate the ability of inflammasome activation to influence AP development and progression are now urgently needed.

A role for IL1β in the inflammasome pathway has been clearly identified in a range of sterile inflammatory human diseases. IL1β is regarded as the major driver of inflammasome-mediated inflammation and so the currently available clinical treatment for NLRP3-related diseases use agents that target this cytokine. The effectiveness of IL1R blockade firmly establishes the value of using additional therapeutics that target the IL1 family of cytokines. Thus, it would be interesting to determine whether addition of IL1 antagonists other than IL1β might prove beneficial in the treatment of AP. In addition, as some cytokines have the ability to compensate the absence of others, combining IL1β and IL18 blockades, for example, may be of interest in order to enhance efficiency.

Inflammasome signaling can also affect biological processes other than inflammatory cytokine production. Among the upstream mechanisms that trigger NLRP3 activation, HMGB1 is of particular interest. HMGB1-targeted therapy has proved highly successful in numerous preclinical experimental models of inflammation but no clinical trials with HMGB1-specific agents have yet been conducted. In severe AP, circulating HMGB1 levels broadly reflect the severity of the disease. In addition, extracellular HMGB1 induces further local pancreatic and systemic injury; therefore, targeting HMGB1 could be an interesting therapeutic approach for AP and other sterile inflammatory conditions.

Moreover, the post-translational modifications of NLRP3 have emerged as important mechanisms in the control of its assembly. Research on the identification of the unknown factors controlling these modifications may help to identify novel pharmacological strategies for inhibiting inflammasome activation.

Collectively, given the vital importance of the inflammatory response in AP, further in-depth studies of the inflammasome are now needed with a view to developing treatments for this disease. The fact that the NLRP3 inflammasome relies on numerous regulatory mechanisms may open up a whole range of therapeutic possibilities.

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Abbreviations

AIM	Absence in melanoma 2
ALRs	Absence in melanoma 2-like receptors
AP	Acute pancreatitis
ASC	Caspase recruitment domain
CAPS	Cryopyrin-associated periodic syndrome
CARD	Caspase activation and recruitment domain
CARS	Compensatory anti-inflammatory response syndrome
CLRs	C-type lectin receptors

COX-2	Cyclo-oxygenase-2
DAMPs	Damage-Associated Molecular Patterns
GSDM-D	Gasdermin-D
HMGB1	High mobility group box 1
IL	Interleukin
IL1R	Interleukin 1 receptor
IRAK	IL1R-associated kinase
IRF	Interferon regulatory factor
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MyD88	Myeloid differentiation primary response 88
NATCH	Nucleotide-binding and oligomerization domain
NF-κB	Nuclear factor-kappa B
NLRP3	NLR pyrin domain containing protein 3
NOD1	NLR nucleotide-binding oligomerization domain-containing protein 1
Nrf2	Nuclear factor erythrocyte-2 associated factor 2
NSAIDS	Non-steroidal anti-inflammatory drugs
PAMPs	Pathogen-Associated Molecular Patterns
PRR	Pattern recognition receptors
PYD	Pyrin domain
RAGE	Receptor for advanced glycation end-products
RLRs	Retinoic acid-inducible gene (RIG)-I-like receptors
ROS	Reactive oxygen species
SIRS	Systemic inflammatory response syndrome
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TRAF	Tumor necrosis factor receptor-activated factor
TRIF	TIR-domain-containing adaptor molecule inducing interferon-beta

References

1. Dumnicka, P.; Maduzia, D.; Ceranowicz, P.; Olszanecki, R.; Drożdż, R.; Kuśnierz-Cabala, B., The Interplay between Inflammation, Coagulation and Endothelial Injury in the Early Phase of Acute Pancreatitis: Clinical Implications. *International journal of molecular sciences* **2017,** 18, (2).
2. Banks, P. A.; Bollen, T. L.; Dervenis, C.; Gooszen, H. G.; Johnson, C. D.; Sarr, M. G.; Tsiotos, G. G.; Vege, S. S., Classification of acute pancreatitis--2012: revision of the Atlanta classification and definitions by international consensus. *Gut* **2013,** 62, (1), 102-11.
3. Zhou, M. T.; Chen, C. S.; Chen, B. C.; Zhang, Q. Y.; Andersson, R., Acute lung injury and ARDS in acute pancreatitis: mechanisms and potential intervention. *World journal of gastroenterology* **2010,** 16, (17), 2094-9.
4. Freeman, M. L.; DiSario, J. A.; Nelson, D. B.; Fennerty, M. B.; Lee, J. G.; Bjorkman, D. J.; Overby, C. S.; Aas, J.; Ryan, M. E.; Bochna, G. S.; Shaw, M. J.; Snady, H. W.; Erickson, R. V.; Moore, J. P.; Roel, J. P., Risk factors for post-ERCP pancreatitis: a prospective, multicenter study. *Gastrointestinal endoscopy* **2001,** 54, (4), 425-34.
5. Yang, Z. W.; Meng, X. X.; Xu, P., Central role of neutrophil in the pathogenesis of severe acute pancreatitis. *Journal of cellular and molecular medicine* **2015,** 19, (11), 2513-20.
6. Janiak, A.; Leśniowski, B.; Jasińska, A.; Pietruczuk, M.; Małecka-Panas, E., Interleukin 18 as an early marker or prognostic factor in acute pancreatitis. *Przeglad gastroenterologiczny* **2015,** 10, (4), 203-7.
7. Takeuchi, O.; Akira, S., Pattern recognition receptors and inflammation. *Cell* **2010,** 140, (6), 805-20.
8. Medzhitov, R., Toll-like receptors and innate immunity. *Nat Rev Immunol* **2001,** 1, (2), 135-45.
9. Sharif, R.; Dawra, R.; Wasiluk, K.; Phillips, P.; Dudeja, V.; Kurt-Jones, E.; Finberg, R.; Saluja, A., Impact of toll-like receptor 4 on the severity of acute pancreatitis and pancreatitis-associated lung injury in mice. *Gut* **2009,** 58, (6), 813-9.

10. Pastor, C. M.; Pugin, J.; Kwak, B.; Chanson, M.; Mach, F.; Hadengue, A.; Frossard, J. L., Role of Toll-like receptor 4 on pancreatic and pulmonary injury in a mice model of acute pancreatitis associated with endotoxemia. *Critical care medicine* **2004,** 32, (8), 1759-63.
11. Sawa, H.; Ueda, T.; Takeyama, Y.; Yasuda, T.; Shinzeki, M.; Nakajima, T.; Kuroda, Y., Role of toll-like receptor 4 in the pathophysiology of severe acute pancreatitis in mice. *Surgery today* **2007,** 37, (10), 867-73.
12. Hoque, R.; Sohail, M.; Malik, A.; Sarwar, S.; Luo, Y.; Shah, A.; Barrat, F.; Flavell, R.; Gorelick, F.; Husain, S.; Mehal, W., TLR9 and the NLRP3 inflammasome link acinar cell death with inflammation in acute pancreatitis. *Gastroenterology* **2011,** 141, (1), 358-69.
13. Tsuji, Y.; Watanabe, T.; Kudo, M.; Arai, H.; Strober, W.; Chiba, T., Sensing of commensal organisms by the intracellular sensor NOD1 mediates experimental pancreatitis. *Immunity* **2012,** 37, (2), 326-38.
14. Kang, R.; Chen, R.; Xie, M.; Cao, L.; Lotze, M. T.; Tang, D.; Zeh, H. J., 3rd, The Receptor for Advanced Glycation End Products Activates the AIM2 Inflammasome in Acute Pancreatitis. *J Immunol* **2016,** 196, (10), 4331-7.
15. Martinon, F.; Burns, K.; Tschopp, J., The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Molecular cell* **2002,** 10, (2), 417-26.
16. Guo, H.; Callaway, J. B.; Ting, J. P., Inflammasomes: mechanism of action, role in disease, and therapeutics. *Nature medicine* **2015,** 21, (7), 677-87.
17. Kayagaki, N.; Warming, S.; Lamkanfi, M.; Vande Walle, L.; Louie, S.; Dong, J.; Newton, K.; Qu, Y.; Liu, J.; Heldens, S.; Zhang, J.; Lee, W. P.; Roose-Girma, M.; Dixit, V. M., Non-canonical inflammasome activation targets caspase-11. *Nature* **2011,** 479, (7371), 117-21.
18. Mason, D. R.; Beck, P. L.; Muruve, D. A., Nucleotide-binding oligomerization domain-like receptors and inflammasomes in the pathogenesis of non-microbial inflammation and diseases. *Journal of innate immunity* **2012,** 4, (1), 16-30.
19. Kummer, J. A.; Broekhuizen, R.; Everett, H.; Agostini, L.; Kuijk, L.; Martinon, F.; van Bruggen, R.; Tschopp, J., Inflammasome components NALP 1 and 3 show distinct but separate expression profiles in human tissues suggesting a site-specific role in the inflammatory response. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* **2007,** 55, (5), 443-52.
20. Latz, E.; Xiao, T. S.; Stutz, A., Activation and regulation of the inflammasomes. *Nat Rev Immunol* **2013,** 13, (6), 397-411.
21. Kelley, N.; Jeltema, D.; Duan, Y.; He, Y., The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation. *International journal of molecular sciences* **2019,** 20, (13).
22. Lopez-Castejon, G.; Luheshi, N. M.; Compan, V.; High, S.; Whitehead, R. C.; Flitsch, S.; Kirov, A.; Prudovsky, I.; Swanton, E.; Brough, D., Deubiquitinases regulate the activity of caspase-1 and interleukin-1β secretion via assembly of the inflammasome. *The Journal of biological chemistry* **2013,** 288, (4), 2721-33.
23. Song, N.; Liu, Z. S.; Xue, W.; Bai, Z. F.; Wang, Q. Y.; Dai, J.; Liu, X.; Huang, Y. J.; Cai, H.; Zhan, X. Y.; Han, Q. Y.; Wang, H.; Chen, Y.; Li, H. Y.; Li, A. L.; Zhang, X. M.; Zhou, T.; Li, T., NLRP3 Phosphorylation Is an Essential Priming Event for Inflammasome Activation. *Molecular cell* **2017,** 68, (1), 185-197.e6.
24. Barry, R.; John, S. W.; Liccardi, G.; Tenev, T.; Jaco, I.; Chen, C. H.; Choi, J.; Kasperkiewicz, P.; Fernandes-Alnemri, T.; Alnemri, E.; Drag, M.; Chen, Y.; Meier, P., SUMO-mediated regulation of NLRP3 modulates inflammasome activity. *Nature communications* **2018,** 9, (1), 3001.
25. Bose, S.; Segovia, J. A.; Somarajan, S. R.; Chang, T. H.; Kannan, T. R.; Baseman, J. B., ADP-ribosylation of NLRP3 by Mycoplasma pneumoniae CARDS toxin regulates inflammasome activity. *mBio* **2014,** 5, (6).
26. He, Y.; Hara, H.; Núñez, G., Mechanism and Regulation of NLRP3 Inflammasome Activation. *Trends in biochemical sciences* **2016,** 41, (12), 1012-1021.
27. Lu, B.; Wang, H.; Andersson, U.; Tracey, K. J., Regulation of HMGB1 release by inflammasomes. *Protein & cell* **2013,** 4, (3), 163-7.
28. Rathinam, V. A.; Vanaja, S. K.; Waggoner, L.; Sokolovska, A.; Becker, C.; Stuart, L. M.; Leong, J. M.; Fitzgerald, K. A., TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-negative bacteria. *Cell* **2012,** 150, (3), 606-19.
29. Pellegrini, C.; Antonioli, L.; Lopez-Castejon, G.; Blandizzi, C.; Fornai, M., Canonical and Non-Canonical Activation of NLRP3 Inflammasome at the Crossroad between Immune Tolerance and Intestinal Inflammation. *Frontiers in immunology* **2017,** 8, 36.

30. Kayagaki, N.; Stowe, I. B.; Lee, B. L.; O'Rourke, K.; Anderson, K.; Warming, S.; Cuellar, T.; Haley, B.; Roose-Girma, M.; Phung, Q. T.; Liu, P. S.; Lill, J. R.; Li, H.; Wu, J.; Kummerfeld, S.; Zhang, J.; Lee, W. P.; Snipas, S. J.; Salvesen, G. S.; Morris, L. X.; Fitzgerald, L.; Zhang, Y.; Bertram, E. M.; Goodnow, C. C.; Dixit, V. M., Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature* **2015,** 526, (7575), 666-71.
31. Gurung, P.; Anand, P. K.; Malireddi, R. K.; Vande Walle, L.; Van Opdenbosch, N.; Dillon, C. P.; Weinlich, R.; Green, D. R.; Lamkanfi, M.; Kanneganti, T. D., FADD and caspase-8 mediate priming and activation of the canonical and noncanonical Nlrp3 inflammasomes. *J Immunol* **2014,** 192, (4), 1835-46.
32. Malmstrøm, M. L.; Hansen, M. B.; Andersen, A. M.; Ersbøll, A. K.; Nielsen, O. H.; Jørgensen, L. N.; Novovic, S., Cytokines and organ failure in acute pancreatitis: inflammatory response in acute pancreatitis. *Pancreas* **2012,** 41, (2), 271-7.
33. Mayer, J.; Rau, B.; Gansauge, F.; Beger, H. G., Inflammatory mediators in human acute pancreatitis: clinical and pathophysiological implications. *Gut* **2000,** 47, (4), 546-52.
34. Fu, Q.; Zhai, Z.; Wang, Y.; Xu, L.; Jia, P.; Xia, P.; Liu, C.; Zhang, X.; Qin, T.; Zhang, H., NLRP3 Deficiency Alleviates Severe Acute Pancreatitis and Pancreatitis-Associated Lung Injury in a Mouse Model. *BioMed research international* **2018,** 2018, 1294951.
35. Hoque, R.; Farooq, A.; Ghani, A.; Gorelick, F.; Mehal, W. Z., Lactate reduces liver and pancreatic injury in Toll-like receptor- and inflammasome-mediated inflammation via GPR81-mediated suppression of innate immunity. *Gastroenterology* **2014,** 146, (7), 1763-74.
36. Wu, B. U.; Hwang, J. Q.; Gardner, T. H.; Repas, K.; Delee, R.; Yu, S.; Smith, B.; Banks, P. A.; Conwell, D. L., Lactated Ringer's solution reduces systemic inflammation compared with saline in patients with acute pancreatitis. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association* **2011,** 9, (8), 710-717.e1.
37. de-Madaria, E.; Herrera-Marante, I.; González-Camacho, V.; Bonjoch, L.; Quesada-Vázquez, N.; Almenta-Saavedra, I.; Miralles-Maciá, C.; Acevedo-Piedra, N. G.; Roger-Ibáñez, M.; Sánchez-Marin, C.; Osuna-Ligero, R.; Gracia, Á.; Llorens, P.; Zapater, P.; Singh, V. K.; Moreu-Martín, R.; Closa, D., Fluid resuscitation with lactated Ringer's solution vs normal saline in acute pancreatitis: A triple-blind, randomized, controlled trial. *United European gastroenterology journal* **2018,** 6, (1), 63-72.
38. Shen, A.; Kim, H. J.; Oh, G. S.; Lee, S. B.; Lee, S. H.; Pandit, A.; Khadka, D.; Choe, S. K.; Kwak, S. C.; Yang, S. H.; Cho, E. Y.; Kim, H. S.; Kim, H.; Park, R.; Kwak, T. H.; So, H. S., NAD(+) augmentation ameliorates acute pancreatitis through regulation of inflammasome signalling. *Scientific reports* **2017,** 7, (1), 3006.
39. Xue, J.; Habtezion, A., Carbon monoxide-based therapy ameliorates acute pancreatitis via TLR4 inhibition. *The Journal of clinical investigation* **2014,** 124, (1), 437-47.
40. Wu, X. B.; Sun, H. Y.; Luo, Z. L.; Cheng, L.; Duan, X. M.; Ren, J. D., Plasma-derived exosomes contribute to pancreatitis-associated lung injury by triggering NLRP3-dependent pyroptosis in alveolar macrophages. *Biochimica et biophysica acta. Molecular basis of disease* **2020,** 1866, (5), 165685.
41. Algaba-Chueca, F.; de-Madaria, E.; Lozano-Ruiz, B.; Martínez-Cardona, C.; Quesada-Vázquez, N.; Bachiller, V.; Tarín, F.; Such, J.; Francés, R.; Zapater, P.; González-Navajas, J. M., The expression and activation of the AIM2 inflammasome correlates with inflammation and disease severity in patients with acute pancreatitis. *Pancreatology : official journal of the International Association of Pancreatology (IAP) ... [et al.]* **2017,** 17, (3), 364-371.
42. Sendler, M.; van den Brandt, C.; Glaubitz, J.; Wilden, A.; Golchert, J.; Weiss, F. U.; Homuth, G.; De Freitas Chama, L. L.; Mishra, N.; Mahajan, U. M.; Bossaller, L.; Völker, U.; Bröker, B. M.; Mayerle, J.; Lerch, M. M., NLRP3 Inflammasome Regulates Development of Systemic Inflammatory Response and Compensatory Anti-Inflammatory Response Syndromes in Mice With Acute Pancreatitis. *Gastroenterology* **2020,** 158, (1), 253-269.e14.
43. Calogero, S.; Grassi, F.; Aguzzi, A.; Voigtländer, T.; Ferrier, P.; Ferrari, S.; Bianchi, M. E., The lack of chromosomal protein Hmg1 does not disrupt cell growth but causes lethal hypoglycaemia in newborn mice. *Nature genetics* **1999,** 22, (3), 276-80.
44. Oppenheim, J. J.; Yang, D., Alarmins: chemotactic activators of immune responses. *Current opinion in immunology* **2005,** 17, (4), 359-65.
45. Shen, X.; Li, W. Q., High-mobility group box 1 protein and its role in severe acute pancreatitis. *World journal of gastroenterology* **2015,** 21, (5), 1424-35.

46. Yasuda, T.; Ueda, T.; Takeyama, Y.; Shinzeki, M.; Sawa, H.; Nakajima, T.; Ajiki, T.; Fujino, Y.; Suzuki, Y.; Kuroda, Y., Significant increase of serum high-mobility group box chromosomal protein 1 levels in patients with severe acute pancreatitis. *Pancreas* **2006,** 33, (4), 359-63.
47. Kocsis, A. K.; Szabolcs, A.; Hofner, P.; Takács, T.; Farkas, G.; Boda, K.; Mándi, Y., Plasma concentrations of high-mobility group box protein 1, soluble receptor for advanced glycation end-products and circulating DNA in patients with acute pancreatitis. *Pancreatology : official journal of the International Association of Pancreatology (IAP) ... [et al.]* **2009,** 9, (4), 383-91.
48. Lindström, O.; Tukiainen, E.; Kylänpää, L.; Mentula, P.; Rouhiainen, A.; Puolakkainen, P.; Rauvala, H.; Repo, H., Circulating levels of a soluble form of receptor for advanced glycation end products and high-mobility group box chromosomal protein 1 in patients with acute pancreatitis. *Pancreas* **2009,** 38, (8), e215-20.
49. Yasuda, T.; Ueda, T.; Shinzeki, M.; Sawa, H.; Nakajima, T.; Takeyama, Y.; Kuroda, Y., Increase of high-mobility group box chromosomal protein 1 in blood and injured organs in experimental severe acute pancreatitis. *Pancreas* **2007,** 34, (4), 487-8.
50. Sawa, H.; Ueda, T.; Takeyama, Y.; Yasuda, T.; Shinzeki, M.; Nakajima, T.; Kuroda, Y., Blockade of high mobility group box-1 protein attenuates experimental severe acute pancreatitis. *World journal of gastroenterology* **2006,** 12, (47), 7666-70.
51. Luan, Z. G.; Zhang, X. J.; Yin, X. H.; Ma, X. C.; Zhang, H.; Zhang, C.; Guo, R. X., Downregulation of HMGB1 protects against the development of acute lung injury after severe acute pancreatitis. *Immunobiology* **2013,** 218, (10), 1261-70.
52. Yuan, H.; Jin, X.; Sun, J.; Li, F.; Feng, Q.; Zhang, C.; Cao, Y.; Wang, Y., Protective effect of HMGB1 a box on organ injury of acute pancreatitis in mice. *Pancreas* **2009,** 38, (2), 143-8.
53. Zhang, Z. W.; Zhang, Q. Y.; Zhou, M. T.; Liu, N. X.; Chen, T. K.; Zhu, Y. F.; Wu, L., Antioxidant inhibits HMGB1 expression and reduces pancreas injury in rats with severe acute pancreatitis. *Digestive diseases and sciences* **2010,** 55, (9), 2529-36.
54. Yang, Z. Y.; Ling, Y.; Yin, T.; Tao, J.; Xiong, J. X.; Wu, H. S.; Wang, C. Y., Delayed ethyl pyruvate therapy attenuates experimental severe acute pancreatitis via reduced serum high mobility group box 1 levels in rats. *World journal of gastroenterology* **2008,** 14, (28), 4546-50.
55. Chen, R.; Hou, W.; Zhang, Q.; Kang, R.; Fan, X. G.; Tang, D., Emerging role of high-mobility group box 1 (HMGB1) in liver diseases. *Molecular medicine (Cambridge, Mass.)* **2013,** 19, (1), 357-66.
56. Li, G.; Wu, X.; Yang, L.; He, Y.; Liu, Y.; Jin, X.; Yuan, H., TLR4-mediated NF-κB signaling pathway mediates HMGB1-induced pancreatic injury in mice with severe acute pancreatitis. *International journal of molecular medicine* **2016,** 37, (1), 99-107.
57. Yanai, H.; Ban, T.; Wang, Z.; Choi, M. K.; Kawamura, T.; Negishi, H.; Nakasato, M.; Lu, Y.; Hangai, S.; Koshiba, R.; Savitsky, D.; Ronfani, L.; Akira, S.; Bianchi, M. E.; Honda, K.; Tamura, T.; Kodama, T.; Taniguchi, T., HMGB proteins function as universal sentinels for nucleic-acid-mediated innate immune responses. *Nature* **2009,** 462, (7269), 99-103.
58. Nover, L.; Scharf, K. D., Heat stress proteins and transcription factors. *Cellular and molecular life sciences : CMLS* **1997,** 53, (1), 80-103.
59. Pilon, M.; Schekman, R., Protein translocation: how Hsp70 pulls it off. *Cell* **1999,** 97, (6), 679-82.
60. Singh-Jasuja, H.; Hilf, N.; Arnold-Schild, D.; Schild, H., The role of heat shock proteins and their receptors in the activation of the immune system. *Biological chemistry* **2001,** 382, (4), 629-36.
61. Asea, A.; Kraeft, S. K.; Kurt-Jones, E. A.; Stevenson, M. A.; Chen, L. B.; Finberg, R. W.; Koo, G. C.; Calderwood, S. K., HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nature medicine* **2000,** 6, (4), 435-42.
62. Asea, A.; Rehli, M.; Kabingu, E.; Boch, J. A.; Bare, O.; Auron, P. E.; Stevenson, M. A.; Calderwood, S. K., Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. *The Journal of biological chemistry* **2002,** 277, (17), 15028-34.
63. Rakonczay Jr, Z; Takács, T; Boros, I; Lonovics, J., Heat shock proteins and the pancreas. *J Cell Physiol* **2003,** 195, 383-91.
64. Balog, A.; Gyulai, Z.; Boros, L. G.; Farkas, G.; Takács, T.; Lonovics, J.; Mándi, Y., Polymorphism of the TNF-alpha, HSP70-2, and CD14 genes increases susceptibility to severe acute pancreatitis. *Pancreas* **2005,** 30, (2), e46-50.
65. Song, J. M.; Liu, H. X.; Li, Y.; Zeng, Y. J.; Zhou, Z. G.; Liu, H. Y.; Xu, B.; Wang, L.; Zhou, B.; Wang, R., Extracellular heat-shock protein 70 aggravates cerulein-induced pancreatitis through toll-like receptor-4 in mice. *Chinese medical journal* **2008,** 121, (15), 1420-5.

66. Somensi, N.; Brum, P. O.; de Miranda Ramos, V.; Gasparotto, J.; Zanotto-Filho, A.; Rostirolla, D. C.; da Silva Morrone, M.; Moreira, J. C. F.; Pens Gelain, D., Extracellular HSP70 Activates ERK1/2, NF-kB and Pro-Inflammatory Gene Transcription Through Binding with RAGE in A549 Human Lung Cancer Cells. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* **2017,** 42, (6), 2507-2522.
67. Idzko, M.; Ferrari, D.; Eltzschig, H. K., Nucleotide signalling during inflammation. *Nature* **2014,** 509, (7500), 310-7.
68. Elliott, M. R.; Chekeni, F. B.; Trampont, P. C.; Lazarowski, E. R.; Kadl, A.; Walk, S. F.; Park, D.; Woodson, R. I.; Ostankovich, M.; Sharma, P.; Lysiak, J. J.; Harden, T. K.; Leitinger, N.; Ravichandran, K. S., Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature* **2009,** 461, (7261), 282-6.
69. Dixit, A.; Cheema, H.; George, J.; Iyer, S.; Dudeja, V.; Dawra, R.; Saluja, A. K., Extracellular release of ATP promotes systemic inflammation during acute pancreatitis. *American journal of physiology. Gastrointestinal and liver physiology* **2019,** 317, (4), G463-g475.
70. Frossard, J. L.; Steer, M. L.; Pastor, C. M., Acute pancreatitis. *Lancet (London, England)* **2008,** 371, (9607), 143-52.
71. Mithöfer, K.; Fernández-del Castillo, C.; Ferraro, M. J.; Lewandrowski, K.; Rattner, D. W.; Warshaw, A. L., Antibiotic treatment improves survival in experimental acute necrotizing pancreatitis. *Gastroenterology* **1996,** 110, (1), 232-40.
72. Fritz, S.; Hartwig, W.; Lehmann, R.; Will-Schweiger, K.; Kommerell, M.; Hackert, T.; Schneider, L.; Büchler, M. W.; Werner, J., Prophylactic antibiotic treatment is superior to therapy on-demand in experimental necrotising pancreatitis. *Critical care (London, England)* **2008,** 12, (6), R141.
73. Foitzik, T.; Fernández-del Castillo, C.; Ferraro, M. J.; Mithöfer, K.; Rattner, D. W.; Warshaw, A. L., Pathogenesis and prevention of early pancreatic infection in experimental acute necrotizing pancreatitis. *Annals of surgery* **1995,** 222, (2), 179-85.
74. Luiten, E. J.; Hop, W. C.; Endtz, H. P.; Bruining, H. A., Prognostic importance of gram-negative intestinal colonization preceding pancreatic infection in severe acute pancreatitis. Results of a controlled clinical trial of selective decontamination. *Intensive care medicine* **1998,** 24, (5), 438-45.
75. Luiten, E. J.; Hop, W. C.; Lange, J. F.; Bruining, H. A., Controlled clinical trial of selective decontamination for the treatment of severe acute pancreatitis. *Annals of surgery* **1995,** 222, (1), 57-65.
76. Dinarello, C. A., IL-1: discoveries, controversies and future directions. *European journal of immunology* **2010,** 40, (3), 599-606.
77. Lukens, J. R.; Gross, J. M.; Kanneganti, T. D., IL-1 family cytokines trigger sterile inflammatory disease. *Frontiers in immunology* **2012,** 3, 315.
78. Watanabe, T.; Kudo, M.; Strober, W., Immunopathogenesis of pancreatitis. *Mucosal immunology* **2017,** 10, (2), 283-298.
79. Hoque, R.; Mehal, W. Z., Inflammasomes in pancreatic physiology and disease. *American journal of physiology. Gastrointestinal and liver physiology* **2015,** 308, (8), G643-51.
80. Dinarello, C. A.; Simon, A.; van der Meer, J. W., Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nat Rev Drug Discov* **2012,** 11, (8), 633-52.
81. Netea, M. G.; van de Veerdonk, F. L.; van der Meer, J. W.; Dinarello, C. A.; Joosten, L. A., Inflammasome-independent regulation of IL-1-family cytokines. *Annual review of immunology* **2015,** 33, 49-77.
82. Fields, J. K.; Günther, S.; Sundberg, E. J., Structural Basis of IL-1 Family Cytokine Signaling. *Frontiers in immunology* **2019,** 10, 1412.
83. Banerjee, S.; Bond, J. S., Prointerleukin-18 is activated by meprin beta in vitro and in vivo in intestinal inflammation. *The Journal of biological chemistry* **2008,** 283, (46), 31371-7.
84. Ueda, T.; Takeyama, Y.; Yasuda, T.; Matsumura, N.; Sawa, H.; Nakajima, T.; Ajiki, T.; Fujino, Y.; Suzuki, Y.; Kuroda, Y., Significant elevation of serum interleukin-18 levels in patients with acute pancreatitis. *Journal of gastroenterology* **2006,** 41, (2), 158-65.
85. Wereszczynska-Siemiatkowska, U.; Mroczko, B.; Siemiatkowski, A., Serum profiles of interleukin-18 in different severity forms of human acute pancreatitis. *Scandinavian journal of gastroenterology* **2002,** 37, (9), 1097-102.
86. Rau, B.; Baumgart, K.; Paszkowski, A. S.; Mayer, J. M.; Beger, H. G., Clinical relevance of caspase-1 activated cytokines in acute pancreatitis: high correlation of serum interleukin-18 with pancreatic necrosis and systemic complications. *Critical care medicine* **2001,** 29, (8), 1556-62.

87. Pastor, C. M.; Morel, D. R.; Vonlaufen, A.; Schiffer, E.; Lescuyer, P.; Frossard, J. L., Delayed production of IL-18 in lungs and pancreas of rats with acute pancreatitis. *Pancreatology : official journal of the International Association of Pancreatology (IAP) ... [et al.]* **2010,** 10, (6), 752-7.
88. Zhang, X. H.; Zhu, R. M.; Xu, W. A.; Wan, H. J.; Lu, H., Therapeutic effects of caspase-1 inhibitors on acute lung injury in experimental severe acute pancreatitis. *World journal of gastroenterology* **2007,** 13, (4), 623-7.
89. Ueno, N.; Kashiwamura, S.; Ueda, H.; Okamura, H.; Tsuji, N. M.; Hosohara, K.; Kotani, J.; Marukawa, S., Role of interleukin 18 in nitric oxide production and pancreatic damage during acute pancreatitis. *Shock (Augusta, Ga.)* **2005,** 24, (6), 564-70.
90. Sennello, J. A.; Fayad, R.; Pini, M.; Gove, M. E.; Ponemone, V.; Cabay, R. J.; Siegmund, B.; Dinarello, C. A.; Fantuzzi, G., Interleukin-18, together with interleukin-12, induces severe acute pancreatitis in obese but not in nonobese leptin-deficient mice. *Proc Natl Acad Sci U S A* **2008,** 105, (23), 8085-90.
91. Carriere, V.; Roussel, L.; Ortega, N.; Lacorre, D. A.; Americh, L.; Aguilar, L.; Bouche, G.; Girard, J. P., IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Proc Natl Acad Sci U S A* **2007,** 104, (1), 282-7.
92. Lüthi, A. U.; Cullen, S. P.; McNeela, E. A.; Duriez, P. J.; Afonina, I. S.; Sheridan, C.; Brumatti, G.; Taylor, R. C.; Kersse, K.; Vandenabeele, P.; Lavelle, E. C.; Martin, S. J., Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases. *Immunity* **2009,** 31, (1), 84-98.
93. Lefrançais, E.; Roga, S.; Gautier, V.; Gonzalez-de-Peredo, A.; Monsarrat, B.; Girard, J. P.; Cayrol, C., IL-33 is processed into mature bioactive forms by neutrophil elastase and cathepsin G. *Proc Natl Acad Sci U S A* **2012,** 109, (5), 1673-8.
94. Pinto, S. M.; Subbannayya, Y.; Rex, D. A. B.; Raju, R.; Chatterjee, O.; Advani, J.; Radhakrishnan, A.; Keshava Prasad, T. S.; Wani, M. R.; Pandey, A., A network map of IL-33 signaling pathway. *Journal of cell communication and signaling* **2018,** 12, (3), 615-624.
95. Kotsiou, O. S.; Gourgoulianis, K. I.; Zarogiannis, S. G., IL-33/ST2 Axis in Organ Fibrosis. *Frontiers in immunology* **2018,** 9, 2432.
96. Jiang, Y.; An, Y.; Jiang, D.; Wu, B.; Yang, Y.; Sun, D., TNF-α Regulating Interleukin-33 Induces Acute Pancreatic Inflammation in Rats. *Annals of clinical and laboratory science* **2016,** 46, (1), 54-9.
97. Xiang, H.; Tao, X.; Xia, S.; Qu, J.; Song, H.; Liu, J.; Shang, D., Emodin Alleviates Sodium Taurocholate-Induced Pancreatic Acinar Cell Injury via MicroRNA-30a-5p-Mediated Inhibition of High-Temperature Requirement A/Transforming Growth Factor Beta 1 Inflammatory Signaling. *Frontiers in immunology* **2017,** 8, 1488.
98. Kempuraj, D.; Twait, E. C.; Williard, D. E.; Yuan, Z.; Meyerholz, D. K.; Samuel, I., The novel cytokine interleukin-33 activates acinar cell proinflammatory pathways and induces acute pancreatic inflammation in mice. *PloS one* **2013,** 8, (2), e56866.
99. Ouziel, R.; Gustot, T.; Moreno, C.; Arvanitakis, M.; Degré, D.; Trépo, E.; Quertinmont, E.; Vercruysse, V.; Demetter, P.; Le Moine, O.; McKenzie, A. N.; Delhaye, M.; Devière, J.; Lemmers, A., The ST2 pathway is involved in acute pancreatitis: a translational study in humans and mice. *The American journal of pathology* **2012,** 180, (6), 2330-9.
100. Sesti-Costa, R.; Silva, G. K.; Proença-Módena, J. L.; Carlos, D.; Silva, M. L.; Alves-Filho, J. C.; Arruda, E.; Liew, F. Y.; Silva, J. S., The IL-33/ST2 pathway controls coxsackievirus B5-induced experimental pancreatitis. *J Immunol* **2013,** 191, (1), 283-92.
101. Kaplan, M.; Yazgan, Y.; Tanoglu, A.; Berber, U.; Oncu, K.; Kara, M.; Demirel, D.; Kucuk, I.; Ozari, H. O.; Ipcioglu, O. M., Effectiveness of interleukin-1 receptor antagonist (Anakinra) on cerulein-induced experimental acute pancreatitis in rats. *Scandinavian journal of gastroenterology* **2014,** 49, (9), 1124-30.
102. Hoffman, H. M.; Throne, M. L.; Amar, N. J.; Sebai, M.; Kivitz, A. J.; Kavanaugh, A.; Weinstein, S. P.; Belomestnov, P.; Yancopoulos, G. D.; Stahl, N.; Mellis, S. J., Efficacy and safety of rilonacept (interleukin-1 Trap) in patients with cryopyrin-associated periodic syndromes: results from two sequential placebo-controlled studies. *Arthritis and rheumatism* **2008,** 58, (8), 2443-52.
103. Harrison, S. R.; McGonagle, D.; Nizam, S.; Jarrett, S.; van der Hilst, J.; McDermott, M. F.; Savic, S., Anakinra as a diagnostic challenge and treatment option for systemic autoinflammatory disorders of undefined etiology. *JCI insight* **2016,** 1, (6), e86336.
104. Stahl, N.; Radin, A.; Mellis, S., Rilonacept--CAPS and beyond. *Annals of the New York Academy of Sciences* **2009,** 1182, 124-34.

105. Rondeau, J. M.; Ramage, P.; Zurini, M.; Gram, H., The molecular mode of action and species specificity of canakinumab, a human monoclonal antibody neutralizing IL-1β. *mAbs* **2015,** 7, (6), 1151-60.
106. Fenini, G.; Contassot, E.; French, L. E., Potential of IL-1, IL-18 and Inflammasome Inhibition for the Treatment of Inflammatory Skin Diseases. *Frontiers in pharmacology* **2017,** 8, 278.
107. Landmann, E. C.; Walker, U. A., Pharmacological treatment options for cryopyrin-associated periodic syndromes. *Expert review of clinical pharmacology* **2017,** 10, (8), 855-864.
108. Zahid, A.; Li, B.; Kombe, A. J. K.; Jin, T.; Tao, J., Pharmacological Inhibitors of the NLRP3 Inflammasome. *Frontiers in immunology* **2019,** 10, 2538.
109. York, J. M.; Castellanos, K. J.; Cabay, R. J.; Fantuzzi, G., Inhibition of the nucleotide-binding domain, leucine-rich containing family, pyrin-domain containing 3 inflammasome reduces the severity of experimentally induced acute pancreatitis in obese mice. *Translational research : the journal of laboratory and clinical medicine* **2014,** 164, (4), 259-69.
110. Coll, R. C.; Robertson, A. A.; Chae, J. J.; Higgins, S. C.; Muñoz-Planillo, R.; Inserra, M. C.; Vetter, I.; Dungan, L. S.; Monks, B. G.; Stutz, A.; Croker, D. E.; Butler, M. S.; Haneklaus, M.; Sutton, C. E.; Núñez, G.; Latz, E.; Kastner, D. L.; Mills, K. H.; Masters, S. L.; Schroder, K.; Cooper, M. A.; O'Neill, L. A., A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. *Nature medicine* **2015,** 21, (3), 248-55.
111. Yang, Y.; Wang, H.; Kouadir, M.; Song, H.; Shi, F., Recent advances in the mechanisms of NLRP3 inflammasome activation and its inhibitors. *Cell death & disease* **2019,** 10, (2), 128.
112. Ren, P.; Wu, D.; Appel, R.; Zhang, L.; Zhang, C.; Luo, W.; Robertson, A. A. B.; Cooper, M. A.; Coselli, J. S.; Milewicz, D. M.; Shen, Y. H.; LeMaire, S. A., Targeting the NLRP3 Inflammasome With Inhibitor MCC950 Prevents Aortic Aneurysms and Dissections in Mice. *Journal of the American Heart Association* **2020,** 9, (7), e014044.
113. Shrimali, D.; Shanmugam, M. K.; Kumar, A. P.; Zhang, J.; Tan, B. K.; Ahn, K. S.; Sethi, G., Targeted abrogation of diverse signal transduction cascades by emodin for the treatment of inflammatory disorders and cancer. *Cancer letters* **2013,** 341, (2), 139-49.
114. Xia, X. M.; Wang, F. Y.; Wang, Z. K.; Wan, H. J.; Xu, W. A.; Lu, H., Emodin enhances alveolar epithelial barrier function in rats with experimental acute pancreatitis. *World journal of gastroenterology* **2010,** 16, (24), 2994-3001.
115. Xu, J.; Huang, B.; Wang, Y.; Tong, C.; Xie, P.; Fan, R.; Gao, Z., Emodin ameliorates acute lung injury induced by severe acute pancreatitis through the up-regulated expressions of AQP1 and AQP5 in lung. *Clinical and experimental pharmacology & physiology* **2016,** 43, (11), 1071-1079.
116. Zhang, Q.; Tao, X.; Xia, S.; Qu, J.; Song, H.; Liu, J.; Li, H.; Shang, D., Emodin attenuated severe acute pancreatitis via the P2X ligand-gated ion channel 7/NOD-like receptor protein 3 signaling pathway. *Oncology reports* **2019,** 41, (1), 270-278.
117. Gao, Z.; Sui, J.; Fan, R.; Qu, W.; Dong, X.; Sun, D., Emodin Protects Against Acute Pancreatitis-Associated Lung Injury by Inhibiting NLPR3 Inflammasome Activation via Nrf2/HO-1 Signaling. *Drug design, development and therapy* **2020,** 14, 1971-1982.
118. Bao, X. Y.; Zheng, Q.; Tong, Q.; Zhu, P. C.; Zhuang, Z.; Zheng, G. Q.; Wang, Y., Danshensu for Myocardial Ischemic Injury: Preclinical Evidence and Novel Methodology of Quality Assessment Tool. *Frontiers in pharmacology* **2018,** 9, 1445.
119. Yin, Y.; Duan, J.; Guo, C.; Wei, G.; Wang, Y.; Guan, Y.; Mu, F.; Yao, M.; Xi, M.; Wen, A., Danshensu accelerates angiogenesis after myocardial infarction in rats and promotes the functions of endothelial progenitor cells through SDF-1α/CXCR4 axis. *European journal of pharmacology* **2017,** 814, 274-282.
120. Liu, X. H.; Pan, L. L.; Jia, Y. L.; Wu, D.; Xiong, Q. H.; Wang, Y.; Zhu, Y. Z., A novel compound DSC suppresses lipopolysaccharide-induced inflammatory responses by inhibition of Akt/NF-κB signalling in macrophages. *European journal of pharmacology* **2013,** 708, (1-3), 8-13.
121. Ren, Z.; Li, H.; Zhang, M.; Zhao, Y.; Fang, X.; Li, X.; Chen, W.; Zhang, H.; Wang, Y.; Pan, L. L.; Sun, J., A Novel Derivative of the Natural Product Danshensu Suppresses Inflammatory Responses to Alleviate Caerulein-Induced Acute Pancreatitis. *Frontiers in immunology* **2018,** 9, 2513.
122. Kim, J. H.; Park, Y. M.; Shin, J. S.; Park, S. J.; Choi, J. H.; Jung, H. J.; Park, H. J.; Lee, K. T., Fraxinellone inhibits lipopolysaccharide-induced inducible nitric oxide synthase and cyclooxygenase-2 expression by negatively regulating nuclear factor-kappa B in RAW 264.7 macrophages cells. *Biological & pharmaceutical bulletin* **2009,** 32, (6), 1062-8.

123. Kim, M. J.; Bae, G. S.; Jo, I. J.; Choi, S. B.; Kim, D. G.; Jung, H. J.; Song, H. J.; Park, S. J., Fraxinellone inhibits inflammatory cell infiltration during acute pancreatitis by suppressing inflammasome activation. *International immunopharmacology* **2019,** 69, 169-177.
124. Mohan, R.; Hammers, H. J.; Bargagna-Mohan, P.; Zhan, X. H.; Herbstritt, C. J.; Ruiz, A.; Zhang, L.; Hanson, A. D.; Conner, B. P.; Rougas, J.; Pribluda, V. S., Withaferin A is a potent inhibitor of angiogenesis. *Angiogenesis* **2004,** 7, (2), 115-22.
125. Lee, J.; Liu, J.; Feng, X.; Salazar Hernández, M. A.; Mucka, P.; Ibi, D.; Choi, J. W.; Ozcan, U., Withaferin A is a leptin sensitizer with strong antidiabetic properties in mice. *Nature medicine* **2016,** 22, (9), 1023-32.
126. Lee, S.; Park, J. M.; Jeong, M.; Han, Y. M.; Go, E. J.; Ko, W. J.; Cho, J. Y.; Kwon, C. I.; Hahm, K. B., Korean red ginseng ameliorated experimental pancreatitis through the inhibition of hydrogen sulfide in mice. *Pancreatology : official journal of the International Association of Pancreatology (IAP) ... [et al.]* **2016,** 16, (3), 326-36.
127. Kanak, M. A.; Shahbazov, R.; Yoshimatsu, G.; Levy, M. F.; Lawrence, M. C.; Naziruddin, B., A small molecule inhibitor of NFκB blocks ER stress and the NLRP3 inflammasome and prevents progression of pancreatitis. *Journal of gastroenterology* **2017,** 52, (3), 352-365.
128. Abreu, F. F.; Souza, A. C.; Teixeira, S. A.; Soares, A. G.; Teixeira, D. F.; Soares, R. C.; Santana, M. T.; Lauton Santos, S.; Costa, S. K.; Muscará, M. N.; Camargo, E. A., Elucidating the role of oxidative stress in the therapeutic effect of rutin on experimental acute pancreatitis. *Free radical research* **2016,** 50, (12), 1350-1360.
129. Aruna, R.; Geetha, A.; Suguna, P., Rutin modulates ASC expression in NLRP3 inflammasome: a study in alcohol and cerulein-induced rat model of pancreatitis. *Molecular and cellular biochemistry* **2014,** 396, (1-2), 269-80.
130. Liu, H.; Talalay, P., Relevance of anti-inflammatory and antioxidant activities of exemestane and synergism with sulforaphane for disease prevention. *Proc Natl Acad Sci U S A* **2013,** 110, (47), 19065-70.
131. Dong, Z.; Shang, H.; Chen, Y. Q.; Pan, L. L.; Bhatia, M.; Sun, J., Sulforaphane Protects Pancreatic Acinar Cell Injury by Modulating Nrf2-Mediated Oxidative Stress and NLRP3 Inflammatory Pathway. *Oxidative medicine and cellular longevity* **2016,** 2016, 7864150.
132. Greaney, A. J.; Maier, N. K.; Leppla, S. H.; Moayeri, M., Sulforaphane inhibits multiple inflammasomes through an Nrf2-independent mechanism. *Journal of leukocyte biology* **2016,** 99, (1), 189-99.
133. Xu, J. C.; Zhou, X. P.; Wang, X. A.; Xu, M. D.; Chen, T.; Chen, T. Y.; Zhou, P. H.; Zhang, Y. Q., Cordycepin Induces Apoptosis and G2/M Phase Arrest through the ERK Pathways in Esophageal Cancer Cells. *Journal of Cancer* **2019,** 10, (11), 2415-2424.
134. Wang, Z.; Chen, Z.; Jiang, Z.; Luo, P.; Liu, L.; Huang, Y.; Wang, H.; Wang, Y.; Long, L.; Tan, X.; Liu, D.; Jin, T.; Wang, Y.; Wang, Y.; Liao, F.; Zhang, C.; Chen, L.; Gan, Y.; Liu, Y.; Yang, F.; Huang, C.; Miao, H.; Chen, J.; Cheng, T.; Fu, X.; Shi, C., Cordycepin prevents radiation ulcer by inhibiting cell senescence via NRF2 and AMPK in rodents. *Nature communications* **2019,** 10, (1), 2538.
135. Nallathamby, N.; Guan-Serm, L.; Vidyadaran, S.; Abd Malek, S. N.; Raman, J.; Sabaratnam, V., Ergosterol of Cordyceps militaris Attenuates LPS Induced Inflammation in BV2 Microglia Cells. *Natural product communications* **2015,** 10, (6), 885-6.
136. Yang, J.; Li, Y. Z.; Hylemon, P. B.; Zhang, L. Y.; Zhou, H. P., Cordycepin inhibits LPS-induced inflammatory responses by modulating NOD-Like Receptor Protein 3 inflammasome activation. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* **2017,** 95, 1777-1788.
137. Yang, J.; Zhou, Y.; Shi, J., Cordycepin protects against acute pancreatitis by modulating NF-κB and NLRP3 inflammasome activation via AMPK. *Life sciences* **2020,** 251, 117645.
138. Robles, L.; Vaziri, N. D.; Ichii, H., Role of Oxidative Stress in the Pathogenesis of Pancreatitis: Effect of Antioxidant Therapy. *Pancreatic disorders & therapy* **2013,** 3, (1), 112.
139. Hua, K. F.; Chou, J. C.; Ka, S. M.; Tasi, Y. L.; Chen, A.; Wu, S. H.; Chiu, H. W.; Wong, W. T.; Wang, Y. F.; Tsai, C. L.; Ho, C. L.; Lin, C. H., Cyclooxygenase-2 regulates NLRP3 inflammasome-derived IL-1β production. *Journal of cellular physiology* **2015,** 230, (4), 863-74.
140. Kornasoff, D.; Maisenbacher, J.; Bowdler, J.; Raber, A., The efficacy and tolerability of aceclofenac compared to indomethacin in patients with rheumatoid arthritis. *Rheumatology international* **1996,** 15, (6), 225-30.
141. Nalamachu, S.; Wortmann, R., Role of indomethacin in acute pain and inflammation management: a review of the literature. *Postgraduate medicine* **2014,** 126, (4), 92-7.

142. Luo, H.; Zhao, L.; Leung, J.; Zhang, R.; Liu, Z.; Wang, X.; Wang, B.; Nie, Z.; Lei, T.; Li, X.; Zhou, W.; Zhang, L.; Wang, Q.; Li, M.; Zhou, Y.; Liu, Q.; Sun, H.; Wang, Z.; Liang, S.; Guo, X.; Tao, Q.; Wu, K.; Pan, Y.; Guo, X.; Fan, D., Routine pre-procedural rectal indometacin versus selective post-procedural rectal indometacin to prevent pancreatitis in patients undergoing endoscopic retrograde cholangiopancreatography: a multicentre, single-blinded, randomised controlled trial. *Lancet (London, England)* **2016,** 387, (10035), 2293-2301.
143. Lu, G.; Pan, Y.; Kayoumu, A.; Zhang, L.; Yin, T.; Tong, Z.; Li, B.; Xiao, W.; Ding, Y.; Li, W., Indomethacin inhabits the NLRP3 inflammasome pathway and protects severe acute pancreatitis in mice. *Biochem Biophys Res Commun* **2017,** 493, (1), 827-832.
144. Hou, C.; Zhu, X.; Shi, C.; Peng, Y.; Huang, D.; Li, Q.; Miao, Y., Iguratimod (T-614) attenuates severe acute pancreatitis by inhibiting the NLRP3 inflammasome and NF-κB pathway. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* **2019,** 119, 109455.
145. Jin, H. Z.; Yang, X. J.; Zhao, K. L.; Mei, F. C.; Zhou, Y.; You, Y. D.; Wang, W. X., Apocynin alleviates lung injury by suppressing NLRP3 inflammasome activation and NF-κB signaling in acute pancreatitis. *International immunopharmacology* **2019,** 75, 105821.
146. Zhu, C.; Fuchs, C. D.; Halilbasic, E.; Trauner, M., Bile acids in regulation of inflammation and immunity: friend or foe? *Clinical and experimental rheumatology* **2016,** 34, (4 Suppl 98), 25-31.
147. Guo, C.; Xie, S.; Chi, Z.; Zhang, J.; Liu, Y.; Zhang, L.; Zheng, M.; Zhang, X.; Xia, D.; Ke, Y.; Lu, L.; Wang, D., Bile Acids Control Inflammation and Metabolic Disorder through Inhibition of NLRP3 Inflammasome. *Immunity* **2016,** 45, (4), 802-816.
148. Li, B.; Yang, N.; Li, C.; Li, C.; Gao, K.; Xie, X.; Dong, X.; Yang, J.; Yang, Q.; Tong, Z.; Lu, G.; Li, W., INT-777, a bile acid receptor agonist, extenuates pancreatic acinar cells necrosis in a mouse model of acute pancreatitis. *Biochem Biophys Res Commun* **2018,** 503, (1), 38-44.
149. Jia, L.; Chen, H.; Yang, J.; Fang, X.; Niu, W.; Zhang, M.; Li, J.; Pan, X.; Ren, Z.; Sun, J.; Pan, L. L., Combinatory antibiotic treatment protects against experimental acute pancreatitis by suppressing gut bacterial translocation to pancreas and inhibiting NLRP3 inflammasome pathway. *Innate immunity* **2020,** 26, (1), 48-61.

ESTUDIO 4. EFECTO DEL PEG35 EN LA MODULACIÓN DE LA INFLAMACIÓN MEDIADA POR EXOSOMAS

En este último estudio, evaluamos la interacción entre el PEG35 y los exosomas en células epiteliales y macrófagos.

Estas nanovesículas son secretadas por diferentes tipos celulares e igualmente captadas por distintas "células recipiente". El conocimiento acerca de los exosomas se ha ido ampliando a lo largo de los últimos años, relacionándolos con la progresión de diversas enfermedades inflamatorias, como es el caso de la PA.

De esta manera, a partir de los resultados previos obtenidos sobre la función antiinflamatoria del PEG35 en diferentes modelos de inflamación pancreática, evaluamos la posibilidad de que el PEG35 ejerciera algún efecto tanto en el mecanismo de captación celular de los exosomas como en la respuesta inflamatoria modulada por estos.

Así, observamos que el PEG35 aumentaba la captación celular de exosomas y activaba una respuesta antiinflamatoria a través de la inhibición de la vía de NFκB. El PEG35 también era capaz de disminuir la expresión de citoquinas proinflamatorias inducida por la internalización de estas nanovesículas.

PEG35 MODULATES EXOSOMAL UPTAKE AND FUNCTION

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ABSTRACT

Background: Polyethylene glycols (PEGs) are neutral polymers widely used in biomedical applications due to its hydrophilicity and biocompatibility. Exosomes are small vesicles secreted by various cell types and are involved in many biological functions. There is also significant evidence on critical role played by exosomes in many diseases.

Objectives: To evaluate the potential role of PEG35 in the modulation of exosomes-mediated inflammation in macrophages.

Methods: Human macrophage-like cells THP-1 and epithelial BICR-18 and CAPAN-2 cells were exposed to increasing concentrations of PEG35 (2, 4 and 6%) or 4% PEG35, respectively, prior to incubation with exosomes of different cellular origin. Confocal microscopy was used to evaluate exosomes internalization. In another set of experiments, differentiated THP-1 cells were incubated with increasing concentrations of PEG35 for 30 min before the incubation with the appropriate stimuli: lipopolysaccharide, BICR-18 derived exosomes or exosomes from acute pancreatitis-induced rats. NFκB and STAT3 signaling pathways activation and expression levels of pro-inflammatory genes were determined.

Results: PEG35 administration significantly enhanced internalization of exosomes in both macrophages and epithelial cells lines in a dose-dependent manner. Further, PEG35 ameliorated the inflammatory response induced by acute pancreatitis-derived exosomes by reducing the expression of pro-inflammatory genes and p65 nuclear translocation.

Conclusions: PEG35 promotes the cellular uptake of exosomes and modulates the pro-inflammatory effect of acute pancreatitis-derived vesicles through the inhibition of NFκB. Overall, our observations emphasize the potential value of PEG35 as an anti-inflammatory agent for biomedical purposes.

Keywords: polyethylene glycol, exosomes, pancreatic inflammation, macrophages, cytokines.

INTRODUCTION

Polyethylene glycols (PEGs) are neutral polymers composed of repeated ethylene glycol units with different chain lengths (1). PEGs are one of the best investigated polymers with a large number of applications in different fields. The water-solubility and hydrophilic properties of PEGs combined with a low intrinsic toxicity make the polymers ideally suited for biological applications. Indeed, their use is approved by the Food and Drugs Administration (FDA) and European Medicines Agency (EMEA) in several fields such as pharmaceutical, cosmetic, packing, food and clinical (2).

Evidence from different experimental studies have suggested beneficial effects of PEGs. PEGs have demonstrated membrane protective effects in a variety of cells or organs against various insults. In this sense, PEGs reduce oxidative stress mainly through the preservation of cell membrane integrity and protecting against reactive oxygen species production during ischemia reperfusion injury (3). PEG ameliorated the early and late effects of radiation in mice intestine through the stabilization and preservation of lipid-raft signalling leading to the preservation of membrane integrity (4). PEG has also been shown to reinforce epithelial barrier and reduce inflammation of the colon in experimental colitis (5). The intravenous administration of PEG is effective in minimizing myocardial ischemia-reperfusion injury and preserve the ventricular function through the inhibition of apoptotic signalling and upregulation of cell survival signalling (6). The potential role of PEG in organ preservation has been demonstrated in experimental kidney (3), liver (7) and pancreatic (8) transplantation. The presence of PEG in the preservation solution ameliorated the deleterious consequences of ischemia-reperfusion by triggering protective cell signalling pathways.

However, there is much less information regarding the precise mechanisms involved in the described beneficial effects of PEGs. In particular, little is known about interactions between PEGs and the different biological processes mediated by extracellular vesicles and, in concert, exosomes. Exosomes are the smallest subtype of extracellular vesicles secreted constitutively by fundamentally all cells in physiological conditions into the extracellular space (9). They have received increasing attention since they were discovered to enclose functional proteins, lipids, DNA, mRNA, microRNA and a large variety of other small noncoding RNA species (10,11). Such exosomal cargo can be delivered into neighbouring and distal cell subpopulations to either confer pathogenic

or therapeutic effects through modulation of immune responses. Moreover, exosomes can be localized in many biological body fluids including blood, saliva, ascitic fluid, cerebrospinal fluid and urine, thereby facilitating intercellular communication (12).

Over the past years, a number of studies have provided evidence of exosomes' implication in physiological and pathological cellular events (13). It has been reported that exosomes play relevant roles in processes as cancer, metastasis, neurological disorders or inflammation. Previous studies of our group have determined the involvement of exosomes in the lung damage associated with experimental acute pancreatitis (AP). Circulating exosomes from AP-induced rats reached the alveolar compartment and polarized macrophages to a pro-inflammatory phenotype (14). Hence, exosomes are now recognized as promising targets in a large number of pathologies and any agent with capability to modulate their function can be considered as a potential therapeutic tool.

Interestingly, we have recently reported an anti-inflammatory role of 35-kDa molecular weight PEG (PEG35) in experimental AP and associated lung injury (15). Intravenous administration of PEG35 was able to reduce the severity of the inflammatory damage and improved outcomes when administered following the initiation of AP-associated systemic effects. These effects may be attributed to a direct anti-inflammatory activity of PEG35, but a potential effect of this polymer on circulating exosomes could also be hypothesized.

In the present paper, we aimed to explore the interaction between exosomes and PEG35 in differentiated inflammatory cells in order to determine the potential role of PEG35 on the regulation of exosomes function.

MATERIALS AND METHODS

Cell culture

The human pro-monocytic THP-1 cell line was purchased from Sigma-Aldrich (St. Louis, MO, USA). The cells were grown at 37ºC in suspension in RPMI 1640 medium GlutaMAX™ (Fisher Scientific; Madrid, Spain) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. THP-1 cells were plated at a density of $3x10^5$ in 24-well culture plates and differentiated to macrophages through a first incubation with 100 nM phorbol12-myristate13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO) for 24h. The PMA-containing media was discarded and replaced with fresh media without PMA for a further 24h.

The human epithelial cell line BICR-18 (from larynx squamous cell carcinoma) and CAPAN-2 (from pancreatic ductal adenocarcinoma) were obtained from American Type Cell Collection (ATCC, Manassas, VA, USA) and maintained at 37ºC in DMEM GlutaMAX™ (Fisher Scientific; Madrid, Spain) supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cultures were split every 3 days by trypsinization with 0.1% trypsin in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) containing 0.9 mM EDTA (Sigma-Aldrich; St. Louis, MO, USA).

All cells were cultured at 37 °C in humidified atmosphere of 95% air and 5% CO_2.

Experimental animals

Male Wistar rats weighting 200-250 g were housed in a controlled environment with free access to standard laboratory pelleted formula (A04; Panlab, Barcelona, Spain) and tap water. A period of one week was allowed for animals to acclimatize before any experimentation. All procedures were conducted in accordance with European Union regulatory standards for animal experimentation (Directive 2010/63/EU on the protection of animals used for scientific purposes). The Ethical Committee for Animal Experimentation (CEEA, Directive 396/12, University of Barcelona) approved the animal experiments.

Exosomes isolation

In order to generate exosome-free medium, exosomes present in FBS were removed by an overnight centrifugation at 100 000 ×g followed by filtration through 0.2-µm

syringe-fitted filters (Millipore, Burlington, MA, USA). This exosome-depleted FBS was used for cell culture (DMEM supplemented with 10% exosome-free FBS). For the exosomes' isolation, BICR-18 cell supernatants and plasma samples from AP-induced rats, were collected and centrifuged at 2 000 ×*g* and 10 000 ×*g* for 10 and 30 min, respectively, at 4°C. The last supernatant was filtered through a 0.22 µm syringe filter (Millipore, Burlington, MA, USA) and ultracentrifuged at 120 000 ×*g* for 70 min. After that, the pelleted vesicles were washed with PBS and centrifuged again at 120 000 ×*g* (16).

Quality of exosomes preparations was verified by nanoparticle tracking analysis and by determining the presence of exosomal marker TSG101 and the absence of calnexin (CNX) by Western Blot (Figure 1). The number of exosomes obtained was also quantified by measuring their protein content using a Bradford assay.

Exosomes and cell staining

For internalization assays, exosomes were isolated from BICR-18 cells (ExoB) or from AP-induced rats (ExoAP) and labelled with the PKH26 red fluorescent cell linker dye (Sigma-Aldrich, St. Louis, MO, USA) for 5 min. The staining reaction was stopped with 3% BSA for 1 min. In order to remove the unbound dye, exosomes were washed three times with PBS using 300 kDa Nanosep centrifugal devices (Pall Corporation, New York, NY). Fixed cells were also stained with the DNA-specific blue fluorescent stain 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) for 1-5 min. at room temperature.

Cell treatments

To analyse the exosomes uptake in the presence of PEG35, differentiated THP-1 cells were incubated with increasing concentrations of PEG35 (1, 2, 4 and 6%) diluted in PBS during 30 min prior to treatment with the appropriate stimuli: 10 µg/ml ExoB, 10 µg/ml ExoAP or 0.1 µg/ml LPS (Sigma-Aldrich, St. Louis, MO, USA) for 1.5h. As well, epithelial BICR-18 and CAPAN-2 cells were incubated with 4% PEG35 during 30 min. prior to treatment with ExoB for 1.5 h. The concentration of exosomes was selected according to previous *in vitro* studies (17). Exosomes internalization was analysed by confocal microscopy imaging.

Nanoparticle tracking analysis

The size distribution and concentration of exosomes were measured using a NanoSight LM10 machine (NanoSight, Salisbury, UK). All the parameters of the analysis were set at the same values for all samples and three 1 min-long videos were recorded in all cases. Background was measured by testing filtered PBS, which revealed no signal.

Animal model of AP

The rats were anesthetized with an intraperitoneal injection of pentobarbital at a dose of 50mg/kg. After a midline laparotomy, a polyethylene catheter connected to an infusion pump was inserted through the duodenum, via the Ampulla of Vater, and 3–4 mm into the biliopancreatic duct. A bulldog clamp was applied to the proximal biliopancreatic duct (near the liver) to prevent infusion into the liver. The experimental model of AP was induced in the rats by retrograde injection of 5% sodium taurocholate in saline solution at 1mL/Kg for 1 min using an infusion pump (Harvard Instruments, Edenbridge, UK). Buprenorphine (0.05 mg/Kg) was intravenously administered as an analgesic immediately before surgery. Three hours after AP induction, animals were euthanized and blood was collected in heparinized syringes from the vena cava for exosome isolation.

Confocal microscopy

Cells were imaged using an inverted Nikon Eclipse Ti2-E microscope (Nikon Instruments) attached to the spinning disk unit Andor Dragonfly. For all experiments an oil-immersion objective (Plan Fluor 20×, numerical aperture (NA) 0.75, oil) was used. Samples were excited with 405 nm, 488 nm and 561 nm laser diodes. The beam was coupled into a multimode fibber going through the Andor Borealis unit reshaping the beam from a Gaussian profile to a homogenous flat top. From there it was passed through the 40 µm pinhole disk. Cells were imaged on a high resolution scientific complementary metal oxide semiconductor (sCMOS) camera (Zyla 4.2, 2.0 Andor, Oxford Instruments Company, Concord, MA, USA). Fusion software from Oxford Instruments Company was used for acquisition of images. Image deconvolution was performed after acquisition. Image processing and analysis was performed with Image J/Fiji open source software using Image J Macro Language.

SDS-PAGE and Western blot

Exosomal protein was extracted in ice-cold RIPA buffer (10 mM Tris pH 8.0, 140 mM NaCl, 1% Triton X-100, 1 mM EDTA and 0.1% SDS) in the presence of protease inhibitors. Extracts were then centrifuged at 15 000 ×g for 20 min at 4°C, and the supernatants were collected. Cell lysates, used as a negative control, were obtained from differentiated THP-1 cells. Protein concentration of the supernatants were determined by the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). SDS-PAGE was performed on a 10% gel and proteins were transferred to a PVDF membrane for blotting (Bio Rad, Hercules, CA, USA). Membranes were blocked for 1 h in 5% non-fat milk in PBS, followed by overnight incubation at 4ºC with the following antibodies from Proteintech (Manchester, United Kingdom): TSG101 (1:1000 dilution, reference 14497-1-AP) and Calnexin as negative control (1:1000 dilution, reference 10427-2-AP). Blots were washed and incubated with the corresponding HRP-conjugated secondary antibody. Bound antibodies were detected using enhanced chemiluminescence (ECL) (Bio-Rad Laboratories, Hercules, CA, USA), and were analysed using ChemiDoc™ Touch Imaging System (Bio-Rad Laboratories, Hercules, CA, USA).

Immunofluorescence

To determine Nuclear factor kB (NFκB) or Signal transducer and activator of transcription 3 (STAT3) nuclear translocation, THP-1 macrophages were incubated in coverslips overnight at 37 °C under 5% CO_2 in air. Cells were treated with 4% PEG35 for 30 min before the incubation with 10 μg/ml ExoAP or 0.1 μg/ml LPS for 45 min, fixed with 3.5 % formaldehyde for 5 min at room temperature, and permeabilized with Triton X-100. The cells were stained with the following antibodies from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA, USA): anti-NFκB p65 (1:400 dilution, reference sc-372) and anti-STAT3 antibody (1:400 dilution, reference sc-483). Alexa Fluor 488-conjugated anti-goat antibody (Molecular Probes, Eugene, OR, USA) was used as secondary antibody. Localization of NFκB and STAT3 was examined by Nikon eclipse E1000 fluorescence microscopy (Nikon, Melville, New York, USA).

Real-time qRT-PCR

Total RNA from the cells was extracted with Nucleozol reagent ((Macherey-Nagel, Dueren, Germany) according to the manufacturer's protocol. RNA concentration and

quality were measured with the OD A260/A280 ratio and OD A260/A230 ratio respectively, and the integrity of 18S and 28S ribosomal bands for all RNA preparations was verified by running a 1% agarose gel electrophoresis. Reverse transcription was conducted on a 1 µg RNA sample using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Subsequent PCR amplification was conducted using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) on a CFX Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using 10µL of amplification mixtures containing 50 ng of reverse-transcribed RNA and 250 nM of the corresponding forward and reverse primers. PCR primers for the detection of Interleukin 1β (IL1β), Tumor necrosis factor (TNFα) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were designed with Primer3.0 plus (18). The sequences were as follows: IL1β Forward: 5'-GGACAAGCTGAGGAAGATGC-3' Reverse: 5'-TCGTTATCCCATGTGTCGAA-3', TNFα forward: 5'-AGCCCATGTTGTAGCAAACC-3' Reverse: 5'- GGCACCACCAACTGGTTATC-3', and GAPDH Forward: 5'-GATCATGAGCAATGCCTCCT-3' Reverse: 5'-TGTGGTCATGAGTCGTTCCA-3'. The specificity of the amplicons was determined by melting curve analysis. Reactions were carried out in duplicate and threshold cycle values were normalized to GAPDH gene expression. The ratio of the relative expression of target genes to GAPDH was calculated by the DCt formula.

Statistical analysis

All data were exported into Graph Pad Prism 4 (GraphPad Software, Inc.) and presented as means ± SEM. Statistical analyses were carried out by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test to determine the significance between pairs. The minimal level of statistical significance was considered to be < 0.05.

RESULTS

Biological characterization of isolated exosomes

After collection of ExoB and ExoAP, we confirmed their size by the NanoSight particle tracking system (Figure 1A). The size of all the tested vesicles preparations showed a peak between 100 and 200 nm, consistent with exosomes. Analysis by immunoblotting confirmed the presence of exosome marker TSG101 and the absence of Calnexin in both exosomes from BICR-18 and from acute pancreatitis plasma origin (Figure 1B). Calnexin was readily detectable in the whole cell lysates.

PEG35 increased exosomes uptake in macrophages and epithelial cell lines

To determine the effect of PEG35 in exosome uptake, THP-1 macrophages were incubated with PKH26-labelled ExoB in presence of increasing concentrations of PEG35. As shown in Figure 2A and B, PEG35 enhanced the ExoB uptake capacity of macrophages increased in a dose-dependent manner. Further, PEG35 also raised the macrophages capacity to internalize exosomes from AP-induced rats (Figure 2C and D). Tissue macrophages are known to express a wide range of surface, vacuole and cytosolic molecules for recognition and uptake of host-derived and foreign particles by phagocytosis, and for clearance of soluble molecules by endocytosis (19). Moreover, exosomes are known to internalize more efficiently by phagocytic cells than non-phagocytic cells (20). Hence, we also evaluated the effect of PEG35 in exosome uptake in epithelial BICR-18 and CAPAN-2 cells. As occurs with macrophages, the presence of 4% PEG35 significantly increased ExoB cell internalization (Figure 3A and B).

Effect of PEG35 on the inflammatory activation of macrophages

Previous results of our group support a an anti-inflammatory role for PEG35 against the AP-associated inflammation (15). Thus, we explored whether PEG35 might modulate the inflammatory response induced by AP-derived exosomes in THP-1 macrophages. As expected, treatment of macrophages with ExoAP results in the induction of expression of inflammatory cytokines IL1β and TNFα (Figure 4A). The increase induced by exosomes on these cytokines was inhibited by the presence of PEG35 in a dose-dependent manner, confirming the anti-inflammatory role of PEG35. On the other hand, PEG35 showed a concentration-dependent anti-inflammatory activity when administered to macrophages activated with LPS (Figure 4B). Finally, we treated macrophages with increasing

concentrations of exosomes from AP and observed an evident inflammatory response through the induction of TNFα (Figure 4C).

PEG35 prevented LPS and ExoAP-induced pro-inflammatory response in macrophages through NFκB inhibition

One of the important features of the anti-inflammatory effect of PEG35 is that it counteracts the activation of genes encoding inflammatory mediators, such as TNFα, IL1β or IL6. Because NFκB is highly active both in inflammatory cells, such as macrophages, and in cells found in inflamed tissues, it is recognized as a key mediator of inflammation. In addition to NFκB, STAT3 also plays a pivotal role in inflammatory processes. Both factors are essential signalling molecules that coordinate inflammatory response in several pathologies.

Given that the molecular mechanisms underlying the anti-inflammatory role of PEG35 need to be elucidated, we examined the effect of PEG35 on these pathways in macrophages. As shown in Figure 5, unstimulated THP-1 macrophages showed p65 subunit of NFκB staining localized in the cytosol. After LPS or ExoAP stimulation, staining was mostly transferred to the nucleus, indicating the activation of this signalling pathway. Pre-treatment of cells with 4% PEG35 inhibited p65 accumulating in the nucleus. On the other hand, STAT3 was activated by LPS treatment but not by ExoAP and the activation induced by LPS was not inhibited by PEG35.

DISCUSSION

Over the last decade has been considerable interest in the study of PEG properties, and their use has extended to different fields. Recently, our group has demonstrated the anti-inflammatory effect of prophylactic administration of PEG35 in an experimental model of acute necrotizing pancreatitis and besides, we have showed that the therapeutic treatment with this polymer exerted a significant protection against acute lung inflammation associated with this disease.

In the current study, we explored whether this protective action of PEG35 is related with effects on the cellular uptake of exosomes. It is well known that these nanovesicles are able to trigger the inflammatory response in a broad spectrum of pathologies and, in particular, in the pancreatitis-associated inflammation. Since PEG has the capability to bind biological membranes, it could be hypothesised that its presence could impair the process of exosomes uptake, thus reducing the associated inflammatory response triggered by these nanovesicles. Unexpectedly, our results indicate the opposite mechanism and exosome uptake increased in a dose-dependent manner in the presence of PEG35.

We then explored whether this mechanism was related to the high phagocytic capacity of macrophages and evaluated the effect of PEG35 on different epithelial cell lines. In all cases, the presence of 4% PEG35 increased exosomes uptake, thus pointing out an unspecific mechanism, which probably involves the binding process between exosomal and cell membranes.

Our group first clarified that exosomes are involved in AP and that circulating exosomes transform alveolar macrophages into a pro-inflammatory phenotype. As expected, in our investigation, the presence of exosomes from AP increased the expression of pro-inflammatory IL1β and TNFα on THP-1 macrophages, revealing their activation. Interestingly, PEG35 treatment succeeded in the inhibition of these inflammatory cytokine's expression. This fact indicates that, despite the increase in the internalization of exosomes induced by PEG35, its anti-inflammatory effect is able to prevent the activation induced by these nanovesicles.

Next, we determined the direct anti-inflammatory effect of PEG35 on the activation of macrophages and confirmed that this polymer markedly prevented IL1β expression induced by LPS treatment in a concentration-dependent manner. This is

consistent with the results obtained in a previous study, in which we demonstrated a direct anti-inflammatory effect of PEG35 in pancreatic acinar AR42J cells (21). In this sense, we found that the administration of this polymer attenuated the expression of pro-inflammatory cytokines and associated cell death markers following TNFα or cerulein treatment.

Given that activation of NFκB and STAT3 plays a crucial role in inflammatory processes, it was conceivable that these signalling pathways could be involved in PEG35 inhibition of inflammation. Although triggered by different pathways, activated STATs and NFκB translocate into the nucleus and function either individually or collaboratively in regulating inflammatory gene expression. Our data indicated that macrophages treatment with PEG35 caused a shift in the p65 nuclear localization observed under LPS or ExoAP stimulation. However, STAT3 was not found to be a target for PEG35-mediated attenuation of inflammation in LPS-treated macrophages.

In conclusion, PEG35 facilitates exosomes internalization into cells and is able to prevent the pro-inflammatory effect of acute pancreatitis-derived exosomes by targeting NFκB signalling pathway. Nevertheless, our results indicate that the effects of PEG35 related to exosomes must be carefully considered. The increase in exosome uptake could be attractive in situations that exosomes carry molecules of interest, as occurs in the anti-inflammatory effects of exosomes released by mesenchymal stem cells (22). However, in other pathological situations in which exosomes contribute to the pathogenic process, any increase in exosome uptake could be detrimental. Obviously, further studies are required in order to clarify both the potential and limitations of PEGs when using them in pathologies in which exosomes play a relevant role.

ACKNOWLEDGEMENTS

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REFERENCES

1. Turecek PL, Bossard MJ, Schoetens F, Ivens IA. PEGylation of Biopharmaceuticals: A Review of Chemistry and Nonclinical Safety Information of Approved Drugs. Vol. 105, Journal of Pharmaceutical Sciences. Elsevier B.V.; 2016. p. 460–75.

2. Pasut G, Panisello A, Folch-Puy E, Lopez A, Castro-Benítez C, Calvo M, et al. Polyethylene glycols: An effective strategy for limiting liver ischemia reperfusion injury. Vol. 22, World Journal of Gastroenterology. Baishideng Publishing Group Co., Limited; 2016. p. 6501–8.

3. Dutheil D, Rioja-Pastor I, Tallineau C, Goujon JM, Hauet T, Mauco G, et al. Protective effect of PEG 35 000 Da on renal cells: Paradoxical activation of JNK signaling pathway during cold storage. Am J Transplant. 2006 Jul;6(7):1529–40.

4. Valuckaite V, Zaborina O, Long J, Hauer-Jensen M, Wang J, Holbrook C, et al. Oral PEG 15-20 protects the intestine against radiation: Role of lipid rafts. Am J Physiol - Gastrointest Liver Physiol. 2009 Dec;297(6).

5. Videla S, Lugea A, Vilaseca J, Guarner F, Treserra F, Salas A, et al. Polyethylene glycol enhances colonic barrier function and ameliorates experimental colitis in rats. Int J Colorectal Dis. 2007 Jun;22(6):571–80.

6. Xu X, Philip JL, Razzaque MA, Lloyd JW, Muller CM, Akhter SA. High-molecular-weight polyethylene glycol inhibits myocardial ischemia-reperfusion injury in vivo. J Thorac Cardiovasc Surg. 2015 Feb 1;149(2):588–93.

7. Abbas R, Kombu RS, Dignam D, Gunning W, Stulberg JJ, Brunengraber H, et al. Polyethylene glycol modified-albumin enhances the cold preservation properties of university of wisconsin solution in rat liver and a hepatocyte cell line. J Surg Res. 2010 Nov;164(1):95–104.

8. Garca-Gil FA, Fuentes-Broto L, Albendea CD, Trinidad Serrano M, Roselló-Catafau J, Lampreave F, et al. Evaluation of institut georges lopez-1 preservation solution in pig pancreas transplantation: A pilot study. Transplantation. 2014;97(9):901–7.

9. Mathieu M, Martin-Jaular L, Lavieu G, Théry C. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. Vol. 21, Nature Cell Biology. Nature Publishing Group; 2019. p. 9–17.

10. Hessvik NP, Llorente A. Current knowledge on exosome biogenesis and release. Vol. 75, Cellular and Molecular Life Sciences. Birkhauser Verlag AG; 2018. p. 193–208.

11. Lunavat TR, Cheng L, Kim DK, Bhadury J, Jang SC, Lässer C, et al. Small RNA deep sequencing discriminates subsets of extracellular vesicles released by melanoma cells – Evidence of unique microRNA cargos. RNA Biol. 2015 Jan 1;12(8):810–23.

12. Doyle L, Wang M. Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis. Cells. 2019 Jul 15;8(7):727.

13. Théry C, Zitvogel L, Amigorena S. Exosomes: Composition, biogenesis and function. Vol. 2, Nature Reviews Immunology. European Association for Cardio-Thoracic Surgery; 2002. p. 569–79.

14. Bonjoch L, Casas V, Carrascal M, Closa D. Involvement of exosomes in lung inflammation associated with experimental acute pancreatitis. J Pathol. 2016 Oct 1;240(2):235–45.

15. Ferrero-Andrés A, Panisello-Roselló A, Serafín A, Roselló-Catafau J, Folch-Puy E. Polyethylene glycol 35 (PEG35) protects against inflammation in experimental acute necrotizing pancreatitis and associated lung injury. Int J Mol Sci. 2020 Feb 1;21(3).

16. Edwards JP, Zhang X, Frauwirth KA, Mosser DM. Biochemical and functional characterization of three activated macrophage populations. J Leukoc Biol. 2006 Dec;80(6):1298–307.

17. Bonjoch L, Gironella M, Iovanna JL, Closa D. REG3β modifies cell tumor function by impairing extracellular vesicle uptake. Sci Rep. 2017 Dec 1;7(1).

18. Untergasser A , Cutcutache I, Koressaar T, Ye J,Faircloth BC, Remm M RS. Primer3--new capabilities and interfaces. Nucleic Acids Res. 2012;40(15):e115.

19. Gordon S. Phagocytosis: An Immunobiologic Process. Vol. 44, Immunity. Cell Press; 2016. p. 463–75.

20. Feng D, Zhao WL, Ye YY, Bai XC, Liu RQ, Chang LF, et al. Cellular internalization of exosomes occurs through phagocytosis. Traffic. 2010 May;11(5):675–87.

21. Ferrero-Andrés AP-RAR-CJF-PE. Polyethylene glycol 35 ameliorates pancreatic inflammatory response in cerulein-induced acute pancreatitis in rats. World J Gastroenterol. 2010;In press.

22. Harrell CR, Jovicic N, Djonov V, Arsenijevic N, Volarevic V. Mesenchymal Stem Cell-Derived Exosomes and Other Extracellular Vesicles as New Remedies in the Therapy of Inflammatory Diseases. Vol. 8, Cells. 2019.

FIGURES

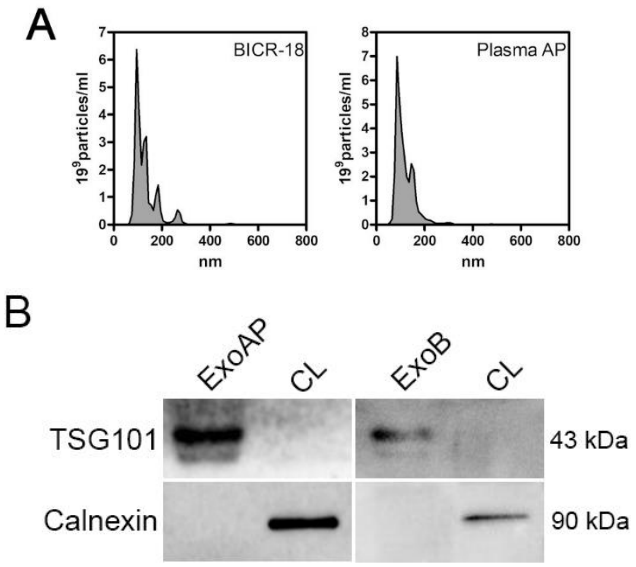

Figure 1. Characterization of extracellular vesicles. A) Size distribution curves, evaluated by NanoSight, indicated that sizes are compatible with exosomes. B) Western blot analysis was performed with whole cell lysates (CL) and pooled exosomes isolated from plasmatic acute pancreatitis-induced rats and from epithelial cell line BICR-18 to confirm the presence of classical exosome marker (TSG101) and the absence of endoplasmic reticulum contamination (Calnexin, CNX). ExoAP, exosomes from plasma of acute pancreatitis-induced rats; CL, Cell lysates; ExoB, Exosomes from BICR-18

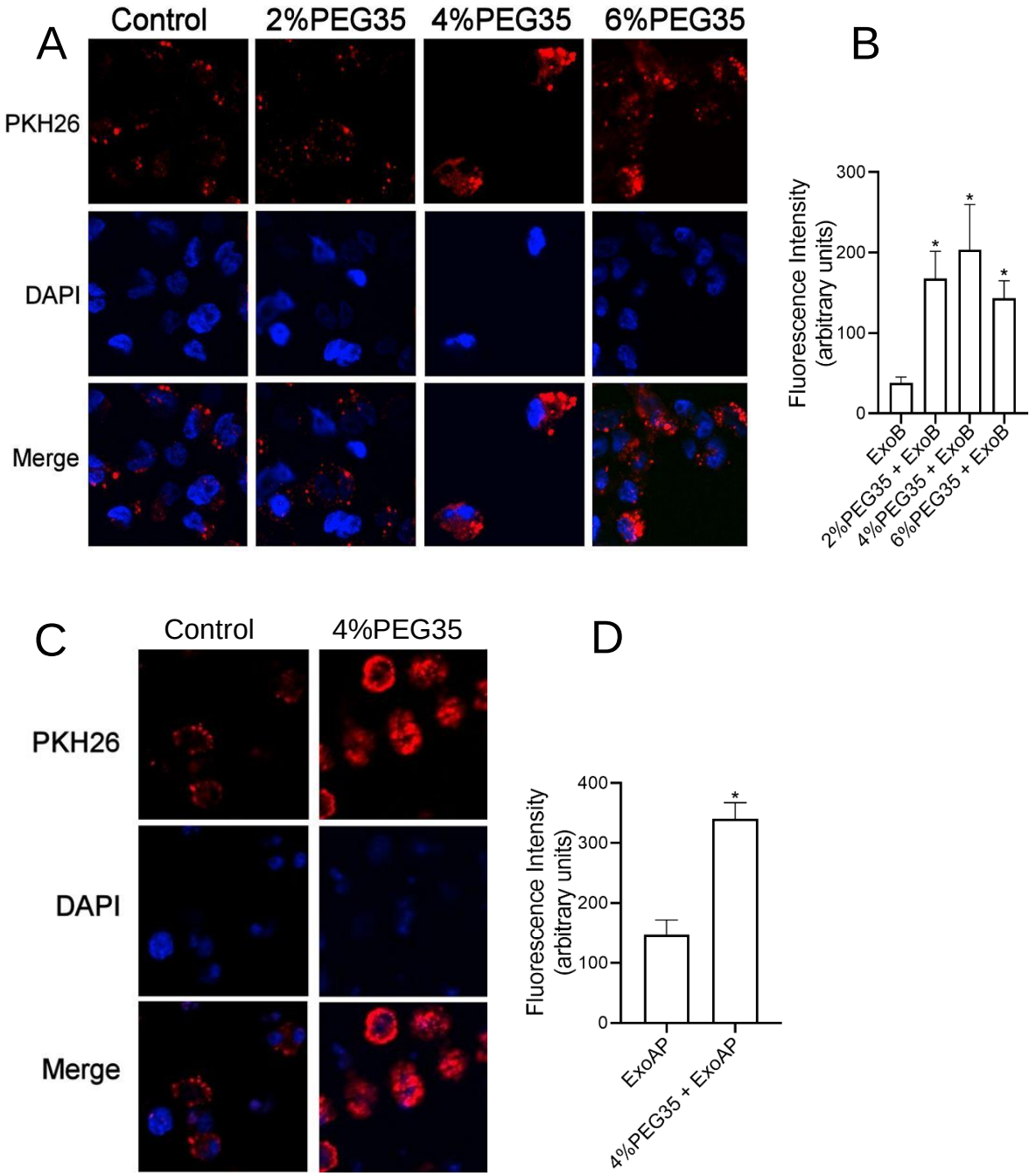

Figure 2. PEG35 enhanced uptake of exosomes in macrophages. A) Representative images showing internalized ExoB in THP-1 macrophages cells at increasing concentrations of PEG35. B) Fluorescence intensity analysis of the PKH26-labelled exosomes from BICR-18 by Image J. C) Representative images showing internalized exosomes from AP-induced rats in THP-1 macrophages incubated with 4%PEG35. D) Fluorescence intensity analysis of the PKH26-labelled exosomes from AP by Image J. Untreated and PEG35-treated cells were fixed and imaged with confocal microscopy (original magnification 20x; blue, DAPI stained nuclei; red, PKH26 stained exosomes).

The values shown represent the mean ± SEM. *P < 0.05 versus ExoB. PEG35, 35-kDa polyethylene glycol; ExoB, exosomes from BICR-18 cells; ExoAP, exosomes from AP-induced rats.

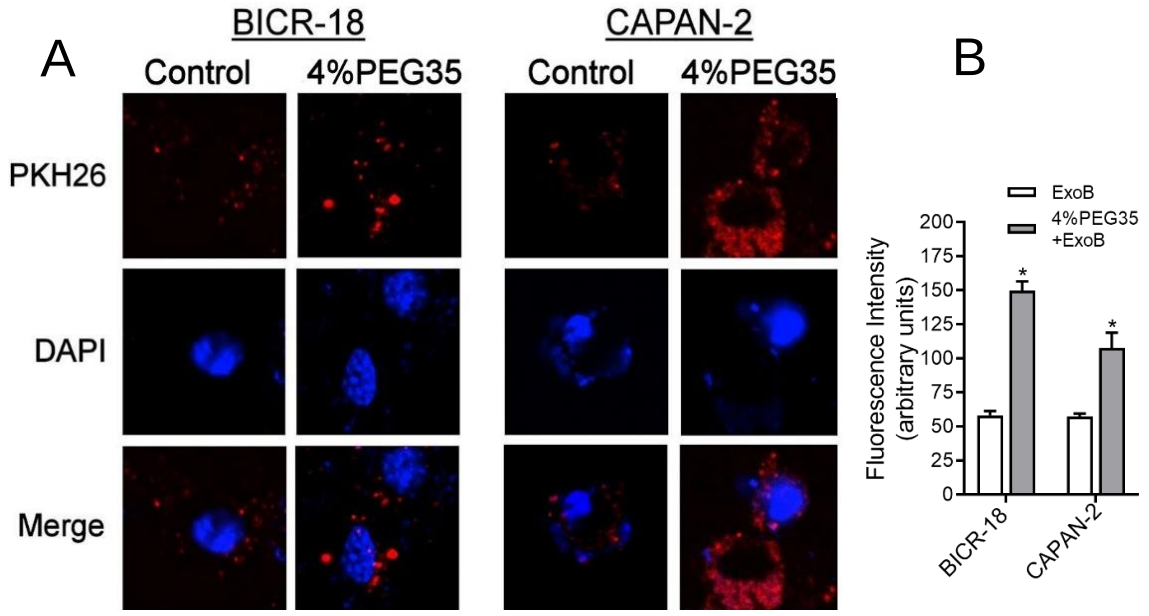

Figure 3. PEG35 enhanced uptake of exosomes in epithelial cells. A) Representative images showing internalized ExoB in epithelial BICR-18 and CAPAN-2 cells under treatment with 4% PEG35. Untreated and PEG35-treated cells were fixed and imaged with confocal microscopy (original magnification 20x; blue, DAPI stained nuclei; red, PKH26 stained exosomes). B) Fluorescence intensity analysis of the PKH26-labelled exosomes by Image J. The values shown represent the mean ± SEM. *P < 0.05 versus ExoB. Data is representative of several repeated experiments. ExoB, exosomes from BICR-18 cells; PEG35, 35-kDa polyethylene glycol.

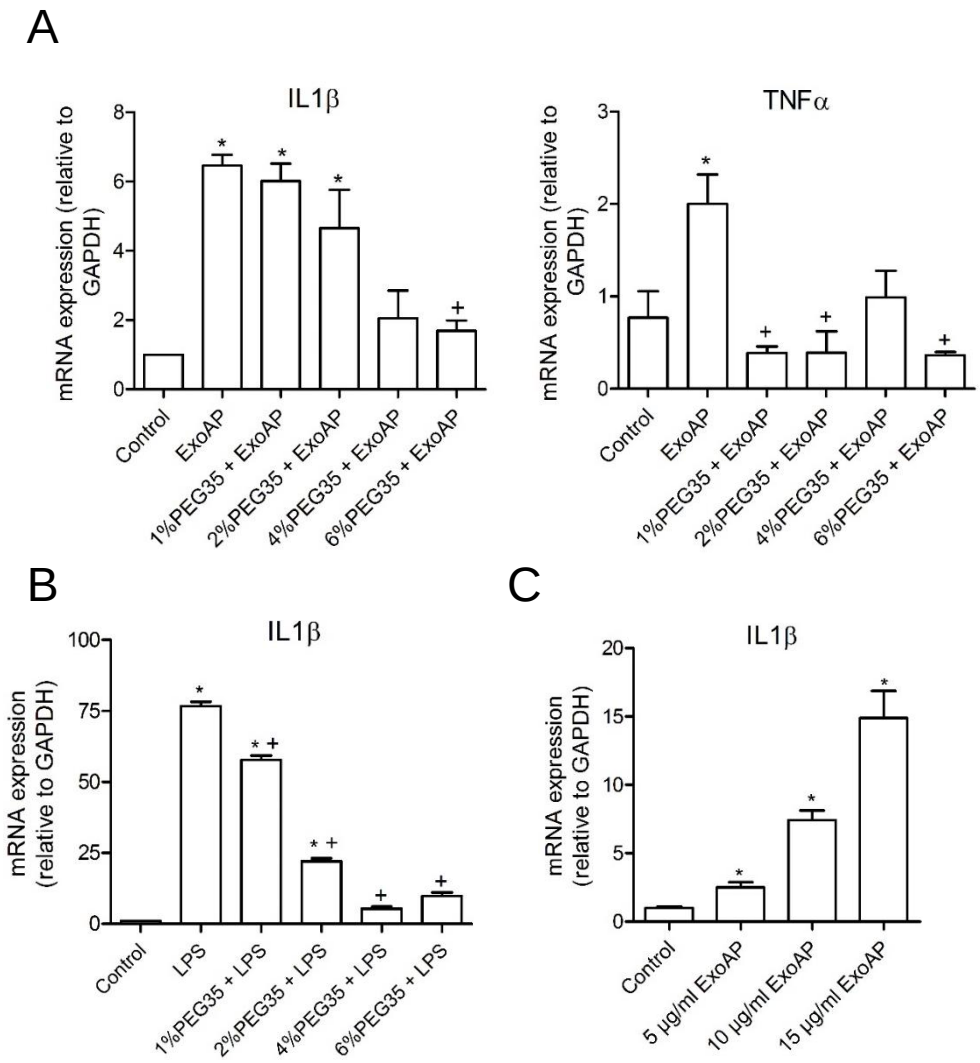

Figure 4. Gene expression of pro-inflammatory cytokines in THP-1-treated cells. A) Gene expression by real-time qRT-PCR of IL1β and TNFα in ExoAP-treated THP-1 cells subjected to increasing concentrations of PEG35. B) Gene expression by real-time qRT-PCR of IL1β in LPS-treated THP-1 macrophages subjected to increasing concentrations of PEG35. C) Gene expression by real-time qRT-PCR of IL1β in THP-1 macrophages treated with increasing concentrations of ExoAP. In all cases, mRNA induction levels were normalized to GAPDH mRNA expression. Bars represent mean values of each group ± SEM. **P* < 0.05 versus Control, +*P* < 0.05 versus ExoAP or LPS. Each determination was carried out in triplicate. ExoAP, exosomes from acute-pancreatitis-induced rats; PEG35, 35-kDa polyethylene glycol; IL1β, interleukin 1β; TNFα, tumor necrosis factor α.

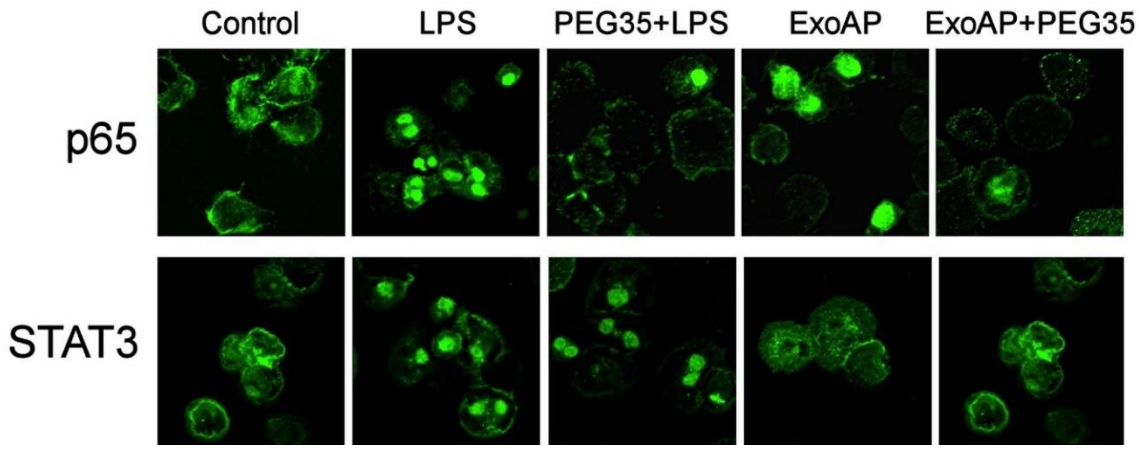

Figure 5. 4%PEG35 suppressed p65 translocation to the nucleus, but not STAT3, in LPS or ExoAP-stimulated macrophages. Representative images of immunofluorescence staining for subcellular localization of p65 subunit of NFκB and STAT3 observed by confocal microscopy. Magnification 20X. Data is representative of several repeated experiments. LPS, Lipopolysaccharide; ExoAP, exosomes from AP-induced rats; PEG35, 35-kDa polyethylene glycol.

RESULTADOS GENERALES

Los PEGs son polímeros hidrosolubles y no tóxicos ampliamente estudiados por las características fisicoquímicas beneficiosas que presentan al ser adicionados a diferentes compuestos dentro de la industria farmacéutica, clínica, alimentaria y cosmética.

En la última década, el estudio de los efectos protectores presentados por estos polímeros en diversos modelos experimentales, tanto *in vivo* como *in vitro*, ha ido incrementando.

Dado los numerosos beneficios fisiológicos que se relacionan con los PEGs de distintos pesos moleculares, decidimos evaluar los efectos del PEG35 en la respuesta inflamatoria asociada a la PA.

Para nuestros estudios nos basamos en varios modelos experimentales *in vivo* e *in vitro* observando los siguientes resultados:

- En un modelo experimental de PAN en rata, la administración profiláctica de PEG35 vía intravenosa redujo la posterior inflamación pancreática y pulmonar. Además, la administración terapéutica por la misma vía disminuyó las complicaciones locales asociadas a la PAN.

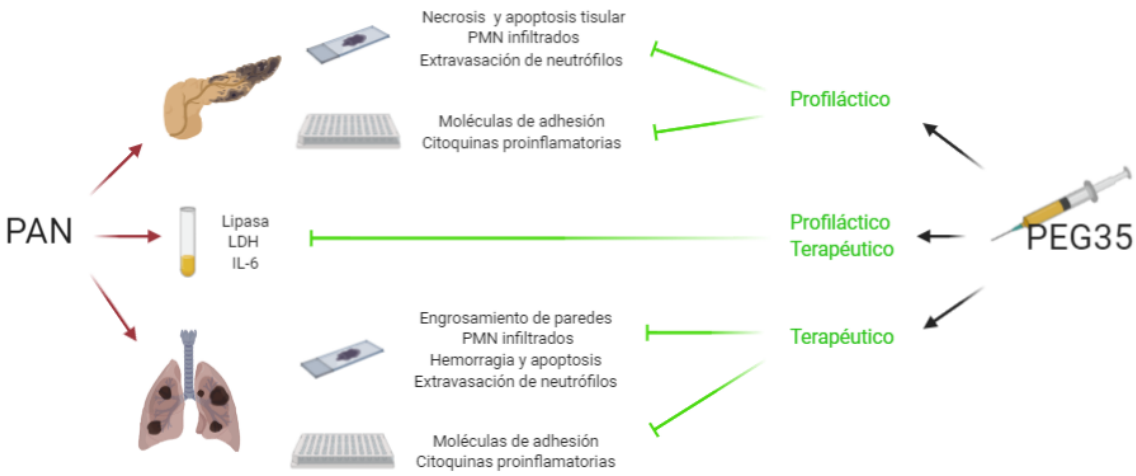

Figura 11. Esquema de los eventos fisiológicos asociados a un modelo experimental de PAN. Efecto de la administración profiláctica y terapéutica del PEG35 sobre ellos.

- En un modelo experimental de PAEI en rata, la administración profiláctica de PEG35 por vía intravenosa redujo el proceso inflamatorio en el páncreas y la muerte celular asociada.

Figura 12. Esquema de las manifestaciones patológicas asociadas a un modelo experimental de PAEI. La administración de PEG35 antes de la inducción de este modelo de inflamación pancreática conseguía reducir el daño inflamatorio.

- En una línea celular acinar pancreática, AR42J, tratada con diferentes estímulos proinflamatorios y proapoptóticos, observamos que el pretratamiento con PEG35 atenúa la inflamación y la muerte celular asociada a estos estímulos.

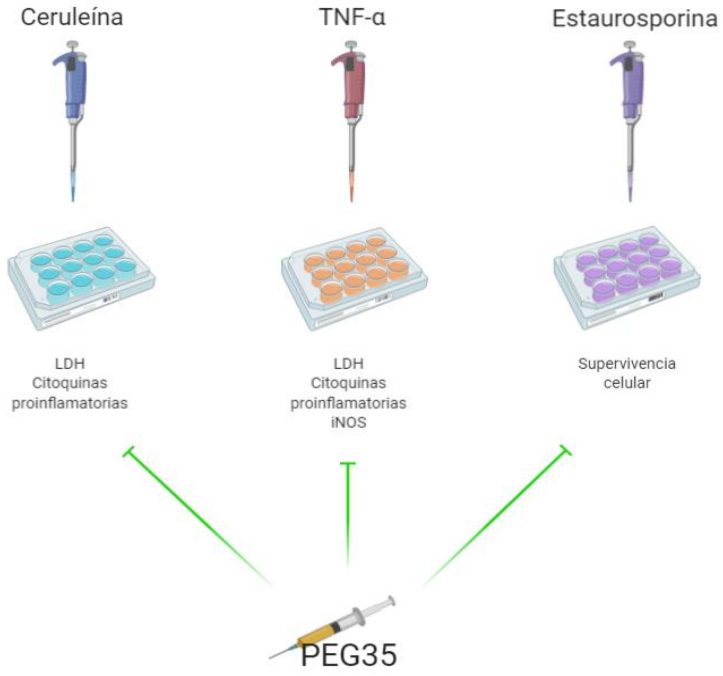

Figura 13. Esquema de modelos *in vitro* de inflamación y muerte celular. El pretratamiento con PEG35 disminuía el daño celular ocasionado por distintos estímulos.

- En líneas celulares epiteliales de carcinoma de laringe, BICR-18, y de adenocarcinoma del conducto pancreático, CAPAN-2, así como en una línea celular promonocítica, THP-1, el PEG35 favorece la captación de exosomas. Asimismo, este polímero es también capaz de reducir el efecto proinflamatorio de los exosomas procedentes del plasma de animales inducidos con una PA.

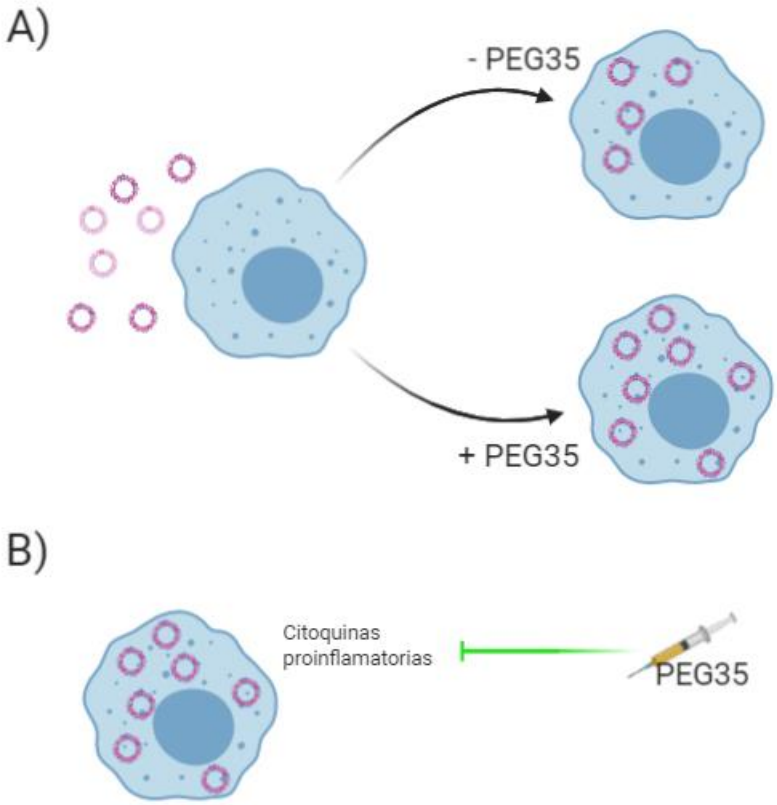

Figura 14. Captación de exosomas y efecto del PEG35. A) Esquema de la internalización de exosomas en macrófagos. B) Esquema del efecto del PEG35 en la respuesta inflamatoria mediada por la captación de exosomas.

Los resultados obtenidos esclarecen el papel del PEG35 en la modulación de los procesos inflamatorios asociados al páncreas.

DISCUSIÓN

"Krat'eroi."

Fueron las últimas palabras que pronunció Alejandro Magno antes de su misteriosa muerte. Desde que esto sucediera en el año 323 a.e.c, los enormes avances en investigación biomédica han permitido relacionar no solo este sino otros tantos casos históricos de enfermedades con lo que a día de hoy conocemos como una PA (172), (173).

Avanzando en la historia, la primera descripción clínica de una PA fue publicada por el médico italiano Iacobo Auberto Vindone en 1579, en la que describe una necrosis del tejido pancreático en un paciente alcohólico (174). Desde entonces, se ha recorrido un largo camino en el que los avances en diagnosis y prognosis, de la mano con las investigaciones clínicas en fisiopatología pancreática, han ido esclareciendo los inicios biomoleculares de esta enfermedad y mejorando su tratamiento (175). Sin embargo, examinando en retrospectiva, el progreso en el manejo clínico de la PA ha ido lento en las últimas décadas. Uno de los mayores cambios relacionados con el tratamiento es que la intervención quirúrgica ha pasado a reservarse para los casos en los que aparecen complicaciones en los estadios más tardíos de esta enfermedad (176).

Debido al número creciente de casos clínicos que han aparecido en las últimas décadas y al elevado índice de mortalidad asociada a la aparición de complicaciones sistémicas, con especial atención en la afección pulmonar, esta enfermedad continúa siendo un reto en investigación, más aún sin la existencia de un tratamiento farmacológico específico.

Numerosos estudios han observado propiedades protectoras de los PEGs frente a daños tisulares por diversas vías de administración (177), (178), (151), (179). La naturaleza no tóxica y su elevada solubilidad le confieren unas características interesantes para su uso en farmacología.

Con estos antecedentes, se sentaron las bases de la investigación llevada a cabo en esta Tesis y de los experimentos que se han descrito en ella.

En el **OBJETIVO UNO** se estudió el efecto protector de la administración intravenosa de PEG35 en un modelo de PAN en rata. Como se ha mencionado, la complicación

sistémica asociada a esta patología pancreática con un mayor porcentaje de mortalidad es la disfunción pulmonar. El objetivo principal que se seguía en estos experimentos era, por tanto, reducir esta afectación sistémica con el uso de PEG35.

En este estudio se demostró que, la unidosis de PEG35 administrada previamente a la inducción de la PAN protegía el tejido pancreático y pulmonar de los efectos deletéreos de esta enfermedad. La primera señal de esta protección se observó al medir la actividad de la enzima lipasa en plasma, uno de los marcadores de daño pancreático más utilizados, junto con la amilasa, para el diagnóstico de la PA. Así, la administración profiláctica de PEG 35 disminuía el daño pancreático al reducir significativamente los elevados niveles asociados a la inducción de la PA.

En la evaluación histopatológica del pulmón, observamos un descenso de los principales marcadores de inflamación y necrosis del tejido bajo el tratamiento de PEG35, tanto profiláctico como terapéutico. En cambio, en tejido pancreático solo disminuían estos marcadores con la administración profiláctica de este polímero. Esta última observación fue determinante para sugerir el efecto protector sistémico del PEG35 al administrarlo posteriormente a la inducción de la PAN.

La PA, como enfermedad de base inflamatoria, desencadena una respuesta del sistema inmune caracterizada por la activación de varias vías de señalización que desembocan en la liberación de citoquinas pro y antiinflamatorias. La medición de los niveles de estas citoquinas refleja la lesión inflamatoria provocada por esta enfermedad. Entre las principales citoquinas proinflamatorias, destaca la acción de IL-6, IL1-β y TNF-α, y de quimioquinas como CXCL-2. En este sentido, el PEG35 administrado por vía profiláctica fue capaz de reducir significativamente la elevada expresión génica en el páncreas de IL-6, IL1-β y CXCL-2 bajo la inducción de PAN. Sin embargo, su administración terapéutica no fue capaz de revertir la respuesta inflamatoria en el páncreas. Probablemente, el daño pancreático causado una vez se activa la cascada proinflamatoria durante la PAN es extremadamente difícil de revertir. En relación al pulmón, la inducción de PAN aumentó los niveles de TNF-α, IL-6, IL1-β y CXCL-2 comparado con el grupo control. La administración profiláctica de PEG35 redujo significativamente la expresión de IL-6, TNF-α e IL1-β mientras que la administración

terapéutica fue capaz de reducir los niveles de IL-6 y TNF-α, aun cuando la lesión pancreática era equivalente a la del grupo inducido con PA sin tratar.

Puesto que IL-6 se ha propuesto como un marcador de la severidad de la PA, así como un indicador del posible desarrollo de complicaciones sistémicas (160), (161) determinamos los niveles de esta interleuquina en plasma. Tras la inducción de la PAN, los elevados niveles de IL-6 indicaban la gravedad de la lesión pancreática en este modelo de pancreatitis. La administración de PEG35, tanto profiláctica como terapéutica, produjo un descenso significativo de los niveles sistémicos de esta citoquina en plasma, sugiriendo que la administración de este polímero está frenando la progresión de la PAN y sus efectos sistémicos.

Tras analizar la respuesta inflamatoria característica asociada a la PAN a través de los niveles de las citoquinas y quimioquinas, se realizó una evaluación de la expresión de moléculas de adhesión implicadas en daño pulmonar y pancreático. La P-selectina y la ICAM-1 son las principales moléculas de adhesión relacionadas con la inflamación aguda del páncreas e implicadas en la progresión sistémica de la PA debido a que median la infiltración de neutrófilos desde el endotelio al tejido pancreático y pulmonar (180), (181), (182), (183). El análisis de los niveles de expresión de P-selectina e ICAM-1 mostró un aumento significativo tras la inducción de la PAN, tanto en páncreas como en pulmón. Estos datos apoyaban la hipótesis de que la sobreestimulación de estas moléculas de adhesión está relacionada con la severidad de la PAN, como ya se había demostrado en otros estudios anteriores (184), (185). Como era de esperar, estos niveles se reducían en ambos tejidos bajo el pretratamiento con PEG 35. Por otro lado, aunque la administración terapéutica de PEG 35 redujo significativamente la expresión de P-selectina y de ICAM-1 en tejido pulmonar, no fue así en el páncreas, demostrando que el tratamiento terapéutico con PEG 35 ejerce un papel protector disminuyendo significativamente la respuesta inflamatoria en el pulmón asociada a la PA.

El reclutamiento y la infiltración de neutrófilos son unas de las principales señales identificadas en procesos inflamatorios, ambas facilitadas por las moléculas de adhesión P-selectina e ICAM-1. Esta característica se midió observando la presencia de la enzima mieloperoxidasa en el tejido pancreático y pulmonar. Esta enzima está almacenada en

los gránulos azurófilos de los neutrófilos por lo que su detección en tejido es indicativa de un proceso inflamatorio. Bajo la administración profiláctica de PEG35 había una menor presencia de neutrófilos infiltrados tanto en tejido pancreático como pulmonar, mientras que el tratamiento terapéutico solo reducía el número de neutrófilos en el pulmón. Estos datos nuevamente mostraban que la terapia con PEG35 estaba ejerciendo un efecto antiinflamatorio sistémico, reduciendo la extravasación de neutrófilos desde las células endoteliales.

En la PA, tanto a nivel experimental como en el curso de la enfermedad en pacientes, son bien conocidos los procesos de muerte celular por los que pasan las células acinares pancreáticas: necrosis y apoptosis. La determinación de la liberación de LDH, relacionada con la necrosis celular, nos permitió sugerir un efecto antinecrótico del PEG35 ya que, tanto la administración profiláctica como terapéutica redujo la actividad plasmática de esta enzima. Por otro lado, la caspasa-3 es una enzima que interviene en procesos apoptóticos celulares, por lo que la evaluación de su presencia en tejido refiere a la activación de este tipo de muerte programada en las células. En tejido pancreático y pulmonar se observó un aumento de su expresión a las 3 horas de la inducción de la PAN. Tanto el pretratamiento como el tratamiento de PEG35 redujo la presencia de la caspasa-3, ejerciendo un efecto protector frente a la apoptosis celular. Estos resultados son consistentes con los de un estudio previo en el que se demostró el papel antiapoptótico del PEG 15-20 en miocitos expuestos a condiciones de isquemia – reperfusión (151).

Tomando en conjunto todos estos resultados, pudimos demostrar un efecto protector del PEG35 en este modelo experimental al reducir la severidad de la PAN y proteger frente la muerte celular asociada a los procesos inflamatorios.

Teniendo en cuanta que la reversibilidad del daño pancreático es complicada de abordar en la práctica clínica, minimizar la respuesta inflamatoria sistémica es el principal objetivo en el tratamiento de esta enfermedad, con el fin de revertir el fallo multiorgánico y la morbilidad y mortalidad asociadas. Nuestros resultados sugieren la utilización de PEG35 como una potencial herramienta terapéutica para modular la progresión de la PAN hacia la letalidad.

Para el **OBJETIVO DOS** evaluamos la administración profiláctica por vía intravenosa del PEG35 en un modelo experimental de PAEI en ratas. Las características principales de este modelo son la formación de edema intersticial y la inflamación local del páncreas, que cursan con una clínica más leve que en el caso de la PAN.

En primer lugar, medimos los niveles plasmáticos de la enzima lipasa y observamos un aumento significativo de estos respecto a los grupos control. Tras la administración profiláctica de PEG35, la actividad de la lipasa pancreática se reducía hasta valores significativos, lo que indica que este polímero está ejerciendo un efecto protector local.

Para estudiar la formación de edema característica de esta patología, se evaluó una ratio de peso fresco-peso seco del órgano pancreático. Los resultados obtenidos indicaron un descenso significativo del contenido de líquido con la administración de PEG35. El edema se presenta como un aumento del volumen tisular ocasionado por la acumulación de líquido intersticial. La formación de edema puede deberse a un aumento de la presión hidrostática en el interior de los capilares sanguíneos, que puede ocasionar el flujo de agua hacia el espacio intersticial, un descenso de la presión oncótica capilar por la menor presencia de biomoléculas en el interior de los vasos que limita la capacidad de reabsorción de agua del espacio intersticial o bien un aumento de la presión oncótica intersticial con una mayor presencia de proteínas en el intersticio. Este desequilibrio entre presiones hidrostáticas y oncóticas es provocado por un aumento de la permeabilidad capilar, que altera el intercambio de fluido entre las membranas celulares. La hipovolemia provocada por el descenso en el volumen intravascular, así como la presencia de fluido intersticial, pueden terminar produciendo una hipoperfusión y necrosis pancreáticas de no poder resolverse mediante reanimación por hidratación (186), (187).

Existen diversos estudios que demuestran las propiedades oncóticas de los PEGs debidas a su naturaleza hidrofílica, y su interacción con la bicapa de fosfolípidos que constituye la membrana celular (188). Los efectos del PEG sobre el edema pueden deducirse de su capacidad para aumentar la presión oncótica en el interior del capilar, al interactuar con la membrana de las células endoteliales y restaurar la barrera

endotelial (189), así como disminuir la presión oncótica intersticial, pudiendo adherirse a las proteínas presentes en el intersticio aumentando su solubilidad (190).

Se ha descrito como principal desencadenante de la PA la activación prematura de las enzimas pancreáticas en las células acinares, con su consiguiente daño tisular y el desencadenamiento de una respuesta inflamatoria. Esta secreción enzimática conduce también a un daño microvascular alterando la permeabilidad de los capilares sanguíneos y a una liberación de citoquinas proinflamatorias (191).

El desencadenamiento de la respuesta inflamatoria comienza tras la activación de las vías de señalización que producen la liberación de las citoquinas proinflamatorias IL-6, IL-1β y TNF-α, principalmente. En el modelo experimental de PAEI, se observó un aumento significativo de los niveles de estas citoquinas en tejido pancreático con respecto a los grupos control. La administración profiláctica de PEG35 ejerció un efecto protector frente a la inflamación local al reducir la expresión génica de las citoquinas proinflamatorias. Además de estas citoquinas proinflamatorias, el aumento de la actividad de la enzima óxido nítrico sintasa inducible (iNOS) también está relacionado con la presencia de inflamación tisular, por lo que sus niveles eran susceptibles de verse afectados durante la PAEI. De esta manera, se examinó la expresión génica de iNOS viéndose un incremento significativo tras la inducción de la PA que disminuía con el pretratamiento de PEG35. Además, se estudió la liberación de factores antiinflamatorios, como IL-10, expresados en páncreas y se observó cómo aumentaban durante la PAEI. Al administrar el PEG35 profiláctico, los niveles de esta citoquina permanecían elevados, pues la señal de protección frente a la inflamación persistía tras la administración de este polímero.

Este modelo de PA, caracterizado por la presencia de edema intersticial local, también lleva asociados procesos de muerte celular que afectan a las células acinares pancreáticas. Así, observamos un aumento significativo en la liberación plasmática de LDH bajo la inducción de la PAEI. Los niveles plasmáticos de esta enzima descendieron con la administración profiláctica de PEG35, corroborando su efecto local antinecrótico. La muerte celular por apoptosis está también relacionada con la respuesta inflamatoria

de las células acinares pancreáticas durante la PAEI. De esta manera, se evaluaron indicadores apoptóticos como la caspasa-3 y la BCL-2 en el páncreas.

BCL-2 es un bloqueador de la liberación de citocromo C. La presencia de este en el citosol desencadena una cascada de señalización apoptótica que termina activando la caspasa-3, una de las principales caspasas efectoras que inician los procesos de degradación de determinadas biomoléculas en el interior de la célula. Los niveles elevados de BCL-2 impiden la salida del citocromo C, por lo que son una señal de protección frente a la apoptosis celular. En cambio, un incremento de la expresión de la caspasa-3 indica que se está activando una respuesta apoptótica en las células. Durante la PAEI, los niveles de la caspasa-3 aumentaron, como cabía esperar ya que la aparición de edema y la inflamación celular están estrechamente relacionadas con la apoptosis, mientras que la expresión proteica de BCL-2 disminuyó. De este modo, con estos resultados pudimos determinar que el PEG35 presenta un efecto antiapoptótico, al mantenerse elevada la expresión de BCL-2 y reducirse los niveles de caspasa-3 en tejido pancreático.

Para completar la investigación de los efectos protectores del PEG35 frente a patologías pancreáticas *in vivo*, se realizaron experimentos *in vitro* con células acinares pancreáticas y macrófagos.

Así, en el **OBJETIVO TRES** observamos los efectos del PEG35 en cultivos de células acinares pancreáticas de la línea AR42J bajo distintos estímulos de inflamación y muerte celular.

En uno de nuestros modelos experimentales en ratas se inyectaron sucesivas dosis de ceruleina para inducir una PA leve. Este es un modelo ampliamente utilizado en experimentación animal que, sin embargo, no se había estudiado mucho en cultivo celular. En las dos últimas décadas, comienza a aparecer bibliografía en la que se describe la utilización de la ceruleína, compuesto análogo a la CCK, en modelos experimentales *in vitro* con células acinares pancreáticas como las AR42J (192), (193).

La administración de ceruleína produce una liberación de citoquinas proinflamatorias desde las células acinares del páncreas. De esta manera, se evaluó la

expresión génica de IL-1β y TNF-α en un modelo experimental con células AR42J en el que se administraron diferentes concentraciones de PEG35 previas al tratamiento con ceruleína. Así, se pudo observar un efecto de dosis-respuesta por parte del PEG35, pues la respuesta inflamatoria correspondiente a los niveles de estas citoquinas disminuía a medida que aumentaba la concentración de este polímero.

Otro modelo experimental *in vitro* que desencadena una respuesta inflamatoria en las células acinares pancreáticas es la administración de TNF-α (194). El pretratamiento de las AR42J con PEG35 disminuía los niveles de expresión de marcadores moleculares proinflamatorios, como TNF-α e iNOS, de manera dosis-dependiente, reafirmando así los resultados obtenidos bajo la administración de ceruleína.

Se ha descrito cómo en los modelos experimentales *in vivo* de PA el PEG35 actúa también sobre las vías de señalización relacionadas con procesos de muerte celular que tienen lugar durante el desarrollo de esta enfermedad pancreática. El control de la muerte celular puede limitar los efectos sistémicos de la PA, ya que se han relacionado los procesos de apoptosis celular con una menor severidad (195).

Durante la PA, las células pancreáticas acinares activan vías apoptóticas y se liberan patrones de daño molecular (DAMPs). Estos suelen ser fácilmente fagocitados por las células, pero cuando la tasa de fagocitosis es superada por la apoptosis celular, los DAMPs se pueden acumular en el interior de las células y llegar a agravar el daño pancreático (196). Este es uno de los motivos por los que resulta interesante pensar en estrategias de tratamiento contra la propagación de la muerte celular asociada a la PA. Para ello, se realizó un estudio de proliferación celular *in vitro*, en el cual se evaluó el efecto de la administración de PEG35 en células AR42J tratadas con estaurosporina, un alcaloide natural aislado de la *Streptomyces staurosporeus* y potente inductor de apoptosis (197). Corroborando los datos sobre muerte celular obtenidos en los experimentos *in vivo*, el pretratamiento con PEG35 mantenía la viabilidad de las células, observándose un aumento en respuesta a concentraciones crecientes de PEG35.

Por último, el efecto antinecrótico del PEG35 se evaluó de igual manera *in vitro* mediante un examen de LDH en el sobrenadante celular, bajo tres tipos de estímulos

proinflamatorios: ceruleína, TNF-α y LPS. La administración de PEG35 reducía los niveles de LDH de manera dosis-dependiente en cada uno de ellos.

Por último, en el **OBJETIVO CUATRO** estudiamos el efecto del PEG35 en la respuesta inflamatoria mediada por exosomas. Estas nanovesículas se han relacionado con la activación de vías de señalización inflamatorias capaces de estimular a los TLRs de las membranas celulares.

La incubación de monocitos humanos THP-1 diferenciados a macrófagos, con concentraciones crecientes de PEG35 y en presencia de exosomas aumentaba la captación de estas nanovesículas. Con la presencia de PEG35 en el medio de cultivo, los macrófagos eran capaces de incorporar un mayor número de exosomas en su interior. Asimismo, debido a que los macrófagos internalizan exosomas con mayor eficiencia que las células no fagocíticas, corroboramos este efecto del PEG35 en líneas celulares epiteliales, BICR-18 y CAPAN-2.

Teniendo en cuenta que los exosomas pueden desencadenar la activación de citoquinas proinflamatorias, estudiamos si este aumento en la captación provocado por el PEG35 venía acompañado también de un incremento de la respuesta inflamatoria en las células. Para ello, utilizamos exosomas extraídos del plasma procedente de ratas en las que se había inducido una PA y evaluamos los niveles de algunas de las principales citoquinas proinflamatorias, IL1-β y TNF-α, en los macrófagos incubados con estos exosomas. De esta manera, determinamos que la incorporación de PEG35 al medio de cultivo disminuía la expresión de estas citoquinas. De acuerdo con lo que ya habíamos observado en otros modelos experimentales, el PEG35 presentaba un efecto antiinflamatorio que también era capaz de reducir la inflamación inducida por los exosomas.

Por otro lado, estudiamos la localización celular del PEG35 en macrófagos y pudimos observar que la incubación de estos con una mayor concentración de PEG35, aumentaba la presencia de este polímero en el citoplasma. Teniendo en cuenta una de las características del PEG35 destacadas anteriormente como es su capacidad de interactuar con las membranas celulares es posible que ésta influya facilitando la

internalización de los exosomas, ya que estos presentan una estructura externa de bicapa fosfolipídica, similar a la superficie celular (198).

En conclusión, el PEG35 modula la respuesta inflamatoria mediada por los exosomas y favorece su captación.

Globalmente, los resultados obtenidos a lo largo de esta Tesis Doctoral nos llevan a proponer un efecto protector del PEG35 frente a la inflamación y la muerte celular asociadas a la PA. Esto, unido a sus interesantes características fisicoquímicas, convierten a este polímero en una molécula biocompatible, cuyo estudio podría ofrecer una nueva herramienta para el tratamiento clínico de patologías inflamatorias del páncreas. De esta manera, el uso del PEG35 podría ser una nueva estrategia de terapia para las complicaciones letales de la PAN, así como para el tratamiento de la inflamación local asociada a la PAEI.

CONCLUSIONES

CONCLUSIONES

Las conclusiones que se pueden deducir de los estudios que componen esta Tesis Doctoral son las siguientes:

1. La administración profiláctica de PEG35 reduce la inflamación local pancreática y pulmonar en un modelo de PAN en rata. El pretratamiento con PEG35 protege también frente a la muerte celular asociada a la PAN.

2. La administración terapéutica de PEG35 reduce la severidad del proceso inflamatorio pulmonar asociado a la PAN, aun cuando la inflamación pancreática es equivalente a la de una PA sin tratar. Asimismo, la muerte celular asociada al proceso inflamatorio también se ve reducida.

3. El PEG35 reduce la lesión local pancreática en un modelo de PAEI en rata a través de la disminución del proceso inflamatorio y la muerte celular asociada.

4. El PEG35 protege frente al daño celular en células acinares pancreáticas AR42J tratadas con estímulos inflamatorios y apoptóticos.

5. EL PEG35 aumenta la captación celular de exosomas, tanto en células fagocíticas, como los macrófagos, como en líneas celulares epiteliales. Además, el PEG35 favorece su propia internalización en macrófagos.

6. El PEG35 modula el proceso inflamatorio inducido por los exosomas procedentes de una PA.

BIBLIOGRAFÍA

1. Agrawal RM, Agrawal ST, Aoun E. The Physiology of the Pancreas. 2014.

2. Bockman DE. The Pancreas: Biology, Pathobiology and Disease. Second. Raven Press; 1993.

3. Latajet M, Ruiz Liard A. Anatomía Humana. 5ª. Editorial Médica Panamericana, editor. 2007.

4. Feldman Mark, Friedman Lawrence S. BLJ. Anatomy, Histology, Embryology, and Developmental Anomalies of the Pancreas: Sleisenger and Fordtran's Gastrointestinal and Liver Disease. Volume 1. Philadelphia, PA: Saunders, Elsevier; 2010. 385–388.

5. Drake RL, Vogl AW, Mitchell AW, M. Gray. Anatomía para estudiantes. 3ª. Elsevier, editor. 2015.

7. Cesmebasi A, Malefant J, Patel SD, Plessis M Du, Renna S, Tubbs RS, et al. The surgical anatomy of the lymphatic system of the pancreas. Clin Anat. 2015 May 1 ;28(4):527–37.

8. Babic T, Travagli RA. Neural Control of the Pancreas. 2016;

9. Eynard AR, Valentich MA, Rovasio RA. Histología y embriología humanas : bases celulares y moleculares con orientación clínico-patológica. Editorial Médica Panamericana; 2016. 800 p.

10. Sastre J, Sabater L, Aparisi L. Physiology of pancreatic secretion. Gastroenterol Hepatol. 2005 Feb 15;28(SUPPL. 2):3–9.

11. Shahid Z, Singh G. Physiology, Islets of Langerhans. StatPearls. StatPearls Publishing; 2019.

12. Bonner-Weir S, Sullivan BA, Weir GC. Human Islet Morphology Revisited: Human and Rodent Islets Are Not So Different After All. J Histochem Cytochem. 2015 Aug 20;63(8):604–12.

13. Da Silva Xavier G. The Cells of the Islets of Langerhans. J Clin Med. 2018 Mar 12;7(3):54.

14. Ehrhardt JD, Gomez F. Embryology, Pancreas [Internet]. StatPearls. StatPearls Publishing; 2020.

15. Rohen JW (Johannes W, Lütjen-Drecoll E. Embriología funcional una perspectiva desde la biología del desarrollo. Médica Panamericana; 2008.

16. Sánchez-Bernal C, San Román García JI, López Rodríguez MA, Calvo Andrés JJ, Navarro S, Pérez-Mateo M GL. Fisiología y bioquímica del páncreas. Tratado de páncreas exocrino. Ediciones Médicas; 2002.

17. Feldman Mark, Friedman Lawrence S. BLJ. Sleisenger and Fordtran's Gastrointestinal and Liver Disease. ninth. Elsevier; 2010.

18. Pandol SJ. The Exocrine Pancreas. Morgan & Claypool Life Sciences; 2011.

19. Hu K, Carroll J, Fedorovich S, Rickman C, Sukhodub A, Davietov B. Vesicular restriction of synaptobrevin suggests a role for calcium in membrane fusion. Nature. 2002 Feb 7;415(6872):646–50.

20. Rinderknecht H. Activation of pancreatic zymogens. Normal activation, premature intrapancreatic activation, protective mechanisms against inappropriate activation. Dig Dis Sci. 1986 Mar;31(3):314–21.

21. Whitcomb DC, Lowe ME. Human pancreatic digestive enzymes. Vol. 52, Digestive diseases and sciences. 2007. p. 1–17.

22. Hall JE, Guyton AC. Guyton y Hall. Tratado de fisiología médica. 2016

23. Tresguerres AF. Fisiología humana. 4ª. McGraw Hill, editor. 2010

24. Williams JA. Pancreatic Polypeptide. 2014.

25. Chey WY, Chang TM. Neural hormonal regulation of exocrine pancreatic secretion. In: Pancreatology. Elsevier B.V.; 2001. p. 320–35.

26. Boden G, Sivitz MC, Owen OE, Essa-Koumar N, Landor JH. Somatostatin suppresses secretin and pancreatic exocrine secretion. Science (80-). 1975;190(4210):163–5.

27. Yamada T, Alpers DH, Kalloo AN, Kaplowitz N, Owyang C, Powell DW. Textbook of Gastroenterology, Fifth Edition. Vols. 1–2, Textbook of Gastroenterology, Fifth Edition. Blackwell Publishing Ltd.; 2009. 1–3551 p.

28. Lenz HJ, Messmer B, Zimmerman FG. Noradrenergic inhibition of canine gallbladder contraction and murine pancreatic secretion during stress by corticotropin-releasing factor. J Clin Invest. 1992 Feb;89(2):437–43.

29. Liddle RA. Regulation of cholecystokinin secretion by intraluminal releasing factors. Vol. 269, American Journal of Physiology - Gastrointestinal and Liver Physiology. 1995.

30. Feldman M, Friedman L, Brandt L. Pancreatic physiology and secretory testing: Sleisenger and Fordtran's Gastrointestinal and Liver Disease. 9th ed. WB Saunders Company, editor. 1998.

31. Lee M, Chung S, Dae YU, Myoung KP. Regulation of zymogen granule exocytosis by Ca2+, cAMP, and PKC in pancreatic acinar cells. Biochem Biophys Res Commun. 2005 Sep 9;334(4):1241–7.

32. Hokin LE. Effects of calcium omission on acetylcholine-stimulated amylase secretion and phospholipid synthesis in pigeon pancreas slices. BBA - Gen Subj. 1966 Jan 25;115(1):219–21.

33. Messenger SW, Falkowski MA, Groblewski GE. Ca2+-regulated secretory granule exocytosis in pancreatic and parotid acinar cells. Vol. 55, Cell Calcium. Elsevier

Ltd; 2014. p. 369–75.

34. H Fitz R. Acute Pancreatitis — A Consideration of Pancreatic Hemorrhage, Hemorrhagic, Suppurative, and Gangrenous Pancreatitis, and of Disseminated Fat-Necrosis. the Middleton Goldsmith Lecture for 1889. Bost Med Surg J . 1889;120.

35. Beger HG, Rau BM. Natural Course of Acute Pancreatitis. In: Diseases of the Pancreas Current Surgical Therapy. Springer; 2008.

36. Domínguez-Muñoz JE. Natural Course of Chronic Pancreatitis. In: Diseases of the Pancreas Current Surgical Therapy. Springer; 2008.

37. Di Sebastiano P, Di Mola FF. Mechanisms of Pain in Chronic Pancreatitis. In: Diseases of the Pancreas Current Surgical Therapy. Springer; 2008.

38. Medicina A DE, Bustamante Durón D, García Laínez A, Umanzor García W, Leiva Rubio L, Barrientos Rosales A, et al. Acute Pancreatitis: Current Evidence. 2017 ;14:4.

39. Campbell F, Verbeke CS, Campbell F, Verbeke CS. Embryology, Anatomy, and Histology. In: Pathology of the Pancreas. Springer London; 2013. p. 3–20.

40. Yadav D, Lowenfels AB. The epidemiology of pancreatitis and pancreatic cancer. Gastroenterology. 2013;144(6):1252–61.

41. Roberts SE, Morrison-Rees S, John A, Williams JG, Brown TH, Samuel DG. The incidence and aetiology of acute pancreatitis across Europe [Internet]. Vol. 17, Pancreatology. Elsevier B.V.; 2017 . p. 155–65. 42. Valverde-López F, Wilcox CM, Redondo-Cerezo E. Evaluation and management of acute pancreatitis in Spain. J Gastroenterol Hepatol. 2018;41.

43. Xiao AY, Tan MLY, Wu LM, Asrani VM, Windsor JA, Yadav D, et al. Global incidence and mortality of pancreatic diseases: a systematic review, meta-analysis, and meta-regression of population-based cohort studies. Lancet Gastroenterol Hepatol. 2016;1(1):45–55.

44. Acharya C, Cline RA, Jaligama D, Noel P, Delany JP, Bae K, et al. Fibrosis reduces severity of acute-on-chronic pancreatitis in humans. Gastroenterology. 2013 Aug [cited 2020 Mar 26];145(2):466–75.

45. Petrov MS, Yadav D. Global epidemiology and holistic prevention of pancreatitis. Vol. 16, Nature Reviews Gastroenterology and Hepatology. Nature Publishing Group; 2019. p. 175–84.

46. Lankish PG, Maisonneuve P, Lowenfels AB. Etiology and Epidemiology of Acute Pancreatitis. In: Diseases of the Pancreas: Current Surgical Therapy. Springer; 2008.

47. Huh JH, Jeon H, Park SM, Choi E, Lee GS, Kim JW, et al. Diabetes Mellitus is Associated with Mortality in Acute Pancreatitis. J Clin Gastroenterol .

2018;52(2):178–83. 48. Wu BU, Johannes RS, Kurtz S, Banks PA. The Impact of Hospital-Acquired Infection on Outcome in Acute Pancreatitis. Gastroenterology . 2008 Sep;135(3):816–20. 49. Gardner TB, Vege SS, Pearson RK, Chari ST. Fluid Resuscitation in Acute Pancreatitis. Vol. 6, Clinical Gastroenterology and Hepatology. 2008. p. 1070–6.

50. Párniczky A, Kui B, Szentesi A, Balázs A, Szűcs Á, Mosztbacher D, et al. Prospective, Multicentre, Nationwide Clinical Data from 600 Cases of Acute Pancreatitis. Einwächter H, editor. PLoS One . 2016 Oct 31 ;11(10):e0165309. 51. Bollen TL. Acute pancreatitis: International classification and nomenclature. Vol. 71, Clinical Radiology. W.B. Saunders Ltd; 2016. p. 121–33.

52. Da Silva S, Rocha M, Pinto-De-Sousa J. Acute Pancreatitis Etiology Investigation: A Workup Algorithm Proposal. Vol. 24, GE Portuguese Journal of Gastroenterology. S. Karger AG; 2017. p. 129–36.

53. Lankisch PG, Apte M, Banks PA. Acute pancreatitis. Lancet. 2015;386(9988):85–96.

54. Dawra R, Sah RP, Dudeja V, Rishi L, Talukdar R, Garg P, et al. Intra-acinar trypsinogen activation mediates early stages of pancreatic injury but not inflammation in mice with acute pancreatitis. Gastroenterology. 2011 Dec;141(6):2210-2217.e2.

55. Testoni PA. Acute recurrent pancreatitis: Etiopathogenesis, diagnosis and treatment. World J Gastroenterol. 2014 Dec 7;20(45):16891–901.

56. Apte M V., Pirola RC, Wilson JS. Mechanisms of alcoholic pancreatitis. J Gastroenterol Hepatol. 2010 Dec;25(12):1816–26.

57. Alexandre M, Uduman AK, Minervini S, Raoof A, Shugrue CA, Akinbiyi EO, et al. Tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone initiates and enhances pancreatitis responses. Am J Physiol - Gastrointest Liver Physiol. 2012 Sep 15;303(6):G696-704.

58. Rossell MC. Historia del Páncreas y de la evolución de los conceptos y la clasificación de Pancreatitis. Rev Gastroenterol del Perú. 2002;22.

59. Talukdar R, Swaroop Vege S. Classification Systems for the Severity of Acute Pancreatitis.

60. Sarr MG. 2012 revision of the Atlanta classifcation of acute pancreatitis. Vol. 123, Polskie Archiwum Medycyny Wewnetrznej. Medycyna Praktyczna; 2013. p. 118–24.

61. Chatila AT, Bilal M, Guturu P. Evaluation and management of acute pancreatitis. Vol. 7, World Journal of Clinical Cases. Baishideng Publishing Group Co; 2019. p. 1006–20.

62. Scallan J, Huxley VH, Korthuis RJ. Capillary Fluid Exchange: Regulation, Functions, and Pathology. Colloq Ser Integr Syst Physiol From Mol to Funct. 2010 Jan

14;2(1):1–94.

63. Zerem E. Treatment of severe acute pancreatitis and its complications. Vol. 20, World Journal of Gastroenterology. WJG Press; 2014. p. 13879–92.

64. Kylänpää L, Rakonczay Z, O'reilly DA. The Clinical Course of Acute Pancreatitis and the Inflammatory Mediators That Drive It. Int J Inflam. 2012;2012.

65. Habtezion A. Inflammation in acute and chronic pancreatitis. Vol. 31, Current Opinion in Gastroenterology. Lippincott Williams and Wilkins; 2015. p. 395–9.

66. Gukovskaya AS, Gukovsky I, Algül H, Habtezion A. Autophagy, Inflammation, and Immune Dysfunction in the Pathogenesis of Pancreatitis. Vol. 153, Gastroenterology. W.B. Saunders; 2017. p. 1212–26.

67. Manohar M, Verma AK, Venkateshaiah SU, Sanders NL, Mishra A. Pathogenic mechanisms of pancreatitis. World J Gastrointest Pharmacol Ther. 2017 Feb 6;8(1):10.

68. Tasci S, Senocak R, Lapsekili E, Kaymak S, Yigit T. The effects of a TNF-alpha inhibitor and HBO combination on the severity of pancreatitis and oxidative response in an experimental model of acute pancreatitis. Bratislava Med J. 2019 ;120(6):417–22.

69. Malleo G, Mazzon E, Siriwardena AK, Cuzzocrea S. Role of tumor necrosis factor-α in acute pancreatitis: From biological basis to clinical evidence. Vol. 28, Shock. 2007. p. 130–40.

70. Zhao H, Jaffer T, Eguchi S, Wang Z, Linkermann A, Ma D. Role of necroptosis in the pathogenesis of solid organ injury. Cell Death Dis. 2015;6(11).

71. Hitomi J, Christofferson DE, Ng A, Yao J, Degterev A, Xavier RJ, et al. Identification of a Molecular Signaling Network that Regulates a Cellular Necrotic Cell Death Pathway. Cell. 2008 Dec 26;135(7):1311–23.

72. Vanlangenakker N, Bertrand MJM, Bogaert P, Vandenabeele P, Vanden Berghe T. TNF-induced necroptosis in L929 cells is tightly regulated by multiple TNFR1 complex i and II members. Cell Death Dis. 2011 Nov 17;2(11):e230.

73. Li J, McQuade T, Siemer AB, Napetschnig J, Moriwaki K, Hsiao YS, et al. The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. Cell. 2012 Jul 20;150(2):339–50.

74. Cai Z, Jitkaew S, Zhao J, Chiang HC, Choksi S, Liu J, et al. Plasma membrane translocation of trimerized MLKL protein is required for TNF-induced necroptosis. Nat Cell Biol. 2014 Jan;16(1):55–65.

75. Tanaka T, Narazaki M, Kishimoto T. Il-6 in inflammation, Immunity, And disease. Cold Spring Harb Perspect Biol. 2014 Oct 1;6(10).

76. Schmidt-Arras D, Rose-John S. IL-6 pathway in the liver: From physiopathology to therapy. Vol. 64, Journal of Hepatology. Elsevier B.V.; 2016. p. 1403–15.

77. Papachristou GI. Prediction of severe acute pancreatitis: Current knowledge and novel insights. Vol. 14, World Journal of Gastroenterology. Baishideng Publishing Group Inc; 2008. p. 6273–5.

78. Wang Y, Bu C, Wu K, Wang R, Wang J. Curcumin protects the pancreas from acute pancreatitis via the mitogen-activated protein kinase signaling pathway. Mol Med Rep. 2019;20(4):3027–34.

79. Wang C. Obesity, Inflammation, and Lung Injury (OILI): The Good. Mediators Inflamm. 2014;2014.

80. Jones SA, Richards PJ, Scheller J, Rose-John S. IL-6 transsignaling: The in vivo consequences. Vol. 25, Journal of Interferon and Cytokine Research. 2005. p. 241–53.

81. Lesina M, Wörmann SM, Neuhöfer P, Song L, Algül H. Interleukin-6 in inflammatory and malignant diseases of the pancreas. Vol. 26, Seminars in Immunology. Academic Press; 2014. p. 80–7.

82. Zhang H, Neuhöfer P, Song L, Rabe B, Lesina M, Kurkowski MU, et al. IL-6 trans-signaling promotes pancreatitis-associated lung injury and lethality. J Clin Invest. 2013 Mar 1;123(3):1019–31.

83. Plotnikov A, Zehorai E, Procaccia S, Seger R. The MAPK cascades: Signaling components, nuclear roles and mechanisms of nuclear translocation. Vol. 1813, Biochimica et Biophysica Acta - Molecular Cell Research. Elsevier; 2011. p. 1619–33.

84. Legendre F, Bogdanowicz P, Boumediene K, Pujol J-P. transcription and mitogen-activated protein kinase/extracellular. Role of interleukin 6 (IL-6)/IL-6R-induced signal tranducers and activators of. Vol. 32, The Journal of Rheumatology Rheumatology The Journal of on July. 2005

85. Kany S, Vollrath JT, Relja B. Cytokines in inflammatory disease. Vol. 20, International Journal of Molecular Sciences. MDPI AG; 2019.

86. Dinarello CA. Immunological and Inflammatory Functions of the Interleukin-1 Family. Annu Rev Immunol. 2009 Apr;27(1):519–50.

87. Agostini L, Martinon F, Burns K, McDermott MF, Hawkins PN, Tschopp J. NALP3 forms an IL-1β-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. Immunity. 2004 Mar;20(3):319–25.

88. Lopez-Castejon G, Brough D. Understanding the mechanism of IL-1β secretion. Vol. 22, Cytokine and Growth Factor Reviews. Elsevier; 2011. p. 189–95.

89. Liu T, Zhang L, Joo D, Sun SC. NF-κB signaling in inflammation. Vol. 2, Signal Transduction and Targeted Therapy. Springer Nature; 2017. p. 17023.

90. Xu B, Bai B, Sha S, Yu P, An Y, Wang S, et al. Interleukin-1β induces autophagy by affecting calcium homeostasis and trypsinogen activation in pancreatic acinar

cells. Int J Clin Exp Pathol. 2014;7(7):3620–31.

91. Harris J. Autophagy and cytokines. Vol. 56, Cytokine. 2011. p. 140–4.

92. Panahi Y, Fakhari S, Mohammadi M, Rahmani MR, Hakhamaneshi MS, Jalili A. Glycyrrhizin down-regulates CCL2 and CXCL2 expression in cerulein-stimulated pancreatic acinar cells. Am J Clin Exp Immunol. 2015;4(1):1–6.

93. De Filippo K, Henderson RB, Laschinger M, Hogg N. Neutrophil Chemokines KC and Macrophage-Inflammatory Protein-2 Are Newly Synthesized by Tissue Macrophages Using Distinct TLR Signaling Pathways. J Immunol. 2008 Mar 15;180(6):4308–15.

94. Boro M, Balaji KN. Coupled Receptor CXCR2 − Inflammasome Activation via G-Protein CXCL1 and CXCL2 Regulate NLRP3. J Immunol Ref. 2020;199:1660–71.

95. Al-Alwan LA, Chang Y, Mogas A, Halayko AJ, Baglole CJ, Martin JG, et al. Differential Roles of CXCL2 and CXCL3 and Their Receptors in Regulating Normal and Asthmatic Airway Smooth Muscle Cell Migration. J Immunol. 2013 Sep 1;191(5):2731–41.

96. Osakabe N, Takano H, Sanbongi C, Yasuda A, Yanagisawa R, Inoue KI, et al. Anti-inflammatory and anti-allergic effect of rosmarinic acid (RA); Inhibition of seasonal allergic rhinoconjunctivitis (SAR) and its mechanism. In: BioFactors. IOS Press; 2004 [cited 2020 Jul 29]. p. 127–31.

97. Sato T, Shibata W, Maeda S. Adhesion molecules and pancreatitis. Vol. 54, Journal of Gastroenterology. Springer Tokyo; 2019. p. 99–107.

98. Staubli SM, Oertli D, Nebiker CA. Laboratory markers predicting severity of acute pancreatitis. Vol. 52, Critical Reviews in Clinical Laboratory Sciences. Taylor and Francis Ltd; 2015. p. 273–83.

99. Donald C, Josep M. Role of P-selectin and ICAM-1 in Pancreatitis-Induced Lung Inflammation in Rats. Emma Julin Pans Emilio Gelp Joan RosellCatafau Piqu Laureano Fernndez sl Ann Surg Vol 230. 1999;6 SRC-B.

100. Panicker SR, Mehta-D'Souza P, Zhang N, Klopocki AG, Shao B, McEver RP. Circulating soluble P-selectin must dimerize to promote inflammation and coagulation in mice. Blood. 2017 Jul 13;130(2):181–91.

101. Perkins LA, Anderson CJ, Novelli EM. Targeting P-selectin adhesion molecule in molecular imaging: P-selectin expression as a valuable imaging biomarker of inflammation in cardiovascular disease. Vol. 60, Journal of Nuclear Medicine. Society of Nuclear Medicine Inc.; 2019. p. 1691–7.

102. Ramachandran V, Yago T, Epperson TK, Kobzdej MMA, Nollert MU, Cummings RD, et al. Dimerization of a selectin and its ligand stabilizes cell rolling and enhances tether strength in shear flow. Proc Natl Acad Sci U S A. 2001 Aug 28;98(18):10166–71.

103. Steyers CM, Miller FJ. Endothelial dysfunction in chronic inflammatory diseases. Vol. 15, International Journal of Molecular Sciences. MDPI AG; 2014. p. 11324–49.

104. Lawson C, Wolf S. ICAM-1 signaling in endothelial cells. 2009.

105. Walling BL, Kim M. LFA-1 in T cell migration and differentiation [Internet]. Vol. 9, Frontiers in Immunology. Frontiers Media S.A.; 2018.

106. Hartwig W, Werner J, Warshaw AL, Antoniu B, Fernandez-Del Castillo C, Gebhard M-M, et al. Membrane-bound ICAM-1 is upregulated by trypsin and contributes to leukocyte migration in acute pancreatitis. Am J Physiol Gastrointest Liver Physiol. 2004;287:1194–9.

107. Kang R, Lotze MT, Zeh HJ, Billiar TR, Tang D. Cell death and DAMPs in acute pancreatitis. Mol Med. 2014;20(JULY-DECEMBER 2014):466–77.

108. Olga A, Stephen J, Anna S. Cell Death in Pancreatitis. Peggy Gukovskaya sl J Biol Chem Vol 281. 2006 SRC.

109. Berghe T Vanden, Linkermann A, Jouan-Lanhouet S, Walczak H, Vandenabeele P. Regulated necrosis: The expanding network of non-apoptotic cell death pathways. Vol. 15, Nature Reviews Molecular Cell Biology. Nature Publishing Group; 2014. p. 135–47.

110. Dondelinger Y, Declercq W, Montessuit S, Roelandt R, Goncalves A, Bruggeman I, et al. MLKL Compromises Plasma Membrane Integrity by Binding to Phosphatidylinositol Phosphates. Cell Rep. 2014 May 22;7(4):971–81.

111. Weinlich R, Oberst A, Beere HM, Green DR. Necroptosis in development, inflammation and disease. Vol. 18, Nature Reviews Molecular Cell Biology. Nature Publishing Group; 2017. p. 127–36.

112. Mareninova OA, Sung KF, Hong P, Lugea A, Pandol SJ, Gukovsky I, et al. Cell death in pancreatitis: Caspases protect from necrotizing pancreatitis. J Biol Chem. 2006 Feb 10;281(6):3370–81.

113. Green DR, Llambi F. Cell death signaling. Cold Spring Harb Perspect Biol. 2015 Dec 1;7(12).

114. Davidovich P, Kearney CJ, Martin SJ. Inflammatory outcomes of apoptosis, necrosis and necroptosis. In: Biological Chemistry. Walter de Gruyter GmbH; 2014. p. 1163–71.

115. Nagata S, Hanayama R, Kawane K. Autoimmunity and the Clearance of Dead Cells. Vol. 140, Cell. Elsevier; 2010. p. 619–30.

116. Vince JE, Silke J. The intersection of cell death and inflammasome activation. Vol. 73, Cellular and Molecular Life Sciences. Birkhauser Verlag AG; 2016. p. 2349–67.

117. Gutierrez KD, Davis MA, Daniels BP, Olsen TM, Ralli-Jain P, Tait SWG, et al. MLKL Activation Triggers NLRP3-Mediated Processing and Release of IL-1β

Independently of Gasdermin-D. J Immunol. 2017 Mar 1;198(5):2156–64.

118. Wang X, Jiang W, Yan Y, Gong T, Han J, Tian Z, et al. RNA viruses promote activation of the NLRP3 inflammasome through a RIP1-RIP3-DRP1 signaling pathway. Nat Immunol. 2014 Nov 18;15(12):1126–33.

119. Lee K-H, Kang T-B. The Molecular Links between Cell Death and Inflammasome. Cells. 2019 Sep 10;8(9):1057.

120. Bhatia M, Fei LW, Cao Y, Hon YL, Huang J, Puneet P, et al. Pathophysiology of acute pancreatitis. Vol. 5, Pancreatology. Elsevier B.V.; 2005. p. 132–44.

121. Bonjoch L, Casas V, Carrascal M, Closa D. Involvement of exosomes in lung inflammation associated with experimental acute pancreatitis. J Pathol. 2016 Oct 1;240(2):235–45.

122. He C, Zheng S, Luo Y, Wang B. Exosome theranostics: Biology and translational medicine. Vol. 8, Theranostics. Ivyspring International Publisher; 2018. p. 237–55.

123. Jiménez-Alesanco A, Marcuello M, Pastor-Jiménez M, López-Puerto L, Bonjoch L, Gironella M, et al. Acute pancreatitis promotes the generation of two different exosome populations. Sci Rep. 2019 Dec 1;9(1).

124. Guo XY, Xiao F, Li J, Zhou YN, Zhang WJ, Sun B, et al. Exosomes and pancreatic diseases: Status, challenges, and hopes. Vol. 15, International Journal of Biological Sciences. Ivyspring International Publisher; 2019. p. 1846–60.

125. Gea-Sorlí S. Role of macrophages in the progression of acute pancreatitis. World J Gastrointest Pharmacol Ther. 2010;1(5):107.

126. Nigeen H, Timothy B. Advances in Management of Acute Pancreatitis. Gardner Janisch sl Gastroenterol Clin North Am Vol 45. 2016;1 SRC-B.

127. Cruz-Santamaría DM. Update on pathogenesis and clinical management of acute pancreatitis. World J Gastrointest Pathophysiol. 2012;3(3):60.

128. Mao E. Intensive management of severe acute pancreatitis. Ann Transl Med. 2019;7(22):687.

129. Alamsyah Siregar G, Putra Siregar G. Management of Severe Acute Pancreatitis the Creative Commons Attribution-NonCommercial 4.0 International License (CC BY-NC 4.0). Maced J Med Sci. 2019;7(19):3319–23.

130. Jennifer K. Acute Pancreatitis: Diagnosis, Prognosis, and Treatment. Teresa Gipson sl Am Fam Physician Vol 75. 2007 SRC.

131. Sainio V, Kemppainen E, Puolakkainen P, Taavitsainen M, Kivisaari L, Valtonen V, et al. Early antibiotic treatment in acute necrotising pancreatitis . Schrder T sl Lancet Vol 346. 1995 SRC.

132. Tenner S, Baillie J, Dewitt J, Vege SS. American college of gastroenterology guideline: Management of acute pancreatitis. Am J Gastroenterol.

2013;108(9):1400–15.

133. Nesvaderani M, Eslick GD, Vagg D, Faraj S, Cox MR. Epidemiology, aetiology and outcomes of acute pancreatitis: A retrospective cohort study. Int J Surg. 2015 Nov 1;23:68–74.

134. Scott K, Jonathan G, Robin D. Polyethylene glycol and solutions of polyethylene glycol as green reaction media. Ji Chen Spear Huddlest Rogers sl Green Chem Vol 7. 2005 SRC.

135. Bohn P, Meier MAR. Uniform poly(ethylene glycol): a comparative study. Polym J. 2020 Jan 1;52(1):165–78.

136. Hauet T, Eugene M. A new approach in organ preservation: Potential role of new polymers. Vol. 74, Kidney International. Elsevier; 2008. p. 998–1003.

137. Zhang P, Sun F, Liu S, Jiang S. Anti-PEG antibodies in the clinic: Current issues and beyond PEGylation. J Control Release. 2016 Dec 28;244(Pt B):184–93.

138. Suk JS, Xu Q, Kim N, Hanes J, Ensign LM. PEGylation as a strategy for improving nanoparticle-based drug and gene delivery. Vol. 99, Advanced Drug Delivery Reviews. Elsevier B.V.; 2016. p. 28–51.

139. Morar AS, Schrimsher JL, Chavez MD. PEGylation of proteins: A structural approach. BioPharm Int. 2006 Apr 1;19:34–49.

140. Abuchowski A, McCoy JR, Palczuk NC, van Es T, Davis FF. Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. J Biol Chem. 1977;252(11):3582–6.

141. Garay RP, El-Gewely R, Armstrong JK, Garratty G, Richette P. Antibodies against polyethylene glycol in healthy subjects and in patients treated with PEG-conjugated agents. Expert Opin Drug Deliv. 2012 Nov;9(11):1319–23.

142. Schellekens H, Hennink WE, Brinks V. The Immunogenicity of Polyethylene Glycol: Facts and Fiction.

143. Kolářová H, Ambrůzová B, Šindlerová LŠ, Klinke A, Kubala L. Modulation of Endothelial Glycocalyx Structure under Inflammatory Conditions. Mediators Inflamm. 2014;2014.

144. Cao RN, Tang L, Xia ZY, Xia R. Endothelial glycocalyx as a potential theriapeutic target in organ injuries. Vol. 132, Chinese Medical Journal. Lippincott Williams and Wilkins; 2019. p. 963–75.

145. Bruinsma R, Behrisch A, Sackmann E. Adhesive switching of membranes: Experiment and theory. Phys Rev E - Stat Physics, Plasmas, Fluids, Relat Interdiscip Top. 2000;61(4):4253–67.

146. Zaouali MA, Bejaoui M, Calvo M, Folch-Puy E, Pantazi E, Pasut G, et al. Polyethylene glycol rinse solution: An effective way to prevent ischemia-reperfusion injury. World J Gastroenterol. 2014 Nov 21;20(43):16203–14.

147. Dutheil D, Underhaug Gjerde A, Petit-Paris I, Mauco G, Holmsen H. Polyethylene glycols interact with membrane glycerophospholipids: Is this part of their mechanism for hypothermic graft protection? J Chem Biol. 2009 Mar;2(1):39–49.

148. Passov A, Schramko A, Mäkisalo H, Nordin A, Andersson S, Pesonen E, et al. Graft glycocalyx degradation in human liver transplantation. PLoS One. 2019;14(8):e0221010.

149. Lopez A, Panisello-Rosello A, Castro-Benitez C, Adam R. Glycocalyx Preservation and NO Production in Fatty Livers-The Protective Role of High Molecular Polyethylene Glycol in Cold Ischemia Injury.

150. Neu B, Armstrong J, Fisher T, Meiselman H. Surface characterization of poly(ethylene glycol) coated human red blood cells by particle electrophoresis. Biorheology. 2003 Feb 1;40:477–87.

151. Malhotra R, Valuckaite V, Staron ML, Theccanat T, Souza KM, Alverdy JC, et al. High-molecular-weight polyethylene glycol protects cardiac myocytes from hypoxia-and reoxygenation-induced cell death and preserves ventricular function. D sl Am J Physiol Hear Circ Physiol Vol 300. 2011;5 SRC-B.

152. Nagelschmidt M, Minor T, Saad S, Nagelschmidt M. Polyethylene glycol 4000 attenuates adhesion formation in rats by suppression of peritoneal inflammation and collagen incorporation. Am J Surg. 1998 Jul;176(1):76–80.

153. Nagelschmidt M, Minor T, Saad S, Nagelschmidt M. Polyethylene glycol 4000 attenuates adhesion formation in rats by suppression of peritoneal inflammation and collagen incorporation. Am J Surg. 1998 Jul;176(1):76–80.

154. Karlsson PC, Hughes R, Rafter JJ, Bruce WR. Polyethylene glycol reduces inflammation and aberrant crypt foci in carcinogen-initiated rats. Cancer Lett. 2005 Jun 8;223(2):203–9.

155. Polyethylene glycol rinse solution: An effective way to prevent ischemia-reperfusion injury. Mohamed Amin Maria Eirini sl World J Gastroenterol Vol 20. 2014;43 SRC-.

156. Milton Harris J, Chess RB. Effect of pegylation on pharmaceuticals. Vol. 2, Nature Reviews Drug Discovery. Nature Publishing Group; 2003. p. 214–21.

157. Yu LMY, Lu JJ, Chiu IWY, Leung KS, Chan YW, Zhang L, et al. Poly(ethylene glycol) enhances the surface activity of a pulmonary surfactant. Colloids Surfaces B Biointerfaces. 2004 Aug 1;36(3–4):167–76.

158. Hutanu D. Recent Applications of Polyethylene Glycols (PEGs) and PEG Derivatives. Mod Chem Appl. 2014;02(02).

159. Hoffman MD, Van Hove AH, Benoit DSW. Degradable hydrogels for spatiotemporal control of mesenchymal stem cells localized at decellularized bone allografts. Acta Biomater. 2014;10(8):3431–41.

160. Dang LTH, Feric NT, Laschinger C, Chang WY, Zhang B, Wood GA, et al. Inhibition of apoptosis in human induced pluripotent stem cells during expansion in a defined culture using angiopoietin-1 derived peptide QHREDGS. Biomaterials. 2014;35(27):7786–99.

161. Shimoshige H, Kobayashi H, Mizuki T, Nagaoka Y, Inoue A, Maekawa T. Effect of polyethylene glycol on the formation of magnetic nanoparticles synthesized by Magnetospirillum magnetotacticum MS-1. PLoS One. 2015 May 20;10(5).

162. Costa EC, Moreira AF, De Melo-Diogo D, Correia IJ. Polyethylene glycol molecular weight influences the ClearT2 optical clearing method for spheroids imaging by confocal laser scanning microscopy. J Biomed Opt. 2018 May 11;23(05):1.

163. "Sniff test" technology for meat and fish products. Available from: https://www.foodprocessing.com.au/content/packaging-labelling-coding/article/-sniff-test-technology-for-meat-and-fish-products-331290260

164. Roskott AMC, Nieuwenhuijs VB, Dijkstra G, Koudstaal LG, Leuvenink HGD, Ploeg RJ. Small bowel preservation for intestinal transplantation: A review. Vol. 24, Transplant International. 2011. p. 107–31.

165. Franco-Gou R, Mosbah I Ben, Serafin A, Abdennebi H Ben, Roselló-Catafau J, Peralta C. New preservation strategies for preventing liver grafts against cold ischemia reperfusion injury. J Gastroenterol Hepatol. 2007;22(7):1120–6.

166. Neuzillet Y, Giraud S, Lagorce L, Eugene M, Debre P, Richard F, et al. Effects of the Molecular Weight of Peg Molecules (8, 20 and 35 KDA) on Cell Function and Allograft Survival Prolongation in Pancreatic Islets Transplantation. Transplant Proc. 2006 Sep 1;38(7):2354–5.

167. Hauet T, Goujon JM, Baumert H, Petit I, Carretier M, Eugene M, et al. Polyethylene glycol reduces the inflammatory injury due to cold ischemia/reperfusion in autotransplanted pig kidneys. Kidney Int. 2002 Aug;62(2):654–67.

168. Mosbah IB, Zaouali M, Martel C, Bjaoui M, Abdennebi HB, Hotter G, et al. IGL-1 solution reduces endoplasmic reticulum stress and apoptosis in rat liver transplantation. Cell Death Dis. 2012 Mar 8;3(3):e279–e279.

169. Bell EA, Wall GC. Pediatric Constipation Therapy Using Guidelines and Polyethylene Glycol 3350. Vol. 38, Annals of Pharmacotherapy. 2004. p. 686–93.

170. Padrissa-Altés S, Zaoualí MA, Roselló-Catafau J. AMP-activated protein kinase as a target for preconditioning in transplantation medicine. Vol. 90, Transplantation. 2010. p. 1241.

171. Chedid MF, Grezzana-Filho TJM, Montenegro RM, Leipnitz I, Hadi RA, Chedid AD, et al. First report of human pancreas transplantation using IGL-1 preservation solution: A case series. Vol. 100, Transplantation. Lippincott Williams and Wilkins; 2016. p. e46–7.

172. Sbarounis CN. Did alexander the great die of acute pancreatitis? Vol. 24, Journal

of Clinical Gastroenterology. 1997. p. 294–6.

173. Bank S. Chronic pancreatitis: clinical features and medical management. Am J Gastroenterol. 1986 Mar;81(3):153–67.

174. Progymnasmata, in Ioan. Fernelii med. Librum de abditis rerum naturalium et medicamentorum causis quibus adduntur quorundam grauissimorum morborum curationes. Autore Iacobo Auberto Vindone, .., de Jacques ; critico letterario> Aubert (; critico letterario>) - Libros en Google Play.

175. Navarro S. Historical review of our knowledge of acute pancreatitis. Vol. 41, Gastroenterologia y Hepatologia. Ediciones Doyma, S.L.; 2018. p. 143.e1-143.e10.

176. Andren-Sandberg A. Clinical pancreatology I: Pancreatic medical history. N Am J Med Sci. 2010;2(11):502–9.

177. Videla S, Lugea A, Vilaseca J, Guarner F, Treserra F, Salas A, et al. Polyethylene glycol enhances colonic barrier function and ameliorates experimental colitis in rats. Int J Colorectal Dis. 2007 Jun;22(6):571–80.

178. Valuckaite V, Zaborina O, Long J, Hauer-Jensen M, Wang J, Holbrook C, et al. Oral PEG 15-20 protects the intestine against radiation: Role of lipid rafts. Am J Physiol - Gastrointest Liver Physiol. 2009 Dec;297(6):G1041-52.

179. Xu X, Philip JL, Razzaque MA, Lloyd JW, Muller CM, Akhter SA. High-molecular-weight polyethylene glycol inhibits myocardial ischemia-reperfusion injury in vivo. J Thorac Cardiovasc Surg. 2015 Feb 1;149(2):588–93.

180. Hackert T, Bchler MW, Werner J. Targeting P-selectin in acute pancreatitis. Vol. 14, Expert Opinion on Therapeutic Targets. 2010. p. 899–910.

181. Zhang X, Wu D, Jiang X. Icam-1 and acute pancreatitis complicated by acute lung injury. JOP. 2009 Jan 8;10(1):8–14.

182. Hackert T, Sperber R, Hartwig W, Fritz S, Schneider L, Gebhard MM, et al. P-selectin inhibition reduces severity of acute experimental pancreatitis. Pancreatology. 2009 Jun 1;9(4):369–74.

183. Zhang X, Wu D, Jiang X. Icam-1 and acute pancreatitis complicated by acute lung injury. JOP. 2009 Jan 8;10(1):8–14.

184. Hilal MA, Ung CT, Westlake S, Johnson CD. Carboxypeptidase-B activation peptide, a marker of pancreatic acinar injury, but not L-selectin, a marker of neutrophil activation, predicts severity of acute pancreatitis. J Gastroenterol Hepatol. 2007;22(3):349–54.

185. Chooklin S. Pathogenic aspects of pulmonary complications in acute pancreatitis patients. Hepatobiliary Pancreat Dis Int. 2009 Apr;8(2):186–92.

186. Gad MM, Simons-Linares CR. Is aggressive intravenous fluid resuscitation beneficial in acute pancreatitis? A meta-analysis of randomized control trials and cohort studies. World J Gastroenterol. 2020 Mar 14;26(10):1098–106.

187. Buxbaum JL, Quezada M, Da B, Jani N, Lane C, Mwengela D, et al. Early Aggressive Hydration Hastens Clinical Improvement in Mild Acute Pancreatitis. Am J Gastroenterol. 2017 May 1;112(5):797–803.

188. Valuckaite V, Seal J, Zaborina O, Tretiakova M, Testa G, Alverdy JC. High molecular weight polyethylene glycol (PEG 15-20) maintains mucosal microbial barrier function during intestinal graft preservation. J Surg Res. 2013 Aug;183(2):869–75.

189. Eddie T. Chiang, Sara M. Camp, Steven M. Dudek, Mary E. Brown, Peter V. Usatyuk O, Zaborina*, John C. Alverdy* and JGNG. Protective Effects of High-Molecular Weight Polyethylene Glycol (PEG) in Human Lung Endothelial Cell Barrier Regulation: Role of Actin Cytoskeletal Rearrangement. Microvasc Res. 2009;77(2):174–86.

190. Polyethylene Glycol (PEG) and Pegylation of Proteins | Thermo Fisher Scientific - ES. Available from: https://www.thermofisher.com/es/es/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/polyethylene-glycol-peg-pegylation-proteins.html

191. Garber A, Frakes C, Arora Z, Chahal P. Mechanisms and Management of Acute Pancreatitis. Gastroenterol Res Pract. 2018;2018.

192. Yu JH, Lim JW, Kim H. Altered gene expression in cerulein-stimulated pancreatic acinar cells: Pathologic mechanism of acute pancreatitis. Korean J Physiol Pharmacol. 2009 Dec;13(6):409–16.

193. Chen WD, Zhang JL, Wang XY, Hu ZW, Qian YB. The JAK2/STAT3 signaling pathway is required for inflammation and cell death induced by cerulein in AR42J cells. Eur Rev Med Pharmacol Sci. 2019;23(4):1770–7.

194. Robinson K, Vona-Davis L, Riggs D, Jackson B, McFadden D. Peptide YY Attenuates STAT1 and STAT3 Activation Induced by TNF-α in Acinar Cell Line AR42J. J Am Coll Surg. 2006 May;202(5):788–96.

195. Mayerle J, Sendler M, Hegyi E, Beyer G, Lerch MM, Sahin-Tóth M. Genetics, Cell Biology, and Pathophysiology of Pancreatitis. Gastroenterology. 2019 May 1;156(7):1951-1968.e1.

196. Gaman L, Dragos D, Vlad A, Robu GC, Radoi MP, Stroica L, et al. Phytoceuticals in Acute Pancreatitis: Targeting the Balance between Apoptosis and Necrosis. Evid Based Complement Alternat Med. 2018;2018.

197. Malsy M, Bitzinger D, Graf B, Bundscherer A. Staurosporine induces apoptosis in pancreatic carcinoma cells PaTu 8988t and Panc-1 via the intrinsic signaling pathway. Eur J Med Res. 2019 Jan 28;24(1):5.

198. Yamamoto T, Teramura Y, Itagaki T, Arima Y, Iwata H. Interaction of poly(ethylene glycol)-conjugated phospholipids with supported lipid membranes and their influence on protein adsorption. Sci Technol Adv Mater. 2016 Jan 1;17(1):677–84.

199. The Pancreas | Boundless Anatomy and Physiology. Available from: https://courses.lumenlearning.com/boundless-ap/chapter/the-pancreas-2/

200. Islets of Langerhans | Definition, Function, Location, & Facts | Britannica. Available from: https://www.britannica.com/science/islets-of-Langerhans

201. Learn human anatomy the fastest, most engaging and guided way @Kenhub Available from: https://www.kenhub.com/