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CARACTERIZACIÓN DE LAS VÍAS METABÓLICAS Y DE SEÑALIZACIÓN INVOLUCRADAS EN LA ACTIVACIÓN PRO-INFLAMATORIA DE MACRÓFAGOS HUMANOS EN RESPUESTA A LA HIPERGLUCEMIA

TESIS

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CONTENIDO

LISTA DE ABREVIATURAS

AGE.- Advanced Glycation End-product (siglas en inglés). Productos terminales de glicación avanzada.

Anti-RAGE.- Anticuerpo neutralizante dirigido contra el receptor de AGEs.

AR.- Aldosa Reductasa. Enzima citosólica que cataliza la reducción de las hexosas, como la glucosa, a sorbitol.

CD11c.- Cluster of differentiation 11c (siglas en inglés), Integrina implicada en la adhesión celular por medio de su unión con CD18.

CD14.- Cluster of differentiation 14 (siglas en inglés), Correceptor de TLR-4 que participa en el reconocimiento de LPS.

CD206.- Cluster of differentiation 206 (siglas en inglés). Receptor de manosa.

CD45.- Cluster of differentiation 45 (siglas en inglés). Tirosina fosfatasa de membrana, presente en todos los leucocitos con diferentes isoformas producidas por splicing alternativo.

CP.- Control of pressure (siglas inglés). Control de presión, condición experimental de presión osmótica similar a la condición HG, establecida con las concentraciones 5 mM de D-glucosa y 10 mM de D-manitol.

DM.- Diabetes Mellitus. Enfermedad metabólica caracterizada por las altas concentraciones de glucosa en sangre.

ELISA.- Enzyme-Linked ImmunoSorbent Assay (siglas en inglés). Inmunoensayo enzimático ó Inmunoensayo ligado a enzimas, técnica utilizada para la detección y cuantificación de ligandos por medio de anticuerpos.

FSC.- Forward Scatter (siglas en inglés). Dispersión frontal, hace referencia a la dispersión de la luz frontal determinada por el tamaño de un objeto (célula) que pasa frente a un haz de luz (láser del citómetro).

HG.- High Glucose (siglas en inglés). Alta concentración de glucosa, condición experimental *in vitro* establecida con la concentración 15 mM de D-glucosa.

HOMA-IR.- Homeostatic Model Assessment of Insulin Resistance (siglas en inglés). Modelo homeostático de evaluación de la resistencia a la insulina, índice de utilidad clínica que evalúa la incidencia de resistencia a la insulina.

IL-1β.- Interleucina 1 beta. Citocina con actividad pro-inflamatoria implicada en la proliferación y diferenciación celular durante la

respuesta inflamatoria, induce la producción de ciclo-oxigenasa 2 a nivel de sistema nervioso central.

IL-3.- Interleucina 3. Citocina coadyuvante de la diferenciación de monocitos hacia macrófagos diferenciados.

IL-6.- Intreleucina 6. Citocina con capacidad quimiatrayente de linfocitos CD4+.

IL-10.- Interleucina 10. Citocina con actividad anti-inflamatoria que regula negativamente la activación de respuestas Th1 y la activación pro-inflamatoria de macrófagos.

IL-12.- Interleucina 12. Citocina que favorece la activación de respuestas Th1 e induce la producción de INF-γ y TNF-α.

IMF.- Intensidad Media de Fluorescencia. Índice determinado por la cantidad de anticuerpos acoplados a fluorocromos que se unen a las células y que es utilizado para determinar de manera semicuantitativa la cantidad de antígeno.

LPS.- Lipopolisacárido, derivado de la pared de bacterias Gram negativas, induce la activación inflamatoria clásica de células inmunes.

M1.- Macrófago con actividad pro-inflamatoria.

M2.- Macrófago con actividad anti-inflamatoria.

M-CSF.- Macrophage Colony Stimulation Factor (siglas en inglés). Factor estimulador de la colonia de macrófagos. Citocina que induce la diferenciación de monocitos a macrófagos.

MDM.- Monocyte-Derived Macrophages (siglas en inglés). Macrófagos derivados de monocitos.

mRNA.- messenger ribonucleic acid (siglas en inglés). Ácido ribonucleico mensajero.

NF-κB.- Nuclear Factor kappa B (siglas en inglés). Factor nuclear kappa B.

NG.- Normal Glucose (siglas en inglés). Concentración fisiológica de glucosa, condición experimental *in vitro* establecida con la concentración 5 mM de D-glucosa.

NLRP3.- NOD, LRR and PYD domains-containing protein 3 (siglas en inglés). Miembro de la familia de proteínas denominada “con dominio de unión a nucleótidos (NOD por sus siglas en inglés) y ricas en leucina” (NLR por sus siglas en inglés).

NO.- Nitric Oxide (siglas en inglés). Óxido nítrico.

PCR.- Reacción en Cadena de la Polimerasa.

PTL.- Partenolide, inhibidor de lKK y por ende de la activación de NF-κB.

RAGE.- Receptor for Advanced Glycation End-product (siglas en inglés). Receptor para productos terminales de glicación avanzada

RNA.- Ribonucleic Acid (siglas en inglés). Ácido ribonucleico.

ROS.- Reactive Oxigen Species (siglas en inglés). Especies reactivas de oxígeno.

SD.- Sorbitol Deshidrogenasa. Enzima de la vía del poliol que utiliza como sustrato al sorbitol y que lo convierte en fructosa.

SOR.- Sornbinil. Inhibidor de la enzima aldosa reductasa.

SSC.- Side Scatter (siglas en inglés). Dispersión lateral, hace referencia a la dispersión de la luz lateral (90º) determinada por la complejidad o granularidad de un objeto (célula) que pasa frente a un haz de luz (láser del citómetro).

TLR-4.- Toll-Like Receptor 4 (siglas en inglés). Receptor de membrana característico del LPS.

TNF-α.- Tumor Necrosis Factor alfa (siglas en inglés). Factor de necrosis tumoral alfa, citocina pro-inflamatoria con funciones autocrinas y paracrinas.

VSMC.- Vascular Smooth Muscle Cells (siglas en inglés). Células de músculo liso vascular.

I. RESUMEN

La hiperglucemia representa la principal característica de la Diabetes Mellitus. El estudio de los efectos deletéreos de este incremento anómalo de las concentraciones sanguíneas de glucosa resulta de vital importancia dado el grave problema de salud que la diabetes mellitus tipo 2 representa en la actualidad. Por ser de carácter sistémico, la hiperglucemia o altas concentraciones de glucosa afectan todo tipo de tejidos y células. Entre los más estudiados son los efectos inflamatorios en células endoteliales y células de músculo liso vascular, estas últimas también ven incrementada su proliferación mientras que las primeras pueden presentar efectos citotóxicos, todo esto lleva al daño y pérdida de la integridad del tejido vascular. Por mencionar otro ejemplo se encuentra el efecto deletéreo que se observa en células del sistema nervioso central y periférico que ocasiona múltiples alteraciones patológicas de observancia clínica como la neuropatía periférica diabética. Es por estos efectos que las altas concentraciones de glucosa no son sólo el resultado de la diabetes mellitus, sino que constituyen el principal agente causal de toda la serie de complicaciones derivadas de la diabetes. En este sentido, trabajos previos de nuestro grupo de trabajo sugieren que estas concentraciones incrementadas de glucosa pudieran inducir la polarización inflamatoria de los macrófagos. Sin embargo, los mecanismos que podrían estar involucrados en este fenómeno aún no se han dilucidado del todo. Por lo tanto, el objetivo de este trabajo fue caracterizar los mecanismos moleculares involucrados en la polarización a un fenotipo inflamatorio de los macrófagos expuestos a altas concentraciones de glucosa. Así como conocer la

participación de la activación de la vía del poliol o sorbitol (iniciada por la aldosa reductasa), la activación de la vía de señalización iniciada por RAGE y la activación de NF-κB. En el presente trabajo utilizamos células mononucleares de sangre periférica de donadores varones, jóvenes, sin sobrepeso u obesidad, ni alteraciones metabólicas del banco de sangre del Hospital General de México “Dr. Eduardo Liceaga”. Posteriormente, con perlas magnéticas seleccionamos los monocitos de sangre periférica. Los monocitos obtenidos fueron diferenciados hacia macrófagos mediante la adición de M-CSF e IL-3 durante 6 días. Subsecuentemente estos macrófagos derivados de monocitos se incubaron bajo diferentes concentraciones de glucosa control fisiológico (5 mM de glucosa), control de presión osmótica (5 mM de glucosa + 10 mM de manitol) y concentración elevada de glucosa (15 mM de glucosa). Las células fueron colectadas a los 3 y 9 días de cultivo para determinar la expresión de CD11c y CD206 en membrana, por citometría de flujo. Además se analizó la producción de TNF-α, IL-10 e IL-1β en sobrenadantes de estos cultivos por ELISA tipo sándwich y se determinó la expresión de iNOS y arginasa 1 a nivel de mRNA. Determinamos que la polarización pro-inflamatoria de macrófagos derivados de monocitos inducida por alta concentración de glucosa se caracteriza por la reducción temprana (3 días de cultivo) de la IL-10 y el posterior incremento en la producción de la IL-1β. Indicadores tempranos de la polarización pro-inflamatoria fueron también el incremento de CD11c que se mantuvo a tiempos largos, el incremento en la expresión de iNOS y la reducción en la expresión de arginasa 1. Por otro lado, tomamos como parámetro de la polarización pro-inflamatoria el incremento de la producción de IL-1β y buscamos inhibir dicho fenómeno con la

adición de partenolide (inhibidor de IKK y de la activación de NF-κB), anti-RAGE neutralizante (para inhibir la unión a sus ligandos) y sorbinil (inhibidor de la aldosa reductasa y de la vía del poliol). Nuestros resultados muestran que en este modelo experimental la vía mediada por RAGE no parece participar en la polarización inflamatoria inducida por alta concentración de glucosa en estos macrófagos. La activación de NF-κB participa en el proceso de polarización pero es mayormente la vía del poliol la que presenta mayor efecto en la producción incrementada de IL-1β.

II. ABSTRACT

Hyperglycemia represents the hallmark of Diabetes Mellitus. The study of harmful effects of increased blood glucose levels is important given the serious health problem that currently represents diabetes mellitus type 2. The systemic feature of hyperglycemia or high glucose concentrations affects all types of tissues and cells. Among the most studied effects is inflammation induced on endothelial cells and vascular smooth muscle cells, associated with cytotoxicity and increased proliferation in these cells, respectively, leading to tissue damage and loss of vascular integrity. Another example is deleterious effect observed on central and peripheral nervous system cells, that causes multiple pathological alterations of clinical relevance such as diabetic peripheral neuropathy. The high glucose concentrations are not only the result of diabetes mellitus, but are the main causative agent of complications derived from diabetes. In this way, our previous work suggests that high glucose concentrations might induce pro-inflammatory polarization in macrophages. However, the mechanisms that could be involved in this phenomenon have not been fully elucidated. Therefore the objective of this work was to characterize the molecular mechanisms involved in the inflammatory polarization of macrophages exposed to high glucose concentrations. Additionally, we studied the role of the polyol or sobitol pathway activation (initiated by aldose reductase), the RAGE pathway and the activation of NF-κB in this inflammatory polarization of macrophages. The peripherial mononuclear cells were obtained from young male donors, without overweight or obesity and without metabolic alterations, from the blood bank of the Hospital General de México “Dr. Eduardo

Liceaga". Then, only peripheral blood monocytes were selected using magnetic beads. The obtained monocytes were differentiated into macrophages with M-CSF and IL-3 during 6 days. Subsequently, these monocyte-derived macrophages were incubated under different concentrations of glucose, such as; physiological control (5 mM glucose), osmotic pressure control (5 mM glucose + 10 mM mannitol) and high glucose concentration (15 mM glucose). The cells were havested at 3 and 9 days of culture to determine CD11c and CD206 proteins in the membrane by flow cytometry. In addition, the TNF, IL-10 and IL-1β productions were analyzed in supernatants of these cultures by sandwich ELISA, as well as iNOS and arginase 1 mRNAs expression were determined. The pro-inflammatory polarization of monocyte-derived macrophages induced by high glucose concentration was characterized by the early (3 days of culture) decrease of IL-10 production and the subsequent increase of IL-1β production. In addition, increased expression of CD11c and iNOS, as well as decreased expression of arginase 1, were also early markers of pro-inflammatory polarization in these monocyte-derived macrophages. On the other hand, increased IL-1β production was taken as parameter of pro-inflammatory polarization, and inhibition assays were carried out to avoid the higher production by the addition of parthenolide (inhibitor of IKK), neutralizing anti-RAGE (inhibition of AGE/RAGE binding) and sorbinil (inhibitor of aldose reductase and polyol pathway). Our results show that in this model, the RAGE-mediated pathway does not seem to participate in the pro-inflammatory polarization induced by high glucose concentrations in these macrophages. Activation of NF-κB participates in the polarization process, but it is primarily the polyol pathway that has the greatest effect on the increase of IL-1β production.

III. INTRODUCCIÓN

La regulación homeostática de la glucosa en sangre es un mecanismo altamente controlado. Las variaciones en la concentración de la glucosa en sangre varían dependiendo del estado nutricional. Inmediatamente después de comer las concentraciones de glucosa en sangre superan los 120 mg/dL, sin embargo, tras varias horas de ayuno estas tienden a bajar hacia los 80 mg/dL (1). La regulación homeostática de estas concentraciones es directamente afectada por la secreción y actividad de hormonas como, la insulina, el glucagón, la somatostatina y las catecolaminas (1, 2).

La ingesta de comida estimula la síntesis y secreción de insulina por parte de las células β pancreáticas (de los islotes pancreáticos o islotes de Langerhans). Dicha hormona estimula la captación de glucosa induciendo la expresión en membrana plasmática del transportador de glucosa GLUT-4 en células insulino-dependientes como las células de musculo esquelético y tejido adiposo. Por lo tanto, provoca la disminución de la concentración de glucosa en sangre. La insulina además estimula la glucolisis en hígado y la producción de ácidos grasos y triglicéridos en hígado (no se almacenan en hígado) y tejido adiposo (1, 3).

Por otro lado, la hormona glucagon se produce en las células α de los islotes pancreáticos en respuesta a bajas concentraciones de glucosa en sangre. La actividad del glucagon se relaciona con mayor producción de adenosina monofosfato cíclico (cAMP), que promueve la gluconeogénesis e inhibe la síntesis de glucógeno, la actividad de la piruvato cinasa (PK) del hígado (inhibiendo el flujo glucolítico y favoreciendo la acumulación de fosfoenolpiruvato). En tejido adiposo,

el glucagon también induce el aumento de cAMP que activa a las lipasas dependientes de hormonas que producen glicerol y ácidos grasos a partir de los triglicéridos (1-4). La somatostatina producida células δ de los islotes de Langerhans (aunque también se puede expresar en hipotálamo) puede inhibir la secreción tanto del glucagon como de la insulina (1, 4).

Por último, las catecolaminas como la adrenalina y la noradrenalina son secretadas de las terminaciones nerviosas presinápticas y actúan como neurotransmisores u hormonas. En respuesta a bajas concentraciones de glucosa, la adrenalina promueve la activación de la adenilato ciclasa (enzima que produce el cAMP), la glucogenólisis en músculo, así como promueve la degradación de triglicéridos en tejido adiposo y la secreción de glucagon. Por otro lado, la adrenalina inhibe la síntesis de glucógeno en hígado, la captación de glucosa en músculo (lo que aumenta la glucosa en sangre) y la secreción de insulina (1, 5). La acción de las catecolaminas es de corta duración en comparación con la actividad del glucagon (1).

No obstante, la incidencia de enfermedades autoinmune o de alteraciones asociadas al desarrollo del síndrome metabólico, pueden alterar la regulación homeostática de la glucosa. Una de estas alteraciones es la resistencia a la insulina y/o la destrucción de las células β, lo que favorece el desarrollo de un estado hiperglucémico y la diabetes mellitus.

3.1 Diabetes Mellitus definición y clasificación

La diabetes mellitus es una enfermedad crónica caracterizada por niveles elevados de glucosa en la sangre, acompañada por un metabolismo alterado de las grasas y las proteínas (6-8). La glucosa en la sangre aumenta porque no se puede metabolizar en las células, debido a la falta de producción de insulina por parte del páncreas o la incapacidad de las células para utilizar de manera efectiva la insulina que se produce (8). La diabetes puede ser diagnosticada con base en la determinación de la concentración de glucosa en plasma en ayuno (FPG por sus siglas en inglés) ≥ 126 mg/dL; la glucosa en el plasma de una prueba de tolerancia a la glucosa oral (OGTT por sus siglas en inglés) con 75 g glucosa de 2 hr. ≥ 200 mg/dL; o bien por el valor de A1C (porcentaje de hemoglobina HbA1c glicada) ≥ 6.5% (7).

La diabetes mellitus se clasifica de acuerdo a los criterios de la Asociación Americana de Diabetes (ADA por sus siglas en inglés) como:

1. Diabetes mellitus tipo 1. Debida a la destrucción auntoinmune de las células β pancreáticas, usualmente conduce a la deficiencia absoluta de la producción de insulina (7).
2. Diabetes mellitus tipo 2. Debida a una pérdida progresiva de la secreción de insulina de las células β pancreáticas, frecuentemente tiene asociado un trasfondo de resistencia a la insulina (7).
3. Diabetes mellitus gestacional. Diabetes diagnosticada en el segundo o tercer trimestre de embarazo, y sin indicios previos de diabetes antes del embarazo (7).

4. Tipos específicos de diabetes como la diabetes juvenil de inicio en la madurez (MODY por sus siglas en inglés) o la neonatal, que son de tipos monogenéticos. Otro tipo específico es la diabetes relacionada a la fibrosis cística, la diabetes es la comorbilidad más común en estos pacientes (20% en adolescentes y 40-50% en adultos). Por último la diabetes mellitus post-trasplante a veces llamada diabetes de novo con aparición después de trasplante (NODAT por sus siglas en inglés) o diabetes mellitus post-trasplante (PTDM por sus siglas en inglés) (7).

3.2 Consecuencias de la Diabetes Mellitus tipo 2 en la Salud Pública

Acorde con los datos proporcionados por el último reporte global de Organización Mundial de la Salud (OMS) de 2016, se tenía registrado que en el año 2014 existían en el mundo 422 millones de personas que padecían diabetes mellitus, lo que representa una incidencia de 8.5% (8, 9). Además, cinco millones de personas murieron en 2015 por causas relacionadas con la diabetes y representaron el 12,8% de la mortalidad global por todas las causas (10). De acuerdo a la OMS el mayor número de casos de diabetes pertenecen a diabetes mellitus tipo 2 (DM2) (8, 9). La incidencia de los trastornos metabólicos como la DM2, se ha incrementado en las últimas tres décadas, y la etiología multifactorial ha sido ampliamente debatida teniendo en cuenta la predisposición genética, los hábitos alimenticios, el ejercicio e incluso la exposición a los disruptores endocrinos (EDC) (11, 12).

Es importante señalar que la hiperglucemia asociada con el advenimiento

de la DM2 juega un papel clave en el desarrollo de complicaciones y por lo tanto en la alta morbilidad y mortalidad que se observa en los pacientes diabéticos (13, 14).

3.3 La hiperglucemia como característica de la DM

La hiperglucemia se define como una elevación anormal en los niveles sanguíneos de glucosa por encima de 100 mg/dl en ayuno, lo cual no necesariamente significa diagnóstico de DM sino solo un estado de glucosa alterado (hay personas con hiperglucemia en estado pre-diabético) (7, 15, 16). La hiperglucemia puede ser de tipo transitoria o de tipo crónica (17-20). Estudios recientes han demostrado que la hiperglucemia transitoria puede presentarse como consecuencia del deterioro metabólico durante enfermedades severas tales como el síndrome de respuesta inflamatoria sistémica (17). Es importante mencionar que la hiperglucemia transitoria revierte a estados normoglucémicos en el 40% de los pacientes que la presentan (18, 19). Por el contrario, la hiperglucemia crónica se presenta únicamente en el contexto del desarrollo de la diabetes, en donde los niveles de glucosa sanguínea se mantienen permanentemente elevados, condición que es frecuentemente acompañada por hiperinsulinemia, dislipidemia, hipertensión y obesidad (20).

Los principales procesos que llevan a la progresión de las complicaciones mediadas por la hiperglucemia son el desarrollo de micro- y macroangiopatías. Las microangiopatías causan discapacidad y pérdida de la calidad de vida de los pacientes con DM2, mientras que las macroangiopatías son la causa de muerte en

un 80% de las personas con DM2 (21). Estas complicaciones afectan crónicamente a los nervios y a los vasos sanguíneos, lo cual se traduce en patologías tales como la retinopatía, la insuficiencia renal crónica, y el infarto al miocardio, mismas que constituyen la primera causa de morbilidad y mortalidad en México desde el año 2005 (22).

Numerosos estudios han propuesto dos mecanismos principales a través de los cuales las altas concentraciones de glucosa pueden ser capaces de generar daño sobre el tejido endotelial vascular que origina estas micro- y macroangiopatías: el primero es la glucotoxicidad intracelular y el segundo la formación de productos terminales de glicación avanzada (AGE, por sus siglas en inglés) (23-25).

La glucotoxicidad intracelular está relacionada con la activación de la vía del poliol, que es capaz de inducir en endotelio la liberación de especies reactivas de oxígeno (ROS, por sus siglas en inglés).

Mientras tanto, los AGEs están involucrados en promover la adhesión leuocitaria al tejido del endotelio vascular, así como el daño macro-y microvascular (25-27). De manera interesante, información reciente señala a la activación inflamatoria del macrófago como un posible mecanismo adicional mediante el cual las altas concentraciones de glucosa pueden con llevar a efectos deletéreos sobre el tejido endotelial vascular. En este sentido, estos dos mecanismos de daño al endotelio afectarían incluso a células como los monocitos y macrófagos, alterando su estado de activación.

3.4 Relación entre el sistema inmune y los trastornos metabólicos como la hiperglucemia

Novedosas líneas de evidencia han señalado una relación entre varios trastornos metabólicos y el desarrollo de un estado inflamatorio sistémico que potencia el desequilibrio metabólico y su complicación. Tal estado de inflamación sistémica también conocido como inflamación de bajo grado o metainflamación ha demostrado tener importantes diferencias con respecto a la respuesta inflamatoria clásica. El concepto de metainflamación se concibió porque se ha demostrado con frecuencia que esta inflamación sistémica está implicada en el aumento del riesgo de desarrollar enfermedad cardiometabólica en individuos obesos e insulino resistentes (28, 29). Esta metainflamación se caracteriza principalmente por niveles circulantes elevados de proteínas de fase aguda y citocinas pro-inflamatorias, incluida la proteína C reactiva (PCR), el factor de necrosis tumoral alfa (TNF-α), interleucina (IL) -1β, IL-6, IL-17 e IL -18 (30-34). Además, la metainflamación implica un mayor reclutamiento e infiltración de macrófagos en los tejidos periféricos principalmente tejido adiposo y tejido endotelial vascular (35-39). Curiosamente, la metainflamación no promueve lesión tisular, por lo que se ha considerado debajo nivel de activación (35, 39-42).

La activación inflamatoria de los macrófagos es un agente clave en el desarrollo de la metainflamación por la infiltración y proliferación de macrófagos sostenidos en el tejido adiposo, como el aumento de la producción local de citocinas pro-inflamatorias (35, 39, 43). Además, los macrófagos inflamatorios están relacionados con el desarrollo de resistencia a la insulina y complicaciones

vasculares en el contexto de trastornos metabólicos (39, 44-46). Por lo tanto, el desarrollo de trastornos metabólicos como la hiperglucemia en el contexto de la DM y sus complicaciones está estrechamente relacionada con la activación de macrófagos de manera causal.

3.5 Las condiciones hiperglucémicas inducen la polarización inflamatoria de macrófagos

La hiperglucemia es un componente clave del síndrome metabólico y el sello distintivo de la DM. Las concentraciones altas de glucosa (HG, por sus siglas en inglés) pueden incrementarse de manera transitoria, como es el caso de los episodios agudos de hiperglucemia (AHG, por sus siglas en inglés) debidos al consumo excesivo de azúcar o la hiperglucemia por estrés (SH, por sus siglas en inglés), una manifestación común de hiperglucemia hospitalaria (HH, por sus siglas en inglés) (47-49). La hiperglucemia postprandial transitoria por ejemplo puede favorecer la producción de especies reactivas de oxígeno y el daño oxidativo (50).

Una plétora de estudios ha demostrado que la HG es el principal factor asociado al daño microvascular y al desarrollo de complicaciones en pacientes con DM2 (51-53). Curiosamente, se ha demostrado que una cantidad cada vez mayor de mecanismos relacionados a las HG podrían inducir respuestas inflamatorias en varios tipos de células. En este sentido, se ha demostrado que los fibroblastos gingivales aumentan la producción de IL-6 e IL-8 en respuesta a la glucosa alta (54). Mientras tanto, las células endoteliales aórticas humanas han

demostrado aumentar la transcripción de IL-1β en condiciones de glucosa alta in *vitro* (55). Por otro lado, la HG en pacientes diabéticos se ha correlacionado inversamente con la producción de IL-10 en macrófagos obtenidos a partir de la placa aterosclerótica (56). Además, se ha informado que los macrófagos presentes en heridas de pacientes diabéticos regulan positivamente el TNF-α, IL-1β (pro-inflamatorios) y la regulación negativa de IL-10 y CD206 (anti-inflamatorios) (57). De forma similar, en el presente trabajo demostramos que la HG pueden inducir características pro-inflamatorias en macrófagos derivados de monocitos (MDM) *in vitro* y en monocitos circulantes de donantes hiperglucémicos (58).

Se ha descrito que la HG que origina la glucotoxicidad en células endoteliales, tiene efecto sobre otros tipos celulares entre ellos algunas células inmunitarias, favoreciendo la producción de mediadores de la inflamación (52). Por lo tanto, la activación de la vía del poliol y la formación de AGEs podrían tener efecto sobre el estado de activación de los macrófagos.

3.6 La activación de la vía del Poliol induce la producción de ROS, el estrés oxidativo y la activación de NLRP3

El primer mecanismo implicado en la polarización inflamatoria de macrófagos inducida por HG, es la vía del poliol. Dicha vía está constituida por dos enzimas: la aldosa reductasa (AR) convierte la glucosa en sorbitol y la sorbitol deshidrogenasa (SD) que transforma el sorbitol en fructosa. Ambos podrían considerarse activadores no convencionales del sistema inmune, ya que genran cambios

metabólicos en células endoteliales e inmunes que conducen a la producción de especies reactivas de oxígeno (ROS) y a la activación inflamatoria. En este sentido, la HG ha estado implicada en el aumento del contenido de sorbitol asociado con la disminución del glutatión reducido, la mejora de la relación NADH / NAD y el desarrollo de estrés oxidativo (52, 59-61).

Es precisamente la producción de ROS derivada de la actividad de la vía del poliol (a su vez inducida por la HG) la que media los efectos citotóxicos, así como la activación de NF-κB y AP1 en la transcripción de citocinas inflamatorias (62, 63). Diversos estudios han demostrado que la activación de la vía del poliol induce la activación inflamatoria de células de músculo liso vascular (VSMC por sus siglas en inglés) y de linaje monocítico (células THP-1). Dicha participación de la vía del poliol ocurre tanto en respuesta a estímulos clásicos como LPS, como en respuesta solo a la hiperglucemia (63-65). De igual manera la actividad de la vía del poliol puede activar al NLRP3 mediante la producción de ROS (66). Si bien no se conoce cómo los ROS pueden activar a NLRP3, se ha observado que la inhibición de la AR puede evitar la activación de NLRP3 y de la caspasa-1 inducida por HG (66).

3.7 Formación de AGEs inducida por HG y vía de señalización mediada por RAGE

El segundo mecanismo implicado en la polarización de macrófagos debido a HG, es la vía de glicación o formación de productos finales de glicación avanzada (AGE). Tales productos resultan de la glicación de proteínas a una alta

concentración de glucosa de manera no enzimática. El aumento de la formación de AGEs en la HG se ha relacionado con el desarrollo de estrés oxidativo y formación de ROS, aumento de la resistencia a la insulina, disfunción de células β y muerte, y promoción del desarrollo de complicaciones como daño vascular (52, 67, 68). De esta forma, se ha demostrado que los AGEs interactúan con el receptor de membrana para productos finales de glicación avanzada (RAGE), para liberar citocinas y producir ROS mediante la activación del NF-κB en varios tipos de células (69-74). Estudios recientes en ratones han mostrado un aumento en la formación de AGE hipotalámica, con dietas altas en carbohidratos que conducen a inflamación hipotalámica mediada por AGE/RAGE (70). Además, los AGE proporcionados por la dieta aumentan el TNF-α y la IL-6 en suero en ratones diabéticos, así como en tejidos pancreáticos, cardíacos, renales y endoteliales dañados (71). También, se ha reportado que la AGE-albúmina favorece la polarización preferencial a las células Th1 y Th17 durante la diferenciación de las células T CD4 (+) humanas vírgenes, así como a la disminución de la función de las células Treg humanas (72). Además, se ha descrito que la AGE de albúmina sérica bovina (AGE-BSA) promueve la secreción de TNF-α, IL-1β e IL-6 en células THP-1 (73). Esta producción de IL-1β indica que la actividad de NLPR3 podría ser inducida por la vía AGE/RAGE. En ratones, se demostró que la administración intraperitoneal a largo plazo de AGE-BSA se asocia a mayor activación de NLRP3 y una expresión aumentada de caspasa y procaspasa-1 asociada con una mayor producción de IL-1β y óxido nítrico en el tejido renal (74). Sin embargo, el receptor soluble circulante (sRAGE), el receptor secretor endógeno (esRAGE) y el receptor escindido (cRAGE) formados a través del corte y empalme alternativo (75),

protegen a las células del reconocimiento de AGE mediante RAGE de cadena móvil. La formación de AGEs se ve aumentada por la HG, y se ha correlacionado positivamente con sRAGE, esRAGE y cRAGE en pacientes con enfermedad renal terminal (69). Sin embargo, el aumento de la producción de AGEs puede ser mayor que sRAGE, esRAGE y cRAGE, lo que contrarrestaría cualquier efecto benéfico (69). La señalización de células AGE/RAGE activa a NF-κB a través de una ruta convergente con TLR (76).

De hecho, estas vías podrían estar estrechamente relacionadas entre ellas, por ejemplo, la inhibición a largo plazo de la vía del poliol podría favorecer la glicación de las proteínas (59). Tal fenómeno que podría considerarse contradictorio respecto de múltiples estudios que han postulado resultados benéficos del uso de inhibidores de la AR (77-79), podría de hecho explicar la falla en el uso de tales inhibidores para tratamientos a largo plazo (80). Tanto la unión de AGEs a TLRs y RAGE en células vasculares, como la inducción de la vía del poliol en monocitos aumentan la producción de ROS que conduce al estrés oxidativo y potencia la actividad PKC (81, 82). Además, el aumento de la actividad de AR durante la HG podría afectar la capacidad antioxidante mediante el uso competitivo de NADPH, que la glutatión reductasa necesita para convertir el glutatión oxidado en glutatión reducido (83-85). Por otro lado, la actividad SD también promueve estrés oxidativo por producción de fructosa y NADH (83). En resumen, la HG pudiera promover a través de mecanismos no convencionales la activación de macrófagos que conduce a una condición de metainflamación sostenida y de estrés oxidativo.

IV. ANTECEDENTES

Diversos estudios sobre el efecto de las altas concentraciones de glucosa en líneas celulares monocíticas de células THP-1 han demostrado la inducción de mayor expresión de quimiocinas a nivel de mRNA (86). Además, estas células THP-1 expuestas a altas concentraciones de glucosa presentan mayor adherencia a células vasculares de músculo liso (VSMC, por sus siglas en inglés) debido al incremento de la expresión de la quimiocina CX3CL1 por estas células (87).

Estudios previos en nuestro laboratorio mostraron menor porcentaje de monocitos CD206+ (marcador de macrófagos anti-inflamatorios) y un mayor porcentaje de monocitos CD11c+ (marcador de macrófagos pro-inflammatorios) en dichos donadores hiperglucémicos (58). Tanto el receptor de manosa CD206 como la integrina CD11c son marcadores de los fenotipos anti- y pro-inflamatorios, respectivamente, útiles para identificar cambios inmunofenotípicos de MDM. Por tal motivo, los consideramos dentro de nuestra caracterización fenotípica.

Posteriormente, nos dimos a la tarea de estudiar si la HG inducía la polarización inflamatoria en MDM (88). Aunque en dicho estudio no observamos el incremento en la producción de las citocinas pro-inflamatorias (TNF, IL-6, IL-12), notamos menor producción de IL-10 y mayor expresión de los RNA mensajeros de TLR-4, NF-κB, AR y SD en estos macrófagos incubados durante tres días en condiciones de HG (88). Es en el presente trabajo donde nos avocamos a enriquecer la caracterización fenotípica y funcional de estos MDM e indagar en las posibles vías de señalización y/o metabólicas involucradas en su polarización pro-inflamatoria inducida por la HG.

V. PLANTEAMIENTO DEL PROBLEMA

Como ya se ha mencionado, la diabetes mellitus es una enfermedad metabólica que representa uno de los mayores problemas de salud a nivel mundial. El principal factor implicado en esta patología es un estado hiperglucémico que propicia una serie de distintas alteraciones vasculares, además de perpetuar el estado de resistencia a la insulina. Ambos aspectos están fuertemente mediados por una condición inflamatoria y en especial, por macrófagos que median dicha actividad. Es decir, el estado de hiperglucemia parece activar los macrófagos de manera inflamatoria, promoviendo el daño vascular y la resistencia a la insulina. Por lo tanto, es de suma importancia conocer los mecanismos moleculares por los cuales la hiperglucemia induce la actividad inflamatoria de dichos macrófagos para en un futuro cercano poder plantear alternativas de tratamiento o nuevos blancos terapéuticos que puedan disminuir tanto la resistencia a la insulina como el daño vascular exacerbado por los macrófagos.

VI. HIPÓTESIS

Las altas concentraciones de glucosa inducen un fenotipo pro-inflamatorio en macrófagos humanos *in vitro* al activar la vías de señalización RAGE, la activación de NF-κB, y la vía metabólica del poliol (sorbitol).

VII. OBJETIVOS

7.1 Objetivo General

Caracterizar las principales vías metabólicas y de señalización involucradas en la polarización pro-inflamatoria de macrófagos humanos expuestos a altas concentraciones de glucosa *in vitro*.

7.2 Objetivos Particulares

1. Determinar el fenotipo de macrófagos humanos, así como el perfil de producción de citocinas y expresión a nivel de mRNA, expuestos a altas concentraciones de glucosa.
2. Evaluar la influencia de la vía de señalización RAGE y la actividad de NF-κB en la activación pro-inflamatoria de macrófagos humanos expuestos a altas concentraciones de glucosa, mediante el bloqueo de la unión AGE/RAGE y con la inhibición farmacológica de NF-κB.
3. Evaluar la influencia de la vía metabólica del sorbitol (poliol) en la activación pro-inflamatoria de macrófagos humanos expuestos a altas concentraciones de glucosa, mediante la inhibición farmacológica de la aldosa reductasa.

VIII. MATERIALES Y MÉTODOS

8.1 Características de los donadores y de las muestras biológicas

Las muestras biológicas para el estudio se obtuvieron a partir de donadores clínicamente sanos que acudieron al Servicio de Banco de Sangre del Hospital General de México “Dr. Eduardo Liceaga”. Los donadores aceptaron participar en el estudio mediante la firma de un consentimiento informado. El protocolo de investigación fue aprobado por la Comisión de Ética en Investigación del Hospital General de México “Dr. Eduardo Liceaga”.

Los extractos leucocitarios fueron obtenidos mediante un sistema de fraccionamiento de sangre validado con ultracentrifugación y extractores semiautomáticos. Los donadores reclutados para el estudio fueron varones, mexicanos, mestizos, en el rango de edad de 18 a 35 años e índice de masa corporal (IMC) entre 18.5 y 24.9 kg/m^2 (sin obesidad o sobrepeso). Los donadores fueron incluidos en el estudio si presentaban química sanguínea de seis elementos en rangos normales (glucosa, colesterol, triglicéridos, ácido úrico, creatinina y urea). Los donadores fueron incluidos en el estudio si no presentaban resistencia a la insulina de acuerdo con la definición del índice de HOMA-IR<3.8 para individuos hispanos (89), y sin diagnóstico previo de intolerancia a la glucosa o DM. Además, los donadores fueron incluidos en el estudio si no presentaban seropositividad para los virus de la hepatitis B (HBV), hepatitis C (HCV) y de la inmunodeficiencia humana (HIV), así como ningún tipo de infección bacteriana o parasitaria al momento de su asistencia al Servicio de Banco de Sangre.

Los donadores que no se consideraron para el presente estudio fueron aquellos

con diagnóstico previo de diabetes tipo 1 y 2, pancreatitis aguda o crónica, insuficiencia renal aguda o crónica. Tampoco se incluyeron donadores con cualquier grado de desnutrición, VIH-SIDA y hepatitis virales; y con infecciones por virus de influenza, bacterianas, parasitarias y micóticas en un lapso menor de 30 días al momento de la toma de la muestra sanguínea. De igual manera se excluyeron donadores con enfermedades autoinmunes, procesos inflamatorios crónicos, neoplasias de cualquier origen o cualquier otro desorden o condición con capacidad de alterar al sistema inmunológico. Se excluyeron también individuos que en un lapso menor de 30 días al momento de la toma de la muestra sanguínea hayan usado anti-inflamatorios esteroides o no esteroides, anti-piréticos, anti-histamínicos u otros fármacos inmunosupresores o inmunomoduladores, o que los usaran de manera regular.

Se eliminaron del estudio a aquellos sujetos que presentaron un resultado positivo a las pruebas confirmatorias para infecciones por HBV, HCV y HIV, posterior al reclutamiento. Se eliminaron también aquellas muestras en donde el extracto leucocitario por parte del Servicio de Banco de Sangre presentara ruptura de la bolsa o algún daño que comprometiera la calidad de la muestra, o por errores en el procesamiento de las mismas.

8.2 Purificación de monocitos

Las células mononucleares de sangre periférica (PBMC) fueron obtenidas de los extractos leucocitarios de donadores. Las PBMC se obtuvieron diluyendo el extracto leucocitario 1:2 con buffer de fosfatos 1X estéril (PBS, por sus siglas en

inglés), separándolas por gradiente de Ficoll usando Histopaque 1077 (Sigma-Aldrich). Las muestras se lavaron 4 veces por 5 min con PBS 1X. Los monocitos fueron obtenidos mediante selección negativa por columna de separación magnética (Miltenyi). La pureza de la separación (>95%) se comprobó por citometría de flujo con la adición de 3 µl de cada uno de los siguientes anticuerpos: anti-CD45 humano acoplado a fluoresceína 5-isotiocianato (FITC, Biolegend) y anti-CD14 humano acoplado a ficoeritrina con Cy7 (PE-Cy7, Biolegend). Las muestras se analizaron en un citómetro de flujo (BD FACSCanto II), considerando 20 mil eventos para la población de monocitos. Los resultados fueron expresados como media de fluorescencia y analizados mediante el programa FACSDiva.

8.3 Obtención de macrófagos derivados de monocitos

Los monocitos $CD14^+$ obtenidos a partir de la separación se cultivaron a una concentración de $2x10^6$ células/mL en placas de 6 pozos (Costar, México). Las células se cultivaron en RPMI 1640 (Gibco) con gentamicina (50 µg/mL), 10% de suero fetal bovino inactivado por calor (Hi-SFB, Gibco), 10 ng/mL de factor estimulante de la colonia de macrófagos (M-CSF, Peprotech, México), 10 ng/mL de Interleucina 3 (IL-3, Peprotech, México) y fue incubado por seis días cambiando el medio de cultivo cada 48 horas.

8.4 Cambios fenotípicos inducidos por la diferenciación de monocitos

La comparación morfológica entre monocitos y macrófagos derivados de monocitos (MDM) se llevó a cabo por citometría de flujo, tomando en cuenta el

tamaño y la complejidad celular, así como la intensidad media de fluorescencia (IMF) de CD14 y CD45 en las células.

8.5 Estímulo con alta concentración de glucosa

Los MDM de cada donador fueron expuestos a distintas concentraciones de glucosa, como se describirá a continuación. La incubación de macrófagos se llevó a cabo durante tres días usando concentración fisiológica de glucosa (5 mM) y concentración elevada de glucosa (15 mM), usando 5 mM de glucosa y 10 mM de D-manitol como control de presión osmótica. La producción de citocinas se determinó por medio del Ensayo por Inmunoabsorción Ligado a Enzimas (ELISA, por sus siglas en inglés) a partir del sobrenadante de los cultivos. Las células se analizaron por citometría de flujo para identificar su fenotipo y se extrajo el RNA de las muestras con el fin de caracterizar su actividad transcripcional por PCR punto final.

8.6 Expresión de CD45, CD14, CD11c y CD206

La caracterización fenotípica se encuentra categorizada en dos fases, la primera reconoce la incidencia de los marcadores fenotípicos que definieron a nuestra población de estudio. El fenotipo de los MDM fue realizado mediante la identificación de la expresión de CD45 y CD14, seguidos de CD11c y CD206. Para la inmunodetección por citometría de flujo se colectaron los MDM con un cosechador manual de células y se colocaron 50 µL de la suspensión celular conteniendo $1x10^6$ células en un tubo Eppendorf. Posteriormente se adicionaron 5

µL de Human TruStain FcX a cada para bloquear las fracciones Fc por 5 minutos, con anticuerpos no marcados y así evitar el pegado inespecífico de los anticuerpos unidos a fluorocromo. Se adicionaron los anticuerpos en las cantidades que se indican en la Tabla 1, se agitó en vortex por 3 segundos y se incubaron durante 15 minutos a 4ºC en oscuridad. Las células se centrifugaron a 2500 rpm por 5 minutos. El sobrenadante fue retirado y la pastilla celular fue suspendida en 400µL de PBS 1X (pH 7.2). Las muestras se analizaron en un citómetro de flujo (BD FACSCanto II), considerando 20 mil eventos para la población de MDM. Los resultados se expresaron como porcentajes y medias de fluorescencia, analizados mediante el software FACSDiva.

Tabla 1. Condiciones para la detección de monocitos-macrófagos por citometría de flujo.

Marcador	**Anticuerpo**	**Cantidad de anticuerpo**	**Función**	**Células que lo expresan**
CD45	Anti-CD45 FITC	1000 ng	Tirosina-fosfatasa	Todos los leucocitos.
CD14	Anti-CD14 PE-Cy7	500 ng	Co-receptor de TLR-4	Monocitos y Macrófagos.
CD11c	Anti-CD11c PE-Cy5	500 ng	Integrina-Adhesión celular	Macrófagos pro-inflamatorios.
CD206	Anti-CD206 APC-Cy7	1000 ng	Receptor de manosa	Macrófagos anti- inflamatorios.

8.7 Producción de TNF, IL-10 e IL-1β

El sobrenadante de los cultivos de MDM se colectó después de 3 y 9 días de cultivo *in vitro*. La producción de citocinas se cuantificó por ensayos por ELISA tipo sándwich. Los MDM cultivados en presencia de concentraciones fisilógica y elevada de glucosa, así como activados por vía clásica o alternativa se analizaron para determinar la producción de TNF, IL-10 e IL-1β. La cuantificación por ELISA tipo sándwich se realizó siguiendo las instrucciones del fabricante (Peprotech, México). Brevemente, a cada pozo se añadieron 100 µl del anticuerpo de captura (1 µg/ml) y se incubó toda la noche a temperatura ambiente. Posteriormente, las placas fueron lavadas cuatro veces con PBS 1X-Tween 20 al 0.05% (Sigma-Aldrich) y se bloqueó la placa con PBS 1X BSA 1% por 1 hora y media, seguido por un lavado adicional con PBS 1X-Tween 20 0.05% (Sigma-Aldrich). Enseguida se añadieron los estándares y los sobrenadantes de las células en cultivo por triplicado a temperatura ambiente, incubando durante 2 horas, seguido de un lavado con PBS 1X-Tween 20 0.05% (Sigma-Aldrich). Posteriormente se adicionó el anticuerpo secundario conjugado a biotina a temperatura ambiente durante 2 horas (Peprotech, México. Después se lavó para eliminar el exceso de anticuerpo secundario libre. Luego, se añadió la solución del conjugado de Avidina-Peroxidasa de rábano (HRP, por sus siglas en inglés) seguido de un último lavado para eliminar el exceso del conjugado enzimático no acoplado al anticuerpo secundario. Por último, se añadió el sustrato ácido 2,2'-azino-bis(3-etilbenzotiazolin-6-sulfónico) (ABTS, Sigma, México). Finalmente, la concentración de proteína se cuantificó por medio de lector de ELISA a 405 nm (Bio-Tek ELx800,

96 pozos, BIO-Tek, USA) con corrección de longitud de onda a 630 nm.

8.8 Expresión de iNOs y arginasa 1 por RT-PCR

Los MDM adheridos a la placa se colectaron con un cosechador manual de células y se colocaron 50 µL de la suspensión celular con $1X10^6$ células en un tubo Eppendorf. Los MDM fueron lisados con 200 µL de Trizol (Invitrogen). Las muestras fueron homogeneizadas y posteriormente se adicionaron 40 µL de cloroformo (Sigma-Aldrich). Las muestras se incubaron 5 min a 4°C y se centrifugaron a 13000 rpm/4°C por 30 min, recuperando la fase acuosa en tubos Eppendorf de 1.5 mL. Enseguida, considerando el volumen de la fase acuosa se adicionó el mismo volumen de isopropanol (Sigma-Aldrich) y se incubó a 4°C toda la noche. Después de este tiempo, el ARN fue precipitado centrifugando a 13000 rpm durante 30 min a 4°C, y se lavó con alcohol absoluto tres veces. El RNA resultante se suspendió en H_2O-DEPC (Sigma-Aldrich) y se cuantificó en el espectrofotómetro a 260 nm. La pureza del RNA se comprobó por medio de la lectura de su absorbancia en el espectrofotómetro a 280 nm, para calcular el indice 260/280 y verificar la pureza del RNA. La integridad del RNA se verificó corriendo una alícuota de las muestras en un gel de agarosa al 1.5%. Posteriormente, se procedió a la retrotranscripción de 1 µg de RNA con oligonucleótido dT (1:5; Invitrogen) y M-MLV retrotranscriptasa (1 µL/20 µL de reacción; Invitrogen), a 37°C/60 min. Usando el cDNA como templado, se realizó el PCR con oligonucleótidos específicos de secuencia para marcadores de activación clásica (NF-κB y TLR-4), alternativa (IL-10) y genes relacionados con la

vía del poliol (sorbitol deshidrogenasa y aldosa reductasa). Se utilizó 18s como gen de expresión constitutiva. Las condiciones del PCR fueron las siguientes: AmpliTaq 0.5 µL/50 µL de reacción (Biotecnologías Universitarias, UNAM, México), temperatura inicial de 95°C por 5 min, 35-45 ciclos de 95 °C por 30 seg (desnaturalización), 57 °C – 63 °C por 30 a 45 seg (dependiendo del gen; alineación), y 72°C por 30-45 seg (elongación), con una temperatura de elongación final de 72°C por 5 min. Posteriormente, el producto de PCR se corrió en gel de agarosa 1.5 %, para, previa tinción con bromuro de etidio 1%, ser visualizado en transiluminador de luz UV de onda corta. La expresión de cada gen fue analizada como la medida de su densidad óptica entre el valor de densidad óptica del gen constitutivo, con el software de análisis Image-J.

8.9 Activación clásica con LPS e inhibición de la activación de NF-κB y de las vías mediadas por RAGE y AR (vía del poliol)

Para realizar estímulos clásicos de activación con LPS estandarizamos, por curvas de concentración y tiempo, que la adición de 10 ng/ml a los cultivos de MDM durante 6 horas era la mejor opción. Lo anterior se determinó ya que después de este tiempo (7-9 horas con LPS) la producción de TNF-α en estos MDM activados no se incrementaba de manera significativa, con respecto a la producción tras 6 horas de cultivo con el estímulo (la cual muestra incremento significativo con respecto a la producción basal de TNF-α). Similar fue la lógica para determinar las concentraciones de trabajo basados en la literatura, probamos concentraciones de 1, 10, 50 y 100 ng/ml.

Inhibimos la actividad de NF-κB, de la vía de señalización iniciada por RAGE y de la vía metabólica iniciada por la actividad de la AR para determinar su participación en la polarización pro-inflamatoria de MDM inducida por la HG. La activación de NF-κB fue inhibida con el uso de partenolide (PTL; Sigma-Aldrich) probando en curvas de concentración desde 0.1 hasta 100 µg/ml (para inhibición de la activación con LPS) y 5 hasta 100 µg/ml (para estímulos con HG). El bloqueo de la vía mediada por RAGE se buscó usando anticuerpos neutralizantes anti-RAGE (ab89911, abcam) se probaron concentraciones desde 1 hasta 50 µg/ml. Por último, se inhibió de la vía del poliol se con sorbinil (SOR; Sigma-Aldrich) en concentraciones desde 5 hasta 100 µg/ml.

8.10 Análisis estadístico

El análisis de los datos se realizó mediante el software GraphPad Prism versión 6. Los análisis fueron comparaciones de dos grupos (caracterización de la diferenciación de monocitos a macrófagos), y de tres grupos (comparación entre concentraciones de glucosa fisiológicas, elevadas y control de presión osmótica). Los resultados de cada variable de salida fueron analizados con pruebas de normalidad Kolmogorov-Smirnov (K-S), para determinar si presentaban una distribución normal. Los resultados de las pruebas de normalidad K-S se confirmaron mediante pruebas de normalidad Shapiro-Wilk (S-K). Al comparar dos grupos cuyas variables de salida presentaban una distribución normal, se utilizó como prueba estadística *t* de Student pareada. Las variables analizadas bajo este esquema fueron las comparaciones de tamaño, complejidad celular e IMF de

CD14 entre monocitos y MDM. Al comparar tres grupos cuyas variables de salida presentaban distribución normal, se utilizó como prueba estadística ANOVA de una vía, seguido de un análisis de comparación múltiple de Tukey. Las variables analizadas bajo este esquema fueron las comparaciones de IMF de CD206, concentración de TNF-α, IL-10 e IL-1β en los MDM expuestos a concentraciones fisiológicas y elevadas de glucosa y de control de presión osmótica. La comparación entre dos grupos cuyas variables de salida no presentaron distribución normal se analizó con una prueba de Wilcoxon. La variable analizada bajo este esquema fue la IMF de CD45 entre monocitos y macrófagos. La comparación entre tres grupos cuyas variables de salida no presentaron distribución normal se analizó con una prueba de Friedman, seguida con un análisis de comparación múltiple de Dunn. Las variables analizadas bajo este esquema fueron porcentaje de células CD11c+, porcentaje de células CD206+, IMF de CD11c, la expresión de los genes de NF-κB, TLR-4, AR y SD entre MDM cultivados en concentración fisiológica, elevada de glucosa y de control de presión osmótica. De acuerdo con literatura previa, se consideró una n de 10 individuos para realizar el análisis estadístico correspondiente.

IX. RESULTADOS

9.1 Obtención de monocitos y de macrófagos derivados de monocitos (MDM)

Las células obtenidas después de la separación por gradiente de Ficoll y selección por columna magnética, son monocitos $CD45^{+}CD14^{+}$ con pureza de 97% (Fig. 1). Son adherentes en plástico y presentan morfología esférica al microscopio, mientras que por citometría de flujo se aprecia que presentan un menor tamaño y complejidad celular, comparada con estas mismas células después de seis días de cultivo con M-CSF e IL-3, cuando ya son macrófagos derivados de monocitos (MDM; Fig. 2).

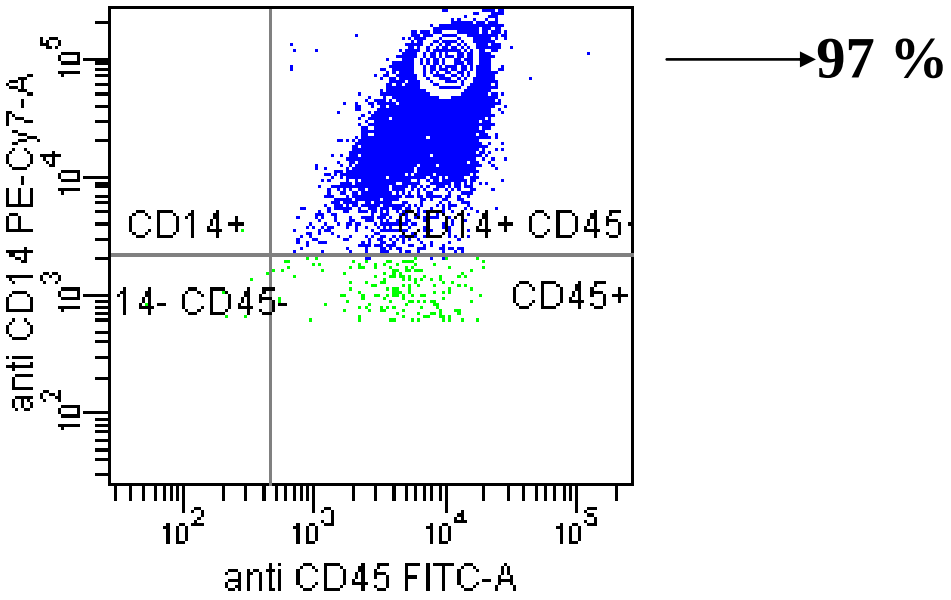


Figura 1. Purificación de monocitos de sangre periférica. Porcentaje de células CD45+CD14+ después de la separación por gradiente de Ficoll y selección por columnas magnéticas.

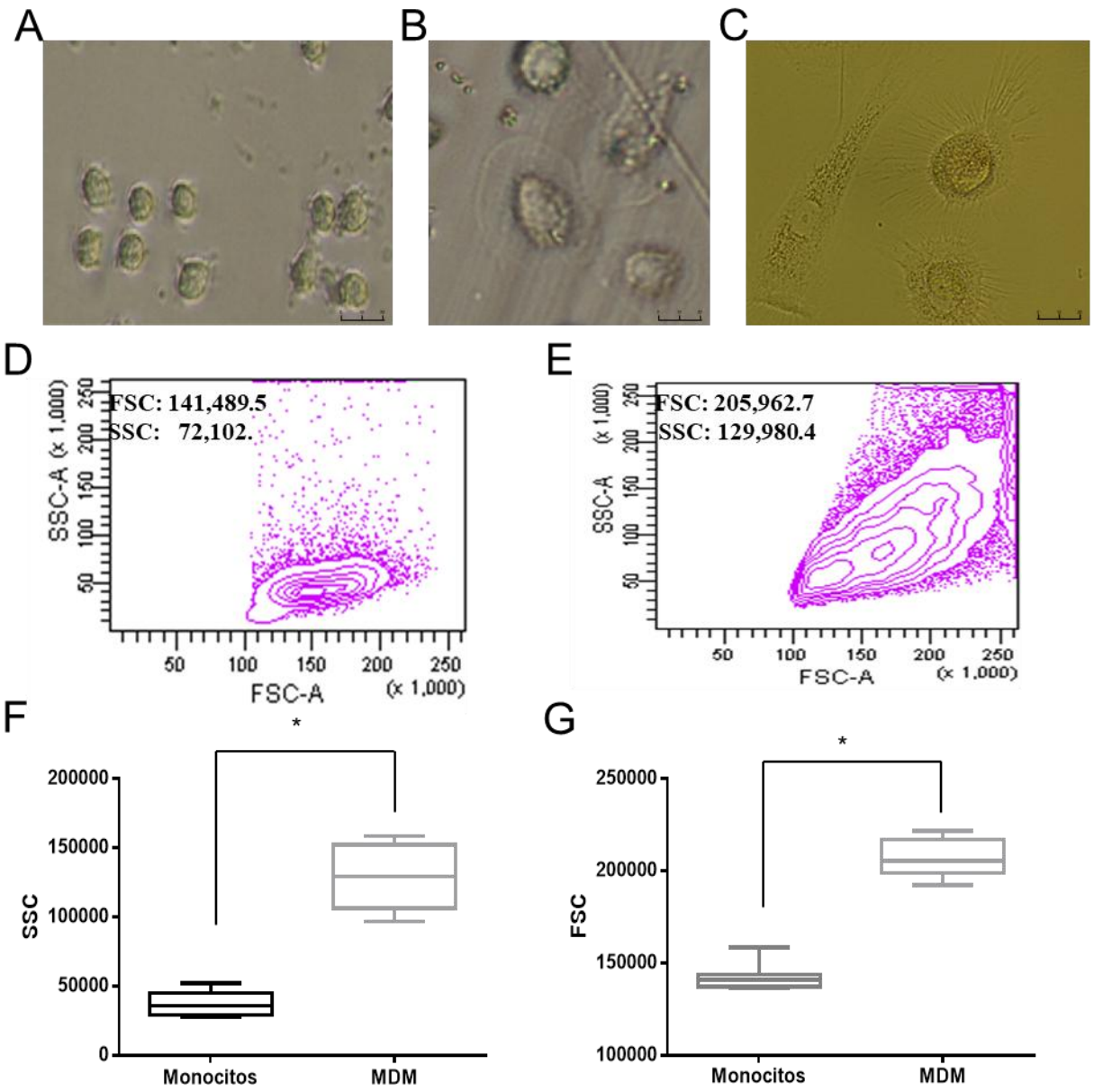


Figura 2. Comparación morfológica de monocitos y MDM. Monocitos recién aislados (A), imagen tomada a 20X. MDM obtenidos después de seis días de diferenciación con M-CSF e IL-3 (B), imagen tomada a 20X. Las proyecciones citoplasmáticas características de los macrófagos diferenciados se aprecian modificando el contraste y reduciendo la cantidad de luz del campo claro del microscopio (C), imagen tomada a 20X. Gráficas de puntos obtenidas por citometría de flujo del tamaño (FSC-A) y granularidad (SSC) de monocitos (D) y sus correspondientes MDM (E) de un experimento representativo. Gráficos de caja que ilustran los datos de granularidad (SSC) de monocitos y sus correspondientes MDM de un conjunto de 10 experimentos (F). Gráficos de caja que ilustran los datos de tamaño (FSC) de monocitos y sus correspondientes MDM de un conjunto de 10 experimentos (G). Análisis con una prueba de Wilcoxon.*P< 0.05. Los datos de las gráficas de cajas de los paneles F y G grafican las medianas de los datos y las barras agrupan los valores inferiores o superiores de los cuartiles 1 y 3, respectivamente.

9.2 Expresión de marcadores fenotípicos de membrana

Los macrófagos cultivados después de seis días de diferenciación y tres o nueve días de cultivo, expuestos a diferentes concentraciones de glucosa, muestran 100% de expresión de CD11c, independiente de la condición glucémica en la cual fueron cultivados (Fig. 3). Sin embargo, existen diferencias en la intensidad en la que los MDM cultivados bajo cada condición expresan el marcador de membrana (Fig. 4). Los macrófagos cultivados en condición HG después de tres días expresan 1.5 veces más la integrina CD11c con respecto a sus respectivos controles en concentración fisiológica de glucosa (Fig. 5). Lo anterior se observó considerando todas las células $CD45^{+}CD14^{+}$. De manera interesante, el incremento de la expresión de CD11c es más marcado después de nueve días de cultivo en HG (4 a 5 veces mayor con respecto a los controles NG; Fig. 5).

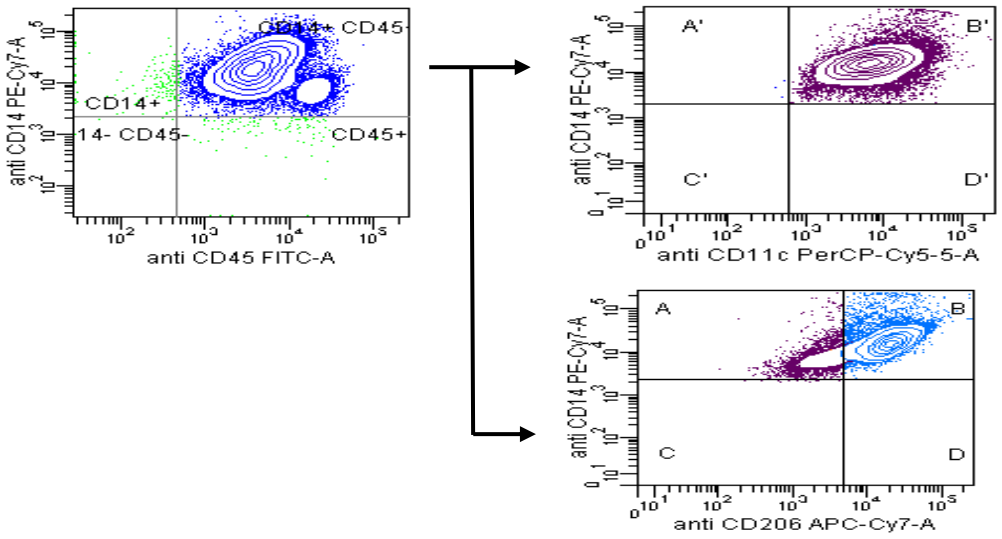


Figura 3. **Estrategia de gating para la citometría de flujo.** A partir de las células dobles positivas para la expresión de la tirosina fosfatasa CD45 (marcador de leucocitos y del Co-receptor para LPS CD14 se estableció la población de MDM analizados posteriormente para la expresión de CD11c y CD206.

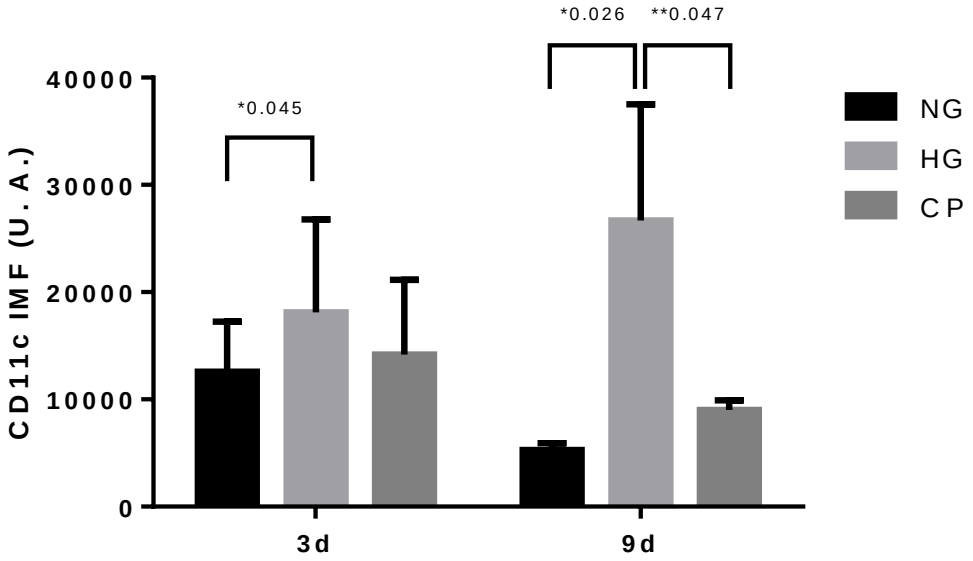


Figura 4. **Expresión de CD11c a 3 y 9 días de cultivo en diferentes concentraciones de glucosa.** La expresión de CD11c se analizó de acuerdo a la intensidad media de fluorescencia. Los MDM cultivados a HG incrementaron su expresión de CD11c de manera significativa Se utilizó una prueba múltiple de T para identificar las diferencias significativas. *P<0.05. La gráficas ilustran la media y desviación estándar de los datos. 5 mM de glucosa (**NG**), 15 mM de glucosa (**HG**) y control de presión osmótica (**CP**).

El marcador CD206 solo se expresa en alrededor del 86-90% de las células en cultivo (Fig. 3), sin embargo a diferencia de como ocurre con CD11c, la expresión de CD206 no muestra diferencias significativas bajo distintas concentraciones de glucosa, ni siquiera después de nueve días de cultivo. Esta observación coincide con lo reportado, ya que no se habían observado diferencias en la expresión a los tres días de cultivo (88).

9.3 Perfil de expresión de iNOS y Arginasa-1

Se determinó la expresión a nivel de RNA mensajero de las enzimas óxido nítrico sintasa inducible (iNOS) y arginasa 1 (Arg-1). La expresión y actividad de dichas

enzimas determinan el fenotipo pro- y anti-inflamatorio de los macrófagos, respectivamente. Por PCR punto final se observó que la expresión a nivel de RNA mensajero de iNOS se incrementó en MDM expuestos a HG de manera significativa (Fig. 5A). En contraste, observamos una tendencia a la baja en la expresión de Arg-1 a nivel de RNA mensajero en estas mismas células (Fig. 5B). Estos resultados son consistentes con la polarización de los macrófagos hacia un fenotipo M1.

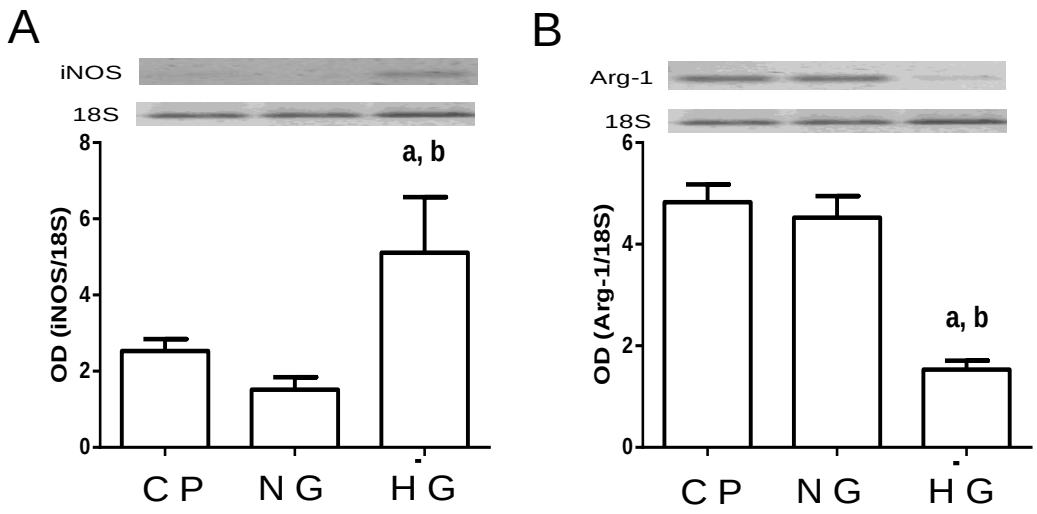


Figura 5. **Expresión del RNA mensajero de iNOS y Arginasa-1 en macrófagos cultivados en diferentes concentraciones de glucosa.** Control de presión osmótica (**CP**), 5 mM de glucosa (**NG**) y 15 mM de glucosa (**HG**). Se utilizó 18s como gen de expresión constitutiva. Se utilizó una prueba múltiple de T para identificar las diferencias significativas. *P<0.05. Las barras representan las medias y desviaciones estándar de los datos. a, diferencia significativa contra CP; b, diferencia significativa contra NG.

9.4 Producción de citocinas

Estudios previos de nuestro laboratorio habían mostrado una menor producción de IL-10 en MDM en HG a los tres días de cultivo (88). El presente estudio confirma dichos resultados además de ofrecer un análisis extensivo durante seis y nueve

días (Fig. 6). Se observa, una tendencia a la baja en la producción de IL-10 a los nueve días de incubación con HG, sin embargo, en este caso las diferencias no son significativas (P=0.058).

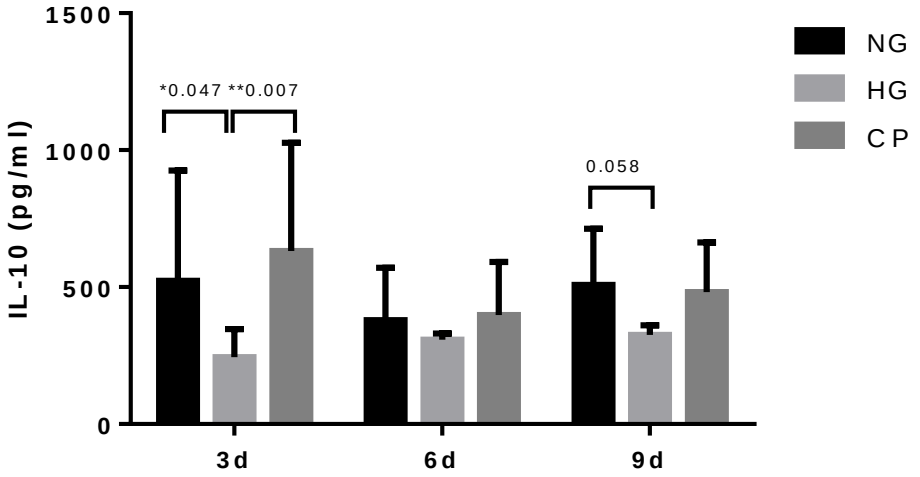


Figura 6. **Producción de IL-10.** Se cuantificó la cantidad de IL-10 en el sobrenadante de los cultivos de MDM a los 3, 6 y 9 días de incubación. Se utilizó una prueba múltiple de T para identificar las diferencias significativas. *P<0.05. Las barras representan la media y desviación estándar de los datos. Condiciones de control de presión osmótica (**CP**), 5 mM de glucosa (**NG**) y 15 mM de glucosa (**HG**).

Previamente se observó que la alta concentración de glucosa no parecía modificar la producción de las citocinas pro-inflamatorias TNF-α, IL-6 e IL-12 de macrófagos, por lo menos durante los primeros tres días de estimulación en condiciones de hiperglucemia (88).

Una de las probables explicaciones que propusimos en su momento fue la restricción de nuestra ventana de observación experimental. Por este motivo, extendimos el tiempo de cultivo para observar si incrementaba la producción de TNF-α como un evento tardío y posterior a la disminución de IL-10 observada a los tres días de cultivo de MDM a 15 mM de glucosa. Sin embargo, tras analizar los resultados de la producción de TNF-α a tres, seis y nueve días, no se observó

diferencia significativa (Fig. 7).

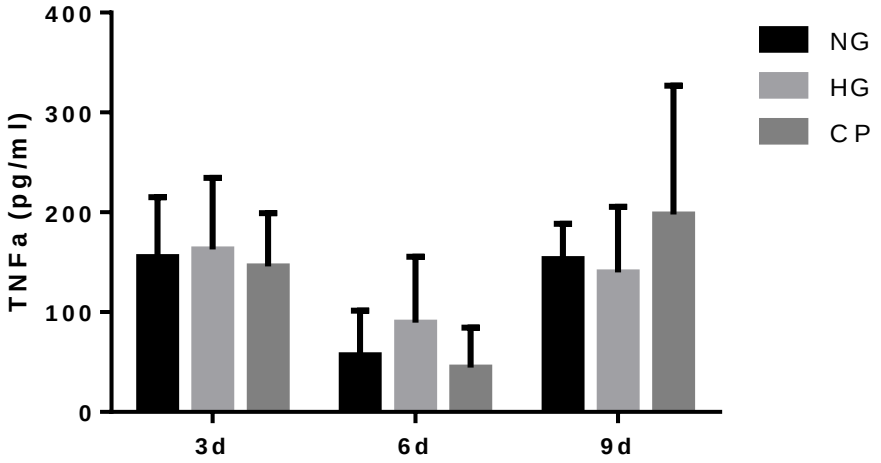


Figura 7. **Producción de TNF-α de MDM a distintas concentraciones de glucosa.** La producción de TNF-α no se incrementó con la exposición a altas concentraciones de glucosa a 3, 6 ni 9 días. Se utilizó una prueba múltiple de T para identificar las diferencias significativas. *P<0.05. Las barras representan la media y desviación estándar de los datos. Condiciones de 5 mM de glucosa (**NG**), 15 mM de glucosa (**HG**) y control de presión osmótica (**CP**).

Por tal motivo, se exploró si la inducción hacia un fenotipo inflamatorio estaría evidenciado por otra citocina inflamatoria no analizada anteriormente, la IL-1β. La producción de IL-1β se determinó por ELISA tipo sándwich. A pesar de los niveles bajos de esta citocina se observó un incremento cuando los macrófagos fueron expuestos a 15 mM de glucosa tras 9 días de cultivo (Fig. 8).

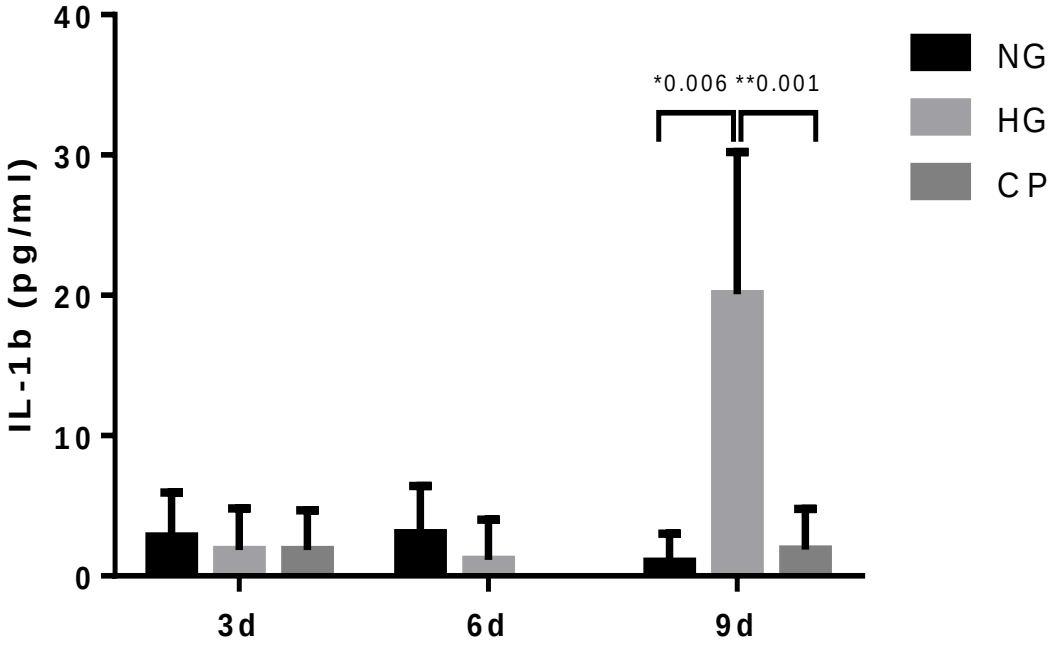


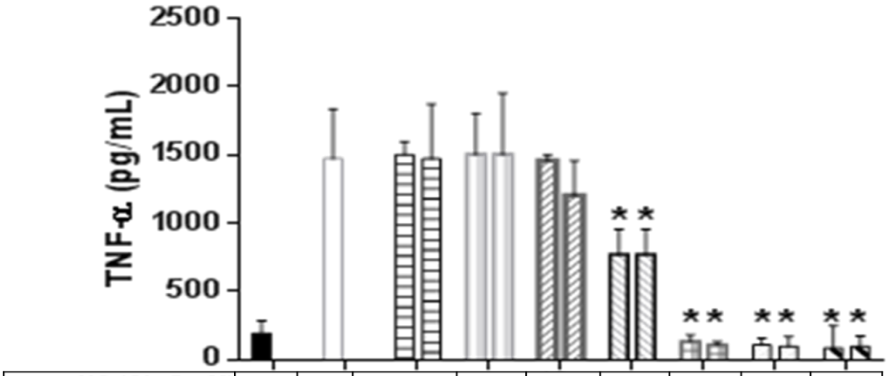
Figura 8. Producción de IL-β de MDM a distintas concentraciones de glucosa. La producción de IL-1β se incrementó con la exposición a altas concentraciones de glucosa a 9 días. Se utilizó una prueba múltiple de T para identificar las diferencias significativas. *P<0.05. Las barras representa la media y desviación estándar de los datos. Condiciones de 5 mM de glucosa (**NG**), 15 mM de glucosa (**HG**) y control de presión osmótica (**CP**).

La IL-1β es producida por macrófagos activados como una pro-proteína que se procesa proteolíticamente en su forma activa por la caspasa 1 (CASP1/ICE). Esta citocina es un mediador importante de la respuesta inflamatoria, y está implicada en diversas de actividades celulares incluyendo proliferación celular, diferenciación y apoptosis

9.5 Participación de las vías mediadas por RAGE y NF-κB

Nuestro estudio previo mostró que la expresión de genes inducidos por NF-κB, como TLR-4 o incluso el mismo mRNA de NF-κB, se incrementaban en

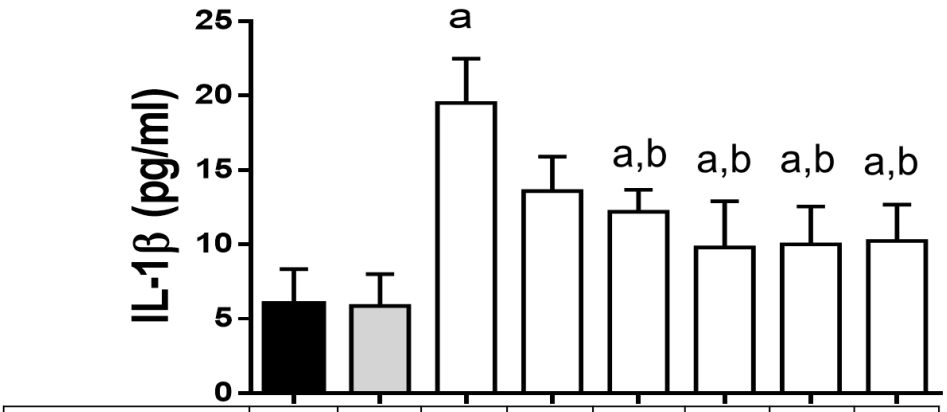
macrófagos expuestos a HG (88). Dicho aumento fue de 2.7 y 2.9 veces, respectivamente, comparado con macrófagos cultivados en condiciones NG y CP (88). Este antecedente inmediato nos hizo analizar la participación de NF-κB en la polarización inflamatoria de MDM inducida por HG. Por lo tanto, para caracterizar la participación de NF-κB en este fenómeno de polarización inhibimos su activación. Para tal efecto, se utilizó un inhibidor farmacológico llamado partenolide (PTL) en un estímulo clásico de activación de la vía de NF-kB, evidenciado por la producción de TNF-α (Fig. 9).



NG (Glu 5 mM)	+	+	+	+	+	+	+	+	+
LPS 10 ng/ml		+	+	+	+	+	+	+	+
PTL 0.1 µM (1 h, 2h)			+						
PTL 1 µM (1 h, 2h)				+					
PTL 5 µM (1 h, 2h)					+				
PTL 10 µM (1 h, 2h)						+			
PTL 20 µM (1 h, 2h)							+		
PTL 50 µM (1 h, 2h)								+	
PTL 100 µM (1 h, 2h)									+

Figura 9. Inhibición de un estímulo clásico de activación (LPS) con PTL. Las concentraciones de PTL superiores a 10 uM son las que muestran tener efecto inhibitorio sobre la activación de NF-kB. Las mejores concentraciones para la inhibición son superiores a los 20 uM, las cuales llegan observarse inhiben hasta concentraciones basales la producción de TNF- α. Se probaron 1 y 2 horas de incubación con PTL previos a la activación con LPS para cada concentración del inhibidor. Las barras representan la media y desviación estándar de los datos.
NG; 5 mM de glucosa, CP; Control de presión osmótica, HG; 15 mM de glucosa. LPS; Lipopolisacárido, PTL; Partenolide. *P< 0.05.

Posteriormente se evaluó si el incremento de la citocina pro-inflamatoria IL-1β en HG, podía evitarse con la inhibición de NF-κB. Sin embargo, a tiempos cortos de 1 y 2 horas de incubación con el inhibidor (previos a colectar las células), no se observó la disminución de la producción de la IL-1β. Por tal motivo se incrementó el tiempo de exposición al PTL a 24 horas (previas a colectar las células), observándose la disminución significativa de la producción de IL-1β debido al efecto de las HG en concentraciones superiores a los 10 μM de PTL en MDM cultivados en HG por 9 días (Fig. 10). No se utilizaron tiempos superiores a 24 horas de incubación con PTL, ya que en experimentos piloto observamos disminución de la viabilidad celular a partir de las 48 horas. Lo anterior ha sido reportado también por otros grupos (90), razón por la que la inhibición con PTL no se aplicó antes del estímulo con HG (ya que implicaría un cultivo de 9 a 10 días con PTL). La producción de IL-1β por MDM en HG también es significativamente mayor a la de MDM en NG. Es decir, la producción incrementada de IL-1β inducida por HG se reduce significativamente, sin embargo, no se logra abatir por completo para alcanzar un estado basal como en el control NG (Fig.10)

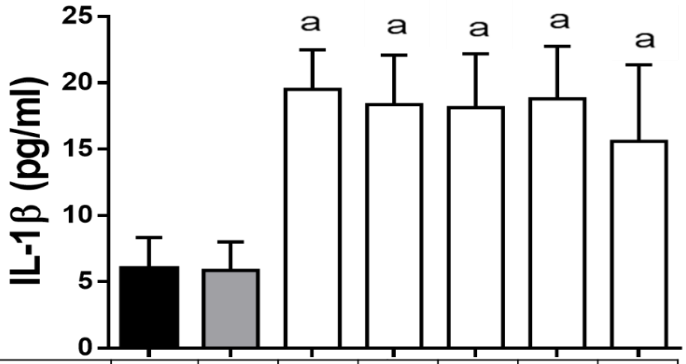


NG (Glu 5 mM)	+							
CP (G+M 15 mM)		+						
HG (Glu 15 mM)			+	+	+	+	+	+
PTL 5 µM				+				
PTL 10 µM					+			
PTL 25 µM						+		
PTL 50 µM							+	
PTL 100 µM								+

Figura 10. Efecto de la inhibición de NF-κB sobre la producción de IL-1β. En cultivos de MDM por 9 días en HG se añadieron diferentes concentraciones de PTL 24 horas previas al final del cultivo celular.
NG; 5 mM de glucosa, CP; Control de presión osmótica y HG; 15 mM de glucosa, PTL; Partenolide. a. Denota diferencia significativa con respecto NG, b. Diferencia significativa con respecto a la condición HG. *P< 0.05. Las barras indican la media y desviación estándar de los datos.

Los resultados arriba mostrados indican que la activación de NF-κB participa en cierta medida en el incremento de la producción de IL-1β inducido por la HG. Una de las posibilidades planteadas inicialmente al inicio de este trabajo experimental fue que la HG indujera la formación de AGEs que pudieran ser reconocidos por su receptor en membrana (RAGE). La unión AGE-RAGE activaría una vía de señalización parecida a la de TLR-4 y activaría a NF-κB. Por lo tanto,

para inhibir el inicio de dicha vía probamos un anticuerpo neutralizante de RAGE y así evaluar su participación en la producción incrementada de IL-1β inducida por HG. La figura 11 muestra los resultados los experimentos con anticuerpos neutralizantes contra RAGE. Se buscó inhibir la producción incrementada de IL-1β, incubando los MDM en HG con diferentes concentraciones de anti-RAGE durante 24 horas previas a colectar las células que se expusieron a HG por nueve días. No observamos inhibición de la producción de IL-1β bajo esas condiciones experimentales, ni en experimentos adicionales cuando extendimos el tiempo de incubación con el anticuerpo neutralizante o cuando los redujimos. Es importante destacar que otros autores han el reportado efecto neutralizante de este anticuerpo desde 1 µg/mL (91).



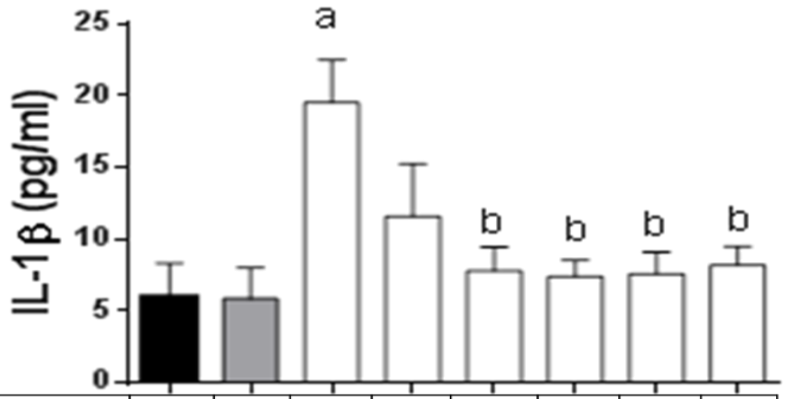
NG (Glu 5 mM)	+						
CP (G+M 15 mM)		+					
HG (Glu 15 mM)			+	+	+	+	+
anti-RAGE 1 µg/mL				+			
anti-RAGE 5 µg/mL					+		
anti-RAGE 10 µg/mL						+	
anti-RAGE 50 µg/mL							+

Figura 11. **Adición de anticuerpo neutralizante anti-RAGE a MDM incubados por nueve días en HG**. Producción de IL-1β a distintas concentraciones de glucosa y con la adición de anti-RAGE por 24 horas. Las barras representan las medias y desviaciones estándar de los datos.
NG; 5 mM de glucosa, CP; control de presión osmótica y HG; 15 mM de glucosa.

9.6 Participación de la vía del poliol

En estudios previos determinamos que los macrófagos expuestos a concentración alta de glucosa incrementan la expresión del mRNA de las enzimas AR y SD (88). Lo anterior puede ser indicativo de un incremento de la actividad de la vía del poliol o vía del sorbitol.

Buscamos determinar la participación de la vía del poliol en el fenómeno de polarización pro-inflamatorio de MDM inducido por HG. Para tal fin utilizamos un inhibidor de la AR (primer y crucial enzima de la vía) llamado sorbinil (SOR). Observamos que los MDM incubados hasta nueve días en HG no incrementaban la producción de IL-1β cuando se adcionó SOR 24 horas antes de la colecta de células (Fig. 12). La inhibición del efecto de la HG sobre los MDM es dosis-dependiente y se observó a partir de 10 μM de SOR. Es importante destacar que la dicha inhibición es total, ya que la producción de la citocina estudiada en MDM expuestos a HG más SOR, no muestran diferencia significativa con respecto a la producción basal (condiciones NG y CP). Los resultados indican que la inhibición de la vía del poliol tiene efecto sobre la producción de IL-1β, que se observa al cultivar MDM a HG (Fig. 12). Por lo tanto, la inhibición de la vía del poliol pudiera inhibir la polarización de estos MDM hacia un fenotipo pro-inflamatorio, aún en presencia de HG.



NG (Glu 5 mM)	+							
OP (G+M 15 mM)		+						
HG (Glu 15 mM)			+	+	+	+	+	+
SOR 5 µM				+				
SOR 10 µM					+			
SOR 25 µM						+		
SOR 50 µM							+	
SOR 100 µM								+

Figura 12. La inhibición de AR evita el incremento de IL-1β en MDM inducido por HG. Producción de IL-1β a distintas concentraciones de glucosa y con el uso de SOR por 24 horas.
NG; 5 mM de glucosa, CP; Control de presión osmótica y HG; 15 mM de glucosa, SOR; Sorbinil. a. Denota diferencia significativa con respecto NG, b. Diferencia significativa con respecto a la condición HG. *P< 0.05. Las barras representan las medias y desviaciones estándar de los datos.

Estos resultados muestran que la activación de la vía del poliol favorece la polarización inflamatoria en MDM inducida por HG.

X. DISCUSIÓN

El modelo experimental del presente estudio fue un sistema *in vitro* de macrófagos derivados de monocitos diferenciados mediante M-CSF e IL-3. Las condiciones de concentración fisiológica y elevada de glucosa fueron reproducidas en cultivo celular, debido a la dificultad de acceder a este tipo de muestras. Consideramos el hecho bien conocido que la diferenciación de monocitos a macrófagos mediante M-CSF induce a estas células a un fenotipo parecido al M2 o "M2-like" (92). Sin embargo, es justo el tipo de fenotipo que presentan los macrófagos residentes de tejidos en condiciones fisiológicas que pretendemos emular en este estudio.

Es importante destacar que en nuestro modelo usamos macrófagos bien diferenciados y los cambios morfológicos y del inmunofenotípo son debidos al proceso de diferenciación (Fig. 2). Es por tal motivo que se observó la reducción significativa de la expresión en superficie de la fosfatasa de tirosina CD45. Dicha fosfatasa de membrana regula negativamente el proceso de diferenciación desfosforilando PKC (93), por lo cual la menor expresión en membrana parece ser un fenómeno que favorece la diferenciación de los monocitos. De manera similar se determinó la eficiencia del proceso de diferenciación debido a la reducción en la expresión de CD14, fenómeno que se ha reportado en células THP-1 (líneas celulares monocíticas), como en monocitos humanos diferenciados y en la diferenciación de células de Langerhans (94, 95). Todo este conjunto de información nos permite establecer que las células bajo estudio en este trabajo son macrófagos bien diferenciados.

La integrina CD11c ha sido frecuentemente utilizada para la identificación de macrófagos humanos pro-inflamatorios en tejido adiposo de sujetos con sobrepeso y obesidad (96, 97). Por otro lado, macrófagos CD11c+ intestinales despliegan respuestas pro-inflamatorias, como la producción de IL-23 activada por *Helicobacter hepaticus* (estímulo de activación clásica) (98). Es por ello que asociamos la expresión de CD11c a la polarización inflamatoria de los MDM. En cuanto a la expresión de esta integrina, se ha descrito que incrementa su expresión entre tres a cuatro veces debido a la adherencia al plástico que favorece la expresión de moléculas de adhesión celular (99). Esta característica de la expresión en células de linaje monocítico es seguramente uno de los factores que observamos en la expresión de CD11c por el100% de los MDM en cultivo. A pesar de lo anterior, pudimos observar diferencias significativas en expresión entre MDM en HG con respecto a MDM en NG. De manera muy interesante, observamos como la exposición a tiempos largos en HG induce una diferencia más notoria en la expresión de esta integrina, con respecto a la expresión basal en condiciones de NG o CP. CD11c no es una integrina normalmente utilizada para estudiar macrófagos inflamatorios ya que, colectivamente, se le ha asociado al fenotipo de células dendríticas. Sin embargo, la expresión de CD11c es ubicua también en los macrófagos y algunos autores reportan que es incluso mayormente expresado en macrófagos (como MDM) que en células dendríticas (100). Además, es importante no perder de vista la función de CD11c como molécula de adhesión celular ya que, el incremento en su expresión en macrófagos debido a HG podría, al menos en parte, influenciar un incremento de la capacidad adherente de estas células. Aunado a ello, otros autores han reportado que la expresión de CD11c se

incrementa en monocitos de sangre periférica debido a estados de hiperglucemia transitoria durante periodos postprandiales (101). En este sentido, las HG también incrementan la capacidad de VSMC y de células endoteliales de adherir monocitos y favorecer su diferenciación (87, 102). Estas últimas observaciones pudieran tener muy importantes implicaciones en el desarrollo de complicaciones vasculares.

La peculiaridad de observar en tiempos más cortos primero la disminución en la producción de IL-10 nos obliga a pensar si es necesario primero disminuir la capacidad anti-inflamatoria de macrófagos expuestos a HG, para que después estos incrementen gradualmente la expresión de citocinas pro-inflamatorias como IL-1β. Esto resultaría de vital importancia para entender por qué los efectos de las HG resultan ser evidentes a nivel clínico después de mucho tiempo. Si consideramos el aspecto de la cronicidad como un factor importante dentro del fenómeno de polarización inflamatoria en condiciones de hiperglucemia y además también del fenómeno de inflamación sistémica sobre la integridad tisular como en el caso del tejido vascular, podremos entender mejor la razón de que las macro- y microangiopatias no sean un evento temprano en el transcurso de la DM. En este sentido se ha descrito como macrófagos de la placa aterosclerótica muestran la disminución en la síntesis de IL-10 en pacientes con estado hiperglucémico crónico que subsecuentemente tiene repercusiones en la estabilidad de la placa aterosclerótica (56).

Estudios previos sobre el perfil de expresión de mRNA en células THP-1 habían mostrado incremento de mensajeros de IL-1β inducido por HG. Sin embargo. estos resultados curiosamente no fueron abordados de manera más

extensa incluso por los autores del trabajo (86). No es sino hasta ahora cuando reportamos el incremento en la producción de IL-1 β en macrófagos humanos (no líneas celulares) secundaria a la exposición a alta concentración de glucosa.

La polarización pro-inflamatoria fue confirmada con la expresión de mRNA de dos enzimas clave en la caracterización de la dicotomía pro- y anti-inflamatorio. La expresión de iNOS y arginasa-1 revela mucho sobre la funcionalidad de los macrófagos, dado su implicación en el metabolismo y producción de NO por parte de macrófagos pro-inflamatorios o bien de ornitina en el caso de estas células con fenotipo anti-inflamatorio (103). Nuestros resultados claramente mostraron que la expresión de arginasa -1 disminuye en MDM expuestos a HG, en contraste, la expresión de iNOS incrementó significativamente, lo cual podría tener implicaciones *in vivo* en la producción de NO. Debido a las características de nuestro modelo, estudiar la producción de NO en los MDM no es la mejor alternativa. Este fue el principal motivo por el cual nuestra aproximación fue a través de caracterizar la expresión de ambas enzimas a través de mRNA. La expresión y actividad de estas enzimas que compiten por la L-arginina como sustrato establecen el punto de inflexión que divide el camino entre la polarización de los macrófagos hacia un perfil anti-inflamatorio (si arginasa 1 es favorecida) o hacia un perfil pro-inflamatorio (si iNOS es favorecida). La expresión de iNOS y arginasa 1 en los macrófagos humanos ha sido controvertida desde hace mucho tiempo, algunos sostienen que no son del todo adecuados para estudios de macrófagos humanos (104-107). Sin embargo, estudios clásicos demostraron que los monocitos y macrófagos humanos producían pequeñas cantidades de NO y aunque no en todos los casos se modificaban mediante la adición de LPS o de

citocinas como INF-γ (108-110), si se observaba que el mRNA de iNOS se podía aumentar mediante LPS (111). Aunque, algunos informes adicionales mostraron que diferentes estímulos promueven mayor expresión de iNOS, como estímulos con polinucleótidos o poli I:C (112), apolipoproteina E, β-amiloide (113) y como células tumorales que sensibilizaban monocitos humanos para la producción de NO (114). Los resultados polémicos o contradictorios en monocitos/macrófagos serían entonces debidos a diferencias fisiológicas entre monocitos, MDM y aislados primarios de macrófagos (considerando los muy diversos tipos de macrófagos humanos provenientes de aislados) y si fuera poco también de los estímulos utilizados para polarizar o activar de manera inflamatoria que se utilizan en diversos estudios.

Lo que queda claro a partir de este estudio es que la inducción de este estado pro-inflamatorio de los MDM, al menos en nuestro modelo experimental, es debida básicamente a la actividad de la vía del poliol. En este sentido, la actividad de la vía del poliol muy seguramente es la responsable de la activación de NF-κB mediante la producción de ROS, y no mediante el inicio de una vía mediada por receptores como RAGE (como nosotros pensábamos en un principio). Dicho proceso encontraría su símil en lo ocurrido en células endoteliales donde la acumulación de sorbitol (metabolito intermedio de la vía del poliol) en el interior de la célula endotelial genera mal plegamiento de proteínas, y subsecuentemente la liberación de radicales libres y citocinas inflamatorias (115). La importancia de la actividad de la vía del poliol parece no sólo restringirse a este proceso de polarización pro-inflamatoria de los macrófagos sino incluso se observa en los procesos de activación clásica. La activación de PKC es necesaria para la

activación pro-inflamatoria de macrófagos en respuesta a LPS (116) y pudiera ser abolida por la inhibición de la AR. Lo anterior es una idea que surge teniendo en cuenta que se ha demostrado que la inhibición de la aldosa reductasa puede evitar la activación de PKC en VSMC (117). LA importancia de la actividad de la vía del poliol en la producción de la IL-1β debido a la DM ya se había empezado a vislumbrar por autores que proponían la participación de NADPH oxidasas (118). Aunque no se conocía bien cuales podrían estar implicadas. Cabe resaltar que precisamente la AR y la SD son NADPH oxidasas por lo cual nuestros resultados concuerdan con estos estudios previos y señalan a la vía del poliol como el mecanismo por el cual se estaría iniciando la polarización inflamatoria.

XI. CONCLUSIÓN

La alta concentración de glucosa inducen la polarización pro-inflamatoria de macrófagos principalmente caracterizada en tiempos cortos (3 días) por mayor expresión de CD11c en membrana y de iNOS a nivel de mRNA, así como de la reducción en la expresión de arginasa 1 y la producción de IL-10. La exposición prolongada (9 días) a concentración elevada de glucosa promueve en estos MDM el incremento en la expresión de CD11c con respecto a MDM en NG. El incremento en la producción de IL-1β es un evento tardío de la exposición de los MDM a HG. Además, este aumento en la producción de IL-1β en MDM expuestos a HG está principalmente favorecido por la actividad de la vía del poliol con la participación sinérgica de la actividad de NF-κB, es probable que la generación de ROS por parte de la vía del poliol sea responsable de la activación de NF-κB (e incluso del inflamasoma NLRP3, responsable de la maduración de IL-1β), sin embargo, esto último debe ser demostrado experimentalmente en estudios posteriores.

XII. REFERENCIAS

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XIII. ANEXO I: ARTÍCULOS GENERADOS DURANTE LOS ESTUDIOS DE POSGRADO

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Human monocytes and macrophages undergo M1-type inflammatory polarization in response to high levels of glucose

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ABSTRACT

Emerging data suggest that elevated glucose may promote inflammatory activation of monocytic lineage cells with the ability to injure vascular endothelial tissue of diabetic patients, however evidence in primary human monocytes and macrophages is still insufficient. We investigated the effect of high glucose concentration on the inflammatory capacity of human macrophages *in vitro* and examined whether similar responses were detectable in circulating monocytes from prediabetic patients. Primary monocytes were isolated from healthy blood donors and differentiated into macrophages. Differentiated macrophages were exposed to normal levels of glucose (NG), high glucose (HG) or high mannitol as osmotic pressure control (OP) for three days. Using PCR, ELISA and flow cytometry, we found that HG macrophages showed overexpression of CD11c and inducible nitric oxide synthase as well as down-regulation of arginase-1 and interleukin (IL)-10 with respect to NG and OP macrophages. Consistent with *in vitro* results, circulating monocytes from hyperglycemic patients exhibited higher levels of CD11c and lower expression of CD206 than monocytes from normoglycemic controls. In subjects with hyperglycemia, elevation in $CD11c^+$ monocytes was associated with increased obesity, insulin resistance, and triglyceridemia as well as low serum IL-10. Our data suggest that human monocytes and macrophages undergo M1-like inflammatory polarization when exposed to high levels of glucose on *in vitro* culture conditions and in patients with hyperglycemia. These results demonstrate that excess glucose has direct effects on macrophage activation though the molecular mechanisms mediating such a response remain to be elucidated.

1. Introduction

Hyperglycemia is a metabolic alteration characterized by abnormally high levels of blood glucose (blood glucose ≥ 5.5 mM or 100 mg/dl) [1]. Hyperglycemia is considered a key component of the metabolic syndrome and hallmark of *Diabetes Mellitus*, a chronic disease with high prevalence and mortality rates worldwide [2–4]. In diabetic patients, hyperglycemia has been shown to adversely affect both large and small blood vessels, leading to macro- and microvascular complications that include coronary artery disease as well as diabetic nephropathy, neuropathy, and retinopathy [5–7].

Abbreviations: AGEs, advanced glycation end products; PKC, protein kinase C; ROS, reactive oxygen species; CD, cluster of differentiation; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor alpha; IL, interleukin; Arg-1, arginase 1; LPS, lipopolysaccharide; PBMCs, peripheral blood mononuclear cells; PCR, polymerase chain reaction; ELISA, enzyme-linked ImmunoSorbent Assay; WBCs, white blood cells; BMI, body mass index; HOMA-IR, homeostatic model assessment for insulin resistance; M, male; F, female; NG, normal glucose levels; HG, high glucose levels; OP, osmotic pressure control; MDM, monocyte-derived macrophages; M-CSF, macrophage-colony stimulating factor; M1, classically activated macrophage; M2, alternatively activated macrophage.

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Numerous studies have proposed three main mechanisms through which high levels of glucose may damage vascular endothelial tissue: intracellular glucose toxicity, production of advanced glycation end products (AGEs), and protein kinase C (PKC) activation [8–10]. Intracellular glucose toxicity (also referred to as the polyol pathway) is able to elicit endothelial release of reactive oxygen species (ROS), while AGEs and PKC activation are involved in promoting leukocyte adhesion to vascular endothelial tissue and subsequent blood vessel injury [10–12]. Interestingly, emerging data have now pointed to macrophage inflammatory activation as a possible additional mechanism through which high levels of glucose may also damage vascular endothelium.

Macrophages are white blood cells that originate from monocytes [13]. In the bone marrow, hematopoietic stem cells are capable of differentiating into monocytes. Monocytes are believed to persist in blood circulation for 4–7 days and afterward migrate into tissues where they will differentiate into tissue resident macrophages [13,14]. Depending on the extracellular milieu, macrophages are capable of carrying out inflammatory or anti-inflammatory actions [15–17]. Macrophages displaying a pro-inflammatory phenotype of activation (also referred to as M1, or classically activated) are characterized by expression of specific cell surface markers such as the cluster of differentiation (CD) 11c [18,19]. Functionally, M1 macrophages are characterized by production of inflammatory mediators including inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF-α), and interleukin (IL)-1β [20]. In contrast, macrophages exhibiting anti-inflammatory activation (also referred to as M2, or alternatively activated) typically show expression of CD206 and arginase 1 (Arg-1) as well as increased production of IL-10, a cytokine with potent immunoregulatory actions [20–22]. A growing body of experimental evidence has recently suggested that excessive amounts of glucose may be capable of acting directly on monocyte-macrophage cell lines by shifting the polarization of these immune cells toward a pro-inflammatory state that resembles an M1 phenotype [17,23–25]. Indeed, RAW264.7 cells (a monocyte-macrophage cell line derived from murine leukemia) significantly increase TNF-α production after being exposed to high levels of glucose [23]. Similarly, exposure to high glucose levels results in stimulation of IL-6 and IL-8 secretion in lipopolysaccharide (LPS)-stimulated U937 monocytes [17]. Furthermore, murine peritoneal macrophages show increased mRNA levels of TNF-α, IL-1β, IL-6, and IL-12 in response to elevated concentrations of glucose [24]. Human peripheral blood mononuclear cells (PBMCs) have been demonstrated to exhibit decreased production of IL-10 when exposed to high glucose conditions [25]. These findings suggest that excess glucose may be associated with an M1/M2 imbalance; however, such a phenomenon has not been explored in primary human monocytes and macrophages to date. To this end, we chose to investigate the effect of high levels of glucose on the inflammatory and anti-inflammatory capacity of human macrophages *in vitro*, while also examining whether a similar response could be seen in circulating monocytes from patients with hyperglycemia.

2. Materials and methods

2.1. Subjects

For *in vitro* studies, ten healthy male volunteers with no metabolic disease were included. For *in vivo* studies, 101 women and men attending the Blood Bank or the Internal Medicine Department of the General Hospital of Mexico were included and divided into normoglycemic or hyperglycemic groups. All of the participants provided written informed consent, previously approved by the institutional ethical committee of the General Hospital of Mexico, which guaranteed that the study was conducted in accordance with the principles described at the Helsinki Declaration. For both *in vitro* and *in vivo* studies, subjects were excluded if they had previous diagnosis of non-communicable or infectious diseases. We also excluded pregnant or lactating women as well as subjects taking any kind of anti-inflammatory, anti-aggregant, or anti-hypertensive medication.

2.2. Monocyte isolation and cell culture

For *in vitro* studies, buffy coat samples were collected from each donor (n = 10) and separately diluted 1:2 with phosphate saline buffer 1X (PBS 1X, Sigma-Aldrich, Mexico) for posterior isolation of PBMC by density gradient centrifugation using histopaque-1077 (Sigma-Aldrich, Mexico). Monocyte cells were then isolated from PBMC by CD14-negative selection using magnetic columns (Miltenyi Biotec, Germany). Purified monocytes were placed in glucose-free RPMI-1640 medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 μg/ml gentamicin, 5.5 mM D-glucose, and 10 μg/ml macrophage-colony stimulating factor (M-CSF) in 6-well cell-culture plates (Costar, USA), at a density of 3×10^6 monocytes per well. Culture media and M-CSF were replaced every other day for six days.

2.3. High glucose stimulation

After six days of *in vitro* culture differentiation, monocyte-derived macrophages (MDM) were exposed to a high glucose environment. Control MDM (NG) were incubated in glucose-free RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine, 50 μg/ml gentamicin, and 5.5 mM D-glucose for three days designed to resemble normal glucose levels observed in healthy subjects. High glucose MDM (HG) were incubated in glucose-free RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine, 50 μg/ml gentamicin, and 15 mM D-glucose for three days designed to resemble sugar levels seen in diabetic patients with uncontrolled hyperglycemia. Control for osmotic pressure (OP) was achieved by incubating MDM in glucose-free RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine, 50 μg/ml gentamicin, 5.5 mM D-glucose, and 9.5 mM D-manitol for three days. After three days in culture, MDM were harvested using sterile cell scrapers (Corning, USA) and supernatant was collected from each culture well. Collected MDM were equally divided into 1 ml of PBS 1X (Sigma-Aldrich, Mexico) or 500 μl of TRIzol Reagent (Life Technologies, USA) for being used in flow cytometry and RT-PCR assays, respectively.

2.4. Characterization of cell surface markers by flow cytometry

After collecting MDM, 1×10^6 cells were resuspended in 50 μl of sterile PBS 1X (Sigma-Aldrich, Mexico). Immediately after, 3 μl of Human TruStrain (BioLegend, Inc., USA) was added and incubated for 5 min on ice. Then, MDM were simultaneously incubated with anti-CD45 FITC, anti-CD14 PE/Cy7, anti-CD11c PE/Cy5, and anti-CD206/Cy7 APC (BioLegend, Inc., USA) for 20 min for posterior analysis on a FACSCanto II flow cytometer (BD Biosciences, Mexico) by means of BD FACSDiva™ software 6.0, acquiring 50,000 events per test in triplicate. FITC mouse IgG1, PE/Cy7 mouse IgG2, APC/Cy7 mouse IgG1, and PE/Cy5 mouse IgG1 (BioLegend, Inc., USA) were used as isotype control antibodies for cell surface staining of CD45, CD14, CD206, and CD11c, respectively.

2.5. Cytokine production by ELISA

In vitro production of TNF-α and IL-10 were measured in MDM culture supernatants by the Enzyme-Linked ImmunoSorbent Assay

(ELISA) (Peprotech, Mexico), using 100 μl of undiluted supernatant per test. All cytokine measurements were performed in triplicate according to manufacturer's instructions.

2.6. Gene expression profile by RT-PCR

After collecting MDM, 1×10^6 cells were resuspended in 500 μl of TRIzol Reagent (Life Technologies, USA) for posterior RNA isolation following manufacturer's instructions. Then, total RNA samples were reverse-transcribed by means of the M-MLV Retrotranscriptase system using dT primer (Invitrogen, USA). Resulting cDNA samples were specifically amplified by polymerase chain reaction (PCR) using TaqDNA polymerase (Biotecnologías Universitarias, UNAM, Mexico) and human-specific primers to detect mRNA levels of iNOS, and Arg-1. 18S-ribosomal RNA was used to control for constitutive expression. Relative expression of each amplified gene was obtained by optical density analysis (OD) using the 18S-ribosomal RNA as the control for constitutive expression.

2.7. Anthropometric, metabolic, and immunological measurements in normoglycemic and hyperglycemic subjects

For *in vivo* studies, body mass index (BMI), waist circumference, and body fat percentage were individually recorded in 101 subjects. Serum glucose levels were measured in triplicate by the glucose oxidase assay, following the manufacturer's instructions (Megazyme International, Ireland). Serum insulin levels were measured in triplicate by ELISA, following the manufacturer's instructions (Abnova Corporation, Taiwan). The estimate of insulin resistance was individually calculated by means of the homeostasis model assessment of insulin resistance (HOMA-IR). Total cholesterol and triglyceride levels were individually measured in triplicate by enzymatic assays according to manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Serum levels of TNF-α and IL-10 were measured in triplicate by ELISA kits (Peprotech, Mexico) using serum samples diluted 1:250 in PBS 1X (Sigma-Aldrich, Mexico). All of the metabolic and immunological measurements were performed at the same time in order to avoid procedural variations.

2.8. White blood cell isolation and characterization of monocyte surface markers

Twenty milliliters of venous blood were obtained from each subject and collected into tubes containing EDTA (Vacutainer™, BD Diagnostics, NJ, USA). Collection tubes were then centrifuged at 1800*g* for 10 min and white blood cells (WBCs) separated using a micropipette. Total WBCs were separately placed into 1.6 ml pyrogen-free eppendorf tubes containing 1 ml of ACK Lysing Buffer (Life Technologies, USA) and incubated at 4 °C for 8 min. Each sample was then centrifuged at 1800*g*/4 °C for 4 min and cell pellets washed twice with PBS 1X (Sigma-Aldrich, Mexico). After an additional centrifugation step and removal of the supernatant, each cell pellet was resuspended in 50 μl of PBS 1X (Sigma-Aldrich, Mexico). On each test, 3 μl of Human TruStrain Reagent (BioLegend, Inc., USA) was added to 2×10^5 WBCs and then incubated for 5 min on ice. Immediately after, WBCs were incubated with anti-CD45 FITC, anti-CD14 PE/Cy7, anti-CD11c PE/Cy5, and anti-CD206/Cy7 APC following the same experimental procedure described above. Assessment of the levels of CD11c and CD206 was performed on $CD45^+CD14^+$ double positive cells, which correspond to circulating monocytes.

2.9. Statistical analysis

For *in vitro* studies, Friedman test was used to compare control, HG, and OP MDM groups in terms of CD11c and CD206 expression, as well as TNF-α and IL-10 *in vitro* production. One-way ANOVA, followed by a post-hoc Tukey test, was used to compare the expression of iNOS and Arg-1 in HG, NG, and OP macrophages. For *in vivo* studies, *t*-test was used to compare normoglycemic and hyperglycemic subjects in terms of BMI, waist circumference, body fat percentage, fasting glucose, fasting insulin, HOMA-IR, total cholesterol, and triglycerides. Mann-Whitney test was used to compare normoglycemic and hyperglycemic subjects in terms of CD11c and CD206 expression as well as TNF-α and IL-10 serum levels. All statistical analyses were performed using the GraphPad Prism 5 software. Differences were considered significant when $P < 0.05$.

3. Results

With regard to M1 macrophage cell-surface markers, all cultured macrophages showed expression of CD11c, irrespective of having been exposed to normal (NG) or high (HG) glucose concentrations (Fig. 1A). In contrast, a 2-fold increase in the intensity of CD11c expression was observed in MDM exposed to high glucose concentrations as compared with MDM exposed to normal glucose concentrations or hyperosmotic (OP) conditions (Fig. 1B). With regard to M2 macrophage cell-surface markers, neither the percentage of MDM producing CD206 nor the intensity of CD206 expression significantly differed in response to high glucose levels or hyperosmolar conditions (Fig. 1C and D, respectively).

In terms of the cytokine production profile associated with macrophage polarization, no significant differences were observed in the secretion level of TNF-α in MDM exposed to high glucose concentrations with respect to NG and OP human macrophages (Fig. 2A). In contrast, a 3-fold reduction in the average secretion of IL-10 was seen in MDM cultured in high glucose concentrations as compared with cells exposed to normal levels of glucose or hyperosmolar conditions (Fig. 2B). With respect to gene expression markers for M1 macrophages, a significant 3-fold increase in the expression of iNOS was observed when MDM were cultured under elevated concentrations of glucose as compared with NG and OP human macrophages (Fig. 2C). In terms of gene expression markers for M2 macrophages, expression of Arg-1 exhibited a clear 3.5-fold decrease in MDM treated with high glucose concentrations in comparison with macrophages exposed to normal levels of glucose or hyperosmolar conditions (Fig. 2D).

After evaluating the effect of high levels of glucose upon human macrophages *in vitro*, we attempted to corroborate our main findings in circulating monocytes of patients with hyperglycemia. No significant differences were observed in the average age and woman/man ratio between hyperglycemic and normoglycemic individuals (Table 1). However, subjects with hyperglycemia showed higher values of BMI (30.77 ± 3.21 *versus* 23.76 ± 2.56 kg/m^2), waist circumference (99.42 ± 10.63 *versus* 82.17 ± 7.46 cm), and body fat percentage (33.17 ± 7.34 *versus* 26.33 ± 8.07) than normoglycemic controls (Table 1). Insulin resistance and triglycerides were also prominently elevated in hyperglycemic subjects when compared to normoglycemic individuals (for HOMA-IR, 4.09 ± 1.62 *versus* 2.51 ± 0.67, and for triglycerides, 219.2 ± 42.56 mg/dl *versus* 147.5 ± 18.75 mg/dl, respectively) (Table 1). Serum insulin and total cholesterol were slightly higher in hyperglycemic subjects than in normoglycemic individuals (for insulin, 15.43 ± 6.05 *versus* 12.67 ± 3.19 mU/l, and for cholesterol, 196.9 ± 9.10 *versus* 192.3 ± 10.47 mg/dl, respectively) (Table 1).

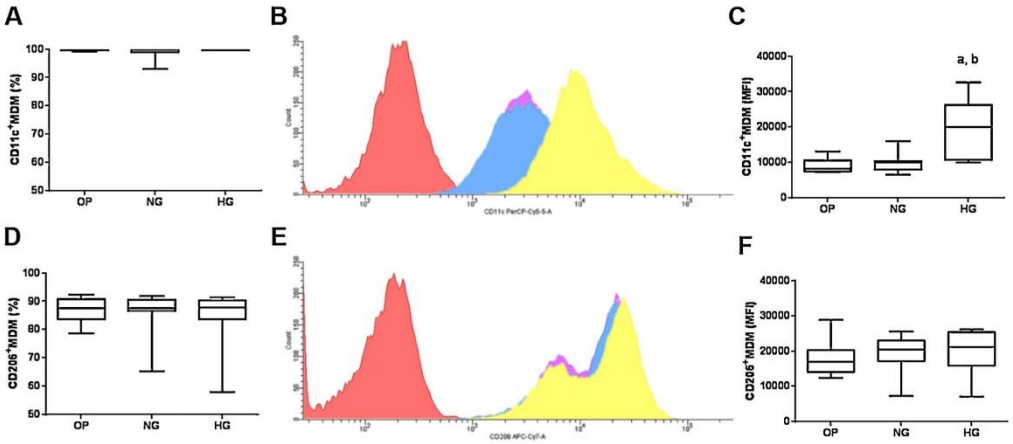

Fig. 1. Cell-surface CD11c and CD206 expression in human macrophages exposed to high levels of glucose. After three days of *in vitro* culture, all cultured monocyte-derived macrophages (MDM) showed expression of CD11c (A). Panel B illustrates representative FACS histograms showing a significant increase in the intensity of CD11c expression in MDM treated with high levels of glucose (yellow) with respect to MDM exposed to normal glucose concentrations (blue) or hyperosmolar conditions (purple). Average values for the intensity of CD11c expression are shown in C. Neither the percentage of MDM expressing CD206 (panel D) nor the intensity of CD206 expression (E and F) significantly differed in response to high levels of glucose or hyperosmolar conditions. Panel E illustrates representative FACS histograms for the intensity of CD206 expression. Panel F shows average values for the intensity of CD206 expression. PE/Cy5 mouse IgG1 and APC/Cy7 mouse IgG1 were used as isotype control antibodies for staining of CD11c and CD206, respectively, and are illustrated in red color. Data are expressed as median and inter-quartile range in a box plot analysis. Significant differences were estimated by means of the Friedman test. Differences were considered significant when $P < 0.05$, as follows: [a]significant difference *versus* the OP group; [b]significant difference *versus* the NG group; [c]significant difference *versus* the HG group. NG, normal glucose levels (5.5 mM D-glucose); HG, high glucose levels (15 mM D-glucose); OP, control of osmotic pressure (5.5 mM D-glucose + 9.5 mM mannitol); MFI, mean fluorescence intensity (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Anthropometric and metabolic characteristics of the study subjects. Subjects were divided into normoglycemic or hyperglycemic individuals depending on the levels of fasting blood glucose. Data are presented as median ± standard deviation. n = 101. Significant differences were estimated by means of the Student's T-test. Differences were considered significant when $P < 0.05$.

	Normoglycemic	Hyperglycemic	*P*-value
	n = 51	n = 50	
Gender (M/F)	20/31	29/21	0.0739
Age (years)	31.08 ± 11.41	35.72 ± 10.40	0.0801
BMI (kg/m^2)	23.76 ± 2.56	30.77 ± 3.21	<0.0001
Waist circumference (cm)	82.17 ± 7.46	99.42 ± 10.63	<0.0001
Body fat (%)	26.33 ± 8.07	33.17 ± 7.34	<0.0001
Fasting blood glucose (mg/dl)	80.34 ± 4.14	107.2 ± 3.28	<0.0001
Serum insulin (mU/l)	12.67 ± 3.19	15.43 ± 6.05	0.0046
HOMA-IR	2.51 ± 0.67	4.09 ± 1.62	<0.0001
Total cholesterol (mg/dl)	192.3 ± 10.47	196.9 ± 9.10	0.0692
Triglycerides (mg/dl)	147.5 ± 18.75	219.2 ± 42.56	<0.0001

Abbreviations: M, male; F, female; BMI, body mass index; HOMA-IR, homeostatic model assessment of insulin resistance.

The percentage of circulating monocytes expressing CD11c was 1.8 times higher in hyperglycemic subjects than in normoglycemic individuals, although no differences were observed in the intensity of CD11c expression between groups (Fig. 3A and B, respectively). Concomitantly, a 3-fold decrease was found in the percentage of circulating CD206$^+$ monocytes in hyperglycemic subjects with respect to normoglycemic controls (Fig. 3C). The intensity of CD206 expression in the total percentage of circulating monocytes from normoglycemic and hyperglycemic individuals did not show any significant difference (Fig. 3D).

The percentage of CD11c$^+$ monocytes showed a significant inverse relationship with the number of circulating monocytes expressing CD206 in our study population ($r = -0.6624$, $P < 0.0001$) (Fig. 4). Ninety four percent of hyperglycemic subjects had a high percentage of circulating CD11c$^+$ monocytes, frequently accompanied by low numbers of CD206$^+$ monocytes. In contrast, sixty three percent of individuals with normal levels of blood glucose exhibited high values of circulating CD206$^+$ monocytes, accompanied by a low percentage of CD11c$^+$ monocytes (Fig. 4).

Consistent with *in vitro* results, systemic levels of TNF-α did not significantly differ between normoglycemic and hyperglycemic subjects (Fig. 5A). However, patients with hyperglycemia exhibited a 1.5-fold decrease in the serum levels of IL-10 as compared with individuals showing normal glycemia (Fig. 5B).

4. Discussion

CD11c is a heterodimeric integrin expressed in monocytes and macrophages exhibiting an inflammatory response [26–28]. CD11c has been shown to mediate monocyte recruitment to the inner lining of arteries, where these cells are able to differentiate into macrophages and foam cells contributing to the formation of atherosclerotic lesions and acute coronary arteritis [29–32]. It is well known that several immunological stimuli can induce CD11c expression in M1 macrophages including interferon gamma (IFN-γ), LPS, lipocalin, and galectin-3 [33–36]. However, little is understood about the role of non-immunological stimuli, such as glucose, in promoting CD11c expression in macrophages [37]. Our data show that elevated levels of glucose have an effect on CD11c expression in human monocytes and macrophages. *In vitro*, CD11c was expressed by all cultured macrophages, regardless of glucose exposure, which is consistent with previous reports demonstrating that adherence to a plastic surface is capable of inducing CD11c expression on macrophages [38,39]. However, the expression of CD11c on each macrophage was considerably increased in response to excess glucose suggesting that the production rate of this integrin may be susceptible to modulation by glucose concentrations

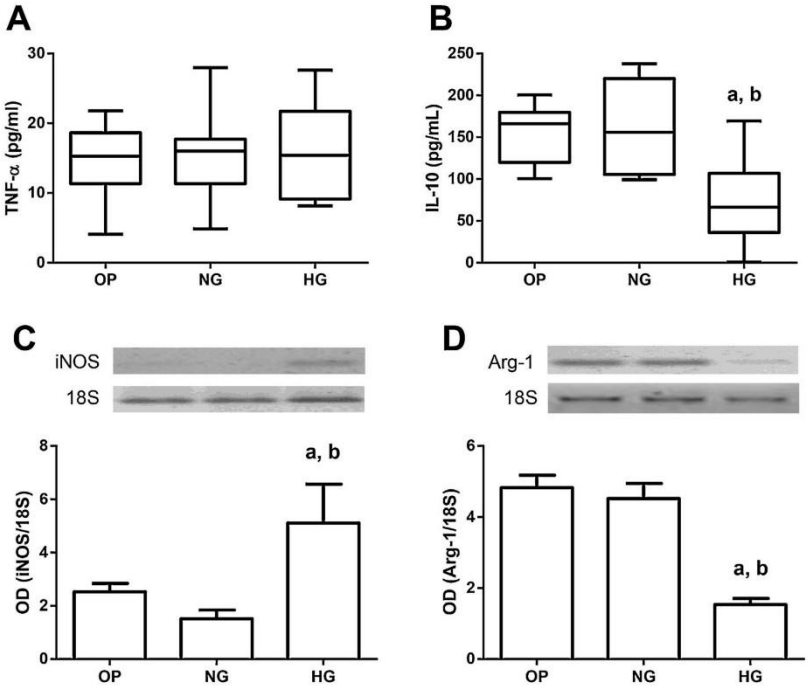

Fig. 2. Cytokine production and gene expression profile of human macrophages exposed to high levels of glucose. After three days of *in vitro* culture, no significant differences were observed in the secretion level of TNF-α (A) in human monocyte-derived macrophages exposed to high levels of glucose with respect to NG and OP macrophages. In contrast, secretion of IL-10 showed a significant reduction in HG macrophages as compared with NG and OP macrophages (B). A significant increase in the expression of iNOS was observed in HG macrophages as compared with NG and OP macrophages (C). In contrast, expression of Arg-1 exhibited a clear decrease in HG macrophages as compared with NG and OP macrophages (D). Representative agarose gels showing differential expression of iNOS and Arg-1 are shown. In A and B, data are expressed as median and inter-quartile range in a box plot analysis. In C and D, data are expressed as mean ± standard deviation. Differences were considered significant when $P < 0.05$, as follows: [a]significant difference *versus* the OP group; [b]significant difference *versus* the NG group; [c]significant difference *versus* the HG group. NG, normal glucose levels (5.5 mM D-glucose); HG, high glucose levels (15 mM D-glucose); OP, control of osmotic pressure (5.5 mM D-glucose + 9.5 mM mannitol.)

independent of the *in vitro* adherence effect. Our *in vivo* findings support the idea that there is a relationship between high levels of glucose and CD11c expression as the number of circulating monocytes expressing CD11c increased in hyperglycemic subjects when compared to euglycemic individuals. These data support the idea that non-immunological stimuli, specifically metabolism-related stimuli such as glucose, have the ability to alter the inflammatory profile of monocytes and macrophages [40]. This notion is consistent with prior studies demonstrating that elevated levels of plasma triglycerides also seem to be capable of stimulating CD11c expression in circulating monocytes from healthy individuals after eating a standardized high-fat meal [41]. Our data show that $CD11c^+$ monocytes are most strongly associated with hyperglycemia, but also exhibit a positive correlation with high triglyceride levels in our study population, which would support the aforementioned findings (data not shown). The implications for understanding the mechanisms through which glucose concentrations and other metabolism-related stimuli are able to induce CD11c expression on human monocytes and macrophages are profound and the idea that further knowledge of these pathways might allow prediction of cardiovascular disease susceptibility adds a compelling degree of urgency to further study.

As described above, increased production of CD11c is strongly indicative of inflammatory polarization in both monocytes and macrophages [26–28]. This possible M1-like phenotype in macrophages exposed to high glucose was also supported by increased mRNA levels of iNOS, a marker consistently expressed by proinflammatory macrophages both *in vivo* and *in vitro* [42,43]. iNOS is known to play a major role in the production of nitric oxide, a free radical that can in turn stimulate NF-κB-dependent inflammatory pathways in M1 macrophages [44–47]. In contrast, M2 macrophages are characterized by high levels of Arg-1, a key marker of alternative activation in these immune cells [48]. Our data demonstrate a relationship between elevated glucose concentration and increased iNOS expression as well as Arg-1 reduction in human macrophages. This relationship suggests a role for excess glucose in biasing macrophages toward an M1-like phenotype. As mRNA expression is not always correlated with protein synthesis and enzymatic activity, it is important to note that iNOS and Arg-1 were assessed by means of mRNA analysis only and the discussion of these results makes no attempt to conjecture beyond that. Further research is needed to draw conclusions regarding the role of high concentrations of glucose on the protein levels and enzymatic activity of iNOS and Arg-1 in human macrophages.

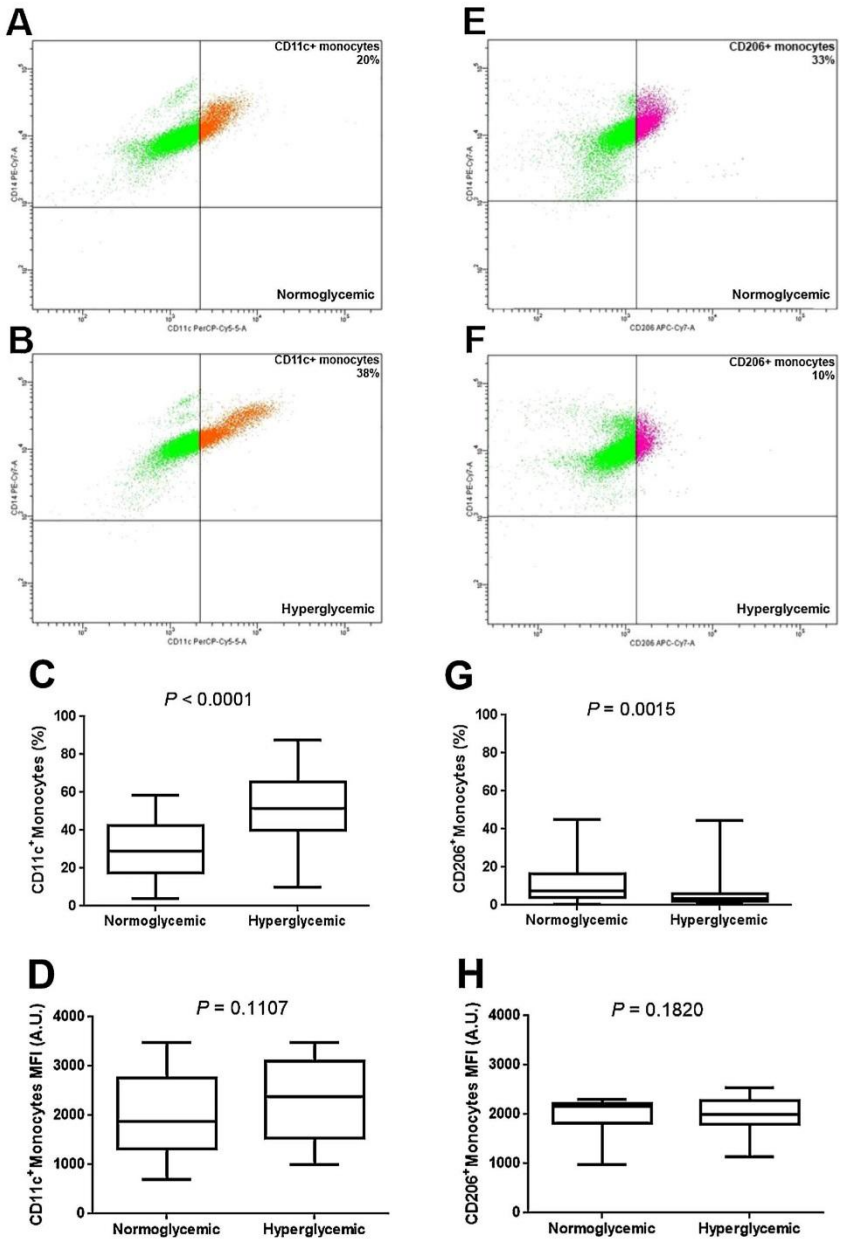

Fig. 3. Cell-surface CD11c and CD206 expression in circulating monocytes of patients with hyperglycemia. Panel A and B illustrate representative dot plots showing CD11c⁺monocytes percentage in normoglycemic subjects and hyperglycemic patients, respectively (orange). Patients with hyperglycemia showed higher levels of circulating CD11c⁺ monocytes than normoglycemic controls (C). The intensity of CD11c expression in circulating monocytes was not significantly different between hyperglycemic patients and controls (D). Panel E and F illustrate representative dot plots showing CD206⁺ monocytes percentage in normoglycemic subjects and hyperglycemic patients, respectively (pink). Patients with hyperglycemia showed lower levels of circulating CD206⁺ monocytes than normoglycemic controls (G). The intensity of CD206 expression in circulating monocytes was not significantly different between hyperglycemic patients and controls (H). Average data are expressed as median and inter-quartile range in a box plot analysis. Differences were considered significant when $P < 0.05$ and calculated using the Mann-Whitney test. MFI, mean fluorescence intensity; AU, arbitrary units (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

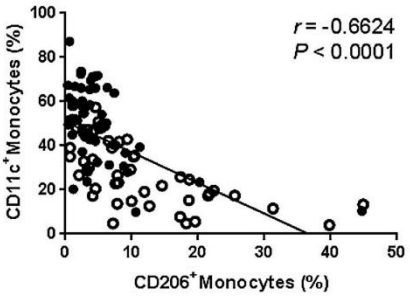

Fig. 4. Relationship between circulating CD11c$^+$ monocytes and CD206$^+$ monocytes in the study population. The percentage of CD11c$^+$ monocytes showed a significant inverse relationship with the number of circulating monocytes expressing CD206 in our study population. Ninety-four percent of hyperglycemic subjects showed a high percentage of CD11c$^+$ monocytes in circulation, frequently accompanied by low numbers of CD206$^+$ monocytes. In contrast, sixty-three percent of individuals with normal blood glucose levels exhibited a high percentage of CD206$^+$ monocytes in the circulation, accompanied by low numbers of CD11c$^+$ monocytes. Assessment of CD11c- and CD206-positive cells was exclusively performed on CD45$^+$CD14$^+$ double positive cells, which correspond to a monocyte cell population. PE/Cy5 mouse IgG1 and APC/Cy7 mouse IgG1 were used as isotype control antibodies for cell surface staining of CD11c and CD206, respectively. Solid dots = hyperglycemic patients; open dots = normoglycemic controls. Coefficients (r) and P values were calculated by the Spearman's correlation model. The correlation level was considered significant when $P < 0.05$.

Consistent with an apparent inclination toward an M1-like phenotype, we found that human macrophages exposed to excess glucose have a reduction in IL-10 secretion. It has been proposed that IL-10-producing macrophages may play a pivotal role in preventing vascular endothelial damage during hyperglycemia as it occurs in atherosclerosis. Sato et al. recently demonstrated that the number of IL-10-producing macrophages decreases in the hemorrhagic atheromatous plaques of patients with acute coronary syndrome (ACS) and hyperglycemia when compared to ACS patients without hyperglycemia who have more stable plaques [49]. Additionally, Chung et al. have shown that adipose tissue macrophages from hyperglycemic mice have less capacity for expressing IL-10 than macrophages derived from adipose tissue of mice exhibiting normal levels of plasma glucose [40]. Our data expand on this body of work by revealing that increased levels of glucose are associated with diminished IL-10 secretion in human macrophages, which may negatively affect their capacity to regulate inflammatory events. These *in vitro* data are congruent with *in vivo* results showing that patients with hyperglycemia exhibit high percentages of circulating CD11c$^+$ monocytes and low systemic values of IL-10. However, further clinical studies are needed to clarify the relationship between CD11c$^+$ monocytes and decreasing serum levels of IL-10 in hyperglycemic patients.

Another phenomenon captured in our study is that macrophages exposed to high glucose concentrations exhibit low production of IL-10 without a concomitant increase in the synthesis of TNF-α. In the presence of prototypic activating stimuli such as IFN-γ/LPS or IL-4/IL-13, macrophages are capable of polarizing toward M1 or M2 phenotypes, which in turn are characterized by increased production of TNF-α and IL-10, respectively [50–52]. However, numerous studies have also reported non-prototypic activating stimuli with the capacity to prime macrophages to assume an M2-like phenotype, as is the case of M-CSF [52–55]. M-CSF has been shown to induce a state in which macrophages appear to be primed for a M2-like profile denoted by high production of IL-10, whereas macrophages exposed to granulocyte-macrophage colony stimulating factor seem to be capable of overproducing TNF-α [56]. M-CSF is produced by a variety of epithelial and stromal cells which drive macrophages to adopt an M2-like phenotype under normal homeostatic conditions [16,57–59]. Our *in vitro* culture system used M-CSF to differentiate human monocytes into MDM and this exposure may largely explain that the resulting macrophages showed improved IL-10 secretion but negligible capacity to produce TNF-α. In this scenario, it is reasonable to consider that prolonged exposure of macrophages to elevated levels of glucose may not only reduce IL-10 secretion, but also favor the production of pro-inflammatory cytokines, which would support the body of work that suggests M2 macrophages initially lose anti-inflammatory capacity prior to adopting an M1 inflammatory profile [60–63].

Consistent with evidence of possible M2 to M1 repolarization, we found that the monocyte subpopulation primed to an inflammatory phenotype (CD11c$^+$ monocytes) is inversely correlated with the subpopulation of monocytes exhibiting an M2 phenotype (CD206$^+$ monocytes) in the circulation of hyperglycemic patients. Despite having been firstly described in obese mice (high-fat diet), CD11c and CD206 are also now recognized as cell surface markers for M1 and M2 macrophages in human beings, respectively [64–66]. In this sense, our results suggest that CD206$^+$ monocytes may be converted into CD11c$^+$ monocytes in response to high glu-

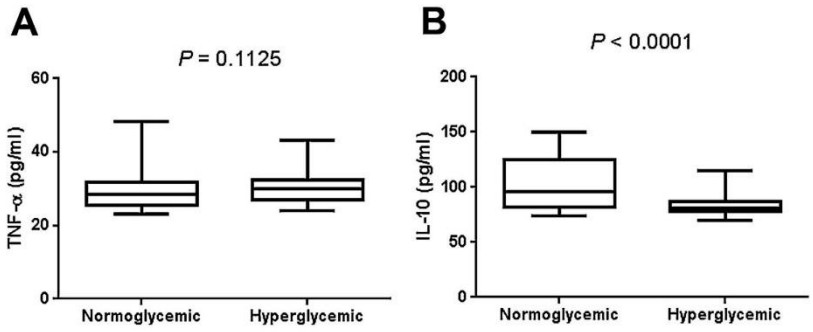

Fig. 5. Serum levels of TNF-α and IL-10 in patients with hyperglycemia. Consistent with *in vitro* results, systemic levels of TNF-α did not show significant differences between hyperglycemic subjects and normoglycemic subjects (A). In contrast, patients with hyperglycemia exhibited a significant decrease in systemic levels of IL-10 with respect to normoglycemic individuals (B). Data are expressed as median and inter-quartile range in a box plot analysis. Differences were considered significant when $P < 0.05$ and calculated using the Mann-Whitney test.

cose concentrations, which supports the idea that macrophages may be primed toward an inflammatory phenotype in the first stages of monocyte differentiation. Interestingly, the imbalance between both monocyte subpopulations was not only associated with hyperglycemia but also with increased insulin resistance, triglycerides, BMI and central obesity. This could suggest that the phenotype of monocytes is influenced by numerous parameters of metabolic dysfunction, which should be taken into account in designing therapeutic interventions focused on monocyte repolarization in prediabetic and diabetic patients.

With regard to the possible cell mechanisms involved in the inflammatory polarization of human macrophages, it has been demonstrated that high levels of glucose are associated with elevation of the glucose efflux into the intracellular space [8]. In endothelial and smooth muscle cells, the increase of the intracellular glucose concentration induces activation of the polyol pathway that results in overproduction of sorbitol and fructose by aldose reductase and sorbitol dehydrogenase, respectively. The excessive activity of both enzymes has been associated with imbalance in the Redox cell mechanisms that in turn leads to free radical overproduction [8,11]. In macrophages, production of free radicals has been shown to activate NFκB-dependent signaling pathways resulting in inflammatory cytokine release [46,47]. Thereby, it is reasonable to consider that high levels of glucose may trigger the polyol pathway in macrophages, which could lead to free radical production, NFκB-dependent signaling pathway activation, and finally macrophage inflammatory polarization. However, to the best of our knowledge there is not yet solid evidence demonstrating the role of the polyol pathway upon free radical production and NFκB activation during macrophage polarization and these speculations should be carefully considered in further studies.

5. Conclusions

Our data demonstrate for the first time that high glucose concentrations have direct effects on the polarization of primary human macrophages toward an M1-like phenotype, characterized by up-regulation of CD11c and iNOS as well as down-regulation of Arg-1 and IL-10. The apparent inclination to M1-like activity was also observed in circulating monocytes in the setting of hyperglycemia, in which we found increased values of $CD11c^{+}$ monocytes accompanied by decreased levels of $CD206^{+}$ monocytes and IL-10. Furthermore, this study included an osmotic pressure control in all of the *in vitro* experiments in order to ensure that the results attributed to elevated glucose concentrations were not due to hyperosmolarity caused by the elevated levels of glucose. The molecular mechanisms through which high glucose concentrations and other non-prototypical metabolic stimuli are able to promote the expression of pro-inflammatory markers in monocytes and macrophages remain unclear and will require further study. In patients with elevated risk of developing vascular endothelial damage, including the vast number of people living with diabetes across the world, the potential impact of such work stands to be profound.

Conflict of interest

The authors have declared that no conflict of interest exists.

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Synergistic Role Among Adipose Tissue Hypertrophy, Dyslipidemia, and Systemic Inflammation in the Development of Atherosclerosis

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Abstract. Recent experimental and clinical evidence has suggested a synergistic causal role among adipose tissue hypertrophy, dyslipidemia, and systemic inflammation in the development and instability of the atherosclerotic plaque. Here, we summarize some of the most recent findings regarding the role of white adipose tissue hypertrophy in the development of dyslipidemia and chronic low-grade systemic inflammation. We also review the contribution of dyslipidemia in releasing free-fatty acids, cholesterol, and oxidized low-density lipoproteins as metabolic ligands able to trigger inflammatory signal pathways in macrophages via pattern recognition receptor (PRR) activation. Finally, we discuss the possible mechanisms through which dyslipidemia-related metabolic ligands and chronic low-grade systemic inflammation are capable of inducing macrophage activation, which in turn is involved in mediating instability and rupture of the atherosclerotic plaque. A better understanding of the inflammatory mechanisms contributing to atherosclerosis may help to identify potential molecular targets to decrease the cardiovascular risk in patients by using anti-inflammatory therapies and PRR blockers.

Keywords: Adipose tissue hypertrophy, chronic low-grade systemic inflammation, dyslipidemia, PAMP, DAMP, tumor necrosis factor alpha, macrophage, atherosclerosis

INTRODUCTION

Recent data in humans and animal models have suggested that atherosclerosis and thus cardiovascular disease may result from a systemic inflammatory response [1–5]. Such a systemic inflammatory state has been shown to have important differences with respect to a classic inflammatory response. Systemic inflammation is mainly characterized by increased circulating levels of acute-phase proteins and proinflammatory cytokines including C reactive protein (CRP), tumor necrosis factor alpha (TNF-α), interleukin (IL-) 1 beta, IL-6, IL-17, and IL-18 [6–10]. It has been also reported that systemic inflammation is associated with recruitment and infiltration of monocyte-derived macrophages into peripheral tissues such as skeletal muscle, liver, and visceral adipose tissue [11–14]. Notably, numerous lines of evidence have also revealed that macrophage infiltration is not directly related to necrotic lesion formation in the aforementioned tissues, which has led to the notion that chronic systemic inflammation has a low-grade activation level [11, 15–17]. Therefore,

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chronic low-grade systemic inflammation can be evidenced by persistently high levels of proinflammatory cytokines and increased macrophage infiltration in insulin dependent-peripheral tissues with no histological manifestation of tissue damage.

Interestingly, chronic low-grade systemic inflammation has been frequently shown to increase the risk to develop metabolic abnormalities and cardiovascular diseases in obese individuals [18–20]. For this reason, systemic inflammation has been also referred to as metabolic inflammation or metainflammation [18, 21]. Although the connection between metainflammation and cardiometabolic disease development appears to be clear, there are still numerous questions to be solved, i.e. is there evidence linking adipose tissue hypertrophy and dyslipidemia to the onset of systemic inflammation? What is the role of dyslipidemia-related metabolic ligands in macrophage activation? Is there a synergistic mechanism linking adipose tissue hypertrophy, dyslipidemia, and macrophage activation that contributes to atherosclerosis development?

In this review we summarize all of the abovementioned questions with a particular interest in describing the molecular mechanisms contributing to trigger chronic low-grade systemic inflammation and promote atherosclerosis development.

ROLE OF THE VISCERAL ADIPOSE TISSUE IN DEVELOPING DYSLIPIDEMIA AND SYSTEMIC INFLAMMATION

A growing body of evidence has pointed out to the visceral adipose tissue as a key contributor in the genesis of dyslipidemia and metainflammation [22–24]. There are two main types of adipose tissue, brown adipose tissue and white adipose tissue [25]. Brown adipose tissue preferentially localizes in the mediastinum and the supraclavicular area and is mainly involved in thermogenesis. On the contrary, white adipose tissue is predominantly localized in intra-abdominal depots surrounding the viscera and represents the most important energy storage in the organism because of its ability to accumulate large amounts of lipids [25]. However, imbalance between energy intake (calories consumed as food) and energy expenditure (calories used during physical activity) has a direct repercussion in the normal architecture of the adipose tissue, especially the visceral adipose tissue. In this regard, when energy expenditure is lesser than energy intake accumulation of visceral adipose tissue occurs, resulting in a pathological condition clinically known as central obesity, abdominal obesity or visceral obesity [26–28].

Central obesity has been demonstrated to play a pivotal role in the development of atherosclerosis and cardiovascular disease [26, 29]. For this reason, numerous efforts to determine normal cut-off points of waist circumference for each population have been done. In this form, a waist circumference higher than 80 centimeters (cm) in women and 94 cm in men are clear indicators of central obesity in Caucasian, Mediterranean, Middle East, and Sub-Saharan African population [30]. On the other hand, a waist circumference higher than 80 cm in women and 90 cm in men have been proposed to diagnose abdominal obesity in Asian as well as Ethnic Central and South American population [30].

Accumulation of visceral adipose tissue resulting in central obesity also leads to white adipocytes to experience an expansion process affecting both their number and size [31]. The increase in the number of white adipose cells is also referred to as adipose tissue hyperplasia -a common feature in obese adults who were not obese in the childhood, whereas the expansion of the adipocyte size is known as adipose tissue hypertrophy [32]. In obese adults with medical history of childhood obesity, hypertrophy is the most important contributor to the expansion and accumulation of visceral adipose tissue. Hypertrophy of white adipose tissue is largely associated with progressive loss of the adipocyte capacity to store fatty acids, which in turn is directly related to increased blood levels of lipids, a pathological entity known as dyslipidemia [33].

Dyslipidemia is an asymptomatic clinical condition characterized by decreased levels of high-density lipoproteins (HDL) and elevation of total cholesterol, low-density lipoproteins (LDL), apolipoprotein B (apoB), triglycerides, and free-fatty acids (FFA) [33]. Such alteration in the circulating levels of plasma lipids is also clinically referred to as atherogenic lipid profile due to its utility as a cardiovascular risk predictor directly involved in the origin and progression of atherosclerosis [34]. Dyslipidemia is a highly prevalent condition in the USA (~35%) and Europe (~5–29%, depending on the country) [35–39]. In Mexico, the burden of dyslipidemia affects more than 26.5% of the total adult population, having a dramatically negative effect on reducing the patient's life expectancy by 3.8-to-8.7 years [40–42].

As previously mentioned, enlargement of hypertrophic adipose tissue is directly linked to reduced ability of storing lipids, which in turn contributes to ectopic accrual of fat in non-adipose tissue that leads to free-radical overproduction and tissue lipotoxicity [43]. Adipocyte hypertrophy has been also shown to associate with remodeling of the adipose tissue stromal vascular fraction and consequently hypoxia-related necrosis of adipocytes that are remotely situated from the blood vessels [15, 44]. Both lipotoxicity and hypoxia-related necrosis are key contributors to the recruitment of peripheral monocytes into the hypertrophic adipose tissue, a decisive step to understand the origin of the proinflammatory milieu observed in this tissue [11, 18].

Histologically, adipose tissue inflammation is mainly characterized by macrophage infiltration with scant presence of other innate immune cells such as granulocyte cells [11, 45]. Infiltrated macrophages seem to surround hypertrophic adipose cells forming crown-like structures, a histological feature of adipose tissue inflammation [44]. Infiltrated macrophages are also an abnormal source of proinflammatory cytokines and chemokines in the hypertrophic adipose tissue including IL-1 beta, IL-6, TNF-α, interferon gamma (IFN-γ), monocyte chemoattractant protein 1 (MCP-1), and macrophage migration inhibitory factor (MIF) [46]. Visceral adipose tissue inflammation (VATI) has two major deleterious consequences impacting both the local and systemic milieu of the organism.

In the first place, VATI has been shown to impair adipocyte insulin sensitivity and affect the normal lipid metabolism in the organism, which in turn leads to dyslipidemia [6, 11, 46, 47]. In the second place, we have previously mentioned that VATI is associated with increased production of inflammatory cytokines and chemokines. Upon release into the blood stream, inflammatory mediators seem to decisively alter the normal balance between inflammatory and anti-inflammatory plasma factors, which in turn is able to bias the organism toward a systemic state of inflammation [19, 48–50]. In obese individuals, such a systemic inflammation state is characterized by high circulating levels of TNF-α, IL-1 beta, IL-6, IL-18, and IFN-γ, as well as decreased plasma concentrations of IL-10 [19, 20, 51, 52]. As it will be discussed, dyslipidemia and systemic inflammation that are related to adipose tissue hypertrophy appear to play a pivotal role in macrophage activation, a major event contributing to atherosclerosis.

ROLE OF METABOLIC LIGANDS IN PROMOTING MACROPHAGE ACTIVATION

Innate immune cells have an extensive repertoire of primitive receptors with the ability to orchestrate immediate responses against both internal and external adverse stimuli. Due to their ability to recognize a wide range of prototype molecules, these highly versatile receptors are known as Pattern Recognition Receptors (PRR) [53].

PRR have been demonstrated to recognize two main types of patterns: pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP). PAMP are molecules mainly derived from microbes including bacteria and protozoa whereas DAMP are released in response to cellular stress or tissue injury [54]. Notably, interaction between PRR with either PAMP or DAMP is able to initiate an innate immune response against infectious agents while also eliciting a healing process in wounded tissues [53, 54]. PRR are categorized in two main subgroups, Toll-like receptors (TLRs) and nucleotide-binding and oligomerization domain containing cytosolic receptors (NOD-like receptors also referred to as NLRs) [55, 56].

Upon PAMP or DAMP activation, TLRs orchestrate numerous signal pathways leading to nuclear factor kappa B (NF-κB) activation and thus production of inflammatory cytokines and chemokines able to recruit both immune and non-immune cells. The role of TLRs in recognizing PAMP has been broadly described in infectious diseases whereas their participation in the recognition of DAMP has been seen in heat-shock cellular responses, endotoxinemia, and cellular exposure to mutagenic stimuli [53, 54]. In parallel, NLRs have been also demonstrated to recognize PAMP allowing production of inflammatory cytokines, chemokines, and anti-microbial peptides via NF-κB and mitogen-activated protein kinase (MAPK) activation [57]. However, NLRs have been also shown to instigate activation of the oligomeric inflammatory complex (also referred to as inflammasome or inflammasome-NLRs) in response to DAMP [58].

The inflammasome-NLRs is a multiprotein oligomer that consists of the NACHT, LRR, and PYD domain-containing protein (NLRP), the apoptosis-associated speck like protein (ASC), and caspase-1 [58]. Following NLRP-ASC-caspase-1 assembly and inflammasome-NLRs activation, caspase-1 exerts its ability to cleave proteolytically the precursor forms

of IL-1 beta and IL-18, thus leading to inflammation-induced programmed cell death also referred to as pyroptosis [59, 60]. Recent experimental data now supports a potential role of both TLRs and NLRs in recognizing DAMP-like metabolic signals that might be associated with macrophage inflammatory activation, systemic inflammation, and atherosclerotic plaque formation and instability.

Such a breaking evidence could lead us to consider the metabolic dysfunction-associated molecular patterns within a new DAMP subcategory characterized by release of molecules that are produced in metabolic diseases and will be subsequently called by us like "metabolic-DAMP" or metabolic ligands.

As previously mentioned, obesity, adipose tissue hypertrophy, and dyslipidemia are dysfunctional metabolic conditions mainly characterized by abnormally high circulating levels of total cholesterol, LDL, and free-fatty acids [15, 32, 43, 44, 46]. These pro-atherogenic molecules have been shown to behave as DAMP since they can be recognized by PRR located on the macrophage cell surface and trigger inflammatory signal cascades [47, 55, 61–63]. Such a revealing information has led us to propose the existence of the "metabolic-DAMP" that are released from tissues experiencing metabolic abnormalities with the aim of inducing inflammation and immune surveillance, cellular debris clearance, and tissue repair [64]. However, inflammation related to metabolic-DAMP appears to have major differences in comparison to the classical inflammatory response mediated by PAMP or conventional DAMP. Indeed, these two proinflammatory scenarios could be distinguished from each other on the basis of the intensity, duration, and location of the response. Inflammation related to metabolic ligands seems to show lesser intensity (exhibiting a low-grade activation level), longer duration (that tends to persist chronically), and systemic dissemination (instead of having a specific anatomical site) with respect to classic inflammation.

In this sense, palmitic acid (a saturated free-fatty acid released from hypertrophic adipose tissue) has been recently demonstrated to bind TLR4 and induce MCP-1 gene expression in human macrophage-like THP-1 cells [65]. Furthermore, palmitate has been also shown to intensify TLR4/LPS-induced IL-6 and TNF-α expression in RAW 264.7 and primary murine macrophages by stimulating the endogenous production of ceramide, a sphingolipid with proinflammatory effects [66–68]. Moreover, it has been also demonstrated that several FFA including arachidonic, lauric, and oleic acids exert the ability to increase TNF-α production in $CD11c^+$ myeloid murine cells (a subset of macrophages capable to infiltrate hypertrophic adipose tissue) via TLR2 and TLR4 [69]. Also, a recent study showed that stearate (a C:18 FFA) is able to enhance the effect of monosodium urate monohydrate (MUM) on the TLR2-dependent IL-1 beta production in peripheral blood mononuclear cells of patients with primary gout and murine macrophages [70, 71]. Interestingly, the authors proposed a synergistic effect between MUM and stearate that could be likely mediated via inflammasome-NLRs activation-dependent proIL-1 beta cleavage [71]. All of this information strongly supports the notion that free-fatty acids may be taken into a new subcategory of metabolic-DAMP because they are released in metabolic dysfunction and have the ability to induce inflammatory cytokine production in macrophages via PRR.

In addition to the role of free-fatty acids like metabolic ligands, other lipid metabolism-associated molecules have been also involved in polarizing macrophages toward a proinflammatory activation profile, as is the case of LDL. As previously mentioned, LDL comprises a set of low-density proteins mostly synthesized in liver tissue with the ability to transport cholesterol throughout the blood stream. Interestingly, it has been well established that LDL-coupled cholesterol can be affected by oxidation process, which is capable to confer inflammatory and pro-atherogenic characteristics to this protein/lipid complex [72–74].

LDL-couple cholesterol can be oxidized (oxLDL) in the presence of free radicals including nitric oxide, hydrogen peroxide, and superoxide anion, all of them largely abundant in hypertrophic adipose tissue, liver steatosis, and most importantly atherosclerotic lesions [74–77]. oxLDL has been already shown to induce IL-1 beta, IL-6, and IL-8 production in THP1 cells and primary human macrophages in a time-dependent fashion [78, 79]. Notably, oxLDL-induced IL-1 beta, IL-6, and IL-8 production is attenuated when inhibiting the TLR2, 4, and 6 gene expression on macrophages, suggesting that oxLDL may promote production of inflammatory cytokines by directly activating TLRs-dependent signal pathways [78, 79].

Furthermore, exposure of primary human macrophages to oxLDL is not only able to stimulate production of inflammatory cytokines but also promote macrophage differentiation into foam cells. As a matter of fact, when human macrophages are *in vitro* exposed to oxLDL they start to differentiate

into foam cells exhibiting markers of macrophage activation including histocompatibility complex class II molecules and CD86 [79]. Interestingly, formation of oxLDL-induced foam cells is abrogated when blocking CD36 and TLR4 on macrophages, suggesting the involvement of oxLDL-uptake and TLR4-dependent signal pathways in mediating foam cell generation [79].

Moreover, oxLDL has not been only related to production of inflammatory cytokines and foam cell formation but also inhibition of the expression of anti-inflammatory mediators in macrophages. In fact, a recent study showed that oxLDL is able to abrogate dose-dependently the IL-10 production (cytokine with potent anti-inflammatory actions) in human monocytes via TLR2 and TLR4 [80]. Additionally, NLRs-dependent signal pathways have been also shown to mediate oxLDL-induced inflammatory activation in macrophages. In this sense, besides promoting foam cell formation, oxLDL-uptake by macrophages is capable of inducing IL-1 beta secretion via NLRP3 (NACHT, LRR, and PYD domain-containing protein 3), ASC, and caspase-1 assembly, and then inflammasome-NLRP3 activation [75]. Notably, inflammasome-NLRP3 activation-dependent IL-1 beta production appears to be directly stimulated by production of reactive oxygen species and cathepsin B releasing (an intracellular DAMP triggering inflammasome-NLRP3 activation by inducing cellular potassium efflux) [75, 76]. Interestingly, cathepsin B has been recently demonstrated to elevate in 45.9% of patients undergoing acute myocardial infarction [77], suggesting a possible participation of the inflammasome-NLRP3 in promoting acute coronary syndrome. However, it is still necessary to determine clinically whether the inflammasome-NLR family could play a key role in increasing the risk to develop sudden cardiac arrest while also evaluating its utility as a potential early marker associated with propensity to acute myocardial infarction.

In parallel, cholesterol crystals have been shown to induce dose-dependently caspase-1/cathepsin B-dependent IL-1 beta production in primary human monocytes and macrophages [81]. In view of the fact that cholesterol crystals are largely abundant in arteriosclerotic lesions, previous findings suggest the involvement of the inflammasome-NLRP3 in mediating some of the proinflammatory and pro-atherogenic effects of cholesterol. Thus, both oxLDL and cholesterol could be also considered into a new subcategory of metabolic ligands with the ability to promote inflammatory activation of macrophages via PRR in response to dyslipidemia-related metabolic ligands.

The latent possibility of having endogenous metabolic signals capable of polarizing macrophage activity via PRR may also have therapeutic implementation as in the case of docosahexaenoic acid (DHA). DHA is a free-fatty acid belonging to the omega-3 fatty acid family largely present in cold-water oceanic fish oils. A growing body of recent evidence has attributed strong anti-inflammatory effects to DHA in multiple pathological scenarios but the involved mechanisms have not been fully elucidated [82–85].

DHA has been recently demonstrated to reduce cyclooxygenase-2 (COX-2) gene expression in murine peritoneal macrophages thus decreasing the resultant production of prostaglandin E2 (PGE2), a substance with well known inflammatory properties [86]. In this sense, it has been demonstrated that DHA is able to abrogate COX-2-dependent PGE2 production via free-fatty acid receptor 4 (FFAR4). As a matter of fact, the DHA/FFAR4 assembly has been shown to inhibit the TLR4-dependent inflammatory signal pathway [86]. Interestingly, DHA has been also shown to exert anti-atherogenic effects in apo-E deficient mice (mice homozygous for the Apoe mutation) by significantly reducing both the plasma cholesterol levels and the extent of aortic atherosclerotic lesions [87]. However, despite of apparently having potential clinical benefit, it is still needed to assess whether some free-fatty acids mimicking metabolic-DAMP may be useful in abrogating PRR activation on macrophages, thus preventing atheroma formation and instability in human beings. In light of this information, it is largely probable that dyslipidemia-related metabolic ligands show the ability to polarize macrophages upon an inflammatory phenotype with capacity to infiltrate vascular endothelial tissue and contribute to the development of atherosclerosis.

DYSLIPIDEMIA, SYSTEMIC INFLAMMATION, AND ATHEROGENESIS

Atherogenesis is the pathological process through which atheromatous plaques (also referred to as atheromas) are formed and deposited in the inner wall of arteries. The atherosclerotic plaque formation is a highly complex and dynamic process comprising numerous cellular and extracellular components including total cholesterol, LDL, oxLDL, extracellular matrix proteins, vascular smooth muscle cells

(VSMC), platelets, and macrophage-derived foam cells [88].

As previously mentioned, dyslipidemia is characterized by increased circulating levels of lipid-derived molecules among which total cholesterol and oxLDL are known to elevate significantly [33]. The excess of cholesterol and oxLDL has been demonstrated to attach and accumulate preferentially in the inner lining of arteries enhancing monocyte recruitment and infiltration into the blood vessel [89]. Upon infiltration in the vascular endothelium, monocyte cells differentiate into macrophages with capacity of clearing out the excess of cholesterol and oxLDL by means of scavenger receptors that exert the ability to uptake extracellular lipids into cytosolic depots [90]. Then, macrophages containing high amounts of cholesterol and oxLDL start to differentiate to foam cells and migrate into the subendothelial space of arteries, constituting in this way the classic process in the atherosclerotic plaque formation [90, 91].

As it can be seen, development of atherosclerotic lesions is principally associated with dyslipidemia and macrophage recruiting. In light of the aforementioned information, an additional synergistic scenario between dyslipidemia-related metabolic ligands and macrophage activation might be also participating in a pivotal process during atherosclerosis complications, the atheromatous plaque instability.

Formation of atheromas is in some extent frequent in the adult life [92]. Presence of atheromatous lesions has been demonstrated to be asymptomatic in most of the patients [92, 93]. Atherosclerotic lesions turn into symptomatic once the stability of the atheromatous plaque has been lost leading to a prothrombotic state, partial or total blockage of the blood supply to vital organs, and imminent threat of developing cardiac or cerebral ischemic events [92–95]. Thus, stability of atherosclerotic lesions has been recognized as a major determinant in stroke, acute myocardial infarction, and sudden death [96–99]. The atheroma stability is a highly complicated and dynamic process depending on multiple factors, among which the synergistic relationship between dyslipidemia and macrophage activity has been recently demonstrated to play a major role [33, 91, 93, 100].

As previously said, free fatty acids including palmitate and stearate appear to have the ability to induce expression of TNF-α, IL-8, and IL-1 beta in human macrophage-like THP-1 cells [101]. Moreover, palmitate has been recently demonstrated to promote osteoblastic differentiation in human aortic smooth muscle cells by means of overproducing osteopontin, a protein with capacity to inhibit IL-10 release in alternatively activated macrophages [102]. In the same sense, the excess of oxLDL has been also shown to induce differentiation of macrophages into foam cells via formyl peptide receptor 2 (FPR2) and CD36 (scavenger receptor acting like a FFA translocase), which eventually leads to TNF-α synthesis [91, 100]. Furthermore, high levels of oxLDL and cholesterol have been also demonstrated to induce inflammatory signal pathways on macrophages via TLRs and NLRs thus leading to increased production of IL-1β, IL-6, IL-18, and TNF-α. Taking all of this information into consideration, a microenvironment rich in FFA, cholesterol, and oxLDL seems to favor inflammatory cytokine production in macrophages via specific and non-specific receptors. Interestingly, such a marked dyslipidemic profile is largely present in the atherosclerotic plaque [88].

In light of previous information, it is clear that atheromas appear to provide a proinflammatory milieu for macrophages, which in turn may be directly involved in instability and rupture of the atherosclerotic plaque. In this sense, both IL-1 beta and TNF-α have the ability to induce apoptosis in vascular smooth muscle cells (VSMC) [103–106]. VSMC are a significant source of extracellular matrix proteins including beta 1 integrins, type I, III, and V collagen, which have been associated with a structurally stable patch in the atheroma [107]. Therefore, upon IL-1 beta and TNF-α-induced apoptosis, the number of atheroma-associated VSMC starts to diminish leading to loss of EMP production and then instability of the atheromatous plaque. In parallel, it has been also reported that IL-1 beta is able to promote expression of metalloproteinase (MMP) 1, 3, 9, and 13 in rabbit condylar chondrocytes, a large family of enzymes with the ability to degrade extracellular matrix proteins [108]. It is then probable that macrophage-derived IL-1 beta may induce loss of atheroma-associated extracellular matrix proteins by directly stimulating MMP production in both macrophages and VSMC, which in turn could contribute to atherosclerotic lesion instability and rupture. A third mechanism possibly participating in promoting instability of atheromatous plaques involves the role of chronic low-grade systemic inflammation.

As previously mentioned, adipose tissue hypertrophy seems to associate with either dyslipidemia or systemic inflammation. While dyslipidemia is largely correlated with increased production of metabolic

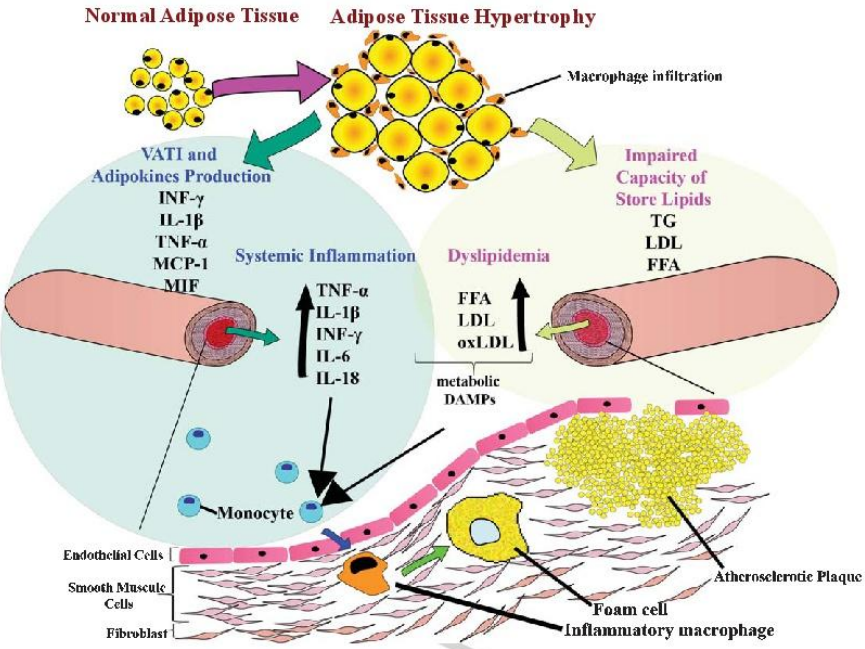

Fig. 1. Synergistic interaction among adipose tissue hypertrophy, chronic low-grade systemic inflammation, macrophage activation, and atherosclerosis development. Hypertrophic adipose tissue is able to release metabolic DAMPs including free-fatty acids (FFA), low-density lipoproteins (LDL), and oxidized low-density lipoproteins (oxLDL). Moreover, adipose tissue hypertrophy is associated with impaired capacity to store lipids in the adipose cells, which dramatically increases the systemic levels of triglycerides (TG), LDL and FFA. Metabolic DAMPs are capable of triggering adipose tissue-resident macrophages under a proinflammatory profile that in turn promotes monocyte recruitment and increased local and systemic levels of proinflammatory cytokines, which leads to local and systemic inflammation. Dyslipidemia-related DAMPs and chronic low-grade systemic inflammation promote monocyte migration into the inner layer of blood vessels, where these immune cells will differentiate into macrophages and foam cells, which in turn contribute to atherosclerotic plaque development, instability and rupture by producing a repertory of proinflammatory cytokines. Abbreviations: DAMPs, damage-associated molecular patterns; FFA, free-fatty acids; LDL, low-density lipoproteins; oxLDL, oxidized low-density lipoproteins; TNF-α, tumor necrosis factor alpha; IL, interleukin; IFN-γ, interferon gamma; MCP-1, monocyte chemoattractant protein 1; MIF, macrophage migration inhibitory factor; VATI, visceral adipose tissue inflammation.

ligands including FFA, cholesterol, and oxLDL, chronic low-grade systemic inflammation is typically characterized by high circulating levels of cytokines with proinflammatory actions such as IL-1 beta, TNF-α, and IFN-γ [19, 20, 51, 52]. Several studies have consistently reported the increase in the systemic levels of IFN-γ in both obese humans and mice [109, 110]. IFN-γ is the most representative T helper 1(Th1) cytokine exerting the ability to induce IL-1 beta, IL-6, and TNF-α production in macrophages [111]. Thus, it is plausible to expect that high plasma IFN-γ levels may be associated with increased macrophage capacity of producing IL-1 beta and TNF-α that might lead to VSMC apoptosis and thus atheroma instability. In parallel, IFN-γ has been also shown to induce apoptosis in human VSMC *in vitro*, which supports a direct role for IFN-γ in atheroma instability without necessarily involving the participation of IL-1 beta and TNF-α [112].

CONCLUDING REMARKS

Atherosclerosis is a dynamic and complex process involving a large number of molecular and cellular events. Here, we have briefly discussed some of the most recent findings regarding the role of adipose tissue hypertrophy in the development of dyslipidemia and systemic inflammation. Also, we have discussed the possible participation of dyslipidemia in promoting the release of metabolic

ligands (free-fatty acids, cholesterol, and oxLDL) that once recognized by either TLRs or NLRs may exert the ability to polarize macrophages toward an inflammatory activation state. Finally, we have also summarized several possible mechanisms through which dyslipidemia-related metabolic ligands and chronic low-grade systemic inflammation may synergistically induce macrophage activation and lead to atherosclerotic plaque instability and rupture (Fig. 1). Despite of having been gaining increased attention in the last years, the study of the mechanisms governing atherogenesis, including formation and instability of the atheromatous plaque, remains unclear. Thus, further clinical and basic research is still needed to fully understand the inflammatory mechanisms contributing to atherosclerosis development while also examining the benefit of using anti-inflammatory therapies such as TLR/NLR blockage in patients with increased cardiovascular risk, as is the case of the vast number of people living with obesity and Type 2 Diabetes around the world.

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RIC

RINCÓN DEL RESIDENTE

Gut microbiota as a key player in triggering obesity, systemic inflammation and insulin resistance

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ABSTRACT

Obesity-related systemic inflammation contributes to develop insulin resistance. The main factors involved in the relationship of obesity with systemic inflammation and insulin resistance have not been completely elucidated. Microbiota includes around 10^{13} to 10^{14} microbes harboring the human gut, which are clustered in approximately a thousand different bacterial species. Several studies suggest that imbalance in the intestinal bacterial population could result in obesity, systemic inflammation and metabolic dysfunction. Here, we review the main bacterial groups observed in obesity as well as their possible role in increasing the intestinal permeability and lipopolysaccharide-related endotoxemia. Furthermore, we point out the role of intestinal dysbiosis in the inflammatory activation of macrophages with the ability to infiltrate in the visceral adipose tissue and induce insulin resistance. Finally, we discuss the apparent beneficial use of prebiotics and probiotics in ameliorating both systemic inflammation and metabolic dysfunction. Present information may be useful in the future design of novel therapies focused on treating obesity and insulin resistance by restoring the gut microbiota balance.

Key words. Gut microbiota. Bacteria. Obesity. Insulin resistance. Inflammation. Macrophages.

La microbiota como agente inductor de la obesidad, la inflamación sistémica y la resistencia a la insulina

RESUMEN

La obesidad se asocia con un estado inflamatorio sistémico que contribuye al desarrollo de resistencia a la insulina. Sin embargo, los factores involucrados en la relación del fenotipo obesogénico con el establecimiento de la respuesta inflamatoria y la pérdida de la sensibilidad a la insulina todavía no han sido identificados completamente. La microbiota está constituida por alrededor de 10^{13} y 10^{14} bacterias que habitan el intestino humano, agrupadas en cerca de 1,000 especies bacterianas distintas. Numerosos estudios sugieren que las alteraciones en las poblaciones bacterianas intestinales podrían conducir a mayor propensión a la obesidad, el desarrollo de un estado de inflamación sistémica y la disfunción metabólica. En esta revisión se exponen los principales filabacterianos asociados con el fenotipo obesogénico, así como su posible papel en el aumento de la permeabilidad intestinal y la generación de la endotoxemia por el lipopolisacárido. Además, discutimos la participación de la disbiosis intestinal en la activación de macrófagos inflamatorios, con capacidad de infiltrar el tejido adiposo visceral induciendo pérdida de la sensibilidad a la insulina. Finalmente, se revisan los aparentes beneficios del uso de prebióticos y probióticos, en el tratamiento de la inflamación sistémica y la disfunción metabólica. En un futuro esta información podría ser útil en el desarrollo de estrategias terapéuticas encaminadas al control de la obesidad y la resistencia a la insulina, a través del restablecimiento del balance poblacional de la microbiota.

Palabras clave. *Microbiota. Bacterias. Obesidad. Resistencia a la insulina. Inflamación. Macrófagos.*

INTRODUCTION

Obesity is a multifactorial disease largely characterized by the excessive accumulation of body fat. Obesity is strongly associated with increased risk of developing metabolic disorders including hyperglycemia and insulin resistance by promoting a systemic inflammatory response.[1] Numerous studies have recently brought to light novel factors involved in the development of obesity and obesity-related systemic inflammation and metabolic dysfunction as is the case of the microbiota. The microbiota has been recently shown to play a key role in inducing obesity, systemic inflammation, and metabolic disease, thus having an enormous impact on both basic and clinical research. Here, we review the most recent basic and clinical evidence concerning the role of microbiota as a possible trigger of obesity, systemic inflammation, and insulin resistance. We also discuss about the use of both prebiotics (non-digestible diet elements working as microbiota's substrate) and probiotics (living microorganisms influencing the microbiota balance) as potential therapeutic agents in ameliorating adiposity and inflammation.

THE HUMAN BEING AND THE MICROBIOTA

The human being has become capable of coexisting in a complex ecosystem as a result of multiple evolutionary mechanisms. A clear scenario exemplifying the intricate relationship between humans and their environment can be seen at the human microbiome. The human intestinal lumen is occupied by 10^{13}-10^{14} microorganisms belonging to more than a thousand different bacterial species that encompass the microbiota.[2-4] The microbiota is involved in numerous important functions as digestion, micronutrient production, restriction of the growth of potentially harmful bacteria, and development of the immune response.[4] However, the microbiota is susceptible to experience changes that may lead to deleterious outcomes in different clinical scenarios including obesity and obesity-related metabolic dysfunction.

In terms of microorganisms, the human gastrointestinal tract is sterile before the birth and starts to be progressively inhabited by thousands of bacteria immediately after having contact with the birth canal. Then, the extra-uterine environment involves multiple immune challenges capable of modifying the newborn's microbiota in a few months thus having a decisive influence on the microbiota composition during the adulthood.[5]

The analytical methods for the study of the microbiota have been shown to have numerous limitations that have led to controversial results. Classical culture methods are only able to identify ~30% of the human intestinal bacteria because of a limited knowledge concerning the nutritional requirements of the majority of gut microorganisms. Nevertheless, the accurate characterization of the human microbiota has been achieved with the advent of novel molecular techniques including the 16S ribosomal ribonucleic acid sequence analysis and both metagenomic and metatranscriptomic tools.[6] Such a remarkable molecular techniques have evidenced the three predominant phyla in human and murine microbiota: firmicutes, bacteroidetes, and actinobacteria, which altogether represent ~75% of the total bacterial diversity at the intestine.[7]

Recent studies have pointed out a potential role of obesity in impairing the balance of the intestinal microbiota.[8,9] In obese animals (leptin-deficient mice or exhibiting mutations in the leptin receptor gene), firmicutes significantly predominate over bacteroidetes.[8] In humans, the study of the microbiota in monozygotic twins revealed that bacteroidetes decrease while actinobacteria increase in the obese twin as comparing with the lean twin.[9] It has been also shown that bariatric surgery-related weight loss in morbidly obese patients promotes an increase in the bacteroidetes population accompanied by reduction in firmicutes.[10] In light of this information, obesity in mice and humans seems to be linked to a microbiota profile largely characterized by increasing in firmicutes and actinobacteria as well as decreasing in bacteroidetes. However, which of the multiple factors associated with obesity may be decisively involved in the microbiota imbalance?

THE MICROBIOTA PROFILE IS MODIFIED IN RESPONSE TO OBESITY-RELATED FACTORS

The microbiota diversity can be modified in response to both genetic and environmental factors that may significantly alter the intestinal bacterial composition. In terms of genetic factors, several studies have indicated that monozygotic twins have a more similar microbiota between them with respect to unrelated people.[11,12] Environmental factors include dietary habits and physical activity that have been shown to play a decisive role in defining the intestinal microbiota profile. In this sense, it has been recently demonstrated that the intake of calories, fiber, and monounsaturated and polyunsat-

urated fatty acids is associated with the percentage of *Bacteroides spp* and bifidobacteria, both of them belonging to bacteroidetes and actinobacteria respectively.[13] Moreover, it has been also shown that transplantation of microbial communities from feces of lean individuals to germ free (GF)-mice promotes an intestinal flora similar to that present in healthy adult human beings. Notably, when these GF mice are fed with a high-fat diet a significant change in the microbiota profile is observed, which at the same time correlates with increased adiposity.[14] Furthermore, normal-fat diet-fed mice show decrease in bacteroidetes and increase in both firmicutes and proteobacteria (including *Escherichia spp*, *Salmonella spp*, and *Helicobacter spp*) when feeding a high-fat diet.[15] The abovementioned experimental findings are consistent with clinical evidence showing a clear increase in the bacteroidetes population after restricting consumption of carbohydrates and fats in obese human beings.[16]

In contrast, Duncan, *et al.*, reported conflicting results showing no differences in the firmicutes/bacteroidetes ratio between lean and obese individuals as well as among subjects exposed to different diets aimed to reduce body weight.[17] However, the aforementioned study did not take into consideration the evidence described by Dr. Marie A. Hildebrandt indicating that increased availability of lipids and carbohydrates are *per se* capable of inducing dysbiosis, even independently of the obesity degree.[15] Thus, the exposure to different diets only aimed in reducing body weight without contemplating the origin of the calorie contents does not resemble the experimental conditions proposed by Hildebrandt and coworkers making necessary to reexamine such controversial data.

Physical activity is another factor that could potentially influence the gut microbiota profile. As a matter of fact, the microbiota profile is significantly different in mice with free-access to work out with respect to animals without having physical activity.[18] In addition, mice with free-access to physical activity also show increased fermentation of prebiotic as well as decreased intestinal and systemic inflammatory response, which are presumably associated with high production of n-butyrate.[18] This study has motivated the development of a research protocol aimed to determine the effect of physical activity upon the microbiota profile in obese patients exhibiting different cardiometabolic disorders.[19] However, it is necessary to perform such studies in a population-specific fashion taking into consideration numerous factors including genetic background, diet contents, and lifestyle habits, which can significantly influence the microbiota profile.

All of this evidence suggests that obesity-related factors including diet contents and sedentary lifestyle may have a significant impact on altering the microbiota composition. However, is it possible to induce or restrict the obesity development by switching the intestinal microbiota profile? Such an intriguing question could lead us to a notion in which microbiota appears to play a central role in the development of obesity and obesity-related inflammatory and metabolic disorders.

THE ROLE OF MICROBIOTA IN TRIGGERING OBESITY

GF-mice do not develop obesity despite being exposed to hypercaloric diets, suggesting that gut bacteria could directly promote obesity.[2,20] Besides confirming this observation, further studies have also revealed that bacteria-dependent prebiotic fermentation is largely associated with production of short chain fatty acids (SCFA) and thus restriction of obesity.[21-24] In addition to represent an alternative energy source for humans, numerous SCFA (including n-butyrate, acetate, and propionate) are also capable of acting as intracellular signaling molecules. Acetate and propionate have been shown to act as ligands for the G protein-coupled receptors GPR41 (free fatty acid receptor 3 or FFAR3) and GPR43 (FFAR2). Both GPR41 and GPR43 are located at enteroendocrine cells within the gastrointestinal tract.[21,22]

Peptide YY (PYY) is an anorexigenic hormone capable of promoting delayed gastric emptying thereby inducing nutrient absorption and suitable digestion.[25] Notably, it has been demonstrated that production of PYY can be stimulated when acetate and propionate bind to GPR41 and GPR43, respectively.[26] In addition, GPR41 activation is associated with increased leptin synthesis that in turn favors an anorexigenic effect and thus decreased fat mass gain.[23] Moreover, acetate and/or propionate-dependent GPR43 activation has been shown to promote lipolysis thus decreasing lipid accumulation and improved glucose metabolism in adipocytes.[24]

Interestingly, the bacteroidetes population appears to be closely related to prebiotic fermentation and production of SCFA. As a matter of fact, the intestinal increase in bacteroidetes has been associated with decreased appetite as well as less body fat gain due to stimulation of leptin and PYY secretion.[27] Furthermore, predominance of bacteroidetes

in the intestinal microbiota has been linked to decreased levels of ghrelin, a hormone with well described orexigenic actions.[27] In contrast, elevation in the firmicutes population has been associated with increased production of free fatty acids (FFA) in the intestinal lumen, augmented levels of ghrelin, increased adiposity and intestinal permeability, and insulin resistance (Figure 1).[27] Also, it has been reported that chronic administration of doxycycline and hydroxychloroquine in humans is capable of leading to decreased number of intestinal bacteroidetes and increased amount of firmicutes, which is in turn associated with body weight gain.[28] All of the abovementioned information indicates that alterations in the gut microbiota could decisively contribute to the establishment of obesity.[20-28] Furthermore, recent evidence suggests that intestinal microbiota modifications could not only predispose to obesity but also trigger a systemic state of inflammation and metabolic dysfunction.

THE ROLE OF MICROBIOTA IN TRIGGERING SYSTEMIC INFLAMMATION

In 2008, Cani, *et al.*, reported that high-fat diet-fed mice showed an increase in the intestinal fraction of firmicutes and proteobacteria, fat mass gain, and endotoxemia due to increased blood concentration of lipopolysaccharide (LPS).[29] In these animals, a more severe LPS-induced endotoxemia has been largely associated with augmented intestinal permeability.[29] The intestinal epithelium of obese mice expresses lower levels of occludin and ZO-1 (epithelial cell-binding proteins), thus resulting in increased gut permeability.[30] Both occludin and ZO-1 have been demonstrated to play a key role in initiating an intestinal inflammatory response, which in turn is capable of modifying the epithelial integrity and permeability thus allowing LPS entry into the organism.[30]

Another consequence of the firmicutes/bacteroidetes imbalance is the intestinal accumulation of

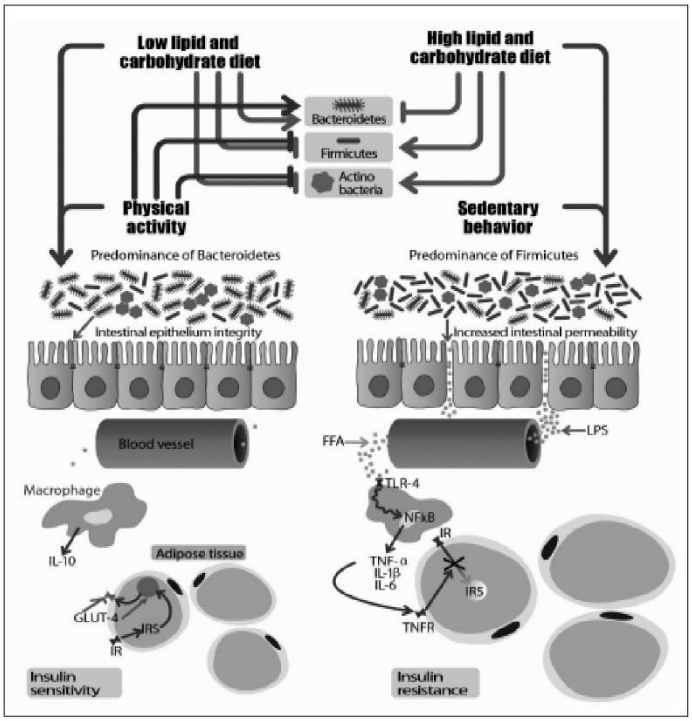

Figure 1. *Possible role of microbiota in the development of insulin resistance. Besides physical activity, a low intake of lipids and carbohydrates favors bacteroidetes, which are associated with decreased levels of LPS and free fatty acids in the intestinal lumen and blood as well as anti-inflammatory activation of macrophages and insulin sensitivity in adipose tissue (left). In contrast, a sedentary behavior accompanied by high intake of lipids and carbohydrates favors firmicutes and actinobacteria thus promoting increased intestinal permeability, LPS-induced endotoxemia, high serum levels of free fatty acids, inflammatory activation of macrophages, and finally insulin resistance (right).*

bacteria-derived metabolic products as is the case of FFA. A recent study showed that bacteria belonging to the phylum firmicutes are able to produce and store a large amount of FFA.[31] Notably, LPS and FFA can be recognized by different Toll-like receptors (TLR) located at the surface of immune cells including T lymphocytes and monocytes-macrophages.[32] Particularly, TLR2 and TLR4 are capable of binding FFA,[33] while LPS recognition is preferentially mediated via TLR4.[34] Both LPS-TLR4 and FFA-TLR2/4 interactions have been shown to elicit an inflammatory signaling cascade characterized by NFκB activation as well as expression of inflammatory cytokines including interleukin (IL) 1β and tumor necrosis factor alpha (TNF-α) in immune cells.[32-34] All of this evidence suggests that obesity-related intestinal dysbiosis could decisively participate in triggering local and systemic inflammatory responses due to endotoxemia that in turn mediates activation of circulating immune cells with capacity of migrating into peripheral tissues including adipose and renal tissue, liver, and muscle.[35]

THE ROLE OF MICROBIOTA IN TRIGGERING SYSTEMIC INFLAMMATION-RELATED INSULIN RESISTANCE

The obesity-related inflammatory process has been largely described as a systemic state of inflammation, characterized by increased circulating levels of inflammatory cytokines and macrophage infiltration in peripheral tissues.[35] In this form, systemic inflammation exhibits major differences with respect to a classically reported local inflammatory response.[35-38] The serum levels of inflammatory cytokines associated with systemic inflammation are below in comparison to those observed during an infectious disease.[36] Furthermore, the tissue-infiltrating macrophages do not induce tissue damage or loss of the normal function on it.[37] Finally, systemic inflammation has been largely associated with developing of metabolic disorders including insulin resistance and hyperglycemia, which has given birth to the term "metainflammation" to refer the systemic inflammatory process related to obesity and metabolic disease.[38]

Interestingly, a growing body of evidence suggests that gut microbiota alterations as well as the risk to become obese and insulin resistant may be linked via metainflammation. As previously mentioned, intestinal dysbiosis may provide the initial step toward establishing metainflammation (Figure 1). Microbiota modification is associated with increased FFA production and LPS-induced endotoxemia.[29-31] Upon TLR-mediated recognition, FFA and LPS are capable of promoting inflammatory activation of monocyte-macrophages, which in turn exert the ability to migrate toward visceral adipose tissue and overproduce IL-1β, IL-6, IL-8, and TNF-α.[32] In parallel, the large amounts of FFA resulting from dysbiosis can be absorbed at the intestinal tissue thus leading to increased FFA storage in visceral adipose cells and consequently fat mass gain.[39] Fat mass gain repeatedly involves accumulation of visceral adipose tissue (also referred to as central obesity) and leads to adipose cells to experience an expansive process affecting both their number and size. The increase in the number of white adipose cells is also called adipose tissue hyperplasia (a frequent histological feature in obese adults who were not obese in childhood), whereas the enlargement in the adipocyte size is known as adipose tissue hypertrophy. Both hyperplasia and hypertrophy have been shown to induce hypoxia, increased lipid peroxidation, oxidative stress, endoplasmic reticular stress, and autophagy in adipose tissue.[40,41] All of these deleterious factors are capable of activating inflammatory signaling cascades thus promoting an accurate microenvironment to attract macrophages and T cells.[42] Macrophages have been demonstrated to surround adipose cells forming crown-like structures (CLS), a distinctive histological feature of metainflammation in hypertrophic fat tissue.[40] Immune cell infiltration is indeed a persistent source of inflammatory cytokines in hypertrophic adipose tissue, which in consequence exhibits high local levels of IL-1β, IL-6, IL-8, and TNF-α, as well as increased chemokine production.[43] Chemokines are cytokines with the ability of recruiting circulating immune cells toward specific tissues. As a matter of fact, the hypertrophic/hyperplasic adipose tissue has been shown to express high levels of macrophage chemoattractant protein-1 (MCP-1) and macrophage migration inhibitory factor (MIF). MCP-1 and MIF have the ability of attracting new monocyte-macrophages toward the hypertrophic fat tissue thus perpetuating the local inflammatory microenvironment. All of the abovementioned immunological events described for visceral adipose tissue are not only restricted to affect in a paracrine fashion but also at the endocrine level. In this sense, obese subjects show increased levels of cytokines, chemokines, and proinflammatory monocytes in circulation.[44-46] Moreover, obesity-induced circulating monocytes seem to be able to differentiate into TNF-α-secreting macrophages at the hypertrophic fat mass thus inducing progressive loss of

the insulin sensitivity in white adipose cells. This tentative physiopathological scenario constitutes one of the latest hypotheses trying to explain the origin of the insulin resistance in the hypertrophic adipose tissue of obese human beings.

In normal conditions, insulin binds to its receptor located on the adipose cell surface thus initiating an intracellular signaling pathway mediated by the activation of the insulin receptor substrate 1 (IRS-1). Then, IRS-1 promotes the downstream activation of Akt via phosphoinositide 3 kinase. Activated AKT can then go on to stimulate translocation of glucose transporter proteins (GLUT-4 in the adipose tissue) from the cytosol to the cell membrane and finally mediate glucose entry into adipose cells. Experimental evidence from *in vitro* cultures of adipose cells has recently demonstrated that TNF-α has the ability to inhibit the activation of the insulin signaling pathway by blocking the IRS-1 and AKT phosphorylation via activation of ERK/JNK, PTP1B, and NFκB (Figure 1).[47] Taking these experimental findings into consideration, infiltration of TNF-α-secreting macrophages in the adipose tissue may represent an initial inflammatory step toward promoting a local environment of insulin resistance. Then, insulin resistance in adipose tissue could chronically generate hyperglycemia, compensatory hyperinsulinemia, pancreatic β-cell exhaustion and apoptosis, finally affecting the insulin signaling pathway in liver and skeletal muscle. In brief, gut microbiota alterations may play a key role in triggering systemic inflammation thus leading to macrophage infiltration in the adipose tissue, increased TNF-α production, and progressive loss of the insulin sensitivity. However, these studies have been almost exclusively performed in murine models so it is necessary to incorporate studies in patients in order to clarify whether changes in the intestinal microbiota could be associated with obesity, systemic inflammation, and insulin resistance in human beings.

ROLE OF PREBIOTICS AND PROBIOTICS IN IMPROVING GUT MICROBIOTA, SYSTEMIC INFLAMMATION, AND METABOLIC DYSFUNCTION

Taking into account the possible role of the intestinal dysbiosis in inducing systemic inflammation and insulin resistance, numerous research groups have attempted to study the effect of the microbiota restoration by means of administering prebiotics and probiotics (Table 1). Prebiotics belonging to the inulin family have been shown to increase the amount of bifidobacteria and *Faecalibacterium prausnitzii* thereby decreasing LPS-induced endotoxemia and systemic inflammation in obese subjects.[48] Inulin and oligofructose supply has been also demonstrated to reduce the amount of hepatic triglycerides and thus the liver steatosis development in rats.[27] Taking into consideration that both simple steatosis and steatohepatitis have been shown as endotoxemia-related inflammatory entities,[30,49,50] prebiotic supply could represent a therapeutic complement to treat such liver disorders in obese patients.

Administration of oligofructose has been also associated with increased bacteroidetes population as well as decreased adiposity and glycemia, and improved insulin sensitivity in rodents, even in animals exposed to hypercaloric diets.[51] In humans, administration of fructooligosaccharide has been described to elevate the plasma level of glucagon-like peptide 1, a hormone capable of inhibiting gastric emptying and glucagon release.[52] Prebiotic supply has been also associated with increased SCFA and leptin production and thus decreased appetite sensation in rodents.[27] Notably, n-butyrate is a SCFA capable of modulating the NFκB activity thus being associated with less severity of intestinal inflammatory entities including inflammatory bowel disease and colorectal cancer.[53,54]

Administration of probiotics (as is the case of *Lactobacillus plantarum)* reduces adipose cell size, adiposity, serum cholesterol, and circulating leptin in animal models.[55] Moreover, supply of probiotics including *Lactobacillus paracasei, L. rhamus,* and *Bifidobacterium animalis* has been also demonstrated to decrease fat mass gain and macrophage infiltration in adipose tissue of mice, even in animals receiving hypercaloric diets.[56] In the same sense, *Lactobacillus gasseri* administration has been shown to reduce body mass index and visceral fat percentage in obese human beings.[57] Furthermore, Lactobacillus acidophilus intake has been observed to promote insulin sensitivity in glucose-intolerant individuals and patients with type 2 diabetes.[58] Probiotic administration has been also associated with decreased serum concentrations of TNF-α and IL-6, NFκB inhibition, reduced liver transaminase levels, and increased insulin sensitivity in obese rodents and humans.[59] In diabetic mice, administration of *Saccharomyces boulardii* has been demonstrated to reduce body weight, adiposity, and lipid contents in the liver. Additionally, supply of *S. boulardii* has been recently associated with decreased macrophage infiltration and IL-1β expression in liver tissue, as

Table 1. Effect of prebiotics and probiotics upon obesity, systemic inflammation, and metabolic dysfunction.

Prebiotic/Probiotic	Study type	Dosage	Duration	Main effect	Reference
Inulin/oligofructose	Randomized, double-blind, placebo-controlled clinical trial	16 g/day, inulin/ oligofructose 1:1	3 months	Increase in bifido bacteria, decrease in adiposity, circulating LPS, and CRP	Dewulf *et al*.[48]
Inulin/oligofructose	Experimental study in JCR:LA-cp rats	Diet containing 10 and 20% of inulin/oligofructose	10 weeks	Decrease in cholesterolemia	Parnell *et al*.[25] and liver triglycerides
Oligofructose	Experimental study in hypercaloric diet-fed ob/ob and C57BL/6 mice	Diet containing 10% of oligofructose	4 weeks	Decrease in firmicutes, adiposity, and systemic levels of TNF-α, IL-1β, and IL-6, as well as increase in bacteroidetes and glucose tolerance	Everard *et al*.[51]
Fructooligo-saccharides (raftilose)	Randomized, double-blind, placebo-controlled clinical trial	20 g/day, raftilose	2 periods of 2 and 7 days	Increase in plasma GLP1 levels	Piche *et al*.[52]
Lactobacillus plantarum	Experimental study in hypercaloric diet-fed C57BL/6 mice	1 x 10^8 CFU/mouse/day	11 weeks	Decrease in adipose tissue hypertrophy, adiposity, serum cholesterol, and leptin levels	Takemura *et al*.[56]
Lactobacillus gasseri	Randomized, double-blind, placebo-controlled clinical trial	200 g/day of fermented milk supplemented with *L. gasseri* SBT2055	12 weeks	Decrease in visceral adiposity, BMI, and waist circumference	Kadooka *et al*.[57]
Lactobacillus acidophilus	Randomized, double-blind, placebo-controlled clinical trial	Lyophilized tablets of *L. acidophilus* NCFM (1 g/day equivalent to 1 x 10^{10} CFU)	4 weeks	Decrease in insulin resistance (HOMA-IR)	Andreasen *et al*.[58]

LPS: lipopolysaccharide. CRP: C-reactive protein. TNF-α: tumor necrosis factor alpha. IL: interleukin. GLP1: glucagon-like peptide 1. CFU: colony forming units. BMI: body mass index. HOMA-IR: homeostasis model assessment of insulin resistance.

well as low serum levels of IL-1β, IL-6, and TNF-α in both obese and diabetic mice.[60]

In summary, administration of prebiotics and probiotics seems to reduce body weight, fat deposition, metainflammation, and metabolic dysfunction in animals and humans.[59] Therefore, restoration of a proper intestinal microbiota by means of supplying prebiotics and/or probiotics could be a promissory therapeutic strategy to treat obesity-related diseases thus preventing systemic inflammation and metabolic complications.

CONCLUDING REMARKS

All of this information indicates that changes in the intestinal microbiota profile might be associated with increased susceptibility to obesity, systemic inflammation establishment, and metabolic dysfunction development. Present knowledge could provide new scientific bases into the development of novel therapeutic strategies to treat obesity and insulin resistance by restoring the balance of the intestinal microbiota. In a near future, a therapy based on administering prebiotics, probiotics, and/or anti-inflammatory drugs (as is the case of recombinant IL-10 or infliximab) could contribute to a better metabolic control of obese patients. However, further clinical research focused on examining the microbiota profile on different ethnic populations is still required to understand the role of microbiota and dysbiosis in the development of chronic noncommunicable diseases in humans.

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Clinical Study

Serum Levels of Interleukin-13 Increase in Subjects with Insulin Resistance but Do Not Correlate with Markers of Low-Grade Systemic Inflammation

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Experimental evidence in mice suggests a role for interleukin- (IL-) 13 in insulin resistance and low-grade systemic inflammation. However, IL-13 serum levels have not been assessed in subjects with insulin resistance, and associations of IL-13 with parameters of low-grade systemic inflammation are still unknown. Our main goal was to examine the systemic levels of IL-13 in patients with insulin resistance, while also studying the relationship of IL-13 with anthropometric, metabolic, and low-grade systemic inflammatory markers. Ninety-two participants were included in the study and divided into insulin-resistant patients and noninsulin-resistant controls. Blood levels of IL-13, glucose, insulin, triglycerides, cholesterol, tumor necrosis factor-alpha (TNF-α), IL-10, proinflammatory (Mon-CD11c$^+$CD206$^-$), and anti-inflammatory (Mon-CD11c$^-$CD206$^+$) monocytes, as well as anthropometric parameters, were measured in all volunteers. Insulin-resistant patients showed 2.5-fold higher serum levels of IL-13 than controls ($P < 0.0001$) and significantly increased values of TNF-α and Mon-CD11c$^+$CD206$^-$, with concomitant reductions in IL-10 and Mon-CD11c$^-$CD206$^+$. Increased IL-13 was extraordinarily well associated with hyperglycemia ($r = 0.7362$) and hypertriglyceridemia ($r = 0.7632$) but unexpectedly exhibited no significant correlations with TNF-α ($r = 0.2907$), IL-10 ($r = -0.3882$), Mon-CD11c$^+$CD206$^-$ ($r = 0.2745$) or Mon-CD11c$^-$CD206$^+$ ($r = -0.3237$). This study demonstrates that IL-13 serum levels are elevated in patients with insulin resistance without showing correlation with parameters of low-grade systemic inflammation.

1. Introduction

Insulin resistance is a key pathophysiological event in the development of type 2 diabetes (T2D), a serious public health problem of global proportions, with alarmingly high morbidity and mortality rates in several countries including USA and Mexico [1, 2]. A growing body of clinical and experimental evidence has consistently shown that insulin resistance is linked to obesity and low-grade systemic inflammation [3, 4]. Specifically, increased body mass index (BMI) and visceral fat accumulation have been shown to augment the risk to develop dyslipidemia, hyperglycemia, and insulin resistance [5]. Low-grade systemic inflammation is characterized by abnormally high serum levels of proinflammatory cytokines (i.e., tumor necrosis factor alpha [TNF-α]) and increased percentage of proinflammatory monocytes such

as monocytes that exhibit high expression of CD11c and no expression of CD206 (Mon-CD11c$^+$CD206$^-$) [6, 7]. Low-grade systemic inflammation is also associated with decreased serum concentrations of anti-inflammatory cytokines such as interleukin- (IL-) 10 and low percentages of monocytes exerting anti-inflammatory abilities as is the case of monocytes that show high expression of CD206 and no expression of CD11c (Mon-CD11c$^-$CD206$^+$) [7, 8]. High amounts of TNF-α have been reported to concur with increased adiposity [9], hypertriglyceridemia [8], and impaired insulin sensitivity in adipose and hepatic tissue [10]. Moreover, Saghizadeh and coworkers have previously demonstrated that TNF-α is actively expressed in skeletal muscle tissue of insulin-resistant patients as compared to subjects with normal insulin sensitivity [11]. In the same sense, TNF-α infusion in healthy individuals is able to induce muscle insulin resistance by increasing phosphorylation of p70 S6 kinase, extracellular signal-regulated kinase −1/2 (ERK-1/2), and c-Jun NH2 terminal kinase (JNK) [12]. Interestingly, phosphorylation of p70 S6 kinase, ERK-1/2, and JNK is associated with decreased activation of the insulin receptor substrate-1 (IRS-1) and Akt substrate 160 [12], which supports the notion that TNF-α is a major contributor of insulin resistance in skeletal muscle. On the other side, decreased IL-10 has been related to elevated serum concentrations of TNF-α, increased proportion of Mon-CD11c^{+-} CD206$^-$ over the Mon-CD11c$^-$CD206$^+$ subpopulation, hyperglycemia, and higher levels of insulin resistance in obese subjects [7, 13]. Therefore, low-grade systemic inflammation has now gained increasing attention since it appears to play a causative role in the development of insulin resistance in liver, adipose tissue, and skeletal muscle of obese patients [14].

IL-13 is a cytokine belonging to the alpha-helix protein family that is mainly produced by activated Th2 cells, mast cells, and basophils and has been widely studied in the scenario of helminth parasite infections and allergic asthma [15, 16]. Nevertheless, recent experimental evidence in mice has now found that IL-13 may also participate in low-grade systemic inflammation and insulin resistance [17, 18]. In this sense, it has been reported that exogenous administration of IL-13 improves insulin sensitivity while also decreasing TNF-α expression and macrophage infiltration in epididymal adipose tissue of C57BL/6J mice fed a high-fat diet (HFD) [17]. Likewise, a further study demonstrated that IL-13 gene transfer plays a protective role during experimental obesity by diminishing adipocyte hypertrophy, glucose intolerance, insulin resistance, and macrophage infiltration into adipose tissue of HFD-fed C57BL/6J mice [18]. Interestingly, IL-13 has been also associated with improved insulin secretion. In this sense, Darkhal and colleagues previously showed that IL-13 gene overexpression concurs with increased insulin serum levels in mice [18]. Additionally, a recent study demonstrated that IL-13 *in vitro* increases insulin secretion in beta-cells of humans and rats [19], supporting the fact that IL-13 also has an impact on insulin production and release. In parallel, IL-13 has been also shown to play a role in the development of insulin resistance in human beings. In this regard, a previous study demonstrated that IL-13 serum levels are reduced in T2D patients that exhibit increased insulin resistance [20]. However, the role of IL-13 in the pathogenesis of insulin resistance in humans is still unclear, and potential associations of this cytokine with parameters of low-grade systemic inflammation and metabolic dysfunction remain obscure.

The main goal of this work was to examine the systemic levels of IL-13 in patients with insulin resistance, while also studying the relationship of IL-13 with parameters of low-grade systemic inflammation such as TNF-α, IL-10, Mon-CD11c$^+$CD206$^-$ percentage, and Mon-CD11c$^-$CD206$^+$ percentage, and other insulin resistance-related metabolic markers including fasting blood glucose and insulin, BMI, central obesity, body fat percentage, waist-to-hip ratio, triglycerides, and cholesterol.

2. Materials and Methods

2.1. Subjects. Ninety-two Mexican adult women and men from the south-central region of Mexico were included in the study. All of the volunteers provided written informed consent, previously approved by the institutional review board of the General Hospital of Mexico, which guaranteed that the study was conducted in accordance with the principles described at the Helsinki Declaration. Volunteers were excluded from the study if they had previous or recent diagnosis of *Diabetes Mellitus*, cardiovascular diseases, chronic renal disease, chronic or acute hepatic disease, blood pressure higher than 135/85 mm Hg, inflammatory or autoimmune disorders, acute or chronic infectious diseases, cancer, and endocrine disorders including hypothyroidism. We also excluded pregnant or lactating women, patients with cardiovascular drug therapy including anti-inflammatory, antiaggregant, and antihypertensive drugs, and subjects without having an overnight fasting of 8–10 hours. All participants enrolled into the study received full medical evaluation, including achievement of clinical history and physical examination by expert physicians.

2.2. Insulin Resistance Assessment. The insulin resistance level was estimated by means of calculating the homeostatic model assessment of insulin resistance (HOMA-IR) in each participant. The HOMA-IR value resulted from multiplying fasting insulin concentration (mU/l) by fasting glucose concentration (mmol/l) and then divided by 22.5. Cut-off points for insulin resistance were given according to previous studies validated in the Mexican population [21], as follows: subjects showing HOMA-IR < 3.8 were considered as the control group by having a normal level of insulin resistance. On the contrary, subjects showing HOMA-IR ≥ 3.8 were considered as the insulin-resistant group by having a significant level of insulin resistance.

2.3. Anthropometric Measurements. BMI resulted from dividing corporal weight (kg) by height squared (m^2) and was recorded in all participants, as follows: BMI 18.5–24.9 kg/m^2, normal weight subjects; BMI 25–29.9 kg/m^2, overweight subjects; and BMI ≥ 30 kg/m^2, obese subjects. Central obesity was estimated in each participant by measuring the midpoint

between the lower rib margin and iliac crest with a conventional tape in centimeters (cm). Cut-off point values for central obesity were given as follows: women showing 80 cm waist circumference or higher were considered to have central obesity, while men showing 90 cm waist circumference or higher were considered to have central obesity. Body fat percentage was individually estimated by means of using a body composition analyzer (TANITA®, Body Composition Analyzer, Model TBF-300A, Tokyo, Japan).

2.4. Metabolic Measurements. Blood samples were collected after overnight fasting and placed into pyrogen-free tubes (Vacutainer™, BD Diagnostics, NJ, USA) at room temperature. Afterwards, collection tubes were centrifuged at 1000g/4°C for 25 min and serum samples obtained and stored at −80°C until use. Serum levels of insulin were individually measured in triplicate by means of the Enzyme-Linked ImmunoSorbent Assay (ELISA), following the manufacturer's instructions (Abnova Corporation, Taiwan). Serum levels of glucose were individually measured in triplicate by the glucose oxidase assay, following the manufacturer's instructions (Megazyme International, Ireland). Total cholesterol and triglyceride levels were individually measured in triplicate by enzymatic assays according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). All biochemical parameters were measured at the same time to avoid procedural variations.

2.5. Assessment of the Serum Levels of IL-13, TNF-α, and IL-10 by ELISA. Blood samples were collected after overnight fasting and placed into pyrogen-free tubes (Vacutainer, BD Diagnostics, NJ, USA) at room temperature. Afterwards, collection tubes were centrifuged at 1000g/4°C for 25 min and serum samples obtained and stored at −80°C until use. Serum levels of IL-13, TNF-α, and IL-10 were determined in triplicate by ELISA, following the manufacturer's instructions (Peprotech, Mexico). All cytokines were measured at the same time to avoid procedural variations.

2.6. Characterization of Monocyte Surface Markers by Flow Cytometry. Blood samples were collected after overnight fasting and placed into pyrogen-free tubes containing EDTA (Vacutainer, BD Diagnostics, NJ, USA). Afterwards, collection tubes were centrifuged at 1800g/8°C for 10 min and white blood cells (WBCs) separated using a micropipette. WBCs were separately placed into 1.6 ml pyrogen-free eppendorf tubes containing 1 ml of ACK Lysing Buffer (Life Technologies, USA) and incubated at 4°C for 5 min. Immediately after, cell suspensions were centrifuged at 1800g/8°C for 5 minutes and resulting cell pellets washed twice using PBS 1X (Sigma-Aldrich, Mexico). After an extra centrifugation step, supernatants were discarded and resulting cell pellets resuspended in 50 μl of PBS 1X (Sigma-Aldrich, Mexico) for posterior cell counting using trypan blue staining with Neubauer chamber. In each case, 3 μl of Human TruStrain Reagent (BioLegend Inc., USA) was added to 1×10^6 WBCs and then incubated for 7 minutes on ice. Afterwards, each WBC sample was simultaneously incubated with human anti-CD14 PE/Cy7, anti-CD11c PE/Cy5, and anti-CD206/Cy7 APC at 8°C for 20 min in the absence of light. Analysis of the cell surface markers CD11c and CD206 was exclusively performed on CD14-positive cells that correspond to monocytes, using a FACSCanto II flow cytometer (BD Biosciences, Mexico) by means of BD FACSDiva™ software 6.0, acquiring 50,000 events per test in triplicate. PE/Cy7 mouse IgG2, APC/Cy7 mouse IgG1, and PE/Cy5 mouse IgG1 (BioLegend Inc., USA) were used as isotype control antibodies.

2.7. Statistical Analysis. Student's t-test was used to compare noninsulin-resistant (NIR) and insulin-resistant (IR) subjects in terms of age, HOMA-IR, BMI, waist circumference, body fat percentage, waist-to-hip ratio, fasting blood glucose, serum insulin, systolic blood pressure, total cholesterol, triglycerides, TNF-α, IL-10, Mon-CD11c$^+$CD206$^-$ percentage, and Mon-CD11c$^-$CD206$^+$ percentage. Student's t-test results are expressed as mean ± standard deviation. Pearson's correlation coefficient was calculated for examining the association of IL-13 with anthropometric, metabolic, and systemic inflammation parameters. Pearson's correlation coefficient results are expressed as coefficients (r) and P values. Differences were considered significant when $P < 0.05$. All statistical analyses were performed using the GraphPad Prism 6.01 software.

3. Results

Ninety-two participants of both sexes were included in the study (47 noninsulin-resistant and 45 insulin-resistant subjects). No significant differences were found in age (for noninsulin-resistant controls mean age 31.02 ± 10.41 years, whereas for insulin-resistant subjects mean age 36.75 ± 11.18 years), woman/man proportion (23 women and 24 men in the noninsulin-resistant group, whereas 22 women and 23 men in the insulin-resistant group), and systolic blood pressure (SBP) (for noninsulin-resistant controls mean systolic pressure 128.40 ± 3.57 mmHg, whereas for insulin-resistant subjects mean systolic pressure 127.9 ± 4.28 mmHg) (Table 1). On the other hand, BMI, waist circumference, and body fat percentage exhibited a significant increase in insulin-resistant subjects as compared to noninsulin-resistant controls (Table 1). Similarly, fasting blood glucose, serum insulin, and HOMA-IR were also higher in the insulin-resistant group than in the noninsulin-resistant group (Table 1). When dyslipidemia was evaluated, triglyceride values showed a clear elevation in insulin-resistant subjects with respect to noninsulin-resistant controls; however, cholesterol levels did not significantly differ between groups (Table 1). In terms of systemic inflammatory parameters, serum levels of TNF-α were significantly increased in the insulin-resistant group as compared to noninsulin-resistant individuals, whereas IL-10 was clearly reduced in insulin-resistant subjects with respect to controls (Table 1). At the same time, insulin-resistant subjects showed a significant increase in the proinflammatory monocyte percentage (Mon-CD11c$^+$CD206$^-$) accompanied by decreasing numbers of monocytes with anti-inflammatory profile (Mon-CD11c$^-$CD206$^+$) as compared to controls. Figure 1 shows representative flow cytometry dot plots illustrating the

Table 1: Demographic, anthropometric, metabolic, and immunological characteristics of the study subjects.

Parameters	NIR	IR	P
Gender (W/M)	23/24	22/23	n.s.
Age (years)	31.02 ± 10.41	36.75 ± 11.18	n.s.
BMI (kg/m^2)	26.82 ± 4.89	32.92 ± 2.34	<0.001
Waist circumference (cm)	86.87 ± 13.00	105.00 ± 6.21	<0.001
Body fat (%)	26.61 ± 8.15	36.99 ± 7.42	<0.001
Waist-to-hip ratio	0.92 ± 0.12	0.97 ± 0.04	n.s.
SBP (mmHg)	128.4 ± 3.57	127.9 ± 4.28	n.s.
Blood glucose (mg/dl)	84.17 ± 9.79	104.9 ± 5.58	<0.0001
Serum insulin (mU/l)	11.16 ± 2.38	21.56 ± 1.89	<0.0001
HOMA-IR	2.28 ± 0.39	5.59 ± 0.64	<0.0001
Total cholesterol (mg/dl)	193.7 ± 10.28	198.0 ± 10.18	n.s.
Triglycerides (mg/dl)	159.40 ± 37.93	256.6 ± 13.19	<0.0001
TNF-α (pg/ml)	8.26 ± 3.45	30.29 ± 3.93	<0.0001
IL-10 (pg/ml)	100.8 ± 26.41	37.04 ± 10.36	<0.0001
Mon-CD11c$^+$CD206$^-$ (%)	28.30 ± 17.18	60.93 ± 20.68	<0.001
Mon-CD11c$^-$CD206$^+$ (%)	24.23 ± 9.46	6.05 ± 5.71	<0.0001

Data are expressed as mean ± standard deviation. Significant differences were estimated by means of performing two-way Student's t-test. Differences were considered significant when $P < 0.05$. W: women; M: men; BMI: body mass index; SBP: systolic blood pressure; HOMA-IR: homeostatic model assessment of insulin resistance; TNF-α: tumor necrosis factor alpha; IL: interleukin; Mon CD11c$^+$CD206$^-$: proinflammatory monocytes; Mon CD11c$^-$CD206$^+$: anti-inflammatory monocytes; n.s.: nonsignificant differences.

amount of Mon-CD11c$^+$CD206$^-$ in (A) noninsulin-resistant controls and (B) patients with insulin resistance, as well as the amount of Mon-CD11c$^-$CD206$^+$ in (C) noninsulin-resistant controls and (D) patients with insulin resistance.

When IL-13 was examined, we found a significant 2.5-fold increase in the serum levels of IL-13 in insulin-resistant subjects as compared to noninsulin-resistant controls (37.69 ± 17.82 versus 15.88 ± 6.71 pg/ml, resp.) (Figure 2).

IL-13 was significantly elevated in the insulin-resistant group. Therefore, our next step was to identify anthropometric, metabolic, and inflammatory parameters that were clearly related to the elevation in the serum concentrations of this cytokine. IL-13 exhibited a strong positive correlation with BMI ($r = 0.6727$, $P < 0.0001$) and waist circumference ($r = 0.6394$, $P < 0.0001$) (Figures 3(a) and 3(b), resp.). Moreover, serum levels of IL-13 were moderately associated with body fat percentage ($r = 0.4310$, $P < 0.001$) and waist-to-hip ratio ($r = 0.2410$, $P = 0.026$) (Figures 3(c) and 3(d), resp.).

In the specific case of metabolic parameters, serum IL-13 showed a strong positive relationship with fasting blood glucose ($r = 0.7362$, $P < 0.0001$) and HOMA-IR ($r = 0.6673$, $P < 0.0001$) (Figures 4(a) and 4(c), resp.). Despite being significant, statistical correlation between IL-13 and serum insulin was positive but tended to be moderate ($r = 0.5468$, $P < 0.0001$) (Figure 4(b)). Furthermore, IL-13 was strongly related to the amount of triglycerides ($r = 0.7632$, $P < 0.0001$) but showed no significant correlation with total cholesterol ($r = 0.2104$, $P = 0.055$) (Figures 5(a) and 5(b), resp.).

In terms of systemic inflammatory markers, circulating levels of IL-13 exhibited no significant correlations with serum TNF-α ($r = 0.2907$, $P = 0.066$) or Mon-CD11c^{+-}CD206$^-$ monocyte percentage ($r = 0.2745$, $P = 0.062$), both of them typical proinflammatory parameters (Figures 6(a) and 6(b), resp.). On the contrary, IL-13 was barely correlated with IL-10 serum levels ($r = -0.3882$, $P = 0.0471$) but showed no significant association with Mon-CD11c$^-$CD206$^+$ monocyte percentage ($r = -0.3237$, $P = 0.0544$), which have been shown to exert anti-inflammatory actions (Figures 6(c) and 6(d), resp.).

4. Discussion

IL-13 is a cytokine that belongs to the alpha-helix protein family and is mainly produced by Th2-activated T lymphocytes, mast cells, and basophils [22]. Besides having been shown to play a critical role in helminth parasite infections and allergic asthma [13, 14], IL-13 has been now suggested to exert additional functions in the development of metabolic alterations such as insulin resistance and hyperglycemia [17, 18]. However, clinical findings in humans regarding the role of IL-13 in metabolic disease are still controversial.

In this sense, it has been previously shown that serum levels of IL-13 are significantly reduced in type 2 diabetic patients with coronary artery disease as compared to healthy controls [23]. Similarly, a recent study demonstrated that type 2 diabetic patients show decreased serum levels of IL-13 with respect to normal-glucose tolerant individuals [20]. On the contrary, it has been also reported that morbidly obese patients with insulin resistance exhibit higher values of serum IL-13 than normal weight controls, and bariatric surgery was able to reduce IL-13 serum concentrations after 1-year of the surgical procedure [24]. Likewise, expression of interleukin-13 receptor subunit alpha-2 (IL-13RA2) has been demonstrated to increase in peripheral blood mononuclear cells (PBMC) of obese children with abnormal insulin sensitivity as compared to normal weight boys [25]. This apparent contradiction could be attributed to the possible role of IL-13 in the insulin resistance pathogenesis that involves the liver, adipose tissue, skeletal muscle, and pancreatic beta-cells. In this regard, a previous study in mice showed that IL-13 gene deficiency concurs with reduced phosphorylation of IRS-1 and AKT in liver, adipose tissue, and skeletal muscle, which was directly related to decreased insulin sensitivity in the aforementioned tissues [26]. Interestingly, IRS-1 and AKT phosphorylation was found to depend on the activation of the signal transducer and activator of transcription (STAT) 3 and STAT6 [26], a well-known family of transcription factors with the ability to elicit anti-inflammatory signaling pathways in response to IL-13. Also, IL-13 has been shown to promote insulin secretion in pancreatic beta-cells [19], which is associated with compensatory hyperinsulinemia aimed to counteract hyperglycemia and insulin resistance. Then, it is reasonable to speculate that IL-13 plays a protective role in insulin resistance by promoting IRS-1 and AKT phosphorylation in insulin-dependent tissues via STAT3 and STAT6 activation as well as improvement of the beta-cell function. However, together with other

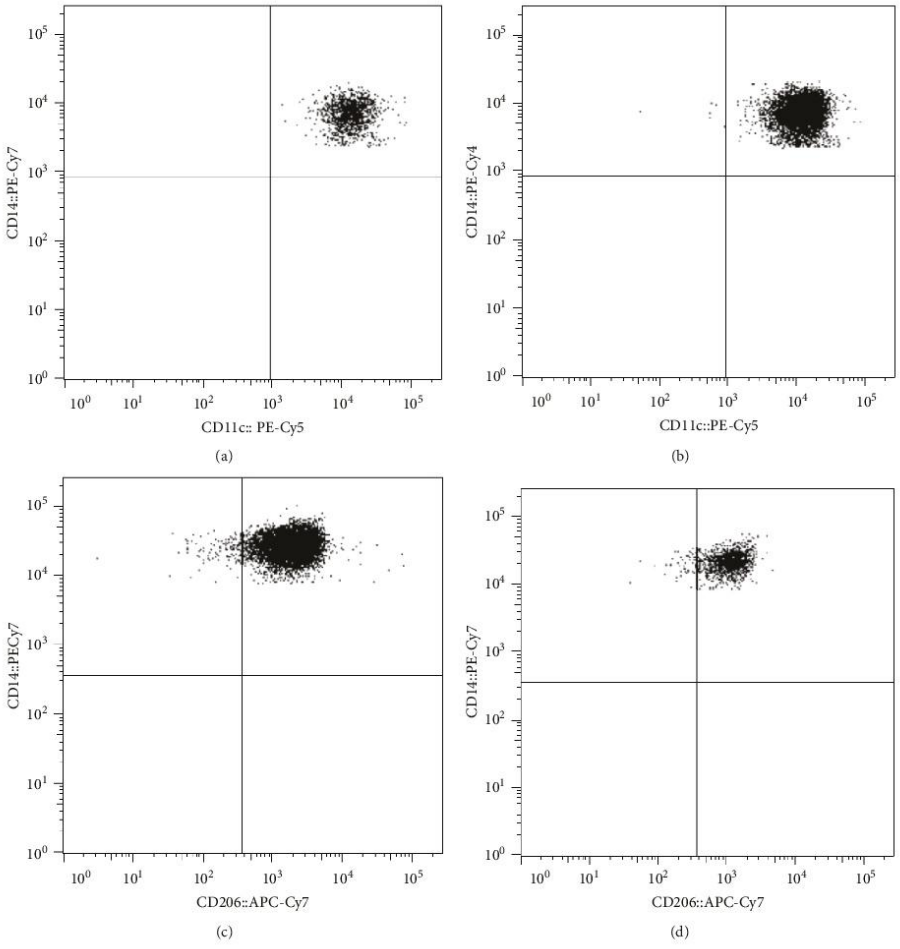

Figure 1: Representative dot plots showing percentages of proinflammatory and anti-inflammatory monocytes in patients with insulin resistance and noninsulin-resistant controls. (a) and (b) illustrate representative flow cytometry dot plots showing percentages of proinflammatory monocytes that express CD11c but do not express CD206 (Mon-CD11c$^+$CD206$^-$) in controls and insulin-resistant patients, respectively. (c) and (d) illustrate representative dot plots showing percentages of anti-inflammatory monocytes that express CD206 but do not express CD11c (Mon-CD11c$^-$CD206$^+$) in controls and insulin-resistant patients, respectively. Dot plot quantification can be seen in Table 1.

studies [17, 24, 25], our work shows a significant increase in the serum levels of IL-13 in insulin-resistant patients, which appears to disagree with previous evidence. In this sense, a previous work showed that IL-13 serum levels significantly increase as the severity of T2D-related chronic heart failure also increases [27]. Similarly, a very recent study demonstrated that IL-13 gene expression tended to increase in the left ventricular free wall of T2D patients with heart failure as compared to healthy donors [28]. Interestingly, despite IL-13 gene tended to be upregulated, the IL-13 receptor subunit alpha 1 (IL-13Rα1) production was significantly decreased in the same cardiac muscle specimens of T2D patients with heart failure, who are by definition insulin-resistant [28]. In some extent, this information is consistent with our findings and suggests a progressive loss of the cellular capacity to respond to IL-13 in the scenario of insulin resistance. Such a state of "IL-13 action resistance" may partially explain the increment of the IL-13 serum levels in

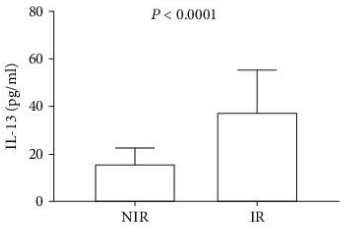

Figure 2: Serum levels of IL-13 in patients with insulin resistance and controls. Systemic levels of IL-13 showed a 2.5-fold significant increase in patients with insulin resistance as compared to noninsulin resistance controls. NIR: noninsulin resistance controls; IR: patients with insulin resistance. A 3.8 cut-off point was used for defining insulin resistance in the study population. Data are expressed as mean ± standard deviation. Differences were considered significant when $P < 0.05$ and calculated using Student's t-test.

several cohorts of patients with insulin resistance, including our own study population. To the best of our knowledge, this is one of the first studies suggesting a state of IL-13 action resistance, characterized by high levels of IL-13, reduced activation of the IL-13-dependent signaling pathway in insulin-dependent tissue, and consequently increased insulin resistance. Nevertheless, we have drawn a speculative hypothesis to explain the apparently contradictory results regarding the role of IL-13 in the development of insulin resistance, and the discussion of this information makes no attempt to conjecture beyond that. For this reason, it is still of enormous importance to study the role of IL-13 in the pathogenesis of insulin resistance, also evaluating the possible existence of a state of IL-13 action resistance in patients with altered insulin sensitivity.

In our study, serum concentrations of IL-13 exhibited a strong correlation with obesity-related anthropometrical parameters including BMI and central obesity. As it has been previously reported, increased BMI and central obesity are key contributing factors to the development of insulin resistance, metabolic syndrome, and type 2 diabetes [29]. Central obesity directly results from the expansion of white adipose cells that accumulate around the viscera of the abdominal cavity [30]. Interestingly, it has been recently shown that increased central obesity and body weight are associated with elevation in the circulating levels of IL-13 [31]. Furthermore, Kwon and coworkers demonstrated that IL-13 is overproduced in the white adipose tissue of HFD-fed mice and obese humans while also reported that adipocytes were the main cellular source of this cytokine [17]. These findings may explain the extremely strong association observed in our study between serum IL-13 levels and obesity-related anthropometrical parameters such as central obesity and, in some extent, BMI. In other words, our data confirm a direct link between fat mass expansion and IL-13 overproduction, which could be supported by the fact that hypertrophic and hyperplasic adipocytes increase their ability to synthesize IL-13. However, we only studied the relationship of obesity-related anthropometrical parameters with IL-13 serum concentrations by means of statistical correlation models and the discussion of these results makes no attempt to conjecture beyond that. Further research is needed to draw conclusions regarding the capacity of white adipose cells to release IL-13 into the bloodstream and the potential role of this cytokine in the development of insulin resistance in obese patients.

Another phenomenon captured in our study is that IL-13 serum levels appear to be extraordinarily correlated with elevated blood values of glucose in the study population, and especially in subjects with insulin resistance. Consistent with our results, Nehete and coworkers previously demonstrated that obese chimpanzees show increased serum levels of IL-13, glucose, and glucagon, a peptide hormone in charge of raising glucose concentration in the bloodstream [32]. This finding suggests a direct link among IL-13, glucagon, and elevated glucose levels. In this sense, glucagon-like peptide-1 (GLP-1) is a peptide hormone able to reduce glucose levels by restricting the secretion of glucagon [33]. Recently, GLP-1 has been also shown to decrease IL-13 production in LPS-treated human eosinophils [34], which supports the idea that increased serum IL-13 could be directly associated with elevated blood glucose levels via glucagon-dependent mechanisms. Nevertheless, it is important to note that IL-13 has been also demonstrated to downregulate the hepatic production of glucose in mice [26] and increase glucose uptake in skeletal muscle cells *in vitro* [20], which seems to disagree with our results. We want to speculate that such a discrepancy could be explained by the fact that protective effects of IL-13 depend on reaching a critical concentration, in which high IL-13 levels are able to counteract the elevation of blood glucose and insulin resistance. In this hypothetical scenario, it is expected to find a positive correlation between IL-13 and insulin, a hormone widely known to counteract the effects of glucagon and decrease glucose levels. Interestingly, our results show a positive correlation between increased IL-13 and elevated values of serum insulin. Consistent with this hypothesis, it has been recently demonstrated that IL-13 promotes beta-cell survival and insulin secretion in human pancreatic beta-cell *in vitro* cultures [19]. However, it is still of great importance to examine the possible effect of IL-13 in regulating glucagon, GLP-1, insulin, and blood glucose levels with the aim of merging apparently controversial data regarding the role of this cytokine in glucose metabolism.

Numerous studies have suggested a direct relationship between IL-13 and lipid metabolism, especially in the scenario of metabolic dysfunction. For instance, a recent work demonstrated that PBMC show increased IL-13 expression in type 2 diabetic nephropathy patients that also exhibit hypertriglyceridemia [35]. Similarly, overweight and obese pregnant women with gestational hypertension show increased IL-13 concentration, which in turn is associated with elevated triglyceride levels [36]. Moreover, metabolic syndrome subjects exhibit increased serum IL-13 that is also correlated with rising concentration of blood sugar and triglycerides [37]. Our data expand on this body of work by revealing that circulating levels of IL-13 are strongly correlated with triglyceride values in insulin-resistant individuals.

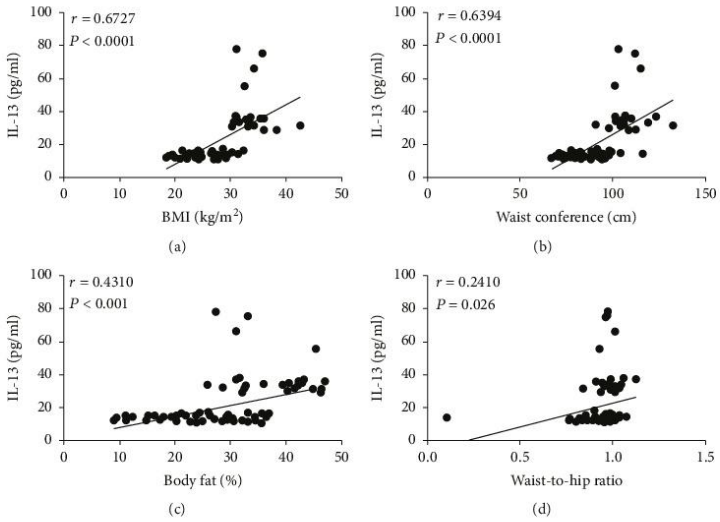

Figure 3: Correlation analysis between IL-13 serum levels and anthropometric parameters in the study population. (a) Correlation analysis between IL-13 serum levels and BMI. (b) Correlation analysis between IL-13 serum levels and waist circumference. (c) Correlation analysis between IL-13 serum levels and body fat percentage. (d) Correlation analysis between IL-13 serum levels and waist-to-hip ratio. Serum levels of IL-13 were moderately associated with BMI and waist circumference and showed to be barely related to body fat percentage and waist-to-hip ratio. BMI: body mass index. Coefficients (r) and P values were calculated by Pearson's correlation model. The correlation level was considered significant when $P < 0.05$.

Interestingly, Tsao and coworkers previously demonstrated that IL-4, another Th2 cytokine with a similar structure and function to IL-13, is able to induce lipolysis in 3T3L1 adipocytes, thus increasing glycerol release and secretion into the culture supernatant [38]. This work concurs with our findings and reveals a novel function of Th2 cytokines in the regulation of triglyceride metabolism; however, there is no evidence yet exploring whether IL-13 may have a similar effect to IL-4. Further research is needed to evaluate the possible role of IL-13 in lipid metabolism and identify novel molecular targets with the aim of reducing triglyceride levels and cardiovascular risk in patients with insulin resistance.

Besides examining its association with insulin resistance-related metabolic markers, IL-13 was also studied in terms of low-grade systemic inflammation. As described above, low-grade systemic inflammation is hallmarked by increased levels of proinflammatory cells and cytokines whereby immune cells and cytokines with anti-inflammatory actions are decreased [39]. Consistent with this notion, we saw a clear elevation in the circulating levels of TNF-α and proinflammatory monocytes Mon-CD11c$^+$CD206$^-$ in insulin-resistant patients with respect to noninsulin-resistant controls. Simultaneously, circulating levels of IL-10 and anti-inflammatory monocytes Mon-CD11c$^-$CD206$^+$ were also significantly diminished in patients with insulin resistance. However, this state of low-grade systemic inflammation did not relate to serum IL-13, although several reports suggest an association of IL-13 with proinflammatory and anti-inflammatory immune responses [17, 18, 40, 41]. In this sense, it has been previously reported that IL-13 does not always correlate with low-grade systemic inflammation parameters. In fact, a recent study conducted in morbidly obese men with metabolic syndrome showed a significant increase in the serum levels of IL-6 and IL-12, both cytokines with proinflammatory actions, without reporting any difference in IL-13 [42]. Similarly, TNF-α soluble receptor levels were shown to raise in plasma of burn-induced systemic inflammatory response syndrome children whereas IL-13 serum levels remained unchanged [43]. Furthermore, Matia-García and coworkers recently reported that young obese subjects with hypertriglyceridemia exhibit low-grade systemic inflammation characterized by increasing levels of C-reactive protein (CRP) and IL-6, accompanied by reduced IL-10 serum concentration [44]. Interestingly, IL-13 showed neither statistical changes between obese and normal weight subjects nor significant correlation coefficients with CRP, IL-6, and IL-10 [44]. Therefore, our results reveal that serum levels of IL-13 elevate in insulin resistance without showing correlation with markers of low-grade systemic inflammation in humans.

Finally, it is important to note that serum IL-13 levels appear to be grouped in two main clusters, characterized by high and low production of this cytokine. Notably, ninety-five percent of low-IL-13 producers showed a HOMA-IR value below 3, whereas an inverse tendency was seen in high-IL-13 producers that exhibited increasing levels of

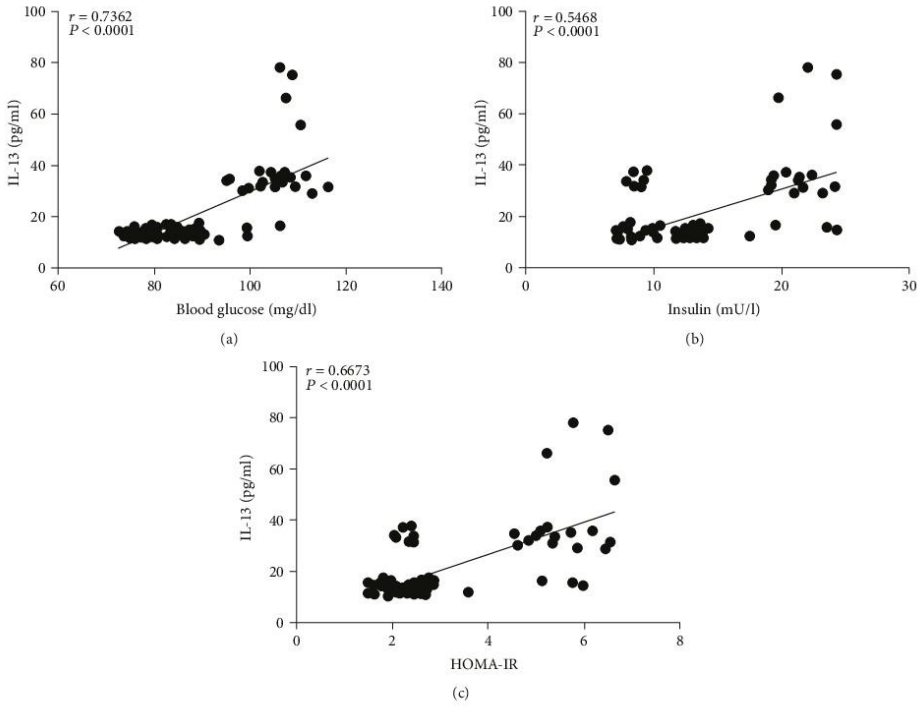

Figure 4: Correlation analysis between IL-13 serum levels and parameters of glucose metabolism in the study population. (a) Correlation analysis between IL-13 serum levels and blood glucose. (b) Correlation analysis between IL-13 serum levels and insulin. (c) Correlation analysis between IL-13 serum levels and HOMA-IR value. Serum levels of IL-13 were strongly associated with blood glucose and showed to be moderately related to insulin and HOMA-IR value. HOMA-IR, homeostatic model assessment of insulin resistance. Coefficients (r) and P values were calculated by Pearson's correlation model. The correlation level was considered significant when $P < 0.05$.

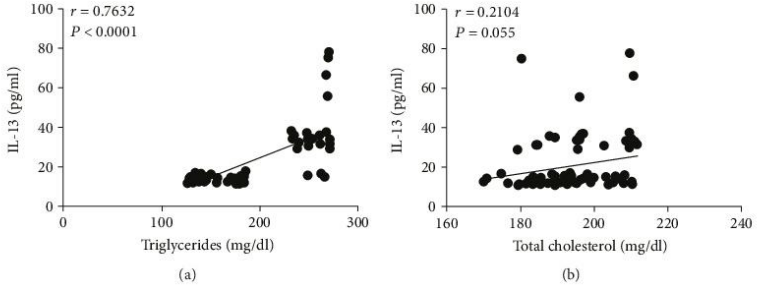

Figure 5: Correlation analysis between IL-13 serum levels and parameters of lipid metabolism in the study population. (a) Correlation analysis between IL-13 serum levels and triglycerides. (b) Correlation analysis between IL-13 serum levels and total cholesterol. Serum levels of IL-13 were strongly associated with blood triglycerides but showed no significant correlation with cholesterol. Coefficients (r) and P values were calculated by Pearson's correlation model. The correlation level was considered significant when $P < 0.05$.

insulin resistance (HOMA-IR > 4.5). Moreover, ninety percent of high-IL-13 producers had central obesity and hyperglycemia, while a similar amount also showed triglyceride levels higher than 200 mg/dl (data not shown), which suggests that increased IL-13 could concur with the development of metabolic syndrome. However, we did not categorize the

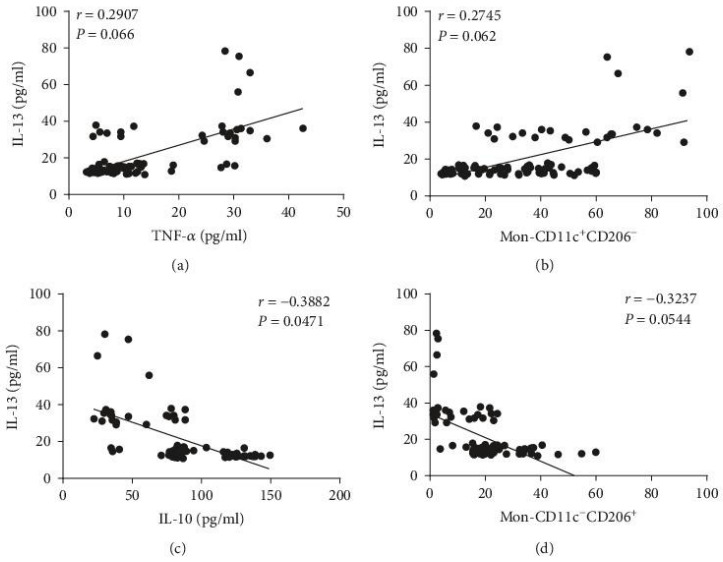

Figure 6: Correlation analysis between IL-13 serum levels and parameters of low-grade systemic inflammation in the study population. (a) Correlation analysis between IL-13 serum levels and circulating concentration of TNF-α. (b) Correlation analysis between IL-13 serum levels and the percentage of proinflammatory monocytes Mon-CD11c$^+$CD206$^-$. (c) Correlation analysis between IL-13 serum levels and circulating concentration of IL-10. (d) Correlation analysis between IL-13 serum levels and the percentage of anti-inflammatory monocytes Mon-CD11c$^-$CD206$^+$. Serum levels of IL-13 were barely associated with IL-10 but showed no significant correlations with TNF-α, Mon-CD11c$^+$CD206$^-$, and Mon-CD11c$^-$CD206$^+$. Mon-CD11c$^+$CD206$^-$, proinflammatory monocytes that express CD11c but do not express CD206; Mon-CD11c$^-$CD206$^+$, anti-inflammatory monocytes that express CD206 but do not express CD11c. Coefficients (r) and P values were calculated by Pearson's correlation model. The correlation level was considered significant when $P < 0.05$.

study subjects according to the number of metabolic syndrome risk factors and the discussion of these results makes no attempt to conjecture beyond that. Further clinical research is needed to understand whether low and high-IL-13 producers have different risks for developing metabolic syndrome and its cardiovascular comorbidities.

5. Conclusions

This study demonstrates that serum levels of IL-13 are significantly elevated in insulin-resistant patients without showing correlation with parameters of low-grade systemic inflammation such as TNF-α, IL-10, and monocytes that show expression of CD11c and CD206. Hyperglycemia and hypertriglyceridemia appear to be strongly linked to the increase in IL-13, which suggest a novel role of this cytokine in the regulation of glucagon-dependent pathways and lipolysis that should be addressed in patients at higher cardiovascular risk such as the vast majority of individuals living with insulin resistance, metabolic syndrome, and T2D. Current results also allow us to speculate regarding the existence of a state of IL-13 action resistance that could be associated with increased serum IL-13 levels in insulin-resistant patients, a notion that needs to be elucidated in basic and clinical research studies. The study of IL-13 in the development of insulin resistance may provide novel insights regarding the role of cytokines in the pathogenesis of metabolic disease and immune hyperactivation. We also remark the urgency of performing clinical studies evaluating whether IL-13 may represent a novel risk marker of insulin resistance in human beings.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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