Universidad Autonoma de Zacatecas
"Francisco García Salinas"
Unidad Académica de Biología

"Dinámica de HMGB1 durante la formación de trampas extracelulares de neutrófilos inducida con suero de pacientes con artritis reumatoide"

T E S I S

Que para obtener el grado de

DOCTOR EN CIENCIAS BÁSICAS

(Orientación en Ciencias Biológicas)

PRESENTA

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DRA. SAMANTHA DECIRÉ BERNAL AYALA
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P R E S E N T E

Por medio del presente le informo que la ***C. FATIMA DE LOURDES OCHOA GONZALEZ,*** egresado del Programa de Doctorado de la Unidad Académica de Ciencias Biológicas con número de matrícula **22301312,** eligió la opción de Titulación por tesis, titulada "**DINÁMICA DE HMGB1 DURANTE LA FORMACIÓN DE TRAMPAS EXTRACELULARES DE NEUTROFILOS INDUCIDA CON SUERO DE PACIENTES CON ARTRITIS REUMATOIDE**" bajo la dirección del Dr. en C. Jesús Adrian López.

Se anexa documentación que hace constar que la ***C. FATIMA DE LOURDES OCHOA GONZALEZ,*** cuenta con todo el procedimiento requerido con control escolar para la realización de este.

Sin otro particular por el momento, aprovecho la ocasión para enviarle un cordial saludo.

A T E N T A M E N T E

Zacatecas, Zac., 10 de junio de 2021

M. en B.E. ROSA GABRIELA REVELES HERNANDEZ
Directora de la Unidad Académica de Ciencias Biológicas

UNIDAD ACADEMICA
CIENCIAS BIOLOGICAS

Dra. en C. Samanta Desiré Bernal Ayala
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Universidad Autónoma de Zacatecas "Francisco García Salinas"

PRESENTE:

Por medio de la presente le informo que la tesis titulada "**Dinámica de HMGB1 durante la formación de trampas extracelulares de neutrófilos inducida con suero de pacientes con artritis reumatoide**" que fue desarrollada por la alumna **Fátima de Lourdes Ochoa González** con matrícula **22301312** para obtener el grado de Dra. en C. del Doctorado en Ciencias Básicas con orientación en Ciencias Biológicas ha sido revisada y aprobada para su impresión.

Sin otro particular por el momento, me reitero de Usted.

Atentamente,

En la ciudad de Zacatecas, Zacatecas a 22 de Junio de 2021

Dr. en C. Jesús Adrián López
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Comité tutoral

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RESUMEN

La artritis reumatoide (AR) es una enfermedad crónica, inflamatoria sistémica que afecta principalmente a las articulaciones sinoviales periféricas que conduce a la destrucción del cartílago, erosión ósea y deformidad articular. La inflamación sinovial se caracteriza por infiltración leucocitaria, los más abundantes son los neutrófilos, durante su activación incrementan la producción de especies reactivas de oxígeno, con ello se libera el calcio intracelular, se activan enzimas que finalmente conducen a la formación de trampas extracelulares de neutrófilos (NET´s). Durante la liberación de NETs se liberan patrones moleculares asociados a daño, entre ellos HMGB1 (High mobility group box 1). Una proteína nuclear que en el espacio nuclear actúa como alarmina incrementando la respuesta inflamatoria. Evaluamos la dinámica de HMGB1 durante la formación de trampas extracelulares de neutrófilos inducida con suero de pacientes con AR y evaluar su relación con diversos marcadores de actividad de la enfermedad característicos del proceso inflamatorio en artritis reumatoide. Demostramos que HMGB1 es liberada al espacio extracelular durante la inducción de NETs con suero de pacientes con AR, los pacientes con AR presentan incremento en la expresión relativa de HMGB1 y se encuentra asociada a las características clínicas de los pacientes con AR.

Palabras clave: Artritis reumatoide, HMGB1, NETs

ABSTRACT

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease that primarily affects the peripheral synovial joints leading to cartilage destruction, bone erosion, and joint deformity. Synovial inflammation is characterized by leukocyte infiltration the most abundant are neutrophils, during their activation they increase the production of reactive oxygen species, thereby releasing intracellular calcium activating enzymes that ultimately lead to the formation of neutrophil extracellular traps (NETs). During release of NETs, damage-associated molecular patterns (DAMPs) including HMGB1 (High mobility group box 1). A nuclear protein that acts as alarmin in the nuclear space, increasing the inflammatory response. We evaluated the dynamics of HMGB1 during the formation of NETs induced with serum from patients with RA and to evaluate its relationship with several markers of disease activity characteristic of the inflammatory process in RA. HMGB1 is released into the extracellular space during the induction of NETs with serum from patients with RA, patients with RA show an increase in the relative expression of HMGB1 and it is associated with the clinical characteristics of patients with RA.

Keywords: Rheumatoid arthritis, HMGB1, NETs

CONTENIDO

CAPÍTULO 1

INTRODUCCIÓN

Los neutrófilos son células del sistema inmune de origen mieloide que representan entre el 60 y 70 % de las células presentes en circulación sanguínea. Son considerados la primera línea de defensa del huésped. Su citoplasma es altamente granulado, posee un característico núcleo multilobulado que le permite migrar con mayor facilidad hacia el sitio de lesión en respuesta a citocinas y quimiocinas. Durante una infección o daño tisular los patrones moleculares asociados a daño (DAMPs) son reconocidos por diversos receptores, con lo que puede comenzar la activación de células residentes e iniciar la producción de mediadores inflamatorios como citocinas y quimiocinas, la capacidad de producción de citocinas en los neutrófilos es mucho menor que la de otras células de origen mieloide, pero a pesar de ello los neutrófilos son tan abundantes en el sitio inflamatorio que las pequeñas contribuciones individuales son potencialmente amplificadas.

Una vez que los neutrófilos llegan al sitio inflamatorio despliegan sus funciones efectoras ya sea de enviar señales para promover o disminuir la respuesta inmunológica, mediante la degranulación, fagositosis de microorganismos o mediante la formación de trampas extracelulares (NETs). Este proceso es definido como la formación de estructuras extracelulares que contienen ADN, histonas y proteínas que comunmente se encuentran presentes en los gránulos citoplasmáticos (como la mieloperoxidasa, elastasa de neutrófilos o calreticulina). Durante la formación de NETs existe un incremento en la generación de ROS que conduce a la activación de la elastasa de neutrófilos. Por otro lado la generación de ROS promueve la liberación de calcio intracelular con lo que se activan proteínas dependientes de calcio como la peptidil arginina deiminasa (PAD) que junto a la elastasa se traslocan a núcleo donde ejercen modificaciones post-traduccionales (citrulinación) o cortes proteolíticos en las histonas y como resultado se descondensa la cromatina que finalmente es liberada al espacio extracelular.

Los neutrófilos se despliegan en sitios inflamatorios donde liberan moléculas que son tóxicas para los microorganismos pero tambien para el huésped, ya que se ha descrito que en el

proceso de defensa antimicrobiana pueden inducir daño colateral ya que las proteasas presentes en los gránulos y la formación de ROS pueden destruir el tejido sano. La externalización de las trampas extracelulares de neutrófilos proporcionan una fuente para la presentación de autoantígenos y el desarrollo de diferentes enfermedades reumáticas.

Las enfermedades reumáticas son un conjunto de trastornos inflamatorios que afectan los tejidos y las articulaciones. Una característica distintiva de estas enfermedades es la generación de autoanticuerpos que reconocen estructuras propias (autoantígenos), dichas estructuras se encuentran principalmente dentro de otras células. Estos autoanticuerpos se generan como resultado de la pérdida de tolerancia, causan inflamación y destrucción de los tejidos. La identificación de la estructuras generadas por los neutrófilos durante la formación de NETs son reconocidas como la principal fuente de liberación de autoantígenos en lupus eritematoso sistemico (LES), vasculitis, artritis reumatoide entre otras.

La artritis reumatoide es una enfermedad inflamatoria, sistémica, de etiología autoinmune que afecta aproximadamente al 1% de la población mundial. Se presenta con mayor frecuencia en mujeres en una proporción 3:1. La artritis es caracterizada por inflamación persistente de la membrana sinovial que conduce a la destrucción del cartílago, erosión ósea, deformidad articular y la presencia de autoanticuerpos. Conforme evoluciona la enfermedad los pacientes comienzan a presentar manifestaciones sistémicas. La inflamación del sinovio producida durante el desarrollo de la enfermedad es caracterizada por infiltración leucocitaria, principalmente neutrófilos, cuya activación esta relacionada directamente con el daño en la articulación. Se han encontrado grandes cantidades de de proteínas granulares muy probablemente debido a la activación excesiva del proceso de NETosis, dicho proceso se ha relacionado directamente como una de las principales fuentes de autoantígenos.

Durante la formación de NETs se citrulinan proteínas que son reconocidas por los anticuerpos presentes en los pacientes con AR. Durante la formación de NETs se genera gran cantidad de especies reactivas de oxigeno (ROS) que promueven la liberación de calcio intracelular activación de proteasas como la elastasa de neutrófilos, la peptidil arginina deiminasa que están implicadas en la descondensación de cromatina y finalmente terminan con la expulsión

al medio extracelular de gran cantidad de moléculas intracelulares consideradas alarminas. Tal es el caso del ADN, histonas y HMGB1, esta es una proteína nuclear no histona de unión a ADN e histonas que ayuda a mantener la homeostasis nuclear.

Se ha descrito que la presencia de proteínas citrulinadas en las estructuras de las NETs pueden ser endocitadas por fibroblastos sinoviales (FS) a traves del receptor de productos de glicosilación avanzados (RAGE)- TLR9. Dicho proceso conduce a la regulación de la expresión del complejo mayor de histocompatibilidad (MHC) clase II por FS y a una subsecuente presentación de péptidos expulsados durante la formación de NETs a linfocitos T^{+} CD4. También, a la subsecuente activación de linfocitos B y generación de anticuerpos anti péptidos citrulinados (ACPAs). En dicho proceso se ha descrito a HMGB1 como la molécula posiblemente responsable de la interación directa entre el receptor RAGE y TLR9. Por otro lado se ha descrito a HMGB1 como una molécula que por si sola es capaz de inducir AR en un modelo murino, además de que se han descrito altas concentraciones de HMGB1 en pacientes con artritis juvenil.

La presencia en el núcleo de HMGB1 está implicada en la regulación de la expresión génica. Sin embargo, una vez secretada ha demostrado ser un mediador de la respuesta inmunológica ya que interacciona con diversos receptores de membrana que conducen a la activación de genes relacionados con la producción y secreción de citocinas. Estas citocinas están implicadas en el mantenimiento y prolongación de la fase inflamatoria. Se desconocen los mecanismos involucrados en la movilización y dinámica intracelular de HMGB1 así como el proceso de expulsión de ésta durante la inducción de NETs.

ANTECEDENTES

Definición y epidemiología de la artritis reumatoide

La artritis reumatoide (AR) es una enfermedad inflamatoria sistémica autoinmune de origen desconocido. Se caracteriza por la presencia de células inflamatorias en la membrana sinovial, que afecta principalmente a las articulaciones periféricas. La inflamación persistente conduce a la hiperplasia sinovial que contribuye a la destrucción del cartílago, erosión ósea y deformidad articular. La artritis reumatoide afecta aproximadamente al 1% de la población mundial con una incidencia entre 5 – 50 casos por cada 100,000 habitantes, esta enfermedad se presenta con mayor frecuencia en las mujeres, con una proporción de 3:1[1].

Existen diversos factores de riesgo asociados al desarrollo de la enfermedad entre los que encontramos los factores genéticos caracterizados por variaciones alélicas que codifican para el antígeno leucocitario HLADRB1 que incrementa hasta en un 30 % el riesgo de desarrollar AR, esta condición conocida como el "epítope compartido" confiere mayor afinidad al reconocimiento a residuos citrulinados [2]. Se ha descrito que en los familiares de pacientes con AR seropositivos se incrementa entre un 40 – 65 % la probabilidad de desarrollar AR [3]. Sin embargo, dicha susceptibilidad solo explica una parte de la etiológica de la enfermedad, por ello se han desarrollado diversas hipótesis que están relacionadas con interacciones ambientales que manejan asociaciones genotípicas con el contexto geográfico y ambiental de los individuos [4].

Fisiopatología de artritis reumatoide

La etiología de la AR aún no se ha esclarecido. Diversos factores de la respuesta inmune innata y adaptativa se han asociado a la fisiopatología de la enfermedad. El proceso inmunológico e inflamatorio en el sinovio es caracterizado por infiltración leucocitaria y posteriormente por proliferación. La migración leucocitaria es activada por incremento en la expresión de moléculas de adhesión y quimiocinas [5].

Diversas células pueden migrar a los tejidos y cada una de ellas es importante para entender las fases iniciales de la enfermedad, se ha descrito la participación de macrófagos implicados en la destrucción del tejido sinovial ya que al ser activados mantienen una respuesta proinflamatoria mediante la producción de citocinas [6-8]. Nuestro grupo recientemente ha descrito un perfil transcripcional asociado a la artritis reumatoide temprana. Claramente

existen diferencias importantes, pero también genes compartidos entre los ACPA+ y AR temprana, lo que sugiere que el proceso de activación transcripcional del proceso inflamatorio precede al inicio de la sintomatología en AR [9].

Recientemente se ha descrito que la huella de interferón tipo I también se asocia con las etapas tempranas de la AR e incluso la fase preclínica autoinmune [10]. Entre las funciones asociadas al inicio del fenómeno inflamatorio por tipos celulares, destacan aquellas mediadas por los neutrófilos [11].

Neutrófilos en artritis reumatoide

Los neutrófilos representan entre el 60 y 70 % de las células sanguíneas y debido a sus características polimorfonucleares presentan gran capacidad para migrar a los tejidos, además de producir diversas citocinas que inducen un incremento en la migración y activación de otras células como es el caso de los osteoclastos [12]. Los neutrófilos se han asociado al daño articular debido a la secreción de productos citotóxicos [13], las alteraciones en la apoptosis de los neutrófilos se han asociado con la prolongación de la fase inflamatoria característica de las articulaciones sinoviales [14].

Los neutrófilos pueden ser activados por diversos estímulos, entre ellos por complejos inmunes vía FcϒRs (receptores a la fracción Fc γ de IgG), DAMPs, patrones moleculares asociados a patógenos (PAMPs). Recientemente se ha descrito que los neutrófilos pueden producir NETs una elaborada forma de suicidio, actualmente conocida como NETosis. Durante este proceso los neutrófilos mueren formando redes de cromatina descondensada y proteínas presentes en los gránulos citoplasmáticos [15].

Recientemente se ha descrito que los neutrófilos de los pacientes con AR son más susceptibles de morir por NETosis, esto es debido a la presencia de ACPAs y factor reumatoide (FR) [16]. Posteriormente, se describió que durante la formación de NETs se genera hipercitrulinación de los componentes intracelulares de neutrófilos y que dicho patrón de citrulinación es compartido con el perfil de péptidos citrulinados presentes en el fluido

sinovial de pacientes con AR [17]. Por esto, es posible que la generación de NETs en tejido sinovial sea la principal fuente del origen de autoantígenos en estos pacientes.

NETs

Los neutrófilos pueden formar NETs en respuesta a diversos estímulos entre los que se encuentran los receptores tipo Toll (TLR) TLR4 [18], TLR2 [19] y FcϒRs [20], estos receptores activan vías de señalización asociadas a un marcado incremento en la actividad de PKC (proteína cinasa C) , la activación de PKC promueve la activación de la NADPH oxidasa (nicotinamidaadenina dinucleotido fosfato oxidasa) [21] con lo que se incrementa la producción de especies reactivas de oxigeno (ROS) [22].

El incremento de ROS promueve la liberación de calcio intracelular [23]. El calcio promueve la activación de enzimas como la PAD4 (peptidil arginin deiminasa 4) (implicada en la citrulinación de proteínas) [24] y de vías de señalización que conducen a la producción de citocinas. Las cuales, finalmente conducen a la liberación en el espacio extracelular de las moléculas características de las moléculas características de las NETs, algunas de ellas conocidas como DAMPs.

DAMPs

Los DAMPs son moléculas implicadas en los procesos inflamatorios ya que pueden iniciar o prolongar la fase inflamatoria, estas moléculas son expulsadas por la célula en respuesta al daño generado por estrés celular [25, 26], por lo tanto, pueden ser de origen metabólico, citosólico o nuclear, entre las moléculas presentes en el contenido nuclear se encuentran el ADN; histonas, HMGB1, entre otras [27].

HMGB1

HMGB1 es una proteína nuclear no histona de 215 aminoácidos implicada en la remodelación de cromatina. Presenta dos sitios de unión a ADN (caja A, caja B) y una cola C-terminal caracterizada por la presencia de aminoácidos aniónicos que se encuentra unida a histonas [28]. Una vez que HMGB1 es liberada al espacio extracelular puede actuar como un potente factor proinflamatorio, con capacidad de interactuar con distintos receptores

(RAGE, TLRs (2,4, 7/9). Puede inducir la producción de citocinas, promover la quimiotaxis y activar diversas células del sistema inmune, células endoteliales y fibroblastos.

La actividad inmunológica de HMGB1 depende del estado redox de las cisteínas 23, 45 y 106 [29]. Se ha descrito un incremento en la concentración de HMGB1 en el líquido sinovial de pacientes con artritis reumatoide idiopática juvenil [30], en un modelo murino de inducción de AR muestran como después de 21 días de haber administrado HMGB1 recombinante los ratones siguen mostrando características inflamatorias y progresión en el daño articular [31].

Se desconocen los mecanismos involucrados en la movilización intracelular de HMGB1 y si las citocinas junto con los autoanticuerpos característicos de los pacientes con AR son capaces de inducir la movilización nuclear y citoplasmática de HMGB1 como un evento temprano durante la inducción de NETs. Como se ha venido mencionando HMGB1 es una molécula que participa de manera activa como mediador del proceso inflamatorio.

Existe poca información acerca de la misma en procesos inflamatorios crónicos, se ha descrito que HMGB1 induce la polarización de linfocitos T colaboradores (Th) tipo 2 y 17 en un modelo de asma en ratón [32], además de que HMGB1 vía RAGE puede inducir la producción de TNF-α (factor de necrosis tumoral-α) y de BAFF (factor activador de células B) [33]. Debido a que la producción de citocinas por células Th2 y la presencia de BAFF son muy importantes en la producción de anticuerpos, es importante evaluar si HMGB1 se asocia activamente en la producción de los autoanticuerpos. Adicionalmente, el incremento de HMGB1 junto con la PCR (proteína C reactiva) y la procalcitonina se encuentran incrementados en pacientes con periodontitis [34], además de que la PCR induce la secreción de HMGB1 en células RAW264.7 [35], por lo que la asociación de HMGB1 y reactantes de fase aguda como PCR y la VSG sería relevante para identificar el papel de HMGB1 en la prolongación del proceso inflamatorio en AR.

PLANTEAMIENTO DEL PROBLEMA

¿Existe movilización de HMGB1 durante la formación de trampas extracelulares de neutrófilos inducida con suero de pacientes con AR?

¿La presencia de HMGB1 en los pacientes con AR se asocia con los niveles de ACPAs y reactantes de fase aguda en sujetos sanos, sujetos en riesgo de desarrollar AR (ACPA+/ACPA-), AR y ARE?

CAPÍTULO 2

HIPÓTESIS

HMGB1 es movilizada durante la formación de trampas extracelulares de neutrófilos inducida con suero de pacientes con artritis reumatoide.

JUSTIFICACIÓN

La artritis reumatoide es una enfermedad multifactorial, caracterizada por inflamación persistente y destrucción del sinovio, dado que el diagnóstico y tratamiento oportuno son la base en el éxito terapéutico, es importante reconocer cada uno de los factores que pudieran estar implicados en el proceso inflamatorio que conduce al deterioro de las articulaciones.

Las células que infiltran al sinovio son las principales células involucradas en el mantenimiento del proceso inflamatorio, como es el caso de los neutrófilos, que en respuesta a las moleculas presentes en el medio responden mediante la formación de NET´s. En dicho proceso hay un marcado incremento en la producción de especies reactivas de oxígeno, que de manera indirecta conducen a la elevación de las concentraciones citoplasmáticas de calcio.

Esto se ha asociado a la liberación de moléculas asociadas a daño como es el caso de HMGB1, histonas e incluso el ADN. HMGB1 al ser una proteína de unión a ADN e histonas implicada en la regulación de la expresión génica ha demostrado ser un mediador de la respuesta inmune ya que interacciona con diversos receptores de membrana que conducen a la activación de genes implicados en la producción y secreción de citocinas relacionadas con el mantenimiento y prolongación de la fase inflamatoria.

Se sabe que los neutrófilos son las principales células implicadas en la generación de autoantígenos y que estas células pueden inducir NETosis en respuesta a las citosinas presentes en el suero de los pacientes con AR. Sin embargo, poco se sabe sobre el proceso de liberación de moléculas inmunomoduladoras como es el caso de HMGB1que puediera estar involucrada en la modulación de la respuesta inflamatoria. Por lo antes mencionado es importante evaluar si el suero de pacientes con artritis reumatoide es capaz de promover la

movilización intracelular de HMGB1 durante la formación de NETs y si la presencia e incremento de HMGB1 se asocia con la presencia de autoanticuerpos y moléculas caracterÍsticas del proceso inflamatorio en pacientes con AR.

Los niveles de HMGB1 se asocian positivamente a la presencia y concentración de ACPAs y reactantes de fase aguda en sujetos sanos, sujetos en riesgo de desarrollar AR (ACPA+/ACPA-), AR y ARE.

OBJETIVO GENERAL

Evaluar la movilización de HMGB1 durante la formación de trampas extracelulares de neutrófilos inducida con suero de pacientes con artritis reumatoide.

Asociar la concentración plasmática de HMGB1 con la de ACPAs y reactantes de fase aguda en pacientes con artritis reumatoide en sujetos sanos, sujetos en riesgo de desarrollar AR (ACPA+/ACPA-), AR y ARE

OBJETIVOS ESPECÍFICOS

Ex vivo

1. Evaluar la movilización intracelular de HMGB1 durante la inducción de trampas extracelulares de neutrófilos con suero de pacientes con AR

Clínico – transversal

1. Determinar la expresión de HMGB1 en pacientes con AR en sangre total de sujetos sanos, sujetos en riesgo de desarrollar AR (ACPA+/ACPA-), pacientes con artritis reumatoide (ART o ARE)
2. Cuantificar los niveles de HMGB1 presentes en suero de sujetos sanos, sujetos en riesgo de desarrollar AR (ACPA +/ACPA-), pacientes con artritis reumatoide ((ART o ARE)
3. Determinar si existe correlación entre los niveles de HMGB1, ACPAs y reactantes de fase aguda en el suero de los pacientes con AR

CAPÍTULO 3

MATERIAL Y MÉTODOS:

Aspectos éticos y de bioseguridad

El material biológico utilizado en el presente proyecto almacenadas a -20°C y – 70°C en la Unidad de Investigación Biomédica del IMSS, al momento de la recolección de las muestras los pacientes firmaron una carta de consentimiento informado en donde proveen su autorización para el uso de dichas muestras.

Es importante destacar que los procedimientos propuestos en el presente proyecto se llevaron a cabo de acuerdo con las normas éticas, el reglamento de la Ley General de salud en materia de investigación en salud y con la declaración de Helsinki de 1975 y sus enmiendas, así como los códigos y normas internacionales vigentes para las buenas prácticas en la investigación clínica y biomédica. Otros datos clínicos fueron tomados del expediente clínico que se formó durante la entrevista, revisión clínica y firma de consentimiento informado.

Los datos recabados se tomaron con apego a la NOM-004-SSA3-2012 en la cual se describen los criterios establecidos de los registros médicos, así como de los servicios médicos, de enfermeras y demás personal del área de la salud obligados en la elaboración, uso, archivo, conservación, propiedad, titularidad y confidencialidad del expediente y que por lo tanto contiene datos personales del paciente en el ámbito sanitario.

Para efectos sobre el manejo de la información contenida en el expediente cada uno fue tratado de manera anónima y sin prejuicios para así preservar la integridad de la persona. Las muestras de los pacientes fueron manejadas de acuerdo con la legislación vigente en el IMSS en materia de bioseguridad (NOM-087-ECOL-SSA1-2002) de acuerdo con prácticas y en las instalaciones adecuadas.

Ex-vivo

Aislamiento de PMN

Se tomaron muestras de donadores sanos de sexo masculino. Cada uno de los participantes firmó una carta de consentimiento informado, el protocolo de investigación con título "DINAMICA DE HMGB1 DURANTE LA FORMACIÓN DE TRAMPAS EXTRACELULARES DE NEUTRÓFILOS INDUCIDA CON SUERO DE PACIENTES CON ARTRITIS REUMATOIDE" fue aprobado por el CNIC con número de registro R-2018-785-099 (Anexo 1). Las muestras fueron usadas para el aislamiento de células polimorfonucleraes por gradiente de densidad. Se realizó una dilución de sangre total-EDTA con un volumen igual de PBS 1x. Se transfirió sobre 3 mL de Limphoprep y se centrifugó a 800 x g durante 15 minutos. El plasma y las células mononucleraes fueron removidas, las células polimorfonucelares y los eritrocitos fueron resuspendidos agregando 12 mL de buffer de lisis ($NaHCO_3$ 14 mM, NH_4Cl 15.6 mM, EDTA 0.126 mM) e incubadas durante 10 minutos en hielo, los detritos celulares fueron retirados por centrifugación (5 minutos a 400 x g). Las células PMN fueron lavadas 3 veces con PBS 1x y posteriormente resuspendidas en 3 mL de Gibco medio RPMI-1640 (sin rojo fenol con 1x de L-glutamina) suplementado con albumina humana al 2%. Se realizó un ensayo de viabilidad celular con azul de tripano indicando una viabilidad mayor al 97% después del aislamiento. La pureza de los neutrófilos se evaluó por citometria de flujo usando un anticuerpo anti CD66 (BD-Biosiences) marcado con CF405 encontrando una pureza de neutrófilos alrededor del 91%.

Inducción de NETs

Los neutrófilos aislados se sembraron a una densidad de 3 x 10^5 cel/pozo sobre cubreobjetos previamente tratados con poli-L-lisina (0.01%, Sigma Aldrich). Se incubaron durante 30 minutos a 37°C para permitir su acondicionamiento, posteriormente fueron estimuladas para la inducción de NETs con PMA (20 nM, Sigma Aldrich), A23187 (25 μM, Sigma Aldrich) y un pool de pacientes con AR que presentan títulos altos de anticuerpos anti peptidos citrulinados al 10%. Este se preparó seleccionando muestras de 5 pacientes con AR establecida que presentaron títulos de anticuerpos anti-peptidos citrulinados mayores a 500 U/mL determinados usando el kit CCP plus de EURO diagnóstica. Se realizaron cinéticas de inducción cada 30 minutos hasta los 180 minutos, o incubaciones continuas de 180 minutos

en RPMI sin rojo fenol suplementado con albumina humana al 2% (Biowest) a 37°C con 5% CO_2 (Thermo Fisher Scientific). El medio de cultivo fue recolectado en un tubo nuevo y centrifugado a 5 minutos a 22 000 x g, el sobrenadante fue transferido a un tubo nuevo y almacenado a -20 °C para su posterior uso. Las células fueron fijadas con paraformaldehido (PFA) al 4%.

Cuantificación de ADN libre de células

El ADN libre de células liberado durante la inducción de NETs fue cuantificado en los sobrenadantes de cultivo celular usando el Kit Quanti-iT PicoGreen (Thermo Fisher Scientific). 50 µL del sobrenadante se transfirió a una placa de 96 pozos, se añadio un volumen igual del reactivo Picogreen 1x, se incubó durante 5 minutos a temperatura ambiente cubriéndolo de la luz. La placa fue analizada en un fluorometro Fluoroskan (Thermo Fisher Scientific) usando una longitud de onda de 485 nm de exitación y 535 nm de emisión. La concentración de ADN liberado fue cuantificado usando una curva estandar con diluciones seriadas (factor de dilución 1:2) de ADN de esperma de salmón.

Inmunofluorescencias

Para la visualización de NETs, los cubreobjetos fueron lavados 3 veces con PBS 1x, seguido de una permeabilización con PBS 1x- Triton X-100 al 0.5% durante 5 minutos, se realizaron 3 lavados con PBS 1x y una incubación de 30 minutos con BSA al 5 % (Biowest), después de ello se incubaron con anticuerpo de conejo anti-HMGB1 (10 µg/mL, Novus biologicals) durante una hora a temperatura ambiente, se realizaron 3 lavados con PBS 1x, se incubó durante una hora con anticuerpo secundario anti-conejo IgG marcado PE (BD-Biosciences), se realizaron tres lavados con PBS 1x, se incubaron durante 15 minutos con un agente intercalante de ADN que puede ser Draq5 (Invitrogen, Thermo Fisher Scientific) o reactivo de Vindelov (Tris buffer 100 mM pH 7.6, 110 mM de yoduro de propidio (Sigma Aldrich), 0.1% de NP40 (Sigma Aldrich)), se realizaron tres lavados con PBS 1x, finalmente se montaron los cubreobjetos con fluoroshiel (Sigma Aldrich). Las laminillas fueron analizadas (Z-Stack: 7 µm, 11 steps, Z step size 0.56 µm) en un microscopio confocal Leica DM2500.

Análisis de imágenes

La intensidad media de fluorescencia de HMGB1-PE y el área nuclear (yoduro de propidio o Draq5) se analizó usando el software FIJI (https://doi.org/10.1038/nmeth.2019). Se aplicó un filtro Gausiano a cada imagen, se realizó inversión del color ajustando una mascara binaria entre el 5 - 15 %. Después de ello se realizó la detección de partículas filtrando por tamaño y eliminando todas las partículas que se encuentran en las esquinas. Se obtuvó el área y la intensidad media de fluorescencia de cada imagen, los datos generados se analizaron posteriormente en GraphPad Prism 7.0 (**figura 2**).

Clínico transversal

Pacientes y donadores

El grupo de trabajo cuenta con una base de datos clínicos y muestras congeladas (sangre total-EDTA/RNA latter (Ambion), ARN, o DNA-copia) derivadas del proyecto "IDENTIFICACIÓN DE BIOMARCADORES ASOCIADOS AL DESARROLLO DE ARTRITIS REUMATOIDE TEMPRANA EN HUMANOS" con número de registro CNCI R-2013_785_009 (Anexo 2), que comprende cerca de 350 muestras. Dichos sujetos con artritis reumatoide fueron evaluados siguiendo los criterios del ACR/EULAR 2010 y posteriormente clasificados según la fase de la enfermedad en artritis temprana o artritis establecida, también se reclutaron familiares en primer grado de los pacientes con artritis reumatoide y donadores sanos siguiendo los criterios descritos en la **tabla 1**. Se recabaron datos clínicos como la edad, el número de articulaciones inflamadas y tabaquismo, tiempo con diagnóstico de AR o con sintomatología para el caso de los pacientes diagnosticados con AR temprana. Se tomaron muestras de sangre por venopunción en tubos suplementados con EDTA, también se recolectó muestra en tubos sin anticoagulantes para la obtención de suero que fueron usadas para la determinación de la velocidad de sedimentación globular (VSG mm/hr), proteina C reactiva (PCR mg/L), factor reumatoide (FR U/mL), concentració de HMGB1 (ng/mL) y de los títulos de anticuerpos anti-péptidos citrulinados (ACPA U/mL). Todos los participantes firmaron una carta de consentimiento informado de acuerdo a la declaración de Helsinki.

Tabla 1. Criterios de inclusión y exclusión de donadores sanos y paciente pacientes con AR temprana, establecida y sujetos con alto riesgo a desarrollar AR.		
Grupos clínicos	Criterios de inclusión	Criterios de exclusión
Donadores sanos	Firmar carta de consentimiento informado. Sin antecedentes heredofamiliares de AR	Pacientes positivos a hepatitis B o C. (activo o inactivo). Pacientes con enfermedades crónico-degenerativas como: diabetes mellitus tipo I, hipo e hipertiroidismo, cirrosis hepática y los distintos tipos de cáncer. Sujetos con enfermedades reumáticas diferentes a AR. Sujetos que no firmen carta de consentimiento informado.
Sujetos con alto riesgo a desarrollar AR- ACPA negativos.	Familiar consanguíneo de paciente con AR. Anticuerpos anti-péptidos citrulinados menor a 25 U/mL (ACPA -). Firmar carta de consentimiento informado.	
Sujetos con alto riesgo a desarrollar AR- ACPA positivos.	Familiar consanguíneo de paciente con AR. Anticuerpos anti-péptidos citrulinados mayor a 25 U/mL (ACPA +). Firmar carta de consentimiento informado.	
Pacientes con AR temprana (ART).	Cumplir los criterios de diagnóstico para AR, propuestos por la ACR/EULAR. Sintomatología con menos de 1 año de evolución. Firmar carta de consentimiento informado.	
Pacientes con AR establecida (ARE).	Cumplir los criterios de diagnóstico para AR, propuestos por la ACR/EULAR. Sintomatología por más de 2 años. Firmar carta de consentimiento informado	

Implicaciones de HMGB1 en pacientes con AR

Para evaluar las implicaciones de HMGB1 en pacientes, se evaluó la expresión de HMGB1, usando muestras de ADN copia sintetizado a partir de ARNm extraido de sangre total que se encontraban almacenadas en el banco de muestras de la Unidad de Investigación Biomédica del IMSS Zacatecas. Se utilizaron primers específicos dirigidos a la secuencia compartida entre las 3 variantes del gen de *hmgb1*, la qPCR se realizó utilizando SsoFast™ EvaGreen® Supermix (Bio-Rad) en un termociclador en tiempo real LightCycler® 480 (Roche). El volumen final de la reacción fue de 10 µL, que incluyeron 2 µL de producto de RT (50 ng/µL), 5 µL de mezcla maestra de PCR SsoFast Evagreen supermix (BIO-RAD) y 0.25 µL de cada uno los primers (Forward y reverse), 2.5 µL de H_2O libre de RNasas. Cada muestra se analizó por triplicado. Se realizó cálculo de expresión relativa con el método de $2^{-\Delta\Delta Ct}$ que describe el cambio de expresión con respecto a un gen de referencia que mantenga una expresión constante. Como gen de referencia se utilizó *hprt* (Hipoxanthine phosphoribosyltransferase). Las secuencias de los primers para *hprt* son: forward 5′- TGA CCT TGA TTT ATT TTG CAT ACC -3′ y reverse 5′-CGA GCA AGA CGT TCA GTC CT-3′, Para la amplificación del gen de *hmgb1* se realizó el diseño de primers basado en la secuencia de las tres variantes reportadas para el gen *hmgb1*, la secuencia de estos tres genes se alinearonusando el software serial cloner 2, se tomó la secuencia conservada entre las tres variantes para el diseño de primers en primer blast. Los primers usados para la amplificación de *hmgb1* son forward 5′- TTG TGT GTC CTG TGT ACC CG -3′ y reverse 5′- GTT TGT GTT ACA TGG GCG CT-3′.

Determinación de HMGB1 y de anticuerpos anti-péptidos citrulinados

Se determinó la concentración de HMGB1, de ACPA, y de anticuerpos anti péptidos carbamilados en muestras de pacientes con ARE, ART, familiares en riesgo de desarrollar AR ACPA+, ACPA- y donadores sanos sin relación familiar. Se determinaron los títulos de ACPA usando el kit CCP2 de Euro-Diagnostica (Malmö, Suecia) de acuerdo con las indicaciones del fabricante. Para la determinación de HMGB1 se diluyeron las muestras usando un factor de dilución 1:5, posteriormente se siguió el procedimiento descrito por el fabricante Novus biological (Cat. NBP2-62766). La determinación de la concentración de

anticuerpos anti-péptidos carbamilados se realizó de acuerdo con el protocolo descrito por Trouw y colaboradores y que ha sido replicado por nuestro grupo [10].

Análisis estadístico

Los datos fueron analizados usando el software GraphPad prism 7.0. La visualización y cuantificación de las NETs se realizó en en tres experimentos independientes por duplicado, se realizó análisis de una vía o Kruskal Wallis, con un post test de Tukey o Dunn´s según la distribución de los datos. Se realizó un anális de correlación para evaluar la asociación de la concentración de HMGB1 y los títulos de ACPA y anti peptidos carbamilados, asi como de los reactantes de fase aguda. Se consideró estadísticamente significativo los valores de p menor o igual a 0.05.

CAPÍTULO 4

RESULTADOS

Inducción de NETs con suero de pacientes con artritis reumatoide

La artritis reumatoide es una enfermedad crónica que afecta principalmente a la membrana sinovial, conforme la enfermedad evoluciona los pacientes presentan manifestaciones sistemicas, debido a esto en sangre periférica se puede encontrar un marcado incremento de citocinas proinflamatorias. Basados en estas observaciones nos preguntamos si el suero de los pacientes con artritis reumatoide es capaz de inducir la formación de NETs, para ello se aislaron neutrófilos de sangre periférica de donadores masculinos sanos, para la inducción se usó un agonista de PKC (phorbol myristate acetate-PMA), un ionóforo de calcio (A23187) y un pool de suero de pacientes con artritis reumatoide (SPAR) al 10% [16]. Una de las principales características del proceso de netosis es la descondensación de la cromatina, para observar estos cambios se realizaron preparaciones para microscopia confocal usando Draq5 a los 30 y 180 minutos de tratamiento.

En la **figura 1A** se observa que los neutrófilos aislados de donadores sanos sin estímulo conservan su núcleo multilobulado, los tratados con A23187 y PMA se observa descondensación de la cromatina y formación de redes de ADN. En el caso de las células tratadas con el pool de SPAR conservan su membrana nuclear aunque esta no muestra sus características multilobuladas. Posteriormente se realizó un análisis del área nuclear utilizando el software FIJI. En la **figura 1B** podemos observar que en las células sin estímulo no existen diferencias diferencias estadísticamente significativas a los 30 y 180 minutos mientras que en las células tratadas con A23187 y PMA hay un marcado incremento del área nuclear mostrando diferencias estadísticamente significativas. Sorprendentemente en las células tratadas con el pool de SPAR se observa disminución del área nuclear. En la **figura 1C** se muestra la cuantificación del ADN libre de células en los sobrenadanetes a los 180 minutos de incubación. En los neutrófilos sin estímulo se encontraron menos de 0.25 µg/ml de ADN libre y se puede observar como la cantidad de ADN incrementa hasta dos veces en los neutrófilos estimulados con A23187 y con PMA, encontrando mayor ADN libre el las células tratadas con PMA.

En los neutrófilos tratados con el pool SPAR se promueve la liberación de ADN mostrando un marcado incremento en la cantidad de ADN liberado al espacio extracelular durante el tiempo de incubación. La respuesta generada de los neutrófilos al pool de SPAR tiene variaciones dependientes del donador ya que en algunos se encontraron grandes cantidades de ADN y en otros la respuesta fue mínima, a pesar de ello podemos observar que existen diferencias estadísticamente significativas en la cantidad de ADN libre de células encontrando en el sobrenadante de células tratadas con el pool de SPAR a los 180 minutos. Cabe resaltar que previo a la inducción se realizó una cuantificación de ADN libre en el pool de SPAR y la cantidad de ADN encontrada fue sustraida en el análisis.

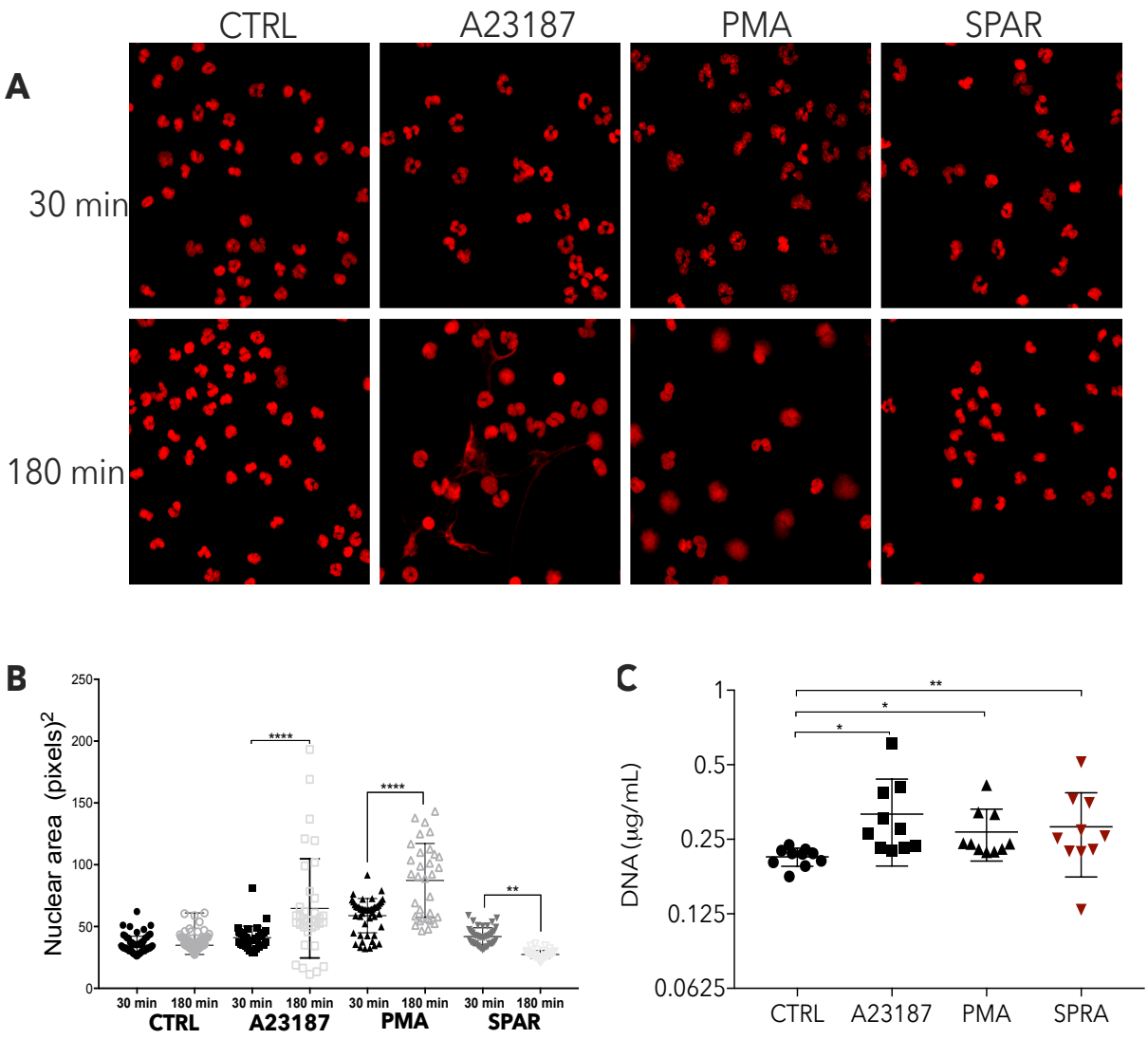

Figura 1. DNA liberado durante la formación de NETs. Neutrófilos aislados de sangre periférica por gradiente de densidad, 3.0 x 10^5 células por condición fueron tratadas con PMA (30 nM), A23187 (25 µM) o SPAR (10 %) durante 180 min. DNA libre de células en el sobrenadante cuantificado con picogreen **A.** Fotomicrografía de tinción de núcleo (Draq 5), imágenes adquiridas por microscopia confocal **B.** Análisis de área nuclear usando el software FIJI, gráfico de puntos muestra media +/- SD. **C.** DNA liberado durante la formación de NETs usando picogreen, DNA-SPAR normalizado. La figura muestra media y desviación estándar. Se realizó una prueba de ANOVA de 1 vía con post test de Tukey y un valor de $p<0.05$ fue considerado como estadísticamente significativo.

Movilización de HMGB1 durante el proceso de NETosis con suero de pacientes con artritis reumatoide.

Durante el proceso de netosis se expulsan junto al ADN gran cantidad de proteínas intracelulares que pueden actuar como DAMPs y con ello exacerbar la respuesta inmunológica, una de ellas es HMGB1, una proteína no histona de unión a ADN que puede ser expulsada en respuesta a diversas citocinas presentes el el suero de pacientes con AR , es por ello que decidimos evaluar si el pool de SPAR es capaz de inducir mobilización de HMGB1 durante el proceso de netosis esto se evaluó mediante una cinética de inducción recolectando y fijando las células cada 30 minutos hasta completar los 180 minutos.

Las fotomicrografias de la **figura 2A** muestran que HMGB1 presenta localización citoplasmatica que coincide con lo previamente reportado [36]. Esto es igual en las células sin estímulo, en las tratadas con A23187, con PMA y con SPAR a los 30 minutos de incubación, se puede observar como la intensidad media de fluorescencia (IMF) cambia con respecto al tiempo y al estímulo.

Las células sin estímulo mantienen constante la intensidad media de fluorescencia de HMGB1 hasta los 90 minuto, a los 135 minutos hay disminución que finalmente es recupera a los 180 minutos a pesar de estas observaciones no se encuentran diferencias estadisticamente significativas, en los neutrófilos tratados con A23187 la intensidad media de fluorescencia aumenta con respecto al tiempo, encontrando diferencias a partir de los 135 minutos ($p=0.0019$) alcazando un máximo a los 180 minutos ($p=0.0008$), cabe mencionar que en las células que formaron NETs, HMGB1 forma parte de las estructuras **figura 2A**.

En los neutrófilos estimulados con PMA presenta variación en cuanto a la intensidad media de fluoresencia de HMGB1 a través del tiempo mostrando un incremento en la señal a partir de los 60 minutos ($p=0.0012$), alcanzando el máximo a los 90 minutos ($p=0.0004$), presenta distribución cercana al núcleo, a partir de los 135 minutos la señal comienza a disminuir como un indicativo del inicio de la liberación del DNA al espacio extracelular ($p=0.0087$). En las células tratadas con el pool de SPAR, se observa un incremento en la intensidad media de fluorescencia de HMGB1 a los 60 minutos ($p=0.0049$), esta comienza a bajar a partir de

los 135 minutos (*p=0.0001*), cabe mencionar que en este tratamiento no se observa de manera evidente en la formación de redes de cromatina. En la **Figura 2C** se observa el análisis de área nuclear, los neutrófilos sin estímulo mantienen el tamaño del área nuclear a través del tiempo, se observa una pequeña caída en el área a los 135 minutos misma que es recuperada a los 180 minutos, a pesar de ello no se encuentran diferencias estadisticamente significativas.

En los neutrófilos tratados con A23187 se puede observar que el área nuclear incrementa con respecto al tiempo, el incremento significativo comienza a los 135 minutos (*p=0.0001*) alcanzando un máximo a los 180 minutos (*p=0.0001*), el área nuclear de las células tratadas con PMA muestra pequeños cambios a los 30 minutos (p=0.0367), incrementa a los 90 minutos (*p=0.0023*), esto se mantiene constante hasta los 135 (*p=0.0001*), a los 180 minutos (*<p=0.0001*) se observa un incremento del área nuclear casi al doble el tamaño que tenia a los 135 min.

En los neutrófilos tratados con el pool de SPAR hay disminución en el tamaño del área nuclear a partir de los 135 minutos (*p=0.0284*), a los 180 minutos se puede observar que el tamaño del núcleo es más pequeño (*p=0.0158*), cabe mencionar que el núcleo no parece estar descondensado, más bien pareciera hipercondensado.

En la **figura 2D** observamos la cuantificación del DNA libre de células en el sobrenadante de cultivo producto de la formación de NETs. Se puede observar que en las células sin estímulo el DNA libre es constante a través del tiempo ya que no se encontraron diferencias estadisticamente significativas, en el caso de los sobrenadantes de A23187 se observa un incremento a partir de los constante durante el estímulo alcanzando el máximo a los 180 minutos (*p=0.0161*), en los sobrenadantes del tratamiento con PMA se observa que el DNA libre se mantiene constante hasta los 135 minutos, al igual que lo observado en el área nuclear, la cantidad de DNA libre incrementa abruptamente a los 180 minutos (*p=0.0017*).

En los sobrenadantes de células tratadas con el pool de SPAR se puede observar un incremento en la cantidad de DNA libre a partir de los 60 minutos alcanzando el máximo a

los 180 minutos (*<p=0.0001*), ésta observación coincide con la disminución del área nuclear por lo que existe una correlación negativa entre la disminución del área nuclear y el incremento del DNA libre de células (R spearman=-1.00, *p=0.017*). Por lo tanto el pool es capaz de inducir la mobilización de HMGB1 **figura 2B** y este proceso va acompañado de la liberación de DNA al espacio extracelular **figura 2C,D** cabe mencionar que en este proceso no es evidente la formación de trampas extracelulares de neutrófilos y este proceso puede ser considerado como una netosis vital.

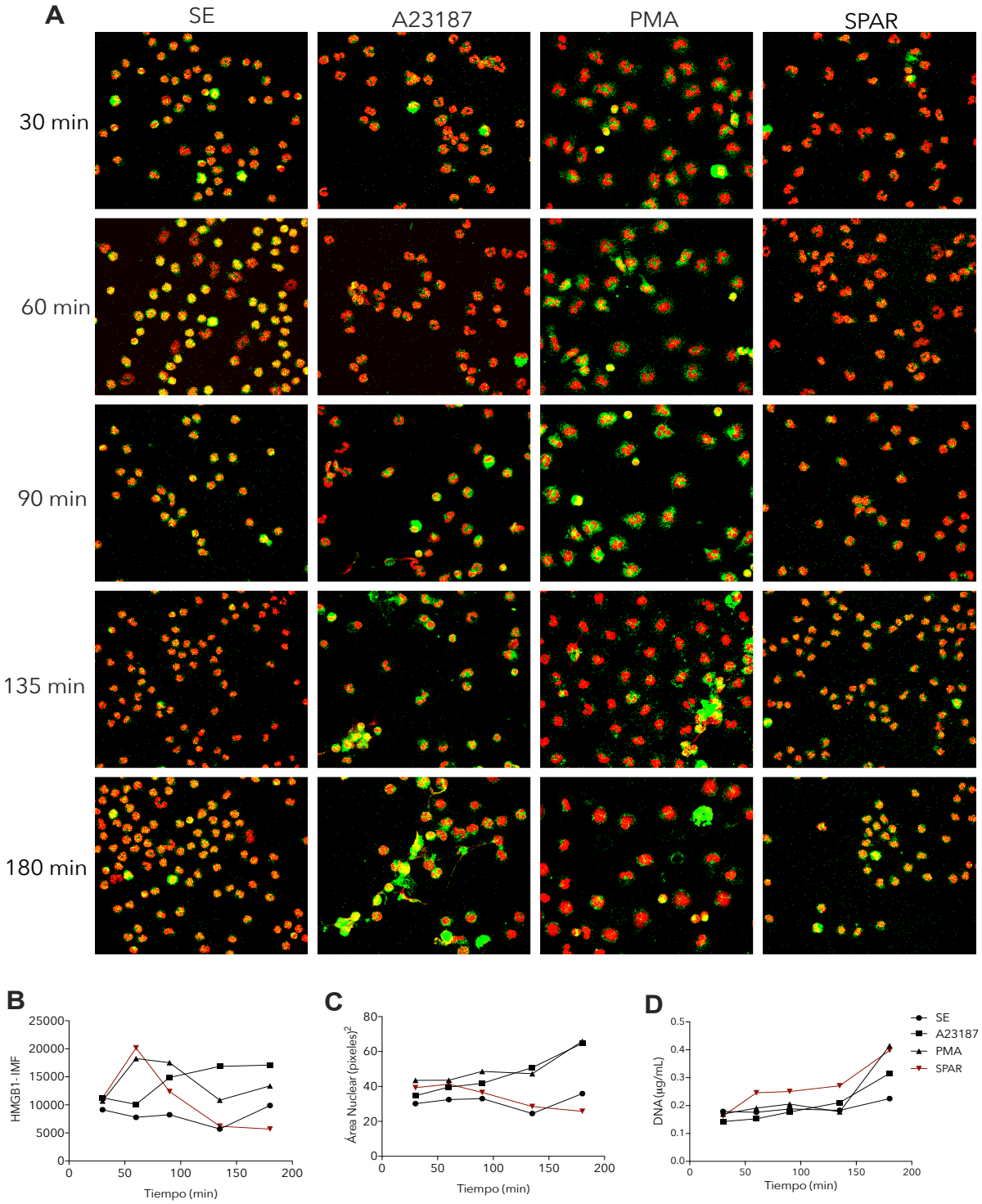

Figura 2. DNA liberado durante la formación de NETs. Neutrófilos aislados de sangre periférica por gradiente de densidad, 3.0 x 10^5 células por condición fueron tratadas con PMA (30 nM), A23187 (25 µM) o SPAR (10 %) durante 180 min. DNA libre de células en el sobrenadante cuantificado con picogreen **A.** Fotomicrografía de inmunodetección de HMGB1-PE y tinción de núcleo-Draq 5, imágenes adquiridas por microscopia confocal **B.** Análisis de intensidad media de fluorescencia-PE usando software FIJI, la figura muestra media **C.** Análisis de área nuclear usando el software FIJI, la figura muestra media **D.** Cuantificación de DNA liberado durante la formación de NETs usando picogreen, DNA-SPAR normalizado. La figura muestra la media. ANOVA de 1 via, post test de Tukey y un valor de $p<0.05$ fue considreado como estadísticamente significativo.

ADN libre-HMGB1 y generación de anticuerpos anti-péptidos carbamilados en pacientes con artritis reumatoide

A partir de este punto nos enfocamos en la identificación de marcadores producto de la activación de los neutrófilos, debido a ello se realizó la cuantificación de ADN libre de células, encontrando que el ADN libre de células es mayor en los pacientes con AR (*M*=0.234 µg/mL) en comparación con la cantidad encontrada en donadores sanos (*M*=0.252 µg/mL) (*p=0.006*) (**Figura 3A**).

Posteriomente se realizó el análisis de *hmgb1* utilizando el cálculo de expresión relativa por el método del $2^{-\Delta\Delta Ct}$ usando como referencia la expresión del gen *hprt*. Se partió de ADN complementario creado a partir de ARN que fue extraído de sangre total de donadores sanos y pacientes con AR. Se encontró que la expresión relativa de *hmgb1* es mayor en los pacientes con AR en comparación de la expresión relativa de los donadores sanos (*p=0.0116*) **Figura 3B**. Posteriormente se evaluó la concentración de HMGB1 en el suero de los pacientes, no se encontraron diferencias estadísticamente significativas en los títulos de HMGB1 entre los pacientes con AR y los donadores sanos **figura 3C**.

Durante la formación de NETs se expulsan al espacio extracelular gran cantidad de proteínas presentes en los gránulos citoplasmaticos entre ellas la mieloperoxidasa que en el sitio inflamatorio se ha visto implicada en el metabolismo del tiocianato al promover su oxidación en presencia de peróxido de hidrógeno, este proceso promueve la generación de cianato que se une a un grupo amino primario o a un tiol para formar grupos carbamil, esto genera lo que se conoce como peptidos carbamilados, estos petidos tiene gran potencial de romper la barrera inmunológica e inducir la formación de anticuerpos, por ello decidimos evaluar los títulos de anticuerpos anti péptidos carbamilados, en la **figura 3D** se observa que los pacientes con AR presentan títulos más altos de anticuerpos (*p=0.0391*).

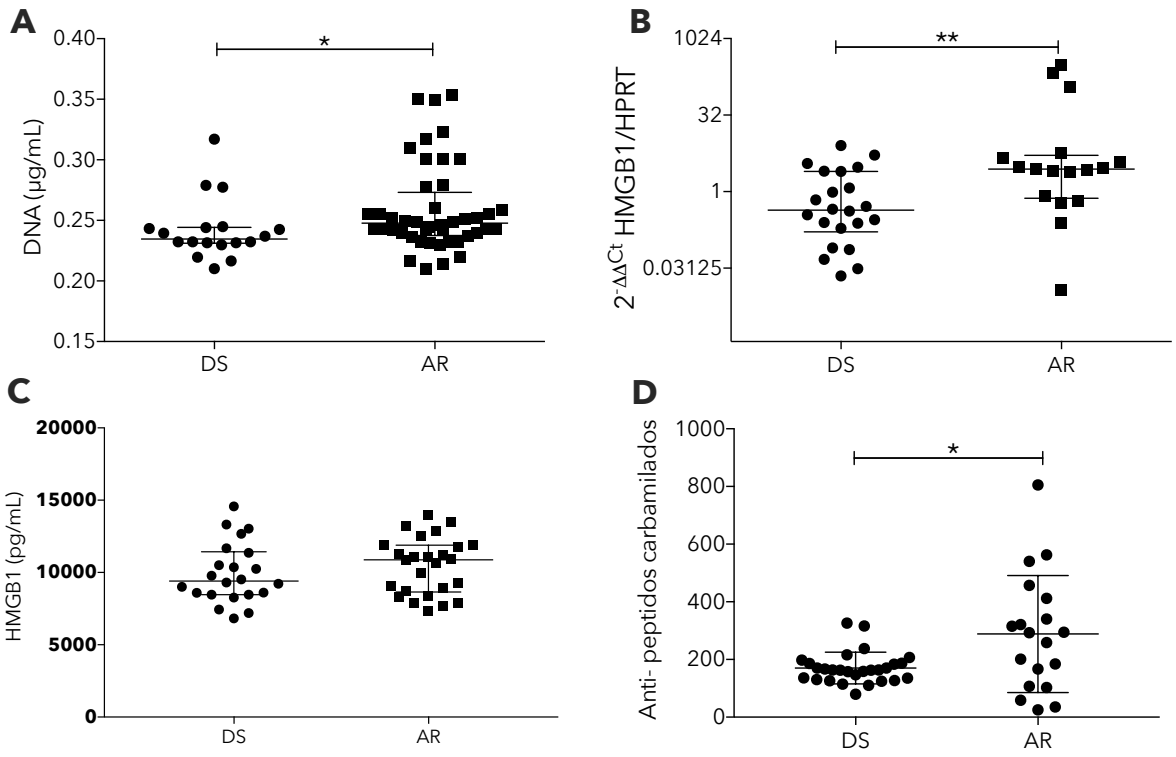

Figura 3. ADN libre-HMGB1 y generación de anticuerpos anti-peptidos carbamilados en pacientes con artritis reumatoide. Gráficos de puntos **A.** Cuantificación de ADN libre de células en suero de pacientes con AR y donadores sanos. La figura muestra mediana con RIQ, Mann-Whitney *(p=0.006)* **B.** Análisis de expresión relativa por el método de $2^{-\Delta\Delta Ct}$ de HMGB1 usando la expresión de HPRT como endogeno. La figura muestra mediana y RIQ, Mann-Whitney (p=0.0116). **C.** Cuantificación de HMGB1 en suero de pacientes con AR y donadores sanos. La figura muestra mediana y RIQ, Mann-Whitney. **D.** Cuantificación de titulos de anticuerpos anti-peptidos carbamilados en pacientes con AR y donadores sanos. La figura muestra mediana y RIQ, Mann-Whitney (p=0.0391).

HMGB1 en sujetos con riesgo de desarrollar AR

La evidencia de que algunos pacientes presentan algunos anticuerpos antes del inicio de las manifestaciones clínicas nos llevó a incluir en el estudios a familiares en primer grado de pacientes con AR ya que se ha descrito que presentan un riesgo dos a tres veces mayor de desarrollar la enferemedad, estos pacientes fueron clasificados deacuerdo a los titulos de anticuerpos anti peptidos citrulinados ciclicos como ACCP negativos (ACCP-) cuando presentaron títulos menores a 25 U/mL y ausencia de sintomatología sugestiva de AR, como ACCP positivos (ACCP+) cuando presentaron títulos mayores de 25.1 U/mL y que no presentaron sintomas sugestivos de AR.

Los pacientes con AR fueron subclasificados como artritis reumatoide temprana (ART) a los pacientes que presentaron un cuadro de evolución menor a un año y con artritis reumatoide establecida (ARE) a los pacientes que presentan más de un año de evolución, ambos grupos cumplieron con los criterios para su clasificación según los criterios del ACR-EULAR [37] cuyas principales características se muestran en la **tabla 2**, en la **figura 4A** se observa que la expresión relativa de *hmgb1* es mayor en los pacientes con ARE que en comparación con los donadores sanos (*p=0.0305*). Por otro lado el análisis de la proteína muestra una tendencia inversa a la mostrada por a expresión del gen.

Tabla 2. Características clínicas por grupo de estudio

	CRTL *(n=9)*	***ACCP-*** *(n=8)*	***ACCP+*** *(n=13)*	***ART*** *(n=7)*	***ARE*** *(n=19)*	***Valor p***
Edad	*35.8 ± 19.9*	*36.3 ± 25.0*	*28.6 ± 16.4*	*29.9 ± 20.6*	*43.1 ± 23.3*	*0.906[a]*
Genero	*78/22*	*83/17*	*82/18*	*90/10*	*91/9*	_____[b]
ACCP	*18.0 (3.0 – 24.6)❖*	*13.2 (10.2 – 15.7)^*	*28.8(25.7- 33.2)*	*157.0 (6.7 – 263.0)*	*312.7 (16.4 – 819.1) ❖^*	*0.0020[a]*
CarP	*187.2 (170.7 – 237.5)*	*160.6 (131.4 – 190.0)*	*158.7 (125.8 – 173.8)**	*201.3 (30.0 – 293.7)*	*340.4 (167.0 – 540.5)**	*0.0437[a]*
FR	_____	*4.4 (0.0 – 6.8)º^*	*6.7 (0.0 – 8.1)❖**	*159.4 (82.9 – 367.0)º❖*	*160.0 (100.8 – 175.4)^**	*0.0004[a]*

[a]Kruscal-Wallis (mediana, IQR), Edad (media ± SD)
[b]Proporción de sujetos por genero % (F/M)
*❖ CTRL vs ARE, * ACCP+ vs ARE, ❖ ACCP+ vs ART, ºACCP- vs ART, ^ACCP- ARE*

En la **figura 4B** se observa que los familiares de pacientes con AR presentan ACCP+ presentan menor concentración de HMGB1 en comparación con los donadores sanos (*p=0.0063*), de igual manera los pacientes con ARE presentan una concentración de HMGB1 más baja que los donadores sanos (*p=0.0170*), tambien se observa que en los pacientes con ART incrementa la concentración de HMGB1 comparado con los familiares en riesgo de desarrollar AR ACCP+ (*p=0.0250*), la AR es una enfermedad que afecta principalmente las articulaciones, basado en las observaciones nos preguntamos si la concentración de HMGB1

en fluido sinovial de pacientes es diferente de las concentraciones séricas, la **figura 4C** muestra que existe una tendencia al incremento en la distribución de la concentración de HMGB1 encontrada en fluido sinovial en comparación con la concentración sérica, sin embargo no se encontraron diferencias estadísticamente significativas (p=0.496).

El daño articular puede ser de tipo inflamatorio como el caso de la AR o no inflamatorio como el caso de diferentes enfermedades articulares como las artrosis, basados en dichas observaciones no preguntamos si la concentración de HMGB1 es diferente en estas condiciones. La **figura 4D** muestra que no existen diferencias estadisticamente significativas en la concentración de HMGB1 encontrada en el fluido sinovial de las artrosis en comparación con la del fluido sinovial de pacientes con artritis reumatoide, cabe mencionar que la distribución es mucho más amplia en los paientes con AR.

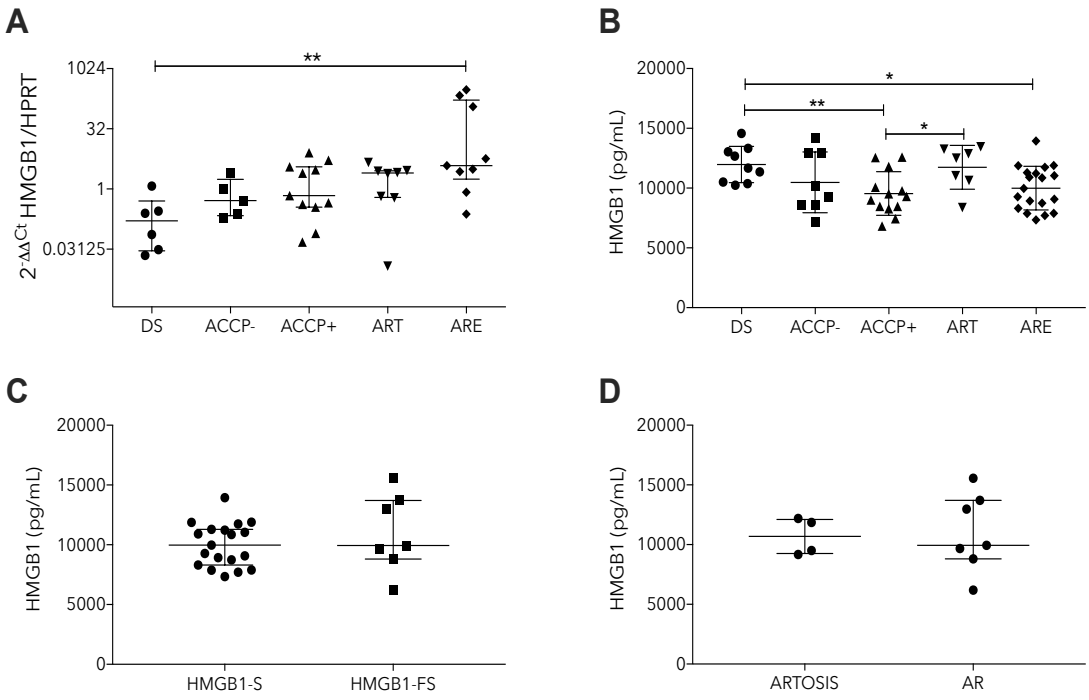

Figura 4. HMGB1 en pacientes con artritis reumatoide y sujetos en riesgo de desarrollar AR. La figura muestras gráficos de puntos con las características clínicas de los participantes asociadas al induccion de NETs **A.** Cálculo de expresión relativa del gen *hmgb1* con el método de $2^{-\Delta\Delta Ct}$ usando la expresión de *hprt* como constitutivo. Donadores sanos (DS) vs sujetos en riesgo de desarrollar artritis por ser familiares en primer grado de pacientes con artritis reumatoide negativos a ACCP (ACCP-), positivos a ACCP (ACCP+), pacientes con atritis reumatoide temprana, pacientes con artritis reumatoide establecida, la figura muestra mediana y RIQ, Kruskal-Wallis (*p=0.0305*) **B.** Cuantificación de HMGB1 en suero de donadores sanos (DS) vs sujetos en riesgo de desarrollar artritis por ser familiares en primer grado de pacientes con artritis reumatoide negativos a ACCP (ACCP-), positivos a ACCP (ACCP+), pacientes con atritis reumatoide temprana, pacientes con artritis reumatoide establecida, la figura muestra mediana y RIQ, Kruskal-Wallis de prueba (*p=0.0262*) **C.** Concentración de HMGB1 sérica (HMGB1-S) vs concentración de HMGB1 en fluido sinovial, la figura muestra mediana y RIQ, Mann-Whitney (*p=0.496*) **D.** Concentración de HMGB1 en fluido sinovial de enfermedades articulares no inflamatorias (ARTROSIS) vs concentracion de HMGB1 en flido sinovial de pacientes con AR, a figura muestra mediana y

HMGB1 como marcador pronóstico:

Uno de los principales objetivos del tratamiento para los pacientes con AR es llevarlos a una fase de remisión con el fin de evitar la progresión del daño articular o incluso la discapacidad, para ésto se evalúan diversos criterios que permitan diferenciar la presencia o ausencia de actividad, para poder establecer la relación entre la formación de NETs y las características clínicas que se recolectan comunmente en los pacientes se realizó un análisis de correlación en el que se puede observar una correlacción positiva entre la edad de los participantes con la inflamación base evaluado atraves de el conteo de el número de articulaciones doloridas, del dolor base evaluado a través del número de articulaciones doloridas, con los títulos de anticuerpos anti-peptidos carbamilados y los títulos de ACPA, estos últimos además presentan correlación con el factor reumatoide, la velocidad de sedimentación globular (VSG) presenta correlación con los títulos de proteina C reactiva (PCR).

Una de las formas más comunmente usadas para evaluar la actividad de la enfermedad es el uso del DAS28-VSG con el que se toma en cuenta la evaluación concreta de 28 de articulaciones definidas para realizar el cálculo por lo tanto este parámetro correlaciona con la inflamación base y el dolor base como era de esperarse, encontramos que la expresión relactiva de HMGB1 correlaciona con el factor reumatoide y los títulos de ACPA, estos últimos son considerados como los principales marcadores pronósticos de la enfermedad, la concentración de HMGB1 en suero correlaciona con el DAS28_VSG, por lo tanto la expresión relativa y la cuantificación de HMGB1 pudieran ser incluidos como un criterio para la evaluación de la actividad y pronóstico de la AR.

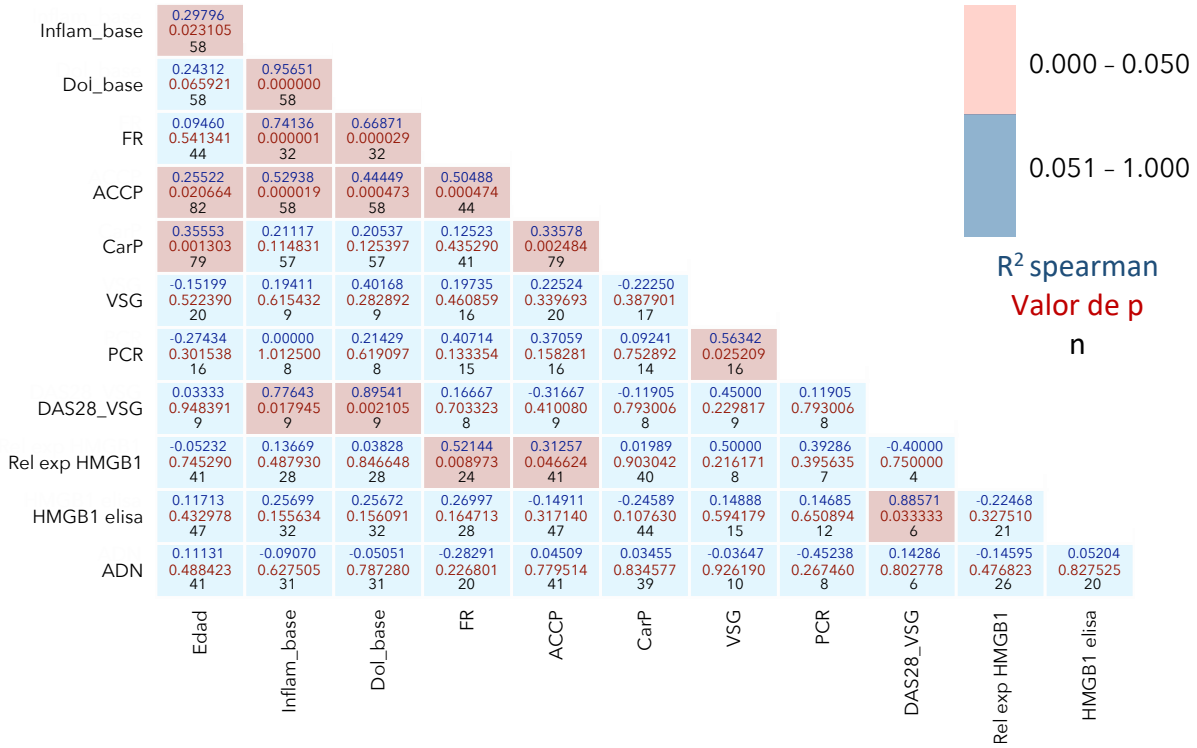

Figura 5. HMGB1 como un marcador pronóstico. Gráfico de correlación de spearman de las características clínicas implicadas en la progresión de la artritis reumatoide, la figura muestra en las celdas color rosa los valores de p menores a 0.05 y en azul todos aquellos que son mayores a 0.051. Los datos estan organizados por colores dentro de cada celda R^2 Spearman, Valor de p y (n) número de la muestra.

CAPÍTULO 5

DISCUSIÓN

Diversos estudios han descrito como el proceso de generación de NETs está implicado en la generación de auto anticuerpos. Sin embargo, las implicaciones de los neutrófilos en la prolongación de la fase inflamatoria se han dejado de lado debido a que la producción de citocinas por parte de estas células es muy limitada. Sin embargo, el gran número de neutrófilos en el tejido sinovial demuestra la participación de los neutrófilos en la patogénesis de la enfermedad [12]. En el presente trabajo planteamos la hipótesis de que HMGB1 puede ser secretada como un evento temprano durante la formación de NETs en respuesta al ambiente pro-inflamatorio caracterizado por la presencia de autoanticuerpos, citocinas proinflamatorias en el suero de los pacientes con AR.

Khandpour et al. describió como los neutrófilos aislados de sangre periférica y de fluido sinovial de pacientes con AR fueron más propensos a formar NETs tanto en la presencia de estímulo como en ausencia de este [16], sin embargo, a este punto no queda claro si la susceptibilidad a la formación de NETs es únicamente en respuesta a los componentes presentes en el suero o si es debido al estado de activación que presentan los neutrófilos de las mujeres [38], ya que recientemente Gupta et al. describió que los neutrófilos presentan diferencias en cuanto al género en su estado de activación [38]. Los datos aquí mostrados hacen evidente que los neutrófilos de donadores sanos del sexo masculino expulsan componentes de las NETs a partir de los 60 min de estímulo en respuesta a los componentes presentes en el SPAR. Como había sido previamente descrito [16]. Sin embargo, el ambiente inflamatorio que contribuye a fomentar la producción de auto anticuerpos contra antígenos citrulinados presentes en dichos sitios no ha sido caracterizado en su totalidad.

En la estructura de las NETs se encuentran diversas moléculas que pueden estar implicadas en la prolongación de la fase inflamatoria, entre ellas HMGB1. Esta es una proteína de origen nuclear que presenta diferentes funciones: 1) en el espacio extracelular puede unirse a diversos receptores como TLRs y RAGE [39, 40]. Nuestros datos demuestran que la dinámica de HMGB1 es dependiente de estímulo, en el caso de los neutrófilos tratados con

SPAR, HMGB1 es secretada al espacio extracelular a partir de los 60 minutos, este fenómeno al parecer es acompañado de la liberación de DNA. Sugiriendo que la activación de dichos receptores extracelulares está mediada por HMGB1 y contribuye de esta manera al proceso inflamatorio sistémico.

Con el fin de evaluar si las características observadas *in vitro*, reflejan una condición fisiológica, es decir si en el suero de los pacientes con AR son capaces de inducir movilización de HMGB1 y liberación del ADN de manera sistémica; se evaluaron las concentraciones de esta molécula en el suero de los pacientes con AR. Hemos demostrado, que los pacientes con AR presentan un incremento en la expresión de HMGB1, sin embargo, al evaluar la proteína no se observó el mismo fenómeno. Esto puede ser debido a que los tratamientos con FARMEs (Fármacos antirreumáticos modificadores de enfermedad) afectan la expresión de HMGB1 en pacientes con AR, como ha sido documentado en el tratamiento con metotrexato [41]. Aunque no se encontró incremento significativo en la concentración sérica de HMGB1, se observa una tendencia al incremento. Estas observaciones no son concordantes con lo previamente reportado [42] y puede deberse a la sensibilidad analítica del kit utilizado (KITs con estatus RUO) o incluso a las características de los mismos pacientes, ya que la mayoría de los pacientes están tratados con FARMEs.

Adicionalmente, y dado que HMGB1 claramente muestra diferencias en su expresión y liberación en células de sujetos expuestos a SPAR y otros inductores de NETs, exploramos la posible utilidad de esta molécula como biomarcador pronostico. Al realizar un análisis de correlación se encontró que existen correlación positiva entre la expresión relativa de HMGB1, el factor reumatoide y los títulos de ACCP. Estos datos sustentan pues, que la formación de NETs esta relacionada con la respuesta inflamatoria a la generación de autoanticuerpos como lo describió previamente Violeta Romero [17] y los títulos de HMGB1 en suero presentan una fuerte relación un uno de los principales marcadores de actividad de la enfermedad como es el DAS28_ESR. Estas observaciones indican que HMGB1 puede ser un importante mediador y un biomarcador en enfermedades reumáticas. Diversos tratamientos pueden afectar la expresión o la actividad de HMGB1 se ha evaluado como un posible blanco terapéutico en enfermedades inflamatorias [43].

CONCLUSIONES

En el presente trabajo hemos descrito la inducción de NETs mediada por las citocinas presentes en el suero de pacientes con AR, la expulsión de HMGB1 al espacio extracelular durante el proceso de inducción. Además de identificar la participación de HMGB1 en la prolongación de la fase inflamatoria de los pacientes con AR y su posible utilidad como un marcador de pronóstico de la enfermedad. Esto es particularmente relevante ya que al encontrarse directamente relacionado a marcadores de actividad de la enfermedad puede ser usado como un blanco terapéutico ya que su expresión se encuentra asociado a las características clínicas de los pacientes.

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ANEXOS

ANEXO 1. Dictamen de aprobación de proyecto investigación "Dinámica de HMGB1 durante la formación de trampas extracelulares de neutrófilos con suero de pacientes con artritis reumatoide" por el Comité de Nacional de Investigación Científica

MÉXICO
GOBIERNO DE LA REPÚBLICA

Dirección de Prestaciones Médicas
Unidad de Educación, Investigación y Políticas de Salud
Coordinación de Investigación en Salud

IMSS

Dictamen de Aprobación

Martes, 28 de agosto de 2018

Ref. 09-B5-61-2800/201800/ 2 2 0 0

Dr. José Antonio Enciso Moreno
Unidad de Investigación Médica Zacatecas (UNIDAD DE INVESTIGACION BIOMEDICA)
Zacatecas

Presente:

Informo a usted que el protocolo titulado: **Dinámica de HMGB1 durante la formación de trampas extracelulares de neutrófilos inducida con suero de pacientes con artritis reumatoide**, fue sometido a la consideración de este Comité Nacional de Investigación Científica.

Los procedimientos propuestos en el protocolo cumplen con los requerimientos de las normas vigentes, con base en las opiniones de los vocales del Comité de Ética en Investgación y del Comité de Investigación del Comité Nacional de Investigación Científica del IMSS, se ha emitido el dictamen de **APROBADO**, con número de registro: R-2018-785-099.

De acuerdo a la normatividad vigente, deberá informar a esta Comité en los meses de enero y julio de cada año, acerca del desarrollo del proyecto a su cargo. Este dictamen sólo tiene vigencia de un año. Por lo que en caso de ser necesario requerirá solicitar una reaprobación al Comité de Ética en Investigación del Comité Nacional de Investigación Científica, al término de la vigencia del mismo.

Atentamente,

Dr. Fabio Salamanca Gómez
Presidente
Comité Nacional de Investigación Científica

Anexo comentarios:
Se anexa dictamen
SNN/ iah. F-CNIC-2018-44

IMSS
SEGURIDAD Y SOLIDARIDAD SOCIAL
4° piso Bloque "B" de la Unidad de Congresos Av. Cuauhtémoc 330 Col. Doctores México 06720 56276900 ext 21210 conis@cis.gob.mx

ANEXO 2. Carta de consentimiento informado "Dinámica de HMGB1 durante la formación de trampas extracelulares de neutrófilos inducida con suero de pacientes con artritis reumatoide"

CARTA DE CONSENTIMIENTO INFORMADO

INSTITUTO MEXICANO DEL SEGURO SOCIAL
UNIDAD DE EDUCACIÓN, INVESTIGACIÓN Y POLÍTICAS DE SALUD
COORDINACIÓN DE INVESTIGACIÓN EN SALUD
CARTA DE CONSENTIMIENTO INFORMADO
(Adultos)

"Dinámica de HMGB1 durante la formación de trampas extracelulares de neutrófilos inducida con suero de pacientes con artritis reumatoide"

Lugar:_____Fecha:_____
Número de registro del proyecto: **R-2018-785-099**
Justificación y objetivo del estudio: La identificación de biomarcadores moleculares (sustancias en la sangre) en individuos con riesgo de desarrollar artritis reumatoide, antes de que presenten síntomas de la enfermedad, puede permitir un tratamiento más oportuno y aumentar el porcentaje curación con el tratamiento convencional, e incluso probar tratamientos de prevención. La función de varias sustancias como HMGB1 en la artritis reumatoide no ha sido claramente definido ni su relación con la presencia de anticuerpos como los ACPA. El presente proyecto pretende evaluar la movilización intra y extracelular de HMGB1 durante la formación de trampas extracelulares de neutrófilos inducida con suero de pacientes con artritis reumatoide y asociar la concentración plasmática de HMGB1 con la de ACPAs en pacientes con artritis reumatoide.
Procedimientos:
Usted ha sido invitado a participar en este estudio en el hospital general Emilio Varela Luján del IMSS en Zacatecas o en el hospital # 51 de Villanueva Zacatecas (IMSS-Prospera). Si acepta, el Dr. Martín Zapata Zúñiga le realizará una evaluación clínica y le programará la toma de muestras de sangre (aproximadamente 20 mililitros, el equivalente a dos cucharadas). Se hará un análisis de laboratorio para medir los biomarcadores moleculares (sustancias en la sangre). El resto de la muestra (aproximadamente 10 mililitros) será congelada y conservada en el laboratorio. Podemos solicitarle en función de los resultados de sus análisis de laboratorio que se le tomara una muestra adicional de sangre (otros 10 mililitros) en los meses posteriores. El tiempo que tomará su evaluación clínica y la toma de sangre será de 30 a 35 minutos en cada una de las visitas.
Las muestras de sangre se mantendrán congeladas y almacenadas hasta su uso para las pruebas de laboratorio por un periodo no mayor a 10 años. Se les pondrá una etiqueta con un código para mantener su anonimato. En caso de que usted lo solicite, sus muestras serán destruidas antes del tiempo estipulado.
Para el caso de los pacientes con AR que asisten a cirugía de rodilla (si es su caso) adicionalmente se les solicitará que nos sea donado el tejido que es retirado durante dicha cirugía. Dichos tejidos y fluido sinovial usualmente son desechados por parte del hospital. Por lo que solo necesitamos su consentimiento para conservarlo en congelación (Una pequeña parte del tejido que es retirado durante la cirugía 1 cm^2 aproximadamente).

Posibles riesgos y molestias:
Las molestias o riesgos asociados con los procedimientos de evaluación clínica (medición de peso, talla, cintura, tensión arterial, etc.) son mínimos. Se trata de estudios clínicos no invasivos que no ocasionan dolor, incomodidad o riesgo alguno.
Las molestias durante la toma de muestra de sangre son mínimas. En algunas ocasiones el procedimiento puede causar un poco de dolor o es posible que se le pueda formar un moretón. Para reducir esas molestias, la toma de sangre se hará por personal capacitado.
Para el caso de la remoción de tejido y fluido sinovial, únicamente las inherentes al procedimiento quirúrgico que le fue programado.
Posibles beneficios que recibirá al participar en el estudio:
Este estudio no implica gasto alguno para usted, y tampoco recibirá un pago por su participación. Usted recibirá una evaluación clínica gratuita y los resultados de las pruebas de laboratorio antes mencionadas. Un posible beneficio de su participación en este estudio es que le proporcionaremos información sobre su estado de salud.
Si bien los beneficios directos para usted pudieran no existir, los resultados del presente estudio contribuirán a la identificación de la función de moléculas relevantes para el desarrollo de la artritis reumatoide.

Información sobre resultados y alternativas de tratamiento:

Durante el transcurso de este estudio, le informaremos de cualquier hallazgo nuevo (ya sea bueno o malo), que sea importante para la decisión de participar o continuar participando en este estudio. Los resultados de sus muestras de sangre estarán disponibles si usted los solicita y le serán proporcionados por un médico especialista en estas enfermedades.

Participación o retiro:

Su participación en este estudio es completamente voluntaria. Si usted decide no participar, seguirá recibiendo la atención médica brindada por el IMSS si es derechohabiente, o por alguna otra institución del Sector Salud a la cual pertenezca. Si usted no desea participar en el estudio, su decisión, no afectará ni su relación con el IMSS o con ninguna otra institución del Sector Salud, ni su derecho a obtener los servicios de salud que reciba en ese momento. Si en un principio desea participar y posteriormente cambia de opinión, usted puede abandonar el estudio en cualquier momento y solicitar que su muestra de sangre que se tiene almacenada sea destruida. El abandonar el estudio no modificará de ninguna manera los beneficios que usted tiene como derechohabiente del IMSS o de otra institución a la cual esté afiliado.

Privacidad y confidencialidad:

Para garantizar su privacidad, la información que nos proporcione para identificarla (o) (como su nombre, teléfono y dirección) será guardada de manera confidencial y por separado al igual que sus respuestas a los cuestionarios y los resultados de sus pruebas clínicas. Las muestras que nos proporcione serán almacenadas en forma codificada, bajo el resguardo de personal de la Unidad de Investigación Biomédica de Zacatecas del IMSS. El equipo de investigadores, su médico y las personas que estén involucradas en el cuidado de su salud sabrán su participación en este estudio. Sin embargo, nadie más tendrá acceso a la información que usted nos proporcione durante su participación en este estudio, al menos que usted así lo desee. Sólo proporcionaremos su información si fuera necesario para proteger sus derechos o su bienestar (por ejemplo, si llegara a sufrir algún daño físico o si llegara a necesitar cuidados de emergencia), o si lo requiere la ley.

Cuando los resultados de este estudio sean publicados o presentados en conferencias, por ejemplo, no se dará información que pudiera revelar su identidad. Su identidad será protegida y resguardada. Para proteger su identidad le asignaremos un número que utilizaremos para identificar sus datos, y usaremos ese número en lugar de su nombre en nuestras bases de datos.

Autorización para conservar la muestra de sangre:

☐ Autorizo que se tome la muestra solo para este estudio.

☐ Autorizo que mis muestras sean guardadas en congelación por un periodo no mayor a 10 años y sean utilizadas para estudios futuros.

Beneficios al término del estudio: Se otorgará a los participantes la evaluación clínica relacionada con Artritis Reumatoide y en caso presentar síntomas de la enfermedad, se les atenderá para iniciar tratamiento inmediato en la Unidad de Artritis y Reumatismo o si lo serán referidos al médico de su preferencia.

En caso de dudas o aclaraciones relacionadas con el estudio podrá dirigirse a:

Investigador Clínico: Dr. Martín Zapata Zuñiga. Hospital Rural # 51 Villanueva Zacatecas. Dr. Cesar Ramos Remus. Unidad de Artritis y Reumatismo Guadalajara Jalisco.

Investigador Biomédico: Dr. José Antonio Enciso Moreno y Dr. Julio Enrique Castañeda Delgado, que es el investigador responsable del estudio, a los teléfonos: 01 492 92 2 60 19, en la Unidad de Investigación Biomédica de Zacatecas ubicada en Interior Alameda número 45, Col. Quebradilla, Zacatecas, Centro , C.P. 98000, Zacatecas, México.

En caso de dudas o aclaraciones sobre sus derechos como participante podrá dirigirse a: Comisión de Ética de Investigación de la CNIC del IMSS: Avenida Cuauhtémoc 330 4° piso Bloque “B” de la Unidad de Congresos, Col. Doctores. México, D.F., CP 06720. Teléfono (55)56 27 69 00 extensión 21230, Correo electrónico: comiteeticainv.imss@gmail.com

_____

Nombre y firma del sujeto

_____

Nombre y firma de quien obtiene el consentimiento

Testigo 1

Nombre: _____

Dirección: _____

Relación: _____

Firma: _____

Testigo 2

Nombre: _____

Dirección: _____

Relación: _____

Firma: _____

ANEXO 3. Carta de consentimiento informado “Identificación de biomarcadores asociados al desarrollo de artritis reumatoide temprana en humanos”

CARTA DE CONSENTIMIENTO INFORMADO PACIENTES
INSTITUTO MEXICANO DEL SEGURO SOCIALUNIDAD DE EDUCACIÓN, INVESTIGACIÓN Y POLÍTICAS DE SALUD COORDINACIÓN DE INVESTIGACIÓN EN SALUD CARTA DE CONSENTIMIENTO INFORMADO (ADULTOS)

CARTA DE CONSENTIMIENTO INFORMADO PARA PARTICIPACIÓN EN PROTOCOLOS DE INVESTIGACIÓN

IDENTIFICACIÓN DE BIOMARCADORES ASOCIADOS AL DESARROLLO DE ARTRITIS REUMATOIDE TEMPRANA EN HUMANOS

Lugar_____ Fecha: _____

Número de registro: 2013-785-009.

Justificación y objetivo del estudio: La identificación de biomarcadores moleculares para identificar individuos con alto riesgo de desarrollar artritis reumatoide (AR), antes de que la enfermedad inicie, permitiría dar un tratamiento más oportuno y por lo tanto aumentar el porcentaje de éxito con la terapia, e incluso eventualmente probar tratamientos profilácticos para evitar la AR. El objetivo de este estudio es evaluar los perfiles transcripcionales asociados al inicio de la AR en sujetos sanos con alto riesgo de desarrollar la enfermedad, cuyo propósito final es la identificación de biomarcadores en sangre que puedan ser útiles en el desarrollo de un método pronóstico temprano para identificar conversión en sujetos con alto riesgo a desarrollar AR.

Procedimientos:Si usted acepta participar ocurrirá lo siguiente:

Durante su cita al servicio de reumatología en el Hospital General de Zona 1- de Zacatecas para su evaluación clínica, se le invitará a participar en el estudio y en caso de aceptar, se le realizará una toma de sangre para la medición de anticuerpos anti-CCP, para IgM-factor reumatoide y medición de velocidad de sedimentación globular. A usted se le tomara una muestra de sangre periférica de 15 ml para la medición de los parámetros antes mencionados y obtención de suero, y 5 ml que serán almacenados en tubos PaxGene (Scanalitics USA) o RNA latter (Ambion) a -70 C para la obtención de material genético.

Se les dará seguimiento durante los primeros 4 meses de su tratamiento con fármacos modificadores de la enfermedad (FARMES) realizando tomas de sangre para su evaluación. Nos tardaremos aproximadamente 5 minutos en la toma de muestras y aproximadamente media hora en su evaluación clínica.

El propósito de realizarle los estudios clínicos y de laboratorio esta contemplado dentro del esquema regular de seguimiento clínico que realiza el servicio de reumatología, para saber más sobre sus condiciones asociadas a AR. Le entregaremos los resultados de anticuerpos y de laboratorio clínico en aproximadamente 72 horas posteriores a la toma de muestras.

Posibles riesgos y molestias:Las molestias o riesgos asociados con los procedimientos de evaluación clínica (medición de peso, talla, cintura, tensión arterial, etc.) son mínimos. Se trata de estudios clínicos no invasivos que no ocasionan dolor, incomodidad o riesgo alguno. Las molestias durante la toma de muestra de sangre son mínimas. En algunas ocasiones el procedimiento para tomarle una muestra de sangre puede causar un poco de dolor o una discreta molestia, es posible que se le pueda formar un moretón

Posibles beneficios que recibirá al participar en el estudio:

No recibirá un pago por su participación en este estudio, ni este estudio implica gasto alguno para usted. No recibirá ningún beneficio directo al participar en este estudio. Un posible beneficio de su participación en este estudio es que los resultados de las pruebas clínicas y de laboratorio que le realizaremos le proporcionarán información sobre su estado de salud. El conocer sobre su estado de salud pudiera ser un beneficio para usted. Si bien los beneficios directos para usted pudieran no existir, los resultados del presente estudio contribuirán a la identificación de biomarcadores moleculares asociados al desarrollo de ARt en familiares consanguíneos de pacientes con AR, los cuales en su momento pueden servir como plataforma para el desarrollo de un método pronostico de ARt.

Información sobre resultados y alternativas de tratamiento:

Durante el transcurso de este estudio, le informaremos de cualquier hallazgo nuevo (ya sea bueno o malo) que sea importante para la decisión de participar o continuar participando en este estudio; por ejemplo, si hubiera cambios en los riesgos o beneficios por su participación en esta investigación o si hubieran nuevas alternativas de tratamiento que pudieran cambiar su opinión sobre su participación en este estudio. Si le llegamos a proporcionar información nueva, nuevamente le solicitaremos su consentimiento para seguir participando en este estudio.

Participación o retiro: Su participación en este estudio es completamente voluntaria. Si usted decide no participar, seguirá recibiendo la atención médica brindada por el IMSS. En caso de ser derechohabiente del IMSS, se le ofrecerán los procedimientos establecidos dentro de los servicios establecidos de atención médica. Es decir, que, si usted no desea participar en el estudio, su decisión, no afectará su relación con el IMSS o con ninguna otra institución del Sector Salud y su derecho a obtener los servicios de salud que reciba en ese momento. Si en un principio desea participar y posteriormente

cambia de opinión, usted puede abandonar el estudio en cualquier momento. El abandonar el estudio en momento que quiera no modificará de ninguna manera los beneficios que usted tiene como derechohabiente del IMSS.
Privacidad y confidencialidad:La información que nos proporcione que pudiera ser utilizada para identificarla/o (como su nombre, teléfono y dirección) será guardada de manera confidencial y por separado al igual que sus respuestas a los cuestionarios y los resultados de sus pruebas clínicas, para garantizar su privacidad.
El equipo de investigadores, su médico en el Servicio de Reumatología del HGZ1 del IMSS en Zacatecas y las personas que estén involucradas en el cuidado de su salud sabrán que usted está participando en este estudio. Sin embargo, nadie más tendrá acceso a la información que usted nos proporcione durante su participación en este estudio, al menos que usted así lo desee. Sólo proporcionaremos su información si fuera necesario para proteger sus derechos o su bienestar (por ejemplo, si llegara a sufrir algún daño físico o si llegara a necesitar cuidados de emergencia), o si lo requiere la ley.
Cuando los resultados de este estudio sean publicados o presentados en conferencias, por ejemplo, no se dará información que pudiera revelar su identidad. Su identidad será protegida y ocultada. Para proteger su identidad le asignaremos un número que utilizaremos para identificar sus datos, y usaremos ese número en lugar de su nombre en nuestras bases de datos.
En caso de colección de material biológico (si aplica):

☐ No autorizo que se tome la muestra.

☐ Si autorizo que se tome la muestra solo para este estudio

☐ Si autorizo que se tomen las muestras para este estudio y estudios futuros

☐ Si autorizo a que mis muestras puedan ser guardadas en congelación por un periodo no mayor a 10 años y que luego sean destruidas.

Beneficios al término del estudio: Se otorgará a los participantes la evaluación clínica relacionada con Artritis Reumatoide y se le atenderá para iniciar tratamiento inmediato.
En caso de dudas o aclaraciones relacionadas con el estudio podrá dirigirse a: **Investigador Responsable**: Dr. JOSE ANTONIO ENCISO MORENO, que es el investigador responsable del estudio, a los teléfonos: 01 492 92 2 60 19, en la Unidad de Investigación Médica-Zacatecas ubicada en Interior Alameda número 45, Col. Quebradilla. **Investigador Clínico** Pedro Martínez Tejada Servicio de Reumatología del HGZ1 de Zacatecas Domicilio: Interior de Alameda # 45, Col. Centro, Zacatecas, Zac, México,C.P. 9800. Teléfono: 01 (492) 922 60 19.
En caso de dudas o aclaraciones sobre sus derechos como participante podrá dirigirse a: Comisión de Ética de Investigación de la CNIC del IMSS: Avenida Cuauhtémoc 330 4° piso Bloque “B” de la Unidad de Congresos, Col. Doctores. México, D.F., CP 06720. Teléfono (55)56 27 69 00 extensión 21230, Correo electrónico: conise@cis.gob.mx

_____
Nombre y firma del sujeto

_____
Nombre y firma de quien obtiene el consentimiento

Testigo 1
Nombre: _____
Dirección: _____
Relación: _____

Firma: _____

Testigo 2
Nombre: _____
Dirección: _____
Relación: _____
Firma: _____

ANEXO 4. Dictamen de procedencia de artículo de investigación

Doctorado en Ciencias Básicas
Universidad Autónoma de Zacatecas
"Francisco García Salinas"

Dictamen de procedencia de artículo de investigación.

Nombre del Estudiante: __**Pasante de Doctora en Ciencias Básicas, Fátima de Lourdes Ochoa González**

Nombre del manuscrito/artículo de investigación: ***"Calpain Participates in Cortical Cytoskeleton Modification and DNA Release during Neutrophil Extracellular Trap Formation"***

¿El estudiante aparece como primer autor?: Si__X__ No_____.

¿Cuántos autores tiene el artículo?: __9__.

¿Aparece algún otro estudiante del programa?: *No*.

¿Los resultados reportados en el artículo de investigación están claramente relacionados con el tema de tesis doctoral? Si:__X__, No:_____.

Estatus actual: Aceptado_ __ o Publicado _X_ (ePub).

Si es aceptado anexar carta de aceptación y caratula del manuscrito, en caso de ser publicado indicar: Volumen___, Pagina__1-11__, año _2021_ y DOI: **10.1159/000515201**

Respecto de la revista:

Nombre de la revista: _***International Archives of Allergy and Immunology***_____
ISSN:_ **1423-0097**__, Factor de impacto (InCites JCR):_2.917 _, SJR cuartil:__1___
Revista indexada: Scopus (Elsevier):__X___, Thomson:__X___, Otro catalogo de indexación (Indique cual): PubMed, MEDLINE, US National Library of Medicine (Web of Science); Science Citation Index, Science Citation Index Expanded, Current Contents Life Sciences, BIOSIS Previews (Clarivate Analytics); Google Scholar (Google); Chemical Abstracts Service (American Chemical Society); FSTA: Food Science and Technology Abstracts (IFIS); CAB Abstracts, Global Health (CABI); Embase, Pathway Studio, CABS (Elsevier); Academic Search, STM Source (EBSCO), Health Research Premium Collection, Medical Database, Health & Medical Collection, ProQuest Central (ProQuest), WorldCat (OCLC).

Comité de validación (CADO Ciencias Biológicas, DCB, UAZ)

	Nombre	Firma
Integrantes del CADO de ...	Dra. Melina Del Real Monroy	
	Dr. Rogelio Rosas Valdez	
	Dra. Lucía Delgadillo Ruíz	

Observaciones:
El artículo se encuentra asociado al Capítulo 3 del documento de tesis de la alumna.
Incluir una copia del artículo en la tesis, esto como un anexo.

Dictamen final (Aprobado/No Aprobado): Aprobado

Asesor

Dr. Jesús Adrián López

Vo. Bo. Coordinador Académico
del Doctorado en Ciencias Básicas

Juan Carlos Mtz

Dr. Juan Carlos Martínez Orozco

ARTÍCULOS PUBLICADOS

Analysis of miRNA expression in patients with rheumatoid arthritis during remission and relapse after a 5-year trial of tofacitinib treatment

International Immunopharmacology 63 (2018) 35–42

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Preliminary report

Analysis of miRNA expression in patients with rheumatoid arthritis during remission and relapse after a 5-year trial of tofacitinib treatment

Julio C. Fernández-Ruiz[a,b], Cesar Ramos-Remus[c], José Sánchez-Corona[d], José D. Castillo-Ortiz[e], José J. Castañeda-Sánchez[e], Yadira Bastian[a,f], María F. Romo-García[a,b], Fátima Ochoa-González[a], Adriana E. Monsivais-Urenda[b], Roberto González-Amaro[b], José A. Enciso-Moreno[a], Julio E. Castañeda-Delgado[a,f,*]

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ARTICLE INFO

Keywords:
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Tofacitinib
JAK
STAT
Firefly Bioworks

ABSTRACT

The physiopathology of rheumatoid arthritis (RA) is mediated by proinflammatory cytokines, some of which are regulated by the JAK/STAT pathway. Tofacitinib is a JAK inhibitor, but its role in the regulation of microRNAs (miRNAs) is unknown. There is also no information regarding the role of miRNAs in the clinical relapse/remission of RA. The present project aims to identify a signature profile of miRNA expression in a subgroup of RA patients who had to discontinue tofacitinib treatment (because of the ending of a 5-year open-label clinical trial) and to describe the expression of miRNAs during RA remission or flare-up. The relative expression of 61 miRNAs was determined in serum samples with the Firefly™ BioWorks assay. Statistical analysis was performed by means of Student's *t*-test and heatmap analysis was performed with Firefly™ Analysis Workbench software and in the software GraphPad® Prism v5.0. Target prediction and Gene Ontology analysis were carried out using bioinformatic tools. We found a distinctive signature of miRNA expression associated with relapse, featuring upregulated expression of hsa-miR-432-5p ($p < 0.05$). We also found upregulation of hsa-miR-194-5p ($p < 0.05$) in samples of patients with RA flare-up. Gene Ontology analysis of the target genes for hsa-miR-432-5p was performed to identify relevant pathways associated with relapse; the implications of these pathways in the physiopathology of RA are discussed. Tofacitinib treatment does not have a direct effect on the expression of measured miRNAs. The changes in hsa-miR-432-5p and hsa-miR-194-5p are associated with the regulation of proinflammatory pathways and RA flare-up.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory and autoimmune disease that affects joints and other systems [40]. The etiology of RA is multifactorial, including genetic and environmental factors (air pollution, infections, etc.). RA predominantly affects women of reproductive age and is estimated to affect about 1% of the world's population [41].

The physiopathological mechanisms of RA are mediated by proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), granulocyte-colony stimulating factor (GM-CSF), interleukin (IL)-6, IL-1β and IL-17, among others [7, 18]; the intracellular signaling of these cytokines is mediated through the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathway. When a cytokine binds to its receptor, it leads to the tyrosine phosphorylation of associated JAKs and to the recruitment, phosphorylation, and dimerization of STATs, which translocate to the nucleus and promote or regulate the transcription of target genes related to inflammation [1, 35].

The therapeutic arsenal in rheumatology has improved drastically

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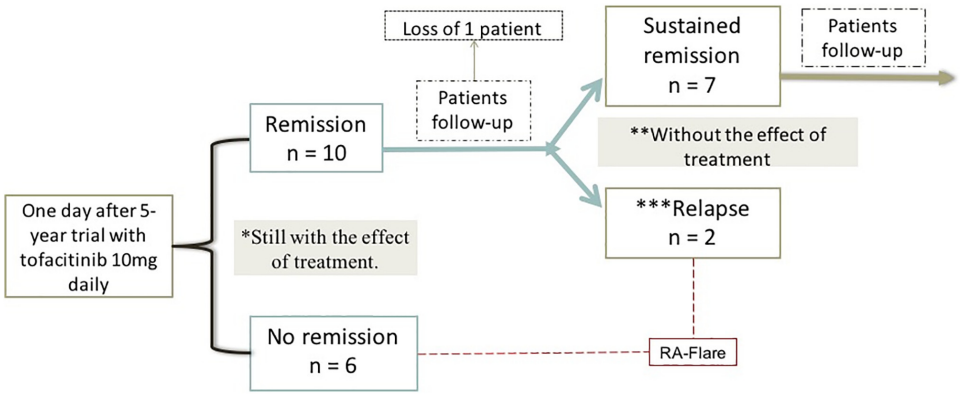

Fig. 1. Diagram of patient follow-up and sample collection (*first sample collection, **second sample collection, ***at least one inflamed joint).

during the last 20 years with the availability of biological and target synthetic disease-modifying anti-rheumatic drugs (bDMARDs and tsDMARDs). Recently, other approaches have been explored, particularly the inhibitors of JAK kinases such as tofacitinib (TOFA), a new kind of tsDMARD. TOFA was recently approved for the treatment of RA in several countries [11, 34, 39]. Smolen et al. found that TOFA was effective at inducing remission after 3 months of treatment using various established and new remission criteria; the remission rates were generally greater with TOFA at 10 mg twice a day [39]. It is known that patients with RA who are in remission can suffer relapse after the suspension of any of the DMARDs; however, the causes of this are unknown, although several risk factors for relapse have been identified [6, 15, 32]. Moreover, little information on the molecular mechanisms involved in RA relapse has been obtained.

There are natural mechanisms that regulate important molecules of signaling pathways, as well as the expression of pro- and anti-inflammatory cytokines. One of the components in these mechanisms is the microRNAs (miRNAs). miRNAs are small noncoding RNA molecules that bind target messenger RNA (mRNA) to negatively regulate the translation of genes involved in the immune system, such as those encoding cytokines, chemokines, and signaling molecules [3, 23]. The changes in the expression of miRNAs and the regulation of their targets during periods of remission and relapse of RA are unknown; how these miRNAs change upon TOFA treatment is also unclear.

Current treatment guidelines clearly define the criteria for using bDMARDs or tsDMARDs for RA treatment; however, there are no current guidelines available as to when to stop therapy. Some studies have described certain clinical variables that can predict relapse, such as methotrexate (MTX) discontinuation, basal anti-citrullinated protein antibody (ACPA) levels, and high rheumatoid factor (RF) [6, 15, 32]. The present project aims to find a signature profile of the miRNAs in a subgroup of RA patients who had to discontinue TOFA because of the ending of a 5-year open-label clinical trial and to describe the expression of miRNAs during RA remission or flare-up.

2. Materials and methods

2.1. Study design

This was a prospective cohort study of patients with RA assembled at the last TOFA intake because of the ending of an open-label, industry-sponsored, long-term extension trial (study number A3921024) conduced in a single research center. All patients included in the study had a failure to MTX treatment. None of them had any previous use of bDMARDs.

2.2. Patients

All patients were enrolled in an open-label extension study using TOFA (10 mg per day) for 5 years at one participating center in Mexico. Patients received treatment with MTX (10 mg per day) during the open-label study. The remission criterion was that, at the last TOFA dose, all patients had a 28-Joint Disease Activity Score (DAS28) < 2.6 and no swollen joints. The inclusion criteria were: patients who failed MTX therapy, patients naive to treatment with bDMARDs and patients with 18 years and older. The exclusion criterion was: subjects with serious medical conditions that would make treatment with TOFA potentially unsafe such as infections.

2.3. Procedures and follow-up

After the end of the extension study, patients no longer received TOFA but continued with MTX at the same dose as before. Given the elimination kinetics of TOFA, serum levels of the drug are detectable 30 days after stopping therapy [17]. We obtained a blood sample at the first month after the last dose of TOFA in order to describe the levels of miRNAs associated with treatment and also to monitor the changes in miRNAs associated with relapse or RA flare-up. Each patient was instructed to communicate with our center if they believed that they had a swollen joint; RA flare-up was confirmed by in-office assessment by the attending rheumatologist. In cases with confirmed RA relapse, a blood sample was taken and the occasion was considered as the last visit for the follow-up (Fig. 1).

2.4. Analysis of miRNA expression by flow cytometry using firefly™ BioWorks technology

We designed a panel of 61 miRNAs to be analyzed in the patients' samples, in which miRNAs related to immune response, inflammatory response, JAK/STAT pathway, and RA were included (Table 1). Measurements were performed using the Firefly™ Circulating miRNA Assay (Abcam, Cambridge, MA) [8]. In general, the protocol consists of adding 40 µl of serum to each well and miRNAs hybridizing to complementary oligonucleotide sequences covalently attached to encoded hydrogel microparticles for target capture. The bound target was ligated to oligonucleotide adapter sequences that served as universal PCR priming sites. The miRNA–adapter hybrid molecules were then dehybridized from the particles and reverse-transcription polymerase chain

Table 1
61plex custom panel microRNA list.

microRNA	Accession number (miRBASE)	Sequence
hsa-miR-432-5p	MIMAT0002814	UCUUGGAGUAGGUCAUUGGGUGG
hsa-miR-424-5p	MIMAT0001341	CAGCAGCAAUUCAUGUUUUGAA
hsa-miR-19a-5p	MIMAT0004490	AGUUUUGCAUAGUUGCACUACA
hsa-miR-19a-3p	MIMAT0000073	UGUGCAAAUCUAUGCAAAACUGA
hsa-miR-21-5p	MIMAT0000076	UAGCUUAUCAGACUGAUGUUGA
hsa-miR-21-3p	MIMAT0004494	CAACACCAGUCGAUGGGCUGU
hsa-miR-155-5p	MIMAT0000646	UUAAUGCUAAUCGUGAUAGGGGU
hsa-miR-155-3p	MIMAT0004658	CUCCUACAUAUUAGCAUUAACA
hsa-miR-216a-5p	MIMAT0000273	UAAUCUCAGCUGGCAACUGUGA
hsa-miR-23a-5p	MIMAT0004496	GGGGUUCCUGGGGAUGGGAUUU
hsa-miR-23b-5p	MIMAT0004587	UGGGUUCCUGGCAUGCUGAUUU
hsa-miR-373-5p	MIMAT0000725	ACUCAAAAUGGGGGCGCUUUCC
hsa-miR-520a-5p	MIMAT0002833	CUCCAGAGGGAAGUACUUUCU
hsa-let-7c-5p	MIMAT0000064	UGAGGUAGUAGGUUGUAUGGUU
hsa-let-7c-3p	MIMAT0026472	CUGUACAACCUUCUAGCUUUCC
hsa-miR-145-5p	MIMAT0000437	GUCCAGUUUUCCCAGGAAUCCCU
hsa-miR-146a-5p	MIMAT0000449	UGAGAACUGAAUUCCAUGGGUU
hsa-miR-146a-3p	MIMAT0004608	CCUCUGAAAUUCAGUUCUUCAG
hsa-miR-146b-5p	MIMAT0002809	UGAGAACUGAAUUCCAUAGGCU
hsa-miR-146b-3p	MIMAT0004766	UGCCCUGUGGACUCAGUUCUGG
hsa-miR-223-5p	MIMAT0004570	CGUGUAUUUGACAAGCUGAGUU
hsa-miR-34b-5p	MIMAT0000685	UAGGCAGUGUCAUUAGCUGAUUG
hsa-miR-125b-5p	MIMAT0000423	UCCCUGAGACCCUAACUUGUGA
hsa-miR-939-5p	MIMAT0004982	UGGGGAGCUGAGGCUCUGGGGGUG
hsa-miR-7977	MIMAT0031180	UUCCCAGCCAACGCACCA
hsa-miR-1202	MIMAT0005865	GUGCCAGCUGCAGUGGGGGAG
hsa-miR-6085	MIMAT0023710	AAGGGGCUGGGGGAGCACA
hsa-miR-5739	MIMAT0023116	GCGGAGAGAGAAUGGGGAGC
hsa-miR-27b-3p	MIMAT0000419	UUCACAGUGGCUAAGUUCUGC
hsa-miR-194-5p	MIMAT0000460	UGUAACAGCAACUCCAUGUGGA
hsa-miR-24-3p	MIMAT0000080	UGGCUCAGUUCAGCAGGAACAG
hsa-miR-23b-3p	MIMAT0000418	AUCACAUUGCCAGGGAUUACC
hsa-miR-6826-5p	MIMAT0027552	UCAAUAGGAAAGAGGUGGGACCU
hsa-miR-199a-3p	MIMAT0000232	ACAGUAGUCUGCACAUUGGUUA
hsa-miR-192-5p	MIMAT0000222	CUGACCUAUGAAUUGACAGCC
hsa-miR-128-3p	MIMAT0000424	UCACAGUGAACCGGUCUCUUU
hsa-miR-215-5p	MIMAT0000272	AUGACCUAUGAAUUGACAGAC
hsa-miR-6749-5p	MIMAT0027398	UCGGGCCUGGGGUUGGGGGAGC
hsa-miR-4286	MIMAT0016916	ACCCCACUCCUGGUACC
hsa-miR-195-5p	MIMAT0000461	UAGCAGCACAGAAAUAUUGGC
hsa-miR-126-3p	MIMAT0000445	UCGUACCGUGAGUAAUAAUGCG
hsa-miR-517a-3p	MIMAT0002852	AUCGUGCAUCCCUUUAGAGUGU
hsa-miR-30c-5p	MIMAT0000244	UGUAAACAUCCUACACUCUCAGC
hsa-miR-148a-3p	MIMAT0000243	UCAGUGCACUACAGAACUUUGU
hsa-miR-29a-3p	MIMAT0000086	UAGCACCAUCUGAAAUCGGUUA
hsa-miR-1-3p	MIMAT0000416	UGGAAUGUAAAGAAGUAUGUAU
hsa-let-7e-5p	MIMAT0000066	UGAGGUAGGAGGUUGUAUAGUU
hsa-miR-4634	MIMAT0019691	CGGCGCGACCGGCCCGGGG
hsa-miR-6165	MIMAT0024782	CAGCAGGAGGUGAGGGGAG
hsa-miR-378a-3p	MIMAT0000732	ACUGGACUUGGAGUCAGAAGGC
hsa-miR-30a-5p	MIMAT0000087	UGUAAACAUCCUCGACUGGAAG
hsa-miR-762	MIMAT0010313	GGGGCUGGGGCCGGGGCCGAGC
hsa-miR-124-3p	MIMAT0000422	UAAGGCACGCGGUGAAUGCC
hsa-miR-516a-5p	MIMAT0004770	UUCUCGAGGAAAGAAGCACUUUC
hsa-miR-9-3p	MIMAT0000442	AUAAAGCUAGAUAACCGAAAGU
hsa-miR-15b-5p	MIMAT0000417	UAGCAGCACAUCAUGGUUUACA
hsa-miR-221-5p	MIMAT0004568	ACCUGGCAUACAAUGUAGAUUU
hsa-miR-203a-5p	MIMAT0031890	AGUGGUUCUUAACAGUUCAACAGUU
hsa-miR-29b-3p	MIMAT0000100	UAGCACCAUUUGAAAUCAGUGUU
hsa-miR-221-3p	MIMAT0000278	AGCUACAUUGUCUGCUGGGUUUC
hsa-miR-203a-3p	MIMAT0000264	GUGAAAUGUUUAGGACCACUAG

reaction (RT-PCR) was performed using a fluorescent forward primer. Once amplified, the fluorescent target was rehybridized to the original capture particles and scanned on a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA).

2.5. Analysis of differential expression of miRNAs

Once acquired, the FCS files were exported and analyzed in the Firefly™ Analysis Workbench. The software requires a plex file to instruct it, the particle code of which corresponds to each miRNA. Data processing, analysis, and visualization were performed with this software. The software was used to compare data quality, and to perform image processing, normalization, and transformation to log ratio for each probe. The bar graphs and heatmap were obtained using GraphPad® Prism v5.0 (GraphPad Software Inc., San Diego, CA) and Firefly™ Analysis Workbench.

2.6. Target prediction and Gene Ontology analysis using bioinformatic tools

The targets of miRNAs differentially expressed between groups were predicted in miRWalk 2.0, a comprehensive database on miRNAs, which hosts predicted as well as validated miRNA binding site information on all known human genes [10]. For the identified target genes, Gene Ontology (GO) analysis and functional classification were carried out in the PANTHER Classification System where *p*-values were adjusted for multiple testing [29, 30].

2.7. Statistical analysis

Normalization of the data was performed with the geNorm algorithm described by Vandesompele [42] included in the Firefly™ Analysis Workbench. Student's *t*-test was performed to determine whether there were significant differences between the groups. In addition, a cluster analysis was carried out and a heatmap was constructed for the miRNAs that were shared between groups (for the samples in relapse). All of the data were analyzed in the software GraphPad® Prism v5.0 and Firefly™ Analysis Workbench.

3. Results

3.1. Clinical features and general characteristics of the patients

Sixteen patients participated in this study, 90% were female, their mean age was 46.25 years at baseline, and 10 patients achieved remission after finishing treatment with TOFA, of the total population we make a sub classification according to the characteristics shared by the patients through the follow-up (Table 2).

3.2. Downregulation of hsa-miR-432-5p in patients achieving clinical remission

To assess the change in miRNA expression of RA patients in and not in remission when finished TOFA treatment, we compared the changes in the expression of the 61 miRNAs measured by the Firefly™ assay and found statistically significant differences ($p < 0.05$) in the expression of hsa-miR-432-5p, with this miRNA being upregulated in those

Table 2
Demographic and clinical features.

Number of patients in the study	16
Number of patients in remission after treatment with tofacitinib	10
Number of patients in relapse after follow-up	2
Patient lost to follow-up	1
Number of patients in sustained remission after follow-up	7
Number of patients with Flare-Up condition[a]	8
Age at diagnosis (years)[b]	35.44 ± 11.2
Age at the time of taking the first sample (years)[b]	46.25 ± 13.64
Diagnosis delay (month)[b]	6.4 ± 10.7
Disease duration (years)[b]	12.7 ± 7.2
Gender (M:F)	1:15
Education (years)[b]	8.75 ± 4.65

M = Male, F = Female.
[a] Includes patients who never achieved remission and the patients in relapse.
[b] Values are expressed as mean ± SD.

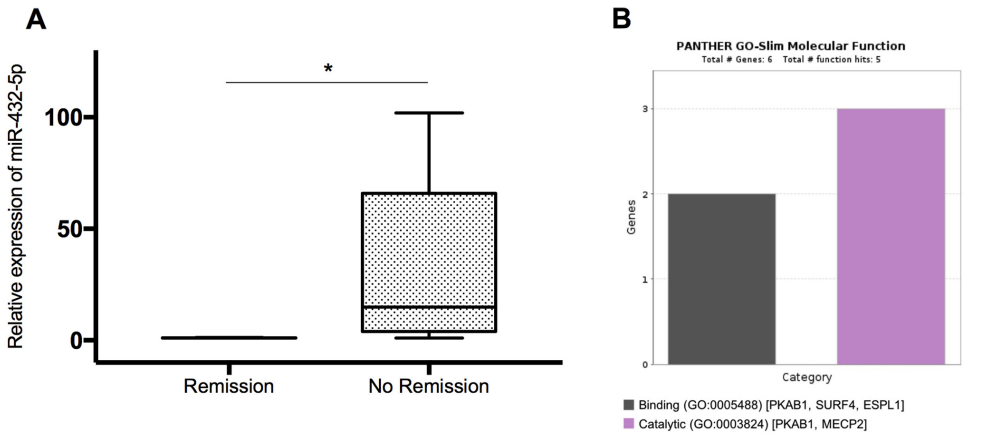

Fig. 2. Relative expression of hsa-miR-432-5p in RA patients with and without remission. A. The relative expression of hsa-miR-432-5p was determined in serum samples with the Firefly™ BioWorks assay; a comparison of hsa-miR-432-5p expression between the remission group ($n = 10$) and the group of patients who never achieved remission ($n = 6$) is shown. The analysis was performed with GraphPad® Prism v5.0 software; the statistical analysis was performed by Student's *t*-test, $^{*}p < 0.05$. B. GO analysis of important pathways related to the validated target genes of hsa-miR-432-5p. Classification of genes per group according to their molecular function was performed for the validated genes. The analysis was performed in the PANTHER Classification System.

patients who never achieved remission (Fig. 2A).

3.3. Target prediction of hsa-miR-432-5p and GO

To determine the targets of hsa-miR-432-5p and the pathways involved in the process of inflammation, we used the miRWalk 2.0 target prediction bioinformatic tool (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/). For our analysis, we only took into account those that had experimentally validated interactions on publications (western blot or qPCR data of the miRNA–target interaction). The analysis showed a total of eight genes with validation data; the relevant miRNA target genes included PRKAB1, ANO5, and SURF4. We also included two additional genes identified from databases that we believed could be important in the pathophysiology of RA (Table 3). After determining the targets of interest, we used the PANTHER GO classification system (http://pantherdb.org/) to perform GO analysis of these genes. The molecular functions associated with the targets were also identified and the functions of the targets were classified as binding and catalytic (Fig. 2B).

3.4. Differences in miRNA expression depending on the effect of the treatment with TOFA

Changes in miRNA expression were measured by the Firefly™ assay. When comparing between subjects who entered clinical remission after a month of follow-up, however, no statistically significant differences were found in this comparison (data not shown). This suggests that treatment does not affect miRNA expression directly.

3.5. Patients with RA relapse have a specific miRNA signature

To evaluate the changes in miRNA expression and identify a miRNA signature characteristic of patients in relapse, we performed cluster and heatmap analyses. We observed that patients in relapse shared an

Table 3
hsa-miR-432-5p target genes.

miRNA	Ensembl ID	Gene symbol	Binding *p*-value	Position	Binding site	N pairings	Validated
hsa-miR-432-5p	ENST00000541640	PRKAB1	0.92	CDS	319,343	18	MIRT650657
hsa-miR-432-5p	ENST00000585156	PDE4DIP	0.92	CDS	779,803	18	MIRT488397
hsa-miR-432-5p	ENST00000618462	PDE4DIP	0.92	CDS	614,638	18	MIRT488397
hsa-miR-432-5p	ENST00000324559	ANO5	1.00	CDS	2150,2179	20	MIRT441220
hsa-miR-432-5p	ENST00000421834	DST	1.00	CDS	1384,1410	19	MIRT440775
hsa-miR-432-5p	ENST00000312431	DST	1.00	CDS	1110,1136	19	MIRT440775
hsa-miR-432-5p	ENST00000361203	DST	1.00	CDS	1003,1029	19	MIRT440775
hsa-miR-432-5p	ENST00000317357	SOBP	1.00	CDS	1045,1070	20	MIRT439569
hsa-miR-432-5p	ENST00000545297	SURF4	0.85	3UTR	2468,2491	18	MIRT439505
hsa-miR-432-5p	ENST00000613129	SURF4	0.85	3UTR	2753,2776	18	MIRT439505
hsa-miR-432-5p	ENST00000618229	SURF4	0.85	3UTR	2805,2828	18	MIRT439505
hsa-miR-432-5p	ENST00000371989	SURF4	0.85	3UTR	2655,2678	18	MIRT439505
hsa-miR-432-5p	ENST00000303391	MECP2	0.92	3UTR	8355,8377	18	MIRT438694
hsa-miR-432-5p	ENST00000257934	ESPL1	1.00	CDS	2229,2266	20	MIRT065695
hsa-miR-432-5p	ENST00000552462	ESPL1	1.00	CDS	2291,2328	20	MIRT065695
hsa-miR-432-5p	–	P55PIK[a]	–	–	–	–	(21)
hsa-miR-432-5p	–	SOCS5[a]	–	–	–	–	(42)

The analysis was performed using the miRiWalk 2.0 bioinformatics tool.
[a] Genes found in the bibliography.

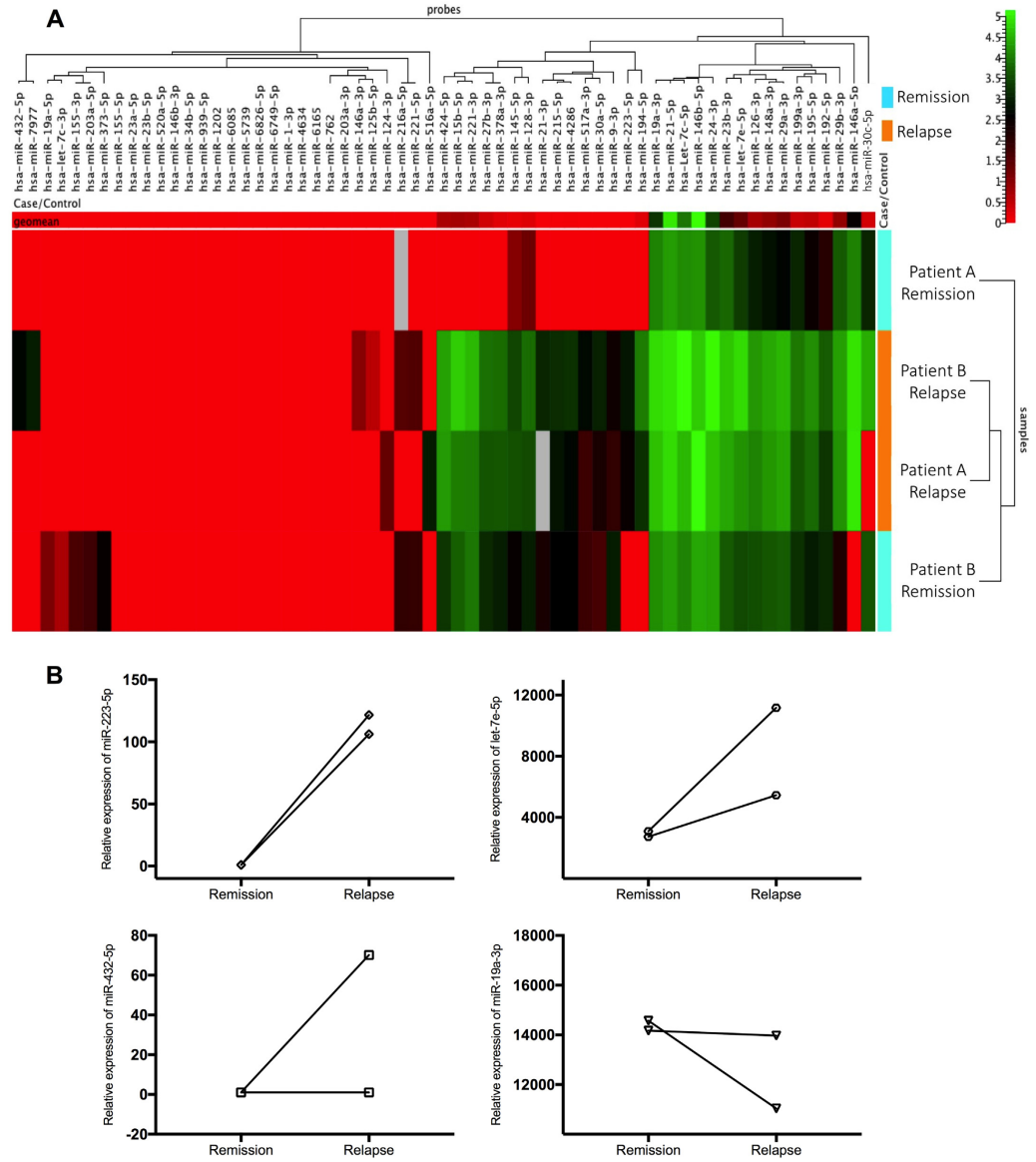

Fig. 3. Heatmap and cluster analyses of differentially expressed miRNAs of two patients before and after relapse. The fold change analysis shows the miRNA differential signature between the two patients before and after relapse. The chart shows the fold change, the color pattern describes the level of expression, the red color those miRNAs with downregulated expression, the black color those with unchanged expression among the samples, and the green color those miRNAs with upregulated expression. The size of the branches represents the Euclidian distance for the average expression of the miRNAs. The analysis was performed using the Firefly™ Analysis Workbench. B. Relative expression of hsa-miR-223-5p, hsa-let-7e-5p, hsa-miR-432-5p, and miR-19a-3p of two patients before and after relapse. The analysis was performed with GraphPad® Prism v5.0 software. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

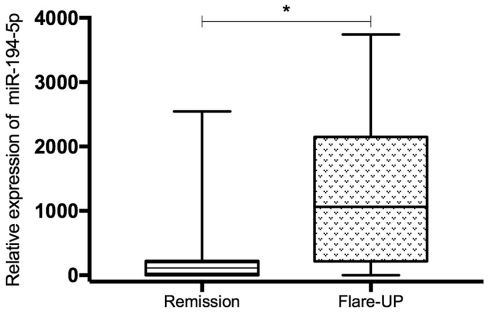

Fig. 4. Relative expression of hsa-miR-194-5p in RA patients in remission/sustained remission and flare-up. The relative expression of hsa-miR-4194-5p was determined in serum samples with the Firefly™ BioWorks assay; a comparison of hsa-miR-194-5p expression between the remission/sustained remission group ($n = 17$) and the group of patients with flare-UP condition ($n = 8$, no remission/relapse) is shown. The analysis was performed with GraphPad® Prism v5.0 software; the statistical analysis was performed by Student's *t*-test, $^{*}p < 0.05$.

expression signature for the 61 miRNAs evaluated. In addition, the miRNA signature of those in remission tended to be different from the signature corresponding to relapse (Fig. 3A). Owing to the small number of patients in relapse, we could not perform statistical analysis of differential expression; however, three miRNAs tended to be over- or underexpressed in the patients with relapse (Fig. 3B). Interestingly, hsa-miR-432-5p had higher expression in relapsed patients, which was a result comparable to the first analysis of those subjects who never achieved clinical remission.

3.6. hsa-miR-194-5p is upregulated during RA flare-up condition

Finally, to evaluate miRNAs related to RA flare-up, we compared the relative expression in those samples with clinical activity versus those with remission, so to make this analysis we include the results of the patients in remission in two different time points (the first 10 samples and the 7 samples after follow-up) and we compare with the flare-up condition that includes patients who never achieved remission and the patients in relapse. We found a statistically significant difference ($p < 0.05$) in the expression of hsa-miR-194-5p (Fig. 4), suggesting that this miRNA could be used as a biomarker of relapse.

4. Discussion

miRNAs are regarded as negative regulators of the translation of mRNA and have been reported to be associated with the pathogenesis of RA. However, the role that these miRNAs might play in the stage of RA relapse after treatment discontinuation is unknown. We found that the expression of hsa-miR-432-5p was higher in those patients who never achieved clinical remission. It seems that the increase of this miRNA occurs in those phases with greater clinical activity of the disease. Regarding the clinical implications of the elevation of this miRNA, it could increase nuclear factor kappa B (NF-kB) activity, given that one validated target of hsa-miR-432-5p is P55PIK, the regulatory subunit of phosphatidylinositol 3-kinase (PI3K) [25]. Since inhibition of p55PIK would stop the downstream cascade of AMP-activated protein kinase/protein kinase B (AMPK/AKT) crosstalk, AMPK can suppress inflammatory responses via sirtuin 1 (SIRT1) (S. R. [44]; Z. [45]), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) [20, 36], and FoxO [22, 46] signaling, which agrees with the analysis of the target genes that showed PRKAB1 as an important target, namely, the gene that encodes AMPK regulatory subunit 5′-AMP-activated protein kinase subunit beta-1 [37]. This specifically inhibits the NF-kB signaling pathway [24]. SIRT1 physically binds to the p65/RelA protein in the NF-kB complex and inhibits the transcriptional activity by deacetylating the p65 component. The role of SIRT1 in the inhibition of inflammation has been verified in different studies [9, 33]. In addition, SIRT1 induces FoxO/DAF-16 signaling, which might further potentiate the inhibition of the NF-kB system [4, 5]. Then, SIRT1 can inhibit the NF-kB pathway directly by itself or indirectly via FoxO. In this way, miR-432-5p can indirectly enhance the NF-kB pathway when targeting P55PIK and promote the chronic inflammatory response (Fig. 5).

The NF-kB signaling pathway plays a crucial role in the inflammation, hyperplasia, and tissue destruction of RA. In the initial states of chronic inflammation of RA, the NF-kB signaling pathway promoted by the expression and activation of several receptors such as receptor activator of nuclear factor kappa-B ligand (RANKL) and CD40 can

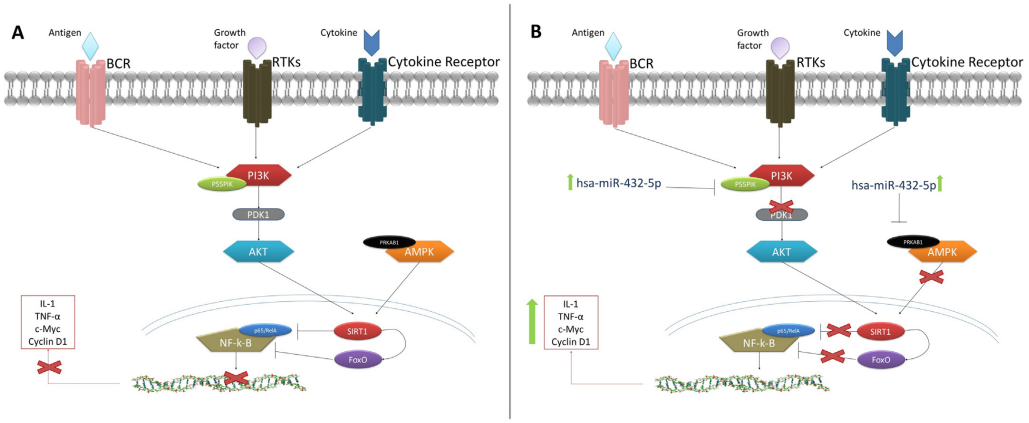

Fig. 5. Proposed mechanism of miR-432-5p activity within the PI3K signaling pathway. A) PI3K and AMPK act by inhibiting the NF-kB signaling pathway, which is mediated by SIRT1 and FoxO. B. The possible mechanism by which miR-432-5p could be involved in overactivation of the NF-kB signaling pathway is through the downregulation of P55PIK and PRKAB1 expression.

upregulate the expression of NF-κB-dependent molecules such as major histocompatibility complex (MHC) class II, CD80, and CD86 [2, 26]. The T-cell receptor and CD28 act synergistically in inducing the expression of the NF-κB-dependent genes required for T-cell activation and proliferation, such as IL-2, IL-2 receptor (IL-2R), and TNFα. This is because the interaction of antigen-presenting cells (APCs) and T cells causes the activation of NF-κB in both cell types [13, 21]. NF-κB also controls the expression of the cytokines IL-1β and TNFα, mediators of inflammation in RA. TNFα and IL-1 are potent inducers of NF-κB activation, thus forming a positive feedback loop for the pathway [12, 31]. NF-κB can also serve as a positive regulator of cell growth and fibroblasts by inducing the expression of c-Myc and cyclin D1, proteins required for cell cycle progression [14, 16] in immune cells [27]. However, functional assays are required to demonstrate the relationship of hsa-miR-432-5p to the PI3K, AKT/AMPK, and NF-κB signaling pathways.

The activity of hsa-miR-432-5p in the JAK/STAT pathway was demonstrated in a previous study in which the overexpression of hsa-miR-432-5p enhanced the phosphorylation of STAT1, resulting in an increased cellular inflammatory response. This is because suppressor of cytokine signaling 5 (SOCS5) is a target of hsa-miR-432 [38] and an important negative regulator of the JAK/STAT signaling pathway.

In the case of hsa-miR-194-5p upregulation in those samples associated with clinical activity of RA, it would be interesting to analyze the possible role of this miRNA in the pathophysiology of RA as a regulator of osteogenesis, since this miRNA is involved in signaling pathways such as the TGF-β and Wnt pathways, promoting the expression of osteogenic markers such as Runx2 and osteocalcin [19, 28, 43].

To the best of our knowledge, this is the first study that links the expression of a miRNA with the relapse of RA. Interestingly, we found a differential pattern of expression before and after relapse, and this differential miRNA signature could be useful to predict the risk of relapse after treatment discontinuation. We must acknowledge that given the exploratory nature of this study the sample size could be a potential caveat of the study.

5. Conclusions

We found that TOFA treatment does not have a direct effect on the expression of measured miRNAs, but there is a distinctive miRNA signature associated with the remission/relapse phenomenon. The changes in hsa-miR-432-5p and hsa-miR-194-5p could be associated with the regulation of proinflammatory pathways and RA flare-up condition, however further investigation is needed to confirm our findings.

List of abbreviations

RA	rheumatoid arthritis
TNF-α	tumor necrosis factor-alpha
GM-CSF	granulocyte-colony stimulating factor
JAK	Janus kinase
STAT	signal transducer and activator of transcription
bDMARD	biological disease-modifying anti-rheumatic drug
tsDMARD	target synthetic disease-modifying anti-rheumatic drug
TOFA	tofacitinib
miRNA	microRNA
mRNA	messenger RNA
MTX	methotrexate
ACPA	anti-citrullinated protein antibody
RF	rheumatoid factor
DAS28	28-Joint Disease Activity Score
RT-PCR	reverse-transcription polymerase chain reaction
GO	Gene Ontology
NF-κB	nuclear factor kappa B
PI3K	phosphatidylinositol 3-kinase
AMPK	AMP-activated protein kinase
AKT	protein kinase B
SIRT1	sirtuin 1
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PRKAB1	5′-AMP-activated protein kinase subunit beta-1
RANKL	receptor activator of nuclear factor kappa-B ligand
SOCS5	suppressor of cytokine signaling
TGF-β	transforming growth factor-beta

Ethics approval and consent to participate

All patients were enrolled in the study with written informed consent under approval from the institutional ethics committee, with registration number A3921024. IMSS-R-2015-785-106.

Consent for publication

Not applicable.

Availability of data and materials

Relevant files or data of this project will be shared on request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceived and designed the experiments: JECD, JAEM, CRR, JDCO, and JSC. Performed the experiments: JCFR, FOG, and MFRG. Analyzed the data: JCFR. Contributed reagents/materials/analysis tools: JECD, YBH, JAEM, RGA, and AEMU. Wrote the paper: JCFR and JECD. Critically reviewed the manuscript: JCFR, CRR, JSC, JDCO, JJCS, YBH, MFRG, FOG, AEMU, RGA, JAEM, and JECD.

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ABSTRACT

The aim of the present work was to evaluate MTX treatment (0.1, 1 and 10 μg mL^{-1}) *in vitro* in order to characterize its effects on cell proliferation alterations in cell cycle of HaCaT keratinocytes and wound healing in a Skh1 mice treated with MTX (low doses 30 mg kg^{-1}, high doses 200 mg kg^{-1} and repeated doses at 1.5 mg kg^{-1}). We analyzed the cytotoxic effect of methotrexate by a resazurin assay. The effects in the proliferation, cell cycle and apoptosis of HaCaT cells were analyzed by flow cytometry. The effects of MTX on wound healing *in vivo* were also analyzed. A trend toward reduction in the resazurin assay was found ($p > 0.05$). Reduced proliferation was also identified in a clonogenic assay and a CFSE assay ($p < 0.05$) due to the MTX treatment. A reduction in the G_2/M and S phases was observed accompanied by apoptosis induction with increased sub G_0 phase and annexin V FITC staining. Effect of MTX was evidenced *in vivo* on the wound closure process after day 10 ($p < 0.05$) with alterations in tissue architecture and remodeling. There is a marked effect of MTX on wound healing *in vivo* in Skh1 mice with implications for long-term therapy and surgical interventions.

1. Introduction

MTX is an analogue of folic acid, with anti-proliferative [1] and anti-inflammatory [2,3] effects. It has been used for long-term treatment schemes in leukemia and rheumatic diseases. Several toxic effects have been described such as acute mucosal ulcers, skin erosions and skin toxicity by keratinocyte dystrophy and alopecia [4–6].

However, for patients who receive MTX treatment and who undergo

Abbreviations: MTX, methotrexate; CFSE, carboxyfluorescein succinimidyl ester; RA, Rheumatoid arthritis; FBS, Fetal Bovine Serum; DMEM, Dulbecco's Modified Eagle Medium; PFA, paraformaldehyde.

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surgical interventions, the clinical practice guidelines lack criteria regarding MTX treatment during the recovery process [7], as there is no clear evidence that MTX could delay or alter the quality of the wound closure process. Studies relating MTX to the healing process have been carried out mainly in fibroblasts and osteoblasts. In a recent report, Navai et al. describe several reports that study the direct effects of MTX on fibroblasts, which show a decrease in the production of type 1 collagen, an increase in MMP-1 and the induction of apoptosis [8]. Also, Malviya et al. made a thorough review of the effects of MTX in osteoblasts and the results do not show effects of MTX on cell proliferation [9]. In this work we evaluated several dosage schemes; high doses, particularly used in neoplasms, repeated doses, used in rheumatoid arthritis and low doses, used in psoriasis. This is to evaluate the impact that different MTX treatment schemes have on the healing process.

Therefore, this study focused particularly on the evaluation of MTX in keratinocytes, since there is very little evidence of the alterations can generate on this cell type, this being one of the most important due to its participation during the healing process. Additionally, there is little information on MTX effects in the wound remodeling process using *in vivo* models that allow a comprehensive evaluation of the impact that this drug may have on the quality of the healing process.

2. Materials and methods

2.1. Reagents

High-glucose DMEM with L-glutamine 2 mM (Corning, USA), heat-inactivated fetal bovine serum (Biowest, USA), penicillin/streptomycin (Gibco, USA), trypsin 0.25%/EDTA (Gibco, USA), gelatine (Sigma, USA) methotrexate (25 mg mL^{-1}) (TEVA, Israel), metformin (Sigma, USA), etoposide 20 mg mL^{-1} (PiSa, Mex), mitomycin (PiSa, Mex), prednisone (Allergan, Ireland), fluoroshield™ (Sigma, USA) sevoflurane (Abbott, Colombia), cycletest Plus reagent Kit (BD Biosciences, USA), BD Pharmingen™ Annexin V-FITC Microscopy Kit (BD Pharmingen, USA) and, CFSE (BD Biosciences, USA).

2.2. Cell culture

HaCaT cells were cultured in 25 cm^2 flasks according to the conditions stipulated in the technical sheet provided by Cell Line Services, GMBH Germany. Subculture was made to 80–90% confluence as verified in an inverted microscope (Leica, DMI-IL Led, Germany). Cells were detached by trypsin and viability assessed by trypan blue exclusion. Number of cells was determined by Neubauer chamber.

2.3. Resazurin assay

HaCaT Cells (1.25×10^4) were seeded in a 96 well plate and serially diluted to make a calibration curve. Metformin (Met 20 mM) was used as a control due to the reported effect on the cell line [10], and the following concentrations were used for a dose-response curve: 0.1 μg mL^{-1} (0.22 μM), 1 μg mL^{-1} (2.2 μM). In addition, 10 μg mL^{-1} (22 μM) of MTX were used and cells were exposed 24/48 h. These concentrations had been reported as circulating in the serum of patients in the steady-state treatment phase [11,12]. Resazurin was added (4.95 mM), incubated for 1 h and IMF determined in a fluorescence plate reader (Fluoroskan Ascent, Thermo Fisher) excitation/492 nm and emission/590 nm for the analysis of resorufin. The number of viable cells was estimated with a calibration curve.

2.4. Clonogenic assay

HaCaT cells (1×10^4) were seeded in 6 well culture plates and after 24 h with the following stimulus: PBS (vehicle), Met 20 mM, MTX 0.1 μg mL-1, 1 μg mL-1 and 10 μg mL-1. Cells were incubated for 5 days to allow cell growth and photographic documentation of cell growth and colony formation recorded for each condition. The cells were fixed on day five with 4% (v/v) p-formaldehyde (PFA). The cells were stained with 0.05% crystal violet/25% methanol (v/v) for 30 min and washed with PBS 1x. Colonies counted (aggregates of >50 cells) in a Leica DMI inverted microscope (Leica, Germany). Micrographs for all conditions are shown.

2.5. CFSE proliferation assay

HaCaT cells were cultured as previously described and synchronized by reduction in FBS to 1% (v/v) and incubated overnight. Trypsin detached cells were labeled with 2 μM CFSE (BD Biosciences, USA). Once labeled, 5×10^5 cells were split for use with each condition and seeded in 6 well plates with fresh medium containing 0.1 μg mL^{-1}, 1 μg mL^{-1}, 10 μg mL^{-1} of MTX. Vehicle (PBS) or Met 20 mM (as a control of inhibition of proliferation) were used as controls. Cells were incubated for 24/48 h. A fraction of synchronized cells (1×10^6) was also fixed and used as loading control (non-proliferation condition). Trypsin detached cells were fixated with 4% PFA. Data was acquired in a FACS CANTOII flow cytometer (BD Biosciences, USA) and analyzed in the FlowJo software v.10.0 (FLOWJO, LLC, USA).

2.6. Cell cycle analysis

5×10^5 HaCaT cells were seeded into 6 well plates after 24 h the cells were then exposed to MTX concentrations of 1 μg mL^{-1} and 10 μg mL^{-1} compared to vehicle (PBS 1x) and Met 20 mM for 48 h. Cells were enzymatically detached from the culture plates and fixed by addition of cold 70% ethanol for 24 h. Cells were then stained with the BD Cycletest DNA Reagent (BD Biosciences, USA) according to manufacturer's instructions. Data was acquired in a FACS CANTO II (BD Bioscience, USA) flow cytometer and the analysis of the data was carried out in the FlowJo software v.10.0 (FLOWJO, LLC, USA).

2.7. Apoptosis assay

HaCaT cells were seeded (2.5×10^5) in 6 well plates as previously described. The cells were then exposed for 48 h to MTX 0.1 μg mL^{-1}, 1 μg mL^{-1} and 10 μg mL^{-1}. Met 20 mM and Etoposide 10 μM were used as apoptosis controls. Cells were enzymatically detached and fixed in 70% (v/v) cold ethanol for 24 h. Cell staining was done with the BD Pharmingen Annexin V-FITC kit according to manufacturer instructions (BD Pharmingen, USA). The cells were adhered to a glass slide and mounted with fluoroshield (Sigma, USA). The slides were then analyzed by confocal microscopy in a Leica DM2500 upright microscope (Leica, Germany).

2.8. In vivo wound healing model

Fifteen 8-week-old male Skh1 mice were used for the experiments. Assays were carried out in the *"Claude Bernard"* animal facility at the Autonomous University of Zacatecas, Mexico. The weight range of the animals per group was 25–30 g/animal. All animals had water and food *ad libitum* and each mouse was kept in a separate polycarbonate cage at 22 ± 3 °C with a humidity of 40–70% and with light and darkness cycles of 12 h each. All technical procedures were carried out according to the NOM062ZOO1999 for the production, care and use of experimental animals. All animals were divided in 5 (n = 5) groups according to treatment: control, prednisone, low single dose of MTX (30 mg kg^{-1}), high single dose of MTX (200 mg kg^{-1}) and repeated doses of MTX (every third day, 1.5 mg kg^{-1}) [13–15]. MTX was administered intraperitoneally one week before the wound was made. The wound was 0.3 cm in diameter. Daily photographic documentation (with reference) was made for all animals as well as weight and glycemia. On day 14, the animals were sacrificed through CO_2 exposure, removing the scar tissue, fixed in 4% formaldehyde for 48 h.

2.9. Histological analysis

The obtained tissue was processed by standard procedures for histological technique by alcohol/xylol and embedded in paraffin. Tissue sheets were mounted on glass slides for staining with Harris-Hematoxylin and Eosin or Mason Trichromatic stain. Microphotographs were taken in a Zeiss M200 inverted microscope (Zeiss, Germany) for histological analysis.

2.10. Statistical analysis

Statistical analysis was done in GraphPad Prism software v.6.0. The normality of data was determined by Kolmogorov-Smirnov test. Two-way ANOVA was used for multiple comparisons and one-way ANOVA or non-parametric Kruskal-Wallis test was used when only treatment effect was considered. Post-hoc Tukey or Dunn was used, respectively. A p value of <0.05 was considered statistically significant.

3. Results

3.1. MTX showed no immediate toxicity but diminished keratinocyte proliferation

MTX has effects on the proliferation of dermal cells. The effect of different concentrations of MTX on the proliferation of keratinocytes (HaCaT) was studied. In Fig. 1, the metabolic capacity of the cell as a sign of cellular oxidative function. Our data show no effect on cell toxicity ($P > 0.05$) when comparing the MTX exposed cells to the control. However, tendency downward at 48 h of exposure was observed when comparing metformin 20 mM and the vehicle control. Through a clonogenic test, long-term exposure to MTX was studied. Cells were followed for 5 days for the formation of colonies. As shown in Fig. 2A, the cells in the control condition (vehicle) were able to proliferate and generate colonies. A marked reduction in the proliferation capacity was shown for metformin (as previously reported [10]) and for the MTX treatment compared to the control condition (number of colonies of >50 cells), however proliferation was not halted completely (Fig. 2B). A clear effect was visible at 72 h after treatment with MTX. Additionally, we analyzed the effect of MTX in proliferation by a CFSE dye dilution assay by flow cytometry. In Fig. 2C, the effect of Metformin 20 mM and MTX (1 and 10 μg mL^{-1}) at 48 h is shown. Untreated cells lost fluorescence at a higher rate due to cell division; cells with a retained fluorescence do not proliferate. This was further quantified as the proliferation ratio shown in Fig. 2D. Statistically significant differences were identified for all conditions at 48 h when comparing between them and for interaction ($p < 0.01$).

3.2. MTX treatment caused cell cycle arrest in keratinocytes

To evaluate whether the cell proliferation reduction was associated to a particular checkpoint in the cell cycle of the HaCaT cells, the cell cycle was analyzed by flow cytometry. As shown in Fig. 3A, several alterations were noted in the MTX treated cells at both 0.1 and 10 μg mL^{-1}. The peak that corresponds to G_2/M phase is practically absent in the exposed cells, suggesting that cell division was altered due to MTX treatment. This was also observable in the Metformin control. Additionally, a contraction of the S phase was observed. This is shown in Fig. 3B, differences compared to the control were identified. In the G_0/G_1 phase there was a significant accumulation of PI fluorescence ($P < 0.05$) in the MTX exposed cells. Significant differences were also identified for the M phase in the control versus MTX treated cells ($P < 0.05$).

3.3. MTX treatment induced apoptosis in HaCaT cells

We noted a sub G_0 population in MTX treated PI histograms Fig. 3A, suggesting that the cells were dying by apoptosis. This was then confirmed by analysis of phosphatidyl serine exposure in the outer layer of the membrane by means FITC labeled Annexin V and analyzed by fluorescence microscopy. As shown in Fig. 4A, the MTX treated cells at both the 1 and 10 μg mL^{-1} concentration exhibited a strong fluorescent signal comparable to that of etoposide (10 μM), a known inducer of apoptosis and contrasting with the vehicle and metformin 20 mM.

3.4. Skh1 mice treated with MTX exhibited a delay in their wound closure capacity

To provide *in vivo* evidence of MTX effects, we evaluated the effects of three dosage schemes in hairless Skh1 mice. These three regimes resemble those of human treatment schemes: A) Low doses 30 mg kg^{-1} (resembling that of inflammatory skin diseases), B) High doses (200 mg kg^{-1} as those used in chemotherapy for cancer) and C) Repeated doses (1.5 mg kg^{-1} every third day) like those used for RA murine models and other rheumatic diseases. As shown in Fig. 5A, the control group of animals had complete closure of the wound at day 10 and the scarring tissue was barely perceptible at day 12. Prednisone was used as control. The wound had complete contraction at day 12 and complete closure at

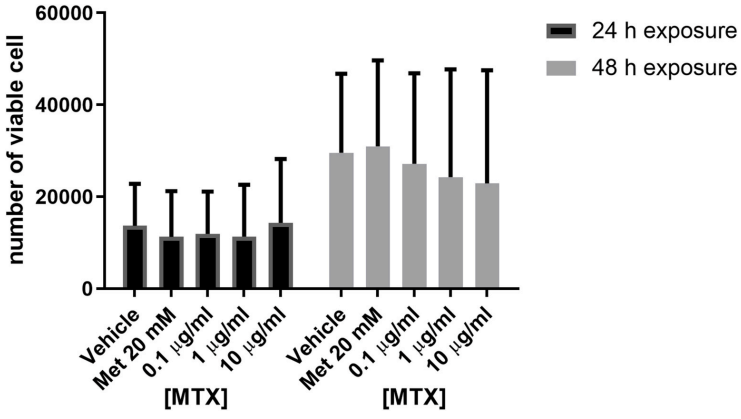

Fig. 1. Effect of methotrexate on HaCaT cells viability. For this purpose, 1.25×10^4 cells were seeded in a 96 well plate: Vehicle (PBS 1x), Metformin 20 mM and MTX (0.1 μg mL^{-1}, 1 μg mL^{-1}, 10 μg mL^{-1}) were used to confirm previous results and broad the concentrations of exposure. The cells were maintained in standard culture conditions for 24 h and 48 h. At each time point, resazurin (4.95 mM) was added to the wells and incubated for 1 h at room temperature. Fluorescence of the resorufin compound was measured in a fluorometer. The number of viable cells was estimated by a calibration curve. The mean and standard error of the mean of the three independent experiments are shown. A two-way ANOVA performed, a p value < 0.05 was considered significant.

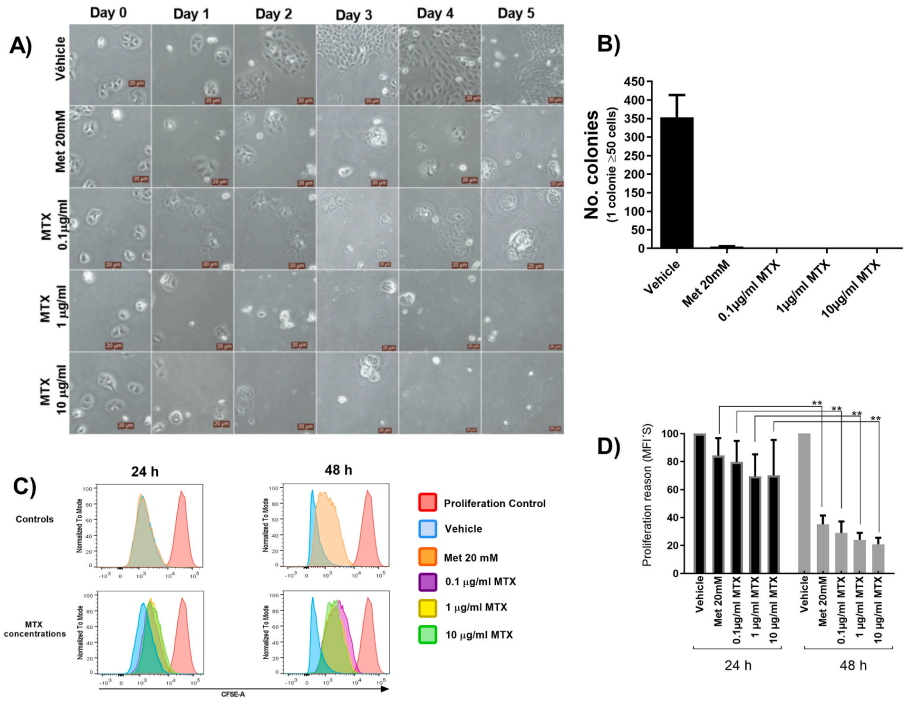

Fig. 2. Methotrexate shows antiproliferative effects on HaCaT cells. A) MTX reduces the development of colonies in HaCaT cells. In the assay 1×10^4 cells were seeded in 12 well culture plates. Several conditions were used: Vehicle, (PBS), Metformin 20 mM and MTX (0.1 µg mL^{-1}, 1 µg mL^{-1}, 10 µg mL^{-1}). The cells were incubated for 5 days in standard culture conditions. The proliferation was documented based on the morphology and size of the colonies. Micrographs taken at 20X **B)** Colonies of more than 50 cells were counted by crystal violet staining in bright field images. **C)** MTX diminishes cell proliferation of HaCaT cells. Cells were synchronized in their cell cycle by FBS depletion to 1%, 3×10^6 cells were labeled with CFSE and divided into several culture conditions: vehicle, Metformin 20 mM and MTX (0.1 µg mL^{-1}, 1 µg mL^{-1}, 10 µg mL^{-1}) and incubated for 24 and 48 h. Normalized histograms are shown for fluorescence retention indicating a reduced proliferation of MTX exposed HaCaT cells. **D)** Ratio of proliferation. The ratios of proliferation are shown based on the quantification of fluorescence in each histogram of CFSE labeled cells exposed to MTX. The mean and standard error of the mean of the three independent experiments are shown; a two-way ANOVA was performed with Tukey posttest, (*) $p < 0.05$ (**) $p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

14 days. When comparing the control and prednisone treated animals, we observed similarities among the three groups treated with MTX and those treated with the steroid. As shown in the three lower lanes of Fig. 5A, there's incomplete wound contraction and closure in the repeated doses treatment scheme (lower-most lane). As shown in Fig. 5B, there was a statistically significant difference from day 6 onward ($p < 0.001$) in the MTX repeated doses compared to the vehicle.

3.5. Tissue remodeling and wound closure was affected by MTX treatment

We analyzed whether MTX treatment affects wound healing by histopathological evaluation. In Fig. 6A, there are demonstrative images of scarred mouse tissue under MTX treatment where H&E staining demonstrates the damage of MTX treatment with respect to the thin layer of remodeled epidermis, this is consistent with those found in the *in vitro* model. While with the Mason's trichromatic stain it became evident that the collagen synthesis and deposition (indicative of maturation and of the tensile strength of the tissue) was severely impaired. Other alterations were noted and highlighted: 1) Alterations in the formation of sebaceous glands, hair follicles, etc. 2) Reduced smooth muscle tissue thickness, 3) reduced thickness in both basal stratum and stratum corneum. In Fig. 6B, quantification of thickness is shown. We observed statistically significant differences in both the high doses and repeated doses MTX treatment schemes ($P < 0.05$).

4. Discussion

MTX therapy is a cornerstone in the treatment for rheumatic diseases and leukemias [16]. As described before, MTX can have toxic effects on different organs like the skin [17] and severely impair the wound closure process at different levels (inflammation, proliferation and remodeling). There is little evidence of the effect of MTX on this process. The improved knowledge of the effects of MTX will lead to better understand the secondary long-term effects of MTX treatment. For this, we evaluated the activity of MTX on keratinocytes (HaCaT), which are keys cells in the wound healing process. The cytotoxic effect of MTX on HaCaT cells is not significant. This may be due to the short exposure time and alternate activation of biosynthetic pathways of purines. Then, by means of clonogenic tests, prolonged exposure to MTX was evaluated. After 48 h, we found a visible effect on the proliferation of cells and the lack of colonies in the MTX exposed condition. We confirmed this effect of MTX and the reduced proliferation by the CFSE dilution assay, proliferation

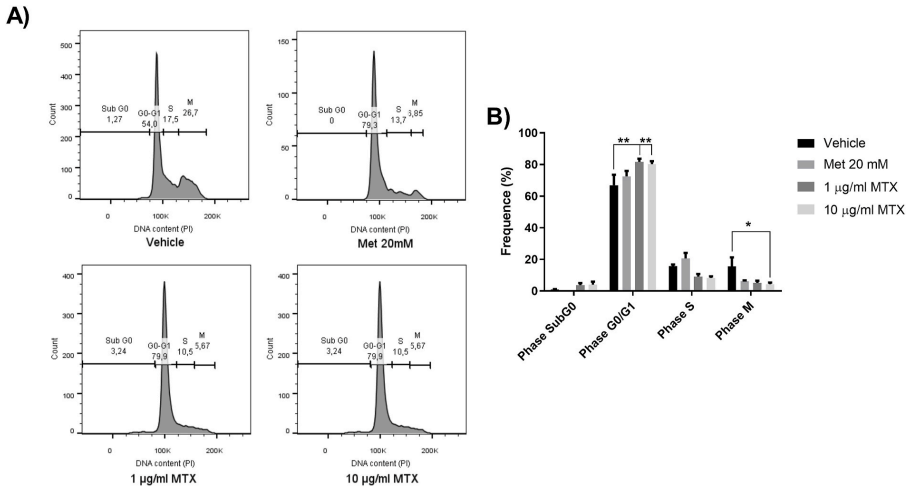

Fig. 3. MTX treatment alters the cell cycle of HaCaT cells. For the assay 5×10^5 cells were seeded in 6-well cell culture plates and stimulated with vehicle (PBS), Metformin 20 mM and MTX (1 µg mL^{-1}, 10 µg mL^{-1}) and incubated during 48 h. Afterwards, cell detachment was made by trypsin treatment, counting and fixation with 4% PFA. The cells were stained with the BD Cycle test DNA Reagent KIT (BD Bioscience, USA) and analyzed by flow cytometry. **A)** DNA Histograms are shown for each condition. Changes in the distribution of G_0/G_1, S and G_2/M are shown when compared to the control. **B)** The percentage of cells in each phase of the cell cycle is shown compared to the control. The mean and the standard deviation of three independent experiments are shown. A two-way ANOVA was performed, (*) $p < 0.05$.

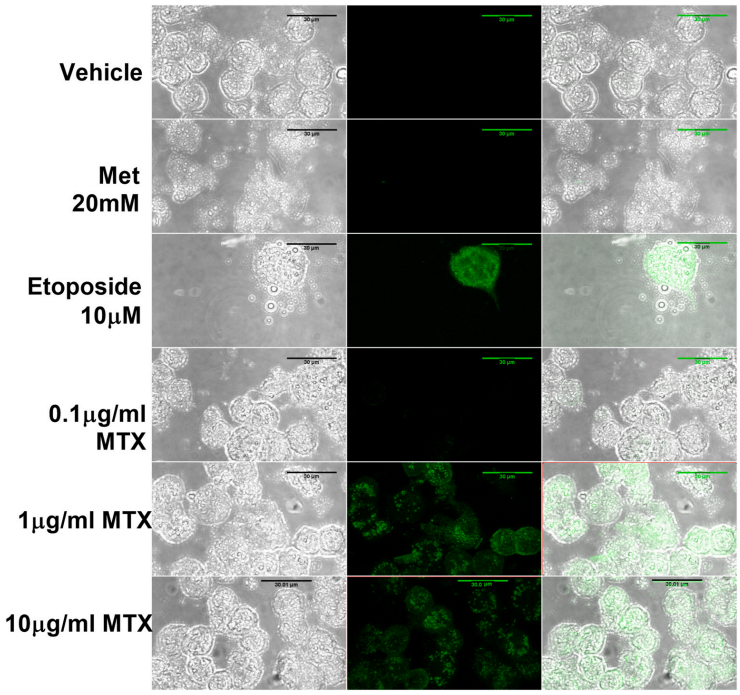

Fig. 4. MTX treatment induces early apoptosis and reduces the migration of HaCaT cells. A) Early apoptosis induction in HaCaT cells by methotrexate treatment. For the assay 2.5 × 105 cells per well were used and exposed to: Vehicle (PBS), Metformin 20 mM, Etoposide 10 µM and MTX (0.1 µg mL^{-1}, 1 µg mL^{-1} y 10 µg mL^{-1}). The cells were incubated during 48 h and detached by trypsin enzymatic digestion and fixed by cold 70% ethanol, then stained with the BD Annexin V-FITC kit (BD Pharmingen, USA). The images were acquired in a Leica DM2500 confocal microscope. The images are representative of three independent experiments.

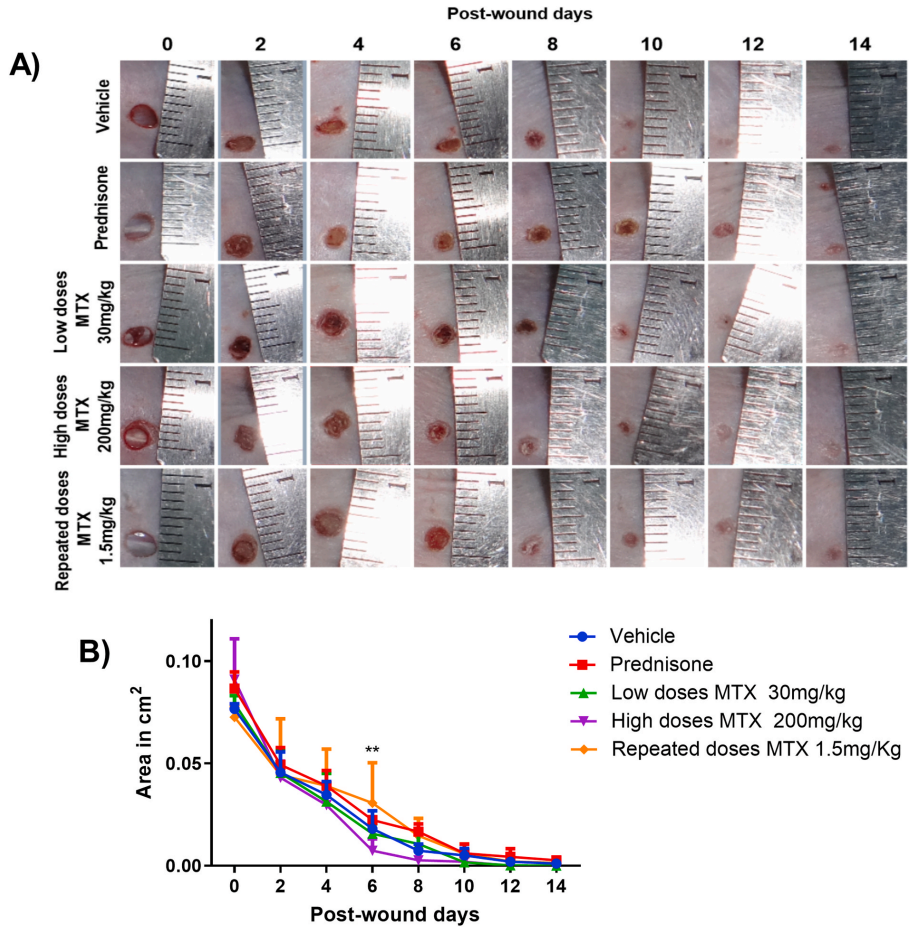

Fig. 5. Wound closure is reduced by repeated doses of MTX in a hairless Skh1 mouse model. A) A panel with representative images of all time points is shown. Five animals per group were used in five different groups: Vehicle (PBS), Prednisone, low doses (30 mg kg^{-1}), high doses (200 mg kg^{-1}) and repeated doses (1.5 mg kg^{-1} every third day). All animals started treatment and after 7 days, an aseptic wound was made on the back of the animal under sevoflurane anesthesia with a 3 mm biopsy punch. Referenced photographic documentation was performed on all animals at days: 0, 2, 4, 6, 8, 10, 12 and 14. **B)** Image analysis and area were calculated with Image J software. The area quantification is shown for all time points after the wound was made an all animals. Mean and standard deviation of area is shown for all time points. The average and standard deviation of the area is shown on each day. The one-way ANOVA test determined the difference between the (●) Vehicle control group and the groups exposed to (▼) High-doses (**) and (◆) Repeated-doses (**) of MTX. The statistical analysis considered the significant differences from (*) $p < 0.05$, (**) $p < 0.001$ and (****) $p < 0.0001$.

arrest was observed, corroborating an antiproliferative effect. Then, we analyzed if proliferation arrest happened at a specific checkpoint of the cell cycle. We found a reduction in the G_2/M phase and also in the S phase suggesting the likely participation of the Cyclin E and CDK2 implicated in the S phase checkpoint [18], further experimental evidence of such activation is needed.

Interestingly, we observed a Sub G_0 population in the histograms of the cell cycle analysis suggesting an increased induction of apoptosis. This is consistent with previous studies, where they report that cells die in an apoptosis dependent mechanism associated with the release of Cytochrome C and therefore the mitochondrial toxicity mechanism is inducing apoptosis [19]. Additionally, we confirmed the induction of early apoptosis by the analysis of phosphatidyl serine translocation by analysis of Annexin V FITC, which confirms this phenomenon in our model.

Dosage schemes like those of humans were used in our animal model to further dissect the effect of MTX in proliferation and wound healing. We found a marked reduction in the wound closure capacity of the animals treated with MTX at high doses and in the repeated doses group. The main findings were: **1)** Delayed proliferation phase with repeated dose administration. **2)** Reduced thickness of the scar/skin in the MTX treated animals (high doses group. **3)** There is a reduced formation and deposition of collagen fibers in the tissue. **4)** Loss of skin associated structures like sebaceous glands and hair follicles. According to our

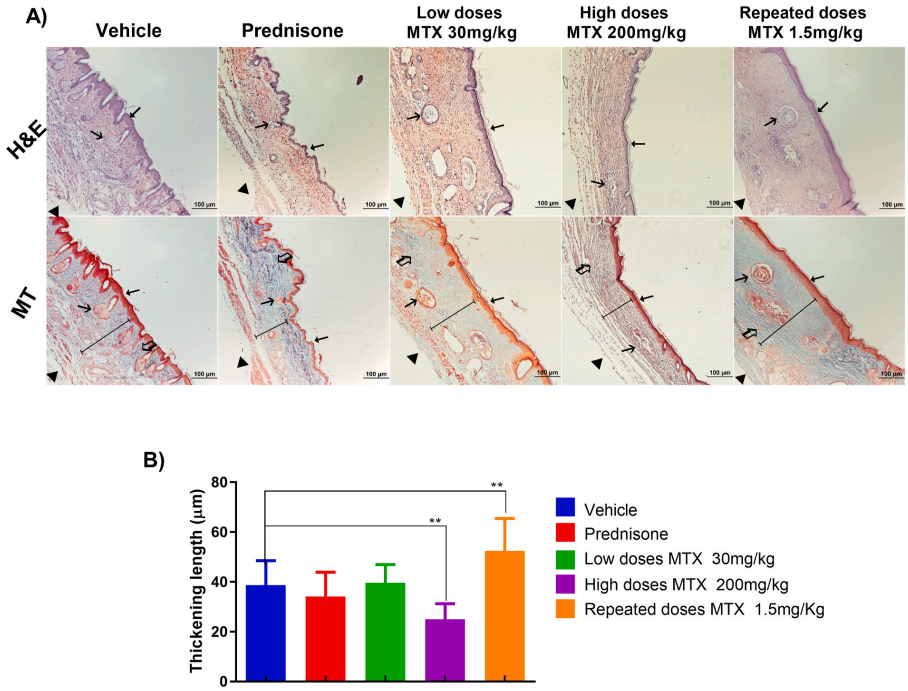

Fig. 6. MTX treatment interferes with extracellular matrix formation and tissue remodeling. The tissue surrounding the wound was collected by complete excision. The tissues were processed for histology analysis and **A)** hematoxylin/eosin staining was performed as well as **B)** Mason's trichromatic stain. Several images from each sample were acquired. Several structures were identified: (↘) This symbol indicates the widening of the epidermis; (↔)This symbol indicates several skin structures such as sebaceous and sweat glands and hair follicles; (◣)this one shows the absence or presence of striated muscle tissue; (⇔)and this one the density of collagen fibers; (I) This indicates the size of the dermis barrier. Images from both the vehicle control and prednisone treated control are shown for comparison purposes. Images and histological findings are representative of the animals included in each group (n = 5). **B)** Measurement of epidermal thickening by image J analysis. In each of the images the scar tissue was measured (n = 5) in the control and the MTX treated groups. The mean ± SD of the epidermis thickness is shown. Statistical analysis was performed by means of one-way ANOVA test average and standard deviation of the epidermis thickness shown. (*) $p < 0.05$, (**) $p < 0.001$ and (****) $p < 0.0001$.

previous results (*in vitro* and *in vivo*), we can hypothesize how MTX can affect healing, through alteration in keratinocytes. At the cellular level we observe a proliferation arrest, caused by cell cycle arrest and a slow induction of apoptosis, these effects can be reflected in the *in vivo* model during the delay of the group of repeated doses during the proliferation phase. Although these activities do not compromise wound closure, they do affect the remodeled tissue. MTX might affect communication pathways such as miRNA/microvesicles that are released by keratinocytes for the genetic regulation of fibroblasts, and this could influence the thickness of scar tissue [20,21]. This study contributes to a better understanding of the effects of MTX on the healing process, as our results in addition to indicating that MTX has anti-proliferative and apoptotic effects on HaCaT keratinocytes, wound closure is severely affected in treated animals with MTX with marked alterations in the remodeling of the skin and a reduced thickness of the basal and corneal stratum. This evidence shows that the use of MTX after surgery must be carefully evaluated to avoid complications in this process.

5. Conclusion

MTX have anti-proliferative effects caused by arrest in the G_0/G_1 phase of the cell cycle and the induction of apoptosis in HaCaT keratinocytes. Wound healing is severely impaired in MTX treated animals with marked alterations in skin remodeling and reduced thickness of basal and corneal stratum.

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Availability of data and material

The data used in this study are available from the corresponding author upon request.

Ethics approval

The experiments in Skh1 animal model has been approved by the ethics committee of the health sciences area in the Autonomous University of Zacatecas, Méx registration number: 068/2017.

Author contributions

MF M-L: performed the *in vitro* and *in vivo* experiments and determinations and participated in the drafting of the manuscript; JE C-D: conception of the idea, experimental design and planning, first draft, provided partial funding, overall supervision and final approval of the draft; JC F-R, performed *in vitro* experiments and determinations by flow cytometry; FL O-G: performed *in vitro* experiments, colorimetric and confocal microscopy determinations; L T-V: performed histopathological analysis and reporting of *in vivo* experiments S G-C: performed *in vivo* experiments and material collection; M Z-Z: conception of the idea, critical review of the manuscript; CJ S, critical review of the manuscript, and revision of final draft; D P–P: critical review of the manuscript, and revision of final draft; JA E-M: experimental, technical and infrastructure support, critical review of the manuscript, and revision of final draft; AR C–V: experimental design, analysis, critical review of the manuscript, and final revision of the draft and provided partial funding, approved final draft.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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REVIEW ARTICLE

Innate Immunity Alterations in Type 2 Diabetes Mellitus: Understanding Infection Susceptibility

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Abstract: Diabetes is a chronic disease characterized by marked alterations in the metabolism of glucose and by high concentrations of glucose in the blood due to a decreased insulin production or resistance to the action of this hormone in peripheral tissues. The International Diabetes Federation estimates a global incidence of diabetes of about 10% in the adult population (20 - 79 years old), some 430 million cases reported worldwide in 2018. It is well documented that people with diabetes have a higher susceptibility to infectious diseases and therefore show higher morbidity and mortality compared to the non-diabetic population. Given that the innate immune response plays a fundamental role in protecting against invading pathogens through a myriad of humoral and cellular mechanisms, the present work makes a comprehensive review of the innate immune alterations in patients with type 2 diabetes mellitus (T2D) as well as a brief description of the molecular events leading or associated to such conditions. We show that in these patients a compromised innate immune response increases susceptibility to infections.

Keywords: Innate response, phagocytosis, Type 2 Diabetes Mellitus, infection, antimicrobial peptide, innate immune cells.

1. INTRODUCTION

Diabetes Mellitus is a group of chronic metabolic diseases characterized by hyperglycemia and abnormalities in the use and metabolism of glucose, proteins and lipids [1]. A sustained and elevated glucose levels and increased resistance to insulin action in peripheral tissues characterize it. Recent estimates by the world health organization (WHO) and international diabetes federation (IDF) suggest that nearly 430 million people live with the disease, and it has been estimated that with an increase of 48 % in 30 years, more than 600 million people worldwide would suffer from diabetes [2] and particularly of type 2 diabetes (T2D). The incidence of diabetes has been rising, associated with increased rates of obesity in the elderly and young individuals [3]. This growing trend in the incidence of diabetes demands an increased awareness and an urgent assessment of disease-associated co-morbidities in order to reduce or mitigate deaths related to this illness. Several complications have been observed in diabetic individuals, such as hyperglycemic emergencies that can lead to death [4]. Other complications that have been frequently observed in these individuals are diabetic retinopathy [5], diabetic foot ulcers [6] and infection [7]. About infections, several reports exist on the matter. Seshasai et al. reported that there is an increased hazard ratio (HR) of 2.37 for pneumonia associated deaths and an HR of 1.67 for premature death in diabetic patients with infectious diseases [8]. Another study from Sha et al performed in Canada on data from the public health system (of approximately 1,000,000 medical records) found an increased risk of death associated with infection with an odds ratio (OR) of 1.92 in diabetic patients [9]. Additionally, in a 12 month prospective study conducted in the Netherlands by Muller et al, the

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authors evaluated through a logistic regression the risk of infection comparing 6712 patients with T2D and 18,911 control subjects finding an increased risk of lower respiratory tract infections (OR 1.42), Urinary tract infections (OR 1.24), bacterial skin an mucous membrane infection (OR 1.44) [10]. Further evidence on infection susceptibility of diabetics has been described in recent years. It has been demonstrated in various populations that reactivation of latent infectious diseases such as tuberculosis is a serious and recurrent complication in diabetic patients and T2D has been identified and associated as an independent risk factor for infection and reactivation of tuberculosis [11-14].

The link between infection susceptibility and diabetes has been hard to explore in fundamental and physiopathology-oriented studies due to varied and frequent comorbidities and complications in diabetic patients; however, the fundamental physiological mechanisms behind this phenomenon have been widely studied. Despite these efforts, the complex mechanisms behind increased susceptibility to infection in diabetic patients remain unclear and also, the underlying mechanisms are still poorly understood. Therefore, the objective of this article is to provide a comprehensive update in our understanding of how innate immune function or dysfunction plays a fundamental role in the susceptibility to infection in diabetic patients. To this purpose, we describe the fundamental mechanisms of innate immune defense such as alterations in mucosal/epithelial barriers, the skin, phagocytosis, complement, chemotaxis, natural killer (NK) cells, innate lymphocytes and their relationship with infection susceptibility through reports on alterations due to diabetes. The adaptive immunity mechanisms are beyond the scope of this review.

2. ALTERATIONS IN SKIN AND MUCOUS MEMBRANES

One of the major components of the innate immune system is the epithelial barrier system. These are physical barriers between microbes in the external environment and the internal milieu of the host. The integrity of these barriers and the lining of epithelial cells (in mucosal surfaces), keratinocytes (in the skin), the secretions and molecules produced by these cells and the intraepithelial lymphocytes contribute to the effectiveness of such barrier. Patients with diabetes have frequent infections, particularly those with diabetic foot ulcers are constantly infected with normally harmless organisms living on the skin such as *S. aureus* [15], it is also very common that T2D patients have become infected with *Candida species* [16, 17]. In a publication by Hu et al, the authors describe a risk analysis assessment of urinary tract infections (UTIs) in postmenopausal women, they identify in such analysis that (among others), the presence of T2D increases the risk (OR 2.78) of UTI [18]. The underlying immune mechanisms affected by hyperglycemia in these patients that explain the increased susceptibility to urinary infection could be due to hyperglycemia conditions that are known to promote the expression of virulence factors in some fungal species [19, 20].

Lan and colleagues also described this altered function in keratinocytes; the authors describe the effects of glucose exposure on keratinocytes. A decreased motility of keratinocytes, expression of proliferation markers and migration mechanisms (such as expression of Matrix metalloproteinase 1) was also decreased by activation of Signal transducer and activator of transcription 1 (STAT1) [21]. This data suggest that epithelial and keratinocyte functions are altered due to high glucose concentrations and, therefore, the integrity and function of the aforementioned barriers.

Other factors that clearly contribute to the integrity of these barriers are the soluble molecules produced by the epithelial cells and keratinocytes. In this regard, it has been described that several soluble molecules known as antimicrobial peptides (AMPs) show a decreased expression in diabetes. AMPs are cysteine-rich small peptides (3 to 5 kDa), which are ubiquitous in all vertebrates and invertebrates. In humans, they are subdivided into two families, α-defensins and β-defensins, based on their structure. Six α-defensins and four β-defensins have been well characterized in humans, but a recent analysis of the human genome revealed 34 defensin genes, which highlights the significance of this family of antimicrobial peptides. Defensins, like most other antimicrobial peptides, are highly cationic, enabling them to bind to the negatively charged cell walls of bacteria and fungi and to kill them by pore formation and increase cell membrane permeability [22]. Rivas-Santiago et al. describe in a study of skin biopsies from diabetic foot ulcers a decreased expression of the AMP LL-37 both at mRNA and protein levels (detected by immunohistochemistry) in the outer layers of the skin particularly the *corneum stratum* and the granulation tissue [23]. This suggests that diabetic patient's susceptibility to infection in these ulcers might be associated with such mechanisms and to the impairment of the barriers function to maintain such surfaces free of microbial colonization. Additionally, the surveillance of epithelial/mucosal surfaces and of the keratinized epithelia could be affected in diabetics. In a study by Gonzalez-Curiel et al., it was reported that patients with T2D have decreased mRNA transcriptional levels of *CAMP* (LL-37), *DEFB4* (HBD-2) and *DEFB103A* (HBD-3) in whole blood leukocytes, additional groups of patients with latent or active tuberculosis infection and T2D showed a decreased expression of such AMP genes [24], antimicrobial peptide expression impairment in blood leukocytes could also affect the antimicrobial capacity of the tissues such as the skin or mucosa where these leukocytes are migrating. Further research on the role of immune mechanisms associated with barrier function needs to be addressed in order to provide a wider picture of how diabetes could affect such processes and therefore contribute to infection susceptibility.

3. ALTERATIONS IN CHEMOTAXIS AND CELL MIGRATION

Cell migration in response to gradients of soluble molecules is known as chemotaxis, an essential process in the immune response. This process happens in response to several proteins collectively known as "chemotactic factors". These are essential for the extravasation or infiltration of leukocytes to the tissues and their proper arrival to the sites of inflammation in response to an insult or stimuli. The importance of this process for infection control has been associated with a decreased leukocyte function in diabetes. It is known that diabetic patients present alterations in the process of adhesion and migration, some studies have shown a decrease in the expression of chemoattractant molecules of neutrophils and monocytes [25], Waltenberger et al. showed in an in vitro migration assay that hyperglycemia, which was induced by either preincubating endothelial cells with serum from hyperglycemic patients or addition of 30 mM glucose, induced the upregulation of Intercellular Adhesion Molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin on endothelial cells, which resulted in an increased adherence of neutrophils [26], or increase in the expression of RAGE, which promotes the production of reactive oxygen species (ROS) mediated by damage-associated molecular patterns (DAMPs) in aortic cells [20], that could be associated to a reduced migration to sites of inflammation and to an increased susceptibility to cardiovascular disease in diabetics. Also, increased expression of molecules like CD11a, CD11b and CD11 were evaluated on neutrophils by flow cytometry, since these molecules are very important for the adherence of leukocytes to activated endothelium an elevation of these cell markers might be indicative of increased intravascular aggregation. In diabetic patients, an enhanced spontaneous adhesion was found. This phenotype of increased "adherence" also provided evidence that neutrophil chemotaxis was impaired in the presence of diabetes, the authors also demonstrated that the reduced chemotaxis of polymorphonuclear phagocytes toward N-formyl-methionyl-leucyl-phenylalanine (fMLP) and phorbol 12-myristate 13-acetate (PMA) in an agarose migration assay [27]. The implications of such alterations in the expression of adhesion molecules in diabetics might be associated with reduced migration and elimination of infectious agents in peripheral tissues.

Stegenga et al., reported in an insulinemic/euglycemic clamp (in six human subjects over a span of 6 hours) that the hyperinsulinemic condition (a common feature in diabetics) enhanced the lipopolysaccharide (LPS)-induced inflammatory response, since interleukin (IL) -1, IL-6, and IL-8 responses were significantly higher than in their respective low-insulinemic controls. Tumor necrosis factor-alpha (TNF-α) responses were only enhanced in the hyperinsulinemic/euglycemic group, but not in the presence of hyperglycemia. When chemotaxis was analyzed under hyperglycemic and hyperinsulinemic conditions, the cells only responded against platelet-activating factor (PAF) but not to the complement molecule C5a or to PMA. Also, the mRNA gene expression of IL-1A, IL-1B and C-C motif ligand 3 (CCL3) were increased upon stimulation with LPS in these subjects [28].

In an animal model of streptozotocin-induced type I diabetes and mycobacterium tuberculosis (MTB) infection, the main mechanisms for the development of immunopathology were evaluated. The main findings suggest that the innate immune cell-mediated inflammatory response is delayed and as a consequence, the interferon-gamma related response is also delayed. When the migration of the dendritic cells was analyzed in this context, no differences were found in the infected animals, only that of alveolar macrophages, highlighting the role of these cells in the association of infection of the respiratory tract in diabetics [29].

The adipose tissue releases a variety of pro-inflammatory and anti-inflammatory factors, including leptin, adiponectin and resistin. Leptin is a protein mainly produced by adipocytes. It has been shown that in db/db (with a non-functional leptin gene) nonfunctional protein, levels of leptin have been associated with alterations in the innate response [30]. The absence of the leptin receptor has a major impact on the production of some inflammatory genes and chemotactic proteins, particularly that of C-motif ligand 1 (XCL1), C-C motif ligand 2 (CCL2), C-X-C motif ligand (CXCL) 1, CXCL2, and CXCL13. Besides, retardation in the early recruitment of immune cells to the sites of infection was observed, indicating reduced chemotaxis and therefore, a lack of bacterial control in diabetic animal models [31]. These findings taken together, suggest that the immune infiltration in response to chemotactic factors is delayed in diabetes after infection, however, the underlying causes of these "lazy" leukocyte behavior are not well understood. However, it has been suggested that advanced glycation end products (AGE's) might be at least partially responsible, given that it has been associated with diabetic complications [32].

4. ALTERATIONS IN COMPLEMENT SYSTEM

The complement system is made up of a large number of distinct plasma proteins that work together to induce a series of inflammatory responses. The complement proteins are circulating proteases found in the bloodstream in an inactive form, which are sequentially activated by protease cleavage on the recognition of molecular components of microorganisms. Several reports have documented complement alterations in T2D, Pan et al, found that the fasting serum of patients with T2D present high concentration of C1q/TNF-related protein, the levels were higher in men than that in women (358.4 vs 232.7 ng/ml) in comparison with healthy subjects and this positively correlates with indicators of glucose metabolism [33], although the authors suggested that glycation can render these molecules inactive.

Regarding alterations in the alternative pathway of the complement system, a few years ago it was described in a prospective cohort of 1220 subjects followed for 3.3 years that the elevation of C3 complement levels was a strong predictor of cardiovascular risk and metabolic syndrome [34] and recently, Wlazlo et al associated the C3 levels with abnormalities in glucose metabolism and with an increased incidence of T2D in metabolic syndrome individuals that were followed for 7 years. A marked elevation of these molecules is observed in T2D and metabolic syndrome patients that the authors associated with increased cardiometabolic risk [35]. The authors suggest that such elevation could be due to increased levels of adipokines and that the increased metabolic risk could be associated with altered production of adhesion molecules in the endothelium. A further literature review found that in a report from 1990, it was described that C3 activity in hyperglycemic conditions was impaired, apparently through binding of glucose, the active site of C3 inhibits the attachment of this protein to the microbial surface [36]. Hair and colleagues recently confirmed this; hyperglycemic conditions inhibited S. aureus activation and deposition of C3 and iC3b on the bacterial surface. This could be affecting phagocytosis and microbial elimination. The authors go even further and determine by mass spectrometry the structural differences in the C3 molecules associated with glycation. The authors suggest that these differences might be associated with the functional impairment of complement activation in T2D patients [37].

In an experimental study in diabetic rats, Mauriello et al. detected a deficiency of the C4 component associated with susceptibility to infection by S. Aureus in T2D as well as with a reduction in C3 and C4. This reduction of C4 is probably associated with polymorphonuclear dysfunction and reduced cytokine response [38].

The binding of specific carbohydrate patterns to mannose-binding lectin (MBL), which are multimeric lectin complexes, can lead to the activation of the complement cascade. A recent study from Axelgaard et al describes that the injection of fluorescently labeled MBL to C57BL6 mice results in the accumulation of such fluorescent protein in the kidney of the animals [39]. This could increase the filtration rate and albumin loss that could lead to mucosal infection in diabetic patients. The mechanisms underlying these observations require further exploration to establish the possible effects of non-specific glycation in the reduced activity of the complement or the diminished signaling of the involved receptors.

5. ALTERATIONS IN PHAGOCYTOSIS AND ANTIGEN PRESENTATION

Phagocytosis is a cellular process that contributes to control intracellular growth and eliminate infectious agents as well as to remove cellular debris. Macrophages are major players responsible for this task as well as polymorphonuclear neutrophils and dendritic cells. Particularly, the phagocytosis process gains importance due to the fact that at least in dendritic cells and macrophages, it will ultimately lead to the generation of the adaptive immune response through antigen presentation.

In a study published by Delamaire et al in 1997, the authors describe in several conditions the altered functions of neutrophils in 61 patients (free of infection, T1D and T2D). In this article, the authors describe an impaired and reduced function of leucocytes for the phagocytosis of opsonized latex microbeads, also the authors describe a reduced bactericidal activity due to a reduction in oxidative killing potential. The results showed a significant association with poor glycemic and metabolic control (Hb1AC%, glucose). Also, a lower response in NO^- and O^- free radicals was detected upon stimulation of cells from diabetic patients compared to controls [27]. More recently, Raposo et al., described in an experimental model of whole blood infected *in vitro* with *Mycobacterium tuberculosis* (MTB) a deficient capacity of phagocytosis in monocytes and neutrophils from patients with T2D [40], the authors suggest that previously reported functional defects in phagocytosis could be mediated by the phagocytic receptors of the Fc gamma family of receptors. Restrepo et al. [40], showed a strong inverse correlation (R=-0.51, p=0.01) between phagocytosis via complement receptor CR3 (CD11c) as well as FCγRI (CD32), FCγRII (CD64) and FCγRIII (CD16) with a high HbA1c [41], further showing that chronic hyperglycemia in T2D patients may lead to generalized defects in the internalization process of phagocytosis. Recently our group has demonstrated that macrophages derived from monocytes that were isolated from persons with diabetes have an altered (reduced) phagocytic response against Mtb compared to those that were isolated from control individuals as well as the alteration in co-stimulatory molecules and a higher expression on the immune checkpoint inhibitor molecule PD-L1 [42].

After internalization, an important step in the phagocytic process is the formation of the phagosome-lysosome, necessary to destroy the pathogens. Recent evidence suggests that under hyperglycemic circumstances, the mechanism involved in its operation fails. Molina et al. reported a decreased expression of ATP6V1H mRNA in peripheral blood from T2D patients. This is an ATPase required to acidify the phagosome-lysosome compartment and facilitate the activation of other hydrolytic enzymes and therefore, such process is impaired in T2D patients. [43, 44]. Another important mechanism that contributes to antimicrobial activities in the phagolysosome is mediated by the oxidative burst, required for pathogen degradation. However, an excessive and ill controlled NOS and ROS production could lead to cytotoxicity. In a study by Ridzuan et al., it was shown that neutrophils isolated from T2D patients and activated with PMA secreted higher ROS and had higher activation of Nicotinamide adenine dinucleotide phosphate (NADPH) compared to the non-diabetic subjects [45]. Additional evidence by Huang et al, showed that

NADPH oxidase is increased as well as its p22phox subunit in monocytes underlies oxidative stress of patients with T2D; while the activity of superoxide dismutase (SOD) was lower in T2D patients than in the healthy controls [46], suggesting an impaired mechanism of oxidants detoxification that could be deleterious to the phagocytic cells and the whole innate immune system.

Further experimental evidence on the role of hyperglycemia and hyperinsulinemia comes from animal models. Park et al, in a db/db mouse model, studied the effects of such diabetic-like conditions. When animals were infected with *Staphyloccocus aureus* in the hind paw, a high bacterial burden developed in these animals and was associated with a diminished infiltration of leukocytes (mainly neutrophils) in the injury sites and with a reduced respiratory burst carried out by these cells. This does not happen in normal animals, further supporting the evidence observed in human observational and correlational studies [47].

S. Fisher et al. suggest a functional relationship between leptin concentrations and resistance to insulin in T2D that leptin and the leptin receptor could modulate several innate immune functions such as phagocytosis, respiratory burst and other adaptive immunity mechanisms (reviewed in [48]). Recently, our group described that diabetes alters the basal phenotype of human macrophages and diminishes their capacity to respond, internalize and control of *Mycobacterium tuberculosis* [42].

6. ALTERATIONS IN CELLS OF THE INNATE IMMUNE SYSTEM

6.1. Natural Killer Cells

Natural killer (NK) cells mediate important effects on other cells such as cell-cell recognition in the case of malignancy but also in the production of cytokines, chemokines and killer factors necessary to lyse target cells infected with a virus or transformed cells. However, whether its functions are compromised during metabolic alterations such as T2D has not been clearly explored. Kumar et al. showed that the frequency of NK cells from patients with TB- T2D is not altered in comparison with active TB only. The authors describe that the frequency of NK cell expressing CD107a like a marker of NK cell functional activity, was lower in response to MTB antigen stimulation in TB-T2D patients. Suggesting that T2D alters the NK cell response against M. tuberculosis [49], this could have relevance for intracellular bacterial pathogens such as MTB and also for viral infections. Also, given that patients with diabetes have a higher incidence of several types of cancer [50-53], it has been proposed that a reduction in the activation capacity of the NK cell compartment could be associated with such susceptibility, however, further evidence on the matter is needed.

Berrou et al. observed an altered NK cell phenotype, where the frequency of NKG2D-expression on NK cells was lower in diabetic patients compared to healthy controls, as well as the frequency of NKp46 positive cells. Both killer activation receptors (KAR) and Killer inhibitory receptors (KIR) expression were unchanged [54]. Given the primary role of NK cells, these changes in activation markers in NK cells could partly explain viral infection susceptibility in diabetics.

Invariant Natural Killer T (iNKT) cells are a subset of innate-like T cells that have an invariant T cell receptor [55]; these are CD1d-restricted NK cells that recognize self and foreign lipids. There is evidence suggesting that iNKT cells have a protective role in microbial infections against bacterial, viral, protozoan and fungal pathogens [56]. There are reports of defects in the iNKT cells population in both T1D) and T2D (Reviewed in [57]). In DM1, Beristan-Covarrubias et al. reported a reduced number of iNKT cells in whole blood (CD3+Vα24+Vβ11+) compared to non-diabetic first-degree relatives [58]**.** Although several reports describe alterations in these cells in animal models, the clinical implications and data from T2D patients are still lacking. Further research is needed to clarify the role of iNKT cells in T2D diabetic patient's innate immune response.

6.2. Alterations of MAIT Cells

Diabetic patients often require medical attention for infection management; medical visits are frequently related to mucosal infections such as urinary tract infections, sepsis, skin, and soft tissue infections and pneumonia [59]. About half the lymphocytes of the immune system are in the mucosa-associated lymphoid tissue (MALT). MALT is situated along the superficies of all mucosal tissues and its main function is to produce and secrete IgA across mucosal surfaces. The IgA promote B-cells clonal expansion; this occurs in response to antigen-specific T-cell activation [60]. A novel class of innate immune cells known as Mucosal-associated invariant T cells (MAIT) has recently been described. These cells are members of the innate T cells subset and are distributed throughout the blood and mucosal sites (Reviewed in [61]). The MAIT cells were first described by Tilloy et al. [62] and represent the most abundant innate T cells in humans. They express a canonical Vα7.2-Jα33 chain of the T-cell receptor (TCR), to probe ligands bound by the highly conserved molecule MHC-I related molecule (MR1) [63]. The MAIT cells possess a naïve phenotype, express the C-type lectin-like receptor CD161 at high levels [64], and about 50% of the MAIT cells express the T cells co-receptor CD8. These CD161++CD8+ T cells are functionally and transcriptionally distinct from other CD8+ T cells because they were identified as the unique population secreting IL-17 [65], and other inflammatory cytokines like TNFα. Moreover, this effector function is completely functional immediately upon egress from the thymus [66], which is not usual for other cell populations of the T cell lineage. MAIT cells are activated in response to ligands of MR1, such

as small organic molecules derived from riboflavin, and folic acid precursor metabolites produced by a variety of bacterial and fungi species, including M. tuberculosis [67]. After TCR activation, MAIT cells produce high amounts of cytokines such as IFN-γ, TNF-α, granzyme B, perforin, IL-17 and IL-22 [30, 68]. Recent reports have shown that female patients of European descent with T2D have alterations in the intestinal microbiota (determined by shotgun sequencing) compared to non-diabetic individuals; this suggests that such changes in microbiota could have major effects on mucosal infiltration of leukocytes [69]. Furthermore, recent studies in diabetic mice with streptozotocin-induced diabetes show a marked relationship between the intestinal microbiota and the frequency of the MAIT cell population. In the same report, it was found that MAIT cell deficiency in $Mr1^{-/-}$ NOD mice exacerbates the development of diabetes and alters the integrity of the gut mucosa [70]. Magalhaes et al reported that T2D patients exhibit several defects in the frequency of MAIT cells, particularly in obese patients. Reportedly, MAIT cells evaluated by flow cytometry from diabetics produce higher levels of IL-17A, as compared to lean control individuals. Additionally, a reduced frequency of MAIT CD161+MR1+ cells was observed for T2D obese individuals that increased in their frequency after bariatric surgery [71]. The mechanism underlying MAIT cell defects and increased IL-17 production remains elusive and further research is needed on this matter.

6.3. Myeloid-derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) have been described as immature monocytic and granulocytic cells with immune suppressive functions. Several reports suggest an elevation in the frequency of MDSCs in some pathologic conditions and it has been described that these cells are producers of interferon gamma (IFN-γ), IL-10 and transforming growth factor beta (TGF-β) [72, 73]. In recent studies, two immunophenotypes of MDSCs were evaluated in T2DM, the first was evaluated by Wang T et al. where they found an increase in the frequency of CD11b + CD33 + MDSCs and probe how these MDSCs inhibits T cell proliferation [74] and previous studies showed that peripheral blood MDSCs from type 1 diabetes mellitus patients inhibit T cell proliferation in a contact-dependent manner in vitro [75]. Recently, our group found an increased frequency of CD33 + HLADRlow/- MDSCs and verified that these MDSCs are producers of immunoregulatory cytokines such as IL-10 and TGF-β [75], such increase could be associated with the expansion of regulatory T (Treg) cells, several reports have described that the production of such cytokines in diabetics and obese people is increased and linked to insulin resistance [76]. MDSCs were linked in the last few years with increased susceptibility to infectious diseases, particularly it was recently described that frequencies of these cells are elevated in patients with tuberculosis [77], a study performed by Knaul et al. observed that functionally suppressive MDSC internalize MTB and promote a pro-inflammatory milieu that stimulates hematopoiesis, MDSC production and recruitment to the lung [78]. Taken together, the evidence of the increased frequency of MDSC suggests the likely participation of this cell population in the increased susceptibility to infection. More research is needed to clarify such an association.

7. INITIAL FEATURES OF DIABETIC PATIENTS AND SUSCEPTIBILITY TO INFECTION

As described previously, the alterations of the innate immune system in diabetic patients clearly have a broad impact on the susceptibility to infection. A summary of such processes is depicted in Figure **1** describing the alterations in innate immunity that have been reported so far in diabetes and that contribute to infection susceptibility in this highly vulnerable group of diabetics. In order to describe the possible implications of such innate immune alterations, we describe in the next paragraphs an example of such implications in several infections (bacterial, viral, etc.) that have been described in diabetes.

7.1. Bacterial Infections

7.1.1. Mycobacterium tuberculosis (Mtb)

People with diabetes may be more easily infected than non-diabetic people leading to a higher risk of latent TB infection. A meta-analysis by Jeon and Murray [79] of 13 observational studies and 1,786,212 participants with 17,698 TB cases found that the relative risk of TB among patients with diabetes was 3.11 (95% CI 2.27-4.26). Providing strong evidence of such an association of TB/diabetes.

During the infection process, *M. tuberculosis* must avoid the initial defensive barriers present within the respiratory tract to gain access to its host cells. The epithelial cells in the airway secrete antimicrobials including lysozyme and antimicrobial peptides such as defensins and cathelicidin (Reviewed in [80]). In vitro studies conducted by our research group show that increasing glucose concentrations correlate with the low expression of cathelicidin in uninfected cells, however, in cells infected with MTB, the expression of LL-37 increased substantially in concentrations of glucose higher, so, the mycobacterial load also increased; this result may be associated with the immunomodulatory activity of cathelicidin [81]. Additionally, humoral innate immune responses would be responsible for the elimination of the pathogen such as that of the complement and as previously described above, the diabetes process affects the complement-mediated immune response. The authors describe in an in vitro infection model of monocytes that the internalization of bacteria was impaired in the T2D group and also that heat inactivation of serum resulted in the loss of such differences, suggesting that the complement might be implicated in such process [82]. As described above in the previous sections of this review, there is strong evidence suggesting that hyperglycemia is associated with the loss of function of several complement molecules. In the natural course of MTB infection, the series of events that follow such

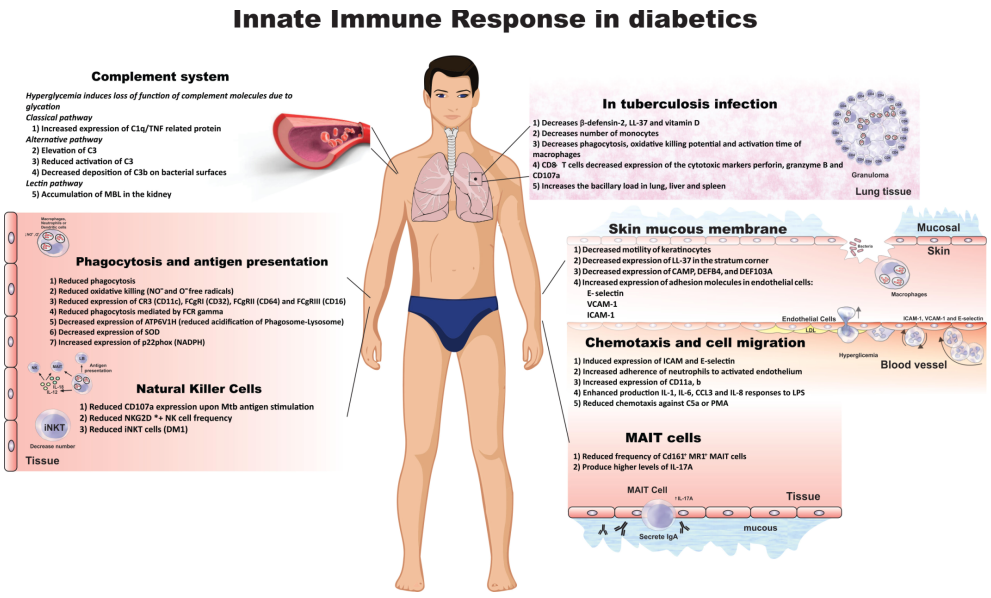

Fig. (1). Innate immunity impairment in Diabetes mellitus. Innate immunity prevents microbial colonization and entry into the host. Several mechanisms of innate immunity and the associated alterations in diabetes are described. The contribution of such alterations to infection is described in the context of TB infection (Right upper corner). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

infection would imply the recognition of MTB molecular patterns by innate receptors in the epithelial cell lining and the secretion of chemokines and cytokines to mount an effective immune response against the pathogen. To our knowledge, no alterations in such chemotactic responses have been reported on MTB infection and diabetes other than those mentioned above in diabetes alone.

If all defenses so far are avoided by MTB and the bacilli gain access to the alveolar space, the last line of defense is alveolar macrophages. It was described by Restrepo et al, that FC gamma receptor-mediated phagocytosis of MTB was also impaired in monocytes derived from T2D patients compared to non-diabetics [41]. On the other hand, in diabetic patients, the results show that the monocytes of T2D patients and low serum vitamin D levels show an altered capacity to control the intracellular growth of M. tuberculosis, which is not associated with a significant decrease in the expression of LL-37 or of human β-defensin-2. These data suggest that vitamin D could be the link between diabetes and susceptibility to tuberculosis [83].

The diabetic patients present alterations in the phagocytosis and a reduced bactericidal activity due to a reduction in oxidative killing potential [84]. Vallerskog et al. have shown that diabetic mice induced with streptozotocin are more susceptible to infection with tuberculosis due to an innate immune response delayed by alveolar macrophages exposed to M. tuberculosis. This phenomenon conditions the delay in antigen presentation to mount an effective adaptive immune response [29]. In addition, the bacillary loads in diabetic mice were significantly higher in the liver, spleen, and lungs compared with non-diabetics. Increased inflammatory lesions and a kinetic alteration of cytokines (TNF-α, MCP-1, IL-12, IFN-γ) in diabetic mice were also observed. The macrophages isolated from these mice had lower absorption of beads coated with mycolic acid and reduced bacterial internalization [85]. This phenomenon is also observed in humans where the association of M. tuberculosis with monocytes was significantly lower in diabetics (19.2 ± 6.1) than non-diabetics (27.5 ± 7.9; p = 0.02) [82]. These findings are in agreement with previous reports suggesting that diabetic rats have an alteration in the monocyte signaling that influences the survival of MTB due to the decrease in the timely activation of alveolar macrophages [86]. To our knowledge, no information regarding macrophage function and their differences in diabetics is available; therefore research on the matter is warranted.

The role of neutrophils in the early stages of MTB infection is still unclear. As described above neutrophils might play an important role in the early stages of MTB infection; however, diabetes mellitus modifies the

adhesion and expression of integrins in neutrophils [87], reduces chemotaxis [88], decreases phagocytosis [40, 89, 90] and reduces microbicidal activity in bacterial infections compared to neutrophils from non-diabetics [84]. Further research is needed on the molecular mechanisms associated with the impaired function of neutrophils in diabetes/tuberculosis comorbidity.

Once infected, cell populations such as CD8+ cytotoxic T cells and natural killer cells are important in the elimination of intracellular MTB. The population of Natural Killer cells in patients infected with tuberculosis suffering from diabetes mellitus is increased and characterized by expanded frequencies of NK cells stimulated by TB antigens expressing type 1 cytokines (TNF-α) and 17 (IL-17A e IL-17F) [49]. In contrast, CD8 (+) T cells were associated with significantly decreased expression of the cytotoxic marker perforin, granzyme B, and CD107a. This data suggests that pulmonary tuberculosis complicated by T2D is associated with an altered repertoire of CD8 (+) T and NK cells that express cytotoxic molecules and produce cytokines, possibly contributing to exacerbating pathology. The lung response to infection by immune and non-immune cells and humoral components is critical in determining whether the host will directly eliminate the pathogen or will undergo the formation of the granuloma.

7.1.2. Klebsiella Pneumoniae

Increased risk of *Klebsiella pneumoniae* live abscess (KPLA) has been reported in East Asia, particularly in Taiwan and Korea. Lin et al. reported that the effects of glycated hemoglobin (Hb1ac% above 7) were associated with increased formation and risk of KPLA. Suggesting that the uncontrolled glycemia was responsible for the effects on bacterial growth and control of the infection [91]**.** This is consistent with the thesis described above in the susceptibility of diabetic patients based on impaired immune mechanisms.

7.1.3. Enteric and Other Pulmonary Infections

An increased risk of enteric infection has been associated with the epithelial barrier dysfunction. Thaiss et al described that an alteration in the epithelial barrier function associated with GLUT-2 expression was responsible for the increased susceptibility of enteric infection. These alterations are associated with increased permeability of the epithelial barrier and TLR4 alterations [92]. Increased susceptibility to multidrug-resistant *Acinetobacter Baumanni* infection was identified in patients with chronic pulmonary failure. An increased odds ratio for infection was identified in patients with diabetes, which was identified as the only independent factor associated with an increased risk of infection [93].

7.2. Viral Infections

7.2.1. HIV Infection

In 2018, 38 million people lived with HIV infection around the globe according to data from the WHO (www.who.org). Identifying and characterizing the risk factors associated to infection is still a major area of research that could contribute to hinder transmission rates and to the proposal of new control strategies in vulnerable groups. It is known that several factors associated with mucosal and barrier immunology determine the outcome of initial contact for the transmission of HIV infection. Several risk factors have been identified for the transmission of infection such as 1) multiple sexual partners, 2) unprotected vaginal or anal sex, 3) having another transmitted infection, 4) sharing contaminated needles, among others. Recently, also a strong association (although causality cannot be properly established) has been reported for other diseases such as diabetes, metabolic syndrome, etc. [94], suggesting that diabetic individuals engaging in such risky behaviors have an increased risk of infection due to impaired immune function.

7.2.2. HCV Infection

Chronic viral liver disease has also been associated with diabetes. In a follow-up study to determine the frequency of the association between viral chronic liver disease by Iovanescu et al (2015), the authors found a significant association between the two [95]. Additionally, it has been reported that in chronic HCV infected patients who also have diabetes as comorbidity, an increased risk of progression to end-stage liver disease and liver cancer exist [96]**,** suggesting that the immune protection mechanisms associated with the elimination of cancerous cells are not working properly (namely NK cell function). The underlying mechanisms of such susceptibility have not been explored in detail, but as summarized in the previous sections, the reduction in the activity of NK cell activity (related to abnormal cell proliferation) and other migration-related mechanisms could contribute to such a process. Further investigation in this matter is needed.

7.2.3. Influenza Infection

A strong correlation and association have been established for influenza susceptibility infection in diabetic patients. Vaccination responses and cellular activation of diabetic individuals vaccinated with an H1N1 vaccine are reduced compared to non-diabetic individuals [97]**.** Also, following the H1N1 influenza pandemic in 2009, a strong association between diabetes and disease severity/ mortality was established and strongly suggested that oscillations in the levels of glucose could be responsible for both alterations in the immune response to the infection and also for an increased replication of the pathogen.

7.3. Other Fungal or Yeast Infections

In a study by Hine et al, the authors investigate the effect of glycemic control (measured as Hb1ac%) and the relationship with the incidence of infection. Sample sizes of 647,000 patients (34278 diabetics and 613052 for controls) were included. This study concluded that bronchitis, pneumonia, skin and soft tissue infections, etc were all increased in diabetic patients and with

worse glycemic control. Almost all infections analyzed were more common in people with T2D [98].

8. MOLECULAR MECHANISMS ASSOCIATED WITH IMMUNE IMPAIRMENT IN T2D

T2D is a complex disease characterized by altered glucose and lipid metabolism that slowly drives to chronic insulin resistance. Therefore, a better understanding of molecular mechanisms underlying defects in this process is crucial for the understanding of the role that such processes play in the immune system impairment in T2D. Few studies have analyzed the implications of metabolic alterations as the cause for immune impairment; however, several signaling pathways and metabolic processes could be affected.

8.1. Insulin Receptor Signaling

In normal conditions, insulin is involved in homeostasis of glucose and lipids by binding to insulin receptor (IR). A very detailed description of the process is known and involves the phosphorylation on serine of insulin receptor substrate (IRS) with subsequent activation of phosphoinositide 3-kinase (PI3K) and mechanistic target of rapamycin (mTOR). Akt also regulates all of this process. The inhibition of Protein kinase Akt can be sufficient to block PI3K dependent activation of mTOR in response to insulin, Akt must be phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) using PI3K-generated PIP3. The signal of Akt inhibits the tuberous sclerosis complex (TSC), the specific GAP for the small GTPase Rheb, this relieves inhibition of Rheb, allowing it to become active and stimulate mTOR kinase activity (reviewed in [99]), the PI3K signaling has been related in many aspects of complications of diabetes mellitus at level cardiovascular, neurological and urinary system the direct link with the function of immune cells is still to be determined but these regulators are known to regulate the metabolic functions of a broad group of immune cell types [100].

8.1.1. ROS Production

Concentrations of free fatty acids and glucose are increased in patients with T2DM [101]; these molecules are important sources for efficient cellular energy production. They are catabolized to generate acetyl-CoA, which serves as a substrate for ATP synthesis into mitochondria. An excess of this process critically induces reactive ROS generation and could be the cause of alterations in the function of several immune-associated functions. The recent advent of metabolomics to study immunological processes will help to delineate the role of immunometabolism in T2D innate immune impairment.

8.1.2. Mitochondrial Alterations

Including density and reduced phosphorylation dysfunction alters insulin action, mainly via subsequent oxidative stress and lipid accumulation, whereas endoplasmatic reticulum (ER) through the activation of the adaptive response is called unfolded protein response (UPR). The UPR signaling sensor of unfolded proteins present in the ER-transmembrane are inositol-required protein 1 (IRE1), activating transcription factor (ATF6) and PKR-like ER kinase (PERK) when these sensors fail to restore the homeostasis, cell death signaling pathways are activated [102]. ER-mitochondria contact sites also support ROS-mediated signals that influence mitochondria functions. Indeed, loss of PERK induced mitochondria-associated membranes (MAM). The physical interaction between ER and mitochondria does not involve membrane fusion but is mediated through protein bridges; space is small up 10 to 25 nm ER [103]. The protein interaction is between the voltage-dependent anion channel (VDAC) at the outer mitochondrial membrane and the inositol 1,4,5-triphosphate receptor (IP3R) at the ER through the molecular chaperone glucose-regulated protein 75 (Grp75), allowing Ca2+ transfer, in addition to a large amount of phospholipid [104, 105] all of these mechanisms could be associated with impairment of superoxide production in phagocytes.

8.1.3. Endoplasmic Reticulum Stress

ER is the first station of the secretory pathway and the site of synthesis for proteins resident or destined for the Golgi compartment, endosomes, lysosomes, the plasma membrane, and extracellular milieu; it also serves as a site of biosynthesis of lipids and Ca^{2+} storage. Disruption of the ER-mitochondria contact sites prevents the formation of ATG14, a unique subunit of the autophagy specific-PI3K complex [106]. Autophagy has been associated with the elimination of intracellular bacteria in macrophages.

8.1.4. Formation of Advanced Glycation End Products

AGE are products of nonenzymatic glycation and oxidation of proteins. AGEs also activate signaling mechanisms via Receptor for advanced glycation end products (RAGE) that cause cell stress contributing to cellular dysfunction. RAGE/AGE production has been associated with damage to target organs leading to complications. The interaction of AGE-RAGE on innate immune cells has a functional modulating role inducing chemotaxis and activation of nuclear factor-κB (NF-κB) and regulating inflammatory cytokines such as TNF-α, IL-1β, and IL-6. Nielsen et al. describe that diabetes causes the host to become hypersusceptible to infections caused by increased inflammation driven by TL4 and continued by RAGE both signaling through a common pathway via MyD88 [107], given that RAGE and its ligands HMGB1 and AGE were higher in T2DM patients [108]. Further studies are needed to delineate the role of AGE/RAGE on immune impairment in T2D.

8.1.5. Alterations in Cytokine Production Due to Hyperglycemia

Neutrophils are glucose-sensitive inflammatory cells. Under conditions of hyperglycemia, these cells secrete S100 calcium-binding proteins A8/A9 (S100A8/A9) that interact with the RAGE on hepatic cells resulting in an increased production of IL-6. Elevated plasma levels of these proteins are related to

chronic inflammatory disorders related to thrombocytosis, atherogenesis and cardiovascular disease in diabetes [109].

CONCLUSION

Diabetes mellitus is a highly prevalent metabolic disease that has several associated complications and is growing at an alarming rate with an expected diabetic prevalence in 2050 of 14% in the world population [110]**.** Among these, infections have been associated with an increased risk of mortality in these subjects. Clearly, the innate immune system plays a fundamental role in the protection of the internal milieu in response to infection, but several of these important mechanisms have been described to be defective or at least partially defective in diabetics. The frequency and functions of several cell populations are also affected such as macrophages, epithelial cells, innate lymphoid cells, and neutrophils. Given that innate immunity and particularly phagocytosis, chemotaxis and cytokine production are of pivotal relevance for the initiation of the adaptive immune response; these subjects suffer from frequent infections that derive from these defective innate immune mechanisms.

LIST OF ABBREVIATIONS

WHO	=	World health organization
IDF	=	International diabetes organization
T2D	=	Type 2 diabetes
HR	=	Hazard ratio
OR	=	Odds ratio
UTI	=	Urinary tract infection
STAT	=	Signal transducer and activator of transcription
AMP	=	Antimicrobial peptide
ICAM-1	=	Intercellular Adhesion Molecule 1
VCAM-1	=	Vascular cell adhesion molecule 1
ROS	=	Reactive oxygen species
DAMP	=	Damage-associated molecular
fMLP	=	N-formyl-methionyl-leucyl-phenylalanine
PMA	=	Phorbol 12-myristate 13-acetate
LPS	=	Lipopolysaccharide
IL	=	Interleukin
TNF-α	=	Tumor necrosis factor-alpha
PAF	=	Platelet-activating factor
CCL3	=	C-C motif ligand 3
MTB	=	Mycobacterium tuberculosis
XCL1	=	C-motif ligand 1
CCL2	=	C-C motif ligand 2
CXCL	=	C-X-C motif ligand
AGE	=	Advanced glycation end product
KAR	=	Killer activation receptors
KIR	=	Killer inhibitory receptors
iNKT	=	Invariant Natural Killer T
MALT	=	Mucosa-associated lymphoid tissue
MAIT	=	Mucosal-associated invariant T cells
TCR	=	T-cell receptor
MR1	=	MHC-I related molecule
MDSC	=	Myeloid-derived suppressor cell
IFN-γ	=	Interferon gamma
TGF-β	=	Transforming growth factor beta
Treg	=	Regulatory T
KPLA	=	*Klebsiella pneumoniae* live abscess
PDK1	=	Phosphoinositide-dependent kinase
TSC	=	Tuberous sclerosis complex
IRE1	=	Inositol-required protein 1
ATF6	=	Activating transcription factor
PERK	=	PKR-like ER kinase
MAM	=	Mitochondria-associated membranes
IP3R	=	Inositol 1,4,5-triphosphate receptor
Grp75	=	Glucose-regulated protein 75
RAGE	=	Receptor for advanced glycation end products
NF-κB	=	Nuclear factor-κB
S100A8/A9	=	S100 calcium-binding proteins A8/A9

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Calpain Participates in Cortical Cytoskeleton Modification and DNA Release during Neutrophil Extracellular Trap Formation

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Keywords
Calpain · Neutrophil extracellular traps · Phorbol myristate acetate · Cytoskeleton · Calcium ionophore

Abstract
Introduction: The formation of neutrophil extracellular traps (NETs) is a process in which several kinds of enzymes participate generating posttranslational modifications of proteins. NETs have been associated with infectious, autoimmune, and inflammatory diseases. Inhibition of several proteases reduces the formation of NETs. In the present work, we analyzed the role of several broad-acting and specific inhibitors of proteases in the formation of NETs. ***Methods:*** Neutrophils were isolated from peripheral blood of healthy individuals by density gradient. The neutrophils were quantified and seeded into cell culture plates. Phorbol myristate acetate and A23187 were used as NETs inducers, and several specific inhibitors of proteases were used. The cells were stained for cytoskeleton or DNA. The cell-free supernatants were used to assess DNA release. Statistical analysis was carried out by a Kruskal-Wallis or ANOVA test. ***Results:*** We observed marked changes in actin organization after the induction of NETs, suggesting that the cytoskeleton is being actively regulated. When we used protease inhibitors, the release of DNA was reduced, suggesting the participation of actin remodeling in the process. Further characterization of the specific proteases revealed that calpain modulates the reorganization of actin cytoskeleton and DNA release. Preservation of part of the actin cytoskeleton suggests that DNA release is not only a mechanic process associated to the chromatin decondensation; rather the process is highly regulated by active proteases that promote cytoskeleton reorganization and chromatin decondensation that culminates in DNA release. ***Conclusion:*** Calpain mediates the DNA release in the NET formation process by the modification of cortical actin cytoskeleton in a calcium-dependent manner.

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Introduction

Takei et al. [1] did the first description of the effects of phorbol myristate acetate (PMA) on neutrophil chromatin decondensation in 1996. The authors described major changes in the morphology of neutrophils caused by the PMA-mediated activation of PKC [1]. Later, in 2004 Brinkmann et al. [2] described in a seminal paper such changes and further characterized the phenomenon. They coined the term "NETs" (derived from neutrophil extracellular traps) that quickly became a topic of interest in the neutrophil biology. Several groups have explored the implications of such mechanism of neutrophil death in infectious and noninfectious diseases [2]. It was later demonstrated by Papayanopoulos et al. [3] that the inner workings of such process involved the generation of reactive oxygen species as well as the role of myeloperoxidase and elastase as necessary steps in the formation of NETs [3].

Further developments and characterizations of the process described how (peptidyl-arginine deiminase 4) PAD4-mediated citrullination was necessary for the relaxation of the histone/DNA complex and the formation of NETs [4, 5]. Since PAD4 and the citrullination process have been associated to the generation of autoantigens, and in consequence to autoantibodies in rheumatoid arthritis, this process was explored and found to be associated with other autoimmune diseases [6, 7]. However, the molecular mechanisms associated with NET formation are still not fully described; thus, further characterization could lead to the identification of other relevant molecular targets. In this regard, the participation of the actin cytoskeleton has not been fully described. The marked changes in the morphology of NET forming neutrophils suggest the participation of the cytoskeleton and accessory proteins [8]. Stojkov et al. [9] describe how the dynamics of actin and tubulin filaments of cytoskeleton are clearly modified during NET formation by a NADPH and glutaredoxin mediated mechanism. Nevertheless, the participation of proteases associated to such processes has not been fully characterized during NET formation.

During NET formation, elastase has been directly linked to histone co-localization to the nucleus and the decondensation of chromatin [3]. It is not known whether the membrane rupture of neutrophils undergoing NET formation is associated with the increasing volume of the nucleus or an enzyme-mediated process.

The participation of the cytoskeleton as well as the events associated to its modification also remains to be elucidated. In this regard, an important group of calcium-dependent proteases called calpains could mediate such process. These proteases (classified as μ and **m** according to the calcium concentration required for their activation) [10] are well known for the regulation and activation of cytoskeleton in neutrophils [11–13]. Therefore, we aimed to describe the changes in the actin cortical cytoskeleton and to identify the proteases that participate in the regulation of actin dynamics as well as their role in nuclear decondensation and DNA release during the NET formation process in neutrophils.

Materials and Methods

Isolation of Neutrophils

Peripheral blood samples from healthy male donors were used for the isolation of polymorphonuclear cells). Anticoagulated blood was diluted in an equal amount of Hanks' Balanced Salt Solution layered on Lymphoprep (Axis Shield) and centrifuged (20 min at 400 *g*). The plasma and mononuclear cells were removed, and the remaining neutrophils and erythrocytes were resuspended in 25 mL of cold lysis buffer ($NaHCO_3$ 14 mM, NH_4Cl 15.6 mM, and EDTA 0.126 mM). Cells were then incubated for 10 min on ice, and cell debris was removed by centrifugation (5 min at 400 *g*). Neutrophils were washed twice with Hanks' Balanced Salt Solution, resuspended in 3 mL of RPMI-1640 (without phenol red, with 1× of L-glutamine), and supplemented with 2% human serum albumin (Gilson). Trypan blue dye exclusion (Sigma-Aldrich) indicated a viability >97% after isolation. The purity of the isolated neutrophils was evaluated by flow cytometry with CD66 (Becton Dickinson) labeled with CF405 (Biotium) and found to be over >91% neutrophils (see online suppl. Fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000515201). Adult donors (age ≥20 years) signed an informed consent in accordance with the declaration of Helsinki.

NETs Induction

3×10^5 neutrophils were seeded onto a 24 well-plates on 0.01% poly-L-Lysine-coated glass slides (Sigma-Aldrich) and incubated at 37°C under 5% CO_2 for 30 min in the following conditions: protease inhibitor cocktail (Calbiochem 539131) or 1 mM phenylmethyl sulfonyl fluoride (PMSF, Sigma-Aldrich) or 5 mM EDTA (Sigma-Aldrich) or 50 μM N-acetyl-Leu-Leu-Norleu-al (ALLN, Santa Cruz Biotechnology) or 100 μM calpeptin (Sigma-Aldrich) before addition of NETs inducers 30 nM PMA (Sigma-Aldrich), 25 μM calcium ionophore A23187 (Sigma-Aldrich) or with 10% of a serum pool of RA patients with high titers of anti-citrullinated antibodies (ACPA positive). Induction kinetics were performed every 30 min up to 180 min or continuous incubations of 180 min as indicated. Then culture medium was transferred to a new tube and centrifuged (5 min at 22,000 *g*), and the cells were fixed with 4% PFA (in PBS). After centrifugation, the cell-free supernatant was transferred into a new tube and stored at −20°C for further analysis. Untreated neutrophils were used as negative controls.

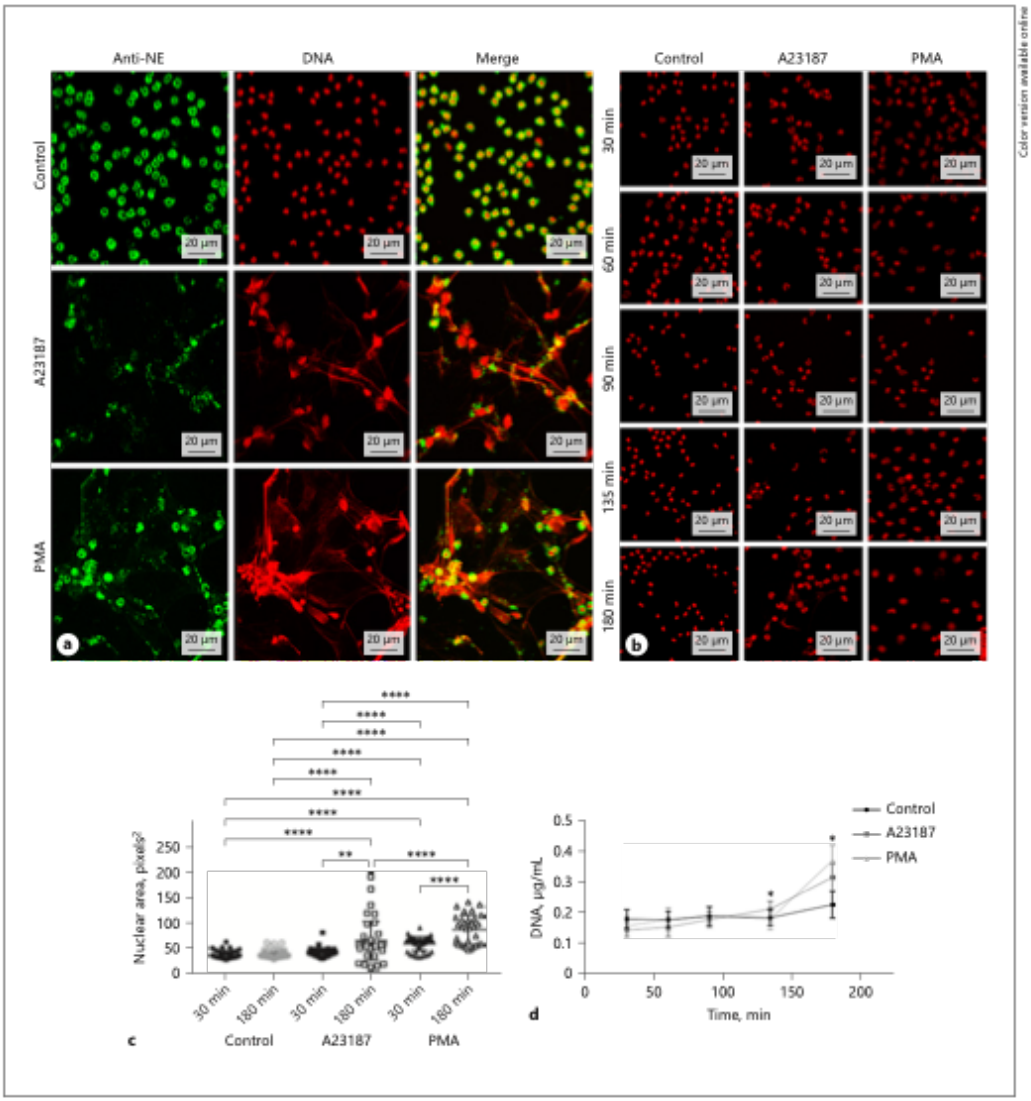

Fig. 1. Nuclear changes and DNA release during the induction of NETs. Neutrophils isolated from healthy donor 3×10^5 cells/well the induction of NETs was performed with. **a** Control, A23187 (25 µM), PMA (30 nM) were incubated for 180 min at 37°C, 5% CO_2, and 95% O_2, fixed with 4% PFA and stained with anti-NE-DyLight 488 and PI, and analyzed by confocal microscopy. **b** The cells stimulated were fixed every 30 min with 4% PFA and stained with Draq5 and subsequently analyzed by confocal microscopy. **c** The nuclear area was analyzed using FIJI software, the graph shows mean and SD, one-way ANOVA ($p = 0.0001$). **d** Quantification of the DNA released during the formation of NETs with PicoGreen. The graph shows mean and SEM, 2-way ANOVA stimulus ($p = 0.0156$) and time ($p = 0.0001$). NET, neutrophil extracellular trap.

Quantification of Cell-free DNA from NETs

Cell-free DNA released from neutrophils by induction of NETs was quantified as extracellular DNA in culture supernatants using the Quanti-iT PicoGreen KIT (Thermo-Fisher Scientific). Briefly, 50 μL of cell-free supernatant were transferred into a 96-well plate, equal volumes of PicoGreen reagent were added to each well, and the plate was incubated for 5 min at room temperature under protection from light. The plate was analyzed in a Fluoroskan fluorometer (Thermo-Fisher) with an excitation wavelength of 485 nm and the emission wavelength of 535 nm. DNA concentration was calculated using a standard curve ranging from 5 μg/mL to 40 ng/mL generated from a serial dilution of salmon sperm DNA.

Cytoskeleton Staining and Visualization of NETs

For the visualization of NETs, glass coverslips were washed 3 times with PBS 1×. The cells were permeabilized with 0.5% Triton X-100 in PBS 1× for 5 min and blocked using 5% BSA for 30 min at room temperature, coverslips were then washed 3 times with PBS 1× (According to Brinkman et al. [14] report). Next, neutrophils were incubated with 2 U/mL phalloidin-AF488 (Santa Cruz Biotechnology), anti-NE (Invitrogen), anti-PAD4 (Novus biologicals), anti-H3cit (Abcam), or anti-calpain (Santa Cruz Biotechnology) for 45 min at room temperature. After 3 washes, cells were incubated for 5 min with Vindelov's reagent without RNAase or DRAQ5 as indicated (DRAQ5 was used in some experiments to avoid spectral overlapping of propidium iodide). Finally, after washing, the glass slides were embedded in Fluoroshield (Sigma-Aldrich). Samples were visualized using a Leica confocal microscope DM2500 (Leica Microsystems).

Image Analysis

The nuclear area (PI or Draq5) and phalloidin-AF488 mean fluorescence intensity (MFI) were analyzed using FIJI software. Briefly, a Gaussian filter was applied to every image, the color was reversed, and a binary mask adjusted to 5–15% was applied. Afterward, the detection of particles was done filtering by size and eliminating those found in the corners. The area value for the events was obtained for each image, and the results were subsequently analyzed (online suppl. Fig. 2 in online suppl. material).

Statistical Analysis

Data were analyzed using GraphPad Prism 7.0 software. NET visualization and quantification were performed in at least 3 independent experiments with 2 replicates. Using repeated-measures one-way ANOVA or Friedman test, according to the distribution of the data, we compared differences among treatments. Posttest (Tukey or Dunn) was used to identify differences among specific conditions. The interaction p value among treatment and time was determined by 2-way ANOVA. Differences were regarded as significant at a level of $p < 0.05$.

Results

DNA Release and Nuclear Decondensation Coincide during NET Formation

We evaluated in neutrophils by the use of time-lapse experiments, the changes in nuclear chromatin condensation levels, and the release of DNA to the culture supernatant in response to the calcium ionophore A23187 or the PKC activator PMA, which are well-known inducers of NETs. The characteristic features of NETosis and granule proteins were verified by detection of neutrophil elastase (Shown in Fig. 1a). Also, the detection of citrullinated histones, PAD4 staining, and calpain presence was made by confocal microscopy (online suppl. Fig. 3 in online suppl. material). As a more physiological inducer, we used the serum of RA patients, with antibodies against citrullinated proteins (online suppl. Fig. 4 in online suppl. material).

As can be observed in Figure 1b, there is a complete chromatin decondensation at 135 min of treatment. This is accompanied by an increase in the nuclear area measured by the FIJI area tool and showing significant differences for the nuclear area when comparing the control condition against the PMA or A23187 conditions (Fig. 1c, $p < 0.001$). Additionally, this is coincident with DNA release measured by PicoGreen which reached its maximum at 180 min ($p < 0.0001$) as shown in Figure 1d, this process is dependent on the stimulus ($p < 0.0156$).

The Dynamics of Actin Cytoskeleton Are Affected during the NET Induction Process

As mentioned before, the role of the actin cytoskeleton during the NET formation process has not been described yet. Therefore, using phalloidin-AF488, we evaluated the changes in the staining pattern of the actin cytoskeleton during the induction of NETs in neutrophils with PMA and A23187. As shown in Figure 2a, clear changes in the distribution of actin can be observed throughout the 3-h period in cells stimulated with PMA or A23187 versus control cells. Also, we observed a reduced phalloidin staining of actin filaments in the case of A23187 stimulation as well as the presence of a speckle-like pattern, which suggests the likely degradation of the actin cortical cytoskeleton. In the case of PMA-induced NETs, the speckle-formation indicates the likely vesiculation of the cell content accompanying the DNA release.

Proteases Are Implicated in Cytoskeleton Dynamics, Chromatin Decondensation, and DNA Release during NET Formation

As previously described, the role of proteases, and in particular the role of elastase, was described by Papayannopoulos et al. [3]. To provide further insight into the participation of other proteases, we evaluated the effects of broad protease inhibitors to inhibit the NET release process. As shown in Figure 2a, a uniform staining of ac-

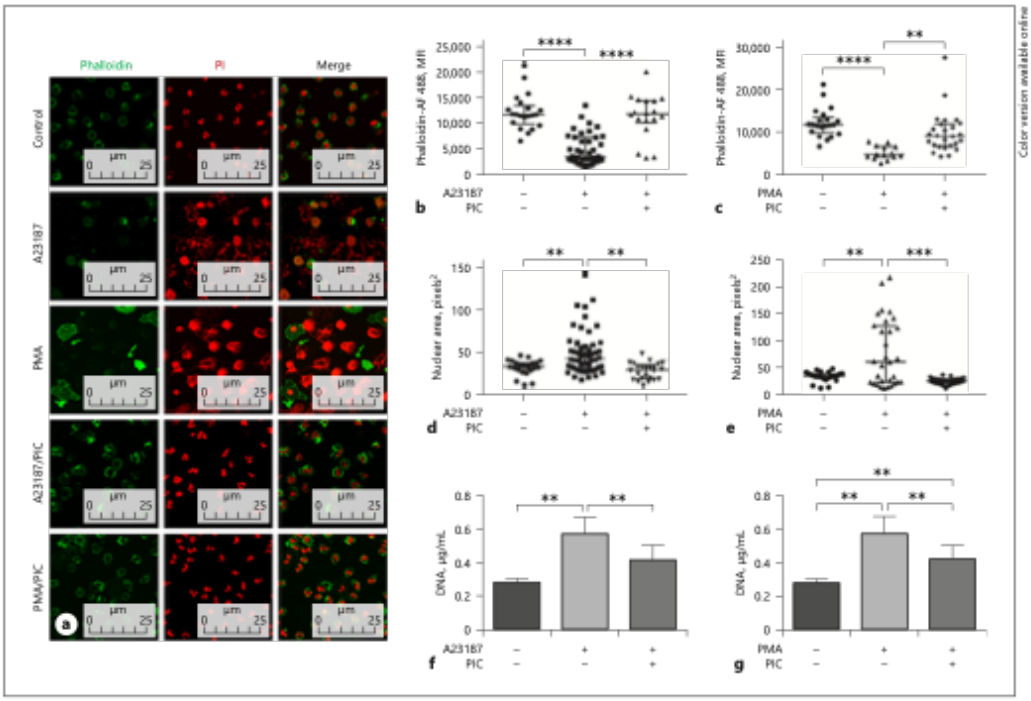

Fig. 2. Proteases participate during the formation of NETs. Neutrophils isolated from healthy donors (3×10^5 cells/well) preincubated for 30 min with PCI were used for the induction of NETs with A23187 (25 µM) and PMA (30 nM) for 180 min at 37°C, 5% CO_2, and 95% O_2. **a** Cells were fixed with 4% PFA, then stained with phalloidin-AF488/PI and subsequently analyzed by confocal microscopy. **b**, **c** Analysis of MFI on FIJI software of cells stimulated with A23187 ($p = 0.0001$) or PMA ($p = 0.0001$), the graph shows median ± IQR, Kruskal-Wallis. **d**, **e** Analysis of changes in nuclear area during the formation of NETs stimulated with A23187 ($p = 0.0001$) or PMA ($p = 0.0002$), the graph shows median ± IQR, Kruskal-Wallis. **f**, **g** Quantification of DNA released during the formation of NETs stimulated with A23187 ($p = 0.005$) or PMA ($p = 0.0001$), the graph shows mean ± SD, one-way ANOVA ($p = 0.005$). NET, neutrophil extracellular trap; MFI, mean fluorescence intensity.

tin filaments by phalloidin is observed in the control neutrophils. However, when A23187 or PMA was used f-actin staining fades, suggesting that actin cytoskeleton is affected during the NET formation. As shown in Figures 2b and c, the staining of actin filaments (MFI) is clearly diminished after induction with A23187 or PMA, which is prevented by the pretreatment with protease inhibitors ($p < 0.001$). However, upon induction with either A23187 or PMA, the characteristic loss of nuclear morphology and formation of DNA fibers appears, and when pretreated with broad protease inhibitors, such changes in the nucleus are prevented from happening as shown in the measurement of nuclear areas (Fig. 2d, e, $p < 0.05$), as well as major changes in the cytoskeleton. This suggests that proteases are implicated in such changes of chromatin decondensation and in the distribution of the filamentous actin throughout the NET induction process. Additionally, as shown in Figure 2f and g, the release of intracellular DNA to the culture media is significantly increased in both the A23187 and PMA treated cells ($p < 0.032$). This increased in the release in DNA is lowered to basal levels when mixtures of serine/threonine, cysteine, and metallo-protease inhibitors are used. This suggests that DNA release is mediated by protease activity over different cellular structures and not only the nucleus as previously described.

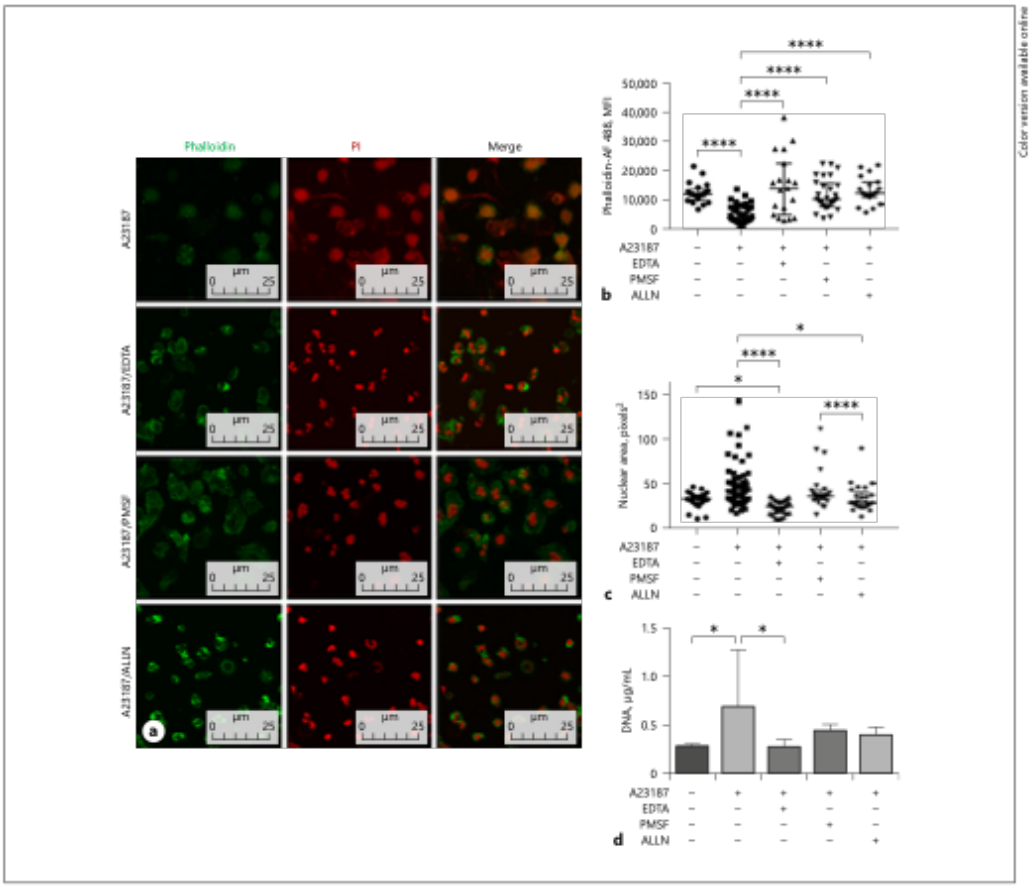

Fig. 3. Calcium-dependent proteases involved in the formation of NETs induced with A23187. Neutrophils isolated from peripheral blood from healthy donors (3×10^5 cells/well) were preincubated for 30 min with PMSF (serine protease inhibitor), ALLN (cysteine-protease inhibitor), or EDTA (metallo-proteases inhibitor). The induction of NETs was performed with A23187 (25 µM) incubated for 180 min at 37°C, 5% CO_2, and 95% O_2. **a** Cells were fixed with 4% PFA, stained with phalloidin-PI, and subsequently analyzed by confocal microscopy. **b** Analysis of MFI on FIJI software, the graph shows median ± IQR, Kruskal-Wallis ($p = 0.0001$). **c** Analysis of changes in nuclear area during the formation of NETs, the graph shows median ± IQR, Kruskal-Wallis ($p = 0.0001$). **d** Quantification of DNA released during the formation of NETs, the graph shows median ± IQR, Friedman test ($p = 0.0032$). NET, neutrophil extracellular trap; ALLN, acetyl-Leu-Leu-Norleu-al; MFI, mean fluorescence intensity.

Calcium-Dependent, Serine/Threonine, and Cysteine Proteases Mediate DNA Release during NET Formation

To further dissect the role of specific proteases, we analyzed specific groups of proteases. A23187 treated cells were analyzed for calcium dependency, effect of serine (PMSF), and cysteine-protease inhibitors (ALLN a broad calpain inhibitor). The changes in the staining for the F-actin cytoskeleton are partially recovered with the divalent cation-chelator and with ALLN but not with PMSF

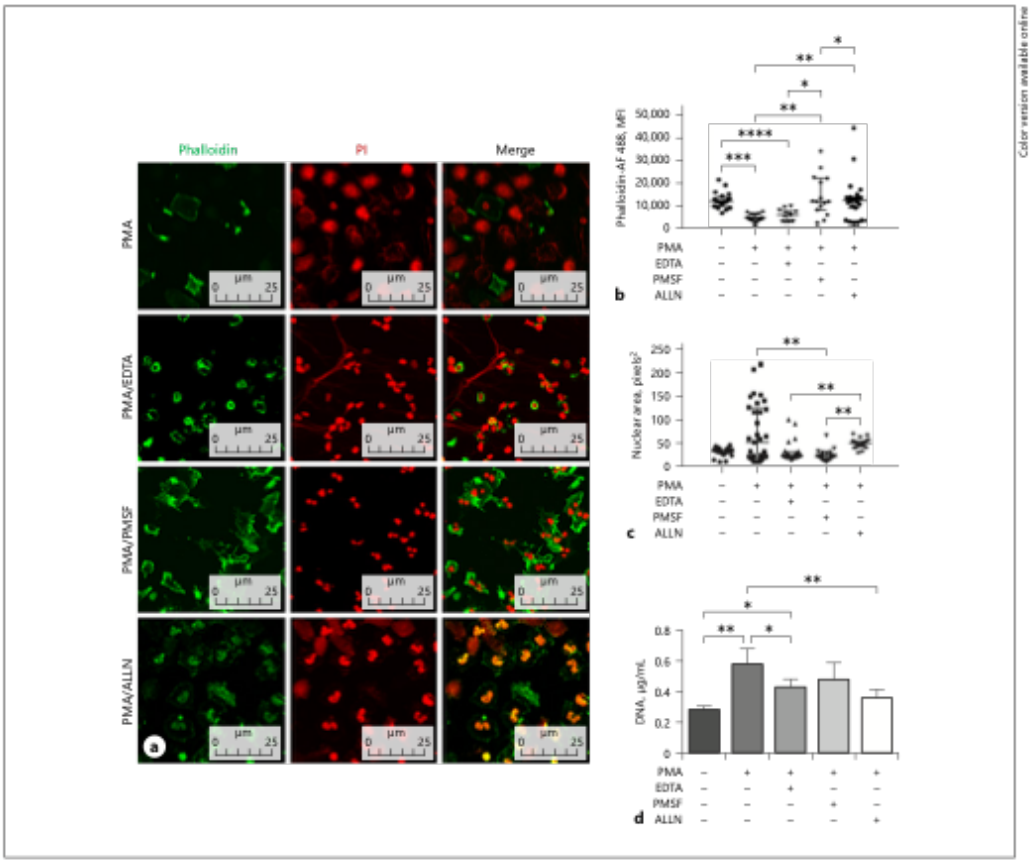

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Fig. 4. Calcium-dependent proteases involved in the formation of NETs induced with PMA. Isolated Neutrophils were preincubated for 30 min with PMSF (serine protease inhibitor), ALLN (cysteine-protease inhibitor), or EDTA (metallo-proteases inhibitor). The induction of NETs was performed with PMA (30 nM) incubated for 180 min at 37°C, 5% CO_2, and 95% O_2. **a** Cells were fixed with 4% PFA, stained with Phalloidin-PI, and subsequently analyzed by confocal microscopy. **b** Analysis of MFI on FIJI software, the graph shows median ± IQR, Kruskal-Wallis ($p = 0.0001$). **c** Analysis of changes in nuclear area during the formation of NETs, the graph shows median ± IQR, Kruskal-Wallis ($p = 0.0001$). **d** Quantification of DNA released during the formation of NETs, the graph shows median ± IQR, repeated-measures one-way ANOVA ($p = 0.0033$). NET, neutrophil extracellular trap; PMSE, phenylmethyl sulfonyl fluoride; ALLN, acetyl-Leu-Leu-Norleu-al; MFI, mean fluorescence intensity.

in A23187-induced NETs (Fig. 3a). In the case of PMA-induced NETs, inhibition by EDTA, ALLN, and PMSF partially reduces F-actin polymerization as shown in Figure 3b ($p < 0.05$). For all inhibitors, a reduced chromatin decondensation was observed compared to controls (Fig. 3c, $p < 0.05$), suggesting that such changes might affect DNA release. To further characterize DNA release, culture supernatants were evaluated by PicoGreen assay. DNA release is reduced when EDTA is used, but in the case of ALLN and PMSF, only a trend toward reduction is observed (Fig. 3d, $p = 0.0032$). In the case of PMA-induced NETs, clear changes in the distribution of phalloi-

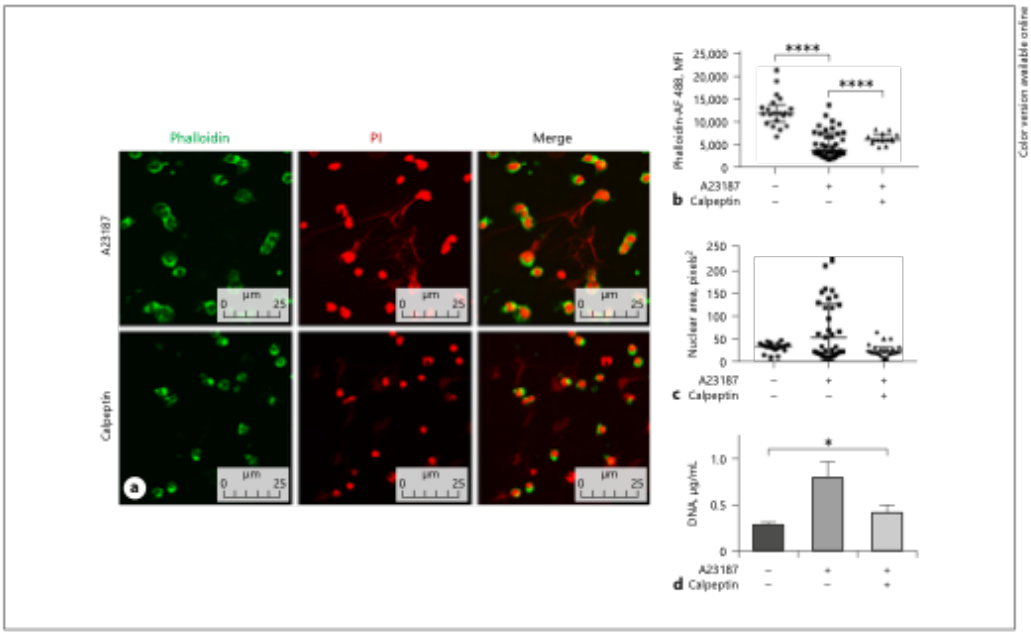

Fig. 5. Participation of calpain during the formation of A23187-induced NETs. Neutrophils isolated from peripheral blood from healthy donors (3×10^5 cells/well) were preincubated with calpeptin (100 µM) for 30 min. Induction of NETs was performed with A23187 (25 µM) incubated for 180 min at 37°C, 5% CO_2, and 95% O_2. **a** Cells were fixed with 4% PFA, stained with phalloidin-PI, and subsequently analyzed by confocal microscopy. **b** Analysis of MFI on FIJI software, the graph shows median ± IQR, Kruskal-Wallis ($p = 0.0001$). **c** Quantification of DNA released during the formation of NETs stimulated with A23187, the graph shows mean ± SD, RM-one-way ANOVA ($p = 0.033$). **d** Analysis of changes in nuclear area during the formation of NETs, the graph shows median ± IQR, Kruskal-Wallis ($p = 0.0001$). NET, neutrophil extracellular trap; MFI, mean fluorescence intensity.

din staining are observed (Fig. 4a), and the MFI quantification shows that such changes in actin loss are reverted with PMSF and ALLN but not with EDTA (Fig. 4b, $p < 0.05$). In the case of the nuclear area quantification, PMSF induces a significant reduction in the nuclear area compared to PMA treated cells (Fig. 4c, $p < 0.05$); EDTA and ALLN show a nonsignificant difference, but a trend is observed. Additionally, a partial reduction in DNA release is observed for EDTA and ALLN treated neutrophils (Fig. 4d, $p < 0.05$). Taken together, these results suggest the participation of calcium in such inhibition and the participation of cysteine proteases (such as the proteasome, calpain, or others) in the changes observed in the cytoskeleton as well as in DNA decondensation and release during NET formation.

Calpain Inhibition Induces the Formation of F-Actin Structures in NETs from Neutrophils and Impair DNA Release

To further characterize the role of calpain in the regulation of DNA release during NET formation, the specific inhibitor calpeptin was used. This is a µ and m calpain inhibitor. As observed in Figure 5a, in neutrophils treated with A23187, the use of calpeptin reduces both chromatin decondensation (lower lane) and the staining of actin filaments. The formation of a strong focal staining of F actin by phalloidin suggests that such inhibition of calpain prevents the degradation of the cortical cytoskeleton (Fig. 5b, $p < 0.05$) and that calpain is partially responsible for the observed nuclear decondensation (Fig. 5c, $p < 0.05$). Also, DNA release was reduced when comparing A23187 alone versus calpeptin-treated cells

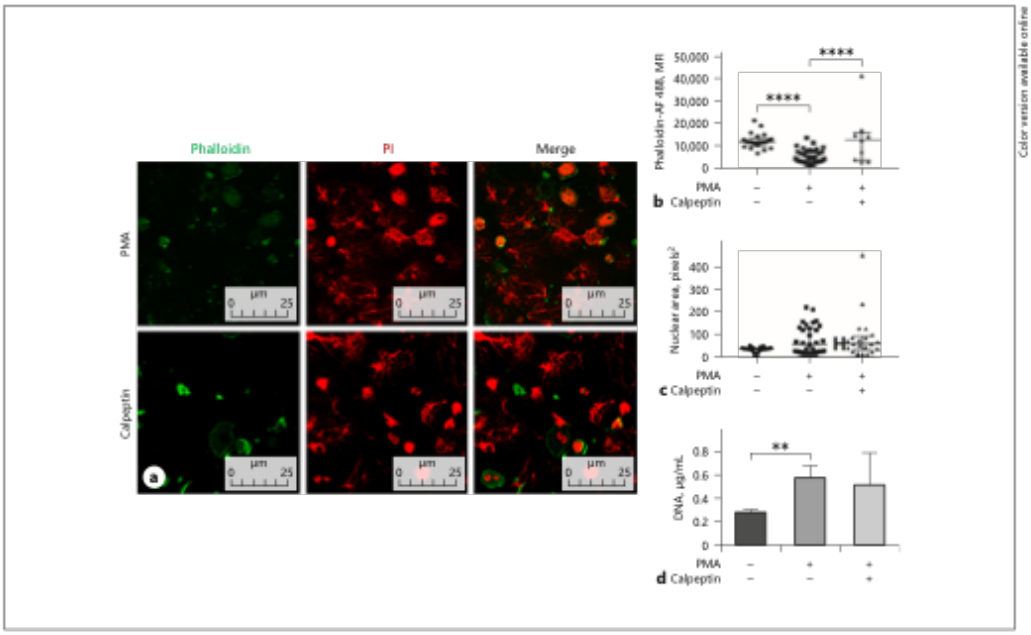

Fig. 6. Participation of calpain during the formation of NETs stimulated with PMA. Neutrophils isolated from peripheral blood from healthy donors (3×10^5 cells/well) were preincubated with calpeptin (100 µM) for 30 min. Induction of NETs was performed with PMA (30 nM) for 180 min at 37°C, 5% CO_2, and 95% O_2. **a** Cells were fixed with 4% PFA, stained with phalloidin-PI, and subsequently analyzed by confocal microscopy. **b** Analysis of MFI on FIJI software, the graph shows median ± IQR, Kruskal-Wallis ($p = 0.0001$). **c** Quantification of DNA released during the formation of NETs stimulated with PMA, the graph shows mean ± SD, RM-one-way ANOVA ($p = 0.038$). **d** Analysis of changes in nuclear area during the formation of NETs, the graph shows median ± IQR, Kruskal-Wallis ($p = 0.0001$). NET, neutrophil extracellular trap; MFI, mean fluorescence intensity.

(Fig. 5d, $p < 0.033$). In the case of PMA induction, although there are not focal points of phalloidin staining as those observed in A23187 induction, the staining of phalloidin partially remains suggesting that calpain inhibition is also preventing the likely degradation or polymerization of actin (Fig. 6a). This was further analyzed by the measurement of phalloidin-AF488 MFI (showing consistent results to those observed with A23187) as shown in Figure 6b ($p < 0.05$). There is also a tendency to a reduced chromatin decondensation (Fig. 6c, $p > 0.05$). This is consistent with DNA release (Fig. 6d, $p > 0.05$) in which no significant differences were observed. Taken all together, our data clearly show that calpain (a calcium-dependent cysteine protease) participates in the regulation of chromatin decondensation, cortical cytoskeleton dynamics, and DNA release during NET formation.

Discusion

The process of NETosis and NET formation is associated to the physiopathology of a wide range of diseases such as rheumatoid arthritis, lupus, cardiovascular disease, vasculitis, and cancer [15–17]. The better understanding of the NET formation process might also identify potential targets for treatment or diagnostic biomarkers. Although elastase has been linked to chromatin decondensation in previous reports by Papayannopoulos et al [3], which suggests that neutrophil elastase is responsible for myeloperoxidase and PAD4 mobilization, the molecular events associated with DNA extrusion might be associated to the mechanic force exerted by the decondensing nucleus on the membrane. Here, we demonstrate that actin cortical cytoskeleton is affected upon neutro-

phil activation by PMA or A23187, and we provide evidence that the molecular mechanisms driving such processes are not merely mechanic but highly regulated. Our data are in accordance with previous observations indicating that protease inhibition reduces the release of DNA by the action of calcium inhibitors or chelators [18]. We explored here the role of calcium-activated enzymes and found that calpain inhibition reduces both DNA decondensation in the nucleus and its release. Our data suggest that the control of the formation and degradation of the actin cytoskeleton are important for the DNA decondensation. Given the participation of actin in the transport of internal content and given that elastase is needed for decondensation, impairment of calpain and other cysteine-protease activity might be associated with reduced transport of elastase to the nucleus. Implications of the loss of F-actin staining need to be further clarified in order to understand whether actin is being actively degraded or prevented to form filaments.

A recent report by Gösswein et al. [18] has suggested the participation of calpain in the NET release process. The authors analyze the effects of recombinant calpain in isolated nuclei from both A549 cell line and mouse embryonic fibroblasts. They provide evidence of calpain activity on the degradation of nuclear laminin. However, they do not provide evidence if the process occurs in a similar manner in neutrophils. Considering that the role of calpain in nuclear decondensation has not been previously analyzed in neutrophils, our observations provide for the first time in neutrophils, strong evidence showing that during NET release, calpain inhibition results in a reduction in chromatin decondensation, degradation of the cortical cytoskeleton, and DNA release. This is clearly different for PMA or A23187 NET induction highlighting how the dependency on calcium is paramount for calpain activity.

As described throughout the results section, the polymerization of actin filaments was evaluated, but not the degradation of actin as a result of protease activity in general. Calpain function has been associated to membrane-associated events but the effects that we see have important and marked functions in chromatin decondensation mediated by PAD4 citrullination and degradation of nuclear laminin. Inductors of NETs such as LPS, IL-8, Fc-activation, and the role of calpain in such processes remain to be described. Also, given the role of actin polymerization in the formation of the leading edge of neutrophils, the participation of such structures in the process of NET-associated events remains elusive. In summary, we describe in a relevant cell model the role of calpain in chromatin decondensation and the implications in DNA release by the reorganization of actin cortical cytoskeleton. This is relevant for the better understanding of the events associated with NETs in several diseases.

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Statement of Ethics

The present study was approved by the national commission on scientific investigation (CNIC) at the Mexican Institute for Social Security as well as the national ethics commission with registration number: R-2018-785-099. All protocols were based on the international declaration of Helsinki and in the principles of not malevolence, justice, and equality. All participants that agreed to participate signed an informed consent and blood samples were drawn from only these individuals.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

J.E.C.D. and Y.B. conceived and designed the experiments; F.L.O.G., R.C.V., and G.T.I.A. performed the experiments; F.L.O.G. analyzed the data and prepared final figures; J.E.C.D. and F.L.O.G. wrote the paper; J.E.C.D., Z.Z.M., E.M.J.A., J.A.M., and L.J.A. provided materials, reagents, and samples for experiments; F.L.O.G., Y.B., R.C.V., G.T.I.A., Z.Z.M., L.J.A., E.M.J.A., J.A.M., and J.E.C.D. critically reviewed and accepted the final manuscript.

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